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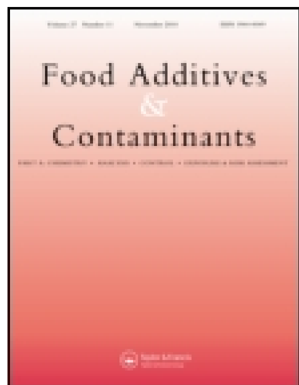

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Natural deoxynivalenol occurrence and genotype and chemotype determination of a field population of the Fusarium graminearum complex associated with soybean in Argentina

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Natural deoxynivalenol occurrence and genotype and chemotype determination of a field population of the *Fusarium graminearum* complex associated with soybean in Argentina

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Soybean (*Glycine max* L.), the main source of protein throughout the world, is used both as a food and a feedstuff. Currently, limited information about the occurrence of *Fusarium* species and mycotoxins in soybean grain and by-products is available. The aims of the present study were: (1) to identify toxigenic *Fusarium* species associated with soybean during crop reproductive stages; (2) to determine the occurrence of deoxynivalenol (DON) and nivalenol (NIV) in soybean seeds; (3) to determine the genotype and chemotype of selected *Fg* complex strains using molecular and chemical analysis, respectively; and (4) to characterize the strains using $AFLP_s$ markers. One soybean field located at Córdoba Province, Argentina, was monitored and samples of soybean tissue were harvested at three reproductive stages: flowering (R2), full seed (R6) and full maturity (R8). A total of 389 *Fusarium* strains *F. equiseti* (40%) was the most frequently species recovered followed by *F. semitectum* (27%) and *F. graminearum* (*Fg*) (11%). From the 40 soybean samples analysed, only two presented detectable DON levels. Based on DON occurrence on soybean seeds at ripening stages, the toxigenic ability of *Fg* complex strains isolated from soybean seeds, pods and flowers were analysed. The trichothecene genotype was determined by a multiplex PCR using primers based on *Tri3*, *Tri5* and *Tri7* toxin genes and then the chemotype was verified by chemical analysis. Most *Fg* complex strains showed 15-ADON genotype and five strains presented a DON/NIV; these also produced both toxins under *in vitro* culture. Neither the NIV nor the 3-ADON genotypes were detected among the members of the population evaluated. All the 15-ADON genotype strains were characterized as *F. graminearum sensu stricto* (lineage 7), while the strains presented a DON/NIV genotype were characterized as *F. meridionale* (lineage 2). The present study contributes new information on the occurrence of *Fusarium* species and trichothecenes toxins on soybean at the pre-harvest stages. Also, this is the first report on the chemotype, genotype and lineages among *Fg* complex isolated from soybean.

Keywords: mycology; molecular biology; PCR; mycotoxins; fungi; trichothecenes; beans

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Introduction

Soybean (*Glycine max* L.), the main source of protein throughout the world, is used both as food and feedstuff. Currently, virtually all soybean crops commercially grown in Argentina are genetically modified (Roundup Ready®-RR resistance to glyphosate). The country ranks third among soybean world producers and most production is exported as seed, meal and oil (Ministerio de Agricultura Ganadería Pesca y Alimentación de la Nación (MAGPyA) 2010).

Previous studies have shown the occurrence of toxigenic *Fusarium* species on soybean seeds in Argentina (Boca et al. 2003; Broggi et al. 2007). However, no information about the occurrence of *Fusarium* mycotoxins in soybean and by-products is available. Although several toxigenic fungi such as *Fusarium* species may infect and colonize the soybean plant from its early reproductive stages, most studies have given more attention to fungi associated with mature soybean seeds than with flowers and pods (Roy et al. 2001).

Fusarium rot of soybeans is caused by a complex of species (Pitt and Hocking 1997), and several are known to produce a broad spectrum of toxins including trichothecenes of A- and B-types (Desjardins 2006). Among B-type trichothecenes, deoxynivalenol (DON) and nivalenol (NIV) are mycotoxins produced by members of the *F. graminearum* species complex (*Fg* complex) (O'Donnell et al. 2008). DON occurs worldwide in cereal crops from both temperate and subtropical regions (Desjardins and Proctor 2011). NIV also occurs in cereals, but is most commonly found in Asian countries, and at relatively lower levels compared with samples from Europe, southern Africa and South America (Placinta et al. 1999). DON is associated with feed refusal, vomiting and suppressed

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immune functions; NIV is of higher toxicity to humans and domestic animals than DON (Ryu et al. 1988).

Fg complex strains usually express one of three main trichothecene metabolites either: (1) nivalenol and its acetylated derivatives (NIV chemotype), (2) deoxynivalenol and 3-acetyldeoxynivalenol (3-ADON chemotype), or (3) deoxynivalenol and 15-acetyldeoxynivalenol (15-ADON chemotype) (Ward et al. 2002). Surprisingly, *Fusarium* isolates that can produce both DON and NIV (NIV/DON chemotype) have been described as 'unknown' chemotypes (Ward et al. 2002; Quarta et al. 2006). The 15-ADON chemotype is predominant in America and in Europe, and the 3-ADON chemotype is predominant in the same areas of China, Australia and New Zealand (Guo et al. 2008). However, several recent studies indicated that genetically differentiated populations of *Fg* have emerged in Canada and the United States. Ward et al. (2008) and Guo et al. (2008) reported a significant increased of isolates with the 3-ADON chemotype in Canada. The newly emerging 3-ADON population appears to be more aggressive and produces a higher level of DON than the 15-ADON populations (Ward et al. 2008; Puri and Zhong 2010; von der Ohe et al. 2010).

Primers based on the sequences of alleles at *Tri3*, *Tri5* and *Tri7* have been designed to differentiate between the three toxin genotypes (Lee et al. 2001; Ward et al. 2002; Chandler et al. 2003; Jennings et al. 2004; Quarta et al. 2006). Interestingly, the region of the TRI cluster has a parallel and independent evolution from the rest of the genome (Ward et al. 2002) and may be useful to give further insight into the structure of a field population. Genotype characterization present in a given region and different crops could be a useful tool to map a population and identify population changes in the field (Karugia et al. 2009) as well as predicting the contamination with different trichothecenes.

Several reports examined trichothecene production by *Fg* complex strains from cereals in Argentina based on chemical analyses (Faifer et al. 1990; Lori et al. 1992; Molto et al. 1997; Fernandez Pinto et al. 2008; Alvarez et al. 2009). However, there is a lack of information on the trichothecene genotypes of *Fg* complex strains associated with soybean in Argentina.

The *Fg* complex (teleomorphs *Gibberella* species) is composed of at least 13 lineages (O'Donnell et al. 2000, 2004, 2008; Starkey et al. 2007; Yli-Mattila et al. 2009), most of which have now been described as species. Molecular markers such as amplified fragment length polymorphism (AFLP) have been used to characterize *Fusarium* populations isolated from wheat and maize in America (Zeller et al. 2004; Schmale et al. 2006; Ramirez et al. 2007). The geographic location often influences the lineage present, e.g. populations from wheat in Argentina are genotypically diverse and belong to lineage 7 (Ramirez et al. 2006, 2007), also named *F. graminearum sensu stricto* (O'Donnell et al. 2000, 2004). Most studies on molecular characterization of the *Fg* complex have focused on populations from wheat, barley and corn, and there is little information on populations from soybean. Surveys of soybean seed grown in South Brazil revealed infection with species belonging to the *Fg* complex and were identified as *F. astroamericanum* (lineage 1), *F. meridionale* (lineage 2) and *F. cortaderiae* (lineage 8) (Martinelli et al. 2004).

The aims of the present study were: (1) to identify toxigenic *Fusarium* species associated with soybean during crop reproductive stages; (2) to determine the occurrence of deoxynivalenol (DON) and nivalenol (NIV) in soybean seeds; (3) to determine the genotype and chemotype of selected *Fg* complex strains using molecular and chemical analysis, respectively; and (4) to characterize the strains using $AFLP_s$ markers. The working hypothesis was that infection with toxigenic *Fusarium* species can occur at the earlier stages of seed development, which would allow sufficient time for toxin production. Also, it is hypothesized that *Fg* complex strains isolated from soybean are similar to those found in wheat populations.

Materials and methods

Field location and sampling procedure

Samples were collected during the 2007/08 growing season in experimental plots conducted at the Universidad Nacional de Río Cuarto, Córdoba, Argentina. The cultivar used, Nidera 4613, is genetically modified for tolerance to glyphosate (maturity group IV) and one of the most planted by growers from Córdoba Province. The field (100 m length × 25 m width) was planted on December 2007 with a row spacing of 0.75 m under a no-till cultivation system and was harvested in April 2008. The samples were taken in two diagonal transects extending from opposing corners. A total of 20 samples (ten from each transect) of either flowers, pods and seeds were taken sequentially at three soybean reproductive stages, respectively: R2 (full bloom), R6 (full seed) and R8 (full maturity). At each sampling point ten plants were randomly selected and from each flowers and pods were collected from the bottom and top canopy heights. In the laboratory seeds from each pod were removed and water activity (a_w) was measured with an Aqualab Series 3 (Decagon Devices, Inc., Pullman, WA, USA). The samples were stored at 4°C until analysis.

Fungal isolation and morphological identification

From each sample 100 flowers, pods and seeds were surface disinfested for 1 min in 1% NaOCl solution,

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rinsed three times in sterile distilled water and transferred to Petri dishes containing dichloran rose Bengal chloramphenicol agar (DRBC) (Pitt and Hocking 1997) and Nash–Snyder medium (Leslie and Summerell 2006). Petri dishes containing DRBC were incubated at 25°C for 5–7 days and Nash–Snyder medium was incubated at 25°C for 7 days under a 12/12-h photoperiod of cold white-and-black fluorescent lamps. The isolation frequency of *Fusarium* spp. was calculated as:

$$(\text{Number of samples where fungi occurred}/\text{total number of samples}) \times 100$$

Single-spored cultures from colonies initially identified as *Fusarium* spp. were transferred to carnation leaf agar and further identified based on morphology (Leslie and Summerell 2006).

Deoxynivalenol and nivalenol determination in soybean seeds

Seeds samples collected at R6 and R8 stages were assessed for DON and NIV contamination by HPLC analysis. The analysis was performed using the method described by Barros et al. (2008). The dried residue was redissolved in 400 µl of water:methanol (88:12, v/v), homogenized in a vortex mixer and injected into the HPLC system (Hewlett Packard model 1100 pump, Palo Alto, CA, USA; Rheodyne manual injector with a 50 µl loop, Rheodyne, Cotati, CA, USA). Chromatographic separations were performed on a stainless steel, C_{18} reversed-phase column (150 mm × 4.6 mm i.d., 5 µm particle size; Luna-Phenomenex, Torrance, CA, USA). The mycotoxins were detected by UV (Hewlett Packard model 1100 programmable UV detector) at 220 nm and quantified by a data module Hewlett Packard Kayak XA (HP ChemStation Rev. A.06.01). The mycotoxin levels were calculated by comparing the area of the chromatographic peak of the sample with those of the standard calibration curve. The mobile phase was water:methanol (88:12, v/v) at a flow rate of 1.5 ml min^{-1} and the detection limit (LOD) was 0.1 µg g^{-1} for DON and 0.2 µg g^{-1} for NIV, based on a signal-to-noise ratio 3:1.

DNA extraction and PCR analysis for Fg *complex strains*

Fg complex strains were grown in complete medium (CM) and incubated on an orbital shaker (150 rpm) for at least 3 days at 25 ± 1°C. The resulting mycelia were harvested by filtration through non-gauze milk filters (Ken AG, Ashland, OH, USA). Excess water was removed by blotting mycelia between clean paper towels, and dried mycelia were stored frozen at −20°C. DNA was extracted with a cetyltrimethylammnonium bromide (CTAB) method (Leslie and Summerell 2006).

Prior to trichothecene genotyping, all isolates were additionally identified using Fg16F (5′-CTCCGGATATGTTGCGTCAA-3′) and Fg16R (5′-GGTAGGTATCCGACATGGCAA-3′) primers which produce polymorphic products with DNA from the *Fg* complex (Nicholson et al. 1998). PCR experiments were conducted with 10–25 ng of fungal DNA in a total volume of 25 µl of 1× reaction buffer containing 1.5 mM $MgCl_2$, 2 U Taq DNA polymerase (Promega), 2 mM dNTPs and 0.6 µM each of the primers. A negative control, containing all reagents and primers but no fungal DNA, was included with every set of reactions. PCR was conducted in a PTC-2000 Thermal Cycler (MJ Research, Inc., Watertown, MA, USA). The PCR conditions were: 95°C, 3 min then 37 cycles of 95°C, 20 s, 62°C, 20 s, 72°C, 45 s, followed by a final extension step of 10 min, 72°C. PCR products were separated by electrophoresis through 2% agarose gels. Gels were stained with 1 µg ml^{-1} ethidium bromide and photographed under UV light.

For trichothecene genotype determination multiplex PCR experiments were conducted with 10–25 ng of fungal DNA in a total volume of 50 µl of 1× reaction buffer containing 1.5 mM $MgCl_2$, 2 U Taq DNA polymerase (Promega), 2 mM dNTPs and 0.2 mM each of the *Tri*3 primers (Tri3F971 5′-CATCATAC TCGCTCTGCTG-3′, Tri3F1325 5′-GCATTGGCTA ACACATGA-3′ and Tri3R1679 5′-TT(AG)TAGTT TGCATCATT(AG)TAG-3′) and 0.1 mM each of primers Tri7F340 (5′-ATCGTGTACAAGGTT TACG-3′), Tri7R965 (5′-TTCAAGTAACGTTCGA CAAT-3′), 3551 H (5′-ACTTTCCCACCGAGTAT TTT-3′) and 4056 H (5′-CAAAAACTGTTGTTCCA CTGCC-3′) (Quarta et al. 2005, 2006). A negative control, containing all reagents and primers but no fungal DNA, was included in every set of reactions. PCR was conducted in a PTC-2000 Thermal Cycler (MJ Research) and the conditions were: 94°C, 3 min then 35 cycles of 94°C, 30 s, 53°C, 30 s, 72°C, 1 min, followed by a final extension step of 10 min, 72°C. PCR products were separated by electrophoresis through 2% agarose gels. Gels were stained with 1 µg ml^{-1} ethidium bromide and photographed under UV light. DNA from strains with known NIV, 3-ADON and 15-ADON chemotypes were used as positive controls. The trichothecene genotypes of the DON/NIV strains also were determined in a PCR reaction with primers for the Tri7 and Tri13 alleles. PCR reactions included 20 ng of genomic DNA as template in a 50 µl reaction composed of 1× reaction buffer, 2 mM MgCl2, 1.25 U Taq DNA polymerase (Promega), 2 mM dNTPs and 2 µM of each primer (GzTri7/p1 5′-GGCTTTACGACTCCTCA ACAATGG-3′, GzTri7/p2 5′-G(A/G)CGG(C/T) AAAGAAAACCAATCAAC-3′, GzTri13/p1 5′-AAT ACT(A/C)AAG(C/T)CTAG(G/T)ACGACGC-3′ and

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GzTri13/p2 5′-GTG(A/G)T(A/G)TCCCAGGATCT GCGTGTC-3′) (Lee et al. 2001, 2002). PCR was performed in a thermal cycler (MJ Research) with an initial denaturation step at 95°C for 2 min; 30 cycles of 94°C (1 min), 55°C (1 min), and 72°C (3 min); and a final extension step at 72°C for 10 min. PCR products were separated by electrophoresis through 2% agarose gels. Gels were stained, photographed and analysed as described above. DNA from strains with known NIV, 3-ADON and 15-ADON chemotypes were used as positive controls.

Chemotype determination of Fg *complex strains*

The *Fg* complex strains were cultured in Erlenmeyer flasks (250 ml) containing 25 g of long grain rice. A total of 10 ml of distilled water was added before autoclaving for 30 min at 121°C, twice. Each flask was inoculated with a 3-mm diameter agar disk taken from the margin of a colony grown on synthetic nutrient agar (SNA) at 25°C for 7 days (Leslie and Summerell 2006). Flasks were shaken once a day by hand for 1 week. These cultures were incubated for 28 days at 25°C in the dark. At the end of the incubation period the contents of the flask were dried at 50°C for 24 h and then stored at −20°C until analysed for toxin.

Toxin analyses were done by using a modified version of that originally reported by Cooney et al. (2001). Each sample was finely ground, in a laboratory grinder, and homogenized. A sub-sample of ground rice (15 g) was extracted by mixing with 40 ml acetonitrile:methanol (14:1, v/v), shaken for 2 h on a oscillatory shaker and then filtered through Whatman No. 1 filter paper. A syringe (3 ml capacity) plugged with glass wool and dry-packed with alumina/carbon (20:1, w/w; 500 mg) was used as a mini-cleanup column. A 2 ml aliquot of extract was applied to the column and allowed to drain under gravity and the eluent collected. The column was washed with 500 μl acetonitrile:methanol:water (80:5:15), and the combined eluents evaporated to dryness under N_2 at 50°C. The cleaned-up residue was dissolved in 500 μl methanol:water (5:95, v/v).

The HPLC system consisted of a Hewlett Packard model 1100 pump connected to a Hewlett Packard 1100 Series variable wavelength detector and a data module Hewlett Packard Kayak XA (HP ChemStation Rev. A.06.01). Chromatographic separations were performed on a Luna™ C18 reversed-phase column (100 × 4.6 mm, 5 μm particle size) connected to a guard column SecurityGuard™ (4 × 3.0 mm) filled with the same phase. The mobile phase consisted of methanol:water (12:88, v/v), at a flow rate of 1.5 ml min^{-1}. The detector was set at 220 nm with an attenuation of 0.01 AUFS. Injection volume was 50 μl. Retention times for NIV and DON were 400 and 900 s, respectively. Quantification was relative to external standards of DON and NIV (Sigma-Aldrich Co., St Louis, MO, USA) of 1–4 μg ml^{-1} in methanol:water (5:95). The quantification limit was 5 ng g^{-1} for each toxin.

For 3-ADON and 15-ADON determination the extract was dissolved in 500 μl of toluene:acetonitrile (95:5, v/v). The standard mycotoxins and the extracts were applied to TLC plates (Merck 5553), and the plates were developed in a solvent system of chloroform:acetone:2-propanol (8:1:1, v/v/v). After development the plates were sprayed with 20% aluminium chloride in ethanol and heated to 120°C for 7 min. The amounts of 3-ADON and 15-ADON (Sigma-Aldrich) were determined by visual comparison with known amounts of standards under 366 nm UV light (Molto et al. 1997).

Characterization of Fg *complex strains using AFLPs markers*

AFLPs reactions were performed as described by Vos et al. (1995), as modified by Leslie and Summerell (2006) in a PTC-2000 Thermal Cycler (MJ Research). All buffers and DNA-modifying enzymes were used following either the manufacturer's instructions or standard protocols (Sambrook et al. 1989). Genomic DNAs digested to completion with *Eco*RI and *Mse*I and ligated to AFLP adapters in a single overnight at room temperature (21–24°C) were used. The digested and ligated templates were diluted in 9 vols of Tris-EDTA buffer prior to pre-amplification. Samples were pre-amplified with the following cycling conditions: initial denaturation at 94°C for 60 s, followed by 20 cycles consisting of 30 s at 94°C, 60 s at 56°C, and 60 s at 72°C and a final extension step of 72°C for 5 min, and then held at 4°C indefinitely. Pre-amplified reactions were diluted 1:50 with water prior to final specific AFLP amplification. Two primer pair combinations (EcoRI + AA/MseI + AT, and EcoRI + TG/MseI + TT) were used. EcoRI primers for specific amplification were end-labelled with [γ-^{33}P] ATP. For final specific AFLP reactions, 1.3 μl of diluted pre-amplification reactions were used and the final volume was 5 μl. The PCR programme for the AFLP amplification was: one cycle of 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s, then this cycle was followed by a 12 cycle step-down protocol in which the annealing temperature was lowered each cycle by 0.7°C from 65°C to 56°C. After that, 23 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s were performed; followed by a final extension step of 72°C for 5 min, and then held at 4°C.

AFLP fragments were separated in denaturing 6% polyacrylamide gels (Long Ranger gel solution, BMA, Rockland, ME, USA) with 1× Tris-borate EDTA

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buffer (pH 8.0) in both the gels and the running buffer. Gels were run at a constant power of 60 W until the xylene cyanol (Sigma) marker had run approximately 22 cm. After that the gels were transferred to 3 MM gel blotting paper (Midwest Scientific, Valley Park, MO, USA) and dried before exposure to X-ray film at room temperature (Classic Blue Sensitive, Midwest Scientific) for 3–7 days to resolve banding patterns. Bands sizes were estimated on polyacrylamide gels against [γ-^{33}P] ATP-labelled BRL low-mass ladder (Life Technologies, Rockville, MD, USA). The presence or absence of polymorphic AFLP bands was scored manually and the data recorded in a binary format. All polymorphic bands in this size range were scored, including those assumed to be homologous and to represent the same allele and locus. Each scored band of differing mobility was treated as a single independent locus with two alleles (present or absent).

To estimate the genetic distances between individuals, similarity coefficients (S) were calculated using the formula:

$$S = 2N_{xy}/(N_x + N_y)$$

where N_x is the number of fragments amplified in isolated x and y, respectively; and N_{xy} is the number of fragments shared by the two isolates (Nei 1978). Genetic distance (D) was derived from similarity coefficients as follows:

$$D = 1 - S$$

Genetic distance matrices were constructed for isolates using the compiled AFLP data. Dendrograms were prepared using the UPGMA (unweighted pair-group method using arithmetic averages) clustering strategy of the NTSYSpc 2.0 (Numerical Taxonomy System) software package (Rohlf 1990).

We included reference strains identified as belonging to the *Fg* complex: *F. austroamericanum* (lineage 1), *F. meridionale* (lineage 2), *F. boothii* (lineage 3), *F. mesoamericanum* (lineage 4), *F. acacia-mearnsii* (lineage 5), *F. asiaticum* (lineage 6), *F. graminearum sensu stricto* (lineage 7), *F. cortaderiae* (lineage 8), and *F. brasilicum*, *F. vorosii*, *F. gerlachii* (no lineage number) (O'Donnell et al. 2000, 2004; Starkey et al. 2007) as standards on each gel.

Statistical analysis

To compare the independent (flowers, pods and seeds and top and bottom of the plant) and dependent variables fungal infection, data were analysed by ANOVA. Means separation and comparison were made with Duncan multiple range test ($p < 0.05$) (*SAS User Guide*, SAS Institute, Inc., Cary, NC, USA).

Results

Fungi associated with flowers, pods and seeds soybean

The mycoflora isolated from flowers, pods and seeds were dominated by two genera: *Alternaria* and *Fusarium*, at similar levels across all stages evaluated. The isolation frequency of *Fusarium* genus in flowers, pods and seeds at different growth stages is shown in Figure 1. Statistical analysis showed no significant differences in *Fusarium* genus infection levels between different sampling heights (bottom and top of the plant) ($p > 0.05$). *Fusarium* contamination across different stages showed higher frequency in pods and seeds at stage R6 (full seed), being the a_w of immature seeds 0.992. At stage R8 (full maturity) the water content of the seeds dropped dramatically to 0.70 and the percentage of *Fusarium* spp. also diminished compared with the R6 stage. Among 389 *Fusarium* isolates recovered, 45% were isolated from pods, 38% from seed and 17% from flowers. Among the *Fusarium* species identified, *F. equiseti* was the most frequently recovered (40%), followed by *F. semitectum* (27%) and *Fg* species complex (11%). Other *Fusarium* species identified were: *F. oxysporum*, *F. solani*, *F.verticillioides*, *F. proliferatum* and *F. sambucinum*.

Our data showed that strains of *Fg* complex were present along three stages evaluated, with higher isolation in pods and seeds at R6 stage (52.5% of isolates), followed by pods and seeds at R8 stage (40% of isolates) and flowers (7.5% of isolates).

Trichothecenes contamination in soybean seeds

Soybean seed samples from each reproductive stage (R6 and R8) were evaluated for DON and NIV contamination. It was found that only one sample from each crop stage, in a total of 40 seed samples analysed, were contaminated with DON at levels of 1.6 $\mu g\,g^{-1}$ (R6) and 0.9 $\mu g\,g^{-1}$ (R8). NIV was not detected.

Molecular identification and trichothecene genotype and chemotype determination

In the PCR reaction using the Fg16F/Fg16R primers, all strains ($n = 40$) produced an expected fragment between approximately 450 or 500 bp, depending on the isolate.

Information for each *Fg* complex strain analysed on amplified fragments by PCR reactions and trichothecene production is shown in Table 1. PCR assays showed that 35 of 40 strains presented the 15-ADON genotype. The remaining five strains presented a DON/NIV genotype, since they amplified two fragments of 525 and 625 bp corresponding to *Tri5* and *Tri7* alleles, respectively (Figure 2). We did not detect the NIV and the 3-ADON genotypes. The genotype of DON/NIV

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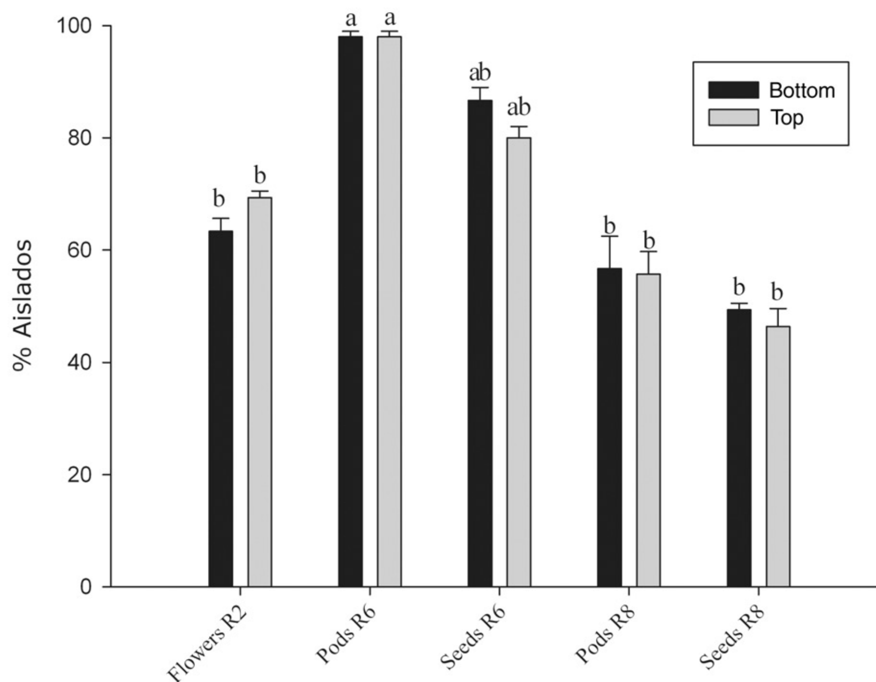

Figure 1. Frequency of *Fusarium* spp. isolated from flowers, pods and seeds sampled at the bottom and top canopy at three reproductive growth stages. Bars not sharing a common letter are significantly different ($p < 0.05$) according to Dunn's test.

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strains was confirmed using the PCR assay according to Lee et al. (2001, 2002). Also these strains showed DON/NIV genotypes since amplification of two fragments 327 bp for DON and 161 bp for NIV was observed.

Chemical analysis confirmed the production of both DON and 15-ADON by the strains that showed 15-ADON genotype. Only one strain did not produce detectable levels of DON. Out of five strains that showed DON/NIV genotype, four were able to produce both DON and NIV and one isolate produced only DON. Similar to results from the PCR assays, the NIV and 3-ADON chemotypes were not observed.

AFLPs analysis

Twenty-two strains (five with DON/NIV genotype and 17 presented 15-ADON genotype selected at random) were analysed using AFLP technique. A total of 86 AFLP bands were identified in the 200–500 bp range from the 22 analysed strains when using the two primer pair combinations. Of these 86 AFLP loci, 77.9% were polymorphic (67 loci). All 15-ADON genotype strains had AFLP profiles typical of *F. graminearum sensu stricto* (lineage 7) and we identified 16 haplotypes and a clone formed by two isolates (F5226 and F5228). Four out of five DON/NIV genotype strains clustered with the standard strain of *F. meridionale* (lineage 2). The remaining strain with DON/NIV genotype (F5036) also grouped with the standard strain of lineage 2, although was genetically distant from the other four strains. Genetic similarity coefficients between strains averaged 0.88 (range from 0.74 to 1.00) for *F. graminearum sensu stricto* strains and 0.80 (range from 0.69 to 0.94) for *F. meridionale* strains.

Discussion

A diverse group of saprophytic and parasitic fungi are able to colonize and infect soybean pods and seeds prior to harvesting (Roy et al. 2001). *Alternaria* and *Fusarium* species are the most commonly isolated fungi from soybean in Argentina (Boca et al. 2003; Broggi et al. 2007) and the United States (Baird et al. 2001; Villarroel et al. 2004). Although previous studies carried out in Argentina evaluated the fungal contamination on freshly harvested soybean or soybean seeds in storage, this is the first report on *Fusarium* species and trichothecene contamination at pre-harvest reproductive stages.

Fusarium spp. were isolated at high frequency during the three reproductive stages evaluated. The isolation frequency of this genus on pods and seeds was greater at later reproductive stage which could be related to the difference in the a_w between crop stages. In contrast, other studies had demonstrated that the rate of moisture loss has been positively correlated with pathogen incidence, such as *Phomopsis* spp., in the interval between the R6 and R8 growth stages (Baird et al. 2001; Villarroel et al. 2004). Most of *Fusarium* species are considered saprophytic fungi causing no direct damage to soybean seed, but some produce

Table 1. Amplified fragments by PCR reactions and trichothecene production in *Fg* complex strains.

		Quarta et al. (2006) protocol amplified fragments (pb)				Trichothecene production (μg g^{-1})			
Isolate	Source/stage	354	525	625	708	DON	NIV	15-ADON	3-ADON
F5030	Flower R2	–	+	–	+	15.7	n.d.	+	n.d.
F5051	Flower R2	–	+	–	+	36.0	n.d.	+	n.d.
F5221	Flower R2	–	+	–	+	0.8	n.d.	+	n.d.
F5031	Pod R6	–	+	–	+	35.4	n.d.	+	n.d.
F5038	Pod R6	–	+	–	+	8.7	n.d.	+	n.d.
F5040	Pod R6	–	+	+	–	18.2	9.1	n.d.	n.d.
F5047	Pod R6	–	+	+	–	16.1	11.9	n.d.	n.d.
F5049	Pod R6	–	+	–	+	18.5	n.d.	+	n.d.
F5052	Pod R6	–	+	–	+	144.0	n.d.	+	n.d.
F5055	Pod R6	–	+	–	+	30.3	n.d.	+	n.d.
F5056	Pod R6	–	+	–	+	48.1	n.d.	+	n.d.
F5057	Pod R6	–	+	–	+	4.8	n.d.	+	n.d.
F5222	Pod R6	–	+	–	+	3.2	n.d.	+	n.d.
F5223	Pod R6	–	+	–	+	81.1	n.d.	+	n.d.
F5225	Pod R6	–	+	–	+	8.0	n.d.	+	n.d.
F5226	Pod R6	–	+	–	+	16.0	n.d.	+	n.d.
F5227	Pod R6	–	+	–	+	7.6	n.d.	+	n.d.
F5036	Seed R6	–	+	+	–	3.5	n.d.	n.d.	n.d.
F5044	Seed R6	–	+	–	+	4.3	n.d.	+	n.d.
F5046	Seed R6	–	+	–	+	15.3	n.d.	+	n.d.
F5184	Seed R6	–	+	–	+	3.6	n.d.	+	n.d.
F5185	Seed R6	–	+	–	+	41.5	n.d.	+	n.d.
F5186	Seed R6	–	+	–	+	4.3	n.d.	+	n.d.
F5187	Seed R6	–	+	–	+	7.9	n.d.	+	n.d.
F5001	Pod R8	–	+	–	+	15.6	n.d.	+	n.d.
F5024	Pod R8	–	+	–	+	16.6	n.d.	+	n.d.
F5028	Pod R8	–	+	–	+	15.3	n.d.	+	n.d.
F5034	Pod R8	–	+	–	+	n.d.	n.d.	+	n.d.
F5048	Pod R8	–	+	+	–	2.3	9.4	n.d.	n.d.
F5050	Pod R8	–	+	–	+	62.7	n.d.	+	n.d.
F5053	Pod R8	–	+	–	+	15.9	n.d.	+	n.d.
F5054	Pod R8	–	+	–	+	3.8	n.d.	+	n.d.
F5058	Pod R8	–	+	–	+	4.2	n.d.	+	n.d.
F5059	Pod R8	–	+	–	+	40.0	n.d.	+	n.d.
F5002	SeedR8	–	+	–	+	16.0	n.d.	+	n.d.
F5005	SeedR8	–	+	–	+	16.6	n.d.	+	n.d.
F5043	SeedR8	–	+	+	–	12.3	5.8	n.d.	n.d.
F5220	SeedR8	–	+	–	+	0.4	n.d.	+	n.d.
F5224	SeedR8	–	+	–	+	8.7	n.d.	+	n.d.
F5228	SeedR8	–	+	–	+	9.8	n.d.	+	n.d.

Note: n.d., Not detected.

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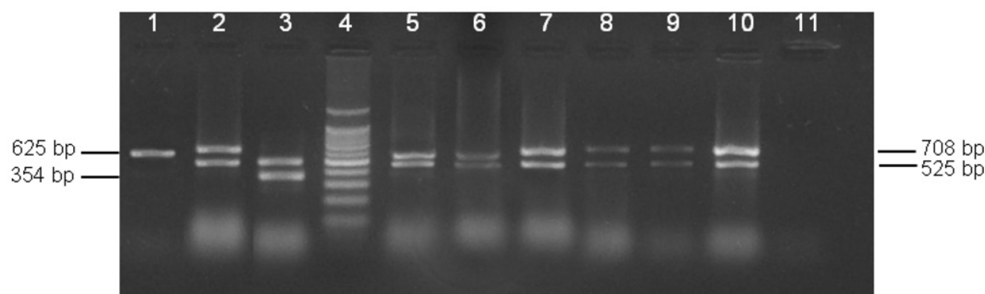

Figure 2. DNA agarose gel of the multiplex PCR assay: lanes 1–3, positive controls of NIV, 15-ADON and 3-ADON genotypes, respectively; lane 4, 100-bp ladder marker; lanes 5 and 6, DON/NIV genotype soybean isolates; lanes 7–10, 15-ADON genotype soybean isolates; and lane 11 negative control (omitting DNA template).

mycotoxins which are harmful to human and animals if consumed (Roy et al. 2001).

The *Fusarium* species identified in this work were similar to those found by Broders et al. (2007) in soybean seed and seedlings in Ohio (USA) and Broggi et al. (2007) from soybean seeds harvested in Entre Ríos Province (Argentina). Among the toxigenic *Fusarium* species, *F. equiseti* was the most frequently isolated. This species is known to produce trichothecenes such as diacetoxyscirpenol, nivalenol and T-2 toxin (Leslie and Summerell 2006). We did not analyse this toxin in our work and further studies are necessary to evaluate the toxigenic ability of the isolates and natural occurrence of those toxins in soybean seeds.

Of the many species of *Fusarium* isolated in the present study, perhaps the most important are members of *Fg* complex. Disease reports in soybean by species within this complex are contradictory and some authors considered them non-pathogenic to soybean (García-Romera et al. 1998; Miller et al. 1998), a secondary colonist of soybean seed damaged by other fungi or by freezing (Wicklow et al. 1987; Osorio and McGee 1992; Jacobsen et al. 1995), and a primary pathogen (Martinelli et al. 2004; Broders et al. 2007; Xue et al. 2007).

The presence of species belonging to the *Fg* complex in soybean may be attributable to the increase of the conservation tillage systems in Argentina. No-tillage cultivation must leave at least 30% of the debris on the soil surface and members of the *Fg* complex has been shown to colonize readily crop debris left behind wheat, corn and soybean (Baird et al. 1997; Osborne and Stein 2007). Inoculum propagules including both ascospores and conidia are likely to be found at nearly any time during the adult stages of the crop when the environmental falls within the wide range of favourable conditions (Beyer and Vereet 2005). Our data showed that strains of the *Fg* complex were present in the three stages evaluated and the isolation frequency was greater on pods than on seeds and flowers. Roy et al. (2001) suggested that fungal colonization of pods and flowers is important in relation to the dynamics of soybean pod and seed infection and to disease control. For example, certain fungi capable of sustained colonisation of maturing and mature pods are more likely to progress into seeds. In this way, a high incidence of the *Fg* complex strains on pods may affect the percentage of infection in soybean seeds, increasing the risk of DON accumulation.

In previous studies DON was detected in soybean, but this contamination occurred in damaged seeds (Wicklow et al. 1987; Jacobsen et al. 1995) or in soybean inoculated under greenhouse conditions (Martinelli et al. 2004). However, to our knowledge this is the first report to demonstrate natural contamination of DON in soybean during grain ripening. Generally, grains with a moisture content equivalent to less than 0.70 a_w (<14.5% moisture by weight) may not be subject to fungal spoilage and mycotoxin production (Alfred and Magan 2004). In the present study, an a_w of 0.7 was observed in soybean seeds at stage R8, thus suggesting that toxin production occurred between full seed and full maturity stages when the a_w level was around 0.99.

Fernandez Pinto et al. (2008) found that 13 of 19 wheat samples were contaminated with DON in a range of 0.3–70 $\mu g\ g^{-1}$ and reported for the first time the natural co-occurrence of DON and NIV in wheat cultivated in Argentina. The incidence and DON levels found in soybean seeds were lower than those observed in wheat from Argentina.

The *Fg* complex strains isolated from soybean were mainly 15-ADON genotypes, which is similar to studies on wheat strains from the Netherlands, Brazil, the UK, China, Luxembourg and Argentina (Waalwijk et al. 2003; Jennings et al. 2004; Ji et al. 2007; Pasquali et al. 2009, 2010; Scoz et al. 2009; Reynoso et al. 2011).

Analysis of trichothecene chemotype among the *Fg* complex isolates revealed that 15-ADON was the dominant chemotype. Similar results were obtained in previous studies that evaluated the toxigenic potential of *Fg* isolates from wheat in Argentina (Alvarez et al. 2009; Reynoso et al. 2011) and south Brazil (Scoz et al. 2009). This finding is not surprising since soybean is routinely rotated with wheat in Argentina and the pool of strains could be the same in the agro-ecosystem. This is in contrast to results obtained by Martinelli et al. (2004) who found that *Fg* complex strains from soybean in Brazil to possess an NIV and 3-ADON chemotype. The prevalence of this chemotype in Brazilian soybean may be related to fact that strains that produce NIV and 3-ADON may be more aggressive than DON/15-ADON producing strains (Ward et al. 2008). Strains with an NIV and/or 3-ADON chemotype were not recovered from soybean in our study.

The DON/NIV genotype from two Italian strains isolated from barley and weeds described by Quarta et al. (2006) and from nine Argentinean strains isolated from wheat by Reynoso et al. (2011) amplified the 525-bp fragment together with the 625-bp fragment. However, the Italian strains expressed the NIV chemotype and the Argentinean strains the DON chemotype. In contrast, Korean strains isolated from rice showing the DON/NIV genotype based on *Tri-7* and *Tri-13* loci produced only 3-ADON by chemical analysis (Lee et al. 2009). Our results showed that among the strains characterized as a DON/NIV genotype according to the methodologies proposed by Quarta et al. (2006) and Lee et al. (2001, 2002), four produced detectable DON and NIV levels and one strain produced only DON by chemical analysis. The *Fg* complex strains with an unusual ability to produce both DON and NIV have also be found previously from wheat populations in Argentina

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(Fernandez Pinto et al. 2008; Reynoso et al. 2011). Soybean is often used in rotation with wheat and other cereal crops in Argentina, therefore the *Fg* complex strains from soybean possessing a distinct chemotype could provide a inoculum source to infections on wheat. Further studies will be necessary in order to characterize the virulence of the hybrid DON/NIV producer's strains in soybean crop. Previous studies demonstrated that NIV-producing isolates are less virulent on wheat than DON-producing isolates (Desjardins et al. 2004). Consequently, without high levels of initial inoculum of DON/NIV-producing isolates, infection cannot cause high NIV levels in harvested seeds. This may explain the no natural occurrence of NIV in soybean seeds observed in the present study.

This work represents the first report on the genotypic and chemotypic characterization of *Fg* complex strains isolated from soybean in Argentina. A strong correlation between multiplex PCR (genotype) and chemical analysis (chemotype) was observed. In this sense, PCR genotyping allowed a rapid and accurate diagnostic and may be a useful tool to determine the chemotype distribution across several crops and to monitor potential shifts in field populations in the future.

F. graminearum sensu stricto (lineage 7) is the most widespread species within of the *Fg* complex described to date. The results obtained in the present study are consistent with those of Zeller et al. (2003) and Scoz et al. (2009) who found that *F. graminearum sensu stricto* was the dominant specie in Uruguay and southern Brazil, respectively, and Ramirez et al. (2007) who detected only *F. graminearum sensu stricto* among populations isolated from wheat in Argentina. In contrast, Martinelli et al. (2004) found that all Brazilian isolates from soybean seeds belonged to *F. austroamericanum*, *F. meridionale* and *F. cortaderiae* (lineages 1, 2 and 8, respectively). Previously, Sampietro et al. (2010) detected one strain of *F. meridionale* in maize of the subtropical region of Argentina. However, this is the first report of *F. meridionale* isolated from soybean in Argentina.

Strains characterized as *F. meridionale* in the present work showed a DON/NIV chemotype. In contrast, strains of this species isolated by Sampietro et al. (2010) and Martinelli et al. (2004) exhibited an NIV chemotype. These results agree with those of Ward et al. (2002) who suggested that lineage and trichothecene chemotype are not correlated.

The lineage composition of *Fg* complex populations does appear to be host and location dependent. In China and the southern provinces of Korea *F. asiaticum* (lineage 6) usually dominates and may be associated with the culture of rice (Gale et al. 2002; Lee et al. 2009). A recent survey of strains on wheat from Brazil suggests that *F. graminearum sensu stricto* is predominant (Scoz et al. 2009), while all Brazilian soybean isolates belong to *F. austroamericanum*, *F. meridionale* and *F. cortaderiae* (Martinelli et al. 2004). This could partially explain the presence of *F. meridionale* strains in soybean and that they have not been recovered from wheat in Argentina. Additional sampling on different soybean-growing regions in Argentina and further sequence analysis will be needed to confirm the role of the soybean crop as a population reservoir of several species within the *Fg* complex.

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