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Pathogenicity of *Fusarium graminearum* and *F. meridionale* on soybean pod blight and trichothecene accumulation

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Soybean (*Glycine max*) is the most important crop in Argentina. At present *Fusarium graminearum* is recognized as a primary pathogen of soybean in several countries in the Americas, mainly causing seed and root rot and pre- and post-emergence damping off. However, no information about infections at later growth stages of soybean development and pathogenicity of *F. graminearum* species complex is available. Therefore, the objectives of this study were to compare the pathogenicity of *F. graminearum* and *F. meridionale* isolates towards soybean under field conditions and to evaluate the degree of pathogenicity and trichothecene production of these two phylogenetic species that express different chemotypes. Six isolates of *F. graminearum* and *F. meridionale* were evaluated during 2012/13 and 2013/14 soybean growing seasons for pod blight severity, percentage of seed infected in pods and kernel weight reduction. The results showed a higher aggressiveness of both *F. graminearum* and *F. meridionale* species during the 2013/14 season. However, the differences in pathogenicity observed between the seasons were not reflected in a distinct trichothecene concentration in soybean seeds at maturity. *Fusarium meridionale* isolates showed similar pathogenicity to *F. graminearum* isolates but they were not able to produce this toxin *in planta* during the two field trials.

Keywords: chemotypes, *Fusarium graminearum*, *Fusarium meridionale*, *Glycine max*, pathogenicity

Introduction

The *Fusarium graminearum* species complex is composed of at least 16 lineages (O'Donnell *et al.*, 2000, 2004, 2008; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009; Sarver *et al.*, 2011), most of which have now been described as species. The species composition of the *F. graminearum* complex population appears to be host- and location-dependent. In Argentina, *F. graminearum sensu stricto* is the only phylogenetic species isolated from wheat in different subregions of the main wheat production area (Ramirez *et al.*, 2007; Alvarez *et al.*, 2011), while *F. meridionale* and *F. boothii* are the most important species isolated from maize in the northwest region (Sampietro *et al.*, 2011). With regards to soybean, *F. graminearum* was the dominant species isolated from seeds, pods and flowers in a field located in Córdoba Province, Argentina, followed by *F. meridionale* (Barros *et al.*, 2012).

The focus of studies on the *F. graminearum* species complex in Argentina has largely been directed to wheat and maize because these species produce fusarium head blight and ear or stalk rot, respectively. However, soybean represents the most important crop in this country with a planted area of 20.3 million ha and production of around 55.7 million tonnes during the 2013/14 growing season (ACSOJA, 2014). Moreover, crop rotation such as maize–soybean or soybean–wheat in combination with reduced-tillage and no-tillage are practices commonly used for producers in the Córdoba soybean growing area. In this way, members of the *F. graminearum* complex that colonize soybean debris could provide inoculum for wheat and maize infections, and those that colonize wheat and maize debris could provide inoculum for soybean infections.

Fusarium rot of soybeans is caused by a complex of species and several of them 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 (O'Donnell *et al.*, 2008). DON has a worldwide occurrence in cereal crops from both temperate and subtropical regions (Desjardins & Proctor, 2011). NIV also occurs in cereals but has been most commonly found in Asian countries, and at relatively lower levels in Europe, southern Africa and South America (Placinta *et al.*, 1999). Both mycotoxins have been associated with feed refusal, vomiting and suppressed immune functions, but NIV has a higher toxicity to humans and domestic animals than DON (Pestka, 2010).

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Members of the *F. graminearum* complex usually express one of three main trichothecene metabolites, either: (i) nivalenol and its acetylated derivatives (NIV chemotype); (ii) deoxynivalenol and 3-acetyldeoxynivalenol (3-ADON chemotype); or (iii) deoxynivalenol and 15-acetyldeoxynivalenol (15-ADON chemotype). At present *F. graminearum* is recognized as a primary pathogen of soybean in several countries in the Americas, mainly causing seed and root rot and pre- and post-emergence damping off (Martinelli *et al.*, 2004; Xue *et al.*, 2006, 2007; Broders *et al.*, 2007; Ellis *et al.*, 2011; Diaz Arias *et al.*, 2013). In Argentina, Pioli *et al.* (2004) were the first to describe a pathogenic association between *F. graminearum* and soybean. Later, Barros *et al.* (2014) demonstrated that different phylogenetic species within the *F. graminearum* species complex such as *F. graminearum*, *F. meridionale* and *F. cortaderiae* were pathogenic to soybean seedlings under controlled conditions. However, no information about infections at later growth stages of soybean development and pathogenicity of the *F. graminearum* species complex is available. Moreover, the pathogenicity of isolates with different mycotoxin chemotypes on soybean and the role that mycotoxins play in the virulence of strains of *F. graminearum* complex toward soybean have not yet been evaluated. The objective of this study was to compare the pathogenicity of *F. graminearum* and *F. meridionale* isolates towards soybean under field conditions and to evaluate the variation in aggressiveness and trichothecene production from isolates of the two phylogenetic species expressing different chemotypes.

Materials and methods

Fungal isolates

Four isolates representing the two species of *F. graminearum* complex most frequently isolated from soybean in Córdoba Province (Argentina) were included in this study: two *F. graminearum* DON-15 ADON producers (F5050, F5051) and two *F. meridionale* DON-NIV producers (F5030, F5043). These genotypes/chemotypes were the only observed among the species of *F. graminearum* complex isolated from soybean in Argentina. These isolates were characterized in previous studies using AFLP markers and by sequencing of the translation elongation factor 1α (*TEF-1α*; Barros *et al.*, 2012, 2014). Moreover, two reference strains, *F. graminearum* GZ3639 (DON-15 ADON producer) and *F. meridionale* GZ11367 (NIV producer) isolated from wheat in the USA were included in this study. All the isolates were maintained as cultures on Spezieller Nährstoffarmer Agar (SNA; Nirenberg, 1976) slants at 4 °C and as spore suspensions in 15% glycerol (w/v) at −80 °C. Chemotype confirmation of isolates was tested prior to the field experiments.

Pathogenicity assays

The field trials were conducted during the 2012/13 and 2013/14 growing seasons in experimental plots at the Universidad Nacional de Río Cuarto, Córdoba, Argentina. The soybean cultivar used, Nidera A 5009 RG, is genetically modified for tolerance to glyphosate (maturity group IV) and is one of the most commonly planted by growers in Córdoba Province. The planting dates were 2 December 2012 and 24 November 2013, in plots with 0.75 m row spacing under no-till conditions. Each plot consisted of three rows 2 m in length, and the inoculum was applied in the middle of the rows. Three replicate rows per isolate were established in a randomized complete block design.

The inoculum of each species was prepared by growing each isolate on liquid SNA medium and incubated on an orbital shaker (150 rpm) for at least 15 days at 25 ± 1 °C. The conidial suspension was filtered through three layers of cheesecloth to reduce the amount of mycelial fragments. The filtered macroconidial concentration was determined in a Neubauer chamber under an optical microscope and diluted to obtain 10^3 spores mL^{-1}.

At the beginning of seed formation (R5 stage), pods were inoculated with the isolates by injecting 10 μL of a spore suspension containing 10^3 conidia mL^{-1}. To determine if the fungus could invade beyond the inoculation point, the pods were inoculated on the mid-lateral surface into a central carpal containing a developing seed. Plants inoculated with sterile water were included as negative controls. At the end of full maturity (R8 stage), plants were removed from each row by hand and transported to the laboratory for more accurate and detailed studies. From each treatment, all seeded pods were collected and 100 pods/replicate were randomly selected (300 pods/treatment) and used for further analysis. The seeded pods were arranged with the peduncle of the pod pointing downward, opened along the suture, and the half-carpel on the left-hand side was discarded. Pods were visually assessed for pod blight severity. Symptoms were rated using a 0–4 scale according to the approximate proportion of pod covered with necrotic lesions: 0, no visible disease symptoms; 1, less than ⅓; 2, less than ⅔; 3, more than ⅔; and 4, completely necrotic pod. Seeds obtained from each treatment were assessed for percentage of seed infected in the pods and kernel weight reduction (%). For this last point, the yield in the control treatment was considered as 100% of weight and the kernel weight of each treatment was calculated as yield reduction. Ten pods with symptoms and seeds by treatment were transferred to Petri dishes containing SNA medium and identified by morphology as members of the *F. graminearum* complex according to Leslie & Summerell (2006).

Mean temperature and precipitation during the period from R5 to R8 stages were recorded for each growing season. Data were obtained from Agro-meteorology Department, Agronomy Faculty, UNRC (Table 1).

Mycotoxin analysis in soybean seeds

Seeds obtained from each treatment were assessed for DON and NIV contamination by HPLC analysis. The analysis was performed using the method described by Barros *et al.* (2008) with

Table 1 Environmental conditions during R5 to R8 soybean stages in 2013 and 2014 seasons

Year	Month	T_{max} (°C)	T_{mean} (°C)	T_{min} (°C)	Rainfall (mm)
2013	January	16.3	23.4	31.6	76
	February	15.4	21.9	29.6	64
	March	12.0	18.1	25.8	101
	April	11.2	17.9	26.1	43
2014	January	16.6	24.1	32.0	126
	February	15.8	20.5	27.3	167
	March	12.1	18.0	25.4	56
	April	10.1	15.8	22.5	33

some modifications. Grains were finely ground in a laboratory grinder and homogenized. A subsample (25 g) was extracted by mixing with 100 mL acetonitrile:water (84:16, v/v), shaken for 30 min in an oscillatory shaker and then filtered through Whatman no. 4 filter paper. Clean-up was carried out with a Mycosep 227 column (Romer Labs Inc.). The filtrate (8 mL) was transferred to a culture tube and slowly pressed into the interior of the tube with the rubber flange end turned down until 6 mL of the extract had passed through the column. Then, 4 mL of the purified extract was transferred to a vial and evaporated to dryness under nitrogen at 60 °C. The dried residue was redissolved in 400 μL water:methanol (88:12, v/v), homogenized in a vortex mixer and injected into the HPLC system (Hewlett Packard model 1100 pump; Rheodyne manual injector with a 50 μL loop). Chromatographic separations were performed on a stainless steel, C_{18} reversed-phase column (150 × 4.6 mm i.d., 5 μm particle size; Luna-Phenomenex). 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:noise ratio of 3:1.

Statistical analysis

Data on pod blight severity and mycotoxin levels were often not normally distributed and were analysed by using the nonparametric Kruskal–Wallis test and then by Dunn's nonparametric multiple comparisons test at a probability level of $P < 0.05$. For the remaining dependent variables (seed infection and kernel weight reduction) the percentage data were arcsine transformed and subjected to an analysis of variance (ANOVA). Treatment means of the transformed and untransformed data were separated by Fisher's least significant difference test at $P < 0.05$ (InfoStat, 2008).

Results

During the 2012/13 field trial, significant differences in pod blight severity ($P < 0.05$) were observed among the isolates and the control treatment, except for the reference strain *F. meridionale* GZ11367. However, no significant variation was observed among *F. graminearum* and *F. meridionale* isolates, which showed a mean disease severity averaged across all isolates of around 1.7. In general, all the isolates were moderately pathogenic and the main symptom observed was pod bleaching in about 60% of pods. The analysis of seeds obtained from these pods showed that the percentages of soybean seed infected by *F. graminearum* and *F. meridionale* were significantly different ($P < 0.05$) among the isolates and the control treatment, with isolate *F. meridionale* F5030 showing the highest infection percentage. *Fusarium meridionale* F5030 and F5043 showed significant reduction in kernel weight compared to the control treatment, while the *F. graminearum* isolates and *F. meridionale* GZ11367 did not cause reduction compared to the control (Table 2). Trichothecene accumulation on harvested seed with symptoms showed that the three *F. graminearum* isolates with 15-ADON chemotype produced detectable levels of both DON and 15-ADON. Surprisingly, no trichothecenes were detected in seeds recovered from pods inoculated with *F. meridionale* isolates (Table 3), although all isolates produced detectable amounts, mainly of NIV, under *in vitro* conditions. No trichothecene occurrence was detected in seeds recovered from control treatments.

Regarding the pod blight severity during the 2013/14 field trial, all isolates caused symptoms in pods. Significant differences ($P > 0.05$) in disease severity were observed among *F. graminearum* and *F. meridionale* isolates, with *F. graminearum* F5051 and *F. meridionale* F5043 and GZ11367 being the most aggressive. The mean disease severity among the isolates was higher than that observed during the 2012/13 growing season (mean 2.3; Table 2). The increase in the pathogenicity of the strains tested was evident compared to the previous year, and many of the isolates were able to colonize the inside of the pods, showing signs of blighting and bleaching. A high percentage of seeds that showed infection near to the site of injection were not able to develop or showed

Table 2 Variation among isolates of *Fusarium graminearum* and *F. meridionale* in relation to the growth parameters evaluated in the pathogenicity assays

	Season 2012/13			Season 2013/14		
Isolate	Disease severity	Kernel weight reduction (%)	Seeds infected within the pod (%)	Disease severity	Kernel weight reduction (%)	Seeds infected within the pod (%)
F. graminearum						
F5050	1.8 b[a]	4.3 a	20.6 ab	1.7 b	30.5 c	36.3 b
F5051	1.6 b	0.0 a	10.3 ab	2.7 d	41.9 c	58.6 b
GZ3639	1.8 b	4.0 a	18.0 ab	2.2 c	28.5 c	61.6 b
F. meridionale						
F5030	2.1 b	17.8 b	46.6 b	2.2 c	14.0 b	48.3 b
F5043	2.0 b	10.6 b	23.3 ab	2.6 d	35.5 c	69.0 b
GZ11367	1.2 a	4.0 a	13.3 ab	2.5 d	31.8 c	72.6 b
Control	1.0 a	0.0 a	2.0 a	1.2 a	0.0 a	4.8 a

[a]Within a column, values followed by a different letter are significantly different ($P < 0.05$) by LSD.

Table 3 Trichothecene production on soybean seeds obtained from pods inoculated with *Fusarium graminearum* and *F. meridionale* in two growing seasons

	Season 2012/13			Season 2013/14		
Isolate	DON	15-ADON	NIV	DON	15-ADON	NIV
F. graminearum						
F5050	6.8 ± 1.7 c[a]	+	ND[b]	8.0 ± 0.4 c	+	ND
F5051	3.1 ± 0.9 b	+	ND	1.6 ± 0.8 b	+	ND
GZ3639	6.9 ± 1.5 c	+	ND	0.5 ± 0.2 a	+	ND
F. meridionale						
F5030	ND	ND	ND	ND	ND	ND
F5043	ND	ND	ND	ND	ND	ND
GZ11367	ND	ND	ND	ND	ND	ND
Control	ND	ND	ND	ND	ND	ND

[a]Within a column, values followed by a different letter are significantly different ($P < 0.05$) by LSD.
[b]ND, not detected; <0.1 μg g^{-1} for DON and 0.2 μg g^{-1} for NIV.

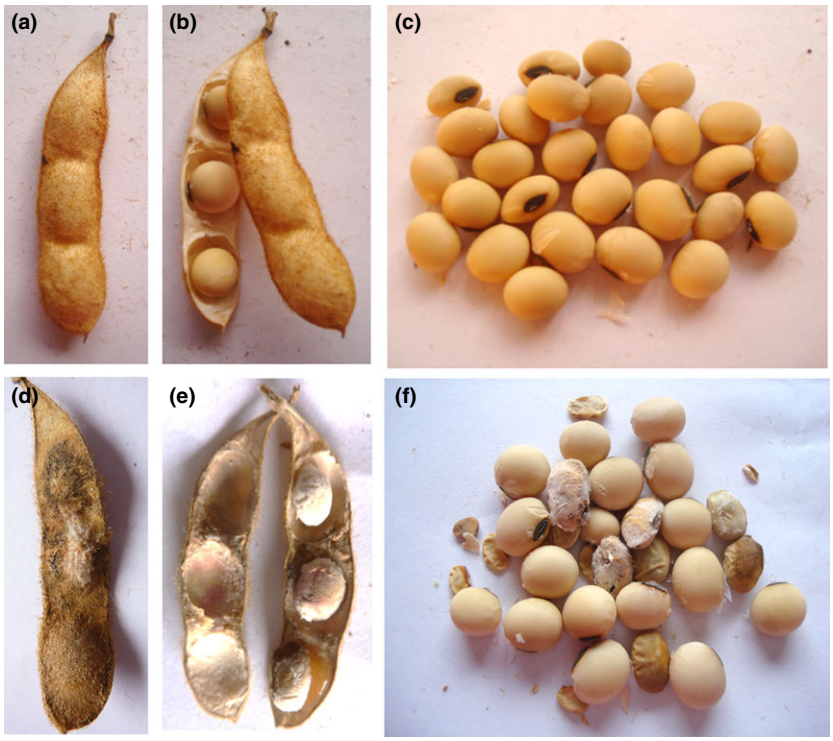

Figure 1 External (a) and internal (b) symptomless pods and symptomless seeds (c) from plants inoculated with sterile water. Symptoms caused by *Fusarium graminearum* F5051 isolate in pod showing tan to brown lesion (d), colonization of the entire interior of the pods (e) and seeds affected in varying degrees from inoculated pods (f).

a reddish discolouration, but many seeds distal to this area were heavily colonized by fungal hyphae (Fig. 1). All *F. graminearum* and *F. meridionale* isolates significantly reduced kernel weight ($P < 0.05$) and increased seed infection relative to the control plants; however, no significant differences were observed among the isolates of each species evaluated. Although difference in the disease severity was observed between seasons, the data on trichothecene contamination were similar. The *F. graminearum* isolates produced DON and 15-ADON contamination in seeds harvested at the R8 stage. *Fusarium meridionale* isolates with DON/NIV and NIV chemotypes were unable to produce detectable mycotoxin levels under the conditions evaluated. Neither DON nor NIV was detected in seeds recovered from the control treatments (Table 3).

Discussion

Members of the *F. graminearum* species complex are important pathogens, mainly of wheat and maize, in the subtropical and temperate regions of Argentina. Numerous studies have evaluated the populations within this species complex isolated from wheat and maize, focusing on species diversity and distribution, trichothecene genotype/chemotype, genetic diversity and molecular

characterization (Chulze *et al.*, 1996; Ramirez *et al.*, 2007; Fernandez Pinto *et al.*, 2008; Alvarez *et al.*, 2011; Sampietro *et al.*, 2011). Rotation of wheat–soybean or maize–soybean is a common practice in Argentina, yet the *F. graminearum* species complex isolated from soybean has been scarcely studied in comparison. Only two previous studies looking at a pathogenic association between these species and soybean plants, mainly related to root rot, have been undertaken (Pioli *et al.*, 2004; Barros *et al.*, 2014). The present work provides new data on the pathogenicity of the *F. graminearum* species complex on soybean pods.

In a previous study, a higher isolation frequency of species within the *F. graminearum* complex was observed on soybean pods than on seeds, which may suggest a certain inability of these strains to penetrate the seeds (Barros *et al.*, 2012). However, the results obtained in the present study suggest that both *F. graminearum* and *F. meridionale* were moderately pathogenic on soybean seeds when they were inoculated by injection. Infection by both species resulted in pod blight, with variation among and within species. Furthermore, a higher percentage of infected seed and kernel weight reduction compared with the control was observed. The same isolates also caused root rot on soybean seedlings under controlled conditions in a previous study (Barros *et al.*, 2014). Therefore, these findings could indicate that *F. graminearum* and *F. meridionale* are pathogenic on different soybean tissues and their effect is higher when the natural barrier is damaged. It has been suggested that different penetration strategies can be used by these pathogens when infecting different tissues and host species (Martinelli *et al.*, 2004; Peraldi *et al.*, 2011; Kazan *et al.*, 2012).

The higher species pathogenicity was not associated with DON contamination in seeds. Ecological conditions that influence the species growth are different from those that allow optimum DON production. The higher rainfall, high humidity and prolonged dew during grain ripening registered during the R5 and R8 soybean stages in the 2013/14 growing season were apparently a more favourable environment for fungal infection compared to the 2012/13 growing season, leading to an increase of symptoms on pod blight and a higher soybean seed infection and kernel weight reduction. However, data on trichothecene contamination in soybean seeds were similar in both evaluated seasons, and lower during the most humid year. These results could be explained by the fact that the mean environmental temperatures were similar in both years (20 °C), and these temperatures were near to the optimal for DON production by *F. graminearum* in soybean (Garcia *et al.*, 2012), despite differences in rainfall. This result agrees with previous findings obtained in wheat pathogenicity assays (Walker *et al.*, 2001; Alvarez *et al.*, 2010).

The differences in aggressiveness observed between the seasons was not reflected in distinct NIV content in soybean seeds at the maturity stage. Even though *F. meridionale* isolates demonstrated similar aggressiveness to *F. graminearum* isolates, none of them was able to produce trichothecene *in planta* during the two field trials. The data obtained suggest that the trichothecene production may not be necessary for development of soybean pod disease. Martinelli *et al.* (2004) demonstrated that Brazilian soybean isolates of the *F. graminearum* species complex were able to produce trichothecene during soybean plant infection at low levels, while high levels were produced during wheat infection. Similar results were obtained by Sella *et al.* (2014) in soybean seedlings, who found that DON contributed to rot symptoms but the disease development seemed to be reduced in comparison to wheat spike. These studies showed that the protein kinase (OS-2) and MAP kinase (Gpmk1) of *F. graminearum* are essential factors for the progress of infection and symptom development in soybean and the OS-2 MAPK also plays an important role, providing resistance to the soybean phytoalexins.

This is the first study to characterize the aggressiveness of the *F. graminearum* species complex with different mycotoxin chemotypes and its relation to the trichothecene production in soybean pods. Further studies using trichothecene nonproducing mutants under greenhouse conditions are in progress and will be relevant to confirm the role of trichothecenes in the aggressiveness of *F. graminearum* and *F. meridionale* on soybean.

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