Genetic diversity and pathogenicity on root seedlings from three soybean cultivars of *Fusarium graminearum* isolated from maize crop residues

Diversidad genética y patogenicidad a nivel de raíz en plántulas de soja de tres cultivares de cepas de *Fusarium graminearum* aisladas de rastrojos de maíz

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Abstract

Crops residues are an important source of maintenance of *Fusarium graminearum* inoculum in the soybean agroecosystem. Given that these populations can interact in the substrate through mechanisms of mycelial recognition and that they can come into direct contact with the implanted seed and cause disease, the following objectives were set: (1) to evaluate the genetic diversity through of the mycelial compatibility of *F. graminearum* strains isolated from maize crop residues; (2) to analyze the pathogenicity of *F. graminearum* strains isolated from crop residues towards soybean seedlings from different cultivars treated and untreated with fungicide. Mycelial compatibility studies showed a unique pattern of mycelial compatibility for each strain, indicating a great heterogeneity in the population evaluated. Pathogenicity tests in all strains tested were capable of causing symptoms of root rot with varying degrees of severity and reductions in the height of seedlings. In the factorial statistical analysis, the greatest effect was marked by the soybean cultivar effect. A clear decline in the severity index was also observed with the fungicide application, so this would be a useful prevention tool to reduce the intensity in soybean seedling diseases.

Keywords

Fusarium graminearum • crop residues • soybean • fungicide • pathogenicity • mycelial compatibility

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Resumen

Los rastrojos de cultivos antecesores son una fuente importante de mantenimiento del inóculo de *Fusarium graminearum* en el agroecosistema de la soja. Teniendo en cuenta que estas poblaciones pueden interactuar en el sustrato a través de mecanismos de reconocimiento micelial y que las mismas pueden entrar en contacto directo con la semilla implantada y causar enfermedad, se plantearon los siguientes objetivos: (1) Evaluar la diversidad genética a través de la compatibilidad micelial en cepas de *F. graminearum* aisladas de rastrojos de maíz; (2) Analizar la patogenicidad de cepas de *F. graminearum* aisladas de rastrojos respecto de la podredumbre de raíz en plántulas de distintos cultivares de soja tratadas y no tratadas con fungicida curasemillas. Los estudios de compatibilidad micelial mostraron un único patrón de compatibilidad micelial para cada cepa, indicando una gran heterogeneidad en la población evaluada. En los ensayos de patogenicidad todas las cepas evaluadas fueron capaces de provocar síntomas de podredumbre de la raíz con distintos grados de severidad y reducciones en la altura de plántulas. El análisis estadístico factorial demostró que el mayor efecto observado en las variables independientes estuvo marcado por el efecto del cultivar de soja evaluado. También se observó una clara disminución en el índice de severidad con la aplicación de un fungicida curasemilla, por lo que esta sería una herramienta útil de prevención para disminuir la intensidad en las enfermedades de plántulas de soja.

Palabras Clave

Fusarium graminearum • rastrojos • soja • fungicida • patogenicidad • compatibilidad micelial

Introduction

Fusarium is a cosmopolitan fungal genus that includes agronomically important plant pathogens. Among them, most relevant pathogens are within four species complex: *F. fujikuroi*, *F. oxysporum*, *F. solani* and *F. graminearum* (5). The *F. graminearum* species complex is composed of at least sixteen lineages separate structural and biogeographically, however previous work on soybean in Argentina have shown that *F. graminearum sensu stricto* is the predominant species (6, 10, 11).

In Argentina, more than 90% of soybean is grown using conservative tillage practices, involving reduced tillage or no-tillage in order to reduce erosion and maintain soil moisture, conserve energy, reduce costs and increase crop yields (21).

As consequence, a greater amount of crop residue on the soil surface after harvest creates a favorable environment for maintaining high inoculum levels of *F. graminearum* which survives in saprophytic way between hosts. During favorable conditions, *F. graminearum* produces perithecia with ascospores (sexual spores) which are expelled and dispersed over long distances by air movements (29) and together with conidia (asexual spores) are the primary sources of inoculum (Shaner, 2003). The conidia are produced in large quantities on the crop residues spreading through the rain, and thus arrive at hosts in which produce disease, mycotoxins, or both (28). According to Chiotta *et al.* (2015a), the crops residues would be the

main source of *F. graminearum* inoculum in soybean agroecosystem compare to the surrounding air crop.

Several studies have analyzed the genetic diversity of *F. graminearum* populations isolated from grains both wheat and soybean (3, 6, 26, 27); however this diversity has not yet been evaluated in populations isolated from crop residues. One approach that has been used successfully in many filamentous fungi to examine the population structure, clonality and potential gene flow is the study of vegetative incompatibility. Mycelial incompatibility assay at macroscopic level is a simple and reliable way to indicate a reaction of self or non-self-recognition and has been used in several fungal genera. Thus, the ability of strains from crop residues to recombine poses the danger of introducing virulence and/or toxigenic genes intolocal pathogen populations (16). Assessing genotypic diversity is therefore important to align plan protection to the existing and potentially changing pathogen population, especially where sexual reproduction play a role in the pathogen life cycle. An understanding of the genetic structure of *F. graminaerum* populations may provide insights into their epidemiology and evolutionary potential and may lead to improved strategies for their management (1).

The focus of studies on *F. graminearum* species complex in Argentina largely has been directed to wheat and maize since these species produce Fusarium head blight and ear or stalk rot, respectively (8). In soybean, reports about the pathogenicity of *F. graminearum* strains have been contradictory. In some cases, these strains were considered to be secondary colonizers of soybean seeds previously damaged by other fungi or by freezing (15, 22). At present different investigations in the America carried out in Brazil (18), United States (9, 12, 14) and Argentina (7, 25) have recognized to *F. graminearum* as a primary pathogen of soybean. Considering that the crop residues are the main source of maintenance of *F. graminearum* inoculum in the soybean agroecosystem, these populations can interact in the substrate and that they may can come into direct contact with the implanted seed and cause disease. For this reason, the active ingredients used in seed-treatment fungicides would be an important strategy to prevent diseases in soybean seedlings. Of these fungicides, fludioxonil was the only one that inhibited mycelial growth (9).

The objective of the present study was: to assess the genetic diversity through the mycelial compatibility of *F. graminearum* strains isolated from maize crop residues; and to analyze the pathogenicity of *F. graminearum* strains toward soybean seedlings from different soybean cultivars treated and untreated with fungicide.

Materials y methods

Evaluation of mycelial compatibility in *F. graminearum* strains from maize crop residues

Mycelial compatibility testing of 50 *F. graminearum* strains isolated from maize crop residues in a previous study in Cordoba Province (Argentina) (10), was carried out following the methodology described by Akinsanmi *et al.* (2008). The mycelial compatibility was evaluated on V8-soybean medium (150 ml V8 juice (Campbell's, UK), 20g agar, 20g of ground soybean seed and distilled water to a liter). A maximum of nine mycelial plugs of the isolates were placed equidistant from one another on agar medium in a single 90-mm Petri dishand incubated at 25°C for 7 days.

Two replicates of each isolate were paired with every other isolate in all possible combinations and themselves in a repeated experiment. A compatible reaction was indicated by mycelium continuity between the interacting colonies without a zone line, while an incompatible reaction was evidenced by the formation of a barrier between the interacting isolates (Figure 1a and 1b).

Mycelial reactions for each strain were recorded in a binary matrix where 1 represented a compatible reaction and 0 an incompatible one. Compatibility was scored 1 because it requires a higher level of matching between mycelial compatibility than incompatibility, which can occur from differences at a few loci. The mycelial compatibility group data were transported to obtain a complete matrix.

The genetic similarity was estimated using the SinQual program (NTSYS.pc version 2.01), taking into account all pairs of strains tested according to Dice coefficient. The dendrogram of genetic similarity was constructed using the method of arithmetic means UPGMA and comparison program NTSYS matrices (NTSYS.pc version 2.01).

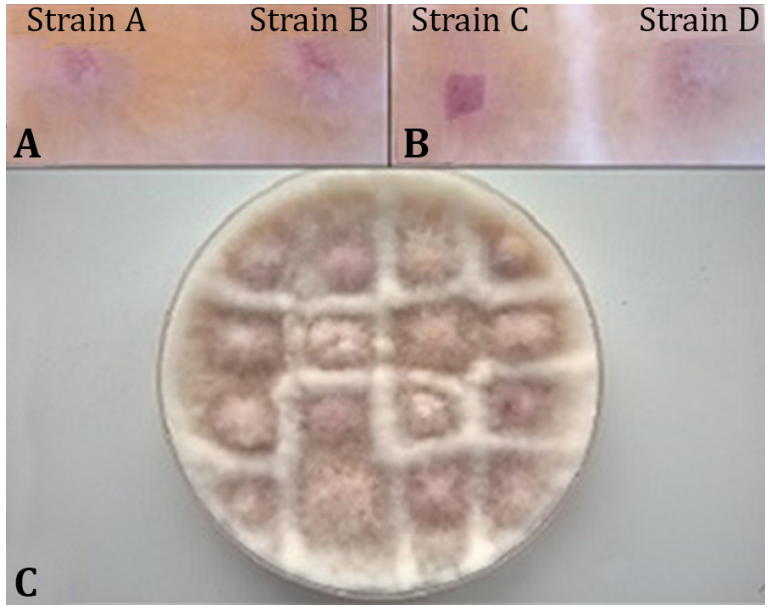


Figure 1. Compatibility (A) and incompatibility (B) reactions between *Fusarium graminearum* strains on V8-soybean medium. Multiple crosses among different *Fusarium graminearum* strains in V8-soybean plate (C).

Figura 1. Reacciones de compatibilidad (A) e incompatibilidad (B) entre cepas de *Fusarium graminearum* sobre medio V8-soja. Placa de medio V8-soja donde se observan múltiples cruzamientos entre distintas cepas de *Fusarium graminearum* (C).

Pathogenicity assays

Out of 50 *F. graminearum* strains included in the compatibility analysis we arbitrary selected 10 strains to perform the pathogenicity tests. Two *F. meridionale* strains (F5043 y F5048) were included as positive control since were highly aggressive in a previous study on soybean seedlings (6). Three soybean cultivars were used in the present study: Nidera 5009, Nidera 5258 and Don Mario 5.1 i. The evaluation of seedlings height and disease severity was performed in a rolled-towel assay described by Xue *et al.* (2007). Soybean seeds were surface disinfected by soaking in 0.5% (v/v) NaOCl for 30 sec and rinsed twice in sterile distilled water.

Then disinfected seeds were spread evenly on two layers of sterilized paper towels moistened with 50 ml of sterilized water and covered with two layers of paper towels in plastic containers to allow the germination. Containers were covered with plastic lids and kept in darkness at 24°C for 3-4 days until plants were at the early VE growth stage and root hairs were visible. Visually healthy seedlings were selected and disinfected as described above. Seedlings were infested by transferring agar plugs (5 mm in diameter) with a sterilized metal needle from 7-day-old fungal cultures to the root-hair zone, about 1.5 cm behind the primary-root tip.

Control plants were inoculated with agar plugs from sterile PDA. Inoculated plants were each placed vertically on precut covering sheets consisting of two layers of the sterilized paper tissue laid on top of an aluminum foil sheet. The entire root along with the attached agar disc of an individual plant was then covered by folding the covering sheets in the middle. A growth unit for each plant was formed by further folding the covering sheets on the opening side.

The growth units were placed in plastic trays containing sterilized distilled water at a depth of 0.5 cm. The open top of the growth unit allowed for plant growth and the open bottom for root development and water absorption. The aluminum foil sheet was used to separate each unit and for moisture retention. The trays were placed in a growth chamber operated at 24°C with a 16 hour photoperiod of fluorescent light.

The water level in the tray was checked daily and the water was added as needed. Ten days after inoculation, plants were removed from the growth unit and visually assessed for root-rot severity. Symptoms were rated using a 0-4 scale (figure 2, page 152): 0, no visible disease symptoms; 1, lesion visible, but infection confined to the inoculation site, with normal seedling growth; 2, lesion size extended and the plant growth retarded; 3, infection of the entire root, and the plant growth halted; and 4, massive infection of the entire root resulting in plant death.

The experiments were repeated twice in independent way. In seedlings subjected to fungicide treatment, the seeds from the three cultivars were not superficially disinfected and were directly treated with the fungicide prior to inoculation of the fungal isolate using the above methodology for the untreated seed with fungicide.

The fungicide used was MAXIM®XL (Syngenta, Brazil) containing Fludioxonil (4- (2,2-difluoro-1,3-benzodioxol-4-yl) -1H-pyrrole-3-carbonitrile) and metalaxyl - M (N- (2,6-dimethylphenyl) -N- (2'-methoxyacetyl) alanine methyl ester-D) as active agents. The application was performed according to the manufacturer's recommendation: per 100 g of seeds, 0.15 ml of fungicide and 0.35 ml of water were added.

0, no visible disease symptoms; 1, lesion visible, but infection confined to the inoculation site with normal seedling growth; 2, lesion size extended and plant growth retarded; 3, infection of the entire root, and plant growth halted; 4, massive infection of the entire root resulting in plant death.

0, sin síntomas visibles de la enfermedad; 1, lesión visible, pero con infección confinada al sitio de la inoculación y crecimiento normal de plántula; 2, tamaño de la lesión extendida y crecimiento de planta retardado; 3, infección de toda la raíz, se detuvo el crecimiento de planta; 4, la infección masiva de toda la raíz, que resulta en muerte de la planta.

Figure 2. Ordinal scale used to evaluate disease severity.

Figura 2. Escala ordinal utilizada para evaluar la severidad de la enfermedad.

Statistical analysis

Data from the two independent pathogenicity experiments were combined and analyzed as one.

Dependent variables seedling height and disease severity were subjected to an analysis of variance (ANOVA) and treatment means were separated by Fisher's least significant difference (LSD) test at a probability level of $P<0.05$ using the INFOSTAT software version 2012 (13). For disease severity, multifactorial analyses across soybean cultivar, isolates, fungicide treatment and their interactions were subjected to an analysis of variance (ANOVA)(13).

Results and discussion

During the 1990s, agriculture changed significantly in Argentina through the adoption of transgenic crops such as soybeans, maize and cotton under no-tillage system (23).

The adoption of this type of conservation tillage was a major change affecting the *F. graminearum* populations that can colonize easily crop residues of wheat, maize and soybeans (10, 24). Soybean is often part of a succession using wheat and other cereal crops in Argentina, therefore, *F. graminearum* strains colonizing soybean residues could provide the primary inoculum for infections wheat and maize and *vice versa*. For this reason, it was considered analyzing genetic diversity and pathogenicity of *F. graminearum* strains that colonize crop residues.

In filamentous fungi, the ability of self-recognition is essential for vegetative growth, sexual reproduction and defense against invading pathogens. Thus, the mycelial compatibility test can serve as a tool to measure the ability of self-recognition and in this study we used that tool to assess the genetic diversity and the potential of different genotypes to interact in the crop residues. A total of 1275 pairings were performed, representing all possible combinations between *F. graminearum* isolates, in which all isolates were self-compatible.

Regardless self-compatibility reactions, out of 1225 pairings between *F. graminearum*s trains, 104 were compatible representing 8.4% of crosses (figure 3, page 154).

In Argentina, similar results were found by assessing the diversity of *F. graminearum* populations from wheat and soybean using VCG analysis (Vegetative Compatibility Groups) (26) and neutral molecular markers $AFLP_s$(polymorphism amplified fragment length) (6), respectively. Other findings in the world have shown that none of *F. graminearum* populations are completely panmictic or clonal (2, 19, 32). Taking into account that the strains included in the mycelial compatibility analysis were isolated from maize crop residues recovered from a single field, the population showed a high degree of genetic diversity. This suggests that sexual recombination is probably the main factor affecting the genetic diversity of this population. Moreover, balancing selection between parasitic and saprophytic subpopulations can generate genetic variation.

Naef and Défago (20) compare the genetic diversity within a saprophytic field population from maize stubbles with a pathogenic population from wheat using SSR markers. The study found that genetic diversity of the saprophytic population was significantly higher than parasitic population. This result may partly explain the high genetic diversity found in this study considering the origin of our strains.

In the present study it was observed that mutually compatible isolates had very different patterns of compatibility with other isolates (figure 1c, page 150), and this fact suggest that many factors are involved in the control of compatibility and incompatibility reactions (2). However, we found the presence of "multi-compatible" strains (figure 3, page 154) that showed hyphal anastomosis with several others strains which could have implications for gene flow through somatic recombination, which could contribute to increase genetic diversity.

In the present study, the pathogenicity of 10 *F. graminearum* strains isolated from maize crop residues and 2 *F. meridionale* used in a previous study, were evaluatedunder controlled conditions in seedlings from three soybean cultivars widely sown by growers in the Cordoba Province. The pathogenicity of each strain was assessed by two parameters: seedling height and severity in terms of root rot.

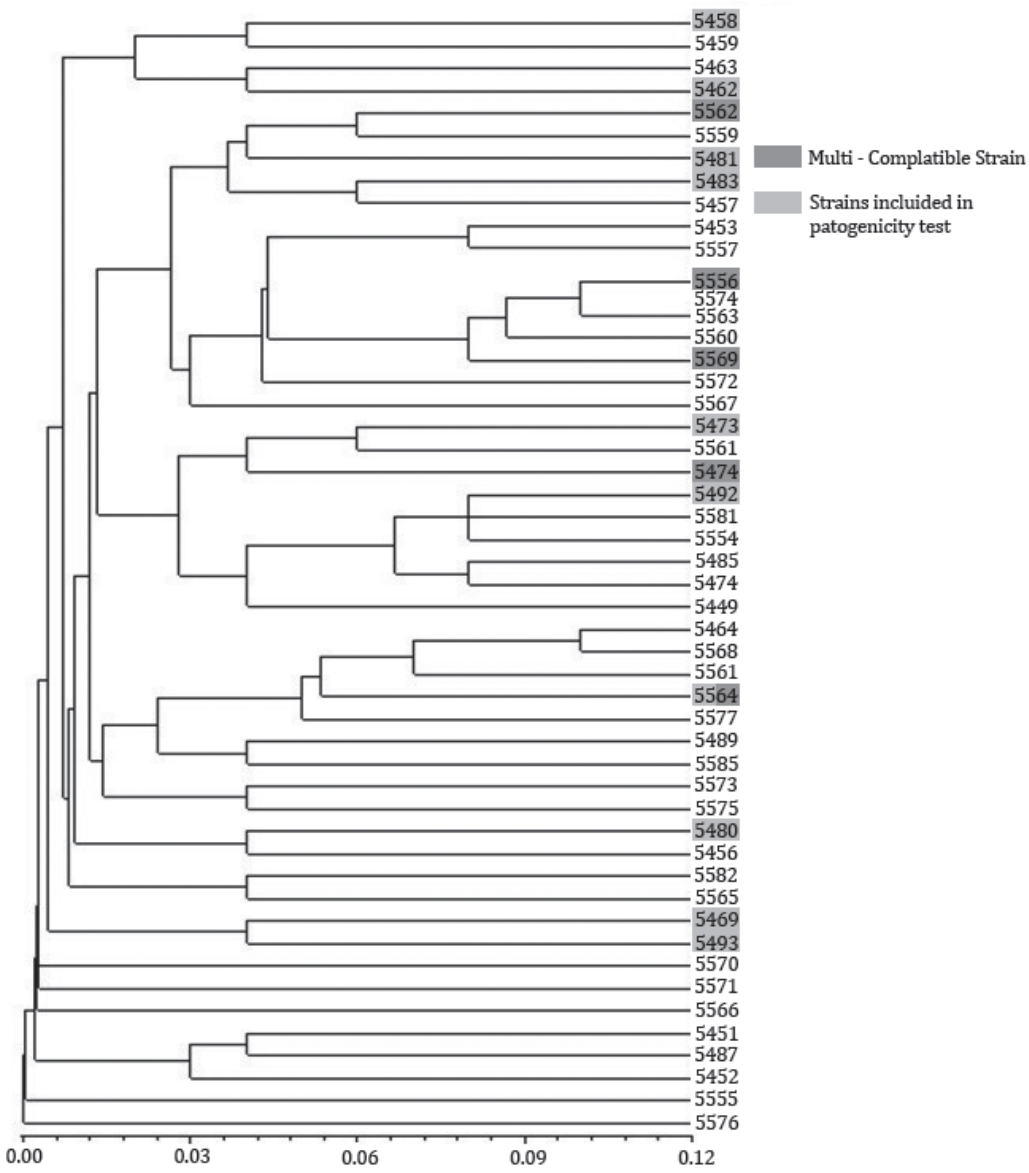


Figure 3. Cluster analyses of mycelial reactions of compatibility and incompatibility of the 50 *Fusarium graminearum* strains.

Figura 3. Análisis de *cluster* con base a las reacciones miceliales de compatibilidad/ incompatibilidad en las 50 cepas de *Fusarium graminearum.*

Regarding the seedling height, it was significantly reduced (P<0.05) by all strains compared to the control non-inoculated seedlings (table 1).

Table 1. Variation among *Fusarium graminearum* and *Fusarium meridionale* strains in relation to the growth parameters evaluated in the pathogenicity assays.

Tabla 1. Variabilidad en las cepas de *Fusarium graminearum* y *Fusarium meridionale* analizadas respecto de las variables altura de plántula y severidad de la enfermedad.

Isolate	Seedling Height (cm)[a]	Severity[b]
F. graminearum 5458	13,0c	1,7c
F. graminearum 5462	14,7d	1,6c
F. graminearum 5464	14,9d	1,3c
F. graminearum 5469	12,4c	1,6c
F. graminearum 5473	9,3a	2,2d
F. graminearum 5480	10,4ab	2,4de
F. graminearum 5481	12,5c	1,9cd
F. graminearum 5483	10,1ab	2,3de
F. graminearum 5492	11,8c	1,9cd
F. graminearum 5493	14,4d	1,2b
F. meridionale 5043	9,61a	3,0e
F. meridionale 5048	10,0ab	2,9e
Control	19,6e	0a

[a] Within a column, values not sharing a common letter are significantly different (p < 0.05).
[b] Disease Severity (root rot): rated on a 0-4 scale described by Xue *et al.* (2007).

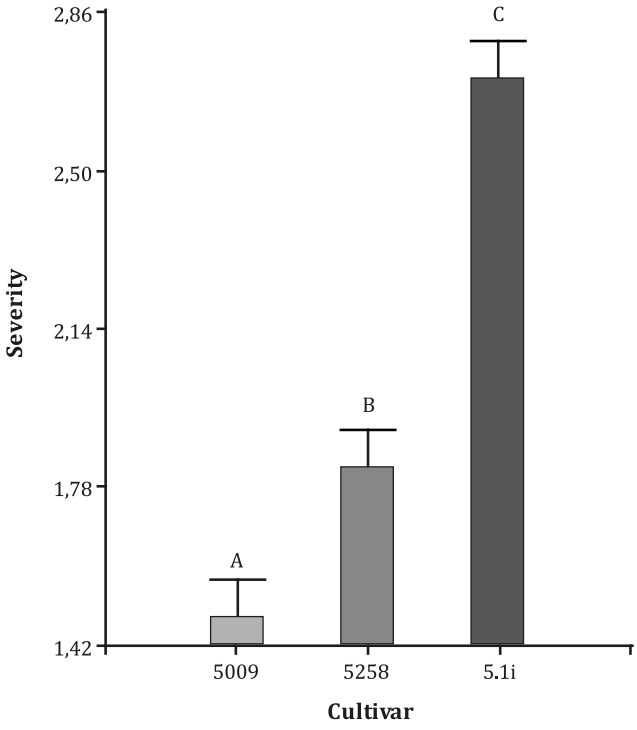
[a] Dentro de una columna, los valores que no comparten una letra común son significativamente diferentes (p < 0,05).
[b] Severidad de la enfermedad (podredumbre de raíz): escala de 0-4 descripta por Xue *et al.* (2007).

Reductions in the seedlings heights ranged from 24% to 52.5%. However, it was not observed in seedling height parameter significant differences among soybean cultivars and treatment with and without fungicides.

By the other hand, significant differences (P<0.001) were detected for cultivar, isolate, fungicide treatment and their interactions for disease severity. All genotypes tested showed symptoms of root rot with all the strains evaluated and were significantly different (P<0.05) respect to control treatment, in which non-inoculated seedlings remained healthy throughout the experience.

Don Mario 5.1i was the most susceptible cultivar, while the cultivar Nidera 5009 showed lower severity index with all strains tested (figure 4, page 156). These results are not coincidental with those obtained by Xue *et al.* (2007), who showed no differences in behavior for root rot in three soybean cultivars widely used in Canada. According to these authors, the lack of differences between cultivars is not compatible with the notion that *F. graminearum* is pathogen to soybean in a strict sense, coinciding with other authors who considered to this fungus secondary colonizer of soybean seeds previously damaged by other fungi or by freezing (15, 22). However, in the present work we observed not only significant differences between cultivars, but also in the multifactorial statistical analysis, the greatest effect was marked by the cultivar effect, with a value F twice that the fungicide treatment (table 2, page 156). The fact that *F. graminearum* generate differences in disease severity among cultivars makes us think that it could be a primary pathogen in roots of soybean seedlings as has been observed by other researchers in Brazil (18), United States (9, 12, 14) and Argentina (7, 25).

The strains included in the pathogenicity assay were scattered throughout the dendrogram (figure 3, page 154) and showed varying degrees of aggressiveness.



Bars showing different letters were significantly different (P<0.05).

Las barras que muestran letras diferentes fueron significativamente diferentes (P<0,05).

Figure 4. Differences in performance of the three soybean cultivars evaluated for the root rot severity.

Figura 4. Diferencias en el comportamiento de los tres cultivares evaluados respecto de la severidad en la podredumbre de raíz.

Table 2. Analyses of variance of the disease severity of soybean cultivars inoculated with *Fusarium graminearum* strains.

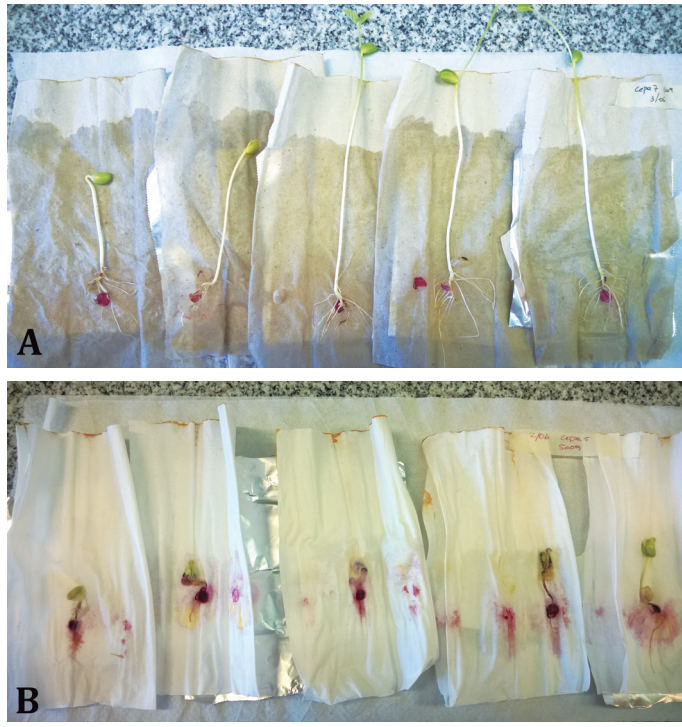
Tabla 2. Análisis de la varianza para la variable severidad en los tres cultivares inoculados con cepas de *Fusarium graminaerum*.

Source	Sum of quares	Freedom Degree	Mean squares	*F* value	*P* value
Fungicide	43.0	1	43.02	45.42	<0.0001
Cultivar	190.4	2	95.20	100.51	<0.0001
Isolate	221.4	11	20.13	21.25	<0.0001
Fungicide*Cultivar	126.2	22	63.11	66.63	<0.0001
Fungicide*Isolate	21.1	11	1.92	2.03	<0.0001
Cultivar*Isolate	95.1	22	4.32	4.56	<0.0001
Fungicide*Cultivar*Isolate	86.8	22	3.95	4.17	<0.0001
Error	613.8	648	0.95		
Total	1397.95	719			

In both experiments, it was found less aggressive strains such as *F. graminearum* F5464 and highly aggressive strains as *F. graminearum* F5480 (figure 5) and the two *F. meridionale* F5043 and F5048 evaluated in previous studies using Don Mario 4613 cultivar (7). However, in the multifactorial analysis it was observed that the isolate effect was significantly lower than the cultivar and fungicide treatment effect.

The mean disease severity averaged over the isolates evaluated in this study was approximately 2. This result indicates that the majority of the isolates, although showed low aggressiveness, produced a decrease in the seedling growth of that could influence the future crop production.

The value of mean disease severity found in the present study is similar to the average reported by Xue *et al*. (31) for Canadian wheat isolates evaluated on different soybean cultivars.



(A) low aggressive strain of *Fusarium graminearum* (5464): infection confined to the site of inoculation and normal growth of seedlings or slightly affected seedling; (B) aggressive strain of *Fusarium graminearum* (5480): highly affected plants, and dead plants with entire root, cotyledons and first pair leaves rot 10 days after inoculation.

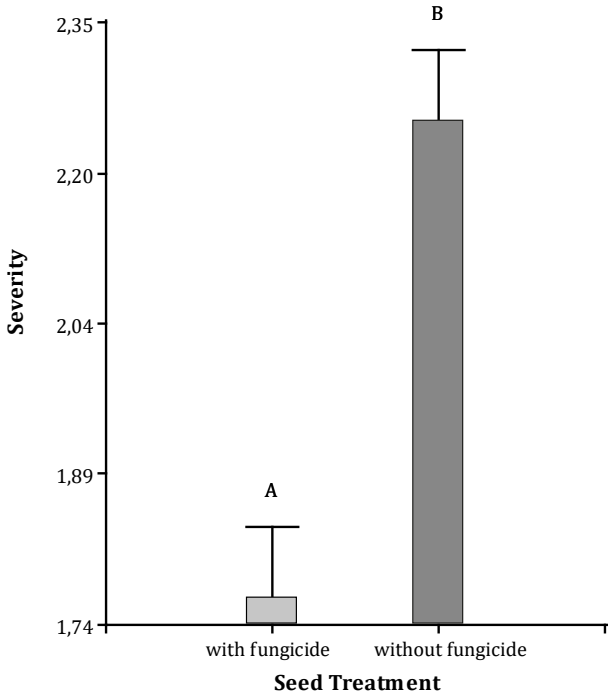
(A) cepa poco agresiva de *Fusarium graminearum* (5464): se observan plántulas con infección confinada al sitio de la inoculación y crecimiento de plántulas normales o levemente afectadas; (B) cepa muy agresiva de *Fusarium graminearum* (5480): se observan plántulas muy afectadas o muertas con podredumbre de toda la raíz, cotiledones y las primeras hojas, 10 días después de la inoculación.

Figure 5 Seedlings with different symptoms at the root level.

Figura 5 Las plántulas con diferentes síntomas a nivel de raíz.

The seed treatment with fungicides is the most common practice for handling problems related to soil-borne, seeds and seedlings pathogens, including pathogenic *Fusarium* species (17). In this study, fungicide treatment of seed was carried out using fludioxonil, the active ingredient recommended to protect soybean against fungi belonging to the *Fusarium* genus (28).

In the present study, disease severity was affected by fungicide treatment, showing higher severity levels the seedlings not treated with the fungicide (figure 6). Similar results were obtained by Broders *et al.* (9) and Ellis *et al*. (14) who tested 4 and 6 fungicides respectively, in the treatment of soybean in the US and found that fludioxonil was more effective fungicide in preventing damage by *F. graminearum*.



Bars showing different letters were significantly different (P<0.05).

Las barras que muestran letras diferentes fueron significativamente diferentes (P<0,05).

Figure 6. Assessment of disease severity according to seed treatment with and without fungicide treatment.

Figura 6. Evaluación de la severidad de acuerdo con el tratamiento de la semilla con y sin fungicida curasemilla.

Conclusions

Given that no-tillage system in wheat/soybean or maize/soybean rotations has allowed an increase in the primary inoculum of *F. graminearum*, the use of fludioxonil as the active ingredient could be seen as a prevention tool to reduce seedling diseases by an emerging potential pathogen of soybean in Argentina. So far four species within the *F. solani* complex (*F. virguliforme, F. tucumaniae, F. crassistipitatum* and *F. brasiliense*) are considered the most important pathogens

in causing sudden death syndrome and root rot of soybean Argentina (4). However, considering the present work, further studies are needed to determine the contribution of *F. graminearum* isolates in these soybean diseases in different agro-ecological zones showing high inoculum rates.

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