AGGRESSIVENESS OF *FUSARIUM* SECTION *LISEOLA* ISOLATES CAUSING MAIZE EAR ROT IN ARGENTINA

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SUMMARY

Developing resistance to species of *Fusarium* in maize (*Zea mays* L.) is important to prevent field mycotoxin contamination. Isolates representative of natural conditions need to be identified to maximize selection responses. Sixty isolates belonging to *Fusarium* section *Liseola* collected from a major maize growing region in Argentina were tested for sexual compatibility with eight standard tester strains (A-H) of the *Gibberella fujikuroi* complex. A twenty-nine isolate sub-sample (MAT-A: 26, MAT-E: 2 and MAT-D: 1) was tested for *in vitro* production of fumonisins and for aggressiveness to two maize hybrids after silk inoculation. Mating population A (*F. verticillioides*) was the most prevalent species (90%) coexisting with some isolates belonging to MAT-D (*F. proliferatum*) and MAT-E (*F. subglutinans*). Fumonisin production varied from 0.4 to 2884 $\mu g\ g^{-1}$ for MAT-A and from 0.3 to 0.6 $\mu g\ g^{-1}$ for MAT-E. The only isolate from MAT-D produced undetectable levels. Most isolates showed mild aggressiveness but two uncommon highly aggressive strains (MAT-A and D) were also identified. No associations between fumonisin production and disease severity were observed. Differences in disease severity between moderately resistant and susceptible hybrids varied across years and isolates suggesting that responses to selection might depend on the isolate used to produce the inoculum. The use of isolate mixtures might reduce genotype-by-isolate interaction although it would hinder identification of resistance to specific strains.

Keywords: maize, *Fusarium,* aggressiveness, *Gibberella fujikuroi* complex, fumonisins, host-pathogen interaction.

INTRODUCTION

Fusarium species belonging to section *Liseola* are common pathogens of maize. This group, whose members have teleomorphs in the *Gibberella fujikuroi* species complex, can be divided into at least eight biological species, also named mating populations (MAT-A to H). Mating population A, *F. verticillioides* (Saccardo) Nirenberg [(= *F. moniliforme* (Sheldon), teleomorph *Gibberella moniliformis* (Wineland) (= *G. fujikuroi* (Sawada Ito in Ito & Kimura, mating population A)] is the most prevalent maize (*Zea mays* L.) ear rotting pathogen in Argentina (Chulze *et al.*, 1996). *Fusarium proliferatum* (Matsushima) Nirenberg (= *G. fujikuroi* mating population D; teleomorph *G. intermedia* (Kuhlman)) and E (*F. subglutinans* (Wollenweber and Reinking) Nelson, Toussoun and Marasas [*G. fujikuroi* mating population E; teleomorph *G. subglutinans* (Edwards) Nelson, Toussoun and Marasas] have also been identified, although less commonly in some maize-growing regions of Argentina including Buenos Aires and the northern provinces (Chulze *et al.*, 1998; Reynoso *et al.*, 2004; Torres *et al.*, 2001).

These fungi cause loss in grain yield and affect grain quality due to contamination with mycotoxins. The most important mycotoxins in naturally infected maize are the fumonisins, mainly FB1, FB2 and FB3 (Chulze *et al.*, 1998; Shephard *et al.*, 1990). FB1 causes equine leukoencephalomalacia (Marasas *et al.*, 1988), liver cancer in rats (Voss *et al.*, 1990), pulmonary oedema in swine (Colvin and Harrison, 1992) and has often been found in regions with high frequencies of oesophageal cancer in humans (Rheeder *et al.*, 1992).

Mating populations vary in their ability to produce fumonisins. Isolates belonging to MAT-A and MAT-D when grown on maize-based media produced higher levels of fumonisins than those belonging to MAT- E (Leslie *et al.*, 1992).

One way to reduce mycotoxin levels in grain is to prevent fungal infections in the field by using less susceptible host plant genotypes. Since disease incidence and disease severity of *Fusarium* ear rot vary across years, selection based on inoculation experiments is preferred to that based on natural infection. According to Reid *et al.* (1993), an isolate or a mixture of isolates properly representing local fungal populations would closely model natural conditions and give adequate evaluation for disease resistance and fumonisin accumulation in grain. Information is needed on prevalence of mating popula-

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tions, fumonisin production and aggressiveness [i.e. the amount of disease induced by a pathogenic isolate on a susceptible host (Van der Plank, 1968)] of local populations of *Fusarium* section *Liseola* in Argentine maize-growing regions, to properly decide on the source of inoculum for selection. Previous research has suggested that fumonisins are not necessary or sufficient to cause maize ear rot and ear infection in maize (Desjardins *et al.*, 2002). Thus, measurement of fumonisin production *in vitro* in this study was made only to estimate the mycotoxigenic potential of the evaluated isolates.

The objective of this study was to assess the aggressiveness of a sub-sample of *Fusarium* section *Liseola* isolates differing in ability to produce fumonisin *in vitro* towards two maize host genotypes differing in disease resistance.

MATERIALS AND METHODS

Maize sampling. Fifty maize ears freshly harvested at random were collected in 2001/2002 from commercial hybrid trials in five locations from the north of the Province of Buenos Aires (9 de Julio, Ferré, Junín, Pergamino and San Antonio de Areco). Ears were naturally dried and shelled. The resulting grain samples were mixed within location.

***Fusarium* isolation and identification.** Maize kernels from each sample were surface-sterilized in a commercial 5% aqueous solution of NaOCl for 3 min, then rinsed with distilled water three times and plated onto PDA (10 kernels per plate) at 25°C for 7 days. *Fusarium* colonies belonging to section *Liseola* that developed from the kernels were then identified according to the key of Nelson *et al.* (1983). Single spore isolates from each colony were passaged on CLA, and the mating population was analyzed.

Mating population determination. Each isolate was crossed with tester strains (+ and –) belonging to each mating population (A-H). Tester strains were obtained from the international collection at the Istituto di Scienze delle Produzioni Alimentari del CNR (Bari, Italy). Crosses were made in triplicate on carrot agar following the methods of Klittich and Leslie (1988) with the standard tester as female parent and the uncharacterized isolate as male parent. After 7 days, each tester strain was fertilized by applying 0.5 ml of a conidial suspension from each isolate under study. Fertilized isolates were grown for 7 days at 25°C in darkness and 21 days at 21°C with fluorescent and near UV (NUV) light. Every seven days, cultures were checked for the appearance of perithecia and asci or ascospores. A cross was scored positive only if perithecia could be seen oozing a cirrus of ascospores.

Production of fumonisins *in vitro*. A twenty-nine isolate random sub-sample, including 26 isolates from MAT-A, two from MAT-E and one from MAT-D, was taken for studies of *in vitro* production of fumonisin and aggressiveness (Table 1). Cultures for assessing production of fumonisins *in vitro* were grown on Petri dishes for 25 days at 25°C in a medium consisting of 75% corn meal and 25% distilled water following the method of Jardine and Leslie (1999). Three replicates were used for each isolate. Colonized media were lyophilized with a Rificor L-6 lyophilizer (Rificor, Argentina), milled with a laboratory mill (Falling Number 3600) and stored in caramel flasks at 4°C. The concentration of fumonisins in each culture was assessed by ELISA (Ridascreen Fast Fumonisin, R-Biopharm AG, Germany). Fumonisins were extracted by blending 5 g of milled culture in 25 ml of 70% methanol. The mix was shaken for 2 min in a Boeco V-1 vortex (Boeckel & Co., Germany), filtered through a Whatman No. 1 filter and diluted 1:14 with sterile distilled water. Diluted extracts and 5 standards at concentrations of 0, 0.22, 0.67, 2.00 and 6.00 μg g^{-1} of fumonisins were subjected to ELISA. Well absorbance was measured at 450 nm with a Biotek ELx 800 microplate reader (Biotek Instruments, USA). Absorbance values of positive standards and samples were divided by the absorbance value of the first standard (standard zero). The concentration of fumonisins in the samples was estimated on the basis of a logit-log function between fumonisin concentration and relative absorbance of the four positive standards. Ridasoft Win software Version 1.10 (R-Biopharm AG, Germany) was used for fumonisin determination. The recovery rate in maize meal samples spiked with fumonisins (ratio FB1:FB2:FB3= 5:2:1, R-Biopharm) was 89.5% with a mean coefficient of variation of 2.4%. The detection limit of the method was 0.246 μg g^{-1}. Fumonisin concentration was measured in duplicate.

Fungal biomass determination. Ergosterol concentration, as an indicator of fungal biomass, was determined by the method of Seitz *et al.* (1977) with some modifications. Fifteen ml of methanol and 1 g of lyophilized milled sample were mixed for 2 min in a 125 ml Erlenmeyer flask. The blend was poured into a 50 ml capped polypropylene centrifuge tube. The remaining blend from the flask was washed off with 15 ml of methanol and poured into the centrifuge tube. The final extract was then centrifuged for 15 min at 3000 *g*. The supernatant was poured off and the residue was re-suspended in 10 ml of methanol, shaken for 30 sec, and centrifuged as described above. Supernatant portions were pooled, mixed with 8.5 g of KOH and 25 ml of ethanol, and refluxed for 30 min at 65°C. The cooled, saponified mixture was diluted with 5 ml of distilled water and extracted three times with 10 ml of hexane. Hexane extracts were combined and evaporated to dry-

ness under nitrogen with heating (35°C) in a rotatory evaporator. The resultant residue was dissolved in 5 ml methanol (HPLC grade). The solution was transferred to vials for HPLC analysis after filtration (0.22 µm). HPLC analysis was carried out with a Hewlett-Packard model 1050 system. Elution was performed at room temperature on a Hipersil ODS C18 microbore column (150 x 2.1cm) 5 µm using an isocratic mobile phase consisting of methanol at a flow rate of 0.3 ml min^{-1} and detection at 282 nm. A volume of 10 µl was injected into the HPLC. The ergosterol peak was eluted at about 6.6 min. Amounts were quantified against an external standard (Ergosterol from Sigma, USA) with a calibration curve range from 1.0 to 150 µg ml^{-1}. The curve was linear and covered the concentration range of the samples. Analyses were performed in duplicate and the results were averaged. The recovery rate was 92%.

Aggressiveness. Two maize hybrids were used, earlier shown to be moderately resistant (Condor) and susceptible (Chalten) to *Fusarium* ear rot (Presello *et al.*, 2006a, 2008). Experiments in a complete randomized

Table 1. Mating population, mean fumonisin (FB) concentration and mean severity of ear rot in two maize hybrids following field inoculation with 29 isolates of *Fusarium* section *Liseola* over two years.

Isolate	Mating population	FBs (µg g^{-1})	Disease severity†			
			2002/2003		2004/2005	
			Condor	Chalten	Condor	Chalten
NJ10	A	0.4	3.0	4.4	4.7	6.1
P319	D	ND‡	5.4	5.2	4.6	4.2
F31	A	0.7	3.3	3.8	2.2	3.5
P327	E	0.6	2.5	3.7	2.9	3.6
F16	A	1.1	3.4	4.1	2.7	4.3
P705	A	1.8	3.2	3.4	2.4	3.6
P332	A	3.5	3.2	3.0	3.0	4.2
P326	E	0.3	3.0	3.4	2.4	4.7
F24	A	28.0	2.6	3.2	2.1	4.2
P364	A	22.0	2.8	3.9	2.9	3.6
P315	A	91.0	2.8	4.6	2.7	4.3
S884	A	149.0	2.4	3.9	2.6	3.5
S888	A	99.0	3.2	4.7	2.5	4.0
P355	A	97.0	2.3	3.6	2.8	3.5
F28	A	118.0	2.6	4.8	2.4	3.6
SPS1	A	438.0	3.4	4.6	3.0	3.5
P318	A	187.0	3.5	3.6	2.5	4.1
SPC1	A	195.0	3.0	4.6	2.6	4.0
NJ5	A	332.0	2.4	4.6	2.3	4.2
S752	A	490.0	2.6	4.4	2.5	3.8
P102	A	591.0	2.8	3.5	2.6	3.8
P307	A	614.0	3.0	3.2	2.1	3.4
J63	A	2548.0	2.3	3.9	3.0	3.9
S8	A	1953.0	2.6	5.5	2.5	4.1
F63	A	2684.0	3.3	3.5	2.5	3.6
NJ6	A	2884.0	2.9	3.7	2.6	3.8
P302	A	no data	3.4	4.6	2.1	3.6
P321	A	no data	2.8	4.4	2.3	4.2
SK1	A	no data	3.5	3.8	3.1	3.9
Uninoculated control		-	1.5	2.7	1.9	2.5
LSD§			1.1		1.0	

† Based on a scale of 1-7, where 1= no symptoms, 2= 1-3%, 3 = 4-10%, 4 = 11-25%, 5 = 26-50%, 6 = 51-75% and 7 = 76-100% of the ear exhibiting ear rot symptoms.

‡ ND: not detected levels

§ Least significant difference to compare two disease severity means at a probability level of 0.05 (*t* test).

block design with two blocks were conducted in Pergamino, Province of Buenos Aires, in 2002/2003 and 2004/2005. Isolates and hybrids were randomized in a 29-by-2 factorial arrangement. Uninoculated treatments of each hybrid were included as a control. Experimental units consisted of single 6 m rows sown at a rate of 5 plants per m. To produce the inoculum, each isolate was grown in PDA for 7 days (Nelson *et al.*, 1983). Microconidia were washed off from Petri dishes cultures with sterile distilled water, and conidial concentration was adjusted to 1×10^6 ml^{-1} with sterile distilled water. Two ml of inoculum were injected into the silk channel using a different syringe for each isolate with a stainless steel needle 4 days after silking. Maize ears were manually harvested at 20% grain moisture, and symptoms were visually assessed according to a scale based on the percentage of symptomatic kernels, as in previous experiments where 1 = no symptoms, 2 = 1-3%, 3 = 4-10%, 4 = 11-25%, 5 = 26-50%, 6 = 51-75% and 7 = 76-100% of the ear showing ear rot symptoms (Presello *et al.*, 2006a).

Statistical analysis. Non-linear ear rot severity scores were re-converted to percentages of the ear exhibiting symptoms by replacing each score with the mid-point of the interval that the score represents on the percentage scale (e.g. 1 = 0%, 2 = 2%, 3 = 7%, 4 = 18%, 5 = 38%, 6 = 63%, 7 = 88%) (Campbell and Madden, 1990). The percentages of disease severity were transformed to ln (disease severity + 1) and subjected to analysis of variance. The conclusions reached by analyzing the transformed percentages and the rating scores were the same. Only rating score results are presented here. Cluster and principal component analyses (PCA) were conducted using disease severity data from both hybrids in both years. Cluster analysis was based on Euclidean distance following the clustering method of Ward (1963). PCA analysis was made by using a correlation matrix. Clusters (I, IIA and IIB) shown in the Bi-plot of PC were made by using clustering method information in combination with PC analysis. All statistical analyses were carried out with SAS 8.2 and INFOSTAT 1.1 at probability level of 0.05.

RESULTS

Mating populations. Fifty four out of the 60 isolates tested matched the tester strain belonging to MAT-A. Five isolates belonging to MAT-E and one belonging to MAT-D were also identified.

Production of fumonisins *in vitro* and fungal biomass assessment. All fungal isolates produced profuse growth. The mean square of isolate from the analysis of variance for ergosterol concentration was not significant indicating no differences among isolates for fungal biomass. Thus, all differences in fumonisin concentration among isolates can be attributed to the ability of each isolate to produce mycotoxin. Fumonisin concentration ranged from 0.4 to 2884 μg g^{-1} for MAT-A, and from 0.3 to 0.6 μg g^{-1} for MAT-E. The only isolate belonging to MAT-D produced undetectable levels (Table 1).

Aggressiveness. Disease severity was affected by year, hybrid, isolate, isolate×hybrid and isolate×year. The main effects were more important than double interaction effects (results not shown). Differences in disease severity means between Condor and Chalten in natural infection were significant only in 2002/2003. Differences in disease severity means between inoculated and uninoculated treatments were significant across hybrids and years for isolates SK1, NJ10 (MAT-A) and P319 (MAT-D) while for the other isolates, results were not consistent across hybrids and years (Table 1).

In inoculated treatments, differences in disease severity between hybrids were significant in both years for isolates SPC1, NJ5, NJ10, F28, S752 S888, S8, P302, P315, P321 and P355, and non-significant in both years for P319. For the other isolates, results were not consistent across years (Table 1).

In the PCA, the first three PCs explained 91% of the variation for disease severity. PC1 (49%) was mainly associated with the effect of isolate, PC2 (22%) with the effect of year, and PC3 (20%) with the effect of hybrid. Cluster analysis grouped isolates into two major clusters (Fig. 1). Cluster I included isolates P319 (MAT-D) and NJ10 (MAT-A) causing the most severe symptoms as accounted for PC1. Cluster II grouped the other 27 isolates (25 belonging to MAT-A and 2 belonging to MAT-E) causing mild symptoms. Isolates grouped in Cluster II were divided into two sub-clusters: Cluster IIA and Cluster IIB, each one containing isolates differing in disease severity, mainly accounted for PC3, between the moderately resistant (IIA) and the susceptible hybrids (IIB) in both years.

DISCUSSION

Members of MAT-A were the most prevalent *Fusarium* spp. in our sample, with isolates that exhibited a wide range of fumonisin production, as shown in previous reports from Argentina and other regions of the world (Leslie, 1991; Chulze *et al.*, 1996). Isolates belonging to MAT-D and MAT-E were found to be less prevalent and were low fumonisin producers. Only one isolate belonged to MAT-D, in contrast to the frequencies reported in previous work from the temperate maize growing region in Argentina (Chulze *et al.*, 1996), probably due to the environmental conditions prevailing at the time of this experiment. This isolate produced

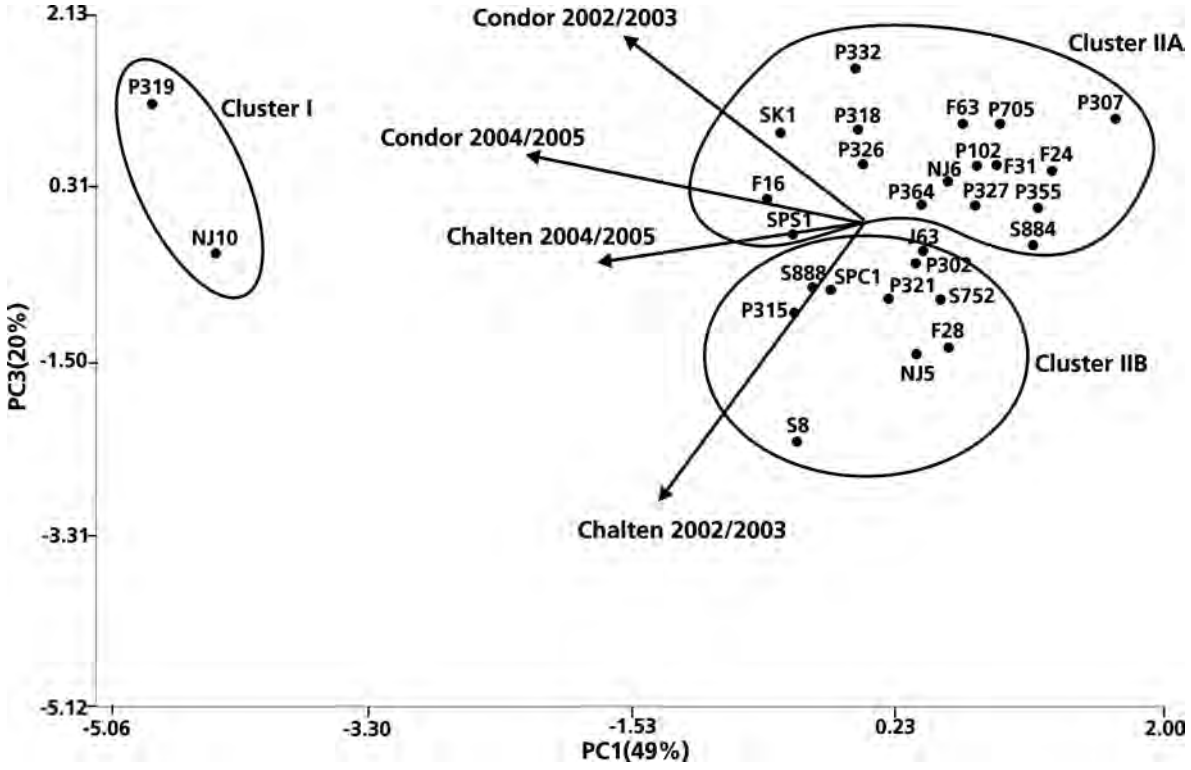

Fig. 1. Bi-plot of principal components computed from disease severity means of 29 isolates belonging to *Fusarium* section *Liseola*, two hybrids and two years.

undetectably low levels of fumonisin. Members of this MAT have been described as important fumonisin producers in maize including some isolates with significant values for the FB2/FB1 ratio (Sydenham *et al.*, 1993; Ross *et al.*, 1992). However, low fumonisin producers within MAT-D were reported on fruit and vegetables in Europe (Desjardins, 2006). Isolates belonging to MAT-E have been reported previously in some other Argentine maize regions such as the provinces of Jujuy and Buenos Aires, including low-fumonisin producers as described above (Torres *et al.*, 2001; Reynoso *et al.*, 2004).

After inoculation of each hybrid, isolates caused differences in ear rot severity, suggesting different levels of aggressiveness. *Fusarium verticillioides*, the most prevalent species from section *Liseola*, has been regarded as a weak maize pathogen (Yates *et al.*, 2005). In this study, mildly aggressive isolates were the most prevalent in local populations of *Fusarium* section *Liseola*. However, two highly aggressive isolates were also identified. Isolates grouped in Cluster I showed very low or no fumonisin production and high aggressiveness. Isolates grouped in Clusters IIA and IIB were mildly aggressive and produced different levels of fumonisins, in agreement with previous reports. This suggests that an isolate's ability to produce fumonisins may not be a prerequisite for aggressiveness to maize (Desjardins and Plattner, 2000). Since strain aggressiveness to ear rot in maize might not be related to the ability to produce fumonisins, and grain fumonisin concentration seems to depend on disease severity (Presello *et al.*, 2006a), aggressive non-toxigenic isolates, such as P319 and NJ10, might be used as a source of inoculum for selection in order to reduce a breeding team's exposure to fumonisins in field experiments.

Relative frequencies of mildly and highly aggressive strains found in this study suggest that low aggressiveness might be a selective advantage for the pathogen. Less aggressive strains causing mild damage to the host might be favored by natural selection and their presence in crops may increase.

In previous work, interactions among isolates of *Fusarium spp* and maize genotypes for disease severity were not found (Jardine and Leslie, 1999) or were not important (Reid *et al.*, 1993), and general resistance mechanisms seemed to be effective across more than one *Fusarium* species (Presello *et al.*, 2006b). Although relative importance of main and interaction effects led to similar conclusions in our experiments, isolate-by-hybrid interaction effects were still important, suggesting that selection responses would be affected by the isolate chosen as source of inoculum. In fact, differences in disease resistance between Condor and Chalten after inoculation with highly aggressive isolates P319 and NJ10 appeared only for NJ10. Similar conclusions arise when

comparing disease severity caused by isolates grouped in Cluster IIA and IIB, suggesting that disease resistance of the moderately resistant hybrid would be effective only for some isolates, regardless of their aggressiveness.

Inoculation of single-spore isolates on these two maize hybrids revealed host-pathogen interactions caused by genetic variations in both organisms and by the environment. Since under natural conditions host genotypes might be challenged by a complex pathogen population, the use of a mixture of isolates representing the most prevalent pathogenic populations for inoculation, as suggested by Reid *et al.* (1993), might be desirable even though it would hinder the identification of some sources of resistance to specific isolates. As species of *Fusarium* may have significant interactions (Reid *et al*., 1999), mixture reaction among isolates should be tested before inoculating.

Our study provides a set of data on populations of *Fusarium* section *Liseola* from maize in the north of Buenos Aires province, which were characterized on the basis of their morphology, fertility, fumonisin production and aggressiveness to maize. The presence of interactions between genotype, pathogen and year highlights the importance of using isolates properly characterized for discrimination of effective resistance across environmental and pathogenic variations. These results may facilitate the selection of new genetic materials able to withstand *Fusarium* infection and mycotoxin contamination in maize, so as to develop disease management strategies aimed at protecting animal and human health.

Collecting information about differences in aggressiveness of *Fusarium* isolates could provide valuable information for the development of quantitative resistance in maize. In addition, because of the huge evolutionary potential of plant pathogens to overcome plant host defenses, it is of primary importance to understand how the development of such resistance would affect aggressiveness in pathogen populations.

ACKNOWLEDGEMENTS

This work was supported by grants from the Instituto Nacional de Tecnología Agropecuaria (INTA), PNCER 1332 and the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) PICTO INTA / SECyT 08-12913. The authors thank the maize breeding and the plant pathology crews at INTA for assistance with field and laboratory experiments and E. Maiola, A. Ivancovich and C. Díaz for reviewing the manuscript.

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Received March 31, 2009
Accepted July 29, 2009