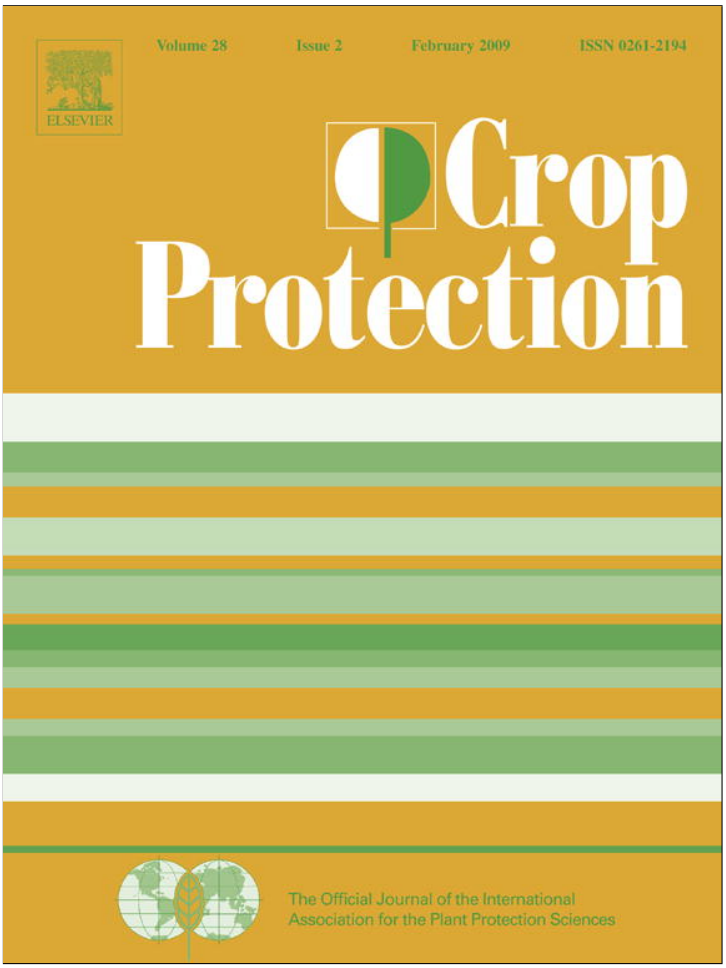

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Short communication

The pericarp and its surface wax layer in maize kernels as resistance factors to fumonisin accumulation by *Fusarium verticillioides*

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ABSTRACT

Fusarium verticillioides causes maize ear rot and contaminates kernels with fumonisin mycotoxins in Argentina. The aim of this work was to elucidate if the kernel pericarp and its surface wax layer are resistance factors to fumonisin accumulation in maize genotypes from Argentina. Fourteen maize genotypes were inoculated with *F. verticillioides* in laboratory assays. Intact kernels of genotypes resistant to fumonisin accumulation in the field had the lowest mycotoxin concentration in the current assays suggesting that kernel factors are involved. Intact kernels of landraces, breeding populations and L4637 inbreed were less susceptible than wounded ones, suggesting that intact kernel pericarp restricted fumonisin accumulation. Removing wax from the pericarp significantly increased fumonisin concentration and a higher wax content on kernels was associated to lower fumonisin accumulation. Our results suggest that the pericarp and its wax content are resistance factors to fumonisin accumulation in most genotypes assayed. Nevertheless, other kernel factors could not be excluded.

1. Introduction

Fusarium verticillioides (Saccardo) Nirenberg [synonym, *Fusarium moniliforme* (Sheldon)], teleomorph *Gibberella moniliformis* (Wineland) is the most important ear and kernel rotting pathogen of maize (*Zea mays* L.) in tropical and temperate regions of Argentina (Chulze et al., 1996). This fungus not only reduces maize yield (Presello et al., 2008) but also contaminates infected kernels with fumonisin mycotoxins. Fumonisins are of greatest concern because of their noxious effects on humans and animals (Harrison et al., 1990; Norred et al., 1992; Wilson et al., 1992). Most fumonisin accumulation occurs in the field before harvest, mainly in early stages of kernel drying (Munkwold, 2003). Although concentration of fumonisins in kernels was positively associated to disease severity caused by *F. verticillioides* in field infections (Desjardins et al., 1998; Presello et al., 2008), in several situations accumulation of these mycotoxins has also been detected in asymptomatic kernels (Miller, 2001). For this reason, maize genotypes included in breeding programs are currently characterized not only for their resistance to infection by *F. verticillioides* but also for resistance to fumonisin accumulation in their kernels (Presello et al., 2008).

Inheritance of maize resistance to *F. verticillioides* is complex (De Leon and Pandey, 1989; Presello et al., 2005). Identifying factors associated with kernel resistance to infection by *Fusarium* and fumonisin accumulation helps in the understanding of genetic mechanisms controlling disease resistance and also facilitates maize breeding. Previous studies indicated that physical factors, such as pericarp thickness (Hoenisch and Davis, 1994) and husk covering (Warfield and Davis, 1996) may be involved in *Gibberella* ear rot resistance. Chemical factors, such as phenolic compounds in the kernel pericarp were also suggested as resistance factors, although current evidence is not conclusive (Bily et al., 2003). Recent studies indicated that population GT-MAS:gk, which is resistant to *Aspergillus flavus* infection/aflatoxin accumulation is also resistant to the infection by *F. verticillioides*. Pericarp wax layers participate in resistance of GT-MAS:gk to *A. flavus* (Brown et al., 2001), and might also be a resistance factor to *F. verticillioides*. Nevertheless, there are no reports relating wax layers in kernel surface with susceptibility to fumonisin accumulation for *F. verticillioides*. Furthermore, mechanisms of resistance to *F. verticillioides* in Argentinian maize genotypes are still unknown.

The aim of this work was to elucidate if kernel pericarp and its surface wax layer are resistance factors to fumonisin accumulation

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by *F. verticillioides* in Argentinian maize genotypes previously characterized for *Gibberella* ear rot resistance and fumonisin accumulation in the field.

2. Materials and methods

2.1. Maize genotypes

Fourteen maize genotypes previously characterized for their resistance to *F. verticillioides* in temperate or subtropical regions of Argentina were assayed (Table 1). Ears of each genotype were harvested at $19 \pm 1\%$ of kernel moisture, naturally dried, and shelled. Kernel samples were stored at 4 °C until used in assays. As asymptomatic kernels may accumulate fumonisins, kernel samples from each genotype were analyzed for fumonisin concentration and fumonisin free samples were used as controls in the assays. All samples were free of fungicides, insecticides, and dyes.

2.2. Fungal strain and inoculum preparation

A local isolate of *F. verticillioides* (P364) known for its aggressiveness to maize and ability to produce fumonisin in maize-based cultures (Presello et al., 2008) was used for inoculum production. The fungus was grown in malt–agar medium (2% w/v malt extract; 0.5% w/v peptone, 2% glucose and 1.8% w/v agar) at 30 °C under a continuous light source (400 $\mu mol\ m^{-2}\ s^{-1}$). After a week, a 1 cm^2 piece of medium was added to a modified Bilay's liquid medium and macroconidial suspensions were obtained according to Reid et al. (1996). Prior to inoculation, the liquid medium containing macroconidia was filtered using a double cheesecloth and the concentration of spores was adjusted to 1×10^6 spores ml^{-1}.

2.3. Kernel wounding and inoculation

Time and equipment limitations precluded simultaneous testing of the 14 genotypes assayed. Therefore, wounded and intact kernels of genotypes were assayed in two groups. ARZ03018, AZRM05040, AZRM04014, L4671, L6856 and Condor were assayed in Test 1 while Poblacion D, Leales 25, L4637 and L4674 were included in Test 2. For consistency and comparison, both tests included two susceptible (Cargill 350 and Chalten) and one resistant (NK120) commercial hybrids.

Kernels from each genotype were immersed in 1% sodium hypochlorite for 5 min. They were then rinsed three times in sterile distilled water, in a laminar flow. Kernels of each genotype were wounded to a depth of 1 mm using a sterile 20 gauge needle. Wounded and intact kernels were immersed into the fungal suspension and transferred to sterile 5 cm Petri dishes. Fourteen kernels of each genotype were placed in each Petri dish. Each treatment was replicated 12 times. After covering in sterile conditions, Petri dishes were transferred to plastic containers ($58 \times 43 \times 25$ cm) containing 1 L of distilled water at the bottom, and placed onto a uniformly perforated-plastic sheet to avoid direct contact with water. Three plastic containers were prepared as indicated above and four Petri dishes of each treatment were placed in each container at random. After sealing, the containers were transferred into a growth chamber at 26 °C for 7 d in the dark. Immediately following this, kernels were dried in a forced-air oven at 60 °C for 2 d to stop further fumonisin accumulation. Three-kernel samples were dried per treatment, each one extracted from a different container. Dry kernels were ground using a Willey mill. After a thorough mix, a 5-g subsample was extracted from each ground sample for fumonisin analyses.

2.4. Fumonisin analysis

Concentration of fumonisins in maize samples was assessed by ELISA (Ridascreen® Fast Fumonisin, R-Biopharm AG, Darmstadt, Germany) according to the procedure described by Presello et al. (2008). Fumonisins were extracted by blending each 5-g subsample in 25 ml of 70% methanol. The mix was shaken for 2 min in a vortex, filtered through filter paper Whatman No. 1 and diluted 1:14 with sterile distilled water. Diluted extracts and five standards, at concentrations of 0, 222, 667, 2000 and 6000 $\mu g\ g^{-1}$ of fumonisins, were subjected to ELISA. Absorbance was measured at 450 nm with a microplate reader BioRad 680 (BioRad, USA). Concentration of total 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 using RIDA® SOFT Win software (R-Biopharm AG, Darmstadt, Germany).

2.5. Removal of wax layer from kernel pericarp and assay of kernel resistance to F. verticillioides

The role of wax layer from the kernel pericarp in limiting colonization and fumonisin production by *F. verticillioides* was evaluated in a subsample of six resistant (ARZM03018, ARZM05040, ARZM04014, L4637, Leales 25 and Poblacion D) and two susceptible (Cargill 350 and Chalten) genotypes exhibiting differences of fumonisin accumulation in wounded and intact kernels. Sixty intact kernels from each genotype were immersed twice in 60 ml of chloroform for 30 s, to remove the surface wax layer. Chloroform fractions from each genotype were pooled and

Table 1
Pedigree, origin, colour and kernel type, and resistance to *F. verticillioides* of the fourteen maize genotypes assayed.

Genotype	Type	Resistance to *F. verticillioides*	Colour and kernel type	Origin	
				Germplasm	Region
Leales 25	Breeding population	Resistant	Yellow semident	Broad-based composite	Subtropical
Poblacion D	Breeding population	Resistant	Yellow semident	Broad-based composite	Subtropical
Condor	Commercial hybrid	Resistant	Yellow flint	Unknown	Temperate
Cargill 350	Commercial hybrid	Susceptible	Yellow semident	Unknown	Subtropical
NK120	Commercial hybrid	Resistant	Yellow semident	Unknown	Subtropical
Chalten	Commercial hybrid	Susceptible	Yellow semident	Unknown	Temperate
L4637	Inbred	Resistant	Yellow flint	(LP561xLP611)F2	Temperate
L1186	Inbred	Susceptible	Yellow semident	(LP915xL3125)F2	Temperate
L4671	Inbred	Susceptible	Yellow flint	(LP561xLP611)F2	Temperate
L4674	Inbred	Susceptible	Yellow semident	R4973 Synthetic	Temperate
L6856	Inbred	Susceptible	Yellow semident	AX888	Temperate
ARZM03018	Landrace	Resistant	White flint (perlita race)	Unknown	Temperate
ARZM04014	Landrace	Resistant	Red pericarp flint (perlita race)	Unknown	Subtropical
ARZM05040	Landrace	Resistant	White dent	Unknown	Subtropical

evaporated under vacuum at 40 °C. Weight of dry residue was determined for each sample and then expressed as wax weight extracted from 60 kernels. Two additional samples, each one of 60 kernels, from each genotype were also extracted and weighed as mentioned before. The assay was performed with intact kernels and kernels after chloroform washing. Fourteen kernels were placed in each Petri dish and each treatment was replicated 12 times. Techniques for inoculation, incubation and sample preparation for fumonisin analysis were as described for Tests 1 and 2. The experiment is presented below as Test 3.

2.6. Statistical analysis

Data of kernel assays were subjected to a one way ANOVA and differences among means were tested by the Tuckey's test. Linear regression between fumonisin ratio in chloroform washed to intact kernels and wax weight was calculated using the regression analysis procedure of SPSS 7.5 for Windows. Statistical tests were performed at a probability level of 0.05.

3. Results

A significant difference in fumonisin accumulation was observed between resistant and susceptible genotypes. In intact kernels, susceptible genotypes accumulated more fumonisins than resistant ones. In Test 1, intact kernels of susceptible genotypes L6856 and L1186 accumulated the highest levels of fumonisin (Fig. 1A). A significantly lower accumulation was detected in susceptible genotypes L4671, Cargill 350 and Chalten followed by resistant genotypes AZRM05040, AZRM04140, Condor and NK120. Kernels from resistant genotype ARZM03018 had the lowest fumonisin accumulation. In Test 2, intact kernels of Cargill 350 and L4674 accumulated higher fumonisin levels than did the other genotypes (Fig. 1B). Differences in fumonisin accumulation among genotypes were still detectable following inoculation of wounded kernels in both Tests 1 and 2. Differences of fumonisin accumulation between wounded and intact kernels were observed for the resistant genotypes ARZM03018, ARZM05040, ARZM04014, Poblacion D, Leales 25, L4637 and for the susceptible genotypes Chalten, L4674 and Cargill 350. Consistency of Tests 1 and 2 was demonstrated by comparing results from genotypes included in the two tests. In both cases, NK120 and Condor consistently accumulated lower fumonisin levels than Cargill 350. Furthermore, wounding increased kernel fumonisin levels of Cargill 350, something not observed for NK120 and Condor in both assays (Fig. 1A and B).

Except for the resistant genotype L4637, chloroform washing increased kernel fumonisin accumulation in both susceptible and resistant genotypes assayed in Test 3 (Fig. 2). The squared correlation (r^2) between wax weight from 60 kernels and fumonisin ratio in chloroform washed to intact kernels was 0.61 ($P = 0.05$, Fig. 3).

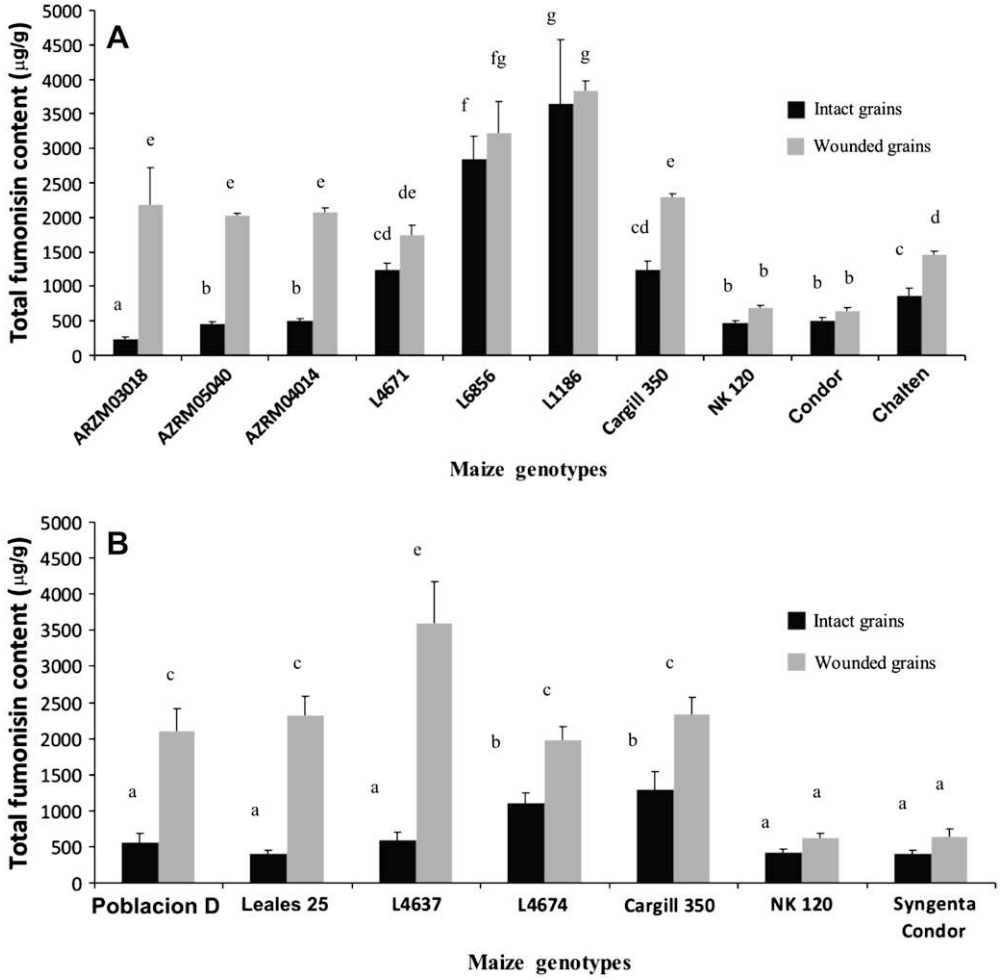

Fig. 1. Levels of total fumonisin in intact and pericarp-wounded kernels from different maize genotypes inoculated with *Fusarium verticillioides*: A) Test 1, B) Test 2. Bar indicates standard deviation. Different letters indicate significant differences between means at the level of $P < 0.05$ (Tuckey's test).

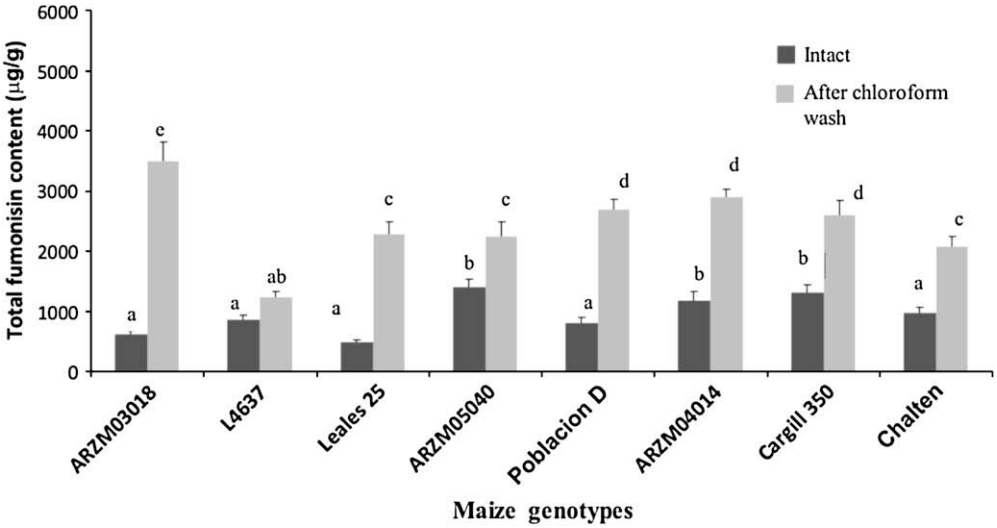

Fig. 2. Total fumonisin accumulated in intact and chloroform washed kernels. Bar indicates standard deviation. Different letters indicate significant differences between means at the level of $P < 0.05$ (Tuckey's test).

4. Discussion

Intact kernels of maize genotypes characterized as resistant to fumonisin accumulation in the field had also the lowest fumonisin concentrations in the current assays. This result suggests that kernel related resistance factors, effective in these genotypes at field conditions, were also functioning in the mature kernels assayed. Kernel wounding increased fumonisin accumulation in most resistant genotypes assayed, suggesting that mechanical or chemical characteristics of their pericarp layer restricted initial fungal penetration or colonization. On the other hand, two resistant genotypes, NK120 and Condor, exhibited no differences in fumonisin accumulation between intact and wounded kernels suggesting that factors in inner components of their kernels such as germ or endosperm may also participate in the observed resistance.

Wax content in outer pericarp layers and wax composition were identified as kernel factors in maize resistance to *A. flavus* infection/aflatoxin accumulation (Russin et al., 1997). Although no studies have addressed the role of surface wax layer in resistance to fumonisin accumulation in maize, thickness of surface wax layer covering the kernel was previously associated with resistance to *F. verticillioides* infection in other cereal crops (Rodriguez-Herrera et al., 2000). In this study, removing kernel wax with chloroform increased fumonisin accumulation in most genotypes suggesting that wax layer restricts fungal infection. Interestingly, a positive relationship was established between kernel wax weight and ratio of fumonisin accumulation in chloroform washed/intact kernels of the assayed genotypes suggesting that thickness of wax layer could regulate fumonisin accumulation.

In conclusion, our results suggest that kernel factors are involved in resistance to fumonisin accumulation in the maize genotypes assayed. Resistance was associated to outer kernel layers and wax content in most of them. High wax content would be a broad base resistance mechanism in maize kernels against mycotoxin production by pathogenic fungi. However, it cannot completely explain the resistance observed, suggesting that other pericarp (i.e., wax composition and phenolic compounds) or inner kernel factors are also functioning in the resistance to fumonisin accumulation by *F. verticillioides*.

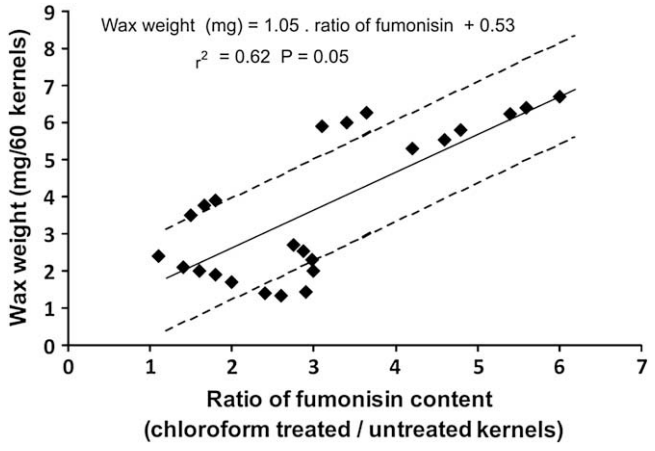

Fig. 3. Relationship between wax content and ratio of fumonisin accumulation in chloroform washed/untreated kernels. Dashed lines show the 95% confidence interval limits. Solid line shows the slope.

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