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Short communication

Influence of water activity and temperature on growth and production of trichothecenes by *Fusarium graminearum sensu stricto* and related species in maize grains

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ABSTRACT

Fusarium meridionale and *F. boothii* cause Gibberella Ear Rot (GER) in maize. This study determined the effects of temperature (5–35 °C) and water activity (0.90–0.995 a_w) on the growth, and deoxynivalenol (DON) and nivalenol (NIV) production of *F. meridionale* and *F. boothii* strains in maize grains. *Fusarium graminearum sensu stricto* strains from wheat were also tested. The three *Fusarium* species grew best at 0.995 a_w and 25 °C. Growth was absent or marginal at 0.90 a_w regardless of temperature. *F. meridionale* and *F. boothii* were sensitive to 30 °C and more affected by water stress than *F. graminearum sensu stricto*. The highest DON levels were at 0.995–0.97 a_w and 30 °C and at 0.97 a_w and 20 °C for *F. graminearum sensu stricto*, and at 0.995–0.97 a_w and 20 °C for *F. boothii*. *Fusarium meridionale* reached maximum NIV accumulation at 0.995 a_w and 20 °C. This produced DON at negligible levels compared to the other two *Fusarium* species. Growth of *F. meridionale* and *F. boothii* was well adapted to the usual autumn high humidity and mild temperatures associated with GER in northwest Argentina. Control strategies during grain development should be taken into account to reduce the risk of the presence of DON and NIV in the harvested grains.

1. Introduction

Fusarium graminearum Schw. is an important ear rot pathogen of cereals worldwide. In Argentina, it has been reported as an etiological agent of Fusarium Head Blight (FHB) on wheat in the center of the country (Ramirez et al., 2006) and of Gibberella Ear Rot (GER) on maize in the northwest (Sampietro et al., 2011). This fungus reduces grain yields and contaminates the grains with B-trichothecenes, responsible for feed refusal, vomiting, and other adverse effects in humans and animals (Pestka, 2010). Individual isolates of *F. graminearum* can produce one of three B-trichothecene chemotypes (Aoki et al., 2012; Miller et al., 1991): nivalenol and acetylated derivatives (NIV chemotype), deoxynivalenol and primarily 3-acetyldeoxynivalenol (3ADON chemotype), and deoxynivalenol and primarily 15-acetyldeoxynivalenol (15ADON chemotype). This variation is important for food safety because B-trichothecenes differ from each other in their toxicities (Forsell and Pestka, 1985; Luongo et al., 2008). It also appears to have important consequences for pathogen fitness (Ward et al., 2002). In the last decade, the use of phylogenetic species recognition based on genealogical concordance indicated that *F. graminearum* Schw. is in fact a complex (Fg complex) of at least 16 phylogenetically distinct species with marked biogeographic structure (Aoki et al., 2012; Gryzenhout et al., 2016). In this context, the FHB in wheat in central Argentina was associated with *F. graminearum sensu stricto* with the 15ADON chemotype (Alvarez et al., 2011). The occurrence of GER in the northwest was associated with a dominant presence of *Fusarium meridionale* and some participation of *F. boothii*. PCR genotyping predicted NIV and 15ADON chemotypes for the *F. meridionale* and *F. boothii* strains, respectively (Sampietro et al., 2011). GER has an important impact in late-planted maize, which has silk emergence and grain maturation in autumn (Aguaysol et al., 2013). It is necessary to understand the impact of interacting environmental factors such as water activity (a_w) and temperature on growth and trichothecene production of *F. meridionale* and *F. boothii* in maize grain in order to predict the possible risk of DON and NIV contamination before harvest and during grain storage. For this reason, the aim of this work was to investigate the

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Table 1
Main features of the fungal strains assayed.

Strain	*Fusarium* species	Cereal host	Geographical origin	Trichothecene genotype
IV II 3	*Fusarium graminearum sensu stricto*	Wheat	Los Hornos (Buenos Aires)	15ADON
44B	*Fusarium graminearum sensu stricto*	Wheat	Pergamino (Buenos Aires)	15ADON
48	*Fusarium graminearum sensu stricto*	Wheat	Concepción del Uruguay (Entre Rios)	15ADON
69[a]	*Fusarium graminearum sensu stricto*	Wheat	Paraná (Entre Rios)	15ADON
F10	*Fusarium boothii*	Maize	Viclos (Tucumán)	15ADON
F46	*Fusarium boothii*	Maize	Trancas (Tucumán)	15ADON
F78	*Fusarium boothii*	Maize	Trancas (Tucumán)	15ADON
F82	*Fusarium boothii*	Maize	Trancas (Tucumán)	15ADON
F5	*Fusarium meridionale*	Maize	Viclos (Tucumán)	NIV
F33	*Fusarium meridionale*	Maize	La Cocha (Tucumán)	NIV
F39	*Fusarium meridionale*	Maize	Trancas (Tucumán)	NIV
F81	*Fusarium meridionale*	Maize	Trancas (Tucumán)	NIV

minimum and optimum conditions for growth and trichothecene accumulation (DON and/or NIV) of *F. meridionale* and *F. boothii* strains on maize grains. *F. graminearum sensu stricto* has not yet been isolated as an etiological agent of GER in maize of northwest Argentina. Hence, *F. graminearum sensu stricto* strains were also assayed, to identify any growth or toxigenic response which could explain its absence from maize. In contrast to a previous study (Rybecky et al., 2018), the influence of water activity and temperature on growth and on DON and NIV production was assessed in maize grains for *F. graminearum sensu stricto*, *F. meridionale* and *F. boothii*.

2. Materials and methods

2.1. Fungal strains

The main features of the strains of the *F. graminearum* complex assayed in this work are listed in Table 1. The strains belonged to *F. graminearum sensu stricto*, *F. meridionale* and *F. boothii* according to a previously published multilocus genotyping (MLGT) assay based on allele-specific primer extensions (Sampietro et al., 2011; Ward et al., 2008). Multiplex PCR assays developed by Ward et al. (2002) indicated that the strains have 15ADON (*F. graminearum sensu stricto*, *F. boothii*) and NIV genotypes (*F. meridionale*). The strains are preserved as spore suspensions in 15% glycerol frozen at −80 °C in the laboratory of bioactive agents and phytopathogens (LABIFITO) of the National University of Tucumán (Argentina).

2.2. Water activity adjustment of maize grain

Maize grain (hybrid DKB 390), with an initial moisture content of 12.5% (0.58 a_w) was gamma irradiated (10–12 KGy) using a cobalt radiation source and stored aseptically at 4 °C. The irradiated grain contained no microbial infection and was absent of NIV and DON contamination. Two kilograms of irradiated maize grain were weighed into sterile beakers and rehydrated to each required a_w (0.90, 0.95, 0.97 or 0.995) by addition of sterile distilled water using a moisture absorption curve. Each flask was refrigerated for 48 h at the temperatures of 5, 15, 20, 25, 30 or 35 °C with periodic shaking to allow absorption and equilibration. Finally, the water activity values were confirmed by using a LabSwift-a_w water activity meter (Novasina, Zurich, Switzerland).

2.3. Inoculation, incubation and growth assessment

The rehydrated maize grain was placed in sterile 9 cm Petri dishes to form single grain monolayers (about 20 g per dish). Then, the twelve fungal strains were inoculated in the monolayers. To do this, 6 mm diameter agar disks were taken with a cork borer from the margin of a 5-day old colony of a fungal strain growing on malt extract (15 g/L; peptone, 5 g/L; sucrose, 20 g/L; and agar, 20 g/L) at 25 °C. Each disk was transferred to the center of a monolayer. The inoculated Petri dishes containing grains fixed to different water activities (0.90, 0.95, 0.97 and 0.995 a_w) were enclosed in plastic boxes in a way that allowed them to be assayed at different temperatures (5, 10, 15, 20, 25, 30 or 35 °C) for 28 days. Vapor pressure was maintained constant in each box by placing a beaker containing an aqueous glycerol solution with the same water activity as that of the Petri dishes in the inside. Assessment of growth was made daily during the incubation period, examining the maize grain cultures using a binocular magnifier (×10). For this assessment, the mean ratio was calculated from two diameters of each growing colony measured daily at right angles to each other until the colony reached the edge of the plate. The mean radii were regressed against time to calculate the growth rates (mm/day). Each strain was represented by three Petri dishes at each combination of water activity/temperature assayed. The whole experiment described was carried out twice. Mean growth rates presented in Fig. 1 were calculated from the growth rates recorded from four strains of the same Fg complex species, including the data of two experiments per strain. At the end of the 28 days, the grain monolayers contained in the Petri plates were dried at 50 °C for 48 h and stored at −20 °C until trichothecene analysis.

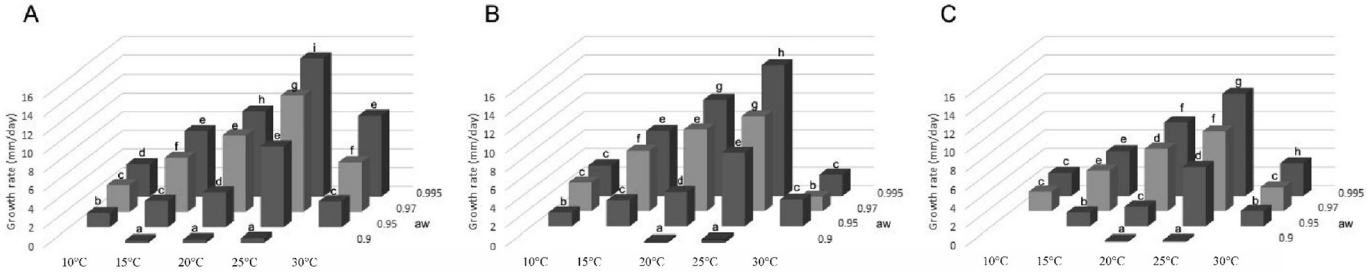

Fig. 1. Effect of water activity and temperature on growth rates of strains of *Fusarium graminearum sensu stricto* (A), *F. boothii* (B) and *F. meridionale* (C) grown on irradiated maize grain. Different letters over bars indicate significant differences in the fungal growth ($p < 0.05$). Each bar is the mean growth rate calculated from data of four strains.

2.4. Quantification of trichothecenes

Each grain monolayer was finely ground in a laboratory grinder and then homogenized. A sub-sample of each ground material (12 g) was extracted by mixing with 40 mL of acetonitrile/water (84:16, v/v), shaken for 1.5 h on an oscillatory shaker (150 rpm), and then filtered through Whatman N° 1 filter paper. The filtered extracts were passed through MycoSep ™225 columns (Romer Labs Inc., Union, MO, USA) and then 5 mL of the cleaned extract was evaporated to dryness at 60 °C under reduced pressure. The residue was dissolved in 0.5 mL of methanol/water (5:95, v/v), pressed through 0.45 µm membrane filters. These filtered samples were injected in a Gilson HPLC (Villiers Le Bel, Val d'Oise, France) equipped with a Luna™ C18 reverse-phase column (100 × 4.6 mm, 5 µm). The mobile phase consisted of methanol/water (5:95, v/v) at a flow rate of 0.7 mL/min. The HPLC was coupled to a Gilson 118 UV-VIS detector set at 220 nm. Injection volume (60 µL) was performed through a Rheodyne injector fitted with a 20 µL loop. The trichothecenes were identified and quantified by comparison with retention times of NIV and DON standards (Sigma-Aldrich Co., St Louis, MO). Four mixed working solutions of NIV/DON were injected at concentrations of 4.0/2.0, 2.0/1.0, 1.0/0.5 and 0.5/0.25 µg/mL. The calibration curves showed good linearity for both mycotoxins (R^2 = 0.990 for DON and R^2 = 0.980 for NIV). The quantification limit was 5 ng/g. The filtered samples were also co-injected with the standard NIV/DON solutions to corroborate the identity of the trichothecene peaks. The mean DON and NIV levels presented in Table 3 were calculated from the DON or NIV levels recorded from four strains of the same Fg complex species, including the data of two experiments per strain.

2.5. Statistical analysis

Growth rates and trichothecene levels were tested for normality using the Shapiro-Wilk test. Due to non-normality, trichothecene data were transformed using log10(x). Then, growth rates and trichothecene levels were subjected to multifactorial analysis of variance (ANOVA) to test for significant differences related to fungal species, temperature, water activity, strain and their interactions. The Tukey's significant difference (Tukey - HSD) multiple range test ($p < 0.05$) was used to check for significant differences in the means of growth rates and trichothecene levels, as shown in Fig. 1 and Table 3, respectively. Statistical analysis was performed using the Infostat® package, version 2008 (Infostat Group, FCA, UNC, Argentina).

3. Results

3.1. Effects of temperature and water activity on fungal growth

Fig. 1 shows that the three *Fusarium* species grew best at 25 °C and 0.995 a_w, with maximum growth rates of about 14 mm/day (*F. graminearum sensu stricto* and *F. boothii* strains) and 11 mm/day (*F. meridionale* strains). At high water activities (0.97–0.995 a_w), growth decreased from 25 °C to 30 °C by a factor of about 2 (*F. graminearum sensu stricto* strains), 6 (*F. boothii* strains) and 3 (*F. meridionale* strains). The three species showed marginal growth at 0.90 a_w in the range 20–25 °C for the *F. boothii* and *F. meridionale* strains, or 15–25 °C for the *F. graminearum sensu stricto* strains. They were not able to grow at 5 °C or at 35 °C regardless of the water activity. The *F. meridionale* strains were also unable to grow at 0.95 a_w/10 °C. A multifactorial analysis of variance (ANOVA) showed water activity accounting for most of the variability in the growth rates of the 12 fungal strains ($p < 0.05$), followed by temperature and to a lesser extent interspecific differences (Table 2). Intraspecific variations of growth rates were not significant. There was a significant triple interaction of water activity × temperature × species.

Table 2
Analysis of variance of the effect of water activity, temperature, species and strain on growth of the fungal strains on irradiated maize grain.

Source of variation	df[a]	MS[b]	F[c]
a_w	3	597.95	79,273.91*
Species	2	63.32	8395.06*
Temperature	4	310.63	41,182.63*
Strain	9	0.01	1.38
a_w × species	6	5.95	788.39*
a_w × temperature	12	34.06	4515.92*
Species × temperature	8	8.94	1184.75*
a_w × species × temperature	24	3.41	451.52*

[a] Degrees of freedom.
[b] Mean square.
[c] F Snedecor.
* Significant at $p < 0.05$ level.

3.2. Effect of temperature and water activity on trichothecene accumulation

Table 3 shows the highest trichothecene levels at 0.97 a_w/20–30 °C and 0.995 a_w/30 °C (21.8–47.5 µg DON/g) for *F. graminearum sensu stricto* strains, at 0.97–0.995 aw/20 °C (3.7 µg DON/g) for *F. boothii* strains, and at 0.995 a_w/20 °C for *F. meridionale* strains (17.2 µg NIV/g). The *F. meridionale* strains also produced negligible DON levels compared to strains of the other two species. The *F. graminearum sensu stricto* strains produced DON in the range 0.95–0.995 a_w/15–30 °C while *F. boothii* and *F. meridionale* strains did so in a narrower interval of 0.97–0.995 a_w/15–25 °C. NIV contamination appeared in the range 0.95–0.995 a_w/15–30 °C. Multifactorial ANOVA performed on the DON levels produced by the 12 strains showed a significant influence of species, followed by temperature and finally water activity (Table 4). A similar result was obtained in multifactorial ANOVA based on the DON levels of the *F. graminearum sensu stricto* and *F. boothii* strains and the NIV levels of the *F. meridionale* strains (not shown). Intraspecific variations in trichothecene production were not significant.

4. Discussion

The strains shared the same optimal growth conditions irrespective of the species assayed. These data agree with reports indicating the highest growth rates at water activity of 0.995 a_w and 25 °C for strains of *F. graminearum sensu lato* on gamma-irradiated wheat grains and cereal-based media (Hope et al., 2005; Neagu and Borda, 2013; Ramirez et al., 2006). However, optimal growth at 30 °C or 25–30 °C in yeast extract-sucrose agar was also reported (Schmidt-Heydt et al., 2011). In the case of *F. meridionale,* strains isolated from Argentinian soybean and Brazilian winter cereals (wheat and barley) showed the highest growth rates at 0.98–0.99 a_w/25 °C in milled soybean agar (Rybecky et al., 2018) and at 1 a_w/25–30 °C in potato-dextrose agar (Spolti et al., 2012), respectively. Although our assays indicated that water activity had the strongest influence on growth of all the fungal strains, the interspecific differences were evidently associated with the interaction of water activity and temperature in marginal growth conditions. In the range of 0.90–0.995 a_w/15–30 °C, the *F. meridionale* strains showed 20 to 100% lower growth rates than the other strains. Assays previously performed with *F. meridionale* and *F. graminearum sensu stricto* strains in a range of 10–35 °C showed lower growth rates for *F. meridionale* strains, but only at 25 °C and 30 °C (Spolti et al., 2012). However, these data cannot be directly compared with our results because they were obtained in an artificial medium (potato-dextrose agar) at 1 a_w. The growth response of the *F. graminearum sensu stricto* strains observed at 30 °C indicates that they have a better adaptation for survival in warm environments than *F. meridionale* or *F. boothii* strains.

Temperature was a major factor driving both NIV and DON production by the fungal strains on the irradiated maize grains. As reported

Table 3
Mean trichothecene production by *Fusarium graminearum sensu stricto*, *F. meridionale* and *F. boothii* on irradiated maize grains.

Species	a_w	15 °C	20 °C	25 °C	30 °C
Deoxynivalenol levels (μg/g) ± SD					
Fusarium graminearum sensu stricto	0.95	1.5 ± 0.1^{b}	3.0 ± 0.1^{c}	3.5 ± 0.1^{f}	4.5 ± 0.3^{d}
	0.97	5.4 ± 0.2^{e}	21.8 ± 0.3^{j}	34.3 ± 0.2^{k}	40.9 ± 0.5^{l}
	0.995	5.9 ± 0.1^{e}	4.7 ± 0.5^{d}	5.8 ± 0.1^{e}	47.5 ± 0.7^{l}
Fusarium boothii	0.95	ND^{a}	ND^{a}	ND^{a}	ND^{a}
	0.97	1.8 ± 0.2^{b}	3.7 ± 0.3^{f}	0.5 ± 0.1^{g}	ND^{a}
	0.995	2.8 ± 0.3^{c}	3.7 ± 0.2^{f}	0.7 ± 0.2^{g}	ND^{a}
Fusarium meridionale	0.95	ND^{a}	ND^{a}	ND^{a}	ND^{a}
	0.97	0.1 ± 0.0^{h}	0.1 ± 0.1^{h}	0.2 ± 0.1^{h}	ND^{a}
	0.995	0.1 ± 0.0^{h}	1.7 ± 0.2^{b}	0.1 ± 0.0^{h}	ND^{a}
Nivalenol levels (μg/g) ± SD					
Fusarium meridionale	0.95	0.4 ± 0.1^{z}	3.1 ± 0.4^{q}	4.5 ± 0.2^{u}	2.1 ± 0.2^{w}
	0.97	3.2 ± 0.3^{q}	2.6 ± 0.1^{q}	5.0 ± 0.3^{u}	2.2 ± 0.1^{w}
	0.995	1.7 ± 0.2^{w}	17.2 ± 0.2^{y}	4.7 ± 0.1^{u}	4.1 ± 0.5^{u}

ND: not detected. Detection limit: 0.005 μg/g. SD: standard deviation. Different letters in a same column indicate significant differences in the trichothecene accumulation ($p < 0.05$). Each value of trichothecene content was calculated from data of four strains.

Table 4
Analysis of variance of the effect of water activity, temperature, species and strain on DON production of the fungal strains on irradiated maize grain.

Source of variation	df[a]	MS[b]	F[c]
a_w	2	0.20	11.61*
Species	2	16.01	928.51*
Temperature	3	0.97	56.05*
Strain	9	0.03	1.52
a_w × species	4	0.83	48.17*
a_w × temperature	6	0.30	17.21*
Species × temperature	6	0.67	38.61*
a_w × species × temperature	12	0.33	19.37*

[a] Degrees of freedom.
[b] Mean square.
[c] F Snedecor.
* Significant at $p < 0.05$ level.

for several *Fusarium* species, the highest production of trichothecenes always occurred under environmental conditions which were stressful for mycelial growth (Medina and Magan, 2011). However, warm temperatures were required by *F. graminearum* strains while the *F. boothii* and *F. meridionale* strains needed mild ones. Data from other reports regarding optimal and marginal conditions for trichothecene production of strains of the Fg complex cannot be directly compared with our results because their strains were not classified at the species level, or the assays differed in incubation times, inocula (i.e. macroconidia), substrates and/or other environmental conditions (Comerio et al., 1999; Llorens et al., 2004; Martins and Martins, 2002; Mylona et al., 2012; Ramirez et al., 2006; Rybecky et al., 2018; Vesonder et al., 1982). For example, Ramirez et al. (2006) found that DON production was favored at 0.995 a_w/30 °C in two strains of *F. graminearum sensu lato* isolated from wheat of central Argentina, presumably *F. graminearum sensu stricto* strains, grown on irradiated wheat grains for 28 days (0.90–0.995 a_w/15–30 °C). However, the highest DON contents (3.5 μg/g) were several times lower than those recorded in this work. Rybecky et al. (2018) assayed three strains isolated from soybean and grown in milled soybean agar for 21 days (0.95–0.995 a_w/20–30 °C) of which two strains were DON producers, with the highest mean value of 33.5 μg/g at 0.96 a_w/25 °C. The three were NIV producers with top levels at water availabilities of 0.98 a_w (55.7 μg/g) or 0.995 a_w (45.0 μg/g) and 20 °C, and at 0.98 a_w/30 °C (28.5 μg/g). To the best of our knowledge, there are no reports regarding the *F. boothii* strains. Regarding the marginal conditions for growth and trichothecene contamination, our results indicate that the risk of trichothecene contamination under specific environmental conditions varies according to the fungal species infecting the maize grains. For example, *F. meridionale* or *F. graminearum sensu stricto* strains should have the same chance to generate significant NIV or DON contamination from 15 °C to 30 °C, while *F. boothii* strains should be a hazard only at higher water activities in a narrower range of temperatures.

In summary, the growth of *F. meridionale* and *F. boothii* strains on maize grains was better adapted to mild temperatures, and more sensitive at low temperatures to water stress. High water availabilities and mild temperatures usually occur during autumn in northwest Argentina (Belizán et al., 2018). The prevalence of these environmental conditions may be the reason for the strong incidence and severity of GER in this part of the country, increasing the risk of trichothecene contamination. The *F. graminearum sensu stricto* strains were adapted to a wider range of environmental conditions than the strains of the remaining species and were high trichothecene producers on maize grains. Both features suggest that *F. graminearum sensu stricto* has a high fitness to generate GER in the northwest, although further research is needed. Control strategies should be considered during grain development to reduce the risk of the presence of DON and NIV in the harvested grains.

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