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Effect of water activity and temperature on growth and trichothecene production by *Fusarium meridionale*

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ABSTRACT

Fusarium meridionale has been frequently isolated from soybean in Argentina and showed similar pathogenicity as *F. graminearum* sensu stricto. However, no data on their growth and mycotoxin production under different environmental conditions are yet available. The aims of this study were: to determine the effect of temperature, water activity (a_W) and strain on growth of *F. meridionale* and to evaluate deoxynivalenol (DON) and nivalenol (NIV) production in a soybean based medium. The results showed that optimal conditions for *F. meridionale* growth were at 25 °C and 0.98–0.99 a_W. Deoxynivalenol production was favored at 25 °C and 0.96 a_W while NIV production was strain-dependent, being 30 °C and 0.98 a_W optimal conditions for *F. meridionale* B2300 strain and 20 °C and 0.98 a_W for *F. meridionale* F5043 and *F. meridionale* 5048 strains. These conditions are similar to those observed at pre-harvest stage in soybean crop, thus control strategies need to be considered to reduce the risk of the occurrence of DON and NIV in harvested grains.

1. Introduction

Argentina is the third worldwide soybean producer and the main country as exporter of processed products such as oils and protein meals. During 2017 season, the area planted reached 20 million hectares with an estimated production of 57.3 million tons and an average yield of 31.9 quintal/ha (Agrofy, 2016). The optimal production depends on the amount of rain water (400–600 mm) during the crop cycle and temperatures ranging from 25 to 30 °C during the day and from 18 to 25 °C at night (Melgar et al., 2011). These conditions could be optimal for *Fusarium* species development and mycotoxin production on grains in the field affecting the final crop yield and/or with a potential risk of mycotoxin accumulation in the harvested grains (Munkvold, 2017).

Fusarium graminearum species complex (FGSC) infect soybean crop showing symptoms of infection such as seed and root rot and pre- and post-emergence damping off (Barros et al., 2012, 2014; Chiotta et al., 2016; Martinelli et al., 2004; Pioli et al., 2004). These species produce type B trichothecenes such as deoxynivalenol (DON), nivalenol (NIV) and its acetylated forms (Desjardins, 2006). 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). In addition, these mycotoxins are phytotoxins and function as virulence factors on some cereals (Jansen et al., 2005; Maier et al., 2006). Due to DON toxicity, international legislations have been set to limit and restrict the contamination of food to protect consumer health. The European Commission set legislative limits for the main mycotoxins produced by *Fusarium* species in grain-based foodstuffs intended for human consumption being the limit for DON between 200 and 750 μg/kg (European Commission 1881, 2006). No legislative limit has been set for NIV as the amount of NIV usually follows closely the levels of DON and thus it is envisaged that the legislation for DON will prevent unacceptable exposure to this toxin.

Several studies carried out in different American countries found that *Fusarium graminearum* sensu stricto is frequently isolated from soybean (Broders et al., 2007; Ellis et al., 2011; Xue et al., 2006, 2007). Recently, a phylogenetic study of FGSC was carried out in Argentina in order to identify trichothecene producers species isolated from soybeans. The data showed that *F. graminearum* sensu stricto was frequently isolated followed by *F. meridionale* (Barros et al., 2012; Chiotta et al., 2015). *Fusarium graminearum* sensu stricto showed the dominant chemotype 15-ADON whereas *F. meridionale* showed an unusual pattern

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of trichothecenes with simultaneous DON and NIV production. Later, Chiotta et al. (2016) compared the pathogenicity of *F. graminearum* and *F. meridionale* isolated from soybean under field conditions and evaluate the trichothecene production of these two phylogenetic species which express different chemotypes. The results indicated that *F. graminearum* strains produced DON and 15-ADON contamination in seeds during two crop seasons, whereas *F. meridionale* strains with DON/NIV chemotype were unable to produce detectable mycotoxin levels under the conditions evaluated. This data suggest that the trichothecene production by *F. meridionale* could not have been favored under the environmental conditions registered during both seasons.

Previously, the influence of environmental factors on *F. graminearum* growth and DON production on different culture media have been evaluated (Garcia et al., 2012; Martins and Martins, 2002; Ramirez et al., 2004, 2006). These studies showed that optimal growth rate for *F. graminearum* was between 20 and 25 °C and 0.995 a_W and optimal DON production was between 15 and 30 °C and 0.97–0.995 a_W. However, no data on *F. meridionale* growth and DON and NIV production are available. The aim of this study was to evaluate the growth and toxin accumulation by *F. meridionale* under different temperatures and a_W conditions on a culture medium based on soybean.

2. Materials and methods

2.1. Fungal strains

Fusarium meridionale F5043 and F5048 strains with DON/NIV genotypes/chemotypes *and F. meridionale* B2300 strain with NIV genotype/chemotype, previously isolated from soybean in Argentina and Brazil respectively, were selected for the present study (Barros et al., 2012). Molecular characterization of these strains was performed by sequencing of 1α elongation factor (EF-1α, 725 bp), 3-*O*-acetyltransferase (Tri101, 1329 bp) and reductase (RED, 993 Pb) genes (Chiotta et al., 2015). All the isolates were maintained as spore suspensions in 15% glycerol (w/v) at −80 °C. Chemotype confirmation of the isolates was tested previous to this study.

2.2. Inoculum and medium preparation

Inocula were prepared by growing colonies of each strain on Spezieller Nährstoffarmer Agar (SNA; Nirenberg, 1976) for 7 days at 25 °C with periods of 12 h of white light and 12 h of black light (Leslie and Summerell, 2006). Each strain was inoculated by seeding an agar plug (5 mm) taken from the margin of SNA colonies onto centre of a plate containing 2% (w/v) milled soybean agar. The initial a_w of medium was 0.99 and glycerol was added to obtain a_w levels of 0.98, 0.96 and 0.95 (AquaLab Series 3, Decagon Devices, Inc., WA, USA). Plates of the same a_w were incubated in low density polyethylene bags to minimize moisture loss while allowing free gaseous exchange. The cultures were incubated at 20, 25 and 30 °C, in combination with the different a_w tested. Temperatures and a_w ranges used in this study simulate those registered in seeds throughout R6 stage (full seed) during previous years in the Río Cuarto - Córdoba region, where *F. graminearum* species complex were isolated more frequently (Barros et al., 2012; Chiotta et al., 2015b). The experiments were done in triplicate and repeated twice.

2.3. Lag phase and growth assessment

Growth was assessed every day during a 21 day period using a binocular magnifier. Two diameters of the growing colonies were measured at right angles in two directions to each other until the colony reached the edge of the plate. The radii of the colonies were plotted against time, the growth rate (mm/day) was calculated by linear regression as the slope of the regression line. The lag phase was defined as the mean time (hours) at which each colony reached 5 mm in diameter for each treatment.

2.4. Deoxynivalenol and nivalenol analysis

For DON and NIV extraction, three agar plugs (diameter 5 mm) were taken along the radius from colonies after 7, 14 and 21 days of incubation, placed in a vial with 1 mL of acetonitrile and shaken for 5 seg. After 60 min, the vials were shaken again and the extracts filtered (Whatman No. 4), blown to dryness under nitrogen and stored at 4 °C until analysis by HPLC. Plug extraction was performed in triplicate. 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 (Waters e2695 separations module, Milford, MA, USA). Chromatographic separations were performed on a stainless steel, C18 reversed-phase column (150 × 4.6 mm, 5 µm particle size; Luna-Phenomenex) connected to pre-column (20 × 4.6 mm, 5 µm particle size, Luna-Phenomenex). Mycotoxins were detected by UV at 220 nm using a Waters 2998 diode array detector. The mobile phase was water:methanol (88:12, v/v) at a flow rate of 1.5 mL/min and the detection limit (LOD) was 0.1 µg/g for DON and 0.2 µg/g for NIV, based on a signal:noise ratio of 3:1. The retention times of NIV and DON were 4.6 min and 10.1 min, respectively.

2.5. Data analysis

Lag phase, growth rate and trichothecene concentration were evaluated by analysis of variance ANOVA test, followed by Tukey mean separation test ($p < 0.05$). Trichothecene data were transformed to $\log_{10}$ to obtain the homogeneity of variance. A factorial design was performed to determine the main parameters affecting growth and trichothecene concentration. Statistical analyses were performed using InfoStat (2008) and SigmaStat version 3.5 (SPSS, Chicago, USA) programs.

3. Results

3.1. Effect of temperature and a_w on lag phase

The effect of temperature and a_W on lag phase of *F. meridionale* is showed in Table 1. The lag phases of all strains showed the same behavior, decreased with increased a_W, except at 0.99 and 30 °C in which *F. meridionale* F5043 and F5048 strains had longer lag phases than 0.98 and 0.96 a_W. As regard temperature, *F. meridionale* B2300 and F5348 strains showed the shortest lag phases at 25 °C while *F. meridionale* F5343 at 20 °C and 25 °C. The longest lag phases were observed at 30 °C

Table 1
Mean lag phase (h) of *Fusarium meridionale* strains on soybean based medium under different environmental conditions.

Strain	a_W	20 °C	25 °C	30 °C
		Lag phase ± SD	Lag phase ± SD	Lag phase ± SD
F. meridionale B2300	0.950	161.3 ± 9.5^{f}	86.1 ± 23.2^{c}	252.7 ± 66.0^{h}
	0.965	114.5 ± 5.2^{d}	60.1 ± 1.0^{b}	141.1 ± 19.5^{e}
	0.982	70.7 ± 2.9^{b}	49.0 ± 5.3^{a}	66.1 ± 4.6^{b}
	0.995	67.8 ± 1.9^{b}	36.6 ± 1.6^{a}	197.1 ± 14.1^{g}
F. meridionale F5343	0.950	133.5 ± 10.2^{c}	72.0 ± 3.7^{b}	176.4 ± 22.1^{f}
	0.965	102.0 ± 1.8^{c}	63.2 ± 2.7^{b}	102.1 ± 5.7^{c}
	0.982	67.95 ± 7.3^{b}	46.8 ± 0.8^{a}	97.9 ± 3.0^{c}
	0.995	55.5 ± 6.9^{a}	53.6 ± 1.5^{a}	116.7 ± 6.3^{d}
F. meridionale F5348	0.950	158.3 ± 8.7^{d}	83.1 ± 24.3^{b}	209.3 ± 14.6^{e}
	0.965	116.8 ± 15.5^{c}	57.9 ± 18.1^{b}	116.9 ± 8.4^{c}
	0.982	74.8 ± 5.3^{b}	39.3 ± 2.4^{a}	109.7 ± 24.7^{c}
	0.995	57.9 ± 1.4^{b}	32.7 ± 0.7^{a}	116.8 ± 3.8^{c}

Values with the same letter do not differ significantly ($p < 0.05$).
SD: standard deviation.

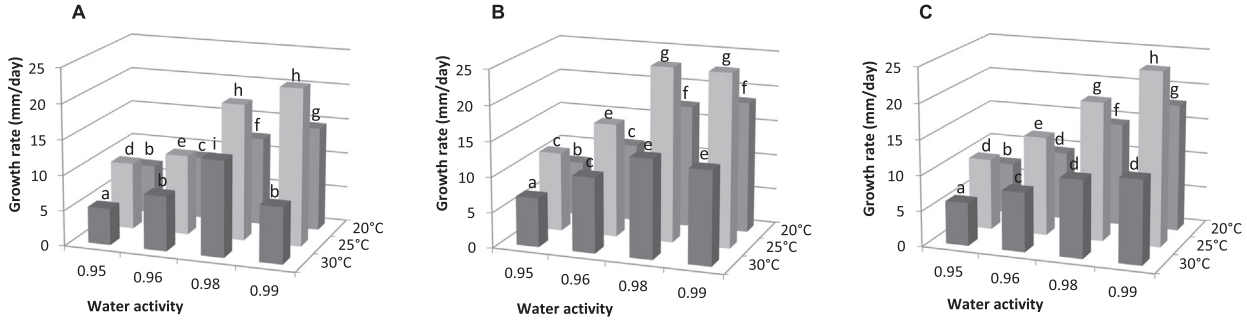

Fig. 1. Effect of different water activities and temperatures on growth rate (mm/day) of *Fusarium meridionale* B2300 (A), F5043 (B) and F5048 (C) strains on soybean based medium. Different letters over bars indicate significant differences in the fungal growth ($p < 0.05$).

for all the strains.

3.2. Effect of temperature and a_w on growth rates

The data obtained on growth rates showed that optimal conditions for *F. meridionale* strains were at 25 °C and 0.98–0.99 a_W (Fig. 1). For all strains, a higher growth was observed at 20 °C than at 30 °C and growth rate increased with increased a_W, except at 0.99 a_W and 30 °C for *F. meridionale* B2300 strain ($p < 0.005$). The analysis of variance showed statistically significant differences between treatments in relation to *F. meridionale* strains growth rates. The analysis on the effect of each factor and the combination of factors showed that both temperature and a_W affect significantly the growth in both species evaluated.

3.3. Effect of temperature and a_w on deoxynivalenol and nivalenol production

The optimal condition for DON production by *F. meridionale* F5043 and F5048 were 25 °C and 0.96 a_W, reaching maximum levels at 21 days of incubation (Table 2). At the other temperatures evaluated, both strains did not show high toxin levels being the production higher at 30 °C than at 20 °C. At 30 °C and high a_W, DON production was favored while different production pattern was observed at 20 °C for each strain. At 20 °C, DON production for *F. meridionale* F5043 was detected at low a_W whereas for *F. meridionale* F5048 was observed at high a_W. No DON production by *F. meridionale* B2300 was detected.

As regard NIV production, *F. meridionale* B2300 produced the highest levels at 30 °C and 0.98 a_W at 21 day of incubation (mean = 28.5 ppm) (Table 3). At 25 °C the highest levels were detected at higher a_W while at 20 °C the levels were low compared with the other temperatures evaluated, a maximum production was reached at 0.98 a_W and 21 days of incubation. The maximum NIV production for *F. meridionale* F5043 and *F. meridionale* F5048 was observed at 20 °C and 0.98 a_W, at 21 and 14 days of incubation, respectively. For *F. meridionale* F5043, the highest a_W was observed at 25 °C and 30 °C, with higher concentrations at 25 °C and increasing with incubation days. Similar results were obtained for *F. meridionale* F5048, but the highest levels were detected at 0.99 a_W.

Statistical analyses showed that temperature and a_W were the most important factors on DON and NIV production by *F. meridionale*.

4. Discussion

The results showed that optimal conditions for *F. meridionale* growth were 25 °C and high water activity. For all strains a decrease in growth rate with reduced a_W was observed but a not characteristic pattern with temperature was obtained. At 20 °C the strain showed high growth than 30 °C. These conditions were different to those described for *F. graminearum* by Garcia et al. (2012), which evaluated the impact of cyclic temperatures on its growth on 2% milled soybean agar. The authors found that *F. graminearum* growth rate was higher in this medium at 20 °C and at temperature cycle of 15/20 °C. No growth was recorded at 30 °C and under the combination of temperatures 25/30 °C. Our results showed that *F. meridionale* grows better at intermediate temperatures, with higher growth capacity at high temperatures in soybean agar in comparison to *F. graminearum*. These results could explain the higher incidence of *F. meridionale* in both soybean and maize samples collected from Brazil and maize samples from Northern Argentina, where these crops are exposed to higher temperatures (Chiotta et al., 2015; Del Ponte et al., 2012; Martinelli et al., 2004; Sampietro et al., 2011). However, in eco-physiological studies performed in wheat was observed that *F. graminearum* growth is favored at 25 °C (Hope et al., 2005; Ramirez et al., 2006) although these strains were not characterized by phylogenetic studies. Thus, it is makes difficult to determine to which species of *F. graminearum* complex these strains belonged. Since the toxin profiles of an individual species vary and define the toxicological potential risk, it is important the precise identification at species level.

Table 2
Mean deoxynivalenol production by *Fusarium meridionale* on soybean based medium.

Strains	Deoxynivalenol levels (μg/g) ± SD									
	a_W	20 °C		25 °C				30 °C		
		7 days	14 days	21 days	7 days	14 days	21 days	7 days	14 days	21 days
F. meridionale F5043	0.950	ND^{a}	6.6 ± 0.3^{f}	11.5 ± 0.6^{j}	1.8 ± 0.2^{c}	2.4 ± 0.2^{c}	17.2 ± 0.3^{n}	ND^{a}	ND^{a}	ND^{a}
	0.965	ND^{a}	2.1 ± 0.1^{c}	6.5 ± 0.2^{f}	6.3 ± 0.4^{f}	35.7 ± 0.5^{v}	32.4 ± 0.5^{t}	ND^{a}	ND^{a}	12.0 ± 1.0^{j}
	0.980	ND^{a}	ND^{a}	ND^{a}	10.3 ± 0.8^{i}	16.4 ± 0.1^{m}	29.6 ± 0.2^{r}	ND^{a}	5.1 ± 0.2^{e}	7.6 ± 0.5^{g}
	0.995	ND^{a}	ND^{a}	5.2 ± 0.1^{e}	9.1 ± 0.5^{h}	21.7 ± 0.7^{p}	31.7 ± 0.2^{s}	ND^{a}	ND^{a}	14.4 ± 0.1^{l}
F. meridionale F5048	0.950	ND^{a}	ND^{a}	ND^{a}	3.6 ± 0.6^{d}	24.7 ± 0.7^{q}	12.9 ± 0.3^{k}	ND^{a}	4.8 ± 1.0^{e}	0.9 ± 0.0^{b}
	0.965	ND^{a}	ND^{a}	ND^{a}	38.5 ± 0.2^{w}	46.6 ± 0.2^{x}	21.1 ± 0.1^{p}	ND^{a}	0.6 ± 0.5^{b}	5.0 ± 0.1^{e}
	0.980	ND^{a}	ND^{a}	2.5 ± 0.5^{c}	15.7 ± 0.2^{m}	38.6 ± 0.2^{w}	34.6 ± 1.5^{u}	ND^{a}	ND ± 0.2^{a}	3.6 ± 0.5^{d}
	0.995	ND^{a}	ND^{a}	7.3 ± 0.3^{g}	18.8 ± 0.3^{o}	12.8 ± 0.4^{k}	11.4 ± 0.8^{j}	ND^{a}	ND ± 0.1^{a}	17.0 ± 0.6^{n}

ND: not detected. Detection limit: 0.1 μg/g. SD: standard deviation. Different letters over bars indicate significant differences in the fungal growth ($p < 0.05$).

Table 3
Mean nivalenol production by *Fusarium meridionale* on soybean based medium.

Strains	Nivalenol levels (μg/g) ± SD									
	a_W	20 °C			25 °C			30 °C		
		7 days	14 days	21 days	7 days	14 days	21 days	7 days	14 days	21 days
F. meridionale B2300	0.95	ND^{a}	ND^{a}	1.4 ± 0.1^{c}	ND^{a}	ND^{a}	3.7 ± 0.1^{e}	ND^{a}	ND^{a}	ND^{a}
	0.96	ND^{a}	2.8 ± 0.1^{d}	3.6 ± 0.5^{e}	ND^{a}	15.2 ± 0.2^{m}	4.4 ± 0.1^{f}	ND^{a}	8.9 ± 1.1^{i}	4.5 ± 0.2^{f}
	0.98	ND^{a}	ND^{a}	6.9 ± 0.7^{gh}	ND^{a}	20.5 ± 0.5^{r}	17.5 ± 0.0^{o}	3.6 ± 0.4^{e}	ND^{a}	28.5 ± 0.5^{t}
	0.99	ND^{a}	ND^{a}	8.0 ± 0.2^{i}	ND^{a}	0.4 ± 0.3^{b}	23.7 ± 0.5^{s}	ND^{a}	0.7 ± 0.2^{b}	7.3 ± 0.4^{h}
F. meridionale F5043	0.95	ND^{a}	ND^{a}	5.0 ± 0.2^{g}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}
	0.96	9.1 ± 0.4^{i}	15.2 ± 0.8^{m}	ND^{a}	ND^{a}	ND^{a}	16.2 ± 0.1^{n}	ND^{a}	ND^{a}	ND^{a}
	0.98	20.4 ± 0.3^{r}	28.7 ± 0.1^{t}	55.7 ± 1.5^{y}	10.5 ± 0.2^{j}	8.7 ± 0.5^{i}	28.2 ± 0.8^{t}	ND^{a}	4.3 ± 0.2^{f}	6.2 ± 0.6^{g}
	0.99	ND^{a}	3.8 ± 0.2^{e}	12.5 ± 0.5^{k}	ND^{a}	ND^{a}	6.9 ± 0.5^{gh}	ND^{a}	ND^{a}	5.1 ± 0.2^{g}
F. meridionale F5048	0.95	ND^{a}	ND^{a}	ND^{a}	2.2 ± 0.3^{c}	2.6 ± 0.2^{d}	1.8 ± 0.5^{c}	ND^{a}	0.5 ± 0.4^{b}	ND^{a}
	0.96	7.0 ± 1.0^{h}	3.8 ± 0.5^{e}	ND	ND^{a}	13.2 ± 0.1^{l}	4.2 ± 0.0^{f}	ND^{a}	ND^{a}	ND^{a}
	0.98	31.8 ± 0.2^{u}	74.2 ± 0.2^{z}	35.7 ± 0.0^{v}	ND^{a}	ND^{a}	5.8 ± 0.9^{g}	ND^{a}	ND^{a}	3.5 ± 0.5^{e}
	0.99	3.7 ± 0.3^{e}	48.3 ± 0.5$^{\times}$	45.0 ± 0.8^{w}	18.3 ± 0.0^{p}	19.4 ± 0.1^{q}	15.4 ± 0.2^{m}	ND^{a}	ND^{a}	10.1 ± 0.3^{j}

ND: not detected. Detection limit: 0.2 μg/g. SD: standard deviation. Different letters over bars indicate significant differences in the fungal growth ($p < 0.05$).

Deoxynivalenol accumulation by *F. meridionale* on soybean agar was higher at 25 °C and 0.96 a_W. Temperature was the key factor affecting DON production. The temperature conditions that favor *F. meridionale* growth were also favorable for DON production, even under a wide range of water activities. Similar results were obtained for *F. graminearum* on soybean based medium (Garcia et al., 2012). In other studies on DON production by *F. graminearum* on wheat grains was also observed that DON was high at temperatures of 15 and 25 °C and at low water activities (0.97) (Hope et al., 2005; Ramirez et al., 2007).

As regards NIV production, the strains had a different toxicogenic production profile with major similarities observed between *F. meridionale* F5043 and F5048 strains. These results could be explained by the fact that both strains were isolated from the same geographical region in Argentina, while *F. meridionale* strain B2300 was isolated from Brazil. Data showed that the optimal conditions for NIV production was dependent on the strain evaluated and water activity significantly influences toxin production on soybean medium. Since this study is the first report on the effect of environmental conditions on NIV production by *F. meridionale* and on soybean medium, there are not data to compare our results. Previous findings where NIV production by *F. culmorum* strains was used, it was founded that toxin production was mostly influenced by temperature, while water activity did not affect its production on a corn-based medium (Llorens et al., 2004). Similar results were obtained by Hope and Magan (2003), who demonstrated that incubation temperature and time significantly influenced the NIV production by *F. culmorum* strains on a wheat-based medium, with higher production at 15 °C than at 25 °C. These differences could be related to the factors inherent to the substrate used and/or the species evaluated.

The conditions under which *F. meridionale* was able to growth and to produce mycotoxins are those observed at pre-harvest stage in soybean crop. Control strategies during grain development need to be taken into account to reduce the risk of the presence of these toxins in harvested grains.

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