Research Article

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Impact of cycling temperatures on *Fusarium verticillioides* and *Fusarium graminearum* growth and mycotoxins production in soybean

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Abstract

BACKGROUND: *Fusarium graminearum* and *F. verticillioides* are two very important mycotoxigenic species as they cause diverse diseases in crops. The effects of constant and cycling temperatures on growth and mycotoxin production of these species were studied on soybean based medium and on irradiated soya beans.

RESULTS: *F. graminearum* grew better when was incubated at 15, 20 and 15–20 °C (isothermal or cycling temperature) during 21 days of incubation. Maximum levels of zearalenone and deoxynivalenol (39.25 and 1040.4 μg g^{-1}, respectively) were detected on soya beans after 15 days of incubation and the optimal temperature for mycotoxin production was 15 °C for zearalenone and 20 °C for deoxynivalenol. *F. verticillioides* grew better at 25 °C in culture medium and at 15/20 °C and 15/25 °C on soybean seeds. Fumonisin B_1 was produced only in culture medium, and the maximum level (7.38 μg g^{-1}) was found at 15 °C after 7 days of incubation.

CONCLUSION: When growth and mycotoxin production under cycling temperatures were predicted from the results under constant conditions, observed values were different from calculated for both species and substrate medium. Therefore, care should be taken if data at constant temperature conditions are to be extrapolated to real field conditions.

Keywords: Fusarium graminearum; Fusarium verticillioides; cycling temperature; growth, mycotoxins

INTRODUCTION

Soybean (*Glycine max* L.) is a species of legume, originally from Asia, which is used particularly for oil and flour production.[1] This commodity is the main source of protein used for food and feedstuffs throughout the world.[2] Argentina is the third largest producer of soybean; however, it is the main exporter of the by-products, where 57% of flour exports are destined for the European Union.[3] About 34% of soybean oil is exported to China, 20% to India and the rest to countries such as Venezuela, Egypt, Peru and South Africa.[4]

Soybean is often infected by fungi during cultivation or postharvest (in transit or in storage), which significantly affects its productivity.[5] Fungal contamination can cause damage in cereal grains and oilseeds, such as low germination, discolouration, heat, wilt, rot and mycotoxin occurrence. Fungal growth and mycotoxin production are influenced by different factors such as temperature, substrate aeration, water activity (a_w), inoculum concentration, microbial interactions, physiological state of mould, etc.[6] *Alternaria* and *Fusarium* species are the most commonly isolated fungi from soybean in Argentina and in other regions of the world.[7–10] *Fusarium* species have a large degree of morphological, physiological and ecological diversity and are considered the most important plant pathogens worldwide.[11] Among the *Fusarium* species a few are responsible for mycotoxin production; *F. graminearum* is the main producer of deoxynivalenol (DON) and zearalenone (ZEA), while *F. verticillioides* is mainly responsible for the production of fumonisins (FBs).[11]

Many studies have been published during the last few years describing the growth of mycotoxigenic fungi as a function of environmental factors. Some of them included predictive models for fungal growth under constant conditions of either temperature or a_w.[12–18] However, variation of temperature occurs during the development of soybean plants at field stage when *Fusarium* species can colonise this legume. Besides, field temperature fluctuation will result in modification of the pattern of growth and mycotoxins production. For this reason, the objectives of the present study were: (1) to determine the impact of different constant temperature regimes and cycling temperatures on growth and mycotoxin production on soybean-based medium and on irradiated soya beans by two strains

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of *F. verticillioides* and *F. graminearum* isolated from soybean in Argentina; and (2) to asses if growth and toxin production at cycling temperatures could be predicted from data at constant temperature.

MATERIAL AND METHODS

Fungal isolates

One strain of *F. verticilliodes* (F5017) and one of *F. graminearum* (F5050) (*F. graminearum sensu stricto* (line 7)) isolated from soybean in Argentina were evaluated. The strains are kept in the culture collection at Department of Microbiology and Immunology, Universidad Nacional de Río Cuarto Culture Collection. The isolate of *F. verticillioides* had proven ability to produce fumonisins, while *F. graminearum* had been proven to produce ZEA and DON.[10] The isolates were sub-cultured on carnation leaves agar at 25 °C for 7 days to enable significant sporulation.

Medium

A 2% (w/v) milled soybean agar was used in this study (0.99 a_w). The medium was autoclaved at 121 °C 1 atm for 20 min and poured into 9-cm sterile Petri dishes. The a_w of the medium was checked with an AquaLab Series 3 (Labcell Ltd., Pullman, WA, USA) with an accuracy of ±0.003.

Preparation of soya beans

Soya beans (14.5% moisture content, 1 kg batches) were gamma irradiated (7 kGy) using a cobalt radiation source and stored aseptically at 4 °C. Beans were adjusted at 0.99 a_w by aseptically adding sterile distilled water simulating a_w at the filling step (1.47 mL g^{-1} to the beans in sterile bottles). The bottles were cooled to 4 °C for 48 h with periodically hand-shaking during this time. Final a_w values of each bottle were checked with an AquaLab Series 3 (Labcell Ltd) with an accuracy ±0.003. Then, approx. 25 g of seeds were placed in single layers into Petri dishes.

Inoculation and incubation conditions

Petri dishes were inoculated centrally with a 4 mm diameter agar disc taken from the margin of a 7-day-old colony of each isolate on synthetic nutrient agar[19] at 25 °C and transferred face down to the centre of each plate. Inoculated plates of the same a_w were sealed in containers along with beakers containing a water glycerol solution of the same a_w as the plates in order to maintain the a_w.[20] All plates were incubated at 15, 20, 25 and 30 °C and at 12-h cycling intervals at temperatures 15 °C/20 °C; 15 °C/25 °C and 20 °C/30 °C. Temperatures were selected based on mean temperature at R6 soybean growth stage (full seed) where 15 °C was the minimum and 30 °C maximum average temperatures registered in previous years in the Río Cuarto, Córdoba, region. The experiments were repeated three times, for both culture medium and soya beans. Moreover, separated sets of treatments were prepared for each sampling period: 7, 15 and 21 days.

Growth assessment

For both soybean seeds and agar medium, fungal growth was observed on a daily basis for an overall period of 21 days and diameter measurements carried out at right angles with the aid of a ruler and a binocular magnifier.[21]

Mycotoxins analysis from soybean-based medium

Extraction

Three agar plugs (diameter 5 mm) of each colony were removed from the inner, middle and outer parts of the colonies after 7, 15 and 21 days of incubation and placed in a vial for each repeated experiment. One millilitre of methanol for FBs and 1 mL of acetonitrile for DON and ZEA were added, and the vials were shaken for 5 s and allowed to rest. After 60 min, the vials were shaken again and extracts filtered (Whatman N° 4) into another vial and stored at 4 °C until analysis by HPLC (Waters, Milford, MA, USA). Plug extraction was performed in duplicate.

Detection and quantification

All mycotoxins were detected and quantified separately by using a HPLC system (Waters 2695, separations module). Chromatographic separations were performed on a stainless steel C_{18} reversed-phase column (250 mm × 4.6 mm i.d., 50 µm particle size; Waters Spherisorb, Dublin, Ireland) connected to a security guard cartridge (10 mm × 4 mm i.d., 5 µm particle size; Waters Spherisorb). Injection volume was 100 µL by automatic injector. For fluorescence detection of fumonisins and zearalenone a Waters 2475 module was used and for absorbance detection of DON a Waters 2487 module was employed. Quantification was always achieved using a software integrator (Empower, Milford, MA, USA). The mycotoxin levels were calculated by comparing the area of the chromatographic peak of the sample with those of the standard calibration curve.

Fumonisins B_1 and B_2. FBs were detected by fluorescence (λ_{exc} 335 nm; λ_{em} 440 nm). The mobile phase was methanol: 0.1 mmol L^{-1} sodium dihydrogen phosphate (77 : 23) solution adjusted to pH 3.35 with orthophosphoric acid. Dried extracts were dissolved in methanol and derivatised with *o*-phthaldialdehyde.[22] Detection limit of the analysis was about 0.01 ng g^{-1} of culture medium for FB_1 and 0.002 ng g^{-1} of culture medium for FB_2, based on a signal-to-noise ratio of 3 : 1. The range of FBs standards used for quantification was 0.015–5 µg mL^{-1}.

Zearalenone. ZEA detection was achieved by fluorescence (λ_{exc} 274 nm; λ_{em} 445 nm). The mobile phase was acetonitrile/water (60 : 40). Samples were dissolved in mobile phase. Detection limit of the analysis was about 0.017 ng g^{-1} of culture medium, based on a signal-to-noise ratio of 3 : 1. The range of ZEA standards used for quantification was 5–25 ng mL^{-1}.

Deoxynivalenol. DON was detected by absorbance (λ 220 nm). The mobile phase was water/acetonitrile/methanol (90 : 5:5). Samples were dissolved in mobile phase. Detection limit of the analysis was about 0.31 ng g^{-1} of culture medium, based on a signal-to-noise ratio of 3 : 1. The range of DON standards used for quantification was 0.5–15 µg mL^{-1}.

Mycotoxins analysis from soya beans

Fumonisins B_1 and B_2

Fumonisins were analysed by using the method of Shephard *et al.*[23] as modified by Doko *et al.*[24] The detection limit of the analytical method was 20 µg kg^{-1} for both mycotoxins. The HPLC system consisted of a Hewlett Packard 1050 pump (Palo Alto, CA, USA) connected to a Hewlett Packard 1046A programmable fluorescence detector and a Hewlett Packard 3395 integrator.

Chromatographic separations were performed on a stainless steel, C_{18} reversed-phase column (150 × 4.6 mm i.d., 5 μm particle size; Luna-Phenomenex, Torrance, CA, USA) connected to a security guard cartridge (4 × 3 mm i.d., 5 μm particle size; Phenomenex).

Deoxynivalenol and zearalenone

DON detection and clean-up was done by using the method described in Barros *et al.*,[25] while ZEA was analysed using the methodology proposed by Silva and Vargas.[26]

For DON, the mobile phase was water/methanol (88 : 12) at a flow 1.5 mL min^{-1}. Mycotoxin was detected by UV absorbance (λ: 220 nm; Hewlett Packard 1100, programmable UV detector, Palo Alto, CA, USA) and quantified by a data module Hewlett Packard Kayak XA (HP ChemStation Rev. A.06.01, Palo Alto, CA, USA). The detection limit of the analytical method was 50 μg kg^{-1}. The HPLC system consisted of a Hewlett Packard 1100 pump (Palo Alto, CA, USA; Rheodyne manual injector with a 50 μL loop; Rheodyne, Cotati, CA, USA). Chromatographic separations were performed on a stainless steel, C18 reverse-phase column (150 × 4.6 mm i.d., 5 μm particle size; Luna-Phenomenex, Torrance, CA, USA).

For ZEA, the mobile phase was methanol/water (80 : 20, v/v) at a flow rate of 0.5 mL min^{-1} and detection was achieved by fluorescence (λ_{exc} 280 nm; λ_{em} 460 nm). The detection limit of the analytical method was 10 μg kg^{-1}. The HPLC system was the same as described above for FBs.

Statistical analyses

Diameters of growing colonies were plotted against time, and the Baranyi and Roberts[27] model was used to estimate growth rate and time to visible growth for each growth condition and isolate (Equations 1 and 2) by using Statgraphics® Plus 5.1 (Manugistics, Inc, Maryland, USA) with the nonlinear regression option. Analysis of variance of growth rates and time to visible growth was used in order to assess significant differences due to growth conditions. Mycotoxins were expressed as μg g^{-1} of agar medium/soybean seeds. LSD test was used to establish the differences among mean values of the variables under the different levels of factors at $P < 0.05$.

$$D = \mu A - \ln 1 + \frac{[\exp(\mu A) - 1]}{\exp D_{max}} \quad (1)$$

where

$$A = t + \left(\frac{1}{\mu}\right) \ln[\exp(-\mu t) + \exp(-\mu \Lambda) - \exp(-\mu t - \mu \Lambda)] \quad (2)$$

where D is the colony diameter (D: cm), D_{max} is the maximal colony diameter (mm), t is the time (days), Λ is the time to visible growth (days), μ is the growth rate (cm day^{-1}).

RESULTS

Effects of constant and fluctuating temperatures conditions on mould growth

There were significant differences on mould growth due to constant temperatures assayed ($P < 0.05$). In general, 20 °C was the best temperature for the growth of *F. graminearum* and 25 °C for *F. verticillioides*.

Figure 1 shows growth rate values, μ (cm day^{-1}) for *F. graminearum*, growing on soya beans and soybean-based medium. Significant differences in the growth rate among the different temperatures and temperature cycles studied were found ($P < 0.05$) on both soya beans and soybean-based medium. On culture medium, the higher growth rate was obtained at 20 °C and at 15/20 °C cycling temperature and on seeds the major growth rate was observed at 15/20 °C. Besides, on soya beans the lower growth rate was observed at 25 °C and 15/25 °C. No growth was found at 30 °C and 25/30 °C in any of the substrates. Regarding time to visible growth, it increased with decreasing growth rates, except at 15/20 °C in soya beans where the higher growth rate was reported together with a relatively long lag time.

Figure 2 shows growth rate, μ (cm day^{-1}) for *F. verticillioides* growing on soya beans and soybean based medium. Significant differences on growth rate for the different temperatures and temperature cycles studied were found ($P < 0.05$). On culture medium the higher growth rate was observed at 25 °C and in temperature cycles, the fastest growth was observed at 15/25 °C. The slowest growth was observed at 30 °C. On the other hand, there was a decrease in growth when *F. verticillioides* grew in soya beans compared to the growth obtained on culture medium. On soybeans, higher growth rates were observed at 15 °C, 15/20 °C and 15/25 °C. As for *F. graminearum*, no growth was observed at 30 °C and 25/30 °C on soya beans; however, at these temperatures growth occurred on soybean based medium.

Regarding time to visible growth, Λ, in general, it increased with decreasing growth rates, except at 15/25 °C in soya beans where delayed growth was observed with a subsequent relatively high growth rate, similarly growth rate and lag time were not negatively correlated at 30 °C in culture medium.

For both species, longer times to visible growth were reported on soy beans compared to agar medium, suggesting an easier initial nutrient uptake in agar medium.

Results obtained under fluctuating temperatures were compared with the sum of 12 h growth under each constant temperature, for both soya beans and soybean based medium (Table 1 and Table 2, respectively). On culture medium, *F. graminearum* showed significant differences ($P < 0.05$) between observed and calculated growth at cycling 25/30 °C and 15/20 °C (the difference was small in this later case) (Table 1), while for soya beans this difference occurred at 15/25 °C and 25/30 °C (Table 2). *F. verticillioides* showed remarkable differences at 15/20 °C and at 15/25 °C between observed and calculated growth on culture medium, as well as at 15/20 °C in soya beans.

As a conclusion, growth at cycling temperatures can not be predicted from data at constant temperature.

Production of zearalenone and deoxynivalenol on culture medium and soybean seeds at different temperatures

Table 3 shows ZEA production by *F. graminearum* under the different conditions studied. When this mould grew in culture medium, it produced the mycotoxin at all temperatures, although the production was low. The major production was observed at 15/25 °C, but the levels did not exceed 0.35 μg g^{-1} agar. Mycotoxin production at 15/20 °C was lower than expected taking into account prediction at constant 15 and 20 °C, while production at 15/25 °C was higher than the expected, in contrast to growth results. Respect to ZEA production on soya beans, this mycotoxin was only detected under the lowest temperatures studied (15 °C and 15/20 °C); however production levels on seeds were higher compared with production on soybean based medium. The highest level (39.25 μg g^{-1}) was observed at 15 °C after 15 days of incubation.

With regard to DON production, the toxin was detected in culture medium under all temperatures at which growth occurred,

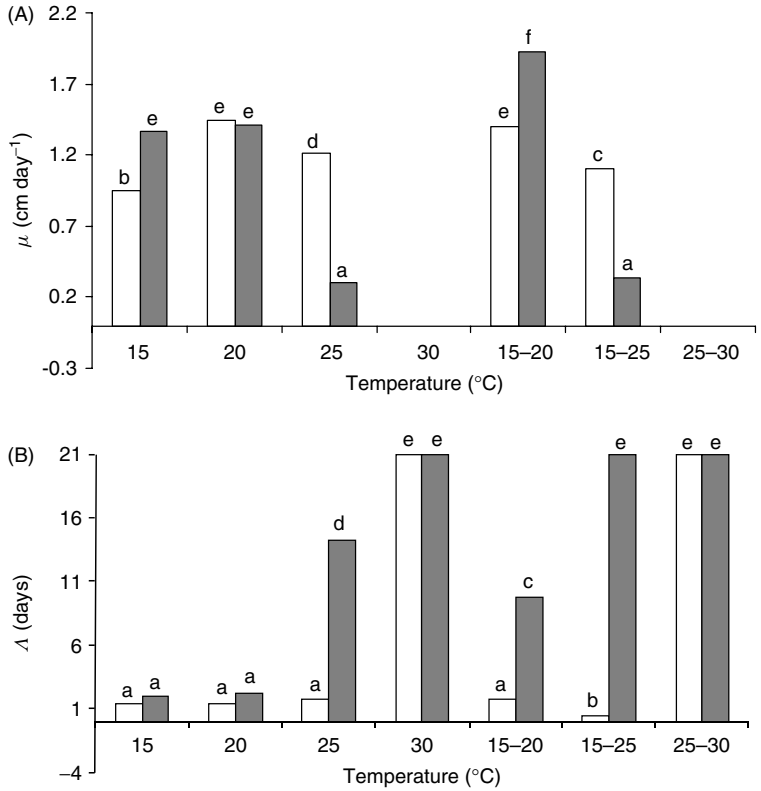

Figure 1. (A) Growth rate, μ (cm day^{-1}) and (B) time to visible growth, Λ (days) of *Fusarium graminearum* on culture medium □ and on soybean seeds ■. Bars with different letters denote significant differences according to the LSD test ($P < 0.05$).

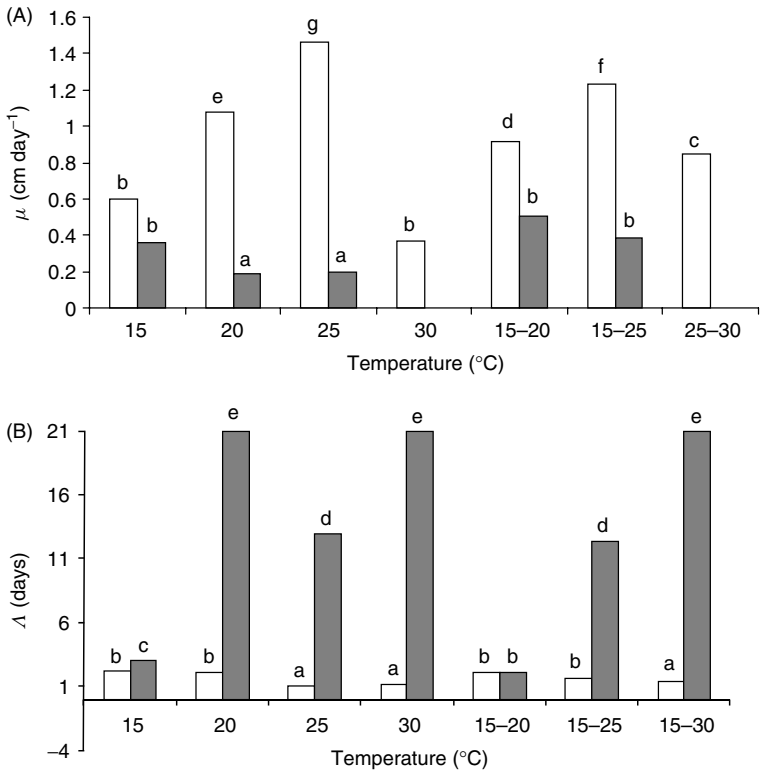

Figure 2. (A) Growth rate, μ (cm day^{-1}) and (B) time to visible growth, Λ (days) of *Fusarium verticillioides* on culture medium □ and on soybean seeds ■. Bars with different letters denote significant differences according to the LSD test ($P < 0.05$).

Table 1. Calculated and observed and growth rates, μ (cm day^{-1}) of *Fusarium* isolates at fluctuating temperatures on culture medium

	F. graminearum			*F. verticillioides*		
Measurement	15/20 °C	15/25 °C	25–30 °C	15/20 °C	15/25 °C	25/30 °C
Calculated	1.20[b]	1.08[a]	0.61	3.02[b]	0.76[b]	0.92[a]
Observed	1.40[a]	1.10[a]	NG	0.91[a]	1.23[a]	0.85[a]

[a,b] Different superscript letters in the same column denote significant differences according to LSD test ($P < 0.05$). NG, no growth.

Table 2. Calculated and observed growth rate, μ (cm day^{-1}) of *Fusarium* isolates at fluctuating temperatures on soybean seeds

	F. graminearum			*F. verticillioides*		
Measurement	15/20 °C	15/25 °C	25–30 °C	15/20 °C	15/25 °C	25/30 °C
Calculated	1.39[a]	0.84[b]	0.15	0.27[b]	0.27[a]	0.10
Observed	1.93[a]	0.34[a]	NG	0.50[a]	0.39[a]	NG

[a,b] Different superscript letters in the same column denote significant differences according to LSD test ($P < 0.05$). NG, no growth.

except at 15 °C (Table 4). The higher production was observed at the temperature cycles, where the highest level was recorded at 15/25 °C at 21 days of incubation. When *F. graminearum* grew on soya beans, the DON levels were higher than those observed on culture medium. Production was detected at all temperatures where growth occurred. High levels of production were observed at 15 days of growth, decreasing at 21 days, except at 25 °C, where the highest concentration occurred after 7 days of incubation. The highest level of DON was found at 20 °C (1040.4 μg g^{-1}).

Production of fumonisins on culture medium and soybean seeds at different temperatures

On culture medium *F. verticillioides* produced fumonisin B_1 at all temperatures tested at 7 days of incubation (Table 5). The

Table 3. Production of zearalenone on culture medium and soybean seeds at different temperatures at 7, 15 and 21 days

	Culture medium (μg g^{-1} agar)			Soybean seeds (μg g^{-1})		
Temperature	7 days	15 days	21 days	7 days	15 days	21 days
15 °C	<LD	0.23 ± 0.23	0.11 ± 0.03	10.7 ± 0.14	39.25 ± 7.85	31.15 ± 9.83
20 °C	0.03 ± 0.04	0.29 ± 0.28	0.06 ± 0.08	<LD	<LD	<LD
25 °C	0.08 ± 0.11	<LD	<LD	<LD	<LD	<LD
30 °C	NG	NG	NG	NG	NG	NG
15/20 °C	0.09 ± 0.12	0.04 ± 0.62	0.08 ± 0.11	<LD	19.95 ± 10.39	21.9 ± 1.23
15/25 °C	0.34 ± 0.45	<LD	0.33 ± 0.35	NG	NG	NG
25/30 °C	NG	NG	NG	<LD	<LD	<LD

NG, no growth; LD, limit of detection.

Table 4. Production of deoxynivalenol on culture medium and soybean seeds at different temperatures at 7, 15 and 21 days

	Culture medium (μg g^{-1} agar)			Soybean seeds (μg g^{-1})		
Temperature	7 days	15 days	21 days	7 days	15 days	21 days
15 °C	<LD	<LD	<LD	158.2 ± 223.73	244.04 ± 192.74	<LD
20 °C	<LD	<LD	0.35 ± 4.98	<LD	1040.4 ± 11.31	0.25 ± 0.07
25 °C	0.11 ± 0.16	0.41 ± 0.58	<LD	708.08 ± 993.1	32.09 ± 37.41	<LD
30 °C	NG	NG	NG	NG	NG	NG
15/20 °C	<LD	<LD	1.03 ± 1.46	<LD	223.48 ± 259.40	1.18 ± 0.67
15/25 °C	<LD	<LD	2.48 ± 3.5	<LD	424.62 ± 600.50	0.18 ± 0.25
25/30 °C	NG	NG	NG	NG	NG	NG

NG, no growth; LD, limit of detection.

Table 5. Production of fumonisin B_1 on culture medium and soybean seeds at different temperatures at 7, 15 and 21 days

	Culture medium (μg g^{-1} agar)			Soybean seeds (μg g^{-1})		
Temperature	7 days	15 days	21 days	7 days	15 days	21 days
15 °C	7.38 ± 4.56	<LD	<LD	<LD	<LD	<LD
20 °C	1.77 ± 0.27	1.75 ± 0.50	<LD	<LD	<LD	<LD
25 °C	0.80 ± 1.14	<LD	0.60 ± 0.86	<LD	<LD	<LD
30 °C	1.77 ± 2.50	2.55 ± 1.22	<LD	NG	NG	NG
15/20 °C	0.62 ± 0.87	0.79 ± 1.12	<LD	<LD	<LD	<LD
15/25 °C	1.27 ± 0.06	0.84 ± 1.19	0.96 ± 0.08	<LD	<LD	<LD
25/30 °C	1.24 ± 0.29	<LD	0.66 ± 0.004	NG	NG	NG

NG, no growth; LD, limit of detection.

toxin was mainly detected after 7–15 days of growth. The highest production was observed at 15 °C after 7 days of incubation. When *F. verticillioides* grew directly on soya beans, no FBs were detected.

DISCUSSION

Great potential exists for infection of different grains with multiple *Fusarium* species, probably resulting in contamination of feed with multiple *Fusarium* mycotoxins. Soybean matrix has been poorly studied compared to other commodities in relation to the mycobiota and their mycotoxin production. However, some research has shown natural contamination with *Fusarium* mycotoxins in soybean and by-products.[7,9,10,28]

There are many published works regarding growth and mycotoxin production by different *Fusarium* species under different conditions.[17,18,29–34] However, the majority of these studies were made under isothermal conditions. Nevertheless, these results cannot necessarily be extrapolated to natural ecosystems where temperature conditions fluctuate during the day.

In the present work, growth and mycotoxin production by *F. graminearum* and *F. verticillioides*, isolated from Argentinean soybean, were evaluated at different constant and cycling temperatures on culture media and soybean seeds. Temperatures assayed in this study simulated field conditions for growing soybean during February, March and April in Argentina. *F. graminearum*, grew better when was incubated at 15/20 °C and 15/25 °C (isothermal or cycling temperature). *F. verticillioides* grew better at 25 °C in culture medium and at 15/20 °C and 15/25 °C on soybean seeds. Our results agree with other studies which found that the optimal temperature for growth of different *Fusarium* species was between 20 and 25 °C.[17,18,29,30,32–35] However, Samapundo *et al*.[17] and Marín *et al*.[31] found that the optimal temperature for *F. proliferatum* and *F. verticillioides* growth on corn was 30 °C; both authors worked with the same strains isolated from Spanish maize. The different optimum temperature reported may be attributed to either the geographical and host origin of the strains which may lead to genetic differences or to the different growth substrate.

On the other hand, in general, the isolates grew better in culture medium compared with soya beans (except in some conditions for *F. graminearum*); for this reason, care should be taken when using only culture medium for fungal growth studies, because in some cases this may lead to overestimated growth values.

F. graminearum is the most important DON and ZEA producer species isolated from soybean growth stages in Argentina[10] and co-occurrence of ZEA and DON has been well documented.[36–41] In our work, *F. graminearum* synthesised both ZEA and DON, and production was affected by temperature, time and substrate. In general, the higher levels of both toxins were recorded in soybean seeds at 15 days of incubation. Besides, levels of DON were higher compared with level of ZEA, and temperatures for higher DON production (15, 15/20 °C) were similar to those for growth, while temperature for optimal ZEA accumulation varied (15 °C, 20 °C; 15/20 °C and 15/25 °C). In some cases DON and ZEA production did not occur at cycling temperature but did so at constant one. Eugenio *et al*.[42] and Mirocha *et al*.[43] concluded that incubation at low temperatures (12–14 °C) following initiation of fungal growth at room temperature resulted in higher amounts of ZEA and DON by some strains of *F. graminearum*. However, Eugenio *et al*.[42] and Vaamonde *et al*.[44] concluded that soybean is not favourable for the production of this mycotoxin. They hypothesised that soybean possess some factors that limits production of ZEA by *Fusarium* isolates.

In contrast, *F. verticillioides* only produced fumonisin B_1 on culture medium and the maximum level was at 30 °C at 15 days of growth. However, at this temperature the species was unable to grow in soybean seeds. As for *F. graminearum*, under isothermal temperatures the level of FBs was higher compared to cycling temperatures. The optimum condition for FB_1 production on corn was reported to be 20 °C at a_w between 0.956 and 0.968.[31,45] Ryu *et al*.,[46] when studying FB_1 production by *F. moniliforme* and *F. proliferatum* in milling rice, with cycling temperatures, found that the optimal condition for fumonisin B_1 production was different for each species: a cycling temperature between 10 and 25 °C for *F. moniliforme* and between 5 and 25 °C for *F. proliferatum*. Thus, toxin production depends, among other factors, on temperature, isolate and matrix.

In summary, when growth obtained under fluctuating temperatures was compared to predicted growth by pooling growth at constant temperature, in general, the observed values were different from the calculated values ($P < 0.05$) for both species and incubation medium with some exceptions. Obviously, the real temperature gradient was not reproduced in this work. Therefore, temperature fluctuation influences the colonisation of *Fusarium* species in the field. Taking into account the results in soybean seeds, predicted growth was faster than the observed for *F. graminearum* and vice versa for *F. verticillioides*, while predicted toxin production was, in general, higher than the observed. Although both of them are fail-safe prediction cases, this highlights the fact that extrapolation from constant conditions to real field conditions must be done with extreme care.

Currently, predictive models on growth contamination and mycotoxin production in cereal crops tend to be on field situations to simulate real conditions.[47,48] To our knowledge, this is the first study on *F. graminearum* and *F. verticillioides* on soybean-based medium and soya beans where growth parameters and mycotoxin production are studied at isothermal and cycling temperatures. Although incubation of fungal cultures at alternating temperature is impractical, it is very important to study ecophysiological behaviour under these conditions, because growth and subsequent mycotoxin production at isothermal conditions seem to be different compared to cycling temperatures.

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REFERENCES

1 Norman AG, *Fisiología, Mejoramiento, Cultivo y Utilización de la Soja*, 1st edition. Ed. Hemisferio Sur, Buenos Aires (1983).
2 Food and Agriculture Organization of the United Nations, Worldwide regulations for mycotoxin, in *Food and Feeds in 2003*. *Food and Nutrition* Paper 81. FAO, Rome (2004).
3 Food and Agriculture Organization of the United Nations, *FAOSTAT Statistical Database* (2009). Available: http://www.faostat.fao.org/[June 2011].
4 Secretaría de Agricultura Ganadería Pesca y Alimentación de la Nación (SAGyP) (2006). Available: http://www.sagyp.mecon.gov.ar [June 2011].
5 Oviedo MS, Ramirez ML, Barros GG and Chulze SN, Effect of environmental factors on tenuazonic acid production by *Alternaria alternata* on soybean-based media. *J Appl Microbiol* **107**:1186–1192 (2009).
6 Garcia D, Ramos AJ, Sanchis V and Marín S, Predicting mycotoxins in foods: A review. *Food Microbiol* **26**:757–769 (2009).
7 Boca RT, Pacín AM, González HHL, Resnik SL and Souza JC, Soja y Micotoxinas: Flora fúngica – Variedades – Prácticas agronómicas. *Aceites y Grasas* **53**:510–515 (2003).
8 Gally T, González B and Pantuso F, Efecto conjunto de *Fusarium* spp. y *Phomopsis* spp., patógenos transmitidos por las semillas en plántulas de soja (*Glycine max* (L.) Merrill). *Rev Mex Fitopat* **24**:156–158 (2006).
9 Broggi LE, González HHL, Resnik SL and Pacin A, *Alternaria alternata,* prevalence in cereal grains and soybean seeds from Entre Ríos, Argentina. *Rev IberoAm Micolog* **24**:48–51 (2007).
10 Barros G, Alaniz-Zanon MS, Abod A, Oviedo MS, Ramirez ML, Torres A, *et al*, Natural deoxynivalenol occurrence and genotype and chemotype determination of a field population of the *Fusarium graminearum* complex associated with soybean in Argentina. *Food Addit Contam* **1**:1–11 (2011).
11 Glenn AE, Mycotoxigenic *Fusarium* species in animal feed. *Anim Feed Sci Technol* **137**:213–240 (2007).
12 Pardo E, Lagunas U, Sanchis V, Ramos AJ and Marín S, Influence of water activity and temperature on conidial germination and mycelial growth of ochratoxigenic isolates of Aspergillus ochraceus on grape juice synthetic medium. Predictive models. *J Sci Food Agric* **85**:1681–1686 (2005).
13 Pardo E, Malet M, Marín S, Sanchis V and Ramos AJ, Effects of water activity and temperature on germination and growth profiles of ochratoxigenic *Penicillium verrucosum* isolates on barley meat extract agar. *Int J Food Microbiol* **106**:25–31 (2006).
14 Pardo E, Marín S, Ramos AJ and Sanchis V, Ecophysiology of ochratoxigenic Aspergillus ochraceus and Penicillium verrucosum isolates. Predictive models for fungal spoilage prevention – A review. *Food Addit Contam Part A* **4**:398–410 (2006).
15 Plaza P, Usall J, Teixidó N and Viòas I, Effect of water activity and temperature on germination and growth of *Penicillium digitatum, P. italicum* and *Geotrichum candidum*. *J Appl Microbiol* **94**:549–554 (2003).
16 Rosso L and Robinson TP, A cardinal model to describe the effect of water activity on the growth of moulds. *Int J Food Microbiol* **63**:265–273 (2003).
17 Samapundo S, Devliehgere F, De Meulenaer B and Debevere J, Effect of water activity and temperature on growth and the relationship between fumonisin production and the radial growth of *Fusarium verticillioides* and *Fusarium proliferatum* on corn. *J Food Prot* **68**:1054–1059 (2005).
18 Samapundo S, Devlieghere F, De Meulenaer B, Geeraerd AH, Van Impe JF and Debevere JM, Predictive modelling of the individual and combined effect of water activity and temperature on the radial growth of *Fusarium verticilliodes* and *F. proliferatum* on corn. *Int J Food Microbiol* **105**:35–52 (2005).
19 Gerlach W and Nirenberg HI, The genus *Fusarium,* a pictorial atlas, in *Mitteilungen aus der Biologischen Bundesanstalt fur Land- und Forstwirtschaft*, vol. 206, ed. by Nirenberg HI. Paul Parey, Berlin–Dahlem, p. 406 (1982).
20 Dallyn H, The effect of substrate water activity on the growth of certain xerophilic fungi. PhD thesis, CNAA Polytechnic of the South Bank, London (1978).
21 Garcia D, Ramos AJ, Sanchis V and Marín S, Modelling the effect of temperature and water activity in the growth boundaries of Aspergillus ochraceus and Aspergillus parasiticus. *Food Microbiol* **28**:406–417 (2011).
22 Sydenham EW, Shephard GS, Thiel PG, Stockenstrom S, Snijman PW and Van Schalkwyk DJ, Liquid chromatographic determination of fumonisins B1, B2, and B3 in corn: an AOAC–IUPAC collaborative study). *J AOAC Int* **79**:688–696 (1996).
23 Shephard GS, Sydenham EW, Thiel PG and Gelderblom WCA, Quantitative determination of fumonisins B_1 and B_2 by high performance liquid chromatography with fluorescence detection. *Liq Chromatogr* **13**:2077–2080 (1990).
24 Doko B, Rapior S, Visconti A and Schjoth J, Incidence and levels of fumonisinas contamination in maize genotypes grown in Europe and Africa. *J Agric Food Chem* **43**:429–434 (1995).
25 Barros GG, García D, Oviedo S, Ramírez ML, Torres A and Chulze S, Deoxynivalenol and nivalenol analysis in soybean and soy flour. *World Mycotoxin J* **3**:263–266 (2008).
26 Silva CMG and Vargas EA, A survey of zearalenone in corn using Romer Mycosep™ 224 column and high performance liquid chromatography. *Food Addit Contam* **18**:39–45 (2001).
27 Baranyi J and Roberts TA, A dynamic approach to predicting bacterial growth in food. *Int J Food Microbiol* **23**:277–294 (1994).
28 Barros GG, García D, Oviedo S, Ramírez ML, Torres A, Lattanzio V, *et al*, Survey of T-2 and HT-2 toxins in soybean and soy meal from Argentina using immunoaffinity clean-up and high performance liquid chromatography. *World Mycotoxin J* **4**:189–197 (2011).
29 Alberts JF, Gelderblom WCA, Thiel PG, Marasas WFO, Van Schalkwyk DJ and Behrend Y, Effects of temperature and incubation period on production of fumonisin B1 by *Fusarium moniliforme*. *Appl Environ Microbiol* **56**:1729–1733 (1990).
30 Marin S, Sanchis V and Magan N, Water activity, temperature, and pH effects on growth of *Fusarium moniliforme* and *Fusarium proliferatum* isolates from maize. *Can J Microbiol* **41**:1063–1070 (1995).
31 Marin S, Sanchis V, Vinas I, Canela R and Magan N, Effect of water activity and temperature on growth and fumonisin B1 and B2 production by *Fusarium proliferatum* and *F. Moniliforme* on maize grain. *Lett Appl Microbiol* **21**:298–301 (1995).
32 Ramirez ML, Chulze S and Magan N, Temperature and water activity effects on growth and temporal deoxynivalenol production by two Argentinean strains of *Fusarium graminearum* on irradiated wheat grain. *Int J Food Microbiol* **106**:291–296 (2006).
33 Mogensen JM, Nielsen KF, Samson RA, Frisvad JC and Thrane U, Effect of temperature and water activity on the production of fumonisins by *Aspergillus niger* and different *Fusarium* species. *BMC Microbiology* **9**:article n° 281 (2009).
34 Medina A and Magan N, Comparisons of water activity and temperature impacts on growth of Fusarium langsethiae strains from northern Europe on oat-based media. *Int J Food Microbiol* **142**:365–369 (2010).

35 Marin S, Sanchis V, Teixido A, Saenz R, Ramos AJ, Vinas I, *et al*, Water and temperature relations and microconidial germination of *Fusarium moniliforme* and *Fusarium proliferatum* from maize. *Can J Microbiol* **42**:1045–1050 (1996).
36 Tanaka T, Yamamoto S, Hasegawa A, Aoki N, Besling JR, Sugiua Y, *et al*, A survey of the natural occurrence of *Fusarium* mycotoxins, deoxynivalenol, nivalenol and zearalenone, in cereals harvested in the Netherlands. *Mycopathologia* **110**:. 19–22 (1990).
37 Kim J-C, Kang H-J, Lee D-H, Lee Y-W and Yoshizawa T, Natural occurrence of *Fusarium* mycotoxins (trichothecenes and zearalenone) in barley and corn in Korea. *Appl Environ Microbiol* **59**:3798–3802 (1990).
38 Gonzalez HHL, Martinez EJ, Pacin AM, Resnik SL and Sydenham EW, Natural co-occurrence of fumonisins, deoxynivalenol, zearalenone and aflatoxins in field trial corn in Argentina. *Food Addit Contam* **16**:565–569 (1999).
39 Ryu D and Bullerman LB, Effect of cycling temperatures on the production of deoxynivalenol and zearalenone by *Fusarium graminearum* NRRL 5883. *J Food Prot* **62**:1451–1455 (1999).
40 Sohn H-B, Seo J-A and Lee Y-W, Co-occurrence of *Fusarium* mycotoxins in mouldy and healthy corn from Korea. *Food Addit Contam* **16**:153–158 (1999).
41 Muthomi JW, Ndung'u JK, Gathumbi JK, Mutitu EW and Wagacha JM, The occurrence of *Fusarium* species and mycotoxins in Kenyan wheat. *Crop Protect* **27**:1215–1219 (2008).
42 Eugenio CP, Christensen CM and Mirocha CJ, Factors affecting production of the mycotoxin F-2 by *Fusarium roseum*. *Phytopathology* **7**:1055–1057 (1970).
43 Mirocha CJ, Christensen CM and Nelson GH, Estrogenic metabolite produced by *Fusarium graminearum* in stored corn. *Appl Microbiol* **15**:497–503 (1967).
44 Vaamonde G and Bonera N, Zearalenone production by Fusarium species isolated from soybeans. *Int J Food Microbiol* **4**:129–133 (1987).
45 Le Bars J, Dupuy H, Boudra R and Cassini R, Biotic and abiotic factors in fumonisin production and accumulation (abstract), in *Proceedings of the 106th Annual AOAC International Meeting*, Aug. 31–Sept. 3, 1992. Cincinnati, OH, p. 106 (1992).
46 Ryu D, Munimbazi C and Bullerman LB, Fumonisin B1 production by *Fusarium moniliforme* and *Fusarium proliferatum* as affected by cycling temperatures. *J Food Prot* **62**:1456–1460 (1999).
47 Stewart DW, Reid LM, Nicol RW and Schaafsma AW, A mathematical simulation of growth of *Fusarium* in maize ears after artificial inoculation. *Phytopathology* **92**:534–541 (2002).
48 Maiorano A, Reyneri A, Sacco D, Magni A and Ramponi C, A dynamic risk assessment model (FUMAgrain) of fumonisin synthesis by *Fusarium verticillioides* in maize grain in Italy. *Crop Protect* **28**:243–256 (2009).

