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Effect of fungicides commonly used for Fusarium head blight management on growth and fumonisin production by *Fusarium proliferatum*

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KEYWORDS

Fungicides;
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FHB management

Abstract Fumonisin occurrence was reported in wheat grains and *F. proliferatum* has been suggested to be the main contributor to its presence in wheat. Thus, a survey was performed in order to study the impact of four commercial fungicides used in Argentina for controlling Fusarium head blight disease (epoxiconazole + metconazole, tebuconazole, pyraclostrobin + epoxiconazole, and prothioconazole) on growth and fumonisin production of two *F. proliferatum* strains in relation to water activity (a_w ; 0.99, 0.97, 0.95) and temperature (15 °C and 25 °C). Most fungicides reduced growth rates when compared to the control (reduction increased as fungicide concentration increased), and reduced fumonisin production when they were used at high doses; however, most fungicides enhanced fumonisin production at sublethal doses, with the exception of prothioconazole. Thus, fungicides used for FHB management could enhance fumonisin production by *F. proliferatum* strains present in wheat grains.

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PALABRAS CLAVE

Fungicidas;
Fusarium
proliferatum;
Fumonisin;
Trigo;
Manejo de FET

Efecto de fungicidas utilizados para el control de la fusariosis de la espiga de trigo sobre el crecimiento de *Fusarium proliferatum* y la producción de fumonisinas

Resumen En los últimos años se ha observado contaminación de granos de trigo con fumonisinas, y se ha postulado a *Fusarium proliferatum* como el principal responsable de la presencia de dicha micotoxina en este cereal. Por este motivo, se realizó un ensayo de manera tal de estudiar el efecto de cuatro formulaciones comerciales de fungicidas utilizadas para el manejo

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y control de la fusariosis de la espiga de trigo en el crecimiento y la producción de fumonisinas de dos cepas de *F. proliferatum*, en relación con la actividad de agua (a_w ; 0,99; 0,97 y 0,95) y la temperatura (15 y 25 °C). La mayoría de los productos fueron capaces de reducir la velocidad de crecimiento de ambas cepas, con una mayor acción a medida que la concentración del fungicida aumentó, y también de reducir la producción de fumonisinas cuando fueron utilizados en altas dosis. Sin embargo, la producción de fumonisinas se elevó cuando estos fueron utilizados a dosis subletales, excepto cuando se aplicó protriocanazol. Se concluye que los fungicidas comúnmente utilizados para el control de la fusariosis de la espiga de trigo en Argentina podrían estimular la producción de fumonisinas en cepas de *F. proliferatum* presentes en los granos de trigo, llevando así a la contaminación de este cereal con estas micotoxinas.

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Introduction

Fusarium head blight (FHB) is one of the most important diseases of wheat and other cereals in many areas of the world. In Argentina, *Fusarium graminearum sensu stricto* is the main pathogen associated with this disease, consequently this cereal could be contaminated with mycotoxins, deoxynivalenol (DON) being the predominant one^{26,39,44}. DON occurrence in wheat grains is of concern because this toxin has been associated with adverse health effects in humans and animals⁴¹. Wheat in Argentina is often grown in rotation with maize, increasing the risk of fungal and mycotoxin accumulation. Fumonisin (FB) contamination of wheat grains and wheat-based products has recently been observed not only in Argentina, but also in other countries^{5,8,9,38}. Moreover, *Fusarium proliferatum* and *Fusarium verticillioides* were isolated from wheat grains, and many authors have suggested that *F. proliferatum* could be the main contributor to FB occurrence in wheat^{2,18,36,38}.

FBs are mycotoxins that can cause various adverse health effects in humans and animals. FB consumption has been associated in humans with esophageal cancer, neural tube defects, and stunting in children^{11,45}. Fumonisin B₁ (FB₁) is the most significant in terms of toxicity and occurrence. It has been classified as a "2B" carcinogen by the International Agency of Research on Cancer²⁰, and the Joint FAO/WHO Expert Committee on Food Additives has determined a provisional maximum tolerable daily intake of 2 µg/kg body weight/day for FB₁, FB₂ and FB₃ alone or in combination⁴⁷.

As wheat is an important grain cereal used for human consumption its safety is of concern. In order to decrease FHB damage and mycotoxin contamination several strategies are used: combinations of agronomical practices, resistant cultivars and fungicides¹⁴. The use of fungicides is a complementary control measure when weather conditions are conducive to fungal infection. Among the fungicides used, azole application is a primary method for FHB management⁴⁰. Azoles block the ergosterol biosynthesis pathway by inhibiting sterol α -demethylase²⁴. These compounds have been shown to reduce FHB symptoms and DON content in wheat; however, the effectiveness of azole

compounds varies and is strongly dependent on different factors (disease severity, resistance level of the wheat cultivar, and spraying technology)^{22,24,35,40}. Strobilurins are also used to manage FHB by blocking electron transport in the mitochondrial respiratory chain, reducing aerobic energy production, and inhibiting fungus growth⁴⁶.

A significant focus has been laid on the development and use of fungicides to prevent and control the infection of pathogenic *Fusarium spp.* of small grain cereal crops, but less attention has been paid to the effect that those fungicide applications may have on mycotoxin production. Moreover, increasing evidence suggest that fungicides might not be as efficient at reducing toxin production, because in certain conditions they may act as stress factors resulting in the induction of toxin biosynthesis. Sublethal doses of some fungicides may lead to a stimulation of mycotoxin production by several *Fusarium* species^{15,16,19,23,32,33,43}. It is surprising that most reports took no account of the interactions between the efficacy of fungicides and key environmental factors, while it has been demonstrated that the main factors that influence fungal growth and mycotoxin production are temperature, water activity (a_w) and the presence of antifungal substances^{1,25,29,33,43}.

As fungicide applications occur in cereal production systems, *Fusarium* species colonizing ripening cereals are exposed to them⁴⁵. Thus, the balance among fungi (toxicogenic and saprophytic) as well as the diversity of toxin producers and the final amount of mycotoxin contamination are affected by fungi sensitivity to fungicides²⁸. Presuming that fungicides used for FHB management are active against all *Fusarium* species present in wheat grains, they could be effective against the accumulation of many *Fusarium* mycotoxins²¹. When Ramirez et al.⁴³ analyzed some fungicide effects on *F. graminearum* growth and DON production, they concluded that further work is needed to understand the combined effects of environmental factors and fungicides on growth and mycotoxin production of fungal pathogens and saprophytic populations present in wheat.

Assuming that *F. proliferatum* strains are responsible for FB contamination of wheat, the aim of the present study was to analyze the impact of four commercial fungicides used in Argentina for controlling FHB on growth and FB production

Table 1 Active ingredient and brand name of fungicides used during the study.

Active ingredient	Brand name
Prothioconazole 480 g/l	RUDIS
Tebuconazole 430 g/l	FOLICUR
Epoxiconazole 37.6 g/l, metconazole 27.5 g/l	DUETT PLUS
Pyraclostrobin 133 g/l, epoxiconazole 50 g/l	OPERA

of two *F. proliferatum* strains in relation to water activity and temperature using a wheat-based medium.

Materials and methods

Strains. Two *F. proliferatum* strains (ITEM 15654; 15664) isolated from Argentinean wheat grains and characterized by molecular, biological and morphological methods were used. Their ability to produce FBs was analyzed³⁸. These strains are registered at the Microbial Culture Collection of ISPA (Institute of Sciences and of Food Production), Italy, and are deposited at the Research Institute on Mycology and Mycotoxicology (IMICO, UNRC-CONICET). Cultures are maintained in 15% glycerol at -80 °C.

Fungicides and media preparation. Four fungicides were used (Table 1). Dilutions of the fungicides were prepared by mixing appropriate amounts of each fungicide in sterile distilled water and stored at 4 °C until used. A 2% (w/v) milled wheat agar was used. The a_w of the basic medium was adjusted to 0.995, 0.97 and 0.95 by the addition of different amounts of glycerol¹³. Media were autoclaved at 120 °C for 20 min before cooling to 50 °C at which time the fungicides were added to obtain the required concentration (0.5, 2.5, 5, and 15 µg/ml). Media without fungicide addition were used as control treatments per each a_w . Flasks of molten media were thoroughly shaken, prior to pouring into 9 cm sterile Petri dishes. The a_w of representative media samples was checked with an Aqualab Series 3 (Decagon Devices, Inc., WA, USA). Uninoculated plates were measured at the end of the experiment in order to detect any significant deviation of the a_w .

Inoculation, incubation and growth assessment. Treatments and control plates were inoculated centrally with a 3 mm diameter agar disk taken from the margin of a 7-day-old colony of each strain grown on synthetic nutrient agar¹⁷ at 25 °C and transferred face down to the center of each plate. Inoculated plates of the same a_w were sealed in polyethylene bags and incubated at 15 and 25 °C for 28 days. A full factorial design was used where the factors were a_w , temperature, fungicide concentration and strain. The experiments were carried out with three replicates per treatment.

Fungal growth assessment was performed every day during the incubation period and two diameters of the growing colonies were measured at right angles to each other until the colony reached the edge of the plate. Colony radii were plotted against time, and linear regression was applied in

order to obtain the growth rate (mm/day) as the slope of the line. FB determination was performed after the incubation period.

Fumonisin extraction. For FB extraction Petri plates of each strain for every treatment condition were used. Toxins were extracted with 40 ml of acetonitrile: water (1:1 v/v) by shaking the whole culture media (~20 g) and mycelia with the solvent for 30 min in an orbital shaker (150 rpm) and then filtering the extracts through filter paper (No.4; Whatman International Ltd., Maidstone, Kent, UK). An aliquot of the extracts (1000 µl) was taken and diluted with acetonitrile: water (1:1 v/v) as necessary for the HPLC-MS/MS analysis.

HPLC-MS/MS analysis. Fumonisin detection was performed as described by Cendoya et al.⁷ with a Waters 2695 separation module (Waters Corporation, Milford, MA, USA) equipped with a 2695 autosampler and interfaced to a Micromass[®]156 –Quattro 157 Ultima[™] Platinum tandem quadrupole mass spectrometer with electrospray ionization (ESI) source. A 158 XBridge[™] C18 (3.5 µm, 2.1 x 150 mm) column with a XBridge BEH C18 Sentry Guard Cartridge (130 159 Å, 3.5 µm, 2.1 x 10 mm). The mobile phase of the chromatographic procedure was a gradient of 160 aqueous 1% formic acid (solvent A) and methanol 1% formic acid (solvent B). The gradient program was performed as described by Cendoya et al.,⁷ The flow rate was 0.2 ml/min. The temperature of the column was kept at 20 °C. The nitrogen flow was adjusted to 109 and 726 l/h for cone and desolvation gases, respectively. Blank matrix extracts were investigated to confirm that no spectrometric interferences came from the matrix. Data acquisition and processing were performed using Mass Lynx V.4.1, Waters INC software. The interface was operated in a positive ion mode. Nebulization and desolvation temperatures were 150 and 200 °C, respectively. The capillary voltage was 3.00 kV. Multiple-reaction monitoring (MRM) was used for toxin determination. The precursor peak $[M+H]^+$ and two product peaks monitored to accomplish both quantification and qualification criteria, as well as the retention times and the detector settings, are shown in Table 2. Trace m/z 722 > 352 was used for the quantification of FB₁, while 706 > 336 was used for both FB₂ and FB₃, respectively. Aliquots of 10 µl of sample extracts were injected into the HPLC unit. Four points of identification were used to identify FB₁, i.e. retention time, the precursor ion $[M+H]^+$ and two product ions (m/z 334 and 352). A calibration curve was obtained injecting 10 µl of a different mixed standard solution (FB₁ + FB₂) (0.25; 0.5; 1.0 and 2.0 mg/ml). Good linearity was obtained for the calibration range with a correlation coefficient of 0.98 and 0.97, for FB₁ and FB₂, respectively. The calculated instrumental LOD (S/N=3) for FB₁ and FB₂ was 0.01 ng/g and LOQ (S/N=5) was 0.05 ng/g, and the relative within day and between-day standard deviations (% RSD) were 6.5.

A recovery experiment was performed in triplicate by spiking 2% milled wheat agar at levels of 1 to 10 µg/g of each fumonisin (FB₁ and FB₂). Mean recovery ranged from 95 to 98% for FB₁ and FB₂, respectively.

Statistical analysis. Growth rates and mycotoxin concentration were evaluated by analysis of variance (ANOVA) using SigmaStat for Windows version 2.03 (SPSS Inc., Chicago, IL). Statistical significance was determined at $p < 0.01$.

Table 2 Analysis of variance of the effects of strain (S), water activity (a_w), temperature (T), fungicide (F), and fungicide concentration (C) on growth rate in two *Fusarium proliferatum* strains on a wheat-based medium.

Source of variation	df	Growth rate	
		ms	f
S	1	21.1	24.2*
T	1	8391	9591*
a_w	2	6880.2	7863.9*
F	2	227.2	260*
C	4	8588	9816*
SxT	1	96.3	110.1*
Sx a_w	2	17	19.4*
SxF	2	5.6	6.4*
SxC	4	28	32*
Tx a_w	2	3333.4	3810*
TxF	2	64.3	73.5*
TxC	4	3973.9	4542*
a_w xF	4	77.3	88.4*
a_w xC	8	4182.8	4781*
FxC	8	35.9	41*
SxTx a_w	2	68	77.7*
SxTxF	2	1.05	1.2
SxTxC	4	101.1	116*
Sx a_w xF	4	2.3	3
Sx a_w xC	8	14	15.4*
SxFxC	8	6	7*
Tx a_w xF	4	30.4	34.8*
Tx a_w xC	8	2143.6	2450*
TxFxC	8	20	23*
a_w xFxC	16	31	35*
SxTx a_w xF	4	0.5	0.5
SxTx a_w xC	8	64.6	73.9*
SxTxFxC	8	7.4	8.5*
Sx a_w xFxC	16	3.9	4.4*
Tx a_w xFxC	16	16.6	19*
SxTx a_w xFxC	16	5	5.7*

* Significant $p < 0.01$; df: degrees of freedom; ms: mean square; f: Snedecor-F.

Results

Effect of a_w , temperature and fungicides on growth rate.

Fig. 1 and Fig. 2. show the effect of a_w , temperature, and fungicides on growth rates of both *F. proliferatum* strains on a wheat-based medium. Both strains showed a similar behavior in all tested conditions. Under control conditions, i.e. without fungicide addition, maximum growth rates were obtained at 25 °C and 0.995 a_w , and decreased as the a_w of the medium and temperature were reduced. All fungicides were able to reduce the growth rates when compared to the control condition, with the exception of tebuconazole (T) at the lowest concentration used at 0.95 a_w regardless of the temperature; however, the same fungicide in other conditions had a considerably effect on fungal growth (at least 50% reduction). This reduction increased as the

fungicide concentration increased. Prothioconazole (P) was able to inhibit fungal growth at 15 °C in all tested conditions, and at 25 °C at 0.97 and 0.95 a_w ; at 0.995 the growth rate was observed just at 25 °C and at the lowest fungicide concentration. The mixture epoxiconazole and metconazole (D) was able to reduce the growth rates of both strains by 50% (sometimes more than 50%) at the lowest concentration used. Pyraclostrobin + epoxiconazole (O) showed the lowest reduction effect in growth rates under all conditions in comparison with control conditions: at high concentrations the growth rate reduction reached 50% while at low concentrations the reduction was about 25%. The ANOVA of the effect of single variables and two- three- four- and five-way interactions revealed that all single variables and some interactions had a significant effect on growth rates. The most significant effect was given by fungicide concentration, followed by temperature, a_w , and fungicide (Table 2).

Effect of a_w , temperature and fungicides on fumonisin production.

Fig. 3 and Fig. 4 show mean FB concentrations obtained on a wheat-based medium inoculated with two *F. proliferatum* strains in relation to a_w , temperature and fungicide type and concentration. Strain ITEM 15654 produced FB₁, FB₂ and FB₃. Minor amounts of FB₃ were produced at all a_w and temperature conditions tested, and higher amounts of FB₁ than FB₂ were produced at 25 °C, while higher amounts of FB₂ than FB₁ were produced at 15 °C. Strain ITEM 15664 produced just FB₁ and FB₂, and higher amounts of FB₂ than FB₁ under all conditions. For both strains as long as the a_w of the medium and temperature were reduced, FB production was reduced. ITEM 15664 was able to produce higher amounts of FBs than ITEM 15654 under all tested conditions. When fungicides were applied, FB production was significantly reduced in comparison with the controls under some conditions, but in other conditions FB production significantly increased. Differences between strains were also observed for fungicide effects on FB production. Most fungicides used (tebuconazole, pyraclostrobin + epoxiconazole and epoxiconazole + metconazole) showed FB stimulation in at least one of the assayed conditions. The mixture pyraclostrobin + epoxiconazole and tebuconazole showed a higher stimulation effect. Prothioconazole was the only fungicide that was able to reduce FB production by both strains under all assayed conditions; with the exception of the lowest doses used (0.5 µg/ml) at a_w 0.995, and 15 and 25 °C for ITEM 15664 and ITEM 15654, respectively. The mixture epoxiconazole + metconazole reduced FB production under all the assayed conditions for strain ITEM 15654, while for strain ITEM 15664, an increase in FB production was observed at 15 °C and 0.995 a_w when fungicide doses were low. Tebuconazole application reduced FBs for strain ITEM 15654 at 25 °C and at 0.995 a_w , while at the same temperature but lower a_w levels, stimulation of FB production was observed when low doses (< 5 µg/ml) of fungicides were applied. Using the same fungicide at 15 °C, stimulation was observed just at the highest a_w level when low fungicide concentrations were used. For the other strain, ITEM 15664 the same behavior was observed at both assayed temperatures: when a_w was 0.995, low levels of fungicide

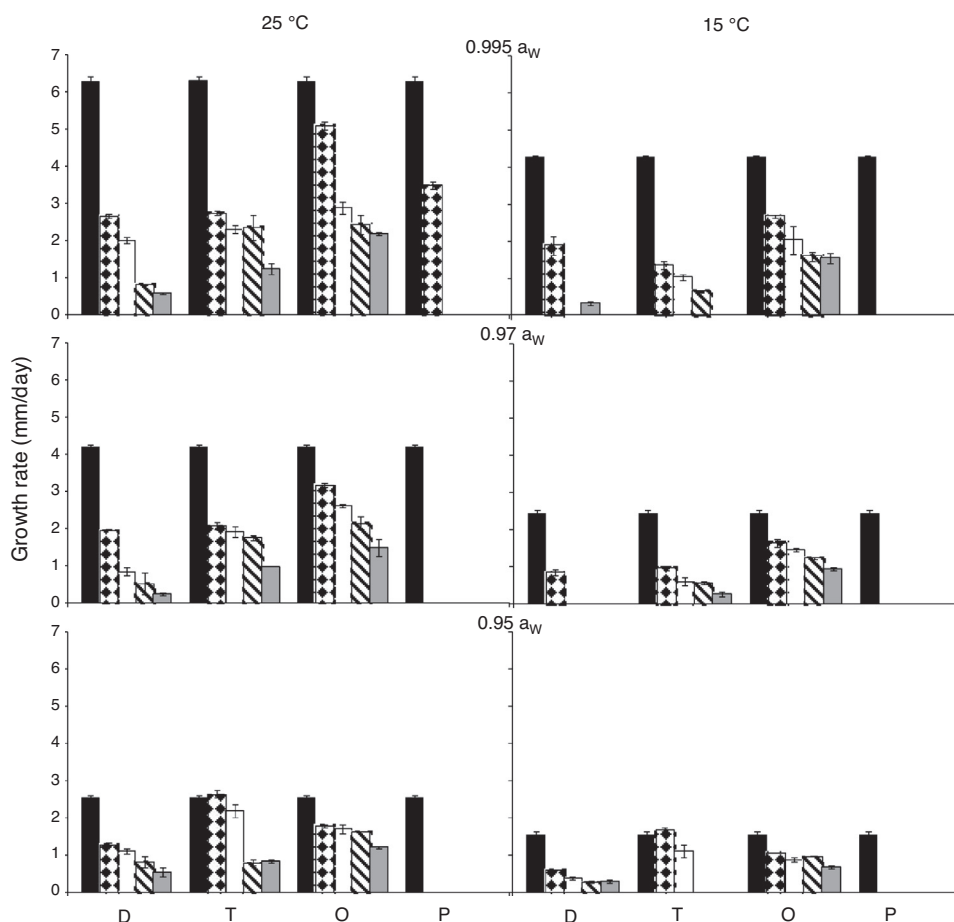


Figure 1 Growth rates (mm/day) for *F. proliferatum* ITEM 15654 under different a_w (0.995; 0.97; 0.95), temperatures (15; 25 °C), fungicides (D: epoxiconazole + metconazole; T: tebuconazole; O: pyraclostrobin + epoxiconazole; P: prothioconazole), and fungicide concentrations: 0 (■); 0.5 (◼); 2.5 (□); 5 (▨); 15 (▩) µg/mL.

(< 5 µg/ml) stimulated FB production, while at lower a_w levels a reduction in FB production was observed. The effect of the pyraclostrobin+epoxiconazole mixture on strain ITEM 15664 was the same at both assayed temperatures: reduction in FB production when a_w levels were low, and stimulation at 0.995 a_w and low fungicide doses. For the other strain the same fungicide stimulated FB production at all a_w levels at 25 °C and 0.995 a_w at 15 °C. Prothioconazole reduced FB production under all assayed conditions for both strains, with an exception at 25 °C and 0.995 a_w for strain ITEM 15654 where toxin production was similar to that observed in the control condition. Statistical analysis showed that all single source of variance and some, two-, three-, and four-way interactions significantly influenced FB production, a_w being the most significant for both *F. proliferatum* strains, with the exception of temperature, which was the most significant for FB₃ and FB₂ production by *F. proliferatum* strain ITEM 15654 (Table 3).

Discussion

Since fungicides used for FHB control are active against all *Fusarium* species present in wheat, and it has been

shown that *F. proliferatum* as well as FBs can be present in wheat, the effect of fungicides on *F. proliferatum* growth and FB accumulation on a wheat-based medium was analyzed. As a result, it was shown that complex interactions occur between abiotic factors (a_w and temperature) and fungicides on growth and FB production by *F. proliferatum* strains.

Growth rate results observed under control conditions are in agreement with previous results reported using the same two strains grown in the same medium and also using irradiated wheat grains^{6,7,10}.

With regard to the effect of fungicides on growth rates, all fungicide applications reduced growth rates (as fungicide concentrations increased, growth rates decreased) of *F. proliferatum* strains when compared to the control condition. Prothioconazole caused the greatest effect in both strains. Differences in fungicide efficacy are related to their action mechanism. Such information could be useful for the effective control of *F. proliferatum* growth and possible FB production on wheat grains. In the present study, little differences between the strains were observed, which is in agreement with other studies²³. The differences in the efficacy of the same fungicide in inhibiting the mycelial growth

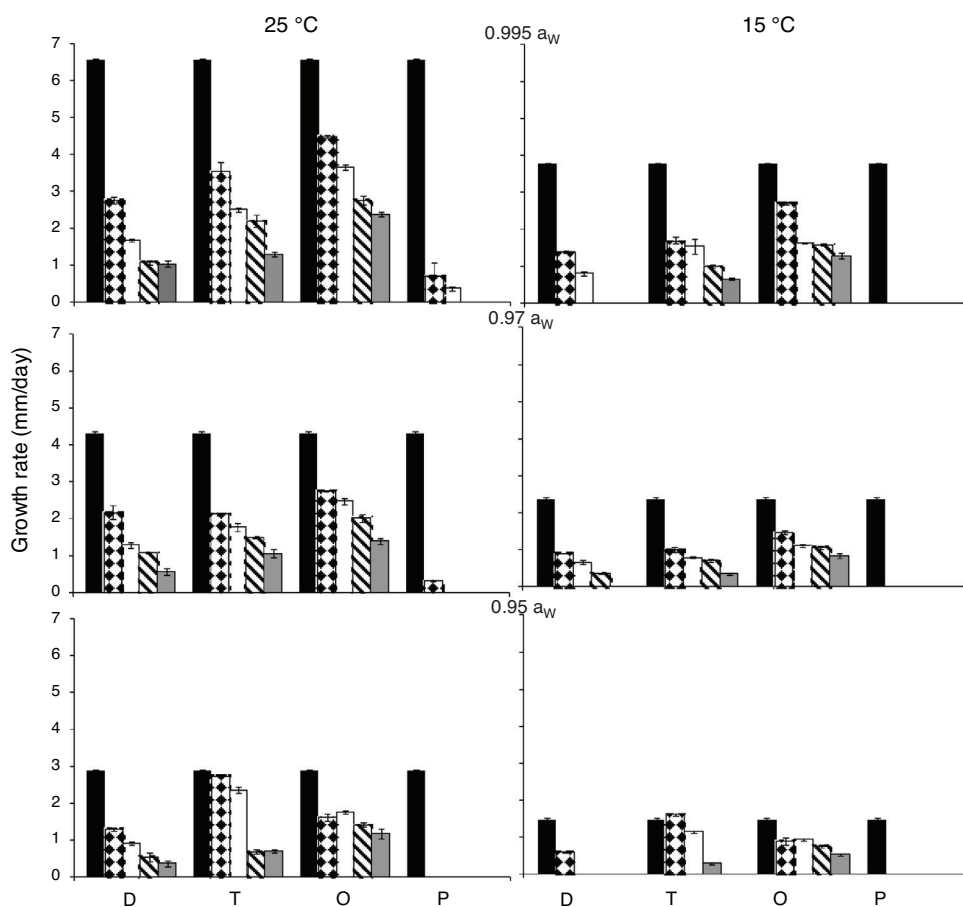


Figure 2 Growth rates (mm/day) for *F. proliferatum* ITEM 15664 under different a_w (0.995; 0.97; 0.95), temperatures (15; 25 °C), fungicides (D: epoxiconazole + metconazole; T: tebuconazole; O: pyraclostrobin + epoxiconazole; P: prothioconazole), and fungicide concentrations: 0 (■); 0.5 (▨); 2.5 (□); 5 (▩); 15 (▧) µg/mL.

Table 3 Analysis of variance of the effects of water activity (a_w), temperature (T), fungicide (F), and fungicide concentration (C) on fumonisin production by two *Fusarium proliferatum* strains on a wheat-based medium.

Source of variation	ITEM 15654						ITEM 15664				
	df	FB ₁		FB ₂		FB ₃		FB ₁		FB ₂	
		ms	f	ms	f	ms	f	ms	f	ms	f
T	1	3318	12*	9873	37*	1363	16*	3972	78*	322759	14*
a_w	2	10247	37*	7448	28*	1070	13*	4606	90*	2697372	120*
F	3	1453	5*	1685	6*	183	2.2*	702	14*	391737	18*
C	4	3582	13*	3635	14*	1010	12*	2295	45*	708640	32*
Tx a_w	2	442	1.5	1950	7*	137	1.7	1241	24*	85647	4*
TxF	3	166	0.6	487	1.8	46	0.6	40	0.8	109874	5*
TxC	4	758	3*	1767	7*	292	3.5*	684	13*	24648	1.1
a_w xF	6	442	1.5	518	2*	23	0.3	221	4*	217711	10*
a_w xC	8	1250	5*	1137	4*	353	4*	1553	30*	513174	23*
FxC	12	272	1	520	2*	41	0.5	121	2*	80445	4*
Tx a_w xF	6	364	1.3	106	0.4	118	1.4	8	0.2	43137	1.2*
Tx a_w xC	8	595	2*	975	4*	240	3*	403	8*	24811	1.1
TxFxC	12	374	1.3	467	2*	48	0.6	41	0.8	23602	1.1
a_w FxC	24	283	1	471	2*	40	0.5	78	1.5*	56427	2.5*
Tx a_w FxC	24	382	1.4	509	2*	61	0.7	44	0.9	22842	1.0

* Significant $p < 0.1$; df: degrees of freedom; ms: mean square; f: Snedecor-F.

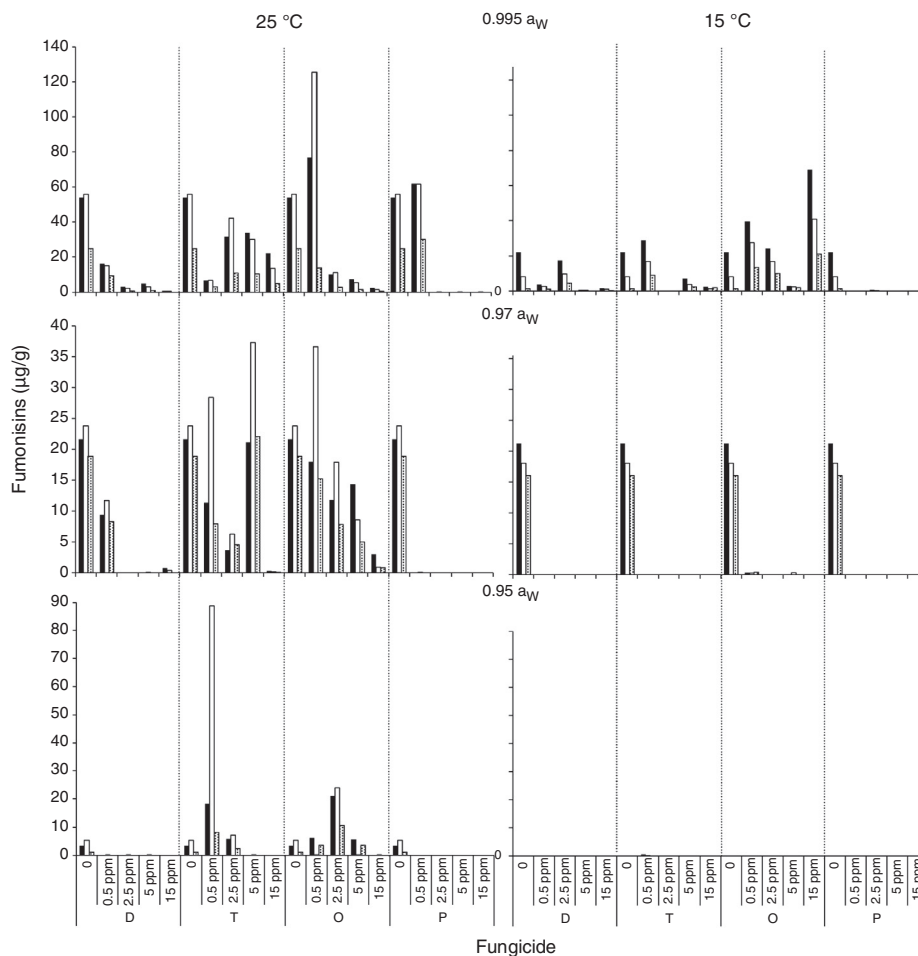


Figure 3 Effect of fungicides (D: epoxiconazole + metconazole; T: tebuconazole; O: pyraclostrobin + epoxiconazole; P: prothioconazole), added to a wheat-based medium at different concentrations (0; 0.5; 2.5; 5; 15 $\mu\text{g/mL}$) on fumonisin production ($\mu\text{g/g}$): FB_1 (■); FB_2 (□) and FB_3 (▨) by *F. proliferatum* ITEM 15654, at different a_w levels (0.995; 0.97; 0.95) and temperatures (15; 25 $^\circ\text{C}$). Means of replicates in experiments.

of different strains of the same *Fusarium* species could be related to their genetic variability (higher or lower sensitivity of the strains)¹⁶. The efficacy of the fungicides is dependent on the fungal species, strain, ecological conditions and interactions among all these factors³³.

Fungicides used in the present study were included because they have been reported to reduce the severity of FHB losses in Argentina⁴. Tebuconazole, metconazole+epoxiconazole and prothioconazole belong to the triazole group. The other fungicide used belongs to the strobilurin fungicide group: pyraclostrobin. Although the fungicides have different modes of action, during the present study they were all able to reduce fungal growth under almost all assayed conditions. Only tebuconazole showed growth stimulation when it was used at the lowest concentration and a_w , 0.95. Marin et al.³² obtained similar results when studying the effect of tebuconazole on *F. proliferatum* and *F. verticillioides* strain growth, and observed that tebuconazole application in situations of high water stress may not be very effective. Furthermore, the authors noted that *F. proliferatum* was less susceptible to

the fungicide than *F. verticillioides*. Tebuconazole is one of the most extensively used antifungal compounds applied in agriculture to control fungal pathogens in cereals and other crops and is considered to be very effective against *Fusarium* species^{15,37}. Mateo et al.³³ observed that azole-based compounds such as prochloraz and tebuconazole were more effective at 15 $^\circ\text{C}$ than at 20- 25 $^\circ\text{C}$, which may be important as some *Fusarium* species are known to cause problems in cooler climatic regions.

The effect of fungicides on growth rates have been observed in many *Fusarium* species. Machado et al.²⁷ determined the sensitivity of *F. graminearum* species complex strains to tebuconazole and metconazole, and concluded that regardless of the species, the isolates were around 4-fold less sensitive to tebuconazole compared to metconazole. Shin et al.⁴⁶ analyzed *F. subglutinans* and *F. temperatum* growth *in vitro* with fungicide addition (tebuconazole, difenoconazole, fluquinconazole, azoxystrobin, prochloraz and resoxim-methyl), and concluded that azoles were more effective than strobilurin fungicides at inhibiting mycelial growth (prochloraz and tebuconazole being

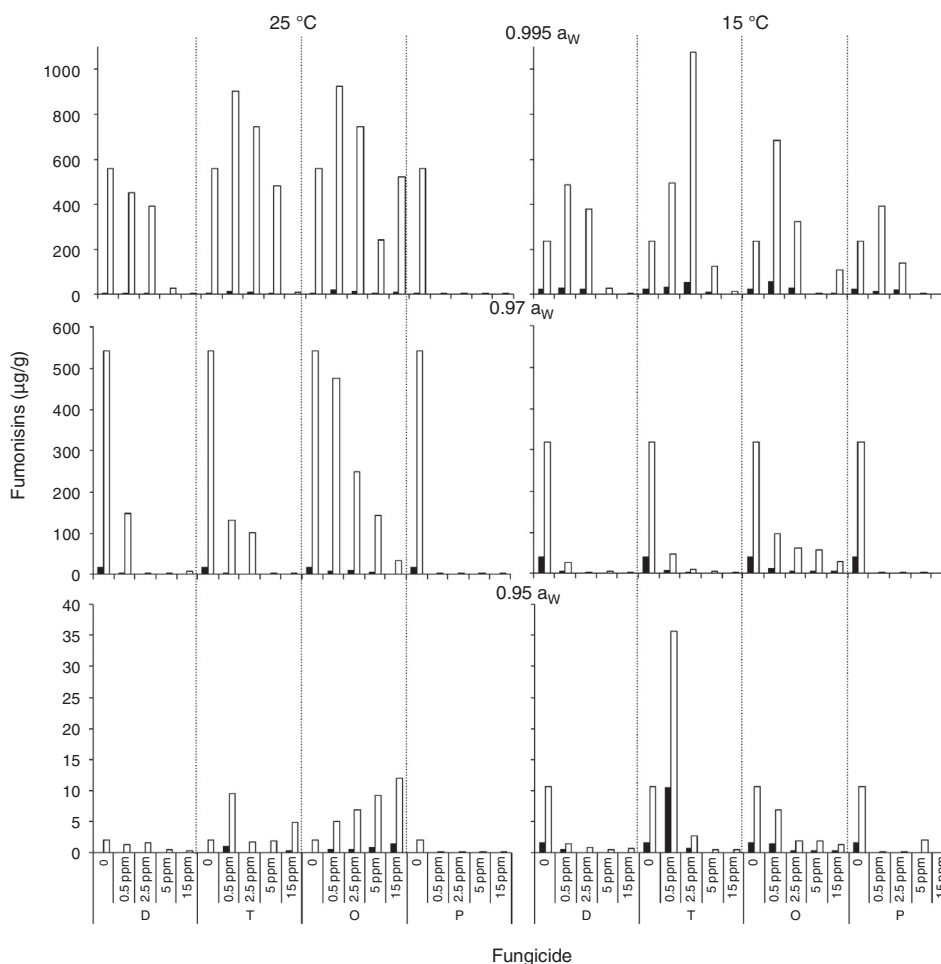


Figure 4 Effect of fungicides (D: epoxiconazole + metconazole; T: tebuconazole; O: pyraclostrobin + epoxiconazole; P: prothioconazole), added to a wheat-based medium at different concentrations (0; 0.5; 2.5; 5; 15 $\mu\text{g}/\text{mL}$) on fumonisin production ($\mu\text{g}/\text{g}$): FB_1 (■) and FB_2 (□) by *F. proliferatum* ITEM 15664, at different a_w levels (0.995; 0.97; 0.95) and temperatures (15; 25 °C). Means of replicates in experiments.

the most effective). As FHB epidemics are common in Croatia and wheat is frequently followed by maize in crop rotation, Ivic et al.²² studied the effect of carbenazamin, tebuconazole, flutriafol, metconazole and prochloraz on *F. graminearum*, *Fusarium avenaceum* and *F. verticillioides* growth, the last one being the most sensitive to the fungicides used (prochloraz and metconazole showed the greatest effect, followed by tebuconazole). However, several field studies recorded higher efficacy of tebuconazole compared to prochloraz in FHB management^{12,21,34}. Thus, many authors remark that the effect of a fungicide *in vitro* may not reflect the efficacy of a product under practical conditions. Some authors observed that prochloraz was most active in decreasing fungal growth than tebuconazole^{32,33,43}.

It has been stated that sublethal doses of some fungicides may lead to an increase in mycotoxin production by *Fusarium* species. Thus, when fungicides are used to control fungal diseases, the implications for mycotoxin production should be considered. FB production patterns and concentrations under the control conditions observed in the present study were similar to those previously observed using the

same two *F. proliferatum* strains^{6,7,10}. When fungicides were applied, different situations were observed. Prothioconazole reduced FB production under almost all assayed conditions. Contrary to our results, Audenaert et al.³ observed that sublethal concentrations of prothioconazole on *F. graminearum* cultures resulted in an increased accumulation of DON.

With respect to mycotoxin stimulation, when low doses of fungicides were applied, similar results were observed in other fungal species by other researchers^{23,30,31,33}. With regard to the effect of fungicides on FB production by *F. proliferatum* and *F. verticillioides*, it has been previously observed by Marin et al.³¹ that tebuconazole treatment did not reduce *FUM1* gene expression (good indicator of FB production). Moreover, *FUM1* induction was higher in *F. proliferatum* than in *F. verticillioides*. They concluded that *FUM1* induction with tebuconazole treatments might represent a potential additional risk of FB contamination. This fact should be remarked because tebuconazole is used for FHB management in wheat and *F. proliferatum* strains could be present in this cereal, consequently wheat grains could

be contaminated with FBs as has been observed worldwide⁵. Marin et al.³² concluded that a direct effect of tebuconazole might exist at a transcriptional level of the toxin biosynthetic enzymes for FB production in *F. proliferatum* and *F. verticillioides*, which might enhance the induction level caused by environmental stress and the combination of those two situations might result in an undesirable increase of mycotoxin levels. Other fungicides (quintozene and fludioxonil + metalaxyl-M) increased mean FB₁ levels when they were added to the culture medium of *F. verticillioides*¹⁶, suggesting the importance of focusing on the effect of fungicides on mycotoxin production as well as on phytopathogen control.

Similarly to tebuconazole, the mixture pyraclostrobin + epoxiconazole reduced FB production under some conditions, and stimulated production in other conditions, with differences between the analyzed strains. Those results are in agreement with previous *in vitro* studies mentioned above where mycotoxin production was enhanced when sublethal doses of fungicides were applied. Furthermore, DON production by *F. culmorum* was significantly increased at reduced a_w in the presence of epoxiconazole and propiconazole¹⁹.

The mechanisms of fungicide stimulation on mycotoxin production are not completely elucidated and can vary according to fungal species and strains, climatic conditions during cropping and fungicide class¹⁶. Edwards and Godley¹⁵ suggested that mycotoxin production may be favored by fungicide applications by eliminating fungal species competitive to *Fusarium spp.*, by affecting the balance between *Fusarium* species differing in fungicide sensitivity, and by a direct stress effect on mycotoxicogenic species increasing the enzyme activity involved in toxin biosynthesis; moreover, fungicides can affect the gene expression of toxin biosynthesis. Therefore, interactions between environmental conditions and fungicide treatments should be carefully considered. Furthermore, Popiel et al.⁴² suggested that increased mycotoxin contamination can be strongly influenced not only by the amount or the type of antifungal compound, but also the timing of fungicide exposure. They observed that even a very effective treatment (complete growth suppression) can still result in the induction of mycotoxin biosynthetic genes and increase mycotoxin accumulation.

There is one comparable study on the effect of fungicides used for FHB control and FB contamination in wheat grains²¹. In that study, the authors determined the relationship between fungicide effectiveness, FHB severity and FB₁ and zearalenone (ZEN) contamination of wheat kernels. As a result, no significant differences between fungicide treatments were found between FB₁ and ZEN levels in the analyzed grains. Additionally, no significant correlation was observed between FB content and FHB severity. The authors concluded that further research is needed to make hypothetical conclusions.

Our results showed that the stimulation or reduction in FB production in the presence of fungicides is influenced by complex interactions between abiotic factors and fungicide concentration in both *F. proliferatum* strains. It can be observed that fungicides used for FHB management have a profound effect on *F. proliferatum* growth and FB

production, leading to FB contamination of wheat grains. However, this study was performed using a wheat-based medium, it would be interesting to perform a similar assay using irradiated wheat grains, in order to exactly reflect the ability of the same strains to grow and produce mycotoxins using a natural substrate. Moreover, field experiments are needed in order to prove the results obtained in this study.

Conflict of interest

The authors declare that they have no conflicts of interest.

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