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ORIGINAL PAPER

Influence of the time of fungicide application in the production of trichothecenes on field inoculated spikes from wheat grown in Argentina

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Abstract

Fusarium graminearum is considered the main causative agent of Fusarium Head Blight in wheat. The fungus produces mycotoxins of the trichothecene family that pose a significant threat to the health of animals and humans. The objective of this work was to analyze the effect of the time of fungicide application on the trichothecene production in 8 inoculated wheat varieties. The fungicide Tebuconazole was applied in two independent stages: immediately after inoculation and after 72 h of the inoculation (infective event derived from 72 h of wetting). The detection of mycotoxins was performed by gas chromatography with electron capture detector and the results were confirmed by gas chromatography coupled to a mass spectrometer. Significant levels of deoxynivalenol were found in all samples and in almost all of them 15 acetyldeoxynivalenol was found in low concentration. Less frequently, 3 acetyldeoxynivalenol was found in higher values. There were not significant differences ($p > 0.05$) between the time of fungicide application (immediately after inoculation in anthesis and after 72 h) in all wheat varieties studied. The expansion of the fungicide application window for effective control of trichothecenes for 72 h represents an economic advantage for the producer avoiding the use of agrochemicals by preventive applications and minimizing the risk on human health and the environment.

Keywords Wheat varieties · *Fusarium graminearum* · Mycotoxins · Tebuconazole · Fungicide application

Introduction

Fusarium Head Blight (FHB) is a main fungal disease of wheat. Spike infection occurs during the opening of flowers and is favored by high humidity or wet weather accompanied by warm temperatures (Bai and Shaner 1994). Worldwide *Fusarium graminearum* is the pathogen with the highest predominance to the development of this disease. The economic impact includes loss of human and animal life, increased health care and veterinary care costs, reduced livestock production, and disposal of contaminated foods and feeds. *Fusarium graminearum* produces mycotoxins, the trichothecenes, that includes deoxynivalenol (DON), its acetyl derivatives 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV), and Fusarenone X (Fernández Pinto et al. 2013). DON is considered by the World Health Organization (WHO) as a teratogenic, neurotoxic and immunosuppressive toxin. The European Union has established maximum limits for DON. However, no country has established limits for the acetylated derivatives, and few studies have been published about their toxicity (FAO/WHO 2011). In Argentina, DON is the main *Fusarium* toxin detected in wheat, and very low levels of NIV and Fusarenone X have been reported (Fernández Pinto et al. 2013). Although DON and NIV are structurally very similar, NIV is 10 times more toxic than DON (Mirocha

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et al. 1976; Kamimura et al. 1981; Ueno 1983; Ueno and Hsieh 1985a).

FHB causes yield losses that can reach 50% in cases of severe epidemics. With moderate epidemics, losses between 10 and 20% can be expected. These losses are related to the sterility of spikelets and the formation of grains which have an altered constitution and low weight (Parry et al. 1995; McMullen et al. 1997). The severity of the FHB epidemics is determined by the abundance of primary inoculum, the susceptibility of the host and the weather conditions (wet duration and temperature) during and after the anthesis (Bai and Shaner 2004). The control methods are fundamentally based on the reduction of the initial inoculum and its dispersion and the protection of the spikes when the inoculum is present (Parry et al. 1995). The application of fungicides aimed at the protection of exposed anthers is a highly feasible alternative (Reis and Carmona 2002). Chemical protection is based on the use of preventive fungicides that suppress the infection when spores are deposited on exposed anthers (Moschini et al. 2013). Chemical control at the field level is not simple and shows erratic behavior. The effectiveness of the chemical treatment depends not only on the active ingredient, but also on the method and the time of application (Parry et al. 1995).

The prediction models advise that the fungicide application decision should be made when the susceptible period is observed (first exposed anthers) and the predisposing meteorological variables are recorded (periods of two days with occurrence of rainfall and high records of relative humidity and temperature) (Moschini and Fortugno 1996). The combined use of chemical control and prediction models would prevent the use of agrochemicals (by preventive applications), contributing to minimize the risks they produce on human and animal health and their impact on the environment.

The objective of this work was to analyze the effect of the time of fungicide application on the trichothecenes production in 8 inoculated wheat varieties in Argentina. The fungicide (tebuconazole) application was performed immediately after the inoculation in anthesis and after 72 hs.

Materials and methods

Fusarium graminearum ss inoculum

The eight wheat varieties were inoculated with four *Fusarium graminearum* sensu stricto strains isolated from wheat samples in the locality of Marcos Juárez, Córdoba in 2010. The four strains used were: LP2A, LP12C, LP16D and LP17B, of the collection of the University of La Plata (Buenos Aires, Argentina) and were available in the collection of the Laboratorio de Microbiología de Alimentos, Instituto de Micología y Botánica. Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, (Buenos Aires, Argentina) (mail: ing.albuquerque@gmail.com).

Table 1 Trichothecene production profiles of the four *Fusarium graminearum* sensu stricto strains in rice grain cultures

Isolates	Trichothecene production profiles (µg/g)		
	DON	3-ADON	15-ADON
LP2A	7118.58	12,743.00	6860.00
LP12C	2641.19	668.91	53.93
LP16D	14,850.86	1125.83	19.96
LP17B	1165.50	ND	15.14

ND not detected. (≤ limit of detection)

The toxicogenic capacity of the isolates used to inoculate in the field was determined. by the methodology descripted by Alvarez et al. 2009 (Table 1).

The mix of isolates of *F. graminearum* were grown for 48 h at 22 °C in 250 mL Erlenmeyer flasks containing 50 mL of an agar culture (Wheat bran 40 g, agar 20 g, distilled water 1 l). The conidia of each Erlenmeyer were extracted with sterile distilled water washes and filtered with sterile gauze. The suspensions of the four isolates were mixed in equal parts and brought to a concentration of 3×10^5 conidia to perform spray inoculation in anthesis.

Field trial

The experiments were performed in the experimental station INTA Marcos Juarez, (Córdoba, Argentina (33° 3′ 35.92" S—64° 25′ 10.17" O), during the wheat growing seasons 2014. The 8 wheat varieties analyzed in these trials were selected based on a previous study (Alberione 2014), where it was evaluated their behavior against FHB (Table 2).

Table 2 Resistance of wheat varieties against FHB

Varieties	Maturity	FI*	Resistance**
ACA 906	Early	18	MR
Bio INTA 1005		21.8	MR
AGP Fast		11.8	MR
SRM LE 2333		5.9	MR
ACA 320	Late	19.6	MR
Baguette P 11		42.8	MS
SRM Nogal		17.3	MR
Bio INTA 3005		30.7	MS

**Fusarium* Index = (Incidence x severity) / 100 (Calculated from data of Alberione 2014)

**MR: moderately resistant (FI 5 to 25%) MS: moderately susceptible (FI 25 to 50%). Scale based on spike severity (Kohli 1989)

Sowing was carried out in the first week of June for cultivars of early maturity and in the last week of June for the ones of late maturity. The experimental plots consisted of two rows (2 m/row, 0.2 m between rows; 8 plants per cultivars) with three replicates per treatment. This area had a humidifier to ensure wetting conditions after inoculation (80% HR) and was enclosed with anti-hail mesh. To favor germination of the macroconidia, the sprinklers were lit to maintain relative humidity (80%) and ensure the long wet period required for infection from inoculation to 72 hours after.

The experiments were done in a random block design. All varieties were artificially inoculated in anthesis when yellow anthers were visible on 50% of spikes. The fungicide Tebuconazole at 25% (250 gr/L) was applied (850 mL/ha) in two stages: immediately after inoculation (IwF) and after 72 h of the inoculation (IwF72) (infective event derived from 72 h of wetting). At harvest, wheat heads were collected to determine trichothecenes concentration in grains from each experimental plot. There was not yield and grain quality measured. After homogenizing the sample, it was stored in polyethylene bags at -18° C until the analysis.

Trichothecenes analysis

The toxins production was evaluated in the samples of the wheat grains harvested.

In the samples, 25 g of each of the previously ground grain samples were weighed in a 250 ml Erlenmeyer flask to continue the extraction process. In order to quantify the toxins, the technique described by Alvarez et al. (2009) was applied. The extraction of DON, NIV, 15-ADON and 3-ADON was carried out with 125 mL of acetonitrile: ethyl acetate: water (50: 41: 9) as extraction of solvent for 1 h at 300 rpm. The clean-up was performed with a column packed with 1.5 g of charcoal: alumina: celite (0.7:0.5:0.3) and dried in Rotavapor ®. Trichothecenes were detected and quantified by gas chromatography with electron capture detection Shimadzu Model GC17, equipped with split/ splitless injector and fitted with RX-5MS capillary column (30 m × 0.25 mm × 0.25 µm). The injector and detector temperatures were 300° C and 330° C respectively. Separation was achieved with a temperature program consisting of 2 min at 90 °C, then an increase from 90 °C to 275 °C at a rate of 23 °C per min, followed by another increase from 275 °C to 290 °C at a rate of 30 °C per min. Nitrogen was both the auxiliary and carrier gas. The derivatization was carried out with trifluoracetic anhydride with the method reported by Croteau et al. (1994). The injection volume was 2 µl and the run time per sample was 18 min. The detection limit value was 0.010 µg / g for DON, 3-ADON and 15-ADON and 0.050 for NIV. Standards of DON, NIV, 15-ADON and 3-ADON were purchased from SIGMA Chemical Company (St Louis, MO, USA).

Confirmatory analysis

The presence of compounds was confirmed by the Gas Chromatography-Mass spectrometer system of representative extracts. Gas chromatography analyses were performed using a Perkin Elmer Clarus 600 gas chromatograph equipped with a single quadrupole mass detector. Sample volumes of 2.0 µL were injected into the programmable split/ splitless injector, in splitless mode with the split outlet opened after 1.5 min with injector port temperature at 250 °C. The capillary column used was DB-5MS (30 m × 0.25 µm × 0.25 mm) (Agilent Technologies). Separation was achieved with a temperature program consisting of 2 min at 90 °C, an increase from 90 °C to 275 °C at a rate of 23 °C per min, two min at 275 °C, and an increase from 275 °C to 290 °C at a rate of 30 °C per min, keeping this last temperature for 5 min. Helium was both the carrier and auxiliary gas. The injection volume was 1 µl and the total running-time was 27 min for each sample. Derivatization was carried out using trifluoracetic anhydride (TFAA) following the methodology reported by Schwadorf and Muller (1991) and Schollenberger et al. (1998). Identity confirmation was based on retention time and mass spectra. Multiple ion chromatograms were built using the most characteristics ions m/z: 153, 229, 244, 257, 358, 371, 525, 598, 696 for NIV; 117, 145, 194, 231, 259, 343, 371 and 584 for DON; 133, 145, 221, 277 and 344 for 15-ADON; and finally, 123, 163, 221 and 344 for 3-ADON. Retention time was 9.01 min for NIV and 9.76 min for DON, while for acetyl derivatives it was 10.86 and 11.30 min for 15- and 3-ADON, respectively.

Statistical analysis

A Student's test was carried out to determine the effect of the time of fungicide application. The software used was SPSS, version 21 (SPSS Inc., Chicago, Ill., U.S.A.). Levene´s test was applied to determine the homogeneity of variance**.**

Results

Toxin production profiles of the strains inoculated in the wheat varieties

Trichothecenes (DON, 3-ADON and 15-ADON) production profile of the isolates is presented in Table 1. The isolates were chosen to ensure that the three toxins were produced in detectable amounts.

Two isolates (LP12C and LP16D) produced high levels of DON, 3-ADON and less 15-ADON. One isolate LP17B produced high levels of DON, less 15-ADON but no 3-ADON. And the other isolate, LP2A, produced high levels of DON, high levels of 3-ADON and less 15-ADON. No detectable values were obtained for nivalenol (Table 1).

Toxin production in the inoculated wheat varieties

DON and 15-ADON were produced in all inoculated samples. Much lower amounts of 15-DON were detected as observed in the toxin production profile of the inoculated strains. The other acetyl derivative 3-ADON was only detected in 44% of the samples, but in much higher quantities also in accordance with the production profiles of the strains. When the effect of wheat varieties on the production of trichothecenes was analyzed, it was observed that ACA 906, a variety considered susceptible to the disease (Table 2) had accumulated the lowest DON value (0.1340 µg / g), in contrast to ACA320 that had accumulated the highest DON value (1.147 µg / g) and was determined as moderately resistant. This discrepancy could also be observed on the other varieties with respect to all the toxins. These results could indicate that the varieties have a different response if their behavior is evaluated with respect to their resistance to the development of the disease or to the accumulation of toxins.

Analysis of the effect of the time of fungicide application on the toxin production.

The statistical analysis carried out by a Student´s Test showed that the time of fungicide application (IwF or IwF72) did not have significant effect ($P > 0.05$) on the toxin production (DON, 3-ADON and 15-ADON) in all wheat varieties studied (Table 3).

Discussion

According to studies based on chemical analyzes conducted in the wheat crop area of Argentina, isolates of *Fusarium graminearum*, simultaneously produced DON, 15-ADON and 3-ADON, and in some cases both acetyl derivatives were produced in similar quantities (Fernández Pinto et al. 2008; Alvarez et al. 2009). This could be evidenced in the results obtained in the present work, since of the total of the 4 isolates used to inoculate, 3 of them (LP2A, LP12C and LP16D) generated the 3 toxins (DON, 3-ADON and 15-ADON) and the remaining one (LP17B) only generated DON and 15-ADON.

In relation to the impact of the time of application of fungicides on the control of the associated disease / mycotoxin complex, other authors found that optimal control of the development of symptoms of FHB was observed when the fungicide metconazole was applied before infection. In contrast, to control contamination with mycotoxins, the optimal time of

Table 3 DON, 3-ADON, and 15-ADON concentration in inoculated wheat varieties after the fungicide application

Varieties	Times application	DON (µg/g)	3-ADON (µg/g)	15-ADON (µg/g)
ACA 320	IwF	1.147 ± 0.067	ND	0.036 ± 0.001
	IwF72	0.446 ± 0.044	ND	0.034 ± 0.001
ACA 906	IwF	0.134 ± 0.078	ND	0.176 ± 0.161
	IwF72	0.280 ± 0.001	1.972 ± 0.678	0.025 ± 0.010
AGP Fast	IwF	0.457 ± 0.029	ND	0.025 ± 0.011
	IwF72	0.992 ± 0.023	ND	ND
Bio INTA 1005	IwF	0.313 ± 0.058	ND	0.034 ± 0.001
	IwF72	0.296 ± 0.020	3.936 ± 1.424	0.034 ± 0.001
Bio INTA 3005	IwF	0.455 ± 0.057	ND	0.035 ± 0.001
	IwF72	0.456 ± 0.008	4.939 ± 1.706	0.038 ± 0.001
SRM Nogal	IwF	0.160 ± 0.014	6.660 ± 0.759	0.034 ± 0.001
	IwF72	1.140 ± 0.066	ND	0.035 ± 0.001
Baguette P 11	IwF	0.521 ± 0.057	4.636 ± 1.601	0.035 ± 0.001
	IwF72	0.207 ± 0.012	ND	0.034 ± 0.001
SRM LE 2333	IwF	0.305 ± 0.151	4.375 ± 0.298	0.034 ± 0.001
	IwF72	0.189 ± 0.113	4.318 ± 0.687	0.036 ± 0.001

IwF(fungicide application immediately after the inoculation) IwF (fungicide application 72 hs after the inoculation)

ND not detected(≤ limit of detection)

Means ± standards deviations

application was in milky grain. Similar results were achieved by analyzing applications of a fungicide (methyl thiophanate: systemic fungicide with preventive and curative activity) in before and up to 30 days post-anthesis (artificial inoculation and humidifier) (Ueno and Hsieh 1985b) (Yoshida et al. 2012).

In the susceptible period in which the spikes had exposed anthers, severe epidemics are associated with the occurrence of long wetting periods (24 to 72 h) and temperatures of 15 to 25° C (Reis and Carmona 2002). Fungicides applied in the susceptible period can help to prevent economic losses but their effectiveness depends on the time of application and the technology to achieve good spike coverage. The fungicides that are available must be applied preventively (before infective events occur) or semi-preventive (maximum 2 to 3 days after the infective event) (Annone 2003). A rational control strategy can be based on the use of predictive models of FHB to alert in real time about the occurrence of favorable weather conditions for infection (at severe levels) and thus be in time for post-infection chemical control. In the present work, especially for varieties that could reach high levels of mycotoxin contamination, the response to the application of triazole post-infection fungicide (up to 72 h) was promising. In agreement with the present results, another author found that applications of triazole fungicides made up to 6 days following anthesis may be an be as effective or more than those made before, in reducing FHB and DON levels (D'Angelo et al. 2014). Under a holistic approach, when designing a rational control strategy of the fungal / mycotoxin complex analysis, it is relevant to know the temporary window of application of a fungicide, for an effective control of the disease / mycotoxins. Although preliminary, the results obtained in this work contribute in this regard. The results of this preliminary work may be used to determine the optimal time of fungicide application to control the synthesis of mycotoxins in grains. Especially when wheat varieties with high toxin accumulation are sown. This work shows the possibility of expanding the window of application of the fungicide at 72 h post infection in anthesis. In this way, the preventive use of agrochemicals would be avoided, contributing to minimizing the risks they produce on human health and its impact on the environment.

Author contributions R. Moschini, N. Pesquero, and V. Fernandez Pinto conceived the project and its components. E. Alberione performed the field trials and selected the *Fusarium* isolates. N. Pesquero, D. Ramirez Albuquerque and D. Rojas were responsible for the toxin analysis. R. Moschini, N. Pesquero, A. Gonzalez and V. Fernandez Pinto analyzed the data. A. Gonzalez designed the statistical analysis. R. Moschini,V. Fernandez Pinto, N. Pesquero and A. Gonzalez wrote the manuscript and all co-authors contributed to manuscript revision.

Declarations

Conflict of interest All authors of the manuscript submitted to Australasian Plant Pathology, point out that we have no potential conflict of interest.

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