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NATURAL OCCURRENCE OF NIVALENOL AND MYCOTOXIGENIC POTENTIAL OF *FUSARIUM GRAMINEARUM* STRAINS IN WHEAT AFFECTED BY HEAD BLIGHT IN ARGENTINA

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ABSTRACT

The principal agents of *Fusarium* head blight in the main cropping area of Argentina were investigated in heavily infected samples. The ability of the isolates to produce trichothecenes was determined by GC and HPLC. *Fusarium graminearum* was the predominant species and of 33 isolates, 10 produced deoxinivalenol (DON) (0.1- 29 mg kg⁻¹), 13 produced both deoxinivalenol (1.0- 708 mg kg⁻¹) and nivalenol (0.1- 6.2mg kg⁻¹), 12 produced 3-acetyldeoxinivalenol (0.1- 14 mg kg⁻¹), 13 produced 15-acetyldeoxinivalenol (0.1- 1.9 mg kg⁻¹), 10 produced Fusarenone X (0.1- 2.4 mg kg⁻¹) and 7 produced zearalenone (0.1- 0.6 mg kg⁻¹). These results suggest that *F. graminearum* strains isolated from the wheat growing regions in Argentina belong to DON chemotype. Although some strains produced both deoxinivalenol and nivalenol, nivalenol was produced in lower levels. The natural occurrence of nivalenol in wheat affected by head-blight collected in the main production area during two years (2001-2002) was also determined. From 19 samples 13 were contaminated with deoxinivalenol in a range of 0.3 to 70 mg kg⁻¹ and 2 samples with both deoxinivalenol (7.5 and 6.7 mg kg⁻¹) and nivalenol (0.05 and 0.1 mg kg⁻¹), respectively. This is the first report of natural occurrence of nivalenol in wheat cultivate in Argentina.

Key words: *Fusarium*, nivalenol, toxicogenic potential, trichothecenes.

INTRODUCTION

Wheat is one of the most important cereal crops in Argentina both for local consumption and as an export commodity, with yields over 12.000.000 tons (1). The cultivated area of 6.000.000 ha is distributed according to agrometeorological conditions into five zones, with a main production area on the Buenos Aires province (Zones IIS, IV and VS), East of La Pampa province (Zone VS) and South of Santa Fe province (Zone IIN). This extended area presents very different conditions of temperature and humidity. The incidence of toxigenic *Fusarium* spp. and their toxins overall this wide cultivated area has been scarcely

studied. *F. graminearum* Schwabe [teleomorph *Gibberella zeae* (Schw.) Petch] is the most common causal agent of *Fusarium* head blight (FHB) in many parts of the world. Recent outbreaks had been reported in USA, Asia, Canada, Europe and South America. This destructive disease affects wheat, barley and other small grains in temperate and semitropical areas. The disease has the capacity to destroy a potentially high- yielding crop within a few weeks of harvest, causing economic losses due to reduced seed quality. Additionally, infected grains may contain significant levels of mycotoxins like trichothecenes and zearalenone. Trichothecenes toxins are sesquiterpenoids that are potent inhibitors of eucaryotic protein biosynthesis. Adverse

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effects of the toxins in animals are food refusal, diarrhoea, emesis, alimentary haemorrhaging, contact dermatitis and immunosuppression. Zearalenone is an estrogenic mycotoxin (11). Some earlier works have shown that *F. graminearum* was the main occurring species and deoxinivalenol (DON) was the main toxin contaminant detected in wheat and wheat-products in Argentina (10,18,19,20). Two main chemotypes were reported in *G. zea* from rice in Japan: the "NIV chemotype" which produces nivalenol (NIV) and Fusarenone X (Fus-X) and the "DON Chemotype" which produces deoxinivalenol (DON) and its acetylated forms (12). Differences in the geographical distribution of DON and NIV chemotypes of *F. graminearum* have been reported. Only the DON chemotype has been found in the United States and Canada (16), while both chemotypes were isolated in Japan (26), Italy (14), South Africa (23), and Australia (2). Successively, Sugiura *et al.* (22) demonstrated that some *G. zea* isolated from rice stubble in Japan produced both NIV and DON. This capability was also confirmed in some strains in Hungary (24) and in strains isolated from maize and wheat in Nepal (6). The ability of Argentinean *Fusarium* isolates to produce trichothecenes is controversial and the pattern of micotoxin production is not already well defined. Faifer *et al.* (8) reported that *F. graminearum* isolated from wheat in Argentina produced DON, 15-acetyldeoxinivalenol (15AcDON), 3-acetyldeoxinivalenol (3AcDON) and zearalenone (ZEA) while Ramirez *et al.* (20) found only DON and 3-AcDON producers. On the other hand, Lori (15) studied the capability of 76 *F. graminearum* strains isolated from Argentinean wheat and 56% produced DON, 3AcDON, 10% NIV, FUS-X and 34% both DON and NIV. All those studies were carried out in crops heavily affected by FHB.

The purposes of this study were to determine:

- a) the pattern of trichothecene production by the isolates of the principal FHB agents
- b) the presence of both NIV and DON producing strains
- c) the natural occurrence of NIV, in Argentinean wheat affected by FHB

MATERIALS AND METHODS

Wheat samples

Nineteen wheat samples affected by head blight were collected from four regions in the major wheat production area in Argentina, 5 in the 2000- 2001 harvest and 14 in the 2001-2002 one. In both harvests the meteorological conditions favour the infection. The regions were: Region II North (IIN) and Region II South (IIS) (North of Buenos Aires province), Region IV (South East of Buenos Aires province) and Region V (South West of Buenos Aires and La Pampa province). Regions IIN and IIS have a temperate humid climate while Regions IV and V have a more continental semiarid one. Samples were also collected from farms in 15 districts of Bs. As. and La Pampa provinces. At least

0.5 kg was collected per sample, selected from heads with spikelets with visible symptoms of FHB. All samples were from locally grown cultivars.

Mycological analyses

For the isolation of the internal microflora, 100 wheat kernels of each sample were surface disinfected in a 2% aqueous solution of commercial sodium hypochlorite for 1 minute and rinsed twice with sterile distilled water. The kernels were placed, 10 kernels per plate, on potato dextrose agar (PDA) with 2% of pentachloro nitrobenzene (PCNB) plates and incubated for 5 days at 28°C under fluorescent and black light lamps (2700 lux; 12 h photoperiod) to stimulate conidial formation and the percentage of infected kernels was calculated. Representative cultures of the species isolated were grown from single conidia for 7 days on petri dishes with carnation leaves agar (CLA) and with potato dextrose agar (PDA) and incubated at 28°C under fluorescent light. The identification of colonies of *Fusarium* species was made according to the criteria and synoptic keys of Leslie and Summerell (13)

Toxin analysis

Sample analysis

A subsample of 25g of wheat was milled at 2500 rpm in a commercial grinder. The extraction of deoxynivalenol (DON), nivalenol (NIV), was done with 125 ml of acetonitrile: ethyl acetate: water (50:41:9) as extraction solvent per 1 hour at 300 rpm. The clean-up was made with a column packed with charcoal: alumina: celite (0.7:0.5:0.3) (19); and the extract was dried in a rotary evaporator. The dry residue was re-dissolved in 500 ml ethyl acetate: methanol (95:5) and transferred to a vial.

Evaluation of toxicogenic capacity of *Fusarium* isolates

To test for trichothecenes and zearalenone production, *Fusarium* isolates were placed in duplicate on 25g of sterilized rice in 500 ml Erlenmeyer flasks. The rice was added with 15 ml of sterile distilled water before sterilization by autoclaving at 121°C for 15 min. Each flask was inoculated with a suspension containing 10^6 conidia ml⁻¹. The conidial suspension was performed by adding 5,0ml of sterile distilled water to a slant of a 7day culture at 28°C on PDA and gently scraping the agar surface with a wire loop to give a turbid suspension.

Toxin extraction

DON, NIV, 15AcDON, 3AcDON, FUSX, and ZEA were extracted with acetonitrile: ethyl acetate: water (50: 41: 9) as extraction solvent, and the clean up was made with a column packed with charcoal: alumina: celite (0.7: 0.5: 0.3) as was previously described (19).

Standards of DON, NIV, 15AcDON, 3AcDON, FUSX, and ZEA were purchased from SIGMA chemical Company (St. Louis, MO, USA).

Toxin detection

Toxins detection for both samples and isolates production was made by gas liquid chromatography (CGL) and the NIV presence was confirmed in all cases by HPLC.

DON, 3-AcDON, 15 AcDON, FUSX and ZEA were also confirmed by TLC (15)

CGL

Trichothecenes and ZEA were detected and quantified by gas liquid chromatography with Ni⁶³ electron capture detection Shimadzu Model GC17, equipped with split/splitless injector and fitted with a RX-5MS capillary column (25 m x 0, 2 mm i.d.). The injector temperature was 250°C and the detector one 300°C. Separation was achieved with a temperature program consisting of 1 min at 80°C, then increase 80°C - 140°C at 30°C per min, followed by 140°C-280°C at 5°C per min. Both the carrier and auxiliary gas were nitrogen. The derivatization was carried out with heptafluorbutyric anhydride with the method reported by Croteau *et al.* (4). The detection limit for DON and its acetyl derivatives was 20 µg kg⁻¹ and for NIV, FUS and ZEA 50 µg kg⁻¹ respectively

HPLC

NIV was confirmed with Shimadzu HPLC (2 pumps LC-10 ADvp, 1 DAD SPD- M10Avp, 1 System controller SCL-10 Avp) with precolumn Phenomenex Wipore C18 (ODS) (4x3 mm)

and column Phenomenex Jupiter 5u C18 (300A, 20x 4,6 mm). Each analysis was done with a 20 ul loop, water (A) + acetonitrile (B) as eluent solvents (gradient: 0 min 10%B, 10min 100% B, 13 min 100%B, 16 min 10%B, 20 min 10%B), total flow 1ml per min at wavelength: 219nm. The detection limit was 50 µg kg⁻¹.

RESULTS

The percentage of infected kernels in the samples is illustrated in Table 1. In 13 of the 19 samples the infection was of 100%. In the other samples the level of infection was always high (69 to 95%). *Fusarium graminearum* was found as the main occurring species and it was found in 17 samples in levels from 4 to 88%. *Fusarium poae* and *F. verticillioides* were also rarely encountered (less of 2%).

All the samples were assayed from the presence of DON and NIV. The results were presented in Table 1, where it is also shown the geographical origin of the samples.

Fifteen of the 19 samples were contaminated with DON and the levels ranged from 0.3 to 70 mg kg⁻¹. In this study 2 samples (Arrecifes from North of Buenos Aires province and Conhelo from La Pampa province) were contaminated with DON (7.5 and 6.7 mg kg⁻¹) and NIV (0.05 and 0.1 mg kg⁻¹) respectively. From the samples of wheat most heavily contaminated were isolated 33 strains of *F. graminearum* and their toxicogenic potential was determined in autoclaved rice.

Table 1. Natural occurrence of DON and NIV in Argentinean wheat affected by head blight.

Zone	Sample	Infected. kernels %	<i>F. graminearum</i> %	DON mg kg ⁻¹	NIV mg kg ⁻¹
IIN	13-Pergamino	100	50	7.8	ND
	14-San Pedro	100	82	70	ND
IIS	1-Arrecifes	100	74	7.5	0.05
	9- Salto	100	88	16	ND
	15- Villegas	100	40	3.0	ND
	19- Lincoln	100	6	3.3	ND
	10-Saladillo	100	85	14	ND
IV	2- Olavarria	100	-	-	ND
	3- Olavarria	100	21	1.3	ND
	4- Olavarria	83	-	0.8	ND
	7- Gral.Pueyrredon	100	12	0.3	ND
	11- Gral. Pueyrredon	100	4	0.4	ND
V	5- Cnel Suarez	85	39	5.4	ND
	8- Conhelo	95	67	6.7	0.1
	12- Conhelo	100	43	5.8	ND
	6- Mt. Nievas	100	39	5.8	ND
	16- Luiggi	86	4	ND	ND
	17- Winifreda	69	7	ND	ND
18- Castex	79	6	ND	ND	

Concentration of trichothecenes and zearalenone produced are illustrated in Table 2.

In this study, 10 strains produced DON ranging between 0.1 and 29 mg kg⁻¹. Of these strains 3 produced also 3 AcDON ranging between 0.7 and 3.0 mg kg⁻¹ and 4 produced 15 AcDON ranging between 0.1 and 0.9 mg kg⁻¹. The results also demonstrated the production of both DON (ranging between 1.0 and 708 mg kg⁻¹) and NIV (ranging between 0.1 and 6.2 mg kg⁻¹) by 13 of 33 *F. graminearum* strains tested. In DON and NIV producers, 9 strains produced FUS X (up to 2.4 mg kg⁻¹), 8 produced 3AcDON (up to 14 mg kg⁻¹) and 9 produced 15 AcDON (up to 1.9 mg kg⁻¹). Of these, 5 strains produced 3 AcDON and 15 AcDON simultaneously. Only one strain produced FUS X

(0.2 mg kg⁻¹), DON (20 mg kg⁻¹) and 3 AcDON (4.1 mg kg⁻¹). Zearalenone was produced by 7 strains in low amounts, ranging between 0.1 and 0.6 mg kg⁻¹ and no exclusive relationship could be observed between ZEA production and the DON and NIV production. Finally, we did not detect trichothecene production from eight strains.

DISCUSSION

These results agree with other reports carried out in Argentina where *F. graminearum* proved to be the main toxigenic *Fusarium* species (10,15,20). All the samples collected in the zones IIN and IIS are 100% infected. On the contrary, zone V can be considered less contaminated because 71.42% of samples don't reach 100% of infection. In fact, *F. graminearum* is the most frequent fungi in the zone IIN and IIS while the percentages are lower in the zones IV and V.

The levels of toxin contamination found in samples in this study are similar to those reported earlier in heavily infected wheat in Argentina (10,19). This is the first report of the natural occurrence of NIV in wheat cultivated in Argentina. DON and NIV are very similar in their chemical structure, but differ markedly in their toxicological properties with NIV considered more mycotoxic than DON (25). Therefore the presence of NIV in Argentinean wheat may have a significant impact on the food safety aspects because its presence can synergize the toxicity of DON. By this study and other reports (5,10,19) Argentina prove to have a high risk of DON wheat contamination due to the high incidence of FHB, particularly in the area with temperate humid climate. This risk seems also to be present in South of Brazil (9) and Uruguay (18).

Overall, the DON production by strains proved to be higher than NIV. All the data analyzed in this study support the fact that the variation among the toxigenic potential of *F. graminearum* strains isolated from Argentinean wheat is quite complex and may contribute to a wide range of mycotoxin content in this cereal. The introduction of novel FHB chemotypes via global trade in agricultural products should be assessed because could have the potential to exacerbate FHB problem (17). In particular the relative frequency of DON and NIV is of concern because recent reports suggest that strains of

Table 2. Production of trichothecenes and zearalenone by *F. graminearum* isolates (mg kg⁻¹).

Strain N°	Sample	Region	NIV	FUSX	DON	3ADON	15ADON	ZEA
4620	13	IIN	ND	ND	25	ND	ND	ND
4614	10	IIS	ND	ND	19	0.9	ND	ND
4601	1	IIS	ND	ND	29	0.7	0.1	ND
4602	1	IIS	ND	ND	8.3	3.0	0.9	ND
4604	1	IIS	ND	ND	10	ND	ND	ND
4622	14	IIS	ND	ND	2.5	ND	0.2	ND
4626	14	IIS	ND	ND	25	ND	0.1	ND
4629	15	IIS	ND	ND	16	ND	ND	ND
4632	9	IIS	ND	ND	6.0	ND	ND	0.1
4617	13	IIN	ND	ND	0.1	ND	ND	
4619	13	IIN	0.8	2.4	120	2.9	0.3	0.1
4615	10	IIS	1.5	2	95	ND	1.8	ND
4616	10	IIS	0.4	0.1	38	2	0.2	0.6
4600	1	IIS	6.2	0.7	708	14	ND	ND
4603	1	IIS	0.2	ND	11	ND	ND	ND
4623	14	IIS	0.1	ND	1.0	0.3	ND	0.1
4634	9	IIS	1.1	ND	14	1.0	0.3	ND
4635	9	IIS	2.1	ND	21	ND	1.9	0.1
4636	9	IIS	0.6	0.1	37	0.3	0.1	ND
4637	9	IIS	0.2	0.1	98	0.1	ND	ND
4638	9	IIS	2.2	0.1	17	ND	0.7	0.1
4640	15	IIS	0.5	0.1	8.5	ND	0.3	ND
4606	7	IV	0.2	0.1	58	1.0	0.4	0.4
4609	8	VS	ND	0.2	20	4.1	ND	ND
4618	13	IIN	ND	ND	ND	ND	ND	ND
4624	14	IIS	ND	ND	ND	ND	ND	ND
4625	14	IIS	ND	ND	ND	ND	ND	ND
4627	14	IIS	ND	ND	ND	ND	ND	ND
4628	14	IIS	ND	ND	ND	ND	ND	ND
4631	9	IIS	ND	ND	ND	ND	ND	ND
4633	9	IIS	ND	ND	ND	ND	ND	ND
4605	7	IV	ND	ND	ND	ND	ND	ND

the *F. graminearum* complex that produce NIV may be more aggressive towards corn but less aggressive to wheat than DON producing strains. These results indicate that there may be important consequences for the fitness and aggressiveness of FHB pathogens of different chemotype on particular hosts (3,7,11). Further studies will be necessary in order to characterize the genetic diversity, chemotype and virulence of *F. graminearum* populations from Argentina, a country where this species is widespread and wheat is cultivated under different agro-meteorological conditions.

RESUMO

Ocorrência natural de nivalenol e potencial micotoxigênico de cepas de *Fusarium graminearum* em trigo afetado por giberela na Argentina

O principal causador de giberela no trigo na Argentina e sua capacidade de produzir tricotecenos foram estudados por GC e HPLC em amostras altamente infectadas. A espécie predominante foi *Fusarium graminearum*, sendo que de um total de 33 isolados, 10 produziram deoxinivalenol (0,1-29 mg kg⁻¹), 13 produziram deoxinivalenol (1,0-708 mg kg⁻¹) e nivalenol (0,1-6,2 mg kg⁻¹), 12 produziram 3-acetildeoxinivalenol (0,1-14 mg kg⁻¹), 13 produziram 15-acetildeoxinivalenol (0,1-1,9 mg kg⁻¹), 10 produziram fusarenona X (0,1- 2,4 mg kg⁻¹) e 7 produziram zearalenona (0,1- 0,6 mg kg⁻¹). Esses resultados sugerem que as cepas de *F. graminearum* isoladas de trigo cultivado na Argentina pertencem ao quimiotipo DON. Embora algumas cepas tenham produzido tanto DON quanto NIV, NIV foi produzido em quantidade inferior ao DON. A ocorrência natural de nivalenol em trigo afetado pela giberela coletado na principal área de produção durante dois anos (2001-2002) foi também determinada. De 19 amostras, 13 estavam contaminadas com deoxinivalenol na faixa de 0,3 a 70 mg kg⁻¹ e 2 amostras continham tanto deoxinivalenol (7,5 e 6,7 mg kg⁻¹) quanto nivalenol (0,05 e 0,1 mg kg⁻¹), respectivamente. Esse é o primeiro relato da ocorrência de nivalenol em trigo cultivado na Argentina.

Palavras-chave: *Fusarium*, nivalenol, potencial toxicogênico, tricotecenos.

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