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PRODUCTION OF MYCOTOXINS BY *FUSARIUM GRAMINEARUM* ISOLATED FROM SMALL CEREALS (WHEAT, TRITICALE AND BARLEY) AFFECTED WITH SCAB DISEASE IN SOUTHERN BRAZIL

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ABSTRACT

Fusarium fungi are known to be pathogenic for plants and mycotoxin producers. The *in vitro* production of deoxynivalenol and zearalenone was qualitatively evaluated in 24 different isolates of *Fusarium graminearum* collected from small cereals associated with the scab disease, in southern Brazil. Isolates were cultivated in rice during 14 days at 28ºC. Cultivates were extracted with methanol:water (40:60 v/v) and analyzed by thin layer chromatography. Other trichothecenes (diacetoxyscirpenol, fusarenon-X, neosolaniol and nivalenol) and zearalenol, often produced by *Fusarium*, were also analyzed. In the conditions used, it was possible to detect zearalenone and deoxynivalenol in 67% and 33% of the isolates, respectively. The presence of zearalenol, diacetoxyscirpenol and fusarenone was also detected. None of the isolates was found to produce nivalenol or neosolaniol.

Key words: *Fusarium graminearum*, mycotoxins, scab, small cereals, Southern Brazil

INTRODUCTION

Fusarium fungi, widely found in nature and well known as pathogenic for plants and producers of mycotoxins, cause major damage in cereals, fruits and vegetables. They are frequently associated with pre-harvest contaminated cereals. Wheat, barley and maize make up almost two-thirds of the world production of cereals and thus liable to contamination (9,10). *Fusarium*-caused diseases in cereals are worldwide and occur in all climatic conditions (6,7).

The *Fusarium* head blight or scab, a disease caused by several species of *Fusarium* (e.g *Fusarium graminearum)*, chiefly in small cereals such as wheat, triticale and barley, inhibits the formation of grains or produces wrinkled, hollow, coarse, rosy grains, contaminated by trichothecenes (mainly deoxynivalenol) and zearalenone (9).

Trichothecenes are secondary metabolites produced by several genera of fungi, including *Fusarium* and form a structurally related mycotoxin group with various degrees of cytotoxicity. They have a sesquiterpenoid structure basic ring and are classified as A, B, C and D, according to the presence or absence of characteristic functional groups. Inhibition of protein synthesis, irritation of the skin, haemorrhage, diarrhoea, nauseas, food reflux and vomiting are the different toxicological characteristics of trichothecenes (16). Deoxynivalenol, fusarenon-X, diacetoxyscirpenol, neosolaniol and nivalenol are the most frequent trichothecenes in *F. graminearum*.

Zearalenone is a mycotoxin produced by the fungus's secondary metabolism through the biosynthetic polycetidic pathway, with estrogenic activity in mammals (6,8,13,16). Zearalenone occurs chiefly during the growing phase of several grains when the fungus attacks and preys on the seeds during periods of heavy rainfall. It proliferates in mature grains, which were not sufficiently dried, owing to humidity, during harvest or storing period. Alternations between low (12-14ºC) and high

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(25-28ºC) temperatures are normally needed to start and maintain zearalenone production in grains (6). Probable primary biochemical lesion and early cell events in the series that direct cell toxicity or zearalenone-caused cell deregulation may be attributed to an initial lesion in the cytosolic estrogen receptor which causes hormone control damage (13,16).

Fusarium scab, associated with deoxynivalenol production in wheat, oats and rye, not only triggers high financial losses in the U.S.A. and in Canada, but is also a great concern for animal and human health (11,12,13,14). Nevertheless, since not all *F. graminearum* produce deoxynivalenol, its world distribution has been mapped by phylogenic studies and molecular biology techniques (10). Scanty information exists in literature on the occurrence of deoxynivalenol in Brazil. Maize contamination and its subproducts in contaminated samples in south Brazil have been already mentioned (12). Current research examined 24 selected *F. graminearum* isolates from southern Brazil, associated to the scab disease, in wheat, barley and triticale. Toxigenicity *in vitro* of these samples could be verified by the qualitative evaluation of trichothecenes and zearalenone production.

MATERIALS AND METHODS

Microorganisms

Monosporic culture of 24 *F. graminearum* isolates identified according to Nelson (7), associated with scab disease in wheat, triticale and barley, harvested in several regions of southern Brazil and preserved at the Phytopathology Laboratory – DCA/UEM, were used as follows:

Isolates numbers: 2 (Campo Mourão, PR), 4 and 5 (Mamborê, PR), 6 (Peabiru, PR), 8 and 10 (Francisco Beltrão, PR), 11 and 12 (Iguaraçú, PR) and 14 (Sertanópolis, PR); isolated from the wheat ear.

Isolates 17 and 21 (Carambeí, PR) from the triticale seed (batch 482) and from the wheat seed (batch 385); isolate 22 (Tibagi, PR) from the wheat seed; isolate 23 (Guarapuava, PR) from the barley seed; isolates 29 and 30 (Abelardo Luz, SC) from the triticale seed and 35 (Pato Branco, PR) from the triticale seed too.

Isolates 39 (Seberi, RS), 40 (Palmeira das Missões, RS), 44 and 45 (Ijuí, RS), 46 and 47 (Não-Me-Toque, RS), 60 (Maringá, PR) and 69 (Londrina, PR), all wheat seeds.

Growth conditions for production of mycotoxins

Substrate rice was employed for DON research, according to method described by Gimeno *et al.* (3). The fungi were grown in PDA (Potato Dextrose Agar) plates for seven days, at 25ºC. A suspension with sterile solution of 0.05% Tween 80 was prepared from these plates to be used as inoculum. The rice (Uncle Ben's®, 0.60 a_w) sterile (twice autoclaved) culture medium (87.5 g rice and 37.5 g distilled water) were inoculated with 10 mL of the inoculum, (10^3 to 10^5 macroconidia/mL in sporulating isolates) and then incubated for 14 days at at 28ºC.

Extraction of mycotoxins

Samples were ground with 350 mL methanol-water solution (40:60 v/v) and filtered through Whatman 1 filter paper; methanol was then evaporated by rotation evaporator, at 65ºC. Sodium chloride was then added to the aqueous extracts till saturation, and rested for 24 h. Extracts were then filtered and extracted three times with ethyl acetate. Five grams of anhydrous sodium sulfate were then added to the organic extracts and maintained at rest for 24 h. Extracts were afterwards filtered and ethyl acetate evaporated by rotation evaporator, at 55ºC. Residues were dissolved in acetone, evaporated, till dried. Final residues were dissolved in a known volume of methanol and kept in the refrigerator at 4ºC until analysis. All cultivation and extraction experiments were done thrice.

Flasks with rice, albeit without fungus inoculum, received the same culture and extraction treatment as samples (controls) for the exclusion of interfering compounds that might be confused with the mycotoxins under analysis. Growth conditions for production and extraction described above were also applied to zearalenone.

Analysis of mycotoxins

Trichothecenes standards (Sigma®), deoxynivalenol (DON), diacetoxyscirpenol (DAS), fusarenon-X (FX), neosolaniol (NS) and nivalenol (NIV) (1 μg/uL) were used in chloroform/methanol (9:1, v/v). Zearalenone (ZEA) and zearalenol (ZOL) standards (Sigma®) were dissolved in methanol (3 μg/μL). Thin Layer Chromatography (TLC) technique was employed in 20x20 cm glass plates with gel silica (Aldrich®), without fluorescence indicators, to detect and identify mycotoxins.

With regard to TLC, 10 μL of extracts were applied, in duplicates, in plates, together with specific standards (separately) or with standard pools, developed and revealed under different conditions. Whereas solvent system (1) was used with chloroform:acetone:isopropanol (8:1;1, v/v) (3) to detect trichothecenes, solvent system (2) with chloroform:methanol (93:7, v/v) detected ZEA, ZOL and DAS. Simultaneous detection procedure of mycotoxins, described by Kamimura *et al*. (5), was used for revelation. Three steps were taken after plate development. First, each plate was analyzed under UV light at 365 and 254 nm. ZEA, alone, is seen as a blue fluorescent spot. Second, $AlCl_3$ solution 20% was sprayed on the same plate, heated for 10 min at 110ºC and seen under UV light at 365 nm. NIV, DON, FX and ZEA appear as bluish fluorescent spots. Third, the same plate was sprayed with a 20% solution of H_2SO_4, heated at 110ºC for 10 minutes and then seen under UV light at 365 nm. NS and DAS appear as green-bluish fluorescent spots. According to Kammimura (5), the minimum detectable concentrations (μk/kg) were 2.0 to DON, FX, NIV; 10 to ZEA and 80 to NS and DAS.

Recoveries of the mycotoxins added to various samples at 1.0-2.0 μg/g were greater than 71% and averaged 85%. Besides the procedure above, confirmation of ZEA and ZOL was obtained by Fast Violet B. ZEA and ZOL appear as orange-colored spots and become of a violet color when the same plate is sprinkled with H_2SO_4 and then heated to 110ºC (1,16).

RESULTS

The twenty-four isolates were analyzed for TLC by means of two different solvent systems and with different revelations. Figs. 1, 2 and 3 showed the procedure used to detect mycotoxins in some of the isolates when solvent 1 is used. Fig. 1 shows: a sample (isolate 10) and the presence of DON; two samples (isolates 11 and 12) with FX, and ZOL in the sample of isolate 12. Fig. 1 also shows extract of isolate 17 with ZOL and a negative sample (isolate 14) in which no mycotoxins could be identified. Fig. 1 (1B) shows NS standard, which appears only after revelations with $AlCl_3$ and H_2SO_4. Fig. 2 shows a sample of wheat (21), one of barley (isolate 23) and two of triticale (isolates 29 and 30). These isolates failed to produce any mycotoxins in the conditions posited. Another sample (isolate 22) in which the production of toxins ZEA, ZOL, DON and FX were produced, comparatively, in a larger quantity than in all the other isolates under analysis, is also shown.

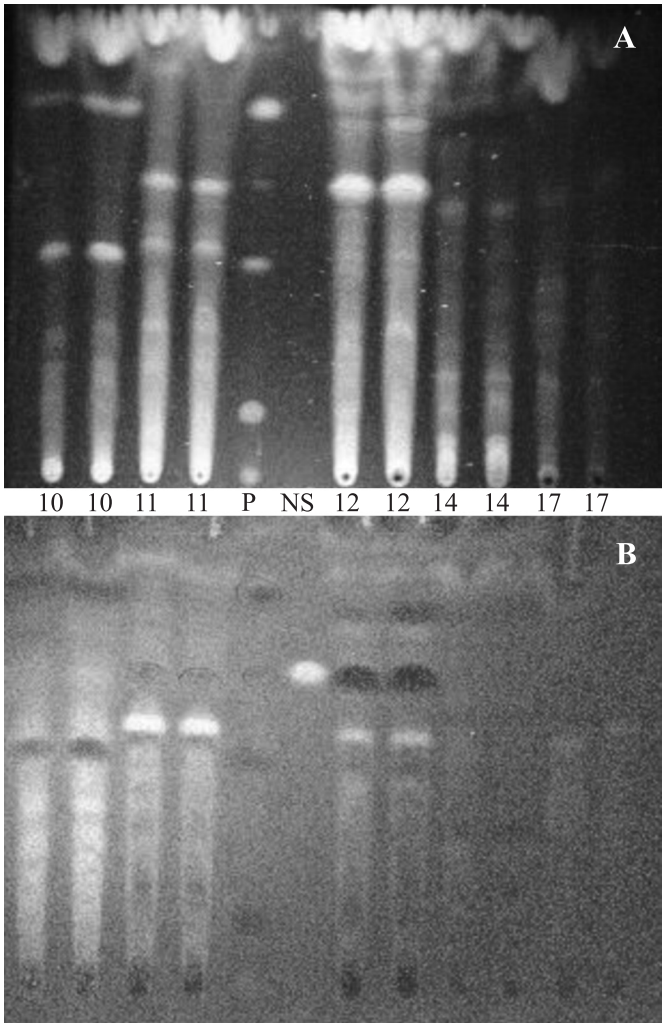

Figure 1. TLC of culture extracts of some isolates under analysis. Solvent System 1: chloroform:acetone:isopropanol (8:1:1, v/v). (A) UV revelation (365 nm), after treatment with $ALCl_3$. P (NIV, DON, FX and ZOL standards, in eluition order). (B) After treatment with H_2SO_4 and UV light (365 nm). NS neosolaniol standard.

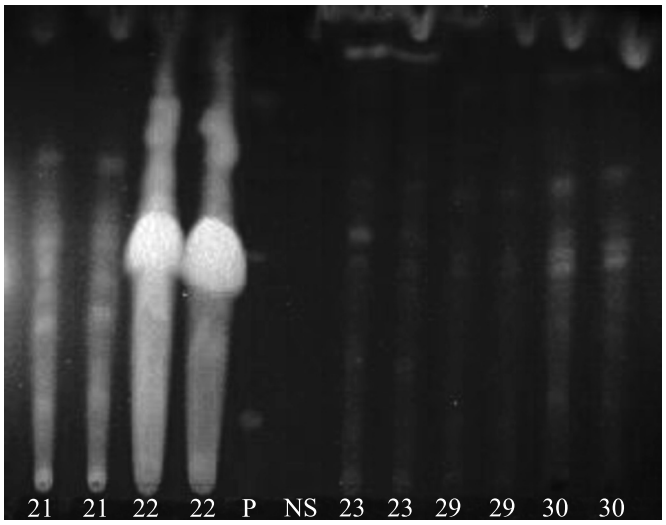

Figure 2. TLC of culture extracts of some isolates under analysis. Solvent system 1: chloroform:acetone:isopropanol (8:1:1, v/v). P (NIV, DON, FX and ZOL standards in eluition order). Revelation by ultraviolet light (365 nm) after $AlCl_3$ treatment.

Fig. 3 exemplifies procedure for ZEA and ZOL detection when solvent 2 and UV (365 and 254 nm) revelations are used, followed by treatment with Fast Violet B and H_2SO_4. Under such conditions ZEA and ZOL standards reveal ratio fronts (rfs) close to 0.73 and 0.50, respectively. ZEA and ZOL were thus produced in isolates 46, 47 and 60. Fig. 3 shows a negative sample too (isolate 69).

In the procedure and under the conditions above, the in vitro production of mycotoxins in all 24 isolates was verified. Briefly given in Table 1, results show that 71% of isolates are toxic. It may be observed that, when DON and ZEA are taken separately, 33% and 67% produced respectively DON and ZEA in the context of the 24 isolates. Several isolates showed multiple productions of mycotoxins, although NS and NIV were produced by none.

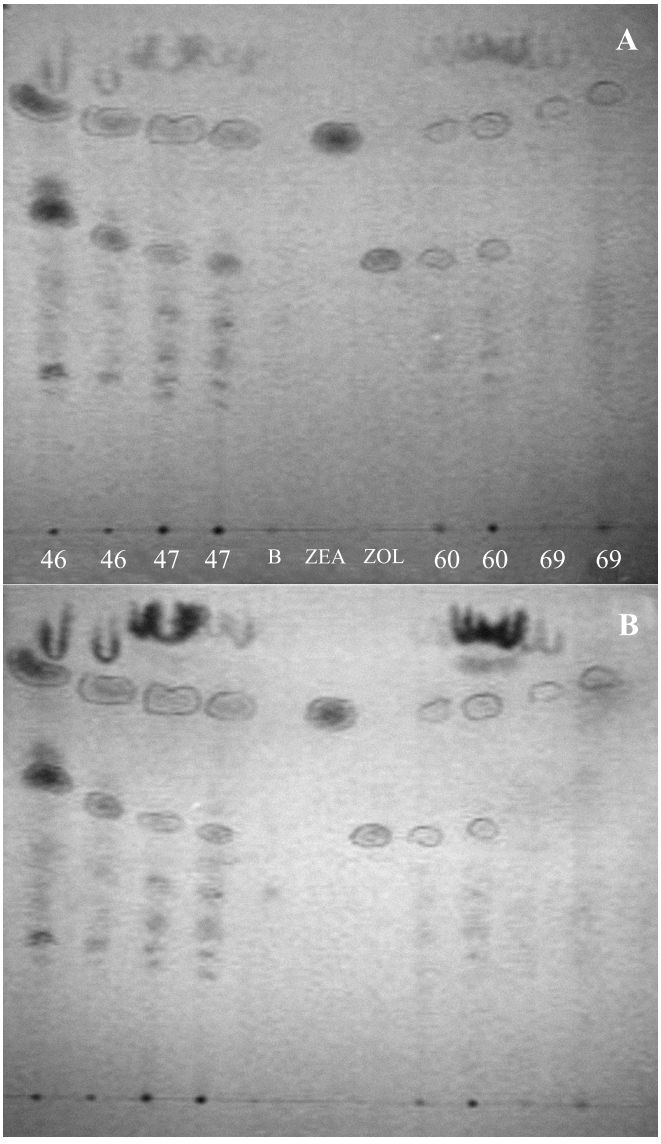

Figure 3. TLC of culture extracts of some isolates under analysis to verify zearalenone and zearalenol production. Solvent system 2: chloroform:metanol (93:7, v/v). (**A**) Revelation by Fast Violet B and (**B**), after treatment with H_2SO_4. (B) Control), (ZEA) zearalenone and (ZOL) zearalenol.

DISCUSSION

Much importance has been given to mycotoxins in the case of animal and human health. Actually mycotoxins are involved in the food chain of animals and humans through the contamination of cereals, food and other commodities, resulting in toxicological and immunologic problems (13,14,15).

Mycotoxins of the genus *Fusarium* are generally produced when cereals, usually rice and maize, are used as a solid substrate for growth. Such cultures have to pass through extraction and purification processes prior to identifications by Thin Layer Chromatography, Gas-Liquid Chromatography, or High Performance Liquid Chromatography (1,2). Due to its low costs and straightforwardness, verifications of mycotoxin production in trials, like those used in current research, are mainly done by TLC.

In our study the fungi were cultivated in rice and conditions were posited to produce trichothecenes. Although conditions were not the best possible, they were also used for ZEA production. Optimization in ZEA production involves alternations in time and temperature (1 to 2 weeks, at 24-27ºC, followed by 4 to 6 weeks, at 12-14ºC). However, a shorter time (14 days) and a constant temperature (25-28ºC), as in current research, have also been employed to verify the detection of ZEA (6).

The two systems of solvents and revelation methodology have identified the mycotoxins produced by the isolates (Table 1). Table 1 shows that 71% of isolates may be toxicogenic; or rather, taken alone, 33% and 67% of fungi produced DON and ZEA respectively.

When the multiple productions of mycotoxins are taken into account, it may be verified that two isolates (4 and 5) simultaneously produced ZEA, ZOL, DON and DAS; two isolates (22 and 46) simultaneously produced ZEA, ZOL and DON; three isolates (12, 44 and 45) simultaneously produced ZEA, ZOL and FX. Whereas one isolate (8) produced ZEA, DON and DAS and another (10) produced ZEA and DON, only ZEA and ZOL were produced by isolates 17, 47 and 60. Likewise, isolates 39 and 40 produced ZEA, and isolate 11 produced FX only. Neosolaniol and nivalenol were not found in any of the 24 isolates and no mycotoxins studied were found in isolates 14, 21, 23, 29, 30, 35 and 69. They may be characterized as non-toxicogenic isolates.

Two items may be enhanced with regard to the distribution of toxicogenic isolates (Table 1): a similar set of characteristics of mycotoxins in isolates 2 to 10 which were collected in a geographically close area; although number of isolates was small, there was a higher incidence of toxicogenic isolates in seeds and ears of wheat when compared to those of triticale and barley. Only two isolates from wheat ears and seeds, out of 19, failed to produce mycotoxins. On the other hand, from four samples of triticale and one of barley seed only isolate 17 produced mycotoxins.

Certain samples with inconclusive TLC (isolates 2, 4, 6, 8, 17, 39, 40, 44 and 60) received confirmation for ZOL and ZEA. When revelators UV and $AlCl_3$ only were used, spots on TLC plates were weak. Confirmation was obtained by Fast Violet B followed by a 30% solution of sulfuric acid. This procedure proved that isolate 11 failed to produce ZEA (Fig. 1) and that isolates 8, 39 and 40 failed to produce ZOL (data not shown).

With regard to other doubtful isolates in ZEA and ZOL production, confirmation was positive, albeit slight, when spot

Table 1. Isolates of *Fusarium graminearum*: Sites of collection and production of mycotoxins.

(Isolates)	Collection site (municipality / state)	Plant part from where isolate was extracted	Host	Mycotoxins						
				ZEA	ZOL	DON	DAS	FX	NS	NIV
2	Campo Mourão, PR	Ear	Wheat	+	+	+	-	-	-	-
4	Mamborê, PR	Ear	Wheat	+	+	+	-	-	-	-
5	Mamborê, PR	Ear	Wheat	+	+	+	+	-	-	-
6	Peabiru, PR	Ear	Wheat	+	+	+	-	-	-	-
8	Francisco Beltrão, PR	Ear	Wheat	+	-	+	+	-	-	-
10	Francisco Beltrão, PR	Ear	Wheat	+	-	+	-	-	-	-
11	Igaraçú, PR	Ear	Wheat	-	-	-	-	+	-	-
12	Igaraçú, PR	Ear	Wheat	+	+	-	-	+	-	-
14	Sertanópolis, PR	Ear	Wheat	-	-	-	-	-	-	-
17	Carambeí, PR	Seed batch 482	Triticale	+	+	-	-	-	-	-
21	Carambeí, PR	Seed batch 385	Wheat	-	-	-	-	-	-	-
22	Tibagi, PR	Seed	Wheat	+	+	+	-	+	-	-
23	Guarapuava, PR	Seed	Barley	-	-	-	-	-	-	-
29	Abelardo Luz, SC	Seed	Triticale	-	-	-	-	-	-	-
30	Abelardo Luz, SC	Seed	Triticale	-	-	-	-	-	-	-
35	Pato Branco, PR	Seed	Triticale	-	-	-	-	-	-	-
39	Seberi, RS	Seed	Wheat	+	-	-	-	-	-	-
40	Palmeira das Missões, RS	Seed	Wheat	+	-	-	-	-	-	-
44	Ijuí, RS	Seed	Wheat	+	+	-	-	+	-	-
45	Ijuí, RS	Seed	Wheat	+	+	-	-	+	-	-
46	Não-Me-Toque, RS	Seed	Wheat	+	+	+	-	+	-	-
47	Não-Me-Toque, RS	Seed	Wheat	+	+	-	-	-	-	-
60	Maringá, PR	Seed	Wheat	+	+	-	-	-	-	-
69	Londrina, PR	Seed	Wheat	-	-	-	-	-	-	-

- not detected.

intensity of samples was compared with those of standards. In the latter case, if cultures have been done with temperature variations and a longer incubation time (6), more ZEA and ZOL could be produced and their detection made easier. Nevertheless, although literature emphasizes the presence of ZEA rather than that of ZOL, attention should be given to the fact that, in the context of 24 isolates, ZEA was produced by 16 and ZOL was produced by 12 out of the 16. Importance should be given to the above since ZOL-produced hyperestrogenic effect is said to be three times higher than that of ZEA (6,15).

Although the aim of current research did not comprise the quantitative determination of mycotoxins, it has been found that isolate 22 (Fig. 2) was predominant since several intense spots appeared on the TLC plate. A high production of mycotoxins in the extract was thus indicated. The *Fusarium* strain from the wheat seed collected in the vicinity of Tibagi, PR needs special attention due to the intensity of its mycotoxin production when compared to that of other samples analyzed.

Owing to a lack of similar data in Brazil as those analyzed in our work and in spite of the small number of samples, current research contributes towards a deeper knowledge on the percentage and distribution of toxicogenically potential isolates in south Brazil.

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RESUMO

Produção de micotoxinas por *Fusarium graminearum* isolados em cereais de inverno (trigo, triticale e cevada) associados com a Giberela na Região Sul do Brasil

Fungos do gênero *Fusarium* são bem conhecidos como patógenos para plantas e como produtores de micotoxinas. O objetivo deste trabalho foi avaliar qualitativamente a produção "in vitro" de desoxinivalenol e de zearalenona, em 24 diferentes

isolados de *Fusarium graminearum* coletados a partir de cereais associados à doença Giberela na Região Sul do Brasil. Os isolados foram cultivados em arroz, durante 14 dias, a 28ºC. Os cultivos foram extraídos com metanol:água (40:60, v/v) e analisados por cromatografia em camada delgada. Outros tricotecenos (diacetoxiscirpenol, fusarenona-X, neosolaniol e nivalenol) e zearalenol, freqüentemente produzidos por *Fusarium*, também foram avaliados. Nas condições utilizadas, foi possível determinar o perfil de produção dessas micotoxinas, sendo que 67% dos isolados produziram zearalenona e 33% dos isolados produziram desoxinivalenol. Também foram detectadas as presenças de zearalenol, diacetoxiscirpenol e fusarenona. Finalmente, em nenhum dos isolados estudados foram encontrados nivalenol e neosolaniol.

Palavras-chave: *Fusarium graminearum*, micotoxinas, Giberela, cereais de inverno, Região Sul do Brasil

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