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Influence of nitrogen sources on growth and mycotoxin production by isolates of *Pyrenophora tritici-repentis* from wheat

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ABSTRACT

The fungus *Pyrenophora tritici-repentis* (Died.) Drechs. infects the leaves and kernels of wheat, causing tan spot and red smudge, respectively. Isolates of *P. tritici-repentis* have been reported to be both phytopathogenic and mycotoxigenic. This research investigates the influence of nitrogen sources on growth and production of mycotoxins by eight different isolates of *P. tritici-repentis*. A synthetic agar medium (SAM) was used with different nitrogen sources, both inorganic [(NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$)] and organic (L-alanine, L-histidine, and L-lysine), at a concentration of 37.5 mmol L^{-1}. Individual isolates exhibited different growth rates that varied according to the nitrogen source added to the medium. The choice of nitrogen source also had a major effect on production of the mycotoxins emodin, catenarin and islandicin. The highest concentrations of emodin, 54.40 ± 4.46 μg g^{-1}, 43.07 ± 23.39 μg g^{-1} and 28.91 ± 4.64 μg g^{-1} of growth medium, were produced on the complex medium (V8-potato dextrose agar) by the isolates Alg-H2, 331-2 and TS93-71B, respectively. A relatively high concentration of emodin also was produced by isolates Az35-5 (28.29 ± 4.71 μg g^{-1} of medium) and TS93-71B (27.03 ± 4.09 μg g^{-1} of medium) on synthetic medium supplemented with L-alanine. The highest concentrations of catenarin (174.54 ± 14.46 μg g^{-1} and 104.87 ± 6.13 μg g^{-1} of medium) were recorded for isolates TS93-71B and Alg-H2 on synthetic medium supplemented with L-alanine and NH_4Cl, respectively. The highest concentration of islandicin (4.64 ± 0.36 μg g^{-1} medium) was observed for isolate 331-2 in the presence of L-lysine. There was not a close relationship between mycelial growth and mycotoxin production by the fungal isolates. This is the first report on the influence of nitrogen sources on the production of mycotoxins by *P. tritici-repentis*.

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1. Introduction

The ascomycete fungus *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.), causal agent of tan spot, is an important foliar pathogen of wheat: *Triticum aestivum* L. and *T. turgidum* subsp. *durum* (Desf.) Husn. This fungus occurs worldwide in all major wheat growing regions [1] and can cause yield losses that range from 3% to 50%, depending on weather conditions and the susceptibility of the host cultivar [2,3]. Moreover, *P. tritici-repentis* also infects the kernels, resulting in blackening of the germ end of affected seeds known as black point, and/or a reddish discoloration termed red smudge [4]. For Canadian Western Amber Durum (CWAD), 0.25% of red smudge or a 10% combination of black point and smudge will lower the grade from #1 to #2, representing an average loss of $12 CAD per ton [5]. Isolates of *P. tritici-repentis* can produce several mycotoxins of the anthraquinone class (polyketides). The most important mycotoxins produced by this fungus are emodin, islandicin and catenarin [6,7]. These mycotoxins have been isolated previously as mold metabolites [8,9]. Emodin is a mutagenic, genotoxic and diarrheagenic mycotoxin [10–12]. A crude extract of emodin administered orally caused severe diarrhea in 1-day-old cockerels and the mean lethal dose of emodin was 3.7 mg kg^{-1} [10]. Clinical symptoms included loss of appetite, accumulation of fecal material with acute epidermal irritation around the cloaca, general debilitation, anorexia, diarrhea, and death within 5 days of ingestion [10,13]. Islandicin is a mutagenic mycotoxin [11] and catenarin also is classified as an undesirable mycotoxin [14,15]. Therefore, *P. tritici-repentis* is not only a phytopathogen, but also a mycotoxigenic fungus [7].

Fungi are important contaminants of food and feed, where they are responsible for spoilage and more importantly the production of dangerous toxins. While the most common mycotoxigenic fungi are *Aspergillus*, *Penicillium* and *Fusarium* [16], several other fungal genera are also able to produce mycotoxins, including *Acremonium*, *Alternaria*, *Bipolaris*, *Byssochlamys* [syn. *Paecilomyces*], *Chaetomium*, *Chrysosporium*, *Cladosporium*, *Claviceps*, *Cochliobolus*, *Cylindrocarpon*, *Gliocladium*, *Helminthosporium*, *Monascus*, *Mortierella*, *Mucor*, *Myrothecium*, *Neotyphodium*, *Phomopsis*, *Pithomyces*, *Stachybotrys*, *Stagonospora*, *Trichoderma*, *Trichothecium* and *Verticimonosporium* [6,16–23]. Little attention has been paid to these fungi, although they could be of concern to public health.

Bouras and Strelkov [7] reported that wheat kernels harvested at maturity contained approximately 0.05 mg catenarin and 0.06 mg emodin per gram of tissue following inoculation with *P. tritici-repentis* at the mid-to-late milk stage. No mycotoxins were detected in non-inoculated tissues. Given the production of several anthraquinone mycotoxins by *P. tritici-repentis*, infection of wheat kernels by this fungus may represent a potential risk for human and animal health [7]. Wheat is an important food commodity worldwide, and is an important ingredient in the production of baked goods. Unfortunately, wheat also is an excellent substrate for growth of several saprophytic, pathogenic and mycotoxigenic fungi.

Fungal contamination is one of the main causes of deterioration of stored grains. Thus, early detection of fungal toxins on food and feedstuffs is crucial for safety purposes and to eliminate mycotoxins from the food chain. The development and application of methods to achieve this detection must be based on knowledge of the genetic features of mycotoxin producers [24], as well as knowledge of their needs for growth and mycotoxin production. Moreover, to further explore the biosynthesis of these mycotoxins, it is necessary to determine the environmental factors that regulate their production. Generally, the biosynthesis of secondary metabolites such as mycotoxins in microfungi is controlled by nitrogen and carbon sources [24]. Several studies found that nitrogen source can have an important influence on mycotoxin production, including kojic acid [25], aflatoxins [26], gibberellin [27], citrinin [28] and ochratoxin A [29].

The objective of this study was to examine the influence of different nitrogen sources on the production of emodin, catenarin and islandicin by *P. tritici-repentis*. Improved knowledge of the factors affecting the production of these mycotoxins is the first step in understanding mycotoxin biosynthesis by this wheat pathogen. This is the first study in which the influence of nitrogen sources on mycotoxin production by *P. tritici-repentis* has been assessed.

2. Material and methods

2.1. Isolates of *P. tritici-repentis*

Eight single-spore isolates of *P. tritici-repentis* were included in this study (Table 1). These isolates were originally collected from infected wheat leaves sampled from four different regions worldwide (Canada, Algeria, Azerbaijan, and the Turkish–Syrian border [30,31]). The isolates (ASC1, 86-124, 331-2, 90-2, Alg 3-24, Alg-H2, Az35-5 and TS93-71B) were selected because they represent all of the known races of *P. tritici-repentis*. All isolates were provided by the late Dr. L. Lamari (University of Manitoba, Winnipeg, Canada). The fungal cultures were maintained on potato dextrose agar (PDA) at 4 °C (Difco Laboratories, Detroit, Michigan, USA) until use.

Table 1 – Isolates of *Pyrenophora tritici-repentis* included in this study.

Isolate	Host	Origin	Reference
ASC1	Winter (bread) wheat *T. aestivum* (Norstar)	Canadian prairies, Manitoba	[32]
86-124	Winter (bread) wheat *T. aestivum* (BH1146)	Canadian prairies, Manitoba	[32]
331-2	Durum wheat *T. durum* (Newton)	Canadian prairies, Manitoba	[32]
90-2	Winter (bread) wheat *T. aestivum*	Canadian prairies, Manitoba	[32]
Alg 3-24	Durum wheat *T. durum*	Guelma (Heliopolis), eastern Algeria	[32]
Alg-H2	Durum wheat *T. durum*	Guelma (Heliopolis), eastern Algeria	[30]
Az35-5	Durum wheat *T. durum*	Azerbaijan	[31]
TS93-71B	Durum wheat *T. durum*	Turkish–Syrian border	[31]

2.2. Inoculum production

Conidial suspensions of each isolate were produced on 15% V8-PDA (V8-potato dextrose agar) [150 mL of V8-juice, 3 g $CaCO_3$, 10 g Difco PDA, 10 g Bacto agar, and 850 mL of sterile deionized water (sd-H_2O)] as per the protocol of Lamari and Bernier [32]. Cultures were incubated in the dark (to prevent conidiophore formation during mycelial growth) at 20 °C for 5 days (or until the colonies reached ca. 4 cm in diameter). The sporulation of *P. tritici-repentis* requires a special treatment [32]. For this purpose, the cultures were flooded with sd-H_2O, the mycelium was flattened with the bottom of a sterile test tube and the excess water decanted. The cultures were subsequently incubated for 20 h under cool white fluorescent light (about 90 μmol m^{-2} s^{-1}) at room temperature (RT; 23 ± 2 °C) to induce the formation of conidiophores, followed by 24 h in the dark at 15 °C to induce the formation of conidiospores. About 30 mL of sd-H_2O was added to each culture and the conidiospores were gently dislodged from each colony with a sterile wire loop, resulting in a conidiospore suspension that was decanted into a 250 mL Erlenmeyer flask. Two or three additional water rinses were made to re-suspend and recover the conidiospores that had settled. After gentle homogenization, the spores were counted with a hemocytometer (Hausser Scientific, Blue Bell, PA, USA) and the final inoculum concentration was adjusted to 1×10^5–3×10^5 spores mL^{-1} with sd-H_2O. The conidial solution was kept on ice to minimize germination before being used to inoculate petri dishes containing various growth media as described below.

2.3. Culture conditions and growth measurement

To investigate the influence of nitrogen sources on growth and mycotoxin production by *P. tritici-repentis*, a synthetic agar medium (SAM) reported by Awad et al. [33] was used. The isolates of *P. tritici-repentis* were grown on SAM with the following composition: 10 mL of mineral solution (containing [per liter of distilled water] 70 mg $Na_2B_4O_7{\cdot}10H_2O$, 50 mg $(NH_4)_6{\cdot}Mo_7O_{24}{\cdot}4H_2O$, 1000 mg $FeSO_4{\cdot}7H_2O$, 30 mg $CuSO_4{\cdot}5H_2O$, 11 mg $MnSO_4{\cdot}H_2O$, and 1760 mg $ZnSO_4{\cdot}7H_2O$) added to 26 g L^{-1} K_2HPO_4, 1 g L^{-1} KCl, 1 g L^{-1} $MgSO_4{\cdot}7H_2O$, 15 g L^{-1} agar and 50 g L^{-1} glucose. The effect of nitrogen source (NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, L-alanine, L-histidine and L-lysine), at a concentration of 37.5 mmol L^{-1}, on colony growth and mycotoxin production was tested in the presence of glucose (30 g L^{-1}) as the only carbon source. The pH of the medium was adjusted to 6.5 with 2 mol L^{-1} HCl, and the medium was autoclaved at 121 °C for 30 min without the glucose; the latter was autoclaved separately and aseptically added before inoculation.

Two complex media, yeast extract sucrose (YES) agar and V8-potato dextrose agar (V8-PDA) also were included in the study. The YES agar contained 20 g of yeast extract, 40 g of sucrose and 15 g of agar per 1 L of d-H_2O [34], and the composition of V8-PDA was as described above. All of the culture media were autoclaved at 121 °C for 30 min. A 10 μL aliquot of spore suspension, prepared as described above, was placed in the center of each petri dish and the cultures were incubated at RT in darkness. The diameters of growing colonies were measured daily in two directions at right angles to each other, over an incubation period of 9 days. Check treatments consisted of petri dishes filled with solid sterilized media without inoculation, incubated under the same conditions.

2.4. Mycotoxin determination

The emodin-, catenarin- and islandicin-producing abilities of the fungal isolates were evaluated after 7 and 15 days of incubation on the various culture media [35], with minor modifications. Briefly, eight agar plugs (9 mm diameter) were excised with a cork borer from points representing the inner, middle and outer areas of each colony. The plugs were weighed (in order to calculate mycotoxin yield per gram of culture medium), and transferred into two microcentrifuge tubes (2 mL). A volume of 750 μL methanol (MeOH) was added to each tube, and the tubes were incubated overnight at RT. The microcentrifuge tubes were centrifuged three times at 13,000 r min^{-1}, and the supernatants (extracts) were collected and filtered through 0.45 μm polyvinylidene difluoride (PVDF) (Millipore Corp., Bedford, Massachusetts, USA) into high performance liquid chromatography (HPLC) vials for analysis. Non-inoculated blanks, consisting of solid sterilized medium incubated under the same conditions, were included as controls in each experiment.

2.5. High-performance liquid chromatography (HPLC)

The HPLC apparatus was comprised an Alliance 2690 Separations Module (Waters, Milford, Massachusetts, USA) and solvent delivery system, connected to a Shimadzu (Kyoto, Japan) SPD-M10A*vp* diode array detector (DAD). The analytical reversed-phase column was a 4.6 mm × 150.0 mm Uptisphere (Varian, Mississauga, ON, Canada), 5 μm C_{18} ODB, fitted with a 4 mm × 10 mm guard column. The samples were loaded in an 80 μL injection volume using an auto-sampler, with the column kept at RT during the analysis. The solvents included 0.2% glacial acetic acid in HPLC grade water (A) and acetonitrile (B). The crude extracts were analyzed with a linear elution gradient over 55 min at a flow rate of 1 mL min^{-1}, beginning from 0% to 100% of solvent B over the first 50 min, followed by an isocratic flow of 100% solvent B for 2 min, and a return to starting conditions over the final 3 min of the run [36]. The UV absorbance of the eluent was monitored concurrently at 220, 250 and 470 nm. The spectrum was measured from 190 to 650 nm with a 2 nm step and a sampling rate of 640 ms. The retention times of emodin, catenarin and islandicin were recorded as 34.84, 37.50 and 40.47 min, respectively, under these conditions. A Class-*VP* 7.2 Shimadzu EZChrom Chromatography Data System was used to record and integrate the chromatographic peaks. The quantification of mycotoxins was based on calibration curves generated with the corresponding external standards at a UV absorbance of 470 nm. The lowest limits of detection were: 20 ng catenarin, 25 ng islandicin and 30 ng emodin (per gram of culture medium), based on a signal-to-noise ratio of 4:1.

2.6. Reagents and standards

All reagents and solvents were of analytical and HPLC grade, respectively. The catenarin and emodin standards were purchased from Apin Chemicals Ltd. (Abingdon, UK) and Fluka Canada (Oakville, Ontario, Canada), respectively. The islandicin standard was obtained from Dr. K.F. Nielsen (Center for Microbial

Biotechnology, Technical University of Denmark, Denmark) and Dr. L.C. Lin (National Research Institute of Chinese Medicine, Taipei, Taiwan).

2.7. Statistical analysis

The data were subjected to analysis of variance with Sigmastat v. 2.03 (SPSS Inc., Chicago, Illinois, USA). The means were compared with Fisher's least significant difference at $P < 0.05$. All tests, with the exception of the growth experiments, were repeated 3 times (with 3 cultures per treatment per isolate). The growth experiments were run four times. The treatments were arranged in a completely randomized design. The means (±standard deviation) are presented in the results.

3. Results

3.1. Influence of nitrogen sources on fungal growth

The growth of the *P. tritici-repentis* isolates was measured on the synthetic medium in the presence of several nitrogen sources (mineral and organic). Individual isolates exhibited variable growth rates depending on the nitrogen source added to the medium. The mean colony diameter ranged from 6.0 ± 0.5 mm to 83.0 ± 7.9 mm after 9 days of incubation on the various media (Fig. 1). Isolate Alg3-24 exhibited the lowest growth when cultured on SAM supplemented with NH_4Cl (diameter of 18.0 ± 3.5 mm), $(NH_4)_2SO_4$ (diameter of 7.0 ± 4.2 mm), L-alanine (7.0 ± 2.0 mm), L-histidine (12.0 ± 0.9 mm), or L-lysine (10.0 ± 0.8 mm), respectively (Fig. 1, C, D, E and F). However, on SAM supplemented with NH_4NO_3 (Fig. 1), the lowest growth was observed for isolate 86-124 (23.0 ± 1.9 mm). The lowest amounts of growth for TS93-71B (7.0 ± 4.9 mm) and ASC1 (6.0 ± 0.5 mm) were recorded on SAM supplemented with $(NH_4)_2SO_4$ (Fig. 1) or L-histidine (Fig. 1E), respectively. On YES medium, the lowest growth was for isolate 331–2 (30.0 ± 2.0 mm) (Fig. 1). On V8-PDA (Fig. 1), however, the growth of isolates Alg-H2 and Az35-5 was the lowest (32.0 ± 2.0 mm and 30.0 ± 2.0 mm, respectively).

In contrast, the growth of isolate 86-124 was very high on SAM in the presence of NH_4Cl as a nitrogen source (83.0 ±7.9 mm) (Fig. 1). The greatest amount of growth for isolate Alg-H2 was in the presence of $(NH_4)_2SO_4$ (77.0 ± 5.4 mm), and in the presence of L-alanine (63.0 ± 4.1 mm) (Fig. 1 and D, respectively). The most

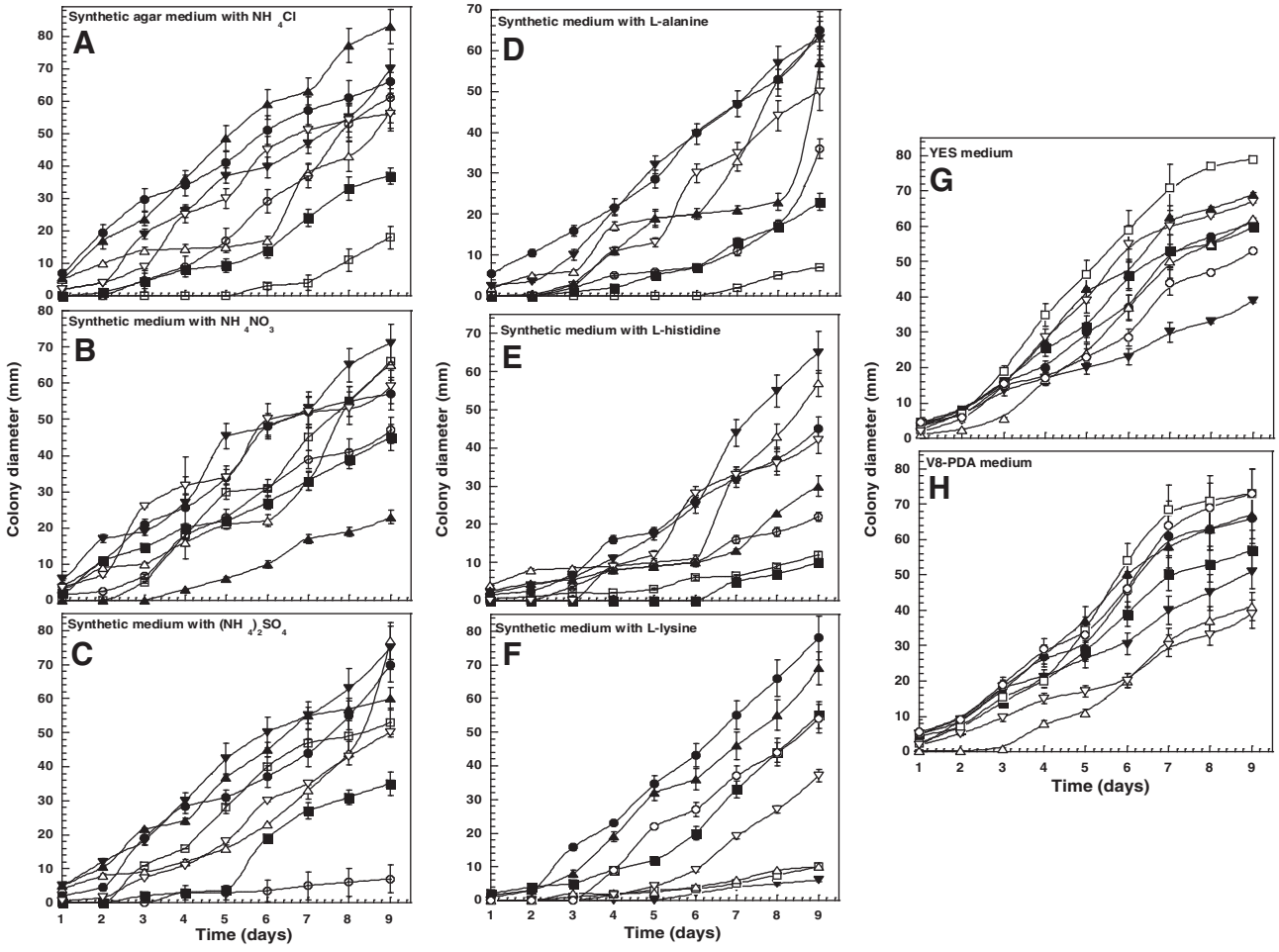

Fig. 1 – Growth of *Pyrenophora tritici-repentis* on different culture media over a 9 day period. (A–F) Modified synthetic agar medium (SAM) containing: NH_4Cl (A), NH_4NO_3 (B), $(NH_4)_2SO_4$ (C), L-alanine (D), L-histidine (E) or L-lysine (F) as nitrogen source; (G) yeast extract sucrose (YES); and (H) V8-potato dextrose agar (PDA) medium. The *P. tritici-repentis* isolates were: ASC1 (■), 86-124 (▲), 331-2 (▼), 90-2 (●), Alg3-24 (□), Alg-H2 (Δ), Az35-5 (▽) and TS93-71B (○).

growth for isolate 90-2 occurred on organic nitrogen sources L-alanine (65.0 ± 4.5 mm) and L-lysine (78.0 ± 6.4 mm), respectively (Fig. 1 and F). In the case of isolate 331-2, the greatest amount of growth was on SAM supplemented with NH_4NO_3 (71.0 ± 5.2 mm), $(NH_4)_2SO_4$ (75.0 ± 3.5 mm), L-alanine (63.0 ± 5.3 mm), L-histidine (65.0 ± 5.5 mm) and L-lysine (69.0 ± 4.9 mm), respectively (Fig. 1, C, D, and F).

3.2. Influence of nitrogen on the production of mycotoxins

The influence of different nitrogen sources on the production of emodin, catenarin and islandicin was investigated. The emodin yields in the *P. tritici-repentis* isolates ranged from 0.06 and 54.40 μg g^{-1} of the medium (Table 2). In SAM supplemented with L-alanine, Az35-5 had the highest emodin production (28.29 ± 4.71 μg g^{-1} medium) measured at day 7. The highest concentrations of emodin (54.40 ± 4.46 μg g^{-1}, 43.07 ± 23.39 μg g^{-1} and 28.91 ± 4.64 μg g^{-1} medium) were detected, respectively, for isolates Alg-H2, 331-2 and TS93-71B on the complex medium (V8-PDA). The lowest concentration of emodin (0.06 ± 0.01 μg g^{-1} medium) was produced by isolate Az35-5 at 15 days with NH_4Cl as the sole nitrogen source. Relatively little emodin was produced by isolate ASC1, only at day 15 in the presence of NH_4Cl (0.41 ± 0.14 μg g^{-1}) and in the presence of L-lysine (0.09 ± 0.02 μg g^{-1} medium). In addition, small amounts of emodin were produced by isolate 86-124 in the presence of L-alanine and NH_4Cl. No emodin was detected from isolate 90-2 under any of the conditions assayed. The amount of toxin produced by specific isolates often declined from day 7 to day 15.

The catenarin concentrations produced by the *P. tritici-repentis* isolates varied from 0.09 to 174.54 μg g^{-1} of the medium (Table 3). The highest concentration of catenarin (174.54 ± 14.46 μg g^{-1} medium) was at day 15 for isolate TS93-71B when L-alanine was the sole nitrogen source. The lowest concentration of catenarin (0.09 ± 0.03 μg g^{-1} medium) was for isolate 86-124 at day 15 in the presence of NH_4Cl. Isolate ASC1 produced very small amounts of catenarin only in the presence of NH_4Cl at day 15 (0.63 ± 0.26 μg g^{-1} medium), and in the presence of L-lysine at day 15 (0.18 ± 0.05 μg g^{-1} medium). Isolate 86-124 produced very low concentrations of catenarin only in the presence of NH_4Cl and L-alanine. No catenarin was detected from isolate 90-2 under any of the conditions tested. In general, some nitrogen sources appeared to favor emodin production and accumulation while others favored catenarin production. The results were closely related to the isolate and the nitrogen source tested. Generally, L-alanine and L-lysine were the most favorable nitrogen sources for emodin production by isolates of *P. tritici-repentis*. Among the different nitrogen sources tested, L-lysine did not provide the best catenarin yields, although significant yields were obtained for some isolates. There was little or no production of emodin and catenarin by the isolates ASC1, 86-124 or 90-2.

Only three isolates (331-2, Alg-H2 and TS93-71B) produced islandicin in detectable amounts (Table 4). Isolate Alg-H2 produced islandicin in the presence of all nitrogen sources, while isolate TS93-71B produced islandicin only in the presence of the two amino acids (L-alanine and L-histidine). Isolate 331-2 produced islandicin in the presence of all nitrogen sources, except NH_4Cl. The islandicin concentrations detected among the producing isolates varied from 0.04 to 4.64 μg g^{-1} medium. The highest concentration (4.64 ± 0.36 μg g^{-1} medium) was for isolate 331-2 at day 15 in the presence of L-lysine, while the lowest concentration (0.04 ± 0.03 μg g^{-1} medium) was from isolate 331-2 at day 7 in the presence of NH_4NO_3. No islandicin was detected in the culture media YES or V8-PDA for any isolate.

Table 2 – Emodin production by isolates of *Pyrenophora tritici-repentis* on yeast extract sucrose (YES), V8-potato dextrose agar (V8-PDA), and synthetic agar medium supplemented with different nitrogen sources. The values are means of three replicates ± standard deviations.

Isolate	Cultivation period (days)	Emodin production (μg g^{-1} medium)								LSD [a]
		Synthetic medium with different nitrogen sources						YES	V8-PDA	
		NH_4Cl	NH_4NO_3	$(NH_4)_2SO_4$	L-alanine	L-histidine	L-lysine			
ASC1	7	ND	ND	ND	ND	ND	ND	ND	ND	–
	15	0.41 ± 0.14	ND	ND	ND	ND	0.09 ± 0.02	ND	ND	0.086
86-124	7	ND	ND	ND	1.59 ± 0.30	ND	ND	ND	ND	0.184
	15	0.13 ± 0.06	ND	ND	0.43 ± 0.12	ND	ND	ND	ND	0.082
331-2	7	0.49 ± 0.18	2.53 ± 0.24	0.37 ± 0.18	16.11 ± 5.89	3.88 ± 0.23	2.06 ± 0.71	ND	43.07 ± 23.39	8.158
	15	0.15 ± 0.03	0.07 ± 0.02	ND	0.40 ± 0.03	1.55 ± 0.38	2.82 ± 0.18	0.65 ± 0.37	13.29 ± 4.65	2.286
90-2	7	ND	ND	ND	ND	ND	ND	ND	ND	–
	15	ND	ND	ND	ND	ND	ND	ND	ND	–
Alg 3-24	7	1.08 ± 0.49	0.47 ± 0.30	0.54 ± 0.34	ND	ND	ND	ND	ND	0.409
	15	8.45 ± 0.55	0.17 ± 0.08	6.42 ± 1.58	ND	0.06 ± 0.01	0.19 ± 0.04	ND	3.62 ± 0.93	1.092
Alg-H2	7	2.43 ± 0.57	1.52 ± 0.48	3.99 ± 0.13	6.71 ± 0.29	3.26 ± 0.74	3.62 ± 0.38	ND	25.93 ± 9.16	3.183
	15	8.95 ± 2.05	6.27 ± 1.23	0.83 ± 0.16	3.55 ± 0.45	3.99 ± 0.13	15.79 ± 1.21	ND	54.40 ± 4.46	1.707
Az35-5	7	0.31 ± 0.24	0.36 ± 0.09	0.37 ± 0.18	28.29 ± 4.71	ND	0.33 ± 0.11	ND	ND	2.890
	15	0.06 ± 0.01	ND	0.10 ± 0.02	25.93 ± 3.07	ND	ND	ND	7.25 ± 4.15	1.951
TS93-71B	7	ND	ND	ND	1.03 ± 0.41	1.34 ± 0.44	ND	2.72 ± 1.68	28.91 ± 4.64	2.536
	15	0.18 ± 0.05	0.17 ± 0.06	0.04 ± 0.01	27.03 ± 4.09	3.25 ± 0.75	0.71 ± 0.06	ND	7.98 ± 3.39	2.621

ND: Not detected.
[a] LSD: Fisher's least significant difference at $P < 0.05$.

Table 3 – Catenarin production by isolates of *Pyrenophora tritici-repentis* on yeast extract sucrose (YES), V8-potato dextrose agar (V8-PDA), and synthetic agar medium supplemented with different nitrogen sources. The values are means of three replicates ± standard deviations.

Isolate	Cultivation period (days)	Catenarin production (μg g^{-1} medium)								LSD [a]
		Synthetic medium with different nitrogen sources						YES	V8-PDA	
		NH_4Cl	NH_4NO_3	$(NH_4)_2SO_4$	L-alanine	L-histidine	L-lysine			
ASC1	7	ND	ND	ND	ND	ND	ND	ND	ND	–
	15	0.63 ± 0.26	ND	ND	ND	ND	0.18 ± 0.05	ND	ND	0.162
86–124	7	ND	ND	ND	6.42 ± 0.69	ND	ND	ND	ND	0.422
	15	0.09 ± 0.03	ND	ND	3.01 ± 0.39	ND	ND	ND	ND	0.239
331–2	7	0.72 ± 0.06	58.43 ± 5.57	3.81 ± 0.19	66.37 ± 4.63	7.76 ± 1.37	4.53 ± 0.61	ND	41.09 ± 8.47	8.067
	15	0.23 ± 0.07	0.63 ± 0.14	0.47 ± 0.09	1.48 ± 0.12	1.47 ± 0.09	6.42 ± 0.58	1.20 ± 0.70	28.98 ± 9.67	2.517
90–2	7	ND	ND	ND	ND	ND	ND	ND	ND	–
	15	ND	ND	ND	ND	ND	ND	ND	ND	–
Alg 3–24	7	13.89 ± 3.11	4.71 ± 0.29	4.09 ± 0.21	ND	ND	4.53 ± 0.47	ND	ND	1.937
	15	77.26 ± 6.74	11.71 ± 2.21	82.33 ± 16.72	ND	0.14 ± 0.03	3.15 ± 0.15	ND	0.63 ± 0.49	11.118
Alg-H2	7	65.63 ± 7.37	19.98 ± 3.02	35.43 ± 5.57	15.96 ± 3.04	65.65 ± 5.35	15.81 ± 3.19	ND	19.05 ± 3.64	7.695
	15	104.87 ± 6.13	30.21 ± 8.79	7.72 ± 2.28	7.79 ± 2.79	4.12 ± 0.88	15.01 ± 1.99	ND	ND	7.046
Az35–5	7	0.33 ± 0.11	0.32 ± 0.07	0.38 ± 0.06	18.13 ± 3.87	ND	0.36 ± 0.07	ND	ND	2.370
	15	0.12 ± 0.04	ND	0.13 ± 0.06	25.17 ± 3.83	ND	ND	ND	ND	2.344
TS93-71B	7	ND	ND	ND	45.71 ± 6.29	2.53 ± 0.46	1.83 ± 0.17	ND	25.95 ± 5.30	5.790
	15	4.17 ± 0.83	0.25 ± 0.08	0.19 ± 0.07	174.54 ± 14.46	18.64 ± 3.64	2.71 ± 0.29	3.62 ± 0.94	19.36 ± 3.09	9.489

ND: Not detected.
[a] LSD: Fisher's least significant difference at $P < 0.05$.

4. Discussion

In this study, the effect of nitrogen source (organic and inorganic) on the growth of, and mycotoxin production by, a collection of *P. tritici-repentis* isolates representing all the known races of the fungus was investigated for the first time.

Propagules of microorganisms get on a grain in various ways, including with dust from the soil, from the surface of plant debris at harvest, and during storage, transportation, and processing [37]. Fungal growth on agricultural commodities is a major problem in throughout the world and may negatively impact grain quality, decreasing germination and significantly affecting nutritional value [38]. Colonization of grain tissues by fungi may also have adverse effects on human and animal health, as a consequence of the production of mycotoxins by these fungi [39]. Little attention has been paid to uncommon mycotoxigenic fungi such as *Pyrenophora*, especially given that many of these fungi are also phytopathogenic and/or endophytic. The importance of studying *P. tritici-repentis* stems from its ability to produce anthraquinone polyketides such as emodin, catenarin and islandicin, which belong to the second major class of carcinogenic mycotoxins [40], and pose a threat to wheat grain quality. More than 10 anthraquinones were detected from cultures of isolate 331-2 (data not shown), but only seven have been characterized from cultures of *P. tritici-repentis* [6].

Catenarin is produced by various species in the genera *Helminthosporium* and *Pyrenophora* [6–8], *Aspergillus* [41] and *Bipolaris* [18]. Islandicin was isolated only from the two fungal species *Penicillium islandicum* Sopp and *P. tritici-repentis* [6,42]. Emodin is a widespread mycotoxin produced by several *Penicillium* and *Aspergillus* species, mainly *P. islandicum* [8,42,43], *P. brunneum* Udagawa [44], *A. ochraceus* var. *alutaceus* Wilh. (formerly *Aspergillus ochraceus*) [45], *A. wentii* Wehmer [10,46], *A.*

Table 4 – Islandicin production by isolates of *Pyrenophora tritici-repentis* on yeast extract sucrose (YES), V8-potato dextrose agar (V8-PDA), and synthetic agar medium supplemented with different nitrogen sources. The values are means of three replicates ± standard deviations.

Isolate	Cultivation period (days)	Islandicin production (μg g^{-1} medium)								LSD [a]
		Synthetic medium with different nitrogen sources						YES	V8-PDA	
		NH_4Cl	NH_4NO_3	$(NH_4)_2SO_4$	L-alanine	L-histidine	L-lysine			
331–2	7	ND	0.04 ± 0.03	0.11 ± 0.06	3.55 ± 0.45	3.15 ± 0.35	1.41 ± 0.59	ND	ND	0.512
	15	ND	ND	ND	ND	ND	4.64 ± 0.36	ND	ND	0.220
Alg-H2	7	0.17 ± 0.06	0.05 ± 0.04	0.33 ± 0.07	0.31 ± 0.09	0.27 ± 0.05	0.18 ± 0.13	ND	ND	0.122
	15	0.12 ± 0.05	0.09 ± 0.04	0.13 ± 0.05	0.21 ± 0.08	0.21 ± 0.06	0.76 ± 0.14	ND	ND	0.116
TS93-71B	7	ND	ND	ND	0.16 ± 0.07	0.09 ± 0.02	ND	ND	ND	0.044
	15	ND	ND	ND	2.94 ± 0.16	0.05 ± 0.02	ND	ND	ND	0.098

ND: Not detected.
[a] LSD: Fisher's least significant difference at $P < 0.05$.

petrakii Vörös-Felkai, *A. terricola* Marchal [47], *A. japonicus* var. *aculeatus* Iizuka and *Pyrenochaeta terrestris* Larson [46].

The production of secondary metabolites may be influenced by changing physical, chemical and biological factors, and by interactions involving these factors, chief among which are carbon and nitrogen sources, temperature and incubation times [48]. The nature and concentration of the carbon and nitrogen sources are primary factors that interact to affect mycotoxin biosynthesis through several phenomena such as catabolic repression [49,50]. Many researchers have examined the effect of culture conditions on the production of several mycotoxins by producing species [51,52]. These studies, which made use of complex media, focused on the identification of culture media that favor mycotoxin production, but it is not possible to evaluate the influence of specific nutrients in such media. A few studies used defined media prepared with chemically pure nutrients to study the production of mycotoxins [24,53]. While these media are very useful in metabolic and biosynthetic studies, the amount of secondary metabolites produced by fungal species is always lower than that produced on complex media, where nutrients and growth factors are plentiful. In the present study, defined media were used to study the influence of nitrogen sources on anthraquinone mycotoxin production by *P. tritici-repentis*.

All isolates tested in the study were able to grow on synthetic media supplemented with different nitrogen sources (including organic and mineral sources). This indicates that isolates of *P. tritici-repentis* are adapted to different nitrogen sources despite their different response to environmental factors. Each isolate exhibited unique and distinct growth characteristics, suggesting that the influence of the different nitrogen sources was not the same on the growth of different isolates. These differences may result from intraspecific variability among the isolates, which were obtained from different regions around the world and represented distinct races [30,31,54].

In the case of isolates Az35-5 and TS93-71B, the presence of amino acid L-alanine in synthetic medium resulted in increased production of emodin relative to its production in the presence of mineral nitrogen sources (NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$). This amino acid could be supplying precursors for the biosynthesis of emodin. These results are consistent with those of Fraleigh et al. [55], who reported that organic nitrogen sources were more favorable for the production of some secondary metabolites, such as kojic acid, compared to inorganic nitrogen sources.

In contrast, the addition of the aromatic amino acid L-histidine had a negative effect on the production of emodin by all tested isolates. Similarly, Medina et al. [56] reported that ochratoxin A concentrations were somewhat lower when L-histidine was added to a defined medium. In the presence of ammonium nitrogen sources (NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$), the production of emodin by all isolates was considerably decreased (Table 2). Catabolite repression by ammonium can negatively regulate the biosynthesis of secondary metabolites by means of enzyme inhibition, thereby enabling or inhibiting the production of important precursors [57]. This effect has been reported for the biosynthesis of α-aminoadipic acid, which is a precursor of cephalosporin in *Cephalosporium acremonium* Corda [58]. The same phenomenon occurred in the production of the antibiotic spiramycin by *Streptomyces ambofaciens* Pinnert-Sindico [59]. In this microorganism, valine deshydrogenase, the first enzyme in the degradation of valine, was sharply repressed by ammonium ions, reducing the availability of the precursor isobutyrate [60–62]. Catabolite repression also can affect the activity of enzymes required in secondary metabolic pathways. This has been found in the repression of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) synthetase and isopenicillin synthetase, enzymes involved in the biosynthesis of penicillin, by ammonium ions in *C. acremonium* and *S. clavuligerus* Higgens & Kastner [63].

In the current study, the production of catenarin by isolate Alg-H2 was highly stimulated in the presence of NH_4Cl and L-histidine (Table 3). Among mineral sources of nitrogen, ammonium is known to be required in the catabolite regulation of secondary metabolites [57,64,65]. Ammonium ions allow weak production of secondary metabolites, but when nitrate or sulfate is associated with ammonium, production is enhanced, as was the case for some isolates of *P. tritici-repentis* such as Alg 3-24 and Alg-H2. In addition, the production of catenarin was supported by the addition of L-alanine or to a lesser extent L-lysine. In contrast, the addition of L-histidine did not stimulate the production of this anthraquinone in any isolate except Alg-H2. Amino acids can stimulate, inhibit, or have no effect on the production of secondary metabolites, according to their capacity to release ammonium ions into the culture medium or to permit the accumulation of direct and/or indirect precursors during assimilation by microorganisms [66,67].

A high level of intraspecies variability was observed when secondary metabolite profiles produced on different culture media were compared [36,68,69]. These observations support the suggestion that medium composition and brands of ingredients should be consistent when applying metabolite profiles to identify fungal species [70,71]. The results obtained also suggest that secondary metabolism is induced differentially under different sources of nutrition.

The production of mycotoxins by *P. tritici-repentis* was strongly dependent on the specific isolate, type of nitrogen source and length of incubation period. Isolates of this fungus cannot utilize inorganic and (or) organic nitrogen sources indiscriminately to biosynthesize emodin and/or catenarin. The isolates showed a large variation in ability to produce emodin and/or catenarin, indicating intraspecific variation between isolates. This was described earlier for ochratoxin A production by other species, including *A. ochraceus*, *A. carbonarius* (Bainier) Thom. and *A. niger* Tiegh. [72,73]. It is still unknown if such variation results mainly from genetic features of the isolates and/or from the manner in which they use the culture media. The results of the present study indicate that nitrogen sources have a considerable impact on isolate growth rate and mycotoxin production, with each isolate responding differently to each nitrogen source.

Generally, the production of mycotoxins by *P. tritici-repentis* was lower on synthetic media. Others found that V8-PDA and other semi-synthetic media were the most favorable for emodin and catenarin production by isolates of *P. tritici-repentis* [7,36]. Usually, the yield of secondary metabolites produced by a microorganism growing in complex media is higher because it has abundant nutrients and growth factors.

Despite the fact that isolates of *P. tritici-repentis* grew well on YES medium (Fig. 1) no islandicin (and very little emodin and catenarin) was detected in that substrate (Table 4),

suggesting that it is not favorable for mycotoxin formation by this fungus. This finding is interesting, since the YES medium is regularly used as a standard medium to obtain profiles of the mycotoxin-producing capacity of fungal species and strains [36,74,75]. In the case of *P. tritici-repentis*, the excess of sugar in the YES medium may have inhibited islandicin biosynthesis by catabolite repression. In addition, anthraquinones can be metabolized and biotransformed to other anthraquinones [41,44,46], or incorporated in the melanin structure [76]. Emodin is frequently converted to more highly substituted and complex metabolites [77], and islandicin to other compounds such as benzophenone [78]. Such changes may also help to explain the observation that the concentration of mycotoxins produced by some of the isolates was lower after a cultivation period of 15 versus 7 days. Given that catenarin and emodin are stable mycotoxins and are unlikely to be degraded by physical factors, it is also possible that these decreases resulted from an active degradation by the fungus as nutritive sources became exhausted in the culture medium [36]. A similar phenomenon was reported for ochratoxin A produced by *A. niger* [79,80].

Genetic and physiological studies to explain differences in the mycotoxin-producing capacity of isolates are very important. These help to explain the distribution and the specificity of these fungi. The yield of emodin produced on complex media by *P. terrestris* and *A. ochraceus* var. *alutaceus* ranged from 0.9 to 2.5 $\mu g\ mL^{-1}$ and from 1.3 to 6.0 $\mu g\ mL^{-1}$, respectively [46]. However, in this study, *P. tritici-repentis* isolate Az35–5 produced more than 28 μg of emodin g^{-1} of medium in the presence of L-alanine as the sole nitrogen source, and more than 54 μg emodin g^{-1} of medium on the complex medium (V8-PDA). Furthermore, more than 104 μg of catenarin g^{-1} medium and 4 μg of islandicin g^{-1} medium were obtained, respectively, from isolate Alg-H2 in the presence of NH_4Cl and isolate 331-2 in the presence of L-lysine. The ability of *P. tritici-repentis* to produce emodin is much greater than that of other known fungal species, which is a cause for concern given its effects as a mycotoxin [13]. The optimal nitrogen source for growth and mycotoxin production by isolates of *P. tritici-repentis* reported in this study can, in part, help to explain trends in wheat kernel contamination.

Infection and contamination of wheat kernels by *P. tritici-repentis* occurs in the field rather than in storage [81]. Nonetheless, fungal growth and the production of mycotoxins during the post-harvest period cannot be entirely discounted, especially if grains are exposed to or stored under conditions of high moisture [81]. Consequently, *P. tritici-repentis* could pose a risk for grain and grain products (especially bread and flour-based foods), and a better understanding of the ecological requirements of this pathogen is needed. This study may promote further research into the physiology and genetics of anthraquinone production by fungal species, as well as in determining cultural conditions leading to biosynthesis of these polyketide mycotoxins.

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