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Short communication

Influence of water activity and temperature on growth and mycotoxin production by isolates of *Pyrenophora tritici-repentis* from wheat

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ABSTRACT

Pyrenophora tritici-repentis is a phytopathogenic fungus that can infect wheat kernels and leaves, causing red smudge and tan spot, respectively. A number of *P. tritici-repentis* isolates have been shown to be mycotoxigenic, producing the anthraquinone mycotoxins emodin, catenarin and islandicin. The influence of water activity (a_w; 0.75–0.99 a_w) and temperature (5–45 °C) on growth and mycotoxin production by five isolates of *P. tritici-repentis* was studied. All isolates grew at 0.95–0.99 a_w and 15–25 °C on a wheat-based medium, with three isolates also producing small colonies at 5 °C. The optimal growth conditions for all isolates consisted of 0.99 a_w and 25 °C, and growth was significantly reduced at 0.95 a_w and/or 15 °C. Emodin and catenarin were detected in cultures of all isolates, at concentrations ranging from 0.06 ± 0.04 to 11.31 ± 2.96 μg emodin/g medium, and from 0.09 ± 0.06 to 53.42 ± 4.36 μg catenarin/g medium. In most isolates, the concentrations of emodin and catenarin declined under suboptimal growth conditions. However, in some isolates, significant increases in the concentrations of both compounds were observed under suboptimal conditions. Islandicin was detected in cultures of only three isolates, at concentrations ranging from 0.07 ± 0.05 to 5.69 ± 0.76 μg/g medium. The results suggest that growth and mycotoxin formation by *P. tritici-repentis* are markedly influenced by a_w and temperature, and that this fungus is hygrophilic. Therefore, infection and contamination of kernels by *P. tritici-repentis* are likely to occur in the field rather than in storage. To our knowledge, this is the first study on the effect of environmental factors on mycelial growth and mycotoxin production by *P. tritici-repentis*.

1. Introduction

The fungus *Pyrenophora tritici-repentis* (anamorph: *Drechslera tritici-repentis*), causal agent of tan spot, is an important foliar pathogen of wheat. This fungus is endemic to major wheat growing areas worldwide (Strelkov and Lamari, 2003), causing yield losses that range from 3 to 50% in the Central Plains of the U.S.A. and Canada (Shabber and Bockus, 1988). In addition to foliar symptoms, *P. tritici-repentis* can also infect wheat kernels, causing a red discoloration known as red smudge, and/or a blackening of the germ end of affected seeds called black point (Menzies and Gilbert, 2003). In Canadian Western Amber Durum wheat, 0.25% red smudge or a 10% combination of black point and smudge will lower the grade from # 1 to # 2 (Fernandez et al., 1998a). While *P. tritici-repentis* is well-known as a producer of host-specific phytotoxins (Strelkov and Lamari, 2003), the fungus has more recently been shown to also be mycotoxigenic. Production of the anthraquinone mycotoxins emodin, islandicin and catenarin has been demonstrated *in vitro* and *in planta* (Bouras and Strelkov, 2008; Wakuliński et al., 2003). Of particular concern is the detection of emodin in wheat kernels infected by *P. tritici-repentis* (Bouras and Strelkov, 2008), as this compound is diarrheagenic, mutagenic, genotoxic, and cytotoxic (Liberman et al., 1980; Morita et al., 1988; Müller et al., 1996; Wells et al., 1975). Although catenarin has also been detected in infected kernels (Bouras and Strelkov, 2008) and is classified as a mycotoxin (Vytřasová et al., 2002), toxicological data for this metabolite are lacking. Nevertheless, the red discoloration associated with red smudge may result from catenarin accumulation in wheat kernels (Bouras and Strelkov, 2008; Wakuliński et al., 2003), and it could therefore contribute to declines in grain quality. Islandicin has been reported to be mutagenic (Liberman et al., 1980). Therefore, *P. tritici-repentis* should be regarded as a contributor to the accumulation of mycotoxins in wheat kernels and their derivatives.

Fungal growth and mycotoxin production are influenced by numerous abiotic and biotic parameters and their complex interactions. Water availability is probably the single most important factor affecting germination, growth and establishment of fungi on nutrient-rich substrates (Magan and Lacey, 1988). The second most important is temperature. Little is known, however, regarding the influence of water activity (a_w) and temperature on growth and mycotoxin production by *P. tritici-repentis*. Information on the effects

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of these parameters is needed to determine whether this fungus can attack wheat kernels only in the field or also in transit or during storage and processing. Preventing germination and growth of *P. tritici-repentis* will necessarily prevent the production of mycotoxins by this species. The main objective of this study was to examine the influence of a_w and temperature on growth and the production of emodin, catenarin and islandicin by isolates of *P. tritici-repentis* collected from wheat. To our knowledge, this is the first examination of the effect of environmental factors on the production of mycotoxins by this fungus.

2. Materials and methods

2.1. Isolates of P. tritici-repentis and inoculum production

A total of five single-spore isolates of *P. tritici-repentis* were used in this study. Isolate 331-2 was obtained from infected wheat leaves collected on the Canadian prairies (Lamari and Bernier, 1989), isolates Alg3-24 and AlgH2 originated from Algeria (Lamari et al., 1995; Strelkov et al., 2002), isolate Az35-5 came from Azerbaijan, and TS93-71B from near the Turkish–Syrian border (Lamari et al., 2003). All fungal cultures were maintained at 4 °C on potato dextrose agar (PDA) medium (Difco Laboratories, Detroit, MI) until use. The formation of conidia was induced on 15% V8-PDA [150 mL V8-juice, 3 g $CaCO_3$, 10 g Bacto agar, 10 g Difco PDA, and 850 mL sterile deionized water (sd-H_2O)] as described by Lamari and Bernier (1989). The spores were harvested by flooding the cultures with sd-H_2O and gently scraping the colony surface with a sterile wire loop. Conidial concentrations in the resulting suspensions were quantified using a hemocytometer (Hausser Scientific, Blue Bell, Pa) and adjusted to $1–3\times10^5$ spores/mL with sd-H_2O.

2.2. Culture conditions

The basic growth medium consisted of 3% (w/v) wheat meal extract agar (WMEA) prepared by homogenizing 30 g of dry ground wheat kernels (cv. Katepwa) in 500 mL d-H_2O for 1 h. The resulting mixture was filtered through a double layer of muslin. Fifteen grams of agar was added to the filtrate and the volume was adjusted to 1 L. The water activity (a_w) of this medium was 0.99. To determine the influence of a_w on mycelial growth and mycotoxin production, appropriate amounts of the non-ionic solute glycerol were added to the basal media (0.99 a_w) to obtain water activities of 0.95, 0.90, 0.85, 0.80 and 0.75 (Pardo et al., 2004). These amounts were based on calibration curves obtained by plotting different concentrations of glycerol vs. resulting a_w. Final a_w values were verified with a water activity meter (AquaLab, Series 3, Model CX2, Decagon Devices Inc., Pullman, Washington, USA). The accuracy of a_w values was ±0.003. The medium was autoclaved at 121 °C for 30 min and poured into 90 mm diameter Petri dishes (≈20 mL/dish).

The WMEA-filled Petri dishes were point-inoculated with 10 µL aliquots of spore suspension produced as described above. The a_w of each spore suspension was adjusted with sterilized glycerol so that it was equal to that of the medium being inoculated (i.e., 0.95, 0.90, 0.85, 0.80 and 0.75). To minimize moisture loss during incubation, Petri dishes with the same a_w were sealed with parafilm and enclosed in polyethylene bags along with beakers containing glycerol–water solutions of the same a_w. Cultures were incubated in darkness at 5, 15, 25, 35 and 45 °C for 15 days.

2.3. Growth measurement and mycotoxin determination

The growth of fungal colonies was monitored daily by measuring the colony diameter in two directions at right angles to each other. These diameter measurements were then averaged over the number of colonies measured for each treatment. Emodin, catenarin and islandicin production was evaluated after 7 and 15 days of growth on WMEA, according to the method of Bragulat et al. (2001), with some minor modifications. Eight agar plugs (9 mm diameter) were excised with a cork borer from different points (representing the inner, middle and outer areas) of each colony, weighed and placed in two microcentrifuge tubes (2 mL). The plugs in each tube were vortexed in 750 µL of methanol (MeOH) and allowed to stand at room temperature (RT) overnight. The tubes were centrifuged at 13,000 rpm a total of three times, and the extracts (supernatants) collected and passed through 0.45-µm polyvinylidene difluoride (PVDF) filters (Millipore Corp., Bedford, MA) into high-performance liquid chromatography (HPLC) vials for analysis. Blanks consisting of sterilized, non-inoculated WMEA medium, incubated under the same conditions, were used as controls in each experiment.

2.4. HPLC

Mycotoxins were detected and quantified by HPLC. The HPLC apparatus consisted of an Alliance 2690 Separations Module (Waters, Milford, MA) coupled to a Shimadzu (Kyoto, Japan) SPD-M10A*vp* diode array detector (DAD), with a solvent delivery system. The analytical reversed-phase column used was a 4.6×150 mm Uptisphere (Varian, Mississauga, Canada), 5 µm C_{18} ODB fitted with a guard column (4×10 mm). Samples were applied with an autosampler in an 80 µL injection volume. During analysis, the column was maintained at RT. The UV absorbance of the eluant was monitored simultaneously 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 solvents used were 0.2% glacial acetic acid in HPLC grade water (A) and acetonitrile (B). The crude extract was analyzed using a linear elution gradient over 55 min at a flow rate of 1 mL/min, starting from 0 to 100% solvent B over the first 50 min, followed by an isocratic flow of 100% solvent B for 2 min, and a return to initial conditions over the last 3 min of the run. Under these conditions, the retention times of emodin, catenarin and islandicin were 34.84, 37.50 and 40.47 min, respectively. Chromatographic peaks were recorded and integrated using a Class-VP 7.2 Shimadzu EZChrom Chromatography Data System. Mycotoxins were quantified based on calibration curves prepared using corresponding external standards at 470 nm. The detection limits (based on a signal-to-noise ratio of 4:1) were 20 ng catenarin, 25 ng islandicin and 30 ng emodin/g of medium.

2.5. Reagents and standards

All reagents were of analytical grade, and all solvents were of HPLC grade. The catenarin standard was purchased from Apin Chemicals Ltd. (Abingdon, UK), and the emodin standard from Fluka (Oakville, Ontario, Canada). Islandicin was generously provided by Dr. L.-C. Lin (National Research Institute of Chinese Medicine, Taipei, Taiwan) and Dr. K.F. Nielsen (Center for Microbial Biotechnology, Technical University of Denmark, Lyngby, Denmark).

2.6. Statistical analysis

All tests were repeated three times with the exception of the growth experiments, which were repeated four times; the means ± standard deviations are given in the results. Data were subjected to analysis of variance using Sigmastat® 2.03 Statistical Software (SPSS Inc., Chicago, IL) and means were compared using Fisher's least significant difference at $P<0.05$.

3. Results

3.1. Influence of water activity and temperature on fungal growth

Growth of *P. tritici-repentis* was detected only at 0.95 and 0.99 a_w and from 5 to 25 °C, with maximum growth for all isolates observed at