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Ochratoxin A production by *Eurotium amstelodami* and *Eurotium* spp. isolated from locally grown barley in Saudi Arabia

MOHAMMED Z. AL-JULAIFI

National Agriculture and Water Research Center, Ministry of Agriculture
P.O.Box 31623, Riyady 11418, Saudi Arabia

ABSTRACT

In a survey of the occurrence of ochratoxin A isolated from locally grown barley in Saudi Arabia, three species of the genera *Eurotium* were obtained. The isolates were screened for their ability to produce ochratoxin A in three different media including Potato Dextrose Agar (PDA), Yeast Extract Sucrose (YES) broth as artificial media and autoclaved barley kernels (*Hordeum vulgare* Linn) as a natural medium. Ochratoxin A production by the isolated species was verified by means of thin-layer chromatography and high-performence liquid chromatography (HPLC). Results showed that all the three isolates were found to produce ochratoxin A in different concentrations. quantities of ochratoxin A detected in YES produced by *E. amstelodami, E. herbariorum* and *Eurotium* spp were 138.21, 302.17 and 245.25 μg 1^{-1}, respectively. In PDA, only *E. amstelodami* and *Eurotium* spp were able to produce ochratoxin A at concentrations of 97.14 μg 1^{-1} and 205.13 μg 1^{-1}, respectively. Ochratoxin A produced on barley medium was 165.14, 121.39 and 214.77 μg kg^{-1} for *Eurotium* spp, *E. amstelodami* and *E. herbariorum*, respectively. This is the first report of ochratoxin A production by *E. amstelodami* and *Eurotium* spp.

Keywords: Barley; *Eurotium amstelodami*; *E. herbariorum*; *Eurotium* spp; Ochratoxin A.

INTRODUCTION

Molds including several fungal species in the genera *Aspergillus*, *Eurotium* and *Penicillium* are widely distributed in nature and commonly contaminate crops and foods. They have the ability to cause significant health problems in animals and humans that consume contaminated foods. Numerous strains of *Aspergillus* and *Penicillium* were found to be toxigenic and have been reported to produce ochratoxins associated with a variety of health problems in animals and humans. Ochratoxins are composed of a group of seven isocoumarin derivatives linked with an amide bond to the amino group of L-β-phenylalanine (Blunden *et al.* 1991). Ochratoxins contaminate varieties of plant, food commodities and animal products, but are most often found on grains, cereals, coffee and bread

(Speijers & Van Egmond 1993). Mycological, chemical and toxicological studies pointed out that ochratoxin A (OTA) {*(R)*-N-[5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl)carbonyl]-L-phenylalanine} is the most prevalent and has clearly been shown to be a toxic substance, with rephrotoxic, immunosuppressive, teratogenic, genotoxic, mutagenic and carcinogenic effects in a wide spectrum of species (Boorman *et al.* 1984, Kittane *et al.* 1984, Kuiper-Goodman & Scott 1989, Gekle & Silbernagl 1993, IARC 1993, Kuiper-Goodman *et al.* 1993, Pohland 1993, Jonsyn *et al.* 1995).

Although OTA was discovered in 1965 as a secondary metabolite of *Aspergillus ochraceus* strains, in subsequent years, several other strains of *Aspergillus*, *Penicillium* and *Eurotium herbariorum* became established as OTA producers (Betina 1984, Chelkowski *et al.* 1987). To our knowledge, there is no information indicating whether isolates of *Eurotium* from Saudi Arabia are capable of producing OTA on barley. Thus, the objective of the present study was to screen quantitatively for the ability of isolates of *Eurotium* from barley locally grown in Saudi Arabia to produce OTA in artifical and natural culture media.

MATERIALS AND METHODS

Eurotium species isolation

Eurotium species were isolated by placing intact seeds of uniform size of barley (*Hordeum vulgare* Linn), after surface-disinfecting with 1% sodium hypochlorite for 10 min, on potato dextrose agar (PDA) and incubating plates at 25, 37 and 45°C for 6 to 10 days. The identity of these species was determined by the method of Raper and Fennell (1965), Klich and Pitt (1988), and Kozakiewicz (1989). Identity of the isolates was confirmed by MS. KEMIN Europa N. V. (Industriezone Wolfstee, 2410 Herentals, Belgium).

OTA production

For OTA production identified species of *Eurotium* were grown on PDA slant, yeast extract sucrose (YES) broth (2% yeast extract, 15% sucrose) and on untreated autoclaved barley grains. Dense conidial suspensions (0.1 ml, 10^6-10^7 spores cm^{-3}) were inoculated onto 5ml of PDA and YES broth contained in 15-ml vials. Barley grains (25g in Erlenmeyer flasks) were inoculated, with 1 ml of conidial suspension, after autoclaving and adjusting the moisture content to 23%. The inoculated media were incubated in the dark at 25°C for 7 days (Al-Julaifi & Al-Khaliel 1993).

OTA extraction and cleanup procedure

After incubation, spores on PDA and YES were killed by autoclaving the vials for 30s at 121°C. OTA was extrated twice with 5ml of hot chloroform and the combined extracts were dried over anhydrous sodium sulfate, then evaporated to dryness (Scott *et al*. 1970, Bullerman 1974, Tsai *et al*. 1984). Barley cultures were dried in the oven at 104°C for 24 hours. Twenty-five grams of the untreated barley sample and the barley cultures were finely ground and extracted as described by Lepom (1986) with modifications as follows. All of the flask content was extracted with 100 ml of methylene chloride plus 12.5 ml H_3PO_4 (0.1%) followed by mechanical agitation for 3 min. The mixture was filtered through Whatman filter paper No. 4. Five milliliter of the extract was pipetted into a 15 ml culture tube, 4 ml of hexane was added and the mixture was vortex for 10s. The mixture was purified by transferring it to a Romer Lab MycoSep column no. C212 (Romer Lab., Inc., Union, MO, USA) attached to a vacuum apparatus. The flow rate was 2 ml min^{-1}. Afterwards, the column was washed with 15 ml methylene chloride and flushed with 20 ml methylene chloride-formic acid (99:1, vol/vol). The eluent was collected and evaporated to dryness at a temperature of 60°C.

Analysis of OTA

Analyses of the purified extracts for OTA were conducted by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The residue was re-dissolved in 500 µl HPLC - grade toluene-acetic acid (99:1, vol/vol). Produced OTA was determined on a Whatman Silica Gel 60 A TLC aluminum sheet (Cat. No. 4861 820) after activation for 1 h at 100°C. Eighty microliters of the extract were spotted on the TLC plate and develped in 100 ml of toluene-methanol-acetic acid (18:1:1, vol/vol/vol). The positive samples were identified by visual comparison of the fuorescence intensity of the sample with appropriate reference standards, including internal standars, at wavelenghs of 366 and 254 nm. OTA formed a blue green fluorescence spot under long wave UV light and bright blue green under short wave UV with an R_f at approximately 0.6. The fluorescence turned bright blue on treatment with ammonia. Spraying the plate with 10% alumminium chloride in methanol increased the fluorescence intensity (Waldi 1969, Scott *et al*. 1970). The identity and amount of OTA was confirmed by HPLC using a HP 1100 system. The instrument consisted of a vacuum degasser (G1322A), binary pump (G1312A), autosampler (G1313A), thermostated column compartment (G1316A), diode array detector (G1315A) and fluorescence detector (G1321A). Separations were achieved on a Lichrospher clolumn (100 RP18 125 by 4-mm id, 5 µm, HP) using water with 2% acetic acid-acetonitrile (1:1, vol/vol) as an isocratic mobile phase

at a flow rate of 1 ml min^{-1} with a column temperature of 40°C. The diode array detector wavelength was recorded at 236 nm with 20 nm bandwidth. The fluorescence detector was recorded at excitation (λ_{Ex}) 236 nm and emission (λ_{Ex}) 464 nm. Quantitation was done by comparison with reference standars.

RESULTS AND DISCUSSION

Three fungal species of the genera *Eurotium* were isolated and identified as *E. amstelodami*, *E. herbariorum* and *Eurotium* spp. Since OTA had not previously been reported as a metabolite of most species in *Eurotium* genera, the three isolates were tested for their ability to product OTA on three laboratory media including PDA, YES and barley. OTA production was quantified using TLC and confirmation was obtained by HPLC. In YES cultures, results showed OTA production by all of the *Eurotium* species tested. *E. amstelodami*, *E. herbariorum* and *Eurotium* spp produced 138.21, 302.17 and 245.25 μg l^{-1}, respectively. Although the OTA produced by these species was low, which means that they are weak producer species, the concentrations produced are quite similar to those reported from the species of *Aspergillus ochraceus* and *Eurotium herbariorum* (Chelkowski *et al.* 1987, Cvetnic & Pepelijnjak 1990, Ranjan & Sinha 1991). In PDA cultures, the OTA concentrations detected were 97.14 μg l^{-1} (*E. amstelodami*) and 205.13 μg l^{-1} (*Eurotum* spp). No detectable amount of OTA was found with *E. herbariorum*. Thus, PDA could be not suitable as a media for OTA production by this isolate.

Although *Eurotium* species are common in homes, stored grains, and rodent dwellings, some of them, including *E. amstelodami*, *E. herbariorum* and *Eurotium* spp, are widely distributed in different varieties of substrates. Moreover, there were no toxic metabolites in naturally occurring cereals produced by most of them (Chelkowski 1991). The results from this study showed quantities of OTA produced on barley medium ranging from 165.14 to 214.77 μg kg^{-1}. *E. herbariorum* produced the highest amount (214.77 μg kg^{-1}) followed by *Eurotium* spp (121.39 μg kg^{-1}).

Since *Eurotium* species including *E. amstelodami* and *Eurotium* spp are fungi considered as allergenic, no toxic or invasive diseases have been reported to date. But of these species, OTA was detected only from cereal contaminated with *E. herbariorum* (Chelkowski *et al.* 1987). This is the first report of *E. amstelodami* and *Eurotium* spp. being capalbe of producing OTA. Further studies of the production of OTA in grains and feedstuffs under natural field conditions should be carried out. Thus, contamination of food and feedstuffs destined for human and animal consumption with the common fungus *E. amstelodami*, *E. herbariorum* and *Eurotium* spp indicates a serious potential danger to both human and animal health. Therefore, monitoring of grains for

OTA determination when *Eurotium* species are isolated should be a matter of concern to local countries.

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إنتاج سم الأوكراتوكسين أ بواسطة فطرتي الإيروشيم أميستيلودامي والإيروشيم سبيشز المعزولة من شعير مزروع محلياً في المملكة العربية السعودية

محمد الجليفي

المركز الوطني لأبحاث الزراعة والمياه
ص.ب. 31623، الرياض، المملكة العربية السعودية

خلاصة

من محصول شعير مزروع محلياً في المملكة العربية السعودية، تم عزل وتعريف ثلاث أنواع فطرية تابعة لجنس الإيروشيم. ولدراسة مقدرة تلك الأنواع الفطرية على إفراز سم الأوكراتوكسين أ، نميت العزلات على ثلاث بيئات مختلفة تمثلت في البيئتين الصناعيتين آجار البطاطس مع الدكستروز (PDA)، مستخلص الخميرة بالسكروز (YES)، وبيئة حبوب الشعير (*Hordeum vulgare* Linn) بعد التعقيم كبيئة طبيعية. ولتعريف وتقدير سم الأوكراتوكسين أ، اتبعت طريقة ألواح الفصل الكروماتوغرافي (TLC) والكروماتوغرافي السائل عالي الإنجاز (HPLC).

أظهرت النتائج مقدرة العزلات الثلاث على إنتاج سم الأوكراتوكسين أ بكميات متفاوتة. حيث قدرت كمية السم المحصول عليها من بيئة مستخلص الخميرة بالسكروز والمفرزة من الأنواع الفطرية إيروشيم أميستيلودامي، إيروشيم هيرباريورم والإيروشيم سبيشز بالكميات 138,21، 302,17 و245,25 ميكروجرام/لتر، على التوالي. وعلى بيئة آجار البطاطس مع الدكستروز، فقط الفطرتين إيروشيم أميستيلودامي والإيروشيم سبيشز تمكنتا من إفراز 97,14 و205,13 ميكروجرام/لتر، على التوالي. أما على بيئة الشعير فقد تمكنت جميع الفطريات المعزولة من إنتاج الكميات 165,14، 121,39 و214,77 ميكروجرام/كجم للإيروشيم سبيشز، إيروشيم أميستيلودامي، إيروشيم هيرباريورم، على التوالي. هذا يعني أنه لأول مرة يتم تسجيل مقدرة الفطرتين إيروشيم أميستيلودامي، والإيروشيم سبيشز على إفراز سم الأوكراتوكسين أ.