Ohcratoxin producing *Aspergillus* spp. isolated from tropical soils in Sarawak, Malaysia

Jaya Seelan Sathiya Seelan [1] & Sepiah Muid [2]

[1] Institute for Tropical Biology and Conservation, Locked bag 2073, Universiti Malaysia Sabah, 88999, Kota Kinabalu, Sabah, Malaysia
[2] Department of Plant Science and Environmental Ecology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia
Email: [1] avinash80us@yahoo.com

Aspergillus spp. has been widely studied for mycotoxin analysis. Mycotoxin is considered one of the chemical groups that causes serious side effects in humans and animals. Although *Aspergillus* spp. contains bioactive compounds, there is a need to screen for mycotoxin metabolites since mycotoxin is hazardous to human health. Ochratoxin is a mycotoxin with nephrotoxic, nephrocarcinogenic, teratogenic and immunosuppressive properties, and has received growing interest in the scientific community and food committees in the last few years (Battaglia et al. 1996; Abarca et al. 2003). Only species belonging to the genera *Aspergillus* and *Penicillium* have been reported as capable of producing ochratoxins. They were initially described by Scott (1965) in *Aspergillus ochraceus* but have also been found in other species of the section *Circumdati: A. alliaceus*, *A. melleus*, *A. ostianus*, *A. petrakii*, *A. sclerotiorum*, *A. sulphureus* (Hesseltine et al. 1972), *A. albertensis*, *A. auricomus* (Varga et al. 1996); as well as in the black aspergilli of *section Nigri: A. niger* var. *niger*, *A. carbonarius* (Samson et al. 2004). In this study, eighteen species of *Aspergillus* isolated from different habitats were selected to screen for ochratoxin producing strains.

Date of publication (online): 26 March 2010
Date of publication (print): 26 March 2010
ISSN 0974-7907 (online) | 0974-7893 (print)

Editor: V.B. Hosagoudar

Manuscript details:
Ms # o2247
Received 01 July 2009
Final received 23 November 2009
Finally accepted 09 January 2010

Citation: Seelan, J.S.S. & S. Muid (2010). Ohcratoxin producing *Aspergillus* spp. isolated from tropical soils in Sarawak, Malaysia . *Journal of Threatened Taxa* 2(3): 762-765.

Acknowledgements: The authors would like to thank Universiti Malaysia Sarawak (UNIMAS) for permission granted to present these findings and MOSTI for providing financial support for this project. The author is grateful to Prof. Maren Klich of the United States Agricultural Department (USDA) for confirming the indentification of the *Aspergillus* species.

Material and Methods

Fungal isolates: Eighteen isolates of *Aspergillus* species were selected (Table 1) and grown on potato dextrose agar (PDA), Czapek's yeast extract agar (CYA), and Malt extract agar (MEA), incubated for 7 days at 25°C. The strains were identified based on Raper & Fennell (1965) and Klich (2002). Colonies were mounted in lactophenol blue and images were taken by using a NIKON digital camera.

Extraction and immunochemical tests by using ELISA kit: Each fungus was cultured in a Czapek's yeast extract broth (CYB) medium for 7 days and incubated at 25°C, in shaker of 120rpm. Crude fermentation broth was blended thoroughly and centrifuged at 4000rpm for 5 minutes. The supernatant was passed through a filtration membrane (0.22µm, Minipore). Then the homogenized broth was extracted with chloroform. The combined organic extract was evaporated under reduced pressure yielding a crude semi-solid (Wang 2002). Then, the extracts were tested by using enzyme-linked immunosorbent assays (ELISAs) test for positive results. ELISA positives were confirmed by HPLC technique.

OA detection by HPLC: The samples were analysed by using a reverse phase HPLC equipped with a Jasco FP-920 fluorescence detector (330nm excitation wavelength, 460nm emission wavelength). Chromatographic separations were performed on an analytical column (symmetry waters C18 ODS2, 150mm x 3.9mm, 5µm) fitted with a precolumn with the same stationary phase. The mobile phase used was pumped at 1.0ml/min and consisted of an isocratic program as follows: acetonitrile/water/acetic acid (99:99:2, v/v). The injection volume was 20µl. Samples were taken as positive for OA presence if they yielded a peak at a retention time similar to the OA standard peak (approximately 4 min), with a height five times higher than the baseline noise.

Results and Discussion

ELISAs test: Altogether, 18 *Aspergillus* strains were tested for OA production by the immunochemical test. Among these, only two strains of *Aspergillus*, namely, *A. carbonarius* and *A. sulphureus* produced OA when tested with ELISA. The other sixteen strains of *Aspergillus* did not produce this toxin (Table 1). In temperate regions, *A. carbonarius* and *A. sulphureus* have been widely

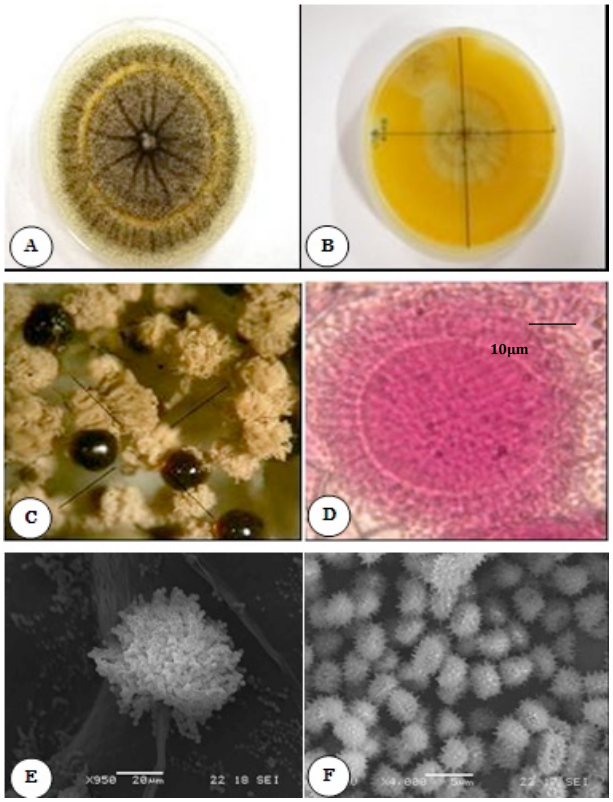

Image 1. *Aspergillus carbonarius* (JS742).
A - Colony surface on CYA media (7 days, 25°C); B - Colony reverse on MEA media (7 days, 25°C); C - Conidial head splitting into diverging columns and producing heavy exudates when mature; D - Conidial head radiate and biseriate; E - SEM of conidial head, (Bar = 20µm); F - Conidia globose, rough and spikes (Bar = 5µm).

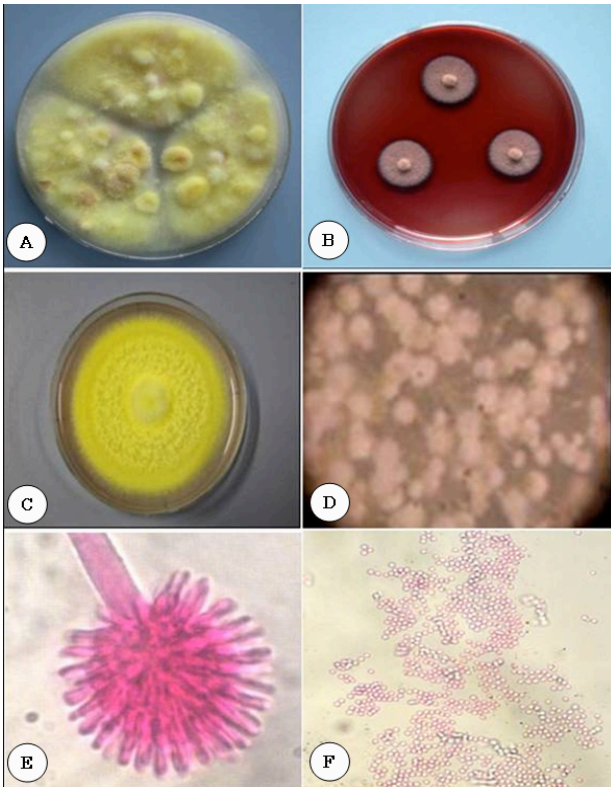

Image 2. *Aspergillus sulphureus* (JS500).
A - Colony surface on CYA25 media (7 days, 25°C); B - Colony surface on CYA37 media (7 days, 37°C); C - Colony surface on MEA(7 days, 25°C); D - Conidial heads white; E - Uniseriate conidial head (Bar = 50µm); F - Conidia (Bar = 10µm).

studied on their OA production. These two species were earlier described as OA producing species (Ciegler 1972; Hesseltine et al. 1972). In this study, we have showed that these tropical strains produce a lower concentration of OA using the czapek's yeast extract broth (CYB). The OA production was influenced by the type of media and the origin of the isolates (Harwig 1974). This was shown when two other *A. carbonarius* were isolated from the peat soil in Bintulu and did not show any OA production under the same liquid media. Thus, not all strains of *Aspergillus* species are ochratoxin (OA) producers.

Morphological identification of OA producers

***Aspergillus carbonarius* (JS742):** Colonies on Czapek's yeast extract agar (CYA) 57-58mm in diameter (7 days, 25°C), wrinkled, dense and velutinous, exudates present, white at first and becomes dark brown with forming of conidial heads, reverse dark yellow. Colonies on malt extract agar (MEA) 52-56mm in diameter (7 days, 25°C) similar to those on CYA but less dense and conidia in duller colors, reverse dirty yellow. No growth at 5°C. Growth at 37°C is exceptionally rapid, colonies on CYA 38-40mm in diameter in three days. This strain can grow at 45°C. Conidial apparatus develops as erect conidiophores. Tips of conidiophores enlarge and form vesicles with many phialides producing conidia in long chains.

Conidial heads are compactly columnar, 40-48*µ*m in diameter, dark brown to black. Conidiophores are unbranched, smooth and dark brown, stipes 200-300 x 3-4 *µ*m. Vesicles are round to globose shaped, 20-30*µ*m in diameter. Phialides crowded dark brown, 5-7*µ*m long. Conidia globose to subglobose, roughened, hyaline, and often decidous spinules when young and verruculose at maturity. Sclerotia occasionally produced on CYA (Image 1).

***Aspergillus sulphureus* (JS500):** Colonies on Czapek's yeast extract agar (CYA) 40-50mm in diameter (7 days, 25°C), wrinkled, showing radial furrowing, sulphur yellow in color, forming white conidial heads, reverse pale yellow, presence of abundant sclerotia shading from white to cream to pale yellow. Colonies on malt extract agar (MEA) 60-70mm in diameter (7 days, 25°C), similar to those on CYA but less dense and conidia dull yellow in color, reverse pale yellow. No growth at 5°C. Growth at 37°C is exceptionally rapid, colonies on CYA 55-60mm in diameter in three days. This strain did not grow at 45°C. Conidial apparatus develops as erect conidiophores.

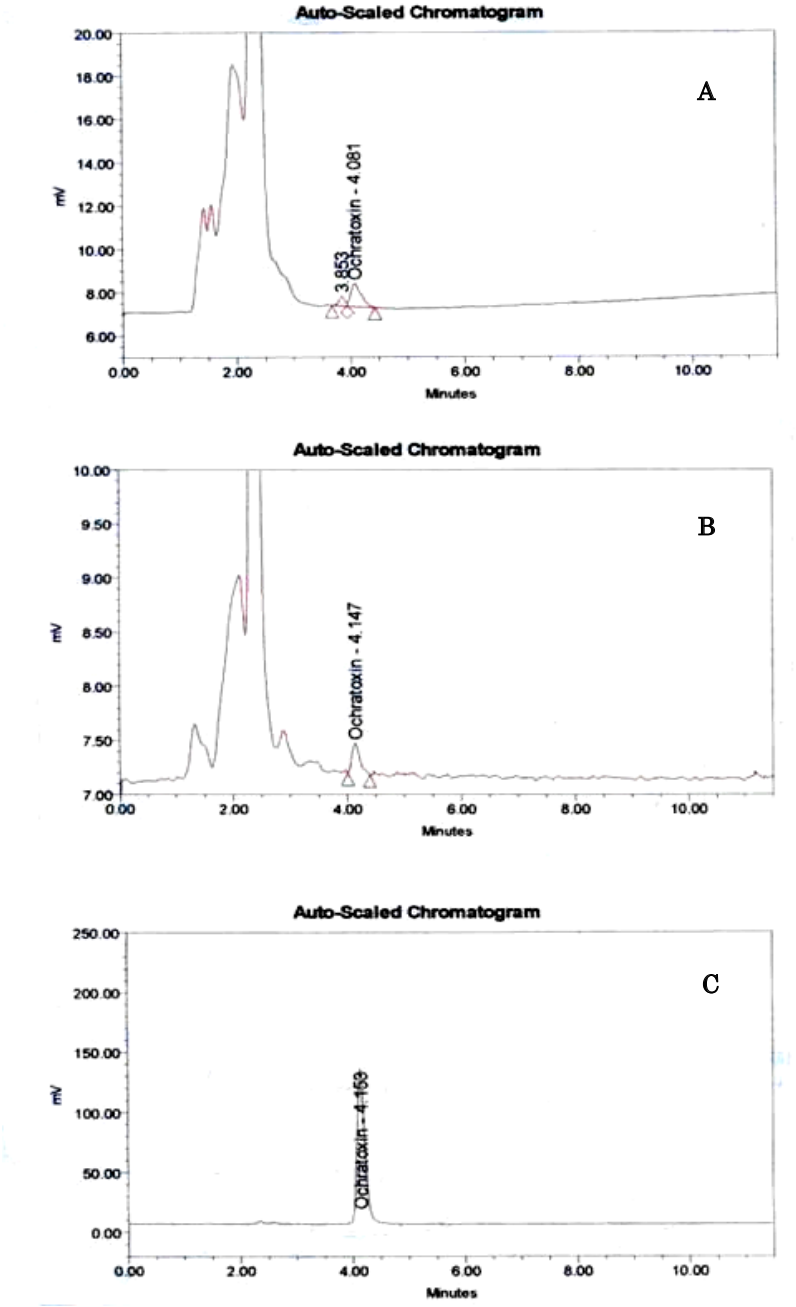

Figure 1. HPLC analyses of crude extracts of (A) - *A. sulphureus* (JS500) grown on CYB at the top; (B) - *A. carbonarius* (742); (C) - The following metabolite (ochratoxin) produced by this isolate can be identified using retention times and comparing UV spectra from analyses of standard Ohcratoxin A.

Conidial heads are loosely radiate, with spore chains adherent into numerous narrow, divergent and tangled columns, 400-450μm in diameter and white in color. Conidiophores up to 1mm long, unbranched, smooth, colorless, stripes 500-650 x 5-7μm. Vesicles hyaline, globose, 12-26μm in diameter. Sterigmata in two series (uniseriate or biseriate). Primaries (uniseriate) 4.5-7.5 x 3.0-4.5 μm, secondaries (biseriate) 6.5-8.0 x 2.0-2.5 μm. Conidia fusiforms and slightly roughened when first formed, but quickly globose, smooth, 2.0-2.5 μm in diameter (Image 2).

HPLC analyses

For quantification of OA, an HPLC apparatus equipped with a fluorescent detector was used. Extracts were considered positive if they yielded a peak at a retention time identical to that of standard OA (Figure 1). The amounts of OA observed for *A. carbonarius* and *A.*

Table 1. List of *Aspergillus* strains examined for ochratoxin metabolite in this study

Species	Ochratoxin content (µg ml^{-1})	Strain (sources and origin)
A. niveus	ND	JS518 (peat soil, UNIMAS)
A. ochraceus	ND	JS517 (peat soil, Loagan Bunut)
A. sulphureus*	0.05	JS500 (peat soil, UNIMAS)
A. niger	ND	JS024 (lake soil, Samarahan)
A. niger	ND	JS025 (citrus leaves, Samarahan)
A. niger	ND	JS022 (peat soil, Loagan Bunut)
A. flavus	ND	JS157 (dead wood, Bau Limestone)
A. flavus Link	ND	JS027 (peat soil, Samunsam)
A. flavus	ND	JS026 (peat soil, UNIMAS)
A. carbonarius*	0.10	JS742 (peat soil, Samarahan)
A. carbonarius	ND	JS744 (peat soil, Bintulu)
A. carbonarius	ND	JS752 (leaf litter, Bintulu)
A. terreus	ND	JS910 (leaf litter, UNIMAS)
A. terreus	ND	JS911 (peat soil, UNIMAS)
A. terreus	ND	JS912 (Riverine, Loagan Bunut)
A. fumigatus	ND	JS1006 (beach soil, Lundu)
A. fumigatus	ND	JS1001 (beach soil, Lundu)
A. fumigatus	ND	JS2003 (soil, Mukah)

* - Ochratoxin (OA) positive for ELISA's Test; ND - Not Detected

sulphureus were 0.05 to 0.10 µg ml^{-1}, respectively (Table 1). The OA concentration that was obtained in this study was lower in concentration compared to the standard OA found in *A. ochraceus* (250 µg ml^{-1}). These two strains from our tropical region proved to be a low OA producer, similar to *A. glaucus* and the black *Aspergillus* strains (Abarca et al. 1994). OA production in *A. carbonarius* and *A. sulphureus* was confirmed by HPLC comparing the UV spectra as recorded with a diode array detector. The retention times (4.417 and 4.081) and UV spectra were similar to that of the OA standard (Figure 1).

Conclusion

The immunochemical method based on the application of a monoclonal antibody preparation against OA proved to be a useful tool for the screening of ochratoxin production among the Aspergilli. The present study helps to eliminate the toxic producing *Aspergillus* strains. It also showed that only minimum concentrations of ochratoxin producing *Aspergillus* occurred compared to the temperate region countries.

References

Abarca, M.L., F. Accensi, M.R. Bragulat, G. Castella & F.J. Cabanes (2003). *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried wine fruits from the Spanish market. *Journal of Food Protection* 66: 504–506.

Abarca, M.L., M.R. Bragulat, G. Castella & F.J. Cabanes (1994). Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. *Applied and Environmental Microbiology* 60: 2650–2652.

Battaglia, R., T. Hatzold & R. Kroes (1996). Conclusions from the workshop on ochratoxin in food. *Food Additives and Contaminants* 13: 1-3.

Ciegler, A. (1972). Biproduction of ochratoxin A and penicillic acid by members of the *Aspergillus ochraceus* group. *Canadian Journal of Microbiology* 18: 631-636.

Harwig, J. (1974). Ochratoxin A and related metabolites, pp. 345-367. In: Purchase, I.F.H. (ed.). *Mycotoxins*. Elsevier Scientific Publishing Company, Amsterdam, 702pp.

Hesseltine, C.W., D.I. Vandergraft, M.L. Fennell, Smith & O.L. Shotwell (1972). Aspergilli as ochratoxin producers. *Mycologia* 64: 539-550.

Klich, M.A. (2002). *Identification of Common Aspergillus Species*. 1st Edition. Centraalbureau voor Schimmelcultures, Utrecht, Netherlands, 122pp.

Raper, K.B. & D.I. Fennell (1965). *The Genus Aspergillus*. Williams and Wilkins, Baltimore, 686pp.

Samson, R.A., E.S. Hoekstra & J.C. Frisvad (2004). *Introduction to Food and Airborne Fungi*. 7th Edition. Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.

Scott, D.B. (1965). Toxigenic fungi isolated from cereal and legume products. *Mycopathologia and Applied Mycology* 25: 213-222.

Teren, J., J. Varga, Z. Hamari, E. Rinyu & E. Kevei (1996). Immunochemical detection of ochratoxin A in black *Aspergillus* strains. *Mycopathologia* 134: 171-176.

Varga, J., E. Kevei, E. Rinyu, J. Teren & Z. Kozakiewicz (1996). Ochratoxin production by *Aspergillus* species. *Applied and Environmental Microbiology* 62: 4461-4464.

Wang, J., Y. Huang, M. Fang, Y. Zhang, Z. Zheng, Y. Zhao & W. Su (2002). Brefeldin A, a cytotoxin produced by *Paecilomyces* sp. and *Aspergillus clavatus* isolated from *Taxus mairei* and *Torreya grandis*. *FEMS Immunology and Medical Microbiology* 34: 51-57.