Evaluation of Brazilian terrestrial *Aspergillus* strains for Mycotoxin production

Avaliação de linhagens terrestres brasileiras de Aspergillus para produção de micotoxinas

Thais V. MILANEZ [1]*
Iracema H. SCHOENLEIN-CRUSIUS[2]
Luci K. OKINO[2]

RIALA6/911

Milanez, Thais V. ; Schoenlein-Crusius, Iracema H.; Okino, Luci K. Evaluation of Brazilian terrestrial *Aspergillus* strains for Mycotoxin production. **Rev. Inst. Adolfo Lutz,** 61(1):7-11,2002

ABSTRACT. Screening tests for aflatoxins B_1, B_2, G_1 and G_2, ochratoxin A and sterigmatocystin production were performed in 13 strains of *Aspergillus* spp, isolated from the terrestrial environment in the Brazilian Atlantic Rainforest (São Paulo State/Brazil). Coconut agar medium and moistened corn were employed as substrates. The fungal extracts obtained from both media were submitted to thin-layer chromatography and the toxins were estimated according to the intensity of their fluorescence observed under UV light. None of the tested strains presented any of the mentioned mycotoxins. Because many unknown fluorescent spots were present, it was necessary to proceed a confirmation step using multiple chromatography, two dimensional chromatography and derivatization. In view of the accuracy of the employed methods and the presence of many unknown fluorescent spots, the need of further studies on the production of others mycotoxins of fungi isolated under tropical conditions is justified.

KEY WORDS. *Aspergillus* spp, toxigenic fungi, aflatoxins, ochratoxin A, coconut agar medium, corn medium.

INTRODUCTION

A great majority of the species of mycotoxin-producing fungi mentioned in the literature were isolated from food and feedstuffs. [1,9,13,17,21,22]. Reports of mycotoxin-producing fungi isolated from non-edible materials or producing mycotoxins when inoculated on non-edible materials are also found. Land and Hult[13] analysed twenty five strains of *Penicillium* spp. isolated from discouloured mould-infected outdoor softwood for mycotoxin production and found patulin being produced by one strain of *Penicilium expansum*. They have also found in some strains the ability to produce mycotoxins on synthetic and natural (wood) substrates. One strain of *P. nordicum* (not wood-associated) produced ochratoxin A when cultured on

*[1] Corresponding author. Mailing address: Instituto Adolfo Lutz, Divisão de Bromatologia e Química, Seção de Química Biológica - Av. Dr. Arnaldo ,355, CEP 01246-902, São Paulo, SP, Brasil.
[2] Instituto de Botânica, Seção de Micologia e Liquenologia . CINP/SMA.

wood chips and one strain of *P. expansum* produced patulin both on wood chips and on wood blocks.

Ruiz et al[17] studied the fungal contamination in a green house used for the production of cucumbers. They analyzed 158 samples of water, sand, air, leaves and fruits, isolating 25 different genera, among them *Aspergillus,, Fusarium* and *Penicillium*. Twenty one strains of *A. flavus* were identified and only eight of them produced aflatoxins on aflatoxin production agar medium, one sample of sand and seven samples of leaves.

Whether mycotoxins are also produced on other natural substrates is not well known. The knowledge about the presence of micotoxigenic fungi in the Brazilian soils has been restricted to the results of some tests using fungi isolated from soil samples, randomly taken from the north and northeast regions of the country by Prof. Dr. Chaves Batista according to Schoenlein-Crusius [19].

Several methods have been proposed to screen fungal isolates for mycotoxins and other secondary metabolites, most of them using a simple method to detect and another for confirmation. Arseculeratne et al.[3] used freshly grated coconut as a medium for aflatoxin production and recommended it as a useful substrate in the preparation of aflatoxins on a large scale. Lin and Dianese[14] developed a coconut–agar medium for fast detection of aflatoxigenic fungi and in the same line. Bastos[4] stablished a method for detection of ochratoxins and sterigmatocystin production by *Aspergillus* sp. Sara et al.[18] reported a method based on ultraviolet detection of aflatoxin on agar medium, even though identification by thin-layer chromatographic should be required for positive identification of aflatoxin as the fluorescing substance. In 1987, Krivobok et al.[12] developed a rapid and sensitive method to identify mycotoxin production (aflatoxins, sterigmatocystin, ochratoxin A, patulin and penicillic acid) from the liquid medium where the fungi were cultivated. Filtenborg and Frisvad [7] developed a simple screening-method for extracellular mycotoxins taking a small plug from the agar substrate that may be applied to the TLC plate. Filtenborg et al.[8] developed a method similar to the agar plug[7] but to detect the intracellular mycotoxins. These methods are fast, simple and the sensitivity may be sufficient to detect the most important toxigenic isolates.

According to the literature, all taxa of fungi tested here may had the ability to produce mycotoxins. For instance, strains of *A. flavus*, one of the tested fungi, have been known to produce aflatoxins B_1 and B_2[6,14] and also sterigmatocystin[25]. The most important producer of sterigmatocystin is *A. versicolor* and it may also be produced by *A. sydowii* and *A. ustus*[25]. *A. versicolor* was also found to be an OTA producer by Abarca et al.[1], in corn culture but not in YES broth (yeast extract sacarose). Bastos[4] tested 92 isolates of *Aspergillus* from seeds. Only one isolate of the *A. niger*, for the first time, was characterized as an OTA producer. Ueno et al.[23] analyzing some *Aspergillus* isolates, verified that two *A. foetidus* isolates, one variety of *A. niger* used in the production of a local alcoholic beverage, produced OTA. Abarca et al.[2] found among 19 isolates of *A. niger var. niger* two OTA producers, not only in liquid medium (yeast and sacarose) but also in moistened corn. Ueno et al.[24] examined 100 fungal isolates and they verified that 26 were OTA producers. Among the *Aspergillus* species producers were: *A. sydowii, A. terreus, A. ustus* and *A. foetidus*.

Having in mind that species of toxigenic fungi may also be found in natural terrestrial environments such as soil, leaf litter, wood and humus. The screening of *Aspergillus* isolated from the Atlantic rainforest soil at the "Reserva Biológica de Paranapiacaba", Santo André Municipality, São Paulo State, Brazil was undertaken to evaluate their ability to produce aflatoxins, ochratoxin A and sterigmatocistin. The aim was to detect as much as possible all toxigenic isolates, irrelevant of the amount of mycotoxin produced. To guarantee the results two substrates, agar coconut and moistened corn, were tested, since one of them could fail, as reported by Abarca et al.[1].

MATERIAL AND METHODS

The strains of *Aspergillus* spp. tested were obtained from the Fungi Culture Collection of the "Instituto de Botânica", in São Paulo (Table 1). The group of fungi was composed by cultures originally isolated from the terrestrial environment of the Brazilian Atlantic Rainforest. The isolation and identification methods used were described by Schoenlein-Crusius[19].

Table 1. *Aspergillus* strains from Brazilian native Atlantic Rainforest.

Aspergillus ustus (1074 e 1110)	*A. sydowii* (1072 e 1073)
A. wentii (MLB 439)	*A. flavus* (MLB 459 e 1112)
A. fumigatus (1082)	*A. clavatus* (1077)
A. niger (1078)	*A. versicolor* (1079)
Aspergillus sp. (1071)	*Aspergillus* sp. (1076)

The ochratoxigenic strain *Aspergillus ochraceus* NRRL 3174[10] and the aflatoxins B_1 and B_2 producer *Aspergillus flavus* NRRL 5940, were donated by Prof. Dr. Benedito Correa (Instituto de Ciências Biomédicas, Universidade de São Paulo) to help in testing the analytical procedures.

The aflatoxins standards B_1, B_2, G_1, G_2 were acquired from SIGMA, ochratoxin A were obtained from Dr. Peter Scott, of the Health Protection Branch, Health Welfare of Canada and sterigmatocystin was obtained from Dr. Lucia Valente Soares, FEA/UNICAMP.

All fungal cultures were purified transfering them from the old plates to new ones, five times successively, until no other microrganism were present on the culture media. Then they were plated on potato dextrose agar (PDA) and incubated at 20 to 22ºC. After seven days the colonies were transferred to specific media to enhance the toxin production.

METHODS

Method 1: coconut agar medium[14] modified by Bastos[4] was used as a specific media for toxin production. The reverse side of the colony was observed daily, during six days, under ultra-violet light, to verify the presence of a blue fluorescent ring, an indication of the presence of aflatoxin or OTA, thus characterizing it as a toxigenic strain. Fluorescent colors in specific media may indicate the presence of mycotoxins. Blue colors refer to aflatoxin; blue-green indicates the presence of ochratoxin A, while orange or red may indicate the presence of sterigmatocystin[4]. In the present study the fungal extracts presented many fluorescent spots making it necessary to use different solvent systems, co-chromatography and two-dimensional chromatography to identify the mycotoxins present

The fluorescent spots in this medium were cut off, transferred to a mortar, macerated with chloroform and defatted with hexane. The chloroform phase was concentrated (fungus extract) and then thin-layer chromatography was performed.

Method 2: corn moistened (20g of corn plus 10 ml of distilled water, sterilized at 121°C for 1 h) was used as the second media for toxin production. The incubation period ranged from 10 to 20 days. Mycotoxins were extracted using the method developed by Soares and Rodriguez-Amaya[20] as modified by Milanez and Sabino[15].

Aflatoxins and OTA identification were conducted on thin-layer chromatography by visual comparison with standards. Confirmation for aflatoxins and OTA were according to Przybylski[16] and Hunt et al.[11], respectively.

The presence of many fluorescent spots justified the use of two dimensional thin-layer chromatography and multiple-chromatography, in addition to the unidimensional thin-layer chromatography, and the use of specially chosen solvents. For the two dimensional thin-layer chromatography the following solvents were used: (1^{st}) toluene + ethyl acetate+ formic acid, (60+30+10); (2^{nd}) chloroform+ acetone (90+10); or (1^{st}) chloroform+hexane+acetone (85+20+15) and (2^{nd}) toluene+ethyl acetate +formic acid (50+40+10); or (1^{st}) chloroform+methanol (98+2) and (2^{nd}) toluene+ethyl acetate +formic acid (60+30+10).

For chromatography with multiple developments the following solvents were used: (1^{st}) chloroform+acetone+hexane (85+15+20) and (2^{nd}) benzene+acetic acid (90+10); or (1^{st}) benzene+hexane (75+24) and (2^{nd}) benzene+acetic acid (90+10) or chloroform+hexane+acetone (85+15+20) for the identification of the aflatoxins, ocratoxin A and sterigmatocystin.

Toluene + ethyl acetate + formic acid (50+40+10) idem (60+40+0,5); benzene+metanol+acetic acid (96+8+4) and chloroform+acetone+hexane (85+15+20) were employed for the unidimensional chromatography.

RESULTS AND DISCUSSION

The chemical analysis of the fungal extracts of moistened corn and of coconut agar required an extra clean-up, in order to improve the observation, resolution and identification of the fluorescents spots on the TLC plates. So a deffating step with hexane has been introduced.

The results presented on Table 2 were all confirmed by

Table 2. Tested strains of *Aspergillus* spp, fluorescence and detection of mycotoxins

STRAIN	Fluorescences and reverse color on agar coconut medium	Detected Mycotoxin by TLC
A. flavus NRRL 5940 *	P-LB	AFB1, AFB2
A. ochraceus NRRL 3174 *	P-GB	OTA
A. fumigatus (1082)	N-BO	Nd
A. wentii (MLB 439)	P-PY	Nd
A. ustus (1110)	P-PY	Nd
A. ustus (1074)	P-PY	Nd
A. clavatus (1077)	N	Nd
A. niger (1078)	P-PY	Nd
A. sydowii (1072)	N	Nd
A. sydowii (1073)	P-GB	Nd
A. versicolor (1079)	N	Nd
A. flavus (1112)	P-B/BG	Nd
A. flavus (MLB 459)	N	Nd
Aspergillus sp (1071)	P-GB	Nd
Aspergillus sp (1076)	N	Nd

Legend: * standard strains, P- positive; N - negative; Color: LB - light blue; B - blue; BG - blue green; GB - greenish blue; PY - pale yellow; BO - orange brown; nd - not detected.

chemical analysis, in addition to the observation of the fluorescence on the reverse side of the colony of the "standard strains", for which toxin production is well-known. The use of agar coconut medium seems to be an easy and fast way to verify the aflatoxigenicity or ochratoxigenicity of suspected isolates. However, it was found necessary to analyze the coconut agar using thin-layer chromatography to get reliable results and to confirm the identities of the toxins by thin-layer chromatography. Although none of the isolates examined produced aflatoxins, OTA or sterigmatocystin (Table 2), the TLC plates revealed many fluorescent spots. There is still a possibility of the presence of other toxigenic metabolites, whose standards were not available at the time, such as ochratoxin B, penicilic acid and citrinin [5,10].

Among the species mentioned in Table 2, *A. clavatus* (1077) and *A. fumigatus* (1082) are considered possible producers of sterigmatocystin[1], but these findings were not confirmed by two dimensional TLC.

Besides producing OTA, *A. clavatus* may produce patulin[25]. According to Bastos[4], the coconut agar media may not be an appropriate media to verify the presence of citrinin and patulin.

The methodology tested in this study, including the substrates used, is considered adequate to the objectives, but further research must be carried out to enhance the knowledge about the metabolites produced by tropical Brazilian terrestrial fungi.

ACKNOWLEDGMENTS

The authors are grateful to Prof. Dr. Benedito Correa of the Universidade de São Paulo for the standard strains, Dr Peter Scott of The Health Protection Branch, Health Canada for the ochratoxin A standard and also to Prof. Lúcia V. Soares of University of Campinas for the standard of sterigmatocystin.

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RESUMO. Testes de triagem para verificar a produção de aflatoxinas B_1, B_2, G_1 e G_2, ocratoxina A e esterigmatocistina foram conduzidos em 13 linhagens de *Aspergillus* spp, isoladas do ambiente terrestre da Mata Atlântica Brasileira (SP/Brasil). Os meios de agar côco e milho umidificado foram os substratos testados neste estudo. Os extratos dos fungos obtidos a partir dos dois substratos foram submetidos à cromatografia em camada delgada e as micotoxinas estimadas de acordo com as fluorescências apresentadas sob luz ultravioleta. Nenhuma das linhagens testadas apresentou produção das micotoxinas mencionadas. Foi necessário acrescentar uma etapa de confirmação, usando múltipla cromatografia, cromatografia bidimensional e derivação. Tendo em vista a eficiência da metodologia aqui empregada e da presença de muitos pontos fluorescentes desconhecidos, justifica-se a necessidade da ampliação dos estudos sobre a produção de outras micotoxinas em fungos isolados de ambientes tropicais.

PALAVRAS-CHAVE. *Aspergillus*, fungo toxigênico, aflatoxinas, ocratoxina A, meio agar coco, milho umidificado.

REFERENCES

1. Abarca, M.L. et al. New ochratoxigenic species in the genus *Aspergillus*. **J. Food Prot.**, 60 (12): 1580-1582, 1997.
2. Abarca, M.L. et al. Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. **Appl. Environ. Microbiol.**, 60: 2650-2652,1994.
3. Arseculeratne, S.N. et al. Coconut as medium for the experimental production of aflatoxin. **Appl. Microbiol.**, 18(1): 88-94, 1969.
4. Bastos, S.T.G. **Método rápido para detecção de produção de ocratoxina A e esterigmatocistina por *Aspergillus* spp em meios de ágar**. Brasília, 1979, 77p. [Master's dissertation - Instituto de Ciencias Biológicas, Universidade de Brasília].
5. Ciegler, A. Bioprodution of ochratoxin A and penicillic acid by members of the *Aspergillus ochraceus* group. **Can. J. Microbiol.**, 18: 631-636, 1972.
6. Detroy, R.W.; Lillehoj, E.B.; Ciegler, A. Aflatoxin and related compounds. In: Lin, M.T.; Dianese, J.C. A coconut-agar medium for rapid detections of aflatoxin production by *Aspergillus* spp. **Phytopathology**, 66: 1466-1469,1976.
7. Filtenborg,O.; Frisvad, J.C. A simple screening-method for toxigenic moulds in pure cultures. **Lebensm. -Wiss. u.-Technol**.,13: 128-130, 1980.
8. Filtenborg, O.; Frisvad, J.C.; Svendsen, J.A. Simple screening method for molds producing intracellular mycotoxins in pure cultures. **Appl. Environ. Microbiol.** 45(2): 581-585, 1983.
9. Frisvad, J.C. Taxonomic approaches to mycotoxin identification (Taxonomic Indication of Mycotoxins Content in Foods). In: Cole,R.J. (**Ed**) **Modern Methods in the Analysis and Structural Elucidation and Mycotoxins**. Academic Press, New York, 1986. p.415-457.

10. Hesseltine, C.W. et al. *Aspergilli* as ochratoxin producers. **Mycologia,** 64: 539-550, 1972.
11. Hunt, D.C.; McConnie, B.R.; Crosby, N.T.; Confirmation of ochratoxin A by chemical derivatisation and high performance liquid chromatography. **Analyst**, 105: 91-92, 1980.
12. Krivobok,S. et al. Screening methods to detect toxigenic fungi in liquid medium. **J. Microbiol. Meth**.,7:23-36,1987.
13. Land, C.J.; Hult, K. Mycotoxin production by some wood-associated *Penicillium* spp. **Lett. Appl. Microbiol.**,4: 41-44,1987.
14. Lin, M.T.; Dianese, J.C. A coconut-agar medium for rapid detection of aflatoxin production by *Aspergillus* spp. **Phytopathology**. 66:1466-1469, 1976.
15. Milanez. T.V.; Sabino, M. Ocratoxina A em feijão comercializado no Estado de São Paulo e sua estabilidade no cozimento. **Rev. Inst. Adolfo Lutz**.,49:131-135, 1989.
16. Przybylski, W. Formation of aflatoxin derivatives on thin-layer chromatographic plates. **J. Assoc. Off. Anal. Chem**., 58: 163-164, 1975.
17. Ruiz, J.A. et al. Aflatoxin-producing strains of *Aspergillus flavus* in the mould flora of different greenhouse substrates for the cultivation of cucumber (*Cucumis sativus*,L.). **Int. J. Food Microbiol**., 29: 193-199,1996.
18. Sara, S.; Fennell, D.I.; Hesseltine, C.W. Aflatoxin-producing strains of *Aspergillus flavus* detected by fluorescence of agar medium under ultraviolet light. **Appl. Microbiol.**, 27:1118-1123, 1974.
19. Schoenlein-Crusius, I. H. **Sucessão fúngica em folhas de *Alchornea triplinervia* (Spreng.) M. Arg. em ambientes aquático e terrestre, na mata atlântica, Reserva Biológica do Alto da Serra de Paranapiacaba, Santo André, SP**. Rio Claro, 1993. 398 p. [Doctor's Thesis – Faculdade de Biologia, Universidade do Estado de São Paulo].
20. Soares, L.M.V.; Rodriguez-Amaya, D.B. Survey of aflatoxins, ochratoxin A, zearalenone and sterigmatocystin in some brazilian foods by using multi-toxin thin-layer chromatographic method**. J.Assoc. Off. Anal. Chem**, 72:22-26,1989
21. Stack, M.E. et al Mycotoxins produced by isolates of *Aspergillus ochraceus* found in coffee. Proceedings of V International IUPAC **Symphosium on mycotoxins and phycotoxins** - Vienna/Technical University/Austria - 1-3/09/82. p.196-199.
22. Trenk, H.L.; Butz, M.E.; Chu, F.S. Production of ochratoxins in different cereal products by *Aspergillus ochraceus*. **Appl. Microbiol.**, 21: 1032-1035,1971.
23. Ueno, Y. et al Use of monoclonal antibodies, enzyme-linked immunosorbent assay and immunoaffinity column chromatography to determine ochratoxin A in porcine sera, coffee products and toxin-producing fungi. In: Castegnaro, M.; Plèstina, R.; Dirheimer,G.; Chiernozemsky,I.N.; Bartsch, H. (**eds**). **Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours**. International Agency for Research on Cancer (IARC), Lyon,1991. p.71-75.
24. Ueno, Y. et al. **Application of Mab-based ELISA and affinity chromatography for surveys of ochratoxin A in swine sera, coffee products and toxin producing fungi.** Meeting on Mycotoxins, Nephropathy and Urinary Tract Tumours, Lyon, France,1991. p.47.
25. Wilson, D.M.; Abramson, D. Mycotoxins. In: Sauer, D.B. (ed) **Storage of cereal grains and their products**. American Association of Cereal Chemists, Inc., St. Paul, MN, 1992. p. 341-391.

Recebido em 07/05/2002; Aprovado em 02/09/2002