ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 2, Issue 4, pp: (37-42), Month: October - December 2014, Available at: www.researchpublish.com

DETECTION OF MYCOTOXIN PRODUCE BY ENDOPHYTIC FUNGI

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Abstract: **The endophytic fungi can be considered the symbiosis relationship between plants and fungi, where the major features of mutualistic symbiosis include the lack of destruction of most cells or tissues, nutrient or chemical cycling between the fungus host, enhanced longevity and photosynthetic capacity of cells and tissues under the influence of infection, enhanced survival of the fungus. But endophytes may produce mycotoxins, or otherwise alter host physiology and morphology of the plants. In this study, studied the ability of endophytic fungi to produce mycotoxin, and identified the mycotoxin. Can be isolated the endophytic fungi from three plants and chosen the dominant fungi and tested for producing mycotoxin and identified the mycotoxin. The chosen fungus is *chaetomium* sp. Firstly study The effect of *Chaetomium* species filtrate on *Artemia salina* to detect the ability of this genus to produce toxins and identify these toxins. Eight *Chaetomium* species out of 28 isolates tested had toxicity (high or moderate) to brine shrimp larvae, toxigenic *Chaetomium* species belonged to five species and these are *Chaetomium atrobrunneum*, *C. causiiforme*, *C. gelasinosporum*, *C. globosum* and *C. hexagonosporum*. Scondly identification of mycotoxin, sterigmatocystin was detected in different toxic extracts (8 extracts) of toxigenic *Chaetomium* species (5 species) in concentration varied between 12-13 µg / 50 ml medium. Four unknown compounds (yellow flouresent) at RF values 0.31, 0.4, 0.43 and 0.53 were detected in different toxic extracts.**

I. INTRODUCTION

Fungi produce a wide variety of low molecular weight fungal secondary metabolites, many of which have been associated with the precipitation of adverse effects in animals and humans. These compounds have been given the term mycotoxins (Schollenberger *et al*., 2007; Ghali *et al*., 2008). They occur in the mycelium of filamentous fungi, but may also be present in the spores of these organisms. The deleterious effects are referred to as mycotoxicoses, classified as mycotoxins (D'Mello & Macdonald, 1997). In nature, mycotoxins are a chemical defense for fungi and have evolved as mechanisms for antiherbivory and to provide a competitive advantage when colonizing new substrata (Abbott, 2002). Mycotoxins are a diverse group of compounds produced by a wide range of different fungi, normally after a phase of balanced growth. However, the production of a particular mycotoxins is generally confined to a retatively small number of fungal species and may be species or even strain specific. There is convincing evidence to suggest that the more intricate the pathways of synthesis of a particular mycotoxins, the fewer number of fungal species producing that compounds (D'Mello & Macdonald, 1997).

II. TOXIGENIC FUNGI

Many species in the genera *Aspergillus*, *Penicillium* and *Fusarium* are known to produce mycotoxins. These three genera of molds are also very common indoors. Other toxigenic molds frequently found indoors are *Alternaria*, *Trichoderma*, *Cladosporium*, *Paecilomyces*, *Chaetomium* and *Acremonium*. *Chaetomium* comprises more than 100 species, but the taxonomic data given by different authors vary greatly, e.g. Udagawa *et al*. (1979) characterized more than 200, Domsch

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et al. (1993) distinguished 160-180, while Von Arx *et al*. (1986) accepted only about 80 species. *Chaetomium* sp. strains are known producers of antibiotics (chaetocins, chaetomin, cochliodiol), cytotoxic chaetoglobosins A-K, hydroxylamines and nitrites, and cytotoxic, antibiotic, carcinogenic, mutagenic and nephrotoxic mycotoxin sterigmatocystin as well (Betina, 1989).

III. MATERIALS & METHODS

Detection of Mycotoxins

a) Cultivation Of The Tested Fungi

A total number of 28 isolates representing 9 fungal species related to genus *Chaetomium* were screened for toxicity and mycotoxin production on liquid medium. The tested fungi were grown on cellulose-Czapek's agar medium. The cultivation was done in 250 ml Erlenmeyer flasks, each containing 50 ml of medium. The flasks were inoculated with a single 6 mm disc, cut-out from the margin of a 5 d colony, of the fungus grown on cellulose - Czapek's agar medium. The flasks were incubated at 28±2°C for 15 days as stationary cultures.

B) Extraction procedures

The contents of each flask (mycelium + medium) were homogenized in 50 ml chloroform for 5 min in a high speed blender (16000 rpm). Extraction was repeated three times. The combined chloroform extract was filtered over anhydrous sodium sulphate,(5g) evaporated to near dryness using a rotary evaporator, and the dry material was transferred to a dry vial with small amount of the solvents which was evaporated to near dryness under the air.

Biological Assay:

Brine shrimp (Artemia salina Linnaeus) test:

The immature stage (nauplii) of brine shrimp was used for toxins bioassay. Larvae of shrimp are suitable for rapid assay of extremely small quantities of toxins. The test has been used for aflatoxins studied by Brown *et al*., 1968 ; Biji *et al*., 1981 ; and for other mycotoxins studied by Eppley & Bailey, 1973 ; Korpinen, 1974 ; Scott *et al*., 1980 ; Jimenez *et al*., 1986 and other.

a) 15-20 drops of brine shrimp eggs were hatched in natural sea water (100 ml, Red Sea water) at 25°C. Air usually conducted into the sea water in small bubbles through a tube in pore made in the plug of the container. Three days after the emergence of the first larve, the hatched larvae were used as test animals.

b) Each extract (0.05 mg) was applied to 0.8 cm diameter filter paper disc (Whatman No.3), each disc was placed in each test tube and about 20-40 shrimp larvae in 1 ml salt water were transferred into the tubes. The tubes were kept at a temperature of 25 ±1°C. A control tube with only sea-water was also made.

c) 24 h later, the mortality was determined using a stereoscopic microscope.

d) The titration of every preparation was repeated twice.

Clean Up Of Crude Extract:

The mycotoxins were cleaned up using a mini column (14×0.8 cm). A small piece of glass wool was put in the bottom of the column and 2.8 g kiesel gel 60, 70/ 230 silica gel were added. The washing and eluting solvent system (8 ml, each) of aflatoxins and sterigmatocystin (chloroform and 3:97 methanol-chloroform, respectively).

Thin-Layer Chromatographic Determination of Mycotoxins:

For qualitative determination, thin-layer chromatographic technique adopted in this laboratory (El-Kady & Moubasher, 1982) was employed.

Solvent systems:

For the purpose of separation of the different mycotoxins, two solvent systems of the following composition were used, all were of reagent grades:

Benzene: methanol : acetic acid (90 : 1 : 15, v/v/v) for sterigmatocystin as described by Naoi *et al*. (1972).

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Application and Development:

The samples to be analyzed, 0.01 ml of solution in chloroform were applied, using micropipettes. The spots were dried during application with a flow of cold air. The plates were developed in a developing tanks of 15 x 30 x 30 cm (Zeiss; Jena; Germany) saturated with solvent vapour. Each substance was chromatographed in two series in all the solvent systems. When the front of the system reached the development was interrupted, the chromatogram was dried in air and then detection was carried out.

Detection and Spraying Reagents:

The developed plates were detected before and after spraying with the different reagents visually, under short wave (254 nm) and long wave (365 nm) ultra violet irradiation (UV lamp, Desaga, Heidelberg, Germany).

Mycotoxins were identified by comparison with appropriate reference standards.

Aflatoxins:

Aflatoxins B_1 and B_2 fluoresce blue and aflatoxin G_1 and G_2 fluoresce green under long wave UV light (Gimeno, 1979).

Sterigmatocystin:

The compound exhibits a dull brick red fluorescence under short wave UV light. Spraying plates with aluminium chloride reagent and heating at 110° C for 10 min changes the fluorescence to light yellow (Gorst-Allman & Steyn, 1979).

UV Spectrophotometeric Analysis:

For quantitative determination of mycotoxin (sterigmatocystin), a spectrophotometer (cecil, model 703) was used at molecular coefficient of 21.800 at 238 nm according to the methods described by Bean *et al.*(1972), and employed by El-Maraghy and El-Maghraby (1987).

IV. RESULTS

Mycotoxin Production by Chaetomium Species:

A total of 28 isolates belonging to 9 species of *Chetomium* (*C. atrobrunneum*, *C. hexagonosporum*, *C. gelasinosporum*, *C. causiiforme*, *C. globosum*, *C. bostrychodes*, *C. dreyfussii*, *C. gangligerum* and *C. vitellinum*) were grown on 2.1 % cellulose – Czapek's agar (strilling growth) at 28°C (Table, 22).

The chloroforme extracts (mycelium medium) of different isolates tested proved that 8 isolates belonging to 5 species of *Chaetomium* namely: *C. atrobrunneum*, *C. hexagonosporum*, *C. gelasinosporum*, *C. causiiforme* and *C. globosum* had toxicity to brine shrimp (*Artemia salina* L) larvae.

Based on thin layer chromatography (TLC) analyses, sterigmatocystin was detected in different toxic extracts of the 8 isolates tested and these isolates belonging to *C. atrobrunneum* (1 isolate), *C. hexagonosporum* (4 isolates), *C. gelasinosporum* (1 isolate), *C. causiiforme* (1 isolate) and *C. globosum* (1 isolate) . UV spectrophotometeric sterigmatocystin analyses revealed that (12-113 µg / 50 mL) was detected in the toxic extracts. Also four unknown compounds (yellow flouresent) at RF, 0.31, RF , 0.4, RF, 0.43 and RF, 0.53 were detected in different extracts. *C. atrobrunneum* and *C. globosum* had the ability to produce the four compounds (++ to +++ flouresent). *C. causiiforme* produced three unknown compounds in low concentration (+).*C. gelasinosporum* produced two compounds in low concentration (+).*C. hexagonosporum* had the ability to produce 1-2 compounds at Rf 0.43 and Rf 0.53.

Table (1): Toxicity and mycotoxins produced by toxigenic *Chaetomium* species.

Chaetomium species	Toxicity brine shrimp test	sterigmatocystin	Comp. 1 yellow spot RF 0.31	Comp. 2 yellow spot RF 0.4	Comp. 3 yellow spot RF 0.43	Comp. 4 yellow spot RF 0.53
C. atrobrunneum	H	10 x 11.3 µg/50 mL	+++	+++	+++	++
C. causiiforme	M	10 x 2.6 µg/50 mL	+	-	+	+

C. gelasinosporum	M	10 x 2 µg/50 mL	-	+	-	+
C. globosum	H	10 x 2.5 µg/50 mL	+++	++	+++	++
C. hexagonosporum	M	10 x 7.1 µg/50 mL	-	-	-	+
C. hexagonosporum	M	10 x 12.4 µg/50 mL	-	-	-	-
C. hexagonosporum	H	10 x 3.8 µg/50 mL	-	-	-	+
C. hexagonosporum	H	10 x 1.2 µg/50 mL	-	-	+	+

H: *highly toxicity, more than 75% dead larvae.*
M: *moderate toxicity, between 50-75% dead larve.*

V. DISCUSSION

Mycotoxin of Chaetomium Species:

Of the most dominant fungal genera (*Fusarium* and *Chaetomium*) as endophytes of the three plants (*Altheae rosea*, *Calotropis procera* and *Nerium oleander*), in different organs (leaves, stems and roots) *Chaetomium* species (9 species) were chosen for mycotoxin production. Several strains of *Chaetomium* are found in soil, plants, debris and endophytic habitats, where they suppress the growth of bacteria and fungi through direct competition, mycoparasitism and antibiosis (Park *et al.,* 2005).

Based on thin layer chromatography (TLC) and spectrophotometric analyses, sterigmatocystin was detected in ethyl acetate extracts of *Chaetomium* species including *C. atrobrunneum*, *C. hexagonosporum*, *C. gelasinosporum*, *C. causiiforme* and *C. globosum* in concentration 12 – 113 mg / 50 mL Czapek's medium .The compounds is much like aflatoxin in it's biological activity, structure and biosynthetic pathway. However, it is unlike aflatoxin that it is rarely encountered in feedstuff naturally occurring (Smith & Moss, 1985; Bullerman, 1986; Eley, 1992). Also, the toxin was produced by some fungal isolates of *A.versicolor* and *A. nidulans* (*Emercilla nidulans*) as reported by El- Maghraby & El-Maraghy, (1988) and was detected naturally in seeds and grains (El- Maraghy & El-Maghraby, 1986; El- Maghraby & El-Maraghy, 1987; El-Maghraby, 1996).

Chaetomium species are also well-known sources of bioactive compounds. For instance, antifungal chaetoatrosin A and fuscoutroside have been isolated (Hwang *et al.*, 2000; Kobayashi *et al.*, 2005) from *C. atrobrunneum*, antibacterial chaetochalsin A from *C. brasiliense* (Oh *et al*., 1998), antibiotic chaetomin from *C. cochlioides* (Geiger, 1949). Novel and diverse compounds with unknown biological activity have also been obtained from fungus *Chaetomium* as listed in Marwah *et al*. (2007). Also, four unknown compounds (yellow flouresent at 254 nm UV light) were detected at Rf values 0.31, 0.4, 0.43 and 0.53 were detected from the toxigenic *Chaetomium* species (5 species). Based on the results obtained, *Chaetomium globosum* and *C. atrobrunneum* produced all of unknown compounds. In this respect, *C. globosum* has been reported to produce chaetoglobosins and chaetomin (Udagawa, 1984). It is the most common species of *Chaetomium* (Andersen & Nissen, 2000). It is known to produce the highly cytotoxin chaetomins and chaetoglobosins that inhibit cell division and glucose transport (Ueno, 1985). Other *Chaetomium* species produced sterigmatocystin. Also, isolates of *C. globosum* produced large quantities of chaetoglobosin A & C and more than to unknown metabolities (Nielsen *et al.*, 1999).

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