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Detection of Ochratoxigenic Potential in some *Aspergillus* and *Penicillium* Isolates from Vineyard Soil, Fresh and Dried Grapes by ELISA

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ABSTRACT

Several species of *Aspergillus* and *Penicillium* were isolated and identified from samples of vineyard soil, fresh grape berries and dried vine fruits collected from grapevine nursery and shops in Duhok province. The ochratoxigenic potential of some of their strains was evaluated by ELISA technique. Ochratoxin A (OA) was detected in cultures of two species of section *Nigri* (*A.carbonarius* and *A.niger* aggreg.), two species of *Aspergillus* section *Circumdati* (*A.ochraceus* and *A.westerdijkia*) and one species of OA was found at levels from 0.64 to 0.72 ng\ ml in *A.carbonarius* isolates, from 0.64 to 0.66 ng\ ml in *A.niger* aggreg. isolates, from 0.23 to 0.65 ng\ ml in *A.ochraceus* isolates and 0.40 ng\ml in *A.westterdijkia* isolate, whereas the level was from 0.61 to 0.64 ng\ml in *Penicillium verrucosum.*

Keywords: Ochratoxin A, *Aspergillus, Penicillium*, grapevine, ELISA.

الكشف عن قابليه إنتاج الاوكراتوكسين في بعض مزارع عزلات من الاسبرجلس والبنيسيليوم المعزولة من ترب حقول الأعناب ومن ثمار الأعناب الطرية والجافة

الملخص

تم عزل وتشخيص العديد من الأنواع الفطرية العائدة للجنسين اسبرجلس و بنيسيليوم من عينات ترب حقول الأعناب فضلا عن ثمار العنب الطرية و الجافة والتي جمعت من محافظه دهوك. تم الكشف عن قابليه إنتاج هذه العزلات لإنتاج السم الفطري اوكراتوكسين باستخدام تقنيه .ELISA تم الكشف عن إنتاج السم من قبل عزلتان من Aspergillus section *Nigri* (*A.carbonarius A.niger* aggreg.), وعزلتان من *Aspergillus* section *Circumdati* (*A.ochraceus A.westerdijkiae* و عزله من *Penicillium* (*P.verrucosum*.

تراوح مستوى إنتاج السم من قبل عزلات *A.carbonarius* ما بين 0.64-0.72 نانوغرام / ملغم وفى عزلات *A.niger* aggreg. مابين 0.64-0.66 نانوغرام/ ملغرام وفى عزلات *A.ochraceus* مابين 0.23-0.65 و في عزله *A.westerdijkiaie* 0.40 نانوغرام/ ملغرام بينما تراوح الإنتاج مابين -0.64-0.61 نانوغرام/ ملغم فى عزلات *Penicillium verrucosum*.

الكلمات الدالة: اوكراتوكسين، الاسبرجلس، البنيسيليوم، حقول الأعناب، ELISA.

INTRODUCTION

Grapevine (*VitisviniferaL.*) is a crop of major economic importance used for the production of table and wine grapes and is taken as a fresh berry, dried vine fruits or as juices. Black aspergilli (*Aspergillus* section *Nigri*) are common saprophytes in soil around the world, particularly in tropical and subtropical regions (Klich, 2002). Several species common in vineyards causes *Aspergillus* rot

in grapes such as *A.aculeatus*, *A.carbonarius*, and *A.niger* (Snowdon, 1990). Black aspergilli are found on the surface of healthy grapes at all stages of maturation and are the main fungi responsible for post-harvest decay of fresh fruits (Snowdon, 1990 ; Saadullah and Abdullah, 2012) OchratoxinA (OA) was originally produced by species of *Aspergillus* section *Circumdati* (*A.ochraceus*) and by *Penicillium* (*P.verrucosum* and *P.nordicum*). However, during the last decade several investigationshave revealed that some species of the *Aspergillus* section *Nigri* (*A.niger*aggr., and *A.carbonarius*) which are usual contaminants of fruits such as grapes and their products were found ochratoxigenic (Abarca *et al*., 1994 ; Hocking *et al.*, 2003).They are considered as the main source of OA contamination of grapes and grapevine products (Abarca *et al*., 2003; Leong *et al.*, 2007) .Ochratoxin A has been shown to be nephrotoxic, teratogenic and immunotoxic to several animal species and has been linked to human populations (IARC, 1993; Petzinger and Ziegler, 2002). OA has been classified as a possible human carcinogen (group 2 B) by the International Agency for Research on Cancer (IARC, 1993).

The study was aimed to determine the ochratoxigenic potential of some isolates of *Aspergillus* and *Penicillium* isolated fromvineyard soil, fresh grape berries and dried vine fruits by using Immunological methods (ELISA).

MATERIALS AND METHODS

Isolation of the Fungi from Soil

Direct plating method as described by (Warcup, 1960). was adopted. About 1g of fine soil collected from grapevine nurserywas sprinkled on the surface of a solidified Dichloran Rose Bengal Chloromphenicol Agar medium (DRBC) (Fluka-Germany) in Petri dishes (13). Plates were incubated in darkness at 25ºC for 7 days.

Isolation of the Fungi from Grape Fruits (berry) Samples

Sixty berries were surface disinfected with sodium hypochlorite 2% for 1 min., then washed twice with sterile distilled water as described by (14). Ten berries put per each plate containing DRBC agar medium. The plates were incubated in darkness at 25ºC for 7 days.

Isolation from Dried Vine Fruits (raisins) Samples

Two different media, DRBC and Dichloran 18% Glycerol Agar (DG18) (peptone, 5.0g; glucose, 10.0g; Kh2PO4, 1.0g; MgSO47H2O,0.5g; Dichloran (0.2%in ethanol), 1ml; glycerol, 220.0g; chloramphenicol, 0.1g; agar, 15.0g; distilled water, 1L) were used for the isolation and enumeration of fungi from dried fruits (King *et al*., 1979; Pitt and Hocking, 1997). Sixty dried vine fruits treated with 2% sodium hypochlorite solution for 2 min then rinsed with sterile water and 10 fruits were aseptically placed in Petridishes. All the plates were incubated in darkness for 7 days at 25 ºC.

Identification of *Aspergillus* and *Penicillium* Species

Samples from soil, fresh berries and dried vine fruits were examined daily with the help of stereomicroscope (AmScope, China) for sporulating fungi. Pure colonies were established on appropriate media for identification. Majority of detected species were identified to species level based on morphological and cultural characteristics. For identification of species of *Aspergillus* and *Penicillium*, pure colonies were grown on four media according to (Klich, 2002; Samson *et al*., 2000). The media were as follows: Czapeck Yeast Extract Agar incubated for seven days at 25˚C (CYA25), Czapeck Yeast Extract Agar incubated for seven days at 37˚C (CYA37), Czapeck Yeast Extract Agar with 20% Sucrose incubated for seven days at 25˚C (CY20S), Malt Extract Agar (MEA) incubated for seven days at 25C˚. Ingredients and preparation of the above five media were mentioned in (Klich, 2002; King, 1979). For each culture four plates were used, two of CYA and one each of CY20S, MEA. Each plate is inoculated at the center and incubated in the dark for seven days. One CYA is incubated at 37˚C. The rest are incubated at 25˚C.

Confirmation Tests for Identification of *Aspergillus* and *Penicillium* Species

1. Ehrlich Test

The Ehrlich test was used by (17) to distinguish taxa of *Penicillium*sub genus *Penicillium* and is based on the detection of alkaloids reacting with Ehrlich reagent [18] using a filter paper method. The Ehrlich reagent consists of 2 g of 4- dimethylamino – benzaldehyde in 96 % ethanol (85 ml) added to 15 ml 10 N HCl. An four mm agar plug is cut out from the center of a colony grown on CYA (incubated for 5-9 d at 25ºC.) and a round piece (1cm diam.) of the wetted filter paper (Whatman No.1) is placed on the mycelia side of the plug. This method is also used to classify some *Aspergillus* species in section *Nigri* by (Samson *et al*., 2007)

2. Growth on Creatine Sucrose Agar (CREA)

In this study, the growth abilities of all *Aspergillus* species in section *Nigri* strains were tested on Creatine Sucrose Agar medium (creatine(1H2O), 3g; sucrose, 30g; KCl, 0.5g; MgSo4.7H2O, 0.5g; FeSO4.7H2O, 0.1g; K2HPO4.3H2O, 1.3g; bromocresol purple, 0.05g; agar, 15.0g and distilled water, 1L). CREA is the semi-selective medium useful for classification of various fungal culture especially *Penicillium* species (17, 20). On CREA characteristics of colonial growth, production of acid (turning of the medium from purple to yellow) and base production can be used as diagnostic feature. CREA can be used as a semi – selective medium for dividing all black aspergilli into groups (20).

All species identifications were done according to the keys and descriptions provided by . (Klich, 2002; Samson *et al*., 2007; Samson *et al.,* 2004; Frisvad *et al*., 2004; Abarca *et al*., 2004).

Extraction of Ochratoxin A from Fungal Cultures

Isolates from *Aspergillus* and *Penicillium* genera were evaluated for their OA producing potential. A rapid method of (Bragulat *et al*., 2001). for extraction of ochratoxin A was adopted. The isolates were grown on Czapek yeast autolysate agar (CYA) medium incubated at 25ºC for 7 days. Three plugs (7-mm diameter) were removed from the inner, middle, and outer areas of each colony. Plugs were introduced into 3-ml vials; 1 ml methanol was added and the vials were shaken and incubated at 25ºC for 60 min. The extracts were centrifuged three times for 5 min at 4000 rpm. The supernatant was passed through a filtration membrane Millipore filter (0.22) µm (Millex GP Filter unit Coringhwohill Co. Ireland). Then the extracts were tested by using Enzyme-linked Immunosorbent Assays (ELISAs) test for positive results.

Ochratoxin Analysis

The quantitative analysis of OA was performed by the Enzyme Linked Immunosorbent Assay (ELISA). This was performed according to instructions provided by the manufacture (Veratox quantitative ochratoxin test, NeogenCorporation, USA).The concentration of OTA was calculated from the standard curve derived from the OTA standards and was expressed in ng\ml.

RESULT AND DISCUSSION

The capacity of the fungal strains for producing OA was determined on solid media (CYA) after 7 days at 25C. A rapid method of Bragulat *et al.,* (2001) for extraction of OA was adopted as described in 2.6. Source, of isolates and their OA producing capacity is represented in (Table 1).

Table 1: List of fungal strains examined for ochratoxin A by ELISA technique and their sources

Fungus (culture)	Ochratoxin A ng/ml	Source of Strain
A.japonicus	0.00	Soil
A.carbonarius	0.68	Soil
A.carbonarius	0.67	Zibib
A.carbonarius	0.68	Zibib
A.ochraceus	0.65	Soil
A.carbonarius	0.67	Berry
P.verrucosum	0.61	Zibib
A.sclerotioniger	0.00	Zibib
*A.niger*aggr.	0.00	Berry
*A.niger*aggr.	0.66	Berry
*A.niger*aggr.	0.00	Soil
A.westerdijkiae	0.40	Zibib
A.carbonarius	0.72	Zibib
P.verrucosum	0.64	Zibib
*A.niger*aggr.	0.64	Soil
P.verrucosum	0.00	Soil
A. sclerotiorum	0.00	Berry
A.ochraceus	0.63	Zibib
A.sclerotioniger	0.00	Zibib
A.ochraceus	0.23	Berry
A.carbonarius	0.64	Berry
*A.niger*aggr.	0.00	Zibib

Ochratoxin A was detected in two species of *Aspergillus* section *Nigri* (*A.carbonarius, A.niger*aggr.), two species of *Aspergillus* section *circumdati* (*A.ochraceus, A.westerdijkaii*) and one species of *Penicillium* (*P.verrucosum*). The percentage of OTA producing strains tested by ELISA technique were 100% for *A.carbonarius*, *A.ochraceus,* and *A.westerdijkiae*, while the percentages of ochratoxigenic potential in *A.niger*aggr. and *P.verrucosum*were 60% and 66.6% respectively (Table 2).

Table 2: Percentage(%) of ochratoxinA producing strains and range of OA detected

Species	No. of isolates tested	OA producing strains (%)	Range ng/ml.
A.carbonarius	6	100	0.67-0.72
A.japonicus	1	0	N.D
*A.niger*aggr.	5	60	0.64-0.66
A.ochraceus	3	100	0.23-0.65
A.sclerotioniger	2	0	N.D
A.sclerotiorum	1	0	N.D
A.westerdijkaii	1	100	0.40
P.verrucosum	3	66.6	0.61-0.64

N.D =Not detected

The reported percentage of the ochratoxigenic strains in the genera *Aspergillus* and *Penicillium* from different parts of the world is quit variable depending on the number of isolates studied and geographical regions. The ochratoxigenic potential of *A.carbonarius* strains have been tested by several authors (Serra *et al.,* 2005; Battilani *et al.,* 2003; Rocha *et al.*,2002). All these studies showed consistent ability of this species to produce OA with a percentage of ochratoxigenic potential of *A.carbonarius* strains ranging from 41.7 to 100%.

The reported percentage of ochratoxigenic isolates in *A.niger* by several authors is ranges from between 0.8 to 18.5% (5,24-26,28). However, De Rocha Rosa (Magnoli *et al*., 2003) reported that 30% of *A.niger* from grapes cultivated in Brazil was OA producers and Magnoli (Teren *et al.,* 1996) found an unusually higher percentage (43.1%) of ochratoxigenic strains in*A.niger* from grapes cultivated in Argentina.

The uniseriate black *Aspergillus japonicas* showed no ochratoxigenic producing ability (Table 1). This is in line with what is mentioned by literature (1, 30, 32). All the three isolates of *A.ochraceus* were positive for ochratoxin A producing ability. However, Serra (Serra *et al*., 2006) reported 50% of *A.ochraceus* strains isolated from Portuguese wine grapes were positive for OA producing abilities. A higher percentage of OA positive isolates among *A.ochraceus* has been reported by some authors in Argentina, Brazil and Spain (Magnoli *et al*., 2003; Hokobyan *et al*., 2010).

Ochratoxin A was not detected from isolates of *A.sclerotioniger* in present study. The species originally isolated from coffee beans proved to be ochratoxigenic (20) and was also isolated from dried vine fruits (Abdullah and Saadullah, 2012).

*A.westerdijkiae*is a member of *A.spergillus* section *circumdati* was originally isolated from *Andropogon sorghum* from South Africa and then it was found more common on coffee bean in several countries in the world (Samson *et al.,* 2004). The fungus is not very common on grapes; however, we have detected it in few occasions (4). Our isolate was previously reported as OA producer (Abdullah and Saadullah, 2012).

Out of 3 strains of *P.verrucosum*, two were positive for OA production. *P.verrucosum* is the major species producing OA in cereals such as wheat and barley in temperate and cold climate (Cabanes *et al.*,2010), however, the fungus was isolated from samples of dried vine grapes (zibib) purchased from Duhokmarket (Saadullah, 2011).

CONCLUSIONS

Based on our results, the incidence of ochratoxigenic strains of *Aspergillus*and *Penicillium* in soil of grapevine nurseries, grapevine berries and dried vine fruits represents special hazards to consumer health. Therefore, such contaminated commodities should be inspected for the presence of such fungi.

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