

Review Paper

1
Aspergillus niger as the Source of Ochratoxin A of Contaminated

3 *Pyrus communis* in Taif Market.

Abstract

5 Fruits are one of the most important agricultural products that supply the
body with vitamins and essential minerals elements, but it is attacked by fungi
during the period of growth, harvesting and storage. *A. niger* is one of the species
that grows on the fruit during the period of storage, and secretes mycotoxins
especially ochratoxin A. This study was concerned with the isolation and
definition of different strains of *A.niger* from 20 samples of pear collected from
Taif markets and the ability of these strains to form ochratoxin A. The results
showed that 19 samples of pear were contaminated with different strains of *A.*
niger. The study showed the ability of this strains to produce ochratoxin A. From
27 isolates of *A. niger* used to test the ability of production ochratoxin A, 10
strains only produced ochratoxin A. The range of OTA in all strains were 0.18 to
9.5 ppb. Representative 27 strains of ochratoxigenic and non ochratoxigenic black
aspergilli isolates were subjected for detection of ochratoxin biosynthesis genes,
by using two sets of primer for two genes involved in ochratoxin biosynthetic
pathway. Bands of the fragments of PKS15C-MeT and PKS15KS genes visualized
at 928 and 776 bp, respectively.

21 **Keywords:-** black aspergilla, ochratoxin A, *Pyrus communis*, DNA
Isolation, PKS15C-MeT and PKS15KS genes

Introduction

24 Fruits are commercially and nutritionally vital food. Fruits play a vital role
in human nutrition by supplied with the required growth factors as vitamins and
essential minerals, fats, and oil within the right proportion to take care of growth
and development on humans, daily diet maintaining a decent and traditional
health¹. Because of environmental condition, pests, inadequate downfall and fungi
attack, fruits and vegetable have serious challenges to their existence¹. Over the

Change to contaminated :[1T]Comment

This study was conducted with :[2T]Comment
the purpose of isolating and identifying different
strains of *A.niger* from 20 samples of pear collected
from Taif markets and to determine the ability of
these strains to form ochratoxin A.

It was observed that showed that :[3T]Comment
out of 20 pear samples collected, 19 samples were
detected to be contaminated with different strains of
A. niger and the strains were able to produce
ochratoxin A. From 27 isolates of *A. niger* which
was used to test the ability of production ochratoxin
A, 10 strains only produced ochratoxin A. The range
of OTA in all strains were 0.18 to 9.5 ppb.

isolated :[4T]Comment

yearly fungal caused several of disease like rot diseases that provoke severe losses of agricultural and horticultural crops each year^{2,3}. One of the most vital limiting factors that impact the economic value of fruits is the comparatively short shelf-life period caused by pathogens. About 20-25% of the harvested fruits are deteriorated by pathogens throughout post-harvest handling even in advanced countries^{4,5}.

Fungi are treated as an important post-harvest losses agent of many different fruits, depended on variety, season and production area amid alternative factors^{6,7}. Many of crop diseases are caused by fungi as the most crucial and common pathogens. Fungi colonized many of fruits and vegetables during storage and transportation⁸. Rotted fungi are considered biological agents that have ability to produce a wide range of enzyme, which able these fungi to colonize the fruit. Mould growth depends on several factors such pH, water activity (aw), temperature, atmosphere, time, etc⁹.

Aspergillus niger is a fungus and one the most widespread species of the genus *Aspergillus*. It causes a disease called black mold on fruits and vegetables similar to grapes, onions and peanuts and is a common contamination of food. *A. niger* is common in soil and many of environments. *A. niger* produce many of mycotoxins such as ochratoxin A^{10,11}, fumonisins B2, B4 and B6^{12,13,14}, as well as numerous other compounds with poorly investigated activities^{15,16}, such as hepatocarcinogenic, nephrogenic which are immunological in nature. In addition, this fungus is also causative agent for many rot diseases in plants¹⁷. Black *Aspergilli* (*Aspergillus* section Nigri) are useful in food mycology, medical mycology and biotechnology, often occurring in indoor environments^{18,19}. several species of fungi cause food spoilage, however are also utilized in the fermentation industry to provide varied enzymes and organic acids²⁰.

Isolates of *A.niger* have the ability to produce OTA, then, many concerns have arisen not only for their biotechnological safety but also for their food safety risk due to their common presence in numerous commodities^{21,22,23, 24}. OTA have properties of a potent nephrotoxin and has teratogenic, immunosuppressive and carcinogenic properties²⁵. OTA entry to humans and animals is caused by cereals

and cereal based food and feed which considered the main contributors, since OTA is stable under traditional food processing operation conditions and it is carried-over from raw materials to processed products²⁶. At recent days, mycotoxin issues has widened, there are many reports showed the ability of *A.niger* to produce fumonisin B2 (FB2) along with OTA^{26,27,28}. The International Agency for Research on Cancer classified OTA as a possible carcinogen to humans (group 2B)²⁵. Many varieties of food product within the markets are reported to be contaminated with OTA. These include tree nuts, peanuts, figs, melon seed, pumpkin seed, sesame seed, sunflower seed, lotus seed, corn seed, red pepper, white pepper, mixed spices, rice, corn, mixed cereals, chilies, and coconut²⁹.

This study aimed for the isolation and definition of different strains of *A.niger* from 320 samples of pear collected from Taif markets and the ability of these strains to form ochratoxin A.

Materials & Methods

Collection of samples:

Twenty samples showing rot symptoms of *Pyrus communis* (Pear) were collected from different markets and vendors in Taif city during October-December 2015 to isolate black aspergilli.

Isolation of black aspergilla

Isolation was performed by serial dilution technique³⁰, 10 g from *Pyrus communis* samples at the margin of diseased/ healthy tissue were removed and soaked in 100 ml sterilized distilled water that have been put in the shaking incubator for 30 min. Thereafter, 1ml aliquots from serial dilution were inoculated onto three plates containing malt extract agar medium (MEA) and then incubated at 27° C for 5-7 days and the developing fungi were counted and identified. At the end of the incubation period, colonies black aspergilli was counted and were conducted following calculations for account of isolates:

Determination of OTA ability of black aspergilli species isolates:

Ochratoxin-producing ability of the isolates was performed by cultivating black aspergilli in czapek yeast autolysate agar medium (CYA) (g/L; sucrose 30.00, sodium nitrate 2.00, magnesium glycerophosphate 0.50, potassium sulfate 0.35, potassium chloride 0.50, ferrous sulfate 0.01, agar- agar 15.00) supplemented with (5.0 g / L) yeast extract³¹ for 5 days at 27° C.

OTA was extracted by grinding the moldy agar (20 g) in blender for 1 min with methanol (100 ml) containing 0.5% NaCl. The mixture was then filtered through a fluted filter paper (24 cm), and the filtrate was diluted (1:4) with 1x 0.1% Tween PBS (Phosphate Buffered Saline) and refiltered through a glass-fiber filter paper. Two milliliters of the glass-fiber filtrate were placed on OchraTest columns (VICAM, Watertown, MA, USA) and allowed to elute at 1-2 drops/sec. The columns were washed two times with 10 ml of 1x 0.1% Tween PBS and 10 ml of Phosphate Buffered Saline (PBS), respectively. Then, OTA was eluted from the column with 1.5 ml OchraTest™ Eluting Solution and OTA concentration was read on a recalibrated VICAMSeries-4 fluorometer after 60 seconds.

Extraction of genomic DNA:

Mycelial cultures were harvested from potato dextrose broth (PDB) grown for 24 h in 10-ml tubes (3 ml of culture) or at 30° C (225 rpm) by filtering them through Whatman paper (Fisher Scientific, Inc., Pittsburgh, Pa.), washed according to the manufacturer's instructions, and then blotted dry.

DNA extraction was performed with an Epicentre kit but with a modification of the manufacturer's protocol. Approximately 200 mg of washed mycelia was added to a 1.7-ml micro centrifuge tube. The step involving grinding in liquid nitrogen was omitted; instead, 450 µl of yeast cell lysis solution and 1 µl of 150-µg/ml concentration of proteinase K were added to the tubes. The tubes were vortexed for 10 s, incubated in a 65° C heating block for 1 h, and then chilled on ice for 5 min. Next, 225 µl of protein precipitation reagent was added, and the tubes were vortexed for 5 s. The suspensions were then centrifuged at 20,800g for 10 min to pellet cellular debris. The supernatant (~500 µl) was transferred to a new tube, spun again to remove any residual cellular material, and then transferred to a new tube. An equal volume of isopropanol was added, and

the tubes were gently inverted several times to precipitate the DNA, which was then pelleted by centrifugation at 20,800g for 10 min. Pellets were washed with 70% ice-cold ethanol, centrifuged, and then vacuum dried. DNA was resuspended in 100 to 100 µl of Tris-EDTA and then treated with 2 µl of a 5-mg/ml concentration of RNase A at 65° C for 1 h³². Finally, the DNA quantity and quality were checked by electrophoresis on a 0.8% agarose gel, revealed with ethidium bromide and visualized by UV trans-illumination.

Molecular detection of OTA biosynthetic genes in ochratoxigenic species of black Aspergelli:

Two published primer sets were used for the specific detection of two OTA genes.

The first one, denoted PKS15C-MeT (5'GCTTTCATGGACTGGATG and 5'CAATTCGTTGATCCCATCG). Reactions were incubated for 2 min at 95° C, followed by 35 cycles of 45s at 94° C, 50s at 62° C and 1 min at 72° C. Amplification cycles finished with 5 min incubation at 72 °C. Expected Results: Amplification ~SIZE 998 bp only on positive strains.

The second pair, named PKS15KS (5'CAATGCCGTCCAACCGTATG and 5'CCATCGCCTCGCCCGTAG). Reactions were incubated for 4 min at 94° C, followed by 35 cycles of 45 s at 94° C, 50 s at 60° C and 1 min at 72° C. Amplification cycles finished with 5 min incubation at 72° C. Expected Results: Amplification ~SIZE 776 bp only on positive strains.

Results

Three species belonging to black Aspergelli were isolated and identified from *Pyrus communis* fruit on MEA medium at 27° C (Table, 1 and 2).

The total counts of fungi from *Pyrus communis* fluctuated between 0-27 isolates with the highest count being estimated in samples number 14 (27 isolates), while the lowest number of isolates were recovered from samples number 3, 6, 7, and 8 (1 isolate), whereas sample number 4 not contaminated by this species (Table, 2). According to the average total counts (ATC) of all black Aspergilli collected from 20 *Pyrus communis* fruit samples, *A.niger* was the most

common species, which recovered from 70% of the samples, matching 73.4% of total black aspergilli. In the individual sample the count of this species ranged from 5-23 colonies and the highest count was observed in sample no. 14 (Tables, 1 & 2).

Quantitative determination of OTA:

All black aspergilli species collected from the investigated samples represented with single isolate from each sample of *Pyrus communis* fruits collectively were tested for OTA potentials. It was detected at varying degrees and estimated by part per billion.

Table (3) showed the results of OTA production, where only two well known ochratoxigenic species were detected (*A. niger* and *A. tubingensis*).

Among isolates of black aspergilli, the ranges of OTA in all strains were 0.18-9.5 ppb. *A. niger* (SNM7 strain) showed the highest level of OTA (9.5 ppb) and *A. niger* (SNM22 strain) showed the lowest level of OTA (0.18 ppb). The production level of OTA from *A. niger* (SNM15 strain), (SNM19 strain), (SNM20 strain) and (SNM25 strain) were 2.4, 2.5, 1.2, and 0.95 ppb, respectively. And the production level of OTA from *A. tubingensis* (SNM13 strain), (SNM16 strain), (SNM17 strain) and (SNM26 strain) were 0.84, 1.2, 0.3 and 0.65 ppb, respectively.

Whereas, OTA disappeared in all *A. awamori* isolates (SNM3 strain), (SNM8 strain), (SNM10 strain) and (SNM18 strain). Also, OTA disappeared from 12 isolates (SNM 1, 2, 4, 5, 9, 11, 12, 14, 21, 23, 24 and 27) of *A. niger* and one isolate (SNM6) of *A. tubingensis*.

Detection of some of OTA biosynthesis genes:

Representative 27 strains of ochratoxigenic and non ochratoxigenic black aspergilli isolates were subjected for detection of ochratoxin biosynthesis genes.

Polymerase chain reaction (PCR) was applied using two sets of primer for two genes involved in ochratoxin biosynthetic pathway. Bands of the fragments of PKS15C-MeT and PKS15KS genes visualized at 998 and 776 bp, respectively (fig. 1). 180

181 Table (4) explained the total ochratoxin and ochratoxigenic genes
(PKS15C-MeT and PKS15KS) detected in 27 strains of ochratoxigenic black
182 aspergilli isolates collected from *Pyrus communis* samples. From those 27 strains,
183 *A. niger* (SNM7, and 19), and *A. tubingensis* (SNM16, and 26) contained the two
184 OTA biosynthesis genes. But, *A. niger* (SNM20, and 22) contained only PKS15C-
185 MeT gene, while *A. niger* (SNM15, and 25) and *A. tubingensis* (SNM17)
186 contained only PKS15KS gene. On the other hand, 17 non ochratoxigenic strains
187 showed no bands, which means that, there is deletion in targeted genes in this
188 isolate. All *A. awamori* strains (SNM3, 8, 10, and 18), *A. niger* strains (SNM1, 2,
189 4, 5, 11, 12, 14, 21, 23, 24, and 27), and *A. tubingensis* strain (SNM6) showed
190 no bands.

191 Discussion

192 Three species belonging to black Aspergilli were isolated and identified from
193 *Pyrus communis* fruit on MEA medium at 27° C. *A. niger* was the most common
194 species, which recovered from 70% of the samples, matching 73.4% of total black
195 aspergilli. In the individual sample the count of this species ranged from 1-23
196 colonies and the highest count was observed in sample no. 14. *A. niger* var. *niger*
197 and *Aspergillus niger* var. *awamori* were isolated in highest frequency from black
198 dried pomegranate fruits on DRBC and DG18 media. Where OTA was found in 74% of
199 the dried vine fruits samples. *A. carbonarius* occupied the first place in the
200 production of OTA, were detected (82.6%). Followed by *Aspergillus* section
201 Nigri so sixty two strains (28 %) have the ability to produce OTA³³. The
202 pomegranate trees are not affected by any serious disease however the fruit are
203 often damaged by heart rot caused by different species fungi and bacteria or after
204 invasion of the insect. Twenty-six samples of splitting pomegranate fruits from
205 different garden close to Cairo, Egypt were examined, and they showed that they
206 contained a reproductive structure of genus *A. niger* which can reach the guts of the
207 fruit throughout the period of growth until harvest the mature fruits³⁴.

208 Among isolates of black aspergilli, the ranges of OTA in all strains were
209 0.18-9.05 ppb. *A. niger* (SNM7 strain) showed the highest level of OTA (9.5 ppb)
210 and *A. niger* (SNM22 strain) showed the lowest level of OTA (0.18 ppb).

Whenas, OTA disappeared in all *A. awamori* isolates (SNM3 strain), (SNM8 strain) (SNM10 strain) and (SNM18 strain). Also, OTA disappeared from 12 isolates (SNM 1, 2, 4, 5, 9, 11, 12, 14, 21, 23, 24 and 27) of *A. niger* and one isolate (SNM6) of *A. tubingensis*.

Fungi producing OTA in Portuguese wine grapes, a survey was conducted in 17 vineyards, from winemaking regions every with different climatic conditions. They isolated 370 strains of *Aspergillus* and 301 strains of *Penicillium* from 650 samples of barriers, the study showed 14% of the aspergilli were OTA-producing strains. None of the penicillia were OTA-producing strains. The black aspergilli were predominant (90%). 97 % of black aspergilla were *Aspergillus carbonarius* and 3% of the *Aspergillus niger* collected in this study were OTA producers³⁵.

many species of fungi were isolated from five grape varieties grown in Spain. The most fungal genera isolated were *Alternaria*, *Cladosporium*, and *Aspergillus*. The study showed that 82% *Aspergillus* sp. section Nigri were OTA-producing strains, was assessed using yeast extract-sucrose broth supplemented with 5% bee pollen. Cultures of 205 isolates from this section appeared that 74.2% of *Aspergillus carbonarius* and 14.3% of *Aspergillus tubingensis* isolates produced OTA ranging from 1.2 to 3,530 mg/ml and from 46.4 to 111.5 mg/ml, respectively. No *Aspergillus niger* isolate had the ability to produce this toxin under the conditions assayed³⁶.

Onion bulbs will become contaminated with different species of fungi during the storage period, *Aspergillus* section Nigri was the most important causal agents. standard morphological methods was not enough to identify black Aspergilli, because this group of *Aspergillus* contain many species that cannot be reliably identified by standard morphological methods. Therefore, it's necessary to identify the fungus inflicting this problem within the onion specifically, particularly since some species of section Nigri are well-known as ochratoxin and fumonisin producers. Sixty fungal isolates belonging to 10 fungal genera were isolated from 40 onion samples collected from the Taif region in Saudi Arabia from 40 samples of onion, Black aspergilli were isolated from 37 onion

243 samples. Using primer pairs (awaspec and Cmd6) designed based on partial
244 calmodulin gene sequence data, 37 isolates were identified as *A. welwitschiae* (*A.*
245 *awamori*). The ochratoxin A and fumonisin B2 contents of the onion samples were
246 examined. No ochratoxins were detected within the collected samples, whereas
247 fumonisin B2 was detected in 37.5 % of the onion samples. Eighteen of thirty
248 seven isolates of genus *Aspergillus welwitschiae* were recognized as potential
249 producers for fumonisin B2. Multiplex polymerase chain reactions designed to
250 detect biosynthetic genes of fumonisins confirmed these results³⁰.

251 the total ochratoxin and ochratoxigenic genes (PKS15C-MeT and
252 PKS15KS) detected in 27 strains of ochratoxigenic black aspergilli isolates
253 collected from *Pyrus communis* samples. From those 27 strains, *A. niger* (SNM7,
254 and 19), and *A. tubingensis* (SNM16, and 26) contained the two OTA biosynthesis
255 genes. But, *A. niger* (SNM20, and 22) contained only PKS15C-MeT gene, while *A.*
256 *niger* (SNM15, and 25) and *A. tubingensis* (SNM17) contained only PKS15KS
257 gene. On the other hand, 17 non ochratoxigenic strains showed no bands, which
258 meant that there is deletion in targeted genes in these isolates. All *A. awamori*
259 strains (SNM3, 8, 10, and 18), *A. niger* strains (SNM1, 2, 4, 5, 9, 11, 12, 14, 21,
260 23, 24, and 27), and *A. tubingensis* strain (SNM6) showed no bands. According to
261 the results of³⁷ study, the aflatoxigenic species of *Aspergillus* has been shown to
262 vary their aflatoxin potentials with the substrate and environmental factors.
263 Whereas, the presence of four tested genes is not sufficient marker for
264 differentiation between aflatoxigenic and non aflatoxigenic isolates.

265 OTA-nonproducing isolates of *A. niger* and *A. welwitschiae* (*A. awamori*)
266 species lacked the ochratoxin A biosynthetic gene (OTA) cluster, analysis of
267 gene sequence data revealed a single pattern of OTA gene deletion in the two
268 species. Phylogenetic analysis suggests that the simplest explanation for this is that
269 OTA cluster deletion occurred in a common ancestor of *A. niger* and *A.*
270 *welwitschiae*, and subsequently both the intact and deleted cluster were retained as
271 alternate alleles during divergence of the ancestor into descendent species. When
272 comparing their results with previous studies indicated that a minority of isolates
273 of both species produce OTA. also, suggested that the relative abundance of each

spec~~274~~ and frequency of OTA-producing isolates can vary with crop and/or
geog~~275~~raphic origin³⁸.

Con~~276~~clusion

277 In this study, black Aspergilli was isolated from 20 samples of *Pyrus*
con~~278~~*com~~279~~munis*. Most of the samples showed to be contaminated with black Aspergilli.
The~~280~~ common black Aspergilli is *A.niger*. In detection of the ability of these fungi
to p~~281~~roduce ochratoxin A, some of them have the ability to form ochratoxin A, the
pro~~282~~duction of these toxins is linked to the presence of one or more genes.

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UNDER PEER REVIEW

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Table 1: Counts (as colonies in every sample) of *Aspergillus* section Nigri recorded from 20 *Pyrus communis* fruit on MEA medium at 27° C.

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Species	Samples																				Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
<i>A. awamori</i>	2	0	0	0	2	0	1	0	0	0	0	0	0	0	1	1	0	4	0	11	
<i>A. niger</i>	10	10	1	0	0	1	0	1	8	0	1	6	13	23	6	11	0	7	0	102	
<i>A. tubingensis</i>	0	0	0	0	0	0	0	0	3	2	1	5	4	4	3	2	2	0	0	26	
Gross total count	12	10	1	0	2	1	1	1	11	2	2	11	17	27	9	14	3	7	4	139	

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Table 2: Average total counts (ATC, calculated per g fresh fruit in all samples), percentage counts (%C, calculated per *Aspergillus* section Nigri), percentage frequency (%F, calculated per 20 samples), number of cases of isolation (NCI, out of 20 samples) and occurrence remarks (OR) of various fungal species collected from *Pyrus communis* fruit samples on MEA medium at 27° C.

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Species	ATC	C%	NCI	OR	F%
<i>A. awamori</i>	36.7	7.9	6	M	30
<i>A. niger</i>	340	73.4	14	H	70
<i>A. tubingensis</i>	86.7	18.7	9	M	45
Total count	463.4	100			

Occurrence remarks: OR (out of 20 samples), H= high occurrence from 10-20 cases, M= moderate occurrence from 5-9 cases, L= low occurrence from 2-4 cases and R= rare occurrence 1 case.

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457(3): Total Ochratoxin A (PPB) produced by different black aspergilli
458 species isolated from different *Pyrus communis* samples in CYA medium at 27° C
459 for 7 days.

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No.	Strain code	Species	OTA level (PPB)
1	SNM3	<i>A. awamori</i>	Nd
2	SNM8	<i>A. awamori</i>	Nd
3	SNM10	<i>A. awamori</i>	Nd
4	SNM18	<i>A. awamori</i>	Nd
5	SNM1	<i>A. niger</i>	Nd
6	SNM2	<i>A. niger</i>	Nd
7	SNM4	<i>A. niger</i>	Nd
8	SNM5	<i>A. niger</i>	Nd
9	SNM7	<i>A. niger</i>	9.5
10	SNM9	<i>A. niger</i>	Nd
11	SNM11	<i>A. niger</i>	Nd
12	SNM12	<i>A. niger</i>	Nd
13	SNM14	<i>A. niger</i>	Nd
14	SNM15	<i>A. niger</i>	2.4
15	SNM19	<i>A. niger</i>	2.5
16	SNM20	<i>A. niger</i>	1.2
17	SNM21	<i>A. niger</i>	Nd
18	SNM22	<i>A. niger</i>	0.18
19	SNM23	<i>A. niger</i>	Nd
20	SNM24	<i>A. niger</i>	Nd
21	SNM25	<i>A. niger</i>	0.95
22	SNM27	<i>A. niger</i>	Nd
23	SNM6	<i>A. tubingensis</i>	Nd
24	SNM13	<i>A. tubingensis</i>	0.84
25	SNM16	<i>A. tubingensis</i>	1.2
26	SNM17	<i>A. tubingensis</i>	0.3
27	SNM26	<i>A. tubingensis</i>	0.65

461 Nd: not detected with the limit of detection

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Table (4): Total ochratoxin A and ochratoxigenic genes (PKS15C-MeT and PKS15KS) detected in 27 strains of black aspergilli isolates collected from pear samples.

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No.	Strain code	Total OTA genes	PKS15C-MeT	PKS15KS
1	SNM1	-	-	-
2	SNM2	-	-	-
3	SNM3	-	-	-
4	SNM4	-	-	-
5	SNM5	-	-	-
6	SNM6	-	-	-
7	SNM7	+	+	+
8	SNM8	-	-	-
9	SNM9	-	-	-
10	SNM10	-	-	-
11	SNM11	-	-	-
12	SNM12	-	-	-
13	SNM13	+	+	+
14	SNM14	-	-	-
15	SNM15	+	-	+
16	SNM16	+	+	+
17	SNM17	+	-	+
18	SNM18	-	-	-
19	SNM19	+	+	+
20	SNM20	+	+	-
21	SNM21	-	-	-
22	SNM22	+	+	-
23	SNM23	-	-	-
24	SNM24	-	-	-
25	SNM25	+	-	+
26	SNM26	+	+	+
27	SNM27	-	-	-

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+ Presence
- Absence
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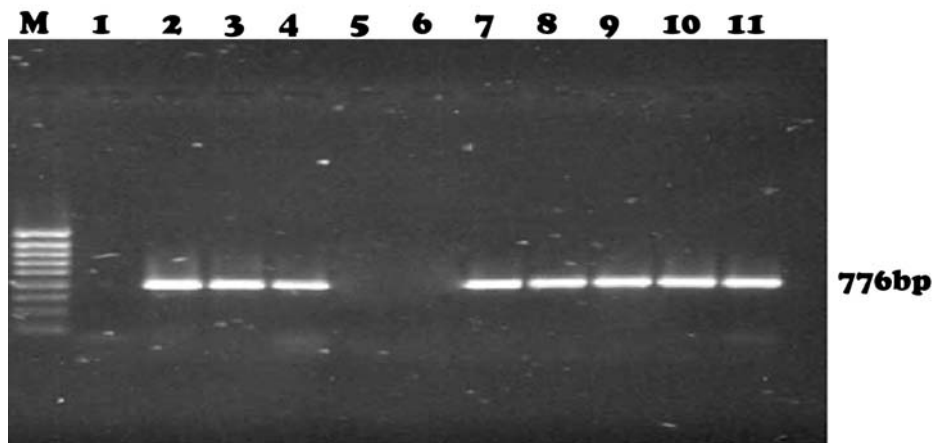
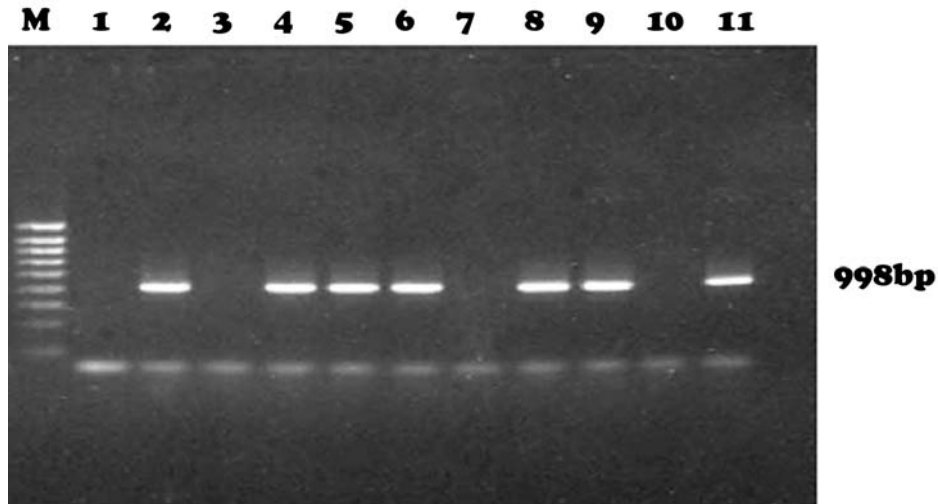


Fig 471): Ochratoxin biosynthesis genes amplifications. (A). PKS15C-MeT gene and (B). PKS15KS gene. M, DNA marker; Lane 1, negative control; Lanes 2-7, *A. niger* (SNM 7, 15, 19, 20, 22, and 25); Lanes 8-11, *A. tubingensis* (SNM 13, 16, 17, and 26).

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