Biotechnol Res 2019; Vol 5(1):1-4
eISSN 2395-6763

ORIGINAL RESEARCH

Isolation and Identification of a Fungus Infecting Egyptian wheat grains Gamiza11 *Triticum aestivum*

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• Received: 28 November 2018 • Revised: 16 December 2018 • Accepted: 30 December 2018 • Published: 24 February 2019 •

ABSTRACT
The *Penicillium* species are among the most commonly occurring and economically important members of micro-fungi family. In this study, Molecular identification of fungal isolates were used to group and determine genetic variability of *Penicillium* isolate from Egyptian Gamiza11 wheat grains *Triticum aestivum* were obtained from Agricultural Administration in Menouf, Governorate of Menoufia, Egypt. The fungal DNA was then sent to SolGent Company, Daejeon South Korea for polymerase chain reaction (PCR) and gene sequencing. PCR was performed using two universal fungal primers ITS1 (forward) and ITS4 (reverse) which were incorporated in the reaction mixture. Molecular analysis also indicated genotype variability between the isolates with little correlation with either the origin of soil or geographical location.

.**KEY WORDS:** *Penicillium verrucosum, Ochratoxin A, fungal isolation, PCR*

INTRODUCTION

Food is the fuel of life, and we are all concerned about the quality and safety of our food (Gratz, 2007).Harmful components in plant derive foods can be either produced by the plant itself or are contaminants deriving from manmade sources or from microorganisms. Among these microorganisms, toxin producing fungi are ubiquitous in the environment and can invade our crops and produce toxin secondary metabolites known as “Mycotoxins” (Gratz, 2007).

In principle, all species of *Aspergillus* and *Penicillium* and indeed many other types of fungi can cause health hazard because of the potential of their spore to act as allergens to those suffering from hay fever or asthma. Further, many species of *Aspergillus* and *Penicillium* produce mycotoxins (Lewis *et al*., 2003)

Mycotoxins are a large group of secondary metabolic products from fungi, or molds, which pose serious risks for human and animal health (Zain, 2011). Fungal growth and mycotoxin production may occur in the field and/or during storage, under suitable temperature and humidity conditions (Bryden, 2012). Mycotoxin contamination occurs widely in feedstuffs of plant origin, especially in cereals, fruits, hazelnuts, almonds, seeds, fodder, and other agricultural feed or food intended for animal or human consumption .It is also worth noting that human exposure to mycotoxins may be caused by not only consumption of plant-derived foods contaminated with toxins, but also the carry-over of mycotoxins and their metabolites in animal products, such as animal tissues, milk and eggs (Stefani and Bérubé,2006).

Moreover, mycotoxins lead to huge economic losses annually, including loss of human and animal life, loss of livestock production, loss of for age crops and feeds, and so on (Robinson *et al*.,2015)).The aim of this study Molecular identification of fungal isolates were determined genetic variability of *Penicillium* isolate from Egyptian Gamiza11 wheat grains *Triticum aestivum* were obtained from Agricultural Administration in Menouf, Governorate of Menoufia, Egypt.

RESULTS AND DISCUSSION

Isolated fungi

The isolated fungus was showed in fig 1.

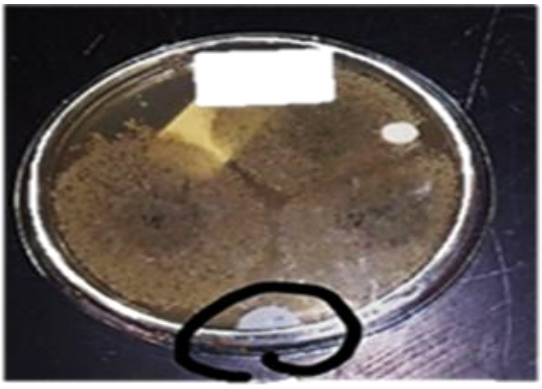

Figure 1: The original sample. The circled fungus was isolated and purified

A Rapid Identification Method using ammonia Vapor

The color change was detected after two days incubation and increased with time, the maximum intensity being observed at third day (fig 2). The color change of the colony reverse to pink by ammonia vapor occur immediately after the ammonia vapor contacted with colony (no specific medium is not necessary for culturing fungi)

Figure 2: Isolated fungus before and after exposing to ammonia vapor

Molecular identification of fungal isolates

Sequences of rDNA of Sample-1 (AUMC-13317) using ITS1 forward primer

GGGAGGCTCTGGGTCCACCTCCCACCCGTGTTTATTTT
ACCTTGTTGCTTCGGCGGGCCCGCCTTAACTGGCCGCC
GGGGGGCTTACGCCCCCGGGCCCGCGCCCGCCGAAG
ACACCCTCGAACTCTGTCTGAAGATTGTAGTCTGAGTGA
AAATATAAATTATTTAAAACTTTCAACAACGGATCTCTTG
GTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAC
GTAATGTGAATTGCAAATTCAGTGAATCATCGAGTCTTT
GAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCAT
GCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTT
GTGTGTTGGGCCCCGTCCTCCGATCCCGGGGGACGGG
CCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGA
GCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCC
GGCGCTTGCCGATCAACCCAAATTTTTATCCAGGTTGAC
CTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATA
TCAATAAGCGGAGGAT

Sequences of rDNA of Sample-1(AUMC-13317) using ITS4 reverse primer

ACTGGCATCTACTGATCCGAGGTCACCTGGATAAAAATT
TGGGTTGATCGGCAAGCGCCGGCCGGGCCTACAGAGC
GGGTGACAAAGCCCCATACGCTCGAGGACCGGACGCG
GTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGGATC
GGAGGACGGGGCCCAACACACAAGCCGTGCTTGAGGG
CAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATAC
CAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCA
CTGAATTTGCAATTCACATTACGTATCGCATTTCGCTGC
GTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGA
AAGTTTTAAATAATTTATATTTTCACTCAGACTACAATCTT
CAGACAGAGTTCGAGGGTGTCTTCGGCGGGCGCGGGC
CCGGGGGCGTAAGCCCCCCGGCGGCCAGTTAAGGCGG
GCCCGCCGAAGCAACAAGGTAAAATAAACACGGGTGGG
AGGTTGGACCCAGAGGGCCCTCACTCGGTAATGATCCT
TCCGCAGGTTCACCCCTACGGAAG

Sample1 showed 99.8 % similarity with *Penicillium chrysogenum*, *Penicllium crustosum* and *Penicillium verrucosum*. Phylogenetic tree of 18S was showed in fig 3. Further studies are required to achieve the perfect identification e.g. toxin profile, culture pigmentation, macro- and microscopical variations

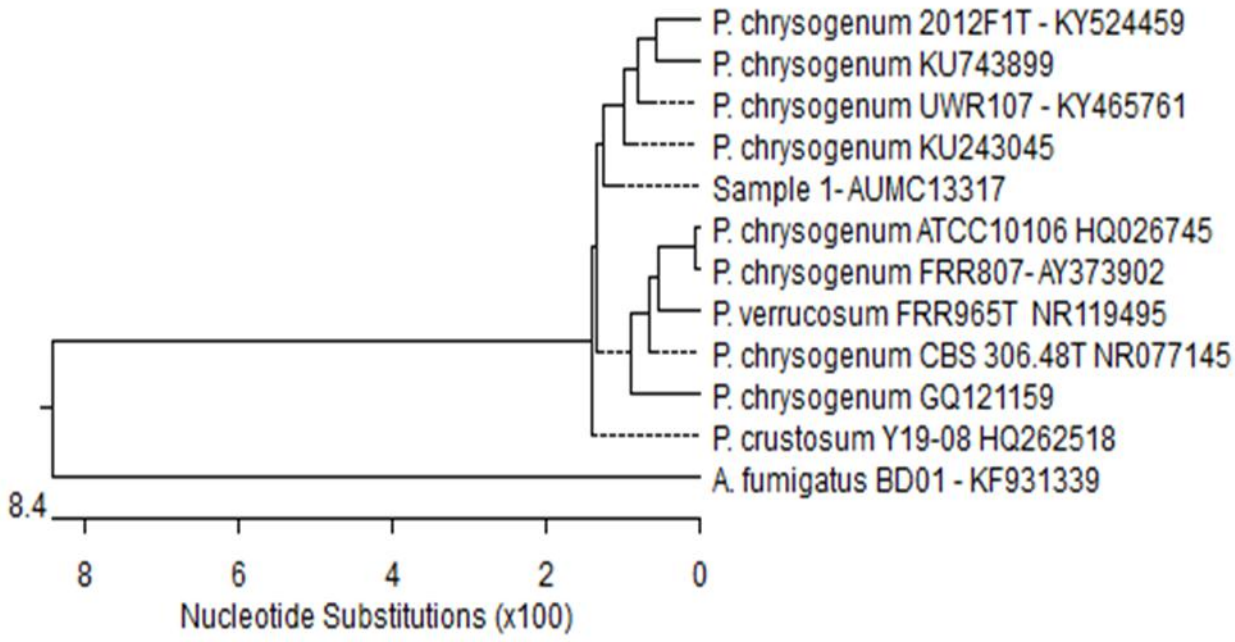

Figure 3: Phylogenetic tree of 18S (rDNA covering ITS1 and ITS2) sequences of the fungal strains isolated in the present study (Sample 1- AUMC13317) aligned with closely related sequences accessed from the GenBank. (P = *Penicillium*). *Aspergillus fumigatus* is included as an outgroup strain

MATERIAL AND METHODS

Wheat Sample:

Egyptian Gamiza11 wheat grains *Triticum aestivum* were obtained from Agricultural Administration in Minuf Governorate of Menoufia, Egypt.

Microbiological media

Potato Dextrose Agar (PDA):

Potato Dextrose Agar is composed of dehydrated Potato Infusion and Dextrose that encourage luxuriant fungal growth. Agar is added as the solidifying agent (Mishra *et al.,* 1994).

Nutrient agar (N A):

Medium was prepared as described in Difco's Manual (1984).

Isolation of the Toxic Fungi

100 gm of Gamiza11 wheat grains (*Triticum aestivum* the most widely grown is common wheat) were obtained from Agricultural Administration in Minuf Governorate of Menoufia, Egypt.

A sample of 5 gm from the wheat grains were added to 15 ml Potato Dextrose Broth (PD Broth) test tube then incubated for 7 days at 28 ºC. One ml of the suspension was transferred after well shacking to a petri dish contains Potato Dextrose Agar (PDA). Re-transfer on Potato Dextrose Agar (PDA) petri dishes and purified until we get pure strain.

The isolated fungus exposed to a rapid identification method by Ammonia Vapor (Saito *et al* 1999). The isolated fungus was inoculated at the center of Nutrient Agar medium in 10 Centimeter (Cm) glass petri dish and incubated at 28ºC for 7 days. To observe the color change of colony reverse after incubation, dish was placed upside down and a drop of 0.2 milligrams (ml) of 25% ammonia solution was put into the lid of petri dish. The color change was checked daily with plates incubated for one-week (1wk) 28 ºC. The isolated fungus was identified at The Central Factories of the Egyptian Armed Forces using (Maldi - Tof) that showed its genus is *Penicillium*. The isolate was confirmed at Assiut University Mycological Centre (AUMC), Egypt.

Molecular identification of fungal isolates:

The fungal strain was cultivated on Potato Dextrose Agar (PDA) medium at 28° Celsius for five days.

A small amount of the fresh culture was scraped and suspended in 100µl autoclaved distilled water in a sterile Eppendorf vial (2ml capacity) and boiled in a water bath at 100° Celsius for 15 minutes. The non-living fungal strain was sent to the Molecular Biology Research Unit, Assiut University, Egypt for DNA extraction using patho gene-spin DNA/RNA extraction kit provided by Intron Biotechnology Company, Korea. The fungal DNA was then sent to SolGent Company, Daejeon South Korea for polymerase chain reaction (PCR) and gene sequencing.

Polymerase chain reaction (PCR) was performed using two universal fungal primers ITS1 (forward) and ITS4 (reverse) which were incorporated in the reaction mixture. Primers have the following composition:

ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3').

The purified PCR product (amplicon) was reconfirmed using a size nucleotide marker (100 base pairs) by electrophoreses on 1% agarose gel. Then these bands were eluted and sequenced with the incorporation of dideoxynucleotides (dd NTPs) in the reaction mixture. Each sample was sequenced in the sense and antisense directions using ITS1 and ITS4 primers (White *et al*., 1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315-322 In: PCR Protocols: (Gelfand, 2012)

Sequences were further analyzed using Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done with the help of MegAlign (DNA Star) software version 5.05. (White *et al* 1990)

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