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Detection of Mycophages Associated with Fungal Strains Isolated from Soil of KSA and Identified *via* 18S rRNA Gene.

Sonya Mohamed[a,b], Mubarak Al-Yami[a], and Atef Sadik[a,c]*.

[a]Department of Biology, Faculty of Science, Taif University, Taif, Al-Haweiah, P.O. Box 888, Zip code 21974, Taif, KSA
[b]Department of Agricultural Microbiology, Soil, Water and Environmental Research Institute, Agricultural Research Center, P.O. Box, 12619, Giza, Egypt
[c]Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, P.O. Box 68, Hadayek Shobra 11241, Cairo, Egypt

ABSTRACT

Fungi are an important part of the Eco world system we live in. Viruses in fungi probably have existed for a long time but they were discovered only recently. This study was designed to isolate and identify some mycophages from some soils collected from different regions of KSA and could be considered as the first record in KSA. A number of six soil samples from different regions (Taif, Jeddah, Makkah) in KSA were collected and subjected to physical, chemical and microbiological analyses. A number of ten fungal isolates were selected, highly purified, and then their cultural and morphological (*via* light microscopy) characters were determined. The nucleotide sequences of the 18S rDNA gene of these fungal isolates was also determined. The presence of viruses (mycophages) in the mycelia of the identified fungal isolates was detected *via* electron microscopy for the partially purified viruses preparation. The highest fungal total count was found in soil sample (JG1) from Jeddah followed by soil (MH1) from Makkah, soil sample (TA1) from Taif. Cultural charcters and light microscopy revealed that the ten fungal isolates were belonging to the following species: *F. oxysporum* (two isolates), *F. solani* (one isolate), *Tricoderma* sp. (one isolates), *Alternaria alternate* (one isolate), *Aspergillus niger* (one isolate), *Penicillium commune* (two isolates), *P. polonicum* (one isolate), and *P. expansum* (one isolate). The lengths of 18S rRNA gene of ten fungal isolates were ranged from 480 to 680 nts except for the *P. expansum* isolate which showed approximately full lenthg of 1568 nts. The similarities between the fungal isolates and those similar in GenBank were ranged from 99 to 100% and confirmed the biological identification of the isolates under investigation as aboveformentioned. Electron microscopy of the virus suspensions prepared from the mycelia of the ten identified strains showed the presence of spherical or isometric virus-like particles in the virus suspension of *Penicillium polonicum* strain only. As a conclusion, combination of cultural growth, light and microscopy and use of 18S rRNA gene were more than effective in identification of the fungal strains. On the other hand, mycoviruses could be considere as hidden particles in the mycelia with low concentrations.

Keywords: Viruses, 18S rDNA gene, Light microscopy, Fungi, KSA, mycoviruses.

Corresponding author

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INTRODUCTION

Mycoviruses (fungal viruses) have been recorded to be widespread in the major taxonomic groups of filamentous fungi, yeasts, and oomycetes [1]. Such fungal-virus systems are valuable for the development of novel biocontol strategies and for gaining an insight into the molecular basis of fungal virulence [2,3]. In many cases, mycovirus infection does not cause any visible abnormal symptoms for host fungi (cryptic infections). However, infection by some mycoviruses can cause abnormal symptoms in their host fungi, including reduced mycelial growth and debilitated virulence [4]. Macroscopic symptoms caused by fungal viruses, e.g., reduction in growth, reductions in pigmentation and sporulation, excessive sectoring, and aerial mycelial collapse, are a consequence of alterations in complex physiological and biochemical processes involving interactions between host and virus factors [5]. Most mycoviruses have RNA genomes, which are either ss or ds, and are now classified into 12 families [2]. Ghabrial *et al*. [3] revealed that mycoviruses were widespread in all major taxa of fungi. They were transmitted intracellularly during cell division, sporogenesis, and/or cell-to-cell fusion (hyphal anastomosis), and thus their life cycles generally lack an extracellular phase. Their natural host ranges were limited to individuals within the same or closely related vegetative compatibility groups. Most known mycoviruses have dsRNA genomes packaged in isometric particles. Kaya *et al*. [6] reported that mycoviruses were common obligate parasites in many organisms including plant pathogenic fungi. Over 80 mycovirus species have been officially recognized from ten virus families [1]. Mycoviruses have been reported in *Botrytis cinerea* [7,8,9], *Alternaria longipes* [10], *Aspergillus fumigatus* [11,12], *Aspergillus foetidus* [13], *Aspergillus ochraceus* [14], *Verticillium alboatrum* [15], *Rosellinia necatrix* [16], *Sclerotinia sclerotiorum* [17,18], *Fusarium virguliforme* [19], *Fusarium graminearum* [20,21], *Fusarium poae* [22], *Rhizoctonia solani* [23], *Helminthosporium victoria* [24], *Saccharomyces cerevisiae* [8], *Phytophthora infestans* [25], *Penicillium stoloniferum* [14], *Penicillium species* [26] and *Leucostoma persoonii* [27]. Ghabrial and Suzuki [3] reported that although the majority of known mycoviruses have dsRNA genomes that are packaged in isometric particles, an increasing number of usually unencapsidated mycoviruses with positive-strand RNA genomes have been reported. Transmission electron microscopy of mycoviruses-infected fungal mycelia showed that fungal viruses were isometric particles and ranging from 23 to 50 nm in diameter [27,28,29,30].

Therefore, the objective of this study is to isolate and identify some fungal strains from some soils collected from different regions of KSA followed by detection of mycophages associated with the fungal isolates.

MATERIALS AND METHODS

Isolation and purification of some fungal isolates

Some soil samples were collected from Taif, Jeddah, Makkah in sterile pages as described by Mohamed [31] for isolation of some fungi and their mycophage(s). The fungal soil-contaminants were isolated on potato glucose agar medium [32]. The purified cultural fungal isolates were sent to The Clinical Plant Center, Department of Plant Pathology, Faculty of Agriculture, Ain Shams University, Cairo, Egypt, for highly purification and then identification up to genus and/or species based on their cultural growth and morphological charaters. Maintenance of the stock fungal isolates was carried out as described by Wu *et al*. [6]. Mycelial agar plugs (6 mm in diameter) was removed from the colony margin of a 3- to 5-day-old culture of each isolate and were placed on rose bengal agar medium in Petri dishes (9 cm in diameter) at one plug per dish. The dishes were incubated at 25-28°C for determination of the mycelial growth rate and for observation of the colony morphology. The fungal mycelia, spores, and other tiny features of fungi you were examined using a microscope capable of at least x 400 magnification [33].

PCR isolation of 18S rDNA gene of the isolated fungal isolates

Fungus and Mold genomic DNA samples were extracted using a InstaGenetm Matrix(BIO-RAD). Both of NS1 primer (5'GTAGTCATATGCTTGTCTC3') and NS8 primer (5'TCCGCAGGTTCACCTACGGA3') were used for the PCR. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30 µl reaction mixture by using a EF-*Taq* (SolGent, Korea) as follows:

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Files	Title	(°C)	Minutes	Cycles
File 1	Denaturation (Activation of *Taq* polymerase)	95	2	1
File 2	Denaturation	95	1	35
	Annealing	55	1	
	Elongation	72	1	
File 3	Final step	72	10	1

Sequencing of 18S rDNA gene of the fungal isolates and its analysis

The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was then incubated at 95°C for 5 min, followed by 5 min on ice. Nucleotide sequences were determined on both strands of PCR amplification products at the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea). NCBI blast tool was used for blast search result. DNA sequence was analyzed using BLASTN 2.2.23+software (http://www.ncbi.nlm.nih.gov/blast/) against the isolates/strains collected from the database for genotyping. The sequence that showed the lowest e-value and maximum identity was considered as the genotype of the sample analyzed.

Detection of mycophage(s) associated with the fungal isolates

Fungal mycelium (20 g wet weight) was added to 40 ml 0.1 M phosphate buffer (pH 7.0) and homogenized three times for five min each. Cell debris was removed by centrifugation at 5,000 rpm for 20 min. The supernatant was overlaid on the top of a 25 % sucrose cushion in a ultracentrifuge tube. Then the tubes were centrifuged at 23,000 rpm using SW 28 rotor for 2 h at 4^{o}C. The pellet was re-suspended in 500 µl of 0.02 M phosphate buffer (pH 7.0). Virus suspension was filtrated with Amicon-100 filter to remove soluble biological macromolecule fragments of host fungi. After washing three times with 0.1 M ammonium acetate solution (pH7.0), the retained mycophage solution was used directly for negative staining as described by Nugent and Cole [34]. Photographs were taken with a JEOL101 transmission electron microscope operating at 100 kV.

RESULTS AND DISCUSSION

Fungi are an important part of the Eco world system we live in. They cause diseases in man, animal, and plants. But some fungi also serve useful functions: they produce food and chemicals and assist in the decomposition of waste products [35]. Viruses in fungi probably have existed for a long time but they were discovered only recently. Nowadays viruses were found in a wide range of major fungal groups [1,2,36]. Fungal-virus systems are valuable for the development of novel biocontol strategies and for gaining an insight into the molecular basis of fungal virulence [2,3]. Increasing numbers of novel fungal viruses (mycoviruses) are being reported from various host fungi [14,25,37,38].

Viruses in fungi are generally latent and rarely cause overt affects; nerveless, they profoundly influence many biological activities of their hosts [35]. This study was designed to isolate and identify some mycophages from some soils of KSA. Therefore, a number of six soil samples from different regions (Taif, Jeddah, Makkah) in KSA were collected and subjected to physical, chemical and microbiological analyses. Results showed that the texture of Makkah's soils (MH1 and MC2) was sandy clay loam and sandy clay, respectively, while, the texture of those collected from Jeddah was sandy and clay loam for JD1 and JG2 soil samples, respectively. The highest total count of fungi (TCF) was found in JG1 ($10X10^3$ - cfu/mL) followed by MH1 ($6.5X10^3$ - cfu/mL), TA1 ($4X10^3$ - cfu/mL) and MC2 ($2.1X10^3$ - cfu/mL). Equal TCF ($1.5X10^3$ - cfu/mL) was recorded for both of JD2 and TF2 soil samples. A number of ten fungal isolates varied in their cultural growth were picked up and re-inoculated on rose Bengal agar medium and highly purified and identified as mentioned in the materials and methods. Results showed that three, three and four isolates were obtained from soil samples of Makkah, Jeddah and Taif, respectively. Based on the characters of cultural growths, these isolates were classified as members of genera *Fusarium* (three isolates), *Tricoderma* (one isolate), *Alternaria* (one isolate), *Aspergillus* (one isolate) and *Penicillium* (four isolates) as shown in Fig. 1. Combination of microscopy and culture gave a positive result in 11 of 30 samples of microbial keratitis [39].

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In this study, results of cultural charcters, and light microscopy revealed that the ten isolates were belonging to the following species: *F. oxysporum* (01 and 02), *F. solani* (03), *Tricoderma* sp. (04), *Alternaria alternate* (05), *Aspergillus niger* (06), *Penicillium commune* (07 and 10), *P. polonicum* (08), and *P. expansum* (09) as shown in Fig. 2.

The ribosomal RNA (rRNA) genes are essential genes existing in all life forms from bacteria to man and they have been subjected to extensive analysis. Nucleotide sequence comparison of rRNA genes of different organisms should provide insight into evolutionary trends [40]. There are two high molecular weight RNA components (18S rRNA and 25-28S rRNA) in eukaryotic ribosomes, and 18S rRNA sequences are known to be more highly conserved than the other high molecular weight rRNA sequences [41,42]. The advent of PCR [43] made rRNA genes even more accessible for sequencing. In addition, the 18S rDNA sequence is an excellent system to study evolutionary trends among various organisms [40].

The nucleotide sequencing of 18S rDNA (rRNA) of the ten fungal isolates were determined using both of NS1 and NS8 primers. The nucleotide sequencing obtained *via* the forward and reverse directions -of each isolate - were complemented to each other to obtain the final confirmed sequencing. The lengths of 18S rRNA gene of ten fungal isolates -which recorded in GenBank with the accession numbers from LC092107.1 to LC092116.1- were ranged from 480 to 680 nts except for the *P. expansum* isolate which showed approximately full lenthg of 1568 nts. The similarities between the fungal isolates and those similar in GenBank were ranged from 99 to 100% and confirmed the biological identification of the isolates under investigation as aboveformentioned. Therefore, the isolates were classified as strains of *Fusarium oxysporum* (ASM-01, LC092107.1), *F. oxysporum* (ASM-02, LC092108.1), *F. solani* (ASM-03, LC092109.1), *Tricoderma sp.* (ASM-04, LC092110.1), *Alternaria alternate* (ASM-05, LC092111.1), *Aspergillus niger* (ASM-06, LC092112.1), *Penicillium commune* (ASM-07, LC092113.1), *P. polonicum* (ASM-08, LC092114.1), *P. expansum* (ASM-09, LC092115.1) and *P. commune* (ASM-10, LC092116.1). Results of phylgentic trees of partial sequencing of 18S rRNA gene of the ten fungal isolates confirmed the sequences producing significant alignments of partial sequencing of 18S rRNA gene of the fungal isolates compared to those similar strains in GenBank. The 18S rRNA gene was chosen as the target gene for this study was in harmony with that reported by Embong *et al*. [39].

As we mentioned before, this study aimed at detecting the presence of some mycophages in some soil samples of different regions in KSA. Results proved that the fungal isolates under investigation were belonging to genera *Fusarium*; *Tricoderma*; *Alternaria*; *Aspergillus*, and *Penicillium*. These genera were reported to be infected with mycophages in a number of researches: *Alternaria* [10]; *Aspergillus* [11,12,14,35,44,]; *Fusarium* [19,20,21,] and *Penicillium* [14,26,35,44].

The presence of mycophages was detected in the partially purified virus suspensions of the ten fungal strains *via* negatively staining. Electron microscopy of the virus suspensions prepared from the mycelia of the ten identified strains showed the presence of spherical or isometric virus-like particles in the virus suspension of *Penicillium polonicum* strain only as shown in Fig. 4. No virus-like particles were occurred in the virus suspensions of the other nine fungal isolates.This results agree with that found by Mier [28] and Newhouse *et al*. [45]. Who reported that mycoviruses or VLPs were cytoplasmic factors, spherical or isometric virus-like nucleoprotein particles, which contain dsRNA and exhibit diam. ranging from 25 to 40 nm. *Penicllium* sp. were recorded to be associated with mycoviruses [14,44].

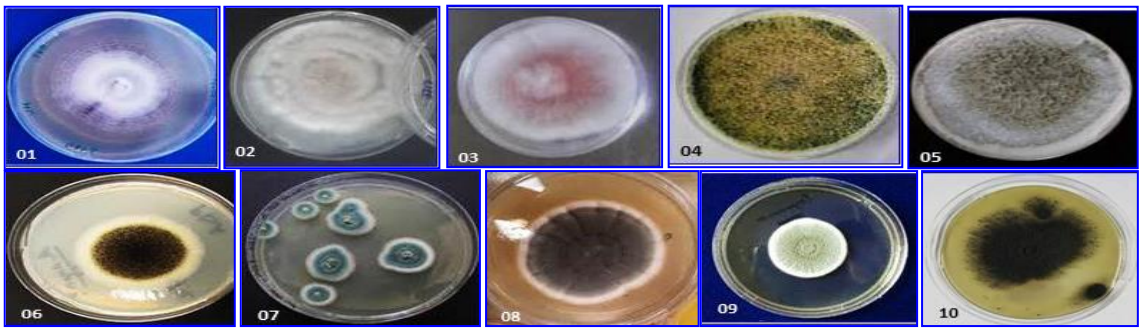

Figure 1: Purified cultural growth of ten fungal isolates which identified as members of genera: *Fusarium* (01, 02 and 03), *Trichoderma* (04), *Alternaria* (05), *Aspergillus* (06), *Penicillium* (07, 08, 09 and 10)

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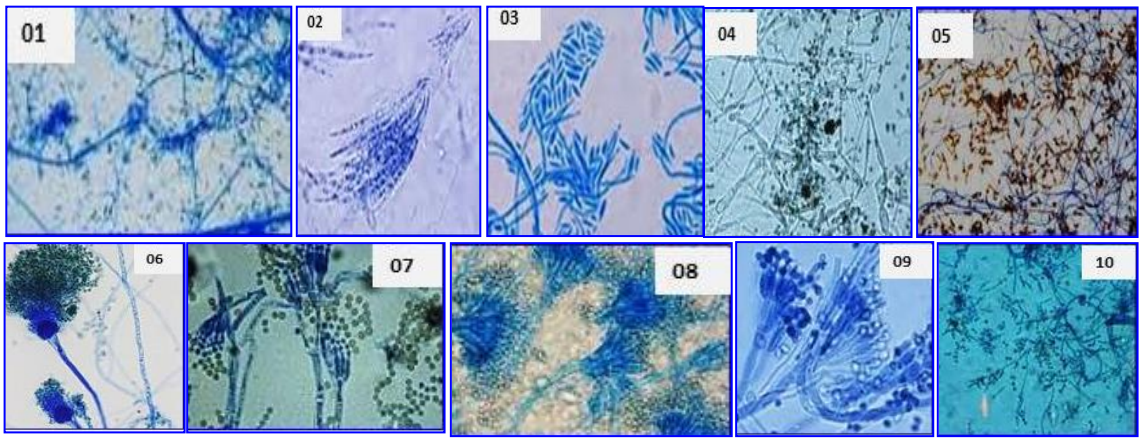

Figure 2: Light microscopy of the ten fungal isolate which identified as as *F. oxysporum* (isolates 01 and 02), *F. solani* (isolate 03), *Tricoderma* sp. (isolate 04), *Alternaria alternate* (isolate 05), *Aspergillus niger* (isolate 06), *Penicillium commune* (isolates 07 and 10), *P. polonicum* (isolate 08), and *P. expansum* (isolate 09)

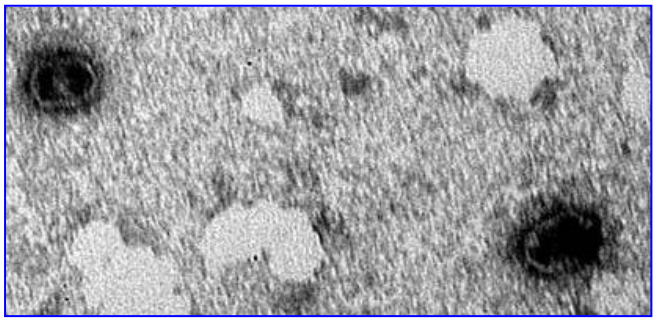

Figure 3: Electron micrograph of virus suspension prepared from the mycelium of the fungal isolate (08) (*Penicillium polonicum*) negatively stained with (2%) uranyle acetate. Note, presence of spherical virus-like particles.

CONCLUSIONS

As a conclusion, combination of cultural growth, light and microscopy and use of 18S rRNA gene were more than effective in identification of the fungal strains. On the other hand, mycoviruses could be considere as hidden particles in the mycelia with low concentrations.

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