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# Molecular And Biological Exploring Studies On Mycovirus Of Egyptian isolate Of Trichoderma hamatum

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**Abstract:**Trichoderma spp. are used extensively in agriculture as biological control agents to prevent soil-borne plant diseases. Seventeen fungal species were isolated from different sites of four governorates Dakahlia, Al-Sharqiya, kafr-Elsheikh and Damietta. The count of each fungal isolate was generally affected by physicochemical features of collected soils during the studies. The isolated fungal strains were morphologically classified into three classes namely; Zygomycetes, Ascomycetes and Hyphomycetes. Hyphomycetes was the most predominant class and was represented by three genera namely, *Alternaria*, *Fusarium* and *Trichoderma*. Ascomycetes was the second dominant class and was represented by two genera namely *Aspergillus* and *Penicillium*. Zygomycetes was represented by only one genera namely *Rhizopus*. Among all fungal isolates of this study, *T. hamatum* contained a single double-stranded RNA (dsRNA) segment of approximately 3 kb, which is typical of mycoviruses. We designated this mycovirus as Trichoderma hamatum mycovirus 1(ThaMV1). Transmission electron microscopy of (ThaMV1) revealed the presence of putative virus-like particles (VLPs) with an isometric structure approximately of 50 nm in diameter. This study represents the first report of isolation of a new unclassified mycovirus infecting *T. hamatum*

**keywords**: Fungi, Physicochemical properties, Double-stranded RNA, Mycovirus

## 1.Introduction

Both bacteria and fungi have been playing a major role in biocontrol activity. In this connection, the *Trichoderma* fungal genus plays a significant role in the prevention of some plant diseases [1]. *Trichoderma* spp. are present in almost all agricultural soils and decaying wood. *Trichoderma* is used primarily as a biocontrol agent against phytopathogenic fungi, and as a biofertilizer due to its ability to make mycorriza-like associations with plants [1-3]. Competition for space and nutrients, production of diffusible and/or volatile antibiotics and hydrolytic enzymes such as chitinase and $\beta$-1, 6- glucanase are major mechanisms used in the biocontrol activity of *Trichoderma* spp. These hydrolytic enzymes partialy degrade the cell wall of the pathogen and contribute to its parasitization. [4]. This mycoparasitism mechanism suppresses the production and activity of plant pathogenic fungi. Mycoviruses (fungal viruses) is occasionally associated with hypovirulence and have been found in all major taxa of filamentous fungi, yeast and mushrooms fruiting bodies [5-7]. Different genome types of fungal viruses are: (A) double-stranded RNA (dsRNA) genomes, taxonomically classified into seven families: *Chrysoviridae, Megabirnaviridae, Quadriviridae, Partitiviridae, Reoviridae, and Totiviridae*; and the recently proposed *Botybirnaviridae* family [8]; (B) Single-stranded genomes of RNA (ssRNA) classified into seven families: *Alphaflexiviridae, Barnaviridae, Gammaflexiviridae, Hypoviridae, Narnaviridae, Endornaviridae* and *Mymonaviridae*; and (C) circular single-stranded genomes of DNA (ssDNA) classified into a newly proposed family: *Genomoviridae* and non-classified genomes[9]. The purpose of the analysis was to isolate certain soil fungi from different sites in Dakahlia, Mediterranean Coasts and Damietta. This research was expanded to isolate mycovirus (ThaMV1) from the isolated fungi.

## 2. Materials and methods

### 1. Collection of soil samples:

Soil samples were collected from different sites which represent two different habitats (cultivated area and Mediterranean coastal regions) within four governorates Dakahlia (Talkha, Aga, El-senbillawin, Sherbin, Salka and Kafr-saad), Al-Sharqiya (Kafr-sakr), kafr-Elsheikh (Baltium) and Damietta (Gammasa and New Damietta) and during the period from January to March, 2017; all samples were collected according to the methods described by Johnson *et al* [10] and modified by Graham & Sticklen [11]**.** In brief, soil samples were taken into a clean sterilized plastic bag. Four replica samples were taken at random from each locality and mixed into one composite sample.

### 2. Isolation of fungi:

The dilution plate technique as described by Johnson *et al* [10] and updated by Graham & Sticklen [11] was used. Ten grams dry air samples were suspended in 100 ml sterile water. Then the mixture was shaken and the soil suspension was left to stand for sedimentation soil. The supernatant soil solution was added into sterile water then shaken; 10 ml of the dilute was added to 90 ml sterile water. One ml of each of the desired dilutions was transferred to Petri dish containing potato dextrose agar (PDA) medium combined with streptomycin as a bacteriostatic agent. After the solidification, the plates were incubated at 30oC for up to 10 days and examined daily. The fungal colonies were isolated and counted and their percentage frequency was calculated. The isolates were purified and stored in slope slants containing PDA medium until fungal identification and then preserved for further studies.

### 3. Morphological identification of fungi:

The developed fungal colonies were examined daily and, according to the following references, the purified fungi were morphologically classified at the species level whenever possible:

- **Raper and Thom (1949)**, for *penicillium* and penicillium-related genera [12].
- **Raper and Fennel (1965)**, for *Aspergillus* species [13].
- **Eills (1971, 1976)**, for Dematiaceous Hyphomycetes [14.15] and **Carmichael *et al*. (1980)**, for the genera of Hyphomycetes [16].
- **Domsch *et al*. (1980)**, Compendium of soil Fungi [17].
- **Nelson *et al*. (1983)**, for *Fusarium* species [18].

### 4. Physico-chemical soil properties:

Physicochemical parameters including organic carbon/nitrogen, pH, water content and electrical conductivity (EC) were determined [19].

### 5 Purification and analysis of dsRNA from fungal isolates

For all the isolated fungal species mycelia were manually separated from culture potato dextrose broth media with forceps and excess moisture was removed by pressing between two paper towels. Mycelia were then used to extract associated dsRNA using a modified method [20, 21]. Briefly, using a mortar and pestle, the fungal mycelium pellet (3-5g) was ground to a fine powder with liquid nitrogen (LN2). The fine powder was provided with 1.0 ml of 10% sodium dodecyl sulphate (SDS), 0.5 ml of bentonite (from a 2% aqueous suspension) and 9.0 ml of 1X Sodium Chloride-Tris-EDTA (STE)-saturated phenol. The tubes were shaken on ice for 30 min before being centrifuged at 8,000 xg for 15 min. Then the upper aqueous phase was collected, completed to 10 ml volume using 1X STE buffer and transferred into a 50 ml centrifuge tube.

2.1 ml of 95% ethanol was carefully added to each 10.0 ml aqueous phase and mixed well. Half gram per sample of cellulose (Whatman CFI1 [Whatman, Clifton, NJ]) were added to each tube and mixed well, then the tubes were vertically kept for 10 to 15 minutes (at room temperature). dsRNA-bound-cellulose was collected by spinning for 10 seconds, and then was flushed gently with 40 ml of IX STE containing ethanol, 16.0% v/v; dsRNA-bound-cellulose was kept re-flushing until the entire washing buffer was used. Then the liquid was discarded and the tube wall was dried using

paper towels then left upside down for 5 minutes to get rid of any washing buffer remained.

6 ml of 1X STE was added and mixed vigorously for 3 minutes then centrifuged for 30 seconds at 1000 xg. The supernatant was transferred to a new tube, 5 ml of 3.0 M sodium acetate (pH 5.5) and 20 ml of 95 % ethanol were added. The sample tube was stored vertically at – 20$^o$C over night to precipitate the dsRNA. At 8000 xg for 25 min, the samples were centrifuged. The ethanol was discarded and the tubes were set upside down for about 15 minutes to dry off. 0.2 ml of (molecular biology grade) pure water was added to each tube and mixed well to resuspend the dsRNA. Finally, the samples were stored at – 20$^o$C for gel electrophoresis [22, 23].

### 6 Virus purification

10-15 grams of virus-infected fungi was harvested and ground in LN2. The mycelia powder was thawed by the addition of 0.05 M Tris [tris (hydroxymethyl) amino methane]-HCl buffer. The homogenate was filtered and re-extracted with 1/4 volume of the same buffer. The two extracts were combined and centrifuged at 4000 rpm for 10 min to remove the pellet. 4 g of polyethylene glycol (PEG 6000), 4 ml of NaCl (20 %) were added to every 100 ml of supernatant and stirred slowly overnight at 4$^o$C. The pellets were collected by centrifugation at 10000 rpm for 15 min and then resuspended in 0.04 M sodium phosphate buffer pH 8.2 and stirred for 45 min at 4$^o$C. The supernatant was examined using the electron microscope after centrifugation at 6500 rpm for 10 min at 4$^o$C. [24].

### 7 Electron microscopy

Morphological feature assessments of the purified virus-like particles (VLPs) were examined by transmission electron microscope (TEM). Purified VLPs were negatively stained by adding a drop of virus suspension on a copper carbon- coated grid (400 meshes) for 3 min and the grid was stained with 2% (w/v) uranyl-acetate [25]. Excess stain was removed by washing with d.$H_2O$, left to dry for 5 min and then examined with TEM (JEOL JEM-2100, Japan) at the Electron Microscope Unit, Faculty of Agriculture, Mansoura University, Mansoura, Egypt

### 8. Molecular identification of the virus-containing *Trichoderma* isolate

Genomic DNA was extracted from 7 days old fungal mycelia grown on cellophane placed on PDA plates, using a commercially available DNA purification kit. DNA was used to amplify and sequence the non-coding internal transcribed spacer (ITS) regions using the primer pair ITS4/ITS5 [26]. Obtained nucleotide sequence was compared with other sequences of the NCBI nucleotide collection using BLAST search. Corresponding sequences were obtained, aligned using MUSCLE alignment and used to construct the neighbor-joining phylogenetic tree using MEGA 5 software [27].

## 3.Results and Discussion

### 1. Physicochemical properties of soil:

The collected soils had different Physicochemical properties; the pH values ranged between 7.09 and 8.25, soil texture varied between sandy and clay and the organic matter (OM) content ranged between 0.17 (Gammasa) and 5.11 % (Salka). The soil salinity (dissolvent salts as EC) appeared to be a limiting factor for the occurrence of isolated fungi (Table 1). Therefore, New Damietta and Baltim sites were found inhabiting a low number of the fungal total count when compared with other samples.

This result is consistent with those obtained by Kumar *et al* [28].who documented a decrease in the total number of fungal isolates with increasing the soil salinity

### 2 Count and Frequency of isolated fungi:

As indicated in table 2 , the highest record of total fungal populations (4500 colonies per g dry humus soil ) was found in sample No. 4 (Sherbin), followed by sample No. 3(El-senbillawin, 4100 colonies per g dry soil ) and the smallest count was observed in sample No. 8 (Baltim,830 colonies per g dry humus soil) . There was no regular correlation between the count and the number of genera or species. This was due to deep fluctuations in response to alternation in condition of the atmosphere, chemical and physical analysis of the soil [29].

**Table 1**: Physiochemical properties of the soil samples collected from four different Egyptian governorates during the period from January to March, 2017.

| | Sample No. | Locality | Soil Texture | pH | EC(µS.cm) | OM (%) | Total count |
|---|---|---|---|---|---|---|---|
| Dakahlia | 1 | Talkha | Clay | 8.25 | 320 | 3.20 | 3660 |
| | 2 | Aga | Clay | 7.78 | 149 | 4.58 | 2850 |
| | 3 | El-senbillawin | Clay | 7.88 | 110 | 2.99 | 4100 |
| | 4 | Sherbin | Clay | 7.90 | 320 | 2.24 | 4500 |
| | 5 | Salka | Clay | 7.95 | 350 | 5.11 | 2050 |
| | 6 | Kafr-saad | Clay | 7.33 | 658 | 2.24 | 2500 |
| Al-Sharqiya | 7 | Kafr-sakr | Sandy | 7.11 | 167 | 0.59 | 1900 |
| kafr-Elsheikh | 8 | Baltim | Sandy | 7.54 | 2820 | 0.27 | 830 |
| Damietta | 9 | Gammasa | Sandy | 7.32 | 1870 | 0.17 | 1020 |
| | 10 | New Damietta | Sandy | 7.09 | 2530 | 0.27 | 985 |

**Table 2:** Count of fungi isolated from the collected soil.

| Soil sample No. | T.C(No./g dry soil) | No. of G. | No.of spp. | Locality |
|---|---|---|---|---|
| 1 | 3660 | 5 | 5 | Talkha |
| 2 | 2850 | 3 | 5 | Aga |
| 3 | 4100 | 6 | 8 | El-senbillawin |
| 4 | 4500 | 6 | 10 | Sherbin |
| 5 | 2050 | 4 | 4 | Salka |
| 6 | 2500 | 4 | 5 | Kafr-saad |
| 7 | 1900 | 5 | 5 | Kafr-sakr |
| 8 | 830 | 3 | 5 | Baltim |
| 9 | 1020 | 3 | 3 | Gammasa |
| 10 | 985 | 3 | 4 | NewDamietta |

**Total count (T.C), Number of Genera (No. of G.), Number of Species (No. of spp.) and Locality of 10 soil sample tested.**

### 3 Identification of the isolated fungi:

Fungal isolates were identified and arranged according to their taxonomical positions as shown in (Table 3)

The present data agree with **Duo-Chun** who isolated *A.flavus*, *A.terreus*, *P. purpurogenum*, *T.hamatum* and *F.oxysporum* from rhizospheric soil. *T.hamatum* exhibited the highest count from this study [30].

### 4 Frequency of isolated fungal species:

Total counts and frequency of occurrence of identified isolated fungi are as shown in Table (4).

- **H= high is isolated more than 6 times out of 10 samples.**
- **M=Moderate, from 5 to 6 times out of 10 samples.**
- **L= Low, from 3 to 4 times out of 10 samples.**

**R= Rare less than 3 times out of 10 samples.**

The results presented in Table 4 show that *T. hamatum* and *T. viridi* were the most frequent species recorded during this survey as compared with other isolated species. That two isolates were represented in all soil samples while *F. equiseti* was represented in one soil sample.

**Table 3:** List of fungal species recorded in this investigation and their classes.

| **I-Ascomycete**<br>**Genus: *Aspergillus***<br>1. *A. flavus* Link |
|---|
| 2. *A. niger* Teigh |
| 3. *A. terreus* Thom |
| 4. *A. ochraceus* Wilhelm |
| **Genus : *penicellim*** |
| 5. *P.chrysogenum* Thom |
| 6. *P. purpurogenum* Stoll |
| 7. *P. citrinum* Thom |
| **II-Zygomycetes** |
| **Genus: *Rhizopus*** |
| 8- *Rhizopus nigricans* Lindit<br>9- *Rhizopus oligosporus* lindit |
| **III Hyphomycetes (Duetromycetes)** |
| **Genus: *Trichoderma*** |
| 10-*T . hamatum* Oudem<br>11-*T. viridi* Pers |
| 12- *T. harzianum* Rifai |
| **Genus : *Alternaria*** |
| 13- *A. alternata* Keissler |
| 14- *A. chlamydospora* Mouchacca |
| **Genus: *Fusarium*** |
| 15- *F. oxysporum* Schledht<br>16- *F. solani* Mart |
| 17- *F. equiseti* Corda |

**Table 4:** Total counts (colonies per g dry samples) and frequency of occurrence of fungal isolates from 10 sources of soil samples.,

| Fungal Species No. | Fungal Species | Total Count | Frequency | Occurrence | |
|---|---|---|---|---|---|
| | | | | % | level |
| 1 | *F.oxysporum* | 1200 | 3 | 30 | R |
| 2 | *F. solani* | 1350 | 5 | 50 | M |
| 3 | *F. equiseti* | 1100 | 1 | 10 | R |
| 4 | *A. flavus* | 1650 | 7 | 70 | H |
| 5 | *A. niger* | 2330 | 8 | 80 | H |
| 6 | *A. ochraceus* | 1530 | 7 | 70 | H |
| 7 | *A. terreus* | 1315 | 6 | 60 | M |
| 8 | *Rhizopus nigricans* | 1310 | 6 | 60 | M |
| 9 | *Rhizopus oligosporus* | 1050 | 5 | 50 | M |
| 10 | *P.chrysogenm* | 3520 | 8 | 80 | H |
| 11 | *P.purpurogem* | 2700 | 6 | 60 | M |
| 12 | *P. citrinum* | 1150 | 4 | 40 | L |
| 13 | *A. alternate* | 1100 | 5 | 50 | M |
| 14 | *A.chlamydospoa* | 860 | 3 | 30 | R |
| 15 | *T. viridi* | 8115 | 8 | 80 | H |
| 16 | *T.harzianum* | 730 | 8 | 80 | H |
| 17 | *T. hamatum* | 9220 | 10 | 100 | H |

### a. Occurrence of mycoviruses in the isolated fungal species isolated from 10 soil samples:

The 17 fungal isolates were screened for the presence of dsRNA using CF11 cellulose chromatography method (Table 5). This study describes a new mycovirus discovered in *T. hamatum* isolate found in the soil of in New Damietta, Egypt, from collected soils. Just three mycovirus isolates have so far been recovered from *Trichoderma* spp. [31]. The overall incidence of dsRNA in fungal strain of this study was (~6%) which is lower than that reported in *Beauveria bassiana* (54.8%) and *Gremmeniella abietina* (14.2%) [32, 33]. For instance it will therefore be of interest to decide what determines the initial introduction of dsRNA and how fast dsRNA will spread among natural inhabitants.

Electrophoresis showed that one segment of ~ 3 kb was the putative mycovirus fragment. DNase 1 and S1 nuclease were added to partially purified dsRNA and the purified product confirmed the size and profile of the dsRNA (Fig 1)

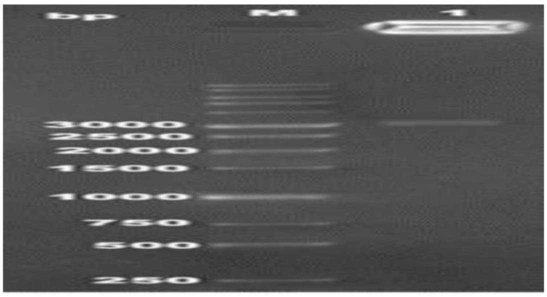

**Fig 1:** Agarose gel electrophoresis showing dsRNA profile of the infected isolate. (M; 10 kb; Nippon) was used as a marker.

**Table 5:** Occurrence of mycoviruses in the isolated fungal species isolated from 10 soil samples

| Fungal Species No. | Fungal Species | Occuranceof mycoviruses |
|---|---|---|
| 1 | *F. oxysporum* | Absent |
| 2 | *F. solani* | Absent |
| 3 | *F. equiseti* | Absent |
| 4 | *A. flavus* | Absent |
| 5 | *A. niger* | Absent |
| 6 | *A. ochraceus* | Absent |
| 7 | *A. terreus* | Absent |
| 8 | *Rhizopus nigricans* | Absent |
| 9 | *Rhizopus oligosporus* | Absent |
| 10 | *P.chrysogenum* | Absent |
| 11 | *P. purpurogenum* | Absent |
| 12 | *P. citrinum* | Absent |
| 13 | *A. alternate* | Absent |
| 14 | *A. chlamydospora* | Absent |
| 15 | *T. viridi* | Absent |
| 16 | *T. harzianum* | Absent |
| 17 | *T . hamatum* | Present |

### b. Mycovirus morphology using transmission electron microscopy

TEM showed symmetrical VLPs of isometric morphology with a diameter of ~50 nm (Fig 2), which is a common morphology and particle size for several mycoviruses [34].

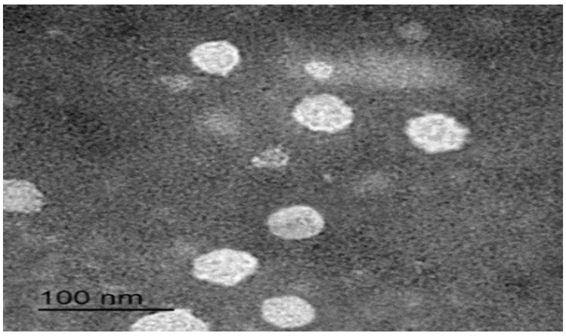

**Fig 2:** Electron micrograph of viral particles. The purified viral particles were negatively stained with 2% urine acetate) and observed on a transmission electron microscope.

## 7 Molecular confirmation of the virus-containing Trichoderma isolate

Fungal molecular identification by sequencing ITS regions is widely used and has been previously utilized to identify several Trichoderma spp. isolates [35].Primers ITS4 and ITS5 amplified a ~500 nts fragment that was 99.8% similar to other sequences of *T. hamatum*. This confirms the results obtained from the morphological identification of the virus-containing isolate of this study. The ITS sequence of the *T. hamatum* identified in our study and corresponding partial sequences of other *Tichoderma* spp. were used to infer the phylogeny of the identified *Tichoderma* isolate. As shown in (Figure 2) the virus-containing *Trichoderma* isolate of this study formed a well-supported clade with other *T. hamatum* isolates.

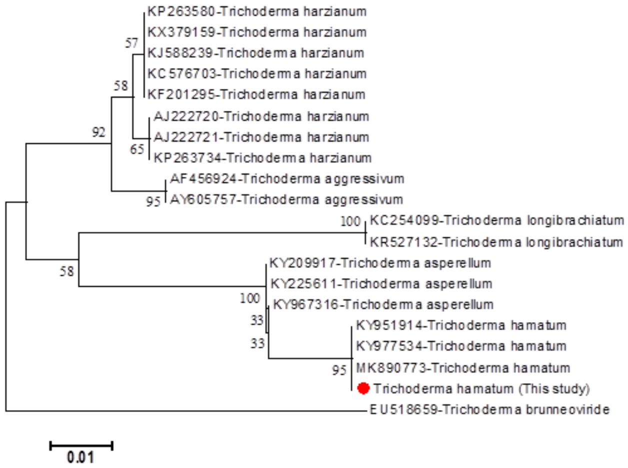

**Fig 2:** Neighbor-joining phylogenetic tree based on multiple alignments of partial ITS sequences of *Trichoderma* species. Values on the branches represent the percentage of 1000 bootstrap replicates.

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