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CHARACTERIZATION AND ELIMINATION OF A TMV ISOLATE INFECTING *CHRYSANTHEMUM* PLANTS IN EGYPT

[25]

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ABSTRACT

An Egyptian isolate of a *Tobamovirus* was isolated and identified from *Chrysanthemum* cultivation in Egypt. Both biological, serological and sequence analysis of the coat protein gene demonstrated that the virus represents an isolate of the *Tobamoviridae* Family. The isolated virus was nominated as TMV *Chrysanthemum* Egyptian isolate (TMV–Ch–EG). This virus isolate caused severe disease symptoms in *Chrysanthemum* plants with mosaic, mottling and flower discoloration. The virus was purified biologically using serial transfer of the single local lesion technique on *Nicotiana gultinosa*. The induced antiserum for the isolated virus had a titer 1\1024. 600 bp DNA fragments from the coat protein gene (CP) of TMV–*Ch*–EG was amplified with Rt-PCR technique. Phylogenetic analysis of the TMV-*Ch*-EG/CP- gene showed 89% nucleotide sequence homology with other published strains of TMV in GenBank and 81% amino acid sequence homology. Tissue culture approach was used to permit the recovery of TMV-Ch-free micropropagated shoots via application of 20 mg/l virazole followed by thermotherapy at 38°C for two weeks and early screening to facilitate the efficient production of virus-free tissue culture derived propagules using the produced antiserum against TMV-*Ch*-EG.

Keywords: *Chrysanthemum*, TMV, ELISA, RT-PCR, DNA sequencing.

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INTRODUCTION

Chrysanthemum is a genus of about 30 species of perennial flowering plants in the Family *Asteraceae*. *Chrysanthemum* is one of the three most important cut flowers in the world. *Chrysanthemum* has an economical importance as ornamental, culinary, environmental and insecticidal uses **(Marongiu *et al.*, 2009).** *Chrysanthemum cinerarifolium* is economically important as a natural source of insecticide with a huge international market **(Liu and Gao, 2007).** *Chrysanthemum* plants are infected with viruses as *Tomato aspermy virus* (TAV) **(Verma *et al.*, 2006)**, *Tobacco mosaic virus* (TMV) **(Zaitlin and Israel, 1975)** and *Cucumber mosaic virus* (CMV), *Chrysanthemum B virus* (CVB) **(Ram *et al.*, 2009)** and viroids as *Chrysanthemum stunt viroid* (CSVd) and *Chrysanthemum chlorotic mottle viroid* (CChMVd) **(Hosokawa *et al.*, 2005)**

The current study aims at identifying and characterizing an Egyptian TMV isolate from naturally infected *Chrysanthemum cinerarifolium* based on its biological, serological, and molecular properties, and the producion of virus free plant materials .

MATERIALS & METHODS

Source of the virus isolate

About 35 *Chrysanthemum* plants were collected from garden and houses plants of Horticulture Dept. Fac. of Agriculture, Ain Shams University. Egypt. Some of these samples showed virus like symptoms.

Biological properties of isolated:

Infected *Chrysanthemum* plants with foliar symptoms were used in mechanical inoculation of indicator *N. glutinosa* plants. Infected *Chrysanthemum* tissues were triturated (1:1, v/v) with 0.05M phosphate buffer pH 7.2, containing 0.2% mercaptoethanol. Homologous single local lesion produced on *N. glutinosa* were cut and macerated on a glass slide and mechanically inoculated on seedlings of *N. tabacum cv.* Samsun as a propagative host.

Infected sap from Samsun tobacco was used in host range testing using (20 plant species belonging to 7 families). Infectivity of inoculated plants was tested with ELISA **(Table 1).**

Physical properties characters as thermal inactivation point (TIP), dilution-end point (DEP) and longevity *in vitro* (LIV) of the TMV isolate was determined

according to **Noordam (1973),** using *Ch. Aum productionmaranticolor* as a local lesion assay host.

The virus was purified from inoculated *Chrysanthemum* plants according to **El Ahdal *et al.* (1984).**

Antiserum production:

An adult New Zealand rabbit was injected with TMV purified preparations seven times at one week intervals according to the method of **Makkouk and Gumpf (1976).** The titer of the antiserum was determined by indirect ELISA technique **(Dijkstra and DeJager (1998).**

Molecular characterization of the TMV isolate:

Total RNA was extracted from healthy and infected *Chrysanthemum* plants using High Pure RNA Tissue Kit (Roche Molecular Biochemicals, Cat. No.2033674).

RT-PCR protocol was as described by **(Chung *et al.*, 2007)** using two primers (TMV1-ATGTCTTACAGTATCACTACTCC/ and TMV2-TCAAGTTGCAGGACCAGAGG for the coat protein gene. Complementary DNA (cDNA) synthesis was accomplished as described by **(Chung *et al.*, 2005).** Amplification of the cDNA included the following cycles: Denaturation at 94°C for 30 sec, primer annealing at 50°C for 1 min and extension at 72°C for 1 min for 40 cycles with a final extension at 72°C for 7 min. The PCR products were analyzed by electrophoresis onto 1.0% agarose gel and the size of the DNA fragment was determined in accordance with the molecular weight markers. The PCR products were visualized on a UV transilluminator (λ=254) and photographed with an UVB laboratory products, Epichemi II Dark room, 3UV transilluminator Pharmacia.

DNA sequencing for the partially amplified coat protein gene (~600nt) of TMV isolate was performed using ABI prism 3100, Genetic Analyzer by using dye-primer and dye terminator method at Gene Link D)NA Sequencing service, New York, USA. The resulting sequence for CP gene was then compared to the published sequences of TMV using the Clustal W method **(Thompson *et al.*, 1997).**

Production of Chrythansmum TMV- free plantlets:

Media used in this investigation was salt mix of **Murashige and Skoog (1962).** The infected explants were aseptically cut into single nodal pieces. The base of

each cutting was cultured in jars contained 25 ml of MS supplemented with 0.25 mg/l BA and at 25 ° C ± 1 ° C under flourescent light. The heat treatment was applied to the *in vitro* micropagated plant material Shoot proliferation were pruned and placed in growth chamber at 37° C ± 1°C for 14 and 28 days at 16 photoperiod .Three different concentration of 10, 20, and 30 mg/l of filter sterilized virazole were tested. Combination between thermotherapy and chemotherapy were applied *in vitro* by subjecting the micropropagated shoots at 37° C ± 1 for 21 days and then transferred to media containing 10, 20 and 30 mg/l virazole. After 2 weeks, shoots were placed in antiviral free media. A period of about 35 days was necessary to have all the cultures well-established and the shoots proliferated: then a few leaves could be sampled for virus detection using produced antiserum by indirect ELISA test.

RESULTS

Biological studies:

Symptomatology and hostrange studies:

Naturally infected Chrysanthemum plants exhibiting symptoms of conspicuous mottling, mosaic and vein banding of leaves are shown in **Figure (1).**

Host range studies showed the formation of systemic mottling, mosaic, and blisters upon virus inoculation on *N. tabacum cv.* Samsun. Whereas chlorotic local lesions surrounded by necrotic tissues were developed on inoculated *N. glutinosa* **(Figure 2).**

According to the response of the tested hostrange plants **(Table 1),** host reactions was classified into four categories as follows: 1) Plants showed local infection including *Cenopodium quinoa, C. murale, Vicia faba cv.* Giza 402 and *C .amaranticolor, Gomphrena .globosa, N. glutinosa* and *N.tabacum cv.* White Burley, *Datora metel* and *D.stramonium,* 2) Plants showed different systemic symptoms includeding, *N. tabacum cv.* Samson, *L. esculentum cv.* Castle rock, *Capsicum annuum, Jasminum mesnyi, Jasmonium multiflorum,* J. mesnyi, *J. grandiflora* and *Chrysanthemum spp.* 3) Plants showed local lesion followed by systemic symptoms including, *N. gluca* and *N.* rustica, and 4) Plants showed no visible reaction with TMV isolate included, *Cucarbita maxima, Cucarbita sativus* and *Plumeria sp.* These

results were confirmed by indirect ELISA

Inclusion bodies:

Light microscopy of the epidermal strips from infected *Chrysanthemum morifolium* leaves, 21 days post TMV inoculation showed cytoplasmic inclusion bodies. The crystalline inclusions induced by TMV were observed in epidermal and hair cell as well as amorphous inclusions stained with bromophenol blue and mercuric chloride. In addition, the virus infection caused decrease in number of stomata and lignifications of stomata gap as well as irregular of epidermal cells compared with healthy **(Figure 3).**

Serological characters

Production of antiserum

The titer of induced antiserum was determined by indirect ELISA was 1/1024. The induced antiserum for the TMV-*Ch*-EG isolate (AS1) was more specific than commercial authentic antiserum (AS2) in detection of TMV in *Chrysanthemum* samples **(Table 1)** and other hosts **(Table 2).**

Molecular characters:

RT-PCR

RT-PCR successfully detected TMV viral RNA from *Chrysanthemum* tissues. Partial amplification of the CP gene about 600 bp in size was detected from TMV infected tissues by using TMV1 and TMV2 specific primer for the coat protein gene **(Figure 4).**

Results for the partially amplified DNA sequence of the CP gene is presented in **Figure (5).**

Phylogenetic analysis:

The partial nucleotide sequence (598 nt) of the TMV/CP-EG was aligned with six coat protein sequences of TMV **(Figure 6)** by using DNAMAN programme (DNAMAN V 5.2.9 package, Madison, Wisconsin, USA). A phylogenetic tree of TMV/CP-EG showed 89% sequence similarity with the other compared accessions.

Deduced amino acids sequence of TMV/CP gene:

Deduced amino acids were determined using computer software. The predicted number of amino acids produced from translation of partial CP gene nucleotide sequence of TMV-*Ch*-EG (GeneBank accsseion ADF47092.1) isolate was 159 amino acids starting with Methionine (M) and ended with Serine **(Figure 7).**

The predicted amino acids sequence (159 amino acids) of the

TMV-*Ch*-EG CP-gene was published in GeneBank accsseion ADF47092.1 and compared with other nine amino acid sequences of TMV published in GeneBank. A phylogenatic tree of TMV-EG presented in **(Figure 8)** revealed that the deduced amino acid sequence of TMV under study has 82% resemblance accsseions (AAK33120_Brazil, AAT45114_ Thailand, P69507_Japan, AAK06750_Korea, AM64218_China, AAQ12020_Finland, CAL69972_Vietnam, ACY412-14_USA and CAD22079 Germany).

Comparisons between the predicted amino acids of partial CP-gene sequence of TMV-*Ch*-EG isolate were showed that, the type of amino acids consists of 18 amino acids. Threonine and (Serine and Leucine) have a high frequency and percentage 17 and 10.69% respectively, when Histidine and Methionine have low frequency and percentage 1 and 0.62 % respectively **Table (3).**

Production of virus free plantlets using tissue culture techniques

Combining chemotherapy and thermotherapy by subjecting the infected shoots to 3^°C for 2 weeks in addition to10, 20 and 30 mg/L virazole produced ratios of 95, 90 and 85% *Chrysanthemum* survival respectively, while the percentages of virus free were 38, 61.5 and 76.8 % respectively **(Table 4).**

Micro propagation stage of *invitro* chrysanthemum plants healthy and infected are shown in **Figure 9** while the effect of thermotherapy and chemotherapy are shown in **Figures 10&11** respectively.

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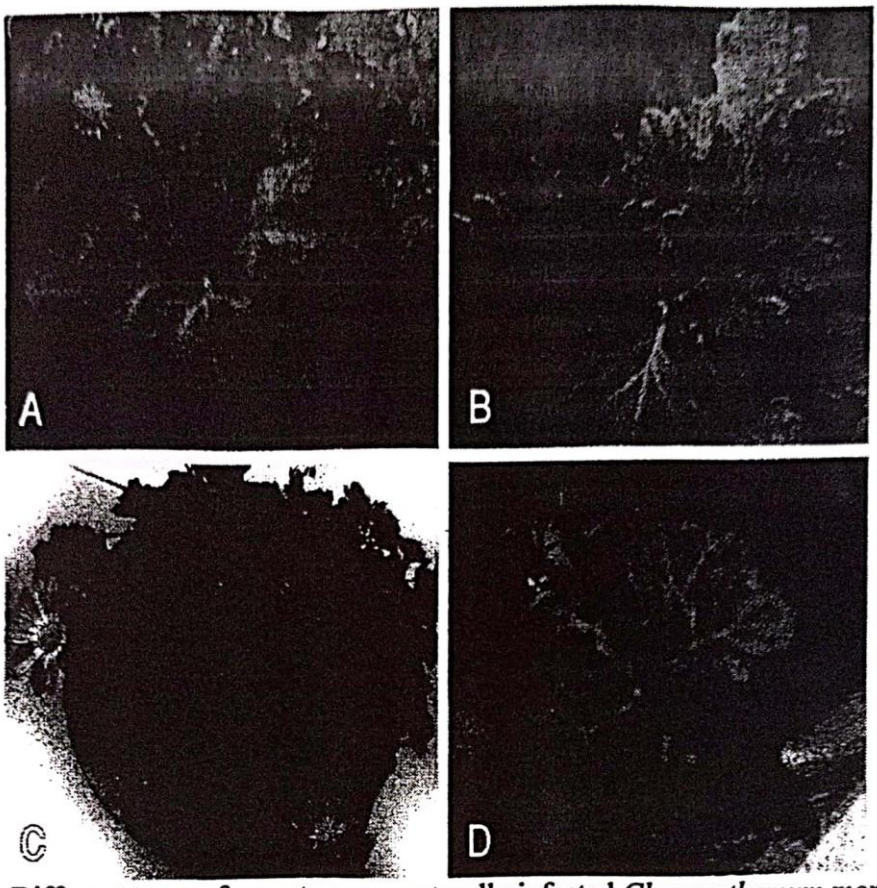

Figure 1. Different types of symptoms on naturally infected *Chrysanthemum morifolium*.
A, Healthy *Chrysanthemum* ; B, leaves showing vein banding
C, flower showing color breaking; D, leaf curling with vein clearing

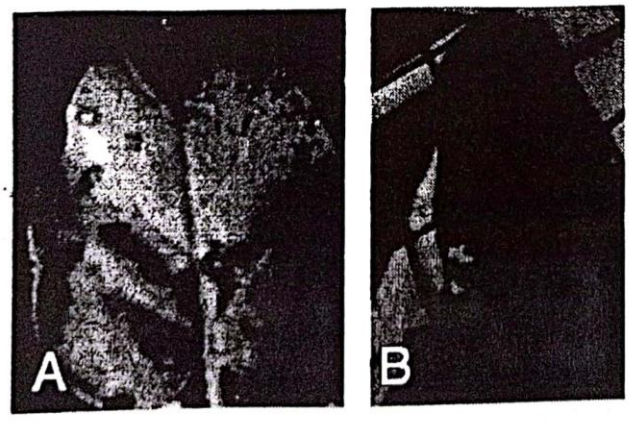

Figure 2. Differential hosts mechanically inoculated with TMV isolate showing: A, Necrotic local lesions with chlorotic spots on *N. glutinosa*; B, Mosaic and mottling on *N. tabacum* cv. Samasun

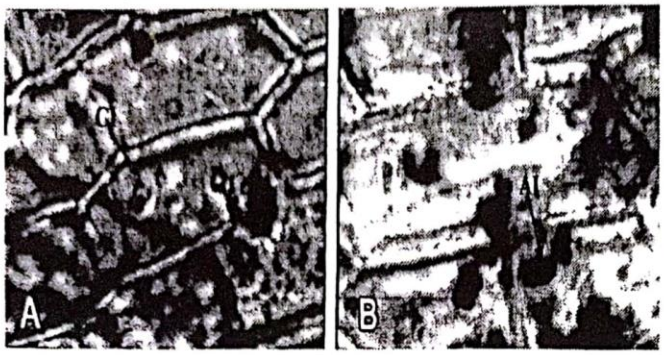

Figure 3. Epidermal cells of Chrysanthemum leaves inoculated with TMV-Ch-EG isolate showing (A) Crystalline (CI) and (B) amorphous (AI) inclusions.

Table 1. Occurrence of TMV in infected *Chrysanthemum* samples using produced antiserum and common antiserum.

Indirect ELISA (Optical Density, O.D., at 405 nm											
Sample Number	**AS1**	**AS2**	**Sample Number**	**AS1**	**AS2**	**Sample Number**	**AS1**	**AS2**	**Sample Number**	**AS1**	**AS2**
1	0.294	0.214	8	0.192	0.195	15	0.177	0.176	22	0.647	0.425
2	0.284	0.213	9	0.246	0.210	16	0.322	0.232	23	0.371	0.256
3	0.162	0.169	10	0.241	0.201	17	0.216	0.198	24	0.234	0.168
4	0.198	0.111	11	0.191	0.184	18	0.211	0.194	25	0.390	0.255
5	0.252	0.202	12	0.106	0.105	19	0.405	0.298	26	0.273	0.212
6	0.231	0.198	13	0.169	0.156	20	0.298	0.225			
7	0.195	0.196	14	0.231	0.204	21	0.229	0.199			

Negative control for Chrysanthemum= 0.106; Positive control for Chrysanthemum = 0.212

Table 2. Occurrence of TMV in infected *Chrysanthemum* samples using produced antiserum and common antiserum

Plant variety	Produced antiserum	Common antiserum
P. hybrida	0.405	0.298
J. multiflorum	0.348	0.226
J. grandiflora	0.335	0.222
J mesnyi	0.329	0.205
Plumeria sp.	0.228	0.108

***Negative control* =0.105 nm**

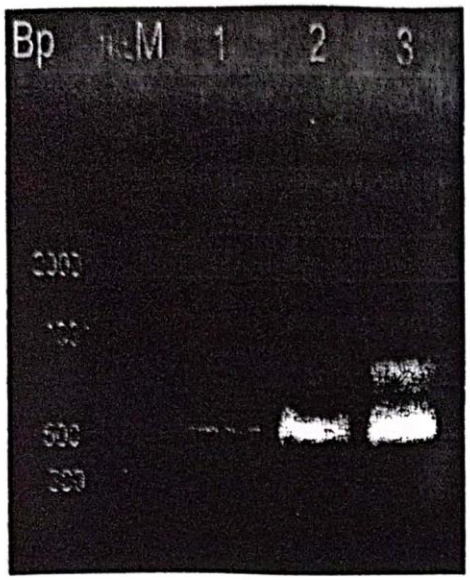

Figure 4. 1.0% Agarose gel electrophoresis showing partially amplified PCR products for the TMV/CP gene. lane at the left represents 1kb DNA markers; lane1, inoculated *Chrysanthemum morifolium*, lane 2&3: naturally infected *Chrysanthemum morifolium.*

1 atgtatcaca gtaaccgtac tccatcacag ttcgtgttct tgtcatcagc gtgggccgac
61 ccaatagagt taattaattt atcaactaat gccttaccaa atcagttaca aacacttcaa
121 gctcgttctg tcgttcaaag acaattcagt gacctgtgga aaccttcacc acttgtaact
181 gttaccaacc ctgacagtct ctttaaggtg tacacctaca atgcggtatt agaggcgcta
241 gtcacagcac tgttaggtgc attcgacact agaaatagaa taatagaagt tgaaaatg
301 gcgaacccca cgactgccga aacgttagat gctactcgta gagtagacga cgcaacggtg
361 gccataacca gcgcgatatt taatttaata gtagaattga tcagaccaac cggatcaaat
421 aatcggagct caaacgagag ctcaactggt ttggtaagga cctctcctcc tgcttcttga
481 ggtagtcttg atccattata aatatcgcat tgtgtgggta atcacacgtg gtgcctacga
541 taacgcatag tgttaaaccc tccacttatt tcgaagggtt gtgtcaagga tcgcgccc

Figure 5. The partial nucleotide sequence (598 nt) analysis of the TMV/CP-gene for the TMV-*Ch*-EG isolate

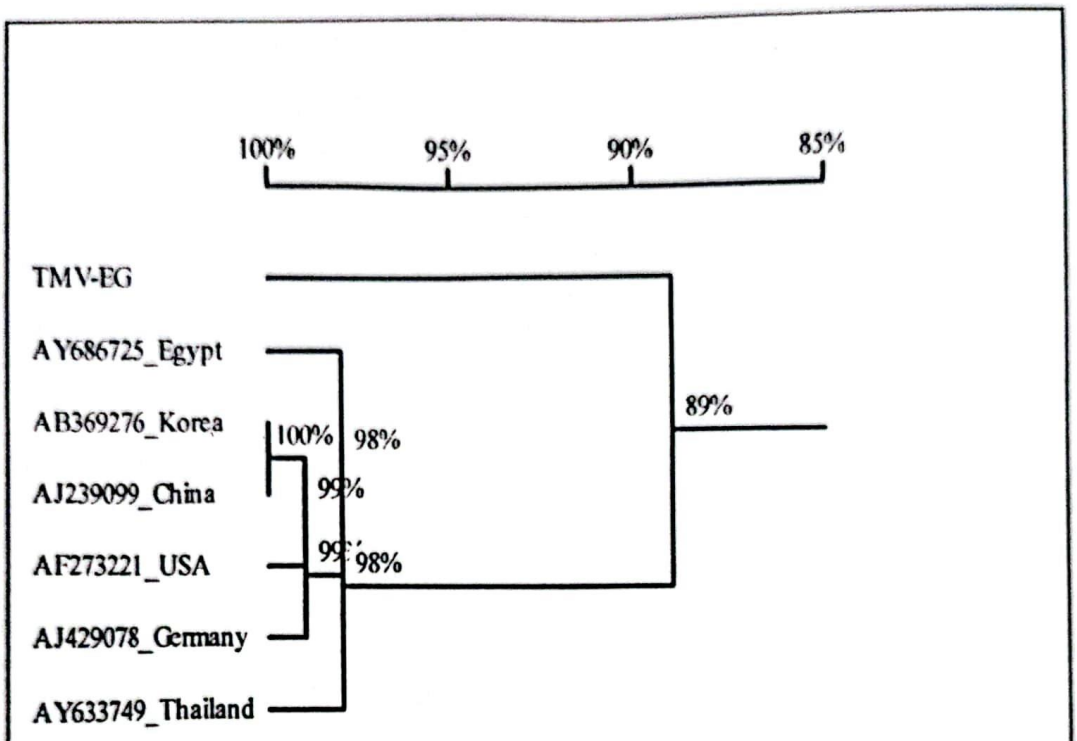

Figure 6. Phylogenetic tree constructed from the multiple alignments of the CP/TMV-*Ch*-EG (GeneBank accessesion GU982315) and six TMV strains (AY686725_Egypt, AB369276_Korea, AJ239099_China, AF273221_USA, AJ429078_ Germany and AY633749_ Thailand).

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1 myhsnrtpsq fvflssawad pielinlstn alpnqlqtlq arsvvqrqfs dlwkpsplvt

61 vtnpdslfkv ytynavleal vtallgafdt rnriievenq anpttaetldatrrvddatv

121 aitsaifnli velirptgsn nrssnesstg lvrtsppas
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Figure 7. Predicted amino acids of partial CP gene of sequence of TMV-*Ch*-EG isolate (GeneBank accsseion ADF47092.1).

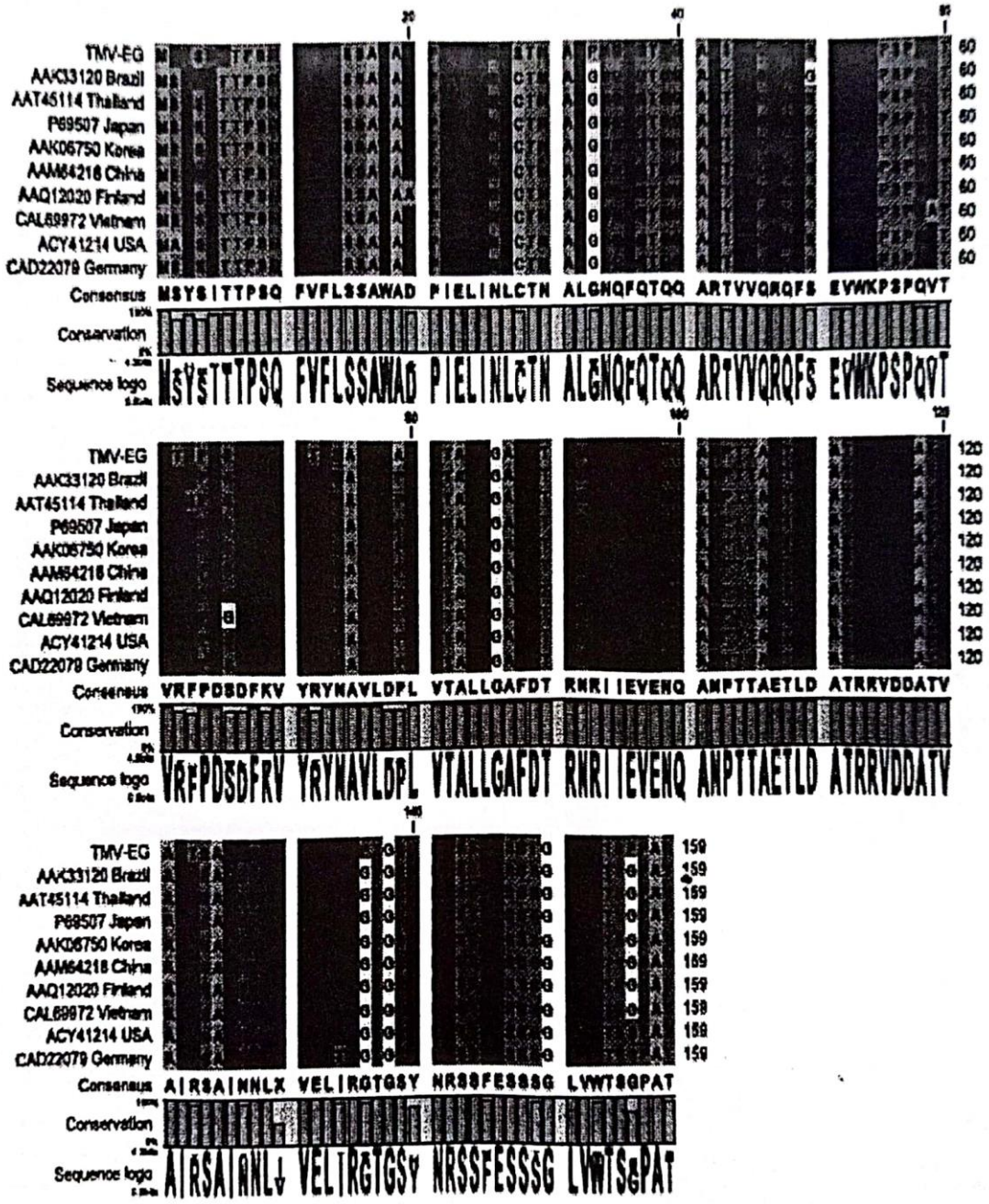


Figure 8. A multiple alignment of predicted amino acid sequences encoding the coat protein (CP) gene of TMV-Ch-EG isolate (GeneBank accession ADF47092.1)

Table 3. Composition of predicted amino acids of partial *cp gene* sequence for TMV-Ch-EG isolate.

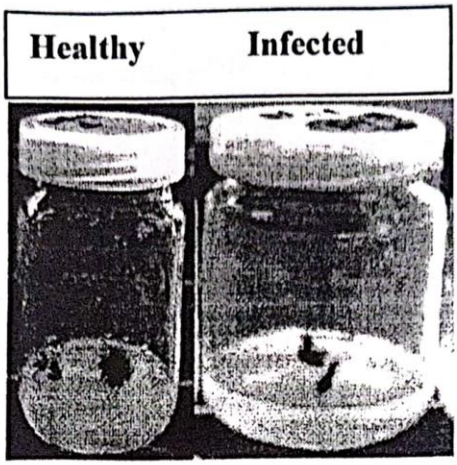
Type of A.A.	Name	No.	%
P	Pro.	10	6.28
V	Val.	13	8.17
E	Glu.	7	4.40
R	Arg.	10	6.28
I	Ile.	8	5.03
S	Ser.	17	10.69
Q	Gln.	7	4.40
L	Leu.	17	10.69
W	Trp.	2	1.25
G	Gly.	3	1.88
Y	Tyr	3	1.88
F	Phe.	6	3.77
D	Asp.	7	4.40
H	His.	1	0.62
M	Met.	1	0.62
T	Thr.	17	10.69
K	Lys.	2	1.25
A	Alan.	15	9.43
N	Asn.	13	8.17
Type of A.A.		159	100%

A.A. = Amino acids.

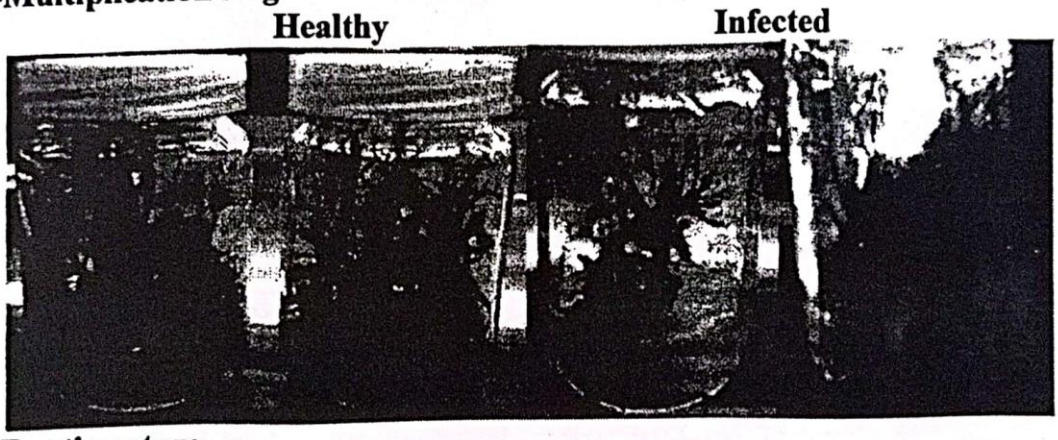
Table 4. The effects of thermotherapy and chemotherapy (virazole) on TMV elimination from infected *C.morifolium* plants

Parameters / Treatments	% Survival	No. of TMV-free plantlets	No. of TMV-infected plantlets	% of TMV-free plantlets
Thermotherapy				
2 weeks(38 °C)	100%	4	10	28.5%
3 weeks(38 °C)	95%	6	8	42.8%
Chemotherapy				
10mg/L	100%	4.11	9.89	29.4%
20mg/L	93%	4.66	9.34	31.3%
30mg/L	86%	5.76	8.23	58%
Chemo/Thermotherapy				
10 mg/L	95%	5.38	8.61	38%
20 mg/L	90%	8.61	5.38	61.5%
30 mg/L	85%	7.36	6.63	76.8%

1-Starting stage



2-Multiplication stage



3-Rooting stage

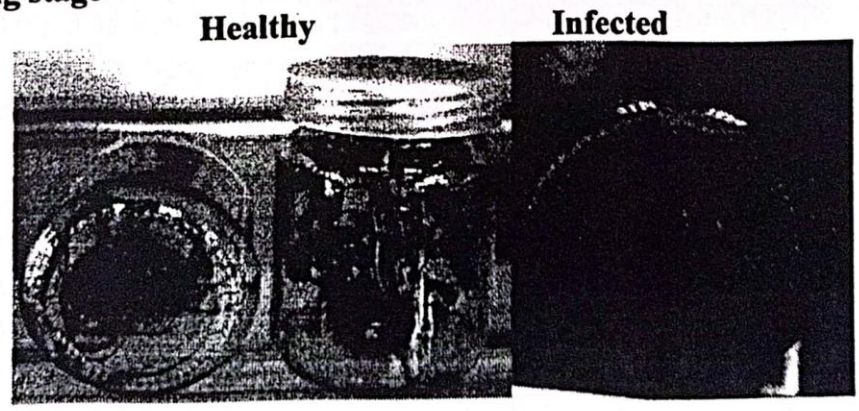


Figure 9. Micropropagation stage of *Chrysanthemum* plants *in vitro* (healthy and infected).

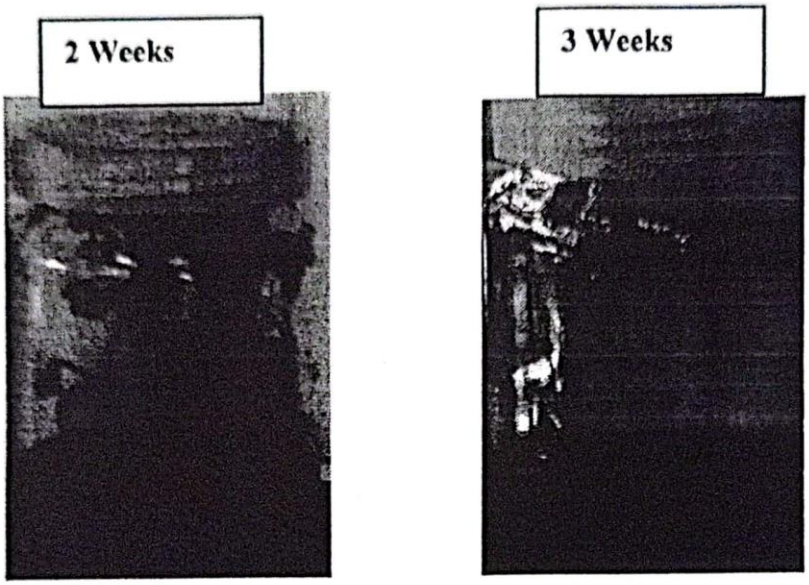


Figure 10. Effect of temperature(thermotherapy) on TMV infected *C. morifolium* plants.

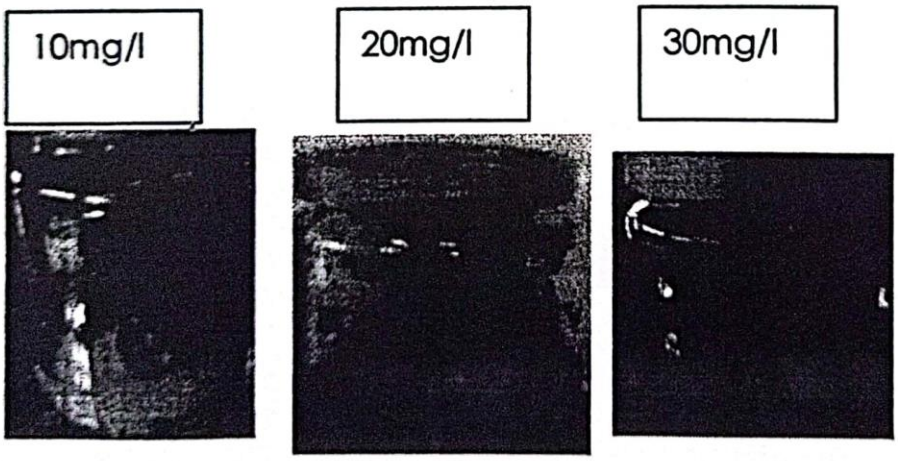


Figure 11. Effect of Virazol on TMV infected *C. morifolium* plants.

DISCUSSION

According to biological and molecular characters, TMV isolated from *Chrysanthemum* (TMV-Ch-EG) is considered as a strain of TMV common (isolated from tobacco plants).

TMV isolate used in this study was found to give necrotic local lesions on *N. tabacum cv.* White Burley plants and chlorotic local lesions surrounded with necrotic tissue on *N. glutinosa.* These findings differed from that obtained by **(El-Ahdal *et al.*, 1984; Brunt *et al.*, 1996 and El- Afifi, 2002)** who described severe mosaic and malformation on *N. tabacum cv.* White Burley and necrotic local lesions on *N. glutinosa.*

The partial nucleotide sequence of the RT-PCR amplified fragment with TMV1 and TMV2 primers for the coat protein cp-gene of TMV isolate was done to determine the relationship with other recommended TMV strains registered in Genebank .

Threonine and (Serine and Leucine) have a high frequency and percentage 17 and 10.69% respectively, when Histidine and Methionine have low frequency and percentage 1 and 0.62 %, respectively **(Chung *et al.*, 2005)**

A phylogenetic tree of TMV isolate revealed 89% a moderate degree of similarity to the other isolate sequences of TMV strains while that obtained by **Cherian *et al.*, (1999),** "TMV (Tom-K)" nucleotide sequence of coat protein *cp-gene* showed 93.1% identity with other TMV strains and by **Chung *et al.*, (2007)**ucleotide sequence of coat protein *cp-gene* of TMV-pet showed 99.0% identity with TMV-potato3-2 (GeneBank accession no. AF318215) isolated from potato showing yellow mosaic and stunt symptom, and with a TMV Korean strain (GeneBank accession no. X68110).

The predicted number of amino acids was produced from translation of partial *cp-gene* nucleotide sequence were 159 amino acids starting with Methionine (M) and ended with Serine (S). Comparison between amino acids composition of partial cp-gene sequence for TMV isolate and nine TMV strains published in GeneBank (accessions (AAK33120_Brazil, AAT45114_Thail-and, P69507_Japan, AAK06750_Korea, AAM64218_China,AAQ12020_Finl and, CAL69972_Vietnam, AC-Y41214_USA and CAD22079_Germany) was done to determine amino acid composition and revealed 82%

amino acids similarity with these TMV strains .

The biological, serological and molecular characters reported in this study confirmed identification of an isolate TMV-*Ch*-EG strain of TMV *Tobamovirus* isolated from *C. morifolium* in Egypt.

In our results shoot tips (explants) were cultivated after sterilization process in media containing 0.25 mg/L BAP . that mainly resulted in the growth of stem, leaves and occurrence of auxiliary shoots. While the micropropagated stage was done by sub cutting of the stem cuttings up to two times every 21 days. These results were in agreement with **Liu and Gao (2007) and Min *et al.* (2004)** who mentioned that rapid propagation technology was established and optimized *in vitro* on MS medium supplemented with 0.3-0.5 mg l-1 benzyl adenine (BA) and 0.3 mg l-1 naphthalene acetic acid (NAA). Root induction and development could be observed within 15 days after inoculation on 1/2 MS medium supplemented with 0.2 mg l-1 indole-3-acetic acid (IAA). Subculture of these shoots on the same medium resulted in similar responses in multiple shoot formation within 4 weeks. Also, the results of **Patil *et al.* (2005)** revealed that the shoot tip explants of chrysanthemum inoculated in MS basal media containing 2 mg BAP and 0.5 mg NAA/L gave better performance in terms of response to shoot initiation, the days to shoot initiation and number of shoots per culture. The best rooting was obtained in MS basal 1.0 mg NAA/L.

Therapeutic treatments were tested since the 1920s to cure valuable cultivars from virus-like diseases.

The obtained results indicated that during *in vitro* heat therapy at 37°C±1 for 14 and 21 days, the percentage of survival was (100%) and (95%) and virus free plantlets was (28.5%) and (42.8%) for *C. morifolium* plants, respectively. These results were in agreement with Ram *etal* , (2005) whom stated that heat treatment (thermotherapy) at elevated temperatures (34-42°C) was used to produce healthy plants from individuals suffering from diseases such as potato witches' broom and aster yellows caused by phytoplasma.

Walkey (1991) reported that for obtaining virus-free materials, addition of antiviral compounds were recommended in the absence of heat treatment and meristem

culture to eliminate viruses especially in the commercial laboratories. Several investigators suggested that the contact of the virus-infected tissues with the culture medium might eliminate viruses present in the tissue. Moreover, the multiplication of viruses was usually decreased when infected plants were treated with chemical analysis to the purine and pyrimidine bases of nucleic acids. These analogues act like heat therapy to increase the success of the apical meristem and tip culture technique.

Harris and Robins (1980); Kim *et al*, (1994) and Ram *et al.* (2005) stated that ribavirin (Virazole or 1-B-Dribofuranosyl-1, 2, 4-triazole - 3-carboxamide), a base nucleotide analogues that has been extensively tested against human viruses, appears to be effective against both RNA and DNA viruses. Many investigators demonstrated that incorporation of ribavirin into culture media resulted in an increased percentage of virus-free progeny.

The obtained results indicated that incorporation of ribavirin (virazole) in culture medium at concentration of 10, 20 and 30 mg/L progressively increase the percentage of virus-free plantlets to (29.4, 31.3 and 58%) in *Chrysanthemum* plantlets respectively. Virazole proved to be somewhat phytotoxic to *Chrysanthemum* plantlets but this effect disappeared gradually 15 days after culture and the plantlets reversed to green color. The percentages of survival by using virazole were (100, 93 and 86%) for *Chrysanthemum* plantlets, while **Albouy *et al.* (1988)** reported that in Orchid cultures, two viral diseases caused by *Odontoglossum ring spot virus* (ORSV) and *Cymbidium mosaic virus* (CyMV) have been reported to limit the production. Attention of virus free orchids by meristem tip culture has proved to be often inadequate. Different chemicals such as virazole have been reported as means of freeing plant culture *in vitro* from viruses. In order to increase the efficiency of eradication of ORSV and CyMV, chemotherapy based on the incorporation of virazole in the solid medium culture has been investigated on Cymbidium protocorms cultured *in vitro*. Factors such as growth regulators and active charcoal are of critical importance. Best results were obtained in shortening the time of transplantation (18 days) allowing a

good excision of Cymbidium new protocorms. After 5 subcultures of 18 days in presence of 25 ppm of virazole we obtained 95 % virus free plantlets.

The obtained results concerning the combination between chemotherapy and thermotherapy by subjecting the infected shoots to 3^°C for 2 weeks produced (95, 90 and 85%) survival for *Chrysanthemum* while the percentages of virus free were (38, 61.5 and 76.8%) for 10, 20 and 30 mg/L virazole, respectively, showed the effects of thermotherapy and chemotherapy (virazole) on TMV elimination from infected *Chrysanthemum* plants.

Finally, the obtained results under this study concerning the combination treatments are promising to be an effective method for TMV eradication from *Chrysanthemum* plants in order to produce TMV-free *Chrysanthemum* plantlets via tissue culture technique.

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