



## Detection and identification of a new isolate of *Grapevine fanleaf Virus* naturally infecting Grapevine plants in Egypt using qReal Time-PCR

Dalia Gamil Aseel<sup>1\*</sup>; Mahmoud Hamdy Abd El-Aziz<sup>2</sup>; E. E. Hafez<sup>1</sup>

<sup>1</sup>Plant Protection and Biomolecular Diagnosis Department, Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Applications, New Borg EL-Arab city, Alexandria, Egypt; <sup>2</sup>Plant Pathology Institute, Agricultural Research Center, Alexandria, Egypt

\*Corresponding author E-mail: [daliagamil52@gmail.com](mailto:daliagamil52@gmail.com)



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### Abstract

*Grapevine fanleaf virus* (GFLV) is a member of the genus *Nepovirus* in the family Comoviridae, a widely distributed virus responsible for grapevine (*Vitis vinifera*) degeneration. This virus causes serious economic losses by reducing grape crop yield. The Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (qReal Time-PCR) assay was carried out on (GFLV) recovered from infected grapevines leaves at Alexandria, Egypt. A 606 bp fragment of the GFLV RNA-2 coat protein (CP) gene was amplified and then sequenced. Results of reactions of diagnostic hosts were observed on *Gomphrena globosa*, which developed systemic mottling, leaves twisting and necrotic spots during spring, whereas *Chenopodium amaranticolor* induced systemic mottling and leaf deformation, and its sap seemed relatively insensitive to the inhibitors of infection. Mottling of *Glycine max* was detected after inoculation, but inoculation of *Nicotiana glutinosa* didn't induce any symptoms. This study aimed to detect and identify a new isolate of GFLV-DA3 from Egypt using biological and molecular tools.

**Keywords:** *Grapevine fanleaf virus*, qReal-Time PCR, Coat protein, Sequencing, Mechanical transmission

### 1. Introduction

Grape is one of the most popular fruits all over the world (Abido *et al.*, 2013). In Egypt, grapes rank the second position in exportation after citrus. The total planted area of the vineyards in Egypt reached 167296 feddan with a production of 1370241 tons according to the latest statistics of the Ministry of Agriculture at (2009). The cultivars in Egypt cover approximately the whole season, these cultivars help in increasing

exports to European, Arab and Asian countries (Ahmed *et al.*, 2012). *Grapevine fanleaf virus* (GFLV) is a member of the genus *Nepovirus* in the family Comoviridae, and is a widely distributed virus responsible for grapevine degeneration. It causes serious economic losses by reducing yield, lowering fruit quality and substantially reducing the longevity of grapevines. Infected grapevines show a range of foliar

symptoms consisting of leaf deformation, yellow mosaic, vein banding, ring and line patterns and flecks (Martelli and Savino, 1990; Andret-Link *et al.*, 2004). GFLV is specifically transmitted by the nematode *Xiphinema index* that feeds on growing root tips (Hewitt *et al.*, 1958; Wyss, 2000). Virus strains were recovered by mechanical inoculation and maintained in *Chenopodium quinoa* (Raski *et al.*, 1983; Bovey *et al.*, 1990; Martelli and Savino, 1990). The genome of GFLV is bipartite and composed of two single-stranded positive-sense RNAs (Pinck *et al.*, 1988). RNA1 encodes the poly protein P1 which matures into the VPg (viral protein genome-linked), the RNA polymerase, the proteinase and the NTP-binding protein. RNA2 encodes the polyprotein P2 that is subsequently cleaved into the movement protein and the 56 kDa coat protein (CP) reported by (Serghini *et al.*, 1990; Gaire *et al.*, 1999; Elbeaino *et al.*, 2011).

The aims of the present study were; a)-to study the symptomology of some host ranges such as; *Chenopodium amaranticolor*, *Gomphrena globosa*, *Nicotiana glutinosa* and *Glycin max*, which were the most readily infected and common test plants routinely employed, b)-to partially characterize the GFLV-Egyptian isolate based on CP gene using; qRT-PCR, amplification of GFLV-CP gene using RT-PCR, sequencing and phylogenetic tree.

## 2. Material and methods

### 2.1. The natural plant source of GFLV

Naturally infected grapevines leaves were collected from several grapevine fields at New Borg El-Arab city, Alexandria, Egypt. The collected leaves were showing typical systemic symptoms of GFLV. Severe deformation of young grapevine leaves and conspicuous vein-clearing of these expanded leaves were observed.

### 2.2. Detection and identification of GFLV isolate

#### 2.2.1. Isolation of total RNA from leaves naturally infected with GFLV

Total RNA was extracted from grapevine leaves using RNeasy Mini Kit (QIAGene, Germany) according to the manufacturer's instructions, and then dissolved in diethyl pyro-carbonate treated water. The obtained RNA was dissolved in diethyl dicarbonate-treated water, incubated with DNase for 1 h at 37°C to remove any DNA residues, and then quantified using a Nano Drop 1000 spectrophotometer (Thermo Scientific, USA).

#### 2.2.2. cDNA synthesis and detection of GFLV-CP gene using the qRT-PCR assay

In a total volume of 25 µl, the reaction mixture contained; 2µg RNA (3 µl), 10 mM dNTPs (2.5 µl), 10x buffer with MgCl<sub>2</sub> (2.5 µl), 10 pmol/ µl reverse GFLV-CP primer (4 µl), and 0.2 µl of reverse transcriptase enzyme (BioLabs, New England). The qRT-PCR reaction mixture was then incubated at 37°C for 2 h, and inactivation period at 65°C for 20 min. The qRT-PCR was carried out using a Sure Cyclor 8800 thermocycler (Agilent Technologies, USA). The current qRT-PCR assay of the GFLV-CP gene was done using the protocol described by Aseel and Hafez, (2017).

#### 2.2.3. Amplification of the GFLV-CP gene using RT-PCR

For PCR amplification, a sense of the GFLV-CP gene (5'- GTGAGAGGATTAGCTGGT-3') and the anti-sense (5'-AGCACTCCTAAGGGCCGT-3') were designed from the CP gene located in the RNA2 of the GFLV infected leaves, according to Fattouch *et al.*, (2001). The PCR amplification was carried out using 10 ng cDNA (1 µl), 10x buffer mix (12.5 µl), 10 pmol/µl of each primers (2 µl), 5U Taq polymerase (0.25 µl, Bioline, Germany), and a final volume up to 25 µl with sterile water. The PCR reaction conditions were: initial denaturation at 95°C for 3 min. followed by 30 cycles; denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, and elongation at 72°C for 1 min. Final elongation at 72°C was done for 5 min. The PCR amplification products were separated by 2 % agarose gel electrophoresis according to Aseel *et al.*, (2019).

#### 2.2.4. Sequencing, phylogenetic analysis of the GFLV-CP gene, and deduced amino acid sequence analysis

The amplified CP gene of the GFLV was sequenced using an automated sequencer (Macrogen Company, Korea), with forward universal primer. The nucleotide sequence was aligned using NCBI-BLAST, and then compared to the other *Nepoviruses* available in the GenBank database (<http://www.ncbi.nlm.nih.gov>). For using amino acids sequence analysis, the DNA sequences were translated to deduced amino acids and aligned using the ClustalW2 Multiple Sequence program (Rice *et al.*, 2000; Larkin *et al.*, 2007). The alignment and phylogenetic analysis were carried out using the Molecular Evolutionary Genetics Analysis as described by Kawanna and Aseel, (2019).

#### 2.3. Mechanical transmission of the GFLV in different herbaceous plants

Grape-leaf sap was manually inoculated to herbaceous plants by the method previously described by Cadman *et al.*, (1960). Approximately, 0.2-0.5 g of young grape leaf was macerated in 5 ml (0.1 M) phosphate buffer (pH 7) and carborandum 600 mesh (Hamza *et al.*, 2018). The herbaceous plants used include *C. amaranticolor* and *Gomphrena globosa*, which were the most readily infected plants and the common test plants routinely employed. All the herbaceous plants were kept in darkness for 1-2 d before inoculation, to increase their susceptibility to infection. After inoculation, the leaves were rinsed with tap water and then kept in daylight in an insect free glasshouse. The grapevine viruses were maintained in *C. amaranticolor* or *Gomphrena globosa* as sources of inoculum, their sap were prepared immediately before use. *C. amaranticolor* was also used for infectivity assays of the host plant. The collected virus sample was therefore inoculated into *N. glutinosa*, *Glycin max*, *C. amaranticolor* and *Gomphrena globosa* plants.

### 3. Results and Discussion

#### 3.1. The natural source of GFLV

The symptoms observed on GFLV naturally infected grapevine leaves collected from New Borg El-Arab city, Alexandria including; vein banding, obscure speckles, small yellow lesions, shortened internodes, leaf deformations, mottling, vein clearing, and fan leaf (Fig. 1), are in agreement with Martelli and Savino, (1990); Andret-Link *et al.*, (2004).

#### 3.2. Detection and identification of GFLV isolate

##### 3.2.1. Quantitative expression of the GFLV-CP gene using qReal-Time-PCR

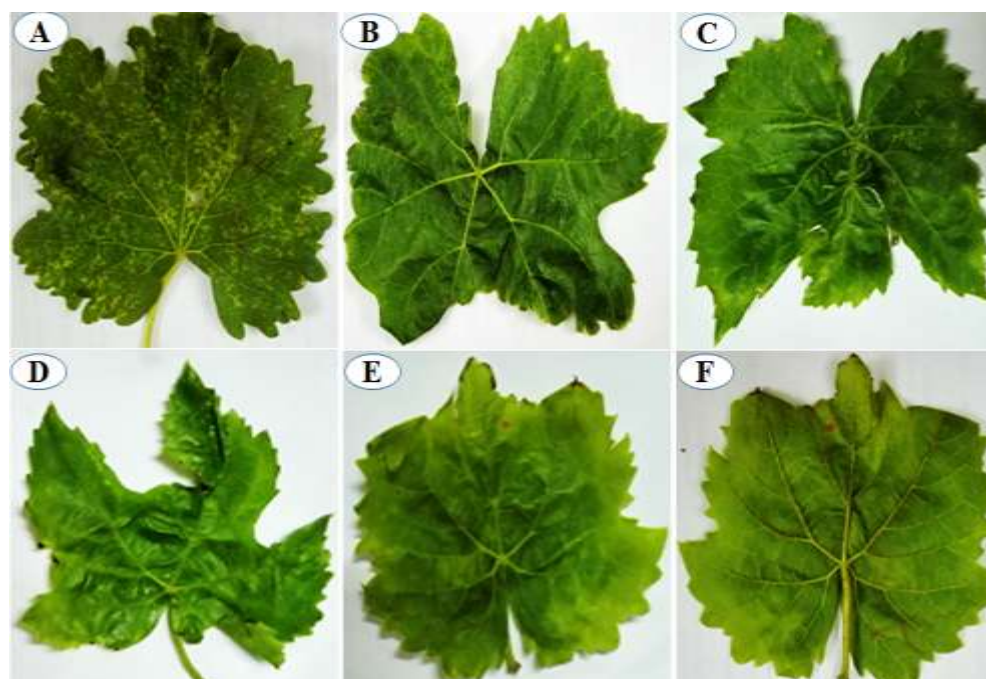
In the current study, measurement of the GFLV-CP gene expression was carried out using qReal-Time-PCR. Results recorded high expression level of about (~35-fold) of the GFLV-CP gene detected in the infected symptomatic leaves; however, no expression of this gene was observed with the healthy grapevine leaves as clear in Fig. (2).

##### 3.2.2. Amplification of the GFLV-CP gene using RT-PCR, phylogenetic tree construction, and amino acid sequence analysis

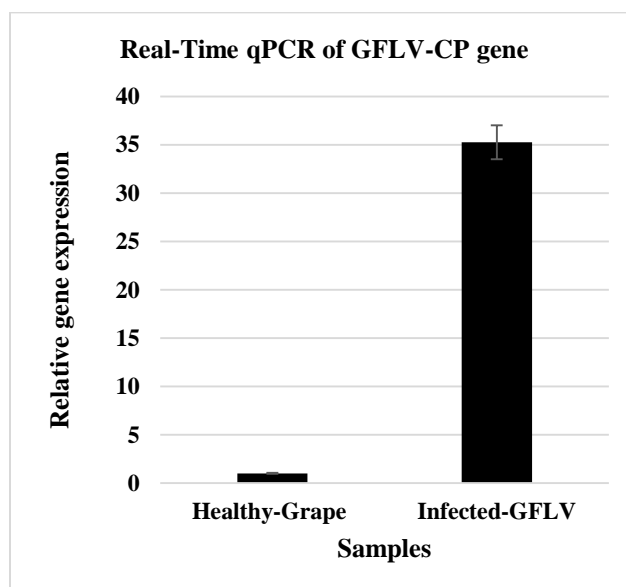
The primers successfully amplified the cDNA product (606 bp) of the viral CP gene recovered from leaves infected with GFLV, whereas, no fragments are detected with the healthy plant (negative control) leaves, these results are shown in Fig. (3A). These results are in agreement with previous findings of Fattouch *et al.*, (2001). Partial sequences of the GFLV-CP gene is aligned and compared with other *Nepoviruses* available in the GenBank database. The phylogenetic relationships were generated using the MEGA4 Bootstrab neighbor joining method. The Egyptian isolate of Grapevine fanleaf virus-DA3 is closely related with the *Grapevine fanleaf virus* GFLV-CP genes (AF4185790; AF304014, and JN585800) from Brazil; USA and Spain, with a nucleotide sequence identity of 94% (Fig. 3B).

The GFLV-CP sequences were aligned with different CP genes available in the GenBank database using the

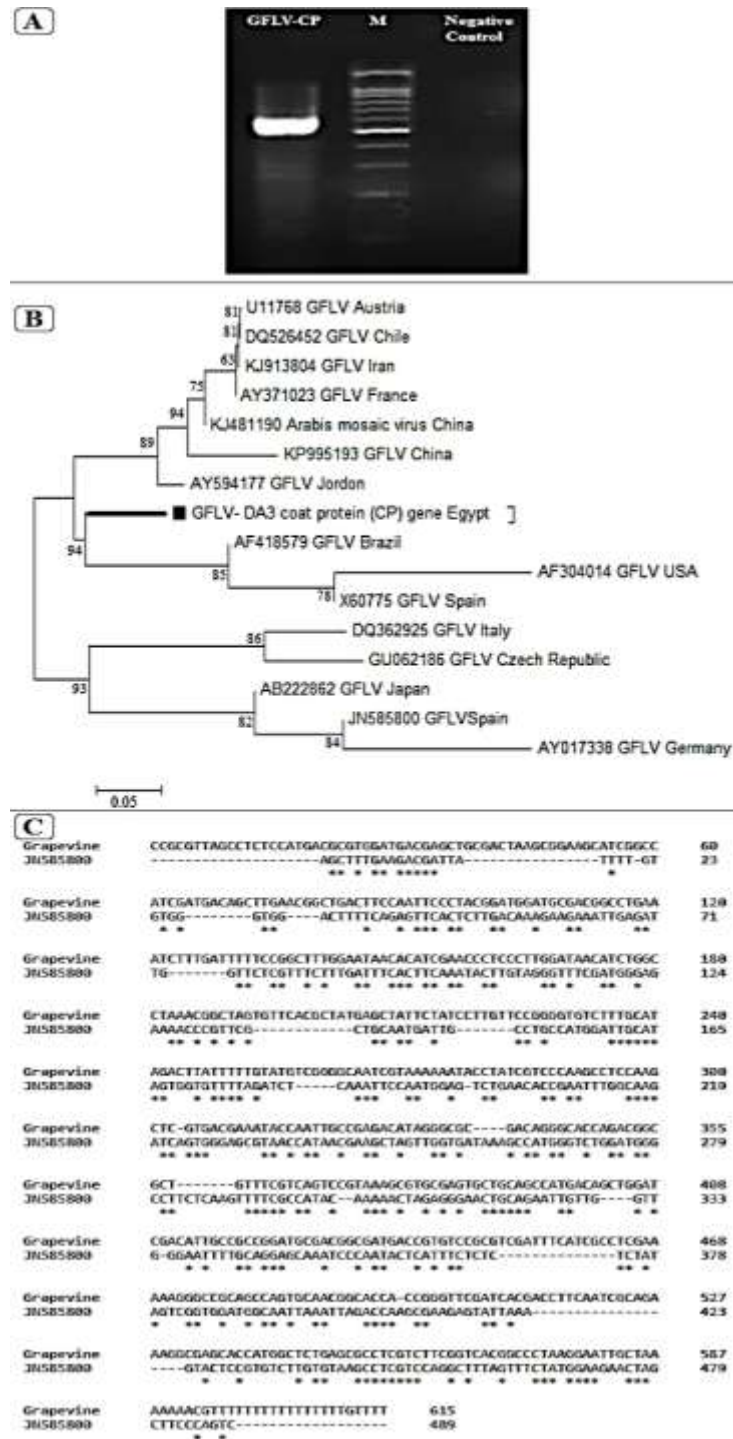
Clustal W2 Multiple Sequence Alignment program (1.83) software (Fig. 3C).



**Fig. 1:** Natural symptoms of grapevine leaves infected with GFLV collected from different localities in Egypt. Where; (A) Systemic mottling; (B, C, D, E) leaf deformation; (F) vein clearing and necrotic spots.



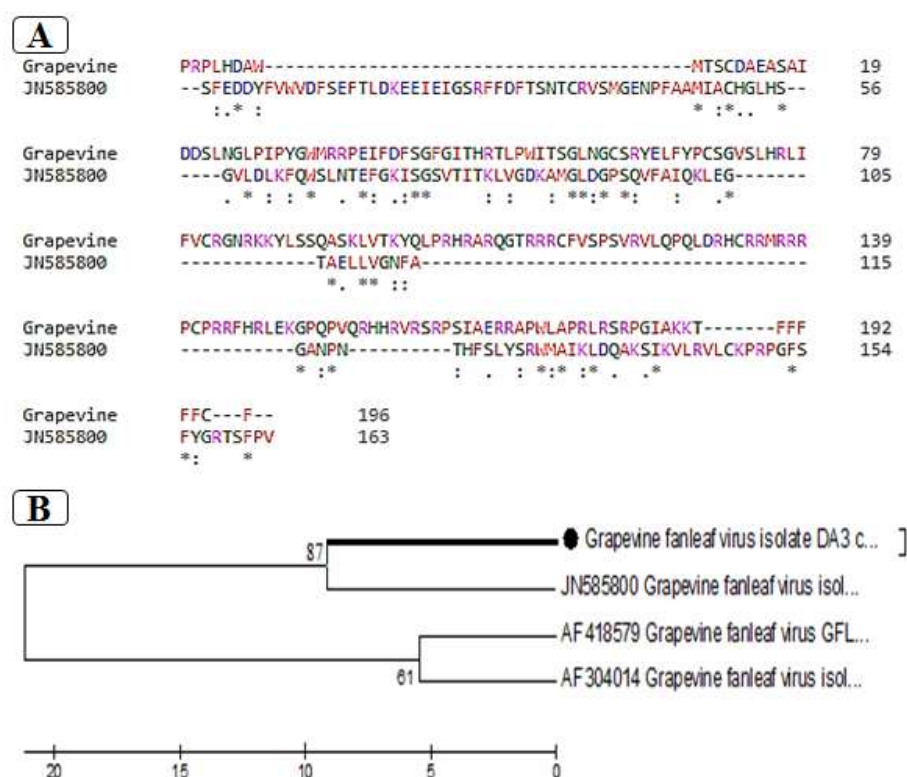
**Fig. 2:** Measurement of Coat protein (CP) gene expression of the *Grapevine fanleaf virus* using Real-Time qPCR.



**Fig. 3:** (A): Detection of GFLV in Egyptian grapevine leaves by RT-qPCR. DNA fragments were analyzed by electrophoresis on 2% agarose gels. Amplified cDNA product (627 bp) with CP: GFLV-CP; M 1.5 Kbp DNA marker; Negative control (Healthy plant). (B): Phylogenetic relationships of CP gene of *Grapevine fanleaf virus* with other *Nepoviruses* available in the GenBank database. This phylogenetic tree was generated using Bootstrab neighbor joining method in MEGA4. The scale for the branch length was given in substitutions per site. (C): Nucleotide sequence alignment of GFLV-CP gene DNA sequences with CP gene of *Grapevine fanleaf virus* isolate from Spain available in GenBank. (\*): Asterisks indicate the consensus sequence. (-): indicate absent nucleotides.

According to the previous results of Fazeli *et al.*, (2000); Youssef *et al.*, (2006), their putative GLRaV-1 sequence is closely related to the current Egyptian GLRaV-1 sequence with an identity of 95%. Moreover, analysis of the phylogenetic tree showed noticeable similarity with the newly Czech isolates sequenced from South Moravia, and relatively high dissimilarity from the rest of the analyzed isolates including the previously sequenced isolate HV5 from South Moravian region as reported by Eichmeier *et al.*, (2010). In the current study, results of the deduced amino acid sequence showed variations of the GFLV

isolate from Spain, i.e. H → E, A → G, R → N, S → E, D → K and S → Q with substitutions in the Egyptian GFLV-DA3 isolate (Fig. 4A). The phylogenetic tree of the deduced amino acid sequence demonstrated that the GFLV-DA3 Egyptian isolate is closely related with *Grapevine fanleaf virus* GFLV from Spain with amino acid sequence identity of 87% (Fig. 4B). Similar results were obtained by Izadpanah *et al.*, (2003), they observed that the inferred amino acid sequences were 96% similar. Where, many of the nucleotide differences either were silent or led to conservative amino acid substitutions.



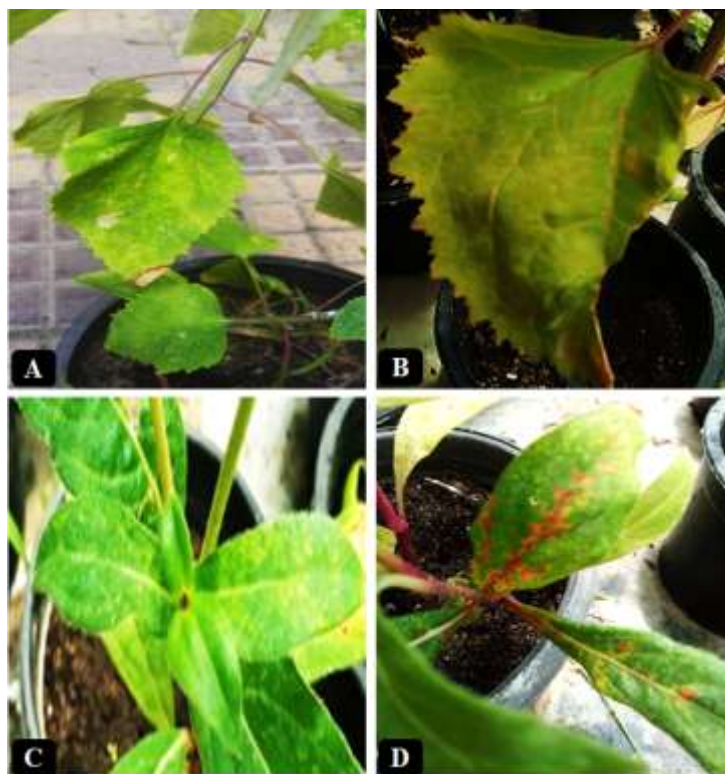
**Fig. 4:** (A): Alignment of deduced amino acid sequence of GFLV-CP gene with *Grapevine fanleaf virus* isolate from Spain available in GenBank. (\*): Asterisks indicate the consensus sequence; whereas, (-): indicates absent amino acids, (:): indicates degeneracy, (.) indicates substitution. (B): Phylogenetic tree for GFLV-CP gene based on the deduced amino acid sequences using Bootstrab, UPMEGA method in MEGA4 program.



### 3.3. Reactions of the diagnostic herbaceous hosts

The isolate of *Grapevine fanleaf virus* is transmitted from the infected plants, but the symptoms produced in *C. amaranticolor* and *Gomphrena globosa* plants are unlike those produced by isolates from other grapevines with yellow mosaic. *Gomphrena globosa* developed several symptoms including; systemic mottling, leaves twisting and necrotic spots during spring, whereas *C. amaranticolor* showed systemic mottling and leaf deformation. The virus seemed relatively insensitive to the inhibitors of infection present in the sap of *C.*

*amaranticolor*. On the other hand, *Glycine max* mottling appeared after inoculation (Fig. 5A-5D); however, *N. glutinosa* doesn't show symptoms after inoculation. The symptoms are developed on the diagnostic hosts on inoculation with the GFLV isolated from naturally infected vitis plants in accordance with Cadman *et al.*, (1960); Raski *et al.*, (1983); Bovey *et al.*, (1990); Martelli and Savino, (1990). GFLV is sap transmitted to a limited range of hosts; these results are in agreement with the finding of Cadman *et al.*, (1960); Dias, (1963). *N. glutinosa* reacted negatively with the GFLV isolate.



**Fig. 5:** (A): Flaking symptoms induced by infecting *Chenopodium amaranticolor* with GFLV; (B): Induced systemic mottling and leaf deformation after mechanical inoculation by GFLV on *Chenopodium amaranticolor*; (C): Systemic mottling developed and leaf deformation appeared on *Gomphrena globosa* leaves infected by GFLV; (D): Leaves twisting and necrotic spots around the main vein due to injury of *Gomphrena globosa* leaves with GFLV.

## Conclusion

To our knowledge, this is the first study carried out in Egypt concerning the detection and identification of a new isolate of *Grapevine fanleaf virus-DA3*, from naturally infected grapevine field. The Real Time-qPCR approach demonstrated high specificity and sensitivity in the detection of this isolate of GFLV.

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## Conflict of interest

The author(s) declare that they have no competing interests.

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