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Research Papers

Occurrence of *Grapevine fanleaf virus* in Algerian vineyards, and complete genome sequencing

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Summary. Prevalence and genetic diversity of *Grapevine fanleaf virus* (*Nepovirus foliumflabelli,* GFLV) were determined in vineyards and grape varieties in Algeria. Samples (414) from different cultivars and viticulture areas were screened using DAS-ELISA and partially confirmed by RT-PCR, revealing 21% infection incidence. In Ahmer Bou Amer the greatest incidence of infection was recorded (61%). Some vines, confirmed to be GFLV-infected, had characteristic symptoms of leaf yellowing, chloroses, and mosaic patterns, reducing vine vigour and fruit quality. High throughput sequencing and bioinformatics analyses of a single GFLV-infected accession obtained a nearly complete grapevine fanleaf virus RNA1 consensus sequence of 5,979 nt, and an RNA2 with complete consensus sequence of 3,711 nt. *Grapevine yellow speckle viroid*, *Hop stunt viroid* and other viruses were also identified in the 'background' virome. Phylogenetic analyses of an amplified fragment of the GFLV coat protein gene from some of the accessions indicated close genetic relationships between Algerian and Russian/United States of America GFLV isolates, suggesting potential shared origins or transmission pathways. These results emphasize the need for implementing strict phytosanitary measures (e.g. use of virus-free planting material) to mitigate GFLV spread and its detrimental effects on grapevine production in Algeria.

Keywords. Grapevine, GFLV, DAS-ELISA, High Throughput Sequencing, RT-PCR.

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INTRODUCTION

Grapevine is the most important fruit crop in the world, with cultivation area of 6.7 million hectares producing 74 million tons of grapes annually. In Algeria, viticulture is carried out on 64,720 ha yielding 627,325 tons each year (FAOSTAT, 2022). Grapevines are susceptible to a several pests and pathogens, among which infectious intracellular agents (viruses, viroids, phloem and xylem limited prokaryotes) cause significant crop losses. Among diseases caused by viruses, the most important are grapevine degeneration and decline caused by nepoviruses, leafroll disease, the rugose wood complex and fleck disease (Fuchs, 2024).

Grapevine fanleaf virus (*Nepovirus foliumflabelli,* GFLV) is one of the 15 viruses implicated in fanleaf degeneration, a severe and widespread grapevine disease. (Schmitt-Keichinger *et al*., 2017). This virus can reduce vineyard yields, fruit quality, and decrease vine lifespans (Krebelj *et al*., 2015). GFLV is a member of the *Nepovirus* (*Secoviridae*), and is transmitted by the nematode *Xiphinema index* (M'rabet Samaali *et al*., 2024), in a nonpersistent, noncirculative manner (Demangeat, 2007; Fuchs *et al*., 2017).

Symptoms of GFLV infections appear in early spring and are in two main syndromes; leaf discolouration (mosaic patterns, vein discolouration, chloroses) and leaf malformation (deformation, close veins, serrated edges, enlarged petiolar sinuses) (Cigsar *et al*., 2003; Schmitt-Keichinger *et al*., 2017). Affected shoots exhibit fasciations, double nodes, and short internodes, with zigzag growth patterns. The fruit bunches on infected vines have clusters with small, unevenly ripened berries, which are few and irregular (Digiaro *et al*., 2017).

GFLV has a genome consisting of two single-stranded positive-sense RNAs (Vigne *et al*., 2008; Sanfaçon et *al*., 2009). Each RNA contains a single open reading frame (ORF) translated as a single polyprotein matured into functional products. The RNA 1 polyprotein (P1) with 7342 nt encodes the five proteins, protease cofactor (1A), helicase (1BHe), genome-linked protein (1CVPg), protease (1DPro), and RNA-dependent RNA polymerase (1EPol) (Vigne *et al*., 2004b). The RNA 2 polyprotein (P2), consisting of length 3774 nt, encodes protein $2A^{HP}$, designated as a putative homing protein necessary for the replication of RNA2, the movement protein (2BMP), and the coat protein (2Ccp) (Vigne *et al*., 2008; Mekuria *et al*., 2009). Both RNAs are polyadenylated at their 3' ends, and have a small covalently attached VPg protein (Mekuria *et al*., 2009; Elbeaino *et al*., 2014).

The GFLV genome has been characterized. High-throughput sequencing (HTS) of GFLV isolates from infected vines in the Champagne region of France showed that most grapevines were infected with multiple genetically diverse variants (Kubina *et al*., 2022). Additionally, *de novo* assembly of three GFLV genomes, along with those of other viruses and viroids, was achieved from four RNAseq datasets using HTS in Colmar, France (Hily *et al*., 2018). The complete RNA1 and RNA2 sequences of a new GFLV isolate (GFLV-SDHN) from northeastern China were determined, revealing unique genetic features, including a distinct insertion and significant sequence divergence compared to other isolates, along with evidence of a recombination event in the RNA2 2AHP region (Zhou *et al*., 2017).

In addition to virus pathogens, viroids are also important infectious agents affecting grapevine (Di Serio *et al*., 2017). Recently, six grapevine infecting viroid were known in the Mediterranean basin (Kaponi *et al*., 2024)

Based on coat protein (CP) genes, genetic diversity of GFLV has been was determined in South Africa (Liebenberg *et al*., 2009), Russia (Porotikova *et al*., 2021), China (Zhou *et al*., 2015), Tunisia (Fattouch *et al*., 2005) and France (Vigne *et al*., 2004a), but genetic diversity of GFLV in Algerian isolates is unknown, although some studies have hinted at presence of the virus in the center and west of Algeria (Tahirine *et al*., 2020).

HTS is an technology for analyzing genomic data (Mokili *et al*., 2012; Massart *et al*., 2014; Brister *et al*., 2015), and identifying virus pathogens *de novo* (Adams *et al*., 2009; Kreuze *et al*., 2009; Barba *et al*., 2014; Al Rwahnih *et al*., 2015). A study on Iranian grapevine cultivars by using HTS identified thirteen viruses and viroids, with grapevine red blotch, satellite, grapevine leafroll associated virus 1, and *Grapevine fanleaf virus* dominating (Gholampour *et al*., 2024).

The main objectives of the present study were to determine the presence and distribution of GFLV among different grapevine cultivars from multiple commercial vineyards, including autochthonous and Saharan grapevine accessions in Algeria, and to compare the phylogenetic relationships within the Algerian isolates with those from other countries.

MATERIALS AND METHODS

Virus sources

The field study and sample collections were carried out during autumn 2021 and winter 2022, in the major grapevine growing areas in western (Aïn Temouchent, Mascara, Sidi Belabes and Mostaganem) and central (Algiers, Tizi-Ouzou, Medea, Blida and Boumerdes) regions of Algeria (Figure 1). A total of 414 samples were

collected from individual vines of 24 different grapevine varieties, including commercial, autochthonous, and Saharan varieties. Mature canes were randomly collected. Each sample, consisting of four dormant cuttings, was split into two subsamples for serological and molecular analyses, and were stored at 4°C. During sampling, some randomly collected grapevine plants exhibited characteristic symptoms of GFLV infection. Approx. 20% of the selected samples showed visible symptoms, and these symptomatic vines were photographed for further documentation.

DAS-ELISA tests

To assess prevalence of GFLV viruses in Algeria, all the samples were tested by double sandwich ELISA (DAS-ELISA) (Adams and Clark, 1977) using the GFLV Commercial kit (Bioreba AG), following the manufacturer's protocol. Extracts were obtained by decorticating phloem tissue macerated in PBS buffer using 2 g per 10 mL. Absorbance was recorded at 405 nm using an automatic microplate reader.

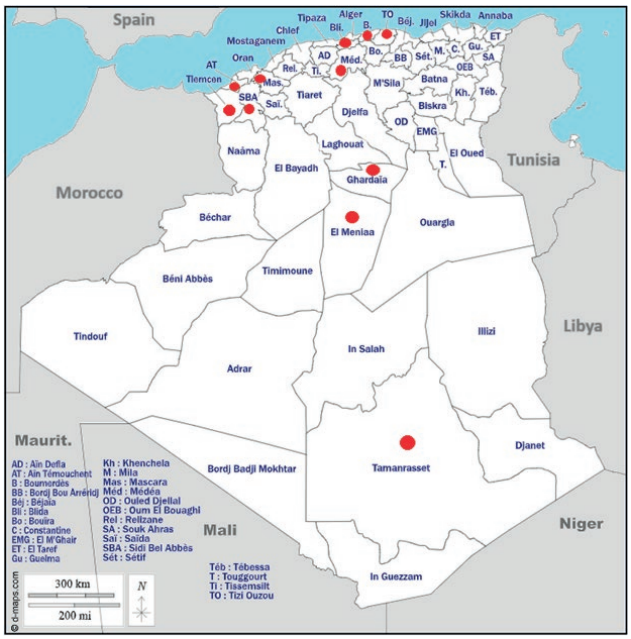

Figure 1. Map of Algeria showing the primary grapevine sampling regions for GFLV assessments. The red circles represent the locations where the samples were collected.

Molecular detection of viruses

Total nucleic acid extraction. Positive samples shown by DAS-ELISA were used for the subsequent molecular analyses, and total nucleic acids were extracted using the protocol of Gambino *et al.* (2008), with modifications. Phloem tissue (0.2 g) was ground in liquid nitrogen, then homogenized with 1 mL of grinding buffer (2% CTAB, 2,5 PVP 40, 2M NaCl, 100mM Tris-HCl pH 8, 25mM EDTA pH 8) prewarmed to 65°CThe solution was then centrifuged, and the supernatant was recovered. An equal volume of a mixture chloroform/isoamyl alcohol (24:1) was added to each tube and shaken for 45 min at low speed. After centrifugation for 15 min at 13,000 rpm, the supernatant from each tube was collected in a new tube. The precipitations were carried out in 2/3 volume of cold isopropanol (-20°C), with incubation for 20 min at -20°C followed by 10 min centrifugation at 13,000 rpm. The washing step was then carried out using ethanol 70%. The resulting pellet was re-suspended in 100 µL of sterile distilled water, and then stored at (-20°C).

Reverse transcription and amplification. Denaturation of total RNA was carried out 70°C by mixing 10 µL of each sample with 1 µL of random primer and 5.5 µL of sterile water. Reverse transcription was carried out using MMLV reverse transcriptase (200 U μL^{-1}). The cDNA was amplified using the Taq polymerase (HOT FIREPol DNA polymerase) and the primer pairs C3310 5′-GATGGTAACGCTCCCGCTGCTCTT-3′ and H2999 5′-TCGGGTGAGACTGCGCAACTTCCTA-3′ designed by MacKenzie *et al.* (1997) were used to amplify a fragment of 312 bp of the coat protein (CP) gene region. PCR cycling conditions used for amplification were: an initial denaturation at 90°C for 12 min, followed by 35 cycles each of denaturation at 90°C for 30s, 52°C annealing for 45 s and 72°C for 1 min, and final elongation at 72°C for 7 min. The PCR was hot-started at 92°C for 10 min, and completed by a 10 -min elongation step at 72°C. Amplified products were electrophoresed on 1.5% agarose gels in 1× tris-acetic-EDTA pH 8.0, and were visualized with gel red under UV light.

Sequence analyses. The amplified true size products were gel-purified using the NucleoSpin Gel & PCR Clean-up Mini kit (MACHEREY-NAGEL), according to the manufacturer's protocol. The purified DNA fragments were then cloned into the pGEM-T Easy Vector System II (Promega) using T4 DNA ligase. The ligation products were then transformed into Escherichia coli JM109 competent cells. Plasmid DNA from positive clones were isolated using the QIAprep Spin Miniprep Kit (Qiagen), following the manufacturer's instructions. The positive clones were sent for sequencing to Eurofins Genomics (Germany), and T7 and SP6 primers were used to obtain the entire sequence for each fragment.

High throughput sequencing

Total RNA extraction and Illumina-HTS. A single grapevine cane sample from the autochthonous cultivar 'Cherchalli' (ALG101) was selected for high-throughput sequencing to obtain the complete virome of the virus in the sample, which was known to be infected by GFLV. The sample was placed in Eppendorf tubes and immersed in RNALater solution, and then sent to the South Korean company JS-link, for further HTS production. QIAGEN's (Hilden, Germany) RNeasy® Plant Mini Kit was used to extract RNA from the plant sample, As part of the Illumina HTS library preparation, total RNA sequencing was carried out using the TruSeq Total RNA Library Prep Kit. After RNA quality was checked with an Agilent 2100 Expert Bioanalyzer (Agilent), the sequences from the RNA library were generated using NovaSeq 6000, 2× 100PE (Illumina, Inc.). Raw reads were trimmed (Kearse *et al*., 2012b) using Trimmomatic-0.39 and BBduk v 37.22 in Geneious Prime® 2024.0.5 (Kearse *et al,*. 2012a)

Map to reference. Mapper Geneious RNA was used in map to reference runs to map RNASeq data to reference sequences of principal grapevine affecting viruses (Sensitivity: Medium-Low). A consensus sequence was extracted from the RNA clean reads after mapping against suspected virus genomes using Geneious Prime® 2024.0.5 (Kearse *et al*., 2012a). The complete RNA reads were also mapped against a concatenated sequence (76,145,671 nt long), representative of all the 5040 plant virus sequences in GenBank. Results are displayed in a report that includes assembled reads, total used reads, coverage, and pairwise identity (Khaffajah *et al*., 2022).

Phylogenetic analysis. Phylogenetic analysis of the CP gene of the retrieved GFLV genome was carried out with sequences from different origins downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). The alignment was carried out using MEGA11, and the phylogenetic relationships were constructed by the neighbor-joining method, with 1,000 bootstrap replications (Tamura *et al*., 2021), with *Grapevine fleck virus* (GFKV) isolate MT48 (NC_003347.1) as the out-group (Figure 3).

RESULTS

Symptoms caused by GFLV

Most of the surveyed vineyards, monitored across various regions in central Algeria during the spring of 2022 exhibited a range of disease symptoms, including leaf deformation, vein banding, mottling, proliferation, and yellow mosaic. The most common symptoms observed were leaf yellowing, vein banding, and deformation (Figure 2). The grapevine cultivars sampled in this study and the sampling locations are listed in Table 1.

In 'Seibel', the leaves each had fanleaf deformation, which is a symptom specific for GFLV. This symptom, along with chloroses, can reduce photosynthetic efficiency of grapevines, reducing overall vigour and productivity. 'Ahmer Bou Amer' exhibited yellow chloroses and mosaic patterns on the leaves, with irregularly distributed discolouration across the leaf surfaces.

GFLV incidence in Algerian grapevines

Results showed that GFLV was present in 87 (21%) of the samples tested by DAS-ELISA assay. The greatest incidence (61%) was observed in the variety 'Ahmer Bou Amer', followed by 'Carignon' (45%), 'Alicante' (40%), 'Muscat Italy' (28%), 'Cardinal' (27%), 'Cinsault' (26%), 'Seibel' (21%), 'Michel Palieri' and 'Ladhari' (20%), 'Gros Noir' (10%), 'Dattier' (6%), and 'Muscat' (5%) (Table 1). In contrast, the varieties 'Mersguerra', 'Valensi', 'Metllili', 'Sebseb', and 'Issers' were free of GFLV (Table 1). In

Figure 2. Grapevine leaf symptoms observed in Centre Algeria. (A) fanleaf with yellow mosaic, (B) chlorosis, and (C) vein discolouration with leaf malformation and yellowing.

Algeria, GFLV incidences were 59% in the central region and 41% in the western region. However, samples from the southern region (Sahara region Tamanrassetm, El Meniaa and Ghardaia) were negative for GFLV (Table 1).

Sequences analysis of the CP gene

Based on geographic origin and grapevine cultivar, 12 GFLV ELISA-positive samples were selected for further RT-PCR analyses. These samples included three from cultivars 'Muscat', 'Ahmer Bou', 'Amer' or 'Seibel' from the central region, and 'Dattier' from the western region. Among these, five cultivars were selected for variability analyses of a 312 bp fragment of the cloned CP gene sequences, to determine genetic diversity across different regions and grapevine varieties. The obtained sequences were submitted to GenBank, and accession numbers was assigned as: 'Ahmer Bou Amer', PP983215; 'Muscat Italy', PP983212; and 'Seibel', PP983213; from central Algeria: 'Dattier', PP983214) from the western region: and 'Cherchalli', PP976050 from central Algeria. These five sequences were selected for NGS analyses.

Table 1. Grapevine virus infection rates of GFLV detected in Algeria using double antibody sandwich (DAS-ELISA).

Grapevine cultivar	Number of samples collected from main viticulture regions in Algeria			Number of infected samples	Infection rate (%)
	Center	Western	South		
Ahmer Bou Amer	34	-	-	21	61.8
Dattier	15	-	-	2	6.7
	-	30	-	1	
Michel Palliri	10	-	-	2	20.0
Muscat Italie	28	-	-	8	28.6
Gros Noir	19	-	-	2	10.3
	-	10	-	1	
Seibel	28	-	-	6	21.4
Cardinal	27	-	-	9	27.0
	-	10	-	1	
Red Globe	20	-	-	0	0.0
Issers	4	-	-	0	0.0
Ladhari	-	20	-	4	20.0
Carignon	-	20	-	9	45.0
Cinsault	-	60	-	16	26.7
Alicante	-	10	-	4	40.0
Mersguerra	10	-	-	0	0.0
Muscat	-	20	-	1	5.0
Valensi	-	20	-	0	0.0
Sebseb	-	-	2	0	0.0
Metlili	-	-	4	0	0.0
Tazrouk Elkahla	-	-	1	0	0.0
Tazrouk Elbeidha	-	-	1	0	0.0
Tazrouk Sans Pepin	-	-	1	0	0.0
Dattier De Beirouth	-	-	1	0	0.0
Vigne Commun	-	-	1	0	0.0
Unknown	-	-	8	0	0.0
Total		414		87	21.0

To infer the phylogenetic relationship of the Algerian isolates, five Algerian coat protein (CP) gene sequences were aligned with homologous sequences from other countries, including 13 from France, two from Russia, eight from the United States of America, one from Iran, and one from Türkiye, all of which were retrieved from the GenBank database. The resulting phylogenetic tree showed that most Algerian isolates, particularly 'Seibel'" and 'Ahmer Bou Amer', exhibited close genetic relationship with European isolates, particularly those from Russia. The 'Muscat' and 'Dattier' isolates clustered with United States of America isolates, while the isolate GFLV ALG 101 clustered with the France isolate (Figure 3).

HTS analysis

The Illumina platform generated 47,034,642 short reads of a total of 101 bases. In Geneious software, all RNA reads were paired and mapped against suspected virus and viroid genomes. Against *Grapevine fanleaf virus* RNA1, 228,166 reads (0.48%) were assembled into a nearly complete consensus sequence contig of 5,979 bp. Two parts were missing, which were not covered by the reads. The first part was 15 bp between 5,980 and 5,994 bp, and the second was 73 bp between 6,801 and 6,873 bp. The coverage was 98.8%, and the GC content was 46.8%. The sequence covered 5,721 bp of 6,855 bp of the whole polyprotein region. This sequence was deposited in GenBank under accession number PP976049, and named ALG100. The partial genome portion reconstructed by reads assembly had one open reading frame coding for RNA helicase and RNA dependent RNA polymerase with length 5,721 nt, which encodes 1,907 amino acids residues. The assembled reads were 301,093 against the sequence of *Grapevine fanleaf virus* RNA2 (0.64%), and produced a complete consensus sequence of 3,711 bp. The coverage was 97.5% and the GC content was 49.9%. The sequence was 3,333 bp long, that encodes 1,111 residues representing the polyprotein P2 region, and was deposited in GenBank under accession number PP976050, and named ALG101. The P2 sequence codes for a typical Nepovirus subgroup A polyprotein and coat protein. In addition to the complete GFLV genome, two other viroids were identified as *Grapevine yellow*

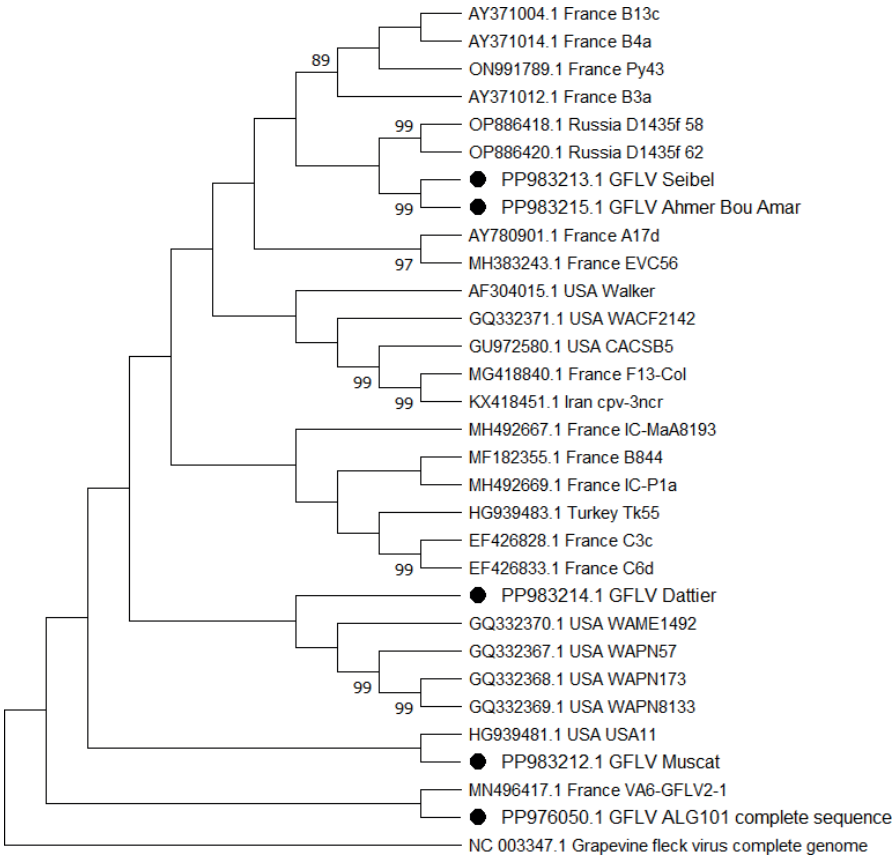

Figure 3. Phylogenetic tree of *Grapevine fanleaf virus*, constructed with sequences of a 312nt fragment of the viral capsid protein (CP), constructed using the neighbor-joining method with 1,000 bootstrap replicates. ● indicates Algerian isolates.

speckle viroid (PP977156) and *Hop stunt viroid* isolate (PP977157). Furthermore, *Grapevine Rupestris stem pitting-associated virus (GRSPaV)* and *Grapevine Pinot gris virus (GPGV)* were also detected. These viruses will be further characterized in a separate study.

DISCUSSION

The survey of vineyards across regions in Algeria revealed prevalence of symptoms suggestive of viral infections in grapevines. The observed symptoms, including leaf deformation, vein banding, mottling and yellow mosaic, were characteristic of virus etiology, aligning with reports from other viticulture regions.

These symptoms are often associated with the *Grapevine fanleaf virus* (GFLV), which have been widely documented as threats to grapevine health and productivity (Andret-Link *et al.,* 2004; Martelli, 2014). The widespread occurrence of these symptoms in Algeria may be influenced by environmental factors and agricultural practices that favour virus persistence and transmission. North Algeria has a moderate climatic conditions and intensive viticulture, which together can create conducive environments for GFLV spread through vectors such as the nematode *Xiphinema index* (Martelli, 2014; El Sayed *et al*., 2023). Additionally, the lack of rigorous virus-free certification programmes for planting material could contribute to dissemination of infected propagation stocks (Panno *et al*., 2021). The observed symptoms in the grapevine samples examined in the present study indicated infections caused only by GFLV. The key symptoms identified included chlorosis or yellowing along the leaf veins and edges, with some cases exhibiting distinct mosaic patterns (Martin *et al*., 2021; Kubina *et al*., 2022). These symptoms were particularly pronounced in 'Seibel' and 'Ahmer Bou Amer' grapevine cultivars.

DAS ELISA test results revealed an infection rate of 21% out of 414 samples, and demonstrated significant presence of GFLV within the surveyed regions. Previously, Tahirine *et al.* (2020) reported a GFLV infection rate of 47% in central and western Algeria. The observed decrease in infection incidence in the present study could

be due to the different vineyards sampled or the limited distribution of the virus in propagation materials.

This study has shown significant regional variations in GFLV incidence across central, western, and southern Algeria. The central region had the greatest infection rate at 59% (out of 159 samples). This could be due to environmental factors or agricultural practices in this region that are conducive to GFLV transmission. In contrast, the western region had a lower GFLV infection rate of 41% (out of 210 samples). While still considerable, this reduced incidence may indicate differences in viticultural practices, or lower presence of GFLV transmission vectors (*Xiphinema index*) (Everaert *et al.,* 2024). In the southern region, no GFLV infections were detected, suggesting that high temperatures may play a significant role in suppressing or eliminating the virus. This observation aligns with the principle underlying thermotherapy, a widely used method for the elimination of grapevine viruses through exposure to elevated temperatures (Panattoni and Triolo, 2010; Miljanić *et al*., 2022; El Sayed *et al*., 2023).

It is important to note that ELISA- and PCR-based methods are complementary in grapevine virus diagnostics. ELISA is widely used as a cost-effective and high-throughput tool for large-scale screening, while PCR offers increased sensitivity and can confirm infections, particularly in asymptomatic plants or when virus titers are low (Erilmez and Kaya, 2016; Vigne *et al*., 2018). In the present study, ELISA was employed to estimate GFLV prevalence across the sampled regions, and then RT-PCR was applied to a subset of samples for molecular confirmation and subsequent sequencing of viruses. Five GFLV isolates were chosen to represent diverse grapevine cultivars and geographic regions, and the phylogenetic analyses showed an absence of correlation between clustering and geographic origins of the samples. The Algerian isolates clustered in different clades, revealing sequence variability among each other and indicating potential differences at origins and spread of a panmictic population. The 'Seibel' and 'Ahmer Bou Amer' isolates grouped with Russian isolates; while 'Dattier', 'Musca't and GFLV ALG 101 isolates grouped with several isolates from the United States of America. These phylogenetic relationships demonstrated that the Algerian GFLV isolates were characterized by high diversity in this country.

A primary objective of the present study was to generate the first complete genome sequences of GFLV from Algeria, using Next-Generation Sequencing (NGS). This technique is important for detecting grapevine viruses due to its high sensitivity and throughput, as well as its capacity to concurrently identify known and novel viruses (Al Rwahnih *et al*., 2015). The resulting genomic data provide a valuable reference for future molecular and epidemiological studies.

Algerian GFLV isolates exhibited considerable genetic similarities to isolates from Russia, the United States of America, and France. These genetic relationships may be related to the past introductions of GFLV-infected propagation material from different viticultural regions, allowing dissemination of the virus across geographically distant host populations. Therefore, it is important to ensure that grafting materials, including scions and rootstocks, which are key sources of GFLV dissemination, are free of viruses. Rigorous procedures must be emphasized throughout production of virus-free plant material to prevent virus transmission. Understanding the genetic diversity within viral populations also facilitates creation of effective diagnostic tools that are essential for pathogen-free plant programmes. Furthermore, analysis of nematode distribution, specifically *Xiphinema index*, is essential for controlling GFLV dissemination in viticulture systems. The present preliminary investigation will continue, aiming to provide insights into the genetic population structure of GFLV in Algeria, and to support development of effective virus containment and management strategies.

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