Development of the diagnoses tools for *Vitis spp*. grown in Taif- Saudi Arabia infected with *Grapevine fanleaf nepovirus*

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Abstract: Grapevine fanleaf virus (GFLV) is the viral agent of one of the most severe diseases in vineyards worldwide. Survey of grapvine farms in Taif governorate, Mecca province, Kingdom of Saudi Arabia resulted in the detection of Grapevine plants (*Vitis vinifera* cv. Perelette) exhibited virus-like symptoms of the GFLV. Symptomatic samples were collected to confirm the viral infection through Double antibody sandwich-enzyme linked immunosorbent (DAS-ELISA) assay with Polyclonal antibodies (PAbs) specific to GFLV. Out of 48 tested samples, 43 samples representing 89.6% gave positive reactions with values ranged between 0.116 and 2,462 compared to values ranged between 0.003 and 0.085 of the 5 negative healthy samples. Grapevine plant with high viral titer showed typical fanleaf symptoms such as abnormal branching, double nodes, short Internodes with zigzag growth and fasciations, reduction of the leaves size with deformities and fan-leaf shape. These results were biologically confirmed following the detection of leaves malformation symptoms, mottling, ringspots and systemic chlorotic mosaic, in viral infected *Nicotiana benthamiana, Phaseolus vulgaris* and *Cucurbita pepo*, respectively. The inoculated plants developed symptoms 21 days post inoculation. The result obtained by ELISA was confirmed by western blot assay. To our knowledge, this is the first study performed in KSA dealing with the detection of grapevine viruses in naturally infected field-grown vines.

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1. Introduction

Among 58 virus species that can infect grapevine plants, Grapevine fanleaf virus (GFLV) **(Martelli, 2006)** is widespread throughout the world, everywhere *Vitis vinifera* is grown. GFLV causes serious economic losses by reducing the yield of grape production by up to 80% and by affecting fruit quality. GFLV is also responsible for the progressive degeneration of grapevines in vineyards worldwide (**Pearson and Goheen, 1991**).

Grapevine fanleaf virus (GFLV, genus *Nepovirus*, family *Comoviridae*) (**Le Gall *et al*., 2005)** Shortly after, **Sanfaçon *et al*. (2009)** classified GFLV to the *Secoviridae* family. Finaly, according to the international committee on taxonomy of viruses (ICTV) GFLV reclassified into Order *Picornavirales, family Secoviridae, subfamily Comovirinae, genus Nepovirus* **(King *et al*., 2012**). They are encapsidated separately in polyhedral virus particles of ≈30 nm in diameter (**Quacquarelli *et al*. 1976; Wetzel, *et al*. 2001**). The genome of GFLV is composed of two single-stranded, positive-sense RNAs (**Wetzel, *et al*. 2001; Bashir *et al*., 2012**), termed RNA1 and RNA2. The size of RNA1 is 7,342 nucleotides (nt) but RNA2 is variable between 3,774 and 3,806 nt (**Serghini *et al*., 1990; Ritzenthaler, *et al*. 1991; Wellink *et al*., 2000**). The symptoms for the infection by GFLV symptoms are green or yellow mosaic, ring and line patterns, flecks and leaf and nodal malformation that appear very seasonally on *Vitis vinifera. V. rupestris* and many other *Vitis spp* and interspecific hybrids (**Brunt *et al*., 1996**).

Grapevine fanleaf virus (GFLV) can be transmitted by several means, incloding the indigenous nematode *Xiphinema index* and *X. Italiea* (**Martelli *et al*., 2003**). GFLV is trasmitted mechanically with difficulty to various hosts. The experimental host range includes over 30 species in seven botanical families. (**El- Kady *et al*., 1991; Brunt *et al*., 1996; Al- Tamimi *et al*., 1998**). and the GFLV was transmitted by grafting; the chip-bud grafts transmitted GFLV form diseased to healthy vines (**El- Kady *et al*., 1991**); symptoms appear as soon as three to four weeks after grafting (**El- Kady, *et al*., 1991; Martelli, 1993; Al- Tamimi *et al*., 1998**).

Grapes are a famous fruit crop of the Kingdom of Saudi Arabia (KSA). They are characterized by there excellent quality and taste. In 2011 The 716 hectares grapevine of the Mekka province produced 19389 tons of grapes, according to the annual agricultural statistical twenty-fourth book published by the Saudi ministry of Agriculture.

In Taif gavernrate (a part of the Mekka province) grapevine is among the most famous fruit crops characterized by its special quality and test.

However, several grapevine diseases caused heavy losses of the local production and affected the exportation of grapes. GFLV was isolated and reported in Ryadah at the first time in 1982 **(Abu-Thuraya 1982; Al-Shahwan, 2003)** but, so far, no study has been performed in KSA to detect grapevine viruses in naturally infected field-grown plants.

Diagnostic tools for the detection of grapevine viruses have evolved since then. Today they include highly sophisticated and sensitive methods from biological indexing using woody indicators and herbaceous hosts, to enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), and real-time PCR (RT-PCR) (**Rowhani *et al*., 1995; Vigne *et al*., 2004; Eichmeier *et al*., 2010).**

In biological indexing, the plant indicators for specific viruses are inoculated with samples taken from the candidate plants and observed for virus symptoms for a period of time. Biological indexing identifies the disease but not the specific virus causing the symptoms (**Martelli *et al*., 2002**).

Antisera have been used for plant virus detection in Western blot, immunosorbent electron microscopy (ISEM), immunocapture-polymerase chain reaction (IC-PCR), and indirect plate-trapped ELISA. It was found that generally polyclonal antibodies produced from recombinant proteins were not suitable for DAS-ELISA (**Nikolaeva *et al*., 1995; Jelkmann and Keim-Konrad, 1997** and **Rubinson *et al*., 1997**).

The present study is the first one performed in the grapevine farms in Taif, KSA to search for the presence of GFLV. It is based on a double-antibody sandwich enzyme-linked immunosorbent assay and GFLV was detected using polyclonal specific antibodies on Western blots. The symptoms characterizing of the GFLV infection of grapevine and *Nicotiana, Phaseolus,* and *Cucurbita* host plants are described.

2.Material and Methods

The experiments have been performed at the Biotechnology and Genetic Engineering Center, Scientific Research Deanship, Taif University, KSA.

Grapevine sampling

Grapevine (*Vitis vinifera*) plants were surveyed in grapevine farms in Taif, KSA. Symptomatic plants of the cultivar Perelette were collected from the King Faisal farm and transferred to the experimental greenhouses at Taif University during the growing season. A total of 48 leaf samples were collected and 1 to 5 gram of mature and of young leaves from each sample.

ELISA assay

The intended purpose of this enzyme-linked immunosorbent assay (ELISA) diagnostic kit, with purchased from Agritest S.r.l., Valanzano, Italy, is the detection in grapevine tissue of grapevine fanleaf virus (GFLV). The method of detection is an enzyme-linked immunosorbent assay (ELISA) based on double-antibody sandwich (DAS) by polyclonal antibodies. Single develops by alkaline phosphatase reaction with p-nitro phenyl phosphate, as described by **Quacquarelli, *et al*. (1976)** and **Clark and Adams (1977).**

DAS-ELISA results were expressed by the mean absorbance at 405 nm of three replicates per sample. Positive and negative controls were supplied with the kit.

Biological studies:

The positive-ELISA samples were used for the mechanical inoculation of *Nicotiana benthemiana*, *Phaseolus vulgaris*, *Cucurbata pepo*, as experimental hosts. The inoculated plants were kept under an insect-proof greenhouse at 24-27ºC (max. day)/18-21ºC, and the symptoms were recorded for 21 days according to **Brunt et al., (1996).**

Mechanical inoculation:

Crude sap was obtained by grinding grapevine symptomatic young leaves in mortars with 2 ml of 0.1 M sodium phosphate buffer, pH 7.2, containing nicotine 2.5 %. Mechanical inoculation was conducted by rubbing sap methods using a cotton swab on the dusted leaves (with 400- mech Carborundum) of the test plants according to **Dias (1963), El- Kady *et al*. (1991) and Hanna *et al*. (2008**). Symptoms appeared three weeks later after inoculation.

Determination of viral protein molecular weight through SDS- PAGE:

Plant samples were prepared by boiling a mixture of loading buffer (125 mM Tris-HCl pH 6.8, 20 % glycerol, 4 % SDS, 10 % β-mercaptoethanol and 0.1 % Coomassie Brilliant Blue R-250) **(Leammli, 1970; Hill and Shepherd, 1987)** for 5 min. immediately put in ice and loaded on the gel. Samples were analyzed on 12% acrylamide gel.

Western blotting analysis:

Western blot analysis was carried out as described by **Quacquarelli, *et al*. (1976)** using two SDS-polyacrylamide gels (12%) for the crude plant

sap of infected grapevine. After electrophoresis, the separated proteins were transferred from SDS-PAGE gel to a nitrocellulose membrane, 0.45 µm pore size from Millipore Corporation (Bedford, MA, USA) by electro blotting in a mini transfer blot system from BioRad with the transfer solution (43.24 g Glycine, 8.7 g Tris base in 3000 ml Water) and transferred at 40 V overnight. The NCM was washed three times for 5 min each, with TBST (TBS, 0.05% Tween 20), and blocked with blocking buffer [2% Triton X- 100, 2% milk in TBS (1- 0.1 M Tris, pH 7.4, 0.5 M NaCl)] for one hour at 37°C. The NCM was washed three times with TBST and incubated with the primary antibody: GFLV polyclonal antiserum diluted at 1: 1000 in antibody buffer (2% non fat milk in TBS pH 7.4) for 1 h at 37°C. After incubation, NCM was washed three times with TBST and incubated for 1 h at 37°C with the secondary antibody: GFLV- IgG alkaline phosphatase conjugated diluted at 1: 1000 in conjugate buffer (1 % non fat milk in TBS pH 7.4.) for 1 h at 37°C. Finally, the NCM was washed three times with TBST and incubated with substrate buffer for color development. After development, the membrane was washed for 10 min in distilled water and then allowed to air dry.

3. Results and Discussion

Symptoms of GFLV infection in Grapevine plants:

Survey of grapevine (*Vitis vinifera)* farms in Taif resulted in the detection of grapevine fanleaf symptoms in the cultivar Perelette. Symptoms appear as abnormal branching, double nodes, short internodes with zigzag growth and fasciations, reduction of the leave size with deformities and fan-leaf shape as shown in **Fig. (1)**. These symptoms are similar to those reported by several studies (**El-Kady, et al., 1991, Garcia-Arenal et al., 2001**) on GFLV-infected grapevines. Moreover, the results are in agreement with those obtained by **Martelli (1993) and Fattouch et al. (2005**) as they reported fanleaf symptoms in vines characterized by malformation, stunting, cane deformations, abnormal branching, double nodes and short internodes with zigzag growth.

Immunological detection of GFLV:

Grapevine seedlings exhibiting symptoms comparable to those of an infection by GFLV were assayed by ELISA using polyclonal antibodies raised against GFLV. The results are listed in **Table 1** and displayed in histogram form in **Fig. 2**. In agreement with **Fiore *et al.* (2008)**, we have considered that the result of the ELISA test was positive if the absorbance at 405 nm was at least three times as high as that of the control done with healthy plants. Assays in which polyclonal antibodies elicited against GFLV bound antigens occurring in leaf extracts had an absorbance at 405 nm between 0.116 and 2.462 while those extracts of healthy plants had an absorbance at 405 nm between 0.003 and 0.085. In total 43 (or 89.5%) of the 48 samples that were analyzed, yielded a positive reaction. Only five grapevine samples gave negative reactions comparable to that of non-infected samples. Several studies have reported the use of DAS-ELISA for the detection of fanleaf virus or other grapevine viruses (**Yan Dun Yui *et al.* 1994; Szychowski *et al.* 1995; and Al- Tamimi *et al.* 1998**). More recently, Bashir and coworkers (**Bashir et al., 2007a, b)** have applied DAS- and/or DAC-ELISA and identified GFLV in 33 out of 126 samples collected **from Iran**. The infectivity assays provided additional evidence for presence of GFLV in the vines. However, only with phosphate buffer pH 8 and at the 4-leaf stage, inoculation resulted in leaf distortion, vein banding and chlorotic spots 15 days post inoculation corresponding to GFLV infection in *C. quinoa* (**Izadpanah et al., 2003**).

Biological Studies:

To confirm the results of the analyses by ELISA, the infected grapevine samples were used for inoculation of various healthy host plants. Seedling of four plant species and varieties belonging to different families were mechanically inoculated and were regularly observed for symptoms development. While non-infected control plants did not show any symptoms, *Nicotiana benthamiana* (*Solanaceae*) displayed leaf malformation, *Phaseolus vulgaris* (*Fabaceae*) displayed mottling and ringspots, and *Cucurbita pepo* (*Cucurbitaceae*) displayed systemic chlorotic mosaic. These symptoms appeared on the inoculated plants within two weeks, as shown in (**Fig. 3**). These plants were back inoculated on Grapevine host plants (*Vitis vinifera* cv. Perelette) the same symptoms were observed.

These results are in agreement with those obtained by **Martelli (1993) and Brunt, *et al.* (1996).** These authors had reported the appearance of fanleaf symptoms in *Nicotiana benthamiana* including systemic mottling and leaves malformation, as well as systemic chlorotic or necrotic mosaic, mottling and ring spots in *Phaseolus vulgaris* and *Cucurbita pepo*. **Hanna *et al.*** (**2008**) used the mechanical inoculation of GFLV suspended in 0.1 M phosphate buffer pH 7.2 containing 2.5% (m/v) nicotine to inoculate *Chenopodium quinoa*, *Chenopodium amaranticolor*, *Cucumis sativus*, *Nicotiana occidentalis*, *Nicotiana benthamiana* and *Nicotiana tabacum* cv. White Burley.

Identification and molecular weight of the viral protein:

To determine the viral protein molecular weight, plant viral preparation was subjected to SDS-PAGE.

The viral protein of GFLV migrated as a single band with a molecular mass of ~ 54 KDa as shown in **Fig. (4)** These results are in agreement with those obtained by.**Quacquarelli, *et al.* (1976)** reported that GFLV capsid contains a single protein species with mol. wt. of about 54000 Dalton.

In addition, **Mayo *et al.* (I97I)** mentioned that the protein preparations from T component or unfractionated GFLV in gel electrophoresis gave a single band containing a polypeptide with a mol. wt. ranging from about 54000 Da. However, **Guan *et al.* (1996)** reported that the entire gene coding for the GFLV coat protein contains 1512 nucleotides which encode a 504 amino acids, with molecular weight approximately 56 kDa.

Western blotting analysis using polyclonal anti-GFLV antibodies confirmed that the polypeptide was the GFLV viral coat protein. The polyclonal antiserum specific for GFLV reacted strongly with the viral coat protein weather crude or eluted from the gel (**Fig. 5**). This confirmed also the Mw of the protein (54 kDa).

To our knowledge, this is the first study performed in KSA on detection of grapevine viruses in naturally infected field-grown vines. Comparing our results obtained with ELISA with that of Real Time PCR in different source materials and sampling is in progress. These results could be the basis for improving detection protocols used to test grapevine propagation material in KSA.

Table (1): DAS- ELISA detection of GFLV in Grapevine samples using the PAbs specific for GFLV from Agritest S.r.l., Valanzano, Italy.

Samples #	ELISA detection		Samples #	ELISA detection	
	EV	R		EV	R
1	**0.22**	+	**25**	**0.480**	+
2	**0.019**	-	**26**	**0.185**	+
3	**0.147**	+	**27**	**0.203**	+
4	**0.064**	-	**28**	**0.313**	+
5	**0.276**	+	**29**	**0.344**	+
6	**0.158**	+	**30**	**0.296**	+
7	**0.367**	+	**31**	**0.326**	+
8	**0.488**	+	**32**	**0.463**	+
9	**0.425**	+	**33**	**0.476**	+
10	**0.142**	+	**34**	**0.085**	-
11	**0.118**	+	**35**	**0.231**	+
12	**0.077**	-	**36**	**0.268**	+
13	**0.274**	+	**37**	**0.273**	+
14	**0.199**	+	**38**	**0.198**	+
15	**0.044**	-	**39**	**0.333**	+
16	**0.428**	+	**40**	**0.450**	+
17	**0.400**	+	**41**	**0.426**	+
18	**0.273**	+	**42**	**0.482**	+
19	**0.116**	+	**43**	**0.425**	+
20	**0.393**	+	**44**	**0.423**	+
21	**0.246**	+	**45**	**0.422**	+
22	**0.192**	+	**46**	**0.309**	+
23	**0.264**	+	**47**	**0.507**	+
24	**2.462**	+	**48**	**0.421**	+

Positive control: **0.116**
Negative control: **0.003**
+ : **Positive**

EV: ELISA values
R: Result
- : **Negative**

Figure (1): GFLV symptoms observed on grapevine seedlings collected in greenhouses of Taif University. (A) is typical GFLV symptoms appears as cane deformations, double nodes, short internodes with zigzag growth and fasciations, the leave were reduced in size with deformities (B) showed the fan-leaf shape and (C) Healthy grapevine plants.

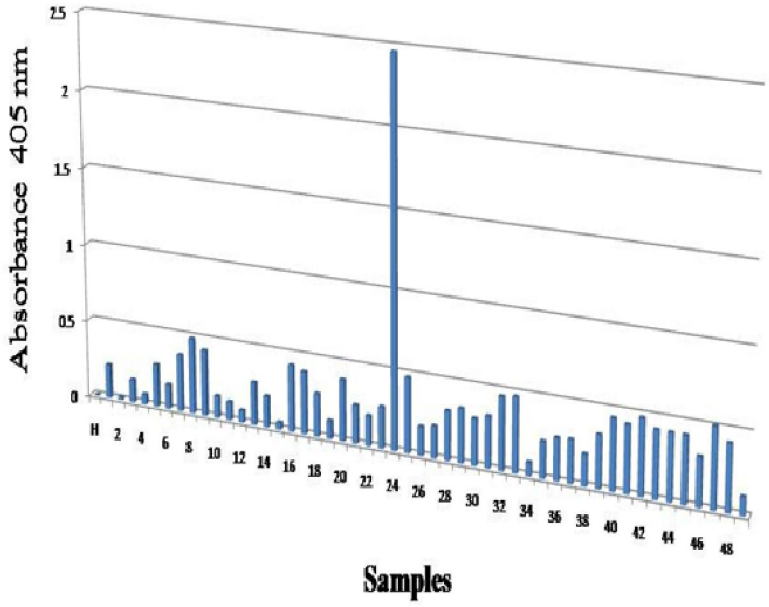

Figure (2): Histogram showing the results of the DAS- ELISA using polyclonal antibodies specific for GFLV from Agritest S.r.l., Valanzano, Italy, with healthy (H) as a (Negative control), Infected as a (Positive control), the samples from 1 to 48 leaf samples of Grapevine (*Vitis vinifera* cv. Perelette) plants.

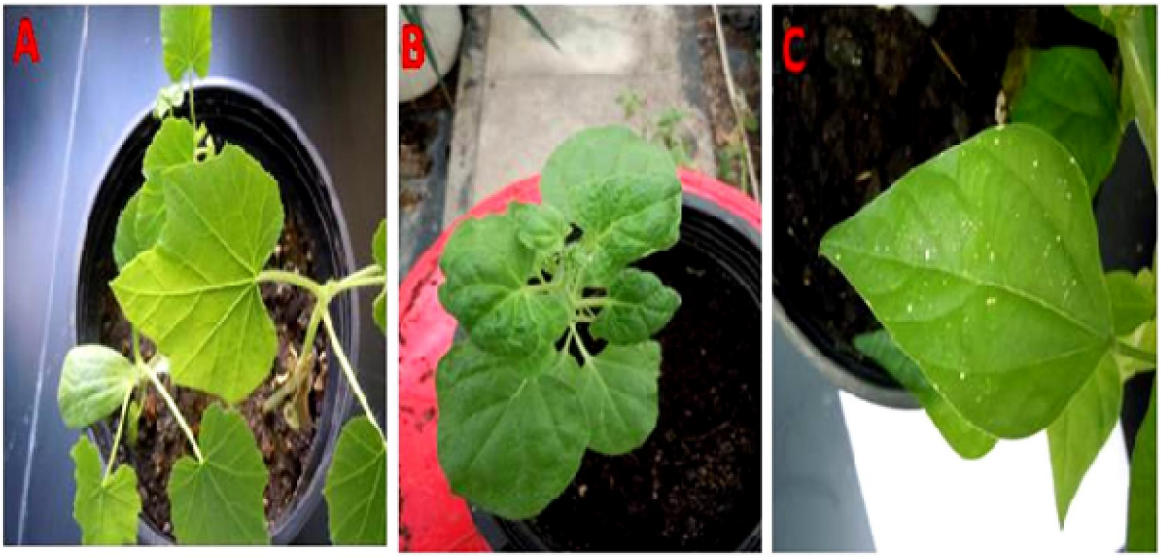

Figure (3): Symptoms observed on diagnostic host plants. (A) *Cucurbata pepo* showed systemic chlorotic mosaic; (B) *Nicotiana benthemiana* showed leaves malformation symptoms. (C) *Phaseolus vulgaris* showed mottling and ringspots; Symptoms development two weeks post mechanical inoculation.

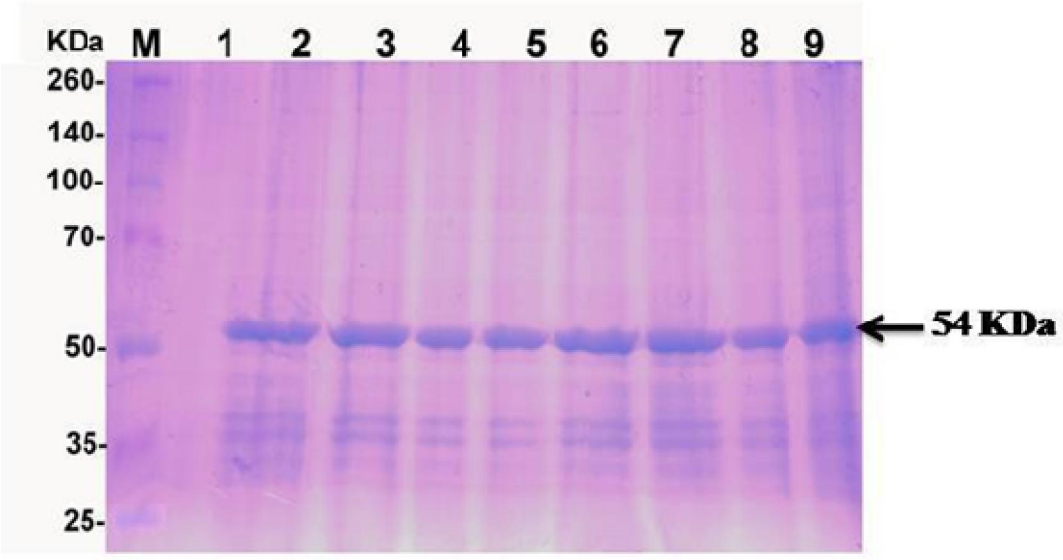

Figure (4): SDS- PAGE for plant viral preparation of GFLV showing the viral coat protein band at MW ~ 54 KDa. Lanes 2- 9 are plant viral preparation. Also, lane 1 is negative control (healthy plant), (Mr) broad range Farmntas protein marker.

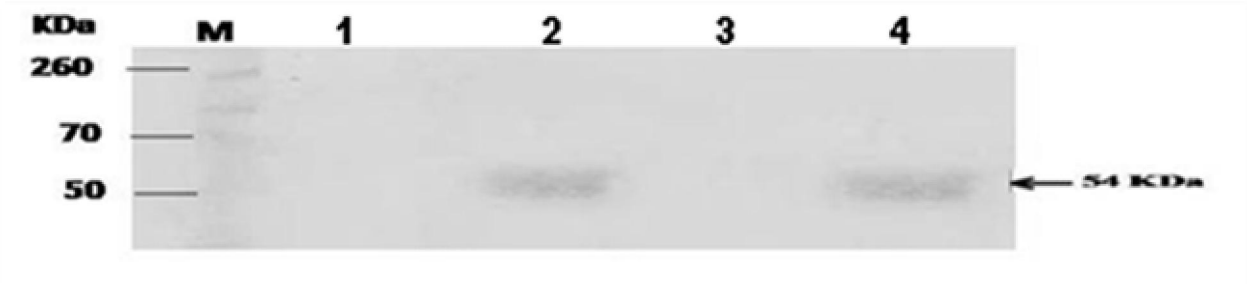

Figure (5): Western blot analysis of plant viral preparation of GFLV against the polyclonal antibodies specific for GFLV from Agritest S.r.l., Valanzano, Italy. Lanes 2- 4 are plant viral preparation, lane 1- 3 is negative control (healthy plant), (Mr) broad range Farmntas protein marker.

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