Enzyme-Linked Immunosorbent Assay to Detect Fanleaf Virus in Grapevines Grown in Containers

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ABSTRACT

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Enzyme-linked immunosorbent assay (ELISA) detected grapevine fanleaf virus (GFLV) in infected glasshouse- or shadehouse-grown grapevines. GFLV was detected in dormant buds in winter and in leaves, internodes, and roots of grapevines sampled in spring. Newly forced leaf tissue gave the most intense color reaction and thus appeared to be the best source of antigen for ELISA tests. In purified preparations, GFLV was detected at concentrations as low as 10 ng/ml. ELISA failed to detect GFLV in leaf samples collected in late spring, summer, and autumn. Bioassays on *Chenopodium quinoa* were as efficient as ELISA in detecting GFLV in leaf samples but failed to detect the virus in dormant buds.

Nepoviruses are rare in Australia, although grapevine fanleaf virus (GFLV) occurs in limited areas, such as Rutherglen in northeast Victoria, but with little evidence of spread (10). However, because grapevine fanleaf is an important disease, all grapevine material passing through quarantine, all clones held in the national repository, and all clones used in certification programs are routinely indexed for GFLV (8).

GFLV is currently indexed by graft-inoculation of the indicator *Vitis rupestris* Scheele 'St. George' (8,12) or by mechanical inoculation of *Chenopodium quinoa* Willd. (13,14). Serology is also used to diagnose GFLV and other nepoviruses infecting grapevine. Taylor and Hewitt (11) and Vuittinez (14), for example, used the gel double-diffusion technique for this purpose, while Bercks (1) found the latex agglutination technique more suitable to reveal GFLV in clarified grapevine sap. Recently, some American workers have tested the enzyme-linked immunosorbent assay (ELISA) technique to detect peach rosette mosaic (6) and tomato ringspot (4) viruses in grapevine, with varying success. In view of the greater sensitivity of ELISA compared with standard methods for detecting a number of plant viruses, we investigated the feasibility of ELISA for detecting GFLV in potted grapevines.

MATERIALS AND METHODS

The virus isolate we used was obtained from grape cultivar Alvarelhao and was a typical fanleaf strain, based on reactions on the indicator cultivar St. George (10). We prepared antiserum to this isolate by

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injecting rabbits intramuscularly with purified preparations (0.06 mg/ml) from *C. quinoa*, made according to the method of Taylor and Hewitt (11), and then cross-absorbed it with healthy plant extract to remove host antibodies. Antiserum titer was 1/128 as determined by gel double-diffusion tests.

The procedures used for ELISA tests resembled those of Clark and Adams (2). The immunoglobulins in 1 ml of antiserum were partially purified by precipitation with ammonium sulfate and dialyzed against 0.005*M* phosphate-buffered saline (PBS), pH 7.4. Purified immunoglobulin was standardized to 1 mg protein per milliliter, and 1 ml was conjugated with 2.5 mg of alkaline phosphatase (Type VII; Sigma Chemical Co., St. Louis, MO 63178). The remaining unconjugated protein was stored in 1-ml portions for coating polystyrene microtiter plates (Cooke Laboratory Products, Alexandria, VA 22314).

Individual wells were coated by adding 200 µl of purified immunoglobulin to each well at a concentration of 10 µg/ml and incubating for 4 hr at 37 C; 0.05*M* sodium carbonate, pH 9.6, was used as a diluent.

Plates were rinsed with PBS containing 0.5% Tween 20, and grapevine extracts (prepared as described below) or virus preparations were added (200 µl per well). Plates were incubated at 4 C for 16 hr. Two wells were filled with each test sample. After further rinsing, 200 µl of enzyme-conjugated immunoglobulin at 1:400 dilution (2.5 µg/ml) in PBS containing 2% polyvinyl pyrrolidone (PVP) (mol wt 44,000) and 0.2% ovalbumin was added to test wells and incubated at 37 C for 6 hr. Plates were rinsed again, and 250 µl of freshly prepared substrate (*p*-nitrophenyl phosphate) was added at a concentration of 0.6 mg/ml in 10% diethanolamine buffer, pH 9.8. After 1 hr, the reaction was arrested by adding 50 µl of 3*M* sodium hydroxide to each well, and plates were scored visually or with a Pye-Unicam spectrophotometer (A405 nm). In the latter case, test samples were recorded as positive if absorbance values exceeded twice that of the healthy control sample (3,7).

Grapevine extracts for ELISA tests were obtained by grinding 1 g of fresh tissue, collected from vines growing in the glasshouse or shadehouse, in 5 ml of PBS containing Tween 20 (0.5%) and PVP (2%). Extracts were filtered through cheesecloth and diluted with the same medium when necessary.

For bioassay of grapevine extracts on *C. quinoa*, 1 g of young leaf tissue was ground in 5 ml of 0.05*M* phosphate buffer (pH 7.0), and the sap was rubbed onto Carborundum-dusted leaves. Ten plants were used in each test. Inoculated plants were observed for 2 wk for symptoms. Virus preparations for bioassay were diluted in PBS containing Tween 20 (0.5%) and PVP (2%) and were rubbed onto Carborundum-dusted *C. quinoa* plants.

Agar double-diffusion tests were conducted in plates of 0.85% ion agar containing 0.85% sodium chloride and 0.02*M* sodium azide.

Detection of GFLV in grapevine tissues. Dormant potted vines, known from previous indexing to be infected, were moved into the glasshouse in late winter (August). In August–September, when two to four vigorous shoots had developed on each vine, samples of bud, young leaf, mature leaf, internode, and root (white) tissues were ground immediately in PBS (1:5, w/v) containing PVP (2%) and Tween 20 (0.5%) and were tested by ELISA. Young leaf tissues from healthy vines were used as controls.

In August, five to 10 dormant buds were collected from potted vines in the shadehouse, ground as described above, and tested. Two infected clones (one each with veinbanding and fanleaf strain), 10 others of unknown virus status, and one healthy clone were tested.

To test for GFLV in newly forced leaf tissues, dormant cuttings of several grapevine clones were collected in July and planted in a cutting bed maintained at 27 C. The cuttings rooted and produced shoots 5–10 cm long with sufficient young leaf tissue for virus assays in about 6 wk, when extracts were prepared and tested. Three test clones were infected with GFLV (one each with fanleaf, veinbanding, and yellow mosaic strains of GFLV), seven were of unknown virus status, and one was healthy.

For detecting GFLV in young leaf tissue at different times of the year, leaf samples from shadehouse-grown plants were collected monthly from September to April, excluding the summer months of December and January, and tested by ELISA.

RESULTS

Detection of GFLV in purified preparations. A purified preparation of the fanleaf strain of GFLV from *C. quinoa* that was used for immunization had an absorbance ratio (260/280) of 1.57 and, using the extinction coefficient for tomato ringspot virus E(0.1%, 1 cm) = 10 (9), contained 0.06 mg of GFLV per milliliter.

Sensitivity of ELISA. To estimate the sensitivity of the ELISA system, a standard dilution curve was constructed by plotting the ELISA values (A405) of a series of dilutions of the above preparation in healthy grapevine sap against virus dilution. The A405 for a GFLV dilution of 1:31,250 (about 2 ng/ml), equaled that

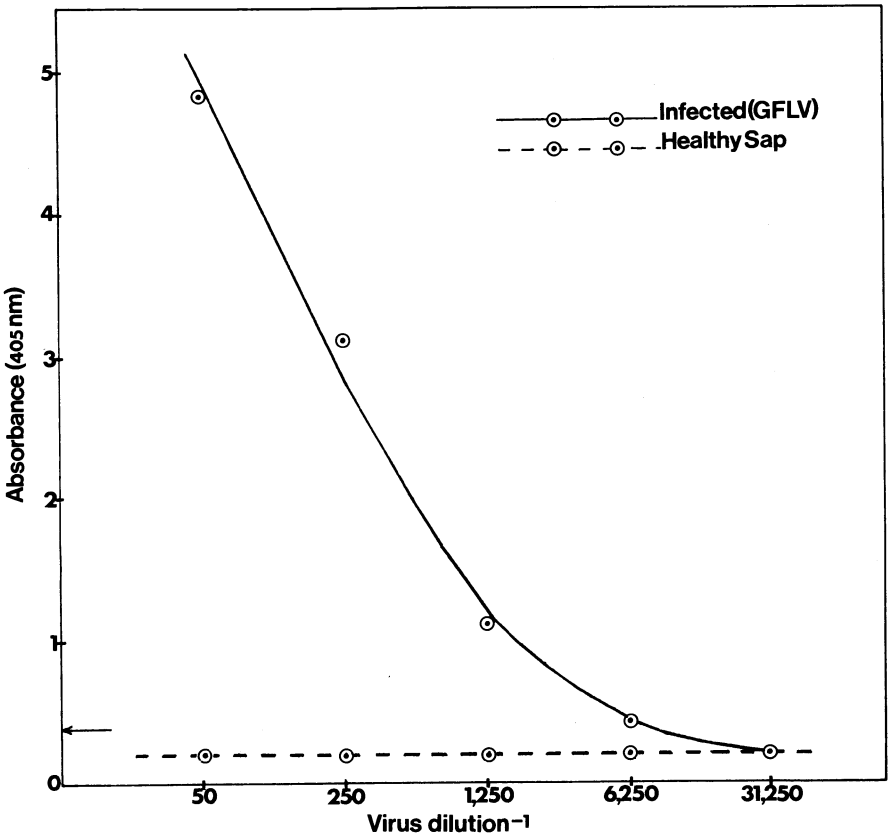

Fig. 1. Enzyme-linked immunosorbent assay absorbance values at 405 nm for grapevine fanleaf virus (GFLV). The solid line represents absorbance of dilutions of purified virus mixed with healthy grapevine sap. The broken line represents the absorbance of dilutions of healthy sap. The arrow indicates the absorbance value considered positive for detection of GFLV. This arbitrarily adopted value is twice the absorbance of healthy control samples.

for healthy sap, when GFLV immunoglobulin was used at 10 μg/ml for coating and at 1:400 for enzyme-conjugate (Fig. 1). The limit of detection, taken as twice the A405 for healthy sap, was thus about 10 ng/ml (indicated by an arrow in Fig. 1). The standard curve was reproducible.

Table 1. Detection by enzyme-linked immunosorbent assay of veinbanding and fanleaf strains of grapevine fanleaf virus in grapevine tissues

Tissue	Virus strain[a]	
	Veinbanding	Fanleaf
Bud	0.86 ± 0.26	1.24 ± 0.18
Young, expanding leaf	1.25 ± 0.41	1.25 ± 0.10
Mature leaf	0.56 ± 0.49	0.62 ± 0.07
Internode	0.19 ± 0.04	0.62 ± 0.20
Roots (white)	0.53 ± 0.28	0.58 ± 0.21
Healthy control (young, expanding leaf)	0.20 ± 0.13	0.20 ± 0.08

[a] Absorbance at 405 nm; mean of four samples with standard errors.

Table 2. Detection by enzyme-linked immunosorbent assay of grapevine fanleaf virus in dormant grapevine buds

Virus status Clone	Test 1[a]	Test 2[a]
Infected		
Alvarelhao (fanleaf)	0.72	0.75
Sultana DIL (veinbanding)	0.61	0.58
Unknown virus status		
Emperor E4V5	0.02	0.08
Peverella D8VI	0.02	0.07
Pinot noir G5V15	0.02	0.06
Waltham Cross B3V12	0.02	0.09
Doradillo SA 140	0.01	0.09
Grenache C4-60	0.01	0.10
Mataro R2V13	0.01	0.05
Perlette EA11V3	0.01	0.05
Rhine Riesling 110V15	0.01	0.08
Waltham Cross E11V4	0.01	0.06
Healthy control		
Mission Seedling 1	0.02	0.08

[a] Absorbance at 405 nm; mean of duplicate samples.

Table 3. Detection of grapevine fanleaf virus in newly forced grapevine leaf tissue by ELISA

Virus status Clone	Absorbance at 405 nm[a]
Infected	
Alvarelhao (fanleaf)	2.44
Sultana DIL (veinbanding)	1.40
R. St. George (yellow mosaic)	1.01
Unknown virus status	
Doradillo SA 133	0.43
Mataro R2V7	0.32
Doradillo SA 140	0.27
Pinot noir G5V15	0.23
Rhine Riesling 110V15	0.18
Semillon SA 45	0.18
Mataro R2V13	0.10
Healthy control	
Mission Seedling 1	0.22

[a] Mean of duplicate samples.

Grapevine sap appeared to have little effect on the ELISA values, as seen by the readings for healthy sap (Fig. 1).

Comparable end points for gel double-diffusion tests and bioassay on *C. quinoa* were 6×10^4 ng/ml (ie, 6,000 times less sensitive than ELISA) and 1.2×10^3 ng/ml (120 times less sensitive than ELISA), respectively.

Detection of GFLV in grapevine tissues. Both the veinbanding and fanleaf strains of GFLV were detected in all tissue samples from actively growing shoots, except one internode sample (veinbanding) and the two control samples (Table 1). Bud and young leaf samples produced the most intense color reaction and thus appeared to contain the highest concentration of virus.

In the tests on dormant buds, only the samples from the two known infected clones gave a positive reaction (Table 2). ELISA values of the infected samples were 30–35 times that of the healthy control samples. Parallel tests on *C. quinoa* using extracts in phosphate buffer gave negative results with all samples tested.

When newly forced leaf tissues were tested, only the three known infected clones gave a positive reaction (Table 3). A repeat test estimated visually gave similar results. Mechanical inoculations of *C. quinoa* confirmed the ELISA results.

When young leaf tissues were tested at different times of the year, GFLV was detected only in the three known infected clones in September and October but not in February, March, or April. All 13 clones of unknown virus status and the two known healthy clones reacted negatively. Parallel inoculations of *C. quinoa* plants in the glasshouse gave identical results; positive assays were obtained only with the three infected clones in September and October.

DISCUSSION

ELISA detected GFLV in most types of infected grape tissues as efficiently as the standard *C. quinoa* bioassay. Our results are thus in agreement with those of Gonsalves (4), who found ELISA as efficient as *C. quinoa* assay for detecting tomato ringspot and tobacco ringspot viruses in grapevine. However, ELISA has been found to be inferior to *C. quinoa* assay for detecting peach rosette mosaic virus, another nepovirus, in Concord grape (6).

The greatest advantage of ELISA is its ability to detect GFLV in dormant grapevine buds. Now we can rapidly index imported grape material in quarantine by testing buds from dormant canes. In addition, the ELISA method is sensitive and rapid. It is about 6,000 times more sensitive than gel diffusion tests and about 120 times more sensitive than bioassay on *C. quinoa*. Moreover, results can be obtained within 48 hr, and as many as 30 samples can be tested on one microtiter plate.

The best source of antigen for ELISA tests appeared to be newly forced leaf tissue in the glasshouse. Uyemoto et al (13) also found that newly forced leaf tissue was a good source of virus for sap inoculation of *C. quinoa*.

The results with samples from shadehouse-grown plants indicate that GFLV can be readily detected in young leaf tissues in September and October; however, leaf samples from field-grown vines were not tested. The failure of the ELISA method to detect GFLV in late spring, summer, and autumn may be the result of very low virus concentration in leaf tissue, and possibly the whole plant, during this period. Other factors such as virus inhibitors in the crude sap could be responsible; improved extraction techniques or partial clarification of the sap might overcome the problem.

An antiserum prepared against one strain of GFLV detected all three strains of GFLV in grape tissues. Gonsalves (4) was able to detect tomato ringspot and tobacco ringspot viruses in young grape leaves from a vineyard using antiserum to single isolates. However, on the basis of our study, it is difficult to conclude that an antiserum to one isolate would be adequate to detect the different strains of GFLV in field-grown vines. The strong reactions observed with the homologous strain, compared with the other two strains, suggest antigenic variations among the strains. Marked differences in ELISA reactions among isolates with different serologic properties have been demonstrated by Koenig (5).

LITERATURE CITED

1. Bercks, R. 1967. Methodisch Untersuchungen uber don serologischen Nachweis pflanzenpathogener viren mit dem Bentonit-Glockungstest, dem Latex-Test und dem Bariumsulfat-Test. Phytopathol. Z. 58:1-17.
2. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-484.
3. Converse, R. H. 1978. Detection of tomato ringspot virus in red raspberry by enzyme-linked immunosorbent assay (ELISA). Plant Dis. Rep. 62:189-192.
4. Gonsalves, D. 1979. Detection of tomato ringspot virus in grapevines: A comparison of *Chenopodium quinoa* and enzyme-linked immunosorbent assay (ELISA). Plant Dis. Rep. 63:962-965.
5. Koenig, R. 1978. ELISA in the study of homologous and heterologous reactions of plant viruses. J. Gen. Virol. 40:309-318.
6. Ramsdell, D. C., Andrews, R. W., Gillett, J. M., and Morris, C. E. 1979. A comparison between enzyme-linked immunosorbent assay (ELISA) and *Chenopodium quinoa* for detection of peach rosette mosaic virus in 'Concord' grapevines. Plant Dis. Rep. 63:74-78.
7. Reeves, J. T., Jackson, A. O., Paschke, J. E., and Lister, R. M. 1978. Use of enzyme-linked immunosorbent assay (ELISA) for serodiagnosis of two maize viruses. Plant Dis. Rep. 62:667-671.
8. Shanmuganathan, N., and Fletcher, G. 1980. Indexing grapevine clones in the Fruit Variety Foundation of Australia for virus and virus-like diseases. Aust. J. Exp. Agric. Anim. Husb. 20:115-118.

9. Stace-Smith, R. 1969. Tomato ringspot virus. Descriptions of Plant Viruses, No. 18. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew, Surrey, England. 4 pp.
10. Taylor, R. H. 1962. Grapevine viruses in Victoria. J. Dep. Agric. Victoria, Aust. 7:336-342.
11. Taylor, R. H., and Hewitt, W. B. 1964. Properties and serological relationships of Australian and Californian soil-borne viruses of the grapevine and arabis mosaic virus. Aust. J. Agric. Res. 15:571-585.
12. Taylor, R. H., and Woodham, R. C. 1972. Indexing grapevine for viruses in Australia. Proc. Int. Council on Viruses and Virus Dis. Grapevine, IV. Inst. Nat. Rech. Agron., Paris. 211 pp.
13. Uyemoto, J. K., Goheen, A. E., Luhn, C. F., and Petersen, L. J. 1976. Use of *Chenopodium quinoa* in indexing for grapevine fanleaf virus. Plant Dis. Rep. 60:536-538.
14. Vuittinez, A. 1970. Fanleaf of grapevine. Pages 217-228 in: Virus Diseases of Small Fruits and Grapevines. N. W. Frazier, ed. University of California Press, Berkeley. 290 pp.