Application of the Enzyme-linked Immunosorbent Assay Procedure to the Detection of Grapevine Fanleaf Virus

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Enzyme-linked immunosorbent assay was used to detect grapevine fanleaf virus (GFV) directly in grapevine leaf tissue. The addition of nicotine or nicotine and sodium diethyldithiocarbamate to the extracting buffer solution greatly enhanced the sensitivity of the procedure and enabled detection of GFV concentrations as low as 12 ng/m ℓ. The conjugated GFV gamma-globulin detected the GFV strains, grapevine vein banding and grapevine yellow mosaic but failed to detect the GFV serotype arabis mosaic in grapevine leaf tissue. The technique makes possible the collection and processing of numerous samples throughout the growing season and is, therefore, particularly suitable for studies on the incidence and spread of GFV in the field. It also facilitates the resolution of disease syndromes with which GFV is associated.

Grapevine fanleaf virus (GFV) has been known to be present in local vineyards for many years (Engelbrecht, 1961, 1963; Van Regenmortel, 1965) but it is difficult to assess its incidence and spread because GFV often causes symptomless infection in local scion and rootstock cultivars (Engelbrecht, 1979; unpublished). Moreover, existing techniques for the detection and identification of latent GFV infection in grapevine are often insensitive or laborious to perform, and this restricts their use (Belli, *et al.*, 1965; Engelbrecht & Wolfswinkel, 1967; Uyemoto, *et al.*, 1976). These limitations have been overcome by using the recently described enzyme-linked immunosorbent assay (ELISA) technique for detecting plant viruses (Voller, *et al.*, 1976). This paper describes the application of ELISA to the detection of GFV in *Vitis*, and considers the suitability of the method for large-scale use in surveys.

MATERIALS AND METHODS

The GFV strain used in antiserum production was isolated from a local *Vitis vinifera* L. cv. Colombar vine showing typical fanleaf symptoms (Engelbrecht, 1972). Virus was cultured in *Chenopodium quinoa* Willd., clarified and concentrated as described by Martelli & Hewitt (1963), before final purification by sucrose gradient zonal electrophoresis in an apparatus similar to that used by Van Regenmortel (1964). Such virus preparations were free of normal host constituents detectable serologically, and elicited, upon injection into rabbits over a 10 week period, an antiserum with a titre of 1/512 as determined by gel double diffusion tests (Ouchterlony, 1958). The antiserum showed that its homologous antigen was serologically indistinguishable from a large number of GFV and grapevine vein banding virus (GVBV) isolates, and distantly related, serologically, to an isolate of arabis mosaic virus (AMV) obtained from an imported *V. vinifera* cv. Regina grapevine. Because of erratic transmission of virus to *C. quinoa*, tests with grapevine yellow mosaic virus (GYMV) were restricted to one isolate from a *V. vinifera* cv. Chenin blanc grapevine.

Unless otherwise stated, purification and conjugation of gamma-globulin, as well as the calibration of the polystyrene microtitre plates were carried out as described by Clark & Adams (1976; 1977). The gamma-globulin fraction of the antiserum was precipitated with ammonium sulphate, and partially purified on a DEAE-cellulose column, followed by exhaustive dialysis. A portion of the purified gamma-globulin preparation with an A_{280} of 1,4 and assumed to have a concentration of 1 mg/m ℓ was conjugated to alkaline phosphate (Sigma type VII, Boehringer, Mannheim) with 0,05% glutaraldehyde. Because of volume changes and possible gamma-globulin losses during the conjugation procedure, concentration of conjugate is given in terms of dilution. The remainder of the gamma-globulin fraction was used for coating the polystyrene microtitre plates. Optimal coating and conjugated gamma-globulin concentrations were determined with GFV-infected *C. quinoa* sap. The conjugated gamma-globulin was incubated for 4 h at 37 °C. The extinction at A_{405} of the contents of each well was recorded *ca.* 1 h after adding the enzyme substrate, *p*-nitrophenyl phosphate, to the wells, and the reaction was stopped with NaOH. All readings were made with a 200 μ ℓ 10 mm path length flow-through cuvette. Results were judged to be positive if the average A_{405} of a test sample exceeded the A_{405} of the healthy control sample in the same test plate by a factor of two or more (Voller, Bidwell & Bartlett, 1977).

Except where stated, all plant extracts for ELISA tests were prepared by grinding *ca.* 0,5 g leaf tissue in a mortar and pestle at 1:10 (m/v) with phosphate-buffered saline solution containing 0,5 m ℓ/ℓ Tween 20. 20 g/ℓ (m/v) polyvinyl-pyrrolidone (Mol. Wt. 44 000) and 2 g/ℓ ovalbumin (Barbara, *et al.*, 1978), and referred to as standard buffer. Ground tissue was usually partially clarified by low speed centrifugation (3 000 *g* for *ca.* 1 min), and was used on the same day or was stored at −20 °C in sealed tubes for later use. Tests were replicated at least twice in each plate. Healthy and GFV-infected *C. quinoa* or grapevine leaf samples and buffer controls were included in each plate.

For sap inoculation, grapevine leaf tissue was ground in 1% nicotine (1:3 m/v) in the presence of 1% (m/v) Celite abrasive. Extracts were then used to inoculate *C. quinoa* leaves, and the plants were kept in a shaded glasshouse at temperatures optimal for successful GFV transmission

(Engelbrecht & Wolfswinkel, 1967). Positive transmissions were usually assessed serologically by double diffusion tests in agar, using undiluted sap.

RESULTS

Purified gamma-globulin calibrated against GFV-infected *C. quinoa* leaf tissue gave excellent differentiation between diseased and healthy leaf tissue at a level of 1,0 μg gamma-globulin/m ℓ coating buffer and an enzyme conjugate dilution of 1:800. Virus was still detectable in *C. quinoa* sap at a dilution of 1:10 000 (A_{405} = 0,18 *cf.* healthy A_{405} = 0,03). In contrast, GFV was detectable in grapevine leaf tissue only when extraction was done at 1:20 (m/v) dilution despite thorough grinding (Thresh, *et al.*, 1977). However, the addition of either 1% (v/v) nicotine or nicotine and 0,2% (m/v) sodium diethyldithiocarbamate to the extracting buffer greatly enhanced the sensitivity of GFV detection in grapevine leaf extract at dilutions of 1:5–1:20 (Table 1). These additives were subsequently incorporated into the standard buffer in all further tests.

ELISA also confirmed the absence of GFV in vines of 26 clones of *V. vinifera* cv. Queen of the Vineyard, showing severe symptoms of grapevine stem-grooving (Engelbrecht, 1973). Similarly, GFV was consistently absent in several *V. vinifera* cultivars, showing symptoms of grapevine yellow speckle and grapevine enations (Engelbrecht, 1979; unpublished).

To determine the distribution of GFV in infected vines, and the minimum leaf sample required for large-scale testing by ELISA, the first fully expanded leaves on growing shoots of GFV-infected *V. vinifera* cv. Colombar vines were collected at random on the vine and from several vines. Each leaf was punched three times, and the resulting 15 mm discs were macerated at a dilution of 1:10 (m/v) in standard buffer. Virus appeared to be evenly distributed in all vines tested, for example all 60 single leaf samples, comprising 3–5 shoots on each of 15 vines, were strongly positive (mean A_{405} = 1,5 *cf.* healthy A_{405} = 0,02) in late spring. Furthermore, the presence of GFV could be established reliably by ELISA in as little as 0,1 g leaf tissue. Similar results were obtained in late autumn but

TABLE 1
Comparison of extracting buffers for the detection of grapevine fanleaf (GFV) virus in grapevine leaf samples[a]

Extracting buffer	Leaf:buffer ratio (m/v)		
	1:5	1:10	1:20
1. Standard buffer (see text)	0,02[b]	0,01	0,10
2. Standard buffer containing 1% (v/v) nicotine	1,15	0,38	0,11
3. Standard buffer containing both 1% (v/v) nicotine and 0,2% (m/v) sodium diethylthiocarbamate	1,08	0,78	0,39

[a] Random 0,5 g aliquots of leaf tissue from a GFV-infected *V. vinifera* cv. Colombar source
[b] Mean A_{405} based on 3 samples of each leaf: buffer ratio

The limit of detectable virus in grapevine leaf tissue was determined in dilutions of purified virus in an extract of healthy grapevine sap, prepared at a 1:10 (m/v) dilution. The purified GFV with an A_{260}/A_{280} ratio and an A_{260} of 1,80 and 1,20 respectively, contained *ca.* 0,12 mg/m ℓ of virus based on an extinction coefficient ($E^{0,1\%}_{1\,cm}$) of 10, suggested by Gibbs & Harrison (1976) for a virus with about 40% RNA (Quacquarelli, *et al.*, 1976). An A_{405} value of 0,03 for a GFV dilution of 1:20 000 (*ca.* 6 ng/m ℓ) equalled that for a healthy grapevine extract (1:10 m/v). Taken as twice the A_{405} for healthy control extracts, the limit of detection of GFV was, therefore, at a virus concentration of *ca.* 12 ng/m ℓ.

A comparison between ELISA and sap transmission to *C. quinoa* showed that as soon as active shoot growth on the vine came to an end by early summer, symptom expression in the herbaceous test plants became erratic and inconsistent, whereas ELISA reacted strongly with extracts from vines with grapevine fanleaf symptoms throughout the growing season (Table 2). Furthermore, the ELISA technique was equally successful in detecting GVBV isolates (mean A_{405}= 0,92) in summer when symptoms were most conspicuous. However, GYMV was only weakly detected in *V. vinifera* cv. Chenin blanc (A_{405} = 0,06 *cf* healthy A_{405} = 0,02) soon after bud-break and before symptoms disappeared on new growth. Extracts from the *V. vinifera* cv. Regina vine, containing AMV, did not react with the GFV antiserum (A_{405} = 0,02).

samples tended to give weaker reactions (mean A_{405} = 0,36 *cf.* healthy A_{405} = 0,03).

To investigate possible spread of GFV in a section of an 18-year-old *V. vinifera* cv. Colombar vineyard, comprising 20 rows of 55 vines each, where the presence of *Xiphinema index* Thorne & Allen, the vector of GFV (Hewitt, Raski & Goheen, 1958) was suspected (P. C. Smith, 1979; personal communication), the positions of healthy and infected vines were recorded. The individual vines either did not react or gave strong positive readings (mean A_{405} = 1,5 *cf.* healthy A_{405} = 0,02), with 140 of the

TABLE 2
Comparison of ELISA (enzyme-linked immunosorbent assay) with sap-transmission to *Chenopodium quinoa* to detect grapevine fanleaf virus in *Vitis vinifera* cv. Colombar vines

Date	No. vines tested	No. vines positive	
		Sap transmission[a]	ELISA[b]
October 1978	10	10	10
November 1978	10	10	10
December 1978	10	4	10
January 1979	10	0	10
February 1979	10	0	10
March 1979	10	2	10
April 1979	10	0	10

[a] Eight *C. quinoa* plants inoculated with extract from each vine
[b] Mean A_{405} at least twice that of healthy grapevine extract

1 049 surviving vines infected. In preliminary bulk sampling tests with some of these vines it was found that antigens could still be detected when only one out of 20 vines was infected (mean A_{405} = 0,25). Moreover, freezing of the extracts for 24 h or longer (8 weeks) did not significantly reduce antigen activity. The null hypothesis of a random distribution of diseased plants was used to compare the observed number of doublets (two adjacent diseased vines) and an expected number d as computed by the expression given by Van der Plank (1947):

$$d = \frac{u\,(u-1),}{n}$$

where n is the total number of vines examined, and u the number of diseased vines. The observed number was 26, the computed expected number 18,5, with a standard error of 4,3.

DISCUSSION

In the past, GFV could not be detected serologically directly in the grapevine unless the virus present was first concentrated or transmitted to *C. quinoa* (Vuittenez, 1970). It was, therefore, difficult to establish the incidence and spread of this soil-borne virus. The modified ELISA procedure described here has demonstrated that GFV can be detected consistently in large numbers of grapevines with negligible background values for healthy extracts. However, the inability of the GFV antiserum to detect AMV, and its weak reaction to GYMV suggest limitations in this assay procedure which can be overcome only by concurrent tests with antisera produced against known serological strains (Barbara *et al.*, 1978). The modified ELISA procedure will also greatly help to resolve uncertainties regarding the possible role of GFV in disease syndromes. For instance, the assumption that grapevine stem grooving disease is caused by a strain of GFV (Hewitt, *et al.*, 1970) has been refuted in the present study. Furthermore, no support could be found for a possible role of GFV in the enations syndrome (Graniti & Martelli, 1970) of local cultivars. The pattern of GFV occurrence in the Colombar vineyard was consistent with random distribution, indicating no spread of GFV and, therefore, also the absence of the nematode vector, *X. index*, from the plot. However, *X. index* has been reported from several vineyard sites in the Western Cape Province (Heyns, 1971; P. C. Smith, 1979; personal communication), and preliminary findings indicate that spread of GFV in a replanted vineyard is correlated with the presence of this nematode (Engelbrecht, 1979; unpublished).

Extracts from several woody plants have shown reduced ELISA reactions (Clark & Adams, 1976). In most cases the inhibition could be overcome by further dilution with the extracting buffer. In the present study the inhibiting effect of the grapevine leaf extract was partially overcome by dilution but the reaction lost most of its sensitivity. This may account for the inability of ELISA to detect peach rosette mosaic virus in some *V. labruscana* Vaily cv. Concord grapevines (Ramsdell, *et al.*, 1979). The beneficial effect of sodium diethylidithiocarbamate in the detection of apple chlorotic leafspot virus in apple has recently been reported (Flegg & Clark, 1979), and explained as a possible stabilisation of the virus against salt-induced breakdown. The ameliorating effect of both nicotine and sodium diethyldithiocarbamate in the present study may be due in part to the action of these substances on tannins and other phenolic compounds present in grapevine tissue, as was pointed out by Kosuge (1965).

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