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/mL. 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and spread because GFV often causes symptomless infection in local scion and rootstock cultivars (Engelbrecht, 1979; unpublished).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 Wild., 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 antisemir with a titre of 1/512 as determined by gel double diffusion tests (Ouchterlony, 1958). The antisemir 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 poly styrene 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 A280 of 1.4 and assumed to have a concentration of 1 mg/mL was conjugated to alkaline phosphate (Sigma type VII, Boehringer, Mannheim) with 0.05% gluteraldehyde. 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 A405 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 μl 10 mm path length flow-through cuvette. Results were judged to be positive if the average A405 of a test sample exceeded the A405 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 Tween 20, 20 g/L (m/v) polyvinyl-pyrrolidone (Mol. Wt. 44 000) and 2 g/L ov albumin (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.
ELISA – Detection of Grapevine Fanleaf Virus

(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 (A405 = 0.18 cf. healthy A405 = 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 diethylthiocarbamate 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 A405 = 1.5 cf. healthy A405 = 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 samples tended to give weaker reactions (mean A405 = 0.36 cf. healthy A405 = 0.03).

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 A260/A280 ratio of 1.10 and 1.20 respectively, contained ca. 0.12 mg/m l of virus based on an extinction coefficient (E1%1cm) of 10, suggested by Gibbs & Harrison (1976) for a virus with about 40% RNA (Quaquarelli, et al., 1976). An A405 value of 0.03 for a GFV dilution of 1:20 000 (ca. 6 ng/ml) equaled that for a healthy grapevine extract (1:10 m/v). Taken as twice the A405 for healthy control extracts, the limit of detection of GFV was, therefore, at a virus concentration of ca. 12 ng/ml.

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 A405 = 0.92) in summer when symptoms were most conspicuous. However, GYMV was only weakly detected in V. vinifera cv. Chenin blanc (A405 = 0.06 cf healthy A405 = 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 (A405 = 0.02).

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 A405 = 1.5 cf. healthy A405 = 0.02), with 140 of the

### Table 1

<table>
<thead>
<tr>
<th>Extracting buffer</th>
<th>Leaf:buffer ratio (m/v)</th>
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<tr>
<td></td>
<td>1:5</td>
</tr>
<tr>
<td>1. Standard buffer (see text)</td>
<td>0,02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2. Standard buffer containing 1% (v/v) nicotine</td>
<td>1,15</td>
</tr>
<tr>
<td>3. Standard buffer containing both 1% (v/v) nicotine and 0,2% (m/v) sodium diethylthiocarbaminate</td>
<td>1,08</td>
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</tbody>
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<sup>a</sup> Random 0.5 g aliquots of leaf tissue from a GFV-infected V. vinifera cv. Colombar source

<sup>b</sup> Mean A405 based on 3 samples of each leaf: buffer ratio

### Table 2

<table>
<thead>
<tr>
<th>Date</th>
<th>No. vines tested</th>
<th>No. vines positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sap transmission&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ELISA&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>October 1978</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>November 1978</td>
<td>10</td>
<td>10</td>
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<td>December 1978</td>
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<td>March 1979</td>
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<tr>
<td>April 1979</td>
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</tr>
</tbody>
</table>

<sup>a</sup> Eight C. quinoa plants inoculated with extract from each vine

<sup>b</sup> Mean A405 at least twice that of healthy grapevine extract

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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