Détection par Elisa du virus de la marbrure de la vigne (GFkV = grapevine fleck virus)

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Elisa detection of grapevine fleck virus (GFkV)

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Summary — The association of grapevine fleck virus (GFkV) with fleck symptoms on grapevine was established using Elisa. The results confirmed that GFkV is probably the virus responsible for fleck. Its detection in vines by Elisa was not reliable throughout the vegetative cycle: under our conditions, in Alsace, June–July was found to be the best period for testing.

grapevine / virus / fleck / Elisa / etiology

Résumé — Détection par Elisa du virus de la marbrure de la vigne (GFkV = *grapevine fleck virus*). *L'association du* Grapevine fleck virus *(GFkV) avec les symptômes de la marbrure de la vigne a été établie par Elisa. La détection par Elisa du GFkV dans les plants de vigne n'est pas possible pendant la totalité du cycle végétatif : dans nos conditions, en Alsace, les mois de juin et juillet constituent la meilleure période pour le dépistage.*

vigne / virus / marbrure / Elisa / étiologie

INTRODUCTION

Detection of pathogens which are transmissible through the wood (viruses, mollicutes, bacteria) is an essential step in the selection of the grapevine (Walter, 1991a). More than 30 major virus and virus-like diseases of grapevines have been described (Bovey and Martelli, 1992). Among them, nepoviruses and closteroviruses are well characterized and can be detected by Elisa or molecular hybridization techniques. The virus-like disease are detected by grafting onto indexing varieties (Walter, 1991b).

Only recently a phloem-limited non-mechanically transmissible isometric virus was demonstrated to be associated with grapevine fleck disease (Boscia *et al,* 1991a,b).

Fleck disease is latent in European grapevine varieties and in most American rootstocks. The following symptoms develop on *Vitis rupestris* used as indexing variety: clearing of veinlets and translucent spots, wrinkling and upward curling of the leaves. The disease can strongly affect root development of rootstocks as well as their grafting ability (Triolo and Materazzi, 1986). No vector is known and dissemination is primarily through infected propagation material. Seed transmission does not occur. The disease can be eliminated by heat therapy or meristem tip culture (Goheen and Luhn, 1973; Ottenwaelter *et al,* 1973; Barlass *et al,* 1982).

In France, sanitary certification presently requires freedom from fanleaf, leafroll, corky bark, *Rupestris* stem pitting and Kober stem grooving for rootstocks and *V vinifera.* In addition, fleck has to be absent from rootstock varieties.

In the present paper we describe the purification of grapevine fleck virus (GFkV) from roots and leaves of infected grapevines and the development of Elisa for routine detection of the virus.

MATERIALS AND METHODS

Source material

Different *Vitis* spp accessions obtained from INRA Vassal were used for purification of the virus. After preliminary assays, *V vinifera* cv Bogaskere (accession Y200) was chosen. Y200 indexed positive on *V rupestris* and was also infected by vein mosaic and vein necrosis but was free from fanleaf and arabis mosaic viruses, leafroll, corky bark, *Rupestris* stem pitting and Kober stem grooving.

For Elisa, we used other *Vitis* spp from collections (INRA Vassal, INRA Cõlmar, INRA Bordeaux) and, in some cases, plants obtained after heat treatment and *in vitro* culture of shoot apices of fleck-diseased mother plants (Bass and Legin, 1981).

Indexing

Detection of fleck disease was made by grafting onto *Vitis rupestris* du Lot, either by using woody cuttings or by green grafting as described by Walter *et al* (1990).

Virus purification

The purification procedure was basically the same as that described by Boulila *et al* (1990) using fresh young and fast growing rootlets or leaves of greenhouse-forced cuttings of the donor vine. After polyethyleneglycol precipitation (PEG 6.000) and high-speed centrifugation, the virus suspension was centrifuged through different types of gradients.

Sucrose gradients were formed by 3 layers of 15, 25 and 35% sucrose in citrate buffer 0.02 M pH 6.1, left to diffuse overnight at 4°C; centrifugation was for 150 min at 24 000 rpm in a Beckman SW 28 rotor at 5°C.

Nicodenz (Sigma) gradients were constituted of 4 layers, each 1 ml, of 60, 50, 40 and 30% with an upper layer of 6 ml 5% Nicodenz in citrate buffer, centrifuged for 18 h at 31 000 rpm at 5°C in Beckman SW 41 tubes.

Cesium sulfate was used at 1.4 g/cm^3 in citrate buffer and centrifuged in a Beckman SW 41 rotor for 17 h at 35 000 rpm at 5°C.

The virus was obtained from the fractions of the gradients after dialysis in citrate buffer and high-speed centrifugation (Beckman 50.2 Ti, 2 h, 250 000 g, 5°C).

Electron microscopy

Carbon-coated pioloform (Agar Scientific R 1275) grids were floated for 15 min on a drop of virus suspension. After dessication, staining was made on a drop of 2% aqueous uranyl acetate. The preparations were observed with a Hitachi HU 11 CS microscope.

Serology

The first assays were made with an antiserum obtained from GP Martelli (University of Bari, Italy).

To prepare antisera, purified virus preparations from sucrose or Nicodenz gradients were emulsified with complete (for the first injection) or incomplete (for booster injections) Freund's adjuvant. Rabbits were injected subcutaneously at 7-d intervals. Antiserum collection began 14 d after the third injection. Immunoglobulins were purified by the rivanol precipitation method (Hardie and Van Regenmortel, 1977). Conjugation of purified immunoglobulins with alkaline phosphatase or biotin was made using 0.06% glutaraldehyde (Avrameas, 1969).

Detection of GFkV in grapevines

Fresh leaves or cortical scrapings were crushed in the presence of 5 vol Tris–HCl 0.2 M buffer pH 8.2 containing 2% polyvinylpyrrolidone, 0.8% NaCl and 0.05% Tween 20. In some cases, this extract was serially diluted from 10^{-1} to 10^{-5} in the same buffer. For Elisa, coating immunoglobulins at 0.25 µg/ml were incubated for 3 h at 37°C. After overnight incubation of the plant extract at 4°C, an immunoglobulin–biotin conjugate (in some cases pre-adsorbed with 5% v/v healthy grapevine extract) was added for 2 h at 38°C. Detection was with a streptavidin–alkaline phosphatase conjugate (Interchim Jackson) at 1/10 000 for 30 min and paranitrophenylphosphate at 1 mg/ml at 38°C.

RESULTS

Virus purification

Elisa (fig 1) and EM observations of sucrose gradient fractions showed that 2 zones were enriched with GFkV particles. An intermediate step in the purification consisting of clarification with ether (1 v/1 v) or butanol (8.5%; v/v) after PEG precipitation and before high-speed centrifugation, yielded more highly purified virus suspensions.

Virus particles were paraspheric, ≈ 30 nm in diameter, some appearing empty, others apparently intact (fig 2).

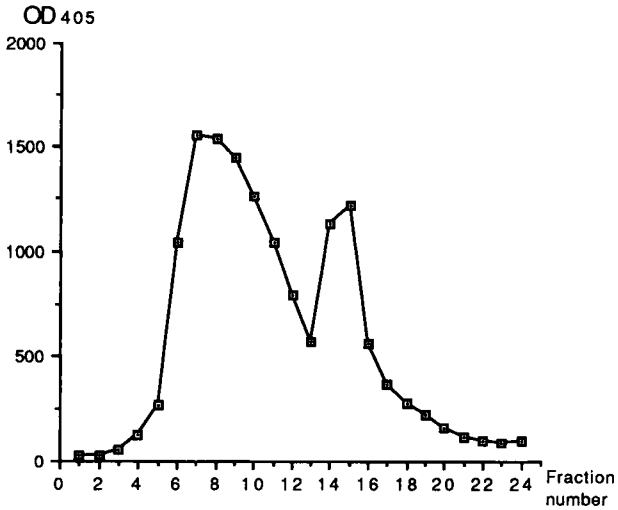

Fig 1. Elisa detection of GFkV in sucrose gradient fractions. For the purification, leaf extract was PEG-precipitated, clarified with butanol and centrifuged at 200 000 *g* for 90 min. The pellet was resuspended in citrate buffer 0.02 M pH 6.1 and 1-ml suspension (corresponding to 250 g leaves) was centrifuged in a Beckman SW 28 rotor at 24 000 rpm for 150 min at 5°C through a gradient obtained after overnight diffusion of 3 layers of 35, 25 and 15% sucrose in citrate buffer. Centrifugation was from left to right. Each gradient fraction was diluted 1/50 in Tris–HCl buffer. Data are OD readings at 405 nm, after 1-h incubation of the substrate.

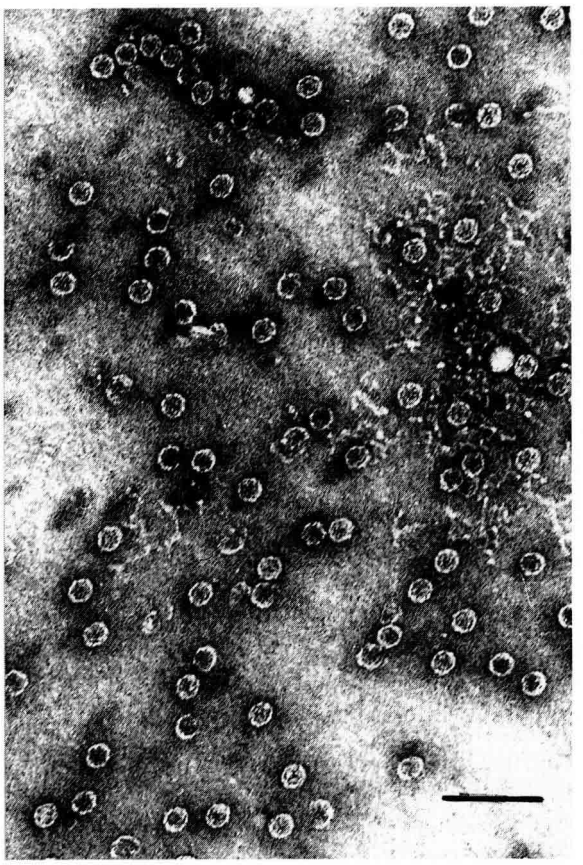

Fig 2. Electron micrograph of a Nycodenz gradient fraction enriched in GFkV after staining with aqueous uranyl acetate. Bar: 100 nm (photo courtesy of G Stocky).

Association of GFkV with fleck symptoms

A very good correlation was found between the presence of fleck symptoms following indexing on *V rupestris* and positive Elisa response: 25 accessions from various geographical origins, which included *V vinifera* and rootstock hybrids, were positive in both tests; 95 were negative in both; 3 were positive by indexing but negative by Elisa and 6 were positive by Elisa but negative by indexing.

Plants obtained after *in vitro* culturing of shoot tips from heat-treated vines (65 *V rupestris* and 23 *V vinifera* Muscat d'Alexandrie) had been found to be free from fleck after > 30 d treatment at 35°C (Bass and Legin, 1981). The absence of fleck as revealed by absence of symptoms (table I) or negative indexing (table II) was highly correlated with the absence of GFkV in Elisa. Only 2 plants of cv Muscat d'Alexandrie indexing negative on *V rupestris* after 50 d heat treatment were positive in Elisa (table II). This difference may be due to a lower sensitivity of indexing compared to Elisa, or to a natural reinfection of these 2 plants that were indexed immediately after planting outdoors, whereas they were checked by Elisa some years later.

Distribution of virus in plants

In the first experiment in May, very young leaves and cortical scrapings of *V vinifera* Muscat d'Alexandrie and *V rupestris* were extracted and

Table I. Presence of GFkV in *Vitis rupestris* with and without symptoms after increasing periods of heat treatment at 35°C.

Heat treatment (d)	*Symptoms*	*Elisa*
0	21+	21+
	3–	3–
26	9+	9+
156	13–	13–
184	6–	6–
382	13–	13–

Table II. Presence of GFkV in *Vitis vinifera* (Muscat d'Alexandrie) that indexed positive or negative on *V rupestris* after increasing periods of heat treatment at 35°C.

Heat treatment (d)	*Index/V* rupestris	*Elisa*
21	3+	3+
36	2+	2+
	2–	2–
50	4+	4+
	9–	7– 2+
426	3–	3–

sap dilutions from 10^{-1} to 10^{-5} were assayed in Elisa. For Muscat, the 10^{-5} dilution of leaf extracts consistently gave a positive reaction, whereas cortical scraping extract only reacted up to 10^{-3}. In contrast, leaves of *V rupestris* reacted to a dilution of 10^{-1} and cortical scrapings up to 10^{-2}. These differences were confirmed when comparing optical density (OD) readings (table III).

In the second experiment, half of a Muscat d'Alexandrie plant was harvested on September 30, 1992 and cortical scrapings, petioles and leaves were analyzed by Elisa. The sample consisted of a main branch from which cortical scrapings were harvested near the beginning of 4 1-yr-old branches that were numbered 1 to 4 from the bottom to the top of the main branch. From each 1-yr-old cane, cortical scrapings, petioles and leaves were collected from the bottom, middle and apical portions (table IV). OD readings ranged from 0.348 to 1.243 in leaf extracts but no conclusion could be drawn about the distribution of the virus in the plant. In petioles and cortical scrapings, Elisa readings were more consistent.

In the third experiment (October 10, 1992) leaves, petioles and cortical scrapings from the bottom, middle and top parts of 4 canes of *Vitis rupestris* were analyzed (table V). Cortical scrapings did not show significant variations, whereas petioles and leaves gave increasing readings from the bottom to the top of the canes.

Table III. Distribution of GFkV in young leaves and wood shavings harvested in May. Data are Elisa OD readings of various dilutions of the extracts.

	V vinifera *Muscat d'Alexandrie*		V rupestris *du Lot*	
	Leaves	*Wood*	*Leaves*	*Wood*
Fleck				
10^{-1}	>2.000	1.673	0.503	0.956
10^{-2}	>2.000	0.778	0.092	0.399
10^{-3}	1.128	0.203	0.046	0.099
Control				
10^{-1}	0.045	0.068	0.094	0.122

Table IV. Distribution of GFkV in leaves, petioles and wood shavings of different portions of 1-yr-old canes and main branch of *Vitis vinifera* Muscat d'Alexandrie.

Portion of cane	*Cane 1*	*Cane 2*	*Cane 3*	*Cane 4*
Top				
Leaves	1.045	0.348	0.590	1.013
Petioles	0.671	0.618	0.701	0.853
Wood	1.000	0.700	0.806	0.963
Middle				
Leaves	1.077	1.015	1.043	0.944
Petioles	1.004	0.868	0.769	0.867
Wood	0.887	0.954	1.040	0.903
Bottom				
Leaves	0.779	0.813	0.746	1.243
Petioles	0.929	0.782	0.917	0.777
Wood	1.014	1.090	0.965	0.901
Main branch	1.082	0.971	0. 689	–

Data are Elisa OD readings of 1/10 extracts after 1-h incubation of nitrophenylphosphate. Healthy controls: leaves = 0.023; petioles = 0.016; wood = 0.015. Buffer control = 0.013.

Table V. Distribution of GFkV in leaves, petioles and wood shavings of different portions of 1-yr-old canes of *Vitis rupestris* du Lot.

Portion of cane	*Cane 1*	*Cane 2*	*Cane 3*	*Cane 4*
Top				
Leaves	0.715	0.847	0.850	0.885
Petioles	0.582	0.800	0.558	0.578
Wood	0.756	0.891	0.754	0.771
Middle				
Leaves	0.779	0.752	0.589	0.529
Petioles	0.516	0.482	0.377	0.439
Wood	0.635	0.848	0.825	0.779
Bottom				
Leaves	0.298	0.398	0.458	0.429
Petioles	0.503	0.371	0.408	0.476
Wood	0.852	0.958	0.796	0.789

Data are Elisa OD readings of 1/10 extracts after 1-h incubation of nitrophenylphosphate. Healthy controls: leaves = 0.098; petioles = 0.045; wood = 0.008. Buffer control = 0.007.

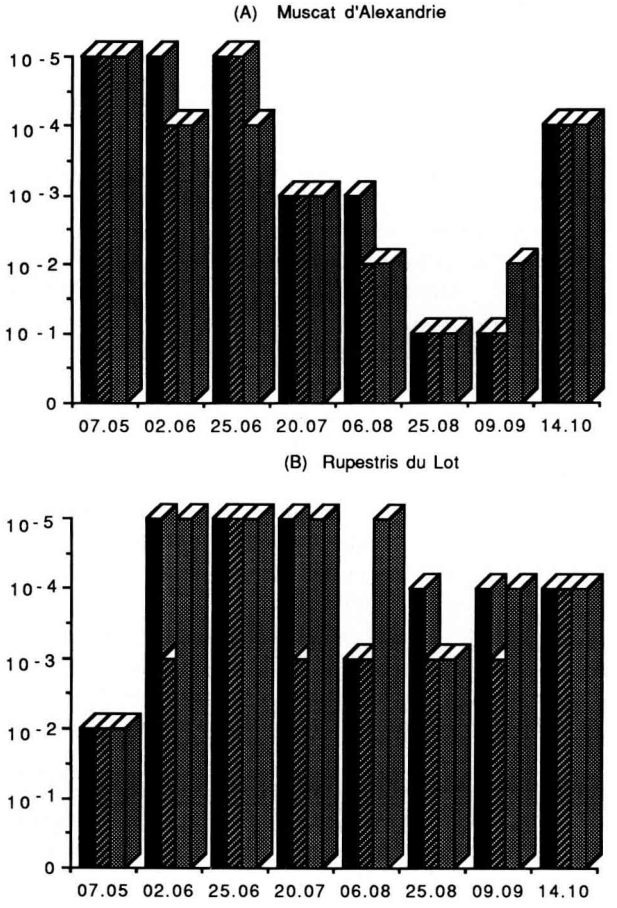

Fig 3. Variation of Elisa end-point dilution for the detection of GFkV over the vegetative period. For detection of GFkV in 3 *V vinifera* Muscat d'Alexandrie plants (A) and 3 *V rupestris* plants (B), leaves were harvested at various dates from May 7th to October 14th. Extracts were diluted from 10^{-1} to 10^{-5} as described in the *Materials and Methods*. The height of the diagrams corresponds to the dilution end-point of extracts reacting in Elisa.

Variation of Elisa response during growth

Large variations in Elisa response were noticed when leaves of vines were analyzed during the entire vegetative period.

The Elisa response decreased rapidly during the hotter summer period, especially in the case of *V vinifera* Muscat d'Alexandrie (fig 3). Under our conditions, the sensitivity of Elisa was maximal during June and July for *V vinifera* and *V rupestris*. This leads to the conclusion that certain periods should be avoided for reliable Elisa detection of GFkV in our climatic conditions: the beginning of the vegetative period for *V rupestris* and late summer (August and September) for *V vinifera* (fig 4).

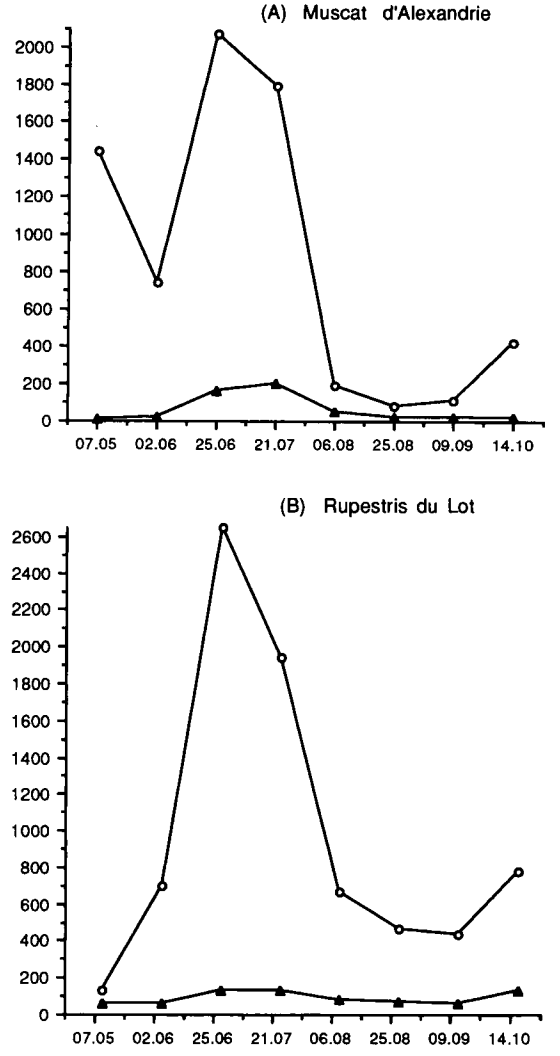

Fig 4. Variation of Elisa response (OD 405 nm) in leaf extract of grapevine during the vegetative period. OD variations in Elisa of a 10^{-1} dilution of the extracts of leaves of *V vinifera* Muscat d'Alexandrie (A) and *V rupestris* (B) harvested at various dates during the vegetative period. Data are OD at 405 nm after 30 min incubation of the substrate for diseased (open circles) and healthy (dark triangles) vines.

DISCUSSION

In this paper, we confirm that GFkV — an isometric virus previously described by Boulila *et al* (1990) and Boscia *et al* (1991b) — is associated with the disease known as fleck (marbrure) and so probably the causal agent of it. Though Koch's postulates have not yet been fully confirmed, we demonstrated a high correlation between the presence of fleck symptoms and the presence of GFkV, both in various grapevine accessions from different geographical origins and in heat-treated vines.

In a series of experiments we have analyzed some factors which could influence reliable detection of GFkV for a routine diagnosis in grapevines, *ie* when, during the year, a vine should be indexed and what type of tissue should be sampled.

In general, leaves and cortical scrapings were comparable as antigen sources for Elisa; under our conditions, there were no significant differences when comparing tissues from the top or bottom of *V vinifera* (Muscat d'Alexandrie). Only petioles and leaves from *V rupestris* showed increasing Elisa readings when harvested from lower to upper parts of the canes. In contrast, Boscia *et al* (1991b) reported that Elisa readings were highest in the basal leaves of LN 33 and Primitivo di Gioia.

Studying Elisa responses in some *V vinifera* and *V rupestris,* we can conclude that the best period for routine testing is, under our conditions, June and July. The dramatic decrease in Elisa readings during the hottest summer period may bring the response very close to the background level.

Considerable seasonal fluctuations have been reported in the efficiency of detection of nepoviruses in grapevines: in some cases, virus detection was impossible in the hottest periods of the year and was most reliable during intensive growth stages (Kölber *et al,* 1985) or at full flowering (Bovey *et al,* 1980; Kölber *et al,* 1981; Lehoczky *et al,* 1983, 1984; Rüdel *et al,* 1983). Under other conditions, detection of nepoviruses was possible by Elisa in grapevine leaves during the whole vegetative period (Walter and Etienne, 1987).

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