

Biotin–Avidin ELISA Detection of Grapevine Fanleaf Virus in the Vector Nematode *Xiphinema index*

D. ESMENJAUD,¹ B. WALTER,² J. C. MINOT,¹ R. VOISIN,¹ AND P. CORNUET²

Abstract: The value of biotin-avidin (B-A) ELISA for the detection of grapevine fanleaf virus (GFLV) in *Xiphinema* was estimated with field populations and greenhouse subpopulations. Samples consisted of increasing numbers of adults ranging from 1 to 64 in multiples of two. Tests with virus-free *X. index* populations reared on grapevine and fig plants as negative controls did not reveal a noticeable effect of the host plant. ELISA absorbances of virus-free *X. index* samples were greater than corresponding absorbances of *X. pachtaicum* samples. Differences occurred between two *X. index* field populations from GFLV-infected grapevines in Champagne and Languedoc. In most tests, 1-, 2-, 4-, and 8-nematode samples of virus-free and virus-infected populations, respectively, could not be separated. Consequently, B-A ELISA was not a reliable method for GFLV detection in samples of less than 10 *X. index* adults, but comparison of the absorbances obtained with increasing numbers may allow differentiation of the viral infectious potential of several populations.

Key words: diagnosis, ELISA, grape fanleaf virus, nematode, virus, *Xiphinema index*, *X. pachtaicum*.

Grapevine fanleaf virus (GFLV) is a nepovirus that causes an economically important disease in vineyards worldwide (15). Certification schemes have greatly limited GFLV dissemination by vegetative propagation; today, transmission is accomplished by the vector of GFLV in the soil, the nematode *Xiphinema index* (Thorne & Allen, 1950). Fallow or nonhost annual crops for 6–7 years eliminate the nematode in the soil, but this control method is not economically feasible in vineyards producing vintage wines (8,9,25). Consequently, soil nematicides are used in these vineyards but are effective for short periods only, particularly in heavy and deep soils (6,10), and have been prohibited in various countries because of adverse environmental effects (19). Plant breeding for resistance to *X. index* or GFLV are complementary approaches to control the disease.

The need for a fast, reliable, and sensitive method for GFLV detection in its vector is obvious. Moreover, a better knowledge of virus survival in its vector would

provide useful data needed for limiting the interval between successive grapevine crops to the minimum required for eliminating the virus. A suitable GFLV assay would also allow detection of the virus in other potential *Xiphinema* vectors.

Immunosorbent electron microscopy (ISEM) is a reliable and sensitive method capable of virus detection in extracts of single nematodes (18) but has limited interest for routine tests because of the complexity of the procedure. Virus detection by infectivity assays on nematodes (24) or by bait-testing field soil (3) are alternative indirect methods that require several weeks. Enzyme-linked immunosorbent assay (ELISA) is a widely used method for routine virus diagnosis in grapevine plants (2,16). Previous studies with DAS (double antibody sandwich) ELISA (1,4,5,22) or B-A (biotin-avidin) ELISA (11) revealed differences between nematodes with and without GFLV in samples of 5–20 nematodes. In the present paper, some factors of variation of B-A ELISA for GFLV detection in *Xiphinema* and their consequences for further practical applications of this technique are evaluated. The existence of differences of viral infectious potential among *X. index* populations is also illustrated.

MATERIAL AND METHODS

Nematode populations: Two geographical populations of *X. index* were selected. Soil

Received for publication 17 February 1993.

¹ Laboratoire de Biologie des Invertébrés, Institut National de la Recherche Agronomique, B.P. 2078, 06606 Antibes Cedex, France.

² Laboratoire de Pathologie Végétale, INRA, B.P. 507, 68021 Colmar Cedex, France.

The authors thank MM. Valentin and Descottes from CIVC (Comité Interprofessionnel du Vin de Champagne) at Epernay, France, and Dr. A. Bouquet and Mr. Pistre from INRA at Villeneuve-les-Maguelonnes, France, for their technical assistance.

samples of the first geographical population (ViC = Viruliferous *X. index* from Champagne) originated from a 14-year old vine ('Chardonnay' over '41B' rootstock in the Mesnil-sur-Oger district, Côte des Blancs, Champagne, France). A first trench had been dug in 1989 in the center of an highly infected area near plants showing marked fanleaf symptoms, with confirmation of GFLV by ELISA serodiagnosis of grape leaves (11). An adjacent trench was dug in 1992 for the present study. In June 1992, soil samples 40–70 cm deep were removed with an auger, transported in plastic bags for 48 hours at ambient temperature, and stored at 5 C for 5 days before nematode extraction by described procedures (11). Samples of the second geographical population (ViL = Viruliferous *X. index* from Languedoc) originated from a severely and homogeneously infected field (8-year old 'Ugni Blanc' on 'Fercal' rootstock) in a French Mediterranean vineyard near Montpellier (Villeneuve-les-Maguelonnes, Languedoc, France). At each test date (October 1991, June 1992, October 1992), soil samples near plant roots were collected with an auger from trenches 20–50 cm deep, stored, and extracted identically to the ViC samples. A test using the ViL population, sampled at 20–50 and 50–80 cm deep respectively, was also made in October 1992.

In a heated (≥ 20 C) greenhouse in Antibes, virus-free (*Vitis vinifera* cv. Aramon \times *Vitis rupestris*) Ganzin no. 1 (= AXR1) and fig plants (*Ficus carica*) obtained from hardwood cuttings of a local clone were grown starting in April 1989 in 10-liter pots filled with 7 liters of a steam-treated soil (27% sand, 40% silt, 33% clay). In April 1990, fig pots and half of the grapevine pots were completed with 3 liters of soil from Languedoc (ViL population) to multiply this population on both plant species. Grapevine pots filled with GFLV-infested soil constituted the ViV population (Viruliferous *X. index* on *Vitis*, actually a greenhouse subpopulation of ViL). After 14 months (June 1991), nematodes reared on fig plants were considered virus free

and constituted the AiF population (Aviruliferous *X. index* on *Ficus*). Three liters of soil containing the AiF population were transferred into the remaining half of the grapevine pots to produce a virus-free population on grapevine (AiV = Aviruliferous *X. index* on *Vitis*). In AiV and AiF pots, ELISA tests (16) on leaves (June 1992) and roots (October 1992) confirmed the absence of GFLV in the plants.

The remaining population used for ELISA was *X. pachtaicum* (Tulaganov, 1938) Kirjanova, 1951 (= *X. mediterraneum*) (ApL = Aviruliferous *X. pachtaicum* from Languedoc) and was obtained from the same field samples as *X. index* at Villeneuve-les-Maguelonnes. *Xiphinema pachtaicum* is very common in most French vineyards (7,11,20) at higher densities than *X. index* and does not vector GFLV (5).

ELISA: Adult nematodes (females) with a dark intestine (i.e., having already fed) were hand picked from the final water suspensions for ELISA. Nematodes of each sample were randomly divided into groups to form one series increasing by multiples of 2 from 1 to 64 individuals. This series was preferred to several replications of an equal number of nematodes because it allowed simultaneous comparison of high numbers of nematodes so as to differentiate populations and comparison of low numbers to estimate the nematode threshold number for virus detection. This method previously provided satisfactory characterization of the viral infectious potential of a given population (11).

The B-A ELISA (DAS ELISA completed with amplification by biotin-avidin) was used. This technique in comparison with DAS ELISA is known to provide higher absorbance readings with comparable backgrounds for GFLV detection in grapevine (26). The immunoglobulins (IgG) were purified from anti-GFLV serum according to the rivanol (6,9-diamino-2-ethoxyacridine lactate) precipitation method (14). In order to limit variability, only one plate was used for each test (same date), and the peripheral wells were not used. The plates were coated with 0.05 $\mu\text{g}/$

ml IgG (titer: 1/8,000 in double immunodiffusion) for 3 hours at 38 C and then rinsed three times with PBS-T (phosphate buffered saline, pH 7.4, containing 0.5 μ l/ml Tween 20). The nematodes were ground in a Potter micro blender in the presence of 100 μ l Tris-HCl (0.2 M, pH 8.2) containing 2% polyvinylpyrrolidone, 0.8% NaCl, 0.05% Tween 20, and carborundum. Homogenates were transferred to the microtitration plate and incubated overnight at 4 C. After three washings with PBS-T, an IgG-biotin conjugate (13) at 1/20,000 was added. After 1 hour at 38 C, streptavidin-phosphatase conjugate (Jackson Immunoresearch Lab., West Grove, PA) was added and samples were incubated at 1/10,000 for 30 minutes. Absorbances at 405 nm were measured with a Titerterk Multiskan MKII spectrophotometer after 0.5, 1, and 2 hours incubation of the substrate (1 mg/ml *p*-nitrophenyl phosphate in 0.01 M diethanolamine, pH 9.8).

RESULTS

Results are presented for the incubation time at which most absorbances of virus-infected samples ranged from 0.5 to 2.5. Figure 1 contains the results from the October 1991 and 1992 ELISA of the AiV, AiF, ViL, and ApL populations. Absorbance curves in AiV and AiF could not be separated. For ViL, the A_{405} values from 8 nematodes in 1991 and from 16 nematodes in 1992 were distinctly greater than those of the other populations. Values for *X. pachtaicum* (October 1991) were very low and regular, and always less than those of any other population. The curves for the ViC and ViL populations were distinctly separate from the 2-adult sample (Fig. 2). Differences between ViL and AiV (i.e., the virus-free reference) increased as adult number increased from 8 to 64. At two dates in 1992, ELISA values were lower in the ViV population that had been transferred from field to greenhouse 24 and 30 months previously than in the ViL population (data not shown). In the sampling depth experiment, absorbances were simi-

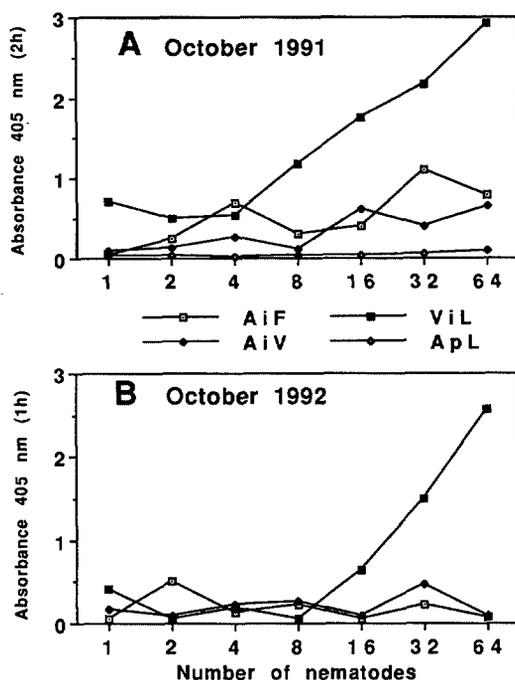


FIG. 1. Comparative ELISA response of virus-free *Xiphinema index* on *Ficus carica* (fig) host (AiF), AXR1 (grapevine) host (AiV), and *X. pachtaicum* (ApL) in tests in October 1991 (A) and October 1992 (B). ViL is the viruliferous parent *X. index* field population from Languedoc.

lar for the highest numbers of nematodes (16 and 32 adults) (Fig. 3).

DISCUSSION

Because absorbance values in virus-free populations were particularly irregular in samples from low numbers of adult nema-

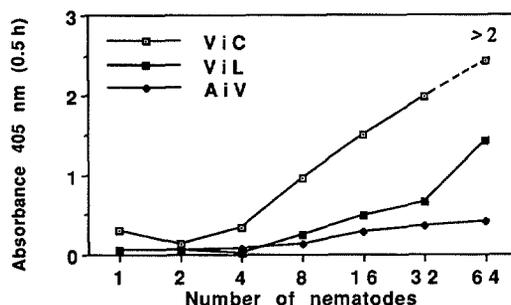


FIG. 2. Comparative ELISA response of viruliferous field populations of *Xiphinema index* from Champagne (ViC) and Languedoc (ViL). AiV is the virus-free greenhouse control population reared on grapevine (AXR1).

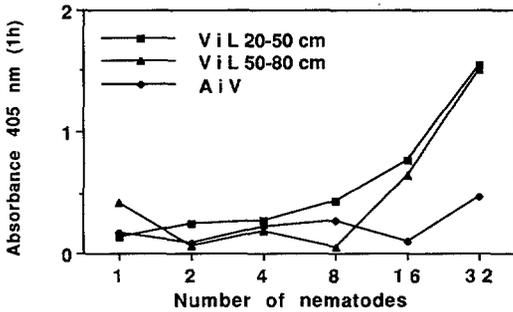


FIG. 3. Comparative ELISA response of a viruliferous *Xiphinema index* population (ViL) sampled at 20–50 cm and 50–80 cm in Languedoc and a virus-free greenhouse control population (AiV) reared on grapevine (AXR1).

todes, the B-A ELISA method, as previously stated by Catalano et al. (4) for DAS ELISA applied to mixed developmental stages, cannot be used for routine analysis of a few nematodes. Differences between nematodes reared on fig vs. grapevine hosts were low and did not reflect a noticeable effect of the host plant. The ELISA response with virus-free *X. index* was markedly higher than that with *X. pachtaicum*. This substantial interference from unknown sources adds to the inability to differentiate between GFLV-positive and -negative nematodes in low numbers.

Prior results (1989) with the ViC population and a virus-free population reared on fig showing distinctly separate absorbance curves from samples of 2–4 nematodes (11) were confirmed in the present study. Apparent differences between the viral infectious potential of the two geographical populations were observed, as indicated by ELISA absorbances. Given that there are very weak differences between isolates tested with polyclonal antibodies (16) as in our study, these differences should not reflect different ELISA reactivity of the two virus isolates; and the most probable explanation would be differential virus numbers in the nematodes. Catalano et al. (4), using DAS ELISA on 20 *X. index* samples, recorded the highest absorbance in the population from a yellow mosaic-affected grapevine. It is possible that both qualitative and quantitative virus

effects are involved in the differences observed. Further investigations involving a range of field populations reared under identical controlled conditions, particularly soil, climate, and rootstock, are needed to determine the possible causes of variation of the ELISA response. Of particular interest is the high response of the Champagne population, which is thus a valuable biological reference material for further studies on nematode–virus–plant interaction.

The ELISA response of the greenhouse-reared ViV subpopulation was less marked than its field parent population (ViL). This difference may be caused by the recent infection of the greenhouse plants, as nematodes were inoculated only 30 months before the last test. Because *X. index* eliminates at each molt the virus adsorbed on its esophagus, young adults are not viruliferous and reacquire GFLV by feeding on the infected grapevine roots (18,23). When a population is maintained in the greenhouse and in the field, important differences observed in samples of few nematodes may be attributed to the possibility that tested adults, even if they had already fed, may have very different ages and thus different virus contents.

Because the present B-A ELISA method, like the DAS ELISA previously tested (1,4,5,22), cannot be used for a reliable detection of GFLV in samples containing fewer than 10 individuals, molecular hybridization methods with homologous probes should be considered (12,17, 21).

LITERATURE CITED

1. Bouquet, A. 1983. Detection immuno-enzymatique du virus du court-noué de la vigne dans son vecteur *Xiphinema index* Thorne et Allen. Comptes Rendus de l'Académie des Sciences Paris Série III 296:271–273.
2. Bovey, R., J. J. Brugger, and P. Gugerli. 1980. Detection of fanleaf virus in grapevine tissue extracts by enzyme-linked immunosorbent assay (ELISA) and immune electron microscopy (IEM). Proceedings of the 7th Symposium of the International Council for the Study of Viruses and Virus Diseases of the Grapevine, Niagara Falls, NY, 259–275.
3. Brown, D. J. F., B. Boag, A. T. Jones, and P. B.

- Topham. 1990. An assessment of soil-sampling density and spatial distribution required to detect viruliferous nematodes (Nematoda: Longidoridae and Trichodoridae) in fields. *Nematologia Mediterranea* 18: 153–160.
4. Catalano, L., V. Savino, and F. Lamberti. 1991. ELISA for the detection of grapevine fanleaf nepovirus in *Xiphinema index*. Proceedings of the 10th Meeting of the International Council for the Study of Viruses, 3–7 September 1990, Volos, Greece. Pp. 243–246.
5. Catalano, L., V. Savino, and F. Lamberti. 1991. ELISA for identifying GFLV-carrying Longidoridae. *Journal of Nematology* 23:523 (Abstr.).
6. Cuany, A., R. Pistre, C. Rennes, P. Truel, A. Dalmaso, J. P. Lacombe, and D. Esmenjaud. 1983. Possibilités d'utilisation de l'aldicarbe en vignoble. *Phytatrie Phytopharmacie* 32:127–138.
7. Dalmaso, A. 1970. Influence directe de quelques facteurs écologiques sur l'activité biologique et la distribution des espèces françaises de la famille des Longidoridae (Dorylaimida). *Annales de Zoologie et d'Ecologie Animale* 2:163–200.
8. Dalmaso, A., and A. Vuittenez. 1978. Problèmes de replantation de la vigne et désinfection du sol dans les pays tempérés. *Bulletin de l'Office International de la Vigne et du Vin* 51:337–351.
9. Esmenjaud, D. 1986. Les nématodes de la vigne. *Phytoma* 374:24–27.
10. Esmenjaud, D., R. Pistre, and M. Bongiovanni. 1988. Activité nématocide de l'aldicarbe sur sol très lourd, en application fractionnée ou non, contre *Xiphinema index*, vecteur du court-noué de la vigne. Mededelingen van de International Symposium over Fytopharmacie en Fytiatrie, Faculteit van de Landbouwwetenschappen Rijksuniversiteit Gent 55:779–786.
11. Esmenjaud, D., B. Walter, G. Valentin, Z. T. Guo, and D. Cluzeau. 1992. Vertical distribution and infectious potential of *Xiphinema index* (Thorne and Allen, 1950) (Nematoda: Longidoridae) in fields affected by grapevine fanleaf virus in vineyards in the Champagne region of France. *Agronomie* 12:395–399.
12. Fuchs, M., M. Pinck, M. A. Serghini, M. Ravelonandro, B. Walter, and L. Pinck. 1989. The nucleotide sequence of satellite RNA in grapevine fanleaf virus, strain F.13. *Journal of General Virology* 70: 955–962.
13. Guesdon, J. L., L. Ternynck, and S. Avrameas. 1979. Use of avidin-biotin interaction in immunoenzymatic techniques. *Journal of Histochemistry and Cytochemistry* 27:1131–1139.
14. Hardie, G., and M. V. H. Regenmortel. 1977. Isolation of specific antibody under conditions of low ionic strength. *Journal of Immunological Methods* 15:305–314.
15. Hewitt, W. B., G. Martelli, H. F. Dias, and R. H. Taylor. 1970. Grapevine fanleaf virus. Descriptions of Plant Viruses, leaflet No. 28. Surrey, England: Commonwealth Mycological Institute and the Association of Applied Biologists.
16. Huss, B., B. Walter, L. Etienne, and M. V. H. Van Regenmortel. 1986. Grapevine fanleaf virus detection in various grapevine organs using polyclonal and monoclonal antibodies. *Vitis* 25:178–188.
17. Ritzenthaler, C., M. Viry, M. Pinck, R. Margis, M. Fuchs, and L. Pinck. 1991. Complete nucleotide sequence and genetic organization of grapevine fanleaf nepovirus RNA 1. *Journal of General Virology* 72:2357–2365.
18. Roberts, I. M., and D. J. F. Brown. 1980. Detection of six nepoviruses in their nematode vectors by immunosorbent electron microscopy. *Annals of Applied Biology* 96:187–192.
19. Rupp, D. 1990. The leaching of nitrate and nematicide from trenched vineyards. *Wein-Wissenschaft* 45:135–140.
20. Scotto La Massè, C. 1985. Atlas of plant parasitic nematodes of France. Distribution of Longidoridae, Xiphinemidae and Trichodoridae. European plant parasitic nematode survey. Dundee, Scotland: Scottish Crop Research Institute & INRA Station de Nématologie et de Génétique Moléculaire des Invertébrés.
21. Serghini, M. A., M. Fuchs, M. Pinck, J. Reinbold, B. Walter, and L. Pinck. 1990. RNA 2 of grapevine fanleaf virus (strain F13): Cloning, sequence, analysis and coat protein cistron location. *Journal of General Virology* 71:1433–1441.
22. Silva, J. F., O. A. Sequeira, M. A. Bravo, M. A. Matos. 1988. Some ecological aspects of the relationship between grapevine fanleaf virus and its nematode vector *Xiphinema index*. Pp. 507–516 in R. Cavaloro, ed. Plant protection problems and prospects of integrated control in viticulture. Proceedings of the CEC/IOBC International Symposium, Lisbon-Vila Real, Portugal, 6–9 June 1988. Ispra, Italy: Joint Research Centre of the Commission of the European Community.
23. Taylor, C. E., and W. M. Robertson. 1970. Sites of virus retention in the alimentary tract of the nematode vectors, *Xiphinema diversicaudatum* (Micol.) and *X. index* (Thorne and Allen). *Annals of Applied Biology* 66:375–380.
24. Trudgill, D. L., and D. J. F. Brown. 1978. Frequency of transmission of some nematode-borne viruses. Pp. 283–289 in P. R. Scott and A. Bainbridge, eds. Plant disease epidemiology. London: Blackwell Scientific Publications.
25. Valloton, R., and J. J. Perrier. 1990. Les nématodes vecteurs de virus dans le vignoble de Suisse romande et le Tessin. *Revue Suisse de Viticulture, Arboriculture et Horticulture* 22:53–58.
26. Walter, B. 1991. New or improved procedures for the detection and identification of viruses or agents of virus-like diseases of grapevine and for diagnosis. Quick detection of virus and virus-like-diseases of the grapevine. Proceedings of the 10th meeting of the International Council for the Study of Viruses, 3–7 September 1990, Volos, Greece. Pp. 243–246.