Stability of ELISA Activity of Barley Yellow Dwarf Virus in Leaf Samples and Extracts

R. M. LISTER, Professor, D. CLEMENT and M. SKARIA, Graduate Research Assistants, and J. A. McFATRIDGE, Technical Assistant, Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907

ABSTRACT

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Extraction and enzyme-linked immunosorbent assay (ELISA) detection of three isolates of barley yellow dwarf virus (P. PAV, MAV, and RPV), as tested over a range of ionic conditions, were optimal at pH 7 and 0.05–0.2 M. ELISA activity of extracts of the P. PAV and RPV isolates was well maintained during storage at −20 C or dialysis to 0.1 M phosphate at pH 7, but that of extracts of the MAV isolate was drastically reduced by these treatments, suggesting differences in stability or solubility between the isolates. In laboratory tests of leaves and in tests of leaf samples mailed worldwide, extractable ELISA activity for all three isolates survived storage better under a variety of conditions in dry leaves than in fresh leaves. Shipment of samples as dry leaves may have wider application in ELISA-based screening and survey programs for plant viruses and could obviate plant quarantine restrictions.

Barley yellow dwarf virus (BYDV) is a general term embracing several luteoviruses that cause varying degrees of yellowing, reddening, dwarfing, and yield reduction in small grains and other Gramineae (10). In the field, such symptoms can readily be confused with other effects, but the problems posed specifically by BYDV in cereal production worldwide are increasingly recognized, especially since the introduction of enzyme-linked immunosorbent assay (ELISA), which has simplified surveys by obviating somewhat the more complex procedure of differential diagnosis by aphid vector transmission studies (5,9).

Unfortunately, antisera to BYDV are not widely available, and the existence of serotypes and serologically distinct isolates adds to this problem by necessitating tests with a range of antisera and the use of virus standards. However, adequate survival of ELISA activity in tissue samples subjected to shipment over long distances would make it feasible to test them at appropriately equipped centers. In recent years, some practical assessment of this possibility has developed through ELISA of cereal leaf samples mailed to our laboratory from various parts of the United States (2). Detection was successful with leaves collected when either actively growing or senescent, with dried leaves, or even with samples that had deteriorated considerably. Because the initial virus status of the samples was unknown, however, these observations did not provide specific information on changes in the extractable ELISA activity of leaves that may have occurred during transit.

The observation that ELISA activity can persist in dried leaf tissue confirmed results of earlier tests in our laboratory (11). This observation is of special interest, because dry cereal leaves can survive for very long periods without obvious deterioration. To define simple procedures for handling survey samples and to predict the chances of successful ELISA with them, we have therefore compared dried and nondried cereal leaves from the same batches of infected tissue as sources of specific ELISA activity after shipment through mailings to various U.S. and other locations worldwide. We also compared the relative efficiencies of buffers of various pHs and ionic strengths in making extracts suitable for ELISA and the differences in ELISA activity of such extracts when stored or dialyzed in various buffers.

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MATERIALS AND METHODS

Viruses and leaf tissue. The BYDV isolates used were the MAV and RPV isolates of Rochow (7) and P. PAV, which is a PAV-like isolate, sensu Rochow, isolated from wheat in Indiana (4). Each was cultured for 2–3 wk in oat seedlings (cultivar Clintland 64) inoculated when about 10 days old by mass infestation with appropriate viruliferous aphids (4), i.e., *Rhopalosiphum padi* L. for the P. PAV and RPV isolates and *Sitobion avenae* Fabr. for the MAV isolate. After harvest, seedlings infected with a particular isolate were cut into pieces (0.5–2.0 cm), which were mixed thoroughly before withdrawing 1-g samples. In preliminary experiments, excellent uniformity in antigen content among samples prepared by this procedure was confirmed.

Sample processing and ELISA. Tissue samples were extracted for ELISA after storage (untreated, air-dried, frozen, or other treatment as described). ELISA was done with polyclonal antisera from rabbits, by a double-antibody sandwich procedure in Dynatech microelisa plates (Dynatech Laboratories Inc., Alexandria, VA). The method was used as described previously (3,5,12), except a "blocking" step consisting of the addition of 1% bovine serum albumin in wash buffer (i.e., phosphate-buffered saline [PBS] containing 0.05% Tween 20) was included after the coating step to reduce background values. Tissue extraction was done by homogenizing each sample in 1:10 (w/v) of 0.1 M potassium phosphate (pH 7) or other buffer in a Polytron homogenizer (Brinkman Instruments, Westbury, NY) for 30 sec, with the PT20ST probe at setting 6. Extracts were then diluted further with 10 volumes of the buffer used initially and were tested directly or after further treatment. Alkaline phosphatase-conjugated antibody was diluted in an extract made in PBS from healthy Clintland 64 oats (1:6, w/v, PBS containing 0.05% Tween 20 and 2% polyvinylpyrrolidone, mol wt 40,000) to reduce background values. Absorbances at 405 nm (ELISA values) of the reacted substrate (*p*-nitrophenyl phosphate) were read after stopping reactions by adding 3 N NaOH after 30–60 min.

Mailed leaf samples. Healthy tissue and tissue infected with the separate viruses were harvested in October 1983, and 1-g samples were prepared as described before. Half of the samples were sealed directly in plastic packets and half were first air-dried by exposure for 4 days at room temperature. Sets comprising duplicate packets of each sample type were then sealed in paper envelopes. These envelopes were sealed in larger envelopes and mailed on 2 November 1983 to various cooperators worldwide with instructions to return the inner envelope unopened so as to avoid

quarantine restrictions. Sets of samples retained as checks in the laboratory were kept at room temperature, in a cold room (4 C), or frozen at −20 or −80 C.

RESULTS

Relative efficiency of various buffers in extracting ELISA activity. Figure 1 shows how leaf extracts made at a ratio of 1:20 (w/v) in phosphate buffers at pH 6, 7, or 8 or in carbonate buffer at pH 9.6 differed in ELISA activity. The phosphate buffers were chosen to cover a commonly used range. The carbonate buffer pH was that of the coating buffer we used in ELISA. Large differences in the ELISA activities of extracts occurred with respect to both buffer molarity and pH. Optimal ELISA activity was obtained at pH 7.0 with each isolate and at about 0.1 M. This was also true when extracts of each isolate made in 0.01 M phosphate at pH 7 were adjusted to a similar range of ionic conditions by 20-fold dilution in appropriate buffers, then tested by ELISA (Fig. 2). To examine the survival of ELISA activity in frozen extracts, two sets of samples from bulks of the replicates of the extracts described before (Fig. 1) were stored at −20 C for 2 wk. Before ELISA, one set was then dialyzed exhaustively at 4 C against 0.1 M phosphate at pH 7 overnight to exclude the observed differential effects of ionic conditions on the ELISA itself (Fig. 2); the other set was simply thawed and kept at 4 C for the same period. In comparisons of assays of these sets, differences in ELISA activity according to extraction buffer used were sustained (Fig. 1). However, although ELISA activities of the frozen extracts of the P. PAV and RPV isolates were little changed, with the MAV isolate this was true only of extracts made at pH 7. Similarly, dialysis to 0.1 M phosphate at pH 7 had little effect on ELISA activity of extracts of the P. PAV and RPV isolates, but such dialysis drastically reduced activity of extracts of the MAV isolate. Freezing was not required to expose the effect of dialysis. In other experiments (results not shown), dialysis to pH 7 in 0.1 M phosphate also reduced the ELISA activity of freshly made, nonfrozen extracts of MAV-infected leaf made in the same range of buffers, from pH 6 to 9.6.

Persistence of ELISA activity in dried leaves. Table 1 summarizes results typical of several experiments in which the ELISA activity of fresh leaves was retained after air-drying in various ways. ELISA activity, though reduced, was readily detectable, and at similar levels, in crude extracts from leaves dried at 25 C (room temperature) or 37 C. Some activity was preserved even in extracts from leaves dried at 60 C. Drying did not necessarily destroy activity, for ELISA

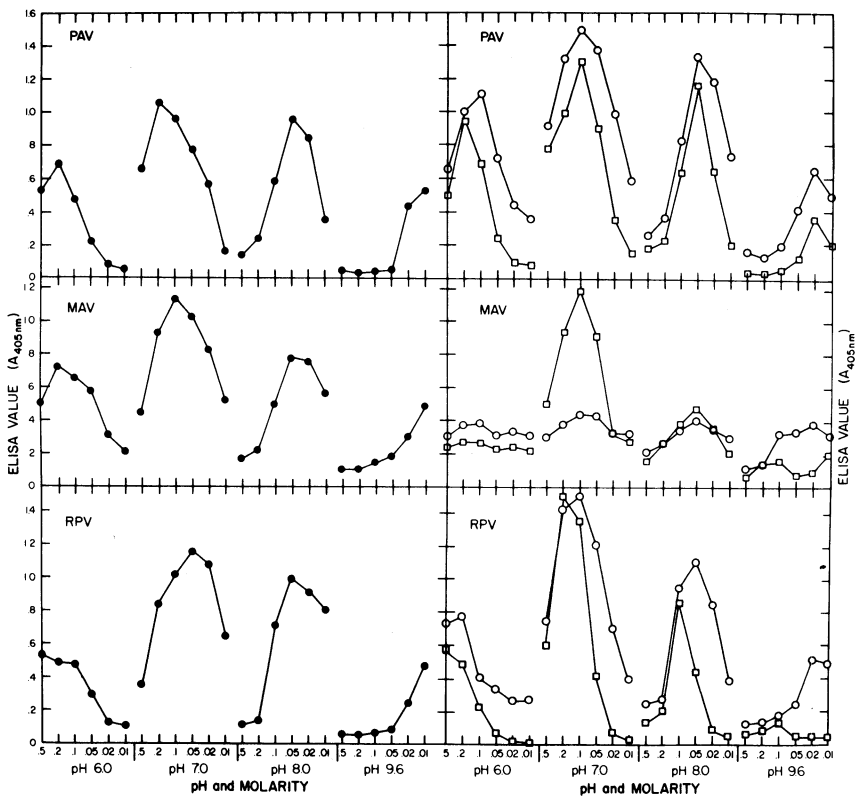

Fig. 1. Comparative ELISA activity of extracts of oat leaf infected with the P. PAV, MAV, or RPV isolates of barley yellow dwarf virus, made in phosphate (pHs 6, 7, or 8) or carbonate (pH 9.6) buffers at the pHs and molarities indicated. Extracts made at 1:10 (w/v) in a Polytron homogenizer were further diluted 1:1 and assayed by ELISA by identical procedures when made or after storage at −20 C for 2 wk with or without subsequent dialysis to 0.1 M phosphate at pH 7. •——• = Averages for quadruplicate freshly made extracts (LSDs were 0.14, 0.13, and 0.11 for the P. PAV, MAV, and RPV isolates respectively); □——□ = values for samples of bulks of the quadruplicate extracts stored 2 wk at −20 C; o——o = values for samples of bulks of the quadruplicate extracts dialyzed after storage at −20 C. Mean values for extracts of healthy tissue were less than 0.0075. When antigen concentration was reduced by one-half, ELISA values were reduced by about one-third.

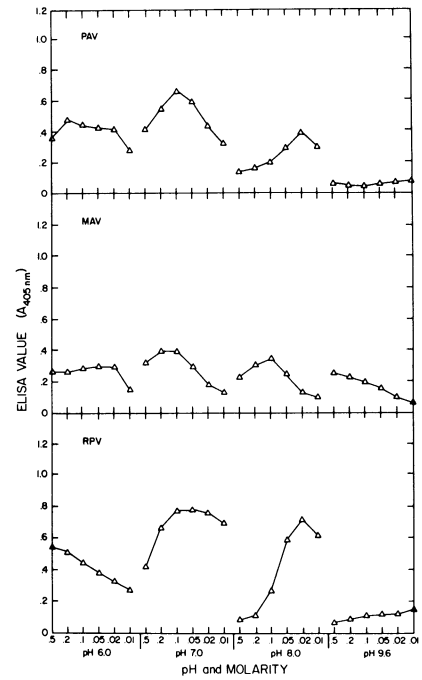

Fig. 2. Comparative ELISA activity of extracts of oat leaves infected with the P. PAV, MAV, or RPV isolates of barley yellow dwarf virus, made in 0.01 M phosphate buffer at pH 7, and adjusted to the pHs and molarities indicated by 20-fold dilutions in phosphate (pH 6, 7, or 8) or carbonate (pH 9.6) buffers.

Table 1. Comparative ELISA values for tissue infected wtih the P. PAV, MAV, or RPV isolates of barley yellow dwarf virus after storage nondried or air-dried at various temperatures[a]

Sample treatment	Mean ELISA values[b] P. PAV	MAV	RPV
Stored at 5 C	0.93	1.31	0.81
Stored at 5 C[c]	···	1.53	1.51
Stored at −20 C	1.00	1.38	0.98
Stored at −80 C	1.12	1.42	1.01
Air-dried at 25 C	0.67	1.19	0.61
Air-dried at 25 C[c]	···	1.68	1.45
Air-dried at 37 C	0.53	1.17	0.80
Air-dried at 60 C	0.24	0.74	0.52
Healthy control tissue stored at −20 C	0.03	0.03	0.04

[a] Three 1-g samples of tissue infected with each isolate were stored as indicated for 4 days, and extracts made in 0.1 M pH 7 phosphate buffer were compared in duplicate ELISA wells.

[b] Bayesian LSDs (13) were 0.14, 0.09, and 0.11 for values for the P. PAV, MAV, and RPV isolates, respectively.

[c] Extracts were clarified by mixing with chloroform, and the aqueous supernatnat was tested after centrifugation at 10,000 *g* for 10 min.

Table 2. ELISA values for extracts of leaf samples infected with the P. PAV, MAV, or RPV isolates of barley yellow dwarf virus that were sent by airmail between Purdue and locations in selected geographical areas during November 1983

Sample description[a]	Mean ELISA values (ranges)[b] for air-dried (A-DL) or nontreated leaves (NL)						Days in transit
	P. PAV-infected		MAV-infected		RPV-infected		
	A-DL	NL	A-DL	NL	A-DL	NL	
Mailed to							
Canada (2)[c]	0.46 (0.39–0.52)	0.08	0.92 (0.71–1.13)	0.10	0.42 (0.40–0.43)	0.07	9–19
Europe (3)	0.37 (0.35–0.43)	0.15	1.07 (0.94–1.16)	0.08	0.41 (0.34–0.55)	0.06	19–26
Australia (2)	0.36 (0.30–0.42)	0.08	0.91 (0.76–1.05)	0.05	0.58 (0.46–0.71)	0.06	14–19
Orient (3)	0.33 (0.32–0.35)	0.11	0.80 (0.68–1.02)	0.07	0.36 (0.29–0.42)	0.07	12–20
United States (7)	0.32 (0.25–0.40)	0.07	0.80 (0.58–0.97)	0.10	0.54 (0.49–0.59)	0.10	6–19
United Kingdom (4)	0.28 (0.21–0.40)	0.12	0.68 (0.53–0.87)	0.09	0.37 (0.30–0.40)	0.09	12–13
South America (4)	0.26 (0.12–0.43)	0.09	0.88 (0.57–1.38)	0.06	0.38 (0.24–0.48)	0.08	14–27
Controls							
Leaf stored at 25 C	0.39	0.16	0.63	0.03	0.44	0.08	
Leaf stored at 5 C	0.36	0.19	0.98	0.05	0.40	0.12	
Leaf stored at −20 C	0.65	0.80	1.39	1.42	0.62	0.74	
Leaf stored at −80 C	0.70	0.96	1.38	1.49	0.53	0.75	
Leaf extract stored at −20 C	0.40	0.84	1.26	1.83	0.50	0.83	
Healthy leaf stored at −20 C	0.02	0.03	0.03	0.04	0.07	0.07	

[a] Duplicate 1-g samples were sealed in polyethylene and either mailed to correspondents in the locations indicated or kept at Purdue as controls in the conditions specified. When returned, the mailed samples were extracted (1:10, w/v, in 0.1 M pH 7 phosphate) and extracts stored at −20 C until tested along with controls 28, 36, or 42 days after the original mailing for the P. PAV, MAV, and RPV-infected samples, respectively.
[b] Means for duplicate samples tested in duplicate wells. Values for mailed healthy control samples were similar to those for samples retained at Purdue.
[c] Number of locations to which samples were mailed are given in parentheses.

values were increased by chloroform clarification of extracts, to a small extent with the MAV isolate and to a much greater extent with the RPV isolate. Improvement of ELISA values after chloroform clarification of the RPV isolate was reported previously (5).

Persistence of ELISA activity in mailed leaf samples. Of 63 packages of leaf samples mailed to cooperators on 2 November 1983, we received 50 after 1–10 wk. Results for those we received during the first 4 wk after mailing are summarized in Table 2. These samples were extracted on receipt and the extracts stored at −20 C until tests could be made. Samples received later were stored intact at −20 C. A representative selection of five of these was subjected to extraction and ELISA 4 mo after the initial mailing date. ELISA values were very similar to those for extracts made on receipt. Overall, improved preservation of ELISA activity occurred in dry leaves compared with nondried leaves when both were subjected to a variety of environmental conditions during transit to and from various locations worldwide. Generally, ELISA values for extracts of the dry leaf samples were similar to those for extracts of dry leaves maintained in the laboratory either at room temperature or in a cold room, and they remained adequate for confident diagnosis. In contrast, deterioration in ELISA activity was obvious in all the nondried leaf samples. This deterioration was often severe enough, even in some samples that were in transit through the mails for only about 1 wk, to make diagnosis uncertain or impossible. For comparable extracts from infected tissue maintained in the laboratory, virus extraction from dry leaves was less efficient than from nondried leaves. Extraction efficiency was improved or activity was better preserved by freezing dry leaves rather than by storing them at 5 or 25 C.

DISCUSSION

Although much can be learned about the stability of ELISA activity of BYDV by laboratory treatment of infected tissue samples, the only way to check stability as affected by the variety of changing environmental conditions likely to be encountered during shipping is by direct tests. We feel that our mailings sampled a wide variety of these conditions, and we conclude that ELISA activity will be maintained at adequate levels for diagnosis of BYDV in infected leaves that are thoroughly air-dried, as described, before transport by mailing or other means. This procedure may be widely applicable with other plant viruses, and it contrasts with other procedures commonly used for transfer of virus-containing samples that are based on attempting to sustain virus infectivity by maintaining tissue in a fresh condition. Air-drying of leaves is simple, and shipment of dry leaves may have wide application in ELISA-based screening of many plant viruses for epidemiological and ecological studies, especially in situations where such work is best done in adequately equipped centers. Presumably, where natural virus is spread only by insects or other vectors from fresh tissue, shipment of dry leaves would eliminate the risk of introducing exotic viruses or isolates, so it could be a valuable procedure in relation to possible quarantine restrictions.

Among the range of buffers compared for extracting and testing leaf samples, 0.1 M phosphate buffer at pH 7 was suitable for use with any of the three isolates studied. It provided not only efficient antigen extraction but also optimum conditions for the ELISA. Therefore, although other buffers may be more appropriate for use with other isolates, this one seems suitable for general use. Carbonate buffers at pH 9.6, as recommended for antigen extraction for the coating step in an indirect ELISA procedure by Lommel et al and others (1,6), gave unsatisfactory results. Clarification of extracts with chloroform, though not essential, can also be a useful, simple way to enhance ELISA values. Freezing of extracts made in 0.1 M buffer at pH 7 provided excellent preservation of ELISA activity.

Other results obtained in this laboratory indicate that our antisera mainly recognize intact virions and that BYDV is dissociated in alkaline conditions (Diaco et al, *unpublished*). We therefore believe that the poor ELISA activity of extracts made in carbonate buffer is due to virion dissociation. The causes of reduced activity under other ionic conditions are not clear, but because of the relative instability of ELISA activity of the MAV isolate in extracts, there may be differences in solubility or stability between its particles and those of the P. PAV and RPV isolates. These differences deserve further investigation because they may be relevant to differences in particle surfaces such as have been invoked to explain vector specificity (8). They could certainly be important in the development of purification procedures.

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LITERATURE CITED

1. Clark, M. F., Lister, R. M., and Bar-Joseph, M. 1985. ELISA techniques. In: Methods in

Enzymology. No. 118. A. and H. Weissbach, eds. Academic Press, New York. In press.
2. Clement, D., Skaria, M., and Lister, R. M. 1984. Screening survey samples for the presence of barley yellow dwarf viruses. Pages 43-51 in: Barley Yellow Dwarf, A Proceedings of the Workshop. P. A. Burnett, ed. CIMMYT.
3. Fargette, D., Lister, R. M., and Hood, E. L. 1982. Grasses as a reservoir of barley yellow dwarf viruses in Indiana. Plant Dis. 66:1041-1045.
4. Hammond, J., Lister, R. M., and Foster, J. E. 1983. Purification, identity and some properties of an isolate of barley yellow dwarf virus from Indiana. J. Gen. Virol. 64:667-676.
5. Lister, R. M., and Rochow, W. F. 1979. Detection of barley yellow dwarf virus by enzyme-linked immunosorbent assay. Phytopathology 69:649-654.
6. Lommel, S. A., McCain, A. H., and Morris, T. J. 1982. Evaluation of indirect enzyme-linked immunosorbent assay for the detection of plant viruses. Phytopathology 72:1018-1022.
7. Rochow, W. F. 1970. Barley yellow dwarf virus. Descriptions of plant viruses. No. 32. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, Surrey, England.
8. Rochow, W. F. 1970. Barley yellow dwarf virus: Phenotypic mixing and vector specificity. Science 167:875-878.
9. Rochow, W. F. 1979. Comparative diagnosis of barley yellow dwarf by serological and aphid transmission tests. Plant Dis. Rep. 63:426-430.
10. Rochow, W. F., and Duffus, J. E. 1981. Luteoviruses and yellows diseases. Pages 147-170 in: Handbook of Plant Virus Infections. Comparative Diagnosis. E. Kurstak, ed. Elsevier/North Holland Biomedical Press.
11. Skaria, M. 1984. Barley yellow dwarf virus synthesis in cereals in relation to resistance and susceptibility. Ph.D. thesis. Purdue University, West Lafayette, IN. 139 pp.
12. Skaria, M., Lister, R. M., and Foster, J. E. 1984. Lack of barley yellow dwarf virus dosage effects on virus content in cereals. Plant Dis. 68:759-761.
13. Smith, W. C. 1978. Bayes least significant differences: A review and comparison. Agron. J. 70:123-127.