Techniques

Quantitative Immunoelectrophoresis of Panicum Mosaic Virus and Strains of St. Augustine Decline

P. H. Berger and R. W. Toler

Research technician and professor, respectively, Department of Plant Sciences, Texas A&M University, College Station 77843.
Texas Agricultural Experiment Station, College Station. Supported in part by U.S. Aid Grant USAID-DSAN-XII-G-0149.
The authors wish to thank Marie Langham and Laura Giorda for technical assistance.
Accepted for publication 19 July 1982.

ABSTRACT

Berger, P. H., and Toler, R. W. 1983. Quantitative immunoelectrophoresis of panicum mosaic virus and strains of St. Augustine decline. Phytopathology 73:185-189.

Panicum mosaic virus (PMV), including seven isolates of St. Augustine decline (SAD) strain, was characterized by "rocket" quantitative immunoelectrophoresis (QIEP) utilizing rabbit antisera. The titer of each strain in a common host could be quantified by QIEP. The slope of the linear regression between log peak area of the rockets and log-relative concentration of plant sap serial dilutions differed between isolates, and this difference was a measure of the serological relationship between the strains. In addition, this method was used to compare virus titers in different hosts or cultivars infected with the same strain. It is a rapid, reliable, inexpensive, and sensitive technique for measuring and characterizing virus from extracts of infected plants.

Additional key word: serology.

Quantitative "rocket" immunoelectrophoresis (QIEP) is a rapid, simple, and accurate method for quantitative determination of antigen concentration. The QIEP technique, developed by Laurell (8–11), has been widely used in the biomedical sciences (19) but has had only limited application in the plant sciences and in plant virology (4–6,16). The commonly used QIEP methods and applications have been thoroughly reviewed by Laurell (12), Axelsen et al (1), and Verbruggen (19).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

Quantitative estimation of antigen concentration in a variety of preparations is often crucial to research in plant virology. Although extremely sensitive techniques are available, including enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA), the instrumentation necessary for these procedures can be expensive. We decided to adopt QIEP in order to quickly and economically identify and differentiate closely related virus isolates or strains and to compare virus titers in breeding lines, with minimal pretreatment of test samples.

The panicum mosaic virus (PMV)/St. Augustine decline strain (SAD) complex has not been assigned to any major virus group at this time. PMV (cryptogram R/1:*/*:S/S:S/*) is approximately 25–30 nm in diameter and frequently has a satellite virus associated with it (13,14). Some SAD isolates show slight symptomatic

differences in common hosts, but the differences are diagnostically unreliable.

Panicum mosaic virus and related isolates of SAD strains were chosen for our model system for several reasons: isolates are available that cannot be readily differentiated; virus isolates, with the exception of PMV, could be maintained for long periods of time in a perennial host with little possibility of cross contamination; the viruses are sap-transmissible; all strains have hosts in common; and the small size of virions means there is no need to degrade them into subunits prior to electrophoresis. Our objective was to examine strain relationships by using a single antiserum with minimal pretreatment of tissue samples.

MATERIALS AND METHODS

Virus sources. A culture of PMV was obtained from C. Niblett (University of Florida, Gainesville) and maintained in *Zea mays* L. 'Oh28.' SAD isolates were obtained as follows: SAD-N (for "normal") was isolated in the Corpus Christi, TX, area; SAD-H (for "homologous") was isolated at the College Station, TX, area as was SAD-S (for "severe"), which was found infecting crabgrass (*Digitaria sanguinalis* L.). SAD-I and SAD-II, SAD-BR, and SAD-A supplied by G. E. Holcomb (Louisiana State University, Baton Rouge) were collected at locations near Lafayette, Baton Rouge, and Alexandria, LA, respectively. All isolates were maintained in foxtail millet (*Setaria italica* (L.) Beauf.) (German strain R) or in St. Augustine grass (*Stenotaphrum secundatum* (Walt.) Kuntze).

Virus purification and antiserum production. Panicum mosaic virus and SAD were purified according to the procedure of Niblett and Paulson (13). Antiserum to SAD-H was obtained from rabbits receiving five weekly intramuscular injections of approximately 1 mg of virus, emulsified with Freund's incomplete adjuvant. The Ouchterlony double-immunodiffusion procedure (DID) was used to test for spur formation and to measure titers of antisera (7).

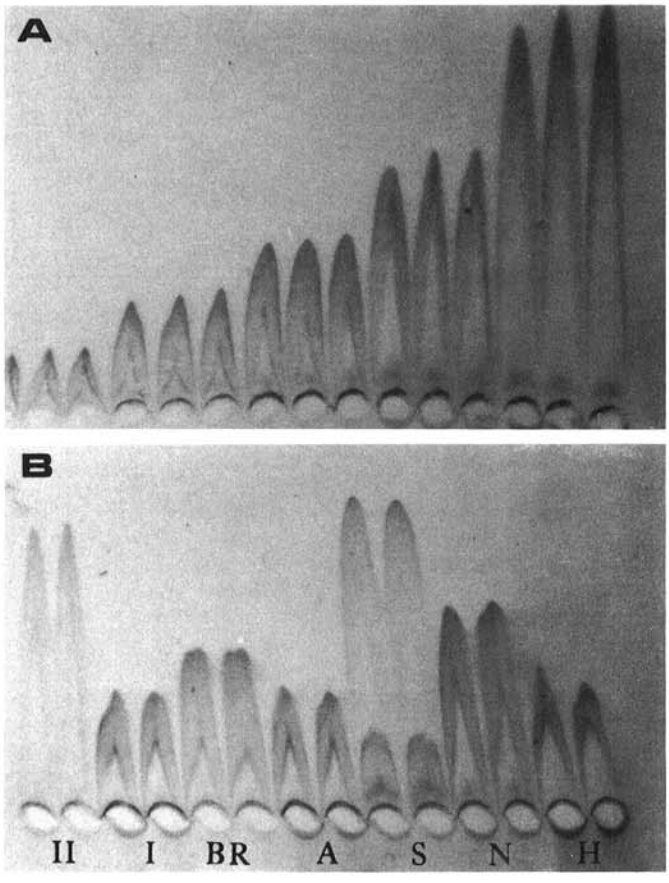

Fig. 1. Reactions of St. Augustine decline (normal strain) (SAD-N) virus in typical quantitative immunoelectrophoresis (QIEP) plates after staining, destaining, and drying. **A,** QIEP of SAD-N twofold serial dilutions from 1:1 to 1:16 (right to left). **B,** All seven strains of St. Augustine decline electrophoresed simultaneously. Antigen was prepared by extracting sap with a sap extraction device, diluting 1:2 (w/v), and clarifying.

Quantitative immunoelectrophoresis. For strain differentiation experiments, all strains were cultured in a common host, foxtail millet. Incubation was for 3 wk following transmission to healthy seedlings. Although it was desirable to subject test sap samples to minimal pretreatment, it was necessary to clarify test samples by low-speed centrifugation, since cellular components plugged gel pores and resulted in jagged peaks that could not be measured. Infected plants were ground 1:2 (w/v) with a mortar and pestle with 0.05 M tris-HCl, pH 7.2 (tris buffer). The sap extract was clarified by low-speed centrifugation (15 min at 5,000 *g*) and serial twofold dilutions were made in tris buffer. The 1:2 (w/v) extract was taken to be 100% relative concentration (hence, log 100% relative concentration = 2).

The QIEP procedures reported are modifications of those of Weeke (20) and Laurell (12). A 1% agarose (Sigma, type 1) solution was made in Svendsen's (1) barbital-glycine/tris buffer, pH 8.6. Ten-milliliter aliquots of the melted solution were dispensed into test tubes and sealed for later use. Gels 1.5 mm thick were formed by melting the agarose, maintaining the tubes at 52 C, adding and thoroughly mixing SAD-H antiserum to 0.1% (v/v) optimum concentration as determined by the procedures of Laurell (12). The melted agarose was poured onto 95 × 85-mm glass plates. Svendsen's buffer was also used as the stem buffer in an LKB Multiphor electrophoresis system with gels connected to the electrode buffers by 95 × 130-mm (Whatman #1) filter paper sheets. Plugs 4 mm in diameter were aspirated from the cathode end of the slabs and a 10-μl test sample was added to each well. This allowed three aliquots from each of the five dilutions to be tested per plate; each group of 15 samples was replicated on two plates. Unless otherwise specified, electrophoresis was done for 18 hr with a constant current of 10 ma (4.4 V/cm) at 5 C. A minimum of six aliquots per dilution were electrophoresed and their resulting peak area was measured. Regressions are based on no less than 30 peak areas.

Following electrophoresis, gels were removed from glass plates and placed in dishes containing 0.15 M NaCl to remove unreacted proteins. After being washed for 2 hr with agitation, gels were stained and destained by the method of Crowle and Cline (3). This staining procedure, which utilizes Crocein scarlet as the primary staining agent, was faster and as sensitive as the standard Coomassie Brilliant blue R250 method (1). Plates could be washed, stained, and destained within 4 hr, with little background staining. Additionally, the staining procedure resulted in better contrast for photographic purposes and a sharper peak boundary (Fig. 1A). If desired, gels were dried on a slab gel dryer (Hoefer Scientific, San Francisco, CA 94107) for a permanent record. Peak areas were determined with a Bio-Rad Peak Height and Area Estimator (Bio-Rad Laboratories, Richmond, CA 94804). Areas rather than simple peak heights were measured, since area measurement should provide more accurate quantification of antigen concentration

TABLE 1. Least squares fits of log-transformed data characterizing panicum mosaic virus and St. Augustine decline virus strains, and the proportion of variance accounted for by the regressions

Virus strain	Slope[a]	Intercept[a]	r^2
SAD-BR	0.559 a	0.645 a	0.935
SAD-A	0.566 ab	0.600 b	0.975
SAD-H	0.630 bc	0.403 c	0.940
SAD-I	0.636 bcd	0.470 d	0.877
PM	0.652 cde	0.546 e	0.965
SAD-S	0.706 def	0.536 f	0.981
SAD-B	0.759 fg	0.234 g	0.982
SAD-II	0.790 g	1.014 h	0.938

[a]Slopes and intercepts not followed by the same letter are significantly different, $\alpha = 0.5$, according to the *F*-test.

(17). Statistical analyses were performed by using the Statistical Analysis System (Cary, NC 27511) program run at the Data Processing Center, Texas A&M University. Regression lines were compared by using the method of Snedecor and Cochran (18).

For purposes of assaying antigen concentration in millet cultivars, sap was expressed from 3–4 g of leaf tissue by using the sap extraction device of Banttari (2). The samples were diluted 1:2 (v/v) in tris buffer, clarified by centrifugation, and electrophoresed as above. The grasses, used for comparing virus concentration listed in Table 3, were inoculated with SAD-H 3 wk after planting and harvested 20 days after inoculation. All plants were grown in the same glasshouse, under ambient conditions.

RESULTS

Strain differentiation. Some isolates of SAD could be readily differentiated by the QIEP method. Although spurs formed in double immunodiffusion tests with some antigen combinations, spur formation was not consistent and could not be used to reliably differentiate virus isolates. If DID tests were inconclusive, the test was repeated using a dilution series of antiserum and antigen. Converting peak areas and relative concentrations to logarithms gives the best fit for linear data and is listed on Table 1. Figure 1B shows all SAD isolates diluted 1:2 (w/v) and electrophoresed simultaneously. In Fig. 2 DID tests for all possible combinations of antigens tested against SAD-H antiserum (titer = 1:64) are shown. Included also is the proportion of variance accounted for by each regression (r^2) and the statistical comparison of the slopes using the F-test at $\alpha = 0.05$. The same F test was used to compare intercepts (18), all of which differed. The relationship between antigen concentration and peak area is exponential. Nontransformed data or semilog-transformed data are not linear; thus, the results reported are log-log transformed (Table 1). The intercept is a term

TABLE 2. Comparison of strain differentiation results obtained by quantitative immunoelectrophoresis (QIEP) and double immunodiffusion (DID) tests. All possible strain combinations are represented

Comb. No.	Strains	Serologically identical? QIEP[y]	DID[z]
1	SAD-BR + SAD-A	yes	yes
2	SAD-BR + SAD-H	no	yes
3	SAD-BR + SAD-I	no	yes
4	SAD-BR + PMV	no	yes
5	SAD-BR + SAD-S	no	no
6	SAD-BR + SAD-N	no	yes
7	SAD-BR + SAD-II	no	no
8	SAD-A + SAD-H	yes	yes
9	SAD-A + SAD-I	yes	yes
10	SAD-A + PMV	no	no
11	SAD-A + SAD-S	no	no
12	SAD-A + SAD-N	no	yes
13	SAD-A + SAD-II	no	no
14	SAD-H + SAD-I	yes	yes
15	SAD-H + PMV	yes	yes
16	SAD-H + SAD-S	no	no
17	SAD-H + SAD-N	no	yes
18	SAD-H + SAD-II	no	no
19	SAD-I + PMV	yes	no
20	SAD-I + SAD-S	yes	no
21	SAD-I + SAD-N	no	yes
22	SAD-I + SAD-II	no	no
23	PMV + SAD-S	yes	no
24	PMV + SAD-N	no	no
25	PMV + SAD-II	no	no
26	SAD-S + SAD-N	yes	no
27	SAD-S + SAD-II	no	yes
28	SAD-N + SAD-II	yes	no

[y] Based on statistical comparisons (see Table 1).
[z] Spur formation test.

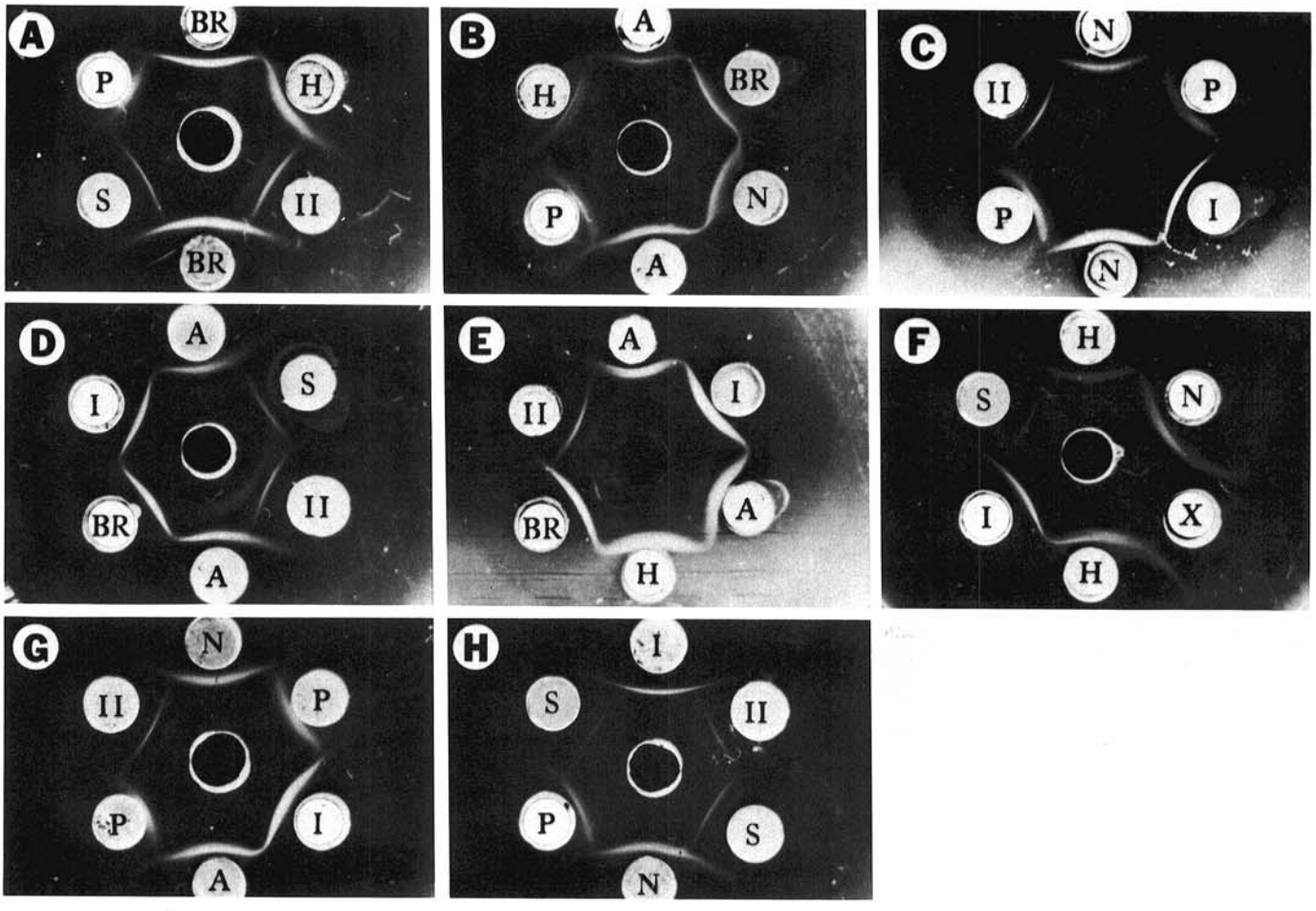

Fig. 2. A–H, Double immunodiffusion spur formation tests for all possible combinations of St. Augustine decline (homologous) (SAD-H) virus isolates and panicum mosaic virus against SAD-H antiserum. **F,** The well designated 'x' is an empty well. 'P' wells are PMV. See text for designations of SAD isolates.

that is related to antigen titer.

Results obtained when the electrophoresis time and voltage were changed from 10 to 15 ma (6.3 V/cm) for 3 hr are shown in Fig. 3. Included also is a standard curve for purified panicum mosaic virus based on a series of optical densities measured at 260 nm. The axes for log-relative concentration and log-optical density coincide. None of the lines in Fig. 3 differ significantly, except that the intercept for the PMV standard curve is significantly greater than the other intercepts. Since the slope of purified PMV is virtually identical with that of sap extracts of PMV-infected tissue, it can be concluded that host sap constituents had no effect upon QIEP.

TABLE 3. Antigen concentration in five grass species infected with St. Augustine decline virus, strain H[w]

Species	P.I. or cultivar	Mean peak area (mm^2)	Standard deviation	Optical density ($OD260$)[x]
Panicum miliare	P.I. 197274	80.65 a[y]	7.09	1.758
Stenotaphrum secundatum	Texas Common	63.65 b	9.76	1.223
Setaria italica	German strain R	56.63 c	6.51	1.021
Panicum bisulcatum	R.I. 286485	51.57 c	10.68	0.884
Setaria sp.[z]	R.I. 202407	19.61 d	1.78	0.201

[w] Based on 12 peak areas.
[x] Based on a panicum mosaic virus (PMV) standard curve.
[y] Duncan's multiple range test. Means with the same letter are not significantly different, $\alpha = 0.05$.
[z] Local lesion host.

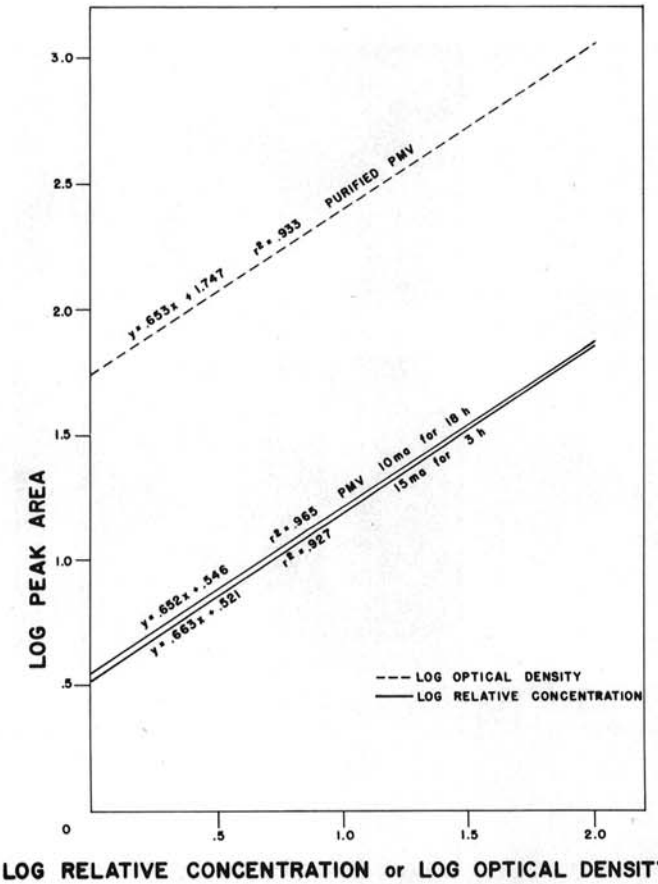

Fig. 3. Least squares linear regressions of log peak area vs log relative concentration or log optical density. The two panicum mosaic virus (PMV) lines compare the effect of different electrophoresis conditions of voltage and time. The uppermost (dashed) line is a standard curve for purified PMV, based on log optical densities. Regression line slopes are not significantly different.

Data for all possible antigen-pair combinations that summarize the effects of using the two methods are shown in Table 2. In the majority of tests, QIEP and DID are in agreement; measurements obtained by QIEP indicate that the reactants are not serologically identical in more cases than DID. Five of the eight viruses for which DID indicated serological identity and QIEP did not, involve SAD-N. The S strain of SAD was involved in five instances in which QIEP indicated serological identity and DID did not. In cases where spurs formed, DID gave a type III reaction of serological partial identity as rated according to the system of Ouchterlony and Nilsson (15).

Virus quantification. Five grasses infected with the same virus strain (SADV-H) are compared for virus concentration (Table 3). *Panicum miliare* "Samai" had the highest titer while a *Setaria* sp. (P.I. 202407), which showed local lesions, had the lowest. Additionally, optical densities (OD_{260}), based on the PMV standard curve (Fig. 3) are noted, although the PMV extinction coefficient may prove to be slightly different than SAD.

DISCUSSION

The spur formation test, although quite useful for providing gross information on serological relationships, is not particularly sensitive. Whether spurs will form and the degree to which they will form is dependent on the concentration of reactants (15). Paul et al (16) used a serological differentiation index (SDI) based on DID end-points to relate some isometric viruses of Graminae (including PMV and SAD). However, when their SDI values were compared to QIEP results, there was no agreement. Our results indicate that QIEP may reduce the problem of highly variable rabbit immune response and the need to standardize antigen concentration, and that (in some cases) the method can detect serological differences when DID produces no spur.

Generally, the QIEP procedure worked well, requiring little time to prepare a single experiment, and staining was rapid and effective. The QIEP technique gave reproducible results when tissue was extracted either with a mortar and pestle or with the Banttari (2) sap extraction device.

These data indicate that a range of electrophoretic conditions may be used with satisfactory results, although it is important to remember that each virus system may require different conditions (ie, buffer, pH, ionic strength, voltage, time, etc.). Using a higher voltage and a shorter running time can be convenient, although our observation is that a longer period (18 hr) at lower voltage yields a more densely staining precipitate. However, QIEP for 3 hr at 15 ma gave results that did not differ statistically from 18 hr, 10 ma QIEP (Fig. 3). The choice of voltage is influenced by the molecular weight of the antigen, the ionic strength of the sample, and the desired bluntness or sharpness of the peaks. Although nonlinearity of antigen concentration to peak area (or height) complicates analysis (6), data may be linearized by a log-log transformation. Routine quantification is a simple matter if viral samples have been cultivated under the same conditions, just as is generally the case if any other characteristic were to be compared.

The results reveal interesting problems that may be inherent in this type of work. Statistical differences may not always agree with observed biological differences. For example, SAD-I and SAD-S are indistinguishable from PMV by QIEP, although spurs will form in the DID test. Panicum mosaic virus and SAD have a different host range.

The slope term, although not independent of intercept (a titer term), is an indicator of serological relationship to other strains. The QIEP test may be a more sensitive indicator of partial serological identity than DID. Ten of the 13 cases in which the two tests do not agree involve SAD-N and SAD-S, suggesting that these isolates may not be homogeneous. In either case, both tests should be applied when examining closely related virus strains (or isolates) for serological relationships.

Our results suggest that a single homologous antiserum can be used to differentiate several closely related virus strains. Testing with homologous antisera to other strains should resolve anamolous results. Work is currently under way to accomplish this.

The QIEP method can be used to quantify any immunogenic substance. It can be used to screen breeding lines, to monitor virus purification results, and to quantitate fractions from column chromatography or gradient centrifugation. Once an extinction coefficient is determined for PMV or SAD, data in Table 3, for example, can be readily converted to antigen concentration.

One of the major reasons QIEP was adopted by our laboratory was to provide rapid and accurate differentiation of maize dwarf mosaic virus (MDMV) and sugarcane mosaic virus (SCMV) strains. The QIEP technique is applicable to the MDMV/SCMV complex (*unpublished*) although degradation of virus particles into subunits prior to electrophoresis is required. We are currently evaluating experimental conditions that will yield optimum results with this potyvirus system.

LITERATURE CITED

1. Axelsen, N. H., Krøll, J., and Weeke, B. 1973. A Manual of Quantitative Immunoelectrophoresis. Methods and Applications. Universitietsforlaget, Oslo, Bergen, and Trømso, Norway. 169 pp.
2. Banttari, E. E. 1980. Sap extraction device for sampling potato leaves and tubers. Am. Potato J. 57:345-348.
3. Crowle, A. J., and Cline, L. J. 1977. An improved stain for immunodiffusion tests. J. Immunol. Meth. 17:379-381.
4. Havranek, P. 1978. Use of quantitative immunoelectrophoresis in cucumber mosaic virus assay. I. Identification of antigenic patterns. Phytopathol. Z. 92:351-358.
5. Havranek, P. 1978. Use of quantitative immunoelectrophoresis in cucumber mosaic virus assay. II. Strain- and isolate-specific modifications of the antigenic complex. Phytopathol. Z. 93:5-11.
6. Havranek, P. 1978. Use of quantitative immunoelectrophoresis in cucumber mosaic virus assay. III. Evaluation of dilution curves. Phytopathol. Z. 93:97-104.
7. Holcomb, G. E. 1974. Serological strains of panicum mosaic virus. (Abstr.) Proc. Am. Phytopathol. Soc. 1:21.
8. Laurell, C. B. 1965. Antigen-antibody crossed electrophoresis. Anal. Biochem. 10:358-361.
9. Laurell, C. B. 1965. Electrophoretic microheterogeneity of serum antitrypsins. Scand. J. Clin. Lab. Investig. 17:271-274.
10. Laurell, C. B. 1966. Quantitative estimation of proteins by electrophoresis in agarose gels containing antibodies. Anal. Biochem. 15:45-52.
11. Laurell, C. B. 1966. Quantitative estimation of protein by electrophoresis in antibody-containing agarose gel. Proc. Colloq. Protides and Biol. Fluids. 14:499-502.
12. Laurell, C. B. 1972. Electroimmunoassay. Scand. J. Clin. Lab. Investig. 20 (Suppl.) 124:21-37.
13. Niblett, C. L., and Paulsen, A. Q. 1975. Purification and further characterization of panicum mosaic virus. Phytopathology 65:1157-1160.
14. Niblett, C. L., Paulsen, A. Q., and Toler, R. W. 1977. Panicum mosaic virus. No. 177 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew, Surrey, England. 4 pp.
15. Ouchterlony, Ö., and Nilsson, L. Å. 1973. Immunodiffusion and immunoelectrophoresis. Chapter 19 in: Handbook of Experimental Immunology. D. M. Weir, ed. Blackwell.
16. Paul, H. L., Querfurth, G., and Huth, W. 1980. Serological relationships of some isometric viruses of the Gramineae. J. Gen. Virol. 47:67-77.
17. Scolari, M., Picard, J. J., and Heremairs, J. F. 1968. Quantitative determination of human α_2-macroglobulin by means of immunoelectrophoresis in antibody-containing gel. Clin. Chem. Acta 19:25-32.
18. Snedecor, G. W., and Cochran, W. G. 1967. Statistical Methods. Sixth ed. Iowa State Univ. Press, Ames. 593 pp.
19. Verbruggen, R. 1975. Quantitative immunoelectrophoretic methods: A literature survey. Clin. Chem. 21:5-43.
20. Weeke, B. 1973. Rocket immunoelectrophoresis. Pages 37–46 in: A Manual of Quantitative Immunoelectrophoresis. N. H. Axelsen, J. Krøll, and B. Weeke, eds. Universitietsforlaget, Oslo, Bergen, and Trømso, Norway.