A TWO-STEP ELISA FOR RAPID, RELIABLE DETECTION OF POTATO VIRUSES

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Abstract

The reliability of the standard double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was compared with a shorter, two-step DAS procedure in which sample and conjugate were mixed and incubated together in one step. The two assays were compared using beet western yellows virus and potato leafroll, M, S, X, and Y viruses. The two-step procedure was more sensitive, *i.e.*, it detected small quantities of virus with greater statistical reliability than the standard procedure. At high virus concentrations, the standard produced stronger ELISA reactions than the two-step assay, but both assays were reliable. Since all of the viruses tested withstood high incubation temperatures, the incubation period for the two-step procedure could be reduced to 1 hr at 30 or 37 C. Therefore, assays could be completed within 2 hr using the two-step procedure compared with 2 days for the standard procedure. Reliable results were achieved with samples prepared by grinding tissues in buffer or, more simply, by adding pure, pressure extracted juice directly to conjugate in assay wells. Coating plates with gamma globulins or with $F(ab')_2$ fragments of gamma globulins gave equally reliable results with all viruses except potato leafroll, where coating with gamma globulins was superior.

Compendio

Se comparó la confiabilidad del método estándar de doble anticuerpo en la técnica de detección por conjugados enzimáticos (DAS-ELISA) con un procedimiento más corto de dos pasos, en el cual la muestra y el conjugado se mezclaron e incubaron al mismo tiempo en un paso. Se compararon las dos pruebas usando el virus occidental del amarillamiento de la remolacha y los virus del enrollamiento de la papa, M, S, X e Y. El

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procedimiento de dos pasos mostró mayor sensibilidad, o sea que detectó pequeñas cantidades de virus con una mayor confiabilidad estadística que el procedimiento estándar. A altas concentraciones del virus, el procedimiento estándar produjo reacciones más marcadas que el procedimiento de dos pasos, pero ambas pruebas fueron confiables. Considerando que todos los virus probados soportaron temperaturas altas, el período de incubación con el procedimiento de dos pasos podría ser reducido a 1 hr a 30 ó 37 C. Por lo tanto usando este procedimiento las pruebas pueden completarse en 2 hr, en lugar de 2 días que requiere el procedimiento estándar. Se consiguieron resultados confiables con muestras preparadas triturando el tejido en bufer o simplemente agregando directamente en los hoyos de prueba jugo puro extraído a presión. El revestimiento de las placas con gama globulinas o con fragmentos de gama globulinas $F(ab')_2$ dió igualmente resultados confiables con todos los virus, con excepción del virus del enrollamiento, donde el revestimiento con gama globulinas fué superior.

Introduction

The general applicability of the standard, double antibody sandwich protocol for the enzyme-linked immunosorbent assay (DAS-ELISA) has been reported for many plant viruses that differ in morphology, stability, and concentration (3, 10). This assay greatly increased the sensitivity (capacity to detect minute quantities of virus) of virus detection and has become the standard assay for routine detection of nearly all plant viruses. However, still greater detection sensitivity is needed for many applications in potato virology. In routine indexing, perhaps the most widely employed assay application, potato leafroll virus (PLRV) infected plants often escape detection because the virus fails for a variety of reasons to reach concentration levels detectable by the standard DAS-ELISA (unpublished data). In another area, a somewhat more sensitive assay that would routinely detect PLRV in migrating aphids could markedly reduce our dependence on insecticides by targeting their use to precisely the times and places needed to kill infective aphids.

The purpose of this study was to find a more sensitive modification of the standard DAS-ELISA that was readily adaptable for mass testing programs. One factor limiting sensitivity of ELISA is the binding affinity between antigen (virus) and antibody (5). Binding affinity, expressed as equilibrium constant, determines the amount of free virus that must dissociate from the antigen-antibody complex to satisfy equilibria requirements each time a new buffer is added to the reaction well after sample application (9). The amount of free virus necessary to satisfy equilibria requirements is a very small percentage of the total bound virus at high antigen-antibody concentrations, but the percentage increases as the amount of bound virus decreases until there is not enough bound virus to satisfy equilibria requirements.

Logically, sensitivity could be increased by decreasing the amount of dissociation from the antigen-antibody complex bound at the surface of the ELISA reaction well following sample application. Dissociation could be decreased by increasing the antigen-antibody binding affinity (5). It seemed probable that dissociation could also be increased by decreasing the number of buffer changes and the length of incubation in each buffer change. Flegg and Clark (6) had previously developed a two-step DAS-ELISA procedure in which sample and conjugate were mixed and incubated together in one step. This eliminated one series of washings and the long conjugate incubation step. The capacity of the two-step assay to detect apple chlorotic leafspot virus, in contrast to the standard assay, was attributed to a stabilizing effect on the structure of the virus by its reaction with antibodies prior to binding on the surface of the reaction well. The relative sensitivity of the procedure was not investigated. Although others (2, 8, 11) have subsequently demonstrated the two-step procedure to be a reliable method to detect several other plant viruses, it has not been commonly adopted in plant virology.

Since the two-step procedure eliminates a long incubation step and a washing series from standard DAS-ELISA, it seemed possible that it could increase sensitivity of the standard assay. We tested this hypothesis using two luteoviruses, two carlaviruses, a potevirus, and a potexvirus. A preliminary report was published (7).

Materials and Methods

Assays were performed using antisera against PLRV (a field isolate from Washington), beet western yellows virus (BWYV) (isolate IY1R from J.E. Duffus, Salinas, CA), potato virus M (PVM) (an isolate from R.W. Goth, Beltsville, MD), potato viruses S (PVS), X (PVX), and Y (PVY) (field isolates from Washington). Antisera were prepared in our laboratory.

Gamma globulins were purified and conjugated with alkaline phosphatase by the methods of Clark and Adams (4). $F(ab')_2$ fragments of gamma globulins were prepared by the procedure of Barbara and Clark (1). Gamma globulins, $F(ab')_2$ fragments, and conjugates were used at end concentrations of 1 μg/ml in all tests.

Virus samples were prepared from infected plant leaves, and control samples were prepared from leaves of healthy plants of the same species. The leaves were ground in extracting buffer (4) with mortar and pestle and assayed at a dilution of 1:20 (w/v). Aliquots of the same sample preparations were used to compare standard and two-step ELISA results.

Standard DAS-ELISA's were conducted as described by Clark and Adams (4). ELISA plates (96-well, polystyrene) were precoated either with purified gamma globulins or with $F(ab')_2$ fragments prepared from gamma globulins. The plates were incubated 18 h at 4 C with 200 μl of sample in each well. After washing, they were incubated for 4 hr at 37 C with 200 μl of

conjugate in each well. Plates were then washed with PBS-tween buffer and incubated at 23 C with 200 μl of p-nitrophenol substrate added to each well. After exactly 30 min, the optical density at 405 nm was measured spectrophotometrically using a BIO-TEK model EL 307 EIA reader. In some instances, optical density was measured again after 1 or 2 hr.

Two-step ELISA's were conducted the same as standard DAS-ELISA's except that the sample was diluted in conjugate in the sample well, and the two were incubated together in one step. To obtain the same end dilutions of sample and conjugate as in the standard procedure, 100 μl of double strength (1:10) sample were added to 100 μl of double strength (2 μg/ml) conjugate directly in plate wells.

Other experiments were conducted to determine whether reliable results could be achieved using pressure-extracted juice as the sample. The raw juice was added directly to plate wells containing either extracting buffer (standard procedure) or conjugate (two-step procedure). Volume and concentration of buffer and conjugate were adjusted according to the volume of juice added to obtain a total volume of 200 μl in each well at an end conjugate concentration of 1 μg/ml. Standard procedures were then performed as usual, and the sample/conjugate mixture was incubated for 4 hr at 30 C in the two-step procedure.

Results

The results of standard DAS-ELISA were compared with those of the two-step procedure in numerous trials over a period of 3 mos. In each comparison, seven different incubation time and temperature combinations were tested ranging from 18 hr at 4 C to 2 hr at 37 C (Table 1). As the species and age of test plants, daylength, and other environmental factors changed during the period of testing, the strength (rate of increase in $OD_{405\ nm}$) of ELISA reactions obtained from virus-infected tissue varied considerably. The background reactions from healthy control plants remained essentially constant. Typical results of three experiments conducted with tissues that produced relatively strong, medium, and weak reactions are presented for each virus in Table 1.

Coating plates with gamma globulins was clearly superior ($P=0.05$) to coating with $F(ab')_2$ fragments for detection of PLRV, but the two coating materials produced equally reliable ($P=0.05$) detection of PVM, PVS, PVX or PVY (Table 1).

Under the same conditions for conjugate incubation (18 hr at 4 C), the two-step and standard assays had the same statistical reliability for all viruses using tissues that produced relatively strong or medium ELISA reactions (Table 1). In contrast, with tissues that produced relatively weak ELISA reactions, the two-step procedure detected all viruses except PVS with greater statistical reliability than the standard assay. This was true whether

TABLE 1. — *Two-step ELISA compared with standard DAS-ELISA at three sample strengths and with ELISA plates coated either with purified gamma globulins or with F(ab')$_2$ fragments of gamma globulins.*

		Gamma Globulin Plate Coating					F(ab')$_2$ Fragment Plate Coating				
		STD[3]	Two-Step ELISA[4]				STD	Two-Step ELISA			
		4 C[5]	4 C	37 C			4 C	4 C	37 C		
Virus[1]	Sample[2] Strength	18-Hr[6]	18-Hr	4-Hr	2-Hr	1-Hr	18-Hr	18-Hr	4-Hr	2-Hr	1-Hr
PLRV	Strong	1.77[7] aba	>2.00 aaa	>2.00 aaa	1.20 aca	0.77 ada	0.63 baa	0.81 baa	0.35 bba	0.31 bba	0.23 bba
	Medium	0.83 abb	1.02 aab	0.49 acb	0.45 acb	0.24 adb	0.42 adb	0.27 bab	0.14 bb-	0.12 abb	0.08 aba
	Weak	0.16 abc	0.23 aac	0.19 a-c	0.16 abc	0.10 abc	0.09 aac	0.14 ba-	0.19 aa-	0.07 bac	0.06 aaa
	Healthy	0.03 aac	0.01 aad	0.01 aad	0.03 aac	0.01 bac	0.04 aac	0.01 aac	0.02 aab	0.03 aac	0.03 aaa
	Buffer	0.02 aac	0.02 aad	0.01 aad	0.02 aac	0.01 aac	0.00 bac	0.00 bac	0.03 bab	0.01 bac	0.00 aaa
BWYV	Strong	1.21 ba	>2.00 aa	>2.00 aa	1.76 aa	1.09 ba					
	Medium	0.83 ba	>2.00 aa	1.64 aa	1.92 aa	0.88 ba					
	Weak	0.28 cb	1.31 ab	0.51 -b	0.84 bb	0.42 -b					
	Healthy	0.06 ab	0.06 ac	0.06 ac	0.06 ac	0.01 ab					
	Buffer	0.00 ab	0.03 ac	0.01 ac	0.02 ac	0.00 ab					
PVM	Strong	>2.00 baa	>2.00 aaa	>2.00 aaa	1.45 aba	1.42 bba	>2.00 aaa	>2.00 aaa	>2.00 aaa	>2.00 baa	1.61 aba
	Medium	>2.00 baa	1.43 abb	1.57 abb	0.71 acb	0.38 adb	>2.00 aaa	1.82 aaa	1.82 aaa	0.84 acb	0.44 adb
	Weak	0.72 abb	1.35 aab	1.24 aac	0.49 a-b	0.21 ac-	0.79 acb	1.53 aab	1.20 abb	0.43 adc	0.22 adc
	Healthy	0.01 aac	0.00 bac	0.01 aad	0.01 aac	0.01 aac	0.01 aac	0.01 aac	0.00 aac	0.01 aad	0.02 aac
	Buffer	0.00 aac	0.00 aac	0.00 aad	0.01 aac	0.01 aac	0.02 aac	0.00 aac	0.01 aac	0.01 aad	0.01 aac
PVS	Strong	1.59 aca	>2.00 aaa	1.88 a-a	1.13 ada	0.76 aea	1.39 bba	1.49 baa	1.49 baa	0.76 bca	0.68 ada
	Medium	1.00 aab	1.02 aab	0.75 abb	0.32 acb	0.17 adb	1.07 abb	1.39 baa	0.99 bbb	0.45 bdb	0.28 aeb
	Weak	0.46 aac	0.25 abc	0.24 a-c	0.14 a-c	0.11 ac-	0.51 aac	0.35 abc	0.30 a-c	0.21 a-c	0.18 adc
	Healthy	0.01 aac	0.00 bac	0.01 aad	0.01 aac	0.01 aac	0.01 aac	0.01 aac	0.00 aac	0.01 aad	0.02 aac
	Buffer	0.01 aad	0.07 aad	0.01 aad	0.01 aad	0.00 aac	0.02 aad	0.07 aad	0.03 bad	0.01 aad	0.01 aac
PVX	Strong	>2.00 aaa	>2.00 aaa	>2.00 aaa	>2.00 aaa	1.84 aba	>2.00 aaa	>2.00 aaa	>2.00 aaa	1.83 baa	1.13 bba
	Medium	>2.00 aaa	>2.00 aaa	1.77 abb	1.11 acb	0.59 adb	>2.00 aaa	>2.00 aaa	1.93 aaa	1.09 acb	0.71 adb

TABLE 1. — Continued.

Virus[1]	Sample[2] Strength	Gamma Globulin Plate Coating: STD[3] 4 C[5] 18-Hr[6]	Gamma Globulin Plate Coating: Two-Step ELISA[4] 4 C 18-Hr	Gamma Globulin Plate Coating: Two-Step ELISA[4] 37 C 4-Hr	Gamma Globulin Plate Coating: Two-Step ELISA[4] 37 C 2-Hr	Gamma Globulin Plate Coating: Two-Step ELISA[4] 37 C 1-Hr	$F(ab')_2$ Fragment Plate Coating: STD 4 C 18-Hr	$F(ab')_2$ Fragment Plate Coating: Two-Step ELISA 4 C 18-Hr	$F(ab')_2$ Fragment Plate Coating: Two-Step ELISA 37 C 4-Hr	$F(ab')_2$ Fragment Plate Coating: Two-Step ELISA 37 C 2-Hr	$F(ab')_2$ Fragment Plate Coating: Two-Step ELISA 37 C 1-Hr
	Weak	0.45 abb	0.74 aab	0.46 abc	0.21 a-c	0.11 adc	0.38 abb	0.83 aab	0.27 abb	0.21 abc	0.13 abc
	Healthy	0.05 aac	0.01 aac	0.03 aad	0.02 aad	0.02 aac	0.05 bac	0.01 aac	0.03 aac	0.03 aac	0.03 aac
	Buffer	0.01 aac	0.00 aac	0.00 aad	0.00 aad	0.01 aac	0.00 bac	0.00 aac	0.00 aac	0.00 aac	0.00 aac
PVY	Strong	>2.00 aaa	>2.00 aaa	>2.00 aaa	1.56 aba	0.91 aca	>2.00 aaa	>2.00 aaa	1.84 aba	1.35 aca	0.78 aca
	Medium	1.59 abb	>2.00 aaa	1.29 acb	0.95 adb	0.50 aeb	1.33 bbb	1.77 bab	0.89 bcb	0.66 bdb	0.45 aeb
	Weak	0.24 abc	0.91 aab	0.39 abc	0.18 a-c	0.08 acc	0.21 a-c	0.74 aac	0.30 abc	0.22 a-c	0.07 adc
	Healthy	0.04 aac	0.05 aac	0.01 aad	0.03 aac	0.01 aac	0.02 aad	0.01 bad	0.02 aad	0.03 aad	0.02 aac
	Buffer	0.01 aac	0.06 aac	0.01 aad	0.02 aac	0.01 aac	0.01 aad	0.00 bad	0.02 aad	0.02 aad	0.02 aac

[1]Virus acronyms are: PLRV (potato leafroll virus), BWYV (beet western yellows virus), PVM, PVS, PVX, PVY (potato viruses M, S, X, and Y, respectively).

[2]Virus source plant samples were selected to produce relatively strong, moderate, or weak virus specific reactions in ELISA. Each set of comparable assays was conducted at the same time and with the same bulk sample.

[3]The DAS-ELISA procedure described by Clark and Adams (4) that involves incubation of sample on precoated plates followed by separate incubation of conjugate on the same plates.

[4]A modification of the standard DAS-ELISA that combines incubation of sample and conjugate in the same step.

[5]Incubation temperature for the sample/conjugate mixture.

[6]Incubation time for the sample/conjugate mixture.

[7]Optical density of reaction wells at 405 nm, 30 min after adding substrate. Each value is the mean of three duplicate assays. Analysis of variance by split plot design and data for each virus were analyzed independently. Means followed by the same letter do not differ significantly using Duncan's multiple range test at $P=0.05$. The first letter after each mean compares coating with gamma globulin or with the equivalent $F(ab')_2$ coating. The second letter compares the five ELISA protocols (left to right) independently under gamma globulin and $F(ab')_2$ coatings. The last letter compares strong, moderate, and weak samples of each virus within each treatment. A "-" in place of a letter indicates no difference between the preceding and succeeding means.

plates were coated with gamma globulins or with $F(ab')_2$ fragments (Table 1).

Since all of the viruses reacted properly at elevated temperatures in the two-step assay, it was possible to reduce the conjugate incubation period to a few hours at high temperature. At 37 C, the conjugate/sample incubation period could be reduced to as little as 4 hr before its reliability fell below that of the standard assay in which conjugate is incubated 18 at 4 C. At shorter conjugate incubation periods, reliability of the two-step assay began to erode when the substrate incubation period was limited to only 30 min, as it was for all of the assays presented in Table 1.

The length of the substrate incubation period had a strong influence on the statistical reliability of assays. With the substrate incubation period limited to only 30 min, assays were not reliable at conjugation incubation periods of 1 or 2 hr, especially for samples that produced relatively weak ELISA reactions (Table 1 and Table 2). However, when the substrate incubation period was extended to 1 hr for these weak reactions, O.D. readings for the virus specific reactions approximately doubled, and the two-step assay was always highly reliable ($P=0.01$) for all of the viruses tested (Table 2). Virus specific readings doubled again in the second hour of substrate incubation. Extending the substrate incubation period beyond 30 min had little, if any, effect on the very low non-specific background readings of healthy tissues (Table 2).

TABLE 2. — *Effect of substrate incubation time on the diagnostic sensitivity of the two-step modification[1] of the standard[2] ELISA with tissue samples that produced relatively weak ELISA reactions.*

	Substrate Incubation Time					
	0.5 hr		1 hr		2 hr	
Virus[3]	Infected	Healthy	Infected	Healthy	Infected	Healthy
PLRV	0.10 c[4]	0.01 c	0.19 b	0.01 c	0.34 a	0.02 c
BWYV	0.24 c	0.06 c	0.54 b	0.06 c	1.04 a	0.06 c
PVM	0.21 c	0.01 d	0.43 b	0.01 d	0.89 a	0.01 d
PVS	0.06 c	0.01 c	0.11 b	0.02 c	0.21 a	0.03 c
PVX	0.12 c	0.02 c	0.23 b	0.01 c	0.45 a	0.02 c
PVY	0.09 c	0.01 c	0.19 b	0.02 c	0.32 a	0.01 c

[1]A modification of the standard DAS-ELISA which combines incubation of sample and conjugate in the same step.

[2]The DAS-ELISA procedure described by Clark and Adams (4) that involves incubation of sample on precoated plates followed by separate incubation of conjugate on the same plates.

[3]Virus acronyms are: PLRV (potato leafroll virus), BWYV (beet western yellows virus), PVM, PVS, PVX, PVY (potato viruses M, S, X, and Y, respectively).

[4]Optical density of reaction mixtures at 405 nm. Each value is the mean of three replicate assays. Data analyzed by analysis of variance, means compared independently for each virus. Optical density values followed by the same letter do not differ significantly using Duncan's multiple range test at $P=0.01$.

There were no differences in detection reliability at conjugate incubation temperatures of 30 and 37 C for any of the viruses.

In both standard and two-step assays, highly reliable results were achieved using, as the sample, pressure-extracted juice added directly to buffer or conjugate in the ELISA plate wells (Table 3). At very high juice concentrations (1:2 juice/total volume), O.D. values were higher for the standard assay than for the two-step assay. With increasing sample dilution however, strength of the standard assay decreased while strength of the two-step assay initially increased before decreasing (Table 3). High juice concentrations tended to increase the healthy control background reactions in both the standard and two-step assays.

Discussion

The two-step variation of the standard DAS-ELISA was a more sensitive assay than the standard, *i.e.*, it detected low concentrations of virus with greater reliability than the standard. Although reliability of the two-step assay decreased as the length of the conjugate/sample incubation period decreased, the length of the conjugate/sample incubation period could be reduced to as little as 4 hr before its reliability fell below that of the standard assay in which conjugate is incubated 18 hr. Highly reliable assays could be performed with the two-step assay using a 1 hr conjugate/sample incubation period. Because the two-step assay eliminated a washing series and an incubation step, it required less labor and was simpler to perform.

The greater sensitivity of the two-step compared with standard ELISA probably results from the elimination of a washing series and an incubation step. Because antibody antigen reactions are reversible and behave according to the concepts of chemical equilibria (9), a small amount of virus dissociates from the antibodies bound at the surface of the reaction well each time the buffer is changed, and it is washed from the well. This amount of virus may constitute a high percentage of the total bound virus in assays where virus concentration is low.

The reduced strength of the two-step ELISA reaction at high virus concentrations is probably the result of competition for a limited supply of conjugate by an excess amount of virus. In standard ELISA, the virus-conjugate reaction involves only virus that is bound by coating gamma globulins at the surface of the reaction well. Therefore, conjugate is in excess and the virus can become completely conjugated. In the two-step assay, the virus-conjugate reaction begins in solution before virus is bound to the well surface. It involves not only that virus that ultimately becomes bound to the well surface but also that which is discarded when the plate is washed following conjugate incubation. Thus, an excess of virus in the sample would result in incomplete conjugation of the virus that ultimately becomes bound on the well surface. Although very high virus concentration in the

TABLE 3. — *Two-step compared with standard DAS-ELISA using as sample pressure-extracted plant juice diluted with buffer or conjugate in reaction wells of ELISA plate.*

		Dilution[2] of plant juice							
		Infected juice					Healthy		Buffer
Virus[1]	Method	1:2	1:4	1:10	1:20	1:40	1:2	1:20	1:2
PLRV	STD[3]	1.51 a[4]	0.92 b	0.51 -	0.29 c	0.18 -	0.05 d	0.00 d	0.02 d
	2-ST[5]	0.42 -	0.63 a	0.62 a	0.40 -	0.22 b	0.06 c	0.01 c	0.00 c
BWYV	STD	0.93 a	0.62 b	0.33 c	0.19 d	0.12 -	0.06 e	0.01 e	0.00 e
	2-ST	0.43 a	0.42 a	0.27 b	0.20 c	0.16 c	0.03 d	0.01 d	0.00 d
PVM	STD	>2.00 a	>2.00 a	>2.00 a	2.00 a	1.59 a	0.06 b	0.01 b	0.01 b
	2-ST	0.68 d	1.23 c	1.53 a	1.47 -	1.39 b	0.03 e	0.00 e	0.00 e
PVS	STD	1.17 a	0.81 b	0.69 -	0.63 -	0.58 c	0.07 d	0.03 d	0.02 d
	2-ST	0.13 -	0.26 b	0.63 a	0.72 a	0.73 a	0.06 d	0.02 d	0.02 d
PVX	STD	>2.00 a	>2.00 a	>2.00 a	>2.00 a	>2.00 a	0.09 b	0.02 b	0.01 b
	2-ST	1.92 a	>2.00 a	1.93 a	>2.00 a	1.79 b	0.06 c	0.01 c	0.00 c
PVY	STD	>2.00 a	>2.00 a	>2.00 a	>2.00 a	1.91 b	0.09 c	0.02 d	0.01 d
	2-ST	>2.00 a	>2.00 a	>2.00 a	>2.00 a	1.83 b	0.11 c	0.00 d	0.00 d

[1]Virus acronyms are: PLRV (potato leafroll virus), BWYV (beet western yellows virus), PVM, PVS, PVX, PVY (potato viruses M, S, X, and Y, respectively).

[2]Parts raw juice to total volume of sample (juice plus buffer or conjugate) in reaction well.

[3]The DAS-ELISA procedure described by Clark and Adams (4) that involves incubation of sample on precoated ELISA plates followed by a separate incubation of conjugate on the same plates.

[4]Optical density of reaction mixtures at 405 nm. Each value is the mean of three replicate assays. Data analyzed by analysis of variance, means compared independently for each horizontal line of values. For each virus, optical density values followed by the same letter do not differ significantly using Duncan's multiple range test at P=0.01. A "-" in place of a letter indicates no difference between the preceding and succeeding means.

[5]A modification of the standard DAS-ELISA that combines incubation of sample and conjugate in the same step.

sample reduced virus-specific reactions of the two-step assay, in practice it never jeopardized its reliability. Furthermore, this limitation was easy to overcome, since near optimum reactions were obtained over a broad dilution range from 1:4 up to 1:40.

Flegg and Clark (6) also observed a reduced response of the two-step assay when testing for CLSV in extracts of *Chenopodium quinoa* Willd. Since a reduced response was not observed with concentrated extracts of woody hosts of the virus, they attributed the phenomenon to an interference by a host constitutent. Based on our results, an alternative possibility is that *C. quinoa* contained a higher concentration of virus than the woody hosts, and the reduction in reaction resulted from competition of the virus for available conjugate.

Except in the case of PLRV, where it was detrimental, we found no advantage or disadvantage to coating plates with $F(ab')_2$ fragments. However the fact that $F(ab')_2$ fragments were satisfactory introduces the possibility that an indirect, protein A-enzyme conjugate system could be utilized with two-step ELISA.

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