Etiology

Clover Yellow Vein Virus in *Limonium sinuatum*

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Preparation of the CYVV antiserum by H. T. Hsu at the American Type Culture Collection and the technical assistance of J. T. McKenzie is gratefully acknowledged. We also thank O. W. Barnett, J. Hammond, R. Koenig, and F. W. Zettler for providing virus cultures and antisera.

Accepted for publication 7 March 1985 (submitted for electronic processing).

ABSTRACT

Lawson, R. H., Brannigan, M. D., and Foster, J. 1985. Clover yellow vein virus in *Limonium sinuatum*. Phytopathology 75:899-906.

A potyvirus from statice, *Limonium sinuatum*, imported from Colombia, was identified as a strain of clover yellow vein virus (CYVV-St) by serology and immunosorbent electron microscopy (IEM). Two isolates of CYVV-St induced reactions of different severity in several hosts, but the two isolates were serologically indistinguishable. The host ranges of both isolates were more similar to the white clover isolate of CYVV from Pratt (CYVV-P) than to bean yellow mosaic virus (BYMV) from gladiolus (BYMV-G, BYMV-G82-25) and red clover (BYMV-204-1). CYVV-St was partially purified from *Chenopodium quinoa* with chloroform, carbon tetrachloride extraction, and differential centrifugation. Concentration of the virus with PEG 8000 resulted in aggregation and reduced yields. The virus was further purified by equilibrium centrifugation in Cs_2SO_4 but was unstable in CsCl. The normal length of purified virions was 777 nm. Infected leaves of *C. quinoa* contained cytoplasmic and nuclear inclusions. In agar gel double-diffusion, indirect ELISA, double antibody sandwich ELISA, and immunosorbent electron microscopy tests CYVV-St showed a closer serological relationship to CYVV-P than to BYMV:204-1 and a more distant relationship to BYMV-G and BYMV-G82-25. Antiserum to statice virus Y (StaVY) from Germany reacted more strongly with CYVV-P and CYVV-St than with the BYMV isolates. We conclude that CYVV-St and StaVY are strains of CYVV related to BYMV-CYVV-pea mosaic viruses and that CYVV-St is more closely related to BYMV-204-1 than to the gladiolus isolates of BYMV.

Statice, *Limonium sinuatum* (L). Mill., is an important commercial floriculture crop produced in the southeastern United States. In recent years, its popularity both as fresh cut and dried flowers has increased. Several viruses have been reported in statice including: turnip mosaic (13); cucumber mosaic (9); broad bean wilt (4); and statice virus Y (StaVY), a potyvirus related to bean yellow mosaic virus (BYMV) and clover yellow vein virus (CYVV) (9). Symptoms similar to the light green mosaic reported in statice infected with StaVY in Germany were observed in statice imported from Colombia, S. A., into the United States.

The purpose of this investigation was to determine the etiology of this disease of statice and to compare the pathogenic agent with those previously reported in statice.

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MATERIALS AND METHODS

Virus cultures. Two virus isolates from a statice plant showing light green mosaic, vein yellowing, leaf malformation, and stunting

(Fig. 1A) were established in *Chenopodium quinoa* Willd. CYVV-St-1 was isolated 1 yr before CYVV-St-2 and maintained in *C. quinoa* (Fig. 1B). BYMV-204-1 (5) from red clover (*Trifolium pratense* L.) and CYVV-P (14) from white clover (*T. repens* L.) and their respective antisera were obtained from O. W. Barnett, Clemson University. BYMV-G (16) from gladiolus and the homologous antiserum were supplied by F. W. Zettler, University of Florida. BYMV-G82-25, also from gladiolus, was obtained from J. Hammond of our laboratory.

Host range. CYVV-St-1 and CYVV-St-2 were isolated in *C. quinoa* inoculated with leaf extracts from statice triturated in 0.05 M Na_2HPO_4-KH_2PO_4, pH 7.0. CYVV-St-1 and -2 were transmitted to *Pisum sativum* L. 'Alaska' (Fig. 1C) from *C. quinoa* only after partial purification and not from crude sap. Inoculum from Alaska pea was used for host range comparisons among CYVV-St-1, CYVV-St-2, CYVV-P, BYMV-204-1, BYMV-G, and BYMV-G82-25. Additional comparisons were made between CYVV-St-1 and CYVV-St-2 by using *Phaseolus vulgaris* L. 'Black Turtle Soup' and 'Bountiful' and Alaska pea as different inoculum sources.

Inoculations were made in a greenhouse at different seasons of the year. In winter, test plants were grown between 18 and 23 C with 20,000 lux fluorescent illumination on a 16-hr photoperiod. In summer, temperatures ranged from 20 to 32 C and plants were grown under shade.

Purification. CYVV-St 1 or -2 was purified from leaves of *C. quinoa* showing necrotic local lesions 7–8 days after inoculation and other isolates from systemically infected leaves of Alaska pea 2–3 wk after inoculation. Plants were darkened for 24–48 hr before harvest. Preparations of CYVV-St-1 and -2 purified from *C. quinoa* contained less host material if excess symptomless tissue was trimmed from infected leaves prior to processing. Leaves were homogenized in 0.1 M Na_2HPO_4–NaH_2PO_4 containing 0.25% Na_2SO_3 and 0.01 M EDTA, pH 7.0, (PO_4 buffer) (5 ml/g), carbon tetrachloride (1 ml/g), and chloroform (1 ml/g). The homogenate was stirred with 2% Triton X-100 and further purified by differential centrifugation and sedimentation through a 30% sucrose pad. Pellets were resuspended manually in 0.5–1.0 ml of 0.05 M boric acid/KCl (borate), 0.001 M dithiothreitol (DTT), and 0.25% Na_2SO_3 at pH 8.0. Following low-speed centrifugation, the virus was mixed with Cs_2SO_4 1.29 g/cc or CsCl 1.37 g/cc in the same borate, DTT, SO_3 medium. Equilibrium gradients were formed by centrifugation at 103,000 *g* for 18 hr, and separated fractions were dialyzed against 0.02 M borate buffer, pH 8.0, and stored at −60 C.

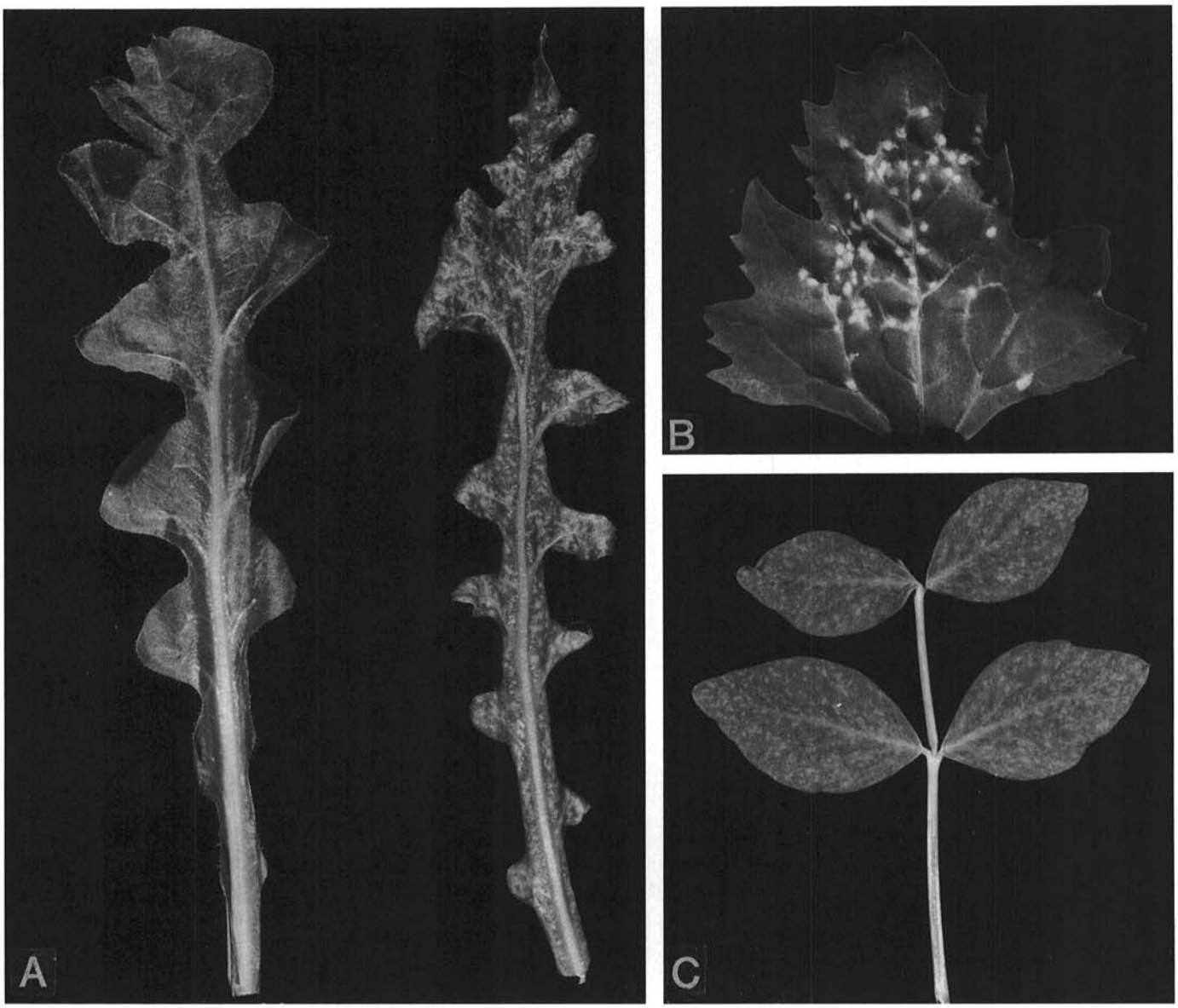

Fig. 1. Symptoms induced by the statice (*Limonium sinuatum*) strain of clover yellow vein virus (CYVV-St). **A,** Leaf strapping and chlorotic mosaic induced by CYVV-St in *L. sinuatum* (right), healthy (left). **B,** Necrotic and chlorotic local lesions and vein necrosis induced by CYVV-St in *Chenopodium quinoa*. **C,** Systemic mosaic induced by CYVV-St in *Pisum sativum* 'Alaska.'

CYVV-St-1 purification was also attempted by polyethlene glycol (PEG 8000) precipitation following organic solvent clarification. The virus was sedimented, after stirring with 4% PEG 8000 for 1 hr at 4 C, by centrifuging at 7,000 *g* and resuspended in 0.1 M borate, 0.25% Na_2SO_3 and 0.001 M DTT, pH 8.0. The virus was either reconcentrated by high-speed centrifugation or precipitated a second time with PEG 8000. A concurrent purification was performed by using two cycles of high-speed centrifugation and separation on Cs_2SO_4 gradients and final yields were compared.

Antiserum preparation. A New Zealand white rabbit was injected intravenously with 150 μg of purified CYVV-St-1, followed in 13 days by an intramuscular (IM) injection of 330 μg of virus mixed with Freund's complete adjuvant. A final IM injection and a subcutaneous injection composed of 215 μg of immunogen each mixed with Freund's complete adjuvant were administered 23 days later. The rabbit was bled 10 and 20 days after the final injection.

Serology. CYVV-St-1 and -2 were tested with antiserum to CYVV-St-1, CYVV-P, BYMV-204-1, and BYMV-G in agar double-diffusion tests, indirect ELISA (7), double antibody sandwich (DAS) ELISA (2), and immunosorbent electron microscopy (IEM) (11). Because CYVV-St-1 and -2 were serologically indistinguishable, and CYVV-St-1 was usually obtained in lower yields, CYVV-St-2 antigen was used to evaluate the serological relationship of CYVV-St with CYVV-P, BYMV-204-1, BYMV-G, and BYMV-G82-25 by testing each antigen against antiserum to CYVV-St-1, CYVV-P, BYMV-G, and BYMV-204-1. Crude sap from healthy *C. quinoa* or Alaska pea was used as a control in each test. ELISA $A_{405\ nm}$ values for healthy controls were less than 0.05 and results were considered positive if $A_{405\ nm}$ values were 0.10 or greater. Each test was replicated twice. All isolates were also tested in indirect ELISA against antiserum to StaVY. StaVY antigen was not available for reciprocal tests.

The standard 0.8% Noble agar, 1.0% NaN_3 and 0.5% SDS agar gel system (15) was modified to reduce nonspecific reactions by changing agar SDS concentrations specifically for each antiserum from 0.5% SDS to 0.075% SDS for CYVV-St and CYVV-P antisera and to 0.15% SDS for BYMV-G and BYMV-204-1 antisera. Undiluted whole antisera were placed in the center wells and, after 0.5–1.0 hr, purified antigens (100 μg/ml) were placed in the peripheral wells.

In indirect ELISA tests, polyvinyl chloride microtiter plates were coated with antigen at 1 μg/ml or 1:50 dilutions of healthy crude sap or of healthy *C. quinoa* or Alaska pea purified in the same manner as purified virus. Two-fold dilutions of whole antiserum (1:500–1:256,000) were incubated in the antigen-coated plate followed by a 1:500 dilution of goat anti-rabbit conjugate. In DAS ELISA tests, microtiter plates were coated with IgG at 1 μg/ml followed by incubation of purified antigen at 1 μg/ml or 1:50 dilution of healthy crude sap and antibody conjugate dilutions of 1:100, 1:200, 1:300, 1:400, 1:500, and 1:600. Coating steps for both DAS and indirect tests routinely involved 18 hr of incubation at 4 C with 2 hr incubation of conjugate. However, in two DAS trials, conjugates were incubated for 18 hr and coating reagents were incubated for 2 hr.

The effects of antigen degradation on $A_{405\ nm}$ values were evaluated in DAS and indirect ELISA tests. BYMV-G preparations were sonicated for 3 min and 6 min in a Heat Systems Ultrasonics Sonicator (Heat Systems-Ultrasonics, Inc., 38 East Mall, Plainview, Long Island, NY) and examined with an electron microscope to determine the extent of particle degradation. Nonsonicated preparations of BYMV-G included as controls were composed predominantly of intact particles. Samples sonicated for 3 min contained both broken and intact particles and those sonicated for 6 min contained few intact particles. Antigen concentrations of 20 , 10, and 1 μg/ml were tested with two-fold dilutions of antiserum in indirect ELISA (1:5,000–1:160,000) and two-fold dilutions of BYMV-G conjugate in DAS ELISA (1:100–1:16,000).

Immunosorbent electron microscopy. CYVV-St antiserum was diluted 1:200 in PO_4 buffer and was incubated on formvar carbon-coated grids for 20 min. The antiserum drop was removed with filter paper and the homologous antigen (10 μg/ml) was incubated on the grids for 20 min. Grids were rinsed with 30 drops of PO_4 buffer and the excess was removed nearly to dryness. Antisera to CYVV-St, CYVV-P, BYMV-204-1, and BYMV-G and normal serum at 1:100 were incubated for an additional 40 min. The rinse procedure was repeated, and the grids were stained with 2% sodium phosphotungstate (PTA), adjusted to pH 7.0 with NaOH. As controls, homologous CYVV-P, BYMV-204-1, and BYMV-G systems were compared. In separate tests, comparisons were made between decorating CYVV-St, CYVV-P, and BYMV-G virions with antisera to StaVY.

Electron microscopy. All virus preparations were negatively stained on formvar-coated grids with 2% PTA. Particle length was determined by measuring 140 purified virions separated on Cs_2SO_4 and photographed at 50,000×; the calibration standard was a waffle grid with 21,600 lines per cm. Virus ultrastructure was

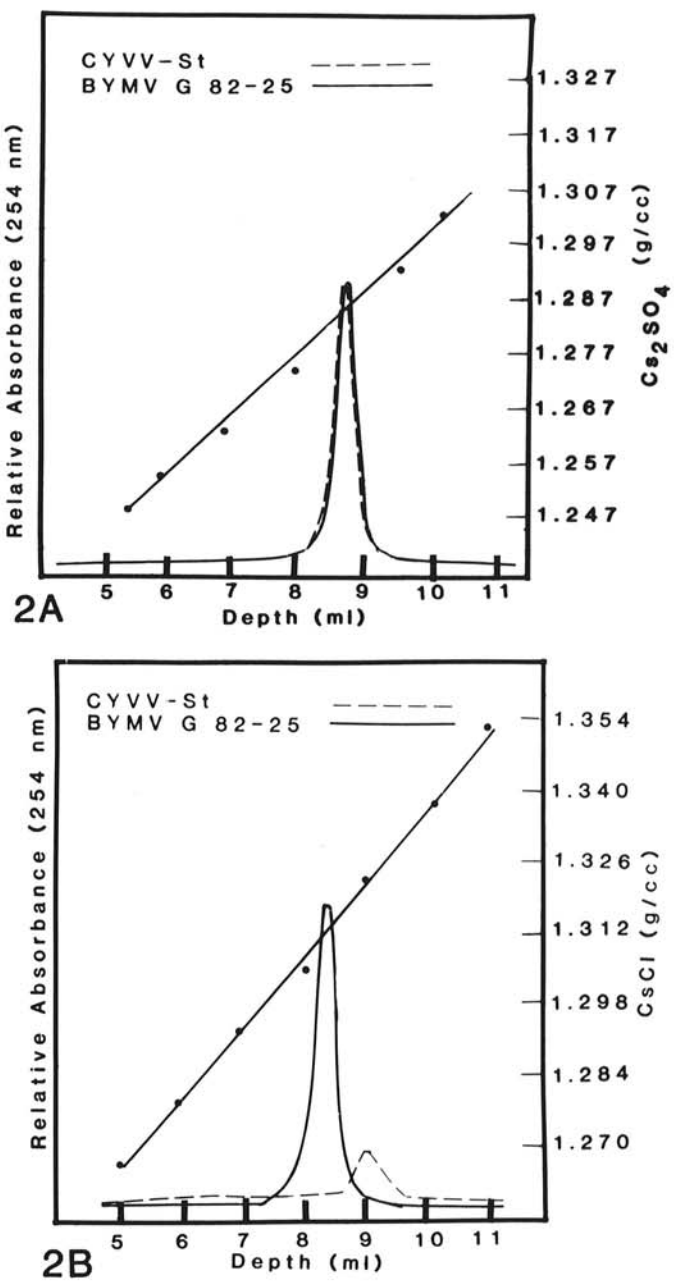

Fig. 2. Density profiles of the statice strain of clover yellow vein virus (CYVV-St) and strain G82-25 of bean yellow mosaic virus (BYMV-G82-25) equilibrium gradient profiles showing **A,** similar single UV-absorbing peaks in Cs_2SO_4 and **B,** instability of CYVV-St in CsCl gradients containing equal quantities of partially purified CYVV-St and BYMV-G82-25.

examined in ultrathin sections of inoculated leaves of *C. quinoa* and statice fixed in glutaraldehyde/acrolein, postfixed in OsO_4 (8), and embedded in LX112.

RESULTS

Host range. CYVV-St-1 and CYVV-St-2 infected the same test plants, however, CYVV-St-2 produced more severe systemic symptoms than CYVV-St-1 in *Lathyrus odoratus* L., *P. sativum* (cultivar Alaska), *Phaseolus vulgaris* (cultivars Black Turtle Soup and Bountiful), *Spinacia oleracea*, *T. repens* (cultivar Tillman), and *Vicia faba* L.; however, CYVV-St-1 produced more severe systemic symptoms in *C. quinoa* and *T. pratense* L. (cultivar Kenland). Reactions produced by CYVV-St-1 and CYVV-St-2 were indistinguishable in *Cucumis sativus* L. 'National Pickling,' and *L. sinuatum* cultivar Blue Bonnee. *T. pratense* L. cultivars Abernathy, Britta, and Red Head were not infected by either isolate.

Infection of Bountiful and Black Turtle Soup beans with inocula of CYVV-St-1 and -2 from different host plants revealed that the number of plants infected by CYVV-St-1 was influenced by the source of inoculum. Bountiful bean was infected with CYVV-St-1 transferred from Black Turtle Soup bean but not from Alaska pea or Bountiful bean. Infections of Black Turtle Soup bean with CYVV-St-1 were established rarely with transfers from Alaska pea but more frequently with transfers from Black Turtle Soup and Bountiful beans. In contrast, the three species were easily infected by CYVV-St-2, regardless of inoculum source.

CYVV-St-1 and -2 had a similar, but wider, host range than the BYMV and CYVV isolates tested concurrently and had different host reactions in common with each isolate. Unlike the BYMV isolates, CYVV-St-1 and -2 and CYVV-P induced severe systemic reactions in *L. odoratus* and *V. faba*. In addition, the occasional systemic reactions induced in *C. quinoa* by CYVV-P and BYMV-204-1 were similar to those regularly induced by CYVV-St-1 and -2. However, similarity between CYVV-P and CYVV-St-1 and -2 was not consistent; BYMV-204-1 and CYVV-St-1 and -2 were the only isolates that infected *L. sinuatum*, and BYMV-G82-25 and CYVV-St-1 and -2 were the only ones that infected Bountiful bean.

Purification. CYVV-St prepared by organic solvent extraction and differential centrifugation produced a single band in Cs_2SO_4 equilibrium gradients with a density of 1.29 g/cc (Fig. 2a) and in CsCl produced a smaller, more diffuse band (Fig. 2b) that occasionally appeared as a double band. In contrast, BYMV-G82-25 showed a single homogenous peak in both CsCl and Cs_2SO_4. In CsCl, unaggregated virions were associated with BYMV-G82-25 fractions while short, apparently broken virions were asssociated with CYVV-St fractions. All isolates were subsequently separated on Cs_2SO_4. Densities of CYVV-P, BYMV-204-1, BYMV-G, and BYMV-G82-25 in Cs_2SO_4 were 1.28, 1.30, 1.28, and 1.29 g/cc, respectively.

Virus concentrated with a single PEG precipitation was aggregated and contaminated with host material and gave an average yield of 15 mg/kg compared to 19 mg/kg obtained with high-speed centrifugation. A second precipitation with PEG further reduced the yield to 1.2 mg/kg, and the remaining virus was highly aggregated.

Average virus yields varied among isolates and were 14.1 mg/kg for CYVV-St-2, 20 mg/kg for CYVV-P, 5.8 mg/kg for BYMV-204-1, 14.8 mg/kg for BYMV-G82-25, and 13.0 mg/kg for BYMV-G. $A_{260\ nm/280\ nm}$ ratios (uncorrected for light scattering) for CYVV-St-2, CYVV-P, BYMV-204-1, BYMV-G82-25, and BYMV-G were 1.27, 1.27, 1.26, 1.24, and 1.27, respectively.

Agar gel double-diffusion tests. Cross-reactivity occurred between all virus isolates. Formation of the weakest heterologous reactions was dependent on SDS concentrations in the gel (Table 1). Heterologous reactions with CYVV-St and BYMV-204-1 antisera appeared at both 0.075% and 0.15% SDS. CYVV-P antiserum developed precipitin bands with all heterologous antigens only at 0.075% SDS, while optimum resolution of heterologous reactions with BYMV-G antiserum occurred at

TABLE 1. Effects[a] of SDS concentrations in agar gel double-diffusion tests

	Antisera															
	CYVV-St % SDS				CYVV-P % SDS				BYMV:204-1 % SDS				BYMV-G % SDS			
Antigen	0.037	0.075	0.15	0.30	0.037	0.075	0.15	0.30	0.037	0.075	0.15	0.30	0.037	0.075	0.15	0.30
CYVV-St	++	+++	+++	++/NS	+	++	−	−	−	+	++	−	−	−	+	NS
CYVV-P	+	+++	+++	++/NS	+	++	−	−	−	+	++	−	−	−	+	NS
BYMV-204-1	−	++	++	++/NS	−	+	−	−	++	++	+++	−	−	−	+	NS
BYMV-G	−	+	++	++/NS	−	+	−	−	+	++	+++	−	+	+	++	NS
BYMV-G82-25	−	+	++	++/NS	−	+	−	−	+	++	+++	−	+	+	++	NS

[a] + = faint positive, ++ = postive, +++ = strong positive, − = no reaction, and NS = nonspecific reaction.

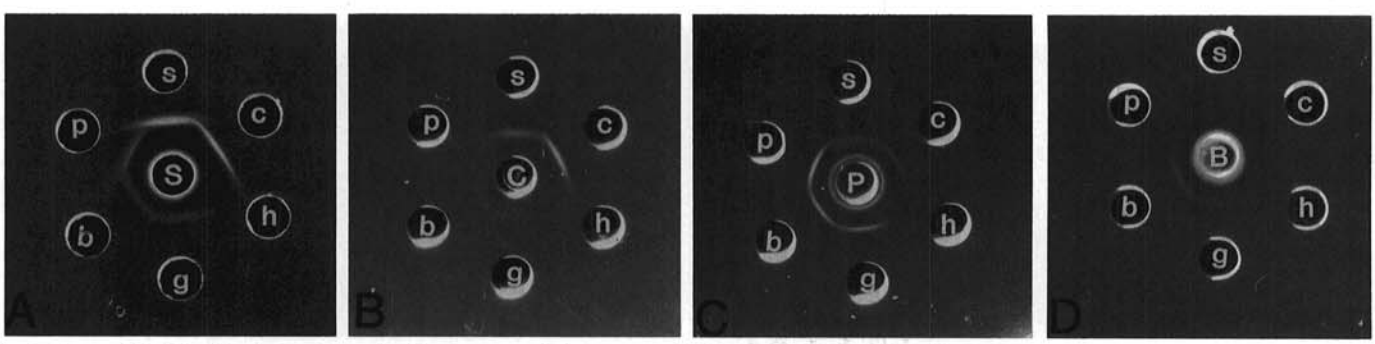

Fig. 3. Agar gel double diffusion serology showing cross-reactivity between CYVV-St, CYVV-P, BYMV-204-1, BYMV-G, and BYMV-G82-25. Peripheral wells contain purified antigens (100 μg/ml): s = CYVV-St, c = CYVV-P, b = BYMV-G, p = BYMV-204-1, g = BYMV-G82-25, and h = crude sap from a healthy plant. Center wells contain undiluted whole antisera: **A,** S = CYVV-St antiserum. Nonidentity is shown between CYVV-St and CYVV-P, CYVV-St and BYMV-204-1, and BYMV-204-1 and BYMV-G. Apparent serological identity is shown between BYMV-G and BYMV-G82-25. **B,** C = CYVV-P antiserum. Nonidentity is shown between CYVV-St and CYVV-P, and between CYVV-St and BYMV-204-1. Apparent identity is shown between BYMV isolates. **C,** P = BYMV-204-1 antiserum. Nonidentity is shown between CYVV-St and BYMV-204-1. Apparent identity is shown between BYMV-204-1, BYMV-G, and BYMV-G82-25 and between CYVV-St and CYVV-P. **D,** B = BYMV-G antiserum. Very faint reactions are shown for CYVV-P, CYVV-St, and BYMV-204-1 relative to BYMV-G and BYMV-G82-25.

0.15% SDS. At SDS concentrations of 0.037 and 0.30%, virus reactions were inhibited or obscured by nonspecific reactions.

CYVV-St showed a stronger reaction with CYVV-P than with the BYMV isolates. With antisera to both CYVV-St and CYVV-P, the heterologous CYVV-St or CYVV-P precipitin bands were stronger than heterologous BYMV reactions and formed at SDS concentrations at which BYMV precipitin bands did not develop. CYVV-St and CYVV-P were differentiated by spur formation at precipitin band intersections between the two antigens reacting with CYVV-St or CYVV-P antisera (Fig. 3a and b), although spurs did not form between the two antigens with BYMV-G or BYMV-204-1 antisera (Fig. 3c and d).

Stronger cross-reactivity occurred among the BYMV isolates than with any single BYMV isolate and CYVV-St or CYVV-P. BYMV-G and BYMV-G82-25 were indistinguishable in tests against all antisera. BYMV-204-1 spurred over BYMV-G when

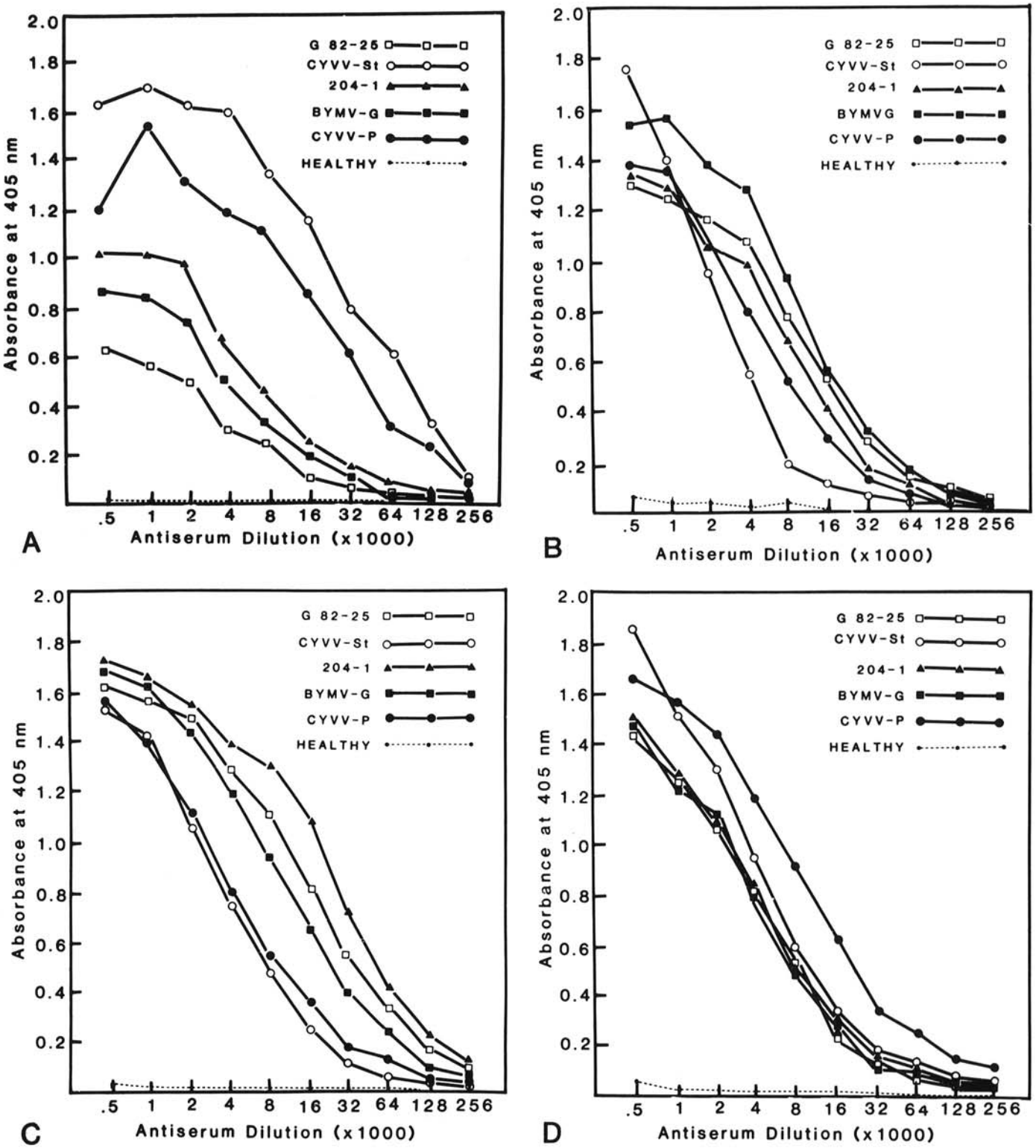

Fig. 4. Indirect ELISA $A_{405\ nm}$ values for CYVV-St, CYVV-P, BYMV-204-1, BYMV-G, and BYMV-G82-25 (1 μg/ml) reacting with: A, CYVV-St whole antiserum, 1:500–1:256,000. Greatest cross-reaction was with CYVV-P. B) CYVV-P whole antiserum, 1:500-1:256,000. Greatest cross-reaction with CYVV-St, between 1:1,000-1:4,000. C) BYMV-204-1 whole antiserum, 1:500-1:256,000. Greatest cross-reaction with BYMV-G82-25 and BYMV-G. D) BYMV-G whole antiserum 1:500-1:256,000. Greatest cross-reaction with BYMV-G82-25 and BYMV-204-1.

tested against antiserum to CYVV-St. A reaction of nonidentity also occurred between these two antigens and BYMV antiserum. BYMV-204-1 formed fainter bands than BYMV-G82-25 with BYMV-G antiserum and stronger bands with CYVV-St antiserum than did the other two BYMV isolates.

Indirect ELISA. A broad cross-reactivity was detected among all virus isolates. Weakest heterologous reactions were easily detected at antisera dilutions of 1:16,000, and many reactions were detected at 1:64,000. Despite the broad cross-reactivity, each virus isolate could be serologically distinguished, although distinctions were not clearly resolved at all antibody dilutions. At antiserum dilutions below 1:2,000, linearity of antigen-antiserum reactions was often reduced, while differences in $A_{405\ nm}$ values between antigens often diminished as antibody dilutions were increased past an optimum dilution (Fig. 4a to d). CYVV-St antigen reacted more strongly than the BYMV isolates with CYVV-P antiserum between dilutions of 1:1,000 and 1:4,000, but $A_{405\ nm}$ values beyond 1:4,000 were similar for all four heterologous antigens. Apparent differences in reactivity between BYMV-G and BYMV-G82-25 antigens reacting with BYMV-G antiserum were not detected at antibody dilutions greater than 1:8,000.

CYVV-St antigen reacted more strongly than the BYMV isolates over only a narrow range of CYVV-P antiserum dilutions; however, in the reciprocal reactions CYVV-P antigen reacted more strongly than the BYMV isolates over a wide range of CYVV-St antiserum dilutions. CYVV-St and CYVV-P antigen reactions with antisera to BYMV-G and BYMV-204-1 were weaker than those of the BYMV antigens (Fig. 4c and d).

Reactions with antibody to StaVY were relatively weak (Fig. 5). The strongest reactions occurred with CYVV-P, which had an end point of 1:2,000, followed by CYVV-St, BYMV-G, BYMV-G82-25, and BYMV-204-1. Similar reactions were obtained with the 10- and 20-day bleedings.

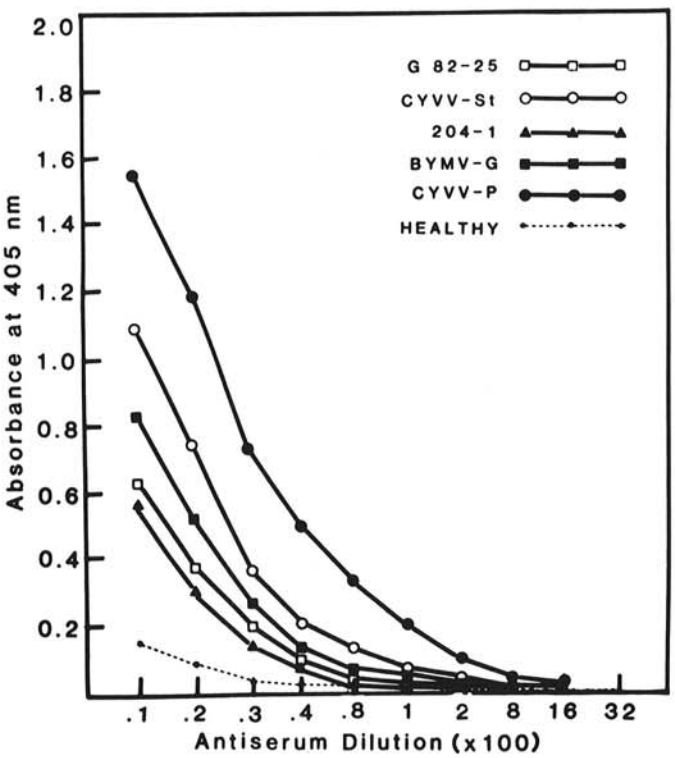

Fig. 5. Indirect ELISA $A_{405\ nm}$ values of CYVV-St, CYVV-P, BYMV-204-1, BYMV-G, and BYMV-G82-25 (1 μg/ml) reacting with statice virus Y (StaVY) whole antiserum 1:100-1:32,000. Greatest cross-reaction was with CYVV-P, followed by CYVV-St.

Double antibody sandwich ELISA. DAS ELISA reactions were more specific than those of indirect ELISA and agar double-diffusion tests. Weaker heterologous cross-reactions were detected at conjugate dilutions of 1:100, if at all, when conjugate was incubated for 2 hr. Heterologous reactions were stronger if conjugate was incubated for 18 hr (Table 2). Incubation for 18 hr also accentuated differences between cross-reactivity of heterologous antigens because dilution end points increased by different amounts for different antigen-antibody combinations. With 18 hr of conjugate incubation, BYMV-204-1 reactions with CYVV-St conjugate increased from an end point of 1:200 to greater than 1:600, whereas BYMV-G reactions with CYVV-St conjugate only increased from 1:100 to 1:200.

Cross-reactivity was greater between CYVV-St and CYVV-P than between either virus and the BYMV isolates (Table 3). As in agar gel double-diffusion and indirect ELISA, CYVV-St antiserum reacted more strongly with BYMV-204-1 than with BYMV-G and BYMV-G82-25. Low cross-reactivity between BYMV-204-1 and BYMV-G confirmed the serological difference between the two BYMV isolates. BYMV-G was further distinguishable from BYMV-G82-25 by different reactivities with antibody conjugates to CYVV-P, BYMV-204-1, and BYMV-G. Unlike the indirect ELISA comparisons, BYMV-G antigen reacted less strongly with its homologous antiserum than did BYMV-G82-25.

Electron microscopy of BYMV-G82-25 and BYMV-G preparations used in ELISA tests revealed that purified BYMV-G82-25 thawed from frozen storage contained more fragmented virions than did similarly handled BYMV-G. Particle breakdown by controlled sonication to disrupt BYMV-G virions indicated no difference in end points and $A_{405\ nm}$ values when identical concentrations of intact and degraded antigen were compared at the same concentration in either indirect or DAS ELISA. However, as observed in previous comparisons, nonsonicated BYMV-G82-25 reacted more strongly than equilivalent concentrations of intact or degraded BYMV-G in DAS, but not indirect, ELISA.

Immunosorbent electron microscopy. All virus cultures tested showed a heavy coating of antibodies with the homologous antiserum. CYVV-St antigen showed a strong antibody reaction in tests with CYVV-P and StaVY antisera. StaVY antibody decorated CYVV-P, but not BYMV-G virions.

Electron microscopy. Purified CYVV-St preparations contained virions with a normal length of 777 nm. Ultrathin sections of leaves of both statice and *C. quinoa* infected with CYVV-St contained cylindrical inclusions with laminated aggregates and crystalline cytoplasmic and nucleolar inclusions (Fig. 6). These inclusions are similar to those induced by BYMV and CYVV, members of subgroup II in the classification scheme of potyvirus inclusions (3).

TABLE 2. Effects of conjugate incubation times on conjugate reciprocal dilution endpoints in double antibody sandwich ELISA

	Conjugate							
	CYVV-St		CYVV-P		BYMV-204-1		BYMV-G	
	Incubation time							
Antigen	2 hr	18 hr	2 hr	18 hr	2 hr	18 hr	2 hr	18 hr
CYVV-St	600	>600	400	>600	100	400	0	300
CYVV-P	300	>600	600	>600	0	200	100	>600
BYMV-204-1	200	>600	0	100	600	>600	200	600
BYMV-G	100	200	0	100	100	300	600	>600
BYMV-G82-25	100	300	100	300	400	>600	600	>600

Scrolls, such as would occur with turnip mosaic virus, a member of subgroup III, were not observed.

DISCUSSION

Host range responses, in conjunction with serological tests, have been used to identify three subgroups of related BYMV and CYVV isolates (5). Interpretation of BYMV host range results is complicated because passages of BYMV isolates through a previous host may influence the infectivity of a test species (6). This influence has been attributed to the production of induced or spontaneous variants which may propogate preferentially in one host only to be diluted out in subsequent passage to a different species by the selective multiplication of other variants. CYVV-St-1 and -2 originated from the same source plant and were serologically identical but showed differences in infectivity depending on the sequence of host passage. Differences between the two isolates were reduced, but not eliminated, by passing CYVV-St-1 through Black Turtle Soup bean and enhancing CYVV-St-1 reactions in Black Turtle Soup bean and Bountiful bean.

CYVV-St could not be identified on the basis of host range comparisons with known CYVV and BYMV isolates described in published reports or in our experimental inoculations. Although CYVV-St-1 and -2 and CYVV-P induced more severe systemic infections in *L. odoratus* and *V. faba* than the BYMV isolates, infections caused by CYVV-St-1 and -2 in *L. sinuatum* were more like those caused by BYMV-204-1, while those in Bountiful bean were more like those caused by BYMV-G82-25. Both CYVV-St-1 and -2 induced systemic infections in *C. quinoa* and infected *C. sativus* and *T. repens*, which are responses associated with infections caused by CYVV rather than BYMV (1,5,10). However, in concurrent tests, CYVV-P, reported to infect *T. repens* (14) and Bountiful bean (12), infected neither, and BYMV-204-1 induced systemic infections in *C. quinoa*. Although differences between reported and observed results of CYVV-P may reflect differences in previous host passage, environmental conditions and/or seed source, the variability observed in host range responses of BYMV/CYVV/PMV (pea mosaic virus) indicate a limited value of host range comparisions for identification.

Results of serological comparisons were less equivocal than host range results. In each procedure, CYVV-St showed the closest serological relationship to CYVV-P, an intermediate relationship to BYMV-204-1, and a more distant relationship to BYMV-G and BYMV-G82-25. BYMV-204-1 was more closely related to BYMV-G and BYMV-G82-25 than to the CYVV isolates, with the two BYMV gladiolus isolates being more closely related to each other than to any of the other isolates. BYMV-G and BYMV-G82-25 were most clearly differentiated in double antibody sandwich ELISA, although faint differences between them were also detected with indirect ELISA.

Comparing the results of different serological procedures was useful in determining the serological relationships among the virus isolates, because relationships were not detected under all test conditions. Each antiserum had a characteristic SDS concentration at which precipitin bands developed with the weakest heterologous antigens in agar gel double-diffusion tests.

TABLE 3. Cross-reactivity of CYVV-St, CYVV-P, BYMV-204-1, BYMV-G, and BYMV-G82-25 in double antibody sandwich ELISA tests

	Virus antiserum			
Antigen	CYVV-St 1:200[a]	CYVV-P 1:100	BYMV-204-1 1:200	BYMV-G 1:300
CYVV-St	2.00[b]	1.280	0.241	0.104
CYVV-P	0.634	2.00	0.142	0.241
BYMV-204-1	0.299	0.134	0.906	0.235
BYMV-G	0.111	0.181	0.174	0.912
BYMV-G82-25	0.147	0.327	0.593	1.135
Healthy crude sap 1:50	0.027	0.023	0.015	0.005

[a] Highest conjugate dilution at which positive reactions were detected for all heterologous antigens with conjugate incubated overnight.
[b] Average $A_{405\ nm}$ values of two trials.

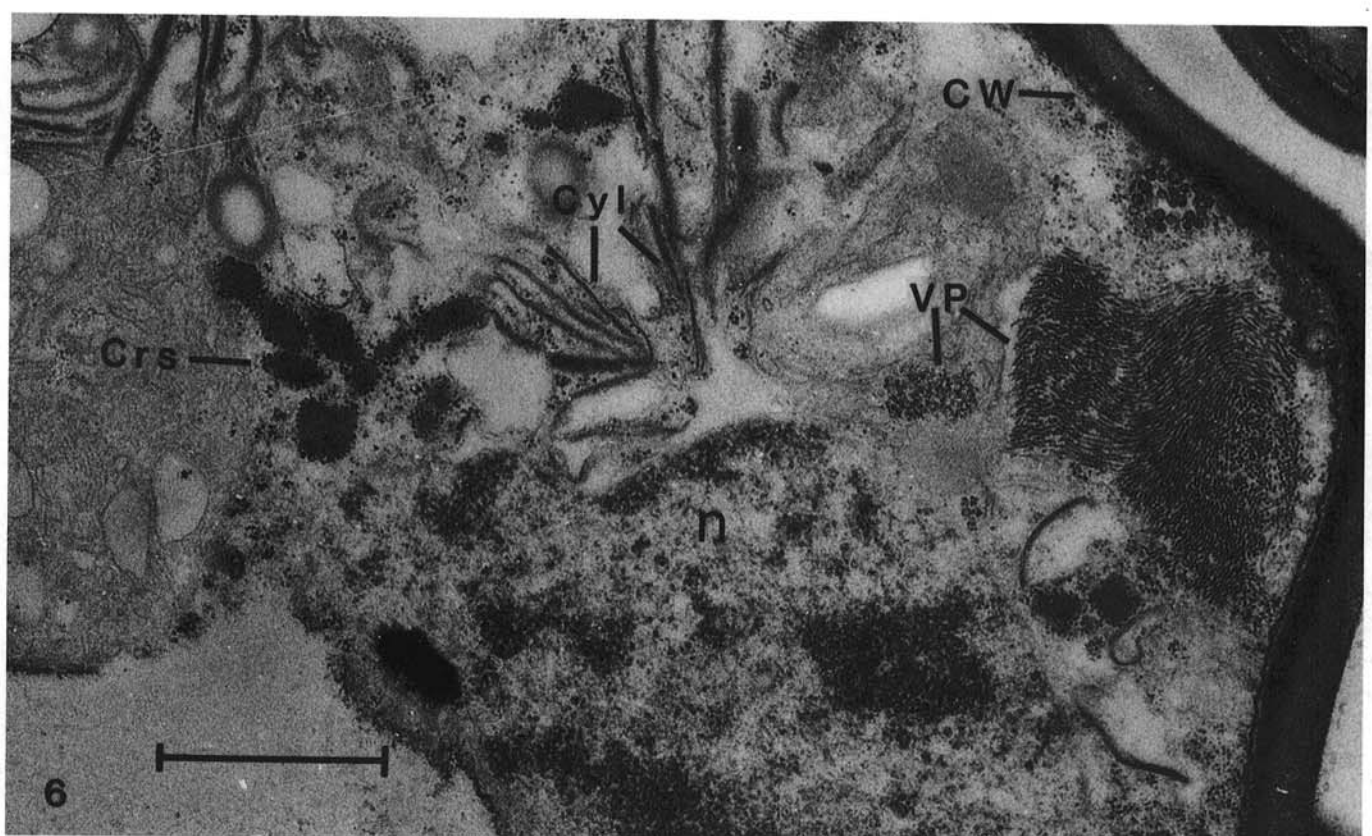

Fig. 6. Cylindrical (Cyl) and crystalline (Crs) cytoplasmic inclusions and virus particles (VP) in systemically infected *C. quinoa*. (N = nucleus, CW = cell wall.) Bar represents 1 μm.

Although recommended SDS concentrations usually range from 0.5 to 1.0% (15), optimum precipitin band formation occurred below 0.25% SDS. A single SDS concentration would not have detected weak heterologous reactions, such as those between CYVV-P antiserum and BYMV antigens or between BYMV-G antiserum and the two CYVV isolates. The validity of these reactions was confirmed by cross-reactivity in indirect and direct ELISA tests. These results indicate it may be useful to evaluate CYVV and BYMV antisera at more than a single SDS concentration in agar gel double-diffusion reactions.

In indirect ELISA tests, recognized for broad spectrum reactivity, it was necessary to compare $A_{405\ nm}$ values over a range of antiserum dilutions rather than to determine relative antigen-antibody reactivity by $A_{405\ nm}$ values at a single dilution or by end points alone. The optimum range of dilutions for resolving differences between isolates was narrow for some antigen-antibody combinations. DAS ELISA, agar gel double-diffusion, and IEM results confirm the validity of this reaction. In strain-specific DAS ELISA tests, incubating conjugates overnight increased reactivity, facilitated detection of weaker heterologous reactions, and accentuated differences between them.

Although serological relationships were clarified by using several tests over a range of conditions, several minor inconsistencies in indirect and DAS ELISA results were noted. BYMV-G reacted more strongly than BYMV-G82-25 with BYMV-G antiserum in DAS ELISA, but less strongly in indirect ELISA. Because this anomaly did not affect identification of CYVV-St, it was not pursued beyond determining that the difference was not due to particle degradation.

Sensitivity of CYVV-St to CsCl was in contrast to the stable, single zones produced by BYMV-G82-25 separated on CsCl. Although further tests should be made to determine the sensitivity of other CYVV isolates to different cesium salts, this property may be a useful criterion in separating and distinguishing different isolates of BYMV and CYVV.

StaVY was described as a new virus more closely serologically related in slide precipitin tests to BYMV than to CYVV (7). However, indirect ELISA and IEM results in the present comparison revealed greater cross-reactivity between StaVY antiserum and CYVV-P and CYVV-St than the BYMV isolates, suggesting that StaVY may be a strain of CYVV. StaVY was distinguished from CYVV by inability to infect *C. sativus* and from BYMV by an inability to infect *P. sativum* (7). Both hosts were infected by CYVV-St; however, different cultivars were utilized in CYVV-St studies than in StaVY comparisions.

From subsequent comparisons between the CYVV, BYMV, and pea mosaic strains of BYMV subgroups (including CYVV-P and BYMV-204-1), we concluded (on the basis of serology and complementary DNA hybridization analysis homologies) that members of subgroup 1 be identified as CYVV, subgroup 2 as BYMV, and subgroup 3 as pea mosaic virus (PMV), elevating PMV from a strain of BYMV to a separate virus and renaming BYMV-204-1, PMV-204-1 (O. W. Barnett, *personal communication*). Results of our comparisons between CYVV-P, PMV-204-1, and BYMV-G correspond to these as well as additional reports that BYMV-G and PMV-204-1 are more closely related to each other than to CYVV-P in agar gel double-diffusion tests but only distantly related to each other or PMV-204-1 in DAS ELISA (12). We conclude that CYVV-St is an isolate of CYVV (subgroup 1) more closely related to PMV than to gladiolus isolates of BYMV, that serological distinctions may exist between different gladiolus isolates of BYMV, and that StaVY may be a strain of CYVV.

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