Survey of Commercial Carnation Cultivars for Four Viruses in California by Indirect Enzyme-Linked Immunosorbent Assay

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ABSTRACT

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An indirect enzyme-linked immunosorbent assay technique was used to survey for carnation ringspot virus, carnation mottle virus, carnation necrotic fleck virus, and carnation etched ring virus in commercial carnations throughout California. A total of 226 samples was collected from 31 locations; statewide incidences were: carnation mottle virus, 78%; carnation necrotic fleck virus, 13%; carnation etched ring virus, 15%; and carnation ringspot virus, 0%. Of the samples surveyed, 21% were infected with two or more of these viruses. The statewide average of plants free from these viruses was 18%. Survey samples were derived from seven propagation sources. Carnations from different propagators varied widely in the incidence of the tested viruses. The data suggested that initial infection of commercial carnations occurred at the propagator level and that only limited spread occurred in production greenhouses, with the exception of carnation mottle virus.

Carnations, *Dianthus caryophyllus* L., are plagued by persistent infection by several viruses, some of which are specific to carnations. Significant economic losses to carnation growers and propagators result from mixed infections by these viruses. The viruses are maintained and spread by vegetative propagation, which frequently results in a situation in which nearly all of a grower's carnations are virus infected. Virus diseases result in reduced hardiness and poor plant productivity and quality, ultimately translating into reduced profits.

Many reports describe the deleterious effects of carnation viruses on quality and quantity of the marketable crop, thus suggesting the need for effective virus disease diagnosis and control. Kassanis (10) reported that carnation ringspot virus (CRSV) is extremely damaging to carnations. The presence of CRSV within the William Sim cultivar causes a marked increase in the number of flowers with split calyx (8). At Fairfield Experimental Horticultural Station in England, there was a 39% increase in yield and a 20% increase in monetary return from a 2-yr virus-free carnation crop as compared with two annual carnation crops that were virus infected, and the number of better quality blooms also increased (9). Brierley and Smith (3) and Paludan and Rehnstrom (16) reported marked loss in yield when commercial carnations are infected with carnation etched ring virus (CERV) and carnation mottle virus (CaMoV).

The recent adaptation of the enzyme-linked immunosorbent assay (ELISA) to plant virus detection (4,18) has created new interest in serological diagnosis of plant viruses. The ELISA procedure is simple, rapid, and extremely sensitive. Generally, the double-antibody sandwich method of ELISA has been used in plant virology. This paper describes the application of the indirect ELISA method to survey for incidence of CRSV, CERV, CaMoV, and carnation necrotic fleck virus (CNFV) (15) in carnations in California. The indirect test has proved to be more sensitive and easier to apply and implement than the sandwich method (5,6,14).

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MATERIALS AND METHODS

A total of 226 samples was collected, by us or by cooperators in the University of California Cooperative Extension, between 13 August and 30 September 1980 from 31 commercial carnation growers in California. Two shoot tips approximately 20 cm in length were collected from each cultivar at each greenhouse visited. The specimens were numbered and stored at −20 C until processed.

Sample preparation. Approximately 1 g of leaf tissue was removed from the shoots and completely minced with 600 µl of water with a sterile applicator stick in a 1.5-ml microfuge tube. The mixture was centrifuged in a Beckman Microfuge centrifuge B for 90 sec. The supernatant was drawn off and stored in another microfuge tube. A total of 150 µl of the preparation was removed for ELISA tests for CRSV, CaMoV, and CNFV. The remaining portion of each sample was processed for the CERV test by precipitation of the virus and viral inclusion bodies with 200 µl of a solution of 40% polyethylene glycol 6000, 1 M sodium chloride. The samples were incubated in ice for 1 hr and centrifuged for 2 min in the Beckman Microfuge B. The pellets were resuspended with 200 µl of coating buffer (0.05 M sodium carbonate, pH 9.6; 0.01% sodium azide) and 4 M urea and allowed to incubate at room temperature for 2 hr before addition to the ELISA plate.

Antisera. CERV antiserum prepared against CERV virions was supplied by R. H. Lawson, U.S. Department of Agriculture. CaMoV antiserum was provided by J. H. Tremaine, Agriculture Canada. CNFV was provided by M. Bar-Joseph, Agricultural Research Organization, Israel. CRSV antiserum was manufactured, purified, and cross-absorbed as described by Lommel et al (14).

ELISA. All tests were performed on the PR-50 EIA automatic analyzer (Gilford Instrument Laboratories, Inc., Oberlin, OH) using polystyrene cuvette plates containing 50 test wells. The indirect ELISA method was performed as described previously (14). Fifty microliters of the test sample was added to 150 µl of coating buffer and serially diluted fivefold. Plates were incubated for 1 hr at 37 C on a rotary shaker at 60 rpm. Virus-specific antibody was added in indirect ELISA buffer (0.01 M phosphate buffered saline, pH 7.4; 0.05% Tween 20; 2% polyvinylpyrrolidone-10; and 0.05% bovine serum albumin). CERV γ-globulin was used at 1/100 (5 µg/ml), CRSV at 1/600 (0.5 µg/ml), CaMoV at 1/400 (1 µg/ml), and CNFV at 1/200 (1 µg/ml) dilution in indirect ELISA buffer. Goat antirabbit IgG conjugated with alkaline phosphatase (Miles Laboratories, Ltd.) was used at 1/5,000 dilution in indirect ELISA buffer. Absorbance readings at 405 nm were monitored 45 min after the alkaline phosphatase substrate was added.

RESULTS

In the initial study, the indirect ELISA

technique and electron microscopy for detection of carnation viruses were compared. Some of the samples were artificially inoculated with CRSV to determine whether it could be detected. The experiment allowed for the independent confirmation of results. Samples were evaluated at the California Department of Food and Agriculture by electron microscopy of negatively stained leaf-dip preparations prior to ELISA analysis in our laboratory. Results obtained by the two methods correlated well (Fig. 1). For each of the four viruses, ELISA values below 0.2 *A* at 405 nm denoted the absence of virions as monitored by electron microscopy.

There were no significant differences in incidence of the four viruses in the three general regions of carnation production, the San Francisco Bay area, Salinas Valley, and the San Diego coast area (Table 1).

The ubiquity of CaMoV infection in commercial carnations has been reported previously (3,10), and we confirmed a high incidence (78%) of this virus in California (Table 1). Statewide, the incidences of CNFV and CERV were 13 and 15%, respectively. CRSV was not detected in any of the samples tested; it can be assumed that CRSV is not present in commercial carnations in California at this time. CRSV was included in the survey because it results in significant yield loss when present (8) and because epiphytotics of CRSV have occurred recently in Belgium, New Zealand, and Israel (1,7,17).

Twenty-one percent of the samples were multiply infected with CNFV, CERV, and CaMoV (Table 1). Only 18% of the commercial carnations tested statewide were free of the four viruses tested.

Age of planting date for the samples ranged from 4 mo to 7 yr, with the average being 18 mo. Incidence of the viruses in the growers' greenhouses was not significantly different in young or old plants, indicating that the viruses did not spread in the greenhouse but rather that the carnations were infected at the time of propagation.

The 226 samples represented a total of 38 different cultivars, with Improved White Sim, Pink Sim, Scania 3C, Tangelo, and Red Sim being the most common. There was no evidence suggesting a higher incidence of virus infections in a particular cultivar.

With respect to the propagators from which samples originated, almost 50% of the samples collected originated from propagator A (Table 2). These samples were infected with the various carnation viruses to approximately the same extent as the statewide average (Tables 1 and 2). Samples from propagator B, however, had a 31% rate of mixed infection, which was about 10% above the state average. A mixed infection incidence of 31% is important when considering the potential for disease loss. Propagator C was below the statewide average for the incidence of CaMoV, CERV, and CNFV separately and in various mixtures (Table 2). A particular greenhouse obtained cuttings from two different propagation sources and maintained the cuttings intermixed; 21 samples were collected from each propagation source and indexed by ELISA. Propagator B's stock had a mixed infection rate of 38%, whereas propagator C's cuttings had no mixed infections and about the statewide average incidence of CaMoV (Table 3). These results support the contention that spread of CERV and CNFV was insignificant at the grower level.

In addition to the 226 samples collected from commercial sources, a sample was taken from a field of seedling carnations for the production of carnation seeds. A few samples of *Gypsophila paniculata* L., a member of the Caryophyllaceae occasionally found growing around the perimeter of carnation greenhouses, were also collected. None of the four carnation viruses being tested was present in these plants as determined by indirect ELISA.

Table 1. Incidence by location of virus infections in commercial carnations in California as determined by indirect enzyme-linked immunosorbent assay

Location[a] (number of properties)	No. of samples	Infection (%)[b]					
		CaMoV[c]	CNFV	CERV	CRSV	Mix	Healthy
Area 1 18	114	77	18	15	0	22	16
Area 2 5	50	76	6	16	0	20	22
Area 3 8	62	81	11	15	0	19	18
Total	226	78	13	15	0	21	18

[a] Area 1 = San Francisco Bay area; area 2 = Salinas Valley; area 3 = San Diego coast.
[b] No significant difference ($P = 0.05$) among areas for each virus using the chi-square test.
[c] CaMoV = carnation mottle virus; CNFV = carnation necrotic fleck virus; CERV = carnation etched ring virus; CRSV = carnation ringspot virus; mix = mixed infection with CaMoV and CERV or CNFV or all three viruses in a particular sample.

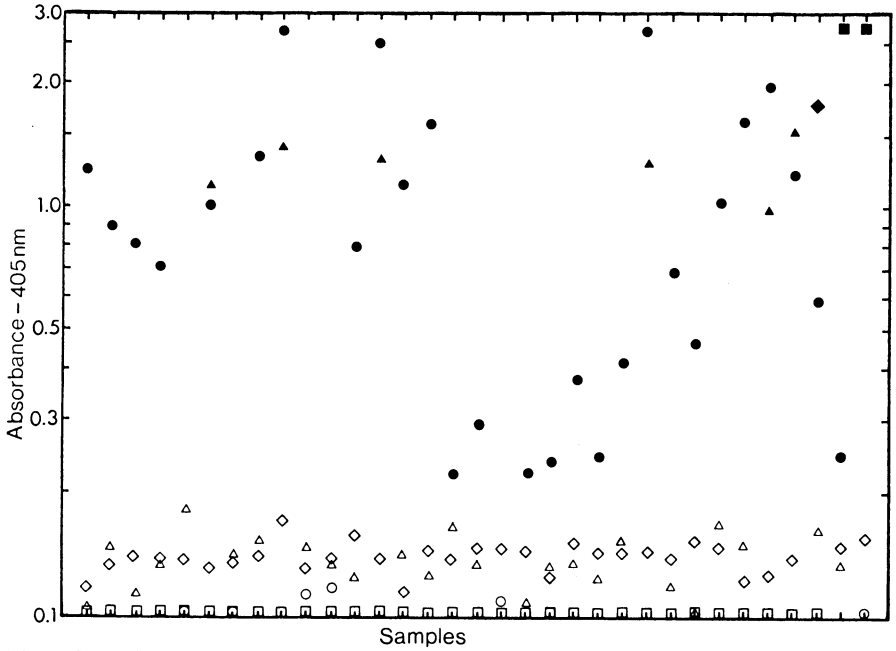

Fig. 1. Detection of four viruses by indirect enzyme-linked immunosorbent assay (ELISA) and election microscopy of 33 selected carnations collected throughout California. ELISA values at 405 nm are plotted on the vertical axis. Solid symbols indicate presence of the particular virus by negative staining, electron microscopy; hollow symbols indicate no observable virions. Circle = carnation mottle virus; triangle = carnation etched ring virus; square = carnation ringspot virus; and diamond = carnation necrotic fleck virus.

DISCUSSION

This study was initiated to evaluate problems associated with the large-scale application of indirect ELISA as a virus detection tool. Our goal was to provide a methodology that could be applied to other crops where viruses are a problem in propagative material and to evaluate the method as a tool for field surveys that would allow for effective epidemiologic studies of virus diseases. Our data indicate that control of carnation viruses could be accomplished with an effective virus-indexing program. The production crop is normally propagated from a small number of nuclear stock plants. The

increase from nuclear stock to production stock frequently involves a millionfold increase. Because spread was not evident at the grower level, with the exception of CaMoV, an efficient ELISA program to monitor virus infection in nuclear stocks could reduce or eliminate the problem viruses at the propagator level.

The simplicity of the indirect ELISA test could result in its use with a minimum level of technical skill. The method required modification of CERV because it occurred in infected plants as large inclusion bodies (13). Detection of CERV in plant extracts by indirect ELISA using inclusion body antisera provided by R. Lawson was not possible because of high backgrounds. However, CERV could be readily detected by virion antiserum in extracts treated with carbonate buffer and 4 M urea. This result indicates the importance of sample preparation for successful implementation of ELISA.

The data in Table 1 suggest that there was no significant difference in carnation virus incidence with respect to geography. Reports in the literature alluded to the idea that warmer temperatures might result in a higher incidence of CaMoV (11) and CRSV (8). Our data do not support this contention for California because the San Diego coast area has a warmer climate than the other two areas.

The high incidence of CaMoV observed in this survey most likely reflects the ease of transmission and possibly the wide host range of this virus (3). Reservoir hosts in the vicinity of greenhouses could be a source (12,17), but our preliminary evaluation of one such host, *G. paniculata*, did not confirm this. CaMoV is readily transmitted mechanically (3), and D. S. Teakle (*unpublished*) working in our laboratory obtained evidence that suggested transmission via root leachates. CaMoV is also the most difficult of the carnation viruses to remove by heat treatment and meristem tip culturing (2). Thus, propagators may supply cuttings with low levels of CaMoV to the growers, and secondary spread could then occur upon handling and cutting with contaminated tools. This is supported by a study where cuttings from propagator A were indexed for CaMoV by ELISA prior to being exposed to growers' greenhouses. The incidence of CaMoV was less than 2% (D. Stenger, *personal communication*), whereas samples originating from propagator A that were analyzed from growers' greenhouses consistently showed 76% CaMoV infection (Table 2). This indicates that CaMoV spreads at the grower level but that CERV and CNFV do not spread significantly at the grower level and thus originate from the propagators (Table 3).

At the time of sample collections, the incidence of CNFV and CERV was approximately 14%. This might be considered an endemic level of infection for these two viruses. There are enough infected plants, presumably at the propagator level, to maintain this degree of infection in the greenhouses. Both of these viruses have a variable incidence in California. This is evidenced by the outbreak of CNFV in 1977 that was severe enough to warrant the imposition of strict sanitation requirements in greenhouses having CNFV-infected plants (15).

It has been clearly demonstrated that some form of virus indexing is needed to maintain a quality carnation crop. Indeed, virus indexing programs have been in existence for many years. These programs initially involved host indexing on indicator plants (8,10). Currently, the two largest California carnation propagators are indexing their nuclear stocks using indicator plant assays in conjunction with leaf-dip, negative staining electron microscopy. A serological assay using the agar gel double-diffusion technique detected CaMoV in crude carnation sap with a detection reliability comparable to indicator plant assays (11).

Indicator plant assays and electron microscopy are very tedious, expensive, and time-consuming techniques. ELISA has all the advantages of the other assay methods with few of the disadvantages. ELISA is sensitive, reliable, accurate, rapid, and comparatively inexpensive and could be valuable, if implemented, in reducing disease incidence.

Table 2. Incidence by propagator of virus infections in commercial carnations in California as determined by indirect enzyme-linked immunosorbent assay

Propagator[a]	No. of samples	Infection (%)[b]					
		CaMoV[c]	CNFV	CERV	CRSV	Mix	Healthy
A	119	76	10	11	0	13	19
B	35	91	14	26	0	31	9
C	34	59	6	3	0	9	41
D	15	67	33	40	0	47	7
E	10	100	10	30	0	30	0
F	9	89	56	11	0	44	0
G	3	100	33	0	0	33	0

[a] Propagator = facility that produces commercial carnation cuttings for sale to individual growers.
[b] Percentage of infection for propagators A, B, and C were significantly different at $P = 0.05$ (heterogeneity chi-square) for CaMoV, CNFV, CERV, mix, and healthy. Propagators D, E, F, and G could not be included in this analysis.
[c] CaMoV = carnation mottle virus; CNFV = carnation necrotic fleck virus; CERV = carnation etched ring virus; CRSV = carnation ringspot virus; mix = mixed infection with CaMoV and CERV or CNFV or all three viruses in a particular sample.

Table 3. Virus incidence for the same California carnation grower utilizing cuttings from two different propagators as determined by indirect enzyme-linked immunosorbent assay

Propagator[a]	Total samples	Infection (%)					
		CaMoV[b]	CNFV	CERV	CRSV	Mix	Healthy
B	21	81	43	19	0	38	5
C	21	71	0	0	0	0	29

[a] Propagator = facility that produces commercial carnation cuttings for sale to individual growers.
[b] CaMoV = carnation mottle virus; CNFV = carnation necrotic fleck virus; CERV = carnation etched ring virus; CRSV = carnation ringspot virus; mix = mixed infection with CaMoV and CERV or CNFV or all three viruses in a particular sample.

LITERATURE CITED

1. Bennet, P. R., and Milne, K. S. 1976. Carnation viruses in New Zealand. Acta Hortic. 59:61.
2. Brierley, P. 1962. Heat treatment restores King Cardinal to health. (Abstr.) Phytopathology 52:163.
3. Brierley, P., and Smith, F. F. 1957. Carnation viruses in the United States. Phytopathology 47:714-721.
4. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of the enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
5. Crook, N. E., and Payne, C. C. 1980. Comparison of three methods of ELISA for baculoviruses. J. Gen. Virol. 46:29-37.
6. Crowther, J. R., and Abu-El Zein, E. M. E. 1979. Detection and quantification of foot and mouth disease virus by enzyme labelled immunosorbent assay techniques. J. Gen. Virol. 42:597-602.
7. Goethals, M., and Verhoyen, M. 1968. Les viroses de l'eoillet en Belgigue. Rev. Agric. (Brussels) 5-6:727-737.
8. Hakkaart, F. A. 1964. Description of symptoms and assessment of loss caused by some viruses in the carnation cultivar 'William Sim.' Neth. J. Plant Pathol. 70:53-60.
9. Jeff, A. E., and Day, M. 1978. Planting systems for virus-free carnation clones. Exp. Hortic. 30:56-60.
10. Kassanis, B. 1955. Some properties of four viruses isolated from carnation plants. Ann. Appl. Biol. 43:103-113.
11. Kemp, W. G. 1967. The reliability of seroassay for the detection of carnation mottle virus in crude sap. Can. Plant Dis. Surv. 47:89-93.
12. Lastra, R., Giannotti, R., and De Uzcategui, R. C. 1980. Virus del clavel in Venezuela. Fitopatologia 15:59-62.
13. Lawson, R. H., and Hearon, S. S. 1980. Carnation etched ring virus inclusions: Serology and ultrastructure of alkaline-treated inclusions. Phytopathology 70:327-332.
14. 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.
15. Mayhew, D. E. 1979. Carnation necrotic fleck

virus in California. Plant Dis. Rep. 63:978-980.
16. Paludan, N., and Rehnstrom, F. 1968. Carnation mottle virus. Influence on the yield and quality of carnation (*Dianthus caryophyllus*). Tidsskr. Planteavl. 72:33-41.
17. Smookler, M., and Loebenstein, G. 1975. Viruses of carnation identified in Israel. Phytopathol. Mediterr. 14:1-5.
18. Voller, A., Bartlett, A., Bidwell, D. E., Clark, M. F., and Adams, A. N. 1976. The detection of viruses by enzyme-linked immunosorbent assay (ELISA). J. Gen. Virol. 33:165-167.