VIRUSES INFECTING CARNATIONS
AND DIANTHUS SPECIES
IN NEW ZEALAND

A thesis presented in partial fulfilment
of the requirements for the degree of
Master of Horticultural Science

at

Massey University

by

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March 1975

ACKNOWLEDGEMENTS

I am greatly indebted to Dr. K.S. Milne for his encouragement and advice during the course of this study and the preparation of this thesis.

I would also like to take this opportunity to thank:

The staff of the Electron Microscope Laboratory, D.S.I.R., Palmerston North.

Mr. J.R. Clouston (Central Photography Unit, Massey University) for photography.

Carnation cut-flower growers and propagators who supplied plant specimens.

Mr. H. Neilson for technical assistance.

The staff of the D.S.I.R. library for obtaining publications.

Miss J.E. Corner, my fiancee, for typing the draft copy and general assistance.

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ABSTRACT

Five viruses were detected in commercial carnations and these and a further four occurred in Dianthus species from gardens. Carnation mottle virus (CarMV) and carnation etched ring virus (CERV-50) were widespread in commercial carnations; arabis mosaic virus (ArMV), carnation latent virus (CLV) and carnation necrotic fleck virus (CNFV) were also detected. In Dianthus species CERV-50, CLV and CarMV were the most prevalent, whereas only a low incidence of ArMV, CNFV, carnation ringspot virus (CRSV) and carnation vein mottle virus (CVMV) was found.

Two new viruses were detected in Dianthus species: an apparently uncharacterized plant rhabdovirus, named carnation bacilliform virus, with particles ca. 260 x 55nm (in ultrathin sections), and an unidentified isometric virus (D 345) ca. 30nm in diameter.

The viruses were characterized by a variety of methods including host range, symptoms, aphid transmission and particle morphology. The three rod viruses CLV, CNFV and CVMV were differentiated by particle morphology and size. Normal lengths for CLV and CVMV were 656nm and 738nm, respectively, while CNFV had particles in the range 1,000-1,450nm. The identity of the polyhedral viruses ArMV, CarMV and CRSV, was confirmed by serology. Carnation etched ring virus was identified by its particle size, ca. 48nm in diameter, and a consistent association with refractile inclusion bodies which were readily observed by light microscopy in epidermal strips stained with phloxine/trypan blue.

Cytological observations were made on ultrathin sections of leaves from plants infected with CBV, CERV-50, CLV and CVMV. Aggregates of CBV and CLV particles were observed in the cytoplasm; CERV-50 infected plants contained typical inclusions and particles of the virus; and CVMV induced cylindrical inclusions typical of the potyvirus group.

CHAPTER 1

VIRUSES AFFECTING CARNATIONS

INTRODUCTION

Carnations (Dianthus caryophyllus L.) are grown for cut flowers throughout the world and are a capital and labour intensive crop with a consequent high cost of production. Correct disease diagnosis and control are therefore of paramount importance in minimising losses in flower production. Because virus infection of carnations has been shown to cause reduction in flower quality and numbers (section 1.3) in other countries, it was considered of value to determine the identity and prevalence of viruses in this crop in New Zealand and to assess methods for their control.

1.1 VIRUSES IDENTIFIED FROM CARNATIONS

Eleven viruses have been definitively identified from carnations. They are: alfalfa mosaic virus (AMV) (Hollings & Stone, 1960); arabis mosaic virus (ArMV) (Hakkaart et al, 1972); carnation etched ring virus (CERV-50) (Hollings & Stone, 1967), which is possibly part of a complex (Hollings, Stone & Bouttell, 1968); carnation Italian ringspot virus (CIRV), a strain of tomato bushy stunt virus (Hollings, Stone & Bouttell, 1970); carnation latent virus (CLV) (Kassanis, 1955); carnation mottle virus (CarMV) (Hollings & Stone, 1964; Kassanis, 1955); carnation ringspot virus (CRSV) (Hollings & Stone, 1965b; Kassanis, 1955; Kowalska, 1972); carnation vein mottle virus (CVMV) (Hollings & Stone, 1971; Kassanis, 1955); cucumber mosaic virus (CMV) (Lovisolo et al, 1968); sowbane mosaic virus (SMV) (Hollings & Stone, 1967); and turnip crinkle virus (TCV) (Hollings & Stone, 1969b).

Recently there have been several reports of viruses with long flexuous particles, resembling beet yellows virus, in carnations. In 1968 Goethals & Verhoyen reported long filaments of about 1,000nm in Belgium and in 1971 Sutton & Taylor in Australia found particles with dimensions of about 1,200 x 12nm. Neither report provided details other than particle morphology and size. However, two more recent reports on viruses of this type in carnations provide more information. Inouye & Mitsuhata (1973) in Japan partially characterised an aphid-borne flexuous rod virus (1,400-1,500nm) which they named carnation necrotic fleck virus (CNFV) while from Israel Smookler & Loebenstein (1974) reported the isolation of an elongated virus (ca. 1,250 x 13nm) which they called carnation yellow fleck virus (CYFV) and which had an identical host range and similar transmission characteristics to CNFV. From these two reports it would appear that CNFV and CYFV are very much alike but distinct from beet yellows virus and other viruses of similar morphology; the earlier records are probably dealing with similar viruses.

There are several records of particles only partially characterised from carnations. Carnation etched ring disease was first described in 1960 by Hollings & Stone (1961) and since then attempts have been made to isolate and characterize the virus or viruses involved. Initially a 28nm diameter polyhedral particle was isolated (Hollings, Stone & Norrish, 1963) but was subsequently reported not to be involved with symptoms of etched ring (Hollings, Stone & Norrish, 1964). Later, two other isometric viruses (25nm and 29nm in diameter) were found in a carnation displaying etched ring symptoms and it was suggested that together with CERV-50 they comprised the full etched ring complex (Hollings & Stone, 1965a; Hollings, Stone & Bouttell, 1968). However, these findings have not been verified.

Symptoms of a streak disease of carnations reported by Hollings (Hollings, Stone & Norrish, 1962) in the United States are reputed to correspond with symptoms found in carnations in Belgium (Verhoyen, 1974). Extracts from plants with streak symptoms contained long filaments about 1,000nm (Goethals & Verhoyen, 1968) but subsequent work by Verhoyen (1974) showed that the disease could be separated into two components by meristem-tip culture. Carnation mottle virus and a 'yellow and necrotic blotch' reaction were implicated, the latter component apparently containing CERV-50 (Horvat & Verhoyen, 1974; Verhoyen, 1974). Symptoms of streak were reproduced in the carnations showing 'yellow and necrotic blotch' by inoculating them with CarMV. Although the component viruses have not been fully detailed, it appears that carnation streak is a complex involving CarMV and the 50nm particle of the etched ring complex at least.

Hakkaart & Olpen (1971) transmitted a 29nm diameter particle from the carnation cultivar Orange Triumph to Saponaria vaccaria L. 'Pink Beauty' on which it caused local white rings; the virus was later named carnation white ring virus (CWRV) (Hakkaart, 1974) but no further details were given. A number of other virus-like diseases and viruses have been partially characterized from carnations. A tobacco ringspot-type virus has been reported (Hollings & Stone, 1960) and there are various communications on carnation mosaic (Ames & Thornberry, 1952; Brierley & Smith, 1955, 1957; Creager, 1943; Jones, 1945; Rumley & Thomas, 1951;

Wright, 1951), carnation streak (Brierley & Smith, 1957; Goethals, 1969; also see above) and carnation yellows (Jones, 1945; Rumley & Thomas, 1948). The information presented was not usually sufficient to be able to ascribe these diseases to the previously mentioned viruses and in many cases mixtures of the known viruses were most likely present. Based on the thermal inactivation data, transmission characteristics and host ranges the mosaic virus of Brierley & Smith (1957) was probably CVMV while those of Noordam et al (1951) and probably Wright (1951) correspond to CRSV. The mosaic virus of Ames & Thornberry (1952) had physical properties similar to those of CVMV but purified preparations contained polyhedral particles (ca. 31nm diameter) when examined by electron microscopy and plants of Dianthus barbatus L. inoculated with these preparations developed mosaic symptoms.

Particles about 19nm in diameter have been observed in ultrathin sections from carnations and in a purified preparation which also contained CarMV (Castro et al, 1971). The size of this virus is similar to that reported for curly top virus (Duffus & Gold, 1973; Esau & Hoefert, 1973; Mumford, 1974).

1.2 WORLD OCCURRENCE

Five of the carnation viruses (CERV-50, CLV, CarMV, CRSV and CVMV) are known to have a wide distribution.

CERV: Since carnation etched ring disease was first recorded in Britain (Hollings & Stone, 1961) it has been found in a number of other European countries as well as in Israel and the United States (Appendix 1). The prevalence of this disease or of the component viruses of the complex has not been studied in carnation crops due to the lack of reliable and efficient methods for detection. However, it appears to be fairly prevalent.

CLV: Carnation latent virus has been infrequently found in cultivated carnations in Britain and Europe (Wetter, 1971) (Appendix 1). The virus has also been reported from Japan (Yora & Yuki, 1965), U.S.S.R. (Procenko & Procenko, 1964) and Australia (Sutton & Taylor, 1971). In Australia CLV was present in most field carnations but not in glasshouse crops.

CarMV: Carnation mottle virus has been recorded in Britain and Europe (Appendix 1) and is also known to be present in the United States (Brierley & Smith, 1957), Canada (Kemp, 1964) and Australia (Sutton & Taylor, 1971). The virus is reported to be prevalent in carnations (Brierley & Smith, 1957; Hollings, 1961; Kowalska, 1973).

CRSV: Like CarMV, CRSV has also been found throughout Europe (Appendix 1) and in the United States (Brierley & Smith, 1955, 1957) and Canada (Kemp, 1964), but though widespread the virus is apparently not prevalent in crops. Kowalska (1973) found 177 out of 1440 plants (12%) were infected with CRSV while Hakkaart (Wasscher, 1965) reported 17.7% of presumably healthy carnations contained CRSV, but the distribution was irregular. Although Kristensen (1957) found that CRSV was very common in Denmark, Paludan (1965) reported only 13% infection. It is probable that this virus is even less common now due to roguing based on the obvious symptoms it produces and the use of 'high-health' planting stock.

CVMV: The vein mottle virus has been recorded in Britain (Kassanis, 1955), Europe (Appendix 1), U.S.S.R. (Korneeva, 1964), the United States (Brierley & Smith, 1957) and Australia (Sutton & Taylor, 1971). However, it is apparently uncommon in N.W. Europe but more prevalent in S. Europe and the United States (Hollings & Stone, 1971). Although common in sweet william (D. barbatus) in gardens in Britain, the incidence of CVMV in glasshouse crops is very low (Hollings & Stone, 1971).

Other viruses characterised from carnations are apparently less widespread.

CNFV/CYFV: Carnation necrotic fleck virus particles were detected in 13 out of 18 varieties of carnation in Japan (Inouye & Mitsuhata, 1973), but the incidence of CYFV in Israel (Smookler & Loebenstein, 1974) was not indicated. The long rod from carnations in Australia was present in both glasshouse and field varieties, but was more prevalent in the latter varieties (Sutton & Taylor, 1971).

Reports for AMV (Hollings & Stone, 1960), ArMV (Hakkaart et al, 1972), CMV (Lovisolo et al, 1968), SMV (Hollings & Stone, 1967) and TCV (Hollings & Stone, 1969b) indicate only isolated cases of the viruses infecting carnations. The widespread occurrence and wide host range of AMV, ArMV and CMV, however, means that they must always be considered potential pathogens of carnation and therefore likely to be present in carnations wherever they are grown. Carnation Italian ringspot virus was isolated from carnations obtained from Italy and the United States but was reported to be extremely rare in Britain and also difficult to mechanically transmit to carnations (Hollings, Stone & Bouttell, 1970).

1.3 THE EFFECT OF CARNATION VIRUSES ON FLOWER QUALITY AND YIELD

Several experiments have been performed to determine the effect of some carnation viruses on flower quality and yield. Both Hakkaart (1964a) and Brierley (1964) found that CarMV, CRSV and CVMV (= Brierley's mosaic virus) depressed yields but the most important consequence was the economic loss resulting from the reduction in flower quality. Carnation ringspot virus was reported to be the most serious virus in the cultivar 'William Sim' (Hakkaart, 1964a) and the reduction in flower quality was even greater when CarMV was also present. However, the loss due to CVMV infection in 'William Sim' was not fully obvious in Hakkaart's results as flowers were graded regardless of colour breaking. Carnation vein mottle virus caused the largest loss in quality and number of saleable flowers of single virus infections in the cultivar 'King Cardinal', but plants infected with all the above viruses plus streak "virus" gave even fewer flowers although the number of rejects was about the same (Brierley, 1964). Streak "virus" alone caused only a slight reduction in quality and yield.

Carnation vein mottle virus is known to cause colour breaking in flowers (Hakkaart, 1964a; Poupet, 1971) which accounts for the severe reduction in flower quality of CVMV-infected carnations. Plants infected with CRSV produced a large proportion of flowers with split calices, but 'King Cardinal' was reported to produce no splits as it has a small calyx. Virus infection also caused a decrease in the average flower weight (Hakkaart, 1964a) which reflects the lower quality.

In contrast to the above findings, Paludan & Rehnstrøm (1968) reported that although CarMV reduced the yield in the first six months, over longer periods it had no effect on number or quality of flowers although the weight per flower was reduced. Trials with carnation cultivars free of known viruses indicate that better yields and quality can be obtained when viruses are absent (Hollings, Stone & Smith, 1972).

1.4 VIRUSES OF CARNATIONS IN NEW ZEALAND

A mosaic disease of carnation was first observed in New Zealand in 1949 and was subsequently found in Auckland, Christchurch and Timaru, although the incidence was comparatively low (Chamberlain, 1954).

Carnation mottle virus is the only virus characterized from carnations in this country (Thomson, 1962; Thomson & Reynolds, 1963) and was detected in all plants from 27 out of the 29 varieties tested (Thomson, 1962).

1.5 SURVEY OF VIRUSES INFECTING CARNATIONS AND DIANTHUS SPECIES IN NEW ZEALAND

An extensive survey involving 410 samples of glasshouse (commercial) and home garden carnations, as well as Dianthus species or hybrids from gardens, was carried out. As a result eight viruses were distinguished using a variety of techniques. The viruses identified were: ArMV, CERV-50, CLV, CarMV, CRSV, CVMV, a very long rod in the same size range as CNFV/CYFV, and a new bacilliform virus (CBV).

1.5.1 Materials and methods

Indicator plants were propagated in a sand-peat-fertiliser medium in a glasshouse in which temperatures ranged between 16-23 C. Pest and disease control was implemented using the insecticide methomyl (Lannate*), the acaracides cyhexatin (Plictran*) and dicofol (Kelthane*), and the fungicide benomyl (Benlate*), while maldison (Malathion*) drenches were used to control fungus gnat (Sciara spp.).

Inoculation: Inoculum was prepared by grinding tissue (usually leaf tissue) in a small volume of buffer plus celite using a pestle and mortar. Indicator plants were inoculated by gently rubbing the leaves with the pestle and then washing the leaves with water. Graft inoculation was carried out by inserting small pieces of leaf tissue, from which the epidermis was removed, into the stem of the test plant.

Electron microscopy: Plant material was indexed rapidly with an electron microscope using negatively stained crude sap. The crushed homogenate method (Walkey & Webb, 1968) was preferred to the epidermal strip (Hitchborn & Hills, 1965) or leaf dip (Brandes, 1957) techniques because of ease and speed of preparation. A small amount of leaf tissue was macerated on a spotting tile in two drops of negative stain and a 300 mesh formvar-carbon-coated copper grid held face down on the solution for a few seconds, after which excess stain was removed by touching the grid to filter paper. The grids were scanned within two days of preparation at a magnification of about 20,000 using a Philips EM-200 electron microscope.

Negative stains used were ammonium molybdate (AM), phosphotungstic acid (PTA) and uranyl acetate (UA). These were prepared as 2% solutions and the pH then adjusted with either 2M potassium hydroxide (for PTA and UA) or 1M ammonium hydroxide (for AM).

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*registered trade names

The size of virus particles was measured from electron micrographs and the magnification calculated using tobacco mosaic virus (TMV) as a standard, assuming a normal length of 300nm for TMV. The normal length (NL) of rod viruses was determined by the method of Brandes & Wetter (1959). Particle measurements were made from electron micrographs of crude homogenates only and after calculating the actual sizes the particles were grouped into 10nm divisions and the NL obtained as the mean of the major peak of the distribution, assuming a 'normal distribution'. The size range of polyhedral viruses was determined and the mean diameter calculated in a manner similar to that used to determine the NL of rod viruses.

Light microscopy: Epidermal strips were removed from leaves and stained for 1-5 minutes in a mixture of trypan blue (0.5% in 0.85% NaCl) and phloxine (0.5% in 0.85% NaCl) in the ratio of 15:1. Strips were then rinsed and mounted in 0.85% NaCl and examined for inclusion bodies of CERV-50 with a light microscope at a magnification of 200 .

Serology: Ouchterlony double diffusion was carried out in 0.75% agar medium with 0.01% sodium azide as preservative in 9cm plastic petri dishes. Usually a pattern with six peripheral wells around a central well was employed, the wells being 5mm in diameter with 6mm between wells. Leaf tissue of virus-infected and healthy plants was respectively ground in 0.1M K/K_2 phosphate buffer, pH 7.5. Serology plates were incubated at 20-24 C in large petri dishes lined with moist filter paper, to prevent drying of the agar, and occurrence of precipitin lines recorded over several days.

1.5.2 Mechanical Transmission

Although CLV, CarMV and CVMV were readily transmissible from carnations the other viruses posed some problems. Arabis mosaic virus was reported not to be transmissible from crude carnation sap to Chenopodium quinoa Willd. or Chenopodium amaranticolor Coste & Reyn. without the addition of bentonite (Hakkaart, Hoof & Maat, 1972). Consequently bentonite was added at the rate of 0.5% in the inoculation buffer (0.1M K/K_2PO_4, pH 7.5). Yarwood's (1966) solution (0.5% bentonite in 0.5% K_2HPO_4) was also found suitable for the transmission of ArMV to C. quinoa (section 2.1).

Carnation etched ring virus was sap transmitted fairly readily, but only to Gypsophila elegans Bieb. and Saponaria vaccaria L. 'Pink Beauty'. Concentric red rings were not induced on the inoculated leaves of S. vaccaria, which contrasts with the results of Hakkaart (1974) (who supplied the seed line). Although the virus did not cause obvious symptoms in G. elegans and S. vaccaria the presence of CERV-50 in these hosts was demonstrable in inoculated and systemic tissues by light and electron microscopy. Graft transmission of CERV-50 to the carnation cultivar 'Joker' occurred readily in the limited number of tests carried out. Symptoms of CERV-50 in 'Joker' are detailed in section 2.3.

The very long flexuous rod was mechanically transmitted by sap inoculation in one case only, a result which is in accord with the reports on CNFV (Inouye & Mitsuhata, 1973) and CYFV (Smookler & Loebenstein, 1974). Carnation bacilliform virus was not transmitted by sap inoculation.

1.5.3 Electron microscopy - indexing

Electron microscopy of negatively stained crude homogenates was found to be a useful method for the detection of both rod and polyhedral plant viruses, despite reports (Brandes, 1966; Schmidt, 1967) to the contrary for polyhedral viruses. However, the results varied depending on the stain, pH, age of infection, the plant host and the particular tissue. Therefore a preliminary trial was carried out with carnation leaf tissue and three common EM stains at varying pH: AM at pH 5.6 and 7, PTA at pH 4 and 7, and UA at pH 4.

Ammonium molybdate gave preparations of poor contrast, but had good spreading properties. Phosphotungstic acid at both pHs provided good contrast generally, although poor spreading occurred at times. With uranyl acetate very poor spreading occurred and this stain was not subsequently used for survey work. Because of the spreading properties of AM, mixtures with PTA at three ratios (AM:PTA at 2:1, 1:1, 1:2) were tried. The best spreading and contrast was obtained with PTA at pH 7 in the ratio of AM:PTA = 1:1 or 1:2 (pH 5.5 and 5.6 respectively). These stain mixtures were subsequently used for most routine indexing of carnations. No obvious damage to virus particles occurred using AM, PTA or the mixtures although this has been observed with some stain and virus combinations (Atkey et al, 1973; Tomlinson et al, 1973).

The carnation rod viruses, CLV, CVMV and CNFV/CYFV and an isometric virus CERV-50, could all be identified by particle size and morphology using electron microscopy. However, the spherical viruses with diameters of about 30nm could not be differentiated on this basis and further definitive tests were required to establish their identity. In the few cases where only ArMV occurred in carnations, particles were seldom observed in the EM.

1.5.4 Light microscopy indexing for CERV-50

The most suitable epidermal strips for light microscopy were obtained from young fully expanded leaves. Inclusion bodies were detected in leaves of various ages, except in very young leaves; in older leaves the inclusion bodies tended to be much less prevalent.

Inclusion bodies have been reported for CVMV (Castro et al, 1971; Norrish, 1962, 1963; Rubio-Huertos, 1959), CLV (Castro et al, 1971) and CNFV (Inouye & Mitsuhata, 1973) but with the methods employed in this study inclusion bodies were correlated only with CERV-50 infection (section 2.3); inclusion bodies were neither found in carnations infected with CLV, CVMV and CNFV/CYFV, nor in plants experimentally infected with these viruses, unless CERV-50 was also present.

There are two other reports of inclusions in carnations, and in both instances only summaries were available. Reiter (1961) found crystalline inclusions, both hexagonal and tetrahedral, amorphous X-bodies and striated inclusions; the identity of the viruses involved is not clear. Thaler et al (1970) used electron microscopy to examine striated virus inclusions which were very similar to those found by Reiter by light microscopy. The inclusions consisted of fibrillose virus particles in regular array. The particles were reported to be about 8-10nm in diameter and 300nm long.

In the present study crystalline bodies were observed in some epidermal strips, but similar crystals were also seen in epidermis from apparently virus-free plants.

1.5.5 Serology

Serology was used to confirm the identity of ArMV and CRSV whenever their presence was suspected. Initially, the identity of CarMV was also checked by serology. The serological tests were generally made with inoculated leaves of *C. quinoa* containing a high concentration of virus.

1.5.6 Survey method

Carnation cuttings or samples were obtained from cut-flower growers, in both the North and South Islands, a major supplier of carnation cuttings and from public and home gardens. One of the cut-flower producers imported his stock plants from Holland.

Virus indexing (Figure 1) involved mechanical inoculation to C. quinoa and one or more of the following hosts: G. elegans, Saponaria vaccaria 'Pink Beauty', Spinacia oleracea L. (spinach); concomitantly grids were prepared for electron microscopy and epidermal strips stained and observed by light microscopy. If the tissue could not be examined immediately, it was held in a refrigerator. Subsequently, tissue from the primary hosts (both inoculated and systemic leaves) was used for sap transmission to the differential hosts (Table 1). The initial host plants were used because the viruses could not be transmitted directly from carnation to some of the differential indicators because of the presence of inhibitors in the carnation sap (Ragetli, 1958; Ragetli & Weintraub, 1962; Want, 1951).

In some cases the symptoms induced by the viruses on the initial hosts were sufficient for identification, for example the reaction of CarMV on C. quinoa, but the reaction of CarMV frequently obscured the reaction of other viruses. The presence of viruses in the primary and differential hosts could be monitored by electron microscopy but the actual identity of most of the icosohedral viruses could not be resolved by this method, and the reactions on differential hosts and serological tests were required to characterize these viruses.

1.5.7 Results and discussion

The results of the present survey are summarized in Table 2. Further details of the incidence of viruses in commercial carnations of which 33 varieties were tested are presented in Appendix 2.

Commercial carnations were almost totally infected with CarMV but the incidence of CERV-50 (75%) may be inflated to some extent as many samples were selected from plants that appeared to have virus symptoms. Not all plants containing CERV-50, however, had obvious symptoms. In particular, plants with light coloured flowers (yellow, salmon and pink) generally expressed fewer leaf symptoms than did darker flowered varieties such as 'Joker' and 'Orchid Beauty'. Also many samples were of very young tissue in which it was more difficult to detect CERV-50 owing to the lower concentration of virus. This negates to some extent the bias in sampling referred to previously. The high incidence of CarMV and CERV-50 is, in all probability, primarily the result of vegetative propagation without an adequate clean stock program. The low incidence of CarMV in garden carnations and Dianthus species, however, may be attributed to propagation of many of them, particularly the species, from seed. Evidence for the likelihood of seed propagation

DIFFERENTIATION OF VIRUSES INFECTING CARNATIONS

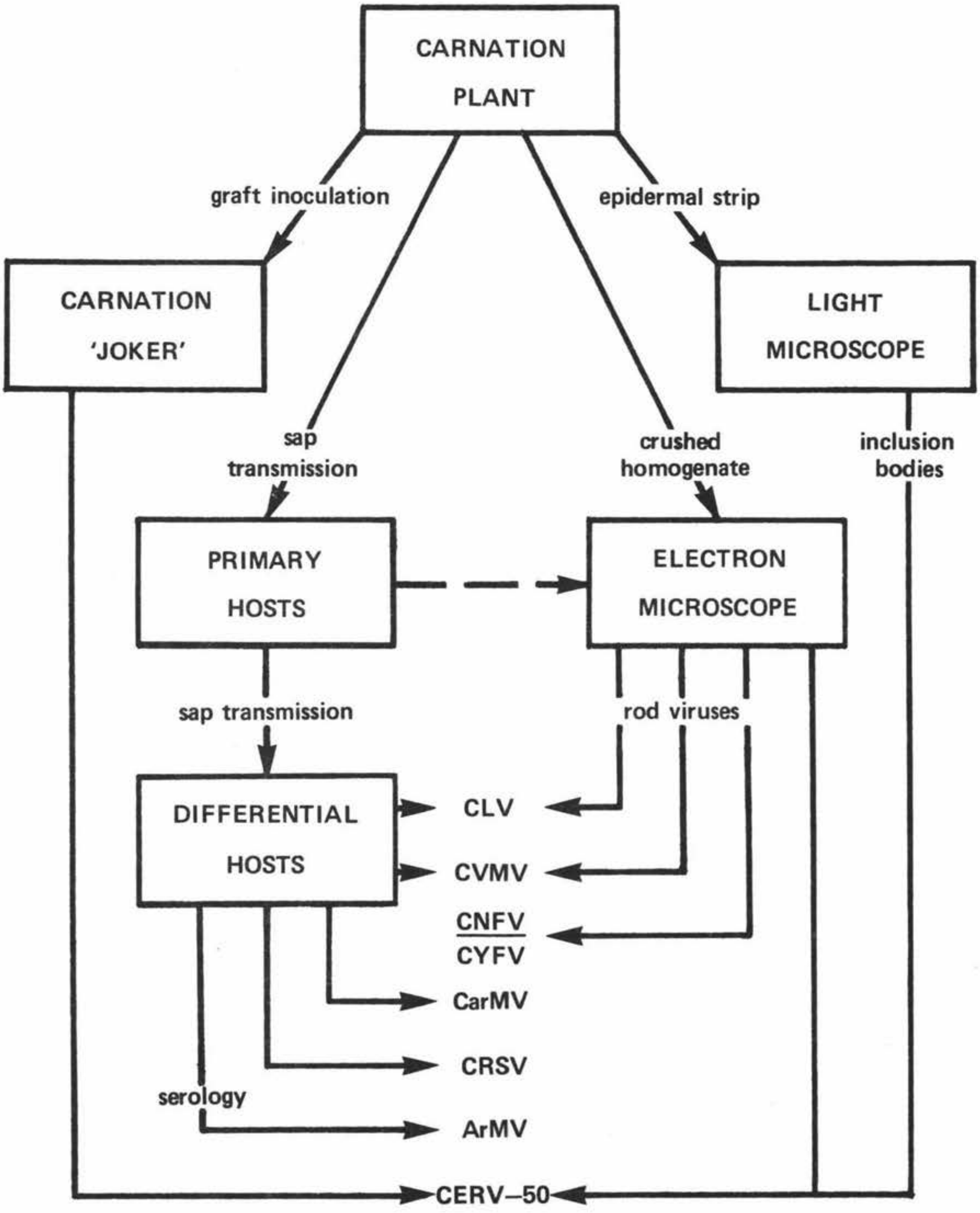


FIGURE 1. Flow chart of the procedure used to differentiate viruses infecting carnations.

TABLE 1. Differential hosts for the identification of six viruses infecting carnations.

Indicator Plants	Viruses					
	ArMV[1]	CERV	CLV	CarMV	CRSV	CVMV
Celosia argentea L. 'Forest Fire'	(L)	NI	NI	(L)	Ln*	NI
Chenopodium amaranticolor Coste & Reyn.	Lc S*	NI	S*	Lc,n	Ln	Lc
Chenopodium quinoa Willd.	Lc S*	NI	Lc S	Lc S	Ln	Lc S
Cucumis sativus L. (cucumber) 'Marketer'	Lc S*	NI	NI	NI	Ln	NI
Dianthus barbatus L. (sweet william)	Lc S	(S)	(S)	Ln	Ln S	S*
Gomphrena globosa L.	L	NI	NI	(L)	Ln S	S
Gypsophila elegans Bieb.	Lc S	(S)	(S)	S	Lr S*	S
Nicotiana clevelandii Gray.	Lr S	NI	(S)	L	Lr S	NI
Nicotiana tabacum L. 'White Burley'	L S	NI	NI	NI	Lr	NI
Saponaria vaccaria L. 'Pink Beauty'	L S	(S)	(S)	S	Lr S	S
Spinacia oleracea L. (spinach) 'Royal Denmark'	Ln S	NI	(L)	L (S)	Ln S	S

Symbols: c=chlorotic; L=local infection; n=necrotic; NI=no infection; r=ringspots; S=systemic infection; ()=symptomless infection. *=important differential reactions.

[1] reactions of ArMV varied with the strain.

(Note: for details of symptoms see the relevant sections in Chapter 2)

TABLE 2. Incidence of viruses detected in carnations and species of *Dianthus*.

Virus	Commercial[1] carnations		Public/home garden			
			carnations		*Dianthus* spp.	
	No	%	No	%	No	%
ArMV	5	1.7	4	6	5	9.6
CBV	-	-	2	3	-	-
CERV-50	$\frac{201}{208}$[2]	75	$\frac{25}{64}$[2]	39	30	58.7
CLV	4	1.4	9	13.4	35	67.3
CarMV	276	96	11	16.4	-	-
attenuated	3		7	10.4	1	2
CNFV/CYFV	6	2	6	9	2	3.8
CRSV	-	-	2	3	2	3.8
CVMV	-	-	2	3	-	-
No detectable virus	7	2.4	22	33	12	23
Number of samples	291		67		52	

[1] Appendix 2 contains more details on commercial carnations.

[2] Not all samples were tested for CERV-50. Total numbers tested are indicated where necessary.

of many garden carnations and Dianthus species can be found in the relatively large number of plants, 33% and 23% respectively, having no detectable viruses in them compared with only 2.4% for commercial carnations. Accordingly the high incidence of CERV-50 (59%) and CLV (67%) in Dianthus species can only be explained on the basis of aphid transmission as neither virus has been reported to be seed-borne. Lack of adequate aphid control probably compounds the incidence of these two viruses and may help account for the high incidence of CERV-50 in commercial plants. However, the very low occurrence of CLV in glasshouse crops (1.4%) is difficult to explain, especially in relation to the high occurrence in Dianthus species (67%).

A hypothesis which needs testing is that carnations (D. caryophyllus) are less susceptible to CLV than other Dianthus species. This could also explain the intermediate levels of CLV in garden carnations (13%), which are subject to a higher infection pressure by aphids than commercial varieties. The incidence of CLV in this country is similar to that in Australia where the virus was found to be much more prevalent in field (outdoor) carnations than in glasshouse crops (Sutton & Taylor, 1971). The higher incidence of CERV-50 in carnations, particularly garden carnations, compared with that of CLV suggests that CERV-50 may be more readily aphid borne than CLV. This appears to be confirmed by results of aphid transmission experiments with these two viruses in the present study (sections 2.3 and 2.4).

The low incidence of CRSV and CVMV in garden plants may be of considerable significance, particularly for CVMV which is aphid borne. In view of the ease with which CVMV is aphid borne the reason for it being less prevalent than CLV in garden carnations is not apparent. In Britain CVMV is reported to be prevalent in sweet william in home gardens (Hollings & Stone, 1971). The two CVMV-infected carnations were from the same source and may be an isolated instance of the virus in this country, but a more extensive survey of home garden plants would help to resolve this. Plants infected with CVMV and CRSV generally exhibit obvious symptoms (sections 2.7 and 2.8) and are therefore unlikely to be used for propagation, which could explain the absence of these two viruses in commercial carnations.

The presence of ArMV in glasshouse carnations would appear to be the result of importing cuttings from overseas as explained in section 2.1, but the infestations in garden carnations and Dianthus species represent natural occurrence of the virus. The garden carnations (3 cultivars) were all from one source and were infected with mild strains of ArMV, while four of the Dianthus species originated from different localities and were infected with severe strains of the virus. These Dianthus plants appeared to be the same species although different cultivars. These plants may either be more susceptible to ArMV than other Dianthus species tested or the virus could be seed-borne as has been reported for a diverse range of plants (Lister & Murant, 1967).

Data on the incidence of CNFV/CYFV and CBV is inconclusive as the methods for detection were not sufficiently sensitive.

Sensitivity of detection methods

The combination of electron microscopy and indexing on indicator plants was sufficiently sensitive for the detection, differentiation and separation of CLV, CRSV, CVMV, and CarMV except the attenuated strain. In this case the virus was seldom detected on *C. quinoa* before transfer through *G. elegans* or *S. vaccaria*. Even then detection was dubious and consequently infections with attenuated CarMV may not have been detected. Carnation latent and vein mottle viruses were differentiated by electron microscopy on the basis of particle length and conformation and could be separated by inoculation to suitable indicator plants.

Carnation etched ring virus was fairly readily detected by both electron and light microscopy but could not be differentiated or separated from other viruses by inoculations to indicator plants. Graft transmission of CERV-50 to 'Joker' did not always cause characteristic symptoms, making this method insufficiently reliable for surveys.

Transmission of ArMV from carnation to indicator plants was the major problem when studying this virus (section 2.1) although the results indicate that the method used was satisfactory. Particles of ArMV were very difficult to detect by electron microscopy in carnation sap indicating that electron microscopy was less sensitive than host inoculations for detecting this virus. This conclusion is also borne out by the results with attenuated CarMV which was rarely observed by electron microscopy.

Detection of CBV and CNFV/CYFV relied solely on electron microscopy and it is possible that both these viruses are more prevalent than indicated. With the long rod virus it was even difficult to obtain reproducible results from the same piece of tissue.

CHAPTER 2

PROPERTIES OF THE VIRUSES INFECTING CARNATIONS AND DIANTHUS SPECIES IN NEW ZEALAND

This chapter deals with the viruses found in carnations and *Dianthus* species during the present study, namely arabis mosaic, carnation bacilliform, carnation etched ring, carnation latent, carnation mottle, carnation necrotic fleck (= carnation chlorotic fleck), carnation ringspot, carnation vein mottle viruses and an unknown virus isolate (D345). Viruses were characterized by a variety of methods and results are discussed under the following headings: host range, vector transmission, purification, electron microscopy of sap (particle morphology and size), physical properties, light microscopy, ultrastructural studies and serology.

METHODS AND MATERIALS

Only those methods and materials common for the viruses are presented here.

Propagation and inoculation of plants, light and electron microscopy are as for section 1.5 unless otherwise stated in the following sections. Plants showing no symptoms in host range studies were back inoculated to *C. quinoa* to check for symptomless infection, except for CBV, CERV-50 and CNFV which did not infect *C. quinoa*. There were no adequate indicator plants for these three viruses, and sap from the hosts was examined for particles by electron microscopy.

A 'MSE Superspeed 65' ultracentrifuge was used for virus purification. Suitable dilutions of purified preparations were mixed with negative stain (1:1) and sprayed onto EM grids for electron microscopical observation.

Thermal inactivation points (TIP) were determined by grinding infected leaf tissue in a small volume of water or buffer, extracting through muslin and pipetting 0.5ml into thin walled tubes 7.5 x 1.2cm that had been equilibrated at the appropriate temperature. The virus solution was then incubated for 10 min at 5C intervals and assayed on an appropriate host. For dilution end point (DEP) determination similar extracts were diluted with water or buffer.

Assays were conducted using 6 half-leaves per treatment; the other leaf halves were inoculated with a suitably diluted control preparation. The number of local lesions per replicate were added and expressed as a percentage of the appropriate control replicate, thereby facilitating comparison between treatments.

Vector transmission studies were conducted using green peach aphids (*Myzus persicae* Sulz.) which had been maintained on chinese cabbage (*Brassica pekinensis* (Lour.) Rupr.). Aphids were removed from these plants with a small brush and placed on moist filter paper in petri dishes and starved (usually 4-8 hours) prior to transmission experiments.

Antisera for serological tests were kindly supplied by Drs. B.D. Harrison, M. Hollings (ArMV); Dr. Wetter (CLV); Mr. J. Sutton (CarMV, CLV, CVMV); Dr. P.R. Fry (CRSV); Dr. J.K. Uyemoto (cherry leaf roll, broad bean wilt, tobacco ringspot and tomato ringspot viruses); Dr. R.A. Goold (raspberry ringspot and tomato black ring viruses). (Refer Appendix 3 for addresses).

Ultrathin sections were prepared by the following schedule: fresh plant tissue was cut into small pieces and vacuum infiltrated in Karnovsky's (1965) fixative (3% glutaraldehyde, 2% formaldehyde in 0.1M Na_2/KPO_4 buffer, pH 7.2). Tissue pieces were washed in buffer (0.1M Na_2/KPO_4, pH 7.2) and postfixed in 1% osmium tetroxide in buffer at 4C for 3-5 hours; the tissue was washed and dehydrated in a graded ethanol series (25, 50, 75, 90 and 100%) and then placed in two changes of propylene oxide. Tissue was then transferred through ascending concentrations of epoxy resin in descending concentrations of propylene oxide and finally embedded in (100%) resin in gelatine capsules and cured for 2-3 days at 60C. Ultrathin sections were cut with an LKB ultramicrotome and picked up on 200 mesh copper grids and stained in saturated uranyl acetate in 50% ethanol for 5-7 min, washed in 50% ethanol, then in distilled water and stained with lead citrate (Venable & Coggeshall, 1965) for 5-7 min. Stained sections were washed in distilled water and observed by electron microscopy.

2.1 ARABIS MOSAIC VIRUS (ArMV)

R/1:*/41:S/S:S/Ne

Arabis mosaic virus, a member of the nepovirus group, has been identified only once from carnations (Hakkaart et al, 1972). However, ArMV infects many cultivated plants and weeds, in some of which it is seed-borne (Lister & Murant, 1967), so that its presence in carnations and *Dianthus* species in New Zealand is not entirely surprising, despite the fact that the virus has previously been reported only in *Cyphomandra betaceae* Sendt. (tamarillo) (Thomas & Procter, 1972), and *Daphne odora* Thunb. 'Leucanthe' and *Daphne* x *hybrida* Sweet (= *D. dauphinii* hort.) (Forster, 1974) in this country. Recently ArMV was also isolated from flowers of *Edgeworthia papyrifera* Sieb. & Zucc. (Dr. K.S. Milne, pers. comm., 1974).

There are numerous strains of ArMV which are serologically closely related and ArMV is also distantly related to grapevine fanleaf virus (Cadman et al, 1960) but is not related to other members of the nepovirus group. The soil-inhabiting nematodes *Xiphinema diversicaudatum* (Micol.) Thorne (Harrison & Cadman, 1959), *Xiphinema coxi* Tarjan (Fritzsche & Schmidt, 1963) and *Longidorus caespiticola* Hooper (Valdez, 1972) are vectors of the virus. Other properties of ArMV summarized by Murant (1970) are:

TIP : 55-61C
DEP : 10^{-3}-10^{-5}

Longevity in vitro

LIV : 1-2 weeks at room temperature
(some strains survive longer)

Transmission from carnation

Hakkaart et al (1972) reported that ArMV could not be transmitted in sap from carnations to *C. quinoa* without partial purification or the addition of bentonite (about 0.2 or 0.1% final concentration) to the inoculum. In the present study experiments with 'Orange Triumph' carnation leaves infected with ArMV revealed that the addition of bentonite (0.5%) to several different buffers aided transmission of ArMV to *C. quinoa* (Table 3). Consequently, buffer containing bentonite or Yarwood's (1966) solution were used for all transmission experiments from carnation. The results also indicate that carnations only support a low level of virus as, with the exception of severe isolates in carnation sap, no localised reaction occurred. Subsequent transfer to another *C. quinoa* resulted in abundant formation of lesions. Bentonite was not necessary for transmission from primary indicator plants to other hosts.

TABLE 3. Comparison of buffers, with and without bentonite, to facilitate transmission of arabis mosaic virus from carnation tissue to *Chenopodium quinoa*.

Buffer	Experiment		
	1	2	3
0.1M K/K_2PO_4, pH 7.5	-	-	-
+ bentonite	+	+	
0.5% K_2HPO_4, pH 8.7 + bentonite		+	+
0.1M K/K_2PO_4, pH 7.5 + 0.01M veronal + 0.007M Na_2 EDTA	-	-	-
+ bentonite		+	+
0.5M K/K_2PO_4, pH 7.5 + 0.1M Na_2SO_3 + 0.1% Na thioglycollate	-	-	-
+ bentonite		-	-
0.06M K_2HPO_4, pH 8.7 + 10% PVP			-

Notes: Bentonite was used at the rate of 0.5% in all buffers. PVP = polyvinyl pyrrolidone; + = infection; - = no infection. Experiments were conducted with ArMV-O.

Host range

Three strains (ArMV-M, ArMV-O and ArMV-S) of ArMV were evident on the basis of the severity of symptoms on indicator plants, although care was necessary in assessment because of the influence of seasonal and plant variation on symptom expression. Most studies were conducted with an isolate (ArMV-O) from the carnation 'Orange Triumph'. These plants originated from Holland, and as ArMV was not detected in other carnation varieties on the same property (section 1.5.7) it is probable that the plants were infected before reaching New Zealand. 'Orange Triumph' (all from the one grower) was the only commercial variety containing ArMV. The two other strains were obtained locally from a home garden carnation and a Dianthus species, respectively; ArMV-M was a milder strain, while ArMV-S was more severe than ArMV-O.

Details of the range of symptoms caused by strains of ArMV on indicator plants follow:

AMARANTHACEAE

Amaranthus caudatus L. and Amaranthus viridis L.: the severe strain (ArMV-S) caused local chlorotic lesions after 5 days followed by systemic chlorosis, particularly along the veins, of young leaves, progressing to interveinal clearing. Later leaves were often symptomless. The milder strains caused symptomless infection.

Celosia argentea L. 'Forest Fire': occasionally local red spots (about 3-4mm) formed after about 8 days (ArMV-S); symptomless systemic infection (all strains).

Gomphrena globosa L.: usually no reaction, but occasionally local necrotic lesions with red peripheries (about 2mm) after 6 days.

CARYOPHYLLACEAE

Dianthus barbatus L. (sweet william): systemic symptomless infection (ArMV-M, ArMV-O), or chlorotic lesions or whitish necrotic ringspots after 5-6 days followed by systemic chlorosis of the young leaves (ArMV-S). Symptoms usually faded and the virus was carried symptomlessly.

Dianthus caryophyllus L. 'Joker': symptoms consisted of downward curling of the leaves and slight stunting of the plants (ArMV-O).

Dianthus chinensis L. 'Bravo': symptomless infection (ArMV-M, ArMV-O), or faint pale green lesions followed by systemic chlorosis and stunting (ArMV-S). Symptoms faded with age.

Gypsophila elegans Bieb.: symptomless infection or faint chlorotic lesions with either diffuse mottling or no symptoms on the younger leaves.

Gypsophila paniculata L.: occasionally symptomless infection.

Saponaria vaccaria L. 'Pink Beauty' (cowcockle): the local reaction ranged from symptomless infection (ArMV-M) to chlorotic lesions after 5 days which subsequently became whitish ringspots (ArMV-O) and to necrotic lesions or ringspots after 5 days (ArMV-S). Systemic infection varied from symptomless (ArMV-M) to mottling (ArMV-O) and vein chlorosis, and sometimes vein necrosis, mottling, deformation and stunting of the young leaves and stunting of the plant (ArMV-S) (Figure 2).

CHENOPODIACEAE

Chenopodium amaranticolor Coste & Reyn.: diffuse chlorotic lesions (after 5 days) (ArMV-M) to necrotic lesions after 3-4 days (ArMV-S); systemic vein clearing and/or chlorotic flecks progressing from the base of the leaves followed by mottling and stunting of the young leaves. Epinasty of the apex and lateral shoots.

Chenopodium quinoa Willd.: chlorotic lesions after 3-4 days (ArMV-M, ArMV-O) to necrotic rings or spots after 3-4 days (ArMV-O, ArMV-S); systemic vein chlorosis, chlorotic and sometimes necrotic flecking of the young leaves. Epinasty of the growing apices frequently accompanied by necrosis of the young leaves. With ArMV-S the apices were frequently killed, while plants infected with the milder strains exhibited some remission of symptoms although leaves remained mottled and the plants stunted.

Spinacia oleracea L. 'Royal Denmark' (spinach): sunken necrotic lesions after about 4 days (the entire leaf became necrotic when infected with ArMV-O, ArMV-S) followed by mild mottling (ArMV-M), chlorotic flecking and severe mottling (ArMV-O) or vein chlorosis with chlorotic and necrotic flecking (ArMV-S) of the young leaves. Complete necrosis of severely affected leaves often occurred. Leaves were distorted and plants stunted. Remission of symptoms sometimes occurred, particularly with milder initial reactions.

COMPOSITAE

Callistephus chinensis Nees (China aster): ArMV-S caused faint chlorotic lesions after 10 days and vein chlorosis and faint chlorotic speckling of the young leaves.

Tithonia speciosa Hook. (Mexican sunflower): chlorotic lesions after about 2 weeks with irregular systemic chlorotic flecks and mottling.

Zinnia elegans Jacq.: local symptomless infection, occasionally.

CUCURBITACEAE

Cucumis sativus L. 'Marketer', 'Polaris' (cucumber): chlorotic lesions on cotyledons after 5 days, the lesions sometimes turning necrotic with ArMV-O and ArMV-S. Infection of the young leaves started as chlorotic spots which expanded to become a mosaic (Figure 3); leaves and plants were stunted and leaves rugose. The severity of symptoms generally depended on the strain of ArMV and remission of the symptoms often occurred.

LEGUMINOSAE

Phaseolus vulgaris L. 'Topcrop' (dwarf bean): no symptoms were observed on inoculated primary leaves (ArMV-O) but local chlorotic lesions formed on the first trifoliate leaves after approximately 10 days. Systemic chlorotic and necrotic flecking, veinal and apical necrosis; distortion and stunting of the young leaves (ArMV-O, ArMV-S).

Vigna sinensis (Torner) Savi 'Blackeye' (cowpea): local necrotic flecks, particularly along veins, after 14 days. Mottling, necrotic flecking and stunting of the young leaves.

SCROPHULARIACEAE

Antirrhinum majus L. (snapdragon): usually ArMV-M and ArMV-O did not cause infection but occasionally symptomless infection resulted. ArMV-S caused pale green lesions after 5 days followed by systemic vein clearing and mottle of the young leaves. Symptoms later abated.

Digitalis purpurea L. (foxglove): systemic symptomless infection in some instances.

SOLANACEAE

Nicotiana clevelandii Gray: chlorotic lesions formed after approximately 7 days, subsequently developing into necrotic ringspots and target spots; systemic chlorotic flecking and mottling occurred with ArMV-M infection, while with ArMV-O and ArMV-S necrotic flecking, and occasionally chlorotic or necrotic ringspots, appeared on the young leaves. Remission of symptoms occurred frequently and the virus was carried symptomlessly in the leaves.

Nicotiana glutinosa L.: symptomless systemic infection.

Nicotiana rustica L.: diffuse local chlorotic spots after 6 days sometimes becoming faint whitish ringspots; diffuse systemic chlorotic spotting and rugosity of young leaves (ArMV-O, ArMV-S).

Nicotiana sylvestris Speg. & Comes: symptomless infection or systemic chlorotic spotting (ArMV-O).

Nicotiana tabacum L. 'Havana 423', 'White Burley' (tobacco): chlorotic spots on inoculated leaves after about 4 days becoming chlorotic and semi-necrotic ringspots (ca. 8 days) (Figure 4); systemic chlorotic spots on young leaves often developed into ringspots and line patterns (Figure 5). Severe infection resulted in necrotic flecks and vein necrosis on the young leaves and also stem necrosis. Plants often showed remission of symptoms when infected with mild strains. 'Havana' appeared to be slightly less sensitive than 'White Burley'.

Petunia hybrida Vilm.: isolates ArMV-M and ArMV-O frequently did not infect this host, but occasionally diffuse chlorotic lesions were formed after 6 days followed by systemic vein chlorosis on young leaves which disappeared with age. ArMV-S caused local necrotic rings after 4 days followed by vein chlorosis of the young leaves which later became symptomless.

Physalis franchetti Mast.: local symptomless infection only.

The following plants were not infected in this study: Apium graveolens L. (celery); Borago officinalis L.; Brassica pekinensis (Lour.) Rupr. 'Chi-Hi-Li' (Chinese cabbage); Capsicum frutescens L. (pepper); Matthiola incana (L.) R.Br. (stock); Mirabilis jalapa L.; Phlox drummondii Hook.; Salvia splendens Ker-Gawl.; Senecio cruentus DC. (cineraria) and Tropaeolum majus L. (nasturtium).

Vector transmission

No tests were carried out to determine the presence of the nematode vectors in the vicinity of virus infected plants. However, Xiphinema diversicaudatum has been recorded in New Zealand associated with ArMV-infected tamarillo (Thomas & Proctor, 1972).

Purification

Highly infectious preparations were obtained from ArMV-O, infected tobacco ('White Burley'), harvested about 21 days after inoculation, and C. quinoa, 14 days after inoculation, by a slight modification of the method employed by Harrison & Nixon (1960). Tissue was homogenized in ascorbate-phosphate buffer (0.1M ascorbic acid plus 0.2M Na_2HPO_4, 1:1, pH 6.5) containing 1% 2-mercaptoethanol at the rate of 1g of tissue per 1ml of buffer and a butanol-chloroform mixture (1:1) poured in while blending (about 3 or 4 volumes of solvent to 1 volume of aqueous phase). Homogenate was expressed through muslin, centrifuged (2,000g/20min) and the aqueous phase drawn off and left at room temperature overnight, after which the solution was clarified by centrifugation (10,000g/10-15min). Pellets were readily resuspended in 0.01M K/K_2 phosphate buffer, pH 7.5, or 0.001M EDTA (1ml/50g tissue for the final pellet).

Electron microscopy

Electron microscopy revealed the presence of icosahedral particles in purified preparations and crude sap from indicator plants but the virus was very difficult to find in carnation sap. Three types of negative stain were tested on purified preparations. Uranyl acetate caused very poor spreading but stained well in small areas (Figure 6a); in phosphotungstate, pH 7, more particles were penetrated by stain and more were disrupted than with UA (Figure 6b). In comparison with UA and PTA, ammonium molybdate provided preparations which were suitably spread and stained (Figure 6c, d) and with all three stains particles showed varying degrees of stain penetration, a situation that has previously been noted for ArMV (Murant, 1970). The 6- and occasionally 5-sided outlines of the particles were evident particularly in arrays which were often present in crude sap. The average diameter of 200 particles was 26.5nm.

Light microscopy

No inclusions were observed by light microscopy in ArMV-infected carnation tissue stained with phloxine-trypan blue and to the author's knowledge no inclusions visible by light microscopy have been reported for ArMV.

Serology

The extensive host range and symptoms, together with particle morphology and size suggested that the virus belonged to the nepovirus group. To confirm this and determine specific identity inoculated leaf tissue from C. quinoa infected with an isolate from 'Orange Triumph' was tested by gel diffusion against antisera to arabis mosaic, tobacco ringspot, tomato ringspot and cherry leaf roll viruses. Positive results were obtained only with ArMV antiserum. Similar results occurred with purified preparations. All suspected isolates of ArMV were tested serologically and no spur formation occurred between the three strains with antisera supplied by Dr. B.D. Harrison and Dr. M. Hollings.

Antiserum to ArMV-O was prepared by injecting a rabbit with a purified virus preparation mixed with an equal volume of Freund's incomplete adjuvant. Three intramuscular injections were given 2-3 weeks apart and the animal was bled 3 weeks after the last injection. Attempts to obtain the titre by microprecipitin tests under paraffin oil with 0.01M Tris buffer, pH 7, with or without 0.85% NaCl, as diluent failed because the virus preparation precipitated with the diluent. However, an antiserum titre of 1/1024 was obtained in gel diffusion tests against a crude sap extract from C. quinoa leaves inoculated with ArMV-O. The antiserum did not react with healthy sap at antiserum dilutions greater than 1/8.

Discussion

The properties reported here confirm the presence of ArMV in carnations and Dianthus species in New Zealand thereby adding to the known natural hosts of the virus in this country. The variation in symptoms in indicator plants inoculated with different isolates is consistent with reports in the literature and again confirms the importance of serology for the provision of conclusive proof of the identity of ArMV (Murant, 1970).

FIGURE 2. Saponaria vaccaria infected with the severe strain of arabis mosaic virus. Local whitish ringspots (bottom leaf) and systemic vein chlorosis (top leaf) 7 days after inoculation.

FIGURE 3. Cucumber (Cucumis sativus) systemically infected with arabis mosaic virus (ArMV-O) 1 month after inoculation.

FIGURE 4. Local ringspots on tobacco 'White Burley' 9 days after inoculation with arabis mosaic virus.

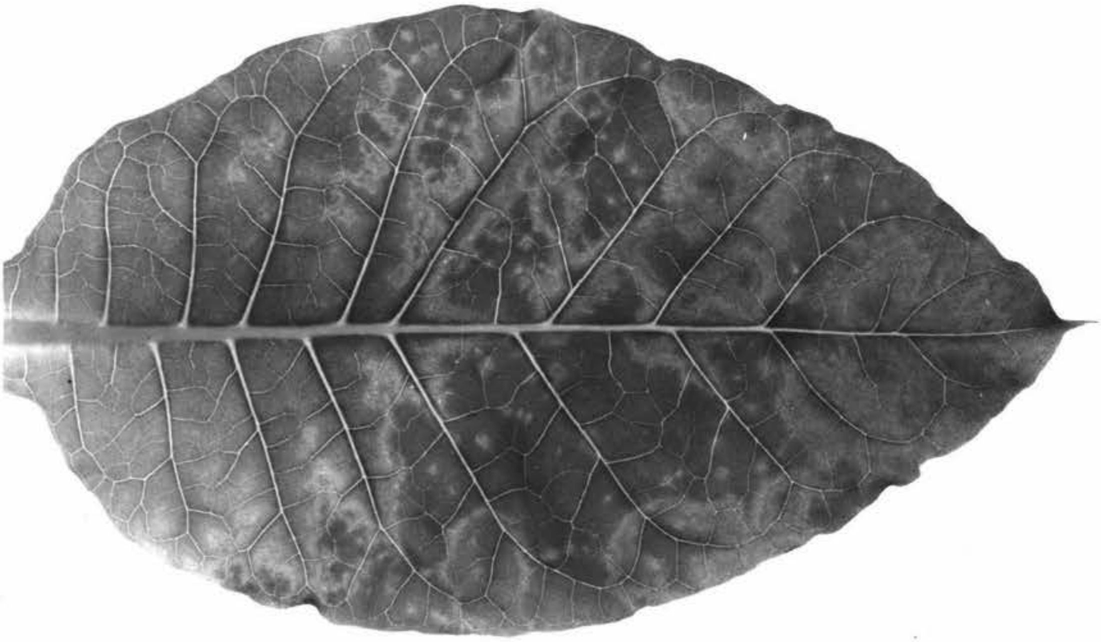


FIGURE 5. Advanced systemic symptoms in a leaf of tobacco 'Havana 423' 1 month after inoculation with arabis mosaic virus.

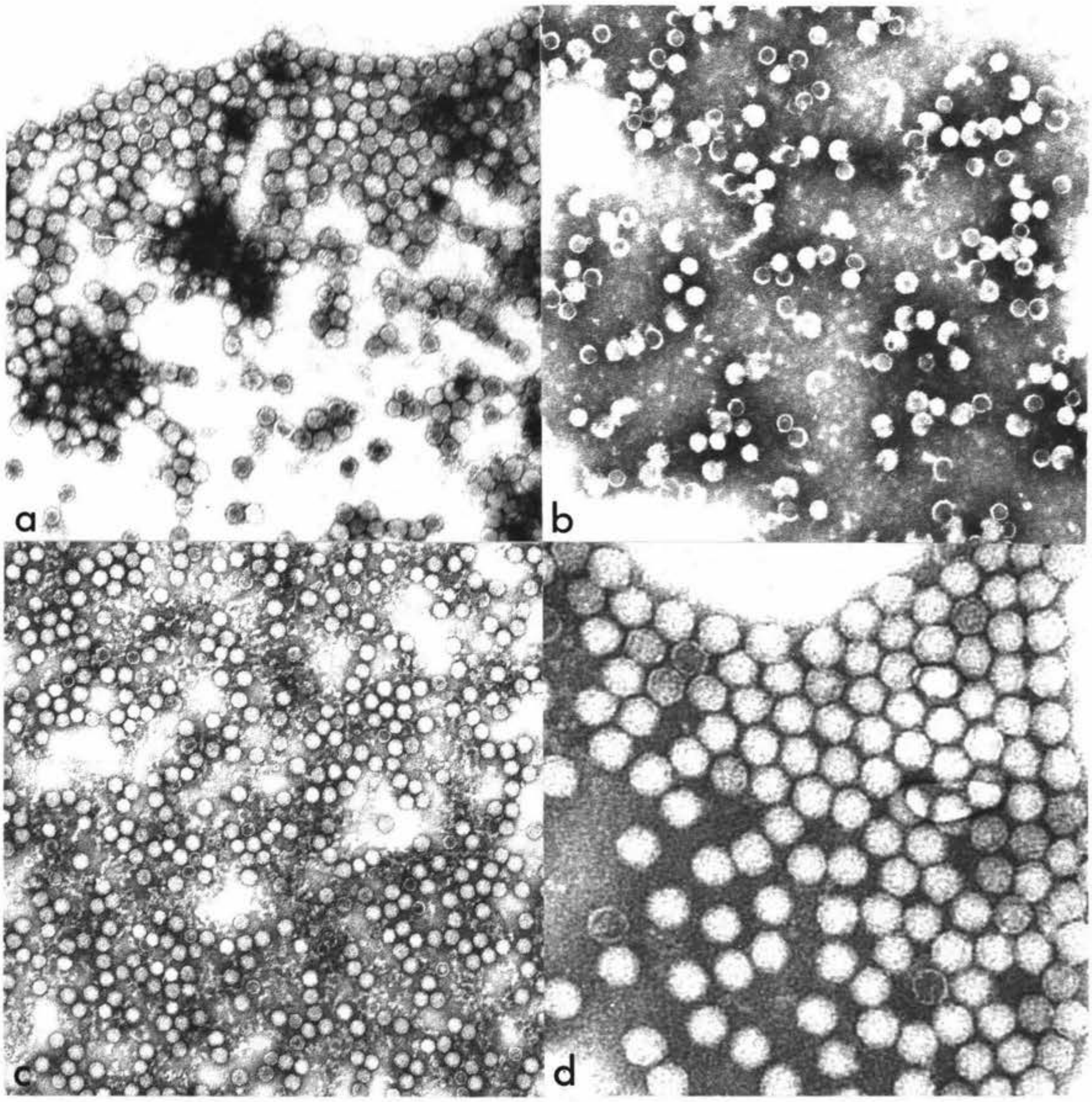


FIGURE 6. Partially purified preparation of arabis mosaic virus stained with (a) uranyl acetate, (b) potassium phosphotungstate, pH 7, and (c) ammonium molybdate, pH 5.4. Note the empty particles, particularly in (b) which also reveals particle breakage, presumably due to the stain. Mag. 85,000. (d) purified preparation in ammonium molybdate showing hexagonal particle conformation. Mag. 200,000.

2.2 CARNATION BACILLIFORM VIRUS (CBV)

/:*/*:U/*:S/*

An apparently new virus, hereby named carnation bacilliform virus, was identified in two cuttings (one a 'Border' carnation and the other an unknown species of *Dianthus*) on the basis of particles observed by electron microscopy in crude sap and ultrathin leaf sections. Particles were bacilliform which places the virus in the plant rhabdovirus group. Plants containing CBV did not show obvious symptoms and the virus was not transmitted by either sap inoculation or aphids (*Myzus persicae*).

Host range

No reaction was observed in any of the plants listed when inoculated with sap from CBV-infected tissue ground in 0.1M K/K_2PO_4 buffer, pH 7.5, containing 0.5% bentonite or Yarwood's solution:

Antirrhinum majus (snapdragon); *Celosia argentea* 'Forest Fire'; *Chenopodium quinoa*; *Dianthus barbatus* (sweet william); *Dianthus chinensis* 'Bravo'; *Digitalis purpurea*; *Gomphrena globosa*; *Gypsophila elegans*; *Momordica balsamina* L.; *Nicotiana sylvestris*; *Nicotiana tabacum* 'Havana 423' and 'White Burley' (tobacco); *Phlox drummondii*; *Saponaria vaccaria* 'Pink Beauty'; and *Spinacia oleracea* 'Royal Denmark' (spinach).

Crude sap from *D. barbatus*, *D. chinensis*, *G. elegans* and *S. vaccaria* was examined for CBV particles by electron microscopy but no particles were observed.

Vector transmission

Aphids, *M. persicae*, were starved for 4 hours, then given 5min, 30min or 24 hour acquisition feeds on carnation leaves containing CBV and subsequently allowed to feed for approximately 24 hours on *D. chinensis*. No symptoms were observed on this host, nor in *D. barbatus* and *G. elegans* after 'inoculation' with aphids given 30min acquisition feeds, and no bacilliform particles were evident in sap from these hosts examined by electron microscopy.

Electron microscopy

Bullet shaped particles were observed in crude sap stained with the ammonium molybdate-phosphotungstate mixture (Figure 7). These particles appeared to consist of an inner component surrounding an axial canal (ca. 12-14nm in diameter) often partially penetrated by stain, and an outer darkly staining capsule approximately 10nm thick (Figures 7a, b). The width of the inner component was about 50nm; the length varied as different sized fragments were present, but most were in the range 120-160nm. In one instance an apparently different type of particle (Figure 7c) was observed in the same sample as the bullet shaped types. These particles exhibited a regular cross banding of the inner component and were approximately 100-130 x 55nm in size. Particles similar to both these types have been found in negatively stained sap, for example Gomphrena virus (Kitajima & Costa, 1966a) and lettuce necrotic yellows virus (Francki & Randles, 1970), and also in partially purified preparations of lettuce necrotic yellows virus (Harrison & Crowley, 1965).

Light microscopy

No inclusion bodes were observed in the epidermal cells of leaves of CBV-infected plants when stained with phloxine-trypan blue.

Ultrastructure

Electron microscopy of ultrathin sections of carnation leaf tissue revealed the presence of aggregates of bacilliform particles (Figures 8-10) in the cytoplasm of phloem parenchyma and sieve cells and in the parenchyma sheath cells surrounding the vascular bundle, but not in the mesophyll or epidermal cells. Particles and aggregates were not numerous. No limiting membrane was evident around the virus aggregates and the particles were not associated with chloroplasts, mitochondria or nuclei. The length of most 'whole' particles (35 out of 50) was in the range 240-280nm with an apparent normal length of 260nm (Figure 11). A secondary peak was present around 220nm and there were also a few extra long (330 and 390nm) particles. The width of particles observed in ultrathin sections ranged between 50-63nm with an average of 55nm for 64 particles from both longitudinal and transverse sections.

Discussion

There are now more than twenty plant rhabdoviruses reported, many of which have particles in the size range 200-300 x 50-100nm. The group can be arbitrarily separated into four divisions :

(a) Aphid transmitted viruses. Includes broccoli necrotic yellows (Campbell & Lin, 1972), lettuce necrotic yellows (Francki & Randles, 1970), sowthistle yellow vein (Peters, 1971) and strawberry crinkle (Richardson et al, 1972) viruses.
(b) Leaf and plant hopper transmitted viruses. These are mainly bacilliform viruses infecting plants of the Gramineae, for example, American wheat striate mosaic (Sinha & Behki, 1972), maize mosaic (Herold, 1972), and rice transitory yellowing (Shikata, 1972) viruses, but potato yellow dwarf virus (Black, 1970) is an exception.
(c) Mealybug transmitted viruses. Cacao swollen shoot virus (Brunt, 1970) and its serotypes are the only examples. The particles of these viruses resemble the bacilliform particles of alfalfa mosaic virus more than other plant bacilliform viruses.
(d) Vector unknown. This division includes plant bacilliform viruses with no known vectors, for example, eggplant mottled dwarf virus (Martelli & Russo, 1973) and plantain virus (Hitchborn et al, 1966).

In ultrathin sections particles of these bacilliform viruses are generally present in association with the nucleus, particularly between the lamellae of the nuclear membrane and sometimes within the nucleus, usually as the inner component. Particles of some are also present in the cell cytoplasm frequently within membrane-bound vesicles which are possibly of endoplasmic reticulum or nuclear membrane origin. However, particles have previously been observed free in the cytoplasm as for instance strawberry crinkle virus (Richardson et al, 1972).

The fact that all CBV particles and aggregates observed were apparently free in the cytoplasm may be due to the relatively few particles present and the age of infection. A study of the multiplication processes, particularly in young tissues, is necessary for a better understanding of the development of CBV within carnations.

The aphids Hyperomyzus lactucae L. (Fry et al, 1973; present study), the vector of lettuce necrotic yellows virus (Francki & Randles 1970) and sowthistle yellow vein virus (Peters, 1971), Macrosiphum euphorbiae Thomas (Cottier, 1953; present study), an inefficient vector of sowthistle yellow vein virus (Behncken, 1973), Aphis idaei Van der Goot (Chamberlain, 1954), the vector of raspberry vein chlorosis virus (Stace-Smith & Lo, 1973), and Brevicoryne brassicae L. (Cottier, 1953), the vector of broccoli necrotic yellows virus (Campbell & Lin, 1972) have been reported in New Zealand. Consequently transmission tests with these vectors may elucidate the possible relationship of CBV to the viruses concerned. In this context it is of interest to note that M. euphorbiae has been found infesting carnation plants in this country (Cottier, 1953; present study). However, lettuce necrotic yellows virus is the only bacilliform virus positively identified in New Zealand (Fry et al, 1973).

The present study is the first report of a plant rhabdovirus infecting Dianthus species and the first in the family Caryophyllaceae. It is therefore likely that CBV is in fact a hitherto unknown virus.

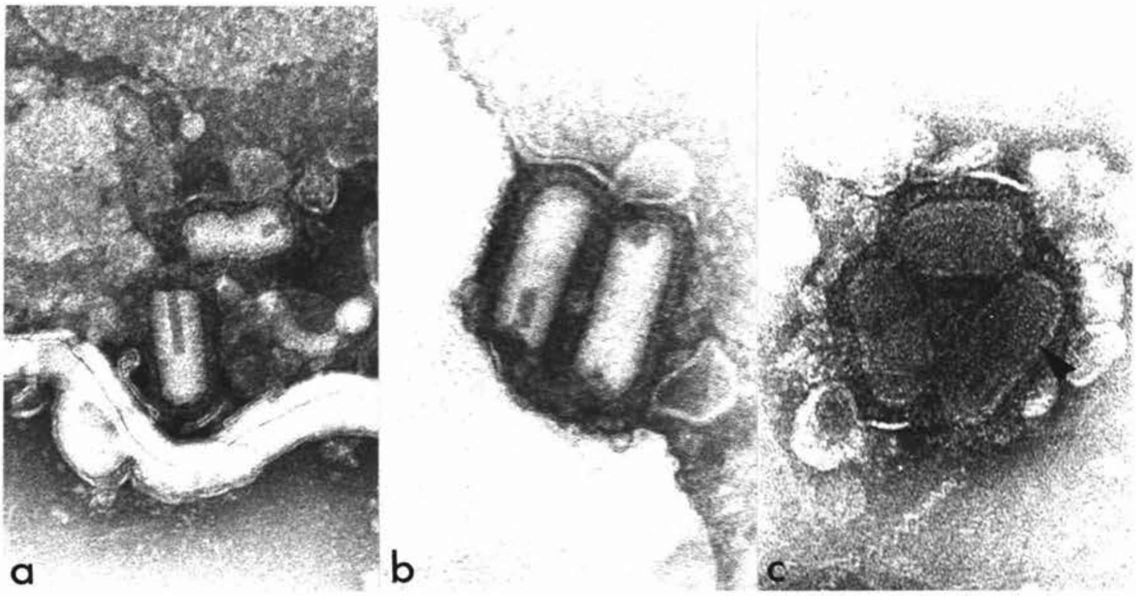


FIGURE 7. Electron micrographs of carnation bacilliform virus in *Dianthus* sp. sap. In (a) and (b) note the axial canal partially penetrated by stain and the surrounding capsid. (c) Particles of CBV showing regular cross-banding of the inner component (arrow).
Mag. (a) 127,000, (b) 166,000, (c) 178,000.
Stain: AM-PTA mixture.

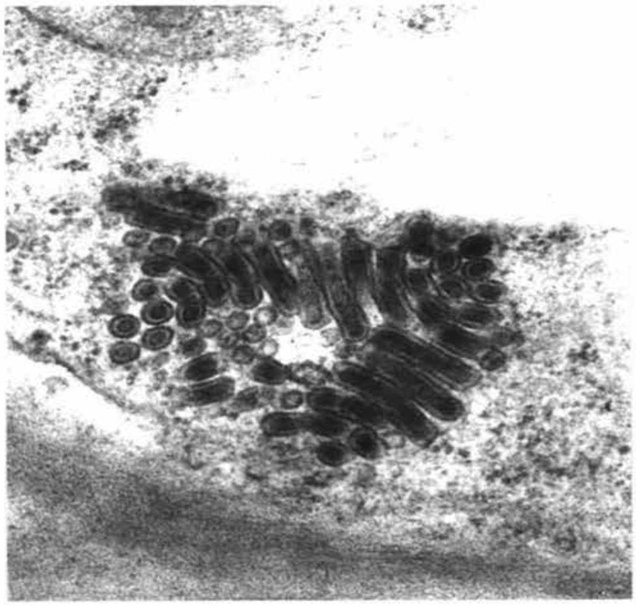


FIGURE 8. Mesophyll cell containing an aggregate of carnation bacilliform virus particles in the cytoplasm of the *Dianthus* sp. Mag. 69,000.

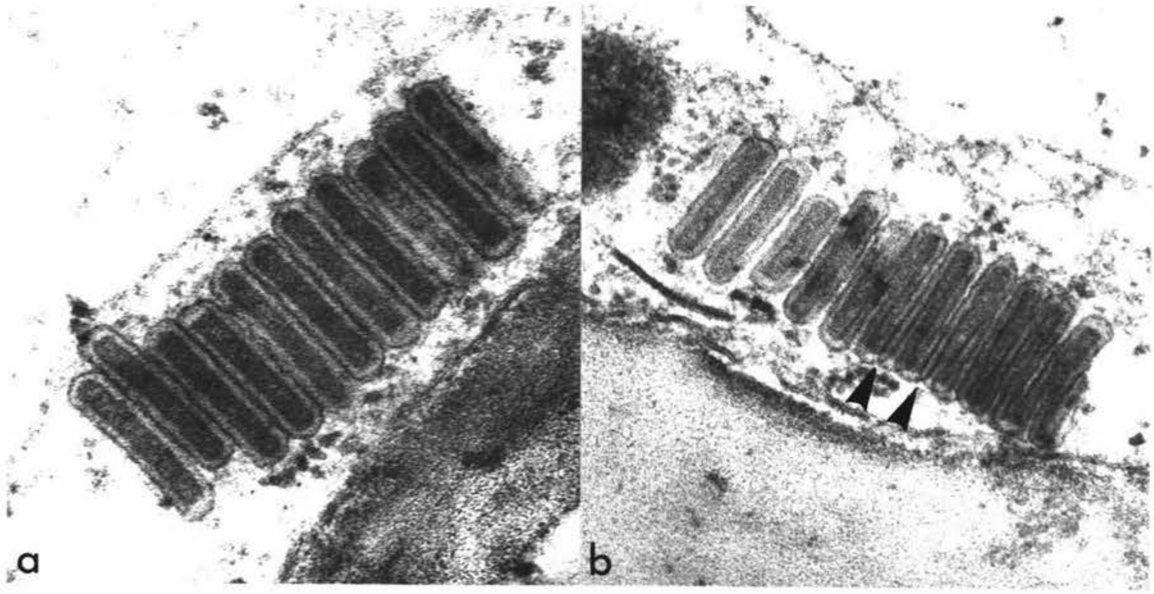


FIGURE 9. Longitudinal section of particles of carnation bacilliform virus in cytoplasm of carnation. In (b) the arrows indicate overlapping of the capsules made obvious by an oblique cut through two layers of particles. Mag. (a) 96,000, (b) 81,000.

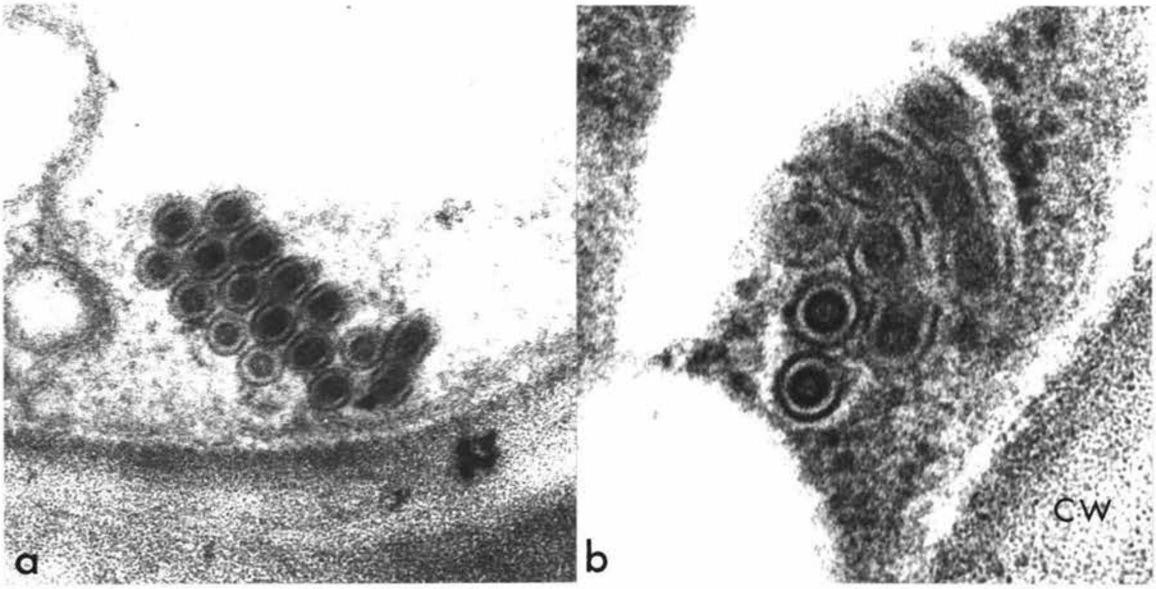


FIGURE 10. Transverse and oblique sections through groups of particles in carnation showing internal organisation of carnation bacilliform virus, particularly the hollow axial canal. Mag. (a) 104,500, (b) 144,500.
CW = cell wall.

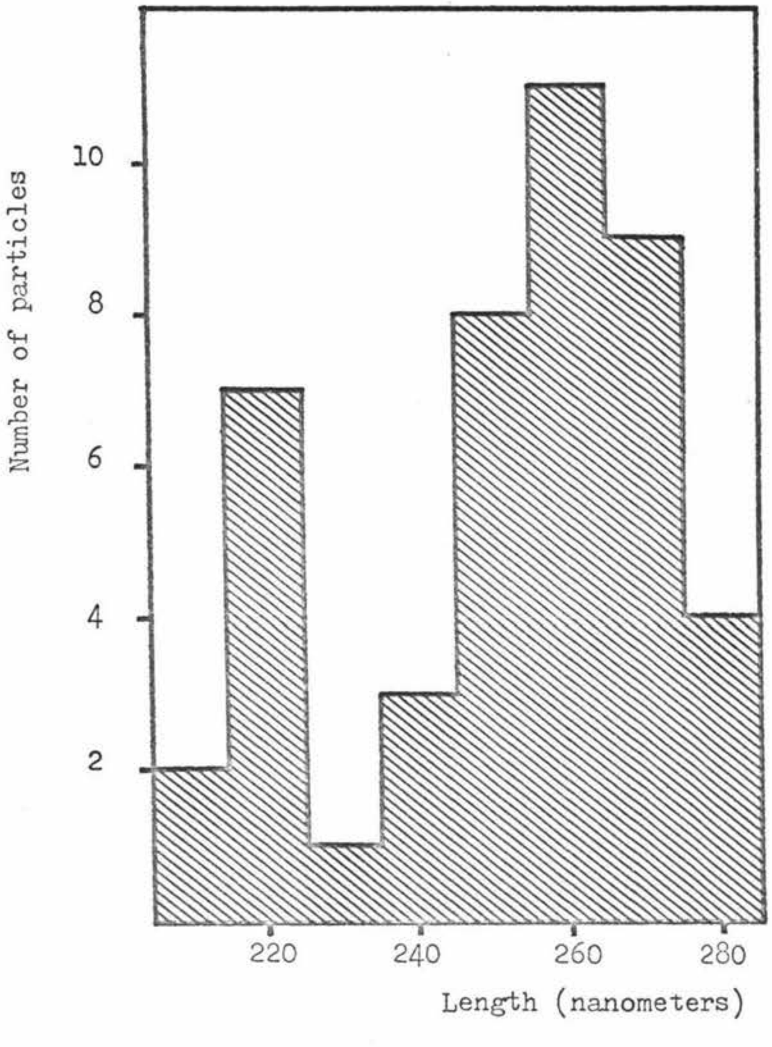


FIGURE 11. Particle length distribution of carnation bacilliform virus in ultrathin sections of carnation leaf. Apparent normal length is 260nm.

2.3 CARNATION ETCHED RING VIRUS (CERV-50)

D/*:*/*:S/S:S/Ap

Carnation etched ring disease was first described by Hollings & Stone (1961). Subsequently an aphid borne polyhedral particle approximately 50nm in diameter was isolated (Hollings & Stone, 1967) and it was suggested that it was associated with two other isometric particles (25nm and 29nm in diameter) in plants exhibiting etched ring symptoms (Hollings, Stone & Bouttell, 1968). The large polyhedral particle, however, is now considered to be the carnation etched ring virus (CERV-50).

Carnation etched ring virus has particles with a diameter in the range of 40-50nm (Fujisawa et al, 1971; Hollings & Stone, 1967), contains DNA as the genetic material (Fujisawa et al, 1971, 1972) and is serologically related to both cauliflower mosaic (ClMV) and dahlia mosaic (DMV) viruses (Hollings & Stone, 1969a) which places CERV-50 in the cauliflower mosaic virus (caulimovirus) group along with ClMV and DMV. Potential members of this group are Mirabilis mosaic (Brunt & Kitajima, 1973), petunia vein-clearing (Lesemann & Casper, 1973), strawberry vein-banding (Kitajima et al, 1973) and cassava vein mosaic (Kitajima & Costa, 1966b) viruses. All the viruses in this group, except the petunia and cassava viruses which have not been tested, are transmitted by aphids (chiefly M. persicae) and they also form similar characteristic types of inclusions in host plants as viewed in ultrathin sections. Generally these inclusions can be observed by light microscopy in epidermal strips stained with phloxine or trypan blue, appearing as elliptical to spherical bodies in the cytoplasm (Brunt, 1971; Fujisawa et al, 1967, 1971).

In the present study CERV-50 was characterized by electron microscopy of both sap and ultrathin sections, light microscopy, aphid transmission and host range.

Host range

Attempts were made to transmit CERV-50 by sap inoculation from carnation, G. elegans and S. vaccaria leaf tissue ground in 0.1M K/K_2PO_4, pH 7.5 buffer with or without 0.5% bentonite. The presence of the virus in hosts was tested by either electron microscopy of sap or light microscopy.

CARYOPHYLLACEAE

Dianthus barbatus (sweet william): only symptomless infection was detected.

Dianthus caryophyllus 'Joker': transmission of CERV-50 to 'Joker' was achieved by graft inserts of carnation or G. elegans leaf tissue. In the few tests made the reaction to CERV-50 varied from symptomless infection to chlorotic spots on young leaves after about two and a half months. These chlorotic spots elongated into chlorotic streaks. When CarMV was also present symptoms were expressed earlier (half to one month) and the chlorotic spots and streaks turned necrotic. Elongated necrotic etched rings were sometimes present on the stems particularly near the leaf nodes. Similar leaf symptoms were observed on naturally infected 'Orange Triumph' from which only CERV-50 and CarMV could be isolated (Figure 12). Naturally infected 'Joker' and 'Orchid Beauty' containing CERV-50 plus CarMV exhibited severe necrosis, particularly towards the tips of older leaves (Figure 13) as well as the milder symptoms. The symptoms of CERV-50 alone were distinct from those induced by other known viruses infecting carnations.

Dianthus chinensis 'Bravo': infection was often symptomless but occasionally reddish blotches appeared on inoculated leaves, particularly when CarMV or CLV was also present in the inoculum. Fujisawa et al (1971) reported local necrotic blotches on D. chinensis plants inoculated with CERV-50 plus CarMV and etched rings on some of the young leaves.

Gypsophila elegans: symptomless systemic infection after about one month.

Saponaria vaccaria 'Pink Beauty': in contrast to the symptoms reported by Hakkaart (1974), plants were usually symptomless, although mild mottling was observed occasionally. This discrepancy may be due to the conditions under which the plants were grown.

The following plants did not appear to be infected as far as could be determined by the methods employed: Amaranthus caudatus, Brassica pekinensis 'Chi-Hi-Li', Celosia argentea 'Forest Fire', Chenopodium amaranticolor, Chenopodium quinoa, Gomphrena globosa, Mirabilis jalapa, Nicotiana clevelandii and Spinacia oleracea 'Royal Denmark'.

Vector transmission

Aphids, M. persicae, were starved for about 8 hours and allowed 5min acquisition feeds on CERV-50 infected G. elegans. Fifteen aphids were then transferred to two D. chinensis plants and one plant each of G. elegans and S. vaccaria and allowed to feed for approximately 8 hours. Both D. chinensis plants and G. elegans were subsequently found to contain CERV-50 particles by electron microscopy. Carnation etched ring virus was also transmitted from carnation to G. elegans in a similar experiment. These results indicate that CERV-50 is readily transmitted by M. persicae.

Purification

Carnation etched ring virus was purified together with CarMV from naturally infected carnation tissue by the method of Fujisawa et al (1971). The leaf tissue (300g) was homogenized in cold 0.5M K/K_2PO_4 buffer, pH 7.5, (1.5ml/g) containing 0.01M Na_2SO_3 and squeezed through muslin. Butanol (8.5%) was added slowly and the extract stirred for about 1 hour after which it was clarified by low speed centrifugation (10,000g/15min). The viruses were then subjected to 2 cycles of differential centrifugation (80,000g/2 hours; 10,000g/15min) and the final pellet resuspended in either 0.01M K/K_2PO_4 buffer, pH 7.5, or 0.01M NH_4 acetate at the rate of 1ml/g of original tissue and briefly centrifuged to remove debris. Both CERV-50 and CarMV particles were observed by electron microscopy in these preparations (Figure 15).

The two viruses were also purified by polyethylene glycol (PEG), MW 20,000, precipitation. An extract was obtained and clarified as before and the virus precipitated with 6% PEG for 2 hours and centrifuged (10,000g/20min). The precipitate was redissolved in 0.001M EDTA and given a cycle of differential centrifugation. The resultant pellet was resuspended in EDTA (1ml/1g tissue). Electron microscopy revealed no obvious difference from the previous method.

When butanol/chloroform clarification (as for ArMV, section 2.1) was used only CarMV was observed in the purified preparation. Purification of CERV-50 alone was not carried out due to lack of sufficient tissue.

Electron microscopy

Particles of CERV-50 were observed in crude carnation sap stained with AM-PTA, or PTA pH 4 or 7 (Figure 14). The ease with which particles could be found depended, apparently, on the concentration of virus in the host; sometimes particles occurred singly and were difficult to find, while at other times groups of particles were present (Figure 14). These particle clusters may be the result of the disintegration of an inclusion. Both G. elegans and S. vaccaria appeared to support a higher concentration of virus than carnation as generally more particles were present in sap from the former plants once the virus had become fully systemic. Purified preparations, also containing CarMV, were suitably spread and stained with 2% AM. An interesting feature of CERV-50 particles in both sap and purified preparations was that they were both positively and negatively stained whereas CarMV particles, when present, were mainly negatively stained (Figures 14a and 15a). The positive staining did not appear to be the result of penetration of stain into 'empty' particles as occurred with ArMV and also CarMV.

A few CERV-50 particles exhibited a hexagonal configuration but frequently they appeared oval in outline which suggested that they were distorted by unequal forces during the drying of grids. Where such particles were used for measurements an average diameter was taken. The diameter of particles measured in sap ranged from 43-56nm with a mean of 48nm for 104 particles. Particle diameters in purified preparations were similar to those in crude sap.

Light microscopy

Cytoplasmic inclusion bodies (X-bodies) of CERV-50 in unfixed epidermal strips have been reported to stain with phloxine (Fujisawa et al, 1971) and both phloxine and trypan blue have been used to stain X-bodies of DMV (Brunt, 1971). Consequently both these stains were tested on epidermal strips from carnation. The fluorescent stain acridine orange was also used in an attempt to determine the nature of the inclusions.

Materials and methods: Unfixed epidermal strips from both healthy and CERV-50 infected carnation leaves were treated for 1-10min periods with various stain solutions: 0.5% phloxine in distilled water, 0.85% NaCl solution or in a methyl cellosolve, ethyl alcohol and distilled water (2:1:1, respectively) mixture (Christie, 1967); 0.5% trypan blue dissolved in near boiling distilled water or 0.85% NaCl solution (McWhorter, 1957) or in the solvent mixture; 0.5% rose bengal; and phloxine/trypan blue mixtures in saline. The epidermal strips were subsequently rinsed and mounted in saline or water according to the stain solution and examined under a light microscope at a magnification of 200x or 400x. A few epidermal strips were fixed in 50% alcohol for approximately 1 hour before staining with phloxine/trypan blue (1:15).

Acridine orange staining was accomplished using the schedule of Hooker & Summanwar (1964). Shorter (5min) rinses, staining and differentiation times were tested. The stain was excited with a high pressure mercury lamp (200w HBO) with a blue filter (Schott BG 12, 5mm thick) transmitting in the range 350-400nm and the epidermal strips were viewed through a yellow barrier filter (K 530).

Results: Carnation etched ring inclusion bodies in unfixed tissue stained intensely with phloxine, rose bengal and trypan blue, the time required depending on the age of the tissue. Periods up to 5min were usually the maximum necessary and longer times often caused plasmolysis of the cells, particularly in very young tissue. The nuclei were also stained but less intensively than the X-bodies, although the nucleoli stained darker than the nuclei. Trypan blue caused the nuclei to swell in unfixed tissue (McWhorter, 1941). Stains dissolved in NaCl appeared to give slightly better results than those in water; the cellosolve-alcohol-water solvent did not give as good results and colours were less intense.

Staining strips sequentially with phloxine then trypan blue was tested (McWhorter, 1941) but a mixture of phloxine and trypan blue (1:15) made prior to use proved as satisfactory, was quicker and simpler to handle, and consequently was adopted for routine use. Best results were obtained with staining times of 1-5min, again depending on the tissue. Where the stains balanced, nuclei were bluish-purple and the inclusion bodies reddish-purple (Figure 16) while the chloroplasts remained unstained. Fixation prior to staining resulted in more intense colouration with less differential between nuceli and X-bodies. There was no advantage in fixation for surveying samples for CERV-50.

Both phase contrast and differential interference microscopy were used to distinguish the X-bodies but the stains gave clearer results and there was no difficulty differentiating the inclusions from plastids or lipid droplets.

Inclusion bodies were also observed in epidermal strips from leaves of G. elegans and S. vaccaria infected with CERV-50. Solubilization of chloroplasts with 5% triton X-100 (Christie, 1971) often aided observation but was unnecessary with carnation tissue. No inclusion bodies were observed in healthy tissues from any of the plants tested.

The X-bodies were ovoid or spherical in outline and highly refractile. They ranged between 1-12µm in diameter and the larger inclusions sometimes appeared to be vacuolate which may correspond with the lacunae observed in ultrathin sections. Generally only one X-body per cell was present but up to about six, usually smaller ones, were occasionally observed in young leaf tissue. Where several smaller inclusions occurred they often appeared to be coalescing. Inclusion bodies were quite often near or opposed to the nuclei, a point that has been reported for ClMV (Martelli & Costellano, 1971).

When stained with acridine orange and excited by far blue light CERV-50 X-bodies fluoresced flame red while nuclei were green to yellow-green, nucleoli flame red and cell walls usually a pale orange-red. The differentiation phase with $CaCl_2$ was not necessary but it did result in appreciably clearer colouration. With unfixed tissue the colours were less intense and inconsistent.

The red fluorescence of the X-bodies shows that single stranded nucleic acid, probably RNA, is associated with them, although the possibility of single stranded DNA cannot be discounted (Schummelfeder, 1958).

Inclusion bodies were consistently correlated with infection of plants by CERV-50, both in mixed virus infections and alone, and this was confirmed by electron microscopy.

Ultrastructure

Inclusions and particles typical for CERV-50 (Fujisawa et al, 1971; Lawson & Hearon, 1974; Rubio-Huertos et al, 1972) and other members of the caulimovirus group were observed in ultrathin sections of leaf tissue from carnation 'Orchid Beauty' naturally infected with

CERV-50 and CarMV (Figures 17 and 18) and in carnation infected with CERV-50 and CLV. Cytoplasmic inclusions were present in mesophyll parenchyma cells and consisted of an electron-opaque matrix in which particles appeared to be embedded. Most particles, however, occurred either at the outer edge of the inclusions or within electron-translucent areas, the lacunae, within the inclusions (Figure 18). The particles appeared as a dark outer shell with an apparently empty core.

No limiting membrane was evident around the inclusions but elements of the endoplasmic reticulum and ribosomes were occasionally associated with them. Size of inclusions ranged from about 0.5 to 3.0µm but few inclusions were observed in the epidermis in the sections under study so that a comparison with the size of X-bodies observed by light microscopy could not be made. Particles of CERV-50 were occasionally present in the cytoplasm (Figure 19) but were not observed in nuclei. Lawson & Hearon (1974) did not find CERV-50 particles in the nuclei of two carnation varieties although particles have been reported in nuclei of D. barbatus (Rubio-Huertos et al, 1972) and S. vaccaria (Lawson & Hearon, 1974).

Discussion

Martelli & Costellano (1971) used cytochemical staining combined with protease digestion of epidermal strips to show that inclusion bodies of ClMV contained both protein and RNA, and possibly DNA. Similar results had been reported for DMV (Kitajima et al, 1969; Robb, 1964). The results of staining CERV-50 X-bodies with acridine orange are in accord with the results for these two viruses, but further cytochemical tests, particularly combined with enzyme digestions, would provide better confirmation of the presence of RNA and could also be used to test for protein.

The use of inclusion bodies as a means of detecting plant viruses is not always reliable but it has been reported to be satisfactory for the detection of DMV in dahlias (Robb, 1963). In the present study staining with phloxine/trypan blue proved to be a very useful and reliable method for detecting CERV-50. Light microscopy of X-bodies in epidermal strips was as sensitive as electron microscopy for detecting CERV-50 particles in sap.

The information reported here demonstrates that carnation etched ring virus is present in Dianthus species, including carnations, in New Zealand and the prevalence (section 1.5.7) and severity of foliar symptoms suggest that it may play a significant role in flower production.

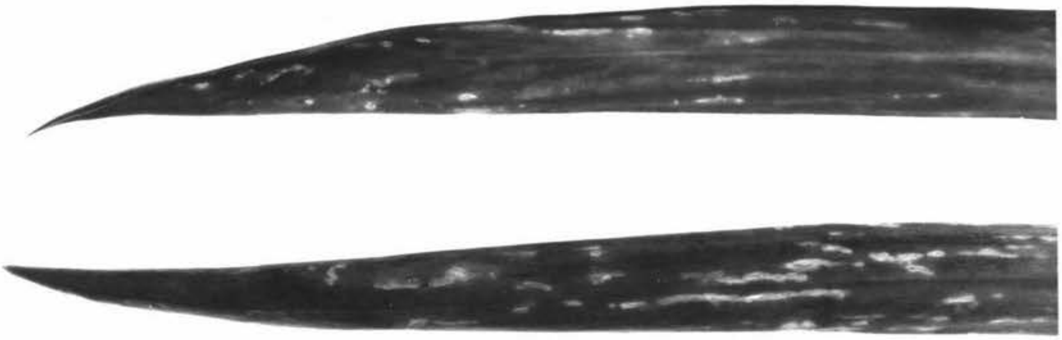


FIGURE 12. Young leaves of carnation 'Orange Triumph' naturally infected with carnation etched ring and carnation mottle viruses.

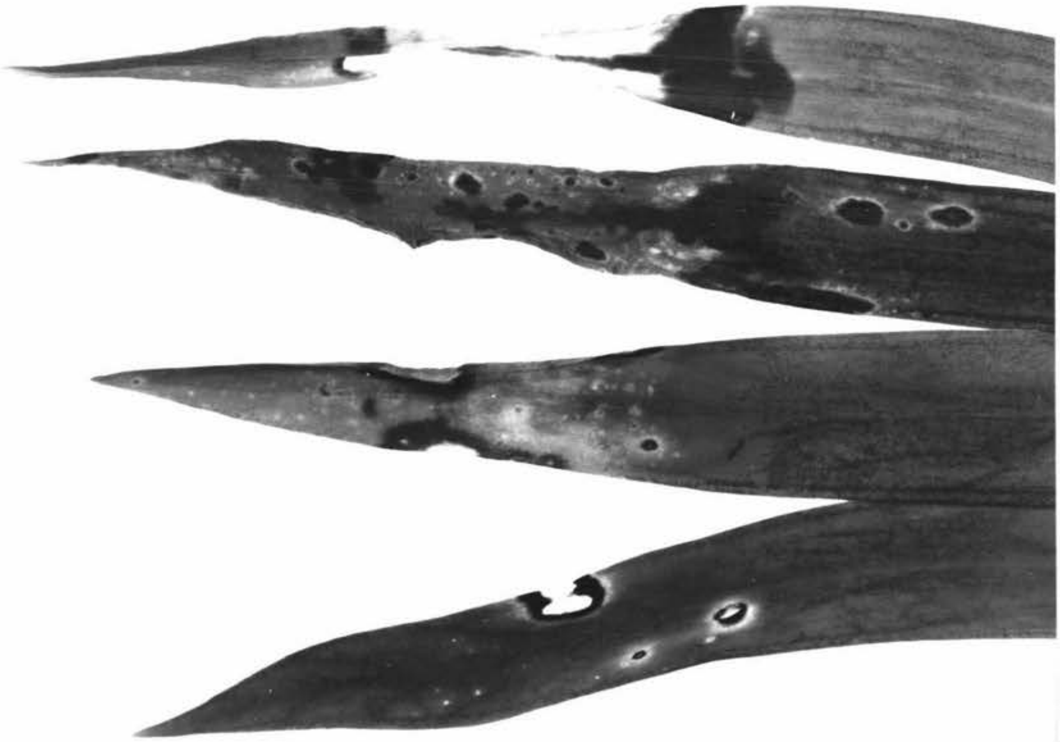


FIGURE 13. Leaves of carnation 'Orchid Beauty' naturally infected with carnation etched ring virus and carnation mottle virus.

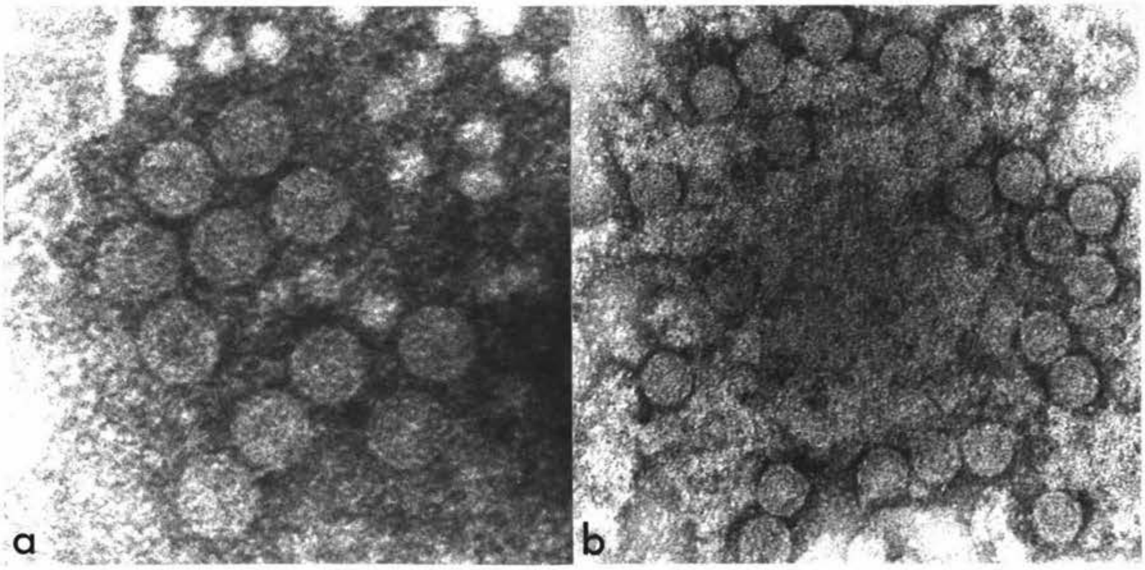


FIGURE 14. Particles of carnation etched ring virus in carnation sap. (a) Also contains carnation mottle virus. Stain: PTA, pH 4; Mag. 210,000. (b) Stained with AM-PTA mixture; Mag. 130,000.

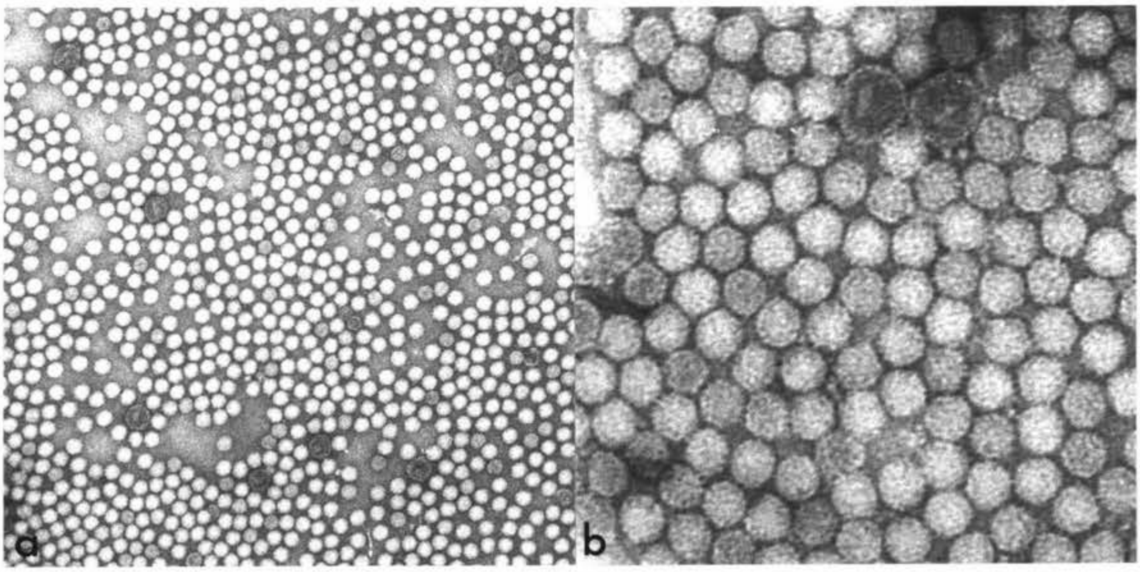


FIGURE 15. Purified preparations from carnation naturally infected with carnation etched ring and carnation mottle viruses. The large particles are etched ring virus. Stain: 2% AM. (a) Mag. 70,000, (b) Mag. 210,000.

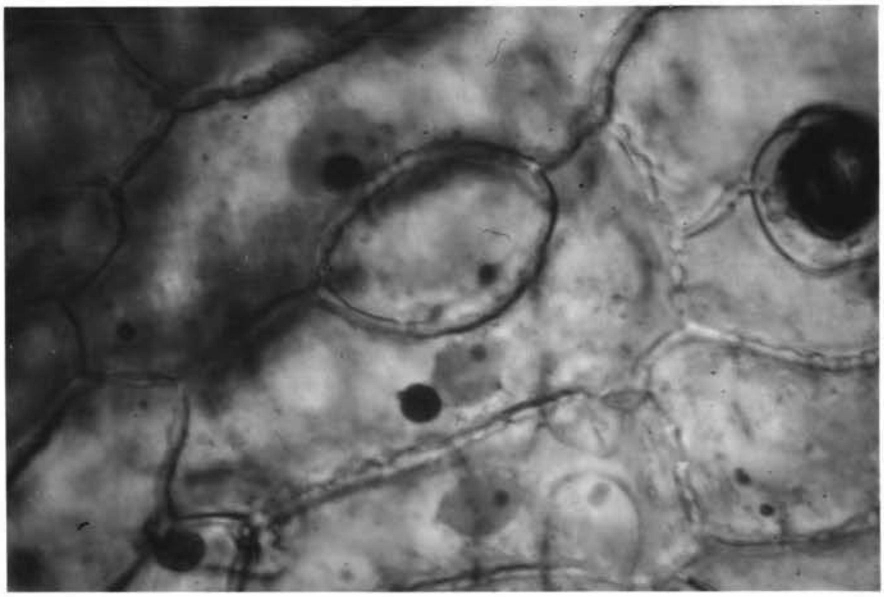


FIGURE 16. Inclusion bodies associated with carnation etched ring virus infection in carnation 'Joker'. Mag. 580.

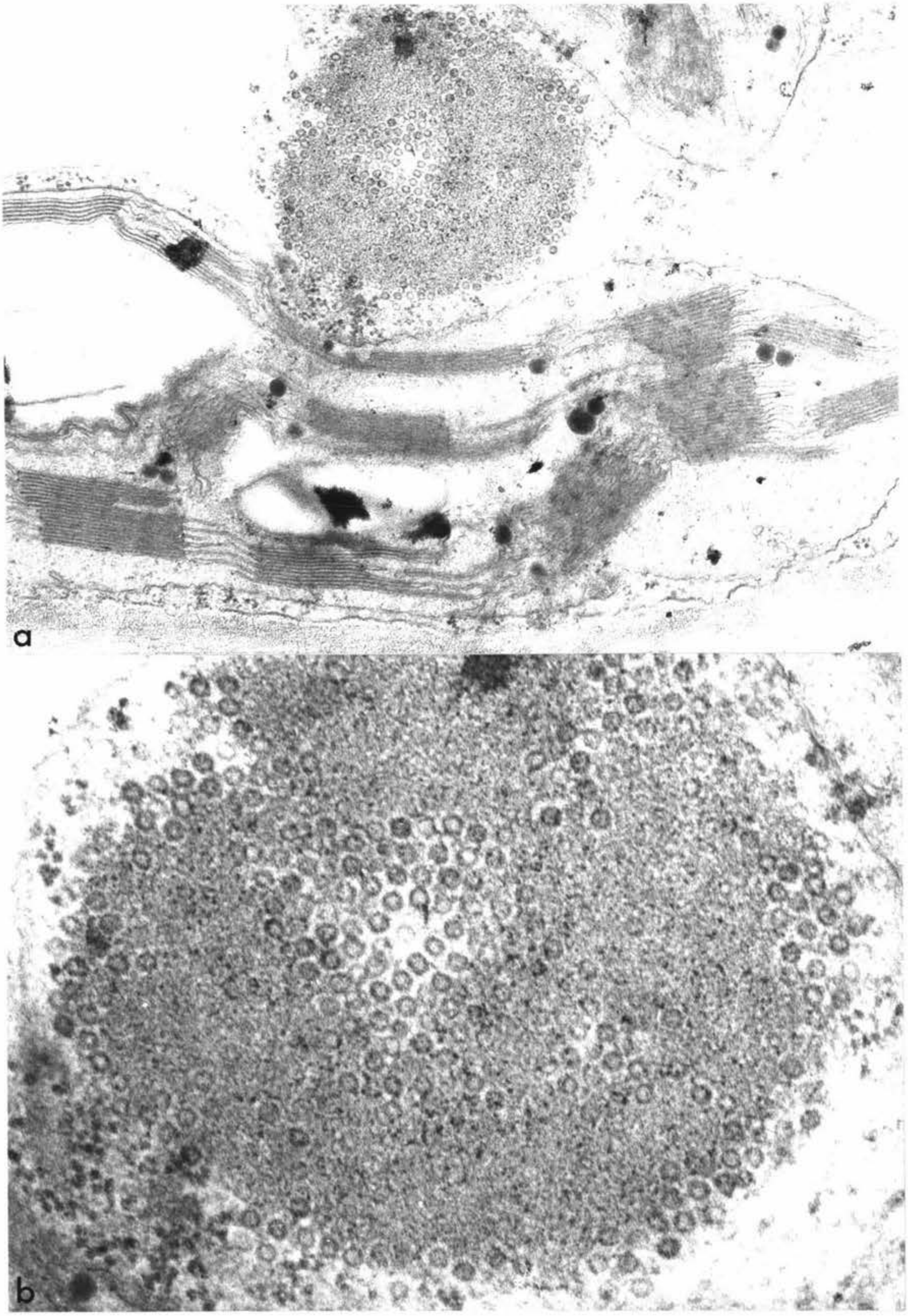


FIGURE 17. An inclusion of carnation etched ring virus in a mesophyll cell of carnation 'Orchid Beauty'.
(a) Mag. 38,000 (b) Mag. 93,000.

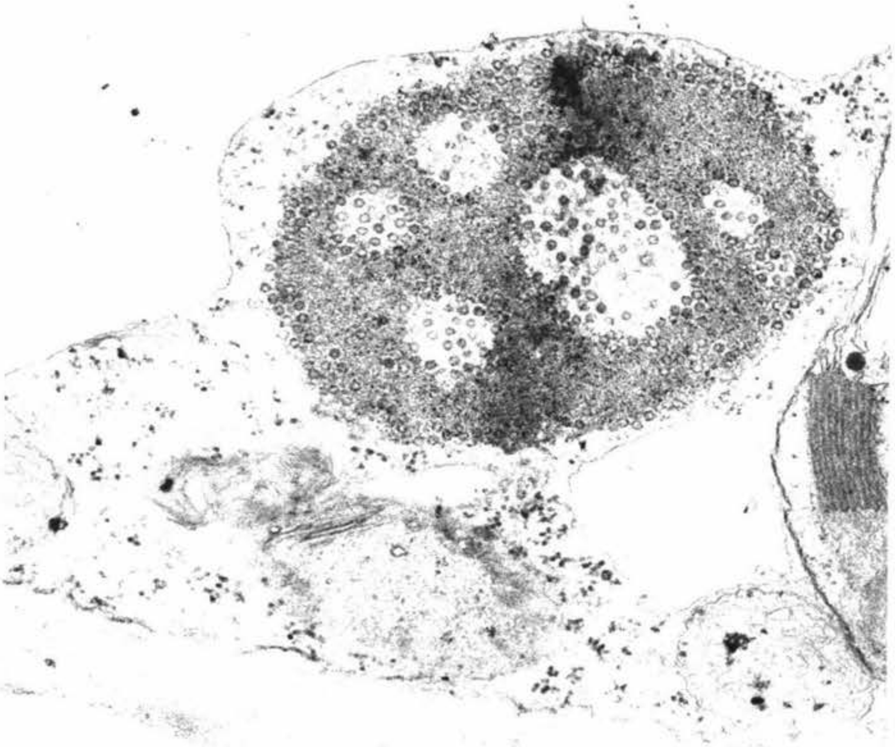


FIGURE 18. An inclusion of carnation etched ring virus in carnation 'Orchid Beauty' showing the 'lacunae'. Mag. 38,000.

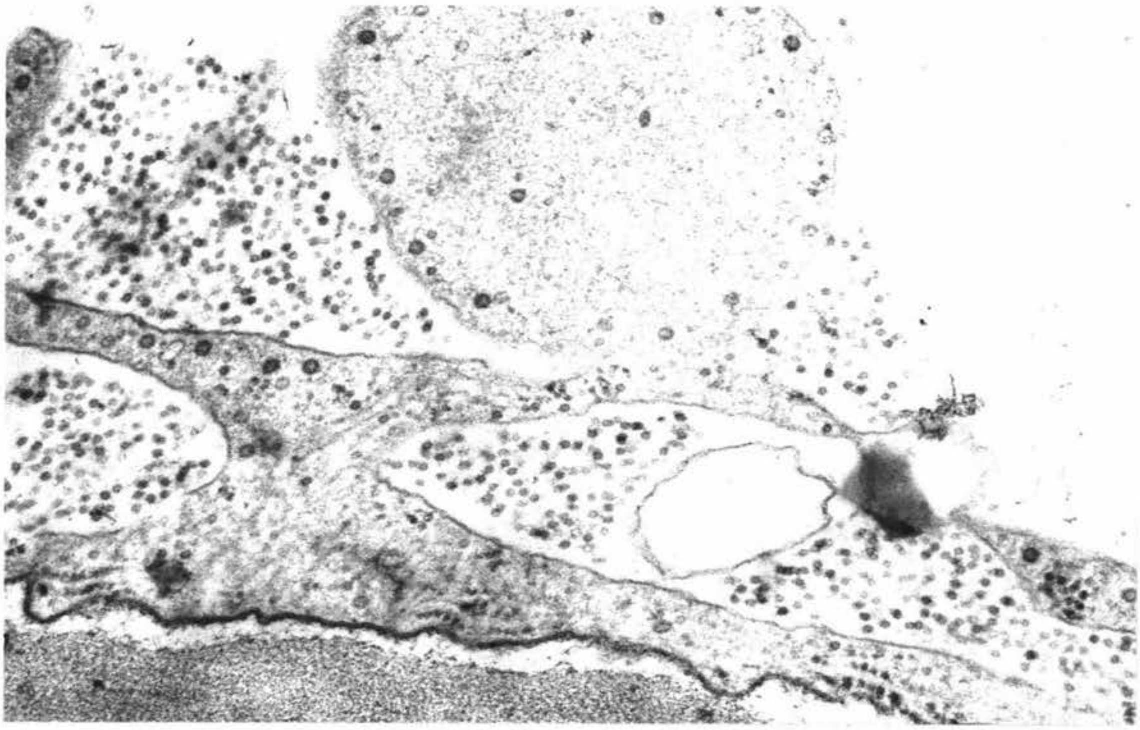


FIGURE 19. Carnation etched ring virus particles in the cytoplasm and carnation mottle virus particles in the vacuole of carnation 'Orchid Beauty'. Mag. 83,000.

2.4 CARNATION LATENT VIRUS (CLV)

R/1:*/6:E/E:S/Ap

Carnation latent virus, first described by Kassanis in 1955, is a member of the carlavirus (potato virus S) group and is serologically related to many of these viruses. Particles are straight to slightly flexuous rods about 650 x 12nm and are readily transmissible by sap inoculation as well as by aphids (*M. persicae*) in a non-persistent manner (Kassanis, 1955; Wetter, 1971). Symptoms on carnations are very slight or non-existent. Wetter (1971) reported the following physical properties of the virus in carnation sap:

TIP : 60-65C
DEP : 10^{-3}-10^{-4}
LIV : 2-3 days/20C

In the present study, characterization of CLV included the study of host range, purification, aphid transmission, physical properties and ultrastructure features.

Host range

Leaf tissue from infected *C. quinoa*, *N. clevelandii* or *S. vaccaria* was used as the virus source for sap inoculation.

CARYOPHYLLACEAE

Dianthus barbatus, *Dianthus caryophyllus* 'Joker', and *Dianthus chinensis* 'Bravo': very faint or symptomless systemic infection.

Gypsophila elegans: slight vein clearing or symptomless systemic infection.

Saponaria vaccaria 'Pink Beauty': slight vein clearing or symptomless systemic infection.

CHENOPODIACEAE

Chenopodium amaranticolor: symptomless local infection or occasionally very diffuse pale green lesions about 2-3mm in diameter after 10 days, followed by mild systemic vein clearing and subsequent interveinal mottling.

Chenopodium quinoa: symptomless local infection but sometimes pale green lesions about 3mm in diameter after 7 to 14 days depending on the isolate and/or the concentration of virus; mild systemic vein clearing followed by subsequent interveinal mottling and leaf stunting.

Spinacea oleracea 'Royal Denmark' (spinach): symptomless local infection.

SOLANACEAE

Nicotiana clevelandii: symptomless systemic infection.

The following plants were not infected:

Amaranthus caudatus; Antirrhinum majus; Brassica pekinensis 'Chi-Hi-Li'; Celosia argentea 'Forest Fire'; Cucumis sativus 'Marketer', 'Polaris' (cucumber); Gomphrena globosa; Lactuca sativa L. 'Webbs Wonderful' (lettuce); Matthiola incana; Nicotiana glutinosa; Nicotiana tabacum 'Havana 423', 'Samsun', 'White Burley' (tobacco); Petunia hybrida; Phaseolus vulgaris 'Scotia' (dwarf bean); Pisum sativum L. 'Greenfeast' (pea); Phlox drummondii; Vigna cylindrica (L.) Skeels; Zinnia elegans.

Vector transmission

Myzus persicae transmitted CLV, using short (ca. 5min) acquisition feeds, from carnations to G. elegans in 2 out of 3 attempts but not to D. chinensis, S. vaccaria or D. barbatus in 3, 2 and 1 attempts respectively. However, these experiments were carried out with tissue known to contain, on the basis of electron microscopy, relatively low concentrations of the virus.

Purification

Three isolates of CLV were purified, from systemically infected C. quinoa leaves one month after inoculation, either by a butanol-centrifugation method similar to the method of Hollings & Stone (1964; also Hollings, Stone & Norrish, 1962) or by a procedure for ArMV adapted from Harrison & Nixon (1960).

(a) Butanol-centrifugation method: tissue was homogenized in 0.5M K/K_2PO_4 buffer, pH 7.5 (1g : 1.5ml), containing 1% 2-mercaptoethanol and expressed through muslin. Butanol (8.5%) was added slowly to the solution while stirring, the mixture stirred for about an hour and then clarified by low speed centrifugation (12,000g/20min). The supernatant was subjected to 2 cycles of differential centrifugation (10,000g/15min and 80,000g/2 hours) and the final pellet resuspended in 0.001M EDTA (1ml/100g tissue).

(b) Modified ArMV procedure: tissue was homogenized in either 0.1M ascorbic acid plus 0.2M Na_2HPO_4 (1:1) or 0.5M K/K_2PO_4, pH 7.5 with 1% 2-mercaptoethanol (as antioxidant) (50-60g/30ml) and a 1:1 mixture of cold butanol plus chloroform added while blending (80ml per 30ml of extraction buffer); homogenate was expressed through muslin and clarified by low speed centrifugation (3,000g/20min). The supernatant was left overnight at about 4C, given a low speed centrifugation (10,000g/15min) and two cycles of differential centrifugation (10,000g/15min and 80,000g/2 hours) and the pellet resuspended in 0.001M EDTA or distilled water (1ml/100g of tissue).

Electron microscopy revealed that the butanol-centrifugation method caused less breakage of the virus rods, although considerable damage was still evident (Figure 21c).

Electron microscopy

Carnation latent virus was readily observed by electron microscopy in crushed homogenates of carnation leaf tissue (Figure 21b) as well as in *D. barbatus*, *D. chinensis* and *N. clevelandii* (Figure 21a), and could be identified by its size and conformation as the particles are less flexuous than those of CVMV (Figure 41).

The concentration of CLV in carnation apparently varied with the season; electron microscopy revealed fewer particles in carnation samples in winter than in spring and early summer. This was also evident in a few CLV-infected carnations grown in the glasshouse.

The normal length of CLV was determined from particles in crude sap of carnation and indicator plants. There was no significant difference in normal length in different hosts or between different isolates; the normal lengths of five isolates of CLV were: 657, 650, 655, 651 and 651nm for 181, 118, 139, 47 and 221 particles respectively. The normal length calculated from all measurements was 656nm for 794 particles with 579 particles in the range 620-700nm (Figure 20). Width of the particles was about 12nm. There were no secondary peaks of significance, even at the double and half normal length positions. Despite the breakage in partially purified preparations a peak was present at approximately the normal length of CLV.

Physical properties

Due to the lack of a sufficiently sensitive and reliable local lesion host it was difficult to assess the TIP and DEP for CLV. Values obtained (TIP : 50-55C; DEP : 10^{-2}-10^{-3}) were in approximate agreement with those cited in the literature (Wetter, 1971).

Serology

Attempts to confirm the identity of CLV isolates by serology in agar double diffusion using 0.5M ethanolamine, pH 10.5, to break the particles into readily diffusible units (Purcifull, 1966) failed. A modification of the method of Langenberg & Ball (1972) in which sodium lauryl sulphate (0.25%) or teepol (0.1%) were used in place of Igepon T-73 (0.1%) in an agar gel containing free ammonia also failed due to non-specific precipitation.

Light microscopy

No inclusions could be correlated with CLV infection of carnations or indicator plants using phloxine/trypan blue stain. Castro et al (1971) reported that amorphous granular inclusions occurred in epidermal strips of carnations naturally infected with CLV. However, because of the possibility of mixed infections the results do not unequivocally demonstrate that these inclusion bodies were induced by CLV.

Inclusion bodies have been reported for other carlaviruses. Pea streak virus induced granular amorphous inclusions in peas (Bos & Rubio-Huertos, 1972) whereas crystalline inclusion bodies were observed in epidermal strips from peas infected with red clover vein mosaic virus (Rubio-Huertos & Bos, 1973).

Ultrastructure

Of the carlaviruses studied so far all exhibit comparable intracellular distribution: carnation latent virus (Castro et al, 1971), lily symptomless virus (Lyons & Allen, 1969), passiflora latent virus (Bos & Rubio-Huertos, 1971), pea streak virus (Bos & Rubio-Huertos, 1972), potato virus M (Tu & Hiruki, 1970), potato virus S (Bokx & Wattereus, 1971; Hiruki & Shukla, 1973) and red clover vein mosaic virus (Rubio-Huertos & Bos, 1973). Particles of these viruses occurred randomly or in aggregates in the cytoplasm of infected cells. The aggregates may consist of particles in variable degrees of order. Aggregates of passiflora latent and pea streak viruses frequently contained particles in parallel arrangement and often were of one particle length only. Particles of the viruses were sometimes observed closely associated or even attached to the membrane of cell organelles, particularly chloroplasts and mitochondria, and the tonoplast, but have not been found in chloroplasts or mitochondria although CLV has been reported in the nucleus (Castro et al, 1971).

In the present study carnation latent virus was observed by electron microscopy in ultrathin sections of naturally infected carnation and sap-inoculated _D. chinensis_ and _G. elegans_. Virus-like particles were present in the cytoplasm of infected mesophyll cells. While some particles were free in the cytoplasm most occurred in aggregates or accumulations in which they were often randomly distributed (Figures 22, 23), although some exhibited more order (Figure 22b). In some instances particles of CLV appeared to be attached to the chloroplast membrane (Figure 23). Smaller virus aggregates were found in the cells of experimentally infected plants (Figures 24, 25) probably due to a shorter period of infection. Aggregates of CLV (Figure 24) are similar to those of passiflora latent virus and pea streak virus.

Discussion

The properties reported in this section positively identify carnation latent virus as infecting _Dianthus_ species, including carnations, in New Zealand.

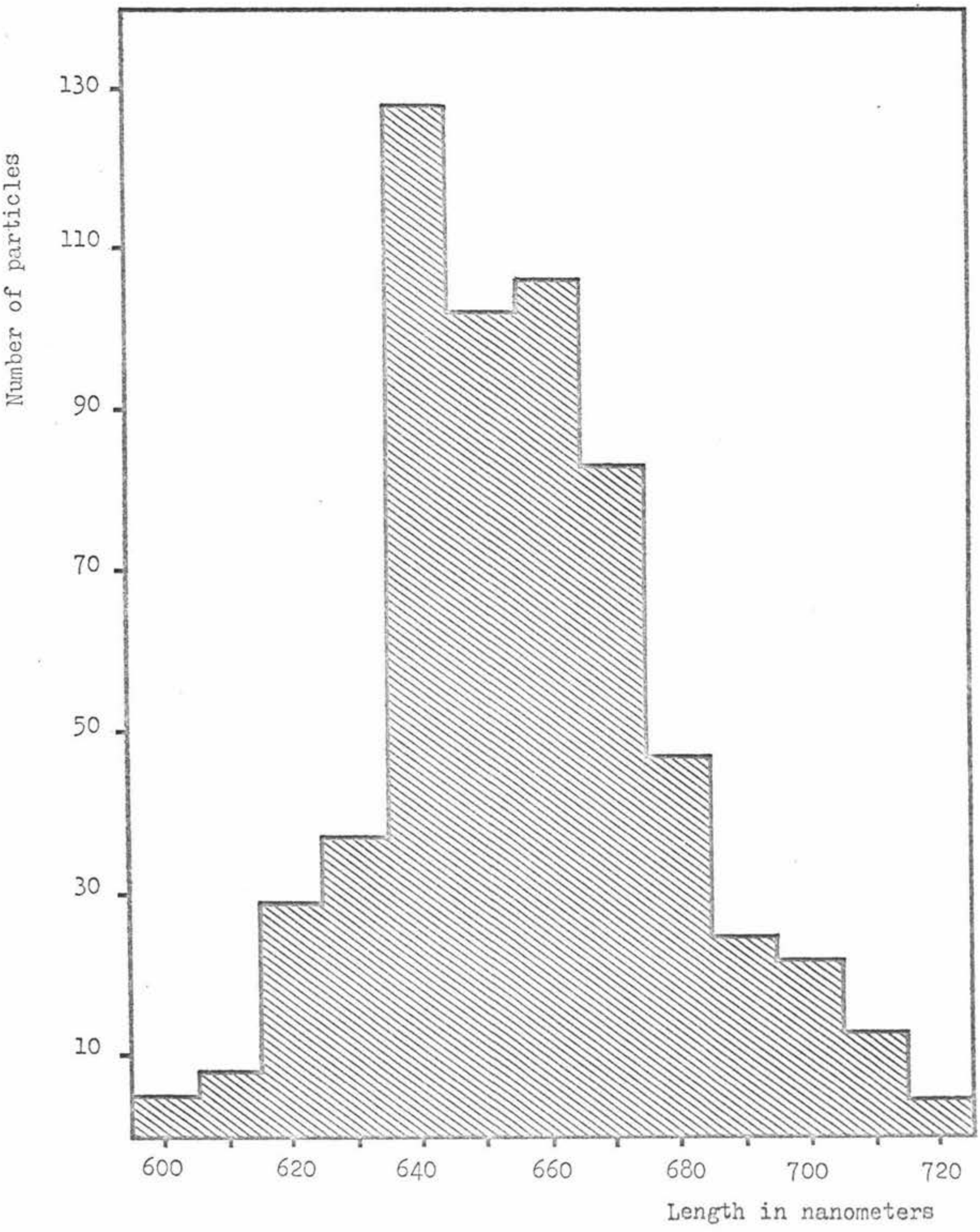


FIGURE 20. Particle length distribution of carnation latent virus in crushed leaves from carnation and crushed hosts. Normal length 656nm.

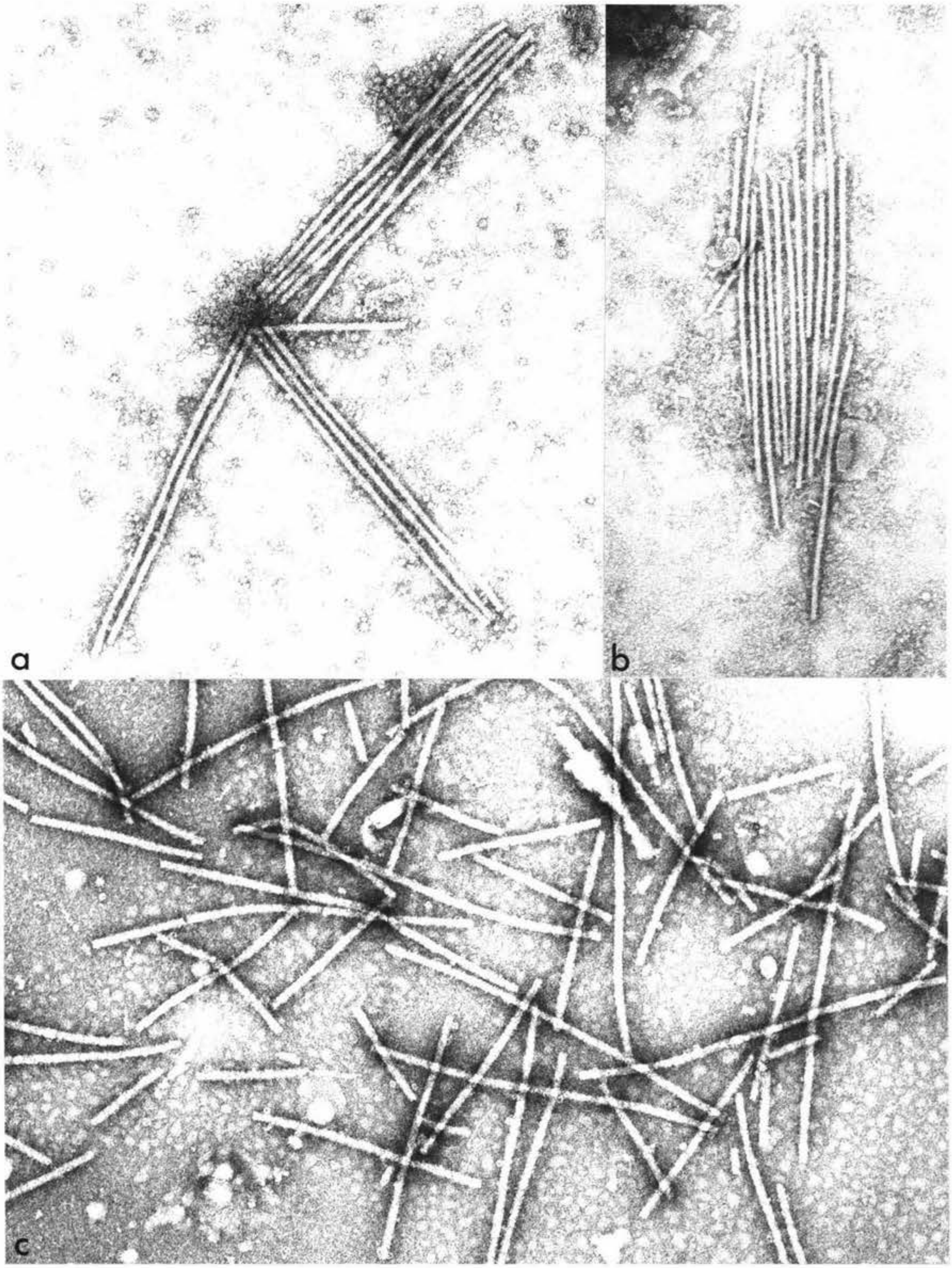


FIGURE 21. Carnation latent virus in (a) sap of Nicotiana clevelandii, Mag. 92,000, (b) carnation sap, Mag. 68,000, and (c) partially purified prep from Chenopodium quinoa, Mag. 92,000.

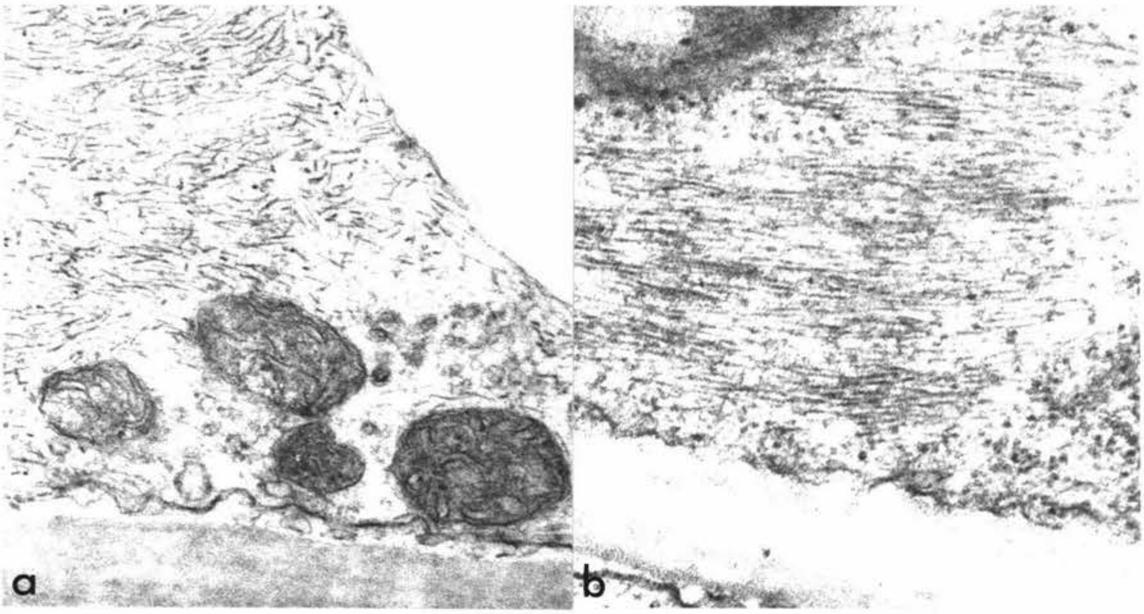


FIGURE 22. Aggregates of carnation latent virus particles in ultrathin sections of carnation leaf tissue. (a) Mag. 35,000, (b) Mag. 50,000.

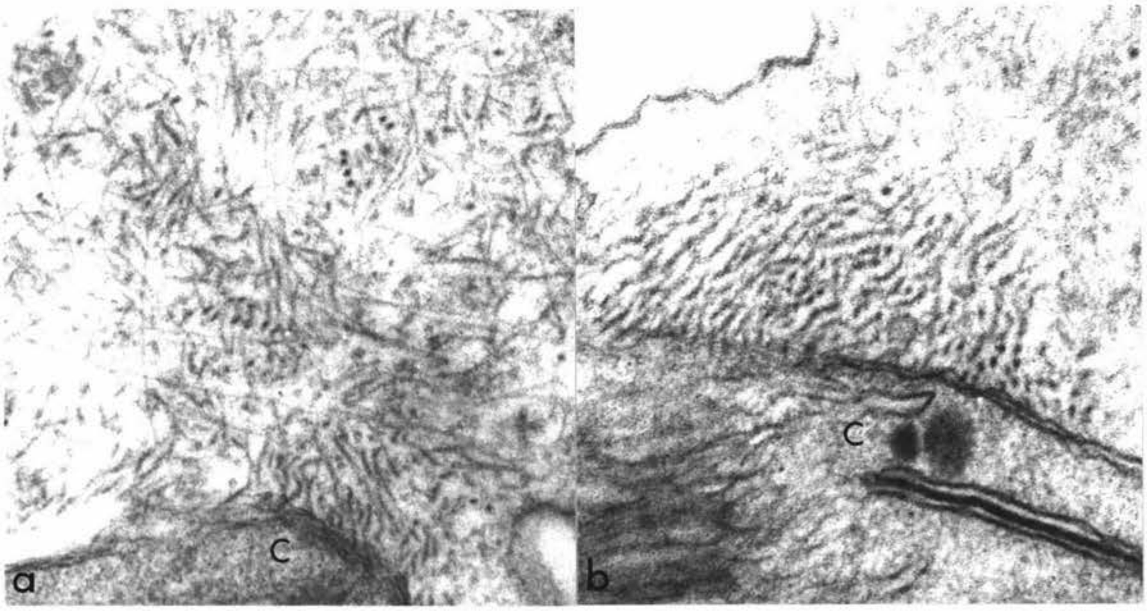


FIGURE 23. Particles of carnation latent virus apparently attached to chloroplast membrane in carnation leaf tissue. (a) Mag. 70,000, (b) Mag. 95,000. C = chloroplast.

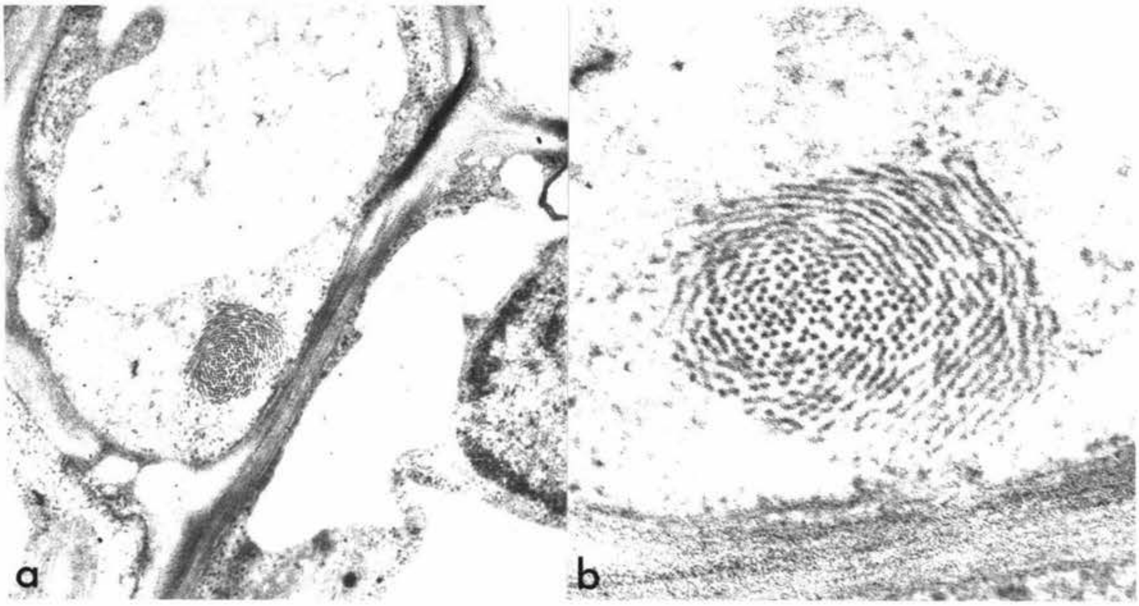


FIGURE 24. An aggregate of carnation latent virus particles in leaf tissue of Dianthus chinensis. (a) Mag. 23,000, (b) Mag. 90,000.

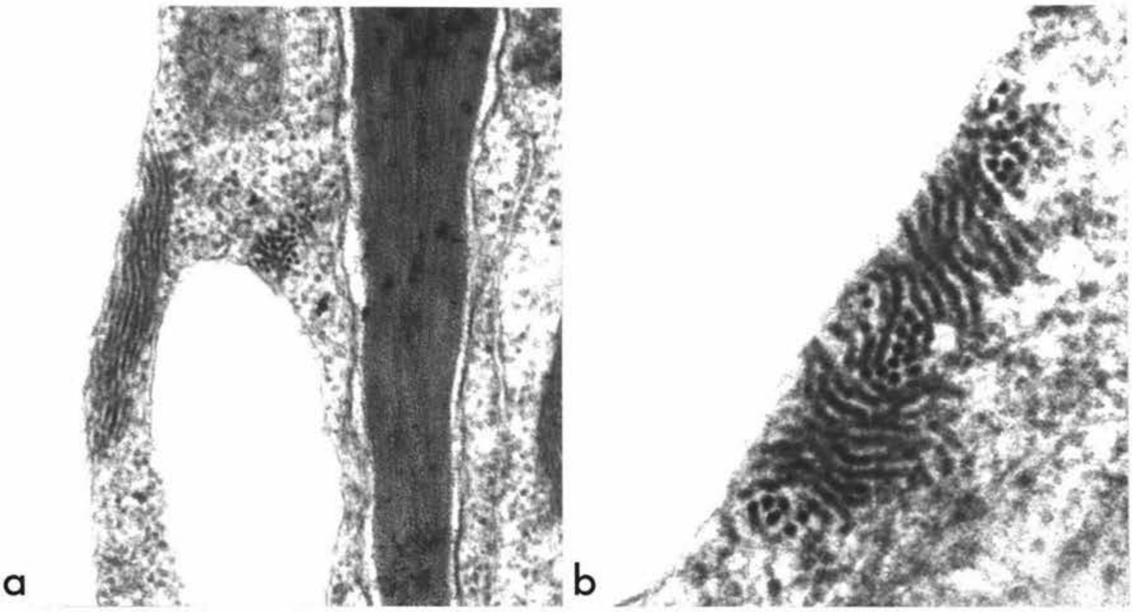


FIGURE 25. Carnation latent virus in Gypsophila elegans leaf tissue. (a) Mag. 58,500, (b) Mag. 125,000.

2.5 CARNATION MOTTLE VIRUS (CarMV)

R/1:*/(20):S/S:S/*

Carnation mottle virus was first characterised by Kassanis in 1955 and subsequently both Hollings & Stone (1964) and Kowalska (1972) reported definitive investigations on host range and other properties of the virus. Strains of the virus are known to occur including a virulent (Hollings & Stone, 1964) and an 'attenuated' (Hollings & Stone, 1962, 1964) form. The attenuated strain is particularly important as it is difficult to detect its presence in carnations (Poupet et al, 1972) although a few large (ca. 4mm) local chlorotic lesions may be formed on C. amaranticolor and C. quinoa plants inoculated with sap from carnations containing the virus (Hollings & Stone, 1964; Poupet et al, 1972). High health stock produced by meristem-tip culture may contain attenuated virus which may later revert or transform to the type strain. It is not known whether this form is due to very low virus concentration or to a slow replicating heat-resistant strain as proposed by Hollings & Stone (1964).

Carnation mottle virus is an isometric particle about 28nm in diameter; it has not been transmitted by the aphid vectors M. persicae and Macrosiphum euphorbiae Thos. (Hollings & Stone, 1964) nor by plant parasitic nematodes (Schindler, 1958), but it can be readily transmitted by horticultural practices involving handling of infected tissue. Other properties reported (Hollings & Stone, 1970a; Kowalska, 1972) include:

TIP : 85-90C
DEP : carnation: more than 10^{-5}
C. amaranticolor: $2\text{-}4 \times 10^{-5}$
LIV : C. amaranticolor sap: 30-35 days at room temperature.
purified preparation: 81 days at about 18C and greater than 3 years at 0C.

Characterization of CarMV was accomplished by studies on host range, purification, electron microscopy, physical properties and serology.

Host range

Several isolates of CarMV were tested on various hosts. Leaves of infected C. quinoa ground in 0.1M K/K_2PO_4, pH 7.5 were used as inoculum.

AMARANTHACEAE

Amaranthus caudatus: local symptomless infection only.

Celosia argentea 'Forest Fire': local infection was generally symptomless but occasionally diffuse chlorotic spots occurred in about 9 days. Systemic infection was symptomless when it occurred.

Gomphrena globosa: local symptomless infection, but occasionally necrotic spots formed as reported by Hollings & Stone (1964). Occasionally limited symptomless systemic invasion occurred.

CARYOPHYLLACEAE

Dianthus barbatus 'Indian Carpet' (sweet william): the reaction of CarMV on this host was variable due to strain differences in the plants. Three basic reactions occurred:
(a) no infection,
(b) local necrotic lesions after 6 days usually followed by symptomless infection or faint mottling of the young leaves,
(c) sparse chlorotic local lesions with subsequent systemic mottling occurred infrequently.

Dianthus caryophyllus 'Joker' (carnation) and 'Chabaud Giants': symptomless systemic infection or faint mottling.

Dianthus chinensis 'Bravo': systemic infection was symptomless or exhibited slight mottling.

Gypsophila elegans: very diffuse chlorotic spots or symptomless infection of inoculated leaves followed by mild mottling or symptomless infection of the young leaves.

Gypsophila paniculata: systemic symptomless infection.

Saponaria vaccaria 'Pink Beauty' (cowcockle): systemic symptoms varied from almost symptomless to veinal chlorosis and mottling; young leaves were often deformed, particularly at the tips and plants were stunted.

CHENOPODIACEAE

Chenopodium amaranticolor: local chlorotic and a few necrotic spots in 4-8 days. Occasionally partial systemic infection occurred as reported by Hollings & Stone (1964).

Chenopodium quinoa: chlorotic local lesions after 4 days followed by systemic chlorotic spotting and mottling with leaf rugosity and stunting of the plant. 'Attenuated' CarMV caused very few large chlorotic spots in about 20 days but on subsequent transmission of the virus to C. quinoa symptoms of the typical strain appeared.

Spinacia oleracea 'Royal Denmark' (spinach): inoculated leaves were usually symptomless but diffuse chlorotic spots occasionally appeared in about 7 days. Symptomless systemic infection often occurred.

CUCURBITACEAE

Cucumis sativus 'Marketer' and 'Polaris': not usually infected but occasionally local symptomless infection occurred.

SOLANACEAE

Nicotiana clevelandii: local infection ranged from symptomless to chlorotic to semi-necrotic lesions in about 8 days. Not systemic.

Nicotiana glutinosa, Nicotiana tabacum 'Havana 423', 'Samsun' and 'White Burley', and Nicotiana sylvestris: no infection.

Petunia hybrida: local symptomless infection.

Local symptomless infection occurred in Antirrhinum majus (snapdragon) and Vinca rosea L.

The following plants were not infected in this study: Apium graveolens (celery); Borago officinalis; Brassica pekinensis 'Chi-Hi-Li' (chinese cabbage); Capsicum frutescens (pepper); Lactuca sativa 'Webbs Wonderful' (lettuce); Lobelia erinus L.; Matthiola incana (stock); Phaseolus vulgaris 'Scotia', 'Topcrop' (bean); Phlox drummondii; Physalis franchetti Mast.; Pisum sativum 'Greenfeast' (pea); Primula malacoides Franch.; Tropaeolum majus (nasturtium); Verbena hybrida Voss.; Vicia faba L. 'Atlas Early' (broad bean); Vigna sinensis 'Blackeye' (cowpea); and Zinnia elegans.

Note: Both G. elegans and S. vaccaria were systemically infected by attenuated strains but no symptoms occurred. However, when the virus was inoculated to C. quinoa from these hosts symptoms of typical CarMV developed. Such a transformation was also reported by Poupet et al (1972) in S. vaccaria.

Vector transmission

No transmission of CarMV was obtained with the aphid M. persicae in six attempts to transfer the virus from carnation or D. chinensis to D. chinensis, D. barbatus and C. quinoa.

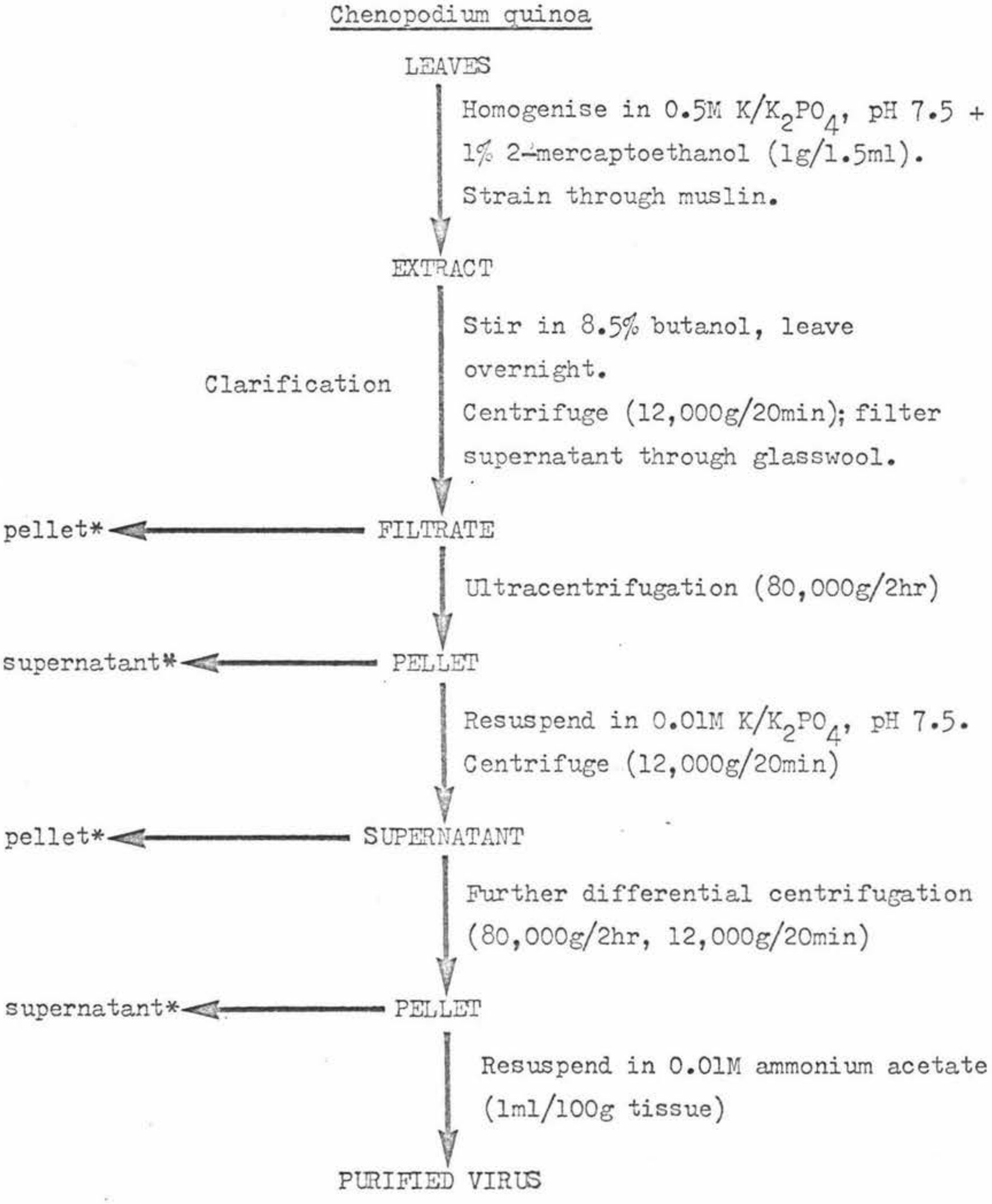
Purification

A purification schedule was based on that used for CarMV by Hollings & Stone (1964) and is summarized in Figure 26. Leaves of locally and systemically infected C. quinoa were harvested 1 or 2 weeks, respectively, after inoculation and homogenized in 0.5M K/K_2PO_4 buffer, pH 7.5 (1.5ml/1g tissue) containing an antioxidant. Butanol was stirred into the expressed homogenate drop by drop to a final concentration of 8.5% and the mixture left overnight at about 4C. The suspension was clarified by low speed centrifugation and the supernatant subjected to two or three cycles of differential centrifugation. Pellets were resuspended in 0.01M K/K_2PO_4, pH 7.5, 0.001M Na_2EDTA or 0.01M ammonium acetate, pH 6.5; the latter two solutions were preferred for final resuspension for electron microscopy as crystals were not formed on the grids.

No significant effect on virus yield, as measured by local lesion assays on C. quinoa, occurred when different antioxidants (0.1M Na_2SO_3, 0.1% thioglycollic acid or 1% 2-mercaptoethanol) or phosphate buffer concentrations (0.05M, 0.1M or 0.5M) were used for extraction of the virus from C. quinoa tissue. The purification procedure finally adopted (Figure 26) was also used successfully to obtain preparations of CarMV from carnation tissue.

Two other methods were tested for purifying CarMV. The method used for ArMV by Harrison & Nixon (1960) gave equally good preparations but this technique had no apparent advantage. Precipitation of the virus with 6% polyethylene glycol (PEG), molecular weight 20,000, from clarified C. quinoa leaf extracts and subsequent differential centrifugation gave preparations of high concentration, but electron microscopy revealed that more apparently empty particles were present than in preparations purified by the standard method.

PURIFICATION OF CARNATION MOTTLE VIRUS



Homogenise in 0.5M K/K_2PO_4, pH 7.5 + 1% 2-mercaptoethanol (1g/1.5ml). Strain through muslin.

Stir in 8.5% butanol, leave overnight. Centrifuge (12,000g/20min); filter supernatant through glasswool.

Ultracentrifugation (80,000g/2hr)

Resuspend in 0.01M K/K_2PO_4, pH 7.5. Centrifuge (12,000g/20min)

Further differential centrifugation (80,000g/2hr, 12,000g/20min)

Resuspend in 0.01M ammonium acetate (1ml/100g tissue)

* discard.

FIGURE 26. Procedure for the purification of carnation mottle virus.

Electron Microscopy

Carnation mottle virus was readily observed by electron microscopy in negatively stained carnation sap (Figure 27 a,b) and in most other hosts of the virus, except tissue containing very low concentrations or 'attenuated' virus. Virus was stable in PTA in the pH range 4 to 7, but the mixture AM-PTA was preferred for general negative staining, although it was less satisfactory than PTA at times, particularly for C. quinoa tissue. Ammonium molybdate (2%) did not stain crude homogenates intensely enough, but it was preferred for purified preparations (Figure 27c) due to the improved spreading obtained with it. In crude homogenates the virus particles sometimes formed arrays (Figure 27a), particularly when in very high concentration.

The sizes of single CarMV particles calculated from crude sap ranged between 25-32nm with an average diameter of 29.6nm for 106 particles. Particles in arrays in sap had an average diameter of 31.5nm (range 30-33nm) for 112 particles. Purified preparations contained particles in the range 27-31nm with an average value of 29.6nm for 80 particles.

Physical Properties

The thermal inactivation point of CarMV was determined in crude sap obtained from infected D. chinensis, D. barbatus and C. quinoa by grinding leaf tissue in either buffer (0.1M K/K_2PO_4, pH 7.0) or distilled water (10% w/v). After heat treatment samples were assayed on C. quinoa.

TIP : 85-90C

Dilution end point was determined from carnation sap diluted in 0.1M K/K_2PO_4 buffer, pH 7.0.

DEP : 10^{-5}-10^{-6}

Serology

The identity of a number of CarMV isolates was confirmed serologically by double diffusion in agar gel. Carnation mottle virus was readily detectable in sap from infected C. quinoa, carnation and D. chinensis. Purified preparations also reacted strongly.

Light Microscopy

No inclusion bodies were correlated with infection by CarMV in carnation, G. elegans or S. vaccaria.

Ultrastructure

Carnation ('Orchid Beauty') tissue used for ultrastructural studies on CERV-50 inclusions was known to also contain CarMV and particles approximately 30nm in diameter were observed in the vacuole of mesophyll cells and occasionally in the cytoplasm (Figure 19, page 48).

Robleda (1973) reported particles of CarMV in the cytoplasm, nucleus and vascular tissues of Atriplex hortensis L., C. amaranticolor, D. barbatus and D. caryophyllus and in the vacuole of cells with broken tonoplasts. However, in this study the tonoplast appeared undamaged.

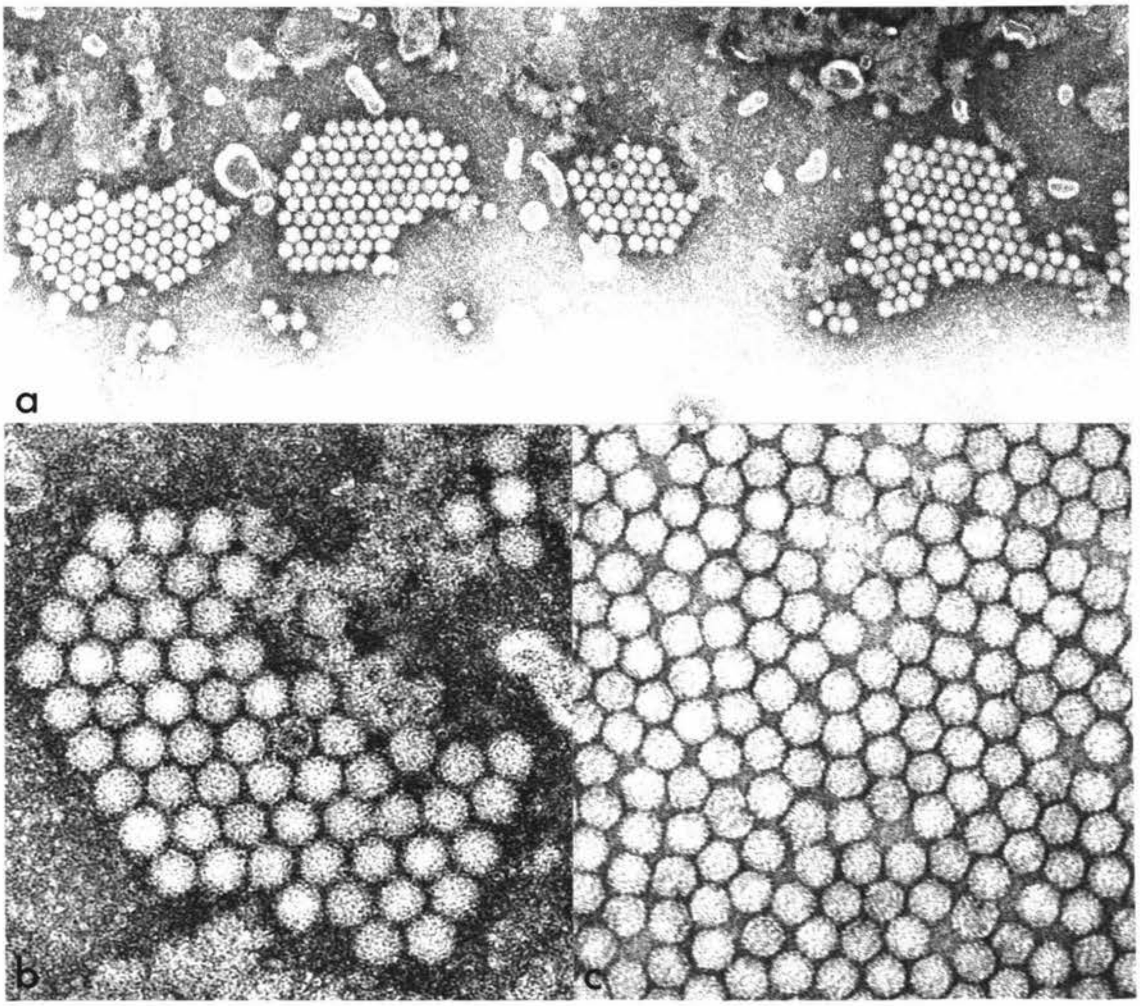


FIGURE 27. Carnation mottle virus: (a,b) carnation sap stained with AM-PTA, Mag. 71,000 and 214,000, respectively. Note the arrays formed in (a). (c) purified preparation from C. quinoa stained with 2% AM, Mag. 180,000. Note the hexagonal appearance of many of the particles.

2.6 CARNATION NECROTIC FLECK VIRUS (CNFV)

(= Carnation yellow fleck virus) */*:*/*:E/E:S/Ap

Carnation necrotic fleck virus was characterized from carnations in Japan (Inouye & Mitsuhata, 1973) and is reported to be a long flexuous rod, ca. 1,400-1,500 x 12-13nm, which exhibits cross banding in leaf dips stained with uranyl formate. Particles of CNFV are therefore similar to beet yellows virus (BYV) in size and morphology (Russell, 1970). Carnation necrotic fleck virus and BYV are also alike in their semi-persistent mode of transmission by aphids. Aphids, M. persicae, required acquisition feeds of 30min or longer and inoculation feeds longer than 10min to transmit CNFV. Efficiency of transmission was increased by longer feeding periods and the aphids retained infectivity for 2 days. Both CNFV and BYV are mechanically transmitted with difficulty and are particularly associated with phloem tissues in which they cause degeneration.

These properties place CNFV in the beet yellows group of viruses along with citrus tristeza virus (Price, 1970), and possibly festuca necrosis (Schmidt et al, 1963) and wheat yellow leaf (Inouye et al, 1973) viruses.

Carnation yellow fleck virus (CYFV)(Smookler & Loebenstein, 1974) is reported to have very similar transmission characteristics, particle morphology and size (ca. 1,250 x 13nm), identical host range (D. barbatus, D. caryophyllus, D. chinensis) and similar symptomatology to CNFV. It is therefore likely that CYFV is the same virus as CNFV.

In the present study CNFV was characterized mainly on particle size and morphology.

Host range

Attempts were made to transmit the long rod to other plants, particularly those in the family Caryophyllaceae, by sap inoculation from carnation using buffer containing bentonite. However, no symptoms were expressed on any plants and the presence or absence of virus had to be monitored by electron microscopy of sap from these plants.

Dianthus chinensis 'Bravo' was the only host found to contain the virus, and then only one plant out of six became infected (Figure 30). Only limited systemic infection was apparent.

Infection could not be detected in the following plants: Celosia argentea 'Forest Fire', Chenopodium quinoa, Dianthus barbatus, Gomphrena globosa, Gypsophila elegans, Saponaria vaccaria 'Pink Beauty' and Spinacia oleracea 'Royal Denmark'.

Vector transmission

Aphid (M. persicae) transmission of the long rod to D. chinensis was obtained in one out of six experiments using 24 hour acquisition and inoculation feeds (Figures 29, 31). Trials using 5-10min and 30min acquisition probes failed to transmit the virus to D. barbatus, D. chinensis or G. elegans.

Electron microscopy

As previously mentioned in the survey (section 1.5.7) it was usually difficult to find CNFV by electron microscopy in negatively stained sap from a sample previously shown to contain the virus. Particles and fragments generally occurred singly (Figure 28) although they were occasionally found in groups (Figures 30, 31). From the photographs (Figures 28-31) it is apparent that there appear to be two types of particles. One type has long rigid rods in which the axial canal was penetrated by stain (Figure 28a, b, 30) and one end of the particle frequently appeared to be disintegrating (Figure 28b). The other type was much more flexuous and cross striations were sometimes evident (Figures 28c, 31). However, both types of particles occurred in sap from carnation and D. chinensis and were occasionally observed on a single grid (Figure 29). It is possible that they are two forms of the same virus, the difference being due to ion effects as reported for some viruses considered to belong to the potato virus Y group (Atkey & Brunt, 1973; Govier & Woods, 1971).

The flexuous particles exhibiting cross striations are similar to those of BYV (Russell, 1970) while the rigid rods resemble those reported for CYFV in aggregates in leaf dips. Particles of CYFV in purified preparations, however, were flexuous and striated like BYV.

Due to fragmentation of particles it was not possible to obtain a normal length for CNFV but a large proportion (38%) of the particles were in the range 1,000-1,450nm. There was another group (14%) in the range 600-800nm; longer particles up to approximately 3,000nm were also present. Width of the particles was about 12nm.

Light microscopy

No inclusion bodies were observed by light microscopy in stained (phloxine/trypan blue) epidermal strips from the few plants that were infected with CNFV but not CERV-50. This contrasts with the report on CNFV in which inclusion bodies were recorded in epidermal strips of D. barbatus stained with phloxine-methylene blue (Inouye, 1974; Inouye & Mitsuhata, 1973). Confirmation of this point, however, is required, particularly as no mention of CERV-50 was made, although other virus particles were present.

Discussion

The results of tests with the long rod strongly suggest that it is CNFV and together with the other reports indicate that CNFV is likely to be of widespread occurrence around the world.

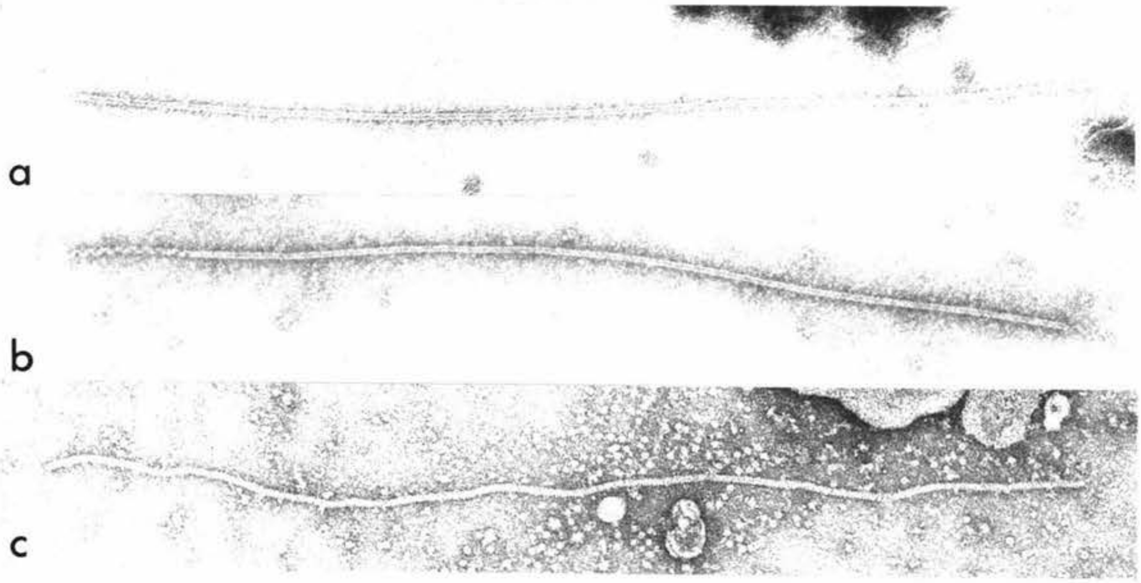


FIGURE 28. Particles of carnation necrotic fleck virus in carnation sap (a) and (b), and in Dianthus chinensis sap (c). In (a) and (b) the particles are quite rigid and exhibit an axial core. Mag. 80,000 and 75,000 respectively. In (c) the particle is more flexuous and cross striations are evident. Mag. 85,000. Stain: AM-PTA mixture.

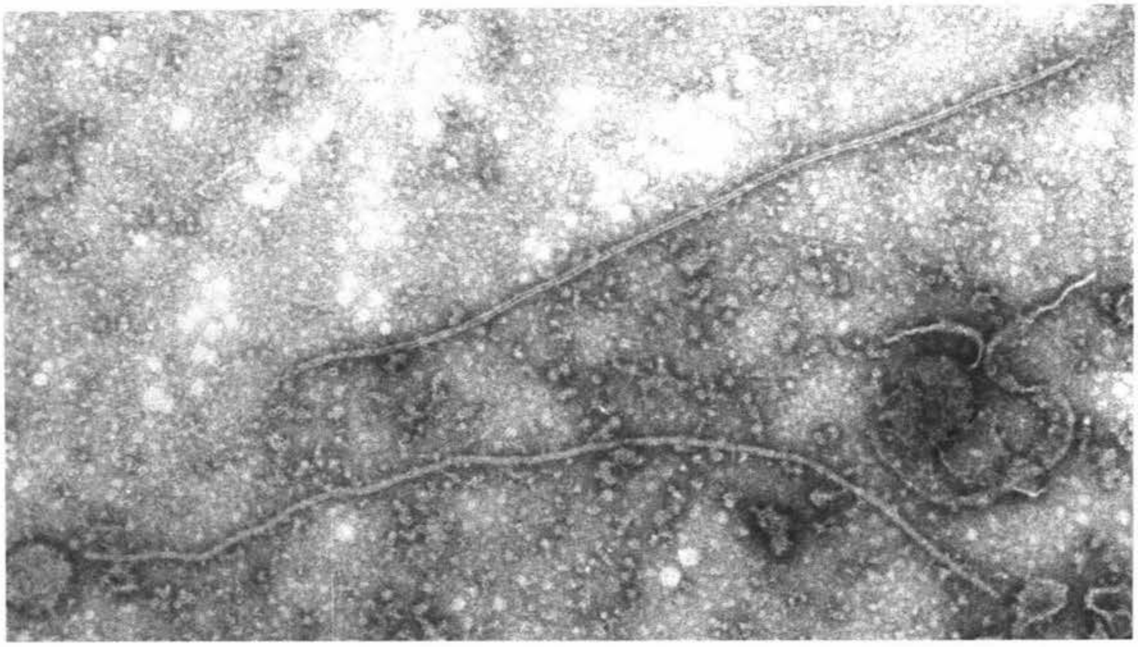


FIGURE 29. Carnation necrotic fleck virus showing both rigid (top) and flexuous types of particle in sap of aphid inoculated Dianthus chinensis. Stain: AM-PTA mixture. Mag. 83,000.

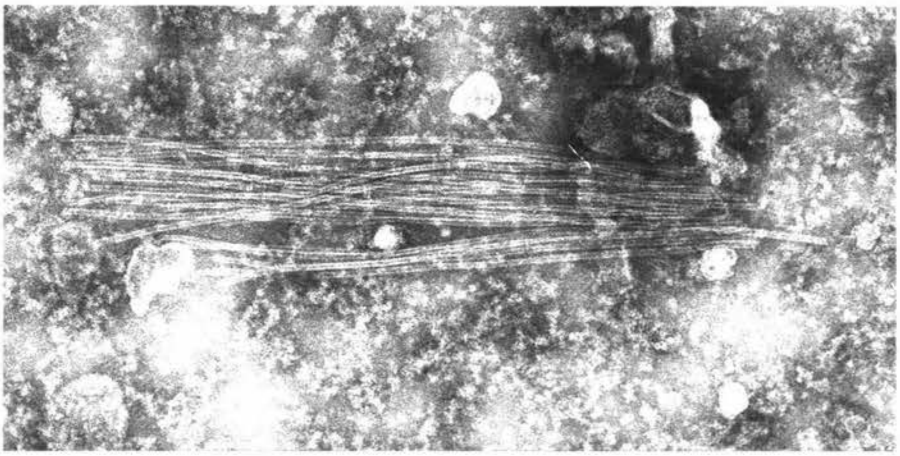


FIGURE 30. A group of rigid particles in sap from Dianthus chinensis mechanically inoculated with carnation necrotic fleck virus. Mag. 85,500. Stain: AM-PTA mixture.

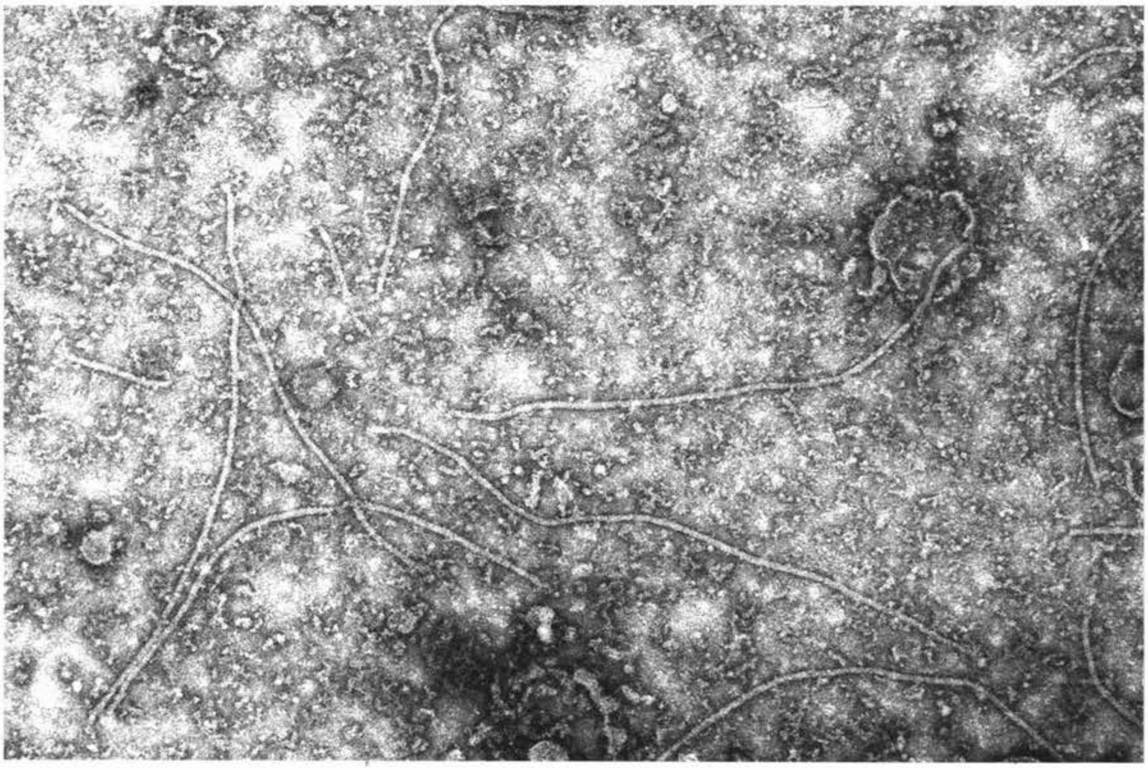


FIGURE 31. Flexuous particles of carnation necrotic fleck virus in Dianthus chinensis inoculated by aphids, Myzus persicae. Mag. 71,600. Stain: AM-PTA mixture.

2.7 CARNATION RINGSPOT VIRUS (CRSV)

R/1:1.4/20:S/S:S/Ne

Carnation ringspot virus has an isometric particle about 30nm in diameter and is reported to be distantly related, serologically, to members of the tymovirus group (Bercks & Querfurth, 1972), but this has not been verified. In addition to mechanical transmission CRSV has been transmitted by the nematodes Longidorus macrosoma Hooper and X. diversicaudatum (Fritzsche & Schmelzer, 1967). The virus occurs naturally in species of Caryophyllaceae but has also been reported in tulip (Slogteren & Asjes, 1970). Physical properties reported (Hollings & Stone, 1965, 1970b) for CRSV in crude sap are:

TIP : 80-90C
DEP : 10^{-5}
LIV : 50-60 days at 20C

The presence of CRSV in samples was confirmed by its reaction on differential hosts, particularly on C. argentea, G. elegans and S. vaccaria, and by comparison with an isolate provided by Dr. P.R. Fry. The identity of isolates was also confirmed by serology.

Host range

AMARANTHACEAE

Amaranthus caudatus and Amaranthus viridis: irregular dark brown necrotic spots after approximately 5 days.

Celosia argentea 'Forest Fire': necrotic dots with red peripheries are formed after 3 days (Figure 33).

Gomphrena globosa: sometimes typical necrotic rings appeared after 4 days, but the local reaction was unreliable. Systemic chlorotic flecking and mottling of the young leaves.

CARYOPHYLLACEAE

Dianthus barbatus: pale green spots, becoming necrotic ringspots, after about 5 days, followed by chlorotic and semi-necrotic flecks, rings and mottle in young leaves. Symptoms may not be expressed in later growth. Variation in symptom expression occurred due to differences between plants and often no local reaction was observed.

Dianthus caryophyllus 'Joker': systemic chlorotic flecks and semi-necrotic rings and line patterns (Figure 32).

Dianthus chinensis 'Bravo': local chlorotic spots after 3 days becoming semi-necrotic ringspots; systemic mottling and irregular semi-necrotic ringspots.

Gypsophila elegans: whitish spots and ringspots in 3-4 days followed by chlorotic flecking, ringspots and distortion in the young leaves (Figure 34).

Gypsophila paniculata: systemic chlorotic flecks and mottling after about 9 days, but sometimes symptomless.

Saponaria vaccaria 'Pink Beauty': chlorotic lesions formed after 3 days and quickly turned into whitish rings (Figure 35); vein clearing and chlorotic flecking of the young leaves occurred after a few more days with subsequent formation of whitish ringspots.

CHENOPODIACEAE

Chenopodium amaranticolor: local necrotic spots after 2-3 days.

Chenopodium quinoa: chlorotic, and sometimes necrotic, lesions after 2 days. Occasionally a few chlorotic flecks and spots formed on the young leaves.

Spinacia oleraceae 'Royal Denmark': chlorotic lesions appeared after 3 days; often these lesions became semi-necrotic rings. Chlorotic flecking of the young leaves was soon evident (4-6 days) followed by mottling and rugosity, and sometimes semi-necrotic ringspots and line patterns. Plants were severely stunted.

CUCURBITACEAE

Cucumis sativus 'Marketer': chlorotic spots appeared on the cotyledons after approximately 5 days and rapidly became necrotic.

Momordica balsamina: chlorotic local spots appeared after 2-3 days.

LEGUMINOSAE

Vicia faba 'Atlas Early': local symptomless infection.

POLEMONIACEAE

Phlox drummondii: faint chlorotic lesions after 4 days followed by faint systemic mottling or symptomless infection.

SCROPHULARIACEAE

Antirrhinum majus (Snapdragon): pale green spots after 3 days becoming semi-necrotic ringspots; symptomless systemic infection occasionally.

Digitalis purpurea: symptomless local infection only.

SOLANACEAE

Nicotiana clevelandii: chlorotic spots developed after 3 days and rapidly became necrotic rings (ca. 4-5 days) (Figure 36); systemic chlorotic and necrotic flecks, ringspots and line patterns occurred on the young leaves. Infection was often very severe.

Nicotiana glutinosa, Nicotiana sylvestris and Nicotiana tabacum ('Havana 423', 'Samsun' and 'White Burley'): at first (3 days) chlorotic spots appeared which subsequently became semi-necrotic rings, ringspots and line patterns. Plants were not systemically infected.

These plants were difficult to infect particularly with inoculum from plants of the Caryophyllaceae and also from the Chenopodiaceae due to inhibitors in extracts from these plants. N. clevelandii was the best source for inoculation.

VERBENACEAE

Verbena hybrida: a few necrotic spots occurred after 3 days.

The following plants were not infected in this study: Apium graveolens (celery); Brassica pekinensis 'Chi-Hi-Li'; Lobelia erinus; Matthiola incana; and Vinca rosea.

Electron microscopy

Polyhedral particles about 30nm in diameter were observed by electron microscopy in crude sap from indicator plants infected with CRSV.

Physical properties

Results of tests carried out with CRSV-infected N. clevelandii to determine the thermal inactivation point were consistent with those in the literature (Hollings & Stone, 1965b; Kowalska, 1972):

TIP : 85-90C

Serology

Isolates that were thought to be CRSV on the basis of reactions on differential hosts reacted positively with antiserum to CRSV.

Light microscopy

No inclusions specific for CRSV were observed either in the naturally infected samples or in 'Joker' carnations mechanically inoculated with the virus. Hollings & Stone (1970b) reported that no inclusion bodes have been observed in CRSV-infected tissues.

Discussion

The results of the reactions on indicator plants, the host range, and serological tests constitute the first report of CRSV in *Dianthus* species in New Zealand.

FIGURE 32. Systemic symptoms in carnation 'Joker' 1 month after inoculation with carnation ringspot virus.

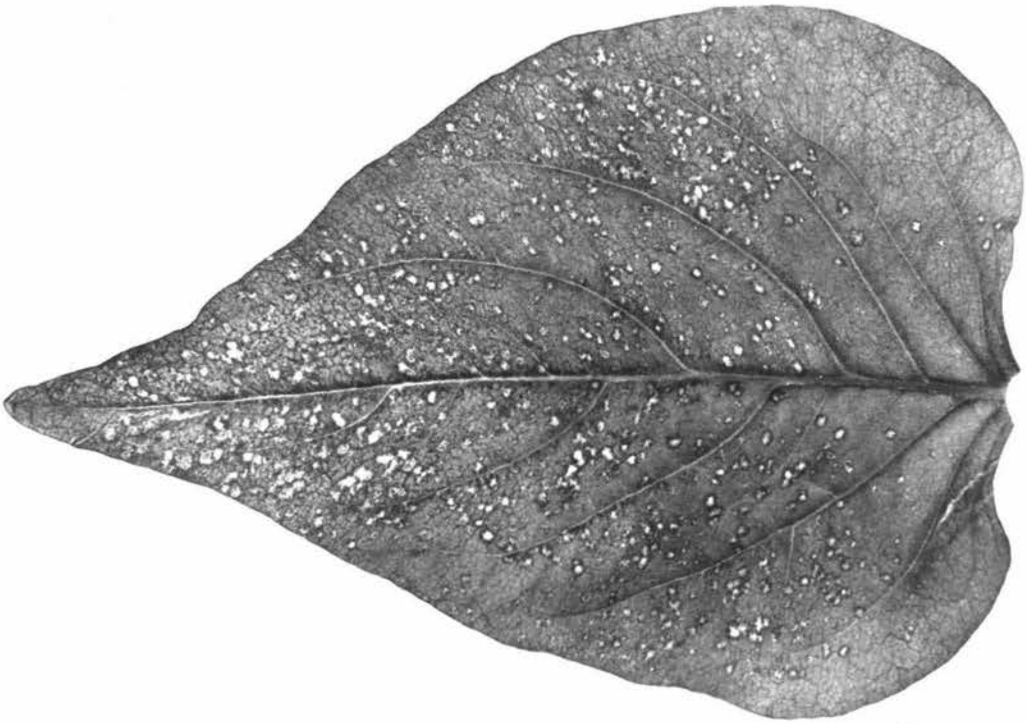


FIGURE 33. Necrotic local lesions induced by carnation ringspot virus in Celosia argentea 6 days after inoculation.

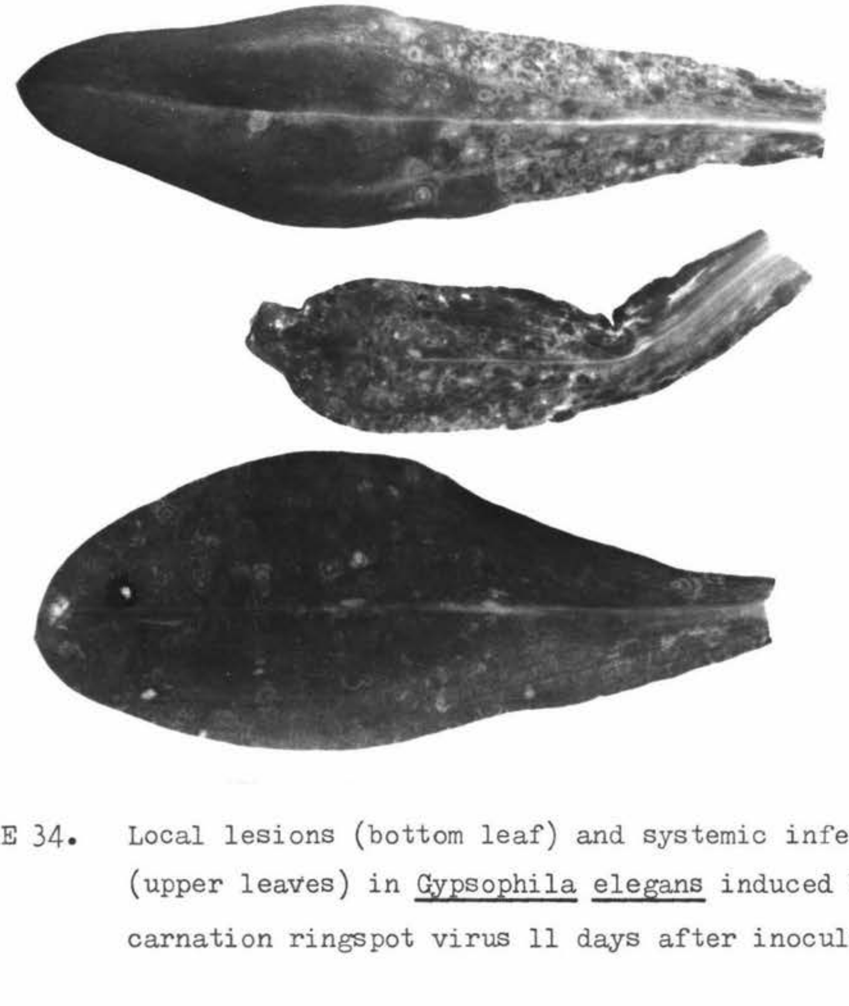


FIGURE 34. Local lesions (bottom leaf) and systemic infection (upper leaves) in Gypsophila elegans induced by carnation ringspot virus 11 days after inoculation.

FIGURE 35. Whitish local rings on Saponaria vaccaria 8 days after inoculation with carnation ringspot virus.

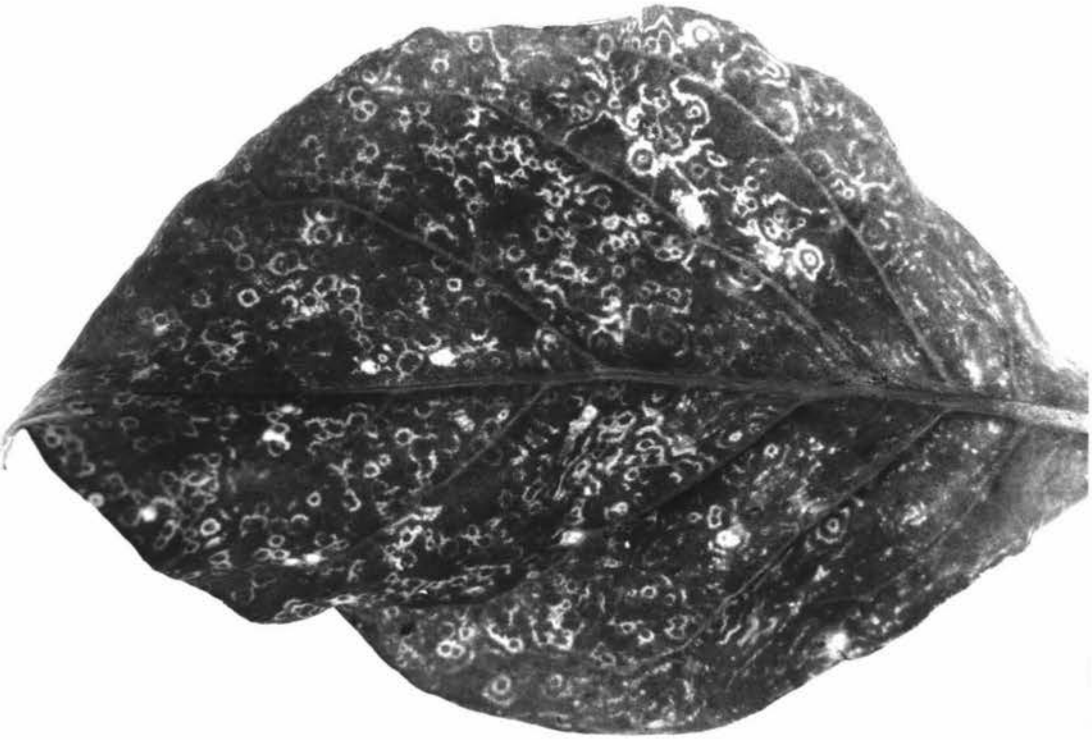


FIGURE 36. Necrotic ringspots and line patterns on Nicotiana clevelandii 14 days after inoculation with carnation ringspot virus.

2.8 CARNATION VEIN MOTTLE VIRUS (CVMV)

R/1:*/*:E/E:S/Ap

Carnation vein mottle virus was first described by Kassanis (1955). It has flexuous rod particles ca. 790 x 12nm and is transmitted by the aphid M. persicae in a non persistent manner and by sap inoculation (Hollings & Stone, 1971). Additionally, a distant serological relationship has been found to bean yellow mosaic, pea mosaic and freesia streak viruses (Hollings & Stone, 1971). Based upon this information carnation vein mottle is a member of the potato Y group of viruses.

The virus has a restricted host range and is known to infect only about 20 species from 7 families (Hollings & Stone, 1971). Castro et al (1971) reported isolating a strain of CVMV from Lychnis dioica L. which belongs to the same family (Caryophyllaceae) as Dianthus species. Slight differences in the stability of CVMV isolates have been reported:

TIP : D. barbatus sap 50-55C (Kassanis, 1955),
60-65C (Brierley & Smith, 1957; Hollings, Stone & Thorne, 1971)
DEP : Carnation sap 10^{-2}-10^{-5}
D. barbatus sap 10^{-3}-10^{-4}
C. quinoa sap 10^{-4}-10^{-5}
LIV : D. barbatus sap 2-10 days/ca. 18C,
22-28 days/ca. 2C
(Hollings, Stone & Thorne, 1971)

Weintraub & Ragetli (1970) reported cytoplasmic inclusions and virus-like particles in ultrathin sections of CVMV-infected D. barbatus; Castro et al (1971) reported pinwheels in carnation.

In the present study CVMV was distinguished by host range, electron microscopy of sap and ultrathin sections, aphid transmission, thermal inactivation and dilution end points.

Host range

Locally infected leaves of C. quinoa were used as the main source of inoculum for host range trials.

AMARANTHACEAE

Amaranthus caudatus: occasionally systemic chlorotic spots, particularly along veins.

Amaranthus viridis: systemic chlorotic vein banding, leaf rugosity (Figure 37).

Gomphrena globosa: faint systemic vein chlorosis (after 12 days) followed by diffuse mottling, leaf distortion and slight stunting. Limited infection only.

CARYOPHYLLACEAE

Dianthus barbatus (sweet william): systemic vein chlorosis after about 10 days followed by chlorotic spotting and mottling.

Dianthus caryophyllus: naturally infected plants exhibited chlorotic splotches, particularly on the young leaves. Symptoms often disappeared as the leaves aged.

Dianthus chinensis 'Bravo': systemic vein chlorosis, chlorotic spotting and distortion of the leaves. Flowers exhibited colour breaking (Figure 38).

Gypsophila elegans: faint chlorotic lesions after approximately 10 days followed by veinal chlorosis and mottling of the young leaves.

Saponaria vaccaria 'Pink Beauty': diffuse local chlorotic spots after 8 days with vein chlorosis and mottling of the young leaves.

CHENOPODIACEAE

Chenopodium amaranticolor: chlorotic lesions after about 7 days. No systemic infection was observed.

Chenopodium quinoa: local chlorotic lesions after 6 days followed by chlorotic spots on the very young leaves, particularly on the axillary shoots. At times CVMV did not infect systemically.

Spinacia oleracea 'Royal Denmark': diffuse systemic chlorotic spots and mild mottling.

Plants not infected by CVMV in this study were:

Beta vulgaris L. (beet root); Borago officinalis; Celosia argentea 'Forest Fire'; Nicotiana clevelandii; N. glutinosa; N. tabacum 'Havana 423', 'Samsun', 'White Burley'; and Physalis franchetti.

Vector transmission

Carnation vein mottle virus was transmitted by *M. persicae* in 2 out of 3 experiments in which aphids (15/plant) were given 5 minute acquisition probes before transfer to *D. chinensis* plants. Symptoms developed about 1 month after inoculation.

Electron microscopy

Particles of CVMV were present in carnation sap (Figure 41) but were often difficult to find. They were observed fairly readily, however, in sap from some of the experimentally infected hosts (e.g. *D. barbatus*) although particles were not usually abundant (Figure 39). In a few instances aggregates of virus particles were observed (Figure 40). Carnation vein mottle virus particles were distinguished from those of CLV by their flexuousness and length (Figure 41).

The length distribution of CVMV particles had a major peak between 710-770nm with a normal length of 738nm for 200 particles. A few apparently double length particles also occurred. Width of particles was ca. 12nm.

Striated plates of varying shapes were observed alone or together with CVMV particles (Figure 42) in sap from *C. amaranticolor*, *C. quinoa* and *S. vaccaria*. The striations had a periodicity of approximately 5.5nm.

Physical properties

Locally infected *C. quinoa* leaves were used to determine thermal inactivation and dilution end points. Leaf tissue was ground in distilled water (1g/5ml) and strained through muslin. Virus solution was either incubated at 5C intervals at temperatures between 45-75C or diluted with distilled water.

TIP : 60-65C
DEP : 10^{-3}-10^{-4}

Light microscopy

Although no inclusion bodies were observed, using light microscopy, in CVMV-infected plants in the present study, a more thorough investigation is required. Castro et al (1971) reported amorphous inclusions of CERV-50 as well as other granular-amorphous inclusions in epidermal strips stained with phloxine. It was reported that CLV and CVMV caused these latter inclusions but there was no evidence presented to show that they were definitely correlated with CLV or CVMV infection. However, inclusions of other potato Y group viruses have been reported to stain with phloxine and/or trypan blue (Berkeley & Weintraub, 1952; Bos, 1969; Oosten & Bakel, 1970).

Ultrastructure

Confusion still exists over the terminology used by various authors for describing inclusions of potato Y group viruses despite a recent review on the subject (Edwardson, 1974). Consequently a summary follows of the terms used. The diagrammatic representations of tobacco etch and watermelon mosaic virus inclusions by Edwardson et al (1968) provide the basis for this summary.

The cylindrical inclusion (CI) is the basic unit or inclusion type diagnostic for the potato Y group (PVY-group) viruses. No viruses proven to belong to other groups have been reported to produce this type of inclusion. Transverse sections of cylindrical inclusions yield what has commonly been called a pinwheel (PW) while longitudinal sections result in bundles (B). The arms of the pinwheels usually appear as single curved sheets or plates radiating from a small central core. With some PVY-group viruses the arms of the pinwheels are partially or wholly comprised of layers of plates making a laminated aggregate (LA). These laminated aggregates exhibit a similar structure in both transverse and longitudinal sections. Small ringlike structures, which are apparently hollow rods in cross section, are occasionally present along either or both sides of the pinwheel arms and also the laminated aggregates. Similar structures also appear free in the cytoplasm (Edwardson, 1974, passionfruit woodiness virus, Figure 43d).

In some instances the component plates of the pinwheels roll up at their ends into loops or scrolls, as observed in transverse section. The loops are either a single plate or laminated aggregate rolled once while the scrolls consist of either a single plate or laminated aggregate rolled several times. In longitudinal section these loops and scrolls appear as tubular structures or tubes. Previously the terms circular inclusion, and tube or tubular inclusion have been used for scrolls (Edwardson et al, 1968) and tubes (Edwardson, 1974; Edwardson et al, 1968), respectively. The plates that constitute the CI, LA and tubes exhibit a striated substructure. Both laminated aggregates and loops may occur separately from cylindrical inclusions in the cytoplasm.

Edwardson (1974) has used the presence or absence of laminated aggregates and tubes/scrolls associated with cylindrical inclusions as a means of separating the PVY-group into three sub-groups:

(1) Viruses which form tubes attached to the central portion of the cylindrical inclusions.
(2) Viruses which form laminated aggregates attached to the central portion of the cylindrical inclusions.
(3) Viruses which induce both tubes and laminated aggregates attached to the cylindrical inclusions.

Although a large amount of information has been amassed and examined for this purpose (Edwardson, 1974) there are exceptions, particularly with virus strains, which do not always appear to fit into a given category. Until more evidence is provided to justify the division of the PVY-group into sub-groups the different forms of the basic cylindrical inclusion must be regarded merely as variations with only intrinsic importance.

Various other types of inclusions, both cytoplasmic and nuclear, have been found in PVY-group infections but so far no diagnostic value has been reported for any of them. Virus particles singly or in aggregates may be present in the cytoplasm. Bridals, consisting of virus-like particles (VLP) in association with membranous sheets or plates, which are usually circular in cross section, occur in some instances.

Results: Inclusions of CVMV were observed in both systemically infected *D. chinensis* (Figure 43) and locally infected *C. quinoa* (Figures 44-48) leaf tissues. Cylindrical inclusions in both transverse (pinwheels) (Figures 43a and 47) and longitudinal (bundles) (Figures 43b and 44) sections were present in the mesophyll cells of both hosts as were loops (Figure 47), tubes (Figure 44) and laminated aggregates (Figures 43b, 44 and 47) which often extended from the arm plates of the pinwheels. At least one end of the CI was frequently associated or attached to host membranes, in particular the plasmalemma (Figure 43) and tonoplast (Figure 44), although in some instances CI were attached to cytoplasmic membranes (Figure 44) which are probably elements of the endoplasmic reticulum. Hollow rods, which appear as rings in cross section, were frequently present along one or both sides of the pinwheel arms (Figure 43a) and laminated aggregates (Figure 47). These hollow rods were approximately 14-18nm in diameter.

Virus-like particles were observed infrequently in the cytoplasm of D. chinensis near the vacuole. In local lesion tissue of C. quinoa, however, the VLP occurred abundantly; they were frequently associated with bridals (Figures 44, 45, 47, 48) and were often observed between two membranous layers (Figures 44, 45) particularly in cytoplasmic strands through the vacuole (Figure 48b). Membranous layers were not usually evident in oblique sections through VLP near the vacuole or through bridals in the cytoplasm (Figures 45-48). Small bridal elements were frequently circular in cross section (Figures 47, 48) and probably represent tubular structures within the cytoplasm. They appeared to contain cytoplasm but in larger bridals vacuolation was apparent and the tonoplast was visible (Figures 47, 48b).

Virus-like particles were roughly circular in cross section and approximately 10nm in diameter, which is close to the diameter of CVMV particles in sap. This strongly suggests that the VLP are particles of CVMV.

Cellular disruption was apparent in chlorotic areas of tissue in C. quinoa leaves and chloroplast grana were disorganised (Figure 48a). Osmiophilic granules were also abundant in the disrupted chloroplasts. Cells of D. chinensis infected with CVMV appeared relatively normal. No nuclear inclusions were observed in either host plant. Similar structures to those induced by CVMV were not observed in healthy plants.

Discussion

Weintraub & Ragetli (1970) did not find pinwheels in ultrathin sections of D. barbatus infected with CVMV but they did report the presence of bundles, loops and scrolls. (Laminated aggregates may have been present). Virus-like particles were also reported in association with membranous layers, particularly in the cytoplasm around the vacuole and in cytoplasmic strands across the vacuole. The authors also noted what appear to be oblique sections of hollow rods. Castro et al (1971) reported the presence of pinwheels associated with natural infection of carnation by CVMV. The findings in the present study are in agreement with the structures previously reported for CVMV.

It is particularly significant that structures induced by CVMV are analogous to those induced by other viruses of the PVY-group. Besides the diagnostic cylindrical inclusions themselves the association of the CI with membrane sheets or elements of the endoplasmic reticulum has been noted for other PVY-group viruses, for instance Datura shoestring (Weintraub et al, 1973), Atropa mild mosaic (Harrison & Roberts, 1971) and wheat spindle streak mosaic (Hooper & Wiese, 1972) viruses. The hollow rods observed in association with PW and LA in CVMV infections do not appear to be common in other PVY-group infections although they have been reported for Datura shoestring (Weintraub et al, 1973), passionfruit woodiness (Edwardson, 1974, Figure 43d), tobacco etch and water-melon mosaic (Edwardson et al, 1968) viruses. The size of the hollow rods is approximately the same as that reported for the hollow rods associated with Datura shoestring virus (ca. 17nm in diameter) (Weintraub et al, 1973). Particles of CVMV have not been observed attached to laminated aggregates as occurs with some viruses (Kamei et al, 1969) but the association of particles with membranous plates or bridals has been reported for pokeweed mosaic virus (Kim & Fulton, 1969) and celery mosaic virus (Edwardson, 1974, Figure 4b). Presence of particles in thin strands of cytoplasm has been observed for beet mosaic (Hoefert, 1969) and pokeweed mosaic (Kim & Fulton, 1969) viruses.

Hoefert (1969) suggested that the cytoplasmic strands were "formed by vesiculation and vacuolation of infected cytoplasm" and Kim & Fulton (1969) also noted vesiculation of the cytoplasm which they said "appeared to be associated with Golgi apparatus and possibly originated from them". This latter statement is supported by the association of potato virus Y around Golgi bodies in tobacco (Edwardson, 1974, Figure 47e).

Vacuolation was evident in CVMV infected tissue in the present study and appeared initially to be associated with bridals in the cytoplasm. Very small bridals (Figures 47 and 48) appeared to contain cytoplasm but larger bridals were vesiculated or vacuolated inside and the tonoplast was evident (Figures 44, 47, 48b). It is possible that these bridals and associated vacuoles enlarge until the cytoplasm becomes thin strands between vacuoles or through a single complex evaginated vacuole. These cytoplasmic strands frequently contained virus particles which in cross section are seen to occur between two membranous layers (Figures 45 and 48b). Oblique and longitudinal sections revealed the extent of the association of particles with the vacuole (Figure 46). Particles of CVMV were not observed in association with Golgi bodies.

Striated plates observed in sap from CVMV-infected hosts were not present in sap either from healthy plants or from plants infected with the other viruses reported in the present study. They are similar to the plates reported for other members of the PVY-group, for example tobacco etch and watermelon mosaic viruses (Edwardson et al, 1968) and plum pox virus (Bakel & Oosten, 1972) and the periodicity of the striations is also in agreement. The striated plates are considered to be fragments of the plates of cylindrical inclusions (Edwardson et al, 1968), a theory which is supported by electron micrographs of purified tobacco etch and potato Y virus inclusions (Hiebert et al, 1971).

The physical properties are in close agreement with those reported and the normal length is within the limits of the variation recorded for CVMV (Edwardson, 1974). Particle aggregates of CVMV observed in sap are comparable with those reported for watermelon mosaic virus (Purcifull et al, 1968).

The characterization of the virus reported here confirms the presence of carnation vein mottle virus in Dianthus plants in New Zealand.

FIGURE 37. Systemic, chlorotic vein-banding of Amaranthus viridis 6 weeks after inoculation with carnation vein mottle virus.

FIGURE 38. Systemic infection of Dianthus chinensis with carnation vein mottle virus. Note the dark stripes in the flower.

FIGURE 39. Particles of carnation vein mottle virus in sweet william (Dianthus barbatus) sap stained with AM-PTA. Mag. 81,500.

FIGURE 40. An aggregate of carnation vein mottle virus particles from local lesions of Chenopodium quinoa stained with AM-PTA. Mag. 81,000.

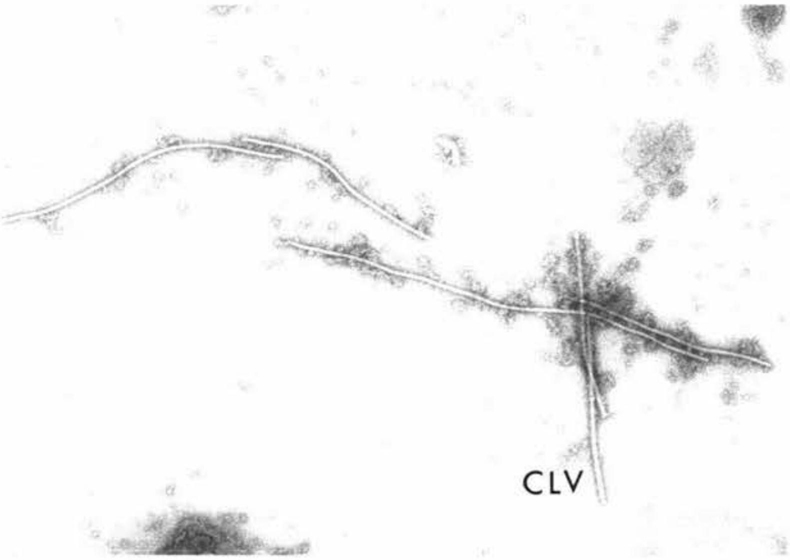


FIGURE 41. Rigid rods of carnation latent virus (CLV) and flexuous particles or fragments of carnation vein mottle virus in carnation sap stained with AM-PTA. Mag. 38,000.

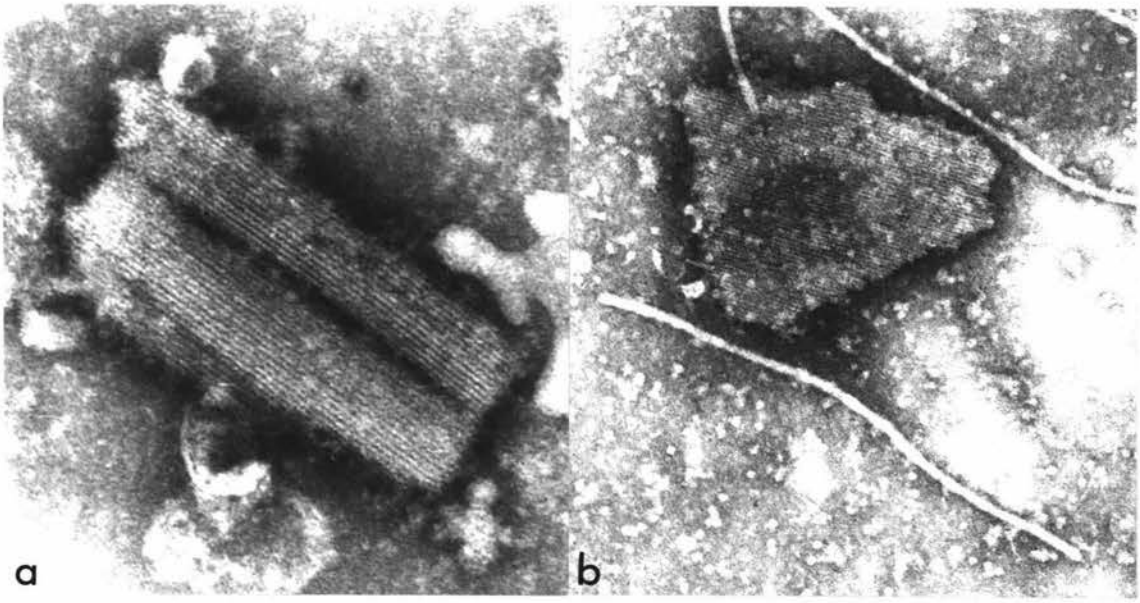


FIGURE 42. Striated plates in (a) sap from locally infected Chenopodium quinoa and (b) sap from systemically infected Saponaria vaccaria infected with carnation vein mottle virus. Stain AM-PTA. Mag. (a) 142,500, (b) 93,500.

FIGURE 43. Cylindrical inclusions in the cytoplasm of mesophyll cells of Dianthus chinensis infected with carnation vein mottle virus, (a) pinwheel with rings along both sides of the arms and a laminated aggregate extending from one arm, Mag. 226,000 and (b) bundles, including a laminated aggregate (LA) above a plasmodesma (P) in the cell wall, Mag. 53,000.

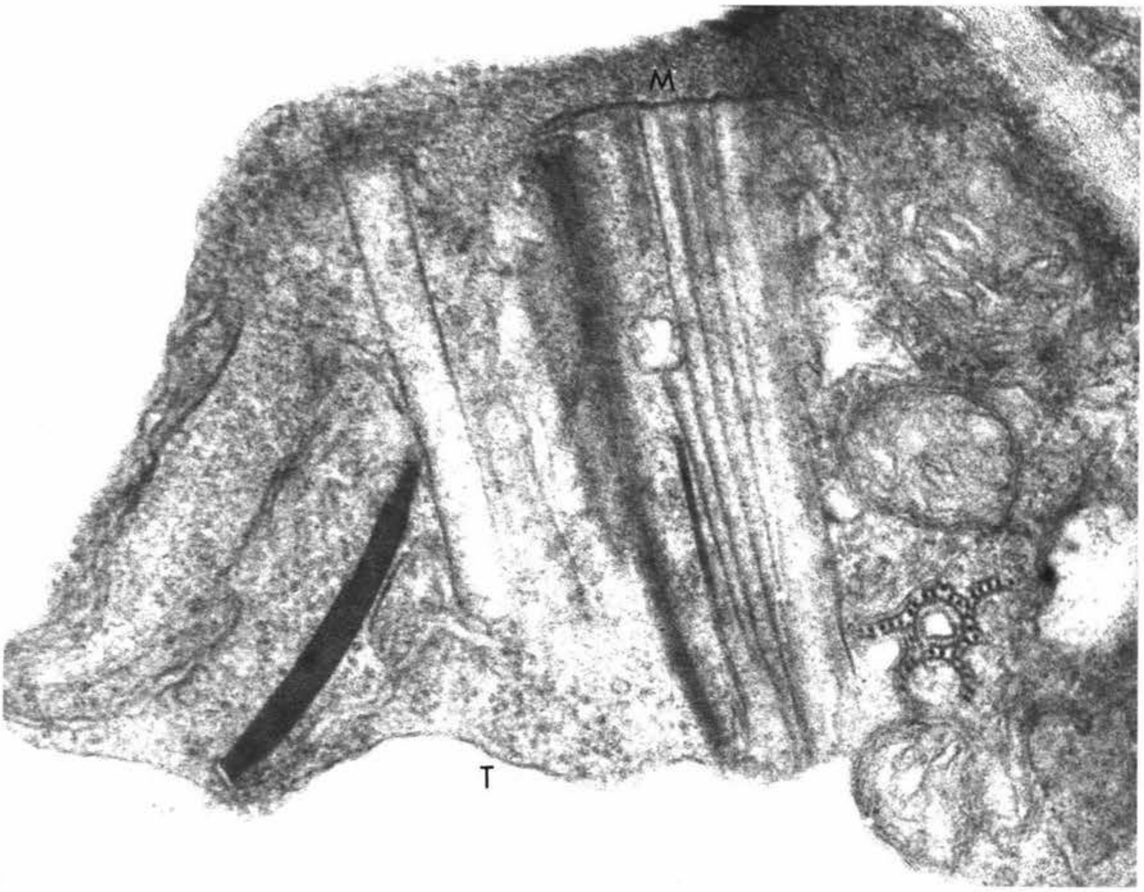


FIGURE 44. Inclusions in the cytoplasm of Chenopodium quinoa infected with carnation vein mottle virus. From left to right: laminated aggregate, tube, bundle associated with membrane (M) and tonoplast (T), and virus-like particles in cross section between two membranous layers. Mag. 79,000.

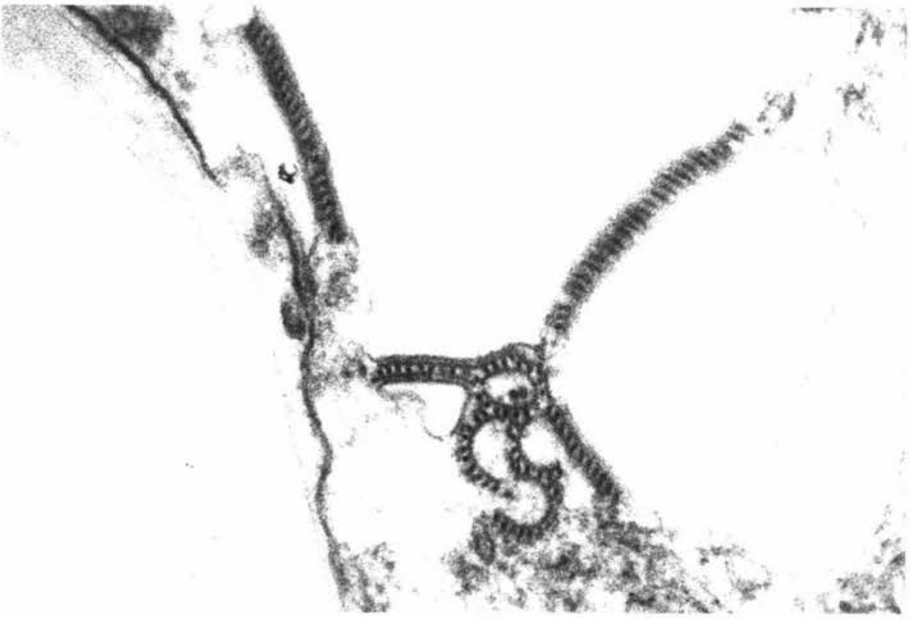


FIGURE 45. Transverse and oblique sections of virus-like particles associated with membranous structures in the cytoplasm of Chenopodium quinoa infected with carnation vein mottle virus. Note that the membranous structures are apparent mainly between the particles and the tonoplast. Mag. 91,000.

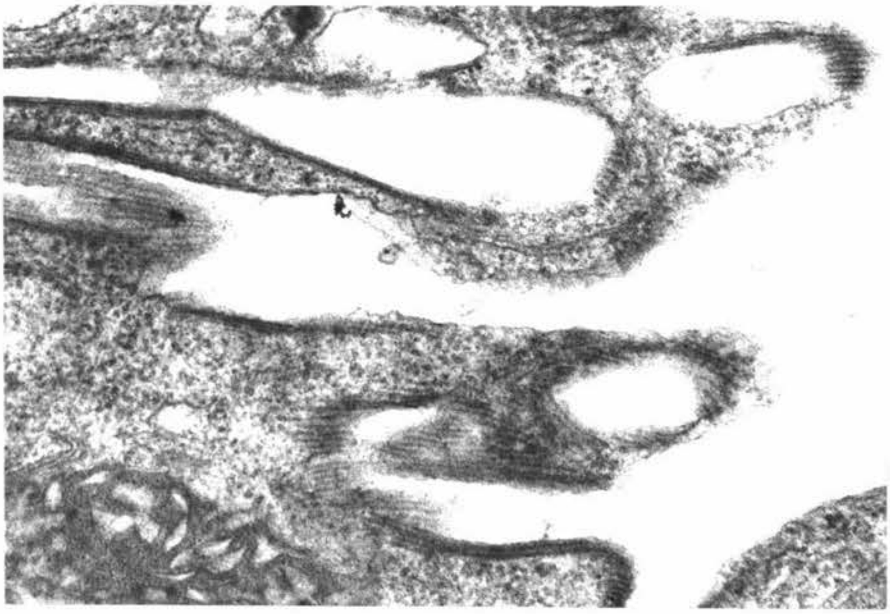


FIGURE 46. Oblique-longitudinal sections of virus-like particles occurring in the cytoplasm near the vacuole in CVMV-infected Chenopodium quinoa. Note the irregularity of the vacuolated areas. Mag. 59,000.

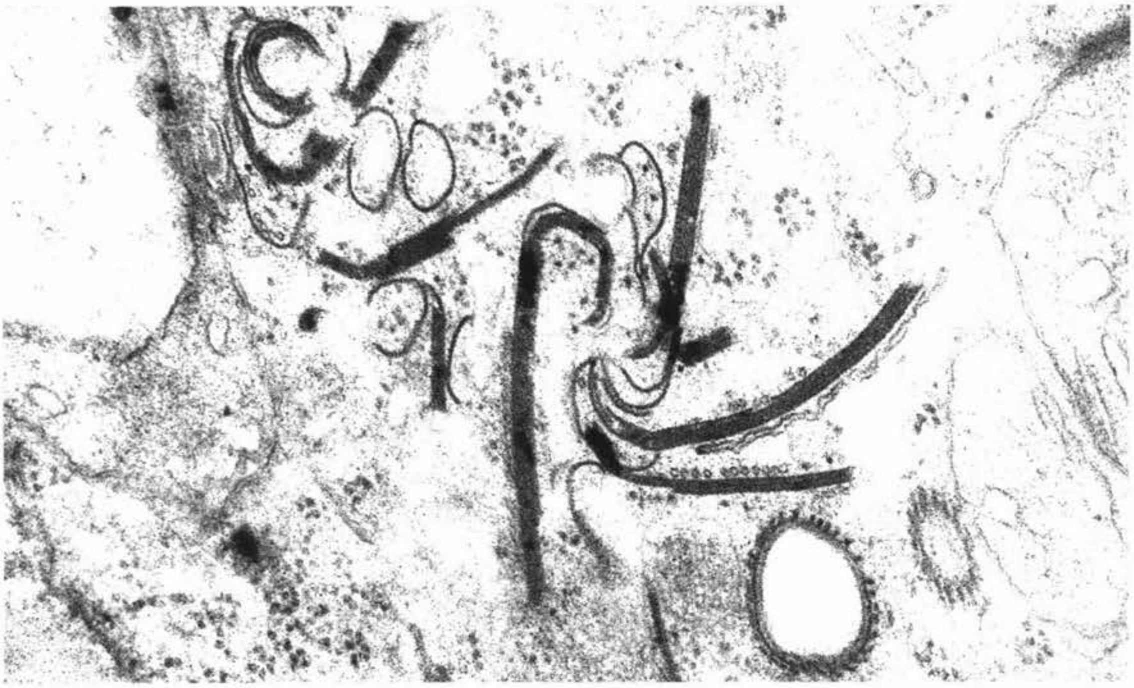


FIGURE 47. Chenopodium quinoa leaf tissue locally infected with carnation vein mottle virus showing laminated plates, some extending from pinwheel arms, loops (top left), and virus-like particles in oblique and cross section associated with bridals (bottom right). Note the rings along the laminated plate. Mag. 73,900.

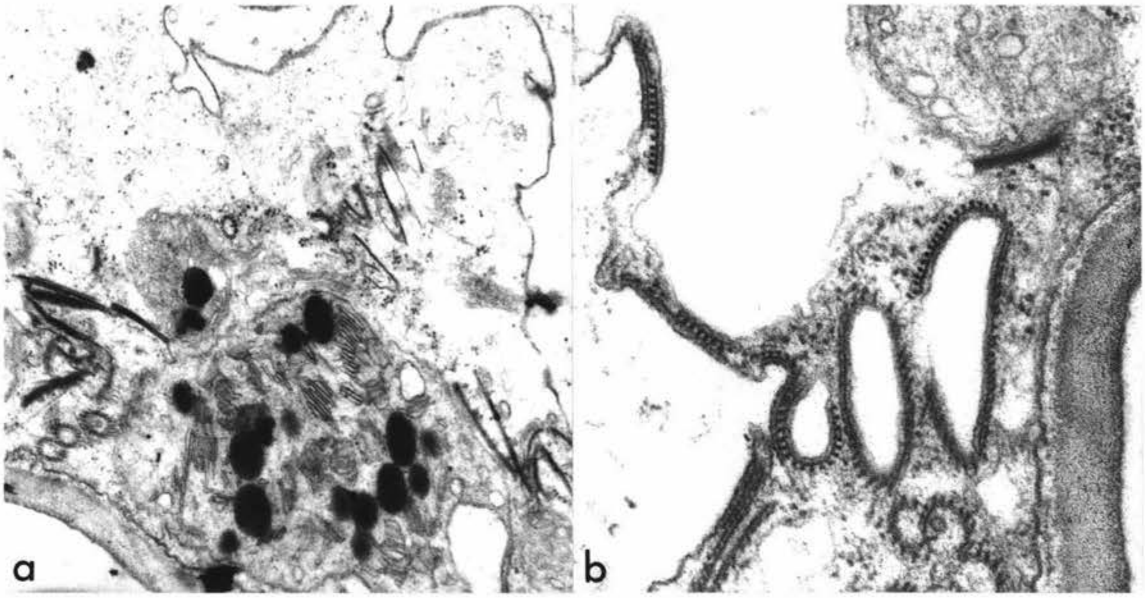


FIGURE 48. Chenopodium quinoa infected with carnation vein mottle virus showing (a) disrupted grana and osmiophilic granules in a chloroplast, Mag. 22,500, and (b) virus-like particles associated with membrane near the vacuole, Mag. 55,500.

2.9 ISOLATE D 345

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Late in the survey a virus was transmitted, using Yarwood's solution, from one Dianthus plant to spinach and G. elegans but not to C. quinoa. Subsequently the virus was sap transmitted to C. quinoa and S. vaccaria in which it caused symptoms similar to those of ArMV infection. The reaction on C. quinoa, however, was more severe than expected for ArMV. As only a few particles, approximately 30nm in diameter, were observed in sap by electron microscopy and serological tests against ArMV antiserum in gel diffusion were negative further host range studies and serological tests were conducted in an attempt to identify the causal virus.

Host range

Tissue from infected C. quinoa or N. clevelandii was used as a source of inoculum.

AMARANTHACEAE

Amaranthus caudatus: local dark brown splotches after 4 days.

Celosia argentea 'Forest Fire': local red rings after 6 days followed by latent systemic infection.

Gomphrena globosa: local necrotic rings after 3 days which became necrotic spots and target lesions (Figure 49). Systemic chlorotic spots and rings subsequently developed; some of the rings became semi-necrotic. Plants were stunted.

CARYOPHYLLACEAE

Dianthus barbatus: a few local chlorotic spots and rings were observed in one instance. No systemic infection.

Dianthus chinensis 'Bravo': not infected.

Gypsophila elegans: faint systemic chlorotic spots and mottling (ca. 9 days).

Saponaria vaccaria 'Pink Beauty': whitish necrotic rings after about 5 days followed by systemic vein chlorosis and necrosis with chlorotic and necrotic flecking.

CHENOPODIACEAE

Chenopodium amaranticolor: local necrotic dots after 3 days followed by systemic necrotic speckle of partially mature leaves, epinasty, necrosis and death of the apices and very young leaves.

Chenopodium quinoa: necrotic local lesions after 2-3 days with severe systemic necrotic speckle, epinasty and apical necrosis usually resulting in the death of the plant.

Spinacia oleracea 'Royal Denmark' (spinach): chlorotic lesions after 3 days followed by systemic mottling and stunting.

COMPOSITAE

Senecio cruentus (cineraria): chlorotic spots after 6 days developed into chlorotic target lesions (Figure 50). Systemic chlorotic vein banding, spotting and irregular rings subsequently formed. Later leaves were symptomless.

Zinnia elegans 'Persian Garden': systemic symptomless infection.

CUCURBITACEAE

Cucumis sativus 'Marketer' and 'Polaris' (cucumber): local necrotic lesions on leaves and cotyledons after 5 days. A chlorotic ring developed around lesions on cotyledons. No systemic infection.

SCROPHULARIACEAE

Antirrhinum majus (snapdragon): faint local chlorotic spots after 3 days followed by systemic chlorotic spots and rings. Infection was occasionally symptomless.

Digitalis purpurea (foxglove): dark reddish-brown local rings after 5 days followed by symptomless systemic infection.

SOLANACEAE

Nicotiana clevelandii: whitish necrotic etched rings and sunken necrotic spots after 3 days with subsequent systemic necrotic speckle, vein necrosis and stunting.

Nicotiana glutinosa: few local necrotic spots and rings after 3 days; systemic symptomless infection.

Nicotiana sylvestris: necrotic rings after 3 days; the lesions later became completely necrotic. Systemic necrotic spotting.

Nicotiana tabacum 'Havana 423' and 'White Burley' (tobacco): sunken green spots (2-3mm) after 2 days which rapidly turned necrotic. Mild systemic interveinal chlorotic and occasionally necrotic lines along the main veins, line patterns and slight distortion of the young leaves.

Petunia hybrida 'Fire Chief' and 'Rosy Morn': systemic vein clearing, chlorotic spots and mottling.

UMBELLIFERAE

Apium graveolens (celery): systemic mottling after about 9 days, the symptoms later abating.

Electron microscopy

Particles of isolate D 345 were difficult to find in sap by electron microscopy, but a few isometric particles approximately 30nm in diameter were observed in sap from N. clevelandii stained with PTA, pH 7. It therefore appears that D 345 is a virus.

Serology

Sap from infected N. clevelandii known to contain a relatively high concentration of virus by infectivity trials was tested in gel diffusion against antisera to arabis mosaic, broad bean wilt, cherry leafroll, raspberry ringspot, tobacco ringspot, tomato ringspot and tomato black ring viruses. No positive reaction resulted, but as few particles were observed by electron microscopy there may be a problem with virus stability.

Discussion

Although the virus did not react with antisera to many of the viruses in the nepovirus group, the reactions on some hosts (e.g. C. quinoa) are very similar to the symptoms reported for nepoviruses. Dianthus species do not appear to be the usual hosts of the virus as it was only isolated in one instance and it was difficult to experimentally infect other Dianthus species. The possibility exists that D 345 is related to the carnation white ring virus of Hakkaart (1974) but CWRV was reported to cause no systemic symptoms in S. vaccaria, although it caused similar local symptoms to D 345 (and also ArMV). A more detailed study of isolate D 345 is required, particularly purification, physical properties and further serological tests.

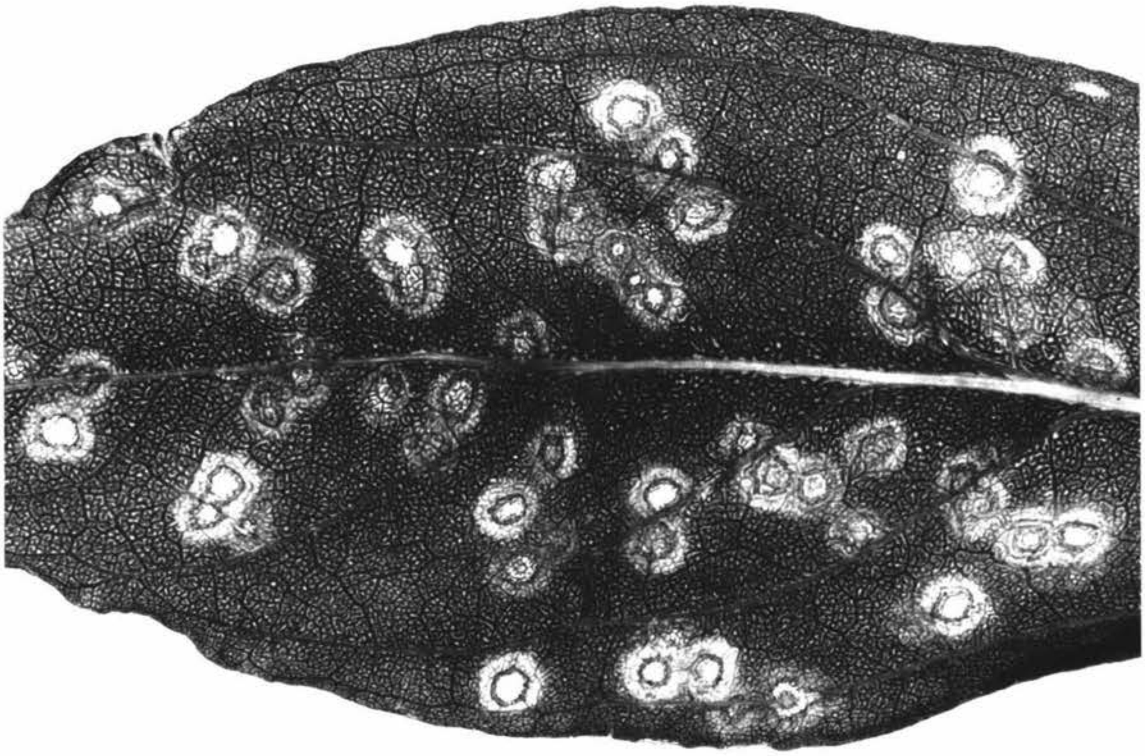

FIGURE 49. Local lesions of isolate D 345 on Gomphrena globosa 9 days after inoculation.

FIGURE 50. Local infection of cineraria (Senecio cruentus) 13 days after inoculation with isolate D 345.

2.10 UNCHARACTERIZED VIRUSES OR VIRUS-LIKE PARTICLES

A number of virus particles were observed by electron microscopy, but because of a lack of transmission or other anomalies these did not generally conform to the known viruses occurring in carnations.

(1) Occasionally isometric virus-like particles (ca. 30nm in diameter) were observed in low concentration in sap of garden carnation or Dianthus species. These particles did not appear to be transmitted to any indicator plants.

(2) In a few instances isometric particles (ca. 30nm in diameter) (Figure 51a) were observed in sap of D. barbatus inoculated with sap of Dianthus species. However, these particles did not appear to be transmissible to other indicator plants.

(3) Ultrathin sections of one Dianthus sp. revealed the presence of spherical particles about 22-25nm in diameter which had electron translucent centres (Figure 52). This particular plant apparently contained attenuated CarMV so that these particles may be the attenuated carnation mottle virion. The particles were present as aggregates in the cytoplasm or surrounding chloroplasts. Similar particles surrounding chloroplasts were also observed in leaf sections of a carnation plant.

(4) Accumulations or aggregates of electron opaque staining particles (ca. 18-23nm) were observed in the cytoplasm of ultrathin sections of a carnation (Figure 51b). The particles were occasionally associated with an electron dense matrix.

(5) On several occasions necrotic local lesions developed on C. quinoa leaves inoculated with sap from Dianthus spp. Carnation latent virus was present in all cases but systemic tissue of C. quinoa containing CLV caused no such necrotic symptoms after back-inoculation to another C. quinoa, whereas locally infected leaves caused further necrotic lesions after back-inoculation. However, it was not possible to maintain the causal agent in C. quinoa as the number of lesions rapidly decreased with serial inoculations. The causal agent was not detected in other indicator hosts even after back-inoculation to C. quinoa. No particles, besides those of CLV, were observed in sap of indicator plants but some of the original host plants contained a few virus-like particles about 30nm in diameter.

It is possible that some of the particles observed are those of attenuated CarMV, that is, besides those already mentioned in (3). Alternatively, they may be related to the two small isometric particles considered to be part of the full etched ring disease syndrome (Hollings, Stone & Bouttell, 1968). The particles mentioned in (4) appear to be similar to the small isometric particles (19-21nm in diameter) associated with an electron dense matrix reported by Castro et al (1971).

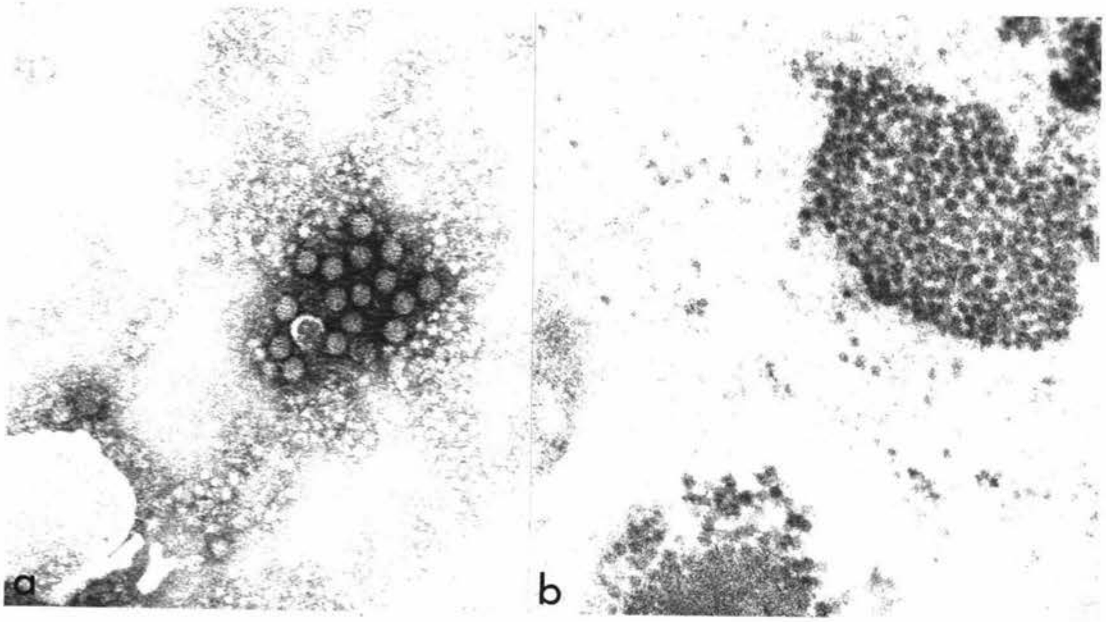

FIGURE 51. (a) Virus-like particles in Dianthus barbatus sap stained with AM-PTA. Mag. 106,000.
(b) Electron dense particles in the cytoplasm of a carnation cell. Mag. 74,000.

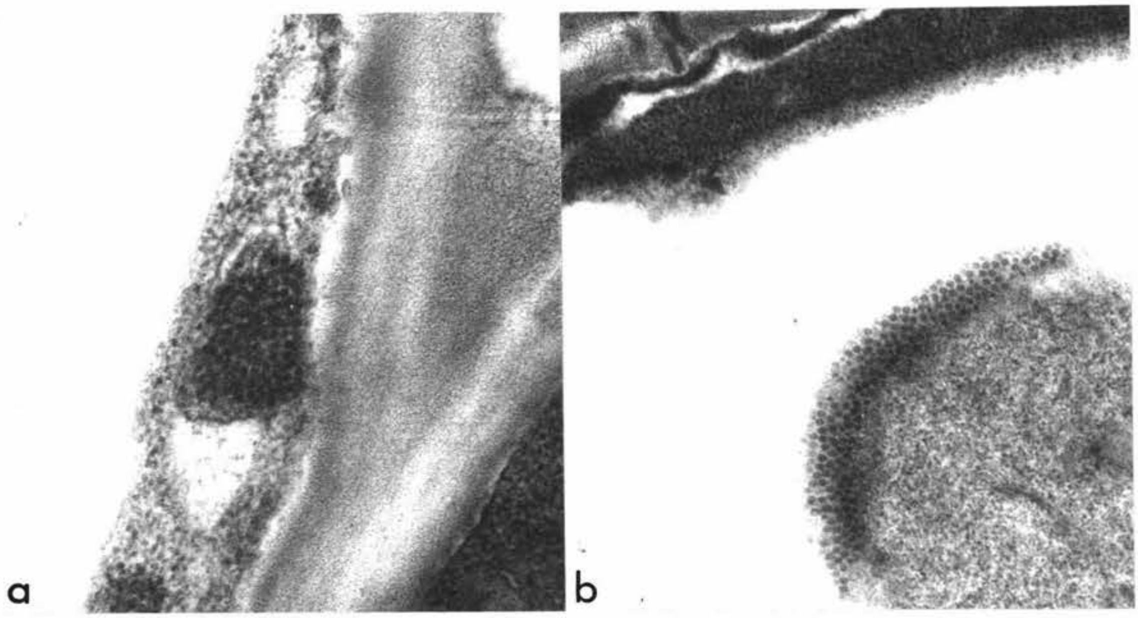

FIGURE 52. Virus-like particles in the cytoplasm of a Dianthus sp. Mag. (a) 55,000 and (b) 41,400.

APPENDIX 1

The presence of some carnation viruses in various countries.

	Australia	Belgium	Britain	Canada
CERV-50		Verhoyen (1974)	Hollings & Stone (1961, 1967)	
CLV	Sutton & Taylor (1971)		Kassanis (1955)	
CarMV	Sutton & Taylor (1971)	Goethals (1969)	Hollings & Stone (1964) Kassanis (1955)	Kemp (1964)
CRSV		Verhoyen (1974)	Hollings & Stone (1965b) Kassanis (1955)	Kemp (1964)
CVMV	Sutton & Taylor (1971)	Verhoyen (1974)	Kassanis (1955)	

	Czechoslovakia	Denmark	France	Holland
CERV-50		Kristensen (1964) Paludan (1970)		Hakkaart (1964b, 1968)
CLV				
CarMV	Mokra (1970)	Paludan (1965)	Devergne & Cardin (1967)	Hakkaart (1968)
CRSV	Mokra (1970)	Kristensen (1957) Paludan (1965)	Devergne & Cardin (1967)	Noordam et al (1951)
CVMV			Poupet (1971) Poupet & Marais (1973)	Hakkaart (1964a, 1968)

APPENDIX 1 continued

	Israel	Italy	Japan	Poland
CERV-50	Smookler & Loebenstein (1973)			
CLV			Yora & Yuki (1965)	
CarMV	Smookler & Loebenstein (1973)	Faccioli & Marani (1967)		Kochman & Kowalska (1971) Kowalska (1972)
CRSV		Bestagno et al (1959)		Kochman & Kowalska (1971) Kowalska (1972)
CVMV		Lovisolo & Luisoni (1968)		

	Spain	Sweden	United States	U.S.S.R.
CERV-50	Fujisawa et al (1971) Castro et al (1971)		Hollings & Stone (1961)	
CLV	Castro et al (1971)			Procenko & Procenko (1964)
CarMV	Castro et al (1971)	Ahman (1969)	Brierley & Smith (1957)	
CRSV		Ahman (1969)	Brierley & Smith (1955, 1957)	
CVMV	Castro et al (1971)		Brierley & Smith (1957)	Korneeva (1964)

APPENDIX 2

The incidence of detectable viruses in commercial carnations.

Varieties	Number tested	NDI*	Viruses				
			ArMV	CERV-50[1]	CLV	CarMV[2]	CNFV CYFV
Alice Sim	2					2	
Apricot sport	2			2		2	
Baurne's Yellow Sim	8			6		7+1	
Bella	2	2					
Cream Sim	16			15		16	
Crowley Sim	7			1		7	
Dusty Sim	10	1		4		9	
Esperance	15			13		15	
Evening Glow	5	1		-/2		4	
Harvest Moon	25			9/24		25	
Joker	20			18		20	
Keefer's Cherry Sim	2			2		1	
Laddie	2			2		2	
Lena	10			10		10	
Le Reve Salmon Pink	6			4	3	6	
Napier Pink	1			1		1	
Orange Triumph	14		5	7		11	
Orchid Beauty	28			19/25		27+1	

APPENDIX 2 continued

Varieties	Number tested	NDI*	ArMV	CERV-50[1]	CLV	CarMV[2]	CNFV CYFV
Persian Pink	10			10		10	
Scania Sim	24			22/23		23	
Shamrock	6			6		6	1
Shocking Pink Sim	2					2	
Sunburst	11			10/10		11	
Tangerine Sim	11	1		9	1	10	
White Sim	12			3/7		12	
William Sim	6	2		4		4	
Yellow Dusty	6			6		6	2
Yellow Sim	10			10		10	
Mixed vars.	6			5		6	3
Aviemore varieties							
Breeze	2			1/1		1+1	
Bushfire	3			2/2		3	
Chiquita	1			NT[3]		1	
Llianne	2			NT		2	
Storm	4			NT		4	
TOTAL	291	7	5	201/268	4	276+3	6
%		2.4	1.7	75	1.4	96	2

* NDI = no detectable infection

[1] Not all plants were tested for CERV-50, and where applicable numbers tested are recorded.

[2] Plus (+) numbers indicate attenuated CarMV (section 2.5)

[3] NT = not tested.

APPENDIX 3

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APPENDIX 4

MERISTEM-TIP CULTURE

Attempts to eliminate viruses from plants rely basically on meristem-tip culture and thermotherapy or a combination of these two methods while chemotherapy has been unsuccessful to date. Because carnations are an important cut flower crop around the world numerous programs have been directed at eliminating viruses from the plants and national programs are in operation in a number of countries. As a result of the number of people involved a variety of media and techniques for meristem-tip culture have been tried for carnations. Most use modifications of several basic media, including Morel's medium (Os, 1964; Quak, 1957; Stone, 1963; Vermeulen & Haen, 1964), Neergaard's medium (Stone 1963, 1968; Sutton & Taylor, 1971) and Murashige & Skoog's medium (Hackett & Anderson, 1967; Marani, 1968; Vermeulen & Haen, 1964). Heat treatment has often been used prior to meristem-tip culture (Os, 1964; Quak, 1957; Sutton & Taylor, 1971) and is reported to give better yields of apparently virus-free plants than using meristem-tip culture alone (Goethals & Hoof, 1971). Media may be either liquid (Baker & Phillips, 1962; Phillips, 1968; Stone, 1963, 1968; Sutton & Taylor, 1971) or solidified with agar (Hackett & Anderson, 1967; Os, 1964; Quak, 1957). Auxin has been used to promote root initiation (Baker & Phillips, 1962; Os, 1964; Phillips, 1968; Stone, 1963, 1968; Sutton & Taylor, 1971), gibberellin to promote shoot growth (Hoof, 1971) and cytokinin to promote growth and/or proliferation of the meristem (Hoof, 1971; Marani, 1968). Other substances, such as vitamins and cofactors, are also used in media. The pH of media is important with the best growth occurring at approximately pH 5-5.5 (Marani, 1968; Stone, 1963; Sutton & Taylor, 1971).

Research has not shown conclusively that any one medium is better than another as there are other factors involved including the time of the year when the meristem tips are taken, the state of growth of the parent plant, and the techniques used.

In the present study meristem-tip culture was conducted with shoots from seven carnation varieties: Cream Sim, Harvest Moon, Lena, Orchid Beauty, Persian Pink, Scania and Sunburst. Shoots were not surface disinfected as this is reported to provide no appreciable benefit in terms of reducing contamination of culture media (Kowalska, 1974; Stone, 1963). The trials were only preliminary tests to indicate the ease and practicability of obtaining high health carnation stock. Tests were also conducted using Phytosan (Consan 20*) (Cheo, 1970) to determine its potential as a virus eradicant.

Materials and methods

The medium is the same as that used by Sutton & Taylor (1971) and is detailed at the end of this Appendix. Leaves were removed by hand from cuttings taken in June from glasshouse grown carnation plants and the meristem-tips (ca. 0.3–0.8mm) excised with a fine-blade sterile scalpel in asceptic conditions. Tips were transferred to filter paper bridges in test tubes containing sterilized medium to which the glucose and naphthalene acetic acid (NAA; a synthetic auxin) were subsequently added. Oxoid caps were used to prevent contamination by microorganisms. The meristem-tips were grown in a 23 hour light - 1 hour dark regime at temperatures between 18–24C. Once roots had initiated shoot tips were transferred to a medium containing half the initial glucose concentration and no NAA as it inhibits root growth. When plants reached about 1cm in height they were potted into a peat-sand-fertilizer medium and maintained in a glasshouse.

Plants were indexed for virus on C. quinoa approximately 1–2 and 3–4 months after planting out. Those that were apparently free of virus were subsequently indexed by light microscopy (section 1.5) for CERV-50.

Phytosan at final concentrations of 250, 500, and 1000ppm was added to the medium after autoclaving.

Results and discussion

In a trial with 46 meristem-tips 15 plants were obtained, although several tips were discarded because of fasciation. Seven of these 15 plants were free of detectable viruses and the other 8 were found to contain mottle, occasionally as the 'attenuated' strain.

Phytosan proved to be phytotoxic even at the lowest concentration (250ppm) used. Furthermore, its ability to denature virus in vivo is doubtful as the chemical did not entirely remove the infectivity from crude carnation sap containing CarMV, even at a final concentration of Phytosan of 4000ppm. Treated sap was inoculated to C. quinoa to assay virus infectivity. Phytosan did exhibit a

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*Trade Name

precipitation effect at high concentrations (> 1000ppm).

The results of the experiment indicate that carnation meristem-tips can be fairly readily cultivated and that it is a practicable means of obtaining plants free from detectable viruses. Thermotherapy prior to meristem-tip culture would probably have increased the yield of "virus-free" plants. Tissue culture techniques now available provide a means of rapid multiplication of crops such as carnations (Hackett & Anderson, 1967; Hoof, 1971).

Sutton & Taylor's (1971) meristem-tip culture medium, a modification of Neergaard's medium:

	Initial solution	Transfer solution
$Ca(NO_3)_2 \cdot 4H_2O$	1.0 g	1.0 g
KNO_3	0.25g	0.25g
$MgSO_4 \cdot 7H_2O$	0.25g	0.25g
$KH_2\ PO_4$	0.25g	0.25g
Modified Berthelot solution	0.5 ml	0.5 ml
Glucose	40.0 g	20.0 g
Naphthalene acetic acid	10^{-3} g	-
Thiamine (Aneurine HCl)	10^{-3} g	10^{-3} g
Iron chelate	3.8 ml	3.8 ml

Make up to 1 litre and adjust pH to 4.4-5.0

Modified Berthelot solution:

$MnSO_4$	2.0 g	H_3BO_3	0.05g
KI	0.5 g	$NiCl_2 \cdot 6H_2O$	0.05g
$CoCl_2 \cdot 6H_2O$	0.05g	$ZnSO_4 \cdot 7H_2O$	0.1 g
$CuSO_4 \cdot 5H_2O$	0.05g	H_2SO_4 (conc.)	1.0 ml

Make up to 1 litre with distilled water

Iron chelate solution:

Na_2EDTA	7.45g
$FeSO_4 \cdot 7H_2O$	5.57g

Make up in separate 400 ml lots of boiling water. Stir and mix well while boiling and make up to 1 litre.

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