VIRUSES INFECTING DAPHNE IN NEW ZEALAND

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by

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ERRATA

p. 15 and 16 Table 2 : substitute under "free of detectable viruses", "0" for "1" in Leucanthe Alba,
"41" for "42" in Sub totals,
"0" for "2" in D. x dauphinii,
"68" for "71" in Totals;
substitute under "DVY (FM)", "3" for "1" in
D. x dauphinii,
"516" for "514" in Totals.

p. 35 line 19 : substitute "Dr. J.K. Uyemoto (tomato ringspot virus and tobRSV)" for "Dr. J.K. Uyemoto (tomato ringspot virus)".

p. 56 line 5 : substitute "Poth were found in Leucanthe" for "Poth were confined to Leucanthe".

p. 56 line 8 : add "Arabis mosaic virus was also found in D. x dauphinii."

p. 88 line 6 : substitute "detected in 65" of specimens" for "detected in all specimens".

p. 88 line 20 : substitute "large (4 - 5mm) or small (0.5 - 1mm) chlorotic local lesions" for "0.5 - 1mm chlorotic local lesions".

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PREFACE

The genus Daphne belongs to the Thymelaeaceae and contains both evergreen and deciduous shrubs which produce attractive, highly fragrant flowers. Plants are neat and shapely and seldom grow more than 3-4 feet high, fitting well into rock gardens or herbaceous borders.

A large number of species of this very popular ornamental are grown in New Zealand, the most common of which is Daphne odora Thunb. This latter species contains several cultivars and one (Daphne odora 'Leucanthe') is almost ubiquitous at least in the North Island. Its importance is illustrated by the fact that several nurseries visited during the study cited production figures for Leucanthe of between 5,000 and 12,000 specimens per annum and one firm has also entered the export trade. Daphne burkwoodii Turrill and Daphne cneorum L. are two other species which are also commonly grown in N.Z.

Most species of daphne are vegetatively propagated, prefer well drained, sunny situations and tolerate a range of soil pH. However, they are intolerant to dry conditions and numerous authors reflect on the often unexplained sudden collapse and death of many daphne specimens. Furthermore, cultivars of D. odora frequently exhibit severe leaf mottling and accompanying lack of vigour. Severely diseased plants are unsaleable due to their unsightly appearance and their acknowledged short life.

These factors have undoubtedly contributed to a lessening in popularity of daphnes and a recent rejection of an export consignment on the basis of foliar mosaic

symptoms has stimulated the concern of nurserymen. In view of the existence of a disease problem, considered at least in part to be caused by virus, a study on the virus aspects was commenced.

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ABSTRACT

Ten viruses were detected in one or more of 20 Daphne species and cultivars in an extensive national survey. Four of these viruses were identified as alfalfa mosaic (AMV), arabis mosaic virus (ArMV), cucumber mosaic virus (CMV) and tobacco ringspot virus (TobRSV), by a variety of methods including serology, host range and symptoms, particle morphology and vector transmission. Purification methods were also developed for AMV and CMV. Three rod viruses, daphne-tobacco mosaic virus (D-TMV), daphne virus X (DVX) and daphne virus Y (DVY), with normal lengths of 300nm, 499nm and 733 nm respectively, were also detected. Daphne virus Y was widespread in several Daphne species while D-TMV and DVX were only found in Daphne cneorum L. and Daphne odora Thunb. Daphne virus X and DVY were characterized by host range and reactions, physical properties, vector studies and a purification method was developed for DVY.

Three other viruses were partially characterized by host range and reactions. Two of these, Leucanthe Variegata virus - 1 (LVV-1) and Leucanthe Variegata virus - 2 (LVV-2), were confined to Daphne odora 'Leucanthe Variegata', while the third, Burkwoodii sphere virus (BurkSV), was only found in Daphne burkwoodii Turrill 'Variegata'. Icosahedral particles were detected in squash homogenates from plants infected with both BurkSV and LVV-1, but particles were not detected in plants infected with LVV-2.

Alfalfa mosaic virus, ArMV and CMV have been reported previously infecting Daphne species but this is the first record of TobRSV and the six other tentatively identified viruses in this genus.

Specimens of Daphne odora 'Leucanthe' and Daphne odora 'Rubra', in which no viruses could be detected, were located during the survey and these are currently being increased for distribution to the nursery industry.

CHAPTER 1

SURVEY OF VIRUSES INFECTING DAPHNE

INTRODUCTION

Although many different species and cultivars of Daphne are known (2), virus infections and 'virus-like' symptoms have been reported for only 2 species, viz., Daphne odora Thunb and Daphne mezereum L. Chamberlain and Matthews (8) reported a mosaic disease of D. odora in New Zealand which was later attributed to cucumber mosaic virus (CMV/7). Both CMV and alfalfa mosaic virus (AMV) are reported to occur in D. odora in the United States of America (20) and Europe (22,24).

In England D. mezereum is regarded as particularly susceptible to CMV (26) while recent reports refer to arabis mosaic virus (ArMV/16,18) and a soil and seed-borne virus (30) occurring in Daphne species. An uncharacterized virus (hardy primrose virus) with a wide host range has also been found in D. mezereum in Germany (17,33).

In view of the concern expressed by nurserymen in N.Z. about the 'virus-like' symptoms frequently exhibited by many daphne plants, and in particular cultivars of D. odora, a national survey was undertaken. The principle objectives of this survey were to determine:

(i) the identity and characteristics of viruses occurring in Daphne species and cultivars;

(ii) the prevalence of these viruses and their influence on plant vigour and appearance;

(iii) whether any plants free of virus were available for the establishment of a 'high-health' nucleus stock.

VIRUSES RECOGNIZED IN THIS STUDY

By the completion of this research four previously described viruses (AMV, ArMV, CMV and tobacco ringspot virus / TobRSV), and six apparently uncharacterized viruses (Burkwoodii sphere virus / BurkSV; daphne - tobacco mosaic virus / D-TMV; daphne virus X / DVX; daphne virus Y / DVY; Leucanthe Variegata virus -1 / LVV-1; Leucanthe Variegata virus -2 / LVV-2 were recognized. Two forms of DVY were recognized, namely a sap transmissible form (DVY-1) and a non sap transmissible form (DVY-2).

MATERIALS AND METHODS

Plants were propagated in a modified UC IIC mix (19) in fan-cooled glasshouses. The temperature was maintained between 17 and 24C with shading provided in summer by means of a white-wash and interior shade cloth. Plants were sprayed 3 - 4 times per month for pest and disease control using lannate and benlate.

Virus inoculum was prepared by grinding selected tissue with a pestle in a mortar containing a small quantity of celite and buffer. Inoculations to test plants were made by gently rubbing leaves with either the pestle or cotton wool budsticks dipped in virus-containing solution. Local lesions from half-leaf assays were counted within 1 - 3 days of appearing.

Electron microscopy. For indexing of plant material by electron microscopy the squash homogenate method of grid preparation (34) was used in this study in preference to the leaf dip (4) or epidermal strip (13) techniques. A small piece of tissue was homogenized in a drop of negative stain and then a 300 mesh copper grid placed face down on the solution for 1 - 2 sec before draining off excess liquid on filter paper. This technique, besides being simple and rapid, was suitable for use with both leaf and flower tissue. Grids were routinely scanned at a magnification of approximately 20,000x using a 'Phillips EM-200' electron microscope.

Particle measurements were taken from micro - graphs using 100 - 200 particles of tobacco mosaic virus (TMV) as a standard (assuming a normal length for the latter of 300 nm).

The pH's of the negative stains, phosphotungstic acid (PTA) and ammonium molybdate (AmMo), were adjusted using 2M KOH and 1M NH_4OH respectively. Single-distilled water was used for these stains but double glass-distilled water was used with uranyl acetate (UrAc).

Serology. The Ouchterlony agar double-diffusion method was used for serology. The medium consisted of Oxoid Ionagar No. 2 (0.75%) in 0.1M K_2HPO_4, with 0.01M sodium azide added as a preservative. Test patterns normally comprised 6 peripheral wells (3 mm diameter) and a central well (4 mm diameter) with 4 mm between central and surrounding wells. Leaf tissue from healthy and virus infected plants was crushed in 0.1M K-K_2 phosphate buffer, pH 7.0 (1g : 2ml). Plates were placed in a large petri dish lined with moist

filter paper and incubated at either 12 or 24 C. The development of precipitin patterns was recorded over several days.

DEVELOPMENT OF TRANSMISSION AND ELECTRON MICROSCOPY METHODS

Because the success of a virus survey is largely dependent on sensitive and reliable detection methods, efforts were made to improve transmission from daphne leaves to differential hosts following inconsistent preliminary results. Specific efforts were also made to improve detection of AMV, rod and icosahedral viruses occurring in daphne.

Transmission

Difficulty was noted transmitting viruses from daphne leaves although flowers always provided an excellent source. A series of trials revealed that reliable transmission of CMV and DVY-1 from young soft leaves was possible using 0.01M K-K_2 phosphate pH 7.0 or Yarwood's bentonite solution (0.5% bentonite plus 0.5% K_2HPO_4, pH 8.7 / 35) respectively. The Chenopodium species, Chenopodium amaranticolor Coste & Reyn. and Chenopodium quinoa Willd., were found to be the most reliable indicators for CMV when inoculated directly from daphne leaves.

Electron microscopy

The electron microscope is useful for the detection of both rod and icosahedral viruses in crude sap (13). Variable results have been recorded in the liter-

ature however on the ease of detection of viruses and their stability in various negative stains. A series of trials was therefore undertaken to compare the effect of commonly used negative stains on several viruses found infecting daphne. Standardization for comparison of different stains was achieved by grinding 0.5 cm square of tissue in a single drop of stain.

Firstly AMV was considered, as a method of detecting this virus in plant sap would enable immediate recognition on the basis of its unique particle morphology. Alfalfa mosaic virus particles have been detected in leaf dips using PTA of an unspecified pH (25) or lithium phosphotungstate and several other stains (15). Hitchborn and Hills (13) however, using neutral PTA, reported great difficulty in detecting AMV from Nicotiana glutinosa L. and Gibbs (11) could only find AMV in purified preparations after fixation with formaldehyde.

In a series of tests AMV was detected in high concentrations in local lesions from C. quinoa and Vigna cylindrica (L.) Skeels and in lower concentrations in several Nicotiana species (Nicotiana clevelandii Gray, N. glutinosa and Nicotiana tabacum L.). Neutral 2% PTA proved the poorest stain giving little contrast with plant material and at magnifications used for routine detection very few particles were found. Ammonium molybdate (2% ; pH 5.6 and 7.0) gave better results with high concentrations of AMV particles being found, although still with some difficulty (Figure 35). Sharp contrast was not achieved with AmMo but the spreading of cellular material

over the grid was always excellent. Particles were more clearly defined in pH 4.0 uranyl acetate but this stain was not favoured because of inconsistent spreading results. Furthermore, with this stain grids could only be prepared by the slower process of crushing tissue in water, draining and then staining and, as with neutral PTA, high concentrations of virus particles were not found (Figure 35). Low pH PTA (pH 3.0 and 4.0) and sodium silicotungstate (pH 6.5) gave the best results, the latter being slightly superior at low magnifications but often giving particles a woolly appearance.

The value of low pH PTA was noted by Uyemoto (pers. comm., 1973) who could not detect AMV from purified preparations stained in neutral PTA whereas in pH 4.0 PTA, particles were readily identified. Finlay and Teakle (10) also noted the value of low pH, compared to neutral, PTA finding both tobacco necrosis virus and sowbane mosaic virus were more stable in PTA at pH 3.0 and pH 4.0 than at pH 7.0.

Arabis mosaic virus and TobRSV were both detected in squash homogenates. High concentrations were found in local lesions of C. quinoa, but Vigna sinensis (Torner) Savi. (cowpea) and N. tabacum also proved useful sources. Considerable variability in the quality of staining was noted with preparations of these viruses but PTA at all values tested gave excellent contrast (Figure 22). In neutral AmMo however TobRSV was rarely observed, most particles being electron dense and only found in areas of high concentrations (Figure 22).

Daphne viruses X and Y were readily observed in

squash homogenates from daphne plants, with very high concentrations of particles being found, especially in flowers. Virus particles however were often poorly defined in preparations from this source. Results with stains were variable but 2% PTA, pH 4.0 usually gave the poorest results (Figure 44). Particles were more clearly defined in neutral 2% PTA and 2% AmMo (Figure 44). Uranyl acetate was unsatisfactory with preparations from flowers due to the formation of a black precipitate immediately upon crushing with tissue. Virus particles were also readily detected in squash homogenates of flowers which had been shadow-cast with platinum (Figure 44), a process by which rod-shaped potato viruses could be detected at greater dilutions than with negative staining (23). In the daphne study however shadow-casting was not favoured because of the time involved.

In daphne leaves rod viruses were readily detected with all stains tested.

As recently as 1966, Brandes (5) alluded to the general thinking that icosahedral viruses could not be differentiated from host plant material. Since then however, there have been many reports of the detection of icosahedral viruses in crude sap and in this study icosahedral (ArMV, BurkSV, CMV, LVV-1, TobRSV), rod (D-TMV, DVX, DVY) and bacilliform (AMV) viruses were readily detected in this source, although the type of negative stain was shown to markedly affect particle definition and spreading of material over the grid. The source plant, nature of tissue and age of infection also influenced

the detection of viruses infecting daphne. The effect of negative stains on particle definition has also been noted by other workers (3, 29).

Alfalfa mosaic virus was readily identified by electron microscopy on the basis of particle morphology but the specific identity of the icosahedral viruses found in daphne was only obtained by applying other definitive tests such as serology, vector transmission and differential host reactions.

SURVEY METHOD

An extensive survey was conducted during late winter and spring to coincide with the flowering periods of most Daphne species. Where possible unopened flowers were indexed with young soft leaves being used only as an alternative.

Representative samples of all available symptom types were collected from each site in separate plastic bags and stored at 4 C until required. Samples from more distant nurseries were mailed and invariably arrived in fresh condition.

Inoculations from daphne flowers were made using 0.01M K-K_2 phosphate buffer pH 7.0 while Yarwood's bentonite solution (35) was used with leaves. Grids for electron microscopy were prepared using neutral 2% AmMo with flower tissue and 2% PTA, pH 4.0 with all leaf tissue.

Each daphne sample was inoculated to C. quinoa and an electron microscope grid prepared concomitantly. Grids were scanned for viruses within 1 to 2 days of

preparation, and enabled identification of D-TMV, DVX and DVY (1 and 2).

Cucumber mosaic virus was detected by the appearance of characteristic rusty necrotic local lesions after 2 - 3 days in C. quinoa (Figure 4). Burkwoodii sphere virus was detected in C. quinoa by the appearance of 4 - 5mm chlorotic local lesions 7 - 12 days after inoculation, but no systemic reaction was produced. Upon reinoculation to a second C. quinoa 1mm chlorotic local lesions were produced after three days followed by systemic chlorotic blotches after 10 - 14 days. All other sap-transmissible viruses from daphne produced systemic symptoms in the primary C. quinoa, but of these only DVX (Figure 42) and DVY (Figure 39) could be identified by their characteristic reactions. The systemic symptoms of AMV, ArMV, LVV-2 and TobRSV appeared initially as chlorotic netting at the base of expanding terminal leaves or over the entire lamina of the smallest leaves. Netted areas subsequently developed a bright-yellow appearance and sometimes necrosis (Figures 6 and 7). From each C. quinoa showing the latter symptoms, systemically infected tissue was separately inoculated to a second C. quinoa with Yarwood's bentonite solution as this worker reported improved transmission with bentonite from another Chenopodium species (C. amaranticolor). High concentrations of local lesions were produced on the second C. quinoa (Figure 5) and these were used as a source of virus for electron microscopy, inoculation to further hosts and serology.

Grids were prepared using 2% PTA, pH 4.0 and examined for the presence of AMV and icosahedral viruses. As well all isolates were inoculated to a series of differential hosts to group the icosahedral viruses and separate any virus mixtures which may have been present.

Details of the differential hosts and their reactions are provided in Table 1.

Isolates reacting on differential hosts in a manner typical for ArMV were run against ArMV antisera kindly supplied by Drs. P. Fry, B. Harrison and M. Hollings. Similarly TobRSV isolates were checked against TobRSV antisera from Dr. J. Uyemoto. Leucanthe Variegata virus -1 was masked by the presence of LVV-2 with which it always occurred. The method to separate these two viruses involved reinoculating infected systemic C. quinoa tissue to a further C. quinoa as soon as net symptoms appeared. This eliminated LVV-1 which systemically invades C. quinoa more slowly. The latter virus was separated by inoculation to tobacco species in which hosts LVV-2 does not produce a systemic reaction. The survey method is summarized in Figure 1.

SURVEY RESULTS AND DISCUSSION

The results of the survey of viruses infecting daphne are illustrated in Table 2.

The results show that D. odora contains a greater number of viruses than any of the other species tested, but this may be biased by the fact that a far greater number of specimens of D. odora were indexed. Viruses only found in this species include AMV, ArMV, LVV-1, LVV-2 and TobRSV. Leucanthe Variegata viruses -1 and -2 were only found in specimens of D. odora 'Leucanthe Variegata'.

Daphne virus Y was common in several Daphne species, DVX was prevalent in D. cneorum while BurkSV was

TABLE 1 Differential hosts for daphne viruses producing systemic net symptoms in *C. quinoa*

Host	Virus			
	AMV	ArMV	LVV-2	TobRSV
Cowpea	necrotic local lesions with irregular outline; no systemic infection	chlorotic local lesions; systemic chlorosis then necrosis	no infection	ditto ArMV
Havana 423	chlorotic local lesions with etched necrotic flecks; systemic mosaic	local deep-green or yellow ring-spots; no systemic infection	symptomless local infection; no systemic infection	chlorotic local lesions; systemic chlorotic blotches or 'oak-leaf'
Nicotiana clevelandii	local chlorotic blotches; systemic vein-net	symptomless or mild systemic chlorosis	———	necrotic local lesions; systemic chlorosis and severe necrosis

DIFFERENTIATION OF VIRUSES
INFECTING DAPHNE

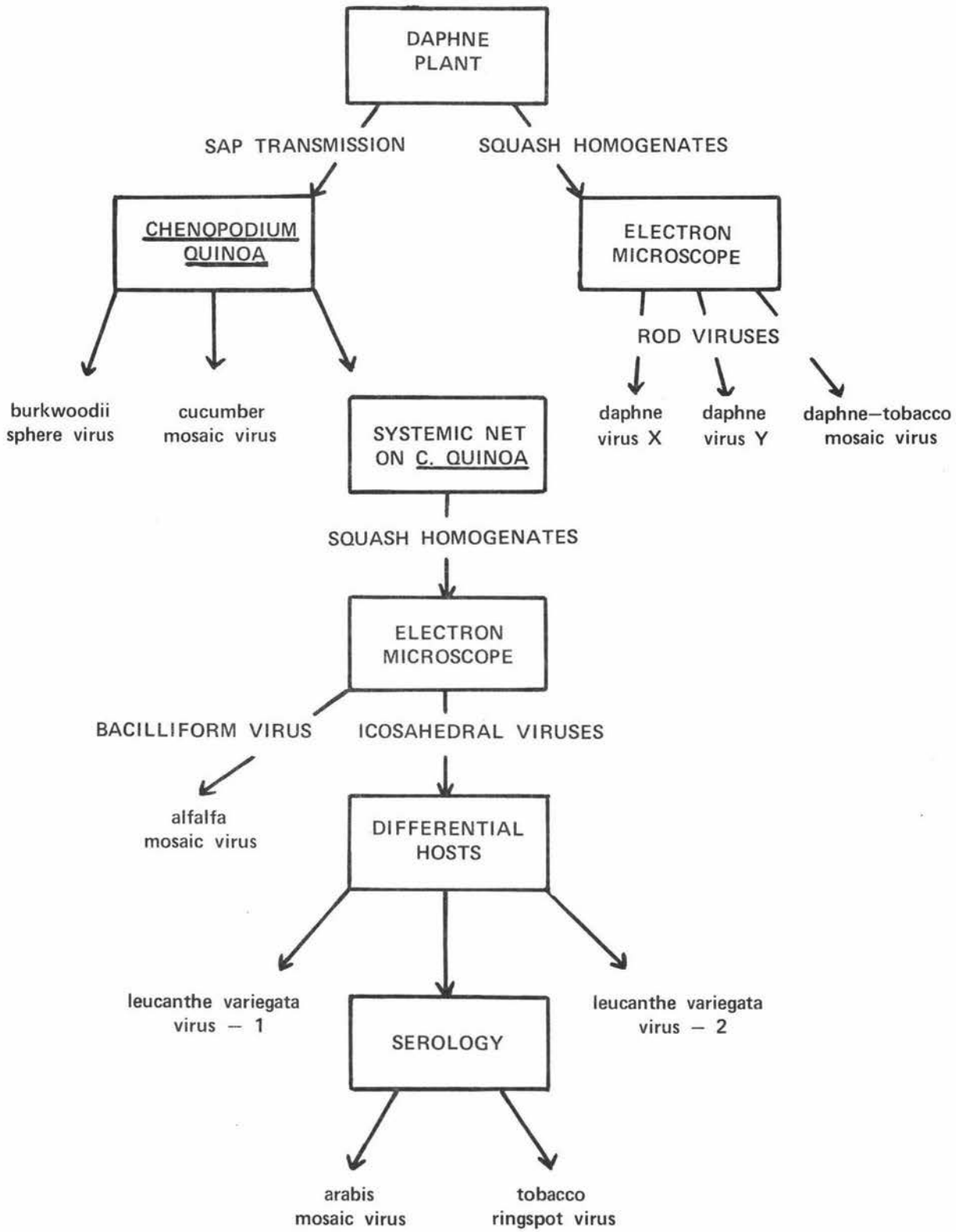

Figure 1 Flow diagram of the procedure used to differentiate viruses infecting daphne.

TABLE 2 Viruses detected in *Daphne* species and cultivars

Daphne species or cultivar	Number tested	Virus											Free of detectable viruses
		AMV	ArMV	Burk SV	CMV	D-TMV	DVX	DVY (EM)*	DVY-1	LVV-1	LVV-2	Tob RSV	
Daphne odora Thunb.													
'Leucanthe'	356	1	33		59	8	2	336	136			3	11
'Leucanthe Alba'	7				1			7					1
'Leucanthe Variegata'	59				5			27	1	3	43		8
'Rubra'	72				5			47	11				21
'Rubra Variegata'	6							5					1
Sub totals	500	1	33		70	8	2	422	148	3	43	3	42
Daphne burkwoodii Turrill													
'Somerset'	37				1			37					
'Variegata'	20			13				20					
Daphne cneorum L.													
'Eximia'	3						3						
'Major'	30					1	30	16	1				
'Variegata'	19				1		19	2					

continued over.....

TABLE 2 Viruses detected in Daphne species and cultivars - continued

Daphne species or cultivar	Number tested	Virus											Free of detectable viruses
		AMV	ArMV	Burk SV	CMV	D-TMV	DVX	DVY (EM)*	DVY-1	LVV-1	LVV-2	Tob RSV	
Daphne blagayana Freyer	2												2
D. collina Smith	1												1
D. x dauphinii Hort.	3		1					1					2
D. genkwa Sieb. & Zucc.	14												14
D. giraldii Nitsche	1												1
D. laureola L.	1							1					
D. mezereum L.	2												2
D. mezereum 'Album'	1												1
D. x neopolitana Lodd.	11							11					
D. retusa Hemsl.	7							4					3
D. tangutica Maxim.	3												3
Totals	655	1	34	13	72	9	54	514	149	3	43	3	71

*DVY(EM) - detected with electron microscope

only detected in D. burkwoodii. Cucumber mosaic virus was found in D. odora and also in single specimens of D. cneorum and D. burkwoodii. The low incidence of CMV in Rubra compared with Leucanthe suggests that Leucanthe is more susceptible than Rubra to CMV and that even with the use of 'clean' planting stock infections of this virus will still present some problems to gardeners.

Species from which virus could not be detected include D. genkwa, D. collina, D. mezereum and D. tangutica, although only a small number of samples of each were tested. A few specimens of other genera of the Thymelaeaceae, Edgworthia and Pimelia, were also free of detectable viruses.

Milbrath and Young (20) conducted a survey of 45 specimens of D. odora. They reported 100% infection with CMV and at least 33% with AMV. These results are in contrast with those from the present study, CMV occurring in only 14% of D. odora and AMV being found only once. Furthermore these authors did not detect viruses in D. cneorum.

During the survey samples were collected from both nurseries and home gardens as it was believed that a comparison of the incidence of viruses in specimens from the two sources could provide information on the origin of viruses in daphne. The results of the survey revealed a difference in virus incidence between nursery and home garden specimens of Leucanthe. No other species or cultivars were considered because of insufficient numbers. Results are presented in Table 3.

TABLE 3 Incidence of viruses in home garden and nursery specimens of Leucanthe

Source	No. of specimens tested	Virus					Free of detectable viruses
		AMV	ArMV	CMV	DVY	TobRSV	
Home gardens	63	2%	12%	57%	94%	0%	5%
Nurseries	293	0%	8%	9%	94%	1%	3%

The results in Table 3 reveal that CMV was the only virus in Leucanthe which increased appreciably in incidence (48%) in garden specimens. This result is probably not surprising considering the many sources of CMV and number of vector aphid species. On the other hand no appreciable difference in the incidence of the nepoviruses ArMV and TobRSV was detected between nurseries and home gardens suggesting that the use of 'clean' stock would provide effective control of these viruses. The high incidence of DVY in both nursery and home garden specimens of Leucanthe precludes any conclusion as to whether this virus has a major reservoir in nature, other than daphne.

Sensitivity of methods. Viruses in this survey were detected and identified using indicator plants, serology and electron microscopy.

(i) Indicator plants. These proved to be a very sensitive method for virus detection. Daphne virus Y and ArMV could be detected in C. quinoa at dilutions of 10^{-6} and 10^{-5}

respectively from daphne flowers. Chenopodium amaranticolor and C. quinoa were both found to be sensitive primary indicator hosts of CMV from daphne leaves and were among the few hosts of DVY-1. For virus identification however, C. amaranticolor does not react characteristically with DVY-1 or CMV, local lesions of the latter taking 3 to 5 days to appear. In contrast both viruses produced characteristic local lesions on C. quinoa, those of CMV appearing 2 to 3 days after inoculation. This was the only virus to react in less than 4 days thus enabling separation on time to react as well as type of reaction. Chenopodium quinoa was also favoured because it is easy to grow, has a relatively long period of sensitivity and is susceptible to a large number of 'woody plant' viruses (in this survey five viruses previously unreported on daphne were first detected on C. quinoa). Hollings (14) noted that C. amaranticolor is susceptible to a wider range of viruses than C. quinoa but that the latter contains less inhibitory material. This was an important consideration enabling direct transmission from C. quinoa to additional hosts for further characterization.

(ii) Electron microscopy. The electron microscope was essential for detection of rod viruses, as isolates of DVY-2 and D-TMV are not sap transmissible and the reactions of both DVX and DVY-1 are masked on C. quinoa in the presence of other viruses causing chlorotic local lesions. The method was very rapid, scanning of each grid requiring only 1 to 2 minutes.

In experiments with potato viruses, Sampson and

Taylor (23) reported that although test plants were the most sensitive virus indicators, rod viruses could be detected by electron microscopy in high concentrations in field material. Using their technique DVY could be observed in daphne flower sap at dilutions of 1:20,000 and the fact that this virus was detected in the great majority of Leucanthe plants indexed also suggests the method is both sensitive and reliable. Besides the sensitivity of electron microscopy for virus detection, it is also considered an accurate method for separation of rod virus groups. Brandes (5) stated that he was able to distinguish between elongated viruses whose normal lengths differed by no more than 10 - 20 nm. Also Sampson and Taylor (23) were able to distinguish potato viruses X, Y, and M on size and morphology as could Brunt and Atkey (6) with 3 filamentous viruses from narcissus. More recently however environmental conditions have been shown to affect the length and morphology of rod viruses. Moore and Guthrie (21) found that the normal length of potato virus X was influenced by air temperature with virus particles in several plants grown at 40 C 2 to 5 times longer than those in plants grown at 20 C. Govier and Woods (12) noted a marked 'environmental' influence on the potyviruses henbane mosaic virus (HMV) and bean yellow mosaic virus (BYMV). Particles of the former in extracted sap were 900 nm long and straight in the presence of magnesium but were shorter (800 nm) and flexuous when exposed to EDTA. A host-dependant variation in length of BYMV has also been noted (31).

The possibility of such length variations

accentuate the need to approach the electron microscopic identification of flexuous rod viruses with care.

Daphne viruses X and Y however, differ sufficiently in length and morphology to be confidently distinguished using electron microscopy (Figure 46). This was illustrated when fresh electron microscope grids were prepared from D. cneorum specimens which, during normal surveying, had been noted to contain DVY alone, DVX alone or a mixture of the two. One hundred and sixty-five particles from the first had a single normal length of 491 nm. Similarily 62 particles from the second had a single normal length of 751 nm while in the specimen noted to contain both viruses two modal peaks 503 nm (131 particles measured) and 739 nm (73 particles measured) occurred representing the 2 distinct virus groups. This separation was confirmed on the basis of other definitive tests (summarized in Table 8).

Antisera to AMV was not available and the virus was identified by electron microscopy from crude sap rather than by differential hosts as different strains vary widely in their host range and reactions.

Limitations of survey method. Although sensitive detection methods were used in this survey there are several limitations affecting the results obtained. Firstly the methods were originally designed for detection of AMV, ArMV, CMV and DVY these being the viruses previously reported in Daphne species or initially found in this study. However as other viruses were detected the method was altered to finally include BurkSV, D-TMV, DVX, LVV-1, LVV-2 and

TobRSV. Daphne virus X was recognised late in the survey and prior to this, electron microscope grids were scanned only for 750 nm rods, shorter particles being considered as fragments. Daphne-TMV was also found late in the survey and occurred in such low concentrations that it was undoubtedly overlooked many times, as was the complex of LVV-1 and LVV-2 which was elucidated after the survey with only a few specimens being rechecked for the presence of either or both viruses. A similar situation occurred for BurkSV.

Secondly the use of single primary indicator hosts and electron microscopy would not necessarily detect non sap-transmissible viruses, other microorganisms or icosahedral viruses or strains of such viruses not infectious to C. quinoa. For example isolates of ArMV from Cyphomandra betacae (Cav.) Sendt. (tamarillo) were not infectious to C. quinoa (32) and TobRSV is reported to be rarely systemic in C. quinoa (27, 28) and thus may be more prevalent than the figures in Table 2 indicate. Isolates of AMV, ArMV, LVV-1 and LVV-2 not systemic in C. quinoa would also have been overlooked.

Finally it is possible that viruses could have been missed in this survey due to variability in sensitivity of individual C. quinoa plants or because distribution of virus in the source plant was incomplete (a phenomenon known for other woody plants).

VIRUS SYMPTOMS IN DAPHNE

Because no virus-free plants were available for inoculation with a known virus or virus combination, all

comments on symptoms are based on the correlation between indexing results and the symptoms of the plants in question. During the survey symptom types were recorded from each source together with details of whether the plant was growing in full sun or shade.

With few exceptions virus disease symptoms were only noted on cultivars of D. odora. A range of leaf symptoms was noted on virus-infected Leucanthe plants and these were categorized into four types, namely:

(i) symptomless - no obvious symptoms, leaves long and straight;

(ii) mild mosaic - leaves with a few pale-green streaks or blotches;

(iii) severe mosaic - light-green areas over most of each leaf blade giving a regular mosaic and in some cases scattered orange blotches. Usually 1 - 2mm sunken spots, chlorotic streaks (sometimes between normal-green lateral veins and at other times as 1 - 2mm wide lateral vein bands) or irregular chlorotic blotches and rings on diseased leaves. (Figures 2 - 3).

(iv) necrosis - necrotic flecks occasionally found, usually in centre of light-green or orange blotches (Figure 3).

Flower symptoms on severely affected plants occurred as a considerable reduction in flower size together with a loss of flower brilliance and unevenness of colour in the form of off-white or grey blotches on the petals.

Plants often displayed varying degrees of decline including a general reduction in leaf size and thin leathery leaves (generally associated with severe mosaic), leaf curling, excessive defoliation during autumn and winter and death of single limbs or whole plants. Flowering on declining plants was often delayed with production of small pallid flowers which failed to open completely.

Often symptoms were most striking in growth associated with the spring flush or in other cases during the hot summer months. In both cases symptoms were noted to recede partially or completely during winter.

Daphne odora 'Rubra' plants usually showed symptoms similar to those on Leucanthe. Slight twisting and rugosity of leaves was common on this cultivar and leaves were often small and narrow with irregular margins.

A similar range of symptoms was noted by other workers. Milbrath and Young (20) found virus in symptomless and diseased plants while Chamberlain and Matthews (8) reported virus only in plants displaying symptoms. The latter authors also noted that symptoms were associated with young growth and Chamberlain (7) recorded stunting of plants, rosetting near the shoot apices and twisting and distortion of the leaves. In the present study Rubra specimens free of detectable viruses also displayed twisting which was usually associated with the presence of aphids. The severe symptoms recorded on D. mezereum by Smith (26) which included distortion and necrosis were not found but single branchlets of only 3 specimens were observed.

An attempt to correlate symptom types on D. odora

with particular viruses was restricted as DVY was the only virus commonly occurring alone. Other viruses almost always occurred in combinations as illustrated in Table 4.

TABLE 4 Virus combinations in Leucanthe and Rubra

Cultivar	Number tested	Virus combinations						Free of detectable viruses
		CMV	DVY	ArMV DVY	CMV DVY	ArMV CMV DVY	CMV DVY TobRSV	
Leucanthe	354	3	263	25	43	10	2	11
Rubra	72	4	40	3	1	0	0	24

The predominance of plants infected with only one virus (DVY) possibly reflects roguing or death of the most severely diseased. Plants infected with DVY-1 alone and grown in an unshaded environment often showed a light green mosaic, blotches and streaks (Figure 2). Plants infected with DVY-2 alone however usually displayed only mild symptoms or were symptomless (most species other than D. odora only contained DVY-2 and lacked symptoms). Often plants with the most severe mosaic symptoms and those plants in the state of decline were infected with CMV (Figure 3). In one instance, for example, where 8 D. odora Leucanthe plants were growing in close proximity to each other, 6 plants infected with CMV and DVY-1 were much more severely diseased than two plants infected with DVY-1 alone.

Necrotic flecks and orange blotches on leaves

and blotches on flowers could not be associated consistently with any particular virus and a totally consistent correlation between virus and symptoms was not found. Furthermore in one instance a single D. odora plant had light-green mosaic on leaves of one limb but no virus could be detected.

While recognising that decline symptoms in daphne could be caused by other factors such as fungal root rots (e.g. phytophthora), agents such as mycoplasmas or poor cultural conditions (e.g. water stress), it is highly likely that virus does play an important part in the development of these conditions.

Environmental influence on symptoms

Daphne species are considered by some writers to prefer cool or shaded conditions (1, 9) and it was in this habitat that plants often exhibited mild symptoms or were symptomless. For example, in one nursery Leucanthe plants infected with DVY-1 alone and maintained in a permanently shaded greenhcuse, produced large symptomless leaves and flowers. However cuttings propagated from this source and subsequently grown in open fields exhibited severe leaf mosaic and indexing again only revealed DVY-1.

A comparison of the degree of severity of foliar symptoms in Leucanthe plants grown in shade with those in full sun is presented in Table 5.

TABLE 5 Effect of growth situations on the symptom expression of Leucanthe infected with DVY-1[a]

Situation	Total plants tested	Severity of foliar symptoms	
		Symptomless or mild	Severe
Shade[b]	84	93%	7%
Sun	105	0%	100%

[a] plants infected with DVY-2 were excluded as they were symptomless in all environments.

[b] less than 1 - 2 h direct sunlight per day

These results indicate a strong correlation between degree of symptom severity and growth situation and emphasize that symptoms are an unreliable guide to the virus status of a plant.

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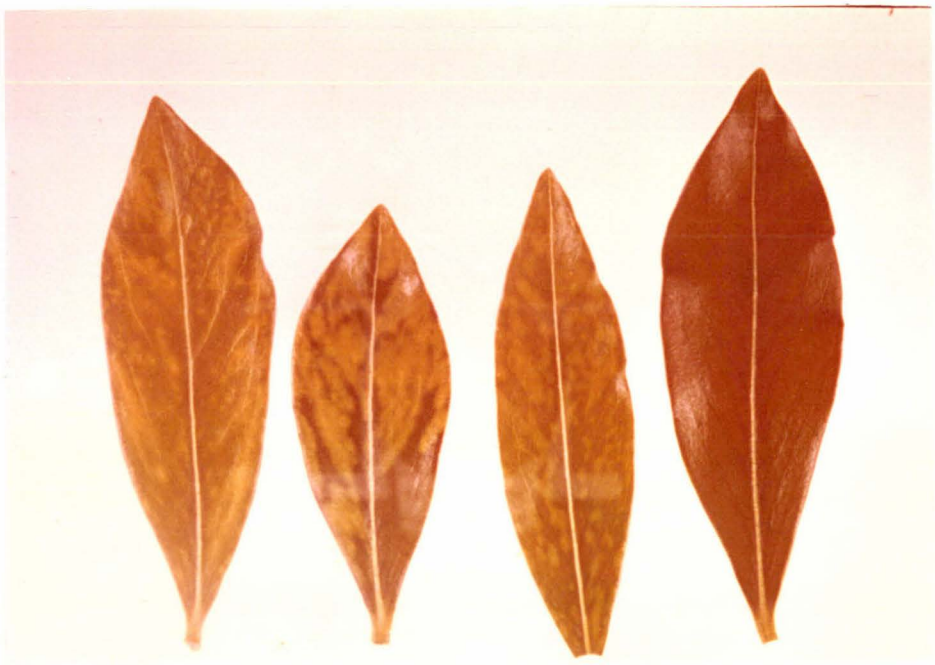


FIGURE 2: Leucanthe leaves infected with DVY-1. Symptomless leaf on right from shaded portion of same plant.

FIGURE 3: Leucanthe leaves infected with DVY-1 and CMV.

FIGURE 4: Local lesions of CMV in C. quinoa, three days after infection.

FIGURE 5: Local lesions of LVV-2 in C. quinoa, five days after infection. Identical symptoms are produced by AMV, ArMV, DVY-1 and TobRSV.

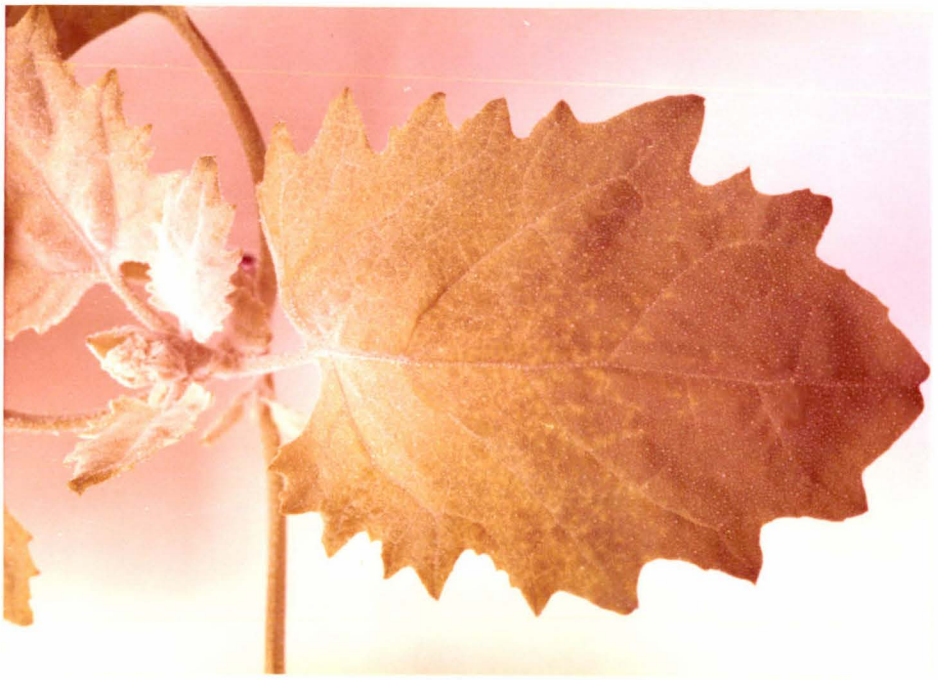


FIGURE 6: Systemic net reaction produced by LVV-2 in C. quinoa seven days after infection.

FIGURE 7: Systemic necrosis on the same plant 10 days after infection.

Identical symptoms are produced by AMV, ArMV and TobRSV.

CHAPTER 2

CHARACTERIZATION OF VIRUSES INFECTING DAPHNE

The viruses found in various Daphne species during the present study are the subject of this section. Each was characterized by various combinations of tests involving host range, particle morphology, vector transmission, physical properties and serology. General materials and methods common to the various sections are presented herein.

Materials and methods

Propagation and inoculation of plants, serology and electron microscopy are as for Chapter 1.

The antisera for serological tests were kindly supplied by: Drs. P.R. Fry; R.D. Harrison; M. Hollings (ArMV): Drs. E. van Slogteren; J.K. Uyemoto (CMV): Dr. J.K. Uyemoto (Prunus necrotic ringspot virus): Dr. J.K. Uyemoto (tomato aspermy virus, chrysanthemum isolate): Dr. J.K. Uyemoto (tomato ringspot virus).

Centrifugation for purification was conducted using an 'MSE Superspeed 65' centrifuge. Unless otherwise stated purified preparations were examined in the electron microscope after mixing 1:1 with negative stain and nebulizing onto grids.

Aphids for vector studies were maintained on Chinese cabbage [*Myzus persicae* (Sulz.)] or *C. quinoa* [*Aulacorthum circumflexum* (Buckt.)]. They were removed from plants with a small, moist artists brush and placed on moist filter paper in petri dishes for preacquisition starvation.

CUCUMBER MOSAIC VIRUS (R/1 : 1/18 : S/S : S/Ap)

Cucumber mosaic virus is a member of the cucumovirus group which includes tomato aspermy virus (including chrysanthemum strains) and peanut stunt virus. At least some strains of each virus are distantly serologically related (97) and all have icosahedral particles (ca. 30nm), a thermal inactivation point of 60 - 70C, a longevity *in vitro* of a few days and a very wide natural host range. The viruses are transmitted in a non-persistent manner by many species of aphid (53).

In this study CMV was commonly detected in *D. odora* and on single occasions in *D. cneorum* and *D. burkwoodii* respectively. The virus was identified from results on particle morphology and size, aphid transmission, host range and reactions, and serology as reported in the following sections.

Host range

Seven isolates of CMV from daphne were each inoculated to differential hosts. Tobacco leaves ground in 0.1M K-K_2 phosphate, pH 6.0 (29) were used as inoculum.

Caryophyllaceae

Dianthus barbatus L. 'Indian Carpet' (sweet-william): off-white or fawn necrotic specks (2-3 days) which expand to 5-10mm lesions composed of concentric fawn rings (Figure 9). Mature lesions assume a 'target-spot' appearance. No systemic infection.

Chenopodiaceae

Beta vulgaris L. 'Yates Early Wonder' (red beet): some isolates produce discrete chlorotic local lesions (6 days) clearly defined by a reddish ring which becomes diffuse. Other isolates produce mild chlorotic blotches. No systemic infection.

Chenopodium amaranticolor Coste & Reyn.: when in high concentrations 0.5-1mm chlorotic local lesions containing pinpoint white necrotic centres are produced (3-5 days). When scattered these appear as 2-3mm necrotic local lesions with a chlorotic halo. No systemic infection.

Chenopodium quinoa Willd.: light-yellow or orange lesions on inoculated leaves on upper parts of the plant (2-3 days) or slightly depressed 1mm water-soaked necrotic spots on first produced leaves which quickly become necrotic (2 days). The chlorotic lesions always contain rusty necrotic areas which exist as a small irregularly shaped central dot, a ring near the periphery, or may include most of the lesion leaving only a chlorotic halo (Figure 4). Tip half of leaves often becomes dessicated with an advancing chlorotic band. No systemic infection.

Spinacea oleracea L. 'Royal Denmark' (spinach): different isolates produce diffuse chlorotic local lesions (5-7 days) which become necrotic or mild local chlorosis. Systemic interveinal chlorosis (6-8 days) with normal green veinlets giving a net-effect. Leaves become twisted and strap-like.

Curcubitaceae

Citrullus vulgaris Schrad. 'Cannonball' (watermelon): 3-4mm necrotic local lesions with a spreading chlorotic halo. No systemic infection.

Cucumis melo L. (cantaloupe): chlorotic local lesions (2-3mm) on cotyledons (3-4 days) expanding to give general chlorosis. Systemic pale-green flecks and blotches (5-6 days).

Cucumis sativus L. 'Marketer' and 'Short Green Prickly' (cucumber): diffuse chlorotic local lesions on cotyledons (4 days) expanding to 5-6mm or more and extending along veins causing chlorotic vein-banding. Systemic symptoms (8-12 days) including vein-clearing and pale-green or bright-yellow blotches.

Cucurbita maxima Duch. 'Buttercup' (pumpkin): faint chlorotic or necrotic local lesions on cotyledons (7-10 days) expanding to 5-6mm blotches or rings. Most isolates rarely or never produce systemic symptoms but when produced occur as large orange blotches with leaf twisting, distortion and stunting.

Cucurbita pepo L. 'Small Sugar' (pumpkin): 2-3mm chlorotic local lesions on cotyledons (4-7 days). Systemic light-green flecks which develop into large chlorotic blotches. Distortion and downcurling of leaves.

Momordica balsamina L. (balsam-apple): chlorotic local lesions (5-6 days) expanding to 5-10mm. No systemic infection (3 CMV isolates tested).

Leguminosae

Dolichos lablab L.: large chlorotic local blotches or rings (3-6 days). Some isolates produce systemic mild mosaic with extreme reduction in leaf and plant size.

Phaseolus vulgaris L. 'Black Turtle', 'Red Kidney' and 'Scotia' (French bean): pinpoint necrotic local lesions (2-3 days). No systemic infection.

Pisum sativum L. 'Bonneville' (garden pea): different isolates produce chlorotic or necrotic local lesions (5-6 days) or no local reaction. Systemic symptoms include chlorotic blotches, necrotic flecks, blackening of the stem (usually beginning about 1mm from the tip) and plant death.

_____ Greenfeast: necrotic rings or irregular patches of veinlet necrosis on inoculated leaves (4-6 days) which become dessicated and abscise. Mild systemic mottle which later recedes or is followed by apical necrosis and death.

Vigna cylindrica (L.) Skeels: reddish-brown pinpoint local lesions (2-3 days). No systemic infection.

Vigna sinensis (Torner) Savi. 'Blackeye' (cowpea): water soaked or necrotic, slightly depressed lesions on inoculated cotyledons (2-3 days) which expand to 1mm dark reddish-brown local lesions with a regular outline. No systemic infection.

Solanaceae

Capsicum frutescens L. 'Sweet Capsicum' (pepper): inoculated leaves are either symptomless or produce chlorotic vein-banding or blotches. Mild systemic chlorotic blotches, downcurling of leaves and stunting of growth.

Lycopersicum esculentum Mill. 'Potentate' (tomato): marked interveinal chlorosis is produced by some isolates followed by slight then severe strapping of leaves but some other isolates produce only mild chlorosis.

Nicotiana clevelandii Gray: inoculated leaves become slightly puckered (4 days). Some isolates produce faint chlorotic local lesions. Systemic chlorotic vein-net or yellow-green cast (5-10 days).

Nicotiana glutinosa L.: different isolates produce scattered chlorotic local lesions (5-8 days) or no local reaction. Systemically infected areas (8-12 days) are light-green or yellow-green with prominent chlorotic veinlets giving a vein-net effect.

Nicotiana tabacum L. 'Havana 423' (tobacco): 3-4mm chlorotic local lesions (4 days) with irregularly scattered etched necrotic dots. Systemic chlorotic vein-banding and blotches (6-9 days) which develop into a severe mosaic. Some isolates also produce scattered etched dots in systemic areas. Later leaves symptomless.

_____ Samsun: 1cm chlorotic blotches (4 days). Etched necrotic dots on older inoculated leaves often forming a pattern of one or more concentric rings (Figure 10) while younger inoculated leaves often display only chlorotic blotches or rings. Systemic symptoms of interveinal chlorosis (6-9 days) with light or dark-brown etched dots. Later leaves symptomless.

_____ White Burley: yellow local lesions (3-4 days) expanding to 1cm or more in diameter as blotches or rings. With some isolates systemic chlorotic blotches appear, often centred on minor veins, and expand to produce a general mosaic; other isolates produce large chlorotic rings sometimes centred on major veins. Later leaves symptomless.

Petunia hybrida Vilm. 'Rose of Heaven': symptomless infection or faint chlorotic local lesions (4-6 days). Mild systemic chlorotic mottle (8-10 days).

Symptomless infection was produced by: Apium graveolens L. 'Dewcrisp Green' (celery); Lactuca sativa L. 'Calmar' and 'Webbs Wonderful' (lettuce); Primula malacoides Franch. 'Carmine Pink'; Primula obconica Hance; Zinnia elegans Jacq. 'Cactus Flowered'.

No infection was detected in: Brassica oleracea botrytis L. 'Deepheart' (cauliflower); Brassica oleracea capitata L. 'Drumhead (cabbage); Brassica pekinensis (Lour.) Rupr. 'Chi-Hi-Li' (Chinese cabbage); Dahlia pinnata Cav. 'Unwins Ideal'; Dianthus caryophyllus L. 'Single Pinks'; Dolichos biflorus L. ; Matthiola incana (L.) R.Br. 'Nice Giants Beauty' (stock); Phaseolus vulgaris L. 'Blue Lake' (French bean); Vicia faba L. 'Atlas Early', 'Coles Early' and 'Exhibition Longpod' (broad bean).

Aphid transmission

Two isolates of the virus were successfully transmitted by M. persicae, one from young soft leaves of Leucanthe and the other from systemically infected Havana 423 leaves, to cucumber and Havana 423 respectively. Aphids maintained on Chinese cabbage were given a pre-acquistion starvation of 24 hours and then a 1 - 2 minute acquistion period.

Purification

A number of problems have been noted by previous workers attempting to purify CMV and because of virus instability different strains often require different procedures (45, 76). Critical factors influencing the success of CMV purification include the virus source, age of infection, extraction medium, clarification method and resuspension medium. The well known tendency of the virus to aggregate upon extraction (83) and to remain attached to cellular material (45), is undoubtedly influenced by environmental conditions during the different stages in extraction, clarification, concentration and in turn affects the yield and infectivity of purified preparations.

Most workers have used tobacco species as a virus source (45, 80, 82). Hollings et al. (45) found the choice of host species was very important for successful purification of high concentrations of virus. These workers could not purify several isolates of CMV from N. glutinosa and only obtained high yields from N. clevelandii.

The importance of age of infection was demonstrated by Tomlinson et al. (93) with a rhubarb strain of CMV in N. tabacum and N. rustica, where peak infectivity was reached 10 - 12 days after inoculation and then declined very rapidly.

Various extraction buffers and additives have been tested. Tomlinson et al. (94) found greater infectivity in extracts in 0.5M K-K_2 phosphate, pH 7.0 than in 0.05M K-K_2 phosphate, pH 7.0 with CMV-Y and since then

high molarity buffers (with a pH near neutrality) have been used in most CMV purification methods. Tomlinson (92) and Scott (80) recorded greater infectivity when extracting in phosphate rather than borate, and citrate has also given useful results (75, 80). Reducing agents have been included to prevent CMV inactivation by leaf polyphenol oxidase systems (41) and chelating agents including citrate, sodium diethydithiocarbonate (Na DIECA) and ethylenediamine tetraacetic acid (EDTA / 93). Tomlinson et al. (93) reported no infectivity of preparations extracted in phosphate without both EDTA and reducing agents, and little infectivity with thioglycollic acid (TGA) alone. However, Hollings (45) found no marked benefit from adding EDTA.

Organic solvents are generally used for clarification and possibly affect the release of virus from cellular material. Scott (80) found that little infectivity of preparations of CMV-Y was retained if the chloroform clarification step was omitted while Hollings et al. (45) recorded increased infectivity by incubating expressed sap with 8.5% n-butanol for up to 14 days. Tomlinson et al.(93) however noted a lowering of infectivity of their rhubarb isolate of CMV when exposed to n-butanol for one hour as compared to infectivity after treatment with chloroform or diethylether. Acidification has also been used for clarification (38) and is more gentle than organic solvents.

Ultracentrifugation is commonly employed for concentration of the virus and pellets have been resuspended in a variety of media. Several workers have apparently disregarded aggregation problems, dilute phosphate being

used for resuspension (64, 94) but Scott (80) overcame the problem by dialysis of the virus-containing extract before high speed centrifugation and by resuspension in dilute borate buffer pH 9.0. The value of EDTA in preventing aggregation and preserving infectivity was demonstrated by Takanami and Tomaru (88) who concluded that aggregation was mainly attributable to the presence of small amounts of free divalent cations in plant sap and this could be completely inhibited by EDTA. Since then EDTA (with high pH borate buffer) has been employed for resuspension (71, 93).

Several daphne isolates of CMV were used to avoid the possibility of variation in sensitivity of different isolates to purification procedures. Scott's method (80) has been employed by several other workers (30, 88) but was not attempted with the daphne isolates because preparations are still coloured (pers. comm., K.S. Milne) contain antibodies to healthy sap (30) and because the process of dialysis is wasteful of virus. Purification was first attempted using the acidification method of Grogan et al. (38) from cucumber, Small Sugar pumpkin or Samsun. The slightly coloured pellets after high speed centrifugation were resuspended in dilute phosphate buffer (0.01M K-K_2 phosphate, pH 7.0) and examined in the electron microscope. Because preparations contained very few virus particles and had only residual infectivity, regardless of the host source tested, the procedure was discontinued.

The method finally adopted is summarized in Figure 8 and involved a slight modification of a procedure recommended by Uyemoto (K.S. Milne, pers. comm., 1973). His

PURIFICATION OF CUCUMBER MOSAIC VIRUS

Infected Havana 423 leaves

HOMOGENIZE

(1 gm : 1.5 ml)
0.5 M $K-K_2$ phosphate pH 7.1
plus 0.01 M Na DIECA
and 0.1% TGA;
filter/muslin cloth.

FILTRATE — **pulp***

Add chloroform (1 : 1);
emulsify in blendor (1 min);
shake 1 h at 4C (at 5 min intervals)
centrifuge 12,000 g / 10 min.

SUPERNATANT — **pellet***

Filter / glasswool;
centrifuge 76,000 g / 2 h.

PELLET — **supernatant***

Resuspend, 0.001M EDTA or
0.005M H_3BO_3 plus 0.005M
EDTA (adjusted to pH 9.0 with
1M NaOH) for 1 h on magnetic
stirrer at 4C;
centrifuge 5,000 g / 10 min.

SUPERNATANT — **pellet***

2 further cycles differential
centrifugation; resuspend as above.

PURIFIED VIRUS

***Discard**

Figure 8 **Flow diagram of the procedure used to purify cucumber mosaic virus.**

method was altered by use of a higher molarity extraction buffer and EDTA in the resuspension buffer however.

A comparison of N. glutinosa, Havana 423 and daphne flowers as virus sources was made. Nicotiana glutinosa (harvested 12 days after inoculation) and daphne flowers, proved most unsatisfactory hosts yielding little virus as judged by electron microscopy. Preparations from N. glutinosa contained large quantities of F1 protein but good results were obtained with Havana 423. A comparison was also made of three-EDTA-containing resuspension buffers. Results are presented in Table 6 and reveal little difference between these media. The chelating agent Na DIECA was not used in these tests but was preferred in the extraction medium because of its great affinity for copper ions, an essential component of the polyphenol oxidase system.

TABLE 6 Infectivity of a daphne isolate of CMV in different resuspension media

Dilution of purified preparation	Resuspension media		
	0.001M EDTA pH 5.0	0.025M K-K_2 phosphate plus 0.01M EDTA, pH 7.0	0.005M H_3BO_3 0.005M EDTA pH 9.0
10^{-3}	312[a]	264	308
10^{-4}	113	84	85
10^{-5}	4	10	2

[a] total local lesions on 5 half-leaves of C. quinoa

Polyethylene glycol (PEG) has been used to purify several plant viruses. This chemical can be employed to remove virus from inactivators and degradative enzymes more rapidly than is possible by ultracentrifugation alone and also has the advantage of avoiding the need to use high speed centrifugation with large volumes of liquid, an important consideration when quantities of virus are required.

In a second method, PEG (MW 20,000) at 6g : 100ml of extract was added to the virus-containing supernatant obtained following clarification with chloroform. The PEG was dissolved in the solution and left at 4C on a magnetic stirrer overnight. The virus was precipitated by low speed centrifugation and then subjected to one or more cycles of differential centrifugation. Virus-containing pellets were resuspended in borate/EDTA.

Havana 423 leaves, infected respectively with three daphne CMV isolates, were each extracted and clarified with chloroform. The three supernatants from low speed centrifugation were halved, the first half of each being subjected to three cycles of differential centrifugation, the second to PEG precipitation followed by two cycles of differential centrifugation. With two of the three isolates excellent infectivity results were obtained with both the PEG method and high speed alone. The results with one of these isolates are presented in Table 7. With the third isolate however only a relatively small, slightly green pellet was obtained by the PEG method, electron microscopic examination revealing very few virus particles whereas excellent preparations were produced by high speed centrifugation alone with this isolate. Similarly poor results were obtained

with PEG precipitation with a CMV isolate from nandina in this laboratory.

TABLE 7 Effect of PEG and differential centrifugation or differential centrifugation alone on infectivity of a daphne isolate of CMV.[a]

Dilution of purified preparation	PEG plus differential centrifugation	Differential centrifugation alone
10^{-3}	299[b]	349
10^{-4}	90	81
10^{-5}	13	6

[a] resuspension in 0.001M EDTA pH 5.0

[b] total local lesions from 5 half-leaves of C. quinoa

The preparations obtained by the high speed method with all three CMV isolates from daphne, as well as the nandina isolate, were highly infectious and little or no contaminating material was revealed by electron microscopy. Pellets from high speed centrifugation were virtually colourless and upon resuspension produced slightly opalescent solutions. The poor results using PEG with one daphne isolate and the nandina isolate again indicates the need to test methods on a number of isolates and is illustrated by the difference in optimum concentration of PEG required to precipitate two strains of potato yellow dwarf virus (4% and 7% respectively / 51).

Electron microscopy

The electron microscope was used to provide information on the quality of purified preparations including the presence of contaminating host material and the relative concentration of virus particles. Early workers used shadow-casting to reveal CMV particles in preparations (83) but this method has been superseded by negative staining which is quicker and provides useful information on the structure of viruses (and contaminants).

Cucumber mosaic virus however has proven difficult to successfully negatively stain. Murant (64) reported very poor contrast of CMV particles in purified preparations stained with PTA, pH 6.0 and often could only detect particles from micrographs. Many of the virus particles observed were disrupted by the stain and preparations exposed to this stain for 1.5 hours contained no intact particles. Various other heavy metals gave better preservation but poorer contrast. Similarly Francki et al. (30) reported rapid disruption of CMV (Q strain) in 2% neutral PTA and obtained best results by fixing in osmium tetroxide and then staining with UrAc.

These results emphasized the need for a study with daphne isolates of CMV to ensure particles could be detected after negative staining and also to find the most suitable stain. Several common stains were compared, including PTA over a range of pH values, UrAc and AmMo. Three CMV isolates were used to avoid the possibility of variations in the effect of the stains on different

isolates. All virus-containing solutions were suspended in borate/EDTA.

In neutral 2% PTA reasonable concentrations of particles were found but the majority were disrupted (Figure 11). A similar result was obtained with PTA, pH 6.0 but in low pH PTA (pH 3.0 and 4.0) much better preservation of particles was noted, little disruption having occurred (Figure 11). Particles in 2% UrAc, pH 4.0 were intact but appeared slightly distorted with a dark staining centre, possibly representing positive staining (Figure 11). The third stain, AmMo gave excellent results, particles being intact, clearly defined and often displaying an hexagonal outline (Figure 11).

Brenner and Horne (9) noted that satisfactory staining results depended upon the ratio of specimen particle concentration to embedding material concentration and in this study with daphne CMV isolates the spreading of preparations was markedly affected by the type of stain. At a standard ratio of virus to strain (1:1) spreading of particles was poor in PTA (at all pH values) being irregular within any one droplet. In UrAc individual particles were often indistinguishable and congealed into thick masses. The latter problem was only avoided when preparations were diluted 1:1 with distilled water (instead of the stain), sprayed onto the grid and then exposed to UrAc for 1 to 2 minutes. In AmMo however spreading was invariably excellent.

From these results it is clear that AmMo is the superior stain for CMV due to the excellent detail provided,

the presence of high concentrations of intact particles and the spreading characteristics. Tomlinson et al. (93) found AmMo was superior to neutral PTA with CMV, but only in preparations suspended in borate/EDTA, those in borate alone being disrupted as with PTA.

The disruptive qualities of neutral PTA noted in this and other studies on CMV have also been found for other icosahedral viruses (55, 66) and is possibly due to dissociation of quaternary protein structures (9). Apparently however few investigators have tested low pH PTA, shown by Finlay and Teakle (27) to enhance preservation of tobacco necrosis virus (TNV) although another stain (UrAc) is normally used at approximately pH 4.0. The latter authors suggested that the greater stability of TNV in acid rather than neutral PTA could have resulted from maintaining the pH close to the isoelectric point (Ip), near which plant viruses display their maximum stability (72). Cucumber mosaic virus has an Ip of 4.7 (33) but the stability of daphne isolates in negative stain is unlikely to represent a pH-effect alone as particles were well preserved in neutral AmMo.

Particle measurements in the various stains were not taken in the present study but have been compared in other reports (30, 87). The observed diameter is known to vary with the thickness of the matrix of negative stain (28) a factor which is likely to vary within and between preparations. Also several workers have found particle diameter is minimum in acid UrAc (30, 54) but Bancroft et al. (1) found that cowpea chlorotic mottle virus

became swollen at pH values near neutrality. If other viruses display this characteristic, the effect of different staining solutions on size may also reflect the different pH values of these stains.

Particle morphology and size. Icosahedral particles were detected in squash homogenates from leaves of daphne, momordica and C. quinoa, as well as from purified preparations. Sixty-eight particles from a purified preparation of one isolate, stained in AmMo measured 29nm (range 24-30nm).

Serology

Several daphne isolates of CMV from inoculated and systemic leaves of Small Sugar pumpkin reacted with antisera in double diffusion tests and spur formation was detected between two of the isolates tested. Purified virus could not be employed in these tests due to non-specific precipitation problems.

Discussion

Host range and symptoms, aphid transmission, particle morphology and size are in general accord with isolates of CMV from other hosts, although the very characteristic reaction of daphne isolates on D. barbatus has not been recorded. The identity of a number of daphne CMV isolates was confirmed by serology.

The virus infects a large number of plant species including weeds in which it may be seed-borne (24). It is inherent from the ubiquity of this virus, the large number of vector species and the efficient manner in which

it is transmitted that healthy daphne plants may very quickly become infected. On the other hand it may be possible to minimize the rate of infection in home gardens with oil sprays or possibly aphid-repellent chemicals and in nurseries by the methods discussed in Chapter 3.

FIGURE 9: Fawn 'target-spots' produced by CMV in sweet-william.

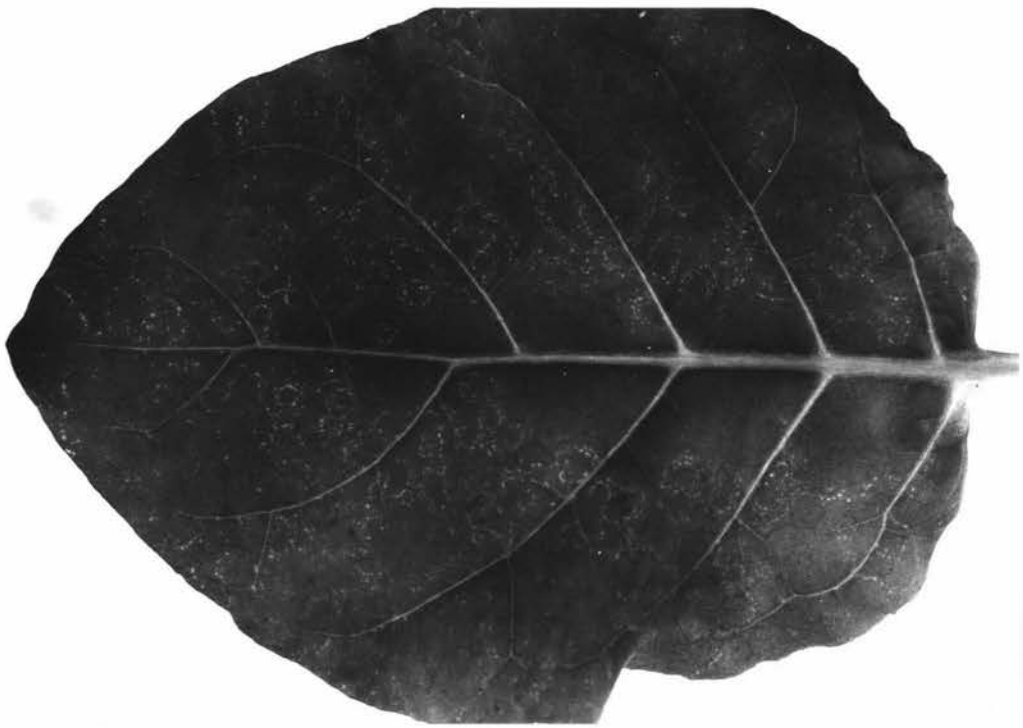


FIGURE 10: Local etched flecks forming concentric rings in Samsun tobacco infected with CMV.

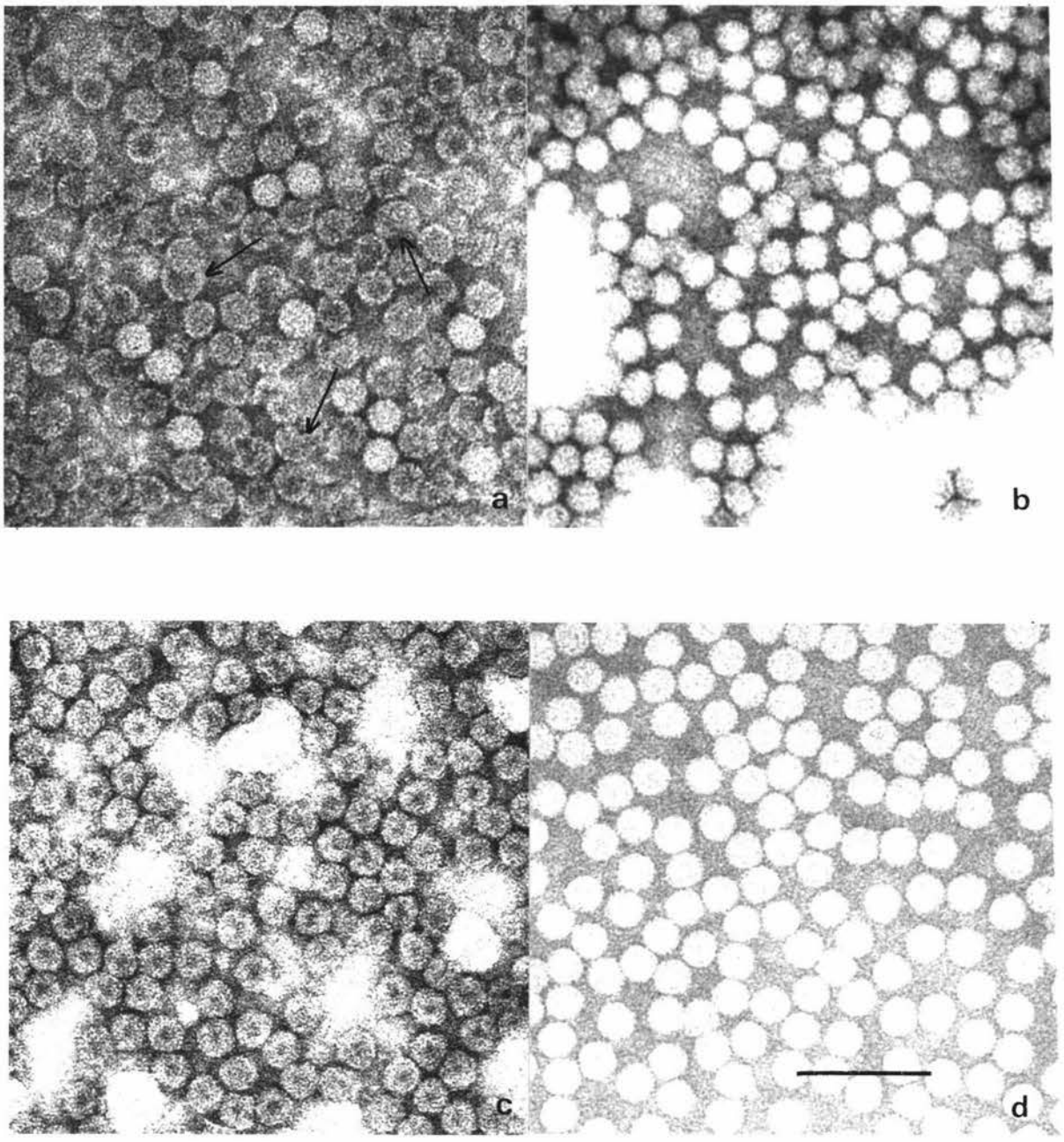


FIGURE 11: Effect of negative stains on purified CMV. (Bar represents 100nm)

(a) distorted and disintergrated particles (arrows) in neutral 2% PTA;

(b) intact particles in 2% PTA, pH 3.0;

(c) distorted particles in 2% UrAc, pH 4.0;

(d) intact particles in neutral 2% AmMo.

NEPOVIRUSES

Introduction

Two nepoviruses, arabis mosaic virus (ArMV) and tobacco ringspot virus (TobRSV), were detected during the present study. Both were confined to Leucanthe, the former occurring in 8% of this cultivar while the latter was only isolated from three infected plants in two nurseries, one in the North Island and one in the South Island.

There are several features which together make this virus group distinctive and include a three-component icosahedral particle system, thermal inactivation points in the range 55 - 70C and mechanical, nematode and seed transmissibility of each virus.

Isolates of each virus from daphne were characterized by a series of definitive tests as reported in the following sections.

ARABIS MOSAIC VIRUS (R/1 : */41 : S/S : S/Ne)

The virus was identified from results obtained with differential hosts, serology and particle size and shape.

Host range

Five isolates of the virus were each inoculated to a series of common indicator hosts from virus-infected cowpea or Havana 423 leaves ground in 0.1M K-K_2 phosphate, pH 7.0.

Amaranthaceae

Gomphrena globosa L. 'Litte Buddy' (globe amaranth): some isolates produce fawn necrotic local lesions (5-6 days) enlarging to red-rimmed halos; one isolate produced systemic reddish blotches and distortion.

Chenopodiaceae

Chenopodium amaranticolor Coste & Reyn. : faint chlorotic local lesions with pinpoint white necrotic centres (4-6 days). Systemic chlorotic blotches or vein-net (8-10 days) with reflexing of leaves. Later leaves partially recovered.

Chenopodium quinoa Willd. : light-green chlorotic local lesions (4-6 days). Systemic chlorotic vein-net (7-8 days) sometimes with necrotic flecks. Netted areas often become bright-yellow then necrotic, usually with plant death.

Spinacea oleracea L. 'Royal Denmark' (spinach): in winter slightly depressed water soaked local lesions (3-4 days) which become necrotic, followed by a bright-yellow systemic vein-net and twisting of leaves (6-8 days); in summer only mild local and systemic chlorosis.

Cucurbitaceae

Cucumis melo L. (cantaloupe): chlorotic local lesions (4-6 days) with systemic chlorotic mottle (7-12 days).

Cucumis sativus L. 'Marketer' and 'Short Green Prickly' (cucumber): chlorotic local lesions (6-10 days) which expand to 3-4mm blotches with a diffuse outer zone. Systemic symptoms (7-15 days) as chlorotic mosaic or bright-yellow 1mm spots. Severe stunting. Some isolates rarely systemically infect this host.

Cucurbita maxima Duch. 'Buttercup' (pumpkin): slightly depressed necrotic local lesions (12-14 days) expanding to 5-6mm. No systemic infection.

Momordica balsamina L. (balsam-apple): chlorotic local lesions (5-6 days) are produced by some isolates.

All isolates produce a systemic chlorotic stipple effect from chlorotic vein-flecks, vein-clearing and vein-banding. (Figure 13)

Leguminosae

Dolichos biflorus L. : chlorotic local lesions (6-8 days) which expand to 2-3mm diffuse blotches. Systemic symptoms produced by only one isolate consisting of a chlorotic mosaic then death of the plant (10 days).

Phaseolus vulgaris L. 'Black Turtle', 'Blue Lake' 'Red Kidney' 'Scotia' and 'Top Crop' (French bean): no local reaction on cotyledons but discrete 1mm chlorotic local lesions on inoculated secondary leaves. Systemic chlorotic mottle (11-15 days) necrotic flecks and vein and apical necrosis.

Pisum sativum L. 'Bonneville' and 'Ranger' (garden pea): necrotic local lesions (6-12 days) with a chlorotic halo. Systemic necrotic flecks (8-16 days) or flagging of leaves and death of plant.

_____ Greenfeast: no local reaction but mild chlorotic mosaic of systemically infected areas (8-10 days). Symptoms usually recede.

Vigna cylindrica (L.) Skeels: faint chlorotic local lesions (4-6 days).

Vigna sinensis (Torner) Savi. 'Blackeye' (cowpea): faint chlorotic local lesions (6-14 days) which expand to 4-6mm chlorotic blotches or rings with occasional patches of local veinlet necrosis. Systemic chlorotic mosaic with necrotic flecks and browning of top 1-2 inches of the hypocotyl (10-20 days). Systemically infected leaves often wilt and die. (Figure 12).

Solanaceae

Nicotiana clevelandii Gray: fawn 2-3mm lesions or symptomless local infection (13 days) and mild systemic mosaic.

Nicotiana glutinosa L. : light reddish-brown lines or rings or symptomless infection of inoculated leaves. No systemic infection.

Nicotiana tabacum L. 'Havana 423' (tobacco): light-yellow or dark-green ringspots surrounding normal-green 'islands' (3-4 weeks) on inoculated leaves (Figure 14). No systemic infection.

_____ Samsun: 2-3mm chlorotic local lesions or large chlorotic blotches (7-10 days), the latter often containing etched necrotic dots. Systemic symptoms rarely produced but occur as scattered chlorotic areas (ca. 1cm) often bounded by etched necrotic dots.

_____ White Burley: 2-3mm chlorotic local lesions (5-6 days) 3-4mm wide yellow ringspots surrounding 1-2cm normal-green areas (3-4weeks). Systemic chlorotic blotches and ringspots (15-30 days) containing etched necrotic lines and flecks. Later leaves almost symptomless.

Petunia hybrida Vilm. 'Rose of Heaven': one isolate reacted to produce 2-3mm necrotic local lesions (10 days) followed by systemic chlorotic vein-net. Necrosis of mid-veins and lateral veins in systemically infected areas followed by dessication of the entire lamina.

Plants not susceptible to mechanical inoculation with ArMV included: Apium graveolens L. 'Dewcrisp' (celery); Brassica oleracea botrytis L. 'Deepheart' (cauliflower); Brassica oleracea capitata L. 'Drumhead' (cabbage); Brassica pekinensis (Lour.) Rupr. 'Chi-Hi-Li' (Chinese cabbage); Capsicum frutescens L. 'Sweet Capsicum' (pepper); Dianthus barbatus L. 'Indian Carpet' (sweet-william); Dianthus caryophyllus L. 'Single Pinks'; Dolichos lablab L. ; Lactuca sativa L. 'Calmar' and 'Webbs Wonderful' (lettuce); Lycopersicum esculentum Mill. 'Potentate' (tomato); Matthiola incana (L.) R.Br. 'Nice Giants Beauty' (stock); Vicia faba L. 'Coles Early', 'Atlas Early' and 'Exhibition Longpod' (broad bean); Zinnia elegans Jacq. 'Persian Carpet'.

Vector transmission

Before the virus was identified as ArMV, unsuccessful transmission attempts were made from C. quinoa to C. quinoa with M. persicae and from Momordica balsamina to C. quinoa with A. circumflexum. Short (1-2 minutes) and long (4-5 days) acquisition periods were used to test for non-persistent and persistent transmission respectively.

These results are consistent with other reports for ArMV which is only known to be transmitted by the free-living nematode Xiphinema diversicaudatum (Micol.) Thorne (39), and Xiphinema coxi (65). The former vector was recently found in N.Z. in tamarillo plantings (90) but was not detected in soil samples from the root zone of Leucanthe stock plants in a nursery demonstrated to contain a high percentage of infection. Bait plants (cowpea and cucumber) were used and Dr. F. Wood (pers. comm., 1973) surveyed samples for nematode populations, but results from the latter method may have been biased as samples were stored at room temperature for two weeks before testing.

Purification

ArMV is readily purified by a variety of methods and is apparently unaffected by aggregation. Most workers have developed a different method for purification of their particular isolates with little regard for other methods (4, 40, 44, 90).

In this study the virus was purified from petunia using the method of Harrison and Nixon (40). High concentrations of particles were obtained after resuspension of

the glassy pellets from two cycles of high speed centrifugation.

Arabis mosaic virus was also purified from Greenfeast pea or C. quinoa by the PEG-precipitation method for AMV but with 6% PEG and two hours high speed centrifugation. The virus was highly infectious from both methods when resuspended in 0.01M K-K_2 phosphate, pH 7.0 or 0.001M EDTA, pH 5.0. Preparations from pea contained less contaminating host material than those from C. quinoa but the PEG method from either host was inferior to the method of Harrison and Nixon.

Electron microscopy

Electron micrographs of purified preparations or squash homogenates stained in neutral or pH 4.0 PTA revealed icosahedral particles, some of which were completely penetrated by stain while others were only penetrated partially or not at all (Figure 21). This situation has been noted for several nepoviruses (21, 22), including ArMV (65).

Particles were often closely aligned in both squash homogenates and purified preparations (Figure 21) in which case the 6- (and sometimes 5-) sided, angular outline of particles was clearly evident. The average diameter of 80 particles was 26nm for single particles and 27.5nm when measuring across clusters. Both figures are within the range of 26-30nm cited for this virus (56).

Serology

The presence of an icosahedral virus with ca. 26nm particles, an extensive host range and lack of transmission by two aphid species suggested that this could be a nepovirus. Because serology is regarded as the only reliable and conclusive means of identifying members of this group (65) an isolate was tested in Ouchterlony gel diffusion tests against antisera to ArMV (from Dr. P.R. Fry), TobRSV and TomRSV. In addition the virus was also tested against antisera to two isolates of CMV, tomato aspermy virus (chrysanthemum isolate) and Prunus necrotic ringspot virus. Positive results were only obtained with ArMV antisera. Subsequently several further daphne isolates gave similar results with two different ArMV antisera (supplied by Dr. M Hollings and Dr. B.D. Harrison).

No spur formation occurred with any combination of daphne isolates and the latter two ArMV antisera (Figure 19). Most other workers have not obtained spur formation with ArMV isolates (13, 44, 90) although an isolate from hops has been reported to differ from the type strain in this respect (4).

Discussion

Following preliminary investigations on host range, transmission and particle morphology and size, ArMV was conclusively identified by serology.

Arabis mosaic virus was first described in Arabis hirsutus (L.) Scop. (85) and is serologically related to

grapevine fanleaf virus (13). Although the virus has been isolated from a number of woody and herbaceous plant species including D. mezereum (56) this is the first report of the virus in D. odora and only the second record of the virus in N.Z., the first being in tamarillo (90).

It is likely that the virus is maintained in daphne by propagation but one of its nematode vectors has been reported in this country and, although not found in one test in soil from a nursery growing daphne, the possibility cannot be excluded that this vector is present in daphne plantings. The virus is maintained in its nematode vectors for up to 8 months (42) and is seed-borne in a number of weed species (57), factors which are important in the ecology of nepoviruses (67).

TOBACCO RINGSPOT VIRUS (R/1 : 2.2/40 : S/S : S/We, Ap, Th)

Tobacco ringspot virus (TobRSV) was identified on the basis of host range, particle morphology and size, and serology as reported in the following sections.

Host range

One isolate of the virus was inoculated to a series of differential hosts from systemically infected tobacco leaves after maceration with 0.1M K-K_2 phosphate, pH 7.0.

Amaranthaceae

Amaranthus hybridus L. : diffuse reddish-brown 2-3mm local lesions (7 days). Systemic 'oak-leaf' pattern around the mid-vein. Later leaves symptomless.

Chenopodiaceae

Chenopodium amaranticolor Coste & Reyn. : semi-necrotic local lesions (5 days). Mild systemic mosaic (9-10 days). Later leaves almost symptomless.

Chenopodium quinoa Willd. : 1-2mm chlorotic local lesions which may contain fawn necrotic dots (4 days). Systemic chlorotic vein-net (7-8 days) with necrotic flecks, flagging and death of laterals above the inoculated leaves and sometimes of the apical areas.

Spinacea oleracea L. 'Royal Denmark' (spinach): mild chlorotic mosaic on inoculated leaves (8 days). Mild systemic mosaic.

Cucurbitaceae

Cucumis melo L. (cantaloupe): local chlorotic spots (7 days) on cotyledons and systemic chlorotic mosaic.

Cucumis sativus L. 'Marketer' and 'Short Green Prickly' (cucumber): 2-3mm diffuse chlorotic local lesions on cotyledons (6 days) followed by scattered systemic chlorotic blotches or ringspots (1-2mm). Stunting.

Cucurbita maxima Duch. 'Puttercup' (pumpkin): necrotic 1mm local lesions (4 days) on cotyledons. Systemic orange blotches (12-15 days) produced by some isolates.

Cucurbita pepo L. 'Small Sugar' (pumpkin): pinpoint necrotic local lesions (6 days) on cotyledons. Systemic chlorotic spotting (12 days).

Leguminosae

Dolichos biflorus L. : diffuse reddish-brown necrotic local lesions (5-7 days) expanding to 3-4mm. No systemic infection.

Dolichos lablab L. : faint chlorotic blotches on inoculated leaves (6 days). No systemic infection.

Phaseolus vulgaris L. 'Red Kidney' and Top Crop' (French bean): discrete 1mm chlorotic local lesions (4 days). Systemic chlorotic mottle with necrotic flecks (9 days) followed by apical necrosis and death of plant.

Pisum sativum L. 'Greenfeast' (garden pea): local chlorotic blotches (2 weeks) with necrotic streaks. Mild, systemic, chlorotic mottle and vein-clearing (3-4 weeks).

Vigna cylindrica (L.) Skeels: pinpoint, reddish-brown necrotic local lesions which expand to 1mm or more. No systemic infection.

Vigna sinensis (Torner) Savi. 'Blackeye' (cowpea): faint, chlorotic, 2-3mm local lesions (5-6 days) some of which become bounded by a fine reddish ring or part ring. Systemic chlorotic mosaic with necrotic flecks, and browning of top 1-2 inches of the hypocotyl (9-10 days). Death of plants.

Scrophulariaceae

Antirrhinum majus L. 'Tom Thumb' (snapdragon): fawn etched rings (5-6 days) which expand to 5-6mm or more with the addition of further concentric rings to give a 'target-spot' appearance. Fawn, systemic ringspots and line patterns (3-4 weeks). Later leaves almost symptomless. (Figure 15).

Solanaceae

Petunia hybrida Vilm. 'Rose of Heaven': 2mm chlorotic local lesions (5 days) followed by systemic chlorotic blotches, ringspots and vein-clearing (10 days). Later leaves symptomless. (Figure 18).

Nicotiana clevelandii Gray: sunken irregular whitish local lesions (3-4 days). Faint chlorotic vein-net (7-8 days) in systemically infected areas, followed one day later by interveinal necrosis (Figure 16). The plant sometimes dies.

Nicotiana glutinosa L. : etched concentric 5-10mm rings and lines (8 days) often forming 'target-spots'. No systemic infection.

Nicotiana glutinosa x clevelandii: puckering of inoculated leaves and fawn, etched, 5-10mm ringspots (6 days). Mild systemic chlorotic cast (8 days) followed by terminal necrosis.

Nicotiana tabacum L. 'Samsun': whitish necrotic local lesions with chlorotic outer (7 days). Systemic zig-zag 'oak-leaf' pattern.

_____ White Burley: sunken fawn or grey local lesions (3-4 days). Systemic chlorotic blotches or ringspots which may contain necrotic flecks. Zig-zag patterns of adjacent lines produce an 'oak-leaf' effect on the main and lateral veins (Figure 17). Later leaves symptomless.

Symptomless infection occurred in the following: Gomphrena globosa L. 'Little Buddy' (globe amaranth); Lactuca sativa L. 'Calmar' (lettuce).

No infection occurred in the following: Capsicum frutescens L. 'Sweet Capsicum' (pepper); Lycopersicum esculentum Mill. 'Potentate' (tomato); Matthiola incana (L.) R.Br. 'Nice Giants Beauty' (stock); Vicia faba L. 'Exhibition Longpod' (broad bean).

Purification

Tobacco ringspot virus is readily purified by a variety of techniques (86) with most isolates giving high yields.

A daphne isolate was purified from inoculated and systemically infected leaves of C. quinoa by a modification of the PEG-precipitation method outlined for AMV. The virus was precipitated with 6% PEG (instead of 8%) and subjected to two hours high speed centrifugation (instead of three hours).

Serology

From host range and reactions it was considered that the virus could be either TobRSV or tomato ringspot virus (TomRSV), both of which induce similar symptoms in a number of hosts (60). However, the lack of systemic necrosis on C. amaranticolor (86) and the severe reaction of snapdragon (35) suggested that the virus was most likely to be TobRSV. In order to confirm this purified preparations or crude sap from inoculated leaves of C. quinoa were tested against antisera to ArMV, TobRSV and TomRSV. A positive reaction was only obtained with the TobRSV antisera confirming results from the host range.

One isolate of TobRSV from daphne was compared by means of double diffusion tests with an isolate from horse radish. The viruses were serologically related, but not identical, as evidenced by spur formation (Figure 20). Spurs were produced by both isolates indicating that each possessed determinant groups not shared by the other.

Unlike ArMV a number of isolates of TobRSV can be distinguished by serology (52, 78, 79) as illustrated by Gooding (36) who separated 100 isolates of TobRSV into four groups on this basis. However there was no correlation between isolates within a strain and the symptoms they produced, or their geographic origin.

Electron microscopy

Squash homogenates from C. quinoa or purified preparations revealed the presence of icosahedral particles (Figure 22), 73 of which had a mean diameter of 28nm (range 24-30nm).

A proportion of particles in PTA were found partially or completely penetrated by the stain. The percentage of penetrated TobRSV particles has been found to be affected by the pH of the PTA (21), and this was noted with TobRSV from daphne; few penetrated particles were found at pH 4.0 and 5.0 (PTA) while at pH 6.0 and 7.0 they were common. Almost all particles were penetrated when stained in neutral AmMo.

Tubular structures containing virus particles were detected in squash homogenates from C. quinoa (Figure 23). These varied in length ranging from 350nm - 1.5 µ and contained closely packed virus particles. Similar structures have been found in plants infected with several nepoviruses (20, 77, 95), maize rough dwarf virus (96) and tymoviruses (43), but those of the latter group are morphologically distinct from the former types (96).

The function of these structures is unknown but they are possibly involved in the dissemination of virus as they have been found to pass through plasmodesmata (20).

Discussion

Tobacco ringspot virus was first reported in tobacco (31) and is now known to cause diseases in a variety of plant species. It is soil-borne by the nematode Xiphinema americanum Cobb (58) in which vector it may be retained for 9 months (59). Insects have also been implicated in transmission (e.g.) nymphs of Thrips tabaci Lind. (61), mites (Tetranychus species: 89), the tobacco flea beetle (81) Epitrix hertipennis Melsh., grasshoppers (Melanoplus species: 25) and aphids (73) M. persicae and Aphis gossypii (Glover).

Tobacco ringspot virus from daphne was identified by serology. Host range and particle size are also in agreement with reports for other isolates (52, 73, 91). The virus was found in only three infected plants but these displayed very severe symptoms and it is possible that roguing is responsible for the small number of infected plants found in this survey. On the other hand figures may have been biased by the indexing method used. The virus was only detected when it produced systemic symptoms in C. quinoa, a reaction also produced by grape isolates (35). Other authors (84, 86) consider that the virus is rarely systemic in this host however.

This is the first report of the virus in N.Z. other than in recently imported horse radish (91) all of which have been destroyed.

The nematode vector has been found in this country(18) and the reported aphid, thrip and mite vectors are all common and providing their ability to vector different TobRSV isolates is confirmed they would represent potentially more dangerous vectors than slow moving nematodes.

FIGURE 12: Cowpea infected with ArMV showing chlorotic local lesions and vein necrosis on cotyledon and brownish-black streaking on upper hypocotyl.

FIGURE 13: Systemic vein chlorosis in momordica infected with ArMV.

FIGURE 14: Yellow ringspots in an inoculated leaf of Havana 423 tobacco, first appearing four weeks after infection with ArMV.

FIGURE 15: Local and systemic fawn ringspots, 'target-spots' and line patterns caused by TobRSV in snapdragon.

FIGURE 16: Large necrotic local lesions and systemic necrosis caused by TobRSV after five days in N. clevelandii.

FIGURE 17: Necrotic local lesions, systemic ringspots and 'oak-leaf' pattern in White Burley tobacco caused by TobRSV.

FIGURE 18: Systemic chlorotic ringspots in petunia infected with TobRSV.

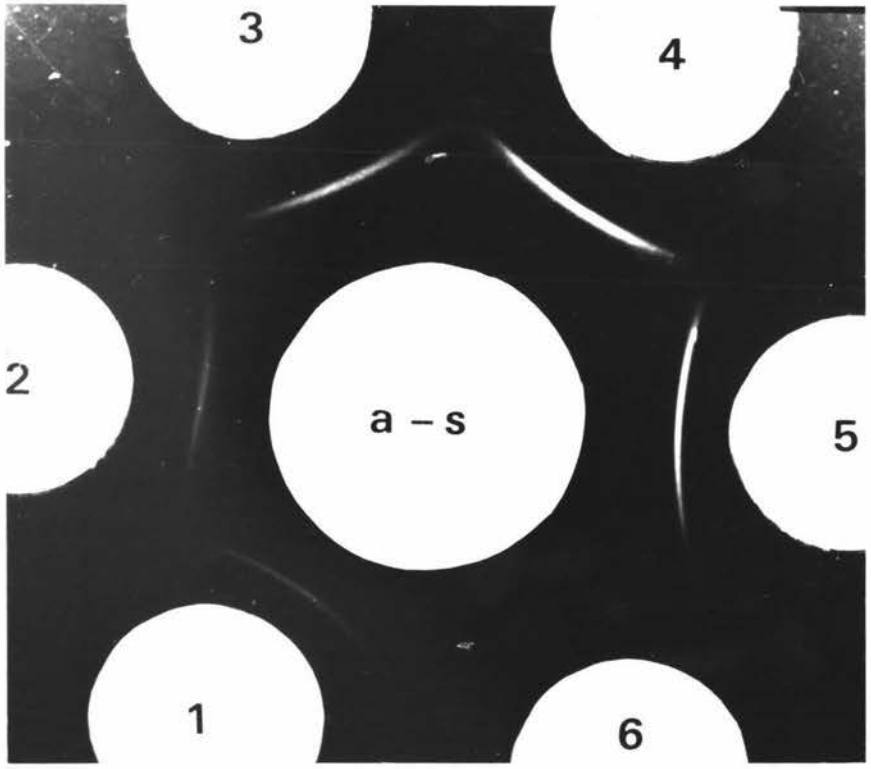


FIGURE 19: Serological reactions between daphne isolates of ArMV in C. quinoa sap (1-5) and ArMV antisera supplied by Dr. P.D. Harrison. Well 6 contains healthy C. quinoa sap.

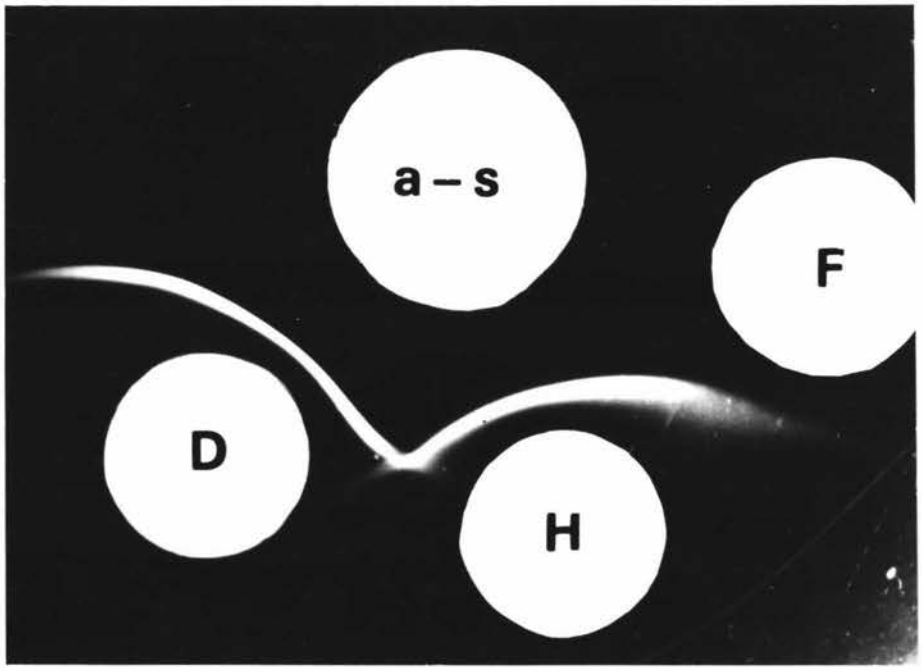


FIGURE 20: Serological reactions between TobRSV from daphne (D) and TobRSV from horse radish (H) in crude C. quinoa sap. Well F contains healthy C. quinoa sap.

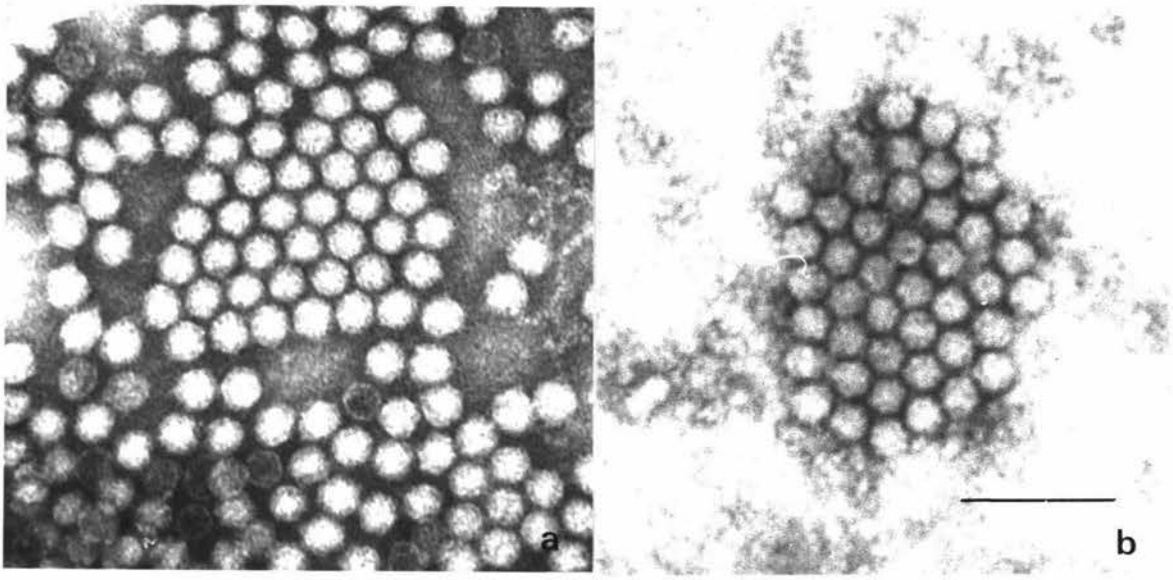


FIGURE 21: ArMV particles negatively stained in PTA, pH 4.0. (Bar represents 100nm)

(a) purified preparation;

(b) squash homogenate from inoculated leaves of C. quinoa.

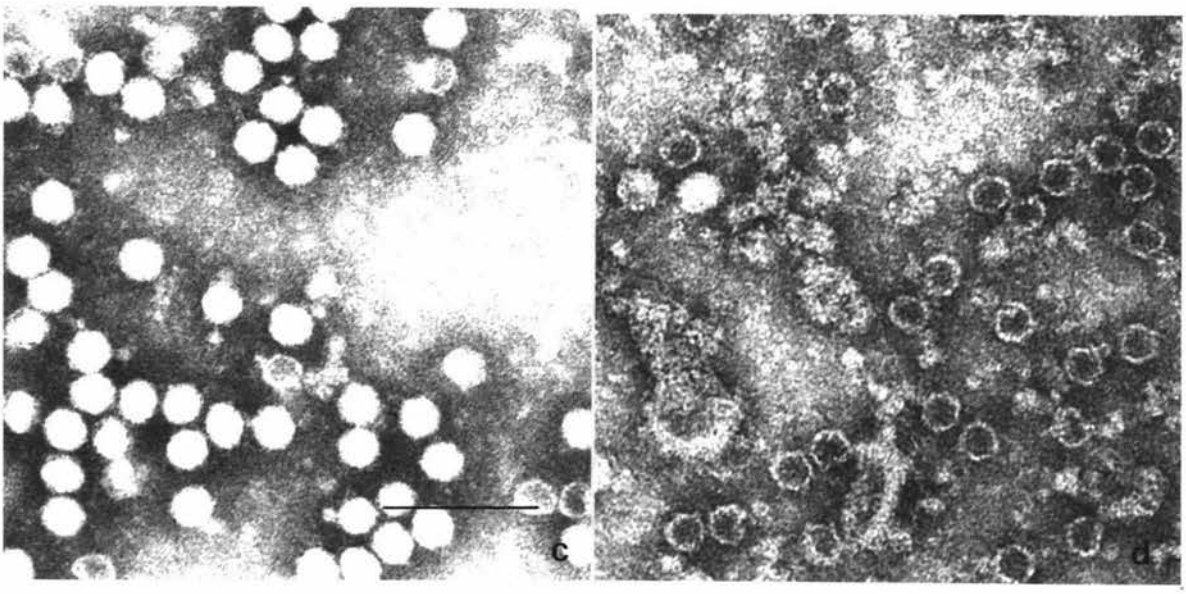
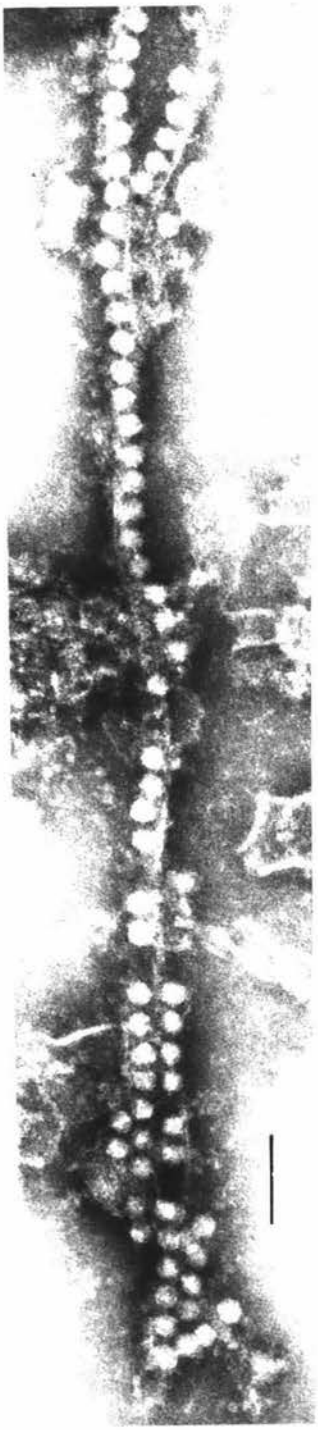


FIGURE 22: Effect of negative stains on TobRSV from inoculated leaves of C. quinoa. (Bar represents 100nm)

(a) clearly defined particles in neutral 2% PTA;

(b) particles penetrated by stain in neutral 2% AmMo.



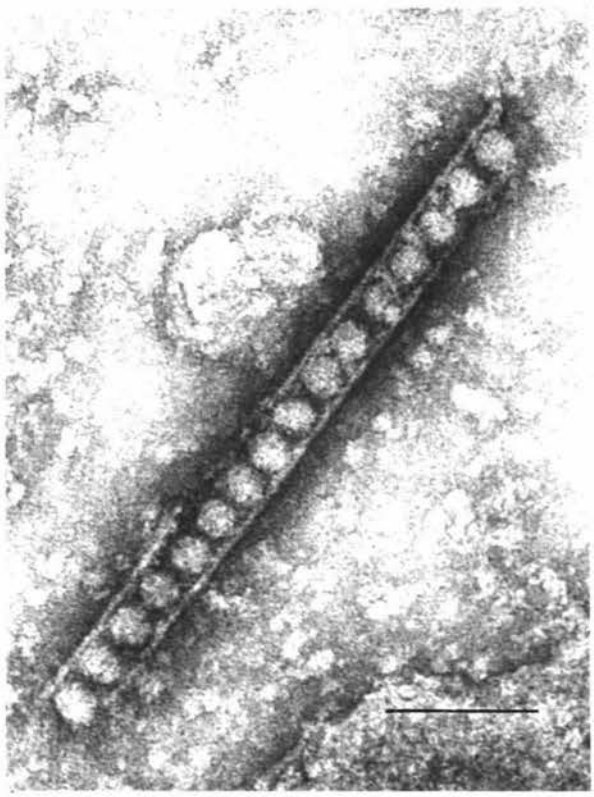


FIGURE 23: Tubular structures containing TobRSV particles in squash homogenates from inoculated leaves of C. quinoa, negatively stained in neutral 2% PTA. (Bars represent 100nm)

LEUCANTHE VARIEGATA VIRUS COMPLEX

Many specimens of Daphne odora 'Leucanthe Variegata' were found to be infected with a virus (Leucanthe Variegata virus - 2 / LVV-2) producing systemic net symptoms and necrosis in C. quinoa. Later, a second virus (Leucanthe Variegata virus - 1 / LVV-1) was detected producing mild systemic chlorotic blotches in C. quinoa which were masked in the presence of LVV-2. The two viruses were partially characterized by several definitive tests.

LEUCANTHE VARIEGATA VIRUS - 1 (*/* : */* : S/S : S/*)

Leucanthe Variegata virus - 1 was detected only in Daphne odora 'Leucanthe Variegata' but its prevalence was not determined.

The virus was partially chacterized on the basis of host range, particle morphology and size.

Host range

Infected leaves of Havana 423 and N. clevelandii were ground in 0.1M K-K_2 phosphate, pH 7.0 and inoculated to indicator plants.

Chenopodiaceae

Chenopodium amaranticolor Coste & Reyn. : 0.5-1mm chlorotic local lesions with pinpoint white necrotic centre. No systemic infection.

Chenopodium quinoa Willd. : 0.5 - 1mm bright-yellow local lesions (2-5 days) which quickly coalesce when present in high numbers. Inoculated leaves become dessicated. Systemic light-yellow or whitish blotches (10 - 15 days).

Spinacea oleracea L. 'Royal Denmark' (spinach): 1-2mm chlorotic local lesions (6 days) which spread and coalesce. Bright-yellow systemic vein-net (7-9 days) which later recedes.

Cucurbitaceae

Citrullus vulgaris Schrad. 'Cannonball' (water-melon): expanding chlorotic local lesions on cotyledons (3-4 days). No systemic infection.

Cucumis melo L. (cantaloupe): 2-3mm chlorotic local lesions on cotyledons (6 days). Systemic chlorotic vein-banding and blotches (7-8 days).

Cucumis sativus L. Marketer' and 'Short Green Prickly' (cucumber): 2-3mm chlorotic local lesions on cotyledons (4-6 days). Systemic mosaic of large diffuse chlorotic blotches. Stunting.

Cucurbita maxima Duch. 'Buttercup' (pumpkin): faint 1-2mm chlorotic local lesions (4 days). Diffuse, orange, systemic blotches (8-10 days).

Cucurbita pepo L. 'Small Sugar' (pumpkin): 1mm discrete chlorotic local lesions (3 days). Systemic chlorotic blotches and 3-4mm patches of chlorotic vein-net (5 days).

Momordica balsamina L. (balsam-apple): 1mm chlorotic local lesions (3 days). Large, diffuse, pale-yellow systemic blotches on outer portion of lamina (Figure 24) with normal-green central areas (6-18 days).

Compositae

Zinnia elegans Jacq. 'Cactus Flowered': diffuse 3-4mm chlorotic local lesions (8 days). Systemic bright-yellow mosaic which later fades (15 days).

Leguminosae

Dolichos lablab L. : faint, diffuse, 3-4mm chlorotic local lesions (6 days). No systemic infection.

Phaseolus vulgaris L. 'Black Turtle', 'Prince', 'Red Kidney', 'Scotia' and 'Top Crop' (French bean):

pinpoint necrotic local lesions (2-3 days) in both winter and summer. No systemic infection.

Pisum sativum L. 'Bonneville' (garden pea): no local reaction. Mild systemic chlorotic mosaic (2-3 weeks).

_____ Greenfeast: chocolate, necrotic spots or rings on inoculated leaves which become dessicated. No systemic infection.

Vicia faba L. 'Atlas Early', 'Coles Early' and 'Exhibition Longpod' (broad bean): water-soaked, slightly depressed local lesions (3 days) which quickly become necrotic. No systemic infection.

Vigna cylindrica (L.) Skeels: light, golden-brown local lesions (2-3 days). No systemic infection.

Vigna sinensis (Torner) Savi. 'Blackeye' (cowpea): reddish-brown slightly depressed necrotic local lesions with a fairly regular outline. No systemic infection.

Scrophulariaceae

Antirrhinum majus L. 'Tom Thumb' (snapdragon): 2-3mm chlorotic local lesions (7 days) which later fade. No systemic infection.

Solanaceae

Nicotiana clevelandii Gray: white diffuse 2-3mm local lesions (6 days). Faint systemic chlorotic vein-net (7 days) which becomes very prominent. Diffuse, fawn vein-banding on systemically infected leaves. Stunting.

Nicotiana glutinosa L. : 2-3mm fawn local lesions (3-4 days) which expand to 4-6mm 'target-spots'. Large systemic yellow blotches or chlorotic mosaic (7-9 days). All subsequent leaves display symptoms.

Nicotiana tabacum L. 'Havana 423' (tobacco): bright-yellow 3-4mm chlorotic local lesions (4 days) which contain etched fawn or brown necrotic spots and lines. Systemic symptoms of chlorotic blotches centred on veins and vein-banding or a bright-yellow interveinal mosaic (Figure 27). Scattered etched dots in systemically infected areas. Subsequent leaves symptomless.

_____ Samsun: fawn necrotic local lesions (4 days) which develop concentric rings, sometimes to give a 'target-spot' effect. Systemic chlorotic vein-banding and blotches (7-9 days). Subsequent leaves symptomless. (Figure 26).

_____ White Burley: 3-4mm chlorotic local lesions with diffuse outer (3-5 days). Systemic chlorotic vein-banding and blotches (7-8 days). Subsequent leaves symptomless.

Petunia hybrida Vilm. 'Rose of Heaven': Sunken water-soaked local lesions (3 days) which expand to 5-6mm fawn necrotic lesions (Figure 25). Bright-yellow systemic mosaic which later fades (12-18 days).

No infection was recorded in the following: Brassica oleracea botrytis L. 'Deepheart' (cauliflower); Brassica oleracea capitata L. 'Drumhead' (cabbage); Brassica pekinensis (Lour.) Rupr. 'Chi-Hi-Li' (Chinese cabbage); Dianthus barbatus L. 'Indian Carpet' (sweet-william); Gomphrena globosa L. 'Little Buddy' (globe amaranth); Matthiola incana (L.) R.Br. 'Nice Giants Beauty' (stock); Phaseolus vulgaris L. 'Blue Lake' (French bean).

Purification

The virus was readily purified by a modification of the method outlined for AMV. Virus infected tissue was homogenized in 0.5M K-K_2 phosphate, pH 7.1 plus 1.0% 2-mercaptoethanol, clarified with either 8% n-butanol (12 h incubation) or a 1:1 mixture of n-butanol plus chloroform (1ml : 1g leaf tissue; 1 min incubation) precipitated with 6% PEG (MW 20,000; 12 h incubation) and 2 cycles of differential centrifugation. Pellets from the latter were glassy with slight pigmentation and upon resuspension in 0.001M EDTA (1ml : 100g tissue) produced an opalescent

solution. Large numbers of particles with little contaminating host material were detected in the electron microscope and preparations were highly infectious to C. quinoa.

Electron microscopy

Purified preparations and squash homogenates negatively stained in 2% PTA, pH 4.0 contained icosahedral particles. Seventy-six particles from a purified preparation had an average diameter of 28nm (range 24-29nm).

Serology

The virus did not react in gel diffusion tests with antisera to TobRSV, TomRSV or ArMV. Non-specific precipitation occurred with virus from purified preparations and consequently all tests were conducted using sap from inoculated leaves of C. quinoa.

Discussion

The information from host range and symptoms, serology, particle morphology and size is insufficient for specific identification of this virus and further characterization by aphid studies, serology and physical properties is necessary.

The information obtained however is sufficient to distinguish this virus from all others in daphne with the possible exception of CMV; both have a wide host range, similar particle morphology and size, but 7 isolates of CMV from daphne did not infect Vicia faba or Dolichos biflorus. Furthermore the daphne CMV isolates did not

produce systemic symptoms in C. quinoa, Momordica balsamina or Zinnia elegans and LVV-1 did not infect Dianthus barbatus, a characteristic of all of a large number of CMV isolates.

LEUCANTHE VARIEGATA VIRUS - 2 (*/* : */* : */* : S/*)

A virus or 'virus-like' agent, tentatively called Leucanthe Variegata Virus - 2 was detected during the present study in a single cultivar (Daphne odora 'Leucanthe Variegata') in which it was apparently very prevalent.

The 'virus' was distinguished from other daphne viruses by its host range and symptoms.

Host range

Virus-infected C. quinoa leaves were ground in Yarwood's bentonite solution and inoculated to indicator plants.

Chenopodiaceae

Chenopodium amaranticolor Coste & Reyn. : 1mm chlorotic local lesions (4 days). Mild systemic mosaic (9-10 days) with slight reflexing of leaves.

Chenopodium quinoa Willd. : 1-2mm chlorotic local lesions (4 days). Systemic chlorotic vein-net (7-9 days), followed by bright-yellow appearance of netted areas then necrosis (Figures 5, 6). Death of lateral shoots and tip.

Spinacea oleracea L. 'Royal Denmark' (spinach): 1-2mm chlorotic local lesions or diffuse local chlorosis (5-6 days). Systemic vein-net (9-12 days).

Cucurbitaceae

Cucumis sativus L. 'Marketer' (cucumber): 2mm chlorotic local lesions (1-2 days) produced by one isolate. No systemic infection.

Nicotiana tabacum L. 'Samsun' (tobacco): fawn etched necrotic lines on inoculated leaves or symptomless local infection.

A mild systemic mosaic was produced in Greenfeast pea but it is uncertain whether this reaction was caused by LVV-1 present initially as a contaminant in low concentrations.

Symptoms were not produced in the following: Brassica pekinensis (Lour.) Rupr. 'Chi-Hi-Li' (Chinese cabbage); Capsicum frutescens L. 'Sweet Capsicum' (pepper); Dianthus barbatus L. 'Indian Carpet' (sweet-william); Gomphrena globosa L. 'Little Buddy' (globe amaranth); Nicotiana glutinosa L. ; Phaseolus vulgaris L. 'Red Kidney' and 'Top Crop' (French bean); Vicia faba L. 'Exhibition Longpod' (broad bean); Vigna sinensis (Torner) Savi. 'Blackeye' (cowpea).

Electron microscopy

'Virus-like' particles were not detected in squash homogenates from systemically infected leaves of C. quinoa or C. amaranticolor ground in 2% PTA, pH 4.0.

Discussion

This 'virus' is probably not rod-shaped due to the ease with which rod virus particles are detected in squash homogenates. The possibility of the virus being icosahedral cannot be excluded however as other icosahedral viruses (ArMV and TobRSV) were rarely detected in systemically infected Chenopodium tissue in the present study. Leucanthe Variegata virus - 2 is distinguished from BurkSV by its characteristic systemic reaction in Chenopodium species and from other daphne viruses by its failure to produce symptoms in many hosts.

FIGURE 24: Systemic chlorotic mosaic caused by LVV-1 in momordica.

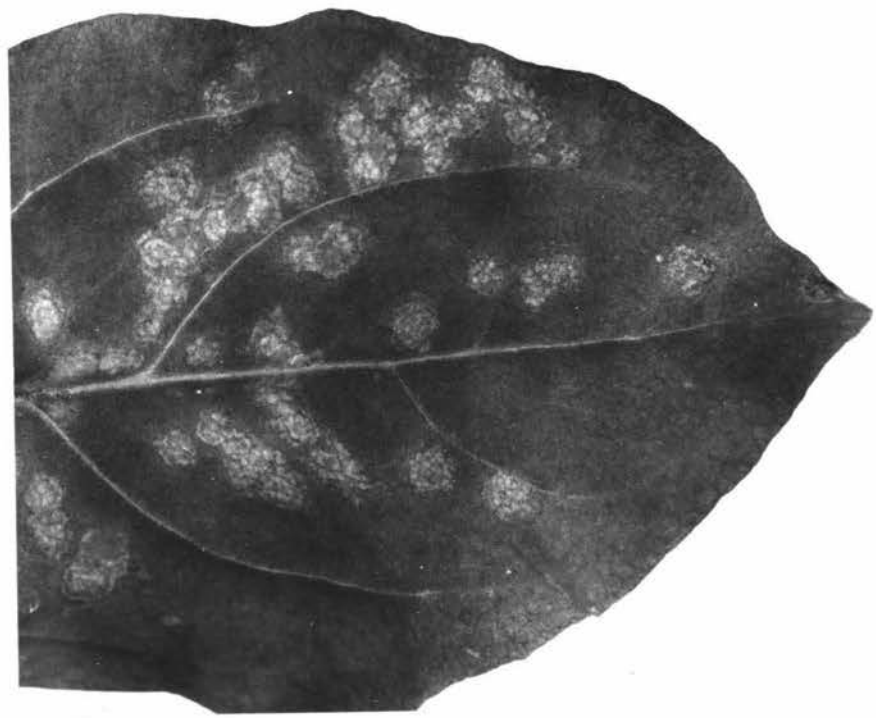


FIGURE 25: Necrotic local lesions in petunia infected with LVV-1.

FIGURE 26: Fawn 'target-spots' and systemic chlorotic mosaic in Samsun tobacco infected with LVV-1.

FIGURE 27: Chlorotic and necrotic local lesions and systemic chlorotic mosaic in Havana 423 tobacco infected with LVV-1.

BURKWOODII SPHERE VIRUS (*/* : */* : S/S : S/*)

An icosahedral virus, tentatively called Burkwoodii sphere virus (BurkSV) was detected in specimens of Daphne Burkwoodii 'Variegata' by the production of large chlorotic local lesions in C. quinoa. The virus was detected in all specimens of the cultivar tested, but because of the late stage at which it was recognized the possibility cannot be excluded that this virus is present in other Daphne species.

Partial characterization was made on the basis of host range and symptoms, particle morphology and size, and serology.

Host range

The virus was inoculated to indicator hosts using locally infected leaves of C. quinoa ground in Yarwood's bentonite solution.

Chenopodiaceae

Chenopodium amaranticolor Coste & Reyn. : faint chlorotic local lesions (3-4 days). No systemic infection.

Chenopodium quinoa Willd. : 0.5-1mm chlorotic local lesions (3-4 days) which coalesce, followed by dehydration of leaves. Systemic chlorotic blotches and vein-streaking (10-14 days) only occurred with highly concentrated inoculum.

Spinacea oleracea L. 'Royal Denmark' (spinach): 1mm chlorotic local lesions (7 days) rapidly coalesce, giving a bright-yellow effect. Symptomless systemic infection.

The virus did not produce symptoms in: Cucumis sativus L. 'Marketer' (cucumber); Nicotiana glutinosa L. ; Nicotiana tabacum L. 'Havana 423' and 'White Burley' (tobacco); Phaseolus vulgaris L. 'Red Kidney' (French bean); Pisum sativum L. 'Greenfeast' (garden pea); Vigna cylindrica (L.) Skeels; Vigna sinensis (Torner) Savi. 'Blackeye' (cowpea).

Electron microscopy

Icosahedral particles were readily detected in negatively stained (2% PTA, pH 4.0) homogenates from inoculated leaves of C. quinoa. Sixty-one particles had an average diameter of 29nm (range 25-31nm).

Serology

The virus did not react with antisera to ArMV, TobRSV or TomRSV using inoculum from locally infected C. quinoa leaves.

Discussion

The identity of BurkSV was not resolved but it was distinguished from rod viruses and AMV by morphology; from ArMV, TobRSV and TomRSV by serology; from CMV and LVV-1 by its restricted host range and from LVV-2 by its characteristic systemic reaction in C. quinoa and failure to systemically infect C. amaranticolor.

ALFALFA MOSAIC VIRUS (R/1 : 1.3+1.1+0.9/18 : U/U : S/Ap)

Alfalfa mosaic virus (AMV) was found on one occasion in Leucanthe. The virus has a multicomponent particle morphology involving a unique combination of bacilliform and spherical components, a TIP of 60 - 65C, a very wide host range and is non-persistently aphid transmitted.

The daphne isolate was purified, and characterized on the basis of host range, symptoms and particle morphology and size.

Host range

Infected tobacco leaves were ground in 0.1M $K-K_2$ phosphate, pH 7.0 and inoculated to indicator hosts.

Amaranthaceae

Gomphrena globosa L. 'Little Buddy' (globe amaranth): fawn necrotic local lesions (3-4 days) which develop a reddish halo. No systemic infection.

Chenopodiaceae

Beta vulgaris L. 'Yates Early Wonder' (red beet): chlorotic local lesions (5 days) later surrounded by a fine reddish ring. No systemic symptoms.

Chenopodium amaranticolor Coste & Reyn. : 1mm semi-necrotic local lesions (3-4 days). Mild systemic chlorotic mosaic and slight reflexing of leaves.

Chenopodium quinoa Willd. : 1-2mm chlorotic local lesions (4 days); systemic chlorotic vein-net (6-7 days), netted areas developing a mild chlorotic cast.

Spinacea oleracea L. 'Royal Denmark' (spinach): 1-2mm chlorotic local lesions (5 days). Mild systemic chlorosis.

Cucurbitaceae

Cucumis melo L. (cantaloupe): 2-3mm chlorotic local lesions (4-5 days). Systemic chlorotic mosaic (7-9 days).

Cucumis sativus L. 'Marketer' (cucumber): 1-2mm chlorotic local lesions (6 days). Systemic chlorotic mosaic (10-14 days).

Leguminosae

Dolichos biflorus L. : slightly sunken 1-2mm necrotic lesions (2-3 days) with irregular outline (Figure 34). No systemic infection.

Dolichos lablab L. : very faint 0.5-1mm chlorotic local lesions (8 days). Some plants become systemically infected with mild mosaic or interveinal chlorosis (2-3 weeks).

Phaseolus vulgaris L. 'Black Turtle', 'Blue Lake', Red Kidney', 'Scotia' and 'Top Crop' (French bean): pin-point necrotic local lesions (2 days) which expand to 1-2mm sometimes with veinlet necrosis (Figure 32). Some plants systemically infected (1-3 weeks) with scattered patches of veinlet necrosis.

Pisum sativum L. 'Bonneville' and 'Ranger' (garden pea): no local reaction. Mild systemic mosaic (2 weeks). No necrosis.

_____ Greenfeast: 2mm necrotic local lesions (6 days) then local veinlet necrosis. Systemic chlorotic mosaic with vein necrosis and necrotic flecks (8-12 days). Plants often die.

Vicia faba L. 'Atlas Early', 'Coles Early' and 'Exhibition Longpod' (broad bean): slightly sunken 2-3mm chocolate, necrotic local lesions (5-7 days). Faint systemic mosaic (9-10 days) with necrotic flecks, streaks and leaf distortion (Figure 33).

Vigna cylindrica (L.) Skeels: faint chlorotic 2mm local lesions (3 days) some with red or fawn etched outline. Systemic chlorotic mosaic sometimes followed by plant death.

Vigna sinensis (Torner) Savi. 'Blackeye' (cowpea): pinpoint necrotic local lesions (2-3 days) which expand to 2-3mm slightly sunken lesions with an irregular outline (Figure 30). No systemic infection.

Solanaceae

Nicotiana clevelandii Gray: 2-3mm diffuse chlorotic local lesions. Systemic chlorotic vein-net (10 days), slight puckering of leaves.

Nicotiana glutinosa L. : no local symptoms. Mild, then strong systemic chlorotic cast (8-9 days).

Nicotiana tabacum L. 'Havana 423' and 'White Burley' (tobacco): fawn or dark-brown etched necrotic dots on inoculated leaves superimposed on 2-3mm chlorotic blotches. Systemic chlorotic rings and blotches often with etched white or brown boundaries or rings. Later leaves symptomless.

_____ Samsun: 2-3mm chlorotic local lesions with scattered etched dots or lines near periphery. Systemic 'oak-leaf' pattern around central and lateral veins (Figure 31).

No infection was recorded in: Dianthus barbatus L. 'Indian Carpet' (sweet-william); Matthiola incana (L.) R.Br. 'Nice Giants Beauty' (stock).

Purification

A variety of methods have been used to purify AMV and are discussed in a recent review (47).

An isolate of AMV obtained from Dr. P.R. Fry was successfully purified in this laboratory by a modification of the PEG-precipitation method developed for tobacco etch virus (19) and employed by Uyemoto for AMV (K.S. Milne, pers. comm., 1973). Alfalfa mosaic virus from daphne however was not successfully purified by this method from

White Burley or Greenfeast pea. Preparations were highly opalescent with no pigmentation in pellets after two cycles of differential centrifugation but did not contain high concentrations of particles (based on electron microscopy). Better concentrations were obtained using 8% PEG MW 20,000 (16) than 4% PEG MW 20,000 and purifying from tobacco rather than pea, but the concentration of particles was still rather low.

It is possible that the long incubation periods with both butanol (12 h) and PEG (12 h) may have allowed degradative enzymic action to which the virus is susceptible (47).

The method which gave the most concentrated preparations is illustrated in Figure 28. The chelating agent EDTA was employed at all stages of purification as it is known to have a stabilizing effect on the infectivity of AMV (5) and may have also reduced aggregation, a recognized problem with this virus (47). Similar concentrations of particles were obtained by resuspending in unadjusted EDTA (pH 5.0) or EDTA, pH 7.0 (adjusted with 0.025M K-K_2 phosphate).

Electron microscopy

Purified preparations and squash homogenates negatively stained with neutral 2% AmMo, unadjusted 2% Na silicotungstate or 2% PTA, pH 4.0 were examined in the electron microscope. The former stain was preferred with purified preparations due to its superior spreading characteristics; poor spreading resulted with the other stains even after addition of bovine serum albumen.

PURIFICATION OF ALFALFA MOSAIC VIRUS

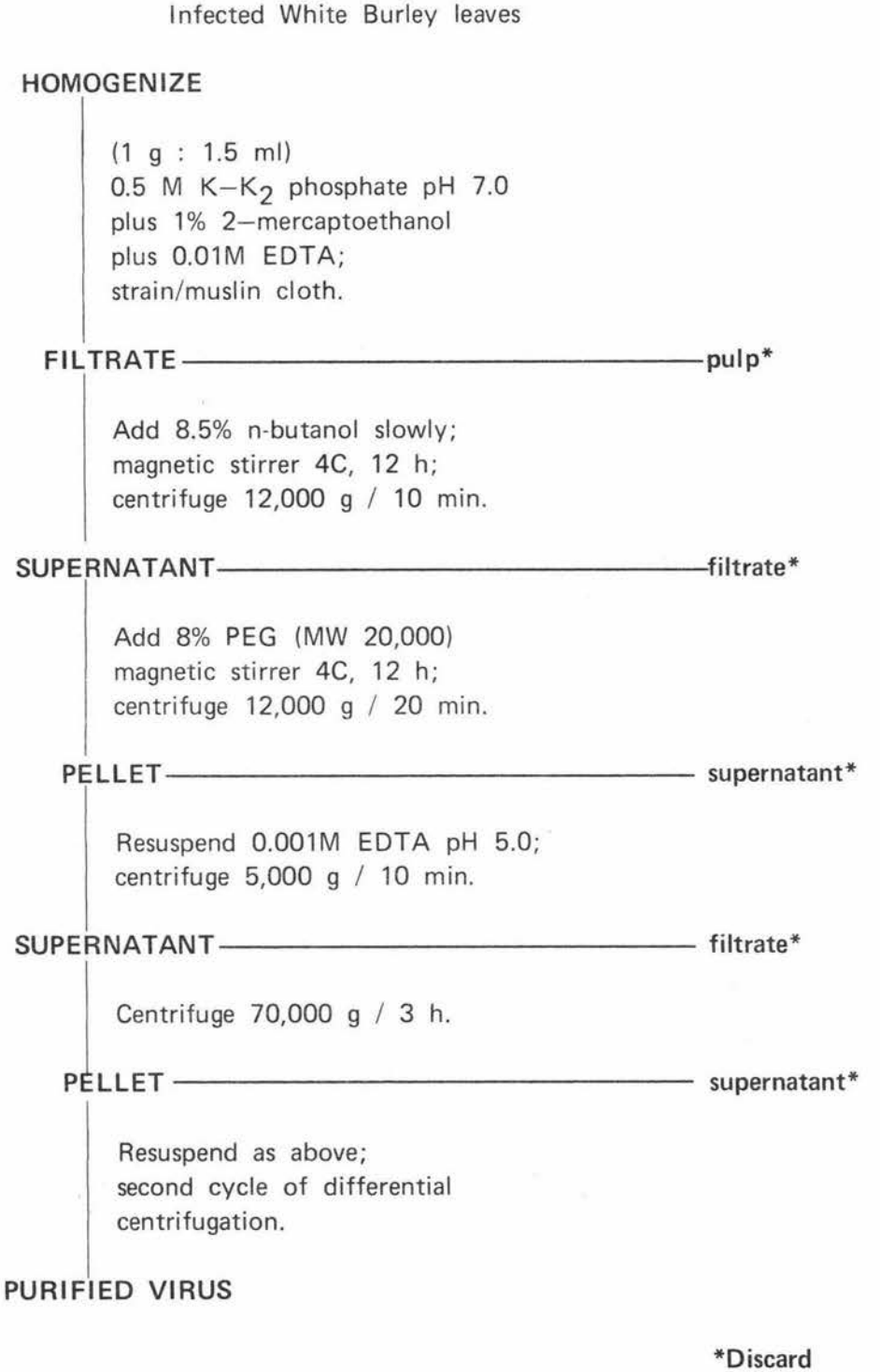


Figure 28 **Flow diagram of the procedure used to purify alfalfa mosaic virus.**

A variety of particle shapes and sizes were found ranging from spherical to bacilliform and from 20 - 145nm (Figure 35) and an average diameter of 18nm (range 17 - 22nm). This range of particle lengths differs from that found for other AMV isolates, most of which have only five major components (7). The daphne isolate possesses particles of similar sizes but in addition contains particles with preferred lengths of 65, 72, 80, 95, and 115nm. (Figure 29).

Discussion

Host range, particle morphology and size were the only criteria used for identification of AMV from daphne. The host range and symptoms are similar to those of a number of strains of AMV (34, 47, 69). Few isolates of AMV are known with a range of particle sizes longer than 'bottom' component (48, 49). One, the VRU strain, was originally isolated from white clover in N.Z. (48) and both the host range and distribution of preferred particle lengths are similar to the daphne isolate. In addition however the VRU strain possesses even longer particles, some up to 1 u or more. Long particles of another AMV strain were detected in lithium phosphotungstate but not in several other stains (49) and it is possible that very long particles are associated with the daphne isolate but were not detected in the stains used.

The reason for the low incidence of AMV in daphne in N.Z. is not known but, apart from being isolated from clovers and lucerne (14), little is known concerning the distribution of AMV in this country.

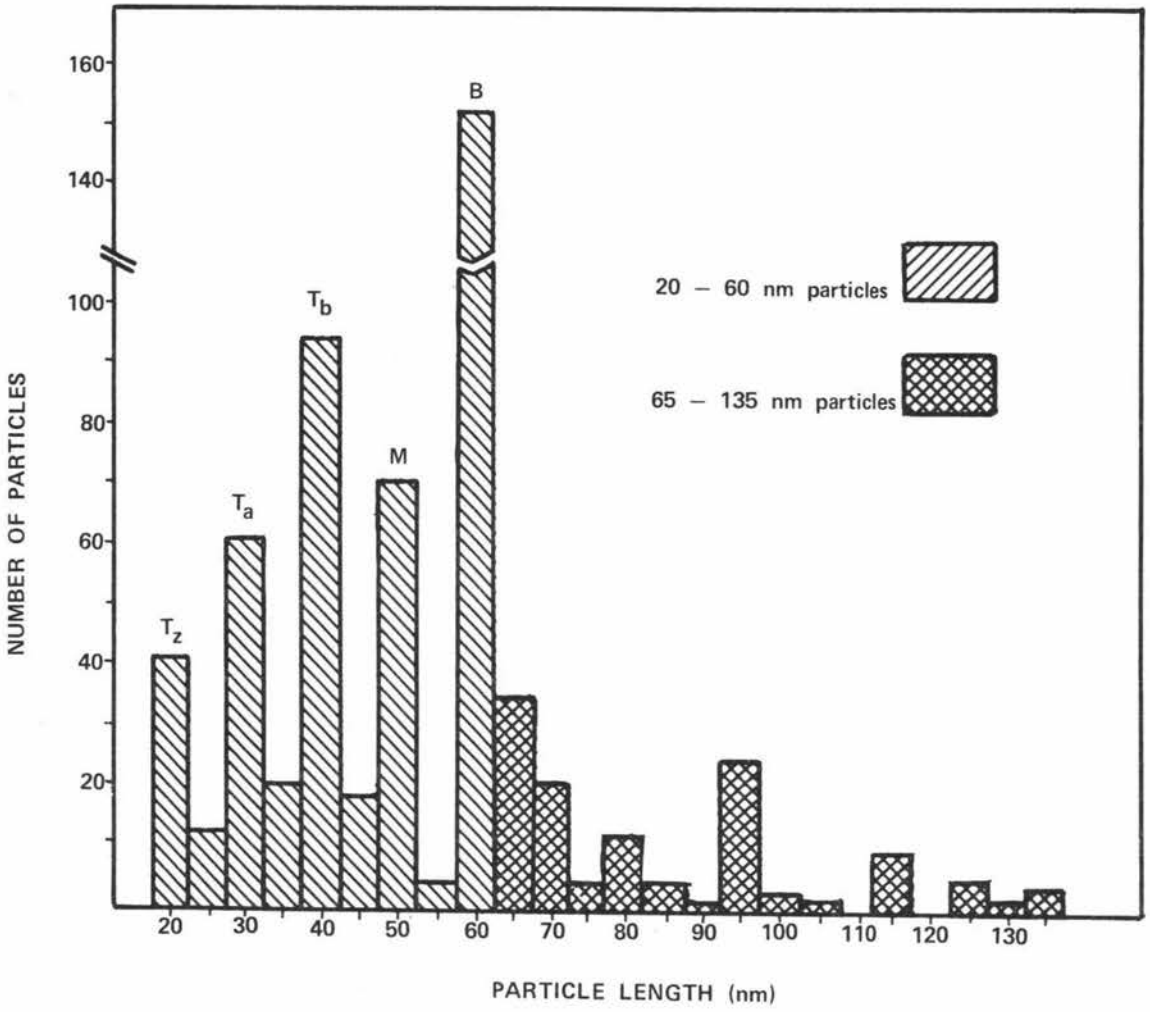


Figure **29** Histogram of particle length distribution of alfalfa mosaic virus in squash homogenates from inoculated leaves of *C. quinoa,* stained in neutral ammonium molybdate.

Named components: T_z, top_z; T_a, top_a; T_b, top_b; M, middle; B, bottom.

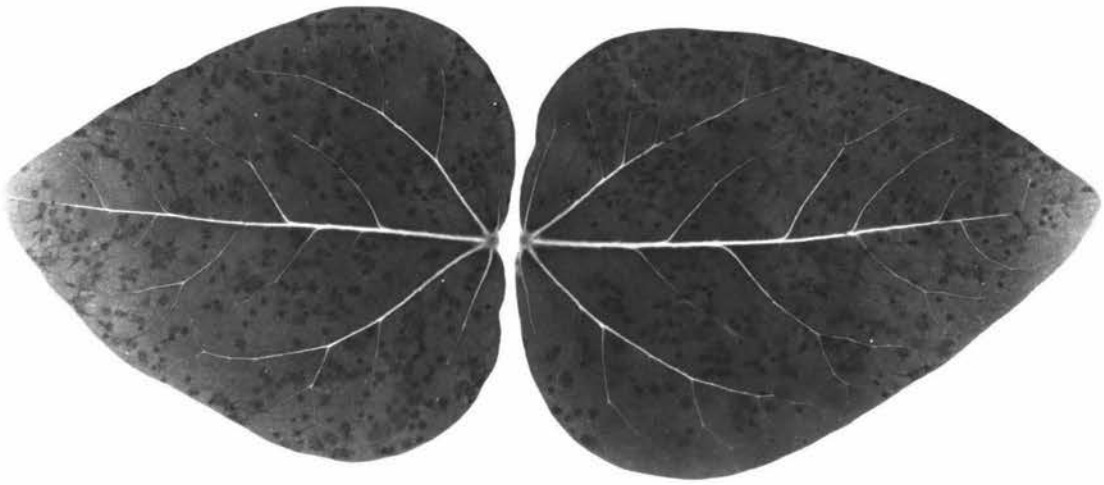


FIGURE 30: Diffuse necrotic local lesions caused by AMV in cowpea.

FIGURE 31: Systemic 'oak-leaf' pattern caused by AMV in Samsun tobacco.

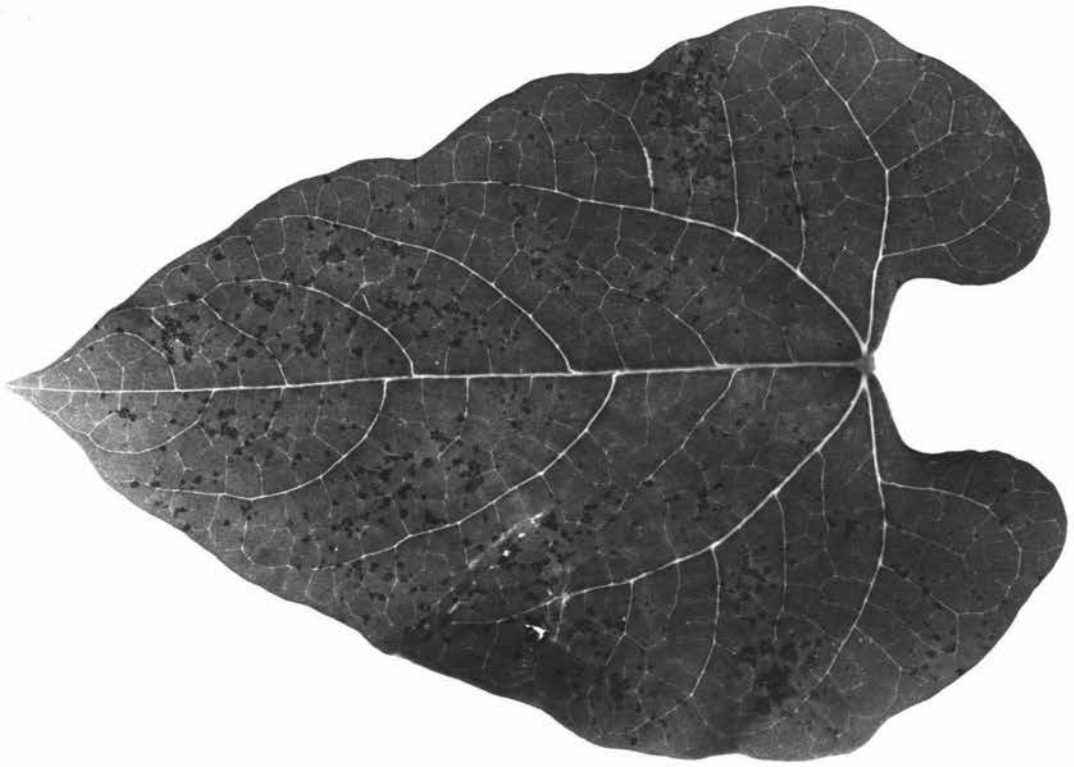


FIGURE 32: Necrotic local lesions in Red Kidney bean infected with AMV.

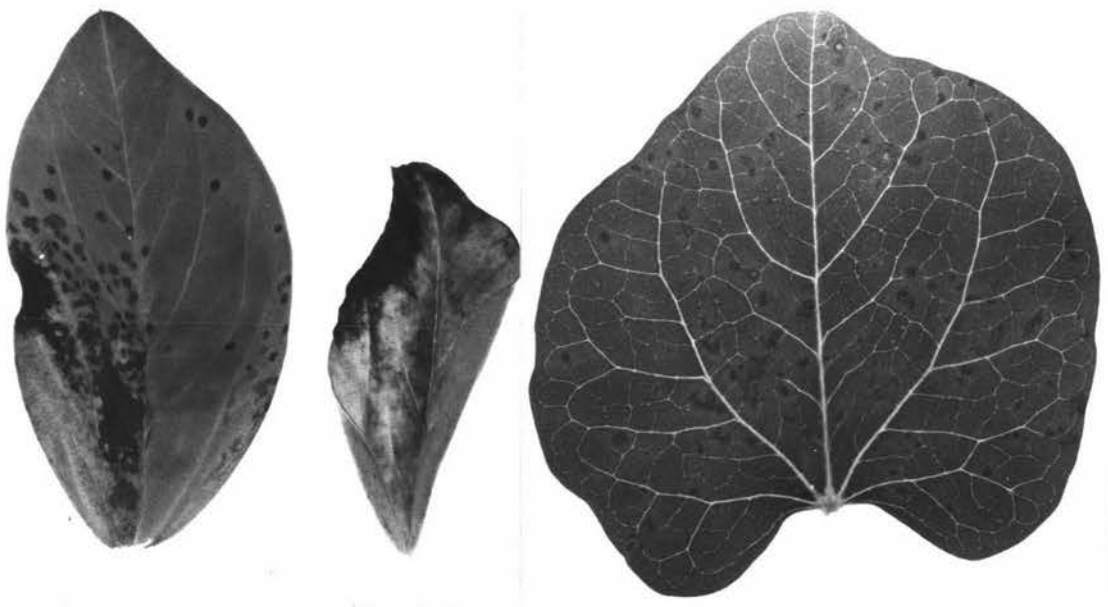
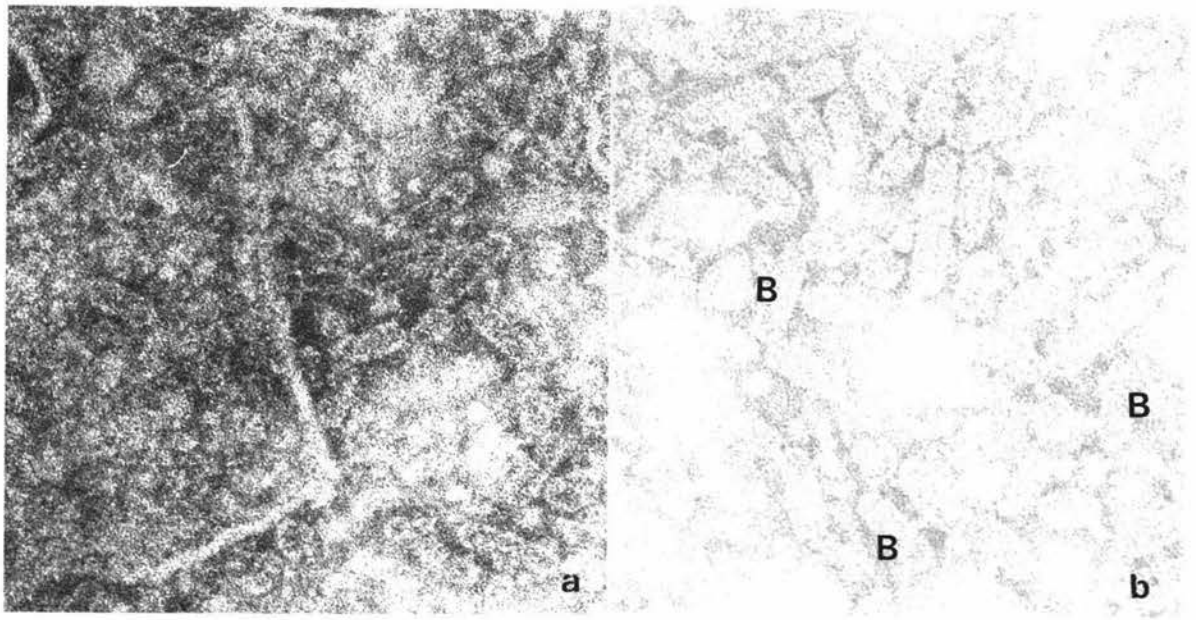
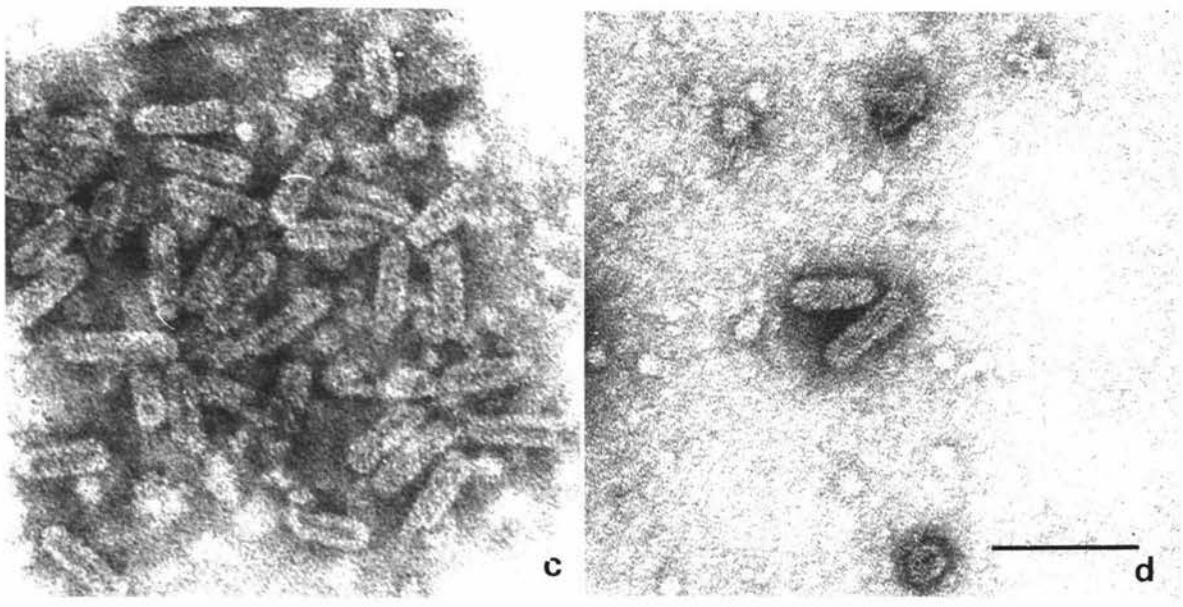


FIGURE 33: Systemic necrosis caused by AMV in Exhibition Longpod broad bean.

FIGURE 34: Necrotic local lesions in D. biflorus infected with AMV.





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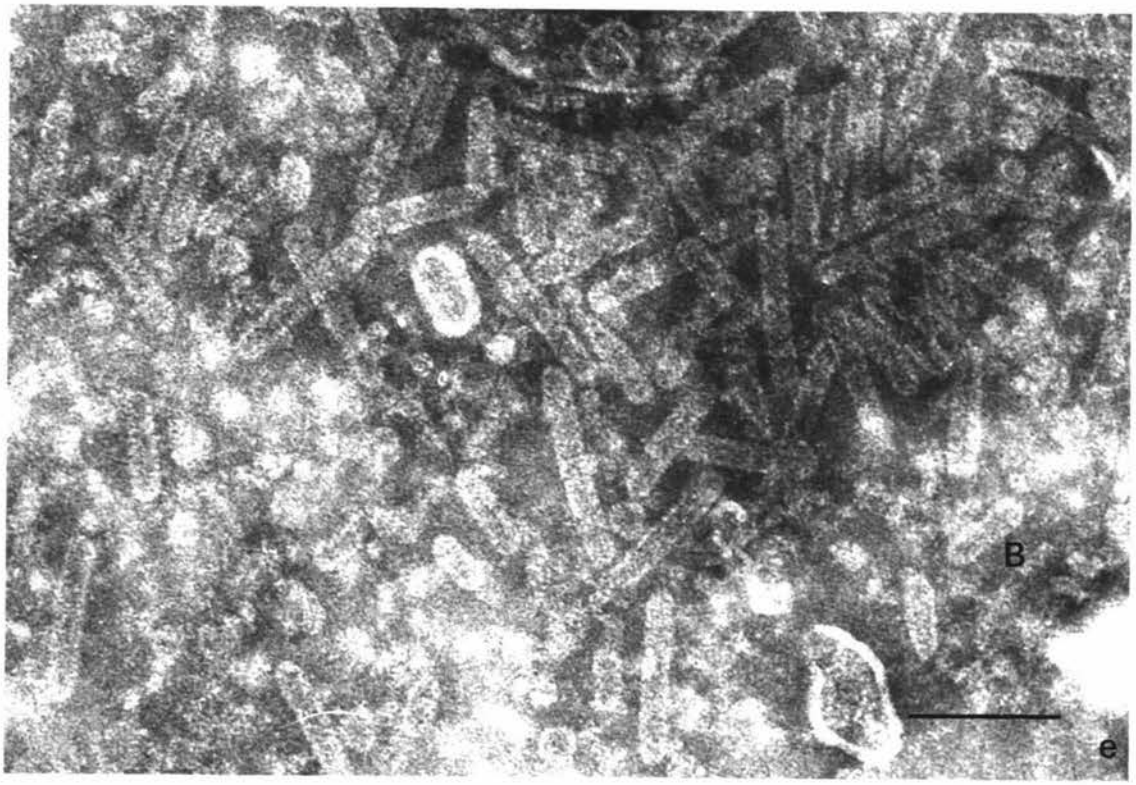

FIGURE 35: Effect of negative stains on the daphne isolate of AMV in squash homogenates from C. quinoa. (Bar represents 100nm)

(a) poorly contrasted particles in neutral 2% PTA;

(b) poorly contrasted particles in neutral 2% AmMo;

(c) clearly contrasted particles in 2% Na silicotungstate, pH 6.5;

(d) clearly contrasted particles in 2% UrAc, pH 4.0;

(e) clearly contrasted particles in 2% PTA, pH 4.0. Note range of particle sizes longer than bottom component (B).

DAPHNE VIRUS Y (*/* : */* : E/E : S/Ap)

A rod virus with ca. 750nm particles was found in large numbers of daphnes in N.Z. For example 100% infection occurred in D. burkwoodii (57 specimens) and 84% in D. odora (500 specimens).

Characterization of the virus, tentatively called daphne virus Y (DVY), was attempted using host range and symptoms, aphid and mechanical transmission and physical properties.

Forms of DVY

Initial investigations revealed the presence of two forms of the ca. 750nm virus, the first (DVY-1) was mechanically transmitted to a few herbaceous hosts and the second (DVY-2) was not transmissible to any common herbaceous indicator plants tested. Inoculations of the latter form were unsuccessful using daphne flowers or leaves as the source, a range of transmission media, purified preparations, dry inoculation (99), a modified agarose column (63) and aphids (M. persicae, A. circumflexum; to C. quinoa, cucumber, Havana 423, spinach).

The detection of the two forms of DVY was dependent on electron microscopy and transmission to C. quinoa but no exact figure for the relative prevalence of each was obtained as isolates of the DVY-1 type would have been overlooked in the presence of other viruses producing chlorotic local lesions in C. quinoa (AMV, ArMV, LVV-2 and TobRSV). Typically, however, the larger nurseries were found to

contain only one form of the virus in any one species or cultivar and DVY-1 was most common in D. odora while DVY-2 was prevalent in many other Daphne species.

Specimens of all Daphne species infected with DVY-2 were symptomless while specimens of Leucanthe infected with DVY-1 were often severely diseased, as detailed in Chapter 1.

Host range of DVY-1

Virus-infected daphne flowers or C. quinoa leaves were ground in Yarwood's bentonite solution and inoculated to indicator plants.

Chenopodiaceae

Chenopodium amaranticolor Coste & Reyn. : faint chlorotic local lesions (4-8 days), expand to 2-3mm with a white, pinpoint necrotic centre, surrounded by a brown necrotic ring and diffuse, outer, chlorotic halo. No systemic infection.

Chenopodium quinoa Willd. : discrete 0.5-1mm chlorotic local lesions (4-6 days) which coalesce when present in large numbers or develop a complete orange or olive outer ring, or a partial or complete necrotic ring. Scattered systemic, chlorotic flecks or blotches (2-4 weeks) which may become bleached or necrotic with time are produced by some isolates while others produce masses of chlorotic flecks or streaks on minor veins (Figure 39).

Spinacea oleracea L. 'Royal Denmark' (spinach): diffuse 2-3mm chlorotic local lesions (7-10 days) which expand to give the entire inoculated leaf a yellow cast. Symptomless systemic infection.

No infection was detected in: Antirrhinum majus L. 'Tom Thumb' (snapdragon); Apium graveolens L. 'Dewcrisp Green' (celery); Brassica pekinensis (Lour.) Rupr. 'Chi-Hi-Li'

(Chinese cabbage); Cassia occidentalis L. (coffee senna); Cucumis melo L. (cantaloupe); Cucurbita maxima Duch. 'Buttercup' (pumpkin): Cucurbita pepo L. 'Small Sugar' (pumpkin); Dianthus barbatus L. 'Indian Carpet' (sweet-william); Gomphrena globosa L. 'Little Buddy' (globe amaranth); Matthiola incana (L.) R.Br. 'Nice Giants Beauty' (stock); Nicotiana glutinosa L. ; Nicotiana tabacum L. 'Samsun' and 'White Burley' (tobacco); Petunia hybrida Vilm. 'Rose of Heaven'; Phaseolus vulgaris L. 'Red Kidney' and 'Top Crop' (French bean); Pisum sativum L. 'Bonneville' (garden pea); Vicia faba L. 'Exhibition Longpod' (broad bean); Vigna sinensis (Torner) Savi. 'Blackeye' (cowpea); Zinnia elegans Jacq. 'Cactus Flowered'.

Means of spread

Daphne virus Y-1 was readily transmitted from C. quinoa or daphne leaves to C. quinoa by M. persicae. Aphids were starved for 24 hours before testing and given a 1-2 minute acquisition period indicating that the virus is non-persistently transmitted.

Physical properties

The only physical properties determined were dilution end point (DEP), longevity in vitro (LIV) and thermal inactivation point (TIP). All local lesion assays were conducted using C. quinoa as the test plant and virus-infected tissue was macerated with 0.01M K-K_2 phosphate, pH 7.0.

The DEP of DVY-1 in daphne flowers was 10^{-6} and in inoculated leaves of C. quinoa, 10^{-3}. The virus was still infectious in C. quinoa sap (1g : 5ml) after 10 days and had a TIP of 55 - 60C.

Purification

Potyviruses are considered very difficult to purify due to relatively low concentrations in hosts, instability in crude sap (82), aggregation and breakage of particles during extraction and purification (6), and loss of virus by adsorption to insoluble plant material (11). Nevertheless, numerous methods have been developed to purify specific members of the group and these have incorporated a wide range of extraction, clarification, and resuspension media.

Distilled water was the most suitable extraction medium for potato virus Y (PVY /23) while high molarity borate was best for pepper veinal mottle virus (PVMV / 11) and turnip mosaic virus (TuMV). Much lower yields were obtained using phosphate instead of borate for PVMV but high molarity phosphate has been used successfully with watermelon mosaic virus (WMV / 62) PVY and tobacco etch virus (TEV / 19). Low rather than high molarity phosphate gave better results with plum pox virus (PPV / 70).

A variety of clarification methods have been employed. Butanol has been successfully used with WMV (62), PVY and TEV and has also been found to enhance the release of the icosahedral viruses including CMV (45) and dahlia mosaic virus (10) from cellular constituents and may act similarly with potyviruses. Considerable losses occurred however when butanol was used with PVMV (11), and bean yellow mosaic virus (6), the latter being severely broken.

Concentration of particles is usually achieved with high speed centrifugation but PEG has also been employed in recent years (19, 70) although it has been reported to cause yield losses with several potyviruses because of resuspension problems (50). A variety of resuspension media have been used to overcome aggregation and include dilute phosphate for WMV (62) and carnation vein mottle (46), dilute borate for TuMV (82) and PVMV (11), dilute tris for several potyviruses (50), phosphate plus urea for TEV and PVY (19) and PPV (70), and phosphate plus EDTA for PVY (23).

The general conclusion from these results is that purification methods used successfully for one potyvirus cannot necessarily be applied for the purification of another. Aggregation is clearly the most serious problem encountered during purification of these viruses and thus requires special attention. Brakke (8) cited high pH, chelating agents such as citrate, and reduction of salt concentrations as means of increasing protein solubility to decrease aggregation. These same conditions however may also make the virus less stable because the secondary valence forces responsible for holding the subunits of a virus together also cause aggregation of the virus. High pH resuspension buffers and chelating agents have been successfully employed with potyviruses (23, 82) as has urea, considered to affect hydrophobic-type interactions which may be responsible for side-to-side and lateral aggregation (19).

The method used to purify DVY was based on the method of Damirdagh and Shepherd (19). Daphne flowers

were used as the source as these contained high concentrations of DVY-1 (DEP 10^{-6}) and were the only suitable source of DVY-2 available. Virus was extracted in phosphate buffer, clarified with n-butanol and precipitated with PEG before high speed centrifugation. Pellets were resuspended in 0.025M K-K_2 phosphate, pH 7.4 containing 0.01M EDTA.

Several modifications were tested before the procedure outlined in the flow diagram (Figure 36) was adopted. Because only DVY-1 isolates could be tested all preparations were checked in the electron microscope for relative content of virus and contaminating host material. Furthermore, the occurrence of two forms of DVY was only elucidated later in this study and as they possibly react differently to a particular treatment a bracketed notation of the virus form in question is included.

Extraction with borate buffer (0.5M H_3BO_3, adjusted to pH 8.0 with 1M NaOH) produced brown-pigmented pellets after butanol clarification, PEG precipitation and two cycles of differential centrifugation. Resuspended pellets in phosphate/EDTA or borate (0.05M H_3BO_3, adjusted to pH 8.0 with 1M NaOH) contained few particles. Clarification with chloroform (1 h incubation) gave preparations which contained only moderate numbers of virus particles and much plant material (DVY-2), while after butanol treatment (12 h incubation) pellets were only slightly coloured and often contained high concentrations of particles. A combination of borate extraction and chloroform clarification gave poor results with very few particles being found.

PURIFICATION OF

DAPHNE VIRUS Y

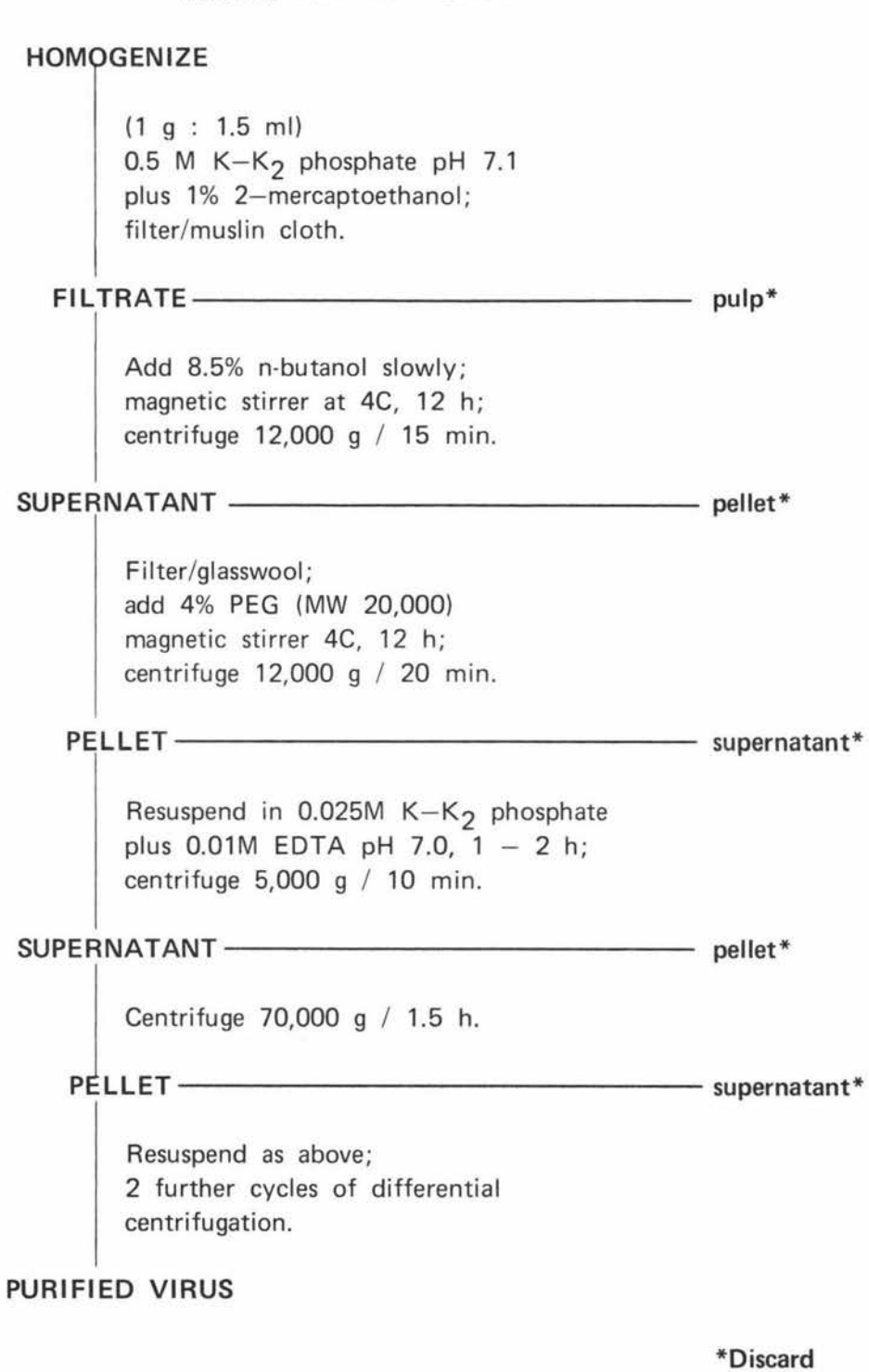

Figure 36 **Flow diagram of the procedure used to purify daphne virus Y.**

Polyethylene glycol was always used for initial concentration to avoid the problem of high speed centrifugation of large volumes of liquid. Low speed centrifugation of the redissolved PEG-precipitate produced extremely large pellets but upon resuspension in borate or 0.001M EDTA these pellets yielded very few virus particles.

Resuspension was initially attempted using phosphate / urea / mercaptoethanol (19) and yielded high concentrations of unaggregated but extensively broken virus particles exhibiting only residual infectivity (DVY-1). Preparations resuspended in dilute phosphate (0.025M K-K_2 phosphate, pH 7.4) and not subjected to low speed centrifugation contained only long, thick masses of highly aggregated particles. Conversely, large quantities of aggregated or broken virus particles were not found in preparations resuspended in borate (0.05M, pH 8.0) or EDTA (0.001M, or 0.01M plus 0.025M K-K_2 phosphate, pH 7.0). Preparations in borate however, showed more colour and appeared in the electron microscope to contain more host contaminants.

Density gradient centrifugation was tested to further purify the virus after PEG treatment and two cycles of differential centrifugation (19). Virus bands failed to separate out after three hours and the method was abandoned.

The destructive action of urea on DVY during purification has not been noted with other potyviruses, but this compound has been tested with only a small number to date (19, 50, 70). The chelating agent EDTA, which prevented aggregation of DVY, has also been used during

the purification of other viruses to remove contaminating metal ions (92) and plant pigments (32), to prevent aggregation (88) and to preserve infectivity (5). Some other viruses however are adversely affected by EDTA, the infectivity of southern bean mosaic virus for example was greatly reduced upon incubation with it. A similar effect has been noted with PPV after exposure to concentrations of EDTA exceeding 0.0005M (70).

Electron microscopy

Electron microscopy of negatively stained squash homogenates from daphne revealed flexuous, filamentous rods (Figure 37). Typically many broken particles were present but the normal length of particles of DVY-1 was 733nm (267 particles) while that of DVY-2 was 726nm (216 particles). No evidence was obtained of a variation in particle morphology with host source, type of stain or purification procedure of the magnitude recorded for BYMV, henbane mosaic virus (37) or PVMV (11).

Discussion

Daphne virus Y is placed in the potyvirus group because of particle length, aphid transmission in a non-persistent manner and mechanical transmissibility. Physical properties are also in agreement with those for other potyviruses.

The virus is tentatively considered a new member of this ever-expanding group based on host range results (for DVY-1) because the host range reported

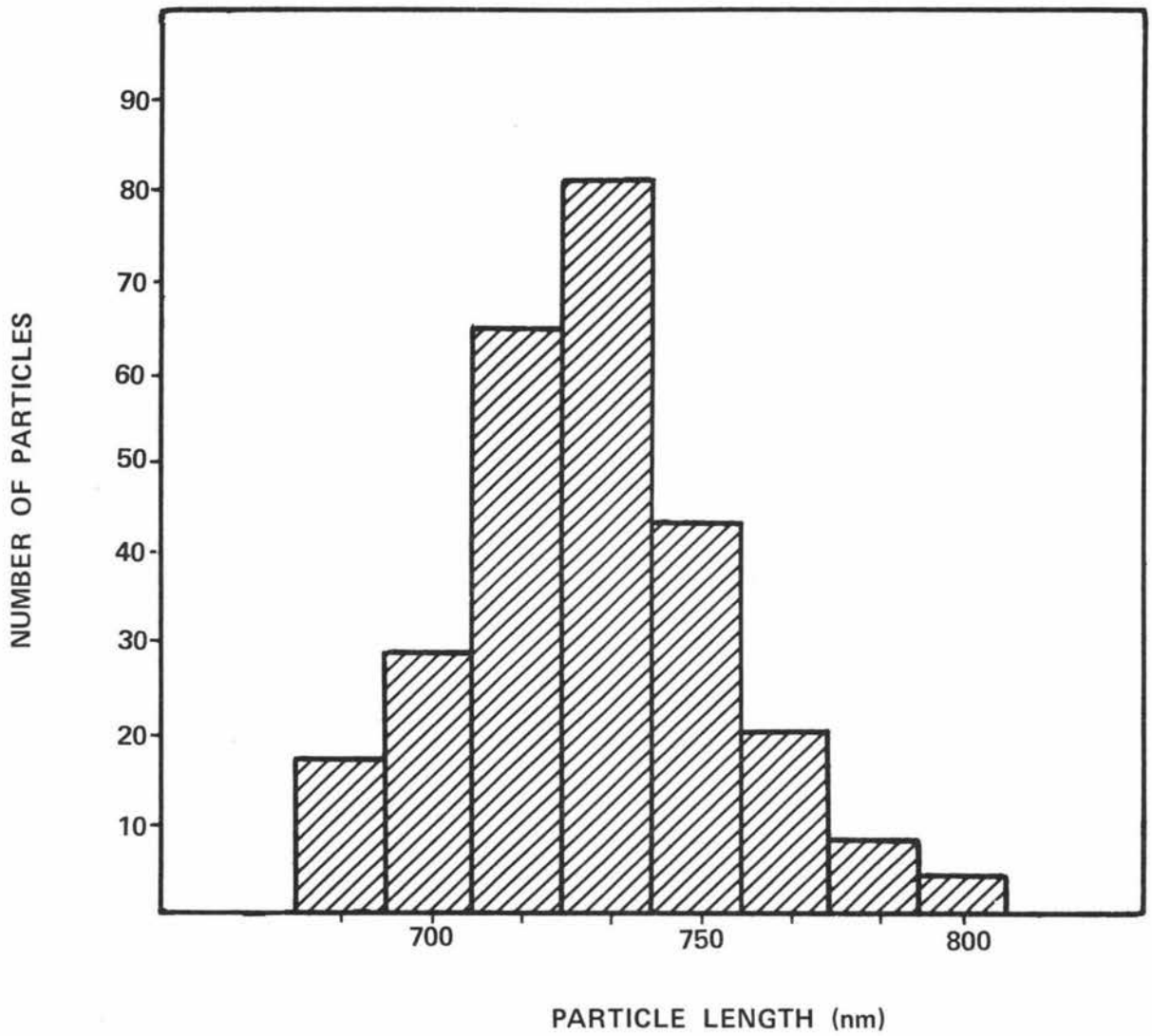

Figure **37** Histogram of particle length distribution of daphne virus Y in squash homogenates of Leucanthe leaves, stained with PTA pH 4.0.

differs from that for at least the common members of the group. Host range alone however is insufficient evidence to make this decision and serology and inclusion body studies (26) could provide further useful criteria.

The two forms of DVY differed only on symptoms in daphne and host range but again this is insufficient evidence for differentiation. Variations in size may result from difficulties associated with accurately measuring long, flexuous particles and the precision of calibration of the electron microscope magnification. The length difference of DVY-1 and DVY-2 are well within the expected range of experimental error.

The fact that at least DVY-1 is transmitted by the ubiquitous M. persicae increases the problem of preventing infection of any 'clean-stock'. Myzus persicae is known to colonize D. odora (17) and during the present study A. circumflexum was found on D. odora, but was not tested as a vector.

DAPHNE VIRUS X (*/* : */* : E/E : S/*)

A rod virus with flexuous particles of similar length to members of the potexvirus group, was found to be widespread in Daphne cneorum ('Major' and 'Variegatum') and was also detected in Daphne cneorum 'Eximia' and Daphne odora 'Leucanthe'.

The virus, tentatively called daphne virus X (DVX), was characterized by host range, aphid transmission, physical properties and particle morphology and size.

Host range

Virus-infected N. clevelandii leaves or D. cneorum flowers were ground in 0.1M K-K_2 phosphate, pH 7.0 and inoculated to indicator hosts.

Amaranthaceae

Gomphrena globosa L. 'Little Buddy' (globe amaranth): dessicated light-brown necrotic flecks (3-4 days) which expand to 1-2mm with a 1-3mm red halo (Figure 41). No systemic infection.

Chenopodiaceae

Chenopodium amaranticolor Coste & Reyn. : very faint chlorotic local lesions (5-7 days). No systemic infection.

Chenopodium quinoa Willd. : irregular faint chlorotic local lesions (4-6 days) which expand to 4-5mm. Systemic symptoms (7-20 days) of mottle or rings, part-rings and blotches (Figure 42).

Cucurbitaceae

Cucumis melo L. (cantaloupe): chlorotic local lesions (4-5 days). Systemic chlorotic blotches (10-12 days).

Cucumis sativus L. 'Marketer' (cucumber): slightly depressed 0.5-1mm chlorotic local lesions (5-6 days). Faint systemic, chlorotic stipple of 0.5-1mm chlorotic dots (7-8 days) on first secondary leaf (Figure 43). Later leaves symptomless but contain virus.

Cucurbita maxima Duch. 'Buttercup' (pumpkin): faint chlorotic local lesions (8-10 days) or no local reaction. Systemic chlorotic mosaic (2-3 weeks).

Leguminosae

Cassia occidentalis L. (coffee senna): 0.5mm orange-brown local lesions (3 days) on cotyledons, expanding to 1-2mm diameter. No systemic infection.

Dolichos biflorus L. : 1-2mm necrotic local lesions (6 days). No systemic infection.

Dolichos lablab L. : 1mm faint chlorotic local lesions (4-5 days).

Phaseolus vulgaris L. 'Red Kidney' and 'Top Crop' (French bean): chocolate pinpoint local lesions (2-3 days). No systemic infection.

Pisum sativum L. 'Bonneville' and Greenfeast' (garden pea): necrotic local lesions or local vein necrosis (4-6 days) followed by wilting and dessication, and blackening of stem in region of inoculated leaves. Systemic chlorotic mottle (10-20 days) sometimes with vein necrosis or necrotic flecks. Severe stunting.

Vigna cylindrica (L.) Skeels: 2-3mm chlorotic local lesions (4-6 days). No systemic infection.

Vigna sinensis (Torner) Savi. 'Blackeye' (cowpea): faint diffuse chlorotic local lesions (6-7 days) which expand to 5-6mm and become more prominent (Figure 40). No systemic infection.

Scrophulariaceae

Antirrhinum majus L. 'Tom Thumb' (snapdragon): 0.5mm chlorotic local lesions (4 days) which expand to 1-2mm and later disappear. No systemic infection.

Solanaceae

Nicotiana clevelandii Gray: 2-3mm chlorotic local lesions (2-5 days) which coalesce when in large numbers and develop ivory or brown necrotic rings or spots. Systemic chlorotic rings or vein-banding (10-14 days) often becoming ivory or brown.

Nicotiana glutinosa L. : 1-2mm chlorotic local lesions (6-8 days) which expand to 1cm. No systemic infection.

Nicotiana glutinosa x clevelandii: 1-2mm chlorotic local lesions (5-6 days) which coalesce when in high concentrations. No systemic infection.

Nicotiana tabacum L. 'Havana 423' (tobacco): Chlorotic 1-2mm local lesions (6 days) which develop into fawn necrotic spots. No systemic infection.

No infection occurred in the following: Apium graveolens L. 'Dewcrisp Green' (celery); Brassica oleracea botrytis L. 'Deepheart' (cauliflower); Brassica oleracea capitata L. 'Drumhead' (cabbage); Brassica pekinensis (Lour.) Rupr. 'Chi-Hi-Li' (Chinese cabbage); Capsicum frutescens L. 'Sweet Capsicum' (pepper); Dianthus barbatus L. 'Indian Carpet' (sweet-william); Dianthus caryophyllus L. 'Single Pinks'; Lactuca sativa L. 'Webbs Wonderful' (lettuce); Matthiola incana (L.) R.Br. 'Nice Giants Beauty' (stock); Medicago sativa L. (lucerne); Nicotiana tabacum L. 'Samsun' (tobacco); Petunia hybrida Vilm. 'Rose of Heaven'; Primula malacoides Franch. ; Saponaria vaccaria L. ; Spinacea oleracea L. 'Royal Denmark' (spinach); Trifolium pratense L. (red clover); Trifolium repens L. 'Grasslands Huia' (white clover); Vicia faba L. 'Atlas Early' and 'Exhibition Longpod' (broad bean); Zinnia elegans Jacq. 'Cactus Flowered'.

Aphid transmission

In a single trial DVX was not transmitted from N. clevelandii to N. clevelandii by M. persicae. The

aphids were starved for 24 hours and given a 1 - 2 minute acquistion period.

Physical properties

The TIP was determined using inoculated leaves of N. clevelandii ground in 0.01M K-K_2 phosphate, pH 7.0 (1g : 5ml). Gomphrena globosa was used as the test plant and the virus had a TIP of 80 - 85C.

Purification

Preliminary studies on purification of DVX were unsuccessful as the virus was either highly aggregated or present only in very low concentrations after purification by the method of Damirdagh and Shepherd (19). The virus was found to be highly aggregated in squash homogenates from N. clevelandii and aggregation from this host was not avoided using EDTA in the extraction buffer (0.01M), or urea (0.5M) or EDTA (0.001M) in the resuspension buffer. Aggregation problems have also been noted for other potex-viruses (2 , 74, 98).

Electron microscopy

Flexuous, filamentous particles were found in negatively stained squash homogenates from daphne and other hosts (Figure 45). The particles were more flexuous than those of DVY (Figure 46).

Size measurements are detailed in Figure 38. Three hundred and twelve particles in D. cneorum flowers stained in neutral 2% PTA had a normal length of 499nm and an average width of 12nm.

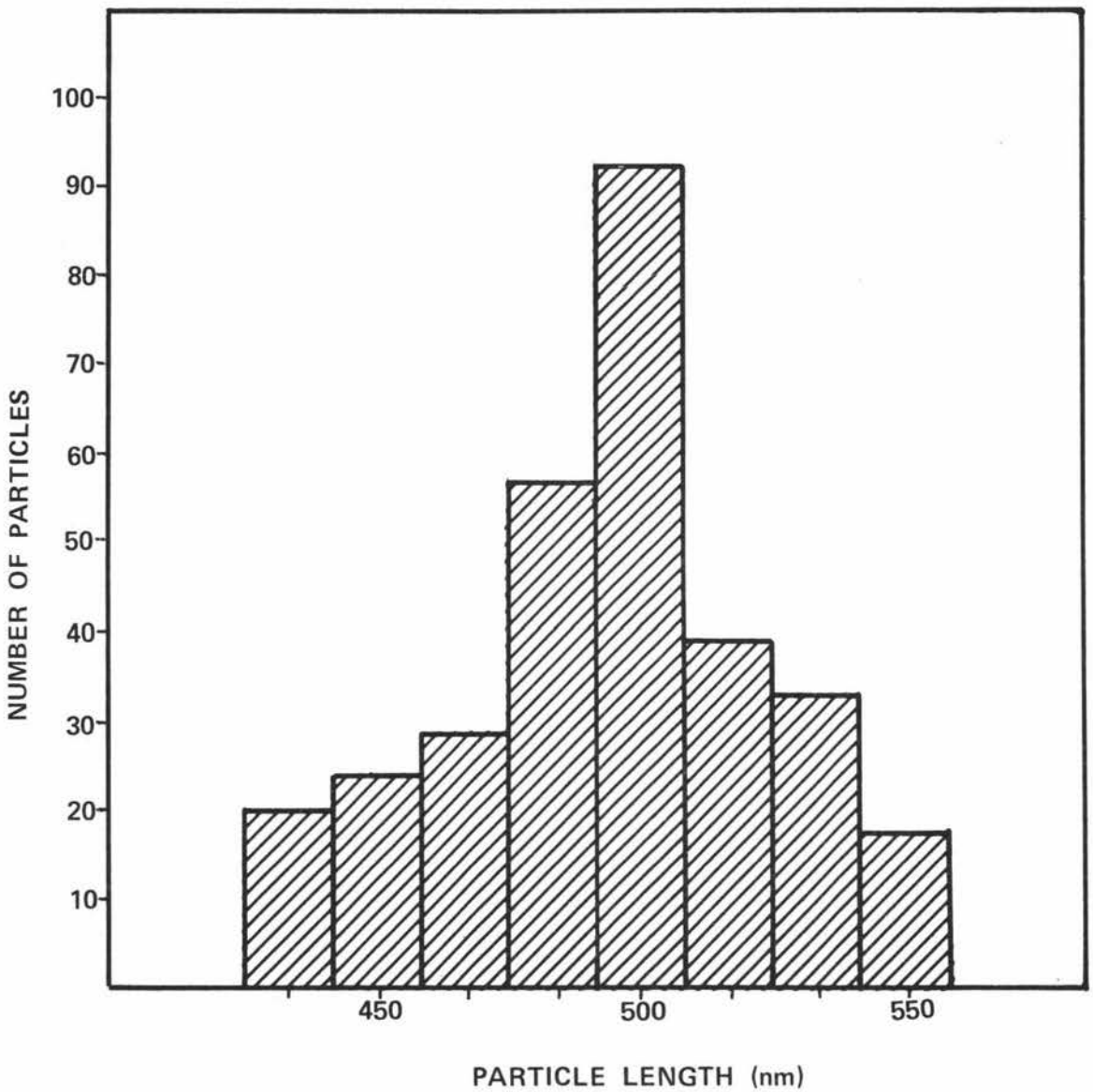

Figure **38** Histogram of particle length distribution of daphne virus X in squash homogenates of *D. cneorum* flowers, stained in neutral PTA.

Discussion

The potexvirus group contains viruses with a normal length of 480 - 580nm and several are serologically related if antisera of high titre is used. The particle size, narrow host range and lack of aphid transmission of DVX are common to potexviruses, most of which also produce similar reactions on G. globosa, snapdragon, and Chenopodium species. The high TIP of DVX is atypical however, but is similar to that for one strain of white clover mosaic virus (3).

Potato virus X, the type member for the potexvirus group is readily transmitted by handling, rubbing of leaves and on knives. The fungus, Synchytrium endobioticum (Schilb.) Perc., has also been reported to transmit potato virus X (68) and the possibility that DVX is transmitted by one or more of these methods cannot be discounted.

The two flexuous rod viruses found in Daphne species were readily distinguished on the basis of particle size but as well there are several other features which facilitate differentiation and these are summarized in Table 8.

TABLE 8 Distinguishing features of DVX and DVY.

Virus	Criteria				
	Morphology & size	Host range	Occurrence in Daphne	Vector	TIP[a] (°C)
DVX	very flexuous 499nm	wide	D. cneorum D. odora	-	80-85
DVY	slightly flexuous 733nm	narrow	many Daphne spp.	aphid N.P.[b]	55-60

[a] thermal inactivation point

[b] nonpersistent

DAPHNE - TOBACCO MOSAIC VIRUS (*/* : */* : E/E : S/*)

A rod virus morphologically identical to tobacco mosaic virus (TMV) was detected in negatively stained squash homogenates from Leucanthe and D. cneorum. The actual prevalence of the virus is unknown however as it occurred in extremely low concentrations and often none or only one or two particles were found in a single electron microscope grid. (Figure 46)

The virus, tentatively called daphne-TMV (D-TMV), could not be increased in herbaceous hosts (N. glutinosa, N. clevelandii, N. tabacum and C. quinoa) by inoculating from daphne leaves or flowers, or from partially purified preparations from daphne leaves. The latter were extracted in 0.5M K-K_2 phosphate, pH 8.0 plus 1.0% 2-mercaptoethanol, incubated with chloroform (1 : 1 ; 20 minutes) and subjected to two cycles of differential centrifugation, pellets being resuspended in 0.01M K-K_2 phosphate, pH 7.0.

Size measurements were limited to a small number of particles from squash homogenates as the mucilaginous nature of the partially purified preparation prevented suitable definition in negative stain. Thirty-two particles in preparations from D. cneorum and Leucanthe had an average length of 300nm and an average width of 15nm. (Figure 46)

Very low concentrations of TMV have been detected in cotton (15) but the significance of such low concentrations in daphne is unknown as many infected specimens of both Leucanthe and D. cneorum were symptomless.

FIGURE 39: Chenopodium quinoa specimens with systemic chlorotic flecks and blotches caused respectively by two different isolates of DVY-1.

(a) three weeks after infection

(b) five weeks after infection

b

a

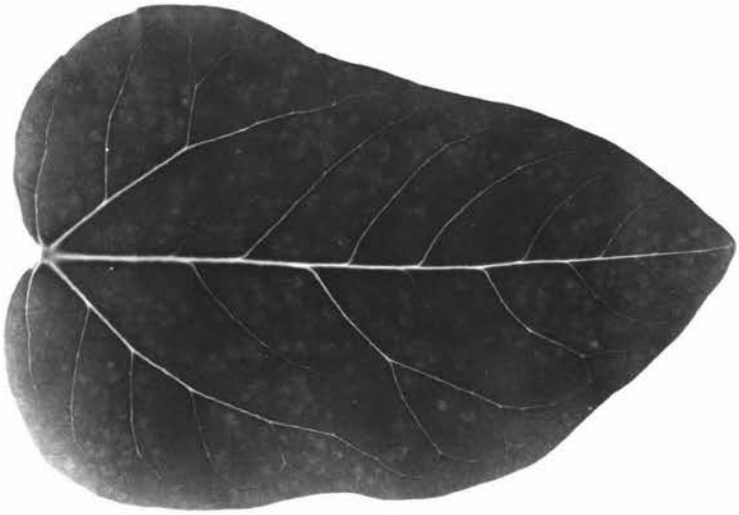

FIGURE 40: Chlorotic local lesions caused by DVX in a cotyledon of cowpea.

FIGURE 41: Irregular necrotic local lesions with red halos in G. globosa infected with DVX.

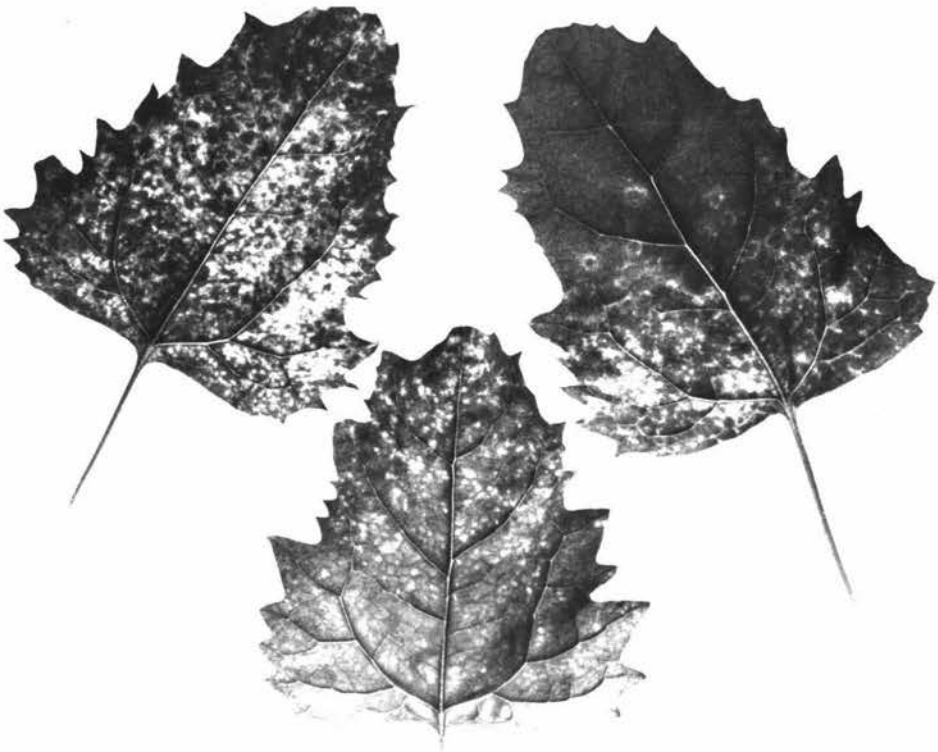

FIGURE 42: Chenopodium quinoa with systemic chlorotic rings and blotches caused by DVX.

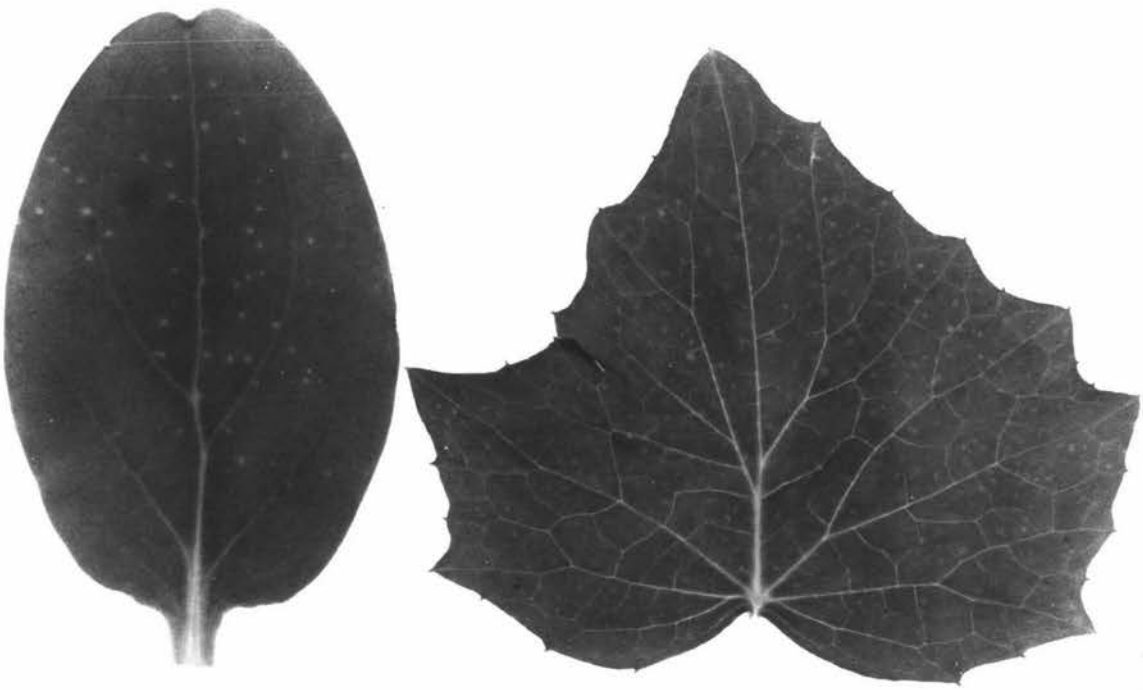

FIGURE 43: Chlorotic local lesions (on cotyledon) and faint systemic chlorotic stipple in first true leaf of cucumber infected with DVX.

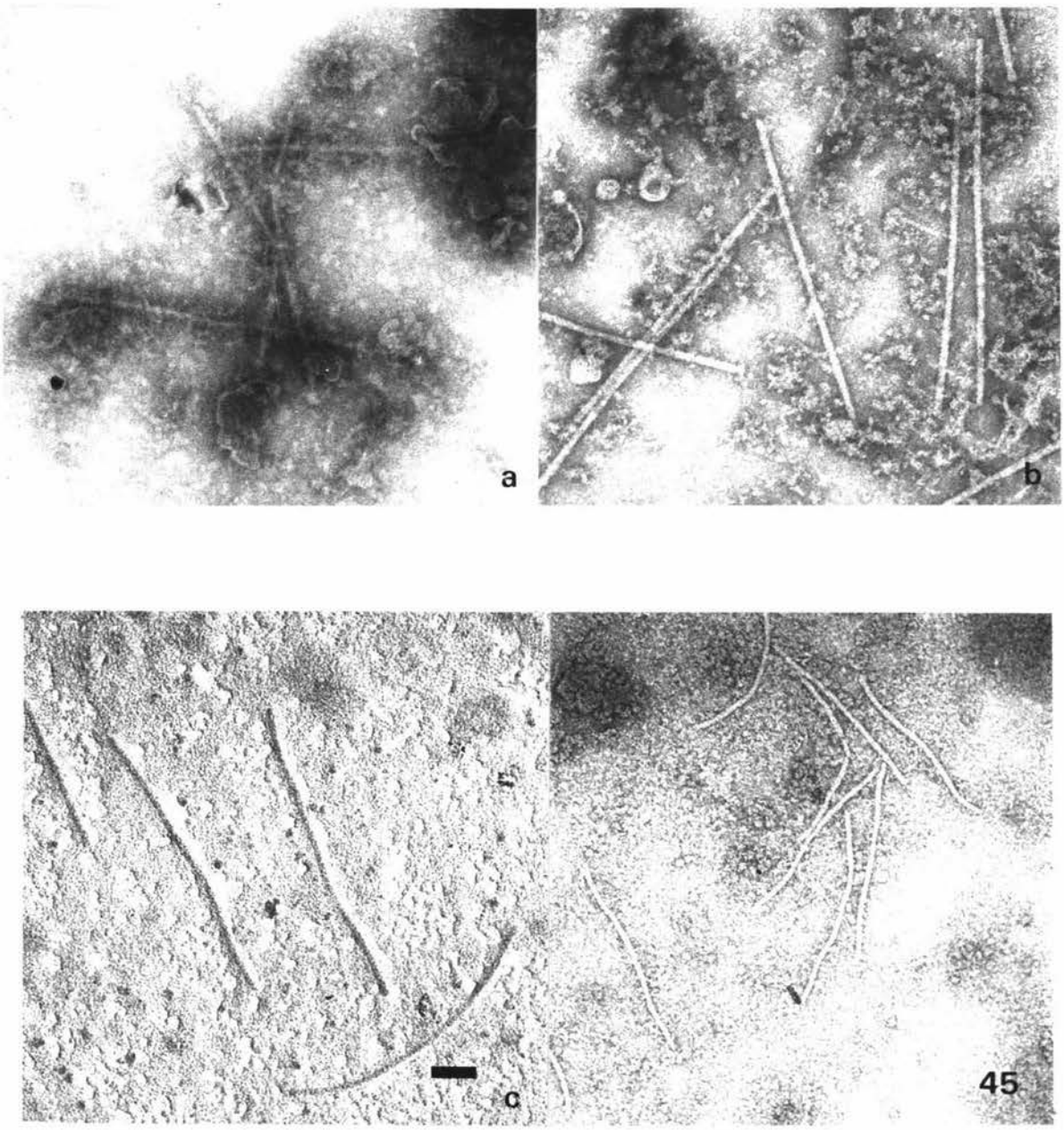

FIGURE 44: Particles of DVY in squash homogenates from daphne flowers.

(a) poor contrast negatively stained in PTA, pH 4.0;

(b) clear contrast negatively stained in neutral 2% AmMo;

(c) clear contrast shadow-cast with platinum.

FIGURE 45: Daphne virus X in squash homogenates from D. cneorum flowers negatively stained with neutral 2% PTA.

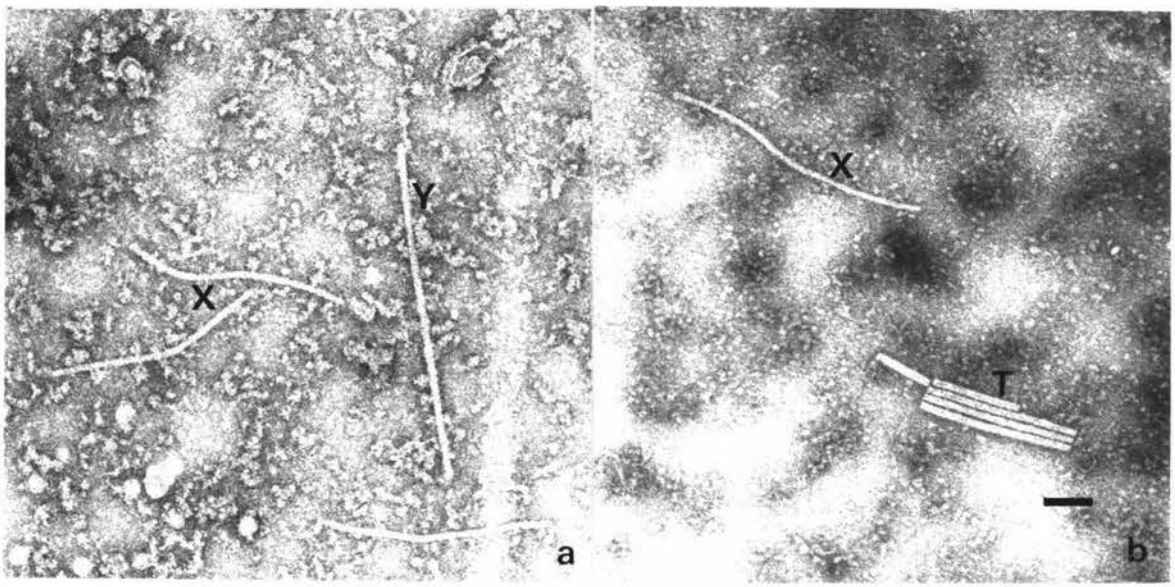

FIGURE 46: Mixed infections of rod viruses in squash homogenates from D. cneorum flowers negatively stained with neutral 2% PTA. (Bar represents 100nm)

(a) DVX (X) and DVY (Y)

(b) DVX (X) and D-TMV (T)

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CHAPTER 3

PRODUCTION AND MAINENANCE OF 'HIGH-HEALTH' STOCK

Production of 'high-health' daphne

Several methods are commonly employed for the production of vegetative plant material free of detectable viruses (hereafter termed 'high-health'). Indexing may reveal the existence of 'high-health' specimens but if a cultivar is wholly infected, 'high-health' plants may be produced by exploiting erratic virus distribution, meristem-tip culture, heat treatment or chemotherapy.

Although erratic virus distribution was not noted in daphnes, 'high-health' specimens of Leucanthe and Rubra were found and these are currently being increased for release to the nursery industry.

Meristem-tip culture has been employed successfully for elimination of viruses in a number of herbaceous plants but in few woody plants. Of the latter group, gooseberry (4), and apple (2) have been successfully propagated from meristem-tips using a stock solution plus the cytokinin, benzyl-amino-purine.

In a single trial meristem-tips from young Leucanthe specimens taken during the autumn growth flush were successfully maintained on filter paper bridges with a stock solution (6) plus 10^{-7}M benzyl-amino-purine (2). The

specimens expanded slightly over several months but failed to initiate roots, a result also noted in trials with apple (2). It is possible however that if the medium could be improved to further increase the size of tips they could be grafted onto seedlings of suitable Daphne species.

Thermotherapy has been employed successfully for the removal of viruses from a large number of plants. Young Leucanthe plants in an active state of growth were exposed to 100C for two weeks following a preliminary acclimatization period at 85C. One to 2cm tips from growth made at the high temperatures were then taken for propagation but at the time of writing these had not been reindexed. A combination of thermotherapy and meristem-tip culture was not attempted, but has been used successfully for other plants (3).

Maintenance of 'high-health' plants

'High-health' plants may become infected with viruses by vectors such as nematodes and aphids. Two of the viruses from daphne (ArMV and TobRSV) are nematode-transmitted and four (AMV, CMV, DVY and TobRSV) are aphid-borne.

Spread by nematodes is very slow and can be avoided by eliminating infected plants or avoiding nematode-infected sites. Conversely, spread by aphids can be rapid and plants may become infected from more distant sources.

All aphid-transmitted daphne viruses are carried in a non-persistent manner. This mode of transmission is characterized by rapid acquisition of the virus (5 - 15 seconds), a short period in which the aphid is viruliferous (1 - 2 h) and rapid reinoculation. Therefore, viruses

transmitted by this method are carried only relatively short distances, and plants can be inoculated before the aphid vector is killed by insecticides.

Absolute aphid control is only possible using aphid-proof houses but this is only economically feasible on a small scale, and could be used for maintaining a nucleus stock of 'high-health' plants. Rooted daphne cuttings are frequently grown in open fields and if originally from a 'high-health' source could be partially protected from infection by isolating from virus sources, use of oil sprays which prevent transmission of non-persistently transmitted viruses (1), or by using aphid repellants, namely chemicals (7) and aluminium foil (5). The latter reflects ultraviolet light which deters air-borne aphids from alighting and would be readily applicable to daphnes, some of which are presently grown through plastic mulch.

The high percentage infection of common Daphne cultivars in nurseries is evidence that vegetative propagation is the main method by which plants infected with DVY and ArMV are produced. Consequently, the availability of 'high-health' stock of common cultivars such as Leucanthe and Rubra should make a significant contribution to the growing of healthier specimens of these cultivars.

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