

PURIFICATION AND PROPERTIES OF A
NEW CARLAVIRUS FROM DANDELION

by

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ABSTRACT

A carlavirus was isolated from naturally-infected dandelions in the Okanagan Valley, B.C. In total, 31 plant species belonging to 12 families were tested as possible hosts for the dandelion virus. In only four families (Amaranthaceae, Chenopodiaceae, Compositae, Solanaceae) were susceptible species found. The virus was contained as local lesions in Gomphrena globosa and Datura stramonium and became systemic in Chenopodium amaranticolor, C. quinoa and Taraxacum officinale. The carlavirus, for which the name Dandelion Virus S (DVS) is proposed, has slightly curved particles with normal length 637 nm and width 12-13 nm. A purification scheme was developed that yielded 20-30 mg of virus per kg of C. quinoa leaf and stem tissue. Partially purified virus preparations had a single nucleoprotein component in rate zonal sucrose and cesium chloride density gradient centrifugation. The UV absorption spectrum has a maximum at 259 nm and a minimum at 245 nm. The ratio of A_{\max}/A_{\min} is approximately 1.1; of A_{260}/A_{280} , 1.4. In sap from infected C. quinoa, DVS had a thermal inactivation point of 75-80°; an infectivity dilution end point of 2×10^{-5} to 2×10^{-6} ; a longevity in vitro of 4-5 days at 23°, 28-56 days at 4° and at least 16.5 months in a lyophilized state at 23°. An antiserum against DVS was prepared by four intramuscular injections of 1 mg each and the maximum homologous titre was 40,960. Two

carlaviruses with similar symptoms in C. quinoa, Peru virus S (PeVS) and Helenium virus S (HVS) were purified for antisera production and comparative serological testing. Antisera to other carlaviruses were also used to determine if serological relationships existed with DVS and other members of the group. Serologically, DVS is related to potato virus S (PVS) and PeVS, and distantly related to chrysanthemum virus B (CVB), Helenium virus S (HVS) and narcissus latent virus (NLV).

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INTRODUCTION

It is becoming increasingly important to recognize the potential reservoir of plant pathogens contained in the weed population, especially when control or eradication programs are being implemented to maximize yields of economically important crops. Virus-infected weeds, volunteer plants and natural vegetation in or near crops, or even at remote distances may contribute to crop infection by harbouring particular viruses and/or their vectors, as well as increasing the number of infective foci in the case of seed-transmitted viruses. The weed hosts are often ignored in crop surveys as they usually show no symptoms when infected (Bos, 1978).

Taraxacum officinale Weber, the common dandelion, is one such potential reservoir of virus. It has become one of the most common weeds in Canada due to its efficient seed dispersal, its long flowering period and its adaptability to a wide range of environmental conditions (Gilkey, 1957; Mulligan, 1976).

Although dandelion is widespread throughout the world, and is frequently associated with agricultural crops, its capacity as a virus reservoir does not adversely affect the majority of crops. The available literature suggests that dandelion is not a host for many viruses that are known to have wide natural host ranges, except those that are transmitted by nematodes. The following nepoviruses have been reported to infect dandelion naturally: arabis mosaic (Harrison, 1958); cherry rasp leaf (Hansen et al., 1974); peach rosette mosaic

(Ramsdell and Myers, 1978); tobacco ringspot (Tuite, 1960; Lister and Murrant, 1967); tomato blackring (Harrison, 1957) and tomato ringspot (Dias, 1977). In all cases, the nepoviruses were seed-transmitted at a low level in dandelion seedlings and the seedlings remained symptomless. It is because of seed transmission and the ubiquitous nature of dandelions that they are implicated in the spread of nepoviruses over larger ranges than is possible simply by movement of infected nematodes through the soil.

There are few reports of other viruses infecting dandelion naturally: chrysanthemum latent (Hollings, 1957); dandelion yellow mosaic (Kassanis, 1944); potato virus Y (Lytaeva, 1971) and tobacco streak (Fulton, 1948).

Although rod-shaped viruses: Bidens mottle (Purcifull et al., 1976), Cassava common mosaic (Costa and Kitajima, 1972), plum pox (Kegler and Schade, 1971) and tobacco mosaic (Zaitlin and Israel, 1975), and bacilliform viruses: lettuce necrotic yellows (Francki and Randles, 1970), potato yellow dwarf (Black, 1970) and sowthistle yellow vein (Peters, 1971), do infect Compositae, few reports have so far been published on the natural occurrence of carlaviruses in dandelion. This probably reflects the low level of interest on the part of the agricultural industry in virus/weed ecology in relation to crops, rather than a true indication of one incidence, as reported here, of a carlavirus naturally infecting dandelion.

When tomato bushy stunt virus (TBSV), (Martelli et al.,

1971), was found in the Okanagan Valley, the potential source of infection was sought by Dr. A.J. Hansen and Ms. L. Green. During their survey, (1972-74), they indexed a variety of indigenous plants growing in and around the infected orchards. Some of the index plants inoculated with dandelion field material showed symptoms of virus infection that were not typical of TBSV infection in those particular hosts. A preliminary examination of a leaf dip from these hosts revealed slightly flexuous rod-shaped virus particules ca. 650 nm (Hansen, personal communication).

Since there was no evidence that the virus from dandelion occurred in any tree fruits in the Okanagan, no further work was done with the virus at the time. In 1977, when I was considering possible projects for a thesis, Dr. Hansen suggested that this problem warranted further investigation and that he would be pleased to provide me with a culture of the virus.

Based on particle size and morphology, the virus was almost certainly a member of the carlavirus group (Harrison et al., 1971). Whether it was a new virus, or whether it was closely or distantly related to a recognized virus was not known. For these reasons, this study was undertaken to determine if the virus from dandelion was a previously-undescribed carlavirus occurring in dandelion or if dandelion was an unrecognized host for a known carlavirus.

The International Committee on the Taxonomy of Viruses (ICTV) has approved the use of various sigla to denote virus

groups and has attempted to classify the groups accordingly (Harrison et al., 1971; Fenner, 1976). Carnation latent virus is the type member of the carlavirus group and when the group was originally described, nine other viruses were included: cactus virus 2; chrysanthemum virus B (CVB); cowpea mild mottle virus; lily symptomless virus; passiflora latent virus; pea streak virus (PSV); potato virus M (PVM); potato virus S (PVS) and red clover vein mosaic virus (RCVMV).

Possible members included: chicory blotch virus; cole latent virus; cynodon mosaic virus; elderberry virus; freesia mosaic virus; hop latent virus; muskmelon vein necrosis virus; narcissus latent virus (NLV) and poplar mosaic virus (PMV) (Harrison et al., 1971; Fenner, 1976).

Since the group has been established, at least seven additional carlaviruses have been reported: alfalfa latent virus (Veerisetty and Brakke, 1977); Chinese yam necrotic mosaic virus (Fukamoto and Tochiwara, 1978); Daphne virus S (Forster and Milne, 1978); eggplant mild mottle virus (EMMV) (Khalil and Nelson, 1977); Helenium virus S (HVS) (Kuschki et al., 1978); lilac mottle virus (Waterworth, 1972) and shallot latent virus (Bos et al., 1978). Complete characterization has not been reported for all of these viruses and in the case of Daphne virus S, further studies are warranted as the size of the particle (704-716 nm) does not fit well within the range for carlaviruses (620-690 nm).

Carlaviruses are characterized by slightly flexuous rod-

shaped particles, 620-690 nm long, containing about 6% single stranded RNA. In general, the longevity in sap at room temperature is a few days and the thermal inactivation point is between 55° and 70°. The concentration in sap is 20-100 mg/l, but because of a tendency to aggregate side-to-side and end-to-end, the yield of purified virus is much lower (Varma et al., 1970; Luisoni et al., 1976; Veerisetty and Brakke, 1978).

The members of this group infect narrow host ranges both naturally and experimentally, causing no symptoms or only mild ones. They are readily transmitted by sap inoculation, except when natural plant inhibitors are present and interspecific transfers are being attempted. In those cases where a vector has been found, it has proven to be an aphid that transmits the virus non-persistently (Harrison et al., 1971; Fenner, 1976; Christie and Edwardson, 1977).

Serological relationships exist between some members of the carlavirus group, although it is not essential that a virus be serologically related to a described member of the carlaviruses in order to be included in the group (Gibbs et al., 1966). For example, poplar mosaic virus is considered a carlavirus even though it is not serologically related to any other known members of this group (Brunt et al., 1976; Luisoni et al., 1976). The main criterion is that particle size and shape must be in the range of values set down for members of this group and on this basis, planned serological

investigations can be undertaken to determine relationships among viruses.

As the study progressed, it became obvious that in order to determine whether in fact the virus from dandelion was an undescribed carlavirus or a strain of a known virus, it was essential to obtain cultures of similar viruses and to undertake direct comparisons under standard conditions. The literature was reviewed to determine which other carlaviruses could produce systemic symptoms in Chenopodium quinoa Willd., similar to those produced by the dandelion virus. From the descriptions in the literature, the virus that seemed most likely to be related was one that had been isolated from Mantaro potatoes in Peru, and was considered to be a strain of potato virus S (Hinostroza-Orihuela, 1973). A culture of this virus was obtained from Dr. A.M. Lekeu, Lima, Peru and the necessary comparisons were made. In order to avoid confusion with potato virus S (PVS) in the tests, the Peru isolate was referred to as Peru virus S (PeVS).

While these studies were underway on the relationship with PeVS, another carlavirus was reported from Helenium amarum hybrids in Germany, that also caused systemic symptoms in C. quinoa (Kucshki et al., 1978). This virus appeared similar to the dandelion virus and warranted inclusion in the comparative studies. A culture of Helenium virus S was obtained from Dr. R. Koenig, Braunschweig, Germany and comparative testing was started.

Carnation latent virus also causes systemic infection of C. quinoa (Kemp and High, 1979), but in view of its relatively mild symptoms and the difficulty with which it is transmitted from carnation to other host species, it was not considered as a potentially close relative of dandelion virus. Consequently, no culture of carnation latent virus was obtained.

When this project was started, the object was to describe some of the biological, physiochemical and serological properties of dandelion virus. (The abbreviation DVS will be used throughout this paper for dandelion virus.) For reasons outlined above, it has evolved into a comparative study of three carlaviruses from three geographically isolated and distinct areas: Canada, Germany and Peru, as well as a description of some of the properties of DVS.

MATERIALS and METHODS

Field occurrence of dandelion virus was determined by indexing dandelions from various sites in British Columbia and to a lesser extent from other parts of Canada. The collected samples were indexed by enzyme-linked immunosorbent assay (ELISA) (Clark and Adams, 1977) and by rub inoculation to C. quinoa.

Host Range and Symptomatology

Leaves from infected dandelions were ground in inoculating buffer, (0.1M KPO₄, pH 7.4 with 1% polyvinyl pyrrolidine (PVP) and 1% nicotine), and rubbed onto carborundum-dusted C. quinoa

leaves. The systemically infected leaves provided the source of inoculum for the host range study. To reduce the effect of Chenopodium spp. inhibitors, the sap was clarified by treatment with calcium phosphate and the virus was concentrated twofold by pelleting through 20% (w/v) sucrose (Veerisetty and Brakke, 1978). The virus pellet was resuspended in inoculating buffer.

Plants from 12 families were lightly dusted with carborundum and the inoculum was applied gently with a foam pad. A negative check was included for each test by rubbing a second plant with inoculating buffer only. Immediately after inoculation, the plants were rinsed with tap water. The test plants were assayed 2 wk after inoculation by back inoculating onto C. quinoa and by ELISA.

Properties in Crude Sap

The longevity in vitro of DVS in C. quinoa sap at 4° and 23° was determined over time. Systemically infected C. quinoa leaves were ground 1:2 (w/v) with inoculating buffer and centrifuged at 5,000 rpm for 5 min in a Sorvall SS 34 centrifuge. The supernatant was dispensed in 1 ml aliquots and stored at 4° or 23° for the appropriate length of time before infectivity was tested. Inoculating buffer served as the negative check in each case, and symptoms were recorded 3 wk after inoculation. Lyophilized tissue was stored at room temperature and the infectivity was checked about every 4 months by inoculation to C. quinoa.

To determine the dilution end point, systemically infected C. quinoa leaves were ground 1:5 (w/v) with inoculating buffer and the resulting sap was diluted in tenfold increments to 10^{-10} . One ml of each dilution was inoculated to five C. quinoa leaves and the symptoms were recorded at 14 and 21 days after inoculation. Inoculating buffer served as the negative check.

The thermal inactivation point was determined with sap from systemically infected C. quinoa leaves, ground 1:2 (w/v) with inoculating buffer. The sap was dispensed in 1 ml aliquots into thin-walled borosilicate test tubes (10 x 75 mm) and incubated in a Thelco Model 81 water bath for 10 min after the sap had reached the treatment temperature. The aliquots were kept on ice before and after treatment. The cooled aliquots were inoculated onto C. quinoa, with inoculating buffer as the negative check. Symptoms were recorded 14 and 35 days after inoculation.

Seed Transmission

Mature C. quinoa seeds were collected 10 wk after inoculation and after a 4-wk aging period, 100 seeds were germinated on moist filter paper. The seedlings and seed coats were separated and ground in inoculating buffer for indexing on healthy C. quinoa.

Seedlings from 100 seeds germinated in soil were transplanted to individual 10 cm pots 10 days after germination. The seedlings that survived this transfer were indexed at the

six leaf stage by inoculation to healthy C. quinoa and by ELISA.

Dandelion seeds were collected from infected dandelions and allowed to age 6 months before testing. Of the 64 seedlings tested by ELISA, 13 were also indexed onto C. quinoa. The rate of germination was determined by placing the seeds from healthy and infected plants on soil or moist filter paper and incubating them at room temperature.

Aphid Transmission

A colony of Myzus persicae (Sulz.) was established on healthy C. quinoa and maintained at 10-15° with a day length of 16 h. The colony was allowed to adapt to C. quinoa before vector studies were undertaken, in order to reduce the effect of anomalous feeding behaviour.

Aphids were collected into a glass Petri dish and starved for 1 hr. Access times from 0-45 min at 5 min intervals were tested with 25 aphids per test, except at time 20 and 35 min, when 50 aphids were used. After exposure to infected C. quinoa leaves, the aphids were transferred to healthy C. quinoa for 18 h after which time they were removed and the plants transferred to the greenhouse. Symptoms were recorded 10 days after inoculation.

Purification

An efficient and inexpensive method of purification was developed to prepare concentrated virus. Systemically infected C. quinoa leaves and stems were homogenized for 3 min in a

Waring blendor with 2 vol (w/v) cold 0.2M sodium borate buffer, pH 9.0, containing 1% mercaptoethanol and 0.2% sodium diethyldithiocarbamate. The homogenate was pressed through nylon cloth and the sap was centrifuged at 10,000 rpm for 20 min (Sorvall GSA rotor). The supernatant was collected through a layer of Miracloth and left at 4° overnight, then centrifuged at 10,000 rpm for 20 min. The resulting supernatant was centrifuged at 26,000 rpm for 90 min (Beckman No. 30 rotor). The pellets consisted of a gelatinous bottom layer containing the virus with a top layer of green debris. The top green layer was allowed to slide off the virus pellet by inverting the centrifuge tubes for about 10 min. Distilled water was used to rinse the remaining green debris off the virus pellet. The virus was resuspended in a small amount of 0.02M borate buffer, pH 8.5 for 1-2 h at 4°. The suspension was centrifuged at 5,000 rpm for 5 min (Beckman No. 40 rotor) and the supernatant was applied to sucrose density gradients. Continuous or linear log gradients were made in polyallomer tubes with 10-40% (w/v) sucrose in 0.02M borate buffer, pH 8.5. The loaded gradients were centrifuged at 38,000 rpm for 90 min (Beckman SW 41 rotor) and then scanned at 254 nm with an ISCO density gradient monitor. All centrifugations were carried out at 4° while all other manipulations were at room temperature, except as noted otherwise.

Cesium chloride gradients were also run using a modified procedure developed for potato virus Y purification (Hiebert

and McDonald, 1973). The high speed pellets obtained by the standard procedure for purification of DVS were resuspended in 5 ml 0.02M KPO_4 buffer, pH 8.5, and layered onto 7 ml cesium chloride, density 1.2858 gm/cc, in 0.02M KPO_4 buffer, pH 8.5. The gradients were centrifuged at 32,000 rpm for 18 h (Beckman SW 41 rotor) and the virus band was collected either by puncturing the cellulose nitrate tube and collecting the opalescent zone by drops or by fractionating the column through the ISCO density gradient monitor at 254 nm.

Changes in concentration and pH of extraction buffer, the use of additives and solvents for clarifying or concentrating the virus preparation and the duration of the initial high speed centrifugation were also investigated. The effectiveness of the various procedures was determined by scanning the sucrose density gradients at 254 nm with the ISCO monitor and comparing the relative yield and purity of the preparations.

Electron Microscopy - Particle Size

Leaf dips were prepared by mincing systemically infected C. quinoa leaves in 2% phosphotungstic acid (PTA), pH 6.7, and placing a drop of the extracted sap onto a carbon-fronted, collodion-coated copper grid (200 mesh) for 1 min. The grid was rinsed with 2% PTA and the excess stain removed with a filter paper wedge. After air drying for a few minutes, the grids were scanned with a Philips EM 200 or EM 300 and photographs taken as required.

Purified and partially purified virus preparations were

applied directly to the grids and then the same procedure was followed as for leaf dips. Varying numbers of particles were measured for the various purification methods as well as for leaf dips.

Infectivity of Gradients

A partially purified DVS preparation was layered onto continuous sucrose density gradients formed with 10-40% (w/v) sucrose in 0.0165M disodium phosphate and 0.0018M trisodium citrate buffer, pH 9.0. After centrifugation at 38,000 rpm for 90 min (Beckman SW 41 rotor), the gradients were scanned at 254 nm and fractionated with an ISCO density gradient monitor and fraction collector. Selected fractions were checked by EM and all fractions were inoculated to C. quinoa. Symptoms were recorded at 12, 14 and 18 days after inoculation.

Absorption Spectrum

Purified virus preparations were scanned in a Beckman DU spectrophotometer over the ultraviolet range from 230-340 nm at 5 nm intervals. The values were plotted and corrected for light scattering (Noordam, 1973). Both the corrected and uncorrected values were used in determining the absorbance characteristics of DVS.

Serology

Antiserum production

A young white New Zealand rabbit was immunized with four intramuscular injections of purified DVS (1 mg/ml) emulsified 1:1 with Freund's complete adjuvant. The first and second

injections were 2 wk apart, with two booster injections at 10 and 14 wk. After the second injection, bleedings were done at weekly intervals over the next 19 wk and tested by the tube precipitin test with the appropriate dilutions of both anti-serum and antigen to determine the titre of the antiserum and the end point of the antigen, ie. the minimum concentration of antigen to produce a visible precipitate. The relative amount of precipitate formed for each reaction was evaluated on a seven unit scale: 4=very dense; 3=dense; 2=moderate; 1=slight; t=trace; s=visible with hand lens and -=no reaction.

Similar schedules were followed for production of antisera to PeVS and HVS. All antisera were stored at 4⁰ with a few crystals of chlorobutanol as preservative.

Agar gel diffusion serology

Healthy and infected leaves of C. quinoa were ground 1:2 (w/v) with 0.85% NaCl and 0.2% sodium azide in distilled water. The sap was collected through Miracloth and then divided: 1 ml sap plus 1 ml grinding buffer; 1 ml sap plus 1 ml 5% pyrrolidine.

The samples were incubated at room temperature for 5 min and then placed in the appropriate wells in the agar gels. The antiserum was prepared at twofold dilutions from 1:80 to 1:10,240 and then added to the appropriate wells in the agar gels. The agar gels were prepared with 0.9% Ionagar, 0.8% NaCl and 0.2% NaN₃ in distilled water; heated for 20 min in a boiling water bath and while the mixture was still hot,

dispensed in 2 ml aliquots onto collodion-coated microscope slides.

The test slides were incubated at room temperature for 48 h in a moist chamber and then checked for precipitin lines.

Tube precipitin serology

In tube precipitin reactions, homologous and heterologous tests were done with dilutions of DVS, PeVS and PVS in combination with dilutions of their antisera. Antisera to DVS and PeVS were produced in the course of this study, while antiserum to PVS from the Plant Virus and Antiserum Bank, Agriculture Canada, Vancouver Research Station, had been stored in glycerol (1:1) and frozen since 1972, and was not as good quality. This difference was taken into consideration when preparing the dilution ranges of antiserum to be tested. Antiserum to HVS was from the first bleeding as HVS had not been included in the comparative studies until recently. All of the antigens at 80 and 20 ug/ml, were tested against the appropriate antisera dilutions and the endpoints determined.

Purified DVS was tested against antisera to other carla-viruses obtained from the Plant Virus and Antiserum Bank, Agriculture Canada, Vancouver Research Station, for which homologous pure antigen preparations were not available. Antisera dilutions were used from 1:20 to 1:320, at twofold intervals against 80 ug/ml DVS.

Sodium dodecyl sulfate agar gel serology

Since flexuous rod-shaped viruses do not migrate

effectively through an agar gel media, unless the particles are in some way degraded into shorter, faster moving components, agents such as SDS were used to fracture the particles (Purcifull and Batchelor, 1977). Sap extracts were obtained by grinding leaf tissue 1:1 (w/v) with water and subjecting this extract to low speed centrifugation, after which the supernatant was collected through Miracloth. SDS was added to a final concentration of 1% and the extract was heated for 90 sec in boiling water.

SDS agar was prepared with 0.6% Ionagar, 0.2% SDS, 0.7% NaCl and 0.1% NaN_3 in 0.1M Tris-HCl buffer, pH 9.0 and 1 ml aliquots were dispensed into small (35 x 10 mm) Petri dishes. Once the gels had set, wells were cut using a template made from discharged 0.22 calibre brass cartridges (Wright and Stace-Smith, 1966) and the agar plugs were aspirated out just prior to filling the wells with the test samples.

To test if DVS antiserum had been produced against whole virus and/or subunits, antigen wells were filled with sap extracts that had not been treated with SDS or heat; with sap extracts that contained 1% SDS, but not heated; and with sap extracts that contained 1% SDS and that had been heated for 90 sec in boiling water.

SDS agar gel diffusion is frequently used to detect relationships among rod-shaped viruses and DVS, (1 mg/ml and 0.5 mg/ml) and PeVS, (2 mg/ml), were tested against full strength DVS antiserum to determine if a spur would form at

the intersection of the homologous and heterologous reactions.

Latex agglutination serology

Antiserum with a titre of 40,960 was prepared at twofold dilutions from 1:100 to 1:12,800 and conjugated with latex beads (Phatak, 1974). About 4 μ l of antiserum-latex dilution was drawn into the capillary tube; the tip was wiped clean and 8 μ l of test sample were drawn up from the same end of the capillary tube; the tip was wiped dry and the tube was taped to a microscope slide. When the range of antiserum dilutions had been used, the microscope slides were taped to an inclined rotating wheel and the contents of the tubes mixed for 15 min at room temperature. The results were read under a stereomicroscope with top lighting of the capillary tubes. Positive results were indicated by an agglutination of the latex beads.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA technique (Clark and Adams, 1977) was adapted for use with DVS. Minor modifications were made to the standard procedure: the immunoglobulin G was concentrated from crude antiserum by two cycles of ammonium sulfate precipitation instead of one cycle and the first major peak eluted through the DEAE-22 Sephadex column was collected; the conjugate was stored at 4° without the addition of bovine serum albumin and the coated microtitre plates (Cooke-Dynatech) were stored at 4° overnight or longer prior to use. A moist environment was maintained throughout the assay by enclosing the plate in a small plastic bag held shut by an elastic band. Test samples

were incubated overnight at 4⁰, rather than at 37⁰ for 4-6 h.

The colour reaction was initially assessed spectrophotometrically at 405 nm with a twofold dilution of the reaction mixture. However, for assay of field material or host range, visual observation was sufficient to distinguish positive and negative results. Black and white prints and colour slides were taken of the plates about one hour after the substrate had been added, so that when no absorbance readings were taken there was still a permanent record of the test.

Transmission electron microscope serology (TEMS)

A drop of antigen was placed on a wax plate and a drop of antiserum was added. The antiserum was used at dilutions over the range 1:10, 1:20 and 1:40. The mixture was incubated at room temperature for 3 min and then a drop of 4% PTA, pH 7.2 was added. After 1 min, a grid was floated on the drop, collodion side down, for 5 min. The grid was dried with a filter paper wedge and scanned. Various combinations of antisera and antigens were used to study the DVS and PeVS reactions in an attempt to distinguish these two carlaviruses by electron microscopy. Tobacco mosaic virus (TMV) was included as a control.

RESULTS

Field Occurrence of DVS

A more extensive survey of dandelions in and around the original collection site near Kelowna was undertaken. A total of 109 plants from Kelowna, Naramata, Summerland and Westbank were indexed and of these, 71 were infected (Table 1). Dandelions growing in other areas of British Columbia, including Vancouver Island, the Fraser Valley, the Chilcotin

Table 1. Results of indexing field collections of dandelions from various areas of Canada for natural infection with DVS

Area	Specific Site	Number of Dandelions	
		Indexed	Positive
Vancouver Island	Saanichton	14	0
Fraser Valley	Abbotsford; Langley;	134	0
	Vancouver Manning Park	13	0
Okanagan Valley	Kelowna; Naramata; Summerland; Westbank	109	71
Kootenay Valley	Castlegar; Christina Lake; Creston; Grand Forks	25	0
Chilcotin	Williams Lake	5	0
Alberta	Calgary	20	0
Saskatchewan	Cut Knife; Maple Creek	108	0
Manitoba	Birds' Hill Park; Winnipeg	75	0
Ontario	Ottawa	15 <u>518</u>	0 <u>71</u>

area and the Kootenay Valley were indexed and found to be negative for DVS. Similarly, dandelions collected from other parts of Canada, as far east as Ottawa were also negative.

In total, 518 dandelions were indexed from 44 collection sites and of these only 71 were found to be naturally-infected with DVS. In Kelowna, 70 of the 92 dandelions indexed were infected, while 1 out of 5 dandelions indexed from Naramata was infected with DVS.

Host Range and Symptomatology

Infected dandelions in the field exhibited no symptoms by which they could be readily identified compared with other noninfected dandelions. Similarly, dandelion seedlings rub-inoculated with DVS, and back indexed to C. quinoa to prove infection, showed no symptoms. Some variations in leaf size and shape were evident in seedlings from both healthy and infected plants but this was attributed to inherent seedling variation rather than to expression of disease symptoms.

C. quinoa was chosen as an assay host because of its obvious symptoms resulting from infection with DVS. Chlorotic local lesions developed 5-7 days after inoculation of plants at the 8-10 leaf stage, and with increasing time, these became more noticeable as necrotic spots on the senescent inoculated leaves. At 10-14 days after inoculation, systemic chlorosis became evident, progressing from mild vein clearing to complete chlorosis and epinasty; axial shoots also exhibited chlorosis (Fig. 1A-D). Inoculation of C. quinoa at the 6-8 leaf stage

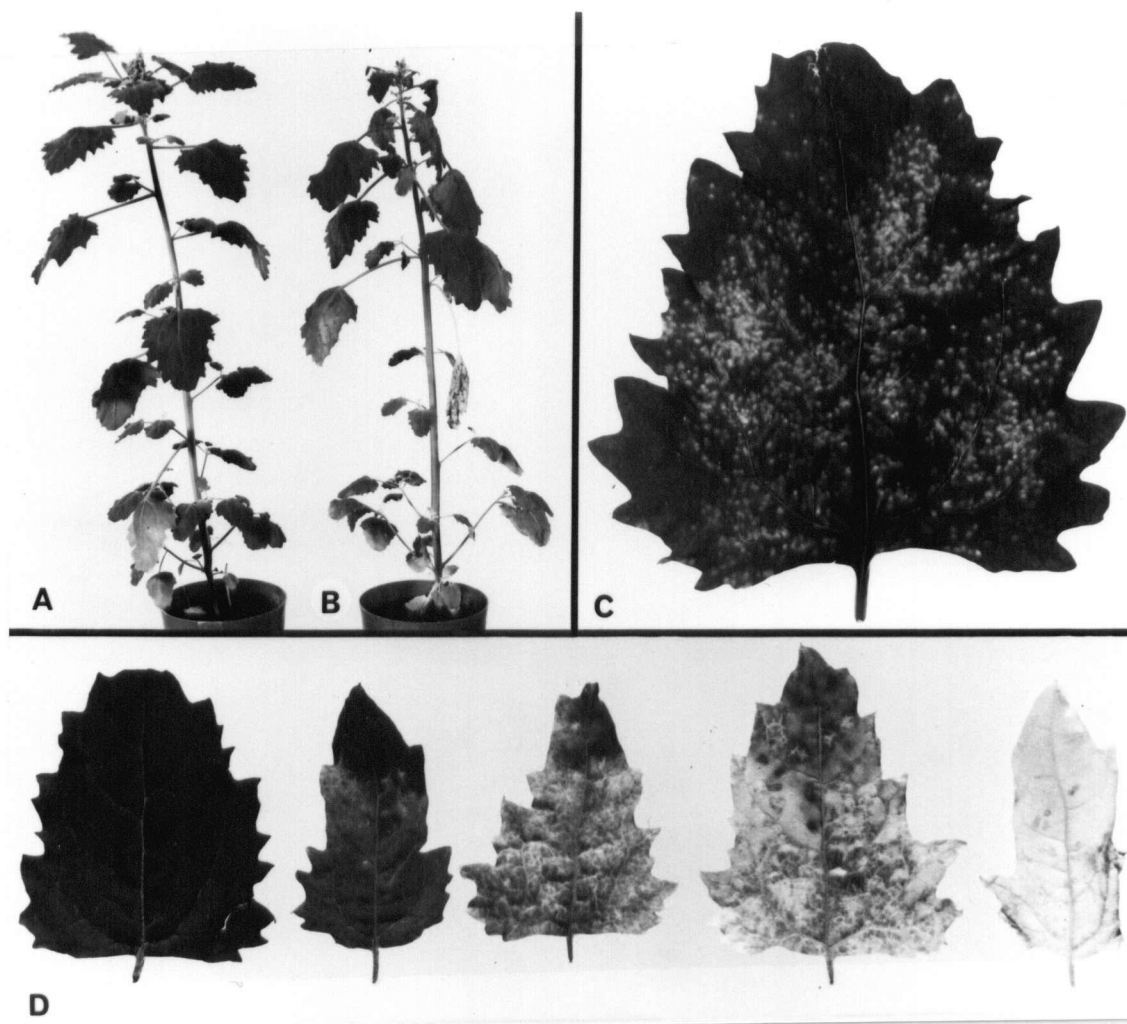


Fig. 1. Symptoms of DVS infection in *C. quinoa*. A. healthy plant; B. infected plant showing vein clearing and epinasty after 14 days; C. local lesions on inoculated leaf after 10 days and D. healthy leaf (left) and progression of symptom development in 4 top leaves of inoculated plant

resulted in mild stunting. Flowering and seed set occurred successfully only if the plant was inoculated at an older age and therefore did not collapse completely prior to flowering.

The inoculum used for host range studies was clarified and concentrated in order to decrease the inhibitor effect of Chenopodium sap, thereby increasing the chance of transmission to other hosts. The preparation was checked by electron microscopy to determine the amount of breakage to the particles. Few particles were found to be broken (Fig. 2A) and the procedure for clarification and concentration did not affect the infectivity of the virus in C. quinoa.

The experimental host range survey included plants from 12 families, 4 of which had members susceptible to infection by DVS. D. stramonium developed some pinpoint necrotic local lesions; G. globosa developed some large necrotic local lesions (Fig. 2B,C); T. officinale showed no symptoms of infection; C. amaranticolor developed many chlorotic local lesions that were emphasized by a red halo as the leaf senesced, and systemic vein clearing, epinasty and stunting (Fig. 2D,E,F). The C. quinoa developed typical symptoms as described above.

Virus infection or lack thereof, in both inoculated and uninoculated leaves was assayed by the ELISA technique and by back inoculation to C. quinoa for all plants in the host range (Table 2). Testing with ELISA detected virus from the inoculated leaves of all the susceptible hosts and also from the systemic leaves of C. amaranticolor, C. quinoa and dandelion.

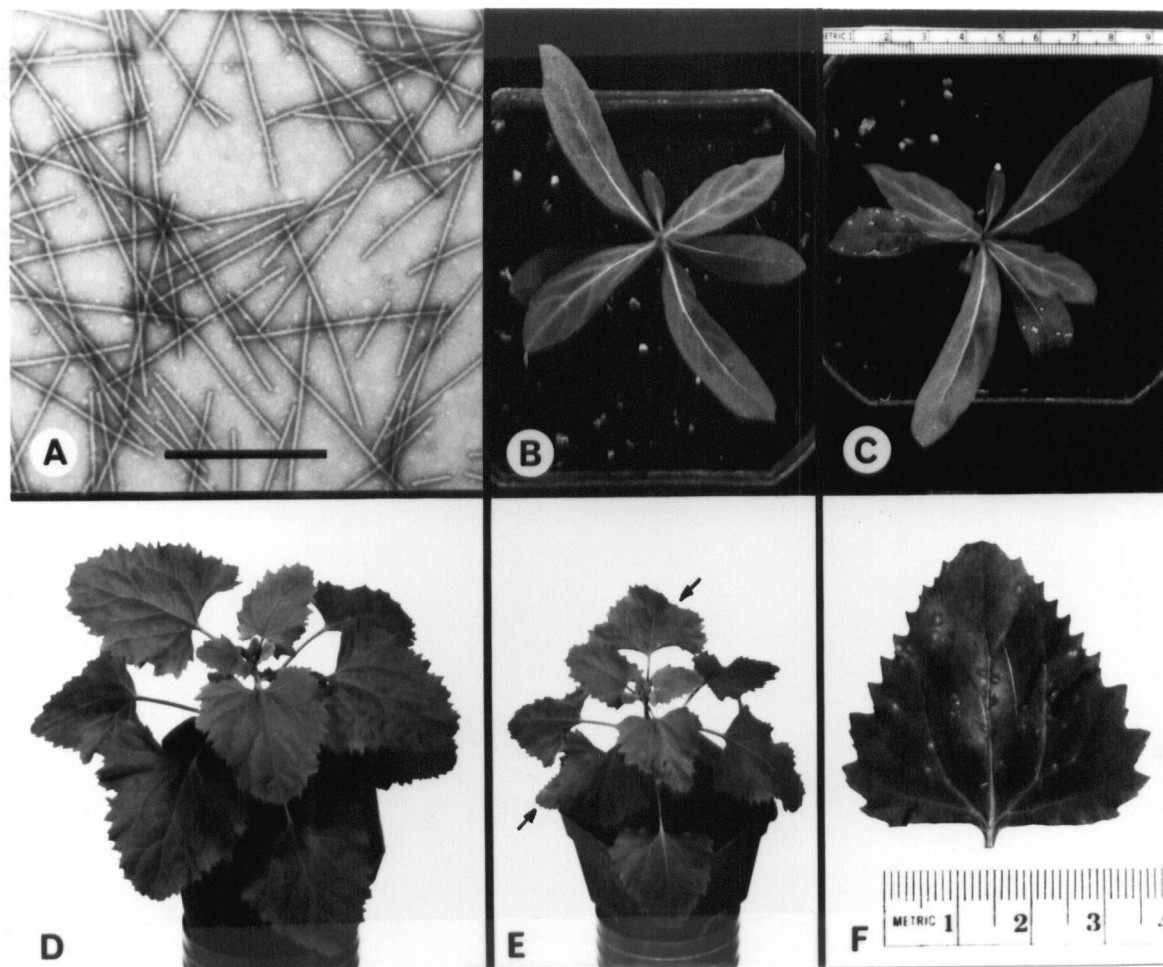


Fig. 2. Electron micrograph of semi-purified DVS used as inoculum for host range studies (A. bar = 500 nm) and symptoms induced on *G. globosa* (B. healthy plant; C. inoculated plant with necrotic local lesions) and on *C. amaranticolor* (D. healthy plant; E. inoculated plant with systemic vein clearing as pointed out by arrows and F. local lesions on inoculated leaf after 14 days)

Table 2. Results of the experimental host range studies as determined by symptoms in the inoculated host and indexing the inoculated (I) and systemic (S) leaves by ELISA and back inoculation to C. quinoa

Family	Species	Symptoms	ELISA		<u>C. quinoa</u>	
			I	S	I	S
Amaranthaceae	<u>Gomphrena globosa</u> L.	+	+	-	-	-
Apocynaceae	<u>Vinca rosea</u> L.	-	-	-	-	-
Caryophyllaceae	<u>Dianthus barbatus</u> L.	-	-	-	-	-
Chenopodiaceae	<u>Chenopodium amaranticolor</u> Coste & Reyn.	+	+	+	+	+
	<u>Chenopodium quinoa</u> Willd.	+	+	+	+	+
Compositae	<u>Helianthus annuus</u> L.	-	-	-	-	-
	<u>Lactuca sativa</u> L.	-	-	-	-	-
	var. capitatum					
	<u>Taraxacum officinale</u> Weber	-	+	+	+	+
	<u>Verbesina encelioides</u> (Car.) Benth. & Hook.	-	-	-	-	-
	<u>Zinnia elegans</u> Jacq.	-	-	-	-	-
Cruciferae	<u>Brassica juncea</u> (L.) Coss	-	-	-	-	-
	<u>Brassica pekinensis</u> (Lour.) Rupr. var Petsai	-	-	-	-	-
Cucurbitaceae	<u>Cucumis sativus</u> L.	-	-	-	-	-
Gramineae	<u>Hordeum vulgare</u> L.	-	-	-	-	-
Leguminosae	<u>Phaseolus vulgaris</u> L. var Bountiful var Pinto	-	-	-	-	-
	<u>Pisum sativum</u> L. var Perfection	-	-	-	-	-
	<u>Vicia faba</u> L.	-	-	-	-	-
	<u>Vigna sinensis</u> Erdl. var Black eye	-	-	-	-	-

Table 2. (cont'd)

Family	Species	Symptoms	ELISA		<u>C. quinoa</u>	
			I	S	I	S
Plantaginaceae	<u>Plantago</u>	-	-	-	-	-
	<u>lanceolata</u> L.					
	<u>Plantago major</u> L.	-	-	-	-	-
Rosaceae	<u>Fragaria vesca</u> L.	-	-	-	-	-
	var Alpine					
Solanaceae	<u>Capsicum</u>	-	-	-	-	-
	<u>frutescens</u> L.					
	var grossum					
	<u>Datura stramonium</u> L.	+	+	-	-	-
	<u>Lycopersicum</u>	-	-	-	-	-
	<u>esculentum</u> Mill.					
	var Rutgers					
	var Subarctic	-	-	-	-	-
	<u>Nicotiana</u>	-	-	-	-	-
	<u>clevelandii</u> Gray					
	<u>Nicotiana debneyi</u>	-	-	-	-	-
	Domin.					
	<u>Nicotiana</u>	-	-	-	-	-
	<u>glutinosa</u> L.					
	<u>Nicotiana rustica</u> L.	-	-	-	-	-
	<u>Nicotiana tabacum</u> L.	-	-	-	-	-
	var Haranova					
	var Havana	-	-	-	-	-
	var Samsun	-	-	-	-	-
	var Silvestris	-	-	-	-	-
	var White Burley	-	-	-	-	-
	var Xanthi	-	-	-	-	-
	<u>Petunia hybrida</u> Vilm.	-	-	-	-	-
	<u>Solanum tuberosum</u> L.	-	-	-	-	-
	var Mirton Pearl					

Presumably, the virus was capable of multiplying in the two local lesion hosts to the extent that the presence of virions could be detected by ELISA, but there were not adequate numbers of infective virions to be detected by back inoculation to C. quinoa.

Properties in Crude Sap

Infectivity was retained in crude C. quinoa sap between 4 and 5 days at 23°; between 28 and 56 days at 4° and more than 16.5 months in a lyophilized state at 23°. Systemic infection of C. quinoa was used as the determining factor for the dilution end point, as sufficient virus was then considered to be present to establish a typical infection. At a dilution of 2×10^{-5} , the inoculum was still able to cause systemic infection, while further dilution did not produce systemic infection even after 21 days, although a few local lesions were present. The temperature range of inactivation for DVS in C. quinoa sap was 75-80°, as determined by the ability of the tested aliquots to cause systemic infection in C. quinoa 35 days after inoculation.

Seed Transmission

The germination rate of seeds from healthy and infected C. quinoa on moist filter paper was 95-100%. Inoculation of the seed coats and the seedlings to C. quinoa produced no symptoms of infection. These seedlings matured with no symptoms and no latent infection was detected by ELISA or by back inoculation to C. quinoa.

The germination rate of seeds from healthy and infected dandelions on filter paper and on soil was 64%. No latent infection was detected by ELISA or by back inoculation to C. quinoa.

Aphid Transmission

Myzus persicae established colonies on C. quinoa, after an adaptation period of about 8 wk, however, the colony became infected with a hymenopteran parasite and was discarded. A second colony was established and no infection by parasites occurred. M. persicae would not colonize dandelions in the rosette stage.

DVS was transmitted to C. quinoa by M. persicae after 5 min access to infected C. quinoa, but not after 35 min. No transmission was obtained when aphids were allowed to feed on healthy C. quinoa.

Purification

Various methods of clarification and concentration were tried and compared on the basis of their absorbance scan patterns. In Fig. 3, the effects of combinations of extracting buffers and additives are shown: with the standard method, the virus peak is sufficiently separated from host contaminants with borate buffer to take advantage of the increased virus yield compared with phosphate buffer extraction (Fig. 3A); chloroform with either buffer did not clarify the preparation and also reduced the virus yield (Fig. 3B); carbon tetrachloride had a similar effect to chloroform, except with phosphate buffer no virus peak was obtained (Fig. 3C); polyethylene glycol did not aid in clarifying or concentrating the virus (Fig. 3D), nor did ammonium sulfate (Fig. 3E); Triton X-100 effectively removed the virus peak (Fig. 3F), as did treatment

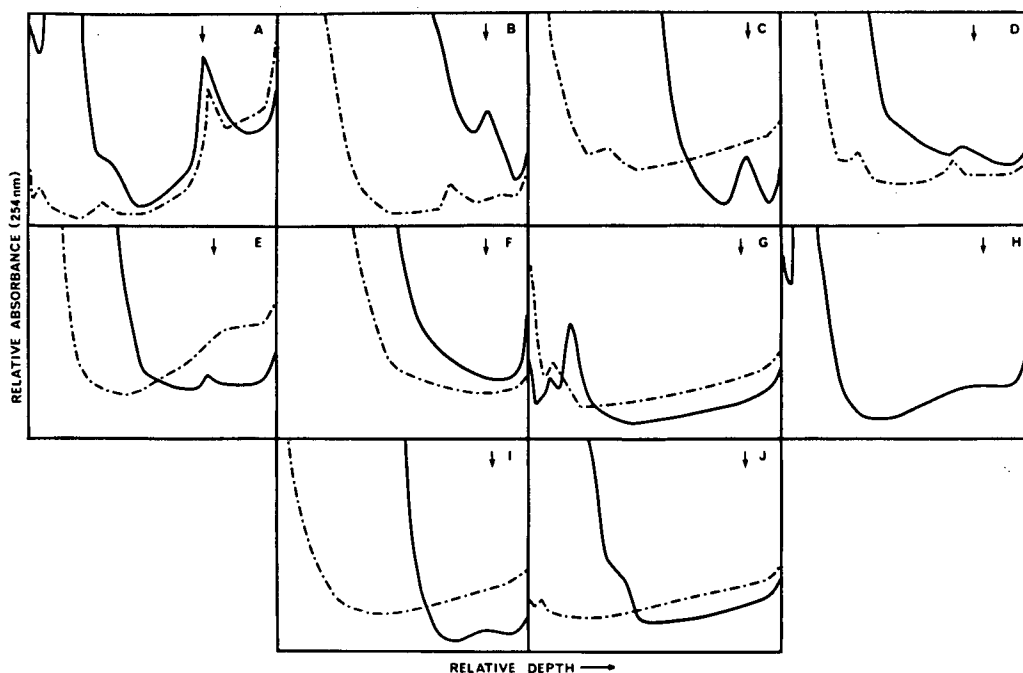


Fig. 3. Absorbance scan patterns of sucrose density gradients showing effects of combinations of extraction buffers and additives on purification of DVS. The extraction buffers were 0.5M borate, pH 8.0 (—) and 0.5M phosphate, pH 7.4 (---). Position of virus peak is indicated by an arrow. Treatments were as follows: A. standard method as outlined in Fig. 10; B. 1:1 chloroform; C. 1:1 carbon tetrachloride; D. 10% polyethylene glycol and 0.1M NaCl; E. 15% ammonium sulfate; F. 1% Triton X-100; G. 1% bentonite; H. adjustment to pH 5.0; I. 1:1 butanol and J. 1:1 chloroform/butanol

with bentonite (Fig. 3G), acidification (Fig. 3H), butanol (Fig. 3I) and chloroform/butanol (Fig. 3J).

No significant increase in purity and yield was obtained with 0.5M borate buffer (Fig. 4), compared with 0.2M borate buffer (Fig. 5), so for economy the lower concentration of buffer was adopted as standard. By comparing the clarification achieved by a range of pH's from 8 to 9.5 (Figs. 5-8), the optimum pH for extraction was determined to be pH 9.0, without readjustment back to pH 9.0 after homogenization (Fig. 7C, E).

The initial clarification was achieved after high speed centrifugation by allowing the green pellet to slide off the underlying virus pellet and the ease with which this was achieved depended on the duration of the centrifugation: after 60 min, the virus pellet was loosely packed and a portion of it tended to slide off with the green pellet; after 90 min, the virus pellet was firm enough that the green pellet could slide off without disturbing the virus pellet; after 120 min the pellets had become too firmly packed to be separated without some force, such as a stream of water, which resulted in loss of virus (Figs. 5-8). Additional clarification was achieved by adding ethylene diamine tetra-acetic acid (EDTA) to the resuspended virus pellets, but the advantage was not great enough to offset the reduction in virus yield to warrant inclusion in the standard method (Figs. 5-8 B,D,G).

Sucrose density gradients were routinely used as the

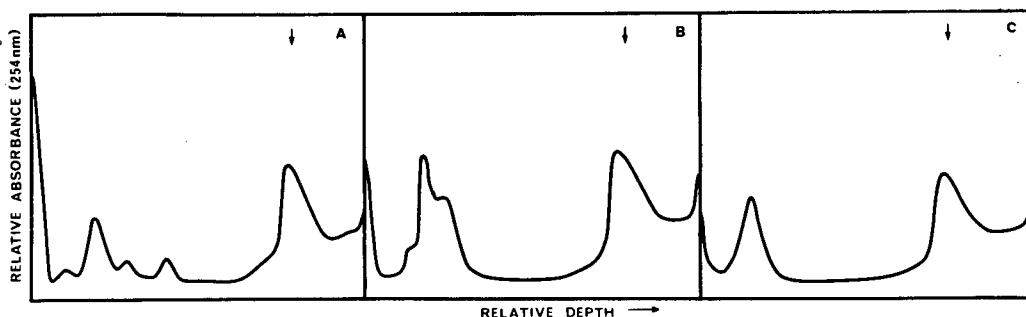


Fig. 4. Absorbance scan patterns of sucrose density gradients showing effects of centrifugation time and addition of EDTA. Position of virus peak is indicated by an arrow. Extraction buffer was 0.5M borate, pH 8.0 and treatments were as follows: A. 60 min centrifugation; B. 90 min centrifugation and C. 90 min centrifugation plus EDTA to 0.01M

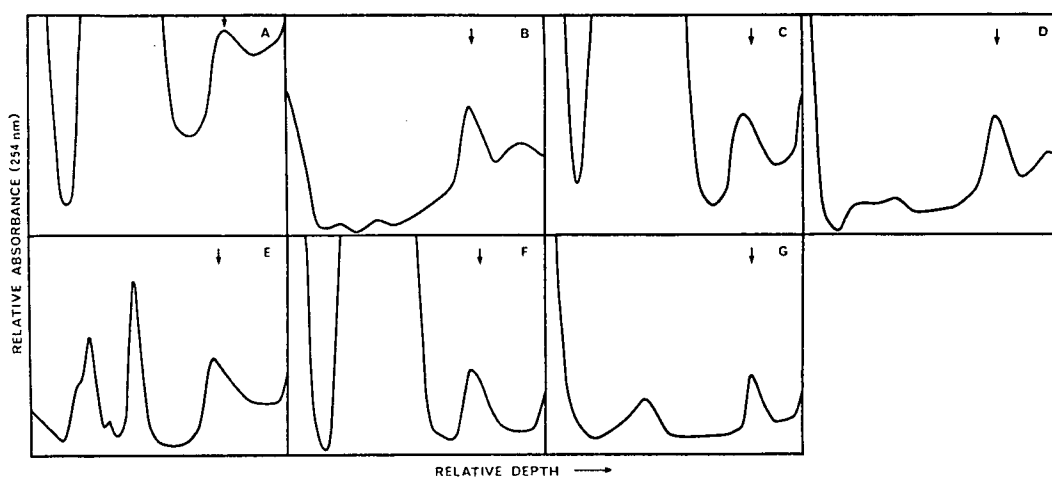


Fig. 5. Absorbance scan patterns of sucrose density gradients showing effects of pH, centrifugation time and addition of EDTA to 0.01M. Position of virus peak is indicated by an arrow. Extraction buffer was 0.2M borate, pH 8.0 and treatments were as follows: A. 60 min centrifugation; B. 60 min centrifugation plus EDTA; C. 90 min centrifugation; D. 90 min centrifugation plus EDTA; E. readjustment of extracted sap to pH 8.0, 90 min centrifugation; F. 120 min centrifugation and G. 120 min centrifugation plus EDTA

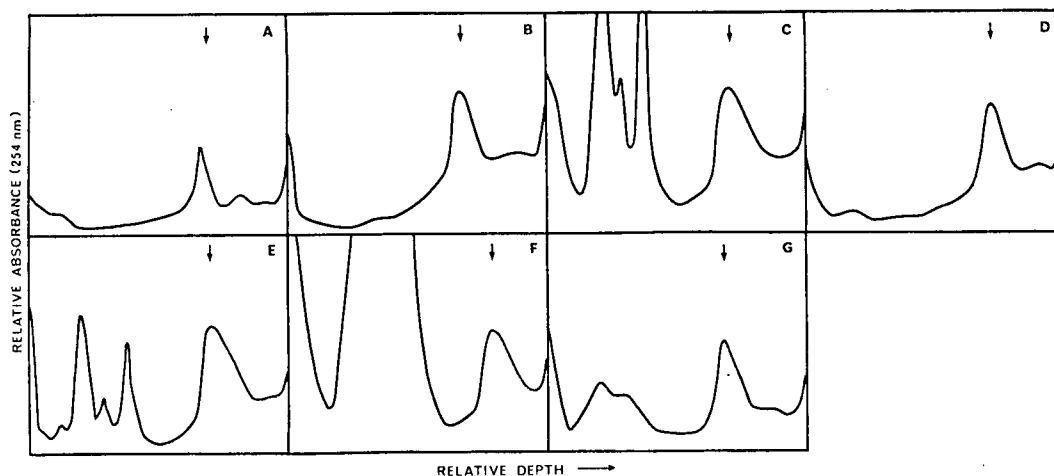


Fig. 6. Absorbance scan patterns of sucrose density gradients showing effects of pH, centrifugation time and addition of EDTA to 0.01M. Position of the virus peak is indicated by an arrow. Extraction buffer was 0.2M borate, pH 8.5 and treatments were as follows: A. 60 min centrifugation; B. 60 min centrifugation plus EDTA; C. 90 min centrifugation; D. 90 min centrifugation plus EDTA; E. readjustment of extracted sap to pH 8.5, 90 min centrifugation; F. 120 min centrifugation and G. 120 min centrifugation plus EDTA

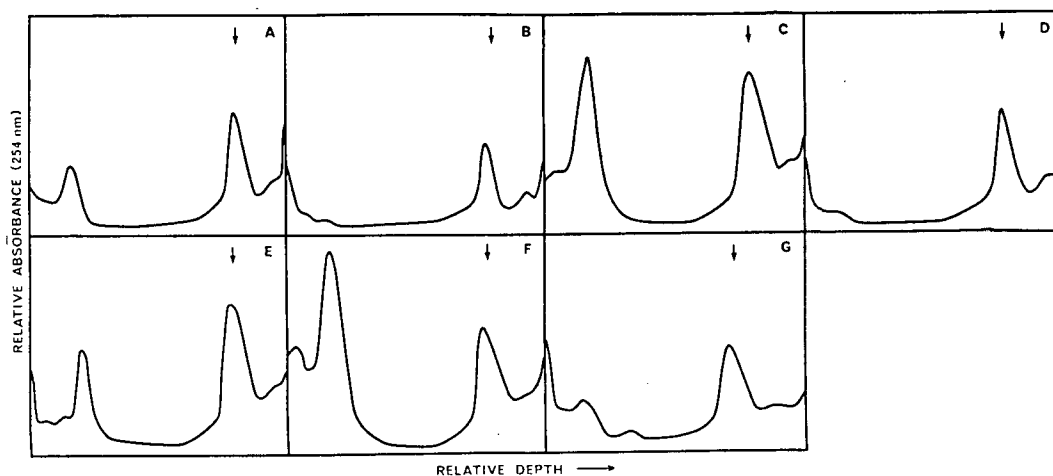


Fig. 7. Absorbance scan patterns of sucrose density gradients showing effects of pH, centrifugation time and addition of EDTA to 0.01M. Position of the virus peak is indicated by an arrow. Extraction buffer was 0.2M borate, pH 9.0 and treatments were as follows: A. 60 min centrifugation; B. 60 min centrifugation plus EDTA; C. 90 min centrifugation; D. 90 min centrifugation plus EDTA; E. readjustment of extracted sap to pH 9.0, 90 min centrifugation; F. 120 min centrifugation and G. 120 min centrifugation plus EDTA

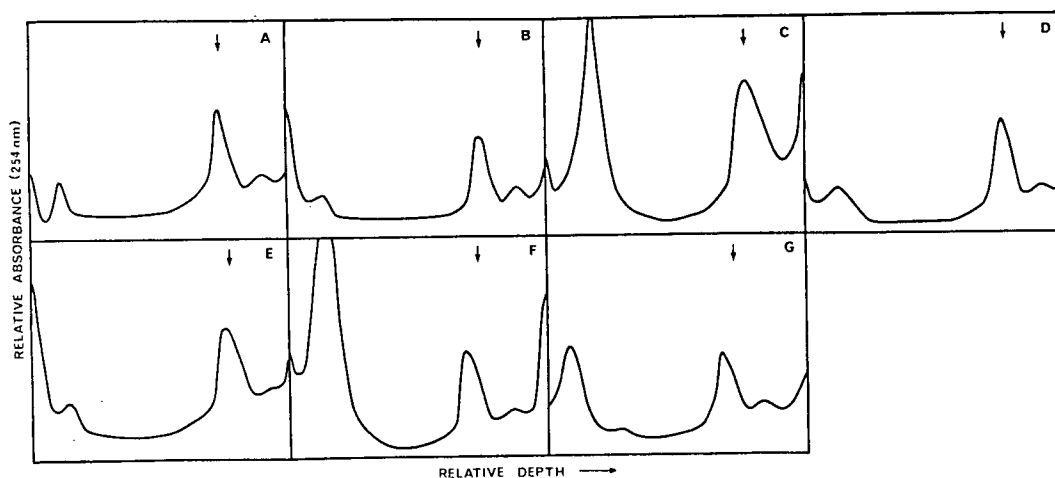


Fig. 8. Absorbance scan patterns of sucrose density gradients showing effects of pH, centrifugation time and addition of EDTA to 0.01M. Position of the virus peak is indicated by an arrow. Extraction buffer was 0.2M borate, pH 9.5 and treatments were as follows: A. 60 min centrifugation; B. 60 min centrifugation plus EDTA; C. 90 min centrifugation; D. 90 min centrifugation plus EDTA; E. readjustment of extracted sap to pH 9.5, 90 min centrifugation; F. 120 min centrifugation and G. 120 min centrifugation plus EDTA

final separation step to purify the virus. The peak obtained was not always sharp, as the dimers and polymers moved much deeper into the gradients (Fig. 9) and consequently, the lower portion of the gradient contained virus particles in various stages of aggregation. Cesium chloride gradients produced a sharp virus band at equilibrium (Fig. 9), but were not routinely used due to the high cost of cesium chloride and the long centrifugation time required.

As a result, a purification schedule was developed that required a minimum of chemicals and that was efficient for purifying DVS, PeVS and HVS from C. quinoa (Fig. 10). Yields obtained by this method were 20-30 mg/kg of plant material.

Electron Microscopy - Particle Size

The effect of various purification treatments on particle size was assessed by comparing the distribution of particle length categories. The standard purification method yielded particles of normal length 645 nm (Fig. 11D), chloroform and EDTA (Fig. 11E), butanol (Fig. 11F), butanol and EDTA (Fig. 11G) or cesium chloride gradients (Fig. 11I) were distributed around a mean of 630 nm. Particles from preparations treated with Triton X-100 had a mean of 650 nm (Fig. 11H). Of the 1 064 particles measured, the average length was found to be 637 nm (Fig. 11J), which was approximately that found with leaf dip preparations, ie. 640 nm (Fig. 11A). The width of the particles in leaf dip preparations was 12-13 nm.

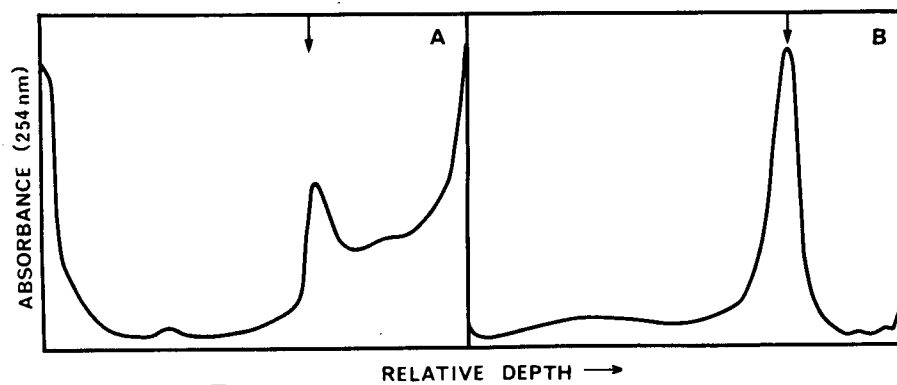
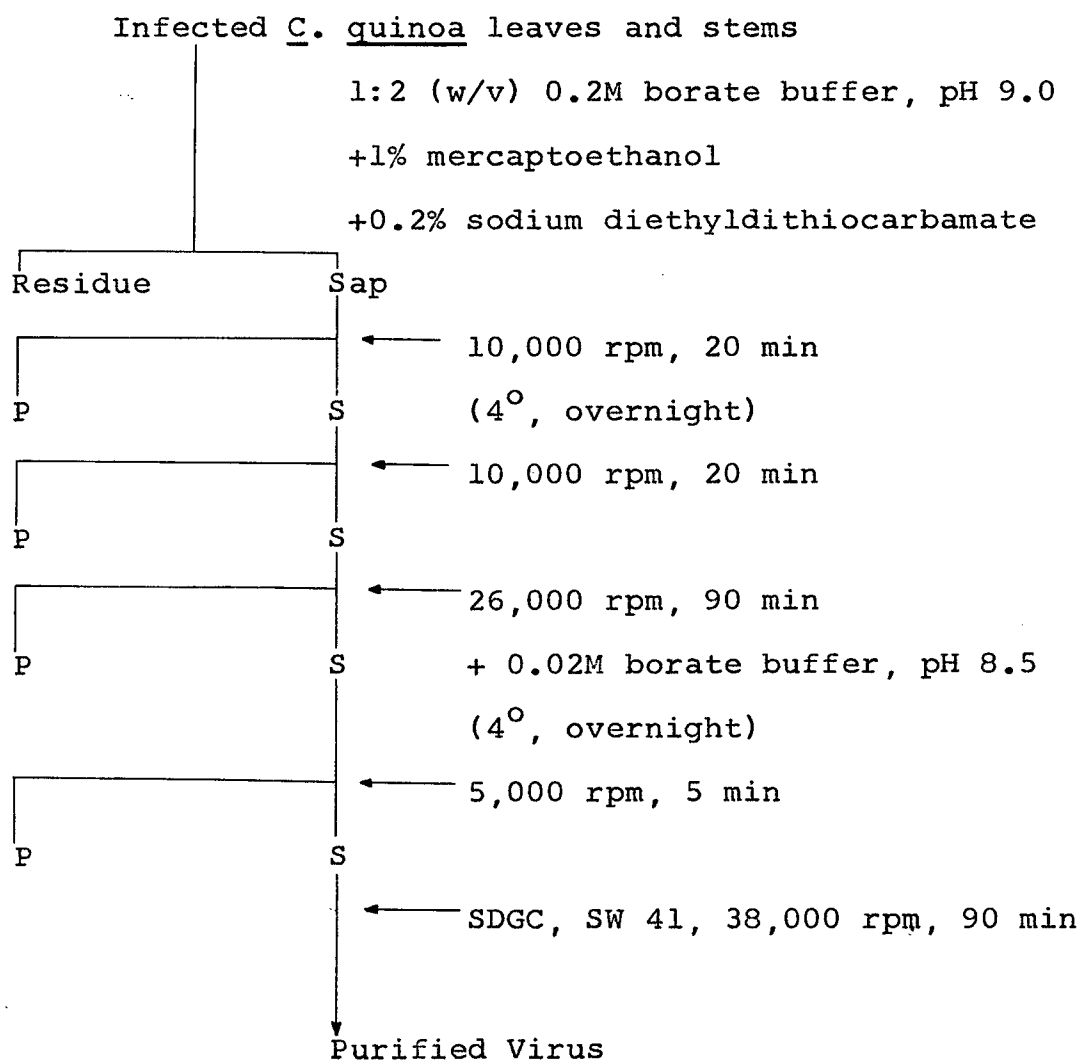


Fig. 9. Absorbance scan patterns comparing the sharpness of the virus peak (indicated by an arrow) obtained with A. sucrose and B. cesium chloride density gradient centrifugation at 38,000 rpm for 90 min and 32,000 rpm for 18 h, respectively in an SW 41 rotor



S: supernatant P: pellet

Fig. 10. Flow diagram of the standard purification method

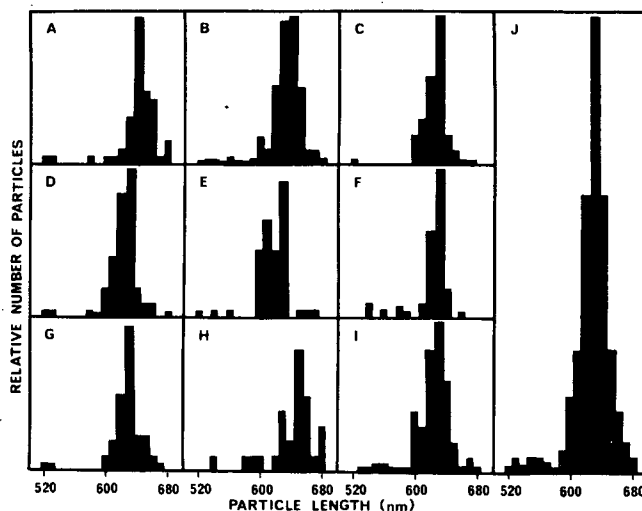


Fig. 11. Effect of purification methods on the particle length distribution of DVS as determined by measurement of particles in electron micrographs: A. leaf dip preparation; B. standard purification method as outlined in Fig. 10; C. 0.01M EDTA; D. 1:1 chloroform; E. 1:1 chloroform and 0.01M EDTA; F. 1:1 butanol; G. 1:1 butanol and 0.01M EDTA; H. 1% Triton X-100 and I. standard purification method with cesium chloride density gradient centrifugation. distribution is obtained when the results from all treatments are combined (J)

Infectivity of Gradients

Fractions from the top quarter of the gradients, as represented on the absorbance scan pattern by the area from the starting meniscus to the end of fraction A (Fig. 12), were not infectious and this was correlated with the absence of virus particles (Fig. 13A). The fractions between A and C (Fig. 12) were slow to cause symptoms in inoculated *C. quinoa*, but were infectious even though broken particles were characteristic of this area (Fig. 13B). Fractions from C to G (Fig. 12) were infectious and the particles ranged from slightly broken

(Fig. 13C,D) to intact virions (Fig. 13E,F). Fractions from G to the bottom of the gradient (Fig. 12) were quick to induce symptoms in inoculated C. quinoa, and the particles in these fractions were found to be polymers or aggregates (Fig. 13G, H,I). The rate and intensity of symptom development in C. quinoa was presumably, a reflection of the concentration of intact infectious virions within the individual fractions used as inoculum.

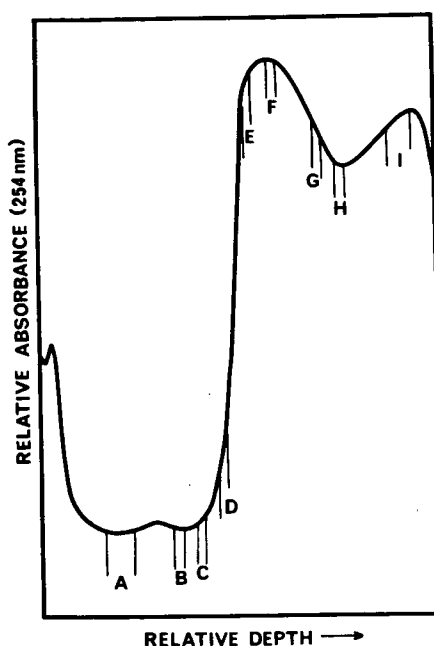


Fig. 12. Absorbance scan pattern of DVS after sucrose density gradient centrifugation for 90 min at 38,000 rpm in an SW 41 rotor. Letters indicate representative fractions that were collected and examined by electron microscopy (Fig. 13)

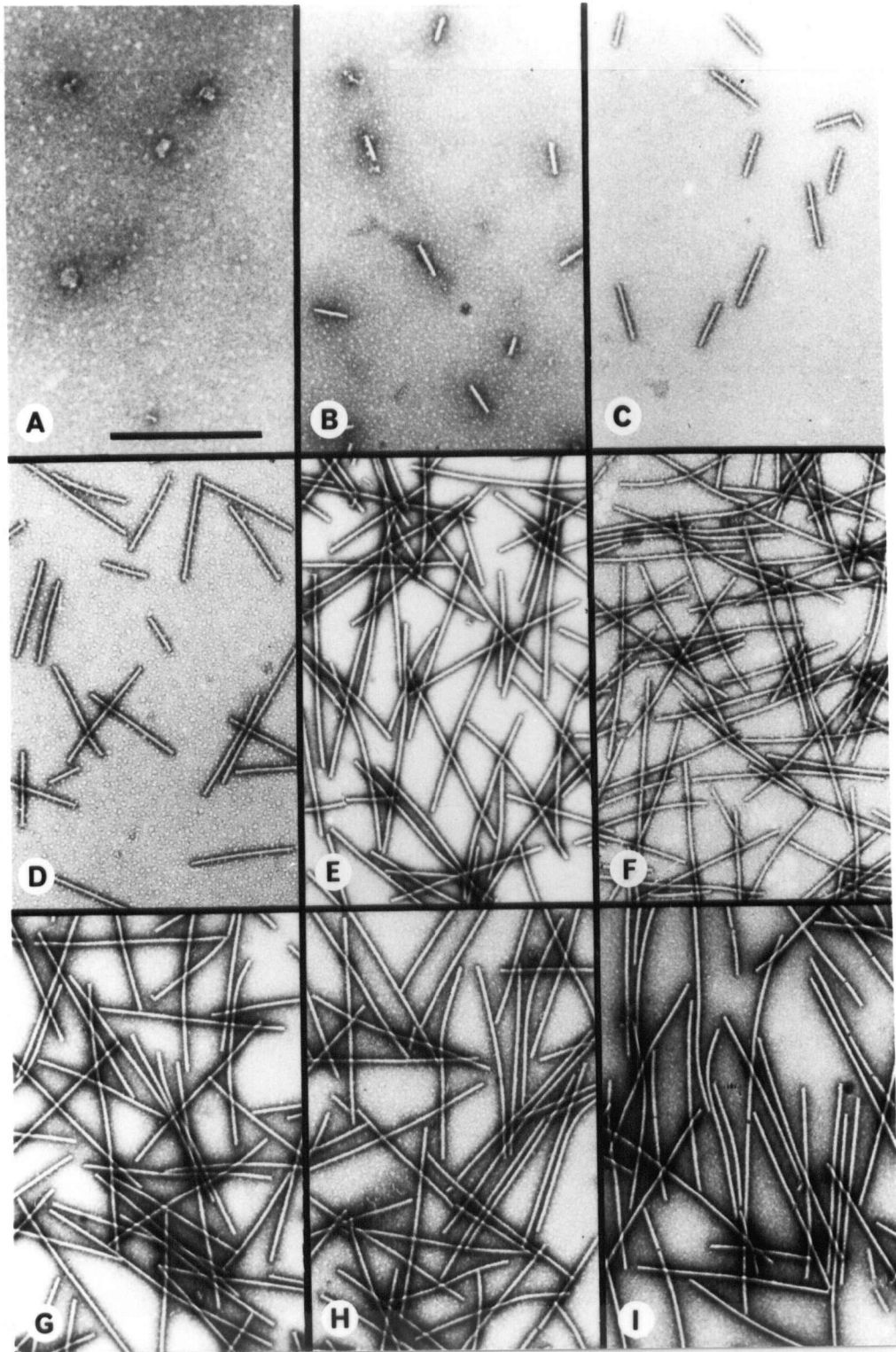


Fig. 13. Electron micrographs of various fractions collected from a sucrose density gradient. A-I correspond to the lettered fractions in Fig. 12. Bar represents 500 nm

Absorption Spectrum

The absorption scan for DVS is shown in Fig. 14, with the corrected scan also drawn in. This pattern is typical for an RNA virus, with a maximum absorption at 259 nm and a minimum at 245 nm. The absorption characteristics were slightly higher when calculated from corrected values than from uncorrected values: A_{\min}^{\max} 1.13 ± 0.04 and 1.09 ± 0.04 ; A_{280}^{260} 1.59 ± 0.11 and 1.41 ± 0.09 , respectively.

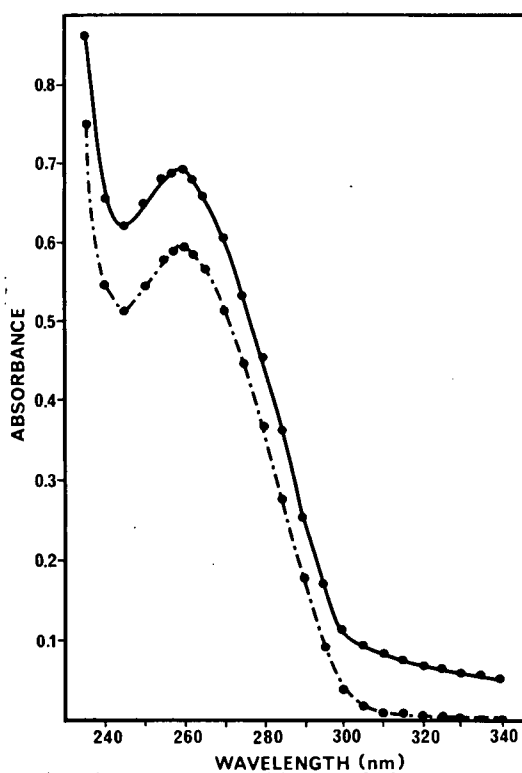


Fig. 14.. Absorbance scan pattern of DVS, from 230-340 nm: uncorrected (—•—•—) and corrected (---•---) for light scattering

Serology

Antiserum production

The homologous titre was determined for each bleeding and the highest titre, 40,960, was obtained after the last booster injection and was maintained until the last bleeding (Fig. 15). The nonspecific titre was 8, indicating that the purified virus preparation used to immunize the rabbit, had only a trace of contamination with host proteins. A representative determination of titre is shown in Table 3, with a titre of 40,960 after 18 h incubation. Only minor differences were noted between readings after 2 h incubation and after 18 h incubation, and the titre remained unchanged. The contour lines emphasize the optimum concentrations of antiserum and antigen (Matthews, 1957). The amount of precipitate formed as

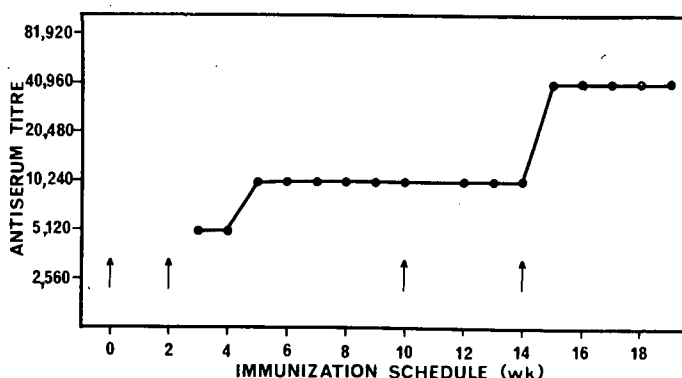


Fig. 15. Antiserum production against DVS antigen, as determined by tube precipitin serology. Arrows indicate injections of antigen

Table 3. Tube precipitin titre and end point determination of DVS using varying concentrations of antigen and antiserum. The contour lines join readings of equal intensity

Time (h)	Antiserum Dilution	Reaction* relative to antigen concentration($\mu\text{g/ml}$)								
		320	160	80	40	20	10	5	2.5	0
18	0	-	-	-	-	-	-	-	-	-
	10	4	4	4	4	3	1	t	-	-
	20	4	4	4	3	3	2	1	-	-
	40	4	4	4	4	3	2	1	t	-
	80	4	4	4	4	3	2	1	t	-
	160	4	4	4	3	2	2	1	t	-
	320	4	3	3	2	2	2	1	t	-
	640	3	3	2	2	2	1	1	t	-
	1,280	4	3	2	2	2	1	t	s	-
	2,560	4	3	2	2	2	1	t	s	-
	5,120	4	3	2	1	2	1	t	s	-
	10,240	1	2	2	1	1	1	t	s	-
	20,480	1	1	1	1	1	1	t	s	-
	40,960	-	-	s	t	1	t	s	-	-
	81,920	-	-	-	s	s	s	-	-	-
	163,840	-	-	-	-	-	-	-	-	-

*: 4: very dense; 3: dense; 2: moderate; 1: slight; t: trace; and s: visible with hand lens

the result of antigen/antiserum interaction in tube precipitin serology was scored on a relative scale: 4: very dense; 3: dense; 2: moderate; 1: slight; t: trace and s: visible with hand lens (Fig. 16). In routine tests, scores as low as 's' were doubtful and, therefore, not considered as meaningful positive reactions.

Similar tests were carried out to determine the titre of PeVS antiserum: the highest homologous titre was 10,280, with a nonspecific titre of 1 and HVS antiserum: the initial bleeding had a homologous titre of 1,280.

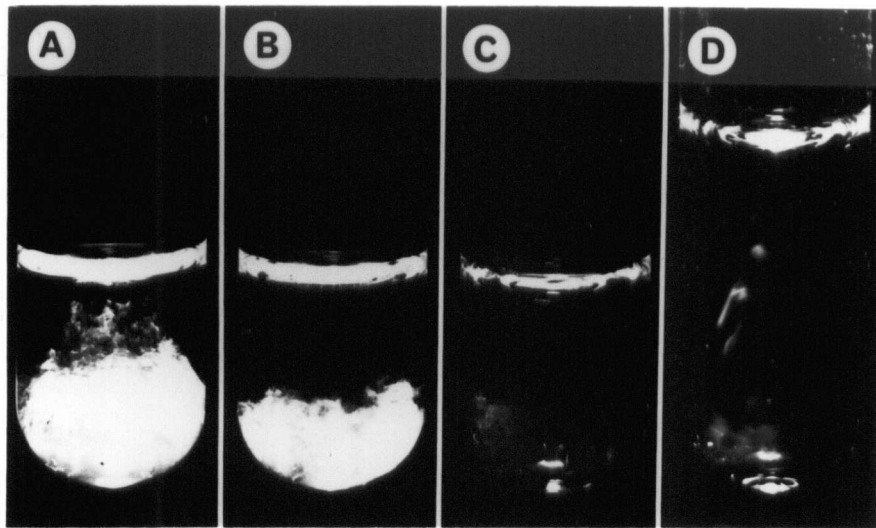


Fig. 16. Relative amounts of precipitate formed with tube precipitin reactions. Precipitation was scored as: A. very dense, 4; B. dense, 3; C. moderate, 2 and D. slight, 1

Agar gel diffusion serology

No reaction was visible between the antiserum and healthy sap. Untreated sap from infected C. quinoa reacted with its homologous antiserum to a dilution of 1:640, whereas the pyrrolidine treated sap did not react under the same conditions. This may have been due to the fact that only old reagent grade

pyrrolidine was available, and it may have contained sufficient contaminants to have destroyed the virus particles.

Tube precipitin serology

The antisera end points determined for homologous and heterologous reactions involving DVS, PVS, PeVS and HVS are summarized in Tables 4 and 5. From these results, it can be seen that 80 $\mu\text{g/ml}$ is too high a concentration of antigen to determine true serological relationships (Table 4), while 20 $\mu\text{g/ml}$ is closer to the optimal concentration (Table 5) and varying degrees of serological relationship among the viruses

Table 4. Precipitin end points in two-way tube precipitin tests including four antisera and their respective antigens at 80 $\mu\text{g/ml}$

Antiserum	Antigen			
	DVS	PVS	PeVS	HVS
DVS	10,240	5,120	1,280	40
PVS	2,560	2,560	1,280	40
PeVS	320	5,120	1,280	20
HVS	40	20	10	320

Table 5. Precipitin end points in two-way tube precipitin tests including four antisera and their respective antigens at 20 $\mu\text{g/ml}$

Antiserum	Antigen			
	DVS	PVS	PeVS	HVS
DVS	20,480	5,120	2,560	80
PVS	1,280	10,240	640	80
PeVS	320	5,120	2,560	80
HVS	40	40	20	1,280

are revealed. DVS is more closely related to PVS than to either PeVS or HVS.

Antisera to several other carlaviruses were obtained, although no pure preparations of homologous antigens were available, so one-way tests were carried out with a constant amount of DVS (80 $\mu\text{g/ml}$) against the test antisera. Antisera to CVB (1:20); NLV (1:20) and PVS (1:80) were still able to produce a visible precipitate. No reactions were observed with antisera to PMV, PVM or RCMV.

Sodium dodecyl sulfate (SDS) agar gel serology

A comparison of precipitin lines for sap with no other SDS treatment, with SDS added to 1% and with SDS added to 1% followed by heating for 90 sec in boiling water, against DVS antiserum showed that equal amounts of precipitate were formed in the SDS gels regardless of the pretreatment given to the sap (Fig. 17A). Consequently, the antiserum was probably formed against a combination of subunits, fragments and intact virus particles.

Greater concentrations of antigen and antisera were required to obtain a visible precipitin reaction in solid phase serology than in liquid. DVS antiserum had a homologous titre of 1:2 against 250 $\mu\text{g/ml}$ purified antigen (Fig. 17B) and a 1:1 dilution of sap from systemically infected C. quinoa.

When DVS and PeVS were reacted against DVS antiserum, the precipitin lines intersected to form a spur (Fig. 17C), indicating a serological relationship. When PVS antiserum was

tested, no spur was formed, however, this may have been due to low titre antiserum that would not detect distant serological relationships (Purcifull and Shepherd, 1964). No further relationships between DVS and other carlaviruses were detected

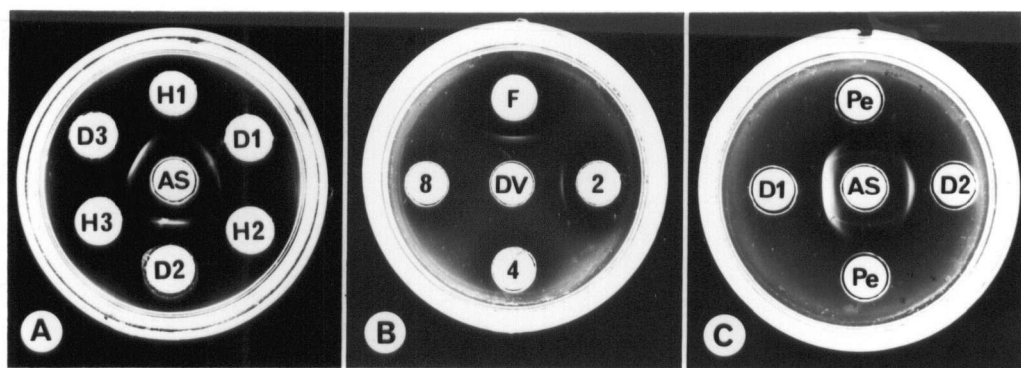


Fig. 17. Sodium dodecyl sulfate (SDS) agar gel serology. A. Effect of treatment steps on relative strength of precipitin lines in healthy (H) and DVS infected (D) sap from *C. quinoa*. 1. no treatment, 2. SDS added to 1% and 3. SDS added to 1% and heated. The centre well contained full strength antiserum to DVS (AS). B. Determination of DVS antiserum titre: the central well contained 250 $\mu\text{g/ml}$ DVS (DV) and the outside wells contained a twofold dilution series of DVS antiserum from full strength (F) to 1:8 (8). C. Determination of relationship between DVS and PeVS. The central well contained full strength DVS antiserum (AS). Outside wells were filled with PeVS at 2 mg/ml (Pe); DVS at 1 mg/ml (D1) and 0.5 mg/ml (D2). Note formation of spur at the intersection of the homologous and heterologous reactions.

by heterologous testing in SDS agar gels with antisera to chrysanthemum virus B, eggplant mild mosaic virus, poplar mosaic virus, pea streak virus, potato virus M and red clover vein mosaic virus.

Latex agglutination serology

The latex agglutination test was quite effective in detecting DVS in crude sap from infected C. quinoa, but because of nonspecific, healthy reactions, it could not be used reliably to detect DVS in dandelion. This may have been due to a latex-like substance found in the sap of the dandelion which could be interfering with the latex reaction.

The latex agglutination test was more sensitive than the tube precipitin test: the end point for the latex test was a dilution of 1:12 800 for the labelled antiserum against a 1:1 280 dilution of the infected C. quinoa sap, while the end point for the tube precipitin reaction was a dilution of 1:2 560 for the antiserum against a 1:160 dilution of the infected C. quinoa sap.

Enzyme-linked immunosorbent assay (ELISA)

IgG from antiserum was purified through a DEAE-22 Sephadex column; the effluent was monitored at 280 nm and the major peak, corresponding to the IgG fraction was collected in the first 2 ml of effluent (Fig. 18). For routine testing optimum dilutions of coating IgG and conjugated IgG were found to be 1:2 000 and 1:800, respectively. With this system, all 96 wells on the plate could be used without any edge effect.

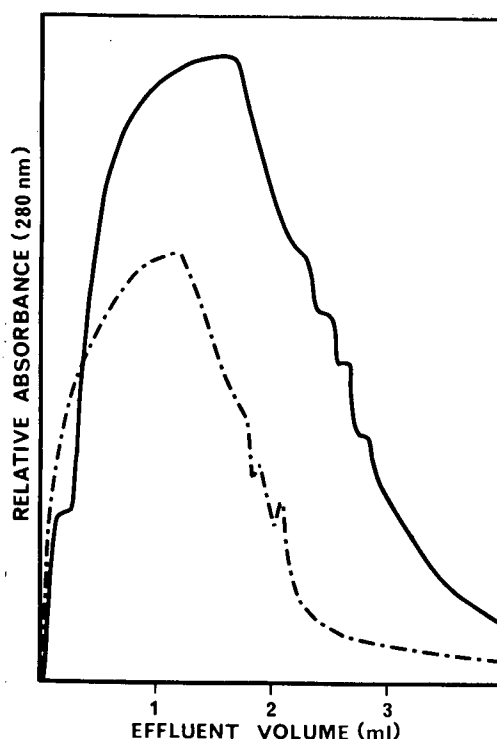


Fig. 18. Absorbance pattern obtained by passing concentrated antiserum from a rabbit immunized with DVS (——) and not immunized (-----) through a DEAE-22 Sephadex column.

Fig. 19 shows the type of results when a grid is set up to determine optimum conditions: rows 1-4 have been coated with 1:1 000 IgG; rows 5-8 have been coated with 1:5,000; rows 9-12 have been coated with 1:10,000. Rows 1,5 and 9 have conjugated IgG at 1:200; rows 2,6 and 10 have conjugate at 1:800; rows 3, 7 and 11 have conjugate at 1:3,200 and rows 4,8 and 12 have no conjugate. In this instance, coating at 1:1,000 is best with conjugate at 1:800, as no nonspecific background is encountered,

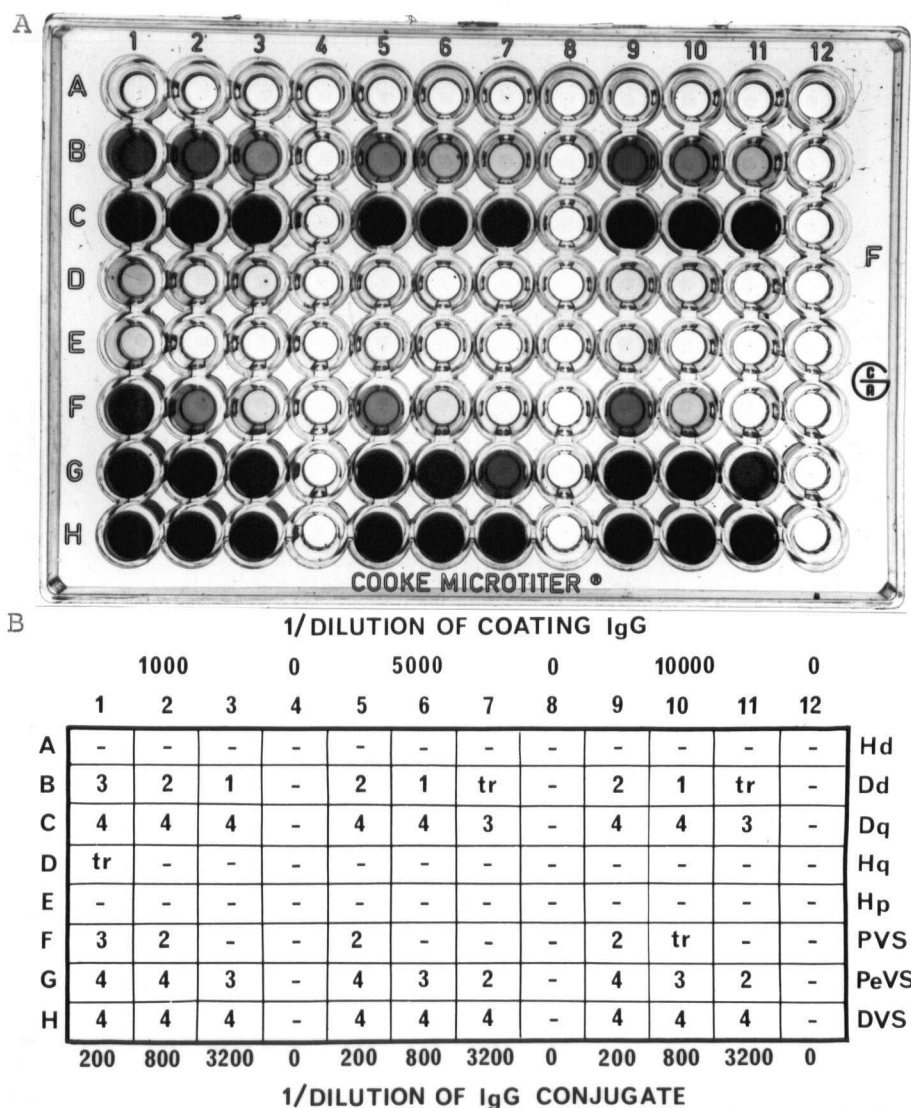


Fig. 19. Determination of optimum conditions for detection of DVS by ELISA. A. shows intensity of enzyme substrate reaction with the different concentrations of coating IgG and conjugated IgG in a microtitre plate. B. shows corresponding concentrations of coating IgG and conjugated IgG; numbers within the grid are visual scores assigned relative to the intensity of reaction in Fig. 19A. Abbreviations to the right of the chart are as follows: Hd, healthy dandelion; Dd, infected dandelion; Dq, infected C. quinoa; Hq, healthy C. quinoa; Hp, healthy potato; PVS, PVS in potato; PeVS, PeVS in C. quinoa and DVS, 1 µg/ml DVS preparation

yet lower levels of virus concentrations are readily detectable.

Occasionally, healthy C. quinoa sap would react slightly depending on the dilution of the sap. Sap from old dandelion leaves would also produce a slight background, possibly due to the nonspecific adsorption of the latex-like substance that was present in higher concentrations in old leaf material than in young leaf material. When C. quinoa plants and old dandelion leaves were assayed, the background of healthy sap reaction was always considered, whether the plates were scored visually or spectrophotometrically.

Sap from infected C. quinoa could be diluted more than 1:150,000, depending on the extent of infection and was still detected by ELISA. Sap from infected dandelions had an end point of 1:31,000 (Fig. 20A). These end points correspond to about 80 ng/ml virus concentration in the sap extracts. Purified DVS had an end point of about 2 ng/ml (Fig. 20B).

Transmission electron microscope serology

A survey of the serological reactions using dilutions of antiserum at 1:10, 1:20 and 1:40 showed that specific coating and aggregation occurred at all three dilutions (Fig. 21). A clearer background was obtained with the 1:40 dilution and this became the standard dilution.

Particles of DVS, PeVS or TMV prepared for scanning by electron microscope by standard negative staining, were readily distinguished in a scan of the grid (Fig. 22 A,E,I).

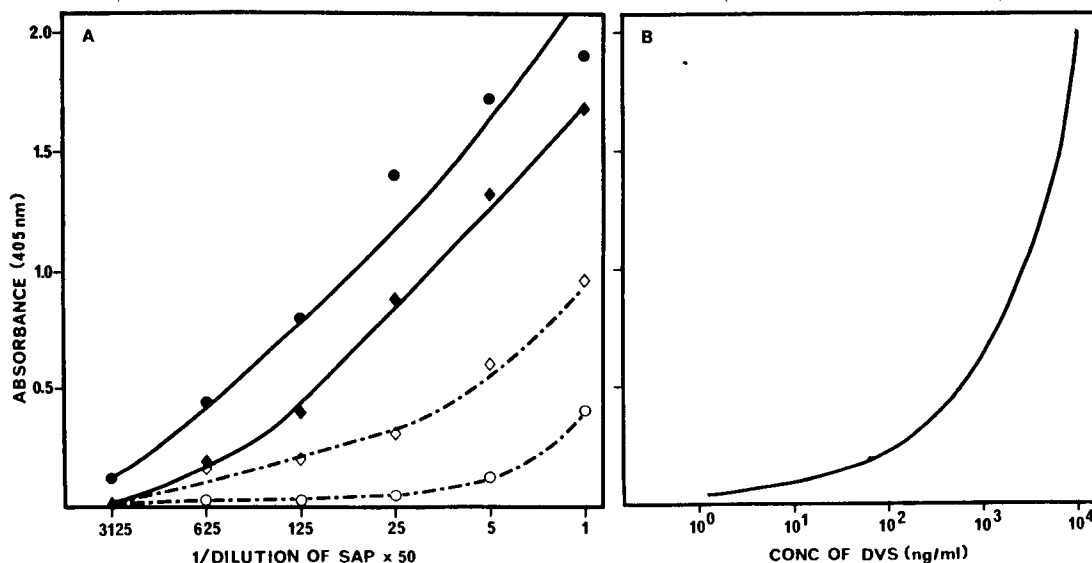


Fig. 20. Standard concentration curves for DVS determined by enzyme-linked immunosorbent assay with A. sap from infected dandelion ($\diamond-\diamond-\diamond$), local lesions of *C. quinoa* ($\diamond-\diamond-\diamond$), moderately infected *C. quinoa* ($\blacklozenge-\blacklozenge-\blacklozenge$) and severely infected *C. quinoa* ($\bullet-\bullet-\bullet$). The values have been corrected for healthy background and the limit of detection of a positive reaction was considered as twice the absorbance of the healthy controls. B. dilution series of purified DVS from 10 $\mu\text{g/ml}$ to 2 ng/ml

When DVS or PeVS was mixed with TMV and prepared by standard negative staining, it was difficult to locate particles of the carlavirus on the grid, without measuring the width of the individual particles (Fig. 22B,F). When TEMS was used with a mixture of DVS or PeVS, its homologous antiserum and TMV, the carlavirus was readily located as coated and clumped particles forming large aggregates on the grid against a background of normally dispersed TMV particles (Fig. 22C,G). The distribution of TMV was not altered by the presence of antiserum to either of the carlaviruses (Fig. 22J,K). Examination of the reaction at higher magnification showed the extent of specific

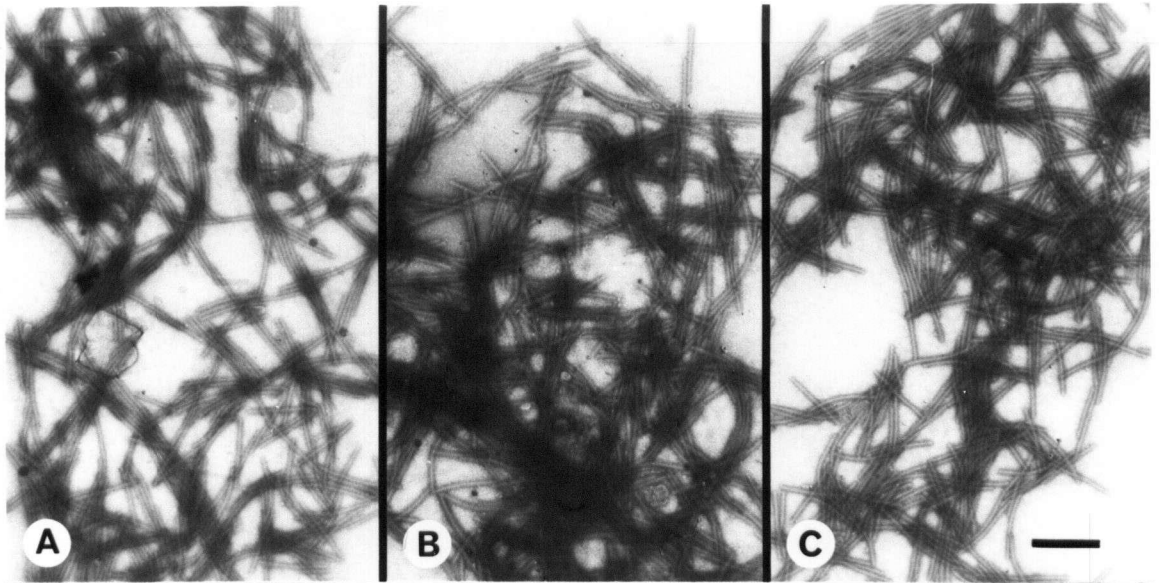
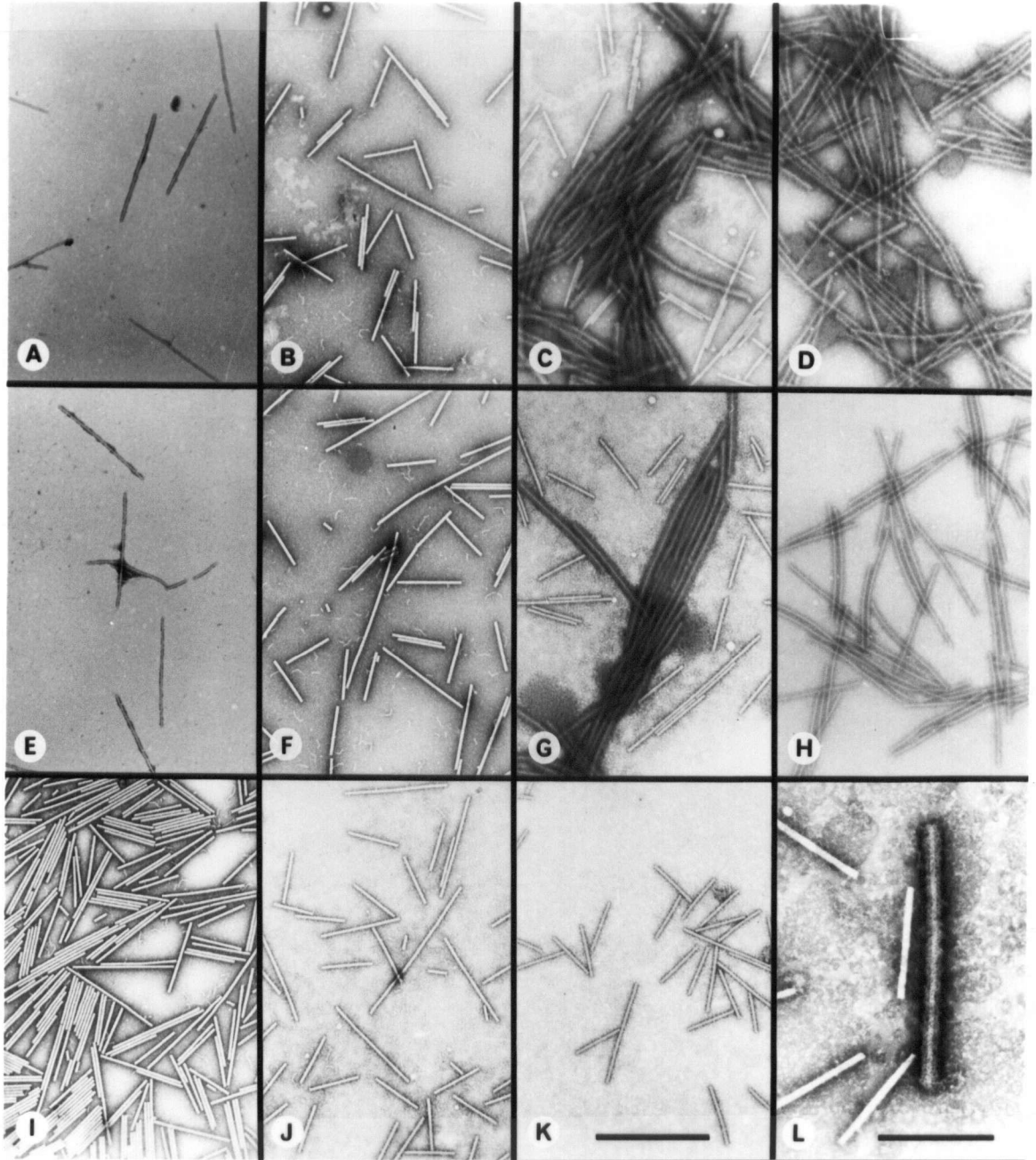


Fig. 21. Electron micrographs of the serological reactions obtained by TEMS, showing the extent of coating and aggregation of DVS caused by varying dilutions of antiserum: A. 1:10; B. 1:20 and C. 1:40. Bar represents 650 nm

coating of eg. DVS by DVS antiserum with TMV remaining uncoated (Fig. 22L). In heterologous reactions between the carlaviruses, the virus particles did not seem as heavily coated with antiserum nor did they aggregate as densely as in homologous reaction (Fig. 22D,H). The individual carlaviruses could not be distinguished in a mixture on the basis of the slight differences in their homologous and heterologous reactions.

Fig. 22. Electron micrographs of the serological reactions obtained by TEMS using combinations of three viruses and two antisera: A. DVS particles without antiserum treatment; B. DVS and TMV are not readily distinguished without antiserum treatment; C. TMV is not affected by DVS antiserum; but DVS particles are coated and clumped and readily distinguishable in a mixture; D. DVS particles treated with PeVS antiserum aggregate less than in the homologous reaction. Similarly, PeVS (E) in a mixture with TMV (F) is readily identified with homologous antiserum (G). PeVS does not react as intensely with DVS antiserum (H). TMV is not affected by the antisera: I. no antisera; J. DVS antiserum; K. PeVS antisera. (Bar = 650 nm for A-K). The intensity of specific coating is shown in L. at a higher magnification of C. (Bar = 300 nm)



DISCUSSION

The dandelion virus is a typical carlavirus in its particle size and morphology, biological properties, ease of detection by electron microscopy, serological relationships with other carlaviruses, narrow host range and latency in its natural host (Fenner, 1976; Harrison et al., 1971).

Most of the carlaviruses are present as latent infections in their natural hosts and as such suggest a long evolutionary involvement between the virus and the host. If this is the case, then DVS would have to be considered as naturally occurring in dandelion, rather than having been introduced recently with the advances of agriculture and the development of a new ecological balance. It is remarkable that DVS had such a limited distribution geographically especially when it was so readily recovered from dandelions within the original orchard surveyed. As the demand for virus-free stock material increases, more latent viruses are being discovered and their effects on crops are being evaluated.

With increasing reports of "new" viruses, it is becoming more important to establish a working definition of what constitutes classification as a new virus vs a new strain of a known virus. Harrison et al. (1971) devised a system of information collection and assessment based on behaviour of the virus in hosts, vector relations, particle properties and particle composition. From this information, they were able to classify the well-characterized viruses and define a virus

group as "a collection of viruses and/or virus strains each of which shares with the type member all or nearly all the main characteristics of the group." Although this system aided in sorting out the classification and nomenclature tangle for plant viruses, it did not make final a distinction between strains and species of viruses. (Shepherd et al., 1975; Harrison et al., 1971)

Differences in serological reactions, host range, symptomatology and physical properties distinguish a virus within a group (Brandes et al., 1959; Brandes and Wetter, 1959). Difficulties in determining symptomatology are encountered with some host plants in that different seed lines and environmental conditions during development can alter the symptoms expressed (Bos et al., 1960; Hiruki, 1975). Comparisons based solely on descriptions in the literature, that are not supplemented by direct comparisons, are always open to erroneous conclusions.

A study of host range, symptomatology, physiochemical properties of crude sap and purified preparations, size and shape of the particle, serological relationships, cross protection and insect transmission will help to determine if the virus is distinct or if it is a strain (Bos et al., 1960).

Based on severity of symptoms on different potato varieties, Kowalska (1978) differentiated 20 isolates of PVM into 14 strains. However, she could not distinguish strains on the same basis with 8 isolates of PVS. The isolate of PVS

from Peru (PeVS) produced systemic infection in C. quinoa rather than local lesions only (Hinostroza-Orihuela, 1973). In both instances, some serological work supported the strain status of these isolates, rather than differences only in symptomatology.

Gibbs (1969) used the unstable or varying characteristics of a virus to distinguish between strains of viruses: details of composition of protein subunits of the particle; the serological specificity; electrophoretic mobility; host range; severity of symptoms; vector specificity and ease of transmission by vector. Myzus persicae (Sulz.) is commonly used to test transmissibility in the laboratory because of its adaptability to a wide host range. M. persicae did not establish colonies on dandelions in the rosette stage, although it did become well established on C. quinoa. Another aphid species, Dactynotus chondrillae Nevsk. does not establish colonies on dandelions in the rosette stage either, but prefers to colonize the flower stalks as nutritive substances such as amino acids and sugars occur more abundantly in the elongating flower stem (Caresche et al., 1974). This may have been the reason M. persicae did not establish colonies on dandelion.

Uroleucon taraxaci Kalt., the dandelion aphid (Blackman, 1974), was not available for transmission studies at the time; M. persicae was tested with infected C. quinoa, although adaptation of the aphids to C. quinoa was slow.

Serological tests are an indirect measure of the

affinities between amino acid sequences exposed on the surface of the protein coat, reflecting the inherent genetic make up of the virus (Gibbs, 1969). Serological resemblance means two viruses are related; lack of resemblance does not imply that the virus should be excluded from the particular virus group.

Biological properties reflect differences in strains that might not be detected in physical or chemical testing of the virus particle. Many naturally-occurring strains of viruses such as TMV, PVS, PVX and PVY have been called "new" viruses on the basis of symptomatology alone. Later, as more information became known, it appeared more logical to classify the so-called "new" virus as a strain of a previously described virus. Serological tests may be usefully supplemented with cross protection tests (Rozendaal and van Slogteren, 1957). However, if antiserum to the virus in question is available, a greater degree of sensitivity and a more meaningful indication of the degree of relatedness can be achieved by serology than with cross protection tests alone.

In order to distinguish related viruses, Matthews (1970) suggested that strains are closely related viruses, while serotypes are distantly related viruses. Viruses with intermediate relatedness exist and cannot be classified as strains and serotypes, thus confusing the problem even further.

The stability of a virus is usually determined by testing its longevity in vitro and its thermal inactivation point. Factors such as storage temperature, presence of oxidizing

agents in the sap, virus concentration, nature and age of the host, pH and ionic strength of the sap can greatly alter the results. Although DVS had a greater stability than most carlaviruses, the values were still close to those expected and no significant difference was noted. The dilution end point was also within range of that expected for a carlavirus. No comparisons as to specific identity of a virus can be made because of the extent of variable factors involved in these in vitro tests, although some indication of how well the virus fits into the group is obtained.

When two viruses are related but not identical, only a proportion of the antigenic sites are common to both viruses. In a tube precipitin test involving a homologous reaction, an antigen excess interferes with the physical formation of the antigen-antibody lattice responsible for the visible precipitate. Consequently, the maximum titre cannot be achieved. In a heterologous test, the antiserum will not react to the same extent as in a homologous test since only a portion of the sites are reactive. A relatively higher concentration of heterologous antigen is required to obtain the maximum precipitation at a given dilution of antiserum.

As outlined in Table 3, the optimum concentration of antigen to obtain maximum titres using homologous antiserum was 10-20 $\mu\text{g/ml}$. If an excess of antigen was used, the end point was reduced 2-4 fold and the true titre was not achieved. In determining heterologous reactions, a two way tube

precipitin test was set up using 80 $\mu\text{g/ml}$ antigen. With this high concentration of antigen, the degree of relationship was obscured (Table 4). For example, the titre of DVS antiserum with DVS antigen was 10,240 and with PVS antigen, 5,120, which is a difference of one dilution, implying a close serological relationship. Of interest is that the homologous titre for PVS was 2,560, which is less than that obtained with DVS or PeVS antiserum. This apparent discrepancy developed by using an excess of antigen for homologous reactions.

When the same tests were run with 20 $\mu\text{g/ml}$ antigen, the true relationships were brought into perspective (Table 5). It is evident that there are marked serological differences among the four viruses tested: for example, DVS antiserum has a homologous titre of 20,480, and against PVS antigen the end point is 5,120, implying about 25% common antigenic determinants; similarly DVS shares about 6% with PeVS and about 0.4% with HVS. The corresponding homologous reactions showed the highest end point compared with the heterologous reactions. These results clearly show that DVS shares slightly more antigenic sites with PVS than with PeVS and that HVS is distantly related to each of the other three viruses tested.

Based on differences in host range, symptomatology and serological reactions, the dandelion virus is distinct from PVS, PeVS and HVS -- the three viruses that were most likely to show a strain relationship or identity with the dandelion virus. Whether the dandelion virus should be classified as a

new virus or as a strain of PVS or PeVS, is largely a subjective decision. In considering all the experimental evidence obtained in the course of this work, it is my opinion that there is sufficient justification to consider the dandelion virus as a distinct entity and as such, a new member of the carlavirus group.

In considering a suitable designation for the dandelion virus, I am proposing that it be called dandelion virus S (DVS) in order to designate the natural host of the virus and to give reference to the group membership. Also, there is a recent example in the literature concerning a new carlavirus from Helenium where the designation Helenium virus S was applied (Kuschki et al., 1978), even though HVS is more closely related to PVM than to PVS. Another reason is that there is historical precedence for considering PVS, rather than CLV as the type member of this group. In a classification of rod-shaped viruses designed by Brandes and Berckes (1965), viruses with rigid to slightly flexible rods, ca. 650 nm in length, were classified in the PVS group. It was not until the Plant Virus Sub-Committee of the International Committee on the Taxonomy of Viruses put forth their classification scheme of 16 virus groups (Harrison et al., 1971) and obtained an acceptable sigla, that PVS was replaced as the type member by CLV with the "carla" sigla.

SUMMARY

The dandelion virus that was the subject of this thesis was a slightly flexuous 637 nm rod and based on its particle size and morphology, it was placed in the carlavirus group. Inclusion in this group was further supported by its biological properties, host range and symptomatology, transmission characteristics and serological relationships.

The biological properties were: thermal inactivation point 75-80°, dilution end point 2×10^{-5} to 10^{-6} and longevity in vitro 4-5 days at 23° and 28-56 days at 4°. The host range and symptomatology were limited as follows: systemic infection of C. amaran-ticolor and C. quinoa, latent systemic infection of T. officinale and local infection of D. stramonium and G. globosa. The virus was readily sap transmitted and also non-persistently transmitted by M. persicae. No seed transmission was obtained.

In determining if the dandelion virus is a new virus or a strain of a previously described virus, serology, host range and symptomatology are important criteria. From the literature, the viruses most likely to be related were PeVS, PVS and HVS. Detailed serological comparisons revealed that the dandelion virus had a close relationship with PVS, a more distant one with PeVS and a very distant one with HVS. As a result of these studies, the dandelion virus was considered to be a new virus and was designated Dandelion Virus S.

LITERATURE CITED

1. Black, L.M. 1970. Potato yellow dwarf virus. C.M.I./A.A.B. Description of Plant Viruses No. 35: 4pp.
2. Blackman, R. 1974. Aphids. Gin and Co., Ltd., London: 100-104.
3. Bos, L. 1978. Role of wild plants in the ecology of virus diseases. Abstract. 3rd Intl. Congr. Plant Pathol.: 22.
4. Bos, L., Hagedorn, D.J. and Quantz, L. 1960. Suggested procedures for international identification of legume viruses. Neth. J. Plant Pathol. 66: 328-343.
5. Bos, L., Huttinga, H. and Maat, D.Z. 1978. Shallot latent virus, a new carlavirus. Neth. J. Plant Pathol. 84: 227-237.
6. Brandes, J. and Bercks, R. 1965. Gross morphology and serology as a basis for classification of elongated plant viruses. Adv. Virus Res. 11: 1-24.
7. Brandes, J. and Wetter, C. 1959. Classification of elongated plant viruses on the basis of particle morphology. Virology 8: 99-115.
8. Brandes, J., Wetter, C., Bagnall, R.H. and Larson, R.H. 1959. Size and shape of the particles of potato virus S, potato virus M and carnation latent virus. Phytopathology 49: 443-446.
9. Brunt, A.A., Stace-Smith, R. and Leung, E. 1976. Cytological evidence supporting the inclusion of poplar mosaic virus in the carlavirus group of plant viruses. Intervirology 7: 303-308.
10. Caresche, L.A., Hasan, S. and Wapshere, A.J. 1974. Biology and host specificity of two aphids Dactynotus chondrillae (Nevsk.) and Chondrillobium blattnyi (Pintera) (Hemiptera) living on Chondrilla juncea. Bulletin Ent. Res. 64: 277-288.
11. Christie, R.G. and Edwardson, J.R. 1977. Light and electron microscopy of plant virus inclusions. Florida Agric. Exptl. Stn. Monograph No. 9: 47-52.

12. Clark, M.F. and Adams, A.N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 34: 475-483.
13. Costa, A.S. and Kitajima, E.W. 1972. Cassava common mosaic virus. C.M.I./A.A.B. Description of Plant Viruses. No. 90: 4pp.
14. Dias, H.F. 1977. Incidence and geographic distribution of tomato ringspot in De Chaunac vineyards in the Niagara peninsula. *Plant Dis. Report:* 61: 24-28.
15. Fenner, F. 1976. Classification and nomenclature of viruses. 2nd Report of the International Committee on Taxonomy of Viruses. S. Karger, Basel. 116pp.
16. Forster, R.L.S. and Milne, K.S. 1978. Daphne virus S: a carlavirus from Daphne. Abstract. *Rev. Plant Pathol.* 57: 497.
17. Francki, R.I.B. and Randles, J.W. 1970. Lettuce necrotic yellows virus. C.M.I./A.A.B. Description of Plant Viruses No. 26: 4pp.
18. Fukomoto, F. and Tochiwara, H. 1978. (Chinese yam necrotic mosaic virus). Abstract. *Rev. Plant Pathol.* 57: 520.
19. Fulton, R.W. 1948. Hosts of the tobacco streak virus. *Phytopathology* 38: 421-428.
20. Gibbs, A.J. 1969. Plant virus classification. *Adv. Virus Research* 14: 263-328.
21. Gibbs, A.J., Harrison, B.D., Watson, D.H. and Wildy, P. 1966. What's in a virus name? *Nature (London)* 209: 450-454.
22. Gilkey, H.M. 1957. Weeds of the Pacific Northwest. Oregon State College, Corvallis: 332-337, 426-427.
23. Hansen, A.J., Nyland, G., McElroy, F.D. and Stace-Smith, R. 1974. Origin, cause, host range and spread of cherry rasp leaf disease in North America. *Phytopathology* 64: 721-727.
24. Harrison, B.C. 1958. Raspberry yellow dwarf, a soil-borne virus. *Ann. Appl. Biol.* 46: 221-229.

25. Harrison, B.C. 1957. Studies on the host range, properties and mode of transmission of beet ringspot virus. *Ann. Appl. Biol.* 45: 462-472.
26. Harrison, B.D., Finch, J.T., Gibbs, A.J., Hollings, M., Shepherd, R.J., Valenta, V. and Wetter, C. 1971. Sixteen groups of plant viruses. *Virology* 45: 356-363.
27. Hiebert, E. and McDonald, J.G. 1973. Characterization of some proteins associated with viruses in the potato virus Y group. *Virology* 56: 349-361.
28. Hinostroza-Orihuela, A.M. 1973. Some properties of potato virus S isolated from Peruvian potato varieties. *Potato Research* 16: 244-250.
29. Hiruki, C. 1975. Factors affecting bioassay of potato virus S in Chenopodium quinoa. *Phytopathology* 65: 1288-1292.
30. Hollings, M. 1957. Investigation of chrysanthemum viruses. II Virus B (mild mosaic) and chrysanthemum latent virus. *Ann. Appl. Biol.* 45: 589-602.
31. Kassanis, B. 1944. A virus attacking lettuce and dandelion. *Nature (London)* 154: 16.
32. Kegler, H. and Schade, C. 1971. Plum pox virus. C.M.I./A.A.B. Description of Plant Viruses No. 70: 4 pp.
33. Kemp, W.G. and High, P.A. 1979. Identification of carnation latent virus from naturally infected hardy garden Dianthus species in North America. *Plant Dis. Rep.* 63: 51-54.
34. Khalil, J.A. and Nelson, M.R. 1977. Isolation and characterization of a carlavirus from eggplant. Abstract. *Proceed. Amer. Phytopath. Soc.* 4: 160.
35. Kowalska, L.A. 1978. Differences among isolates of potato virus M and potato virus S. *Phytopath. Z.* 93: 227-240.
36. Kuschki, G.H., Koenig, R., Düvel, D. and Kühne, H. 1978. Helenium virus S and Y -- Two new viruses from commercially grown Helenium hybrids. *Phytopathology* 68: 1407-1411.

37. Lister, R.M. and Murrant, A.F. 1967. Seed transmission of nematode-borne viruses. *Ann. Appl. Biol.* 59: 49-62.
38. Luisoni, E., Boccardo, G. and Milne, R.G. 1976. Purification and some properties of an Italian isolate of poplar mosaic virus. *Phytopath. Z.* 85: 65-73.
39. Lytaeva, G.K. 1971. (Plants-reservoirs of potato virus Y). Abstract. *Rev. Plant Pathol.* 51: 478pp.
40. Martelli, G.P., Quacquarelli, A. and Russo, M. 1971. Tomato bushy stunt virus. C.M.I./A.A.B. Description of Plant Viruses No. 69: 4pp.
41. Matthews, R.E.F. 1970. *Plant Virology*. Academic Press, New York. 778pp.
42. Matthews, R.E.F. 1957. *Plant Virus Serology*. Cambridge University Press, London. 128pp.
43. Mulligan, G.A. 1976. *Common Weeds of Canada*. McClelland and Stewart, Ltd. in association with Information Canada and Agriculture Canada, Ottawa. 129pp.
44. Noordam, D. 1973. *Identification of Plant Viruses: Methods and Experiments*. Centre for Agricultural Publishing and Documentation, Wageningen. 205pp.
45. Peters, D. 1971. Sowthistle yellow vein virus. C.M.I./A.A.B. Description of Plant Viruses No. 62: 3pp.
46. Phatak, H.C. 1974. Seed-borne plant viruses - Identification and diagnosis in seed health testing. *Seed Sci. Technol.* 2: 3-155.
47. Purcifull, D.E. and Batchelor, D.L. 1977. Immunodiffusion tests with sodium dodecyl sulfate (SDS) - treated plant viruses and plant viral inclusions. *Florida Agric. Exptl. Stn. Tech. Bulletin* No. 788: 39pp.
48. Purcifull, D.E., Christie, S.R. and Zitter, T.A. 1976. Bidens mottle virus. C.M.I./A.A.B. Description of Plant Viruses No. 161: 4pp.

49. Purcifull, D.E. and Shepherd, R.J. 1964. Preparation of the protein fragments of several rod-shaped plant viruses and their use in agar-gel diffusion tests. *Phytopathology* 54: 1102-1108.
50. Ramsdell, D.C. and Myers, R.L. 1978. Epidemiology of peach rosette mosaic virus in a Concord grape vineyard. *Phytopathology* 68: 447-450.
51. Rozendaal, A. and van Slogteren, D.H.M. 1957. A potato virus identified with potato virus M and its relationship with potato virus S. *Proceed. 3rd Conf. on Potato Virus Diseases. Wageningen-Lisse*: 20-36.
52. Shepherd, R.J., Francki, R.I.B., Hirth, L., Hollings, M., Inouye, T., MacLeod, R., Purcifull, D.E., Sinha, R.C., Tremaine, J.H., Valenta, V. and Wetter, C. 1975. New groups of plant viruses approved by the International Committee on Taxonomy of Viruses, Sept. 1975. *Intervirology* 6: 181-184.
53. Tuite, J. 1960. The natural occurrence of tobacco ringspot virus. *Phytopathology* 50: 296-298.
54. Varma, A., Gibbs, A.J. and Woods, R.G. 1970. A comparative study of red clover vein mosaic virus and some other plant viruses. *J. Gen. Virol.* 8: 21-32.
55. Veerisetty, V. and Brakke, M.K. 1978. Purification of some legume carlaviruses. *Phytopathology* 68: 59-64.
56. Veerisetty, V. and Brakke, M.K. 1977. Alfalfa latent virus, a naturally occurring carlavirus in Alfalfa. *Phytopathology* 67: 1202-1206.
57. Waterworth, H.E. 1972. Purification, serology and properties of a virus from lilac, *Syringa oblata affinis*. *Plant Dis. Rep.* 56: 923-926.
58. Wright, N.S. and Stace-Smith, R. 1966. A comparison of the sensitivity of three serological tests for plant viruses and other antigens. *Phytopathology* 56: 944-948.
59. Zaitlin, M. and Israel, H.W. 1975. Tobacco mosaic virus. *C.M.I./A.A.B. Descriptions of Plant Viruses* No. 151: 5pp.