CHARACTERISATION AND DETECTION OF DASHEEN MOSAIC POTYVIRUS IN *ZANTEDESCHIA*

A thesis presented in partial fulfilment of the requirements for the degree of Master of Horticultural Science in Plant Health at Massey University, Palmerston North, New Zealand.

CHRISTINE GRACE MATTHEWS

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ERRATA

Page 11	line 21: "a period of three months with which" should read " a period of three months in which"
Page 14	line 14: "Hendon" should read "Herndon" line 23: "Shawl" should read "Shaw". line 25: "Shukla and Ward, 1989" should read "Shukla and Ward, 1989a".
Page 15	Table 1.1: The first protein should be "P1" not "P3"
Page 20	line 1: "wide geographical" should read "widely separated geographical"
Page 22	line 22: "Dichlorovos®" should read "dichlorvos"
Page 39	line 7: "at 160,800g" should read "at 160,800g for 90 minutes"
Page 40	line 16: "200ul" should read "200ml".
Page 58	Table 3.1, column four, bottom line: "stuntimg" should read "stunting"
Page 67	line 3: "using two virus other isolates" should read "using two other virus isolates".
Page 73	line 18: "Enrlich" should read "Ehrlich"
Page 83	line 10: "feathery mottle potyvirus" should read "taro feathery mottle potyvirus".
Page 84	line 2: "numerable" should read "numerous" line 11: "not unlikely" should read "likely" line 13: "other" should read "some other" line 19: "would clarify whether" should read "would help confirm" line 19: "virus" should read "potyvirus"
Page 113	line 1: should read "Until this study no other potyvirus except DsMV has been ..."
Page 116	line 4: "infection should read "virus infection".
Page 126	Addition to the bibliography: Saiki (1989). The design and optimization of the PCR. In: PCR Technology. Ed. Ehrlich, G.D. M. Stockton Press. p7-16.

ABSTRACT

Four potyvirus isolates believed to be dasheen mosaic potyvirus, the most frequently occurring virus to infect members of the *Araceae*, were obtained from *Caladium*, *Colocasia*, *Xanthosoma* and *Zantedeschia* in world-wide locations. Properties of these isolates such as particle length, serological relatedness, electrophoretic mobility of coat proteins and genomic characteristics were compared.

Serologically distinct strains of dasheen mosaic potyvirus were apparent amongst the isolates. The difference in the serological relationship was coupled with a variation in symptom expression. An isolate from *Colocasia esculenta* (L.) Schott was not serologically related to the other isolates. Further isolates from *C. esculenta* also exhibited no relationship. The modal length was different as well as the ability of complementary deoxyribonucleic acid, produced to the viral ribonucleic acid, to bind with some of the primers used in the polymerase chain reaction. This evidence led to the proposal that the isolate from *C. esculenta* was not dasheen mosaic potyvirus; this virus is tentatively named taro feathery mottle potyvirus.

Cytoplasmic inclusion protein aggregates of dasheen mosaic potyvirus were purified from infected leaf tissue. SDS-polyacrylamide gel electrophoretic analysis of samples revealed a major band with an estimated molecular weight of 68,000 daltons. Such a band was absent from healthy tissue samples.

The ATPase activity in samples from each purification step was determined by measuring the amount of [^{32}P] released from the [γ-^{32}P]ATP during incubation with the cytoplasmic inclusion protein. The level of ATPase activity in each sample showed a strong correlation with the amount of protein that was present.

In a limited survey of commercial plantings twenty nine tubers grown for cutflower or tuber export were obtained from seven properties at different locations in New Zealand and grown on in a greenhouse. Each plant was indexed for virus infection.

Electron microscopy revealed that plants from three of the properties contained 720nm flexuous rods. Samples from all but two plants tested positive to a potyvirus group antiserum using the enzyme-linked immunosorbent assay. The remaining two plants tested positive in microprecipitin and rapid immune electron microscopy tests to an antiserum prepared to a member of the carlavirus group. Particles from these plants were mechanically transmitted to *Nicotiana tabacum* 'Havana'.

Rod-shaped particles of 300nm were observed in plants from four properties and tested positive to tobacco mosaic tobamovirus antiserum using a microprecipitin test. While inoculations to herbaceous indicators resulted in no symptoms, 300nm particles were observed in samples from the indicator plants.

Tomato spotted wilt tospovirus, potato X potexvirus and cucumber mosaic cucumovirus, reported to infect *Zantedeschia* spp, were not detected.

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CHAPTER ONE

INTRODUCTION

Several species of *Zantedeschia*, commonly known as calla lilies or callas, have been grown in gardens throughout the world for many centuries. There is no record of when the first callas were introduced into New Zealand. A number of amateur breeders were active in the early 1900's. In recent years there has been an increasing interest in callas, they have become very fashionable in many of the main flower markets of the world because of their long vase life, attractive shape and long stems making them a popular flower for arranging.

New Zealand breeders and producers have capitalised on this interest with a selected range of superior cut flower and pot plant cultivars. With the use of tissue culture as a means of rapid multiplication New Zealand callas are now available to the world markets as fresh cut flowers or as dormant tubers for flower forcing. Currently New Zealand produces the widest range of callas with flower stems from more than 120 cultivars being exported. Exports have rapidly increased; in 1989 they realised $1.24 million, rising to $5.51 million in 1994[1].

As production of callas has increased so to has the awareness of virus infection. Symptoms observed include leaf distortion, discolourations such as mosaics, mottles and yellowing, and colour break in flowers (see Figures 1.1-1.6). Plants and flowers are often unmarketable.

[1] Statistics NZ, 1994.

Figure 1.1 : Mosaic symptoms on the leaves of *Zantedeschia* sp 'Tinkerbell'.

Figure 1.2 : Distorted leaf of *Zantedeschia* sp 'Pink Persuasion'.

Figure 1.3 : Reduced plant growth of *Zantedeschia* sp 'Pink Persuasion' (healthy plant on the left).

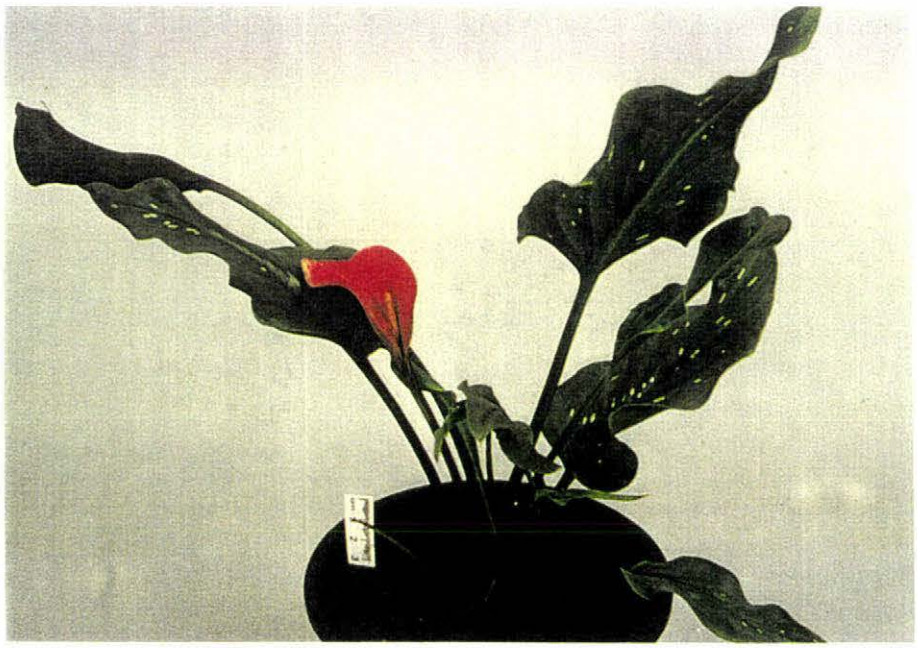

Figure 1.4 : Stunted plant of *Zantedeschia* sp 'Majestic Red'.

Figure 1.5 : Distorted spathe of *Zantedeschia* sp 'Majestic Red'.

Figure 1.6 : Colour break on the spathe of *Zantedeschia* sp 'Pink Persuasion'.

Rod-shaped particles have been observed, using the electron microscope, in plants with virus-like symptoms (Neilson, pers comm). It has been assumed these particles are dasheen mosaic potyvirus (DsMV) (see section 1.3). The symptoms observed resemble those reported for DsMV. Although other viruses are known to infect aroids, DsMV is the most widespread and prevalent.

1.1. THE FAMILY *ARACEAE*.

The family of *Araceae* has more than 110 genera and 2500 species, many of which are of horticultural significance. About one-third of the plants grown commercially world-wide for ornamental foliage belong to this family and include the genera *Aglaonema*, *Caladium*, *Dieffenbachia*, *Epipremnum*, *Philodendron*, *Scindapus*, and *Syngonium*. Species of *Anthurium* and *Zantedeschia* are popular cut flower crops, and several species of *Anubias* and *Cryptocoryne* are grown as aquarium plants. Other aroids, particularly *Colocasia* spp and *Xanthosoma* spp are important food staples of the tropics and subtropics.

The unifying characteristic of *Araceae* is the spathe-and-spadix inflorescence, a floral structure consisting of a petal-like leaf (the spathe) and a flower-bearing protuberance (the spadix). Though technically an inflorescence, because it consists of a spike of several individual flowers, it functions very much like a single flower.

1.2. THE GENUS *ZANTEDESCHIA*.

The genus *Zantedeschia* is native to the African continent, being most prevalent in the south. Letty (1973) recognized six species with two subspecies within the genus *Zantedeschia*; *Z. aethiopica*, *Z. elliottiana*, *Z. rehmannii*, *Z. pentlandii*, *Z. jucunda*, *Z. albomaculata*, *Z. albomaculata* subsp

albomaculata and *Z.albomaculata* subsp *valida*. A seventh species, *Z. odorata* (Perry), has since been proposed (Perry, 1989).

The *Zantedeschia* species have been divided into two distinct sections; section I contains one species *Z. aethiopica*, the white calla lily. It is a robust evergreen plant, with unspotted leaves, a milk-white spathe and bright yellow spadix. Its storage organ is a rhizome, which does not require a resting period. Section II consists of the remaining six species. They exhibit complete foliage senescence in winter and flower during summer. These species possess what is best described as a compact stem, popularly known as a tuber, which requires a resting period. Species within section II can be hybridized with each other and this has led to a collection of zantedeschia hybrids with a great range of colour, size and form.

Over fifty years of crossing and selecting species has produced the present range of cultivars available in New Zealand. Although some native species are widely used, a number of hybrid callas have been developed for commercial objectives such as greater productivity, longer and stiffer stems, uniformity of time to flowering, larger spathes and long lasting vase life. Cultivars have been selected which have a range of flower colours including red, mauve, purple, rosepink, softpink, yellow, cream, gold, orange, apricot and blended shades of these.

Propagation can be done by seed, although this is limited to the true species which have a limited colour range. Hybrid callas have been multiplied by tissue culture to maintain exact trueness to type and subsequently grown on in the field to produce larger flowering sized tubers for the markets. Division of tubers after lifting is a common means of increasing initial stock numbers. This type of propagation is conducive to carry over and spread of viral and bacterial diseases.

Most cultivated aroids are routinely propagated vegetatively, and as such are especially vulnerable to chronic virus infection. As large-scale plantings of callas has increased world-wide so too has the awareness of virus-like symptoms. Symptoms of viral infection have become more prevalent in New Zealand where growers have continuously used the same stock for division.

1.3. VIRUS INFECTION OF CALLAS.

Five different viruses have been reported to infect *Zantedeschia* spp. These are cucumber mosaic cucumovirus (Chamberlain, 1954), dasheen mosaic potyvirus (Zettler, Foxe, Hartman, Edwardson and Christie, 1970), potato X potexvirus (Okuyama and Saka, 1976), tobacco mosaic tobamovirus (Gorter, 1981) and tomato spotted wilt tospovirus (Tompkins and Severin, 1950). With the exception of DsMV, which appears to occur naturally only in aroid species, strains of these viruses are present in a wide range of crops in New Zealand. Two of these, cucumber mosaic cucumovirus (CMV) and tomato spotted wilt virus (TSWV) have been recorded in callas in New Zealand (Chamberlain, 1954).

1.3.1. Dasheen mosaic potyvirus.

Dasheen mosaic potyvirus (DsMV) was first described in 1970 (Zettler et al, 1970). It is the most widespread and prevalent virus reported to infect aroids. As such it provides the basis of study for a considerable portion of this thesis. DsMV is typical of other known viruses of the potato virus Y group in being non-persistently transmitted by aphids, having a mean particle length of 700-800nm and inducing characteristic cylindrical inclusions.

The DsMV genome has been analyzed by *in vitro* translation, and the genomic expression strategy and proposed gene map were determined to be similar to those of other potyviruses (Zettler and Hartman, 1987). The capsid protein

is serologically related to those of certain potyviruses, including blackeye cowpea mosaic, tobacco etch and araujia mosaic, and the helper component protein of tobacco vein mottling potyviruses is related to *in vitro* synthesized gene products of DsMV (Zettler and Hartman, 1986). The cylindrical inclusion protein is serologically related to that of araujia mosaic potyvirus (Zettler and Hartman, 1986).

No strains of DsMV have been distinguished, although minor differences in symptoms induced by single isolates from Florida and Fiji in *Philodendron selloum* have been noted (Abo El-Nil, Zettler and Hiebert, 1977).

The horticultural importance of aroids has contributed greatly to the world-wide distribution of DsMV. It is common in many areas of the world where aroids are cultivated and it infects species in at least 16 genera of *Araceae,* most commonly infecting *Colocasia, Caladium, Dieffenbachia, Zantedeschia* and *Xanthosoma* (Zettler and Hartman, 1986; Zettler and Hartman, 1987). The widespread incidence seems due to frequent use of vegetative means of propagation (Hill and Wright, 1980). Although DsMV is apparently not seedborne (Zettler, Abo El-Nil and Hartman, 1978), obtaining genetically uniform seed of most commercially grown aroids is difficult (Zettler and Hartman, 1986). Tissue culture technology has been successfully applied commercially to control DsMV in several aroids. In those for which this technology has not yet proved economically feasible the incidence of DsMV remains high.

Symptoms of DsMV infection include leaf distortion, mosaic mottling and yellowing of leaves, colour break in flowers and stunting of plants. These are more readily expressed in some cultivars than others (Zettler and Hartman, 1986). In some aroids symptoms have been observed to be seasonal (Rana, Vovlas and Zettler, 1983; Hakkaart and Waterreus, 1976). Reduction in productivity is said to accompany virus infection in *Zantedeschia* spp (Welsh

pers comm.) but no scientific data has been obtained to quantify this. More than 60% yield losses have been recorded in DsMV infected *Philodendron* spp in Florida (Wisler, Zettler, Hartman and McRitchie, 1978).

The aphids *Aphis craccivora* Koch, *A. gossypii* Glover and *Myzus persicae* Sulzer have been found in tests to be vectors of DsMV (Zettler et al 1970; Greber and Shaw, 1986). These three aphid species appear to be natural vectors of DsMV (Zettler and Hartman, 1987) and all three are present in New Zealand (Cottier, 1953). While aphid transmission rates of DsMV are relatively low in comparison to certain other viruses (Morales and Zettler, 1977) DsMV has been observed to spread very rapidly under field conditions in Florida (Zettler and Hartman, 1986). It is not known what role aphids play in the field in New Zealand but it is thought to be minor because of the control obtained from insecticides applied primarily to control thrips. However, they can be prevalent on tubers as they start to shoot during storage before planting (Welsh pers comm).

The natural host range of DsMV appears to be restricted to the family *Araceae* (Zettler and Hartman, 1986) although certain non-aroids are susceptible. DsMV is occasionally transmitted to *Tetragonia expansa* Murr., producing a few chlorotic lesions, (James, Kenten and Woods, 1973). Rana et al (1983) succeeded in mechanically transmitting DsMV to *Chenopodium amaranticolor, C. quinoa, C. ambrosioides, Nicotiana benthamiana* and *Saponaria vaccaria.* Attempts by other investigators to transmit DsMV to non-araceous hosts have been unsuccessful and mechanical transmission seems to be limited to the European isolate in the studies of Rana et al (1983).

The use of tissue culture has enabled the production of virus-free plants. Reinfection can be avoided by ensuring good pest control practices are carried out, and if possible, growing plants in isolation from other infected plantings.

Roguing of any plants showing symptoms will help to eliminate any source of virus inoculum.

When tissue culture is not available careful selection of the most vigorous material for propagation is essential. As DsMV does not appear to be seed transmitted, seed propagation for those species that are true to type is advantageous. Since DsMV may be harboured in apparently healthy plants the continual application of control measures is important.

1.3.2. Tomato spotted wilt tospovirus.

TSWV infection of callas has been reported in a number of locations world-wide and infection can be widespread (Tompkins and Severin, 1950). Symptoms of infection include interveinal chlorotic streaks or circular lesions, which may become necrotic. These can occur on the leaves, petioles, peduncles and spathes. Leaves are often distorted, flowers are frequently of poor quality and plants are usually stunted.

TSWV is apparently not seed-borne (Tompkins and Severin, 1950), but can be perpetuated by vegetative propagation. Transmission by thrips, particularly in greenhouses, has been noted (Tompkins and Severin, 1950). TSWV has been mechanically inoculated from callas to herbaceous indicators such as tomato (Tompkins and Severin, 1950; Fletcher, 1987).

1.3.3. Cucumber mosaic cucumovirus.

CMV infection of callas does not appear to be as prevalent as DsMV or TSWV. Symptoms of infection are a mild chlorotic mottle or a pronounced yellowing of the leaf tissue (Chamberlain, 1954). The Ouchterlony agar double diffusion and a modified agar double diffusion (Tomlinson, Carter, Faithfull and Webb, 1973) tests have been used to confirm the identity of

CMV in callas (Fletcher, 1987). CMV has been transmitted from callas to a range of herbaceous indicators (Fletcher, 1987).

1.3.4. Potato X potexvirus and tobacco mosaic tobamovirus.

Little mention is made in the literature of these two viruses infecting callas. Potato X potexvirus (PVX) was isolated from *Zantedeschia aethiopica* in Japan (Okuyama and Saka, 1976) and tobacco mosaic tobamovirus (TMV) has been reported to infect the same species in South Africa (Gorter, 1981). These appear to be the only recorded incidences of the viruses being isolated from *Zantedeschia* spp. TMV is mechanically transmissible from callas to herbaceous indicators (Chapter 5, section 5.7.4.) as is PVX (Cohen pers comm.).

1.4. DETECTION OF DASHEEN MOSAIC POTYVIRUS.

Bioassay, serology and electron microscopy are routinely used to index aroids for the presence of DsMV. As herbaceous indicators are not generally susceptible to DsMV, *Philodendron selloum* seedlings are usually used as assay plants, but other aroids such as *Anthurium* spp may also be used. *P. selloum* are very susceptible to DsMV, giving mosaic and vein clearing symptoms on the first one or two leaves to unfurl following inoculation. However, sensitivity of test plants was found by Hakkaart and Watereus (1976) to be dependent on the season at the time of inoculation. There was a period of three months with which they found the bioassay using *P. selloum* to be unsuitable under their test conditions.

A limitation of the use of *P. selloum* and other araceous assay plants is that seeds do not retain their viability for long and will not survive prolonged periods of storage. Although *P. selloum* seed is commercially available in the USA, fresh, viable seed is not always readily attainable in New Zealand. *P.*

selloum, like other aroids, has the added disadvantage in that it is very slow to germinate (28 days) and slow growing, needing good advanced planning to ensure plants are available for inoculation when required.

Electron microscopy has been used to detect virus particles in aroids. Greber and Shaw (1986) had difficulty obtaining even stain coverage of extracts on electron microscope grids using 1% sodium phosphotungstic acid (PTA) or ammonium molybdate (AM) apparently due to the characteristics of aroid sap. They concluded that electron microscopy was unlikely to be an efficient method of detection. Investigations into the use of wetting agents and alternate staining conditions may improve the preparation of grids.

Light and electron microscopy may also be used to determine the presence of cylindrical inclusion bodies, which are indicative of the presence of potyvirus infection. Since DsMV is the only potyvirus reported to infect aroids the presence of cylindrical inclusions, or flexuous rod-shaped particles about 750nm in length, is often taken as evidence for its presence. The possibility of yet unidentified potyviruses infecting aroids must not be discounted and thus caution should be exercised when stating specific diagnoses of DsMV using microscopy.

Serological techniques such as immuno-specific electron microscopy (ISEM) and enzyme-linked immunosorbent assay (ELISA) are definitive means which have been used to identify DsMV (Hill and Wright, 1980; Chase and Zettler, 1982; Greber and Shaw, 1986; Anon, 1987; Rodoni and Moran, 1988). However, Chase and Zettler (1982) reported that serological assays were unreliable in detecting DsMV in asymptomatic tissue. Likewise, Anon (1987) could only detect DsMV using ELISA in *Xanthosoma* sp and mechanically infected *P. selloum* leaves that showed symptoms. Variable results have been found using an indirect ELISA test incorporating a monoclonal antiserum to the potyvirus group (Cohen, pers comm). Positive results are consistently

obtained with leaf and flower tissue showing symptoms, however, results have not been as conclusive using tissue which is not showing symptoms but is taken nearby on the same plant. DsMV appears to be intermittently distributed in infected plants. Zettler and Hartman (1986) have noted this to be so in dieffenbachia.

For production of virus-free propagation material originating from *in vitro* cultures, detection of very low concentrations of virus particles or viral RNA is required. More reliable techniques are required. Recently developed molecular biological techniques are showing promise in the detection of plant viruses and are being applied to the development of rapid, specific and sensitive tools for their detection and classification.

1.5. THE POTYVIRUSES.

The potyvirus group, named after its type member, potato virus Y (PVY), is generally recognized to be the largest and most important of the plant viruses. Thirty percent of all known plant viruses belong to this group. A wide range of plants, both monocotyledonous and dicotyledonous, are susceptible to potyvirus infection and the viruses cause significant losses in agricultural and horticultural crops.

The potyvirus group comprises four subgroups based on the vector type and a fifth subgroup containing possible members of the group (Milne, 1988). The largest subgroup is the aphid transmitted potyviruses and it is this subgroup to which DsMV belongs.

Potyviruses have slightly flexuous filamentous particles 680-900nm long and 11-15nm wide. The particles consist of *c*95% protein and *c*5% nucleic acid (Hollings and Brunt, 1981). The genomes of aphid transmitted potyviruses

are a linear, single stranded, positive-sense RNA, encapsidated in a single protein species of M_r 29-37,000.

Those aphid transmitted potyviruses that have been characterized contain a single stranded positive sense RNA genome of M_r 3.0-3.5 x 10^6 (8.8-10.25 kb), which is polyadenylated (20-160 adenosines) at its 3' terminus and with a virus protein (VPg) covalently linked to the 5' terminus. The genomes encode or have the potential to encode eight proteins, the positions of which have been mapped for some viruses (Riechmann, Lain and Garcia, 1992) (see Figure 1.7).

Potyviral RNA is translated into a polyprotein which is proteolytically processed into at least eight proteins by three virus-encoded proteinases, the N-terminal protein (P1) (Verchot, Koonin and Carrington, 1991), the helper component-proteinase (HC-Pro) (Carrington, Cary, Parks and Dougherty, 1989; Carrington and Hendon, 1992), which is also involved in aphid transmissibility, and the nuclear inclusion a protein (NIa) (Carrington and Dougherty, 1987; Chang, Hiebert and Purcifull, 1988; Hellman, Shaw and Rhoads, 1988; Garcia, Riechmann and Lain, 1989; Ghabrial, Smith, Parks and Dougherty, 1990). Other viral proteins with known function are the cytoplasmic inclusion protein (CI) which displays an RNA-dependent ATPase activity characteristic of RNA helicases (Lain, Riechmann and Garcia, 1990; Lain, Martin, Riechmann and Garcia, 1991); VPg which has been determined to constitute the N-terminal domain of NIa (Murphy, Rhoads, Hunt and Shawl, 1990; Dougherty and Parks, 1991), the nuclear inclusion b protein (NIb) thought to be the core replicase (Dougherty and Carrington, 1988) and the capsid protein (Shukla and Ward, 1989). Other putative proteins are less well characterised: the P3 protein has no known function, likewise the small proteins of 6K (Nicolas and Laliberté, 1992).

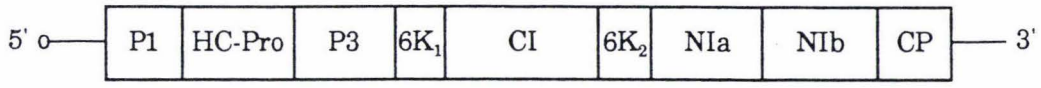

Figure 1.7 : Genome map of potyvirus. The open boxes indicate the positions of the known and putative proteins in the polyprotein. The open circle at the 5' terminus represents the genome-linked protein. (Modified from Garcia, Martin, Cervera and Riechmann, 1992). Table 1.1 outlines the size and function of each protein.

Table 1.1 : Potyvirus proteins and their function. (Modified from Shaw, Hunt, Pirone and Rhoads, 1990).

Protein	**Size (kDa)**	**Function**
P3 protein	28-34	putative movement protein, protease
Helper component (HC-Pro)	50-56	aphid transmission, protease
P3 protein	29-42	unknown
$6K_1$ protein	6	unknown
Cylindrical inclusion protein (CI)	*c*70	helicase, nucleotide binding protein, replication
$6K_2$ protein	6	unknown
Nuclear inclusion a protein (NIa)	49-52	protease, VPg
Nuclear inclusion b protein (NIb)	56-58	replication, polymerase
Coat protein (CP)	29-37	RNA packaging, aphid transmission

The non-coding region of the 3' end of the potyviral genome differs considerably in length, sequence and predicted secondary structure for different potyviruses. In related virus strains this region is similar. Frenkel, Jilka, Shukla and Ward (1992) compared several strains of distinct potyviruses with respect to their 3' terminal nucleotide sequence. Their comparisons revealed a degree of homology between strains in the range of 83-99% whereas between distinct viruses the homology was only 39-53%.

Distinct potyviruses share 40 to 70% homology and strains of the same virus more than 90% homology in their coat protein sequence (Shukla and Ward, 1988, 1989a, 1989b). A high degree of conservation is observed in the core region of the capsid nucleotide and amino acid sequences, but the N-terminal sequences of coat proteins are variable among potyviruses (Atreya, 1992). The termini of the coat proteins are present on the surface of the virion and are the major virus-specific antigenic determinants (Shukla, Strike, Tracy Gough and Ward, 1988).

A feature shared by all potyviruses is the induction of characteristic pinwheel or scroll- shaped inclusion bodies in the cytoplasm of the infected cells (Brunt 1992). These cytoplasmic inclusion (CI) bodies are formed by a virus encoded protein and their presence is the determining factor which allows a virus to be assigned to the potyvirus group. Many potyviruses also induce cytoplasmic amorphous inclusion bodies and some form nuclear inclusions (Lesemann 1988).

Although little is known about the molecular mechanisms of aphid transmissibility, transmission is known to be dependent on the presence within infected plants of a virus-coded helper component protein of M_r 50-56k and also, with some viruses at least, the composition of the capsid protein (Atreya, Raccah and Pirone, 1990).

The relationships among potyviruses is complex. Several attempts have been made to rationalize their taxonomy based on serology, amino acid composition of the whole coat protein and the N-terminal region, the amino acid composition of the cytoplasmic inclusions and nucleic acid hybridisation, with varying success.

Until complete genome sequences from a large number of potyviruses are available and their value in taxonomy determined, coat protein sequences and 3' non-coding regions are proving to be useful taxonomic indicators. Their cloning and sequencing are less complicated and time consuming and they show significant differences among definitive members.

1.6. THE POLYMERASE CHAIN REACTION.

Sensitivity of detection can be improved, using the polymerase chain reaction (PCR) (Sakai, Gelford, Stoffel, Scharf, Higuchi, Horn, Mullis and Erlich, 1988), by increasing the number of target nucleic acid molecules in the original sample. The PCR can be used to enzymatically amplify specific nucleic acid sequences *in vitro* in an exponential fashion and with high fidelity. Vunsh, Rosner and Stein (1990) suggested that the PCR method may detect plant viruses at levels 10^{-4} - 10^{-5} lower than the sensitivity limits of ELISA or direct dot-spot hybridization methods.

PCR is based on repeated cycles of denaturation, oligonucleotide primer annealing and primer extension by DNA polymerase. It involves two oligonucleotide primers that flank the DNA segment to be amplified. These primers hybridize to opposite strands of the target sequence and are orientated so that DNA synthesis by the polymerase proceeds across the region between the primers, effectively doubling the amount of that DNA segment in each cycle. Since the extension products are also complementary

to and capable of binding primers, the cycle can be repeated after a denaturation step.

By repeated cycles of denaturation, priming and extension there is a rapid exponential accumulation of the specific target fragment. Using PCR it is possible to selectively increase DNA by factors of 10^6 - 10^7 or more. After only 10 cycles the target sequence will have been amplified 1000-fold. Equipment has been developed that fully automates the procedure allowing PCR to be carried out speedily and efficiently.

The prerequisites for PCR are that the sequences flanking the DNA region of interest must be known and that primers that recognise these flanking sequences must be available. In addition, for plant pathogens with RNA genomes, such as plant viruses, satellites or viroids, it is necessary to prepare cDNA clones of the RNA genome or part of the RNA genome of interest first, as the technique only amplifies DNA.

Primers for use in PCR are usually unique oligonucleotides designed from a known genome sequence. However, where genome sequence data are not available degenerate primers designed from protein sequence data, to include base redundancies representative of all the known protein sequences, have been used (Rybicki and Hughes, 1990; Rizos, Gunn, Pares and Gillings, 1992; Rojas, Gilbertson, Russell and Maxwell, 1993).

Knowledge of conserved viral gene sequences has been useful in the construction of oligonucleotide primers for use in group-specific polymerase chain reaction amplification. Available potyvirus sequence data has made possible the development of a method for the identification of potyviruses. Langeveld, Dore, Memelink, Derks, van der Vlugt, Asjes and Bol (1991) demonstrated that primers derived from conserved regions of potyvirus coat

protein (CP) genes could be used to amplify CP genes from a wide variety of potyvirus genomes, and could be potentially of great diagnostic value.

Pappu, Brand, Pappu, Rybicki, Gough, Frenkel and Niblett (1993) developed a method for the amplification of part of the coat protein and the whole 3' non-coding region of potyvirus genomes. The method combines the group-specific PCR detection method of Langeveld et al (1991) with the strain specific approach of Frenkel et al (1992). This method has the benefit of providing information that could potentially differentiate potyviruses. The DNA fragments generated may be typed by subsequent hybridization analysis or sequencing or by restriction enzyme cleavage. Frenkel et al (1992) illustrated the use of this method for the detection of dasheen mosaic potyvirus in infected plant tissue.

PCR has a number of advantages over other detection methods, (1) it is very sensitive, (2) it is frequently not affected by plant compounds that interfere with many other detection methods, (3) virus group, virus and strain specific primers can be used and (4) once PCR is set up it is easy and rapid to perform.

1.7. AIMS OF THIS STUDY.

Dasheen mosaic potyvirus infects several members of the *Araceae* family. It has a world-wide distribution and is present wherever members of the *Araceae* are grown. A number of the members of the *Araceae* family have been cultivated for many centuries, especially those of the *Colocasia* and *Xanthosoma* genera. As DsMV appears to be associated with aroid species it is possible over the long time period that aroids have been cultivated that isolates of DsMV could have developed that have some degree of uniqueness.

A study of isolates from wide geographical locations was proposed to determine if any variations do occur. Properties such as particle length, serological relatedness, electrophoretic mobility of coat proteins, genomic characteristics and symptom expression were compared. Any variations in viral properties could make the detection of DsMV complex. The recently developed method of PCR was applied in this study with the intention of investigating its use in detecting DsMV in callas.

An ATPase activity associated with the cytoplasmic inclusion (CI) protein of plum pox potyvirus (PPV) and tamarillo mosaic potyvirus (TaMV) has been reported by Lain, Riechmann and Garcia (1990) and Eagles, Balmori-Melián, Beck, Gardner and Forster (1994) respectively. As cytoplasmic inclusion bodies are characteristic of potyviruses a similar ATPase activity should be determined for the CI protein of DsMV. Investigations into the mode of action of viral encoded proteins will help to understand the functions of plant viruses. Such understandings should ultimately lead to improved means of detection and control of plant viral pathogens.

Virus-like symptoms are frequently observed in plantings of callas in New Zealand. A limited survey of commercial plantings was undertaken to obtain an indication of the incidence of virus infection and which viruses were prevalent in callas.

CHAPTER TWO

A COMPARATIVE STUDY OF FOUR ISOLATES OF DASHEEN MOSAIC POTYVIRUS - MATERIALS AND METHODS.

2.1. PLANT MATERIAL.

Healthy and virus infected tubers of *Caladium candidum* cv candidum were supplied by Dr F.W. Zettler, University of Florida, Gainesville, Florida. The virus infected tubers were infected with the Florida isolate of dasheen mosaic potyvirus (DsMV) (Abo El-Nil, Zettler and Hiebert, 1977).

Healthy and virus infected tubers of *Xanthosoma* sp were also supplied by Dr Zettler. These tubers were of uncertain heritage, from either Florida, Costa Rica or Puerto Rico; it was not known if the virus infected tubers were infected with any known isolate of DsMV.

Tubers of taro, *Colocasia esculenta* cv 'Haapu', infected with a virus believed to be DsMV were obtained from Rarotonga by Dr R. Fullerton, Horticulture and Food Research Institute Ltd, Auckland.

Tubers of New Zealand commercially bred hybrid *Zantedeschia* spp were supplied by Mr T.E. Welsh, Department of Plant Science, Massey University, Palmerston North. Tubers were rogued from either plantings for commercial sale or from trial plots and plants originating from these tubers showed symptoms indicative of DsMV infection.

Extracts of leaf tissue originating from the tubers were observed with the electron microscope as described in section 2.3.1. to confirm their virus status.

Each virus isolate will subsequently be referred to by the following abbreviations; DsMV-FL, Florida isolate; DsMV-X virus isolated from *Xanthosoma* sp; DsMV-H virus isolated from *C. esculenta* cv 'Haapu'; DsMV-Z virus isolated from *Zantedeschia* sp 'Galaxy'.

Zantedeschia spp seedlings, used as healthy controls, were provided by Mrs K. Hill, Department of Plant Science, Massey University, Palmerston North. These were subsequently indexed using the electron microscope or enzyme-linked immunosorbent assay before use in experiments. No symptoms were observed over three years in any of these plants held as healthy controls.

All tubers were potted in a bark-based media containing supplemental fertilizers and conditioners (see Appendix 1). Tubers of *Zantedeschia* spp were maintained in a temperature controlled greenhouse unit at $15^0C \pm 5^0C$. All other tubers were maintained in a temperature controlled greenhouse unit at $20^0C \pm 5^0C$.

Herbaceous indicator plants selected for propagation of virus isolates were raised in a modified bark mix (see Appendix 1). Plants were maintained in a temperature controlled glasshouse unit at $20^0C \pm 5^0C$ until required. After inoculation they were maintained in a temperature controlled glasshouse unit at $15^0C \pm 5^0C$.

All plants were exposed to a gaseous formulation of Lannate® or Dichlorovos® at fourteen day intervals to control aphids and whitefly.

2.2. HOST RANGE STUDIES.

Symptoms of virus infection associated with each virus isolate were investigated. Virus inoculum was prepared by grinding freshly harvested selected leaf tissue with a pestle in a mortar containing a small quantity of Celite® and a ten times volume of 0.2M phosphate buffer pH 8.0 and 2% sodium sulphite (w/v). Inoculations to test plants were made by gently rubbing leaves with the pestle dipped in the virus-containing solution. The leaves were then rinsed with tap water to remove excess inoculum. Plants were tested after 3-4 weeks using ELISA (section 2.4.2.3.) or electron microscopy (section 2.3.1.) to determine the presence of virus infection. If no infection could be detected the inoculation procedure was repeated.

Healthy plants of *Zantedeschia* sp 'Pink Persuasion' were inoculated separately with one of each of DsMV-FL, DsMV-H, DsMV-X and DsMV-Z. Healthy plants of *Caladium candidum* cv. candidum were inoculated separately with one of each of DsMV-H, DsMV-X and DsMV-Z. Healthy plants of *Xanthosoma* sp. were inoculated separately with one of each of the isolates DsMV-FL, DsMV-H and DsMV-Z. No healthy plants of *Colocasia esculenta* were available, therefore study of symptoms of virus infection by differing isolates of DsMV in this host was not possible.

Each isolate was inoculated as described above to a range of herbaceous indicators as listed in Table 5.1. (Chapter 5, section 5.6.). Plants were observed every second day to record the presence or absence of symptoms. To detect possible latent infections leaf samples of each test plant were prepared for electron microsocopy as described in section 2.3.1. or macerated in 0.2M potassium phosphate buffer, pH 8.0 and 2% sodium sulphite (w/v) and inoculated to *Chenopodium quinoa.*

2.3. ELECTRON MICROSCOPY.

2.3.1. Direct investigation of leaf tissue.

A small amount of leaf tissue (< 1 cm^2) was macerated on a spotting tile in two drops of negative stain. One drop of the resultant extract was placed onto a 200 mesh formvar-carbon-coated copper grid. The drop was left on the grid for one minute before the excess stain was removed by touching the edge of the grid to filter paper. Grids were examined with a Philips 201C electron microscope.

Initially, the negative stain potassium phosphotungstic acid (PTA) pH 7.0 was routinely used. However, difficulty in obtaining even stain coverage of extracts on electron microscope grids was found with aroid species. Similar problems were reported by Greber and Shaw (1986), who proposed that this was due to the characteristics of aroid sap. A wetting agent, bacitracin, was added to the PTA to improve its spreading characteristics. One drop of bacitracin solution (300ug/ml) was added to 7 drops of PTA. Particles were readily observed in extracts from *Zantedeschia* spp, *Xanthosoma* sp and *Colocasia esculenta*. Results were poor for extracts obtained from *Caladium candidum*, and no viral particles could be observed (see Figure 2.1). Consequently a range of negative stains were evaluated.

Negative stains used were ammonium molybdate (AM) pH 5.6, PTA pH 7.0 and uranyl acetate (UA) pH 4.0. These were prepared as 2% solutions and the pH adjusted with either 2M potassium hydroxide (for PTA and UA) or 1M ammonium hydroxide (for AM).

Leaf extracts were prepared using PTA combined with either UA or AM. A mixture of equal volumes of PTA and UA did not improve the quality of

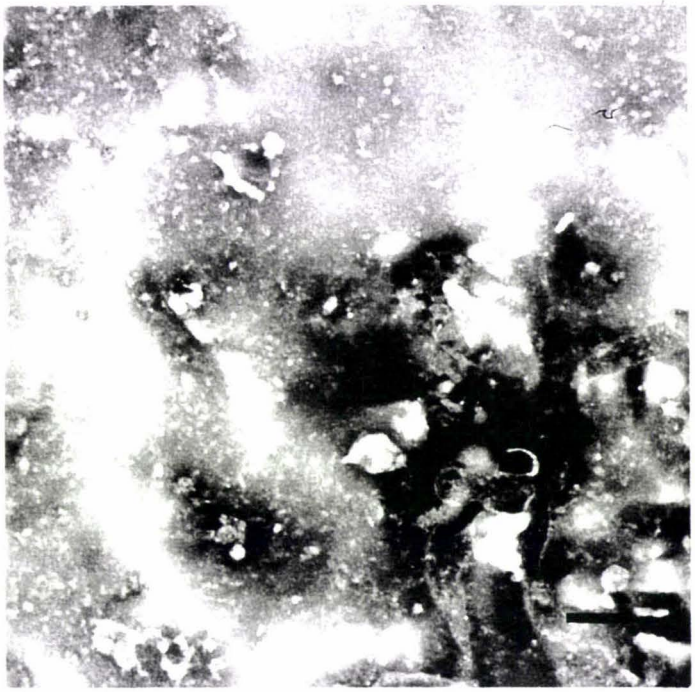

Figure 2.1: Leaf dip of DsMV infected *Caladium candidum* tissue in 2% potassium phosphotungstic acid (PTA) pH 7.0: bacitracin (300ug/ml) (7:1). Bar = 100nm.

Caladium candidum extracts and again no particles could be observed (see Figure 2.2).

Ammonium molybdate is reported to have good spreading properties, (Bennett, 1975). Mixtures with PTA at three ratios (AM:PTA at 2:1, 1:1, 1:2) were tried. Virus particles could be observed with each mixture used. The best spreading and contrast was obtained with the ratio of AM:PTA at 1:1 or 1:2 (see Figures 2.3 - 2.5). The ratio of 1:1 was subsequently used.

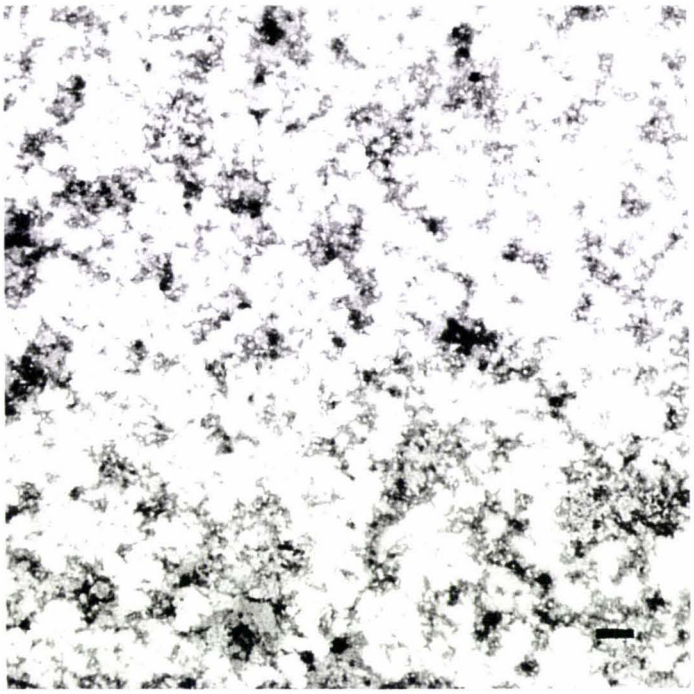

Figure 2.2: Leaf dip of DsMV infected *Caladium candidum* tissue in 2% potassium phosphotungstic acid (PTA) pH 7.0 : 2% uranyl acetate (UA) pH 4.0 (1:1).
Bar = 100nm.

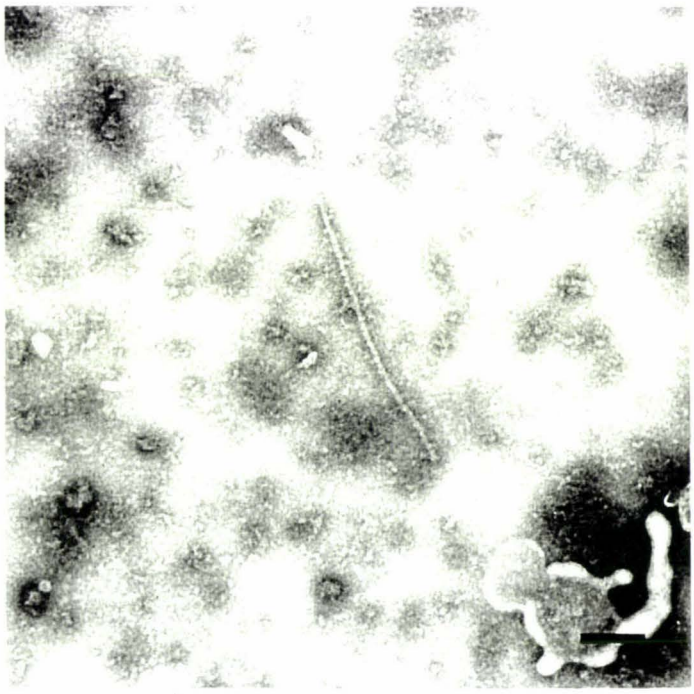

Figure 2.3 : Leaf dip of DsMV infected *Caladium candidum* tissue in 2% potassium phosphotungstic acid (PTA) pH 7.0 : 2% ammonium molybdate (AM) pH 5.6 (2:1).
Bar = 100nm.

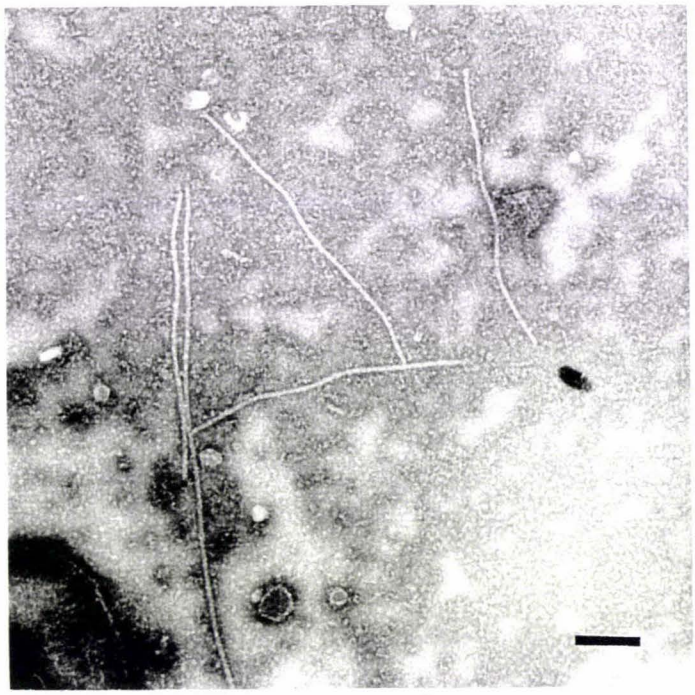

Figure 2.4 : Leaf dip of DsMV infected *Caladium candidum* tissue in 2% potassium phosphotungstic acid (PTA) pH 7.0 : 2% ammonium molybdate (AM) pH 5.6 (1:1).
Bar = 100nm.

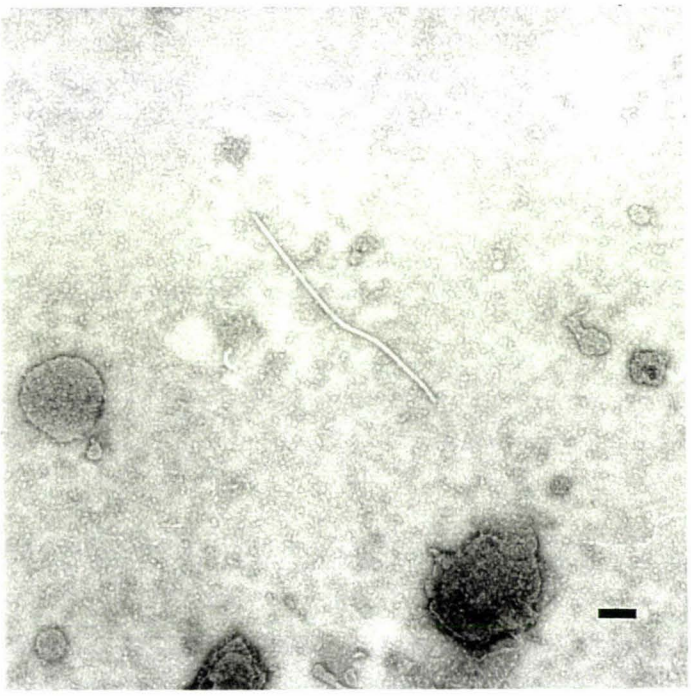

Figure 2.5 : Leaf dip of DsMV infected *Caladium candidum* tissue in 2% potassium phosphotungstic acid (PTA) pH 7.0 : 2% ammonium molybdate (AM) pH 5.6 (1:2).
Bar = 100nm.

2.3.2. Investigation of purified viral preparations.

Viral preparations were routinely dialysed to remove unwanted materials such as sucrose, caesium sulphate or EDTA using the method described by Roberts (1986). A drop of the purified virus preparation was placed on a formvar-carbon-coated copper grid. The grid was then placed, specimen side up, on a filter paper wick. One end of the filter paper wick was placed in deionized water and the other end onto three layers of filter paper. The contaminants (e.g. sucrose, EDTA) were eluted for 20 minutes, during which time they were removed from the grid surface by capillary washing leaving the virus particles attached to the support film.

After elution the grid was removed and a drop of negative stain was applied. The drop was left on the grid for one minute before the excess stain was removed by touching the edge of the grid to filter paper. The grids were examined as before.

2.3.3. Determination of viral particle length.

Low virus concentration in some leaf extracts meant that a crude sap preparation applied directly to grids was not suitable. It would have meant a large number of photographs would have to be taken in order to obtain micrographs of the required number of particles for measurement. Consequently, an immunosorbent electron microscope technique was used (Milne, 1986).

A drop of homologous antiserum diluted 1:1000 in 0.02M sodium phosphate buffer pH 6.5 was applied to a formvar-carbon-coated copper grid. The drop was left on the grid for 10 minutes before the excess was removed by touching the edge of the grid to filter paper. A drop of leaf sap diluted in 0.02M sodium phosphate buffer pH 6.5 was then applied. The drop was left on the

grid for 20 minutes before the excess was removed by gently washing the grid with distilled water for approximately 20 seconds. A drop of negative stain was subsequently applied to the grid and left for one minute before the excess was removed by touching the edge of the grid to filter paper. The grids were examined with a Philips 201C electron microscope.

The length of the virus particles were measured from electron micrographs. The magnification was calculated using the orchid strain of tobacco mosaic tobamovirus (TMV-O) as a standard, assuming a normal length of 300nm for TMV-O.

2.4. SEROLOGY

2.4.1. Antiserum production.

Antiserum to the DsMV-H, DsMV-X and DsMV-Z isolates of dasheen mosaic potyvirus were produced separately in New Zealand white rabbits. Each rabbit was given three intramuscular injections at two weekly intervals followed by an intravenous injection 7 - 10 days later. The intramuscular injections comprised 0.5ml freshly purified viral preparation (0.7 mg/ml) emulsified with 0.5ml of Freund's incomplete adjuvant. The intravenous injection comprised 0.7ml freshly purified viral preparation (0.5 mg/ml). Each rabbit was bled 10 - 14 days later, collecting approximately 40ml of blood by cardiac puncture.

The collected blood was allowed to clot at room temperature for two hours. A glass rod was run around the top of the collecting tube to free the clot from the sides. The tube was then left to stand overnight at 4^0C to allow the clot to contract before the serum portion was decanted off. The tube was centrifuged at 12,000g for 15 minutes, the serum decanted off and combined with that previously collected then centrifuged at 27,000g for 20 minutes.

The resultant antiserum was stored as 2 ml aliquots either 1:1 in glycerol at -20°C or at 4°C with the addition of 0.05% sodium azide (w/v) to aid preservation.

Antiserum to the Florida isolate of DsMV was supplied by Dr F.W. Zettler.

2.4.2. Enzyme-linked immunosorbent assay (ELISA).

2.4.2.1. Antiserum purification for ELISA tests.

The gamma-globulin fraction of each antiserum was prepared by ammonium sulphate precipitation, and purified by ion-exchange chromatography in a DE 52 cellulose column (Whatman Ltd, Catalogue No. 4057-050). One millilitre of antiserum was made up to 10ml with distilled water and 10ml of saturated ammonium sulphate (76.9g $(NH_4)_2SO_4$ per 100ml H_20) was added. The solution was allowed to sit at room temperature for 30-60 minutes, the precipitate was collected after centrifugation at 6000g for 10 minutes. The pellet was dissolved in 2 ml of half-strength phosphate buffered saline (PBS: 8g NaCl, 0.2g KH_2PO_4, 1.14g Na_2HPO_4, 0.2g KCl made up to 1l with H_20, final pH 7.4) then dialysed three times at 4°C against 500 ml of half-strength PBS, the third time overnight.

For each antiserum to be purified a column of cellulose was prepared using 2 g mixed into 12 ml of half-strength PBS buffer. The pH was adjusted to 7.4 with KH_2PO_4 (60g/l). The cellulose was allowed to settle and the supernatant was poured off. Further half-strength PBS buffer was added and the pH readjusted to pH 7.4 with KH_2PO_4. This procedure was repeated two or three times, before the cellulose slurry was left sitting at 4°C overnight. The pH was then checked and readjusted to 7.4 if necessary. This pH is below the isoelectric point for most immunoglobulins, so they become positively charged. Other serum proteins carry a negative charge at this pH and become bound

to the positively charged diethylaminoethyl (DEAE) groups of the column matrix (van Regenmortel, 1982). The positively charged immunoglobulins remain in suspension enabling them to be eluted from the column.

Each column was set up using a 10ml pipette in which glass wool was placed at the narrow end to support the cellulose. The cellulose slurry was poured into the pipette and allowed to settle, giving a column of approximately 10cm in length. Half-strength PBS was washed through the column once or twice. The meniscus of the buffer was lowered until it was just above the slurry. The dialysed gamma-globulin was added taking care that the slurry was not disturbed. The liquid from each column was collected in ten, 1ml aliquots. When the meniscus of the gamma-globulin was just above the slurry approximately 0.5ml of half-strength PBS was added again, taking care not to disturb the slurry. During collection of the aliquots further half-strength PBS was added to prevent the slurry from drying out.

The UV absorbance of each 1ml aliquot was determined spectrophotometrically at 280 nm. The concentration of gamma-globulins was calculated on the basis of $E^{0.1\%}_{1cm,280nm} = 1.4$ (where 0.1% = 1mg/ml gamma-globulin, 1cm = optical path length of radiation). The tubes with the highest readings were pooled for each antiserum. The concentration of purified gamma-globulins was adjusted to 1 mg/ml of PBS buffer. If the concentration of the purified gamma-globulin was less than 1 mg/ml (i.e. the absorbance was less than 1.4 at 280 nm), the resultant concentration was noted and the gamma-globulins were stored undiluted. The purified gamma-globulins were stored in 2 ml aliquots in silicone-coated tubes at - 20^{0}C.

2.4.2.2. Enzyme conjugation of purified gamma-globulins.

Calf intestinal alkaline phosphatase (Sigma, Type VII-T) was conjugated to the respective gamma-globulins using the one-step glutaraldehyde method

(Avrameas, 1969). Alkaline phosphatase was added to purified gamma-globulins at the rate of 2.5 mg/ml before adding glutaraldehyde (EM grade) to a final concentration of 0.06% (v/v). The preparation was left at room temperature for 4 hours, then dialysed against 500ml half-strength PBS three times at 4^0C, the third time overnight, to remove excess glutaraldehyde. To aid in stabilizing the conjugate, bovine serum albumin (RIA grade) was added at a concentration of 5 mg/ml. The conjugated gamma-globulins were stored at 4^0C in silicone-coated tubes. Because of the volume changes and possible gamma-globulin losses during the conjugation procedure all references to the use of the conjugates are in terms of dilutions of the conjugate rather than as absolute concentrations of gamma-globulins.

2.4.2.3. ELISA Methods.

The ELISA tests were based on the double antibody sandwich method of Clark and Adams (1977) as follows :

1. 200ul aliquots of the appropriate dilution (section 2.4.2.4.) of the purified gamma-globulins, diluted in coating buffer (1.59g Na_2CO_3, 2.93g $NaHCO_3$, made up to 1l with H_20, final pH 9.6), were applied to the required number of wells of the microtitre plates (Nunc-Immuno Plate Maxisorp™ Catalogue No. 442404).

2. The plates were incubated at 37^0C for 3 hours in an airtight container lined with a wet paper towel.

3. The plates were washed by flooding with PBS-tween (PBS + 0.05% tween 20 (v/v)) and left for 3-5 minutes before emptying. This was repeated twice before the plates were left to drain on a paper towel.

4. Samples were prepared in extraction buffer (1.3g Na_2SO_3, 20.0g polyvinyl pyrrolidone (PVP) MW 44,000, 2.0g ovalbumin, 20.0g tween 20 made up to 1l with PBS-tween, final pH 7.4) and 200ul aliquots were applied to the appropriate wells of the microtitre plates.

5. The plates were stored at 4^0C overnight in an airtight container lined with a wet paper towel.

6. The plates were washed as described in 3.

7. 200ul aliquots of the appropriate dilution (section 2.4.2.4.) of enzyme-conjugated gamma-globulins diluted in reagent buffer (PBS-tween + 2% PVP, (MW 44,000) (w/v) + 0.2% bovine serum albumin (w/v)) were added to the appropriate wells of the microtitre plates.

8. The plates were placed in an airtight container lined with a wet paper towel and incubated at 37^0C for 3 hours.

9. The plates were washed as described in 3.

10. 200ul aliquots of the enzyme substrate (0.67mg/ml ρ-nitrophenyl phosphate (Sigma 104®) in 10% diethanolamine (v/v), pH 9.8) were added to each well. The plates were left at room temperature for 1-3 hours and then the absorbance of each well was determined at 410 nm using a Dynatech MR5000 ELISA reader.

To test for non-specific hydrolysis of the substrate, additional treatments were included which omitted the addition of test or control samples but included the addition of the appropriate sample extraction buffer.

2.4.2.4. Optimisation of levels of ELISA reactants.

The optimum levels of coating and conjugated gamma-globulins were determined for each antiserum by a multifactorial layout on microtitre plates. Three concentrations of coating gamma-globulins (2,5,10 ug/ml), with four concentrations of enzyme conjugated gamma-globulins (1:200, 1:400, 1:800, 1:1600), were used in combination. Test samples comprised infected sap and healthy sap diluted 1:20 with the extraction buffer (section 2.4.2.4.). As healthy taro plants were not available, healthy xanthosoma sap was used as a control when optimising antiserum to DsMV-H. ELISA procedures were carried out as described in section 2.4.2.4. The combinations that gave the highest ELISA index (ratio of A_{410} of test and control samples) were selected as the optimal level of reactants and are outlined in Table 2.1.

2.4.2.5. Homologous and heterologous reactions of each isolate to individual antisera.

The ELISA procedure was carried out using separate plates coated with the predetermined optimum level of one of each of the four antisera to DsMV. Samples were prepared from infected and healthy leaf tissue. Leaf tissue from *Zantedeschia* sp seedlings was used as healthy controls. Leaf tissue originating from tubers infected with the various isolates of DsMV was used as test samples. To ensure the results for each antiserum are comparable, the same tissue extracts were reacted against each antiserum. All samples were diluted in two-fold steps commencing with a dilution of 1:5. Duplicate aliquots of each dilution of healthy and test samples were added to the appropriate wells. The homologous conjugate was used as the final serum. The absorbance of duplicate healthy and test wells were averaged and graphs for each antiserum were plotted.

Table 2.1 : Concentration of coating gamma-globulins and dilutions of conjugated gamma-globulins of dasheen mosaic potyvirus (DsMV) antisera used in ELISA studies.

Antiserum	Concentration of coating gamma-globulins	Dilution of conjugated gamma-globulins
DsMV ex *Caladium candidum* cv candidum (DsMV-FL)	5 ug/ml	1:200
DsMV ex *Colocasia esculenta* cv 'Haapu' (DsMV-H)	10 ug/ml	1:400
DsMV ex *Xanthosoma* sp (DsMV-X)	2 ug/ml	1:400
DsMV ex *Zantedeschia* sp 'Galaxy' (DsMV-Z)	2 ug/ml	1:800

2.5. ELECTROPHORESIS.

2.5.1. SDS-polyacrylamide gel-electrophoresis (SDS-PAGE).

A discontinuous gel system was employed based on the method of Laemmli (1970). The gel was cast between the glass plates of a vertical gel slab apparatus (Bio-Rad Mini-PROTEAN™ II Dual Slab Cell, Catalogue No. 165-2940). The resolving mix of 12% acrylamide (11.68% acrylamide (w/v), 0.32% N',N' methylene-bisacrylamide (w/v) in 0.375M Tris-HCl pH 8.8) with the addition of 0.1% TEMED (N,N,N',N'- tetramethylethylenediamine) (v/v) and 0.1% freshly prepared ammonium persulphate (w/v) was made and rapidly pipetted into the gel former leaving about 1.5cm at the top for the stacking gel. The solution was overlaid with about 1ml of deionized water to ensure a flat surface and prevent inhibition of polymerisation by oxygen. The gel was left for 30 - 40 minutes to polymerise. The water was then poured off

prior to the 4% stacking gel (3.89% acrylamide (w/v), 0.11% N',N' methylene-bisacrylamide (w/v) in 0.125M Tris-HCl pH 6.8, with the addition of 0.1% TEMED (v/v) and 0.1% ammonium persulphate (w/v)) being poured on top. A teflon gel comb was inserted taking care not to trap any bubbles. After setting of the gel, the comb was removed and running buffer (3.0g Tris, 14.5g glycine, 1.0g SDS made up to 1l with H_20, final pH 8.3) was added to the top of the gel. The gel was left for 30 - 45 minutes to polymerize before being placed in the electrophoresis tank and covered with running buffer prior to the samples being loaded. The gel was run at 200V for 45 minutes until the tracking dye migrated to 5mm from the bottom edge of the gel.

The gel was removed from the glass casting plates and placed in a solution of 0.1% Coomassie Brilliant Blue R-250 (w/v) in 40% methanol:10% acetic acid (v/v) for approximately 60 minutes. The gel was destained in 40% methanol:10% acetic acid (v/v) which was changed approximately every 30 minutes until the bands were clearly differentiated and the background a pale blue.

2.5.2 Preparation of samples for SDS-PAGE.

Samples consisted of purified virus preparations. Equal volumes of sample and loading buffer (62.5mM Tris pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.1% bromophenol blue) were heated at 95^{0}C for five minutes prior to loading. Size comparisons were made with Bio-Rad broad range SDS-PAGE molecular weight standards (Catalogue No. 161-0317).

2.5.3. Non-denaturing electrophoresis of nucleic acids.

Purity and size of nucleic acids were determined by electrophoresis in an agarose gel. One percent agarose (w/v) (Sigma Type 1-A:low EEO) prepared in TBE buffer (10.8g Tris, 5.5g boric acid, 0.93g EDTA, made up to 1l with

H_20, final pH 8.2) was placed in a boiling water bath until dissolved and then cooled briefly before being poured into a horizontal gel casting slab. A comb was placed at one end of the slab, and removed after 15 minutes when the agarose was set. The gel was covered with 150ml TBE and left to cure for a further 45 minutes.

DNA samples were diluted 1:10 with tracking dye (0.1% bromophenol blue (w/v), 10% glycerol (v/v) in TBE buffer) prior to loading. Size comparisons were made with Pharmacia 100 Base Pair Ladder (Code No. 27-4001-01).

RNA samples were diluted 1:10 with tracking dye (0.25% bromophenol blue (w/v), 25% Ficoll (Sigma F-2637) (w/v) in TBE buffer) prior to loading. Size comparisons were made with a 0.24 - 9.5 kb RNA Ladder (Gibco BRL Catalogue No. 5620SA).

Gels were run at 80-100V for 1 hour until the tracking dye had migrated three-quarters of the way through the gel. The gel was then removed from the electrophoresis tank and stained by soaking in 0.05% ethidium bromide (w/v) solution for 10-20 minutes. The gel was destained by soaking in deionized water for 10 minutes prior to viewing with UV light. Gels were photographed with a Hoeffer DS 34 Polaroid direct screen instant camera.

2.6. PURIFICATION OF VIRUSES.

2.6.1 Purification of Dasheen mosaic potyvirus.

In preliminary studies, purification of DsMV was by the method of Abo El-Nil et al (1977). One hundred grams of leaf tissue was homogenized with a prechilled Waring Blendor in a chilled mixture of 200 ml 0.1M sodium citrate pH 7.2 containing 0.6g sodium sulphite and 0.01M EDTA. Once the leaf tissue was fully homogenized 45 ml of chloroform and 45 ml carbon

tetrachloride were added and the tissue further homogenized for one minute.

The homogenate was then centrifuged at 13,000g for 10 minutes in a Sorvall® RC5 centrifuge using a GSA rotor. The aqueous phase was filtered through glass wool, 8% polyethylene glycol (PEG) 6000 (w/v) was added, and the solution stirred for 4 hours at 4°C. The PEG precipitate was pelleted by centrifugation at 13,000g for 10 minutes. The pellet was resuspended by stirring overnight at 4°C in TE buffer (20mM Tris, 10mM EDTA, final pH 7.2) and 0.1% 2-mercaptoethanol (v/v).

The suspension was then centrifuged at 18,800g for 10 minutes in a Sorvall® RC5 centrifuge using a SS34 rotor. Five hundred microlitres of the clarified virus suspension was layered upon 7ml 30% CsCl (w/v) gradient in 20mM Tris pH 7.2 (mean density = 1.28 g/cm^3) in a 17ml tube. The gradient was overlaid with paraffin before centrifugation at 115,900g for 17 hours in a Sorvall® OTD-Combi ultracentrifuge using an AH629 rotor.

After centrifugation no bands were obvious. Approximately 1ml was removed by tube piercing 1.5cm below the paraffin. This was diluted with TE buffer and centrifuged at 160,800g for 90 minutes in a Sorvall® OTD-Combi T1270 rotor. The resultant pellet was resuspended in TE buffer overnight at 4°C.

Samples from the resuspended pellet were observed in the electron microscope. Grids were prepared as described in section 2.3.2. In some preparations many intact particles were observed, whilst at other times few if any particles were observed. The absence of discernible bands in the CsCl gradient after centrifugation made the position for tube piercing difficult to determine. This may have led to the inconsistent results. Consequently, the following alternative purification methods were investigated:

a) Method 1: The method of Abo El-Nil et al (1977) was essentially followed, however, 8ml of clarified virus suspension was mixed with 8ml 60% CsCl (resultant mean density = 1.28gcm^{-3}) and centrifuged at 115,900g for 17 hours using a AH629 rotor. After centrifugation two distinct bands were observed 1cm and 2cm from the bottom of the tube. The lower band was approximately twice as wide as the upper. The bands were removed by tube piercing, diluted in TE buffer and centrifuged separately at 160,800g in a T1270 rotor. Resultant pellets were resuspended in TE buffer overnight at 4^{0}C. Samples from the resuspended pellets were prepared and observed in the electron microscope as described in section 2.3.2. Samples resulting from the upper band contained only a few particles. Samples from the lower band contained a large number of particles, many of which were fragmented.

b) Method 2: The initial procedures of the method of Abo El-Nil (1977) were followed. After PEG 6000 precipitation, the pellet was resuspended in approximately 50ml TE buffer by stirring overnight at 4^{0}C. The suspension was centrifuged at 13,000g for 10 minutes in a GSA rotor. The clarified suspension was ultracentrifuged at 162,600g for 90 minutes on a 5ml 25% sucrose cushion in a Sorvall® OTD-Combi T865 rotor. The resultant pellet was resuspended in 1-2 ml TE buffer and then overlaid onto 10% - 40% sucrose gradients, formed in TE buffer by freezing the buffer containing 25% sucrose (w/v) and then thawing overnight.

Gradients were fractionated using a ISCO model 640 density gradient fractionator and a model UA-5 UV absorbance monitor. The resultant curve, upon fractionation, of absorbance at 254 nm was relatively long and flat (see Figure 2.6). The fractions containing virus particles were collected from a broad area of the response curve, pooled and diluted in TE buffer prior to centrifugation at 162,600g for 90 minutes in a T865 rotor. The resultant pellets were resuspended in TE buffer overnight at 4^{0}C.

Samples from the resuspended pellets were observed in the electron microscope as described in section 2.3.2. Few particles were observed, whereas electron microscopic investigation showed there were many particles in the viral suspension overlaid on the sucrose gradient prior to centrifugation. Many of these particles were laterally aggregated. It is possible they were carried to the bottom of the gradient where they remained uncollected.

c) Method 3: Method 2 was essentially followed but the sucrose gradients were formed in 0.005M borate and 0.2% saline sodium citrate (SSC) (w/v) pH 8.0 (Leiser and Richter, 1978). A single distinct peak occurred when fractionating the gradients (see Figure 2.7). This peak was collected and centrifuged as described for method 2. Examination of the resultant preparation in the electron microscope revealed many virus particles.

d) Method 4: The procedure of Reddick and Barnett (1983) was also investigated. One hundred grams of leaf tissue was homogenized with a prechilled Waring Blendor in 200ul of chilled buffer made up of 0.5M sodium/potassium phosphate buffer, pH 7.0, containing 1M urea (PU) to which was added 0.01M sodium diethyldithiocarbamate (DIECA) and 0.5% thioglycollic acid (TGA) (v/v). Once the leaf tissue was fully homogenized 0.8 volumes chloroform (w/v) was added and the tissue was further homogenized for one minute. The homogenate was then centrifuged a 10,400g for 15 minutes in a GSA rotor. The aqueous phase was filtered through glass wool and then 4% PEG 6000 (w/v) and 0.25M NaCl were added and the solution stirred for two hours at 4^0C. The PEG precipitate was pelleted by centrifugation at 10,400g for 15 minutes. The pellet was resuspended in 50ml PU buffer containing 1% Triton X-100 (v/v) by stirring at 4^0C overnight. Four percent PEG 6000 (w/v) and 0.25M NaCl were added and the solution stirred for two hours at 4^0C. The PEG precipitation was pelleted by centrifugation at 10,800g for 15 minutes in a SS34 rotor. After resuspension in 6 ml PU

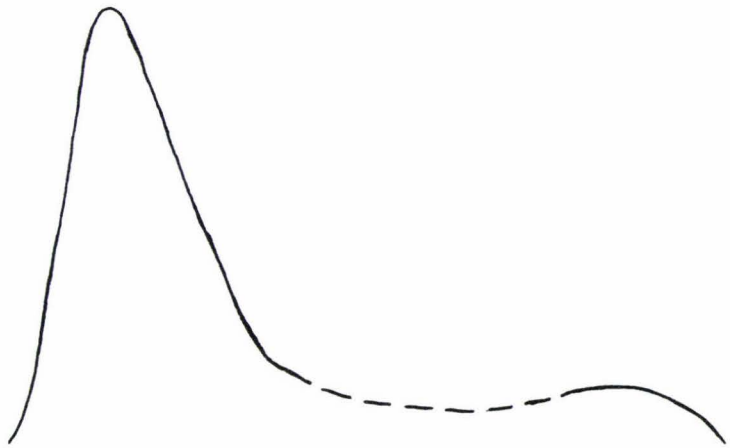

Figure 2.6 : Optical density profile of sucrose density gradients made up in 20mM Tris and 10mM EDTA of a preparation of DsMV purified by a modification of Abo El-Nil et al (1977). Sedimentation is left to right. Relative absorbance at 254nm. The dotted line indicates area where fractions were collected.

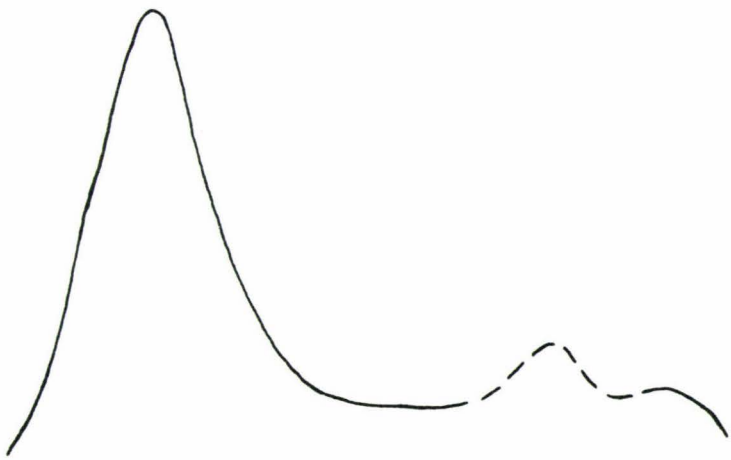

Figure 2.7: Optical density profile of sucrose density gradients made up in 0.005M borate and 0.2% SSC of a preparation of DsMV purified by a modification of Abo El-Nil et al (1977). Sedimentation is left to right. Relative absorbance at 254nm. The dotted line indicates area where fractions were collected.

buffer overnight at 4°C, 1.5g Cs_2SO_4 was added and the volume was made up to 7.5 ml with PU buffer. The solution was shaken at 4°C for 30 minutes to dissolve the Cs_2SO_4. The suspension (3.75 ml per 17ml AH629 rotor tube) was layered onto a cushion of 0.8ml 53% Cs_2SO_4 (w/v) in 20mM Tris pH 7.4. The tubes were then filled with PU buffer prior to centrifugation at 115,900g for 16 hours using a AH629 rotor.

No bands were apparent. The tubes were pierced in two places in the area where a band should have occurred. The collected solution was diluted in TE buffer and centrifuged as described above. Samples from the resultant resuspended pellet were observed in the electron microscope as described in section 2.3.2. Very few particles were present and most of these were fragmented.

e) Method 5: The procedure of Reddick and Barnett (1983) incorporating caesium sulphate and sucrose gradients as described by Shukla, Jilka, Tosic and Ford (1989) was examined. No distinct bands were apparent after the gradients were centrifuged. Electron microscopic investigation showed only a small number of particles were present in the resultant viral preparation and many of these were fragmented.

f) Method 6: Method 4 incorporating 10%-40% sucrose gradients (w/v) made in PU buffer, and prepared as described for method 2, was investigated. Upon fractionation a broad flat peak was observed. Electron microscopy showed many laterally aggregated particles in the virus suspension overlaid onto the gradient (see Figure 2.8). Although urea is reported to reduce lateral aggregation of viral particles (Damirdagh and Shepherd, 1970) it appeared to have little effect when used here. Aggregation of the virus particles may have caused them to migrate to the bottom of the tube where they remain uncollected.

Figure 2.8 : Partially purified preparation of DsMV, after resuspension of a polyethylene glycol 6000 (PEG) precipitate, showing lateral aggregation stained with 2% potassium phosphotungstic acid (PTA) pH 7.0 : 2% ammonium molybdate (AM) pH 5.6 (1:1).
Bar = 100nm.

g) Method 7: Method 4 incorporating 10%-40% sucrose gradients prepared in 0.005M borate and 0.2% SSC (w/v) pH 8.0 was explored. A broad peak was observed upon fractionation of the gradients. Many particles were observed in the pelleted fractions collected from the gradient after centrifugation. A large number were aggregated either laterally and/or end-to-end.

The results of the purification methods are summarised in Table 2.2. Many particles were observed in samples from the resuspended final pellet of methods 1, 3 and 7. Method 1 resulted in a large number of fragmented particles and was consequently discarded. Spectrophotometric examination

Table 2.2 : Comparison of various methods used to purify dasheen mosaic potyvirus (DsMV).

Method	Gradient Bands	Resultant No. of Particles	Particles showing a high degree of Fragmentation
Abo El-Nil et al, (1977)	none	inconsistent	no
Method 1	two	many	yes
Method 2	none	few	no
Method 3	one	many	no
Method 4	none	few	yes
Method 5	none	few	yes
Method 6	none	few	no
Method 7	one	many	no

of viral preparations purified using the method of Reddick and Barnett (1983) incorporating sucrose gradients made up in 0.005M borate and 0.2% SSC (method 7) showed virus yield to be 0.5-2mg/100g plant tissue (assuming an extinction coefficient of 2.38 at 260nm ($E^{0.1\%}_{1cm,260nm}$), Abo El-Nil et al (1977)) and the $A_{260/280nm}$ to be 1.07-1.80. In comparison, a virus yield of 2-5mg/100g plant tissue and an $A_{260/280nm}$ of 1.10-1.20 was obtained using the method of Abo El-Nil et al (1977) incorporating the same type of sucrose gradient (method 3). Consequently method 3 was subsequently employed and was found to be suitable for each isolate being studied.

In all methods trialed many particles were observed in suspensions overlaid onto gradients. Frequently the number of virus particles observed after gradient centrifugation was greatly reduced. Consequently, viral preparations were only taken through a gradient step when this was considered absolutely necessary (RNA extraction, antiserum production).

2.6.2. Purification of cucumber mosaic cucumovirus.

A modification of Lot (1972) was used for cucumber mosaic cucumovirus (CMV) purification. One hundred grams of systemically infected *Nicotiana tabacum* 'Havana' leaves were homogenized with a prechilled Waring Blendor in a chilled mixture of 200ml 0.5M sodium citrate pH 6.5 and 0.1% thioglycollic acid (v/v). Once the leaf tissue was fully homogenized 30 ml of chloroform was added and the tissue was further homogenized for one minute. The homogenate was then centrifuged at 6000g for 10 minutes in a GSA rotor. The supernatant was removed and the remaining leaf fibre was further extracted in 100 ml 0.5M sodium citrate pH 6.5 and 0.1% thioglycollic acid. The homogenized leaf fibre was centrifuged as before. The supernatants were combined and filtered through glass wool. Ten percent PEG 6000 (w/v) was added and the solution stirred for 15 minutes and then stood for 30-40 minutes at 4°C. The PEG precipitate was pelleted by centrifugation at 7000g for 20 minutes in a GSA rotor. The pellet was resuspended in 0.05M sodium citrate pH 7.0 and 2% Triton X-100 (v/v) overnight at 4°C.

The suspension was centrifuged at 14,500g for 20 minutes in a SS34 rotor and then the clarified suspension was centrifuged at 124,500g for 2.5 hours through a 5ml 25% sucrose (w/v) cushion in a T865 rotor. The pellet was resuspended in 5mM EDTA overnight at 4°C and the suspension was overlaid onto a 10%-40% sucrose (w/v) gradients prepared in 5mM EDTA as described in section 2.6.1. Gradients were centrifuged at 115,900g for 2 hours in an AH629 rotor then fractionated as described in 2.5.1. A very distinct peak was observed during fractionation. The fractions containing virus particles were pooled and diluted in 5mM EDTA buffer prior to centrifugation at 162,600g for 90 minutes in a T865 rotor. The resultant pellets were resuspended in TE buffer overnight at 4°C.

2.7. RNA EXTRACTION.

Due to the extreme sensitivity of single-stranded ribonucleic acids (ssRNA) to ribonuclease (RNase) digestion, special precautions were taken to avoid the introduction of this enzyme into RNA preparations.

All glassware and spatulas were baked at 180^{0}C for a minimum of 8 hours (usually overnight). All plasticware was soaked in 0.1% diethyl pyrocarbonate (DEPC) (v/v) overnight at room temperature prior to autoclaving.

Electrophoresis tanks were cleaned with detergent, rinsed with water, dried with ethanol, and then filled with a 3% solution of hydrogen peroxide (v/v) for ten minutes before rinsing with autoclaved DEPC treated water.

All solutions were made in Barnstead® nanopure water which had been treated with 0.1% DEPC and then autoclaved. All chemicals used were reserved for RNA extraction procedures only.

Gloves were worn during all manipulations to prevent the introduction of ribonucleases from the hands.

Care in the isolation and purification of the virus before attempting to isolate the viral nucleic acid also helps to minimize nuclease contamination and subsequent degradation of viral nucleic acids (Ralph and Berquist,1967). Consequently, all viral preparations were taken through a sucrose gradient step to reduce possible contaminants from the plant material.

The procedure used to extract the viral nucleic acid from the viral preparation involved a proteinase K digestion followed by phenol extraction. Proteinase K is a broad-specificity serine protease which is widely used in the isolation

of purified nucleic acids (Tullis and Rubin, 1980). A preliminary incubation of the viral preparation with proteinase K in the presence of 1% sodium dodecyl sulphate (SDS, w/v) was undertaken. This led to the denaturation of the viral coat protein, aided by the SDS, to release the viral nucleic acid, as well as the destruction of nucleases.

Once released from the protective viral protein coat, the viral nucleic acid is extremely susceptible to enzymatic degradation. Consequently, it was important to minimize enzyme activity. This was achieved by avoiding the conditions of pH, ionic strength, metal ions etc, under which the enzymes are most active, and by working at low temperatures (0^0C-4^0C).

After the preliminary incubation with the proteinase K and SDS, water-saturated phenol was added. The phenol, a protein denaturant which causes proteins to precipitate, removes the proteinase K and protein digestion products. Viral nucleic acid was recovered in the upper aqueous phase after centrifugation. Addition of ethanol to the aqueous phase precipitated the RNA which was subsequently recovered by centrifugation.

The following procedure was used to extract RNA from viral preparations :

One volume of 20mM Tris and 10mM EDTA pH 8.0 was added to one volume of 2% SDS. To this was added 5 mg/ml proteinase K (Sigma P-2308). This mixture was then incubated at 37^0C for 30 minutes.

Ten microlitres of 5M NaCl and 190ul 2% SDS (w/v) were added to 200ul of virus preparation resuspended in 20mM Tris and 10mM EDTA pH 8.0. To this was added 400ul of the incubated buffer and proteinase K mixture. This gave a viral preparation diluted in 800ul of 10mM Tris, 5mM EDTA, 125mM NaCl, 0.95% SDS and 2.5 mg/ml proteinase K. This viral preparation was then incubated at 37^0C for 30 minutes.

One volume of phenol/chloroform (1:1) was immediately added to the viral preparation after the incubation period was complete. (Water saturated phenol was equilibrated to pH 8.0 with 1M Tris prior to use). The solution was mixed by inversion before centrifugation at 14,000g for 10 minutes at 4°C. The aqueous phase was removed and an equal volume of phenol/chloroform (1:1) added as before. After mixing by inversion the solution was centrifuged at 14,000g for 10 minutes at 4°C. The aqueous phase was removed, 5M NaCl was added to give a final concentration of 150mM then 2 volumes of ethanol was added to precipitate the RNA. After mixing by inversion the solution was stored at -20°C overnight or until required.

The precipitated RNA was recovered by centrifugation at 14,000g for 15 minutes at 4°C. The ethanol was removed taking care not to dislodge the pelleted RNA. Freshly prepared 70% ethanol (v/v) was gently added to wash the pellet. The ethanol was removed after a centrifugation step at 14,000g for 5 minutes. The tubes were left to drain on a Kleenex® tissue for 2-3 minutes prior to the pellet being dried in a vacuum desiccator. The pellets were resuspended on ice for 20 minutes in 10-20ul water. Two microlitre aliquots of the resuspended RNA were stored at -75°C until required. Purity and size of ribonucleic acids were determined by electrophoresis in an agarose gel as described in section 2.5.3.

The proteinase K digestion step was omitted when extracting RNA from CMV.

2.8. PRODUCTION OF FIRST STRAND cDNA.

The preparation of cDNA requires a template (the viral RNA), a primer (an oligonucleotide complementary to sequences within the template) and a retroviral RNA-dependent DNA polymerase, reverse transcriptase. The

primer used for the cDNA reaction, RLFS-9 was complementary to the polyadenylated 3' end of potyvirus genomes and its sequence was 5' AGC TGT CGA $C(T)_{25}$ 3'.

The experimental procedure used to produce first-strand cDNA was as follows :

Two microlitres of RNA (4 ug) resuspended in water were added to the cDNA reaction mix consisting of 10ul 5X first strand buffer (250mM Tris-HCl, pH 8.3 at 25°C, 375mM KCl, 15mM $MgCl_2$), 5.0ul 5mM dNTP's (dATP, dCTP, dGTP, dTTP) (Pharmacia Ultrapure® dNTP Set, Catalogue No. 27-2035-01), 5.0 ul 0.1M dithiothreitol (DTT), 1.0ul (40 units) RNasin (Promega, Catalogue No. N211/1-4), 1.5ul (300 units) reverse transcriptase (Gibco BRL, Superscript™ II RNase H⁻, Catalogue No. 8064SA), 1.0ul (20 pmols) primer and 24.5 ul H_20. The first strand reaction buffer and the 0.1M DTT were supplied by Gibco BRL with the reverse transcriptase.

This gave a total reaction mix of 50ul in a sterile eppendorf® tube. The contents were mixed by inversion and then incubated at 42°C for 1 hour. After incubation 100ul phenol:chloroform (1:1), 47 ul H_20 and 3ul 5M NaCl were added, the tube inverted several times then centrifuged at 14,000g for 5 minutes. The aqueous phase was recovered and 250ul ethanol added to precipitate the cDNA. The solution was mixed by inversion and stored at -75°C for 15 minutes or -20°C overnight. The precipitated cDNA was recovered by centrifugation at 14,000g for 20 minutes. The ethanol was removed, the pellet washed with 50ul 70% ethanol (v/v) and centrifuged for 5 minutes at 14,000g. The ethanol was removed and the pellet dried in a vacuum desiccator then resuspended in 9ul 10mM Tris and 1mM EDTA pH 8.0 at room temperature for 20-40 minutes. Residual RNA was removed with the addition of 1ul 10ug/ml RNase A (Sigma Type 1-AS No. R-5503) prior to

incubation at 65°C for 15 minutes. cDNA of each isolate of DsMV was stored at -18°C until required.

2.9. THE POLYMERASE CHAIN REACTION.

cDNA was amplified using the polymerase chain reaction (PCR) (Saiki et al, 1988). Two 5' oligonucleotide primers were used for the PCR reaction. One was a degenerate primer designed by Nicolas and Laliberté (1991). This primer, JF3, is complementary to a region having a high percentage of homology amongst published potyviral sequences and which was within the coding region for the nuclear inclusion b (NIb) protein. The sequence of JF3 is described in Table 2.3.

The second 5' oligonucleotide primer was a degenerate primer designed by Langeveld et al (1991). This primer U335 is complementary to a region coding for the conserved amino acid sequence, MVWCIENG, in the potyviral coat protein and its sequence is described in Table 2.3.

Two 3' oligonucleotide primers were used. One was a degenerate primer designed by Langeveld et al (1991) and its sequence is described in Table 2.3. This primer, D335, is complementary to a region coding for the conserved AHFQMKTA residues within the potyviral coat protein.

The other 3' oligonucleotide primer was RLFS-9, as described in section 2.8. All primers were supplied by Dr R.L.S.Forster, Horticulture and Food Research Institute Ltd, Auckland.

The PCR reaction mix consisted of 2.0ul (10ug) cDNA, 1.0ul (20 pmols) 5' primer, 1.0ul (20 pmols) 3' primer, 4ul 1.25mM dNTP's (dATP, dCTP, dGTP, dTTP), 0.6ul 50mM $MgCl_2$, 2.0ul 10X reaction buffer (200mM Tris-HCl, pH 8.4, 500mM KCl), 0.2ul (1 unit) *Taq* DNA polymerase (Gibco BRL, Catalogue

Table 2.3 : Sequences of oligonucleotide primers used in the polymerase chain reaction (PCR).

Primer	Nucleotide Sequence
JF3	5' TAYTGYGAYGCYGATGGYTC 3'
U335	5' GAATTCATGRTNTGGTGYATHGANAAYGG 3'
D335	5' GAGCTCGCNGYYTTCATYTGNRHDWKNGC 3'
RLFS-9	5' AGCTGTCGAC$(T)_{25}$ 3'
Nucleotides at degenerate positions are represented by a single letter code : R = A and G; W = A and T; Y = C and T; K = G and T; H = A,C and T; D = A, G, and T; N = A,C,G, and T.	

No. 18038-018) and 9.2 ul H_20. The reaction buffer and $MgCl_2$ were supplied by Gibco BRL with the *Taq* DNA polymerase. This gave a total reaction mix of 20ul. The reaction mix was prepared in 500ul thin-wall tubes, overlaid with a drop of mineral oil to prevent evaporation of reagents during heating, and placed in a DNA thermal cycler (Perkin-Elmer Cetus 480). A negative control consisting of the reaction mix without the addition of the target cDNA was included in all PCR procedures. The secondary structure of the cDNA was removed by heating to 94°C for five minutes, followed by a 35 cycle programme of denaturation, annealing and extension with an additional 72°C for five minutes after the last cycle.

After completion of the cycling the mineral oil was removed, and the PCR products were analyzed by gel electrophoresis as described in section 2.5.3. The PCR products were stored at -20°C.

2.10. EXTRACTION OF DNA FROM AGAROSE GELS.

Two methods were used to extract DNA from agarose gels; one involved the use of a commercial kit especially designed for this purpose, the other the use of low melting point agarose.

2.10.1. Extraction of DNA from standard agarose.

After electrophoresis the agarose gel was stained as described in section 2.4.3. The gel was examined using a UV light and the appropriate band was cut out in as thin a slice as possible using a new scalpel blade for each separate DNA sample. The DNA was eluted from the gel piece using a BRESA-CLEAN™ DNA Purification Kit (Bresatec Ltd, Catalogue No. BRC 1) as follows:

One-half volume of TBE MELT™ and 4.5 volumes of BRESA-SALT™ was added to the excised gel piece then incubated at 55°C for 5 minutes to melt the agarose. BRESA-BIND™ was vortexed well then 5*u*l was added to the melted gel piece. The mixture was incubated for 5 minutes at room temperature to bind the DNA, mixing regularly to ensure the BRESA-BIND™ remained in suspension.

The BRESA-BIND™/DNA complex was pelleted by centrifugation at 14,000g for 5 seconds. The supernatant was removed and set aside. The pellet was washed with BRESA-BIND™ equivalent in volume to the BRESA-SALT™ used previously. The pellet was vortexed to ensure it was well resuspended prior to centrifugation at 14,000g for 5 seconds. The BRESA-BIND™ was removed by aspirating with a narrow pipette tip ensuring no residues of the BRESA-BIND™ remained. The pellet was dried by vacuum desiccation for 1 minute.

The DNA was recovered by resuspending the pellet in 10ul water, mixing well, then incubated at 55°C for 5 minutes. The resuspended DNA was

centrifuged at 14,000g for 1 minute. The supernatant containing the DNA was removed avoiding contamination from the silica pellet.

2.10.2. Extraction of DNA from low melting point agarose.

A method based on that described by Thuring, Sanders and Borst (1975) was used as follows:

The DNA sample was loaded onto a 0.7% Seaplaque® agarose gel (w/v) (FMC Bio Products, Catalogue No. 50101-3) in TAE buffer (40mM Tris-HCl, 20mM sodium acetate and 1mM EDTA pH 7.8) and run at 60V for 2 hours until the tracking dye had migrated two-thirds of the way through the gel. After electrophoresis the DNA band was cut out of the gel as described in section 2.10.1.

The gel slice was placed in an eppendorf® tube and melted at 65°C. Five hundred microlitres of phenol was added, vortexed to mix, then the tube was placed at
-20°C for at least 2 hours. After centrifugation at 14,000g for 10 minutes, the aqueous phase was removed and an equal amount of chloroform added, vortexed to mix, then centrifuged at 14,000g for 10 minutes. The aqueous phase was removed, 0.1 volumes of 3M Na acetate and 2.5 volumes 95% ethanol (v/v) added, mixing gently and thoroughly, before being placed at -20°C for at least 2 hours.

After centrifugation at 14,000g for 5 minutes, the ethanol was removed and an equal volume of 95% ethanol (v/v) added, centrifuged for 2 minutes, and then removed. The DNA pellet was well drained then dried by vacuum desiccation for 15-20 minutes prior to resuspension in water.

2.11. RESTRICTION ENDONUCLEASE DIGESTION.

Restriction endonucleases are enzymes that recognise and cleave DNA at specific nucleotide sequences, thereby generating a mixture of discrete DNA fragments. Products of the restriction enzyme digestion can be separated on the basis of fragment size by electrophoresis. DNA molecules can, therefore, be compared by examining the number and size of fragments generated by digestion with specific restriction enzymes.

Five microlitres of DNA was added to 2.5 ul 10X buffer, 0.5ul (5 units) restriction enzyme and 12.0ul H_2O to give a final reaction mix of 20ul. The buffer used was that supplied by the manufacturers of the particular restriction enzyme. The reaction mix was incubated at 37^{0}C for 60 minutes. A 5ul aliquot of the digested reaction mix was added to 5ul SDS tracking dye (50mM Tris pH 6.8, 100mM dithiothreitol, 2% SDS (w/v), 0.1% bromophenol blue (w/v), 10% glycerol (v/v)) and run on a 1% agarose gel (w/v) as described in section 2.5.3.

2.12. CLONING OF PCR PRODUCTS.

2.12.1. Ligation.

A Ready-To-Go™ T4 DNA Ligase system (Pharmacia, Catalogue No. 27-0361-01) was used to ligate the DNA into the plasmid vector as follows:

Two microlitres of DNA and 1ul of the plasmid vector (pGEM-T, Promega, Catalogue No. A3600) were added to water to give a final volume of 20ul. The 20ul DNA/vector solution was added to a tube of the Ready-To-Go™ T4 DNA Ligase and incubated at room temperature for 3-5 minutes. The solution was mixed gently by pipetting up and down several times followed

by a brief centrifugation to remove any bubbles. The mixture was then incubated at room temperature for 30 minutes.

2.12.2. Transformation.

One hundred microlitres of competent cells were aliquoted into a chilled eppendorf® tube. Two microlitres of the ligation reaction (see section 2.12.1) were added to the cells, gently shaking the tube to mix. The cells were incubated on ice for 30 minutes, heatshocked at 42°C for 1 minute (without shaking) and placed on ice for 2 minutes. A 0.25ml aliquot of room temperature 2x TY medium (1.6% tryptone (w/v), 1% yeast extract (w/v) and 0.5% NaCl (w/v)) was added and the tube shaken at 225 rpm for 1 hour at 37°C. The cells were plated out onto an AMP plate (1% tryptone (w/v), 0.5% yeast extract (w/v), 1% NaCl (w/v), 1.5% agar (w/v) and 100ug/ml ampicillin) and incubated overnight at 37°C.

2.12.3. One tube plasmid DNA minipreparation.

A single colony was removed from the AMP plate (see section 2.12.2) and inoculated into 3.0mls terrific broth (TB; 1.2% bacto tryptone (w/v), 2.4% bacto yeast extract (w/v), 0.4% glycerol (v/v), 17mM KH_2PO_4 and 72mM $K_2HPO_4.3H_20$), containing 50ug/ml ampicillin, in 15 ml disposable tubes and shaken at 37°C overnight. The resultant cells were spun out at 14,000g for 3 minutes then aspirated to remove the broth.

Two hundred microlitres STET buffer (8% sucrose (w/v), 0.1% Triton X-100 (v/v), 50mM EDTA, pH 8.0 and 50mM Tris-HCl, pH 8.0), containing 10ul RNase A (10mg/ml) and 125ul lysozyme (40mg/ml), were added to the cells, then vortexed to resuspend, prior to incubation at room temperature for 5 minutes. The mixture was boiled for 45 seconds, then centrifuged at 13,000g

for 30 minutes. The 'bogey' (disrupted) cells were removed using a sterile toothpick.

Fifty microlitres of 1% cetyl trimethyl ammonium bromide (CTAB) was added, centrifuged at 13,000g for 5 minutes, drained well to remove the supernatant, then 300ul of 1.2M NaCl added and vortexed to resuspend the pellet.Seven hundred and fifty microlitres of ethanol was added, inverted to mix, then held on dry ice for 5 minutes prior to centrifugation at 14,000g for ten minutes. The tube was drained well and the pellet vacuum dried for 5 minutes. The pellet was resuspended in 50ul water, vortexing briefly, then held on ice. Ten microlitres of the resultant cloned DNA was analyzed by electrophoresis as described in section 2.5.3. The DNA was digested with Pst I as described in section 2.11. to determine if inserts were present.

PCR amplified cDNA of each isolate of DsMV was cloned as described above.

CHAPTER THREE

A COMPARATIVE STUDY OF FOUR ISOLATES OF DASHEEN MOSAIC POTYVIRUS - RESULTS AND DISCUSSION.

3.1. HOST RANGE STUDIES.

Healthy aroid plants were inoculated with one of each of the isolates as described in Chapter 2 (section 2.2.). Inoculated plants were tested after 3-4 weeks using ELISA as described in Chapter 2 (section 2.4.2.3.) or electron microscopy (Chapter 2, section 2.3.1.).

All isolates were successfully transmitted to each of the three *Araceae* species inoculated. Symptoms of virus infection associated with each isolate are summarised in Table 3.1. No isolates were transmitted to the herbaceous indicator plants tested.

Symptoms of DsMV-FL, DsMV-X and DsMV-Z infection in *Caladium candidum* cv candidum were very similar. In contrast, no symptoms were apparent in *C. candidum* inoculated with DsMV-H.

All plants of *Xanthosoma* sp died back after inoculation. Subsequent new growth was stunted, emerging leaves being very small (Figure 3.1). This stunting was much more severe than that occurring in naturally infected *Xanthosoma* sp (Figure 3.2).

Table 3.1 : Symptoms of infection in various *Araceae* associated with four isolates of dasheen mosaic potyvirus.

Host	Isolate of DsMV			
	DsMV-FL	DsMV-H	DsMV-X	DsMV-Z
Caladium candidum cv candidum	mottle on leaves, stunting of plants	no apparent symptoms	mottle on leaves, stunting of plants	mottle on leaves, stunting of plants
Colocasia esculenta cv 'Haapu'	-	chlorotic feathering on leaves, stunting of plants	-	-
Xanthosoma sp	severe stunting	severe stunting	slight chlorotic feathering of leaves, severe stunting	severe stunting
Zantedeschia sp 'Pink Persuasion'	mottle on leaves, distorted leaves, colour break of flowers, stunting of plants	no mottling or mosaic on leaves, no distortion, colour break in flowers, stunting of plants	distinct yellow mosaic on leaves, no leaf distortion, colour break in flowers, stuntimg of plants	mottle on leaves, distorted leaves, colour break in flowers, stunting of plants

Figure 3.1: *Xanthosoma* sp inoculated with DsMV-Z showing severe stunting of emergent leaves.

Figure 3.2: *Xanthosoma* sp infected with DsMV; plant inoculated with DsMV-Z on left showing severe stunting, and a naturally infected plant on the right containing DsMV-X.

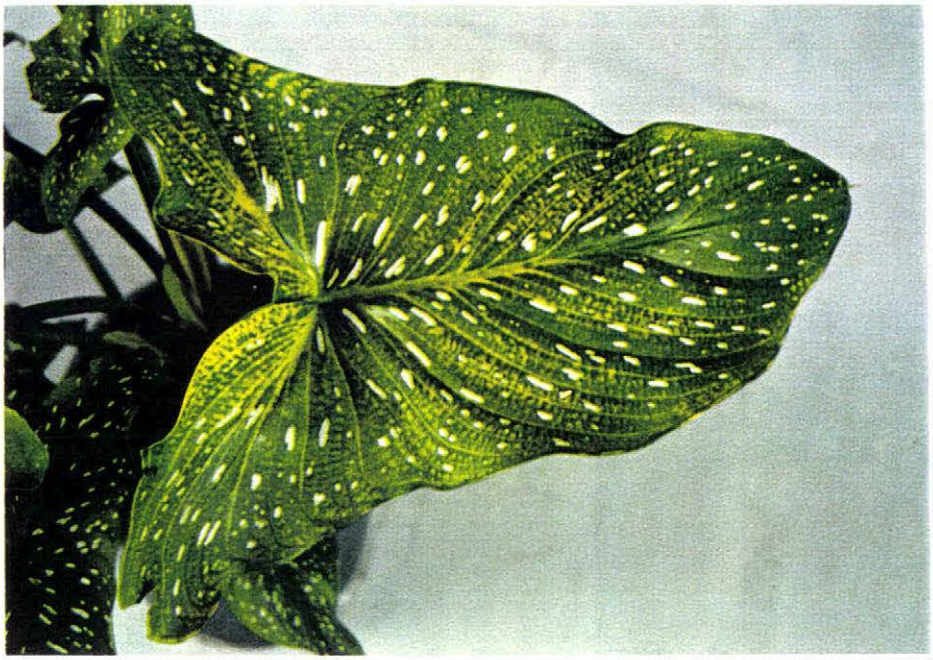

Figure 3.3: *Zantedeschia* sp 'Pink Persuasion' inoculated with DsMV-X showing distinct yellow mosaic symptoms on leaves.

Figure 3.4: *Zantedeschia* sp 'Pink Persuasion' inoculated with DsMV-H showing necrotic leaf margins and reduced growth.

Symptoms expressed by *Zantedeschia* sp 'Pink Persuasion" infected with DsMV-FL were comparable with those resulting from DsMV-Z infection. Mottling of leaves, colour breaking in flowers and stunting of plants were the predominant symptoms. In contrast, DsMV-X infection caused a dominant bright yellow mosaic patterning of the leaves (Figure 3.3). This symptom was not observed in any other DsMV-infected calla held at Massey University. *Zantedeschia* sp 'Pink Persuasion' infected with DsMV-H were stunted and leaf margins necrotic (Figure 3.4), no other symptoms such as leaf mosaics or distortion were apparent.

3.2. ELECTRON MICROSCOPY.

Fifty virus particles of each isolate of dasheen mosaic potyvirus (DsMV) were measured as described in Chapter 2 (section 2.3.3.). After calculating the actual sizes, the particle dimensions were grouped into 30nm divisions and plotted as a histogram (Figures 3.5 - 3.8). The average length for each isolate was determined and are as follows: DsMV-FL, 740nm; DsMV-H, 745nm; DsMV-X, 735nm; DsMV-Z, 730nm.

The routine accuracy of particle size measurements is no better than ± 5% (Roberts, 1986). Given this level of accuracy there is no meaningful difference in the average length of each isolate. The difference between the longest average length (745nm for DsMV-H) and the shortest (730nm for DsMV-Z) is 15nm or 2%.

The modal length, often called the "normal" length of virus particles, is the length which is used to characterise plant viruses (Buchen-Osmond, Crabtree, Gibbs and McLean, 1988). With the exception of DsMV-H, the modal length for each isolate is 720nm. The modal length of DsMV-H is 780nm, 7-8% greater than the other isolates. It can then be assumed the modal length of DsMV-H is longer than the other isolates.

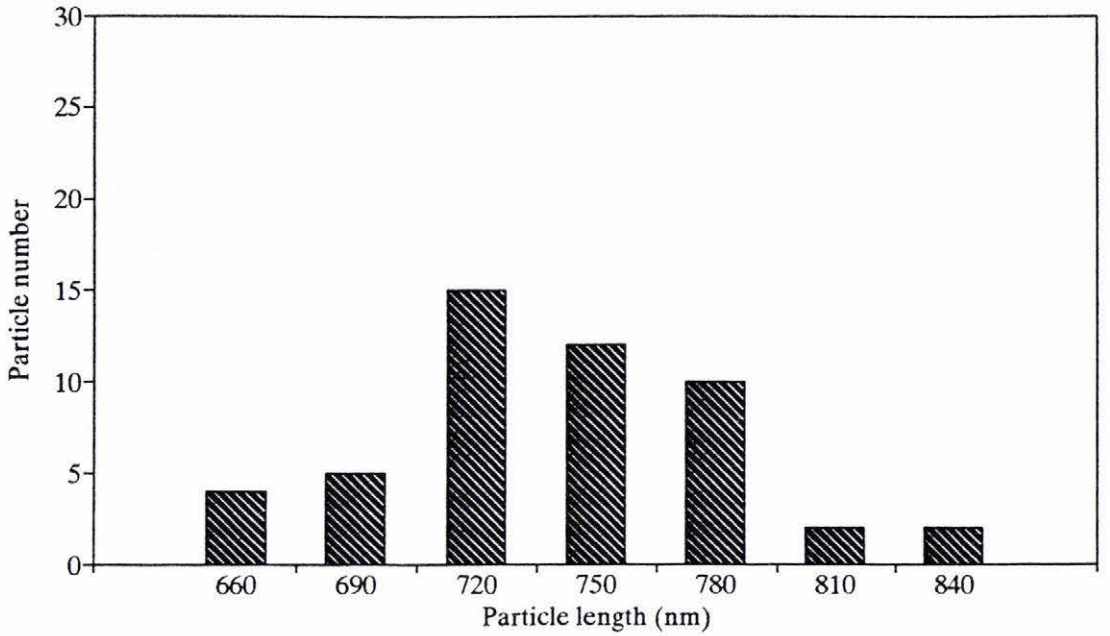

Figure 3.5 : Particle length distribution of dasheen mosaic potyvirus isolate DsMV-FL in leaf sap, negatively stained in 2% potassium phosphotungstic acid (PTA) pH 7.0 : 2% ammonium molybdate (AM) pH 5.6 (1:1).

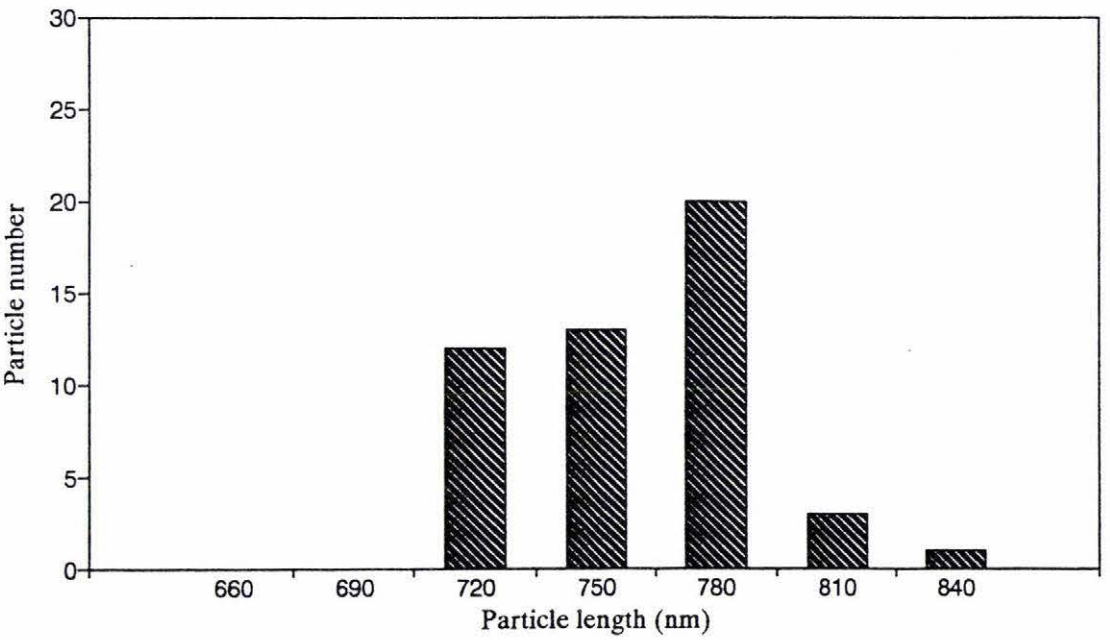

Figure 3.6 : Particle length distribution of dasheen mosaic potyvirus isolate DsMV-H in leaf sap, negatively stained in 2% potassium phosphotungstic acid (PTA) pH 7.0 : 2% ammonium molybdate (AM) pH 5.6 (1:1).

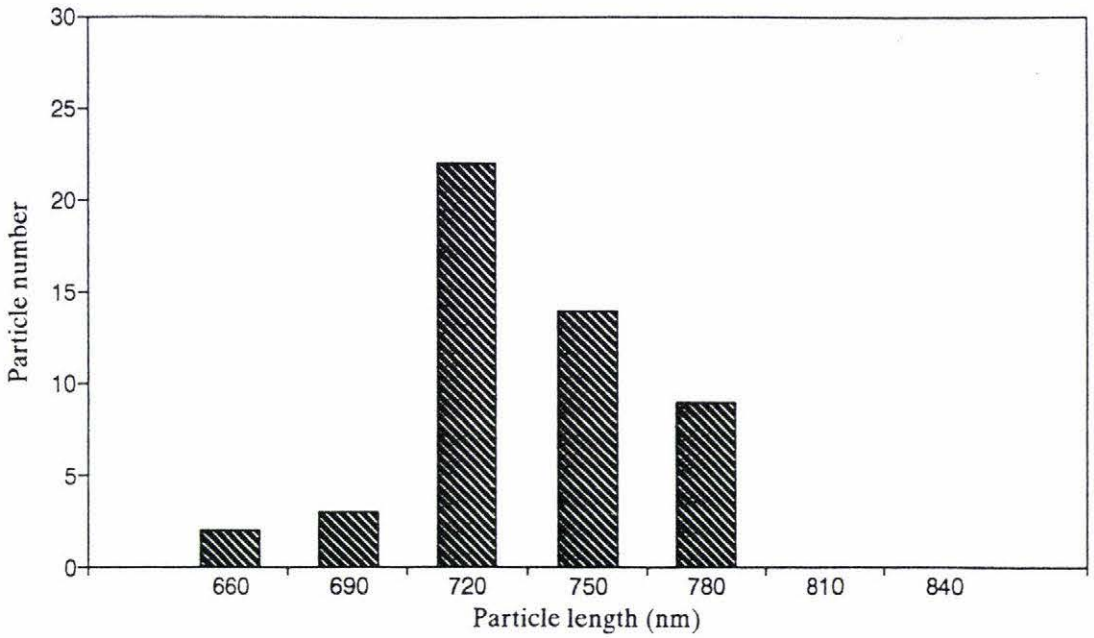

Figure 3.7 : Particle length distribution of dasheen mosaic potyvirus isolate DsMV-X in leaf sap, negatively stained in 2% potassium phosphotungstic acid (PTA) pH 7.0 : 2% ammonium molybdate (AM) pH 5.6 (1:1).

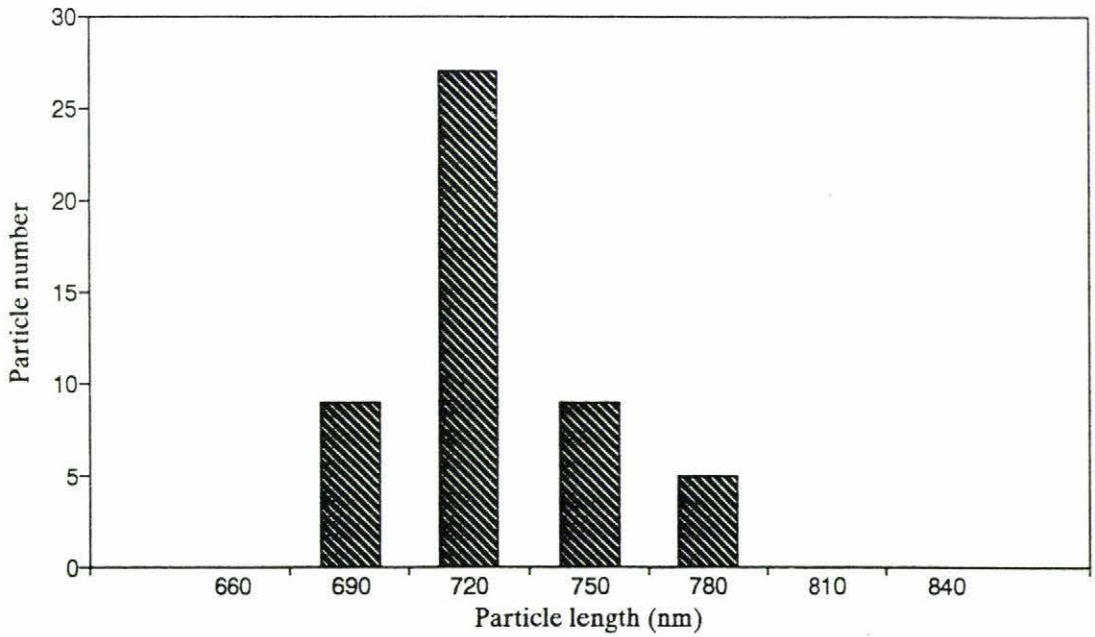

Figure 3.8 : Particle length distribution of dasheen mosaic potyvirus isolate DsMV-Z in leaf sap, negatively stained in 2% potassium phosphotungstic acid (PTA) pH 7.0 : 2% ammonium molybdate (AM) pH 5.6 (1:1).

3.2. SEROLOGY.

Enzyme-linked immunosorbent assay (ELISA) procedures were carried out as described in Chapter 2 (section 2.4.2.5.). Line graphs were plotted from the average absorbance of duplicate test wells to show the homologous and heterologous reactions of each isolate to individual antisera (Figures 3.9 - 3.12).

The antiserum to DsMV-FL reacted clearly with the homologous isolate, DsMV-X and DsMV-Z (Figure 3.9) and gave a very low background with healthy sap, confirming these two isolates are dasheen mosaic potyvirus. DsMV-H did not react at any dilution to this antiserum.

The antiserum to DsMV-Z reacted strongly with the homologous isolate and with DsMV-FL, but did not react with DsMV-H, DsMV-X and healthy sap (Figure 3.10).

The DsMV-X antiserum reacted with the homologous isolate and possibly with DsMV-FL (Figure 3.11). This antiserum apparently did not react with DsMV-Z or DsMV-H. The high background reaction with healthy sap indicated that this antiserum contained high levels of antibodies to plant proteins. This made it difficult to interpret the resultant absorbance plots. Once the dilution of test samples increased to 1/20 a difference between DsMV-X and DsMV-FL with DsMV-H and DsMV-Z became more apparent.

When a comparison is made of the absorbances of each isolate against the antiserum to DsMV-H (Figure 3.12) only the homologous reactant (DsMV-H) reacted positively. The absorbances for the other three isolates is similar to the healthy control. Coupled with this, DsMV-H did not react positively to the antisera prepared against the other three isolates. This suggests that DsMV-H may be a virus distinct from DsMV.

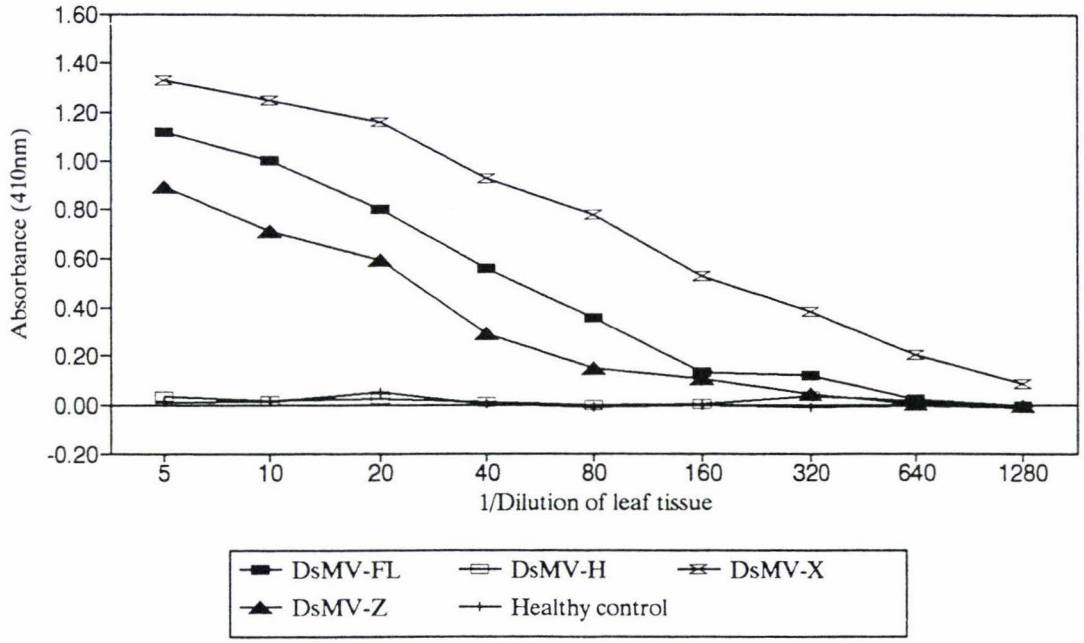

Figure 3.9 : Absorbance of a dilution series of four isolates of dasheen mosaic potyvirus (DsMV) in leaf sap with a homologous/heterologous ELISA test using antiserum to the DsMV-FL isolate.

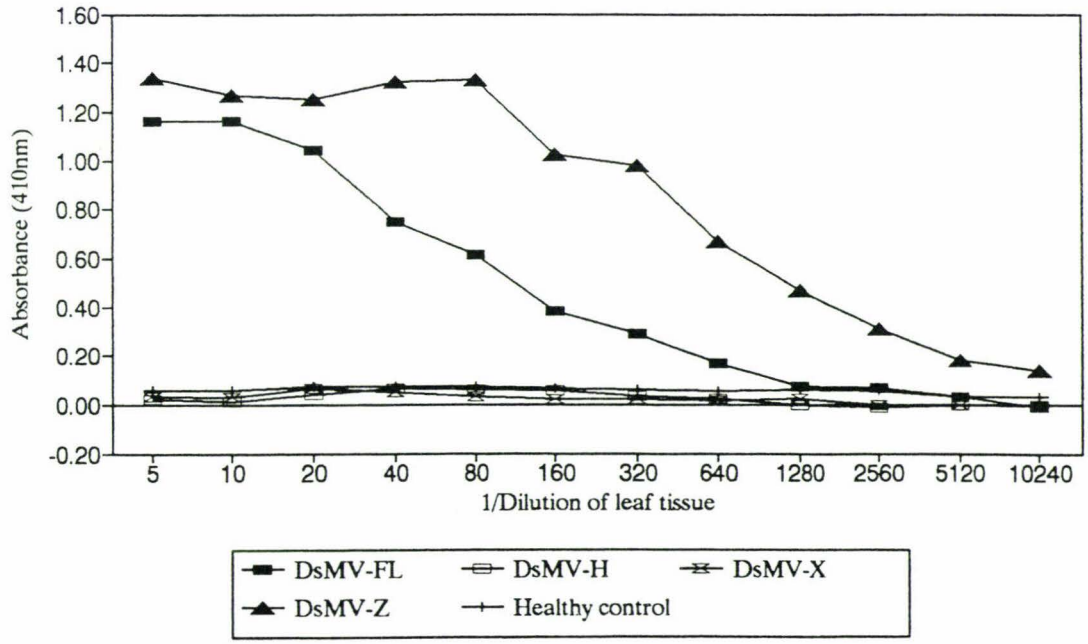

Figure 3.10 : Absorbance of a dilution series of four isolates of dasheen mosaic potyvirus (DsMV) in leaf sap with a homologous/heterologous ELISA test using antiserum to the DsMV-Z isolate.

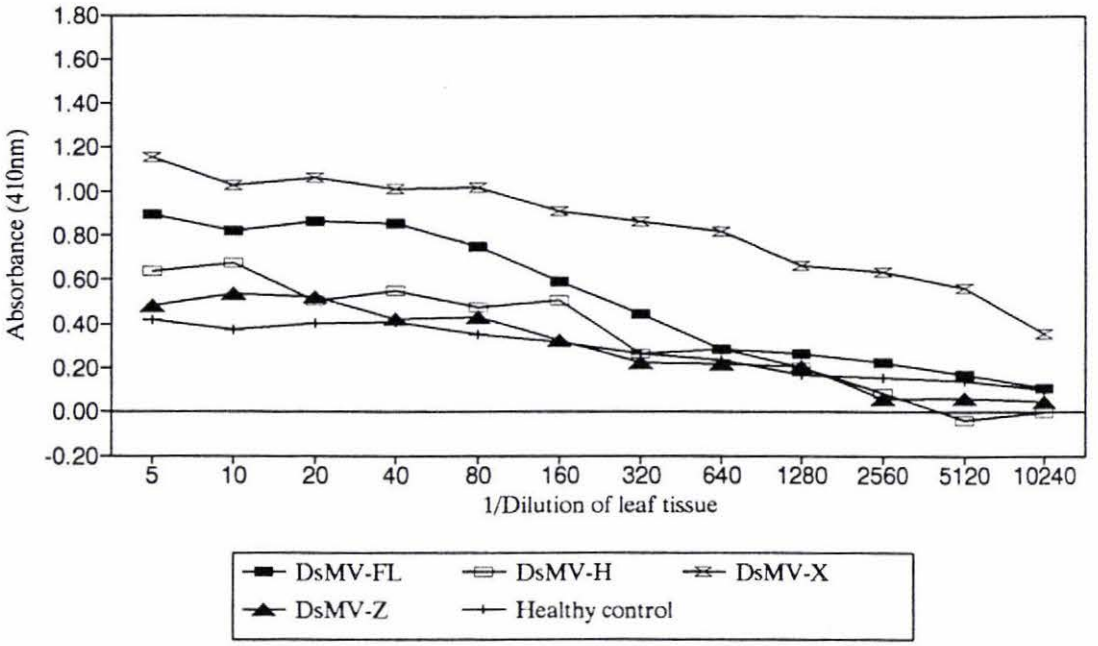

Figure 3.11 : Absorbance of a dilution series of four isolates of dasheen mosaic potyvirus (DsMV) in leaf sap with a homologous/heterologous ELISA test using antiserum to the DsMV-X isolate.

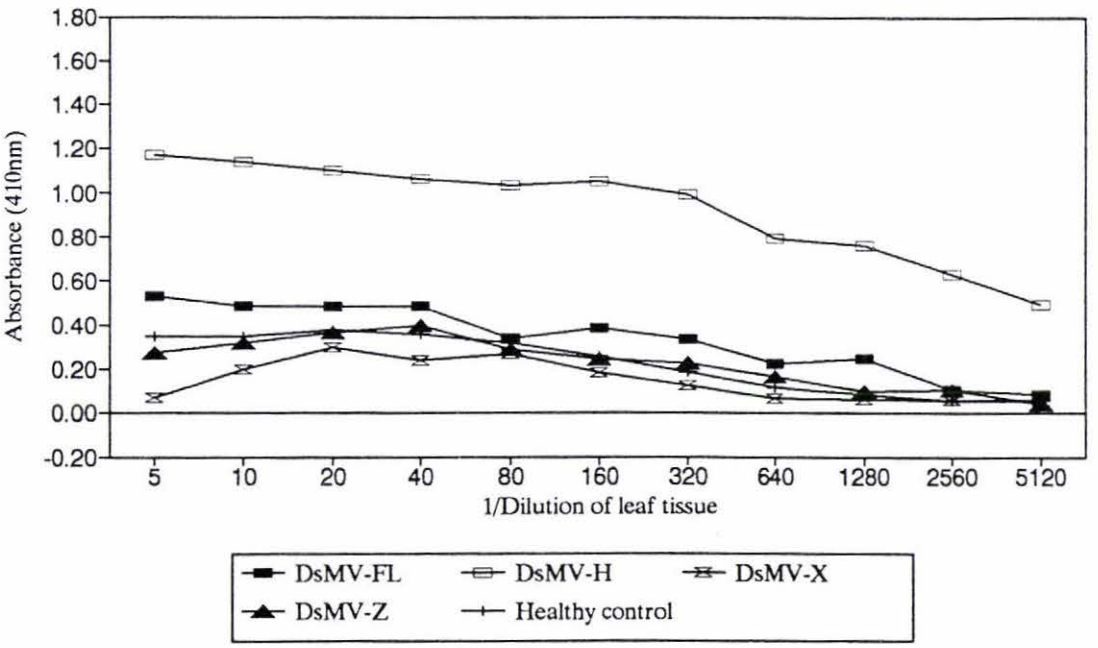

Figure 3.12 : Absorbance of a dilution series of four isolates of dasheen mosaic potyvirus (DsMV) in leaf sap with a homologous/heterologous ELISA test using antiserum to the DsMV-H isolate.

To determine the relationship between the isolate from *C. esculenta* cv 'Haapu' and isolates from other cultivars of *C. esculenta*, a further ELISA test was carried out using two virus other isolates. One isolate DsMV-A, also from Rarotonga, was present in the cultivar *C. esculenta* cv 'Alafua Sunrise'. The other isolate (DsMV-T) was present in a taro plant grown in a home garden in Palmerston North. The origin of this cultivar is unknown.

Leaf tissue of *Nicotiana benthamiana* infected with tamarillo mosaic potyvirus (TaMV) (Mossop, 1977) was included in the assay to determine if there was any relationship to another member of the potyvirus group.

Two healthy controls were included; healthy *Zantedeschia* sp (healthy control 1) and healthy *Nicotiana benthamiana* (healthy control 2).

The two isolates from the additional cultivars of taro reacted positively against the antiserum to DsMV-H (Figure 3.13). This indicates that the virus in the cultivar 'Haapu' may be common in taro. TaMV did not react with the DsMV-H antiserum. The absorbance of TaMV, a distinct potyvirus, against the antiserum to DsMV-H is similar to that for the other isolates of DsMV (Figure 3.12) confirming there is no relationship between DsMV-H and the other isolates of DsMV.

All four isolates together with the virus isolate in *C. esculenta* cv 'Alafua Sunrise' were tested against an antiserum to the potyvirus group as described in Chapter 5 (section 5.4.2.). The resultant absorbance plot is shown in Figure 3.14. The two virus isolates from taro and the healthy control did not react positively whereas the other virus isolates reacted strongly.

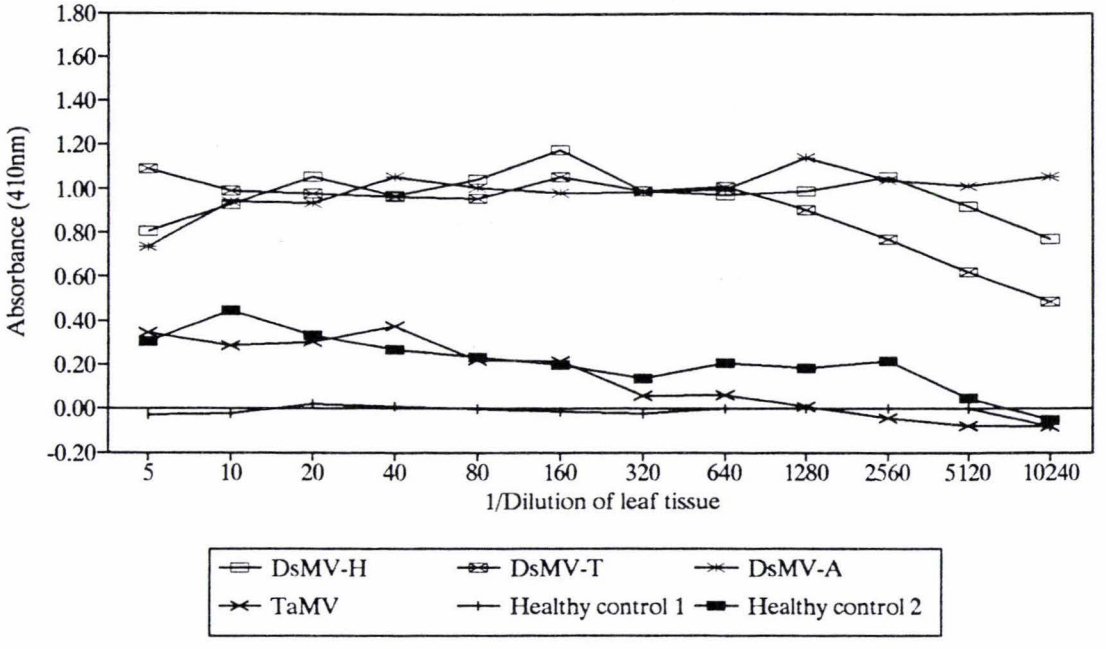

Figure 3.13 : Absorbance of a dilution series of three dasheen mosaic potyvirus (DsMV)-like isolates from *Colocasia esculenta* leaf sap and tamarillo mosaic potyvirus (TaMV) in *Nicotiana benthamiana* leaf sap with an ELISA test using antiserum to the DsMV-H isolate of DsMV.

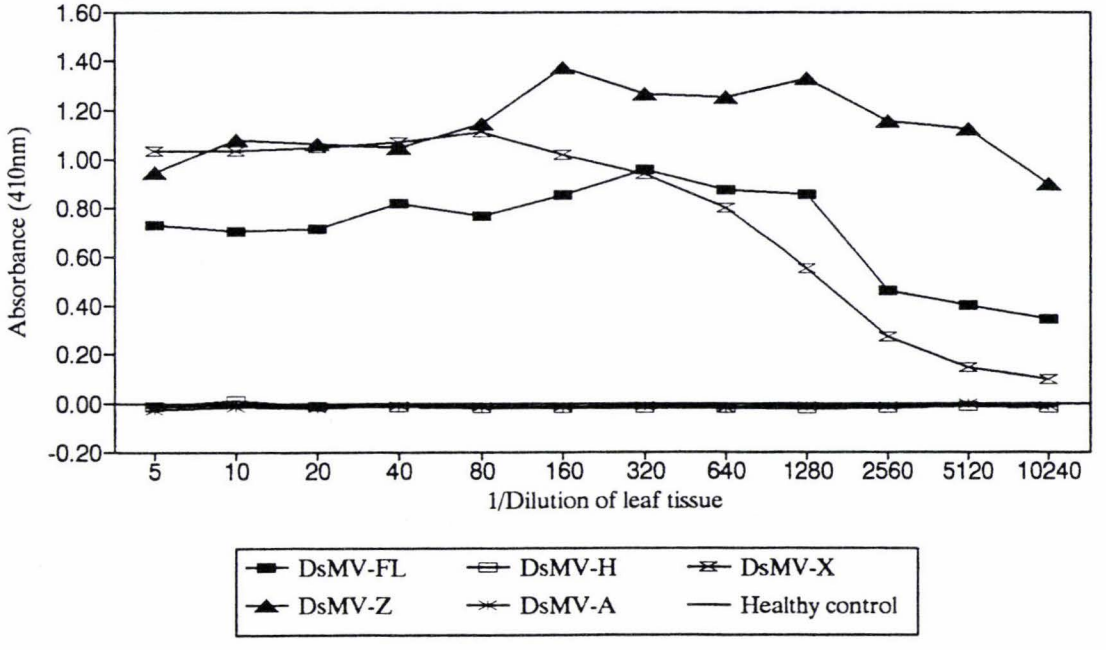

Figure 3.14 : Absorbance of a dilution series of four isolates of dasheen mosaic potyvirus (DsMV) and a DsMV-like isolate from *Colocasia esculenta* cv 'Alafua Sunrise' in leaf sap using antiserum to the potyvirus group.

3.4 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS.

SDS-degraded coat protein of each isolate were analyzed by SDS-PAGE as described in Chapter 2 (sections 2.5.1 and 2.5.2.) Electrophoresis in a 12% polyacrylamide gel resolved at least seven major electrophoretic components for DsMV-X, five for DsMV-H, four for DsMV-FL and one for DsMV-Z (Figure 3.15). The molecular weights of each component was estimated from a standard curve plotted from the log of the molecular weights of the Bio-Rad broad range SDS-PAGE molecular weight standards against their mobility in the polyacrylamide gel (see Appendix 2). The calculated molecular weights are presented in Table 3.2.

A 33 x 10^3 dalton component is common with all isolates and is within the range estimated for the size of the coat protein of potyviruses (Hollings and Brunt, 1981). A number of other components are present for some isolates especially DsMV-X which has at least seven major components as well as a number of other minor components. As coat proteins of potyviruses are susceptible to degradation during viral preparation and storage (Francki, Milne and Hatta, 1985) some of these components are likely to be products of viral coat protein degradation.

A component larger than the expected size of potyvirus coat protein (29-37 x 10^3 daltons) was present with the isolates DsMV-X and DsMV-H. Abo El-Nil et al (1977) also reported large coat protein components for DsMV. No explanation was given for this. They suggested that since other physical and morphological properties of DsMV were compatible with those reported for potyviruses, the coat protein size was likely to be similar to other potyviruses. However, Francki, Milne and Hatta (1985) mention that the molecular weight of some potyviruses may be more than 40 x 10^3 daltons.

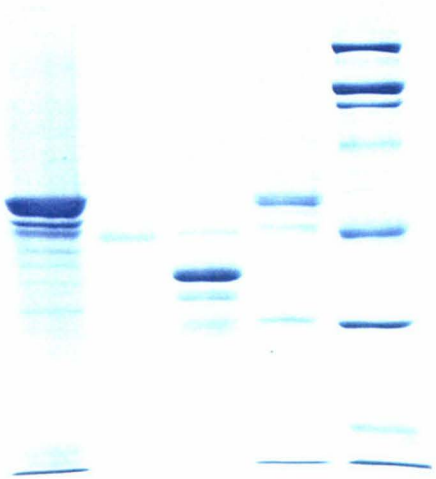

Figure 3.15 : SDS-PAGE analysis of degraded coat proteins of four isolates of dasheen mosaic potyvirus (DsMV) : lane 1, DsMV-X; lane 2, DsMV-Z; lane 3, DsMV-FL; lane 4, DsMV-H; lane 5, Bio-Rad broad range SDS-PAGE molecular weight markers.

Table 3.2. Molecular weight estimates of SDS-degraded coat proteins of four isolates of dasheen mosaic potyvirus (DsMV) by electrophoresis in an SDS-polyacrylamide gel.

	DsMV-X	DsMV-Z	DsMV-FL	DsMV-H
Molecular Weight Estimates ($\times 10^3$)	40	-	-	40
	36	-	-	36
	31	31	31	31
	29	-	-	-
	28	-	-	-
	27	-	27	-
	23	-	23	-
	-	-	21	21
	-	-	-	18

3.5. GENOMIC CHARACTERISTICS.

3.5.1. Isolation of RNA.

Single-stranded RNA (ssRNA) was extracted from purified viral preparations of each isolate as described in Chapter 2 (section 2.7.). Extracted RNA was analyzed by gel electrophoresis to determine its purity and size.

A single RNA species of approximately 10 kilobases was observed under non-denaturing conditions in a 1% agarose gel for each isolate (Figure 3.16). RNA was successfully isolated with minimal degradation for DsMV-X, DsMV-H and DsMV-Z. Considerable degradation occurred during isolation of RNA from DsMV-FL. Despite several attempts, no improvement in the condition of the resultant RNA was achieved.

The amount of RNA present was quantified spectrophotometrically at an absorbance of 260nm using a 1:1000 dilution. The concentration of nucleic acid was calculated assuming an OD of 1 corresponds to approximately 40 ug/ml ssRNA (Sambrook, Fritsch and Maniatis, 1989). Approximately 1.6 to 4.8 ug/ul of RNA was routinely obtained from 200ul virus preparation containing approximately 3.0mg/ml RNA. An OD_{260}/OD_{280} value of greater than 1.8 was achieved, indicating little or no contamination with protein or phenol (Sambrook et al, 1989).

3.5.2. Synthesis of first strand cDNA.

Viral RNA from each of the isolates under study was reverse transcribed into cDNA as described in Chapter 2 (section 2.8.). The quantity and purity of the cDNA was determined spectrophotometrically as described in section 3.5.1 assuming an OD of 1 corresponds to approximately 40 ug/ml ssDNA (Sambrook et al, 1989). Approximately 5 ug/ul cDNA was routinely obtained.

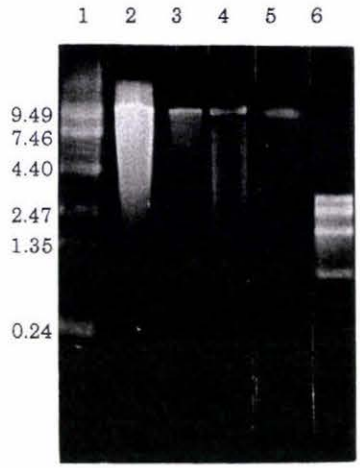

Figure 3.16. ssRNA isolated from purified viral preparations of four isolates of dasheen mosaic potyvirus. Lane 1, BRL 0.24 - 9.49kb RNA molecular weight markers; lane 2, RNA ex DsMV-FL; lane 3, RNA ex DsMV-H; lane 4, RNA ex DsMV-X; lane 5, RNA ex DsMV-Z; lane 6, RNA ex cucumber mosaic cucumovirus (CMV).

3.5.3. The polymerase chain reaction.

First strand cDNA of isolates DsMV-FL, DsMV-H, DsMV-X and DsMV-Z, were each amplified using the polymerase chain reaction (PCR) as described in Chapter 2 (section 2.9.) using four different primer pairs: (a) U335 and D335; (b) U335 and RLFS-9; (c) JF3 and D335; and (d) JF3 and RLFS-9. Each individual primer is described in Chapter 2 (section 2.9).

3.5.3.1. The primers D335 and U335.

The primers U335 and D335 were designed by Langeveld et al (1991) and the expected product size when they are used together as the upstream and downstream primers in PCR is 335 bp. Langeveld et al (1991) used a primer annealing temperature of 60°C for their PCR protocol and succeeded in obtaining the desired product size of 335 bp. Additional fragments were also obtained at this temperature.

Innis and Gelgand (1990) state the temperature required for primer annealing depends upon the length and base composition of the primers. They suggest an applicable annealing temperature is 5°C below the T_m[1] of the amplification primers. The T_m for the primers were estimated using the following formula:

$$T_m = 2 \times (A+T) + 4 \times (G+C)$$ (Thein and Wallace, 1986).

The estimated T_m for D335 was 80°C and 76°C for U335. Hence the theoretical temperature at which primer annealing should occur for this primer pair during PCR is 71°C. At this temperature priming is likely to be highly specific.

Enrlich and Sirko (1994) suggests that an annealing temperature of 55°C could be used initially for most primers. Such a low temperature may allow primers to amplify additional regions in the DNA other than the target segments (Myers, Sheffield and Cox, 1989). From this point the temperature can be raised so the signal strength is increased and less non-specific primer binding occurs, until a temperature is reached where the signal strength begins to decrease, as the primer no longer binds efficiently. The

[1] T_m = melting temperature; the temperature at which 50% of sequences are denatured (Matthews, 1991), or in this case 50% of the base pairing between the target sequence and the primer are dissociated.

temperature optimum is that point at which non-specific priming is minimized and signal intensity is not compromised due to incomplete annealing of the primer to its intended template.

PCR was initially carried out with a primer annealing temperature of 58°C, but without success. Pappu et al (1993) using primers designed from the same conserved potyvirus sequences used an annealing temperature of 42°C. Hence the annealing temperature was lowered to 41°C. At this temperature a distinct band occurred of the expected size of 335 bp. However, in addition to this expected band, other bands were present (Figure 3.17 (a)), the most prominent being 800bp.

The annealing temperature was raised to 45°C to increase the primer specificity. At this temperature the 800 bp band was prominent but the 335 bp was only just discernable (Figure 3.17 (b)). At 47°C no bands were present. Since 41°C gave the strongest signal for the 335 bp fragment this primer annealing temperature was used despite the presence of other bands.

Sakai (1989) stated that the concentration of $MgCl_2$ can have a profound effect on the specificity and yield of an amplification. Excess Mg^{2+} results in the accumulation of non-specific amplification products and insufficient Mg^{2+} will reduce yield. $MgCl_2$ concentration was varied in 0.5mM steps up to 5mM $MgCl_2$ but no improvement was achieved. The subsequent concentration used was 1.5mM, at which nearly all amplification procedures are efficient (Clackson, Güssow and Jones, 1991).

Excess primer usually gives more extraneous amplified products (Kawasaki 1990). The amount of primer used in the PCR reaction mix was diluted up to ten-fold without any increase in specificity or yield. Likewise the cDNA used as the template in the reaction mix was diluted up to one thousand-fold. The possibility that cDNA of varying length was obtained during the reverse

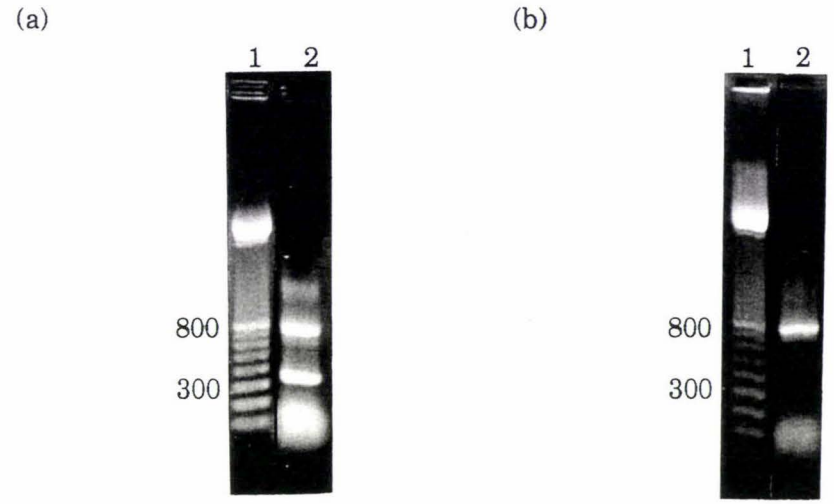

Figure 3:17 (a) : Amplification of cDNA of DsMV-Z using the primers D335 and U335, and a primer annealing temperature of 41°C. Lane 1, Pharmacia 100 base-pair ladder, lane 2, DsMV-Z. (b) Amplification of cDNA of DsMV-Z using the primers D335 and U335, and a primer annealing temperature of 45°C. Lane 1, Pharmacia 100 base-pair ladder, lane 2 DsMV-Z.

transcription reaction, some with the desired target sequences and others without the target sequence but with sequences that will anneal with the degenerate primers was considered. It was thought that by diluting the cDNA, unwanted sequences would be diluted to a level where they did not predominate during the PCR reaction, and hopefully sufficient cDNA containing the target sequence would be present that would then dominate the PCR reaction. Unfortunately, diluting the cDNA did not result in any improvement in the PCR reaction, in fact, yield was greatly reduced.

Too many amplification cycles can lead to more non-specific amplification products (Kawasaki 1990). The number of cycles was varied between 25 and 35 cycles without any difference in the number and relative yield of the resultant bands. Consequently 35 cycles was normally used, as this would result in a greater amount of DNA produced. Any increase over this number of cycles increases the amount of time required and became less convenient.

Despite attempts to increase the specificity of the primer by increasing the annealing temperature, changing the $MgCl_2$ concentration, lowering the primer concentration, decreasing the amount of template and reducing the number of cycles of the PCR reaction, the 800 bp band predominated.

U335 and D335 are degenerate primers, their total degeneracy in combination exceeds 10^7 (384 x 36864) (Langeveld et al, 1991). It is possible that suitable sequences exist that flank the target segments. If this is the case, then the desired target sequences may be present within the 800 bp fragment. If this is so, then reamplifying the PCR product should result in a 335 bp product, (see Figure 3.19 (a)).

Reamplification was carried out using U335 and D335 as primers. This resulted in a high yield of a 335 bp product but in some cases other bands were present including the 800 bp fragment (Figure 3.18). Reamplification of PCR products was subsequently performed to obtain good yields of the desired product size. The desired band was excised from the gel and the DNA extracted as described in Chapter 2 (section 2.10.2.).

An alternative scenario is that the D335 primer is not amplifying efficiently, and that the 800 bp product results from U335 and RLFS-9. Excess RLFS-9 could be present with the cDNA from the reverse transcriptase step. The expected product of U335 and RLFS-9 is 800 bp. Reamplification with U335

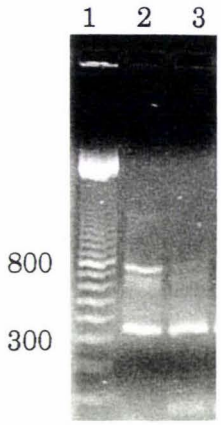

Figure 3.18 : Reamplification of PCR products using the primers D335 and U335. Lane 1, Pharmacia 100-base pair ladder, lane 2, previously amplified DsMV-Z, lane 3, previously amplified DsMV-X.

and D335 could then result in a 335 bp product (Figure 3.19 (b)). To test this a PCR reaction was performed using U335 only and no downstream primer. If there was sufficient RLFS-9 remaining with the cDNA from the reverse transcriptase step then an 800 bp product would be expected. No product resulted.

A c800 bp product was obtained when carrying out one amplification step using cDNA of each isolate under study as a template. After a reamplification step a 335 bp product was obtained for DsMV-FL, DsMV-X and DsMV-Z only. No 335 bp product could be obtained for DsMV-H. The annealing temperature was further lowered to 37^{0}C but no 335 bp could be obtained. At no time was a 335 bp product obtained using cDNA

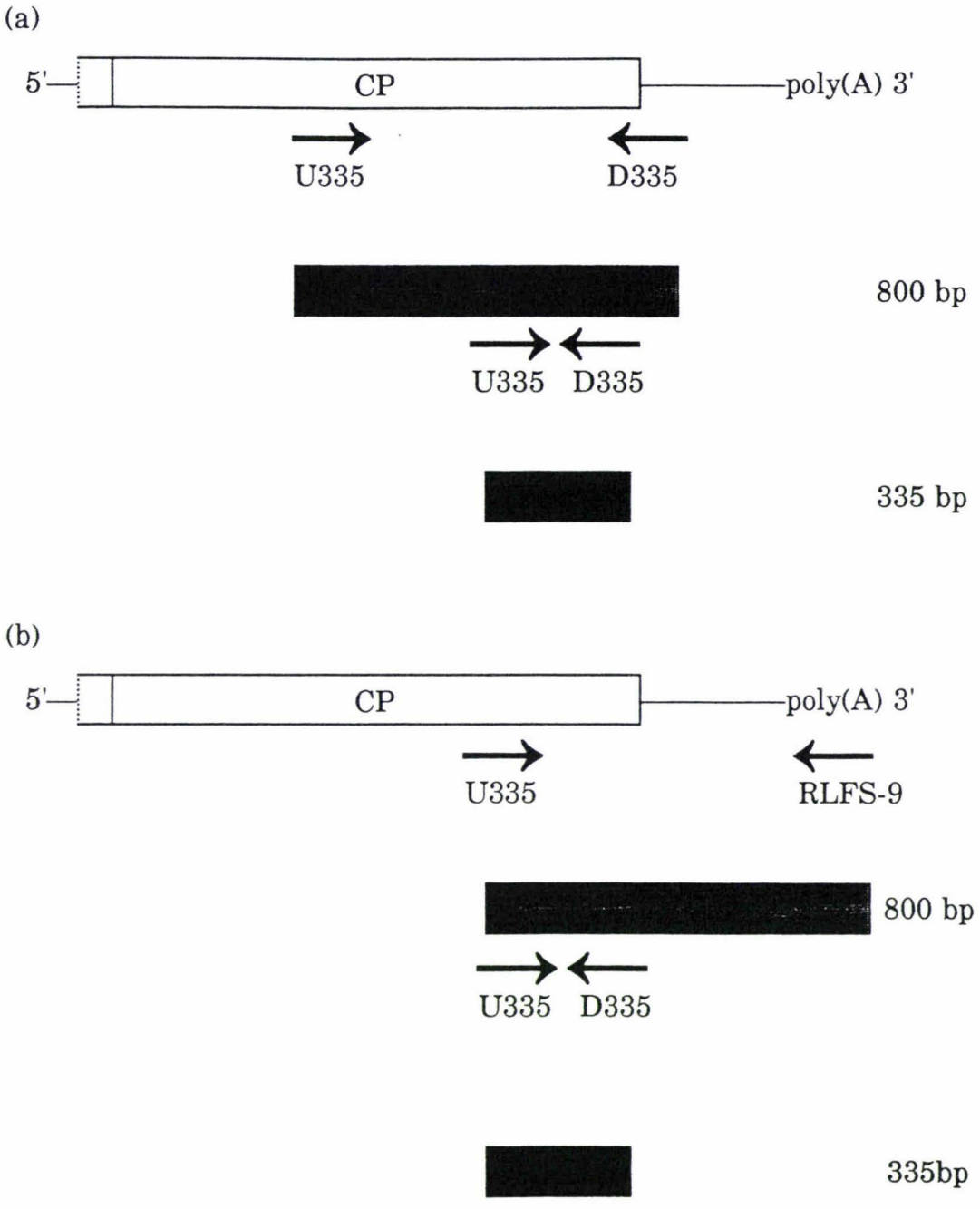

Figure 3.19 : Proposed binding sites of the primers U335 and D335 to cDNA prepared from DsMV RNA. (a) Primers U335 and D335 bind to the cDNA template to give a 800bp fragment. On this fragment U335 and D335 react to give a 335bp fragment. (b) D335 fails to bind, a 800bp fragment results from the binding of U335 and excess RLFS-9 from cDNA preparation to give an 800bp product and on this U335 and D335 react to give a 335bp fragment.

complementary to the viral RNA of DsMV-H, even when reamplification was carried out. This suggests some difference in the genome sequence of DsMV-H.

3.5.3.2. Primers U335 and RLFS-9.

The expected product size was calculated from the published sequences of potyviruses and was estimated to be 800bp. An annealing temperature of 41^{0}C was used as with the primer pairs U335 and D335. The T_m of RLFS-9 was estimated to be 82^{0}C using the formula of Thein and Wallace (1986), well above this annealing temperature.

A 800 bp product was obtained when cDNA of each isolate under study was used as a template. Other bands were also present with some PCR products (Figure 3.20). The desired band of 800 bp was extracted as described in Chapter 2 (section 2.10.2.).

3.5.3.3 Primers JF3 and D335 or RLFS-9.

The expected product size for the primer pairs JF3/D335 and JF3/RLFS-9 were calculated from the published sequences of potyviruses and were estimated to be 1500 bp and 1900 bp respectively.

Several attempts at PCR, with various preparations of cDNA, using these primer combinations, had limited success. A product of the expected size using the primer combination JF3/D335 was obtained when cDNA of DsMV-Z was used as a template (Figure 3.21), but none were obtained when cDNA of DsMV-FL, DsMV-H and DsMV-X were used. No products were obtained using JF3/RLFS-9 as a primer pair.

The absence of PCR products may be due to either incomplete cDNA synthesis (Langeveld et al, 1991) or a difference between the upstream primer

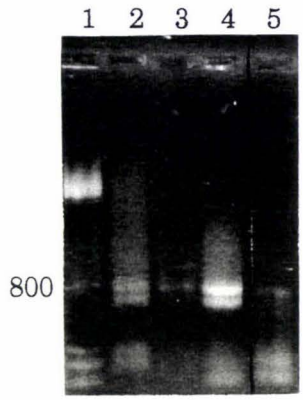

Figure 3.20 : Amplification of cDNA of DsMV-FL, DsMV-X, DsMV-Z and DsMV-H using the primers U335 and RLFS-9. Lane 1, Pharmacia 100 base-pair ladder, lane 2 DsMV-FL, lane 3,DsMV-X, lane 4, DsMV-Z and lane 5, DsMV-H.

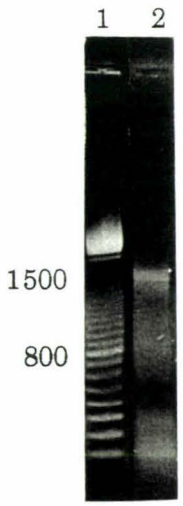

Figure 3.21 : Amplification of cDNA of DsMV using the primers JF3 and D335. Lane 1, Pharmacia 100 base pair ladder, lane 2, DsMV-Z.

(JF3) sequence and the viral sequences. A potential problem with the reverse transcriptase reaction is the premature termination along the RNA template which leads to the production of incomplete cDNA molecules (Nicolas and Laliberté, 1991). However, this is unlikely to be the reason for the absence of PCR products using the primer pair JF3/RLFS-9 with RNA of DsMV-Z since this cDNA did provide a suitable template for the JF3 primer when using the primer pair JF3/D335. It is possible the PCR parameters such as annealing temperature, the length of time of each cycle step, $MgCl_2$ concentration or the concentration of the dNTPs were unsuitable and led to an inefficient reaction.

Premature termination of cDNA synthesis could explain the lack of products with the JF3 primer for the isolates DsMV-FL, DsMV-X and DsMV-X. It was previously noted that the primer D335 failed to bind to cDNA of DsMV-H and therefore the possibility of JF3 not binding due to differences in the viral genome of DsMV-H must be considered.

3.5.4. Cloning and sequencing of PCR products.

Given the differences that were observed with the serological relationships among the four isolates studied and the lack of a PCR product when using cDNA of DsMV-H as a template in conjunction with the primer pair U335 and D335 it was decided to clone and then sequence the 800 bp product resulting from the PCR using the primer pair U335 and RLFS-9. The product will contain a portion of the coat protein sequence and the entire 3' non-coding region.

The non-coding region of the 3' end of the potyviral genome differs considerably in sequence for different potyviruses but has a high degree of homology between strains (Frenkel et al, 1992). By determining the degree of homology between the sequences of each isolate a relationship between them may be determined. A difference in the coat protein sequence in the

region where the primer D335 is complementary is possible for DsMV-H and sequencing should pinpoint any dissimilarities.

The PCR products were cloned as described in Chapter 2 (section 2.12.). DNA was obtained from the minipreparations and was screened for successful insertion of PCR product by cutting the vector with the restriction enzyme Pst I. The linearised DNA was analyzed by agarose gel electrophoresis. Any plasmids containing PCR product will be larger and hence migrate slower. No difference was observed in the migration rate of the linearised DNA, hence these initial attempts to clone the PCR products were unsuccessful. There was insufficient time to repeat the procedure.

3.6. DISCUSSION.

The ELISA studies suggest there are distinct serological differences between the four virus isolates studied. The direct double-antibody sandwich method used has a narrow specificity that allows the differentiation of strains and sometimes other forms of the same virus (Koenig and Paul, 1982). Figure 3.22 summarises a proposed relationship between the isolates based on the ELISA results.

There was no obvious relationship between DsMV-X and DsMV-Z (Figure 3.10 and 3.11) although both appear to be related to DsMV-FL (Figure 3.9). It appears that DsMV-X and DsMV-Z have epitopes in common with DsMV-FL but not with each other. Variations in symptom expression in *Zantedeschia* sp 'Pink Persuasion' infected with DsMV-X and DsMV-Z were noted. Coupling this with the serological differences is it is possible these are two separate strains of DsMV.

Differences were also reflected in the coat protein components resolved by SDS-PAGE (Figure 3.15). However, the outcome of the SDS-PAGE analysis needs to be treated with caution as potyvirus coat proteins are susceptible to

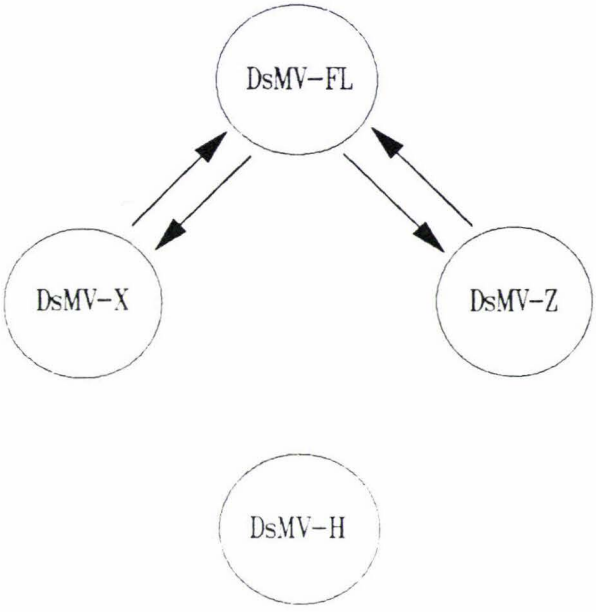

Figure 3.22 : Proposed serological relationship between four isolates of dasheen mosaic potyvirus (DsMV) isolated from *Caladium candidum* cv candidum (DsMV-FL), *Colocasia esculenta* cv 'Haapu' (DsMV-H), *Xanthosoma* sp (DsMV-X) and *Zantedeschia* sp 'Galaxy' (DsMV-Z).

degradation during viral preparation and storage (Francki, Milne and Hatta, 1985).

No serological relationship was observed between DsMV-H and the other three isolates (Figure 3.12). In addition, genomic diversity is apparent. At no time did the primer D335 anneal with cDNA of DsMV-H despite changing the cycling parameters and the reaction components. The modal length of DsMV-H was notably longer and variations in symptom expression accompanying DsMV-H infection were apparent. Given this evidence it appears that the virus present in *Colocasia esculenta* is different from dasheen mosaic potyvirus and is tentatively named feathery mottle potyvirus.

The edible aroids such as *C. esculenta* are an exceptionally diverse group of plants, consisting of numerable cultivars grown by many different ethnic groups throughout the world (Plucknett, de la Pena and Obrero, 1970). Although the isolate observed in *C. esculenta* in this study appears to differ, DsMV has been identified serologically in *C. esculenta* cultivars in wide geographical locations including Queensland (Greber and Shaw, 1986), South Africa (van der Meer, 1985), Egypt (Mostafa, Abo El-Nil and Zettler, 1976), the United Kingdom (Hill and Wright, 1980), China (Zettler, Tsai, Faan, Ke and Lu, 1987) and Florida (Abo El-Nil et al ,1977). Given that *C. esculenta* has been cultivated for many centuries, in locations that are often isolated from other plantings, it is not unlikely that another potyvirus has been identified.

Although this virus did not react in the Agdia potyvirus group test other members of the potyvirus group have not tested positive. Langeveld et al (1991) confirmed that nerine yellow strip virus and nerine virus Y were members of the potyvirus group using PCR despite both viruses not reacting positively to the Agdia potyvirus group serum.

Obtaining the sequence for coat protein portion and 3' untranslated region (UTR) of the genome would clarify whether DsMV-H is another distinct virus. The sequencing of the 3' UTR of DsMV-X and DsMV-Z for comparison with the published 3' UTR sequence of DsMV-FL (Pappu et al, 1993) would confirm whether they are distinct strains.

CHAPTER FOUR

CYTOPLASMIC INCLUSION PROTEINS OF DASHEEN MOSAIC POTYVIRUS

4.1 INTRODUCTION.

Cytological studies of virus-infected tissue show that many plant viruses induce recognisable structures in the cytoplasm or nucleus. These structures, called inclusion bodies, may accumulate in substantial amounts in infected plant cells.

Potyviruses induce the formation of characteristic pinwheel or scroll-shaped inclusions in the cytoplasm. These inclusions are an aggregation of a protein monomer of approximately 70 kDa (Matthews, 1992). Purcifull, Hiebert and McDonald (1973) demonstrated that these are virus-specific proteins distinct from the respective virion capsid protein and from host proteins.

A significant proportion of the genetic content of potyviruses encodes for the inclusion protein. Consequently, it could be assumed that the inclusion proteins have essential functions and the elucidation of these functions may be important in understanding how viruses act as pathogens.

The binding (and hydrolysis) of nucleoside triphosphates such as adenosine triphosphate (ATP) is an essential step for the function of proteins and protein complexes involved in diverse biochemical processes. Adenosine triphosphatase (ATPase) activity was shown by Lain et al (1991) and Eagles et al (1994) to be expressed in plants infected with plum pox virus (PPV) and tamarillo mosaic virus (TaMV) respectively, members of the potyvirus group

of positive-strand RNA viruses. In each instance the ATPase activity copurified with the cytoplasmic inclusion protein of the virus.

In this study, cytoplasmic inclusion (CI) protein from dasheen mosaic potyvirus (DsMV) was purified and the ATPase activity investigated to determine whether a similar association occurred for this potyvirus.

4.2 MATERIALS AND METHOD.

4.2.1. Plant material and virus isolates.

The source of plant material and isolates of DsMV are as described in Chapter 2 (section 2.1.). At the time of this study the isolate of DsMV from *Colocasia esculenta* cv 'Haapu', that is DsMV-H, had not been acquired.

Forty to fifty grams of mature leaves were collected from both healthy and infected plants, for CI purification.

4.2.2. CI protein purification.

Dasheen mosaic potyvirus CI proteins of each isolate, DsMV-FL, DsMV-X and DsMV-Z, were purified using a modified technique of Hiebert et al (1984). This method is based on the large size of the cytoplasmic aggregates formed by the potyvirus CI proteins and on their stability to shear forces and organic solvents.

All stages of the purification procedure were performed at 4^0C. Leaf tissue was homogenized in a Waring blender for two minutes with 100ml of a cold solution containing 0.33M potassium phosphate buffer, pH 7.5, 0.17% (w/v) sodium sulphite (w/v) and 0.53M urea. Twenty five millilitres of carbon

tetrachloride and 25 ml chloroform were then added and the tissue homogenized for a further minute.

The homogenized material was then centrifuged at 1000g for 5 minutes in a Sorvall® RC5 centrifuge using a GSA rotor. The pellet was discarded. The supernatant (S1) was centrifuged at 13,000g for 15 minutes using a GSA rotor.

The resultant supernatant (S2) was maintained on ice and the pellet (P2) was resuspended in 25 ml of a cold solution containing 50 mM potassium phosphate buffer, pH 8.2 and 0.1% 2-mercaptoethanol (v/v). The resuspended pellet (P2) was centrifuged at 27,000g for 15 minutes in a Sorvall® RC5 centrifuge using a SS34 rotor.

The resultant supernatant (S3) was maintained on ice and the pellet (P3) was resuspended in 1.0 ml of 20mM potassium phosphate buffer, pH 7.5, + 0.1% 2-mercaptoethanol (v/v).

Two hundred microlitres of the resuspended pellet (P3) were layered onto 5.2ml sucrose gradients made up of 1.30 ml each of 80%, 70%, 60% and 50% sucrose (w/v) in a Beckman SW 55 tube (Catalogue No. 326819). The gradients were centrifuged at 70,000g for one hour then fractionated by dropwise collection of 200 ul aliquots.

Healthy leaf tissue was purified until the resuspended P3 pellets were obtained.

4.2.3. Protein determination.

The protein concentration of S1, S2, S3, P2, P3 and every second sucrose fraction for each isolate were determined using the Bio-Rad Protein Assay Kit

II (Catalogue No. 500-0002). This kit provided a microassay procedure using a bovine serum albumin (BSA) standard. The samples and diluted Bio-Rad Protein Assay Dye Reagent were loaded into a microtitre plate and the absorbance read on a microplate reader at 595 nm. The protein concentrations were determined using the resultant standard curve from the (BSA) protein standards.

4.2.4. Gel electrophoresis.

Samples from each purification step; S1, S2, P2, S3 and P3, for both healthy and infected tissue were analyzed by electrophoresis on a SDS-polyacrylamide gel (4% stacking, 12% running), as described in Chapter 2 (section 2.5.1). For infected tissue a sample collected from the gradient where the peak was estimated to occur was also included.

Twenty microlitres of S1, S2, S3 and 10ul of P2, P3 and the sucrose fraction were diluted with loading buffer containing 62.5mM Tris, pH 6.8 + 10% glycerol + 2% SDS + 5% 2-mercaptoethanol + 0.01% bromophenol blue to give a final volume of 30ul. The sample mixture was held at room temperature for 10 minutes prior to loading on to the gel. Size comparisons were made with a Bio-Rad SDS-PAGE protein standard set (Catalogue No. 161-0309).

Gels were stained in 0.1% Coomassie Brilliant Blue R-250 (w/v) in 40% methanol/10% acetic acid (v/v) for approximately 60 minutes. The gels were destained in 40% methanol/10% acetic acid (v/v) until the bands were clearly differentiated and the background a pale blue.

Samples from sucrose gradient fractions were analyzed by SDS-PAGE in the same manner. Gels were silver stained using a Bio-Rad Silver Staining Kit (Catalogue Nos 161-0443 - 7).

4.2.5. ATPase activity assay.

The ATPase activity was determined in S1, S2, S3, P2, P3, for each isolate and every second sucrose fraction for DsMV-X. An aliquot from each purification step equivalent to 2.5 ug CI inclusion protein was diluted in 20mM Tris-HCl, pH 8.2 to give a total volume of 20ul. For each sucrose fraction a 20 ul aliquot was taken. To each sample was added 25 ul of buffer consisting of 300mM NaCl, 10mM KCl, 3mM $MgCl_2$, 40 mM HEPES (*N*-2 hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) pH 7.0, 2mM dithiothreitol, and 2 mM PMSF (phenylmethylsulfonyl fluoride). Five microlitres of 1.0 uM [γ-^{32}P]ATP was added giving a final volume of 50 ul. The ATPase reaction was carried out at 37^{0}C for 30 minutes. Four samples containing no protein (20ul H_20, 25 ul buffer and 5 ul 1.0 uM [γ-^{32}P]ATP) were also incubated.

The reaction was stopped by adding to each sample 400 ul of 7% activated charcoal (w/v) in 50 mM HCl and 5 mM orthophosphoric acid. Samples were then centrifuged at 14,000g for 10 minutes in a Heraeus Sepatech Biofuge before duplicate 50 ul aliquots were removed.

The ATPase activity was determined by measuring the amount of [^{32}P] released from the [*Y*-^{32}P]ATP during incubation with the CI protein using a Beckman LS 2800 open window scintillation counter.

The average of background counts per minute recorded from the reactions containing no protein were subtracted from the results of all other reactions.

4.3 RESULTS AND DISCUSSION.

SDS-PAGE analysis of samples from each step of the purification process of DsMV-infected tissue revealed a major band in P2 and P3 and the sucrose fraction as shown in Figure 4.1. The position of the bands are very closely

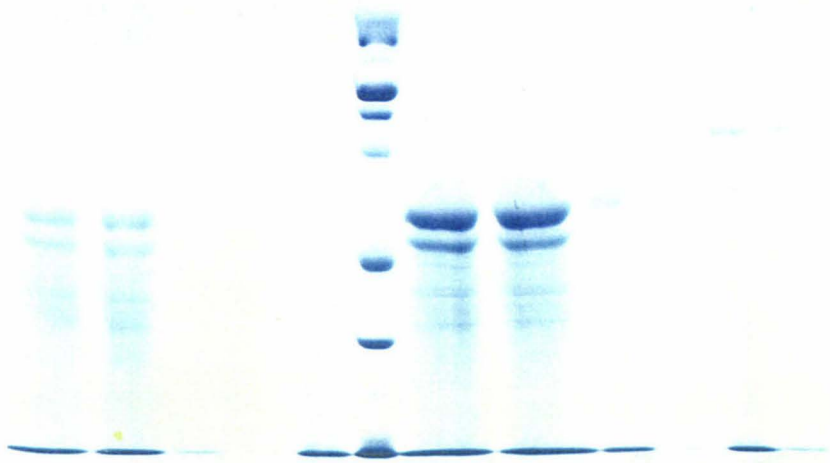

Figure 4.1 : SDS-PAGE analysis of samples from healthy and DsMV-Z infected *Zantedeschia* sp leaf tissue collected from each step during purification of CI proteins. Lanes 1-5: S1, S2, P2, S3 and P3 fractions from the purification of healthy *Zantedeschia* sp tissue. Lane 6: Bio-Rad SDS-PAGE broad range protein standard set. Lanes 7-12: S1, S2, P2, S3, P3 and sucrose fraction 19 from the purification of DsMV-Z infected *Zantedeschia* sp 'Galaxy' leaf tissue.

aligned with the protein standard of MW 66,000. Such a band was absent from healthy tissue samples.

Figures 4.2 - 4.4 show the protein concentration and ATPase activity in each purification step up to the P3 fraction for each isolate of DsMV.

The protein concentration is high in the S1 fraction, probably due to the presence of plant and other viral proteins in addition to the viral CI proteins. Most of these additional proteins are discarded in the supernatant of the next purification step (S2) leaving a much smaller amount of protein in the pellet (P2).

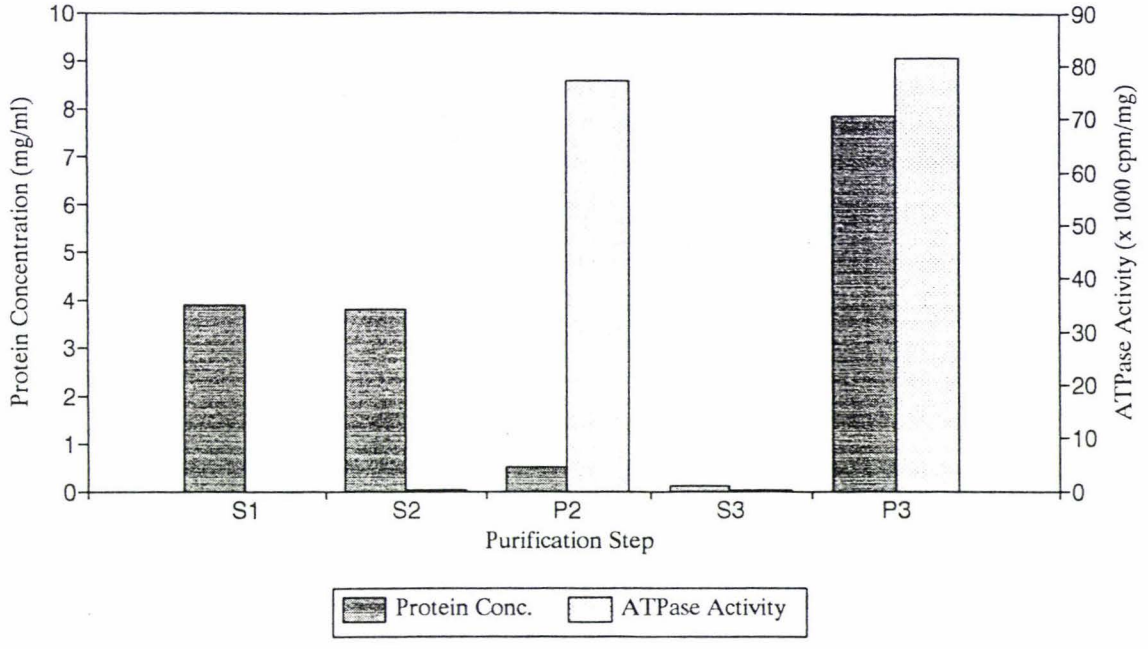

Figure 4.2: Protein concentration and ATPase activity in samples collected from each step during purification of CI proteins from DsMV-FL infected *Caladium candidum* leaf tissue.

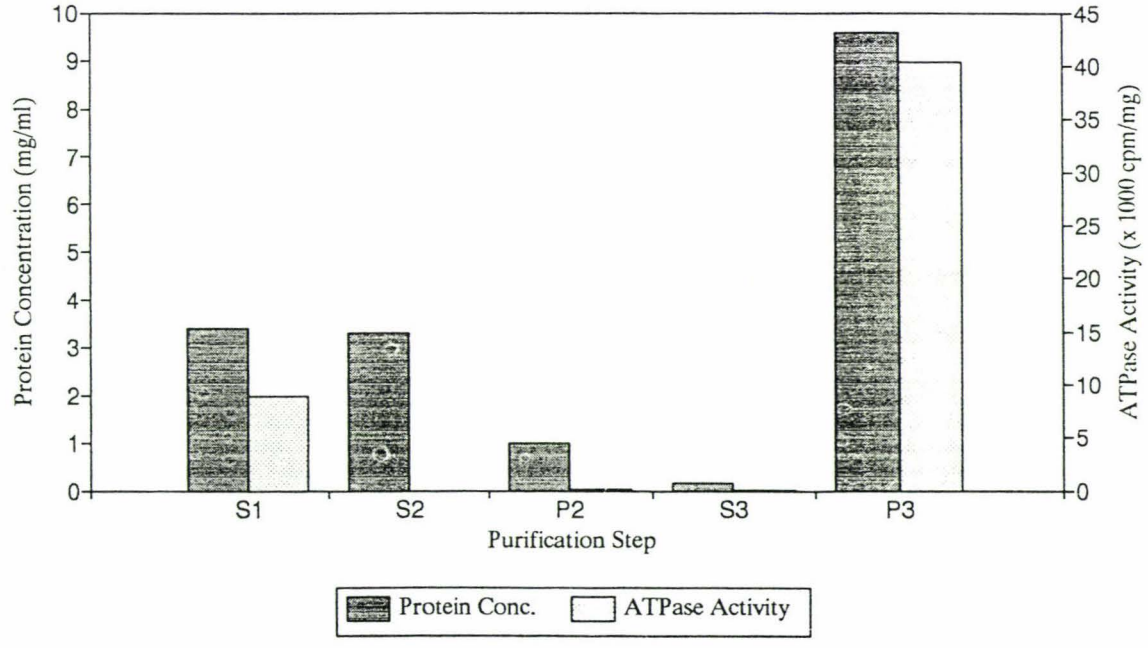

Figure 4.3: Protein concentration and ATPase activity in samples collected from each step during purification of CI proteins from DsMV-X infected *Xanthosoma* sp. tissue.

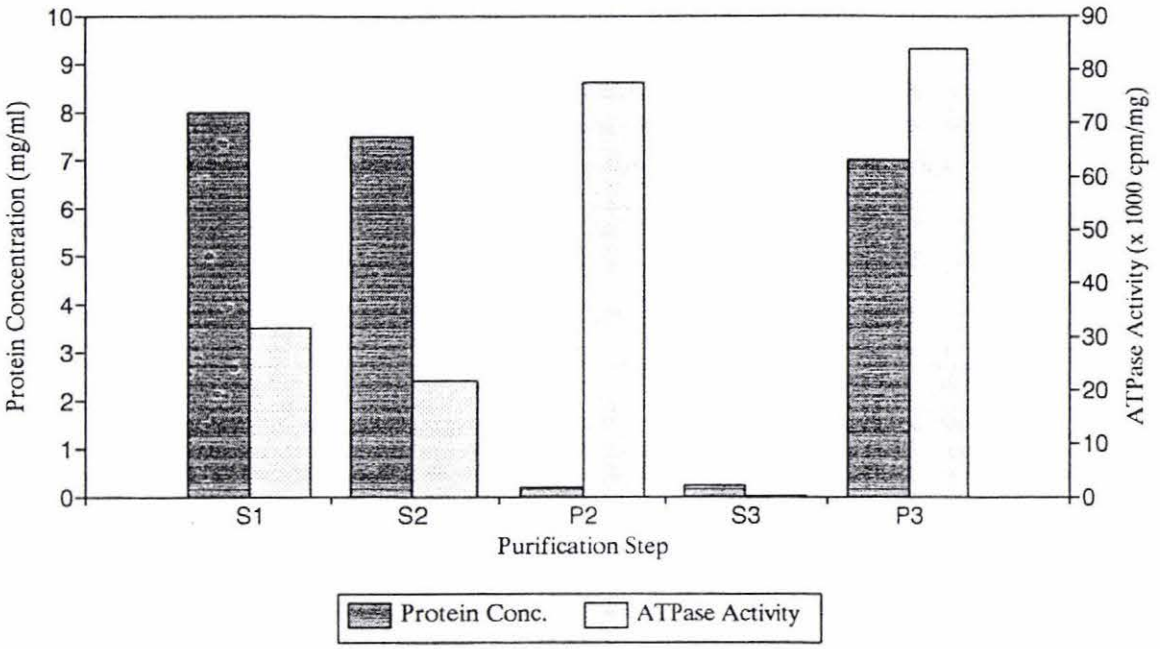

Figure 4.4: Protein concentration and ATPase activity in samples collected from each step during purification of CI proteins from DsMV-Z infected *Zantedeschia* sp. leaf tissue.

The next centrifugation step serves to further concentrate the remaining proteins. This can be seen in Figures 4.2 - 4.4 with the dramatic increase in protein per millilitre of sample in the pellet P3. The SDS-PAGE analysis of the purification steps (Figure 4.1) shows that in addition to the major band of approximate MW 68,000 other proteins are also present in the P3 pellet. Further purification of the P3 pellet by sucrose density gradient centrifugation resulted in a reduction of these additional bands.

Some ATPase activity is present in the S1 fraction from tissue infected with DsMV-X and DsMV-Z (Figure 4.3 and 4.4). This activity is reduced or absent in the next supernatant (S2), indicating that the discarded proteins have little or no ATPase activity associated with them. Figure 4.2 shows no activity in the S1 fraction from tissue infected with DsMV-FL. The ATPase activity may not be detectable due to low levels.

ATPase activity is much higher in the P2 and P3 pellets and the ATPase enzyme appears to copurify with the proteins in the sample. However, in Figure 4.3 it is observed that the ATPase activity is low in the P2 fraction.

This is unexpected and does not agree with the results obtained for the other two isolates. Since the ATPase level is high in the P3 fraction, it is possible that the P2 sample was prepared incorrectly.

The protein concentration and ATPase activity in samples from each purification step of healthy tissue was negligible (data not shown).

Figure 4.5 illustrates the relationship between protein concentration and ATPase activity in the sucrose fractions collected from the gradient overlaid with 200ul of the P3 sample from the tissue infected with DsMV-X. The level of ATPase activity appears to be related closely with the level of protein in each fraction. The higher than expected ATPase activity detected in fractions from the top of the gradient could be due to small amounts of non-aggregated CI protein and/or to another protein component in the sample (Lain et al, 1991).

Figure 4.6 shows the SDS-PAGE analysis of sucrose fractions collected from the gradient overlaid with 200ul of the P3 sample obtained from the tissue infected with DsMV-X. The intensity of the major bands corresponds to the protein concentration determined for each fraction as shown in Figure 4.5.

The data presented shows that an association between CI protein and ATPase activity occurs with dasheen mosaic potyvirus in a similar manner to that found by Lain et al (1991) with plum pox potyvirus CI protein and Eagles et al (1994) with tamarillo mosaic potyvirus CI protein.

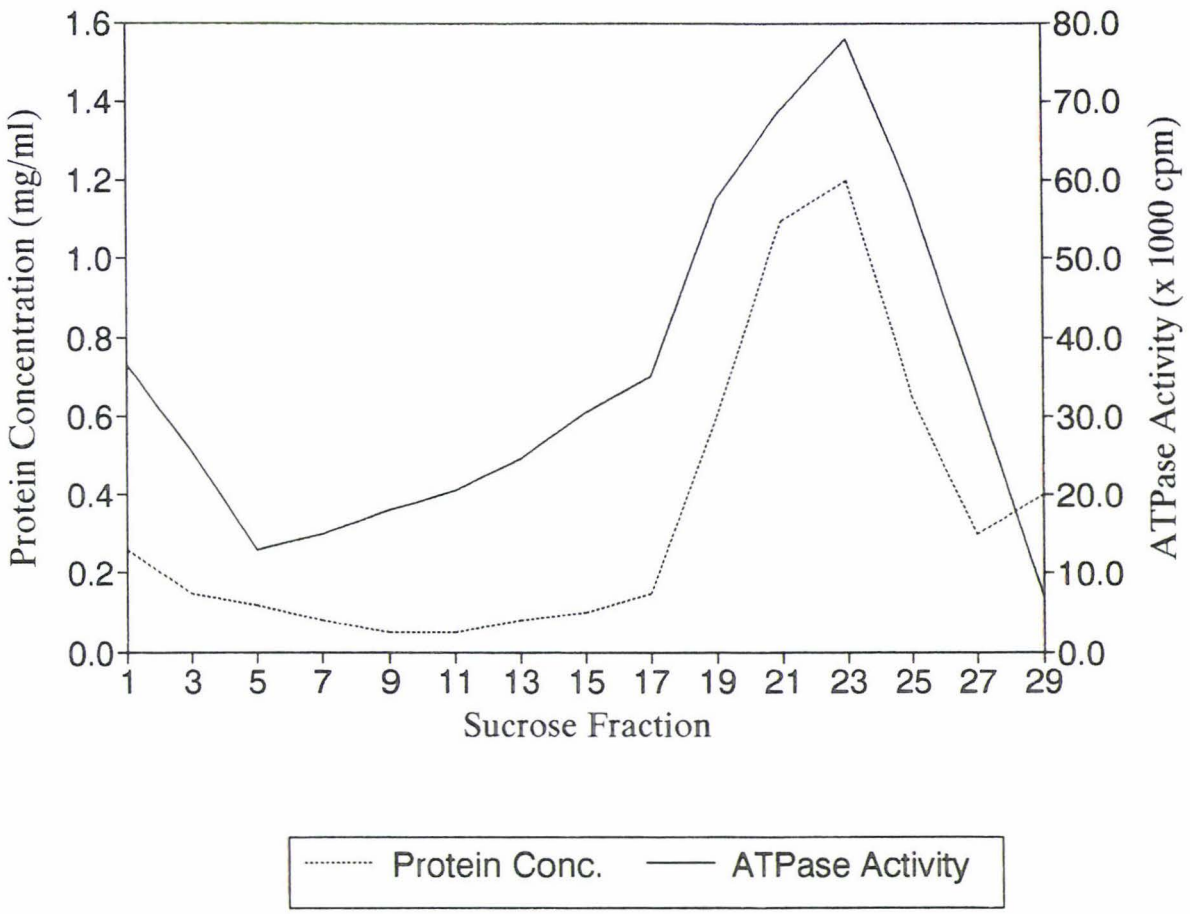

Figure 4.5: Protein concentration and ATPase activity in samples collected from sucrose gradient fractions during purification of CI proteins from DsMV-X infected *Xanthosoma* sp. leaf tissue.

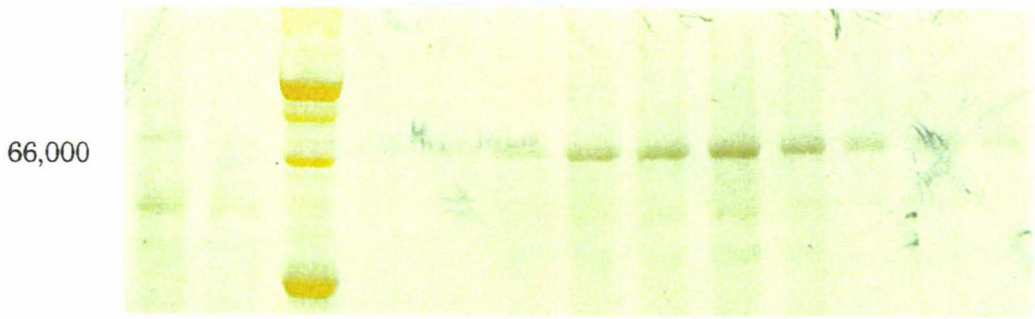

Figure 4.6: SDS-PAGE analysis of samples collected from sucrose gradient fractions during purification of CI proteins from DsMV-X infected *Xanthosoma* sp. leaf tissue. Lanes 1 and 2: Sucrose fractions 1 and 5. Lane 3: Bio-Rad SDS-PAGE broad range protein standard set. Lanes 4-13: Sucrose fractions 9, 11, 15, 17, 19, 21, 23, 25, 27, and 29.

Essentially all the main biochemical processes including DNA replication, recombination, repair and transcription, protein synthesis, membrane transport and signal transduction are coupled to nucleoside triphosphate (NTP) hydrolysis (Gorbalenya and Koonin, 1989). Enzymes catalysing the hydrolysis of NTP to NDP + P*i*, to provide the energy source essential for such biochemical processes, play an important role in the control of these biochemical activities.

The amino acid sequence of proteins encoded by many RNA viruses of plants and animals have been determined. A number of these proteins contain what is called a nucleoside triphosphate binding motif (NTBM) (Walker, 1982). In many instances proteins that contain this motif are believed to possess helicase activity. That is, they are involved in the unwinding of RNA duplexes using nucleoside triphosphate hydrolysis as an energy source (Geider and Hoffmann-Berling, 1981).

Potyvirus CI proteins contain a putative nucleoside triphosphate binding motif (Gorbalenya and Koonin, 1989). To determine whether these CI proteins have the ability to unwind RNA, Lain et al (1990) and Eagles et al (1994), incubated ^{32}P labelled double-stranded RNA with CI proteins and ATP. SDS-PAGE and autoradiography showed that the dsRNA was separated into two strands.

Potyviruses have single-stranded(ss) RNA genomes. However, all nucleic acids replicate by engendering a complementary copy of themselves. With positive-sense ssRNA viruses such as potyviruses, the viral polymerase mediates the synthesis of a complementary strand of RNA resulting in the presence of a double strand of RNA. The unwinding and separation of the nucleic acid strands during replication is thought to be aided by proteins exhibiting helicase activity.

The evidence presented above showing ATPase activity associated with the CI protein of DsMV, and the studies reported by Lain et al (1990) and Eagles

et al (1994) suggest that the NTBM-containing CI proteins have a helicase activity. That is, they are involved in RNA unwinding using nucleoside triphosphate hydrolysis as an energy source. This activity indicates that CI proteins of potyviruses are involved in viral genome replication.

CHAPTER FIVE

SURVEY

5.1. INTRODUCTION.

Five different viruses have been recorded as occurring in *Zantedeschia* spp. These are cucumber mosaic cucumovirus (Chamberlain, 1954), dasheen mosaic potyvirus (Zettler, Foxe, Hartman, Edwardson, and Christie, 1970), potato X potexvirus (Okuyama and Saka, 1976), tobacco mosaic tobamovirus (Gorter, 1981) and tomato spotted wilt tospovirus (Tompkins and Severin, 1950). With the exception of dasheen mosaic potyvirus (DsMV), which appears to be limited to aroid species, strains of all these viruses are present in a wide range of crops in New Zealand.

Virus-like symptoms have been observed in commercial crops of zantedeschias in New Zealand. A limited survey of commercial plantings was carried out to give an indication of the incidence of virus infection, and which of the above viruses or previously unrecorded viruses could be detected.

5.2. SURVEY METHOD.

Seven properties where zantedeschias were grown for cutflower or tuber production were chosen for study. Three properties were in the Bay of Plenty, the others were in Auckland, Manawatu, Horowhenua and Christchurch. At each property at least three tubers were chosen at random by the grower for virus testing. These tubers were forwarded to the author and subsequently raised in a greenhouse at the Plant Growth Unit, Massey University. The tubers were grown as described in Chapter 2 (section 2.1.). Two tubers received from the Bay of Plenty were infected with *Penicillium* spp and failed to grow. One tuber from Christchurch developed soft rot

caused by *Erwinia carotovora*. Consequently, these tubers were not tested (see Table 5.2).

Plants were tested for the presence of virus infection by :

a) Electron microscopy.

b) Enzyme-linked immunosorbent assay (ELISA).

c) Isolation of dsRNA.

d) Mechanical inoculation to herbaceous indicator plants.

5.3. ELECTRON MICROSCOPY.

5.3.1. Direct investigation of leaf tissue.

Samples from each plant were prepared for observation under the electron microscope as described in Chapter 2 (section 2.3.1.). Most filamentous plant viruses including potato X potexvirus (PVX), tobacco mosaic tobamovirus (TMV) and DsMV should be readily detected using this method.

5.3.2. Rapid immune electron microscopy.

The Derrick technique (Derrick, 1972, 1973) combined with decoration (Yanagida and Ahmad-Zadeh, 1970), as developed by Milne and Luisoni (1977), was used to confirm the identity of certain virus particles observed in survey samples.

Antiserum diluted 1:100 was applied to a formvar-carbon-coated copper grid and left for 5 minutes. The grid was washed with 20 drops of 0.1M sodium phosphate buffer pH 7.0, then a drop of leaf sap diluted in the same buffer was applied and held on the grid for 15 minutes. The grid was then rinsed once more in 20 drops of buffer and the edge of the grid was touched to filter paper to remove excess buffer. A further drop of antiserum diluted 1:100 as

before was added to the grid, held for 15 minutes, washed with buffer as before, followed by washing with 30 drops of water. Five drops of 2% PTA, pH 7.0 : 2% AM, pH 5.6 (1:1) was added, held on the grid for one minute, then the excess removed by touching the edge of the grid to filter paper. The grid was then observed in a Philips 201C electron microscope.

5.4. ENZYME-LINKED IMMUNOSORBENT ASSAY.

5.4.1. Double antibody sandwich ELISA (DAS-ELISA).

The double antibody sandwich method of Clark and Adams (1977) was used to test samples for tomato spotted wilt tospovirus (TSWV). The DAS-ELISA tests were conducted as described in Chapter 2 (section 2.4.2.3.). Coating and conjugated gamma-globulins to the lettuce strain of TSWV were obtained from Agdia Inc., Indiana, USA. These were used at the recommended dilution of 1:1000.

5.4.2. Indirect ELISA.

An indirect method of ELISA (Koenig, 1981) was used to test samples for the presence of potyviruses, specifically DsMV. The procedure, using the Agdia potyvirus group test (Jordan, 1989), involves coating plates with plant sap and detecting the virus with a potyvirus specific immunoglobulin (IgG), followed by an anti-IgG enzyme conjugate.

The indirect ELISA method was as follows. (Note : all buffers were the same as those described in Chapter 2, section 2.4.2.3.) :

1. Samples were extracted in 100 volumes of coating buffer. Duplicate 100ul aliquots of each sample were added to the appropriate wells.

The plates were incubated at room temperature for 60-90 minutes in an airtight container lined with wet tissue paper.

2. The plates were washed by flooding the wells with PBS-tween and left for 3-5 minutes before emptying. This was repeated twice before the plates were left to drain on a paper towel.

3. A 100ul aliquot of gamma-globulin diluted 1:100 in reagent buffer was added to each well. The plates were incubated at 4^0C overnight in an airtight container lined with wet tissue paper.

4. The plates were washed as described in 2.

5. A 100ul aliquot of the alkaline phosphatase conjugated anti-mouse IgG (Silenus Laboratories, Catalogue No. 5041DAP0591) diluted 1:500 in reagent buffer was added to each well. The plates were incubated at room temperature for 60 minutes in an airtight container lined with wet tissue paper.

6. The plates were washed as described in 2.

7. 150ul of the substrate buffer containing 0.67mg/ml *p*-nitrophenyl phosphate was added to each well. The plates were left at room temperature for 60 minutes and the absorbance of each well was determined at 410nm using a Dynatech MR5000 ELISA reader.

5.5. ISOLATION OF DOUBLE-STRANDED RNA (dsRNA).

A modification of the method of Morris, Dodds, Hillman, Jordan, Lommel and Tamaki (1983) was used to isolate dsRNA from leaf tissue harvested from plants used in this survey. Leaf tissue was powdered in liquid air using a

mortar and pestle, and stored at -20°C until required. Fifteen millilitres of extraction buffer [STE (0.05M NaCl, 0.025M Tris, 0.5mM EDTA) containing 1% SDS (w/v), 0.1% 2-mercaptoethanol (v/v)and 0.5mg/ml bentonite], 15ml STE-saturated phenol (500g phenol, 100ml STE and 0.6g 8-hydroxy-quinilone) and 2ml chloroform:pentanol (25:1) was added to 4g powdered tissue.

The mixture was shaken vigorously at 4°C for 30-60 minutes prior to centrifugation at 12,000g for 10 minutes in a Sorvall® SS34 rotor. The aqueous phase was removed and 0.18ml ethanol and 0.0125g Whatman CF11 cellulose per millilitre of aqueous phase was added, shaken at 4°C for 10 minutes, then centrifuged at 6000g for 10 minutes in a Sorvall® SS34 rotor.

After centrifugation the supernatant was decanted to waste, 5ul of 15% ethanol in STE added, shaken, then poured into a 10ml disposable syringe with a circle of miracloth at the base. The cellulose held in the syringe by the miracloth was washed with 30-35ml 15% ethanol in STE. dsRNA was then eluted from the cellulose with the addition of 4ml of STE at 37°C. The dsRNA was precipitated with the addition of two volumes of ethanol containing 0.2M sodium acetate followed by storage at -20°C overnight or until required.

The dsRNA was pelleted by centrifugation at 12,000g for 10 minutes in a Sorvall® SS34 rotor. The ethanol was removed and the pellet dried under vacuum. The pellet was resuspended in 200ul TAE buffer (Chapter 2, section 2.10.2.), 6% glycerol (v/v) and 0.1% bromophenol blue (w/v) and stored at -20°C.

The dsRNA was subsequently analyzed by gel electrophoresis as described in Chapter 2 (section 2.5.1.) using a continuous 5% acrylamide gel. This method is capable of readily detecting the presence of cucumber mosaic cucumovirus (CMV) dsRNA (Morris et al, 1983).

5.6. MECHANICAL INOCULATION TO HERBACEOUS INDICATORS.

Indicator plants that were generally sensitive to a wide range of viruses were selected for mechanical transmission and were known to be local lesion hosts of, or capable of becoming systemically infected by some of the viruses reported as infecting zantedeschias. Plants were raised in a modified bark mix (see Appendix 1) and maintained in a temperature controlled glasshouse unit at $20^0C \pm 5^0C$ until required. After inoculation they were maintained in a temperature controlled glasshouse unit at $15^0C \pm 5^0C$. Information on the indicator plants used, including their age and stage of development at inoculation, is given in Table 5.1.

Virus inoculum was prepared by grinding freshly harvested selected leaf tissue with a pestle in a mortar containing a small quantity of Celite® and 10 volumes (w/v) of 0.2M potassium phosphate buffer, pH 8.0 and 2% sodium sulphite (w/v).

Inoculations to test plants were made by gently rubbing leaves with the pestle dipped in the leaf macerate. Inoculated leaves were then rinsed with tap water to remove residual inoculum. At least three plants of each species were mechanically inoculated from each source. Plants were observed every second day to record the presence or absence of symptoms.

To detect possible latent infections leaf samples of each test plant were prepared for electron microscopy as described in Chapter 2 (section 2.3.1.) or macerated in 0.2M potassium phosphate buffer, pH 8.0 and 2% sodium sulphite (w/v) and inoculated to *Chenopodium quinoa*.

Table 5.1: Indicator plants used in mechanical inoculation tests of survey material for detection of viruses in *Zantedeschia* spp.

Indicator Plants	Age[1]	Development
Capsicum frutescens L. Sweet capsicum. [Solanaceae]	5w	2-4 leaves
Chenopodium quinoa L. [Chenopodiaceae]	8w	6 leaves
Cucumis sativus L. Cucumber. [Cucurbitaceae]	10d	cotyledons
Datura stramonium L. Jimson weed. [Solanaceae]	5-8w	1-2 leaves
Gomphrena globosa L. 'Little Buddy'. [Amaranthaceae]	10w	2-4 leaves
Lycopersicum esculentum Mill. [Solanaceae]	5w	2-4 leaves
Nicotiana tabacum L. Tobacco, 'Havana'. [Solanaceae]	5w	3-4 leaves
Nicotiana tabacum L. Tobacco, 'Samsun'. [Solanaceae]	5w	3-4 leaves
Nicotiana tabacum L. Tobacco, 'White Burley'. [Solanaceae]	5w	3-4 leaves
Phaseolus vulgaris L. French bean. [Leguminosae]	10d	2 leaves
Pisum sativum L. Garden pea. [Leguminosae]	12d	4 leaves
[1] Age at inoculation : w = weeks, d = days.		

5.7. RESULTS.

A summary of the results is presented in Table 5.2.

Table 5.2: Results of a survey to detect viruses in commercial plantings of *Zantedeschia spp.*

Origin		EM	ELISA		dsRNA	Indicator Plants
			Potyvirus	TSWV		
Auckland	# 1	-	-	-	-	-
	# 2	-	-	-	-	-
	# 3	-	-	-	-	-
	# 4	+[1]	-	-	-	+[1]
	# 5	+[1]	-	-	-	+[1]
	# 6	-	-	-	-	-
	# 7	-	-	-	-	-
	# 8	+[1]	-	-	-	+[1]
	# 9	+[1]	-	-	-	+[1]
	# 10	+[1]	-	-	-	+[1]
Bay of Plenty - Site A	# 1	+[2]	+	-	-	-
	# 2	NT	NT	NT	NT	NT
	# 3	NT	NT	NT	NT	NT
Bay of Plenty - Site B	# 1	-	-	-	-	-
	# 2	-	-	-	-	-
	# 3	+[1]	-	-	-	+[1]
Bay of Plenty - Site C	# 1	-	+	-	-	-
	# 2	+[1]	-	-	-	+[1]
	# 3	-	-	-	-	-
Christchurch	# 1	+[2]	+	-	-	-
	# 2	+[2]	+	-	-	-
	# 3	NT	NT	NT	NT	NT
Horowhenua	# 1	+[2]	-	-	-	+[2]
	# 2	+[2]	+	-	-	-
	# 3	+[2]	+	-	-	-
	# 4	+[2]	-	-	-	+[2]
Manawatu	# 1	+[1]	-	-	-	+[1]
	# 2	+[1]	-	-	-	+[1]
	# 3	+[1]	-	-	-	+[1]

[1]Virus particles of 300nm length. [2]Virus particles of 690-750nm length. NT = not tested.

5.7.1. Electron microscopy.

Rod-shaped particles c300nm in length, with similar morphology to TMV (see Figure 5.1), were observed in plant samples from four of the seven properties surveyed. A microprecipitin test (Ball,1990) was used to confirm that particles observed were TMV. Positive microprecipitin reactants were analyzed by electron microscopy as follows:

A drop of positive microprecipitin reactant was placed on a formvar-carbon-coated copper grid and left for one minute. Excess liquid was removed by touching the edge of the grid to filter paper. A drop of negative stain (2% PTA pH 7.0:2% AM pH 5.6 (1:1)) was placed on the grid and left for one minute. Excess liquid was removed by touching the edge of the grid to filter paper. Grids were examined with a Phillips 201C electron microscope. Examination of positive microprecipitin reactants by electron microscopy showed particles coated with antiserum (see Figure 5.2).

Tobacco mosaic tobamovirus is known to be present in the seed coat of tomato seed (Taylor, Grogan and Kimble, 1961). The possibility of TMV being present in the seed coat of zantedeschia seed was investigated.

Ten batches of ten seed were soaked overnight in 0.5% $Na_2HPO_4.7H_20$ (w/v). The seed coats were removed and ground up in a mortar and pestle with 0.5% $Na_2PO_4.7H_2O$ (w/v). A drop of liquid from each batch was mixed with a drop of negative stain and applied to separate formvar-carbon-coated copper grids as described in Chapter 2 (section 2.3.1.). Grids were examined with a Phillips 201C electron microscope. Particles 300nm in length (see Figure 5.3) were observed on each grid prepared.

Figure 5.1 : Leaf dip of tissue in a 1:1 mixture of 2% potassium phosphotungstic acid (PTA) pH 7.0: 2% ammonium molybdate (AM) pH 5.6 from a *Zantedeschia* sp grown for commercial production in the Bay of Plenty. Note the particles morphologically similar to tobacco mosaic tobamovirus. Bar = 100nm.

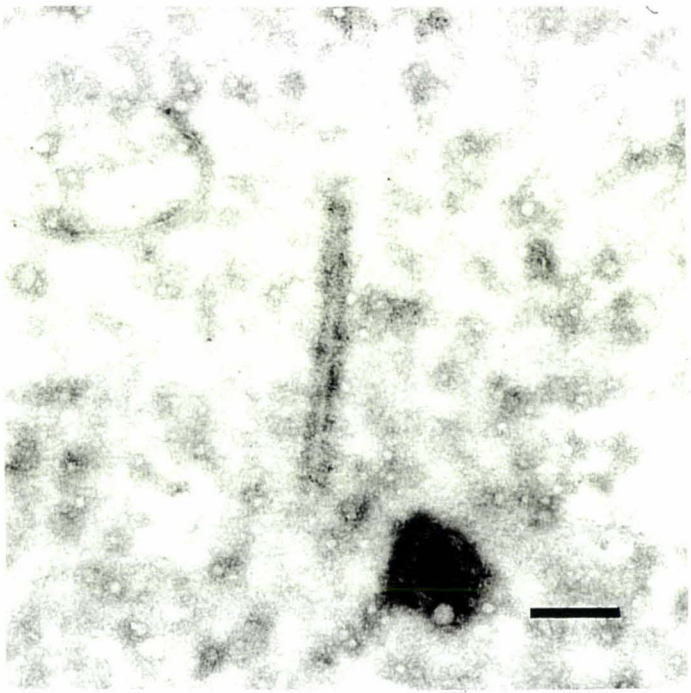

Figure 5.2 : Particle from *Zantedeschia* sp leaf tissue coated with TMV antiserum negatively stained with 2% potassium phosphotungstic acid (PTA) pH 7.0 : 2% ammonium molybdate (AM) pH 5.6 (1:1). Bar = 100nm.

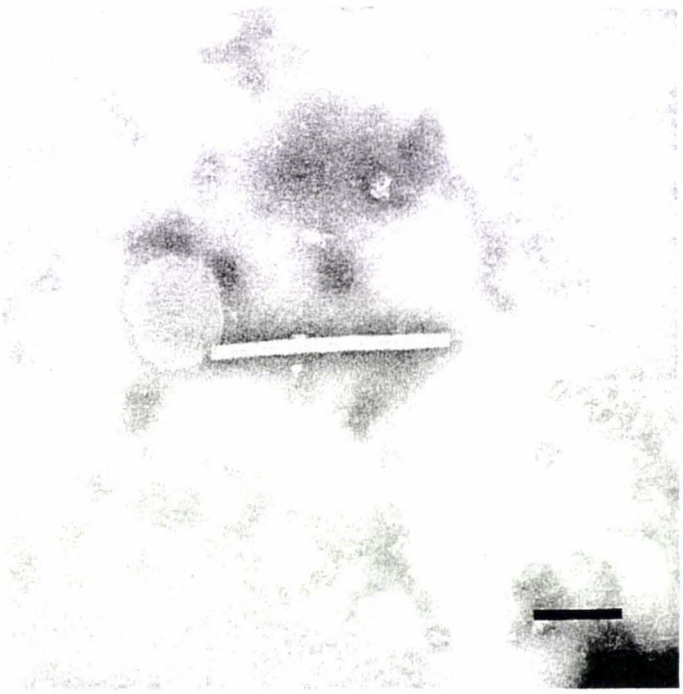

Figure 5.3 : Particle from *Zantedeschia* sp seed coat negatively stained with 2% potassium phosphotungstic acid (PTA) pH 7.0 : 2% ammonium molybdate (AM) pH 5.6 (1:1).
Bar = 100nm.

Larger flexuous rod-shaped particles 690-750nm in length, similar in morphology to that described for potyviruses (Tollin and Wilson, 1988) were observed in plant samples from the remaining three properties surveyed.

There was no site where both particle types were observed in the samples tested.

5.7.2. Enzyme-linked immunosorbent assay.

No samples tested positively to the TSWV antiserum. Samples from plants from three sites tested positive using the anti-potyvirus antiserum. These correlated with plants in which 690-750nm flexuous rods were observed using the electron microscope. This virus was presumed to be DsMV; subsequent

studies with an antiserum to an isolate of DsMV supplied by Dr Z.W. Zettler (Chapter 2, section 2.4.1) confirmed this.

At one property electron microscopic observations revealed flexuous rods *c*700nm in length in samples from two plants. Samples from these plants did not test positive with the Agdia potyvirus antiserum. These particles were mechanically transmissable, and subsequently identified as belonging to the carlavirus group (see section 5.7.4.).

5.7.3. dsRNA extraction.

Samples were loaded onto an acrylamide gel along with dsRNA from known CMV infected plant tissue. No banding patterns matching that for CMV were observed in any survey samples. A banding pattern comparable to that of dsRNA of CMV was obtained for extracts from CMV-infected *Caladium candidum* confirming the technique does work for aroid species.

5.7.4. Mechanical inoculation.

No symptoms were observed in any indicator plants inoculated including *Chenopodium quinoa* used to back test for latent infection. However, electron microscopic observations showed the presence of rod-shaped particles in *N. tabacum* 'Havana', *N. tabacum* 'Samsun', *N. tabacum* 'White Burley', *Phaseolus vulgaris*, and *Datura stramonium* plants as indicated in Table 5.2.

Two distinct types of rod-shaped particle were observed. One type was c300nm in length and occurred in indicator plants inoculated with samples shown by the electron microscope to contain similar particles. Longer particles (*c*700nm in length) were found in *N. tabacum* 'Havana' (Figure 5.4)

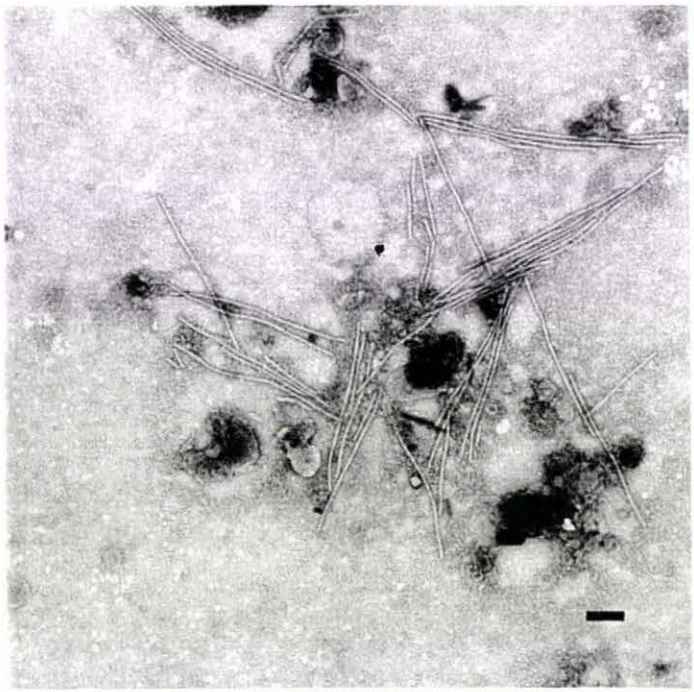

Figure 5.4 : Leaf dip of tissue in 2% potassium phosphotungstic acid (PTA) pH 7.0 : 2% ammonium molybdate (AM) pH 5.6 (1:1) from *Nicotiana tabacum* 'Havana' inoculated with leaf sap from a *Zantedeschia* sp grown for commercial production at a Horowhenua property. Bar = 100nm.

inoculated with samples shown by electron microscopy to be infected with particles of a similar length. As DsMV is not readily inoculated to plants outside the *Araceae* family (Chapter 1, section 1.3.1.) and samples from the plants used to inoculate the indicator plants did not react positively to the anti-potyvirus antiserum (see Table 5.2), it appears that a virus previously unrecorded in *Zantedeschia* spp was present. The particles were of a similar length to carlaviruses. A microprecipitin test using carnation latent carlavirus antiserum (Antiserum No. 4. Department of Plant Science, Massey University), the 'type' member of the carlavirus group was performed. After 48 hours flocculent precipitates were observed in all reactant combinations up to a dilution of 1:128. Slight precipitates were observed in reactant combinations containing healthy sap up to an antiserum dilution of 1:4.

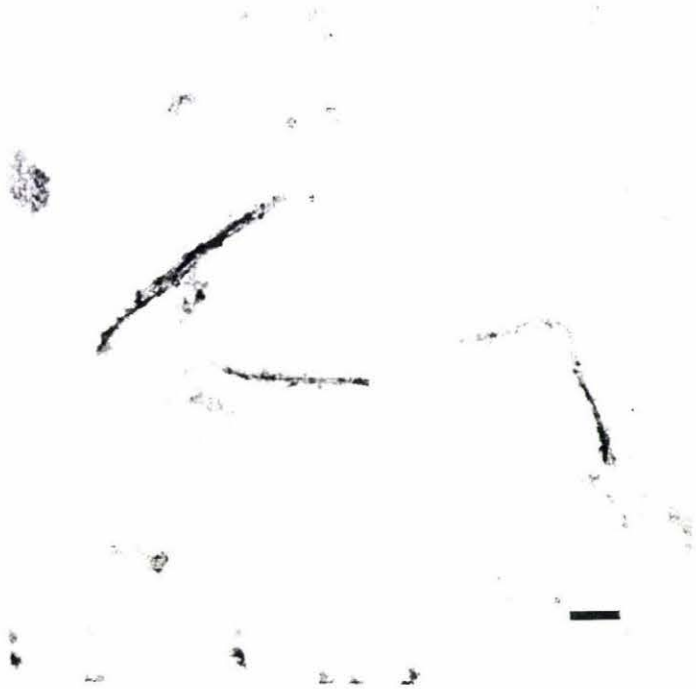

Figure 5.5: Leaf tissue extracts, from a *Zantedeschia* sp. grown for commercial production at a Horowhenua property, treated according to the decoration technique of Milne and Luisoni (1977) using antiserum to carnation latent carlavirus. Note the particles decorated with antiserum. Bar = 100nm.

To further confirm the identity of the particles a rapid immune electron microscopy technique was performed using the antiserum described above. Particles were decorated with antiserum (Figure 5.5).

5.8. DISCUSSION.

Although only a limited survey was carried out virus infection was detected in samples from all sites sampled, including the detection of two viruses previously unrecorded in zantedeschias in New Zealand.

DsMV was found in samples from three of the seven sites surveyed. At two of these sites the zantedeschias were grown for cutflower production.

Division of tubers was used to increase plant numbers, a system which can lead to an increase in virus infection (Wright and Hill, 1980). At the other property zantedeschias were grown for tuber production. Tissue culture was used as a means of increasing tuber numbers. This technique is reported to eliminate DsMV (Zettler and Hartman, 1986) and so the incidence of DsMV should be unlikely. However, the tissue culture techniques used may not have been sufficient to eliminate virus infection, or the tubers could have been subsequently infected by vectors carrying DsMV.

At the sites where DsMV was not detected plants were hybrid callas propagated by tissue culture or native species grown from seed. DsMV is apparently not seedborne (Zettler and Hartman, 1986).

Although TSWV and CMV are reported to occur in New Zealand neither of these were detected in any of the samples collected.

TMV was found in samples from four sites surveyed. Little mention is made in the literature of this virus infecting *Zantedeschia* spp. Gorter (1981) reported the infection of *Z. aethiopica* with TMV, and no other record is apparent of TMV infecting any other member of the *Araceae*.

At three of the properties where TMV was detected plants were propagated by tissue culture. It is possible that TMV is not eliminated by this technique; indeed its spread could be facilitated by mechanical means during the excision of material for culture. At the remaining property plants were propagated from seed and it was this seed source in which TMV particles were found in the seed coat. Its presence in the seed coat of zantedeschias provides a potentially effective way for widespread distribution in the crop of this virus.

The occurrence of TMV in zantedeschia should not be a surprise, given the whole host range and resilience of this virus. Smith (1972) suggests that TMV has an almost unlimited host range and is the cause of many plant virus diseases.

The detection of a carlavirus was unexpected. No viruses from this group have been reported in any member of the *Araceae* family.

CHAPTER SIX

DISCUSSION

Until this study, no other potyvirus has been reported to infect members of the *Araceae*. Strong evidence was obtained to show that the virus isolated from *Colocasia esculenta* was distinct from dasheen mosaic potyvirus (DsMV) and is tentatively named taro feathery mottle potyvirus. Genome sequencing of at least the 3' untranslated region or preferably the region encoding the coat protein would confirm this. It is often assumed that virus particles observed in the electron microscope in *Araceae* extracts resembling potyviruses are DsMV. This assumption is no longer valid and a more definitive means is required to determine the identity of such particles. Added to this, a carlavirus was detected in *Zantedeschia* sp during the survey described in chapter five. The particles are very similar to that of potyviruses when observed in the electron microscope. Serological techniques would be necessary to distinguish these two groups of viruses.

Serologically distinct strains of DsMV were apparent amongst the isolates investigated. No strains of DsMV have been previously noted, although minor differences in symptoms induced by single isolates from Florida and Fiji in *Philodendron selloum* were observed (Abo El-Nil et al, 1977). With the antisera prepared in this study no serological relationship could be detected between the isolate from *Zantedeschia* sp 'Galaxy' and the isolate from *Xanthosoma* sp. This was coupled with a distinct variation in symptom expression.

Care in the choice of antiserum would be required when using the direct double antibody sandwich method of ELISA. This technique has a narrow specificity (Koenig and Paul, 1982), and could fail to detect some strains. In

this study the antiserum to the Florida isolate of DsMV detected all three isolates of DsMV studied. Conversely, the antiserum to the isolate from *Xanthosoma* sp only reacted to the homologous and Florida isolates whereas the antiserum to the isolate from *Zantedeschia* sp 'Galaxy' only reacted to its homologous isolate and the Florida isolate.

Indirect ELISA methods detect a broader range of serologically related viruses (Koenig and Paul, 1982). The Agdia anti-potyvirus group test (Jordan, 1989) detected all three isolates. However, it did not detect the virus present in *C. esculenta* cultivars. Certain other potyviruses also fail to react to this serum (Langeveld et al, 1991).

The Agdia anti-potyvirus group test is the major means by which callas are tested in New Zealand for the presence of DsMV (Cohen, pers comm). Given that variable results have been obtained due to variations in virus titre, that it has been found that DsMV is not the only potyvirus present in *Araceae* species, and that taro feathery mottle potyvirus does not react to the Agdia anti-potyvirus serum, an alternative needs to be sought.

A considerable improvement in the detection of virus particles in aroid sap using the electron microscope was obtained by using a mixture of negative stains. However, at times virus titre was very low and considerable searching on the electron microscope grid was required. The use of the electron microscope as a routine technique for potyvirus detection is not viable. It is very labour intensive and the purchase and operation of equipment is expensive.

The polymerase chain reaction (PCR) was successfully used in this study to amplify portions of the DsMV genome. Once the technique is set up it is simple and quick to perform and results are easily obtained within a day. However, the setting up of the technique is complex and time consuming.

The conservative approach used in this study of viral purification, RNA extraction and production of first strand cDNA before PCR is not viable for routine indexing.

A highly sensitive assay in which viral RNA obtained from antibody-captured viral particles is used as the template for cDNA synthesis prior to PCR amplification was developed by Wetzel, Candresse, Macquaire, Ravelonandro and Dunez (1992) for the detection of plum pox potyvirus. Low levels of virus and uneven distribution in infected stone fruit trees led to the need for a highly sensitive detection method. This immunocapture method was found to be 5000 times more sensitive than ELISA. It also eliminated the need to purify the virus and extract the viral RNA thereby considerably simplifying the preliminary procedures necessary for PCR and largely reducing the time required. If this technique can be developed for the detection of potyviruses in callas it would be more suited for routine indexing. The PCR amplification step has already proved to be suitable for the detection of DsMV and taro feathery mottle potyvirus.

A considerable portion of the genetic content of potyviruses encodes for cytoplasmic inclusion protein. The elucidation of their function may be important in understanding how viruses act as pathogens.

The cytoplasmic inclusion (CI) proteins of DsMV had an ATPase activity associated with them, indicating that the role of these proteins in RNA replication is similar to that proposed for the CI proteins of tamarillo mosaic potyvirus (Eagle et al, 1994) and plum pox potyvirus (Lain et al, 1991). The binding and hydrolysis of nucleoside triphosphates is an essential step for the function of protein and protein complexes involved in diverse biochemical processes.

Although a limited survey was undertaken in this study, at all sites surveyed virus infection was detected. This included the detection of two viruses, tobacco mosaic tobamovirus and a carlavirus, not previously recorded in New Zealand as infecting callas. It appears that infection of calla tubers is widespread despite the use of tissue culture for propagation.

To maintain the quality of calla exports and avoid reductions in productivity control measures are needed to, if not eliminate, then limit infection. The practice of increasing tuber numbers by natural division will require monitoring to ensure viral infection is not also propagated.

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APPENDIX ONE

Composition of bark-based growing media used to raise indicator and araceous plants.

Use	Fertiliser			Substrate blend
	Brand Name	Rate (kg/m^3)	Rate (g/100l)	
Indicator plants	Dolomite	3.0	300	100% bark
	Agricultural lime	3.0	300	
	Iron sulphate	0.5	50	
	Ca ammonium nitrate	1.0	100	
	Osmocote Plus 15-4.8-11.3	4.5	450	
Araceous plants	Dolomite	3.0	300	100% bark
	Agricultural lime	3.0	300	
	Iron sulphate	0.5	50	
	IBDU	1.0	100	
	Osmocote Plus 16-3.5-10.8	4.0	400	

APPENDIX TWO

Curve generated by plotting the log of the molecular weight of the broad range standards versus their mobility in 12% acrylamide.