PURIFICATION, CHARACTERIZATION AND cDNA CLONING OF TWO FILAMENTOUS VIRUSES FROM *NERINE*

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ABSTRACT

Virus-like symptoms were evident in many of the clonally mass propagated lines of the bulbous ornamental *Nerine*, cultivated in New Zealand for cut flower and bulb production. Although filamentous virus particles were common in electron microscope investigation of *Nerine* tissue, cucumber mosaic virus was the only virus mechanically transmitted to herbaceous indicators.

Two of the filamentous viruses from *Nerine* which were not amenable to mechanical transmission were purified from field-infected *Nerine* tissue, characterized and cDNA cloned in this study. An isolate of nerine virus X (NeVX), a potexvirus, was purified from *Nerine fothergilli* 'Major' leaf tissue showing no virus-like symptoms. An isolate of a hitherto unnamed and uncharacterized potyvirus was purified from clonally mass propagated *Nerine sarniensis* hybrid leaf tissue showing severe yellow mosaic symptoms prior to senescence. Nerine virus Y (NeVY) is the name proposed for this potyvirus.

The NeVX isolate was a slightly flexuous filamentous particle with a normal length of 540nm. The molecular weight of the single coat protein subunit was 29.5kd and the size of the genomic RNA was 6.3kb. The NeVX RNA *in vitro* translation profile resembled those of the potexviruses potato virus X and daphne virus X with a major 180kd nonstructural protein band and a number of minor bands without a viral coat protein band.

Double-stranded cDNA to the 6.3kb genomic RNA was cloned into the *Pst* I site of the plasmid vector pBR322. Nick-translated pBR322 DNA containing a 1.8kb insert, representing 28.5% of the viral genome, was found to be highly specific and sensitive to NeVX isolates in dot-blot assays. The cloned cDNA probe did not hybridize to seven other potexviruses, including a ca 540nm potexvirus from *Agapanthus* which, on the basis of serological reactivity, was previously described as an agapanthus strain of NeVX. A survey using the cloned cDNA in dot-blot assays of *Nerine* species indicated that NeVX was prevalent in *Nerine* plants cultivated in different parts of the North Island of New Zealand.

Nerine virus Y was found to be a typically flexuous potyvirus with a normal length of 800nm, a single coat protein subunit with a molecular weight of 33.26kd and a genomic RNA of 10.0kb. Nerine virus Y-infected tissue had characteristic Type I potyvirus cylindrical inclusion bodies. Size fractionated viral RNA was cloned into the lambda vectors gt10 and L47AB. Two cDNA clones of size 1.54kb and 0.56kb derived from lambda gt10 and

a 9.8kb cDNA clone derived from lambda L47AB were subcloned into the plasmid vector pGEM3. In dot-blot assays cDNA from all clones hybridized to the homologous virus but not to nerine yellow stripe virus, another more common potyvirus in nerines. The 1.54kb cDNA cloned probe was found to be highly specific and sensitive and did not hybridize in dot-blot assays to four other potyviruses. A survey using the 1.54kb cloned cDNA probe in dot-blot assays indicated that NeVY was not common in *Nerine* species cultivated in different parts of the North Island of New Zealand.

The use of recombinant technology made it possible to develop highly sensitive and specific diagnostic tools for two filamentous viruses from *Nerine*. These cloned cDNA probes were found to be well suited for conducting field surveys to study the prevalence of the virus. The probes could be used in subsequent studies in screening for viral resistance and in virus elimination studies. Further, the cloned cDNAs may in the future prove useful in the characterization of the genomes of NeVX and NeVY.

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CHAPTER 1

VIRUSES IN THE GENUS *NERINE*

1.1 INTRODUCTION

Nerine Herb. is a genus of perennial bulbous plants belonging to the Amaryllidaceae family. The Amaryllidaceae is quite a diverse family with some 85 genera and about 1100 species (Hickey & King,1981). Some of the other genera of this family of principally ornamental plants include: *Narcissus, Hippeastrum, Alstroemeria, Hymenocallis, Crinum, Galanthus, Brunsvigia, Vallota, Lycoris, Zephyranthus, Leucojum, Cyrtanthus, Spiloxene,* and *Sternbergia*. Although members of the Amaryllidaceae have originated from a range of locations around the world, the 40 or so *Nerine* species are all indigenous to southern Africa. Details on the botanical and historical aspects of *Nerine* are presented in Sections A1 and A2 of Appendix I.

Some of the *Nerine* species have a high ornamental value as cut flowers with good keeping quality. The best known of these species are *N.bowdenii* W. Wats. and *N.sarniensis* L. ('Guernsey Lily'), comprising 85% of the nerines grown in the Netherlands (Fortanier *et al*,1979), the world's largest producer of this relatively new commercial crop. The total area under cultivation in the Netherlands is about 60 hectares (WPI,1987). On a much smaller scale, *Nerine* is also grown in Western Europe, Peru, Portugal, Columbia, South Africa, United States, Japan, Australia and New Zealand. Further information on the commercial cultivation of *Nerine* is given Sections A3 and A4 of Appendix I.

In New Zealand there has been increasing recent interest in the cultivation of various *Nerine* species and their hybrids for cut flower production for the local and export market. Like many other bulbous ornamentals (eg *Lilium* and *Narcissus*) selected *Nerine* cultivars are propagated vegetatively through daughter (offset) bulbs, twin-scales or micropropagation. However, as in most other vegetatively propagated bulbous species studied, pathogens are frequently disseminated on bulbs and virus diseases are prevalent (Moore,1979; Chastagner & Byther,1985).

1.2 VIRUSES IN *NERINE* - A LITERATURE REVIEW

1.2.1 Chronological presentation of research

The literature on viruses occurring in *Nerine* is somewhat scanty with only six published papers (Hakkaart,1972; Koenig *et al*,1973; Hakkaart *et al*,1975; Maat,1976; Maat *et al*, 1978 and Phillips and Brunt,1980). All of these were published by researchers in the Netherlands and in England. There have been several minor sections on viruses of nerines in the annual reports of the Glasshouse Crops Research Institute (GCRI) in Littlehampton, England and K S Milne (Department of Plant Health, Massey University, New Zealand) has corresponded with F A Hakkaart (Research Institute for Plant Protection (IPO), Wageningen, Netherlands), D Z Maat (IPO), and A A Brunt (GCRI, now known as the Horticultural Research Institute) respectively on this subject. The following section considers the research findings chronologically.

Brunt *et al* (1970) reported that electron microscopic investigations of several *N.bowdenii* and *N.sarniensis* hybrid cultivars revealed the presence of three classes of viruses with particle lengths of: ca 550nm, 650nm and 750nm. It was noted that these filamentous viruses often occur as a complex. The 750nm virus was assumed to be narcissus yellow stripe virus (NaYSV), previously detected in *Narcissus*.

Koenig *et al* (1973) reported finding 550nm and 660nm particles in naturally infected *N.bowdenii* 'Rose Queen'. Several attempts to transmit viruses from nerines showing conspicuous leaf mottling to indicator plants, using crude sap as the inoculum, failed. Purified virus preparations, however, consistently induced a few local lesions in *Gomphrena globosa* L. which on subculture induced local and systemic infection in *G.globosa* and local infections in *Chenopodium amaranticolor* Coste & Reynier and *Phaseolus vulgaris* L. The symptoms in these indicator plants were typical of those induced by narcissus mosaic virus (NaMV). The normal length of particles from infected *G.globosa* leaves was almost identical to NaMV in *G.globosa*. The purified virus from *N.bowdenii* and those from mechanically inoculated *G.globosa* were serologically indistinguishable from NaMV. The purified virus from *N.bowdenii* did not react with an antiserum to NaYSV, nor to antisera to eight carlaviruses; namely cactus virus 2, carnation latent, chrysanthemum B, narcissus latent, passiflora latent, potato virus M, potato virus S and red clover vein mosaic. Using SDS-PAGE Koenig *et al* also showed that the coat protein of the 550nm virus from *N.bowdenii* behaved

identically to those of NaMV but differed in coat protein mobility from a number of other potexviruses. It was concluded that the 550nm potexvirus in *N.bowdenii* was an isolate of NaMV.

Hakkaart *et al* (1975) reported that electron microscopic studies of clarified sap and partially purified preparations mainly from *N.bowdenii* revealed filamentous particles of ca 545, 660 and 780nm long. For the 660nm particle the name nerine latent virus (NeLV) was proposed earlier (Hakkaart,1972), because they were first found in *N.bowdenii* plants without virus symptoms. In this study NeLV was present in both leaves with mosaic symptoms, and symptomless leaves. An antiserum was prepared to this virus and it was found to react with clarified extract from nerine containing NeLV. Clarified extract containing the 545nm and 780nm particles did not react to antisera to NaMV and to NaYSV, respectively. From leaf samples containing the 545nm particles no virus other than cucumber mosaic virus (CMV) could be transmitted to *G.globosa*, *C.amaranticolor*, *Chenopodium quinoa* Willd., or *Nicotiana clevelandii* Gray., plants which are hosts to NaMV. No consistent association was found between any of these viruses and the symptoms observed. In *N.bowdenii* seedlings, none of the virus particles mentioned in this study were found. However, virus particles were found in meristem tip cultures of the infected nerine plants.

Maat (1976) described two potexviruses in nerines. The source plants in this study were *N.sarniensis* and *N.manselli* Hort. showing various types of symptoms. The virus particles in *N.sarniensis* had an average particle length of 541nm. From crude preparations of nerine tissue no virus could be transmitted. Seven partially purified preparations of *N.sarniensis* were tested on *G.globosa*, *C.amaranticolor*, *C.quinoa*, and *N.clevelandii*. No symptoms were induced and only in extracts of *C.quinoa* some PVX-type particles were observed. With one exception, the virus could not be further transmitted to *C.quinoa*. Virus particles were also observed in two out of ten *N.bowdenii* seedlings inoculated with partially purified preparations.

The filamentous virus in *N. manselli* had an average particle length of 554nm. Two partially purified virus preparations from *N.manselli*, at pH 7 and pH 9, were tested for infectivity. *G.globosa*, *C.amaranticolor*, *C.quinoa* showed local lesions 10-12 days after inoculation. Symptoms on *N.clevelandii* were described as not clear. *G.globosa* and *N.clevelandii* became systemically infected by the virus.

Serological studies using micro-precipitin tests showed that the homologous antisera for the two nerine viruses could be used to detect viruses in clarified extracts of leaves as well as

flowers of their respective nerine hosts. The two viruses were found to be distantly related to each other and to PVX, and the virus in *N.sarniensis* also reacted to clover yellow mosaic virus. The virus in *N.manselli* was closely related to NaMV. Based on host plant reactions and on serological tests the virus from *N.sarniensis* differed from all other well-known potexviruses and in the absence of any symptom association, the name nerine virus X (NeVX) was proposed by Maat. Conversely, the virus from *N.manselli*, on the basis of particle size, host range and serological reactivity, was considered a strain of NaMV.

Brunt (1977) reported that one or more viruses were detected in plants of fourteen out of nineteen *N.sarniensis* cultivars tested. Eight cultivars were found to have NeVX, arabis mosaic virus (ArMV) and/or an undescribed potyvirus. Four cultivars contained NeLV alone, one contained both NeVX and NeLV and another contained NeLV and a potyvirus. NaMV was not detected in any of the plants. It was stated that in the United Kingdom, as in the Netherlands (Maat,1976), NeLV and NeVX are the carlavirus and potexvirus respectively prevalent in nerines.

Phillips and Brunt (1978) reported that NeVX was detected in three *Agapanthus africanus* Hoffmanns plants. Isolates from *Nerine* and *Agapanthus* were reported to be serologically indistinguishable and had identical but restricted host ranges. The virus was described as being very stable *in vitro*, was readily purified and had particles mostly ca 550nm long. No serological relationship could be established with seven similar viruses: clover yellow mosaic virus, cymbidium mosaic, hydrangea ringspot, narcissus mosaic, potato X, viola mottle and white clover mosaic. It was stated that this virus is probably a newly recognised member of the potexvirus group.

Maat *et al* (1978) found virus particles with an average length of 664nm and 780nm in *N.bowdenii* '63' showing no virus symptoms and *N.bowdenii* 'Van Roon' with severe mosaic. Particles with an average length of 660nm, named earlier as nerine latent virus(NeLV), were also found in *N.flexuosa* 'Alba', *N.manselli*, *N.sarniensis* and in *Hippeastrum*. Usually the highest concentration of this class of rods were found in *Hippeastrum*. The virus concentration was found to be much lower in naturally infected *N.bowdenii* '63' than in *N. bowdenii* 'Van Roon'. Mechanical inoculations with crude extracts from *N.bowdenii* were sometimes found to cause 'non-viral yellowing' of test plants thus possibly masking virus symptoms. Local lesions were occasionally induced with crude sap inoculations on *C.amaranticolor*, *G.globosa* and *C.quinoa* but judged to be too inconsistent to be suitable as diagnostic test plants. With partially purified virus preparations the following local lesion symptoms were observed two to three weeks after inoculation:

C.quinoa - small dark green rings or points which became visible three weeks after inoculation on inoculated leaves which turned yellow

C.amaranticolor - tiny, chlorotic, dark-edged lesions developed in the older, still green inoculated leaves three weeks after inoculation

G.globosa - small brown necrotic rings might develop two weeks after inoculation

Nicotiana clevelandii became locally infected without showing symptoms. *Hippeastrum* seedlings were readily systemically infected symptomless hosts, and mechanically inoculated *N.bowdenii* '63' seedlings had a very low level of infection. Antisera was prepared to virus preparations from *N.bowdenii*. The first antiserum prepared was found to contain antibodies to CMV and normal plant antigens. Antisera prepared from virus preparations which had been subjected to two sucrose gradient cycles and one caesium chloride cycle minimized the level of contaminating antibodies. Serologically, using ELISA and the micro-precipitin test, NeLV was found to be indistinguishable from the partially characterized hippeastrum latent virus (HLV) (Brolman-Hupkes,1975). It was closely related to carnation latent virus (CLV) but differed from the latter in host plant reactions. On the basis of serological and host range criteria, Maat *et al* considered HLV to be an isolate of NeLV, and because the description of HLV by Brolman-Hupkes was somewhat tentative, suggested that the name nerine latent virus should have priority to hippeastrum latent virus. This study also highlighted the cultivar differences, in terms of virus susceptibility, between *N.bowdenii* '63' and *N.bowdenii* 'Van Roon'. *Nerine bowdenii* '63' was more resistant to infection, not only to NeLV but also to NeVX, NaMV and two potyviruses. Nerine latent virus was described in this study as having an average particle length of 664nm, a sedimentation coefficient of 155 S and buoyant density in caesium chloride of 1.298g/cm^3. Nerine latent virus was considered as a member of the carlavirus group.

Phillips and Brunt (1980) reported that a potexvirus in *Agapanthus praecox* Leighton subsp. *orientalis* from S.E. England was serologically indistinguishable from NeVX isolated from *N.sarniensis*. The particle size was given as ca 11 X 525-550nm. Unlike NeVX this virus was readily sap transmissable, maintained in test plants and purified without difficulty. It was mechanically transmitted to 14 species, inducing systemic infections in *A.praecox* subsp. *orientalis*, *N.sarniensis* and

C.quinoa and local infections in 11 other susceptible herbaceous species of the following families: Chenopodiaceae, Amaranthaceae, Aizoaceae, Solanaceae, Compositae and Papilionaceae. Based primarily on the serological relationship, Phillips and Brunt concluded that this potexvirus in *Agapanthus* was an isolate of NeVX, although it differed markedly in its ease of isolation from plants and its experimental host range. It was referred to as the *Agapanthus* strain of NeVX (NeVX-A). Because NeVX-A was readily isolated and purified it was partially characterized. The particles sedimented as a single component with a sedimentation coefficient of 120 S, and had a buoyant density in caesium chloride of 1.31g/cm^3. They contained a ca 5% single-stranded RNA with a molecular weight of 2.5 million and a single polypeptide of 25,600. NeVX-A was found to be serologically distantly related to potato virus X, viola mottle virus, hydrangea ringspot virus and commelina virus X and unrelated to nine other distinct potexviruses including NaMV.

Maat (pers comm to Milne,1983) reported that CMV, tobacco rattle virus (TRV) and two flexuous elongate particles of ca 740nm and 800nm were detected in nerines. The 740nm virus was noted to produce local lesions in *Hyoscyamus niger* L. 'Pallides' and reacted with antisera to ornithogalum mosaic virus, potato virus A, celery mosaic virus and lettuce mosaic virus but not with antisera to 14 other potyviruses. The 800nm virus caused yellow stripe symptoms in *N.sarniensis* hybrids. An antiserum was prepared to the 740nm virus. No serological relationship was found between NeLV and NaMV or between NaMV and NeVX.

Brunt (pers comm to Milne,1983) stated that the 750nm virus particle, originally believed to be NaYSV, was recognised as quite distinct and designated it as nerine yellow stripe (NeYSV). He noted that NeLV, NeVX and NeYSV, were common in some stocks, and NaMV and narcissus latent virus (NaLV) were found only rarely. Arabis mosaic virus was found in plants of one cultivar. It was also stated that Olwen Stone (GCRI), obtained virus-free plants of *N.bowdenii* 'Gill' by meristem tip culture, and these grew "superbly well".

1.2.2 Analysis of research

Electron microscopic investigations in most of these studies have revealed filamentous particles in the potexvirus, carlavirus and potyvirus groups. Two distinct potexviruses with particle lengths in the 530-555nm range have been clearly identified. These were NeVX (Maat,1976) and NaMV (Koenig *et*

al,1973; Maat,1976). Reports from the Netherlands and the United Kingdom indicate that NeVX is common in *N.bowdenii* and *N.sarniensis* stocks, whereas NaMV is found rarely. Narcissus mosaic virus has received greater attention in Britain, not only because it is widespread in an economically important crop such as *Narcissus*, but also because it has been found to be amenable to mechanical transmission to a number of herbaceous diagnostic and propagation hosts (Brunt,1966; Mowat,1971). Nerine virus X from nerines, however, has not been readily transmitted to herbaceous hosts. There is only one reported instance of the mechanical transmission of NeVX from *Nerine* tissue to *C.quinoa* in which it multiplied poorly and could not be readily further transmitted to *C.quinoa* (Maat,1976). Researchers have not been able to characterize this virus from nerines.

The finding by Phillips and Brunt, initially reported in 1978 (Phillips and Brunt,1978) and subsequently in 1980 (Phillips and Brunt,1980), that NeVX was serologically indistinguishable from a more amenable potexvirus in *Agapanthus* led to further work on what was believed to be an isolate of NeVX. There are however some inconsistencies between these two reports. In the 1978 report, Phillips and Brunt indicated that the potexvirus detected in three *A.africanus* plants was serologically indistinguishable from NeVX. It was also stated that the viruses from *Nerine* and *Agapanthus* had identical but restriced host ranges. No serological relationship was found to seven other potexviruses including viola mottle virus and hydrangea ringspot virus. In the 1980 publication the potexvirus from *A.praecox* subsp.*orientalis*, which was also serologically indistinguishable from NeVX and therefore considered an isolate, had a different experimental host range. Further this isolate was reported to be distantly related serologically to a number of potexviruses including viola mottle virus and hydrangea ringspot virus. These apparent contradictions highlight the difficulties that confront researchers on viruses in nerines due primarily to a lack of specific and sensitive diagnostic techniques.

The situation with the potyviruses in nerines is even more confused. The 750nm particle, originally believed to be NaYSV on the basis of particle size by Brunt *et al* (1970), has not been supported by serological relationships (Hakkaart *et al*,1975). The evidence strongly suggests the presence of two distinct potyviruses in nerines, one in the 740-750nm particle length range and the other in the 780-800nm range. The 750nm particle has been referred to as NeYSV by Brunt (pers comm to K S Milne,1985) whereas Maat (pers comm to K S Milne,1983) has reported that the 800nm causes yellow stripe symptoms in *N.sarniensis* hybrids. For the purposes of the present study the 740-750nm particles from nerines will be taken as NeYSV.

The evidence presented by Maat *et al* (1978) on the relationship between NeLV and HLV is supported by the dual criteria of close serological association and an identical host range. Thus, it seems likely that NeLV is an isolate of HLV but the former name should take priority.

Although the emphasis on nerine virus research has been focused on filamentous viruses, primarily because they are readily detected by electron microscopy, the presence of CMV is reported in more than one study (Hakkaart *et al*,1975; Maat *et al*,1978) and antisera supposedly prepared to the filamentous viruses has been found to be contaminated by antibodies for this virus. The difficulties associated with detecting CMV in nerine crude sap or in clarified preparations, electron microscopically or serologically, have resulted in the incidence of this virus in nerines being somewhat neglected. Little can be determined from the literature on the effects of CMV in terms of symptom expression, prevalence, or the effects of its association with other nerine viruses on the crop. Similarly, ArMV has been rarely found in nerines (Brunt,1976) but its prevalence has not been thoroughly investigated.

Studies of viruses infecting nerines so far have established the presence of nine distinct viruses in various *Nerine* cultivars. This information is summarized in Table 1.

Research on viruses infecting nerines reflects the relatively minor economic importance of this comparatively new commercial ornamental crop, compared to other bulbous ornamentals such as *Narcissus*. Indeed, the study of nerine viruses, at least in Britain, was undertaken mainly to determine whether there was any epidemiological association between viruses infecting *Nerine* and *Narcissus* (Brunt, pers comm to Milne,1983). Further, these research efforts have been severely hampered by many of the nerine viruses proving to be most intransigent in mechanical transmission trials. The failure to readily transmit the filamentous viruses from nerine crude sap into herbaceous diagnostic or propagation hosts was, in many cases, not resolved by using partially purified or even purified preparations. This applies to NeVX, NeLV and to the two potyviruses.

This problem was compounded by the finding that in many instances viruses in nerines were present as a complex, with two or more distinct viruses present in the same plants. Thus, purification of viruses directly from nerine tissue to a homogeneous state, which in some cases involved separating particles of similar sizes, proved to be most demanding using conventional purification protocols. Consequently, antisera prepared were often not specific to one class of antigens. Further characterization of these viruses has only been possible

Table 1: The relative prevalence of viruses reported in association with *Nerine* spp.

Virus Group	Virus	Prevalence	*Nerine* spp.	Reference
Carlavirus	nerine latent (hippeastrum latent)	common	*N.bowdenii* *N.sarniensis* *N.manselli* *N.flexuosa* 'Alba'	Maat *et al*, 1978
	narcissus latent	uncommon	*N.sarniensis*	Brunt, 1977
Cucumovirus	cucumber mosaic	common	*N.sarniensis*	Hakkaart *et al*, 1975
			N.bowdenii	Maat *et al*, 1978
Nepovirus	arabis mosaic	uncommon	*N.sarniensis*	Brunt, 1977
Potexvirus	nerine virus X	common	*N.sarniensis*	Maat, 1976
	narcissus mosaic	uncommon	*N.bowdenii* *N.manselli*	Koenig *et al*,1973 Maat, 1976
Potyvirus	nerine yellow stripe (750nm)	common	*N.sarniensis*	Brunt,pers comm, 1985
	uncharacterized virus (800nm)		*N.sarniensis*	Maat,pers comm, 1983
			N.bowdenii	Brunt,pers comm, 1987
Tobravirus	tobacco rattle	uncommon		Maat,pers comm, 1983

in instances where strong serological associations were found between viruses in nerines and viruses in other bulbous species such as *Narcissus*, *Hippeastrum* and *Agapanthus*. The isolates of more amenable viruses from these other bulbous species such as NeVX from *Agapanthus* (Phillips & Brunt,1980) and NeLV from *Hippeastrum* (Maat *et al*,1978), have been investigated further and at least partially characterized. The potyviruses from nerines are least understood and remained uncharacterized. Considerable gaps exist in the knowledge of viruses in nerines in terms of virus etiology, symptom associations, rate of infection and effects on the crop. Most of the studies reported were carried out in the 1970's with little evidence of current interest by scientists in the northern hemisphere. No routine diagnostic procedures exist for the large scale testing for specific viruses in this crop.

1.3 OBJECTIVES OF THIS RESEARCH PROJECT

Interest at Massey University, Palmerston North, New Zealand in viruses occurring in *Nerine* was a result of an expanding local nerine industry with its concomitant demands for pathogen identification, elimination and control. Although all *Nerine* stocks in New Zealand originally came from Britain or the Netherlands, no studies on viruses in New Zealand nerines had been undertaken prior to this study. The primary objectives of this research project were to attempt to determine the identity of viruses infecting nerines and to develop specific detection methods for some of the filamentous viruses so that the diagnostic techniques could subsequently be used in rapid virus indexing procedures and for virus elimination studies. Attempts at virus elimination do not comprise part of this study.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Source of nerine virus isolates for preliminary studies and cDNA tests

Nerine sarniensis hybrids from a commercial nursery in Palmerston North, and, *N.fothergilli* Roem. 'Major' and *N.bowdenii* types from home gardens in the Manawatu region were the source of the virus isolates used in the preliminary electron microscope survey and in mechanical transmission studies. Dot-blot assays using cloned cDNA probes were conducted with samples of nerine leaves obtained from commercial growers in Auckland, New Plymouth and Palmerston North. The *Nerine* species assayed were *N.bowdenii*, *N.sarniensis* hybrids, *N.flexuosa* 'Alba', *N.fothergilli* 'Major', *'N.corusca* 'Major' and *N.manselli*.

2.1.2 Source of other virus isolates used in this study

Table 2 details the source of potexvirus and potyvirus isolates tested against the cloned cDNA probes developed in this study.

Table 2: Source of potexvirus and potyvirus isolates tested against cloned cDNA probes.

Virus Group: Potexvirus

Virus member	Host plant	Supplied by
Cymbidium mosaic virus	*Cymbidium* sp. (hybrid)	R L S Forster[1]
Daphne virus X	*Nicotiana clevelandii*	R L S Forster
Narcissus mosaic virus	*N.clevelandii*	R L S Forster
Nerine virus X (Agapanthus strain)	*Chenopodium amaranticolor*	R L S Forster
Potato virus X	*Nicotiana glutinosa*	R L S Forster
Tulip virus X	*C.amaranticolor*	R L S Forster
White clover mosaic virus	*N.clevelandii*	R L S Forster

Virus Group: Potyvirus

Bean yellow mosaic virus	*Pisum sativum*	R L S Forster
Hippeastrum mosaic virus	*Hippeastrum* sp.	A A Brunt[2]
Nerine yellow stripe virus (750nm particle)	*Nerine sarniensis*	A A Brunt
Potato virus Y	*N.glutinosa*	K S Milne[3]
Uncharacterized potyvirus from nerines	*Nerine bowdenii*	A A Brunt

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[2] A A Brunt, Horticultural Research Institute, Littlehampton, Sussex, England.
[3] K S Milne, Department of Plant Health, Massey University, Palmerston North, New Zealand.

2.1.3 Indicator plants

Indicator plants which were generally sensitive to a wide range of viruses, belonging to a number of host families, were selected for mechanical transmission trials. Some of the test plants used were known to be local lesion hosts of, or capable of becoming systemically infected by, some of the viruses in nerines. Information on the indicator plants used is given in Table 3.

2.1.4 Source of antisera

Antisera to cucumber mosaic virus was obtained from K S Milne, and to nerine virus X (agapanthus strain) from A A Brunt.

2.1.5 Biochemicals

The names and sources of some biochemicals used are given in the text. Other chemicals used were of analytical reagent grade.

Table 3: Virus indicator species tested in mechanical transmission studies.

Host Family	Species	Virus[1]	Symptoms[2]	References
Aizoaceae	*Tetragonia expansa* Murr.	NaMV	LL	Brunt, 1966
		NaYSV	LL (unreliable in summer)	Brunt, 1971
		NaLV	LL	Brunt, 1976
Amaranthaceae	*Gomphrena globosa* L.	NaMV	LL	Maat, 1976
		NaMV	SI	Brunt, 1966
		HLV (syn. NeLV)	LL	Brolman-Hupkes, 1975
Chenopodiaceae	*Chenopodium amaranticolor* Coste & Reyn.	TRV	LL	Harrison, 1970
		NeLV	LL	Maat *et al*, 1978
		ArMV	LL	Murant, 1970
		NaMV	LL	Maat, 1976
		CMV	LL	Francki *et al*, 1979
	Chenopodium quinoa Willd.	HLV	LL	Brolman-Hupkes, 1975
		CMV	LL	Francki *et al*, 1979
		NaMV	LL	Maat, 1976
		ArMV	LL	Murant, 1970
		TRV	LL	Mowat, 1980
Cucurbitaceae	*Cucumis sativus* L.	CMV	SI	Francki *et al*, 1979
Leguminosae	*Phaseolus vulgaris* L.	TRV	LL	Harrison, 1970
		NaMV	LL	Koenig *et at*, 1973
Solanaceae	*Nicotiana clevelandii* Gray.	HLV	SI	Brolman-Hupkes, 1975
		NaMV	SI	Brunt, 1966
		ArMV	SI	Murant, 1970
		NaLV	SI	Brunt, 1977
		TRV	SI	Harrison, 1970
	N. glutinosa L.	CMV	SI	Francki *et al*, 1979
	N. tabacum L. 'White Burley'	ArMV	LL	Murant, 1970
	N. tabacum L. 'Samsun'	CMV	LL	Forster, 1974
	Petunia hybrida (Hook) Viln.	ArMV	LL	Murant, 1970
		ArMV	SI	Murant, 1970

[1] Virus abbreviation: ArMV = arabis mosaic virus; CMV = cucumber mosaic virus; HLV = hippeastrum latent virus; NaLV = narcissus latent virus; NaMV = narcissus mosaic virus; NaYSV = narcissus yellow stripe virus; NeLV = nerine latent virus; TRV = tobacco rattle virus.

[2] Coded symptom description: LL=local lesion; SI=systemic infection.

2.2 METHODS

2.2.1 Nerines

Nerines with various virus-like symptoms were collected from a commercial nursery and home-gardens, potted in pumice/peat media (80:20),and maintained in a temperature controlled glasshouse unit at 20C (+/- 4C).

N.bowdenii seeds were collected from a home-garden and grown on in a different glasshouse under similar growth conditions to that used for the infected plants. This minimized the possibility of cross infection to seedlings.

2.2.2 Plants for experimental host range tests

Test plants were grown in a temperature-controlled glasshouse at 20C (+/- 4C), or in a growth cabinet maintained at an average temperature of 20C. (+/-2 C).

2.2.3 Inoculation

Inoculum was prepared by grinding freshly harvested and diced nerine leaf or flower tissue in a small volume of cold buffer containing additives and celite in a pre-chilled sterile mortar and pestle. Indicator plants were inoculated by gently rubbing the leaves with the pestle or a sterilized cotton bud dipped in the inoculum, and then washing the leaves with water. In some tests 400-mesh carborundum was used as the abrasive. This was dusted onto leaves rather than mixed with the inoculum.

Partially purified, or purified virus preparations of nerine virus X and the hitherto unnamed 800nm potyvirus, and, viral nucleic acids (RNA) extracted from both these viruses were also used as inoculum. Viral RNA preparations were diluted with 0.02M phosphate buffer pH 7.2, containing disodium ethylene-diamine-tetra-acetate (EDTA) treated bentonite (Fraenkel-Conrat *et al*,1961) and dispensed with a micro-pipette on to young leaves which, prior to applying RNA, had been gently abraded with carborundum and rubbed with sterile cotton-buds. The leaves were then rinsed with tap water to remove excess inoculum.

2.2.4 Electron microscopy

The presence, integrity and purity of filamentous viruses in nerines from crude sap, partially purified and purified preparations was determined with a Philips model 201C transmission electron microscope (EM). Formvar- and carbon-coated 200 mesh copper grids were used in all cases. The following stains were tested:

(a) 2% potassium phosphotungstic acid (PTA), pH 7
(b) 2% sodium silicotungstate
(c) 2% uranyl acetate

The most commonly used stain in this study was 2% PTA, pH 7.

2.2.4.1 Electron microscope grid preparation

(a) Crude sap

Samples of nerine tissue were diced and crushed in chilled mortars and pestles with about three volumes (w/v) of 0.1M potassium phosphate buffer, pH 7. The homogenates were left to settle for about 1/2 hour at 4C. About 250ul of the supernatant was drawn out with a Gilson pipetman with disposable tips and placed in numbered wells of a porcelain spotting tile. Formvar-coated copper grids were floated face down on the solution. The tile was covered with polyethylene film ('GLAD-Wrap') or parafilm "M" and left in the refrigerator at 4C for 1-2h. The grids were removed, excess liquid drained by touching the side on a Whatman No 4 filter paper and gently attached to the edge of a double-sided adhesive tape fixed on a slide. A drop of distilled water was placed on each grid and drained by touching the side with a filter paper wick. This procedure was repeated at least three times. The final rinse was with a drop of stain, usually PTA pH 7, left on for about 2min. The grids were air-dried at room temperature and viewed with the electron microscope as soon as possible, but usually within a few hours.

The same procedure was used for electron microscopy of the indicator plants used in the experimental inoculation studies.

(b) Partially purified and purified virus preparations

The grids were attached to the edge of a double-sided tape as described earlier. A drop of diluted virus preparation was placed on the grid and left for about five minutes. Excess fluid was drained, the grids rinsed with distilled water and stained as before.

2.2.4.2 Particle size determination

The approximate size of filamentous virus particles in preliminary surveys was determined by measuring particle length from electron micrographs at a magnification of X48,600.

For accurate size determination virus particles in crude sap preparations were prepared using the method described by S R Christie (pers comm 1986, University of Florida, Gainesville, USA). Infected nerine tissue was diced with a new razor blade on a clean glass slide containing several drops of 0.2M neutral potassium phosphate buffer. A drop of this suspension was transferred to a supported EM grid for 2-3min and blotted dry. The grids were rinsed dropwise with 20 drops of distilled water and stained for 1min with PTA, pH7.

A Polaron cross-line diffraction grating with 2160 lines/mm was used as a standard for accurate determination of magnification of electron micrographs. After calculating particle sizes from electron micrographs, the normal length of the filamentous viruses was determined by the method of Brandes and Wetter (1959). The particles were grouped into 40nm divisions and the normal length obtained as the mean of the distribution, assuming a normal distribution.

2.2.4.3 Ultrathin sectioning and staining procedure

Leaf tissue was fixed in 3%glutaraldehyde + 2%formaldehyde in 0.1%phosphate buffer, pH 7.2 (Karnovsky,1965), vacuum infiltrated, and stored in the primary fixative at 4C for 24h. Specimens were then transferred to fresh buffer (three buffer washes in 30min) and postfixed for 1h in 1% osmium tetroxide at 4C.

Following three buffer washes, the fixed material was dehydrated in an acetone series (25,50,75,95,100%), treated with propylene oxide (15min), infiltrated, embedded in Polarbed 812 and cured at 60C for 72h. Before electron microscopy, grid-mounted sections were stained 5-7min with saturated uranyl acetate in 50% ethanol, washed in 50% ethanol and then distilled water, and stained 5-7min with lead citrate (Venable & Coggeshall,1965); they were then washed with distilled water. Transmission EM sections (85nm thick) were cut with a diamond knife on a Reichert-Jung Ultracut E microtome and mounted on formvar- and carbon-coated 200 mesh copper grids.

2.2.4.4 Light microscopy

Light microscopic recognition of inclusion bodies was by the method described by Christie and Edwardson (1986). Epidermal strips from the lower surface of nerine leaves were obtained by inserting the tips of sharp pointed tweezers under the epidermis and stripping it at an acute angle from the underlying tissue. The epidermal strips were floated with the torn surface in contact with the staining solution (Azure A or O-G, a combination of calcomine orange 2RS and Luxol brilliant green BL dyes; E. I du Pont de Nemours & Co) in a watch glass. After five to ten minutes, the stain was removed with a micropipette. Excess stain was eliminated by several quick changes of 95% ethanol (five to ten seconds per change), for a total of ca 30 seconds. The epidermal strips were carefully lifted without folding and mounted in a drop of Euparal (Carolina Biological Supply) on a glass slide. Regular Euparal was used with Azure A and Euparal 'Vert' with O-G combination. A coverslip was placed over the tissue, excess medium removed, and the tissue flattened by gentle blotting.

For detecting cylindrical inclusions of the potyvirus group the plastids were removed before staining by floating the epidermal strips on a 5% solution of Triton X-100 for 5min before staining with the O-G combination. The staining time after Triton X-100 treatment was about one-half that for untreated tissue. After staining, the tissue was dehydrated and mounted as previously described.

The tissue was examined with a light microscope, at a magnification of X1000 under oil immersion, for the presence of inclusions. The coloured light micrographs were recorded on Polacolor 2 type 58 Land film using appropriate filters.

2.2.5 Virus purification

Failure to readily transmit the filamentous viruses from nerines into herbaceous hosts left no option but to attempt to purify viruses directly from systemically infected nerine tissue. In the preliminary phase of this project several purification methods were attempted to gain familiarity with the protocols and to assess virus yield, aggregation and fragmentation characteristics.

The various clarification and purification procedures used are outlined below. In all cases 3ml of buffer was used for each gram of tissue.

(a) Buffer: 0.5M potassium dihydrogen orthophosphate and di-potassium hydrogen phosphate (KPO_4), pH 7.6
Solvent: Chloroform (0.5 volume)
Additives: 0.1% 2-mercaptoethanol (2-ME),
0.05M ethylene-diamine-tetra-acetate (EDTA),
0.01M sodium diethyl-dithiocarbamate (DIECA),
1% Triton X-100
Centrifugation: two cycles of differential centrifugation 8,000g, 10min; 80,000g, 90min
sucrose density gradient centrifugation
25% sucrose freeze-thaw gradient (Davis and Pearson,1978)
Reference: modification of Mossop,1977

(b) Buffer: 0.5M KPO_4, pH 7.6
Solvent: Chloroform (0.5 volume)
Additives: 0.1% 2-ME, 0.05M EDTA, 0.01M DIECA,
1% Triton X-100
Precipitation: 5% polyethylene glycol MW 6000 (PEG 6000)/ 1.75% NaCl (w/v)
Centrifugation: two cycles of differential centrifugation
sucrose density gradient centrifugation
Reference: modification of (a)

(c) Buffer: 0.5M KPO_4, pH 7.6
Solvent: Chloroform (0.5 volume)
Additives: 0.1% 2-ME, 0.05M EDTA, 0.01M DIECA,
1% Triton X-100
Precipitation: 5% PEG 6000/1.75% NaCl
Centrifugation: isopycnic, caesium chloride, directly after PEG precipitation (Hiebert, pers comm,1986)

(d) Buffer: 20 mM N-2-hydroxyethylpiperazine-N-2'-ethanesulfonic acid (HEPES), pH 7.5
Solvent: chloroform/carbon tetrachloride (1:1;0.5 volume) or 8% n-butanol
Additives: 0.1% sodium sulphite, 1% Triton X-100,
Precipitation: 4% PEG 6000/0.1M NaCl
Centrifugation: isopycnic, caesium chloride
Reference: based on Dougherty & Hiebert,1980

(e) Buffer: 0.1M (hydroxymethyl)aminoethane (Tris)-citric acid, pH 9
Solvent: chloroform (0.5 volume)
Additives: 0.1% sodium thioglycolate, 0.02M DIECA
Precipitation: 5% PEG 6000/0.2M NaCl
Centrifugation: one cycle of differential centrifugation followed by sucrose density gradient centrifugation
Reference: Maat *et al*,1978

(f) Buffer: 0.1M Tris-HCl, pH 9
Solvent: chloroform/carbon tetrachloride (1:1; 0.5 volume)
Additives: 0.1% thioglycollic acid
Centrifugation: two cycles of centrifugation (relatively low speed; 8,000g, 10min; 26,500g, 90min)
Reference: Huttinga,1973

By monitoring the virus preparations by electron microscopy, procedures were selected which, at the correct physiological state of the infected plants (young leaves from plants grown in a glasshouse at about 20C), sufficient unfragmented filamentous virus particles could be obtained for RNA extraction. These methods varied with the type of virus particle to be purified. The procedures described below are the simplest ones successfully used to yield sufficient virus for RNA extraction from 150-200g of systemically infected nerine tissue.

2.2.5.1 Purification of nerine virus X

The following procedure was used to extract viruses from one clump of *N.fothergilli* 'Major' tissue systemically infected with NeVX.

Approximately 150-200g of freshly harvested systemically infected nerine tissue (leaves or flowers) were cut into 2-3cm pieces and homogenized in a pre-chilled blender with three parts (w/v) of cold 0.5M KPO_4 buffer, pH 7.6, containing 0.05M EDTA, 0.01M DIECA and 0.1% 2-ME. The resulting extract was filtered through a double layer of cheese-cloth into a flask placed on ice. Half volume chloroform and 1% Triton X-100 was added and the mixture gently stirred for 3h at 4C. The coagulated green debris obtained was removed by low speed centrifugation at 12,000g in a Sorvall Centrifuge (Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge) for 15min. The coagulated interphase was re-extracted by adding two volumes of buffer, half volume of chloroform and 1% Triton X-100 and stirred for 2h at 4C. After separating the phases by low speed centrifugation the two supernatants were combined and the virions precipitated by the addition of 5% (w/v) powdered PEG 6000, 1.75% sodium chloride and placed on a shaker at low speed for 3h at 4C. The precipitated virions were collected by centrifugation at 13,500g for 20min. The resulting pellet was resuspended in a small volume (about 1ml/100g of tissue) of cold 0.02M KPO_4 buffer, pH 8.2 containing 0.01M EDTA and subjected to gentle agitation overnight at 4C. The virus was separated from the host components by isopycnic centrifugation in caesium chloride (CsCl).

Gradients were prepared according to the method of Brunk and Leick (1969). A stock solution of CsCl at approximately 1.75g/ml was diluted in the buffer to give a 12ml bottom layer with a density of 1.45g/ml and a 12ml top layer containing the virus with a density of 1.25g/ml. The centrifuge tubes with a nominal capacity of 38.5ml were overlayed with paraffin oil. Centrifugation was at 120,000g in a Beckman SW 28 rotor for 16h. After centrifugation the virus zone was located with a diffuse beam of light about 4.3cm from the bottom of the tube and collected manually with a hypodermic syringe. The virus was diluted with 0.02M KPO_4, pH 8.2 containing 0.1% 2-ME and clarified by centrifugation at 12,000g for 10min and reconcentrated by centrifugation at 85,000g for 90min. The final virus pellet was resuspended in water for RNA extraction. The virions were also checked for purity and integrity by electron microscopy.

2.2.5.2 Purification of nerine virus Y

The procedure outlined above had to be modified for the purification of a potyvirus from leaves of one clone of a systemically infected *N.sarniensis* hybrid. Preliminary investigations indicated that when the virus concentration in the tissue was relatively high it was closely associated with much larger proteinaceous inclusion bodies. Failure to separate the virus from the inclusion bodies resulted in considerable loss of virus in the early stages of the purification procedure. The simplest procedure developed for the purification of potyviruses in nerines, which yielded unfragmented particles for RNA extraction, is described below.

About 200g of freshly harvested systemically infected nerine leaves were cut into 2-3cm pieces and homogenized in a pre-chilled blender with three parts of 0.5M KPO_4, pH 7.6 containing 5mg of sodium sulphite per gram of tissue and 0.05M EDTA. Half a volume of a 1chloroform:1carbon tetrachloride mixture and 1% Triton X-100 was added to the blender and re-homogenized at low speed using a power controller for 1-2min. The mixture was left in a flask on ice with gentle agitation for 2-3h. The mixture was centrifuged in a Sorvall Centrifuge using a GSA rotor at 4000g for 5min and the pellet containing the organic solvents was discarded. The aqueous phase was centrifuged at 13,200g for 15min to precipitate the virus inclusion bodies. Virus particles in the supernatant were PEG precipitated and purified as described in Section 2.2.5.1. The pellet containing the inclusions was resuspended in 0.05M KPO_4, pH 8.2, containing 0.1% 2-ME, briefly vortexed and Triton X-100 was added to make a final concentration if 5% (v/v). After stirring for one hour at 4C this mixture was subjected to a low speed centrifugation of 27,000g for 15min to precipitate the inclusion bodies. The pellet was resuspended in 10ml of 0.02M KPO_4, pH 8.2, containing 0.1% 2-ME vortexed, and inclusion bodies further purified according to the method described by Lima *et al* (1979). The inclusion bodies were resedimented by centrifugation at 27,000g for 15min, resuspended in the same buffer and layered on a sucrose step gradient made up of 10ml of 80%, 7ml of 60%, and 7ml of 50% (w/v) sucrose in 0.02M KPO_4, pH 8.2. The gradient was centrifuged for one hour at 45,000g. The inclusions layered on top of the 80% sucrose zone were collected with a hypodermic syringe. To remove the sucrose, the inclusion bodies were diluted in 0.02M KPO_4, pH 8.2, and precipitated by centrifugation at 27,000g for 15min. The pellet was resuspended in 0.02M Tris-HCl, pH 8.2 and used for electron microscopic observations and for sodium dodecyl sulphate (SDS)-polyacrylamide gel analysis.

2.2.5.3 Clarification and concentration of viruses from small samples for dot-blot assays

Two procedures were developed for the clarification and concentration of viruses from nerines and other species for use in dot-blot assays. Both these procedures were modifications of the clarified viral concentrates (CVC) method developed by S R Christie (pers comm, 1986) at the University of Florida, Gainesville, USA.

(a) Procedure for the clarification and concentration of viruses from 'mini-samples'.

About 5g of nerine leaf tissue were cut into small pieces and placed into boiling tubes held on ice. Approximately 50ml of cold 0.5M KPO_4, pH 7.6, and 5ml of a 10% sodium sulphite stock solution was added to each tube. The tissue was homogenized in a PCU-2 polytron (Kinematica) and poured into250ml flasks. Twenty-five ml of chloroform was added and the flasks placed on a shaker for one hour at 4C. Twenty-five ml samples were drawn out from the top of the flasks and centrifuged at 12,000g for 10min. To the supernatant, 30%PEG/0.6M sodium chloride stock solution was added to give a final concentration of 6%PEG and 0.12M sodium chloride. The suspension was allowed to stand for one hour on ice and centrifuged at 12,200g for 5min. The pellet was resuspended in 400ul of 0.1M KPO_4, pH 7.5. The resuspended pellet was spun in a microfuge for 5min and the supernatant loaded on Zeta-Probe membranes for dot-blot assays.

(b) Procedure for the clarification and concentration of viruses from 'micro-samples'.

About 15 nerine leaf discs (approximately 0.1g) were punched out with a 4mm cork borer and placed into 1.5ml microfuge tubes. Two hundred ul of cold 0.5M KPO_4, pH 7.6 containing 1% sodium sulphite was added and the mixture quickly homogenized in the microfuge tubes using a araldite moulded homogenizing cone attached to a metal shaft which fitted into a power drill. A clean homogenizing cone was used for each sample. An equal volume of chloroform was added to the mixture and it was briefly vortexed and left on ice for 1h. The microfuge tubes were centrifuged for 10min in a microfuge. To the supernatant, one fourth volume of 30%PEG/0.6M NaCl stock was added, mixed by inverting and left on ice for 30min. The suspension was

centrifuged in a microfuge for 5min and the pellet was retained and resuspended in 200ul of 0.05M KPO_4, pH 7.5 and left on ice for 1h. The suspension was thoroughly vortexed and re-centrifuged for 5min. The supernatant was loaded on to a Zeta-Probe membrane for dot-blot assays.

2.2.6 Molecular weight determination of viral coat and inclusion body proteins

2.2.6.1 Protein dissociation

Purified virus particles or inclusion bodies were dissociated by the SDS method (Weber & Osborn,1969). To 100ul of protein (conc 1mg/ml) in a microfuge tube was added 6ul of 0.3M NaH_2PO_4-Na_2HPO_4, pH 7.0, 20ul of 10% SDS stock, 2ul of 2-ME and 0.096g of urea. The mixture was briefly vortexed and made up to 200ul with water. It was boiled for 2min and an aliquot used for polyacrylamide gel analysis of the protein. The remainder was stored at -20C until required.

2.2.6.2 Analysis of proteins on SDS-polyacrylamide gels

Discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a system based on that of Laemmli (1970). The apparatus was constructed according to the method described by Slack *et al* (1985). The running gel was prepared by adding 33.5 ml of stock solution of 36% acrylamide, to 37.5ml 1M Tris-HCl (pH 8.8) and 27 ml of water and the mixture briefly degassed under vacuum in a 250ml round-bottomed flask. One ml of 10% SDS, 0.05ml of TEMED and 2.5ml of 2% ammonium persulphate was added, the solution mixed and polymerized into a slab gel 1.5mm thick, 15.5cm long and 13.5cm wide. A 0.1% SDS solution was layered above the acrylamide solution to ensure an even top and to exclude air. The running gel was left to polymerize for about 90min. The SDS solution was then poured off, the surface of the gel washed with distilled water and the gel inverted.

The stacking gel was prepared by mixing 6ml of a 21% stock acrylamide solution with 6ml of Tris-HCl (pH 6.8), 12.2ml of water and degassed as before. Then 0.25ml of 10% SDS, 0.02ml TEMED and 0.8ml of freshly made 2% ammonium persulphate was mixed into the solution and layered above the running gel to

within 0.5cm of the top. A 1.5mm teflon sample-slot former with 0.4cm wide combs was immersed into the stacking gel so that the base of the slots was about 1cm from the top of the running gel. After about 90min the comb mould was removed and the slots rinsed carefully three times with the electrode buffer consisting of 0.025M Tris, 0.19M glycine, and 0.1% SDS. The electrode buffer was poured into the upper and lower electrode baths and air bubbles removed from the bottom of the running gel.

Five and 15ul aliquots of the protein samples were mixed with an equal volume of running buffer (15ul of 0.05% bromophenol blue in water, 5 drops of glycerol, 25ul of mercaptoethanol and 250ul of electrode buffer; Weber & Osborn,1969) and loaded into the sample slots using a 100ul hypodermic syringe. Current was held at 20mA/gel for 1h and then raised to 35-40mA/gel for 4-5h until the tracking dye had reached the bottom of the gel. Gels were run at room temperature or at 4C.

Following electrophoresis, the gels were immersed in 5methanol:5water:1acetic acid (v/v/v), for 30min to precipitate proteins, then washed with at least three changes of distilled water for 30min with gentle agitation to remove SDS which interferes with staining of the proteins. Proteins were then stained with solution containing 0.02% Coomassie Blue R, 5% absolute ethanol, 6% trichloroacetic acid and 25% methanol in water. Precipitated dye from the gel surface was removed by rapidly rinsing the gel with methanol and then transferring the gel to water. After washing the gel overnight in water, it was double stained with Coomassie Blue G-250 (Blakesley and Boezi, 1977) to reduce background colour and to intensify the staining of the bands. This stain was prepared in bulk by mixing overnight 1000ml of water containing 2g Coomassie Blue G-250 with 1000ml 2M sulphuric acid. The solution was filtered through Whatman No.1 paper, and, 220ml 10M potassium hydroxide and 300g trichloroacetic acid in 300ml water was subsequently added. This mixture was filtered through Whatman No.1 paper and stored in the dark.

The gels were photographed on frosted glass, lit from below, using Kodak 35mm colour film.

2.2.7 RNA extraction

Several RNA extraction procedures were attempted for NeVX and the potyvirus in nerines, but especially for the potyvirus. These included the methods described by Xu *et al* (1986) and the lithium chloride method of Francki and McLean (1968) and the procedures described here.

2.2.7.1 RNA extraction from nerine virus X

The virus was purified as shown in Section 2.2.5.1 and resuspended in water. The RNA was extracted by the SDS-phenol-chloroform method described by Palmiter (1974). Purified virus was mixed with an equal volume of 2X extraction buffer consisting of 0.2M NaCl, 0.02M Tris-HCl pH 8, 0.01M EDTA, 2% SDS and 0.1% EDTA-treated bentonite. Two volumes of phenol/chloroform (1:1) were added per one volume of resuspended virus. The suspension was briefly vortexed and heated at 37C for 20min. The suspension was re-vortexed and spun at 10,000g for 20min in a microfuge. To the supernatant an equal volume of phenol/chloroform was added and immediately respun at 10,000g for 10min. Two volumes of 100% ethanol kept at -20C were added, gently mixed and stored at -20C overnight. The ethanol precipitated RNA was pelleted by centrifugation at 12,000g for 40min. The RNA pellet was washed with 70% ethanol, vacuum dried, resuspended in a small volume of sterile double-distilled water and stored at -70C until required for molecular weight determination or cDNA cloning.

To minimize ribonuclease degradation of RNA, all RNA extraction procedures were performed in glassware that had been rinsed with 10% hydrochloric acid, thoroughly rinsed with sterilized distilled water and heated to 200C overnight. The 1.5ml polypropylene eppendorf microfuge tubes were treated with dimethyldichlorosilane to increase the hydrophobicity of the plastic surface to minimize losses of RNA by adsorption. This was done by introducing a 5% solution of dimethyldichlorosilicane in chloroform into individual tubes for several minutes. The solution was removed, the tubes rinsed thoroughly with distilled water, autoclaved and oven-dried. Disposable gloves were used in all procedures involving viral RNA to prevent contamination of the sample with skin RNase.

2.2.7.2 RNA extraction from nerine potyvirus

The following procedure was used to extract RNA from potyviruses in nerines. The freshly purified virus, resuspended in water (described in Section 2.2.5.2), was extracted by dissociating it with an equal volume of 200mM ammonium carbonate pH 9, 2mM EDTA, 2% SDS, 0.2% sodium diethyldithiocarbamate (Brakke and Van Pelt, 1970) and 100ug EDTA-treated bentonite per millilitre of virus solution (Taiwo *et al*,1982). Proteinase K at a concentration of 10ug/ml was added and the mixture gently vortexed and left at room temperature for 20min. The mixture was deproteinized by phenol/chloroform extraction and the RNA was ethanol precipitated as described in Section 2.2.7.1.

2.2.8 Agarose gel electrophoresis of nucleic acids

2.2.8.1 Non-denaturing gels

(a) Agarose gel electrophoresis of DNA

The standard method used for the analysis of DNA in this study was by gel electrophoresis on 1% agarose (low EEO,Type 1,Sigma). 0.7g of agarose was completely dissolved in 70ml of Tris-acetate buffer (40mM Tris-OH, 20mM acetic acid, 2mM EDTA pH 8.1) containing 0.5ug/ml ethidium bromide. The agarose was cooled to about 60C and cast into a slab gel in clear polystyrene trays measuring 12.5 X 8.5 X 0.5cm with a teflon gel comb set in position. The agarose was left to set at room temperature for about 1h before the comb was carefully removed and the gel tray placed in the electrophoresis apparatus. The buffer reservoirs were filled with Tris-acetate buffer until the gel was just submerged. DNA samples dissolved in sterile TE were made up to 10-20ul in the following 'stop mix': 30% Ficoll (Pharmacia Fine Chemicals), 0.25M EDTA, 0.1% SDS, 0.25% bromophenol blue and 10X electrophoresis buffer pH 8. The samples in microfuge tubes were briefly vortexed, heated at 65C for 5min, loaded with disposable micropipette tips into the wells and electrophoresed at about 30V for approximately 20min. When the tracking dye had migrated out of the well the voltage was increased to 100V and electrophoresis continued until the tracking dye had migrated to within 2-3cm of the end of the gel. The gel was gently rinsed with sterile distilled water and viewed under a UV transilluminator (Ultra-Violet Products Inc) and photographed with a Polaroid MP-4 land camera using a red

filter. If the background illumination was too high the gel was immersed in sterile distilled water for about half an hour.

(b) Non-denaturing gel for viral RNA concentration determination

The method used for the determination of the concentration of viral RNA was essentially the same as the procedure described for DNA with the exception that TBE buffer (0.089M Tris-borate, 0.089M boric acid and 0.002M EDTA pH 8.3) was used instead of Tris-acetate and the electrophoresis equipment was treated with 0.1% diethylpyrocarbonate solution overnight and thoroughly rinsed with sterile distilled water as a precaution against contaminating ribonucleases.

2.2.8.2 Denaturing agarose gel electrophoresis

(a) Alkaline agarose gels for DNA

Alkaline agarose gels were run according to the procedure outlined by Maniatis *et al* (1982) to determine the size of the first and second strands in the cDNA synthesis process. The standard protocol outlined in Section 2.2.8.1(a) was modified by using an alkaline agarose buffer consisting of 30mM NaOH and 1mM EDTA and the agarose was dissolved in 50mM NaCl and 1mM EDTA. The buffer was allowed to soak into the set gel for at least 30min before loading the [^{32}P]-labelled DNA samples. The ethanol precipitated DNA samples were dissolved in 10-20ul of alkaline loading buffer consisting of 50mM NaOH, 1mM EDTA, 2.4% Ficoll and 0.025% bromocresol green as the tracking dye. The gel was removed from the tank at the end of the run and mounted on a three layers of Whatman 3MM paper and covered with a polyethylene sheet ('Cling-film') and dried on a gel drier. The dried gel was then autoradiographed with Kodak XAR-5, at -70C with an intensifying screen.

(b) Denaturing gels for viral RNA

Two denaturing gel systems were used in this study. Formaldehyde gels were used for molecular weight determination of viral RNAs and methylmercuric hydroxide gels were used for size-fractionation and purification of viral RNA.

(i) Formaldehyde gels for molecular weight determination of viral RNA

The procedure described by Gerard and Miller (1986) was used for sizing viral RNAs. Ethanol precipitated, vacuum dried RNA samples (1-3ug) in microfuge tubes were dissolved in 2.2ul of Buffer A. This consisted of 294ul of 10X MOPS/EDTA (0.5M MOPS pH 7, 0.01M EDTA pH 7.5) and 706ul of water. Then 4.8ul of formaldehyde/formamide (final concentrations of 2.2M formaldehyde and 50% formamide) was added and the mixture heated to 70C for 10min and quenched on ice. A 1.5ul aliquot of gel loading buffer (mixture of 322ul of Buffer A, 5mg xylene cyanol, 5mg bromocresol green, 400mg sucrose, 178ul of 37% formaldehyde and 500ul of formamide) was added and lightly vortexed. Electrophoresis was performed in a horizontal submarine gel described in Section 2.2.8.1. A 1% agarose gel was prepared in 1X MOPS/EDTA buffer by mixing 0.5g of agarose, 5ml of 10X MOPS/EDTA buffer and 36ml of water. After dissolving the agarose, the solution was allowed to cool to 60C and 9ml of 37% formaldehyde (final concentration 2.2M) was added. The gel was mixed, allowed to set and pre-electrophoresed in the electrophoresis buffer at 60V for 30min. The RNA samples including markers (Bethesda Research Laboratories RNA ladder) were electrophoresed at 60V for 1h and 100V for about 2h until the leading dye had migrated at least 9cm. Constant buffer circulation was carried out using a pump. The gel was stained in the dark for a maximum of 5min in 5ul/ml ethidium bromide in water and destained for 2h in sterilized distilled water. The gel was viewed on a short-wave UV transilluminator and photographed as described earlier.

(ii) Methylmercuric hydroxide gel for size fractionation of viral RNAs

The methylmercuric hydroxide gel method for size fractionating RNA developed by Bailey and Davidson (1975) was used to purify the 10 kilobase(kb) potyviral RNA for cDNA cloning.

A 1.2% low melting point agarose (FMC Seaplaque) gel was prepared in the running buffer consisting of 50mM boric acid, 5mM sodium borate, 10mM sodium sulphate and 5mM methylmercuric hydroxide (Serva). Equal volumes of viral RNA dissolved in water was mixed with 2X loading buffer containing 25ul methylmecuric hydroxide, 500ul 4X running buffer, 200ul 100% glycerol, 275ul water and 0.2% (w/v) bromophenol blue. Twenty ul aliquots (2-3ug/sample) were loaded into six wells, three wells were left empty and wells 10 and 11 were filled with an RNA sample and markers respectively. Electrophoresis was at 25V for about 16h with buffer recirculation. After electrophoresis the gel strip containing wells 10 and 11 were carefully cut out and stained for 30min in 0.5M ammonium acetate and 0.5ug/ml ethidium bromide. The gel strips were viewed under the UV transilluminator to locate the high molecular weight potyviral RNA and the position marked with a fine syringe needle containing Indian ink. The gel strip was lined up with the rest of the gel slab and a narrow horizontal strip in line with the marked position was excised and treated as described by Maniatis *et al* (1982). The gel pieces were soaked in 0.1M dithiothreitol for 30-40min. Approximately four volumes (w/v) of 0.5M ammonium acetate preheated to 65C was added. The mixture was heated to 65C until all the gel melted and gently vortexed. The mixture was phenol extracted at room temperature, centrifuged at 2000g for 10 min at 4C. The aqueous phase was re-extracted twice more with chloroform and the RNA ethanol precipitated and washed with 70% ethanol and 0.05M ammonium acetate. The vacuum dried size-fractionated RNA was dissolved in 20ul of sterile distilled water and stored at -70C. An aliquot (2 ul) was re-run on a formaldehyde gel to check the RNA for concentration and integrity.

The highest safety precautions were observed in all procedures involving methylmercuric hydroxide usage. The gel was run in a fume-hood. All electrophoretic equipment was treated as described earlier for possible RNase contamination.

2.2.9 Transfer of nucleic acids from agarose gels to solid supports

2.2.9.1 Southern blots

Southern blotting is a technique used to transfer DNA from its position in an agarose gel to a solid support such as a nitrocellulose (NC) filter or Zeta-Probe nylon membrane (Biorad). In this study Zeta-Probe membranes were used. The DNA from the gel was denatured and transferred to the membrane by the capillary action of an alkaline solution. The denatured single-stranded DNA covalently bound to the membrane and was subsequently hybridized to a radiolabelled probe to detect complementary DNA species. The hybridization pattern was then established by autoradiography.

The following procedure based on the protocols developed by Reed (1986) specifically for Zeta-Probe membranes was used in this study.

Agarose gel electrophoresis of DNA was carried out according to the procedures outlined earlier. The gel was first photographed and then transferred from the supporting tray to a glass plate. The unused areas of the gel were trimmed away with a razor blade. A Zeta-Probe membrane was cut to the dimensions of the gel and marked with a soft pencil to identify both the gel and the orientation of the membrane. The membrane was first wetted by carefully floating it onto distilled water and then completely immersing it.

The gel transfer apparatus consisted of two gel casting trays glued end-to-end. The trays were filled with 0.4M NaOH. An inverted gel casting tray was placed lengthwise over the well trays for supporting the gel. A Whatmann 3MM filter paper was cut to serve as a wick and transfer medium for the alkaline denaturing agent. When the filter paper was completely saturated the gel was carefully transferred on to the middle of the support tray with its well-side up. The pre-wetted Zeta-Probe membrane was placed over the gel and overlaid by two layers of 3MM paper. Strips of parafilm were placed along the edge of the gel to prevent direct transfer of the fluid. A stack of folded paper towels, cut to the appropriate size, was placed on top and held in place by a 200g evenly distributed weight.

After about 6-8h the stack of absorbent paper was removed, the Zeta-Probe membrane gently peeled from the gel surface, rinsed in 2X SSC (20X stock: 3M NaCl, 0.3M sodium citrate) and blotted dry. For short-term storage (less than 3 days) the membrane was

sealed in a plastic bag between two layers of 3MM paper. For prolonged storage, the membrane was first baked in an oven at 80C for 1.5-2h.

2.2.9.2 Northern blots

The transfer of denatured RNA from agarose gels, to a solid phase such as Zeta-Probe membrane or NC filter is known as northern blotting. The RNA can subsequently be hybridized with a radiolabelled probe of interest.

The procedure used for northern blots in this study was essentially the same as for southern blots except 50mM NaOH was used as the transfer medium instead of 0.4M NaOH. The RNA was electrophoresed on a formaldehyde gel as described earlier and transferred to a Zeta-Probe membrane.

2.2.9.3 Dot-blotting procedure

The technique of nucleic acid hybridization or dot-blot hybridization involves the formation of double-stranded molecules between the viral nucleic acid under test and and a complementary probe nucleic acid. In this case viral nucleic acids or intact virus containing nucleic acids were directly loaded on to a solid matrix such as NC or Zeta-Probe membrane as spots or dots. The single-stranded nucleic acids were bound to the solid phase by baking at 80C for 2h. The remaining nucleic-acid-binding sites were then blocked by prehybridization with a non-specific nucleic acid (eg salmon sperm DNA) and a protein (eg non-fat dissolved milk powder). The specific cloned cDNA radioactively labelled probe was added and allowed to hybridize to any homologous sequences in the immobilized nucleic acids forming a cDNA:RNA hybrid. After washing off the unhybridized probe, the spots to which it hybridized were detected by autoradiography.

The dot-blotting protocols used in this study were based on those developed by Maule *et al* (1983) and Reed (1986). A locally made microfiltration apparatus similar to the Bio-Dot apparatus (Bio-Rad,1985) was used in most of the dot-blotting.

A Zeta-Probe membrane was cut to the appropriate size and immersed in sterile distilled water. The microfiltration apparatus was assembled with the pre-wetted membrane in position

and all screws tightened under vacuum to prevent or minimize cross-well contamination. One hundred microlitres of clarified viral preparation or purified virus or viral RNA was loaded into the wells under low vacuum. The wells were left until all the fluid had passed through the membrane. The wells were rinsed with 100ul of sterile TE, again under low vacuum until all the wells were just dry. The vacuum was disconnected, the microfiltration apparatus disassembled and the membrane removed and left to completely air dry before being placed in an oven at 80C for 1h. The membrane was briefly rinsed in 2X PE (0.133 sodium phosphate, pH 6.9; 0.001M EDTA) containing 1% SDS, air dried, sealed in a plastic bag and used the next day for hybridization analysis. If hybridization was not going to be undertaken on the next day the membrane was baked dry at 80C for 30min and stored between two pieces of filter paper in a sealed plastic bag at 4C.

2.2.10 Preparation of cDNA and molecular cloning

Different strategies were adopted for cDNA preparation and molecular cloning of the two filamentous viruses from nerines. cDNA for nerine virus X was prepared and cloned into plasmid pBR322 using procedures described by Baulcombe & Buffard (1983) and Maniatis *et al* (1982). cDNA for nerine virus Y was prepared by a modification of the procedure described by D'Alessio *et al* (1987) and Watson & Jackson (1985).

2.2.10.1 cDNA synthesis and molecular cloning of nerine virus X RNA

Oligo dT(12-18) priming

All reactions were carried out in microfuge tubes. Vacuum-dried NeVX RNA was primed for reverse transcriptase activity by annealing to an oligo dT primer (Boehringer Mannheim) by incubating 5ul oligo $dT_{(12-18)}$ (1ug/ul), 0.5ul 2mM EDTA and 5ul viral RNA (2.5 ug) at 70C for 10min and then leaving it at room temperature for 10min.

First strand cDNA synthesis

The cDNA reaction mixture and reaction conditions were based on the procedure outlined by Maniatis *et al* (1982). The 10.5ul of the oligo dT primed viral RNA was added to a separate microfuge tube containing 30ul of the cDNA reaction mixture consisting of the following components:

0.5 ul	placental ribonuclease inhibitor (30u/ul) (Bethesda Research Laboratories)
0.5 ul	bovine serum albumin (50ug/ul) (Bethesda Research Laboratories)
2.5 ul	1M Tris-HCl, pH 8.1
5.0 ul	100mM $MgCl_2$
5.0 ul	100mM DTT
5.0 ul	40mM sodium pyrophosphate
1.25ul	50mM dATP
1.25ul	50mM dGTP
1.25ul	50mM dTTP
1.25ul	50mM dCTP
6.5 ul	water

All the components were gently vortexed and 7.5ul of 1M KCl (150mM final conc) and 2ul of avian myeloblastosis virus reverse transcriptase (Life Sciences;40 units) were added, mixed and the total volume of 50.0ul, was incubated at 42C for 2h and the reaction stopped by adding EDTA (pH 7) to a final concentration of 20mM. The products were extracted with an equal volume of one-to-one mixture of phenol/chloroform, using a microfuge to separate the phases. The phenol/chloroform phase was re-extracted by the addition of half-volume water. The supernatants were pooled and re-extracted with an equal volume of chloroform. The RNA:cDNA hybrid was recovered by ethanol precipitation using an equal volume of 4M ammonium acetate and two volumes of ethanol.

Second strand cDNA synthesis

The RNA:cDNA hybrid was vacuum dried and dissolved in 62ul of water. Second strand synthesis was carried out by adding 38ul of a reaction mixture consisting of:

5 ul	1M Tris-HCl, pH 7.5
5 ul	0.1M $MgCl_2$
10 ul	1M KCl
1 ul	BSA (50mg/ml)
10 ul	20mM each deoxynucleotide triphosphate mixture (dNTP's; dATP,dGTP,dTTP & dCTP)
1 ul	8.5 units/ml ribonuclease H (Bethesda Research Laboratories)
5 ul	230 units/ml DNA polymerase I (Bethesda Research Laboratories)
1 ul	10 units/ml T4 DNA ligase (Bethesda Research Laboratories)

The 100ul total reaction volume was sequentially incubated for 2h at 14C and 2h at 21C. EDTA was added to a final concentration of 20mM to stop the reaction. The double-stranded cDNA reaction products were phenol/chloroform extracted and ethanol precipitated using ammonium acetate as shown above.

dCTP-tailing of the double-stranded DNA

The doubled-stranded cDNA was poly dC-tailed so that it could be annealed to the *Pst* I cut dG-tailed plasmid pBR322 DNA. The dC-tailing procedure involved resuspending the vacuum dried dsDNA in 14ul of water and adding the following components:

4.0 ul 5X Bethesda Research Laboratories tailing buffer
(1X=100mM potassium cacodylate pH 7.2, 2mM $CoCl_2$, 0.2mM DTT)
0.5 ul 50mM dCTP stock
0.5 ul BSA (45-50mg/ml)

This mixture was incubated at 37C for about 10min to warm the solution before starting the timed run with the enzyme. One ul of Bethesda Research Laboratories terminal deoxynucleotidyl transferase (15 units) was added to the 19ul mixture and the reaction allowed to proceed at 37C for 12min. The reaction was stopped by the addition of 3ul of 0.5M EDTA pH 8.0 and 2ul 1M NaCl and heated to 65C for 10min to inactivate the enzyme.

Annealing of double-stranded dC-tailed cDNA to vector

The low background transformation cloning vector pBR322 (Gubler & Hoffman,1983) was used. A 0.5ul volume of Bethesda Research Laboratories *Pst* I-cut dG-tailed pBR322 vector (100ng) was added to the following components:

25	ul	dC-tailed double-stranded DNA (from above)
199.5	ul	water
25	ul	10X annealing buffer (5M NaCl, 0.5M Hepes pH 7.6 and 0.5M EDTA pH 7.5)

The 250ul of the annealing reaction mixture was sequentially incubated at 70C for 25min and at 37C for 3h. The recombinant plasmids were then left at room temperature overnight and cooled to 4C for at least 30min before use in transformation.

Transformation of *E. coli* RRI

The recombinant pBR322 plasmid DNA is a circular molecule containing selectable markers for antibiotic resistance. The plasmid contains resistance genes for tetracyline and ampicillin. Cloning into the *Pst* I site inactivates the ampicillin gene but not the tetracycline gene.

The procedure used to transform bacteria essentially involves making logarithmically growing cells permeable to DNA by incubation in $CaCl_2$ (Maniatis *et al*,1982). The uptake of plasmid DNA from the surrounding medium is facilitated by a brief heat shock treatment. Bacterial cells which have successfully taken up the plasmid DNA are then selected for growth on an agar plate containing the antibiotic. Cells not containing the plasmid will not grow on these plates, and the successful transformants will form bacterial colonies.

Three ml of prewarmed BHI broth was inoculated with *E. coli* RRI and vigorously shaken overnight at 37C. This overnight bacterial culture was used to inoculate 100ml of prewarmed BHI broth in a 500ml flask and the cells were grown by vigorous shaking at 37C for about 1h (cells are in mid-logarithmic phase of growth; A_{660} = 0.4). The cell suspension was centrifuged at 4000g in autoclaved Sorvall SS34 tubes for 10min at 4C. The supernatant was discarded and the cells were resuspended in 10ml (per tube) of ice-cold, sterile solution of 50mM $CaCl_2$. The cell suspension was placed on an ice bath for 20min and then re-centrifuged at

4000g for 5min at 4C. The supernatant was discarded and the cells were resuspended in 2ml (per tube) of ice-cold, sterile solution of 50mM $CaCl_2$ and stored on ice until used.

Two hundred ul of competent cells were added to 50ul of annealed plasmid and placed on ice for 30min. The cells were heat treated at 42C for 1.5min in sterile siliconized corex tubes. One ml of BHI broth per 250ul of cells was added and the cells incubated at 37C for 30min without shaking. Using the spreading technique (Maniatis *et al*,1982) the cells were plated on freshly made LB agar plates (250ul/plate) containing a final concentration of 15ug/ml tetracyline. The plates were inverted and incubated at 37C overnight.

Colonies were subsequently checked for tetracyline resistance and ampicillin sensitivity by the replica plating method using a final concentration of 50ug/ml ampicillin. Colonies which were found to be tetracyline resistant and ampicillin sensitive were then cultured for plasmid DNA extraction.

2.2.10.2 cDNA synthesis and molecular cloning of the potyvirus from nerines

Initial attempts to obtain cDNA clones from the potyviral RNA in pBR322 failed to yield transformants with detectable inserts. To overcome this problem the strategy of cloning into phage vectors was attempted to increase the transformation efficiency of the cloning system. The phage vectors used were lambda gt10 and L47AB.

cDNA synthesis

A modification of the one-tube cDNA synthesis protocol derived from the Gubler and Hoffman method (1983), described earlier, was used for cDNA synthesis. The one-tube reaction procedures were a modification of those described by D'Alessio *et al* (1987) and Watson and Jackson (1985).

To approximately 5ug of vacuum dried poty-viral RNA, which was assumed to be polyadenylated, 19ul of water was added and heated to 65C for 5min. The tube was ice chilled and the following reagents added:

10 ul	5X RT buffer(250mM Tris-HCl pH 8.3 at 42C, 40mM $MgCl_2$, 250mM KCl)
5 ul	5mM dNTP's
5 ul	oligo dT (200ug/ml)
11 ul	reverse transcriptase (9u/ml, Promega-Biotec) [Avian myeloblastosis virus]

The mixture was briefly vortexed and incubated at 42C for 2h to synthesize the first cDNA strand. To synthesize the second cDNA strand the following reagents were added to the 50ul cDNA mixture from above:

64 ul	second strand buffer (5X stock: 94mM Tris-HCl pH 8.3,470mM KCl, 20mM $MgCl_2$, 19mM DTT)
8 ul	DNA polymerase 1 (New England BioLabs, 10u/ul)
1 ul	ribonuclease H (Bethesda Research Lab., 2-3u/ul)
12 ul	5 mM dNTP's
185 ul	water

This mixture was incubated at 16C for 2h. The double-stranded cDNA was phenol/chloroform extracted as described previously and further purified by using 'GENECLEAN' (Bio 101)- a powdered glass preparation which binds DNA only at high salt concentrations- and eluted with 25ul of water. Any 'ragged ends' to the cDNA were filled in by adding the following reaction reagents:

25 ul	cDNA
1 ul	5mM dNTP's
1 ul	T4 DNA polymerase (Promega-Biotec, 10u/ul)
3 ul	10X T4 Polymerase buffer

The mixture was incubated at 37C for 30min, phenol/chloroform extracted and further purified with 'GENECLEAN'.The blunt-ended cDNA was eluted from the 'GENECLEAN' in 10ul of water.

To protect internal *Eco* RI sites within the cDNA from subsequent clevage with *Eco* RI, the cDNA was methylated by using *Eco* RI methylase. The following methylation reagents were added to the 10ul of DNA in water:

2 ul	800 uM S-adenosyl-methionine (New England Biolabs)
4 ul	5X methylase buffer (0.5M NaCl,0.5M Tris-HCl pH 8, 5mM EDTA, 500ug/ml BSA).
0.5 ul	*Eco* RI methylase (New England Biolabs, 20u/ul)
3.5 ul	water

The mixture was incubated at 37C for 30min, phenol/chloroform extracted and 'GENECLEAN' treated. The methylated cDNA was eluted from the 'GENECLEAN' with 5ul of polynucleotide kinase-treated linkers (500ng; following procedure described by Watson & Jackson,1985) and eluted with 5ul of water. The 10ul volume of kinase-treated linkers and double-stranded DNA were combined with the following ligation mixture:

2 ul	5X ligase buffer (Bethesda Research Laboratories)
1.2 ul	T4 DNA ligase (Bethesda Research Lab.; 0.72u/ul)

The mixture was incubated at 14C overnight. The ligated cDNA/linkers was heat treated at 65C for 10min to inactivate the T4 DNA ligase and centrifuged briefly in a microcentrifuge to collect the condensate. To this was added 1.5ul of 1M Tris-HCl pH 7.5, 1.0ul of 1M NaCl and 5.6ul of water.

The linkered cDNA was cleaved with *Eco* RI to remove all but the terminal unit of the oligomeric linker addition to generate cohesive *Eco* RI termini on the molecules. This was achieved by adding 130 units of *Eco* RI (Boehringer Mannheim) to the linkered cDNA from above and incubated at 37C for 2h. The mixture was phenol/chloroform extracted and 'GENECLEAN' treated. The cDNA was eluted twice with 48ul of TE, 4ul of 5M NaCl was added and excess linkers removed by passing the it through a PREPAC mini-column (Bethesda Research Laboratories). The cDNA with cohesive cloning ends was eluted from the PREPAC mini-column with 2X100ul of 1M NaCl in TE (10mM Tris-HCl, 1mM EDTA, pH 8.0) followed by 2X100ul of 2M NaCl in TE.

The cDNA was co-precipitated with the vector lambda gt10 arms by adding the 400ul of cDNA to 10ug of lambda gt10 arms. This was ethanol precipitated, centrifuged in a microfuge for 20min and vacuum dried. Ligation of the cDNA into the lambda gt10/*Eco* RI arms was achieved by resuspending the pellet in 3.5ul of water and holding it at 42C for 5min to anneal lambda cos ends and adding the following components:

0.5 ul 10X ligase buffer (Bethesda Research Laboratories)
0.5 ul 10mM ATP
0.5 ul T4 DNA ligase (200U; Bethesda Research Laboratories)

The reaction mixture was left at 14C overnight. The enzyme was inactivated by heat treating the mixture at 65C for 5min. The recombinant DNA (linkered and ligated) was packaged into lambda gt10 particles *in vitro* using the 'Packgene' (Promega-Biotec) extract system. Five ug of recombinant DNA was added to one vial of 'Packgene' extract and incubated at 22C for 2h. Then 0.5ml of SM (50mM Tris-HCl pH 7.5, 100mM NaCl, 10mM $MgCl_2$ and 0.1% gelatin) and 25 ul of chloroform was added, mixed by vortexing and debris removed by centrifuging for 30 seconds in an eppendorf centrifuge.

The lambda gt10 recombinants were diluted with SM. Infection of *E. coli* C600 was carried out by mixing 10ul of diluted phage (10^2/ml) with 100ul of concentrated overnight culture of cells in 100mM $MgCl_2$ in capped sterile 16mm pyrex tubes and incubated at 37C for 15min. To each tube 3 ml of LAM medium (Hohn,1979) containing melted 0.7% agar warmed at 55C was added, poured onto a labelled plate containing 30-35ml of hardened agar bottom medium. The plates were gently swirled to ensure an even distribution of bacteria and top agar. The plates were closed and left for a few minutes for the top agar to harden and inverted and incubated at 37C for 6h.

On C600, both recombinant and nonrecombinant phage form plaques. Insert-bearing lambda gt10 forms a clear plaque and non-insert bearing lambda gt10 forms a turbid plaque.

Eight of the clear plaques that appeared were randomly picked and each transferred into 40ul of PSB (10mM Tris-HCl pH 7.5, 100mM NaCl, 10mM $MgCl_2$ and 0.1% gelatin) and 100ul of *E. coli* and incubated at 37C for 15min. The mixture was transferred into 30ml of prewarmed LB and incubated at 37C overnight with vigorous shaking. 600ul of chloroform, 30ug of DNase I, 30ug of RNase and 1.7g of NaCl was added and incubation continued at 37C for 15min with shaking. The mixture was centrifuged at 6000g for 10min at 4C. To the supernatant 3g of PEG (MW6000) was added, dissolved by gentle shaking and incubated on ice for 1h. The mixture was centrifuged at 6000g for 20min at 4C to pellet the

DNA. The pellet was resuspended in 1 ml of PSB and redigested with 5ug/ml DNase I and 100ug/ml RNase by incubating at 37C for 30 min. Half a ml of a 30%PEG stock and 2.5M NaCl was added, gently mixed and centrifuged at for 5min in a microfuge and the pellet resuspended in 200ul of PSB. Ten ul of 10% SDS, 4ul of 0.5M EDTA, pH 8, 20ul of 1mg/ml proteinase K was added and heated to 68C for 30min. The mixture was extracted once with phenol, once with phenol/chloroform and finally with chloroform. One hundred ul of 7.5M ammonium acetate and two volumes of ethanol was added and centrifuged in a microcentrifuge for 15min to pellet the DNA. The pellet was rinsed with 70% ethanol, spun in microfuge for two minutes and the supernatant carefully removed without disrupting the pellet. The pellet was dried under vacuum and resuspended in 50ul TE buffer. A two ul aliquot of the suspension was run on a gel to check integrity and purity. The rest was used for analysis of insert size and for subcloning into a plasmid vector.

Cloning into lambda L47AB

The bacteriophage lambda L47AB was designed for cloning large fragments of DNA (Loenen & Brammar,1980). It has a lower limit of 8kb for the fragment that can be cloned into it. It was used in this study to attempt to attain near full length inserts of cDNA derived from the viral genome of 10kb.

Half the double-stranded cDNA synthesized above was cloned into lambda L47AB. The procedure used was the same as used above with gt10 except 400ul of double-stranded cDNA was co-precipitated with lambda L47 arms instead of gt10 arms.

Restriction analysis of the inserted cDNA clones

The size of the cDNA inserts was determined by *Eco* RI digestion of the recombinant DNA. About 3ul (0.5ug) of insert-containing lambda gt10 was digested in a 20ul volume containing high salt buffer (100mM NaCl), 10 units of *Eco* RI and water. The mixture was incubated for 2h at 37C and run on an agarose gel as described in Section 2.2.8.1 with *Hind* III cut lambda DNA as markers.

Subcloning of lambda gt10 insert into plasmid pGEM3.

Although lambda vectors generally have a higher cloning efficiency than plasmids, plasmid vectors are useful for the amplification and purification of relatively large quantities of cloned cDNA. Further, the ratio of 'insert' to vector DNA is substantially lower with plasmids, enabling one to use the entire recombinant DNA (vector plus insert) in nick-translation reactions.

The lambda gt10 insert were found to belong to two classes (1.54kb and 0.56kb), on the basis of size. Both of these were subcloned into the plasmid pGEM3 (Promega-Biotec).

Ten ul of recombinant gt10 DNA was digested with 2ul of *Eco* RI (40 units) and 2ul of 10X *Eco* RI digest buffer (1M Tris-HCl, pH 7.5; 0.5M NaCl, 50mM $MgCl_2$) and incubated at 37C for 2h. The digested DNA was run on 0.7% agarose and the inserts were excised, extracted with phenol and further purified with 'GENECLEAN' (described in Section 2.2.10.2) and eluted twice with 5ul of water. The ligation reaction was carried out as described by King and Blakesley (1986) in a 20ul volume containing 10ul of insert DNA in water, 1ul of *Eco* RI-cleaved pGEM3-blue DNA, 4ul of 5X T4 DNA ligase buffer (supplied by Bethesda Research Laboratories), 1.4ul (1 unit) of T4 DNA ligase (Bethesda Research Laboratories) in a sterile polypropylene microcentrifuge tube. The components were mixed briefly, centrifuged for 5sec, then incubated at room temperature for 4h. One ul of 0.5M EDTA (pH 8) was added, the reaction was mixed, and then stored at 4C.

DNA transformation was performed essentially as described earlier for pBR322 using the $CaCl_2$, heat shock treatment method. A 5ul (1-2ng) sample was removed from the completed ligase reaction and added to 200ul of competent *E. coli* JM109 cells in a pre-chilled polypropylene tube, mixed gently and incubated on wet ice for 30min. The tube was placed at 42C for 45sec and then immediately returned to wet ice. After several minutes 800ul of LB medium was added and the tube was incubated at 37C for 1h in a shaking incubator, spun down and resuspended in 50ul of LB and spread onto to LB agar containing 100ug/ml ampicillin and previously treated with 30ul of X-Gal and IPTG (20mg/ml) to serve as indicators. Bacterial cells harbouring recombinant pGEM3-blue vector were white whereas those containing pGEM3-blue vector lacking inserts were blue.

Rapid plasmid preparations were made from the colonies as described earlier, and the recombinant DNA from the plasmid was digested with *Eco* RI (New England Biolabs) and characterized as

previously described. Large scale plasmid DNA preparations were made by inoculating 250ml of LB containing 50ng ampicillin with a single colony of JM109 transformants and incubated at 37C overnight with vigorous agitation and the DNA extracted by the Promega-Biotec method described below.

The 1.54kb insert DNA was nick-translated and used as a probe for screening the L47 library for positive clones. The procedures used were essentially those described by Davis *et al* (1986). From the L47 library one clone with a 9.8kb insert was subcloned into pGEM3 and DNA extracted for use in dot-blots.

Plasmid DNA preparation

Recombinant plasmid DNA for use as a probe was extracted by the LiCl-boiling method (Wilimzig,1985) for analytical purposes and the method developed by Kreig & Melton of Harvard University and described in the Promega-Biotec catalogue (1985/'86) for large-scale preparations.

LiCl-boiling method for plasmid mini-preparations

An *E. coli* pellet from a 1ml overnight culture was resuspended in 100ul of TELT buffer (50mM Tris-HCl pH 7.5, 62.5mM EDTA, 0.4% Triton X-100, 2.5M LiCl). Ten ul of freshly prepared aqueous solution of lysozyme (Sigma, 10mg/ml) was added, briefly vortexed and left at room temperature for about 10min and placed in a boiling water bath for 1min. After cooling on ice for 5min, the precipitate was removed by centrifugation in a microcentrifuge for 8min at room temperature. The DNA in the supernatant was precipitated with ethanol, vacuum dried and resuspended in a small volume (about 50ul) of water or TE (10mM Tris-HCl, pH 7.5, 1mM EDTA).

Large-scale preparations

The *E. coli* cells, grown overnight in 250ml of the appropriate selective medium, were centrifuged at 4000g for 15min at 4C. The culture pellet was resuspended in 6ml of freshly prepared solution consisting of 25mM Tris-HCl pH 7.5, 10mM EDTA, 15% sucrose and 2mg/ml lysozyme. The cells were resuspended

thoroughly by pipetting them up and down with a 10ml pipette. The mixture was incubated in ice water for 20min and 12ml of 0.2M NaOH and 1% SDS was added to it, mixed carefully but thoroughly by inversion and incubated in ice water for a further 10min. To this was added 7.5ml of 3M sodium acetate, pH 4.6, mixed by inversion and incubated in ice water for 20min. The mixture was centrifuged at 12,000g for 15min and the supernatant removed to another tube avoiding the white precipitate. Fifty ul of boiled RNase A (1mg/ml) was added to the supernatant and incubated at 37C for 20min. The DNA was extracted twice with an equal volume of 1phenol:1chloroform, the phases separated by centrifugation at 12,000g for 10min and ethanol precipitated by adding 2.5 volumes of ethanol and one-tenth of one volume of 3M sodium acetate. The precipitate was collected by centrifugation at 12,000g for 10min, vacuum dried and resuspended in 1.6ml of water. To this was added 0.4ml of 4M NaCl and 2ml of 13% PEG (MW6000), mixed and incubated on ice water for 1h. The DNA pellet was collected by centrifugation at 9,000g for 10min, washed with 70% ethanol, vacuum dried and resuspended in a appropriate volume (usually 0.5ml) of water or TE. A small aliquot was diluted and the integrity and purity of the DNA checked on a standard agarose gel as previously described.

Nick-translation of cloned cDNA

The nick-translation reaction is used to introduce radioactive nucleotide phosphates into unlabelled DNA for the purpose of making a probe. The reaction depends on the ability of the enzyme DNA polymerase I to initiate DNA synthesis at free 3'OH groups, which are exposed as nicks in the unlabelled DNA. The nicks are generated in random locations by the limited digestion of DNA with DNase I. The DNA polymerase synthesizes new DNA in a 5' to 3' direction, incorporating labelled triphosphates.

The protocol used for the nick-translation reaction in this study was based on the procedures developed by Rigby *et al* (1977). Substrate DNA at a concentration of about 0.2ug/ml was pre-incubated at 14C for 15min with 1ng of DNase I (bovine pancreas, Sigma) in a total volume of 35ul containing 50mM Tris-HCl (pH 7.5), 7.5mM magnesium acetate, 4mM dithiothreitol, 100 ug/ml of nuclease-free BSA (Boehringer Mannheim), 25mM each of dATP, dGTP, dTTP and 20uCi of [a-^{32}P]dCTP (Nuclear Supplies Ltd). The DNase I was then inactivated by heating at 70C for 5min and chilled on ice. Repair synthesis was started by the addition of 1.5ul of DNA polymerase I (5 units 'nuclease-free', Boehringer Mannheim) and continued at 14C for 15min. Reaction was stopped by the addition of EDTA and SDS to 20mM and 5%(w/v)

respectively.

Immediately before use the labelled probe was fragmented by incubating with 3ul of 4M HCl (final conc of 0.25M) at room temperature for 5-10min (Reed & Mann,1985). The cDNA probe was denatured by adding 9ul of 4M NaOH, briefly vortexed and left to stand at room temperature for 10min.

Hybridization reactions on Zeta-Probe membrane

Zeta-Probe membranes were dot-blotted as described in Section 2.2.9.3. The pre-hybridization solution was freshly prepared and consisted of the following components: 50% deionized formamide, 2X PE (0.133M sodium phosphate pH 6.9, 0.001M EDTA), 7% (w/v) SDS, 0.5% (w/v 'Anchor' skim milk) and 0.5mg/ml sonicated salmon sperm DNA. The dot-blotted membrane was sealed in a plastic bag with the appropriate volume of pre-hybridization solution (varied with the size of the membrane, generally about 10ml), taped to a metal plate and placed on a shaker in an incubator at 50C for at least 1h.

The pre-hybridization solution was drained and replaced with the hybridization solution consisting of the same components as above except the skim milk and the salmon sperm DNA were omitted. The probe was added and the sealed bag was enclosed in a second bag and sealed. The bags were placed in an incubator at 50C overnight for hybridization to occur.

Note: Since formamide is highly toxic and the probe was radioactive standard safety procedures were adopted.

After hybridization the membrane was carefully removed and washed in the following washing solution preheated to 42C: two half-hour cycles of 2XSSC (SSC= 0.15M NaCl, 0.015M sodium citrate), 0.1% SDS with vigorous agitation followed by two half-hour cycles with 0.1X SSC, 0.1% SDS. The membrane was placed on a paper towel and wrapped with a polyethylene film (eg 'Glad-Wrap' and autoradiographed with Kodak XAR-5 film using an intensifying screen, at -70C.

If the membrane was to be re-used for additional hybridization analysis, the probe was removed by heating the membrane in 0.1X SSC, 0.1% SDS to boiling in a microwave oven and checked for probe removal by exposure to X-ray film under the standard conditions used in this study.

2.2.11 *In Vitro* translation of nerine virus X RNA

Translations in messenger-dependent rabbit reticulocyte lysates (Pelham & Jackson,1976; Forster *et al*,1987) of nerine virus X RNA was done in the presence of [^{35}S]methionine. Following translation at 30C for 50min, products were separated on 12.5% polyacrylamide gels and then autoradiographed (Forster *et al*,1987).

2.2.12 Serology

2.2.12.1 Ouchterlony double-diffusion test

The two-dimensional immunodiffusion test (Ouchterlony,1962) was conducted to confirm the identity of isometric particles in a number of test plants showing virus symptoms. The procedure used was based on Morris-Krsinich *et al* (1978) using 0.9% agar in 0.05M phosphate buffer pH 7.8 containing 5mM EDTA. The antiserum was loaded in a 4mm diameter central well and the antigens including appropriate controls were loaded in the outer wells.

2.2.12.2 Immuno-dot blotting

Purified nerine virus X diluted in 0.1M sodium phosphate buffer, pH 7.5 was loaded in a 5cm square Zeta-Probe membrane using the method described in Section 2.2.9.3. After air-drying the membrane was placed in a plastic bag and incubated with gentle agitation in 5ml of TBS (0.02M Tris-HCl pH 7.5, 0.5M NaCl) containing 1.25% 'Anchor' non-fat milk powder for 3h at 37C to block non-specific binding sites. The bag was drained and the solution replaced with TTBS (0.02M Tris, 0.5M NaCl, 0.2% Tween 20) containing 30ul of purified primary antibody (nerine virus X - agapanthus strain ex A A Brunt), 1% BSA and incubated with agitation for 2h at room temperature. The membrane was removed from the plastic bag and washed three times (10min/wash) with 50ml of TTBS. The membrane was placed in a new bag and incubated with TTBS containing 3ul of alkaline phosphatase conjugated secondary antibody (goat anti-rabbit IgG (Immuno-Chemical Products), 1% BSA and incubated for 2h at toom temperature with gentle agitation. The membrane was removed from the bag, washed with three cycles of TTBS and developed by adding the enzyme substrate, containing 15mg of Fast-red (Sigma) dissolved in 5ml of 0.2M Tris, pH 8 and 200ul of Napthol/AS-MX (Sigma), until the colour change occurred.

CHAPTER 3

SYMPTOMATOLOGY, ELECTRON MICROSCOPY AND MECHANICAL TRANSMISSION STUDIES

3.1 INTRODUCTION

A visual inspection of commercial and home-grown nerines undertaken at the commencement of this study suggested that viruses were prevalent in nerines in New Zealand. This chapter provides details of virus-like symptoms in various *Nerine* species and cultivars in New Zealand, and results of surveys for viruses by electron microscopy and by mechanical transmission to herbaceous indicator plants.

3.2 SYMPTOMATOLOGY

Figure 1[A-E] shows some of the more obvious virus-like symptoms seen on leaves, flower stems and flowers, especially in *N.sarniensis* hybrids and in *N.bowdenii* types. These symptoms included distortion of the flower stem (Figure 1[A]); colour-break in the petals (Figure 1[B]); mottling, streaking, yellowing, marginal chlorosis and bleaching of leaves (Figure 1[C], [D] & [E].

The most striking virus-like symptoms on leaves and floral parts were seen in nerine plants infected with more than one class of filamentous viruses. Mosaic and streaking was evident even in very young leaves of a *N.bowdenii* hybrid infected with a 540nm potexvirus (later described as nerine virus X [NeVX] and a 740nm potyvirus, presumably nerine yellow stripe virus [NeYSV], Figure 1[C] & [D]. No virus-like symptoms were seen in the floral parts of this cultivar. However, colour-break in the petals was evident (Figure 1[B]) in a *N.sarniensis* hybrid with pink flowers infected with the same two virus groups, although no obvious virus-like symptoms were seen in the leaves.

Figure 1: Some virus-like symptoms in nerine flowers, flower stems and leaves.

Figure 1[A]: Distortion of flower stem of a *Nerine sarniensis* hybrid infected with 800nm potyvirus particles.

Figure 1[B]: Colour-break in petals of a *Nerine sarniensis* hybrid infected with a 540nm potexvirus and a 740nm potyvirus.

Figure 1[C]

Figure 1[D]

Figure 1[C] & [D]: Mottling, streaking and yellowing of *Nerine bowdenii* hybrid leaves infected with a 540nm potexvirus and a 740nm potyvirus.

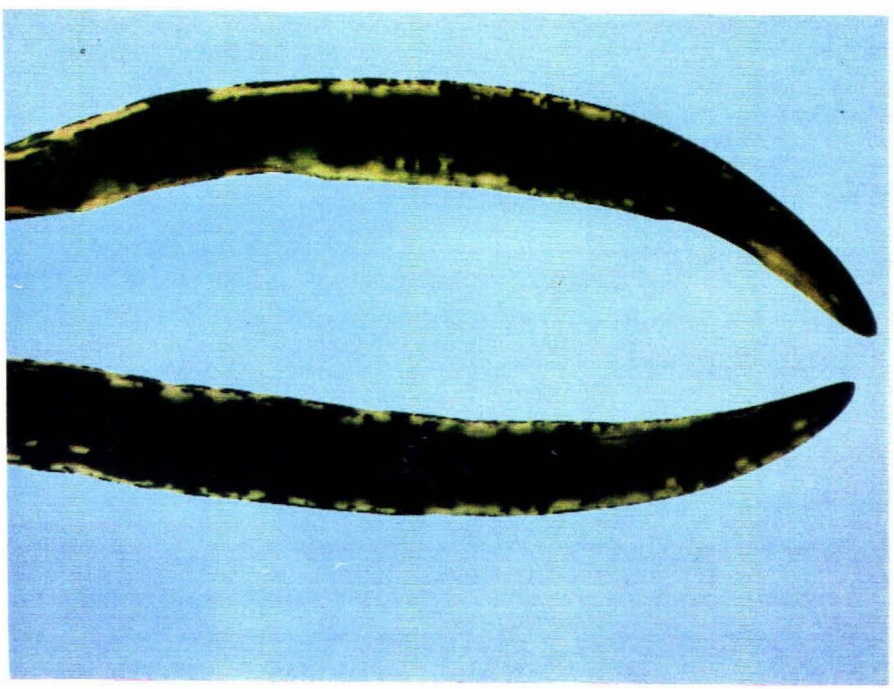


Figure 1[E]: Marginal chlorosis and bleaching in *Nerine bowdenii* leaves infected with 540nm potexvirus particles.

3.3 ELECTRON MICROSCOPY

A range of tissue types and procedures for preparing nerine tissues for electron microscopy was tested. This included the dip method (Noordam,1973) of slicing the leaf with a razor blade and squeezing a drop of sap directly onto the grid; grinding leaf, petal or bulb tissue with a mortar and pestle in a low molarity buffer (eg 0.02M potassium phosphate, pH 7) and placing a drop of the crude homogenate on the grid; and, using the procedure outlined in Section 2.2.4.1 (a). The high level of mucilaginous material in nerine leaf tissue interfered with, and reduced the level of virus trapped on the grids using the leaf dip method. Unacceptably high levels of tissue components were trapped when a drop of crude homogenate was loaded directly on the grid. The procedure described in Section 2.2.4.1.(a) of floating grids in spotting tiles containing crude homogenates and negatively staining with 2% PTA, pH 7.0, proved to be a satisfactory method for batch indexing crude sap with the electron microscope. Potassium phosphotungstic acid gave better contrast for the filamentous viruses from nerines than either 2% sodium silicotungstate or 2% uranyl acetate. In most nerine plants tested the concentration of filamentous virus was higher in floral and leaf tissue than in bulb tissue. Although floral tissue was a good source of virus with minimal levels of host components, the seasonal nature of this tissue precluded it from being used more extensively in the electron microscope survey. Thus, most of the electron microscope indexing for filamentous viruses was conducted using nerine leaf tissue.

A survey of more than 160 commercial and home-garden leaf tissue samples from 14 cultivars of three *Nerine* spp (Table 4) was undertaken. With very few exceptions, filamentous virus particles were observed in varying concentrations on the electron microscope grids. Only in a group of *N.bowdenii* seedlings were filamentous virus particles not detected.

Table 4 shows the number of cultivars and samples processed and results of particle length ranges observed during the electron microscope survey. Although a relatively small number of particles below 480nm were evident, these were assumed to be fragmented rods and are not shown in Table 4.

Table 4: Particle length ranges for filamentous viruses from three *Nerine* species and hybrids indexed by electron microscopy.

Nerine spp.	No. of cultivars	No. of samples	No. of particles measured	Particle length range (nm)
N.sarniensis (hybrids)	9	119	486	480-840
N.bowdenii and hybrids	4	32	143	480-760
N.fothergilli 'Major'	1	17	97	480-580

Two nerine cultivars were consistently found to have filamentous virus particles belonging only to a single size class; a clump of *N.fothergilli* 'Major' from a home-garden had virus particles with a modal length of 540nm (Figure 2) and a *N.sarniensis* hybrid which had been mass propagated by twin-scaling had particles with a modal length of 800nm.

A histogram of the length distribution of flexuous filamentous virus particles, classified at 40nm class intervals, is presented in Figure 3. The results of the electron microscope survey, besides showing the prevalence of filamentous particles in many of the cultivars surveyed, also revealed that virus(es) belonging to the potexvirus group were quite common. Further, in many of the older *N.sarniensis* hybrids, mixed infections of viruses probably belonging to the potexvirus and potyvirus groups were also evident.

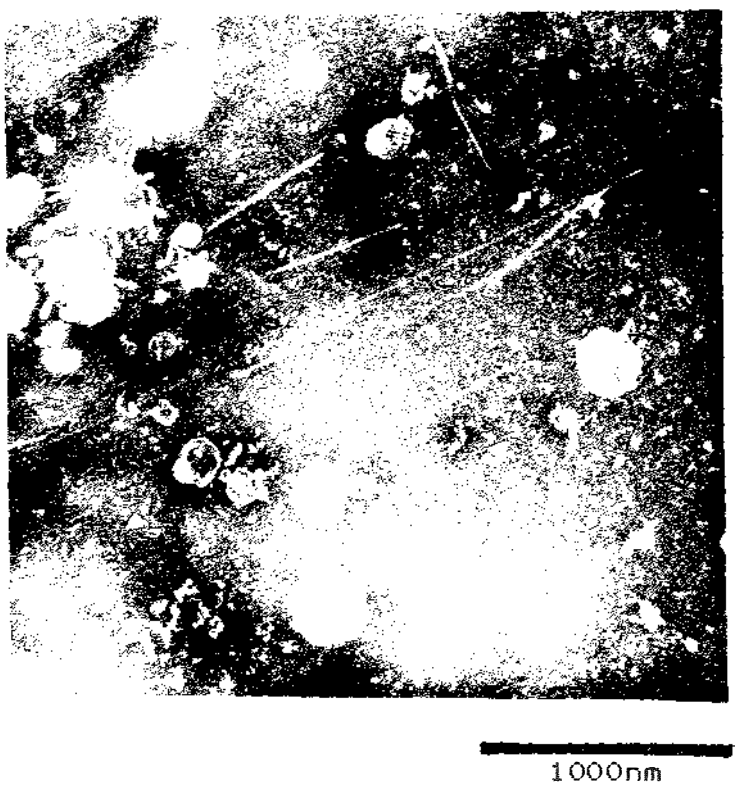


Figure 2: Potexvirus-like particles, in crude sap homogenate of nerine leaf tissue, with a modal length of 540nm. Stain: 2% potassium phosphotungstic acid, pH 7.

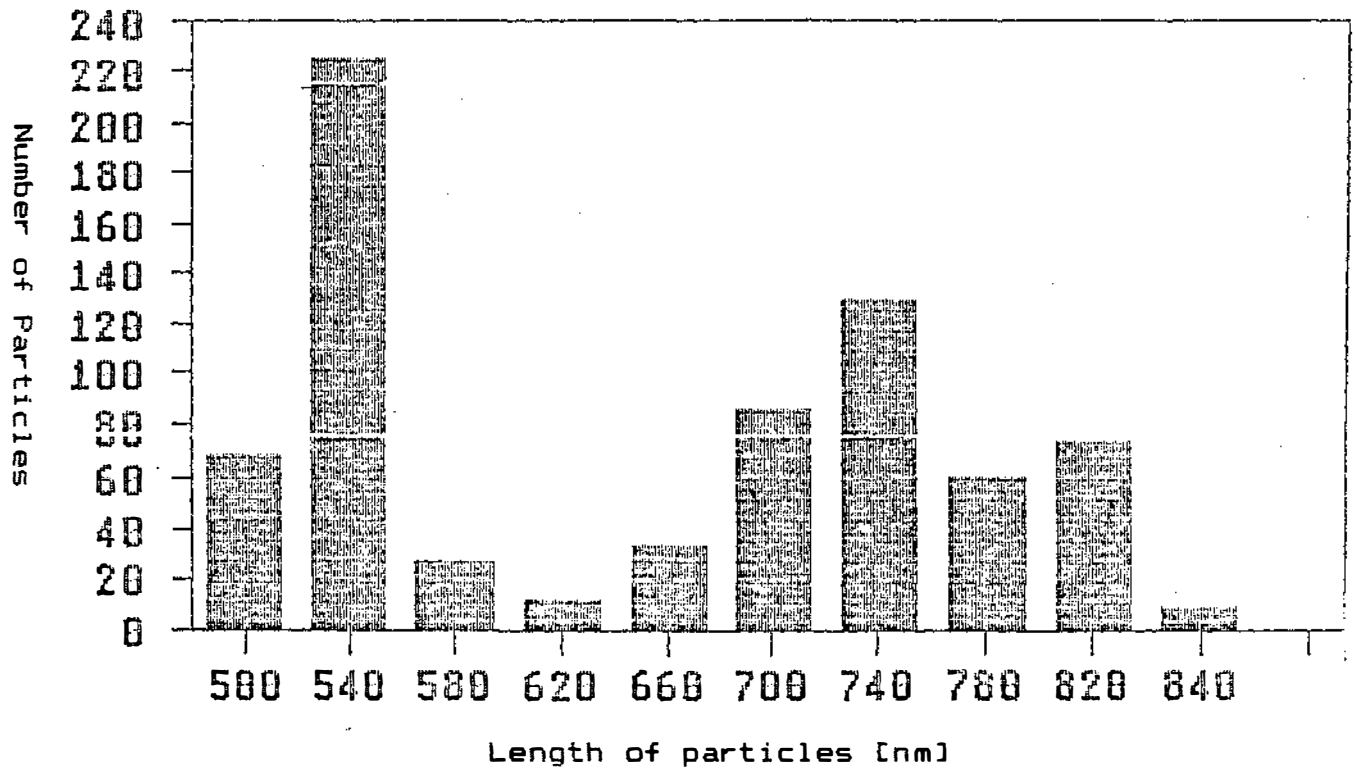


Figure 3: Length distribution of filamentous virus particles found in crude homogenates of nerine leaf tissue tested during surveys using the electron microscope (April-November,1985). Class interval: 40nm.

3.4 MECHANICAL TRANSMISSION

Three methods were tested for preparing leaf tissues for mechanical transmission. These were:

(a) crushing tissue in potassium phosphate buffer containing bentonite (Yarwood,1972)
(b) 2% nicotine solution (Thung & Van der Want,1951)
(c) partial purification by clarification with chloroform followed by polyehtylene glycol precipitation and resuspension in 0.01M potassium phosphate buffer.

The results of these trials using the test plant species mentioned in Section 2.1.4 (Table 3), are given in Table 5. All attempts to transmit the filamentous viruses from nerine tissue using the two buffer systems, and from partially purified preparations, failed to induce any virus-like symptoms on herbaceous test plants. Further, electron microscope investigations of leaf samples of test plants for the possibility of latent infections did not reveal any filamentous particles. Both inoculated leaves and new growth were tested for filamentous virus particles approximately two weeks after inoculation. In contrast, cucumber mosaic virus (CMV) was succesfully transferred from *N.sarniensis* hybrid leaf tissue to a number of herbaceous indicators using partially purified virus preparations which also contained filamentous virus particles. Cucumber mosaic virus was transferred from *N.sarniensis* hybrid leaf crude sap to *N.debneyi* and *N.tabacum* 'Samsun' in two inoculation tests (Table 5). Further details on the mechanical transmission and serological tests for CMV isolates from nerines are presented in Chapter 4.

3.5 DISCUSSION

Maat (1976) mentioned virus-like disease symptoms on leaves, flower stalk and flowers of nerines infected with various viruses in the Netherlands but provided no details. In England, Norris (1974) reported three virus-like symptoms in *Nerine* species. These were:

(a) mosaic and streaking of the leaves
(b) striping or fine irregular lines running across the width of the leaves
(c) colour break or blotchiness in the flowers.

Although filamentous virus particles, belonging mainly to the potexvirus and potyvirus groups, were commonly observed by electron microscopy in tissue with the virus-like symptoms described, it was not possible to definitely establish symptom association because of the possibility of mixed infections.

Table 5: Results of mechanical transmission studies with *Nerine* crude sap or partially purified virus preparation.

Species	Number Inoculated	Inoculum Type[1]	Inoculum Source[2]	Sample Number	Buffer & Additives	Symptoms[4]
Tetragonia	9	c.s.	1,2,3	4	Yarwood's[3]	Nil
expansa	4	c.s.	1	2	2% nicotine	Nil
	6	p.p.	1	2	0.01M KPO_4	Nil
Gomphrena	17	c.s.	1,2,3	8	Yarwood's	Nil
globosa	6	c.s.	1	3	2% nicotine	Nil
Chenopodium	46	c.s.	1,2,3	18	Yarwood's	Nil
amaranticolor	17	c.s.	1	9	2% nicotine	Nil
	6	p.p.	1	2	0.01M KPO_4	Nil
	4	p.p.	1	2	0.01M KPO_4	LL[5]
Chenopodium	69	c.s.	1,2,3	34	Yarwood's	Nil
quinoa	28	c.s.	1	16	2% nicotine	Nil
	4	p.p.	1	2	0.01M KPO_4	Nil
	5	p.p.	1	2	0.01M KPO_4	LL[5]
Cucumis	13	c.s.	1,2,3	6	Yarwood's	Nil
sativus	4	c.s	1	2	2% nicotine	Nil
'Slicemaster'	3	p.p.	1	1	0.01M KPO_4	Nil
	2	p.p.	1	1	0.01M KPO_4	LL & S[5]
Phaseolus	12	c.s.	1,2,3	4	Yarwood's	Nil
vulgaris	3	c.s.	1	2	2% nicotine	Nil
Nicotiana	89	c.s.	1,2,3	42	Yarwood's	Nil
clevelandii	26	c.s.	1	19	2% nicotine	Nil
	4	p.p.	1	2	0.01M KPO_4	Nil
	8	p.p.	1	2	0.01M KPO_4	S[5]
Nicotiana	18	c.s.	1,2,3	6	Yarwood's	Nil
debneyi	4	c.s.	1	2	Yarwood's	S[5]
	6	p.p.	1	2	0.01M KPO_4	Nil
	4	p.p.	1	2	0.01M KPO_4	S[5]
Nicotiana	36	c.s.	1,2,3	21	Yarwood's	Nil
glutinosa	8	c.s.	1	3	2% nicotine	Nil
	5	p.p.	1	2	0.01M KPO_4	Nil
	4	p.p.	1	2	0.01M KPO_4	LL[5]
Nicotiana	14	c.s.	1,2,3	8	Yarwood's	Nil
tabacum	4	c.s.	1	2	2% nicotine	Nil
'White Burley'	6	p.p.	1	2	0.01M KPO_4	Nil
	2	p.p.	1	1	0.01M KPO_4	S[5]
Nicotiana	8	c.s.	1,2,3	3	Yarwood's	Nil
tabacum	2	c.s.	1	1	Yarwood's	LL[5]
'Samsun'	3	p.p.	1	1	0.01M KPO_4	Nil
	2	p.p.	1	1	0.01M KPO_4	LL[5]
Petunia	8	c.s.	1,2,3	3	Yarwood's	Nil
hybrida	2	p.p.	1	1	0.01M KPO_4	Nil

Key

[1]: c.s.=crude sap ; p.p.=partially purified preparation.
[2]: 1=*N. sarniensis*; 2=*N. bowdenii* & hybrids; 3=*N. fothergilli* 'Major'.
[3]: Yarwood's=potassium phosphate buffer containing bentonite (Yarwood, 1966).
[4]: Nil=no visible symptoms; LL=local lesions; S=systemic symptoms
[5]: Virus confirmed as cucumber mosaic virus by Ouchterlony double diffusion tests.

However, some tentative evidence for symptom association is presented in Chapter 6 for an 800nm potyvirus found in *N.sarniensis* hybrid.

There are some reports in the literature of possible symptom association. Koenig *et al* (1973) reported that *N.bowdenii* cv 'Rose Queen' infected with narcissus mosaic virus (NaMV) displayed conspicuous leaf mottling. Further, *N.bowdenii* cv 'Van Roon' infected with nerine latent virus (NeLV) was reported by Maat *et al* (1978) to express severe mosaic symptoms although in other cultivars such as *N.bowdenii* '63' no apparent symptoms were evident. In the instances where symptom association was implicated, the possibility of the simultaneous occurrence of more than one infectious entity was not established.

The literature on nerine viruses reviewed in Chapter 1 indicates that several of the filamentous viruses have not been amenable in mechanical transmission studies (eg nerine strain of nerine virus X (NeVX); nerine yellow stripe virus (NeYSV). However, there are a number of viruses in nerines which have been found to be mechanically transmissible, especially from partially purified preparations. In this category are such viruses as CMV, NeLV, NaMV, and arabis mosaic virus (ArMV).

Yarwood's solution was chosen for mechanical transmission tests because it has been observed to increase the infectivity of many plant viruses (Yarwood,1972). The high pH (pH 8.7) may prevent activity of inhibitors or inactivators of virus infection. Further the additive bentonite in Yarwood's solution binds and inhibits ribonucleases which may inhibit virus infection (Francki & McLean,1968; Singer & Fraenkel-Conrat,1961). A 2% nicotine solution was also used because nicotine has been shown to combine with and precipitate tannins, which can also be inhibitors of virus infection (Thung & Van der Want,1951). Partially purified virus preparations resuspended in 0.01M potassium phosphate, pH 7, were also used as inoculum to to reduce or eliminate inhibitors or inactivators that may have been present in crude nerine sap. Further, potassium phosphate buffers have been found to increase the infectivity of some viruses (Thornberry,1935; Yarwood,1952).

The results of the mechanical transmission studies confirmed the findings reported in the literature (Chapter 1) that a number of filamentous viruses commonly found in nerines are not transmissible to a range of herbaceous indicators (Table 5). It seems unlikely that inhibitors or inactivators prevented mechanical transmission because extracts were prepared in solutions containing additives to minimize these. Furthermore, CMV was successfully transmitted, especially from partially purified virus preparations which were shown by electron microscopy to contain filamentous virus particles.

The failure to transmit any of the filamentous viruses from nerine tissue showing conspicuous virus-like symptoms and in which virus particles could readily be detected by electron microscopy would suggest that sap-transmissible viruses such NaMV, NeLV and tobacco rattle virus were not prevalent in the cultivars indexed. Thus, the 540nm potexvirus found to be quite prevalent in the cultivars indexed was unlikely to be NaMV on the basis of its lack of sap-transmissibility to common herbaceous indicator plants (Table 5).

Although some carlavirus-like particles (620-690nm; Wetter & Milne,1981) were detected in the electron microscope survey (Figure 3) it was not possible to determine whether these were fragmented potyviruses. The mechanical transmission results would again suggest that NeLV, the sap-transmissible carlavirus found to commonly infect *Nerine* species overseas (Hakkaart,1972; Maat *et al*,1978) was not common in the cultivars indexed.

As mentioned in Section 3.2, a 740nm potyvirus (presumably NeYSV) was found in a *N.bowdenii* hybrid cultivar which was also infected with the 540nm potexvirus, and, an unnamed 800nm particle was detected in one of the *N.sarniensis* hybrids. The failure to mechanically transmit any of these viruses to herbaceous test plants is consistent with the findings in the literature reviewed in Chapter 1.

CHAPTER 4

IDENTIFICATION OF CUCUMBER MOSAIC VIRUS IN *NERINE* IN NEW ZEALAND

4.1 INTRODUCTION

The presence of cucumber mosaic visrus (CMV) in nerines has been reported in studies in Europe (Hakkaart *et al*,1975; Maat *et al*,1978) and was found to be common in *N.sarniensis* and *N.bowdenii* cultivars (Table 1, Chapter 1). Hakkaart *et al* (1975) reported that from leaf samples containing 545nm filamentous particles no virus other than CMV could be transmitted to *G.globosa*, *C.amaranticolor*, *C.quinoa*, and *N.clevelandii*.

This chapter provides evidence that the mechanically transmissible virus isolated from naturally infected *N.sarniensis* hybrids is a strain of CMV.

4.2 SYMPTOMATOLOGY IN HERBACEOUS INDICATORS

Although 12 different herbaceous indicator species were used in the mechanical transmission studies, the indicators used most often were *C.quinoa*, *C.amaranticolor*, *N.clevelandii*, *N.glutinosa*, *N.debneyi* and *N.tabacum* 'White Burley'(Table 5, Chapter 3). A total of 42 nerine crude sap inoculations using Yarwood's solution and 19 using 2% nicotine was attempted. Only on two occasions was CMV transferred to *N.debneyi* and once to *N.tabacum* 'Samsun'. In all these instances the inoculum was obtained from *N.sarniensis* hybrid leaf tissue using Yarwood's solution (Table 5). Cucumber mosaic virus was also transmitted to a range of indicator species from two out of four partially purified virus preparations (chloroform clarified and polyethylene glycol precipitated; Section 2.2.5 [c]), from *N.sarniensis* hybrid leaf tissue containing filamentous virus particles.

The nerine isolates of CMV induced local lesion reactions on *C.quinoa*, *C.amaranticolor*, *Cucumis sativus* 'Slicemaster', *N.glutinosa* and *N.tabacum* 'Samsun', and, systemic symptoms in *N.clevelandii*, *N.debneyi* and *N.tabacum* 'White Burley'. Figure 4[A] shows necrotic local lesions on *C.quinoa*, Figure 4[B] shows local etched flecks forming concentric rings on *N.tabacum* 'Samsun', Figure 4[C] shows local lesions and systemic necrosis in *Cucumis sativus* 'Slicemaster', and Figure 4[D] shows fawn 'target-spots' in *N.glutinosa*.

Figure 4: Local lesions in test plants inoculated with virus preparation containing the nerine isolate of cucumber mosaic virus (CMV).

Figure 4[A]: Necrotic local lesions in *Chenopodium quinoa* 15 days after inoculation with partially purified virus preparation from *Nerine sarniensis* leaf tissue containing CMV.

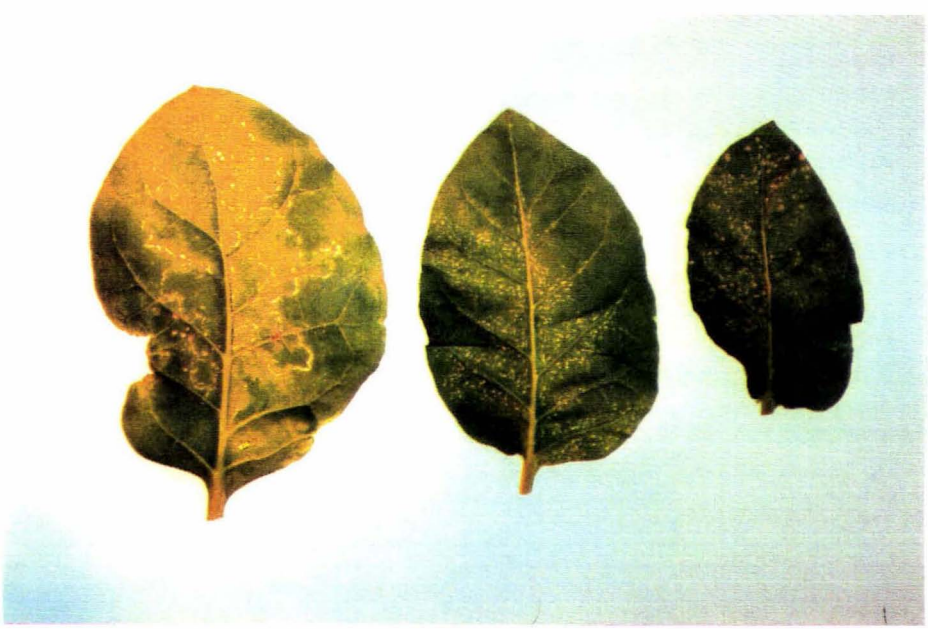


Figure 4[B]: Local etched flecks forming concentric rings in *Nicotiana tabacum* 'Samsun' 23 days after inoculation with nerine crude sap preparation containing CMV.

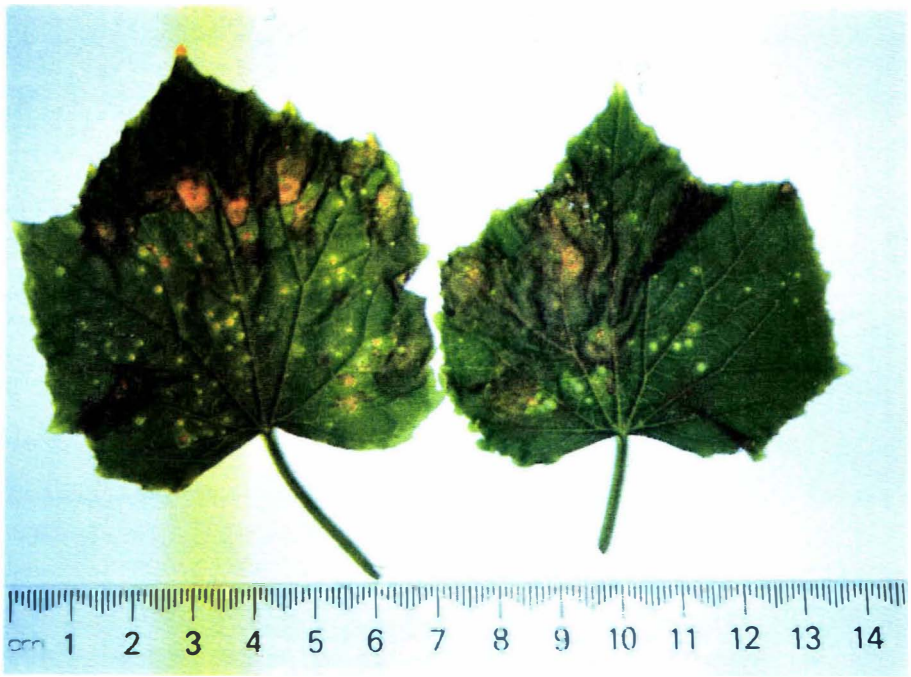


Figure 4[C]: Local lesions and systemic necrosis in *Cucumis sativus* 'Slicemaster' 22 days after inoculation with partially purified nerine virus preparation containing CMV.

Figure 4[D]: Fawn 'target-spots' in *Nicotiana glutinosa* 10 days after inoculation with partially purified nerine virus preparation containing CMV.

4.3 SEROLOGY

Ouchterlony double diffusion tests were used to confirm that the symptoms expressed in the test plants were caused by CMV (Figure 5). The gel diffusion tests show that the precipitin lines from wells 4,5 and 6 in Figure 5 are confluent indicating that CMV was present in the sap of the three inoculated species; *N.debneyi*, *N.tabacum* 'White Burley' and *N.tabacum* 'Samsun'.

4.4 PARTIAL PURIFICATION

A nerine isolate of CMV was partially purified from *N.tabacum* 'White Burley' by clarification with chloroform, polyethylene glycol precipitation (Section 2.2.5 [c]) and resuspension in 0.01M potassium phosphate buffer containing 0.05M EDTA. Electron microscopy revealed the presence of isometric CMV-like particles (Figure 6).

4.5 DISCUSSION

The results of mechanical transmission studies, serology and electron microscopy confirmed the presence of CMV in nerines in New Zealand. The finding that CMV was transferred at a much lower rate from *N.sarniensis* hybrid crude sap preparations compared to partially purified preparations suggests that CMV occurs at a relatively low concentration in the nerine tissue tested. Because this study was focused on filamentous viruses in nerines the incidence of CMV in locally grown *Nerine* spp and cultivars was not pursued any further.

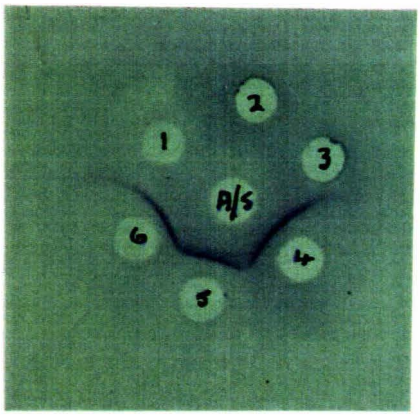


Figure 5: Ouchterlony double-diffusion test in agar. Centre well was charged with cucumber mosaic virus antiserum. Peripheral wells 1, 2 and 3 contained sap from non-inoculated *Nicotiana tabacum* 'White Burley', *N.tabacum* 'Samsun' and *N.debneyi* respectively. Wells 4, 5 and 6 contained sap from test plants inoculated with nerine crude sap or partially purified virus preparation.

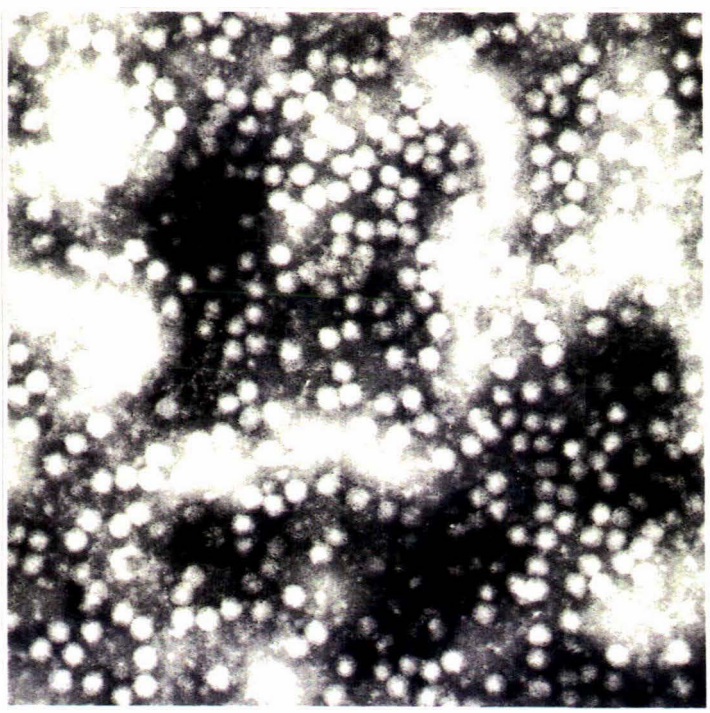


Figure 6: Partially purified preparation of a *Nerine sarniensis* isolate of cucumber mosaic virus from *Nicotiana tabacum* 'White Burley'.

CHAPTER 5

PRELIMINARY PURIFICATION STUDIES OF FILAMENTOUS VIRUSES FROM SYSTEMICALLY INFECTED *NERINE*

5.1 INTRODUCTION

The failure to transmit any of the filamentous viruses from nerine tissue to herbaceous test plants presented no option but to attempt to purify the virus(es) directly from field-infected nerine tissue. However, this gave rise to several problems. It was not possible to obtain substantial quantities of nerine leaf tissue from commercially cultivated nerines or from valuable collections in home-gardens. Thus, virus purification procedures had to be developed which optimized virus yield from relatively small quantities, in most cases less than 200g, of leaf tissue. Further, nerine leaf tissue was found to be highly mucilaginous and fibrous which could have resulted in substantial loss of virus if exploratory studies were not undertaken to minimize this loss.

5.2 PURIFICATION STUDIES

The preliminary purification studies were undertaken with leaves from a clonally mass propagated *N.sarniensis* hybrid cultivar found to be severely infected with ca 800nm filamentous virus. Leaves, showing severe yellow mosaic symptoms just prior to senescence were harvested so that the effects on bulblet and flower production would have been minimal. The leaves were stored either at -70C or at -20C for a period of up to six weeks while the various purification methods described in Section 2.2.5 were attempted using about 100g of leaf tissue on each occasion. Table 6 summarizes the main findings from these studies.

Initial attempts were made to purify viruses from about 100g of infected leaf tissue. The dicing of nerine leaves into 2-3cm pieces prior to homogenization was found to be an important step to prevent fibrous leaf material being tangled in the blades of the Waring blender. The use of Triton X-100 was found to reduce contamination by mucilaginous fibrous host material as determined by electron microscopy. Re-extraction of the

Table 6: Purification studies on potyvirus-like particles from field-infected *Nerine sarniensis* leaf tissue.

	Extraction Buffer	Solvent	Additives	Concentration Procedure	Electron microscopic assessment of purity, integrity, aggregation and yield
(a)	0.5M potassium phosphate, pH 7.6	0.5 vol chloroform	0.1% 2-ME 0.05M EDTA 0.01M DIECA 1% Triton X-100	2 cycles of differential centrifugation	Heavily contaminated with host material; Fragmentation of virus particles; Particles not aggregated; Relatively low virus yield.
(b)	0.5M potassium phosphate, pH 7.6	0.5 vol chloroform	0.1% 2-ME 0.05M EDTA 0.01M DIECA 1% Triton X-100	5% PEG/NaCl, 2 cycles of differential centrifugation, sucrose gradient	Relatively free of host contaminants; Fragmentation of virus particles; End-to-end aggregation; Relatively low virus yield.
(c)	0.5M potassium phosphate, pH 7.6	0.5 vol chloroform	0.1% 2-ME 0.05M EDTA 0.01M DIECA 1% Triton X-100	5% PEG/NaCl, isopycnic caesium chloride centrifugation	No detectable host contaminants; Relatively low fragmentation; End-to-end aggregation; Relatively high virus yield.
(d)	20mM HEPES, pH 7.5	0.5 vol chloroform/carbon tetrachloride (1:1) or 8% n-butanol	0.1% sodium sulphite, 1% Triton X-100	4% PEG/NaCl, isopycnic caesium chloride centrifugation	No detectable host contaminants; Relatively low fragmentation; End-to-end aggregation; Relatively low virus yield.
(e)	0.1M Tris-Citric acid, pH 9.0	0.5 vol chloroform	0.1% sodium thioglycolate	5% PEG/NaCl, 1 cycle of differential centrifugation, sucrose gradient	No detectable host contaminants; Some particles fragmented; End-to-end aggregation; Relatively low virus yield.
(f)	0.1M Tris-HCl	0.5 vol chloroform/carbon tetrachloride (1:1)	0.1% thio-glycolic acid	2 cycles of relatively low speed centrifugation	Heavily contaminated with host material; Relatively low fragmentation; Particles not aggregated; Very low virus yield.

2-ME = 2-mercaptoethanol
EDTA = ethylene-diamine-tetra-acetate
DIECA = sodium diethyl-dithiocarbamate
PEG = polyethylene glycol, molecular weight 6000

coagulated interphase after the first low speed centrifugation was found to increase virus yield. From electron microscopic observations it was estimated that up to 20% of the virus could be lost in the fibrous interphase if re-extraction was not attempted. Overnight chloroform clarification of the preparation on a slow shaker at 4C as opposed to 3h treatments was found to have no apparent deleterious effects on the integrity of the particles.

The most suitable virus purification procedure for maximizing virus yield of unfragmented particles involved the use of a high molarity (0.5M) potassium phosphate buffer (pH 7.6) with polyethylene glycol([PEG];MW6000) precipitation followed directly by isopycnic caesium chloride centrifugation (procedure [c], Table 6). This formed the basis of the purification methods subsequently used in this study. Procedures involving high speed centrifugation to pellet the virus (procedures [a], [b] and [e]; Table 6), usually resulted in fragmentation of many of the virus particles as shown in Figure 7. The use of sucrose gradients after PEG precipitation did not give the sharp separation obtained by using isopycnic caesium chloride centrifugation. One hundred grams of systemically infected leaf tissue was sufficient in most cases to form a distinct opalescent band in caesium chloride (Figure 8). Although the use of PEG was found to be a convenient and relatively rapid technique for the purification of unfragmented filamentous virus particles from nerine tissue, it was found to induce severe end-to-end aggregation in many of the preparations (Figure 9).

5.3 DISCUSSION

The failure to transmit any of the filamentous viruses to herbaceous indicator plants combined with the possiblilty of mixed infections, presented the greatest challenge in this study. The preliminary electron microscope survey provided a useful starting point to determine the morphological and size characteristics of some of the filamentous viruses in locally cultivated *Nerine* species. The finding of two *Nerine* cultivars with predominantly one class of filamentous virus particles proved useful for determining which of the filamentous viruses in nerines were selected for further study. Thus the 540nm potexvirus found in *N.fothergilli* 'Major' and the 800nm potyvirus found in a *N.sarniensis* hybrid were chosen for more intensive study. Since the *N.sarniensis* hybrid was clonally mass propagated for commercial cut flower production, sufficient quantities of leaf material were available during the end of the growing season to undertake purification studies from field-

infected leaf material.

Procedures outlined in Section 2.2.5 were followed in attempts to purify filamentous viruses from nerine leaf tissue. Most of the methods were not suitable for yielding sufficient quantities of unfragmented virus for characterization. Further, the mechanical transmission studies reported in Chapter 3 indicated that cucumber mosaic virus could be a low level contaminant in the virus preparations. The possibility of mixed virus infections gave two options for the development of specific virus indexing methods from field-infected tissue. These were monoclonal antibodies or nucleic acid probes from cloned viral cDNA. Although both these approaches would have given rapid, standardized and sensitive diagnostic probes which could be propagated indefinitely, the cloned viral cDNA method was selected in this study, due to the availability of the required laboratory equipment.

For the cloning of viral cDNA, the virus had to be purified in sufficient quantities, without fragmentation, so that adequate quantities of undegraded viral RNA could be obtained for cDNA synthesis. The purification studies attempted in this chapter were aimed at obtaining maximum quantities of unfragmented virus particles. Gentle procedures using polyethylene glycol precipitation and isopycnic caesium chloride centrifugation were found to yield optimum quantities of unfragmented virus particles suitable for RNA extraction. The phenomena of end-to-end aggregation observed in some virus preparations was of no practical significance. The procedures reported in this chapter to be effective in maximizing virus yield were used in the purification schedules adopted for the purification of NeVX (Chapter 6) and NeVY (Chapter 7).

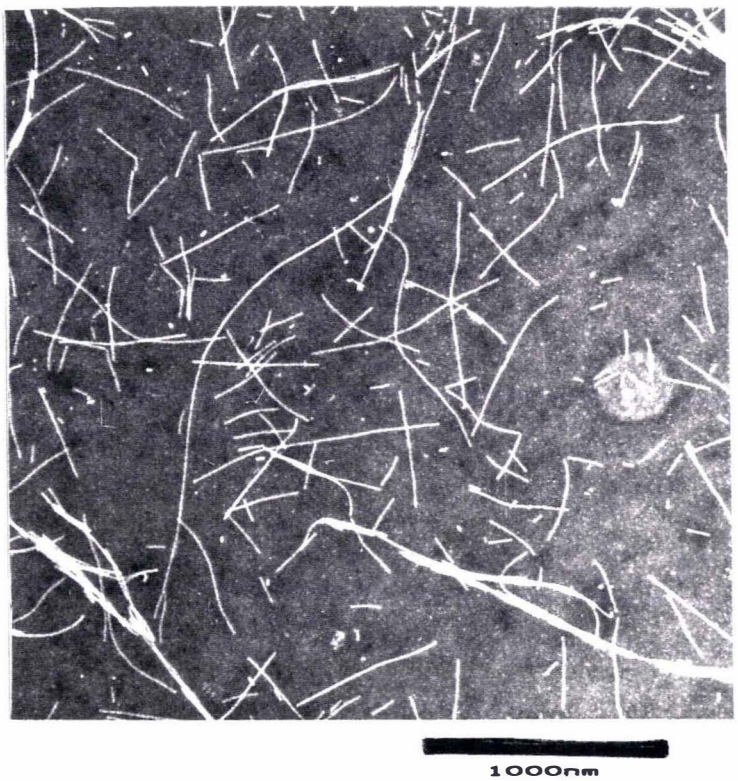


Figure 7: Fragmentation of filamentous virus particles after two cycles of differential centrifugation. Stain: 2% potassium phosphotungstic acid, pH 7.

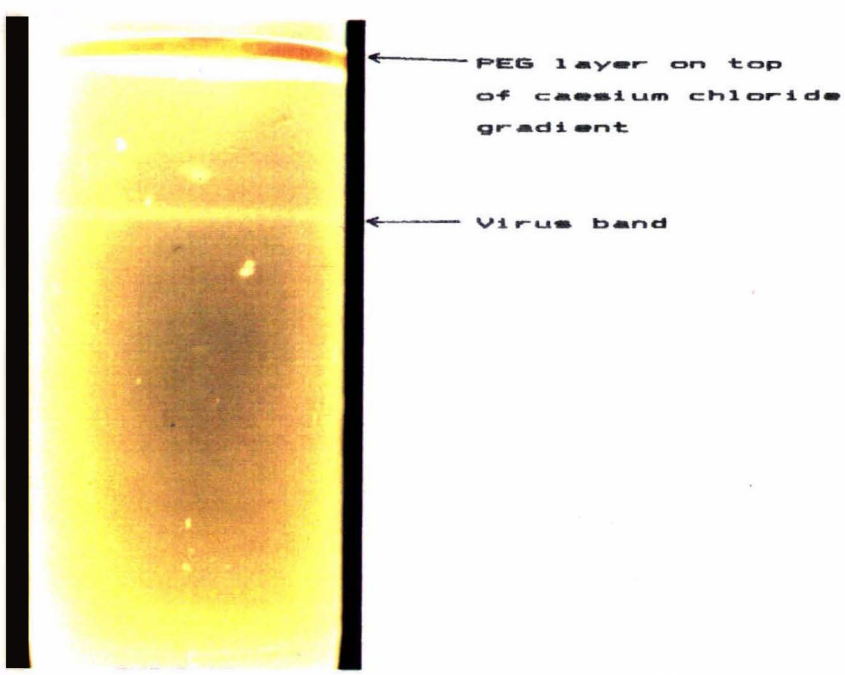


Figure 8: A distinct opalescent virus band after isopycnic caesium chloride centrifugation.

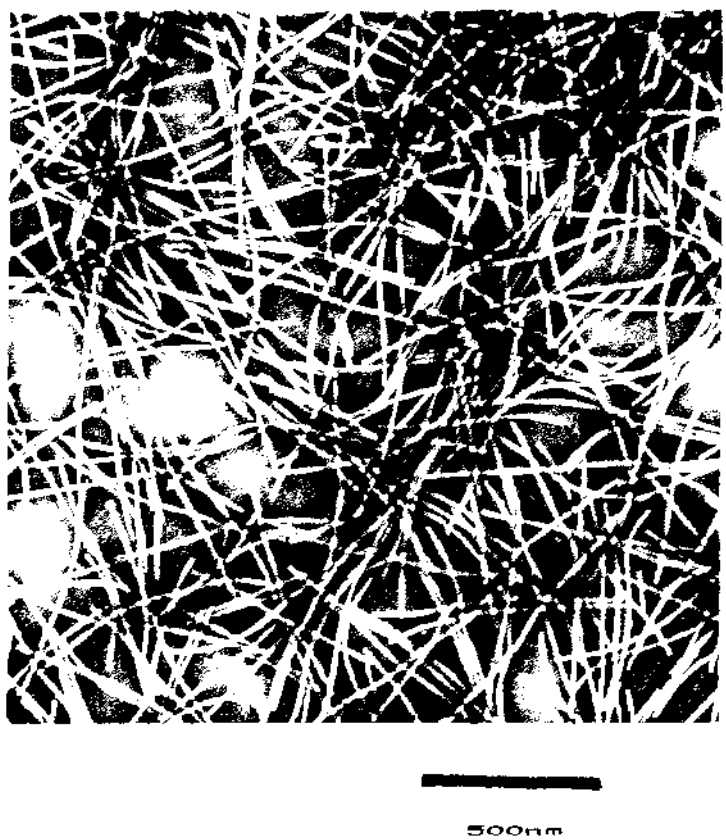


Figure 9: End-to-end aggregation of virus particles following treatment with polyethylene glycol (MW 6000). Stain: 2% potassium phosphotungstic acid, pH 7.

CHAPTER 6

PURIFICATION, CHARACTERIZATION AND cDNA CLONING OF NERINE VIRUS X

6.1 INTRODUCTION

Two potexviruses have been reported in nerines from the Netherlands and the United Kingdom (Maat,1976; Phillips & Brunt,1980). These are nerine virus X (NeVX), with an average particle length of 541nm and narcissus mosaic virus (NaMV) with a particle length of 554nm (Maat,1976). Nerine virus X has been reported to be relatively common in nerines whereas NaMV was found to be relatively uncommon (Table 1).

Due to the failure to mechanically transmit NeVX from nerines into herbaceous hosts (Maat,1976), nerine isolates of the virus remained poorly characterized. However, a strain of NeVX (on the basis of close serological relationships), isolated from *Agapanthus praecox* subsp. *orientalis* (NeVX-A) which was mechanically transmissible to herbaceous indicator plants, was partially characterized (Phillips and Brunt,1980).

In contrast, NaMV in nerines was found to be readily sap transmissible to a number of herbaceous hosts (reviewed in Section 1.5). Narcissus mosaic virus has been well characterized (Brunt,1966;Koenig *et al*,1973; Short &Davies,1983) and it's complete nucleotide sequence recently determined (Zuidema *et al*,1989).

A number of *N.fothergilli* 'Major' plants (Figure 10), from a home garden in Palmerston North, derived from a single mother bulb were found to be infected with one class of filamentous virus particles belonging to the potexvirus group. On the basis of particle morphology, sap transmission characteristics and serology, the New Zealand potexvirus was identified as an isolate of NeVX. This isolate of NeVX was purified from field-infected *N.fothergilli* 'Major' tissue, characterized and cDNA cloned in this study.

Figure 10: Cluster of *Nerine fothergilli* 'Major' bulbs infected with an isolate of nerine virus X.

6.2 PARTICLE MORPHOLOGY

Crude sap extracts of leaf and floral tissue from the infected *N.fothergilli* 'Major' plants were found to contain one distinct population of filamentous virus particles with a normal length of 540nm. When grouped into 20nm categories 80% of the 97 particles measured had a length distribution of 510 to 550nm and 64% within the 530 to 550nm range (Figure 11). The particle width was estimated to be ca 11nm.

The particles were slightly flexuous and no ultrastructural details, such as an axial channel or subunits, were observed when stained with 2% PTA (pH 7), 2% sodium silicotungstate, or 2% uranyl acetate.

Slightly flexuous particles 11-13nm in diameter and length ranging from 480-580nm have been defined as belonging to the potato virus X or potexvirus group (Brandes & Bercks,1965; Lesemann & Koenig,1977; Purcifull & Edwardson,1981).

6.3 MECHANICAL TRANSMISSION EXPERIMENTS

Further to the preliminary mechanical transmission studies using crude sap from leaf and floral parts of *N.fothergilli* 'Major' reported in Chapter 3 (Table 5), repeated efforts were made to transmit the potexvirus using purified preparations to the following indicators: *G.globosa*, *C.amaranticolor*, *C.quinoa*, *N.clevelandii*, *N.glutinosa*, *N.tabacum* 'White Burley', *N.benthamiana* Domin. and *Cassia occidentalis* L. Inoculations on a total of nine test plants on three separate occasions failed to induce any virus-like symptoms on any of the indicators. Electron microscope investigation of crude sap preparations of leaf tissue of all test plants failed to show the presence of any filamentous particles.

An RNA preparation from the potexvirus purified from the *N.fothergilli* 'Major' was also used as inoculum on two *N.clevelandii* and three *C.quinoa* plants. Again no virus-like symptoms were induced and no particles were observed with the electron microscope.

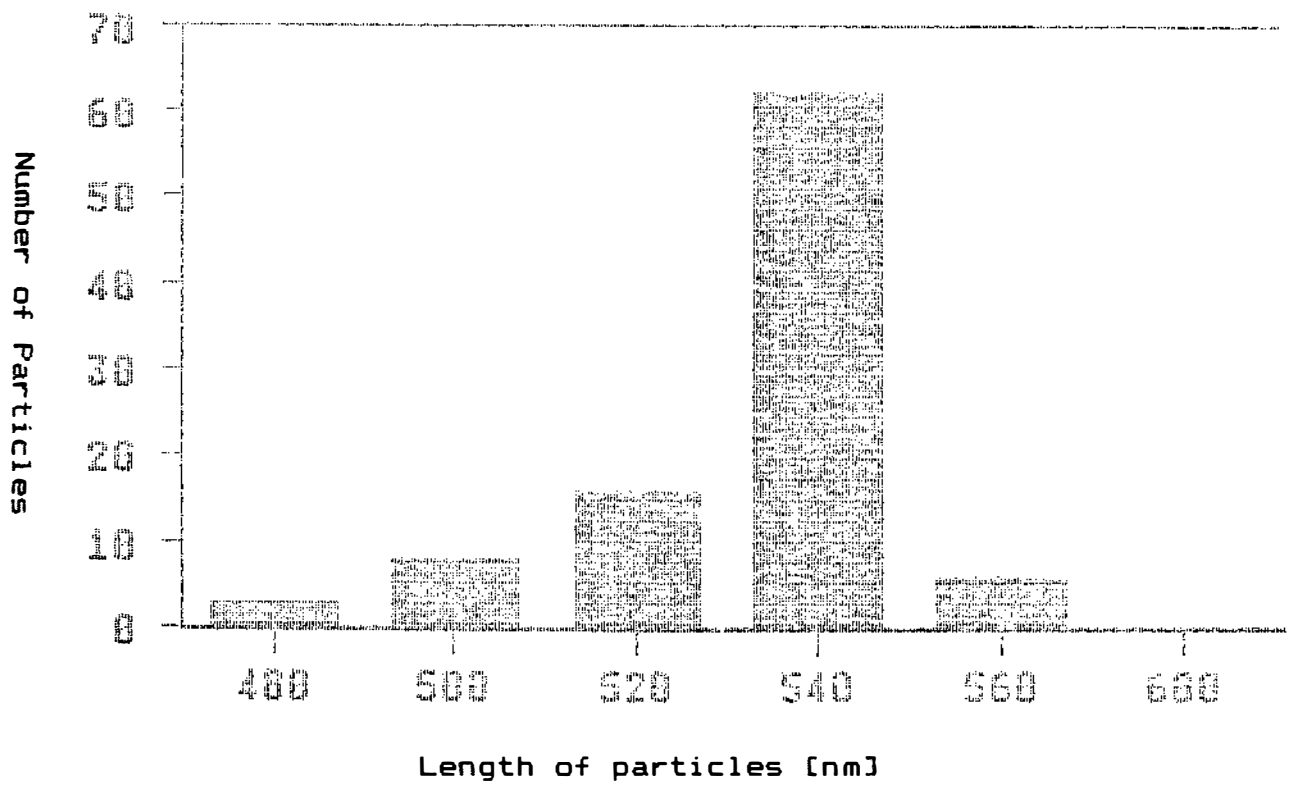


Figure 11: Length distribution of an isolate of nerine virus X found in *Nerine fothergilli* 'Major'.

6.4 SYMPTOMATOLOGY

The *N.fothergilli* 'Major' plants infected with the potexvirus showed no virus-like symptoms on the leaves or floral parts (Figure 12[A] & [B]).

6.5 CYTOPATHOLOGY BY LIGHT AND ELECTRON MICROSCOPY

Inclusion bodies, such as the banded viral aggregates associated with many other potexviruses (Edwardson & Christie,1978; Hiebert *et al*,1984), were not observed in field-infected *N.fothergilli* 'Major' epidermal tissue from young and older leaves after five independent attempts, using the light microscopy techniques developed by Christie (described in Section 2.2.4.4). A similar result was obtained by R G Christie (University of Florida, Gainesville, Florida) using the same tissue (pers comm).

Ultrathin sections of virus-infected nerine leaf tissue also failed to show any characteristic potexvirus inclusion bodies in the epidermal or mesophyll cells.

Figure 12[A]: Close-up of *Nerine fothergilli* 'Major' leaves systemically infected with an isolate of nerine virus X but showing no virus-like symptoms.

Figure 12[B]: Flowers of *Nerine fothergilli* 'Major' infected with an isolate of nerine virus X showing no virus-like symptoms.

6.6 PURIFICATION

The purification procedure outlined in Section 2.2.5.1 was used to purify NeVX. The use of the polyethylene glycol precipitation procedure followed by isopycnic centrifugation using caesium chloride described in Section 2.2.5.1 gave a distinct opalescent virus band which could be readily collected manually with a hypodermic syringe and reconcentrated by centrifugation at 85,000g for 90min. This was a simple and rapid procedure which gave a relatively high yield of unfragmented virus particles with no detectable levels of host components in electron microscope observations (Figure 13). End-to-end aggregation however, was evident in some of the preparations.

In routine preparations about 0.16-0.29mg (3 determinations) of relatively unfragmented virus (Figure 13) was recovered from about 100g of freshly harvested field infected nerine leaf tissue during late winter and early spring (assuming an extinction coefficient at 260nm ($A^{0.1\%}_{1cm}$) of 3 by analogy with other potexviruses [Mowat,1982]).

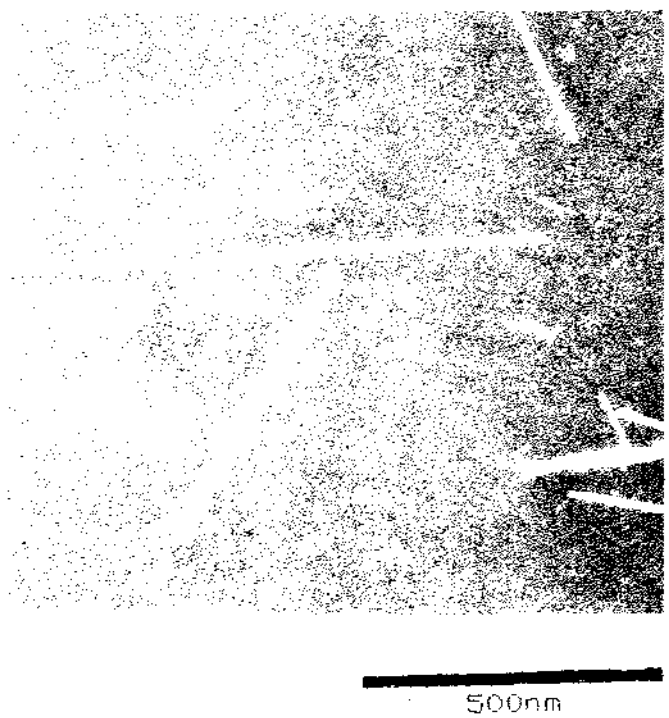


Figure 13: Particles of nerine virus X isolate purified from *Nerine fothergilli* 'Major'; stained with 2% PTA, pH 7.

6.7 MOLECULAR WEIGHT OF COAT PROTEIN

Sodium dodecylsulphate (SDS) dissociated NeVX coat protein from purified virus particles was electrophoresed in SDS-Page slab gels using a SDS-70L molecular weight kit (Sigma) as standards (Figure 14). The mobility of the markers gave the molecular weight calibration data shown in Table 7.

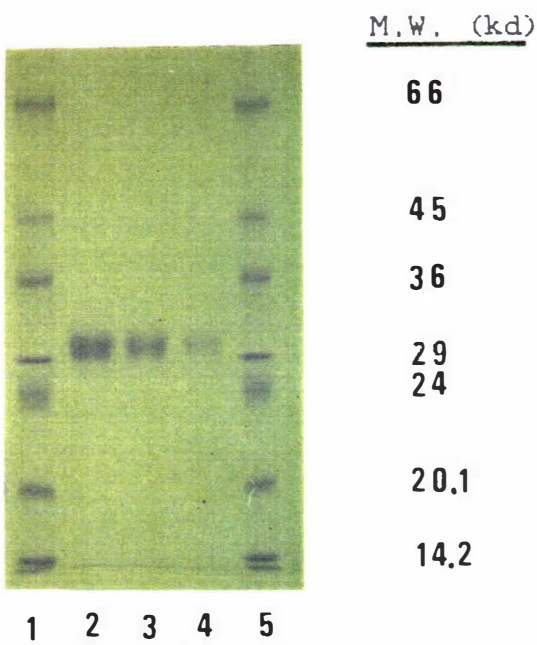


Figure 14: Separation of SDS-dissociated marker proteins (lanes 1&5) and viral coat protein of nerine virus X isolate (lanes 2-4) electrophoresed on a 12.5% polyacrylamide gel. The gel was stained first with Coomassie Blue R and double stained with Coomassie Blue G-250 and photographed with Kodak 35mm film.

Table 7: Calibration data used for molecular weight (MW) determination of nerine virus X coat protein.

Component	MW(kd)	Log_{10} MW	Distance migrated(mm)
(a) Albumin (bovine serum)	66	4.819	20
(b) Ovalbumin	45	4.653	41.5
(c) Glyceraldehyde -3-phosphate dehydrogenase (rabbit muscle)	36	4.556	53
(d) Carbonic anhydrase (bovine erythrocytes)	29	4.462	67.5
(e) Trypsinogen (bovine pancreas)	24	4.380	75
(f) Trypsin inhibitor (soybean)	20.1	4.303	92.5
(g) α - Lactalbumin (bovine milk)	14.2	4.152	107

A calibration curve of distance migrated versus molecular weight (log scale) was plotted (Figure 15) and the molecular weight of the viral coat protein determined by inverse estimation from the regression of the electrophoretic mobility of the markers.

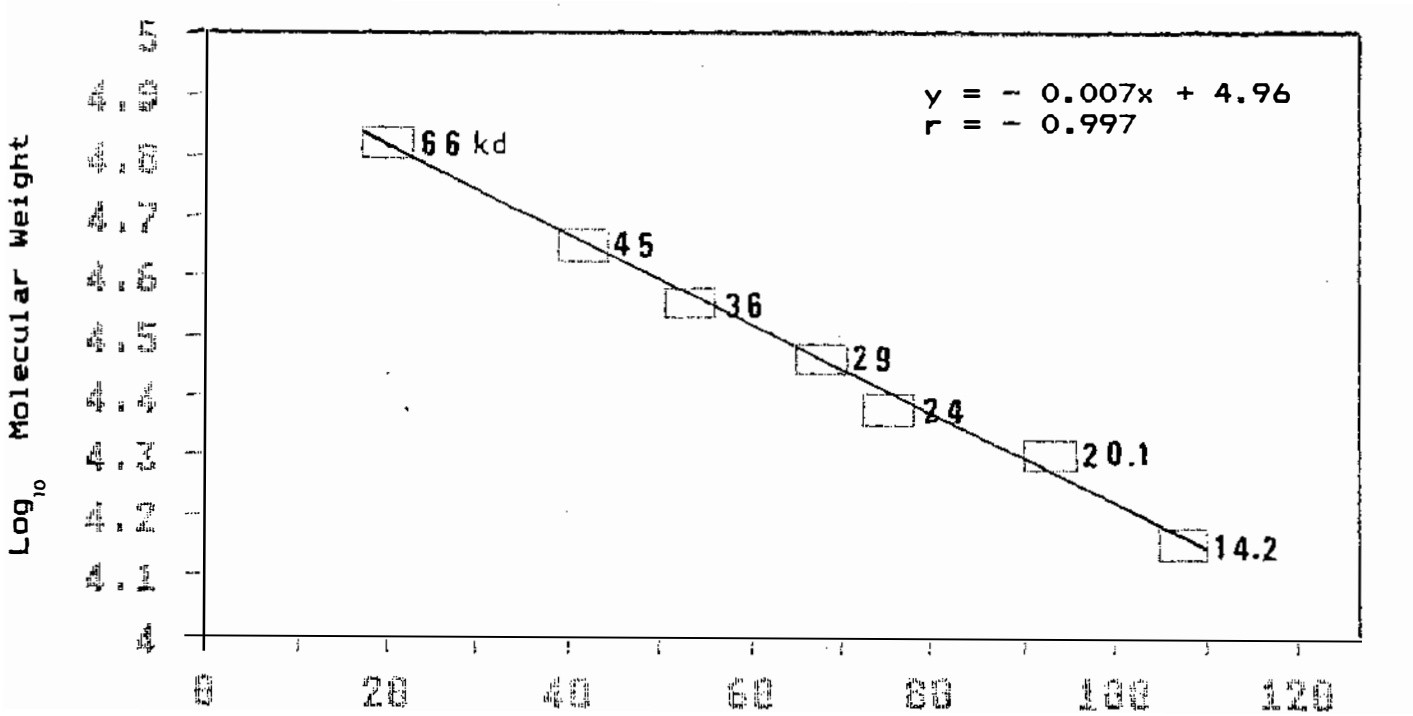


Figure 15: Calibration curve for molecular weight determination of nerine virus X coat protein. The distance migrated by the marker proteins (SDS-70L, Sigma) was plotted against the molecular weight (log scale).

A single viral coat protein band (Figure 14) with an estimated molecular weight of 29.5kd was obtained.

6.8 ANALYSIS OF NERINE VIRUS X NUCLEIC ACID

A single RNA species was observed by agarose gel electrophoresis under non-denaturing conditions (Figure 16) of RNA extracted from purified virus particles. The molecular weight of this RNA was determined by the formamide/formaldehyde method described in Section 2.2.8.2. An RNA ladder (Bethesda Research Laboratories) was used to obtain the molecular weight data presented in Table 8. The calibration curve of distance migrated plotted against molecular weight (log scale) is shown in Figure 17.

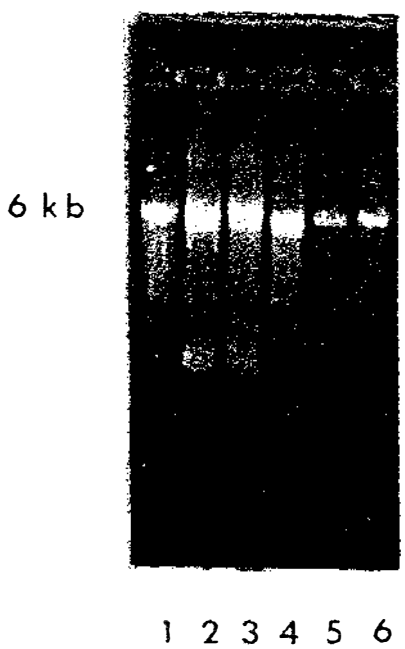


Figure 16: Agarose gel electrophoresis of viral RNAs under non-denaturing conditions. The gel was stained with ethidium bromide and photographed using short wave ultraviolet (254nm) transillumination.
Lane 1: white clover mosaic virus RNA
Lane 2 & 3: narcissus mosaic virus RNAs
Lane 4,5 & 6: nerine virus X.

Table 8: Calibration data for molecular weight determination of nerine virus X RNA. A RNA ladder (Bethesda Research Laboratories) was co-migrated with viral RNA on a 1% agarose gel containing formaldehyde.

Molecular Weight (bases)	Log_{10} MW	Distance migrated (mm)
9490	3.977	12.50
7460	3.873	15.20
viral RNA		*17.6*
4400	3.643	23
2370	3.375	35
1350	3.130	41
330	2.519	70.5

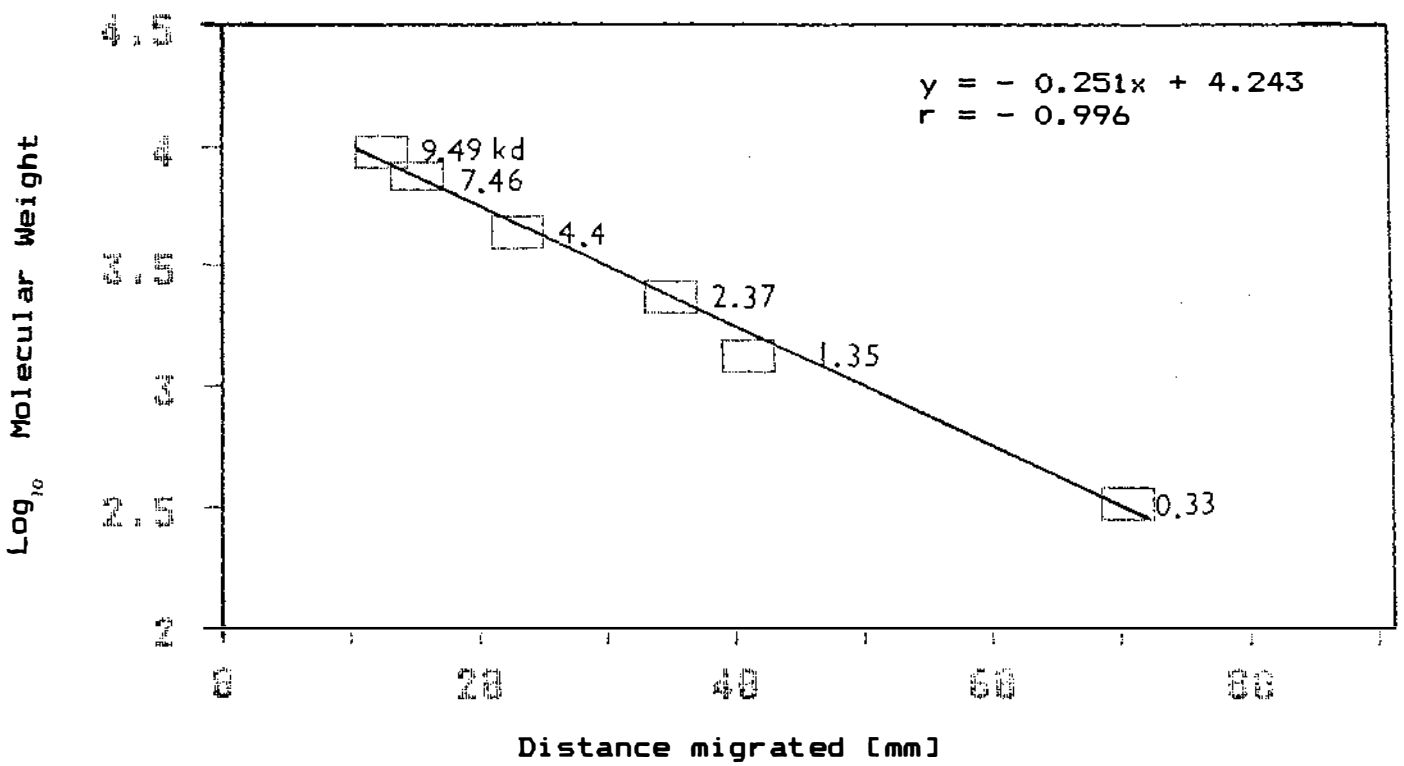


Figure 17: Calibration curve obtained by plotting distance migrated by RNA species in Bethesda Research Laboratories RNA ladder versus molecular weight (log scale).

A single genomic RNA species with an estimated molecular weight of 6.3kb was obtained for the NeVX isolate.

6.9 *IN VITRO* TRANSLATION PRODUCTS

Translation of NeVX RNA in rabbit reticulocyte lysates *in vitro* (as described in Section 2.2.11) led to the synthesis of a large nonstructural protein of molecular weight 180kd (Lane 1, Figure 18) similar to those observed for a number of other potexviruses (Bendena *et al*,1985; Bendena & Mackie,1986; Mackie & Bancroft, 1986; Guilford & Forster,1986; Forster *et al*,1987).

A range of minor products was also observed (Lane 1,Figure 18). The NeVX RNA translation profile resembles the profiles of the potexviruses, daphne virus X (Lane 2,Figure 18) and potato virus X (Lane 3,Figure 18) but differs from narcissus mosaic virus (Lane 4,Figure 18) and white clover mosaic virus [isolate M] (Lane 5,Figure 18) which can direct the synthesis of their respective coat proteins *in vitro* (Short & Davies,1983; Forster *et al*,1987). Narcissus mosaic virus and white clover mosaic virus RNAs direct the synthesis of a 26kd and a 25kd coat protein respectively (Lanes 4 & 5,Figure 18).

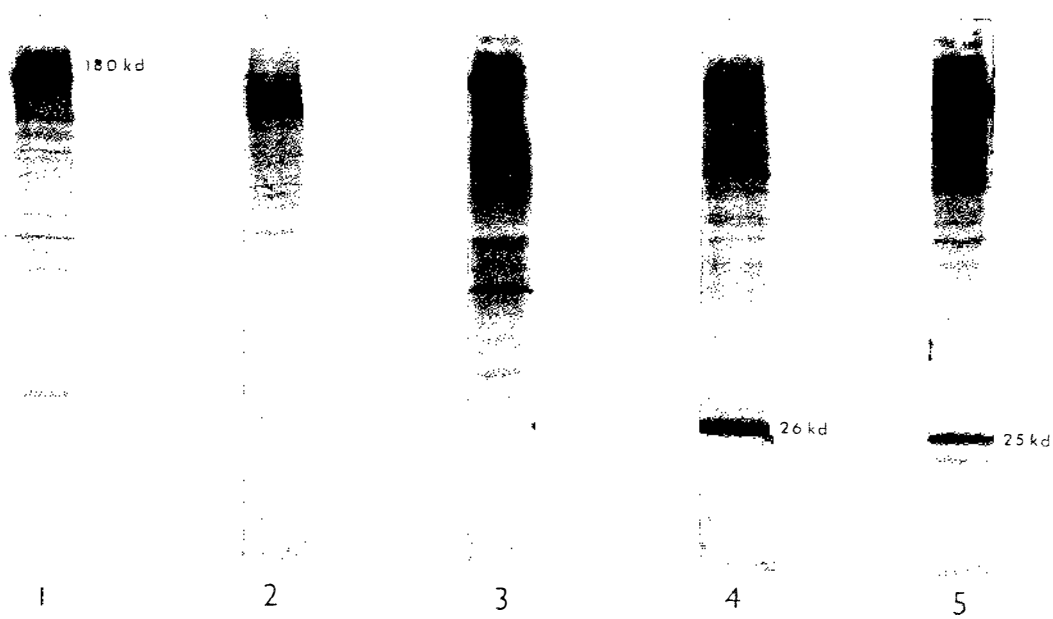


Figure 18: Autoradiogram of translation products made in rabbit reticulocyte lysate of RNAs from purified virus particles of nerine virus X (lane 1), daphne virus X (lane 2), potato virus X (lane 3), narcissus mosaic virus (lane 4) and white clover mosaic virus [isolate M] (lane 5).

6.10 SEROLOGICAL RELATIONSHIP TO THE *AGAPANTHUS* STRAIN OF NERINE VIRUS X

Immunodot-blots were carried out using the methods described in Section 2.2.12.2. Antiserum to the agapathus strain of NeVX (gift from A A Brunt) was found to react with the isolate of NeVX purified from *N.fothergilli* 'Major' in this study (Figure 19). This confirms the serological relationship using tube precipitin tests between the ca 540nm potexvirus in agapanthus and nerines observed by Phillips & Brunt (1980).

Figure 19: Immunodot-blot assays using antiserum prepared to the agapanthus strain of nerine virus X (NeVX-A) against: NeVX-A (A1 & A2); healthy nerine sap (B1 & B2); and NeVX from *Nerine fothergilli* 'Major' (C1 & C2).

6.11 cDNA SYNTHESIS AND MOLECULAR CLONING OF VIRAL RNA

Details of the cDNA cloning procedures used are given in Section 2.2.10. The cDNA was made double-stranded with DNA polymerase I, dC-tailed using terminal deoxynuclotidyl transferase, and cloned into *Pst* I cut, dG-tailed plasmid pBR322 (4.363kb;Figure 20).

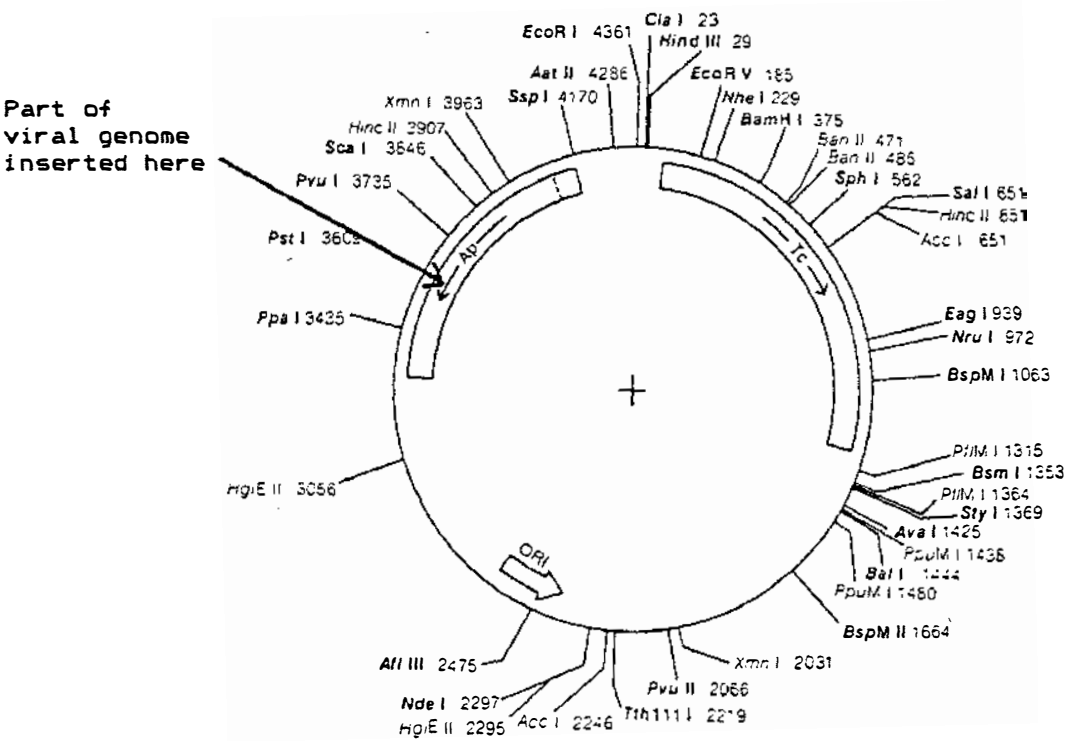


Figure 20: Restriction endonuclease map of pBR322 DNA, showing *Pst* I cloning site.

Recombinant clones were identified on the basis of ampicillin sensitivity and tetracyline resistance. The cDNA clones were analyzed by *Pst* I digestion and agarose gel electrophoresis using *Hind* III cut lambda DNA as markers. One clone, containing a 1.8kb (+/- 0.04 kb; 3 determinations) insert, was selected for further characterization (Figure 21). The size of the remaining linearized pBR322 DNA was estimated to be 4.36kb indicating that no viral cDNA remained attached to the plasmid.

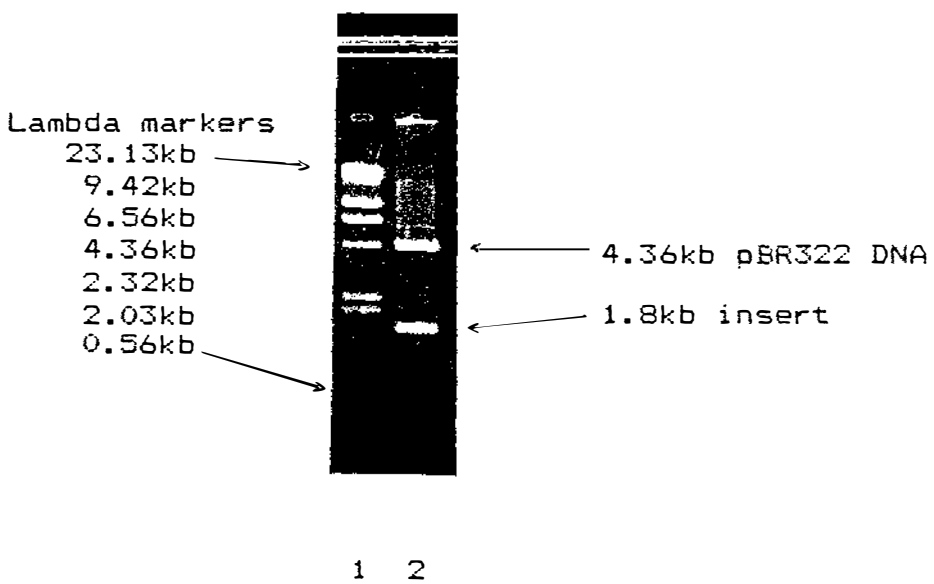


Figure 21: Agarose gel electrophoresis for size determination of cDNA cloned inserts. Lane 1: Size markers using *Hind* III digested lambda DNA. Lane 2: *Pst* I digest of recombinant plasmid DNA. Electrophoresis was on 1% agarose gel run for 2.5h at 60V and stained with ethidium bromide.

Based on an estimated molecular weight of 6.3kb for the genomic RNA, the size of the cDNA insert cloned into pBR322 represented about 28.5% of the viral genome.

6.12 RESTRICTION ANALYSIS OF THE cDNA CLONED INSERT

Three restriction endonucleases; *Eco* RI, *Bam* HI and *Hind* III, all with single recognition sites in pBR322, were used to determine whether there were any internal cutting sites in the viral cDNA insert. Only *Hind* III yielded two fragments approximately 4.26kb and 1.90kb indicating an internal cleavage site within the viral cDNA insert (Figure 22 & 23).

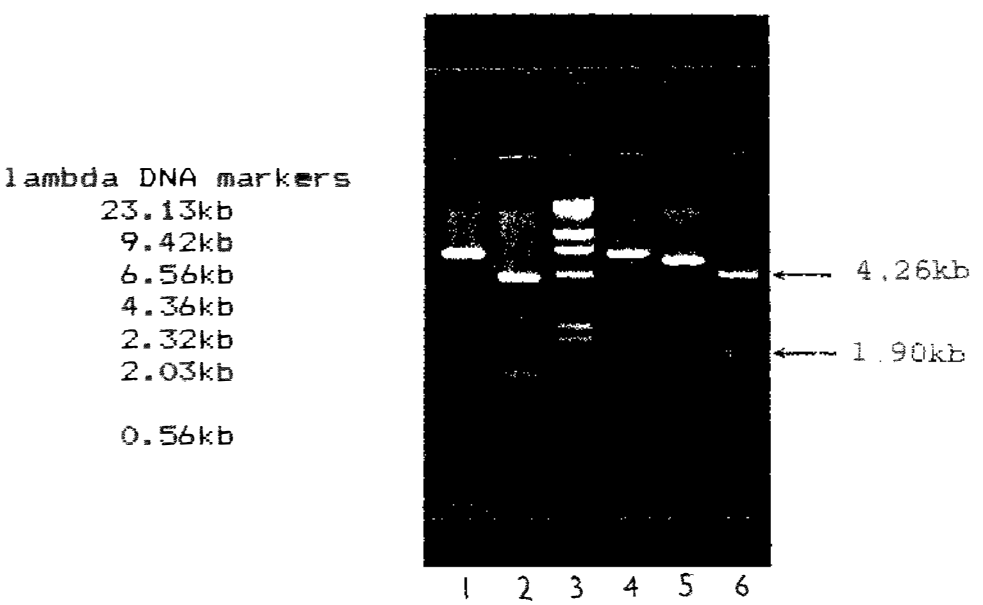


Figure 22: Restriction endonuclease analysis of cDNA clone of nerine virus X RNA. Electrophoresis of digests of recombinant pBR322 DNA using *Eco* RI, *Bam* HI and *Hind* III. Lane 1 & 4: uncut recombinant pBR322 DNA. Lane 2: *Pst* I digest. Lane 3: *Hind* III digest of lambda DNA (marker). Lane 5: 5.26kb DNA (marker). Lane 6: *Hind* III digest. Electrophoresis was for 2h at 60V on 1% agarose gel and stained with ethidium bromide.

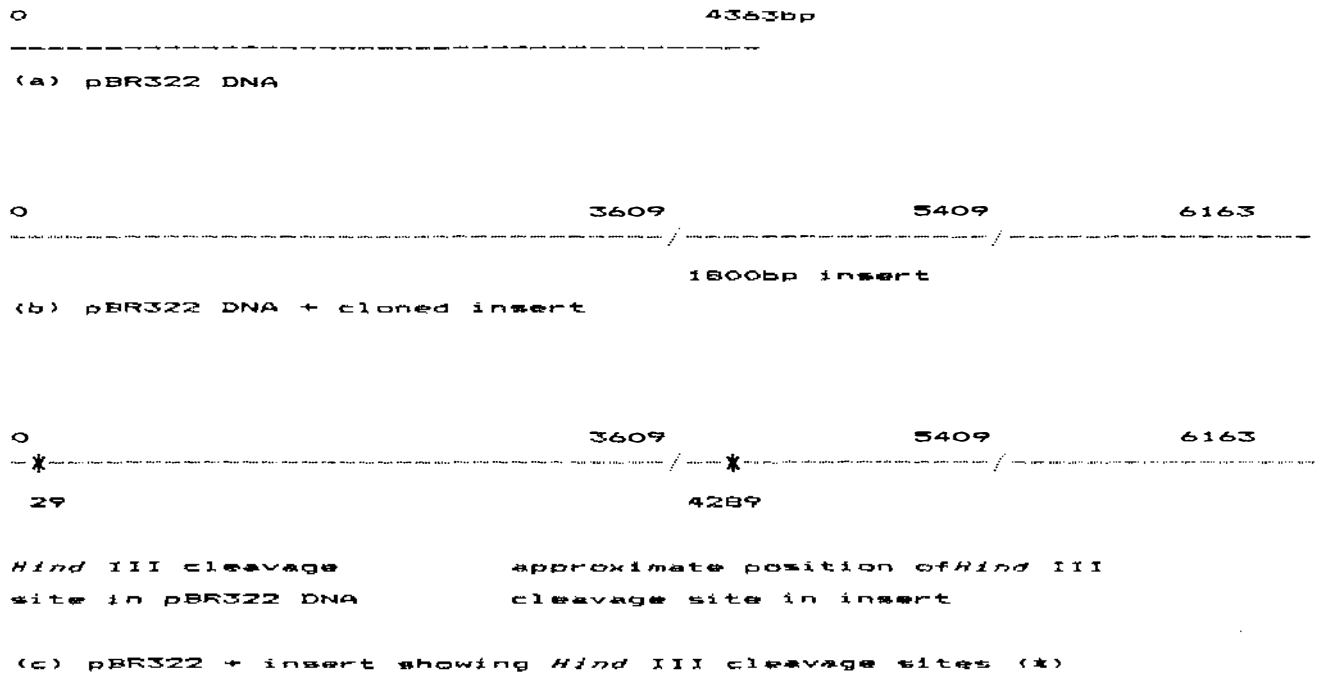


Figure 23: Approximate position of *Hind* III restriction endonuclease site in nerine virus X cDNA clone.

6.13: DETERMINATION OF HYBRIDIZATION SPECIFICITY OF cDNA CLONED PROBE

In order to establish the identity of the clone as a copy of nerine virus X RNA the cloned cDNA was labelled with [^{32}P]-dCTP by nick-translation and hybridized to RNA which had been fractionated on a denaturing gel and blotted onto Zeta-Probe membrane. The membrane was washed after hybridization and autoradiographed to show that the cloned cDNA hybridized strongly to the 6.3kb viral RNA (Figure 24).

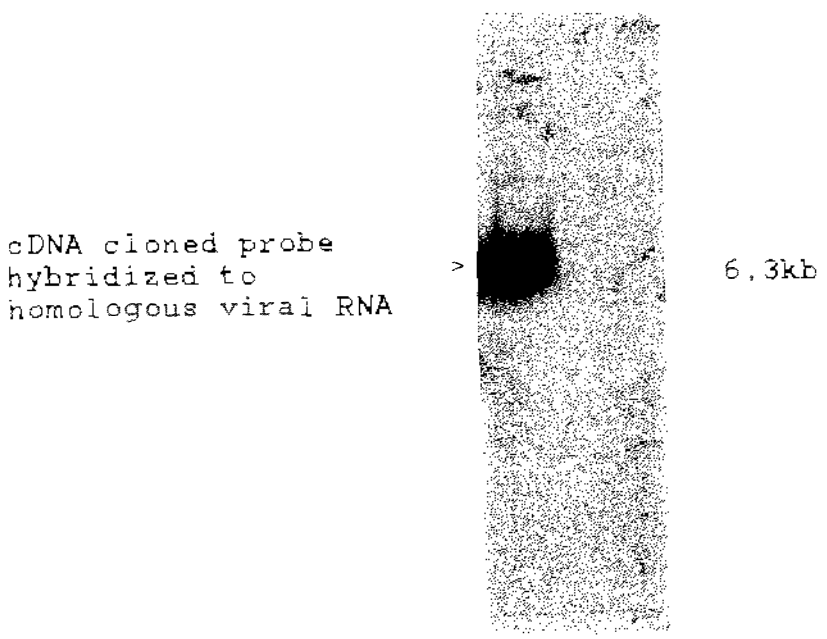
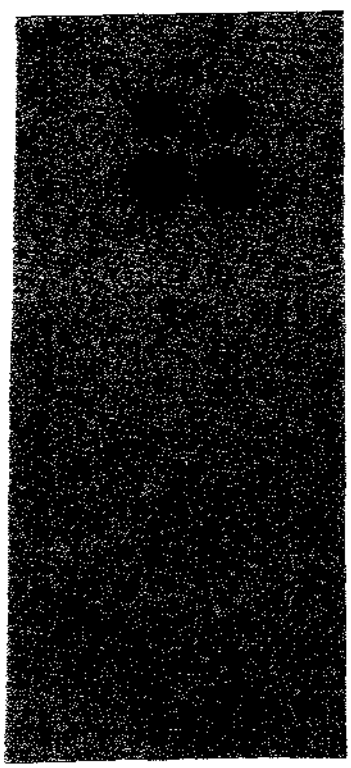


Figure 24: Hybridization of cloned cDNA [^{32}P]-labelled nick-translated probe with homologous genomic viral RNA. The RNA was electrophoresed on a 1% agarose formaldehyde denaturing gel, blotted on to Zeta-Probe membrane (Northern blot) and hybridized to the probe.

The specificity of the cloned cDNA probe was determined by testing the probe against seven other potexviruses: narcissus mosaic virus, white clover mosaic virus, potato virus X, tulip virus X, cymbidium mosaic virus, daphne virus X and the agapanthus strain of NeVX. Clarified viral concentrates of each of these potexviruses were extracted and loaded onto Zeta-Probe membranes by the method described in Section 2.2.9.3.

Repeated hybridization analysis using dot-blot assays (4 experiments) under high stringency conditions failed to show any evidence of hybridization to any of the other potexviruses except to the homologous nerine potexvirus (Figure 25). The cloned cDNA probe was thus found to be highly specific to the homologous potexvirus from nerines indicating that there was limited sequence homology between the cloned viral RNA and the those of the other potexviruses tested.



Purified nerine virus X

CVC of nerine virus X

Agapanthus strain of nerine virus X

Narcissus mosaic virus

Tulip virus X

Cymbidium mosaic virus

White clover mosaic virus

Daphne virus X

Potato virus X

Figure 25: Autoradiograph of dot-blot hybridization of a cloned cDNA [^{32}P]-labelled probe with clarified viral concentrates (CVC) of seven other potexviruses. Kodak XAR-5 film was exposed to the membrane in a intensifying screen at -70C for 12h.

6.14 DETERMINATION OF SENSITIVITY OF THE cDNA CLONED PROBE FOR DETECTION OF NERINE VIRUS X IN SAP

The sensitivity and limits of detection of the cloned cDNA probe was estimated using purified virus preparations and virus extracted from measured quantities of field-infected nerine leaf tissue.

The purified homologous virus was loaded on to Zeta-Probe membrane at various dilutions and the limits of detection of the cDNA probe at approximately 1 X 10^7cpm was determined in three separate experiments. The results (Figure 26) show that the probe was able to detect less than 0.08ug of the homologous purified virus. Since only about 5-6% of the virus is RNA (by analogy with other potexviruses; Purcifull & Edwardson,1981) the limit of detection of the viral RNA would be approximately 2-4ng.

Hybridization analysis of the cloned cDNA probe to sap extracted from microsamples of systemically infected nerine tissue containing the homologous virus (using methods described in Section 2.2.5.3) showed that the probe (specific activity 1X10^7cpm) was able to detect virus from an equivalent of less than 0.3mg of tissue (Figure 27) after 24h exposure of the membrane to Kodak XAR-5 film in an intensifying screen at -70C. Exposure of the autoradiographic film for 48h increased the sensitivity to the equivalent of 12ug of leaf tissue. No background problems or non-specific hybridization was observed using Zeta-Probe nylon blotting membranes and 'Anchor' skim milk as a blocking agent for non-specific binding sites.

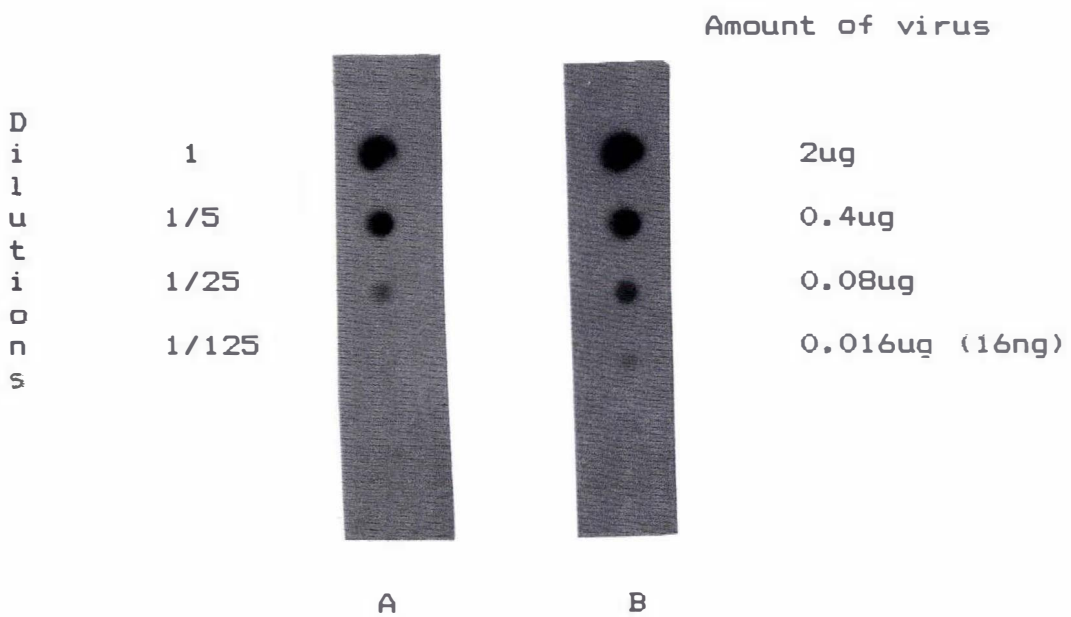


Figure 26: Autoradiograph of dot-blot hybridization of purified nerine virus X at various amounts. Dilution 1 equates to 2ug of purified virus. The virus preparation was loaded on to Zeta-Probe membranes and hybridized with nick-translated [^{32}P]-labelled cloned nerine virus X cDNA probe. X-ray film was exposed to the membrane for 12h(A) and 24h(B) at -70C with intensifying screens.

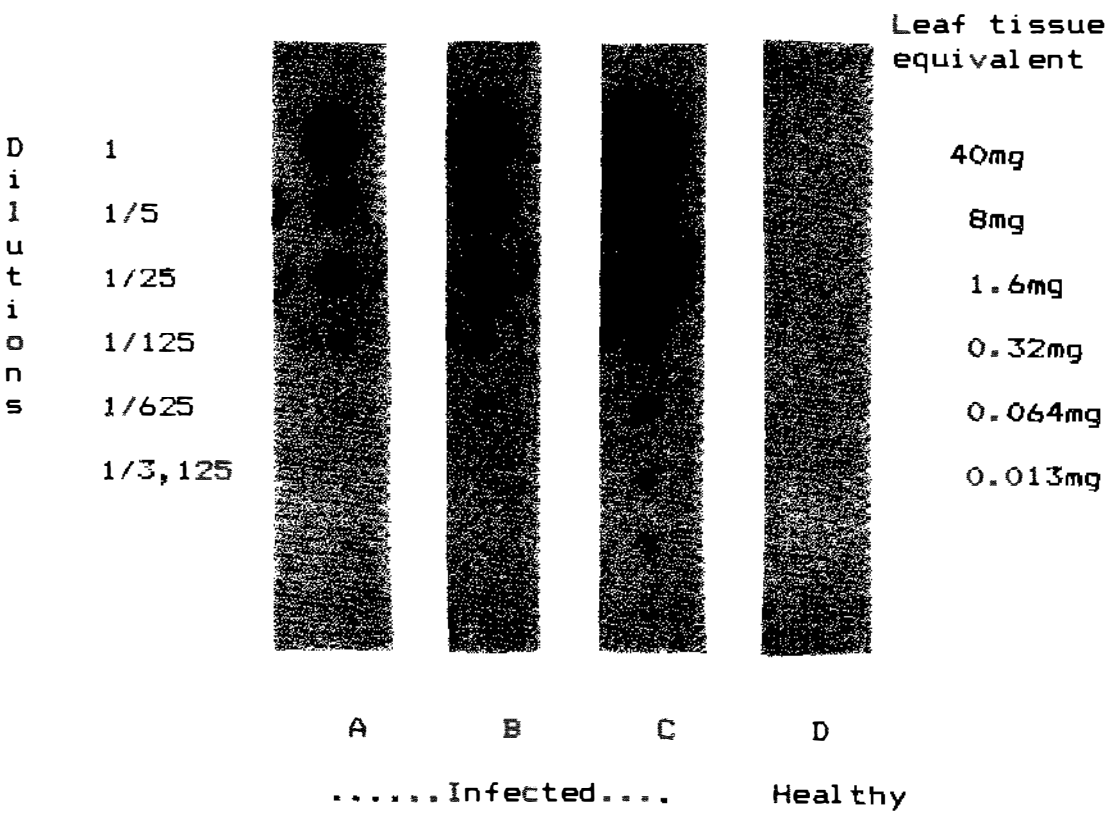


Figure 27: Autoradiograph of dot-blot hybridization of sap extracted from field-infected *Nerine fothergilli* 'Major' leaf tissue. Dilution 1 equates to clarified viral concentrates from an equivalent of 0.04g of infected (A,B & C) and healthy tissue (D). The samples were loaded on to Zeta-Probe membranes and hybridized with nick-translated [^{32}P]-labelled cloned nerine virus X cDNA probe at a specific activity of 1 X 10^{7}cpm. Kodak XAR-5 film was exposed to the membrane for 6h(A), 12h(B) and 48h(C) at -70C with intensifying screens.

6.15 DISCUSSION

On the basis of particle size and morphology, mechanical transmission characteristics, serological relationship and hybridization analysis, the potexvirus characterized and cDNA cloned in this study is believed to be an isolate of NeVX.

Unlike most potexviruses (eg narcissus mosaic virus, cymbidium mosaic virus, potato virus X; see review by Purcifull & Edwardson,1981), NeVX from nerines proved to be intransigent in mechanical transmission trials, both in the present study (see Section 6.3 and Table 5, Chapter 3) and in studies overseas (Maat,1976; Phillips & Brunt,1980).

Preliminary purification efforts to optimize virus yield from limited quantities of nerine leaf tissue, combined with the finding of NeVX in an 'old' *N.fothergilli* 'Major' clone which had multiplied naturally by offset formation, resulted in sufficient quantities of virus being purified for characterization and cDNA cloning.

Relatively unfragmented NeVX particles were purified from systemically infected nerine leaf tissue showing no 'virus-like' symptoms by polyethylene glycol precipitation of the chloroform clarified sap, followed by isopycnic caesium chloride centrifugation. Nerine virus X was found to be typically slightly flexuous rods with a 'normal length' of ca 540nm, a single coat protein subunit with a molecular weight of 29.5kd and a single RNA species of 6.3kb.

No potexvirus-like inclusion bodies were detected either by electron microscopy or light microscopy from systemically infected nerine tissue. This was somewhat surprising because all other potexviruses studied by light microscopy have been found to have conspicuous and characteristic inclusion bodies (Christie & Edwardson,1977). However, the failure to sap transmit NeVX to herbaceous indicators prevented attempts to detect inclusions during the early phases of the infection cycle when inclusion body levels are known to be highest (Christie & Edwardson,1977). Further, some potexvirus inclusions are known to be susceptible to destruction by some of the fixing and staining procedures used including, alcohol, methoxyethanol solutions, water, buffer and aqueous stains (Christie & Edwardson,1977). Thus, it is possible that NeVX induced inclusions were present in the nerine tissue examined but were particularly sensitive to one or more of the fixing or staining treatments used.

The profile of *in vitro* translation products of NeVX RNA in rabbit reticulocyte lysate were similar to those of daphne virus X and potato virus X. A large nonstructural protein of 180kd was synthesized with a range of minor products but without a predominant coat protein band like those synthesized from NaMV and one isolate (M) of WClMV.

The 1.8kb cDNA cloned probe (representing 28.5% of the viral genome) was found to be a highly specific and sensitive diagnostic method for detecting the homologous virus in dot-blot assays using Zeta-Probe membranes. At a specific activity of about 1 X 10^7cpm, the [^{32}P]-labelled nick-translated pBR322 recombinant DNA probe was able to detect NEVX from less than 0.08ug of purified virus and from an equivalent of less than 0.3mg of systemically infected nerine leaf tissue making it a very suitable diagnostic tool for rapidly screening large numbers of tissue samples.

In tube precipitin serological tests, using polyclonal antiserum prepared to the agapanthus strain of nerine virus X, Phillips and Brunt (1980) found that NeVX-A and NeVX were serologically indistinguishable and thus assumed that these were the same virus despite widely differing host range characteristics and ease of isolation from plants.

Using Brunt's antiserum to NeVX-A, the serological relationship between NeVX-A and NeVX (New Zealand isolate) was confirmed in immunodot-blot assays. However, the failure of the 1.8kb cloned NeVX cDNA probe to hybridize to NeVX-A indicates a lack of nucleotide sequence homology suggesting there are genomic differences between NeVX and NeVX-A in spite of some serological relationships.

Although serological relationships between some members of the potexvirus group has been reported (Koenig & Lesemann, 1978), and attempts have been made to classify potexviruses, among other plant viruses, on the basis of amino acid compositions of their capsid protein (Fauquet *et al*,1986), Bendena and Mackie (1987) using random primed cDNA probes found that there was a lack of homology among the RNAs of five members of the potexvirus group. This is not surprising given that serological relatedness is based on the capsid protein, whose coding sequences would constitute only about 10% of the genomes of typical potexviruses (Bendena & Mackie,1987).

The failure of the cDNA cloned NeVX probe to hybridize to NeVX-A combined with the different host range characteristics of these two viruses suggests that these are two distinct viruses sharing some morphological and coat protein (epitopes on the surface of the virus particles) similarities. It was beyond the scope of this project to research this further. Data on nucleotide and RNA sequences would be required to establish the relationship of NeVX and NeVX-A.

The primary objective of this section was to characterize the virus and prepare a highly sensitive, specific and relatively rapid diagnostic method. This was achieved by using recombinant DNA technology. The development of a cloned cDNA probe circumvents the need for repeated purification of an intransigent virus like NeVX. Further, the probe is well suited for conducting field surveys to study the prevalence of the virus, screening for viral resistance and in virus elimination studies.

CHAPTER 7

PURIFICATION, CHARACTERIZATION AND cDNA CLONING OF NERINE VIRUS Y.

7.1 INTRODUCTION

Two distinct potyviruses with particle lengths of ca 750nm and 800nm have been found in nerines in the Netherlands and in the United Kingdom (reviewed in Section 1.5). The ca 750nm virus particle which was associated with yellow stripe symptoms in *N.bowdenii* cultivars was originally named narcissus yellow stripe virus (NaYSV) on the basis of particle length and symptomatology (Brunt *et al*,1970). Narcissus yellow stripe virus was known to be a 750nm particle associated with yellow stripe symptoms in *Narcissus* (Brunt *et al*,1970). The 750nm virus was subsequently found to be serologically distinct from NaYSV (Hakkaart,1975) and renamed nerine yellow stripe virus (NeYSV). The ca 800nm unnamed virus particle found in *N.sarniensis* hybrids was also found to cause yellow stripe symptoms (Maat,1983 pers comm to K S Milne). There are no reported instances of either of these potyviruses being sap transmissible to herbaceous experimental species and both have remained uncharacterized.

Results of the electron microscope survey reported in Chapter 3 indicated that filamentous virus particles in the 680-840nm range (potyviruses; Matthews,1979) were present in some *Nerine* species. Further, one of the *N.sarniensis* hybrid cultivars from Nerine Nurseries Ltd in Palmerston North had flexuous filamentous virus particles with a normal length of 800nm. This virus was purified from systemically infected nerine tissue, characterized and cDNA cloned in this study. Nerine virus Y (NeVY) is the name proposed for this virus.

7.2 PARTICLE MORPHOLOGY

Crude sap extracts from leaf tissue of a field-infected *N.sarniensis* hybrid which had been clonally mass propagated by twin scaling for commercial cut-flower production was found to contain filamentous virus particles with a normal length of 800nm (Figure 28). The particle width was estimated to be 11nm.

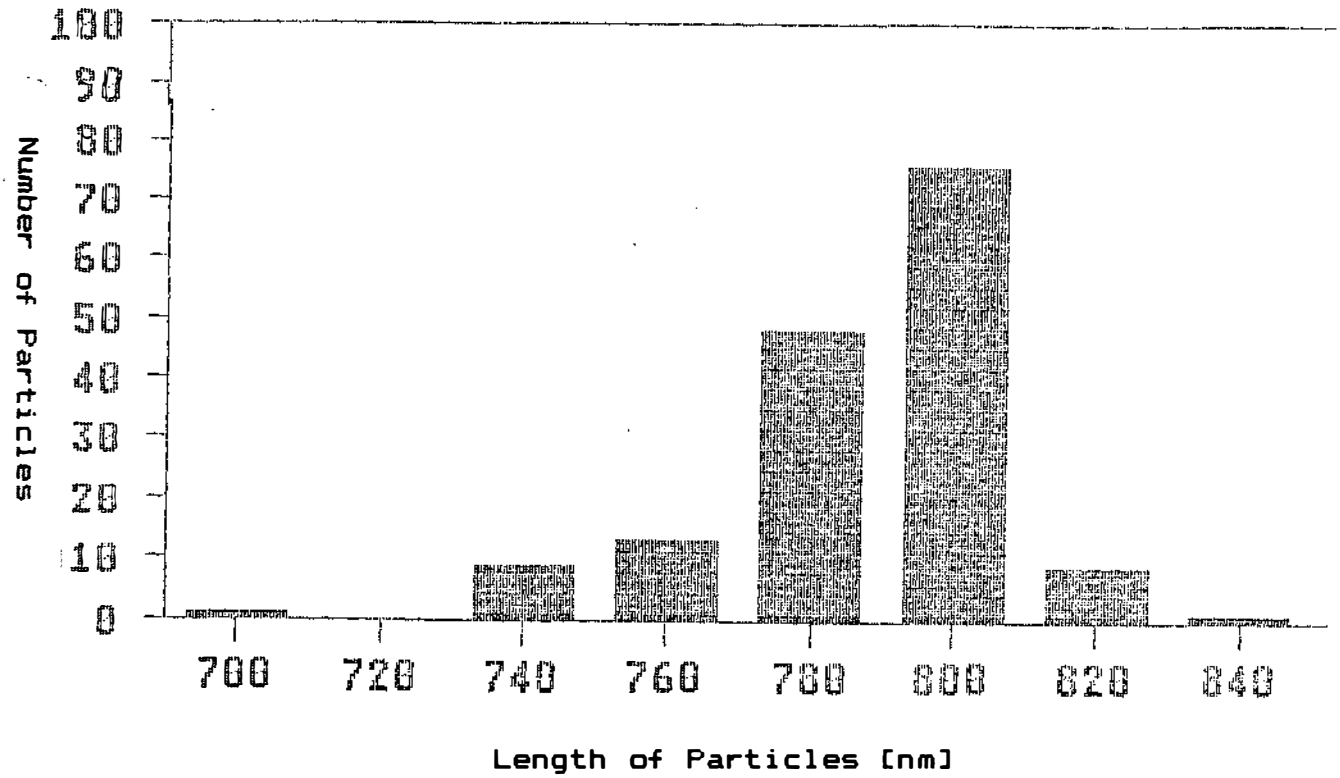


Figure 28: Particle length distribution of nerine virus Y in a field-infected *Nerine sarniensis* hybrid cultivar clonally mass propagated for commercial cut-flower production.

Virus particles of NeVY were typically flexuous. Purified virus preparations showed little surface detail when stained with 2% PTA, pH 7 (Figure 29), 2% sodium silicotungstate or 2% uranyl acetate. However, sub-structural details were evident in storage-degraded virions. Purified virus preparations stored in 0.2M potassium phosphate buffer at pH 7.0 (Kaftanova *et al*,1975) for about 3 months at 4C and stained with 2%PTA, pH 7, showed darkly stained cores (Figure 30). Some virus particles were also found to uncoil from one end showing a helical structure (Figure 31).

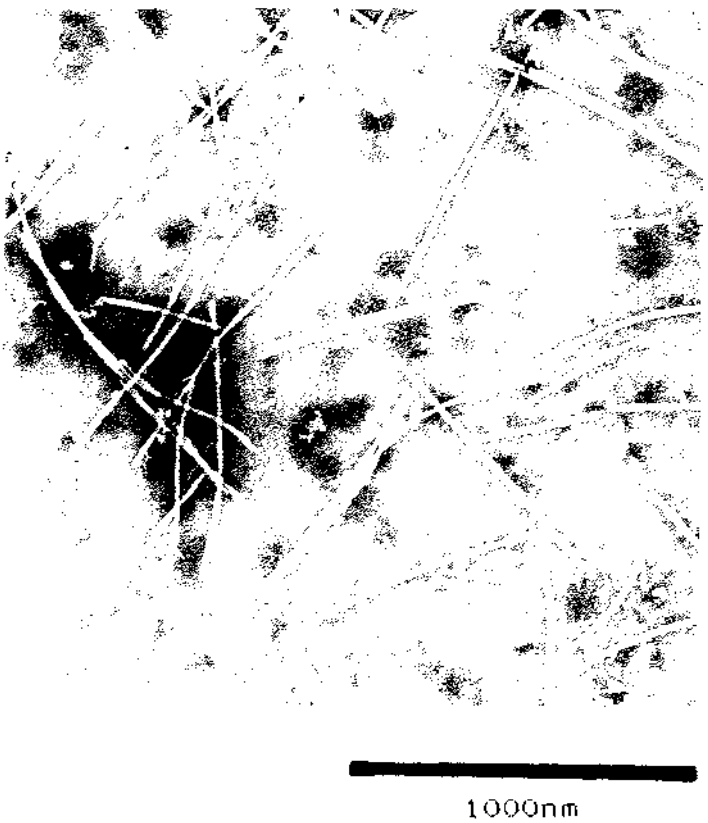


Figure 29: Purified virus preparation of nerine virus Y isolate from *Nerine sarniensis* hybrid stained with 2% PTA, pH 7.

Figure 30: Storage-degraded nerine virus Y particle showing darkly stained core or axial canal when stained with 2% PTA, pH 7. Magnification: X 162,400

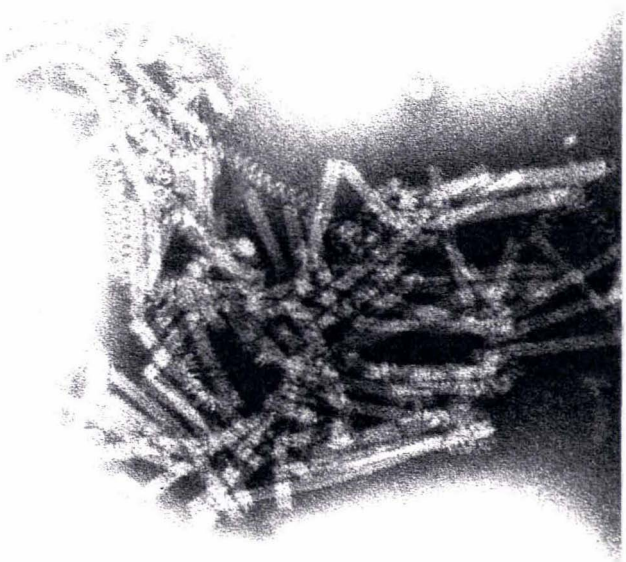


Figure 31: Storage-degaded nerine virus Y particles showing helical structure when stained with 2% PTA, pH7. Magnification: X 103,600

Flexuous filamentous virus particles mostly measuring 11 X 660-900nm have been described as belonging to the potato virus Y or potyvirus group (Matthews,1979;Hollings & Brunt,1981). On the basis of particle length, NeVY can be classified as a potyvirus.

7.3 MECHANICAL TRANSMISSION EXPERIMENTS

Further to the preliminary mechanical transmission experiments using crude sap and partially purified preparations of NeVY infected tissue (Table 7,Chapter 3), repeated efforts were made to transmit the virus from caesium chloride purified preparations to the following indicators: *C.amaranticolor*, *C.quinoa*, *N.clevelandii*, *N.glutinosa*, *N.tabacum* 'White Burley' and *N.benthamiana*. Inoculations on a total of six to eight plants on three separate occasions failed to induce any virus-like symptoms on any of the test plants. Electron microscope investigations of crude sap preparations from inoculated and uninoculated leaves of test plants failed to show the presence of any filamentous virus particles.

7.4 SYMPTOMATOLOGY

The *N.sarniensis* hybrid cultivar growing under shade house conditions in Palmerston North showed no obvious virus-like symptoms for most of the growing season. However, late in the season, when the leaves were beginning to senesce prior to dormancy, severe yellow mosaic symptoms were evident (Figure 32). Leaves of most of the clonally propagated *N.sarniensis* plants showed the virus-like symptoms. Although no obvious colour-break symptoms were seen in the pink petals of the NeVY infected clones, distortion of the flower stalk was common in most of the plants (Figures 33 & 1[A] in Chapter 3).

Nerine virus Y infected bulbs when lifted, potted, and grown under glasshouse conditions at an average temperature of about 20C showed light mosaic symptoms on some of the leaves within three or four weeks after leaf emergence (Figure 34). A relatively high concentration of virus particles and inclusion bodies were evident in light and electron microscope investigations as described in Section 7.5. Leaves from NeVY infected bulbs grown under glasshouse conditions were used in the purification of the virus (Section 7.6).

Figure 32: Severe yellow mosaic symptoms in senescing leaves of a commercially cultivated *Nerine sarniensis* hybrid cultivar systemically infected with nerine virus Y.

Figure 33: Distorted flower stalk of nerine virus Y infected *Nerine sarniensis* hybrid.

Figure 34: Light mosaic symptoms in young leaves of nerine virus Y infected *Nerine sarniensis* hybrid grown in a glasshouse at an average temperature of 20C.

7.5 ANALYSIS OF INCLUSION BODIES

7.5.1 Light Microscopy

Epidermal strips of NeVY infected *N.sarniensis* leaf tissue contained cytoplasmic inclusion bodies (Figure 35[A,B &C]). The epidermal strips were first immersed in 5% Triton X-100 for about 5min to clear the cells of all plastids and other cytoplasmic constituents so that the inclusions would be more readily visible and, stained with a combination of calcomine orange 2RS/Luxol brilliant green BL dye (E.I. du Pont de Nemours & Co) as described by Christie and Edwardson (1986 & pers comm). No cytoplasmic inclusion bodies were seen in epidermal strips of leaf tissue from *N.bowdenii* seedlings not infected with any filamentous virus particles.

The characteristic cylindrical inclusions of potyviruses are known to first appear at the cell periphery where they increase in size and number at the early stage of infection and then move to more central regions of the cell where they form a relatively large and distinct mass in the later stages of the infection cycle (Christie & Edwardson,1977). In NeVY infected nerine leaf tissue the peripheral inclusions were extremely small, widely scattered and not clearly visible in light micrographs. By contrast, inclusion body complexes in more central parts of the cell and near the nucleus were clearly visible as shown in Figure 35.

Figure 35[A],[B] &[C]: Light micrographs of nerine virus Y inclusion bodies in epidermal cells. The nerine leaf epidermal cells were treated with Triton X-100 and stained with a combination of calcomine orange 2RS and Luxol brilliant green BL.

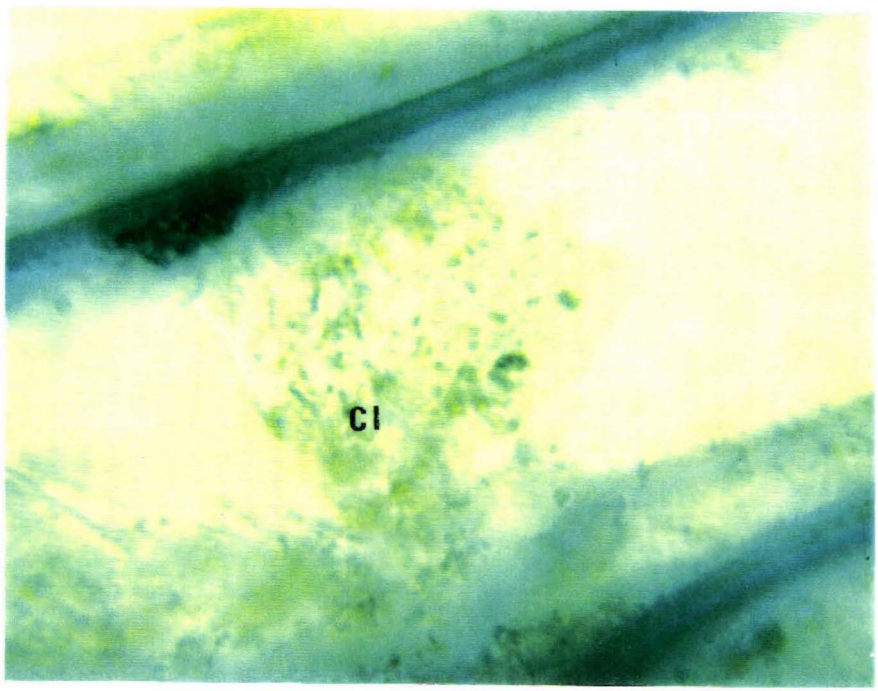


Figure 35[A]: An epidermal cell containing a loose mass of cylindrical inclusions (CI). Magnification: X 970.

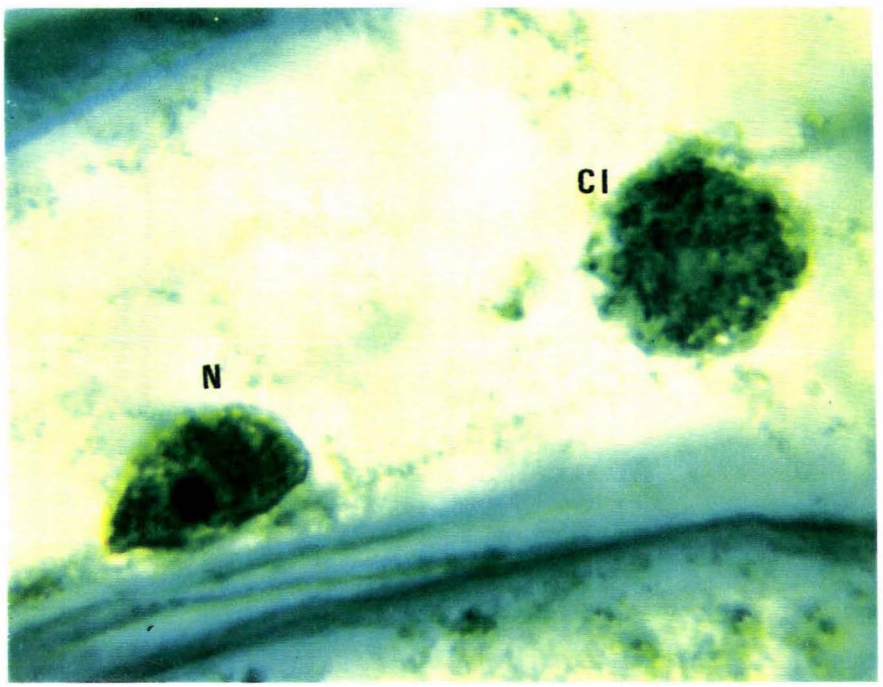


Figure 35[B]: An epidermal cell containing a compact mass of cylindrical inclusions (CI) and nucleus (N). Magnification: X 970

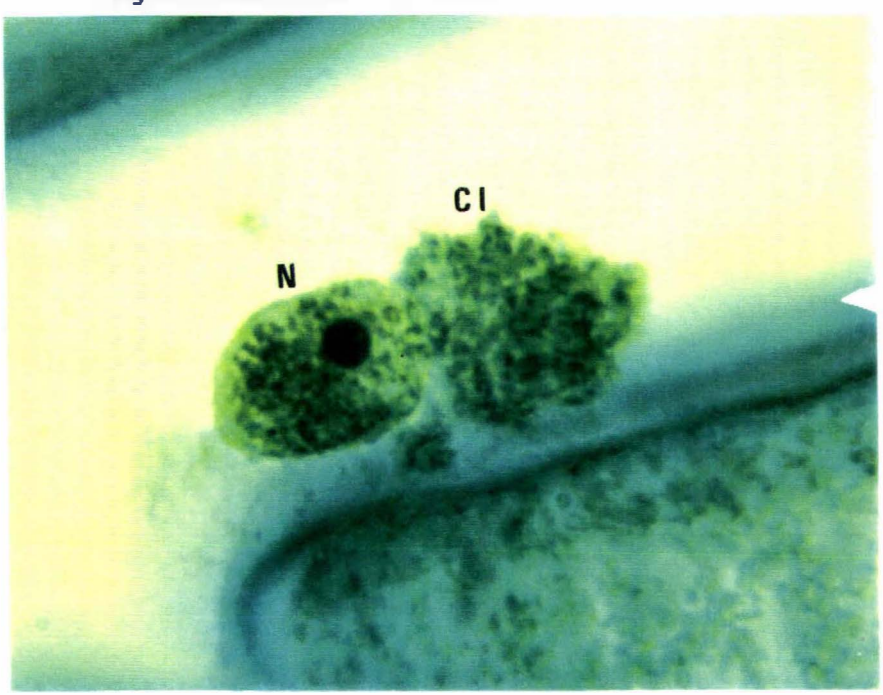


Figure 35[C]: A compact mass of cylindrical inclusions (CI) near the nucleus (N) of the epidermal cell. Magnification: X 970

7.5.2 Electron Microscopy

Fragments of cylindrical inclusion bodies were observed in crude sap preparations from NeVY infected tissue. Inclusion bodies purified by the procedure outlined in Section 2.2.5.2. showed that the rectangular inclusion body plates had a pattern of fine linear striations with a periodicity of approximately 5nm (Figure 36).

Ultrathin (85nm) sections of NeVY infected tissue embedded in Polarbed 812, cured and stained as described in section 2.2.4.3, contained characteristic potyvirus intracellular cytoplasmic inclusions (Figure 37). Approximately three to four weeks after leaf emergence, a large number of the cylindrical inclusions were seen in ultrathin sections cut through mesophyll cells from leaves of the NeVY infected *N.sarniensis* hybrid cultivar grown in the glasshouse.

The basic morphology of the NeVY induced cylindrical inclusion is shown in Figure 37. Figure 37[A] shows masses of cytoplasmic cylindrical inclusions in between chloroplasts and mitochondria. The cylindrical inclusions have been sectioned in different planes to show bundles in longitudinal section (Figure 37[A],[B] & [C]), and, scrolls and pinwheels in cross-section (Figure 37[D] & [E]. In some mesophyll cells masses of cylindrical inclusions were associated with filamentous virus particles (Figures 37[C]). At very high magnifications (162,400 X) 8-10 curved lamella or arms could be seen radiating out of a central core of the pinwheel (Figure 37[E]). The diameter of the pinwheels was estimated to be ca 268nm +/-39.8nm (16 measurements). The length of the curved lamella was estimated to be ca 200nm +/- 23nm (12 measurements). The length of the bundles varied widely extending to more than 2000nm in some cases. Studies of the sequential development of cylindrical inclusions have shown that size, shape, number and location changes with duration of infection (Lawson *et al*,1971; Andrews & Shalla,1974). The evidence from the light and electron micrographs suggests that the tissues sampled contained inclusions in the middle to late phase of the infection cycle.

No cylindrical inclusions were evident in ultrathin sections of *N.bowdenii* seedling leaf tissue not infected with filamentous viruses (Figure 38).

Based on inclusion body morphology NeVY induced cylindrical inclusions, depending on the plane of sectioning, mainly appeared as tubes or scrolls without any laminated aggregates. Nerine virus Y can therefore be categorized in Edwardson and Christie's (1986) Type 1 potyvirus subdivison.

Figure 36: Electron micrograph of a rectangular striated portion of a cylindrical inclusion induced in *Nerine sarniensis* leaf tissue infected by nerine virus Y. Stain: 2% PTA, pH 7. Magnification: X 72,100.

Figure 37[A]-[E]: Ultrathin sections of nerine virus Y cylindrical inclusions induced in *Nerine sarniensis* hybrid leaf cells. The leaf cells have been sectioned in different planes and electron micrographs taken at different magnifications to show inclusion body morphology.

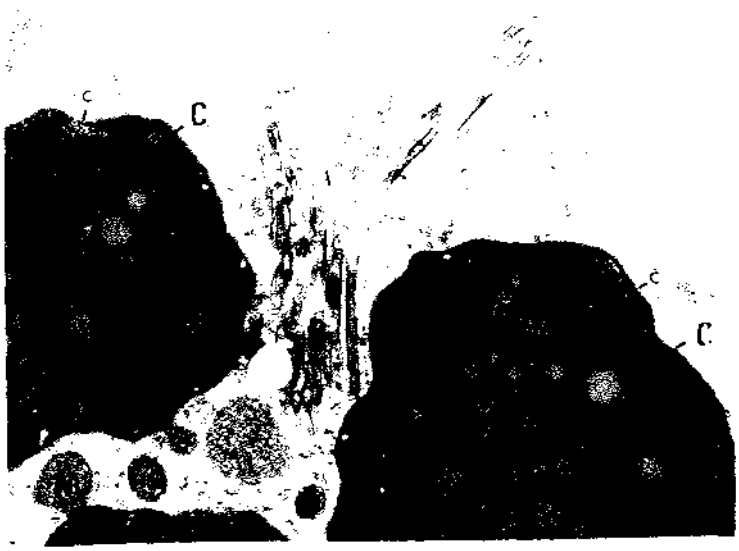


Figure 37[A]: Longitudinal section of cytoplasmic cylindrical inclusions or bundles (B) between chloroplasts (C) and mitochondria (M) in mesophyll cells. Magnification: X 11,200.

Figure 37[B]: Mass of bundles (B) in the cytoplasm of a leaf cell adjacent to the cell wall (CW). Magnification: X 31,800.

Figure 37[C]: Bundles (B) associated with longitudinal sections of filamentous virus particles (V). Magnification: X 48,600.

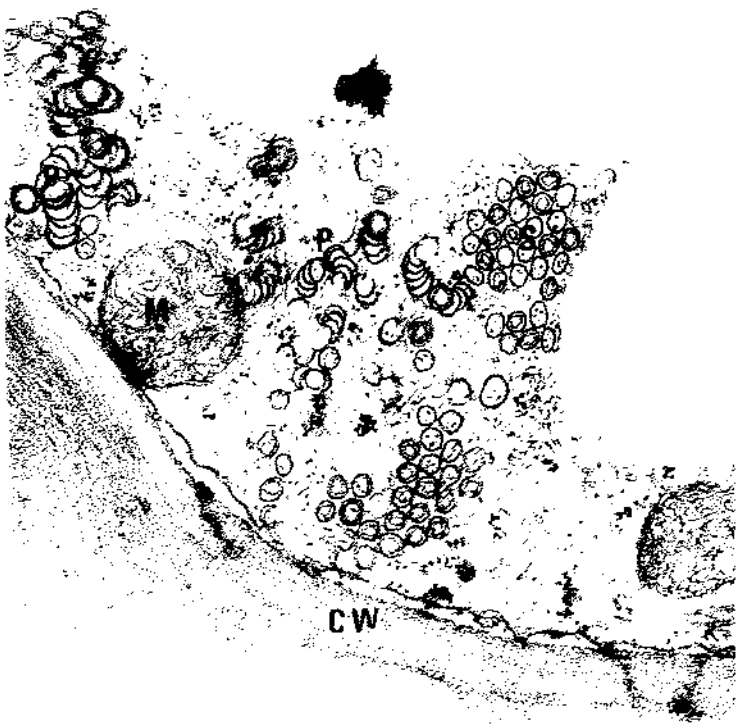


Figure 37[D]: Cytoplasmic cylindrical inclusions in cross-section showing scrolls (S) and pinwheels (P). M = mitochondrion; CW = cell wall. Magnification: X 31,800.

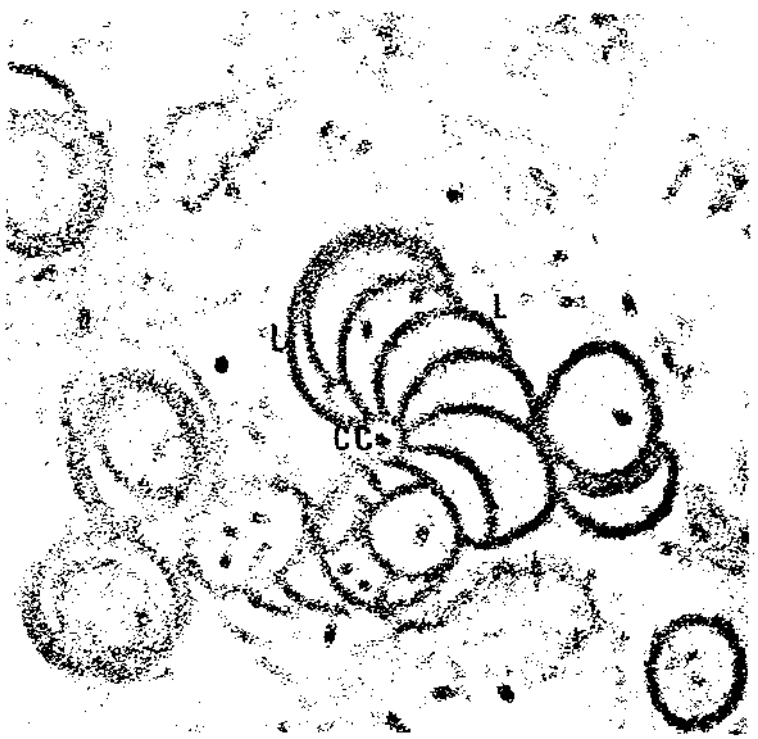


Figure 37[E]: Pinwheel showing curved lamella (L) radiating out of the central core (CC). Magnification: X 162,400.

Figure 38: Ultrathin sections of healthy *Nerine bowdenii* seedling leaf tissue showing chloroplasts (C) and mitochondria (M). Magnification: X 15,300.

7.5.3 SDS-Page Gel Electrophoresis of Cylindrical Inclusions

Electrophoretic separations of SDS-dissociated cytoplasmic inclusions, purified from *N.sarniensis* leaf tissue as described in Section 2.2.5.2, gave two polypeptide bands with estimated molecular weights of 70.6kd and 67.3kd. The 70.6kd main band (Figure 39) is believed to be the inclusion body band by analogy with other potyviruses (Hiebert *et al*,1984), with the 67.3kd minor band being a degradation product of the former.

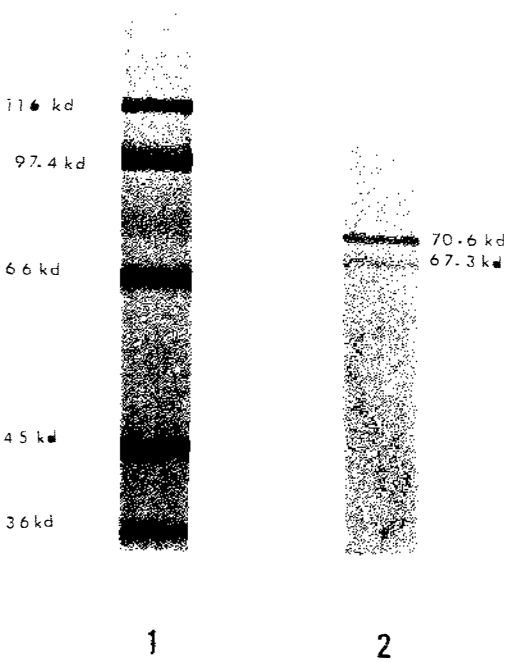


Figure 39: Separation of SDS-dissociated marker proteins (lane 1) and nerine virus Y cylindrical inclusion protein (lane 2) electrophoresed on a 12.5% polyacrylamide gel. The gel was stained first with Coomassie Blue R and double-stained with Coomassie Blue G-250 and photographed with Kodak 35mm colour film.

The calibration data used for molecular weight determination of the cylindrical inclusion protein is given in Table 9 and the calibration curve of distance migrated versus molecular weight (log scale) is shown in Figure 40.

Table 9: Calibration data used for molecular weight determination of nerine virus Y induced cylindrical inclusions.

Component	MW(kd)	Log_{10} MW	Distance migrated (mm)
(a) α-galactosidase (*E. coli*)	116.0	5.06	18
(b) phosphorylase B (rabbit muscle)	97.4	4.98	22.5
(c) albumin (bovine)	66.0	4.81	32.3
(d) albumin (egg)	45.0	4.65	46
(e) glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle)	36.0	4.55	53

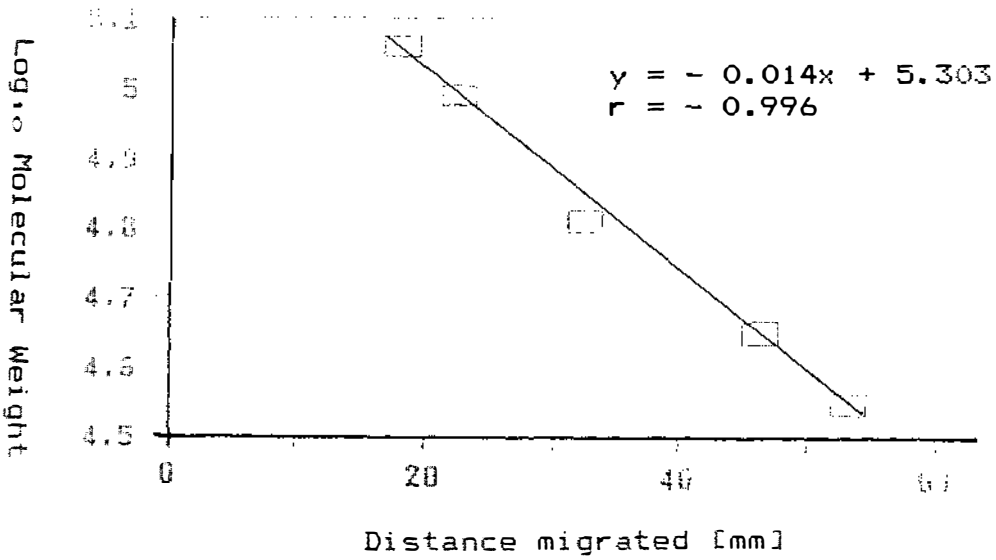


Figure 40: Calibration curve for molecular weight determination of nerine virus Y induced cylindrical inclusions. The distance migrated by the marker proteins was plotted against the molecular weight (log scale).

7.6 PURIFICATION OF NERINE VIRUS Y

The procedure outlined in Section 2.2.5.2. was used to purify NeVY. The virus concentration of NeVY infected *N.sarniensis* leaf tissue grown under shade house conditions in the ground and late in the season, when yellow mosaic virus-like symptoms were apparent, was relatively low. The virus yield in four separate preparations varied from 0.008 to 0.023mg per 100 g of leaf tissue assuming an extinction coefficient of $A^{0.1\%}_{1cm}$ at 260nm of 2.4 by analogy with other potyviruses (Damirdagh & Shepherd,1970; Van Oosten,1972; Derks *et al*,1982; Hunst & Tolin,1982).

However, bulbs potted after their dormant phase and grown under glasshouse conditions had a much higher concentration of NeVY after leaf emergence. The higher virus concentration was also associated with masses of proteinaceous inclusion bodies reported in Sections 7.5.1 and 7.5.2. Nerine virus Y was thus mainly purified from leaves harvested from potted *N.sarniensis* plants grown under glasshouse conditions. Initially considerable loss of virus occurred in the first low speed centrifugation (12,000g), due to a failure to separate the virus from the inclusion bodies. Figure 41 shows cylindrical inclusions and virus particles recovered from the solvent/aqueous interphase after centrifugation at 12,000g. This loss was minimized by homogenizing the freshly harvested nerine leaves in 0.5M potassium phosphate buffer, pH 7.6 containing 5mg of sodium sulphite per gram of tissue and 0.05M EDTA. Half a volume of chloroform:carbon tetrachloride and 1% Triton X-100 was added before re-homogenizing at very low speed with a power controller prior to gentle agitation for 2-3h at 4C. The virus and inclusion bodies were then purified as described in Section 2.2.5.2. Virus yields of between 0.11-0.28mg (3 determinations) from 100mg of young leaves, freshly harvested from glasshouse grown *N.sarniensis*, was obtained within four weeks of leaf emergence.

The use of gentle purification procedures, involving polyethylene glycol precipitation and caesium chloride centrifugation, yielded relatively unfragmented virus particles (Figure 29).

Figure 41: Electron micrograph of cylindrical inclusions (CI) associated with nerine virus Y particles (V) stained with 2% PTA, pH7. Magnification: X 31,800.

7.7 MOLECULAR WEIGHT OF NERINE VIRUS Y COAT PROTEIN

Sodium dodecyl sulphate (SDS)-dissociated NeVY coat protein from purified virus particles was electrophoresed in SDS-Page slab gels using as standards a SDS-70L molecular weight kit (Sigma). The mobility of the markers gave the molecular weight calibration data shown in Table 10. A calibration curve of distance migrated versus molecular weight (log scale) was plotted (Figure 42) and the molecular weight of the viral coat protein determined by inverse estimation from the regression of the electrophoretic mobility of the markers.

Table 10: Calibration data used for molecular weight determination of nerine virus Y coat protein.

Component	MW (kd)	Log_{10} MW	Distance migrated (mm)
(a) Albumin (bovine serum)	66	4.819	21
(b) Ovalbumin	45	4.653	31
(c) Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle)	36	4.556	36
(d) Carbonic anhydrase (bovine erythrocytes)	29	4.462	43
(e) Trypsinogen	24	4.380	46
(f) Trypsin inhibitor (soybean)	20.1	4.303	55

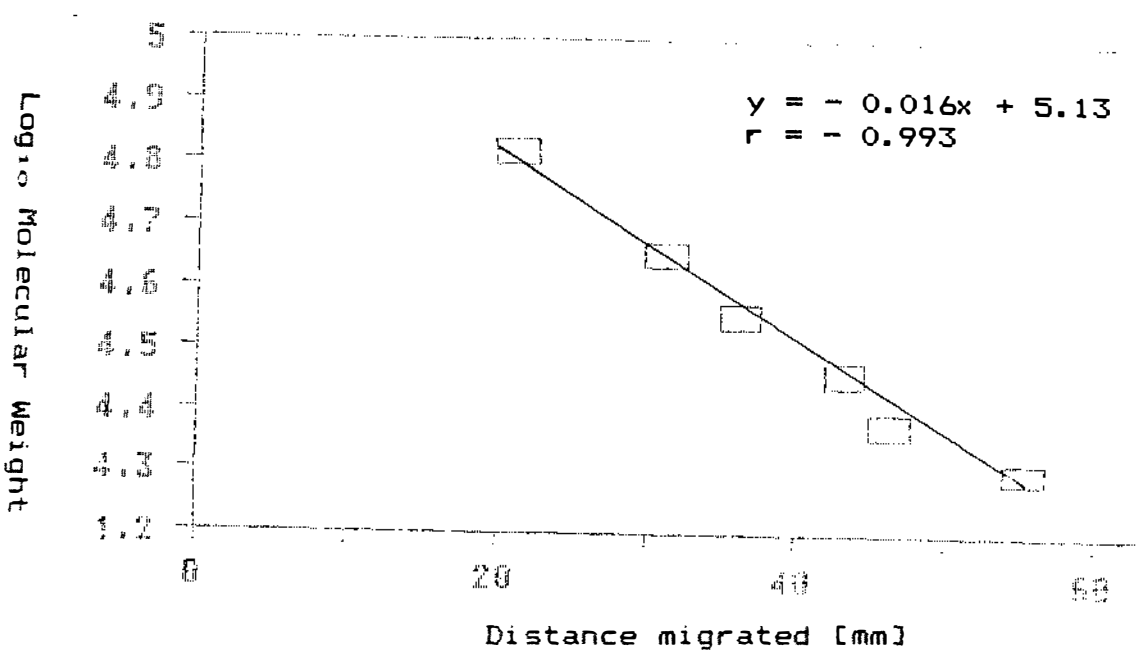


Figure 42: Calibration curve for molecular weight determination of nerine virus Y coat protein. The distance migrated by the marker proteins was plotted against the molecular weight (log scale).

A single viral coat protein band (Figure 43) with an estimated molecular weight of 33.26kd was obtained.

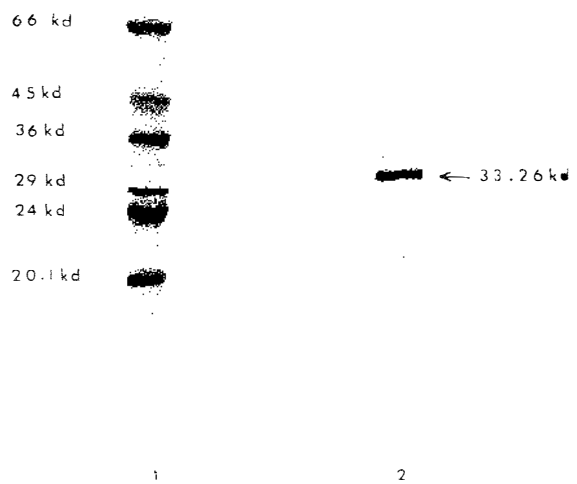


Figure 43: Separation of SDS-dissociated marker proteins (lane 1) and nerine virus Y coat protein (lane 2) electrophoresed on a 12% polyacrylamide gel. The gel was stained with Coomassie Blue R stain and photographed with a Polaroid MP-4 land camera.

7.8 EXTRACTION AND ANALYSIS OF NERINE VIRUS Y RNA

Several methods were attempted to extract RNA from purified virus preparations. These included the use of: proteinase K and phenol as the protein denaturing agents(Xu *et al*,1986); SDS in ammonium carbonate buffer, pH 9 and proteinase K (Taiwo *et al*,1982); SDS in ammonium carbonate buffer, pH 9 (Hiebert & Charudattan,1984); and SDS-phenol-chloroform (Palmiter,1974). The SDS-ammonium carbonate-proteinase K treatment described in Section 2.2.7.2 was found to be the most satisfactory method for yielding relatively undegraded NeVY RNA.

A single RNA species was observed by agarose gel electrophoresis under denaturing conditions (Figure 44) using the formaldehyde/formamide procedure (Gerard & Miller, 1986) described in Section 2.2.8.2. An RNA ladder (Bethesda Research Laboratories) was co-migrated with the genomic RNA to obtain the data presented in Table 11.

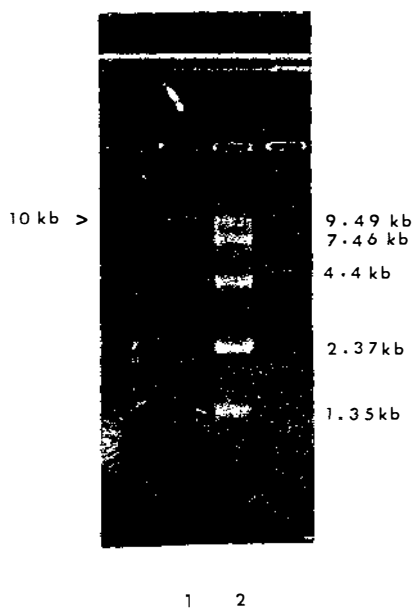


Figure 44: Agarose gel electrophoresis of nerine virus Y RNA under (formaldehyde/formamide) denaturing conditions. Lane 1: nerine virus Y RNA, Lane 2: RNA ladder (Bethesda Research Laboratories). The gel was stained with ethidium bromide, destained in water and photographed with a Polaroid MP-4 land camera using a short-wave ultraviolet(254nm) transilluminator.

Table 11: Calibration data obtained by gel electrophoresis of an RNA ladder (Bethesda Research Laboratories) on a 1% agarose gel under denaturing conditions. The calibration curve of the distance migrated by RNA species plotted against molecular weight, is given in Figure 45.

Molecular weight (bases)	Log_{10} MW	Distance migrated (mm)
viral RNA		*8.5*
9490	3.977	10.0
7460	3.873	12.3
4400	3.643	18.2
2370	3.375	26.8
1350	3.130	35.0

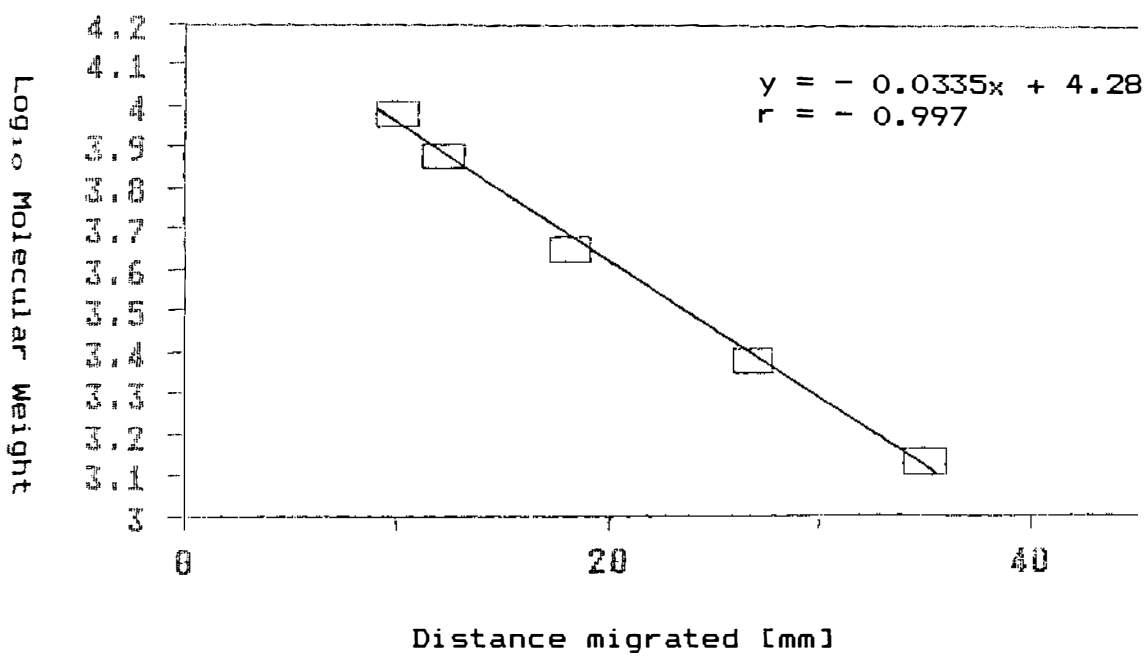


Figure 45: Calibration curve obtained by plotting distance migrated by RNA species in RNA ladder (Bethesda Research Laboratores) versus molecular weight (log scale).

A single genomic species with an estimated molecular weight of 10.0kb was obtained for the nerine virus Y isolate.

7.9 SIZE FRACTIONATION, cDNA SYNTHESIS AND MOLECULAR CLONING OF NERINE VIRUS Y RNA

To minimize possible contamination with RNA from nerine tissue or other RNA viruses in the host plant, the 10.0kb NeVY RNA was size fractionated on methylmercuric hydroxide gels as described in Section 2.2.8.2. Methylmercury is a reversible denaturing agent for nucleic acids (Bailey & Davidson,1976). Therefore the nucleic acids can be recovered by removing the methylmercury and used in cDNA cloning reactions. This is a useful technique for purifying viral RNA, especially from field-infected material where the possibility of mixed viral infection exists. By denaturing the viral RNA so that conformational and intermolecular aggregation is minimized, size fractionating prior to cDNA cloning effectively ensures that only the selected RNA is finally cloned. This strategy minimizes difficulties which may arise from the presence of positive cDNA recombinant clones derived from other than the expected specific viral RNA. Figure 46 shows NeVY RNA and an RNA ladder electrophoresed on a 1.2% low melting point agarose gel.

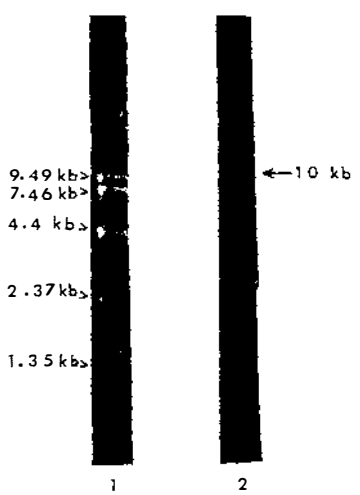


Figure 46: Low melting point 1.2% agarose (FMC Seaplaque) gel electrophoresis of nerine virus Y RNA and RNA markers denatured with methylmercuric hydroxide. Lane 1: RNA ladder, Lane 2: nerine virus Y RNA. The gel was stained with ethidium bromide, destained in water and photographed with a Polaroid MP-4 land camera using a shortwave ultraviolet (254nm) transilluminator.

The cDNA for NeVY was prepared by a modification of the procedures described by D'Alessio *et al* (1987) and Watson and Jackson (1985) and detailed in Section 2.2.10.2. The 10kb NeVY RNA was assumed to be polyadenylated by analogy with other potyviruses (Rosner *et al*,1986). Lambda gt10 was used as the cDNA cloning vector because NeVY RNA was only available in very limited quantities and lambda gt10 is known to be a high efficiency cloning vector, offering cloning efficiencies exceeding $2X10^7$ plaque forming units/ug double stranded cDNA (Watson & Jackson,1985). Lambda gt10 is a 43.34kb insertion vector (Figure 47) which can accept DNA insert fragments of up to 7.6kb (Huynh *et al*,1985).

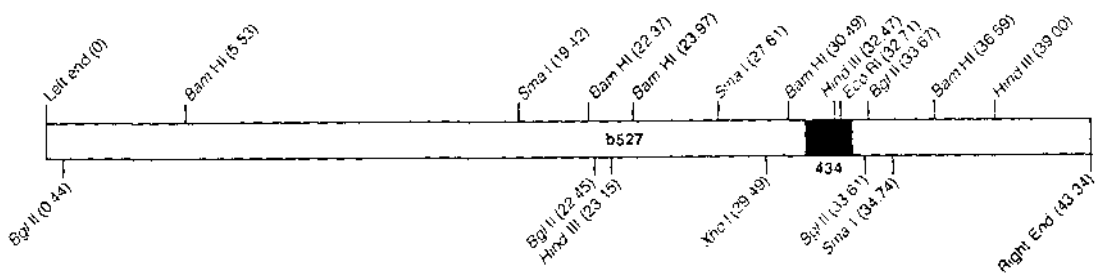


Figure 47: Map of bacteriophage lambda gt10. Restriction endonuclease cleavage sites are designated in kilobase pairs from the left end.

Lambda gt10 inserts were analysed by agarose gel electrophoresis following digestion with *Eco* RI. Inserts from the eight clones were found to belong to two size classes, 1.54kb (five clones) and 0.56kb (three clones). DNA from both these classes were subcloned into the plasmid vector pGEM3 (Figure 48) because plasmid vectors are very useful for the amplification and purification of relatively large quantities of cloned cDNA. Further, while the lambda gt10 vector was 43.34kb, the plasmid pGEM3 (Figure 49) was only 2.752kb giving a substantially lower insert to vector DNA ratio and thus enabling the use of the entire recombinant DNA in nick-translation reactions. The DNA was extracted and purified according to methods described in Section 2.2.10.2. and used in dot-blots assays.

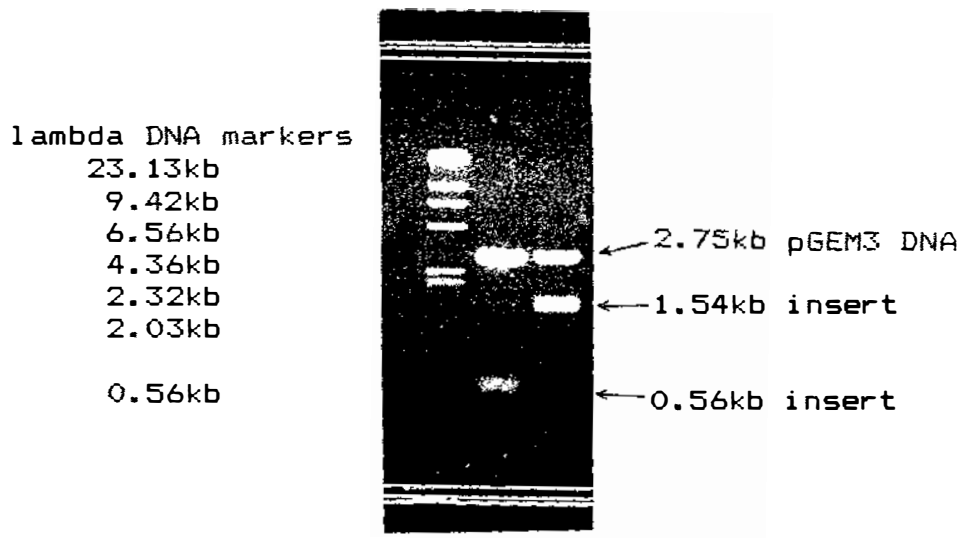


Figure 48: *Eco* RI digests of pGEM3 to show insert size.
Lane 1: *Hind* III digest of lambda DNA used as markers.
Lane 2: pGEM3 DNA and 0.56kb insert.
Lane 3: pGEM3 DNA and 1.54kb insert.

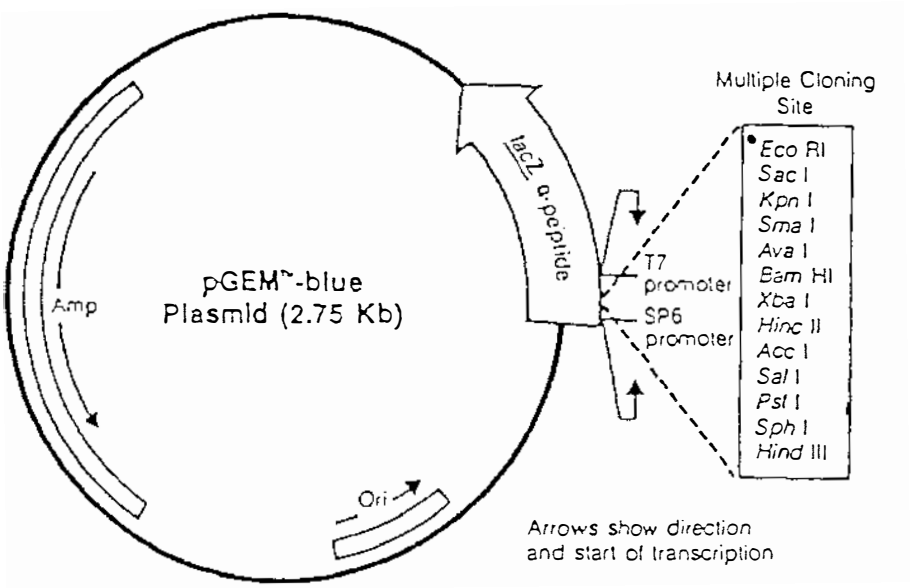
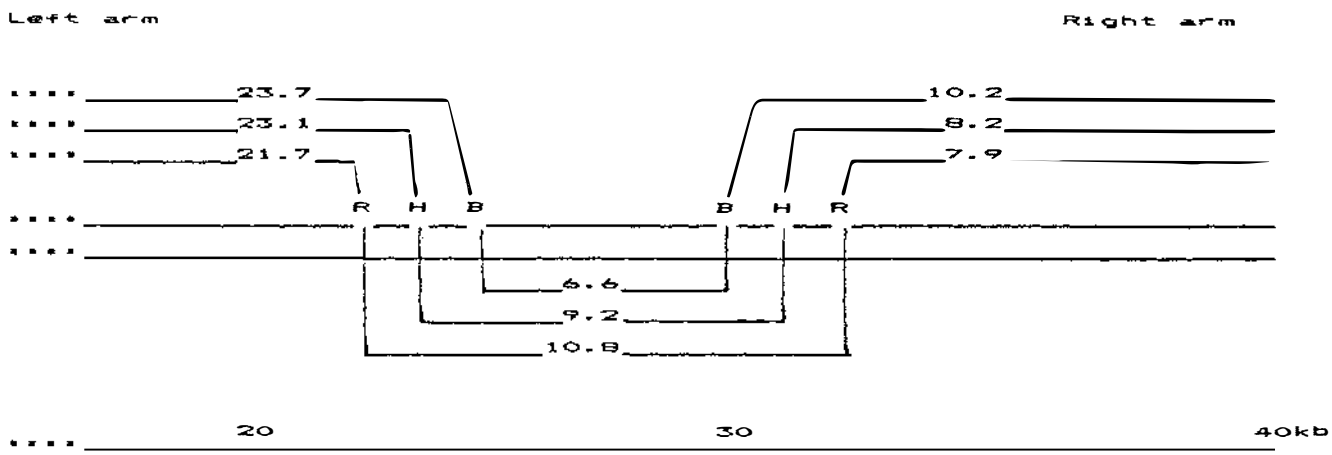


Figure 49: Map of 2.752kb pGEM3 vector showing *Eco* RI• cloning site.

Further to cloning into lambda gt10, which was able to accept cDNA inserts up to a maximum size of 7.6kb pairs, double stranded cDNA was also cloned into the bacteriophage lambda L47AB (Loenen & Brammar, 1980). Lambda L47AB is a 40.4kb replacement vector (Figure 50), into which fragments of at least 8kb can be cloned. Thus L47AB was used in an attempt to obtain near full-length clones of NeVY. A L47AB library of NeVY clones was screened with nick-translated lambda gt10 DNA containing the 1.54kb insert using procedures described by Davis *et al* (1986). One clone with a 9.8kb insert was subcloned into pGEM3 (Figure 51) and the DNA extracted for use in dot-blot assays.



R = *Eco* RI, B = *Bam* HI, H = *Hind* III

Figure 50: Map of part of the 40.4kb bacteriophage lambda L47AB showing location of *Eco* RI, *Bam* HI and *Hind* III restriction sites.

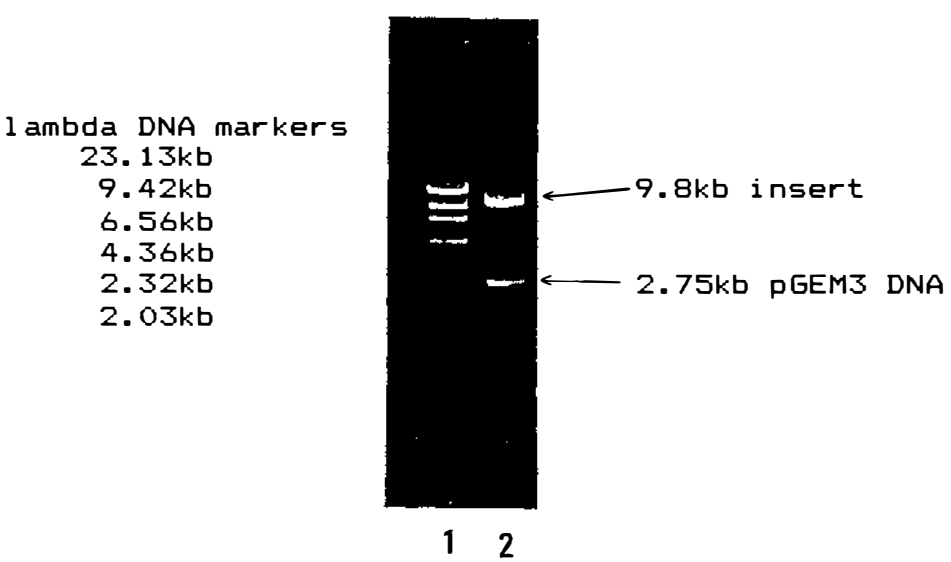


Figure 51: *Eco* RI digest of plasmid pGEM3 to show 9.8kb insert subcloned from lambda L47AB.
Lane 1: *Hind* III digest of lambda DNA used as markers.
Lane 2: pGEM3 DNA and 9.8kb insert.

7.10 ESTABLISHMENT OF IDENTITY AND HYBRIDIZATION SPECIFICITY OF cDNA CLONED PROBES

Three cDNA cloned probes, two derived from lambda gt10 and one from lambda L47AB were used to check that hybridization occurred to the homologous NeVY RNA. cDNA was labelled with [^{32}P]-dCTP by nick-translation, acid-depurinated, and hybridized on Zeta-Probe membrane to sap containing NeVY, healthy sap and sap containing NeVX. The probes were depurinated to decrease the length of the probe because other researchers had established that the optimal probe length for hybridization to nucleic acids immobilized on membranes was 200-1000 bases (Wahl *et al*,1979; Reed and Mann,1985). Fragment sizes outside these limits gave unacceptably poor resolution with high background.

The depurination procedure was checked by agarose gel electrophoresis of a 3ul sample of the nick-translated cDNA probe before and after depurination by transferring it onto a Zeta-Probe membrane prior to autoradiography (Figure 52). To ensure that depurination did not result in complete breakdown of the cDNA, a 2ul aliquot was taken after depurination, spotted, air-dried and chromatographed on polyethyleneimine-cellulose coated plastic sheets (PEI-cellulose; Merck #5579) using as a solvent a 0.75M potassium phosphate solution, pH 3.5. Figure 53 shows an autoradiograph of the PEI-cellulose chromatogram. Disintegration of the DNA into triphosphates and monophosphates would have resulted in migration upwards on the PEI-cellulose strip (Reed & Mann,1987).

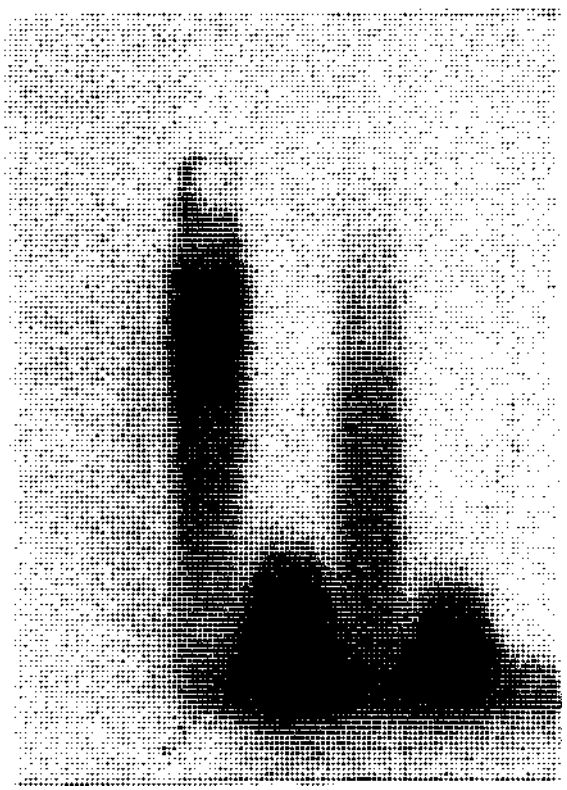


Figure 52: Autoradiograph of unfragmented and depurinated [^{32}P]-labelled nick-translated NeVY cDNA cloned probes.
Lane 1: 1.54kb unfragmented probe.
Lane 2: 1.54kb depurinated probe.
Lane 3: 0.56kb unfragmented probe.
Lane 4: 0.56kb depurinated probe.

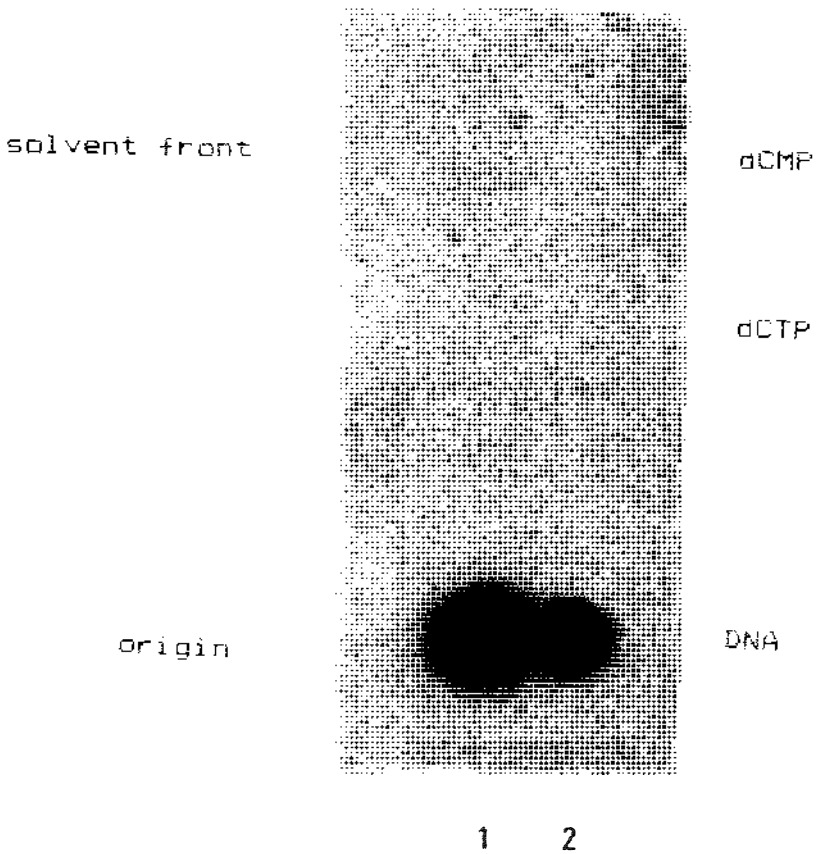


Figure 53: Autoradiographs of PEI-cellulose chromatogram of depurinated [^{32}P]-labelled cDNA cloned probes. The position of the origin and the solvent front is indicated. The expected position of the triphosphate (dCTP) and monophosphate (dCMP), if parts of the DNA had disintegrated is shown.
Lane 1: 2ul sample of depurinated 1.54kb probe.
Lane 2: 2ul sample of depurinated 0.56kb probe.

All three depurinated [^{32}P]-labelled nick-translated cDNA cloned probes only hybridized to the homologous virus preparations (Figure 54). The 1.54kb cDNA cloned probe derived from lambda gt10 was selected for further use in dot-blot assays. The specificity of the probe was determined by testing it against five other potyviruses; namely nerine yellow stripe virus, hippeastrum mosaic virus, an uncharacterized 800nm nerine potyvirus (from A A Brunt), potato virus Y and bean yellow mosaic virus. The probe hybridized only to the uncharacterized 800nm nerine potyvirus obtained from A A Brunt (Figure 56).

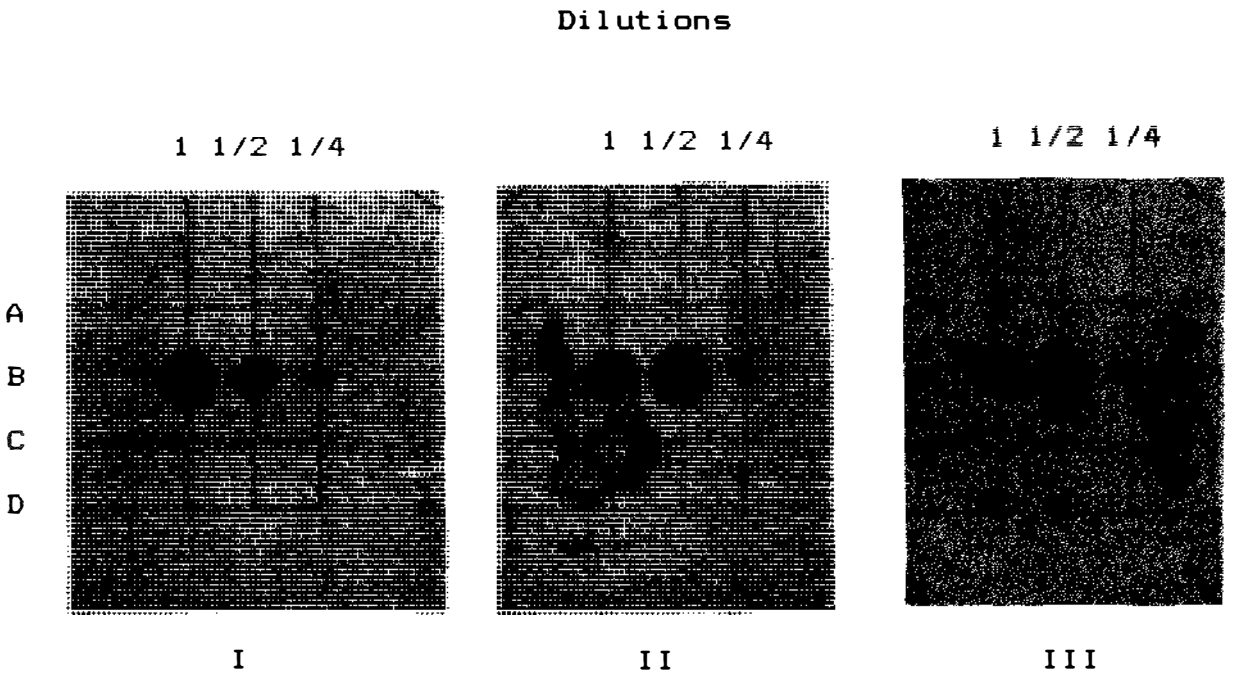
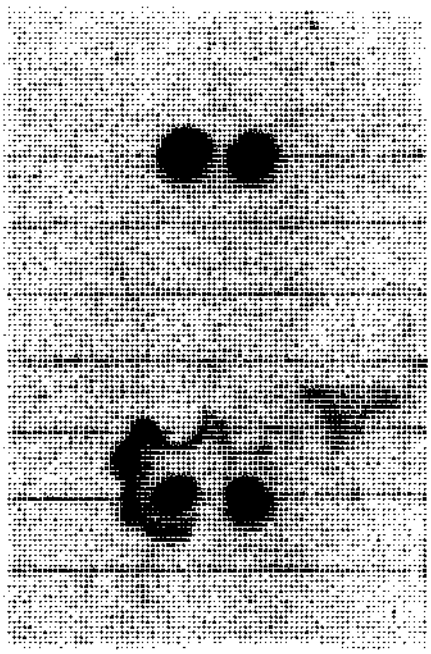


Figure 54: Autoradiographs of dot-blot hybridization of the 1.54kb (I), 0.56kb (II) and 9.8kb (III) [^{32}P]-labelled nick-translated cloned cDNA probes with:

A: purified nerine virus X at twofold dilutions
B: purified nerine virus Y at twofold dilutions
C: clarified sap from healthy nerine leaf tissue diluted in buffer
D: partially purified virus preparation from nerine tissue infected with nerine virus X and a ca 750nm potyvirus (presumably nerine yellow stripe virus).



purified nerine virus Y

nerine healthy sap

CVC of nerine yellow stripe virus

CVC of hippeastrum mosaic virus

CVC of bean yellow mosaic virus

CVC of nerine virus Y

CVC of potato virus Y

CVC = clarified viral concentrates

Figure 55: Autoradiograph of nerine virus Y and four other potyviruses hybridized with cloned cDNA [^{32}P]-labelled probe. Kodak XAR-film was exposed to the Zeta-Probe membrane in an intensifying screen at -70C for 48h.

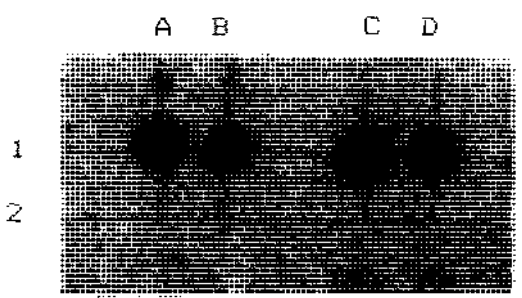


Figure 56: Autoradiograph of clarified viral concentrates of nerine virus Y (A1 and B1) and an uncharacterized ca 800nm nerine potyvirus obtained from A A Brunt (C1 and D1) hybridized with a cloned cDNA [^{32}P]-labelled probe. A2, B2, C2 and D2 were spotted with healthy sap. Kodak XAR-film was exposed to the Zeta-Probe membrane in an intensifying screen at -70C for 48h.

7.11 DETERMINATION OF SENSITIVITY OF THE 1.54kb LAMBDA gt10 DERIVED cDNA CLONED PROBE FOR DETECTION OF NERINE VIRUS Y IN SAP

The sensitivity and limits of detection of the cloned 1.54kb cDNA probe was estimated by using virus extracted from measured quantities of NeVY field-infected nerine leaf tissue.

Hybridization analysis of the cloned cDNA probe (specific activity $1X10^{7}$cpm) to sap extracted from microsamples of systemically infected tissue containing the homologous virus (using methods described in Section 2.2.5.3), loaded at various dilutions onto a Zeta-Probe membrane, showed that the probe was able to detect virus from an equivalent of less than 8mg of tissue after 6h exposure of the membrane to Kodak XAR-5 film using an intensifying screen at -70C (Figure 57). Exposure of the autoradiographic film for 48h increased the sensitivity to less than 0.32mg of leaf tissue. No background problems or nonspecific hybridization to healthy sap was observed.

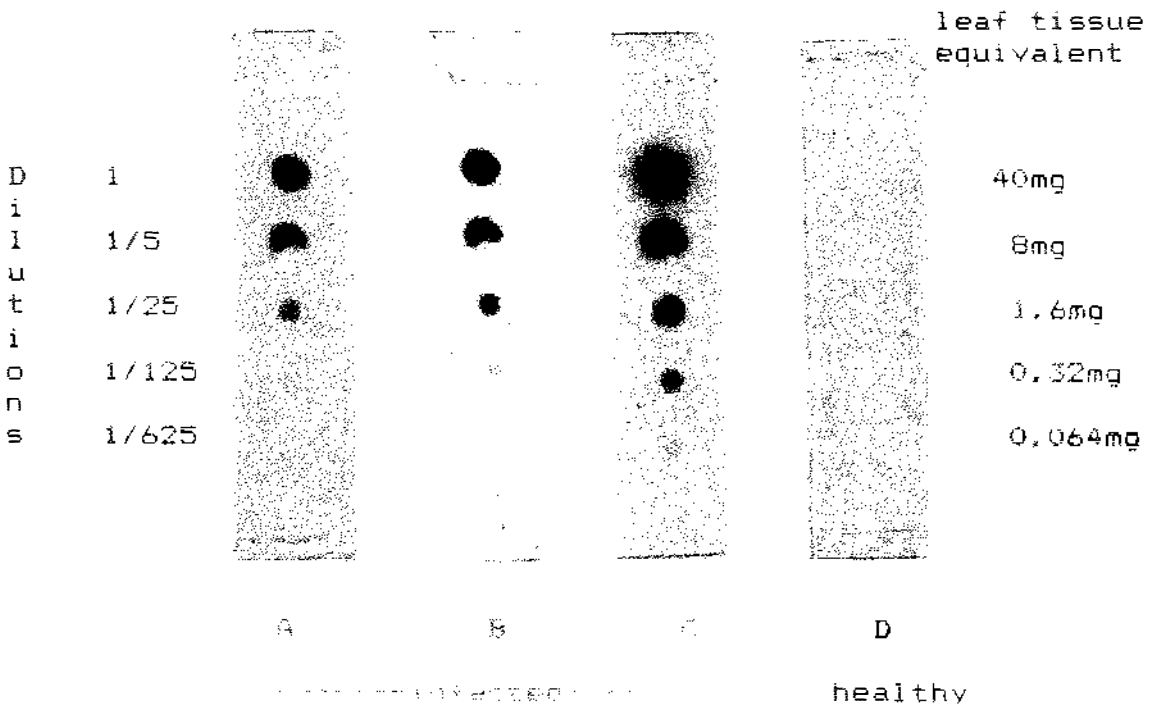


Figure 57: Autoradiograph of sap extracted from field-infected *Nerine sarniensis* hybrid leaf tissue hybridized with a cloned 1.54kb nerine virus Y cDNA [^{32}P]-labelled probe (specific activity 1 X 10^{7}cpm). Dilution 1 equates to clarified viral concentrates from an equivalent of 0.04g of infected and healthy tissue. Kodak XAR-5 film was exposed to the Zeta-Probe membrane for 6h (A), 12h (B) and 48h (C) at -70C with intensifying screens.

7.12 DISCUSSION

On the basis of particle size, intransigence to mechanical transmission and hybridization analysis the potyvirus purified from *N.sarniensis*, characterized and cDNA cloned in this study is believed to be an isolate of the 800nm potyvirus previously found overseas (Maat, pers comm to K S Milne,1983; Brunt, pers comm to K S Milne,1985). The hybridization between cDNA clones of this virus with the 800nm nerine potyvirus from A A Brunt but not with the ca 750nm NeYSV indicates that there are two distinct potyviruses in nerines. The name nerine virus Y is proposed for the 800nm virus.

Nerine virus Y has remained unnamed and uncharacterized presumably because it was not found to be transmissible to herbaceous indicator and because it appears to be relatively uncommon in nerines. The finding of a NeVY-infected *N.sarniensis* cultivar which had been clonally mass propagated during the electron microscope survey combined with the research conducted to optimize virus yield from limited quantities of infected nerine leaf tissue in the early phase of this study (Chapter 5) made it possible to purify, characterize and cDNA clone this hitherto unresearched virus.

The *N.sarniensis* hybrid infected with NeVY showed no virus-like symptoms in the leaves in the early phases of the growth cycle under shade-house conditions. However, severe chlorosis of the leaves was apparent prior to senescence. Under glasshouse conditions, at an average temperature of 20C, light mosaic symptoms were visible soon after the first leaves emerged. No virus-like symptoms were observed in the pink flowers.

Nerine virus Y was a typically flexuous filamentous potyvirus with a normal length of 800nm and particle width of ca 11nm. The molecular weight of the single coat protein subunit of NeVY (32.3kd) is within the range of 32-36kd reported for other potyviruses (Milne,1988). Similarly the genomic RNA of NeVY (10.0kb) is close to the values reported for other potyviruses (Allison *et al*,1986; Domier *et al*,1986; Maiss *et al*,1989).

Nerine virus Y induced characteristic proteinaceous potyvirus cylindrical inclusions, which in ultrathin sections appeared as pinwheels, scrolls and bundles. The cylindrical inclusions were also seen as large masses in leaf epidermal cells by light microscopy. The molecular weight of the cylindrical inclusions was estimated to be 70.6kd. Based on inclusion body morphology, NeVY inclusions were catagorized as belonging to Edwardson and Christie's (1986) Type 1 potyvirus subdivision.

Edwardson and Christie's (1986) Type 1 potyvirus subdivision.

Two lambda gt10 recombinants with cDNA inserts of 1.54kb and 0.56kb, and a 9.8kb cDNA insert derived from lambda L47AB were found to hybridize to the homologous virus RNA but not to several other potyviruses, NeVX, or to nerine healthy sap. The 1.54kb cDNA insert was able to detect NeVY from an equivalent of less than 0.3mg of systemically infected nerine leaf tissue.

The use of recombinant DNA technology enabled the development of a highly sensitive and specific diagnostic tool. Because each cDNA clone is synthesized from a single RNA molecule, it is possible to produce highly specific probes from field-infected material which may contain more than one virus. Once cloned, large quantities of DNA for probes can be obtained without the need for repeated purification of the virus. The cloned cDNA probe can be used for conducting field surveys to study the relative prevalence of NeVY, screening for viral resistance and in virus elimination studies.

CHAPTER 8

TESTS FOR NERINE VIRUS X AND NERINE VIRUS Y USING cDNA PROBES

8.1 INTRODUCTION

Tests of various *Nerine* species from Palmerston North, New Plymouth and Auckland was conducted using cloned cDNA probes to nerine virus X and NeVY. One hundred and nine nerine leaf samples were processed using the clarified viral concentrates method outlined in in Section 2.2.5.3.

8.2 TESTS FOR NERINE VIRUS X USING THE CLONED cDNA PROBE

Figure 58 shows a typical autoradiograph of dot-blot assays obtained in this survey using the 1.8kb cloned cDNA NeVX probe.

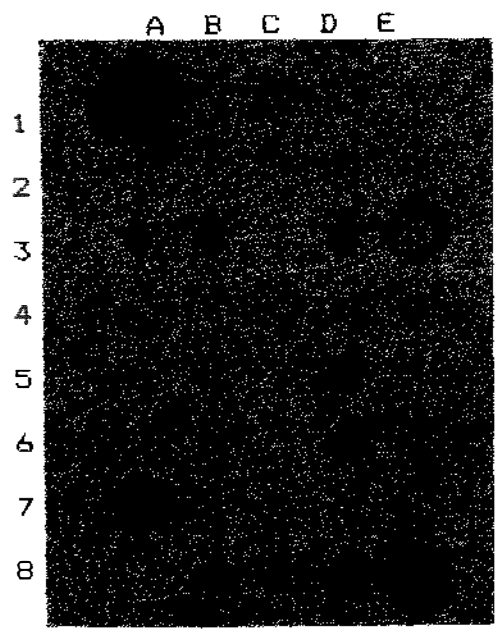


Figure 58: Autoradiograph of clarified viral concentrates (CVC) of nerine leaf tissue hybridized with cloned nerine virus X cDNA [^{32}P]-labelled probe. X-ray film was exposed to the membrane for 48h at -70C with intensifying screens. Spots A1 and E8 are CVC of leaf tissue infected with nerine virus X (positive controls) and A8 and E1 are healthy nerine sap (negative controls). On this membrane spots A3,A7,B3,B8,C1,C4,D1,D3,D5,D6, D8,E2,E3 & E7 were marked as positive. Samples from some of the wells (eg E3 & E7) have leaked due to an imperfect seal in the dot-blot apparatus. The positive samples were also found to have potexvirus-like particles by electron microscopy.

A summary of the results of tests for NeVX is presented in Table 12.

Table 12: Summary of results of tests using the cDNA cloned nerine virus X probe.

Nerine spp.	Source	No. of cultivars	No. of specimens	No. of positive hybridization
N.sarniensis (and hybrids)	P.N.	8	76	13
	N.P.	4	12	2
N.bowdenii (and hybrids)	P.N.	2	26	7
	Auck.	1	16	3
N.fothergilli 'Major'	P.N.	1	6	6
N.corusca 'Major'	Auck.	3	5	1
N.manselli	Auck.	2	6	0
Total		21	147	32

P.N. = Palmerston North N.P.= New Plymouth Auck. = Auckland

While only a small number of samples were processed, the results indicate that NeVX is quite common and infects a number of *Nerine* species grown in different parts of the North Island.

8.3 TESTS FOR NERINE VIRUS Y USING THE CLONED cDNA PROBE

The 1.54kb cDNA cloned NeVY probe was used with clarified viral concentrates in the NeVX tests. Figure 59 shows a typical autoradiograph of dot-blot assays.

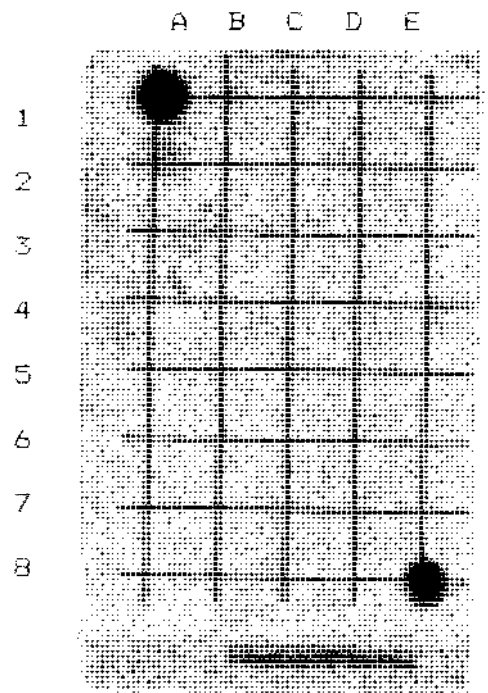


Figure 59: Autoradiograph of clarified viral concentrates (CVC) of nerine leaf tissue hybridized with cloned nerine virus Y cDNA [^{32}P]-labelled probe. X-ray film was exposed to the membrane for 48h at -70C with intensifying screens. Spots A1 and E8 are CVC of leaf tissue infected with nerine virus Y (positive controls) and A8 and E1 are healthy nerine sap (negative controls).

A summary of the results of these tests is presented in Table 13.

Table 13: Summary of results of tests using cDNA cloned nerine virus Y probe.

Nerine spp.	Source	No. of cultivars	No. of specimens	No. of positive hybridizations
N.sarniensis (and hybrids)	P.N.	8	76	9 *
	N.P.	4	12	0
N.bowdenii (and hybrids)	P.N.	2	26	0
	Auck.	1	16	0
N.fothergilli 'Major'	P.N.	1	6	0
N.corusca 'Major'	Auck.	3	5	0
N.manselli	Auck.	2	6	0
Total		21	147	9

P.N.= Palmerston North N.P.= New Plymouth Auck.= Auckland

* all samples were from the same *N.sarniensis* hybrid cultivar mass propagated by twin-scaling.

The results of this survey indicates that NeVY is very uncommon in *Nerine* stocks in New Zealand.

8.4 DISCUSSION

The prevalence of NeVX in all of five *Nerine* species in New Zealand is consistent with the findings reported in the literature (Table 1). Vegetative propagules such as bulbs are well known to be sources of virus(es) and since nerines in New Zealand originated from collections in England or the Netherlands, it is not surprising to find many infected with the same viruses.

Although the potexvirus potato aucuba mosaic virus is transmitted by aphids in the presence of potyviruses (Kassanis & Govier,1971; Mossop,1982), and a potexvirus associated with strawberry mild yellow edge virus is aphid transmitted possibly in the presence of a luteovirus (Jelkman *et al*,1989), most potexviruses lack arthropod vectors (Purcifull & Edwardson,1981).

Because aphids have not been commonly observed on *Nerine* in New Zealand by growers (Hollows, pers comm, 1986), and none were seen on nerines during the course of this study, one can only speculate that the widespread occurrence of NeVX has been brought about by vegetative propagation (including natural bulblet formation, twin-scaling and micropropagation) of infected bulbs and by handling and using contaminated cutting tools during flower stem harvesting and twin-scaling operations.

Nerine virus Y was only detected in one *N.sarniensis* hybrid cultivar. Overseas, an 800nm flexuous filamentous virus has only been found sporadically by electron microscopy. In view of the fact that most potyviruses are efficiently transmitted in a non-persistent manner by aphids, it is to be expected that NeVY is also aphid transmissible. However, the lack of colonization of nerines in New Zealand by aphids suggests that aphid transmission is probably of minor significance. Again, vegetative propagation of NeVY infected stock is the most likely means of spread.

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

The purification, characterization and cDNA cloning of two filamentous viruses of *Nerine* are described in this study. These viruses, nerine virus X (NeVX) and nerine virus Y (NeVY), have hitherto remained uncharacterized primarily because of the inability to mechanically transmit them to herbaceous test plants.

The strategy of conducting an extensive electron microscopic survey in the initial phases of this project concurrently with the mechanical transmission trials led to the discovery of nerine cultivars which were predominantly infected with one class of filamentous virus particles. The potexvirus NeVX was found in an 'old' cultivar of *N.fothergilli* 'Major'. The potyvirus NeVY was detected in a *N.sarniensis* hybrid which had been clonally mass propagated for commercial cut-flower production.

Modifications and refinements to previously used purification procedures played a critical role in optimizing virus yield from limited quantities of field-infected nerine leaf tissue. This, combined with the precautions taken to obtain undegraded, and, for NeVY, size-fractionated viral RNA, made it possible to characterize the viruses and prepare sensitive diagnostic probes.

Due to the lack of mechanical transmissibility of NeVX and NeVY to herbaceous indicators and the limited availability of infected nerine tissue, a diagnostic method was required which did not necessitate the repeated purification of these viruses. The two options available, which met this condition and could be used to produce sensitive and specific diagnostic probes from field-infected material which possibly contained mixed virus infections were monoclonal antibodies and cloned cDNA probes.

Recombinant DNA technology using cloned cDNA probes was selected as the method to be attempted in this project. DNA recombinant technology had been used by a number of researchers for the development of relatively rapid, sensitive and specific diagnostic probes for plant viruses (eg Baulcombe *et al*,1984[a & b]; Linthorst *et al*,1986; Rosner *et al*,1986; Boulton *et al*,1986). Further, large quantities of cloned cDNA can be obtained relatively rapidly.

Although the commonly used plasmid vector pBR322 was successfully used in the cDNA cloning of the 6.3kb NeVX RNA, the use of lambda gt10 and L47AB, prior to subcloning into the plasmid pGEM3, was regarded as an important refinement to increase the cloning efficiency of the 10.0kb NeVY RNA. The use of lambda vectors for initial cDNA cloning of plant viral RNAs is not common, although molecular biologists preparing cDNA libraries for plant RNAs routinely use lambda vectors. Further, the near full-length (9.8kb) cDNA clones produced to NeVY in this study using lambda L47AB were significantly longer than cDNA clones produced to plasmid vectors to other potyviruses such as tobacco vein mottling virus (Domier *et al*,1986), tobacco etch virus (Allison *et al*,1986) and plum pox virus (Maiss *et al*,1989). Full-length cDNA clones would enable researchers to study viral replication, gene expression, identify viral genes involved in specific virus-host plant interactions and hence determine pathogenicity, host range and symptom development (Ahlquist & Janda, 1984; Dawson *et al*, 1986, Meshi *et al*, 1986; Vos *et al*, 1984; Vos, 1987).

The two cloned cDNA probes selected for characterization and development as diagnostic tools for NeVX and NeVY proved to be sensitive and specific to the homologous viruses in hybridization assays. Further, both probes were suitable for use in large-scale detection (surveys) of NeVX and NeVY and for use in virus elimination studies.

Modern molecular cloning techniques have made it possible to develop sensitive, specific and relatively rapid diagnostic methods for two intransigent filamentous viruses from nerines. The strategies used in this project could be adopted for other viruses which have not been found to be readily amenable to mechanical transmission.

Although the NeVX and NeVY cDNA clones were developed primarily as diagnostic tools, they may in the future prove useful for characterization of the genomes of these viruses and for controlling the viruses in a similar manner to that currently being used for viruses of dicotyledonous plants such as tobacco mosaic virus (Abel *et al*,1986).

APPENDIX I

THE GENUS *NERINE*

A.1 BOTANICAL BACKGROUND

A.1.1 Classification

Nerine is a genus of perennial bulbous plant belonging to the Amaryllidaceae family. The Amaryllidaceae is quite a diverse family with some 85 genera and about 1100 species (Hickey & King,1981). There is considerable disagreement as to which genera should be included in the Amaryllidaceae. The current tendency is to include the genus ***Allium*** and its relatives (eg. ***Brodiaea***, ***Agapanthus***) in the Liliaceae because they have a superior ovary although they have an umbellate inflorescence like the Amaryllidaceae (Heywood,1978). Thus, following more recent classifications (Willis,1973;Heywood,1978), the principal diagnostic features of this family include:

- a bulbous rootstock, although some have rhizomes or a bulb with a short rhizome attached to the base
- an umbellate inflorescence subtended by one or more spathes
- 6 free or fused stamens
- leaves which are more or less linear, elongated and strap-like
- an inferior ovary with 3 fused carpels
- a fruit which is either a loculicidally dehiscing capsule or a fleshy berry

Some of the other genera of this family of principally ornamental plants include: ***Narcissus***, ***Hippeastrum***, ***Alstroemeria***, ***Hymenocallis***, ***Crinum***, ***Galanthus***, ***Brunsvigia***, ***Vallota***, ***Lycoris***, ***Zephyranthus***, ***Leucojum***, ***Cyrtanthus***, ***Spiloxene*** and ***Sternbergia***. Although members of the Amaryllidaceae have originated from a range of locations around the world, the 40 or so ***Nerine*** species are all indigenous to southern Africa.

Following modern systems of classification (e.g. Heywood, 1978) nerines can be placed into the following taxonomic groups:

Class:	Angiospermae
Subclass:	Monocotyledoneae
Superorder:	Liliidae
Order:	Liliales
Family:	Amaryllidaceae
Genus:	***Nerine***
Species:	40 different species with a large number of hybrids

A.1.2 Growth Cycles

The growth cycles of the various ***Nerine*** species vary dependant upon their place of origin in the wild state. On the basis of their growth cycle, nerines can be divided into three groups:

(a) Flowering occurs before leaf emergence.
These bulbs are 'dormant' in summer and foliage appears after flowering in late summer. These winter foliage forms are not able to withstand severe frost and have to be grown under glasshouse conditions in Europe and in frost protected situations in New Zealand. Examples of this group are ***N.sarniensis*** and their hybrids, ***N.curvifolia***, ***N.pudica***, and ***N.humuilis***. These species originated from the extreme south of South Africa.

(b) Flowering occurs after leaf emergence.
These bulbs are 'dormant' in winter and the flowers emerge after the foliage in summer. Since these are summer growing the leaves are not likely to be damaged by frost in winter. These are generally hardier species. ***N.bowdenii*** is the best known example of this group. Also in this group are: ***N.angustifolia, N.appendiculata, N.filamentosa, N.filifolia, N.gracilis, N.kirgei, N.masonorum*** and ***N.undulata.*** These species originated from the northern parts of South Africa.

(c) Foliage appears in flushes all year round and flowering is independent of this. The foliage thus appears with the flowers. There is no distinct rest period unless imposed. Examples of this group are ***N.flexuosa,N.manselli***, and a number of hybrids such as ***N.bowdenii*** 'Pink Triumph'. Many of these species originated from the central region of South Africa.

A.1.3 Bulb Structure, Morphology, Development and Propagation

Rees (1972) described three main bulb types - the hippeastrum and the narcissus bulb types (both Family Amaryllidiceae), and the tulip bulb type (Family Liliaceae). The hippeastrum bulb type is composed entirely of leaf bases and the flowers do not emerge from the centre of the leaves. The bases of the old inflorescence stems do not become swollen and store food reserves. In contrast, the tulip bulb type is composed entirely of scales (non-photosynthetic leaf-like organs which are distinct from foliage) and the narcissus bulb type is composed of both scales and leaf bases.

Nerine bulbs are of the hippeastrum bulb type. The bulb is globose at the base with a long thin neck. Growth occurs from the basal plate which is comprised of a group of localised meristematic cells at the centre of the bulb (Figure 60[A]). Organs are initiated in a sympodial branching pattern, each unit of which is composed of four to fifteen leaves, depending on the ***Nerine*** species, and a terminal inflorescence. At the beginning of the growth period the apex at the centre of the bulb initiates leaf buds, 4-15 in ***N.flexuosa*** 'Alba' (Fortanier ***et al***,1979), 10-14 leaf buds in ***N.bowdenii*** (Systema,1975). These leaf buds will emerge as leaves in the following year. The apex then makes a transition to an inflorescence primordia. In ***N.bowdenii*** for example, the time period between floral initiation and anthesis is approximately 24 months. This causes floral emergence to occur in a position lateral to the leaves as the flower bud was initiated in an earlier growth cycle.

Figure 60(B) shows a longitudinal section through a ***N.bowdenii*** bulb. Three growth cycles are evident, the next cycles inflorescence bud just being initiated from the apex and surrounded by unemerged leaves, an outer present cycles inflorescence bud with a number of emerged leaves with fleshy leaf bases and a naked flower stalk from the last flowering season. Since ***N.bowdenii*** and ***N.sarniensis*** initiate their inflorescence buds more than one year before flowering the maximum number of buds in these periodic species is two, while in a non-periodic species such as ***N.flexuosa***, which have a much shorter period between inflorescence bud initiation, it may be four or more (Fortainer ***et al***,1979).

Root systems of mature plants are entirely adventitious, the primary root of the seedling being lost during the first season's growth (Rees,1972). In periodic bulbs roots are initiated and emerge from the basal plate more or less together. This means that damage to the root system at an early stage

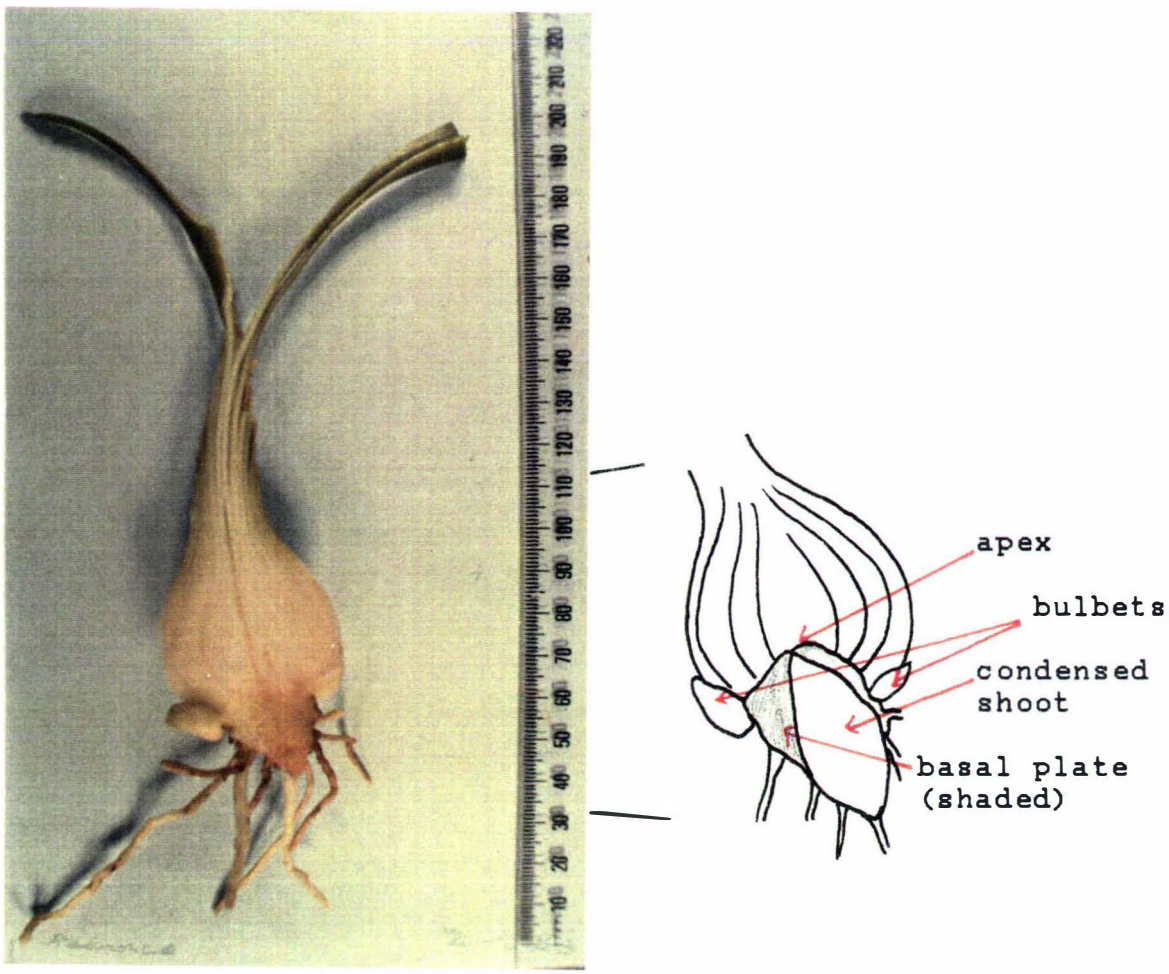


Figure 60 (A): Longitudinal section of nerine bulb

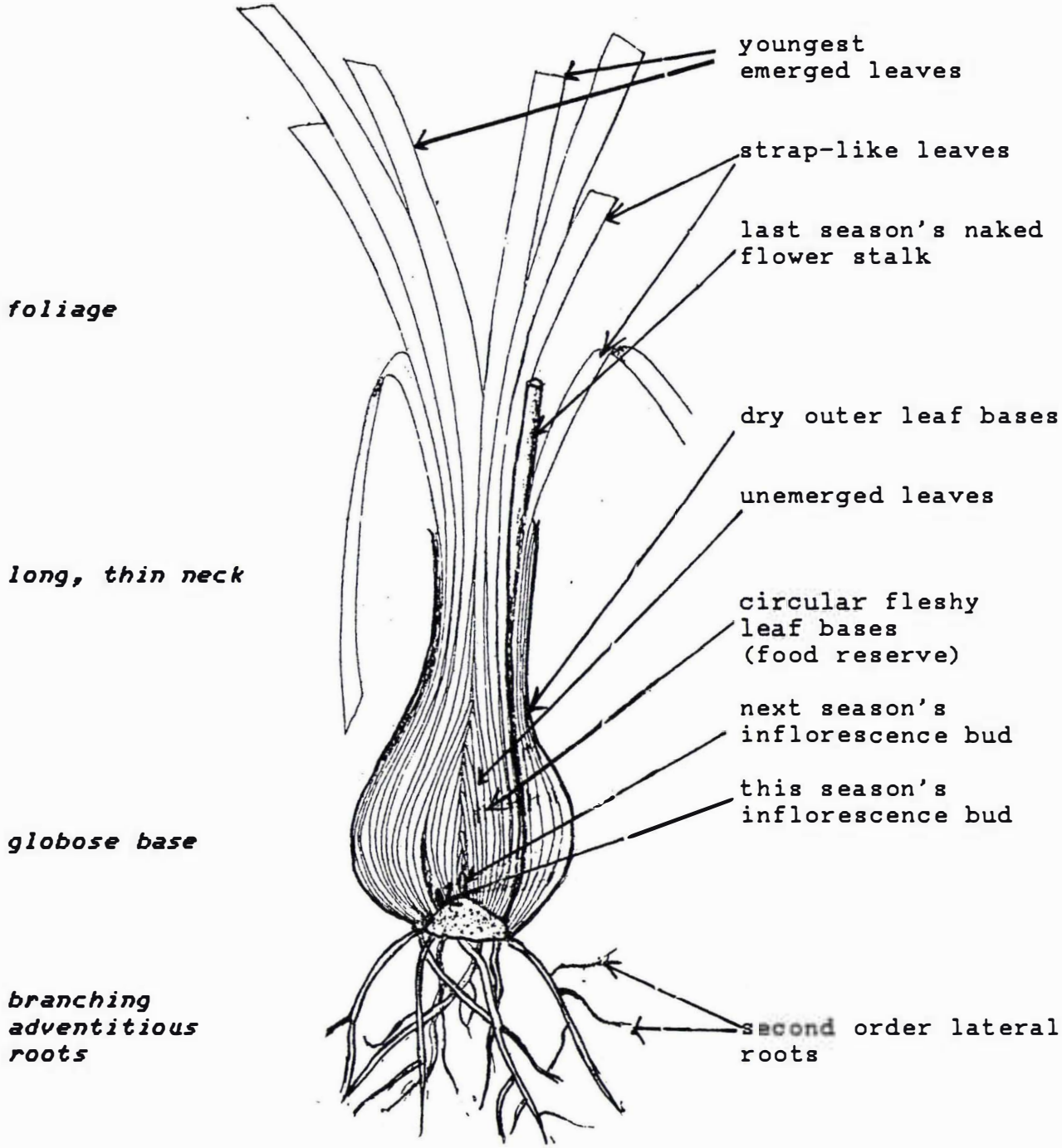


Figure 60(B): Longitudinal section of nerine bulb showing anatomical and morphological features

cannot be compensated for by the formation of new adventitious roots. It is thought that in non-periodic bulbs roots are produced throughout the year. Nerine roots branch and have second-order laterals. Figure 61 shows a typical nerine plant in flower.

Propagation by seeds is generally only practised for breeding purposes. Selected bulbs are multiplied by vegetative means to obtain clonal material which maintain the desirable horticultural characteristics such as flower colour, form and floral stem length. This can be attained by a number of means. Firstly, offsets or bulblets are formed at the base of the circular scales and begin to grow as they reach the outside of the bulb. The number of offsets formed is quite variable, from around five in some ***N.bowdenii*** types to twelve or more in some ***N.sarniensis*** hybrids (see Figure 62). Little is known about offset development while attached to the mother bulb (Pierik & Ippel,1977). Using this method alone, a grower may be faced with a relatively long period for building up stock for commercial production.

The second commonly used method for clonal propagation is the technique of twin-scaling. The nerine bulb is sliced into numerous segments, each with a small part of the meristematic basal plate. About 40 to 60 twin-scales can be obtained from each bulb, and under suitable conditions will develop into a bulb. Figure 63 shows nerine twin-scales.

The third and most modern technique involves using ***in vitro*** methods to attain a higher propagation rate. Excised bulb scale explants (micro twin-scales) have been used to form bulblets (see for example Pierik & Ippel,1977). Flower stem explants from some cultivars have also been successfully propagated by using tissue culture methods. In this case, a callus develops from the cut surfaces of the explants and morphogenesis occurs resulting in shoot production (Pierik & Steegmans,1986). The shoots can be separated and subcultured to develop into rooted bulblets. Figure 64 shows nerine bulblets, derived from floral stem explants, in culture. Although tissue culture procedures are labour-intensive and therefore more expensive, the rate of propagation is much higher than by any of the other methods (Hussey,1984).

Figure 61: Typical nerine plant in flower

Figure 62: Nerine 'mother-bulb' with bulblets (*Nerine sarniensis* hybrid)

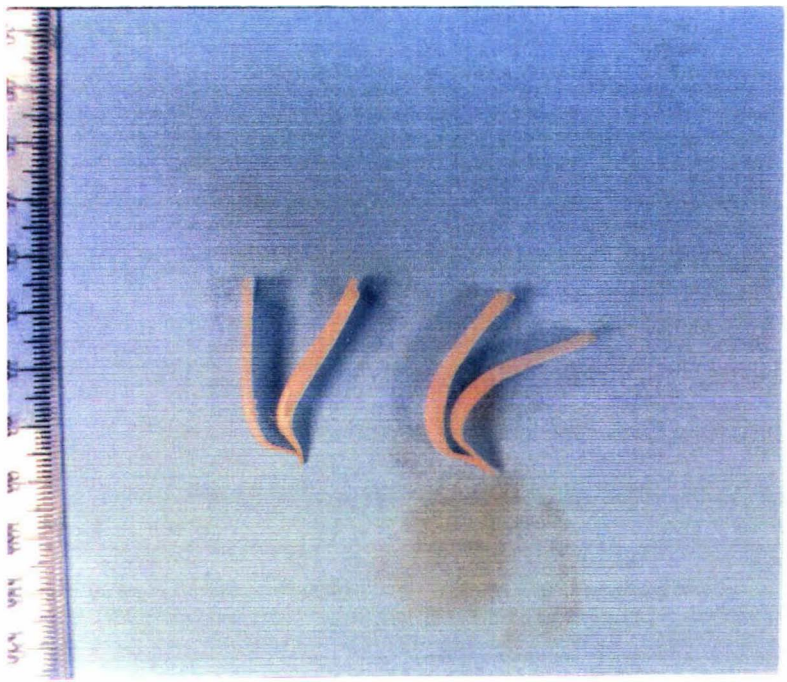


Figure 63: Twin-scales of a ***Nerine sarniensis*** hybrid bulb

Figure 64: ***Nerine sarniensis*** hybrid cultivar in culture, derived from floral stem explants

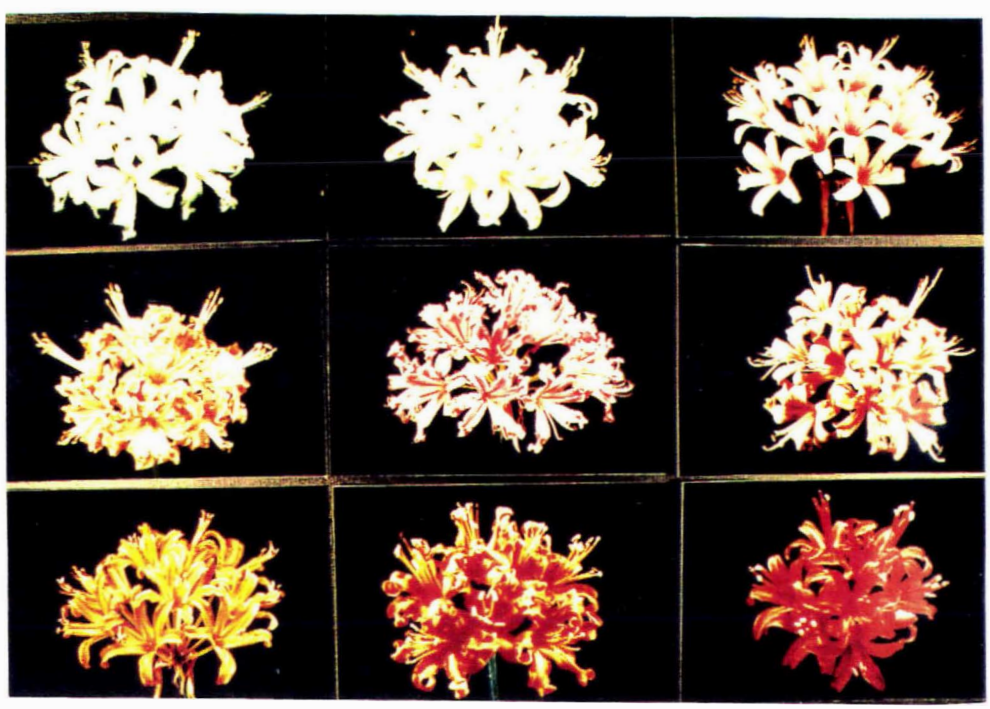


Figure 65: Range of flower colours of modern *Nerine sarniensis* hybrids

A.2 HISTORICAL REVIEW

There is a great deal of myth and mystery about the early history of nerines in Britain. An historical study by Mrs Mary Baxter (Baxter,1986) of Lancashire, England, reveals the following likely sequence. Nerines are believed to have been introduced into Britain in the early-1620s. It is recorded that John Tradescant of Lambeth, London, obtained some ***N.sarniensis*** bulbs from the English Chartered Companies which operated in foreign parts. In the mid-1625's nerines were introduced into France from England and the first recorded flowering of ***N.sarniensis*** was on October 7th 1634 in the garden of Rene Morin of Paris.

In about 1680 Nerine bulbs were part of a cargo of a sailing ship bound from South Africa to Britain. The ship was stranded near the Channel Islands and the bulbs were taken ashore to Guernsey (Latin~Sarnia). It is reported that six bulbs were given to and raised by Mr de Sausmarez. By the end of the 17th century ***Nerine sarniensis*** was well established in Guernsey and the bulbs were exported back to England. Because Guernsey was at that time the sole supplier, the term 'Guernsey Lily' was used to refer to it. Descendants of the de Sausmarez family are still the islands largest exporters of bulbs and flowers from Guernsey (Baxter,1986).

The first book devoted to Guernsey Lily called "Lilium sarniense" was published by Dr J. Douglas in the 1720's. In the 1730's Linneaus classified it as ***Amaryllis sarniensis***. In the mid-1800s the English botanist Dr William Herbert, when classifying members of the Amaryllidaceae family, named it ***Nerine*** after Nereis, daughter of the sea nymph Nereus - a form of floral gift to Guernsey from the sea! The species was named ***N.sarniensis***. It is still commonly referred to as 'Guernsey Lily'. The other commercially important nerine species, ***N.bowdenii***, was sent by Mr Athelstan Cornish Bowden, Surveyor-General of the Cape, to his mother in England in 1900.

Many of the ***Nerine*** species in their natural habitat in South Africa were observed to be relatively small xerophytic plants with a limited colour range, most being pink (Norris 1975,1982). A few had white (e.g. ***N.flexuosa*** 'Alba'), or deeper coloured forms such as bright red (e.g. ***N.appendiculata***). It was through the hybridisation efforts of a number of breeders, mainly in Britain, that the range in forms evident today came into existence. Gallagher(1966) notes that Dr William Herbert in the middle of the last century crossed different species of ***Nerine*** in order to obtain information for his treatise Amaryllidaceae.

Herbert's work was continued by Mr H.J. Elwes who, at the turn of the century, bred and named many ***Nerine*** hybrids which are still in cultivation (Gallagher,1966). These early hybridisation efforts were continued by numerous other enthusiasts such as Sir Ralph Stephenson-Clarke, Sir Frederick Stern and Mr Lionel de Rothschild. Mr Lionel de Rothschild of Exbury, near Southampton, is reported to have had the most extensive and systematic ***Nerine*** breeding programme in the early to mid-1900s. From some 145 recorded crosses, using ***N.sarniensis*** as the primary parent in most of them, he was reported to have obtained a remarkable range of flower colours (Gallagher,1966; Langdon,1976). Many of the commercially important ***N.sarniensis*** hybrids in various parts of the world today, including New Zealand, had their origins in what is commonly referred to as the 'Exbury Collection'.

Up to about the last two decades, ***Nerine*** cultivation and breeding has remained within the hands of a relatively small number of enthusiasts or connoisseurs. Early attempts to exploit its commercial potential by the English firm, Blackmore and Langdon, when they purchased the bulk of the comprehensive Exbury collection of about 2300 bulbs in all, including about 1,000 un-named and unflowered seedlings (Langdon,1976), were not sustained and was subsequently sold to Mr Ambrose Congreve of Ireland to form part of a private collection (Smithers,1984).

There were a number of problems that confronted the early growers in the large scale production of this novel crop. A slow multiplication rate, irregular flowering of some of the varieties, non-hardiness of the more interesting ***N.sarnienis*** hybrids requiring glasshouse conditions to survive the frosts in Europe, lack of information on optimal cultivation conditions, diseases and an uncertain market demand were all possible contributing factors. The three primary factors which, in the last two decades, have had a significant impact on exploiting the potential for the commercial cultivation of nerines can be outlined as follows:

(a) Expeditions to South Africa in 1971, 1976 and 1978 by Mr C A Norris of England (Norris,1982) to collect new species and to study the cultural conditions of nerines in their natural habitat. By broadening the genetic base for ***Nerine*** hybridisation, modern breeders like Norris, Smee (1984) and others have been able to improve on the quality of the ***Nerine*** hybrids, especially of the ***N.sarniensis*** and the ***N.bowdenii*** types. ***N.sarniensis*** hybrids have been developed to produce flowers from white across the various shades of pink, orange and red through to purple. The most recent and commercially interesting development is the success achieved with the more hardy ***N.bowdenii*** hybrids - extending the colour range to reds,

salmon, white and lavender (Norris,1982). An increase in the size of the floral stems, flowerheads and hardiness of the bulbs has also been achieved. Thus, nerines of today possess some of the finest qualities of flowering ornamentals in terms of flower form, shape, symmetry and range of colours. Figure 65 illustrates some the ***N.sarniensis*** hybrids cultivated today for commercial cut-flower production.

(b) The development of two new methods for bulb multiplication. The techniques of twin-scaling and micropropagation have made a significant impact in the rapid multiplication of the bulbs for commercial operations.

(c) Research on some aspects of flowering, bulb growth and disease control, especially in the Netherlands. Scientists based at the Agricultural University in Wageningen and the Research Station for Floriculture at Aalsmeer, through their extensive research programme initiated in the late 1960's, have made a major contribution to the commercial cultivation, especially of ***N.bowdenii*** cultivars, in the Netherlands.

A.3 COMMERCIAL CULTIVATION OF NERINES

The Netherlands is presently the world's largest producer of nerines. The cultivation of nerines for cut flower production in the Netherlands had its beginnings in the 1960's. Between 1968 and 1980 there was an 8-fold increase in the sale of nerine flowers in Dutch auctions, amounting to some 7 million guilders in 1980 (FCIB,1982). The flowers were mainly exported to West Wermany, France, Italy, Switzerland, Austria and Belgium. More recently there has been an increasing demand for nerine cut flowers from the United States and Japan (WPI,1987). Nerine flowers have been mainly used in floral arrangements and in bouquets (FCIB,1982).

Although in 1984 about 96% of the Dutch nerine flower sales were of the hardy pink flowered ***N.bowdenii*** (WFTM,1984), several other varieties are grown in the Netherlands including the following: ***N.undulata*** ***(N.crispa)***, ***N.sarniensis*** 'Corusca Major', ***N.flexuosa*** 'Alba', ***N.bowdenii*** 'Pink Triumph' and ***N.manselli*** (WPI,1987).

Dutch exports of ***Nerine*** bulbs grew from 733,000 in the 1979-80 season to 1.8 million in the 1983-84 season. The bulbs were exported to a wide range of countries including the United States, Columbia, Peru, Portugal and South Africa (WFTM,1984). In 1986 New Zealand imported about 800,000 ***N.bowdenii*** bulbs

from the Netherlands.

Exact figures on the cultivation of nerines on a world-wide scale are difficult to obtain. The approximate areas under nerine cultivation in various parts of the world, obtained from several sources, are presented in Table 14.

Table 14: Approximate areas of nerines in cultivation in several countries.

Country	Area under cultivation (hectares)	Source
Netherlands	60	WPI, 1987
Western Europe	6	WFTM, 1985
Peru	4.5	"
Portugal	3	"
Columbia	3	"
South Africa	3	"
New Zealand	3	Hollows *
United States	0.5	"
Japan	0.5	"
Australia	1	"

* pers comm, 1987

Although nerines are a relatively new commercial ornamental and presently occupy a modest place in the range of commercially cultivated bulbous and tuberous ornamental crops such as tulips, daffodils and lilies, the statistics do indicate an international interest in this crop.

A.4 NERINE CULTIVATION IN NEW ZEALAND

Although the red flowered ***N.fothergillii*** 'Major',the pink ***N.bowdenii*** and the white ***N.flexuosa*** 'Alba' are found in New Zealand home gardens, it is not known when they were first introduced into New Zealand. The earliest ***N.sarniensis*** hybrids are believed to have been first imported into New Zealand by Coopers of Lower Hutt (near Wellington) in the 1920s, from Barrs Nursery in England. These were sold to local enthusiasts and some, like Mr H.J. Poole of Wellington, had a collection of these hybrids in clay pots and displayed them at flower shows around Wellington in the 1940s (Hollows, pers comm, 1987).

In the mid-1950s about 50 varieties of ***N.sarniensis*** hybrids of various colours were purchased from the Exbury collection by R.E. Harrison of Harrison's Nurseries in Palmerston North and kept in pots under glasshouse conditions. One of these was the very fine hyper-triploid (2n=36;x=11) 'Alice'. 'Alice', which is now lost to cultivation, was a cross between 'Aurora' (derived from ***N.bowdenii x N.fothergill***)) and ***N.flexuosa*** 'Alba'. Hart, W working for Harrison, carried out many crosses between selected Exbury lines. 'Alice', which had pale lilac flowers under glasshouse conditions, was selected for many of the crosses (Hart, pers comm, 1987).

In about 1958, about 100 seedlings from these crosses were planted out in the open ground, usually under partial tree canopy cover. Most of the seedlings survived the somewhat mild Palmerston North winters. Selective breeding from the Harrison collection, planted in open ground, was continued by Hart and by 1966 there were 250-300 varieties. Many of these New Zealand bred varieties are reputed to be much hardier and more vigorous than those of English breeding (Smithers,1984). Because no records were kept of the many crosses that were carried out, the lineage of the Harrison collection is not known. In the late 1960s about 50 of these varieties were exported to a number of countries, including the Netherlands, England, Austria, U.S.A., Australia, Japan, Switzerland and South Africa. Breeding was continued on a limited scale, by Hart for Harrison, until 1981.

Much of the Harrison collection is now owned by Nerine Nurseries Ltd of Palmerston North. Maurice (Monty) Hollows is the Managing Director and the major shareholder of this company. Hollows was previously a large scale chrysanthemum grower who started purchasing nerine bulbs from the Harrison collection in 1975. By 1980 he had a few thousand nerine bulbs of about 200 varieties from this collection. Hollows, through an intensive

bulb multiplication effort, mainly by twin-scaling, amassed a collection of more than a million ***N.sarniensis*** hybrid bulbs by 1986. To further expand his nerine operations and to fully exploit the commercial potential of this crop, Nerine Nurseries Ltd was formed in 1986 with venture capital from the Development Finance Corporation of New Zealand. In 1987, this company purchased the entire elite ***N.sarniensis*** hybrid and ***Nerine bowdenii*** hybrid stock from the best known contemporary nerine breeder and enthusiast, C.A. Norris of Welland, Worcestershire, England.

A survey of Nerine Nurseries Ltd stock reveals more than 1500 varieties. Although the vast majority of these are ***N.sarniensis*** hybrids, the Hollow's collection now includes 33 species. Many of these species and their hybrids are currently being evaluated for their commercial cut-flower potential. The Hollow's collection is now believed to be the most extensive one in existence, with a continuing effort being made to breed new varieties. The estimated number of bulbs in early 1989 is in excess of five million. The bulbs are planted under plastic shade house conditions (Figure 66) on a one hectare site, with potential for further expansion.

The second largest operation, Manakau Horticulture, is based in Auckland and owned by Agri-Systems Ltd. They have close to one million bulbs, 95% of which are ***N.bowdenii*** purchased from Holland in 1986. A summary of the main growers of nerines in New Zealand and the approximate size of each operation, as of January 1987, is given in Table 15.

Figure 66: Nerine Nurseries Ltd, Palmerston North

Table 15: Major nerine growers in New Zealand, their location, size of operation and species grown.

Name of Company or Individual	Location	Approximate number of flowering bulbs	Main Nerine species
Nerine Nurseries	Palmerston North	1,000,000 +	*N. sarniensis*
Manakau Hort	Auckland	1,000,000	*N. bowdenii*
Ensor	Blenheim	215,000	*N. bowdenii*
Sherman	Auckland	70,000	*N. bowdenii*
Lott	Blenheim	48,000	*N. bowdenii*
Hatch	Auckland	30,000	*N. sarniensis*
Abraham	Levin	30,000	*N. bowdenii*
Boyes	Blenheim	25,000	*N. bowdenii*
Roberts	New Plymouth	20,000	*N. sarniensis*
Cato	Te Kuiti	20,000	*N. bowdenii*
Harrison	Palmerston North	5,000	*N. sarniensis*

Nerine cultivation in New Zealand is thus at the initial growth phase of what could develop into a significant ornamental industry. Coupled with an anticipated dramatic increase in nerine cut flower and bulb production is the requirement for research efforts towards an increase in quality, yield and pathogen control.

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