

**CHARACTERIZATION OF A TYMOVIRUS CAUSING DISEASE IN
DIASCIA ORNAMENTAL PLANTS**

By

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A dissertation submitted in partial fulfillment of
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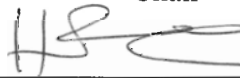
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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of AMOGELANG THETHE SEGWAGWE find it satisfactory and recommend that it be accepted.



Chair



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CHARACTERIZATION OF A TYMOVIRUS CAUSING DISEASE IN DIASCIA ORNAMENTAL PLANTS

Abstract

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Two tymoviruses were isolated from *Diascia ×hybrida* ‘Sun chimes™ coral’ plants exhibiting chlorotic mottling of the leaves and reduced growth. The nucleotide sequence of the entire genome from one isolate was determined. The genome is 6,290 nucleotides long and contains three potential open reading frames (ORFs). The 5'-untranslated region is 154 nucleotides long and RNA folding into four hairpin structures is predicted in this region of the genome. The putative products of ORF I, II and III are 637, 1,790, and 192 amino acids, respectively. Based on homology to other tymovirus sequences, it is predicted that ORF I encodes the movement protein, ORF II the replicase and ORF III the coat protein. Secondary structure analysis of the 3'-terminus show that the RNA can form a transfer-like RNA structure that has an anticodon specific for histidine. The name *Diascia yellow mottle virus* (DiaYMV) is proposed for this novel virus. Based on the coat protein sequence analysis, it was revealed that the plants are also infected by a strain of the *Nemesia ring necrosis virus* (NeRNV). The isolate of NeRNV from Washington (NeRNV-WA) and DiaYMV share 99.5% and 94.5% amino acid sequence identity to the coat protein of NeRNV from *Nemesia fruticans* (NeRNV-Nf). The product of ORF II from DiaYMV shares 54.0% amino acid sequence identity with ORF II of NeRNV-Nf. When the entire genomic nucleotide sequences are compared, 77.8% identity to NeRNV-Nf is revealed.

DiaYMV could not be distinguished from NeRNV by serology using a murine monoclonal antibody M3-7-A6-1 developed in this study. DiaYMV and NeRNV commonly occur in the Pacific Northwest in diascia and nemesia ornamental plants with the exception of *Diascia ×hybrida* 'Coral belle'. DiaYMV host range includes many representatives within the family Scrophulariaceae and Solanaceae with the exceptions of *Datura stramonium*, *Chenopodium quinoa*, some members of *Nicotiana tabacum* and members of the genus *Antirrhinum*. A reverse transcription-polymerase chain reaction and enzyme linked immunosorbent assay based diagnostic assays were developed for rapid detection of DiaYMV and NeRNV in nurseries. The developed diagnostic assays, however, did not discriminate between the two viruses.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	iii
ABSTRACT.....	iv
LIST OF TABLES.....	viii
LIST OF FIGURES.....	x
DEDICATION.....	xiii
CHAPTER 1	
CHARACTERIZATION OF A TYMOVIRUS CAUSING DISEASE IN DIASCIA ORNAMENTAL PLANTS	
GENERAL INTRODUCTION.....	1
REFERENCES.....	7
CHAPTER 2	
MOLECULAR CHARACTERIZATION OF A TYMOVIRUS CAUSING DISEASE IN DIASCIA ORNAMENTAL PLANTS	
SUMMARY.....	11
INTRODUCTION.....	12
MATERIALS AND METHODS.....	13
RESULTS AND DISCUSSION.....	27
CONCLUSION.....	61
REFERENCES.....	62
CHAPTER 3	
MONOCLONAL ANTIBODY PRODUCTION AND SEROLOGICAL DIFFERENTIATION OF THE DIASCIA YELLOW MOTTLE VIRUS	
SUMMARY.....	67
INTRODUCTION.....	67
MATERIALS AND METHODS.....	69
RESULTS.....	78
DISCUSSION.....	84
CONCLUSION.....	89

REFERENCES	90
CHAPTER 4	
HOST RANGE STUDIES AND SYMPTOMATOLOGY OF DIASCIA YELLOW MOTTLE VIRUS	
SUMMARY	93
INTRODUCTION	94
MATERIALS AND METHODS	95
RESULTS	102
DISCUSSION	126
CONCLUSION	129
REFERENCES	131
CHAPTER 5	
GENERAL CONCLUSIONS	134

LIST OF TABLES

TABLE	PAGE
1. List of names, acronyms and accession numbers of viruses used in the multiple sequence alignment and phylogenetic comparisons of tymoviruses	29
2. Percentage identities of the amino acid sequences of the open reading frames (ORF) I, ORF II and ORF III of selected tymoviruses of the <i>Ononis yellow mosaic virus</i> cluster.....	41
3. Detection of immunoglobulin heavy and light chains in hybridoma culture supernate M3-7·A6·1	79
4. Botanical names and common names of plants used in the study	96
5. Reverse transcription-polymerase chain reaction (RT-PCR) and double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) on diascia and nemesia species purchased from nurseries in Washington and Oregon	103
6. <i>Impatiens necrotic spot virus</i> (INSV) detection and symptom expression on plants kept in the greenhouse Prosser-Washington State (Spring, 2005).....	108
7. <i>Impatiens necrotic spot virus</i> (INSV) detection in plants purchased from nurseries in the States of Washington and Oregon (Spring, 2006)	110
8. Symptom expression and detection of <i>Diascia yellow mottle virus</i> on leaves by triple antibody sandwich-enzyme linked immunosorbent assay (TAS-ELISA) on nemesia and diascia plants following mechanically inoculation with <i>DiaYMV</i>	112
9. Detection of <i>Diascia yellow mottle virus</i> (<i>DiaYMV</i>) by triple antibody sandwich-enzyme linked immunosorbent assay in leaf samples of various cultivars of the genus <i>Antirrhinum</i>	113

10. Symptom expression and virus detection by triple antibody sandwich-enzyme linked immunosorbent assay (TAS-ELISA) on leaves of various <i>Nicotiana</i> plants inoculated with Diascia yellow mottle virus (DiaYMV).....	118
11. Diascia yellow mottle virus (DiaYMV) detection by triple antibody sandwich-enzyme linked immunosorbent assay (TAS-ELISA) on leaves of different plants following mechanical inoculation with DiaYMV.....	120
12. Mean absorbance values for triple antibody sandwich-enzyme linked immunosorbent assay (TAS-ELISA) on Diascia yellow mottle virus (DiaYMV) inoculated leaves of various plants	121
13. Temperature effect on healthy and Diascia yellow mottle virus (DiaYMV) infected <i>Diascia barberae</i> ‘Apricot queen’ plants growing in temperature controlled growth chambers.....	124

LIST OF FIGURES

FIGURE	PAGE
1. Gel analysis of double strand RNA (dsRNA) extracted from healthy and virus infected plant tissues.....	28
2. Virus profile from infected <i>Diascia ×hybrida</i> ‘Sun chimes™ coral’ following linear log density gradient centrifugation.....	30
3. Locations of cDNA clones used in the sequencing of the entire genome of <i>Diascia</i> tymovirus of the <i>Diascia</i> yellow mottle virus (DiaYMV).....	32
4. Nucleotide sequence of the deduced genome organization of <i>Diascia</i> yellow mottle virus (DiaYMV) isolated from <i>Diascia ×hybrida</i> ‘Sun chimes™ coral’.....	33
5. Deduced genome organization of <i>Diascia</i> yellow mottle virus (DiaYMV) showing locations of the 5’-untranslated region (UTR), open reading frames (ORF) I, ORF II, ORF III and 3’-untranslated region (UTR) in reference to the entire genome.....	40
6. Predicted secondary structures of the 5’-terminus of <i>Diascia</i> yellow mottle virus (DiaYMV) genomic RNA.....	43
7. Cluster dendograms based on the amino acid similarity of the movement proteins of <i>Diascia</i> yellow mottle virus (DiaYMV) and other tymoviruses from GenBank.....	47
8. Cluster dendograms based on the amino acid similarity of the replicase proteins of <i>Diascia</i> yellow mottle virus (DiaYMV) and other tymoviruses from GenBank.....	49
9. Cluster dendograms based on the amino acid similarity of the coat proteins of <i>Diascia</i> yellow mottle virus (DiaYMV) and other tymoviruses.....	50

10. Nucleotide sequence alignment of open reading frame (ORF) III and the 3'-untranslated region (UTR) of <i>Nemesia ring necrosis virus</i> (NeRNV) and Diascia yellow mottle virus (DiaYMV) isolates.....	52
11. Amino acid sequence alignment of the ORF III of <i>Nemesia ring necrosis virus</i> (NeRNV) and Diascia yellow mottle virus (DiaYMV) isolates	54
12. Secondary structure model of the 3'-terminus of RNA from tymoviruses found in members of the Scrophulariaceae	55
13. The effect of antibody concentration and sample dilution on the mean absorbance values (A_{405}) of Diascia yellow mottle virus (DiaYMV) in a plate trapped antigen-enzyme linked immunosorbent assay (PTA-ELISA) using virus infected <i>Nicotiana occidentalis</i>	81
14. Time course of the plate trapped antigen-enzyme linked immunosorbent assay (PTA-ELISA) with monoclonal antibody M3-7·A6·1	82
15. Comparison between double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) and triple antibody sandwich-enzyme linked immunosorbent assay (TAS-ELISA) on the virus detection in different host plants	83
16. The effect of sample dilution on the mean absorbance value of triple antibody sandwich-enzyme linked immunosorbent assay (TAS-ELISA) results using monoclonal antibody M3-7·A6·1	85
17. Western blot analysis using purified monoclonal antibody M3-7·A6·1 to detect protein in purified immunoglobulin and Diascia yellow mottle virus (DiaYMV) in infected plant tissue extracts	86

18. Electron micrograph of tymovirus infected <i>Diascia ×hybrida</i> ‘Sun chimes™ coral’ showing the distribution of double membrane vesicles in the cytoplasm	104
19. Symptoms on <i>Diascia ×hybrida</i> ‘Sun chimes™ coral’ infected with <i>Diascia</i> yellow mottle virus (DiaYMV) and <i>Impatiens necrotic spot virus</i> (INSV)	107
20. Virus induced symptoms on <i>Nicotiana occidentalis</i> observed one week after inoculation with <i>Diascia</i> yellow mottle virus (DiaYMV).....	114
21. Virus induced symptoms on <i>Diascia barberae</i> ‘Apricot queen’ and <i>Nicotiana benthamiana</i> one week after inoculation with <i>Diascia</i> yellow mottle virus (DiaYMV).....	115
22. Plants showing symptoms on <i>Nicotiana</i> plants following mechanical inoculation with <i>Diascia</i> yellow mottle virus (DiaYMV).....	116
23. Effect of sample dilution on the mean absorbance values (A_{405}) of <i>Diascia</i> yellow mottle virus (DiaYMV) using monoclonal antibody M3-7·A6·1 at 1/200 dilution.....	119
24. Absorbance values of plate trapped antigen-enzyme linked immunosorbent assay (PTA-ELISA) of <i>diascia</i> plants over time after the addition of substrate in a PTA-ELISA.	123
25. Effect of temperature on growth and symptom expression on healthy and <i>Diascia</i> yellow mottle virus (DiaYMV) inoculated <i>Diascia barberae</i> ‘Apricot queen’ seedlings growing in a 15°C growth chamber	125

Dedication

To my son Tefo, my mother and all the women in Botswana who are trying to make it in Science.

CHAPTER 1

GENERAL INTRODUCTION

Plants in the genera *Diascia* and *Nemesia* belong to the snapdragon family Scrophulariaceae. *Diascia* are native to the southern and eastern parts of Africa, and many are native to the Drakensburg Mountains of South Africa and Lesotho (Batten, 1986; Benham, 1987; Garbutt, 1994). *Diascia* is a desirable ornamental plant because it provides a range of colors from “scarlet through salmon and coral into pink” (Schoellhorn, 2003) and possess larger, open canopies and larger flowers relative to *nemesia*. It is believed that they were introduced to Europe in the 1800s (Grey-Wilson, 1986; Boulby, 2001) and were bred and modified to suite different growing conditions of the world. There are several major species of *diascia* currently available for commercial use as well as many hybrids. Some of the currently popular species of *diascia* include *Diascia barberae*, *D. cordata*, *D. integerrima* and *D. rigescens* Benth. (Batten, 1986; Grey-Wilson, 1986; Garbutt, 1994), however there seems to be confusion of naming species (Boulby, 2001). *Diascia* species are characterized very generally as disease resistant, but of the few reports of disease problems in this genera and the most commonly cited diseases include *impatiens* necrotic spot, *arabis* mosaic, *nemesia* ring spot (APS/APHIS Virology working group, 2005) and a fungal disease caused by *Botrytis cinerea* (Boulby, 2001).

The ornamental industry in the USA is rapidly increasing and nursery crops have been one of the fastest growing sectors with horticultural sales estimated at \$45 billion. Floral and nursery crops contributed about a \$15 billion in 2003 (Hammond and Dodds, 2005). Floriculture crop sales increased to \$5.4 billion in 2005 (Jerardo, 2006). The 2006 ornamental crop sales were projected to reach \$16.5 billion with \$100 million from

floriculture and \$200 million from nursery crops. In Washington State, annual gross income contributed by potted flowering plants and bedding plants was \$69 million in 1999 (Copes, 2000). The USA imports 14.1% of its floriculture consumption, mainly in the form of unrooted cuttings and slips of plants (Jerardo, 2005).

The price of ornamental plants is directly related to their physical appearance and disease problems in nurseries or flowering plant production systems cause significant revenue losses due to failure to meet market standards (Copes, 2000). Virus-induced diseases of greenhouse grown floral crops result in substantial economic losses to growers each year, and it has been shown that a variety of viruses that infect floral crops are very often moved from plant to plant by insects (Nameth, 2006). Symptoms associated with floral crop viruses vary substantially with the virus and the host that is affected. Symptoms usually include, but are not limited to, general yellowing, stunting, wilting, mosaics, necrotic spots, ringspots, leaf blistering and deformation. These generic symptoms complicate diagnosis as they could be associated with other floral crop pathogens such as fungi or physiological disorders. Lack of clinically verified diagnostics procedures complicates survey information (Copes, 2000). The USA imports planting material from all over the world, and while there are mechanisms in place to test incoming material for diseases, this is challenging when the host plants do not show any symptoms and when diseases that are not listed for quarantine inspection are extant. This in turn poses danger and risk for disease spread between different crops, countries and States.

The potential for new virus diseases increases as different crops are introduced and grown in new areas, hence methods for reliable and rapid detection and identification of these viral agents are essential to facilitate production of virus free plants. At least 125

different viruses have been identified that infect and cause disease in ornamental plants (Hammond and Lewanodowski, 2005). There are few sources of virus resistance in the available germplasm of ornamental crops even though virus diseases are a major production constraint in the industry. It is possible to generate certain plant species free of some viruses by propagation through seed, but this option is limited to a few crops. The majority of the ornamental plants are hybrids, self-incompatible and vegetatively propagated. Therefore, the use virus-free stock and effective methods to control vectors are essential to limit the spread of virus diseases for these plants and to other potential crop plants. It is common for virus-free plants to become infected in commercial settings mainly due to vectors and mechanical transmission from infected tools. It is important to study and understand mechanisms of infection and transmission for new viruses so that effective control measures can be instituted to reduce the spread of diseases and revenue loss.

Diascia and nemesia plants obtained from nurseries were found to display virus-like symptoms which included a mild mosaic and vein yellowing. At the time the plants were first suspected of disease problems, little was known about the pathogens that affect diascia and nemesia ornamental plants. It soon became evident that the symptoms were a result of a virus infection and efforts were then made to identify and characterize the virus. Proper diagnosis is important to preserve the aesthetic value of these plants. In 2005, there were reports published of a tymovirus that causes diseases in diascia, nemesia and verbena ornamental plants in Europe (Skelton *et al.*, 2004; Barends *et al.*, 2005) and North America (Mathews and Dodds, 2006). This appears to be a recent infection and there is little information available on the causal agent, so it is difficult to ascertain the impact of the disease. The increased studies in virus diseases of ornamental crops in the USA will

eventually increase disease awareness due to viruses, and this trend is already gaining popularity as shown by the number of USDA-ARS projects that are focusing on ornamental crops (USDA-ARS, 2006). Some of the pathogens studied in nemesia and diascia include tymoviruses, tobamoviruses, carmoviruses, potyviruses and tospoviruses. Recent identification of *Nemesia ring necrosis virus* (NeRNV) in diascia, nemesia and verbena is evidence of the increased importance of the study of new and emerging viral diseases of nursery crops (Mathews and Dodds, 2006). Since partial genomic sequences are available for both the European and North American isolates of the tymovirus(es) infecting diascia and nemesia (Koenig *et al.*, 2005; Mathews and Dodds, 2006), the disease may soon receive more attention as there may be rapid assays for detection.

The available detection assay systems available for the tymovirus infecting diascia and nemesia ornamental plants include the use of a double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) that also detects *Scrophularia mottle virus* (ScrMV) and NeRNV. ScrMV, *Ribgrass mosaic virus* and *Impatiens necrotic spot virus* have been reported on diascia and nemesia plants (APS/APHIS Virology working group, 2005). Nemesia ring necrosis is a newly reported disease caused by NeRNV that belongs to the family *Tymoviridae*, genus *Tymovirus* and it has been found to cause disease in susceptible *Nemesia fruticans* Benth. and some diascia hybrids. NeRNV was first characterized in 2005 (Koenig *et al.*, 2005). NeRNV is a virus with isometric tymovirus-like particles which has been found to be widely spread in various ornamental plant species belonging to the Scrophulariaceae and Verbenaceae (Barends *et al.*, 2005). Symptoms displayed on *Diascia ×hybrida* ‘Sun chimes™ coral’ often resemble those associated with infections by viruses within the genus *Tymovirus*. The type species of the tymovirus group is *Turnip yellow*

mosaic virus (TYMV), and it is among the first discovered of the spherical viruses and perhaps the most thoroughly studied by physico-chemical methods (Hirth and Givord, 1988). Tymoviruses typically infect a narrow range of host plants (Gibbs, 1994; Larson *et al.*, 2000), and geographical distribution of individual species varies from restricted to widespread (Martelli *et al.*, 2002). Purified preparations of most members of the genus *Tymovirus* contain two components when analyzed by sucrose density centrifugation (Scott and Moore, 1972; Bercks, 1973), consisting of the bottom component of complete virions and the top component of empty capsids (Gibbs, 1994). Transmission electron micrographs of tymoviruses often show numerous nucleoprotein particles and empty capsids 25 to 28 nm in diameter, thin sections from infected tissues show chloroplasts that developed small peripheral vesicles bounded by double membranes (Bercks, 1973; Lesemann, 1977; Koenig and Lesemann, 1979; Rana *et al.*, 1988). Tymoviruses are transmissible by mechanical inoculation and in vegetatively propagated crops. Virus dissemination is primarily via infected propagating material (Martelli *et al.*, 2002). The majority of tymovirus virions are acquired from the parenchyma cells by coleoptera (beetles) in the families of Chrysomelidae and Curculionidae, which then transmit the virus in a semi-persistent manner.

Classification of NeRNV was based on molecular data and sequence comparisons that showed this virus was a new species in the genus *Tymovirus*. Nemesia ring necrosis disease has been reported in ornamental plants such as diascia, nemesia, verbena and alonsoa (Skelton *et al.*, 2004; Koenig *et al.*, 2005; Mathews and Dodds, 2006). There is limited information on the host range and symptoms displayed in susceptible hosts. Lack of detailed descriptions of the nemesia ring necrosis disease creates problems for disease diagnosis as symptoms alone may be similar to those caused by other viruses. Moreover, the causal agent

is being reported as either NeRNV or ScrMV based on ELISA results with a polyclonal antibody. The two viruses are distinct species that are serologically related (Koenig *et al.*, 2005). This situation makes accurate assessment of the occurrence of the two viruses difficult. Currently, NeRNV and ScrMV can be differentiated by reverse transcriptase polymerase chain reaction using primers that are specific to NeRNV (Mathews and Dodds, 2006).

A viral disease of ornamental plants may be the source of future infections of food crops and hence it is important to study new diseases so that there is a thorough understanding of the biology and epidemiology of the pathogens. The major focus of our study was to understand the mechanisms of infection, pathogenicity and transmission of a viral disease in diascia. The overall objectives of this study were to characterize the virus(es) associated with disease in diascia, use molecular data and physico-chemical properties of the virus(es) to describe and classify the virus, and to develop diagnostic assays for rapid detection of the virus.

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CHAPTER 2

MOLECULAR CHARACTERIZATION OF A TYMOVIRUS CAUSING DISEASE IN DIASCIA ORNAMENTAL PLANTS

SUMMARY:

Diascia ×hybrida ‘Sun chimes™ coral’ developed virus-like symptoms of mild yellowing and mottling. Infected plants also displayed reduced vigor. RNA was extracted from infected tissues, and the entire genome was cloned and sequenced. A sequence consistent with a member of the genus *Tymovirus* was obtained. The genome consists of a positive sense, single-stranded RNA molecule and is 6,290 nucleotides (nt) long. Three potential open reading frames (ORFs) were identified. ORF I overlaps the 5'-terminus of ORF II and its predicted product is similar to the movement protein of tymoviruses. The putative product of ORF II is a viral replicase. The smallest ORF, ORF III, encodes the putative coat protein. The 3'-terminus has the potential to form a transfer RNA-like structure and the 5'-terminus can potentially form secondary structures similar to those formed by other tymoviruses. When the entire genome is considered, it shows the 77.8% nucleotide sequence identity to *Nemesia ring necrosis virus-Nemesia fruticans* isolate (NeRNV-Nf). Sequence alignments from cloned reverse transcription-polymerase chain reaction (RT-PCR) products using primers for the coat protein region revealed the presence of a mixture of two tymovirus-like sequences. The two isolates are denoted as *Nemesia ring necrosis virus* – Washington State strain (NeRNV-WA) and *Diascia yellow mottle virus* (DiaYMV). Analysis of the predicted coat protein amino acid sequence of NeRNV-WA showed 99.5% sequence identity to NeRNV-Nf while DiaYMV showed 94.5% sequence identity to

NeRNV-Nf. Amino acid sequence comparison of the DiaYMV movement protein showed 54.0% identity to NeRNV-Nf. Cluster analysis of ORF I, ORF II and ORF III places DiaYMV in the same cluster as the NeRNV-Nf. Analysis of genomic sequence data suggests that DiaYMV is a unique virus in the genus *Tymovirus*.

INTRODUCTION:

The use of nucleic acid sequences to classify and characterize plant viruses is becoming an essential tool in virus differentiation and identification. The use of other virus criteria such as serological relationships has proved to be limiting and less discriminating in cases where several species in a genus are serologically related. This has been the case in many genera and the genus *Tymovirus* is no exception. Isolation and characterization of viral specific double-stranded RNA (dsRNA) has also been used for the rapid detection and diagnosis of viral infections directly from host tissue (Morris and Dodds, 1979; Valverde *et al.*, 1990). Nucleotide sequence based on the genotype of the virus has been helpful in discriminating viruses into species and identifying the emergence of new viruses, as well as virus variants that exist in natural virus populations (Lin *et al.*, 2003).

Sequences of the coat protein (CP), the replicase protein (RP) and the tRNA-like structure with the corresponding sequences of other tymoviruses have been used to show that *Physalis mottle virus* (PhyMV) is a separate species that was previously identified as the Iowa strain of *Belladonna mottle virus* [BDMV-I] (Jacob *et al.*, 1992). There has been added emphasis on molecular criteria as the most reliable for demarcating new virus species in the genus *Tymovirus* and in many other plant virus genera. These criteria have been used to differentiate *Scrophularia mottle virus* (ScrMV), *Plantago mottle virus* (PlMoV), *Ononis*

yellow mosaic virus (OYMV), *Anagyris vein yellowing virus* (AVYV) and the recently named *Nemesia ring necrosis virus* (NeRNV) (Koenig *et al.*, 2005a; 2005b). The NeRNV isolates obtained in Europe from *Nemesia fruticans* and *Diascia* species will be referred to as NeRNV-Nf and NeRNV-Dia, respectively. An isolate of NeRNV that was reported from infected diascia species in California is referred to as NeRNV-isolate 3 (Mathews and Dodds, 2006). In order to understand the biology of a disease associated with *Diascia ×hybrida* ‘Sun chimes™ coral’, molecular data was used to further understand how virus isolates in these plants relate to other confirmed viruses causing disease in diascia elsewhere.

Tymovirus genomes have three open reading frames (ORF), a tRNA-like structure at the 3'-terminus, and a conserved sequence that serves as a subgenomic RNA (sgRNA) promoter known as the “tymobox” (Ding *et al.*, 1990; Gibbs, 1994; Edwards *et al.*, 1997). The physical-chemical criteria for genus demarcation include genome size (6.0 to 6.5 kilo bases) and molecular mass of the CP (20kDa) (Martelli *et al.*, 2002; Dreher *et al.*, 2004). The criteria for species demarcation within the genus *Tymovirus* include: overall sequence identity of less than 80%; capsid protein identity less than 90%; differential host range; differences in the 3'-terminal structure, and serological specificity. The objectives of this study were to use molecular data to characterize a virus causing disease in diascia ornamental plants collected in States of Oregon and Washington in 2001.

MATERIALS AND METHODS:

Plant source: *Diascia ×hybrida* ‘Sun chimes™ coral’ plants showing virus like symptoms were used in this study. Initially, plants were received from the Oregon State University Plant Diagnostic Clinic (M. Putnam) for diagnosis, and subsequently, plants were

obtained from regional nurseries. Plants were kept in glasshouses with 16 hr daylight and 8 hr night cycle. In the winter months, day length was supplemented with sodium vapour lamps. The virus isolated from *Diascia ×hybrida* ‘Sun chimes™ coral’ will be referred to as Diascia yellow mottle virus (DiaYMV) based on symptoms displayed on the host.

Double stranded RNA (dsRNA) extraction: Plant tissue (7 g) was frozen overnight at -20°C before use. Liquid nitrogen was added to tissue in a pre-cooled mortar and pestle and allowed to evaporate; the tissue was then triturated. The ground tissue was added to 9 ml glycine-phosphate saline buffer (GPS) [0.2 M glycine, pH 9.5; 0.1 M Na₂HPO₄; 0.6 M NaCl], 9 ml water-saturated phenol containing 0.01% 8-hydroxyquinoline and 0.5 ml 2-mercaptoethanol. The contents were briefly shaken and 0.5 ml 20% sodium dodecyl sulfate (SDS) was added and then mixed at room temperature for one hour. Using a swinging bucket rotor (Jouan SWM 180.5; Thermo Fisher Scientific, Waltham, MA), the mixture was centrifuged at 3,071xg for 20 min at 4°C. The aqueous layer was collected, and the organic layer was re-extracted with 10 ml GPS buffer and centrifuged as above. The aqueous layer was collected and combined with the previous aqueous layer. The combined layers were re-extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged as above. The aqueous layer was collected and 95% ethanol was added to a final concentration of 18%. One gram cellulose (CC-41; Whatman Inc, Florham Park, NJ) was added and the suspension was allowed to shake on ice for 45 min and centrifuged for 2 min in a swinging bucket rotor at 1,428xg (IEC clinical centrifuge; Needham Heights, MA). The supernate was discarded and the cellulose was resuspended in 20 ml cold STE [1x STE= 0.1 M NaCl; 10 mM Tris-HCl, pH 8.0; 1 mM EDTA·Na₂,] containing 18% ethanol (referred to as 18% STE). The mixture was centrifuged and the supernate discarded; this step was

repeated three times. The pellet was resuspended in 15 ml cold 18% STE, and the slurry was poured onto a 1.5 cm x 15 cm chromatography column (Bio-Rad, Hercules, CA). The packed column was washed with 300 ml 18% STE. The column was purged with air and the dsRNA was eluted onto a 50 ml sterile centrifuge tube with 3 ml, 2 ml and 2 ml of 0.5 mM EDTA·Na₂, pH 8.0. The dsRNA was precipitated by the addition of 0.1 volume 5 M sodium acetate, pH 5.1, mixed well and then three volumes 95% ethanol were added. The mixture was stored overnight at -70°C. The pellet was collected by centrifugation at 4°C at 21,252×g for 45 min. After carefully decanting off the solution, the pellet was drained and allowed to air dry for 10 min. The dried pellet was resuspended in 30 µl 0.5 mM EDTA·Na₂, pH 8.0; 10 µl of the dsRNA was analyzed by electrophoresis in 0.8% agarose (SeaKem LE; Cambrex Bio Science, Rockland, ME) with 1X TAE (0.04 M Tris-acetate, pH 8; 1 mM EDTA) at 70 V for 2 hr. The gel was stained with ethidium bromide (1 µg/ml) and dsRNA was visualized with a UV transilluminator.

Virus purification: Virus was isolated from infected *Diascia ×hybrida* ‘Sun chimes™ coral’ tissue using a modification of the bentonite extraction procedure outlined by Dunn and Hitchborn (1965). Five grams of tissue were ground in liquid nitrogen with a mortar and pestle. The ground tissue was transferred to a 50 ml centrifuge tube to which 10 ml Buffer 1 (0.01M Tris, pH 7.6; 10 mM MgSO₄) was added and mixed thoroughly. The mixture was centrifuged at 1,428×g for 10 min. The collected supernate was mixed with 0.2 volumes of bentonite suspension (0.01M Na-PO₄, pH 7.5; 10 mM MgSO₄, 40 mg/ml bentonite). The mixture was centrifuged at 10,842×g for 10 min, and the supernate was collected and brought to a final concentration of 0.2 M NaCl by the addition of 5 M NaCl. Polyethylene glycol (PEG) was added to a final concentration of 8% by stirring in finely

ground powder (PEG-6000; Sigma-Aldrich, St. Louis, MO). The mixture was shaken gently at 4°C for 1 hr and centrifuged at 10,842×g for 45 min. The pellet was resuspended in 4 ml Buffer 1 overnight at 4°C and then stored at -20°C for use in cDNA synthesis.

For extraction from a larger mass of tissue, the volumes of reagents described above were increased proportionately. The PEG precipitation step was omitted but the supernate was centrifuged at 50,000×g for 1 hr at 4°C and the pellet was resuspended in 1ml Na/K-PO₄ buffer (0.05M sodium-potassium phosphate, pH 7.4) at 4°C overnight. The partially purified virus preparation was further purified using a 0 to 30% sucrose log-linear gradient (Brakke and Van Pelt, 1970) and centrifuged at 104,630×g for 2 hr at 4°C. The top and bottom translucent layers were collected separately, diluted five-fold in Na/K-PO₄ buffer and centrifuged at 104,630×g for 3 hr at 4°C to pellet the virus. The pellet was resuspended in Na/K-PO₄ buffer and the A₂₆₀ and A₂₈₀ absorbance readings were measured (UV160U Visible recording spectrophotometer; Shimadzu Corp., Japan). An estimate of virus concentration was based on the extinction coefficient of ScrMV as a reference for which an A₂₆₀ reading of 8 was equivalent to a virus concentration of 1 mg/ml (Bercks, 1973).

A butanol:chloroform virus extraction procedure was adapted from Koenig *et al.*, (2005b) protocol for isolation of NeRNV. Infected *Nicotiana occidentalis* tissue (100 g) was homogenized in a blender with 200 ml 0.1 M NaH₂PO₄, pH 7.5 containing 0.02 M NaSO₃. The mixture was strained through four layers of cheesecloth and then 0.2 volumes butanol:chloroform (1:1 v/v) were gradually added while stirring at room temperature for 15 min. The mixture was centrifuged at 21,252×g for 15 min at 4°C. The supernate was collected and centrifuged as above for another 15 min, and the supernate was centrifuged at 104,630×g for 2 hr. The supernate was discarded and 100 ml 0.01 M Tris-HCl, pH 7.5 was

added to the pellet and allowed to dissolve the pellet overnight at 4°C. The virus suspension was centrifuged at 21,252×g to pellet insoluble debris. The supernate was centrifuged at 104,630×g for 2 hr. The glassy pellet was re-dissolved in 0.01 M Tris-HCl, pH 7.5 overnight at 4°C. The partially purified virus preparation was further purified using the log-linear sucrose gradient procedure as described above.

RNA extraction: RNA was isolated from intact virus by treatment with proteinase K. For a 78 µl reaction; 60 µl virus preparation was mixed with 4 µl proteinase K (100 mg/ml; Sigma-Aldrich), 10 µl 10% SDS and 4 µl 0.5 M dithiothreitol (DTT) and incubated at 37°C for 10 min followed by incubation at 65°C for 10 min. An equal volume of phenol:chloroform (1:1 v/v) was added to the mixture, mixed briefly and centrifuged at 16,000×g for 5 min to separate phases. The upper aqueous layer was transferred to a new microcentrifuge tube. The nucleic acid was precipitated by adding 3 µl linear polyacrylamide (LPA) carrier (Gaillard and Strauss, 1990), 0.5 volumes 3 M sodium acetate (NaOAc), pH 5.4 and 3 volumes 95% ethanol. The mixture was stored at -80°C for 1 hr, and then centrifuged at 16,000×g for 3 min. The supernatant was discarded and the pellet was overlaid with 70% ethanol. The mixture was centrifuged at 16,000×g for 2 min and the supernate was decanted, followed by a pulse spin; excess ethanol was removed. The pellet was air dried and used for cDNA synthesis.

cDNA synthesis and cloning: The RNA pellet from above was dissolved in 24 µl sterile double distilled water (ddH₂O), and the following were added to a final concentration of: 1X first strand buffer (Invitrogen, Carlsbad, CA) containing 50mM Tris-HCl (pH 8.3), 75 mM KCl and 3 mM MgCl₂, 0.4 mM deoxyribonucleotide triphosphate (dNTP) mix (0.4 mM each dATP, dCTP, dGTP, dTTP), 0.01 M DTT and 10 µM primer Tymo c6300

(5'-GGAGTTGCACCC-3') designed from aligned sequences of members of the genus *Tymovirus*. The mix was incubated at 65°C for 5 min, placed on ice water and 25 Units (U) Superscript™ II reverse transcriptase (Invitrogen) and 0.25 U RnaseOUT™ (Invitrogen) were added. The reagents were gently mixed and incubated for 2 hr at 42°C. The entire 40 µl first strand cDNA mixture was used for the second strand DNA synthesis.

For a 150 µl second strand cDNA synthesis reaction, 60 µl ddH₂O was added to the 40 µl first strand cDNA reaction, and a mixture containing second strand synthesis buffer (Invitrogen), [20mM Tris-HCl (pH6.9); 90 mM KCl; 4.6 mM MgCl₂; 0.15 mM β-NAD⁺; 10 mM (NH₄)₂SO₄]; 0.2 mM dNTPs each of dATP, dCTP, dGTP, dTTP; 0.133 mM NAD; 0.133 U *Escherichia coli* (*E. coli*) DNA polymerase I (Invitrogen); 6.67 mU RNase H (Invitrogen) and 1.33 mU *E. coli* DNA ligase (Invitrogen) was added. The contents were briefly mixed, and incubated at 16°C overnight. The second strand cDNA mix was extracted with phenol:chloroform, and precipitated with ethanol as above. The cDNA-containing pellet was used for the blunt end cloning reaction.

For blunt-end cloning, the pellet was resuspended in 14.5 µl ddH₂O, and the following reagents were added to a final concentration of: T4 DNA polymerase buffer [33 mM Tris-acetate, pH 7.9; 66 mM NaOAc; 10 mM MgOAc₂; 1 mM DTT] (Invitrogen), 0.625 mM dNTPs (Invitrogen), 0.125 U T4 DNA polymerase (Invitrogen) and 0.25 U T4 polynucleotide kinase (Invitrogen). The 20 µl mixture was incubated at 37°C for 1 hr. The phosphorylated cDNA mix was heated at 70°C to inactivate the T4 polynucleotide kinase. For ligation, 3 µl phosphorylated cDNA were added to 0.5 µl EcoRV digested pBluescript II KS+ dephosphorylated vector (Stratagene, Cedar Creek, TX), T4 ligation buffer [50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 5% weight /volume (w/v) PEG

8000] (Invitrogen), and 0.025 U T4 DNA ligase (Invitrogen). The final volume was brought to 20 μ l with ddH₂O. The combined reagents were mixed gently and incubated at 37°C for 3 hr. *E. coli* DH5 α competent cells (Invitrogen) were transformed by the addition of 3 μ l of the ligation reaction and incubating on ice for 30 min. The cells were subjected to heat shocking at 42°C for 90 sec, and placed back on ice for 5 min. One ml Luria-Bertani Broth (LB), [10 g/L tryptone (Difco Laboratories, Detroit, MI), 5 g/l yeast extract (Difco), 10 g/l NaCl and 5 g/l glucose (Sigma-Aldrich)] media containing 50 μ g/ml ampicillin (LB+amp) was added to the transformed *E. coli* cells and shaken for 1 hr at 37°C. Transformed cells (200 μ l) were plated onto LB+amp+X-gal agar plate [LB broth with 15.0 g/l agar (Difco), 50 μ g/ml ampicillin, 2% (w/v) 5-bromo-chloro-3-indoyl- β -D-galactopyranoside (X-gal) and 100 mM isopropylthiogalactoside (IPTG)]. The plates were incubated at 37°C overnight. Thirty white colonies were picked with sterile toothpicks and transferred to a grid-labeled LB+amp+X gal agar plates and the same toothpick was then used to inoculate 1 ml of LB+amp media. The bacterial cells were allowed to grow overnight at 37°C and then the plasmid DNA (pDNA) was extracted (Sambrook *et al.*, 1989). An aliquot (1 μ l) of the pDNA was digested with 0.5 U EcoR I (Invitrogen) at 37°C for 1 hr and the restriction digestion products were resolved by electrophoresis in a 1% agarose gel and visualized on a UV transilluminator. Colonies that contained substantial inserts as shown by restriction analysis were transferred to LB+amp+X-gal agar grid plate and were simultaneously used to inoculate 5 ml liquid LB amp media. Liquid cultures were grown overnight at 37°C and plasmid DNA was extracted (Sambrook *et al.*, 1989). An aliquot of plasmid DNA (1 μ l) was digested with restriction enzyme 0.5 U EcoR I (Invitrogen) and the restriction products were characterized as above. One clone was found to have a 5,500 base pair (bp) insert and is

referred to as clone Diascia #14. The remaining 49 μ l of the Diascia #14 clone were extracted with phenol:chloroform and precipitated by ethanol as outlined earlier. The pellet was resuspended in 10 μ l diethylpyrocarbonate (DEPC)-treated water. An aliquot (0.5 μ l) of the purified plasmid DNA was used for sequencing the plasmid DNA. The sequences derived from Diascia #14 clone were used as the basis for subsequent cloning reactions.

Rapid amplification of cDNA ends (RACE): RNA from a mini-virus preparation was isolated by treatment with the proteinase K above. For 5'-RACE, first strand cDNA synthesis was synthesized using a virus specific primer Amo1904di-466c designed from the Diascia #14 sequence. The first strand cDNA reaction was initiated by dissolving the RNA pellet in 18 μ l ddH₂O and adding 1 μ l 100 μ M Amo1904di-466c (5'-GTTGGTGAGT-TCGTCGAAGT-3'), the mixture was heated to 70°C for 10 min and quickly chilled in ice water, and then mixed with the following cDNA reagents to a final concentration of: 1X first strand buffer (Invitrogen), 0.01 M DTT (Invitrogen), 0.4 mM each dNTP (Invitrogen). The contents were mixed briefly, incubated at 37°C for 2 min, and then placed on ice. Twenty five units Superscript™II reverse transcriptase (Invitrogen) and 0.25 U RNaseOut™ (Invitrogen) were then added to the mixture. The mixture was gently mixed and incubated at 42°C for 2 hr. One microliter RNase mix (1/1 volume/volume Rnase H and Rnase T1) was added to the first strand cDNA and incubated at room temperature for 30 min. The first strand cDNA was purified with a QIAquick spin column (Qiagen Inc, Valencia, CA) following the manufacturer's protocol and eluted in 20 μ l ddH₂O. A homopolymeric tail was added to the 3'-terminus of the cDNA using terminal deoxynucleotidyl transferase (TdT) (Invitrogen) and dCTP (Invitrogen). The tailing reaction contained the following: 16.5 μ l first strand cDNA; 2 mM Tris-HCl, pH 8.4; 5 mM KCl; 0.3 mM MgCl₂; and 0.5 mM dCTP.

After mixing gently, the solution was heated to 94°C for 3 min, and quick chilled in ice water after which 10U TdT (Invitrogen) were added and the 25 µl reaction was incubated at 37°C for 2 hr. The reaction was stored at -20°C until needed. An aliquot was used for PCR with the 5'-RACE primer (5'-GGCCACGCGTCGACTAGTACGGGGGG-3') and a virus specific primer Amo1904di-171c (5'-TCTCGGTGAGAGGTGGTGGGA-3') designed from the sequence of Diascia #14. The 5'-RACE reaction contained the following: 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton-X, 2 mM MgCl₂, 0.15 mM each dNTP, 0.6 µM primer Amo1904di-171c, 0.6 µM 5'-RACE primer, 0.025 U *Taq* DNA polymerase (Invitrogen) and 2 µl of 5'-terminus tailing reaction in a final volume of 20 µl. The amplification conditions were: 1 cycle of 94°C for 10 min followed by 50 cycles (denaturing 94°C for 30 s, annealing 54°C for 20 s, extension 72°C 1 min) and a final extension of 30 min at 72°C. After amplification, the 5'-RACE products were cloned into pCRII-TOPO vector (Invitrogen) following a modification of the manufacturer's specifications as follows: 4 µl 5'-RACE PCR product, 1 µl salt solution (0.2 M NaCl; 0.01 M MgCl₂ (Invitrogen)) and 1 µl pCRII-TOPO vector (Invitrogen). The mixture was incubated at room temperature for 15 min. Two microliters of ligation reaction were added to thawed TOPO 10 one shot chemically competent *E. coli* cells (Invitrogen) and mixed gently before incubating on ice for 30 min. The *E. coli* cells were then heat shocked at 42°C for 30 s and returned to ice for 20 min. Two hundred fifty microliters SOC medium (2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM Glucose) (Invitrogen) were added to the transformed bacterial cells and held at room temperature for 10 min prior to shaking at 37°C for 1 hr. Transformed cells (50 µl) were mixed with 50 µl SOC medium and spread on prewarmed LB+amp+X gal plates. The plates were incubated at 37°C overnight. The following day,

twenty white colonies were picked and transferred with toothpicks onto LB+amp+X gal agar grid plates and the toothpick then placed in 50 μ l ddH₂O at 65°C. An aliquot (1 μ l) of the suspension was used in PCR amplification using 20 μ M M13 forward primer and 20 μ M M13 reverse primer provided with the cloning kit following manufacturer's instructions. The PCR products were checked for DNA by electrophoresis as outlined above. Bacterial colonies containing plasmid inserts were selected from agar and were grown in 5 ml LB+amp media with gentle shaking overnight at 37°C. The plasmid DNA was extracted (Sambrook *et al.*, 1989), and 1 μ l was digested with restriction enzyme 0.5 U EcoR I (Invitrogen) following manufacturer's suggestions. The restriction products were analyzed by electrophoresis in a 1% agarose gel at 100 V for 2 hr. The agarose gel was stained with ethidium bromide (5 μ g/ml) and the DNA bands were visualized using a transilluminator at ultra violet light (260 nm). The remaining plasmid DNA was extracted with phenol: chloroform and precipitated with ethanol as outline earlier. The pellet was dissolved in 10 μ l ddH₂O and 1 μ l aliquot used for sequencing.

Double-stranded RNA was used for determining the 3'-terminal end of the virus. An oligonucleotide CLSc377 primer (5'-GTGGTTCTCCAGAGTTTT-3') was phosphorylated prior to use. The 25 μ l polynucleokinase reaction to phosphorylate the primer was made of the following components: 80 μ M primer CLSc377, T4 polynucleotide kinase forward buffer (70 mM Tris-HCl, pH 7.6; 10 mM MgCl₂; 100 mM KCl; 1 mM 2-mercaptoethanol) (Invitrogen), 10 mU ATP, and 20 U T4 polynucleotide kinase (PK). The mixture was incubated at 37°C for 1 hr, followed by phenol:chloroform extraction and ethanol precipitation with 2 μ l 5 M NaCl and 62.5 μ l 95% ethanol. The pellet from the 5'-phosphorylated primer was resuspended in 19 μ l ddH₂O, and the following reagents were

added to a final concentration of: TdT buffer (0.1 M potassium cacodylate, pH7.2; 2 mM cobalt chloride (CoCl₂); and 0.2 mM DTT) and 10U TdT (Invitrogen). The mixture was incubated at 37°C for 1 hr and the reaction was terminated by adding 1.6 µl 0.25 M EDTA. The mixture was ethanol precipitated as above, and the pellet was resuspended in 10 µl ddH₂O. An aliquot (1 µl) of the 5'-phosphorylated 3'-blocked primer mixture was used in a ligation reaction containing T4 RNA ligase buffer [100 mM Tris-HCl, pH 7.5; 4 mM MgCl₂; 2 mM DTT; 80 µM ATP] (New England Biolabs, Ipswich, MA), 15 mg/ml bovine serum albumin (BSA), 40 µU rATP, 1 U RnaseOut™ and 20 U T4 RNA ligase (Invitrogen). The contents were mixed with 14 µl dsRNA to make a total volume of 20 µl, and incubated at 37°C for 2 hr. First strand cDNA synthesis was set up with the following: 5 µl aliquot of the ligation mix, 1X first strand buffer (Invitrogen), 0.5 mM dNTPs, 0.01 M DTT and 5 µM CLSV377 primer (5'-AAAACCTCTGGAGAACCAC-3'). The mixture was heated at 70°C for 3 min, then placed in an ice bath to which 5 U Superscript™ II reverse transcriptase and 0.5 U of RnaseOUT™ were added and the cDNA mix was incubated at 42°C for 3 hr. The first strand cDNA mixture was diluted five fold and 2 µl of the diluted product was used for PCR. The general PCR protocol was followed; 2 µl of the diluted cDNA was mixed with 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.001% gelatin, 0.15 mM dNTPs, 0.6 µM AmoCP-41C (5'-GATCTCCATTCCTCTGC-3'), 0.6 µM CLSV377, 0.25U *Taq* DNA polymerase (Invitrogen) and brought to 20 µl with ddH₂O. The reaction mixture was incubated in a thermocycler (Model *icycler*; Bio-Rad) with the following conditions: 1 cycle at 94°C for 10 min, followed by 50 cycles of (denature 94°C for 30 s, annealing 54°C for 20 s and extension 72°C 30 s) and a 1 cycle final extension at 72°C for 30 min and holding at 4°C. The PCR products were resolved by electrophoresis in 3% low melting temperature

agarose (NuSieve GTG agarose; Cambrex BioScience) gel to confirm the presence of the desired product.

Ligation and cloning were carried using the TOPO-TA cloning kit (Invitrogen). The procedures were as described above. Two independent RT-PCRs were performed and three clones from each were selected for sequencing in both directions.

In order to obtain sequence information between clone *Diascia* #14 and the 3'-terminus product, a forward RT-PCR primer was designed from the 3'-terminus of *Diascia* #14 and the reverse primer was designed from the aligned sequence of 5'-RACE of the complement of 3'-terminal end. The procedure for first strand cDNA synthesis was followed as described earlier, but 1 μ l of 100 μ M TymoCPR (5'-CCATTCCTCTGCCATGAACTC-3') was used. The cDNA was amplified by PCR in a 20 μ l reaction containing the following final concentrations: 0.6 μ M each of primers Di1865-188c (5'-AGACTCTAGCTCTGATGCACGA-3') and TymoCPR (5'-CCATTCCTCTGCCATGAACTC-3') while the rest of the components were not changed. A PCR program of 1 cycle of 94°C for 10 min followed by 40 cycles of 94°C (30 s), 55°C (1 min), 72°C (1 min) and one cycle of 72°C final extension for 30 min was performed. The PCR products were analyzed in a 2.5 % (w/v) low gelling temperature agarose (NuSieve GTG agarose) in 1X TAE buffer, and stained in ethidium bromide for visualization with a transilluminator (UV light box at 260nm). A DNA product of approximately 800 bp was excised from the gel and purified using Gel Elute Ethidium bromide minus gel purification kit (Sigma-Aldrich) following the manufacturer's protocol. An aliquot (1 μ l) of the purified DNA product was used for cloning using the TOPO TA cloning kit (Invitrogen) following the manufacturer's

guidelines for ligation, cloning and analysis of clones. Two independent RT-PCR assays were carried out and for each assay, three positive clones were selected for sequencing.

Sequencing: Plasmid DNA was extracted from bacteria and screened for inserts of the correct size as described earlier. The purified plasmid DNA was dissolved in 10 μ l Rnase-free water and prepared for automated sequencing in both directions using Sanger's dideoxy chain termination sequencing method (Sambrook *et al.*, 1989). A 10 μ l sequencing reaction mixture was made with 0.5 μ l pDNA, 0.5 μ l 20 μ M M13 forward or reverse primer (provided with TOPO TA cloning kit), 4 μ l sequencing reaction mix (Laboratory for Biotechnology and Bioanalysis (LBB), Washington State University, Pullman, WA) and 5 μ l ddH₂O. The reagents were briefly mixed well and a PCR was conducted with the following parameters: 50 cycles of denaturing at 96°C for 30 s, annealing at 50°C for 15 s and extension at 60°C for 4 min. Ten μ l ddH₂O was added to the final product and the mixture was then eluted through a performaldehyde gel filtration cartridge spin column (Edge Biosystems, Gaithersburg, MD) at 800 rpm in a microcentrifuge (Eppendorf, Model 5415C) for 2 min. The eluted DNA was dried using a speed vacuum at medium heat (SpeedVac Model DNA110; Thermo Savant; Waltham, MA) for 30 min. The DNA samples were analyzed at LBB.

Following the initial cloning and DNA sequencing, the nucleotide sequences of the virus found in diascia were aligned and assembled using the software program Xesee version 3.2 (Carbot & Beckenback, 1989). The complete genome sequence was designated Diascia tymovirus 1 and was used to design primers for RT-PCR, cloning and sequencing. The primers were such that overlapping clones of about 600 nt were produced. Twelve sequences each of 5'- and 3'-terminal sequences were generated using 5'-RACE procedures as outlined

above. For each overlapping clone, two independent RT-PCRs were performed using different RNA templates from the same host. For each RT-PCR and cloning procedure, three clones of the expected size were isolated and processed for sequencing in forward and reverse directions, thus generating 12 sequences for each overlapping cloning reaction. The sequence that occurred most frequently was regarded as the representative sequence; this process was repeated to assemble the entire genome. The sequence from the second set of sequencing reactions was designated Diascia tymovirus 2. The final genome sequence was aligned together based on sequence information from both Diascia tymovirus 1 and 2. In cases where a primer pair did not yield reliable sequence, a new primer pair was designed to produce clones overlapping existing sequences.

Sequence alignments and analyses: Sequence fragments were aligned with the aid of Pairwise BLAST search (Altschul *et al.*, 1997) and then combined manually by using the Xesee sequence editor version 3.2. Completed sequences were compared to the GenBank database sequences using advanced BLAST algorithm search program from the National Center for Biotechnology Information, Bethesda, MD (NCBI) website. Clustal W version 1.83 (Thompson *et al.*, 1994) was used to generate multiple sequence alignments with other viruses for use in phylogenetic analysis and subsequent primer designs. The open reading frames (ORFs) search was done with the ORFinder program from NCBI. Aligned sequences were analyzed with PHYLLIP (Felsenstein, 1993) programs to infer phylogeny and place confidence limits on phylogeny. The program SEQBOOT was used to generate 100 bootstrap sequence alignments from the Clustal W aligned sequences. The bootstrapped samples were then analyzed by the maximum parsimony method using the program

PROTPARS. The trees generated by PROTPARS were analyzed by the program CONSENSE to deduce a consensus tree.

RESULTS AND DISCUSSION:

The acronym DiaYMV was used here to denote the virus being studied even though no formal classification has been proposed at this time. DsRNA extraction from infected diascia samples revealed a major band corresponding to between 6.0 to 7.0 kilobase pairs (kbp) (Fig. 1). DsRNA from *Nicotiana benthamiana* yielded a more intense band of this size. The bands found in the dsRNA profile of infected samples were not evident in preparations from healthy *Nicotiana benthamiana*. Viruses known to infect plants in the family Scrophulariaceae and produce a predominant dsRNA with a size between 6 and 8 kbp include *Maracuja mosaic virus*, *Papaya mosaic virus*, *Poplar mosaic virus*, *Scrophularia mottle virus* and *Plantago mottle virus*. RT-PCR with broad spectrum universal primers for potexviruses and carlaviruses did not give a positive reaction suggesting that members of these genera were not present in the infected tissue. Since the virus associated with disease in diascia and nemesia plants had a genome size of 6.0 to 7.0 kbp, induced double membrane vesicles in the cytoplasm and yielded two bands in the sucrose density gradient profile of purified virus, it was speculated that the virus may be a member of the genus *Tymovirus* (Table 1). At the start of this study, there was no sequence information published for the tymoviruses ScrMV or PIMoV.

Virus purification yielded a banding pattern with two visible layers in the sucrose density gradients (Fig. 2). The top band was less opaque compared to the bottom band. Virus recovery estimates from 100 g of plant tissue for the bottom layer ranged from 6.0 mg

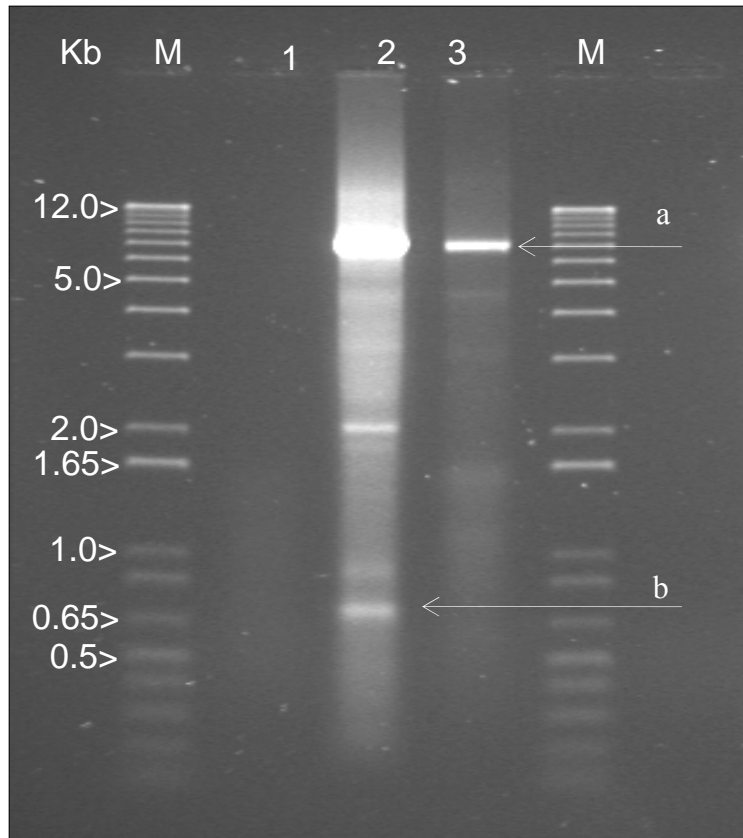


Figure 1. Agarose gel analysis of double stranded RNA (dsRNA) extracted from healthy and infected plant tissues. Lane M: 1 kb plus molecular weight marker (Invitrogen); 1: healthy *Nicotiana benthamiana*, 2: *N. benthamiana* inoculated with Diascia yellow mottle virus (DiaYMV); 3: Infected *Diascia x hybrida* ‘Sun chimes™ coral’. Band “a” is the putative genomic dsRNA and “b” is subgenomic dsRNA.

Table 1. List of names, acronyms, and accession numbers of virus sequences used in multiple sequence alignments and phylogenetic comparisons. Virus sequences were acquired from the National Center for Biotechnology Information (NCBI) – GenBank database.

<i>Virus</i>	<i>Acronym</i>	<i>Accession number</i>
<i>Anagyris vein yellowing virus</i>	AVYV	AY751780
<i>Andean potato latent virus</i>	APLV	AF035402
<i>Belladonna mottle virus</i>	BeMV	X54529
<i>Calopogonium yellow vein virus</i>	CalYVV	U91413
<i>Chayote mosaic virus</i>	ChMV	NC002588
<i>Desmodium yellow mottle virus</i>	DYMV	AF035201
Diascia yellow mottle virus	DiaYMV	<i>this study</i>
<i>Dulcamara mottle virus</i>	DuMV	AY789137
<i>Eggplant mosaic virus</i>	EMV	NC001480
<i>Erysimum latent virus</i>	ErLV	NC001977
<i>Kennedy yellow mosaic virus</i>	KYMV	NC001746
<i>Nemesia ring necrosis virus</i>	NeRNV-Nf	AY751778
<i>Nemesia ring necrosis virus</i>	NeRNV-Dia	AY751781
<i>Nemesia ring necrosis virus</i>	NeRNV-D1 isolate 1 (Diascia)	DQ648150
<i>Nemesia ring necrosis virus</i>	NeRNV-D2 isolate 2 (Diascia)	DQ648151
<i>Nemesia ring necrosis virus</i>	NeRNV-D3 isolate 3 (Diascia)	DQ648153
<i>Nemesia ring necrosis virus</i>	NeRNV-N1 isolate 1 (Nemesia)	DQ648154
<i>Nemesia ring necrosis virus</i>	NeRNV-WA	<i>this study</i>
<i>Okra mosaic virus</i>	OkMV	AF035202
<i>Ononis yellow mosaic virus</i>	OYMV	NC1513
<i>Petunia vein banding virus</i>	PetVBV	AF210709
<i>Physalis mosaic virus</i>	PhyMV	NC003634
<i>Plantago mottle virus</i>	PlMoV	AY751779
<i>Scrophularia mottle virus</i>	ScrMV	AY751777
<i>Turnip yellow mosaic virus</i>	TYMV	NC4063

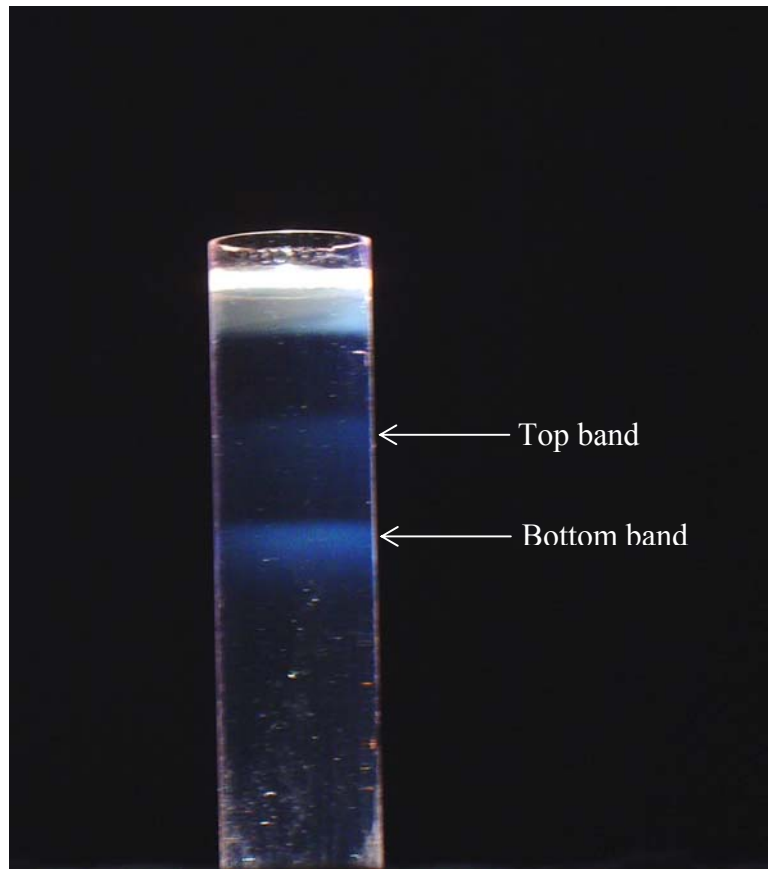


Figure 2. Density gradient profile of virus from infected *Diascia x hybrida* ‘Sun chimes™ coral’ following sucrose linear log density gradient centrifugation.

from *Nicotiana occidentalis* to 0.1 mg from *Diascia ×hybrida* ‘Sun chimes™ coral’. The concentrations were estimated based on the calculation used for virus concentration of ScrMV (Bercks, 1973).

The initial cloning of the viral genome yielded clone Diascia #14 and this clone was 5,500 bp in length. A sequence of 800 bp was derived from clones 28 and 30 which overlap some parts of Diascia #14 and the 3'-RACE sequences. The 5'-RACE clones overlapped Diascia #14 and the sequences were about 500 nt in length while products of the 3'-RACE overlapped clones 28 and 30 and the sequence was also about 500 nt long. All together, these clones represented the complete viral genome (Fig. 3).

Pairwise blast comparison (Altschul *et al.*, 1997) showed that the nucleotide sequence of DiaYMV is similar to members of the genus *Tymovirus* (*data not shown*). The complete sequence of DiaYMV is 6,290 bp (Fig. 4). Its similarity to tymoviruses suggests that the genome is a monopartite, positive-sense, single-stranded RNA molecule. The positive-sense nature of the genome is supported by the presence of large open reading frames in this orientation only.

The entire sequence of the virus associated with disease in *Diascia ×hybrida* ‘Sun chimes™ coral’ was determined, thus giving insight into the possible uniqueness of the virus and its relationship with other viruses in the genus *Tymovirus*. Three putative ORFs were detected (Fig. 5). Genome organization comparison with other members of the tymovirus (Table 2) led to the following deductions: ORF I is 1,914 nt long and encodes the putative movement protein (MP); ORF II is 5,373 nt and encodes the putative replicase protein (RP), often referred to as the overlapping protein (OP) in other tymoviruses since the sequence starts seven nucleotides downstream from the start of ORF I; ORF III is 579 nt and encodes a

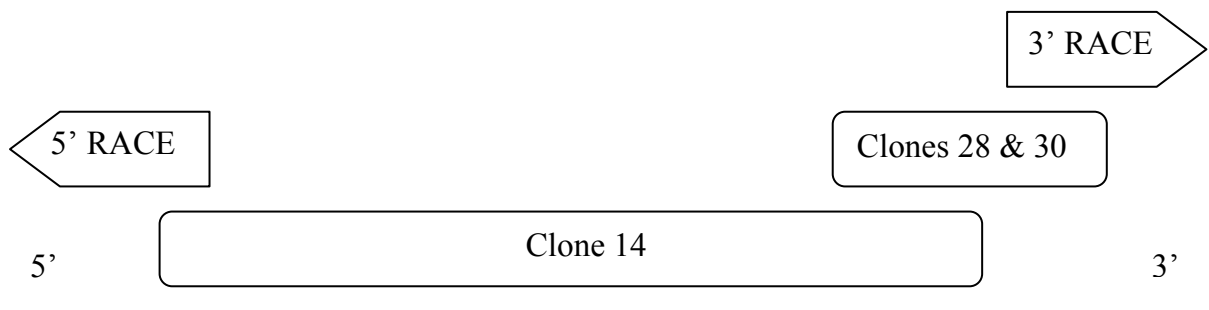


Figure 3. The locations of the cDNA clones used in the sequencing of the entire genome of *Diascia tymovirus* of the *Diascia* yellow mottle virus (DiaYMV). The locations of the clones are not drawn to scale.

Figure 4. Sequence and deduced genome organization of the Diascia yellow mottle virus (DiaYMV) isolated from *Diascia ×hybrida* ‘Sun chimes™ coral’. The numbers on the right side indicate either nucleotide or amino acid positions respectively. An asterisk indicates the stop codon. The open reading frames are capitalized and the 5’- and 3’-untranslated regions (UTR) are in lower case.

DiaYMV	gtaattcgtttccagctcagccccgattcatttcgcacctgagttttcttttcttaccttgcttagctctccggccctaagcaccttttcc	90
DiaYMV	gcttcccagcttcgctctccggccccgaagctttacctctaacagaccgactcgtccctctgttaATGGACCATGGCCTTCCAGCTTGCCC	180
ORFI		9
ORFII	M D H G L P A C P M A F Q L A	6
DiaYMV	TCGAAGCCCTGTCCACCACCTCTCACCGAGATTCTTCCCTGAATCCGGTTCTGTATCACTCCGTGAGTCCGCTCCAGCGCTCTCTCGACC	270
ORFI	R S P V H H L S P R F F P E S G S V S L R E S A P A L S R P	39
ORFII	L E A L S T T S H R D S S L N P V L Y H S V S P L Q R S L D	36
DiaYMV	TCTTTCCTTGGATAATCACCAGGGACGCTCTCCCCTTCCCTCAACTCATGCGGCATTCTGTCTCCGGATTTGGAACCACCCCGCACCCCCc	360
ORFI	L S L D N H Q G R S P L P Q L M R H S C L R I W N H P A P P	69
ORFII	L F P W I I T R D A L P F L N S C G I P V S G F G T T P H P	66
DiaYMV	ATGCTGTCCACAAGGCCATAGAGACCTTCCCTCTTTTCTCCCACTGGAGTTCTCTGGCCTCCACTCTCAGCTCCGTCATGTTTATGAAAC	450
ORFI	C C P Q G H R D L P P F L P L E F S G L H S Q L R H V Y E T	99
ORFII	H A V H K A I E T F L L F S H W S S L A S T L S S V M F M K	96
DiaYMV	CTTCCAAGTTTCGAAAACCTGGCTGCCATCAACCCAAACTTCGACGAACTCACCAACTTTCGTCTCACTCCC GCCGACACCGTTCCGGTACC	540
ORFI	F Q V S K T G C H Q P K L R R T H Q L S S H S R R H R S V P	129
ORFII	P S K F R K L A A I N P N F D E L T N F R L T P A D T V R Y	126
DiaYMV	CGACGACCTCCTCATCACTCCCCAACACGAGATCGTCTTCATGCACGACGCTCTCATGTACTTCACCCCTCATCAAATCGTCGATCTTT	630
ORFI	D D L L I T P P T R D R L H A R R S H V L H P S S N R R S F	159
ORFII	P T T S S S L P Q H E I V F M H D A L M Y F T P H Q I V D L	156
DiaYMV	TCGAACGTTGCCCTCTGCTCAACAGGCTCCACTGCAGCCTCGTTGTCCCTCCAGAGAGCAGCTTCACGGATCTCTCTCTTCCCAACCA	720
ORFI	R T L P S A Q Q A P L Q P R C P S R E Q L H G S L S L P N H	189
ORFII	F E R C P L L N R L H C S L V V P P E S S F T D L S L F P T	186
DiaYMV	TCTACACATACCAACTAAACTCGAACAACCTCCACTACACCCCCGAAGGGCATCACGCCGGAAGCTACGACCAACCTCGGTCCGCCATCC	810
ORFI	L H I P T K L E Q P P L H P R R A S R R K L R P T S V R H P	219
ORFII	I Y T Y Q L N S N N L H Y T P E G H H A G S Y D Q P R S A I	216
DiaYMV	GGTGGTTGAAGCTCCGACGCATCACCAGCCCCTTCTTCAACCTGTCCGTGACCATTCTGGAATCTTGGGGCCCTGTCCACTCGATTCTCA	900
ORFI	V V E A P T H H Q P L L Q P V R D H S G I L G P C P L D S H	239
ORFII	R W L K L R R I T S P F F N L S V T I L E S W G P V H S I L	236

DiaYMV TTCAACGTGGGCTTCCGATCAGCGATTCTCTCGCGCCCCCGCACGCATCCTTCAAGGTGCCCCAGGCCCGAGTGCTGCCAGAAGCCACGT 990
 ORFI S T W A S D Q R F S R A P A R I L Q G A P G P S A A R S H V 279
 ORFII I Q R G L P I S D S L A P P H A S F K V P Q A R V L P E A T 276

DiaYMV TTCTCAACCAACCTCTCCGCCATCGGATGGTCCCTTCCGAGGTGTACGACGCCCTGTTACGTACACTCGAGCCGTCCGCACCCTCCGAA 1080
 ORFI S Q P T S P P S D G P F R G V R R P V H V H S S R P H P P N 309
 ORFII F L N Q P L R H R M V P S E V Y D A L F T Y T R A V R T L R 306

DiaYMV CCTCCGATCCAGCCGGCTTCGTCAGGACCCATTCAAACAAGCCGAAGCACGCCTGGGTCACTCCCCAAGCGTGGGACAACCTTCAAACCT 1170
 ORFI L R S S R L R Q D P F K Q A E A R L G H S P S V G Q P S N L 339
 ORFII T S D P A G F V R T H S N K P K H A W V T P Q A W D N L Q T 336

DiaYMV ACGCCCTCCTAAACGCGCCGGTACGCCCAAGAGTGGTGTACAACCTTCTTCTCTCCCCATTGAGAGGCCATCTCTACTTGGCCCAGC 1260
 ORFI R P P K R A G T P K S G V Q L L P L P H S E G P S L L G P A 369
 ORFII Y A L L N A P V R P R V V Y N F F L S P I Q K A H L Y L A Q 366

DiaYMV ACCTCAACCCCTTCTGGTACGAGCGCTTCCATTCTTGGGCCTCGTCCTCCCTGCCTTGAAGGTGCTCACCCTGGCTTACCAGTCCCCA 1350
 ORFI P Q P L P G T S A S I L G P R P P C L E G A H H W L T S P Q 399
 ORFII H L N P F L V R A L P F L G L V L P A L K V L T T G L P V P 396

DiaYMV GGGTATCGTCCCTTCAAGTTGCTCATCGTCAACTGGTCCGGCCCCACCCGAGCCCTCTCCACAGCTCACCCATTCTCCGCCAAATCG 1440
 ORFI G I V P S S C S S S T G R A P P R A L S H S S P H S P P N R 429
 ORFII R V S S L Q V A H R Q L V G P H P E P S P T A H P I L R Q I 426

DiaYMV TTGCGTTCTCTCAGCAGAATCCCTTCTTTTTGGAACCTCTGCCAAAGAACTCCGTTCTCAACAGGTTCTTCCGGTCCGCTTGTGCGCCAC 1530
 ORFI C V L S A E S L L F G T L P K N S V P Q Q V L P V R L S A T 459
 ORFII V A F S Q Q N P F F L E L C Q R T P F L N R F F R S A C R P 456

DiaYMV CGTCGGCCTTCCGCTTGGAGCTCCAACGATCCACACTCCCCCTCCCATCGTGGCTAGCTGTGCTGCTGATCTCCATGATCCCAGTGATGG 1620
 ORFI V G L P L G A P T I H T P P P I V A S C A A D L H D P S D G 489
 ORFII P S A F R L E L Q R S T L P L P S W L A V L L I S M I P V M 486

DiaYMV CCTCTCCAACCTCTCACCTCGTGCTGCCGAGTCGGTCCAAAGCCTTACGACGGCTATCACACACACCTTCAACCCCTCCGAGTTCAACC 1710
 ORFI L S N S H P R A A A V G P K P S R R L S H T P S P L R V Q P 519
 ORFII A S P T L T L V L P Q S V Q S L H D G Y H T H L H P S E F N 516

DiaYMV TGAGCTGGCCGCTGGAATCCTTCCACGTGCCACTCGTCGGAACCTTCTGCCCCGGGCATGGCTTCCAACCCGAGCCCGCCCCAACTC 1800
 ORFI E L A A G I L P R A H S S E L S A P G H G F Q P E P A P N S 549
 ORFII L S W P L E S F H V P T R R N F L P P G M A S N P S P P P T 546

DiaYMV CCTTCGTGGCACCTCCCTTCGCCACCAACTCTCCCCATCCACCTCCCCCGAGCCCCCACCAGCAAGCCTaGGTCCGAGTCAGCCCCTCG 1980
ORFI L R G T S L R H Q L S P S T S P R A P H R K P R S E S A P R 579
ORFII P F V A P P F A T N S P H P P P P E P P T A S L G P S Q P L 576

DiaYMV CGCCTCATCACCCGACGCCACCGACAAACCCGCCAGCTCCAGCGAGCTCTTCCGCTCCCCCGAGCTCTCGTTGTCCAGAACCCCGCT 1980
ORFI A S S P D A T D K P A S S S E L F R S P G A L V V P E P P L 609
ORFII A P H H P T P P T N P P A P A S S S A P P E L S L S Q N P R 606

DiaYMV CCGAGACCTCGAGCCCCCCTCTCAGCCCTCTCCTTCTCCTTCCCAAAGTTCCCCGCTTCCAGCCGGGCCTCCACCTCACCCGTCTGACG 2070
ORFI R D L E P P L S A L S F S F P K F P A S S R A S T S P V * 637
ORFII S E T S S P P S Q P S P S P S Q S S P L P A G P P P H P S D 636

DiaYMV GTTCCTGCAGGTGCGCCGACTGCCAGCCAGAGCATCAGCACCTCCGCTCCATAAACGAGTTTGGTGCCTCAACCAAAATCCAGCTCCCA 2160
ORFII G S C R C A D C Q P E H Q H L R S I N E F G A L N Q N P A P 666

DiaYMV CCCCCAGTCCCTTGCTCAACCGTTTCGGAGCCTAAGGCCGATCTCCCTGGTTCGGACCTGCTCTCCGATCCGTCTTGCGTGGGCCCGGTG 2250
ORFII T P S P L L N R S E P K A D L P G S D L L S D P S C V G P V 696

DiaYMV TGGAGTTCGAGTGTCTCTTTCCCGAGTCTACCATAACTCAAACGGAACCTTTCTCACTCGACAACGGGCTCAGGCCAGCTCCTCCGCTC 2340
ORFII V E F E C L F P G V Y H N S N G T F L T R Q R A Q A S S S A 726

DiaYMV CCCTACCAGCCAAGAGATGTCTGCTCACATCCATCTCACCTCAGTTAGGTGTTCCGGAGGCAACTCTCTGGAATTACCTCTGCGAGATGC 2430
ORFII P L P A K R C L L T S I S P Q L G V P E A T L W N Y L C E M 756

DiaYMV TGCCAGACAGTCTCTTGGACAACCCAGAGATCAGGAACTATGGTCTCTCCACCGACCACCTCACGGCTTTGGCGTCCCGCTCAACTTTG 2520
ORFII L P D S L L D N P E I R N Y G L S T D H L T A L A S R L N F 786

DiaYMV AATGCGTGATTCACACTGGACACACAACCCCTCCCTTACAGCTGTGTTGGTGCATCCACTCGCGTTCAGATCACATTCCATCCGGGCCCGC 2610
ORFII E C V I H T G H T T L P Y S C V G A S T R V Q I T F H P G P 816

DiaYMV CCAAGCACTTCTCCCCAACATCCGTCTTTCCGCTCCGCCCCTGGATCCAACCCCTCCAAGTCCCCACTGGTTTCGGGCCGCTCTCCGTT 2700
ORFII P K H F S P N I R L S A S A P G S N P S K S P L V R A A L R 846

DiaYMV TCCAGCTGAACGGGGACTTCCTTCCGTTTCATGAACTCTCACAAACACAAGGTCTCAGTTCCCCACGCCAAGAATCTTGTGTCCAACATGA 2790
ORFII F Q L N G D F L P F M N S H K H K V S V P H A K N L V S N M 876

DiaYMV AGAACGGCTTTGACGGCATCACATCCCAGCTCTCTGAGTCTCTGGCCGCTCCCCAAGCAAAAACCTCTAGAGCTTGACGCGACCATCG 2880
ORFII K N G F D G I T S Q L S E S S G R S P K Q K L L E L D A T I 906

DiaYMV ACGTTGCCTTCCCTCGCGAAGTAGACGTGATCCACATAGCTGGTTTTCCCTGGCTGCGGCAAATCCCACCCAGTCCAAAACTCCTGCAGA 2970
 ORFII D V A F P R E V D V I H I A G F P G C G K S H P V Q K L L Q 936

DiaYMV CCAAGGCTTTCAGACACTTCCGCCTTTCTGTGCCACTAACGAGCTCAGAACCAGTGGAAATCGGACATGTCCTTGCCTGAGTCCGAAA 3060
 ORFII T K A F R H F R L S V P T N E L R T E W K S D M S L P E S E 966

DiaYMV TTTGGCGCCTCTGCACCTGGGAAACCAGCCTCTTCAAGAGCTCCAGTATTCTCGTGATTGATGAGATTTACAAGCTCCCCGAGGGTATT 3150
 ORFII I W R L C T W E T S L F K S S S I L V I D E I Y K L P R G Y 996

DiaYMV TGGATCTGGCCCTTCTTGCTGACCCCTCTACATCCCTCGTTATCCTTCTCGGAGATCCACTCCAAGGAGAGTACCACTCCTCTCACCCCT 3240
 ORFII L D L A L L A D P S T S L V I L L G D P L Q G E Y H S S H P 1026

DiaYMV CTTTCATCCAACAACCGCCTCGAGTCTGAAACCTCTCGCCTCTCCAAGTACATTGATTGCTATTGCTGGTGGACTTACCGCTGCCCCGGG 3330
 ORFII S S S N N R L E S E T S R L S K Y I D C Y C W W T Y R C P R 1056

DiaYMV CAGTGGCCGACCTCTTTGGCGTCAAGACGTTCAACACCGAGCAGGGTTTCATTCGAGGTGAGCTCTCCCATCCTCAAGGTCTTCCGAACC 3420
 ORFII A V A D L F G V K T F N T E Q G F I R G E L S H P Q G L P N 1086

DiaYMV TTGTCAACAGTATAGCCACCGCCACAACCATGCAAAACCTTGGCCATCACGCGCTCACGATCTCAAGCAGCCAAGGCATGACCTACTCTT 3510
 ORFII L V N S I A T A T T M Q N L G H H A L T I S S S Q G M T Y S 1116

DiaYMV CTCCGACTACCATCCTCCTCGACCGCCACAGCACCTGCTTTTCGCCTCAAAACTGTTTTGTGGCCCTCACCCGAGCCGCAAGGGCATCA 3600
 ORFII S P T T I L L D R H S T L L S P Q N C F V A L T R S R K G I 1146

DiaYMV TCTTCATCGGTAACATGTAcCAAGCGTCCGGTtACTTTGGCaCTTCGTACATGTTCAcCAAGCACTCACAGGCTCTCCGGTGCACCTGA 3690
 ORFII I F I G N M Y Q A S G Y F G T S Y M F T Q A L T G S P V D L 1176

DiaYMV TGTGTGCTTTTTCTTTGTATCACACCCTGCCGCTCATCTTCGACCCTATCAAATCCCGTCGACAACGACTGGTCGCCGGCGATCCCCTTC 3780
 ORFII M C A F P L Y H T L P L I F D P I K S R R Q R L V A G D P L 1206

DiaYMV CCATTTCCACCTCCGAATCTGCCATACGCAACTATGGCCGACTCCCACCTCACATTCCCACAGACTACGCCAAAGACTGCTTTGTCTCCT 3870
 ORFII P I S T S E S A I R N Y G R L P P H I P T D Y A K D C F V S 1236

DiaYMV CACAAGTGGTTTTCTCCGAAGGCGAGGACCGCACCTTGCCTACCCTCCACCTTCCACCAAGTCGCCTCCCACTCCACTTGCTCACTGAAC 3960
 ORFII S Q V V F S E G E D R T L P T L H L P P S R L P L H L L T E 1266

DiaYMV CGGCTGCCCTTCCGAAGTCTCCTCTCCGAGACCGAGCCCTCCAAGAGCCCCATCACCTTGGCCCTCCTCGGAGAGTCTTTCGAGGAGC 4050
 ORFII P A A P S E V L L S E T E P S K S P I T L A L L G E S F E E 1296

DiaYMV TGGCAGCTCACTTCCTTCCCGCACACGACCCCGAACTCAAGGAAATCATCTTTGCTGACCAGAGCAGCTCCCAGTTTTCCCTTCCTTGATG 4140
 ORFII L A A H F L P A H D P E L K E I I F A D Q S S S Q F P F L D 1326

DiaYMV TTCCCTTCGAACTGTCGTGCCAACCATCTTCCCTCCTCGCAGCTTCTCACAGGCCTGCTTCGGATCCGACTCTTCTGGTGTCTCCATCA 4230
 ORFII V P F E L S C Q P S S L L A A S H R P A S D P T L L V S S I 1356

DiaYMV AGAAGCGACTCAGATTCAGGCCATCCGACTGCCCTACTTCATTTCTCGAATGACATTCTACTGGGGCAGCACCTCTTCAACTCCCTCT 4320
 ORFII K K R L R F R P S D C P Y F I S S N D I L L G Q H L F N S L 1386

DiaYMV GCAGGGCGTTCAAAGAGCCCCCTGGAAGTCATTCCCTTTGATCCTGTGCTGTTTCGCGGAGTGCATTGCTCTCAACGAGTACGCACAGC 4410
 ORFII C R A F N R S P L E V I P F D P V L F A E C I A L N E Y A Q 1416

DiaYMV TCTCTTCCAAAACCAAGGCCACCATCGTCGCCAATGCTTCTCGCTCCGACCCGGACTGGCGCTTACC GCCGTTTCGGATATTTGCGAAAG 4500
 ORFII L S S K T K A T I V A N A S R S D P D W R F T A V R I F A K 1446

DiaYMV CTCAGCACAAGGTCAATGACGGGTCCATTTTTGGGTCCTGGAAAGCTTGCCAGACTCTAGCTCTGATGCACGACTACGTCATCATGACGC 4590
 ORFII A Q H K V N D G S I F G S W K A C Q T L A L M H D Y V I M T 1476

DiaYMV TCGGCCCTGTCAAGAAATACCAGCGAATTTTGGACCACCAAGATCGCCCCTCACACATCTACACCCACTGCGGCAAGACCCAGCTCAGC 4680
 ORFII L G P V K K Y Q R I L D H Q D R P S H I Y T H C G K T P A Q 1506

DiaYMV TTTCTTCGTGGTGCCAGAAATTTTCCCTGGATGGTCCCTCCCTGTGCAACGACTACACTTCTTTTCGATCAGAGTCAACACGGAGAAGCCG 4770
 ORFII L S S W C Q K F S L D G P S L C N D Y T S F D Q S Q H G E A 1536

DiaYMV TCGTTCTCGAGTGTCTCAAGATGCGCCGGTGCAGTATCCCGGACAACCTCATCCAGCTTACCTGCACCTGAAGACCAACATATCCACCC 4860
 ORFII V V L E C L K M R R C S I P D N L I Q L H L H L K T N I S T 1566

DiaYMV AGTTCGGTCCGCTCACCTGCATGCGCTTAACTGGAGAGCCCGGCACTTATGATGACAACACGGACTACAATCTTGCTGTCATCTACTCTC 4950
 ORFII Q F G P L T C M R L T G E P G T Y D D N T D Y N L A V I Y S 1596

DiaYMV AGTATCAGATGGGCGCCACCCCTGCATGGTCTCTGGTGACGACTCCGTTCATCTTCGCCAACCCACCGATCCATCCCCTTGCCAGCCG 5040
 ORFII Q Y Q M G A T P C M V S G D D S V I F A N P P I H P T W P A 1626

DiaYMV TTGAGAACCTCCTTCATCTTCGATTCAAACTGAGTCAACCACCCAACCTCTGTTCTGCGGTTACTATGTTCGGACCCTCCGGCTGCTGCC 5130
 ORFII V E N L L H L R F K T E S T T Q P L F C G Y Y V G P S G C C 1656

DiaYMV GCAACCCTCTCGCCCTTTTCGCCAAGCTCATGATCACCACCGACAAGGGCAATCTTGACGACACTCTCTCCTCATACTTGATGAGTACT 5220
 ORFII R N P L A L F A K L M I T T D K G N L D D T L S S Y L Y E Y 1686

DiaYMV CGATCGGTCATCGTCTCGGCGACGCCTGCCTCAGTCTCTTGCCAGCCATCTCCACTCTTACCAAAGCGCCTGCTTCGATTTCTTCTGTC 5310
 ORFII S I G H R L G D A C L S L L P S H L H S Y Q S A C F D F F C 1716

 DiaYMV GGAAGGCCTCCCCATTGCAGAAAACCTCTTCTCAGCTTTGAAGAACCTTCTCCCTCTTTGTTGAAGAAGCTCGCCTCGAGTTCGGCCTGGG 5400
 ORFII R K A S P L Q K T L L S F E E P S P S L L K K L A S S S A W 1746

 DiaYMV CCTCTGGTCCACTTCTAGCCCAACTCGACAACGATTGCTACAAAGCCTTCTTGAACGCTCCAATCTCCCAAGCTCCCATCTTGACGCCA 5490
 ORFII A S G P L L A Q L D N D S L Q S L L E R S N L P S S H L D A 1776

 DiaYMV GGGTTCAAAGACTTGAGTCTGAATTGCTTCACTCATTTCAATAACATGGAAGAACTCAAGCCGATCAGCGTCAAGCAGCCCTCTATCCCG 5580
 ORFII R V Q R L E S E L L H S F Q * 1791
 ORFIII M E E L K P I S V K Q P S I P 15

 DiaYMV GCACCTGGCACCAAGCTGCCCCCTGCTCCTGGCCAGCAATCGTCCGCGATCGTCCAACCTTTTCAGGTCTCTGTAGCTGACCTCGGCGTC 5670
 ORFIII A P G T K L P P A P G Q Q S S A I V Q P F Q V S V A D L G V 45

 DiaYMV TCCGAAGTCAACGTGCAGATAACCCTCTCGTCCGATCCCACCCTTTCTCAGCTCACCGCTTTCTACCGCCACGCTGATCTCGTTCGAGTGC 5760
 ORFIII S E V N V Q I T L S S D P T L S Q L T A F Y R H A D L V E C 75

 DiaYMV TCCGCCGTTCTGTTTCCGAATTTCACTTCTTCCAACCCAACCCATTGCGACCTCGCCTGGGTGCCTGCGAATTCGACTGCGTCTCCC 5850
 ORFIII S A V L F P N F T S S S N P T H C D L A W V P A N S T A S P 104

 DiaYMV AAGAACATCTTGAAGACCTACGGCGGCAACCGCTTACCCTCGGCGGCCGATCACCGCCAACGAGGTCATATCCGTGCCCTGCCCATG 5940
 ORFIII K N I L K T Y G G N R F T L G G P I T A N E V I S V P L P M 135

 DiaYMV AACTCTGTCAACTGCACCATCAAGGACAGCGTTCTGTACACGGACTCCCCCGACTCTTGGCTCACTCGCCCGCCCTCTCACGGCCAAG 6030
 ORFIII N S V N C T I K D S V L Y T D S P R L L A H S P A P L T A K 165

 DiaYMV ACGATCCCCTCTGGCACCTTAGTCATTTCGTGGCAAGGTTTCGCTTTCTTCTCCCCTCCCTCAGCCTCTCACAGCTTCTTCGTGAgggcgg 6120
 ORFIII T I P S G T L V I R G K V R L S S P L P Q P L T A S S * 192

 DiaYMV cgcatagccataatgcgagtggttcggcttccacttaaatcgaaagccaaccctccagcccgtctgggtggtccctggatcactacgtc 6210

 DiaYMV aacttgctttgtgtaagcaagcggttgggaacacaaaattgtgaggagttcgaatctccccctgtccccgggtaggggaacc 6290



Figure 5. Genome organization of the Diascia yellow mottle virus (DiaYMV) showing the locations of the 5'-untranslated region and cap \odot , Open reading frames (ORF) I ▨ , ORF II ▩ , ORF III ⊠ , and 3'-UTR region with the tRNA-like structure ⌘ . The numbers refer to the nucleotide positions for each region.

Table 2. Percentage identity of the amino acid sequences of the movement protein (Open reading frame (ORF I)); replicase protein (ORF II) and the coat protein (ORF III) of selected tymoviruses of the ‘*Ononis yellow mosaic virus* (OYMV) cluster’ using Clustal W multiple sequence alignment .

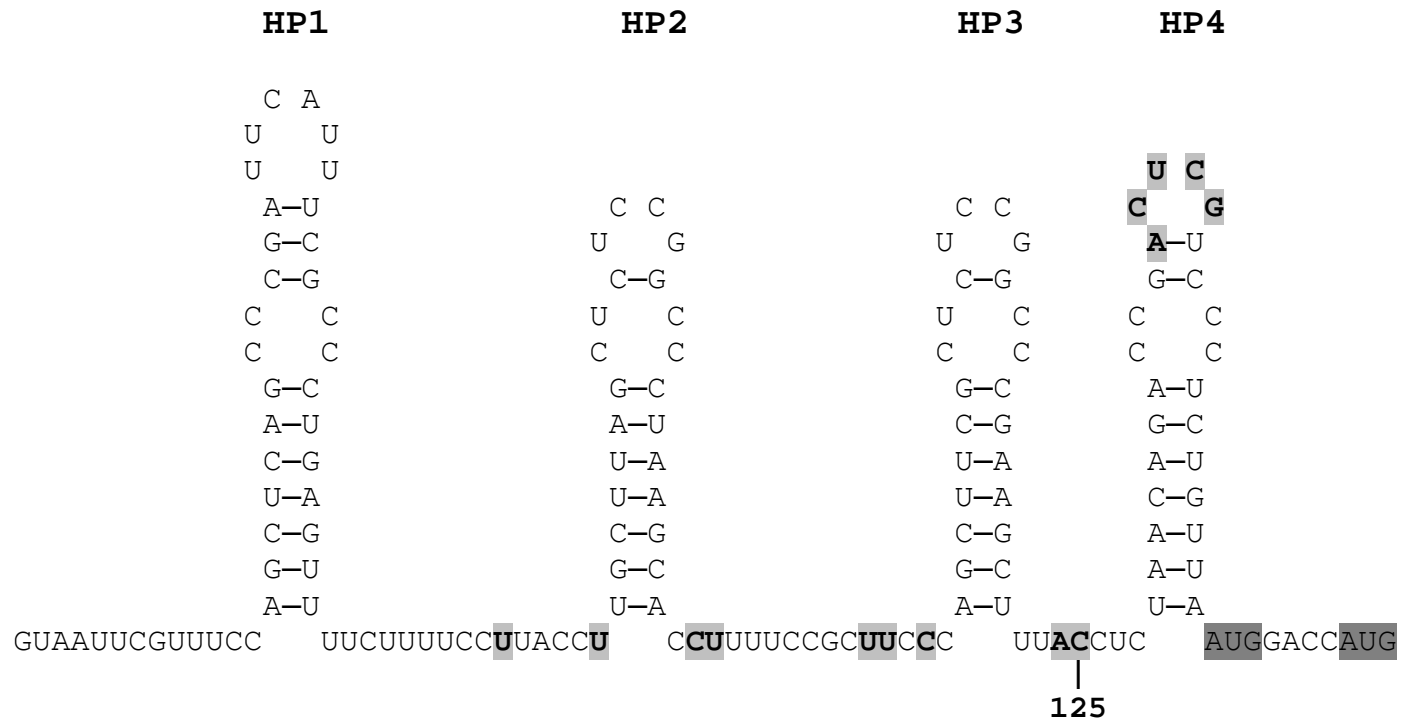
<i>Species name</i>	<i>ORF</i>	<i>DiaYMV</i>	<i>NeRNV</i>	<i>ScrMV</i>	<i>PIMoV</i>	<i>AVYV</i>	<i>OYMV</i>
DiaYMV	I	100	54	29	31	24	28
	II	100	87	65	63	62	64
	III	100	95	74	72	68	71
NeRNV-Nf	I		100	33	31	32	35
	II		100	70	67	62	69
	III		100	74	72	67	69
ScrMV	I			100	31	33	39
	II			100	67	70	69
	III			100	72	65	71
PIMoV	I				100	31	34
	II				100	67	67
	III				100	61	65
AVYV	I					100	35
	II					100	69
	III					100	62
OYMV	I						100
	II						100
	III						100

putative CP with a predicted molecular weight of 21 kDa. In addition to the ORFs, there are 5'- and 3'-untranslated regions (Fig. 4). The DiaYMV total genome size is 6,290 nt long and shares 77.8% nucleotide sequence identity to NeRNV-Nf. The 5'-untranslated region (UTR) of DiaYMV is a region 154 nt long compared the same region in NeRNV that is 153 nt long. Nucleotide sequence comparison for the DiaYMV 5'-UTR is 90% identical to NeRNV-Nf. Tymoviruses have been shown to have a 5'-7-methyl guanosine cap (Ding *et al.*, 1989; Gibbs, 1994; Bernal *et al.*, 2000; Koenig *et al.*, 2005a). In this study, the existence of a 5'-7-methyl guanosine cap structure is inferred solely by the similarity of DiaYMV to other tymoviruses.

The 5'-terminal secondary structure and RNA folding was predicted using Structure analysis of RNA software (STAR version 5: Leiden University, The Netherlands) followed by manual alignment of the nucleotides using NeRNV and OYMV RNA folding as guides (Koenig *et al.*, 2005a). Based on the high sequence similarity of DiaYMV with NeRNV-Nf, it was observed that the 5'-terminus RNA of DiaYMV can be folded into four potential hairpin (HP) structures labeled 1 through 4 (Fig. 6) as predicted by STAR.

The occurrence of an additional nucleotide at position 125 of the DiaYMV 5'-UTR sequence did not change the RNA folding or the occurrence of hairpin 4 (HP4). Although there are nucleotide differences between the sequences of the two RNAs, the predicted secondary structures are similar. The hairpins in tymoviruses are characterized by internal loops that contain C-C or U-C mismatches. The U-C mismatch is found only in members of the 'OYMV serological cluster' and is highly conserved within that group (Koenig *et al.*, 2005a). DiaYMV has U-C mismatches in hairpin loops 2 and 3 which are also found in NeRNV-Nf. Based on the 5'-UTR, DiaYMV is similar in RNA secondary structure to

Figure 6. Predicted secondary structures of the 5'-UTR of Diascia yellow mottle virus (DiaYMV) genomic RNA. The potential hairpin (HP) structures are labeled HP1 to 4. Bold letters in gray background represent the nucleotides that differ from the *Nemesia ring necrosis virus* (NeRNV) nucleotide sequence. The highlighted nucleotides with dark gray background show the start codons of open reading frame (ORF) I and ORF II sequences.



NeRNV and OYMV with the occurrence of four hairpin loops while ScrMV, AVYV, and PIMoV have three (Koenig *et al.*, 2005b).

The putative movement protein (MP) coding region (ORF I) is 1,914 nt long, starting at nucleotide number 155. Amino acid (aa) sequence comparison showed that the MP is 54% identical to the NeRNV-Nf, and 38 and 33% identical to PIMoV and ScrMV, respectively. However, ORF I of DiaYMV is 637 aa long compared to the same region of NeRNV-Nf that is 632 aa long. This protein is also referred to as the overlapping protein (OP) (Morch *et al.*, 1988; Ding *et al.*, 1989; Ranjith-Kumar *et al.*, 1998) since ORF I overlaps the 5'-terminus of ORF II in a manner similar to that found in several animal viral genomes (Keese *et al.*, 1989). Ranjith-Kumar *et al.* (1998) observed that MP sequences of tymoviruses are variable in length and exhibit a sequence identity of 26 to 34% when compared across species. The same authors noticed that there is a tetra-peptide "GILG" found in all tymoviral ORF I for which sequence is reported and this is also true for DiaYMV. This sequence is located at aa positions 239 to 242 of DiaYMV ORF I. It is suggested that the tetrapeptide may have a structural or functional role. NeRNV ORF I is thought to have a role as a suppressor of RNA silencing (Koenig *et al.*, 2005a). Studies of TYMV showed that the MP is involved in the systemic spread of virus within the plant and possibly also in cell-to-cell movement (Bozarth *et al.*, 1992). Thus, the MP may play a major role in host susceptibility to virus infection and ultimately in determining host range.

The MPs of tymoviruses are variable at the amino acid level, even within the 'OYMV cluster' (Table 2). Pairwise comparison of the amino acid sequences of the MP of four strains of TYMV showed that their sequence identities ranged from 89 to 97%. This range of amino acid identities is high relative to the aa identities revealed when the MPs of DiaYMV

and NeRNV-Nf are compared (54%). This analysis suggests that sequence differences between DiaYMV and NeRNV-Nf are greater than those expected between strains of the same tymovirus. Thus, DiaYMV and NeRNV-Nf represent different and unique virus species. Cluster analysis of ORF I amino acid sequences showed that DiaYMV and NeRNV-Nf are in the same cluster, and associated with the other serologically related viruses such as ScrMV, PlMoV and AVYV of the 'OYMV cluster' (Fig. 7). Phylogenetic analysis of the same region as reported by Koenig *et al.*, 2005b places NeRNV in the 'OYMV cluster'. Sequence variability within the MP is not currently considered as one of the criteria for species demarcation in the genus *Tymovirus*, however it is worth noting this large variation. There is an apparent high rate of mutation within the movement protein genes and this has led to limited sequence conservation even within compact groups of viruses that share common genome organization and functional properties (Mushegian and Koonin, 1993). This is why the MP genes are not a viable criterion for use in the species demarcation of many viruses.

The putative ORF II, also called the replicase protein (RP), spans from nucleotide position 162 to 5,536 terminating with TAA codon. The ORF II starts 7 nt downstream of the ORF I, a feature that has been observed in most members of the genus *Tymovirus* (Ding *et al.*, 1990; Rajith-Kumar *et al.*, 1998 and Koenig *et al.*, 2005a). This is the longest ORF in the DiaYMV genome encoding a protein of 1,790 aa and this region is regarded as the most conserved of all the proteins in tymoviruses (Gibbs, 1994). This protein is also referred to as the polyprotein (Keese *et al.*, 1989, Koenig *et al.*, 2005a,) as it encodes a protein some parts of which show sequence similarity to the possible RNA replicase and nucleotide binding sites of other viruses. Sequence analysis of the DiaYMV coding region shows the highest

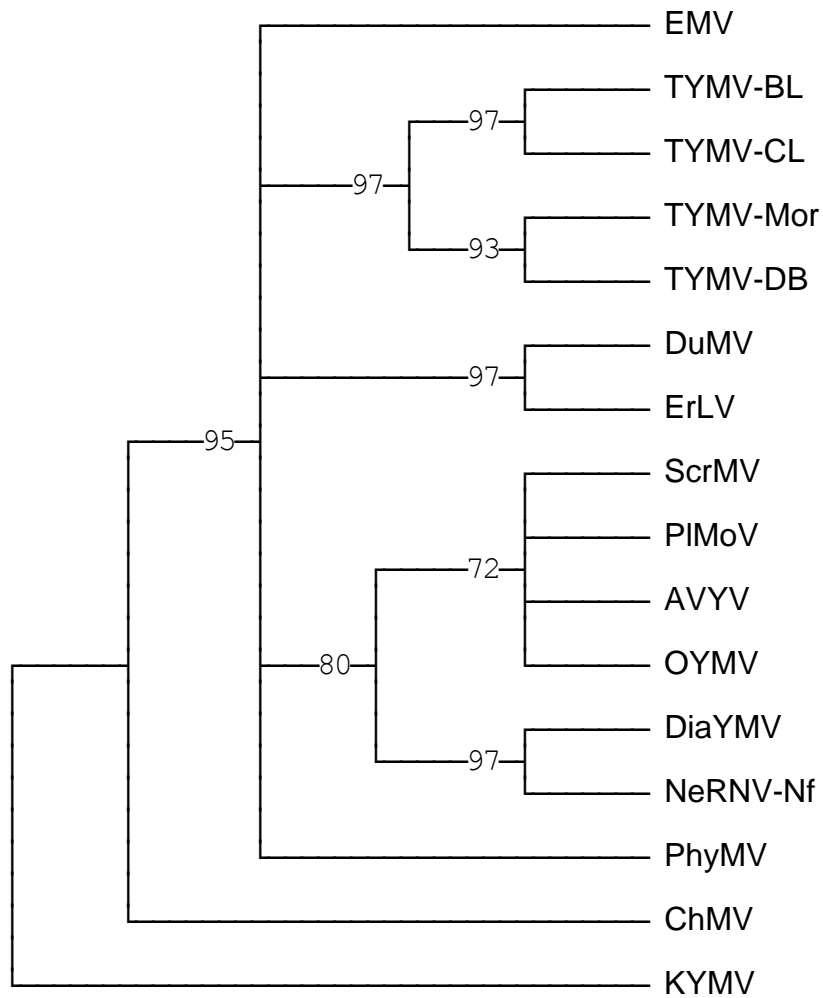


Figure 7. Cluster dendrogram based on amino acid similarity of the movement proteins of *Diascia yellow mottle virus* (DiaYMV) and other known tymoviruses. Percentage values show the unrooted bootstrap values and only branches exceeding 65% are shown. Virus acronyms are defined in Table 1.

amino acid sequence identity of 87% with NeRNV-Nf and 70% identity with ScrMV (Table 2), whereas the average amino acid sequence identity for the RP in tymoviruses is reported to be in the range from 48% to 60% (Rajith-Kumar *et al.*, 1998) or 65% to 71% (Koenig *et al.*, 2005a). The RP for DiaYMV has a highly conserved 16 nucleotide “tymobox”, a feature found in all tymoviruses. The tymobox sequence for the DiaYMV is 5'-GAGTCTGAA-TTGCTTC-3' and it is located in the 3'-terminal region of ORF II. The initiation box CAAT, is located 8 nt downstream of the tymobox and this region has been found in many tymoviruses with the exception of CYVV and the Port Davis strain of KYMV (Ding *et al.*, 1990). The last nucleotide of the initiation box is also the first nucleotide of ORF III in DiaYMV, and hence the initiation box is thought to initiate the synthesis of CP by a subgenomic RNA (Schirawski *et al.*, 2000).

Phylogenetic analysis of the RP amino acid sequences of tymoviruses also places DiaYMV in the same cluster as NeRNV within the ‘OYMV cluster’, again showing the close relationship of DiaYMV with NeRNV (Fig. 8). The NeRNV RP is 1,789 aa long whereas the RP of DiaYMV is 1,790 aa long reflecting a single amino acid addition in the sequence of DiaYMV.

The CP (ORF III) sequence of DiaYMV starts at nucleotide position 5,538 and terminates at 6,114 of DiaYMV, and there is a single nucleotide between the stop codon of ORF II and the start codon of ORF III. However, in AVYV, OYMV, ScrMV and PIMV, the stop codon of the ORF II is located downstream of the start codon of ORF III (Koenig *et al.*, 2005a). DiaYMV ORF III has 95% aa identity with NeRNV-Nf and NeRNV-Dia while the average amino acid sequence identity to other tymoviruses is between 36% and 74%. Phylogenetic analysis of ORF III amino acid sequences of tymoviruses places DiaYMV in the

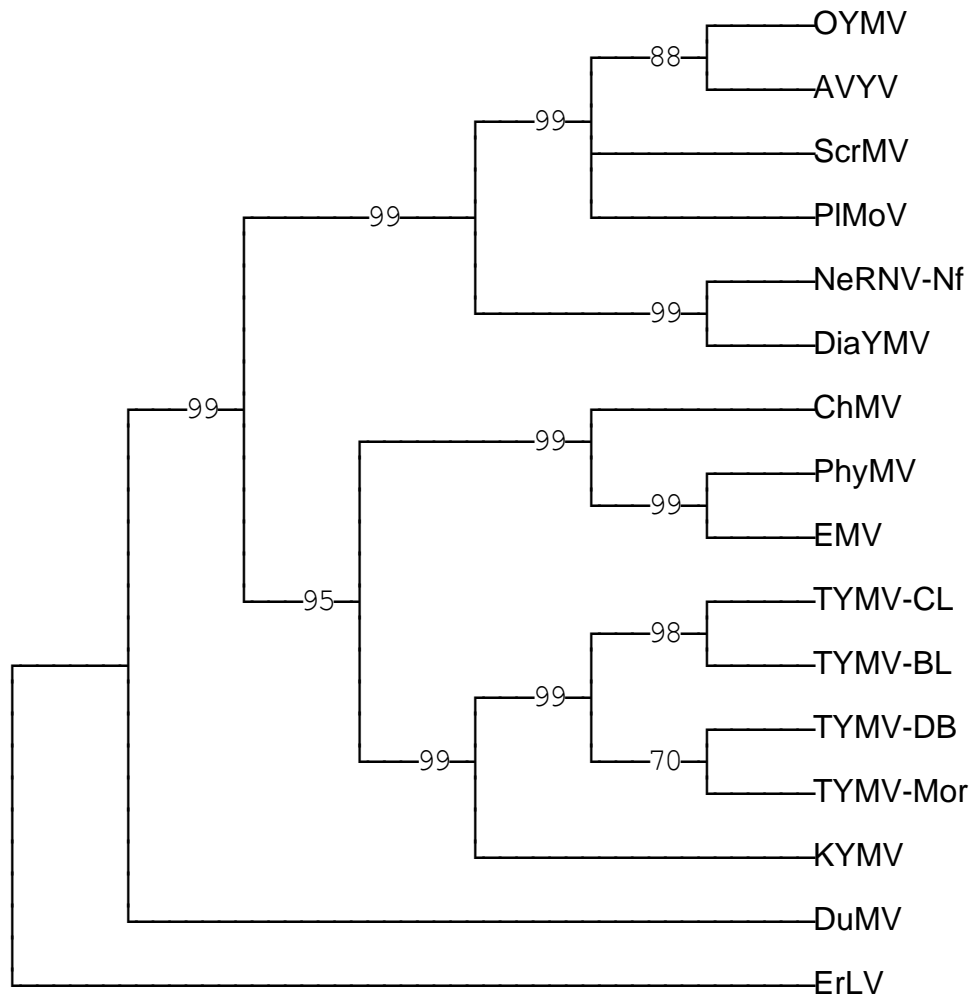


Figure 8. Cluster dendrogram based on amino acid similarity of the replicase proteins of Diascia yellow mottle virus (DiaYMV) and other known tymoviruses. Percentage values show the unrooted bootstrap values and only branches with bootstrap values greater than 65% are shown. Acronyms as listed in Table 1.

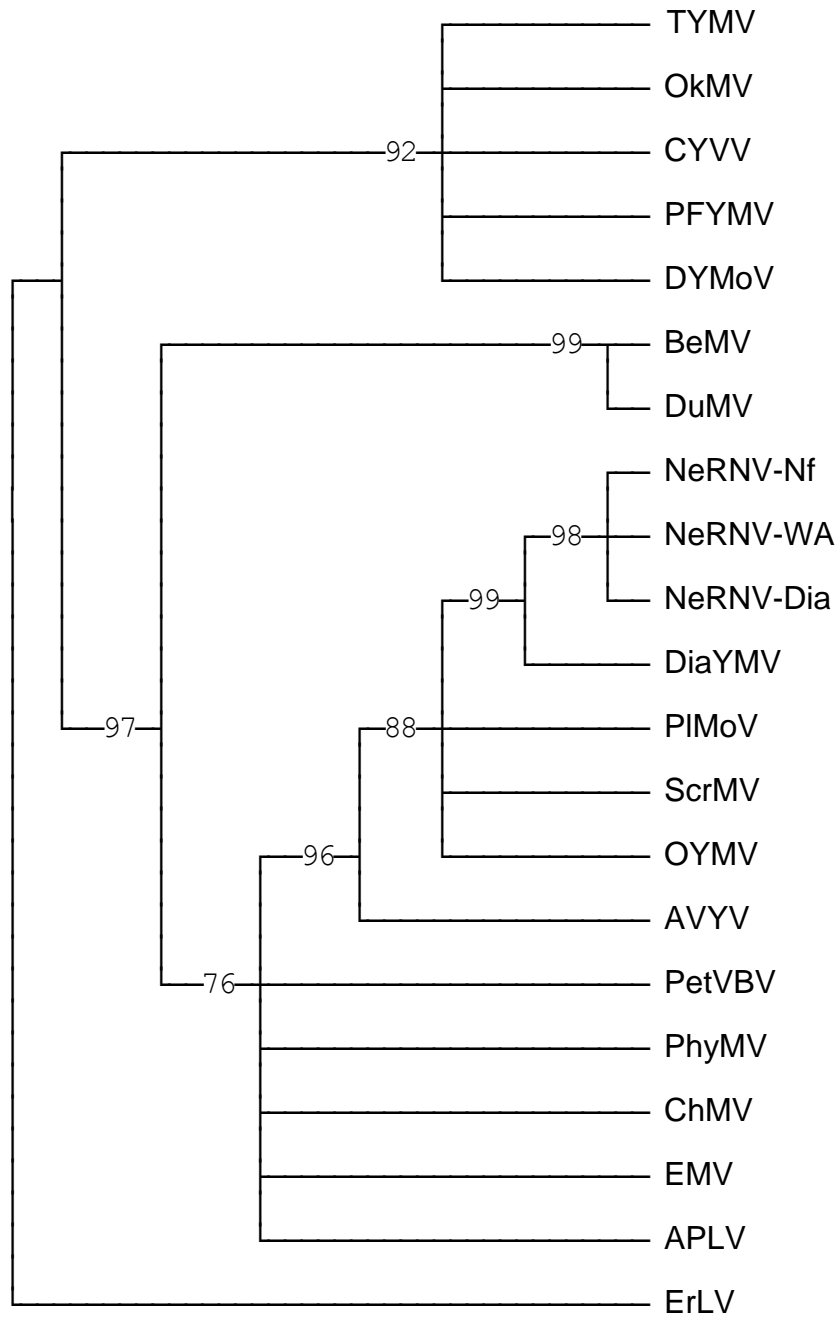


Figure 9. Cluster dendrogram based on amino acid similarity of the coat proteins of Diascia yellow mottle virus (DiaYMV) and other known tymoviruses. Percentage values show the unrooted bootstrap values and only branches with bootstrap values greater than 65% are shown. Acronyms as listed in Table 1.

same cluster with NeRNV-Nf within the ‘OYMV cluster’ (Fig. 9), indicating the close relatedness of the tymoviruses in this cluster. Based on the CP sequence identity with other tymoviruses, it is speculated that DiaYMV ORF III is also synthesized via a subgenomic RNA, even though it was difficult to visualize a corresponding band in this region of the dsRNA profile (Fig. 1). The tymobox is thought to be the promoter element for the subgenomic RNA synthesis (Ding *et al.*, 1990; Rajith-Kumar *et al.*, 1998; Martelli *et al.*, 2002). It is proposed that the separated conserved regions of the tymobox and the initiation box in tymoviruses may have different functions in which the tymobox may act as a signal sequence to be recognized and/or bound by viral RNA replicase whereas the initiation box may help in the precise start of the subgenomic RNA transcription (Ding *et al.*, 1990).

Analysis of virus-specific sequences obtained from these plants revealed the occurrence of two distinct genotypes. This phenomenon suggested that there might be a mixed infection by either two viruses or different strains of the same virus. In addition to the sequence reported here for DiaYMV, a commonly occurring sequence corresponding to ORF III was 99.5% identical at the amino acid level to NeRNV-Nf and NeRNV-Dia. Because of this high degree of identity, it is proposed that these plants are also infected with a Washington isolate of NeRNV (NeRNV-WA) (Fig. 10 and 11).

The 3'-UTR extends from position 6,115 to the end of the sequence at nucleotide 6,290. Sequence comparison of the DiaYMV 3'-UTR revealed highest nucleotide identity of 86% with NeRNV-Nf. The 3'-UTR of DiaYMV is 176 nt long while the NeRNV-Nf 3'-UTR is 175 nt long. Despite several differences in nucleotide sequence, compensating base changes in the sequence of DiaYMV would maintain the secondary structure predicted for NeRNV-Nf (Fig. 12). The pattern of RNA folding for DiaYMV was redrawn based on the

DIAYMV	ATGGAAGAACTCAAGCCGATCAGCGTCAAGCAGCCCTCTATCCCGGCACCTGGCACCAAG	60
NeRNV-Nf	ATGGAAGAACTCAA ACC GATCAGCGTCAA AC AGCCCTCTAT TCCCGCTCCCGGCACCAAG	60
NeRNV-WA	ATGGAAGAACTCAA ACCTGT CAGCGTCAA AC AGCCCTCTAT TCCCGCTCCCGGCACCAAG	60
NeRNV-D1	ATGGAAGAACTCAA ACCT TATCAGCGTCAA AC AGCCCTCTAT TCCCGCTCCCGGCACCAAG	60
NeRNV-D2	ATGGAAGAACTCAA ACCT TATCAGCGTCAA AC AGCCCTCTAT TCCCGCTCCCGGCACCAAG	60
NeRNV-D3	ATGGAAGAACTCAAGCC TAT CAGCGTCAA AC AGCCCTCTAT TCCCGCTCCCGGCACCAAG	60
NeRNV-N1	ATGGAAGAA AT CAAGCC TAT CAGCGTC	27
DIAYMV	CTGCCCCCTGCTCCTGGCCAGCAATC GT CCCGCATCGTCCAACCTTTTCAGGTCTCTGTA	120
NeRNV-Nf	CT TCCCT CCTGC ACCGGGT CAGCAAT CAT CCGCCATCGT TCA ACC CTTCC AGATCT CCGT C	120
NeRNV-WA	CT TCCCT CCTGC ACCGGGT CAGCAAT CAT CCGCCATCGT TCA ACC CTTCC AGATCT CCGT C	120
NeRNV-D1	CT TCCCT CCTGC ACCGGGT CAGCA	83
NeRNV-D2	CT TCCCT CCTGC ACCGGGT CAGCA	83
NeRNV-D3	CT TCCCT CCTGC ACCGGGT CAGCAAT CAT	88
NeRNV-N1		
DIAYMV	GCTGACCTCGGCGTCTCCGAAGTCAACGTGCAGATAACCCTCTCGTCCGATCCCACCCCTT	180
NeRNV-Nf	ACCG ACCTCGGCGT CGCC GAAGTCAACGT TC AGAT CACC CTCT CT CGG ACCC ACT CTCTC	180
NeRNV-WA	ACCG ACCTCGGCGT CGCC GAAGTCAACGT TC AGAT CACC CTCT CT CGG ACCC ACT CTCTC	180
NeRNV-D1		
NeRNV-D2		
NeRNV-D3		
NeRNV-N1		
DIAYMV	TCTCAGCTCACCGCTTTCTACCGCCACGCTGATCTCGTCGAGTGCTCCGCCGTTCTGTTT	240
NeRNV-Nf	TCTCAGCTCAC AGCT TTCTACCG TCAT GCTGACCTCGT GGA ATGCTCCGCT GTACT CT CTC	240
NeRNV-WA	TCTCAGCTCAC AGCT TTCTACCG TCAT GCTGACCTCGT GGA ATGCTCCGCT GTACT CT CTC	240
NeRNV-D1		
NeRNV-D2		
NeRNV-D3		
NeRNV-N1		
DIAYMV	CCGAATTTCACTTCTCTTCCAACCCAACCCATTGCGACCTCGCCTGGGTGCCTGCGAAT	300
NeRNV-Nf	CC AAACT TTCA CTTCT CT CA TCCAACCC CACTCA CTGCGATCTCGCCTGGGT TCCCG CCAAT	300
NeRNV-WA	CC AAACT TTCA CTTCT CT CA TCCAACCC CACTCA CTGCGATCTCGCCTGGGT TCCCG CCAAT	300
NeRNV-D1		
NeRNV-D2		
NeRNV-D3		
NeRNV-N1		
DIAYMV	TCGACTGCGTCTCCCAAGAACATCTTGAAGACCTACGGCGGCAACCGCTTACCCTCGGC	360
NeRNV-Nf	TCC ACCG CGT CA CCCAAG GCA AT CCTTCGA ACCTACGGTGGCA ATCG CTTACCCTCG GA	360
NeRNV-WA	TCC ACCG CGT CA CCCAAG GCA AT CCTTCGA ACCTACGGTGGTA ATCG CTTACCCTCG GA	360
NeRNV-D1		
NeRNV-D2		
NeRNV-D3		
NeRNV-N1		
DIAYMV	GGCCCGATCACCGCCAACGAGGTCAATATCCCGTGGCCCTGCCCATGAACTCTGTCAACTGC	420
NeRNV-Nf	GGCC CCAT CACCGCGA ACG AGGT GATCT CC ATTCC CTGCCCATGAACTCTGTCAACTGC	420
NeRNV-WA	GGCC CCAT CACCGCGA ACG AGGT GATCT CC ATTCC CTGCCCATGAACTCTGTCAACTGC	420
NeRNV-D1		
NeRNV-D2		
NeRNV-D3		
NeRNV-N1		
DIAYMV	ACCATCAAGGACAGCGTCTTGTACACGGACTCCCCCGACTCTTGGCTCACTCGCCCGCC	480
NeRNV-Nf	ACCATCAAGGACAGCGT CA TGTACAC CG ACT CA CCCC GTCTCT AG CTACTCT CGGCC	480
NeRNV-WA	ACCATCA AA AGACAGCGT CA TGTACAC CG ACT CA CCCC GTCTCT AG CTACTCT CGGCC	480
NeRNV-D1		
NeRNV-D2		
NeRNV-D3		
NeRNV-N1		

DIAYMV	CCTCTCACGGCCAAGACGATCCCCTCTGGCACCTTAGTCATTTCGTGGCAAGGTTGCCTT	540
NeRVN-Nf	CCTCTCACGGCCA AAACCA CCCC TTCC GGCAC CTCT TGTCATTTCGTGGCAAGG TCCTCTC	540
NeRVN-WA	CCTCTCACGGCCA AAACCA CCCC TTCC GGCAC CTCT TGTCATTTCGTGGCAAGG TCCTCTC	540
NeRVN-D1		
NeRVN-D2		
NeRVN-D3		
NeRVN-N1		
DIAYMV	TCTTCTCCCTCCCTCAGCCTCTCACAGCTTCTTCGTGAgggcgcgcatgccataatgc	600
NeRVN-Nf	TCTT CCCCCTCCTC CAGCCTCTCACAGCTT CTTC GTGAggg tg ggcgca ca cca ca gtgc	600
NeRVN-WA	TCTT CCCCCTCCTC CAGCCTCTCACAGCTT CTTC GTGAggg tg ggcgca ca ccat ag gc	600
NeRVN-D1		
NeRVN-D2		
NeRVN-D3		
NeRVN-N1		
DIAYMV	gcagtgtttcggcttccacttaaatcgaaagccaaccctccagcccgctcgtggtgttcc	660
NeRVN-Nf	g ta gtgtttcggcttccacttaaatcgaaagccaaccctccag tttt ctggtgttcc	659
NeRVN-WA	g ta gtgtttcggcttccacttaaatcgaaagccaaccctccag tttt ctggtgttcc	659
NeRVN-D1		
NeRVN-D2		
NeRVN-D3		
NeRVN-N1		
DIAYMV	ctggatcactacgtcaacttgcttgtgtaagcaagccggtgggaacacaaaattgtgag	720
NeRVN-Nf	t ggat tg ctacgt tc accag att gtgtaat ct ggccggt ag gaact tt aaaatt gc gcg	719
NeRVN-WA	tc ggat cg ctacgt tc accag att gtgtaat ct ggccggt ag gaact tt aaaatt gc gcg	719
NeRVN-D1		
NeRVN-D2		
NeRVN-D3		
NeRVN-N1		
DIAYMV	gagttcgaatctccccctgtcccgggtagggaacc	755
NeRVN-Nf	gagttcgaatctccccctgtcccgggtagggaacc	754
NeRVN-WA	gagttcgaatctccccctgtcccgggtagggaacc	754
NeRVN-D1		
NeRVN-D2		
NeRVN-D3		
NeRVN-N1		

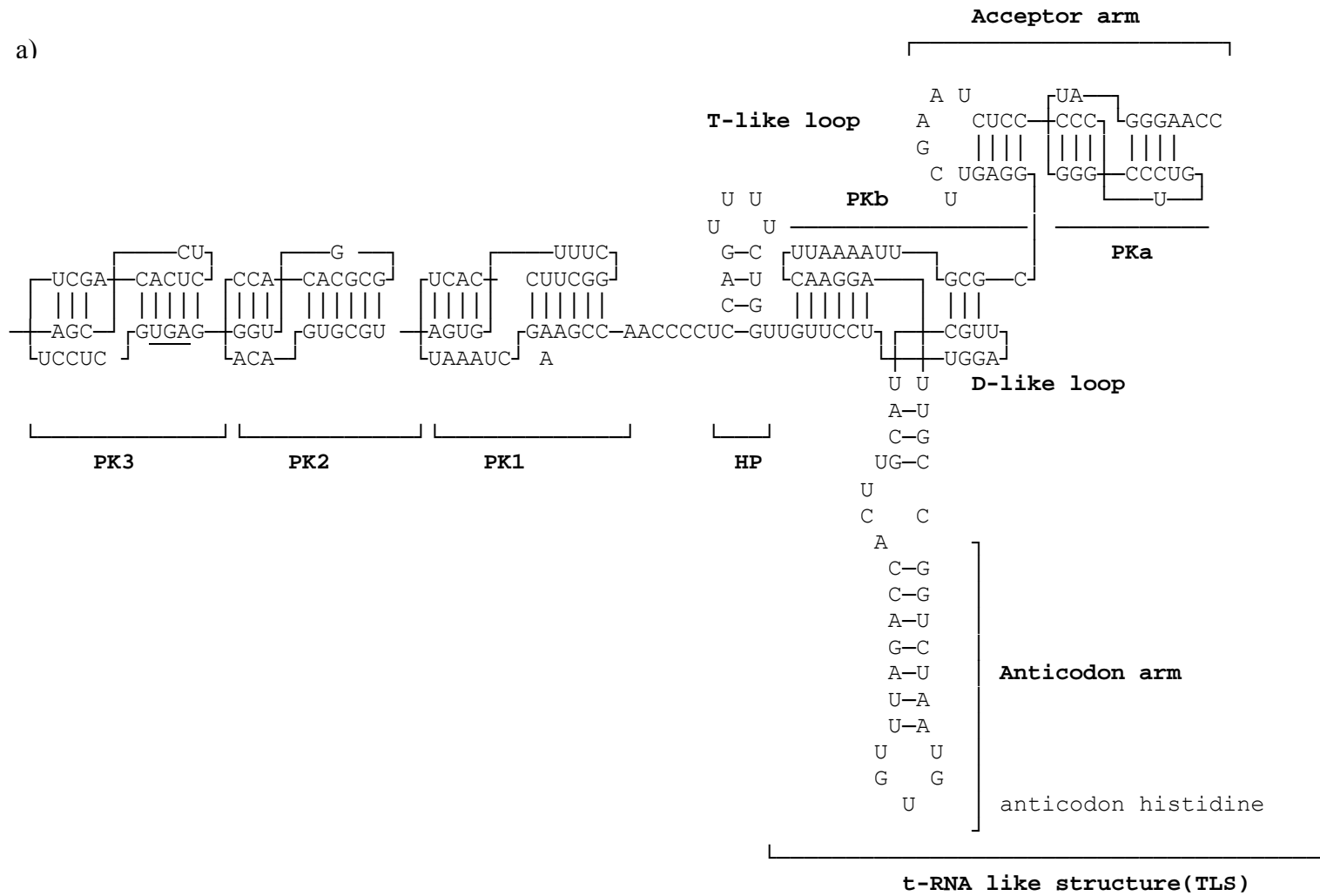
Figure 10. Nucleotide sequence alignments of open reading frame (ORF) III and the 3'-untranslated region of *Nemesia ring necrosis virus* (NeRVN-Nf); NeRVN-WA; Diascia yellow mottle virus (DiaYMV); NeRVN-D1 isolate 1 from diascia; NeRVN-D2 isolate 2 from diascia; NeRVN-D3 isolate 3 from diascia and NeRVN-N1 isolate 1 from nemesia. Bold letters show the base changes relative to DiaYMV. Partial sequences for NeRVN-D1, NeRVN-D2, NeRVN-D3 and NeRVN-N1 provided (Mathews and Dodds, 2006). The numbers on the right side indicate nucleotide positions. The open reading frame (ORF) III is capitalized and the 3' untranslated region (UTR) is in lower case.

DiaYMV	MEELKPISVKQPSIPAPGTKLPPAPGQQSSAIVQPFQVSVADLGVSEVNVQITLSSDPTL	60
NeRNV-Nf	MEELKPISVKQPSIPAPGTKLPPAPGQQSSAIVQPFQ ISV TDLGV AEV NVQITLSSDPTL	60
NeRNV-WA	MEELKPVSVKQPSIPAPGTKLPPAPGQQSSAIVQPFQ ISV TDLGV AEV NVQITLSSDPTL	60
NeRNV-D1	MEELKPISVKQPSIPAPGTKLPPAPGQ	27
NeRNV-D2	MEELKPISVKQPSIPAPGTKLPPAPGQ	27
NeRNV-D3	MEELKPISVKQPSIPAPGTKLPPAPGQQS	29
NeRNV-N1	MEEIKPISV	9
DiaYMV	SQLTAFYRHADLVECSAVLFPNFTSSSNPTHCDLAWVPANSTASPKNILKTYGGNRFTLG	120
NeRNV-Nf	SQLTAFYRHADLVECSAVLFPNFTSSSNPTHCDLAWVPANSTASPK AILR TYGGNRFTLG	120
NeRNV-WA	SQLTAFYRHADLVECSAVLFPNFTSSSNPTHCDLAWVPANSTASPK AILR TYGGNRFTLG	120
NeRNV-D1		
NeRNV-D2		
NeRNV-D3		
NeRNV-N1		
DiaYMV	GPITANEVISVPLPMNSVNCTIKDSVLYTDSPRLLAHSPAPLTAKTIPSGTLVIRGKVRL	180
NeRNV-Nf	GPITANEVIS I PLPMNSVNCTIKDSV MY TDSPRLL AYS PAPLTAKT TP SGTLVIRGKVRL	180
NeRNV-WA	GPITANEVIS I PLPMNSVNCTIKDSV MY TDSPRLL AYS PAPLTAKT TP SGTLVIRGKVRL	180
NeRNV-D1		
NeRNV-D2		
NeRNV-D3		
NeRNV-N1		
DiaYMV	SSPLQPLTASS*	192
NeRNV-Nf	SSPL L QPLTASS*	192
NeRNV-WA	SSPL L QPLTASS*	192
NeRNV-D1		
NeRNV-D2		
NeRNV-D3		
NeRNV-N1		

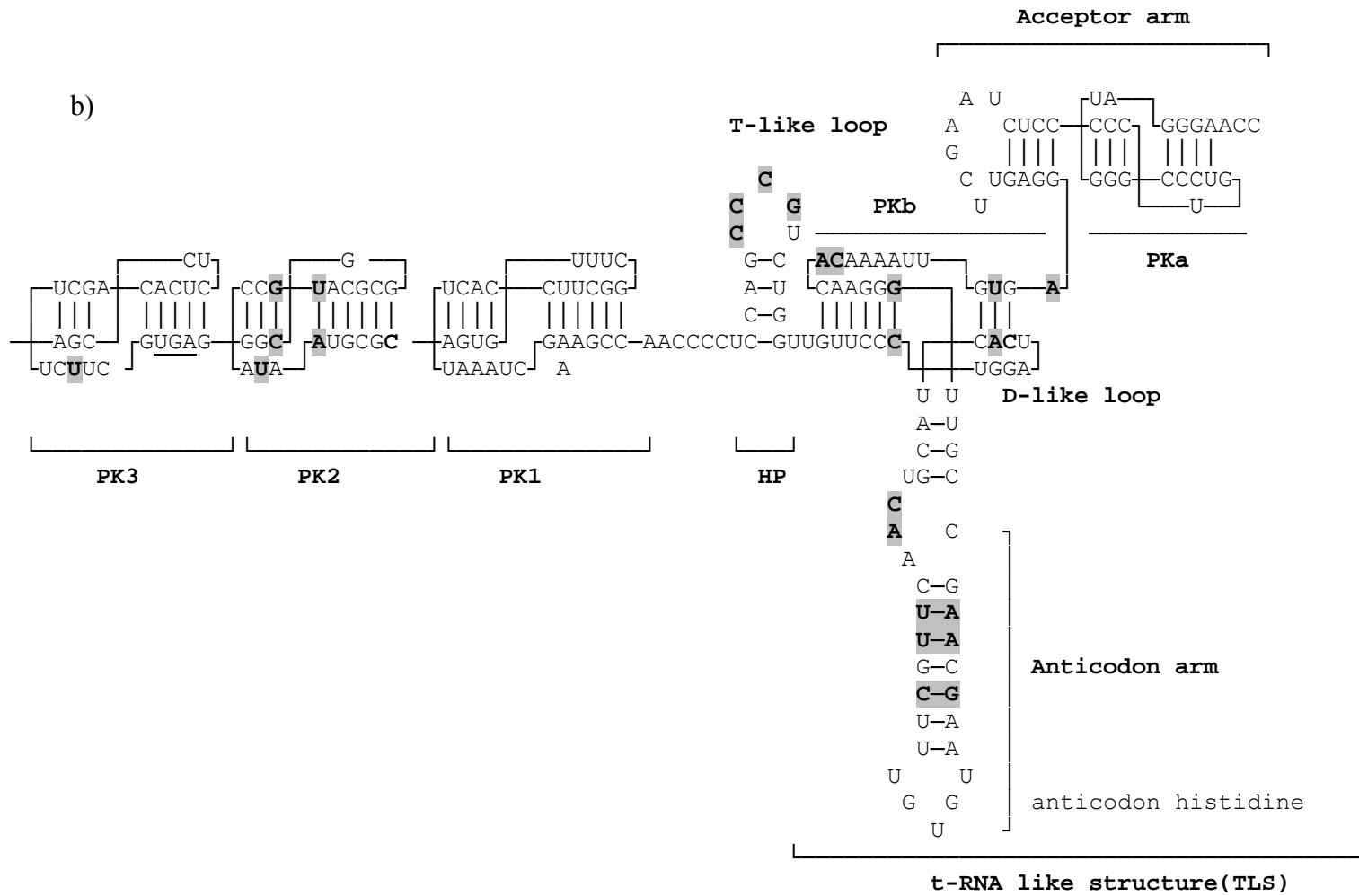
Figure 11. Amino sequence alignment of the open reading frame (ORF) III of *Nemesia ring necrosis virus* (NeRNV-Nf); NeRNV-WA; Diascia yellow mottle virus (DiaYMV); NeRNV-D1 isolate 1 from diascia; NeRNV-D2 isolate 2 from diascia; NeRNV-D3 isolate 3 from diascia and NeRNV-N1 isolate 1 from nemesia. Bold letters show the differences in amino acids relative to DiaYMV. An asterisk indicates the stop codon. Partial sequences for NeRNV-D1, NeRNV-D2, NeRNV-D3 and NeRNV-N1 provided (Mathews and Dodds, 2006).

Figure 12. Secondary structure model of the 3'-terminus of RNA from tymoviruses found in members of the Scrophulariaceae. a) *Nemisia ring necrosis virus* (NeRNV) RNA secondary structure redrawn from Koenig *et al.*, 2005a. b) Secondary structure model of the 3' end of the RNA of *Diascia yellow mottle virus* (DiaYMV) as drawn based on nucleotide sequence identity with *Nemisia ring necrosis virus* (NeRNV) RNA folding and predicted secondary structures (Koenig *et al.*, 2005a). Bold highlighted areas are differences in nucleotides between NeRNV and DiaYMV.

a)



b)



model for NeRNV-Nf (Koenig *et al.*, 2005a). There is a high degree of similarity in the potential for RNA folding of the two viruses which is different from the tRNA-like structure found in other tymoviruses. DiaYMV appears to have a 3'-terminus similar to NeRNV-Nf which can accept histidine while TYMV and other tymoviruses have a tRNA-like structure that can accept valine (Dreher, 1999; Dreher, 2004; Koenig *et al.*, 2005b). The anticodon sequence GUG of DiaYMV is different from CAC found in TYMV and GUU found in *Tobacco mosaic virus* (Dreher, 1999), but similar to GUG anticodon sequence in NeRNV-Nf. The GUG is the histidine codon in the anticodon sequence of both DiaYMV and NeRNV. Some of the similarities of the DiaYMV and NeRNV-Nf include: three upstream pseudo-knot domains (UPD) labeled PK 1 to 3 (Figs. 12a and 12b); sequence of the acceptor arm sequence, and the locations of the D-loop and the T-loop. The hairpin loop is located between the UPD and the tRNA-like structures; despite a few nucleotide changes between DiaYMV and NeRNV-Nf, the structural organization of the HP was not changed. A similar HP structure has been found in some furovirus and pomovirus RNAs, although it was not found in tobamovirus RNAs and its presence is thought to ensure that there is a proper mutual orientation of the UPD and the tRNA-like structure (Koenig, *et al.*, 2005a). One interesting observation was that despite the differences in the base pairing, the overall tRNA-like structure was maintained, suggesting the importance of the structure in the genomes of positive strand RNA viruses. The 3'-terminus is thought to be the site for regulating important events leading to the onset of the replication of the genome since the minus strand must be synthesized from this region, and it is also the site of events that maintain an intact 3' end (Dreher, 1999). Differences in the 3'-terminal structure is another criterion that is used in the classification of tymoviruses, and many of the tymoviruses have been shown to

have their 3'-terminus folded into a tRNA structure and the amino-acyl arm of the tRNA can be a pseudo knot structure. The 3' terminal secondary structure is further supporting the evidence that DiaYMV is more closely related to NeRNV than to other members of the 'OYMV cluster'. The occurrence of sequences similar to NeRNV-Nf is highly suggestive of a mixed infection by NeRNV and DiaYMV in *Diascia ×hybrida* 'Sun chimes™ coral'. A tymovirus isolated from diascia and verbena plants in California was recently identified as NeRNV. There is an 88% aa identity between DiaYMV and the NeRNV-isolate 1 from variety 'Red Ace' (Fig. 11) isolated from diascia plants in California (Mathews and Dodds, 2006). However, the amino acid sequence being compared consists of only 267 amino acids representing 14% of ORF II and 27 amino acids of ORF III. Hence it is difficult to make a meaningful comparison based on this small sequence. There have been two separate occurrences of NeRNV isolated from different cultivars of diascia in Europe (Skelton *et al.*, 2004; Koenig *et al.*, 2005a). Based on the sequence of 890 nucleotides from ORF II and ORF III, Mathews and Dodds (2006) demonstrated the presence of NeRNV in three diascia cultivars in California. This confirmed the presence of NeRNV in North America.

Diascia plants 'Red Ace' and 'Hannah Rose' are reported to be infected with viruses that are not tymoviruses based on dsRNA patterns (Mathews and Dodds, 2004). In this study it was observed that the plants were also susceptible to *Impatiens necrotic spot virus* (INSV). This study has also shown that *Diascia ×hybrida* 'Sun chimes™ coral' is infected with both NeRNV and another tymovirus for which a name Diascia yellow mottle virus is being suggested. Due to limited literature on NeRNV and its impact on its hosts, it has been difficult to ascertain if mixed infections with other tymoviruses are common. It is not clear what impact or role the mixed infection with two tymoviruses would have on the replication

of the viruses, and how this might impact symptomatology and general growth of the diascia plants. Diascia and nemesia plants belong to the family Scrophulariaceae and are often raised under similar management practices in nurseries; hence, it is possible that the plants are exposed to the same pathogens. Although this could not be confirmed for all the nurseries, diascia and nemesia plants collected from one nursery in Oregon all tested positive for NeRNV-Nf and DiaYMV and the nursery had the same supplier for both plants. This observation suggests that cultural practices in nurseries may help disseminate the two viruses.

Virus species classification can be cumbersome, and it is the polythetic nature of viruses that drives us to believe that DiaYMV is a new species. The ICTV guidelines for species demarcation for the tymovirus (Martelli *et al.*, 2002) includes capsid protein sequences less than 90% identity, and overall sequence identity of less than 80%. The occurrence of quasi-species has not been ignored, and variations in the sequences are thus expected. For this reason, molecular criteria alone should not be the criteria used to classify tymoviruses. Other criteria used to classify viruses have been employed and the results of those findings will be discussed in subsequent chapters. On the other hand, it is not uncommon to find nucleotide differences in tymoviruses, the occurrence of variations within plants by hybrid mismatch polymorphisms and also by the use of sequence data of the TYMV isolates have been reported (Skotnicki *et al.*, 1993; Skotnicki *et al.*, 1996). In the genus *Pomovirus*, nucleotide differences have been reported between clones that varied considerably in different parts of the genome of *Beet soil-borne pomovirus* (BSBV) (Koenig *et al.*, 2000). These differences were also found in plantlets which were grown in the same

soil sample. The report however ruled out PCR artifacts and maintained that the high variability in parts of the BSBV genome were most probably due to higher mutation rates.

CONCLUSION:

Based on the analysis of overall genome data, it is evident that DiaYMV is a unique virus in the genus *Tymovirus*. This proposal for a new species is based primarily on the sequence differences observed in ORF I, despite amino acid identities of the putative coat protein to that of NeRNV. This report however acknowledges that NeRNV also occurs in *Diascia ×hybrida* ‘Sun chimes™ coral’.

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CHAPTER 3

MONOCLONAL ANTIBODY PRODUCTION AND SEROLOGICAL DIFFERENTIATION OF THE DIASCIA YELLOW MOTTLE VIRUS

SUMMARY:

Three mice (BALB/c) were immunized with virus purified from *Diascia ×hybrida* 'Sun chimes™ coral' infected with a tymovirus. Hybridoma cell lines were identified following somatic cell fusion between the myeloma cell line FOX-NY and spleen cells from immunized mice. A total of 35 antibody-secreting hybridoma cell lines were isolated after screening by triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA). One cell line designated M3-7·A6·1 produced an antibody of isotype IgA and the specificity of this antibody was determined. The purified antibody M3-7·A6·1 reacted strongly with virus-infected tissue but not with healthy tissue demonstrating that it discriminates between virus infected and non-infected samples in a TAS-ELISA as well as in plate trapped antigen-ELISA (PTA-ELISA). The antibody detected DiaYMV in various plant samples tested. Monoclonal antibody M3-7·A6·1 will be useful for rapid detection of this virus.

INTRODUCTION:

Serological techniques have long been used for detecting plant viruses and enzyme-linked immunosorbent assay (ELISA) is the most convenient, reliable, fast and relatively inexpensive procedure (Nikolaeva *et al.*, 1998). The introduction of ELISA into plant virology (Clark and Adams, 1977) in combination with the use of monoclonal antibodies

permits detection of virus in nanogram quantities (Sander and Dietzgen, 1984). The production of monoclonal antibodies is based on the fusion of antibody-secreting spleen cells with myeloma cells, and the resulting hybrid cells (hybridomas) are cloned so that cell lines are established from a single hybridoma. Each cell line or clone produces antibodies of only one type and valency (Barrett, 1983; Sander and Dietzgen, 1984). To increase the fraction of hybridomas producing antibodies against the desired antigen, the splenocyte donor is immunized with highly purified immunogen. However, partially purified antigen also can be used but more vigorous screening may be required to obtain hybridomas of interest (Dietzen and Sander, 1982).

Monoclonal antibodies have some advantages and disadvantages over polyclonal antibodies. Since hybridomas secreting monoclonal antibodies are immortal, they can be stored and revived almost indefinitely to provide a stable source of antibody. This feature ensures uniform results, better comparison over time and allows for protocol standardization. Polyclonal antibodies do not provide a consistent supply of the same antiserum due to inherent variability of individual bleedings from an immunized animal (Van Regenmortel and Dubs, 1993). Another advantage in plant virology is that a less pure immunogen is acceptable with monoclonal antibodies whereas this leads to high backgrounds and difficulties in interpreting assays with polyclonal antibodies. Some of the limitations of monoclonal antibodies are that they are time consuming to develop, and they tend to be more assay specific than polyclonal antibodies. Production of monoclonal antibodies was selected for this research because of the fact that there was a mixed virus infection in the diascia host plants used in this study, and this technique may provide a means to obtain serological reagents that would discriminate between the two viruses found in diascia and nemesia.

Koenig *et al.* (2005) has shown that NeRNV and ScrMV belong to a cluster of tymoviruses that are serologically related and cross-react. Consequently, producing a polyclonal antibody would provide limited options if we wished to differentiate the two viruses based on serology. The primary objective of the study was to develop a robust serologically based diagnostic assay that can be used in nurseries and for general detection of tymoviruses causing disease in diascia and nemesia ornamental plants.

MATERIALS AND METHODS:

Virus Purification: Purified virus was isolated from *Diascia ×hybrida* ‘Sun chimes™ coral’ using the protocol for purifying the virus from *Nicotiana occidentalis* described in Chapter 2. Previous results have shown that *Diascia ×hybrida* ‘Sun chimes™ coral’ was infected with DiaYMV and NeRNV-WA.

Immunizations: Three six-week old BALB/c female mice (Taconic: Hudson, NY) were immunized with purified virus. Each mouse was administered an intraperitoneal injection of 50 µg purified virus in phosphate-buffered saline (PBS) (1.5 mM KH₂PO₄; 4 mM Na₂HPO₄; 137 mM NaCl; 0.2 mM KCl) pH 7.4, and emulsified in an equal volume of Freund’s complete adjuvant (Sigma Aldrich, St. Louis, MO). After three weeks, each mouse was given an intraperitoneal injection of 50 µg virus in Freund’s incomplete adjuvant (Sigma), followed by two more booster injections every two weeks. Serum was collected by tail bleeding method as described by Harlow and Lane (1988). The final dose of 50 µg virus in PBS was delivered intravenously. Five days after the final injection, spleen cells were extracted from euthanized mice and used for the cell fusion.

Hybridoma production: One week before the fusions, FOX-NY myeloma cells (ATTC: Manassas, VA) were cultured in 15 ml Dulbecco's modified Eagle's medium (DMEM with sodium pyruvate, L-Alanyl-L-Glutamine and high glucose) (Cat no. 10569: Invitrogen, Carlsbad, CA) containing 20% certified, heat-inactivated fetal bovine serum (FBS) (Cat no.10082-139: Invitrogen) and 50 µg/ml gentamicin (Invitrogen). This tissue culture media was referred to as 20% DMEM. Cells were placed in a humidified incubator at 37 °C with 7% CO₂ and maintained so that approximately 10⁶ actively growing myeloma cells were available at the time of each fusion.

Cell fusion protocols were adapted from established protocols (Harlow and Lane, 1988; Goding, 1996) and modified to our conditions. All procedures were performed at room temperature. On the day of the fusion, 200 µl myeloma cells were collected by centrifugation at 800×g for 10 min for each mouse. The supernate was carefully decanted and the cells were consolidated into a single 50 ml tube and gently resuspended in serum-free DMEM and set aside to be used for cell fusion.

Each of three spleens were obtained from immunized BALB/c mice and processed separately. Each spleen was surgically removed, extraneous connective tissue was removed and the spleens were rinsed three times in serum-free DMEM. Spleen cells were isolated by placing the spleen into 10 ml of serum-free DMEM and covered with four layers of cheese-cloth mesh. The spleen cells were gently released by pressing the spleen with a sterile blunt end of a 20 ml syringe plunger. The spleen cells were released into the media by repeatedly washing the mesh with media. The splenocytes were collected in a 50 ml centrifuge tube and gently centrifuged at 400×g for 10 min at room temperature. The supernate was discarded and the cells were washed again in serum-free DMEM and the myeloma cells were also

centrifuged in a separate tube as above. After the final wash, the two cell pellets were resuspended in 10 ml serum-free DMEM pre-warmed to 37°C and combined at equal ratios of packed cell volumes. The cells were centrifuged together at 800×g for 5 min. Excess liquid was removed and the cells in the dry pellet were mixed by gentle agitation. One ml of 50% polyethylene glycol (PEG 4000) in Dulbecco's phosphate buffered saline (Cat. No. 14030-035: Invitrogen) was slowly added over 1 min by stirring with the end of the pipette, and then stirred for an additional minute. A 10 ml pipette was filled with pre-warmed serum-free DMEM; 1 ml was added to the cell suspension over the next minute while stirring with the end of the pipette. The remaining 9 ml were added over a 2 min period while stirring. The cells were centrifuged at 500×g for 5 min at room temperature. The supernate was removed and the cells were resuspended in 10 ml 20% DMEM supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine (1×HAT) (Invitrogen). The cells were transferred to 200 ml of 20% DMEM containing 1×HAT and dispensed into ten 96-well microtiter plates (Nalge Nunc International, Rochester, NY), and the plates-maintained in the 7% CO₂ humidified incubator at 37°C.

Cells were nourished with 10% DMEM containing 1×HAT as necessary depending on cell density. Two weeks after the fusion, the cells were supplemented with 100 μl per well of 10% DMEM containing 1×HAT, and screened for antibody secreting hybridomas.

Hybridoma screening: Flat bottom 96-well Maxisorp plates (NUNC) were used to screen for antibody-producing hybridomas by TAS-ELISA. The wells were coated with 125 μl DiaYMV antiserum solicited against expressed coat protein and produced in sheep (provided by K. C. Eastwell) diluted 1:250 in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6). Wells were washed three times with PBS containing 0.05%

polyoxyethylene sorbitan monolaurate (Tween-20) (PBST). Plates were tapped dry and the wells were blocked with 300 μ l of blocking buffer (PBST plus 3% dried non-fat milk) for 30 min at room temperature.

Young upper leaves of healthy and infected *Nicotiana occidentalis* plants were collected separately into sample grinding bags (Agdia Inc, Elkhart, IN), and the tissue was ground at a ratio of 1 part tissue to 10 volumes of sample extraction buffer (20 mM NaSO₃, 0.5 mM polyvinylpyrrolidone molecular weight 40,000, 0.2 % powdered egg (chicken) albumin, in PBST). Each well was loaded with 125 μ l sample extract; the plates were incubated at 4°C for 2 hr, followed by washing three times with PBST. The wells were blocked with 300 μ l blocking buffer for 30 min at room temperature. The blocking solution was discarded and 25 μ l blocking buffer was added to each well followed by 100 μ l tissue culture supernate. Plates were incubated at 4°C overnight in a humidified container. Plates were washed as above and wells were blocked with blocking buffer for 30 min as described above. Phosphatase-labeled affinity purified antibody to mouse (GAM) (Goat anti-mouse IgG+IgM+IgA: Kirkegaard and Perry Laboratories, Gaithersburg, MD) at a dilution of 1:3000 (v/v) in blocking buffer was added at 125 μ l per well. The plates were then incubated at room temperature for 2 hr and washed as before. After the final wash, wells were filled with PBST and allowed to stand for 2 min. The assay was completed by adding 125 μ l substrate buffer mixture (0.1% p-nitrophenyl phosphate in 9.7% diethanolamine, pH 9.6; 1 mM MgCl₂). The plates were incubated at room temperature and absorbance at 405 nm (A_{405}) was measured (Multiskan Ascent: Thermo electron Corporation, Waltham, MA) at 30 min, 60 min and 120 min intervals. Positive wells were defined as those with A_{405} twice the average reading of a healthy control sample (Sutula *et al.*, 1986). Positive control sample

was DiaYMV-infected *Nicotiana occidentalis* while the negative control was non-inoculated *Nicotiana occidentalis* that had tested negative by RT-PCR; a buffer-only well was also used as a check.

Cells in wells that yielded positive ELISA results in the first screen were transferred to 48-well plates and re-screened. Cells that yielded positive results in a TAS-ELISA were transferred to 24-well plates. The positive wells from 24-well plates were transferred to 6-well plates and screened as described for 48-well plates. Cells from positive wells in 6-well plates were single cell cloned and the bulk of the cells were frozen and kept in vials in liquid nitrogen for future use.

The procedure for freezing cells for storage was outlined in Harlow and Lane (1988). Healthy and rapidly dividing cells were transferred to a sterile centrifuge tube and centrifuged at 400×g at 4°C for 5 min. All the supernatant was removed and cells were gently resuspended in cold 8% dimethyl sulphoxide (DMSO) (Sigma) in FBS. The cell suspension was dispensed into 1 ml cryotube vials (Nalgene Nunc) and transferred to a pre-chilled freezing container to cool the cells at 1°C per minute (Nalgene Nunc). The freezing container was kept at -80°C overnight and the vials were then transferred to liquid nitrogen for long term storage.

Single cell cloning: A 3% agarose gel was made by dispersing 1.5 g ultra-low gelling temperature agarose (Sea Prep: Cambrex, Rockland, ME) in 50 ml glass distilled water and autoclaved. DMEM was prepared at double standard concentrations (2×DMEM) by stirring powdered DMEM (Cat. No. D-7777; Sigma-Aldrich) into 450 ml of sterilized glass distilled water until dissolved, then 3.7 g of sodium bicarbonate (Sigma) was added and the solution was diluted to 500 ml. The 2×DMEM was sterilized by filtering by positive

pressure. Prior to use, the 3% agarose was melted by heating in a microwave and kept in a 37°C water bath, 2×DMEM was supplemented with 20% FBS, 20 mM HEPES, pH 7.5, 100 µg/ml gentamicin, 0.1 mM sodium hypoxanthine and 0.016 mM thymidine and equilibrated at 37°C in the 7% CO₂ incubator until use.

The agarose and 2×DMEM were mixed in a ratio of 1:1 (v/v) and kept in a 37°C water bath. Single cell cloning was then carried out by serial dilution of cell cultures (5^{-1} , 5^{-2} , 5^{-3} , 5^{-4} , and 5^{-5}) in 5 ml soft agar in 60 mm × 15 mm Petri-dishes that were then incubated at 37°C in a 7% CO₂ incubator for approximately 2 wks until colonies were large enough to pick under a dissecting microscope. Colonies were individually picked for each hybridoma cell line and cultured in 96-well plates containing 200 µl 20% DMEM until ready to screen by TAS-ELISA as described above. One hybridoma cell line that tested positive from each cloning was then subjected to a second round of single cell cloning.

Monoclonal antibody production: The cell-line M3-7-A6.1 was transferred to 50 ml culture flasks in 30 ml 20% DMEM supplemented to a final concentration of 1% glucose and 25 mM HEPES. Cultures were allowed to overgrow but not die and the media was collected and replaced with fresh media. Cells were removed from the media by centrifugation and the collected monoculture supernate was used for screening DiaYMV infected plants and also purified for further analysis.

Immunoglobulin isotype determination: Antibody isotype produced by hybridoma cell line M3-7-A6.1 was determined in tissue culture supernate using a TAS-ELISA procedure (mouse monoclonal ID kit (AP): Zymed Laboratories, San Francisco, CA) following the manufacturer's recommended protocol.

Immunoglobulin purification: A protocol for hydroxyapatite chromatography was followed with slight modifications from published procedures (Harlow and Lane, 1988; Gagnon, 1998). The culture supernate was dialyzed against 50 mM Na₂HPO₄, pH 6.8 overnight at 4°C. A column (1.5 cm × 50 cm) was packed with hydroxyapatite (HA Ultrogel: Sigma) and the column was allowed to settle. The column was washed with 500 ml 300 mM Na₂HPO₄, pH 6.8 at a flow rate of 2 ml/min (BioLogic LP: BioRad, Hercules, CA). The column was equilibrated with 10 column volumes (CV) 50 mM 3-(N-morpholino) propane sulfonic acid (MOPS) buffer, pH 6.8, at a flow rate of 2 ml/min. The dialyzed culture supernate was loaded onto the column (2 ml/min) followed by washing with 5 CV 50 mM MOPS, pH 6.8 with 100 ml 50 mM Na-PO₄, pH 6.8 at a flow rate of 5 ml/min and the eluate was collected in 20 tubes. The column was developed sequentially with 100 ml of 100 mM Na-PO₄, 100 ml of 150 mM Na-PO₄ in the next 20 tubes and 100 ml 300 mM Na-PO₄ buffer. After collection, the column was regenerated with 300 mM Na-PO₄ buffer, pH 6.8.

The collected fractions from the columns were tested for the presence of the antibody using a PTA-ELISA. Maxisorp plates were coated with column fractions diluted 50:50 in carbonate buffer, the plates were incubated at room temperature for 4 hr and washed in PBST buffer three times and tapped dry. After washing and blocking as described above, 100 µl GAM is added to each well. The plates were incubated for 2 hr at room temperature, washed and tapped dry. Fractions containing immunoglobulin were detected adding substrate and the absorbance reading were recorded after 30 min and 1 hr.

Fractions with high ELISA readings ($A_{405} \geq 0.200$) were pooled and immunoglobulin was concentrated by (NH₄)₂SO₄ precipitation (Harlow and Lane, 1988). One-half volume of cold saturated (NH₄)₂SO₄ was slowly added to the pooled column fractions and maintained at

4°C overnight. The suspension was centrifuged at 3,000×g for 30 min, and the supernate was transferred to a beaker. Another one-half volume of cold saturated (NH₄)₂SO₄ was added drop wise with stirring and then stored at 4°C overnight. The precipitated protein was collected by centrifugation at 3,000×g for 30 min. The pellet was resuspended in one-tenth volume PBS and dialyzed (Slide-A-Lyzer dialyzing cassette 10K: Pierce, Rockford, IL) against three changes of PBS buffer every 3 hr, and final buffer change held at 4°C overnight. The absorbance of the immunoglobulin was measured (Shimadzu UV-160A spectrophotometer: Shimadzu Corporation, Koyto, Japan). The protein concentration was adjusted with PBS buffer to 1 mg/ml assuming an absorbance of 1.4 at 280 nm.

Standard ELISA conditions: For PTA-ELISA, plant tissue was extracted in carbonate buffer (1 g in 10 ml buffer) in sample grinding bags (Agdia). For each plant sample two wells were coated with 100 µl sample extract and the plates were incubated overnight at 4°C. Wells were washed three times with PBST tapped dry and blocked with blocking buffer for 30 min and the blocking buffer was discarded. A 1:500 v/v dilution of purified antibody in blocking buffer was added and incubated at room temperature for 2 hr, washed and blocked again with blocking buffer. A 1:3000 dilution of alkaline phosphatase labeled GAM in blocking buffer and detection was carried out as outlined earlier.

To optimize reaction conditions, purified virus from *Nicotiana occidentalis* was diluted in healthy plant extract using four fold serial dilutions. A four fold dilution series of the monoclonal antibody were also done. During virus detection, absorbance readings were taken at 10 min intervals for up to 3 hr.

A commercially available polyclonal antibody detection kit for NeRNV was obtained (Agdia Inc., Elkhart, IN). The manufacturer's recommended protocol was followed.

Western blot analysis: The procedure for preparation and casting of a 12% gel for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the BioRad mini protean II apparatus was followed as outlined in the manufacturer's manual (BioRad, Hercules, CA). The total protein extract of healthy or infected leaf sample was obtained by homogenizing plant samples in 4 ml/g 0.5 M Tris-HCl buffer, pH 6.8 (1:4 w/v) in a pre-cooled mortar and pestle. Extracts were centrifuged at 14,000 rpm in a microcentrifuge (Eppendorf Model 5415C: Brinkmann) for 3 min. Twenty microliter of the supernate was added to 5 μ l loading buffer to yield a final concentration of 62.5 mM Tris-HCl, pH 6.8; 10% glycerol, 2% SDS, 5% 2-mercaptoethanol (2-ME) and 0.05% bromophenol blue. The sample was boiled for 5 min and allowed to cool before loading onto the gel. Each lane was loaded with 12 μ l sample or 5 μ l Benchmark protein ladder (Invitrogen). Electrophoresis was performed at 100 V until the tracking dye reached the bottom of the gel. The gel was stained in 45% methanol, 10% glacial acetic acid, 45% double distilled water (ddH₂O), 0.25% Coomassie Brilliant Blue R-250 overnight and destained in 45% methanol, 10% glacial acetic acid in ddH₂O (Sambrook *et al.*, 1989). The proteins resolved in SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane (0.45 μ M, Micron Separations Inc., Westborough, NJ) using a MiniTransblot apparatus (BioRad) following the manufacturer's recommendations. The blot was washed three times with 0.1 M Tris buffer, pH 6.5 containing 0.9% NaCl and 0.1% Tween (TBS-T) for 15 min followed by incubation in a solution of 3% non-fat milk in TBS-T at room temperature for at least 0.5 hr and then rinsed with TBS-T. The membrane was incubated with the antibody diluted at 1:1000 in TBS-T plus 3% non-fat milk for 2 hr at room temperature or overnight at 4°C and then washed as above. The membrane was incubated at room temperature for 2 hr in a solution of

GAM diluted 1:3000 in TBS-T plus 3% non-fat milk. The membrane was washed with TBS-T as above and finally incubated in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (SigmaFast BCIP/NBT: Sigma). The reaction was stopped by transferring the membrane to ddH₂O after sufficient intensity of the signals was attained. The nitrocellulose membrane was air dried and preserved.

RESULTS:

Cell fusion and hybridoma isolation: Initially, two fusions were performed. After screening 1,600 wells, no hybridomas producing antibodies that reacted strongly and specifically with DiaYMV were identified. Fibroblast contamination further hampered selective screening of positive cell lines. At later stages of screening, fungal contamination also occurred. Consequently, only one hybridoma cell line was recovered from these fusions. Antibodies from hybridoma cell line M3·C1·6 reacted weakly with extracts of infected DiaYMV plants

A second set of three fusions yielded 2,400 wells, of which a large proportion contained multiple hybridomas. Screening against healthy and virus infected tissue yielded a total of 35 hybridoma cell lines secreting antibodies that reacted with virus infected tissue only, of which, hybridoma cell line M3-7·A6·1 was selected for further characterization. An ELISA assay used to identify the isotype of this antibody yielded the highest absorbance values for the IgA heavy chain and kappa light chain (Table 3). A weak reaction was also evident with antibodies for isotype IgG₁.

Evaluation of monoclonal antibody M3-7·A6·1: A preliminary test comparing TAS-ELISA and PTA-ELISA showed that the TAS-ELISA assay was more sensitive than

Table 3. Detection of immunoglobulin heavy and light chains in hybridoma culture supernate M3-7·A6·1

<i>Type of Immunoglobulin</i>	<i>Absorbance ratio:</i>
Heavy chain	
IgG ₁	0.204 ± 0.410
IgG _{2a}	0.076 ± 0.004
IgG _{2b}	0.074 ± 0.001
IgG ₃	0.076 ± 0.001
IgA	1.659 ± 0.127
IgM	0.082 ± 0.002
Light chain	
Kappa	1.679 ± 0.247
Lambda	0.085 ± 0.004
Controls	
Buffer	0.071 ± 0.001
Native rabbit serum	0.071 ± 0.001

PTA-ELISA. The signal-to-background ratio for TAS-ELISA absorbance was higher than for PTA-ELISA. However, PTA-ELISA reliably distinguished between healthy and infected tissues, and eliminated an extra step in the assay; hence further evaluation of the antibody was done using the PTA-ELISA.

In an experiment to show the effect of monoclonal antibody concentration and sample dilution on DiaYMV absorbance values, it was shown that purified virus can be detected by PTA-ELISA to as low as 6.25 μg per well when the antibody concentration was 0.39 $\text{ng}/\mu\text{l}$ (Fig. 13). By increasing the antibody concentration to 100 $\text{ng}/\mu\text{l}$, the absorbance values of the ELISA increased linearly, but there is no appreciable increase in the lower limit of virus detection. At the highest level of antibody concentration (100 $\text{ng}/\mu\text{l}$) used in this trial, the assay was not saturated suggesting that further increases in antibody concentration may produce higher ELISA readings. At 1.563 $\text{ng}/\mu\text{l}$ monoclonal antibody concentration and with 10 μg purified virus per well diluted in healthy plant extract, the PTA-ELISA yielded a linear time course until the absorbance value reached 4.0 (Fig. 14). There were no significant differences between the replicate wells indicating that assay was reproducible. Comparison of the purified virus solution and healthy sap showed that the purified virus solution reached optimal absorbance value ($A_{405}=2.0$) in just after 30 min following the addition of the substrate and the reaction was more gradual with the sample dilution at 1:100 (10 μg purified virus) (Fig. 14).

The commercial NeRNV antibody detected both the DiaYMV and *Scrophularia mottle virus* (ScrMV) whereas the M3-7-A6-1 detected only NeRNV and not ScrMV (Fig. 15). In both assays there were significant difference in the absorbance values between the TAS-ELISA and DAS-ELISA.

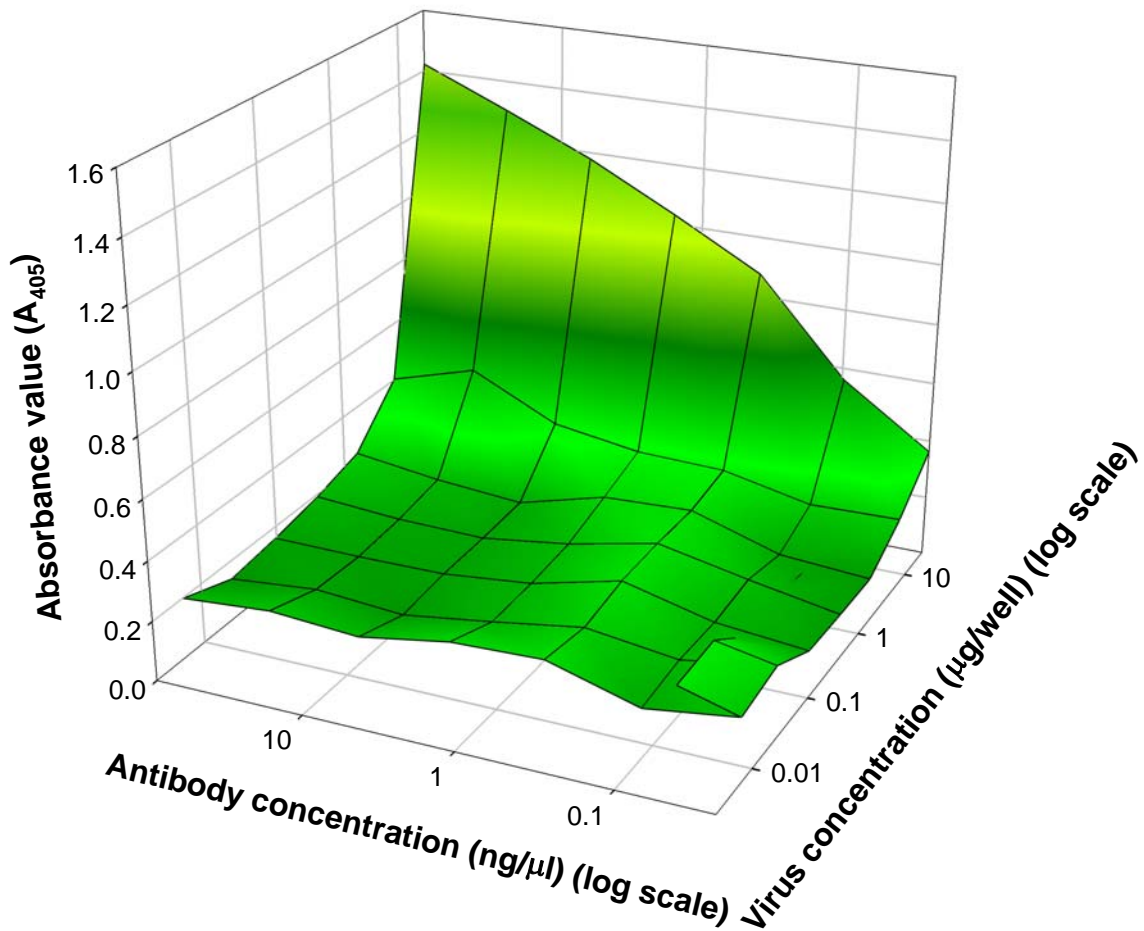


Figure 13. A graph showing the effect of antibody concentration and sample dilution on the mean absorbance values of a plate trapped antigen–enzyme linked immunosorbent assay (PTA-ELISA) using virus infected *Nicotiana occidentalis*. Absorbance readings (A_{405}) were recorded 30 min after the addition of the substrate and the readings were calculated as a difference from the healthy control readings.

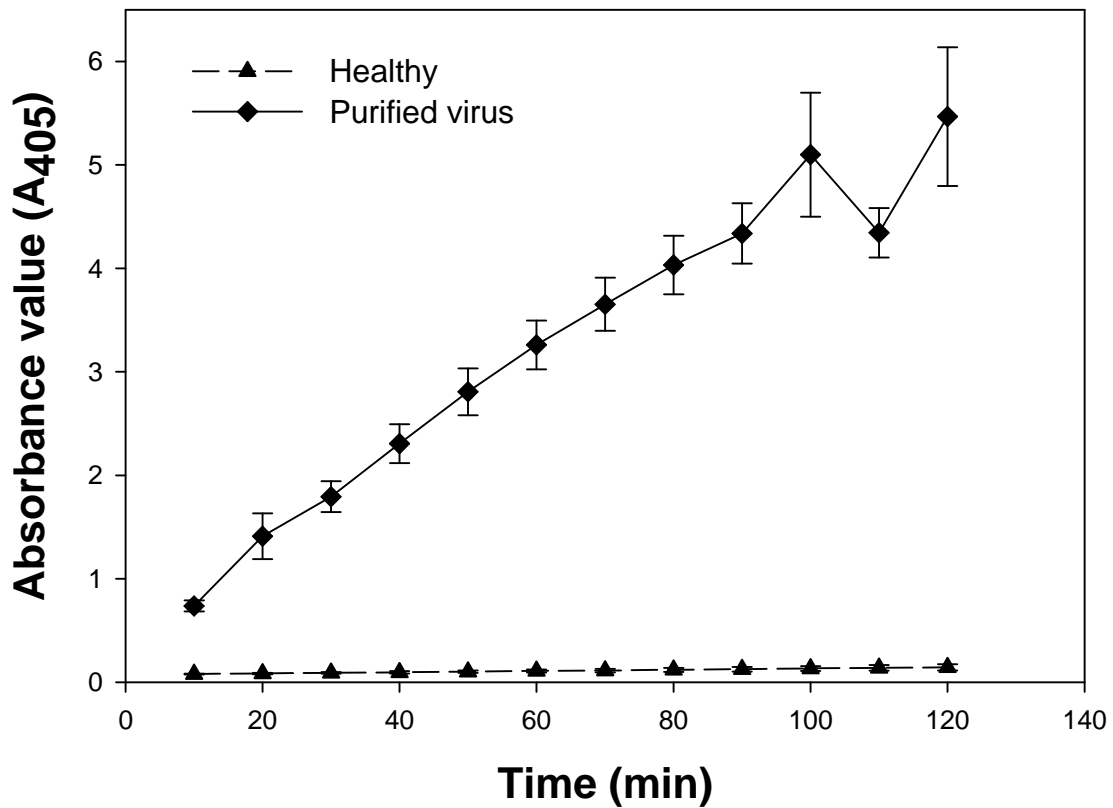


Figure 14. Graph showing course of the plate trapped antigen-enzyme linked immunosorbent assay (PTA-ELISA) with monoclonal antibody M3-7-A6-1. Absorbance values at 405 nm were measured for samples tested by PTA-ELISA. The samples were healthy extracts of *Nicotiana occidentalis* (\blacktriangle); and 10 μg purified tymovirus from *Diascia \times hybrida* ‘Sun chimes™ coral’ (\blacklozenge). The monoclonal antibody concentration was 1.6 ng/ μl .

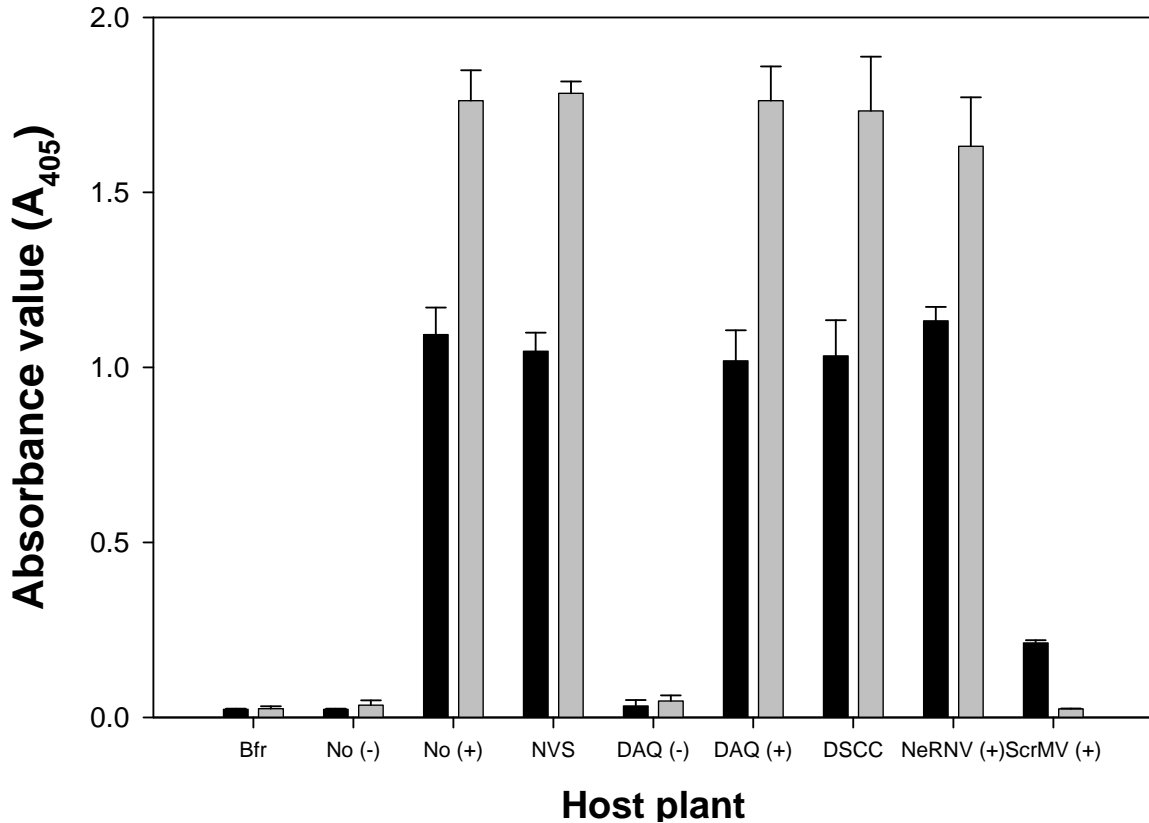


Figure 15. Comparison between double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) using commercial *Nemesia ring necrosis virus* (NeRNV) antibody conjugate, and triple antibody sandwich-enzyme linked immunosorbent assay (TAS-ELISA) using monoclonal antibody M3-7·A6·1 for virus detection in different host plants. Absorbance values were measured 30 min after the addition of the substrate. Black bars are values from DAS-ELISA and gray bars are from TAS-ELISA.

Bfr : Buffer only (negative check); No (-) : Healthy *Nicotiana occidentalis*; No (+) : tymovirus infected *Nicotiana occidentalis*; NVS : Infected *Nemesia ×hybrida* ‘Vanilla sachet™’; DAQ (-): Healthy *Diascia barberae* ‘Apricot queen’; DAQ (+): *tymovirus* infected *Diascia barberae* ‘Apricot queen’; DSCC : tymovirus infected *Diascia ×hybrida* ‘Sun chimes™ coral’; NeRNV (+): NeRNV positive control from Agdia ; ScrMV (+): *Scrophularia mottle virus* positive control.

Extracting the virus from infected tissue using a 1 in 100 extraction buffer for TAS-ELISA did not negatively affect the absorbance values but the reaction was slower and reached the optimal absorbance values later. There were significant differences on absorbance values of different hosts infected with the DiaYMV, when the TAS-ELISA was performed at different sample dilutions. Generally a 1 in 10 sample dilution reached the optimal absorbance value of 2.0 in 20 min after adding the substrate in assays of DiaYMV-infected *Nicotiana occidentalis* whereas the DiaYMV-infected *Diascia barberae* ‘Apricot queen’ yielded an absorbance value of 1.0 within the same time period (Fig. 16). There was a gradual linear decline in the absorbance values with *Nicotiana occidentalis* when samples were diluted further, whereas there were no significant differences in the absorbance values for *Diascia barberae* ‘Apricot queen’ when samples were diluted 1 in 10 or at 1 in 100.

Results of the Western blot analysis showed that the monoclonal antibody was not able to detect DiaYMV in the infected plant extracts (Fig. 17, Lane 3-7). However, the alkaline phosphatase labeled GAM detected the purified immunoglobulin M3-7·A6·1 (Fig. 17, Lane 1-2). Two protein products of the IgA were detected in the lanes with the purified monoclonal antibody; their estimated molecular masses were ~55 kilodaltons (kDa) for heavy chain and ~25 kilodaltons for the light chain.

DISCUSSION:

The immunoglobulin (Ig) isotype detected in cell culture supernate from cell line M3-7·A6·1 was an IgA heavy chain with a kappa light chain. The culture supernate also yielded a weak positive reaction for the IgG₁ heavy chain in ELISA. The most common immunoglobulins detected in culture supernate typically are the IgG subclasses; however,

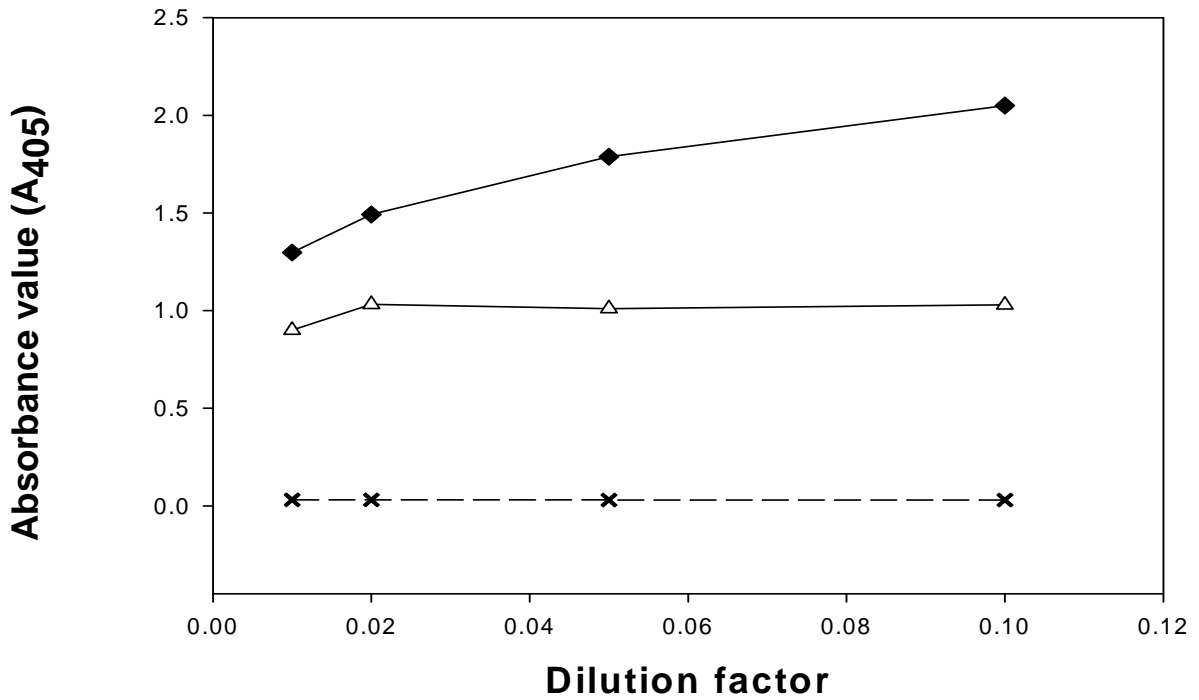


Figure 16. The effect of sample dilution on the mean absorbance (A_{405}) value of triple antibody sandwich-enzyme linked immunosorbent assay (TAS-ELISA) results using monoclonal antibody M3-7·A6·1. Absorbance values (A_{405}) were measured 20 min following the addition of the substrate. Samples were uninfected *Diascia barberae* ‘Apricot queen’ (×), tymovirus infected *Diascia xhybrida* ‘Sun chimes™ coral’ (Δ) and *Nicotiana occidentalis* inoculated with the tymovirus from *Diascia xhybrida* ‘Sun chimes™ coral’ (◆).

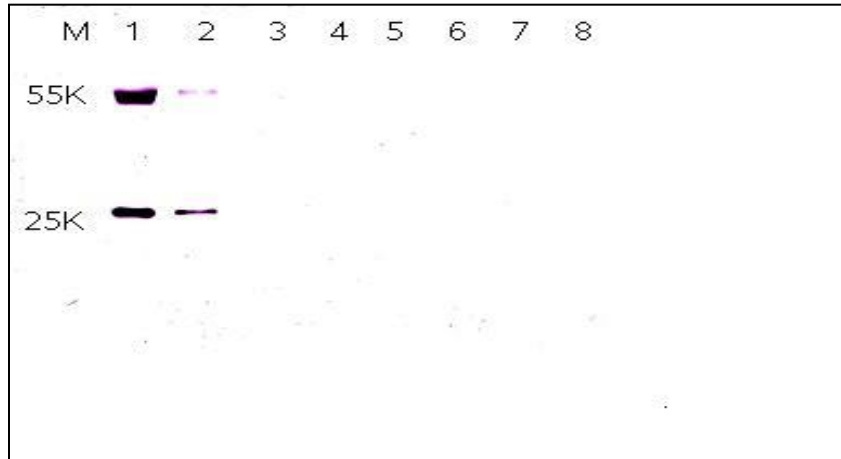


Figure 17. Western blot analysis of the purified monoclonal antibody of M3-7-A6-1 and *Diascia* yellow mottle virus (DiaYMV) infected plant tissue extracts. Lane M: benchmark ladder (Invitrogen); lane 1: 1/50 dilution of the purified immunoglobulin; lane 2: 1/500 dilution of the purified immunoglobulin; lane 3: 1/100 dilution of the purified DiaYMV from infected *Nicotiana occidentalis*; lane 4: 1/5 dilution of the healthy *Nicotiana occidentalis*; lane 5: 1/5 dilution of the DiaYMV infected *Diascia ×hybrida* ‘Sun chimes™ coral’; lane 6: 1/5 dilution of the healthy *Diascia ×hybrida* ‘Coral belle’; lane 7: 1/5 dilution of the DiaYMV infected *Nemesia ×hybrida* ‘Vanilla sachet™’; lane 8: 1/5 dilution of the healthy *Diascia barberae* ‘Apricot queen’. The molecular weights of the two protein products of IgA are 25 and 55 kilodaltons.

other immunoglobulins such as IgA and IgM can occur. Kappa light chain is the predominant light chain class (Sander and Dietzgen, 1984). Wang (1985) found that 2% of the hybridoma cell lines for *Bean common mosaic virus* (BCMV) produced the IgA heavy chain. Wang (1985) also reported that 25% of the hybridoma cultures produced after fusion contained mixed cell lines producing different immunoglobulin heavy chains and with different serological specificity. In the current study, the cell line M3-7-A6.1 was subjected to two cycles of single cell cloning before being characterized, so the possibility of a mixed cell culture was minimized.

During the screening of the supernate of the growing cell cultures, it was found that TAS-ELISA was more sensitive in detecting antibody secreting hybridomas than using the PTA-ELISA method. The increased sensitivity of TAS-ELISA increases the possibility of detecting hybridomas that produce low concentrations of antibody and/or antibodies with low avidity for the immunizing antigen (Al Moudallal *et al.*, 1984). The antibodies secreted by M3-7-A6.1 reacted specifically with virus infected samples only and not to healthy tissue.

DAS-ELISA absorbance values are relatively low for ScrMV (PV 269: ATCC) when the commercial NeRNV antibodies are used, but still significantly different from the healthy control (Fig. 15). This recognition of NeRNV and ScrMV by polyclonal antiserum indicates that they share common epitopes. In TAS-ELISA, M3-7-A6.1 did not detect ScrMV in *Scrophularia nodosa*, whereas M3-7-A6.1 recognizes both DiaYMV and NeRNV. Therefore, monoclonal antibody M3-7-A6.1 recognizes an epitope present on DiaYMV and NeRNV that is not displayed on ScrMV. Failure to detect ScrMV with the monoclonal antibody is supporting evidence that DiaYMV is not a strain of ScrMV. These results further support the findings in Chapter 2 that NeRNV, NeRNV-WA and DiaYMV have high amino

acid identities at the coat protein level (94-99%) and so the chances of possessing similar antigenic sites are very high. It has been shown that there are two major distinct serological groups within members of the genus *Tymovirus*, and that the serogroup to which NeRNV is a member includes the other tymoviruses AVYM, ScrMV, OYMV and PIMV (Koenig *et al*, 2005). It is not clear at this stage whether monoclonal antibody M3-7·A6·1 could discriminate between other tymoviruses of this serogroup; perhaps this could be a feature worth exploring in the future in order to further understand the antigenic structure of the virus and studying more on the serological relationships among closely related tymoviruses.

Although M3-7·A6·1 monoclonal antibody reacted with virus proteins in PTA-ELISA, the monoclonal antibody M3-7·A6·1 did not detect coat protein from infected samples nor purified virions in Western blot analysis. This suggests that the antibody does not recognize denatured virus. Failure to detect virus in Western blots has been found in other viruses such as *Carnation ringspot virus* (Tremaine and Dodds, 1985; Lommel, 2000). Due to the specificity of monoclonal antibodies, small alterations in the conformation of a viral polypeptide brought about by test conditions may alter the epitope to the extent that it is no longer recognized by the monoclonal antibody (Van Regenmortel and Dubs, 1993; Goding, 1996).

In Western blot analyses of the purified monoclonal antibody, two peptides of approximately 55 kilo Daltons (KDa) and 25 KDa reacted with the GAM, consistent with identification of the antibody as an IgA isotype.

CONCLUSION:

The monoclonal antibody produced in this study can detect the virus(es) isolated from *Diascia ×hybrida* ‘Sun chimes™ coral’ plants both in the original diascia virus source material and *Nicotiana occidentalis* plants inoculated with extracts from *Diascia ×hybrida* ‘Sun chimes™ coral’ and purified virus. The immunoglobulin possesses an IgA heavy chain and kappa light chain. A weak reaction to reagents for IgG₁ heavy chains was also noted. Serological evidence shows that the viruses detected in diascia and nemesia species are distinct from ScrMV.

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CHAPTER 4
HOST RANGE STUDIES AND SYMPTOMATOLOGY OF
DIASCIA YELLOW MOTTLE VIRUS

SUMMARY:

Most of the diascia and nemesia ornamental plants purchased from nurseries around Washington State and Oregon were found naturally infected by a tymovirus. Symptoms observed on infected leaves of diascia were vein yellowing and mottling. Reverse transcription-polymerase chain reaction (RT-PCR) was used to test plants bought in nurseries while both RT-PCR and an enzyme linked immunosorbent assay (ELISA) were used to test plants for DiaYMV following inoculation with extracts from virus infected plants. Natural infection was confirmed in six accessions of *Nemesia ×hybrida* and seven accessions of *Diascia ×hybrida*. The only diascia tested that was not infected with tymovirus was ‘Coral belle’. Susceptible hosts to DiaYMV include *Nicotiana benthamiana*, *Nicotiana clevelandii* and *Nicotiana occidentalis* and several species and cultivars in the genus *Nemesia* namely *Nemesia caerulea*, *Nemesia cheiranthus* ‘shooting stars’, *Nemesia strumosa* ‘Nebula F1 hybrid’ and ‘KLM’. *Diascia barberae* ‘Apricot queen’ and ‘Rose queen’ grown from seed also showed systemic infections when inoculated with extracts from *Diascia ×hybrida* ‘Sun chimes™ coral’. Systemic infection was not detected in mechanically inoculated *Nicotiana alata* ‘Nicki Red’, *Nicotiana tabacum* ‘Bright yellow’ and ‘423’, *Chenopodium quinoa*, *Vicia faba* L. ‘Broad Windsor’ or *Datura stramonium*.

Ultrastructural studies of symptomatic diascia tissues showed the presence of double membrane vesicles that are frequently associated with tymovirus replication in chloroplasts.

Murine monoclonal antibody M3-7·A6·1 was developed and detected tymovirus in infected host tissue diluted 100-fold.

INTRODUCTION:

The initial description of a virus is often based on phenotypic characters such as symptomatology, host range, vector type, serology and virion shape (Hull, 2002). Details of its genetic information are used to refine the initial characterization. Currently, more emphasis is placed on initial characterization by sequencing gene fragments (Gibbs and Gibbs, 2006). Biological properties are important even when nucleotide sequences have been determined.

There is limited information on the host range and symptom expression induced by the tymoviruses infecting diascia, including the newly described *Nemesia ring necrosis virus* (NeRNV). Its host range needs to be explored beyond members of the genera *Nicotiana*, *Diascia*, *Nemesia*, *Verbena* and *Lobelia* (Mathews and Dodds, 2004; Mathews *et al.*, 2004; Skelton *et al.*, 2004; Mathews and Dodds, 2006). There is no published information on methods of natural dissemination for NeRNV, and speculation is that the virus of ornamental plants raised in nurseries is mainly disseminated by vegetative propagation from infected germplasm.

The use of enzyme-linked immunosorbent assay (ELISA) has been enormous in large scale testing of perennial and vegetatively propagated crops (Van Regenmortel and Dubs, 1993) and the international recognition of its potential as a practical sero-diagnostic technique has been very rapid (Clark, 1981). Even with the increased use of molecular diagnostics to detect and classify viruses down to the species level, ELISA is still a viable

option in many cases. ELISA is not as sensitive as RT-PCR but it is adequately sensitive for routine use in many diagnosis and virus detection situations (Van Regenmortel and Dubs, 1993). A valuable feature of ELISA is that it can be used to provide quantitative data when used in kinetic studies, and perhaps the data generated can be used to advance our knowledge on the complex interactions between plants and pathogens (Clark, 1981).

The objectives of the study were to study the host range of Diascia yellow mottle virus (DiaYMV), to develop serological methods of its detection, and to study the effect of temperature on the accumulation of virus infected plants.

MATERIALS AND METHODS:

Virus sources: Nemesia and diascia plants were purchased from nurseries in Oregon and Washington at different times throughout the study period. Plants were tested for the presence of the tymovirus by RT-PCR assay using primers specific to the coat protein (CP) region (*see below*).

Virus purification: Initially, virus isolation and purification from infected *Diascia* × *hybrida* ‘Sun chimes™ coral’ used bentonite in aqueous extracts and polyethylene glycol precipitation (Dunn and Hitchburn, 1965) followed by further purification by linear-log sucrose gradient centrifugation (Brakke and Van Pelt, 1970) (see also Chapter 2). In later studies, a butanol:chloroform extraction method was used (Koenig *et al.*, 2005b).

Virus propagation: Plants in the families Solanaceae Juss., Chenopodiaceae L. and Fabaceae L. (Table 4) were grown from seed until they reached the two true leaf stage. Two true leaves were dusted with silicon carbide (grit size 600) and then inoculated with partially purified virus from *Diascia* × *hybrida* ‘Sun chimes™ coral’ using a cotton swab. The leaves

Table 4. Botanical and common names of plants used in this study.

Botanical name	Cultivar name	Common name
<i>Nemesia</i> × <i>hybrida</i>	Blueberry sachet™	Nemesia ¹
<i>Nemesia</i> × <i>hybrida</i>	Sunsatia peach	Nemesia ¹
<i>Nemesia</i> × <i>hybrida</i>	Vanilla sachet™	Nemesia ¹
<i>Nemesia caerulea</i> Heirn.	NS ³	Nemesia ²
<i>Nemesia cheiranthus</i> E.Mey.ex.Benth	Shooting stars	Nemesia ²
<i>Nemesia fruticans</i> Benth.	Safari plum	Nemesia ¹
<i>Nemesia fruticans</i> Benth.	Safari Pink	Nemesia ¹
<i>Nemesia fruticans</i> Benth.	Compact innocence	Nemesia ¹
<i>Nemesia strumosa</i> Benth.	KLM	Nemesia ²
<i>Nemesia strumosa</i> Benth.	Nebula hybrid mixed colors	Nemesia ²
<i>Antirrhinum nanum</i>	Peaches ad cream	Snapdragon ²
<i>Antirrhinum nanum</i>	Tequila sunrise	Snapdragon ²
<i>Antirrhinum majus</i> L.	Brazilian carnival	Snapdragon ²
<i>Antirrhinum majus</i> × <i>nanum</i>	Frosted flames	Snapdragon ²
<i>Antirrhinum majus</i> × <i>nanum</i>	LaBella red and white	Snapdragon ²
<i>Nicotina alata</i> Link and Otto	Nicki red	Tobacco ²
<i>Nicotiana benthamina</i> Domin.	NS	Tobacco ²
<i>Nicotiana clevelandii</i> A.Gray	NS	Tobacco ²
<i>Nicotiana occidentalis</i> Wheeler	37B	Tobacco ²
<i>Nicotiana tabacum</i> L.	Bright yellow	Tobacco ²
<i>Nicotiana tabacum</i> L.	423	Tobacco ²
<i>Datura stramonium</i> L.	NS	Datura ²
<i>Chenopodium quinoa</i> Willd.	NS	Quinoa ²
<i>Vicia faba</i> L.	Broad Windsor	Fava bean
<i>Diascia barberae</i> Hook. F.	Apricot queen	Twinspur ²
<i>Diascia barberae</i> Hook. F.	Rose queen	Twinspur ²
<i>Diascia</i> × <i>hybrida</i>	Coral belle	Twinspur ¹
<i>Diascia</i> × <i>hybrida</i>	Sun chimes™ coral	Twinspur ¹
<i>Diascia</i> × <i>hybrida</i>	Sun chimes™ blush	Twinspur ¹
<i>Diascia</i> × <i>hybrida</i>	Sun chimes™ rose	Twinspur ¹
<i>Diascia</i> × <i>hybrida</i>	Little charmer	Twinspur ¹
<i>Diascia</i> × <i>hybrida</i>	Flying colors – Apple blossom	Twinspur ¹
<i>Diascia</i> × <i>hybrida</i>	Flying colors - Apricot	Twinspur ¹

1. Plants purchased from nurseries in Oregon and Washington State.
2. Plants sown from seed and used in the mechanical transmission studies.
3. NS=Cultivar name not specified.

were allowed to partially dry for 20 min after inoculation at which time the leaves were sprayed with water to wash off residual virus and buffer salts. Plants were monitored for symptom development and seedlings were tested for DiaYMV by RT-PCR at 10 and 21 days after inoculation. Plants that tested positive for DiaYMV were used as source of inoculum for subsequent transfers of virus to healthy seedlings.

Host range studies: Seeds of plants from families Solanaceae Juss. and Scrophulariaceae Juss. (Table 4) were planted into 10 cm pots of vermiculite and germinated in a humid chamber at 25°C. Seedlings were transplanted individually after 4 to 6 weeks into 10 cm pots of potting mix. Seeds of *Vicia faba* L. 'Broad Windsor', *Datura stramonium* L. and *Chenopodium quinoa* Willd. were sown directly in 10 cm pots of potting mix, four seeds per pot; after germination, seedlings were thinned to one seedling per pot for *V. faba* and *Datura stramonium* and two seedlings per pot for *C. quinoa*. Inoculum was prepared from symptomatic leaves of *Nicotiana occidentalis* collected 3 weeks after inoculation with DiaYMV and ground with a pre-cooled sterilized mortar and pestle containing 0.05 M K/Na-PO₄, pH 7.4. Inoculations were carried out as described above. Plants were monitored for symptom development and tested for DiaYMV by RT-PCR using primers that amplify the CP region of the virus and by ELISA using a commercially available polyclonal antibody for NeRNV (Agdia Inc, Elkhart, IN). Mechanical inoculations were repeated on two occasions. Plants in the second replication of the host range study were tested for virus by triple antibody sandwich-ELISA (TAS-ELISA) with monoclonal antibody M3-7-A6-1.

For each host range experiment, six plants were inoculated with the virus sap and another six plants were mock inoculated with K/Na-PO₄ buffer pH 7.4 as the control treatment. Samples collected 10 days and 21 days after inoculation consisted of either the

inoculated leaf or leaves that were potentially infected systemically. Samples from mock inoculated plants were a composite of mock inoculated and newly developed leaves.

Temperature studies: Two plants each of virus infected *Diascia ×hybrida* ‘Sun chimes™ coral’ and virus-free *Diascia ×hybrida* ‘Coral belle’ were selected for the study. Plants were grown in a growth chamber at either 15°C or 25°C with a 16 hr light: 8 hr dark cycle. Each growth chamber contained a virus-infected plant and a virus-free plant; plants were monitored for symptom development and general growth. Plants were tested for DiaYMV by both RT-PCR and plate trapped antigen –enzyme linked immunosorbent assay (PTA-ELISA) after 8 weeks.

The experiment was repeated using *Diascia barberae* ‘Apricot queen’ plants in which each growth chamber contained three plants inoculated with partially purified DiaYMV from *Nicotiana occidentalis* and the other three plants were mock inoculated. Seedlings were acclimatized for a week prior to inoculation. After 12 wks, five side shoots were randomly selected from each plant and measurements of leaf area and fresh weight were collected. The tissue was transferred to brown paper bags and dried in a 60°C incubator for a week and their dry weights measured. Plants were also tested for DiaYMV by RT-PCR and PTA-ELISA. Data analysis was done with Statistical analysis software (SAS version 9.1.3).

RNA extraction: RNA was extracted from samples using commercial kits (Qiagen RNeasy mini plant kit: Qiagen, Inc., Carlsbad, CA). Manufacturer’s specifications were followed for extraction of samples of less than 0.5 g. For more than 0.5 g, tissue was ground in sample bags (Agdia) containing 2 ml water with 30 µl/ml 2-mercaptoethanol. The plant extract (150 µl) was mixed with 450 µl RLT buffer (Qiagen); incubated at 56°C

for 3 min and then processed following the manufacturer's protocol. The eluted RNA was stored at -70°C and aliquots were used for RT-PCR.

RT-PCR: RT-PCR was initially done using a two tube RT-PCR protocol as previously outlined (Chapter 2). For first strand cDNA synthesis, 2.5 µM complementary primer TymoCP-R (5'TTATGGATCCTCACGAGGAAGCTGT3') (Integrated DNA Technologies Inc., Coralville, IA) was used. PCR used primers TymoCP-R and TymoCP-F (5'-TTTTTTCATATGGAAGAACTCAAGCCGA-3'). PCR conditions were: denaturation at 94 °C for 10 min; thirty five cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec and extension at 72°C, for 1 min and final extension at 72°C for 30 min followed by cooling to 4°C. PCR products were analyzed by electrophoresis through 2% low melting agarose (NuSieve GTG: Cambrex, Rockland, ME) in TAE. The gel was stained with ethidium bromide (1 mg/ml) for 30 min and visualized on an ultraviolet transilluminator at 260 nm. Reactions yielding amplicons of the expected size of 600 bp were designated positive for DiaYMV. Where results were ambiguous, other primer pairs were used to confirm the results. NeRNV-WA primers Amo5642 (5'-CAACCTTTTCAGGTCTCTGT-AGC-3') and Amo5854c (5'-TCAAGATGTTCTTGGGAGACG-3') yielded an amplicon with the predicted size of 212 bp. DiaYMV primer pair Amo5721 (5'-CATGC-TGACC-TCGTGGAATG-3') and Amo5993c (5'-CGGAGAGTAGGCTAGAAGACG-3') were derived from the DiaYMV sequence and would amplify a product of 272 bp. Another primer pair designed from DiaYMV sequence was DiaYMV1338 (5'-GTGCTCACCACTGGC-TTACC-3') and DiaYMVc2033 (5'AGAAGGAGAGGGCTGAGAGG3') with the amplicon size of 695 bp.

ELISA: DAS-ELISA was done in the 2006 growing season when a commercial polyclonal antibody for NeRV became available (Agdia Inc.) and manufacturers' instructions were followed. For TAS-ELISA, wells of flat bottom 96-well Maxisorp polystyrene plates were coated with 100 μ l NeRV-capture antibody (Agdia) diluted 1:200 in carbonate buffer, pH 9.6. The plates were incubated at room temperature for 4 hrs and then washed three times with PBST. The remainder of the protocol for TAS-ELISA was followed as previously described (Chapter 3).

Transmission electron microscopy: Thin leaf sections of virus-infected *Diascia* \times *hybrida* 'Sun chimes™ coral' were fixed overnight at 4°C in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.05 M Na₂HPO₄, pH 7.4. The tissue was rinsed three times for 10 min each with 0.05 M Na₂HPO₄, pH 7.4. Leaf samples were post fixed with 1% osmium tetroxide for 2 hr, and rinsed two times for 10 min each with 0.05M Na₂HPO₄, pH 7.4. Samples were dehydrated in graded dilutions of ethanol (30, 50, 70, 80, and 95%) for 10 min each and then dehydrated with 100% ethanol three times for 10 min each. This was followed by another dehydration step in 1:1 (vol:vol) ethanol:acetone for 10 min, followed by dehydration with 100% acetone twice for 10 min each. Tissue infiltration with Spurr's resin was performed in a series of Spurr's:acetone of 1:3, 1:2, 1:1 and 3:1 (vol:vol) overnight at each step. Finally, samples were infiltrated with three daily changes of 100% Spurr's resin. Individual leaf sections together with labels were embedded in 100% Spurr's resin and the molds kept at 60°C until the resin polymerized. Leaf samples were thin sectioned (silver sections) using an ultramicrotome (Reichert-Jung, Leica Microsystems Holdings AG, Wetzlar, Germany). Ultrathin sectioned samples were transferred to formvar coated nickel grids and stained with 2% uranyl acetate and Reynold's lead. Grids were examined with a

transmission electron microscope (JOEL 1200 EX: JOEL Ltd, Tokyo, Japan). During the time when this study was done, all the *Diascia* plants were infected and hence there was no negative control.

Impatiens necrotic spot virus detection: In the spring of 2005, symptomatic plants were tested for *Impatiens necrotic spot virus* (INSV) using INSV Immunostrips (Agdia, Inc.) as per manufacturers' instructions.

Primers were designed for INSV detection by RT-PCR, the primers used were adapted from Tanina *et al.* (2001). A Superscript™ III one –step RT-PCR system with Platinum® *Taq* DNA polymerase kit was used (cat no: 12574-026) (Invitrogen). One microliter of RNA and 0.3 µl of Superscript™ III RT/ Platinum® *Taq* mix were added to following reaction mix at a final concentration of 0.2 mM each dNTP, 1.6 mM MgSO₄, 0.2 µM Forward primer (5'-TAACACAACACAAAGCAAACC-3'), and 0.2 µM reverse primer (5'-CCAAATACTACTTTAACCGCA-3'). The reaction tubes were placed in a thermal cycler (Bio-Rad) that was preheated to 55°C, and programmed such that cDNA synthesis was followed immediately by PCR amplification. Complementary DNA (cDNA) synthesis proceeded at 55°C for 30 min, and PCR conditions were: denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 15 sec, annealing at 50°C for 30 sec and extension at 68°C, for 1 min and final extension at 68°C for 5 min followed by cooling to 4°C. PCR products were analyzed by electrophoresis through 2% low melting agarose (NuSieve GTG: Cambrex, Rockland, ME) in TAE. The PCR amplicon predicted for a positive sample was 906 bp.

All incoming plants in the spring of 2006 were regularly tested for INSV by RT-PCR and by DAS-ELISA using the commercial polyclonal antibody purchased from Agdia Inc. Manufactures guidelines were followed.

RESULTS

Natural infections: Most diascia and nemesia plants purchased from nurseries in Oregon and Washington State tested positive for tymovirus by RT-PCR. The only exceptions were *Diascia* ×*hybrida* ‘Coral belle’ and one out of two plants of *Nemesia* ×*hybrida* ‘Blueberry sachet[™]’ (Table 5). All primer combinations used in this portion of the study could not distinguish between the DiaYMV and NeRNV, so the identity of the tymovirus was not confirmed. Cytopathology studies on leaf sections of *Diascia* ×*hybrida* ‘Sun chimes[™] coral’ showed the occurrence of double membrane vesicles in the cytoplasm and no virus particles could be identified (Fig. 18)

All plants purchased in 2005 were determined by RT-PCR to be infected with *Impatiens necrotic spot virus* (INSV) two months after the initial tests with the INSV immunostrips determined that the plants were not infected (Table 6). Symptoms included necrotic ring spots and necrotic stem lesions (Fig. 19). *Diascia barberae* ‘Apricot queen’ seedlings growing in the 15°C growth chamber tested negative for INSV by immunostrips but tested positive by RT-PCR, while those plants kept at 25°C tested positive for INSV by both methods. Plants purchased in 2006 were determined to be free from INSV by DAS-ELISA and RT-PCR (Table 7). Plants purchased in 2006 were free from INSV and they were regularly tested throughout the summer of 2006 to insure that they had not become infected with INSV.

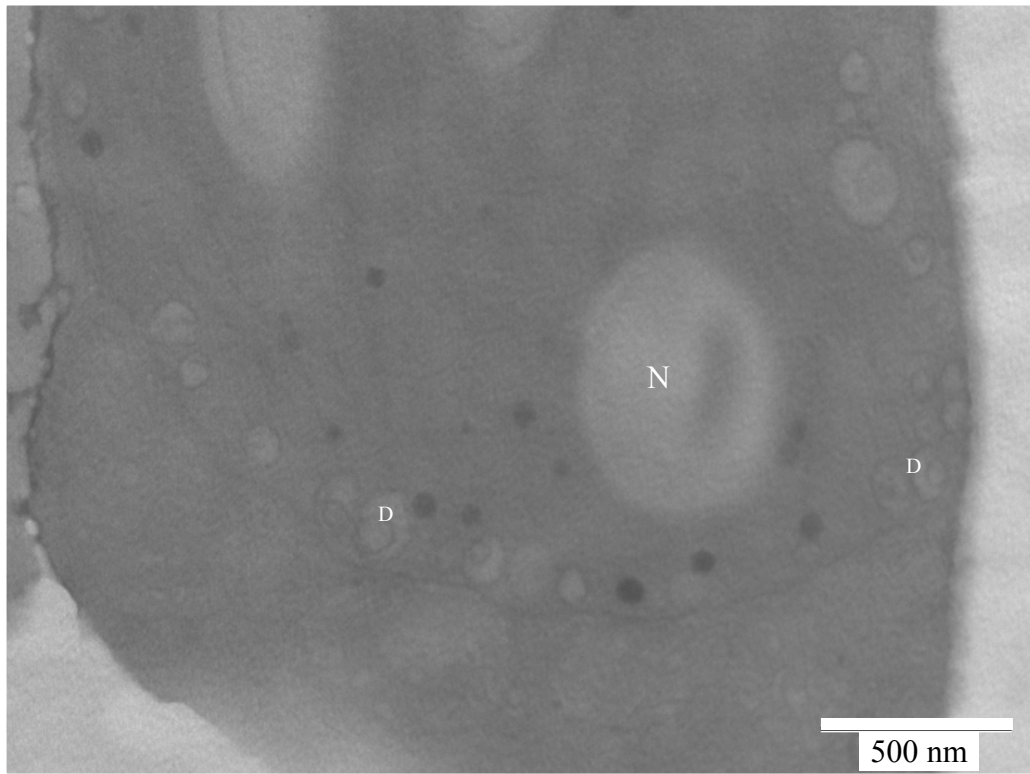
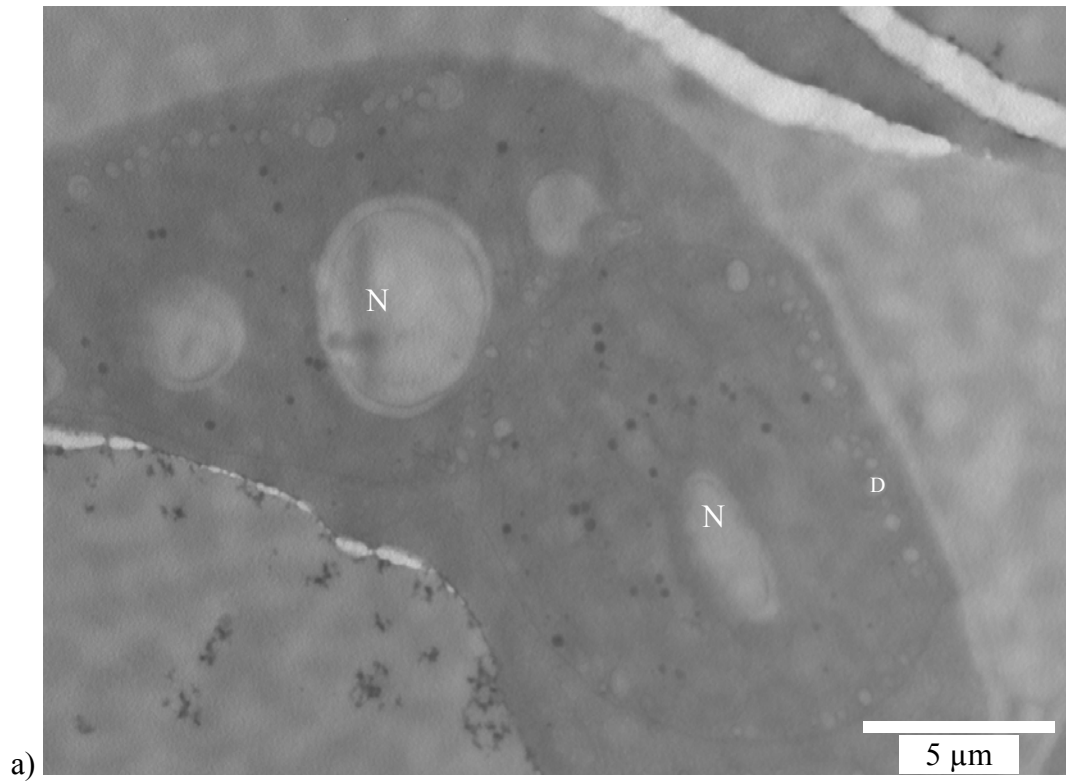
Experimental host range: All nemesia cultivars tested displayed similar systemic symptoms of mild mosaic with distinct yellow veins, but plant vigor was not obviously impacted. *Nemesia* ×*hybrida* ‘Nebula F1 mixed hybrid’ also displayed necrotic spots in leaves as well as reddening of the veins and leaves later in the season. The reddening was

Table 5. Plants purchased from nurseries in the States of Washington and Oregon were tested for the presence of tymoviruses *Nemisia ring necrosis virus* (NeRNV) and Diascia yellow mottle virus (DiaYMV) by reverse transcription polymerase chain reaction.

Host	Symptoms	RT-PCR¹ <i># positive</i> <i># tested</i>	DAS-ELISA^{2,3}	Origin State (County)
<i>Nemisia ×hybrida</i>				
‘Blueberry sachet TM ’	tip necrosis; chlorosis	1/2	+++	OR (Multnomah)
‘Sunsatia peach’	chlorosis; vein yellowing	2/2	NT	WA (Benton)
‘Vanilla sachet TM ’	mild mosaic; necrotic spots	30/30	+++	OR (Multnomah) WA (Benton, Skagit)
<i>Nemisia fruticans</i>				
‘Compact innocence’	chlorosis; stem lesions	2/2	NT	WA (Benton)
‘Safari pink’	chlorosis; stem lesions	3/3	NT	WA (Benton)
‘Safari plum’	chlorosis; stem lesions	3/3	NT	WA (Benton)
<i>Diascia ×hybrida</i>				
‘Coral belle’	no symptoms	1/48	-	WA (Skagit)
‘Flying colors–Apple blossom’	mild mosaic; vein yellowing	2/2	NT	WA (Benton)
‘Flying colors–Apricot’	mild mosaic; vein yellowing	2/2	NT	WA (Benton)
‘Little charmer’	mild mosaic; vein yellowing	2/2	NT	WA (Benton)
‘Sun chimes TM blush’	mild mosaic; vein yellowing	2/2	+++	OR (Multnomah)
‘Sun chimes TM coral’	mellow mottle; stunting	40/40	+++	OR (Multnomah)
‘Sun chimes TM rose’	mild mosaic; vein yellowing	2/2	+++	OR (Multnomah)

1. RT-PCR based on primers designed from the CP region of DiaYMV.
2. DAS-ELISA was performed with a commercial NeRNV assay (Agdia Inc.).
3. Absorbance reading (A₄₀₅) greater than 2.0 after 30 min with substrate were positive (+++); readings below 0.100 were negative (-).

Figure 18. Electron micrographs of tymovirus-infected *Diascia ×hybrida* ‘Sun chimes™ coral showing the distribution of double membrane vesicles in the cytoplasm. a) Magnification 5,000×, Bar = 5 μm showing the distribution of double membrane vesicles (D) in the cytoplasm; b) The same double membrane structure at magnification at 50,000×, Bar = 500nm; c) Plant cell showing the location of double membrane vesicles (D), nucleus (N) and chloroplast (C), magnification 75,000×, Bar = 0.2 μm; d) Higher magnification of electron micrograph of the double membrane vesicle Bar = 0.3 μm.



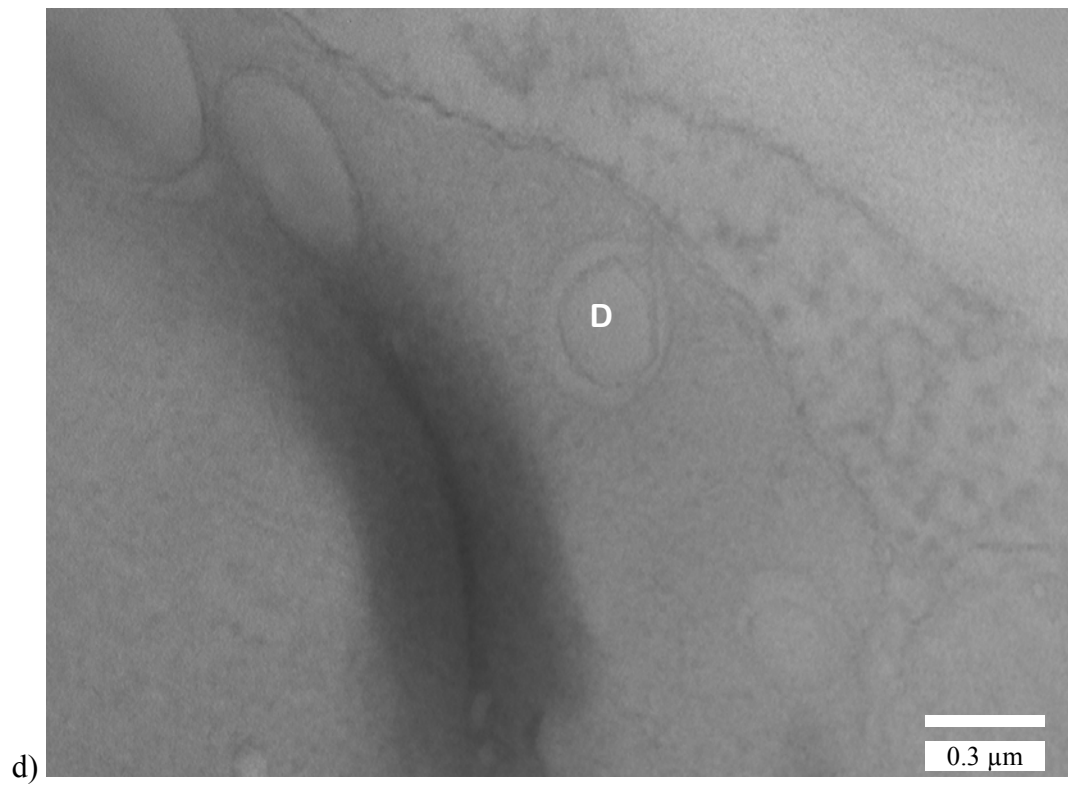
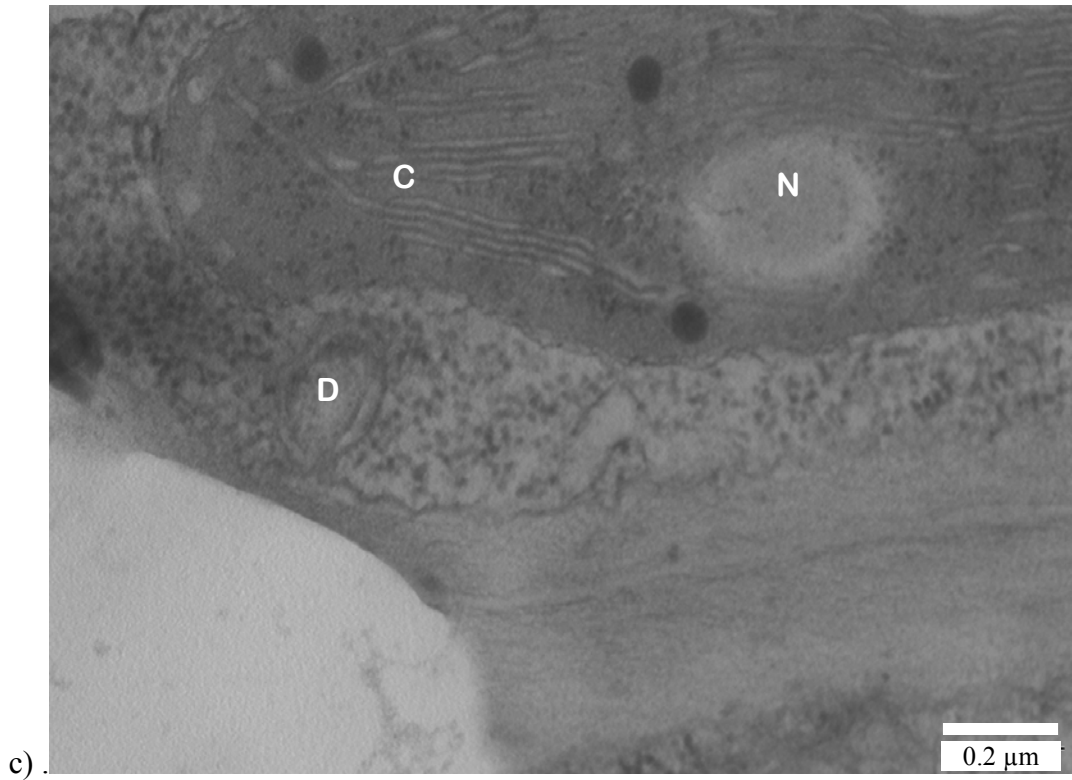




Figure 19. *Diascia x hybrida* ‘Sun chimes™ coral’ infected with *Diascia* yellow mottle virus (DiaYMV) and *Impatiens necrotic spot virus* (INSV). A and B) Mild mosaic and yellowing typical of DiaYMV infection. A and C) Necrotic lesions associated with INSV infection.

Table 6. *Impatiens necrotic spot virus* (INSV) detection and symptom expression on plants kept in the greenhouse at Prosser–Washington State (Spring, 2005).

Host	Symptoms	RT-PCR ¹ # positive # tested	Serology ^{2, 3, 4}
<i>Nemesia ×hybrida</i>			
‘Sunsatia peach’	necrotic lesions on leaves and stems	2/2	+ ²
‘Vanilla sachet™’	necrotic lesions on leaves and stems	20/20	+ ²
<i>Nemesia fruticans</i>			
‘Compact innocence’	leaf ringspots; stem necrotic lesions	2/2	+ ²
‘Safari pink’	leaf ringspots; stem necrotic lesions	3/3	+ ²
‘Safari plum’	leaf ringspots; stem necrotic lesions	3/3	+ ²
<i>Diascia ×hybrida</i>			
‘Flying colors–Apple blossom’	leaf ringspots; stem necrotic lesions	2/2	+ ²
‘Flying colors- Apricot’	leaf ringspots; stem necrotic lesions	2/2	+ ²
‘Little charmer’	leaf ringspots; stem necrotic lesions	2/2	+ ²
‘Sun chimes™ coral’	leaf ringspots; stem necrotic lesions	20/20	+ ^{2, 3}
‘Coral belle’	no symptoms	1/24	- ³
‘Sun chimes™ blush’	leaf ringspots; stem necrotic lesions	2/2	+++ ^{3, 4}
‘Sun chimes™ rose’	leaf ringspots; stem necrotic lesions	2/2	+++ ^{3, 4}
<i>Diascia barberae</i>			
‘Apricot queen’ 15 °C	leaf necrotic ringspots	2/2	- ²
‘Apricot queen’ 25 °C	leaf necrotic ringspots	2/2	+ ²

<i>Antirrhinum spp</i>	Leaf necrotic ringspots	2/2	+ ²
<i>Datura stramonium</i>	Leaf necrotic ringspots	2/2	+ ²
<i>Nicotiana occidentalis</i>	Necrotic spots and lesions	0/2	- ²
<i>Nicotiana clevelandii</i>	Necrotic lesions and lesions	2/2	+ ²
<i>Nicotiana tabacum</i> 'Samsun'	No symptoms	0/2	- ²

1. Reverse transcription-polymerase chain reaction (RT-PCR) based on primers designed from the nucleocapsid protein (N) segment of the RNA.
2. INSV assay was performed with a commercial INSV Immunostrips assay (Agdia Inc.).
3. Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) was performed with a commercial INSV assay (Agdia).
4. Absorbance reading (A_{405}) greater than 2.0 after 30 min with substrate were positive (+++); readings below 0.100 were negative controls (-) for DAS-ELISA only.

Table 7. *Impatiens necrotic spot virus* (INSV) detection in plants purchased from nurseries in the States of Washington and Oregon (Spring, 2006)

Host	RT-PCR¹ <i># positive</i> <i># tested</i>	Serology^{2,3}
<i>Nemesia ×hybrida</i>		
‘Vanilla sachet TM ’	0/16	- ^{2,3}
‘Blueberry sachet TM ’	0/2	-
<i>Diascia ×hybrida</i>		
‘Sun chimes TM coral’	0/24	-
‘Coral belle’	0/24	-

1. Reverse transcription-polymerase chain reaction (RT-PCR) based on primers designed from the nucleocapsid protein (N) segment of the RNA.
2. Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) was performed with a commercial INSV assay (Agdia).
3. Absorbance reading (A_{405}) greater than 2.0 after 30 min with substrate were positive (+++); readings below 0.100 were negative controls (-) for DAS-ELISA only.

also observed on non-inoculated leaves and so is not associated with infection by DiaYMV. *Diascia barberae* ‘Apricot queen’ and ‘Rose queen’ exhibited chlorotic spots on inoculated leaves and a mild yellow mosaic on systemically infected leaves; the leaves were generally smaller than the non-inoculated leaves (Table 8).

Mechanical inoculations of *Antirrhinum majus*, *Antirrhinum nanum* and *Antirrhinum majus* × *nanum* hybrids (snapdragons) resulted in no apparent symptom development. Sporadic chlorotic spots appeared on inoculated leaves as well as non-inoculated plants, hence, the symptom could not be associated with any cultivar and/or virus inoculation and could be a result of mechanical or insect damage. The inoculated plants however yielded positive TAS-ELISA readings only on inoculated leaves (local) but not on systemic leaves (Table 9).

Symptom expression in genera other than Scrophulariaceae: *Nicotiana occidentalis* ‘37B’ (Fig. 20) displayed local necrotic lesions that expanded with time resulting in necrosis, while the systemically infected leaves showed necrotic spots accompanied by yellow netting and leaf distortion, and the plants were stunted. *Nicotiana clevelandii* showed more severe yellow mosaic and necrotic lesions on inoculated leaves, and systemically infected leaves had a severe yellow mosaic, and the plants were stunted. *Nicotiana benthamiana* (Fig. 21) displayed fewer necrotic spots on inoculated leaves and systemically infected leaves displayed a mosaic that later became chlorotic. Symptoms were not as severe as those observed in *Nicotiana occidentalis* and *Nicotiana clevelandii* although the TAS-ELISA absorbance values were equal for both *Nicotiana occidentalis* and *Nicotiana clevelandii*. *Nicotiana alata* ‘Nicki red’ and *Nicotiana tabacum* ‘Bright yellow’ and ‘423’ (Fig. 22) both exhibited no symptoms on inoculated leaves and were symptomless on

Table 8. Symptom expression and detection of Diascia yellow mottle virus (DiaYMV) on leaves by triple antibody sandwich-enzyme linked immunosorbent assay (TAS-ELISA) on nemesia and diascia following mechanical inoculation with DiaYMV.

Host	Leaf	Symptoms	TAS-ELISA ^{1,2} (<i>A</i> ₄₀₅)
<i>Nemesia caerulea</i>	Healthy	None	0.033 ± 0.002
	Systemic	Yellow veins; mild mosaic	0.339 ± 0.104
<i>Nemesia cheiranthus</i> 'Shooting Stars'	Healthy	None	0.039 ± 0.015
	Systemic	Yellow veins; mild mosaic	0.595 ± 0.226
<i>Nemesia strumosa</i> 'KLM'	Healthy	None	0.034 ± 0.002
	Systemic	Yellow vein; mild mosaic	0.869 ± 0.094
<i>Nemesia strumosa</i> 'Nebula Hybrid'	Healthy	None	0.037 ± 0.006
	Systemic	Yellow mosaic, necrotic spots; red veins	0.508 ± 0.100
<i>Diascia barberae</i> 'Apricot queen'	Healthy	None	0.037 ± 0.006
	Systemic	Yellow veins, stunting; mild mosaic	0.976 ± 0.007
<i>Diascia barberae</i> 'Rose queen'	Healthy	None	0.041 ± 0.009
	Systemic	Yellow veins, stunting; mild mosaic	0.852 ± 0.030
<i>Nicotiana benthamiana</i>	Negative	None	0.044 ± 0.010
	control		
	Positive control	Yellow mosaic	0.550 ± 0.105

1. TAS-ELISA was performed with the monoclonal antibody M3-7·A6·1 one week after inoculations with DiaYMV.
2. Absorbance reading (*A*₄₀₅) greater than 0.088 after 30 min with substrate were positive.

Table 9. Detection of Diascia yellow mottle virus (DiaYMV) by triple antibody sandwich-enzyme linked immunosorbent assay (TAS-ELISA) in leaf samples of various cultivars of the genus *Antirrhinum*.

<i>Plant</i>	<i>Cultivar</i>	<i>Leaf status</i>	<i>Absorbance</i> <i>A</i> ₄₀₅ ^{1,2}	<i>Result</i> ³
<i>Antirrhinum majus</i>	‘Brazilian carnival’	Healthy	0.036 ± 0.003	-
		Inoculated	0.623 ± 0.161	++
		Systemic	0.033 ± 0.004	-
<i>Antirrhinum nanum</i>	‘Tequila sunrise’	Healthy	0.042 ± 0.009	-
		Inoculated	1.285 ± 0.279	+++
		Systemic	0.037 ± 0.005	-
<i>Antirrhinum nanum</i>	‘Peaches and Cream’	Healthy	0.037 ± 0.003	-
		Inoculated	0.589 ± 0.257	++
		Systemic	0.036 ± 0.005	-
<i>Antirrhinum majus</i> × <i>nanum</i>	‘Frosted flames’	Healthy	0.035 ± 0.009	-
		Inoculated	0.658 ± 0.195	++
		Systemic	0.027 ± 0.003	-
<i>Antirrhinum majus</i> × <i>nanum</i>	‘La Bella red and white’	Healthy	0.035 ± 0.003	-
		Inoculated	0.447 ± 0.076	+
		Systemic	0.030 ± 0.002	-
<i>Nicotiana</i>		Negative control	0.032 ± 0.003	-
<i>benthamiana</i>		Positive control	1.000 ± 0.080	+++

1. Absorbance values at A_{405} measured 30 min after the addition of substrate
2. Data values are mean absorbance readings ± standard deviation. The threshold value for positive samples was $A_{405} = 0.064$
3. Positive absorbance values at A_{405} ; (+) Absorbance value ≥ 0.064 but ≤ 0.500 ; (++) Absorbance values ≥ 0.5 and < 1.00 ; (+++) Absorbance values greater than 1.00

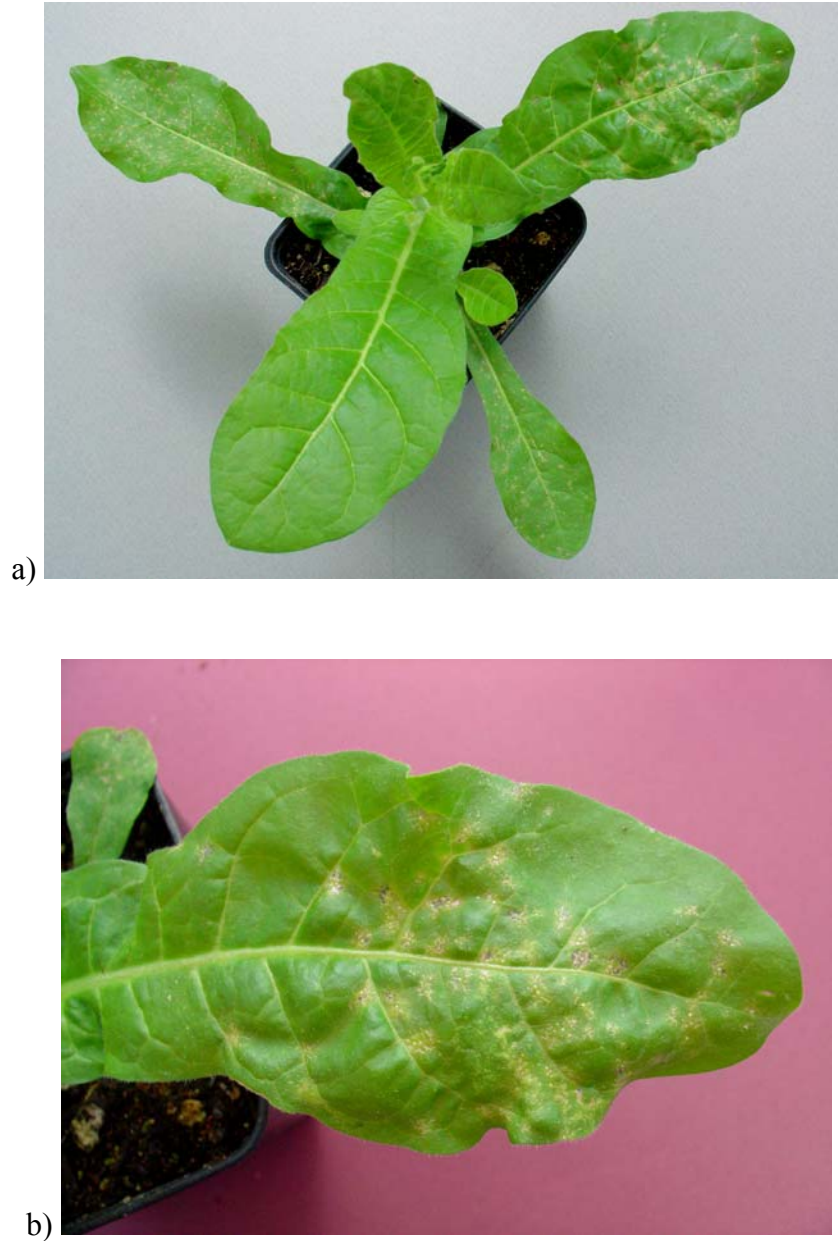


Figure 20. Diascia yellow mottle virus (DiaYMV) infected *Nicotiana occidentalis* '37B' showing virus induced symptoms one week after inoculation. a) Necrotic lesions and leaf distortion of inoculated leaf; b) Close-up view of an inoculated leaf showing the necrotic lesions and the extent of leaf distortion.

a)



b)



Figure 21. Seedlings of *Diascia barberae* ‘Apricot queen’ and *Nicotiana benthamiana* showing virus-induced symptoms. a) *Diascia barberae* ‘Apricot queen’ inoculated with Diascia yellow mottle virus (DiaYMV); b) DiaYMV-infected *Nicotiana benthamiana* systemic infected leaf.

a)



b)



c)



d)



Figure 22. Plants showing symptoms on *Nicotiana* plants following inoculation with buffer only (healthy) and plants inoculated with *Diascia* yellow mottle virus (DiaYMV). a) Healthy *Nicotiana tabacum* 'Bright yellow'; b) DiaYMV-infected *Nicotiana tabacum* 'Bright yellow'; c) Healthy *Nicotiana alata* 'Nicki red'; d) DiaYMV-inoculated *Nicotiana alata* 'Nicki red'.

potentially systemically infected leaves, however, a positive ELISA was obtained from inoculated leaves only (Table 10).

Preliminary studies on the effect of sample dilution on DiaYMV detection by ELISA was performed. It was determined that extracting the samples at a concentration of 1 g in 10 ml extraction buffer yielded higher absorbance values in both of the inoculated samples of *Nicotiana benthamiana* and *Nicotiana occidentalis*. PTA-ELISA of *Nicotiana benthamiana* may yield a false negative result if the sample is prepared at the rate of 1 g per 50 ml extraction buffer although the sample gave a positive ELISA reading at 1g tissue per 10 ml extraction buffer (Fig. 23). Sample extraction at 1 g per 1000 ml extraction buffer resulted in negative ELISA results for both *Nicotiana occidentalis* and *Nicotiana benthamiana* plants. The plants tested positive by RT-PCR prior to the ELISA test.

Mechanical inoculations on *Datura stramonium*, *Vicia faba* ‘Broad Windsor’ and *Chenopodium quinoa* failed to produce systemic infections. TAS-ELISA was positive for the inoculated leaves but not systemic leaves (Table 11).

In an effort to distinguish between residual virus on inoculated leaves and virus produced by localized infections, inoculated leaves were tested by TAS-ELISA 1 hr after inoculation and again after 1 week. It is evident that the virus quantities measured by TAS-ELISA were high when inoculated leaves were tested 1 hr following inoculation, and were higher than that of the remaining half leaves tested 1 week later (Table 12). The high ELISA values obtained from leaves 1 hr after inoculation precluded making definitive conclusions about virus replication in inoculated leaves.

Effect of temperature on virus accumulation and symptom development: The initial temperature studies using *Diascia ×hybrida* ‘Sun chimes™ coral’ and *Diascia ×hybrida*

Table 10. Symptom expression and virus detection by triple antibody sandwich-enzyme linked immunosorbent assay (TAS-ELISA) on leaves of various *Nicotiana* plants inoculated with *Diascia* yellow mottle virus (DiaYMV).

Host ¹	Leaf	Symptoms ²	TAS-ELISA ^{3,4} (A_{405})
<i>Nicotiana alata</i>	Healthy	N/A	0.033 ± 0.002
	Inoculated	no symptoms	0.430 ± 0.020
	Systemic	no obvious symptoms	0.042 ± 0.008
<i>Nicotiana benthamiana</i>	Healthy	N/A	0.033 ± 0.010
	Inoculated	chlorotic, irregular spots	2.329 ± 0.210
<i>Nicotiana clevelandii</i>	Systemic	yellow veins; mosaic	2.356 ± 0.125
	Healthy	N/A	0.062 ± 0.040
<i>Nicotiana occidentalis</i> ‘37B’	Inoculated	yellow mosaic	0.725 ± 0.155
	Systemic	yellow veins; severe mosaic	2.205 ± 0.241
<i>Nicotiana tabacum</i> ‘Bright yellow’	Healthy	N/A	0.035 ± 0.008
	Inoculated	necrotic spots that coalesce with time, yellow netting	1.855 ± 0.220
<i>Nicotiana tabacum</i> ‘423’	Systemic	mosaic, yellow netting; small curled leaves	2.205 ± 0.105
	Healthy	N/A	0.032 ± 0.007
	Inoculated	no symptoms	0.520 ± 0.200
<i>Nicotiana occidentalis</i> PV	Systemic	no obvious symptoms	0.031 ± 0.007
	Healthy	N/A	0.041 ± 0.006
	Inoculated	no symptoms	0.625 ± 0.175
<i>Nicotiana occidentalis</i> PV	Systemic	no obvious symptoms	0.027 ± 0.004
	Negative control	N/A	0.044 ± 0.010
	Positive control	N/A	2.725 ± 0.050

1. PV is the purified DiaYMV isolated from infected *Nicotiana occidentalis* (10 µg)

2. N/A = Healthy plants, no symptoms observed

3. Absorbance values (A_{405}) measured 30 min after substrate addition

4. Threshold value for positive samples is Absorbance value (A_{405}) = 0.088

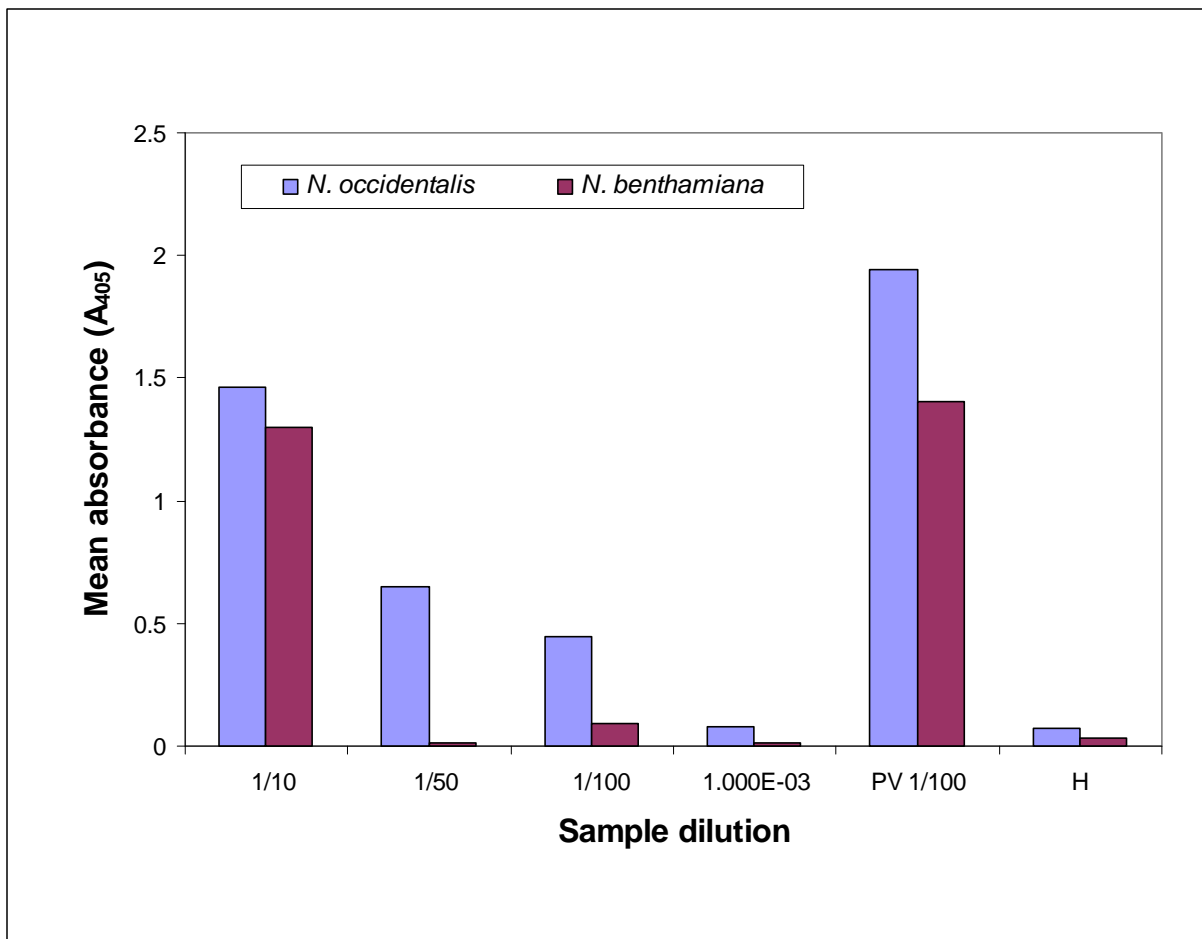


Figure 23. The effect of sample dilution on the mean absorbance value of Diascia yellow mottle virus (DiaYMV) using monoclonal antibody M3-7-A6-1 at 1/200 dilution. Samples used were from systemically infected leaves one week post-inoculation with purified virions of DiaYMV. PV 1/100 = Purified virus diluted in plant extracts to give final virus concentrations of 6.5 ng/well in *N. occidentalis* or 3.5 ng/well in *Nicotiana benthamiana* extract; H = Non-inoculated tissue at 1/10 dilution in general sample extraction buffer.

Table 11. Diascia yellow mottle virus (DiaYMV) detection by triple antibody sandwich-enzyme linked immunosorbent assay (TAS-ELISA) on leaves of different hosts mechanically inoculated with DiaYMV.

<i>Plant</i>	<i>Leaf</i>	<i>Absorbance</i> <i>A</i> ₄₀₅ ^{1,2,3}	<i>Result</i>
<i>Datura stramonium</i>	Healthy	0.062 ± 0.080a	-
	Inoculated	ND	ND
	Systemic	0.042 ± 0.009a	-
<i>Chenopodium quinoa</i>	Healthy	0.036 ± 0.005a	-
	Inoculated	ND	ND
	Systemic	0.034 ± 0.027a	-
<i>Vicia faba</i> 'Broad Windsor'	Healthy	0.032 ± 0.004a	-
	Inoculated	0.518 ± 0.186b	++
	Systemic	0.039 ± 0.008a	-
<i>Nicotiana</i>	Negative control	0.044 ± 0.010a	-
<i>benthamiana</i>	Positive control	0.550 ± 0.105b	++

1. Absorbance readings ± standard deviation measured 30 min after adding the substrate
2. ND = not determined. At the time the assay was done inoculated leaves were not tested.
3. The threshold value for positive samples was $A_{405} = 0.088$. Samples with the same letter were not significantly different and plants with different letters were significantly different from the negative control ($p < 0.001$)

Table 12. Mean absorbance values for triple antibody sandwich-enzyme linked immunosorbent assay (TAS-ELISA) for Diascia yellow mottle virus (DiaYMV) on inoculated leaves of various plants.

<i>Host</i>	<i>Status</i>	<i>Mean Absorbance value (A 405)¹</i>	<i>Mean Absorbance value (A405)^{2,3}</i>
<i>Nicotiana benthamiana</i>	Healthy	0.044 + 0.002	0.035 + 0.021
	Inoculated leaf	1.733 + 0.0100	1.948 + 0.056
<i>Chenopodium quinoa</i>	Healthy	0.023 + 0.012	0.045 + 0.002
	Inoculated leaf	1.284 + 0.008	0.445 + 0.004
<i>Antirrhinum majus</i>	Healthy	0.029 + 0.005	0.045 + 0.003
'LaBella red and white'	Inoculated leaf	1.391 + 0.015	0.398 + 0.045

1. Mean absorbance value (A_{405}) of TAS-ELISA on leaves tested one hour after mechanical inoculation.
2. Mean absorbance value (A_{405}) of TAS-ELISA on leaves tested one week after mechanical inoculation.
3. The threshold value for positive samples was $A_{405} = 0.090$

‘Coral belle’ showed no obvious symptom differences in plants maintained at 15°C or 25°C, but there were noticeable differences in virus titers as measured in TAS-ELISA. Infected *Diascia ×hybrida* ‘Sun chimes™ coral’ kept at 25°C yielded higher TAS-ELISA absorbance values compared to those grown at 15°C (Fig. 24).

Diascia barberae ‘Apricot queen’ seedlings grown at 15°C were smaller than those seedlings kept at 25°C, and the inoculated plants were smaller in size compared to healthy plants kept at the same temperature (Table 13). Virus infected plants showed the typical mild vein yellowing and mosaic on systemically infected leaves, however these symptoms were similar in plants kept at both temperatures. Other parameters of plant growth such as mean leaf area, fresh weight and dry weight per branch were used to evaluate the differences in growth patterns due to temperature and inoculation with the virus. There were highly significant differences in mean leaf area per branch at the two temperatures ($p < 0.0001$), and significant differences due to treatments (healthy versus virus inoculated) ($p < 0.05$) (Table 13). The only significant difference imposed by temperature was noticed when plants were maintained at 15°C; virus inoculated plants were smaller and had relatively small leaves (Fig. 25), while plants kept at 25°C showed no differences in growth as a result of virus inoculation. Regardless of virus status, plants grown at 25°C grew more vigorously than plants kept at 15°C. There were no significant differences in the mean leaf area per branch for plants kept at 25°C. TAS-ELISA results showed that DiaYMV-infected plants kept at 25°C had higher absorbance values (A_{405}) than plants kept at 15°C although the differences were not statistically significant (Table 13). All plants used were not infected by INSV.

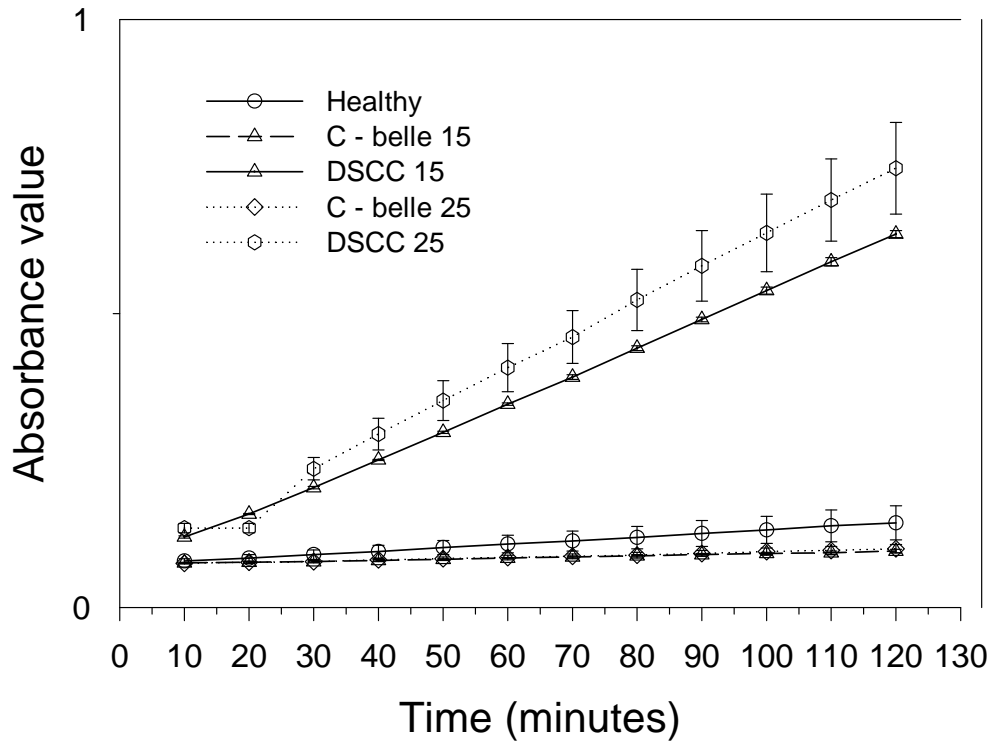


Figure 24. A graph showing the absorbance values (A_{405}) of plate trapped antigen-enzyme linked immunosorbent assay (PTA-ELISA) of diascia plants over time after the addition of substrate in a PTA-ELISA. The plants were grown at 15°C and 25°C in growth incubators for a period of eight weeks prior to analysis by ELISA. Purified virus (100 ng) isolated from infected *Nicotiana occidentalis* was used as a positive control and healthy *Nicotiana occidentalis* was used at the normal 1:10 sample dilution as a negative control. *Diascia* × *hybrida* ‘Coral belle’ (C-belle) represented the non-infected plants since there were no available healthy *Diascia* × *hybrida* ‘Sun chimes™ coral’ (DSCC).

C-belle 15: *Diascia* × *hybrida* ‘Coral belle’ maintained at 15°C

C-belle 25: *Diascia* × *hybrida* ‘Coral belle’ maintained at 25°C

DSCC 15: *Diascia* × *hybrida* ‘Sun chimes™ coral’ maintained at 15°C

DSCC 25: *Diascia* × *hybrida* ‘Sun chimes™ coral’ maintained at 25°C

Table 13. Temperature effect on healthy and Diascia yellow mottle virus (DiaYMV) infected *Diascia barberae* ‘Apricot queen’ plants growing in temperature controlled growth chambers.

Temperature (°C)	Treatment	Mean leaf area (cm ²) ^{1,2}	Mean fresh weight/branch ³	Mean dry weight/ branch ⁴	Absorbance value (A ₄₀₅) ⁵
15	Healthy	0.669 + 0.203 a	0.201 + 0.134 a	0.028 + 0.005 a	0.037 + 0.005 a
	Infected	0.346 + 0.036 b	0.146 + 0.013 b	0.015 + 0.002 b	1.006 + 0.069 b
25	Healthy	1.764 + 0.104 c	0.554 + 0.144 c	0.061 + 0.017 c	0.045 + 0.011 a
	Infected	1.788 + 0.221 c	0.621 + 0.153 c	0.065 + 0.209 c	0.980 + 0.017 b

1. Average size of an individual leaflet measured in cm²
2. Numbers with the same letter(s) per column have non-significant differences at p≤0.001
3. Mean fresh weight per branch, measured as an average of five branches per plant.
4. Mean dry weight per branch, measured as an average of five branches per plant.
5. Mean absorbance value (A₄₀₅) in a triple antibody sandwich–enzyme linked immunosorbent assay.



Figure 25. Healthy and Diascia yellow mottle virus (DiaYMV)-infected *Diascia barberae* ‘Apricot queen’ seedlings growing in a growth chamber maintained at 15°C. Photographs were taken eight weeks after inoculation with virus or buffer only (healthy). Virus-infected plants are stunted in growth compared to healthy seedlings.

DISCUSSION:

Most diascia and nemesia plants purchased from the nurseries in Oregon and Washington were infected with a tymovirus as determined by RT-PCR and DAS-ELISA using a NeRNV-specific commercial kit. Plants of each cultivar were uniformly infected with the exceptions of *Diascia* ×*hybrida* ‘Coral belle’ in which one plant of 48 plants tested positive by RT-PCR but all were negative by DAS-ELISA, and one of two plants of *Nemesia* ×*hybrida* ‘Blueberry sachet™’ were positive by both RT-PCR and TAS-ELISA. Since 2005, there have been reports of diascia, nemesia and verbena plants infected with NeRNV (Skelton *et al.*, 2004; Koenig *et al.*, 2005a; Mathews and Dodds, 2006). There are also reports of *Scrophularia mottle virus* (ScrMV) being detected in diascia and nemesia ornamental plants in eight other US States, and in other plants in the genera *Verbena*, *Torenia*, *Phlox*, *Petunia* and *Ageratum* (APS/APHIS Virology working group-Report from Agdia, Inc. 2005). The status of the disease in the USA is unclear because the only commercially available ELISA diagnostic test detects ScrMV, NeRNV and DiaYMV and does not discriminate between the three viruses. Until there is a discriminatory assay for these viruses, it will be unclear to determine which virus is more prevalent. An earlier report from California associated the disease in diascia plants with ScrMV (Mathews *et al.*, 2004), but this was later confirmed as NeRNV (Mathews and Dodds, 2006). NeRNV in ornamental plants from California were identified by RT-PCR (Mathews and Dodds, 2006). The confusion created by the DAS-ELISA assay is evidence that there is a need to improve the diagnostic assays for both ScrMV and NeRNV. Evidence based on serological studies of tymoviruses in the OYMV subgroup have shown that it is common for antibodies of these viruses to fail to distinguish

between specific tymoviruses even when coat protein sequences of the two viruses infer that the viruses are distinct (Koenig *et al.*, 2005a). This situation was observed with several tymoviruses such as NeRNV and ScrMV; CalYVV and CYVV; and also in ScrMV and AVYV (Koenig *et al.*, 2005a). A thorough evaluation of members of the OYMV subgroup has shown that although the viruses in this subgroup are serologically indistinct, amino acid sequences of their CP regions show that they are distinct species. The increased reporting of both NeRNV and ScrMV in the USA and Europe is evidence for the importance of the viruses associated with the tymovirus disease in the ornamental plant industry.

Nicotiana benthamiana and *Nicotiana occidentalis* were used as propagation hosts for the virus because of the higher virus titer in systemically infected leaves. Symptoms on the inoculated leaves were severe in the genus *Nicotiana* but relatively mild in *Nemesia* and *Diascia* species. Observations made on diascia and nemesia plants showed that the symptoms became mild in the winter, and became more intense in the spring as young tissue developed. Symptoms on *Nicotiana occidentalis* plants were more severe after one passage through *Nicotiana occidentalis* compared to inoculation directly from in *Diascia* × *hybrida* ‘Sun chimes™ coral’. This suggests that the virus titer builds to higher levels in *Nicotiana occidentalis* than in *Diascia* × *hybrida* ‘Sun chimes™ coral’ as also corroborated by the PTA-ELISA results.

Mechanical inoculation of *Diascia barberae* ‘Apricot queen’ resulted in mild symptoms on systemically infected leaves and, in the summer, growth was as vigorous as non-inoculated plants indicating that the virus may go unobserved in the nursery. There were no systemic infections of *Nicotiana alata* or the two cultivars of *Nicotiana tabacum*

tested, although the plants showed chlorotic lesions on inoculated leaves. *Chenopodium quinoa*, *Datura stramonium* and *Vicia faba* 'Broad Windsor' have been reported as diagnostic hosts for ScrMV (Becks, 1973; Honetslegrova *et al.*, 1994; Brunt *et al.*, 1996), however DiaYMV was not successfully transmitted to these hosts suggesting that it is a distinct virus from ScrMV. *Datura stramonium* and *Chenopodium quinoa* were also identified as non-hosts to a tymovirus detected in diascia, nemesia and verbena plants in California now identified as NeRNV (Mathews *et al.*, 2004). There was strong serological cross-reactivity between antibodies produced against ScrMV and NeRNV and other viruses such as *Plantago mottle virus* (PIMV), *Anagyris vein yellow virus* (AVYV) and *Ononis yellow mottle virus* (OYMV) (Koenig *et al.*, 2005a). The results from this study are in agreement and have shown that a commercial NeRNV antibody reacts with both NeRNV and ScrMV. However, this trend was not observed in PTA-ELISA with murine monoclonal antibody M3-7·A6·1. The monoclonal antibody M3-7·A6·1 detected NeRNV but not ScrMV (Chapter 3).

It is still not clear why inoculated leaves tested positive for NeRNV and DiaYMV while the systemic leaves were negative. One possible explanation may be that the virus inoculum was not completely washed from the leaves following inoculations, and the positive test was the residual virus, even though this could not be determined. Plants in the greenhouse are normally watered from the base of the stem and not directly onto leaves, hence, the assumption that there might be residual virus on leaves if not completely washed off. Another possibility could be that the virus could be replicating in the inoculated leaves but fail to move systemically. In this experiment, our definition of successful infection was measured by the ability of the virus to infect leaves systemically.

Tymovirus infected *Diascia ×hybrida* ‘Sun chimes™ coral’ contained double membrane vesicles in the chloroplasts (Fig. 18) and the chloroplasts appear clustered together, a feature that has been associated with tymovirus infections (Lesemann, 1977; De Souza *et al*, 1990; Gibbs, 1994; Alexandre *et al.*, 2000; Hull, 2002). The role of DiaYMV insect vector(s) was not included in this study and there is no current literature that demonstrates involvement of a vector in NeRNV transmission. Studies on vector transmission may provide valuable information of disease epidemiology.

It is difficult to ascertain if DiaYMV is a different species distinct from NeRNV based on serology alone. This uncertainty could be resolved if there was a local lesion host identified that would facilitate single lesion inoculations and infection. The scope of the host range needs to be expanded to determine other plant families that might be susceptible to both viruses and those unique to DiaYMV only.

CONCLUSION:

The results of the natural infection studies indicated that DiaYMV as well as NeRNV are present in nurseries. It was evident from the results that not all members of the family Scrophulariaceae are susceptible to DiaYMV. This has been shown by the failure to induce systemic infection in members of the genus *Antirrhinum*. Other hosts susceptible to DiaYMV include *Nicotiana occidentalis*, *Nicotiana benthamiana* and *Nicotiana clevelandii* while *Nicotiana tabacum* ‘Bright yellow’, *Nicotiana tabacum* ‘423’ and *Nicotiana alata* ‘Nicki red’ were not susceptible. Cytopathological studies only confirmed the presence of a tymovirus. Temperature does not have an impact on virus

titer as determined by PTA-ELISA A_{405} values, but the DiaYMV infected plants showed reduced shoot development and were stunted compared to healthy plants.

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CHAPTER 5

GENERAL CONCLUSIONS

Studies on the diseased ornamental plants *Diascia ×hybrida* ‘Sun chimes™ coral’ have shown the disease is caused by tymoviruses. In the nursery, the disease is expressed as a mild mosaic accompanied by vein yellowing in susceptible diascia plants. The original concern about the occurrence of disease in diascia arose when symptoms appeared in early spring regrowth. Hence, temperature may be a factor in symptom development. However, there were no significant differences in absorbance values of PTA-ELISA between inoculated plants grown at 15°C and 25°C suggesting that temperature did not affect the virus concentration in the plant. In studies with *Diascia barberae* ‘Apricot queen’, there were no visible differences between virus infected and virus-free plants at 25°C, but at 15°C the virus infected plants were stunted compared to virus free plants. This result suggests that warmer temperatures favor disease expression. In this growth chamber experiment, there were no mottling symptoms on the infected plants. Immunohistological studies may provide more insight into the effects of temperature as it relates to virus replication and localization. A more quantitative assay that can measure virus titer such as real-time reverse-transcription polymerase chain reaction could also be employed in the future to study virus titer differences in plants grown at different temperatures.

This study revealed that tymoviruses were widely distributed in nemesia and diascia ornamental plants from Washington and Oregon nurseries. A notable exception is *Diascia ×hybrida* ‘Coral belle’. The virus was not detected in either of two separate

years. Mechanical inoculation of *Diascia* ×*hybrida* ‘Coral belle’ resulted in infection showing that these plants are susceptible to infection. Different cultivars from the same supplier were positive for tymoviruses. This suggests that the origin of propagation material and propagation techniques used in nurseries may be determining factors in the distribution and widespread occurrence of these viruses. In most cases clonal propagation from vegetative tissue is employed in nurseries for production of these ornamental plants. Efforts to eliminate the disease should focus on using virus-free stock for clonal propagation. Nursery management practices such as meristem tip culture could be used to eliminate the disease from propagation stock that is already infected.

Members of genus *Antirrhinum* could not be confirmed as hosts for DiaYMV since virus movement into non-inoculated leaves was not detected by ELISA. DiaYMV was detected in inoculated leaves only but not in non-inoculated leaves two weeks after inoculations. This could imply that the virus is replicating but not moving systemically. Our studies on host range could be expanded to include more hosts such as wild species in the family Scrophulariaceae and other families of important crops. We established that some members of the Solanaceae, mainly in the genus *Nicotiana*, were susceptible. This study has demonstrated that the virus from *Diascia* ×*hybrida* ‘Sun chimes™ coral’ causes mild symptoms in its natural *diascia* and *nemesia* species hosts, but can reach very high titers in *Nicotiana occidentalis* and *Nicotiana benthamiana* where it causes severe mosaic and necrosis. Based on host range studies, it was established that the disease of *Diascia* ×*hybrida* ‘Sun chimes™ coral’ was not caused by *Scrophularia mottle virus* (ScrMV) because no symptoms developed on the ScrMV indicator hosts *Datura stramonium* and *Chenopodium quinoa*, and because the monoclonal antibody developed against the virus

from *Diascia ×hybrida* ‘Sun chimes™ coral’ failed to detect ScrMV in *Scrophularia nodosa*. These serological results support the conclusion based on host range that ScrMV is distinct from the viruses found in *Diascia ×hybrida* ‘Sun chimes™ coral’. Because of the similarity between the coat protein regions of the two viruses, monoclonal antibody M3-7·A6·1 could not distinguish between DiaYMV and NeRNV. Since there are ten amino acid differences out of 192, it may be possible to identify a monoclonal antibody that distinguishes between these viruses but many more hybridoma cell lines would have to be screened. Alternatively, antibodies can be prepared against synthetic peptides centered on these amino acid differences. On the other hand, a monoclonal antibody that can detect more species and strains of a virus group in a single assay would be advantageous and less expensive for use in nurseries to screen plants.

In *Diascia ×hybrida* ‘Sun chimes™ coral’, two viruses were identified, a strain of NeRNV (NeRNV-WA) and a second tymovirus DiaYMV. DiaYMV is closely related to NeRNV with respect to the nucleotide sequences of the coat protein (>90 % identity), and the 3'-untranslated region (UTR), features that are used to classify members of the genus *Tymovirus*. However the movement protein (MP) region is different between DiaYMV and NeRNV-Nf showing only 54% amino acid sequence identity. The movement protein (MP) is responsible for virus movement and consequently, has a major role in symptomatology, disease development and host range. It has been shown elsewhere that the MP is a variable region of the tymovirus genome (Koenig *et al.*, 2005) and it is not considered a better indicator of speciation in tymoviruses and other viruses because of higher mutation rates. The overall genome size of DiaYMV and NeRNV-Nf are different. DiaYMV (6,290 nt) has five additional nucleotides in its genome relative to

NeRNV (6,285 nt) and the two genomes differed in many regions, especially ORF I. The overall nucleotide identities for the two viruses was 77.8%, while the tymovirus species demarcation criteria is >80% for same species.

Criteria for species demarcation in the genus *Tymovirus* indicate that DiaYMV is a variant (strain) of NeRNV based on ORF III (CP) amino acid identity (>90%). The 3'-UTR is more closely related to NeRNV-Nf than other tymoviruses in the *Ononis yellow mosaic virus* (OYMV) cluster. Within members of the OYMV cluster, only NeRNV accepts a histidine at its 3'-terminus while other members accept a valine. DiaYMV is capable of accepting a histidine and further analysis showed that despite base changes in the sequences between DiaYMV and NeRNV, there is no change in RNA folding of this region.

Other factors used in species demarcation of tymoviruses include the host range and serological relatedness of the viruses. In host range studies it has been shown that DiaYMV and NeRNV share similar hosts and the range of symptoms are variable within hosts. Host range and symptomatology cannot be used as reliable indicators because it is well known that symptoms can be similar for different viruses or can be different even for strains of the same virus. A wider host range needs to be explored.

Members of the 'OYMV cluster' are serologically related. It has been shown that tymoviruses can be divided into four serological groups, and members within a group very often cannot be differentiated into species based on serology although amino acid sequences of the CP regions suggest that they are distinct species. The monoclonal antibody produced in this study did not differentiate between DiaYMV and NeRNV and, CP sequence comparisons show that DiaYMV and NeRNV are similar. Criteria based on

the CP sequence favor classification of DiaYMV and NeRNV as the same species, while other data support DiaYMV as a distinct species. MP has not been formally considered as an option for species demarcation in tymoviruses, partly because for many viruses the MP has not fully sequenced. Perhaps as more complete viral sequences of plant viruses become available, the contribution of MP sequences to species demarcation maybe accessed differently in the future.

In conclusion, diascia and nemesia in nurseries in the Pacific Northwest are frequently infected with tymoviruses. A newly described virus, DiaYMV, and a strain of NeRNV were identified. It was shown that these two viruses can occur in mixed infections. Because the assays used in the study do not differentiate between DiaYMV and NeRNV, no conclusions on the relative frequencies of these two viruses can be developed. These viruses degrade the quality of ornamental hosts, but they likely pose little threat to agronomic crops since tymoviruses typically have a limited host range. Until further studies are completed, it should be considered that wild members of the family Scrophulariaceae could be impacted by the introduction and wide spread distribution of these viruses.