MOLECULAR CHARACTERISATION OF VIRUSES IN TARO
[*Colocasia esculenta* (L.) Schott]

by

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(2009-09-120)

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2014

DECLARATION

I hereby declare that the thesis entitled **"Molecular characterization of viruses in taro [*Colocasia esculenta* (L.) Schott]"** is a bonafide record of research done by me and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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"I can no other answer make, but, thanks, and thanks."

- William Shakespeare

Dedicated to my mother...

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LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percentage
µg	Microgram
µl	Microlitre
µ*M*	Micromolar
bp	Base pair
cm	Centimetre
m	Meter
CTAB	Cetyl trimethyl ammonium bromide
CTCRI	Central Tuber Crops Research Institute
DNA	Deoxyribo nucleic acid
RNA	Riboxy nucleic acid
dNTPs	Deoxy nucleotide tri phosphates
EDTA	Ethylene diamine tetra acetic acid
FP	Forward primer
RP	Reverse primer
FAOSTAT	Food and Agriculture Organization Statistical Database
g	gram
h	Hour
ha	Hectare
kbp	Kilo basepair
kg	Kilogram
M	Molar
mg	milligram
min	Minute
ml	Millilitre
mm	Millimeter

m*M*	Millimolar
NaCl	Sodium chloride
DsMV	Dasheen Mosaic Virus
CBDV	Colocasia Bobone Disease Virus
TaBV	Taro Bacilliform Virus
TaVCV	Taro Vein Chlorosis Virus
TaRV	Taro Reovirus
GBNV	Groundnut Bud Necrosis Virus
KoMV	Konjac Mosaic Virus
ssRNA	Single stranded RNA
nm	Nanometer
ORF	Open Reading Frames
polyA	Polyadenelation
mRNA	Messenger RNA
PNG	Papua New Guinea
ELISA	Enzyme Linked Immunosorbent Assay
PCR	Polymerase Chain Reaction
NASH	Nucleic Acid Spot Hybridisation
NCM	Nitrocellulose membrane
DIBA	Dot Immuno Binding Assay
RT	Reverse transcription
CP	Coat protein
BLAST	Basic Local Alignment Search Tool
kDa	Kilo Dalton
RNaseH	Ribonuclease H
M-MuLV	Moloney Murine Leukemia Virus
TANSAO	Taro Network for Southeast Asia and Oceania
TaroGen	Taro Genetic Resources: Conservation and Utilisation

Introduction

1. INTRODUCTION

Colocasia esculenta (L.) Schott is an important tuber crop belonging to the monocot family Araceae. The crop, commonly known as taro, is primarily grown for its corm. Taro is a major root crop in the tropics has its origin and genomic home in Southeast Asia. Worldwide, taro ranks fifth among root crops and fourteen among staple vegetable crops with about 12 million tonnes produced globally from about 2 million hectares with an average yield of 6.5 t/ha (FAOSTAT 2010). Due to the ease with which it adapts to diverse farming systems and food cultures, taro has played a central role in the evolution of agro-ecosystems in many countries (Rao, 2010). In addition to its use as a food staple, the crop also has significant cultural and export importance. The Central Tuber Crops Research Institute (CTCRI) has been identified as the National Active Germplasm Site (NAGS) for tuber crops and is maintaining around 590 accessions of taro collected from different locations in India as well as a few collections from abroad.

Plant viruses are important economically in developing countries that are heavily dependent on agricultural production for food security, employment and export earnings, These viruses affect numerous crops in the tropics causing losses that are usually substantial and sometimes devastating, Their identification and characterization represent a vital task for plant virologists'working in tropical regions before developing any strategy to control them.

About 130 pests and diseases of taro have been documented, with impacts ranging from mild to lethal. These pests and diseases do not, however, occur in all countries in the region. Indeed, the majority of severe pathogens have restricted distributions, occurring in only a few locations (Braidotti, 2006). Research programmes dealing with taro mainly aims to combat factors that can ruin taro production. These include an increasing risk of exposure to lethal pathogens and susceptible plant varieties, inadequate diagnostic, screening and pest management

procedures. The main effect of virus infection is a reduction in corm size and quality, with yield losses of up to 60 percent (Zettler and Hartman, 1986). Virus infection spreads primarily through infected planting material since most of the commercially cultivated hosts are vegetatively propagated. Production of virus free planting material is essential for effective management of the viral diseases. Among several viruses reported from taro, such as members of genus Potyvirus, Badnavirus, Rhabdovirus etc., only Dasheen mosaic potyvirus (DsMV) has been well studied and characterized. The lack of data on virus occurrence and virus diversity, some diseases with unknown virus aetiology, lack of validated diagnostic strategies etc., are some of the challenges we are looking at in taro studies.

In order to improve taro production and to ensure quality taro planting material, effective diagnostic method is a pre-requisite. Development of a breeding program first requires the correct identification of infecting viruses. To fulfill this requirement the present study was undertaken with an objective of **identification and characterisation of viruses implicated in taro.**

Review of Literature

2. REVIEW OF LITERATURE

2.1 ROOT AND TUBER CROPS

Root and tuber crops form the means of sustenance for millions of people in the tropical and sub-tropical world. They have the history of saving mankind in times of famine. The world's total harvested area of tubers is nearly 51 million hectares with one third found in Africa and one third in Asia and Pacific regions. These crops are recognized as the most efficient converters of solar energy. Apart from that these tuber crops are known to supply cheap source of energy especially for the weaker sections of the population (Hutabarat and Maeno, 2002).

2.2 TARO [*Colocasia esculenta* (L.) Schott]

Taro [*Colocasia esculenta* (L.) Schott] is a vegetatively propagated crop with edible tubers and leaves belonging to the family Araceae. It is one of the world's oldest food crops, dating back over 9,000 years (Bayliss-Smith 1996; Denham *et al.*, 2003). This ancient crop, first domesticated in Southeast Asia, has continued to spread throughout the world and is now an important crop in Asia, Pacific, Africa and the Caribbean. It is a crop that has been maintained by farmers for millennia and taro genetic resources have remained largely under the control of local communities (Rao *et al.*, 2010). Due to the ease with which it adapts to diverse farming systems and food cultures, taro has played a central role in the evolution of agro-ecosystems and helped to maintain food security in many countries.

Worldwide, taro ranks fourteenth among staple vegetable crops with about 12 million tonnes produced globally from about 2 million hectares with an average yield of 6.5 t/ha. (FAOSTAT 2010). Taro is perhaps the most widely grown of the edible aroids, is a perennial herb consisting of a cluster of smooth, heart-shaped leaves, rising a foot or higher from underground tubers. In temperate areas it is grown as an ornamental plant for its large, glossy leaves (Nelson, 2008). Different parts of the taro

plant, corms, cormels, leaves, petioles and even flowers are consumed as vegetables or used for other purposes, depending on the cultivars and cultures (Xixiang *et al.*, 2010)

Two principal botanical varieties of taro recognized are: *C. esculenta* var. *esculenta*, referred agronomically as the *dasheen* type of taro, and is characterised by the possession of a large cylindrical central corm, and very few cormels. And *C. esculenta* var. *antiquorum*, referred to agronomically as the *eddoe* type of taro and has small globular central corm, with several relatively large cormels arising from the corm (Purseglove, 1972). The sterile tip of the spadix of *antiquorum* is usually much longer than that of *esculenta*. The variety of taro most grown in the Asia/Pacific region is of the dasheen type. Chromosome numbers reported for taro include 2n = 22, 26, 28, 38, and 42. The disparity in numbers may be due to the fact that taro chromosomes are liable to unpredictable behavior during cell divisions. The most commonly reported results are 2n = 28 or 42.

2.2.1 Origin and distribution of taro

Taro is one of the oldest crops, said to have originated in India or Southeast Asia (Barrau, 1965; Plucknett, 1976; Kuruvilla and Singh, 1981), though this is still debated (Matthews, 1990; Yen, 1991, 1993; Loy *et al.*, 1992).

Before its modern introduction to Central and South America, taro was cultivated at temperate and tropical latitudes in both hemispheres, and at longitudes from West Africa to Eastern Polynesia. Taro was thus the world's most widely distributed starchy food plant before the modern era of rapid international transport. Early cultivation and domestication of taro could have started independently in many places over a large geographical range, from India and southern China to Indonesia and Papua New Guinea. In these regions, apparently natural populations of wild taro can be found today. Little is known about the relationships between wild and

cultivated forms of taro, but the species has clearly had a long and complex history of domestication and dispersal (Matthews, 2010).

2.2.2 Morphology

Taro is an herbaceous plant which grows to a height of 1-2 m. The plant consists of a central corm (lying just below the soil surface) from which the leaves grow upwards; roots grow downwards, while the cormels and runners (stolons) grow laterally. The root system is fibrous and lies mainly in the top one meter of soil. In the dasheen type of taro, the corm, which constitutes the main edible part of the plant, grows up to 30 cm long and 15 cm in diameter. The cormels alone constitute a significant proportion of the edible harvest in eddoe taro. The surface of each corm is marked with rings showing the points of attachment of scale leaves or senesced leaves. Axillary buds are present at the nodal positions on the corm (Deo *et al.*, 2009). Diagramatic representation in Figure 1.

Leaf laminae can be from 30 to more than 80 cm long and from 20 to more than 50 cm wide. Leaf petioles are stout, clasping at the base. Petiole length varies depending on genotype; from less than 30 cm to more than 1.5 m. Leaf size is strongly influenced by the environment. Maximal dimensions of taro leaves are usually associated with the beginning of flowering. Most accessions have a semi-erect position of the leaf lamina and an undulate leaf lamina margin. The colour of leaves is genetically controlled and represents one of the most useful traits for describing genotypes. It varies from a whitish yellow to a very dark purple, depending on the genotype. It can be uniform or show variations (lines, spots or blotches of different pigmentations). Leaf petioles and leaf laminae do not always have the same colour. The basic colour of the petiole is extremely variable and tremendous variation of the patterns (lines, stripes, blotches, dots, etc.) (Lobot *et al.*, 2010).

2.2.3 Nutritional value

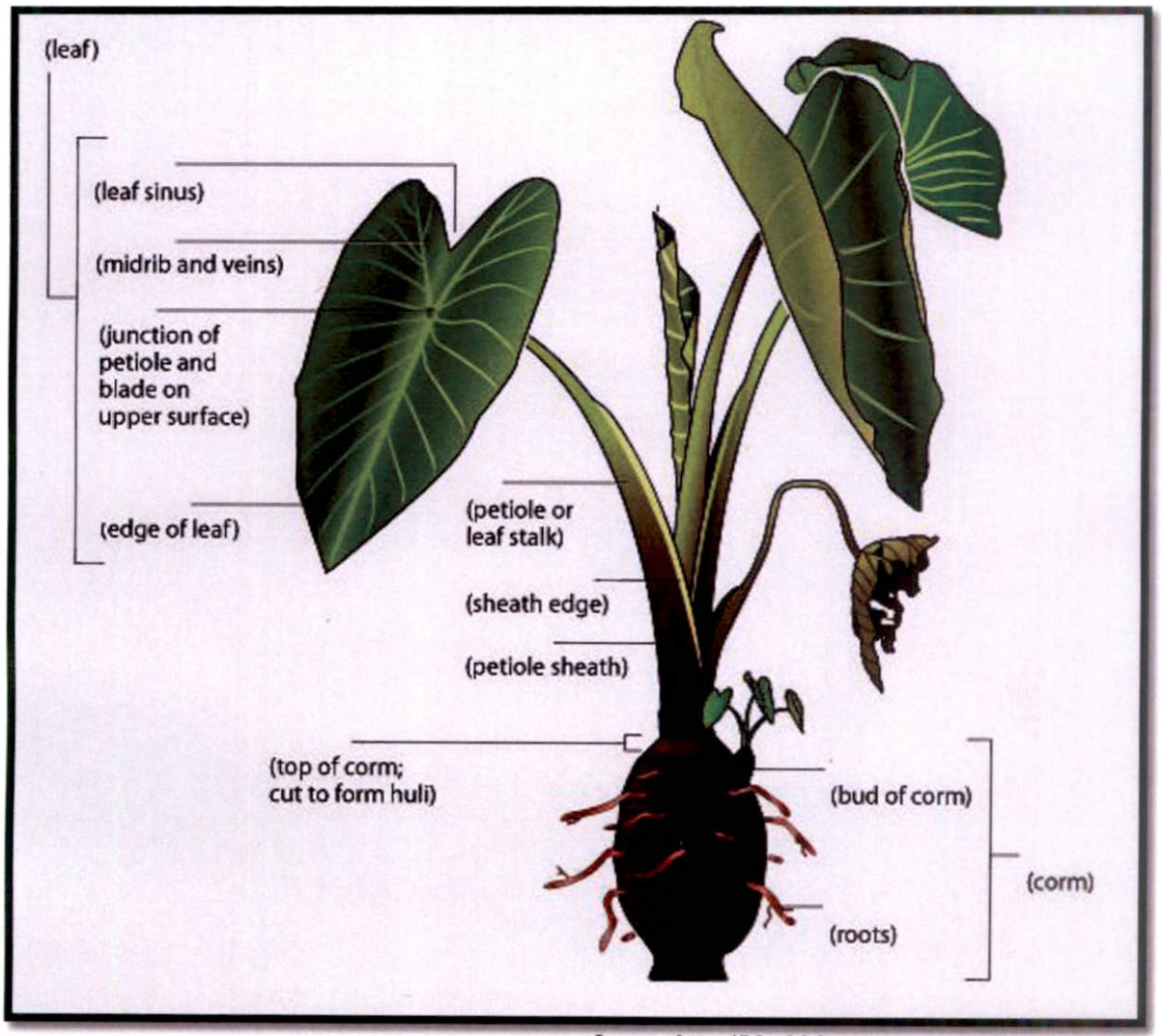


Image: http://hbs.bishopmuseum.org/botany/taro

Figure 1: Taro morphology

Taro is embedded in many cultures as a result it is selected for a wide variety of uses and is a nutritional food. Only the skin of the taro corm, and the true anatomical roots, has not been reported as food. All other parts of the plant are potentially edible (corms, blades, petioles and inflorescences). The corms and leaves are also used for medicinal purposes (Rao, 2010).

Its corms are baked, roasted, or boiled and the leaves are frequently eaten as a vegetable and represent an important source of vitamins, especially folic acid. The corms are primarily a source of energy in the form of easily digested starch. They are high in carbohydrates and low in fat and protein. Fresh corms are composed of about 69% moisture, 25% starch, 1.5% dietary fibre, 1.1% protein and 1% sugar (Bradbury and Holloway, 1988). The corms also provide a good range of vitamins, amino acids and minerals. Among the essential amino acids, phenylalanine and leucine are relatively abundant. Among minerals, potassium is abundant and iron is lacking (Mattlhews. 2010). Standal (1983) reported that the petiole contains fewer nutrients than the corms and leaves (blades), but also has a generous amount of potassium. Taro leaf blades are a good source of protein, minerals and vitamins. The protein content (average 4.2% of fresh weight) is much higher than in the corms (Table 1).

Table 1: Proximate Composition of the Taro Corm on a Fresh Weight Basis

Component	Content
Moisture	63-85%
Carbohydrate (mostly starch)	13-29%
Protein	1.4-3.0%
Fat	0.16-0.36%
Crude Fibre	0.60-1.18%
Ash	0.60-1.3%
Vitamin C	7-9 mg/100 g
Thiamine	0.18 mg/100 g
Riboflavin	0.04 mg/100 g
Niacin	0.9 mg/100 g

Source: Onwueme, 1994

2.3 VIRAL DISEASES IN TARO

Taro, like other root and tuber crops propagated vegetatively, poses special problems not only in collecting and storing, but also from a germplasm health point of view (Rodoni *et al.*, 1995). In vegetatively propagated crops, viruses present in the mother plant inevitably will be found in derived vegetative parts, such as tubers and roots. Viruses are one of the most important pathogens of taro, with some infections resulting in severe yield reductions and plant death.

2.3.1 Viruses infecting taro

The five main viruses reported infecting taro are, *Dasheen mosaic virus* (DsMV), *Colocasia bobone disease virus* (CBDV), *Taro bacilliform virus* (TaBV), *Taro vein chlorosis virus* (TaVCV) and *Taro reovirus* (TaRV). Apart from DsMV, these viruses have been poorly characterised and there is confusion in the literature concerning their distribution and the symptoms associated with infection (Revill *et al.*, 2005a). Some of the viruses occur as latent infections, and all can be found in various combinations; this means that it is difficult to ascribe symptoms to infection by individual viruses.

2.3.2 Viruses infecting taro in India

Sivaprasad *et al.* (2011) reported Groundnut Bud Necrosis Virus (GBNV) infecting taro (*C.esculenta*) for the first time. GBNV is a member of the genus Tospovirus; Tospoviruses are among the most damaging and economically important group of plant viruses causing significant crop losses in wide range of ornamental and food crops in many regions of the world (Mumford *et al.*, 1996). For the first time in India, Konjac mosaic virus (KoMV) belonging to the genus *Potyvirus* was reported to be infecting three aroid plant species *C. esculenta*, *Caladium spp*, and *Dieffenbachia spp* (Manikonda *et al.*, 2010). The associated symptoms were mosaic, chlorotic feathery mottling, chlorotic spots, leaf deformation and chlorotic ring spots.

2.3.3 Potyvirus

The family *Potyviridae* is the largest family of positive-sense, single-stranded RNA (ssRNA) plant viruses currently recognized, many of which cause significant losses in agricultural, pasture, horticultural and ornamental crops. Based on their transmission vectors and genomic characteristics, the members of the family are classified into eight genera, *Potyvirus, Ipomovirus, Macluravirus, Tritimovirus, Bymovirus, Rymovirus, Brambyvirus* and *Tritimovirus*. Among these, the genus *Potyvirus* containing the largest number of plant virus species, including 111 recognized species and 86 tentative species currently assigned to it by the International Committee on Taxonomy of Viruses (ICTV, 2013).

Potyviruses cause significant losses in a wide range of crop plants and are transmitted by aphids in a non-persistent manner. Members of the genus *Potyvirus* have flexuous filament virions, 680-900 nm long and 11-13 nm wide. The single-stranded, positive-sense monopartite or bipartite RNA genome of potyviruses (~10 kb) is polyadenylated at the 3' end and has a viral genome-linked protein (VPg) covalently linked to the 5' end. The whole genome encodes a single polyprotein subsequently processed into 9-10 proteins by three virus-encoded proteinases (van Regenmortel *et al.*, 2000).

2.3.3.1 Dasheen Mosaic Virus (DsMV)

Dasheen mosaic virus (DsMV), first described by Zettler *et al.* 1970, from dasheen (*Colocasia esculenta* (L) Schott), is an aphid-transmitted virus assigned to the genus *Potyvirus* of the family *Potyviridae*. *Dasheen mosaic virus* has a worldwide distribution, but has been found naturally occurring only in members of the family *Araceae* (Zettler *et al.*, 1978; Zettler and Hartman, 1986; Zettler and Hartman, 1987), infecting at least 16 genera of *Araceae* family.

Dasheen mosaic potyvirus (DsMV) is the most important viral pathogen of cultivated edible and ornamental aroid plants worldwide and is ubiquitous in commercial plantings of *Colocasia* and *Xanthosoma* spp (Shimoyama *et al.,* 1992a; Zettler *et al.,* 1978). In India, a mosaic disease on Colocasia has been reported from Thiruvananthapuram in the early 80's, with a typical symptom resembling that of DsMV (Malathi and Shanta, 1981). Occurrence of mosaic disease on *C. esculenta* in north eastern states of India has also been recorded (Swamy *et al.*, 2002).

Infected plants usually display a conspicuous feathery mosaic pattern although cultivars vary considerably in symptom expression. Although typical feathery mosaic symptoms were associated with many infections, the virus was also detected in a large number of plants that appeared healthy and plants that were infected with other viruses (Fig. 2). The main effect of virus infection is a reduction in corm size and quality, with yield losses of up to 60 percent having been reported (Zettler and Hartman, 1986). Like other potyviruses, DsMV has flexuous, filamentous non-enveloped particles about 750nm in length, which contains a positive sense, single-stranded RNA of *Mr* 3.2-3.4x10^6 (Abo El-Nil *et al.*, 1977; Kositratana, 1985; Li *et al.,* 1992). The virus is transmitted by several widely distributed aphid species, including *Myzus persicae* and *Aphis gossypii* in a non-persistent manner. There are no effective control measures for DsMV. Removal and subsequent burning or burial of infected plants has been recommended, as these plants may serve as sources of infection.The virus is reportedly eliminated from taro plants using meristem tip culture, but reinfection is rapid (Carmichael et al., 2008).

2.3.4 Badnavirus

Badnaviruses belongs to the family *Caulimoviridae* and are pararetroviruses which typically have bacilliform-shaped virions approximately 30-130 nm encapsulating a circular dsDNA genome of approximately 7.1–7.6 kbp (Hull *et al.,* 2000). The genomes of all badnaviruses have a similar organisation and contain three

open reading frames (ORFs) and one large intergenic region (Pooggin *et al.*, 1999), although TaBV contain an additional one ORF (Yang *et al.*, 2003). The genome also contains a single transcription start site and a polyadenylation (polyA) signal which allows the synthesis of a larger than unit length terminally redundant transcript. This transcript serves both as a polycistronic mRNA for translation into functional proteins, including aspartic protease, reverse transcriptase, ribonuclease H, movement protein and coat protein, and also as a template for replication via reverse transcription (Hohn and Fütterer 1992; Rothnie *et al.*, 1994).

2.3.4.1 Taro Bacilliform Virus (TaBV)

Taro bacilliform virus (TaBV) is a pararetrovirus of the genus *Badnavirus*, family *Caulimoviridae* which infects the monocotyledonous plant, taro (Purseglove, 1988; Yang *et al.*, 2003). Transmission experiments have indicated that the virus is mealybug transmitted. Infection of taro with TaBV alone is thought to result in a relatively mild disease characterized by vein clearing, stunting and down-curling of the leaf blades (Jackson, 1978) (Fig. 3). In contrast, plants infected with both TaBV and *Colocasia bobone disease virus* (CBDV), a putative rhabdovirus, is thought to develop the lethal alomae disease (James *et al.*, 1973; Kenten and Woods, 1973). The symptoms associated with TaBV are still unclear because many of the plants testing positive for this virus were also shown to be co-infected with other viruses. Alomae is the most economically important virus disease affecting taro and appears to be restricted to Papua New Guinea and the Solomon Islands while TaBV appears to be widely distributed throughout the Pacific (Gollifer *et al.*, 1977; Gollifer and Brown, 1972; Jackson and Gollifer, 1975; Jackson, 1978; Rodoni *et al.*, 1994). In the case of TaBV no control measures are known to prevent the spread because the virus is latent.

2.3.5 Rhabdovirus

2.3.5.1 Taro Vein Chlorosis Virus (TaVCV)

Taro Vein Chlorosis Virus (TaVCV) is a definitive rhabdovirus approximately 210 x 70 nm with most similarity to members of the genus *Nucleorhabdovirus*. TaVCV, causes a distinctive veinal chlorosis in diseased taro and is thought to occur in Fiji, Vanuatu, Tuvalu, Philippines and possibly PNG (Pearson *et al.*, 1999). The complete sequence of genes encoding the matrix and glycoproteins, and partial sequence of the L-gene (polymerase) is known. The conserved intergenic sequences and amino-terminus of the viral glycoprotein has been identified. Its effect on plant growth appears to be minimal and symptoms are commonly seen on plants when they are at maximum growth, rather than after planting or at maturity as with other taro viruses. Leaves show a distinct vein chlorosis (Fig. 4), more pronounced than TaBV (Revill *et al.*, 2005b).

Taro growers do not normally apply control measures for this disease as the taro plants recover and produce symptomless leaves. There is no indication that infection affects corm yield. However, removal and subsequent burning or burial of infected plants are recommended in Papua New Guinea and Solomon Islands, as these plants may serve as sources of infection for alomae. It is not yet clear which viruses interact to cause alomae, but taro vein chlorosis virus (TaVCV) is a candidate.

2.3.5.2 Colocasia Bobone Disease Virus (CBDV)

Brunt *et al.* (1990) reported that Colocasia Bobone Disease Virus (CBDV) as a possible rhabdovirus as it possessed morphologically characteristic bullet-shaped or bacilliform particles measuring 300-335 x 50-55 nm. Among the viruses, the colocasia bobone rhabdovirus appears to be the most difficult one to work with and it is imperative that better identification and testing methods be developed. The virus has the greatest potential to impact on taro production.

CBDV is spread by plant hoppers, *Tarophagus* spp. Infection of taro with CBDV alone is thought to result in the disease known as bobone, a disease only reported from PNG and the Solomon Islands, which is characterised by stunting, leaf

distortion and presence of galls on the petioles (Jackson, 1978). A combination of CBDV with TaBV is found to cause "alomae" disease (James *et al.,* 1973), although there is still a considerable amount of confusion regarding the etiology of this disease. Alomae disease is considered the most destructive virus disease of taro and has only been reported from the Solomon Islands and PNG (Jackson and Gollifer, 1975; Rodoni *et al,* 1994). Symptoms include crinkling of young leaves that fail to develop normally, the presence of thickened veins and lamina, shortening of the petioles and the presence of irregularly shaped outgrowths on the petioles (Fig. 5). Infected plants ultimately die due to the development of a systemic necrosis (Rodoni *et al.,* 1994). As a disease management measure removal and subsequent burning or burial of infected plants is recommended since CBDV is known to be one of the viruses causing alomae.

2.3.6 Reovirus

2.3.6.1 *Taro Reovirus (TaRV)*

Devitt *et al.* (2001) detected *Taro Reovirus* (TaRV) and genome has been partially characterised from taro in PNG while screening taro germplasm for new viruses using dsRNA analysis and found to have greatest similiarity to the viruses in the genus *Oryzavirus* in the family *Reoviridae.* Although virions have not yet been purified, they are typically spherical particles, approximately 65-75 nm in diameter. TaRV has been identified only in *Colocasia esculenta* and only in plants infected with at least one other virus (Devitt *et al.,* 2001; Revill *et al.,* 2005a).

This virus appears to have a genome comprising 10 segments of dsRNA. Based on comparisons with other reoviruses, degenerate primers were designed to conserved regions of S4 to amplify a 1.7 kbp fragment. This previously un-described virus infecting taro although detected in several countries throughout the Pacific, the symptoms associated with infection are unknown since the virus was detected in apparently symptomless plants and in plants that were co-infected with other viruses.

Figure 2: Symptom of Dasheen mosaic virus

Figure 3: Symptom of Taro bacilliform virus

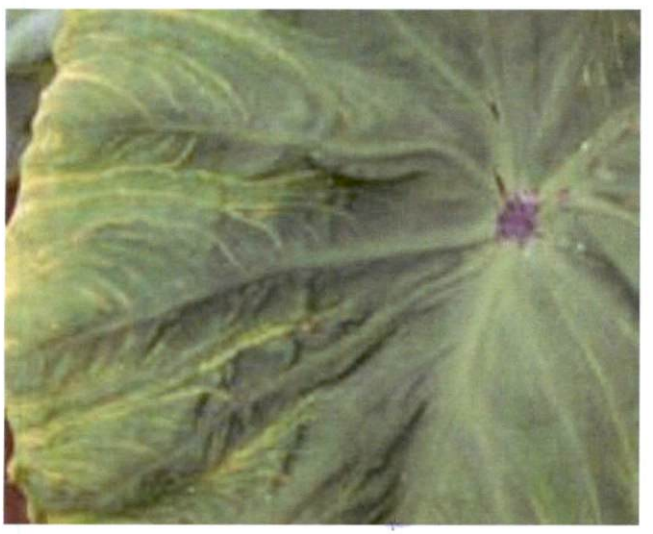


Figure 4: Symptoms of Taro vein chlorosis virus

Figure 5: Symptoms of Colocasia bobone disease virus

Fig. 4 & 5 Source: TaroPest-An illustrated guide to pests and diseases of taro in the South Pacific

As such, the impact of this virus on taro is unknown and there is no information on the significance of TaRV.

2.4 METHODS OF VIRUS DETECTION AND DIAGNOSIS

The most common methods of virus detection and identification include serological methods of detection like enzyme linked immunosorbent assay (ELISA) and nucleic acid based techniques like polymerase chain reaction (PCR), nucleic acid spot hybridization (NASH) etc. The correct diagnosis of virus is an important effort and a prerequisite to virus elimination or development of virus free plants through tissue culture, chemotherapy etc.

2.4.1 Enzyme linked immunosorbent assay (ELISA)

Since the 1970s, serological methods like enzyme linked immunosorbent assay (ELISA) have been used widely and successfully for detection of plant viruses and diagnosis of plant viral diseases (Clark and Adams, 1977; Flegg and Clark, 1979). ELISA techniques include NCM-ELISA and Dot Immuno Binding Assay (DIBA). But it has major limitations such as its low sensitivity during periods of low virus titre. Moreover serological diagnosis of potyviruses is often imprecise, because of frequent serological cross-reactions between species and biological indexing is very cumbersome (Brunt, 1992).

Both double antibody sandwich ELISA (direct ELISA) and indirect ELISA can be used for indexing, identification, and characterization (Hu *et al.*, 1995; Shimoyama *et al.*, 1992b; Rodoni and Moran 1988). With the advancement in molecular biology techniques, viral specific genes are subjected to protein expression and the produced protein are used as a source of antigen for antibody production. Kim *et al.* (2004) produced polyclonal antibodies specific to DsMV infecting *C. esculenta*, using the complete purified virus as source of antigen. Li *et al.* (1998) cloned the coat protein gene of the DsMV-ch isolate from *Caladium hortulanum* and

expressed in bacterial expression vector for the production of specific coat protein which was purified and then subsequently used for antibody production of suitable DsMV antiserum for its diagnosis and detection. The antisera to DsMV were prepared using recombinant capsid proteins as the antigens (Huang *et al.,* 2005, 2007; Hu *et al.,* 2007).

2.4.2 Polymerase chain reaction (PCR)

In the 1990s, nucleic acid-based methods such as reverse transcription (RT) and the polymerase chain reaction (PCR) began to be used in plant virus detection (Wetzel *et al.,* 1991; Rowhani *et al.,* 1995; Thomson and Dietzgen, 1995). The PCR-based methods are fast, highly sensitive and useful for accurate detection, quantification and characterization of plant pathogens. However, PCR and RT-PCR can be carried out for a limited number of samples because after PCR amplification, products need to be analysed by tedious agarose gel electrophoresis. Several degenerate primers have been designed to recognize the conserved regions of viral genomes of many virus species or the whole virus genus or family (Langeveld *et al.*, 1991; Bateson and Dale, 1995; Tian *et al.,* 1996; Gibbs and Mackenzie, 1997; Chen *et al.,* 2001a; Posthuma *et al.,* 2002).

PCR-based methods for the detection and identification of potyviruses are primarily based on the use of degenerate primers to conserved sequences in the viral genomes. The vast majority of these primers have been designed to sequences at the 3' end of the genome, such as the CP- and NIb-coding regions. The use of degenerate primers has not only facilitated the rapid detection of many potyviruses but has also enabled partial genomic sequencing for taxonomic purposes (Ha *et al.*, 2008). Double-stranded RNAs, usually present in detectable amounts in plants infected with RNA viruses (either as a genomic molecule or a product of viral replication), are increasingly becoming preferred templates for detection and characterization of these viruses. They are more stable than single-stranded molecules and, combined with the

proper choice of downstream approaches (cloning, sequencing), represent ideal starting material for detection and identification of novel plant viruses (Sabanadzovic *et al.*, 2010). The group-specific PCR and subsequent sequence analysis of the amplified regions has been used for rapid detection and identification of Potyvirus and is appeared to be the most suitable method for identification of viruses which are difficult to purify and/or occurring in mixed infections (Colinet *et al.*, 1998).

Marie-Jeanne *et al.* (2000) *and* Grisoni *et al.* (2006) used MJ1(F)-5'-TGGTHTGGTGYATHGARAAYGG- 3' and MJ1 (R) 5'-TGCTGCKGCYTTCATY TG-3' as the universal *potyvirus* group specific primers. PCR using MJ1 and MJ2 primers on the infected leaf samples of *Colocasia esculenta* plants showing severe whitish feathery symptoms resulted in an amplicon of 327 bp, encoding the core region of the coat protein gene. Babu *et al.* (2011a) proved RT–PCR with MJ1 and MJ2 primers useful for the detection and identification of the potyvirus infecting *C. esculenta* in India.

Due to the characteristic poly (A) tail at the 3' end of potyvirus genome, the first-strand cDNA of potyviruses was synthesized using oligo(dT)12–18 primer or random hexamers with RNA as the template (Wen-Chi Hu *et al.*, 2010; Li *et al.*, 1998). Hsu et al. (2005) developed an RT-PCR based method, which has the potential to detect members of the genus *Potyvirus* by using new designed potyvirus degenerate primers. Combining the RT-PCR technique and degenerate primers, it is possible to detect many virus species of the same genus or family in a single test, but it cannot distinguish the virus species. Currently, rapid identification of a plant virus is based on ELISA, RT-PCR with specific primers, or cloning and sequencing methods. These methods are facilitated when some information about the target viruses is available.

Three degenerate primers, located at the NIb and CP gene regions, were designed for *potyvirus* detection. Using these primer pairs, 1.0-1.2 kb cDNA

fragments of the 3'-terminal region of six potyviruses were successfully amplified from infected plant tissues. RT-PCR products were sequenced and found to be derived from the expected viruses. To identify further these potyviruses, sequences located between the 3' end of the NIb gene and the 5' end of the CP gene were chosen to design a series of species-specific probes (Hsu *et al.*, 2005). The probes were prepared by PCR with species-specific primers, immobilized onto nylon membrane, and then hybridized with DIG-labeled RT-PCR products amplified by potyvirus degenerate primers. The results suggested that species-specific cDNA probes plus reverse dot blot hybridization was able to identify correctly different species of potyviruses in single as well as mixed infections. Babu *et al.*. (2012) designed DsMV-specific primers for the amplification of partial coat protein and the 3' untranslated region of *Dasheen mosaic virus* infecting *Amorphophallus paeoniifolius.*

Due to *Badnavirus* genomes being highly variable, most of the primers that have been designed are to the ORF III conserved regions (Medberry *et al.*, 1990), i.e. the tRNAMet binding domain and the RNase H and RT regions (Lockhart and Olszewski, 1993; Geering *et al.*. 2000). Based on the sequences of the TaBV RT/RNaseH-coding region, a PCR-based diagnostic test that specifically detects all known TaBV isolates has been developed (Revill et al., 2005a). A sequence showing approximately 50% nucleotide identity to TaBV in the RT/RNaseH-coding region was also detected in all taro plants tested and may represent either an integrated sequence or the genome of an additional badnavirus infecting taro.

2.4.3 Nucleic Acid Spot Hybridisation (NASH)

Nucleic acid hybridization and RT-PCR detection are more sensitive methods for the detection of virus, (Seal and Coater, 1998; James, 1999). Nucleic acid spot hybridization (NASH) is more convenient than RT-PCR for specific and large-scale detection of the viruses (Craig *et al.*, 2004). Babu *et al.* (2011b) prepared cRNA

probes which were successfully used for the diagnosis of the DsMV infecting *A. paeoniifolius* through nucleic acid spot hybridization by RNA-RNA hybridisation. The probes detected only the DsMV infecting *A. paeoniifolius*, while they did not detect the DsMV from *Colocasia esculenta* or *Xanthosoma.*

2.5 CHARACTERISATION OF VIRUSES INFECTING TARO

2.5.1 Dasheen Mosaic Virus (DsMV)

The genome of DsMV infecting taro was outlined by Chen et al., (2001b). It consisted of 9991 nucleotides for its single stranded positive-sense RNA coding ten conserved proteins. Potyvirus isolates with 85% sequence identity or more over the whole genome are usually considered to be from the same species (Fauquet *et al.,* 2005). This was re-evaluated by Adams *et al.,* in 2005, who suggested that a value of about 76% was most appropriate for the entire polyprotein sequence and that slightly different values might be used for the different regions of the potyvirus genome.

The first molecular level characterization of the DsMV infecting *C. esculenta* in India was carried out by Babu *et al.* (2011a). Reverse transcription polymerase chain reaction of the infected leaf samples of *C. esculenta* plants showing severe whitish feathery symptoms were carried out using Potyvirus group specific primers, resulting in an amplicon of 327 bp, encoding the core region of the coat protein gene. Sequencing and BLAST analysis showed that the virus is distinct, closely related to Dasheen mosaic virus (DsMV). Sequence analysis revealed 86 and 96 percent identity at the nucleotide and amino acid level respectively with the DsMV isolate SY1 (accession Number AJ628756).

Diversity in the CP sequences of the DsMV isolates and strains of other potyviruses occurred predominantly at the N-terminal regions. This variable region of the potyviral CP is useful for clarifying relationships among closely related strains (Shukla *et al.,* 1988). Although the CPs of potyviruses are, for the most part, highly

conserved, they can differ somewhat in sequence and length at the N-terminal region (Shukla *et al.,* 1994).

2.5.2 Taro Bacilliform Virus (TaBV)

Yang *et al.*, (2003) characterized the complete nucleotide sequence of a PNG isolate of Taro Bacilliform Virus (TaBV) confirming that it is a pararetrovirus of the genus *Badnavirus*, family *Caulimoviridae*. Two degenerate primers, BadnaFP and BadnaRP, were designed based on the consensus sequences of the reverse transcriptase (RT) region and RNaseH regions of published badnavirus sequences, and these were used in a PCR. The 7458 bp genome and genome organisation of TaBV was similar to that of most other published badnaviruses. The putative amino acid sequence of TaBV open reading frame (ORF) 3 contained motifs that are conserved amongst badnavirus proteins including aspartic protease, reverse transcriptase (RT) and ribonuclease H (RNaseH). Phylogenetic analysis using the amino acid sequence of ORF 3 showed that TaBV branched most closely to *Dioscorea bacilliform virus*. Sequence comparisons between TaBV and other badnaviruses, at both the nucleotide and amino acid levels, revealed low sequence identities. The genome organisation of TaBV differs slightly from most other badnaviruses by the presence of an additional small fourth ORF that encodes a putative protein of 13.1 kDa.

2.5.3 Taro vein chlorosis virus (TaVCV)

Revill et al. (2005b) sequenced the monopartite RNA genome of a Fijian isolate of *Taro vein chlorosis virus* (*TaVCV*) confirming that it is a definitive rhabdovirus with most similarity to members of the genus Nucleorhabdovirus. The TaVCV 12020 nt negative-sense RNA genome contained six ORFs in the antigenomic sequence. Primers were designed based on the G and L genes of similar viruses. The high level of TaVCV variability observed suggested that the introduction of TaVCV to the Pacific Islands was not a recent occurrence.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Molecular characterization of viruses in taro [*Colocasia esculenta* (L.) Schott]" was carried out at the Division of Crop Protection, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2013-2014. Details pertaining to the experimental material and procedures used in the study are elaborated in this chapter.

3.1 SAMPLE COLLECTION

Taro samples belonging to various accessions were collected from various Indian states and from the germplasm collection maintained at CTCRI. 100 leaf samples exhibiting viral symptoms (i.e. plants showing characteristic mosaic, veinal chlorosis, puckering, cupping and curling of leaves) and suspected of virus infection was collected from various parts of CTCRI, Thiruvananthapuram, Regional Centre-CTCRI, Bhubaneshwar and farmers fields in Jharkhand. From these, 22 samples with various symptoms were selected as the representative sample set for the present study after initial serological screening. Some samples of *Xanthosoma sagittifolium* (Tannia) were also collected from the CTCRI germplasm repository which exhibited similar severe virus infection symptoms as seen in taro. Plants showing no observable symptoms were also sampled to check the possibility of latent infection. The samples were photographed, symptoms were recorded and stored. The geographical origin of the selected accessions is represented in Table 3.

3.2 SEROLOGICAL METHODS OF DETECTION

Enzyme Linked Immunosorbent Assay (ELISA) and Dot Immunobinding Assay (DIBA) were employed for the detection of virus infection in *Colocasia esculenta* leaf samples. This procedure was carried out to screen the samples for *DsMV* infection using *DsMV* polyclonal antibody AS-1021 obtained from DSMZ,

Germany. Samples which showed positive results in these methods were subjected to RT-PCR based detection also.

3.2.1 Enzyme Linked Immunosorbent Assay (ELISA)

Double antibody sandwich ELISA (DAS-ELISA) for DsMV detection was carried out on fifty five *C. esculenta* leaf samples showing different symptoms of virus infection. A healthy non host sample collected from the field was used as the negative control.

Wells of ELISA microtitre plates were coated with 200 µl of purified primary antibody (Polyclonal Taro DsMV IgG) diluted to 1:1000 in coating buffer (Appendix) and incubated at 37°C for 4 hours. Leaf sap extract (200 µl) prepared by grinding 100 mg test leaves in sample extraction buffer (Appendix) and centrifuged at 8000 rpm for 10 minutes was loaded in duplicate wells and incubated overnight at 4°C. Similarly, 200 µl of secondary antibody (enzyme labelled anti-rabbit) diluted to 1:500 in conjugate buffer (Appendix) was added to the wells and plates were incubated at 37°C for 4 hours. 200 µl aliquots of freshly prepared substrate (Appendix) was added to each well and incubated at room temperature in dark condition.

The plates were washed with PBS-Tween-20 thrice at three min interval after each incubation step. The A405 for sample in each well was measured in a BIO-RAD iMark Microplate Reader (USA). The readings were taken at 0 h, 5 min, 30 min, 1 h, 2 h and overnight.

3.2.2 Dot Immuno Binding Assay (DIBA)

Twenty two representative leaf samples and a healthy non host were screened for DIBA using Taro *DsMV* antibody. A desired size of NCM was cut and 1 cm^2 squares were drawn on it. The NCM was wetted by floating it in TBS and was air dried. 5 µl of partially purified *C. esculenta* leaf samples were spotted on respective

squares. After air drying, the membrane was immersed in blocking solution (Appendix) with gentle shaking for 1 h at room temperature. It was then rinsed once in TBS for 10 min. This was followed by incubating the NCM with primary antibody (Polyclonal Taro DsMV IgG) diluted to 1:1000 in TBS-SDM for 1 h at room temperature or overnight at 4°C. Then the membrane was washed thrice with TBS at 10 min interval and incubated with secondary antibody (enzyme labeled anti-rabbit IgG (ALP- conjugate)) diluted 1:1000 in TBS-SDM for 1 h at room temperature or overnight at 4°C. After rinsing thrice with TBS, the NCM was incubated in substrate solution (BCIP/NBT) at room temperature in dark condition for 10 to 15 min. It was then observed for color development. The membrane was rinsed with distilled water and then air dried. The color formation and intensity analyzed.

3.3 NUCLIEC ACID EXTRACTION

For carrying out PCR and RT-PCR based detection of the viruses infecting *C. esculenta*, DNA and total RNA isolation is a prerequisite. RT-PCR was performed on RNA isolated from infected leaf samples using genus specific and virus specific primers.

3.3.1 Isolation of total RNA

Leaf samples of virus infected *C. esculenta* plants showing prominent mosaic, feathery mosaic and prominent puckering symptoms were taken for RNA isolation. RNA was isolated using SIGMA TRI reagent based method and Lithium Chloride Method. The RNA isolated by all methods was stored at -20°C. The RNA pellet was solubilized in 30 µl of DEPC treated water.

3.3.1.1 RNA isolation using TRI Reagent (SIGMA)

Tri reagent (SIGMA) was used for extraction of total RNA. Samples (80-100 mg) were ground into a fine powder in liquid nitrogen using mortar and pestle. The ground sample was immediately homogenized in 1 ml of TRI Reagent. Mixture was

transferred into a micro centrifuge tube and centrifuged at 12000 x g for 10 minutes at 4°C and supernatant was collected. Followed by 5 min incubation at room temperature, 0.2 ml of chloroform was added and the tubes were shaken vigorously for 15 s. The tubes were incubated for 15 min at room temperature. The samples were centrifuged at 12,000 x *g* for 15 min at 4°C. The aqueous phase of the sample was transferred into a fresh tube. 0.5 ml of chilled 100 percent isopropanol was added to the aqueous phase and mildly inverted to mix and incubated at room temperature for 10 min. The sample was then centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was removed leaving only a pellet. The pellet was washed with 1 ml of 75 percent ethanol. Discarded the supernatant and the RNA pellet was air dried for 10-15 min. The RNA pellet was then resuspended in 50 µl RNase free sterile water followed by incubation in a water bath at 55-60 °C for 10 min.

The integrity of the total RNA was determined by running 4 µl aliquot of RNA on agarose gel (1.2%) as described in section 3.3.2.1

3.3.1.2 RNA isolation using Lithium Chloride Method

Sample weighed around 100 mg was ground into fine powder in liquid nitrogen and it was transferred into a fresh tube and 1 ml of CTAB RNA extraction buffer (pre-warmed at 65°C) (Appendix) was added, vortexed and incubated at 65°C (Lab ROTEK, India) for 10 min. The tube was centrifuged at 15,000 rpm (Hermle, Table top refrigerated centrifuge) for 15 min at room temperature and supernatant was transferred to a fresh 2 ml tube. Equal volume of chloroform: isoamyl alcohol (24:1) was added to the supernatant and centrifuged at 20,000 x *g* for 10 min at 4°C. After transferring the supernatant into fresh tube, equal volume of chloroform: isoamyl alcohol was added to the supernatant. After centrifugation at 20,000 x *g* for 10 min at 4°C, the supernatant was transferred to a fresh tube. Then, 0.25 volume of ice cold 10 *M* lithium chloride was added, mixed well and incubated overnight at -20°C. Following centrifugation at 30,000 x *g* for 30 min at 4°C, the pellet was

washed with 75 percent ethanol by centrifuging at 10,000 x *g* for 10 min at 4°C. The washing was repeated with 200 µl of 75 percent ethanol. RNA pellet obtained was air dried at 37°C for 30 min and then dissolved in 30 µl DEPC water. After incubating at 37°C for 1 h while tapping intermittently, the RNA was stored at -20°C (Vest frost Low Temperature Cabinet, India).

3.3.2 Analysis of extracted RNA

3.3.2.1 Agarose gel electrophoresis

The most common method adopted to evaluate the integrity of the RNA preparation is to run an aliquot of the sample on agarose gel. An agarose gel of 1.2 percent was prepared in 1X TAE buffer (Appendix) and ethidium bromide was added, 0.5 µl per litre. An aliquot of the RNA sample (3 µl) mixed with the loading dye was loaded in each of the wells of the gel. The gel was run at 5 Vcm^{-1} (Consort Powerpack, Belgium) for 30 min. The gel was then visualized under UV light and the image was documented using Alpha Imager (Alpha Innotech, USA).

3.3.3 cDNA SYNTHESIS

3.3.3.1 First strand cDNA synthesis

The RNA isolated from leaf samples were subjected to cDNA conversion using M-MuLV Reverse Transcriptase (GeNei, Bangalore). The components of the reaction mix were as follows:

RNA	:	5 µl
Oligo (dT) Primer	:	1 µl
Nuclease free water	:	6 µl

The mixture was incubated at 65°C for 5 min and chilled on ice. Followed by a brief centrifuge, the following components were added to the mix.

5x reaction buffer	:	4 µl
dNTP mix (10 mM)	:	2 µl
GeNei M-MuLV RT ($20\ U\mu l^{-1}$)	:	2 µl
Total volume	:	20 µl

All the reagents were added, mixed by vortexing and flashed down. The cDNA conversion reaction was carried out in Eppendorf Mastercycler (Germany). The reaction conditions comprised of a reverse transcription step at 37°C for 1 h followed by an extension step at 70°C for 10 min.

3.3.4 STANDARDIZATION OF DNA ISOLATION PROTOCOL

The extraction of good quality DNA from taro was difficult owing to the presence of high amounts of polyphenol and mucilage.

3.3.4.1 DNA Isolation

CTAB method of DNA extraction (Doyle and Doyle, 1990) with slight modifications was adopted for genomic DNA isolation. β-mercaptoethanol was added fresh to the CTAB extraction buffer (Appendix) to give a final concentration of 2 percent (v/v). The buffer was pre-heated to 60°C in water bath (ROTEK, India). The samples (100 mg) were chilled and pulverized to a fine powder in liquid nitrogen using a sterile mortar and pestle and transferred in to a sterile 2 ml centrifuge tubes containing 1 ml of freshly prepared warm extraction buffer. The content was homogenized by gentle inversion and then incubated at 60°C in water bath for 30 min with intermittent shaking. Then it was centrifuged at 10,000 rpm for 10 min at room temperature. The supernatant was transferred to another sterile microfuge tubes with a sterile pipette tip. To this 10 µl RNase was added and incubated at 37°C for 1 h. The homogenate was then extracted with an equal volume of 24: 1 (v/v) chloroform/isoamyl alcohol and mixed well by inversion for 5-10 min and

centrifuged (Hermle, Table top refrigerated centrifuge) at 15000 rpm for 10 min at room temperature. To the aqueous phase, 0.8 volume of chilled isopropanol was added and mixed by inversion. The mixture was then incubated at -20°C for at least 1 h or overnight to precipitate the nucleic acid. After incubation, the precipitated DNA was pelletized by centrifugation at 15000 rpm for 10 min at 4°C. The supernatant was decanted and the pellet was washed in 0.5 ml ethanol (70 percent) twice, each time centrifuging at 12000 x *g* for 5 min at RT and discarding the supernatant. The pellet was air dried for 30-40 min and dissolved in 50 µl of sterile distilled water. The extracted DNA samples were then stored at -20°C (Vest frost Low Temperature Cabinet, India).

3.3.5 ANALYSIS OF THE EXTRACTED DNA

3.3.5.1 Agarose gel electrophoresis

The integrity and quality of the extracted DNA was checked in agarose gel electrophoresis. An agarose gel of 0.8 percent was prepared in 1X TAE buffer and ethidium bromide was added, 0.5 µl per litre. An aliquot of the DNA sample (5 µl) mixed with the loading dye was loaded in each of the wells of the gel. The gel was run at 5 Vcm^{-1} for 30-45 min. The gel was then visualized under UV light and the image was documented using AlphaImager gel documentation system.

3.4 MOLECULAR DETECTION OF VIRUSES

3.4.1 Polymerase Chain Reaction Based Detection

Polymerase chain reaction (PCR) analyses was carried out with the isolated DNA and cDNA using genus specific primers (Potyvirus, Badnavirus, Nucleorhabdovirus) and virus specific primers (DsMV, TaVCV, TaBV etc). The primers (Table 2) were synthesised from Eurofins (India). The synthesized primers (100 µ*M*) were diluted to a final concentration of 5 µ*M* with sterile water to obtain the

Table 2: List of primers used for virus screening of the samples.

Primer Name	Virus	Amplified Region	Amplicon Size
MJ1 / MJ2	Dasheen Mosaic Virus	Coat Protein	327 bp
DSMV 3F / DSMV 3R	Dasheen Mosaic Virus	Partial CP region and 3' UTR	540 bp
DR0 / DR2	Dasheen Mosaic Virus	Partial CP region and 3' UTR	457 bp
P2F / P2R	Dasheen Mosaic Virus	Partial CP region and 3' UTR	1 kbp
DMV-F / DMV-R	Dasheen Mosaic Virus	Partial CP region and 3' UTR	1.2 kbp
TaVCV Cap2A / TaVCV Cap2B	Taro Vein Chlorosis Virus	Nucleocapsid (N) Gene	1.1 kbp
Taro P3F / Taro P3R	Colocasia bobone disease virus	Coat Protein	150 bp
HafF / HafR	Taro Bacilliform Virus	Badnavirus Reverse Transcriptase	530 bp
TaBV1 / TaBV4	Taro Bacilliform Virus	RT/RNaseH-coding region	320 bp
PNG BadnaF / PNG BadnaR (TaBV Like)	Taro Bacilliform Virus	RT/RNaseH-coding region	250bp

working solution. In order to standardize the annealing temperature of the primers, gradient PCR was performed.

3.4.1.1 PCR standardization

For standardization of PCR conditions, the genomic DNA of few taro samples was used with the TaBV 1/TaBV 4 primers. The composition of the reaction mixture was as follows:

10X Taq buffer (with 15 m*M* $MgCl_2$)	:	2.5 µl
dNTPs (10 m*M* each)	:	0.5 µl
Forward primer (10 µ*M*)	:	0.5 µl
Reverse primer (10 µ*M*)	:	0.5 µl
Template DNA (10 ng μl^{-1})	:	2 µl
Taq DNA polymerase (5 U μl^{-1}	:	0.5 µl
Water	:	13.5 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Mastercycler (Germany) programmed for an initial denaturation at 95°C for 5 min followed by 35 cycles with denaturation at 95°C for 2 min, annealing at 55°C for 2 min and extension at 72°C for 2 min. The final extension was performed at 72°C for 10 min followed by hold at 4°C. The amplified products were resolved in a 1 per cent agarose gel using 100 bp ladder for checking amplification and visualized under the AlphaImager gel documentation system.

3.4.1.2 Gradient PCR for T_a optimization

Based on the data from preliminary primer screening at an annealing temperature of 55°C, it was found necessary to perform gradient PCR to standardize

the annealing temperature for the primer TaBV 1 and TaBV 4. Gradient PCR was carried out with a temperature range of 57-60°C to choose the most appropriate annealing temperature giving specific bands with minimum ambiguity.

3.4.1.3 Reamplification of PCR products

The product obtained after PCR was reamplified using the same reaction mix as in section 3.4.1.1 (except that instead of DNA, PCR amplified product was used) under similar cycling conditions. Products of re-amplification were checked on agarose gel (1%).

3.4.1.4 Analysis of amplicon by agarose gel electrophoresis

The most common method to analyse the PCR product is to to run an aliquot of the sample on agarose gel. Aliquot of PCR mix (10 µl) was loaded on agarose gel (1 percent) made of 1 X TAE buffer. The gel was run at 5 Vcm^{-1} until the dyes migrated 3/4th of the distance through the gel. The gel was visualized and documented under the gel documentation system (Alpha Innotech) using 'Alpha Imager Software'.

3.4.2 PCR Analysis with *MJ1 and MJ2* primers

The components of the mixture were optimized as listed below:

Water	:	12.2 µl
10x Taq buffer A	:	2.5 µl
dNTP	:	1 µl
Forward primer	:	1 µl
Reverse primer	:	1 µl
Template DNA (cDNA)	:	2 µl
Taq polymerase	:	0.3 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min. Final extension was done at 72 °C for 10 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1 percent). The gel was viewed under gel documentation system. The amplicon showing expected band size were purified by gel elution method as described in section 3.6.1.

3.4.3 PCR Analysis with *DsMV 3F and DsMV 3R* primers

The components of the mixture were optimized as listed below:

Water	:	12.2 µl
10x Taq buffer A	:	2.5 µl
dNTP	:	1 µl
Forward primer	:	1 µl
Reverse primer	:	1 µl
Template DNA (cDNA)	:	2 µl
Taq polymerase	:	0.3 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 68 °C for 1 min. Final extension was done at 68 °C for 5 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1 percent). The gel was viewed under gel

documentation system. The amplicon showing expected band size were purified by gel elution method.

3.4.4 PCR Analysis with *DF2 and DR0* primers

The components of the mixture were optimized as listed below:

Water	:	12.2 µl
10x Taq buffer A	:	2.5 µl
dNTP	:	1 µl
Forward primer	:	1 µl
Reverse primer	:	1 µl
Template DNA (cDNA)	:	2 µl
Taq polymerase	:	0.3 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 35 sec, annealing at 56 °C for 35 sec and extension at 72 °C for 1.5 min. Final extension was done at 72 °C for 7 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1 percent). The gel was viewed under gel documentation system. The amplicon showing expected band size were purified by gel elution method.

3.4.5 PCR Analysis with *P2F and P2R* primers

The components of the mixture were optimized as listed below:

Water	:	12.2 µl
10x Taq buffer A	:	2.5 µl

dNTP	:	1 µl
Forward primer	:	1 µl
Reverse primer	:	1 µl
Template DNA DNA (cDNA)	:	2 µl
Taq polymerase	:	0.3 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 41 °C for 1 min and extension at 72 °C for 1 min. Final extension was done at 72 °C for 10 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1 percent). The gel was viewed under gel documentation system. The amplicon showing expected band size were purified by gel elution method.

3.4.6 PCR Analysis with *DMV-F and DMV-R* primers

The components of the mixture were optimized as listed below:

Water	:	12.2 µl
10x Taq buffer A	:	2.5 µl
dNTP	:	1 µl
Forward primer	:	1 µl
Reverse primer	:	1 µl
Template DNA (cDNA)	:	2 µl
Taq polymerase	:	0.3 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 54.3 °C for 1 min and extension at 72 °C for 1 min. Final extension was done at 72 °C for 5 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1 percent). The gel was viewed under gel documentation system. The amplicon showing expected band size were purified by gel elution method.

3.4.7 PCR Analysis with *TaVCV Cap2A and TaVCV Cap2B* primers

The components of the mixture were optimized as listed below:

Water	:	12.2 µl
10x Taq buffer A	:	2.5 µl
dNTP	:	1 µl
Forward primer	:	1 µl
Reverse primer	:	1 µl
Template DNA (cDNA)	:	2 µl
Taq polymerase	:	0.3 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 94 °C for 2.5 min followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 68 °C for 1 min. Final extension was done at 68 °C for 10 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1 percent). The gel was viewed under gel

documentation system. The amplicon showing expected band size were purified by gel elution method.

3.4.8 PCR Analysis with *Taro P3F and Taro P3R* primers

The components of the mixture were optimized as listed below:

Water	:	12.2 µl
10x Taq buffer A	:	2.5 µl
dNTP	:	1 µl
Forward primer	:	1 µl
Reverse primer	:	1 µl
Template DNA (cDNA)	:	2 µl
Taq polymerase	:	0.3 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 68 °C for 1 min. Final extension was done at 68 °C for 10 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (low range) from 'Genei Bangalore' were separated on agarose gel (1 percent). The gel was viewed under gel documentation system. The amplicon showing expected band size were purified by gel elution method.

3.4.9 PCR Analysis with *HafF and HafR* primers

The components of the mixture were optimized as listed below:

Water	:	12.2 µl
10x Taq buffer A	:	2.5 µl

dNTP	:	1 µl
Forward primer	:	1 µl
Reverse primer	:	1 µl
Template DNA	:	2 µl
Taq polymerase	:	0.3 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 94 °C for 3 min followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 1 min and extension at 72 °C for 1 min. Final extension was done at 72 °C for 10 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1 percent). The gel was viewed under gel documentation system. The amplicon showing expected band size were purified by gel elution method.

3.4.10 PCR Analysis with *TaBV1 and TaBV4* primers

The components of the mixture were optimized as listed below:

Water	:	12.2 µl
10x Taq buffer A	:	2.5 µl
dNTP	:	1 µl
Forward primer	:	1 µl
Reverse primer	:	1 µl
Template DNA	:	2 µl
Taq polymerase	:	0.3 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 94 °C for 2 min followed by 50 cycles of denaturation at 94 °C for 30 sec, annealing at 57.3 °C for 30 sec and extension at 72 °C for 1 min. Final extension was done at 72 °C for 10 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1 percent). The gel was viewed under gel documentation system. The amplicon showing expected band size were purified by gel elution method.

3.4.11 PCR Analysis with *PNG BadnaF and PNG BadnaR* primers

The components of the mixture were optimized as listed below:

Water	:	12.2 µl
10x Taq buffer A	:	2.5 µl
dNTP	:	1 µl
Forward primer	:	1 µl
Reverse primer	:	1 µl
Template DNA	:	2 µl
Taq polymerase	:	0.3 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 94°C for 2.5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 1 min. Final extension was done at 72°C for 5 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1 percent). The gel was viewed under gel

documentation system. The amplicon showing expected band size were purified by gel elution method.

3.5 DETECTION BY NUCLEIC ACID SPOT HYBRIDIZATION

3.5.1 Probe preparation

Probe was prepared with Thermo Scientific™ North2South™ Biotin Random Prime Labelling Kit.

The random prime labelling method is based on the procedure of (Feinberg and Vogelstein, 1983) wherein random hepta nucleotides containing all possible sequences anneal to a denatured DNA template and act as primers for complementary strand synthesis by DNA Polymerase (Klenow fragment, 3′-5′ exo). Inclusion of biotinylated nucleotides in the reaction mixture ensures that the newly synthesized DNA strands are labelled with biotin.

3.5.1.1 Labelling reaction

In a micro centrifuge tube, ~100ng DsMV CP primer was diluted for labelling to a final volume of 24µl in nuclease-free water. For the positive control, 0.5 µl of control DNA (125 ng) was diluted to 24 µl. 10 µl of hepta nucleotide mix was added and the template was denatured by boiling the tube for 5 minutes. Quickly froze the denatured solution by placing the tube on ice for 5 minutes. Briefly centrifuged the tube to collect liquid in the bottom of the tube & the tube was placed on ice.

In the order stated, following components were added to the prepared sample solution on ice, the final volume being 50 µl:

5 X dNTP mix	: 10 µl
10 X Reaction Buffer	: 5 µl
Klenow fragment	: 1 µl

Contents were mixed by flicking or brief vortexing of the tube. Centrifuged briefly to collect liquid in the bottom of the tube. The labelling reaction was incubated for 60 minutes at 37 °C and the enzyme was inactivated by adding 2 µl of 500 mM EDTA, pH 8.0. The reaction was adjusted to contain 0.5M ammonium acetate by adding 5 µl of 5M NH_4OAc to 50 µl labelling reaction and mixed well. 2 volumes of 100 percent ethanol was added to the above reaction mixture and mixed well. Chilled the tube at -20°C for 15 minutes followed by centrifugation at 4°C for 30-60 minutes at maximum speed (> 10,000 x *g*). The supernatant was discarded carefully and washed the DNA pellet once by adding ice-cold 70 percent discarded the supernatant and the pellet was dissolved in 100 µl of 1 X TE and store at -20°C.

3.5.1.2 Estimation of probe labelling efficiency

Probe labelling efficiency was estimated using 'Thermo Scientific Chemiluminescent Nucleic Acid Detection Module'. It is a complete system for the detection of biotin-labelled nucleic acids for various blotting applications including Northern/Southern blots.

3.5.1.3 Dot Blot by hand spotting

A positively-charged nylon membrane was equilibrated in TE Buffer for at least 10 minutes. Dilutions of the labeled DNA were prepared from both the control DNA and the experimental probe with TE from 10^{-1} to 10^{-7}. The equilibrated membrane was placed on to a clean, dry paper towel and allowed excess buffer to absorb into the membrane, but did not allow the membrane dry out. 4 µl of cDNA of the representative sample set and DNA of *Badna* positive samples were spotted onto the hydrated membrane and allowed the samples to absorb into the membrane. Immediately, UV cross linked the membrane by placing the nucleic acid side facing the UV radiation for 66 s.

For the detection and analysis of UV cross linked DNA, the membrane was blocked by adding blocking buffer and incubated for 15 minutes with gentle shaking.

Conjugate/blocking buffer solution was prepared by adding 50 µl of the stabilized Streptavidin-Horseradish Peroxidase conjugate to 16 ml blocking buffer (1:300 dilution) and it was a added to the container after decanting blocking buffer from the membrane and incubate for 15 minutes with gentle shaking. Membrane was transferred to a new container and rinsed briefly with 20 ml of 1 X wash solution and it was washed four times for 5 minutes each in 20 ml of 1 X wash solution with gentle shaking. Then the membrane was incubated for 5 minutes with gentle shaking in 30 ml of substrate equilibration buffer in a new container. Chemiluminescent substrate working solution was prepared by adding 6 ml Luminol/Enhancer Solution to 6 ml stable peroxide solution. The membrane was removed from substrate equilibration buffer and carefully blotted an edge of the membrane on a paper towel to remove excess buffer. The membrane was placed in a clean container or onto a clean sheet of plastic wrap placed on a flat surface. Then the membrane was placed nucleic acid side down onto a puddle of the working solution and incubated for 5 minutes without shaking. The membrane was removed from the working solution and blotted on a paper towel for 2-5 seconds to remove excess buffer without allowing the membrane to become dry. Moist membrane was wrapped in plastic wrap and placed in film cassette. X-ray film was exposed to membrane for 4 minutes and then the film was developed by immersing in developer solution for 3 minutes followed by 1 minute rinsing in distilled water and 3 minutes in Fixer solution. Finally, the developed X- ray film was washed in running tap water and air dried.

3.5.2 Nucleic acid spot hybridization

Nucleic acid spot hybridization was performed with North2South® Chemiluminescent Hybridization and Detection Kit. This system combines an enhanced luminol substrate for horseradish peroxidase (HRP) with optimized

hybridization and blocking conditions that ensure consistent results with sensitivity equal to or exceeding ^{32}P.

Positively charged nylon membrane of appropriate size was used for hybridization and it was equilibrated in TE Buffer for at least 10 minutes. 4 µl of cDNA of the representative sample set and DNA of *Badna* positive samples were spotted on the membrane and UV cross linked by the nucleic acid side facing the UV radiation for 66 sec. The blot was placed in hybridisation bottles and sufficient hybridisation buffer was added to completely cover the blot. The blot was prehybridised according to at least 30 minutes at 55°C for DNA hybrids and 65°C for RNA: RNA hybrids. At the end of prehybridisation reaction, the biotinylated DsMV CP probe was denatured by heating at 100°C for 10 minutes and quickly placed in ice for 5 minutes. After pre-hybridisation, the denatured probe (10 µl) was added to the hybridisation buffer and incubated overnight with shaking at 55°C.

On the next day, the North2South® Hybridization stringency wash buffer (2 X) was equilibrated to RT and prepared 1 X concentration of the same in sterile ultrapure water. Stringency washes were performed by washing the blot three times with 1 X stringency wash buffer for 15-20 minutes per wash with gentle agitation at 55°C.

The stringency wash buffer was decanted and sufficient blocking buffer was added generously to cover the membrane and incubated for 15 minutes with shaking or rotating at RT. Next, streptavidin-HRP conjugate (1:300) was added to the blocking buffer and incubated for 15 minutes at RT with agitation. After the addition of streptavidin-HRP conjugate, the membrane was washed four times for 5 minutes with 1 X wash buffer at RT with agitation. Substrate equilibration buffer was added (0.25 ml cm^{-2} of membrane) to new container and placed the blot and incubated for 5 minutes at RT with agitation.

The moist membrane was placed on a tray or a piece of plastic wrap and covered with the substrate working solution and incubated for 5 minutes at RT and ensured that the membrane was fully covered with substrate. Substrate solution was drained from the membrane surface and the moist membrane was transferred to a cassette and covered with plastic wrap and then exposed to X-ray film and developed as described for estimation of probe quality.

3.6 CHARACTERISATION OF VIRUSES

3.6.1 Gel elution of PCR amplified fragments

Extraction of the PCR products was carried out with PureLink™ Quick Gel Extraction kit (Invitrogen, USA). The PCR products were resolved on agarose gel (1 %) and the amplicon was excised from the gel using a clean sharp scalpel. The gel slice was placed into a pre-weighed 2 ml tube and its weight was recorded. Then, thrice the volume gel solubilising buffer was added to the gel slice and it was incubated at 50°C for 10 min occasionally inverting it every 3 min. Then the contents of the tube were incubated for an additional 5 min. One gel volume of isopropanol was added and inverted to mix. The Quick Gel Exraction column was placed into the tube and the dissolved gel pipette into the column. Centrifuge at 20,000 x g for one min and the flow through discarded. The wash buffer (500 µl) containing ethanol was added to the column followed by a centrifugation at 20,000 x g for one min. flow through discarded and the centrifugation step repeated. The column is air dried after discarding the flow through. The air dried column is placed into the recovery tube and 50 µl of the elution buffer added to the centre of the column. Incubate for 2 min at room temperature followed by a centrifugation at 20,000 x g for a min. After elution; the column was discarded and the purified DNA was stored at -20 °C.

The gel elute was subjected to PCR using the same reaction mix under required cycling conditions. The products of PCR were analyzed using agarose gel (1 %).

3.6.2 Cloning and Transformation

The amplified region was cloned and transformed into *E. coli* DH5α using InsTA Clone PCR Cloning Kit (Fermentas, USA). The recombinant clones obtained were analyzed by colony PCR method.

The cloning and transformation protocol was performed in three consecutive days.

Day 1:

E. coli DH5α cells revived in Luria agar medium (Appendix) were used for the transformation procedure. The ligation mix was prepared with the components as listed below.

Vector pTZ57R/T	: 3 µl
5x ligation buffer	: 6 µl
PCR product	: 4 µl
Nuclease free water	: 16 µl
T4 DNA ligase	: 1 µl
Total volume	: 30 µl

After vortexing, the ligation mix was centrifuged for a few seconds and incubated overnight at 4 °C. A control reaction mix was also prepared with 4 µl of control PCR fragment and incubated under similar conditions.

Day 2:

To 1.5 ml C-medium (pre-warmed at 37 °C for at least 20 min), a portion of freshly streaked bacterial culture was transferred using inoculation loop and gently mixed to resuspend the cells. After incubating the medium at 37 °C with shaking for 2 h, the bacterial cells were pelleted by one min centrifugation. The pellet was resuspended in 300 µl of T solution (Appendix) and incubated on ice for five min.

The cells were again pelleted by centrifugation, resuspended in 120 µl of T solution and incubated on ice for five min.

2.5 µl of ligation mix or control reaction mix was added to new microfuge tubes and chilled on ice for two min. 50 µl of prepared cells were added to each tube containing DNA, mixed, incubated on ice for five min and plated immediately on pre-warmed LB Ampicillin X gal/ IPTG agar plates (Appendix). The plates were incubated overnight at 37 °C. Untransformed DH5α cells were plated on an LB Ampicillin X gal/ IPTG agar plate to serve as negative control.

Day 3:

The plates were observed for the presence of recombinants. The number of transformants obtained in the sample as well as positive control plate was determined.

3.6.2.1 Analysis of recombinant clones- colony PCR

The recombinant clones were analyzed for the presence and orientation of the sequence insert by colony PCR using their respective primers (Eurofins, India). The PCR reaction mix was formulated as:

Water, nuclease free	:	14.2 µl
10x Taq buffer A	:	2.5 µl
dNTP	:	1 µl
Forward primer (10 µ*M*)	:	1 µl
Reverse primer (10 µ*M*)	:	1 µl
Taq polymerase	:	0.3 µl
Total volume	:	20 µl

A single white colony was selected and resuspended in the PCR reaction mix. A short strike was made over the culture plate (containing selective medium) in order

to save the clone for repropagation. The PCR was performed at the required PCR condition of each primer. The products of colony PCR were resolved on agarose gel (1%).

3.6.3 DNA sequencing

Gel elutes of PCR products were sequenced at the Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology (RGCB). Nucleotide BLAST of the obtained sequences was performed in order to find out the similar sequences.

3.6.3.1 Sequence analysis

The electropherogram obtained by the capillary sequencing was first edited with BioEdit Sequence Alignment Editor programme version 7.2.5. The edited sequence was compared to known viral sequences using NCBI BLAST. Phylogenetic tree was constructed from BioEdit aligned sequences using NCBI BLAST analysis tool.

Results

4. RESULTS

The results of the study entitled "Molecular characterisation of viruses in taro (*Colocasia esculenta* (L.) Schott)" carried out at the Division of Crop Protection, Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram during 2013-2014 are presented in this chapter.

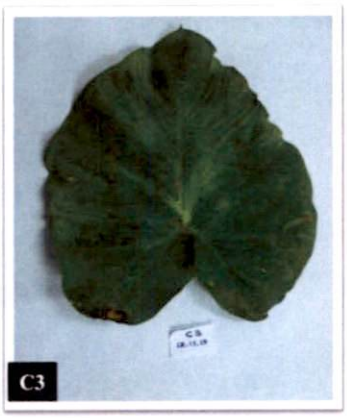
4.1 SAMPLE COLLECTION

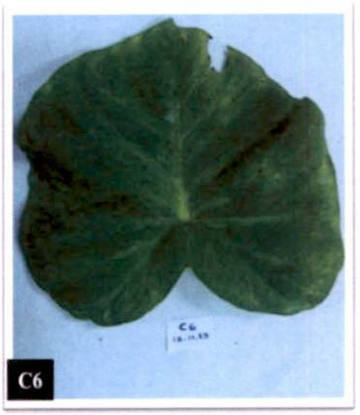
Taro leaf samples (100 no.) were collected and visually analyzed. Most of the samples collected were symptomatic with mosaic; prominent whitish or faint feathery mosaics; mild vein clearing and chlorosis on the midrib and leaf margins; deformed, crinkled or curling, leaf puckering, mottling were also observed. Asymptomatic samples showed no prominent symptoms other than sometimes which seemed to be mild indistinct chlorosis or variegation. After an initial serological screening by ELISA, the samples were grouped according to the symptoms they exhibited and a representative sample set of 22 samples (Plate 1) were made which includes few samples from *Xanthosoma sagittifolium* (Tannia) which showed similar severe virus infection symptoms. Symptom duplicates were eliminated for a more specific sample set. The sample details and symptoms observed are represented in Table 3. This representative sample set was used for further tests and analysis.

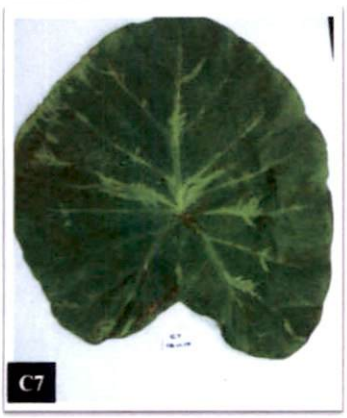
4.2 SEROLOGICAL METHODS OF DETECTION

The serological methods employed for the initial screening of the samples were ELISA and DIBA. This procedure was carried out to screen the samples for *DsMV* infection using *DsMV* polyclonal antibody AS-1021 obtained from DSMZ, Germany.

4.2.1 Enzyme Linked Immunosorbent Assay (ELISA)







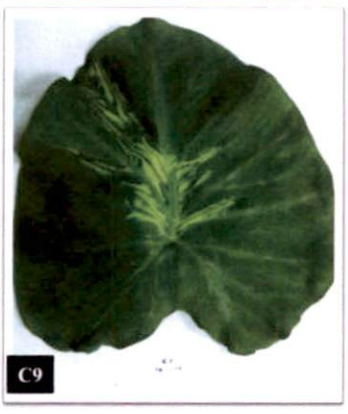


Plate 1: Representative sample set
(Symptoms detail in Table 3)

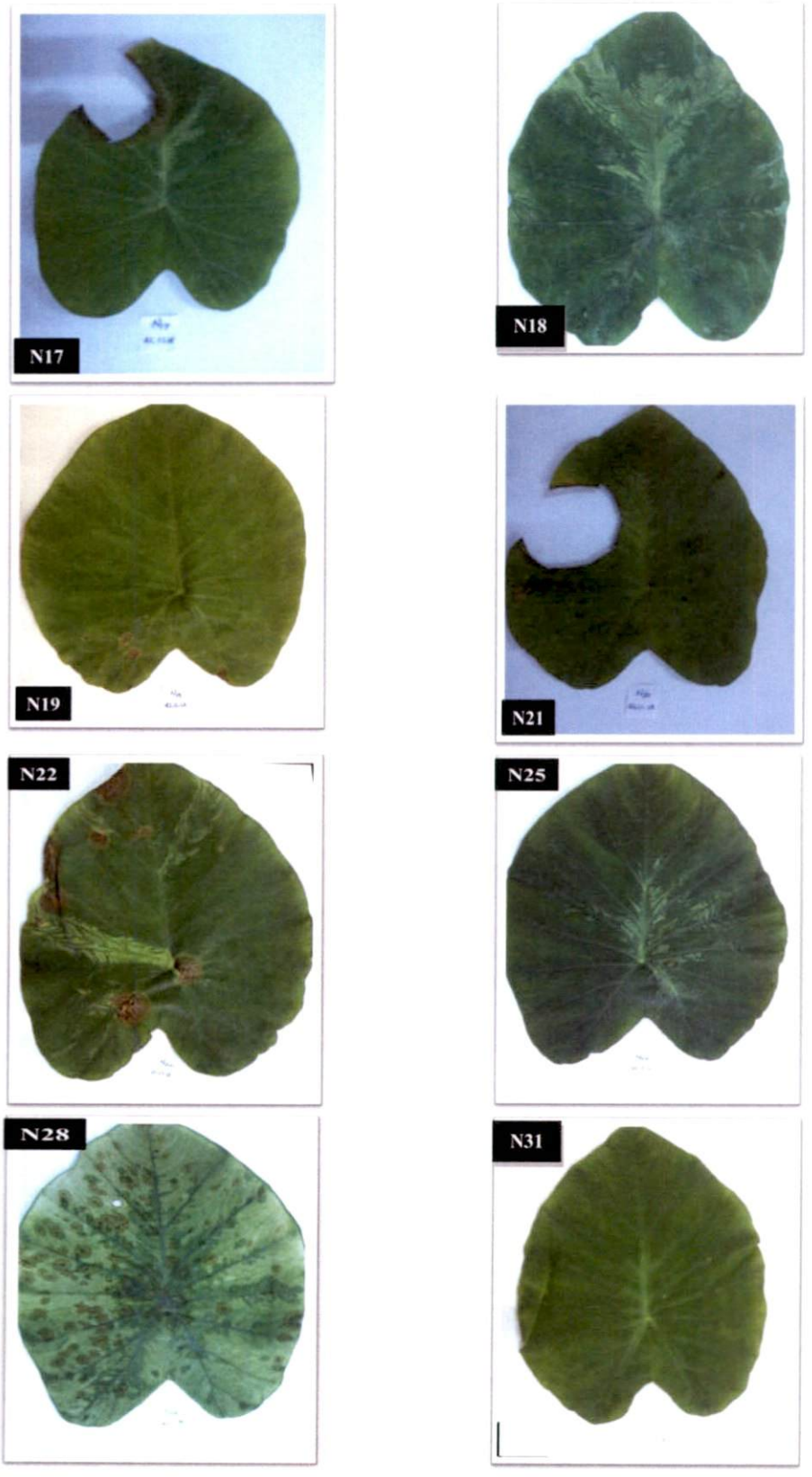


Plate 1(continued): Representative sample set
(Symptoms detail in Table 3)

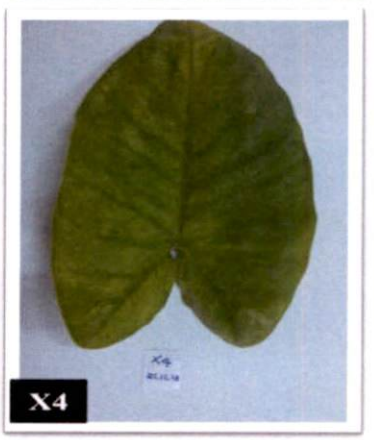


Plate 1(continued): Representative sample set
(Symptoms detail in Table 3)

Table 3: Representative sample set, location and symptoms observed

Sl. No.	Sample ID	Place of collection	Symptoms
1	C1	CTCRI	Severe feathery mosaic, slight distorted leaf margin
2	C2	CTCRI	Mild feathery mosaic
3	C3	CTCRI	Mosaic and puckering
4	C6	CTCRI	Mild mosaic, chlorosis and puckering
5	C7	CTCRI	Feathery mosaic along veins and slight puckering
6	C8	CTCRI	Mild feathery mosaic and chlorosis along margin
7	C9	CTCRI	Severe mosaic along midrib and in some veinal region
8	N3	Bhubaneswar	Chlorosis and mild mosaic
9	N17	CTCRI	Feathery mosaic
10	N18	CTCRI	Symptomless, variegation
11	N19	CTCRI	Indistinct vein and margin chlorosis
12	N21	CTCRI	Feathery mosaic along midrib
13	N22	CTCRI	Feathery mosaic
14	N25	CTCRI	Irregular mosaic and indistinct chlorosis on the margin
15	N28	CTCRI	Prominent vein chlorosis
16	N31	CTCRI	Severe mosaic along midrib and veins
17	N34	CTCRI	Mosaic, puckering and distortion
18	N36	CTCRI	Mosaic, chlorosis, puckering, deformation
19	M3d	CTCRI	Vein chlorosis and puckering
20	X1	CTCRI	Mosaic and severe puckering
21	X2	CTCRI	Mosaic, chlorosis, puckering and deformation
22	X4	CTCRI	Mosaic, chlorosis and puckering

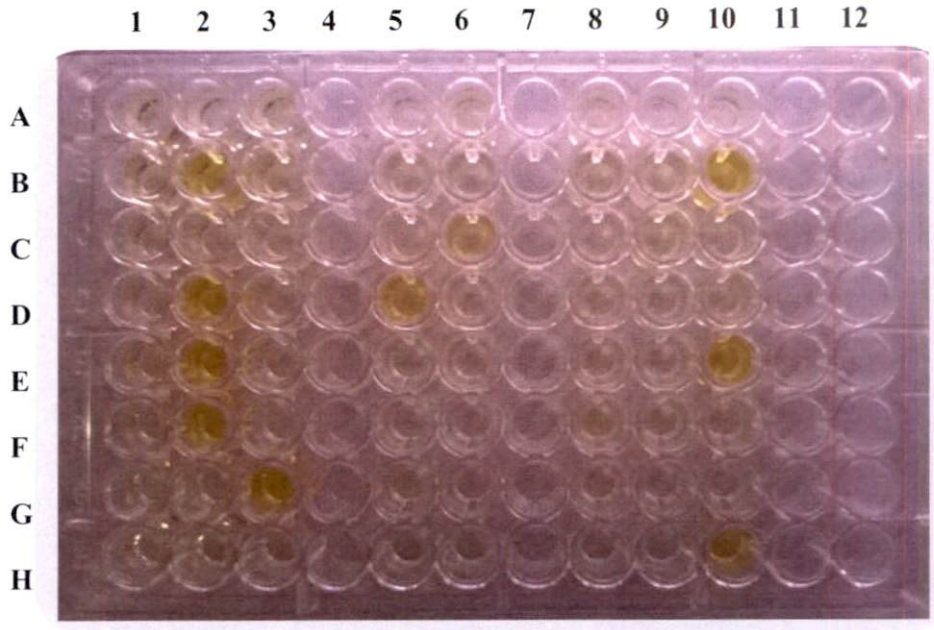
DAS ELISA was carried out for 55 samples (Plate 2a) selected from the whole sample collection. Most of the sample selected was the ones showing symptoms of DsMV infection and few differing and asymptomatic samples were taken. The negative control was a non host and previously confirmed DsMV infected taro leaf as the positive control. The readings were taken at 405 nm in a BIO-RAD iMark Microplate Reader (USA) and the chart plotted (Figure 6). ELISA results (Table 4) showing high positive values and symptoms were further screened with PCR. Out of 55 samples tested, seven of them showed negative for DsMV infection and three samples (C1, N36 and X1) showed high OD value (above 1.5) which showed that these samples were highly susceptible to DsMV infection. Rest of the samples had moderate infection of DsMV. Samples showing feathery mosaic, distortion and puckering were more reactive towards the DAS-ELISA. Samples showing duplicate symptoms were eliminated from the core sample set and based on the ELISA reaction 22 samples were selected for the representative sample set which showed both positive and negative for DsMV.

4.2.2 Dot Immuno Binding Assay (DIBA)

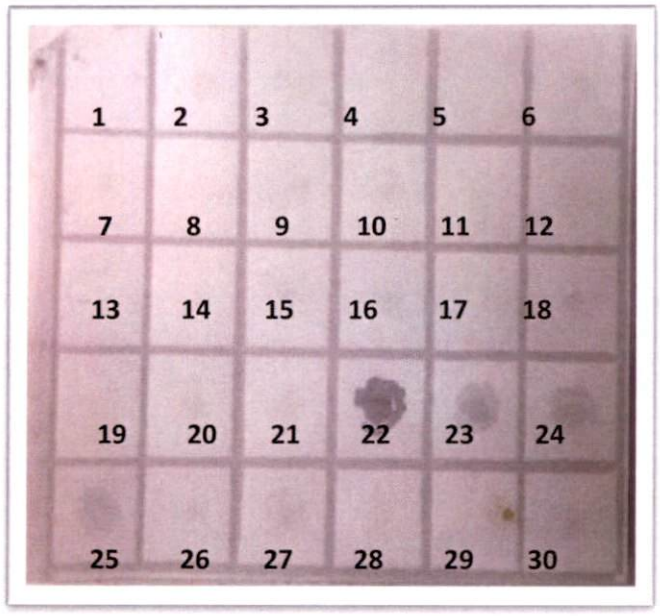
DIBA was carried out with 22 representative samples using the *DsMV* polyclonal antibodies acquired from DSMZ, Germany. Out of 22 samples tested (Plate 2b), fifteen of them have showed positive for *DsMV* infection and seven samples showed negative for *DsMV*. The DIBA reaction is represented in Table 5. Compared to ELISA, DIBA reaction was less and faint; due to the low virus concentration in the sap extract taken; the sap extract used for DIBA being only 5 µl compared to 200 µl used in ELISA. The positive samples exhibited mosaic symptoms varying from mild to severe and puckering symptoms.

4.3 NUCLIEC ACID EXTRACTION

4.3.1 Isolation of total RNA



(a) ELISA reaction of field collected samples tested for DsMV infection



(b) Dot immuno binding assay for DsMV with representative samples
1, 11 and 19 – Buffer control; 8, 14 and 29 – Negative control;
10 and 25 – Positive control.

Plate 2: Serological analysis of representative samples using (a) ELISA and (b) DIBA

Figure 6: ELISA reactions of samples at 405 nm

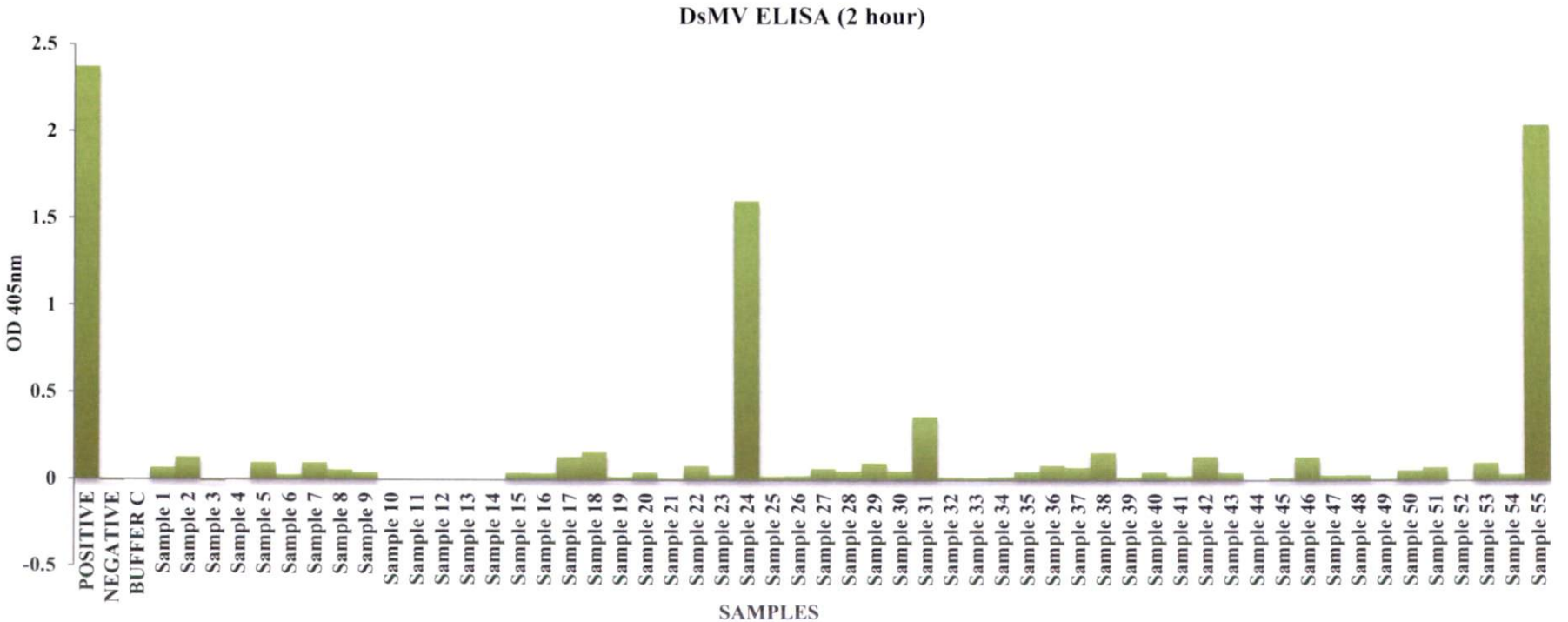


Table 4: DAS-ELISA reading of the samples with varying symptoms using DsMV specific polyclonal antibody

Sl. No.	Samples with symptoms	DAS-ELISA	A_{405} Reading
1	Severe feathery mosaic, slight distorted leaf margin	+	1.608
2	Feathery mosaic	+	0.128
3	Mosaic and severe puckering	+	2.048
4	Mild mosaic, chlorosis and puckering	+	0.103
5	Irregular mosaic and indistinct chlorosis on the margin	+	0.098
6	Symptomless, variegation	-	0.058
7	Mosaic, chlorosis, puckering and deformation	+	0.368
8	Severe mosaic along midrib and veins	+	0.134
9	Chlorosis and mild mosaic	-	0.017
10	Prominent vein chlorosis	-	0.076

Table 5: Reaction of samples to DIBA

Samples	Reaction	Samples	Reaction
1.	*Buffer control*	**16. N21**	Positive
2. C1	Positive	**17. N19**	Negative
3. C2	Positive	**18. N22**	Positive
4. N25	Negative	**19.**	*Buffer control*
5. C6	Negative	**20. C3**	Positive
6. C9	Positive	**21. N17**	Positive
7. C7	Positive	**22. X2**	Positive
8.	*Negative control*	**23. N34**	Positive
9. C8	Positive	**24. N36**	Positive
10.	*Positive control*	**25.**	*Positive control*
11.	*Buffer control*	**26. X2**	Positive
12. N28	Negative	**27. X4**	Positive
13. N3	Negative	**28. N31**	Negative
14.	*Negative control*	**29.**	*Negative control*
15. M3d	Negative	**30. N18**	Positive

RNA isolation from samples was carried out using two methods viz. TRI Reagent method and LiCl protocol. The RNA of the 22 representative samples was isolated. RNA extraction was successful in both but TRI reagent method was a better method for yielding good quality stable RNA. Gel pictures of RNA isolated by both methods represented in Plate 3a and Plate 3b. Samples that were stored too long or samples starting to dilapidate failed to yield good quality RNA and most of the time no RNA was obtained from such samples. The isolated RNA was checked in 1.2 percent agarose gel by electrophoresis and visualized under UV to observe three band pattern of good total RNA. TRI reagent protocol proved to be better method for good quality RNA isolation than LiCl protocol.

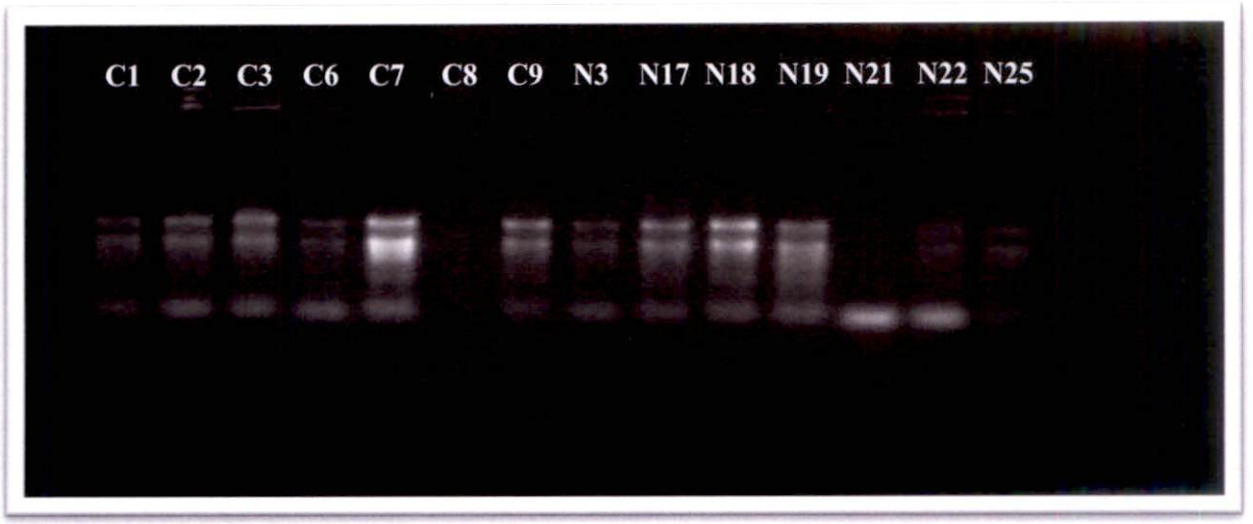
4.3.1.1 cDNA synthesis

The RNA isolated from the 22 samples was converted into cDNA for further PCR based screening. cDNA synthesis is an essential prerequisite for conducting PCR based virus detection. Moreover isolated RNA being less stable for long period of storage, cDNA conversion ensures that the sample is not lost and is available for further tests.

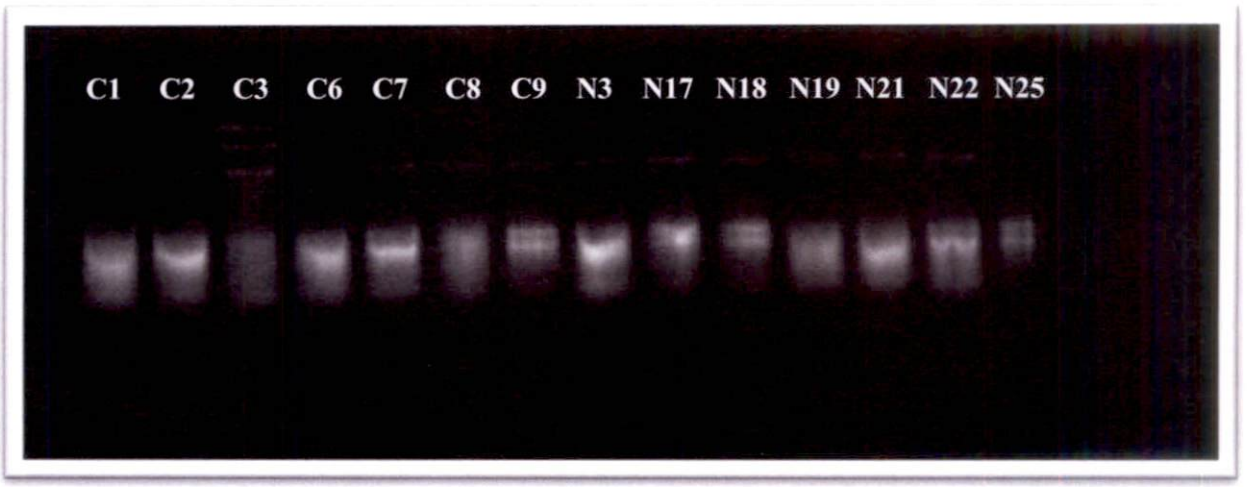
The first strand cDNA conversion was carried out using MMuLV Reverse Transcriptase and oligo-dT primer. The synthesized cDNA was checked on one percent agarose gel to ensure successful conversion and they were visualized in gel under UV as bright sheared band (Plate 3c). cDNA synthesis was positive even from low quality RNA in most cases.

4.3.2 DNA isolation

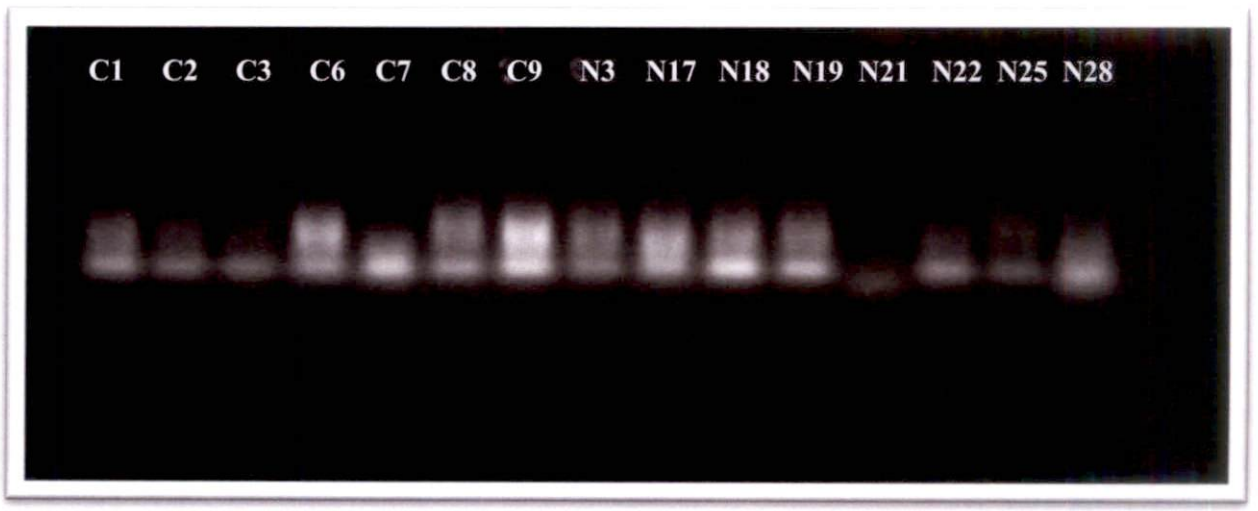
CTAB (2 percent) protocol of DNA isolation was carried out using representative samples. The DNA of the 22 representative samples and 35 other samples from the ELISA set was isolated. The extracted genomic DNA was run on an agarose gel (0.8%) and visualized under UV to observe the bands. In case of protein



3a: RNA isolated by TRI reagent method



3b: RNA isolated by LiCl method



3c: cDNA synthesized visualized as sheared bands

contamination or sheared bands the protocol was slightly modified and grinding was carried out with 2 gram of PVPP and about 80-100 mg amount of leaf sample respectively. The agarose gel profile is represented in Plate 4a.

4.4 MOLECULAR DETECTION OF VIRUSES

As part of the molecular detection and diagnosis, PCR and NASH techniques were employed; PCR was done using the primers (Table 6) for group specific and virus specific which were got synthesized from Eurofins, India.

4.4.1 Polymerase Chain Reaction Based Detection

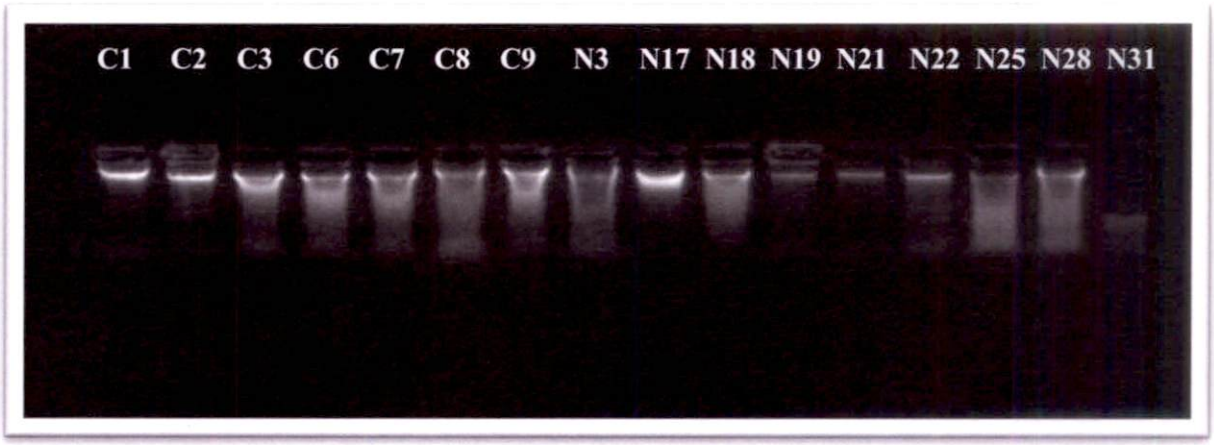
This analysis was carried to detect infections using group specific and virus specific primers and also to analyze the best primer set for efficient diagnostic purposes for taro plants.

4.4.1.2 Gradient PCR for T_a optimization

PCR optimization for the annealing temperature was required for TaBV 1 and TaBV 4 primers which did not yield expected results. The primer annealing step being an important step of PCR, the annealing temperature of the TaBV1 and TaBV4 primers were optimized by conducting a gradient PCR from 57 °C to 60 °C using sample N19 and the optimum Tm value for better amplification was observed at 57.4 °C (Plate 4b).

4.4.1.2 PCR Analysis with MJ1 and MJ2 primers

Detection of *Potyvirus* infection in all the samples were carried using MJ1 and MJ 2 *potyvirus* group specific primers which amplifies the region corresponding to the partial CP gene. A control (non template) was also set during PCR which contains all the components of a typical PCR but no template DNA. PCR conditions were as mentioned in 3.4.2. An amplicon of size 327 bp was observed as a single band for virus positive samples in agarose gel (1 %) electrophoresis. No amplification



Plate(4a): Gel profile of DNA isolated by CTAB (2%) protocol

Plate (4b): Gel profile of gradient PCR to optimise T_a of TaBV 1 /TaBV 4

Lane 1: 57°C, **2:** 57.1°C, **3:** 57.3°C, **4:** 57.7°C, **5:** 58.1°C, **6:** 58.6°C

Lane 7: 59.1°C, **8:** 59.6°C, **9:** 60.1°C, **10:** 60.5°C, **11:** 60.8°C, **12:** 60.9°C

Table 6: Details of the primers used for the detection of viruses in *Colacasia esculenta*

Virus	Sl. No.	Primers	Sequence (5'-3')	T_a	Amplicon size	Reference
Potyvirus	1	**MJ 1**	5'-ATGGTHTGGTGYATHGARAAYGG-3'	50°C	327 bp	Marie-Jeanne *et al.*, 2000
		MJ 2	5'-TGCTGCKGCYTTCATYTG-3'			
	2	**DSMV 3F**	5'-AGTACAAACCTGARCAGCGTGAYA-3'	55°C	540 bp	Maino et al., 2003
		DSMV 3R	5'-TTYGCAGTGTGCCTYTCAGGT-3'			
	3	**DF2**	5'-GACTTCTATGAGGTCAATTC-3'	56°C	457 bp	Huang *et al.* 2005
		DR0	5'-TTGAACACCGTGCACGAAGCATC-3'			
	4	**P2F**	5'-AGGTTGTATTGCAGGCAGATG-3'	41°C	1 kb	Reyes et al., 2009
		P2R	5'-GCCAATAACTGTGGCCTGTT-3'			
	5	**DMV-F**	5'-AGTACAAACCTGARCAGCGTGAYA-3'	54.3°C	1.2 kb	Li *et al.*, 2002
		DMV-R	5'-TTYGCAGTGTGCCTYTCAGGT-3'			
Nucleorhabdovirus	6	**TaVCV Cap2A**	5'-CKSTGYAARSAACATGGTCTTG-3'	55°C	1.1 kb	Revill et al., 2005
		TaVCV Cap2B	5'-TAATCAAGYGGWGGGAGYTTCTC-3'			
	7	**Taro P3F**	5'-ACTGCTGGAAACGGTTTCTTTGCC-3'	55°C	150 bp	Dowling *et al.*, 2005 (Unpublished Data)
		Taro P3R	5'-ATTGCTGTGGAGTTCCCAGCAACA-3'			

Table 6 continued.

Virus	Sl. No.	Primers	Sequence (5'-3')	T_a	Amplicon size	Reference
Badnavirus	8	**Haf F**	5'-ATGCCITTYGCIITIAARAAYGCICC-3'			
		Haf R	5'-CCAYTTRCAIACISCICCCCAICC-3'	50°C	530 bp	Yang *et al.*, 2003
	9	**TaBV1**	5'-CKSTGYAARSAACATGGTCTTG-3'			
		TaBV4	5'-TAATCAAGYGGWGGGAGYTTCTC-3'	57.3°C	320 bp	Revill et al., 2005
	10	**PNG Badna F**	5'-GCAAAAAGAATGGATTGATACTGAGCAG-3'			
		PNG Badna R	5'-CATTTTTCTTTCTCCAGTTGGGCTTAC-3'	57°C	250 bp	Revill et al., 2005

was observed in the non-template control, which indicates that there is no non-specific binding and primer dimer formation in PCR. The PCR results with *MJ1* and *MJ2* primers are shown in Plate 5a. Out of the 22 samples screened with MJ1/MJ2, 11 samples of taro (C1, C2, C3, C7, C8, C9, N17, N21, N22, N34 and N36) as well as 3 samples of tannia (X1, X2, and X4) showed amplification. The samples showing positive exhibited various symptoms like severe whitish feathery mosaics, distorted leaf margins, mild mosaics and puckering.

4.4.1.3 PCR Analysis with DsMV 3F and DsMV 3R primers

Detection of *DsMV* infection in all the samples were carried using *DsMV 3F* and *DsMV 3R*, *DsMV* specific primers which amplifies the partial coat protein gene and 3' untranslated region which gives amplified product of 540 bp. A control (non template) was also set during PCR which contains all the components of a typical PCR but no template sample. PCR conditions were as mentioned in 3.4.3. An amplicon of size 540 bp was observed as a single band for virus positive samples in agarose gel (1 %) electrophoresis. No amplification was observed in the non-template control, which indicates that there is no non-specific binding and primer dimer formation in PCR. The PCR results with *DsMV 3F* and *DsMV 3R* primers are shown in Plate 5b. Out of the 22 samples screened with DsMV 3F and DsMV 3R, eleven samples of taro (C1, C2, C3, C7, C8, C9, N17, N21, N22, N34, and N36) as well as 3 samples of tannia (X1, X2, and X4) showed virus infection. The samples showing positive exhibited various symptoms like severe whitish feathery mosaics, distorted leaf margins, mild mosaics and puckering.

4.4.1.4 PCR Analysis with DF2 and DR0 primers

Detection of DsMV infection in all the samples were carried using DF2 and *DR0*, DsMV specific primers which amplifies the partial coat protein gene and 3' untranslated region. A control (non template) was also set during PCR which contains all the components of a typical PCR but no template sample. PCR conditions were as

mentioned in 3.4.4. An amplicon of size 457 bp was observed as a single band for virus positive samples in agarose gel (1 %) electrophoresis. No amplification was observed in the non-template control, which indicates that there is no non-specific binding and primer dimer formation in PCR. The PCR results with *DF2* and DR0 primers are shown in Plate 5c. Out of the 22 samples screened with DF2 and DR0, only two samples of taro (N18, N22) samples showed virus infection. The samples showing positive exhibited various symptoms like feathery mosaics and mild chlorosis.

4.4.1.5 PCR Analysis with P2F and P2R primers

Detection of DsMV infection in all the samples were carried using *P2F* and *P2R*, DsMV specific primers which amplifies the partial coat protein gene and 3' untranslated region. A control (non template) was also set during PCR which contains all the components of a typical PCR but no template sample. PCR conditions were as mentioned in 3.4.5. No amplification was observed in the non-template control, which indicates that there is no nonspecific binding and primer dimer formation in PCR. The PCR results with *P2F* and *P2R* primers are shown in Plate 6a. Out of the 22 samples screened with P2F and P2R, only one taro (N22) sample as well as one tannia (X2) sample showed bands even though the expected amplicon of size 1 kbp was not observed as a single band for the samples in agarose gel (1 %) electrophoresis.

4.4.1.6 PCR Analysis with DMV-F and DMV-R primers

Detection of DsMV infection in all the samples were carried using *DMV-F* and *DMV-R*, DsMV specific primers which amplifies the partial coat protein gene and 3' untranslated region. A control (non template) was also set during PCR which contains all the components of a typical PCR but no template sample. PCR conditions were as mentioned in 3.4.6. An amplicon of size 1.2 kbp was observed as a single band for virus positive samples in agarose gel (1 %) electrophoresis. No amplification

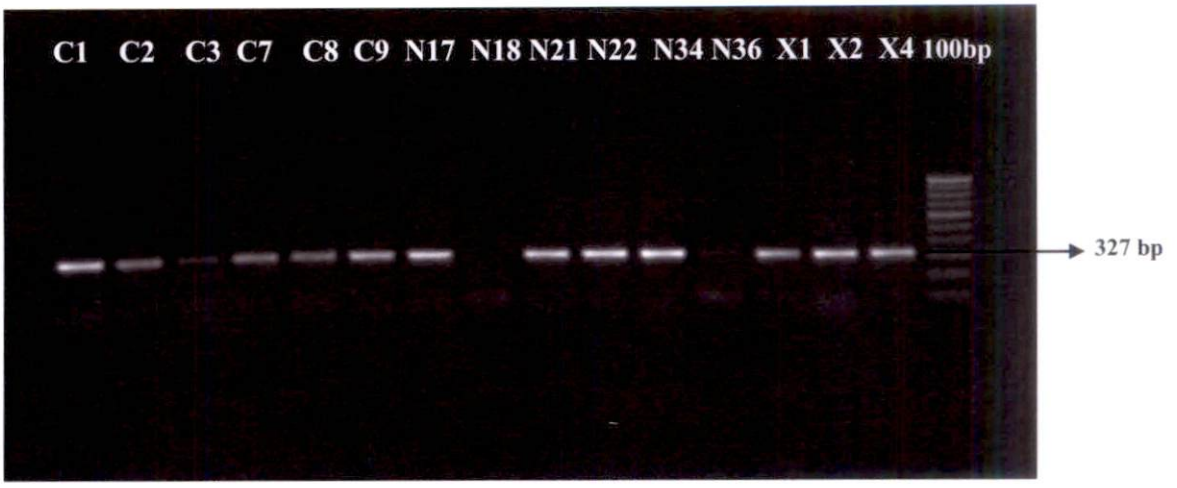


Plate 5a: PCR amplification of taro samples with MJ1/MJ2 primers.

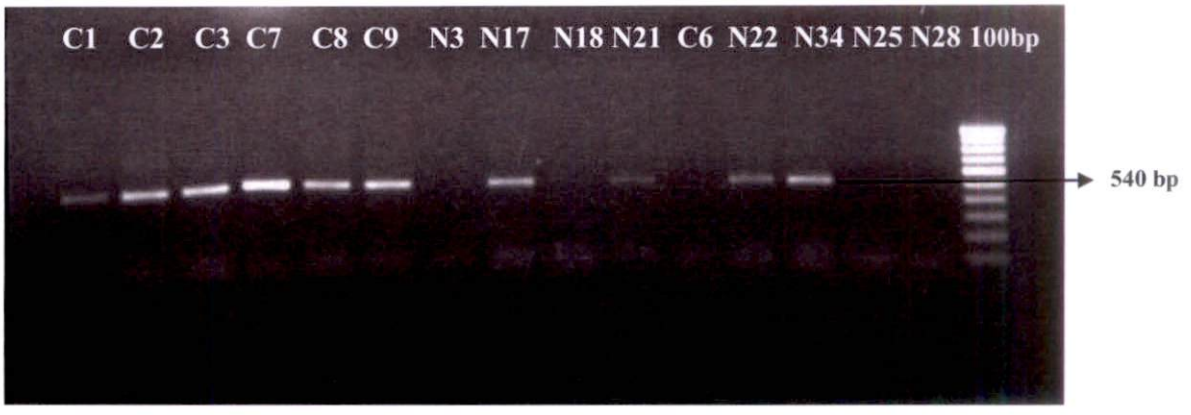


Plate 5b: PCR amplification of taro samples with DsMV3F/DsMV3R primers.

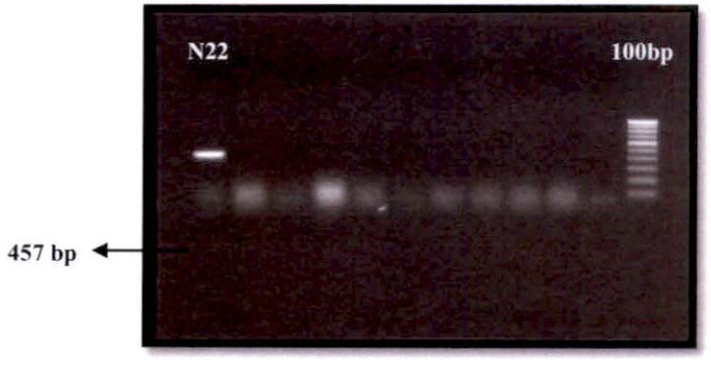


Plate 5c: PCR amplification of taro samples with DF2/DR0 primers.

was observed in the non-template control, which indicates that there is no nonspecific binding and primer dimer formation in PCR. The PCR results with *DMV-F* and *DMV-R* primers are shown in Plate 6b. Out of the 22 samples screened with DMV-F and DMV-R, only two taro (C1, N22) samples as well as three tannia (X1, X2 and X4) samples showed virus infection. The samples showing positive exhibited various symptoms like severe feathery mosaics and puckering.

4.4.1.7 PCR Analysis with TaVCV Cap2A and TaVCV Cap2B primers

Detection of TaVCV infection in all the samples were carried out using *TaVCV Cap2A* and *TaVCV Cap2B*, TaVCV specific primers which amplifies the nucleocapsid (N) gene. A control (non template) was also set during PCR which contains all the components of a typical PCR but no template sample. PCR conditions were as mentioned in 3.4.7. No amplification was observed in the non-template control, which indicates that there is no nonspecific binding and primer dimer formation in PCR. The PCR results with *TaVCV Cap2A* and *TaVCV Cap2B* was negative for all samples. Out of the 22 samples screened for TaVCV, only two tannia (X1 and X2) samples showed indistinct bands even though the expected amplicon of size 1 kbp was not observed in any.

4.4.1.8 PCR Analysis with Taro P3F and Taro P3R primers

Detection of CBDV infection in all the samples was carried using *Taro P3F* and *Taro P3R*, CBDV specific primers which amplifies the nucleocapsid (N) gene. A control (non template) was also set during PCR which contains all the components of a typical PCR but no template sample. PCR conditions were as mentioned in 3.4.8. No amplification was observed in the non-template control, which indicates that there is no nonspecific binding and primer dimer formation in PCR. The PCR results with *Taro P3F* and *Taro P3R* was negative for all samples. Out of the 22 samples screened for CBDV, only two tannia (X1 and X2) samples showed indistinct bands even though the expected amplicon of size 150 bp was not observed in any.

4.4.1.9 PCR Analysis with HafF and HafR primers

Detection of Badnavirus infection in all the samples was carried using *HafF* and *HafR*, *badnavirus* specific primers which amplifies the Badnavirus Reverse Transcriptase. A control (non template) was also set during PCR which contains all the components of a typical PCR but no template sample. PCR conditions were as mentioned in 3.4.9. An amplicon of size 530 bp was observed as a single band for virus positive samples in agarose gel (1 %) electrophoresis. No amplification was observed in the non-template control, which indicates that there is no nonspecific binding and primer dimer formation in PCR. The PCR results with *HafF* and *HafR* primers are shown in Plate 6c. Out of the 22 samples screened with HafF and HafR, six taro (N17, N18, N19, N21, N31 and M3d) samples as well as three tannia (X1, X2, and X4) samples showed virus infection. The samples showing positive exhibited various symptoms like feathery mosaics, chlorosis along margins, vein clearing and puckering. Samples N17, N18, N21, X1, X2, and X4 had showed positive for DsMV infection too.

4.4.1.10 PCR Analysis with TaBV1 and TaBV4 primers

Detection of TaBV infection in all the samples was carried using *TaBV1* and *TaBV4*, TaBV specific primers which amplifies the RT/RNaseH-coding region. A control (non template) was also set during PCR which contains all the components of a typical PCR but no template sample. PCR conditions were as mentioned in 3.4.10. An amplicon of size 320 bp was observed as a single band for virus positive samples in agarose gel (1 %) electrophoresis. No amplification was observed in the non-template control, which indicates that there is no nonspecific binding and primer dimer formation in PCR. The PCR results with *TaBV1* and *TaBV4* primers are shown in Plate 6d. Out of the 22 samples screened with TaBV1 and TaBV4, six taro (N17, N18, N19, N21, N31 and M3d) samples as well as three tannia (X1, X2, and X4) samples showed virus infection. The samples showing positive exhibited various

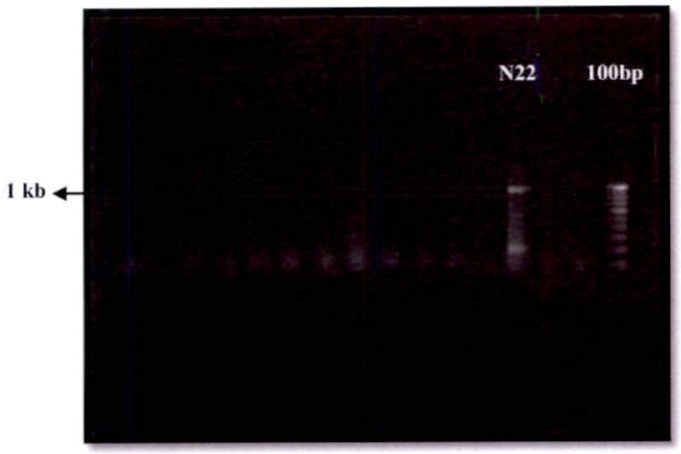


Plate 6a: PCR amplification of taro samples with P2F/P2R

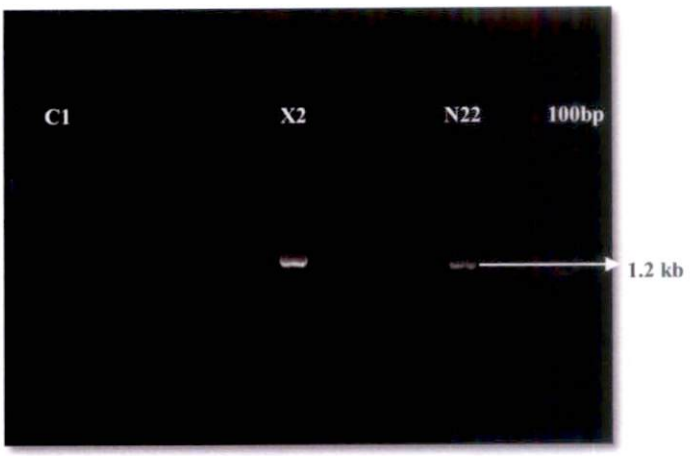


Plate 6b: PCR amplification of taro samples with DMV F/DMV R

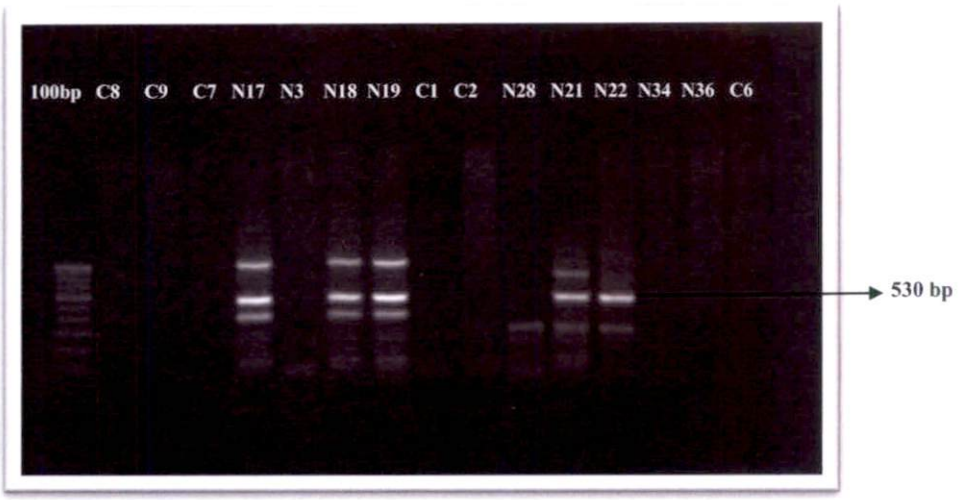


Plate 6c: PCR amplification of taro samples with HafF/HafR

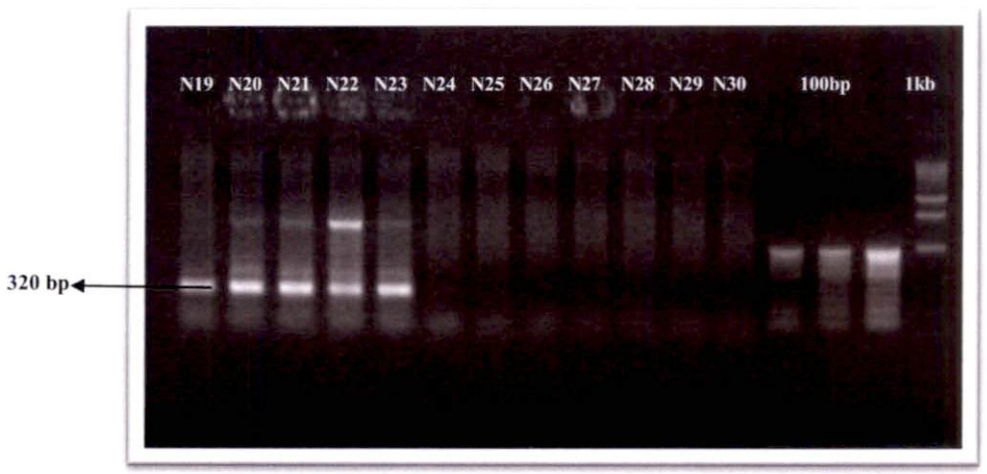


Plate 6d: PCR amplification of taro samples with TaBV1/TaBV4

symptoms like feathery mosaics, chlorosis along margins, vein clearing and puckering. Samples N17, N18, N21, X1, X2, and X4 had showed positive for DsMV infection too.

4.4.1.11 PCR Analysis with PNG BadnaF and PNG BadnaR primers

Detection of TaBV infection in all the samples was carried using *PNG BadnaF* and *PNG BadnaR*, TaBV specific primers which amplifies the RT/RNaseH-coding region. A control (non template) was also set during PCR which contains all the components of a typical PCR but no template sample. PCR conditions were as mentioned in 3.4.11. An amplicon of size 250 bp was observed as a single band for virus positive samples in agarose gel (1 %) electrophoresis. No amplification was observed in the non-template control, which indicates that there is no nonspecific binding and primer dimer formation in PCR. The PCR results with *PNG BadnaF* and *PNG BadnaR* primers are shown in Plate 7. Out of the 22 samples screened seven taro (N17, N18, N19, N21, N28, N31 and M3d) samples as well as one tannia (X1) samples showed virus infection. The samples showing positive exhibited various symptoms like feathery mosaics, chlorosis along margins, vein clearing and puckering. Samples N17, N18, N21, N22, X1, and had showed positive for DsMV infection too.

All the samples screened and viruses detected with the respective primers are represented in Table 7.

4.5 DETECTION BY NUCLEIC ACID SPOT HYBRIDIZATION

The presence of *DsMV* in the samples was confirmed by Nucleic acid spot hybridization analysis.

4.5.1 Probe preparation

Plate 7: PCR amplification of taro samples with PNG BadnaF/ PNG BadnaR

Table 7: Samples screened and viruses detected

No	Sample	MJ1/MJ2	DSMV3 F/ DSMV 3R	DMV-F/ DMV-R	DR0/DR2	P2F/P2R	TaVCV Cap2 A/ TaVCV Cap2B	Taro P3F/ Taro P3R	TaBV1/ TaBV4	Haf F/Haf R	PNG Badna F/ PNG Badna R
1	C1	✓	✓	✓	-	-	-	-	-	-	-
2	C2	✓	✓	-	-	-	-	-	-	-	-
3	C3	✓	✓	-	-	-	-	-	-	-	-
4	C6	-	-	-	-	-	-	-	-	-	-
5	C7	✓	✓	-	-	-	-	-	-	-	-
6	C8	✓	✓	-	-	-	-	-	-	-	-
7	C9	✓	✓	-	-	-	-	-	-	-	-
8	N3	-	-	-	-	-	-	-	-	-	-
9	N17	✓	✓	-	-	-	-	-	✓	✓	✓
10	N18	-	-	-	✓	-	-	-	✓	✓	✓
11	N19	-	-	-	-	-	-	-	✓	✓	✓
12	N21	✓	✓	-	-	-	-	-	✓	✓	✓
13	N22	✓	✓	✓	✓	✓	-	-	-	-	-
14	N25	-	-	-	-	-	-	-	-	-	-
15	N28	-	-	-	-	-	-	-	-	-	✓
16	N31	-	-	-	-	-	-	-	✓	✓	✓

Table 7 continued: Samples screened and viruses detected

No	Sample	MJ1 / MJ2	DSMV 3F / DSMV 3R	DMV-F /DMV-R	DR0 / DR2	P2F / P2R	TaVCV Cap2A / TaVCV Cap2B	Taro P3F / Taro P3R	TaBV1/ TaBV4	Haf F/ Haf R	PNG Badna F/ PNG Badna R
17	N34	✓	✓	-	-	-	-	-	-	-	-
18	N36	✓	✓	-	-	-	-	-	-	-	-
19	X1	✓	✓	✓	-	-	-	-	✓	✓	✓
20	X2	✓	✓	✓	-	-	-	-	✓	✓	-
21	X4	✓	✓	✓	-	-	-	-	✓	✓	-
22	M3d	-	-	-	-	-	-	-	✓	✓	✓

Biotin labelled probe was prepared and the probe quality was checked with "Chemiluminescent Nucleic acid Detection Module". A biotin-labelled probe targeting the CP gene was prepared by random prime labelling. Dilutions of each were spotted on a nylon membrane and analyzed by the direct detection procedure (Plate 8a). For reference, dilutions of a biotin-labelled control DNA were included on the same membrane. Chemiluminescent luminol substrate was used to visualize the DIG signal in the spots with an exposure time of 3 minutes. The probe (10^{-2}) was used to detect the presence of DsMV in the select samples.

4.5.2 Nucleic acid spot hybridization

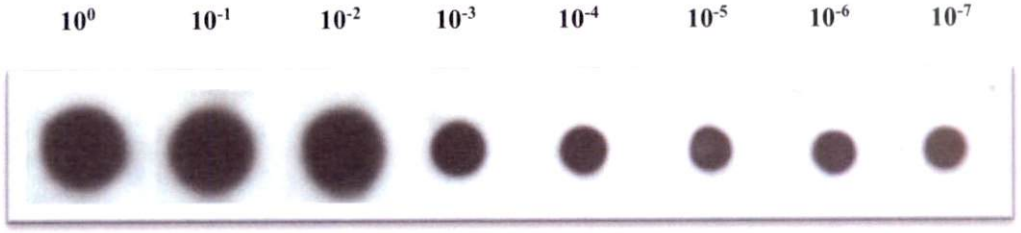
cDNA of the 22 representative samples and DNA of other samples which showed differing symptoms or *Badna* infection in PCR (to check for mixed infection with DsMV) was spotted (4 µl) on positively charged nylon membrane after denaturation and hybridised with biotin labelled *DsMV CP* probe. The positive samples corresponds to the numbers 1, 9, 10, 12, 13, 14, 17, 24, 25, 26, 27, 28, 29, 34 and 35 in Plate 8b. Twelve samples of taro (C1, C2, C3, C7, C8, C9, N17, N18, N21, N22, N34, and N36) as well as 3 samples of tannia (X1, X2, and X4) gave signals on exposure to X- ray film with varying intensity, which indicated the presence of DsMV in the samples. The intensity of the spots was very low compared to the signals from the probe spotted. The DNA spotted showed no signals.

4.6 CHARACTERISATION OF VIRUSES

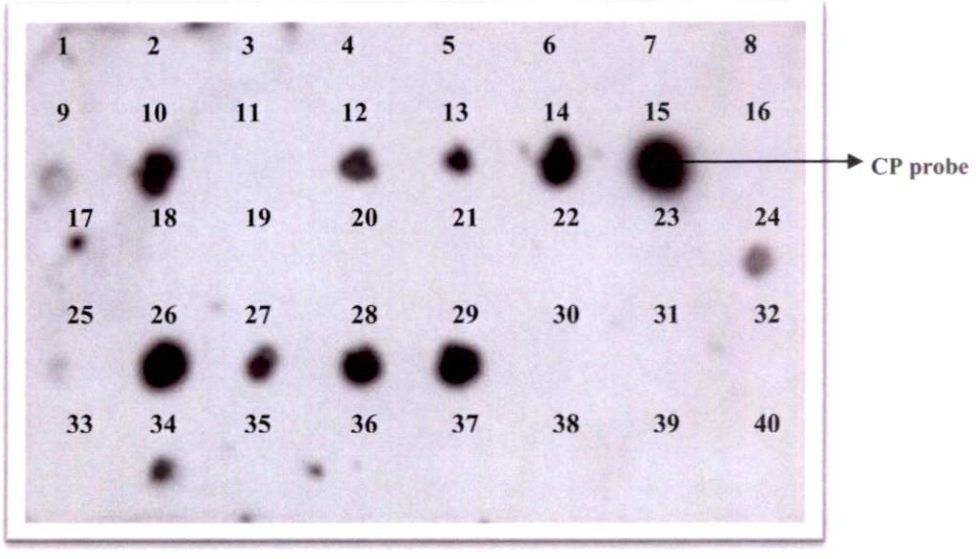
Samples N22 and N31 showing consistent results for DsMV and TaBV screening with different virus specific primers respectively were selected for further characterization through sequencing.

4.6.1 Gel elution of PCR amplified fragments

PCR was done with sample N22 using *DsMV 3F* and *DsMV 3F* primers and for sample N31 with *TaBV* 1 and *TaBV* 4 primers. Amplified products were run on



(a)



(b)

Plate 8: (a) CP probe quality check by serial dilution.
(b) Detection of DsMV infection using NASH method.

1% agarose gel and the amplified product was eluted with PureLink™ Quick Gel Extraction kit (Invitrogen, USA) and the elute was checked for quality in 1 percent agarose gel (Plate 9).

4.6.2 Cloning and sequencing

The gel elute was proceeded with cloning for better sequencing results since both primer sets, DsMV 3F/DsMV 3R and TaBV 1/TaBV 4 are degenerate primers. The eluted amplified products were cloned using InsTA Clone PCR Cloning Kit (Fermentas, USA) and transformation was carried out in *E. coli* DH5α. The transformed colonies were distinguished by blue-white screening in LB Ampicillin X gal/ IPTG plates (Plate 10a). Around 80-100 colonies were obtained in each plate. Grid plates were prepared to maintain the transformed white colonies (Plate 10b). The white colonies were selected for further analysis using colony PCR (Plate 11). The recombinant clones were analysed by colony PCR using respective primers (DsMV 3F/DsMV 3R and TaBV 1/TaBV 4) under appropriate conditions and they were analysed in 1% agarose gel. Gel elution was carried out with one colony PCR positive clone each for DsMV and TaBV. Gel elute amplified product samples of N22 and N31 was sent to the Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology (RGCB) with their respective primers. The sequencing results were obtained as electropherogram resulting from capillary sequencing in *.abi* format.

The *DsMV* sequence obtained is given below (410 nt):

CAGTTTGAGGTCTGGTACAATGCAGTCAAGAGGGAATATGATCTTGAGGA
TGAACAGATGCACATAGTAATGAATGGATTCATGGTTTGGTGCATCGATA
ATGGAACATCACCCGATATTAACGGGGCTTGGGTGATGATGGACGGAAA
TGATCAAATTGAATACCCGTTAAAGCCAATTGTGGAAAATGCAAAACCAA
CCTTGCGTCAGATAATGCATCACTTCTCTGACGCAGCAGAGGCATATATT
GAACTGAGAAATGCGGAGAGACCATATATGCCTAGATATGGTCTTATTCG

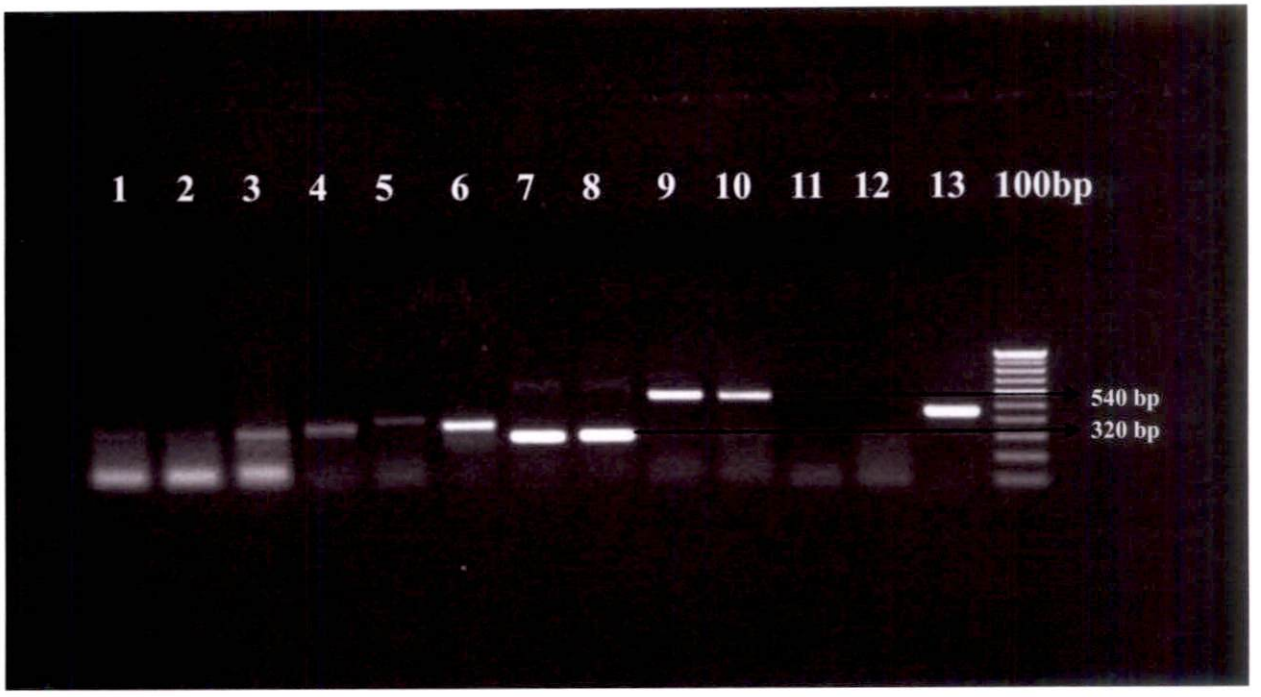


Plate 9: Gel elute of samples. Lane 3, 4, 5, 6, 7, 8 – TaBV; Lane 9, 10, 13 - DsMV

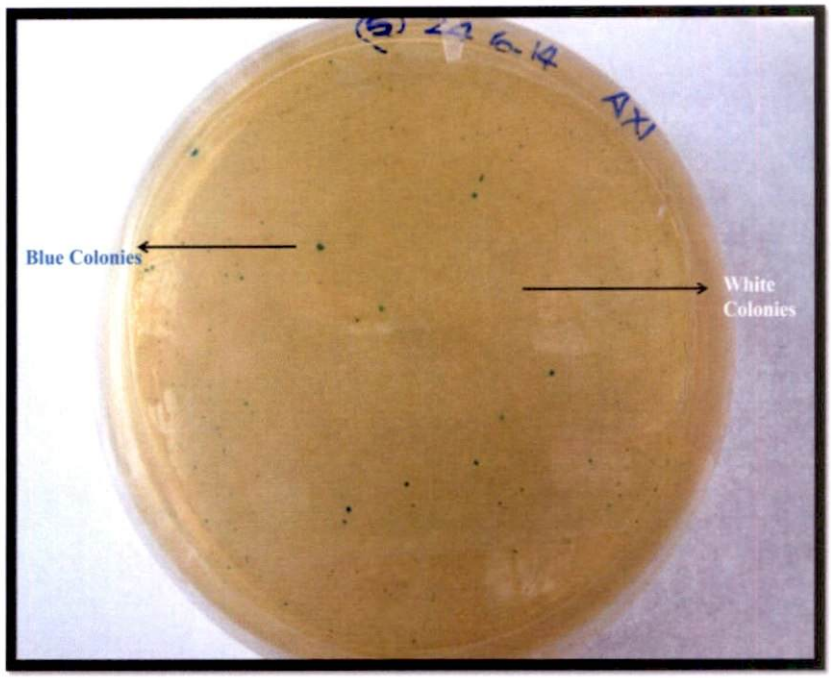


Plate 10(a): Blue-white colony screening in LB-AXI plates

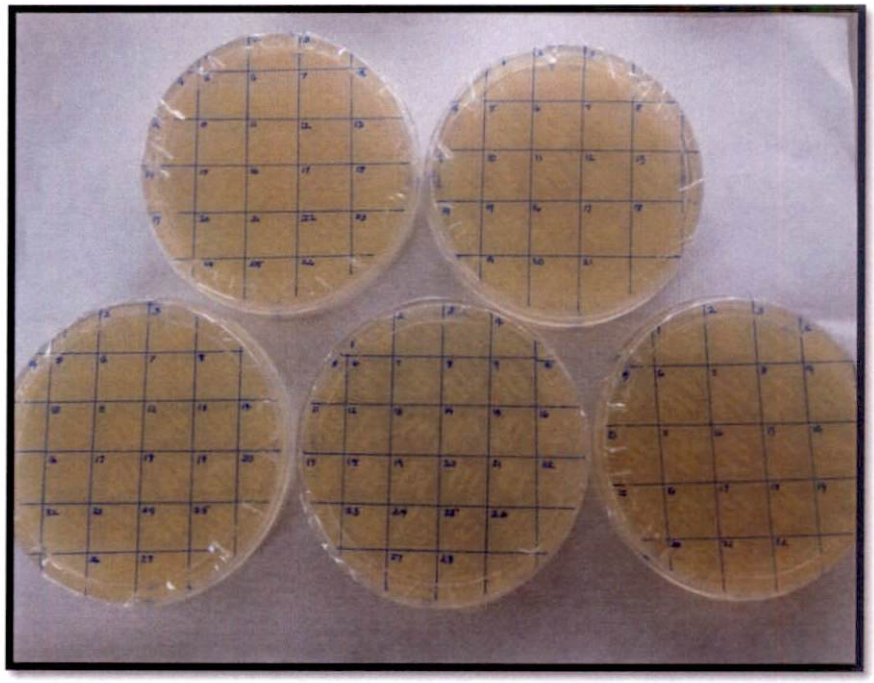


Plate 10(b): Grid plates containing transformed colonies

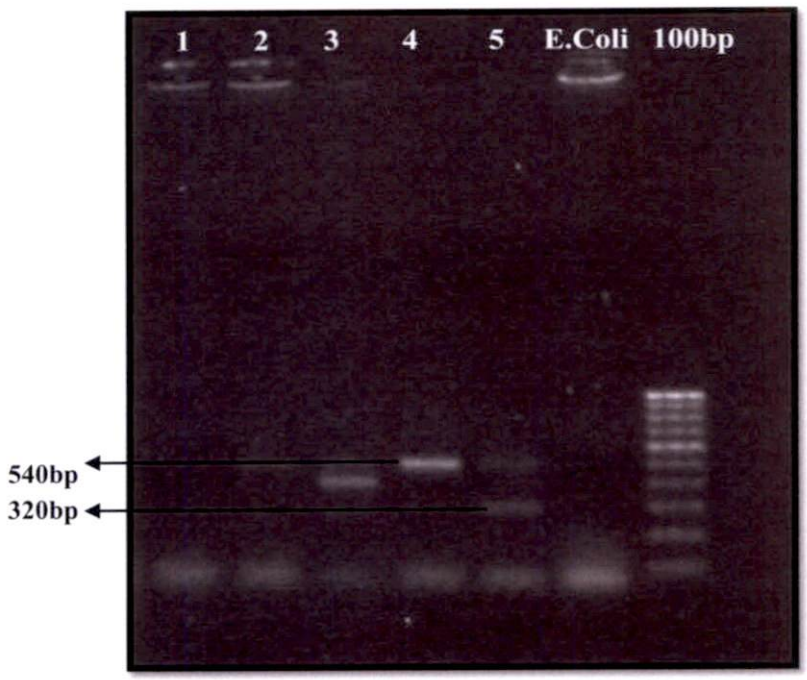


Plate 11: Colony PCR to confirm insert of amplicon
Lanes 1-4: clones specific to DsMV
Lane 5: Clone specific to TaBV

TAACTTACGTGATGCAAGTCTCGCCCGGTATGCCTTCCACTTCTATGAGGT
CAATTCCAAAACACCGGTTCGAGCAAGGGAGGCAGTTGCGCAAATGAAG
GCCGCTGCACT.

The *TaBV* sequence obtained is given below (334 nt):

CATGGTCTTGTCTTATCACCCTCAAAGATGAATATTGCAGTCAAGGAAGT
GAATTTTCTTGGAGCTACTATTGGCAGCAGAAAGGTTAAGCTTCAAGAAA
ATATTATAAAGAAGATCTTGGACTTCAATGAAGAAAATCTTCAATCAAAG
AAAGGTCTTCGGTCTTTTCTGGGAATTCTCAATTATGCCCGAAATCATATT
CCTAATCTCGGGAAAATAGCTGGTCCTTTATATTCCAAGACTTCCATCTAC
GGTGATATCAGATTTTCAGCATCTGATTGGAAGCTTATTAAAGAGATCAA
GAATATTGTTGAGAAACTCCCACCACTTGATT.

4.6.4 Sequence analysis

The sequence results were initially analyzed and edited using BioEdit Sequence Alignment Editor program version 7.2.5 and the obtained sequence was run through the online BLAST program of NCBI. The BLAST results (Figure 7 and 8) were analysed and sequence similiarity was studied.

BLAST Map of the query sequence (410) gave 100 hits related to DsMV similarity with reference to region 383-866, accession number HQ207530. The obtained 410 nt *DsMV* sequence showed maximum similiarity of 93% to *dasheen mosaic virus* isolate *DsMV-Amp3 polyprotein gene*, *DsMV* isolate T10 (Accession KJ786965) and *DsMV* partial CP gene for coat protein of NiNG1 (Accession AM910398) and NiNG4 isolate (Accession AM910400).

BLAST Map of the query sequence (334) gave 100 hits of Badna virus similarity with reference to region 136-424, accession number AY186614. Whereas the 334 nt TaBV sequence showed maximum sequence similiarity of 92% to TaBV isolates (NC1, SI2 and S17) polyprotein gene.

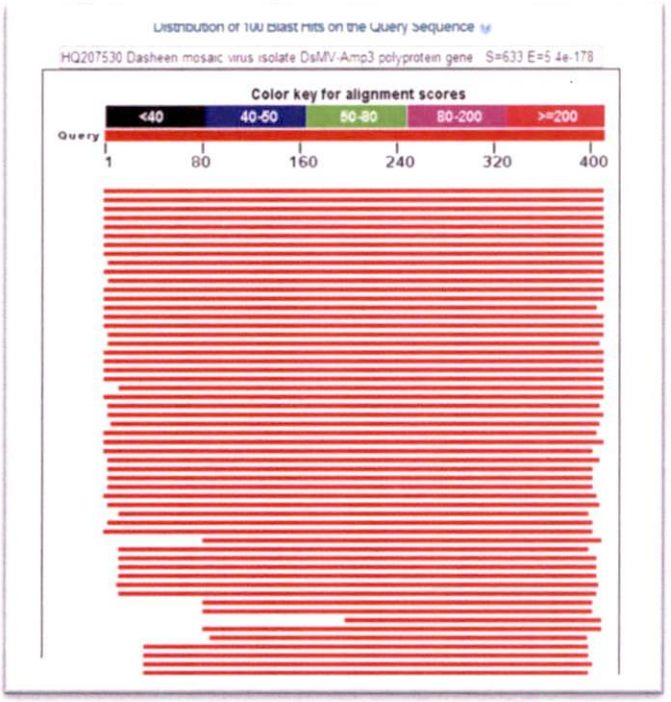


Figure 7: BLAST analysis of the DsMV sequence

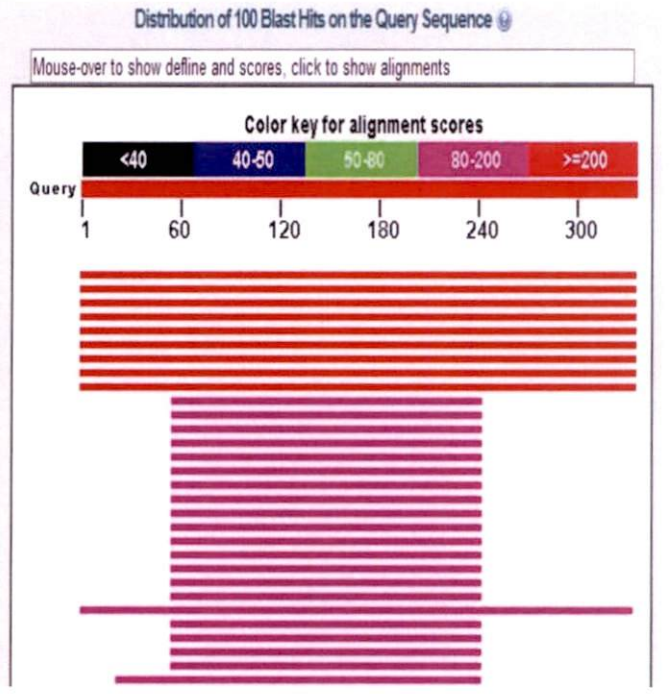


Figure 8: BLAST analysis of the TaBV sequence

The phylogenic tree (Figure 9 & 10) was constructed with similar sequences using online NCBI blast analysis software. The trees constructed at 100 bootstrap replicates showed similiarity with the CP region of different DsMV isolates and the RT region of TaBV of different isolates respectively. Phylogenetic analysis clearly revealed that the sequences obtained in this study belongs to *DsMV* for the sample N22 and *TaBV* for the sample N31 as they grouped along with their respective virus sequences used for comparison analysis.

After the both serological and nucleic acid based screening, 15 samples showed *DsMV* infection and 10 samples showed *TaBV* infection. About 6 samples showed mixed infection of *TaBV* and *DsMV*. Both TaVCV and CBDV were not detected in any of the samples hence they are absent in India. The combined test results of all the methods executed for virus detection is represented in the Table 8.

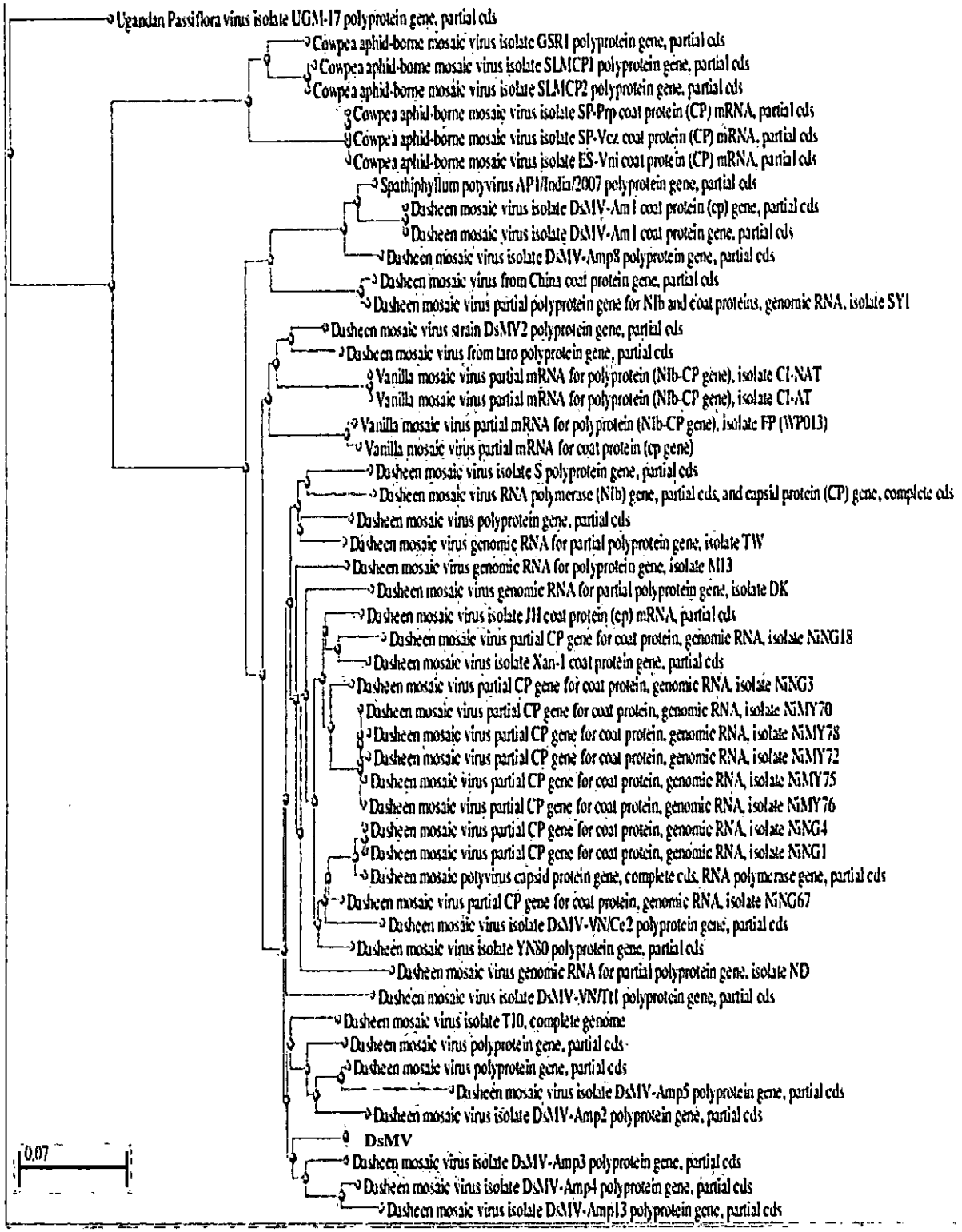


Figure 9: Phylogenetic tree construction of DsMV sequence

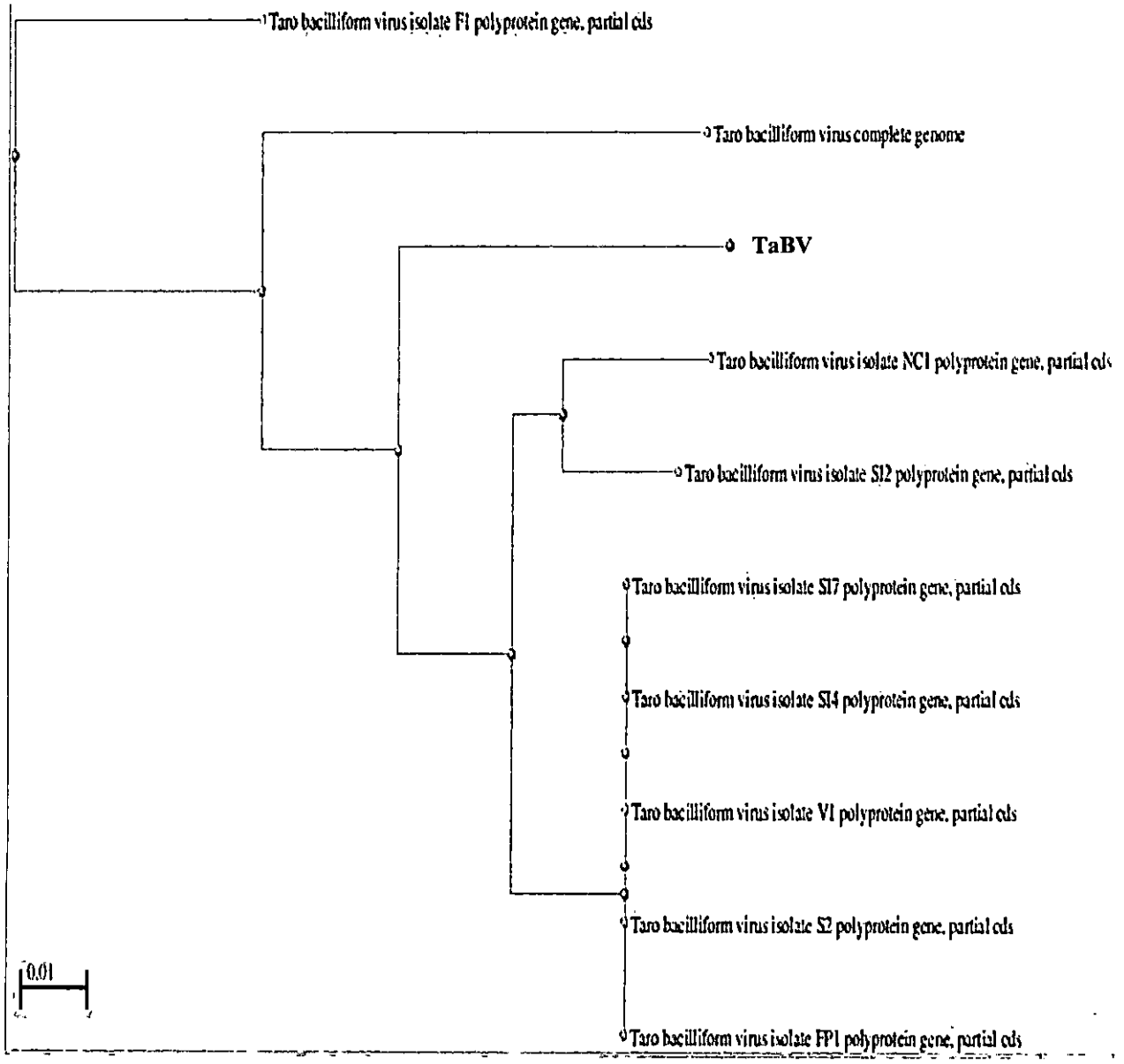


Figure 10: Phylogenetic tree construction of TaBV sequence

Table 8: Sample reactions for each test to detect virus infection.

No	Sample	ELISA (DsMV)	DIBA (DsMV)	PCR				NASH (DsMV)
				DsMV	TaBV	TaVCV	CBDV	
1	C1	✓	✓	✓	-	-	-	✓
2	C2	✓	✓	✓	-	-	-	✓
3	C3	✓	✓	✓	-	-	-	✓
4	C6	-	-	-	-	-	-	
5	C7	✓	✓	✓	-	-	-	✓
6	C8	✓	✓	✓	-	-	-	✓
7	C9	✓	✓	✓	-	-	-	✓
8	N3	-	-	-	-	-	-	
9	N17	✓	✓	✓	✓	-	-	✓
10	N18	✓	✓	✓	✓	-	-	✓
11	N19	-	-	-	✓	-	-	
12	N21	✓	✓	✓	✓	-	-	✓
13	N22	✓	✓	✓	-	-	-	✓
14	N25	-	-	-	-	-	-	
15	N28	-	-	-	✓	-	-	
16	N31	-	-	-	✓	-	-	
17	N34	✓	✓	✓	-	-	-	✓
18	N36	✓	✓	✓	-	-	-	✓
19	X1	✓	✓	✓	✓	-	-	✓
20	X2	✓	✓	✓	✓	-	-	✓
21	X4	✓	✓	✓	✓	-	-	✓
22	M3d	-	-	-	✓	-	-	

Discussion

5. DISCUSSION

Taro (*Colocasia esculenta* (L.) Schott), a tuber crop belonging to the family *Areaceae* is considered to be one of the oldest crops in the world. Even though the origin of this aroid crop is still debated, taro is believed to have originated in Southeast Asia. In spite of being referred to as "an orphan crop" due to the lack of research attention this crop received, the international community soon noticed the role of taro in food security in economically and culturally very important in parts of the globe such as India, China, Africa, Pacific Islands and so on (Rao *et al.*, 2010; Matthews, 2010). Major research projects were taken up such as the TANSAO and TAROGEN projects to study taro and the challenges faced by the crop and the populations depending on it.

In the past, several taro growing countries have been hit with many diseases such as Taro Leaf Blight, Alomae, Bobone disease, Dasheen Mosaic etc. being the major ones, which had ruined the taro production, caused huge economical loss and made food security of the region insecure. Taro being a plant which has almost all of its parts edible, such as the corms, leafs, petioles etc. is also appreciated for its high nutritional content. Since the disease outbreaks and huge economical losses sustained, the international movement of taro germplasm is restricted due to taro viruses and other destructive pathogens. This has serious implications, since many countries are denied access to agronomically elite lines including selected traditional cultivars, lines produced in breeding programs such as those in Samoa and PNG and tissue-cultured plantlets in germplasm collections. To address this problem, characterization of the viruses infecting taro has been taken up by the international research society to enable the development of sensitive and reliable diagnostic tests. In the short term, such tests would be useful to determine the geographical distribution of the viruses and allow informed decisions to be made by countries regarding the risks of taro importation. In the longer term, the development of such tests for all taro viruses for inclusion in an indexing scheme will enable the exchange of virus-tested taro

germplasm between countries. However, since the diagnosis of virus infections based on symptoms is unreliable due to complicated mixed infections in taro with multiple viruses and isolates, it is necessary sensitive diagnostic tests are developed region wise to confront this issue. As a prerequisite to this virus detection and identification has to be carried out in taro to determine the viruses of taro geographically.

Taro, like other root and tuber crops propagated vegetatively, poses special problems not only in collecting and storing, but also from a germplasm health point of view (Rodoni *et al.*, 1995). Viruses are one of the most important pathogens of taro, with some infections resulting in severe yield reductions and plant death. According to Revill et al. (2005a) the five main viruses reported infecting taro are, *Dasheen mosaic virus* (DsMV), *Colocasia bobone disease virus* (CBDV), *Taro bacilliform virus* (TaBV), *Taro vein chlorosis virus* (TaVCV) and *Taro reovirus* (TaRV). Apart from DsMV, these viruses have been poorly characterised and there is confusion in the literature concerning their distribution and the symptoms associated with infection. In this study, the viruses DsMV, TaBV, TaVCV and CBDV were screened for their presence in the collected samples. Both serological and nucleic acid based methods of virus detection were carried out. Initial screening using ELISA was used to detect DsMV infection in both symptomatic and asymptomatic samples. Since the 1970s, serological methods like enzyme linked immunosorbent assay (ELISA) have been used widely and successfully for detection of plant viruses and diagnosis of plant viral diseases (Clark and Adams, 1977; Flegg and Clark, 1979). ELISA techniques include NCM-ELISA and Dot Immuno Binding Assay (DIBA). But it has major limitations such as its low sensitivity during periods of low virus titre. Moreover serological diagnosis of potyviruses is often imprecise, because of frequent serological cross-reactions between species and biological indexing is very cumbersome (Brunt, 1992). Out of 55 samples screened for DsMV by DAS-ELISA, only 5 samples showed negative while all 50 samples showed DsMV infection.

Followed by ELISA screening, samples were subjected to PCR based virus detection using group specific and virus specific primers. In the 1990s, nucleic acid-based methods such as reverse transcription (RT) and the polymerase chain reaction (PCR) began to be used in plant virus detection (Wetzel *et al.,* 1991; Rowhani *et al.,* 1995; Thomson and Dietzgen, 1995). The PCR-based methods are fast, highly sensitive and useful for accurate detection, quantification and characterization of plant pathogens. This study used degenerate primers to detect DsMV, TaBV and TaVCV. Degenerate primers have been designed to recognize the conserved regions of viral genomes of many virus species or the whole virus genus or family (Langeveld *et al.,* 1991; Bateson and Dale, 1995; Tian *et al.,* 1996; Gibbs and Mackenzie, 1997; Chen *et al.,* 2001a; Posthuma *et al.,* 2002).

Out of 22 samples in the core sample set, 15 samples showed DsMV infection in PCR based detection while 50 out of 55 samples showed positive for DsMV in ELISA. *Dasheen mosaic virus* has a worldwide distribution, but has been found naturally occurring only in members of the family *Araceae* (Zettler *et al.,* 1978; Zettler and Hartman, 1986; Zettler and Hartman, 1987), infecting at least 16 genera of *Araceae* family. The common symptoms exhibited by these samples in this study are mild to severe mosaic and feathery mosaics, puckering, leaf distortion and chlorosis. Samples showing no prominent symptoms were also identified being infected with *DsMV.* Zettler and Hartman (1986) reported that infected plants usually display a conspicuous feathery mosaic pattern although cultivars vary considerably in symptom expression. Although typical feathery mosaic symptoms were associated with many infections, the virus was also detected in a large number of plants that appeared healthy and plants that were infected with other viruses.

In this study PCR based diagnostics carried out using potyvirus group specific primer MJ1 /MJ2, and DsMV specifc primers DsMV 3F /DsMV 3R, amplifying the partial CP region and 3'UTR giving an amplicon of 327 bp and 540 bp respectively was found to be a robust of detecting DsMV infecting taro in India. Ha *et al.* (2008)

stated that PCR-based methods for the detection and identification of potyviruses are primarily based on the use of degenerate primers to conserved sequences in the viral genomes. The use of degenerate primers has not only facilitated the rapid detection of many potyviruses but has also enabled partial genomic sequencing for taxonomic purposes. The group-specific PCR and subsequent sequence analysis of the amplified regions has been used for rapid detection and identification of Potyvirus and is appeared to be the most suitable method for identification of viruses which are difficult to purify and/or occurring in mixed infections (Colinet *et al.,* 1998). Babu *et al.* (2011a) proved RT–PCR with MJ1 and MJ2 primers useful for the detection and identification of the potyvirus infecting *C. esculenta* in India.

There were 10 samples showing TaBV infection and 6 of them showed mixed infections. In every case the co-infection was found along with DsMV. TaBV infection was commonly characterized by symptoms like mild chlorosis and vein clearing. And it was found out that although symptoms such as mild vein clearing or chlorosis and sometimes what seems to be variegation, could be associated with *TaBV*. According to Revill et al. (2005a), some of the viruses occur as latent infections, and all can be found in various combinations; this means that it is difficult to ascribe symptoms to infection by individual viruses. Harding et al. (2004) described the symptoms associated with TaBV to be still unclear because many of the plants testing positive for this virus were also shown to be co-infected with other viruses.

None of the samples showed symptoms similar to Alomae disease and no samples showed CBDV infection in PCR. Though *TaBV* is one of the viruses involved; it can be said that alomae is absent in India. Alomae is the most economically important virus disease affecting taro and appears to be restricted to Papua New Guinea and the Solomon Islands while TaBV appears to be widely distributed throughout the Pacific (Gollifer *et al.,* 1977; Gollifer and Brown, 1972; Jackson and Gollifer, 1975; Jackson, 1978; Rodoni *et al.,* 1994).

Badnavirus and TaBV detection carried out with both HafF/HafR and TaBV1/TaBV4 gave almost similar results confirming all badna viruses infections were caused by TaBV. Though slight variations in the results were obtained in the case of primers PNG BadnaF/PNG BadnaR used to detect Badna like sequences. But the former gave positive for all TaBV infected samples too except for tannia (X2 and X4) but showed positive for one taro sample (N28) which was negative for TaBV. Revill et al. (2005a) had reported that sequence showing approximately 50% nucleotide identity to TaBV in the RT/RNaseH-coding region was also detected in taro plants tested and may represent either an integrated sequence or the genome of an additional badnavirus infecting taro. This study also found that BadnaF / BadnaR *badnavirus* group specifc primer and TaBV 1/TaBV4 TaBV specific primer amplifying the RT/RNaseH-coding region giving an amplicon of 530 bp and 320 bp respectively proved to be an efficient and consistent method in detecting TaBV infections.

Even though symptoms similar to TaVCV were observed, none of the samples showed positive for virus infection in PCR. According to Revill *et al.* (2005b) TaVCV infected leaves show a distinct vein chlorosis, more pronounced than TaBV. During this study some of such samples showed positive for TaBV in PCR while others did not. The screening for TaVCV and CBDV was negative for all samples and since the viruses weren't detected in any samples it should be understood that *TaVCV* and *CBDV* presence is mostly confined to PNG and other pacific islands. CBDV disease is only reported from PNG and the Solomon Islands, and is characterised by stunting, leaf distortion and presence of galls on the petioles (Jackson, 1978).

NASH confirmed the DsMV positive samples in this study and the CP probe proved to be better for specific detection of DsMV. Nucleic acid hybridization and RT-PCR detection are more sensitive methods for the detection of virus, (Seal and Coater, 1998; James, 1999). Nucleic acid spot hybridization is more convenient than

RT-PCR for specific and large-scale detection of the viruses (Craig *et al.*. 2004). Incourse of this study it was also found out that tannia (*Xanthosoma sagittifollium*) which is often suggested as a better alternative to taro due to its presumed lesser susceptibility to viruses, was identified with mixed infections on a higher rate than in taro giving positive for most of the primers used in PCR based diagnostics.

The 410 nt DsMV sequence obtained in this study showed maximum similiarity of 93% to *dasheen mosaic virus* isolate *DsMV-Amp3 polyprotein gene*, *DsMV* isolate T10 (Accession KJ786965) and *DsMV* partial CP gene for coat protein of NiNG1 (Accession AM910398) and NiNG4 isolate (Accession AM910400). According to Fauquet *et al.* (2005) potyvirus isolates with 85% sequence identity or more over the whole genome are usually considered to be from the same species. The 334 nt TaBV sequence showed maximum sequence similiarity of 92% to TaBV isolates (NC1, SI2 and S17) polyprotein gene.

Summary

6. SUMMARY

The study entitled "Molecular characterisation of viruses in taro (*Colocasia esculenta* (L.) Schott)" was carried out at the Division of Crop Protection, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2013-2014. The objective of the study was to diagnose, clone and characterise viruses implicated in mixed infections of taro (*Colocasia esculenta* (L.) Schott). The important findings of the above studies are summarised in this chapter.

Taro samples with various virus infection symptoms were collected from the germplasm repository of CTCRI, Trivandrum and field samples from Bhuvaneshwar & Jharkhand. After initial serological screening for DsMV, samples exhibiting duplicate symptoms were eliminated and a representative sample set of 22 taro leaf samples was made based on the common symptoms such as whitish feathery mosaics, mild mosaics, chlorosis, vein clearing, puckering, leaf distortion and mottling. This sample set contained both DsMV positive and negative samples. The samples were mainly screened for *Dasheen Mosaic Virus, Taro Bacilliform Virus, Taro vein chlorosis virus* and *Colocasia Bobone disease virus* using both genus and species specific primers. Fifteen samples out of 22 showed DsMV infection in PCR and ten samples showed TaBV infection. While mixed infection by DsMV and TaBV was found in 6 samples there was a lone case of positive for TaBV-like sequence in one sample (N28). Both TaVCV and CBDV screening through PCR gave negative results for all samples.

This study found out Dasheen Mosaic and taro bacilliform virus to be the most common virus infecting taro in India, the former being ubiquitous in taro everywhere The samples characterized suggests feathery mosaic symptoms, vein clearing, mild chlorosis etc. to be the common symptoms associated with *DsMV* while cases of samples showing no prominent symptoms were also identified being infected with *DsMV*. The symptoms and occurrence of *TaBV* is still not clear since

TaBV was mostly detected along with mixed infections viz. *DsMV*. Though symptoms such as mild vein clearing or chlorosis and sometimes what seems to be variegation could be associated with *TaBV*. Fortunately *TaVCV* and *CBDV* infection was not detected during virus screening and this should be because *TaVCV* and *CBDV* presence is mostly confined to PNG and other pacific islands. Incourse of this study it was also found out that tannia (*Xanthosoma sagittifollium*) which is presumably less susceptible to viruses, was identified with mixed infections on a higher rate than in taro giving positive for most of the primers used in PCR based diagnostics.

PCR based diagnostics carried out using MJ1 /MJ2, potyvirus group specific primers and DsMV 3F /DsMV 3R, DsMV specifc primers amplifying the partial CP region and 3'UTR giving an amplicon of 327 bp and 540 bp respectively was found to be an robust of detecting DsMV infecting taro in India. Whereas BadnaF / BadnaR badnavirus group specifc primer and TaBV 1/TaBV4 tabv specific primer amplifying the RT/RNaseH-coding region giving an amplicon of 530 bp and 320 bp respectively proved to be an efficient and consistent method in detecting TaBV infections. PNG BadnaF and PNG BadnaR for detecting TaBV like virus sequences also gave several positives.

One sample each for DsMV and TaBV, were cloned and sequenced. The sequence data was analysed through BLAST and sequence similiarity was studied. The *DsMV* sequence obtained is 410 nt and it showed maximum similiarity of 93% to *dasheen mosaic virus* isolate *DsMV-Amp3 polyprotein gene*, *DsMV* isolate T10 (Accession KJ786965) and *DsMV* partial CP gene for coat protein of NiNG1 (Accession AM910398) and NiNG4 isolate (Accession AM910400). Whereas the 334 nt TaBV sequence showed maximum sequence similiarity of 92% to TaBV isolates (NC1, SI2 and S17) polyprotein gene. The phylogenic tree was constructed with similar sequences using online NCBI blast analysis software. Phylogenetic analysis clearly revealed that the sequences obtained in this study belongs to *DsMV*

for the sample N22 and *TaBV* for the sample N31 as they grouped along with their respective virus sequences used for comparison analysis.

Out of the major 5 taro affecting viruses worldwide, *Dasheen Mosaic Virus* belonging to *Potyvirus* and *Taro Bacilliform Virus* belonging to *Badnavirus* is widely found regionally. Sample studies showed mixed infections caused by DsMV & TaBV on both Taro and Tannia. *Taro vein chlorosis virus* and *Colocasia Bobone Disease Virus* affecting taro in the Pacific islands is absent in India.

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Appendices

8. APPENDIX

ELISA Coating Buffer

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.20 g

Adjust pH to 9.6 with HCl and make up to 1L. Autoclave and store at 4°C.

ELISA Sample Extraction Buffer

PBS-T (1X PBS + 0.5ml Tween 20/L) + 2% PVP

ELISA Conjugate Buffer

PBS-T (1X PBS + 0.5ml Tween 20/L) + 2% PVP + 0.2% egg albumin

ELISA Substrate Buffer

Diethanolamine	9.7 ml
Distilled Water	80 ml
NaN_3	0.2 g

Adjust the pH to 9.8 with HCl and make up to 100ml.

ELISA Blocking Buffer

PBS-T (1X PBS + 0.5ml Tween 20/L) + 2% dried skimmed milk

CTAB RNA Extraction Buffer

Tris- HCl (pH 8.0)	100 m*M*
EDTA	25 m*M*
NaCl	2 *M*
CTAB	2 %

β-mercaptoethanol	2 % (v/v)	freshly added prior to RNA
PVP	2 % (w/v)	extraction

Prepared in DEPC treated water.

TAE Buffer (50X)

Tris base	242g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

CTAB DNA Extraction Buffer

Tris- HCl (pH 8.0)	100 m*M*	
EDTA	20 m*M*	
NaCl	1.4 *M*	
CTAB	2 %	
β-mercaptoethanol	0.2 % (v/v)	freshly added prior to DNA
PVP	2 % (w/v)	extraction

Luria Agar Medium

35.0 grams of LA (HiMedia) in 1000 ml distilled water

T Solution

Mix 250 µl T- Solution (A) and 250 µl T- Solution (B)

LB Medium

20 g of LB (HiMedia) in 1000 ml distilled water

LB Ampicillin X gal/ IPTG Agar Plates

LB + Ampicillin 0.1 mM +X-gal 40 µg/ml + IPTG 0.1 mM

Abstract

MOLECULAR CHARACTERISATION OF VIRUSES IN TARO
[*Colocasia esculenta* (L.) Schott]

by

ADIL HAKKIM

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Abstract of the thesis
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ABSTRACT

The study entitled "Molecular characterization of viruses in taro [*Colocasia esculenta* (L.) Schott]" was carried out at the Division of Crop Protection, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2013-2014. The objective of the study was to diagnose, clone and characterize viruses implicated in mixed infections of taro hence identifying an effective diagnostic strategy to detect virus infections in taro.

Taro leaf samples with various virus infection symptoms were collected from Jharkhand, Bhuvaneshwar and the germplasm repository of CTCRI. The samples were mainly screened for *Dasheen Mosaic Virus, Taro Bacilliform Virus, Taro vein chlorosis virus* and *Colocasia Bobone disease virus* using both genus and species specific primers. This study found out Dasheen Mosaic and taro bacilliform virus to be the most common virus infecting taro in India, the former being ubiquitous in taro everywhere. Fortunately *TaVCV* and *CBDV* infection was not detected during virus screening and this should be because *TaVCV* and *CBDV* presence is mostly confined to PNG and other pacific islands. PCR based diagnostics carried out using MJ1 /MJ2, potyvirus group specific primers and DsMV 3F /DsMV 3R, DsMV specifc primers amplifying the partial CP region and 3'UTR giving an amplicon of 327 bp and 540 bp respectively was found to be an robust of detecting DsMV infecting taro in India. Whereas BadnaF/BadnaR badnavirus group specifc primer and TaBV 1/TaBV4 TaBV specific primer amplifying the RT/RNaseH-coding region giving an amplicon of 530 bp and 320 bp respectively proved to be an efficient and consistent method in detecting TaBV infections. PNG BadnaF/PNG BadnaR for detecting TaBV like virus sequences also gave several positives. One sample each for DsMV and TaBV, were cloned and sequenced. The BLAST results were analysed and sequence similiarity was studied. The obtained 334 nt *DsMV* sequence showed maximum similiarity of 93% to *dasheen mosaic virus* isolate *DsMV-Amp3 polyprotein gene*, *DsMV* isolate T10 (Accession KJ786965) and *DsMV* partial CP gene for coat protein of NiNG1 and

NiNG4 isolate. Whereas the 410 nt TaBV sequence showed maximum sequence similiarity of 92% to TaBV isolates (NC1, SI2 and S17) polyprotein gene. The phylogenic tree was constructed with similar sequences. The trees constructed at 100 bootstrap replicates showed similiarity with the CP region of different DsMV isolates and the RT region of TaBV of different isolates respectively.

Since the diagnosis of virus infections based on symptoms is unreliable due to complicated mixed infections in taro with multiple viruses and isolates, it is necessary sensitive diagnostic tests are developed region wise to confront this issue. As a prerequisite to this virus detection and identification has to be carried out in taro to determine the viruses of taro geographically.