# MOLECULAR CHARECTERIZATION OF *TARO BACILLIFORM VIRUS* (TaBV)

*by*

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**THESIS**

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## DECLARATION

I hereby declare that the thesis entitled **"MOLECULAR CHARACTERIZATION OF *TARO BACILLIFORM VIRUS* (TaBV)"** 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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***Aarathy M. B.***

DEDICATED TO MY PARENTS

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| | |
|---|---|
| % | Percentage |
| ~ | Approximate |
| °C | Degree Celsius |
| µg | Microgram |
| µl | Microlitre |
| µ*M* | Micromolar |
| 3' | Three prime |
| *5'* | Five prime |
| A | Adenine |
| $A_{260}$ | Absorbance at 260 nm wavelength |
| $A_{280}$ | Absorbance at 280 nm wavelegth |
| BLAST | Basic Local Alignment Search Tool |
| bp | Base pair |
| BSV | Banana streak virus |
| C | Cytosine |
| CBDV | *Colocasia bobone disease virus* |
| cm | Centimeter |
| $cm^2$ | Centimeter square |
| ComYMV | *Commelina yellow mottle virus* |
| CSSV | *Cacao swollen shoot virus* |
| CTAB | Cetyl trimethyl ammonium bromide |
| CTCRI | Central Tuber Crops Research Institute |
| CYMV | *Citrus yellow mosaic virus* |
| DBV | *Dioscorea bacilliform virus* |
| DNA | Deoxyribo nucleic acid |
| dNTPs | Deoxy nucleotide tri phosphates |
| dsDNA | Double stranded DNA |

| | |
|---|---|
| DsMV | *Dasheen mosaic virus* |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylene diamine tetra acetic acid |
| F | Forward primer |
| FAOSTAT | Food and Agriculture Organization Statistical Database |
| G | Guanine |
| g | Gram |
| h | Hour |
| ha | Hectare |
| kb | Kilobases |
| kDa | Kilo Dalton |
| kg | Kilogram |
| L | Litre |
| m | Meter |
| M | Molar |
| mg | Milligram |
| min | Minute |
| ml | Millilitre |
| mm | Millimeter |
| mM | Millimolar |
| NCBI | National Centre for Biological Information |
| nm | Nanometer |
| nts | Nucleotides |
| ORF | Open Reading Frames |
| PCR | Polymerase Chain Reaction |
| p*M* | Picomolar |
| PNG | Papua New Guinea |
| PVBV | *Pellargonium vein banding virus* |

| | |
|---|---|
| PYMV | *Piper yellow mottle virus* |
| R | Reverse primer |
| Rpm | Revolutions per minute |
| RT | Reverse transcription |
| RTC | Root and Tuber crops |
| SCBV | *Sugarcane Bacilliform Virus* |
| Sec | Second |
| SNP | Single nucleotide polymorphism |
| T | Thymine |
| t | Tonnes |
| $T_a$ | Annealing temperature |
| TaBCHV | *Taro bacilliform CH virus* |
| TaBV | *Taro bacilliform virus* |
| TaRV | *Taro reo virus* |
| TaVCV | *Taro vein chlorosis virus* |
| TLB | Taro leaf blight |
| Tris HCl | Tris (Hydroxy Methyl) aminomethane hydrochloride |
| U | Unit |
| V | Volt |
| v/v | volume/volume |
| w/v | weight/volume |
| Ml | Milliliter |

# INTRODUCTION

# 1. INTRODUCTION

Root and tuber crops, which comprise cassava, sweet potato, yams and aroids play an inevitable part of agriculture providing staple food for millions of people in different parts of the world. For a large number of populations in the tropics and subtropics it has now become the source of most of the carbohydrate intake. Besides this, it is also used as a raw material for industrial use and animal feed.

*Colocassia esculenta* (L.) Schott, a member of the family Araceae and commonly known as taro ranks fifth among root crops. It is the fourteenth most consumed vegetable and serves as staple source of diet for people around the world (Rao, 2010). Because of its important role in subsistence economics and crop diversification, taro has a valuable place in the world agricultural scenario. Owing to its adaptability, taro is a widely sought after vegetable. As it can develop under more adverse conditions such as poor soils, excess water, shade and extreme climate stress, taro is being cultivated in tropical and subtropical climates (Nunes *et al.*, 2012). In addition to its use as a staple food, taro has significant cultural as well as economic importance.

Almost about 130 pests and diseases in taro have been documented, and the impacts caused by them may vary, from mild to lethal. However, these pests and the diseases associated with them do not occur in all countries in the region. Majority of severe pathogens indeed have restricted distributions, occurring at very few locations (Braidotti, 2006).

Taro is being replaced by cassava and sweetpotato in many countries mainly due to the pest and disease problems (Ivanic, 1992). It largely affects taro production. Four viruses affecting taro have been identified namely *Dasheen mosaic virus* (DsMV), *Colocassia bobone disease virus* (CBDV), *Taro bacilliform virus* (TaBV), and *Taro vein chlorosis virus* (TAVCV) (Brunt *et al.*, 1990). A fifth virus has been

partially characterized and it was reported to have similarity with viruses belongs to the genus *Oryzavirus* in the family *Rheoviridae* (Devitt *et al.,* 2001). Recently, two isolates of *Taro bacilliform CH virus* (TaBCHV) were determined for the first time in China (Kazmi *et al.*, 2015).

As reported by Wagih *et al.* (1994), the taro production, however has been declining in the recent past years. Taro leaf blight (TLB), caused by the fungus *Phytophthora colocasiae Racib*, taro beetles (Papuana spp.), the Alomae – Bobone virus complex (ABVC) and declining fertility of soil etc have negative impact on yield. These impacts have contributed, in part, to the dwindling production of the crop (Sar *et al.*, 1998). Changes in the dietary habits, preferences for exotic foods, and also the introduction of many crop species with better comparative advantages like Chinese taro (*Xanthosoma sagittifolium*) and sweet potato have also had some negative influences on the taro production (Waddell, 1972; Bourke, 1982; Joughin and Kalit, 1986). The impact of these factors has unfortunately led to the loss of traditional cultivars in some parts of PNG (Wagih *et al.*, 1994).

The major effect of virus infections are severe yield reduction, decrease in the size of corm and its quality (Zettler and Hartman, 1986). The Production of planting material free from virus is very essential for effective management of viral diseases. Several viruses were reported from taro, among them only *Dasheen mosaic virus* has been studied well. The lack of data on virus diversity, occurrence, symptamatology and validated diagnostic strategies are the main challenges facing in taro studies.

Pest and pathogens, particularly plant viruses largely affect many tropical crops and the losses caused by the virus infections are usually substantial and sometimes devastating. Viruses are of particular importance, especially in the developing countries, that are heavily dependent on agricultural production mainly for food security, export earnings and employment. It affects productivity and profitability of many tropical crops. Among several viruses reported from taro, such

as members of genus Potyvirus, Badnavirus, Rhabdovirus etc., only *Dasheen mosaic potyvirus* (DsMV) has been studied very well and characterized.

The lack of data about the virus diversity, its occurence, some diseases with unknown virus aetiology along with validated diagnostic strategies etc are the main challenges mainly focusing on taro studies. Inorder to improve the productionby ensuring quality taro planting material, effective diagnostics is a pre-requisite. Developments in breeding program mainly require correct and specific identification of viruses infecting plant material. Since plant viruses are becoming widespread and there are real threats of new virus epidemics, identification and characterization of *Taro bacilliform virus* has to be done which is necessary for developing specific diagnostic techniques and formulating control strategies. Hence the specific objectives of this work were formulated as:

- To detect and identify the presence of *Taro bacilliform virus*.
- To design primers for whole genome characterization.
- Analyze the phylogenetic relationship with other badnaviruses

# REVIEW OF LITERATURE

# 2. REVIEW OF LITERATURE

## 2.1 ROOT AND TUBER CROPS (RTC) AND ITS IMPORTANCE

Tropical root and tuber crops which includes cassava, sweet potato, yams and aroids are relished as vegetables, used as a raw material for small scale industries, and consumed as staple food especially in the under developed countries (Ravi *et al.*, 1996). During the early evolution of mankind, they were critical components in the diet and the most important food crops associated with human existence, survival and socioeconomic history (Asha and Nair, 2002). Approximately 55% of root and tuber production is consumed as food and the remainder being used as animal feed or for industrial processing for different products like starch,distilled spirit etc, on a global basis. The total harvested area of root and tubers among the world is almost about 51 million hectares, with one third found in Africa and one third in Asia and the Pacific. These crops are also recognized as most efficient converters of solar energy. For the weaker section of population, tuber crops can also supply cheap source of energy (Hutabarat and Maeno, 2002).

The annual world production of rot and tuber crops is about 83.8153483 million tones (mt) consisting of potatoes (385074114 mt), cassava (270278871 mt), sweet potatoes (104453966 mt), and taro and other aroids (10232012 mt) (FAOSTAT 2014).

Root and tuber crops are found in a wide variety of production systems and it do well under various levels of management, from very low input to high input systems. This distinctive feature makes them important inorder to improve the productivity and richness of agro-systems. However, compared to other crops of equivalent economic importance, they are extremely under-researched.

## 2.2 TARO (*Colocasia esculenta* (L.) Schott)

Taro (*Colocasia esculenta* (L.) Schott) is one of the most ancient and important vegetatively propogated root crop species which belongs to the monocotyledonous family Araceae (Matthews, 2006). The family is extremely heterogenous and consists of about 110 genera with over 2500 species (Brown, 1988). In terms of consumption, after sweet potato, taro is the second most important root staple crop (Singh *et al.,* 2007) and in terms of its production by weight, taro is the fourth root crop after sweet potato, yam and cassava, with an estimated annual production of over 229,088 tonnes (Bourke and Vlassak, 2004). This ancient crop was first domesticated in Southeast Asia, it has then continued to spread throughout the world, and now an important crop in Asia, Africa, Pacific and the Caribbean. Being a crop that has been maintained by farmers for millennia the genetic resources of taro have remained largely under the control of local communities (Rao *et al.,* 2010). According to food and agricultural organization 9.1 million metric tonnes of corms are produced annually over an area of 2 million ha (Mishra *et al.,* 2009).

Among the staple vegetable crops, taro ranks fourteenth wotrldwide with about 12 million tonnes produced globally from almost about 2 million hectares with an average yield of 6.5 t/ha. Taro extends to the temperate zones of East Asia, southern Africa, Australia and New Zealand (Jianchu *et al.*, 2001). There is a diversity of cultivars adapted to wide range of microenvironment from swidden field, rain fed upland and home gardens, to paddy fields and swamps (Attri *et al.*, 2013). This taro is vegetatively propogated like most other root and tuber crops, seed production is although possible. Natural breeding as well as population spread have been reported for wild taro (Hunt *et al.*, 2013).

Due to the ease with which taro adapts to the diverse farming systems and food cultures, it has played a central role particularly in the evolution of agro-

ecosystems and helped to maintain food security in many countries. Perhaps, taro is the most widely grown among the edible aroids, is a perennial herb which consists of a cluster of smooth, heart shaped leaves, rising a foot or higher from the underground tubers. In temperate areas taro is grown as an ornamental plant for its large, glossy leaves (Nelson, 2008). Different parts of the taro plant like corms, leaves, petioles and even flowers were consumed as vegetables or used for other purposes, depending mainly 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 cornels. And *C.esculenta var. antiquorum*, referred to agronomically as the eddoe type of taro and it has small globular central corm, with several relatively large cormels arising from the corm (Purseglove, 1972).

### 2.2.1 ORIGIN AND DISTRIBUTION OF TARO

Taro (*Colocasia esculenta* (L.) Schott) is one among the ancient crops originated in the Indo-Malayan region, probably in the eastern India and Bangladesh (Yen and Wheeler, 1968) and it is now being grown throughout the tropical regions. From the Indo-Malayan center of origin, it is thought to have spread from eastward into Southeast Asia, eastern Asia, and the Pacific Islands; westward to Egypt and to the eastern Mediterranean regions; finally southward and then westward from there into Africa, and moved to Carribbean and the Americas (Chang, 1985; Yen and Wheeler, 1968). Spreading over the tropical regions, taro has great genetic diversity (Coates *et al.*, 1988). Various studies have reported two center of origin for taro-one in South-east Asia and another in Malaysia (Lebot and Aradhya, 1991; Lebot *et al.*, 2003; Kreike *et al.*, 2004; Sardos *et al.*, 2012).

Before the modern introduction of taro to Central and South American regions, it was cultivated at temperate and tropical latitudes as well as longitudes in

both the hemispheres, from West Africa to Eastern Polynesia. Thus, before the modern era of rapid international transport, taro was the world's most widely distributed starchy food crop. In many places, over a large geographical area, early cultivation as well as domestication of taro could have started in an independent manner, from India and Southern China to Papua New Guinea and Indonesia. Today apparently, the natural populations of wild taro can be found in these regions. Eventhough the species clearly had a very long and comparatively complex history in terms of domestication and dispersal, but little is known about the relationships among wild and cultivated forms of taro (Matthews, 2010).

### 2.2.2 PRODUCTION

The production of taro is estimated to be 11.8 million tones per annum worldwide (Vishnu *et al.*, 2012). Majority of the global production is contributed by the developing countries and it is characterized by the small holder production systems which mainly relies on minimal external resource input (Singh *et al.,* 2012). Comparitively, production is skewed majorly to West Africa than East Africa; eventhough taro is a crop that could be very well adapted to the different agro-ecological zones of Sub-Saharan Africa nations. West and Central African countries were contributed to almost about seventy four percent of taro production globally (FAO report 2012).

Taro production suffers low productivity and it is mainly due to the low quality planting material, low-level of value addition as well as processing (Wanyama and Mardell, 2006). In India, taro is grown throughout as it is an important vegetable and is sometimes called "potato" of the humid tropics. It is cultivated in localized pockets in different states of Uttar Pradesh, Madhya Pradesh, Odissa, Andhra Pradesh, West Bengal and Kerala.

### 2.2.3 MORPHOLOGY

Taro is an herbaceous plant, grows to a height of about 1-2 m. The plant consists of a central corm (lying just below the soil surface) from which the leaves grow upwards; root grow downwards and the cornels and runners (stolons) which 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 upto 30 cm long and 15 cm in diameter. The cormels alone can constitute to a significant proportion of the edible harvest in eddoe type of taro. The surface of each corm is marked with rings, which indicates the points of attachment of senesced leaves or scale leaves. At the nodal positions of the corm, axillary buds are present (Deo *et al.*, 2009).

Corms and cormels are quite much similar in terms of internal structure. The outermost layer is a thick brownish periderm, which covers the starch-filled ground parenchyma. Leaf laminae can be about 30 to more than 80 cm in length and from 20 to more than 50 cm in width. Leaf petioles were stout and clasping at the base. The length of petiole varies depending on the genotype; from less than 30 cm to more than about 1.5 m. The leaf size is strongly influenced by the different environmental conditions. Maximal dimensions of taro leaves are usually associated with the beginning of flowering. The leaf colour is genetically controlled and it represents one of the most useful traits inorder to describe the genotypes. Depending on the genotype, it varies from whitish yellow to very dark purple. It can be uniform or can show variations (spots, lines or blotches of different pigmentations). It is not necessary that leaf petioles and leaf laminae always have the same colour. The basic petiole colour is extremely variable and tremendous variations in the patterns (lines, dots, blotches, stripes etc.) (Lebot *et al.*, 2010).

### 2.2.4 GENETIC DIVERSITY IN TARO

The assessment of genetic diversity, prevalent in the germplasm needs an immediate attention for the better improvement of taro. Reports regarding the analysis of this crop diversity are scanty. Very few reports are only available, which uses various molecular markers to analyze the genetic diversity of taro. It includes restriction site variations in rDNA and mitochondrial DNA (Matthew *et al.*, 1992). Cytologically, though, separation of taro varieties into diploid and triploid types, there is unpredictable changes in the chromosomes during cell division and thus resulting in the uniformity of this crop (Onyilagha *et al.*, 1987). The indigenous taro germplasm in India has exhibited considerable morphological variability in over 400 accessions from different regions of the country and they have been classified into subjective morphotypes supportrd by variation in nearly 40 morpho-agronomic traits (Velayudhan *et al.*, 1993).

### 2.2.5 NUTRITIONAL VALUE

Apart from providing food security and income, taro is a nutritional source for many people. Only the true anatomical roots and the skin of the taro corm, has not been yet reported as food. Potentially, all other parts of the plant are edible including corms, petioles, blades and inflorescences. The leaves and corms are also sometimes used for medicinal purposes (Rao *et al.,* 2010). It is one among the main staple foods where the leaf as well as the underground parts are much important in the human diet (Akwee *et al.*, 2015).

As far as considering the acceptance of consumers, *C.esculenta* commonly known as taro or cocoyam is one among the important staple food of Africa, Pacific region and Asia. Taro corms are considered as an effective source for supplying nutrients since it can provide carbohydrates and potassium. As this is a carbohydrate rich tuber crop ranging in between 73 to 80 %, it represents one of the effective source of energy for more than 400 million people in many parts of the tropical and

sub-tropical region providing about a third of the food intake (Soudy *et al.*, 2010). The taro leaves can be an excellent source of carotenes, riboflavin, calcium, phosphorous, niacin, pottasium, vitamin A, vitamin C and dietary fibres (Opara, 1999).

## 2.3 CONSTRAINTS TO TARO PRODUCTION

A number of constraints hamper the increased and sustainable production of cocoyams. The major problems associated with taro production have been identified so far are the maturity (time to harvest) and pest/disease problems. Other factors include Taro leaf blight (TLB), pests as taro beetle, presence of different viruses affecting the yield, poor soil management practices and the declining fertility, lack of value addition to production along with the lack of efficient marketing systems. Of the various constraints, TLB and taro beetle are of prime importance and they reduce the yield upto 50% with reduction in corm quality (Prana, 2000).

## 2.4 DISEASES AND PESTS OF TARO

Like other root and tuber crops that are propogated through vegetatively, taro poses special problems not only in collection and storage, but also in terms of germplasm health point of view (Rodoni *et al.*, 1995). In case of vegetatively propogated crops, the viruses present in the mother plant will inevitably be found in derived vegetative parts, including tubers and roots. Among the fungal diseases, the most important is the TLB which is caused by *Phytophthora colocasiae* Rac, result in considerable crop losses. Taro planthoppers are the another major pests of taro. Viruses are one of the other important pathogens of taro, with some of the infections may resulting in severe yield reductions and ultimately leads to the death of the plant.

## 2.5 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, other viruses have been only poorly characterized and there is no confusion in the literature regarding the distribution and the associated symptoms with infection (Revill *et al.*, 2005a). Recently, in 2015 two isolates of *Taro bacilliform CH virus* (TaBCHV) has also been reported from China. Some of these viruses may occur as latent infections, and they can be found in various combinations; which means that it is very difficult to ascribe the symptoms to infections arised by individual viruses.

DsMV, an aphid transmitted virus belongs to the genus *Potyvirus* of the family *Potyviridae*. It is distributed world wide, but can be found naturally occuring only in members of the family Araceae (Zettler *et al.*, 1978; Zettler and Hartman, 1986), infecting about atleast 16 genera of the family. It affects both edible as well as ornamental aroids (Zettler and Hartman, 1986). With a typical symptom resembling that of DsMV, in India a mosaic disease on Colocasia has been reported from Thiruvananthapuram in the early 80's (Malathi and Shanta, 1981). The plants infected by DsMV usually has a conspicuous feathery mosaic pattern, eventhough the cultivars varies in the expression of symptoms. The main effect of virus infection being reduction in size of the corm and its quality, with yield loss of upto 20 % being reported. In the north eastern states of India, the occurrence of mosaic disease on *C. esculenta* has also been recorded (Swamy *et al.*, 2002).

TaBV, a putative badnavirus is transmitted by mealybugs (Gollifer *et al.*, 1977). Yang *et al.* (2003b) reported the widespread distribution of TaBV in the South Pacific Islands. Infection by TaBV alone in taro results in a range of mild symptoms which includes stunting, mosaic and down-curling of leaf blades (Jackson, 1978; Macanawai *et al.*, 2003). When it is co-infected with other virus like CBDV, it results

in lethal alomae disease (Shaw *et al.*, 1979). This alomae disease is considered as the most destructive viral disease of tro in PNG and Solomon Islands (Gollifer and Brown, 1972; Rodoni *et al.*, 1994).

*Taro vein chlorosis virus* (TaVCV) is a definitive Rhabdovirus approximately 210 x 70 nm with most similarity to members of the genus Nucleorhabdovirus. In diseased taro, TaVCV causes a distinctive veinal chlorosis and is thought to occur in Fiji, Philippines, Tuvalu, Vanuatu and possibly PNG (Pearson *et al.*, 1999). The complete gene sequence encoding the matrix and glycoproteins, and partial sequence of the L-gene (polymerase) is also known. The effect on these viruses on plant growth appears to be minimal and sometimes symptoms are commonly seen on plants only when they attain maximum growth, rather than after planting or at maturity like other taro viruses (Revill *et al.*, 2005b).

Brunt *et al.* (1990) reported CBDV as a possible Rhabdovirus because of the presence of morphologically characteristic bullet shaped or the bacilliform particles measuring about 300-335 x 50-55 nm. CBDV is spread through a plant hopper, *Tarophagus spp.* Infections in taro by CBDV alone is thought to result in a disease known as bobone, commonly characterised by stunting, distortions on leaves and presence of galls on petioles (Jackson, 1978). A combination of CBDV with TaBV is found to result in "alomae" disease (James *et al.*, 1973; Attoui, 2000), although there is still exists a considerable amount of confusion regarding the etiology of this disease.

Devitt *et al.* (2001) detected TaRV while screening for the presence of new viruses by dsRNA analysis and genome was partially characterised from taro in PNG and it is found to have greatest similarity with viruses belongs to the genus Oryzavirus in the family Reoviridae. Although the virions have not yet been purified, they are believed to be typically spherical particles, approximately having 65-75 nm in diameter. TaRV has been identified only in *Colocasia esculenta* and only in the

plants infected with atleast one or more viruses (Devitt *et al.*, 2001; Revill *et al.*, 2005a).

### 2.5.1 VIRUSES INFECTING TARO IN INDIA

Sivaprasad *et al.* (2011) reported *Groundnut bud necrosis virus* (GBNV) infecting taro for the first time. GBNV is a member belongs to the genus Tospovirus; Tospoviruses are one of the most damaging as well as economically important group of plant viruses, causing significant amount of losses in wide range of ornamental and food crops in different 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.

## 2.6 BADNAVIRUS

Badnaviruses, belongs to the family *Caulimoviridae* are characterized by non-enveloped bacilliform shaped DNA viruses having a monopartite genome containing 7.2 to 9.2 kb dsDNA. These viruses contain almost three to seven ORFs. Badnaviruses are one of the most threatening group of plant virus, emerged as serious pathogen affecting several tropical crops (Borah *et al.*, 2013). Some members of this group are known as endogenous badnaviruses, integrated into the host genome. This integration is considered to have taken place as a result of illegitimate recombination into the host genome but the presence of virus need not to be necessarily associated with virus infection. The presence of these endogenous viruses pose a serious challenge for the diagnosis, taxonomy, safe germplasm movement as well as the management of viral diseases (Hohn *et al.*, 2008)

Depending on the host and the virus species, the symptoms may vary accordingly, from mild to lethal. Badnaviruses infects both monocots and dicots,

eventhough they have a limited host range. Different species causes high economic loss in various crops and it varies between 10% and 90%. Majorly, badnavirus infect vegetatively propogated perennial hosts (Staginnus *et al.*, 2009).

## 2.7 *TARO BACILLIFORM VIRUS*

*Taro bacilliform virus* (TaBV) is considered as a Pararetrovirus belongs to the genus *Badnavirus*, family *Caulimoviridae* infecting the monocotyledonous plant taro (Purseglove, 1988; Yang *et al.*, 2003b). The complete nucleotide sequence of an isolate from Papua New Guinea comprised 7458 bp with four ORFs (*Yang et al.*, 2003a).

### 2.7.1 Symptomatology and host range

Badnaviruses infects both monocotyledonous and dicotyledonous plants and most of the species were having only limited host range. The infections caused by TaBV alone causes relatively mild symptoms like mosaic, stunting and down curling of the leaf blades (Jackson, 1978, Macanawai *et al.*, 2003). Co-infection of TaBV along with another putative rhabdovirus CBDV, is thought to result in a lethal disease called "Alomae" (Kenten, 1973). This is regarded as the most destructive viral diseases of taro in PNG and Solomon Islands. Symptoms of this alomae disease includes chlorosis, stunting, thickened leaf blades and veins and formation of irregularly shaped galls on the petioles. The infected plant leaves may sometimes fail to unfurl and naturally die as a result of systemic necrosis (Rodoni *et al.*, 1994). The asymptomatic nature as well as masking of symptoms during certain stages of plant growth is common among badnaviruses.

Vein clearing

Puckering

Backward bending of leaf blades

Chlorosis

Figure 1: Symptoms of *Taro bacilliform virus* infection on taro leaves

2.7.2 Vector transmission

TaBV is not transmitted by mechanical means, grafting or by the aphids and they have a natural host range which is restricted to taro (Brunt *et al.,* 1990). TaBV is transmitted by seeds and mealybugs (*Pseudococcus solomonensis*). There exists a possibility that the mealybug feeding may caused the test plants to activate an integrated sequence of TaBV, which cannot be excluded eventhough this is considered highly impossible. From the seed transmission studies, it is clear that this virus can be both seed borne and seed transmitted in taro like other badnaviruses like KTSV, BSV etc (Daniells *et al.,* 1995; Martin and Kim, 1987). TaBV can also be

pollen transmitted as its presence was also detected in the pollen of taro flowers eventhough the role of pollen transmission in virus is unknown (Macanawai *et al.*, 2003)

Gollifer *et al.* (1997) reported the presence of *Planococcus.citri* as a vector of TaBV, but in South pacific there are no records suggesting the infection of *P.citri* on taro and other edible aroids, despite the records that this is the most common mealybug species found in pacific area (Cox, 1981). Normally, Badnaviruses are vectored by several mealybug species including *Planococcus*, *Ferrisia Pseudococcus* etc, so there is a possibility that the vector range of TaBV extends than the previous reports (Lockhart and Olszewski, 1999).

### 2.7.3 Genomic Organization of TaBV

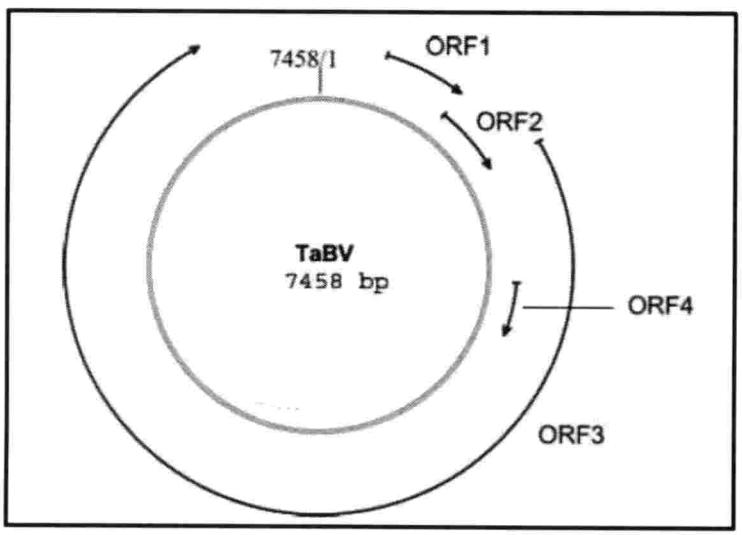



Figure 2: Genome organization of TaBV. The approximate position and sizes are indicated.

The genome of TaBV contains four ORFs on the plus strand, the strand which contains the putative tRNA $^{met}$ – binding site. It potentially encodes proteins of 16.7, 15.8, 214.4 and 13.1 kDa respectively. The organisation and size of ORFs 1-3 is similar when compared to the genome of other badnaviruses. In TaBV the ORF 1 and 2 overlap using the sequence TCATG, different from other badnavirus which uses the sequence ATGA. The main functions of gene products of ORF 1 and ORF 2 are poorly understood, while it has been postulated as the ORF 1 gene product is responsible for mealybug transmission. The start codon ATG of ORF 2 is overlapped by the stop codon TGA of ORF 1. The presence of 2 nucleotides seperates the ORF 2 and ORF 3. Within the ORF 3, ORF 4 was approximately located one fifth of the way along the ORF 3. ORF 3 encodes a putative large protein containing several motifs and they are conserved amongst badnaviruses. It includes a cysteine-rich region, zinc finger-like RNA binding motif, which is commonly found in the coat protein of virus and another cysteine rich region, whose function is unknown but uniquely conserved in badnaviruses (Yang *et al.*, 2003a).

In addition to this, other motifs are also present including Aspartic protease, RNase H, Reverse transcriptase. The gene product of ORF 3 encodes a polyprotein which is cleaved by the virus encoded enzyme, Aspartic Protease and produce proteins. This protein is mainly involved in the virus movement and its assembly as well as replication. The presence of an additional small fourth ORF encodes a putative protein of 13.1 kDa. This makes the genome organization of TaBV differ slightly from other badnaviruses The presence of ORF 4 within ORF 3 is similar to the the ORF 4 of CYMV and CSSV. There is very low sequence identity between TaBV and other badnavirus at both nucleotide and aminoacid level. In case of pararetroviruses, within the intergenic region there is a putative plant cytosolic tRNA-met binding site, which is highly conserved in the TaBV genome (Yang *et al.*, 2003).

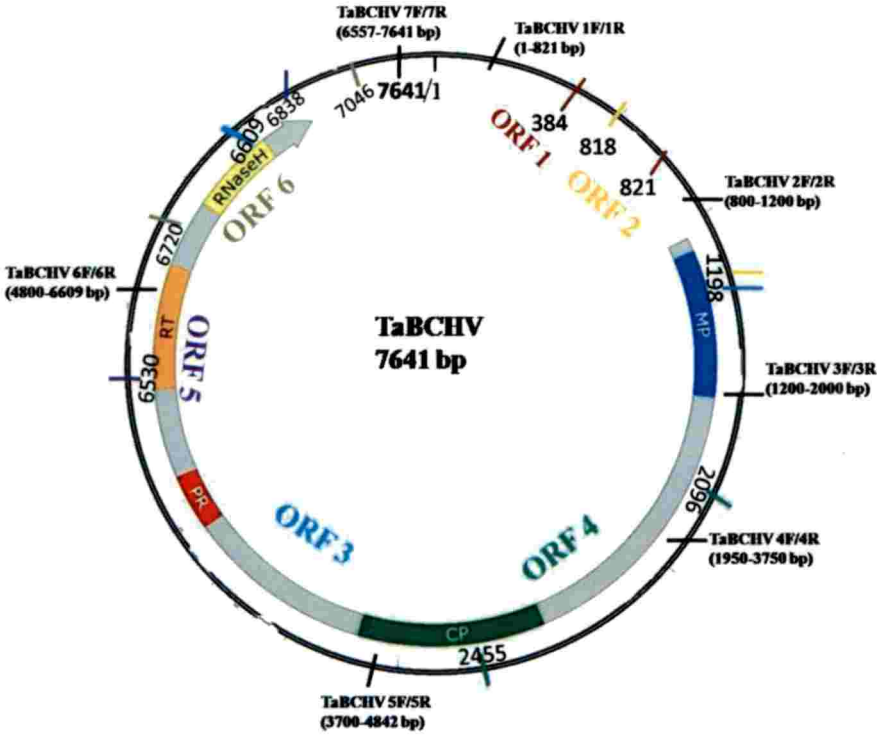
Table 1: Comparison of nucleotide & aminoacid identity between TaBV and other badnaviruses

| Virus | Size | Nucleotide Identity | Amino acid Identity | | | |
|---|---|---|---|---|---|---|
| | | | ORF1 | ORF2 | ORF3 | ORF4 |
| DBV | 7415 | 50.9 | 31.2 | 20.5 | 36.7 | - |
| CSSV | 7161 | 49.7 | 36.8 | 23.3 | 34.9 | ND |
| CYMV | 7559 | 47.0 | 34.7 | 23.2 | 35.5 | ND |
| BSV | 7389 | 48.4 | 25.2 | 14.8 | 32.1 | - |
| ComYMV | 7489 | 46.7 | 22.4 | 13.2 | 30.7 | - |
| SCBV | 7568 | 46.5 | 20.4 | 21.0 | 30.4 | - |

## 2.8 *TARO BACILLIFORM CH VIRUS*

*Taro bacilliform CH virus* (TaBCHV), a new member of badnavirus was recently reported on taro in China and it is considered as the second badnavirus infecting taro after TaBV. Symptoms exhibited includes mild feathery mosaics and brown spots. Recently, the presence of TaBCHV was reported in Hawaii, USA for the first time on taro plants infected with feather like chlorosis and mosaic symptoms (Wang *et al.*, 2017). The two isolates, TaBCHV-1 and TaBCHV-2 were having 7641 bp, within the badnavirus genome range and shared 98% overall genomic identity. With other reported badnaviruses, TaBCHV-1 and TaBCHV-2 shared 44% and 55.8% similarity with RYNV and Fig Badnavirus respectively (Kazmi *et al.*, 2015).

### 2.8.1 Genomic organization of TaBCHV




**Representative genome organization of TaBCHV**

Genomic structure of TaBCHV is different from that of TaBV. On the sense strand of TaBCHV genome, there are 6 ORFs, among which 2 ORFs (ORF5 & ORF6) were absent in the TaBV genome. Position of these two ORFs are also different from that of some other badnaviruses. All the five ORFs overlapped with each other, while ORF4 lies within ORF3. In TaBCHV, the intergenic region comprised of 981 nts and possess conserved nucleic acids like dsDNA viruses. The presence of TATA box at position (7503-7508 nt) identical to CSSV and a downstream polyadenylation signal at position (7624-7630 nt) makes the TaBCHV different from TaBV genome (Kazmi *et al.*, 2015).

associated with other virus infections. Serological and genomic heterogeneity are always present within badnaviruses and this accounts for the low sensitivity of serological detection (Johnson *et al.*, 2014). Alternative methods have to be devised inorder to identify the newly identified viruses. As a more rapid, sensitive and reliable detection methods for badnaviruses, PCR and loop mediated isothermal based assays are also reported (Bhat *et* al., 2014). Endogenous badnaviruses pose serious challenges in diagnostic testing. Due to sequence similarities used for designing of primers, an assay which detects episomal virus sequence may help to detect integrated sequences (Bhatt *et al.*, 2013).

## 2.10 METHODS OF TaBV DETECTION

Serological methods like ELISA and nucleic acid based detection like PCR is very efficient accurate in virus detection. In order to improve taro production and to ensure the quality of planting material, effective diagnoses have to be done.

### 2.10.1 Nucleic acid based technique

The most common nucleic-acid based technique is the polymerase chain reaction (PCR). First described in the 1980's by Mullis *et al.* (1986), PCR has become a powerful technique with great impact on molecular biology. It was first used for the amplification of β-globulin genomic sequences for diagnosis of sickle cell anaemia (Saiki *et al.*, 1985). But has been employed for the detection and differentiation of plant pathogens (Lopez *et al.*, 2003).

#### 2.10.1.1 Polymerase chain reaction (PCR)

The three major steps, *viz.* denaturation, annealing and extension are involved in the PCR technique. DNA is initially denatured at high temperatures (from 90-97°C). The annealing phase is the most important step and occurs at 50-60°C for 1-2 min. The extension of the primers by DNA polymerase occurs at approximately 72°C

for 2-5 min. Depending on both the DNA polymerase itself as well as the length of the fragment needed to be amplified, the time for the last step varies. As a thumb rule, 1 min is allowed for the synthesis of 1 Kb fragment. After the completion of last cycle, the samples are usually incubated for 5 min at 72°C inorder to fill in the protruding ends of the newly formed PCR products. The three stages are repeated 25-40 times in a typical PCR procedure (Joshi and Deshpande, 2010). Specific primers should be ideally 15 to 25 bases long, containing 40-60 per cent G-C with an annealing temperature of about 55°C, at which the primers anneal to the template, slightly below the melting temperature ($T_m$) (Weising *et al.*, 2005).

The method of virus detection by PCR was first published in the early 1990s (Vunsh *et al.*, 1990). Theoretically, it offered the user, the exquisite levels of virus detection in a more sensitive, specific and reliable manner by utilizing agarose gel electrophoresis for the better resolution of results (Martin *et al.*, 2000). With the advancement in the field of molecular biology, various nucleic methods such as RT and PCR began to be used for the detection of plant viruses (Hsu *et al.*, 2005).

There is considerable variability in the symptoms and infections caused by badnaviruses and transmission to the indicator plants is often difficult due to the narrow host range of these viruses. Since the symptoms are periodic and sometimes can get confused with the symptoms produced by other viruses, visual inspection of these viruses are unreliable. The serological as well as the genomic heterogeneity of the Badnaviruses is considered to be the main reason for low sensitivity of viral detection by serological methods. Hence various workers have devised alternative methods. As a more rapid and reliable detection, PCR has been used for the detection of badnaviruses infecting various crops and also vectors (Muller *et al.*, 2001). Foer the detection of some badnaviruses, Real-time PCR and loop-mediated isothermal based assays have also been recently reported (Johnson *et al.*, 2014).

Endogenous badnaviruses pose a serious challenge to the diagnostic testing of virus as mentioned above. An assay that could detect the presence of episomal virus sequences can also detect integrated sequences because of the similarity in the sequences that are used to design primers. Thus, inorder to allow the detection of episomal virus DNA alone, different techniques like IC-PCR or multiplex immuno-capture polymerase chain reaction (IC-PCR) can also be effectively used (Geering *et al.*, 2000).

# MATERIALS AND METHODS

# 3. MATERIALS AND METHODS

The study entitled "Molecular characterisation of *Taro bacilliform virus*" is carried out at the Division of Crop protection, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2016-2017. 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 the germplasm collection maintained at CTCRI and also from some Indian states. 45 leaf samples exhibiting viral symptoms (i.e. plants showing characteristic mosaic,venial chlorosis, puckering, cupping and curling of leaves) and suspected of virus infection was collected from various parts of CTCRI, Thiruvananthapuram, Regional Centre – CTCRI, Bhubaneshwar. Some samples of Xanthosoma sagittifolium (Tannia) were also collected from the CTCRI germplasm repository which exhibited severe virus infection symptoms as seen in taro. The samples were photographed, symptoms were recorded and stored. The samples were photographed, symptoms were recorded and put it in sterile polythene bags and stored at -80°C for subsequent study. The geographical origin of selected accessions were represented in table: 4

## 3.2 NUCLEIC ACID EXTRACTION

For carrying out PCR based detection of the Taro bacilliform virus infecting taro, DNA isolation is a prerequisite. PCR was performed on DNA isolated from infected samples using genus specific and virus specific primers.

### 3.2.1 Standardisation of DNA isolation protocol

Leaf samples of TaBV infecting taro plants showing symptoms were taken for DNA isolation. DNA was isolated using three protocols viz., CTAB Method I, CTAB

Method II and DNeasy Plant Mini Kit. The extraction of good quality DNA from taro was difficult owing to the presence of high amounts of polyphenol and mucilage.

### 3.2.1.1 DNA isolation using DNeasy Plant Mini Kit

Freshly collected infected taro leaf samples weighed upto 100 mg were powdered in liquid nitrogen using sterile mortar and pestle. The powdered samples were homogenized in 400 µl buffer AP1 to make a fine paste and transferred to two ml centrifuge tubes. 4 µl of RNase A solution was added and mixed 5-6 times by inversion. The homogenate was then incubated in a waterbath (ROTEK, India) set at 65°C for ten minutes with intermittent mixing. 130 µl of buffer AP2 was added and mixed. This mixture was then incubated on ice for 5 minutes. The tubes were allowed to cool down to room temperature and centrifuged at 14000 rpm for 5 minutes.The lysate was then pippetted into a QIAshredder Mini spin column in a 2 ml collection tube and then it was centrifuged for 2 minutes at 14000 rpm. Without disturbing the pellet, the flow-through fraction was transferred to a new tube. 1.5 volumes of buffer AP3/E was then added to it and mixed well by pipetting. 650 µl of the mixture was then transferred into a DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for 1 min at 8000 rpm. The flow-through was discarded and this step was repeated with the remaining sample. The spin column was again placed into a new 2 ml collection tube and 500 µl buffer AW was added. The mixture was centrifuged for 1 min at 8000 rpm and the flow through was discarded. Then transferred the spin column to a new 1.5 ml microcentrifuge tube, and 100 µl buffer AE for elution was added. Incubated at room temperature for 5 minutes. Then it is centrifuged for 1 minute at 8000 rpm and repeated the step. The DNA isolated was then stored at -20°C (Vest Frost Low Temperature Cabinet, India).

### 3.2.1.2 DNA isolation using CTAB Method I

Modified Lodhi et al. (1994) method of DNA extraction based on CTAB was used.

Freshly collected infected leaves upto 100 mg was powdered finely in liquid nitrogen using sterile mortar and pestle. A pinch of PVP and ascorbic acid was added and mixed. Before the sample thawed, 2 ml of extraction buffer (refer appendix of vinutha) pre-warmed at 65°C was added and gently homogenized. The lysate was transferred to a 2 ml tube, to which 5 µl RNase was added and mixed well by inversion. The sample was incubated in a water bath maintained at 37°C for 30 min with frequent mixing. To this, 5 µl proteinase K was added, mixed and incubated at 37°C for 30 min with intermittent shaking. The supernatant was collected followed by centrifugation at 10,000 rpm for 15 min and mixed with equal volume of chloroform:isoamyl alcohol (24:1) for 3-5 min. The chloroform:isoamyl alcohol treatment was repeated with the aqueous phase obtained after centrifugation. Isopropanol was added to 0.8 volumes for precipitating DNA followed by centrifugation at 10,000 rpm for 10 min. The resultant pellet was washed in 70 percent ethanol and centrifuged at 10,000 rpm for 5 min. After air-drying the pellet to ensure the complete removal of ethanol traces, 50-100 µl of 1X TE buffer was added to dissolve the pellet. The extracted DNA was stored at -20°C (Vest Frost Low Temperature Cabinet, India) until further use.

### 3.2.1.3 DNA isolation using CTAB Method II

The CTAB extraction method (Sharma et al., 2008a), developed for tuber crops, was adopted with slight modifications.

The extraction buffer was prepared by adding 2 percent PVP and 2 percent β-mercaptoethanol to the freshly prepared buffer preheated at 65°C. Freshly collected infected leaves weighed upto 150 mg was ground to form a fine powder in liquid nitrogen using autoclaved mortar and pestle. Before the powdered samples thawed, 2 ml of preheated extraction buffer was added and gently homogenized. The homogenate was transferred to sterile 2 ml microfuge tubes, labeled with the appropriate accession names. Five µl of RNase (10 mg/ml) was added to the above

samples and mixed gently by inversion. The samples were then incubated at 37°C for 1 h in a water bath with intermittent shaking followed by the incubation at 65°C for 30 min with frequent mixing. The homogenate was centrifuged at 10,000 rpm for 10 min and the supernatant was treated with equal volume of 24:1 (v/v) chloroform/isoamyl alcohol. The upper aqueous layer was collected after centrifuging at 10,000 rpm for 10 min and treated with 2/3$^{rd}$ volume of isopropanol. The precipitated DNA was pelletized by centrifuging at 10,000 rpm for 15 min and washed with 70 percent ethanol by spinning at 10,000 rpm for 5 min. The DNA pellets were air dried to remove the ethanol traces completely followed by suspension in 50-100 µl of 1X TE buffer (Appendix III of vinutha). The DNA suspension was stored at -20°C till further use.

## 3.3 ANALYSIS OF EXTRACTED DNA

### 3.3.1 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 0.5 µl per litre ethidium bromide (EtBr) was added. An aliquot of the DNA sample (5 µl) was mixed 1 µl 6X loading dye was loaded in each of the wells of the gel. The gel was run in a horizontal gel electrophoresis unit for 45 min at 75V. The run was stopped after the dye front reached 3/4$^{th}$ of the gel length and visualized under UV light and the image was documented using Alpha Imager gel documentation system.

### 3.3.2 Quantification of DNA

The DNA yield and purity was determined by spectrophotometric method. To quantify the DNA isolated NanoDrop$^{TM\ 8000}$ spectrophotometer readings at 260 nm were used. Sterile distilled water was used to calibrate the spectrophotometer to blank i.e. zero absorbance at the above mentioned wavelengths. Inorder to assess the DNA purity, the ratio of absorbance at 260 nm and 280 nm is taken. Generally, a ratio of

~1.8 is accepted as DNA. Purity of isolated DNA was checked at absorbance ratio of 260 nm and 280 nm (Table:5).

3.4 MOLECULAR DETECTION OF TaBV

In order to detect the Taro bacilliform virus associated with taro and characterisation of identified virus through PCR, different set of primers were used and their details were provided in the Table 2. These primers were synthesised from Eurofins (India). The synthesised primers (100 pM) were diluted to a final concentration of 10 pM with sterile distilled water to obtain the working solution.

**3.4.1 Polymerase chain Based detection**

Polymerase chain reaction (PCR) analysis was carried out with the isolated DNA using genus specific (badnavirus) and virus specific primers.

**3.4.1.2 PCR analysis with badnavirus (HafF / HafR) specific primers**

The components of the mixture were optimised as listed below:

| | |
|---|---|
| Water | : 12.2 |
| 10x Taq buffer | : 2.5 |
| 200 µm dNTP | : 1 |
| 10 pmol Forward primer | : 1 |
| 10 pmol Reverse primer | : 1 |
| Template DNA | : 2 |
| *Taq* polymerase | : 0.3 |
| Total volume | : 20 |

PCR was carried out in BioRad C100 Touch Thermocycler (Germany). PCR programme was set with 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 simplified products along with PCR marker from 'Geni, Bangalore' were separated on agarose gel (1%). The gel was viewed under gel documentation system. The amplicon showing expected band size were purified by gel elution method.

### 3.4.1.3 PCR analysis with PNG BadnaF and PNG BadnaR primers

The components of the mixture were optimised as listed below:

| | |
|---|---|
| Water | : 12.2 |
| 10x Taq buffer | : 2.5 |
| 200 µm dNTP | : 1 |
| 10 pmol Forward primer | : 1 |
| 10 pmol Reverse primer | : 1 |
| Template DNA | : 2 |
| *Taq* polymerase | : 0.3 |
| Total volume | : 20 |

PCR was carried out in BioRad C100 Touch Thermocycler (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 seperated on agarose gel (1 %). The gel was viewed under gel documentation system. The amplicon showing expected band size were purified by gel elution method.

### 3.4.1.4 PCR analysis with TaBV1 and TaBV4 primers

The components of the mixture were optimised as listed below:

| | |
|---|---|
| Water | : 12.2 |
| 10x Taq buffer | : 2.5 |
| 200 µm dNTP | : 1 |
| 10 pmol Forward primer | : 1 |
| 10 pmol Reverse primer | : 1 |
| Template DNA | : 2 |
| *Taq* polymerase | : 0.3 |
| Total volume | : 20 |

PCR was carried out in BioRad C100 Touch Thermocycler (Germany). PCR programme was set with denaturation at 94°C for 3 min followed by 50 cycles of denaturation at 94°C for 30 sec, annealing at 57.3°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 simplified products along with PCR marker from 'Geni, Bangalore' were separated on agarose gel (1%). The gel was viewed under gel documentation system. The amplicon showing expected band size were purified by gel elution method.

Table 2: Details of primers used for the detection of Badnavirus and TaBV in *Colacasia esculenta*

| Virus | Sl.No | Primers | Sequence | Ta | Amplicon size | Reference |
|---|---|---|---|---|---|---|
| **Badnavirus** | 1 | **Haf F**<br>**Haf R** | 5'ATGCCITTYGCIITIAARAAYGCICC3'<br>5'CCAYTTRCAIACISCICCCCAICC3' | 50°C | 530 bp | Yang et al., 2003 |
| | 2 | **TaBV 1**<br>**TaBV 4** | 5'CKSTGYAARSAACATGGTCTTG3'<br>5'TAATCAAGYGGWGGGAGYTTCTC3' | 57.3°C | 320 bp | Revill et al., 2005 |
| | 3 | **PNG BadnaF**<br>**PNG BadnaR** | 5'GCAAAAAGAATGGATTGATACTGAGCAG3'<br>5'CATTTTTCTTTCTCCAGTTGGGCTTAC3' | 57°C | 250 bp | Revill et al., 2005 |

## 3.5 PRIMER DESIGNING

Primers always function in pairs, the so-called forward primer and the reverse primer except in case of rapid amplification of polymorphic DNA (RAPD). The primer pairs can chosen in such a way that they will be extended towards each other inorder to cover the particular target region (Kampke *et al.*, 2001). Proper primer designing is important for applications in PCR, DNA sequencing, and hybridization. The specificity of primers to avoid mispriming, and the efficiency of primers to be able to amplify a product exponentially are the two main goals to be balanced while designing a primer (Dieffenbach *et al.*, 1993). Usually primer of 20-24 bases and GC content between 45-60 percent with $T_m$ of 52-58°C works best in most applications. The annealing temperature is generally calculated as 5°C lower than the estimated $T_m$. Within a primer pair, the GC content and $T_m$ should be well matched (Dieffenbach *et al.*, 1993; Abd-Elsalam, 2003).

Eight sets of primers were initially designed for the whole genomic characterization of *Taro bacilliform virus*. Though analysis with PNG Badna F/PNG BadnaR Primers leads to the identification of *Taro bacilliform CH virus*, another seven sets of primers for the whole genomic characterization of TaBCHV were designed. All seven pair of primers were designed in such a way it overlapped each other by few nucleotides inorder to amplify the full genome.

### 3.5.1 Primer evaluation

Prior to synthesis, the primers were subjected to in-silico analysis for determining specificity and other characteristics. The synthesized primers (100 μM) were diluted to a final concentration of 5 μM with sterile water to obtain the working solution. In order to standardize the annealing temperature of the primers, gradient PCR was performed. The DNA synthesized from symptomatic as well as non-symptomatic leaf samples were used for PCR amplificatio

Table 3: Details of TaBCHV genome specific primers designed for the molecular characterisation

| Primer name | Sequence | Position in the whole genome | Amplicon size | |
|---|---|---|---|---|
| TBCHV1F/TBCH1R | 5'TGGTATCAGAGCTTTGTTTT3'<br>3'CATGAACGAGTCCACTCTTC3' | 1-821 region | 779 bp | **Developed in this study** |
| TBCHV2F/TBCH2R | 5'AGCACATCTTGAAGAAAGTA3'<br>3'GTAGTCGAGGTTGTCATCTC3' | 800-1200 region | 400 bp | |
| TBCHV3F/TBCH3R | 5'AAAGCGAGGAAGTGTTGATG3'<br>3'AGCCTTGAAGTTCTTGGACT5' | 1200-2000 | 800 bp | |
| TBCHV4F/TBCH4R | 5'ATGAAGTCCAGAGTGTGGTA3'<br>3'GGCAGGTCCAGATTTTCAA5' | 1950-3750 | 1.81 kb | |
| TBCHV5F/TBCH5R | 5'ATGCGGCCAAGAAGGACACT3'<br>3'GCTGCAATGATAGGAACTGT5' | 3700-4842 | 1142 bp | |
| TBCHV6F/TBCH6R | 5'TCCGAATCGAAGGAGACACA3'<br>3'CGTCAGTCTCTAGGACGAT5' | 4800-6609 | 1.2 kb | |
| TBCHV7F/TBCH7R | 5'ACGCGGCAGACAACTACTGC3'<br>3'AAATAAGGCCCTTATTTTGA5' | 6557-7641 | 1065 bp | |

### 3.5.1.1 Gradient PCR for Ta optimization of TBCHV 1F / TBCHV 1R primers

It is necessary to perform gradient PCR inorder to standardize the annealing temperature for the primers TBCHV 1F and TBCHV 1R. Gradient PCR was carried out with a temperature range of **49.2°C - 59.2°C** to choose the most appropriate annealing temperature giving specific bands with minimum ambiguity. PCR was then confirmed with a particular annealing temperature within the range.

**PCR confirmation with TBCHV 1F and TBCHV 1R primers**

The components of the reaction mixture were as follows:

| | |
|---|---|
| Water | : 12.2 |
| 10x Taq buffer | : 2.5 |
| dNTP | : 1 |
| 10 pmol Forward primer | : 1 |
| 10 pmol Reverse primer | : 1 |
| Template DNA | : 2 |
| *Taq* polymerase | : 0.3 |
| Total volume | : 20 |

PCR was carried out in BioRad C100 Touch Thermocycler (Germany). PCR programme was set with denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 50.2°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 simplified products along with PCR marker from 'Geni, Bangalore' were separated on agarose gel (1%). The gel was viewed under gel documentation system. The amplicon showing expected band size were purified by gel elution method.

### 3.5.1.2 Gradient PCR for Ta optimization of TBCHV 2F/ TBCHV 2R primers

It is necessary to perform gradient PCR inorder to standardize the annealing temperature for the primers TBCHV 2F and TBCHV 2R. Gradient PCR was carried out with a temperature range of **50°C - 60°C** to choose the most appropriate annealing temperature giving specific bands with minimum ambiguity.

**PCR confirmation with TBCHV 2F and TBCHV 2R primers**

The components of the mixture were optimised as listed below:

| | |
|---|---|
| Water | : 12.2 |
| 10x Taq buffer | : 2.5 |
| 200 µm dNTP | : 1 |
| 10 pmol Forward primer | : 1 |
| 10 pmol Reverse primer | : 1 |
| Template DNA | : 2 |
| Taq polymerase | : 0.3 |
| Total volume | : 20 |

PCR was carried out in BioRad C100 Touch Thermocycler (Germany). PCR progamme was set with initial denaturation at 95 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 50.4 °C for 30 sec and extension at 72 °C for1 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 simplified products along with PCR marker from Geni, Bangaluru were separated on agarose gel (1%) the gel was viewed under gel documentation system. The amplicon showing expected band size were purified by gel elution method.

### 3.5.1.3 Gradient PCR for Ta optimization of TBCHV 3F/ TBCHV 3R primers

It is necessary to perform gradient PCR inorder to standardize the annealing temperature for the primers TBCHV 2F and TBCHV 2R. Gradient PCR was carried out with a temperature range of **50°C - 60°C** to choose the most appropriate annealing temperature giving specific bands with minimum ambiguity.

**PCR confirmation with TBCHV 3F and TBCHV 3R primers**

The components of the mixture were optimised as listed below:

| | |
|---|---|
| Water | : 12.2 |
| 10x Taq buffer | : 2.5 |
| 200 µm dNTP | : 1 |
| 10 pmol Forward primer | : 1 |
| 10 pmol Reverse primer | : 1 |
| Template DNA | : 2 |
| Taq polymerase | : 0.3 |
| Total volume | : 20 |

PCR was carried out in BioRad C100 Touch Thermocycler (Germany). PCR progamme was set with initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 45 sec, annealing at 55.1 °C for 1 min and extension at 72 °C for 60 sec. 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 simplified products along with PCR marker from Geni, Bangaluru were separated on agarose gel (1%) the gel was viewed under gel documentation system. The amplicon showing expected band size were purified by gel elution method.

### 3.5.1.4 Gradient PCR for Ta optimization of TBCHV 4F/ TBCHV 4R primers

It is necessary to perform gradient PCR inorder to standardize the annealing temperature for the primers TBCHV 2F and TBCHV 2R. Gradient PCR was carried out with a temperature range of **50°C - 60°C** to choose the most appropriate annealing temperature giving specific bands with minimum ambiguity.

**PCR confirmation with TBCHV 4F and TBCHV 4R primers**

The components of the mixture were optimised as listed below:

| | |
|---|---|
| Water | : 12.2 |
| 10x Taq buffer | : 2.5 |
| 200 µm dNTP | : 1 |
| 10 pmol Forward primer | : 1 |
| 10 pmol Reverse primer | : 1 |
| Template DNA | : 2 |
| Taq polymerase | : 0.3 |
| Total volume | : 20 |

PCR was carried out in BioRad C100 Touch Thermocycler (Germany). PCR progamme was set with initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 45 sec, annealing at 55.1 °C for 1 min and extension at 72 °C for 60 sec. 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 simplified products along with PCR marker from Geni, Bangaluru were separated on agarose gel (1%) the gel was viewed under gel documentation system. The amplicon showing expected band size were purified by gel elution method.

### 3.6 Analysis of amplicon by agarose gel electrophoresis

The most common method to analyse the PCR products is to run an aliquot of the sample on agarose gel. Aliquot of PCR mix (10µl) was loaded on agarose gel (1%) made of 1X TAE buffer. The gel was run 80 v/cm until the dyes migrated 3/4$^{th}$ of the distance through the gel. The gel was visualised and documented under the gel documentation system (Alpha Innotech) using Alpha imager software.

3.6 CHARACTERISATION OF VIRUS

### 3.6.1 Gel elution of PCR amplified fragments

Extraction of PCR products was carried out with QIAEX 11 gel extraction kit (QIAGEN). The PCR products were resolved on agarose gel (1%) and the amplicon was excised from the gel using a clean scalpel. The gel slice as placed into pre weighed 2ml tube and weight was recorded. Then, add thrice the volume QX I buffer to the gel slice. Add 30µl of QIAEX II suspension and the tube was incubated at 50°C for 10 minute occasionally inverting it every 2 min to solubilise the gel. The sample was centrifuged at 13,000 rpm, 15- 25°C for 30 s and the flow through was discarded. The pellet was washed with qx1 buffer (500µl), vortex, centrifuged for 13,000rpm, 15-25°C and the flow through was discarded. Again the pellet was washed twice with PE buffer (500µl), vortex, centrifuged for 30 s and the flow through was discarded. The sample tube was air dried for 30 min until the pellet become white. TE buffer (20µl) was added to the tube, vortexed and incubated at room temperature for 5 minute. Then it was centrifuged for 30 s at 13,000 rpm, 15-25 °C band the pellet was discarded. Finally the supernatant containing the purified DNA was stored in a clean tube at -20 °C.

The gel elute was subjected to PCR using the same reaction mix under required conditions. The products of PCR were analysed 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 analysed 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) where used for the transformation procedure. The ligation mix was prepared with the components as listed below.

Vector pTZ57R/T : 3µl

5x ligation buffer : 6 µl

PCR product : 4 µl

Nuclease free water : 16 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 1 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 resuspended the cells. After incubating the medium at 37°C with shaking for 2 h, the bacterial cells were pelleted by 1 min centrifugation. The pellet was resuspended in 300 µl of T solution and then incubated for 5 min. The cells were again pelleted by centrifugation, resuspended in 120 µl of T solution and incubated

on ice for 5 min. 2.5 µl of ligation mix or control mix was added to a new microfuge tubes and then chilled on ice for 2 min. Almost 50 µl of prepared cells were added to each of the tubes containing DNA, mixed and incubated on ice for 5 min and plated immediately on pre warmed LB Ampicillin Xgal/IPTG agar plates (appendix 8). The plates were kept overnight at 37°C for incubation. Untransformed DH5α cells were plated on 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 transformed obtained in the sample as well as positive control plate was determined.

### 3.6.3 Analysis of recombinant clones –colony PCR

The recombinant clones were analysed 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:

| | |
|---|---|
| Nuclease free water | : 14.2 µl |
| 10x Taq buffer A | : 2.5 µl |
| 200 µM dNTP | : 1.0 µl |
| 10 pmol Forward primer | : 1.0 µl |
| 10 pmol reverse primer | : 1.0 µl |
| Template DNA | : 2.0 µl |
| *Taq* polymerase (500U) | : 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 re propagation. The PCR was performed at the required PCR

condition of each primer. The products of colony PCR were resolved on an agarose gel (1%)

### 3.6.4 Plasmid isolation from transformed white colonies

Colonies which showed positive in colony PCR were selected for plasmid isolation for carrying out the downstream applications like further confirmation for the presence of inserts through restriction and sequencing.

Plasmid isolation was done using the manual method formulated as follows.

A single colony (transformed white colony) was inoculated on 2 ml LB broth with appropriate antibiotic [Here, ampicilin (50 µg/ml)] and incubated overnight with shaking of 150 rpm at 37°C. Next day, bacterial cells were pelletized by centrifuging at 10,000 rpm for 10 min at room temperature. After discarding the supernatant, the pellet was resuspended in 100 µl buffer P1 (Appendix 9(1) ) and vortexed. To this, 10 µl actvated RNase (10 mg/ml) was added and incubated for 5 min at 37°C. To this 200 µl buffer P2 (Appendix 9 (2)) was added and gently mixed by inverting 6 to 7times and incubated for 5 min. To this, 150µl P3 (Appendix 9 (3)) was added, mix gently by inverting 6 to 7 times and incubated on ice for 5 min. The mixture was centrifuged at 15,000 rpm for 30 imn. The supernatant was collected and again centrifuged at 15,000 rpm for 20 min. To the collected supernantant, 0.8 volume of isopropanol was then added and incubated on -20 °C for 1 hr. After incubation, the mixture was washed with 70% ethanol by centrifuging for 15,000 rpm for 15 min. After discarding the supernatant, the pellet was air dried, resuspende in sterile distilled water and stored at -20°C.

### 3.6.5 Confirmation of recombinant clones using restriction analysis

Restriction digestion using particular enzymes was performed for the confirmation of insert in the particular white colony

The components of the restriction mixture were optimised as listed below:

| | |
|---|---|
| Cutsmart buffer (10 X) | : 1µl |
| *Hind*III | : 0.5 µl |
| *EcoR1* | : 0.5 µl |
| Water | : 6.5 µl |
| Plasmid DNA | : 2.0 µl |
| Total volume | : 10 µl |

Restriction digestion was carried out at 37°C for 25 min followed by incubation at 65 °C for 10 min for enzyme inactivation. The restricted products along with PCR marker (1kb plus) from 'Thermo Scintific" were separated on agaroise gel (1%). The gel was viewed under gel documentation system.

### 3.6.6 DNA sequencing

Plasmids containing TaBCHV specific DNA were sequenced at Eurofins Pvt. Ltd (Bangalore). Nucleotide BLAST of the obtained sequence was performed in order to find out the similarity with the published sequences.

### 3.6.7 Sequence analysis

The Electrophoregram obtained by the sequencing was analysed and blasted using the basic alignment tools (BLASTn) in the NCBI website. The sequences having more than 80% identity were selected from NCBI and multiple sequence alignment was done using CLUSTAL W. Phlogenetic tree was constructed from Bio Edit aligned sequences using MEGA 7.0.26 analysis tool.

# RESULTS

# 4. RESULTS

The results of the study entitled "Molecular characterization of *Taro bacilliform virus* (TaBV)" conducted at the ICAR- Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram during 2016-2017 are presented in this chapter.

## 4.1 COLLECTION OF SAMPLES AND SYMPTOMATOLOGY STUDIES

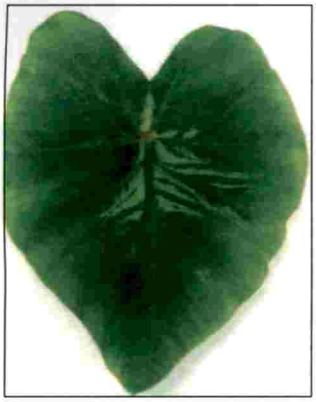
Taro leaf samples belonging to various accessions were collected randomly from the germplasm maintained at CTCRI, Thiruvananthapuram and visually analyzed. Most of the samples collected were symptomatic with mosaic; prominent whitish or faint feathery mosaics; mild vein clearing and chlorosis, curling, leaf puckering, mottling was also observed. Asymptomatic samples showed no prominent symptoms other than sometimes which seemed to be mild indistinct chlorosis or variegation. 33 leaf samples exhibiting viral symptoms were collected, serially labeled and symptomatology was recorded by visual observation. The sample details and symptoms observed are represented in Table.4 and representative samples are shown in Fig. 2. This representative sample set was used for further tests and analysis.

## 4.2 NUCLEIC ACID EXTRACTION

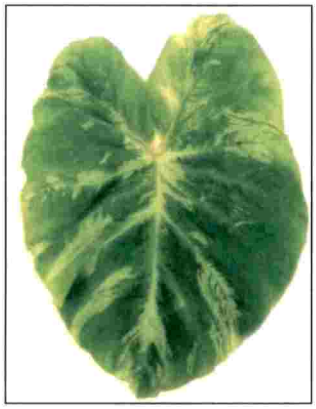
### 4.2.1 Isolation of DNA

DNA isolation from representative 33 samples were carried out using DNeasy Plant Mini Kit, CTAB method I and CTAB method II. The extracted DNA was run on an agarose gel (0.8 %) and visualised under UV to observe the bands. Comparatively more quality DNA is obtained with the third protocol. The spectrophotometric readings and the gel images of isolated DNA were as shown below:

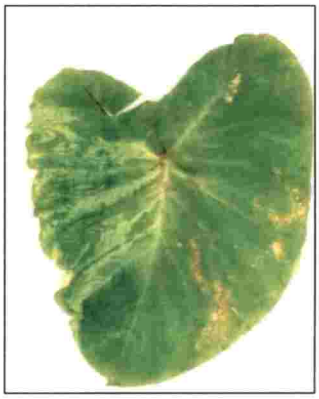
Figure 2: Representative sample set (symptom details in table. 4)



Sample 1



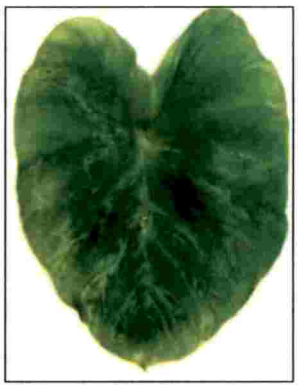
Sample 2



Sample 3

Sample 4

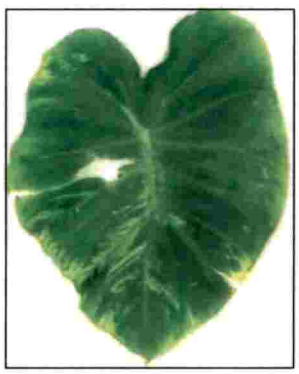
Sample 5



Sample 6

Sample 7

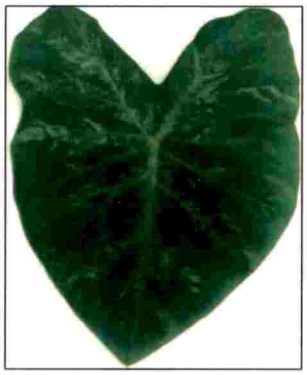
Sample 8



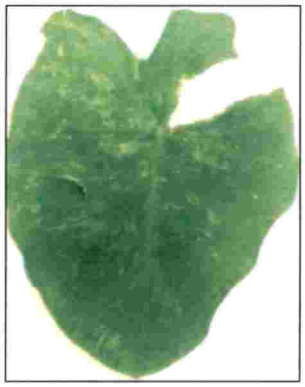
Sample 9

Figure 2: Representative sample set (symptom details in table. 4)

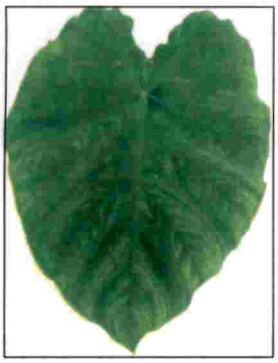
Sample 10



Sample 11



Sample 12

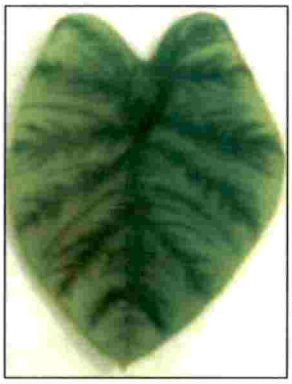


Sample 13

Sample 14

Sample 15

Sample 16



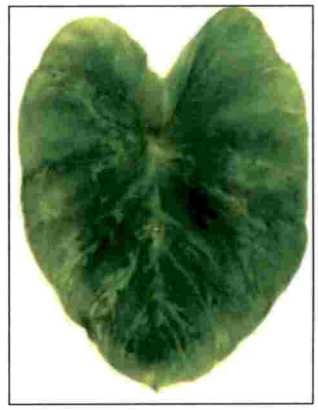
Sample 17

Sample 18

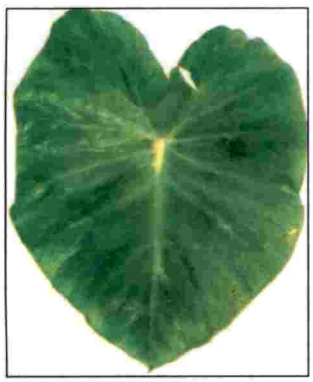
Figure 2 (continued): Representative sample set (symptom details in table. 4)

Sample 19

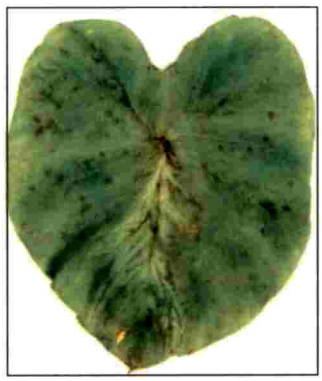
Sample 20



Sample 21



Sample 22



Sample 23

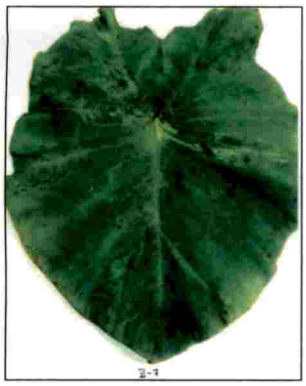
Sample 24

Sample 25

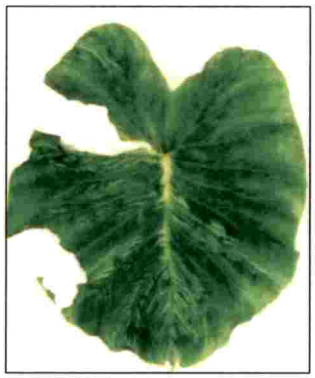
Sample 26

Sample 27

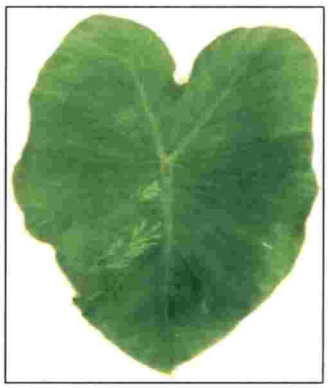
Figure 2 (continued): Representative sample set (symptom details in table. 4)



Sample 28

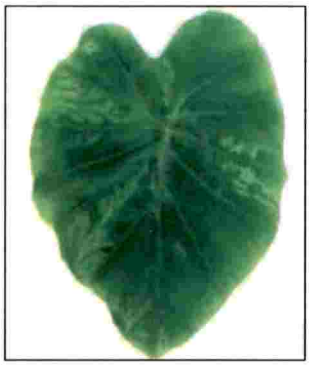


Sample 29



Sample 30

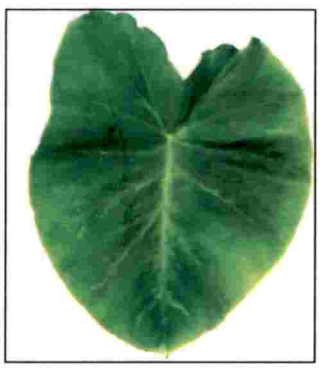
Sample 31



Sample 32

Sample 33

Sample 34

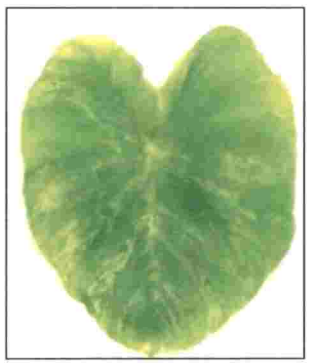


Sample 35

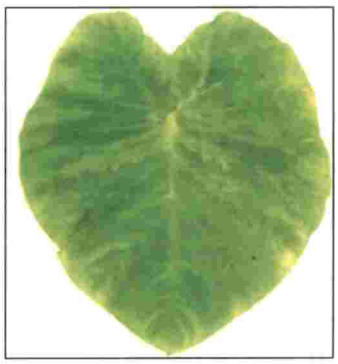
Sample 36

Figure 2 (continued): Representative sample set (symptom details in table. 4)

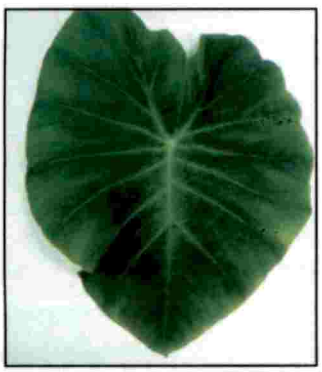
Sample 37



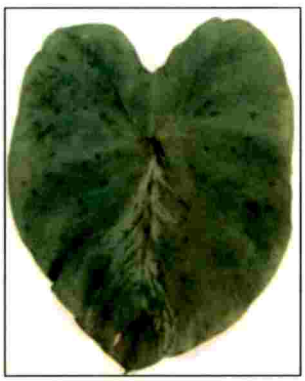
Sample 38



Sample 39



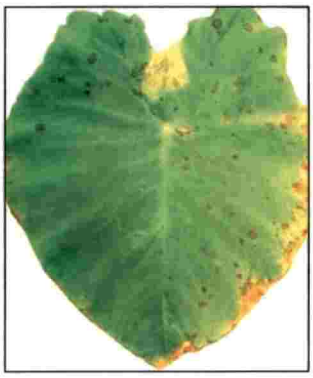
Sample 40



Sample 41

Sample 42

Sample 43



Sample 44

Sample 45

Table 4: Representative sample set, symtoms observed

| Sl. No | Sample ID | Symptoms |
|---|---|---|
| 1 | Line-21 | Mosaic and indistinct chlorosis on the margin |
| 2 | Jhankri (Bhu krpa) | Severe feathery mosaic along the veins |
| 3 | TCR 819 | Slightly distorted leaf margin, feathery mosaic |
| 4 | TCR 545 | Mosaic along midrib & in some veinal region |
| 5 | TCR 389 | Irregular feathery mosaic |
| 6 | IC087165 | Irregular mosaic & indistinct chlorosis on the margin |
| 7 | U-57 | Severe feathery mosaic, distorted leaf margin |
| 8 | 482-1 | Mosaic, presence of black spots |
| 9 | RNC A1 | Mosaic along some veins and leaf margins |
| 10 | D-6 | Feathery mosaic along the veins and margins |
| 11 | IC089624 | Mild feathery mosaic symptoms |
| 12 | JASM 11 | Mild mosaic, formation of yellow leaf spots |
| 13 | NEH-8 | Symptomess, leaf variegation |
| 14 | 464 | Feathery mosaic, chlorosis |
| 15 | NEH-6 | Indistinct mosaic throughout the leaf , chlorosis |
| 16 | DT-2 | Feathery mosaic along the veins |
| 17 | TCR 276 | Symptomless, leaf variegation |
| 18 | 294 | Severe puckering and mosaic |
| 19 | NEH-32 | Mosaic, chlorosis and slight puckering |
| 20 | Line-46 | Mild mosaic , chlorosis |
| 21 | NEH-122 | Mosaic, feathery like symptoms throughout veins |

Table 4 (continued): Representative sample set, symtoms observed

| Sl. No | Sample ID | Symptoms |
|---|---|---|
| 22 | RNC A1 | Slightly chlorotic and mosaic symptoms |
| 23 | Line-55 | Chlorosis& feathery mosaic |
| 24 | Line-42 | Severe feathery mosaic, slight distorted leaf margin |
| 25 | RNC A1 | Mosaic symptoms |
| 26 | Line 33 | Feathery mosaic and chlorosis |
| 27 | RNC A1 | Chlorosis and mosaic |
| 28 | Line-14 | Slight puckering, chlorosis |
| 29 | TCR 683 | Distorted leaf and mild mosaic symptoms |
| 30 | 628 | Mild feathery mosaic |
| 31 | 524 | Severe mosaic infection throufhout the leaf blades |
| 32 | Line-33 | Feathery mosaic symptoms in veins |
| 33 | H-45-75 | Chlorosis on the margins and slightly distorted leaf |
| 34 | Line 21 | Chlorosis on the leaf marg, feathery mosaic |
| 35 | RNC A1 | Mild feathery mosaic on veins |
| 36 | RNC A1 | Leaf deformations and chlorosis on the margin of leaf |
| 37 | Line 36 | Mild feathery mosaic |

Table 4 (continued): Representative sample set, symtoms observed

| Sl. No | Sample ID | Symptoms |
|---|---|---|
| 38 | NEH 122 | Mosaic symptoms throughout the leaf |
| 39 | MNSM/14-1 | Chlorosis and mosaic |
| 40 | 464 | Leaf deformations and severe vein chlorosis |
| 41 | 218 | Mild mosaic |
| 42 | Line 46 | Feathery mosaic symptoms |
| 43 | Line 43 | Mosaic symptoms |
| 44 | DT-1 | Brown spots and deformations |
| 45 | Line 33 | Symptomless |

Table 5: Yield and absorbance of DNA isolated by three methods

| Sample ID | Methods of DNA isolation | | | | | |
|---|---|---|---|---|---|---|
| | DNeasy Plant Mini Kit | | CTAB method I | | CTAB method II | |
| | DNA yield | Absorbance (A260/A280) | DNA yield | Absorbance (A260/A280) | DNA yield | Absorbance (A260/A280) |
| JASM (11-1) | 60 | 2.22 | 80 | 2.07 | 1190 | 2.00 |
| TCR 545 | 400 | 2.32 | 40 | 2.20 | 1000 | 2.34 |
| RNC A1 | 55 | 2.00 | 65 | 1.33 | 980 | 0.34 |
| I-16 | 800 | 2.04 | 30 | 0.77 | 560 | 2.12 |
| II-3 | - | 0.22 | 40 | 2.33 | 660 | 0.88 |
| IC 7056875 | - | 0.13 | - | - | 1230 | 0.32 |

Table 6: Quantification of DNA of representative sample set (CTAB method II)

| Sl.No | Sample ID | Amt. Of DNA (µg/ml) | Purity ($A_{260}/A_{280}$) |
|---|---|---|---|
| 1 | TCR 819 | 366.4 | 1.88 |
| 2 | TCR 545 | 499.5 | 2.11 |
| 3 | TCR 389 | 1120 | 1.42 |
| 4 | IC087165 | 890 | 1.66 |
| 5 | U-57 | 129 | 2.00 |
| 6 | 482-1 | 445.3 | 1.76 |
| 7 | RNCA1 | 565.3 | 2.35 |
| 8 | D-6 | 1340 | 2.17 |
| 9 | ICO89624 | 150 | 2.08 |
| 10 | JASM 11 | 1680 | 2.43 |
| 11 | NEH-8 | 330 | 2.13 |
| 12 | NEH-6 | 667 | 1.89 |
| 13 | DT-2 | 990 | 2.08 |
| 14 | TCR 276 | 1100.5 | 2.30 |
| 15 | Line-55 | 235.5 | 1.34 |
| 16 | Jhankri (Bhu Kripa) | 1221.5 | 2.21 |
| 17 | TCR 873 | 267.5 | 2.00 |
| 18 | TCR 683 | 1046.1 | 2.28 |
| 19 | NEH-34 | 110 | 1.22 |
| 20 | DT-1 | 180 | 2.3 |

Table 6: Quantification of DNA of representative sample set (CTAB method II)

| Sl.No | Sample ID | Amt. Of DNA (µg/ml) | Purity ($A_{260}/A_{280}$) |
|---|---|---|---|
| 21 | 557 | 267 | 2.33 |
| 22 | DT-3 | 220.0 | 1.99 |
| 23 | Line-14 | 1124.5 | 2.06 |
| 24 | Line-42 | 660.8 | 2.23 |
| 25 | TCR 276 | 786.1 | 2.0 |
| 26 | 294 | 235 | 2.12 |
| 27 | NEH 32 | 1056 | 2.00 |
| 28 | Line-46 | 670.3 | 2.12 |
| 29 | NEH-122 | 220 | 1.78 |
| 30 | Line-55 | 1309 | 2.31 |
| 31 | Line 43 | 1240.2 | 0.13 |
| 32 | Line-33 | 988 | 0.22 |
| 33 | H-45-75 | 560.3 | 2.16 |
| 34 | Line 21 | 450 | 1.89 |
| 35 | RNC A1 | 1200 | 2.23 |
| 36 | RNC A1 | 120 | 1.90 |
| 37 | Line 36 | 540 | 2.33 |
| 38 | NEH 122 | 400 | 1.05 |
| 39 | MNSM/14-1 | 1420 | 2.22 |
| 40 | 464 | 1300 | 0.32 |

Table 6: Quantification of DNA of representative sample set (CTAB method II)

| **Sl.No** | **Sample** | **Amt. Of DNA (µg/ml)** | **Purity ($A_{260}/A_{280}$)** |
|---|---|---|---|
| 41 | 218 | 980 | 0.87 |
| 42 | Line 46 | 870 | 1.03 |
| 43 | Line 43 | 650.3 | 2 |
| 44 | DT-1 | 760.3 | 2.32 |
| 45 | Line 33 | 450.2 | 2.01 |

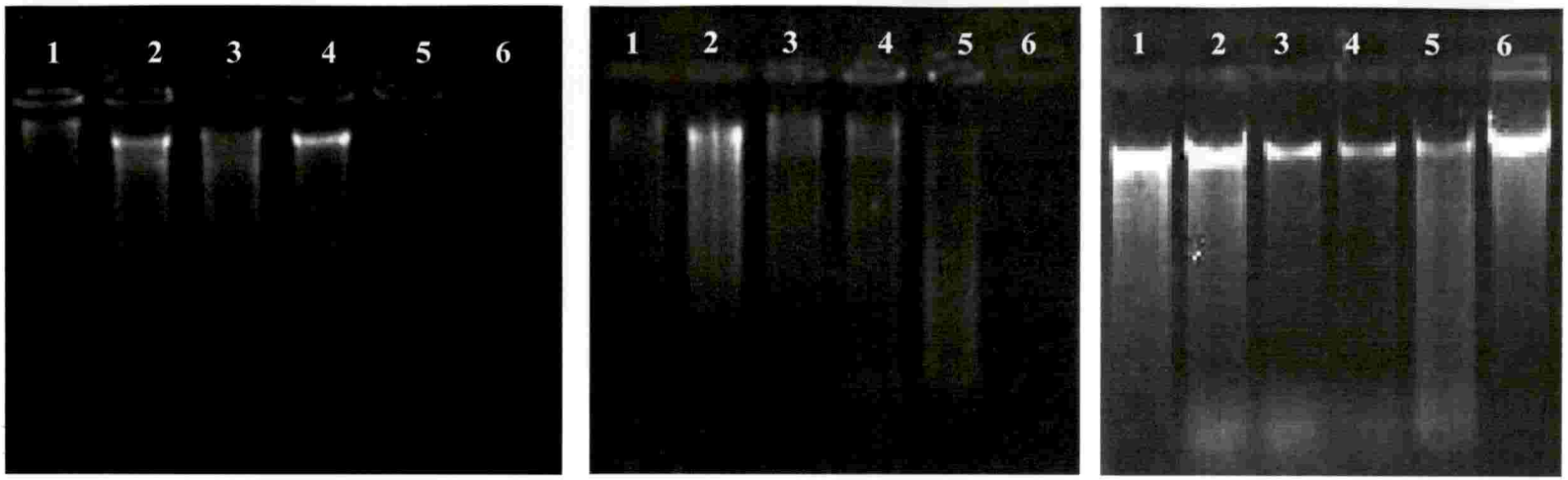



Fig 3: Gel profile of DNA isolated by A) DNeasy Plant Mini Kit B) CTAB method I C) CTAB method II

Lanes: JASM 1, 2= TCR 545, 3= RNC A1, 4=I-16, 5=II-3, 6=IC7056875

## 4.3 MOLECULAR DETECTION OF VIRUSES

Polymerase chain reaction was employed as a part of molecular detection and diagnosis. Group specific and virus specific primers were used for the detection of *Taro bacilliform virus* infecting taro. Primers were got synthesised from Eurofins, India.

### 4.3.1 PCR for detection of badnavirus

Detection of badnavirus infection was carried out using badnavirus group specific primers (Table 3) providing corresponding PCR conditions. A non template was used as control template was used as control having all components of a typical PCR but no template DNA. PCR was done for the representative 45 samples for detection of badnaruses. Band representing the amplicon size 530 for HafF/HafR were observed for virus positive samples in agarose gel (1%) electrophoresis. No amplification was observed in non template control, which indicates there is no non-specific binding in PCR.

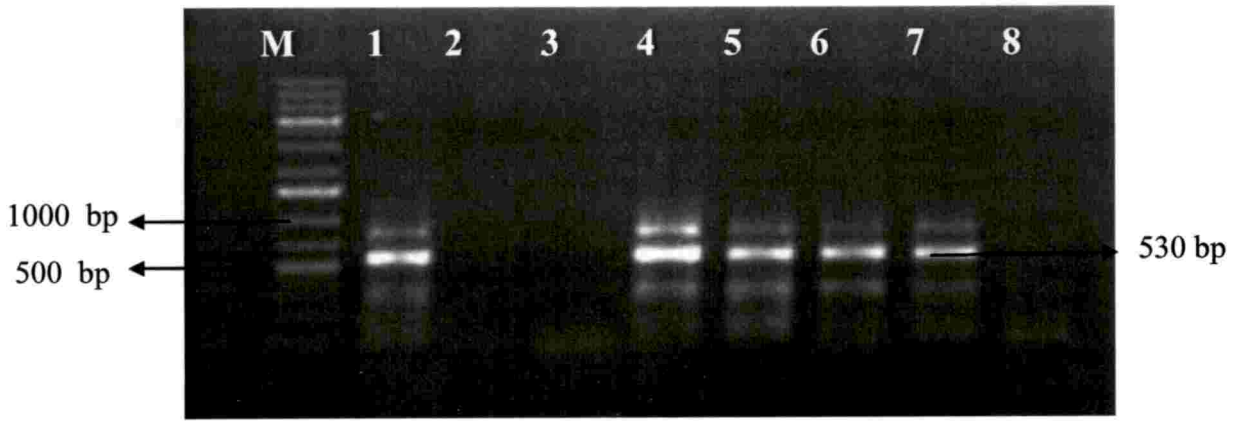



Figure 4: Sample showing badna positive for HafF/HafR primer

M=1kb plus DNA ladder

Lanes: 1=RNC A1, 2=II-4, 3=II-8, 4=RNC A1, 5=TCR 545, 6=II-3, 7=I-2, 8=NTC

### 4.3.2 PCR for detection of TaBV

Detection of TaBV infection was carried out using TaBV specific primers (Table 3) providing corresponding PCR conditions. A non template was used as control template was used as control having all components of a typical PCR but no template DNA. PCR was done for the representative 45 samples for detection of badnaruses. Band representing the amplicon size 250 bp for PNG BadnaF/PNG BadnaR were observed for virus positive samples in agarose gel (1%) electrophoresis. No amplification was observed in non template control, which indicates there is no non-specific binding in PCR.

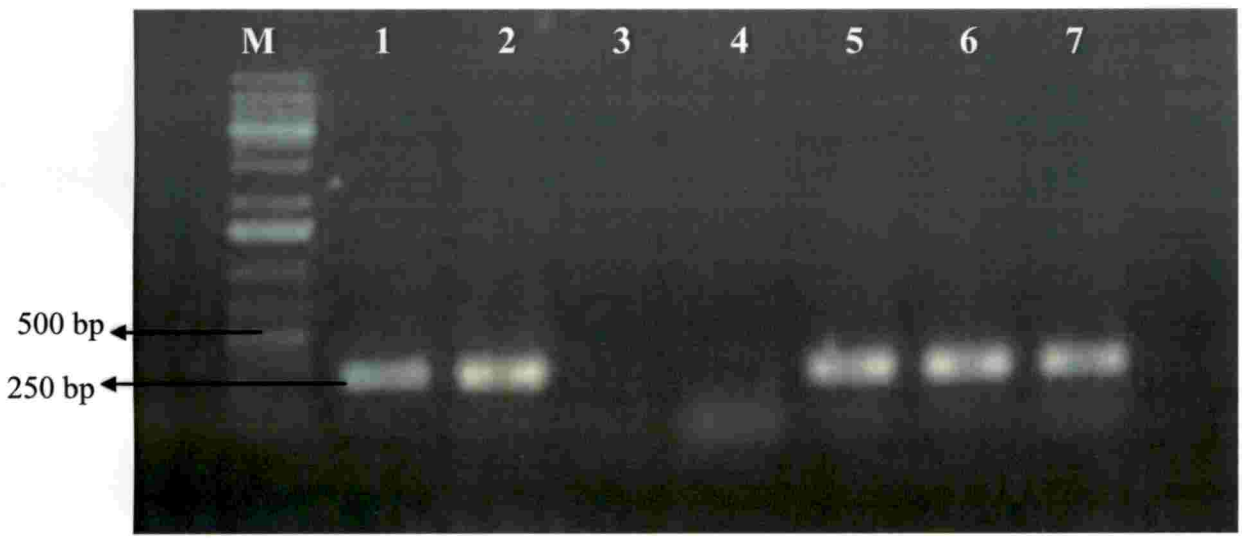



Figure 5: Amplification of TaBV RT/RNase H region with PNG BadnaF/PNG BadnaR

M=1kb plus DNA ladder
Lane 1=RNC A1, 2= RNC A1, 3=II-8, 4=II-4, 5=TCR 545, 6=II-3, 7=I-2, 8=NTC

### 4.3.3 Primer designing

From the NCBI nucleotide database, available nucleotide sequence of TaBCHV whole genome were taken in FASTA format and forward (F) and reverse (R) primers were designed for the whole genome amplification of TaBCHV. Seven sets of primers

#### 4.3.3.1 Gradient PCR analysis


Fig 6 : Gradient PCR with TaBCHV 1F/TaBCHV 1R

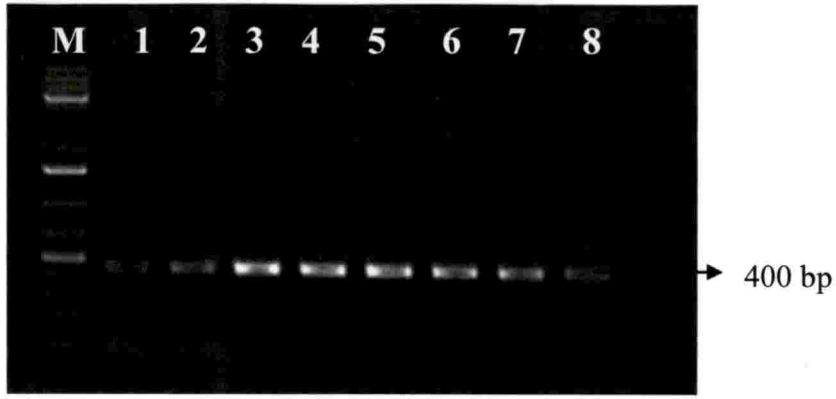



Fig 7: Gradient PCR with TaBCHV 2R/TaBCHV 2R

M-1 kb plus DNA ladder, Sample-RNC A1

Fig 8: Lanes: 1=49.2°C, 2=50.1°C, 3=51.5°C, 4=53.2°C, 5=55.5°C, 6=57.3°C, 7=58.5°C, 8=59.2°C

Fig 9: Lanes: 1=49.2°C, 2=50.1°C, 3=51.5°C, 4=53.2°C, 5=55.5°C, 6=57.3°C, 7=58.5°C, 8=59.2°C

**4.3.3.1 (continued): Gradient PCR analysis**

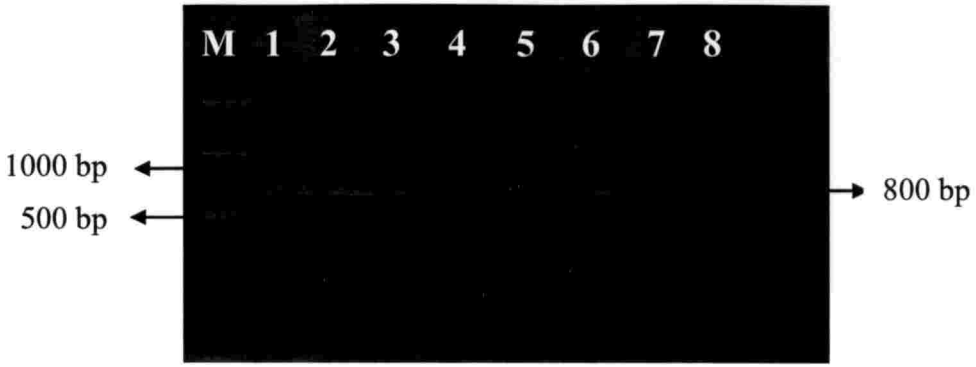



Fig 8: Gradient PCR with TaBCHV 3F/TaBCHV 3R

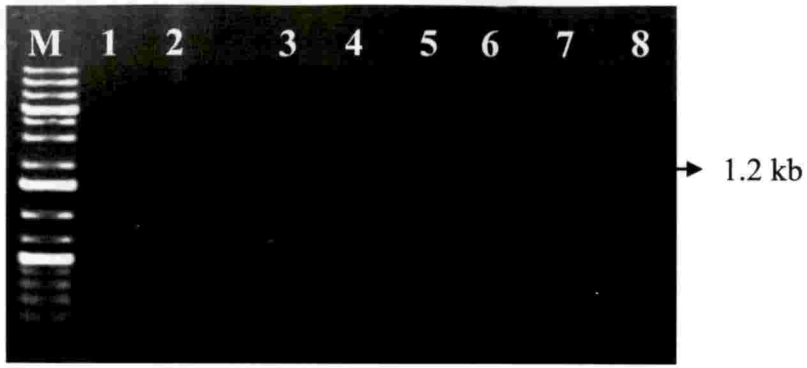



Fig 9: Gradient PCR with TaBCHV 4F/TaBCHV 4R

M-1 kb plus DNA ladder, Sample-RNC A1

Fig 10: Lanes: 1=50.3°C, 2=51.2 °C, 3=52.6°C, 4=54.3°C, 5=56.6°C, 6=58.4°C, 7=59.6°C, 8=60.3°C

Fig 11: Lanes: 1=49.9°C, 2=50.8°C, 3=52.2°C, 4=53.9°C, 5=56.2°C, 6=58°C, 7=59.2°C, 8=59.9°C

were designed for the whole genomic characterization in such a way it overlapped each other by few nucleotides inorder to cover the full genome.

### 4.3.4 PCR confirmation with virus specific primers

Virus specific primers are used for the characterization of TaBCHV infecting taro. These primers amplify the specific regions covering the whole genome of TaBCHV genome

#### 4.3.4.1 PCR analysis with TaBCHV 1F/TaBCHV 1R primer

Detection of TaBCHV infection in representative samples were carried out using TaBCHV 1F/TaBCHV 1R specific primers which amplifies the region from 1-821 bp which gave product of 779 bp. A non template having all the components of a typical PCR was used as negative control. PCR conditions were mentioned in 3.4.3. An amplification size 779 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 there is no non-specific binding in PCR.

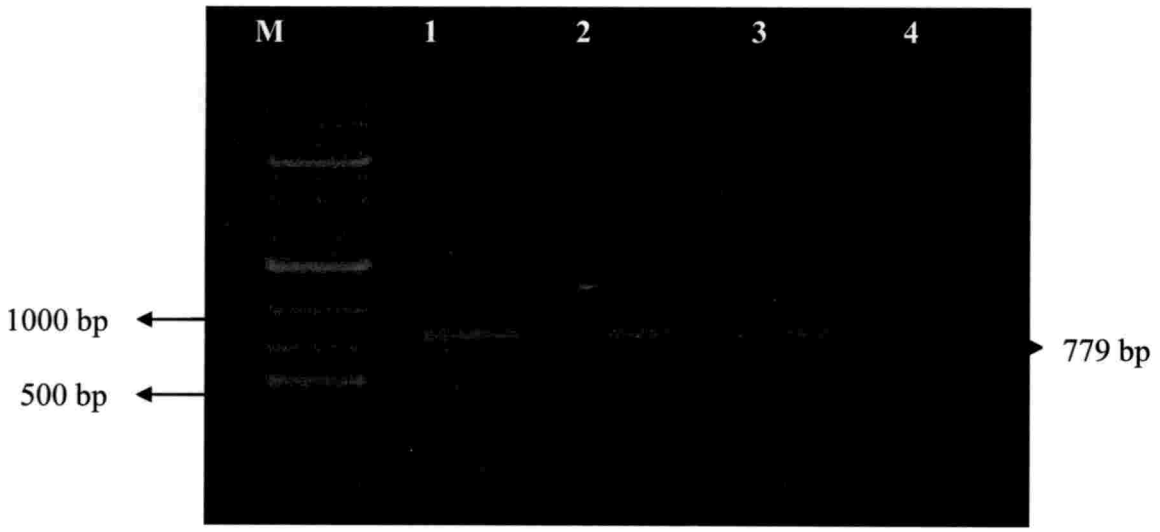



Figure 10: Amplification of TaBCHV with TaBCHV 1F/TaBCHV 1R primer

M=1 kb plus DNA ladder

Lanes: 1=RNC A1, 2=TCR 545, 3= NTC

### 4.3.4.2 PCR confirmation with TaBCHV 2F/TaBCHV 2R primer

Detection of TaBCHV infection in representative samples were carried out using TaBCHV 2F/TaBCHV 2R specific primers which amplifies the portion from 800-1220 bp which gave product of 400 bp. A non template having all the components of a typical PCR was used as negative control. PCR conditions were mentioned in 3.4.3. An amplification size 400 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 there is no non-specific binding in PCR.

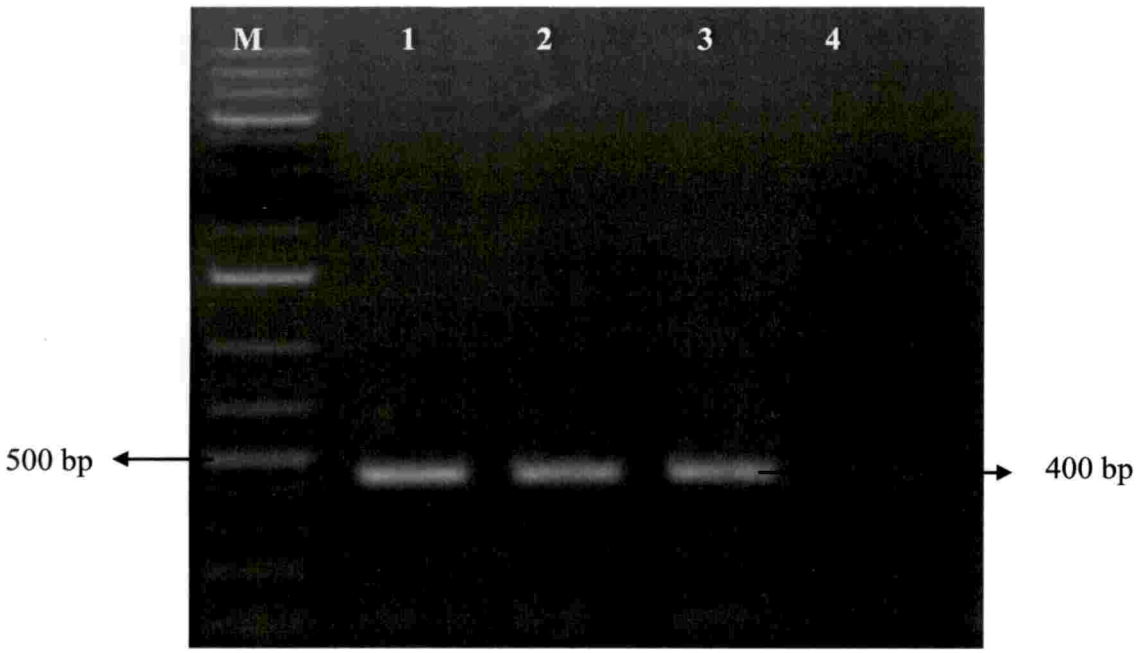



Figure 11: Amplification of TaBCHV with TaBCHV 2F/TaBCHV 2R primer

M=1 kb plus DNA ladder

Lanes: 1=RNC A1, 2=TCR 545, 3=JASM 11, 4=NTC

### 4.3.4.3 PCR confirmation with TaBCHV 3F/TaBCHV 3R primer

Detection of TaBCHV infection in representative samples were carried out using TaBCHV 3F/TaBCHV 3R specific primers which amplifies the portion from 800-1220 bp which gave product of 800 bp. A non template having all the components of a typical PCR was used as negative control. PCR conditions were mentioned in 3.4.3. An amplification size 800 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 there is no non-specific binding in PCR.

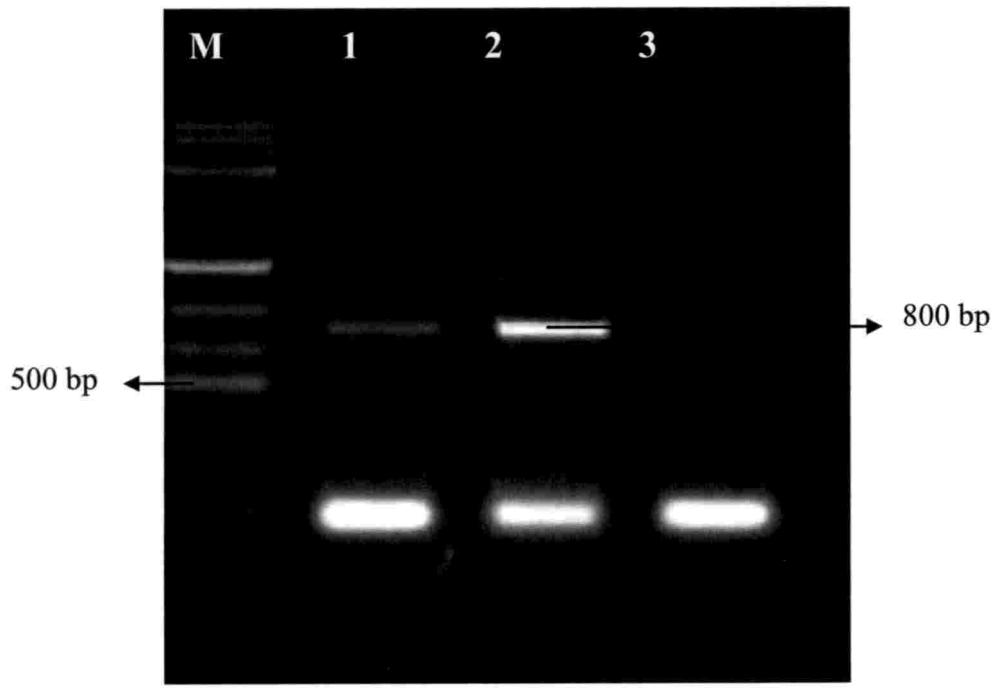



Figure 12: Amplification of TaBCHV with TaBCHV 3F/TaBCHV 3R primer

M=1 kb plus DNA ladder

Lanes: 1=RNC A1, 2=TCR 545, 3= NTC

4.4.2 Analysis of recombinant clones

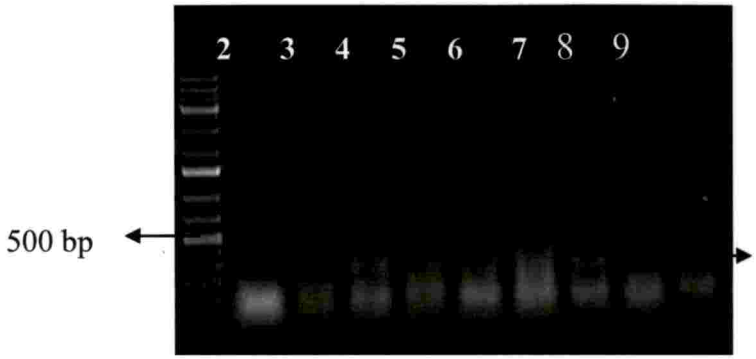



Figure: 14


Figure: 15

Fig 14: Amplification of recombinant clones with PNG BadnaF/PNG BadnaR primer showed amplicon size of 250 bp in Lanes 3,4, 7

Fig 15: Amplification of recombinant clones with TaBCHV 1F/TaBCHV 1R primer showed amplicon size of 779 bp in Lanes 1-3 and 5-14.

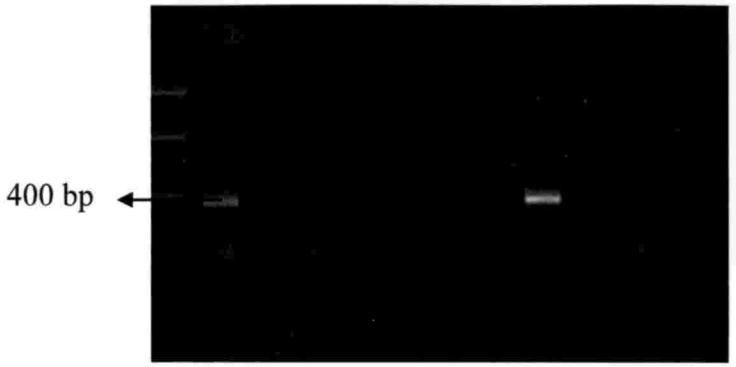



Figure: 16


Figure: 17

Fig 16: Amplification of recombinant clones with TaBCHV 2F/TaBCHV 2R primer showed amplicon size of 400 bp in Lane 1, 3, 7

Fig 17: Amplification of recombinant clones with TaBCHV 3F/TaBCHV 3R primer showed amplicon size of 800 bp in Lane 1, 6, 8, 9.

4.4.3 Plasmid isolation

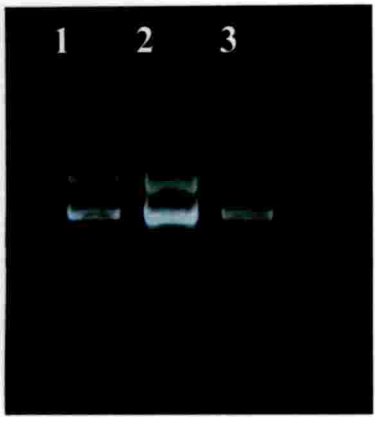



Figure: 18


Figure: 19

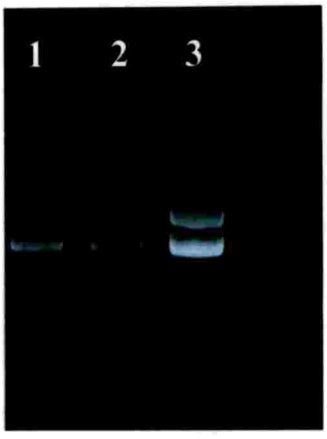



Figure: 20

Fig 18: Isolated plasmid from TaBCHV 1F/TaBCHV 1R positive recombinant clones

Fig 19 : Isolated plasmid from TaBCHV 2F/TaBCHV 2R positive recombinant clones

Fig 20: Isolated plasmid from TaBCHV 3F/TaBCHV 3R positive recombinant clones

4.4.4 Restriction analysis of PNG Badna F/PNG BadnaR primers

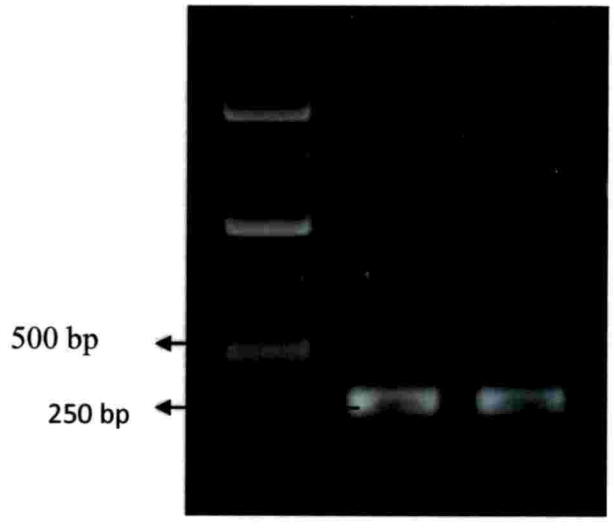



Figure 21: Restriction digestion of *E.coli* plasmid

M =1kb plus DNA ladder
Restriction enzymes = *EcoRI* and *HindIII*
Lane 1 and 2 represents the restricted plasmid of PNG Badna positive clones

## 4.4 CHARACTERISATION OF THE VIRUSES

Cloning and transformation was employed as a part of molecular characterization of virus and further sequence analysis was done for the confirmation.

### 4.4.1 **Cloning and transformation**

The amplicons of 250 bp confirmed for badnavirus and amplicons of 779 bp, 400 bp, 800 bp for TaBCHV 1F/TaBCHV 1R, TaBCHV 2F/TaBCHV 2R and TaBCHV 3F/TaBCHV 3R respectively, confirmed for TaBCHV genome specific primers was proceeded with cloning for better sequencing results. The eluted amplified products where 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 (Fig. 10). Grid plates were prepared to maintain the transformed white colonies.The white recombinant colonies were selected for further analysis using colony PCR (Fig. 18) using respective primer pairs under appropriate conditions and they were analysed in 1% agarose gel. The positive clones were used for plasmid isolation. The insertion of gene of interest is confirmed by restriction release of the isolated plasmids using restriction enzymes *(EcoR1* and *Hind111*) for amplicon of 250 bp and colony PCR for others. The positive clones were sent to Eurofins for DNA sequencing, with their specific primers. The sequencing results were obtained as electrophoregram.


Figure 13: Colonies obtained for transformation using PCR products in LB-AX1 plate

## 4.5 SEQUENCE ANALYSIS

### 4.5.1 Sequence analysis of PCR products

The positive plasmid clones were sent to Eurofins for DNA sequencing, with the specific primers. The sequencing results were obtained as electrophoregram. From the sequencing results, it was found that the sequences showed 100% similarity with new virus, TaBCHV. These sequences were used for further analysis and are listed below.

Table 7: NCBI BLAST results of inserts sequences

| Virus | Sequence Identity | Accession Number |
|---|---|---|
| TaBCHV -1 | 91% | KP710178 |
| TaBCHV-2 | 89 % | KP710177 |
| DBV (isolate NG3Da) | 75% | AM944573 |
| DBV (isolate NG2Da) | 74 % | AM944572 |

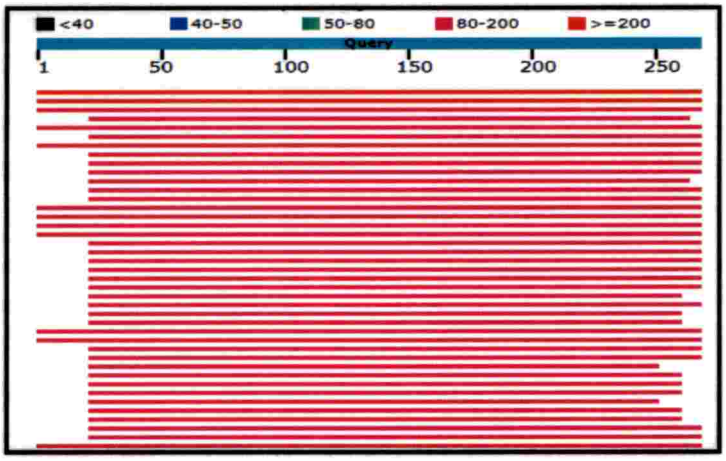



Figure 22: Blast analysis result for PNG Badnaf/PNG Badna R primer

The sequence has 91% identity with TaBCHV isolate TaBCHV-1. The sequence obtained is given below (250 nt):

ATGGATTGATACTGAGCAGCTCAAAAATGAAAATTGCAGTACCAACCATTGA
CTTCCTAGGAGCCACAATAGGTAACCGCAAAATAAAATTGCAGGAGCACATA
ATAAAGAAAATTGCGGATTACCCTGAGAAAGAACTCATGAACACAAAGGGA
TTAAGATCATGGCTAGGAATCCTAAACTACACCAGGAGTTACATCCCTTACCT
GGGTAAACAATTAGGCCCATTATACTCCAAGGTAAGCCCAACTGGAGAAAGA
AAAATG

Table 7: NCBI BLAST results of insert sequences

| Virus | Sequence Identity | Accession Number |
|---|---|---|
| TaBCHV -1 | 94% | KP710178 |
| TaBCHV-2 | 92 % | KP710177 |

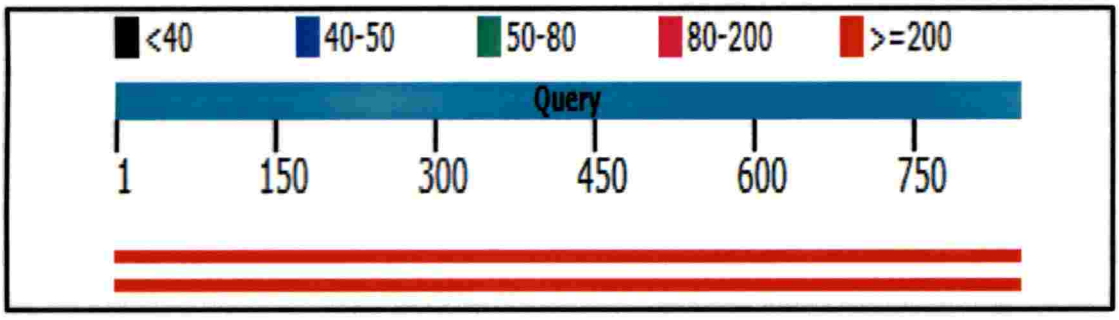



Figure 23: Blast analysis result for TaBCHV 1F/TaBCHV 1R

Sequences obtained for TaBCHV 1F/TaBCHV 1R has 94 & 92% identity with TaBCHV isolates TaBCHV-1 and TaBCHV-2 complete genome. The sequence obtained is given below (779 nt):

CGGTATCGAAGCTTTGTTTCTACTTTCTCCTGGTCGTGCTCTACGCTCTTTAGC
TTCTTTTCTATGCCTGCCTTGACTGGATCCGCTGGTCTGACCTGATTATTGGTC
GTGCCGTCCGCCCCTTACTGATGACATGAATATCGGAACTCGAGCTATAGTCT
TCAAAGACTTGGATAAGGATTTGAAAATTCTGGAACTTACGAATGCCCTCTC
ACTTTTCTGACCCAATGCTTCAGGAATATCGAGTTGGACTTGTCCATTGAGAA
ATGAACTCAGCTAGACTAGGATAAGAAATCGGTCGAAAGAGATGTTGAGTCA
GGAAAATCTTGCGGGTCCGAGTCTTTTTCTTCTGGAATTTTAATGAGTGATAG
ATGGGAAAATGCTGTACAAGAATGAAATTCATCCTTGACTGCCATTCTTGCGT
CCCTAAATCTATCACCTCCACAGCATAAAAGGCCATCCCAGAAAGAATTATT
CCTGACTCTATCAGTTGTTTATGATCATGTCTGCTCATCTACCACATACTGTCT
CAAAAACTTCAAGTCAGTTCTTGAACCACATGCCGTTCTTAAGTCAGATACTG
CATAGCTCATGTATAAGCTCTGTACCTACTTCATTTATTCTTGGTTTGCCACTT
CGATTACTAGATTCCGAACTGCATGCTCCTGTGAATCAAATACTTGCACAACC
TTATCTGGTGAAATAACGTCCGATCCAGATCTCTTTGAACTGGAAAAGAAGC
TCTACCGATTGAAGCACATCCTGATTATAGTAGAATAGTGACTCGTTCATGAC
TCTAGACGCATTCTCGA

Table 8: NCBI BLAST results of inserts sequences

| Virus | Sequence Identity | Accession Number |
|---|---|---|
| TaBCHV -1 | 99% | KP710178 |
| TaBCHV-2 | 96% | KP710177 |
| Hibiscus bacilliform virus | 72% | KF875586 |

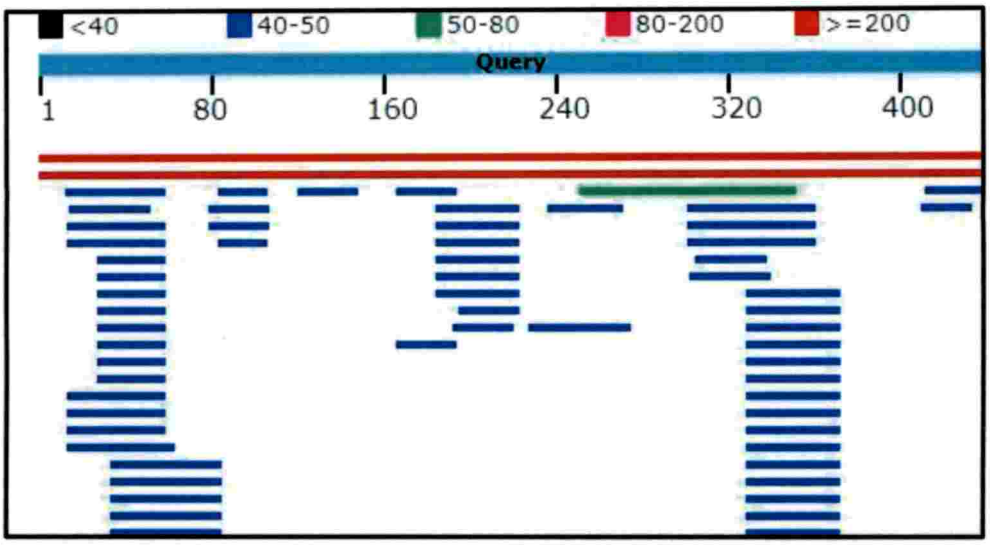



Figure 24: Blast analysis result for TaBCHV 2F/TaBCHV 2R

Sequences obtained for TaBCHV 2F/TaBCHV 2R has 99 & 96% identity with TaBCHV isolates TaBCHV-1 and TaBCHV-2 complete genome. The sequence obtained is given below (400 nt):

GTAGTCGAGGTTGTCATCTCCATCAAGACTTCCTCGCTTTTTCTTTCTTGTATT
CTTCTAGTGGGTTCTTGAATACCCTTAGTTTGCCAGCAGCTTCTCTAGGCTTCT
GATCGGATAGCTTCAAGTTCTTTAATCCTGAAATAAGATCGTCAGGGATAGA
TGATACCTGACCTTTCTCCTTTATGAACTGAGCTAGCTCTTCTCTCAGAGCCTT
CACTTCCTCTGTTAACCTTACAAGCAACTCGATTTGAGTGTTGTTTTGCTTGAT
TAAGGTTGAAGTTCCTGAGTTGGGTCCTTTGTTGTCGGCAGGTTTGGCAAAGC
CGACTGCTGGTGCTTCTATTTCTTCAGTGGCCTGTAGGGCGGAGGTGTAGGTT
TCAGTAGCCTGAGTGTTTATATGACTCATGAACGAGTCCACTCTTCTACTTTC
TTCAAGATGTGCT

Table 9: NCBI BLAST results of inserts sequences

| Virus | Sequence Identity | Accession Number |
|---|---|---|
| TaBCHV -1 | 94% | KP710178 |
| TaBCHV-2 | 92% | KP710177 |
| CSSV (isolate GH67 ) | 72% | KX592571 |
| PYMV (isolate PB) | 68% | KJ873042 |

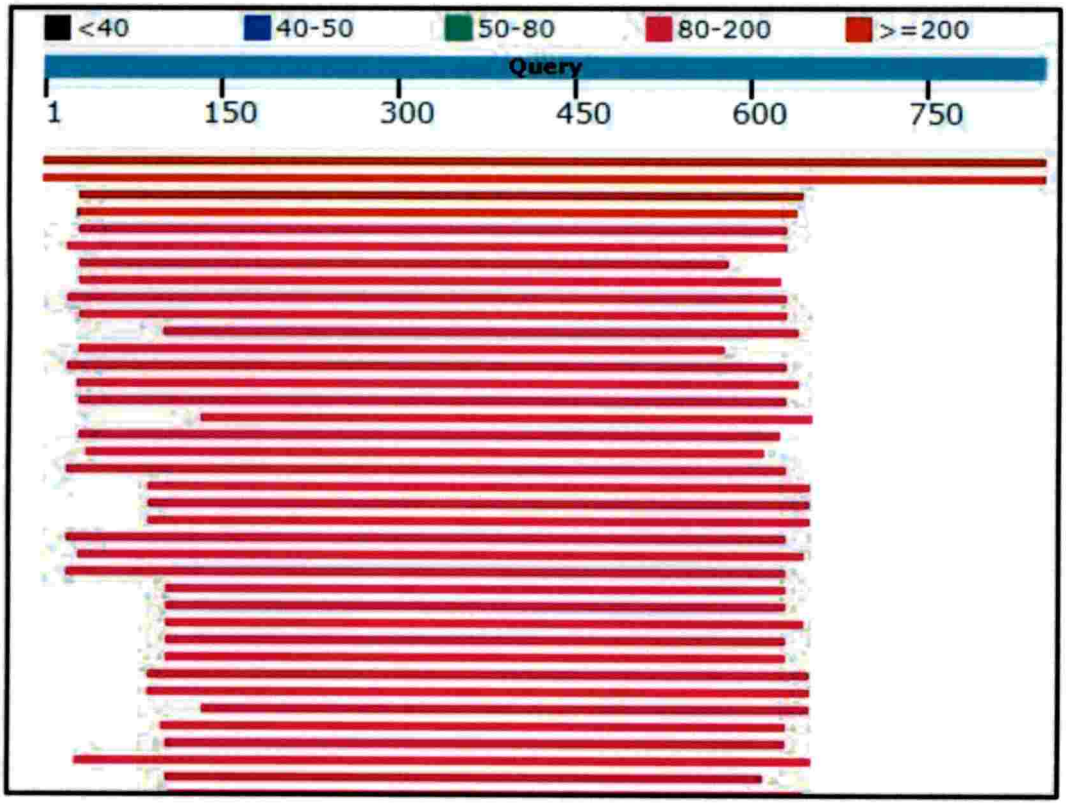



Figure 25: Blast analysis result for TaBCHV 3F/TaBCHV 3R

Sequences obtained for TaBCHV 3F/TaBCHV 3R has 94 & 92% identity with TaBCHV isolates TaBCHV-1 and TaBCHV-2 complete genome. The sequence obtained is given below (800 nt):

AGCCTTGCAGTTCTTGGACTGTTCGTCGTCTTCCAGGGATTGCTTGAACGCCG
TGGCTGACTAAGTAGTCTACCACGCTCTGAACTTCATATGCGAATCCAACATT
TGGGGTGTTGGATAGTCTTCCAACCAATCCTCTGGTGATGAGGAGATTGGCTT
CTCCTCTCCAGGCTTCATGTCCTCTAGTGAGGATTGAGACTTGGACGTTGTTG
TAGAAATCCCCGGTTGTCATCATTGTGTCAGGGATTACATACACCAACTAGCT
TCCCTGTGACAGATCTACCTCCATTGTGGCGAACAGGGAGCGATCATCCGGC
CGCCTGTTGTCACGGAAAACAACAAGAGCCAGCGTACCTTCGTCGTGTCGAT
GCAGTGCTTGAATTCAGAGTTGAATCACTCCAATACGTATGAACTGCATTCTG
CTCCTCTGCAATTGTCAAAAACTGTCTTCTTGGATGAAGATCTTGTCTTGTTAC
TGGTTGTCGGTGCAGAGGATAGCTTCTTCCGATCTGTGTACATAAACTCGATG
ATGCTCATCATCACGACGTGAGTGGTACAGGACTTCTGCAGGGACGATCGCC
GCTCGTTCCTGCATAGAATTCCTCAGTTGCACCTGAGGATCAATTTGCTGCTC
CAAAGTCTGATTATGGAGCTGGCTCCTTCCAGTGATCCGTCGTTGCAGACGTC
CTACTACTTGACGTGCATTATGTAGACGTCTCTGCCTTTGCCGATAGTTGCGG
ATCTGATCCTCGAACAGTGGAGTTGTTTCGGGACGCTGAAGGTTGGTCTTTGA
GTTCTGGCTGTAGTCGAGGTTGTCATCTCCATCAACACTTCCCTCGCTTTT

Construction of phylogenetic tree

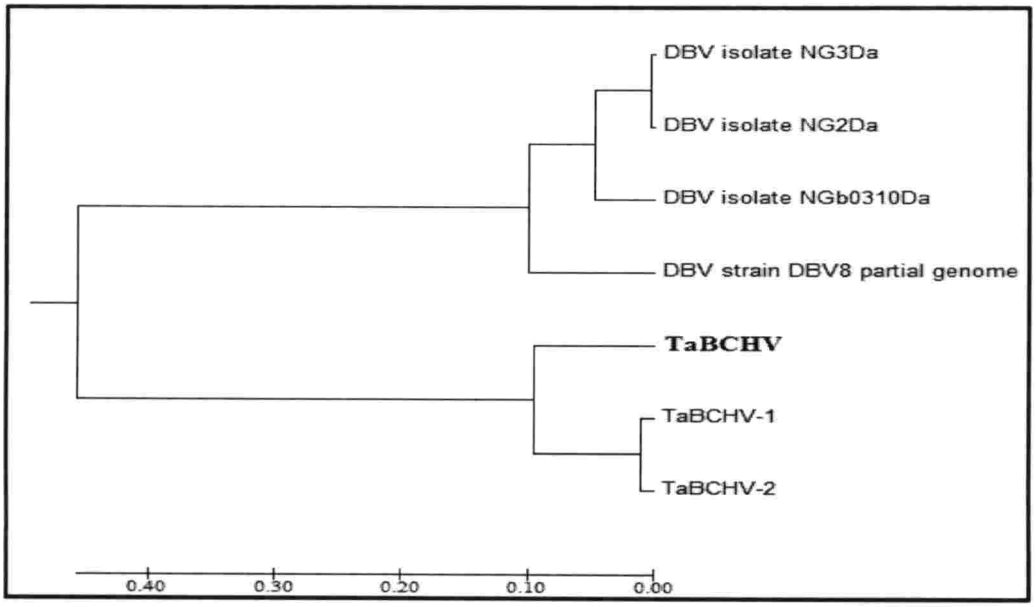



Figure 26: Phylogenetic tree based on RT/RNase H region of Taro bacilliform CH virus and other selected isolates of Diascorea bacilliform virus constructed by MEGA 7.0.26. The scale bar indicates the substitution/site.

Table 10 : Accession number and sequence identity of selected virus used for phylogenetic tree construction

| Virus | Sequence Identity (%) | Accession number |
|---|---|---|
| TaBCHV-1 | 91 | KP710178 |
| TaBCHV-2 | 89 | KP710177 |
| DBV-NG2Da | 74 | AM944572 |
| DBV – NGb0310Da | 74 | KX008583 |
| DBV-8 | 74 | KF829952 |

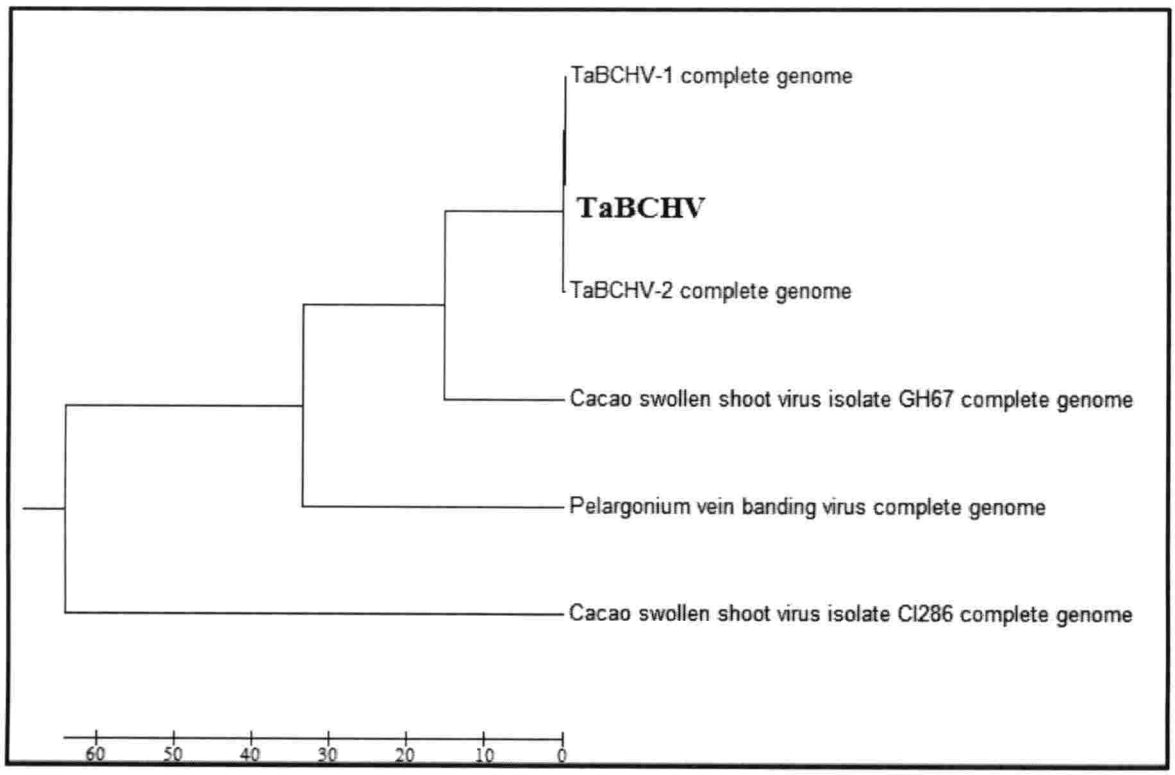



Figure 27: Phylogenetic tree based on the sequenced region of TaBCHV (1-821 bp) with the two isolates of TaBCHV constructed by MEGA 7.0.26.

Table 11: Accession number and sequence identity of selected virus used for phylogenetic tree construction.

| Virus | Sequence Identity (%) | Accession number |
|---|---|---|
| TaBCHV-1 | 100 | KP710178 |
| TaBCHV-2 | 98 | KP710177 |
| CSSV GH67 | 73 | KX592571 |
| CSSV C1 286 | 71 | KX592584 |
| PVBV | 86 | GQ428156 |

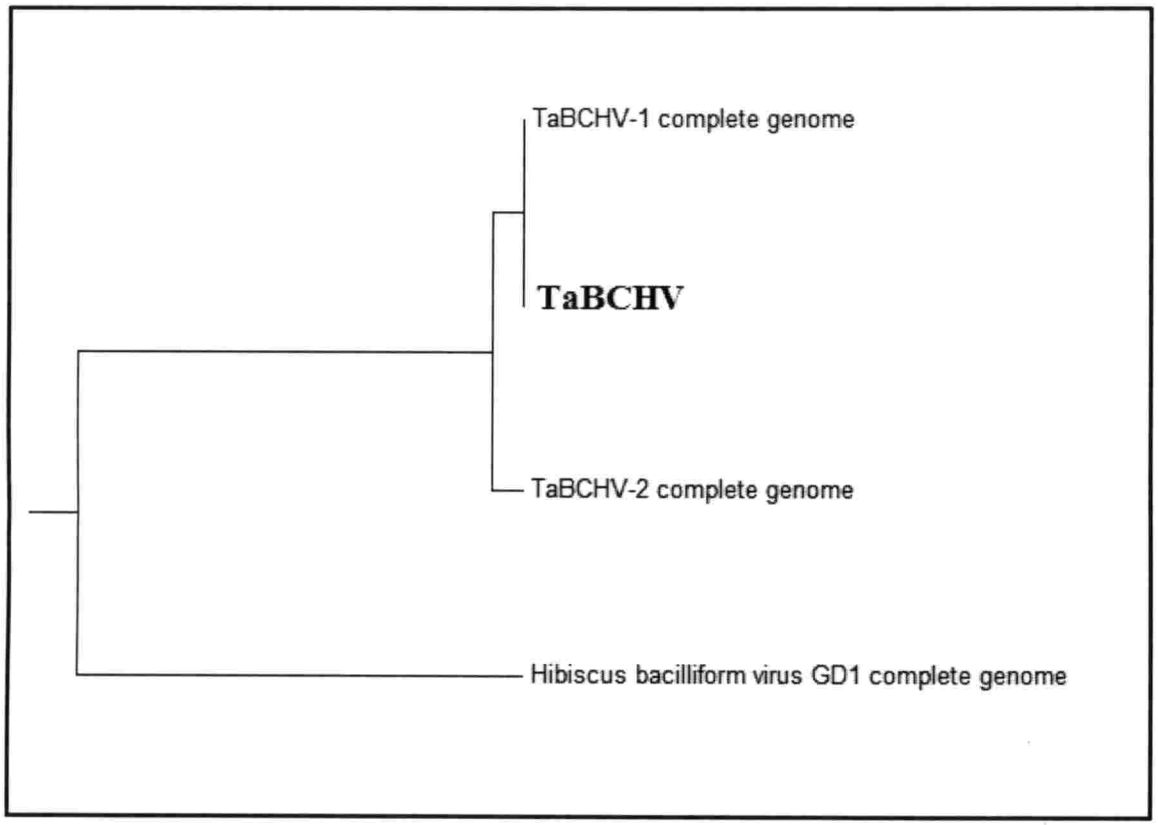



Figure 28: Phylogenetic tree based on the sequenced region of TaBCHV (800-1220 bp) with the twoisolates of TaBCHV constructed by MEGA 7.0.26.

Table 12: Accession number and sequence identity of selected virus used for phylogenetic tree construction.

| Virus | Sequence Identity (%) | Accession number |
|---|---|---|
| TaBCHV-1 | 99 | KP710178 |
| TaBCHV-2 | 96 | KP710177 |
| Hibiscus bacilliform virus | 71 | KF875586 |

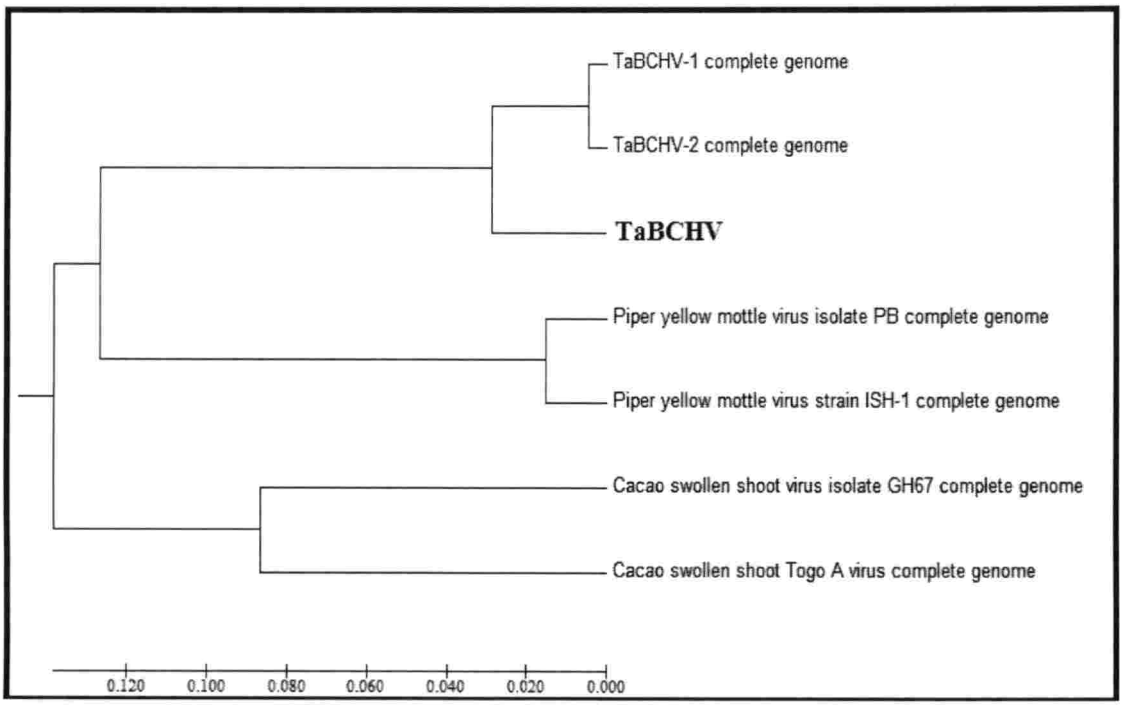



Figure 29: Phylogenetic tree based on the sequenced region of TaBCHV (1200-2000 bp) with the two isolates of TaBCHV constructed by MEGA 7.0.26.

Table 13: Accession number and sequence identity of selected virus used for phylogenetic tree construction.

| Virus | Sequence Identity (%) | Accession number |
|---|---|---|
| TaBCHV-1 | 94 | KP710178 |
| TaBCHV-2 | 92 | KP710177 |
| CSSV isolate GH67 | 69 | KX592571 |
| CSSV Tongo A virus | 67 | AJ781003 |
| PYMV isolate PB | 68 | KJ873042 |
| PYMV strain ISH-1 | 68 | KC808712 |

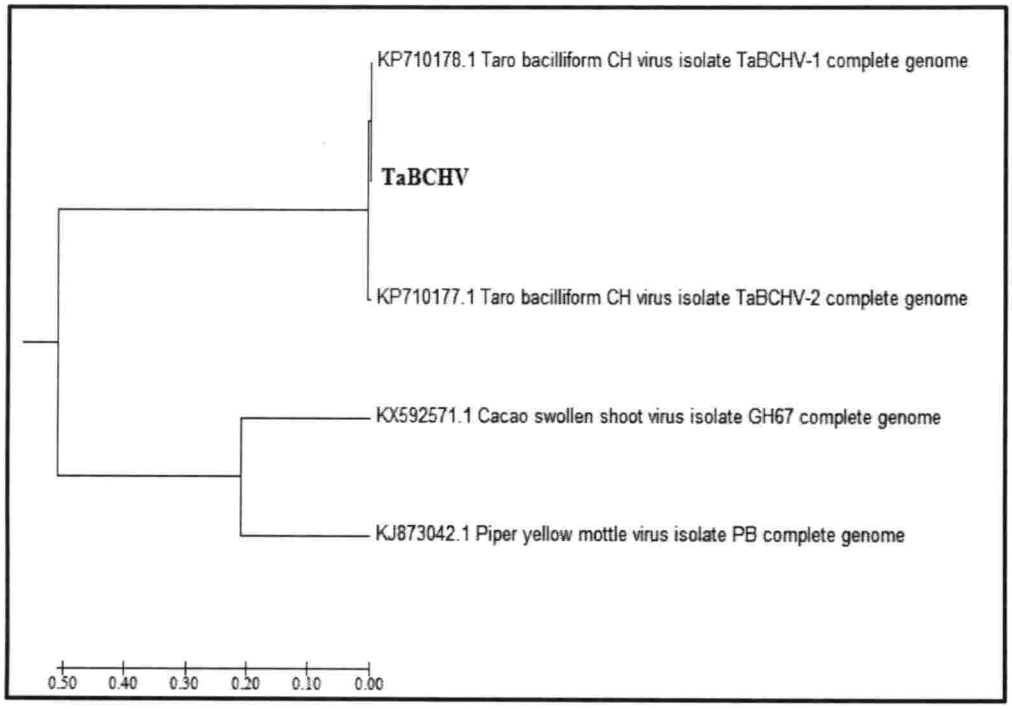



Figure 30: Phylogenetic tree constructed by combining all the three sequences (1-2000 bp) with other selected badnaviruses using MEGA 7.0.26. The scale indicates substitution/site.

Table 14: Accession number and sequence identity of selected virus used for phylogenetic tree construction.

| Virus | Sequence Identity (%) | Accession number |
|---|---|---|
| TaBCHV-1 | 99 | KP710178 |
| TaBCHV-2 | 98 | KP710177 |
| CSSV GH67 | 69 | KX592571 |
| PYMV PB | 68 | KJ873042 |

# 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. Instead of being referred to as "an orphan crop" due to the lack of research attention received by this crop, the international community noticed the role of taro in food security economically as well as culturally (Rao *et al.*, 2010; Matthews, 2010). Viruses infecting taro are poorly characterized, which is a hindrance to the safe movement of taro germplasm.

Diagnostics is very much important for the development and implementation of different strategies for pest management. These tools can effectively be categorized into three main groups: biological characterization which uses symptomatology on indicator hosts, serological based detection method using specific antigen-antibody recognition and molecular characterization using the nucleic acid of virus.

The present study was undertaken inorder to assess the incidence and distribution of *Taro bacilliform virus* infecting taro. Serological detection methods were not yet reported to identify the *Taro bacilliform virus*. Hence nucleic acid based detection method was only reliable to identify the virus.

In our study, *Taro bacilliform CH virus* was detected for the first time in taro in India. TaBCHV was detected in the leaf samples collected from CTCRI fields only, where the virus incidence was found to be more. The CTCRI experimental fields contain taro germplasm obtained from different regions across the world. The presence of TaBCHV infection in the samples collected from these fields only indicate that the virus was introduced through infected germplasm and that it has not been introduced to other regions. Leaf samples from Bhubaneswar and palakkad although showed obvious symptoms of virus infection, tested negative by PCR to the

virus indexed for. The symptoms observed on these plants might be caused by the presence of other virus(es) for which the tests were not done or that are not yet identified. Symptoms may also be due to abiotic agents causing virus-like symptoms, such as nutrient disorder and senescence. On the other hand, the viral detection on non-symptomatic leaf samples showed that absence of visual symptoms on taro leaf samples may not be an indicative or absence of virus infection. Effective laboratory diagnosis can serves as a more sensitive and conclusive way of affirming the health status of potential breeding or planting materials.

Obtaining good quality DNA is a prerequisite for reliable PCR reaction. Although there are many methods for DNA isolation, there is no universal method for all plant species or organs. Even identical tissues at different developmental stages may require special protocols for isolation depending on the crop. Mucilage, a highly viscous secondary metabolite which is composed of a polar polymer consisting of glycoprotein, present in tubers, seeds, and stems, can co-precipitates with DNA and inhibits the action of Taq polymerase used in the polymerase chain reaction (Jose and Usha, 2000; Ghosh *et al.*, 2009).The presence of mucilage also hindered the accurate pipeting of DNA, leading to volumetric errors, and it accounts for the aberrant spectrophotometric readings. CTAB method of DNA isolation which is mostly accepted in plant species was used in this study. The method by Lodhi *et al.* (1994) was found unsuitable for taro probably because of the specific crop characteristics. However, DNA extraction protocol specific to tuber crops by Sharma *et al.* (2008a) was adopted successfully. An effective method for removal of polysaccharides was obtained by increasing the concentration of CTAB along with NaCl in the extraction buffer (Syamkumar *et al.*, 2005; Abdellaoui *et al.*, 2011; Sahu *et al.*, 2012).

Since taro is rich in secondary metabolites, a careful and quck processing of the sample was required mainly during tissue homogenization. The inclusion of PVP and β-mercaptoethanol in the extraction buffer which are known to prevent the

oxidation of secondary metabolites in the disrupted plant material (Prittila *et al.*, 2001), avoided the brown pigmentation in the sample, and increased the yield and quality of DNA. Phenol: chloroform: isoamyl alcohol (25:24:1) has been reported to give high purity DNA in many plant species (Aras *et al.*, 2003; Sablok *et al.*, 2009). To ensure the long term storage of the isolated DNA, TE buffer was used. The absorbance ratio ($A_{260}/A_{280}$) in the range of 1.8 to 2.0 indicates a high level of purity of DNA (Weising et al., 2005). The isolated genomic DNA from young taro leaves confirmed high purity with A260/A280 value ranging from 1.8-2.22.

Brunt *et al.* (1990) reported that the transmission of TaBV is by mechanical means, grafting or by the aphids and is reported as having a natural host range restricted to taro. According to the vector transmission tests conducted by Gollifer *et al.* (1977) showed that TaBV was transmitted by the mealybug, *Pseudococcus lingispinus*, in the SolomonIslands and by *Planococcus citri* at Rothamsted, UK. But we could not trace out any vector in the fields from which the samples were collected. It has been also recently reported that the virus is transmitted by seeds as well as mealybug (*Pseudococcus solomonensis*).

For the study, a pair of badnavirus group specific primers was used to detect the presence of Badnavirus in the virus infected leaf samples which yielded an expected amplicon size of ~530 bp in some of the collected samples. Based on this, another pair of degenerate primers, TaBV 1F/TaBV 1R based on the consensus sequences of the RT/RNase H regions of published Badnavirus sequences were used in a PCR based strategy to characterize the TaBV. TaBV specific primer was used to amplify the RT/RNase H coding region of TaBV which yielded the expected amplicon size of ~320 bp in only one or two samples. The presence of wobble bases in the primer makes the sequencing results difficult. There is sequence variability in the RT/RNase H and CP-coding regions between the different Pacific isolates of TaBV and there is a preliminary evidence that a TaBV related sequence is integrated into the TaBV genome.

So, with respect to this another pair of PNG BadnaF/PNG Badna R primer (TaBV- like sequences) was used to detect the *Taro bacilliform virus* by PCR based detection, which yielded the expected amplicon size of ~250 bp in some of the collected samples which were detected positive for badnavirus infection. Sequence analysis was performed using the computer software MEGA 7.05 further reveals the presence of a new member of the badnavirus, *Taro bacilliform CH virus* (TaBCHV). TaBCHV belongs to the same genus of TaBV. It shows 100 % similarity to the *Taro bacilliform CH virus* isolates, TaBCHV-1 and TaBCHV-2 as well as with *Dioscorea bacilliform virus* partial gene for polyprotein as the amplified product was only 250 bp.The sequence analysis even shows only 8% similarity to the TaBV complete genome and only 4 % to the partial polyprotein gene of TaBV.

Genomic size of the two isolates, TaBCHV-1 and TaBCHV-2 (Genbank Accession Nos: KP710178 & KP710177) were 7641 bp i.e; within the range of badnavirus genome. The overall genomic identity between these two isolates was 98 %. However, the genomic sequence of TaBCHV-1 and TaBCHV-2 shared similarities of about 44% & 55.8 % with other reported badnaviruses like RYNV and Fig Badnavirus respectively (Kazmi *et al.*, 2015).

Based on the sequence analysis results, seven set of primers for the whole genome characterization of TaBCHV were designed. Among the seven pair of primers, three pair of primers were used to characterize the three portions of TaBCHV successfully. Further molecular characterization of the virus with remaining pair of primer requires more specific primers as the presence of multiple bands in the expected size as well as non specific sites makes the further characterization difficult. PCR analysis with the first three pair of primers (TaBCHV 1F/TaBCHV 1R), (TaBCHV 2F/TaBCHV 2R), (TaBCHV 3F/TaBCHV 3R) yielded the expected amplicon size at 779 bp, 400 bp, and 800 bp respectively. PCR analysis with the fourth and fifth pair of primers yielded the expected amplicon size of 1.81 kb and 1220 bp respectively with the bands at the non specific sites also. The sequences

obtained with the first three pair of primers were analyzed by BLAST (Basic Local Alignment Search Tool).

The sequences obtained with first pair of primer showed 100% nucleotide similarity to the two isolates of TaBCHV (TaBCHV-1 and TaBCHV-2) and 52% amino acid similarity to the hypothetical protein of TaBCHV and Hibiscus bacilliform virus. No putative conserved domains were found in the sequence obtained for TaBCHV 1F/TaBCHV 1R. For the second pair of primers, the obtained sequences shared high similarity with the two isolates of TaBCHV (TaBCHV-1 & TaBCHV-2 – 100%). This sequences shared 85% similarity with the hypothetical protein of TaBCHV and 84% similarity with *Piper yellow mottle virus* ORF2. Putative conserved domains were also present. Analysis of sequences obtained for third pair of primer, TaBCHV 3F/TaBCHV 3R also shared 100% similarity with TaBCHV-1 and TaBCHV-2 complete genome and almost 63-72 % similarity with other badnaviruses like *Piper yellow mottle virus* and *Cacao swollen shoot Tongo A virus* complete genome.

Analysis of TaBCHV sequences and genomic structure revealed that it possess unique characteristics. TaBCHV possess six ORFs on the sense strand, ofwhich additional 2 ORFs (ORF5 and ORF6) were not present in the TaBV genome.

The influence of *Taro bacilliform CH virus o*n yield and growth of taro plants has not been well studied yet. Different viruses and strains commonly infect taro and other aroids and the nutrient and trace element deficiencies mimic/enhance the symptoms caused by virus infections. Hence, it has been extremely difficult to assess how much yield or quality reduction is caused by a specific virus. Multiple infections caused by more than one virus are also relatively common. Generally, the effect of the mixed infections were greater than infection with a single virus. According to the studies, the presence of *Taro bacilliform CH* virus was also identified in the samples which showed the specific symptoms of other viruses normally infecting taro, a clear

knowledge of the actual dynamics of the various interplaying factors that ultimately results in the occurrence of mixed infections in taro fields is crucial to proffering effective taro virus control strategies.

As taro is a vegetatively propogated crop, there is an increased chance of virus introduction into new areas through the planting material. Naturally infected taro plants may also became sources of inoculums when used as planting materials the following year. The present study helped to detect the recently reported badnavirus, *Taro bacilliform CH virus* in taro. Since no serological detection methods were yet reported, more standardized diagnostic techniques have to be identified for routine virus indexing. The development of different diagnostic techniques as well as variation studies in the future will help to formulate broad spectrum management strategies.

# SUMMARY

## 6. SUMMARY

The study entitled "Molecular characterization of *Taro bacilliform virus*" in taro (*Colocasia esculenta (L) Schott*) was carried out at the Division of Crop Protection, Central Tuber Crops Research Institute, Sreekaryam, Thiruvananthapuram during 2016-2017. The objective of the study was to clone and characterize the *Taro bacilliform virus* and analyze the phylogenetic relationship with other members implicated in mixed infections of taro. The important findings of the above studies are summarized in this chapter.

Taro samples with virus infection symptoms were collected from the germplasm repository of CTCRI, Trivandrum and field samples of Bhubaneswar. DNA was isolated from the infected leaf samples. Samples exhibiting common symptoms such as whitish feathery mosaics, mild mosaics, chlorosis, vein puckering, leaf distortion etc are mainly selected. The DNA extraction protocol specific to tuber crops by Sharma *et al.* (2008a) was adopted successfully. Serological detection methods were not reported for Taro bacilliform and *Taro bacilliform CH virus*. So nucleic acid based technique (PCR) were performed to detect the presence of *Taro bacilliform virus*.

For nucleic acid based detection, two pair of primers, Badnavirus reverse transcriptase specific as well as Taro bacilliform virus RT/RNase H coding region primer were used expected to produce amplicons at 530 bp and 320 bp respectively. Badnavirus specific primer produced amplicons of ~530 bp in most of the samples, but TaBV specific primers did not produce the amplification at the expected size. Instead another pair of primer, PNG Badna F/PNG Badna R (TaBV like sequences) were used to detect the presence of TaBV, which produced amplicons of ~250 bp in some of the samples tested positive for badnavirus. The positive samples were gel eluted and sequenced to obtain the RT/RNase H region of Taro bacilliform virus and was cloned into *E.coli* DH5α cells. The presence of the insert in the transformed

colonies was confirmed by colony PCR. The recombinant plasmids were isolated and PCR was performed which yielded amplicons of expected band size (~250 bp).

Blast analysis of RT/RNase H coding region revealed that the sequences obtained has maximum similarity to *Taro bacilliform CH virus* isolate (TaBCHV-1) and TaBCHV isolate TaBCHV-2 (100% nucleotide similarity), whereas it shows only 4 % similarity to the expected *Taro bacilliform virus*. It also shows 100 % similarity to the *Dioscorea bacilliform virus* partial gene for polyprotein. The virus RT/RNasre H region exhibited only 75 to 80 percent nucleotide sequence identity with other badnavirus indicating that it is a putative new species.

Further, seven set of primers for the whole genome amplification was designed for the *Taro bacilliform CH virus*. Among them, the first three set of primers, TaBCHV 1F/TaBCHV 1R, TaBCHV 2F/TaBCHV 2R, TaBCHV 3F/TaBCHV 3R produced the amplicons at the expected size of ~779 bp, 400 bp and 800 bp respectively. The positive samples with the three sets of primers were gel eluted and sequenced to obtain the respective portions of the whole genome. These virus specific portions were cloned into *E.coli* DH5α cells and the presence of the insert in the transformed colonies was confirmed by colony PCR. The recombinant plasmids were isolated and further PCR confirmations yielded amplicons at the correct band size in all the three set of primers.

Blast analysis of sequences obtained from the first three set of primers revealed that in the first pair of primer TaBCHV 1F/TaBCHV 1R, the virus has maximum similarity to *Taro bacilliform CH virus* isolate (TaBCHV-1) and (TaBCHV-2) (100 % nucleotide similarity). The second set of primer TaBCHV 2F/TaBCHV 2R, shared 81 % similarity with *Taro bacilliform CH virus* isolates (TaBCHV-1) and (TaBCHV-2). Analysis with third set of primer, TaBCHV 3F/TaBCHV 3R revealed that the sequences obtained has maximum similarity to *Taro bacilliform CH virus* isolates (TaBCHV-1) and (TaBCHV-2) (100 % nucleotide

similarity). These region exhibited only 65 to 70 % nucleotide sequence identity with other viruses further confirming that this is a new member among the Badnagroup. The phylogenetic tree was constructed with similar sequences using online NCBI blast analysis software. Phylogenetic analysis clarly revealed that the sequences obtained in this study belongs to *Taro bacilliform CH virus* isolates TaBCHV-1 and TaBCHV-2.

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# REFERENCES

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# APPENDICES

# APPENDIX

**1. CTAB DNA Extraction Buffer (CTAB method I)**

| | | |
|---|---|---|
| Tris HCl (pH 8.0) | : | 100 mM |
| EDTA | : | 25 mM |
| NaCl | : | 1.5 M |
| CTAB | : | 2.5 % |
| PVP | : | 2 % (w/v) |
| β-mercaptoethanol | : | 0.2 % (v/v) |

(Freshly added prior to RNA extraction)

Autoclave and store at room temperature

**2. Chloroform-Isoamyl alcohol (24: 1)**

| | | |
|---|---|---|
| Chloroform | : | 24 ml |
| Isoamyl alcohol | : | 1 ml |

**3. TE Buffer**

| | | |
|---|---|---|
| Tris HCl (pH 8.0) | : | 10 mM |
| EDTA | : | 1 Mm |

**4. CTAB Extraction Buffer (CTAB method II)**

| | | |
|---|---|---|
| Tris HCl (pH 8.0) | : | 100 mM |
| EDTA | : | 20 mM |
| NaCl | : | 2 M |
| CTAB | : | 2 % |
| PVP | : | 2 % (w/v) |
| β-mercaptoethanol | : | 0.2 % (v/v) |

(Autoclave and store at room temperature)

**5. TAE Buffer (50 X)**

| | | |
|---|---|---|
| Tris base | : | 242g |
| Glacial acetic acid | : | 57.1 ml |
| 1.5 M EDTA (pH 8.0) | : | 100 ml |

***6. Luria Agar Medium***

35 g of LA (HiMedia) in 1000 ml distilled water. Autoclave and store at room temperature

***7. Luria Broth Medium***

20 g of LB (HiMedia) in 1000 ml distilled water. Autoclave and store at room temperature

***8. Preparation of LA Ampicilin/Xgal/IPTG Plates***

**1. Ampicillin stock (50 mg/ml)**

Dissolve 2.5 g Ampicillin in 50 ml deionized water. Filter sterilize and store at -20°C in the dark

**2. X gal stock (20 mg/ml)**

Dissolve 0.2 g X gal in 10 ml N, N Dimethylformamide. Store at -20°C in the dark

**3. IPTG stock (100 mM)**

Dissolve 1.7 g IPTG in 50 ml deionized water. Filter sterilize and store at 4°C in the dark.

Before pouring the plates, allow the LA medium (1L) to cool to 55°C, then add 1ml ampicillin stock (making the final concentration to 50µg/ml). Mix gently and pour the plates. Dry the plates opened at room temperature under UV light for 30 min. Add 40µl X gal stock solution to the plate and spread evenly using a sterile spatula. Allow it to diffuse completely into the medium for about 10 min. Then add 40µl IPTG stock solution, spread evenly using a sterile spatula.

Pre-warm the LA ampicillin /X gal /IPTG Plates at 37°C for 20 min before use.

***9. Reagents required for plasmid isolation***

**1. Buffer P1**

| | | |
|---|---|---|
| Tris (pH 8.0) | : | 50 mM |
| EDTA(pH 8.0) | : | 10 mM |

Autoclave and store at 4°C

**2. Buffer P2 (freshly prepared)**

| | | |
|---|---|---|
| NaOH | : | 200mM |
| SDS | : | 1 % |

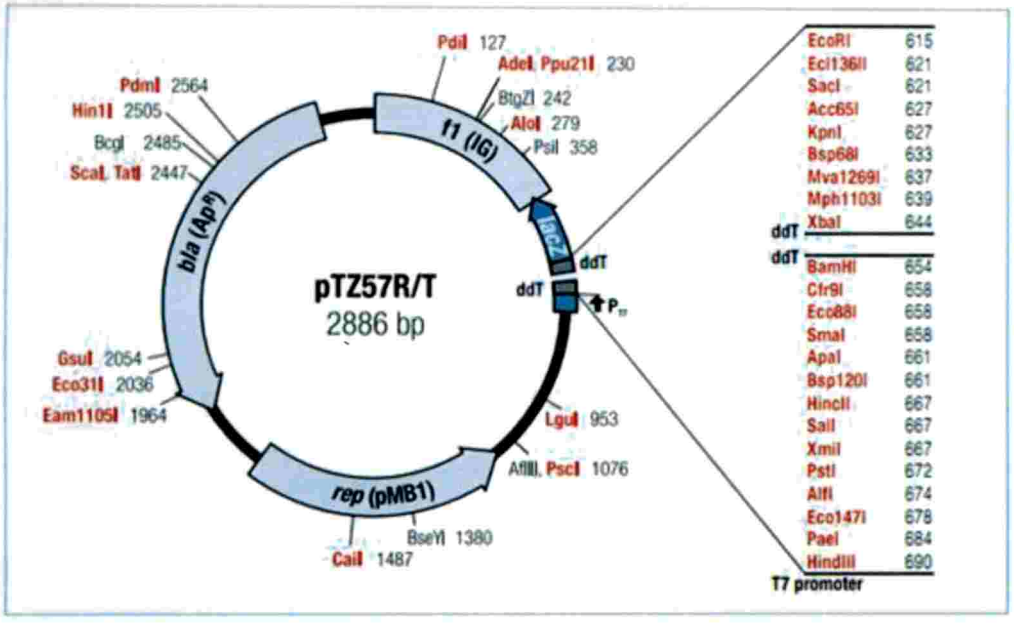
Autoclave and store at room temperature

**3. Buffer P3**

| | | |
|---|---|---|
| Potassium acetate | : | 3 M |

Adjust the pH to 5.5 with glacial acetic acid. Do not autoclave. Use autoclaved distilled water. Store at 4°C

# VECTOR MAP OF pTZ57R/T



# ABSTRACT

# MOLECULAR CHARACTERISATION OF *TARO BACILLIFORM VIRUS* (TaBV)

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**Abstract of the thesis**
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## ABSTRACT

Taro is one of the world's oldest food crop maintained by farmers for millennia and it has continued to spread throughout the world, as an important crop in Asia, Pacific, Africa and the Caribbean. Viral pathogens are one of the important factors threatening production of this vegetatively propogated crop. Taro viruses are poorly characterized, which is a hindrance to the safe movement of germplasm. The overall aim of this study was to detect the Taro bacilliform virus infecting taro and to characterize the virus at molecular level. Serological detection methods were not employed for the detection of TaBV. Molecular analysis with specific primers (TaBV like sequences) identified a new member among the badnavirus, Taro bacilliform CH virus, which was not previously detected in India.

During the study 55 samples from different fields of CTCRI are selected to identify the different symptoms of Taro bacilliform virus infection. The symptoms and occurrence of TaBCHV is still not clear eventhough the samples shows different type of infections with infections in non-symptomatic ones also. Molecular analysis with badnavirus specific primers and further confirmation with PNG BadnaF/PNG Badna R reveals the presence of Taro bacilliform CH virus. It reveals that the RT/RNase H region was highly similar for Taro bacilliform and Taro bacilliform CH virus with <80 % similarity. Sequence and phylogenetic analysis confirms the sequences were more closely related to TaBCHV and exhibited 100 % similarity to TaBCHV isolates TaBCHV-1 and TaBCHV-2. From the Seven pair of primers were specifically designed inorder to characterize the whole genome of Taro bacilliform CH virus three pair of primers were used to successfully characterize the virus partially.

To identify the phylogenetic relationship of the sequenced samples with that of available accessions, dendrograms were made using MEGA 5.0 software and the tree showed that sequences has variability eventhough lies within the group. It s clear

from the study that the genome sizes of TaBCHV isolates, TaBCHV-1 and TaBCHV-2 (Gen Bank Accession Nos: KP710178 and KP710177) were 7,641 bp, and it was within the badnavirus genomic range. The two islates almost shared about 98% genomic nucleotide identity. With other reported badnaviruses the genome sequence of TaBCHV-1 and TaBCHV-2 showed similarity to RYNV (44.1 %) and Fig Badnavirus (55.8 %)