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IDENTIFICATION AND CHARACTERIZATION OF VIRUSES INFECTING LESSER YAM

(*Dioscorea esculenta* (Lour.) Burkill)

by

SUDHEER K. S.

(2010-09-113)

THESIS

Submitted in partial fulfilment of the requirements for the degree of

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Department of Plant Biotechnology

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM - 695 522

KERALA, INDIA

2015

DECLARATION

I, Sudheer K. S., hereby declare that this thesis entitled **"IDENTIFICATION AND CHARACTERIZATION OF VIRUSES INFECTING LESSER YAM (*Dioscorea esculenta* (Lour.) Burkill)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar title, of any other university or society.

Place: Vellayani

Date: 21-12-2015

SUDHEER K. S.

(2010-09-113)

Produce tuber reduce hunger

भाकृ अनुप-केन्द्रीय कन्द फसल अनुसंधान संस्थान
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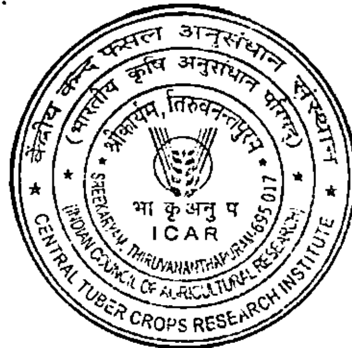

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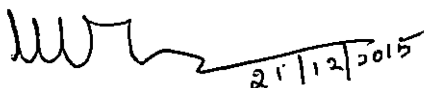

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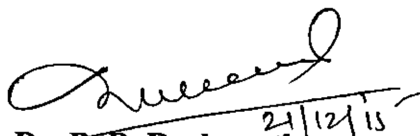

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Dedicated to my dear parents

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µg	Microgram
µl	Microlitre
µ*M*	Micromolar
A	Adenine
A405	Absorbance at 405 nm wavelength
Bp	Base pair
C	Cytosine
cDNA	Complementary DNA
cm	Centimeter
CP	Coat protein gene
CTAB	Cetyl trimethyl ammonium bromide
DAS-ELISA	Double antibody sandwich ELISA
DNA	Deoxyribonucleic acid
TAS-ELISA	Triple antibody sandwich-ELISA
dNTPs	Deoxy nucleotide tri phosphates
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
F	Forward primer
G	Guanine
g	gram
g	standard acceleration due to gravity at the earth's surface
h	Hour
ha	Hectare
kb	Kilo bases

kcal	Kilo calories
kDa	Kilodalton
kg	Kilogram
$LiCl_2$	Lithium chloride
m	Meter
M	Molar
mg	milligram
min	Minute
ml	Milliliter
mm	Millimeter
m*M*	Millimolar
mRNA	Messenger ribonucleic acid
Mt	Metric tonnes
NaCl	Sodium chloride
NCBI	National center for biotechnology information
ng	Nanogram
nm	Nanometer
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
PBS-Tween 20	Phosphate buffered saline- Tween 20
PCR	Polymerase chain reaction
PVP	Polyvinyl pyrrolidone
R	Reverse primer
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RT	Reverse transcriptase

RT-PCR	Reverse transcription-polymerase chain reaction
s	Second
SDS	Sodium dodecyl sulfate
sp.	Species
spp.	Species (plural)
t	Tonnes
T	Thymine
Tm	Melting temperature
Tris HCl	Tris (Hydroxy Methyl) aminomethane hydrochloride
U	Enzyme unit
V	Volt
v /v	volume/volume
w/v	weight/volume

INTRODUCTION

1. INTRODUCTION

Yam is a common name for several species of *Dioscorea* which are tuberous starchy food cultivated and consumed in the developing countries of the tropics and subtropics as staple or subsidiary food. They are ranked the third most important tuber crop after cassava and sweet potato (Fu *et al.*, 2005). The principal edible yams are cultivated mainly in three different regions such as Asia, Africa and South America and also in the temperate regions (Lebot, 2009). The most important edible yams are D. *alata, D. rotundata, D. esculenta D. bulbifera, D. nummularia, and D. pentaphylla.* However they are one of the most widely distributed taxon which are found almost everywhere in the tropics and the subtropics. They are vegetatively propagated crops and the species are characterized by weak, climbing stems and underground tubers or rhizomes but some species produce aerial tubers or bulbils also. The tubers are an important source of carbohydrate for millions of people in the world, although some species are of medicinal and ornamental value (Hou *et al.*, 2002).

In India the three principal edible yam species cultivated are *D. alata L, D. esculenta (Lour).Burk.* and *D. rotundata poir.* They are popular throughout the country, but their major cultivation and consumption are largely in the South and North Eastern states. In addition to the cultivated edible yams, a number of minor edible yams are also eaten by the tribal and poor people in almost every part of the country. The minor edible yams are not yet domesticated and collected from the forests and wild habitats for consumption and sale in the village markets. They are mainly starchy food with essential dietary nutrients which make a significant contribution to the diets of tribal and poor people. Published reports highlight about 19 wild edible yam species consumed by the local and tribal people of the 13 states of India. Among the different states the largest number of wild yam species is utilized in the states of Kerala, where a dozen of them are consumed by the tribal people in the Wayanad district, which contains parts of the Western Ghats forests, where live the different communities of

tribal people (Balakrishnan *et al.*, 2003) followed by those in Nilambur, Pathanamthitta, Parambikulam and Idukki.

Lesser yam, *Dioscorea esculenta* (Lour.) Burk is an ancient crop domesticated in the tropics. Among yams, it ranks third in production and utilization. Although *Dioscorea esculenta* had been extensively cultivated in India before the introduction of potatoes it is now largely confined to certain pockets in Bihar, Madhya Pradesh, Orissa, Tamil Nadu and Kerala, but with limited cultivation in homestead in almost all states. Its tubers are extremely palatable (sometimes sweet), soft in texture, nonfibrous and are free of toxins. It is a comparatively hardy crop which can be grown even in marginal lands with very little input.

Yam can be stored longer than most other tropical fresh products, and therefore stored yam represents stored wealth with increased commercial exploitation and processing. However, production is adversely affected by pests and pathogenic diseases. Virus diseases are of particular importance because they reduce vigor and subsequently cause a reduction in tuber yield and quality (Amusa *et a*l., 2003). The more commonly encountered viruses have been reported to infect yams belonging to the genera of *Potyvirus, Badnavirus, Cucumovirus, Potexvirus* and *Macluravirus* (Kenyon et al., 2001) but information about the presence of such viruses in India is scanty, while the survey conducted at ICAR-Central Tuber Crops Research Institute (ICAR-CTCRI) revealed the presence of *Yam mild mosaic virus* (YMMV) and *Dioscorea alata badna virus* (DaBV) in different yam growing regions of the country.

Use of infected planting materials is a major means of spread of yam viruses and may restrict the international movement of improved or selected germplasm due to quarantine restrictions. Therefore, it is necessary to control the spread of the virus and produce virus free healthy planting material. Diseases caused by viruses cannot be controlled by the use of chemical applications unlike those caused by fungi and bacteria

(Walkey, 1991). Management of yam virus diseases is mainly through the principle of exclusion by using healthy planting materials and prevention of virus infection through cultivation of virus-resistant varieties (Ng, 1992). Both of these methods greatly depend on the availability of sensitive and specific virus diagnostic tools for virus monitoring in plants and plant parts. Effective diagnostic tools are crucial to minimize the risk of yam virus spread and to ensure that distributed planting materials are tested and certified negative for known viruses.

Identification and characterization of viruses infecting lesser yam (*Dioscorea esculenta* (Lour.) Burkill) have to be done, in order to improve lesser yam production and to ensure quality of lesser yam planting materials. For this, effective diagnostic methods are pre-requisite. The correct identification of infecting viruses is needing for the development of a breeding program and formulating control strategies. Hence the specific objectives of this work were formulated as:

1. To detect the presence of different viruses infecting lesser yam
2. To identify the virus
3. To characterize the viruses
4. To design virus specific primers for detection

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 ROOT AND TUBER CROPS

Root and Tuber Crops are the most important food crops after cereals. They have the highest rate of dry matter production per day and are major calorie contributors. Tuber crops find an important place in the dietary habits of small and marginal farmers especially in the food security of tribal population. It is not only enrich the diet of the people but also possess medicinal properties to cure many ailments or check their incidence. Tropical root and tuber crops including cassava, sweet potato, yams and aroids are enjoyed as vegetables, used as raw materials for small-scale industries, and consumed as staple food especially in the less developed countries (Ravi *et al.*, 1996). They are found in a wide variety of production systems and do well under various levels of management from low to high input systems. This is a distinctive feature which makes them important for improving the productivity and richness of agro-systems. Even though their agronomic properties have been well documented, their food and industrial quality characteristics have not been studied extensively. The full potential of these staples is being realized in growing regions and they would continue to contribute to energy and nutrient requirements for the increasing population.

India holds a rich genetic diversity of tropical root and tuber crops viz. cassava, sweet potato, aroids, yams and several minor tuber crops. The Indo-Burma region is the centre of origin of taro and Asiatic edible yams. The two hot spots of global biodiversity viz. North Eastern Himalayas and Western Ghats are particularly rich in wild relatives of tropical root and tuber crops. Safe conservation and sustainable use of plant biodiversity is essential for meeting the present and future needs of tuber crops improvement in India (Edison *et al.*, 2006).

2.2 YAMS

Yam is a multi-species crop belonging to the family Dioscoreaceae, genus *Dioscorea* and serves as a staple crop in West Africa (Asiedu *et al.*, 1992). They are originated principally from Africa and Asia before spreading to other parts of the world (Hahn *et al.*, 1987). The yam tuber, which is the most important part of the plant, can be stored longer than other root and tuber crops, ensuring food security even at times of general scarcity. The crop is of major importance in the diet and economic life of people in West Africa, the Caribbean islands, parts of Asia, and Oceania. It is the third most important tropical root and tuber crop after cassava and sweet potato (Fu *et al.*, 2005).

The word "yam" is believed to have originated from the tribal African word, "niam" (Coursey, 1967a), meaning "to sample" or "taste". It is also known as "igname" (French) and "name" (Spanish). They contain over 600 species, of which only about ten are considered as edible (O'Hair, 1988). Cultivated species include *D. alata*, *D. cayenensis*, *D. rotundata*, *D. esculenta*, *D. bulbifera*, *D. nummularia*, *D. pentaphylla*, *D. hispida*, *D. trifida* and *D. dumetorum*. Among these species D. *rotundata* and *D. cayenensis* are the most commonly cultivated yams and represent 95 percent of yam production worldwide with Nigeria being the world's largest yam producer (FAOSTAT, 2012). Most of the varieties cultivated are accessions selected by farmers from the existing landraces. A few minor cultivated species are known for their medicinal properties like *D. zingiberensis* with high diosgenin content and *D. villosa* containing cortisone precursors (Baah, 2009; Zhou *et al.*, 2013). It is an elite crop, preferred over other root and tuber crops in West Africa and a choice during ceremonies and festivities and also has ritual and socio-cultural significance in West and Central Africa.

In India twenty-six species of *Dioscorea* have been reported (Abruna *et al.*, 1981). At present thirteen species are available in Orissa, Out of them two species are

cultivated and rest eleven are wild (Maharana, 1993). In 1978, Arora and Singh reported that several wild yams are used as food in the Eastern Ghat region but only six *Dioscorea* species from Orissa were used as food source (Niswass, 1985). From the southern peninsular parts of India, 14 minor edible yam species were reported and *Dioscorea vexans* is another minor yam species collected from the Andamans and introduced to the mainland (Abraham *et al.*, 2008). The various wild edible yams (*Dioscorea spp*) serve as a 'life saving' plant group for the marginal farming and forest dwelling communities, during periods of food scarcity and also ethnically important as a secondary staple and a delicacy for tribal people in the interior areas in Western Ghats.

2.2.1 LESSER YAM

Dioscorea esculenta is commonly known as Lesser Yam, which was one of the first yam species cultivated. Lesser yam is the least studied of the major staple yam species, although it is widely cultivated in Southeast Asia, the pacific and is the dominant or co-dominant staple food in parts of India, Papua New Guinea, Philippines, and southern Vietnam. In India it is known as Kayu, Cheruvallikizhangu, Cherukizhangu and Nana kizhangu which is cultivated in various parts of the state such as Bihar, Madhya Pradesh, Orissa, Tamil Nadu and Kerala.

The crops is almost always grown in low input system, where soil fertility is a major limitation to yield but in most areas, cropping intensity has increased in recent years due to land pressure. Its vines seldom reach more than 3 m (10 feet) in length and produce a cluster of fairly small tubers 4-20 per plant in most varieties (Kay, 1987).

Tubers are oblong to fusiform in shape with creamy white flesh and greyish brown skin covered with thin hairs containing 18.4% starch and staple food in most parts of West Africa including Nigeria. Externally, the tuber has been applied to ulcers, boils and abscesses because it contains allantoin, a cell-proliferant that speeds up the

healing processes. Apart from these properties they uses as an antifatigue, antiinflammatory, anti-stress, anti-spasmodic and immune deficiency remedies in various ethno-medicines. Although traditionally used as a contraceptive, treatment of menopausal symptoms and various disorders of the genital organs. The peel has been reported to possess anticancer and antifungal properties.

The ICAR-Central Tuber Crops Research Institute (ICAR-CTCRI) is the nodal organization in India for planning, conducting, promoting and co-ordinating research activities on tropical tuber crops. In *Dioscorea esculenta*, natural variability is munch restricted compared to other tuber crops mainly due to the absence of sexual reproduction and genetic erosion of many ancient clones. Therefore, collection, conservation and evaluation of varieties and clones are of prime importance in the improvement of this underutilized crop.

Unlike other edible yams, viz., white yam (*D. rotundata*) and greater yam (*D. alata)*, lesser yam is not widely cultivated world wide. The literature on cultivation and pathology of the crop is meager internationally and not available in India except some preliminary studies at ICAR-Central Tuber Crops Research Institute (ICAR-CTCRI). Lesser yam is also an edible species of yam and widely cultivated in Kerala, West Bengal, North Eastern India and the pathogens infecting other yams also found infecting this crop. So the available literature on various aspects of work done on other yams also reviewed in this thesis.

2.3 TAXONOMY AND MORPHOLOGY OF YAMS

Yams are annual or perennial tuber-bearing and climbing plants belonging to the order Liliflorae, family *Dioscoreaceae*, and genus *Dioscorea*. It is considered to be the most primitive of the angiosperms of about 600 species identified (Coursey, 1967a; Burkill, 1960). Morphological characteristics of the genus *Dioscorea* were first used for classification and divided the genus into six different sections (Alexander and

Coursey, 1969). However, the morphological characters do not allow the distinction between species such as *D. cayenensis* and *D. rotundata*, and also between cultivars.

Dioscorea esculenta has thin, cylindrical, pubescent spiny stems and vine-like thus usually requiring support from either neighbouring plants or stakes on which they twine clockwise or anticlockwise directions (Johnson, 2003). The emerging stem are brownish green in colour but mature stems has diffused purple to dark purple colouration. Lesser yam has thorns on almost of its stem particularly at the nodes and at the base of the petioles. It is also covered with hairs that can be seen with the naked eye. Leaves are small, entire, cordate and alternate in arrangement. The petioles are thickened at the base with four sharp prickles.

Male flowers are occasionally produced in long spikes but there is no female flowers produced. *Dioscorea esculenta* as an economic plant is a species of great interest subjected to very intense selection by man that it has probably lost its power of producing female flowers and seeds. The tuber, the economically important part of the lesser yam plant, are small and are borne in clusters of 10-20 produced at the ends of stolons. Tubers have thin light brown skin and white flesh.

2.4 ORIGIN AND DISTRIBUTION OF YAMS

Yam is believed to have originated in Asia and carried across the world in the second century AD. Evidence indicates that different yam species originated in three independent areas of the tropics namely Asia, West Africa and tropical America. The species *Dioscorea alata* L. and *D. esculenta* (Lour) Burk originated from Asia and were introduced to West Africa while D. *rotundata* Poir, *D. cayenensis* Lam., and *D. dumetorum* Pax originated from West Africa and are among the few truly West African domesticated plants. *D. trifida* L. originated from tropical America (Ayensu and Coursey, 1972). In general, there was an eastward movement of yam species during the domestication process (Hahn *et al.*, 1987).

Lesser yam is a crop of great antiquity. It is believed to have originated in Indo-China and now cultivated throughout the tropics. The centre of origin is stated by various authors as India, Vietnam or Papu New Guinea and Philippines. As "Madhavaluka" or sweet yam, it is mentioned in the "Susruta Samhita" a treatise of medicines written in the Gangetic plains between 600 and 700 AD. Later in Akbar's great gazetter-the Aini- i-Akbari of about 1590 AD., lesser yam is mentioned as a crop of upper Gangetic plains in the name "Pindalu". It is also documented as a staple food in Southern China from the 2nd and 3rd centuries. It is now widely distributed throughout the tropics especially in South East Asia where it ranks third in production and utilization of yams after *Dioscorea rotundata/cayenensis* and *Dioscorea alata.*

2.5 CULTIVATION OF LESSER YAM.

Knowledge of the special cultural practices peculiar to yam is required for its successful cultivation. Lesser yam grows well in warm and humid conditions with a mean temperature of 30 °C and a well distributed annual rainfall of 1200-2000 mm. The planting materials used for cultivation are referred to as medium sized tubers (100-150g). The seed tubers are usually grown in mounds formed over pits at a spacing of 75cm × 75cm and approximately 1800-2700 kg of seed materials are required to plant one hectare. They requires deep, loose, well-drained, fertile soil and does not come up well in water-logged conditions. Good drainage and high organic matter content are essential for higher yield as well as for the production of tubers of larger size and good shape.

Generally March-April is the ideal time for planting lesser yams and takes 7-8 months for attaining maturity. The tubers are completely covered with soil and mulching materials to hasten germination, control weed growth and regulate soil temperature and also to retain soil moisture. With regard to the plant nutritional requirement, application of farmyard manure @ 10 t/ha^{-1} is incorporated in to the soil during land preparation and NPK @ 80:60:80 kg/ha^{-1} is found to be optimum. After

sprouting, the vines are sometimes trained onto stakes for adequate exposure of leaves to sunlight. It is done within 15 days by coir rope attached to artificial supports in the open area or to the trees where it is raised as an intercrop. The tubers have a large sink capacity and continue to grow and store food reserves throughout the year as long as conditions remain favourable. After harvest, tubers are stored in barns and have relatively long storage life of 4-6 months at a mean ambient temperature of 25 °C or provision of planting materials for the following season. Yam scales are sometimes found to occur on the tubers both under field and storage conditions. As a prophylactic measure the planting material is dipped in monocrotophos 0.05% suspension for 10 minutes. The tubers ae carefully dug out without causing injury when the vines are completely dried up (Sheela *et al.,* 2000).

2.6 PRODUCTION

Yam is one of the most important staple starchy food crops grown extensively in Africa especially in West Africa with over 90 percent of the world's production coming from the areas called "the yam zone of Africa" comprising of Cameroon, Nigeria, Ghana, Benin, Togo and Côte d Ivoire with over 93% of total yam production (FAOSTAT, 2013). World annual production of yam, in 2010, was estimated to be 48.3 million tonnes covering over 5 million hectares of land. According to FAO statistics 2013, 56.6 million tonnes of yams were produced worldwide. West and Central Africa account for 52.5 million tonnes, Nigeria produced 37 million tonnes while Cameroon produced only 0.5 million tonnes and is ranked 6th in the world. The remaining was produced mainly in Central and South America, Oceania and Asia.

West Indies, the second most important yam-producing region, is reported to produce over 0.2 million tonnes of yam; approximately 5% is exported, resulting in an annual export earning of over $15 million. Sub-Saharan Africa is expected to produce 98.1% of total world production of yam by 2020 (Scott *et al.*, 2000a).

In India, the two most important species of yams are *Dioscorea esculenta* (lesser yam) and *Dioscorea alata* (greater yam). *Dioscorea alata* is cultivated practically in all the states but the area and production details are not available for lesser yam. However the major yam producing states are Kerala, Gujarat, Uttar Pradesh, Andhra Pradesh, Maharashtra, Orissa and West Bengal. The total area under cultivation in 2009 was 7756 ha with a yield of 115618 Mt. Consumption is relatively more in Orissa, West Bengal and Kerala. In Kerala, during 2010-11, the area under yam production in Kollam district was 1858 ha. Kollam is the major yam growing district in Kerala with 34 percent area under yam cultivation followed by Pathanamthitta, Idukki, Alappuzha and Kottayam (Srinivas *et al.*, 2012).

2.7 ECONOMIC AND SOCIAL IMPORTANCE OF YAM

Yam has a dormancy period naturally, unlike most tropical crops, which gives the advantage of a longer storage period, ensuring a food supply even at times of general scarcity. It can be stored for up to 6 months or even longer depending on the means of storage. Stored yam is stored wealth and can be sold when prices are higher.

Yam is second to cassava as the most important tropical root crop but from a nutritional point of view, it is better than cassava on account of its higher vitamin C (40-120 mgg^{-1} edible portion) and crude protein content (40-140 gkg^{-1} dry matter). Information on the nutritive value of yam has been highlighted by several authors in their work on yam (Afoakwa and Sefa-Dedeh, 2001). In the South Pacific, yam is a significant food crop, accounting for over 208.1, and 4.6 percentage of the total dietary calorie intake in the Kingdom of Tonga, Solomon Islands, and Papua New Guinea, respectively (Opara, 1999).

Processed yam products have a very high market price internationally if well produced. Until recently yams were mainly considered subsistence crops in Africa, but are now grown as cash crop for both local and export. Report from a survey in Nigeria

revealed that when purchasing power improved at all expenditure levels, there was a corresponding increase in household yam consumption (Nweke *et al.*, 1992). This means that yam will continue to have a high market potential in Nigeria.

Yam also has ritual, medicinal and socio-cultural significance. It is the choice during ceremonies and festivities (Hahn *et al.*, 1987). In some parts of Southeastern Nigeria, the meals offered to gods and ancestors consist principally of mashed yam. Some traditional ceremonies are celebrated with yam as the major food item such as the New Yam Festival in parts of West Africa. In parts of Igboland in Southeastern Nigeria, it is customary for the parents of a bride to offer her seed yams for planting as a resource to assist her in raising a family. However the yam harvest is a traditional festivity with masquerade dancing in the villages and prayers offered to thank the ancestral gods for blessings on the land (Degras, 1993).

2.8 NUTRITIONAL COMPOSITION

Yam, apart from providing basic food security and income, is a source of nutrition for millions of people. It is a rich source of carbohydrate and also contributes to vitamins and minerals especially where it is consumed in large quantities. They are relatively good sources of some minerals. Generally the ash content of yam gives an indication of its mineral status. They contain appreciable amount of potassium, a mineral that helps to control blood pressure so yam is recommended for people with high blood pressure but is not suitable for people with renal failure (Osagie, 1992).

Yam tubers are comprised of approximately 75.6-83.3 % carbohydrate, 3-7.4 % protein, 0.5-1.5% fibre, 0.7-2.0% ash, and 0.05-0.02% fat. A large proportion (65-75%) of the yam tuber is made up of water (Opara, 1999). They are starchy staple food, rich in carbohydrates and are also valuable sources of some vitamins, particularly vitamin C. Yam tubers contain about 13-24.7 mg/100g ascorbic acid and most of it is retained during cooking (Wanasundera and Ravindran, 1994). The high potassium and

low sodium content of yam produces good potassium-sodium balance in the human body and so protect against osteoporosis and heart disease (Walsh, 2003). Yam products are also reported to have lower glycemic index than potato products. Slow break down of carbohydrates and gradually release of glucose into the blood stream means that they will provide more sustained form of energy, and give better protection against obesity and diabetes (Holford, 2008). (Peroni *et al.,* (2006)) reported higher amounts of phosphorus in yam (0.022%) compared to other tropical root and tuber crops such as cassava and sweetpotato. The amount of this minerals or nutrients in yams depends on the type of soil it was harvested from, moisture content and maturity of the crop.

In addition to their use as human food, some wild species of yam have been used as the production of pharmaceutically active compounds for e.g. *Dioscorea villosa* contain steroidal saponins and sapogenins which are precursors for cortisone used medicinally for the management of menopausal symptoms and treatment of arthritis and menstrual disorders (Albrecht and McCarthy, 2006). Other wild species are cultivated for extraction of diosgenin, a female hormone precursor used in the manufacture of contraceptive pills and sex hormones for birth control (Uadia, 2003). Another species, the cinnamon vine, *D. batatas, deene* is cultivated as a decorative plant and other wild species are also of ornamental value (Komesaroff *et al.*, 2001; Hou *et al.*, 2002). The powdered tubers of *Dioscorea* were used as an ingredient of medicines for the treatment of cholera, constipation, piles, skin diseases, intestinal worms and obesity (Natraj *et al.*, 2009). Its pharmacological activities include antitumor, antiallergic, anti-inflammatory and anti-HIV activity (Zhou *et al.,* 2013). In India, *Dioscorea deltoidea* is the major species which is used as a source of diosgenin production followed by *Dioscorea spicata, and Dioscorea hispida (*Asha and Nair, 2005).

Table 1: The nutrient contents of Lesser yam species per 100 g fresh edible tuber portions.

Constituents	Quantity per 100g
Moisture	67-81
Carbohydrate	17-25
Starch	25
Free sugar	0.6
Protein	1.3-1.9
Crude fat	0.04-0.3
Fibre	0.2-1.5
Ash	0.5-1.5
Phosphorous (mg)	35-53
Calcium (mg)	12-62
Vitamin C (mg)	-
Iron (mg)	0.8
Food energy (kcal)	112
β-carotene (μg)	-
Thiamine (mg)	0.1
Riboflavin (mg)	0.01
Niacin (mg)	0.8

(Source: Baah, 2009)

2.9 CONSTRAINTS TO PRODUCTION

The principal problems in yam production that have been identified are the lack of availability and high cost of planting materials, pests and diseases, weeds, cost of labour (labour cost during planting, weeding, staking, and harvesting) and storage problems as well as high post-harvest losses. The Use of edible yam mother tubers as planting materials constitutes very serious problems for low income farmers since the tubers are needed for consumption or for sale (Akoroda and Hahn, 1995). Another major constraint to yam production is the limited processing technologies. About 30% of harvested yam tubers are lost as waste because limited technologies exist for the production of shelf-stable processed yam products and productivity of yams in smallholder's conditions is low soil fertility (O'Sullivan and Ernest, 2008). The transmission of diseases and pests through tuber also a major problem. Although munch studies have not been conducted worldwide on *Dioscorea* of lesser yam. Preliminary survey showed that generally the pathogens infecting other yams infect lesser yam also.

2.10 PESTS AND DISEASES OF YAM

Pests and diseases of yam constitute a major constraint to yam production and storage. Over 25% of yield losses are due to diseases and pests during growth as well as post-harvest. Important yam pests include nematodes, beetles, termites, weevils, scale insects and rodents (Emehute, 1998). Diseases include those caused by fungi, bacteria and viruses (Hughes *et al.*, 1997).

The most serious nematode pest of yam are yam nematode (*Scutellonema bradys* Steiner and LeHew), *Pratylenchus* spp. and root knot nematodes (*Meloidogyne* spp.) (Caveness, 1992; Kwoseh *et al.*, 2007). They cause lesions beneath the tuber skin which are yellow at first, developing into dark brown dry rots which may cover the tuber surface in heavily infested tubers. The importance of infection depends on the

varietal susceptibility, with *D. alata* and *D. esculenta* being the most susceptible to nematode attack. Infection often starts before harvest and continues in storage, leading to a loss of food and planting material for the next season's crop.

The major insects known to attack yam are yam tuber beetles (*Heteroligus spp*), Froth beetle (leaf beetle) (*Criceris libido* Dalm), scale insect (*Aspidiella hartii*) and mealybug (*Planococcus citri*) (O'Hair, 1988). They feed on the yam tuber, reducing tuber quality and quantity, and leaving holes that serve as entry point for pathogens. Wounded or damaged tubers are susceptible to decay. The yam scale insect, infests tubers, and sometimes foliage, causing poor growth. Stored tubers are particularly susceptible to attack and large numbers of scale insects cause shrivelling.

The major fungal disease of yam, anthracnose, is caused by *Colletotrichum gloeosporioides* Penz. (Onwueme and Charles, 1994). It is widespread throughout the tropical countries and is the most prevalent fungal disease infecting yam worldwide. Brown spots occur on young leaves then enlarge and sometimes coalesce, as leaves approach maturity. Epidemics occur during prolonged rains. Young plant growth is infected and destroyed by rapidly expanding black lesions, and mature leaves of anthracnose-susceptible varieties rapidly blacken in response to sunlight and the presence of numerous *C. gloeosporioides* spores, which germinate (Winch *et al.*, 1993). Other fungal diseases of yam are leaf spot and leaf blight caused by *Curvularia* spp. (Onwueme, 1978).

A few bacterial pathogens have been reported, but only *Corynebacterium* species are pathogenic in the field (Emehute *et al.*, 1998). These bacteria are found in association with the yam nematode *S. bradys* and cause dry rot in storage (Ikotun, 1989). However, the main problematic bacterium in storage is *Erwinia carotovora* pv. *carotovora*, which causes tuber rot under high relative humidity and cooler temperatures and is also generally found in association with *S. bradys* (Ekundayo and Naqui, 1972).

2.10.1 Yam viruses

Yam viruses have been reported infecting different yam species in all the tropical regions where yam is grown. The first viral disease of yam was identified in Sierra Leone and Puerto Rico in 1936. Subsequent description of yam virus infection was in 1957 (Robertson, 1961). Viruses belonging to the genera of *Potyvirus*, *Badnavirus*, *Cucumovirus*, *Comovirus*, *Potexvirus* and *Maclura*virus infect yam in different parts of the world (Kenyon *et al.*, 2001). Symptoms associated with yam virus diseases include severe leaf chlorosis, green vein banding, mosaic, shoe stringing, interveinal chlorosis, stunting and distortion. These symptoms result either in the reduction of dry matter, the number of tubers, the size or the weight of the tubers depending on the pathosystem and in some instances, death of the plants (Jones *et al.*, 2008). Furthermore, the presence of yam viruses in tubers hinder the international trading of yam tubers and the international movement of yam germplasm required for research and improvement purposes.

Many surveys have reported the presence of several viruses and serious virus diseases on yams in the yam belt of West Africa, and other yam-growing areas of the world (Eni *et al.*, 2010). *Yam mosaic virus* (YMV), genus *Potyvirus*, family *Potyviridae* is a ubiquitous pathogen (Thouvenel and Fauquet, 1979) found in several species of *Dioscorea* and it is the only well characterized virus infecting yams. Other commonly encountered yam viruses are *Dioscorea alata virus* (DAV) or *Yam mild mosaic virus* (YMMV), *Dioscorea dumetorum virus* (DdV), genus *Potyvirus* (Hughes, 1986) *Cucumber mosaic virus* (CMV), genus *Cucumovirus* (Eni *et al.*, 2008a) and *Dioscorea alata bacilliform virus* (DaBV), genus *Badnavirus* (Kenyon *et al.*, 2008; Eni *et al.*, 2008b). These viruses are spread through seasons and fields, mostly through infected planting material, and their accumulation paralyses yam germplasm movement worldwide, by hindering national and international exchange of selected yam varieties, and also reduces yam production and productivity (Eni *et al.*, 2008c). They are also be

reported to be widespread in the major yam producing countries occurring as single as well as mixed infections (Odedara *et al.*, 2011).

Mixed-infections of viruses from the same or a different genus have been reported in yams. *D. alata potyvirus* (DAV, genus *Potyvirus*) was found in mixed infection with *Yam mosaic virus* (YMV, genus *Potyvirus*) or with *D. dumetorum potyvirus* (DDV, genus *Potyvirus*) in *D. alata* from Nigeria (Hughes *et al.*, 1997). Virus-virus interaction within a host during mixed infection may result in either synergism or antagonism (Carrillo-Tripp *et al.*, 2007; Murphy and Bowen, 2006), however, co-infection of a plant by different species of viruses often results in synergistic interactions that may result in more severe leaf symptoms culminating in greater yield losses (Vance *et al.*, 1995). Furthermore, mixed infections enhances the probability of genomic recombination which may result in more virulent strains of existing viruses or entirely new virus species. Synergistic interaction between yam viruses may frustrate efforts aimed at engineering virus resistance in yam. The presence of mixed-infections of viruses is considered to complicate identification of viruses by the disease symptoms as well as to increase the yield losses.

2.11 FAMILY: *POTYVIRIDAE*

Potyviridae family is the largest plant virus groups contains over 218 definite and tentative species found in all known taxonomical families of cultivated and non-cultivated plants (López-Moya and García, 2008). They account for about 20 percent of all the classified plant viruses and cause significant losses in agricultural, pasture, horticultural and ornamental crops. The viruses are non-enveloped flexuous filamentous particles of 200-300 nm length and 11 to 15 nm diameter. The length of the viruses range from 650 to 950 nm for those with monopartite genomes and 500 to 600 nm for those with bipartite genomes. Each virion comprises 1700 to 2000 coat protein subunits arranged in a helical manner around a single molecule of viral RNA (Shukla *et al.*, 1998; Ha, 2007).

All members of the Potyviridae family form pinwheel or scroll-shaped inclusion bodies in the cytoplasm of infected cells, but they are unique in the diversity of inclusion bodies. This cylindrical inclusions (CI) bodies are formed by a virus-encoded protein and can be considered as the most important phenotypic criterion for assigning viruses to the *Potyvirus* group. Most of the Potyviruses induce cytoplasmic amorphous inclusion bodies and some form nuclear inclusions (NIa or NIb) (Shukla, 1991).

Based on the amino acid sequences of their coat proteins, the *Potyviridae* are now divided into eight genera (ICTV, 2011) and distinguished on the basis of their transmission and genomic relatedness: *Potyvirus* (Gibbs *et al.*, 2008) and *Macluravirus* (Berger *et al.*, 2005) (transmitted by aphids); *Bymovirus* (Zheng *et al.*, 2002) (with two genomic RNAs and transmitted by plasmodiophorids); *Rymovirus* (French and Stenger, 2005) (transmitted by *Abacarus* mites); *Tritimovirus* (Stenger and French, 2004) and *Poacevirus* (Tatineni *et al.*, 2009) (transmitted by *Aceria* mites); *Ipomovirus* (Li *et al.*, 2008) (transmitted by whitefly); and *Brambyvirus* (Susaimuthu *et al.*, 2008) (vector unknown).

2.11.1 Genus: *Potyvirus*

Potyviruses (named after *Potato virus Y*) are one of the most economically important group of plant viruses and pose a threat to crops around the world (Larsen *et al.*, 2003). They are widespread in cultivated plants throughout all regions of the world, but as recent metagenomics studies have indicated, they are also found abundantly in the wild (Roossinck, 2012). Plants infected by potyviruses are more susceptible to fungal pathogens and occurs in complex with other unrelated viruses, the effect on yield quality and quantity are more severe.

The genome of potyviruses are flexuous rods about 750 nm long, 11-15 nm in diameter consist of capsid proteins arranged in helical symmetry around a single

stranded monopartite positive sense RNA of approximately 10,000 nucleotides (Dougherty and Carrington, 1988). The RNA genome has a 3' poly A tail and a genome-linked virion protein, VPg, that is covalently bound to the 5'end. The viral RNA is translated into a polyprotein which is cleaved by proteases encoded by the viral genome, into at least nine mature proteins (Jayaram *et al.*, 1992). Their life cycle consists of entry into cells, uncoating, translation, polyprotein processing, genome replication, virus particle assembly, and movement to other cells. During the life cycle, the viral genome is translated into one protein and then processed into eight different gene products. The genome-linked protein (VPg) and the coat protein (CP) are the only two protein products of the viral genome that are present in the virion (Shukla *et al.*, 1994).

Potyviral genome includes the P1, HC-Pro, P3, 6K1, CI, 6K2, NIa, NIb and CP encoding genes from the amino to the carboxy terminus. These genes function in genome amplification and bind RNA except for P3, 6K1 and 6K2. Proteins encoded by genes at the amino terminal region function in virus movement; those at the carboxy terminal regions are involved in replication. P1 is the amino terminal gene and encodes a trypsin-like serine proteinase that function in autocleavage from the polyprotein, but Helper component proteinase (HC-Pro) is a multicomponent proteinase involved in aphid transmission of the virus, genome amplification, polyprotein processing and long-distance transport of the virus in the host. However the cell-to-cell movement of virus in host plants is mediated by CI genes. The Nia is most important proteinase responsible for the cleavage of all proteins from polyproteins. They contain two domains: the VPg domain at its N terminal and the NIa protease at the C-terminal. NIb is a protein coded by a cistron from the *potyvirus* genome which is located between NIa C terminus and CP N-terminus and generally forms inclusions in the nucleus of infected plants. They interacts with NIa through the VPg domain and if mutations occurs causing loss of interaction between those two proteins it will led to temperature sensitive genome amplification (Urcuqui-Inchima *et al.*, 2001).

Coat protein (CP) is a well characterized potyviral protein, and is roughly divided into 3 domains: the N domain which is highly variable and contains the major virus specific epitopes; the core and C domains which are conserved. The variation in the core region is similar to that of the whole genome and, therefore, is a reliable index for genetic relatedness (Shukla *et al.*, 1998). CP plays a significant role in aphid transmission, cell to cell and systemic movement, virus encapsidation and in the regulation of viral RNA synthesis. The interactions between TVMV Nib protein and two other encoded proteins like NIa protein and the viral coat protein (CP) found that CP interacts with the NIb is sensitive in changes in the highly conserved GDD motif. However the functions of NIb protein or the CP is unclear, but CP is involved in the regulation of viral RNA synthesis in infected cells (Hong *et al.*, 1995).

A number of viruses belonging to the genus *Potyvirus* have been reported in yam: *Yam mosaic virus* (YMV), *Dioscorea alata potyvirus* (DaV), *Dioscorea green-banding mosaic virus* (DGBMV), *Nigerian yam mosaic* (YV-N), *Dioscorea green-banding virus* (DGBV), *Dioscorea trifida potyvirus* (DTV), *Dioscorea alata ring mottle virus* (DARMV), *Japanese yam mosaic virus* (JYMV) and *Dioscorea dumetorum potyvirus* (DDV). *Chinese yam necrotic mosaic virus* (CYNMV) is the only known yam virus associated to the genus *Macluravirus* (lebas, 2002).

Yam mosaic virus (YMV), which is the most fully characterized yam virus, was first reported infecting *D. cayenensis* in Côte d'Ivoire and also known to infect other yam species (Bousalem *et al.*, 2000b). It is a single stranded RNA virus, have rod shaped virion particles of 785 nm in length, transmitted by both mechanically and aphid vectors. They causes severe chlorosis, green vein banding, shoe-stringing and severe stunting that lead to a reduction in the photosynthetic ability of the crop, with deleterious effects on tuber yield (Odu *et al.*, 2001). Based on monoclonal antibodies reactivity, YMV isolates has been divided into two serogroups and into four groups by Western immunoblotting assay. The complete nucleotide sequence of YMV

Côted'Ivoire isolate has been published and further sequence analyses of other YMV isolates and other potyviruses have revealed the presence of consensus motifs characteristic of the *potyvirus* genus supporting the classification of YMV as a *potyvirus* (Aleman *et al.*, 1996; Duterme *et al.*, 1996). Genetic variability among YMV isolates is reported to be high and has been attributed to recombination events and the differential accumulation of mutations (Bousalem *et al.*, 2000b). Several potyviruses are related to YMV both in host range and serologically. One example is DGBMV from West Africa was found to be serologically related to YMV and seems to be identical with YV-N but it was not found in *D. trifida* and never in combination with CMV (Porth *et al.*, 1987). Among all the viruses identified to infect yam, YMV is the causal agent of the most important virus disease and causes very severe yield loss of over 50 % reported in *D. rotundata* (Amusa *et al.*, 2003).

Yam mild mosaic virus (YMMV) is the second most prevalent virus in yam after YMV and reported in in *D. alata* from Africa, Asia and Oceania (Atiri *et al.*, 2003). It was originally described as *yam virus I*, but is synonymous to *Dioscorea alata virus* (DaV) (Odu *et al.*, 1999), (Mumford and Seal (1997)) provided the first molecular evidence for the classification of YMMV as a distinct *potyvirus*. Analysis of the nucleotide sequence of the coat protein gene of YMMV revealed that it differs substantially from both YMV and *Japanese yam mosaic virus* (JYMV). It has a coat protein molecular weight of 32,100 daltons and is transmitted by *Aphis craccivora* Koch. DaV is serologically related to DGBMV and YMV but is clearly distinct from both potyviruses in terms of host range and transmissibility (Porth *et al.*, 1987). The partial cloning of the CP of DaV isolate from Martinique, showed a high percentage of similarity (94-97% amino acid) with the DaV isolate from Papua New Guinea (Fuji *et al.*, 1999a) and from Colombia (Dallot *et al.*, 2001). Widespread amongst *D. alata* in West African countries, strains of YMMV have also recently been identified in Sri Lanka and the South Pacific (unpublished data). However first molecular

characterisation showed a significant molecular divergence between isolates from Colombia, Martinique and Papua New Guinea (Dallot *et al.*, 2001).

2.11.2 Genus: *Macluravirus*

Members of the genus *Macluravirus* are characterized by the possession of slightly shorter filamentous particles (650-675 nm) than other members of *Potyviridae.* They are transmitted by aphids, even though the conserved amino acid motifs DAG or DTG involved in the aphid transmission being absent (Atreya *et al.*, 1990). The genome size is approximately 8 kb but the available sequence information about macluraviruses is limited to their 3'-terminal portion of the genome (except ChYNMV) (Kondo, 2001; Liou *et al.*, 2003). *Maclura mosaic virus* (MacMV) and *Narcissus latent virus* (NLV) are the only described viruses belonging to the genus. Then the 3'-terminal sequence analysis of *Cardamom mosaic virus* (CdMV) revealed that it is a new member of the genus *Macluravirus* (Badge *et al.*, 1997; Jacob *et al.*, 2001).

Narcissus latent virus (NLV) was first described in 1966, as a member of the *Carlavirus* genus (Brunt, 1967). They induces cylindrical cytoplasmic inclusions (CCIs) in infected cells and the nucleotide sequence of NLV 3' terminal region made it clear that it belonged to the genus *Macluravirus* (Mowat *et al.*, 1991).

Maclura mosaic virus (MacMV) was reported to cause mosaic symptoms on the leaves of the ornamental tree *Maclura pomifera* (Plese & Milicic, 1973) and to induce CCIs (Plese and Wrischer, 1978). MacMV has flexuous filamentous particles of 650±710 nm long with monopartite genomes of coat protein size 45 kDa (Plese *et al.*, 1979). It was identified as a member of *Macluravirus* genus of the *potyviridae* family by the 3'-terminal sequence analysis of the RNA genomes of the virus (Badge *et al.*, 1997).

Chinese yam necrotic mosaic virus (ChYNMV) causes necrotic mosaic disease in yams with 30-45% yield reduction (Tochihara, 1993). It was first presumed to be a

member of the genus *Carlavirus.* However, nucleotide sequencing of the 3 - terminal portion of the genome revealed that it is a member of the genus *Macluravirus* in the family *Potyviridae.* They are flexuous, filamentous particle, of 660 nm in length with a single 38 kDa coat protein and is transmitted by aphids in a non-persistent manner (Shirako and Ehara, 1986). ChYNMV coat protein showed 41.3-47.2% identity with the macluraviruses while low identity with all the other genera in the *Potyviridae* (16.0–22.9%) (Badge *et al.*, 1997). ChYNMV is currently the only *Macluravirus* whose complete genome has been sequenced and its genome size (8224 bp) indicates that macluraviruses have the smallest genomes in the family *Potyviridae* (Kondo and Fujita, 2012). The ChYNMV genome contains one long open reading frame encoding a large polyprotein of 2620 amino acids. ChYNMV has no counterpart to the P2 cistron and a short HC-Pro cistron located at the 5' side of the *Potyvirus* genome (Kondo and Fujitha, 2012).

2.12 FAMILY: *CAULIMOVIRIDAE*

The family Caulimoviridae encompassing plant pararetroviruses, replicating through an obligatory RNA intermediate. They possess an open-circular double-stranded DNA (dsDNA) genome of 7-8.3 kbp, replicating via a reverse transcription. It is divided into six genera: *Caulimovirus*, *Soymovirus*, *Cavemovirus*, *Petuvirus*, *Badnavirus* and *Tungrovirus* (Fauquet *et al.* 2005). Out of six genera, three genera have exist as integrated forms under conditions of genomic or environmental stress, excise from the host genome and generate episomal virus infection (Hansen and Heslop, 2004). Such endogenous pararetroviruses (EPRVs) form a distinct class of retroelements found in a variety of crop and ornamental plants.

2.12.1 Genus: *Badnavirus*

Badnaviruses are the second genus of plant viruses considered as emerging pathogens in tropical countries (Borah *et al.*, 2013). They possess a circular double

stranded DNA genome encapsidated in nonenveloped bacilliform particles of 130 x 29-30 nm in size associated with *Internal brown spot bacilliform virus* (IBSV) and *Dioscorea alata bacilliform virus* (DaBV) (Hull *et al.*, 2005). *Badnavirus*-like particles were first reported in yam in 1970s, associated with internal brown spot diseases in *D. alata* and *D. cayenensis* plants from Caribbean and serologically related *badnavirus*-like particles are also detected in other *Dioscorea* species.

The genome sizes of DaBV and DsBV were shown to be approximately 7.4 kb and 7.26 kb, respectively, with 61.9% nucleotide identity. Compared with other previously analysed badnaviruses, both viruses were found to share the highest level of nucleotide identity with *Cocoa swollen shoot virus* (CSSV) (Seal and Muller, 2007). DABV show sequence variability of 11% due to the replication by reverse transcription, which is typical to the genus *Badnavirus*. All badnaviruses have three ORFs with three discontinuities (gaps) at specific sites (Hagen *et al.*, 1993). The functions of ORFs I and II remain unclear. However, the ORF I protein of CoYMV is associated with immature virions whereas ORF II is found with both mature and immature virions. The predicted amino acid sequence of ORF III which encodes a putative polyprotein contains several conserved motifs characteristic of caulimoviruses and badnaviruses (Briddon *et al.*, 1999).

The first yam *badnavirus* was characterised in *D. alata* from Nigeria, designated as *Dioscorea alata bacilliform virus* (DaBV) [Phillips *et al.*, 1999]. It is transmitted mechanically as well as mealybug *Planococcus citri* from *D. alata* to some other *Dioscorea* species causes leaf distortion, crinkling, mottiling and veinal chlorosis. Internal brown spot disease of yam is believed to result from a mixed infection caused by a *Bacillivirus* particle and a *Potyvirus*. It was first reported in *D. alata* and later in *D. cayenensis* (Mohamed and Mantel, 1976). DaBV is serologically related to a distinct *Bacilliform virus* from *D. bulbifera* designated as *Dioscorea bulbifera badnavirus* (DbBV) detected in Puerto Rico and the South Pacific Islands. However, there is no

sequence information is publicly available for DbBV and only DaBV has been assigned badnavirus species status (Hull *et al.*, 2005). Recently the complete genome of a second yam *badnavirus* species from D. sansibarensis has been sequenced and named as *D. sansibarensis bacilliform virus* (DsBV) (Seal and Muller, 2007). The wide geographical distribution of yam badnaviuses is possibly due to international exchange of infected germplasm. Molecular studies have revealed very high genetic variability within the *badnavirus* genus (Lockart and Olszewski, 1993; Geering *et al.*, 2000). Immunocapture PCR was good method for detection of *badnavirus* because it is reported to considerably decrease non-specific PCR amplifications favouring the amplification of DNA contained within capsids and excluding DNA derived from other genomes thus ensuring that only episomal virus sequences are amplified. This is particularly important for the detection of badnaviruses whose sequences have been reported to be integrated into host genomes (Geering *et al.*, 2001). IC-PCR also has the advantage of eliminating, in the washing step, host contaminants that may interfere with PCR.

2.13 DIAGNOSTIC TECHNIQUES

Virus infections affect quality and quantity of agricultural products around the world. Unlike other plant pathogens, there are no direct methods available yet to control viruses. The current measures rely on indirect tactics to manage viral diseases. Hence, methods for detection and identification of viruses play a critical role in virus disease management. Diagnostic techniques for viruses fall into two broad categories: biological properties related to the interaction of the virus with its host and/or vector (eg. symptomatology and transmission tests) and intrinsic properties of the virus itself (coat protein and nucleic acid). Detection methods based on coat protein include precipitation or agglutination tests, enzyme-linked immunosorbent assays, and immunoblotting. Viral nucleic acid-based techniques like dot-blot hybridization assays and polymerase chain reaction are more sensitive than other methods. Diagnosis of

yam virus infection and identification of the causal agent(s), either/both in the field and the laboratory, is based on symptomatology, use of indicator plants, vector transmission, electron microscopy and protein and nucleic acid-based diagnostics (Mumford and Seal, 1997). Knowledge of viral infection is a prerequisite for the safe germplasm movement and to control the spread of viruses. Diagnostic techniques for virus identifications should be sensitive, reliable and also rapid and easy to use (lebas, 2002).

2.14 DIAGNOSIS OF YAM VIRUSES

Accurate and rapid detection of yam viruses is vital for disease management and control, but the diagnosis of yam viruses poses a number of problems. Variability of symptoms caused by changes in environmental factors, differences in the yam cultivars or varieties and/or the strains of the virus (es) make field diagnoses unreliable. Nutrient deficiency symptoms are also sometimes confused with virus symptoms and asymptomatic infections are also known to occur (Bock, 1982; Brunt *et al.*, 1990). Recent advances in immunology, biotechnology and molecular biology have played a significant role in the development of rapid, specific and sensitive assays for the detection of plant viruses. The assays currently available for yam virus diagnosis can broadly be divided into: bioassays (biological tests), immunoassays and nucleic acid-based techniques.

2.14.1 Bioassays

Biological methods of yam virus detection and diagnosis include symptomatology and transmission studies (Terry, 1976). Prior to the development of protein and nucleic acid-based detection methods, visual observation and herbaceous indicator plants were used to diagnose virus infections (De Leeuw, 1972). While these methods are still useful and are useful in the preliminary stages of research on new viruses, they are neither efficient nor consistent enough to be routinely used for virus

identification and detection, and are therefore not useful for the certification of yam planting materials.

2.14.1.1 Symptomatology

Diagnosing virus diseases begins with the proper identification of plant viruses associated with the disease. One of the earliest methods in plant virus diagnosis, which is still practiced today, is the differentiation of plant viruses using a range of symptom expressions and biological activities on inoculated indicator test plants. It is very important to determine if the symptoms are local or systemic and they need to be differentiated from similar symptoms caused by insect pest, nutrient deficiency or herbicide injury (Matthews, 1991).

The most common external symptoms in plants caused by plant viruses are color deviations which result from chloroplast disorders. As the viral infection progresses, the tissue adjacent to the site of infection could become yellow. It cause a wide variety of symptoms, including mosaic/mottling, stunting, leaf deformation, petal-color breaking, chlorotic and necrotic lesions, ringspots, vein banding, reduction in yield, wilting and in many cases, combinations of these symptoms (Gonsalves, 1989). The most commonly used indicator plants to distinguish plant viruses are from the families *Chenopodiaceae, Solanaceae, Cucurbitaceae* and *Fabaceae.* Virus strains can also react differently to different cultivars of a crop and vice versa. Strains are often defined by the reaction of a set of differential cultivars of the same crop species. For example, *Soybean mosaic virus* (SMV) strains can be characterized based on severity and pathogenicity on different soybean cultivars (Ma *et al.*, 2003). However, symptomatology do not give definitive answers on virus identification and must be used in conjunction with other diagnostic procedures. Nevertheless, these biological approaches play a significant role by detecting differences between strains and pathotypes of plant viruses that may not be detected by other methods (Chang, 2009).

2.14.2 Immunoassays

Immunoassays are useful tool for detecting and monitoring virus diseases, particularly for those infecting yam, and for routine testing of yam samples for the presence of viruses. The most common serological tests for plant viruses include precipitation and agglutination tests, immunosorbent electron microscopy, enzyme linked immunosorbent assays (ELISA), tissue blot immunosorbent assay (TBIA) and dot blot immunoassay. These techniques are based on an antigen-antibody binding reaction between epitopes on the surface of virus particles and the binding sites of specific antivirus antibodies. Among the serological tools, ELISA is often the method of choice because of its high sensitivity, simplicity, reproducibility and versatility in screening a large number of samples (Cho, 1990).

2.14.2.1 Enzyme-Linked Immunosorbent Assay (ELISA)

The sensitivity of antigen-antibody reaction can be greatly increased with the addition of a labeled probe. This was the premise for the enzyme-linked immunosorbent assay (ELISA) (Clark and Adams, 1977). The assay is conducted in microtiter plates, commonly with alkaline phosphatase and a substrate that is catalyzed to a yellow color relative to the amount of antigen present. The principle of ELISA is to immobilize an antigen (usually in this case, the virus) from extracted sap onto the surface of wells of polystyrene or polyvinyl plates and to wash off unbound substances from the plate, leaving only the specific antigen of interest. The antigen is then detected by enzyme-linked antibodies. ELISA is used mainly to confirm the presence or absence of viral protein(s) and can be adapted to estimate the concentration of a virus protein in plant sap (Copeland, 1998). An antigen, which usually consists of purified virus nucleoprotein particles, is injected into an appropriate animal to induce the production of antibodies. Two types of antibodies can be made: polyclonal antibodies (PAbs) that consist of a population of antibodies that bind to different regions of the antigen protein and monoclonal antibodies (MAbs) that consist of one type of antibody that binds to

one specific region on the antigen protein. MAbs have been widely used, to study antigenic differences and relationships (potyviruses that infect cucurbits) among virus strains while Polyclonal antisera are specific for several virus species and strains that infect tropical crops (Baker *et al.*, 1991). Protein-based diagnostics are suitable for routine identification of many yam viruses (Hughes *et al.*, 1998) but require high quality polyclonal antibodies that are free from contaminating antibodies against plant proteins and specific monoclonal antibodies due to the diverse strains of different yam viruses, particularly *Yam mosaic virus* (YMV) (Bousalem *et al.*, 2000a).

Although many variations have been used, there are mainly two variants. The direct ELISA procedure involves the direct detection of an antigen by an enzyme-labeled specific antibody, while the indirect procedure involves the detection of an antigen by a specific antibody, which is then detected by an enzyme-labeled, anti-immunoglobulin antibody. The direct methods are highly serotype specific whereas indirect methods are less specific (Van Regenmortel and Dubs 1993). Direct ELISA of double antibody sandwich (DAS)-ELISA and indirect ELISA like protein A sandwich (PAS)-ELISA and triple antibody sandwich (TAS)-ELISA antisera are available for the detection of yam viruses.

DAS-ELISA was first used for the detection of *Arabis mosaic virus* (AMV), an isometric virus, and *Plum pox virus* (PPV), a filamentous virus (Voller *et al.*, 1976). The antibodies (IgG) bound to the well surface of microtitre plate capture the virus in the test sample. The captured virus is then detected by incubation with an antibody-enzyme conjugate followed by addition of color development. However capturing and detecting antibodies can be the same or from different sources. This is highly strain-specific and requires each detecting antibody to be conjugated to an enzyme. TAS-ELISA, is more specific and sensitive than PAS-ELISA because of the use of monoclonal antibodies (Mabs), which allow the detection of specific virus strains. They are used for the detection plant viruses such as *Peanut clump virus* (PCV) in groundnut

(Manohar *et al.*, 1995), BSV in banana (Thottappilly *et al.*, 1998). TAS-ELISA entails the combination of PAbs and MAbs, where the virus is trapped to the specific MAbs. ELISA assays employ alkaline phosphatase or horseradish peroxidase as antibody–enzyme conjugates.

ELISA are also very useful for routine diagnosis of badnaviruses. This screening technique indicated the presence of *badnavirus* in *D. alata* samples taken from seven south specific islands (Kenyon *et al.*, 2008). MacMV is a member of the potyvirus genus due to the presence of CCIs in infected tissue and weak cross-reaction with antisera to *Bean yellow mosaic potyvirus* (Plese *et al.*, 1979). To establish the serological features of RLV and *R. asiaticus* double-antibody sandwich (DAS)-ELISA, antigen coated plate (ACP)-ELISA, and western blot analysis were used (Turina *et al.*, 2006). Serological methods are not particularly successful when used for potyviruses, and determining the relationships of *potyvirus* species. This is because the dominant epitopes of *potyvirus* virions are the variable terminal parts of the virion protein, especially the N-terminal part, which has a repetitive sequence in some species (Ward *et al.*, 1995).

2.14.3 Nucleic acid based techniques

Nucleic acid-based approaches are used extensively for detection and identification of plant viruses, since the advent of the polymerase chain reaction (PCR). First described in the 1980s by Mullis and has become a powerful technique with great impact on molecular biology. It was first used for the amplification of ß-globulin genomic sequences for diagnosis of sickle cell anemia (Saiki *et al.*, 1985) but has been employed for the detection and differentiation of plant pathogens (Lopez *et al.*, 2003). This sensitive *in vitro* method that amplifies trace amounts of DNA to detectable levels using generic or gene-specific primers to the region of amplification, and Taq DNA polymerase (Hadidi *et al.* 1995). The advent of PCR in the diagnosis of plant viruses

has greatly improved the sensitivity, rapidity, specificity and reliability of virus detection (Choi *et al.*, 1999).

The principle of PCR amplification requires deoxynucleotide triphosphates (dNTPs) for the DNA extension, an optimised buffer containing magnesium, reverse and forward primers, which are complementary to the opposite strands of DNA and a thermostable DNA polymerase enzyme (*Thermus aquaticus* (*Taq*)) (Gelfand and White, 1990). Isolated DNA goes through temperature cycles. First DNA strands are disassociated at high temperature (94-96 °C). The temperature is then lowered (50-55 °C) to allow primers to anneal to the DNA strands. Finally, the DNA is subjected to the optimum extension temperature for the polymerase (usually 72 °C). The three steps are repeated 30-45 times, allowing an exponential accumulation of DNA fragments and the total amount of product synthesized would be between 1.65^{n} and 1.85^{n}. Amplified products are analyzed by agarose gel electrophoresis with DNA markers to allow the size of the PCR products to be estimate.

PCR will only work directly for the amplification of DNA viruses such as *Caulimo, Gemini,* and *Badnavirus*. *Badnavirus* genomes being highly variable, so primers were designed from conserved regions in ORF III (Medberry *et al.*, 1990). It was used for the detection, of BSV, ScBV, RTBV and DABV (Briddon *et al.*, 1999). The use of primers designed from the alignment of conserved BSV sequences (Lockhart and Olszewski, 1993) allowed the identification of new *Badnavirus* such as *Citrus mosaic badnavirus* (CMBV), *Piper yellow mottle virus* (PYMV) and *Gooseberry vein banding associated virus* (GVBAV) (Jones *et al.*, 2001).

However, *Potyvirus* genomes are RNA based so that viral RNA is first reverse transcribed into complementary DNA (cDNA). The cDNA provides a suitable DNA target for subsequent amplification called reverse transcription-PCR (RT-PCR). Primers used in PCR can be species-specific or group-specific, such as *potyvirus* degenerate primers (Webster *et al.*, 2007). Distinct potyviruses were identified from

mixed-infected sweet potato by RT-PCR using degenerate primers designed to amplify the variable 5'-terminal region of the CP together with the 3'-terminal region of the NIb protein of potyviruses (Colinet and Kummert, 1993). These primers have also been used for the characterization of YMV isolates (Bousalem *et al.*, 2000a).

The PCR technique can also be combined with serological procedures in the form of immunocapture (IC-PCR), combining the advantages of serology with the sensitivity of PCR to detect diseases and identify the causal agents in which the washing steps eliminate plant contaminants (Nemchinov *et al.*, 1995). It has been developed for the detection of yam infecting potyviruses by Mumford and Seal (1997), YMV (Bousalem *et al.*, 2000b) and *Badnavirus* (Hoffmann *et al.*, 1997). They decreases the non-specific reactions due to high degeneracy of primers with increase the specificity of the test and so would not amplified integrated genome of badnaviruses (Geering *et al.*, 1997). The use of IC-PCR for the detection of episomal virus was efficient because the antisera did not capture any *Musa* nuclear, mitochondrial or chloroplasts genomes, which may contain BSV integrated sequences (Harper *et al.*, 1999a). The IC-RT-PCR technique was five times more sensitive than the purely protein based serological detection methods. TAS-ELISA, DTBI and symptomatology were more suitable in detecting YMV in yam tubers than ICRT-PCR and ISEM because of their sensitivity and reliability. The DTBI assays were very useful for the detection of YMV in yam tubers and tuber sprouts (Njukeng *et al.*, 2005).

Recently, a novel real-time quantitative PCR assay (TaqMan technology) was developed for the detection and quantification of plant viruses (Eun *et al.*, 2000; Roberts *et al.*, 2000). However Multiplex-(RT)-PCR (M-(RT)-PCR) allows the detection of more than one virus. It was developed for the simultaneous detection of RNA and/or DNA viruses. They require more than one pair of primers and the concentration of dNTPS, magnesium and *Taq* polymerase are high. M-(RT)-PCR has been successfully used for the detection of four viral genera (Nie and Singh, 2000).

Moreover, M-(RT)-PCR was combined with an immunocapture step for the detection of RNA and DNA viruses infecting banana (Sharman *et al.*, 2000).

2.15 PRIMER DESIGNING

Primers are short oligonucleotide sequences amplifying the target region of the template and anneal exclusively to that DNA target locus (Lexa, 2001). Primer pair is one of the most important parameters namely forward and reverse, will be extended towards each other to cover the given target region. Selection of oligonucleotide primers is useful for polymerase chain reaction (PCR), oligo hybridization and DNA sequencing. The efficiency of primers to be able to amplify a product exponentially to be balanced while designing a primer. However designing a primer should fulfill certain criteria such as primer length, GC%, annealing and melting temperature, 5' end stability, 3' end specificity etc (Dieffenbach *et al.*, 1993).

Primers of typically 18-30 nucleotides in length are the best and at least 18 nucleotides in length to minimize the chances of problems with a secondary hybridization site on the vector or insert. The optimal melting temperatures for a primer pair is 52-58 °C while above 65 °C should leads to secondary annealing (Wallace *et al.*, 1979). Usually the GC content of a primer is between 45-60 percent while high G/C content in the 3'ends of primers is not desirable as it will lead to mispriming. So a "G" or "C" is desirable at the 3' end of primers since this will reduce "breathing" and thereby increase yield. GC content, melting temperature and annealing temperature are strictly dependent on one another (Rychlik *et al.*, 1990), thus the annealing temperature is generally calculated as 5 °C lower than the estimated Tm. Within a primer pair, the GC content and Tm should be well matched (Abd-Elsalam, 2003). The intra-primer or inter-primer homology should be kept as low as possible to avoid formation of hairpin structures or primer dimers which will interfere with annealing of primer to the DNA template.

Several degenerated primers have been designed to recognize the conserved regions of viral genomes of many virus species or the whole virus genus or family (Chen *et al.*, 2001). Universal primers and species-specific probes (Harrington *et al.*, 2000) have been designed to identify potyviruses based on the conserved sequences of CP gene (Zerbini *et al.*, 1995). WCIEN primers (Pappu *et al.*, 1993), were routinely used for detection and characterization of *potyvirus* in research and diagnostic laboratories. However degenerate oligonucleotide primers to clone the conserved amino acid sequences of the virion proteins together with the 3' untranslated region of the genome, as sequences of this region are useful for discriminating *Potyvirus* strains. Thus the same primers have also been used for the characterization of the YMV isolates (Bousalem *et al.*, 2000b).

2.16 NUCLEOTIDE SEQUENCING

Nucleotide sequencing is the most reliable technique for the detection of mutations or polymorphisms in viral genomes. Genome replication is the most fundamental and conserved function in positive-stranded RNA viruses, and so sequencing is extensively used to elucidate evolutionary relationships between different RNA viruses and their geographical distribution. Cloning is the method whereby individual DNA molecules can be faithfully copied in vivo to produce numerous identical molecules or clones. The DNA molecule containing the sequence of interest can be isolated from a mixture and propagated in a suitable host cell. This yields a limitless supply of this molecule and enables the gene or genes contained within it or any protein product to be studied. Blue white screen is one of the most common molecular techniques that allow detecting the successful ligation of gene of interest in vector or screening the recombinants. The cloned DNA is sequence by different methods, dideoxy chain-termination reaction method is an enzymatic method using DNA polymerase for DNA sequencing first introduced by Sanger (Sambrook *et al.*, 1989). The sequenced nucleotides are then compared to related virus species or

strains by the alignment of the nucleotide sequences and finally phylogenetic trees can be construct which estimate the evolution of viruses (Astier *et al.*, 2001e).

Among characterised potyviruses in *Dioscorea* species, nucleotide sequences of YMV, DAV, JYMV and DDV have become available in GenBank database. The complete nucleotide sequence of YMV isolate from the Ivory Coast was first determined by (Aleman *et al.* (1996)). More recently, the genetic variability of the C terminal part of the NIb, CP and the 3'-untranslated region (3'-UTR) of 27 YMV isolates collected in three *Dioscorea* species from Africa, the Caribbean and French Guyana was assessed (Bousalem *et al.*, 2000a). A fourth YMMV isolate from Japan was partially sequenced (Fuji *et al.*, 1999, direct submission to GenBank). The sequence differences of YMMV isolates showed that it is a different species from YMV and JYMV. The complete nucleotide sequences of JYMV-J1 and JYMV-M have also been determined (Fuji and Nakamae, 2000). JYMV is a member of the genus *Potyvirus* but is distinct to YMV and of YMMV isolates from Martinique (Bousalem and Dallot, 1999) and Papua New Guinea (Fuji *et al.*, 1999).

The sequencing of CYNMV genes has shown that the amino acids of the CP displays 41.3-47.2% with macluraviruses, but only 16.0-22.9% with those of other genera on the *Potyviridae*, and thus it was concluded to be a *Macluravirus* (Kondo, personal communication). The complete nucleotide sequence of DABV isolate was determined and has a genome size of 7.4 kbp. However, the presence of a different yam bacilliform virus was indicated by DaBV being serologically related to a *Badnavirus* from *D. bulbifera* named *Dioscorea bulbifera badnavirus* (Briddon *et al.*, 1999).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 LOCATION

The study entitled "Identification and characterization of viruses infecting lesser yam (*Dioscorea esculenta* (Lour.) Burkill)" was conducted at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute (CTCRI), Sreekariyam, Thiruvananthapuram during September 2014-June 2015. Details regarding the experimental materials and methodology used in the study are elaborated in this chapter.

3.2 SAMPLE COLLECTION

The leaf samples and tuber samples used in this study were collected from the lesser yam (*Dioscorea esculenta* (Lour.) Burkill) growing fields at ICAR-CTCRI, Sreekariyam, Thiruvananthapuram. Young leaves showing symptoms such as mosaic, puckering, cupping and curling of leaves were collected, recorded and stored for analysis and further studies. Plants without observable symptoms were also sampled to check the possibility of latent infection.

3.3 SEROLOGICAL METHODS OF DETECTION

Enzyme Linked Immunosorbent Assay (ELISA) such as Double Antibody Sandwich ELISA (DAS-ELISA) and Triple Antibody Sandwich ELISA (TAS-ELISA) were employed for detection of virus infection in *Dioscorea esculenta* leaf samples. DAS-ELISA was used for the detection of YMMV and *Yam macluravirus* while TAS-ELISA was used for the detection of *Yam badnavirus*. Virus specific antibodies obtained from DSMZ, Germany was used for this study.

3.3.1 Double Antibody Sandwich ELISA (DAS-ELISA) for YMMV

Two hundred microlitre of purified *Yam mild mosaic virus* IgG polyclonal antibody diluted to 1:1000 in coating buffer (Appendix I) was coated in the wells of microtitre plate and incubated at 37 °C for 4 h. Leaf sap extract (200 µl) prepared by grinding 0.5 g of virus infected leaves in 1ml sample extraction buffer (Appendix I) followed by centrifugation at 8000 rpm for 10 min was loaded in triplicate wells and incubated overnight at 4 °C. After incubation 200 µl of YMMV IgG Alkaline Phosphate (AP) diluted to 1:500 in conjugate buffer (Appendix I) was added to the wells and the plates were incubated at 37 °C for 3 h. Similarly, 200 µl aliquots of freshly prepared substrate (Appendix I) was added to each well and incubated at room temperature for 30-60 min or as long as necessary to obtain clear reactions.

The plates were washed with PBS-Tween-20 thrice at three min interval after each incubation step and blotted dry by tapping upside down on tissue paper. The A_{405} for sample in each well was measured in a BIO-RAD i Mark Microplate Reader (USA). The readings were taken at 0 h, 30 min, 1 h, 2 h and overnight.

3.3.2 Double Antibody Sandwich ELISA (DAS-ELISA) for *Yam Macluravirus*

The wells of microtitre plates were coated with 200 µl of purified Yam Maclura IgG polyclonal antibody diluted to 1:1000 in coating buffer (Appendix I) and incubated at 37 °C for 2-4 h. After incubation, the plates were washed with PBS-Tween-20 thrice at three min interval and blotted dry by tapping upside down on tissue paper. 200 µl of leaf sap extract prepared by grinding 0.5 g test leaves in 1ml sample extraction buffer (Appendix I) followed by centrifugation at 8000 rpm for 10 min was loaded in duplicate wells and incubated overnight at 4 °C. On the next day, the above washing step was repeated until the whole sample had been processed. Similarly, 200 µl of *Yam Maclura* IgG Alkaline Phosphate (AP) diluted to 1:500 in conjugate buffer (Appendix I) was

added to the wells and the plates were incubated at 37 °C for 3 h with washing using PBS -Tween-20. Then 200 µl aliquots of freshly prepared substrate (Appendix I) was added to each well and incubated at room temperature in dark condition. The A_{405} for sample in each well was measured in a BIO-RAD i Mark Microplate Reader (USA). The readings were taken at 0 h, 30 min, 1h, 2 h and overnight.

3.3.3 Triple Antibody Sandwich ELISA (TAS-ELISA) for *Yam Badnavirus*

Twenty five leaf samples showing symptoms of virus infection and a healthy non host were taken for TAS-ELISA using Badna antibody. Wells of microtitre plates were coated with 200 µl of purified Badna IgG polyclonal antibody diluted to 1:1000 in coating buffer and incubated at 37 °C for 2-4 h. After incubation the plates were washed with PBS-Tween-20 thrice at three min interval and blotted dry by tapping upside down on tissue paper. The plates were blocked with 200 µl of blocking solution (Appendix I) for 30 min at 37 °C. It was then removed and blotted dry by tapping upside down on tissue paper. Leaf sap extract (200 µl) prepared by grinding test leaves in sample extraction buffer (Appendix I) was loaded in triplicate wells and incubated overnight at 4 °C. Then the plate was washed thrice with PBS-Tween-20 at 3 min interval and dry. This was followed by incubating the plates with 200 µl secondary antibody (MAb) diluted to 1:100 in conjugate buffer at 37 °C for 2-4 h. It was then washed thrice with PBS-Tween-20 at 3 min interval and incubated with 200 µl RaM-ap diluted 1:1000 in conjugate buffer at 37 °C for 2 h to each wells of microtitre plates. After rinsing thrice with PBS-Tween-20 and dry , the microtitre plates were added with 200 µl of freshly prepared substrate (Appendix I) to each well and incubated at room temperature for 30-60 min or as long as necessary to obtain clear reactions.

The A_{405} for sample in each well was measured in a BIO-RAD i Mark Microplate Reader (USA). The readings were taken at 0 h, 30 min, 1 h, 2 h and overnight.

3.4 NUCLEIC ACID BASED DETECTION

For the detection of yam viruses like *Yam mild mosaic virus, Badnavirus,* and *Macluravirus,* infecting *Dioscorea esculenta,* PCR and RT-PCR was performed on DNA and RNA isolated from infected leaf samples using species specific primers.

3.4.1 Isolation of total DNA

Virus infected samples of *Dioscorea esculenta* plant were taken for DNA isolation. CTAB method were used to isolate genomic DNA from samples collected at ICAR-CTCTRI lesser yam growing fields.

3.4.1.1 CTAB Method of DNA Extraction (Lodhi *et al.*, 1994)

Virus infected leaf sample of 100 mg was chilled and pulverized to a fine power in liquid nitrogen using a sterile mortar and pestle and transferred into a sterile 2 ml centrifuge tube. Followed that 1 ml of extraction buffer (pre-warmed at 65 °C) (Appendix II) was added to the tissue powder. Crude extract (750 μl) was transferred into a fresh 2 ml tube and incubated at 65 °C (Lab Companion BS-31 shaking waterbath, Canada) for 30 min to lyse cells. The tube was centrifuged at 10000 rpm (Kubota High Speed Refrigerated Centrifuge 6500, Japan) for 10 min at 27 °C and supernatant was transferred to a fresh 2 ml tube. After transferring the supernatant into fresh tube, 10 μl of RNase was added and incubated at 37 °C for 1 h. Equal volume of chloroform: isoamyl alcohol (24:1) was added to the supernatant and centrifuged at 15000 rpm for 10 min at 4 °C. Then transferring the aqueous layer or supernatant into fresh tube, 0.8 volume of ice cold isopropanol was added to the supernatant and incubated at -20 °C for at least 1h or overnight. Following centrifugation at 12,000 *g* or 15000 rpm for 10 min at 4 °C, the pellet was washed with 0.5 ml of 70 percent ethanol by centrifuged at 12,000 *g* for 5 min at 4 °C. The washing was repeated with 0.5 ml of 75 percent ethanol. DNA pellet obtained was air dried at 37 °C for 30 min

and then dissolved in 50 µl sterile distilled water or 1X TE buffer. After incubating at 37 °C for 1 h, the DNA was stored at -20 °C. The quality of the isolated DNA was checked on 0.8% agarose gel.

3.4.2 Isolation of total RNA

Leaf and tuber sample of lesser yam plants showing severe mosaic, puckering symptoms were taken for RNA isolation. From the leaf samples RNA was isolated using Ambion Purelink RNA Mini Kit and TRIzol based method while $LiCl_2$ based method were used to isolate RNA from tuber samples. The isolated RNA was stored at -20 ^{0}C.

3.4.2.1 Ambion Purelink RNA Mini Kit (Life Technologies, USA)

Leaf sample weighed 80-100 mg was taken and ground into fine power with liquid nitrogen using mortar and pestle. The powdered tissue was transferred to an RNase free micro centrifuge tube and 1000 µl of lysis buffer with 10 µl of β-mercaptoethanol was immediately added to it and vortexed (Labnet Vortex Mixer, USA) briefly. After centrifugation at 20,000 *g* (Kubota High Speed Refrigerated Centrifuge 6500, Japan) for 7 min at room temperature, the supernatant was transferred to a fresh tube. To the supernatant, equal volume of 70 percent ethanol was added and mixed thoroughly to dissolve the precipitate. Then, 700 µl of the sample was transferred to the RNA spin cartridge inserted in a collection tube. Following centrifugation at 12,000 g for 30 s at room temperature, the flow through was discarded and the cartridge was reinserted in a collection tube. The above step was repeated until the whole sample had been processed. Then 700 µl of the wash buffer 1 was added to the spin cartridge and centrifuged at 12,000 g for 30 s. The flow through was discarded and the spin cartridge was placed into a clean collection tube provided in the kit. 500 µl of the wash buffer II was added to the spin cartridge and centrifuged at 12,000 g for 30 s at room temperature. The flow through was discarded and cartridge was reinserted

and repeated the above step once again. Then the empty spin column was centrifuged at 12,000 g for one minute at room temperature. The collection tube was discarded and the cartridge was inserted into a recovery tube and air dried for 10 to 15 min. Then 30 μl of the elution buffer (RNase free water) was added to the center of the spin cartridge and was incubated at room temperature for one minute followed by centrifugation at 12,000 g for one minute to obtain the first elute of RNA. Finally 20 μl of the elution buffer was added to the spin cartridge and centrifuged at 12,000 g for one minute at room temperature to obtain the second elute. The quality of the isolated RNA was checked on agarose gel (1.5%).

3.4.2.2 Manual methods of RNA isolation

3.4.2.2.1 TRIzol method

The leaf sample (100 mg) was taken and ground into fine power with liquid nitrogen using mortar and pestle. The powdered tissue was transferred to a fresh tube to which 1 ml of TRIzol (Life Technologies, USA) reagent was added and incubated at room temperature for five minute. Then 200 μl of chloroform was added, shaken vigorously and incubated at room temperature 2-3 min. After centrifugation at 12,000 g (Kubota High Speed Refrigerated Centrifuge 6500, Japan) for 15 min at 4 ^{0}C, three layers appear the colourless upper aqueous phase was transferred into a new tube. Flowing that 500 μl of isopropanol was added, incubated at 12000 g for 10 min at 4^{0}C. The supernatant was discarded and to the RNA pellet, 1 ml of 75 percent ethanol was added and vortexed (Labnet Vortex Mixer, USA), the tubes was centrifuged at 7,500 g for five minutes at 4 ^{0}C. This washing procedure was repeated until the RNA pellet obtained is pure. The RNA pellet was air dried for 5 to 10 min then resuspended in 40 μl of nuclease free sterile water and incubated at 55-60 ^{0}C (Lab Companion BS-31 shaking waterbath, Canada) for 10-15 min and the quality of the isolated RNA was checked on agarose gel (1.5%).

3.4.2.2.2 Lithium Chloride Method

The tuber tissue was sliced with sterile razor blade and 500 mg of fresh tissue was ground into fine powder using liquid nitrogen. The tissue powder was transferred into a sterile 2 ml centrifuge tube. One ml of extraction buffer (pre-warmed at 65 °C) (Appendix II) was added, vortexed (Labnet Vortex Mixer, USA) and incubated at 65 °C (Lab Companion BS-31 shaking waterbath, Canada) for 10 min. After incubation the tube was centrifuged at 15,000 rpm (Kubota High Speed Refrigerated Centrifuge 6500, Japan) for 15 min at 4 °C and supernatant was transferred to a fresh 2 ml tube. Equal volume of chloroform: isoamyl alcohol (24:1) was added to the supernatant and centrifuged at 20,000 *g* for 10 min at 4 °C. After transferring the supernatant into fresh tube, equal volume of chloroform: isoamyl alcohol was added, centrifuged at 20,000 *g* for 10 min at 4 °C and the supernatant was transferred to a fresh tube. Then, 0.25 volume of ice cold 10 M lithium chloride was added, mixed well and incubated overnight at 4 °C. Following centrifugation at 30,000 *g* for 30 min at 4 °C, the pellet was washed with 75 percent ethanol by centrifuged at 10,000 *g* for 10 min at 4 °C. The washing was repeated with 200 µl of 75 percent ethanol. RNA pellet obtained was air dried at 37 °C for 30 min and then dissolved in 30 µl DEPC water. After incubating at 37 °C forl h, the RNA was stored at -20 °C.

3.4.3 Agarose gel electrophoresis

The most common method used to assess the integrity of total nucleic acid (DNA / RNA) preparation is to run an aliquot of the sample on a denaturing agarose gel stained with ethidium bromide (EtBr). Around 0.8-1.5 percent agarose gel was prepared in 1X TAE buffer (Appendix II), then heated in microwave oven until completely melted and 0.5 µl ethidium bromide, facilitate the visualization of DNA/RNA. After cooling the solution (60 °C), it was poured into casting tray containing sample comb and allowed to solidify at room temperature. Then the comb was removed followed by inserting gel into the electrophoresis chamber and covered

with buffer. An aliquot of the DNA/RNA sample (3 µl) mixed with the loading dye was loaded in each of the wells of the gel. The gel was run at 5 V cm-1 (BIO RAD Power Pac HV, USA) för 30 min. The gel was then visualized under UV light and the image was documented using Alpha Imager (Alpha Innotech, USA).

3.4.4 Amplification of viruses from infected leaf and tuber samples of lesser yam

3.4.4.1 First strand cDNA synthesis

The RNA isolated from infected leaf samples of lesser yam *(Dioscorea esculenta)* were subjected to cDNA conversion using Revert Aid FIRST strand cDNA synthesis kit (Thermo scientific, USA).

The components of the reaction mix were as follows:

5x Reaction buffer	:	4 µl
10mM dNTP mix	:	2 µl
Oligo $(dT)_{18}$ primers (100 *µM*)	:	1 µl
Revert Aid M-MuLV RT (200 U µl-1)	:	1 µl
Ribolock RNase inhibitor (200 U µl-1)	:	1 µl
Water (nuclease free)	:	8 µl
Template RNA	:	3 µl
Total volume	:	20 µl

All the components were added, mixed by vortexing (Labnet vortex mixer, USA) and flashed down. The cDNA conversion reaction was carried out in Agilent

Technologies sure Cycler 8800 (USA). The reaction conditions comprised of a reverse transcription step at 42 °C for 1 h followed by an extension step at 72 °C for five min.

3.4.4.2 Uncoupled RT-PCR for Virus Partial CP Amplification

3.4.4.2.1 Detection of *Yam mild mosaic virus* (YMMV)

The cDNA synthesized from the RNA of infected leaf samples was used for PCR amplification with specific *Yam mild mosaic virus* forward and reverse primers (unpublished). Primer sequence and reaction mix was optimized as listed below: Yam mild mosaic virus 1c (YMMV 1c) and Yam mild mosaic virus 1s (YMMV ls) (18mers)

YMMV 1c (forward primer) sequence: 5' TCTTATATGDTTCCTGTTC 3'

YMMV ls (reverse primer) sequence: 5' CACTCTTATGGTCTTGTT 3'

The reaction mix was:

2x PCR Master Mix (Thermo Scientific, USA)	: 13 µl
Forward primer (YMMV 1c) (10 µ*M*)	: 1 µl
Reverse primer (YMMV ls) (10 µ*M*)	: 1 µl
cDNA	: 2 µl
Nuclease free water	: 8 µl
Total volume	: 25 µl

After the preparation of the reaction mix, it was vortexed (Labnet vortex mixer, USA) and flashed down. The PCR was carried out in Agilent Technologies sure Cycler 8800 (USA). The PCR programme was set with an initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 1

min and extension at 72 °C for 1 min. The final extension was carried out at 72 °C for 10 min. The PCR products were resolved on agarose gel (1.5%), along with Gene Ruler 1 kb DNA ladder visualized under UV light and the image was documented using Gel DOC system (Alpha imager, Alpha Innotech, USA).

3.4.4.2.2 Detection of *Yam Macluravirus*

The cDNA synthesized from the RNA of infected leaf samples was used for PCR amplification. The PCR was carried out with specific yam macluravirus primers (obtained from DSMZ, Germany). Primers sequence and reaction mix was optimized as listed below:

Yam Maclura 1s (YMac1s) and Yam Maclura 1c (YMac1c) primers (18mers)

YMac1s (forward primer) sequence: 5' GATGAAGCCATTGAAGAA 3'

YMac1c (reverse primer) sequence: 5' GTTGATTGTTGTTGTTGT 3'

The reaction mix was:

2x PCR Master Mix (Thermo Scientific, USA)	: 13 µl
Forward primer (YMac1s) (10 µ*M*)	: 1 µl
Reverse primer (YMac1c) (10 µ*M*)	: 1 µl
cDNA	: 2 µl
Nuclease free water	: 8 µl
Total volume	: 25 µl

After the preparation of the reaction mix, it was vortexed (Labnet vortex mixer, USA) and flashed down. The PCR was carried out in Agilent Technologies sure Cycler

8800 (USA). The PCR programme was set with an initial denaturation at 94 °C for 4 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 52°C for 1min and elongation at 72 °C for 1 min. The final extension was carried out at 72 °C for 10 min. The PCR products were resolved on agarose gel (1.5%), along with Gene Ruler 1 kb plus DNA ladder visualized under UV light and the image was documented using Gel DOC system (Alpha imager, Alpha Innotech, USA).

3.4.4.2.3 Detection of *Yam Badnavirus*

The DNA isolated from both leaf and tuber samples of *Dioscorea esculenta* were used to PCR amplification. The PCR was carried out with one pairs of specific yam *badnavirus* primers. Primer sequence (Hafner *et al.*, unpublished) and components of the reaction mix were as follows:

Badnavirus F (Badna F) and Badnavirus R (Badna R) primers (26 and 24 mers)

Badna F (forward primer) sequence: 5'ATGCCITTYGCIITIAARAAYGCICC 3'

Badna R (reverse primer) sequence: 5' CCAYTTRCAIACISCICCCCAICC 3'

The reaction mix was:

PCR Master Mix	: 13 µl
Nuclease free water	: 8 µl
DNA	: 2 µl
Forward primer (10 µ*M*)	: 1 µl
Reverse primer (10 µ*M*)	: 1 µl
Total volume	: 25 µl

The reaction mix, was vortexed (Labnet vortex mixer, USA) and flashed down. The DNA template was amplified in Agilent Technologies sure Cycler 8800 (USA). PCR programme was set with an initial denaturation at 94 °C for 4 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 ^{0}C for 1 min and extension at 72 °C for 2 min. The final extension was carried out at 72 °C for 10 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer formation. The PCR amplified products along with Gene Ruler 1 kb plus DNA ladder (Thermo Scientific, USA) were separated on agarose gel (1.5%). The gel was viewed using Alpha Imager (Alpha Innotech, USA).

3.4.4.3 One Step RT-PCR for *Yam Mild Mosaic Virus* Partial CP Amplification

In single step RT-PCR, the initial reverse transcription step and the latter amplification steps are coupled. Coupled RT-PCR was also used to detect the presence of virus infection in various *D. esculenta* RNA samples. Prime Script ^{Tm} One Step RT-PCR kit were used to the detection of Yam mild mosaic virus (YMMV) with specific primers. The primers sequence (Mumford and Seal, 1997) and RT- PCR reaction mix was optimized as given below:

Yam Virus 1F (YV1-F) and Yam Virus 1R (YV1-R) primers (20 and 21 mers)

YV1- F (forward primer) sequence: 5' CGCACACATGCAAATGAAAGC 3'

YV1- R (reverse primer) sequence: 5' CACCAGTAGAGTGAACATAG 3'

The reaction mix was:

Prime script 1Step enzyme mix : 2 µl

2x 1 Step buffer : 25 µl

Forward primer (YV1F) (10 µ*M*) : 1 µl

Reverse primer (YV1 R) (10 μ*M*)	: 1 μl
Template RNA	: 2 μl
RNase free dH2o	: 19 μl
Total volume	: 50 μl

The reaction mix, was vortexed (Labnet vortex mixer, USA) and flashed down. The RNA template was amplified in Agilent Technologies sure Cycler 8800 (USA). RT-PCR programme was set with a reverse transcription at 42 °C for 45 min and initial denaturation at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 ^{0}C for 30 s and extension at 72 °C for 1 min. The final extension was carried out at 72 °C for 10 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer formation. The PCR amplified products along with Gene Ruler 1 kb plus DNA ladder (Thermo Scientific, USA) were separated on agarose gel (1.5%). The gel was viewed using Alpha Imager (Alpha Innotech, USA).

3.4.4.4 Gel Elution of PCR Amplified Fragments

Extraction of the PCR products was achieved with Gene JET Gel Extraction kit (Thermo Scientific, USA). The PCR products were resolved on agarose gel (1.2 %) and the amplicon was excised from the gel using a clean sharp scalpel. The gel slice was placed into a pre-weighed 1.5 ml tube and its weight was recorded. Then, twice the volume binding buffer was added to the gel slice and it was incubated at 50-60 °C for 10 min or until the gel slice was completely dissolved. In order to facilitate the melting process, the contents of the tube were mixed by inversion every three min. Up to 800 μl of the solubilized gel solution was transferred to the Gene JET purification column followed by centrifugation at 14,000 rpm for one min. The flowthrough was discarded and the column was placed back into the same collection tube. After addition

of 100 μl of binding buffer to the column, it was centrifuged at 14,000 rpm for one min. Again, the flow-through was discarded and the column was placed back into the collection tube. Then, 700 μl of the wash buffer was added to the column, followed by centrifugation for one min. The flow-through was discarded and the empty column was centrifuged for an additional one min to completely remove any residual wash buffer present in the column.

After the column was transferred into a new collection tube, it was air dried for 10 min. The purified DNA was eluted by adding 50 μl of the elution buffer to the centre of the purification column membrane followed by centrifugation for one min. After elution, the column was discarded and the purified DNA was stored at -20 °C. The gel elute was subjected to PCR using the same reaction mix under required cycling conditions. The products of PCR were assessed using agarose gel (1.2%).

3.5 PRIMER DESIGNING

Primers were designed based on the partial CP gene of YMMV and *Yam Macluravirus*. From the NCBI nucleotide database, available nucleotide sequences of YMMV and *Yam Macluravirus* were taken in FASTA format. The sequences collected from NCBI along with the partial CP gene sequences of YMMV and *Yam Macluravirus* obtained using YMMV1C/YMMV1S and YMac1s /YMac1c primers were aligned using BioEdit sequence alignment editor (Hall, 1999). Then the specific primers were designed by using NCBI Primer BLAST.

The designed primers were subjected to in-silico analysis for determining specificity and other characteristics. FastPCR programme were used to study the length of primers as well as the PCR product, self-complementarity and possibilities for primer dimer formation, GC content, melting temperature and feasible annealing temperature. The primer sequence thus validated by in-silico methods were sent to Sigma Aldrich (USA) for synthesis.

3.5.1 Gradient PCR for Standardizing Annealing Temperature

The two sets of synthesized primers were used to test their efficiency in virus infection in *Dioscorea esculenta*. They are listed below:

1) Yam Mild Mosaic Virus F1 (YMMV F1) and Yam Mild Mosaic Virus R1 (YMMV R1) (21and19 base)

YMMV F1 (forward primer) sequence: 5' GAGGGAGCATGTACTCGACAC 3'

YMMV R1 (reverse primer) sequence: 5' CTCCGTTCTGCTCCACTCG 3'

2) Yam Macluravirus F (YMac F) and Yam Macluravirus R (YMac R) (20 base)

YMV F (forward primer) sequence: 5' CTGCAACACTCGGAGGCTTA 3'

YMV R (reverse primer) sequence: 5' AGGCTCTCTGGACTCCACTT 3'

The synthesized primers (100 μ*M*) were diluted to a final concentration of 10 μ*M* with sterile water to obtain the working solution. In order to standardize the annealing temperature of the primers, gradient PCR was performed. The cDNA synthesized from the RNA of infected leaf samples was used for PCR amplification.

The reaction mix was optimized as listed below:

2x PCR Master Mix (Thermo Scientific, USA)	:13 μl
Forward primer (10 μ*M*)	: 1μl
Reverse primer (10 μ*M*)	: 1 μl
cDNA	: 2 μl
Nuclease free water	: 8 μl
Total volume	: 25μl

After the preparation of the reaction mix, it was vortexed (Labnet vortex mixer, USA) and flashed down. The PCR was carried out in Agilent Technologies sure Cycler 8800 (USA). The same PCR programme was set as described in section 3.4.4.2.1 and 3.4.4.2.2 except that the annealing temperature gradient was from 55 °C to 65 °C for 30 s and 45 s respectively. Control reactions were carried out to distinguish the target products from non-target products and primer dimer formation. The PCR amplified products along with Gene Ruler 1 kb plus DNA ladder (Thermo Scientific, USA) were separated on agarose gel (1.5%). The gel was viewed using Alpha Imager (Alpha Innotech, USA).

3.5.2 Uncoupled RT-PCR of Total RNA Using Synthesized Primers

After standardizing the annealing temperatures of designed primers for each viruses like YMMV and *Yam Macluravirus*, the cDNA samples were subjected to uncoupled RT-PCR using the reaction mixture described in section 3.6.1. PCR was carried out in Agilent Technologies sure Cycler 8800 (USA) and the same cycling conditions were set as described in section 3.4.4.2.1 and 3.4.4.2.2 but the annealing temperature was set at 55 °C for 30 s used for YMMV while 62.2 °C for 45 s used for *Yam Macluravirus*. The PCR amplified products along with Gene Ruler 1 kb plus DNA ladder (Thermo Scientific, USA) were separated on agarose gel (1.2%). The gel was viewed using Alpha Imager (Alpha Innotech, USA).

3.6 PCR PURIFICATION OF AMPLIFIED PRODUCTS OF *YAM MILD MOSAIC VIRUS, YAM MACLURAVIRUS* AND *YAM BADNAVIRUS*

The purification of PCR amplified product was carried with Gene JET PCR Purification kit (Fermentas life sciences, UAS). The amplified PCR product was added to the equal volume of binding buffer and mixed thoroughly until the PCR product was completely dissolved. Then 800 µl of the sample was transferred to the Gene JET PCR Purification column. Following centrifugation at 12000 g for 30-60 s at room

temperature, the flowthrough was discarded and the column was placed back into the same collection tube. After addition of 700 µl of wash buffer to the column it was centrifuged at 12000 g for 30-60 s at room temperature. The flowthrough was discarded and the column was placed back into the same collection tube. The empty column was centrifuged for an additional one minutes to completely remove any residual wash buffer present in the column. After the column was transferred into a new collection tube it was air dried for 15-20 min. The purified product was eluted by adding 30 µl of the elution buffer to the centre of the purification column membrane followed by centrifugation for one min. After elution the column was discarded and the purified PCR product was stored at -20 °C. The quality of purified PCR product was checked on agarose gel (1.2%).

3.7 CLONING AND TRANSFORMATION

The amplified region of *Yam mild mosaic virus (*YMMV*), Macluravirus* and *Badnavirus* was cloned and transformed into *E. coli* DH5α using InsTA Clone PCR Cloning Kit (Fermentas, USA). The recombinant clones obtained were analyzed by colony PCR method. Plasmid DNA was isolated from the recombinant clones and checked for PCR amplification. A recombinant plasmid was sequenced to confirm the presence of amplified region of particular virus.

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

Day 1: Ligation of plasmid vector and insert DNA

E. coli DH5α cells revived in Luria agar medium (Appendix III) were used for the transformation procedure.

The ligation mix was prepared with the components as listed below,

pTZ57R/T Cloning vector,(0.17pmol ends)	: 3 µl
5x Ligation Buffer	: 6 µl
PCR product	: 4 µl
Nuclease free water	: 16 µl
T4 DNA Ligase	: 1 µl
Total volume	: 30 µl

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

Day 2: Transformation of DNA into Bacteria

To 1.5 ml C-medium (pre-warmed at 37 °C for at least 20 min), a portion of freshly streaked bacterial culture was transferred using inoculation loop and gently mixed to resuspend the cells. After incubating the medium at 37 °C with shaking for 2 h, the bacterial cells were pelleted by one min centrifugation. The pellet was resuspended in 300 µl of T solution (Appendix III) and incubated on ice for five min. The cells were again pelleted by centrifugation, resuspended in 120 µl of T solution and incubated on ice for five min.

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

Untransformed DH5α cells were plated on an LB Ampicillin X gal/ IPTG agar plate to serve as negative control.

Day 3: Observation of Transformation

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

3.7.1 Analysis of Recombinant Clones-Colony PCR

The recombinant clones were analyzed for the presence and orientation of the DNA insert by colony PCR using their respective primers as well as M13 primers (IDT, USA).

The PCR reaction mix was formulated as:

2x PCR Master Mix	: 10 μl
Water, nuclease free	: 8.8 μl
M13 sequencing primer (10 μ*M*)	: 0.6 μl
M13 reverse primer (10 μ*M*)	: 0.6 μl
Total volume	: 20 μl

A single white colony was selected and resuspended in the PCR reaction mix. A short strike was made over the culture plate (containing selective medium) in order to save the clone for repropagation. The PCR was performed with an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 30 s and extension at 72 °C for 1 min. A final extension step for 5 min was also given. For colony PCR using specific primers, the same reaction

mixture and cycling conditions were used as the required PCR conditions of each primers. The products of colony PCR were resolved on agarose gel (1.2%).

3.7.2 Plasmid DNA Isolation

The recombinant plasmid DNA was isolated using Gene JET Plasmid Miniprep kit (Thermo Scientific, USA). From a freshly streaked selective plate, a single bacterial colony was selected and inoculated in 1.5 ml of LB medium supplemented with ampicillin. The tube was incubated at 37 °C for 12-16 h in shaking waterbath (Lab Companion BS-31 shaking waterbath, Canada). After incubation, the bacterial cells were harvested by centrifugation at 8000 rpm for two min at room temperature. The pelleted cells were resuspended in 250 μl of the resuspension solution and vortexed until no cell clumps remain. Two hundred and fifty μl of the lysis solution was then added and mixed thoroughly until the solution becomes viscous and slightly clear. Three hundred and fifty μl of the neutralization solution was added next and mixed immediately. The cell debris was pelleted by centrifugation for five min. The supernatant obtained was transferred to a Gene JET spin column and centrifuged for one min. The flow-through was discarded and 500 μl of wash solution was added. After centrifuging the column for one min, the flow-through was discarded and the wash procedure was repeated once. An additional one min centrifugation was done to remove any residual wash solution present in the column. The spin column was then transferred into a new 1.5 ml microfuge tube and air dried. Thirty μl of the elution buffer was added to the center of the column to elute the plasmid DNA. The column was incubated at room temperature for two min followed by centrifugation for two min. A second elution step was carried out with 20 μl of the elution buffer. The purified plasmid obtained was stored at -20 °C.

The isolated plasmid DNA was checked on agarose gel (1%). In order to check the presence of insert DNA in the plasmid, a PCR was carried out using specific primers of each viruses.

The reaction mix used was:

2x PCR Master Mix	: 13 µl
Forward primer (10 µ*M*)	: 1µl
Reverse primer (10 µ*M*)	: 1µl
Plasmid DNA	: 2µl
Water, nuclease free	: 8µl
Total volume	: 25 µl

The PCR was performed with the same cycling conditions as described in section 3.4.4.2.1 for YMMV and 3.4.4.2.3 for *Badnavirus*. The products of PCR amplification were analyzed on agarose gel (1%) to confirming the presence of DNA insert.

3.8 DNA SEQUENCING

Plasmid DNA (YMMV and *Yam Badnavirus*), Gel elute of PCR product (*Yam Macluravirus*) were sequenced at the Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology (RGCB).

3.8.1 Sequence Analysis

The sequence obtained was first edited with BioEdit Sequence Alignment Editor Programme version 7.2.5. The edited sequence was compared to known viral sequences using NCBI BLAST. Phylogenetic tree was constructed from BioEdit aligned sequences using MEGA version 6.06 (Tamura *et al.*, 2013) using a Neighbor-Joining method.

RESULTS

4. RESULTS

The result of the study entitled "Identification and characterization of viruses infecting lesser yam (*Dioscorea esculenta* (Lour.) Burkill)" carried out at the division of crop protection ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2014-2015 are presented in this chapter.

4.1 SAMPLE COLLECTION

Fifty leaf samples and 25 tuber samples were collected from lesser yam growing field at ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram and visually analyzed. Most of the samples collected were symptomatic with mild mosaic which was the common symptom observed in all experimental plants. The other symptoms observed include puckering, cupping, leaf crinkling, mottling, leaf distortion and chlorosis (Plate 1). Asymptomatic samples showed no prominent symptoms except some mild indistinct chlorosis on variegation. This representative sample set was used for further test and analysis.

4.2 SEROLOGICAL METHODS OF DETECTION

The serological methods employed for the initial screening of the samples were DAS-ELISA and TAS-ELISA. This procedure was carried out to screen the samples for *Yam mild mosaic virus, Yam macluravirus and Yam badnavirus* infection using antisera obtained from DSMZ, Germany.

4.2.1 DAS-ELISA

DAS-ELISA was carried out for screening *Yam mild mosaic virus* and *Yam macluravirus.* A total of 25 leaf samples were selected from the whole sample collection. Asymptomatic and the samples showed viral symptoms were taken for the test. The previously confirmed *Yam mild mosaic virus* and *Yam macluravirus* infected yam leaf in the diagnostic kit was the positive control and negative control was a non-

Plate 1. Viral Symptoms of lesser yam: (A) lesser yam field (B) Chlorosis (C) Leaf distortion (D) Mosaic (E) Puckering and Cupping (F) Lesser yam tuber

host. The readings were taken at A_{405} in a BIO-RAD i Mark Microplate Reader (USA) and the chart plotted (Figure 1 and Figure 2).

Total of 25 leaf samples were tested for detection of *Yam mild mosaic virus* infection in lesser yam (Plate 2A). Out of 25 leaf samples 22 (88%) were positive for *Yam mild mosaic virus* infection among them 3 samples (CTDe 64, CTDe 79 and CTDe 2) showed high OD value (> 1) which showed that these samples were having high concentration of *Yam mild mosaic virus*. Rest of the sample had moderate infection of YMMV. A summary of the result is presented in Table 2. Samples showing mild to severe mosaic, puckering, cupping and chlorosis symptoms were found to be positive for YMMV. The samples which recorded thrice or more mean absorbance values at 405 nm (A_{405}) than that of the non-host were considered positive for the virus tested. The colour development was prominent after 1 h which has been taken for comparison.

Out of the 25 leaf samples were tested 9 (36%) of them showed positive for *Yam macluravirus* (Plate 2B). Four of them (CTDe 44, CTDe 163, CTDe 72, CTDe 79) showed high OD value 0.656, 0.443, 0.544 and 0.598 compared to non-host (0.036) which indicated that these samples were highly susceptible to *Yam macluravirus* infection. Rest of the samples had moderate infection of the virus. A summary of the result is presented in Table 3. The samples with mild to severe mosaic, puckering, cupping and curling symptoms were found positive for *Yam macluravirus*.

4.2.2 TAS-ELISA

TAS-ELISA was carried out for 25 samples selected from the whole sample collection. Most of the samples selected were showing symptoms of viral infection and some asymptomatic samples were also taken. The previously confirmed DBV infected *Dioscorea* leaf as kept as the positive control and non-host was negative control. The reading were taken at A_{405} in a BIO-RAD i Mark Microplate Reader (USA) and the chart plotted (Figure 3). The samples which recorded thrice or more mean

absorbance values at 405 nm (A_{405}) than that of the non-host were considered positive for the virus tested. The colour development was prominent after 1 h.

A total of 25 leaf samples were tested for DBV infection by TAS-ELISA using specific antibody (Plate 2C). Out of the 25 samples tested 10 (40%) were positive, and 4 of them showed highly susceptible to DBV infection (CTDe 81c, CTDe 47, CTDe 10, and CTDe 72). Other samples had moderate infection of DBV. The samples with mosaic, puckering and cupping symptoms were found to be positive for DBV. A summary of the result is presented in Table 4.

4.4 NUCLEIC ACID BASED DETECTION

4.4.1 Isolation of total DNA

CTAB method of DNA isolation was carried out using lesser yam leaf and tuber samples from ICAR-CTCRI field. Out of the 50 leaf samples of *Dioscorea esculenta* plants 37 showing mosaic, puckering, cupping and chlorosis symptoms of virus infection were taken for DNA isolation. The extracted genomic DNA was run on an agarose gel (0.8%) and visualized under UV to observe the bands (plate 3).

4.4.1 Isolation of total RNA

RNA isolation from samples of *Dioscorea esculenta* leaf showing symptoms of various infection were carried out using three methods, viz Ambion Purelink RNA Mini kit, TRIzol reagent method and lithium chloride protocol. The RNA of 50 leaf samples and 20 tuber samples was isolated. There was a large difference in the yield and quality of total RNA obtained from leaf and tuber samples depending on the method used. RNA extraction was successful in all the 3 methods but the Ambion Purelink RNA Mini kit method provided higher quality RNA from leaf samples.

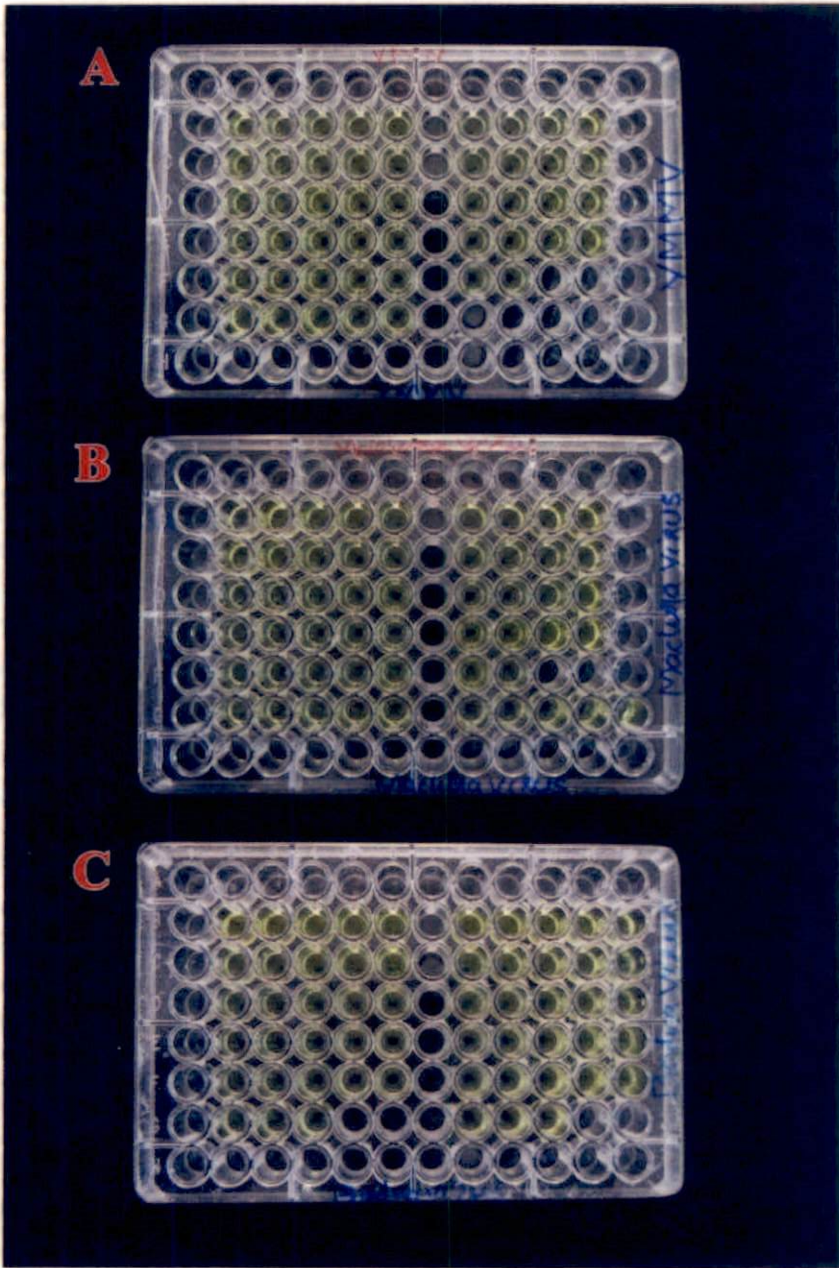


Plate 2. Serological detection of YMMV, *Yam Macluravirus* and *Yam Badnavirus* in lesser yam samples (A) DAS-ELISA for YMMV, (B) DAS-ELISA for *Yam Macluravirus* and TAS-ELISA for *Yam Badnavirus*.

Table 2. Detection of *Yam Mild Mosaic Virus in Dioscorea esculenta* leaf samples by DAS – ELISA

Sample	Absorbance at 405 nm#		Sample	Absorbance at 405 nm#	
	1 hour	Reaction (+/-)		1 hour	Reaction (+/-)
Blank	0.004	-	CTDe 81E	0.086	+
Negative control	0.038	-	CTDe 110	0.111	+
CTDe 1	0.101	+	CTDe 114	0.202	++
CTDe 2	1.212	++++	CTDe 121	0.371	+++
CTDe 3	0.168	++	CTDe 124	0.184	++
CTDe 4	0.034	-	CTDe 132	0.126	++
CTDe 5	0.328	++	CTDe 133	0.247	++
CTDe 7	0.125	++	CTDe 135	0.253	++
CTDe 64	1.214	++++	CTDe 150	0.079	+
CTDe 72	0.102	++	CTDe 151	0.387	+++
CTDe 73	0.021	-	CTDe 153	0.079	++
CTDe 77	0.172	++	CTDe 168	0.029	-
CTDe 79	1.161	++++	CTDe 172	0.399	+++
CTDe 80	0.238	++	_	_	_

(<0.038= Negative, 0.038-0.114(+), 0.114-0.342(++), 0.342-1.026(+++), 1-026-3.076(++++))

Table 3. Detection of *Yam macluravirus in Dioscorea esculenta* leaf samples by DAS – ELISA

Sample	Absorbance at 405 nm#		Sample	Absorbance at 405 nm#	
	1 hour	Reaction (+/-)		1 hour	Reaction (+/-)
Blank	-0.065	-	CTDe 110	-0.091	-
Negative control	0.036	-	CTDe 114	-0.068	-
CTDe 2	-0.142	-	CTDe 124	0.193	+
CTDe 5	-0.008	-	CTDe 130	-0.041	-
CTDe 7	0.126	++	CTDe 133	0.082	-
CTDe 35	-0.056	-	CTDe 135	-0.094	-
CTDe 44	0.656	+++	CTDe 147	-0.065	-
CTDe 72	0.544	+++	CTDe 151	0. 076	+
CTDe 77	-0.037	-	CTDe 153	-0.019	-
CTDe 79	0.598	+++	CTDe 158	-0.098	-
CTDe 80	0.165	++	CTDe 160	-0.054	-
CTDe 81E	-0.333	-	CTDe 163	0.443	+++
CTDe 97	-0.184	-	CTDe 165	0.102	+
CTDe 107	-0.029	-	_	_	_

(<0.036= Negative, 0.324-0.108 (+), 0.108-0.324(++), 0.324- 0.972(+++), 0.972 - 2.916(++++))

Table 4. Detection of *Yam badnavirus in Dioscorea esculenta* leaf samples by TAS – ELISA

Sample	Absorbance at 405 nm[#]		Sample	Absorbance at 405 nm[#]	
	1 hour	Reaction (+/-)		1 hour	Reaction (+/-)
Blank	0.018	-	CTDe 80	-0.053	-
Negatie control	0.031	-	CTDe 81C	0.9	++++
CTDe 1	0.021	-	CTDe 102	-0.035	-
CTDe 2	0.101	+	CTDe 110	0.068	-
CTDe 3	-0.03	-	CTDe 113	-0.053	-
CTDe 5	0.079	+	CTDe 116	-0.029	-
CTDe 7	0.341	+++	CTDe 121	-0.016	-
CTDe 10	0.907	++++	CTDe 132	0.013	-
CTDe 44	0.179	+	CTDe 150	-0.083	-
CTDe 47	1.132	++++	CTDe 151	0.058	-
CTDe 64	-0.112	-	CTDe 158	-0.031	-
CTDe 72	0.853	++++	CTDe 162	-0.028	-
CTDe 77	0.394	+++	CTDe 165	0.022	-
CTDe 79	0.382	+++	_	_	_

(<0.031= Negative, 0.031- 0.093(+), 0.093-0.279(++), 0.279-0.837(+++), 0.837-2.61(++++))

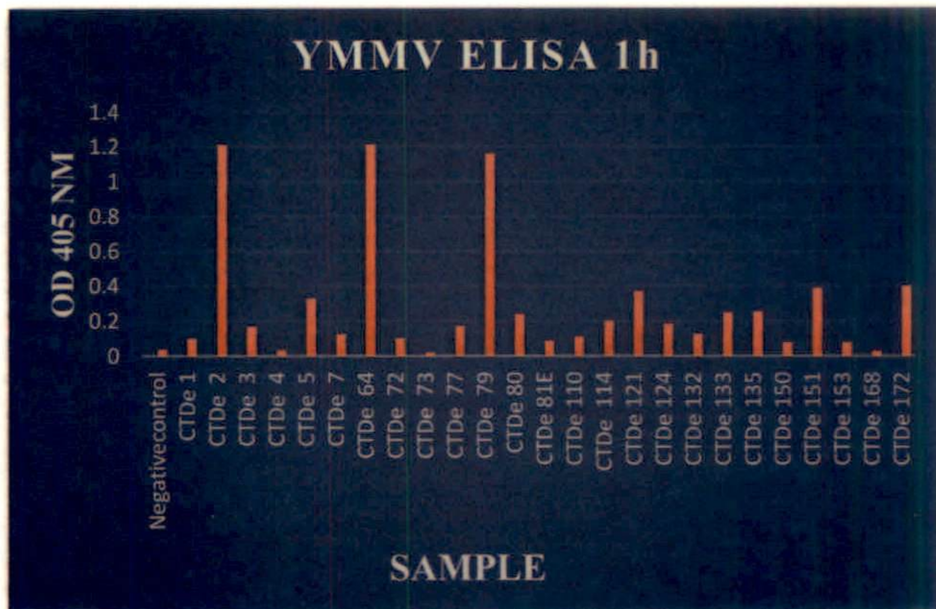


Fig.1 Detection of YMMV in different lesser yam accessions through ELISA

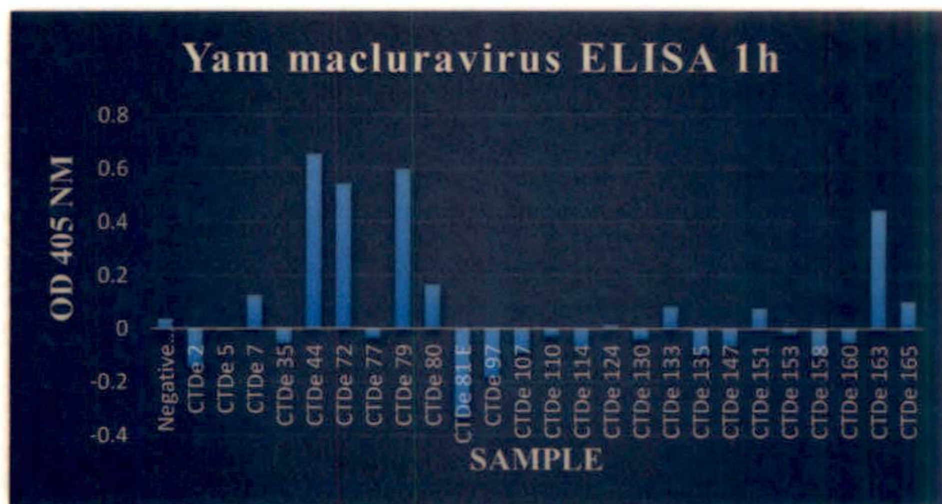


Fig. 2. Detection of *Yam Macluravirus* in different lesser yam accessions through ELISA

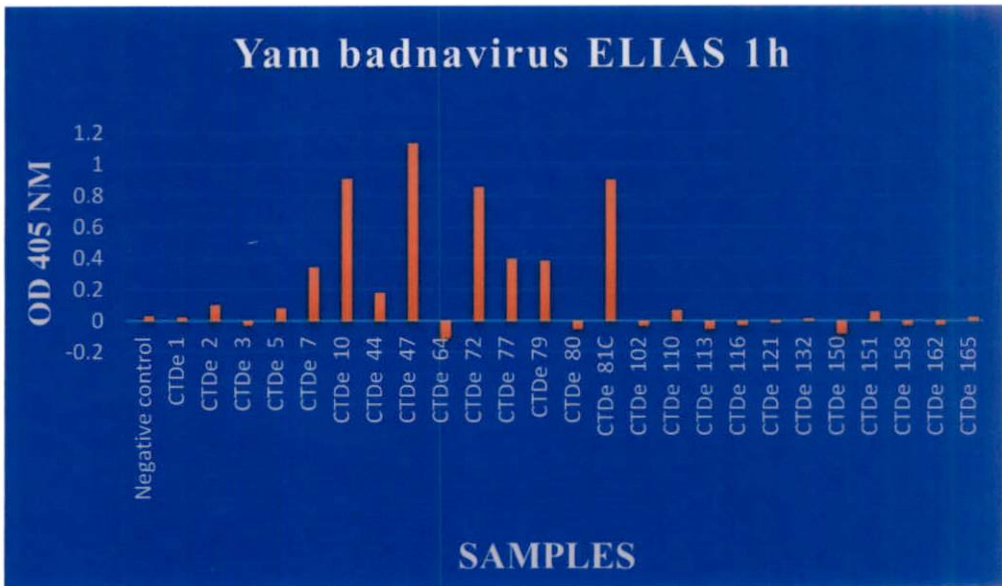


Fig.3. Detection of *Yam Badnavirus* in different lesser yam accessions through ELISA

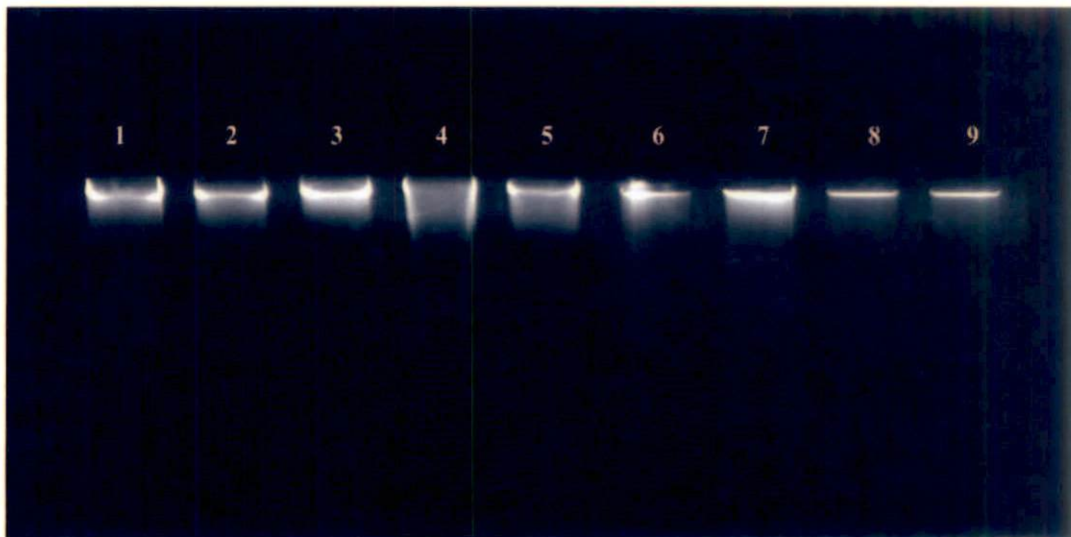


Plate 3. Total DNA isolated from *Dioscorea esculenta* leaf sample using CTAB method.

Among the 3 different methods evaluated for RNA isolation from tubers lithium chloride method provided quality of stable RNA. The isolated RNA was checked in 1.2 percent agarose gel by electrophoresis and visualized under UV to observe band of good total RNA and documented. Gel pictures of RNA isolated by each methods represented in plate (Plate 4_A, 4_B and 4_C).

4.3.2 cDNA synthesis

The RNA isolated from 50 leaf samples and 20 tuber samples was converted into cDNA for further PCR based screening. Isolated RNA is less stable for long period of storage, however cDNA conversion ensure that the sample is not lost and was essential prerequisite for conducting PCR based virus detection. The First strand cDNA conversion was carried out using Revert Aid First strand cDNA synthesis kit (Thermo Scientific, USA). cDNA synthesis was positive even from low quality RNA in most cases.

4.3.4. Uncoupled RT-PCR for YMMV, *Yam macluravirus,* and *Yam badnavirus*

4.3.4.1 Detection of *Yam mild mosaic virus* (YMMV)

After first stranded cDNA synthesis, the detection of YMMV infection in all the samples were carried using YMMV 1c and YMMV 1s specific primers. Uncoupled RT-PCR analysis with YMMV 1c and YMMV 1s specific primers which amplifies the partial coat protein gene yielded amplicons of 500 bp in samples positive for YMMV infection. A control (non-template) was also set during PCR which contains all the components of a typical PCR but no template sample. PCR conditions were mentioned in 3.4.4.2.1. An amplicon of 500 bp was observed as a single band for virus positive samples in 1.5 percentage agarose gel electrophoresis. There was no amplification observed in the control which indicate that, no non- specific binding and primer dimer formation in PCR. The PCR results with YMMV 1c and YMMV 1s primers are shown

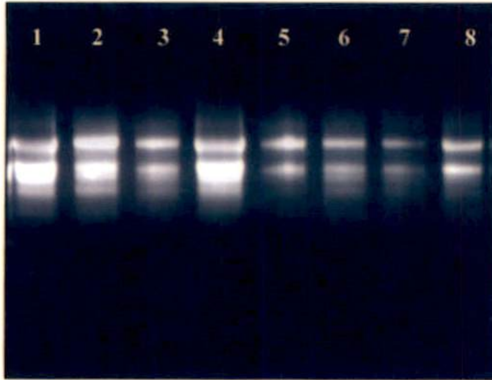
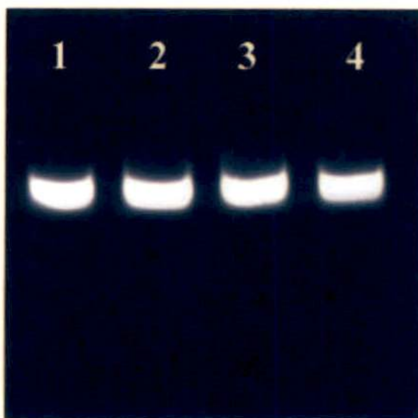


Plate 4. (A) Isolation of total RNA from leaf samples using Ambion Purelink RNA Minikit method.

Plate 4. (B) Isolation of total RNA from leaf samples using TRIzol reagent method.



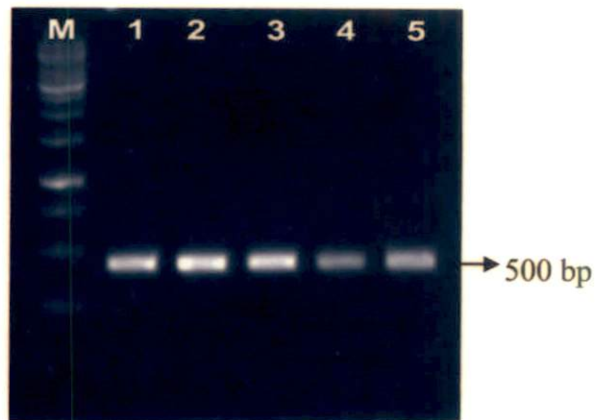


Plate 4. (C) Isolation of total RNA from leaf samples using Lithium chloride method.

Plate 5. Detection of YMMV by uncoupled RT-PCR. M: marker: Lane 1 and 5 lesser yam samples.

in Plate 5. Out of the 35 samples collected from the ICAR-CTCRI field, 27 were found to be positive for virus infection (Table 5).

4.3.4.2 Detection of *Yam Macluravirus*

After first stranded cDNA synthesis, the detection of *Yam macluravirus* infection in all the samples were carried using Ymac 1s and Ymac 1c specific primers. Uncoupled RT-PCR analysis with Ymac 1s and Ymac 1c specific primers which amplifies the partial coat protein gene yielded amplicons of 200 bp in samples positive for *Yam macluravirus* infection. A control (non-template) was also set during PCR which contains all the components of a typical PCR but no template sample. PCR conditions were mentioned in 3.4.4.2.2. An amplicon of 200 bp was observed as a single band for virus positive samples in 1.5 percentage agarose gel electrophoresis. There was no amplification observed in the control which indicate that, no non- specific binding and primer dimer formation in PCR. The PCR results with Ymac 1s and Ymac 1c specific primers are shown in Plate 6. Out of the 35 samples collected from the ICAR-CTCRI field, 14 were found to be positive for virus infection (Table 5).

4.3.4.4 Detection of *Yam Badnavirus*

After extraction of the DNA, detection of *Yam badnavirus* infection in the leaf and tuber samples were carried using Badna F and Badna R specific primers. PCR analysis with Badna F and Badna R specific primers which amplifies the partial coat protein gene yielded amplicons of 579 bp in samples positive for *Yam badnavirus* infection. A control (non-template) was also set during PCR which contains all the components of a typical PCR but no template sample. PCR conditions were mentioned in 3.4.4.2.1.4. An amplicon of 579 bp was observed as a single band for virus positive samples in 1.5 percentage agarose gel electrophoresis. There was no amplification observed in the control which indicate that, no non-specific binding and primer dimer formation in PCR. The PCR results with Badna F and Badna R specific primers are

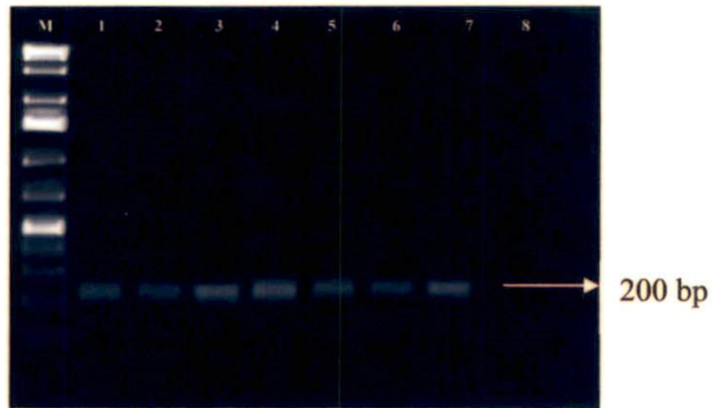


Plate 6. Detection of *Yam Macluravirus* by uncoupled RT-PCR. M: 1kb plus marker: Lane 1 to 7 *Dioscorea esculenta* samples; 8 Negative

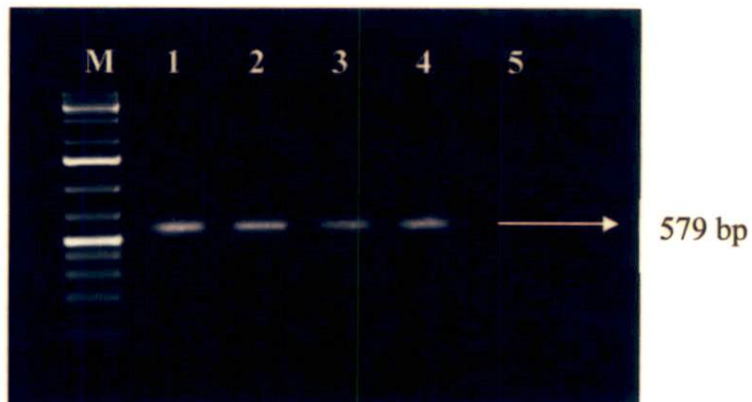


Plate 7. Detection of *Yam Badnavirus* by PCR M: 1kb plus marker: Lane 1 to 4 *Dioscorea esculenta* samples; 5 Negative

shown in Plate 7. Out of the 35 samples collected from the ICAR-CTCRI field, 15 were found to be positive for virus infection (Table 5).

4.3.4.3 One step RT-PCR for YMMV CP Amplification

The initial reverse transcription step and the latter amplification steps are coupled for the detection of YMMV virus infection in all the samples were carried using YV1R and YV1F specific primers. Coupled RT-PCR analysis with YV1R and YV1F specific primers which amplifies the partial coat protein gene yielded amplicons of 260 bp in samples positive for YMMV infection. A control (non-template) was also set during PCR which contains all the components of a typical PCR but no template sample. PCR conditions were mentioned in 3.4.4.3. An amplicon of 260 bp was observed as a single band for virus positive samples in 1.5 percentage agarose gel electrophoresis. There was no amplification observed in the control which indicate that, no non- specific binding and primer dimer formation in PCR. The PCR results with YV1R and YV1F specific primers are shown in Plate 8. Out of the 35 samples collected from the ICAR-CTCRI field, 11 were found to be positive for virus infection (Table 5).

4.4. PRIMER DESIGNING

The virus specific primers were synthesized from Sigma Aldrich (USA). There were 2 sets of forward (F) and reverse (R) primers designed for the amplification of partial CP gene of YMMV and *Yam macluravirus* based on the most favorable combination of conserved regions in the multiple aligned nucleotide sequences.

The analysis of primers using FastPCR programme revealed good GC content and annealing temperature, and also the designed primers did not exhibit hairpin formation and 3' complementarity.

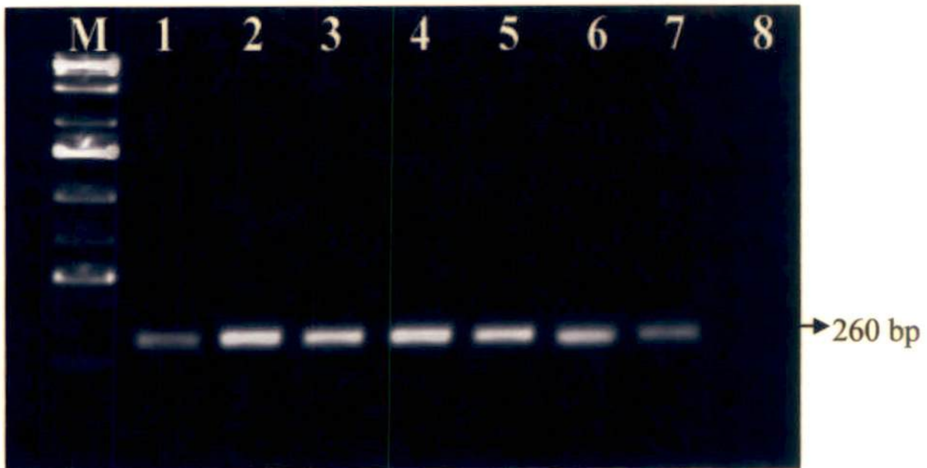


Plate 8. Detection of YMMV by coupled RT-PCR M: 1kb plus marker: Lane 1 to 7 *Dioscorea esculenta* samples; 8 Negative

Table 5. PCR and RT-PCR analysis of YMMV, *Yam Macluravirus* and *Yam Badnvirus*

Sl.No.	Acession number	Single step RT-PCR	Two step RT-PCR		PCR
		YMMV	YMMV	*Yam maclura virus*	*Yam badnavirus*
1	CTDe 1	-	+	-	-
2	CTDe 2	+	+	-	-
3	CTDe 3	+	-	-	+
4	CTDe 4	-	-	+	-
5	CTDe 5	-	+	-	+
6	CTDe 7	-	+	-	-
7	CTDe 10	+	+	+	+
8	CTDe 44	+	+	+	+
9	CTDe 47	-	+	+	+
10	CTDe 64	+	+	-	+
11	CTDe 72	+	+	+	-
12	CTDe 77	+	+	-	-
13	CTDe 79	-	+	+	+
14	CTDe 81C	-	+	-	+
15	CTDe 132	+	+	+	+
16	CTDe 151	+	+	-	+
17	CTDe 163	-	+	+	-
18	CTDe 165	-	-	-	-
19	CTDe 172	-	-	+	-
20	CTDe 173	-	-	-	+
21	FS 1	-	+	-	+
22	FS 2	-	+	+	-
23	FS 3	-	+	+	-
24	FS 4	-	+	-	-
25	FS 5	-	-	-	-
26	FS 6	-	-	-	+
27	FS 7	-	+	-	-
28	FS 8	-	+	+	-
29	FS 9	+	+	-	-
30	FS10	+	+	+	-
31	FS 11	-	+	-	+
32	FS 12	-	-	-	+
33	FS 13	-	+	-	-
34	FS 14	-	+	+	-
35	FS 15	-	+	-	-

The synthesized primers were entitled as;

1) YMMV F1 (forward primer) sequence: 5' GAGGGAGCATGTACTCGACAC 3'

   YMMV R1 (reverse primer) sequence: 5' CTCCGTTCTGCTCCACTCG 3'

2) YMac F (forward primer) sequence: 5' CTGCAACACTCGGAGGCTTA 3'

   YMac R (reverse primer) sequence: 5' AGGCTCTCTGGACTCCACTT 3'

4.4.1 Gradient PCR for T_m Optimization

After synthesized the primers, the annealing temperature of the primers was standardized by using gradient PCR. Being an important step of PCR the annealing temperature of the YMMV F1, YMMV R1 and YMac F, YMac R primers were optimized by conducting a gradient PCR from 55 ^{0}C-65 ^{0}C and the optimum T_m value for better amplification was observed at 55 ^{0}C for YMMV and 62.2 ^{0}C for *Yam macluravirus.*

4.4.2 Uncoupled RT-PCR of Total RNA Using Synthesized Primers

Detection of the virus infection in *Dioscorea esculenta* plants were done using synthesized primers of YMMV F1, YMMV R1 and YMac F, YMac R respectively. The primer pairs amplifies the partial coat protein gene yielded amplicons of 193 bp size for YMMV and 237 bp size for *Yam macluravirus*. A control (non-template) was also set during PCR which contains all the components of a typical PCR but no template sample. PCR conditions were mentioned in 3.6.2. An amplicon of 193 bp and 237 bp size was observed as a single band for virus positive samples in 1.5 percentage agarose gel electrophoresis. There was no amplification observed in the control which indicate that, no non-specific binding and primer dimer formation in PCR. The PCR results with the specific primers are shown in Plate 9a and 9b.

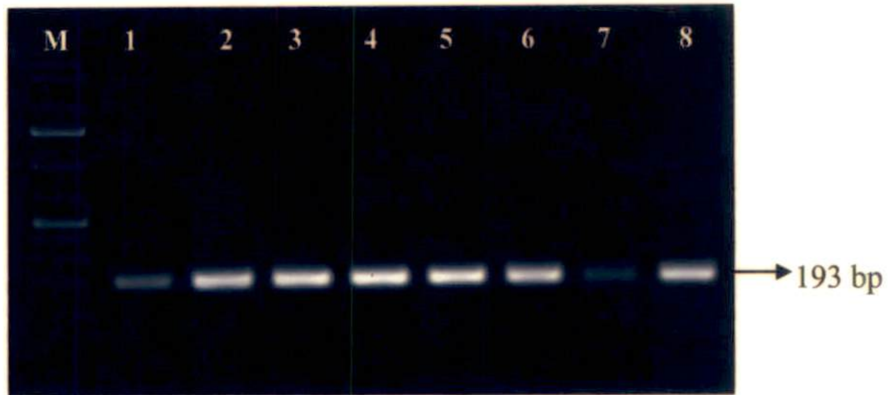


Plate 9. (A). Detection of YMMV by uncoupled RT-PCR using YMMV F1 and YMMV R1 primers. M: 1kb plus marker: Lane 1 to 8 *Dioscorea esculenta* samples.

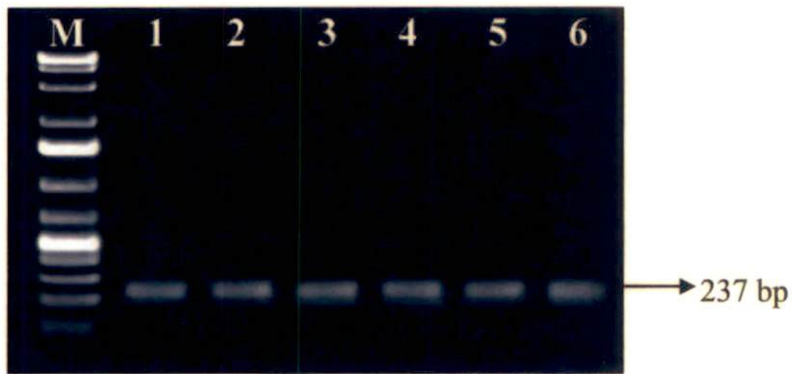


Plate 9. (B). Detection of *Yam Macluravirus* by uncoupled RT-PCR using YMac F and YMac R primers M: 1kb plus marker: Lane 1 to 6 *Dioscorea esculenta* samples.

4.5. PCR PURIFICATION OF AMPLIFIED PRODUCTS OF *YAM MILD MOSAIC VIRUS, YAM MACLURAVIRUS AND YAM BADNAVIRUS.*

The isolated DNA and RNA were subjected to PCR describe in the section 3.4.4.2.1, 3.4.4.2.2 and 3.4.4.2.3. Amplified product of each viruses were purified with Gene JET PCR Purification kit (Fermentas life sciences, UAS). The PCR purified product was checked for quality in 1.2 percentage agarose gel electrophoresis (Plate 10).

4.6 CLONING AND TRANSFORMATION

The amplified PCR purified product of YMMV, *Yam macluravirus*, and *Yam badnavirus* was proceeded with cloning and transformation for better sequencing result. The amplified product were cloned using InsTA Clone PCR Cloning Kit (Fermentas, USA) and transformed into *E. coli* DH5α cells. The transformed colonies were distinguished by blue-white screening in LB Ampicillin Xgal IPTG plates (Plate 11). The white recombinant colonies obtained were selected for further analysis using colony PCR with respective primers under appropriate conditions. The expected product of size 500 bp, 200 bp and 579 bp was obtained from most of the colonies analyzed by colony PCR in 1.2 percent agarose gel (Plate 12_A, 12_B and 12_C). Grid plate were also prepared to maintain the transformed white colonies (plate 13). The plasmid DNA was isolated from the recombinant clones of YMMV and *Yam badnavirus*. The product size of 500 bp and 579 bp was obtained upon analyzing PCR product of plasmid DNA (plate 14_A and 14_B). Gel elution was carried out with single colony PCR positive clones of *Yam macluravirus*.

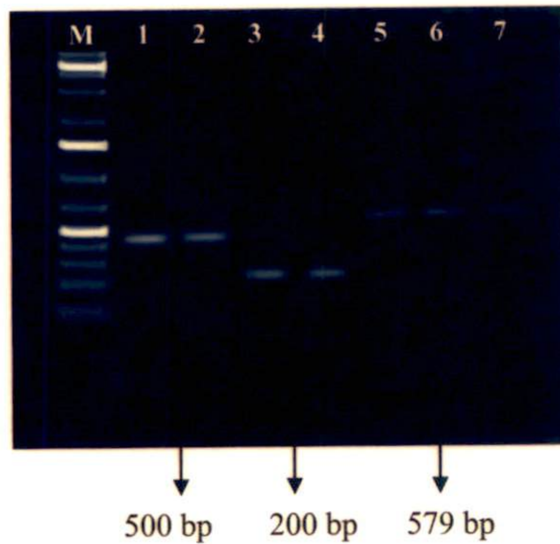


Plate 10. PCR purification of YMMV, *Yam Macluravirus* and *Yam Badnavirus* M: 1kb plus marker: Lane 1 to 2 YMMV, Lane 3-4 *Yam Macluravirus* and Lane 5 to 7 *Yam Badnavirus*.

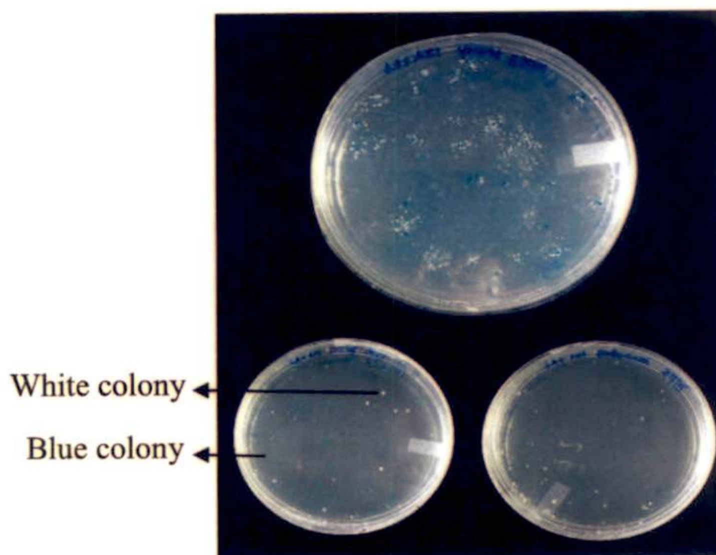


Plate 11. Luria-Bertani agar plate showing white (transformed) and blue (non-transformed) colonies of (A) YMMV (B) *Yam Macluravirus* (C) *Yam Badnavirus*

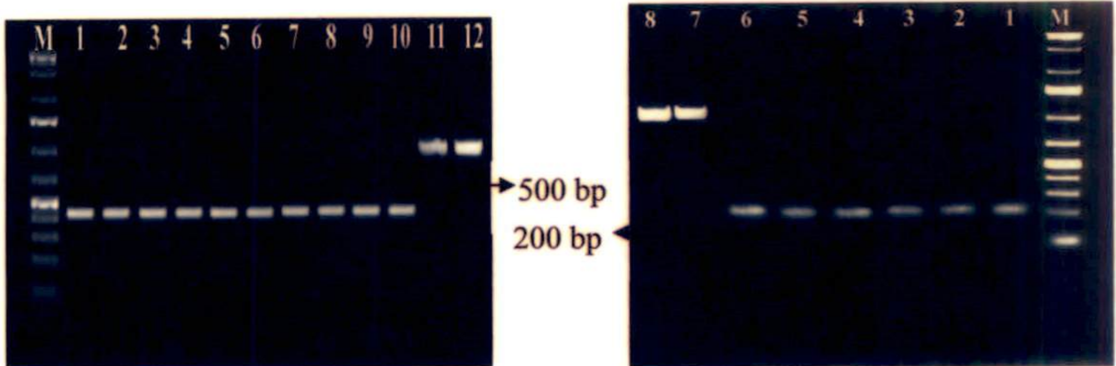


Plate 12. (A) Colony PCR result of YMMV; Lane 1 to 10 colony PCR products; lane 11, 12 control PCR product; M: marker; **Plate 12.** (B) Colony PCR result of *Yam Macluravirus*; Lane 8 to 7 control PCR product; Lane 1-6 colony PCR products; M: marker

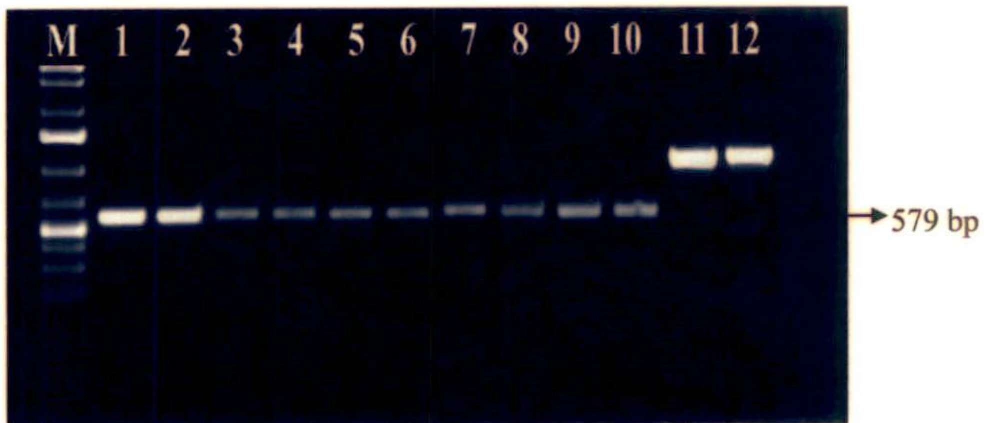


Plate 12. (C) Colony PCR result of *Yam Badnavirus*; Lane 1 to 10 colony PCR products; Lane 11-12 control PCR product; M: marker

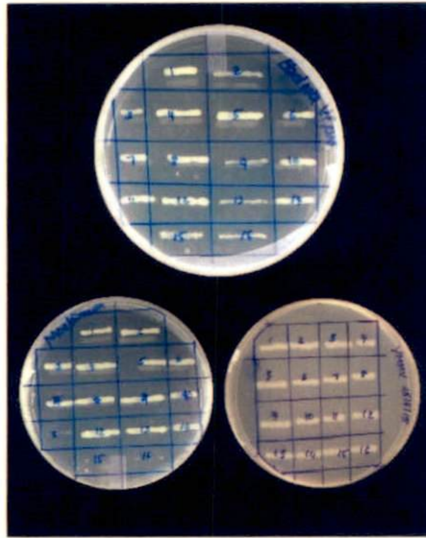


Plate13. Grid plate containing the transformed colonies of *Yam Badnavirus, Yam Macluravirus* and YMMV

Plate 14. (A). PCR amplified plasmid DNA of YMMV (M; marker: Lane 1 to 5 recombinant plasmid. **Plate 14 (B).** PCR amplified plasmid DNA of *Yam Badnavirus* (M; marker: Lane 1 to 6 recombinant plasmid)

4.7 DNA SEQUENCING

A recombinant plasmid DNA of YMMV and *Yam badnavirus* with gel elute amplified product sample of *Yam macluravirus* was sent to the Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology (RGCB) with their respective primers. The sequence result were obtained as electopherogram resulting from capillary sequencing in .abi format.

The YMMV sequence obtained is given below:

CCAGCCGTATTTCACGCGGAAGATGGATAACTGCTTTAAAGGTACT GAAGATTTTATTGCAGTTTACATTGATGATATTCTAGTGTTTTCAGACAAT GTTCGTGATCACAAAAGACACCTGCATATCATGTTATCCATATGCAAAAA GAACGGTCTGGTTCTCAGTCCTACCAAGATGAAGGTTGGGACATCTAGCA TTGATTTCCTCGGATCCACAATCTGCAATAGTAAGATCAAATTGCAGCCTC ACATTATAAGGAAAGTCGCTGACTTCCCCGAGGAAAGCTTGAAGACGAC AAAAGGGCTTAGGTCTTGGCTGGGCTTACTTAACTATGCCCGCAACTATA TCCCTAAGCTCGGAAAAATGTTGGGCCCGTTGTATTCCAAGGTCAGCCCA AATGGAGAGAAGCGAATGAACACACAGGACTGGGCTCTAGTGCGTGACA TGAAGAAGCAGATCCAGAATCTGCCCGATTTAGAACTATCTCCAGAGGAT GCATACATTGTCATTGAAA

The *Yam macluravirus* sequence obtained is given below:

TGTTGATTGTTGTTGTTGTCACCAGTTTCAATCGGTTGGGTGGACG GTCCTGGCCTGCCATCTCGCCTCTGCTCATGCTGCTTCCCTTTATCTTTCTG AGTTGTTTGCTACTGTGGGAGCGAGTGTTAGATCCATCTCTAAAACTTGTG ACCCCTCTGCCTCATCAAAACCTCAAACCGTAACCCTTTTCCATTCATATA TTGATTTTCTTCAATGGCTTCAT

The *Yam badnavirus* sequence obtained is given below:

ATGGTTCCTGTTCCAAAACCCAGCTGTAGAACAAGCGTATCTGCCT TAACAGTTCATCGTATCCCCATGCTTCAATCATCGCTGCACAGATGGCTTC AAGTCTGTGTTCATAATTCTCCGTTCTGCTCCACTCGAGTATTGCAACAAT TCGTTCCTCTTCGAGCTTTGGGATGAGGATGCCTTCCCTTTCAATTGCTTT GTGTGACATAAACCAAATGTCCTCTTTATGTTTGTGTCTATTTCCGAAGTC ATATGAAAGACCAAGTTCTGCGAAAGATTCAGACATTGTGTCGAGTACAT GCTCCCTCTCTGGAGGTATCGCAATCCACCAAATCCATCCCCATTTGCAAA ATAGACACACTCCTTTTGCTTGATGTCGTTTATTCCGTTCTTCAGCATTGC ATATTGTACAGCCAACAAGACCATAAGAGTG

4.7.1 Sequence Analysis

Sequence analysis of the cloned fragment confirmed the presence of YMMV, *Yam macluravirus* and *Yam badnavirus*. The sequence results were initially analysed and edited using BioEdit Sequence Alignment Editor Program version 7.2.5 and the obtained sequence was run through the online BLAST program of NCBI. The blast results (Figure 4, 5 and 6).

BLAST Map of the query sequence (433 nt) of YMMV sequence showed maximum similarity of 86% to *Yam mild mosaic virus* isolate CN20, complete genome (Accesssion KC473517). In the case of 223 nt *Yam macluravirus* sequence obtained in this study showed maximum similarity of 95% to *Macluravirus* YMCTCRI-01 polyprotein gene, partial cds (Accesssion KM099684). While the 515 nt *Yam badnavirus* sequence obtained in this study showed maximum similarity of 99% to *Dioscorea bacilliform virus* 1 gene for polyprotein, isolate FJ65c De (Accession AM072661).

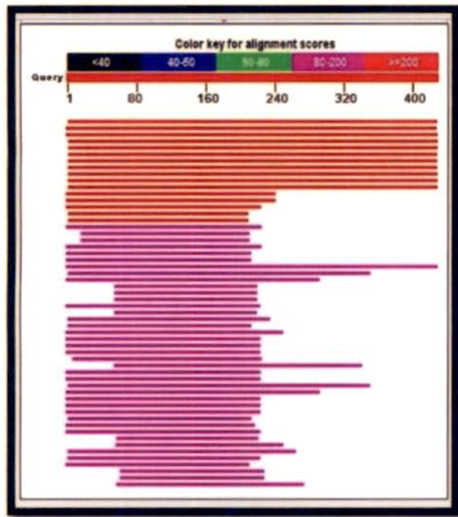


Figure 4: BLAST analysis of the YMMV sequence

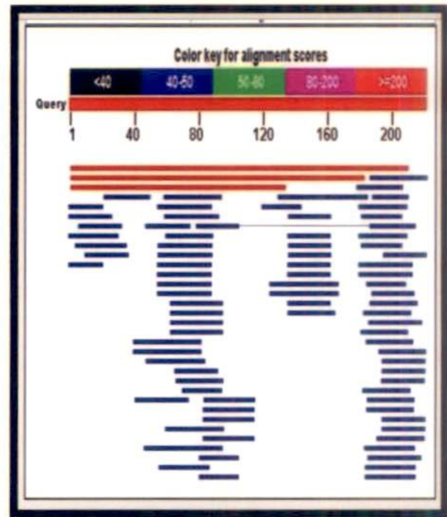


Figure 5: BLAST analysis of the *Yam Macluravirus* sequence

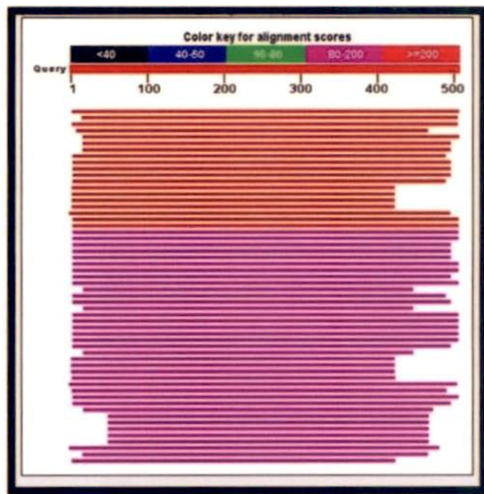


Figure 6: BLAST analysis of the *Yam Badnavirus* sequence

Phylogenic tree (fig 7, 8 and 9) was constructed with similar sequences using online NCBI blast analysis software. The trees constructed at 100 bootstrap replicates showed similarity with the complete genome of YMMV isolates, polyprotein gene of partial CP region of *Yam macluravirus* isolates and complete genome of *Yam badnavirus* isolates respectively.

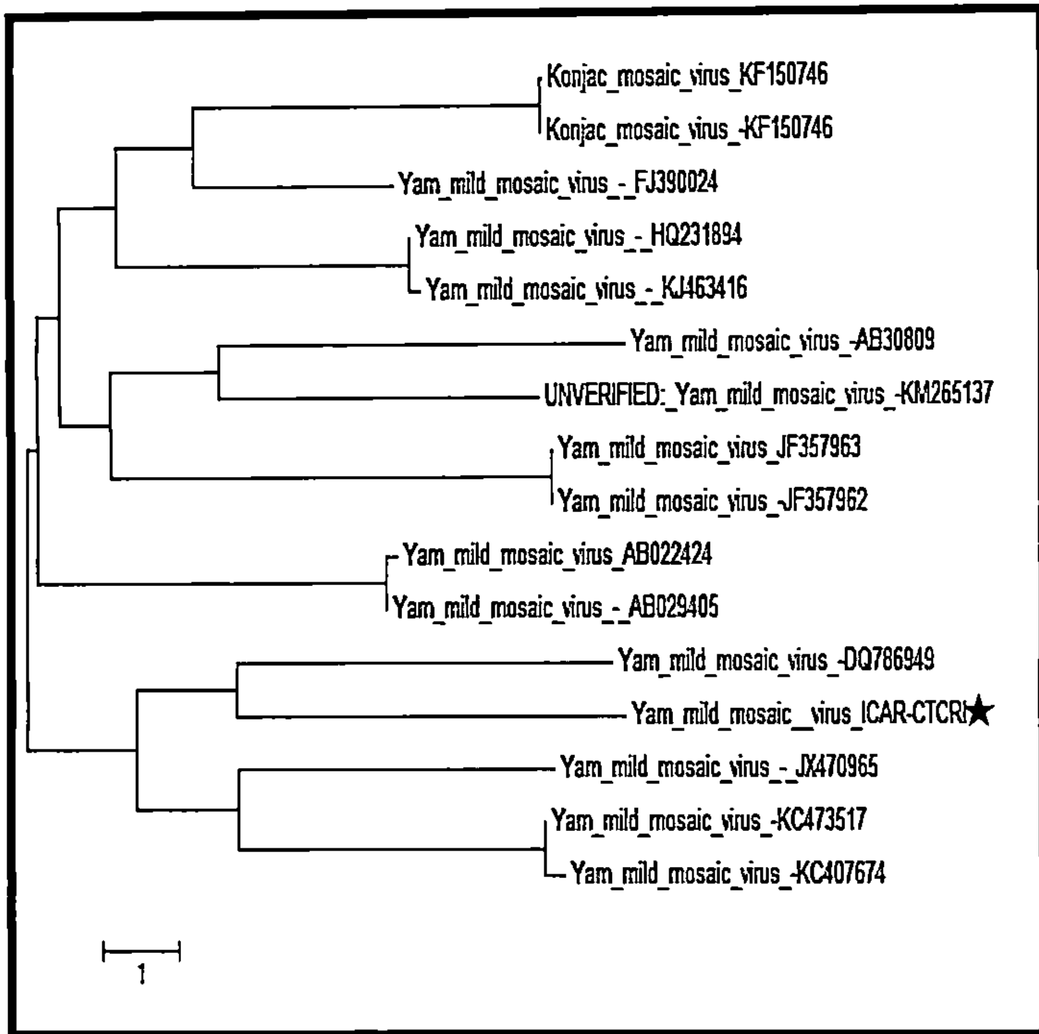


Figure 7: Phylogenetic tree based on the nucleic acid sequence of the partial coat protein coding region of *Yam Mild Mosaic Virus* and other YMMV isolates. The tree was constructed by the Neighbor-joining method using MEGA 6.06. the scale bar indicates 1 substitutions/site.

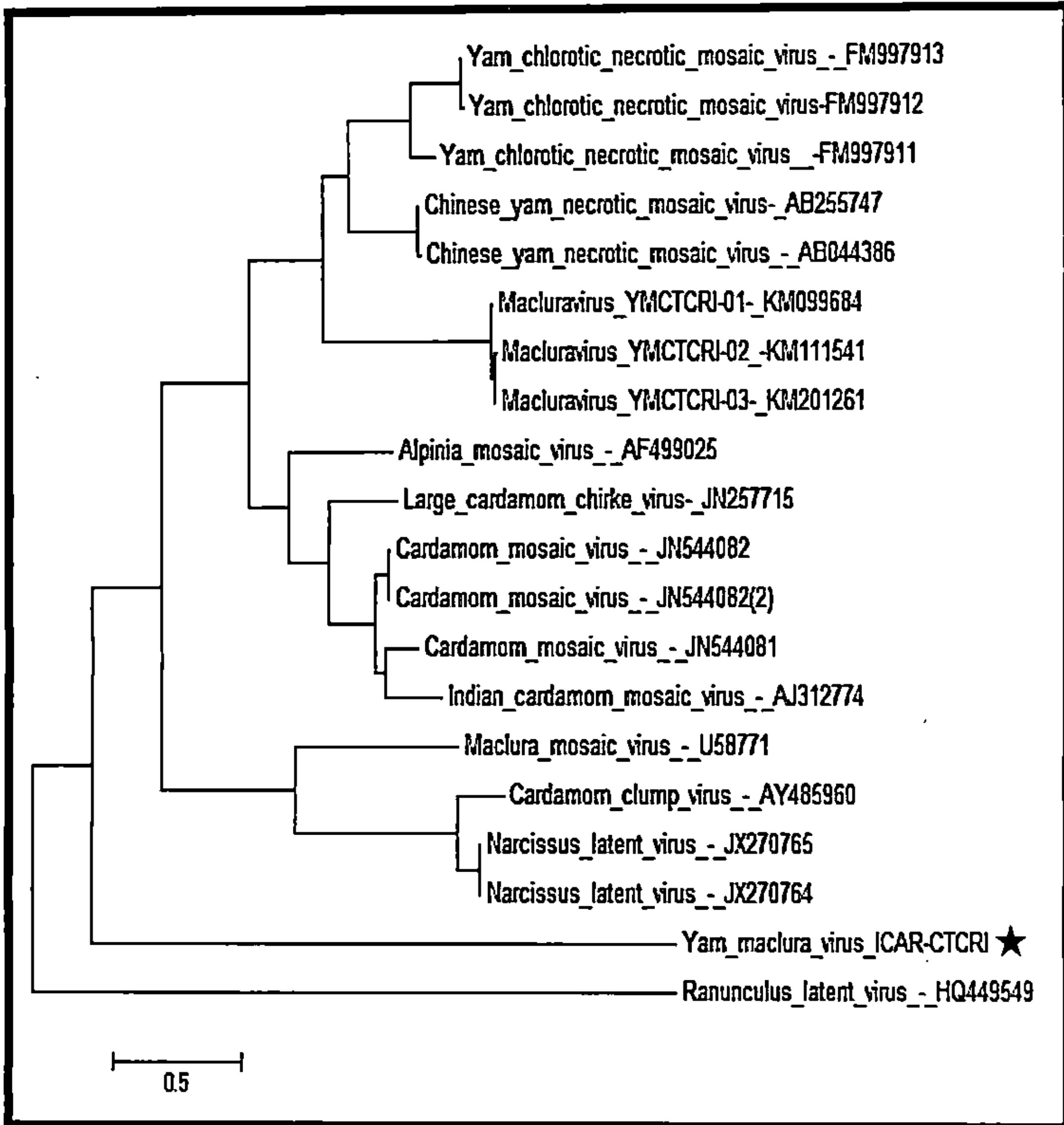


Figure 8: Phylogenetic tree based on the nucleic acid sequence of the partial coat protein coding region of *Yam Macluravirus* and other macluraviruses. The tree was constructed by the Neighbor-joining method using MEGA 6.06. the scale bar indicates 0.5 substitutions/site.

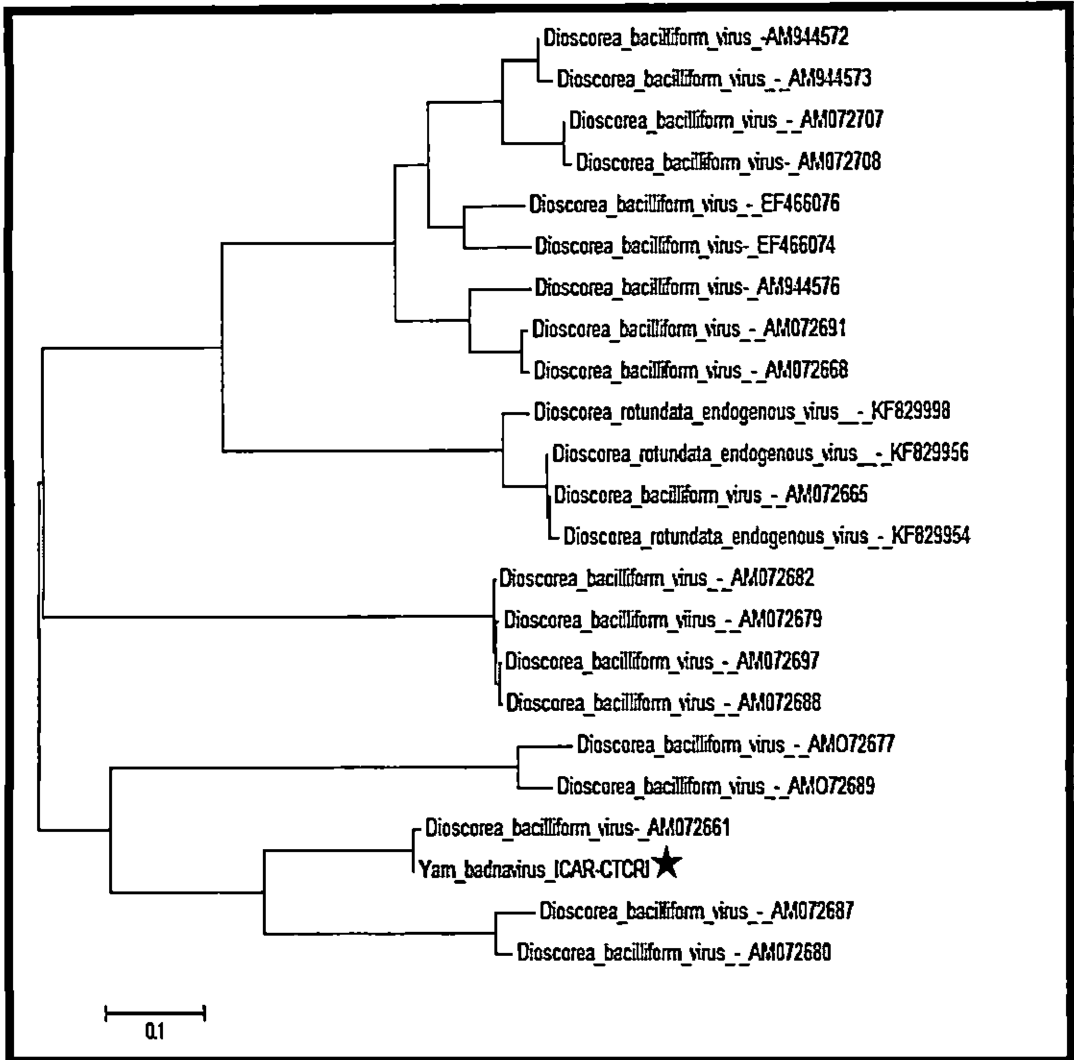


Figure 9: Phylogenetic tree based on the nucleic acid sequence of the partial coat protein coding region of *Yam Badnavirus* and other DBV isolates. The tree was constructed by the Neighbor-joining method using MEGA 6.06. The scale bar indicates 0.1 substitutions/site.

DISCUSSION

5. DISCUSSION

Lesser yam (*Dioscorea esculenta* (Lour.) Burkill), a vegetatively propagated tuber crop belonging to the family *Dioscorea,* is considered to be an ancient crop domesticated in the tropics. The tubers are rich in carbohydrate, beside a few vitamins and minerals. Yam production is adversely affected by pests and pathogenic diseases. Diseases caused by viruses, fungi, nematodes and bacteria, either singly or in combination are responsible for yield losses (Hughes *et al.*, 1997). The widespread distribution of viruses in yams is due to vegetative propagation, insect transmission and uncontrolled introduction of infected yam germplasm. Viruses infecting yams belonging to the genera of *Potyvirus, Badnavirus, Cucumovirus, Potexvirus* and *Macluravirus* (Kenyon *et al.*, 2001), and are poorly characterized, which is a hindrance to the safe movement of planting materials and exchange of germplasm.

The present study was undertaken to assess the prevalence and spreading of different viruses infecting lesser yam in India. In our study three viruses (*Yam mild mosaic virus* (YMMV), *Yam macluravirus* and *Yam badnavirus*) were detected in both from the leaf and tuber samples collected from ICAR-Central Tuber Crop Research Institute (CTCRI) lesser yam growing fields. The germplasm of lesser yam at present in ICAR- Central Tuber Crop Research Institute (CTCRI) experimental field includes accessions from USA, Africa and from different states of India viz., Kerala, Tamil Nadu, Maharashtra, Assam, Nagaland and Goa. The samples collected from ICAR-Central Tuber Crop Research Institute (ICAR-CTCRI) showed different types of viral symptoms. The presence of different symptoms viz., leaf coiling, mosaic, mottle, leaf distortion, vein banding, vein clearing puckering, cupping, chlorosis and leaf bleaching has been reported by different authors (Hughes *et al.*, 1997; Eni *et al.*, 2008; Séka *et al.*, 2009a; CTCRI 2005).

Symptoms represent important characteristics for plant virus identification and characterization. Generally the degree of symptom expression in a plant-virus interaction will depend on the virus and plant genotype, the age of the plant at time of infection and the local environmental conditions. Generally, symptom severity is proportional to virus titre, with severe symptoms associated with a high virus titre and vice versa. In this study diverse symptoms were observed on the lesser yam plants surveyed at ICAR-CTCRI fields. They include mild to severe mosaic, mottling, leaf distortion, leaf crinkling, puckering, cupping and chlorosis. Though there was much reports on the symptoms of lesser yam similar symptoms were previously reported in Nigeria, Benin and Côte d'Ivoire (Eni *et al.,* 2008; Séka et al., 2009a). However the symptoms were well documented in greater yam where the same viruses have been reported (Odu *et al.*, 2001; Amus *et al.*, 2003).

The detection of YMMV, *Yam macluravirus* and *Yam badnavirus* infections on non-symptomatic leaf samples, shows that absence of visual symptoms on yam leaves may not be indicative of absence of virus infection, but laboratory diagnosis serves as a more sensitive and conclusive way of affirming the health status of potential breeding or planting materials. On the other hand some plants showing virus-like symptoms tested negative, by both ELISA and PCR, to all three viruses indexed. The possible explanations for symptoms observed on these leaf samples are of symptoms may be caused by other virus(es) for which tests were not done or that are yet unidentified or symptoms may also be due to abiotic factors causing virus-like symptoms, such as nutrient disorder and senescence.

For screening viruses infecting lesser yam using nucleic acid based method good quality of DNA and RNA is a prerequisite for a reliable PCR and RT-PCR reaction. The present study for isolation of DNA (CTAB method) result revealed that, lesser yam leaves showed poor quality and quantity of extracted DNA. Lodhi *et al.*, (1994) also reported that DNA extraction from very young grapevines leaves result in

poor yields. Therefore fully opened younger leaves were used. While for RNA isolation from leaves, younger yam leaves are most suitable than older leaves. This is because a reduced amount of tannins and phenolics in the younger leaves compared with the older leaves. Although the RNA yield obtained from leaf samples using Ambion Purelink RNA Mini kit method proved to be better method for good quality RNA isolation than TRIzol reagent, the lithium chloride method provided highest quality and quantity of the RNA from tubers.

Viruses are of particular concern because, apart from causing significant reduction in tuber yield and quality, they restrict international exchange of germplasm. There are three viruses reported infecting other edible yams in India, viz., *Yam mild mosaic virus*, *Yam badnavirus* and *Yam macluravirus* (CTCRI 2009 and 2014). In this study the viruses YMMV, *Yam macluravirus* and *Yam badnavirus* were screened for their presence using serological (DAS-ELISA & TAS-ELISA), and nucleic acid based methods. (PCR and RT-PCR) techniques were employed to detect the presence of YMMV, *Yam macluravirus* and *Yam badnavirus* in lesser yam plants. DAS-ELISA, TAS –ELISA were protein based and relied on the use of polyclonal antiserum. Whereas PCR and RT-PCR are entirely based in the nucleic acid only. Initial screening using ELISA was used to detect YMMV, *Yam macluravirus* and *Yam badnavirus*. But it has major limitations such as its low sensitivity during periods of low titre. Although the specificity of the antisera used for the detection of these viruses has been previously evaluated and determined, all leaf samples were also tested by PCR and RT-PCR to ensure that plants with low virus load were not wasted by Njukeng *et al*., (2002).

In the present study, ELISA result revealed that YMMV was the most common virus infecting lesser yam. Similar observations of YMMV has also been reported in *Dioscorea esculenta* from the Solomon Islands (Mumford and seal, 1997). *Badnavirus* and *Macluravirus* was also detected at low incidence in greater yam plants from ICAR-CTCRI fields. The detection of *Badnavirus* through ELISA have already been reported

in many yam species from different countries (Briddon *et al.*, 1999; phillips *et al.*, 1999). The wide spread distribution of these viruses is most probably due to past exchange of infected yam tubers before the implementation of quarantine rules.

In this study molecular characterization of samples is done by RT-PCR with YMMV, *Yam macluravirus* and *Yam badnavirus* specific primers. The primers YMMV 1c/YMMV 1s, YV1-F/YV1-R, Ymac1s/ Ymac1c, and Badna F/Badna R which were used to amplify the partial CP gene had been successfully used for the detection of YMMV, *Yam macluravirus* and *Yam badnavirus* from greater yam plants in ICAR-CTCRI fields. Two sets of primers were used to confirm *Yam mild mosaic virus*. The primers YMMV 1c/YMMV 1s and YV1-F/YV1-R were used to amplify the virus from leaf samples and the tuber samples of lesser yam respectively. Of PCR and RT-PCR analysis with these primers yielded an expected product of size 500 bp, 260 bp, 200 bp and 579 bp respectively in most of the positive samples and YMMV, *Yam macluravirus* and *Yam badnavirus* respectively. Langeveld *et al.*, 1991 reported that detection of viruses with specific primers were 100-1000 fold more sensitive than the degenerate primers.

The present investigation revealed the number of virus infections detected by PCR and RT-PCR was more than the corresponding ELISA tests, possibly due to the greater sensitivity of PCR and RT-PCR. The specificity and sensitivity of both assays for the detection of YMMV, *Yam macluravirus* and *Yam badnavirus* have been previously evaluated and determined (Njukeng *et al.*, 2005). The lower sensitivity observed with the ELISA tests is similar to the findings of Mumford and Seal (1997) and could also be due to low virus concentration in yam (Brunt *et al.*, 1990) or due to interference of polyphenols and glutinous polysaccharides contained in yam leaves (Rossel and Thottappilly, 1985). Nevertheless, some ELISA positive leaf samples were not detected by PCR and RT-PCR. Lebas *et al.* (1999) reported similar results while working with yam from the South Pacific islands. This may suggest some variability

in the virus genomes or may be due to failure of PCR techniques due to inhibiting compounds found in yam (Wilson, 1997).

In this study two pairs of species specific primers (YMMV F1 /YMMV R1 and YMac F /YMac R) was designed to detect YMMV and *Yam macluravirus* infecting lesser yam. RT-PCR analysis with these primers amplifying the partial CP gene giving an amplicon of 193 bp and 237 bp respectively was found to be robust of detecting YMMV and *Yam macluravirus* in lesser yam. The specific primers are generally designed for many viruses and other pathogens to increase the specificity (Mumford and Seal (1997)).

It was found that, the 433 nt YMMV sequence obtained in this study showed maximum similarity of 86 % to Yam mild mosaic virus isolate CN20, complete genome (Accesssion KC473517). The 223 nt *Yam macluravirus* sequence obtained in this study showed maximum similarity of 95 % to *Macluravirus* YMCTCRI-01 polyprotein gene, partial cds (Accesssion KM099684). The 515 nt *Yam badnavirus* sequence obtained in this study showed maximum similarity of 99 % to *Dioscorea bacilliform virus* 1 gene for polyprotein, isolate FJ65c De (Accession AM072661). According to Fauquet *et al.,* (2005) *Potyvirus* isolates with 85 % sequence identity or more over the whole genome are usually considered to be from the same species.

The study suggests that here is an increased chance of virus introduction in to new areas through the infecting planting material because lesser yam is a vegetatively propagated tuber crop. As a result the present study helped to detect different viruses (YMMV, *Yam macluravirus* and *Yam badnavirus)* infect in lesser yam and standardize diagnostic techniques required for routine virus indexing. This will prevent yield loss in future by the use of healthy planting material and safe movement/ exchange of germplasam.

SUMMARY

6. SUMMARY

The study entitled "Identification and characterization of viruses infecting lesser yam (*Dioscorea esculenta* (Lour.) Burkill)" was conducted at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute (CTCRI), Sreekariyam, Thiruvananthapuram during 2014-2015. The objective of this study was to identify and characterize viruses infecting lesser yam at molecular level. The significant findings of the above studies are summarized in this chapter.

The leaf and tuber samples with various virus like symptoms were collected from the lesser yam growing fields of ICAR-CTCRI. General symptoms observed were chlorosis, mosaic, puckering, leaf distortion and mottling. The total nucleic acid (DNA and RNA) was isolated from virus infected samples. CTAB method was effective in isolating DNA from both leaf and tuber. The Ambion PureLink RNA Mini kit and Lithium chloride method were found to be most appropriate for RNA isolation from lesser yam leaf and tuber samples respectively. Serological and nucleic acid based techniques were performed to identify the presence of virus infection. DAS-ELISA and TAS-ELISA were used as serological method for the detection of YMMV, *Yam macluravirus* and *Yam badnavirus* with specific antibodies obtained from DSMZ, Germany. In the initial serological screening, out of 25 samples, 88%, 36% and 40% samples showed positive to YMMV, *Yam macluravirus* and *Yam badnavirus* respectively. Mixed infection of viruses were also observed (YMMV and *Yam macluravirus* (34.28%); YMMV and *Yam badnavirus* (31.42%); YMMV, *Yam maclura* and *Yam badnavirus* (14.28%); *Yam maclura* and *Yam badnavirus* (14.28%).

Nucleic acid based (PCR and RT-PCR) diagnostics were carried out using YMMV 1c/YMMV 1s, YV1- F/YV1- R, YMac1s/YMac1c, and Badna F/Badna R, the virus specific primers. The first two sets of primers amplified YMMV with amplicon size of 500 bp and 260 bp respectively whereas the third and fourth sets gave amplicon

size of 200 bp and 579 bp respectively for *Yam macluravirus* and *Yam badnavirus*. Based on the sequence two pairs of species specific primers *viz.,* YMMV F1/YMMV R1 and YMac F/YMac R were designed to amplify the partial coat protein gene of the viruses of YMMV and *Yam macluravirus*. The annealing temperature was standardized and RT-PCR performed with these primers provided amplicon size of 193 bp and 237 bp respectively.

The current study indicated that YMMV is the most common virus infecting lesser yam plant. The samples characterized, suggest mild to severe mosaic, puckering, cupping, mottling and chlorosis are the common symptoms associated with YMMV. The samples with no prominent symptoms were also detected with YMMV. Since mixed infection of these viruses also showed similar symptoms, the specific symptoms of individual virus could not be established. The *Yam macluravirus* and *Yam badnavirus* were also detected in lesser yam, but compared with YMMV the viruses (*Yam macluravirus* and *Yam badnavirus*) occur at lower incidence and concentration in plant tissues.

Screening of leaf and tuber samples of lesser yam for different viruses using PCR and ELISA, showed the presence of *Yam mild mosaic virus, Yam macluravirus and Yam badnavirus* in 88%, 36% and 48% for ELISA and 74.28%, 40%, 42.85% for samples respectively.

The amplified PCR sample (partial coat protein gene) of YMMV, *Yam macluravirus* and *Yam badnavirus* were cloned into *E. coli* DH5α cells. The presence of gene insert in the transformed colonies was confirmed by colony PCR. The recombinant plasmids DNA were isolated from the transformed colonies of YMMV and *Yam badnavirus* and RT-PCR was performed which yielded amplicons of expected band size of 500 bp and 579 bp respectively. For *Yam macluravirus* the colony PCR product was gel eluted. The plasmid DNA and gel elute were sequenced.

The sequence data was analyzed through BLAST and sequence similarity was studied. The BLAST analysis of the partial CP coding region revealed that YMMV, has maximum similarity of 86% to *Yam mild mosaic virus* isolate CN20, complete genome (Accesssion KC473517), whereas *Yam macluravirus,* 95% to YMCTCRI-01 polyprotein gene, partial cds (Accesssion KM099684) and *Dioscorea bacilliform virus*, 99% to isolate FJ65c De (Accession AM072661). The phylogenetic tree was constructed with similar sequences using. The phylogenetic tree was constructed with similar sequences using mega software. Phylogenetic analysis clearly revealed that the sequences obtained in this study is similar to *Yam mild mosaic virus*, *Yam macluravirus* and *Dioscorea bacilliform virus.*

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7. REFERENCE

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* Original not seen

APPENDICES

APPENDIX I

DAS-ELISA and TAS-ELISA

Coating buffer

Sodium carbonate - 1.59 g
Sodium bicarbonate - 2.93 g
Sodium azide - 0.20 g

Dissolve in 900 ml water; adjust pH to 9.6 with hydrogen chloride, make up to 1 L. Autoclave and store at 4 °C.

PBS (Phosphate Buffer Saline) p H – 7.4 (10 X), 1000 ml

Sodium chloride - 80 g
Dibasic sodium phosphate - 11.6 g
Monobasic potassium phosphate - 2.4 g
Potassium chloride - 2 g
Sodium azide - 2 g

Dissolve in 900 ml water; adjust pH to 7.4 with hydrogen chloride and make up to 1L. Autoclave and store at 4 °C.

PBS – 1 X (1000 ml)

100 ml (10 X) PBS+ 900 ml water

PBS – Tween

1 X PBS+ 0.5 ml Tween-20 1 L

Sample Extraction Buffer

PBS-T + 2% PVP

Conjugate Buffer

PBS-T+ 2% PVP+ 0.2% Egg albumin

Substrate buffer (pH-9.8) 100 ml

Diethanol amine	- 9.7 ml
Distilled water	- 80 ml
Sodium azide	- 0.2 g

Adjust the pH to 9.8 with hydrogen chloride and make up to 100 ml with distilled water.

Substrate Solution

10 mg p-nitrophenyl phosphate dissolved in 10 ml of substrate buffer.

Blocking Buffer

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

APPENDIX II

DNA ISOLATION

CTAB DNA Extraction Buffer

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

RNA ISOLATION
LITHIUM CHLORIDE METHOD

Extraction buffer

CTAB	- 2%
PVP	- 2%
Tris HCl (pH 8)	- 100 m*M*
EDTA	- 25 m*M*
NaCl	- 2 *M*
β-Mercaptoethanol	- 2%

EDTA was dissolved using sodium hydroxide pellets and pH was adjusted to 8. Then it was heated for complete dissolution of EDTA. The pH of Tris was adjusted using concentrated HCl. The reagents were autoclaved and stored. Ice cold 10 *M* lithium chloride was added before the overnight incubation step (4 °C) during RNA isolation.

TAE Buffer, 50 X (pH 8.0)

Tris-base	- 242 g
Glacial acetic acid	- 57.1 ml
0.5*M* EDTA	- 100 ml

Dissolve in 600 ml of distilled water, adjust the pH to 8.0 and make up to 1L with distilled water.

TAE buffer, 1 X

2 ml 50 X TAE + 98 ml distilled water

Agarose Gel (1.5%)

Agarose	- 1.5g
1 X TAE Buffer	-100 ml

Ethidium Bromide (10 mg ml-1)

1 g of ethidium bromide added to 100 ml of water, stirred vigorously and transferred to dark bottle and stored at room temperature.

APPENDIX III

CLONING AND TRANSFORMATION

Luria Agar

Suspend 3.5 g luria agar (HiMedia, Mumbai) in 100 ml distilled water, boil to dissolve and sterilize the medium by autoclaving.

T solution

Thaw T-solution (A) and T-solution (B), mix contents thoroughly. Combine 250 µl each of T-solution (A) and (B) in a separate tube and keep on ice.

Ampicillin stock solution (50 mg ml-1)

Dissolve 2.5 g of ampicillin sodium salt in 50 ml of deionized water. Filter sterilize and store in aliquots at -20 °C.

X-Gal stock solution (20 mg ml-1)

Dissolve 200 mg X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in 10 ml N,N-dimethylformamide. Store at -20 °C in the dark.

IPTG stock solution (100 m*M*)

Dissolve 1.2 g IPTG (isopropyl-β-D-thiogalactopyranoside) in 50 ml deionized water.Filter sterilize, aliquot and store at 4 °C.

LB- ampicillin X-Gal/IPTG plates

Suspend 4 g Luria Agar in 100 ml distilled water, boil to dissolve and sterilize the medium by autoclaving. Before poring the plates, allow the medium to cool to 55 °C. Then add 1 ml of ampicillin stock solution to a final concentration of 50 μg ml-1. Mix gently and pour the plates. Allow the LB-ampicillin agar medium to solidify. Dry plates under UV light for 30 minutes. Add 40 μl each of X-Gal stock solution and IPTG; spread evenly with a sterile spatula. Pre warm the plates at 37 °C for at least 20 minutes before using.

ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF VIRUSES INFECTING LESSER YAM

(*Dioscorea esculenta* (Lour.) Burkill)

SUDHEER K. S.

(2010-09-113)

Abstract of the thesis

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Department of Plant Biotechnology

COLLEGE OF AGRICULTURE

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ABSTRACT

The study entitled "Identification and characterization of viruses infecting lesser yam (*Dioscorea esculenta* (Lour.) Burkill)" was carried out at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute (CTCRI), Sreekariyam, Thiruvananthapuram during 2014-2015 with an aim to identify and characterize the viruses of lesser yam at molecular level and to design virus specific primers for detection.

Lesser yam is a vegetatively propagated crop, grown for its high calorific value tubers. Yam viruses causing significant reduction in tuber yield and quality are poorly characterized which is restrict the international exchange of germplasm. Lesser yam leaf and tuber sample with different virus symptoms were collected from the germplasm repository and experimental fields of ICAR-CTCRI. The serological and nucleic acid based methods were employed for the detection of *Yam Mild Mosaic Virus* (YMMV), *Yam Macluravirus* and *Yam Badnavirus* which were reported in other yams from India. The leaf and tuber samples of lesser yam were indexed for YMMV, *Yam Macluravirus* by double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) and reverse transcription (RT-PCR) whereas *Yam Badnavirus* was by triple antibody sandwich enzyme linked immunosorbent assay (TAS-ELISA) and polymerase chain reaction (PCR). *Yam Mild Mosaic Virus* is the most prevalent virus detected in 74.28% of the samples followed by *Yam Badnavirus* (42.85%) and *Yam Macluravirus* (40%). Mixed infections of YMMV-*Macluravirus* (34.28%), YMMV-*Badnavirus* (31.42%), *Macluravirus-Badnavirus* (14.2%) and combination of these three viruses (14.2%) were also observed. Two pairs of novel species specific primers were developed to amplify the partial coat protein gene of YMMV and *Yam Macluravirus*. After identification, one sample each for YMMV, *Yam Macluravirus* and *Yam Badnavirus* were cloned and sequenced. The sequence data was analyzed through BLAST and

sequence similarity was studied. YMMV has maximum similarity of 86% to *Yam Mild Mosaic Virus* isolate CN20, complete genome, whereas *Yam Macluravirus*, 95% to YMCTCRI-01 polyprotein gene and *Yam Badnavirus*, 99% to *Dioscorea bacilliform virus* 1 gene for polyprotein, isolate FJ65c De. The result obtained from this study will be useful for indexing the planting materials which helps in production of healthy planting material and germplasm there by helping the farming communities to get potential yield.