DETECTION AND CHARACTERIZATION OF *Macluravirus* INFECTING GREATER YAM (*Dioscorea alata* L.)

by

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(2009-09-114)

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DECLARATION

I, Manasa V. G., hereby declare that this thesis entitled **"DETECTION AND CHARACTERIZATION OF *Macluravirus* INFECTING GREATER YAM (*Dioscorea alata* L.)"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µg	Microgram
µl	Microlitre
µ*M*	Micromolar
A	Adenine
A_{230}	Absorbance at 230 nm wavelength
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
A_{405}	Absorbance at 405 nm wavelength
AFLP	Amplified fragment length polymorphism
APS	Ammonium persulfate
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
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
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
NBT	Nitro blue tetrazolium
NCBI	National center for biotechnology information
NCM	Nitrocellulose membrane
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
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
s	Second
SDM	Spray dried milk
SDS	Sodium dodecyl sulfate
sp.	Species
spp.	Species (plural)
SSR	Simple sequence repeat
t	Tonne
T	Thymine
TBS	Tris buffer saline
TEMED	N,N,N',N' –tetramethylethylenediamine
T_m	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

Edible yams are economically important staple food in tropical and subtropical regions of the world. Yam is a common name for several species belonging to the genus *Dioscorea,* family *Dioscoreaceae* and order *Dioscoreales.* These annual or perennial herbaceous vines with edible underground tubers (Nweke *et al.*, 1991) rank the third most important tuber crop after cassava and sweet potato (Fu *et al.*, 2005). Yam tubers are an important source of carbohydrate for millions of people in the world particularly in Africa, the Caribbean, parts of Asia, South and Central America and the Pacific. Some species of yam are of medicinal and ornamental value (Hou *et al.*, 2002). Yam can be stored longer than most of the other tropical fresh products and has a potential for increased commercial exploitation and processing (O'Hair, 1990a). However, yam production is adversely affected by pests and pathogenic diseases (Wilson, 1982; Nweke *et al.*, 1991; Degras, 1993). Virus diseases are of particular importance because they reduce vigor and subsequently cause a reduction in tuber yield and quality (Coursey, 1967a; Amusa *et al.*, 2003). They may also restrict the international movement of improved or selected germplasm due to quarantine restrictions (Amusa *et al.*, 2003).

Nearly ten different viruses have been reported to infect yams (*Dioscorea* sp.) around the world and seven infecting *D. alata*, *viz., Yam mosaic virus* (YMV), *Yam mild mosaic virus* (YMMV), *Dioscorea alata badna virus* (DABV), *Dioscorea alata ring mottle virus* (DARMV), *Dioscorea trifida virus* (DTV), *Dioscorea dumetorum potyvirus* (DDV) and *Chinese yam necrotic mosaic virus* (ChYNMV) (Lebas, 2002). But information about the presence of such viruses in India is scanty.

Survey conducted at Central Tuber Crops Research Institute (CTCRI) revealed the presence of YMMV and DABV in yam growing regions in India. Preliminary studies showed the presence of a *Macluravirus* also in some of the greater yam samples. The genus *Macluravirus*, belonging to the family *Potyviridae*,

is characterized by its flexuous filamentous particles, a polyprotein genome strategy and formation of inclusion bodies in infected plant cells. It has shorter particles (650-660 nm in length) and apparently smaller genome.

Natural transmission of these viruses is mainly through infected planting materials, by vectors and through mechanical transmission (Thouvenel and Fauquet 1986; Brunt *et al.*, 1989; Matthews, 1991). Since yam is propagated vegetatively by means of tubers (seed yam or planting setts), viruses may accumulate in the plant over the years (Brunt *et al.*, 1989). Use of infected planting materials is a major means of spreading yam viruses. Therefore it is necessary to control the spread of 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 material and prevention of virus infection through cultivation of virus resistant varieties. Both of these methods greatly depend on the availability of sensitive and specific virus diagnostic tools for monitoring the viruses in plants and plant parts.

Since plant viruses are becoming widespread and there are real threats of new virus epidemics, the identification and characterization of *Macluravirus* has to be done which is necessary for developing specific diagnostic techniques and formulating control strategies. Hence the specific objectives of this work were formulated as:

- To detect the presence of *Macluravirus* in greater yam (*Dioscorea alata*).
- To identify the virus.
- To design virus specific primers for detection.
- To study the degree of genetic diversity of the virus present in yam collected from different regions of Kerala.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 ROOT AND TUBER CROPS

Tropical root and tuber crops including cassava, sweet potato, yams and aroids are relished as vegetables, used as raw materials for small scale industries, and consumed as staple food especially in the under developed countries (Ravi *et al.*, 1996). During the early evolution of mankind, they were critical components in the diet and the most important food crops associated with human existence, survival and socio-economic history (Asha and Nair, 2002). Apart from providing basic food security, a source of income and diversity in diet, they also serve as an additional source of proteins, essential vitamins and minerals. Even though they belong to different botanical families, they are grouped together owing to their vegetative mode of propagation, production of underground food and bulky perishable nature. Their production, with an exception of sweet potato, is limited to the warmer regions because of lack of tolerance to freezing temperatures (O'Hair, 1990b). But the vegetative propagated nature of these crops exposes them to a plethora of pests and diseases that even impedes the international exchange of germplasm.

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

2.2 YAMS

Yams, belonging to the genus *Dioscorea*, family *Dioscoreaceae*, are important food crops in the tropical and subtropical regions (Ayensu and Coursey, 1972). Yam plants are dioecious monocotyledonous vines, cultivated for their starchy tubers in widely varying agro-ecological zones. The multi-species crop originated principally from Africa and Asia before spreading to other parts of the

world (Hahn *et al.*, 1987). 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 (Girardin *et al.*, 1998). World annual production of yam, in 2010, was estimated to be 48.3 million tonnes covering over 5 million hectares of land (FAOSTAT, 2012).

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). Yam domestication started at least 5000 years ago (Dumont, 1982) and the process is continuing, providing new sources of diversity.

Of the 600 and more species of *Dioscorea* recognized, only about 10 are grown for food purposes. About 30 other species are grown on a small scale for extraction of the pharmaceutical compounds dioscorin and diosgenin (Kenyon *et al.*, 2001). Out of the 10 cultivated yam species, *D. rotundata* Poir., *D. cayenensis* Lam., *D. alata* L. and *D. trifida* L. are the major species, while the six others are often referred to as minor ones. The 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). *D. trifida* is the most important edible yam in tropical America and its flesh is considered to be the most flavorful of all the yams (Martin and Degras, 1978).

Minor cultivated species are important in specific regions or countries. *Dioscorea esculenta* (Lour) Burk is commonly cultivated in Southeast Asia and Melanesia; *D. opposita- japonica* in China and Japan; *D. transversa* in Australia and Melanesia; *D. nummularia* in Indonesia and the Pacific. There are also several wild species that are still a major source of food for some communities, such as the endemic species of Madagascar, *D. soso*, *D. nako*, *D. bemandry*, *D. alatipes*, *D. hombuka*, and the species *D. hamiltonii* consumed by forest based communities in India. 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).

In India, many of the *Dioscorea* species serve as a 'life saving' plant group for the marginal farming and forest dwelling communities, during periods of food scarcity. Most of the species are ethnically important as a secondary staple and a delicacy for tribal people in the interior regions of Western Ghats.

2.2.1 Greater Yam

Dioscorea alata, also known as water yam or greater yam, is the most widely cultivated yam species throughout the tropics. It is an important food in West Africa and the Caribbean than in Asia and America where it originated. It is next to *D. rotundata* in terms of production and extent of utilization. Also, it is the highest yielding yam species and can be stored for a relatively longer period (5-6 months) after harvest (Baah, 2009). Due to the ease of propagation and high multiplication ratio, it has better agronomic flexibility than other cultivated yam species (Petro *et al.*, 2011). It is also known for its high nutritional content with crude protein content of 7.4 percent, starch content of 75-84 percent and vitamin C content ranging from 13.0-24.7 mg 100 g^{-1} (Osagie, 1992).

Greater yam was never found in the wild and its hybridization with other *Dioscorea* species is unknown. But it is believed that two Asian species (*D. hamiltonii* and *D. persimilis*) could be part of its origin (Burkill, 1960) and water yam is a true cultigen derived from these wild forms in Indo-China region through human selection, although there is no concrete evidence to support this claim (Hahn, 1995).

Dioscorea alata is a polyploid and its ploidy level has been found to be correlated with growth vigor, increased tolerance to abiotic and biotic stress, and higher tuber yield (Abraham and Arnau, 2007; Lebot, 2009). The potential yield of *D. alata* has been estimated to be between 60 and 75 t ha^{-1} per year (Zinsou,

1998). Generally, breeding for desired traits in water yam, a heterozygous crop with a long growth cycle and erratic flowering is a difficult and long process.

2.3 ORIGIN AND DISTRIBUTION OF YAMS

Dioscorea spp. is found in a wide range of tropical and subtropical ecologies from semiarid scrub land to tropical rain forests. The different yam species originated in three independent areas of the tropics: Asia, West Africa and tropical America. Some originated from Africa before spreading to other parts of the world while some originated from Asia and have spread to Africa (Hahn *et al.*, 1987). *D. alata* and *D. esculenta* originated from Asia and were introduced to West Africa. *D. rotundata, D. cayenensis* and *D. dumetorum* Pax. originated from West Africa, while *D. trifida* originated from tropical America (Coursey, 1967b; Ayensu and Coursey, 1972; Hahn *et al.*, 1987). In general, there was an eastward movement of yam species during the domestication process.

Today, yams are grown widely throughout the tropics and have a large biological diversity including more than 600 species worldwide (Burkill, 1960; Coursey, 1967b). Most are climbing vines found in tropical forests and forest margins.

2.4 PRODUCTION

Food yams are 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". World annual production of yam, in 2010, was estimated to be 48.3 million tonnes covering over 5 million hectares of land. About 94 percent of this production was from Western and Middle Africa, with Nigeria being the world largest producer (38 million tonnes). The remaining was produced mainly in Central and South America, Oceania and Asia. Nigeria is the leading producer followed by Ghana (6.6 million tonnes), Côte d'Ivoire (5.7 million tonnes), and Bénin (2.7 million tonnes) (FAOSTAT, 2012). Sub-Saharan Africa is expected to

produce 98.1 percent of total world production of yam by 2020 (Scott *et al.*, 2000).

In India, greater yam is cultivated practically in all the states but the major yam producing states are Andhra Pradesh, Assam, Bihar, Gujarat, Kerala, 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. During 2010-11, the area under yam production in Kollam district of Kerala 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).

Yam production has increased steadily in the last two decades, from 18 million metric tonnes in 1990 to recent estimates of over 48 million (FAOSTAT, 2012). This increase has been achieved mainly through the use of traditional landraces and can be explained by the rapid increase in acreage of yam fields into marginal lands and non-traditional yam growing areas. However, per hectare yield of yams has not increased over time.

2.5 MORPHOLOGY

Dioscorea alata, *D. rotundata* and *D. cayenensis* belong to the botanical section Enantiophyllum, which includes species with an anticlockwise twinning stem and entire leaves. *D. trifida* belongs to the section Macrogynodium and is characterized by a clockwise twinning stem and lobed leaves.

Yam shoots are vine-like climbers thus requiring support from either neighboring plants or stakes on which they twine clockwise or anticlockwise depending on the species. Shoots can be round, winged or ridged, some bearing spines at the base. Leaves are opposite, heart shaped, ovate to oblong, with a deeply cordate base. They are 3-6 inches long and have 7-9 veins that begin at the leaf base. Yam is dioecious, producing small inconspicuous flowers about 2-4 mm in diameter. Flowers are white, cream, greenish or brown depending on the

species and they are wind or insect pollinated. Female flowers are borne in small numbers on auxiliary spikes while male flowers are borne in large numbers on panicles. Male and female plants must be grown if seeds are required but time of flowering of male and female plants is difficult to synchronize. Yam fruits are dry capsules, 1-2 cm long and usually broader than long. Seeds are flattened, hard and encircled by a wing.

Yam tubers, which are enlarged storage organs containing food reserves in the form of starch, vary greatly in size ranging from that of a small potato to over 2.5 m in length, and may weigh up to 7.5 kg. They are round, cylindrical, oval or flattened (Toyohara *et al.*, 2000) and are usually produced underground. Depending on the variety, the tuber flesh may be various shades of off-white, yellow, purple, or pink, and the skin can be off-white to dark brown. Some species of yam, such as *Dioscorea bulbifera,* produce aerial tubers. Yam tubers may be annually renewable organs, or may be perennial increasing in size and progressively becoming lignified year after year to form rhizomes (Osagie, 1992).

2.6 GENETIC DIVERSITY

There is tremendous genetic variability in yam. Contrary to the situation in other crops where the deployment of improved varieties has led to loss of diversity and a narrowing of the genetic base, the domestication of semi-wild yam species is still on going in West African countries, which continually augments the germplasm diversity (Mignouna and Dansi, 2003). One major limitation to maximizing the full potential of various yam species has been the poor understanding of their genetics. Among the major staple food crops, yam genetics is one of the least understood (Zoundjihekpon *et al.*, 1994). Little is known about the inheritance of important traits in yam and understanding of the number of chromosomes has been reversed several times (Gedil and Sartie, 2010).

Earlier cytological studies had shown the existence of different ploidy levels (tetraploid, hexaploid and octoploid) in yam species (Abraham and Nair, 1991) with x = 10 as basic chromosome number. The basic chromosome number of x =

10 is reported in all the Asian species. The African and American species are considered to have a chromosome number of x = 10 or 9.

Despite a growing interest in water yam, published data on molecular characterization and genetic diversity of this crop are scanty (Siqueira, 2011). *Dioscorea alata* is a polyploid, with several ploidy levels and reveals a predominance of tetraploidy (Arnau *et al.*, 2009; Obidiegwu *et al.*, 2009). This species includes accessions with 2n = 40, 60 and 80 chromosomes (Abraham and Nair, 1991; Egesi *et al.*, 2006; Arnau *et al.*, 2009). Siqueira *et al.* (2012) studied about the genetic diversity of 36 *D. alata* accessions from Brazil, Puerto Rico and the Democratic Republic of Congo using SSR markers to infer about possible origins of accessions commercialized in Brazil.

Dioscorea rotundata was first considered a tetraploid species (2n = 40) (Dumont and Vernier, 2000). However, segregation studies of microsatellite markers have revealed that it is a diploid with the basic chromosome number of x = 20 (Scarcelli *et al.*, 2005). In segregating populations of guinea yam (*D. rotundata*) and water yam, the observed segregation of AFLP markers reflected a disomic inheritance (Mignouna *et al.*, 2002a, b). Another report based on SSR marker segregation studies has revealed that the American yam *D. trifida* (2n = 80) (Essad, 1984), belonging to the section Macrogynodium, which was considered to be octoploid, is actually a tetraploid with the basic chromosome number of x = 20 (Bousalem *et al.*, 2006, Hochu *et al.*, 2006).

2.7 AGRONOMIC CHARACTERISTICS

Yam grows well in tropical climate and does not tolerate frosty conditions. Temperature between 25 and 30 °C is needed for normal development and growth is hindered below 20 °C. Light intensity also affects growth and tuber formation. Short days with 10 to 11 h promote tuber formation, while days longer (with more than 12 h) promote vine growth. An annual rainfall of about 1000 mm (or an equivalent amount of irrigation water) spread over five to six months and deep, fertile, friable and well-drained soils of pH 6 to 7 are ideal for yam cultivation

(Hahn *et al.*, 1987; Osagie, 1992). Most food yams give the highest yields in areas where long rainy seasons prevail. Yam is also able to survive long dry periods, though yield is reduced considerably (Baah, 2009).

Traditionally, yams are propagated vegetatively from whole tubers (seed yams), large tuber pieces (sets) or from minisetts. The growth of yam starts with a sprout from the post dormant tuber (Onwueme, 1984) and they exhibit a sigmoidal growth pattern common to most annual plants (Craufurd *et al.,* 2001). A period of slow growth during establishment is followed by a phase of rapid exponential growth as the canopy reaches maximum area and, finally, growth rates decline as the canopy senesces. Edible yams are annual crops with basically two growth cycles. During the tropical wet season, the first stage of vegetative growth of vine extension and flowering takes place. During the dry season, most aerial growth stops and the nutrients are moved to the tuber, which grows before entering into a dormant state (Onwueme and Charles, 1994). The tubers have a large sink capacity and continue to grow and store food reserves throughout the year as long as conditions remain favorable. Yam tubers have relatively long storage life of 4–6 months after harvest at a mean ambient temperature of 25 °C.

2.8 NUTRITIONAL VALUE

Apart from providing basic food security and income, yam is a nutritional source for millions of people. It is rich in carbohydrate and is a good source of vitamins and minerals. The tubers have diverse traditional uses but little industrial use (Albrecht and McCarthy, 2006).

Yam tubers are known for their high nutritional content (Table 1), with approximately 75.6-83.3 percent carbohydrate, 3-7.4 percent protein, 0.5-1.5 percent fibre, 0.7-2.0 percent ash, 0.05-0.02 percent fat and vitamin C content ranging from 13.0 to 24.7 mg 100 g^{-1} (Degras, 1993; Wanasundera and Ravindran, 1994; Opara, 1999). The tubers have high moisture content, dry matter and starch. A large proportion (65-75%) of the yam tuber is made up of water. They are also a good source of minerals and are high in dietary fibre, vitamin B6,

potassium and manganese and low in saturated fat, sodium and cholesterol (Woolfe, 1987; Walsh, 2003). The high potassium and low sodium content of yam produces good potassium-sodium balance in the human body and hence 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 gradual 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 (Brand-Miller *et al.*, 2003; 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 sweet potato.

Some wild species of yam, 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 like *D. zingiberensis* are cultivated for extraction of diosgenin, a female hormone precursor used in the manufacture of contraceptive pills and sex hormones (Ulbricht *et al.*, 2003). Its pharmacological activities include antitumor, anti-allergic, anti-inflammatory and anti-HIV activity (Zhou *et al.*, 2013). Some species contain alkaloids (e.g. dioscorine $C_{13}H_{19}O_2N$) and steroid derivatives, which render the tuber bitter and sometimes poisonous if consumed without proper processing (Purseglove, 1976). Brown yam flour is reported to have antioxidant activity which may mediate in oxidative damage and diseases caused by environmental chemicals (Farombi *et al.*, 2000) and could be utilized to stabilize bulk oils, emulsions and biological membranes against lipid peroxidation. The major yam species contain polyphenol oxidase enzyme, the activity of which varies even within a given species (Muzac- Tucker *et al.*, 1993). Specific phenolics in yam play a key role in disease resistance of the tuber.

Table 1: Nutrient contents of yam species (*Dioscorea* spp.) per 100 g fresh edible tuber portions

Nutrient	*D. alata*	*D. rotundata*	*D. cayenensis*	*D. esculenta*	*D. dumetorum*
Moisture (%)	65-78.6	50.0-80	60-84	67-81	67-79
Carbohydrate (%)	22-31	15-23	16	17-25	17-28
Starch (%)	16.7-28	26.8-30.2	16.0	25	18-25
Free sugar (%)	0.5-1.4	0.3-1	0.4	0.6	0.2
Protein (%)	1.1-3.1	1.1-2.3	1.1-1.5	1.3-1.9	2.8
Crude fat (%)	0.1-0.6	0.05-0.1	0.06-0.2	0.04-0.3	0.3
Fibre (%)	1.4-3.8	1.0-1.7	0.4	0.2-1.5	0.3
Ash (%)	0.7-2.1	0.7-2.6	0.5	0.5-1.5	0.7
Phosphorous (mg)	28- 52	17	17	35-53	45
Calcium (mg)	28 -38	36	36	12-62	52
Vitamin C (mg)	2.0-8.2	6.0-12.0	-	-	-
Iron (mg)	5.5-11.6	5.2	5.2	0.8	-
Food energy (kcal)	140	142	71	112	122
β-carotene (µg)	5-10	-	-	-	-
Thiamine (mg)	0.05- 0.10	-	-	0.1	-
Riboflavin (mg)	0.03- 0.04	-	-	0.01	-
Niacin (mg)	0.5	-	-	0.8	-

Source: Baah, 2009

2.9 CONSTRAINTS TO YAM PRODUCTION AND STORAGE

A number of constraints hamper the increased and sustainable production of yams. The major problems associated with yam production have been identified as the lack of availability and high cost of seed yam, high labor requirement (labor cost during planting, weeding, staking, and harvesting), diseases and pests, weeds as well as high post-harvest losses (Tschannen *et al.*, 2003).

2.10 DISEASES AND PESTS OF YAM

Yam is susceptible to a variety of pest and diseases during growth as well as postharvest. Over 25 percent of yield losses are due to diseases and pests. Important yam pests include nematodes, beetles, termites, weevils, scale insects and rodents (Onwueme, 1978; Onwueme and Charles, 1994). Diseases include those caused by fungi, bacteria and viruses (Degras, 1993).

The major insects attacking yam are beetles, termites and scale insects. 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 (Adeniji, 1970). The yam nematode (*Scutellonema bradys* Steiner and LeHew) and the root knot nematodes (*Meloidogyne* spp.) are the most serious nematode pests of yam and are wide spread in West Africa (Caveness, 1992; Kwoseh *et al.*, 2007).

The main fungal disease of yam, anthracnose, is caused by *Colletotrichum gloeosporioides* Penz. (Onwueme and Charles, 1994). It is widespread throughout tropical countries and is the most prevalent fungal disease in yams, especially *Dioscorea alata* (Jacqua *et al.*, 2005; Onyeka *et al.*, 2006). The disease causes leaf necrosis and shoot die-back. Severe production loss has been reported from the Caribbean, South Pacific, West Africa and India. Yield loss of up to 90 percent has been reported under conditions favorable for disease development, while the disease is believed to be responsible for the disappearance of some popular cultivars of *D. alata* in the Caribbean (McDonald *et al.*, 1998). Other fungal

diseases of yam are leaf spot and leaf blight caused by *Curvularia* spp. (Onwueme, 1978).

Yam anthracnose and virus diseases constitute major pathological problems in *Dioscorea alata* production in all yam growing regions of the world. Yam anthracnose disease has been identified as the most important biotic constraint to *D. alata* production worldwide (Egesi *et al.*, 2007).

The most serious pests affecting *Dioscorea rotundata* and *D. cayenensis* are nematodes (Kwoseh *et al.*, 2007) and potyviruses (Bousalem *et al.*, 2000a, b, 2008). Potyviruses are the main limiting factor for the production of *D. trifida*. Potyviruses cause significant economic damage and seriously impede the development of this yam as a viable crop. They are directly involved in the regression of *D. trifida* in the Caribbean and French Guyana (Degras, 1993; Bousalem *et al.*, 2000b, 2003).

2.10.1 Viruses infecting yams

Virus diseases of yam were first identified in Sierra Leone and Puerto Rico in 1936 (Cook, 1978). Subsequent description of yam virus infection was in 1957 (Chant, 1957; Miège, 1957) and 1961 (Robertson, 1961). Moderate to severe mosaic symptoms were later reported in *Dioscorea rotundata* in Nigeria (Terry, 1976), Côte d'Ivoire (Thouvenel and Fauquet, 1979) and Togo (Reckhaus and Nienhaus, 1981). Yam viruses have been reported infecting different yam species in all the tropical regions where yam is grown (Odu *et al.*, 2004; Seal and Muller 2007). Viruses infecting yam are in the *Potyvirus, Badnavirus, Potexvirus, Carlavirus, Cucumovirus, Comovirus, Potexvirus* and *Macluravirus* genera (Kenyon *et al.*, 2001). Symptoms associated with yam virus diseases include severe leaf chlorosis, green vein banding, mosaic, shoe string, mottle, vein clearing, interveinal chlorosis, stunting and distortion. These symptoms, which mainly affect the foliage, lead to a reduction in the photosynthetic ability of the infected plant with deleterious effects on the tuber yield, quality and, in some instances, death of the plants (Odu *et al.*, 2001; Amusa *et al.*, 2003). Furthermore,

the presence of viruses in yam tubers hinder the international trading of yam tubers and the international movement of yam germplasm required for research and improvement purposes (Brunt *et al.*, 1989).

In sub-Saharan Africa, where more than 90 percent of the world's yams are produced, *Yam mosaic virus* (YMV), genus *Potyvirus,* family *Potyviridae* is a ubiquitous pathogen (Thouvenel and Fauquet, 1979). YMV has been identified in all the areas of production (Africa, the Caribbean, Latin America and the South Pacific) and found in several species of *Dioscorea.* It is the only well characterized virus infecting yams. Potyviruses constitute the largest and economically most important genus of plant viruses (Bos, 1992; Shukla *et al.*, 1994). They seem well adapted to the intensive, modern agriculture of temperate regions, but also flourish in crops cultivated in more traditional ways in the tropics.

Other commonly encountered yam viruses are *Dioscorea alata virus* (DAV), *Dioscorea dumetorum virus* (DdV), *Yam mild mosaic virus* (YMMV), genus *Potyvirus* (Hughes, 1986) *Cucumber mosaic virus* (CMV), genus *Cucumovirus* (Eni *et al.*, 2008a) and *Dioscorea alata bacilliform virus* (DaBV), genus *Badnaviruses* (Seal and Muller, 2007; Kenyon *et al.*, 2008; Eni *et al.*, 2008b). These viruses are reported to be widespread in the major yam producing countries occurring as single as well as mixed infections (Lebas, 2002; Eni *et al.*, 2008c, 2010; Odedara *et al.*, 2011). Although tuber yield losses have been attributed to virus infection, the influence of these viruses on yield and growth of the yam plant has not been well studied. However, tuber yield losses accounting for about 30 to 45 percent have been reported in the case of *Chinese yam necrotic mosaic virus* (ChYNMV), a *Macluravirus* infecting Nagaimo variety of Chinese yam (Tochihara, 1993). *Macluravirus* is one of the genera in the family *Potyviridae* characterized by its flexuous filamentous particles, polyprotein genome strategy and inclusion bodies in infected plant cells. It differs from most of the other genera by having shorter particles (650-660 nm in length) and insect transmission (Adams *et al.*, 2012).

Virus-virus interaction within a host during mixed infection may result in either synergism or antagonism (Murphy and Bowen, 2006; Carrillo-Tripp *et al.*, 2007). Nevertheless, co-infection of a plant by different species of viruses often results in synergistic interactions which may lead to more severe leaf symptoms culminating in greater yield losses. The probability of genomic recombination is also enhanced which may result in more virulent strains of prevailing viruses or emergence of entirely new virus species, meddling with the efforts aimed at engineering virus resistance in yam.

2.10.1.1 Potyviridae Family

Potyviridae (named after *Potato virus Y*) is the largest and most economically important family of plant viruses currently recognized containing 218 definite and tentative species (Berger *et al.*, 2005). They account for about 20 percent of all the classified plant viruses and cause significant losses in agricultural, pasture, horticultural and ornamental crops.

The virus comprises flexuous filamentous particles of 200-300 nm length and 11 to 15 nm diameter. The lengths 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).

Cytopathologically, all members of the family characteristically induce the formation of 3D crystalline cytoplasmic inclusions within infected cells. These are seen as pinwheels in transverse section or as bundles in longitudinal section. These cylindrical inclusion (CI) bodies are formed by a virus encoded protein and can be considered as the unique phenotypic criterion for assigning viruses to the family. Some members of the potyvirus genus induce the formation of crystalline nuclear inclusions (NI) that consists of 2 proteins, NIa and NIb (Shukla *et al.*, 1998).

Many members of the family are important pathogens on plants. *Yam mosaic virus* (YMV) genus *Potyvirus* is the most important virus infecting both cultivated and wild yams especially *Dioscorea rotundata*, *D. alata* and *D. cayenensis* in the yam-growing areas of the world (Odu *et al.*, 2011). *Yam mild mosaic virus* (YMMV) also known as *Dioscorea alata virus* (DaV) infects *D. alata* in the South Pacific, Africa, the Caribbean and South America (Bousalem *et al.*, 2003).

2.10.1.1.1 Taxonomy

Initially, the family *Potyviridae* was divided into 4 genera on the basis of vector transmission (Barnett, 1992). Currently, eight genera are recognized (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).

2.10.1.1.2 Genome organization

All members of the *Potyviridae* family have a linear, single stranded positive sense RNA genome of 8,500-12,000 nucleotides with a poly (A) tail at the 3'-terminus and probably a genome-linked protein (VPg) at its 5'-terminus. The genome or genome segments are translated into polyproteins which are subsequently processed by virus encoded proteases into functional proteins (Adams *et al.*, 2005a).

The viruses of the genus *Bymovirus* have a bipartite genome whereas all others have a monopartite genome (Shukla *et al.*, 1998). Further sequence analysis revealed that rymoviruses shared strong sequence identity with the potyviruses

and therefore should be included in the genus *Potyvirus* (Adams *et al.*, 2005a; Shukla *et al.*, 1998).

The monopartite genera have a genome of ~10kb in length, characterized by a 5' untranslated region (5' UTR), a major single ORF and a 3' UTR region terminated by a poly-A tail. The major ORF encodes a large polyprotein that is co-translationally processed into ten functional proteins (Adams *et al.*, 2005b). In the descending order (5'-3'), these proteins are the first protein (P1), helper component protein (HC-Pro), third protein (P3), 6K1, cylindrical inclusion protein (C1), 6K2, viral protein genome- linked (VPg), major protease of small nuclear inclusion protein- NIa (NIa-Pro), large nuclear inclusion protein (NIb) and coat protein (CP) (Shukla *et al.*, 1998). The polymerase regions of the polyprotein have sequences that are most similar to those of coma-, nepo-, and picornaviruses (Zanotto *et al.*, 1996).

By contrast, in the genus *Bymovirus*, RNA1 corresponds to the 3' section (P3 to CP, eight mature proteins) of the genome of other members. The smaller RNA (RNA2) of the bymoviruses has less sequence identity with other members of the family, although its *P2-1* gene has some similarities with the C-terminal region of the *HC-Pro* of monopartite viruses (Adams *et al.*, 2005b).

2.10.1.1.3 Function of genes

Large nuclear inclusion protein (NIb) is a RNA dependent RNA polymerase (RdRp). This function was demonstrated in *Tobacco vein mottling virus* (TVMV) in which the TVMV NIb had poly (U) polymerase activity and was able to utilize full length TVMV RNA as a template for RNA synthesis. The uryldylation activity of NIb has also been demonstrated in *Pepper veinal mottle virus* (PVMV) (Anindya *et al.*, 2005).

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 coat protein N- terminal region that is exposed on the virion surface contains a highly conserved DAG motif located near the N- terminus. Site directed mutagenesis analyses showed that the motif is essential for aphid transmission (Atreya *et al.*, 1995). Mutation analysis by Dolja *et al.* (1994) showed that the N- and C- terminal regions of *Tobacco etch virus* (TEV) CP were indispensable for systemic viral movement, while the core region was essential for cell to cell movement. Later on, it was observed that maintenance of the CP N-terminal neutralized net charge, but not primary sequence, was essential for systemic infectivity of *Zucchini yellow mosaic virus* (ZYMV) (Kimalov *et al.*, 2004). Although initial studies suggested that CP N and C- terminal regions are not necessary for particle assembly (Shukla *et al.*, 1994; Voloudakis *et al.*, 2004), further studies showed both regions are vital for assembly (Anindya and Savithri, 2003; Kang *et al.*, 2006). The interaction between the CP and the NIb through the GDD motif of NIb (Hong *et al.*, 1995) suggested that the CP may be involved in regulation of RNA synthesis.

2.10.1.1.4 Macluravirus

Members of the genus *Macluravirus* are characterized by the possession of slightly shorter filamentous particles (650-675 nm) than other members of *Potyviridae* with monopartite genomes (680-950 nm) (Adams *et al.*, 2012). They are transmitted non-persistently by aphids, but are more closely related to species in the genus *Bymovirus* transmitted by fungi (Badge *et al.*, 1997). This genus includes eight species (including tentative species). However, the available sequence information about Macluraviruses is limited to their 3′- terminal portion of the genome (except ChYNMV) (Kondo, 2001; Liou *et al.*, 2003; Wang *et al.*, 2009; Ciuffo *et al.*, 2011; Jebasingh *et al.*, 2011; Mandal *et al.*, 2012). Also,

details of the geographical distributions, host range, symptoms, and transmission characteristics of each of these remain sparse.

Chinese yam necrotic mosaic virus (ChYNMV) causes necrotic mosaic disease in yams (Fukumoto and Tochihara, 1978). The host range of ChYNMV is restricted to *Dioscorea* spp. It causes a yield loss of about 30-45 percent if the seed tubers have been infected (Tochihara, 1993). ChYNMV is a 660 nm long, flexuous, filamentous virus with a single 38 kDa coat protein and is transmitted by aphids in a non-persistent manner (Shirako and Ehara, 1986). Based on these characteristics, 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.* Identification of cylindrical cytoplasmic inclusions, as well as the morphology and length (660 nm) of purified virus particles, supported this claim (Kondo, 2001). 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). Other monopartite and bipartite viruses typically contain 9–11 kb and 7.5–8.0 kb (RNA1), and 3.5–4.0 kb (RNA2), respectively (Adams *et al.*, 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).

Narcissus latent virus (NLV) was first described in 1966 and initially classified as a *Carlavirus* (Brunt, 1976), but the finding of 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; Badge *et al.*, 1997).

Maclura mosaic virus (MacMV), the type member of *Macluravirus* genus, was reported to cause mosaic symptoms on the leaves of the ornamental tree

Maclura pomifera (Plese & Milicic, 1973) and to induce CCIs (Plese & Wrischer, 1978). MacMV 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).

Ranunculus latent virus (RLV) was first isolated from diseased *Ranunculus asiaticus* plants growing in Imperia Province (Italian Riviera-Liguria Region) (Turina *et al.*, 2006). *Yam chlorotic necrotic mosaic virus* (YCNMV) was identified from *Dioscorea zingiberensis* and *D. parviflora* in Yunnan province of China (Wang *et al.*, 2009).

2.11 DIAGNOSIS OF YAM VIRUSES

Swift and accurate 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 (Rossel and Thottappilly, 1985; 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 main methods available to detect and identify plant viruses currently include symptom diagnosis and host range studies of experimental host plants, transmission electron microscopy (TEM), antisera-based immunoassay (e.g. enzyme-linked immunosorbent assay- ELISA and dot blot immunoassay- DIBA), reverse transcription polymerase chain reaction (RT-PCR) for RNA viruses or polymerase chain reaction (PCR) for DNA viruses, cloning and nucleic acid sequencing, mass spectrometry (MS) and next generation sequencing (NGS). Often, two or more assays are combined as part of the diagnostic process, especially for novel viruses (Cooper *et al.*, 2003). Currently available assays for

yam virus diagnosis can broadly be divided into: bioassays (biological tests), immunoassays and nucleic acid-based techniques.

2.11.1 Bioassays

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). Biological methods of yam virus detection and diagnosis include symptomatology and transmission studies. Symptoms on the natural host are considered, as are symptoms on a range of indicator plant species after mechanical or vector transmission (Terry, 1976). While these methods are still 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.

Transmission studies show that YMV, DAV, DaBV, and CMV are mechanically transmissible between yam plants. All these viruses are transmitted by aphids, except DaBV, which is transmitted by mealybugs (*Planococcus citri*) (Kenyon *et al.*, 2001).

2.11.1.1 Symptomatology and Host Range

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. The selection of an indicator plant for a virus is based mainly on the ease of infection after inoculation or after exposure to the vectors, the time taken to show symptoms and the consistency of visual symptoms on the leaves. Experimental host plants are chosen as they are susceptible to a range of viruses, or they permit high titre infections or are species which are readily infected and maintained (propagation hosts), or they show a strong physical response to infection, such as local lesions (local lesion hosts). The most commonly used indicator plants to distinguish plant

viruses are from the families *Chenopodiaceae, Solanaceae, Cucurbitaceae* and *Fabaceae* (Hull, 2002). Symptoms of plants infected by different viruses in the field are usually inadequate to distinguish one virus responsible for the disease from another, although some virus infections have characteristic symptoms. In other cases, different virus isolates in a species could result in completely different symptoms and susceptibility (Lee *et al.*, 2007).

However, symptomatology and host range 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).

2.11.1.2 Transmission of plant viruses

The spread and transmission from one host to another is necessary for virus to survive. However, the presence of cell walls and the immobility of plants are barriers to plant virus transmission. Some viruses are transmitted via seeds or via pollen. However, most plant viruses are spread by vectors which include fungi, nematodes, arthropods and arachnids (Hull, 2002). Mechanical inoculation and insect transmission are the most commonly used methods for virus transmission in laboratory or glasshouse studies (Dijkstra and de Jager, 1998).

2.11.1.2.1 Mechanical inoculation

Spread of plant viruses by direct contact between infected and healthy plants rarely takes place in nature, and is mainly found in roots. However, in glasshouse studies, mechanical inoculation is the method used more often for plant virus diagnosis, host range tests, infectivity assessment and symptom characterization. Effective and quick manipulation during inoculation is important since viruses do not remain infective for a long period after isolation (Luo, 2012).

Kuroda *et al.* (2010) studied the host range of *Soybean leaf rugose mosaic virus* (SLRMV) belonging to the family *Potyviridae*. The virus was found to

infect 10 plant species in four families. Seed and aphid transmission was absent, but soybeans became diseased when grown in virus-infested soil. Soybean was the only host plant that showed severe symptoms after systemic infection.

Mechanical inoculation studies have played an important role in the identification of macluraviruses. The NLV, initially classified as a *Carlavirus*, was found to systemically infect *Nicotiana clevelandii* and that in it induces CCIs characteristic of *Potyviridae* family (Mowat *et al.*, 1991). RLV readily infects *Nicotiana benthamiana* systemically (Turina *et al.*, 2006) and MacMV infects *Chenopodium amaranticolor* producing cylindrical (pinwheel-type) inclusions in the infected cells (Plese *et al.*, 1979).

2.11.1.2.2 Insect transmission

Insects are the most common vectors of plant viruses. Seven orders of the class Insecta transmit plant viruses, most in Thysanoptera and Hemiptera. They have piercing and sucking mouthparts. Other vectors are in Orthoptera, Dermaptera, Coleoptera, Lepidoptera and Diptera, which have chewing mouthparts (Raccah and Fereres, 2009).

Depending on the way they are transmitted, plant viruses are divided into three groups: non persistent, semi persistent and persistent. Non persistent viruses have a short retention time (usually a few minutes to hours) in the vector, in which virus particles are attached to the stylet of the insect and are transmitted to the next plant it probes or feeds on. Persistent viruses remain viable in the insect salivary gland to either replicate (propagative) or not (circulative). Semi persistent viruses have an intermediate retention time in their vector.

The CP is usually important for virus transmission by insects. Mutations in the CP gene can often change the transmissibility of the virus (Perry *et al.*, 1998). Many viruses encode helper components (HC) to facilitate the interaction between virions and their vectors. Pure virions of potyviruses and caulimoviruses cannot

be transmitted by their vectors unless they are present in the mixture of non-structural virus-encoded protein (HC) (Raccah and Fereres, 2009).

Piercing and sucking mouthparts, the polyphagous nature and global distribution, make aphid vectors of many plant viruses. Fifty percent of insect-vectored plant viruses were reported to be transmitted by aphids (Ng & Perry, 2004), such as the majority of potyviruses.

ChYNMV was shown to be aphid transmitted (Fukumoto and Tochihara, 1978). In *Alpinia mosaic virus* (AlpMV), aphid transmission was shown to be aphid specific (Liou *et al.*, 2003), whereas in the case of NLV, MacMV and *Cardamom mosaic virus* vector specificity was not strict (Badge *et al.*, 1997; Gonsalves *et al.*, 1986). Aphid transmission by *Myzus persicae* was demonstrated in a controlled environment for RLV and three potyviruses isolated from *Ranunculus asiaticus* plants, whereas no transmission through seed was observed (Turina *et al.*, 2006). The aphid *Aulacorthum solani* was used to study the cross-protection ability of an attenuated strain of ChYNMV against virulent strains infecting Chinese yam (Kondo *et al.*, 2007).

2.11.2 Immunoassays

Immunoassays are a useful tool for detecting and monitoring virus diseases, particularly in 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) and dot blot immunoassay. Immunoassays utilize the ability of antibodies raised in animals to bind to the virus of interest.

2.11.2.1 ELISA

Before the ELISA method had been developed, immunoassays were done mainly using radioactively labelled antigens and antibodies (Yalow &Berson, 1960). The principle of ELISA is to use the specific binding of antibodies to antigens, with an enzyme linked to the antibodies. From a subsequent enzyme-

substrate reaction, colour or fluorescence is generated, which can be interpreted both qualitatively and quantitatively. There are two main variants of ELISA. 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.

Since ELISA was first described (using polyclonal antibodies) as a detection tool for plant viruses, it has become one of the most widely used and reliable techniques in plant virus research (Clark & Adams, 1977). It is versatile, reproducible, relatively sensitive, specific and easy to automate (Cho, 1990). The development of monoclonal antibodies (MAbs) instead of polyclonal (PAbs) ones, which are more specific and sensitive (Matthews, 1991; Brattey and Burns, 1998) helped researchers to develop many plant virus antibodies specific to various levels: isolates, species, strains, sub-groups and genera (e.g. potyvirus) (Jordan & Hammond, 1991). This technique is mainly used 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).

PAbs and MAbs are currently available at both the laboratory and commercial levels against most economically important potyviruses and are widely used in the diagnosis of potyviruses (Balamuralikrishnan *et al.*, 2002; Desbiez *et al.*, 2002).

The ELISA screening technique indicated the presence of *Badna* virus in *D. alata* samples taken from seven south specific islands (Kenyon *et al.*, 2008). MacMV was tentatively classified as a member of the potyvirus genus due to the presence of CCIs in infected tissue and its weak cross-reaction with antisera to *Bean yellow mosaic potyvirus* (Plese *et al.*, 1979). Later on, it was demonstrated that NLV particles cross-react in ELISA and IEM with MacMV antiserum, results establishing a link between them (Mowat *et al.*, 1991). Double-antibody sandwich (DAS)-ELISA, antigen coated plate (ACP)-ELISA, and western blot analysis

were used to establish the serological features for RLV and potyviruses infecting *R. asiaticus* (Turina *et al.*, 2006).

Dot immuno blotting assay (DIBA) can be used to detect virus both in plants and vectors (Lange and Heide, 1986; Makkowk *et al.*, 1993). NCM-ELISA is the technique similar to ELISA except that the plant extracts are spotted on the membrane. Tissue Immuno Blotting Assay (TIBA) is a variation of DIBA in which a freshly cut edge of a leaf is detected with labelled antibodies (Makkowk *et al.*, 1993).

Serological methods have been used widely and successfully for over half a century to identify the virions of different viruses, and to determine the relationships between them. However, these methods are not particularly successful when used for potyviruses (Shukla *et al.*, 1994). 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) probably caused by polymerase slippage (Hancock *et al.*, 1995). Although serological tests are often not useful for determining the relationships of potyvirus species, group-specific monoclonal antibodies have been produced that react with the virions of more than 90 percent of tested potyviruses, but not other potyvirids (Jordan and Hammond, 1991) except *Ryegrass mosaic rymovirus* (Salm *et al.*, 1994).

2.11.3 Nucleic acid based techniques

The most common nucleic acid-based technique is the polymerase chain reaction (PCR). First described in the 1980s by Mullis *et al.* (1986), PCR has become a powerful technique with great impact on molecular biology. It was first used for the amplification of β-globulin genomic sequences for diagnosis of sickle cell anemia (Saiki *et al.*, 1985) but has been employed for the detection and differentiation of plant pathogens (Lopez *et al.*, 2003). The advent of PCR in the diagnosis of plant viruses has greatly improved the sensitivity, rapidity, specificity and reliability of virus detection (Martin *et al.*, 2000; Hull, 2002).

Potyvirus genomes are RNA based so that viral RNA is first reverse transcribed into complementary DNA (cDNA). Primers used in PCR can be species-specific or group-specific, such as potyvirus degenerate primers (Langeveld *et al.*, 1991; Webster *et al.*, 2007). RT- PCR is a much more sensitive diagnostic method than ELISA and new PCR primers are also much easier to design and synthesize than novel antibodies. The use of RT-PCR to amplify DNA transcribed from genomic RNA has been successfully applied to a range of pathogens including several potyviruses (Nicholas and Laliberte, 1991; Thomson *et al.*, 1995).

The PCR technique can also be combined with serological procedures in the form of immunocapture (IC) PCR, conjoining the advantages of serology with the sensitivity of PCR to detect diseases and identify the causal agents (Mumford and Seal, 1997, Žižytė *et al.*, 2006).

Triple antibody sandwich (TAS) ELISA, dot (DBI) and direct tissue blot immunoassays (DTBI), immunosorbent electron microscopy (ISEM) and immunocapture (IC)RT-PCR techniques were evaluated for detecting *Yam mosaic virus* (YMV), genus *Potyvirus*, in *Dioscorea rotundata* stems, leaf petioles, leaves and tubers. Younger leaves were more suitable for the detection of YMV using TAS-ELISA and DBI. 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 IC-RT-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.*, 2004, 2005).

Most of the current virus detection tools require some previous knowledge of the virus, and this is a drawback for identifying unknown or unexpected viruses. For antisera-based techniques such as ELISA and DIBA, a wide range of species specific and group specific antibodies are commercially available. However, such antibodies are not available for all known viruses, and certainly

not for unknown viruses. The most widely used nucleic acid-based assay for virus identification is enzymatic amplification of fragments of the virus genome by PCR (preceded by RT in the case of RNA viruses), often followed by nucleotide sequencing. Microarrays are less commonly used because of inherent sensitivity and reliability issues (Boonham *et al.*, 2007). In both cases primer/probes are designed from known sequences of the target genome to be either highly species/strain specific or group specific, depending on whether identification of one specific virus or a group of related viruses is desired (Gibbs & Mackenzie, 1997; Webster *et al.*, 2007; Wylie *et al.*, 2008). Where primer sets amplify a range of viruses, restriction fragment length polymorphisms (RFLPs) within PCR amplicons can be used to identify specific genotypes by electrophoresis or mass spectrometry (Michael *et al.*, 2009).

2.12 PRIMER DESIGNING

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

High G/C content in the 3'ends of primers is not desirable as it will lead to mispriming. However, a "G" or "C" is desirable at the 3' end of primers since this will reduce "breathing" and thereby increase yield (Sheffield *et al.*, 1989; Abd-Elsalam, 2003). When designing primers, it is important to have a minimum of

intra-molecular or inter-molecular homology to inhibit the formation of either hairpins or primer dimers (Abd-Elsalam, 2003).

Langeveld *et al.* (1991) designed degenerate oligonucleotide primers from conserved amino acid sequences of the virion proteins, and showed that they amplified DNA fragments transcribed from several potyviruses in bulb crops, and that they did not amplify Carla or potexvirus sequences. Furthermore, Pappu *et al.* (1993) used primers to clone the same region together with the 3' untranslated region of the genome, as sequences of this region are useful for discriminating *Potyvirus* strains. Colinet and Kummert (1993) and Colinet *et al.* (1994) used similar degenerate primers to separate different viruses from sweet potato. These primers have also been used for the characterisation of the YMV isolates (Bousalem *et al.*, 2000b).

Universal primers have been designed to identify potyviruses based on the conserved sequences such as WCIEN box or QMKAA motif in the CP gene (Bateson and Dale, 1995; Zerbini *et al.*, 1995). The consensus motif (GNNSGQPSTVVDN) in the NIb gene has been shown to be highly conserved among members of the family Potyviridae (Gibbs *et al.*, 2003). The forward degenerate primers corresponding to the GNNSGQP sequence of this motif are specific for numerous members of the family (Gibbs and Mackenzie, 1997; Chen and Adams, 2001). For specific detection of NLV isolates, a one-step RT-PCR procedure was developed (Berniak *et al.*, 2013).

2.13 INTRASPECIFIC VARIATION STUDIES

RNA virus diversity results from the accumulation of mutations due to frequent errors in RNA synthesis. Recombination events are also a major evolutionary factor for RNA plant viruses. However, recombination has rarely been observed in natural populations and the frequency of recombinants has been reported for only a few viruses.

The complete sequence of the genome of DaBV has been determined and the genetic variability of the virus was found to be very high (Brunt *et al.*, 1990). Partial sequences from serologically characterized YMV isolates were determined in conserved (*HC*; *NIb*) and variable (*P1*; *P3*; *CP*) regions of the potyviral genome in order to investigate the intraspecies molecular diversity of YMV. Two levels of diversity were observed among YMV isolates: above 90 percent nucleotide sequence identities were found between YMV isolates of the same group (intragroup) regardless of the region considered, whereas identities between isolates from different groups (intergroup) were lower and depended upon the protein chosen (Aleman-Verdaguer *et al.*, 1997).

Sequencing of the C- terminal part of the *NIb*, *CP* and the 3'-UTR of YMV isolates collected from *Dioscorea alata*, *D. cayenensis*, *D. rotundata* and *D. trifida* showed that it has the most variable coat protein relative to other *potyviruses*. This high variability was structured into nine distant molecular groups, as revealed by phylogenetic analyses and validated by assessment of the molecular evolutionary noise. Phylogenetic topological incongruence and complementary statistical tests highlighted the fact that recombination events, with single and multiple crossover sites, largely contributed to the evolution of YMV. It was hypothesized that YMV has an African origin from *D. cayenensis* and *D. rotundata*, followed by independent transfers to *D. alata* and *D. trifida* during virus evolution (Bousalem *et al.*, 2000b). Amino acid sites in the coat proteins of *Yam mosaic virus* was found to be under diversifying selection mode of evolution (Morel *et al.*, 2002).

2.13.1 Restriction fragment length polymorphism (RFLP)

Molecular markers are powerful tools for identification and determination of genetic diversity of plant viruses. Their major advantages are that they detect polymorphism at the genetic level and are not influenced by environmental factors. The genetic variation obtained through molecular marker is directly related to the number of polymorphisms detected and their reproducibility.

RFLP involves restriction enzyme digestion of the pathogen DNA, followed by separation of the fragments by electrophoresis in agarose or polyacrylamide gels to detect differences in the size of DNA fragments. RFLPs have their origin in the DNA rearrangements that occur due to evolutionary processes, point mutations within the restriction enzyme recognition site sequences, insertions or deletions within the fragments, and unequal crossing over.

Even a single nucleotide alteration can create or destroy a restriction site; hence mutations cause variations in the number of sites. Thus there is variation, or polymorphism, between entities in the positions of cutting sites and the lengths of DNA between them, resulting in restriction fragments of different sizes. The main advantages of RFLP markers are their co-dominance and high reproducibility. Drawbacks as compared with PCR based techniques are the tedious experimental procedures, and the requirement for microgram amounts of pure DNA. RFLPs arise as mutations that alter restriction sites, but the events giving rise to them, over evolutionary time, are as stable as the mutations giving any other form of allelic variation. PCR-RFLP combines the amplification of a target region with the further digestion of the PCR products obtained (Jones *et al.*, 2009).

For molecular characterization of *Ranunculus mosaic virus* (a potyvirus), RFLP analysis with the restriction enzymes *Eco*ICRI and *Hpa*I was carried out in order to ensure a homogeneous RT-PCR product (Turina *et al.*, 2006). A RT-PCR-RFLP method was developed to select and discriminate an attenuated strain of ChYNMV, designated KM3, from other isolates in the field for cross-protection (Kondo *et al.*, 2007).

2.13.2 DNA PAGE

Polyacrylamide gel electrophoresis (PAGE) offers high resolution of low-molecular-weight nucleic acids. In particular, small DNA fragments (< 500 bp) that are poorly resolved by ordinary agarose gels are easily separated on polyacrylamide gels. Depending on the pore size of the gel (3.5% to 20% polyacrylamide), a separation from 10 to 1000 bp can be achieved.

Polyacrylamide gels are chemically cross-linked gels formed by the reaction of acrylamide with a bi-functional crosslinking agent such as N,N′-methylene bisacrylamide (Bis) (Heuer *et al.,* 2003). Polymerization of a polyacrylamide gel is initiated by free radicals provided by ammonium persulfate and stabilized by *N,N,N′,N′*-tetramethyl ethylenediamine (TEMED).

The pore size of a polyacrylamide gel is determined by the total percentage of acrylamide (the sum of the weights of the acrylamide monomer and cross-linker). Historically, this has been expressed as % T. For example, a 5% T gel would contain 5 percent (w/v) of acrylamide plus bisacrylamide. As the % T increases, the pore size decreases. DNA mobilities observed in polyacrylamide gels are essentially independent of the electric field strength used for electrophoresis (Stellwagen and Stellwagen, 1990). The migration distance (D) of double stranded DNA through a non-denaturing gel is inversely proportional to the log of its molecular weight. Also, the base composition of a sequence affects its electrophoretic mobility (Stellwagen, 2009).

MATERIALS AND METHODS

2. MATERIALS AND METHODS

3.1 LOCATION

The study was conducted at the Division of Crop Protection, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during September 2013-June 2014. Details regarding the materials used and methodology adopted for various experiments are presented in this chapter.

3.2 SAMPLE COLLECTION

The leaf samples used in this study were collected from different greater yam growing regions in Thiruvananthapuram, Kollam, Pathanamthitta, Alappuzha and Wayanad districts of Kerala. Five yam growing fields were surveyed for virus incidence in each district. Young leaves of symptomatic plants (i.e. plants showing characteristic mosaic, puckering, cupping and curling of leaves) were collected from each field and stored for further studies. For each sample collected, the symptom type(s) was recorded. Plants showing no observable symptoms were also sampled to check the possibility of latent infection. The tuber samples used for this study were collected from the greater yam fields at Central Tuber Crops Research Institute (CTCRI), Thiruvananthapuram.

3.3 SEROLOGICAL METHODS OF DETECTION

Enzyme Linked Immunosorbent Assay (ELISA) and Dot Immuno Binding Assay (DIBA) techniques were employed for detection of virus infection in *Dioscorea alata* leaf and tuber samples. Virus specific antibody obtained from DSMZ, Germany was used for this study. Samples which showed positive results in these methods were subjected to RT-PCR based detection also.

3.3.1 Enzyme Linked Immunosorbent Assay (ELISA)

Forty six *D. alata* leaf samples showing symptoms of virus infection, eighteen tuber samples and a healthy non host collected from fields were tested for *Macluravirus* infection using Double Antibody Sandwich ELISA (DAS-ELISA).

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 4 h. Leaf sap extract (200 µl) prepared by grinding test leaves in sample extraction buffer (Appendix I) was loaded in duplicate wells and incubated overnight at 4 °C. 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. 200 µl aliquots of freshly prepared substrate (Appendix I) was added to each well and incubated at room temperature in dark condition.

The plates were washed with PBS-Tween-20 thrice at three min interval after each incubation step 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, 15 min, 30 min, 1.0 h, 1.5 h and overnight.

3.3.2 Dot Immuno Binding Assay (DIBA)

Twelve tuber samples and a healthy non host were taken for DIBA using *Yam macluravirus* antibody. A desired size of NCM was cut and 1 cm^2 squares were drawn on it. The NCM was wetted by floating it in TBS and was air dried. 5 µl of partially purified *D. alata* tuber samples were spotted on appropriate squares. After air drying, the membrane was immersed in blocking solution (Appendix I) with gentle shaking for 1 h at room temperature. It was then rinsed once in TBS for 10 min. This was followed by incubating the NCM with primary antibody (Polyclonal Yam Maclura IgG) diluted to 1:1000 in TBS-SDM for 1 h at room temperature or

overnight at 4 °C. Then the membrane was washed thrice with TBS at 10 min interval and incubated with secondary antibody (enzyme labeled anti-rabbit IgG (ALP-conjugate)) diluted 1:10000 in TBS-SDM for 1 h at room temperature or overnight at 4 °C. After rinsing thrice with TBS, the NCM was incubated in substrate solution (BCIP/NBT) at room temperature in dark condition for 10 to 15 min. It was then observed for color development. The membrane was rinsed with distilled water and then air dried.

3.4 TRANSMISSION STUDIES

In order to find out the host range, two methods, *viz.* mechanical inoculation and insect transmission of the virus have been carried out. The inoculated plants were observed for development of viral symptoms. The presence/absence of the virus was determined by RT-PCR of RNA isolated from the leaves.

3.4.1 Mechanical inoculation

D. alata (Sree Keerthi) leaves showing symptoms of virus infection were collected. Sap extracts were prepared by homogenizing the leaves in 1:5 ratio (W/V) in Yam buffer (Appendix I) (Odu *et al.,* 2011) and filtered using a layer of cotton. The following methods of mechanical inoculation were attempted.

3.4.1.1 Hand inoculation

Ten plants each of *Nicotiana benthamiana*, *N. tabacum* and *Vigna unguiculata* were inoculated by this method. The upper leaf surface was dusted with carborandum (abrasive) and inoculated with the sap extract using forefinger by applying gentle pressure from the base to the tip of leaf. After inoculation, the leaves were rinsed with water to wash off excess inoculum. Plants inoculated with buffer alone served as control.

3.4.1.2 Syringe inoculation

Injecting the inoculum using syringe was also attempted in *N. benthamiana* plants. A small quantity of the prepared sap was taken in a 2 ml syringe and injected into the stem/ leaf vein region, while taking care not to cause much damage to the plant. Similarly, virus containing plant sap was inoculated into other *N. benthamiana* plants using needleless syringe. In this method, a small quantity of the sap was taken in the syringe. After removing the needle, the syringe was used to inoculate the upper leaf surface by applying gentle pressure.

The plants were regularly observed for a period of one month for symptom expression.

3.4.2 Insect transmission

Insect transmission of the *Yam macluravirus* was attempted on *N. benthamiana* and *V. unguiculata* plants using aphids. Aphids constitute an important group of virus vectors transmitting a large number of diverse plant viruses. For this study, *Aphis craccivora* reported as vectors for virus transmission (Eni, 2008) was collected and maintained on healthy cowpea plants in glasshouse. Using a camel paint brush, aphids were gently transferred from the plant to a petriplate. The aphids were starved for about 2 h and then allowed to feed on virus infected *D. alata* leaves for five min and transferred to young healthy *N. benthamiana* and *V. unguiculata* plants. After 24 h of feeding, the plants were sprayed with insecticide (0.1% Malathion) to remove the aphids. The plants were maintained in insect free glasshouse and regularly examined for symptom development.

Total RNA was isolated from a few aphids after virus acquisition using Ambion Purelink RNA Mini Kit (section 3.5.1.1). Coupled RT-PCR of the Aphid RNA was performed using Phusion RT-PCR kit (Thermo Scientific, USA) and the PCR product obtained was resolved on agarose gel (1.5%).

3.5 NUCLIEC ACID BASED DETECTION

For detection of the *Yam macluravirus* infecting *D. alata*, RT-PCR was performed on RNA isolated from infected leaf and tuber samples using species specific primers. Primers specific for the Coat Protein (*CP*) gene were synthesized based on the partial *CP* sequences obtained. These primers were validated by RT-PCR amplification of isolated RNA samples.

3.5.1 Isolation of total RNA

Leaf and tuber samples of virus infected *D. alata* plants showing mosaic and prominent puckering symptoms were taken for RNA isolation. From the leaf samples, RNA was isolated using Ambion Purelink RNA Mini Kit and TRIzol based method. Three methods were evaluated for RNA isolation from tubers (Ambion Purelink RNA Mini Kit, Mohan Kumar *et al.*, 2007 protocol and $LiCl_2$ based method). The RNA isolated by all methods was stored at -20 °C.

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

Leaf/tuber sample (100 mg) was taken and ground into fine powder 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 the tube. The above step was repeated until the whole sample had been processed. Then, 700 µl of the wash buffer

I 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 the cartridge was reinserted. The empty spin column was centrifuged at 12,000 *g* for one min at room temperature. Then 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 centre of the spin cartridge and was incubated at room temperature for one min followed by centrifugation at 12,000 *g* for one min 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 min to obtain the second elute. The quality of the isolated RNA was checked on agarose gel (2%).

3.5.1.2 Manual methods of RNA isolation

3.5.1.2.1 TRIzol method (Life Technologies, USA)

The leaf sample weighed 80 to 100 mg was taken and ground into fine powder using liquid nitrogen. The tissue powder was transferred to a fresh tube to which 1 ml of TRIzol reagent was added and incubated at room temperature for five min. 200 µl of chloroform was added, shaken vigorously and incubated at room temperature for 2-3 min. It was then centrifuged at 12,000 *g* (Kubota High Speed Refrigerated Centrifuge 6500, Japan) for 15 min at 4 °C. Out of the three layers that appear, the colorless upper aqueous phase was transferred into a new tube using cut tips. Following that, 500 µl of isopropanol was added, incubated at room temperature for 10 min and centrifuged at 12,000 *g* for 10 min at 4 °C. The supernatant was discarded and to the RNA pellet, 1 ml of 75 percent ethanol was added. After gentle vortexing (Labnet Vortex Mixer, USA), the tube was centrifuged at 7,500 *g* for five min at 4 °C. This washing procedure is repeated until the RNA pellet obtained is pure. The

pellet was then air dried for 5 to 10 min. The RNA pellet was resuspended in 40 µl of nuclease free sterile water and incubated at 55-60 °C (Lab Companion BS-31 shaking waterbath, Canada) for 10-15 min. The quality of the isolated RNA was checked on agarose gel (2%).

3.5.1.2.2 Lithium Chloride Method

Tuber sample weighed 500 mg was ground into fine powder using liquid nitrogen. The tissue powder was transferred into a fresh tube. 1 ml of extraction buffer (pre-warmed at 65 °C) (Appendix III) was added, vortexed (Labnet Vortex Mixer, USA) and incubated at 65 °C (Lab Companion BS-31 shaking waterbath, Canada) for 10 min. 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 to the supernatant. After centrifugation at 20,000 *g* for 10 min at 4 °C, the supernatant was transferred to a fresh tube. Then, 0.25 volume of ice cold 10 *M* lithium chloride was added, mixed well and incubated overnight at 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 for 1 h, the RNA was stored at -20 °C.

3.5.1.2.3 Mohan Kumar et al., 2007 protocol

The tuber tissue was sliced with sterile razor blade (pre-rinsed with RNAse Zap) and 2 g of fresh tissue ground into fine powder using liquid nitrogen was transferred to a 15 ml falcon tube. RNA was extracted by the sequential addition of 1 ml 5M NaCl, 0.5 ml of 10 percent (w/v) SDS, 1.65 ml of 1.95 percent (w/v) Na_2SO_3,

1.75 ml of Borate Tris Buffer (Appendix III) and 0.1 ml of β- mercaptoethanol. The extract obtained was vortexed (Labnet Vortex Mixer, USA), incubated at 65 °C (Lab Companion BS-31 shaking waterbath, Canada) for five min followed by centrifugation at 1800 *g* (Kubota High Speed Refrigerated Centrifuge 6500, Japan) for five min at 23 °C. The supernatant is transferred into a fresh tube to which equal volume of Tris saturated Phenol (pH 7.9) was added and mixed. The mixture was centrifuged at 1800 *g* for five min at 23 °C to achieve phase separation. If phase separation did not occur, additional Borate Tris buffer was added in 1 ml increments until centrifugation resulted in satisfactory phase separation. Upper phase was transferred into a new tube and extracted with equal volume of chloroform- isoamyl alcohol (24:1) followed by centrifugation to facilitate phase separation. One ml of upper phase was transferred to 2 ml tube containing 0.9 ml of isopropyl alcohol. The extract was then incubated at 4 °C for 1 h. RNA pellet was obtained by centrifugation at 20,000 *g* for 15 min at 4 °C. RNA pellet was washed with 70 percent ethanol 4 to 5 times to remove salts. The residual ethanol was evaporated at 65 °C on a block heater. The RNA pellet was solubilized in 30 µl of DEPC treated water and the quality of isolated RNA was checked on agarose gel (2%).

3.5.2 Agarose gel electrophoresis

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

3.5.3 Spectrophotometer analysis

The absorbance of RNA samples was recorded to determine the quantity and quality of RNA preparation. The optical density (OD) of RNA samples was measured using NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). The spectrophotometer was calibrated to blank (zero absorbance) with 1 µl of nuclease free sterile water. Then, the concentration of each of the RNA samples (1 µl) was recorded. The quality of RNA preparation was determined by analyzing the A_{260}/A_{280} and $A_{260}/_{230}$ readings.

3.5.4 Amplification of *Yam macluravirus* partial coat protein gene

3.5.4.1 First strand cDNA synthesis

The RNA isolated from both leaf and tuber samples 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:

RNA	:	2 µl
Oligo $(dT)_{18}$ Primer	:	1 µl
Nuclease free water	:	9 µl
5x reaction buffer	:	4 µl
RiboLock RNase Inhibitor (200 U μl^{-1})	:	1 µl
dNTP mix (10 m*M*)	:	2 µl
Revert Aid M-MuLV RT (200 U μl^{-1})	:	1 µl
Total volume	:	20 µl

All the reagents 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.

3.5.4.2 Gradient PCR for standardizing annealing temperature

Two primer sets (obtained from DSMZ, Germany) were used to identify virus infection:

1) 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'

2) Yam Maclura s (YMacs) and Yam Maclura c (YMacc) primers (18mers)

YMacs (forward primer) sequence: 5' AACAAGATGATTCACTTA 3'

YMacc (reverse primer) sequence: 5' CTTTGCTTTGATTTAGTT 3'

In order to standardize the annealing temperature of the primer pairs used, gradient PCR was performed for both primer sets. 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):		10 µl
Forward primer	:	0.4 µl
Reverse primer	:	0.4 µl
cDNA	:	2 µl

Nuclease free water	:	7.2 µl
Total volume	:	20 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 temperature gradient from 49 °C to 60 °C for one min and extension at 72 °C for one 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).

3.5.4.3 Uncoupled RT-PCR for virus CP amplification

Once the annealing temperatures were standardized for the primer pairs, the cDNA samples were subjected to uncoupled RT-PCR using same reaction mixture as described in section 3.5.4.2. PCR was carried out in Agilent Technologies sure Cycler 8800 (USA) and the same cycling conditions were set as given in section 3.5.4.2 with an annealing temperature of 52 °C for YMac1s and YMac1c primers; 50 °C for YMacs and YMacc primers. The PCR products were analyzed on agarose gel (1.5%). The amplicons showing expected band size were purified by gel elution method.

3.5.4.3.1 Reamplification of PCR products

The product obtained after uncoupled RT-PCR was reamplified using the same reaction mix as in section 3.5.4.2 (except that instead of cDNA, PCR amplified product was used) under similar cycling conditions. Products of reamplification were checked on agarose gel (1.5%).

3.5.4.4 One step RT-PCR for virus CP amplification

Coupled RT-PCR was also used to detect the presence of virus infection in various *D. alata* RNA samples. In single step RT-PCR, the initial reverse transcription step and the latter amplification steps are coupled. The PCR reaction mix was optimized as given below:

10X Robust RT buffer	:	2 µl
dNTP mix (10 m*M*)	:	0.5 µl
Magnesium chloride (50 m*M*)	:	0.7 µl
Oligo $(dT)_{21}$ primer	:	1 µl
Forward primer	:	1 µl
Reverse primer	:	1 µl
AMV (5 U μl^{-1})	:	0.25 µl
Dynazyme	:	1 µl
Sample RNA	:	3 µl
RNase inhibitor	:	0.25 µl
Sterile distilled water	:	9.3 µl
Total volume	:	20 µl

The PCR cycling conditions were set as follows:

Reverse Transcription	:	42 °C for 30 min
Initial denaturation	:	94 °C for 4 min

Denaturation	:	94 °C for 30 s	40 cycles
Annealing	:	52 °C for 1 min	
Elongation	:	72 °C for 1 min	
Final elongation	:	72 °C for 10 min	

The PCR products were resolved on agarose gel (1.5%), visualized under UV light and the image was documented using Gel DOC system (Alpha imager, Alpha Innotech, USA). The amplicons showing expected band size were further eluted to get more purified product.

3.5.4.5 Gel extraction of PCR amplified fragments

Extraction of the PCR products was achieved with GeneJET Gel Extraction kit (Thermo Scientific, USA). The PCR products were resolved on agarose gel (1.5%) 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 flow-through 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 (except that instead of cDNA, gel elute was used) under similar cycling conditions as described in section 3.5.4.3. The products of PCR were assessed using agarose gel (1.5%).

3.5.4.6 Immunocapture RT-PCR (IC-RT-PCR)

D. alata leaf samples showing symptoms of virus infection were collected and ground in sample extraction buffer (Appendix I) in 1:5 dilution. Sample extract was collected by centrifugation at 10,000 *g* for five min.

Fifty µl of the polyclonal antibody diluted to 1:100 in carbonate buffer (Appendix IV) was added to PCR tubes and incubated at 37 °C for 2 h. After washing once with PBS-Tween-20 (Appendix I), 50 µl of the sample was added to the tubes and incubated overnight at 4 °C. The tubes were washed twice with PBS-T and subjected to RT-PCR after the addition of reaction mix.

The RT-PCR was performed using Phusion RT-PCR kit (Thermo Scientific, USA). The components of the reaction mix were optimized as given below:

Sterile Distilled water	:	12.4 µl
10x RT buffer	:	2 µl
5x Phusion HF buffer	:	1 µl
dNTP mix (10 m*M*)	:	0.3 µl
RT enzyme mix	:	2 µl
Oligo $(dT)_{15}$ primers	:	1 µl

Forward primer	:	0.5 µl
Reverse primer	:	0.5 µl
Phusion Hot Start II DNA polymerase:		0.3 µl
Total volume	:	20 µl

PCR cycling conditions:

Reverse Transcription	:	43 °C for 1 h	
Initial denaturation	:	94 °C for 4 min	
Denaturation	:	94 °C for 30 s	40 cycles
Annealing	:	50 °C for 1 min	
Elongation	:	72 °C for 1 min	
Final elongation	:	72 °C for 10 min	

The products of IC-RT-PCR were resolved on agarose gel (1.5%).

3.5.5 DNA sequencing

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

3.6 AMPLIFICATION OF FULL COAT PROTEIN (*CP*) REGION OF VIRUS

A set of species specific primers were synthesized for the amplification of full coat protein (*CP*) region of the virus. The primers were designed using sequences obtained from National Centre for Biotechnology Information (NCBI) database and

the partial *CP* region of sequenced samples. The primer pair thus synthesized was examined for its specificity by uncoupled RT-PCR in leaf and tuber RNA samples.

3.6.1 Primer designing

Primers were designed for the CP gene and the adjoining *NIb* and 3' UTR of the *Yam macluravirus*. From the NCBI nucleotide database, available nucleotide sequences for the CP gene in different macluraviruses were taken in FASTA format. CP gene sequences of *Cardamom mosaic virus, Chinese yam necrotic mosaic virus, Maclura mosaic virus, Narcissus latent virus* and *Alpinia mosaic virus* were obtained from NCBI. The sequences collected from NCBI along with the partial CP gene sequences of *Yam macluravirus* obtained using Maclura1s/1c and Macluras/c primers were aligned using BioEdit sequence alignment editor (Hall, 1999). The general consensus was identified and used for sequence annotation. Hence, the start and end points of the CP gene of *Yam macluravirus* was recognized. Primers were then designed for the *CP* flanking regions using NCBI Primer BLAST.

3.6.1.1 Primer evaluation

Prior to synthesis, the primers were subjected to in-silico analysis for determining specificity and other characteristics. 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 were assessed using FastPCR programme. The primer sequence thus validated by in silico methods were send to Sigma Aldrich (USA) for synthesis.

The synthesized primers (100 μM) were diluted to a final concentration of 5 μM with sterile water to obtain the working solution. In order to standardize the annealing temperature of the primers, gradient PCR was performed. The cDNA synthesized from the RNA of infected leaf samples was used for PCR amplification. The PCR mix was prepared as described in section 3.5.4.2 and the PCR was carried out in

Agilent Technologies sure Cycler 8800 (USA). The same PCR programme was set as described in section 3.5.4.2 (except that the annealing temperature gradient was from 53 °C to 63 °C for one min). The PCR amplified products along with Gene Ruler 1 kb plus DNA ladder (Thermo Scientific, USA) were separated on agarose gel (2%). The gel was viewed using Alpha Imager (Alpha Innotech, USA).

3.6.2 Uncoupled RT-PCR of total RNA using synthesized primers

Once the annealing temperatures of the primers were standardized, the cDNA samples were subjected to uncoupled RT-PCR using the reaction mixture described in section 3.5.4.2. PCR was carried out in Agilent Technologies sure Cycler 8800 (USA) and the same cycling conditions were set as described in section 3.5.4.2 (except that the annealing temperature was set at 59 °C). The products of PCR were analyzed using agarose gel (1.5%). The amplicons showing expected band size were gel eluted and sequenced to get the full *CP* sequence. Nucleotide BLAST of the full *CP* was done to obtain related sequences.

3.7 CLONING AND TRANSFORMATION

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

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

Day 1:

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

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

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

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

Day 2:

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

incubated overnight at 37 °C. Untransformed DH5α cells were plated on an LB Ampicillin X gal/ IPTG agar plate to serve as negative control.

Day 3:

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

3.7.1 Analysis of recombinant clones- colony PCR

The recombinant clones were analyzed for the presence and orientation of the DNA insert by colony PCR using M13 primers (IDT, USA) as well as CP gene specific primers. The PCR reaction mix was formulated as:

M13 sequencing primer (10 μ*M*)	:	0.6 μl
M13 reverse primer (10 μ*M*)	:	0.6 μl
2x PCR Master Mix	:	10 μl
Water, nuclease free	:	8.8 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 two 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 one min. A final extension step for five min was also given.

For colony PCR using CP gene specific primers, the same reaction mixture and cycling conditions were used as described in section 3.6.2. The products of colony PCR were resolved on agarose gel (1.2%).

3.7.2 Plasmid DNA isolation

Recombinant plasmid DNA was isolated using GeneJET 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 (Appendix V) supplemented with ampicillin. The tube was incubated at 37 °C for 12-16 h with shaking. 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. 250 µl of the lysis solution was then added and mixed thoroughly until the solution becomes viscous and slightly clear. 350 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 GeneJET 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. 30 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 CP gene specific primers.

The reaction mix used was:

Plasmid DNA	:	1 µl
Forward primer	:	0.6 µl
Reverse primer	:	0.6 µl
2x PCR Master Mix	:	10 µl
Water, nuclease free	:	7.8 µl
Total volume	:	20 µl

The PCR was performed with the same cycling conditions as described in section 3.7.1. The products of PCR amplification were analyzed on agarose gel (1%).

After confirming the presence of DNA insert by PCR method, the plasmid was send for sequencing.

3.8 SEQUENCE ANALYSIS

The full *CP* 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. Amino acid sequences were deduced using ExPASy translate tool. Phylogenetic tree was constructed from BioEdit aligned sequences using MEGA version 6.06 (Tamura *et al.*, 2013) using a Neighbor-Joining method and a p-distance model for estimating the distances and conducting the bootstrap analysis (1000 replicates).

3.9 INTRASPECIFIC VARIATION ANALYSIS

The variation present within the *Yam macluravirus* was analyzed using Restriction Fragment Length Polymorphism (RFLP).

The restriction sites present in the full *CP* sequence was obtained using the software Geneious (version 6.0.4). The number of recognition sites present for each restriction enzyme was identified and *Taq*1 and*Mse*1 (*Tru*I 1) enzymes were chosen for RFLP analysis based on the number of cut sites present. RFLP was carried out using *Taq*1/*Tru*I 1 enzyme (Fermentas, USA) and PCR products. The reaction mixture used for restriction digestion was optimized as listed below.

PCR product	:	10 µl
Nuclease free water	:	18 µl
10x Buffer R	:	2 µl
Taq1/TruI 1	:	2 µl

After mixing and flashing, the tube was incubated at 65 °C overnight. The digestion reaction was stopped by adding 0.5 *M* EDTA, pH 8.0 to a final concentration of 20 m*M*.

3.9.1 DNA PAGE

Electrophoretic separation of RFLP products was carried out on polyacrylamide gel. Acrylamide solution (5%) (Appendix VI) was casted in between the glass plates and a clean comb was immediately inserted on the top. More acrylamide solution was added to fill the spaces of the comb completely.

After polymerization, the gel was mounted in the electrophoresis unit. 1X TAE buffer was added into the tank until the gel was completely immersed and the comb was removed. The samples were mixed with 6x loading buffer (bromophenol blue) in the ratio 2:1 and loaded into the wells. Gene Ruler 1 kb plus ladder (Thermo Scientific, USA) was also loaded in one of the wells. A voltage of 120 V was applied to the gel and the gel was allowed to run until the dye front reached ¾ the distance.

3.9.1.1 Staining with ethidium bromide

Bands were stained with ethidium bromide (10 mg ml^{-1}) for five min. Excess stain was removed by washing in a tray of distilled water for 10 min. Band pattern was visualized using UV light and the image was captured with Alpha Imager.

The banding profiles were analyzed for the presence of polymorphism.

RESULTS

4. RESULTS

4.1 SAMPLE COLLECTION

Out of the 97 samples collected for virus screening, 72 were leaf samples collected from different greater yam growing regions in Thiruvananthapuram, Kollam, Pathanamthitta, Alappuzha and Wayanad districts of Kerala. The year of collection, number of fields sampled, sample size and presence of virus infection in the various regions are summarized in Table 2. All the 25 tuber samples were collected from the CTCRI experimental fields.

Five different kinds of symptoms were recorded in different proportions in all the fields surveyed. Mild mosaic was the most common symptom observed in all fields. Other symptoms observed include puckering, chlorosis, cupping and curling, and leaf crinkling and distortion (Plate 1).

4.2 SEROLOGICAL METHODS OF DETECTION

4.2.1 ELISA

A total of 46 leaf samples and 18 tuber samples were tested for *Yam macluravirus* infection by DAS-ELISA using specific antibody. The occurrence of *Yam macluravirus* was detected in the samples collected from CTCRI experimental fields only. DAS-ELISA analysis of 28 leaf samples collected from the other four locations gave negative results indicating absence of virus infection. Three of the 46 leaf samples (6.5%) and two of the 18 tuber samples (11.1%) were positive for *Yam macluravirus* (Plate 2A). A summary of the results is presented in Table 3 and 4. The samples with mild to severe mosaic, puckering, cupping and curling symptoms were found to be positive for *Yam macluravirus*. 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.

Table 2. Occurrence of *Yam macluravirus* in greater yam leaf samples in five yam producing regions of Kerala in 2013

Region of collection	No. of fields sampled	Sample size	No. of samples infected
Thiruvananthapuram	3	27	11
Kollam	1	6	Nil
Pathanamthitta	5	14	Nil
Alappuzha	4	15	Nil
Wayanad	10	10	Nil
Total	**23**	**72**	**11**

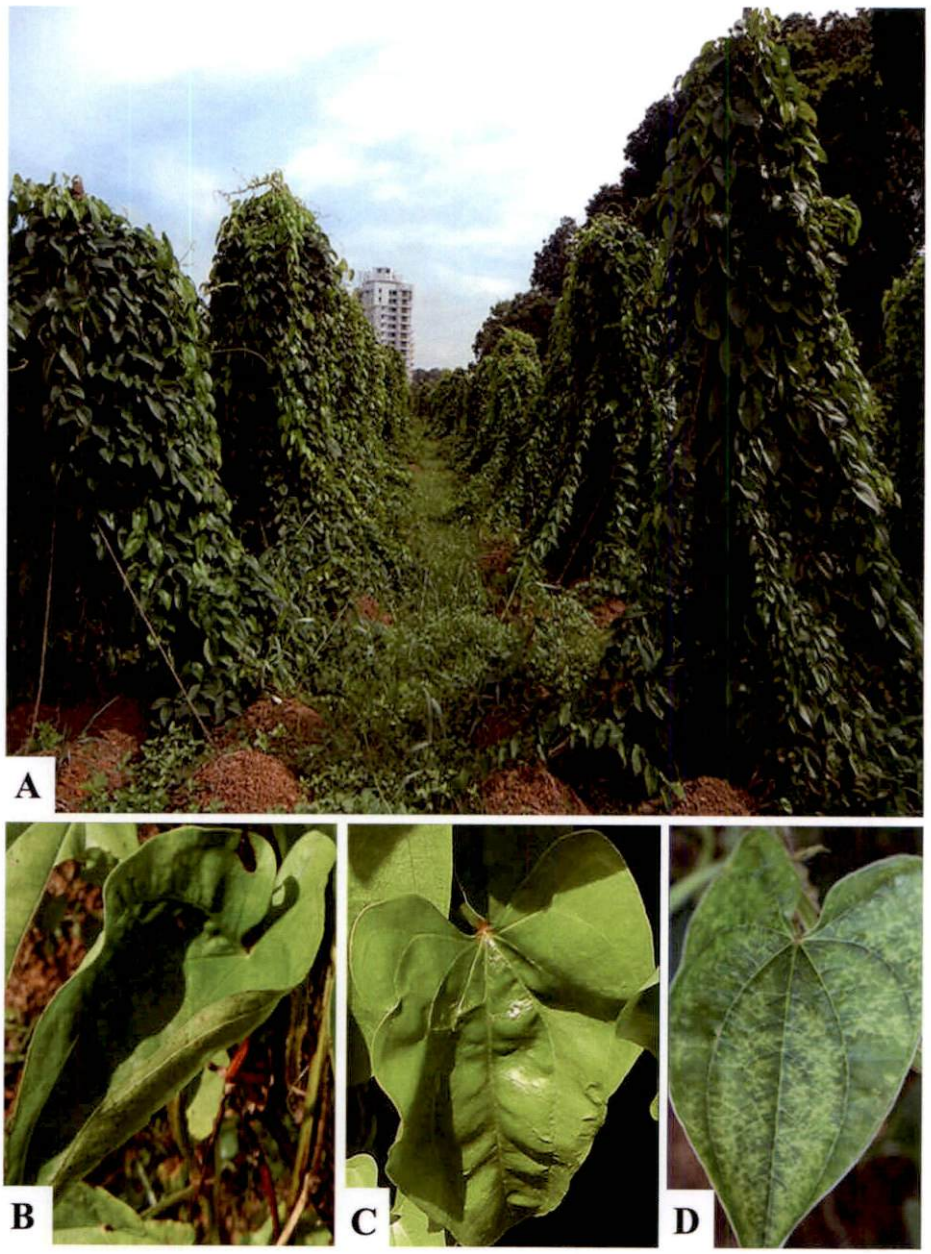

Plate 1. Symptoms of *Yam macluravirus* infection in greater yam: (A) greater yam field (B) Cupping and curling of leaf (C) Puckering on leaf (D) Mosaic on leaf

Table 3. Detection of *Yam macluravirus* in *Dioscorea alata* leaf samples collected from Thiruvananthapuram district by DAS-ELISA

Sample	Absorbance at 405 nm#		Sample	Absorbance at 405 nm#	
	1 hour	Reaction (+/-)		1 hour	Reaction (+/-)
Blank	0.209	-	S_9	-0.102	-
Non host	0.282	-	S_{10}	-0.117	-
S_1	1.528	+++++	S_{11}	-0.122	-
S_2	1.034	+++	S_{12}	-0.097	-
S_3	-0.011	-	S_{13}	0.323	+
S_4	-0.02	-	S_{14}	0.263	-
S_5	-0.087	-	S_{15}	-0.08	-
S_6	0.361	+	S_{16}	-0.094	-
S_7	0.857	+++	S_{17}	-0.01	-
S_8	-0.105	-	S_{18}	-0.108	-

(<0.28 = Negative; 0.28- 0.56 = +; 0.56-0.84 = ++; 0.84-1.12 = +++; 1.12-1.4= ++++; 1.4-1.7=+++++)

Table 4. Detection of *Yam macluravirus* in *Dioscorea alata* tuber samples collected from CTCRI fields by DAS-ELISA

Sample	Absorbance at 405 nm#		Sample	Absorbance at 405 nm#	
	1 hour	Reaction (+/-)		1 hour	Reaction (+/-)
Blank	-0.094	-	5T	-0.130	-
Non host	0.086	-	6T	-0.137	-
OE_1	-0.131	-	7T	0.069	-
OE_2	0.036	-	8T	-0.136	-
OE_3	-0.151	-	9T	-0.152	-
OE_4	-0.150	-	14T	-0.128	-
OE_5	-0.155	-	$R_{18}T$	0.233	++
OE_6	-0.125	-	CJ_2T	0.097	+
OE_7	0.270	+++	CJ_3T	-0.144	-
1T	0.312	+++	T_2T	0.030	-

(<0.086 = Negative; 0.086- 0.17 = +; 0.17-0.26 = ++; 0.26-0.34 = +++)

4.2.2 DIBA

For DIBA, the development of purple colored spots on the nitrocellulose membrane was considered positive for virus infection. Out of the 12 samples (66.7%) analyzed, eight reacted positively to *Yam macluravirus* polyclonal antibody (Plate 2B).

4.3 TRANSMISSION STUDIES

4.3.1 Mechanical inoculation

The mechanically inoculated *Nicotiana benthamiana, N. tabaccum* and *Vigna unguiculata* plants were observed for symptom expression over a period of one month. None of the inoculated plants showed characteristic symptoms (mosaic, puckering, cupping and curling of leaves) of *Yam macluravirus* infection. The RT-PCR analysis of the inoculated leaf samples confirmed negative result which ruled out the chances of any latent infection present.

4.3.2 Insect transmission

Insect transmission of *Yam macluravirus* with *Aphis craccivora* was unsuccessful. The test plants, *viz., Nicotiana benthamiana, N. tabaccum* and *Vigna unguiculata* did not develop any of the characteristic symptoms even after one month of insect transmission. The characteristic symptoms of virus infection was absent in all the test plants.

A few aphids were taken for RNA isolation after virus acquisition. Distinct intact rRNA bands with no apparent RNA degradation and no genomic DNA contamination were observed on agarose gel (1.5%). Coupled RT-PCR analysis of aphid RNA with *Yam macluravirus* specific primers (YMacs/c) yielded a faint band of expected amplicon size (800 bp) indicating virus acquisition by aphids (Plate 3).

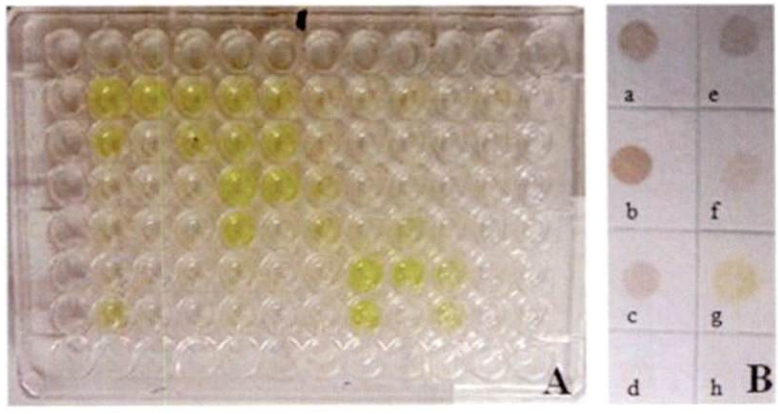

Plate 2. Serological detection of *Yam macluravirus* in *Dioscorea alata* samples: (A) DAS-ELISA (B) DIBA (a to f: *D. alata* tuber samples; g: non host; h: buffer control)

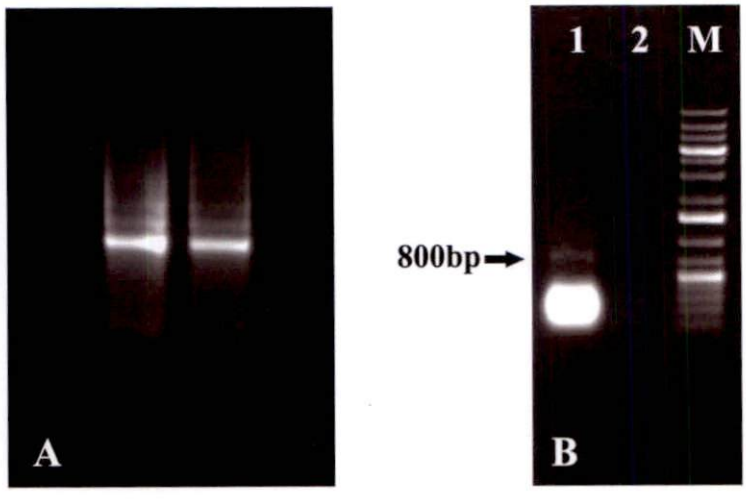

Plate 3. Aphid RNA isolation and RT-PCR analysis: (A) Total RNA isolated from *Aphis craccivora*. (B) Coupled RT-PCR products of Aphid RNA using YMacs/s primers. Lane 1: PCR product of aphid RNA; Lane 2: Negative control; M: 1kb plus DNA marker

4.4 NUCLIEC ACID BASED DETECTION

4.4.1 Isolation of total RNA

Fifty four out of the 72 leaf samples and 25 tuber samples of *D. alata* plants showing symptoms of virus infection were taken for RNA isolation. There was large difference in the yield and purity of total RNA obtained from the leaf and tuber samples depending on the method used.

Although the yield obtained from leaf samples using Ambion Purelink RNA Mini kit and TRIzol reagent was similar, the kit method provided higher quality RNA (Table 5). The isolated RNA was resolved on agarose gel (1.5%) and both methods yielded two distinct rRNA bands which showed no apparent RNA degradation (Plate 4). Among the three different methods evaluated for RNA isolation from tubers, lithium chloride method provided highest quality and quantity of the RNA as revealed by spectrophotometer analysis and gel electrophoresis (Table 6; Plate 5).

4.4.2 Amplification of *Yam macluravirus* partial coat protein gene

4.4.2.1 RT-PCR for virus CP amplification

After first strand cDNA synthesis, the annealing temperatures of YMacs/c and YMac1s/1c primers were standardized as 50 °C and 52 °C respectively. Uncoupled RT-PCR analysis with YMac1s/1c and YMacs/c primers yielded amplicons of ~200 and 800 bp respectively in samples positive for virus infection (Plate 6). Similar results were obtained with coupled RT-PCR analysis of RNA samples. The amplicons of positive samples were gel eluted to obtain purified product. Reamplification of RT-PCR products as well as gel elutes produced amplicons of expected band size confirming the presence of virus infection. *Yam macluravirus* was detected in the leaf samples collected from CTCRI fields only. Out of the 27 leaf samples and 25 tuber samples collected from the fields, 11 and 15 were found to be positive for virus infection respectively.

Table 5. Yield and absorbance ratio of total RNA isolated from greater yam leaf samples by two methods

Method	Sample	RNA yield (ng μl^{-1})	$A_{260/280}$	$A_{260/230}$
Ambion Purelink RNA Mini kit	1	2675.15	2.00	1.68
	2	1392.25	2.08	2.17
	3	1221.65	2.11	2.16
TRIzol reagent	1	2517.03	1.42	1.36
	2	1010.94	1.66	1.22
	3	1250.84	1.76	1.32

Table 6. Yield and absorbance ratio of total RNA isolated from greater yam tuber samples by three methods

Method	Sample	RNA yield (ng μl^{-1})	$A_{260/280}$	$A_{260/230}$
Ambion Purelink RNA Mini kit	1	640.07	1.96	1.61
	2	727.83	1.83	1.54
	3	768.56	1.93	1.79
Mohan kumar *et al.* protocol	1	1344.14	1.62	1.52
	2	931.36	1.44	1.40
	3	1733.95	1.62	1.71
Lithium chloride method	1	2658.47	1.91	1.89
	2	1387.68	2.02	1.92
	3	1401.56	2.04	2.04

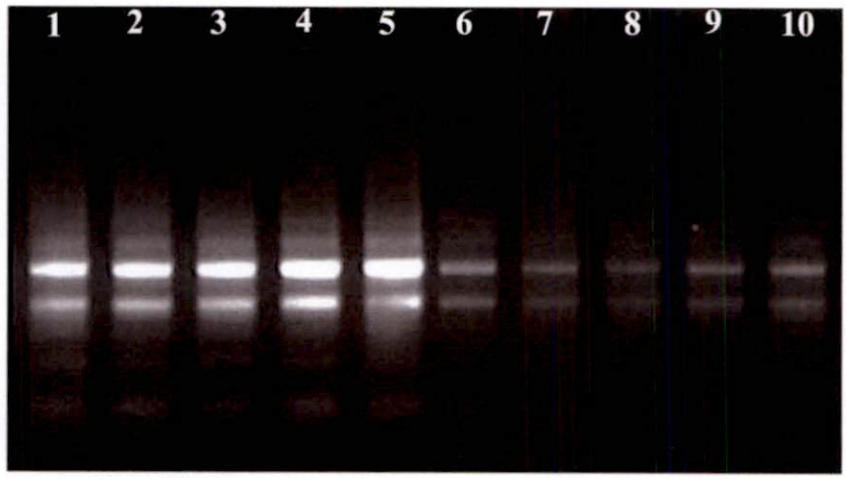

Plate 4. Total RNA isolated from *Dioscorea alata* leaf samples using Ambion Purelink RNA Minikit (lane 1 to 5) and TRIzol reagent (lane 6 to 10)

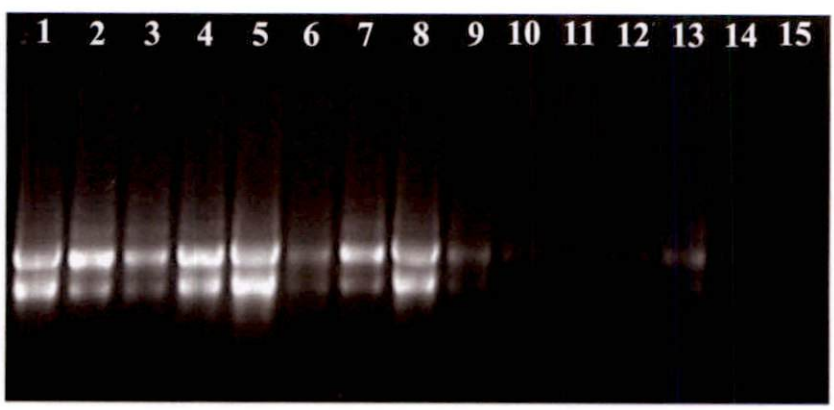

Plate 5. Total RNA isolated from *Dioscorea alata* tuber samples using lithium chloride method (lane 1 to 5), Mohan kumar *et al.* protocol (lane 6 to 10) and Ambion Purelink RNA Minikit (lane 11 to 15)

4.4.2.2 Immunocapture RT-PCR (IC-RT-PCR)

IC-RT-PCR analysis of *D. alata* leaf samples using YMacs/c primers and *Yam macluravirus* specific antibody produced amplicons of ~800 bp size indicating the presence of virus infection (Plate 7).

4.4.3 DNA sequencing

Partial *NIb* and partial *CP* regions of the virus genome were obtained by sequencing the gel elutes of PCR products. Nucleotide BLAST analysis revealed that *Yam macluravirus* was more similar to *Chinese yam necrotic mosaic virus* (ChYNMV) than other viruses of the genus *Macluravirus*. The nucleotide sequences were submitted in the GenBank database under accession numbers KM099684 and KM111541.

4.5 AMPLIFICATION OF FULL COAT PROTEIN (*CP*) REGION OF VIRUS

4.5.1 Primer designing

Forward (F) and reverse (R) primers were designed for the CP gene of *Yam macluravirus* based on the most favorable combination of conserved regions in the multiple aligned nucleotide sequences and named as YMacF1 and YMacR1 respectively.

YMacF1 sequence: 5' ACCTTTGTTGGATGAAGCCAT 3'

YMacR1 sequence: 5' TGGGTTGCTGAGCATTGGAA 3'

4.5.1.1 Primer evaluation

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. The annealing temperature of the primers was empirically standardized as 59 °C by gradient PCR.

4.5.2 Uncoupled RT-PCR of total RNA using synthesized primers

The primer pair amplified a product of ~1100 bp size (Plate 8). The positive samples were gel eluted and sequenced to get the full CP gene sequence along with partial *NIb* and UTR region. The *CP* nucleotide sequence was submitted in the GenBank database under accession number KM201261. The full CP gene (867 bases) of *Yam macluravirus* obtained after sequencing is given below.

5' ATGGATCTAACACTCGCTCCCACAGAGCAAACAACTCAGAAAGACAAA
GGGAAGCAGCATGAGCAGAGGCGAGATGGCAGGCCAGGACCATCCACCC
AACCGATTGAAACTGGTGACAACAACAACAATCAACCGATCGAACAATT
GGGTGACATGCACGACGAAGAAATTGAATGGAGGATTCCTGCAATTGCG
AAATCATTAAGGCATATATCAATTCCTCGGGTTAAAGGCAAGGCTGTTTG
GAATCAGAAAATTTTGAAGAAGATCTCCAAAGAACAGTATTACGAGACG
ACACAAATGGCGACGAGTGAGAATTTTGAAAAATGGATGGAAGCAATAA
GAAAGAACTTGGGAACAACAACTGAGGCAGATTTCCAAATATGTTTGAC
ATCCTGGTGCTTGTGGACAGCAAATAATGGCACATCACCCGAGCTTGATC
CATCGCAGGTCATGGAAGTTCATGCGAACGGGCAGATCATTGAAGTACCA
ATTTCAATCTTCATAGAACCTGCTGCAACACTCGGAGGCTTAAGGAAAAT
AATGCGAAGATTGAGTGGAGTGACTAGCAAGATTCTTGAGGAAGGAGGA
GTGATGACTGCTTGGGGAAAGAAACGTGGATTCACACAAAGGACAATGA
TTCCATATGCGTTTGACGCATACATGCAAACTGATTCAACACCAAAGACA
GTGCGGGAACAATTAAATCAAAGCAAAGCAGCAGCGATTGGAAGTGGAG
TCCAGAGAGCCTTGCTACTTGATGGAAAATTGCACAGAAGCAAGGTTAGT
TATGAAAGACATACGGATGAGGATCGCGATGAGTATGAACACAGTGATA
ATGGCGATCAGGGGCCATCTTTATATTAA 3'

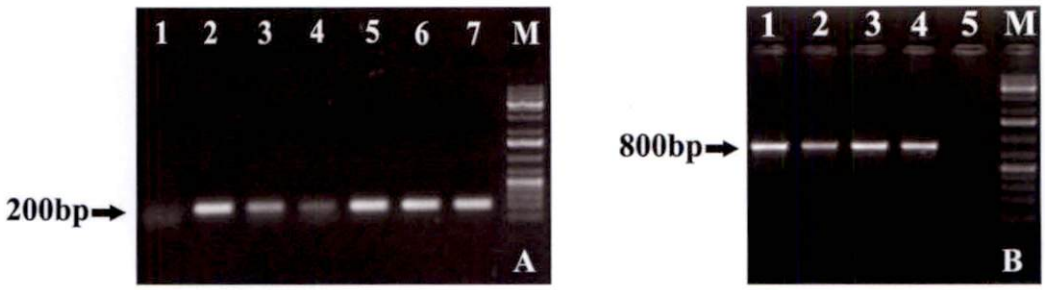

Plate 6. Detection of *Yam macluravirus* by uncoupled RT-PCR: (A) Using YMac1s/1c primers. Lane 1: negative control; lane 2 to 6: *Dioscorea alata* samples; M: 1 kb plus DNA marker. (B) Using YMacs/c primers. Lane 1 to 4: *D. alata* samples; lane 5: negative control; M: 1 kb plus DNA marker.

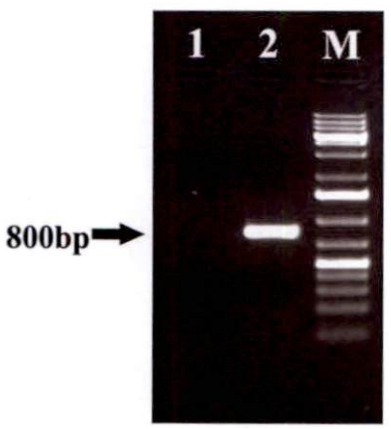

Plate 7. Detection of *Yam macluravirus* by IC-RT-PCR. Lane 1 and 2: *Dioscorea alata* samples; M: marker

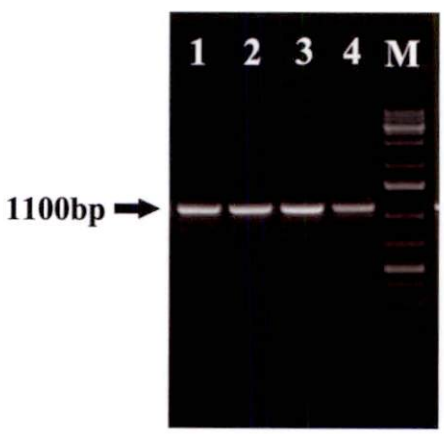

Plate 8. Amplification of full CP region by YMacFI/R1 primers. Lane 1 to 4: *Dioscorea alata* samples; M: marker

4.6 CLONING AND TRANSFORMATION

The amplicon of ~1100 bp was cloned and transformed into *E. coli* DH5α cells. The white recombinant colonies obtained (Plate 9) were confirmed by colony PCR. The expected product of size ~1100 bp was obtained from most of the colonies analyzed (Plate 10A). Similar result was obtained upon analyzing PCR products of plasmid DNA isolated from the recombinant clones (Plate 10B). A recombinant plasmid was further sequenced to confirm the presence of full *CP* region.

4.7 SEQUENCE ANALYSIS

Sequence analysis of the cloned fragment confirmed the presence of *Yam macluravirus*. The CP gene contains one long open reading frame, which consists of 867 nucleotides encoding a protein of 288 amino acid residues. BLAST analysis of the entire CP-coding region revealed that the coat protein exhibited only 65 to 70 percent sequence identity and 42 to 60 percent amino acid identity with other macluraviruses in the database (Table 7, 8). Phylogenetic analysis revealed that the virus was more similar to *Chinese yam necrotic mosaic virus* (ChYNMV) (71% nucleotide sequence similarity) than other viruses of the genus *Macluravirus*, clustering with ChYNMV and *Yam chlorotic necrotic mosaic virus* (YCNMV) sequences (Fig. 1). Analysis of the amino acid sequence of the CP region revealed the presence of few motifs that are conserved for the genus *Macluravirus* like the 'NGTS' motif. But the possible 'LQ/M' cleavage site between the NIb and CP region was not found. The tripeptide motif 'DAG' that is probably involved in the aphid transmission of the virus was not found, although an analogous 'DKG' was found. Similarly the conserved amino acid residues 'AFDF' was absent, although an analogous 'AFDA' was found (Fig. 2).

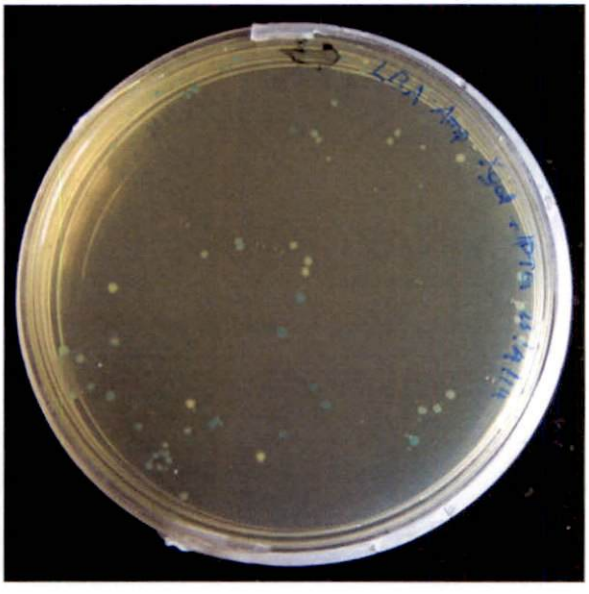

Plate 9. Luria-Bertani agar plate showing white (transformed) and blue (non-transformed) colonies

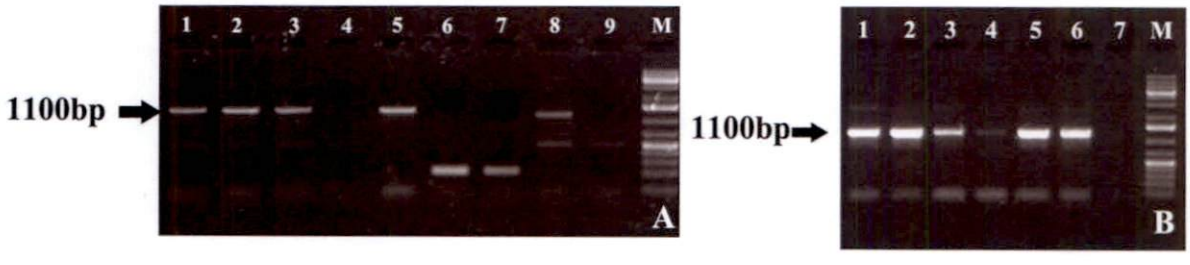

Plate 10. Analysis of recombinant clones: (A) Colony PCR (Lane 1 to 7: colony PCR products; lane 8, 9: control PCR fragment; M: marker) (B) PCR amplified plasmid DNA (Lane 1 to 6: recombinant plasmid; lane 7: negative control; M: marker)

Table 7. Nucleotide sequence identity between *Yam macluravirus* and selected macluraviruses

Virus*	Coat protein sequence identity (%)	Accession number
ChYNMV	71	AB098345
YCNMV	67	FM997911
AlpMV	67	AF499025
CdMV	67	JN544078

**Chinese yam necrotic mosaic virus* (ChYNMV), *Yam chlorotic necrotic mosaic virus* (YCNMV), *Alpinia mosaic virus* (AlpMV) and *Cardamom mosaic virus (CdMV)*

Table 8. Amino acid sequence identity between *Yam macluravirus* and other macluraviruses

Virus*	Coat protein sequence identity (%)	Accession number
ChYNMV	60	BAD16696
YCNMV	58	CAX36860
CdMV	54	AFN85100
AlpMV	59	AAP30734
LCCV	56	AER38178
NLV	45	AFA26138
RLV	42	AAZ79372
MacMV	43	AAB02823

**Chinese yam necrotic mosaic virus* (ChYNMV), *Yam chlorotic necrotic mosaic virus* (YCNMV), *Cardamom mosaic virus* (CdMV), *Alpinia mosaic virus* (AlpMV), *Large cardamom chirke virus* (LCCV), *Narcissus latent virus* (NLV), *Ranunculus latent virus* (RLV) and *Maclura mosaic virus* (MacMV)

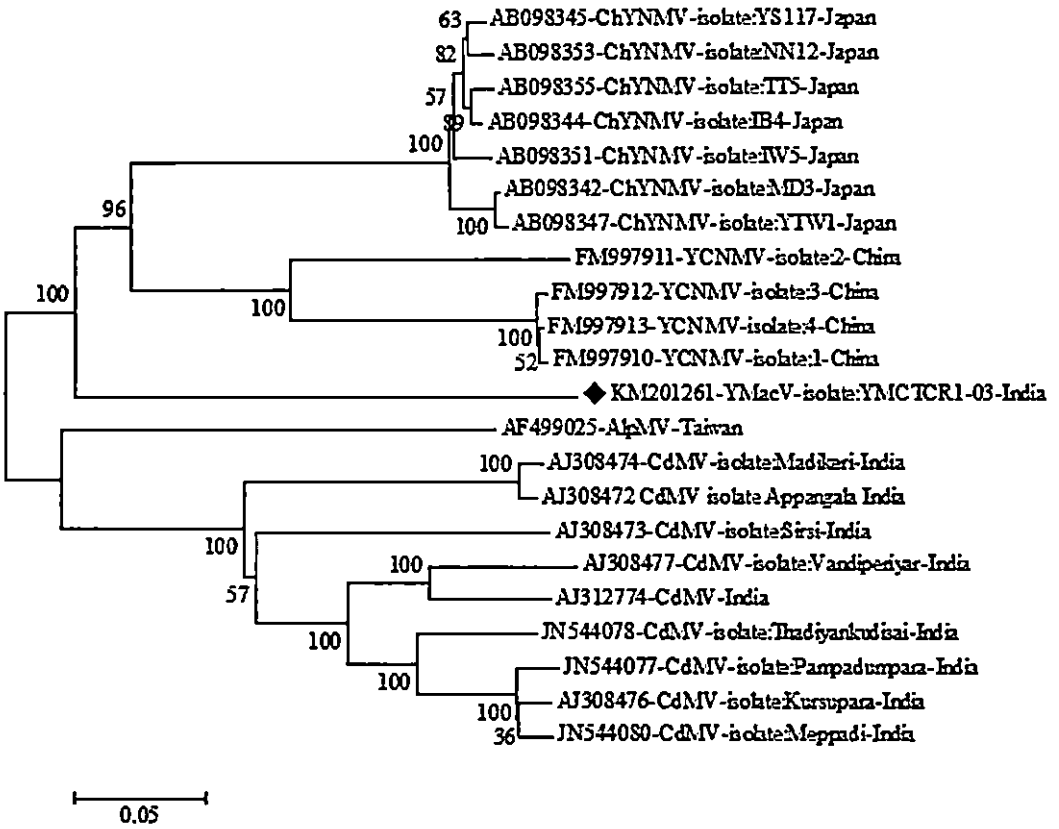

Fig. 1. Phylogenetic tree based on the nucleic acid sequences of the coat protein coding regions of *Yam macluravirus* (YMacV) and other macluraviruses. The tree was constructed by the Neighbor-Joining method using MEGA 6.06. Numbers are percentage support of branching based on bootstrap analysis (1000 replications). The scale bar indicates 0.05 substitutions/site. *Chinese yam necrotic mosaic virus* (ChYNMV), *Yam chlorotic necrotic mosaic virus* (YCNMV), *Alpinia mosaic virus* (AlpMV) and *Cardamom mosaic virus* (CdMV)

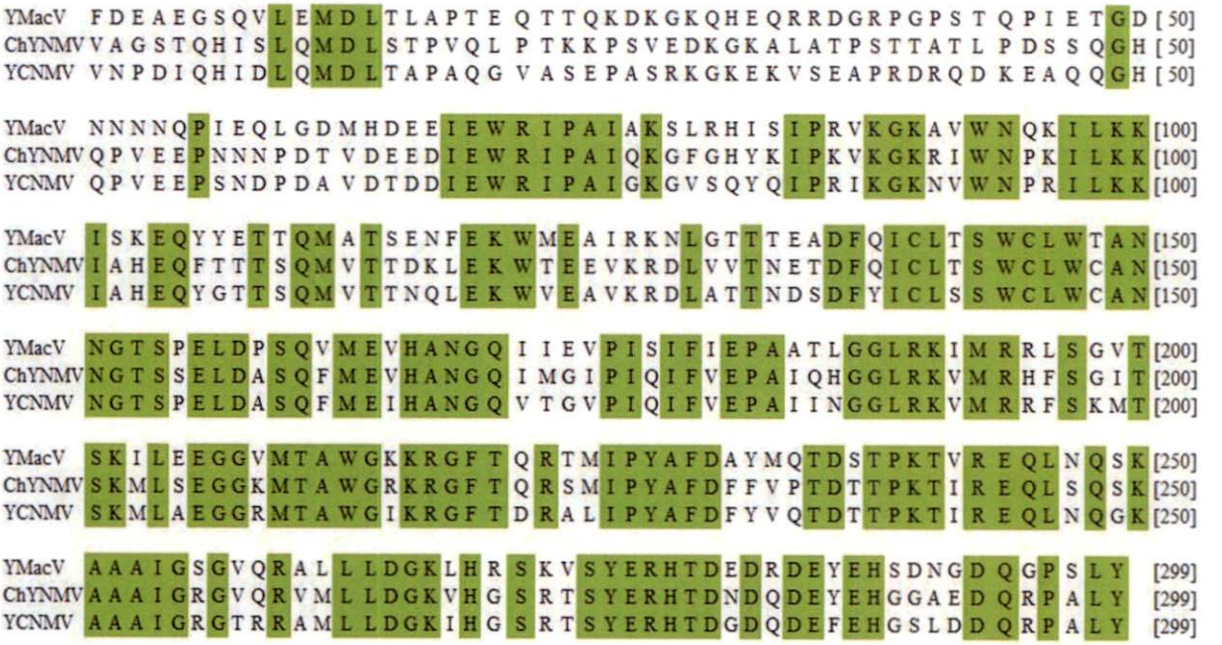

Fig. 2. Alignment of amino acid consensus sequences of the coat protein of *Yam macluravirus* (YMacV) with the corresponding regions of *Chinese yam necrotic mosaic virus* (ChYNMV) and *Yam chlorotic necrotic mosaic virus* (YCNMV). Residues conserved in the three viruses are boxed in green

4.8 INTRASPECIFIC VARIATION ANALYSIS

The variation present within the *Yam macluravirus* was analyzed using Restriction Fragment Length Polymorphism (RFLP). The products of RFLP were first analyzed on agarose gel (5%), which did not yield adequate band separation. Hence electrophoretic separation of RFLP products was carried out on polyacrylamide gel (5%). RFLP analysis with *Taq*1 enzyme produced 3 fragments but none of the samples digested showed intraspecific variation (Plate 11). Restriction digestion with *Mse*1 enzyme produced seven bands and one sample showed presence of polymorphism (Plate 12).

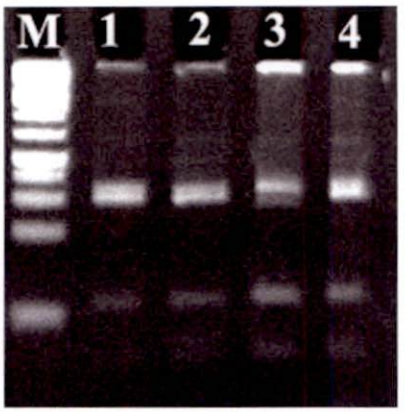

Plate 11. RFLP analysis with *Taq*1 enzyme (Lane 1 to 4: products of restriction digestion, M: 1 kb plus DNA ladder)

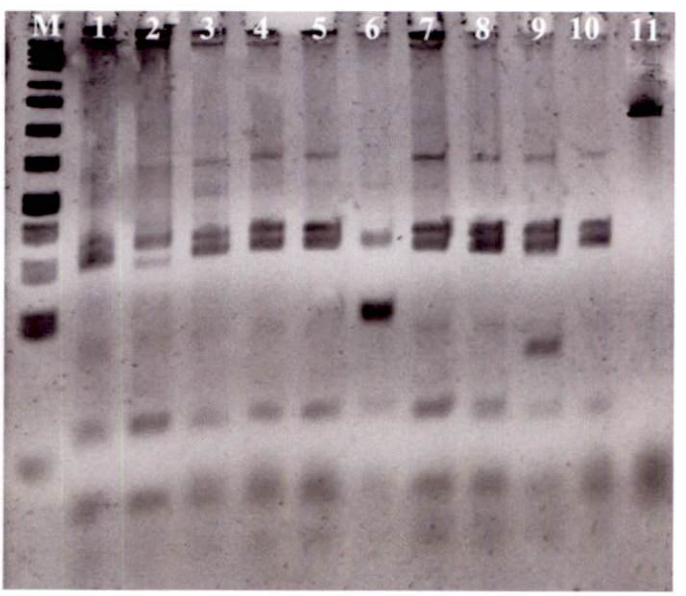

Plate 12. RFLP analysis with *Mse*1 enzyme (Lane 1 to 10: products of restriction digestion, M: 1 kb plus DNA ladder. Lane 6 shows sample with variation)

DISCUSSION

5. DISCUSSION

Yam is a vegetatively propagated crop, grown for its high calorific value tubers. Vegetative propagation, insect transmission and uncontrolled introduction of infected yam germplasm have contributed to the widespread distribution of viruses in this crop. Plant viruses cause some of the world's leading plant diseases, resulting in the loss of billions of dollars annually, yet viruses are one of the most difficult to detect plant pathogens. Even though greater yam is the most widely distributed yam species globally, the high susceptibility to diseases is a major limitation to its profitable and sustainable production. Viruses infecting yams are poorly characterized, which is a hindrance to the safe movement of germplasm.

Diagnostics is important in the development and implementation of pest management strategies. These tools can be categorized into three main groups: biological characterization using symptomatology on indicator hosts, serological detection using specific antigen-antibody recognition and molecular characterization using virus nucleic acids.

The present study was undertaken to assess the incidence and distribution of *Yam macluravirus* infecting greater yam in different regions of Kerala. *Chinese yam necrotic mosaic virus* (ChYNMV) and *Yam chlorotic necrotic mosaic virus* (YCNMV), two distinct members of the *Macluravirus* genus infects yams cultivated in Japan and China respectively (Kondo *et al.*, 2007; Wang *et al.*, 2009). *Cardamom mosaic virus* (CdMV), another member of this genus has been reported to infect cardamom cultivated in South India (Jebasingh *et al.*, 2011). However, there are no reports of macluraviruses infecting yams in India.

In our study, *Macluravirus* was detected for the first time in yams in India. With regard to distribution, *Yam macluravirus* was detected in both the leaf and tuber samples collected from CTCRI fields only (where the virus incidence was found to be high). The CTCRI experimental fields contain greater yam germplasm obtained from

different regions across the world. The presence of *Macluravirus* infection in the samples collected from these fields only indicate that the virus was introduced through infected germplasm and that it has not been introduced to other regions. Leaf samples collected from other districts although showed obvious symptoms of virus infection, tested negative, by both ELISA and PCR, to the virus indexed for. Symptoms observed on these plants might be caused by other virus(es) for which tests were not done or that are yet unidentified. Symptoms may also be due to abiotic agents causing virus-like symptoms, such as nutrient disorder and senescence. On the other hand, virus detection on non-symptomatic leaf samples shows that absence of visual symptoms on greater 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.

Obtaining good quality RNA is a prerequisite for a reliable RT-PCR reaction. Although there are many methods for RNA isolation, there is no universal method for all plant species or organs. Even identical tissues at different development stages may require a special protocol of RNA isolation due to the variability of chemical compositions and content (Wang *et al.*, 2007). For RNA isolation from leaves, younger yam leaves are most suitable than older leaves. This may be due to 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 and TRIzol reagent was similar, the kit method provided higher quality RNA as indicated by the $A_{260/280}$ and $A_{260/230}$ ratios. The traditional TRIzol kit which is the most common RNA isolation method is easy to operate and saves time and labor. However, the low A_{260}/A_{230} ratio (1.22-1.36) indicated that RNA isolated by this method may contain many chemical substances, such as phenols, polysaccharides and salt, which could lead to negative effects. Among the three different methods evaluated for RNA isolation from tubers, lithium chloride method provided highest quality and quantity of the RNA. Tattersall *et al.* (2005) also reported that the

modified Tris-LiCl was as a good choice for large quantities of RNA because it provided a good yield and excellent quality for low cost in several plants.

DAS-ELISA, DIBA, RT-PCR and IC-RT-PCR techniques were used to detect the presence of *Yam macluravirus* in various leaf and tuber samples. Of these 4 techniques employed, two (DAS-ELISA and DIBA) were protein-based and relied entirely on the use of polyclonal antiserum. IC-RT-PCR has a protein-dependent step where the virus particles are bound to the surface of the PCR tube, but from then on the reaction is entirely nucleic acid-based to amplify the viral RNA for subsequent separation and visualization: RT-PCR is entirely based in the nucleic acid only.

DIBA was found to be more effective than DAS-ELISA for the detection of *Yam macluravirus* in yam tuber samples. It was very convenient because sample preparation and the subsequent procedures for virus detection were simple and also it did not require sophisticated instruments such as an ELISA-reader or a Thermo cycler. Both DIBA and ELISA are solid phase serological detection methods and depend on the concentration of the virus particles. The sensitivity of DIBA relative to ELISA may depend on the ELISA format, and is affected by the plant tissue sampled, the components of the plant sap as well as the virus itself (Njukeng *et al.*, 2005).

Although the specificity of the antisera used for the detection (by ELISA and DIBA) of these viruses has been previously evaluated and determined (Winter, S., personal communication, 2013; unreferenced), leaf samples were also tested by RT-PCR and IC-RT-PCR in our study to ensure that plants with low virus load were not neglected. IC-RT-PCR is an important method for the detection of low titre RNA viruses (Mumford and Seal, 1997) like macluraviruses. The primers YMacs/c and YMac1s/1c which were used to amplify the partial CP gene had been successfully used for the detection of yam infecting *Macluravirus* from the CTCRI fields (Winter, S., personal communication, 2013; unreferenced).

Detection by IC-RT-PCR can theoretically be achieved from the RNA of a single particle; The IC-RT-PCR technique has been reported to exhibit a thousand-fold increase in detection sensitivity compared to existing ELISA assays (Mumford and Seal, 1997). Further increase in sensitivity in the detection of virus in yam tissues by nucleic acid-based techniques may be affected adversely by the sap components which may interact with the PCR reaction reagents in spite of stringent washing of the PCR tubes after binding of the virus to the adsorbed antibodies.

It was found that virus detection by RT-PCR and IC-RT-PCR was more than the corresponding ELISA tests, possibly due to the greater sensitivity of RT-PCR and IC-RT-PCR (Njukeng *et al.*, 2005). The lower sensitivity observed with the ELISA tests is similar to the findings of Njukeng *et al.*, (2005) and Eni (2008) and could be due to low virus concentration in yam leaf and tuber samples (Brunt *et al.*, 1990) or due to interference of polyphenols and glutinous polysaccharides contained in yam leaves (Rossel and Thottappilly, 1985). On the other hand, a few DIBA positive tuber samples were not detected by RT-PCR. Eni (2008) obtained similar results with ELISA while working with yam leaf samples 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.

Although macluraviruses are reported to be transmitted by mechanical inoculations and insect vector to numerous plant species (Berger *et al.*, 2005), several attempts to transmit the virus to herbaceous test plants by mechanical and vector transmission were unsuccessful. Moreover, we could not trace out any vectors in the fields from which the samples were collected. But coupled RT-PCR analysis of RNA isolated from aphids which were allowed to feed on the virus infected plants yielded a faint band of expected amplicon size (800 bp, i.e. partial *NIb* and partial *CP* region). This indicates that the virus was acquired from the infected leaf samples by the aphids, but was not transferred to the test plants. Although no other hosts were

identified for *Yam macluravirus*, further research is required to identify whether the virus has a wider natural host range than previously known.

In this study, a pair of species specific primers (YMacF1/R1) was designed to detect *Macluravirus* and used them in a PCR based strategy to characterize the entire *CP* region of the virus. RT-PCR analysis with these primers yielded an expected product of size ~1100 bp (containing partial *NIb*, full *CP* and partial UTR regions) in most of the positive samples. The full *CP* sequence obtained with these primers was used for phylogenetic analysis.

To date, the CP coding region of members of *Potyviridae* has been mainly used to establish evolutionary relationships at both species and strain levels primarily because the majority of potyvirid sequences on the databases are derived from this region (Adams *et al.*, 2005a). In the family Potyviridae, species demarcation criteria are based on genetic information, host range, mode of transmission, and antigenicity (Berger *et al.*, 2005). The genetic information criteria are as follows: (A) the amino acid sequence identity of the CP should be less than ~80 percent, (B) the nucleotide sequence identity should be less than 85 percent over the whole genome, and (C) the polyprotein cleavage site should be different from that of other species. In this study, the *CP* of the virus isolate differs from that of its nearest known relative by 29 percent, sufficient to be discriminated as new viral species.

Based on sequence comparisons between *Yam macluravirus* and other macluraviruses, and according to the molecular criteria for discrimination of members within the family potyviridae, *Yam macluravirus* is a different virus species and hence may be considered as a putative nineth member of *Macluravirus* genus. The lower level of sequence identity (71% nucleotide and 60% amino acid sequence identity with Chinese yam necrotic mosaic virus) is also very similar to the 69 percent nucleotide and 72–73 percent amino acid identity between Yam chlorotic necrotic mosaic virus and ChYNMV (Wang *et al.*, 2009). Phylogenetic analysis revealed that

Yam macluravirus was more similar to ChYNMV than other viruses of the genus *Macluravirus*, clustering with ChYNMV and YCNMV sequences. This may be because the above three have the same natural host (i.e. *Dioscorea* spp.). *Yam macluravirus* was not related to other viruses; a reflection of the low identities of the *Yam macluravirus* CP coding region, at nucleotide and amino acid levels. This finding is consistent with the results of Adams *et al.* (2005a) who reported that, using phylogenetic analyses based on the CP coding region of 89 viruses in the genus Potyvirus, only 20 species received bootstrap values greater than 75 percent. Further, the use of the entire ORF sequences for analyses provided stronger bootstrap support and much clearer relationships. Also, no correlation was observed between the different Macluravirus species reported from the same region. This is evident from the fact that *Yam macluravirus* showed maximum similarity to ChYNMV reported from Japan and not with CdMV which was reported from the Southern states of India (Kerala and Karnataka).

The alignment of amino acid consensus sequences of the coat protein of *Yam macluravirus* with the corresponding regions of *Chinese yam necrotic mosaic virus* (ChYNMV) and *Yam chlorotic necrotic mosaic virus* (YCNMV) generated 22 conserved regions. Upon analysis of the molecular features, some of the expected motifs could be identified. The well conserved motif 'NGTS' was found in *Yam macluravirus* as in other members of the *Potyviridae* family.

The polyprotein cleavage site between the NIb and CP sequences was predicted by comparing the amino acid sequence with those of ChYNMV and YCNMV because they were the most closely related viruses in the database analyses. Prediction of the NIb/CP cleavage site allowed a comparative study of CP amino acid sequences and revealed amino acid motifs implicated in aphid-transmission or conserved in the family Potyviridae.

Alignment of the 3' NIb and full CP region sequence suggests such putative cleavage site (LQ/M) is not present in *Yam macluravirus*. Instead of the possible LQ/M cleavage site suggested by Badge *et al.* (1997), an LE/M site was found. The LQ/M cleavage site is reported to be conserved for ChYNMV, YCNMV, NLV and MacMV. Whereas, *Large cardamom chirke virus* and CdMV have FQ/M cleavage site. The tripeptide motif 'DAG' reported to assist in aphid transmission also was absent in the N-terminal region of *Yam macluravirus* CP region. In its place an analogous DKG motif was present. ChYNMV is also reported to have a DKG motif instead of DAG (Kondo, 2001). Two motifs similar to 'DAG' were found directly after the cleavage site in NLV and MacMV also. NLV has the sequence 'DVG', and MacMV has the sequence 'DAE'. The mutation from 'DAG' to 'DAE', observed in MacMV, gave a non-transmissible product, and that a mutation to 'DVG', which is found in NLV, left only minimal transmission function (Badge *et al.*, 1997). The difficulties observed with the insect transmission of *Yam macluravirus* could be the result of a different transmission mode, particularly taking into account the lack of a DAG motif in the putative CP amino acid sequence. Moreover, aphid transmission in *Alpinia mosaic virus* was shown to be aphid specific (Liou *et al.*, 2003), and the lack of success could be explained by high virus-vector specificity not taken into account in this experiment. ChYNMV also was shown to be aphid transmitted (Kondo and Fujitha, 2012) as well as the other macluraviruses, where in some instances vector specificity was not strict (Turina *et al.*, 2006).

RT-PCR-RFLP technique was used to study intraspecific variation. It has been used to discriminate attenuated strains of ChYNMV from other isolates (Kondo *et al.*, 2007). Intraspecific variation studies could hence be used to identify any attenuated strains of the virus (if present) which could be used for cross protection to control virus spread in the fields. But this also depends on the genetic diversity of the virus in the region. The genetic variability among *Yam macluravirus* isolates in this study was revealed by the existence of two MseI digestion patterns. Restriction digestion with

MseI enzyme produced 7 bands but one sample showed presence of polymorphism. Among the different macluraviruses, CdMV shows the highest level of intraspecific diversity. A wide range of differences between isolates of CdMV in their coat proteins (75.4 to 98.4% nucleotide identity) has been reported (Jacob *et al.*, 2003).

The influence of *Yam macluravirus* on yield and growth of the yam plant has not been well studied. However, tuber yield losses accounting for about 30 to 45 percent has been reported in the case of ChYNMV infection in Nagaimo variety of Chinese yam (Tochihara, 1993). A great range of virus species and strains commonly infect yams and nutrient or trace element deficiencies mimic/enhance the symptoms caused by virus infections. Hence, it has been extremely difficult to assess how much yield or quality reduction is caused by a specific virus. Multiple infections with different species or strains of a virus are also relatively common. Generally, the effect of the mixed infections is greater than infection with a single virus. As *Macluravirus* is often seen as mixed infections with other yam infecting viruses, a clear knowledge of the actual dynamics of the various interplaying factors that ultimately results in the occurrence of mixed infections in yam fields is crucial to proffering effective yam virus control strategies.

As greater yam is a vegetatively propagated crop, there is an increased chance of virus introduction into new areas through the planting material. Naturally infected yam plants may also became sources of inoculum when used as planting materials the following year. The present study helped to detect *Yam macluravirus* in greater yam and standardize diagnostic techniques required for routine virus indexing. Virus characterization as well as intraspecific variation studies carried out will help to formulate broad spectrum management strategies.

SUMMARY

6. SUMMARY

The study entitled "Detection and characterization of *Macluravirus* infecting greater yam (*Dioscorea alata* L.)" was conducted at the Division of Crop Protection, Central Tuber Crops Research Institute (CTCRI), Sreekariyam, Thiruvananthapuram during September 2013- June 2014. The objective of this study was to detect *Macluravirus* infecting greater yam and to characterize the virus at molecular level. Leaf samples used in this study were collected from Thiruvananthapuram, Kollam, Pathanamthitta, Alappuzha and Wayanad districts of Kerala. The tuber samples were taken from the greater yam growing fields in CTCRI.

The total RNA was isolated from both leaf and tuber samples. PureLink RNA Mini kit and Lithium chloride method were found to be most appropriate for RNA isolation from greater yam leaf and tuber samples respectively. Serological (DAS-ELISA and DIBA) and nucleic acid based (RT-PCR and IC-RT-PCR) techniques were performed to detect the presence of *Macluravirus* infection. *Yam macluravirus* specific antibodies were used for serological methods of detection. For nucleic acid based detection, two pairs of *Yam macluravirus* specific primers (YMac1s/1c and YMacs/c) were used which produced amplicons of ~200 and 800 bp (partial NIb-partial CP gene) respectively in virus infected samples. Presence of virus infection was detected only in the samples collected from the CTCRI fields.

A pair of species specific primers (YMacF1/R1) was designed to amplify the full coat protein gene of the virus. RT-PCR analysis with these primers provided an amplicon of ~1100 bp. The positive samples were gel eluted and sequenced to obtain the full CP gene of *Yam macluravirus*. The full CP gene was cloned into *E. coli* DH5α cells. The presence of gene insert in the transformed colonies was confirmed by colony PCR. The recombinant plasmids were isolated and RT-PCR was performed which yielded amplicons of expected band size (~1100 bp).

BLAST analysis of the CP coding region revealed that the virus has maximum similarity to *Chinese yam necrotic mosaic virus* (ChYNMV) (71% nucleotide identity). Sequence and phylogenetic analyses revealed considerable variability; and the virus was found to be in the same cluster as ChYNMV and *Yam chlorotic necrotic mosaic virus* (YCNMV). The virus coat protein gene exhibited only 65 to 70 percent nucleotide sequence identity with other macluraviruses indicating that it is putative new species.

RFLP studies were conducted with two restriction enzymes *(Taq*I and *Mse*I). RFLP analysis with *Taq*I enzyme produced 3 fragments but none of the virus samples digested showed intraspecific variation. Restriction digestion with *Mse*I enzyme produced 7 bands and one sample showed presence of polymorphism. Mechanical inoculation and insect transmission of the virus on *Nicotiana benthamiana, N. tabaccum* and *Vigna unguiculata* plants yielded negative results.

REFERENCES

7. REFERENCES

Abd-Elsalam, K.A. 2003. Bioinformatic tools and guideline for PCR primer design. *Afr. J. Biotechnol.* 2(5): 91-95.

Abraham, K. and Arnau, G. 2007. *Use of DNA Markers for Genetically Improving the Productivity, Palatability, Storability and Dry Matter Content of Tubers of Greater Yam.* Mid-term Report of IFCPAR Project Number 3000-B1, New Delhi, India, p.26.

Abraham, K. and Nair, P.G. 1991. Polyploidy and sterility in relation to sex in *Dioscorea alata* L. *Genetica* 83: 93-97.

Adams, M.J., Antoniv, J.F., and Fauquet, C.M. 2005a. Molecular criteria for genus and species discrimination within the family *Potyviridae. Arch. Virol.* 150: 459-479.

Adams, M.J., Antoniv, J.F., and Beaudoin, F. 2005b. Overview and analysis of the polyprotein cleavage sites in the family *Potyviridae. Mol. Plant Pathol.* 6: 471-487.

Adams, M.J., Zerbini, F.M., French, R., Rabenstein, F., Stenger, D.C., and Valkonen, J.P.T. 2012. Family *Potyviridae.* In: King, A.M.Q., Adams, M.J., Carstens, E.B., and Lefkowitz, E.J. (eds), *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses.* Academic Press, London, pp. 1069-1089.

Adeniji, M.O. 1970. Influence of moisture and temperature on yam decay organisms. *J. Phytopathol.* 60: 1698-1699.

Albrecht, M.A. and McCarthy, B.C. 2006. Seed germination and dormancy in the medicinal woodland herbs *Collinsonia Canadensis* L. (*Lamiaceae*) and *Dioscorea villosa* L. (*Dioscoreaceae*). *Flora* 201: 24-31.

Aleman-Verdaguer, M., Fauquet, C., Goudou-Urbino, C., Dubern, J., and Beachy, R.N. 1997. Analysis of the sequence diversity of the P1, HC, P3, NIb and CP genomic regions of several *Yam mosaic potyvirus* isolates: implications for their intraspecies molecular diversity of potyviruses. *J. Gen. Virol.* 78: 1253-1264.

Amusa, N.A., Adegbita, A.A., Muhammed, S., and Daiyewu, R. 2003. Yam diseases and its management in Nigeria. *Afr. J. Biotechnol.* 2: 497-502.

Anindya, R. and Savithri, H.S. 2003. Surface-exposed amino- and carboxy- terminal residues are crucial for the initiation of the assembly in *Pepper vein banding virus*: a flexuous rod-shaped virus. *Virol.* 316: 325-336.

Anindya, R., Chittori, S., and Savithri, H.S. 2005. Tyrosine 66 of *Pepper vein banding virus* genome-linked protein is uridylated by RNA-dependent RNA polymerase. *Virol.* 336: 154-162.

Arnau, G., Nemorin, A., Maledon, E., and Abraham, K. 2009. Revision of ploidy status of *Dioscorea alata* L. (*Dioscoreaceae*) by cytogenetic and microsatellite segregation analysis. *Theor. Appl. Genet.* 118: 1239-1249.

Asha, K.I. and Nair, M.C. 2002. Ethnic knowledge system on wild *Dioscorea* (yams) by the Kanikkars of Southern Western Ghats, Kerala. *Indian J. Plant Genet. Res.* 15: 146-149.

Atreya, P.L., Lopez-Moya, J.J., Chu, M., Atreya, C.D., and Pirone, T.P. 1995. Mutational analysis of the coat protein N-terminal amino acids involved in *Potyvirus* transmission by aphids. *J. Gen. Virol.* 76: 265-270.

Ayensu, E.S. and Coursey, D.G. 1972. Guinea yams. The botany, ethnobotany, use and possible future of yams in West Africa. *Econ. Bot.* 26: 301-318.

Baah, F.D. 2009. Characterization of water yam (*Dioscorea alata*) for existing and potential food products. Ph.D. thesis, Kwame Nkrumah University of Science and Technology, Ghana, 193p.

Badge, J., Foster, G.D., Robinson, D.J., and Brunt, A.A. 1997. 3'-terminal sequences of the RNA genomes of narcissus latent and maclura mosaic viruses suggest that they represent a new genus of the *Potyviridae*. *J. Gen. Virol.* 78: 253-257.

Balamuralikrishnan, M., Doraisamy, S., Ganapathy, T., and Viswanathan, R. 2002. Serological specificity and titre of *Sugarcane mosaic virus* polyclonal antisera raised under varying immunization procedures and bleeding time. *J. Plant Dis. Prot.* 109: 646-654.

Barnett, O.W. 1992. A summary of potyvirus taxonomy and definitions. *Arch. Virol. (Suppl.)* 5: 435-444.

Bateson, M.F. and Dale, J.L. 1995. *Banana bract mosaic virus*: characterization using potyvirus specific degenerate PCR primers. *Arch. Virol.* 140: 515-527.

Berger, P.H., Adams, M.J., Barnett, O.W., Brunt, A.A., Hammond, J., Hill, J.H., Jordan, R.L., Kashiwazaki, S., Rybicki, E., Spence, N., Stenger, D.C., Ohki, S.T., Uyeda, I., van Jaayen, A., Valkonen, J., and Vetten, H.J. 2005. *Potyviridae.* In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., and Ball, L.A. (eds), *Virus Taxonomy. VIIIth Report of the International*

Committee on Taxonomy of Viruses. Elsevier/Academic Press, London, pp. 819-841.

Berniak, H., Komorowska, B., and Sochacki, D. 2013. Detection of *Narcissus latent virus* isolates using one-step RT-PCR assay. *J. Hortic. Res.* 21(1): 11-14.

Boonham, N., Tomlinson, J., and Mumford, R. 2007. Microarrays for rapid identification of plant viruses. *Annu. Rev. Phytopathol.* 45: 307-328.

Bos, L. 1992. *Potyvirus*, chaos or order? In *Potyvirus* taxonomy. *Arch. Virol. (Suppl.)* 5: 31-46.

Bousalem, M., Arnau, G., Hochu, I., Arnolin, R., Viader, V., Santoni, S., and David, J. 2006. Microsatellite segregation analysis and cytogenetic evidence for tetrasomic inheritance in the American yam *Dioscorea trifida* and a new basic chromosome number in the *Dioscoreae*. *Theor. Appl. Genet.* 113: 439-451.

Bousalem, M., Douzery, E.J., and Fargette, D. 2000a. High genetic diversity, distant phylogenetic relationships and intraspecies recombination events among natural populations of *Yam mosaic virus*: a contribution to understanding potyvirus evolution. *J. Gen. Virol.* 81: 243-55.

Bousalem, M., Dallot, S., and Guyader, S. 2000b. The use of phylogenic data to develop molecular tools for the detection and genotyping of yam mosaic virus. Potential application in molecular epidemiology. *J. Virol. Methods* 90: 25-36.

Bousalem, M., Dallot, S., Fuji, S., and Natsuaki, K.T. 2003. Origin, world-wide dispersion, bio-geographical diversification, radiation and recombination: an evolutionary history of *Yam mild mosaic virus* (YMMV). *Infect. Genet. Evol.* 3: 189-206.

Bousalem, M., Douzery, E.J.P., and Seal, S.E. 2008. Taxonomy, molecular phylogeny and evolution of plant reverse transcribing viruses (family *Caulimoviridae*) inferred from full length genome and reverse transcriptase sequences. *Arch. Virol.* 153: 1085-1102.

Brand-Miller, J., Burani, J., and Foster-Powell, K. 2003. *The New Glucose Revolution- Pocket Guide to the Top 100 Low GI Foods.* Marlowe and Company, Washington, 158p.

Brattey, C. and Burns, R. 1998. Antibody production. In: Foster, G.D. and Taylor, C. (eds). *Plant Virology Protocols: From Virus Isolation to Transgenic Resistance. Methods in Molecular Biology.* Humana Press, New Jersey. pp. 279-286.

Brunt, A., Crabtree, K., and Gibbs, A. 1990. *Viruses of Tropical Plants: Descriptions from the VIDE Database.* CAB International, UK, 707p.

Brunt, A.A. 1976. *Narcissus latent virus. CMI/AAB Descriptions of Plant Viruses, vol. 170.* CMI/AAB, Kew, UK, p.4.

Brunt, A.A., Jackson, G.V.H., and Frison, E.A. 1989. *Technical Guidelines for the Safe Movement of Germplasm.* FAO, Rome, 20p.

Burkill, I.H. 1960. Organography and evolution of *Dioscoreaceae*, the family of yams. *J. Linn. Soc.* 56: 319-412.

Carrillo-Tripp, J., Lozoya-Gloria, E., and Rivera-Bustamante, R.F. 2007. Symptom remission and specific resistance of pepper plants after infection by *Pepper golden mosaic virus. Phytopathol.* 97: 51-57.

Caveness, F.E. 1992. Nematological research at IITA 1969-1988. A summary of investigations. In: Lowe, J. (ed.), *Plant Health Management Research Monograph 2*. IITA, Ibadan, Nigeria, p.52.

Chang, P.S. 2009. Plant virus diagnostics: comparison of classical and membrane-based techniques for immunoassay and coat protein sequence characterization for *Cucumber mosaic virus* and three potyviruses. Ph.D. thesis, Virginia State University, Blacksburg, Virginia, 175p.

Chant, S.R. 1957. Plant pathology. In: *Annual report 1954/55*. Federal Department of Agricultural Research, Nigeria, 24p.

Chen, J. and Adams, M.J. 2001. A universal PCR primer to detect members of the *Potyviridae* and its use to examine the taxonomic status of several members of the family. *Arch. Virol.* 146: 757-766.

Cho, S.N. 1990. Contemporary approaches to the serology of tuberculosis. *Int. Union Tuberc. Lung Dis.* 66: 59-62.

Ciuffo, M., Testa, M., Lenzi, R., and Turina, M. 2011. *Ranunculus latent virus*: a strain of artichoke latent virus or a new *Macluravirus* infecting artichoke? *Arch. Virol.* 156: 1053-1057.

Clark, M.F. and Adams, A.N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 34: 475-483.

Colinet, D. and Kummert, J. 1993. Identification of a *Sweet potato feathery mottle virus* isolate from China (SPFMVCH) by the polymerase chain reaction with degenerate primers. *J. Virol. Methods* 45: 149-159.

Colinet, D., Kummert, J., Lepoivre, P., and Semal, J. 1994. Identification of distinct potyviruses in mixedly-infected sweet potato by the polymerase chain reaction with degenerate primers. *Phytopathol.* 84: 65-69.

Cook, A.A. 1978. *Diseases of Tropical and Sub Tropical Vegetables and Other Plants*. Macmillan Publishing Co. Inc., Hafner Press, New York, 381p.

Cooper, B., Eckert, D., Andon, N.L., Yates, J.R., and Haynes, P.A. 2003. Investigative proteomics: identification of an unknown plant virus from infected plants using mass spectrometry. *J. Am. Soc. Mass Spectrom.* 14: 736-741.

Copeland, R. 1998. Assaying levels of plant viruses by ELISA. In: Foster, G.D. and Taylor, C. (eds), *Plant Virology Protocols: From Virus Isolation to Transgenic Resistance. Methods in Molecular Biology*. Humana Press, New Jersey, pp. 455-460.

Coursey, D.G. 1967a. Internal brown spot, a condition in yams in Barbados. *J. Agric. Soc.* 67: 473-482.

Coursey, D.G. 1967b. *Yams: An Account of the Nature, Origins, Cultivation, and Utilization of the Useful Members of Dioscoreaceae*. Longmans, Green and Co. Ltd., London, 230 p.

Craufurd, P.Q., Summerfield, R.J., Asiedu, R., and Vara Prasad, P.V. 2001. Dormancy in yams. *Exptl. Agric.* 37: 147-181.

De Leeuw, G.T.N. 1972. *Hyoscyamus niger*, a useful local lesion host for mosaic virus in *Hippeastrum*. *Neth. J. Plant Pathol.* 78: 107-109.

Degras, L. 1993. *The Yam, a Tropical Root Crop*. Macmilan, London, UK, 408p.

Desbiez, C., Wipf-Scheibel, C., and Lecoq, H. 2002. Biological and serological variability, evolution and molecular epidemiology of *Zucchini yellow mosaic virus* (ZYMV, *Potyvirus)* with special reference to Caribbean islands. *Virus Res.* 85: 5-16.

Dieffenbach, C.W., Lowe, T.M., and Dveksler, G.S. 1993. General concepts for PCR primer design. *Genome Res.* 3: S30-S37.

Dijkstra, J. and de Jager, C.P. 1998. *Practical Plant Virology: Protocols and Exercises*: Springer-Verlag GmbH, Germany, 459p.

Dolja, V.V., Haldeman, R., Robertson, N.L., Dougherty. W.G., and Carrington, J.C. 1994. Distinct functions of capsid protein in assembly and movement of *Tobacco etch potyvirus* in plants. *EMBO J.* 13: 1482-1491.

*Dumont, R. 1982. Ignames spontanées et cultivées au Bénin et en Haute-Volta. In: Miège, J. and Lyonga, S.N. (eds), *Yams – Ignames*. Clarendon Press, Oxford, pp. 31-43.

Dumont, R. and Vernier, P. 2000. Domestication of yams (*Dioscorea cayenensis-rotundata*) within the Bariba ethnic group in Benin. *Outlook Agric.* 29(2): 137-142.

Egesi, C.N., Asiedu, R., Ude, G., Ogunyemi, S., and Egunjobi, J.K. 2006. AFLP marker diversity in water yam (*Dioscorea alata* L.). *Plant Genet. Resour.* 4: 181-187.

Egesi, C.N., Onyeka, T.J., and Asiedu, R. 2007. Severity of anthracnose and virus diseases of water yam (*Dioscorea alata* L.) in Nigeria: Effects of yam genotype and date of planting. *Crop Prot.* 26: 1259-1265.

Eni, A.O., Hughes, Jd'A., Asiedu, R., and Rey, M.E.C. 2010. Survey of the incidence and distribution of viruses infecting yam (*Dioscorea* spp.) in Ghana and Togo. *Ann. Appl. Biol.* 156(2): 243-251.

Eni, A.O. 2008. Epidemiology, diagnostics and molecular studies of yam viruses in Ghana, Togo and Benin. Ph.D. thesis, University of the Witwatersrand, Johannesburg, 183p.

Eni, A.O., Kumar, P.L., Asiedu, R., Alabi, O.J., Naidu, R.A., Hughes, Jd'A., and Rey, M.E.C. 2008a. First report of *Cucumber mosaic virus* in yams (*Dioscorea* spp.) in Ghana, Togo, and Republic of Benin in West Africa. *Plant Dis.* 92(5): 833.

Eni, A.O., Hughes, Jd'A., Asiedu, R., and Rey, M.E.C. 2008b. Sequence diversity among badnavirus isolates infecting yam (*Dioscorea* spp.) in Ghana, Togo, Benin and Nigeria. *Arch. Virol.* 153(12): 2263-2272.

Eni, A.O., Hughes, Jd'A., and Rey, M.E.C. 2008c. Survey of the incidence and distribution of five viruses infecting yam in the major yam producing zones in Benin. *Ann. Appl. Biol.* 153(2): 223-232.

*Essad, S. 1984. Variation ge'ographique des nombreschromosomiques de base etpolyploi¨diedans le genre Dioscorea a` propos du de'nombrement des espe`cestransversa Brown, pilosiuscula Bert et trifida. *Agron.* 4: 611-617.

FAOSTAT – Food and Agriculture Organization of the United Nations. 2012. *Production: Crops* [on line]. Available: http://faostat.fao.org/ [08 Mar. 2014].

Farombi, O.E., Britton, G., and Emerole, O.G. 2000. Evaluation of the antioxidant activity and partial characterization of extracts from browned yam flour diet. *Food Res. Int.* 33: 493-499.

French, R. and Stenger, D.C. 2005. Genome sequences of *Agropyron mosaic virus* and *Hordeum mosaic virus* support reciprocal monophyly of the genera *Potyvirus* and *Rymovirus* in the family *Potyviridae. Arch. Virol.* 150: 299-312.

Fu, Y.C., Huang, P.Y., and Chu, C.J. 2005. Use of continuous bubble separation process of separating and recovering starch and mucilage from yam (*Dioscorea pseudojaponica* Yamamoto). *LWT.* 38(7): 735-744.

Fukumoto, F. and Tochihara, H. 1978. *Chinese yam necrotic mosaic virus. Ann. Phytopathol. Soc. Japan* 44: 1-5.

Gedil, M. and Sartie, A. 2010. Perspectives on molecular breeding of Africa's main staple food crops- cassava and yam. *Asp. Appl. Biol.* 96: 123-135.

Gibbs, A.J. and Mackenzie, A. M. 1997. A primer pair for amplifying part of the genome of all potyvirids by RT-PCR. *J. Virol. Methods* 63: 9-16.

Gibbs, A.J., Mackenzie, A.M., and Gibbs, M.J. 2003. The 'potyvirid primers' will probably provide phylogenetically informative DNA fragments from all species of *Potyviridae. J. Virol. Methods* 112: 41-44.

Gibbs, A.J., Ohshima, K., Phillips, M.J., and Gibbs, M.J. 2008. The prehistory of potyviruses: their initial radiation was during the dawn of agriculture. *PLoS One 3*:e2523. doi:10.1371/journal.pone.0002523.

Girardin, O., Nindjin, C., Farah, Z., Eshcher, F., Stamp, P., and Otokore, D. 1998. Effect of storage on system and sprout removal on post-harvest yam (*Dioscorea* spp.) fresh weight losses. *J. Agric. Sci.* 130: 329-336.

Gonsalves, D., Trujillo, E., and Hoch, H.C. 1986. Purification and some properties of a virus associated with cardamom mosaic, a new member of the *Potyvirus* group. *Plant Dis.* 70: 65-69.

Ha, C.V. 2007. Detection and identification of potyviruses and geminiviruses in Vietnam. Ph.D. thesis, Queensland University of Technology, Vietnam, 265p.

Hahn, S.K., Osiru, D.S.O., Akoroda, M.O., and Otoo, J.A. 1987.Yam production and its future prospects. *Outlook Agric.* 16(3): 105-110.

Hahn, S.K. 1995. Yams, *Dioscorea* spp. (*Dioscoreaceae*). In: Smartt, J., Simmonds, N.W. (eds), *Evolution of crop plants*. Longman Group Limited, London, pp. 112-120.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Ser.* 41: 95-98.

Hancock, J.M., Chaleeprom, W., Chaleeprom, W., Dale, J., and Gibbs, A. 1995. Replication slippage in the evolution of potyviruses. *J. Gen. Virol.* 76: 3229-3232.

Heuer, D.M., Saha, S., and Archer, L.A. 2003. Topological effects on the electrophoretic mobility of rigid rod like DNA in polyacrylamide gels. *Biopolym.* 70: 471-481.

Hochu, I., Santoni, S., and Bousalem, M. 2006. Isolation, characterization and cross-species amplification of microsatellite DNA loci in the tropical American yam *Dioscorea trifida. Mol. Ecol. Notes* 6: 137-140.

Holford, P. 2008. The glycemic load of common food [on line]. Available: http://www.patrickholford.com/content.asp?id_Content=1245 [08 Feb. 2014].

Hong, Y., Levay, K., Murphy, J.F., Klein, P.G., Shaw, J.G., and Hunt, A.G. 1995. A potyvirus polymerase interacts with the viral coat protein and VPg in yeast cells. *Virol.* 214: 159-166.

Hou, W.C., Hsu, F.L., and Lee, M.H. 2002. Yam (*Dioscorea batatas*) tuber mucilage antioxidant activities in-vitro. *Planta Medica* 68(12): 1072-1076.

Hughes, Jd'A. 1986. Viruses of the *Araceae* and *Dioscorea* species: Their isolation, characterization and detection. Ph.D. thesis, University of Reading, UK. 345p.

Hull, R. 2002. *Matthew's plant Virology* (4th Ed.). Academic Press, San Diego, 1056p.

ICTV [International Committee on Taxonomy of Viruses]. 2011. Virus Taxonomy: 2011 Release [on line]. Available: http://www.ictvonline.org/virusTaxonomy.asp?version=2011 [06 Jan. 2014].

Jacob, T., Jebasingh, T., Venugopal, M.N., and Usha, R. 2003. High genetic diversity in the coat protein and 3' untranslated regions among geographical isolates of *Cardamom mosaic virus* from South India. *J. Biosci.* 28: 589-595.

*Jacqua, G., Fre´zal, L., Neema, C. 2005. Evaluation de la sensibilite´ de ge´notypesd'ignamesa` l'anthracnose (*Colletotrichum gloeosporioides*): test in vitro surfeuillesisole´es. In: Huyez-Levrat, M. (ed.), *Methodesd'appre´ciation du comportementvarie´talvis-a-vis des bioagresseurs.* Le Cahier des Techniques de l'Inra, INRA, Paris, pp. 139-142.

Jebasingh, T., Jose, M., Yadunandam, A.K., Backiyarani, S., Srividhya, K.V., Krishnaswamy, S., and Usha, R. 2011. Molecular modeling and conformational analysis of native and refolded viral genome-linked protein of *Cardamom mosaic virus*. *Indian J. Biochem. Biophys.* 48: 336-340.

Jones, N., Ougham, H., Thomas, H., and Pašakinskienë, I. 2009. Markers and mapping revisited: finding your gene. *New Phytol.* 183: 935-966.

Jordan, R. and Hammond, J. 1991. Comparison and differentiation of *Potyvirus* isolates and identification of strain-, virus-, subgroup-specific and *Potyvirus* group-common epitopes using monoclonal antibodies. *J. Gen. Virol.* 72: 25-36.

Kampke, T., Kieninger, M., and Mecklenburg, M. 2001. Efficient primer design algorithms. *Bioinforma.* 17(3): 214-225.

Kang, S.H., Lim, W.S., Hwang, S.H., Park, J.W., Choi, H.S., and Kim, K.H. 2006. Importance of the C-terminal domain of *Soybean mosaic virus* coat protein for subunit interactions. *J. Gen. Virol.* 87: 225-229.

Kenyon, L., Lebas, B.S.M., and Seal, S.E. 2008. Yams (*Dioscorea* spp.) from South Pacific islands contain many novel badnaviruses: implications of international movement of yam germplasm. *Arch. Virol.* 153: 877-889.

Kenyon, L., Shoyinka, S.A., Hughes, J.d'A., and Odu, B.O. 2001. An overview of viruses infecting *Dioscorea* yams in Sub-Saharan Africa. In: Hughes, J.d'A. and Odu, B.O. (eds), *Plant Virology in Sub-Saharan Africa*. IITA, Ibadan, Nigeria, pp. 432-439.

Kimalov, B., Gal-On, A., Stav, R., Belausov, E., and Arazi, T. 2004. Maintenance of coat protein N-terminal net charge and not primary sequence is essential for *Zucchini yellow mosaic virus* systemic infectivity. *J. Gen. Virol.* 85: 3421-3430.

Kondo, T. 2001. The 3′ terminal sequence of *Chinese yam necrotic mosaic virus* genomic RNA: a close relationship with *Macluravirus*. *Arch. Virol.* 146: 1527-1535.

Kondo, T. and Fujita, T. 2012. Complete nucleotide sequence and construction of an infectious clone of *Chinese yam necrotic mosaic virus* suggest that macluraviruses have the smallest genome among members of the family *Potyviridae*. *Arch. Virol.* 157: 2299-2307.

Kondo, T., Kasai, K., Yamashita, K., and Ishitani, M. 2007. Selection and discrimination of an attenuated strain of *Chinese yam necrotic mosaic virus* for cross-protection. *J. Gen. Plant Pathol.* 73: 152-155.

Kuroda, T., Nabata, K., Hori, T., Ishikawa, K., and Natsuaki, T. 2010. *Soybean leaf rugose mosaic virus*, a new soil borne virus in the family *Potyviridae*, isolated from soybean in Japan. *J. Gen. Plant Pathol.* 76: 382-388.

Kwoseh, C.K., Plowright, R.A., Bridge, J., and Asiedu, R. 2007. Search for *Scutellonema bradys* resistance in yams (*Dioscorea* spp.). *J. Sci. Technol.* 27(3): 33-42.

Lange, L. and Heide, M. 1986. Dot immuno binding (DIB) for detection of virus in seed. *Can. J. Plant Pathol.* 8: 373-379.

Langeveld, S.A., Dore, J.M., Memelink, J., Derks, A.F., van der Vlugt, C.I., and Asjes, C.J. 1991. Identification of potyviruses using the polymerase chain reaction with degenerate primers. *J. Gen. Virol.* 72: 1531-1541.

Lebas, B.S.M. 2002. Diversity of viruses infecting *Dioscorea* species in the South Pacific. Ph.D. thesis, University of Greenwich, Medway, p.45.

Lebot, V. 2009. *Tropical root and tuber crops: cassava, sweet potato, yams and aroids*. Crop Production Science in Horticulture Series No. 17, CABI Press, Wallingford, 413 p.

Lee, J.A., Choi, S.K., Yoon, J.Y., Hong, J.S., Ryu, K.H., and Lee, S.Y. 2007. Variation in the pathogenicity of lily isolates of *Cucumber mosaic virus*. *J. Plant Pathol.* 23: 251-259.

Li, W., Hilf, M.E., Webb, S.E., Baker, C.A., and Adkins, S. 2008. Presence of P1b and absence of HC-Pro in *Squash vein yellowing virus* suggests a general feature of the genus *Ipomovirus* in the family *Potyviridae*. *Virus Res.* 135: 213-219.

Liou, R.F., Yan, H.Z., and Hong, J.L. 2003. Molecular evidence that aphid-transmitted *Alpinia mosaic virus* is a tentative member of the genus *Macluravirus*. *Arch. Virol.* 148: 1211-1218.

López, M.M., Bertolini, E., Olmos, A., Caruso, P., Gorris, M.T., Llop, P., Penyalver, R., and Cambra, M. 2003. Innovative tools for detection of plant pathogenic viruses and bacteria. *Int. Microbiol.* 6: 233-243.

Luo, H. 2012. Using new tools to detect and characterize plant viruses. Ph.D. thesis, Murdoch University, Perth, Western Australia. 244p.

Makkowk, K.M., Hsu, H.T., and Kumari, S.G. 1993. Detection of three plant viruses by dot blot and tissue blot immunoassays using chemiluminescent and chromogenic substrates. *J. Phytopathol.* 139: 97-102.

Mandal, B., Vijayanandraj, S., Shilpi, S., Pun, K.B., Singh, V., Pant, R.P., Jain, R.K., Varadarasan, S., and Varma, A. 2012. Disease distribution and characterization of a new *Macluravirus* associated with chirke disease of large cardamom. *Ann. Appl. Biol.* 160: 225-236.

Martin, F.W. and Degras, L. 1978. *Dioscorea trifida*. In: *Tropical Yams and their Potential*. Part 5 of Agriculture Handbook No. 522. US Department of Agriculture, Washington, DC, USA, pp. 1-3.

Martin, R.R., James, D., and Levesque, C.A. 2000. Impacts of molecular diagnostic technologies on plant disease management. *Ann. Rev. Phytopathol.* 38: 207-239.

Matthews, R.E.F. 1991. *Plant Virology* (3rd Ed.). Academic Press Inc.. London. 835p.

McDonald, F.D., Alleyne, A.T., Ogarro, L.W., and Delauney, A.J. 1998. Yam anthracnose in the English-speaking islands of the Eastern Caribbean-successes and research advances in disease management. *Trop. Agric.* 75: 53-57.

Michael, K., Harder, T.C., Mettenleiter, T.C.. and Karger. A. 2009. Diagnosis and strain differentiation of avian influenza viruses by restriction fragment mass analysis. *J. Virol. Methods* 158: 63-69

*Miege, J. 1957. Influence de quelques caractères des tubercules semences sur la levée et le rendement des ignames cultivées. *J. d'Agric. Trop. Bot. Appl.* 4 (3): 15-34.

Mignouna, H.D., Dansi, A. 2003. Yam (*Dioscorea* spp.) domestication by the Nago and Fon ethnic groups in Benin. *Genet. Resour. Crop Evol.* 50: 519-528.

Mignouna, H.D., Mank, R.A., Ellis, T.H.N., van den Bosch, N., Asiedu, R., Abang, M.M., and Peleman, J. 2002a. A genetic linkage map of guinea yam (*Dioscorea rotundata* Poir.) based on AFLP markers. *Theor. Appl. Genet.* 105: 716-725.

Mignouna, H.D., Mank, R.A., Ellis, T.H.N., van den Bosch, N., Asiedu, R., Abang, M.M., and Peleman, J. 2002b. A genetic linkage map of water yam (*Dioscorea alata* L.) based on AFLP markers and QTL analysis for anthracnose resistance. *Theor. Appl. Genet.* 105: 726-735.

Mohan Kumar, G.N., Iyer, S., and Knowles, R.N. 2007. Extraction of RNA from fresh, frozen, and lyophilized tuber and root tissues. *J. Agric. Food Chem.* 55: 1674-1678.

Morel, C., Jacquemond, M., Moury, B., and Johansen, E. 2002. Evidence for diversifying selection in *Potato virus Y* and in the coat protein of other potyviruses. *J. Gen. Virol.* 83: 2563-2573.

Mowat, W.P., Dawson, S., Duncan, G.H., and Robinson, D.J. 1991. Narcissus latent, a virus with filamentous particles and a novel combination of properties. *Ann. Appl. Biol.* 119: 31-46.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., and Erlich, H. 1986. Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harb. Symp. Quant. Biol.* 51: 263-273.

Mumford, R.A. and Seal, S.E. 1997. Rapid single-tube immunocapture RT-PCR for the detection of two yam potyviruses. *J. Virol. Methods* 69: 73-79.

Murphy, J.F. and Bowen, K.L. 2006. Synergistic disease in pepper caused by the mixed infection of *Cucumber mosaic virus* and *Pepper mottle virus*. *Phytopathol.* 96: 240-247.

Muzac-Tucker, I., Asemota, H.N., and Ahmad, M.H. 1993. Biochemical composition and storage of Jamaican yams (*Dioscorea* sp.). *J. Sci. Food Agric.* 62: 219-224.

Ng, J.C.K. and Perry, K.L. 2004. Transmission of plant viruses by aphid vectors. *Mol. Plant Pathol.* 5: 505-511.

Nicholas, O. and Laliberte, J.F. 1991. The use of PCR for cloning of large cDNA fragments of *Turnip mosaic potyvirus*. *J. Virol. Methods* 32: 57-66.

Njukeng, A.P., Atiri, G.I., and Hughes, J.d'A. 2005. Comparison of TAS-ELISA, dot and tissue blot, ISEM and immunocapture RT-PCR assays for the detection of *Yam mosaic virus* in yam tissues. *Crop Prot.* 24: 513-519.

Njukeng, A.P., Atiri, G.I., Hughes, J.d'A., and Winter, S.S. 2004. Development of serological procedures for rapid, sensitive and reliable detection of *Yam mosaic virus* genus *Potyvirus* in yam tissues. *Trop. Sci.* 44: 136-147.

Nweke, F.I., Ugwu, B.O., Asadu, C.L.A., and Ay, P. 1991. *Production Costs in the Yam-Based Cropping Systems of South-Western Nigeria.* Resource and Crop Management Division. Research monograph No. 6, IITA, Ibadan, 29p.

Obidiegwu, J.E., Rodriguez, E., Ene-obong, E.E., Loureiro, J., Muoneke, C.O., Santos, C., Kolesnikova-allen, M., and Asiedu, R. 2009. Estimation of the nuclear DNA content in some representative of genus *Dioscorea*. *Sci. Res. Essay* 4: 448-452.

Odedara, O.O., Ayo-John, E.I., Gbuyiro, M.M., Falade, F.O., and Agbebi, S.E. 2011. Serological detection of yam viruses in farmers' fields in Ogun State, Nigeria. *Arch. Phytopathol. Plant Protect.* 45(7): 840-845.

Odu, B.O., Asiedu, R., Shoyinka, S.A., and Hughes, J.d'A. 2011. Analysis of resistance to *Yam mosaic virus,* genus *Potyvirus* in white guinea yam (*Dioscorea rotundata* Poir.) genotypes. *J. Agric. Sci.* 56(1): 1-13.

Odu, B.O., Hughes, J.d'A., Asiedu, R., Ng, N.Q., Shoyinka, S.A., and Oladiran, O.A. 2004. Responses of white yam (*Dioscorea rotundata*) cultivars to inoculation with three viruses. *Plant Pathol.* 53: 141-147.

Odu, B.O., Shoyinka, S.A., Hughes, J.d'A., Asiedu, R., and Oladiran, O.A. 2001. Yam viruses of Nigeria. In: Akoroda, M.O. and Ngeve, J.M. (eds), *Proceedings of 7th Triennial Symposium of the International Society for Tropical Root Crops – African Branch*. ISTRC-AB and Government of Benin, Benin, pp. 12-16.

O'Hair, S.K. 1990a. Tropical root and tuber crops. *Hortic. Rev.* 12: 157-196.

O'Hair, S.K. 1990b. Tropical root and tuber crops. In: Janick, J. and Simon, J.E. (eds), *Advances in New Crops*. Timber Press, Portland, USA, pp. 424-428.

Onwueme, I.C. 1984. Yam. In: Goldsworthy, P.R. and Fisher, N.M. (eds), *The Physiology of Tropical Field Crops*. John Wiley and Sons, Chichester, pp. 569-588.

Onwueme, I.C. 1978. *The Tropical Tuber Crops. Yams, Cassava, Sweet Potato and Cocoyams*. John Wiley and Sons, Chichester, 234p.

Onwueme, I.C. and Charles, W.B. 1994. *Tropical Root and Tuber Crops: Production, Perspectives and Future Prospects*. FAO, Rome, Italy, 288p.

Onyeka, T.J., Petro, D., Ano, G., Etienne, S., and Rubens, S. 2006. Resistance in water yam (*Dioscorea alata*) cultivars in the French West Indies to anthracnose disease based on tissue culture-derived whole-plant assay. *Plant Pathol.* 55: 671-678.

Opara, L.U. 1999. Yam storage. In: Bakker-Arkema (ed.), CIGR Handbook of Agricultural Engineering Volume IV Agro Processing. The American Society of Agricultural Engineers, St. Joseph, MI, pp. 182-214.

Osagie, A.U. 1992. *The yam tuber in storage.* Postharvest Research Unit, University of Benin, Benin City, 247 p.

Pappu, S.S., Brand, R., Pappu, H.R., Rybicki, E.P., Gough, K.H., Frenkel, M.J., and Niblett, C.L. 1993. A polymerase chain reaction method adapted for selective amplification and cloning of 3' sequences of potyviral genomes- application to *Dasheen mosaic virus*. *J. Virol. Methods* 41: 9-20.

Peroni, F.H.G., Rocha, T.S., and Franco, C.M.L. 2006. Some structural and physicochemical characteristics of tuber and root starches. *Food Sci. Tech. Int.* 12 (6): 505-513.

Perry, K.L., Zhang, L., and Palukaitis, P. 1998. Amino acid changes in the coat protein of *Cucumber mosaic virus* differentially affect transmission by the aphids *Myzus persicae* and *Aphis gossypii*. *Virol.* 242: 204-210.

Petro, D., Onyeka, T.J., Etienne, S., and Rubens, S. 2011. An intraspecific genetic map of water yam (*Dioscorea alata* L.) based on AFLP markers and QTL analysis for anthracnose resistance. *Euphytica* 179: 405-416.

Plese, M. and Milicic, D. 1973. Two viruses isolated from *Maclura pomifera*. *Phytopathol. Z.* 77: 178-183.

Plese, M. and Wrischer, M. 1978. Light and electron microscopy of cells infected with *Maclura mosaic virus*. *Acta Bot. Croat.* 37: 47-51.

Plese, M., Koenig, R., Lesemann, D.E., and Bozarth, R.F. 1979. *Maclura mosaic virus* - an elongated plant virus of uncertain classification. *Phytopathol.* 69: 471-475.

Purseglove, J.W. 1976. *Tropical Crops. Monocotyledons*. Longman Group Ltd., London, pp. 97-117.

Raccah, B. and Fereres, A. 2009. Plant virus transmission by insects. In: Harper, D. (ed.), *Encyclopedia of Life Sciences*. John Wiley and Sons Ltd., Chichester, p.9.

Ravi, V., Ked, J., and Balagopalan, C. 1996. Review on tropical root and tuber. In: Critical Reviews. *Food Sci. Nutr.* 36 (7): 360-730.

Reckhaus, P. and Nienhaus, F. 1981. Etiology of a virus disease of a white yam (*Dioscorea rotundata*) in Togo. *J. Plant Dis. Prot.* 88: 492-509.

Robertson, D.G. 1961. *Annual report 1959-1960*. Federal Department of Agricultural Research, Nigeria, p.21.

Rossel, H.W. and Thottappilly, G. 1985. *Virus Diseases of Important Food Crop in Tropical Africa.* International Institute of Tropical Agriculture, Ibadan, Nigeria. 67p.

Saiki, R.K., Sharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., Arnheim, N. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230: 1350-1354.

Salm, S.N., Rey, M.E.C., and Wolfson, M.M. 1994. A South African isolate of *Ryegrass mosaic virus. Plant Pathol.* 43: 709-712.

Scarcelli, N., Dainou, O., Agbangla, C., Tostain, S., and Pham, J.L. 2005. Segregation patterns of isozyme loci and microsatellite markers show the diploidy of African yam *Dioscorea rotundata* (2n = 40). *Theor. Appl. Genet.* 111: 226-232.

Scott, G.J., Best, R., Rosegrant, M., and Bokanga, M. 2000. Roots and tubers in the global food system: A vision statement to the year 2020 (including annex). A co-publication of the International Potato Center (CIP), Centro Internacional de Agricultura Tropical (CIAT), International Food Policy Research Institute (IFPRI), International Institute of Tropical Agriculture (IITA), and International Plant Genetic Resources Institute (IPGRI). International Potato Center, Lima, Peru, 111p.

Seal, S. and Muller, E. 2007. Molecular analysis of a full-length sequence of a new yam badnavirus from *Dioscorea sansibarensis*. *Arch. Virol.* 152: 819-825.

Sheffield, V.C., Cox, D.R., Lerman, L.S., and Myers, R.M. 1989. Attachment of a 40 base pair G+C rich sequence (GC-clamp) to genomic DNA fragments by polymerase chain reaction results in improved detection of single base changes. *Proc. Natl. Acad. Sci.* 86: 232-236.

Shirako, Y. and Ehara, Y. 1986. Rapid diagnosis of *Chinese yam necrotic mosaic virus* infection by electro-blot immunoassay. *Ann. Phytopathol. Soc. Japan* 52: 453-459.

Shukla, D.D., Ward, C.W., and Brunt, A.A. 1994. *The Potyviridae.* CAB International, Wallingford, UK, 516p.

Shukla, D.D., Ward, C.W., Brunt, A.A., and Berger, P.H. 1998. *Potyviridae Family.* AAB/CMI Description of Plant Viruses No. 366. Association of Applied

Biologists (AAB), Kew, Surrey, England. Available: http://www.dpvweb.net/dpv/showdpv.php?dpvno=366 [12 Mar. 2014].

Siqueira, M.V.B.M. 2011. Yam: a neglected and underutilized crop in Brazil. *Hortic. Bras.* 29: 16-20.

Siqueira, M.V.B.M., Dequigiovanni, G., Corazon-guivin, M.A., Feltran, J.C., and Veasey, E.A. 2012. DNA fingerprinting of water yam (*Dioscorea alata*) cultivars in Brazil based on microsatellite markers. *Hortic. Bras.* 30: 653-659.

Srinivas, T., Nedunchezhiyan, M., and Misra, R.S. 2012. *Marketing System of Aroids and Yams in India*. Technical Bulletin Series: 53. CTCRI, Thiruvananthapuram, 82p.

Stellwagen, A. and Stellwagen, N.C. 1990. Anomalously slow electrophoretic mobilities of DNA restriction fragments in polyacrylamide gels are not eliminated by increasing the gel pore size. *Biopolym.* 30: 309-324.

Stellwagen, N.C. 2009. Electrophoresis of DNA in agarose gels, polyacrylamide gels and in free solution. *Electrophoresis* 30: 188-195.

Stenger, D.C. and French R. 2004. Complete nucleotide sequence of *Oat necrotic mottle virus*: a distinct *Tritimovirus* species (family *Potyviridae*) most closely related to *Wheat streak mosaic virus*. *Arch. Virol.* 149: 633-640.

Susaimuthu, J., Tzanetakis, I.E., Gergerich, R.C., and Martin, R.R. 2008. A member of a new genus in the *Potyviridae* infects *Rubus*. *Virus Res.* 131: 145-151.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30: 2725-2729.

Tatineni, S., Ziems, A.D., Wegulo, S.N., and French, R. 2009. *Triticum mosaic virus*: a distinct member of the family *Potyviridae* with an unusually long leader sequence. *Phytopathol.* 99: 943-950.

Tattersall, E.A.R., Ergul, A., AlKayal, F., DeLuc, L., Cushman, J.C., and Cramer, G.R. 2005. Comparison of methods for isolating high-quality RNA from leaves of grapevine. *Am. J. Enol. Vitic.* 56 (4): 400-407.

Terry, E.R. 1976. Incidence, symptomatology, and transmission of a yam virus in Nigeria. In: Cock, J., McIntyre, R., and Graham, M. (eds), *Proceedings of the 4th Symposium of the International Society for Tropical Root Crops.* CIAT, Cali, Columbia, pp. 170-173.

Thomson, K.G., Dietzgen, R.G., Gibbs, A.J., Tang, Y.C., Liesak, W., Teakle, D.S., and Stackebrandt, E. 1995. Identification of *Zucchini yellow mosaic potyvirus* by RT-PCR and analysis of sequence variability. *J. Virol. Methods* 55: 83-96.

Thouvenel, J.C. and Fauquet, C. 1986. *Yam mosaic virus*. AAB/CMI Description of Plant Viruses No. 314. Association of Applied Biologists (AAB), Kew, Surrey, England, p.4.

Thouvenel, J.C. and Fauquet, C. 1979. Yam mosaic, a potyvirus infecting *Dioscorea cayenensis* in the Ivory Coast. *Ann. Appl. Biol.* 93: 279-283.

Tochihara, H. 1993. Yams. In: Tsuchizaki, T., Tochihara, H., Kameya, M., and Yanase, H. (eds), *Plant Virus Diseases in Japan*. Zenkoku Noson Kyoiku Kyokai, Tokyo, pp. 431-435.

Toyohara, H., Irie, K., Ding, W., Iwata, H., Fujimaki, H., Kikuchi, F., and Ukai, Y. 2000. Evaluation of tuber shape of yam (*Dioscorea alata* L.) cultivars by image analysis and elliptic Fourier descriptors. *J. Breed. Genet.* 32(1): 31-37.

Tschannen, A.T., Girardin, O., Nindjin, C., Dao, D., Farah, Z., Stamp, P., and Escher, F. 2003. Improving the application of gibberellic acid to prolong dormancy of yam tubers (*Dioscorea* spp.). *J. Sci. Food Agric* 83: 787-796.

Turina, M., Ciuffo, M., Lenzi, R., Rostagno, L., Mela, L., Derin, E., and Palmano, S. 2006. Characterization of four viral species belonging to the family *Potyviridae* isolated from *Ranunculus asiaticus*. *Phytopathol.* 96: 560-566.

Ulbricht, C., Basch, E., and Sollars, D. 2003. Wild yam. *J. Herb. Pharmacother.* 3(4): 77-91.

Voloudakis, A.E., Malpica, C.A., Aleman-Verdaguer, M.E., Stark, D.M., Fauquet, C.M., and Beachy, R.N. 2004. Structural characterization of *Tobacco etch virus* coat protein mutants. *Arch. Virol.* 149: 699-712.

Walkey, D.G.A. 1991. *Applied Plant Virology*. Chapman and Hall. London, 338p.

Walsh. S. 2003. *Plant Based Nutrition and Health*. Vegan Society Ltd., Toledo. 240p.

Wanasundera, J.P.D. and Ravindran, G. 1994. Nutritional assessment of yam (*Dioscorea alata*) tubers. *Plant Foods Hum. Nutr.* 46 (1): 33-39.

Wang, J.G., Zou, X.J., Zheng, H.Y., Adams, M.J., Chen, H.R., and Chen, J.P. 2009. Molecular characterization of a new *Macluravirus* from yam in Yunnan, China. *Arch. Virol.* 154: 1379-1380.

Wang, W., Liang, G.L., and Xie, J.H. 2007. A study of the methods for total RNA extraction from different tissues of banana. *J. Southwest China Normal Univ.* 32(1): 62-68.

Ward, C.W., Weiller, G., Shukla, D.D., and Gibbs, A.J. 1995. Molecular systematics of the *Potyviridae*, the largest plant virus family. In: Gibbs, A.J., Calisher, C.H.,

and Garcia-Arena, F. (eds), *Molecular Basis of Virus Evolution.* Cambridge University Press, Cambridge, pp. 477-500.

Webster, C.G., Coutts, B.A., Jones, R.A.C., Jones, M.G.K., and Wylie, S.J. 2007. Virus impact at the interface of an ancient ecosystem and a recent agroecosystem: studies on three legume-infecting potyviruses in the southwest Australian floristic region. *Plant Pathol.* 56: 729-742.

Wilson, E. 1982. Recent developments in the propagation of yam (*Dioscorea* spp.). In: Miege, J. and Lyonga, S.N. (eds), *Yams – Ignames.* Oxford Science Publications, pp. 55-59.

Woolfe, J.A. 1987. *The Potato in the Human Diet.* Cambridge University Press, Cambridge, UK, 231p.

Wylie, S.J., Coutts, B.A., Jones, M.G.K., and Jones, R.A.C. 2008. Phylogenetic analysis of *Bean yellow mosaic virus* isolates from four continents: relationship between the seven distinct groups found and their natural isolation hosts and geographical origins. *Plant Dis.* 92: 1596-1603.

Yalow, R.S. and Berson, S.A. 1960. Immunoassay of endogenous plasma insulin in man. *J. Clin. Invest.* 39: 1157-1175.

Zanotto, P.M.deA., Gibbs, M.J., Gould, E.A., and Holmes, E.C. 1996. A re-evaluation of the higher taxonomy of viruses based on RNA polymerases. *J. Virol.* 70: 6083-6096.

Zerbini, F.M., Koike, S.T., and Gilbertson, R.L. 1995. Biological and molecular characterization of *Lettuce mosaic potyvirus* isolates from the Salinas valley of California. *Phytopathol.* 85: 746-752.

Zheng, T., Chen, J., Chen, J.P., and Adams, M.J. 2002. The complete sequence of *Oat mosaic virus* and evidence for deletion and duplication in RNA2. *Arch. Virol.* 147: 635-642.

Zhou, Q., Shi, W., Meng, X., and Liu, Y. 2013. Studies on the morphological, crystalline, thermal properties of an underutilized starch from yam *Dioscorea zingiberensis* C.H. Wright. *Starch* 65: 123-133.

Zinsou, C. 1998. In: Berthaud J., Bricas, N., and Marchand J.L. (eds), *Actes du Séminaire International surl'igname*. Montpellier, France, pp. 213-222.

Žižytė, M., Staniulis, J., and Zitikaitė, I. 2006. Identification of *Beet necrotic yellow vein virus* isolate detected in Lithuania. *Agron. Res.* 4: 475-478.

Zoundjihekpon, J., Hamon, S., Tio-Toure, B., and Hamon, P. 1994. First controlled progenies checked by isozymic markers in cultivated yams *Dioscorea cayenensis- rotundata*. *Theor. Appl. Genet.* 88: 1011-1016.

* Original not seen

APPENDICES

APPENDIX I

DAS-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 1 l. 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 l^{-1}

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.

DIBA

Antigen Extraction buffer

1 l TBS containing 50 m*M* DIECA (Add 11.25 g DIECA to TBS)

TBS (Tris Buffer Saline) pH -7.5 (5 X)

Tris - 1.210 g

NaCl - 14.61 g

Adjust pH to 7.5 with HCl and make up to 100 ml with distilled water.

1 X TBS (pH 7.5)

Tris - 0.02 *M*

NaCl - 0.5 *M*

Adjust pH to 7.5 with HCl

Blocking solution

Add 5 g of spray dried milk (SDM) to 100 ml TBS (5% SDM in TBS)

APPENDIX II

MECHANICAL INOCULATION

Yam buffer

0.1 *M* Phosphate buffer (pH 7.7)

DIECA

Preparation of Phosphate buffer

0.1 *M* KH_2PO_4

0.1 *M* K_2HPO_4

Mix 10 ml of A and 90 ml of B to get 0.1 *M* Phosphate buffer (pH 7.7)

APPENDIX III

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.

MOHAN KUMAR *et al.* PROTOCOL

Borate Tris Buffer (0.2 *M*, pH 8.0 containing 10 m*M* EDTA)

Tris - 54 g

Boric acid - 27.5 g

EDTA (0.5*M*) - 20 ml

Dissolve in 900 ml of distilled water; adjust pH to 8.0 with HCl and make up to 1 l. Autoclave and store.

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 (2%)

Agarose - 2 g

1 X TAE Buffer -100 ml

Ethidium Bromide (10 mg ml^{-1})

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

APPENDIX IV

IC-RT PCR

Carbonate buffer/ coating buffer (pH 9.6)

Na_2CO_3 - 1.59 g

$NaHCO_3$ - 2.93 g

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

APPENDIX V

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.

APPENDIX VI

5% Acrylamide gel

Water	-8.8 ml
30% Acrylamide mix	-2.5 ml
1.5M Tris (pH 8.8)	-3.74 ml
10% APS	-0.15 ml
TEMED	-0.03 ml

ABSTRACT

DETECTION AND CHARACTERIZATION OF *Macluravirus* INFECTING GREATER YAM (*Dioscorea alata* L.)

by

MANASA V. G.

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ABSTRACT

Yams constitute a group of *Dioscorea* species cultivated mainly in Asia, Africa and South America for their edible, underground tubers. Viral pathogens are one of the most important factors threatening production of this vegetatively propagated crop. Yam viruses are poorly characterized, which is a hindrance to the safe movement of germplasm. The overall aim of this study was to detect *Macluravirus* infecting greater yam and to characterize the virus at molecular level. Serological and nucleic acid based methods were employed for the detection of *Macluravirus* infecting greater yam, which was not previously detected in India. Analysis of greater yam leaf and tuber samples by double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA), dot immunobinding assay (DIBA) and reverse transcription polymerase chain reaction (RT-PCR) followed by sequence analysis revealed the occurrence of *Macluravirus*. Novel species specific primers were developed to amplify the full coat protein gene of the virus. Sequence and phylogenetic analyses, based on the entire CP- coding region revealed considerable variability and the virus was found to be more similar to *Chinese yam necrotic mosaic virus* (ChYNMV) than other viruses of the genus *Macluravirus*. The virus coat protein gene exhibited only 65 to 70% nucleotide sequence identity with other macluraviruses indicating that it is putative new species. Hence the name *Yam macluravirus* is proposed for the new sequence reported. Restriction digestion with *Mse*1 enzyme followed by DNA polyacrylamide gel electrophoresis (DNA PAGE) showed presence of intraspecific variation within the virus isolates obtained from CTCRI field. Mechanical inoculation and insect transmission of the virus on *Nicotiana benthamiana, N. tabaccum* and *Vigna unguiculata* plants yielded negative results. These results will be useful for reliable virus indexing to facilitate the safe movement of greater yam planting material and exchange of germplasm within and outside India.