Epidemiology, Diagnostics and Molecular Studies of Yam Viruses in Ghana, Togo and Benin

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A thesis submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy

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DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the Degree of Doctor of Philosophy in the School of Cell and Molecular Biology at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

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This_____day of _____20_____

ABSTRACT

Yam (*Dioscorea* spp.) is one the most important food crops cultivated in the West African yam zone comprising Cameroon, Côte d'Ivoire, Ghana, Nigeria, Benin and Togo, which account for over 90% of the 4.59 million hectares of yam cultivation worldwide. Viral pathogens are amongst the most important factors threatening yam production and productivity and safe movement of germplasm. *Cucumber mosaic virus* (CMV, genus *Cucumovirus*), *Dioscorea bacilliform alata virus* (DaBV, genus *Badnavirus*), *Yam mosaic virus* (YMV, genus *Potyvirus*) and *Yam mild mosaic virus* (YMMV, genus Potyvirus) have been reported to infect yam in West Africa. More recently a second bacilliform virus, *Dioscorea sansibarensis bacilliform virus* (DsBV), isolated from *Dioscorea sansibarensis* in Benin was characterized. DaBV and DsBV are serologically related. Besides, occurrence of several uncharacterized isometric, flexuous rod shaped and bacilliform viruses have been described in the literature. Information on the occurrence and distribution of yam viruses is limited to Nigeria and Côte d'Ivoire. In this study, surveys were conducted to document viruses occurring in major yam producing zones in Benin during 2004 and 2005, and in Ghana and Togo during 2005. Analysis of a total of 1632 yam leaves obtained from these surveys by enzyme-linked immunosorbent assay (ELISA), immunocapture polymerase chain reaction (IC-PCR) and/or IC-reverse transcription-PCR (IC-RT-PCR) revealed the occurrence of CMV, YMV, YMMV, yam-infecting badnaviruses (DaBV and/or DsBV), but not Dioscorea mottle virus (DMoV, Como-like virus). Incidence of the yam-infecting badnaviruses was highest, followed by YMV and YMMV. Incidence of CMV was lowest, but this finding is the first official report of

its occurrence in these countries. Polyclonal antibodies against a yam isolate of CMV and an isolate of yam-infecting badnavirus from a *D. alata* in Nigeria were produced in rabbits. CMV antisera had an end-point titre of 1:25,600 and 1:64,000 by PAS- and ACP-ELISA, respectively; whereas badnavirus antisera had a titre of 1:1280 using both the methods. The antibodies of each virus detected homologous antigen in infected yam leaves from Ghana, Togo, Benin and Nigeria. Diversity among 19 badnavirus isolates representing diverse production zones in Ghana, Togo, Benin, and Nigeria was assessed by analyzing nucleotide sequence of partial RT/RNaseH-coding region. Homology between the deduced amino acid sequences of the 19 isolates ranged from 73 to 100%. Phylogenetic analysis revealed that the badnavirus isolates analyzed in this study correspond to two distinct species, *Dioscorea alata bacilliform virus* (DaBV) and *Dioscorea sansibarensis bacilliform virus* (DsBV). One isolate from Benin had 77% and 75% amino acid identities with DaBV and DsBV, respectively and may represent a third species. This result suggests the occurrence of three different bacilliform viruses in yam in West Africa. Information on the aetiology, geographical distribution and variability of viruses naturally occurring in yam in Ghana, Benin and Togo and the diagnostic tools developed in this study would enhance germplasm monitoring and disease control efforts.

DEDICATION

To my husband Ukpabi Eni for his love and support and to the memory of my father late Chief Julius Nnabuenyi Nzekwe

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ABBREVIATIONS

%	Percent
µg	Microgram
µl	Microliter
aa	Amino acid
A	Absorbance
cm	Centimeter
CMV	*Cucumber mosaic viru*s
DaBV	*Dioscorea alata bacilliform*
DMoV	*Dioscorea mottle virus*
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide phosphates
DsBV	*Dioscorea sansibarensis bacilliform*
ELISA	Enzyme-linked immunosorbent assay
et al.	And others
g	grams
h	hour(s)
IgG	Immuno-γ-globulin
IITA	International Institute of Tropical Agriculture
JYMV	*Japanese yam mosaic virus*
kb	Kilo base
kDa	Kilo Dalton
l	Liter
M	Moles
mg	Milligram
min	minutes
ml	Milliliter
mM	Millimoles
nm	Nanometer

^{o}C	Degree centigrade
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pH	Hydrogen ion concentration
rpm	Revolutions per minute
RT-PCR	Reverse transcription-polymerase chain reaction
sec	Seconds
TAS-ELISA	Triple antibody sandwich-ELISA
v	Volume
w	Weight
YMMV	*Yam mild mosaic virus*
YMV	*Yam mosaic virus*

CHAPTER 1 – GENERAL INTRODUCTION

1.1 Introduction

Yam is a common name for several species of the genus *Dioscorea* (family *Dioscoreaceae*) used for food purposes. They are annual or perennial herbaceous vines with edible underground tubers and are the world's second most important tuber crop (Nweke *et al*., 1991). Yam is cultivated on 4.59 million hectares worldwide with a world production of 51.4 million tonnes (mt) in 2006 (FAO 2007). Tubers of domesticated yam are an important source of carbohydrate for millions of people in the tropics and sub-tropical regions of 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 (FAO 2000; Hou *et al*., 2002).

Yam can be stored longer than most other tropical fresh products, and therefore stored yam represents stored wealth. Furthermore, yam has been identified as a crop with potential for increased commercial exploitation and processing (O'Hair, 1990). However, 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 vigour and subsequently cause a reduction in tuber yield and quality (Coursey, 1967b; Amusa *et a*l., 2003).

Yam breeding and selection work which aims to collect, conserve and utilise the genetic diversity of yam for the benefit of people throughout the world, particularly in

sub-Saharan Africa (SSA), and the use of tissue culture techniques for mass production of vegetative planting materials, have resulted in an increased exchange of planting materials internationally. The international movement of germplasm involves the risk of accidentally introducing pests of quarantine importance with the plant material. Pathogens such as viruses pose serious risks and may restrict the international movement of improved or selected germplasm due to quarantine restrictions (Hughes *et al*., 1998; Amusa *et a*l., 2003).

Natural transmission of viruses is mainly through infected planting materials, by vectors and through mechanical transmission (Thouvenel and Fauquet 1986; Brunt *et al*., 1989; Matthews, 1991). Yam is propagated vegetatively by the use of tubers (seed yam or planting setts) from previous year's harvest, except for breeders who require true botanical seeds for genetic improvement work (Akoroda, 1985). This results in an accumulation of viruses over the years since yam viruses are transmitted through planting materials (Brunt *et al*., 1989). Use of infected planting materials is therefore a major means of spread of yam viruses.

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 (Ng, 1992). Both of these methods greatly depend on the availability of

sensitive and specific virus diagnostic tools for virus monitoring in plants and plant parts. Effective diagnostic tools are crucial to minimize the risk of yam virus spread and to ensure that distributed planting materials are tested and certified negative for known viruses.

1.2 The yam plant

1.2.1 Taxonomy and morphology of yam

Yam belongs to the order Liliflorae, family *Dioscoreaceae*, genus *Dioscorea*. About 600 species of yam have been identified (Coursey, 1967a; Burkill, 1960). Yam tubers, which are enlarged storage organs containing food reserves in the form of starch, vary greatly in size ranging from small potato size to over 2.5 m in length, and may weigh up to 7.5 kg (RHS, 1992). They are round, cylindrical, oval or flattened (Toyohara *et al*., 2000) and are usually produced underground (Figure 1.1). 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 *D. 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).

Figure 1.1 A Tongan farmer showing off his prize yams Source: (James Foster, 1986)

Yam shoots are climbing and vine-like thus usually requiring support from either neighbouring plants or stakes on which they twine clockwise or anticlockwise depending on the specie (Johnson, 2003). 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 seed is 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.

1.2.2 Origin and distribution of yam

Yam is believed to have originated in Asia and to have been carried across the savannah to West Africa by Malaysians populating Madagascar in the second century AD. From West Africa, yam was brought across the Atlantic to the Caribbean during the slave trade in the late 1450s (Johnson, 2003). Evidence indicates that different yam species originated in three independent areas of the tropics: Asia, West Africa and tropical America. *Dioscorea alata* L. and *D. esculenta* (Lour) Burk originated from Asia and were introduced to West Africa. *D. rotundata* Poir, *D. cayenensis* Lam., and *D. dumetorum* Pax. originated from West Africa and are among the few truly West African domesticated plants. *D. trifida* L. originated from tropical America (Coursey, 1967a; Ayensu and Coursey, 1972; Alexander and Coursey, 1969; Hahn *et al*., 1987; IITA, 1995). In general, there was an eastward movement of yam species during the domestication process (Hahn *et al*., 1987). Of the 600 known species of yam, only ten species are cultivated for their edible tubers (Coursey, 1967a; Malaurie *et al*., 1998). The most common food yams are *D. rotundata* (white yam), *D. alata* (water yam), *D. cayenensis* (yellow yam), *D. dumetorum* (trifoliate yam), *D. bulbifera* L. (aerial yam) and *D. esculenta* (Chinese yam). These account for over 90% of all the food yams grown in the tropics (Hahn *et al*., 1987). Less common

species are *D. hispida* Dennst., *D. opposita* Thunb., *D. japonica* Thunb., and the cush-cush yam *D. trifida* (Coursey, 1967a; Ikotun, 1989).

1.2.3 Yam production, cultivation, composition, uses and consumption

Production

Yam is one of the most important staple starchy food crops of the tropics, and is particularly important in West Africa, where approximately 93% of the world's annual yam production of about 51.4 mt (FAO 2007) is produced. The remaining 7% of the world's annual yam production is grown in other parts of Africa, the West Indies and parts of Asia, South and Central America and the Pacific. Over four million hectares of land is cultivated worldwide annually for yam production. The 'yam belt' of West and Central Africa lies between latitudes 25°N and 15°S and is restricted peripherally to the forest and savannah areas of Nigeria, Ghana, Côte d'Ivoire, Cameroon, Benin and Togo (FAO, 2000). Major yam producing countries in the world are listed in table 1.1.

Cultivation

Knowledge of the special cultural practices peculiar to yam is required for its successful cultivation. 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).

Table 1.1 Ten major yam producing countries in the world ranked by 2006 production data (FAO, 2007)

Country	**Production Quantity (Tonnes)**	**Area Harvested (Hectares)**
Nigeria	36720000	3035000
Côte d'Ivoire	4800000	540000
Ghana	3600000	300000
Benin	2239757	195747
Togo	621055	60246
Central African Republic	350000	58000
Colombia	332862	28011
Cameroon	300000	40000
Brazil	236325	25736
Chad	230000	24000

Yam requires a fairly high level of soil fertility and well drained soil of pH 6-7. A minimum of 1000 mm rainfall or an equivalent amount of irrigation water is required over the 5-6 months during the aerial growth stage (Hahn *et al*., 1987; Osagie, 1992). Land preparation for yam cultivation in West Africa begins with clearing of the vegetation. Mounds are prepared after clearing from early December to about mid

March. Mounds are 0.6-3 m in length and 0.9-1.3 m apart. These operations are completed before the onset of the major rains. Farmers normally plant whole yam called seed yam or cut pieces of yam, which are referred to as setts. Cut pieces used for planting usually have at least 2-3 'eyes' (buds) to ensure sprouting. Cut surfaces of the pieces (setts) are allowed to dry before planting and are sometimes treated with fungicides. They are planted with the cut surface tilted up at an angle of 45º. Planting is done by making a hole of about a third to half way down the mound and the seed yam or sett placed in it after which the hole is filled with soil.

Once planted, the yam remains dormant for some time or may sprout and send out a bare vine which will develop leaves when the rains come. After sprouting, the vines are sometimes trained onto stakes for adequate exposure of leaves to sunlight. Mounds are sometimes destroyed after heavy rains and may need to be re-shaped. Weeding is required and can be done manually with cutlasses and hoes, or by use of herbicides. The tubers have a large sink capacity and continue to grow and store food reserves throughout the year as long as conditions remain favourable. After harvest, yam tubers are stored in barns and have relatively long storage life of 4–6 months at a mean ambient temperature of 25°C.

Composition

Yam tubers are comprised of approximately 75.6-83.3 % carbohydrate, 3-7.4 % protein 0.5 -1.5% fibre, 0.7-2.0% ash, and 0.05-0.02% fat. A large proportion (65-75%) of the yam tuber is made up of water (Oyenuga, 1968; Francis *et al*., 1975;

Bell, 1983; Degras, 1993; Wanasundera and Ravindran, 1994; Opara, 1999). They are a starchy staple food, rich in carbohydrates and are also valuable sources of some vitamins, particularly vitamin C. Yam tubers contain about 13-24.7 mg/100g ascorbic acid and most of it is retained during cooking (Coursey, 1969; Wanasundera and Ravindran, 1994). They also very good source of minerals and are high in dietary fibre, vitamin B6, potassium and manganese and low in saturated fat, sodium and cholesterol (Omonigho, 1988; Wanasundera and Ravindran, 1994; Walsh, 2003). The high potassium and low sodium content of yam produces good potassium-sodium balance in the human body and so protect against osteoporosis and heart disease (Walsh, 2003). Yam products are also reported to have lower glycemic index than potato products. Slow break down of carbohydrates and gradually release of glucose into the blood stream means that they will provide more sustained form of energy, and give better protection against obesity and diabetes (Brand-Miller *et al*., 2003; Holford, 2008).

Uses

Yam is important for food, income and socio-cultural activities in many countries in West Africa, South-East Asia, the Pacific and Caribbean islands and part of Brazil (Nweke *et al*., 1991; FAO 2000). Yam may be boiled, baked, mashed, roasted or fried and eaten with stew made with vegetables, fish or meat. In West Africa, yam is often pounded into a thick paste ('fufu') after boiling and is eaten with soup (Omonigho, 1988) or processed into flour; the flour is cooked in boiling water to make a paste called 'Amala' or 'Kokonte' and is also eaten with stew. Yam peels are used for

traditional animal feed as a source of carbohydrate and wild species make up part of the diet of omnivorous and herbivorous burrowing animals in the humid tropics (Degras, 1993).

In the yam-producing areas of West Africa, many important cultural values are attached to the yam (Purseglove, 1992; Degras 1993), especially during weddings and other social and religious ceremonies. The Yam Harvest is a traditional festivity with masquerade dancing in the villages and prayers offered to thank the ancestral gods for blessings on the land (Degras, 1993).

In addition to their very important use as human food and their ceremonial significance, some wild species of yam such as *D. villosa* are known to 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 (Komesaroff *et al*., 2001). Other wild species are cultivated for extraction of diosgenin, a female hormone precursor used in the manufacture of contraceptive pills and sex hormones (Coursey, 1967a; Eka, 1985; Kay, 1987 Ulbricht *et al*., 2003). Another species, the cinnamon vine *D. batatas* Deene., is cultivated as a decorative plant and other wild species are also of ornamental value (Komesaroff *et al*., 2001; Hou *et al*., 2002)

1.2.4 Constraints to yam production

The major constraints to yam production are the lack of availability and cost of planting materials, pests and diseases, weeds, cost of labour, and storage problems (Wilson, 1982; Degras, 1993). Use of edible yam tubers as planting materials constitutes very serious problems for low income farmers since the tubers are needed for consumption or for sale (Akoroda and Hahn, 1995).

1.2.5 Diseases and pests of yam

Yam is susceptible to a variety of pest and diseases during growth as well as post-harvest. Diseases and pests of yam have been reported in most yam growing areas and they constitute a major constraint to yam production and storage (Degras, 1993; Mantell, 1993). Over 25% of yield losses are due to diseases and pests (Ezeh, 1998; FAO, 1998). Important yam pests include nematodes, beetles, termites, weevils, scale insects and rodents (Onwueme, 1978; Onwueme and Sinha, 1991; Ng, 1992; Purseglove, 1992; Onwueme and Charles, 1994; Emehute, 1998). Diseases include those caused by fungi, bacteria and viruses (Nwakiti and Arene 1978; Degras, 1993; Hughes *et al*., 1997).

The major insects known to attack 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 greater yam beetle, *Heteroligus meles* Billberg, is widespread in tropical Africa while the lesser yam beetle, *H. appius* Burmeister,

occurs in southern Nigeria (Coursey, 1967a). The yam scale insect, *Aspidiella hartii* Cockerell, infests tubers, and sometimes foliage, causing poor growth (Akinlosotu and Kogbe, 1986). Stored tubers are particularly susceptible to attack and large numbers of scale insects cause shrivelling. Scales insects are widespread in Africa, Asia, Central America, the Pacific islands and the West Indies (CABI, 1966).

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 (Bridge, 1972; Caveness, 1982; Caveness, 1992). Various *Pratylenchus* spp. are also known to infest yam and have been reported in Puerto Rico and in several countries of the South Pacific (Kermarreca *et al*., 1991). Nematodes cause lesions beneath the tuber skin which are yellow at first, developing into dark brown dry rots which may cover the tuber surface in heavily infested tubers. Infection often starts before harvest and continues in storage, leading to a loss of food and planting material for the next season's crop (Siddiqi, 1972). Major sources of losses in storage can be attributed to an interaction between nematodes (*S. bradys, Meloidogyne* spp. and *Pratylenchus* spp.), fungi and bacteria, and moderated by physical factors of the environment such as temperature and humidity (Bridge, 1972).

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 infecting yam worldwide (Adebanjo and Onesirosan, 1986). Brown spots occur on young leaves then enlarge,

and sometimes coalesce, as leaves approach maturity. Epidemics occur during prolonged rains. Young plant growth is infected and destroyed by rapidly expanding black lesions, and mature leaves of anthracnose-susceptible varieties rapidly blacken in response to sunlight and the presence of numerous *C. gloeosporioides* spores, which germinate (Winch *et al*., 1993). Other fungal diseases of yam are leaf spot and leaf blight caused by *Curvuloria* spp. (IITA, 1995; Onwueme, 1978).

1.3 Yam viruses

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 *D. rotundata* in Nigeria (Craige, 1964; 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 (Thouvenel and Fauquet, 1979; Goudou-Urbino *et al*., 1996a; Goudou-Urbino *et al*., 1996b; Hughes *et al.*, 1997; Aleman *et al*., 1997; Phillips *et al*., 1999; Odu *et al*., 2004; Seal and Muller 2007). Viruses infecting yam are in the *Potyvirus, Badnavirus, Potexvirus* and *Cucumovirus* genera. Symptoms associated with yam virus diseases include severe leaf chlorosis, green vein banding, mosaic, shoe stringing, 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 (Thouvenel and Dumont, 1988; Odu *et al*., 2001).

1.3.1 Family: Potyviridae, Genus: *Potyvirus*

The most completely characterized yam viruses are the potyviruses; they are also reported to be the most widespread (Hughes *et al*., 1997; Brunt *et al*., 1996; Kenyon *et al*., 2001). *Yam mosaic virus* (YMV), which is the most fully characterized yam virus, was first reported infecting *D. cayenensis* in Côte d'Ivoire and has been reported to be widespread in *D. rotundata* in Africa (Thouvenel and Fauquet, 1979; Goudou-Urbino *et al*., 1996a). YMV is also known to infect other yam species (Bousalem *et al*., 2000b; Goudou-Urbino *et al*., 1996b). The virus is a single-stranded RNA virus known to be transmitted both mechanically and by a wide range of aphid vectors (Thouvenel and Fauquet, 1979; Odu *et al*., 2004). Based on monoclonal antibodies reactivity, YMV isolates has been divided into two serogroups and into four groups based on eletrophoretic mobility by Western immunoblotting assay (Goudou-Urbino *et al*., 1996b). The complete nucleotide sequence of YMV Côte d'Ivoire isolate has been published (Aleman *et al*., 1996) and further sequence analyses of other YMV isolates and other potyviruses have revealed the presence of consensus motifs characteristic of the potyvirus genus supporting the classification of YMV as a potyvirus (Aleman *et al*., 1996; Duterme *et al.*, 1996). Genetic variability among YMV isolates is reported to be high and has been attributed to recombination events and the differential accumulation of mutations (Aleman *et al*., 1997; Bousalem *et al*., 2000b). Several potyviruses that are related to YMV both in host range and serologically has been described, YMV is synonymous with Dioscorea green banding mosaic virus (Reckhaus and Nienhaus, 1981) and YV-N, (Terry, 1976).

Yam mild mosaic virus (YMMV) was originally described as yam virus 1 (YV1) (Hughes, 1986) and subsequently as Dioscorea alata virus (DAV) (Odu *et al*., 1999). Mumford and Seal (1997) provided the first molecular evidence for the classification of YMMV as a distinct potyvirus. Analysis of the nucleotide sequence of the coat protein gene of YMMV revealed that it differs substantially from both YMV and *Japanese yam mosaic virus* (JYMV) (Fuji *et al*., 1999). It has a coat protein molecular weight of 32,100 daltons and is transmitted by *Aphis craccivora* Koch (Odu *et al*., 1999). YMMV has been reported in *D. alata* from Africa (Hughes *et al*., 1997; Odu *et al*., 1999), Asia and Oceania (Hughes, 1986; Mumford and Seal, 1997; Fuji *et al*., 1999) and has been described as the second most prevalent virus in yam after YMV (Atiri *et al*., 2003).

JYMV, a single stranded RNA virus, was first isolated from *D. japonica* in Japan in 1974 and reported as a strain of YMV (Okuyama and Saka, 1978). Infected plants show mosaic and green vein banding symptoms similar to those induced by YMV, but comparative studies of the genomic nucleotide sequence of JYMV and YMV (Côte d'Ivoire isolate) have revealed considerable differences, indicating that JYMV is a different potyvirus species (Aleman *et al*., 1996; Fuji and Nakamae, 1999). A mild strain of JYMV, which causes very mild or no symptoms at all, has also been described and its complete genomic nucleotide sequence published. It is used for cross protection against the severe strain (Fuji and Nakamae, 2000).

1.3.2 Family: Caulimoviridae, Genus: *Badnavirus*

Badnaviruses possess circular double stranded DNA genome encapsidated in non-enveloped bacilliform particles (Fauquet *et al*., 2005). They are typically 7.3 -8.0 kbp in size (Briddon *et al*., 1999). Bacilliform virus particles of approximately 130 x 29-30 nm in size have been associated with *Internal brown spot bacilliform virus* (IBSV) and *Dioscorea alata bacilliform virus* (DaBV) (Brunt *et al*., 1996; Phillips *et al*., 1999). DaBV is the more common of these two and is reported to infect mostly *D. alata*. It is transmitted by the mealybug, *Planococcus citri* Risso and is also mechanically-transmissible (Phillips *et al*., 1999; Odu *et al*., 2004). DaBV causes severe leaf distortion, crinkling and mottling in infected *D. alata* and is reported to be widely distributed (Phillips *et al*., 1999; Briddon *et al*., 1999). Internal brown spot disease of yam is believed to result from a mixed infection caused by a bacilliform virus particle and a potyvirus (Harrison and Roberts, 1973; Mohamed and Mantell, 1978). It was first reported in *D. alata* cv. White Lisbon from Barbados (Harrison and Roberts, 1973) and later in *D. cayenensis* (Mohamed and Mantel, 1976). The complete nucleotide sequences of a Nigerian isolate of DaBV and a bacilliform virus isolated from *D. sansibarensis* from Benin, DsBV, have been analysed (Briddon *et al*., 1999; Seal and Muller 2007). The genome sizes of DaBV and DsBV were shown to be approximately 7.4 kb and 7.26 kb, respectively, with 61.9 % nucleotide identity. Compared with other previously analysed badnaviruses, both viruses were found to share the highest level of nucleotide identity with *Cocoa swollen shoot virus* (CSSV) (Briddon *et al*., 1999; Seal and Muller 2007). Three and four open reading frames (ORF) were identified for DaBV and DsBV respectively. The predicted amino acid

sequence of ORF 3 of both viruses encodes a putative polyprotein which contains several conserved motifs characteristic of caulimoviruses and badnaviruses (Figure 1.2) (Medberry *et al*., 1990; Bouhida *et al*., 1993; Briddon *et al*., 1999; Seal and Muller 2007).

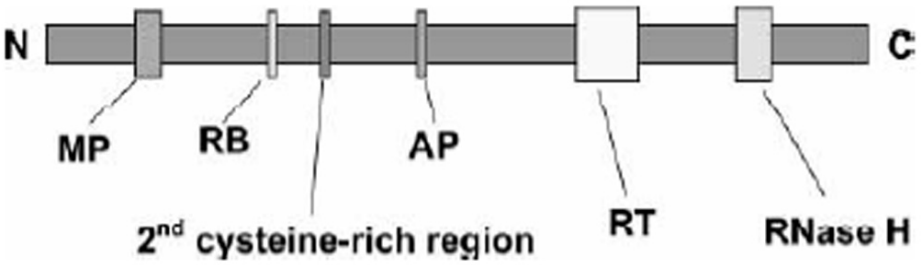

Figure 1.2 Approximate locations of motifs in the amino acid sequence of badnavirus ORF 3. MP = movement protein; RB = cysteine-rich zinc finger-like RNA binding region; AP = Aspartic protease; RT = reverse transcriptase: RNase H = ribonuclease H

1.3.3 Family: Bromoviridae, Genus: *Cucumovirus*

Cucumber mosaic virus (CMV), an isometric virus particle which is about 30 nm in diameter, is one of the most economically important plant viruses and has a very wide host range including plants from approximately 365 genera and at least 85 families (Palukaitis *et al*., 1992). CMV has been reported worldwide in a wide range of plant species (Palukaitis *et al*., 1992), but reported in *D. alata* only in Guadeloupe, Côte d'Ivoire and Nigeria (Migliori and Cadilhac, 1976; Fauquet and Thouvenel, 1987; Hughes *et al*., 1997). The virus is efficiently transmitted in a non-persistent manner by many aphid species and mechanically transmissible to a wide range of test plant

species of which cucumber (*Cucumis sativus* L.), *Nicotiana glutinosa* L., *N. benthamiana* L. and *Chenopodium quinoa* Willd. are diagnostically most useful. It is a positive-sense RNA virus with a tripartite genome consisting of RNA 1, RNA 2 and RNA 3 (Peden & Symons, 1973; Lot *et al.,* 1974). A subgenomic coat protein messenger RNA (RNA 4) is thought to be generated during replication of RNA 3 (Schwinghamer & Symons, 1977; Gould & Symons, 1982). CMV is divided into two main subgroups, I and II, based on serological relationships, peptide mapping of the coat protein (CP), nucleic acid hybridization and nucleotide sequence identity (Palukaitis *et al*., 1992). Serologically, the two subgroups are closely related, as shown by the cross reactions of polyclonal antibodies (Wahyuni *et al*., 1991). Some monoclonal antibodies produced against the coat proteins of subgroups I and II can differentiate the two, indicating the presence of unique epitopes for each subgroup (Porta *et al*., 1989; Wahyuni *et al*., 1991). More recently, phylogenetic analysis of a number of CMV isolates led to a further subdivision of subgroup I into subgroups IA and IB (Roossinck, 2002; Roossinck *et al*., 1999).

1.3.4 Unclassified yam viruses

Several virus isolates from yam are yet unclassified because research has yet to conclude their status as separate species or strains, or that they are same as those previously recognized in yam. Some of these viruses include *Yam internal brown spot virus*, a possible member of the genus *Badnavirus* which was first reported in *D. alata* from Barbados by Coursey (1967b), consists of non-enveloped bacilliform virions that are 130 nm in length and 29 nm wide. It causes brown spots in the tubers

of infected plants. *Dioscorea mottle virus* (DMoV), a possible member of the genus *Comovirus* and *Dioscorea dumetorum virus* (DDV) which may be a member of the genus *Potyvirus*, were reported in Nigeria by Hughes *et al*., (1997). Others include *Dioscorea latent virus* (DLV), which was first reported in *D. composita* and *D. floribunda* from Puerto Rico (Waterworth *et al*., 1974). DLV has been reported to show some characteristics of some of the viruses belonging to the Potexvirus genus (Waterworth *et al*., 1974). The DLV genome is reported to be a single stranded RNA and the virions are filamentous, non-enveloped, flexuous particles with a clear modal length of 445 nm and 11 nm wide (Phillips *et al*., 1986). *Chinese yam necrotic mosaic virus* (ChYNMV), a filamentous particle of about 12-13 x 660 nm, was reported in *D. batatas* from Japan. ChYNMV is wide spread in Japan (Fukumoto and Tochihara, 1978). *Dioscorea trifida virus* (DTV), another unclassified virus, was first reported in *D. trifida* L. from Guadeloupe inducing mosaic symptoms, chlorotic flecking, vein-banding and leaf deformation of naturally infected plants. It is transmitted in a non-persistent manner by *Aphis gossypii* and is also transmitted by mechanical inoculation (Migliori and Cadilhac 1976).

1.4 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 (Bock, 1982; 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. Currently available assays for yam virus diagnosis can broadly be divided into: bioassays (biological tests), immunoassays and nucleic acid-based techniques.

1.4.1 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 (Maramorosch, 1955; Christie, 1969; De Leeuw; 1972). Biological methods of yam virus detection and diagnosis include symptomatology and transmission studies (Terry, 1976; Hearon *et al*., 1978). Symptoms on the natural host are considered, as are symptoms on a range of indicator plant species after mechanical or vector transmission (Hollings, 1959; Terry, 1976). While these methods are still useful and are useful in the preliminary stages of research on new viruses, they are neither efficient nor consistent enough to be routinely used for virus identification and detection, and are therefore not useful for the certification of yam planting materials.

1.4.2 Immunoassays

Immunoassays are a useful tool for detecting and monitoring virus diseases, and particularly those infecting yam, and for routine testing of yam samples for the

presence of viruses. The most common serological tests for plant viruses include precipitation and agglutination tests, immunosorbent electron microscopy, enzyme-linked immunosorbent assays (ELISA) and dot blot immunoassay. Immunoassays utilise the ability of antibodies raised in animals to bind to the virus of interest. Among the serological tools, ELISA is often the method of choice because of its high sensitivity, simplicity, reproducibility and versatility in screening a large number of samples (Cho, 1990). The use of ELISA as a serological virus detection method using polyclonal antibodies improved the sensitivity of virus detection (Clark and Adams, 1977). This was further enhanced by the development of monoclonal antibodies, which are more specific and sensitive (Matthews 1991; Brattey and Burns, 1998).

The principle of ELISA is to immobilize an antigen (usually in this case, the virus) from extracted sap onto the surface of wells of polystyrene or polyvinyl plates and to wash off unbound substances from the plate, leaving only the specific antigen of interest. The antigen is then detected by enzyme-linked antibodies. ELISA is used mainly to confirm the presence or absence of viral protein(s) and can be adapted to estimate the concentration of a virus protein in plant sap (Copeland, 1998). Although many variations have been used, there are two main variants. The direct ELISA procedure involves the direct detection of an antigen by an enzyme-labeled specific antibody, while the indirect procedure involves the detection of an antigen by a specific antibody, which is then detected by an enzyme-labeled, anti-immunoglobulin antibody.

1.4.3 Nucleic acid-based techniques

The most common nucleic acid-based technique is the polymerase chain reaction (PCR). It was first used for the amplification of ß-globulin genomic sequences for diagnosis of sickle cell anemia (Saiki *et al*., 1985) but has been employed for the detection and differentiation of plant pathogens (Lopez *et al*., 2003). The advent of PCR in the diagnosis of plant viruses has greatly improved the sensitivity of virus detection (Choi *et al*., 1999). The technique can also be combined with serological procedures in the form of immunocapture (IC) -PCR, combining the advantages of serology with the sensitivity of PCR to detect diseases and identify the causal agents (Wetzel *et al*., 1992; Nemchinov *et al*., 1995; Mumford and Seal, 1997). PCR can be highly sensitive and as specific as the primer design permits.

1.5 Motivation

Yam contributes more than 200 dietary calories per person each day for millions of people in West Africa, where approximately 93% of the world's annual production of 51.4 mt is grown (FAO, 2007). Major yam producers in West Africa are Nigeria, Ghana, Côte d'Ivoire, Benin and Togo. Average yam consumption per capita per day is highest in Bénin (364 kcal) followed by Côte d'Ivoire (342 kcal), Ghana (296 kcal), and Nigeria (258 kcal) (IITA 2007). The use of infected vegetative propagules and uncontrolled introductions of infected germplasm by farmers through porous land borders have resulted in the presence of yam viruses in all yam growing areas of West Africa (Thouvenel and Fauquet, 1979; Goudou-Urbino *et al*., 1996a; Hughes *et al*., 1997; Olatunde, 1999). Yam virus symptoms are mainly chlorotic-mosiac symptoms

on leaves, shoe stringing, and other leaf deformation and discoloration. These leaf deformation and discoloration leads to patchy loss of photosynthetic activity and generally increases the rate of chloroplast senescence (Balachandran *et al*., 1994), leading to reduced sugar formation and minimal starch storage. Viruses are a threat to yam production, causing significant reduction in tuber yield and quality (Mantell and Haque, 1978; Thouvenel and Dumont, 1990). Yam viruses are of substantial economic importance not only because of yield losses they cause, but also due to the high cost of preventive measures (Degras, 1993).

Although several viruses have been reported to infect yam in West Africa (Thouvenel and Fauquet, 1979; Goudou-Urbino *et al*., 1996a; Hughes *et al.*, 1997; Aleman *et al*., 1997; Phillips *et al*., 1999; Odu *et al*., 2004; Seal and Muller 2007), earlier work on yam viruses mainly reported the identification and characterization of these viruses (Hearon *et al*., 1978; Thouvenel and Fauquet, 1979; Goudou-Urbino *et al*., 1996b; Odu *et al*., 1999). Thottapilly (1992) indicated the presence of several viral diseases in yam in Africa however, information on the geographical distribution of yam viruses in many West African countries is limited and incomplete and mainly limited to Nigeria and Côte d'Ivoire (Thouvenel and Fauquet, 1979; Porth *et al.*, 1987; Thouvenel and Dumont, 1988; Hughes *et al*., 1998; Thouvenel and Dumont, 1990; Odu *et al*., 2001; Gumedzoe *et al.*, 2001; Njukeng *et al*., 2002) Although there have been reports of virus-like symptoms and virus diseases infecting yam in some parts of Ghana and Togo (Olatunde 1999; Phillips *et al*., 1999; Gumedzoe *et al*., 2001), information on the incidence and geographical distribution of these viruses is

lacking. Previous surveys of yam fields in Benin had concentrated on assessing farmer's knowledge and practices on the domestication of yam and on identification of cultivated species and studies of morphological diversity within cultivated species (Dansi *et al.*, 1998, Dansi *et al*., 1999, Dansi *et al*., 2000, Dumont and Vernier, 2000). A survey of the major yam producing agro-ecological zones in Ghana, Togo and Benin will therefore, identify the viruses naturally occurring in yam in these countries, determine their geographical distribution and assess the potential for enhanced production through distribution of virus-tested germplasm.

Potyviruses infecting yam in West Africa have been well characterized (Thouvenel and Fauquet, 1979; Duterme *et al*., 1996; Aleman-Verdaguer *et al*., 1997; Odu *et al*., 1999; Fuji *et al*., 1999). However, knowledge of the genomic variability among badnaviruses infecting yam in West Africa is limited. Molecular studies of badnaviruses infecting other crops have revealed that extremely high levels of genetic variability occur within this group of viruses (Lockhart and Olszewski, 1993; Geering *et al*., 2000; Lebas, 2002). Furthermore, integrated badna-like sequences in host genomes were able to give rise to infectious episomal genome via homologous recombination (Ndowora *et al*., 1999). These integrated sequences and the high sequence variability among badnaviruses complicates the development of reliable molecular detection tests (Braithwaite *et al*., 1995; Smith *et al*., 1996; Geering *et al*., 2000). Two badnaviruses infecting yam in West Africa, *Dioscorea alata bacilliform virus* (DaBV) and *Dioscorea sansibarensis bacilliform virus* (DsBV) have been fully sequenced. Analysis of the complete nucleotide sequence revealed up to 38.1%

sequence variability (Briddon *et al*., 1999; Seal and Muller, 2007). More isolates of yam badnaviruses from West Africa needs to be studied to establish the sequence variability among yam-infecting badnaviruses occurring in West Africa. Information of the diversity of badnaviruses infecting yam in West Africa is needed for the development of reliable diagnostic tests which will detect and differentiate between yam- infecting badnaviruses and their strains.

The crucial role of timely and accurate diagnosis of yam viruses cannot be over emphasized. Certification of yam planting materials will give a guarantee of the health status of the propagules, will simplify plant quarantine and reduce yield losses due to viruses. Mass production of virus-free planting materials using tissue culture techniques also depends greatly on effective diagnosis of yam viruses for choosing the material to be multiplied and subsequently in verification of the health status (indexing) of the multiplied materials before distribution. Data on the health status of the plant material is also very useful to breeders who must monitor the progress of their breeding work and will assist extension workers in advising the farmers on the choice of healthy planting materials.

Diagnosis of yam virus infection and identification of the causal agent(s), either/both in the field and the laboratory, is based on symptomatology, use of indicator plants, vector transmission, electron microscopy and protein and nucleic acid-based diagnostics (Goudou-Urbino *et al*., 1996b; Mumford and Seal, 1997; Hughes *et al*., 1998). Protein-based diagnostics are suitable for routine identification

of many yam viruses (Hughes *et al*., 1998) but require high quality polyclonal antibodies that are free from contaminating antibodies against plant proteins and specific monoclonal antibodies due to the diverse strains of different yam viruses, particularly *Yam mosaic virus* (YMV) (Fuji and Nakamae, 2000; Bousalem *et al*., 2000a; Bousalem *et al*., 2000b). These antibodies are not readily available (only available in a few laboratories and not in large volumes) and the financial investment required for many of the laboratory-based diagnostic procedures makes it difficult for national agricultural research systems (NARS) and farmers in developing countries to get accurate and timely diagnosis of virus diseases. These tests are therefore not routinely available, and most farmers do without the information, with potentially devastating economic effects.

There is therefore a need to assess the geographical distribution of yam viruses in the major yam producing agro-ecological zones in Ghana, Togo and Benin and to establish the genetic variability within badnaviruses infecting yam, which are under studied. Furthermore there is a need to make diagnostics for yam viruses, particularly antibodies, available to NARS and to simplify the diagnostic procedures while maintaining and/or improving the sensitivity of the tests in order to take the diagnostics out of the laboratory and make them available to the end users, whether diagnosticians, extension officers, quarantine officers or farmers.

1.6 Objectives

The specific objectives of this research are:

1. To conduct surveys for the occurrence and distribution of viruses infecting yam in major yam growing agro-ecological zones in Ghana, Togo and Benin and to produce yam virus distribution maps of this countries.

2. Produce polyclonal antibodies to some previously characterized viruses infecting yam in West Africa.

3. To establish genetic diversity among badnavirus isolates infecting yam in Ghana, Togo, Benin and Nigeria and to characterise any other uncharacterised yam virus encountered in this study.

1.7 Thesis outline

This introductory chapter will be followed by chapter 2 describing the occurrence and distribution of yam viruses in the four major yam-producing agro-ecological zones in Benin. The incidence and distribution of viruses across the various yam species and the incidence of mixed infection will be discussed. Chapter 3 reports the survey of yam fields in Ghana and Togo. The occurrence and distribution of viruses infecting yam in the major yam producing zones in the two countries are highlighted.

In Chapter 4, the production of rabbit polyclonal antibodies to two of the viruses infecting yam will be presented. The deduced amino acid sequences of partial RT/RNaseH-coding region of badnavirus isolates infecting yam in Ghana, Togo, Benin and Nigeria are analysed in Chapter 5; sequence diversity among isolates and phylogenetic analysis will be presented. Chapter 6 presents a summary of the main findings of this study.

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CHAPTER 2 – SURVEY OF THE INCIDENCE AND DISTRIBUTION OF VIRUSES INFECTING YAM (DIOSCOREA SPP.) IN BENIN

Eni A.O., J d'A Hughes and M.E.C. Rey (2008) Survey of the incidence and distribution of five viruses infecting yam in the major yam producing zones in Benin. Annals of Applied Biology Published online: 27-May-2008. doi: 10.1111/j.1744-7348.2008.00253.x.

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CHAPTER 2 – SURVEY OF THE INCIDENCE AND DISTRIBUTION OF VIRUSES INFECTING YAM (DIOSCOREA SPP.) IN BENIN

Abstract

Surveys were conducted in 2004 and 2005 to determine the incidence and distribution of viruses infecting yam in four major yam-producing agro-ecological zones in Benin. Yam leaf samples collected from 69 fields and one experimental screen-house were indexed for *Cucumber mosaic virus* (CMV), *Dioscorea mottle virus* (DMoV), *Yam mild mosaic virus* (YMMV), *Yam mosaic virus* (YMV) and yam-infecting badnaviruses (DaBV and DsBV) by enzyme-linked immunosorbent assay (ELISA), immunocapture polymerase chain reaction (IC-PCR) and/or IC-reverse transcription-PCR (IC-RT-PCR). Eighty-two percent and 66% of leaf samples tested in 2004 and 2005, respectively, were infected with CMV, YMMV YMV and/or badnavirus. DMoV was not detected. Yam-infecting badnaviruses were the most prevalent virus infection, detected in 45% of the total leaves sampled followed by YMV (31%), YMMV (27%) and CMV (2%). Although CMV incidence was low, this is the first record of CMV in yam in Benin. Mixed virus infections were detected in 48% (2004) and 39% (2005) of the infected leaves. A mixture of YMMV and badnaviruses (DaBV or DsBV) was the most common mixed infection detected. *Dioscorea alata,* with a higher incidence of badnavirus infection (81%), YMMV (51%) and CMV (8%), was more heavily infected than *D rotundata*.

2.1 Introduction

Yam occupies a very important position as a food crop in Benin and was ranked first among 20 most important food and agricultural commodities of the country in 2005. Benin is the fourth largest producer of yam in the world, cultivating 195,747 hectares of yam in 2006 (FAO, 2007). Due to the continued and increasing dependence on yam for food in Benin, its importance for food security and the need for improvement in yam production, farmers are continually boosting the diversity of their plots by domestication of wild species (Dumont and Vernier, 2000). This is also encouraged by a developed yam chips production system that has emerged for storage and preservation of the valuable tubers (Vernier, 1998). Major yam production is in Zone Vivrière du Sud Borgou and Zone Ouest-Atacora and yam is also produced in Zone Cotonnière du Nord Bénin and Zone Cotonnière du Centre Bénin. Very little production (0 - 1 %) is observed in the southern zones (Zone des Terres de Barre, Zone des Pêcheries and Zone de la Dépression), and yam is not produced in the Zone Extrême Nord Bénin (Akker 1999).

Yam production is adversely affected by pests and pathogenic diseases. Diseases caused by viruses, fungi, nematodes and bacteria, either singly or in combination are responsible for yield losses (Nwankiti and Arene 1978; Onwueme, 1978; Ng, 1992; Hughes *et al*., 1997). Viruses are of particular concern because, apart from causing significant reduction in tuber yield and quality, they restrict international exchange of germplasm.

Yam viruses have been reported in most of the yam-growing areas of West Africa (Thouvenel and Fauquet, 1979; Thouvenel and Dumont 1990; Goudou-Urbino *et al*., 1993; Goudou-Urbino *et al*., 1996; Hughes *et al*., 1997; Phillips *et al*., 1999; Olatunde, 1999; Odu 2002). Viruses infecting yam belong to the *Potyvirus, Badnavirus,* and *Cucumovirus* genera, while a number of yam viruses remain unclassified. Viruses reported to infect yam in West Africa include *Yam mosaic virus* (YMV), genus *Potyvirus, Yam mild mosaic virus* (YMMV), genus *Potyvirus*, *Dioscorea dumetorum virus* (DdV), genus *Potyvirus, Dioscorea alata bacilliform virus* (DaBV), genus *Badnavirus*, *Dioscorea sansibarensis virus* (DsBV), genus *Badnavirus, Cucumber mosaic virus* (CMV), genus *Cucumovirus*, and *Dioscorea mottle virus* (DMoV), genus *Comovirus.*

Although there have been reports of yam viruses in Benin (Goudou-Urbino *et al*., 1996; Phillips *et al*., 1999), knowledge of the incidence, distribution and virus identities implicated in these yam virus diseases is lacking. Previous surveys of yam fields in Benin had concentrated on assessing farmer's knowledge and practices on the domestication of yam and on identification and morphological diversity of cultivated species (Dansi *at al.*, 1998, Dansi *et al.,* 1999, Dansi *et al* 2000, Dumont and Vernier, 2000). The objective of this study was to determine the occurrence and distribution of viruses infecting yam in the major yam-producing zones in Benin.

Immunoassays are a useful tool for detecting and monitoring virus diseases. They utilise the ability of antibodies raised in animals to bind to the virus of interest. The

advantage offered by the enzyme-linked immunosorbent assay (ELISA) over other immunoassays is the ability to test crude plant extracts (Clark, 1981). The principle of ELISA is to immobilize an antigen (usually in this case, the virus) from extracted sap onto the surface of wells of polystyrene or polyvinyl plates and to wash off unbound substances from the plate, leaving only the specific antigen of interest. The antigen is then detected by enzyme-linked antibodies. ELISA is used mainly to confirm the presence or absence of viral protein(s) and can be adapted to estimate the concentration of a virus protein in plant sap (Copeland, 1998). The most common nucleic acid-based technique is the polymerase chain reaction (PCR). The advent of PCR in the diagnosis of plant viruses has greatly improved the sensitivity of virus detection (Choi *et al*., 1999). The technique can also be combined with serological procedures in the form of immunocapture (IC) -PCR, combining the advantages of serology with the sensitivity of PCR to detect diseases and identify the causal agents (Wetzel *et al*., 1992; Mumford and Seal, 1997). For this study, ELISA was chosen because antisera against several yam viruses were available from the International Institute of Tropical Agriculture (IITA), Nigeria. IC-PCR was employed because it favours the amplification of DNA contained within capsids and excluding DNA derived from other genomes thus ensures that only episomal virus sequences are amplified. This is particularly important for the detection of badnaviruses whose sequences have been reported to be integrated into host genomes (Harper *et al*., 1999a; Geering *et al*., 2005; Harper *et al*., 1999b; Geering *et al*., 2001). Furthermore, IC-RT-PCR is reported as an important method for the detection of low titre RNA viruses (Wetzel *et al*., 1992) and most yam viruses are RNA viruses. Immuno-capture

PCR also has the advantage of eliminating, in the washing step, host contaminants that may interfere with PCR.

2.2 Materials and methods

2.2.1 Survey

Surveys of the four major yam-producing agro-ecological zones in Benin were conducted in October 2004 shortly after milking for early maturing yam cultivars and August 2005 before the milking period. In 2004, yam fields were surveyed in each of the location by walking through the fields and inspecting yam plants for symptoms of virus infection. Young leaves of symptomatic plants were collected moving diagonally across the field. Three plants with no obvious symptoms of virus infection were also sampled in two of the location visited. Leaves were collected from these plants for indexing to exclude the possibility that plants were infected but tolerant (Cooper and Jones, 1983). The species of each plant was recorded and for symptomatic plants, symptom type(s) was recorded. Leaf samples were also collected from 23 plants (Benin landraces) in an experimental screen-house of the International Institute of Tropical Agriculture (IITA) in Cotonou, Benin. In 2005, yam fields were surveyed by walking diagonally across and scoring plant for the presence or absence of virus symptoms. The species of each plant was recorded and for symptomatic plants, symptom type(s) was recorded. Young leaves were collected both from symptomatic and non-symptomatic plants along the diagonal, five to ten paces apart, depending on the field size. Leaf samples from symptomatic samples outside the diagonal survey path showing symptoms not observed along the diagonal were also

collected. Leaf samples were stored in a mobile refrigerator at 4ºC while in the field. Some leaves from each plant were later transferred into Eppendorf tubes and stored in liquid nitrogen for PCR while others were dried over calcium chloride for later processing. All the yam sampled were mostly at the mid-developmental stage and were of four different varieties, *Dioscorea alata* L*, Dioscorea cayenensis* Lam, *Dioscorea dumetorum* (Kunth) Pax *and Dioscorea rotundata* Poir.

2.2.2 Enzyme-linked immunosorbent assay (ELISA)

Triple antibody-sandwich (TAS) ELISA was used for the detection of YMV and DMoV while protein-A sandwich (PAS) ELISA was used for the detection of badnavirus (DaBV and DsBV), YMMV and CMV. Rabbit polyclonal antisera and monoclonal antibodies against YMV (YMV-M24), known to detect YMV isolates from seven West African countries were used for YMV detection (Njukeng *et al*., 2002). For YMMV detection, rabbit polyclonal antibody working at 1:250 dilutions for trapping and 1:6400 for detecting in PAS-ELISA and known to show no cross reactivity with other potyviruses was used (Odu *et al*., 1999). Rabbit polyclonal antisera against CMV, DaBV, and DMoV, and monoclonal antibodies to DMoV routinely used for the certification of virus-tested plantlets at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, were used for the detection of these viruses.

The rabbit polyclonal antibody against DaBV is now known to detect, but not differentiate, the two characterised yam-infecting badnavirus, DaBV and DsBV (Chapter 5). Therefore subsequent mention of badnavirus, in this chapter, would refer to DaBV and/or DsBV.

TAS-ELISA was carried out as previously described by Thottappilly *et al*. (1998) with slight modifications. Wells of microtitre plates were coated with 100 µl of YMV or DMoV polyclonal antibodies diluted 1:1000 in coating buffer (0.05 M sodium carbonate buffer, pH 9.6) and incubated at 37^{o}C for 2 h. The plates were blocked with 200 µl of 5% skimmed milk powder (Marvel; Premier International Foods, UK) for 1 h at 37^{o}C and 200 µl of leaf sap extract, prepared by grinding the test leaves in grinding buffer (phosphate buffered-saline containing 0.05 % (v/v) Tween-20 (PBS-T), 0.5 mM polyvinyl pyrrolidone (PVP)-40 and 79.4 mM Na_2SO_3) was loaded in duplicate wells and incubated overnight at 4^{o}C. Hundred µl of YMV or DMoV mice monoclonal antibodies diluted in PBS-T was added to the wells and the plates were incubated at 37^{o}C for 2 h. Goat anti-mouse alkaline phosphatase diluted at 1:40,000 in conjugate buffer (half strength PBS containing 0.05% (v/v) Tween-20, 0.02% (w/v) egg albumin, 0.005 mM PVP-40) was added to each well and incubated at 37 ^{o}C for 2 h. Then 200 µl p-nitrophenylphosphate substrate (pNPP) (1 mg ml^{-1} in 10% diethanolamine, pH 9.8) was added into each of the wells to detect the antigen-antibody reactions.

Protein-A sandwich (PAS)-ELISA followed the method described by Edwards and Cooper (1985). Wells of a microtitre plate were coated with 100 µl of protein-A (Sigma, UK) diluted at 1 µg ml^{-1} in coating buffer and incubated at 37°C for 2 h. CMV, YMMV and badnavirus rabbit polyclonal antibodies were diluted in PBS-T buffer to 1:3000, 1:250 and 1:1000, respectively, and 100 µl of these were added into the respective wells of ELISA plate and they were incubated at 37°C for 2 h. Leaf sap extracts (~200 µl) prepared as described previously for TAS-ELISA were added into the wells in duplicate and incubated at 4°C overnight. Then, 100 µl of CMV, YMMV or badnavirus rabbit polyclonal antibodies at 1:3000, 1:6400 and 1:1000, respectively, were added into the respective wells and plates were incubated at 37°C for 2 h. Protein-A alkaline phosphatase diluted at 1:50,000 in conjugate buffer was added to each well and the plate incubated for 2 h at 37°C. The antigen-antibody reactions were detected using pNPP substrate as described before.

The plates were washed three times at 3 minute intervals with PBS-T after each incubation step for both TAS- and PAS-ELISA, but not after the milk blocking step in TAS-ELISA. Leaf sample extract from healthy yam was used as negative controls and virus isolates of CMV, DMoV, YMMV and DaBV available at IITA-Ibadan, were used as positive controls. The A_{405} for the substrate in each well was measured in a DYNEX MRX microplate reader 1 h after the addition of pNPP substrate.

2.2.3 Immunocapture-polymerase chain reaction (IC-PCR)/immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR)

IC-PCR was used for the detection of badnavirus and IC-RT-PCR was used for the detection of CMV (only in 2005), YMV and YMMV. Leaf samples were not tested for DMoV by PCR-based methods. Immunocapture was carried out using polyclonal antibodies diluted 1:3000 for CMV, 1:250 for YMMV, 1:1000 for both badnavirus and YMV. The following primer sets were used;

CMV Primer 1 5′ GCC GTA AGC TGG ATG GAC AA 3′, Primer 2 5′ TAT GAT AAG AAG CTT GTT TCG CG 3′, Wylie *et al.* (1993)

BadnaFP 5′-ATG CCI TTY GGI ITI AAR AAY GCI CC-3′, Badna RP 5′-CCA YTT RCA IAC ISC ICC CCA ICC-3′, Seal and Muller (2007)

YMMV Forward 5′-GGC ACA CAT GCA AAT GAA RGC-3′, Reverse 5′-CAC CAG TAG AGT GAA CAT AG-3′, Mumford & Seal (1997)

YMV Forward 5′-ATC CGG GAT GTG GAC AAT GA-3′, Reverse 5′-TGG TCC TCC GCC ACA TCA AA-3′, Mumford & Seal (1997)

Immunocapture was done using a modification of the coating and trapping method by Clark and Adams (1977). PCR tubes (200 µl, ABgene, UK) were coated with antibodies, incubated and washed as described above for ELISA. The coated tubes were loaded with 200 µl of sap prepared as in TAS-ELISA above and incubated at 4°C overnight. After washing three times with PBS-T and once with distilled water, 25 µl of reaction cocktail consisting of 1 x Taq reaction buffer (10mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl, 0.01% Triton-X 100), 0.25 mM of each dNTP, 50

pmol each of badnavirus forward and reverse primers and 1.25 U of Taq DNA polymerase (Promega, USA) was added to each tube and PCR carried out for the detection of badnavirus (DaBV and DsBV) in a PTC-200 Peltier Cycler (MJ Research Inc., USA) using the following themocyclic regime; 94°C for 4 min, 94°C for 30 s, 50°C for 30 s, 72°C for 30 s. The cycle was repeated 40 times excluding the first step, and then a final extension was done at 72°C for 5 min.

For the detection of YMV and YMMV in 2004, after the immunocapture step as described above, RT-PCR was done as described by Mumford and Seal (1997) by including 5 U of M-MLV reverse transcriptase (Promega, USA) to the reaction cocktail described above and replacing the badnavirus primers with 50 pmol each of the forward and reverse YMMV primers or 25 pmol each of YMV forward and reverse primers. Twenty-five µl of the cocktail was dispensed into each tube and the following thermocyclic regime used: 50°C for 10 min for reverse transcription, 95°C for 4.5 min, 95°C for 30 s, 55°C for 1 min and 72°C for 1 min. The cycle was repeated 35 times from the third step followed by a final extension step of 72°C for 10 min. In 2005, after the immunocapture step as described above, RT-PCR for the detection of CMV, YMV and YMMV, was done using QIAGEN Onestep RT-PCR kit (Qiagen, Germany) according to the manufacturer's protocol. Twenty-five pmol each of CMV, YMV or YMMV forward and reverse primers was added to the cocktail and 25 µl of the cocktail was dispensed into each pre-coated tube. Reverse transcription was done at 50°C for 30 min followed by a PCR activation step at 95°C for 15 min. PCR continued with 40 cycles of 94°C for 1 min, 60°C for 1 min and

72°C for 1 min. This was followed by a final extension step of 72°C for 10 min. Ten µl of IC-PCR and IC-RT-PCR products were analysed on a 1.5% agarose gel in TAE (40 mM Tris-acetate pH 8.3, 1 mM disodium ethylenediaminetetraacetate (EDTA)) buffer at 100 V for 1 h. The 1-kbp plus DNA ladder (Invitrogen) was ran along with the PCR products and used for size estimation. The gels were stained in ethidium bromide and observed on a UV-transilluminator.

2.3 Results

2.3.1 Virus survey

A total of 204 yam leaf samples were collected from 24 of the 29 locations visited in 2004. Leaf samples were not collected from the other five locations because obvious symptoms of virus infection were not observed in the fields. Twenty-three leaf samples were collected from the screen house. In 2005, 349 leaf samples were collected from symptomatic yam plants, and 210 were collected from non-symptomatic plants. A total of 22 fields were sampled in Zone Cotonnière du Centre Bénin, eight in Zone Cotonnière du Nord Bénin, 18 in Zone Ouest-Atacora and 26 in Zone Vivrière du Sud Borgou in 2004 and 2005 (Figure 2.1). Thirteen different symptom phenotypes occurred at different proportions in 83% of the fields surveyed in 2004 and 100% of fields in 2005. Leaf mosaic (Figure 2.2c) and chlorosis/chlorotic spots (Figure 2.2a) were the most common symptoms observed in both years accounting for 75% and 58% of the field symptoms in 2004 and 2005 respectively. Bleaching, crinkling, distortion, leaf curl, mottle, puckering, shoe stringing, vein

banding, vein clearing, whitish bleaching and leaves showing retarded growth, accounted for the remaining 25% and 42% of the field symptoms.

Distribution of symptom types varied across species both in 2004 and 2005. In 2004, chlorosis and mosaic were the major symptoms observed in all three yam species though very few *D. cayenensis* (8 plants) were assessed. In 2005, puckering was the most common symptom type observed in *D. alata* followed by chlorosis, mottling and mosaic. Chlorosis and mosaic remained the more common symptom types in *D. rotundata*. The proportion of the other symptom types varied across the different species but leaf distortion, leaf curl, leaf retardation and vein clearing were observed only in *D. rotundata* but not in the other two yam species. Most of the symptom types were associated with the four viruses detected, however, except for one leaf sample that tested positive to all four viruses, puckering in *D. alata* was always associated with badnavirus and/or YMMV and 20/30 (66.6%) *D. rotundata* leaf samples that tested positive to single infection of YMMV were non-symptomatic.

2.3.2 ELISA/PCR

For ELISA, leaf samples with mean absorbance values at 405 nm (A_{405}) that were twice or more than that of the healthy leaves were considered to be virus infected (Thottappilly *et al*., 1998). Leaf samples that showed the expected band sizes of 500 bp, 249 bp 586 bp and 579 bp, on gel after PCR, were considered positive for CMV, YMMV, YMV and badnavirus (DaBV or DsBV) respectively (Mumford & Seal 1997; Wylie *et al*., 1993; Seal and Muller, 2007).

One hundred and eighty-six of the 227 leaf samples (81.9%) tested in 2004 and 369 of 559 leaf samples (60.0%) tested in 2005, were positive by ELISA and/or by PCR for CMV, YMMV, YMV and/or badnavirus (DaBV and/or DsBV). DMoV was not detected in any of the leaf samples tested in both years (Table 2.1).

Badnavirus was the most prevalent virus infection in 2004 detected in 161/227 leaves samples (70.9%) in 23 of the 24 locations and in the screen-house. The occurrence of YMV in the 2004 growing season was low, being detected in only 23/227 leaf samples (10.1%) from 14 locations, and no detection in the screen-house. In comparison, YMV was the most prevalent virus infection in 2005, and was detected in 222/559 leaf samples (39.7%) and was present in all fields in all locations. Cucumber mosaic virus was the least prevalent virus in both years detected only in 11/227 leaf samples (4.8%) in 2004 and in 4/559 leaf samples (0.7%) in 2005 (Table 2.1).

Similar to the observation in 2004, when two of the three non-symptomatic leaf samples tested positive to badnavirus and/or YMMV, 98/210 non-symptomatic leaf samples (46.6%) tested in 2005 reacted positively to single or mixed infection of badnavirus, YMMV or YMV. Forty out of 224 symptomatic leaf samples (17.8%) tested in 2004 and 79/349 symptomatic leaf samples (22.6%) from 2005, tested negative to all five viruses indexed for (Table 2.1).

2.3.3 Distribution across species and across zones

Of the two major yam species tested for viruses during this study, incidence of YMV was higher in *D. rotundata* 232/659 (35.2%) compared to *D. alata* 12/116 (10.3%) in all four zones in both years (Table 2.1). Incidence of badnavirus (DaBV and/or DsBV) was higher in *D. alata* 95/116 (81.9%) compared to *D. rotundata* 250/659 (37.9%) in most of the zones where both species were tested besides the observation in Zone Ouest-Atacora in 2004 where the incidence of badnavirus was higher in *D. rotundata* (Table 2.1). As observed for badnavirus, the incidence of YMMV was also higher in *D. alata* 60/116 (51.7%) than in *D. rotundata* 154/659 (23.4%) in all the zones in both years except for Zone Vivrière du Sud Borgou in 2004 (Table 2.1). Both in 2004 and 2005, incidence of CMV across the four zones varied greatly however, incidence was higher in *D. alata* 10/116 (8.6%) compared to *D. rotundata* 5/659 (0.8%) (Figure 2.3). Five of the eight *D. cayenensis* leaves testes in 2004 were positive to badnavirus while two were positive to YMMV. One of the three *D. dumetorum* leaves tested in 2005 had a mixed infection of badnavirus and YMMV while the other two tested negatively to all five viruses indexed for (Table 2.1).

2.3.4 Mixed infections

In 2004, 90 of the 186 infected leaf samples (48.3%) were infected with a mixture of two or more viruses (Table 2.2). As observed in 2004, 145 of the 369 infected leaf samples (39.2%) in 2005 also had mixed virus infection. Percentage of mixed infections encountered in various yam species in each zone in relation to the number

of infected leaf samples ranged from 17-75% in 2004 and 0-100% in 2005 (Table 2.2). Distributions of mixed infection across the zones were similar in both years with Zone Cotonnière du Centre Bénin having the highest incidence of mixed infection in 2004 (46%) and 2005 (30%). Followed by Zone Vivrière du Sud Borgou (44% in 2004 and 26% in 2005), Zone Cotonnière du Nord Bénin (32% in 2004 and 25% in 2005) and Zone Ouest-Atacora (29% in 2004 and 20% in 2005) (Table 2.1).

Six mixed infections detected in 2004 were also detected in 2005. The mixture of CMV and YMMV was detected in 2004 but not in 2005 while the mixture of CMV, YMMV, YMV and badnavirus was detected in two leaf samples in 2005 but not in any leaf samples tested in 2004. Dual infections were more common than triple infections in 2004 (78 leaf samples and 12 leaf samples respectively) and 2005 (122 leaf samples and 21 leaf samples respectively). The most frequent mixed infection detected in 2004 was badnavirus and YMMV followed by badnavirus and YMV. In 2005, badnavirus and YMV was the most common mixed infection followed by badnavirus and YMMV (Table 2.3).

A new severe leaf symptom, which the authors describe as whitish bleaching/patches was observed on six yam plants in the field and two yam plants in the screen-house in 2004 and in five yam plants in 2005. Leaf samples taken from eight out of the 13 (61.5%) yam plants showing this symptom had mixed infection of badnavirus, CMV and YMMV or badnavirus and YMMV or badnavirus and YMV, 3/13 (23%) had single infection of badnavirus, YMMV or YMV while 2/13 (15.4%)

tested negative to all the viruses indexed for. This symptom was observed only in *D. alata* and *D. rotundata* but not in *D. cayenensis* or *D. dumetorum*.

2.4 Discussion

This study provides information on the incidence and distribution of five viruses infecting yam in four major yam producing agro-ecological zones in Benin. CMV, YMMV, YMV and badnavirus (DaBV and/or DsBV) were detected in all the four agro-ecological zones, but DMoV was not detected in any of the leaf samples tested. All four viruses that were detected in Benin have been reported in other countries in the West African yam zone. (Thouvenel and Fauquet 1979; Porth *et al*., 1987; Hughes *et al*., 1997; Phillips *et al*., 1999; Odu *et al*., 1999)

Although the specificity of the antisera used for the detection of these viruses has been previously evaluated and determined (Hughes unpublished; Odu *et al*., 1999; Njukeng *et al*., 2002) all leaf samples were also tested by IC-PCR to ensure that plants with low virus load were not missed and that badnavirus detection is correctly reported. IC-RT-PCR is an important method for the detection of low titre RNA viruses (Wetzel *et al*., 1992) and three of the viruses tested for (CMV, YMMV and YMV) are RNA viruses. Secondly, IC-PCR favours amplification of DNA contained within capsids excluding DNA derived from other genomes thus ensures that only episomal virus sequences are amplified. This considerably decreases non-specific PCR amplifications particularly, for the detection of badnaviruses whose sequences have been reported to be integrated into host genomes (Harper *et al*., 1999a; Harper *et*

al., 1999b; Geering *et al*., 2001; Geering *et al*., 2005). We found that virus detection by IC-RT-PCR/IC-PCR was more than the corresponding ELISA tests (data not shown), possibly due to the greater sensitivity of IC-RT-PCR/IC-PCR (Olatunde 1999; Mumford and Seal 1997; Njukeng *et al*., 2005; Agindotan *et al*., 2006). The lower sensitivity observed with the ELISA tests is similar to the findings of Mumford and Seal (1997) and Njukeng *et al*., (2005) and could be due to low virus concentration in yam leaf samples (Brunt *et al*., 1990) or due to interference of polyphenols and glutinous polysaccharides contained in yam leaves (Rossel and Thottappilly, 1985).

The detection of badnavirus in 67 of the 70 locations (95.7%) where viruses were detected and in all four yam species encountered in this study, and its presence in six of the eight mixed infections, makes the badnaviruses (DaBV and DsBV) very important and widely spread yam viruses in Benin. The observed higher incidence of badnavirus and YMMV in *D. alata* compared to *D. rotundata*, in Benin, is similar to the findings of Hughes *et al.* (1997) on the distribution of these viruses in Nigeria. The occurrence of a higher incidence of YMV in *D. rotundata* compared to the other yam species sampled is also similar to the distribution of YMV in Nigeria (Hughes *et al.*, 1997) and in Ghana (Olatunde, 1999). The extensive spread of CMV, YMMV, YMV and badnavirus and the high incidence of mixed infection observed in all the four zones surveyed in this study may be attributed to the exchange of infected planting materials both locally and internationally through permeable land borders.

These exchanges may also account for the similarities in the incidences and distribution of these viruses in Nigeria (Hughes *et al.*, 1997).

The high incidence of badnavirus and YMMV mixed infections in all four yam species tested and its detection in almost all the locations visited in both years, is of concern. Some leaf samples tested positive for badnavirus but not for YMMV, in ELISA and PCR, and vice versa. This shows that the tests conducted accurately discriminates between the two viruses in single and mixed infections. The occurrence of two types of mixed infection in the screen-house in comparison with the field, where eight different types were detected, can be attributed to the controlled environment of the screen-house which excludes insect vectors, particularly aphids. Three (CMV, YMMV, and YMV) of the four viruses detected in Benin during this study, are reported to be aphid-transmitted (Terry, 1976; Odu *et al*., 1999; Odu *et al*., 2004), except the badnaviruses, which are transmitted by the mealybug *Planococcus citri* Risso (Kenyon *et al.*, 2001; Phillips, 1999; Odu *et al*., 2004).

Dioscorea alata was found to be the most heavily infected yam species in this study. Even though it is the most widely distributed yam species globally, its high susceptibility to diseases is a major limitation to the profitable and sustainable production of *D. alata*, which is also a favoured alternative to *D. rotundata* for diabetic patients (Abang *et al.*, 2003, Riley *et al*., 2006).

Leaf samples from some plants showing obvious symptoms of virus infection, tested negative to all the viruses indexed for. Symptoms observed on these plants may 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. The detection of badnavirus, YMMV and YMV infections on non-symptomatic leaf samples, shows that absence of visual symptoms on yam leaves may not be indicative of absence of virus infection, but laboratory diagnosis serves as a more sensitive and conclusive way of affirming the health status of potential breeding or planting materials. Most of the symptoms observed during this study have been reported (Thouvenel and Dumont, 1988; Odu *et al*., 2001), but the previously unrecorded virus symptom of whitish bleaching/patches described in this paper has not been reported. The severity of this symptom and its effect on the green pigmentation of yam leaves may lead to a severe reduction in the photosynthetic ability of affected plants, with a consequent reduction in tuber yield.

The extensive spread of yam viruses within the West African sub-region needs to be addressed. Restricting movement and exchange of planting materials across land bothers by enforcing registration and certification programs, and the use of planting materials (seed tubers) obtained from healthy plants for yam cultivation would be a good initial approach to solving this problem. Some of the major challenges for farmers and researchers in choosing healthy planting materials and in screening of yam genotypes for multiplication as clean planting material both for local and international distribution, include multiplicity of viruses and symptom types,

confounding of virus symptoms with those of nutritional disorders, mixed virus infections, and the absence of methods for rapid and reliable field diagnosis. A rapid field test for the certification of yam planting materials will be major progress for the control of yam viruses.

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Table 2.1 Occurrence of four viruses infecting yam (*Dioscorea* spp.) in four agro-ecological zones sampled in Benin in 2004 and 2005

Agro-ecological zone	N[a]	Yam species	N[b]	Viruses present[c]				
				CMV	Badna virus	YMMV	YMV	Mixed
2004								
Zone Cotonnière du Centre Bénin	7	*D. alata*	21	2	19	15	2	15
		D. cayenensis	8	0	5	2	0	1
		D. rotundata	45	1	34	16	8	18
Zone Cotonnière du Nord Bénin	2	*D. rotundata*	22	1	13	9	2	7
Zone Ouest-Atacora	8	*D. alata*	5	0	3	1	0	1
		D. rotundata	12	1	9	1	2	4
Zone Vivrière du Sud Borgou	12	*D. alata*	13	1	12	5	0	5
		D. rotundata	78	1	57	42	9	35
Screen house	1	*D. alata*	23	4	9	2	0	4
2004 total	**29**		**227**	**11**	**161**	**93**	**23**	**90**
2005								
Zone Cotonnière du Centre Bénin	15	*D. alata*	29	1	27	18	5	17
		D. dumetorum	1	0	1	0	1	1
		D. rotundata	166	1	54	30	77	40
Zone Cotonnière du Nord Bénin	6	*D. alata*	11	1	11	8	3	9
		D. rotundata	56	0	8	9	22	8
Zone Ouest-Atacora	10	*D. alata*	3	1	3	3	0	3
		D. dumetorum	2	0	0	0	0	0
		D. rotundata	108	0	29	20	25	20
Zone Vivrière du Sud Borgou	14	*D. alata*	11	0	11	8	2	9
		D. rotundata	172	0	46	27	87	38
2005 total	**45**		**559**	**4**	**190**	**123**	**222**	**145**
2004 and 2005 total		*D. alata*	**116**	**10**	**95**	**60**	**12**	**63**
2004 and 2005 total		*D. cayenensis*	**8**	**0**	**5**	**2**	**0**	**1**
2004 and 2005 total		*D. rotundata*	**659**	**5**	**250**	**154**	**232**	**170**
2004 and 2005 total		*D. dumetorum*	**3**	**0**	**1**	**0**	**1**	**1**

[a]number of fields sampled; [b]number of plants sampled; [c]number of plants infected

Table 2.2 Multiple infections detected in yam in four agro-ecological zones sampled in Benin in 2004 and 2005

Agro-ecological zone	Yam species	Occurrence[a]	Frequency [b]	Most abundant multiple infections[c]
2004				
Zone Cotonnière du Centre Bénin	*D. alata*	15/21 (71.4)	15/20 (75.0)	A (12/15)
	D. cayenensis	1/8 (12.5)	1/6 (16.7)	A (1/1)
	D. rotundata	18/45 (40.0)	18/39 (46.2)	A (11/18)
Zone Cotonnière du Nord Bénin	*D. rotundata*	7/22 (31.8)	7/16 (43.8)	A (4/7)
Zone Ouest-Atacora	*D. alata*	1/5 (20.0)	1/3 (33.3)	A (1/1)
	D. rotundata	4/12 (33.3)	4/9 (44.4)	A(2/4), B (2/4)
Zone Vivrière du Sud Borgou	*D. alata*	5/13 (38.5)	5/12 (41.7)	A (4/5)
	D. rotundata	35/78 (44.9)	35/72 (48.6)	A (28/35)
Screen-house	*D. alata*	4/23 (17.4)	4/9 (44.4)	C (2/2), D (2/2)
2005				
Zone Cotonnière du Centre Bénin	*D. alata*	17/29 (58.6)	17/29 (58.6)	A (12/17)
	D. dumetorum	1/1 (100.0)	1/1 (100.0)	B (1/1)
	D. rotundata	40/166 (24.1)	40/115 (34.7)	B (18/40)
Zone Cotonnière du Nord Bénin	*D. alata*	9/11 (81.8)	9/9 (100.0)	A (6/9)
	D. rotundata	8/56 (14.3)	8/31 (25.8)	A (4/8)
Zone Ouest-Atacora	*D. alata*	3/3 (100.0)	3/3 (100.0)	A (2/3)
	D. dumetorum	0/2 (0.0)	0/2 (0.0)	NA
	D. rotundata	20/108 (18.5)	20/51 (39.2)	B (7/20)
Zone Vivrière du Sud Borgou	*D. alata*	9/11 (81.8)	9/11 (81.8)	A (7/9)
	D. rotundata	38/172 (22.1)	38/117 (32.4)	B (22/38)

[a] Number of plants infected with more than one viruses/number of plants tested (%)
[b] Number of plants infected with more than one viruses/number of plants infected (%)
[c] A = badnavirus + YMMV, B = badnavirus + YMV, C = CMV +badnavirus, D = CMV +badnavirus +YMMV (Number of plants with the specified multiple infection/total number of multiple infection)
NA = not applicable

Table 2.3 Combination of multiple infections encountered in four yam species in four agro-ecological zones and in the screen-house in Benin in 2004 and 2005

Combinations	**Frequency**	**Zones[a]**					**Species[b]**		
		1	**2**	**3**	**4**	Screen-house	**1**	**2**	**3**
2004									
Cucumber mosaic virus + Badnavirus	5	1	0	1	1	2	2	0	3
Cucumber mosaic virus + *Yam mild mosaic virus*	1	0	1	0	0	0	0	0	1
Badnavirus + *Yam mild mosaic virus*	62	4	32	2	24	0	17	1	44
Badnavirus + *Yam mosaic virus*	8	0	3	2	3	0	0	0	8
Yam mild mosaic virus + *Yam mosaic virus*	2	0	1	0	1	0	0	0	2
Cucumber mosaic virus + Badnavirus + *Yam mild mosaic virus*	5	0	1	0	2	2	5	0	0
Badnavirus + *Yam mild mosaic virus* + *Yam mosaic virus*	7	2	2	0	3	0	1	0	6
2005									
Cucumber mosaic virus + Badnavirus	1	0	0	0	1	-	0	0	1
Badnavirus + *Yam mild mosaic virus*	43	7	10	9	17	-	27	0	16
Badnavirus + *Yam mosaic virus*	54	4	23	7	20	-	3	1	50
Yam mild mosaic virus + *Yam mosaic virus*	24	4	8	3	9	-	0	0	24
Cucumber mosaic virus + Badnavirus + *Yam mild mosaic virus*	1	0	0	1	0	-	1	0	0
Badnavirus + *Yam mild mosaic virus* + *Yam mosaic virus*	20	1	6	3	10	-	5	0	15
Cucumber mosaic virus + Badnavirus + *Yam mild mosaic virus* + *Yam mosaic virus*	2	1	0	0	1	-	2	0	0

[a] 1 = Zone Cotonnière du Nord Bénin, 2 = Zone Vivrière du Sud Borgou,
3 = Zone Ouest Atacora, 4 = Zone Cotonnière du Centre Bénin

[b] 1 = *Dioscorea alata*, 2 = *Dioscorea cayenensis (2004) or Dioscorea dumetorum (2005)*,
3 = *Dioscorea rotundata,*

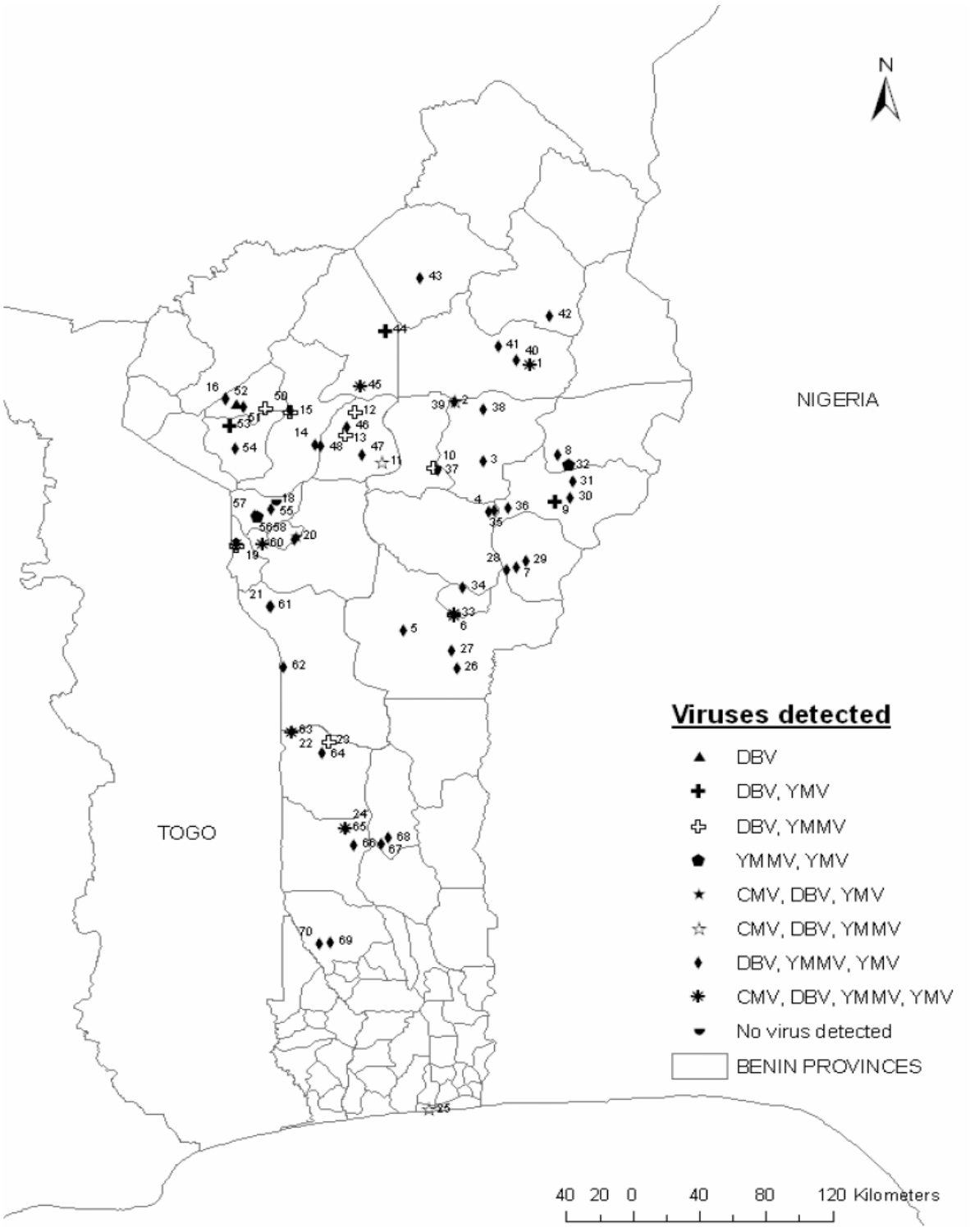

Figure 2.1 Map of Benin showing the locations sampled and viruses detected in each location. 1-29 = 2004 locations, 30-74 = 2005 locations

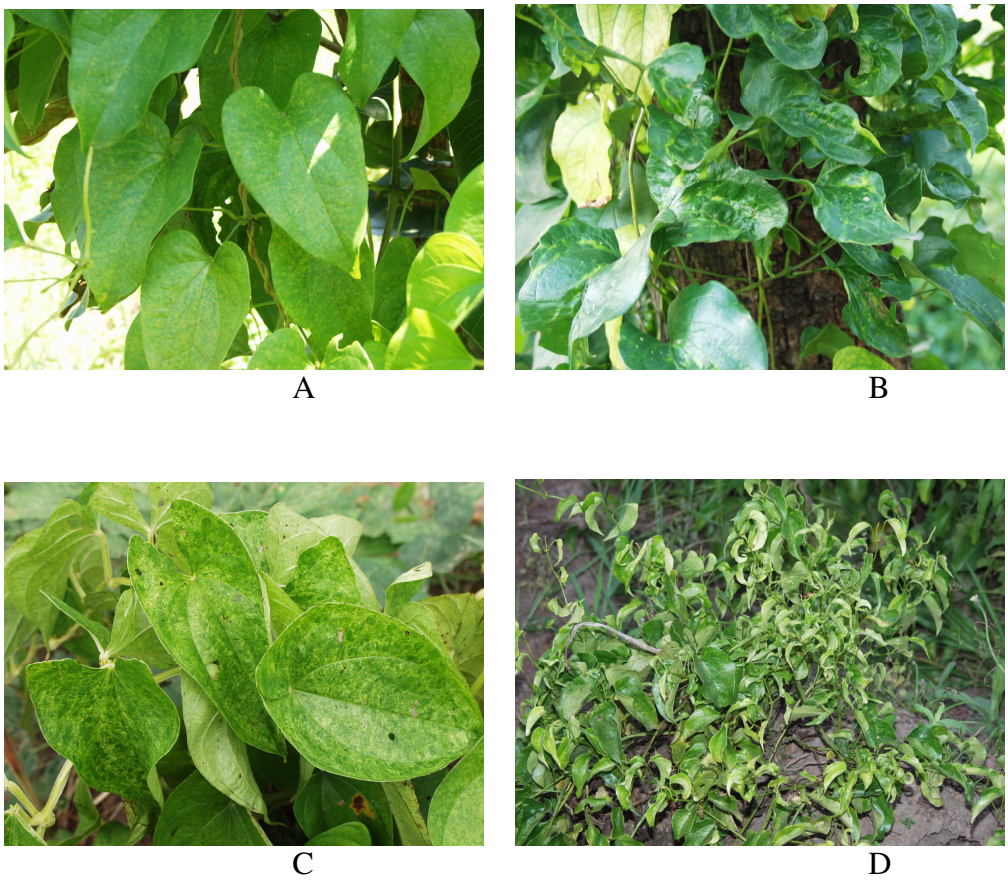

Figure 2.2 Symptoms of virus infection of yam in Benin
A. Chlorotic spots on leaf sample with mixed infection of badnavirus and YMMV B. Mosaic and puckering on leaf sample with mixed infection of badnavirus and YMV C. Mosaic on leaf sample with mixed infection of badnavirus and YMV D. Shoe stringing on leaf sample with YMV

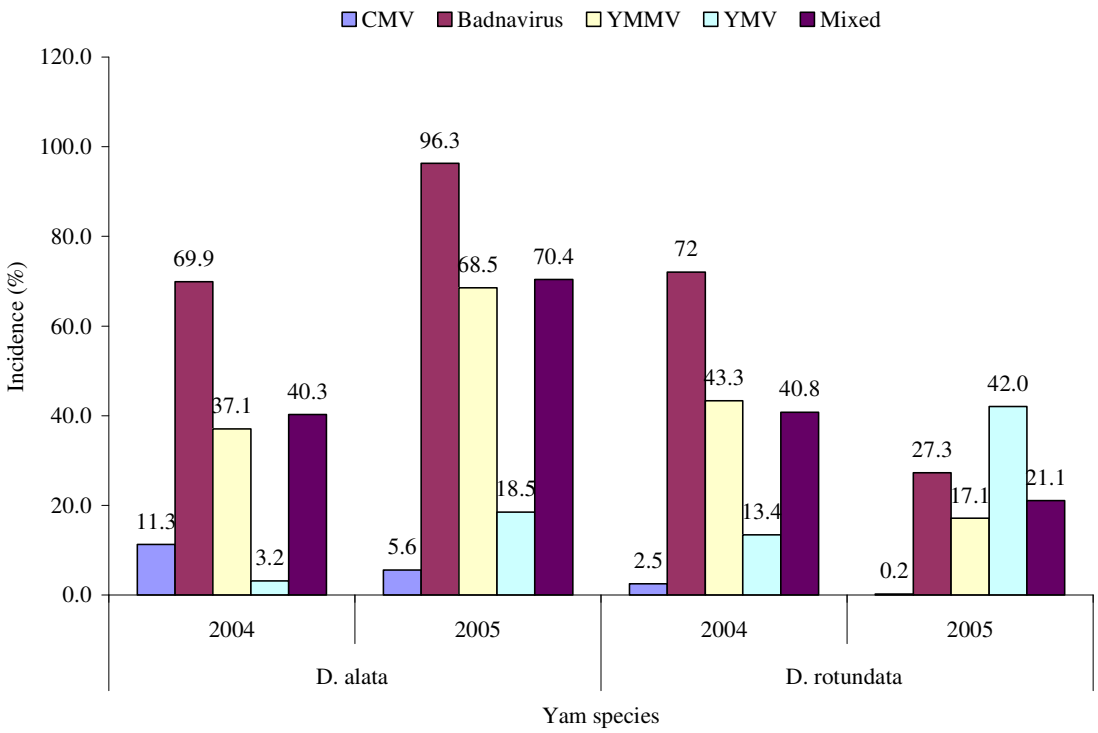

Figure 2.3 Prevalence (%) (2004) and incidence (%) (2005) of *Cucumber mosaic virus* (CMV), *Yam mild mosaic virus (YMMV)*, *Yam mosaic virus* (YMV), yam-infecting badnaviruses (DaBV or DsBV) and mixed infections in *Dioscorea alata* and *Dioscorea rotundata* in 70 locations sampled in Benin.

CHAPTER 3 – SURVEY OF THE INCIDENCE AND DISTRIBUTION OF VIRUSES INFECTING YAM IN GHANA AND TOGO

Eni, A.O., P. Lava Kumar, R. Asiedu, O.J. Alabi and R.A. Naidu, J.d'A. Hughes, M.E.C. Rey (2008) First Report of Cucumber mosaic virus in yams (*Dioscorea* spp.) in Ghana, Togo, and Republic of Benin in West Africa. *Plant Disease* **92(5)**:833 DOI: 10.1094/PDIS-92-5-0833B

Eni A.O., J d'A Hughes, R. Asiedu, P. Lava Kumar and M.E.C. Rey. Survey of the incidence and distribution of viruses infecting yam in the major yam producing zones in Ghana and Togo. Manuscript in preparation

CHAPTER 3 – SURVEY OF THE INCIDENCE AND DISTRIBUTION OF VIRUSES INFECTING YAM IN GHANA AND TOGO

Abstract

Yam leaves from fields in major yam producing agro-ecological zones in Ghana (N = 628) and Togo (N = 218) were collected during surveys in 2005. Leaf tissues were analyzed for *Cucumber mosaic virus* (CMV), *Dioscorea mottle virus* (DMoV), *Yam mild mosaic virus* (YMMV), *Yam mosaic virus* (YMV) and yam-infecting badnaviruses (DaBV and DsBV) by enzyme-linked immunosorbent assay (ELISA), immunocapture polymerase chain reaction (IC-PCR) and/or IC-reverse transcription-PCR (IC-RT-PCR). Eighty-one percent (370/459) and 78.9% (127/161) of symptomatic leaf samples from Ghana and Togo, respectively and 56.2% (95/169) and 57.9% (33/57) of non-symptomatic leaf samples, reacted positive to CMV, YMMV, YMV and/or badnavirus, but DMoV was not detected. Yam-infecting badnavirus (DaBV and/or DsBV) and YMV had the highest incidence in the Guinea Savannah and Forest-Savannah transition agro-ecological zones (AEZ) of Ghana respectively. Badnavirus was detected in 176 of 372 leaves tested and YMV was detected in 119 of 256 leaves tested in the two AEZ. Incidence of badnavirus was between 50 to 57.9% across the zones in Togo. Highest incidence of badnavirus (57.9%), YMV (34.2%) and CMV (7.9%) in Togo, was found in Savane Derivée Seche AEZ. *Dioscorea alata* was more heavily infected than *D. rotundata* in both countries. Mixed infection by badnavirus and YMMV was the most frequent (105 of 276 mixed infections) in the two countries.

3.1 Introduction

Yam is important for food, income and socio-cultural activities in many countries in West Africa (Nweke *et al*., 1991; Degras, 1993) which contributed over 93% of the world's production of over 51.4 million tonnes (mt) in 2006 (FAO, 2007). Yam was ranked first among 20 most important food and agricultural commodities (ranked by value) in Ghana and Togo in 2005 followed by cassava, cocoa beans and plantain in Ghana and maize, cassava and fresh vegetables in Togo (FAO, 2006). Ghana exports the largest quantity of yam (about 12 000 t) annually but yam production in Ghana has been on the decline despite the increasing demand for local consumption and for export (Tetteh and Saakwa, 1991). Analysis of responses to questionnaires sent to scientists in 14 sub-Saharan African (SSA) countries in 2001 revealed that diseases caused by viruses are major production constraint on yam in their countries. However, knowledge of type of viruses occurring in the West Africa sub-region is limited (Offie, 2001). Although there have been reports of virus-like symptoms and virus diseases on yam in Ghana and Togo (Olatunde 1999; Gumedzoe *et al*., 2001, Phillips *et al*., 1999), information on the virus identities implicated in some of these virus disease infections and the incidence and distribution of these viruses is lacking. In Ghana yam is grown in the Guinea Savannah and the Forest-Savannah Transition agro-ecological zones while in Togo major yam production is in the Savane Derivée Humide, Forêt Decidue et semi decidue de montagne and Savane Derivée Seche. Information on disease incidence is necessary for researchers, policy makers and donors in order to determine research priorities. Yam virus distribution maps for these countries will assist extension workers and growers in making decisions on

procurement and production of virus-free planting materials and assist national agricultural programs on the dissemination of healthy planting material to farmers. In this chapter, data from yam virus surveys conducted in major yam producing agro-ecological zones in Ghana and Togo during 2005 is presented.

3.2.1 Materials and methods

3.2.1 Survey

A survey was conducted between July and August 2005 to determine the incidence and distribution of viruses infecting yam in the Guinea Savannah and the Forest-Savannah Transition agro-ecological zones (AEZ) in Ghana and the Savane Derivée Humide, Forêt Decidue et semi decidue de montagne and Savane Derivée Seche AEZ in Togo. One yam field in the Savane Cotière AEZ was also sampled in Togo. In each of the locations, extension workers with the national agricultural research stations (NARS) identified major yam fields in their districts particularly those having disease problems. Yam fields were surveyed by walking across a diagonal and scoring plant for the presence or absence of virus symptoms. The species of each plant was recorded and for symptomatic plants, symptom type was recorded. Young leaves were collected both from symptomatic and non-symptomatic plants along the diagonal, five to ten paces apart, depending on the field size. Leaf samples from symptomatic plants outside the diagonal survey path that showed symptoms that were not encountered along the diagonal were also collected for virus testing. Leaf samples were stored in a mobile refrigerator at 4ºC while in the field. Some leaves from each

plant were later transferred into Eppendorf tubes and stored in liquid nitrogen for PCR while others were dried over calcium chloride for later processing. All the yam sampled were mostly at the mid developmental stage. *Dioscorea alata* L*, Dioscorea cayenensis* Lam, *Dioscorea dumetorum* (Kunth) Pax, *Dioscorea rotundata* Poir *Dioscorea bulbifera* L. *and* an unidentified wild species were sampled in Ghana while. *D. alata, D. cayenensis*, *D. dumetorum*, and *D. rotundata* were sampled in Togo.

3.2.2 Enzyme-linked immunosorbent assay (ELISA)

Triple antibody-sandwich (TAS) ELISA was used for the detection of YMV and DMoV while protein-A sandwich (PAS) ELISA was used for the detection of badnavirus (DaBV and DsBV), YMMV and CMV. Rabbit polyclonal antisera and monoclonal antibodies against YMV (YMV-M24), known to detect YMV isolates from seven West African countries were used for YMV detection (Njukeng *et al*., 2002). For YMMV detection, rabbit polyclonal antibodies working at 1:250 dilutions for trapping and 1:6400 for detecting in PAS-ELISA and known to show no cross reactivity with other potyviruses was used (Odu *et al*., 1999). Rabbit polyclonal antisera against CMV, DaBV, and DMoV, and monoclonal antibodies to DMoV routinely used for the certification of virus-tested plantlets at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, were used for the detection of these viruses.

The rabbit polyclonal antibody against DaBV is now known to detect, but not differentiate, the two characterised yam-infecting badnavirus, DaBV and DsBV (Chapter 5). Therefore subsequent mention of badnavirus, in this chapter, would refer to DaBV and/or DsBV.

TAS-ELISA was carried out as previously described by Thottappilly *et al*. (1998) with slight modifications. Wells of microtitre plates were coated with 100 µl of YMV or DMoV polyclonal antibodies diluted 1:1000 in coating buffer (0.05 M sodium carbonate buffer, pH 9.6) and incubated at 37^{o}C for 2 h. The plates were blocked with 200 µl of 5% skimmed milk powder (Marvel; Premier International Foods, UK) for 1 h at 37^{o}C and 200 µl of leaf sap extract, prepared by grinding the test leaves in grinding buffer (phosphate buffered-saline containing 0.05 % (v/v) Tween-20 (PBS-T), 0.5 mM polyvinyl pyrrolidone (PVP)-40 and 79.4 mM Na_2SO_3) was loaded in duplicate wells and incubated overnight at 4^{o}C. Hundred µl of YMV or DMoV mice monoclonal antibodies diluted in PBS-T was added to the wells and the plates were incubated at 37^{o}C for 2 h. Goat anti-mouse alkaline phosphatase diluted at 1:40,000 in conjugate buffer (half strength PBS containing 0.05% (v/v) Tween-20, 0.02% (w/v) egg albumin, 0.005 mM PVP-40) was added to each well and incubated at 37 ^{o}C for 2 h. Then 200 µl p-nitrophenylphosphate substrate (pNPP) (1 mg ml^{-1} in 10% diethanolamine, pH 9.8) was added into each of the wells to detect the antigen-antibody reactions.

Protein-A sandwich (PAS)-ELISA followed the method described by Edwards and Cooper (1985). Wells of a microtitre plate were coated with 100 µl of protein-A (Sigma, UK) diluted at 1 µg ml^{-1} in coating buffer and incubated at 37°C for 2 h. CMV, YMMV and badnavirus rabbit polyclonal antibodies were diluted in PBS-T buffer to 1:3000, 1:250 and 1:1000, respectively, and 100 µl of these were added into the respective wells of ELISA plate and they were incubated at 37°C for 2 h. Leaf sap extracts (~200 µl) prepared as described previously were added into the wells in duplicate and incubated at 4°C overnight. Then, 100 µl of CMV, YMMV or badnavirus rabbit polyclonal antibodies at 1:3000, 1:6400 and 1:1000, respectively, were added into the respective wells and plates were incubated at 37°C for 2 h. Protein-A alkaline phosphatase diluted at 1:50,000 in conjugate buffer was added to each well and the plate incubated for 2 h at 37°C. The antigen-antibody reactions were detected using pNPP substrate as described before.

The plates were washed three times at 3 minute intervals with PBS-T after each incubation step for both TAS- and PAS-ELISA, but not after the milk blocking step in TAS-ELISA. Leaf sample extract from healthy yam was used as negative controls and virus isolates of CMV, DMoV, YMMV and DaBV available at IITA-Ibadan, were used as positive controls. The A_{405} for the substrate in each well was measured in a DYNEX MRX microplate reader 1 h after the addition of pNPP substrate.

3.2.3 Immunocapture-polymerase chain reaction (IC-PCR)/immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR)

IC-PCR was used for the detection of badnavirus (DaBV and DsBV) and IC-RT-PCR was used for the detection of CMV, YMV and YMMV. Leaf samples were not tested for DMoV by PCR-based methods. Immunocapture was carried out using polyclonal antibodies diluted 1:3000 for CMV, 1:250 for YMMV, 1:1000 for both badnavirus and YMV. The following primer sets were used;

CMV Primer 1 5′ GCC GTA AGC TGG ATG GAC AA 3′, Primer 2 5′ TAT GAT AAG AAG CTT GTT TCG CG 3′, Wylie *et al.* (1993)

BadnaFP 5′-ATG CCI TTY GGI ITI AAR AAY GCI CC-3′, Badna RP 5′-CCA YTT RCA IAC ISC ICC CCA ICC-3′, Seal and Muller (2007)

YMMV Forward 5′-GGC ACA CAT GCA AAT GAA RGC-3′, Reverse 5′-CAC CAG TAG AGT GAA CAT AG-3′, Mumford & Seal (1997)

YMV Forward 5′-ATC CGG GAT GTG GAC AAT GA-3′, Reverse 5′-TGG TCC TCC GCC ACA TCA AA-3′, Mumford & Seal (1997)

Immunocapture was done using a modification of the coating and trapping method by Clark and Adams (1977). PCR tubes (200 µl, ABgene, UK) were coated with antibodies, incubated and washed as described in Section 3.2.2. The coated tubes were loaded with 200 µl of leaf sap prepared as described in Section 3.2.2. Tubes were incubated at 4°C overnight. After washing three times with PBS-T and once with distilled water, 25 µl of reaction cocktail consisting of 1 x Taq reaction buffer (10mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl, 0.01% Triton-X 100), 0.25 mM of each dNTP, 50 pmol each of badnavirus forward and reverse primers and 1.25

U of Taq DNA polymerase (Promega, USA) was added to each tube and PCR was carried out for the detection of badnavirus in a PTC-200 Peltier Cycler (MJ Research Inc., USA) using the following themocyclic regime; 94°C for 4 min, 94°C for 30 s, 50°C for 30 s, 72°C for 30 s. The cycle was repeated 40 times excluding the first step, and then a final extension was done at 72°C for 5 min.

For the detection of CMV, YMV and YMMV, after the immunocapture step as described above, RT-PCR was done using QIAGEN Onestep RT-PCR kit (Qiagen, Germany) according to the manufacturer's protocol. Twenty-five pmol each of CMV, YMV or YMMV forward and reverse primers was added to the cocktail and 25 µl of the cocktail was dispensed into each pre-coated tube. Reverse transcription was done at 50°C for 30 min followed by a PCR activation step at 95°C for 15 min. PCR continued with 40 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. This was followed by a final extension step of 72°C for 10 min.

Ten microliters of IC-PCR and IC-RT-PCR products were analysed on a 1.5% agarose gel in TAE (40 mM Tris-acetate pH 8.3, 1 mM EDTA) buffer at 100 V for 1 h. The 1-kbp plus DNA ladder (Invitrogen) was ran along with the PCR products for size estimation. The gel was stained in ethidium bromide and observed on a UV-transilluminator.

3.3 Results

3.3.1 Virus survey

In Ghana, 27 and 19 fields were surveyed in the Guinea savannah zone and Forest-Savannah Transition zone, respectively (Figure 3.1). In Togo, 8, 7, 4 and 1 yam fields were surveyed in the Savane Derivée Humide, Forêt Decidue et semi decidue de montagne, Savane Derivée Seche and Savane Cotière zones, respectively (Figure 3.1). A total of 628 and 218 yam leaf samples were collected for virus screening from Ghana and Togo, respectively. Sample size and viruses detected in the various zones and in the various yam species are summarized in Table 3.1 a & b. Both in Ghana and in Togo, leaf mosaic and chlorosis were the most common of the 13 symptoms types observed. Other symptoms observed were bleaching, crinkling, distortion, leaf curl, mottle, puckering, shoe stringing, vein banding, vein clearing, whitish bleaching and leaves showing retarded growth (Figure 3.2). The distribution and proportion of the various symptom types observed in the fields varied across the different species in both countries. In Ghana, leaves with retarded growth and leaf curl were only observed in *D. rotundata*; while leaf symptoms of crinkling, distortion, shoe stringing and whitish bleaching on leaf lamina were observed only on *D. rotundata* and *D. alata* but not in any of the other yam species. One *D. dumetorum* plant and all 15 wild *Dioscorea species* sampled were asymptomatic. In Togo, leaf curl was not observed in any of the plants sampled and leaves with retarded growth, crinkling, distortion, shoe stringing, vein banding and whitish bleaching of leaf lamina were observed only in *D. rotundata* but not any of the other species. Similar to the

observation in Ghana, all the three *D. dumetorum* plants sampled in Togo were asymptomatic.

3.3.2 Virus incidence

Leaf samples with mean absorbance values at 405 nm (A_{405}) twice or more than that of the healthy leaves were considered as virus positive by ELISA (Thottappilly *et al*., 1998). Leaf samples that showed the expected band sizes of 500 bp, 249 bp 586 bp and 579 bp, for CMV, YMMV, YMV and badnavirus, respectively, were considered to be virus infected in PCR assay (Mumford & Seal 1997; Wylie *et al.,* 1993; Seal and Muller, 2007).

Eighty-one percent (370/459) and 78.9% (127/161) of symptomatic leaf samples from Ghana and Togo, respectively, reacted positively by ELISA and/or PCR to at least one of the five viruses tested. Among the asymptomatic leaf samples, 95/169 (56.2%) from Ghana and 33/57 (57.8%) from Togo, tested positive to at least one virus. CMV, YMMV, YMV and badnavirus were detected but DMoV was not detected in any of the leaf samples tested. A summary of results is presented in Table 3.1 a & b. Badnavirus had the highest incidence in both countries (43.8% in Ghana and 52.3% in Togo). CMV was detected in 13/628 (2.0%) and 6/218 (2.8%) in Ghana and Togo, respectively, and was found in both agro-ecological zones in Ghana and in three of the four zones in Togo. CMV occurred in both *D. alata* and *D. rotundata* in both countries but not in any of the other yam species. Except in one *D. rotundata* leaf sample in each country, CMV occurred in mixed infection with badnavirus,

YMMV and/or YMV. Mild chlorosis was observed in the leaf sample that had a single infection of CMV in Ghana while the leaf with the single infection of CMV in Togo, was from an asymptomatic plant. Symptoms associated with other CMV positive leaf samples were bleaching, chlorosis and crinkling in Ghana, and bleaching and crinkling in Togo. Fifty-three percent (7/13) and 50% (3/6) of CMV positive leaf samples in Ghana and Togo, respectively, were from asymptomatic plants. This is the first confirmed report of CMV infection in yam in Ghana and Togo.

3.3.3 Distribution across zones

CMV, YMMV, YMV and badnavirus (DaBV and/or DsBV) were detected in all major yam producing agro-ecological zones surveyed in both countries. In Ghana, incidence of CMV (2.3%) and YMV (46.5%) were higher in the Guinea savannah zone while the incidence of badnavirus (47.3%) and YMMV (28%) were higher in the Forest-Savannah Transition zone (Table 3.1 a & b). In Togo, incidences of badnavirus in all four zones were similar ranging from 50% – 57.9%. Highest incidence of badnavirus (57.9%), YMV (34.2%) and CMV (7.9%) in Togo, was in the Savane Derivée Seche AEZ. The highest incidence of YMMV (35.3%) was recorded in Savane Derivée Humide whereas the lowest incidence of YMV (8.3%) was observed in Savane Cotière though this zone is not a major yam producing zone and only one yam field was sampled in this zone (Table 3.1 a & b).

3.3.4 Virus distribution across species

Very few plants of *D. cayenensis* (twelve in Ghana, nine in Togo), *D. dumetorum (*one in Ghana, three in Togo), *D. bulbifera* (six in Ghana) found in some fields and 15 unidentified wild species found near some yam fields in Ghana were collected and tested but due to the limited number, virus incidences in these species (Table 3.1 a & b) are not compared with those in *D. alata and D. rotundata* which are the two major yam species sampled. Both in Ghana and Togo, incidence of CMV, badnavirus and YMMV was higher in *D. alata* than in *D. rotundata* (Figure 3.3). Of the 107 *D. alata* leaves sampled in Ghana, 11, 96 and 78 tested positive for CMV (10.3%), badnavirus (89.7%) and YMMV (72.9%), respectively. In Togo, CMV was detected in two of the 28 (7.1%) *D. alata* leaves sampled while badnavirus and YMMV were detected in 25 (89.7%) and 27 (96.4%) *D. alata* leaves, respectively (Figure 3.3). The incidence of YMV was higher in *D. rotundata* in Ghana (214/487 leaf samples) and in Togo (62/178 leaf samples) compared to *D. alata* (Figure 3.3).

3.3.5 Mixed infections

A considerable number of leaf samples were infected with a mixture of more than one virus. Of the 465 infected yam leaves from Ghana, 203 (43.6%) had a mixture of two or more viruses while in Togo 73 of the 160 infected leaf samples (45.6%) had mixed infections (Table 3.1a and 3.1b). Double infections occurred more frequently both in Ghana (166/203 (81.7%)) and Togo (58/73 (79.4%)) compared to triple infections and only four *D. alata* leaf samples from Ghana and one *D. rotundata* from Togo were simultaneously infected with CMV, YMMV, YMV and badnavirus (Table

3.2a and 3.2b). The combination of badnavirus and YMMV was the most frequent double infection encountered in Ghana (78 of 203 mixed infections) and Togo (27 of 73 mixed infections), followed by the combination of badnavirus and YMV (Table 3.2 a & b). A mixture of badnavirus, YMMV and YMV was the most frequent triple infection combination in both countries (Table 3.2 a & b). Incidence of mixed infection was concurrently higher in *D. alata* compared to *D. rotundata* in all the zones in both countries and the most abundant double infection detected in *D. alata* was badnavirus and YMMV while badnavirus and YMV was the most frequently double infection detected in *D. rotundata* (Table 3.3 a & b).

3.4 Discussion

This study was undertaken to assess the incidence and distribution of five major viruses known to infect yam in West Africa. CMV, YMMV, YMV and badnavirus were detected in both countries, but DMoV was not detected in any of the leaf samples tested. Occurrences of badnavirus and YMV have been reported previously in Ghana and Togo (Olatunde, 1999; Gumedzoe *et al*., 2001; Phillips *et al*., 1999) however, information on the other viruses infecting yam in these countries was unavailable. CMV was detected for the first time in yam in these countries. Although the incidence of CMV in both countries was low, CMV infection was found to be widespread and was detected in 12 fields in Ghana and 5 fields in Togo and was detected both in *D. alata and D. rotundata*. With regards to distribution, CMV was present in the two and three major yam producing agro-ecological zones in Ghana and Togo, respectively. CMV is known to have the widest host range of over 800

species (Palukaitis *et al*., 1992), and is spread naturally by more than 60 aphid species hence its ability to cause virus epidemics in many economically important crops.

The detection of badnavirus in all the fields sampled and in all yam species encountered during this survey, makes badnavirus the most widely spread yam virus in Ghana and Togo. Badnavirus can therefore be considered a prominent viral threat in the yam system in both countries, although its actual effect on yield needs to be investigated. The higher incidence of badnavirus and YMMV infections in *D. alata* compared to *D. rotundata* in Ghana and Togo is similar to the findings of Hughes *et al.* (1997) on the distribution of these viruses in Nigeria. Furthermore, higher incidence of YMV observed in *D. rotundata* compared to *D. alata* in both countries, is also similar to the distribution of YMV in Nigeria (Hughes *et al.*, 1997) and in some parts of Ghana (Olatunde, 1999). The high incidence of virus infection observed in *D. alata* in Ghana and Togo was similarly observed in *D. alata* leaf samples from Benin (Eni *et al*., in press). Though the most widely distributed yam species globally, its high susceptibility to diseases is a major limitation to the profitable and sustainable production of *D. alata*, which is also a favoured alternative to *D. rotundata* for diabetic patients (Abang *et al.*, 2003; Riley *et al*., 2006).

The number of virus infections detected by IC-RT-PCR/IC-PCR was more than the corresponding ELISA tests, possibly due to the greater sensitivity of IC-RT-PCR/IC-PCR. The specificity and sensitivity of both assays for the detection of these viruses have been previously evaluated and determined (Njukeng *et al*., 2002; Odu *et al*.,

1999; Hughes *et al*., unpublished; Njukeng *et al*., 2005; Mumford and Seal 1997). The lower sensitivity observed with the ELISA tests is similar to the findings of Mumford and Seal (1997) and Njukeng *et al*., (2005) and could also be due to low virus concentration in yam (Brunt *et al*., 1990) or due to interference of polyphenols and glutinous polysaccharides contained in yam leaves (Rossel and Thottappilly, 1985). However, some ELISA-positive leaf samples were not detected by IC-RT-PCR/IC-PCR. Lebas *et al.* (1999) obtained similar results while working with yam from the South Pacific islands. This may suggest some variability in the virus genomes or may be due to failure of PCR techniques due to inhibiting compounds found in yam (Lebas *et al*., 1999; Wilson, 1997).

The very high frequency of mixed infections observed in all the yam species sampled and their detection in almost all the fields sampled both in Ghana and Togo is of concern. However, the occurrence of single infections of each virus and the varying mixtures observed in both countries, show that the tests conducted accurately discriminates between these viruses in single and mixed infections. Most of the leaf samples with mixed virus infections (185/203 in Ghana and 61/73 in Togo) were from plants showing virus-like symptoms.

There were no clear correlations between the symptom phenotypes observed and the specific viruses detected since most of the symptom types were associated with the several viruses detected. Some plants showing virus-like symptoms tested negative, by both ELISA and PCR, to all five viruses indexed. The possible

explanations for symptoms observed on these leaf samples are (i) symptoms may be caused by other virus(es) for which tests were not done or that are yet unidentified; (ii) Symptoms may also be due to abiotic factors causing virus-like symptoms, such as nutrient disorder and senescence. It is also notable that a high percentage of leaves from non-symptomatic plants (56.2% and 57.8% from Ghana and Togo, respectively), tested positive to CMV, YMMV, YMV and/or badnavirus. This shows that absence of visual symptoms on yam leaves may not be indicative of absence of virus infection, but laboratory diagnosis serves as a more sensitive and conclusive way of affirming the health status of potential planting materials. Use of planting materials (seed tubers) obtained from healthy plants for yam cultivation remains a major way of limiting the spread of yam viruses. The results of this study indicate a wide distribution of badnavirus, YMMV and YMV and high incidence of mixed infections in both countries surveyed. This may be attributed to the local use of virus-infected planting materials. *Dioscorea mottle virus* which was not detected in any of the leaf samples tested is therefore a quarantine pest in Ghana and Togo. Exchange of planting and breeding materials should take this into consideration. Efforts should also be made to monitor the spread of CMV infections in yam particularly around the Upper West and the Volta regions in Ghana where CMV was not detected.

3.5 References

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Table 3.1a. Occurrence of four yam viruses in five yam species in two yam producing agro-ecological zones in Ghana

				Viruses present[c]				
Agro-ecological zone	**N[a]**	**Yam species**	**N[b]**	**CMV**	**Badna virus**	**YMMV**	**YMV**	**Mixed**
Forest-Savannah Transition	19	*D. alata*	52	5	44	31	13	32
		D. bulbifera	1	0	1	1	0	1
		D. cayenensis	10	0	2	1	4	0
		D. rotundata	185	1	47	31	98	49
		Unidentified wild	8	0	5	1	4	4
		Sub total	256	6	99	65	119	86
Guinea savannah	27	*D. alata*	55	6	52	47	12	45
		D. bulbifera	5	0	2	5	0	2
		D. cayenensis	2	0	0	0	0	0
		D. dumetorum	1	0	1	0	1	1
		D. rotundata	302	1	121	52	116	69
		Unidentified wild	7	0	0	0	4	0
		Sub total	372	7	176	104	133	117
		Total	**628**	**13**	**275**	**169**	**252**	**203**

[a]number of fields sampled; [b]number of plants sampled; [c]number of plants infected

Table 3.1b Occurrence of four yam viruses in four yam species in three yam producing agro-ecological zones in Togo

Agro-ecological zone	N[a]	Yam species	N[b]	Viruses present[c]				
				CMV	Badna virus	YMMV	YMV	Mixed
Forêt Decidue et semi decidue de montagne	7	*D. alata*	8	1	6	8	0	6
		D. cayenensis	9	0	3	1	2	2
		D. rotundata	66	1	33	8	26	17
		Sub total	83	2	42	17	28	25
Savane Derivée Humide	8	*D. alata*	16	0	15	16	3	15
		D. rotundata	68	1	29	13	22	16
		D. dumetorum	1	0	0	1	0	0
		Sub total	85	1	44	30	25	31
Savane Derivée Seche	4	*D. alata*	3	1	3	2	0	2
		D. rotundata	33	2	18	8	13	11
		D. dumetorum	2	0	1	1	0	1
		Sub total	38	3	22	11	13	14
Savane Cotière*	1	*D. alata*	1	0	1	1	0	1
		D. rotundata	11	0	5	3	1	2
		Sub total	12	0	6	4	1	3
		Total	**218**	**6**	**114**	**62**	**67**	**73**

[a]number of fields sampled; [b]number of plants sampled; [c]number of plants infected
*Not a major yam producing zone

Table 3.2a Combination of multiple infections encountered in three yam species in two agro-ecological zones in Ghana

Combinations	**Frequency**	**Zones[a]**		**Species[b]**					
		1	**2**	**1**	**2**	**3**	**4**	**5**	**6**
Cucumber mosaic virus + *Yam mosaic virus*	1	1	0	0	1	0	0	0	0
Badnavirus + *Yam mild mosaic virus*	78	29	49	50	24	0	3	0	1
Badnavirus + *Yam mosaic virus*	59	27	32	2	53	0	0	1	3
Yam mild mosaic virus + *Yam mosaic virus*	28	15	13	1	27	0	0	0	0
Cucumber mosaic virus + Badnavirus + *Yam mild mosaic virus*	5	1	4	5	0	0	0	0	0
Cucumber mosaic virus + Badnavirus + *Yam mosaic virus*	1	1	0	1	0	0	0	0	0
Cucumber mosaic virus + *Yam mild mosaic virus* + *Yam mosaic virus*	1	1	0	1	0	0	0	0	0
Badnavirus + *Yam mild mosaic virus* + *Yam mosaic virus*	26	9	17	13	13	0	0	0	0
Cucumber mosaic virus + Badnavirus + *Yam mild mosaic virus* + *Yam mosaic virus*	4	2	2	4	0	0	0	0	0
Total	203	86	117	77	118	0	3	1	4

[a] 1 = Forest-Savannah Transition, 2 = Zone Guinea savannah

[b] 1 = *Dioscorea alata*, 2 = *Dioscorea rotundata*, 3 = *Dioscorea cayenensis,* 4 = *Dioscorea bulbifera,* 5 = *Dioscorea dumetorum,* 6 = *Species unknown*

Frequency = number of leaf samples with the specified mixed infection

Table 3.2b Combination of multiple infections encountered in four yam species in four agro-ecological zones in Togo

Combinations	**Frequency**	**Zones[a]**				**Species[b]**			
		1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Cucumber mosaic virus + Badnavirus	1	0	0	0	1	0	1	0	0
Cucumber mosaic virus + *Yam mosaic virus*	1	0	0	0	1	0	1	0	0
Badnavirus + *Yam mild mosaic virus*	27	7	2	14	4	19	7	0	1
Badnavirus + *Yam mosaic virus*	24	14	0	8	2	0	22	2	0
Yam mild mosaic virus + *Yam mosaic virus*	5	1	0	2	2	0	5	0	0
Cucumber mosaic virus + Badnavirus + *Yam mild mosaic virus*	2	1	0	0	1	2	0	0	0
Badnavirus + *Yam mild mosaic virus* + *Yam mosaic virus*	12	1	1	7	3	3	9	0	0
Cucumber mosaic virus + Badnavirus + *Yam mild mosaic virus* + *Yam mosaic virus*	1	1	0	0	0	0	1	0	0
Total	73	25	3	31	14	24	46	2	1

[a] 1 = Forêt Decidue et semi decidue de montagne, 2 = Savane Cotière,
3 = Savane Derivée Humide, 4 = Savane Derivée Seche

[b] 1 = *Dioscorea alata*, 2 = *Dioscorea rotundata*, 3 = *Dioscorea cayenensis, 4 = Dioscorea dumetorum*

Frequency = number of leaf samples with the specified mixed infection

Table 3.3a Frequency of multiple infections detected in five yam species in two yam producing agro-ecological zones in Ghana

Agro-ecological zone	Yam species	Occurrence[a]	Frequency[b]	Most abundant multiple infections[c]
Forest-Savannah Transition	*D. alata*	32/52 (61.5)	32/50 (64.0)	A (21/32)
	D. bulbifera	1/1 (100.0)	1/1 (100.0)	A (1/1)
	D. cayenensis	0/10 (0.0)	0/7 (0.0)	NA
	D. rotundata	49/185 (26.5)	49/123 (39.8)	B (23/49)
	Unidentified wild	4/8 (50.0)	4/6 (66.7)	B (3/4)
Guinea savannah	*D. alata*	45/55 (81.8)	45/55 (81.8)	A (29/45)
	D. bulbifera	2/5 (40.0)	2/5 (40.0)	A (2/2)
	D. cayenensis	0/2 (0.0)	0/0 (0.0)	NA
	D. dumetorum	1/1 (100.0)	1/1 (100.0)	B (1/1)
	D. rotundata	69/302 (22.8)	69/213 (32.4)	B (30/96)
	Unidentified wild	0/7 (0.0)	0/4 (0.0)	NA
	Total	**203/628 (32.3)**	**203/465 (43.7)**	

[a] Number of plants infected with more than one viruses/number of plants tested (%)
[b] Number of plants infected with more than one viruses/number of plants infected (%)
[c] A = badnavirus + YMMV, B = badnavirus + YMV, NA = not applicable (Number of plants with the specified multiple infection/total number of multiple infection)

Table 3.3b Frequency of multiple infections detected in four yam species in three yam producing agro-ecological zones in Togo

Agro-ecological zone	Yam species	Occurrence[a]	Frequency[b]	Most abundant multiple infections[c]
Forêt Decidue et semi decidue de montagne	*D. alata*	6/8 (75.0)	6/8 (75.0)	A (5/6)
	D. cayenensis	2/9 (22.2)	2/4 (50.0)	A (2/2)
	D. rotundata	17/66 (25.8)	17/48 (35.4)	B (12/17)
Savane Derivée Humide	*D. alata*	15/16 (93.8)	15/16 (93.8)	A (12/15)
	D. rotundata	16/68 (23.5)	16/45 (35.6)	B (8/16)
	D. dumetorum	0/1(0.0)	0/1 (0.0)	NA
Savane Derivée Seche	*D. alata*	2/3 (66.7)	2/3 (66.7)	NA
	D. rotundata	11/33 (33.3)	11/27 (40.7)	C (3/11)
	D. dumetorum	½ (50.0)	1/1 (100.0)	A (1/1)
Savane Cotière*	*D. alata*	1/1 (100.0)	1/1 (100.0)	A (1/1)
	D. rotundata	2/11 (18.2)	2/6 (33.3)	NA
	Total	**73/218 (33.5)**	**73/160 (45.6)**	

[a] Number of plants infected with more than one viruses/number of plants tested (%)
[b] Number of plants infected with more than one viruses/number of plants infected (%)
[c] A = badnavirus + YMMV, B = badnavirus + YMV, C = badnavirus + YMMV + YMV, NA = not applicable (Number of plants with the specified multiple infection/total number of multiple infection)
* Not a major yam producing zone

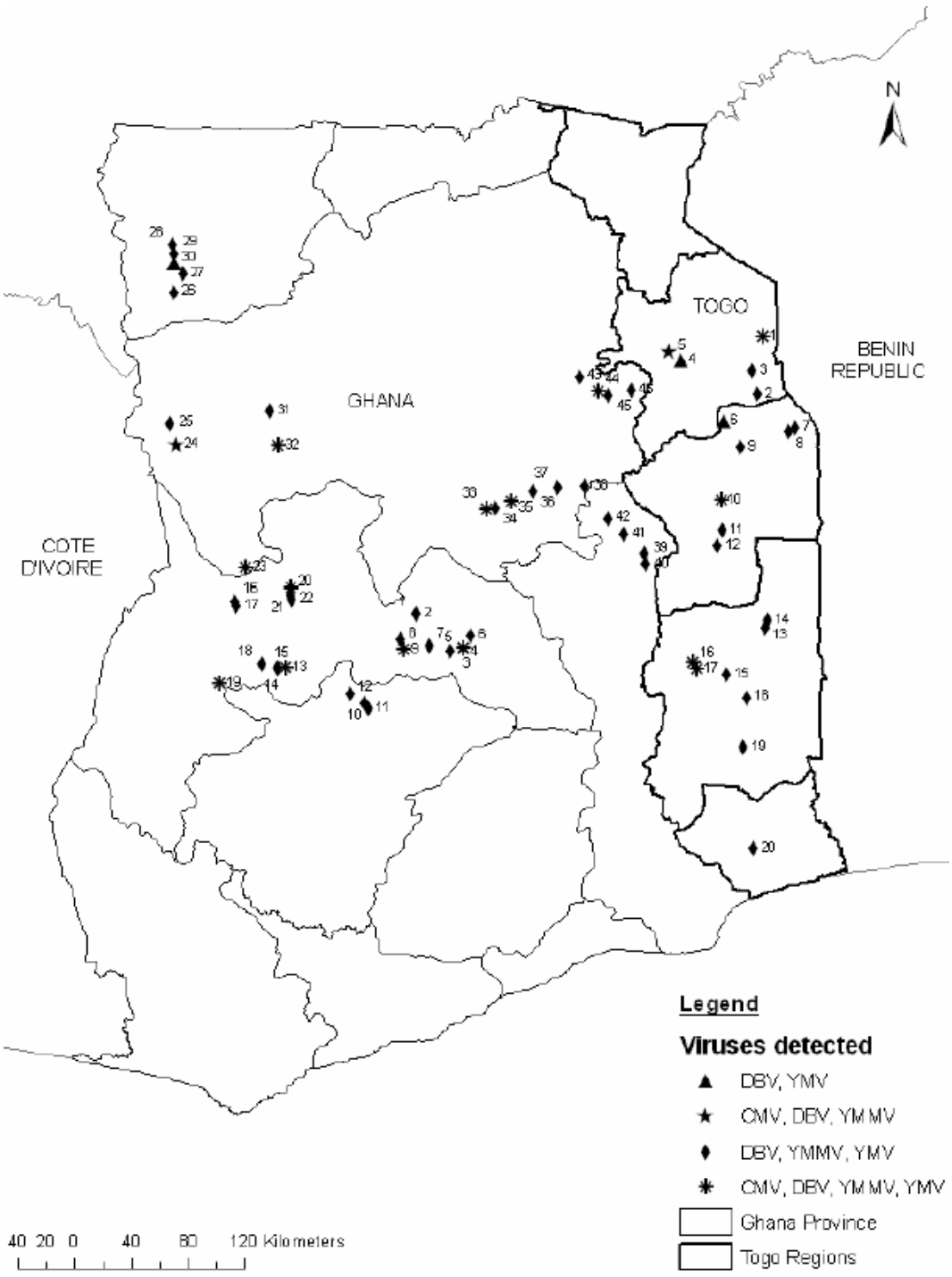

Figure 3.1 Maps of Ghana and Togo Benin showing the locations sampled and viruses detected in each location. 1-46 = Ghana locations, 1-20 = 2005 locations

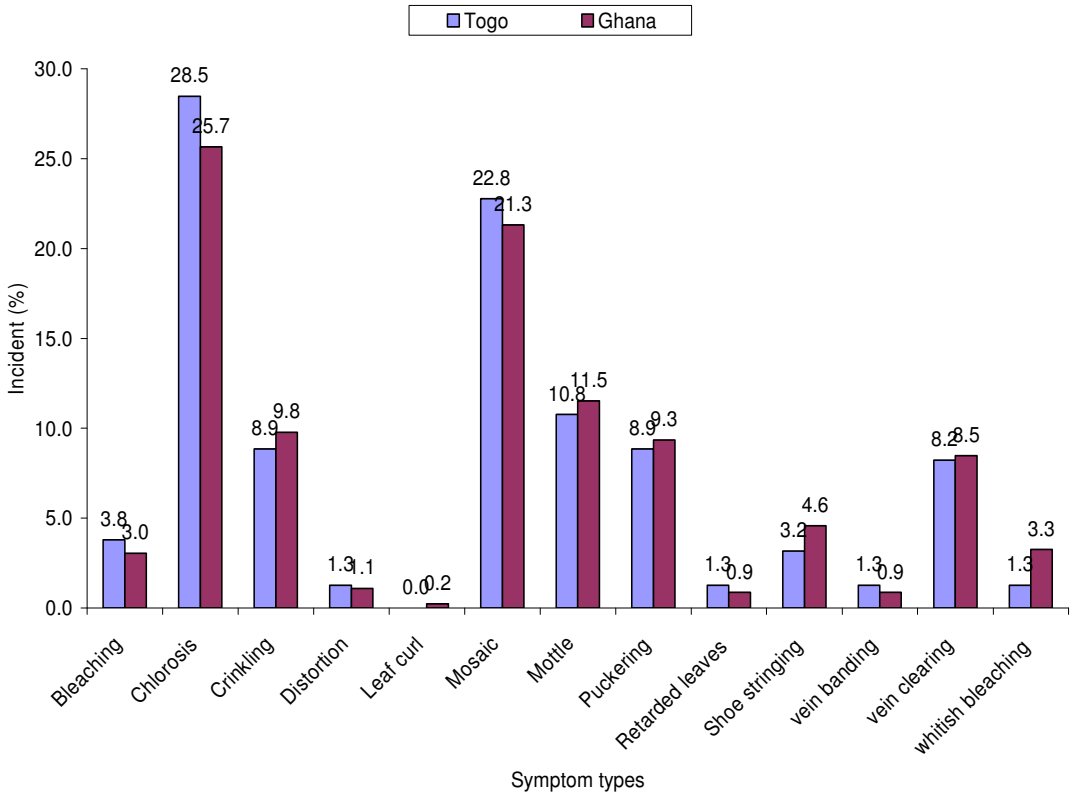

Figure 3.2 Proportion of samples exhibiting the major symptoms observed in yam in Ghana and Togo

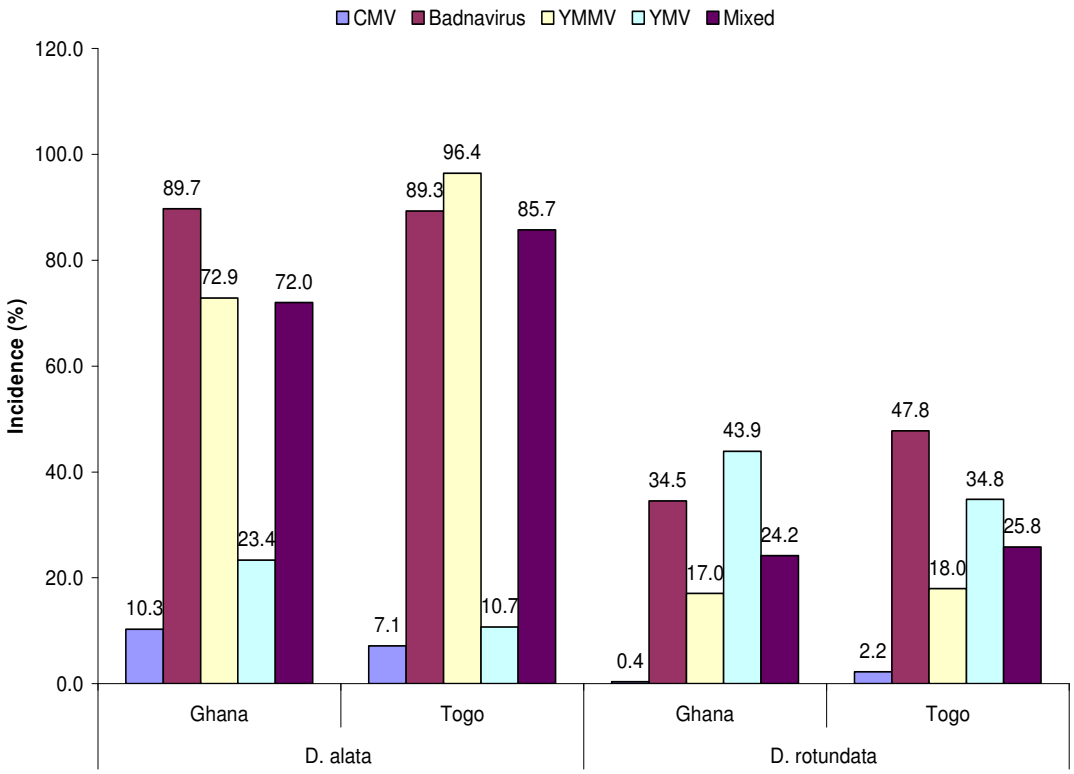

Figure 3.3 Incidence (%) of *Cucumber mosaic virus* (CMV), Badnavirus (DaBV or DsBV), *Yam mild mosaic virus* (YMMV), *Yam mosaic virus* (YMV) and multiple infections in *Dioscorea alata and Dioscorea rotundata* in Ghana and Togo

CHAPTER 4 – PRODUCTION OF POLYCLONAL ANTIBODIES TO DIOSCOREA BACILLIFORM VIRUS AND AN ISOLATE OF CUCUMBER MOSAIC VIRUS INFECTING YAMS (DIOSCOREA SPECIES)

Abstract

Serological techniques are of great importance for plant virus identification and characterization. The major limiting factor to the use of these techniques for yam viruses is the limited availability of antibodies. Antibodies against yam viruses are not readily available (only available in a few laboratories and not in large volumes). Furthermore, the financial investment required for many of the other laboratory-based diagnostic procedures makes it difficult for national agricultural research systems (NARS) and farmers in developing countries to get accurate and timely diagnosis of virus diseases. Rabbit Polyclonal antibodies were produced against purified preparations of a yam isolate of *Cucumber mosaic virus* (CMV) and an isolate of yam-infecting badnavirus from *D. alata* in Nigeria. Protein-A sandwich (PAS) enzyme-linked immunosorbent assay (ELISA) and antigen coated plate (ACP) ELISA were used to determine the titre of the two antibodies. The CMV antiserum had a titre of 1:25,600 and 1:64,000 by PAS- and ACP-ELISA, respectively. The badnavirus antiserum had a titre of 1:1280 both by PAS-ELISA and ACP-ELISA. The detection limits of the two polyclonal antibodies were determined by PAS-ELISA using serial dilutions of infected sap. The sap dilution end point was 1:160 and 1:20 for CMV and the badnavirus, respectively. The antibodies of each virus detected homologous antigen in infected yam leaves from Nigeria, Ghana, Togo and Benin. The antibodies produced in this study will enhance the monitoring of the

spread of both viruses and contribute to prevention of virus spread particularly for CMV infection which is rapidly spreading in yam.

4.1 Introduction

Virus disease infections are one of the major constraints to yam production and have been reported in all yam producing areas of the world (Thouvenel and Fauquet, 1979; Duterme *et al*., 1996, Hughes *et al.*, 1997; Aleman-Verdaguer *et. al.*, 1997). Results of surveys conducted in Ghana, Togo and Benin in 2004 and/or 2005 (Chapters two and three) revealed that badnaviruses were the most important of the four yam viruses detected in all three countries. They had the highest incidence (detected in 45.3% of 1632 yam leaves tested) and the widest distribution (present in 97.7% of 136 locations sampled). Results of these surveys also revealed for the first time, the occurrence of *Cucumber mosaic virus* (CMV) in yam in these three countries. CMV infection in yam has previously been reported only in Guadeloupe, Côte d'Ivoire and Nigeria (Migliori and Cadilhac, 1976; Fauquet and Thouvenel, 1987; Hughes *et. al.*, 1997). CMV has the widest host range of any known plant virus, infecting over 800 plant species worldwide (Palukaitis *et. al*., 1992). It is a potential cause of virus epidemics in many economically important crops and up to 60% yield losses have been reported (Jones, 2000).

Swift and accurate diagnosis is vital for monitoring and control of plant viruses and for certification and breeding purposes. Diagnosis of yam viruses in the field and the laboratory is based on symptomatology, use of indicator plants, vector transmission,

electron microscopy and protein and nucleic acid-based diagnostics (Goudou-Urbino *et al*., 1996; Mumford and Seal, 1997; Hughes *et al*., 1998). The use of symptomatology, either in the natural yam host or herbaceous indicator plant species, for diagnosis of yam viruses is unreliable due to the variability of symptoms caused by changes in environmental factors, differences in the yam cultivars or varieties of test plants and/or the strains of the virus(es). Also, nutrient deficiency symptoms are sometimes confused with virus symptoms and symptomless infections are known to occur (Rossel and Thottappilly, 1985; Brunt *et. al*., 1990). Very high levels of mixed virus infections observed in yam leaves (Chapter two and three) further complicates the possibility of using visual symptoms for virus identification. Nucleic acid-based diagnostics are very sensitive, and can be used to test for 3-4 viruses at a time. However, sample preparation procedures for these nucleic acid-based tests are lengthy and the reagents and equipment used are expensive and dependent on services such as electricity, refrigeration and maintenance which may not always be available in developing and under-developed countries. Enzyme-linked immunosorbent assay (ELISA) is more routinely used for the diagnosis of yam viruses (Hughes *et al*., 1998) and ELISA analysis requires antibodies with broad reactivity to ensure detection of all or most strains of a virus.

The objective of this study was to produce polyclonal antibodies against a yam isolate of CMV and an isolate of yam-infecting badnavirus. Serological and molecular differences have been reported for CMV isolates (Palukaitis *et al.,* 1992; Roossinck *et al*., 1999) and antibody against the yam isolate of CMV is currently not

available. Furthermore, one of the central objectives for which Gatsby Charitable Foundation UK funded this research, was to produce and disseminate yam virus antibodies to national agricultural research stations (NARS) in sub-Saharan Africa.

4.2 Materials and methods

4.2.1 Virus propagation

Yam leaves that tested positive by PAS-ELISA to CMV using antibodies described in chapter two were used for mechanical inoculation. CMV infected yam leaves were ground in inoculation buffer (0.1 M phosphate buffer pH 7.7, containing 10 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM L-cystein) at a ratio of 1:5 w/v (5 ml/g of leaf tissue) using a sterile mortar and pestle. The sap extract was mechanically inoculated on to carborundum (600 mesh) dusted upper surface of fully expanded cotyledons leaves of one-week-old *Cucumis sativus* L. The inoculated cotyledons were immediately rinsed with tap water. All inoculations were done in an insect proof screen house. After seven days, symptomatic leaves from the inoculated plants were tested by ELISA for CMV, badnavirus, *Yam mosaic virus* (YMV), *Yam mild mosaic virus* (YMMV), and *Dioscorea mild mottle virus* (DMoV) using antibodies available at the International Institute of Tropical Agriculture (IITA), Nigeria. Test plants that were positive only to CMV were propagated on *C. sativus*. Symptomatic leaves were harvested and used for CMV purification.

Tubers from *D. alata* cv. Sagbe (TDr 92-2) plants infected with badnavirus were planted in an insect proof screen house (28-32°C) for virus isolation. Symptomatic

leaf samples were tested by ELISA for badnavirus, YMV, YMMV, DMoV and CMV. Source plants that were positive only to badnavirus were selected for the virus purification.

4.2.2 Virus Purification

Purification of Cucumber mosaic virus (CMV)

CMV purification was done using the method described by Roossinck and White (1998). Freshly harvested CMV infected *C. sativus* leaves were placed in a chilled blender jar and blended for two minutes using 1 ml of buffer A (0.5 M sodium citrate, pH 7.0, 5 mM EDTA, 0.5% (v/v) thioglycolic acid) and 1 ml of cold (4°C) chloroform for each gram of tissue. The homogenate was transferred into chloroform - resistant centrifuge tubes and centrifuged at 4°C in a Hermle Z232K refrigerated centrifuge at 15,000 *g* for 10 minutes. The aqueous phase was filtered through cheese cloth dampened with buffer A and the filtrate was transferred into clean 50 ml centrifuge tubes, under laid with 10 ml of cushion I (0.5 M sodium citrate, pH 7.0, 5 mM EDTA, 10% (w/v) sucrose) using a 10 ml glass pipette and centrifuged at 212,000 *g* for 1.5 h at 4°C in a Beckman L5-50B ultracentrifuge. The supernatant was quickly removed and 5 ml of buffer B (5 mM sodium borate, pH 9.0, 5 mM EDTA, 2% (v/v) triton X-100) was added to the pellet in each tube and the pellets were allowed to re-suspend overnight at 4°C. The pellets in buffer B were vortexed briefly and allowed to stir for 2 h at 4°C. After stirring, the suspension was centrifuged at 7500 *g* for 10 minutes at 4°C. The supernatant was removed into an ultracentrifuge tube, under-laid with 5 ml of cushion II (5 mM sodium borate, pH 9.0, 5 mM EDTA,

10% (w/v) sucrose) and centrifuged again for 212,000 *g* for 1.5 h at 4°C. The supernatant was poured off and 2 ml of autoclaved buffer C (5 mM sodium borate, pH 9.0, 5 mM EDTA) added to each of the pellets. Pellets were allowed to re-suspend at 4°C overnight. The virus preparation was stored in buffer C at -20°C before use.

Using 5 as the extinction coefficient for CMV (Francki *et al*., 1966), the concentration of the purified virus preparation was calculated by spectrophotometry assuming $E^{0.1\%}_{260} = 5 = 1$mg CMV/ml

Purification of yam-infecting badnavirus

Badnavirus isolation followed a modification of the methods by Adomako *et al*. (1983). Infected leaves were ground in a chilled blender in 600 ml of extraction buffer (0.1M phosphate buffer pH 7.2 containing 0.01M diethyldithiocarbamate (DIECA) and 0.5% (w/v) hemicellulase). The homogenate was incubated for 2.5 h at room temperature and then at 4°C overnight. The mixture was then filtered through cheese cloth and centrifuged at 3,000 *g* for 20 min. Six percent (w/v) NaCl and 9% (w/v) polyethylene glycol (PEG) was added to the supernatant, dissolved at room temperature and stirred for 2.5 h. The mixture was centrifuged at 2,500 *g* for 3 min and the precipitated virus preparation was re-suspended in 200 ml of re-suspension buffer I (0.05M phosphate buffer pH 7.0 containing 0.01 M DIECA, 0.2 M NaCl and 0.01 % (v/v) 2- mercaptoethanol). After overnight incubation at 4°C, the mixture was centrifuged at 8,000 *g* for 20 min, the supernatant was retained. The pellet was re-suspended in re-suspension buffer I, incubated at room temperature for 30 min and centrifuged at 8000 *g* for 20 min. The two supernatants were combined and 2 g of

pre-washed celite (Fluka, Switzerland) was added and the mixture was filtered under vacuum through Whatman No. 1 filter paper to remove the celite (Fluka, Switzerland). The filtrate was centrifuged at 6,000 *g* for 2 min to sediment all remaining celite (Fluka, Switzerland). The supernatant was then centrifuged at 785,000 *g* for 2.25 h. The pellet in each centrifuge tube was re-suspended in 0.5 ml of re-suspension buffer II (0.02 M citrate buffer pH 6.1) and allowed to re-suspend properly at 4°C overnight. The virus preparation was stored at -20°C before use.

4.2.3 Molecular weight determination

To determine the protein molecular weight of the purified virus preparations and to confirm their purity and the absence of other contaminating protein before use for antibody production, the purified preparation of CMV and badnavirus were subjected to a discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the protocol described by Laemmli (1970). A 12.5% resolving gel and a 4% stacking gel were used (Sambrook *et al*., 1989).

A 12.5 % resolving gel was prepared by mixing 2.5 ml of 1.5 M Tris-HCl, pH 8.8, 100 µl of 10% SDS, 4.0 ml of 30% acrylamide/bis-acryamide (Sigma, USA) and 3.5 ml distilled water. The solution was degassed for 15 minutes and 50 µl of freshly prepared 10% ammonium persulphate and 5µl N, N, N, N-tetramethylethylenediamine (TEMED) (Sigma, Germany) were added for polymerisation. The 4% stacking gel was prepared by mixing 2.5 ml of 0.5M Tris-HCl, pH 6.8, 100 µl 10 % SDS, 1.3 ml of 30% acrylamide/bis-acryamide acrylamide

(Sigma, UK) and 6.1 ml distilled water. After degassing for 15 min at room temperature, 50 µl of 10% freshly prepared ammonium persulphate and 10 µl TEMED were then added to mixture. Fifteen µl of the denatured CMV or badnavirus preparation was loaded into four wells alternated with 3 µl of low range protein molecular weight markers (Sigma, UK) with a Hamilton syringe. Protein separation was achieved by applying 100 V to the electrophoresis tank for 1.5 h. The gels were stained with 0.1% Coomassie brilliant blue R-250 (Sigma, USA) in fixative (45% methanol, 10% acetic acid and 45% distilled water) at room temperature for 30 minutes. The gels were destined overnight in 45% methanol, 10% acetic acid and 45% distilled water.

The molecular weight of the virus coat proteins were estimated by comparing their mobility in the gel with that of the other proteins of known molecular weight as reference molecular weight markers.

4.2.4 Antibody production

Purified CMV and badnavirus preparations from section 4.2.2 were used as antigen for antiserum production in New Zealand white rabbits.

Prior to antigen injection, the rabbits were injected with Ivomectrin (ICOMEC, USA) at the rate of 0.1 ml/kg of animal weight to rid them of any internal and/or external blood sucking parasites that may reduce the quantity of serum obtained after

the experiment. The animals were allowed two weeks after Ivomectrin injection before commencement of antigen injection.

To elicit an immune response in the rabbits, four intramuscular injections of the purified virus preparations were administered over an eight-week period. The first three injections were given at two weeks intervals while the fourth one was given 28 days after the third injection. The injections were alternated between the left and right thigh muscles. The first injection consisted of 1 ml of purified virus preparation emulsified with 1 ml of Freund's complete adjuvant (Sigma, USA), subsequent injections of 1 ml purified virus were emulsified with 1 ml of incomplete Freund's adjuvant (Sigma, USA).

Test bleeds were taken from one of the ear veins of the animals two weeks after the second and third injections for titre determination. A final bleed was done two weeks after the last booster injection. After each bleeding, the collected blood was incubated in a glass beaker at 37°C for 1 h. The clot was ringed with a spatula and further incubated at 4°C overnight to allow the clot to shrink and the serum to completely separate. The serum was collected into micro-centrifuge tubes and centrifuged at 6000 *g* for 5 min to further clarify the serum of remaining red blood cells. The antisera were kept at 4°C before titre determination.

4.2.5 Characterization of antisera

Two ELISA formats, PAS-ELISA and ACP-ELISA, were used for antisera titration. For PAS-ELISA, an initial dilution of 1:100 and 1:10 was made for CMV and badnavirus antisera respectively, a double-fold serial dilutions of each was made and used for titration. Wells of a microtitre plate were coated with 100 µl of protein-A (Sigma, UK) diluted at 1 µg ml^{-1} in coating buffer and incubated at 37°C for 2 h. Each dilution of antisera (100 µl) was loaded into four wells and the plate was incubated at 37°C for 2 h. Sap (200 µl) prepared by grinding CMV or badnavirus infected leaf tissues and healthy yam leaves in grinding buffer (PBS-T containing 0.5 mM polyvinyl pyrrolidone (PVP)-40 and 79.4 mM Na_2SO_3) was loaded in duplicate wells for each antisera dilution and incubated at 4°C overnight. 100 µl of each antisera dilution were incubated in the wells again at 37°C for 2 h for detection. Protein-A alkaline phosphatase diluted at 1:50,000 in conjugate buffer (half strength PBS containing 0.05% v/v Tween-20, 0.02% w/v egg albumin, 0.005 mM PVP-40) was added to each well and the plate incubated for 2 h at 37°C. The alkaline phosphatase conjugates were detected by adding 200 µl p-nitrophenyl phosphate (pNPP) substrate diluted 1 mg ml^{-1} in 10% diethanolamine, pH 9.8, to each of the wells. The A_{405} for the substrate in each well was measured in a DYNEX MRX microplate reader after 1 h.

For ACP-ELISA antisera dilutions were made in conjugate buffer. Initial antisera dilutions of 1:500 or 1:100 were made for CMV and badnavirus, respectively, followed by double-fold serial dilutions of each diluted antisera. Wells of microtitre

plates were coated with 100 µl of sap obtained by grinding CMV or badnavirus infected leaf tissues and healthy yam leaves in coating buffer containing 0.5 mM PVP-40 and 79.4 mM Na_2SO_3. The plates were incubated at 37°C for 2 h and washed as described for PAS-ELISA. The plates were blocked by adding 200 µl of skimmed milk powder (Marvel; Premier International Foods, UK) to each well and incubating for 30 min. at 37^{o}C. After the incubation step, the plates were tapped dry (not washed) and 100 µl of each antisera dilution was loaded into duplicate wells pre-coated with diseased and healthy sap and incubated at 37°C for 2 h. After the washing step, 100 µl of goat anti-rabbit alkaline phosphatase diluted 1:40,000 in conjugate buffer were added to each well and incubated37°C for 2 h. The alkaline phosphatase conjugate was detected as described for PAS-ELISA above.

The detection limit of the two antisera was determined using serial dilutions of CMV or badnavirus-infected sap. Infected leaf tissues were ground in 10 volumes of grinding buffer and then diluted in a two-fold series to 1:640. Two hundred microlitres of infected diluted sap was added to four wells of pre-coated ELISA plate and PAS-ELISA carried out as earlier described. The test for each virus was replicated four times

To assess the reactivity of the antibodies for the detection of CMV and badnavirus isolates, CMV and badnavirus infected yam leaves from Ghana, Togo, Benin and Nigeria were tested by PAS-ELISA using the prepared antibodies. CMV-infected leaves of *C. sativus* and *Nicotiana tabaccum* L. were also tested for CMV and sap

from *Musa* sp. infected with *Banana streak virus* (BSV), genus Badnavirus, were tested using the badnavirus antisera.

4.3 Results

4.3.1 Virus propagation

About 50% of the leaves of the mechanically inoculated *C. sativus* showed mosaic symptoms (Figure 4.1) three days after inoculation. The symptoms intensified progressively with over 70% infected leaves by the seventh day when the plants were harvested for virus purification. Puckering, cricking and intervienal chlorosis were observed on leaves of *D. alata* cv. Sagbe two months after planting. Puckering and crinkling persisted and intensified while the chlorosis became less visible.

4.3.2 Virus purification

Clear glassy pellets were obtained after the final high-speed centrifugation during purification of CMV. The concentration of the purified virus preparation was 0.28 mg/ml using 5 as the extinction coefficient for CMV (Francki *et al*., 1966). The pellets resulting from badnavirus purification were tiny and cloudy, spectrophotometer absorbance value would be unsatisfactory, so spectrophotometry was not done for the badnavirus.

4.3.3 Molecular weight determination

The molecular weight of the purified CMV coat protein was estimated from the known protein molecular weight of the proteins contained in the low range marker. A

single protein band of about 29 KDa was observed (Figure 4.2). The purified badnavirus preparation repeatedly failed to yield any protein band on SDS-PAGE, possibly due to low concentration of the virus in the purified preparation but the purified preparation tested positively for badnavirus by PAS-ELISA using the badnavirus antibody previously described in chapter two.

4.3.4 Characterization of antisera

The titres of the first two test bleeds taken were low for both antisera (result not shown). The titre of the third bleed taken after the forth injection was determined by PAS-ELISA and ACP-ELISA. For CMV detection by PAS-ELISA, the mean absorbance value of infected sap at antibody dilution of 1:100 was 0.798 and that of the healthy sap was 0.340. The A_{405} of the infected sap continued to be more than two times that of the healthy sap up to 1:25,600 dilutions (Figure.4.3). CMV detection by ACP-ELISA continued up to 1:64,000 dilutions (Figure.4.4). The mean absorbance value of badnavirus-infected sap by PAS-ELISA at 1:10 antibody dilution was 2.950 and that of the healthy sap was 1.154. Although the A_{405} of the healthy sap was high initially, the value decreased progressively as the dilution increased. Both for PAS-ELISA and ACP-ELISA, the A_{405} of the infected sap continued to be more than two times that of the healthy sap up to 1:1,280 dilutions (Figure 4.5 and 4.6).

Sap dilution end point was used to determine the sensitivity of both antisera. The mean A_{405} value of 0.879 obtained for CMV infected sap at 1:10 dilution decreased to 0.625 at 1:20 sap dilution. CMV was not detected after 1:160 sap dilutions. Mean

A_{405} value of 0.695 was obtained at 1:10 dilutions of badnavirus-infected sap and 0.301 at 1:20 dilutions relative to 0.146 obtained for the healthy sap. Badnavirus was not detected at further dilutions (Table 4.1 and 4.2)

The antibodies produced detected CMV and badnavirus in infected yam leaves from Nigeria, Ghana, Togo and Benin (Table 4.3). Both viruses were detected in *D. rotundata* and *D. alata*. CMV was also detected in *C. sativus* and *N. tabaccum* while the badnavirus antisera detected BSV in infected *Musa* sp leaf.

4.4 Discussion

Cucumis sativus was used as a propagative host for CMV in this study because the occurrence of yam viruses in low titre and the presence of mucilaginous substances contained in yam leaves are a problem to yam virus purification and antibody production (Thouvenel and Fauquet, 1979; Rossel and Thottappilly, 1985; Brunt *et al*., 1990; Mumford and Seal, 1997). Although DaBV is reported to be transmitted by mechanical inoculations and insect vector to several yam species (Philips *et al.*, 1999; Odu *et al*., 2004), several attempts to transmit the yam infecting badnavirus to herbaceous test plants by mechanical and vector transmission were unsuccessful. Mild mosaic symptoms were observed on leaves of *Nicotiana occidentalis* L. and *Nicotiana benthamiana* L. mechanically inoculated with the badnavirus but subsequent transmission from these test plants were unsuccessful. Transmission experiments using *Planococcus citri* Risso., known vector of DaBV (Philips *et al.*, 1999; Odu *et al*., 2004) and three other mealybug species, *Planococcus solani* Ferris,

Dysmicoccus brevipis Cockerell and *Ferrisia virgata* Cockerell, were unsuccessful. Very low transmission rate (5 of 25 plants inoculated tested positive by PAS-ELISA but showed no visible symptoms on the leaves) was found with *P. citri*. The yam-infecting badnavirus was purified from infected yam leaves from *D. alata* in Nigeria due to the challenges outlined above.

Although badnavirus coat protein band (56 kDa) (Phillips *et al*., 1999) could not be visualized in SDS-PAGE gels, the purified virus preparations reacted to heterologus DaBV antibodies (available at IITA-Ibadan) in ELISA, suggesting that concentration of virus in the purified preparations could be very low. The low virus yields maybe due to interfering mucilaginous substances contained in yam leaves which were used for the purification of the yam-infecting badnavirus in this study and/or due to the occurrence of yam viruses in low titre in yam leaves. Phillips *et al*. (1999) reported low virus yield while extracting DaBV from yam leaves. Similarly in spite of several attempts to purify Taro bacilliform virus (TaBV), Yang *et al*. (2003) reported very low badnavirus-like particles in their purified preparations. Despite the low virus yield, the purified preparations of the yam-infecting badnavirus were used for immunization and as a consequence of low badnavirus yields, antibodies produced had low titre.

For the purified CMV preparation, a single clear band of estimated size 29 kDa was observed in SDS-PAGE. This band size differs from the 24, 25 or 26 kDa previously reported for CMV coat protein (Haq *et al*., 1996; Raj *et al*., 2002). The observed

difference in coat protein size may be due to incomplete denaturation of the coat protein or due to variation in gel concentration used in the various studies. However, positive reactions were obtained when sap from previously tested CMV-infected leaf tissues from IITA were tested using the CMV antibody produced with this virus preparation.

The results obtained for the sensitivity of both antibodies, in relation to sap dilutions, are similar to those obtained for the detection of YMV and YMMV in yam sap by Mumford and Seal (1997). The low sensitivity (1:160 for CMV-infected sap and 1:20 for badnavirus-infected sap), can be attributed to low virus titre and/or inhibitory substances found in yam leaves (Rossel and Thottappilly, 1985; Brunt *et al*., 1990).

The antibodies produced detected CMV and badnavirus in yam leaves from Nigeria, Ghana, Togo and Benin. The CMV polyclonal antibody also detects other CMV isolates from *C. sativus* and *N. tabaccum* and badnavirus polyclonal antibody detected BSV from *Musa sp*. This reactivity of both antibodies is useful in detecting all or most strains of both viruses particularly for CMV which has been reported to consist of two subgroups based on serological and nucleic acid properties (Palukaitis *et al.,* 1992; Roossinck *et al*., 1999).

Phillips *et al*. (1999) reported serological relationships among badnaviruses infecting yam and among badnaviruses in general. In their study, DaBV was trapped

and decorated, in immunosorbent electron microscopy (ISEM), by two antisera raised against Dioscorea bulbifera badnavirus (DbBV), ?genus Badnavirus, *Sugarcane bacilliform virus* (ScBV), *genus Badnavirus*, and *Banana streak virus* (BSV) *genus Badnavirus*. Furthermore, he found that immunoglobulin (IgG) extracted from two DbBV antisera detected both DbBV and DaBV by ELISA though IgG from DaBV detected only the homologous virus. Seal and Muller (2007) have also reported the use of a general badnavirus antibody for the detection of *Dioscorea sansibarensis bacilliform virus* (DsBV) *genus Badnavirus* by ISEM and PAS-ELISA. Results from current studies confirm that badnaviruses infecting yam are serologically related to each other and to other badnaviruses. The polyclonal antibody produced in this study, is useful for reliable virus indexing test(s) for badnaviruses infecting yam both for disease monitoring in field survey samples and for routine laboratory use by national agricultural research stations. However, except a yam-infecting badnavirus is clearly identified by use of specific primers and/or monoclonal antibody, its actual identity remains unknown and it is therefore generally called a yam-infecting badnavirus.

The detection of CMV in yam sap from Ghana, Togo Benin and Nigeria confirms that CMV infects yam naturally possibly through one or more of its numerous aphid vector (Choi *et al*., 1999). CMV infection is reported to be widespread (Palukaitis *et al.,* 1992) and causes virus epidemics and yield losses in many economically important crops. Its recent detection in yam in Ghana, Togo and Benin needs to be monitored as recent results indicate an increase in number of infections and spread in yam (Chapter two and three). Yam is usually intercropped with melon, okra, potatoes,

cowpea and other vegetables which are known CMV host species. These CMV infected crops may act as sources for vector transmission and will complicate efforts to control CMV infection by use of virus-free planting materials. Naturally infected yam plants may also became sources of inoculum when used as planting materials the following year. To monitor the spread of CMV infection in yam and to prevent CMV accumulation in planting stock, farmers need to be adequately advised and health status of planting materials ensured by adequate yam virus indexing.

The antibodies produced in this study will improve the diagnostic capability for detecting and monitoring CMV and badnaviruses in West Africa and contribute to preventing virus spread.

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Table 4.1 Detection of *Cucumber mosaic virus* (CMV) isolates in leaves of yam and other plant species from Ghana, Togo, Benin and Nigeria by Protein-A sandwich enzyme-linked immunosorbent assay (PAS-ELISA)

Country of origin	Yam species	symptom	PAS-ELISA A_{405}	Detection factor[a]
Ghana	*D. rotundata*	Asymptomatic	0.282	2.7
Ghana	*D. alata*	Asymptomatic	0.293	2.8
Togo	*D. alata*	Asymptomatic	0.241	2.3
Togo	*D. rotundata*	Puckering	0.349	3.3
Benin	*D. alata*	Chlorosis, crinkling	0.245	2.3
Benin	*D. rotundata*	Mottle	0.408	3.9
Nigeria	*D. alata*	Chlorosis	0.330	3.1
Nigeria	*D. alata*	Asymptomatic	0.452	4.3
Nigeria	*D. alata*	Healthy	0.105	0.0
Nigeria	*C. sativus*	Mosaic	3.296	29.4
Nigeria	*N. tabaccum*	Mosaic	1.766	15.8
Nigeria	*N. tabaccum*	Healthy	0.112	0.0

[a] A_{405} of infected sap/A_{405} of healthy sap. Value >2 is considered positive

Table 4.2 Detection of badnavirus isolates in yam leaves from Ghana, Togo, Benin and Nigeria; and BSV in leaves of *Musa species* by Protein-A sandwich enzyme-linked immunosorbent assay (PAS-ELISA)

Country of origin	Yam species	Symptoms	PAS-ELISA A_{405}	Detection factor[a]
Ghana	*D. rotundata*	Vein clearing	0.438	3.5
Ghana	*D. alata*	Mottle	0.341	2.7
Togo	*D. alata*	Vein clearing	0.259	2.1
Togo	*D. alata*	Chlorosis	0.257	2.1
Benin	*D. alata*	Asymptomatic	0.630	5.0
Benin	*D. alata*	Puckering	0.436	3.5
Nigeria	*D. alata*	Puckering	0.460	3.7
Nigeria	*D. rotundata*	Chlorosis	0.286	2.3
Nigeria	*D. alata*	Healthy	0.125	0.0
Nigeria	*Musa species*	Chlorotic streak	0.375	2.6
Nigeria	*Musa species*	Chlorotic streak	0.336	2.3
Nigeria	*Musa species*	Healthy	0.146	0.0

[a] A_{405} of infected sap/A_{405} of healthy. Value >2 is considered positive

Table 4.3 Sensitivity of *Cucumber mosaic virus* (CMV) and badnavirus antisera in the detection of CMV and badnavirus in infected yam sap by Protein-A sandwich enzyme-linked immunosorbent assay (ELISA).

Sap dilution	Absorbance at 405 nm		Detection factor[b]	
	CMV	Badnavirus	CMV	Badnavirus
1/10	0.879	0.695	7.4	4.8
1/20	0.625	0.301	5.3	2.1
1/40	0.504	0.233	4.3	1.6
1/80	0.451	0.216	3.8	1.5
1/160	0.304	0.196	2.6	1.3
1/320	0.214	0.191	1.8	1.3
1/640	0.187	0.191	1.6	1.3
Healthy	0.118	0.146	0.0	0.0

[b] A_{405} of infected sap/A_{405} of healthy sap. Value >2 is considered positive

H **LS** **US**

Figure 4.1 A leaf of *Cucumis sativus* infected with a yam isolate of *Cucumber mosaic virus* (CMV). H = Healthy, LS = Lower surface of infected leaf, US = Upper surface of infected leaf

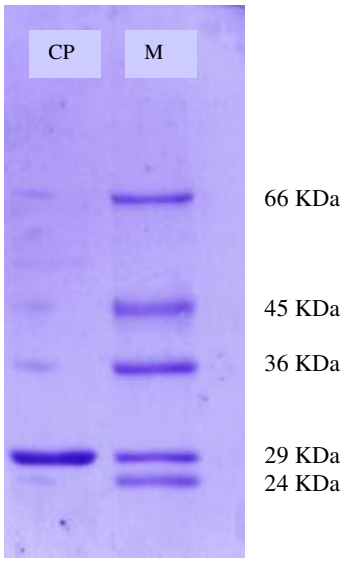

Figure 4.2 An SDS-polyacrylamide gel showing coat protein band from purified preparation of *Cucumber mosaic virus* (CP) and Sigma low range protein molecular weight marker (M).

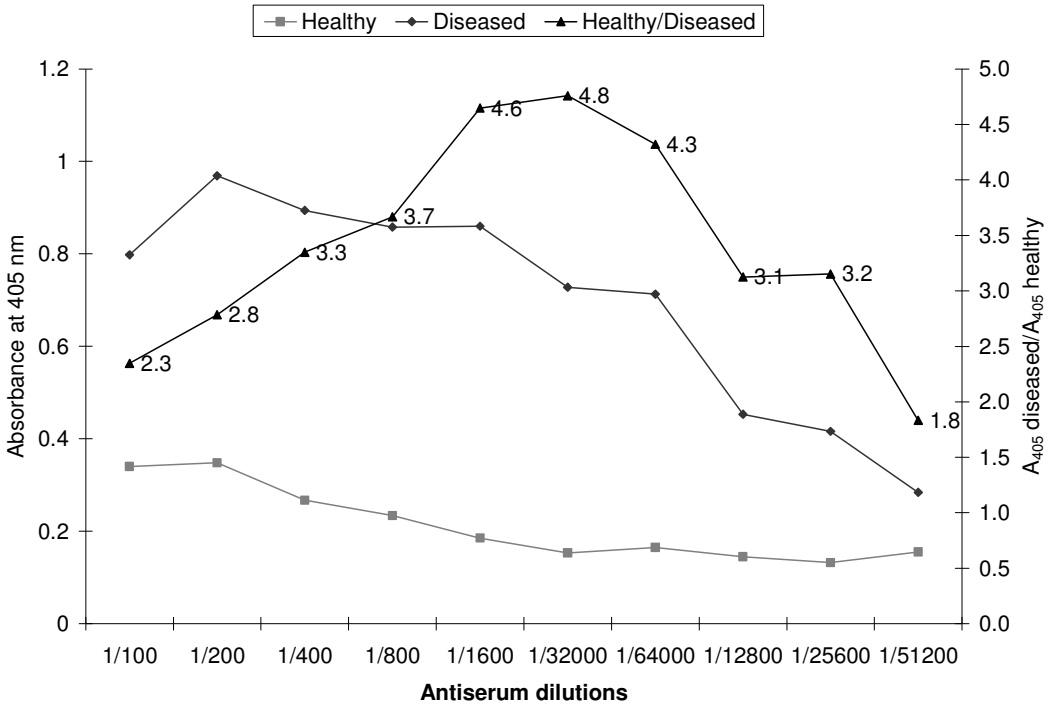

Figure 4.3 Detection of *Cucumber mosaic virus* (CMV) in serially diluted infected and healthy sap (for titre determination) by Protein-A sandwich enzyme-linked immunosorbent assay (PAS-ELISA). A_{405} of infected sap/A_{405} of healthy sap ratio > 2 is considered positive.

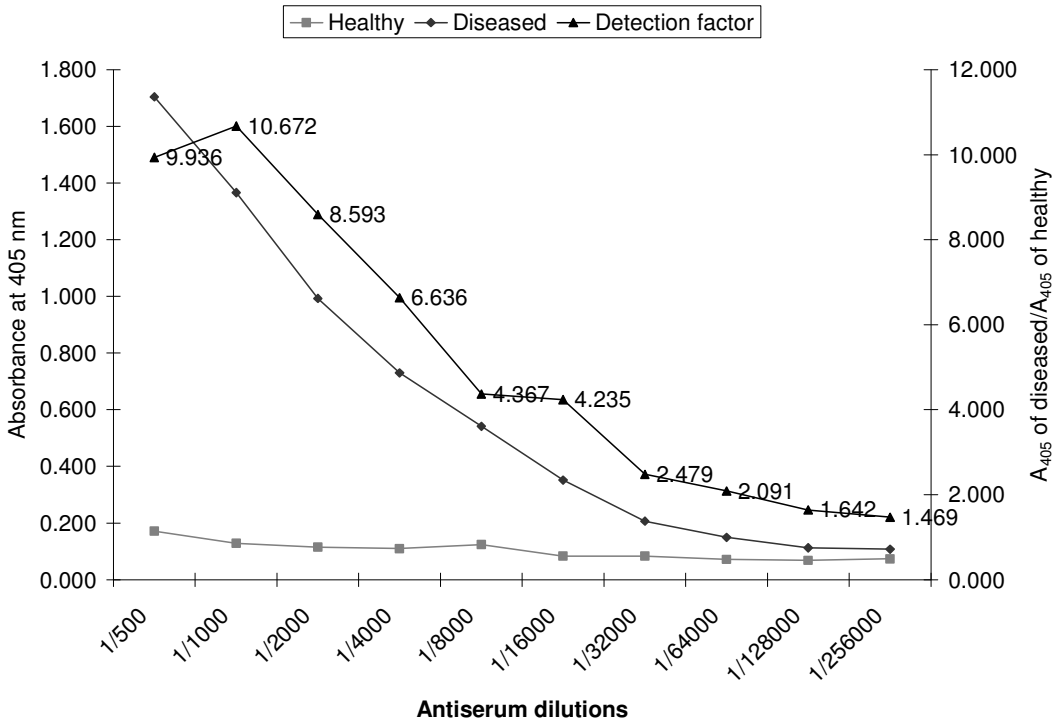

Figure 4.4 Detection of *Cucumber mosaic virus* (CMV) in serially diluted infected and healthy sap (for titre determination) by Antigen coated plate enzyme-linked immunosorbent assay (ACP-ELISA). A_{405} of infected sap/A_{405} of healthy sap ratio > 2 is considered positive.

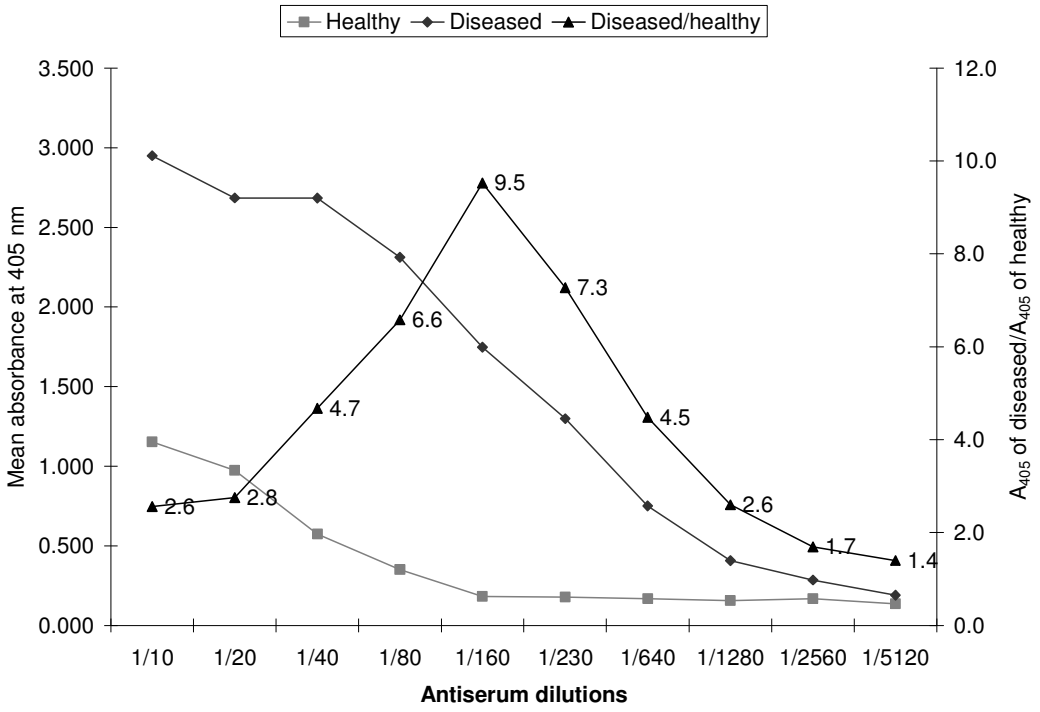

Figure 4.5 Detection of badnavirus in serially diluted infected and healthy sap (for titre determination) by Protein-A sandwich enzyme-linked immunosorbent assay (PAS-ELISA). A_{405} of infected sap/A_{405} of healthy sap ratio > 2 is considered positive.

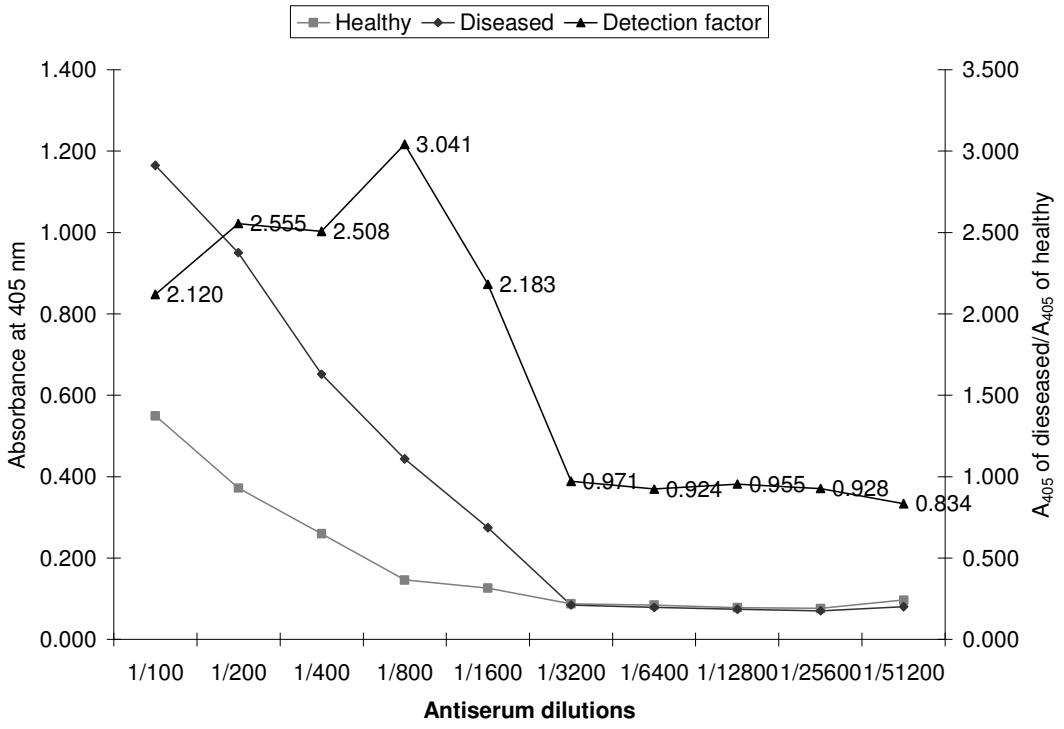

Figure 4.6 Detection of badnavirus in serially diluted infected and healthy sap (for titre determination) by Antigen coated plate enzyme-linked immunosorbent assay (ACP-ELISA). A_{405} of infected sap/A_{405} of healthy sap ratio > 2 is considered positive.

CHAPTER 5- SEQUENCE DIVERSITY AMONG BADNAVIRUS ISOLATES INFECTING YAM IN GHANA, TOGO, BENIN AND NIGERIA.

Eni A.O., J d'A Hughes, R. Asiedu, and M.E.C. Rey. Sequence diversity among Badnavirus isolates infecting Yam in Ghana, Togo, Benin and Nigeria. Archives of Virology (Submitted)

CHAPTER 5 - SEQUENCE DIVERSITY AMONG BADNAVIRUS ISOLATES INFECTING YAM IN GHANA, TOGO, BENIN AND NIGERIA.

Abstract

Analysis of 1632 yam leaves obtained during surveys conducted in 2004 and 2005 in Ghana, Togo and Benin revealed the occurrence of badnaviruses in 45.3% of the leaves tested. Incidence of badnavirus was highest of the four viruses detected and it was the most widely distributed virus in the three countries, detected in 97.7% of 136 locations sampled. To assess the diversity within the badnaviruses infecting yam, nucleotide sequence of the RT/RNaseH-coding region of 19 badnavirus isolates infecting yam in Ghana, Togo, Benin, and Nigeria were amplified by IC-PCR, cloned and sequenced. Phylogenetic analysis of the deduced amino acid sequences revealed that the isolates are broadly divided into two distinct species, each clustering with one of the two previously published yam-infecting badnaviruses, *Dioscorea alata badnavirus* (DaBV) and *Dioscorea sansibarensis bacilliform virus* (DsBV). Isolates within the DaBV cluster had 96-100% amino acid identity to each other and 90-96% amino acid identity with DaBV. The isolates within the DsBV cluster had 94-99% amino acid identity with each other and 83-84% amino acid identity with DsBV. An isolate, designated BN4Dr, from Benin was distinct and had 77% and 75% amino acid identities with DaBV and DsBV, respectively and may be a third badnavirus species infecting yam. Besides the Nigerian isolates which clustered together with DaBV, the two main species were present in Ghana, Togo and Benin and were observed to infect both *D. alata* and *D. rotundata* indiscriminately. Results of this

study demonstrate that two distinct species of badnaviruses infect yam in the West African yam zone and suggests a putative third species, BN4Dr. Results also conclude that these species are not related to geographic origin or host species. However, the three species are serologically related. Further genomic sequencing of these isolates is necessary to confirm their actual taxonomic status.

5.1 Introduction

Badnaviruses are reported to infect a wide range of economically important tropical crops such as cacao (Muller and Sackey, 2005), banana (Harper and Hull, 1998), yam (Briddon *et al*., 1999), sugarcane (Bouhida *et al*., 1993), citrus (Huang and Hartung, 2001), and rice (Bouhida *et al*., 1993). They are pararetroviruses, characterised by non-enveloped bacilliform particles containing circular dsDNA genomes of 7.0-7.6 kb (Fauquet *et. al*., 2005). Badnaviruses were first reported in yam in association with a potyvirus, as the causative agent of mosaic symptoms observed in yam leaves infected with internal brown spot disease of *D. alata* in Barbados (Harrison and Roberts, 1973). A similar virus was later detected in *D. cayenensis* in the Caribbean (Mohamed and Mantell, 1976). Badnaviruses infecting yam from several countries were partially characterised by Phillips *et al.* (1999), and two viruses were tentatively designated DaBV and Dioscorea bulbifera badnavirus (DbBV). The complete nucleotide sequences of a Nigerian isolate of DaBV and a bacilliform virus isolated from *D. sansibarensis* from Benin, DsBV, have been analysed (Briddon *et al*., 1999; Seal and Muller 2007). The genome sizes of DaBV and DsBV were shown to be approximately 7.4 kb and 7.26 kb, respectively, with 61.9 % nucleotide identity.

Compared with other previously analysed badnaviruses, both viruses were found to share the highest level of nucleotide identity with *Cocoa swollen shoot virus* (CSSV) (Briddon *et al*., 1999; Seal and Muller 2007).

Molecular studies have revealed very high genetic variability within the badnavirus genus (Lockart and Olszewski, 1993; Geering *et al*., 2000). It is therefore important to determine the sequence variability among the badnaviruses infecting yam in West Africa to ensure that diagnostics are robust and reliable.

The aim of this work was to establish sequence variability in the reverse transcriptase (RT) and ribonuclease H (RNaseH) regions of badnaviruses infecting yam in Ghana, Togo, Benin and Nigeria. This region is reported to be highly conserved among badnaviruses (Bouhida *et al*., 1993; Harper and Hull 1998; Huang and Hartung 2001).

Immunocapture PCR was employed in this study because it is reported to considerably decrease non-specific PCR amplifications favouring the amplification of DNA contained within capsids and excluding DNA derived from other genomes thus ensuring that only episomal virus sequences are amplified. This is particularly important for the detection of badnaviruses whose sequences have been reported to be integrated into host genomes (Harper *et al*., 1999a; Geering *et al*., 2005; Harper *et al*., 1999b; Geering *et al*., 2001). IC-PCR also has the advantage of eliminating, in the washing step, host contaminants that may interfere with PCR.

5.2 Material and methods

Nineteen badnavirus-infected yam leaves (tested previously using antibodies and IC-PCR described in Chapters two and three) from Nigeria, Ghana, Benin and Togo were selected and used for this work. Leaf tissues were dried and stored over calcium chloride prior to use. Sample numbers, host species and country of origin for each sample is summarised in Table 5.1.

The rabbit polyclonal antisera raised against DaBV routinely used for the certification of yam virus-tested plantlets at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, were used for immunocapture. Degenerate badnavirus primer pair Badna FP (5'-ATGCCITTYGGIITIAARAAYGCICC-3'), which binds from nucleotide 5833-5859 of the DsBV genome, and Badna RP (5'-CCAYTTRCAIACISCICCCCAICC-3'), which binds from nucleotide 6388-6411 of the DsBV genome, were used for PCR amplification (Seal and Muller 2007).

5.2.1 Immunocapture-polymerase chain reaction (IC-PCR)

Immunocapture followed the coating and trapping method by Clark and Adams (1977). PCR tubes (200 µl, ABgene, UK) were coated with 100 µl of badnavirus polyclonal antisera diluted 1:1000 in coating buffer (0.05 M sodium carbonate buffer, pH 9.6) and incubated at 37^{o}C for 2 h. The tubes were washed three times at 3 minute intervals with phosphate buffered-saline containing 0.05 % (v/v) Tween-20 (PBS-T) and once with distilled water. PCR reaction cocktail consisting of 1 x Taq reaction buffer (10mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Triton-X 100),

0.25 mM of each dNTP, 50 pM each of forward and reverse primers and 1.25 U of Taq DNA polymerase (Promega, USA) was made and 25 µl was added to each pre-coated tube. PCR was carried out in a PTC-200 Peltier Cycler (MJ Research Inc. USA) using the following thermocyclic regime; 94°C for 4 min, 94°C for 30 s, 50°C for 30 s, 72°C for 30 s. The cycle was repeated 40 times excluding the first step, and then a final extension was done at 72°C for 5 min. Ten µl of IC-PCR products were resolved on a 1.5% (w/v) agarose gel containing ethidium bromide (50ug/ml) using TAE (40 mM Tris-acetate pH 8.3, 1 mM EDTA) at 100 V for 1 h. The 1-Kb plus DNA ladder (Invitrogen) was ran along with the PCR products and used for size estimation.

All amplicons were purified using Wizard SV Gel and PCR clean up system (Promega, USA). PCR products were mixed with 10-15 µl of membrane binding solution (1:1 volume with PCR product) and transferred to the mini-columns. The columns were centrifuged at 16,000 x *g* for 1 minute and the flow-through discarded. The membranes were washed by adding 700 µl of membrane wash solution to the mini-columns and centrifuging again at 16,000 x *g* for 1 minute. The wash step was repeated with 500 µl of membrane wash solution. After discarding the flow-through, the column was re-centrifuged again with the micro-centrifuge lid open to allow evaporation of residual ethanol. The mini-column was transferred into a sterile 1.5 ml microcentrifuge tube and 50 µl of nuclease free water added to the centre of the min-column. The column was incubated at room temperature for 1 minute and centrifuged at 16,000 x *g* for 1 minute. The purified DNA was stored at -20°C before use.

5.2.2 Cloning and sequencing

Purified DNA was ligated into the pGEM T-easy (Promega, USA) cloning vector following the manufacturer's protocol. Briefly, standard ligation reactions consisting of 2.5 µl of 2x rapid ligation buffer, 0.5 µl of pGEM T-easy vector (50ng), 1 µl of purified PCR product, 0.5 µl of T4 DNA ligase (3 Weiss units/ µl) and 0.5 µl of water were set up and incubated overnight at 4°C. Positive control and background control reactions were also set up by replacing the PCR product in the standard ligation reaction with control insert DNA for the positive control and omitting both the PCR product and the control insert DNA in the background control.

The plasmids were transformed into *E. coli* JM 109 high competent cells as follows. One µl of each ligation reaction was placed in a 1.5 ml micro-centrifuge tube on ice, 25 µl of competent cells were transferred into each tube and allowed to stand on ice for 20 minutes. The tubes were then placed in a water bath for 45-50 s at 42°C to heat-shock the cells and immediately returned to ice for 2 min. 425 µl of SOC medium (100 ml is made by mixing 2g Bacto-tryptone, 0.5g Bacto-yeast extract, 1 ml of 1 M NaCl, 1 ml of 1M KCl, 1 ml of 2M Mg^{2+} stock and 1 ml of 2M glucose in distilled water pH 7.0) was added into each tube containing transformed cells and the tubes incubated for 1.5 h at 37°C with shaking. Fifty µl of each transformation culture was plated on Luria-Bertani agar plates (10g Bacto-tryptone, 5g Bacto-yeast extract, 5g NaCl, 15g agar in 1 litre distilled water) supplemented with Ampicillin (100 µg/ml), isopropyl-ß-$_D$-thiogalactopyranoside (IPTG, 25 µg/ml), and 5'-bromo-4-chloro-3-indolyl-ß-$_D$-galactopyranoside (X-Gal plates 80 µg/ml). The plates were

incubated at room temperature for 30 minutes and then overnight (12-16 h) at 37°C for selection of transformants. The plates were stored at 4°C and screened for positive clones.

The pGEM-T Easy Vector System allows identification of recombinant clones by blue/white color screening on indicator plates. White colonies generally contain inserts. The tip of a sterile tooth pick was inserted into a selected white colony and transferred into a 12 ml falcon tube containing 3 ml of Luria- Bertani broth supplemented with Ampicillin (100 µg/ml). The tubes were incubated at 37°C overnight. After overnight incubation, 0.85 ml of the growing cell solution from each tube was mixed with 150 µl of glycerol, vortexed to mix and stored at -20°C for long term storage.

5.2.3 Plasmid purification

The remainder of the growing cells were centrifuged at 1,000 rpm for 10 minutes and plasmid DNA purified from the cells using QIAprep Spin Miniprep kit (Qiagen) according to manufacturer's instruction. The pelleted bacterial cells were re-suspended thoroughly in 250 µl of buffer P1 by vortexing and transferred to micro-centrifuge tubes. Equal volume (1:1) of buffer P2 was added and each tube mixed gently but thoroughly by inverting six times. Buffer N3 (350 µl) was added to each tube and the mixture was centrifuged for 10 min at 16,000 *g*. Supernatants were applied to QIAprep spin columns, the columns were centrifuged for 60 s at 16,000 *g*. Flow-through were discarded and the columns were washed by adding 0.75 ml of

buffer PE and centrifuging for 60 s at 16,000 *g*. The columns were centrifuged for an additional 60 s, after discarding the flow-through, to remove residual wash buffer. The QIAprep columns were then placed in sterile 1.5 ml micro-centrifuge tubes and 50 µl of sterile distilled water was added to the centre of each column and allowed to stand for 1 min. Plasmid DNA was eluted by centrifuging the columns for 60 s at 16,000 *g*.

Purified plasmids were then digested using *Eco*R1 to determine if the inserts are of the expected band size. For *Eco*R1 digestion, 8 µl of a digestion cocktail consisting of 1 x restriction enzyme buffer (90mM Tris-HCl (pH 7.5), 50 mM NaCl, 1.0 mM $MgCl_2$), 2 µg of acetylated bovine serum albumin (BSA) and 5 U of restriction enzyme was added to 2 µl of purified plasmid DNA in PCR tubes and incubated for 2 h at 37°C. Restriction digest products were resolved on a 1.5% agarose gel using TAE (40 mM Tris-acetate pH 8.3, 1 mM EDTA) at 100 V for 1 h.

5.2.4 Sequencing and sequence analysis

Purified plasmids were sequenced in both direction using SP6 and T7 primers in an automated sequencer at Inqaba Biotechnical Industries, South Africa. Sequences were edited and consensus sequence for each isolate was made working with the SP6 and T7 sequences along with their electropherograms. Vector sequences were identified using the VecScreen program (Altschul *et al*., 1997) and they were removed prior to sequence analysis. Sequence similarity searches were made in the GenBank databases using the BLAST program (NCBI). Amino acid sequences were deduced using the

Transeq program (Rice *et al*., 2000). Multiple alignments were done using the CLUSTAL W programs (Chenna *et al*., 2003), and phylogenetic trees were created using the neighbour-joining method (Saitou and Nei 1987). The tree was viewed using the njplot program (Perrière and Gouy 1996) and the robustness of the tree was determined by bootstrap using 1000 replicates. Two previously sequenced badnaviruses infecting yam, DaBV and DsBV, and five members of the *Badnavirus* genus *Banana streak virus* (BSV), *Cocoa swollen shoot virus* (CSSV), *Commelina yellow mottle virus* (CoYMV), *Sugarcane bacilliform virus* (ScBV), and *Taro bacilliform virus* (TaBV) were used for phylogenetic analysis. *Rice tungro bacilliform virus* (RTBV) was defined as outgroup (Table 5.2).

5.3 Results

The expected product of size 579 bp was amplified from all the 19 badnavirus isolates and there was no amplification in healthy control (Figure 5.1). Over 50% of the recombinant colonies were white suggesting high cloning efficiency (Figure 5.2).

*Eco*R1 digest of purified plasmids revealed two restriction patterns. Fourteen plasmids released inserts of the expected band size of 579 bp while the three isolates from Nigeria (NG1Da, NG2Da, NG3Da) and two isolates from Ghana (GN4Da and GN5Dr) had an internal *Eco*R1 site at nucleotides 224-229 and as a consequence, inserts were further cleaved into two fragments of 224 and 355 bp (Figure 5.3, Table 5.1).

Analysis of the deduced amino acid sequences of the RT/RNaseH-coding region of the badnavirus isolates in this study and published sequences of other badnaviruses, showed some conserved regions typical of *Caulimoviridae* family (Medberry *et. al*., 1990; Bouhida *et al*., 1993; Harper and Hull, 1998), and revealed that all the badnavirus isolates were more closely related to each other and to the two published badnaviruses infecting yam, than to any other known badnaviruses (Figure 5.4). Phylogenetic analysis revealed that the 19 isolates formed two main groups, the DaBV group clustering with the previously published sequence of DaBV and the DsBV group clustering with the previously published sequence of DsBV (Figure 5.4). The DaBV group was further branched into two subgroups. The three Nigerian isolates (NG1Da, NG2Da, NG3Da), two Benin isolates (BN5Dr and BN7Dr) and one isolate each from Ghana (GN4Da) and Togo (TG3Da) clustered with the previously published sequence of DaBV Nigerian isolate in subgroup I and showed 96 - 100% amino acid identity to each other and 90-96% amino acid identity with DaBV. The virus isolates in subgroup II showed 95-99% amino acid identity to each other and consisted of three isolates from Ghana (GN2Dr, GN3Dr and GN5Dr), two isolates from Benin (BN6Dr and BN2Dr) and one isolate from Togo (TG1Dr).

Two isolates from Togo (TG2Dr and TG4Da) and one isolate each from Ghana (GN1Dr) and Benin (BN1Dr) clustered with the previously published sequence of DsBV from Benin in the DsBV group. The isolates in the DsBV group had 94-99% amino acid identity to each other and shared 83-84% amino acid identity with DsBV (Table 5.3). One isolate from Benin (BN4Dr) did not cluster with any isolates in the

DsBV group and remained as separate branch (Figure 5.4). The nucleotide sequence of BN4Dr varied from those of the other isolates by 25-29% while the deduced amino acid sequence varied by 20-23%. This isolate was more closely related to DaBV (72% nucleotide identity and 77% amino acid identity) than to DsBV (70% nucleotide identity and 75% amino acid identity) (Table 5.3).

Nucleotide sequence variability between the two virus groups ranged from 28-31%, while the two groups showed 73-79% amino acid identity (Table 5.3). Single amino acid differences were observed at positions 10, 25, 39 and 41 of the reverse transcriptase. The major differences between the two groups were observed between positions 46-100 and positions 124-178 with patches of conserved and semi-conserved regions for all isolates scattered between these two stretches (Figure 5.5). All the yam-infecting badnavirus isolates in this study and the two previously sequenced yam badnaviruses, DaBV and DsBV, showed an 18 consecutive amino acid identical match at positions 104-121 (Figure 5.5). This and the conserved region at amino acid positions 59-71 could be potential sites for specific primers for the amplification of all badnaviruses infecting yam (Figure 5.5). Some conserved regions observed at the nucleotide level could also be used for designing specific primers for differentiating between the DaBV and the DsBV groups (Figure 5.6).

The two subgroups of the DaBV group varied both at the nucleotide and amino acid sequences, with variability ranging from 13-18% and 4-7%, respectively (Table 5.3). The major differences between the amino acid sequences of isolates in the two sub

groups were found at positions 21, 46, 56, 101 and 102 where arginine, glutamic acid, lysine, and two glutamic acids, are replaced with lysine, arginine, glutamic acid, and two aspartic acids subgroups I and II, respectively (Figure 5.5).

The deduced amino acid of all the badnavirus isolates in this study and the two previously published badnaviruses infecting yam, DaBV and DsBV, were closer to BSV (69-74% amino acid identity), than to CSSV (64-68% amino acid identity), CoYMV (63-67% amino acid identity), TaBV (61-67% amino acid identity), ScBV (63 -66% amino acid identity), and RTBV (44-47% amino acid identity) (Table 5.4).

5.4 Discussion

The BadnaFP and BadnaRP primers used for this study were designed from the ScBV sequence (G. Hafner, John Innes Centre, Norwich NR4 7UH, UK). The BadnaFP primer differs by the insertion of ITI from that reported by Yang *et al*., (2003) for the amplification of TaBV. The primers used in this study had been used successfully for the detection of yam-infecting badnaviruses from Ghana, Togo and Benin (Chapter 2 and 3) and have been used for the amplification of DsBV, another yam-infecting badnavirus (Seal and Muller, 2007).

The presence of some conserved and semi-conserved regions observed in the amino acid sequences of the yam-infecting badnaviruses in this study and other members of the *Badnavirus* genus, particularly within the previously described FIAVYIDDILVFS stretch of the reverse transcriptase in the C-terminal region of the

open reading frame (ORF) III polyprotein, confirms that all 19 virus isolates in this study belong to the *Badnavirus* genus (Medberry *et al*., 1990; Bouhida *et al*., 1993; Seal and Muller 2007).

The badnaviruses infecting yam in Ghana, Togo, Benin and Nigeria can clearly be distinguished into two distinct virus species. One group was more closely related to the published sequenced of the Nigerian DaBV isolate (90-96% amino acid identity) while the second group was more related to the published sequence of the Benin DsBV isolate (83-84% amino acid identity). Sequence variability observed among the isolates in each of the two species in this study, may be attributed to the inaccurate replication by reverse transcription reported for Caulimoviridae (Medberry *et al*., 1990; Harper and Hull, 1998) and to the fact that yam is vegetatively propagated (Briddon *et al*., 1999).

The lower level of amino acid identity observed between one isolate from Benin, BN4Dr, and DaBV (77%) and DsBV (75%) strongly suggest that BN4Dr is a different virus species hence a putative third badnavirus specie infecting yam in West Africa. The 74% amino acid identity between DaBV and DaBV, in the same RT/RNaseH region analysed in this study, is similar to the observed aa identity between BN4Dr, DaBV (77%) and DsBV (75%). Furthermore, nucleotide sequence identity between BN4Dr and DaBV (72%) and DsBV (70%) is also very similar to the 70.8% nucleotide identity reported between DaBV and DsBV in the same RT/RNaseH region (Seal and Muller 2007). The 100% amino acid match in the 13

highly conserved FIAVYIDDILVFS stretch of the reverse transcriptase of badnaviruses (Medberry *et al*., 1990; Bouhida *et al*., 1993; Seal and Muller 2007) observed in BN4Dr and the 18 consecutive identical amino acids present in DaBV, DsBV and at positions 104-121 of all the badnavirus isolates in this study, confirms that BN4Dr is a yam-infecting badnavirus. In order to further confirm the identities and variabilities among yam-infecting badnaviruses, further ORFs need to be sequenced. Indeed, Seal and Muller (2007) noted that the partial RT/RNaseH data obtained in their study, suggest the presence of many more badnaviruses in *Dioscorea* species. As badnavirus strains within a single leaf can be highly diverse (Geijskes *et al*., 2002; Harper *et al*., 2004; Muller and Sackey, 2005), mixtures of the three badnavirus species detected in this study maybe present in some yam leaves but this cannot be confirmed unless many clones are screened.

The deduced amino acid sequences of the virus isolates within the DaBV group in this study correspond to the amino acid sequences between positions 1538 and 1730 of the highly conserved amino acids sequence of the putative polyprotein encoded by ORF 3 of DaBV and other badnaviruses (Harper and Hull, 1998; Huang and Hartung, 2001; Briddon *et al*., 1999). The first 38 aa sequences of the members of the DaBV group, correspond to the last 38 aa sequences of the reverse transcriptase of DaBV and were mostly identical across the whole length. Amino acid changes observed were either conserved or semi conserved except for the three aa changes at positions 24, 27 and 35. The last 18 aa sequences of the virus isolates in the DaBV group which corresponds to the first 18 aa sequences of the RNaseH of DaBV were exact

match in 15 positions with three semi conserved amino acid changes (Figure 5.7). The 193 deduced amino acid sequences of the virus isolates within the DsBV group correspond to the amino acids sequences between positions 1530 to 1722 of the putative polyprotein of DsBV ORF 3 (Seal and Muller 2007). Both the 38 aa of the reverse transcriptase and the 18 aa of the RNaseH of the isolates in the DsBV group and DsBV are mostly identical across the whole length, six amino acid changes observed in the RT and RNaseH regions were conserved or semi-conserved (Figure 5.7). Similarly conserved 38 and 18 aa sequences of the RT and RNaseH regions were present in the deduced aa sequence of BN4Dr (Figure 5.7). The presence of these two important functional motifs, located at the C-terminal region of ORF 3 of DaBV and DsBV, in all the virus isolates in the DaBV and DsBV groups in this study and in BN4Dr, further establishes that the three virus species in this study are yam-infecting badnaviruses and confirms previous reports that the aa sequences in the RT and RNaseH regions of badnaviruses are highly conserved (Medberry *et al*., 1990; Bouhida *et al*., 1993).

Further genetic variability among the badnavirus isolates in this study was revealed by the existence of two *Eco*R1 digestion pattern (Figure 5.3). Three Nigeria isolates and two Ghana isolates were digested into two fragments of 224 and 355 bp while fourteen isolates were undigested. Sequence data confirmed the presence of the *Eco*R1 digestion site (5' GAATTC 3') at nucleotides 224-229 in the five sequences that were digested. The *Eco*R1 digestion site was also present, in the same position, in the previously published sequence of DaBV Nigeria isolate. Phylogenetic analysis

revealed that four of the five isolates (NG1Da, NG2Da, NG3Da and GN4Da) which had the *Eco*R1 site clustered with DaBV. However, the *Eco*R1 digestion pattern may not be a very useful tool for differentiating isolates of yam-infecting badnavirus since other isolates lacking the *Eco*R1 digestion site clustered in the DaBV subgroup I and GN5Dr the fifth isolate with the EcoR1 digestion site clustered in different subgroup (Figure 5.4).

The integration of badnavirus-like sequences into host genomes have been reported (Harper *et al*., 1999b; Geering *et al*., 2005; Yang *et al*., 2003). The high level of amino acid identity observed between the badnavirus isolates in this study and the sequences of the two published yam-infecting badnaviruses DaBV and DsBV (74% and above), strongly indicates that genomic virus-like sequences were likely not amplified, since Yang *et al*., (2003) reported approximately 50-60% similarity between TaBV and TaBV-like sequences amplified from Taro plants. Furthermore, the use of immunocapture as employed in this study was found efficient for the detection of episomal BSV as the antisera did not capture any *Musa* nuclear, mitochondria, or chloroplast genomes, which may contain BSV integrated sequences (Harper *et al*., 1999a).

Besides the Nigerian isolates which clustered together, there was no correlation between isolates and country of origin or host species. The two badnavirus species are widespread and were present in Ghana, Togo and Benin and were observed to infect both *D. alata* and *D. rotundata* indiscriminately. Furthermore, BN7Dr from a

D. rotundata plant in Benin and NG2Dr from a *D. alata* plant in Nigeria both had identical amino acid sequences. This suggests that different badnavirus species may spread from one yam host species to another.

The distribution of the two badnavirus species across Ghana, Togo and Benin and the possible presence of a third virus species based on the results of this study, is possibly due to unrestricted exchange of planting materials through permeable land borders, these countries being located next to each other. There is a need to enforce more stringent quarantine laws on the movement of yam germplasm across the West African yam region. Knowledge on the actual yield losses due to yam-infecting badnaviruses will also help in convincing the farmers of the damage that could result from such unrestricted movement of planting materials.

5.5 References

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Table 5.1 Host species, geographical origin, *Eco*R1 digestion pattern and accession numbers of 19 yam-infecting badnavirus isolates from Ghana, Togo, Benin and Nigeria.

Isolate number	Host species	Country	Size of released insert	EMBL Accession Number
NG1Da	*Dioscorea alata*	Nigeria	224, 355*	AM944571
NG2Da	*Dioscorea alata*	Nigeria	224, 355*	AM944572
NG3Da	*Dioscorea alata*	Nigeria	224, 355*	AM944573
GN1Dr	*Dioscorea rotundata*	Ghana	579	AM944574
GN2Dr	*Dioscorea rotundata*	Ghana	579	AM944575
GN3Dr	*Dioscorea rotundata*	Ghana	579	AM944576
GN4Da	*Dioscorea alata*	Ghana	224, 355*	AM944577
GN5Dr	*Dioscorea rotundata*	Ghana	224, 355*	AM944578
TG1Dr	*Dioscorea rotundata*	Togo	579	AM944579
TG2Dr	*Dioscorea rotundata*	Togo	579	AM944580
TG3Da	*Dioscorea alata*	Togo	579	AM944581
TG4Da	*Dioscorea alata*	Togo	579	AM944582
BN1Dr	*Dioscorea rotundata*	Benin	579	AM944583
BN2Da	*Dioscorea alata*	Benin	579	AM944584
BN3Dr	*Dioscorea rotundata*	Benin	579	AM944585
BN4Dr	*Dioscorea rotundata*	Benin	579	AM944586
BN5Dr	*Dioscorea rotundata*	Benin	579	AM944587
BN6Dr	*Dioscorea rotundata*	Benin	579	AM944588
BN7Dr	*Dioscorea rotundata*	Benin	579	AM944589

*Isolates with internal *Eco*R1 site

Table 5.2 Details of viruses used for comparative sequence analysis

Virus name	Host species	Country of origin	GenBank accession number
Dioscorea alata bacilliform virus (DaBV[a])	*Dioscorea alata*	Nigeria	X94575-X94582
Dioscorea sansibarensis bacilliform virus (DsBV)	*Dioscorea sansibarensis*	Benin	DQ822073
Banana streak virus (BSV)	*Musa species*	Nigeria	AJ002234
Cocoa swollen shoot virus (CSSV)	*Theobroma cacao*	Togo	AJ781003
Commelina yellow mottle virus (CoYMV)	*Commelina diffusa*	unknown	X52938
Rice tungro bacilliform virus (RTBV)	*Oryza sativa*	Philippines	X57924
Sugercane bacilliform virus (ScBV)	*Saccharum officinarum*	Morocco	M89923
Taro bacilliform virus (TaBV)	*Colocasia esculenta*	Papua New Guinea	AF357836

Table 5.3 Nucleotide sequence identity and amino acid sequence identity of pair-wise combinations of the RT/RNaseH-coding region of yam-infecting badnavirus isolates, DaBV and DsBV

NG1	NG2	NG3	GN1	GN2	GN3	GN4	GN5	TG1	TG2	TG3	TG4	BN1	BN2	BN3	BN4	BN5	BN6	BN7	DsBV	DaBV	
100	90	90	71	87	86	90	87	87	70	87	70	69	84	86	75	85	84	89	71	95	NG1
100	**99**	**99**	**78**	**96**	**95**	**98**	**95**	**95**	**78**	**99**	**79**	**79**	**93**	**97**	**80**	**98**	**94**	**99**	**76**	**96**	
	100	98	71	85	86	89	84	84	71	87	70	69	83	84	74	87	83	88	72	90	NG2
	100	**98**	**77**	**96**	**96**	**97**	**95**	**94**	**77**	**98**	**78**	**79**	**93**	**96**	**80**	**98**	**94**	**100**	**76**	**96**	
		100	70	84	85	89	84	84	71	87	70	69	82	84	74	87	82	88	71	89	NG3
		100	**77**	**96**	**95**	**97**	**95**	**94**	**77**	**98**	**78**	**79**	**93**	**96**	**80**	**98**	**93**	**98**	**75**	**95**	
			100	72	72	70	72	72	93	71	89	89	72	72	71	71	72	70	77	68	GN1
			100	**76**	**76**	**76**	**75**	**75**	**97**	**77**	**94**	**95**	**73**	**76**	**79**	**77**	**76**	**77**	**84**	**74**	
				100	95	85	91	92	71	85	71	70	91	84	72	84	91	85	68	84	GN2
				100	**98**	**96**	**98**	**98**	**76**	**97**	**77**	**78**	**96**	**96**	**79**	**97**	**97**	**96**	**76**	**93**	
					100	84	91	92	72	85	71	71	91	84	73	85	91	85	70	84	GN3
					100	**95**	**97**	**97**	**76**	**96**	**77**	**77**	**95**	**95**	**79**	**96**	**97**	**96**	**76**	**93**	
						100	85	85	70	88	69	69	83	87	73	88	83	90	69	88	GN4
						100	**95**	**94**	**76**	**98**	**77**	**78**	**93**	**96**	**79**	**98**	**93**	**97**	**74**	**94**	
							100	94	72	84	70	70	94	86	73	84	94	84	70	84	GN5
							100	**99**	**75**	**96**	**76**	**77**	**97**	**96**	**78**	**96**	**97**	**95**	**76**	**92**	
								100	72	85	70	70	93	85	72	85	93	84	70	84	TG1
								100	**75**	**95**	**76**	**76**	**98**	**95**	**78**	**95**	**97**	**94**	**75**	**91**	
									100	70	88	88	70	72	72	70	72	71	77	68	TG2
									100	**77**	**94**	**94**	**73**	**76**	**79**	**77**	**75**	**77**	**84**	**74**	
										100	71	71	83	91	72	94	83	91	68	85	TG3
										100	**78**	**79**	**94**	**97**	**80**	**99**	**94**	**98**	**75**	**95**	
											100	99	70	72	72	70	70	72	76	68	TG4
											100	**99**	**75**	**76**	**79**	**78**	**76**	**78**	**83**	**75**	
												100	69	71	72	70	70	71	76	67	BN1
												100	**75**	**77**	**80**	**78**	**76**	**79**	**84**	**76**	
													100	85	72	83	97	83	67	82	BN2
													100	**94**	**76**	**94**	**96**	**93**	**74**	**90**	
														100	71	91	85	91	69	84	BN3
														100	**78**	**97**	**94**	**96**	**75**	**93**	
															100	73	72	73	70	72	BN4
															100	**79**	**77**	**80**	**75**	**77**	
																100	83	91	69	84	BN5
																100	**94**	**98**	**75**	**95**	
																	100	84	68	82	BN6
																	100	**94**	**75**	**91**	
																		100	70	87	BN7
																		100	**76**	**96**	
																			100	70	DsBV
																			100	**74**	
																				100	DaBV
																				100	

% of nucleotide sequence identity, **% of amino acid sequence identity**

Table 5.4. Nucleotide sequence identity and amino acid sequence identity of pair-wise combinations of the RT/RNaseH regions between yam-infecting badnavirus isolates, other badnaviruses and *Rice tungro bacilliform virus* (RTBV)

	DaBV	DsBV	BSV	CSSV	TaBV	CoYMV	SCBV	RTBV
NG1	95	71	70	65	63	64	64	56
	96	**76**	**74**	**67**	**67**	**66**	**66**	**47**
NG2	90	72	69	66	63	64	65	54
	96	**76**	**74**	**68**	**66**	**65**	**66**	**46**
NG3	89	71	69	67	63	64	65	55
	95	**75**	**73**	**67**	**66**	**65**	**66**	**46**
GN1	68	77	66	64	62	63	64	53
	74	**84**	**69**	**64**	**62**	**66**	**64**	**44**
GN2	84	68	67	61	60	63	61	53
	93	**76**	**73**	**66**	**66**	**65**	**64**	**46**
GN3	84	70	68	61	61	63	62	34
	93	**76**	**73**	**67**	**65**	**65**	**64**	**45**
GN4	88	69	69	67	63	63	65	55
	94	**74**	**72**	**66**	**66**	**65**	**65**	**46**
GN5	84	70	66	62	62	63	64	55
	92	**76**	**72**	**66**	**65**	**65**	**64**	**45**
TG1	84	70	66	62	62	62	63	56
	91	**75**	**72**	**66**	**65**	**64**	**64**	**45**
TG2	68	77	67	63	62	62	64	55
	74	**84**	**70**	**64**	**61**	**66**	**65**	**44**
TG3	85	68	69	67	62	66	65	56
	95	**75**	**73**	**67**	**66**	**65**	**66**	**47**
TG4	68	76	67	65	61	64	63	55
	75	**83**	**70**	**64**	**63**	**66**	**65**	**46**
BN1	67	76	68	64	62	64	63	55
	76	**84**	**70**	**64**	**63**	**66**	**65**	**46**
BN2	82	67	67	60	61	62	62	34
	90	**74**	**70**	**65**	**64**	**63**	**63**	**44**
BN3	84	69	69	65	62	65	66	55
	93	**75**	**71**	**66**	**64**	**63**	**64**	**46**
BN4	72	70	67	64	64	64	65	48
	77	**75**	**73**	**67**	**66**	**67**	**65**	**45**
BN5	84	69	69	65	63	67	63	55
	95	**75**	**73**	**67**	**66**	**65**	**65**	**47**
BN6	82	68	67	61	61	62	63	55
	91	**75**	**72**	**66**	**64**	**64**	**64**	**45**
BN7	87	70	69	64	63	67	65	55
	96	**76**	**74**	**68**	**66**	**65**	**66**	**46**
DaBV	100	70	69	65	63	62	63	55
	100	**74**	**73**	**67**	**65**	**63**	**65**	**46**
DsBV		100	66	65	61	63	63	38
		100	**69**	**67**	**64**	**65**	**63**	**43**

% of nucleotide sequence identity, **% of amino acid sequence identity.**

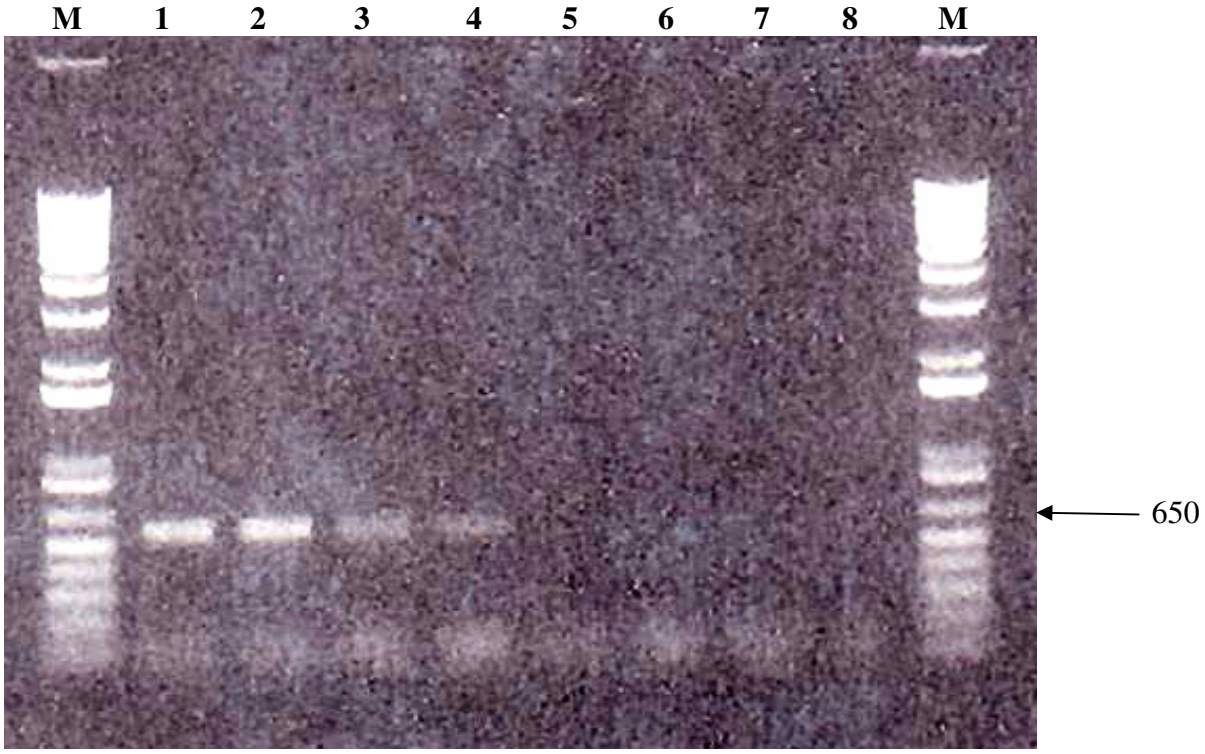

Figure 5.1 Immunocapture polymerase chain reaction (IC-PCR) detection of yam-infecting badnaviruses. Lanes 1 to 4 IC-PCR products from badnavirus infected sap. Lanes 5 to 8 IC-PCR products from healthy sap.

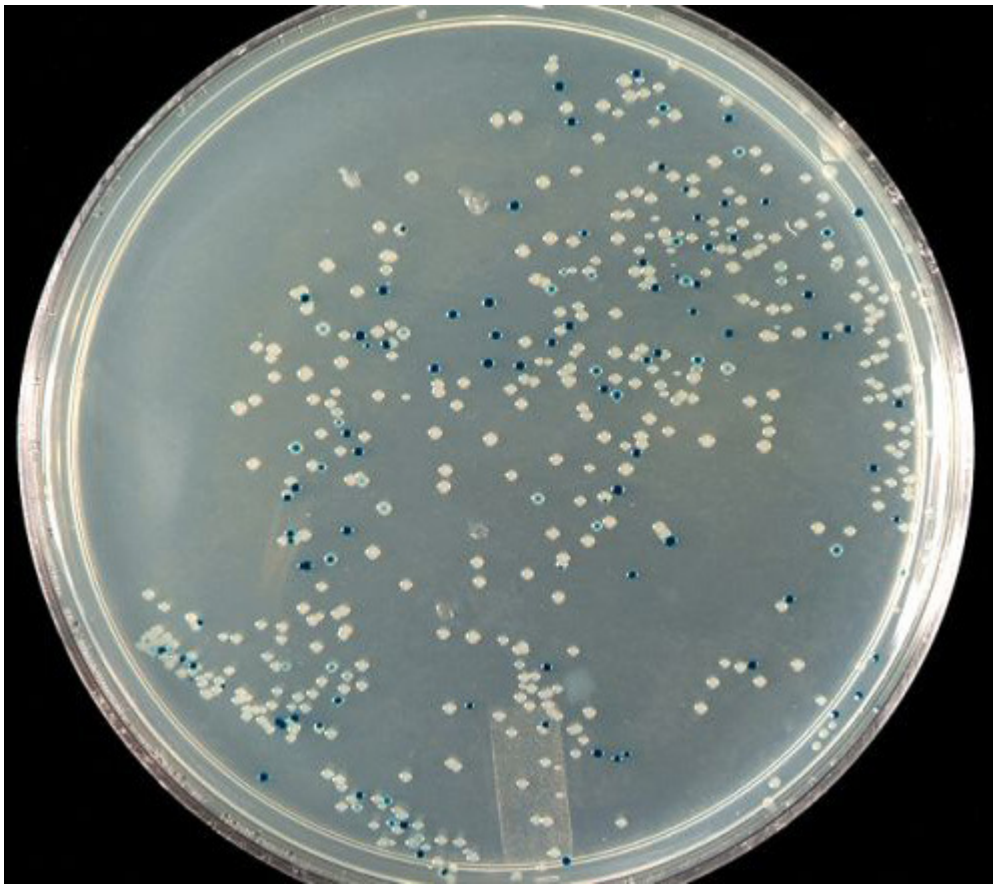

Figure 5.2 Luria-Bertani agar plate showing white (transformed) *E. coli* colonies and blue (non-transformed) *E. coli* colonies.

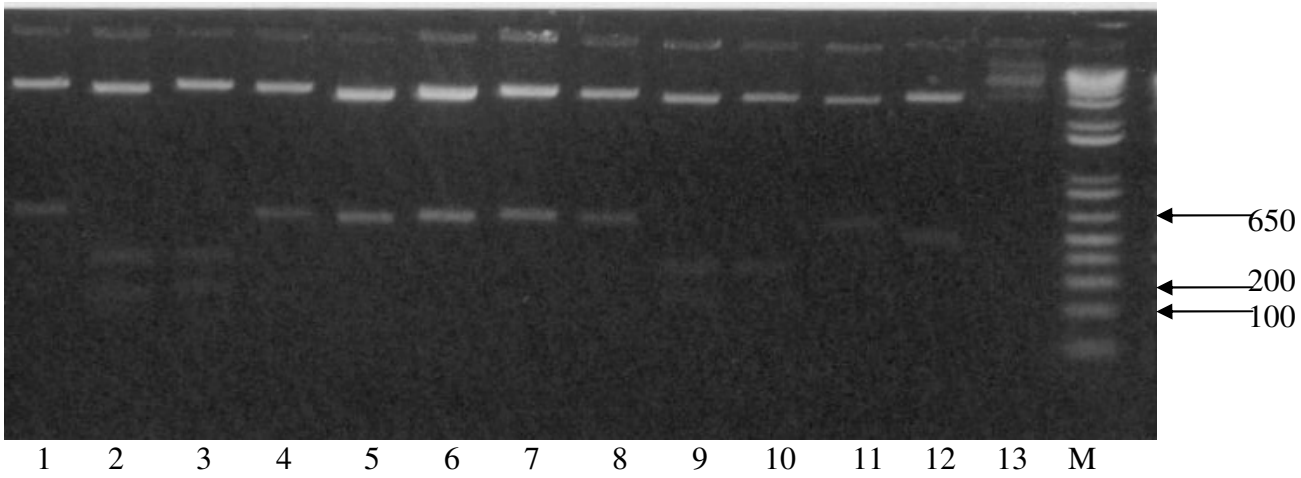

Figure 5.3 *Eco*R1 digest of purified plasmid DNA. Lanes 1, 4, 5, 6, 7, 8, and 11, digestion product from isolates without the internal EcoR1 digestion site. Lanes 2, 3, 10 and 11, digestion products with the internal *Eco*R1 site. Lane 12 digestion product from the positive control, lane 13 digestion product from the background control. M= 1kb plus DNA marker

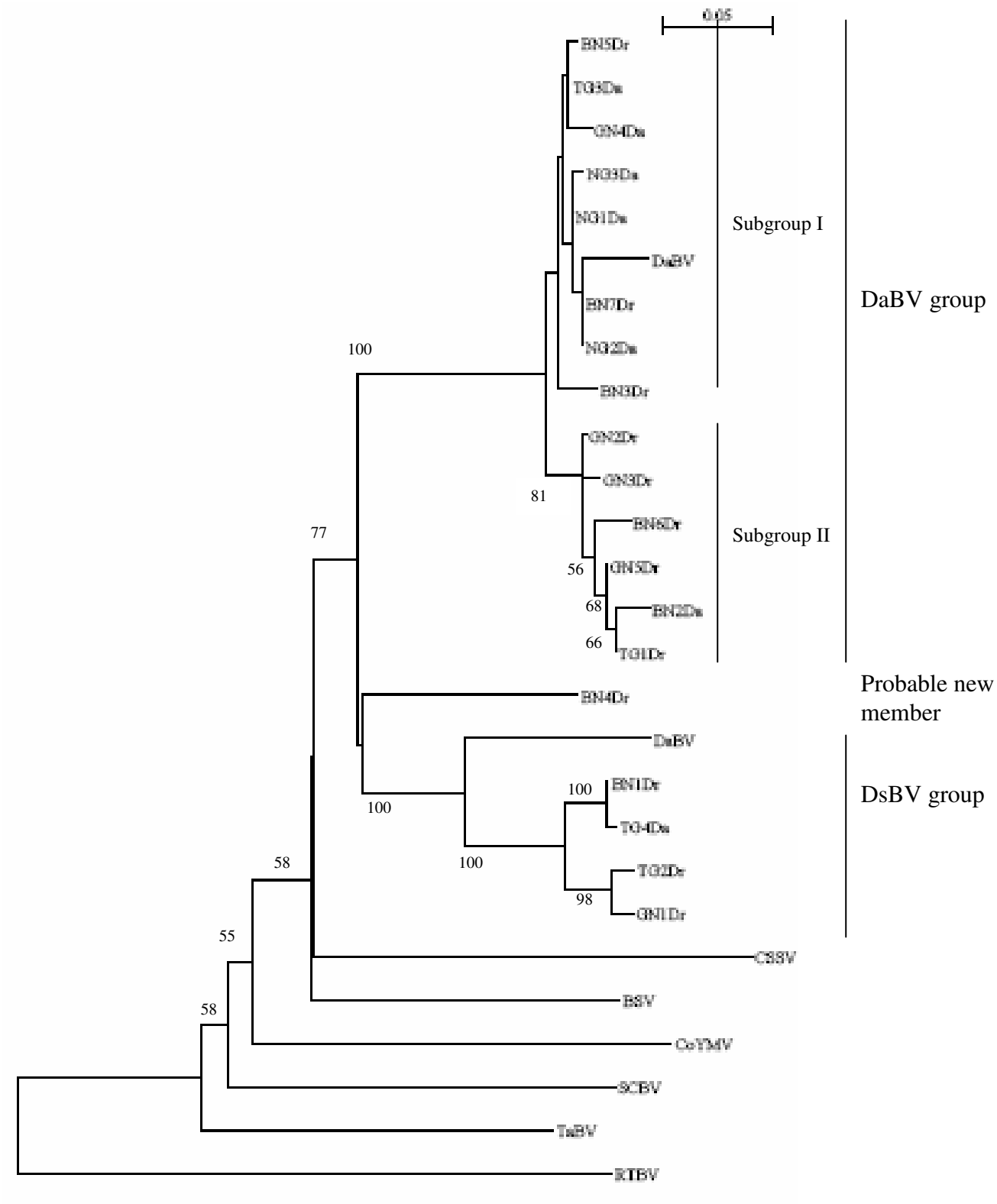

Figure 5.4 Neighbour-joining phylogenetic tree based on the amino acid sequences of the RT/RNaseH-coding regions of yam-infecting badnavirus isolates, other badnaviruses and *Rice tungro bacilliform virus* (RTBV).

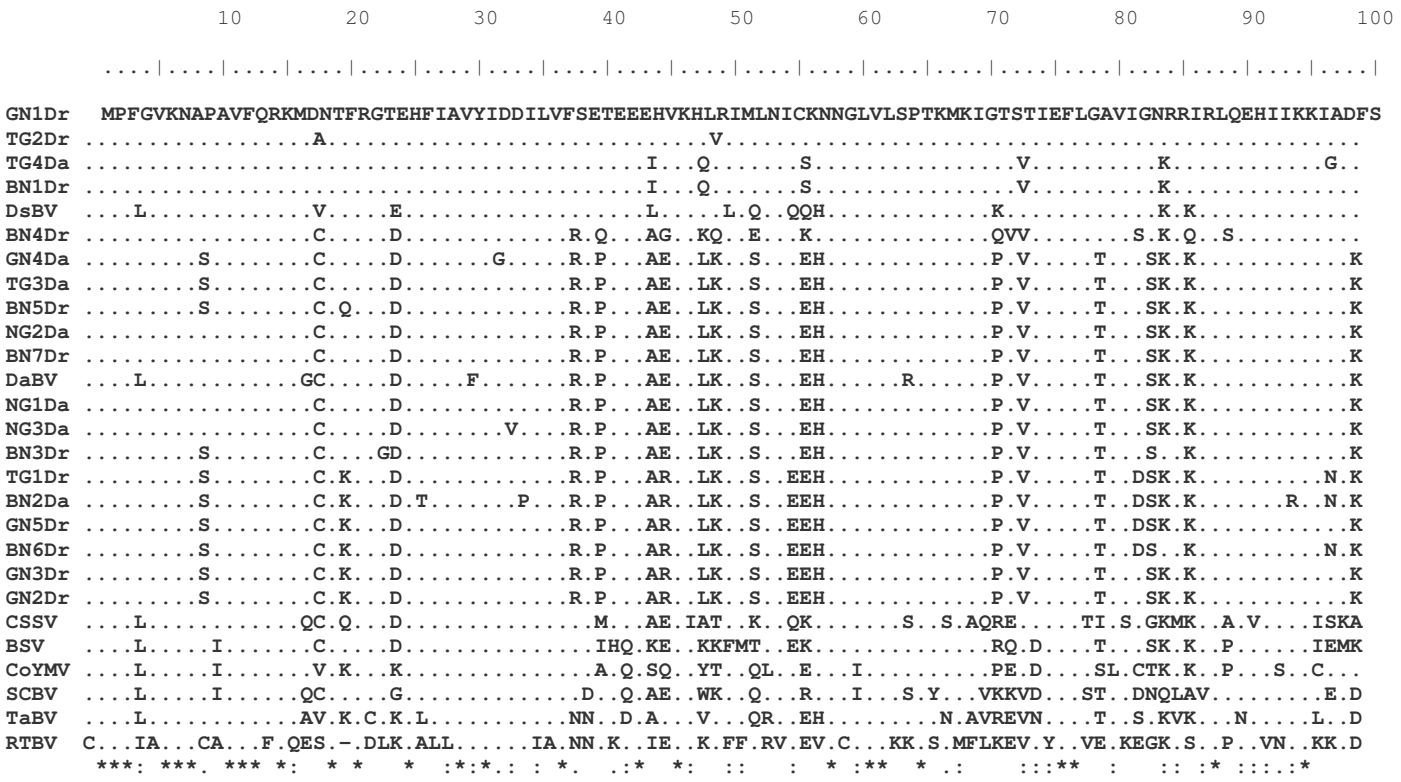

Figure 5.5 Amino acid sequence of section of the RT/RNaseH-coding regions of *yam-infecting badnavirus* isolates, other badnaviruses and Rice Tungro bacilliform virus (RTBV). An asterisk denotes an exact match, and double or single dots denote positions of conserved or semi-conserved amino acid changes, respectively.

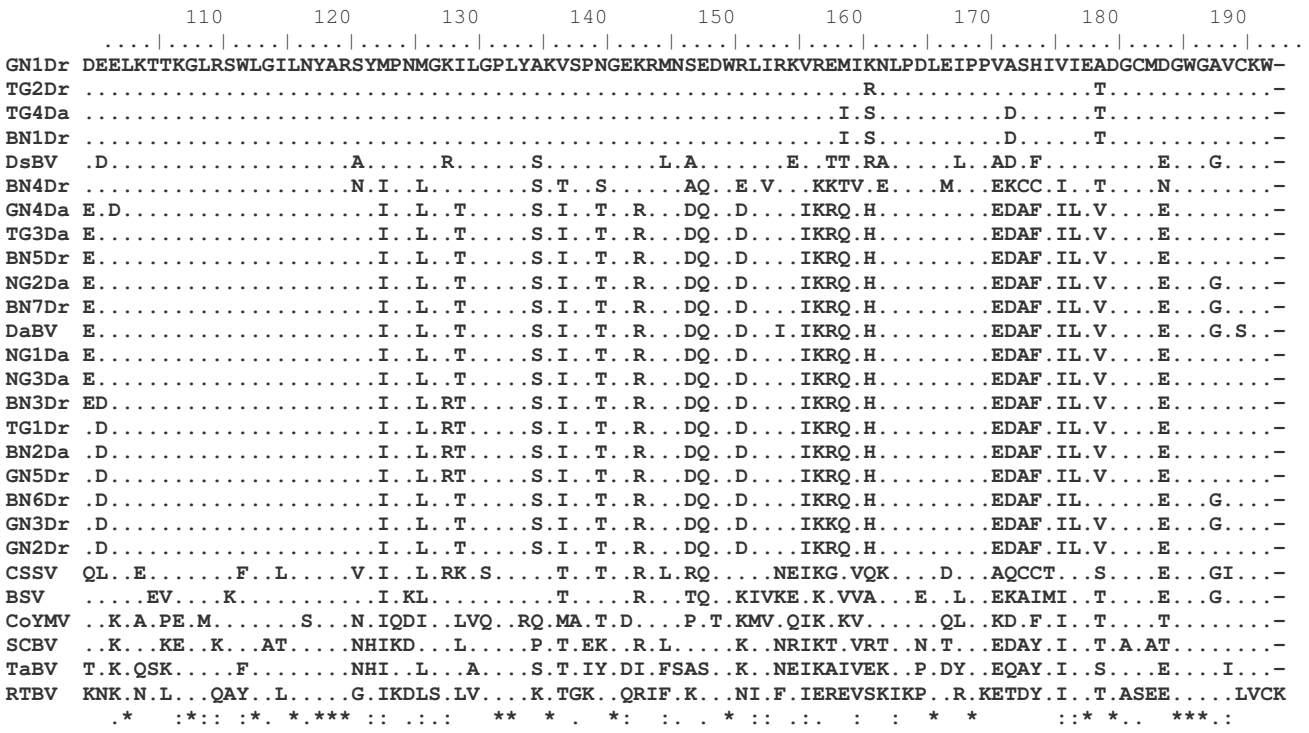

Figure 5.5 (continued) Amino acid sequence of section of the RT/RNaseH-coding regions of *yam-infecting badnavirus* isolates, other badnaviruses and Rice Tungro bacilliform virus (RTBV). An asterisk denotes an exact match, and double or single dots denote positions of conserved or semi-conserved amino acid changes, respectively.

```
      104                          126
NG2   CTTGTGTTTTCCAGAACACCAGA
NG3   CTTGTGTTTTCCAGAACGCCAGA
DaBV  CTTGTGTTTTCCAGAACACCAGA
NG1   CTTGTGTTTTCCAGAACACCAGA
TG4   CTTGTGTTTTCCAGAACACCAGA
BN5   CTTGTGTTTTCCAGAACACCAGA
BN3   CTTGTGTTTTCCAGAACACCAGA
BN7   CTTGTGTTTTCCAGAACACCAGA
GN4   CTTGTGTTTTCCAGAACACCGGA
GN2   CTTGTGTTTTCCAGAACACCAGA
GN3   CTTGTGTTTTCCAGAACACCAGA
BN2   CCTGTGTTTTCTAGAACACCAGA
GN6   CTTGTGTTTTCTAGAACACCAGA
GN5   CTTGTGTTTTCTAGAACACCAGA
TG1   CTTGTGTTTTCCAGAACACCAGA
```

A

```
      485                                  510
NG2   CACAATCTGCCTGATCTCGAGATACC
NG3   CACAATCTGCCTGATCTCGAGATACC
DaBV  CACAATCTGCCTGATCTCGAGATACC
NG1   CACAATCTGCCTGATCTCGAGATACC
TG4   CACAATCTGCCTGATCTCGAGATACC
BN5   CACAATCTGCCTGATCTCGAGATACC
BN3   CACAATCTACCTGATCTCGAGATACC
BN7   CACAATCTACCTGATCTCGAGATACC
GN4   CACAATTTGCCTGATCTCGAGATACC
GN2   CACAATTTGCCAGATCTTGAGATACC
GN3   CACAATCTTCCCGATCTCGAGATACC
BN2   CACAATTTGCCAGATCTCGAGATACC
GN6   CACAATTTGCCAGATCTCGAGATACC
GN5   CACAATTTGCCAGATCTCGAGATACC
TG1   CACAATTTGCCAGATCTCGAGATACC
```

B

```
        62                                  90
GN1   CGAGGTACTGAACATTTTATTGCAGTATA
TG2   CGAGGTACTGAACATTTTATTGCAGTATA
TG5   CGAGGTACTGAACATTTTATTGCAGTATA
BN1   CGAGGTACTGAACATTTTATTGCAGTATA
DsBV  CGAGGTACTGAAGAATTTATTGCTGTATA
```

C

```
      490                                  508
GN1   TCTTCCTGATCTCGAGATTCCACCTGTAG
TG2   TCTTCCTGATCTCGAGATTCCACCTGTAG
TG5   TCTTCCTGATCTCGAGATTCCACCTGTAG
BN1   TCTTCCTGATCTCGAGATTCCACCTGTAG
DsBV  TCTTCCTGATCTCGAACTACCACCAGCGG
```

D

Figure 5.6 Conserved nucleotide sequences for isolates in the DaBV and DsBV groups of yam-infecting badnavirus isolates infecting yam in Ghana, Togo, Benin and Nigeria, (Potential sites for specific primers)
A = bp 103-125; B = bp 484-509 of DaBV group
C = bp 62-89; D = bp 489-507 of DsBV group

```
                          10        20        30
                   ....|....|....|....|....|....|....|...
TG1Dr            1 MPFGVKNAPSVFQRKMDNCFKGTEDFIAVYIDDILVFS
BN6Dr            1 ....................................
GN5Dr            1 ....................................
GN3Dr            1 ....................................
GN2Dr            1 ....................................
BN2Da            1 ........................T.......P...
BN5Dr            1 ......................Q.............
GN4Da            1 ......................R...........G.....
TG3Da            1 ......................R.............
BN3Dr            1 ......................R..G..........
BN7Dr            1 .........A..........R.............
NG2Da            1 .........A..........R.............
NG1Da            1 .........A..........R.............
NG3Da            1 .........A..........R...........V....
DaBV          1358 ....L....A.......G..R.........F.......
Clustal Consensus ****:****:*******.**:** ** ***:*.: ***
```

Reverse transcriptase (DaBV group)

```
                          10
                   ....|....|....|...
BN3Dr          176 IILEVDGCMEGWGAVCKW
BN5Dr          176 ..................
BN2Da          176 ..................
TG3Da          176 ..................
TG1Dr          176 ..................
GN5Dr          176 ..................
GN4Da          176 ..................
GN2Dr          176 ..................
NG3Da          176 ..................
NG1Da          176 ..................
DaBV          1713 ............G.S..
NG2Da          176 ............G....
GN3Dr          176 ............G....
BN6Dr          176 ....A........G....
BN7Dr          176 ............G....
Clustal Consensus  ****.********.*.**
```

Ribonuclease H (DaBV group)

Figure 5.7. Alignments of partial reverse transcriptase and ribonuclease H domain of the open reading frame 3 polyprotein of DaBV and DsBV with the corresponding amino acid sequences of the yam-infecting badnaviruses in the DaBV and DsBV groups. The number of the starting amino acid for DaBV and DsBV are indicated. An asterisk denotes an exact match, and double or single dots denote positions of conserved or semi-conserved amino acid changes, respectively

```
                          10        20        30
                   ....|....|....|....|....|....|....|...
TG4Da         1    MPFGVKNAPAVFQRKMDNTFRGTEHFIAVYIDDILVFS
BN1Dr         1    .....................................
GN1Dr         1    .....................................
TG2Dr         1    ................A....................
DsBV       1530    ....L.............V.....E.............
Clustal Consensus  ****:*************.*****.*************
```

Reverse transcriptase (DsBV group)

```
                          10
                   ....|....|....|...
TG4Da         176  IVIETDGCMDGWGAVCKW
BN1Dr         176  ..................
TG2Dr         176  ..................
GN1Dr         176  ....A.............
DsBV          1705 ....A....E...G....
Clustal Consensus  ****:****:***.****
```

Ribonuclease H (DaBV group)

```
                          10        20        30
                   ....|....|....|....|....|....|....|...
BN4Dr         1    MPFGVKNAPAVFQRKMDNCFRGTEDFIAVYIDDILVFS
DaBV       1538    ....L.............G.............F.......
DsBV       1530    ....L.............V.....E.............
Clustal Consensus  ****:************. *****:*****:*******
```

Reverse transcriptase (BN4Dr)

```
                          10
                   ....|....|....|...
BN4Dr        176   IIIETDGCMNGWGAVCKW
DsBV         1705  .V..A....E...G....
DaBV         1713  ..L.V....E...G.S..
Clustal Consensus  *::*.****:***.*.**
```

Ribonuclease H (BN4Dr)

Figure 5.7 (Continued) Alignments of partial reverse transcriptase and ribonuclease H domain of the open reading frame 3 polyprotein of DaBV and DsBV with the corresponding amino acid sequences of the yam-infecting badnaviruses in the DaBV and DsBV groups. The number of the starting amino acid for DaBV and DsBV are indicated. An asterisk denotes an exact match, and double or single dots denote positions of conserved or semi-conserved amino acid changes, respectively

CHAPTER 6 – SUMMARY

The occurrence and distribution of five viruses infecting yam in major yam-producing agro-ecological zones in Ghana, Togo and Benin were assessed and virus distribution in relation to four yam species was also studied. Rabbit polyclonal antibodies against a yam isolate of CMV and an isolate of yam-infecting badnavirus from *D. alata* in Nigeria were produced. Furthermore, genetic variability among badnaviruses infecting yam in Ghana, Togo, Benin and Nigeria was investigated.

The results of this study provide further evidence that yam viruses are widespread in *Dioscorea* spp. in the yam zone of West Africa. Results of field surveys carried out in Ghana, Togo and Benin (Chapters 2 and 3) indicate that CMV, badnavirus (DaBV and/or DsBV), YMMV and YMV infect yam in these countries. An isometric virus, Dioscorea mottle virus (DMoV, genus ?*Comovirus*), reported to infect yam in Nigeria (Hughes *et al*., 1997) was not found in any of the three countries during this study. Prior to this study, DaBV, YMMV and YMV were the only viruses reported to infect yam in these countries (Olatunde, 1999; Phillips *et al*., 1999; Gumedzoe *et al.,* 2001; Oppong *et al*., 2007). Current results reveal that CMV also infects yam in all three countries. Although previously reported only in *D. alata* (Fauquet and Thouvenel, 1987; Hughes *et al*., 1997), this study indicates that CMV infects both *D. rotundata* and *D. alata* but CMV was not detected from natural infection of *D. bulbifera*, *D. cayenensis*, *D. dumetorum* or any of the wide species tested. With previous documentation of CMV infection in yams in Côte d'Ivoire and Nigeria (Fauquet and

Thouvenel, 1987; Hughes *et al*., 1997), results of this study add to existing knowledge on the distribution of CMV in yam, with implications for yam production and germplasm distribution in West Africa.

Current studies indicate that badnavirus (DaBV and/or DsBV) is the most commonly found of the four viruses detected in Ghana, Togo and Benin, and was the most widely distributed yam virus in the three countries. This is contrary to previous reports of potyviruses, particularly YMV, being the most widespread (Hughes *et al*., 1997; Brunt *et al*., 1996; Kenyon *et. al*., 2001). Badnaviruses generally occur at lower concentration in plant tissue than potyviruses which are often present in higher concentration (Seal and Muller, 2007). Previous studies have relied solely on serological detection methods which may have failed to detect viruses present in low concentration. The use, in this study, of more sensitive nucleic acid-based techniques, may have detected viruses occurring in low concentrations (Mumford and Seal, 1997; Njukeng *et al.,* 2005). The detection of badnavirus, YMMV and YMV in all agro-ecological zones in Ghana, Togo and Benin in this study, may have resulted from past international exchange of infected germplasm or exchange of planting materials through porous land borders, these three countries being located next to each other. Distribution may also have been due to spread by vectors.

The higher incidence of the eight different types of mixed virus infections found in the field during this study compared to the screen-house in Benin, where only two mixed infection types were observed, suggest that besides virus transmission through

vegetative planting materials, other methods of virus transmission, such as vector transmission, are very effective in the spread of yam viruses in the field. Three (CMV, YMMV and YMV) of the four viruses detected during this study are transmitted by aphids (Terry, 1976; Odu *et al*., 1999; Choi *et al*., 1999; Odu *et al*., 2004). Improvement in phytosanitation particularly during seed (sett) cutting, preparation and planting, and possibly reducing vector populations by crop management measures, will reduce virus spread and consequently reduce tuber losses due to yam viruses.

During the current study, *D. alata* was found to be the most heavily infected yam species in the three countries surveyed and had the highest incidence of CMV, badnavirus and YMMV infections. Although it is the most widely distributed yam species globally, its high susceptibility to diseases (Abang *et al.*, 2003) is a major limitation and may be partly responsible for the preference of *D. rotundata* by most farmers, as observed in fields during this study.

Unavailability of antibodies and diagnostics is a limiting factor for virus identification, characterization and monitoring in yam. Antibodies against yam viruses are only available in a few laboratories and not in large volumes. Furthermore, the occurrence of yam viruses in low titre and the mucilaginous substances contained in yam leaves are a problem to virus purification and antibody production (Thouvenel and Fauquet, 1979; Rossel and Thottappilly, 1985; Brunt *et al*., 1990; Mumford and Seal, 1997). During the course of this study, rabbit

polyclonal antibodies were raised against a yam isolate of CMV and an isolate of DaBV from a *D. alata* in Nigeria (Chapter 4). The antibodies produced detected CMV and yam-infecting badnaviruses (DaBV and/or DsBV) in yam leaves from Ghana, Togo, Benin and Nigeria. The CMV polyclonal antibody produced also detected CMV in infected leaves of *C. sativus* and *N. tabaccum* and the badnavirus polyclonal antibody detected *Banana streak virus* (BSV) genus *Badnavirus* in *Musa* sp. This reactivity of both antibodies is useful in detecting most or all strains of both viruses, the antibodies can therefore be used for testing field samples and will detect most, if not all, of the strains of these two viruses. This will improve the diagnostic capability for detecting these viruses in West Africa, increasing the ability to detect the viruses in field samples, thus will permit the occurrence and distribution of both viruses to be monitored, thus contributing to prevent virus spread.

Analysis of the sequences of the RT/RNaseH coding region of badnavirus isolates in this study revealed that two distinct badnaviruses species (DaBV and DsBV) infects yam in Ghana, Togo, Benin and Nigeria. Sequence data strongly suggest that the single isolate from Benin (BN4Dr) may be a distinct third species (Chapter 5). Sequencing of further regions in the ORF 3 will be needed to confirm this. These results confirm previous research indicating that more than one badnavirus species infects yam (Lebas, 2002; Seal and Muller, 2007). The two main species, DaBV and DsBV were detected in Ghana, Togo and Benin and infected both *D. alata* and *D. rotundata* indiscriminately. This is the first report of DsBV in Ghana and Togo. The sequence information obtained from this study can be used to develop PCR-based

diagnostics to detect the various species and/or strains of badnaviruses infecting yam in West Africa. Further sequencing of the isolates is required to ascertain the divergence of the badnaviruses infecting yam so that reliable virus-indexing tests can be developed.

Based on the results of this study, it is recommended that virus-tested planting materials be distributed to farmers in the West African region, to systemically replace current infected planting stock and that farmers should be trained on the importance of using healthy planting materials. Furthermore, stringent quarantine laws, which are already in place, need to be enforced in the West African yam zone, particularly with respect to the consequences of porous land borders on the containment of plant diseases. Indexing of yam germplasm, and certification of planting materials, should employ both the use of ELISA and PCR as serological and molecular differences are known to occur. Yam breeding programs should target multiple virus resistance since many viruses occur in yam and incidences of mixed virus infections are observed to be high. Studies of tuber yield loss due to badnaviruses will ascertain the importance of these widely spread group of viruses on yam yield. Information from yield loss studies will assist extension and agricultural officers in convincing and advising yam farmers in West Africa, on the damage caused by unrestricted movement of possibly infected planting materials and on the use of healthy planting materials. These efforts are geared towards improving yam production and ensuring high and stable yield of yam tubers both for consumption and for income.

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