UNIVERSITY OF CAPE COAST

PREVALENCE OF VIRUS DISEASES, DISTRIBUTION AND CHARACTERIZATION OF VIRUSES INFECTING CUCURBIT CROPS IN SIX AGRO-ECOLOGICAL ZONES IN CÔTE D'IVOIRE

BY

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DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this university or elsewhere.

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Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

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ABSTRACT

Viral diseases are a worldwide problem of cucurbits and a major limiting factor for they production. A field survey was conducted through six agro-ecological zones of Côte d'Ivoire during the dry and rainy seasons in 2014 to assess disease prevalence and severity, identify associated viruses, and evaluate the effect of temperature and growing seasons on virus diseases. Viruses were detected on sampled leaves displaying virus-like symptoms using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and reverse transcription polymerase chain reaction (RT-PCR). The phylogenetic relationships of the new detected virus were also studied. High disease prevalence and severity was observed in both dry and rainy seasons. Significant difference ($P < 0.05$) was observed in rainy season varying between 0.6% and 56.8%. *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV) and *Papaya ringspot virus* (PRSV) were detected out of five. CMV was more prevalent in dry season at an incidence of 31.5% whereas ZYMV was more prevalent in rainy season at an incidence of 34.3%. Pepo aphid borne yellows virus (PABYV) was detected by RT-PCR in Côte d'Ivoire as a Polerovirus based on the sequence similarity and phylogenetic analyses. CMV was detected at significantly enhanced levels in plants when co-infected with ZYMV; plant height was reduced compared to the single infection at 20°C and 25°C. During growing seasons, high incidence and severity were recorded with the highest mean disease severity in growing season 2. It is concluded that co-infection with ZYMV enhance the titer of CMV, which could have epidemiological significance.

KEY WORDS

Agro-ecological zones

Growing season

Seasons

Severity

Temperature

Viruses

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DEDICATION

This work is dedicated to my parents, who always let me believe that I could achieve anything.

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LIST OF ACRONYMS

AEZ	Agro-ecological zone
ANOVA	Analysis of variance
AVRDC	Asian Vegetable Research and Development Center
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary Deoxyribonucleic acid
CGMMV	Cucumber green mottle mosaic virus
CMV	Cucumber mosaic virus
DAS-ELISA	Double antibody sandwich enzyme linked immunosorbent assay
dATP	d-amino triphosphate
dCTP	d-cytosine triphosphate
dGTP	d-guanosine 5'-triphosphate
DNA	Deoxyribonucleic acid
DnaSP	DNA sequence polymorphism
dNTP	d-Nitritriphosate dTTP d-Thymine triphosphate
DP	Disease prevalence
DSI	Disease severity index
FAO	Food and Agriculture Organization
GDP	Gross domestic Product
IC	Incidence
ICTV	International committee Taxonomy on virus
LSD	Least significance difference
MEGA	Molecular Evolutionary Genotype Analysis

MMLVRT	Moloney mukina leukine virus reverse transcriptase
OD	Optical density
PABYV	Pepo aphid borne yellows virus
PRSV	Papaya ringspot virus
RDP	Recombination Detection program
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase chain reaction
WMV	Watermelon mosaic virus
ZYMV	Zucchini yellow mosaic virus

CHAPTER ONE

INTRODUCTION

Background to the study

Agricultural activities are the basis of human development (Bi, Koffi, Baudoin, & Bi, 2011). In Sub-Saharan Africa (SSA), these activities are important since 62% of active population are engaged and represent 27% of Gross Domestic Product (GDP) (Wackernagel, Kitzes, Moran, Goldfinger, & Thomas, 2006; Morris, Kelly, Kopicki, & Byerlee, 2007). Despite this important role of agriculture, SSA populations are suffering from hunger, food insecurity, high mortality, and low economic productivity (Biesalski, 2013). The strategies to solve this problem are the diversification of dietary and the fortification of foods (Grubben et al., 2014), through the development of household enterprises and the production of vegetables (Proctor, 2014).

Vegetables being an effective alternative to protective food have become an essential component of human diet. They are the best resource for overcoming micronutrient deficiencies and provide smallholder farmers with much higher income and more jobs per hectare than staple crops. The worldwide production of vegetables has increased from 15 million tons in 1950 to more than 146 million tons in current years, exceeding cereals production (Bhardwaj, 2012).

The *Cucurbitaceous* or cucurbits are important vegetable family of 925 species with 125 genera (Schaefer & Renner, 2011) cultivated worldwide.

They are important in the diversification of dietary foods and also play a role in rural population economy in Sub-Saharan Africa and in Côte d'Ivoire in particular. They are among most important crops since they have been recorded as second in economic importance to *Solanaceous* crops (Romay et al., 2014). The most popular cucurbits include watermelon (*Citrullus lanatus (Thunb*.) *Matsum. & Nakai*), cucumber (*Cucumis sativus* L.), melon (*Cucumis melo L*.) and squashes (*Cucurbita* spp.). Other important cucurbit crops include Loofa (*Luffa acutangula (L.)* Roxb.), bottle gourd (*Lagenaria siceraria (Molina*) Stand.), chayote (*Sechium edule* (Jacq.) Swartz), wax gourd (*Benincasa hispida (Thunb*.) Cogn.) and bitter melon (*Momordica* spp.) (Robinson & Decker-Walters, 1997; Jeyadevi et al., 2012). Theses crops supply human with edible fruits and valuable fibers essential for their nutritional and medicinal values (McGrath, 2004; Jeyadevi et al., 2012). Their productions also create employments and generate incomes for small-holder farmers. In addition, the growth of cucurbits might be seen as a strategy to adapt to climate change since it can be adapted to the new technologies of irrigation system (Bhardwaj, 2012)

Despite this importance of the cucurbits, their production is under threats because of the reduction of yields and production losses. Several constraints including abiotic factors namely, climate change and variability, lack of improved well-performing varieties; and humankind activities and biotic factors such as pests and diseases (Villareal, 1979; Ladipo & Roberts, 1988) are reported to be the major constraints.

Problem statement

Viruses are a major factor militating against cucurbit production worldwide (Ibada, Laing & Gubba., 2015). The number of viruses naturally infecting cucurbit crops has increased to more than 59 (Lecoq & Desbiez, 2012). In addition to the virus diseases, the changing climate prevalent is a serious threat. The interaction of these two factors affects drastically the production of cucurbits. The losses between 50% and 94% (Blua & Pering, 1989; Müller et al, 2006) are estimated to cost more than $30 billion annually (Sastry & Zitter, 2014).

Information for the best management of virus diseases in Côte d'Ivoire is needed. However, little is known about the causative agents, the impact of the variability of climate on viruses then their geographical distribution, and the influence of climate virus-host interaction. Such information may be essential for the best understanding of virus disease epidemiology and useful in developing effective management strategies.

Objectives of the study

General objective

The study aimed to improve cucurbits production in Côte d'Ivoire with emphasis on virus diseases and the seasonal variability effect of climate on disease incidence.

Specifics objectives

1. To estimate the prevalence and severity of virus diseases
2. To identify viruses associated with cucurbits and their distribution, in six agro-ecological zones in Côte d'Ivoire

3. To characterize Pepo aphid-borne yellows virus, a novel virus infecting cucurbits in Côte d'Ivoire
4. To assess the effect of temperature on virus and its host in single and mixed infection in controlled condition
5. To assess the effect of cropping season on disease incidence and severity in Zucchini and cucumber crops

Hypotheses

1. Several viruses are involved in cucurbits crops infection in Côte d'Ivoire among which are reported CMV, ZYMV and PRSV
2. The seasonal variation of climate influences the distribution of viruses
3. The temperature influences the interaction between the virus and the host plant
4. The growing seasons affect the occurrence and the development virus diseases

Significance of the study

Agriculture is an important sector in Côte d'Ivoire. It accounts for 27% of gross domestic product (GDP), and employs more than two-thirds of the active populations. Despite the important contribution of agriculture to the growth of the economy, the rate of poverty of rural population was estimated to 62.5% in 2008 against 29.4% in urban areas (www.uncdf.org). Among the diverse causes of this poverty, virus diseases in addition to the variability of climate might be the major factors. These two factors account for up to 50% to the losses of production of cultivated crops (Jones, 2009; Bhardwaj, 2012). The necessity to inform stakeholders in order to raise the awareness about the

virus disease and changing environmental threat are needed for the efficient control and management of viral diseases and to adapt to climate variability situation. Overcoming this fact may help improving household life in rural areas.

Considering socio-economic importance of agricultural activities, the management and the control of viral disease can help increasing yield and farmers' incomes, which could be a solution to alleviate poverty and food insecurity.

Cucurbitaceae is a plant family commonly known as melons, gourds or cucurbits and includes crops like cucumbers, squashes (including pumpkins), luffas, melons (including watermelons). The family is predominantly distributed around the tropics, where those with edible fruits were amongst the earliest cultivated plants in both old and new world. Introduced since the independent days, for the diversification and fortification of foods, cucurbits were produced for the high demand of foreign communities. Now they have integrated population diets and the productions seems to have increased over the time due to high demand and consumer awareness on the health benefits of cucurbit fruits (Jeyadevi et al., 2012).

The impacts of this study are in many levels such as socio-economic environmental and awareness reaction.

The economic dimension of the study is that the results might be a contribution of the increase the incomes from smallholders farmers and the improvement of the life. The social impact may be an indication for the politics and the international organization and the no-governmental

organization (NGO) to initiate the programs for training and knowledge's on news technologies to alleviate food insecurity and poverty in rural areas.

The environmental impact of the study is the fact that the best management of virus disease in a context of climate variability can help to reduce the overuse of pesticides in order to keep healthy the consumers and the environment, but also may contribute to reduce the greenhouses gases.

Concerning the learning, the study may help the smallholder farmers through participatory action research (PAR) which is ultimately about the improvement of practice and the creation of knowledge in social groups. It creates new ways of working, interacting, and knowing.

Delimitation

The study was conducted across Côte d'Ivoire situated between the latitudes **4°30' and 10°30' N.** Four eco-climatic zones regimes namely, a humid coastal forest zone, following by a wide zone forest greatly affected by a deforestation with variable rainfall, about 2599 mm/ year, a wooded savannah zones and grasses savannah zone more warm and dry. These four eco-climatic zones covers the six agro-ecological zones surveyed with the variable annual mean temperatures and rainfall as following:

AEZ I with the mean annual rainfall ranged from1400 mm to 2500 mm; and a mean annual temperature of T= 29˚C (5.6); AEZ II with the mean annual rainfall ranged from1300 mm to 1750 mm. The mean annual temperature is about 23.5˚C (13.4), AEZ III with the mean annual rainfall ranged from1300 mm to 2300 mm, a mean annual temperature of T= 24.5˚C (7.7); AEZ IV with the mean annual rainfall ranged from 1300 mm to 1750

mm and a mean annual temperature of T= 23.5˚C (13.4); AEZ V with the mean annual rainfall ranged from1300 mm to 1750 mm; and a mean annual temperature of T= 23.5˚C (13.4), and finally the AEZ VI with the mean annual rainfall ranged from11500 mm to 1350 mm and a mean annual temperature of T= 26.7˚C (1.1).

Limitations of the study

Although there is much remains to be done our work generates, important information concerning virus diseases on cucurbits crops. Having acknowledged the limitations of data management, it can nevertheless be confirmed that there are some limitations of this study. Although the present study has yielded some preliminary findings, its design is not without imperfections. A number of limitations need to be noted regarding the present study.

The first limitation concerns the vectors which transmit the viruses. The main vectors were observed but their identification, the assessment of dynamism of their population related to the changing environmental conditions and the control measures are significant factors in the development of strategies of virus disease management. Based on this importance further work would have been conducted on these factors to complete the strategies of virus disease control.

The second limitation is about the molecular characterization of the three other viruses i.e. CMV, PRSV, and ZYMV. Comprehensive knowledge of virus genetic diversity is necessary to elucidate plant–pathogen interactions

and also help to identify the pathogenic determinants of the viruses being studied.

Such information on plant–pathogen interactions will also be useful in breeding programmes, especially when gene-for-gene interactions occur or are suspected, and to assess the possible risks of resistance breakdown when plants are challenged by virulent strains (Thresh & Fargette, 2003).

Definition of terms

Prevalence: Prevalence is the proportion of a population living with a specific health outcome within a specified time (Alexander, Lopes, Ricchetti-Masterson, & Yeatts, (2009).

Severity: Plant disease severity can be defined as the area or volume of plant tissue that is (visibly) diseased, usually relative to the total plant tissue. It is a continuous variable, typically bound by 0 and 1, and a measure of the quality of plant tissue, not the number of plant units affected (Campbell & Madden, 1990).

Incidence: Plant disease incidence can be defined as the number of plant units that are (visibly) diseased, usually relative to the total number assessed (Campbell & Madden, 1990; Madden & Hughes, 1995).

Climate variability: Climate variability refers to variations in the mean state and other statistics (such as standard deviations, the occurrence of extremes, etc.) of the climate on all temporal and spatial scales beyond that of individual weather events. Variability may be due to natural internal processes within the climate system (internal variability), or to variations in

natural or anthropogenic external forcing (external variability) (Griggs, & Noguer, 2002).

***Cucurbitaceous*:** *Cucurbitaceae* is a plant family commonly known as melons, gourds or cucurbits and includes crops like cucumbers, squashes (including pumpkins), luffas, melons (including watermelons). The family is predominantly distributed around the tropics, where those with edible fruits were amongst the earliest cultivated plants in both the old and the new world (Jeyadevi et al., 2012).

Organization of the study

The Thesis is divided into seven chapters.

Chapter one is the introduction which presents the background to the study, the statement of the problem. The chapter includes also the objectives, the hypotheses, the significance of the study, the delimitation, the limitation, the definition of terms and organization of the study. Chapter two refers to the literature review on the *Cucurbitaceae*, the significance of the cucurbits presenting the socio-economic, nutritional and medicinal values of cucurbits, the important diseases reported on these crops and several viral disease symptoms observed on the leaves. Based on this literature review, the methods used for sampling during survey, the methods used to identify viruses through the serological and the molecular procedures, and the management of virus diseases put end to this chapter. Chapter three is the development of the specific objective one which is the cross-sectional survey and data collection in the fields: Prevalence, disease severity index. The identification of virus diseases using serological method and their distribution, in Côte d'Ivoire

across the six agro-ecological zones was also determined. The infection per individual virus was considered and weeds hosts of viruses were identified. Chapter four goes into characterization of *Pepo aphid-borne yellows virus* (PABYV) a novel virus detected infecting cucurbit crops in Côte d'Ivoire. Chapter five is the experimentation conducted in controlled environment to assess the effect of varying temperatures (20°C, 25°C, and 30°C) on the multiplication of CMV and ZYMV in single and mixed infection, then on inoculated cucumber plants height. Chapter six is field experiments to evaluate the effect of different growing seasons on disease incidence and severity. Finally, Chapter seven is the final chapter including the general discussion, conclusion and recommendations and the perspectives.

Summary of Chapter one

The chapter one describes the interaction between the climate variability, the virus diseases and the agriculture. It shows how the changing environmental condition through the seasons and the diverse agro-ecological zones, affects viral disease occurrence and its distribution. Moreover, the chapter gives an overview on the vegetables of interest which are the cucurbits, and the information regarding the virus diseases in Sub-Saharan Africa, the lacking, the methods of detection and the measures of management. The general and the specifics objectives, problem statement, the hypotheses, the significance of the study, the delimitation, limitations, and the definitions of the terms and the organization of the study are presented in this chapter.

CHAPTER TWO

LITERATURE REVIEW

Overview on *Cucurbitaceae*

In developing world in particular in Sub-Saharan Africa (SSA), the hidden hunger and the insecurity of food and the low productivity are the limiting factors of development for the inhabitants (Biesalski et al., 2013) because of the increasing population of 4.473 billion out of the total world population of 5.5767 billion (Rybicky & Pietersen, 1999). The consumption of vegetables as dietary supplement is one of the strategies to solve this problem.

The *Cucurbitaceae* is one of the most important families of plants that supplies human with edible fruit and useful fibers (Weng & Sun, 2012). The family is predominantly distributed around the tropics, where those with edible fruits were amongst the earliest cultivated plants in both the old and new world (Jayadevi et al., 2012). The important cucurbits in the tropical and subtropical regions include *Citrullus* spp, *Cucumis* spp., *Cucurbita* spp. (Lebeda et al., 2011).

History and production of cultivated cucurbits

The production of cucurbits seems to have increased over the time due to high demand and consumer awareness on the health benefits of cucurbit fruits. According to Pitrat et al., (1999), the four leading countries are Turkey, China, Iran, and USA. These countries produce 50% of world's watermelon production estimated at 29.9 million tons; 66% of world's cucumber

production, estimated at 23.3 million tons; and 57% of global production of melon estimated at 16.2 million tons.

Concerning the origin of these cucurbit species, watermelon may be originated from Africa and introduced in Europe. The cucumber was likely domesticated in India and then introduced in Europe at the early times. The melon is from Africa but the diversity center is in Asia. It was introduced in Europe by 3 independent ways during the Greek or Roman period.

Significance of cucurbits

Socio-economic importance of cucurbits

Cucurbits production is one of the best resource for providing smallholder farmers with much higher income and more jobs per hectare than staple crops since they can be grown several times in a year. They are cultivated for their fruits, leaves and roots, for therapeutic and nutritional use. The species belonging to the genera *Citrullus*, *Cucumis*, and *Cucurbita* are extensively cultivated by commercial and subsistence farmers throughout South Africa (Trench et al., 1992). In Senegal, the growth and the commercialization of watermelon in the period of money issues is a strategy to earn incomes. In Côte d'Ivoire, the cultivation of cucumber is increased by the great demand by the foreigner communities and the population for which is included in the diets. Ninety percent of women are engaged in the marketing of these products to increase their incomes improve their livelihood.

Nutritional and cultural uses of cucurbits

The cucurbits are reported to be rich in nutrients such as dietary fibers, minerals, vitamins necessary to combat hunger by overcoming nutrient deficiencies (Kumar, Raidu, & Atyam, 2010,; Ayo-John et al., 2014).

They can be eaten fresh as salad and pickled or cooked.

Indigenous species are prized for their oleaginous seeds consumed as thickeners of traditional soup called *Egusi* soup in Nigeria or Benin and pistachio soup in Côte d'Ivoire (Akobundu et al., 1982; Badifu, 2001; Zoro BI, Koffi, & Djè, 2003). Cultivated cucurbits for seed consumption are also reported to be rich in nutrients. The oily seeds are consumed as soup thickener. Thus, seeds are extracted, washed, dried, shelled, and winnowed to obtain the kernels. Then the kernels are slightly roasted and ground for use as thickener preferentially during popular fetes and prestigious ceremonies.

Medicinal uses of cucurbits

The phytochemical contained in cucurbits is an opportunity to use them as medicine. These phytochemicals (carotenoids, flavonoids, terpenes saponins, cardiacglusosides, etc, are cardioprotective, (Sharma, Katoch, & Rana, 2016; Rao, Kesavulu, Giri, & Rao., 1999), hepatoprotective (Kumar, Lakshmayya, & Setty., 2008), nephroprotective (Kumar et al., 2011), antidiabetic (Kameswararao, Kesavulu, & Apparao, 2003); Schreinemachers, Ebert, & Wu, 2014), and anti-ulcer (Dhasan, Jegadeesan, & Kavimani, 2010) activities. *Telfairia occidentalis* is a tropical vine grown in West Africa. The leaves are rich sources of iron (Fe) and can therefore be used for treatment of

anaemia (Alada, 2000). The bitter gourd immature fruit is a good source of Vitamin C, and also contains Vitamin A, phosphorus, and iron.

The tender vine tips are an excellent source of Vitamin A, and a fair source of protein, thiamin, and Vitamin C. Bitter gourd is a blood purifier, activates spleen and liver and is highly beneficial in diabetes. It is a purgative, appetizer, digestive, anti-inflammatory and has healing capacity. The bitter gourd is very much helpful in curing the diabetes, leucorrhoea, jaundice, liver troubles, stomach worms, piles, and constipation (Janssen et al., 2002 & 2003). Also, some reports on the antioxidants indicate their importance in the treat of some diseases. Thus, based on clinical studies, Mo*mordica cymbalaria* was reported to have very good inhibitory effect against *Staphylococcus aureus, Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa*, and *Aspergillus niger* (Sangeetha, Chetana, Paarakh, & Vedamurthy, 2010) when compared to standard antibiotics.

Important diseases of Cucurbit crops

Fungal diseases

Several fungal diseases have been reported on the cucurbits. The most frequent are *Alternaria* leaf spot or *Alternaria* blight; powdery mildew, downy mildew, anthracnose, scab or gummosis, and *fusarium* wilt etc.

Alternaria leaf spot occurs throughout the world and can infect most cucurbits. It is also known as target leaf spot or *alternaria blight*, caused by *Alternaria cucumerina* [(Ellis & Everh.) Elliot] and *Alternaria alternata* [(Fr.) Keisslar]. The disease is seed and soil-borne. For disease development, high humidity

and temperature in the range of 15°C–32°C is quite favorable for its development.

Powdery mildew affects almost all cucurbits under field and greenhouse conditions. It is caused by airborne fungi *Sphaerotheca fuliginea* [(Schlect. ex. Fr.) Poll.] and *Erysiphe cichoracearum* (D. ex. Merfat), the latter being the perfect stage of the fungi. It was also reported that Powdery mildew is caused by *Leveillula taurica* (Joi et al., 1980).

Downy mildew caused by *Pseudoperonospora* cubensis [(Berk. Curt.) Rostow zew] is airborne and the major foliar disease that infects most of the cucurbits. It is particularly serious in warm weather conditions or tropical environment. High humidity, fog, and heavy dew favor the disease development (Lebeda et al., 2011; Sharma et al., 2016).

Anthracnose caused by *Colletotrichum orbiculare* [(Berk & Mont.) Arx.] is both air- and seed-borne fungal disease that occur on most cucurbits during warm and moist seasons. High humidity and temperature of 24°C is optimum for disease multiplication though conidia do not germinate below 4.4°C or above 30°C or under non-humid conditions. It is caused by seed-borne fungi *Cladosporium cucumerinum*. The disease appears on leaves, petioles, stems, and fruits (Ogorek, Lejman, Pusz, Miłuch, & Miodynska, 2012). The optimum temperature for disease development is 17°C–27°C along with high humidity. The optimum temperature for disease development is 20°C–24°C along with free moisture. *Fusarium wilt* of cucurbits is caused by seed- and soil-borne fungi of the genus Fusarium, that is, *Fusarium oxysporum* f. sp. *niveum* (EF Smith Snyder & Hansen) affects watermelon, *Fusarium*

*oxyspor*um f. sp. *melonis* affects muskmelon, and *Fusarium oxysporum* f. sp. *cucumerinum* affects cucumber (Owen, 1955).

Bacterial disease on cucurbits crops

Angular leaf spot

Angular leaf spot is the most widespread bacterial disease of cucurbits that causes reduction in fruit number, fruit yield, and quality (Pohronezny et al., 1977). This disease is caused by the bacteria *Pseudomonas syringae* pv. *lachrymans*. Angular leaf spot is most active between 24℃ and 28℃ and is favoured by high humidity.

Bacterial leaf spot

Bacterial leaf spot disease development is favoured by high temperatures in the range of 25℃–30℃ and high relative humidity in the range of 90%. The disease is not common but can occur during persistent warm and humid conditions on different cucurbits. This disease is caused by the seed- and soil-borne bacterium *Xanthomonas campestris* pv. *cucurbitae* (Bryan) (Sharma et al., 2016).

Bacterial fruit blotch

Bacterial fruit blotch is caused by the bacterium *Acidovorax avenae* subsp. *citrulli*. It is a serious problem in watermelon, though all cucurbits are susceptible. Under favorable conditions, the bacterium spreads rapidly in the field, leading to seedling blight or fruit rot at later stages. The initial symptoms appear on seedlings after 5–8 days of planting as greasy (Walcott, 2005)

Bacterial Rind Necrosis

The disease occurs sporadically and is thought to be caused by bacteria that are naturally present in watermelon fruits. The external symptoms are usually absent, with only misshapen fruits in few severely affected melons. The symptoms include brown, dry, and hard necrosis of the rind that rarely extends into the flesh (Sharma et al., 2016).

Nem*a*todes

Most cucurbits are susceptible to root-knot nematodes (RKN) (Fassuliotis, 1971) causing losses in many growing areas (Edelstein, Oka, Burger, Eizenberg, & Cohen 2010; Wesemael, Viaene, & Moens, 2011; Talavera et al., 2012). Hence, it was reported in South-eastern Spain RKN damage on Zucchini under protected cultivation, a yield losses estimated to 649504 Euros (Talavera et al., 2012).

Viral diseases in cucurbits crops

The cucurbitaceous are submitted to virus diseases which induced the enormous production losses. Currently, the number of viruses infecting naturally the cucurbits has increased currently to more than 59 species. The symptoms induced are variable including mosaic, deformation, chlorosis, curling and yellowing of the leaves, vein clearing and vein banding, stunting of the plants and deformation of fruits. The most frequent viruses reported include *Cucumber Mosaic Virus (CMV), Squash Mosaic Virus* (SqMV), *Zucchini Yellow Mosaic Virus* (ZYMV), *Papaya Ringspot Virus* (PRSV) formerly known as *Watermelon mosaic virus* 1, *Watermelon Mosaic virus-2* (WMV -2) (Yuki et al., 2000; Fattouh, 2003; Choi et al., 2007; Massumi et

al., 2007; Yardimci and Ozgonen, 2007) and *Moroccan Watermelon Mosaic Virus* (Lecoq et al., 2001; Owolabi et al., 2011). The viruses which occurring less frequently but also of economic significance includes *Cucurbit Aphid-borne Yellows Virus* (CABYV), *Cucurbit Yellows Stunting Disorder Virus* (CYSDV), *Cucumber Vein Yellowing Virus* (CVYV), *Cucumber Green Mottle Mosaic Virus* (CGMMV) and *Zucchini Lethal Chlorotic Virus* (ZLCV) (Shim *et al*., 2005; Bananej et al., 2006; Safaeizadeh, 2008; Wintermantel, Hadky, Cortez, & Natwick, 2009; Moradi, & Jafarpour, 2011; Webster et al., 2011; Gil-Salas et al. 2011; Moradi, 2011; Abdalla, Bruton, Fish, & Ali, 2012; Finetti-Sialer., Mascia, Cillo, Vovlas, & Gallitelli, 2012).

Three of these viruses namely, *Cucumber mosaic virus* (CMV), *Zucchini, yellowing mosaic virus* (ZYMV), Papaya ringspot virus (PRSV) have been reorted in Côte d'Ivoire. For the first time, CMV was reported in 1987 (Fauquet & Thouvenel, 1987); PRSV was detected by Diallo et al., in 2007, and then ZYMV reported by Koné et al., in 2010. All these viruses are aphid-borne viruses and are transmitted by the non-persistent manner (Francki, Fauquet, Knudson, & Brown, 1991; Katis et al., 2006). Other vectors transmitting viruses to cucurbits include whiteflies, beetles (Spence, 2001; Ayo-John et al, 2014) and nematodes (*Xiphinema americanum*) (Sharma et al., 2016).

Cucumber mosaic virus (CMV)

Cucumber mosaic virus (CMV) (genus *Cucumovirus*, family *Bromoviridae*) is one of the most economically important viruses causing hundreds of diseases worldwide in more than 1,000 botanical species

(belonging to 100 families) (Van Regenmortel et al., 2000). It is transmitted by aphids (about 75 species) in a non-persistent manner and is also seed-borne in different host plants such as *Stellaria media* and *Ecballium elaterium*. Its ubiquitous nature may be attributed to its broad host range, non-persistent transmission by more than 86 aphid species in the field (Edwardson & Christie, 1991) and transmission through seed in some hosts (O'keefe, Berryman, Coutts, & Jones, 2007).

It was reported in Côte d'Ivoire, infecting *Citrullus* Sp. and *lagenaria siceraria* with the incidence estimated to 30.77% (Agneroh, Kouadio, Soro, & Pohé, 2012). It was also reported on Plantain causing the burn of young plantain plants with an incidence of 66.2% (Aka, Kouassi, Agneroh, Amancho, & Sangaré, 2009).

Zucchini yellow mosaic virus (ZYMV)

Zucchini yellow mosaic virus (ZYMV) is among the worldwide viruses known for their economically importance. It was reported for the first time in Italy in 1973 (Lisa, Boccardo, D'Agostino, Dellavalle, & D'Aquilio., 1981). It widely spread and can be found in Temperate, Tropical and Mediterranean zones because of its ability to adapt to any agroecosystems. Symptoms include yellowing, stunting, leaf deformations, and misshaped and discolored fruits (Figure 1), which often render the fruits unmarketable, drastically reducing agricultural yields (Blua & Perring, 1989; Desbiez & Lecoq, 1997; Gal-On, 2007).

ZYMV was first reported in Côte d'Ivoire in zucchini squash, cucumber in southern country during a rainy season (Koné et al., 2010). It was also detected in other cultivated crops (tomatoes, sweet pepper).

Papaya ringspot virus (PRSV)

Papaya ringspot virus (PRSV) is by far the most widespread and damaging virus that infects papaya. Trees infected with PRSV develop a range of symptoms: mosaic and chlorosis of leaf lamina, water-soaked oily streaks on the petiole and upper part of the trunk, and distortion of young leaves that resembles mite damage. PRSV is transmitted by numerous species of aphids in a non-persistent manner to a limited host range of cucurbits and papaya. PRSV is grouped into two types (Purcifull et al., 1984): Type P (PRSV-p) infects cucurbits and papaya, whereas type W (PRSV-w) infects cucurbits but not papaya. The latter type was previously referred to as WMV-1. PRSV is among the viruses economically important infecting cucurbits. It was reported firstly in Côte d'Ivoire in 1987 (Diallo, Monger, Kouassi, Yoro, & Jones, 2007), and now it spread in all cucurbits growing areas. It incidence on theses crops was estimated to 58.46% (Agneroh et al., 2012).

The symptoms caused by the viruses infecting cucurbits

The infections of plants by the viruses are variable consequences, ranged from the attenuated to severe symptoms. These symptoms are important for numerous reasons. Thus, they can be used for virus identification, to name the disease. However, it cannot be used in solo to characterize a virus as affected by many factors. These factors can be the environment, virus strain/ mixed infections, host varieties, nutrition, age, and

stage of infection (Hull, 2009). The susceptible plants showed symptoms on the leaves and the fruits (Desbiez & Lecoq, 1997; Astier et al., 2001); which includes the abnormalities in the colours and the growth characterizing the trouble of metabolism or the death or tissues. The abnormal coloration is mosaic, mottle, yellowing, chlorosis, vein clearing, vein banding Green vein banding, yellowing vein banding), leaf roll, leaf curl, streak, and blistering.

The abnormal growth includes phyllody, enation, witches broom, proliferation, stunting, ringspot, wilt, tumors or galls, and necrosis.

Mosaic symptom is the abnormal distribution of the pigments in the leaves weather the stains are present unclear small ringspots this correspond to the mottle mosaic. In the case of the intensive color of the nervures it is called vein banding while, the color is clear it is called clear banding (Hull, 2009). Some symptoms observed during the field survey were described in figure 1. Cucumber infected by CMV, showing swelling skin (blue arrow) on cucumber fruit (A); Zucchini plant leaves presenting Green vein banding (B); Zucchini leaf showing yellowing (red arrow) associated to the necrosis (black arrow); (C); vein banding; Shoe-string symptom (D); vein clearing associated to leaf roll (E).

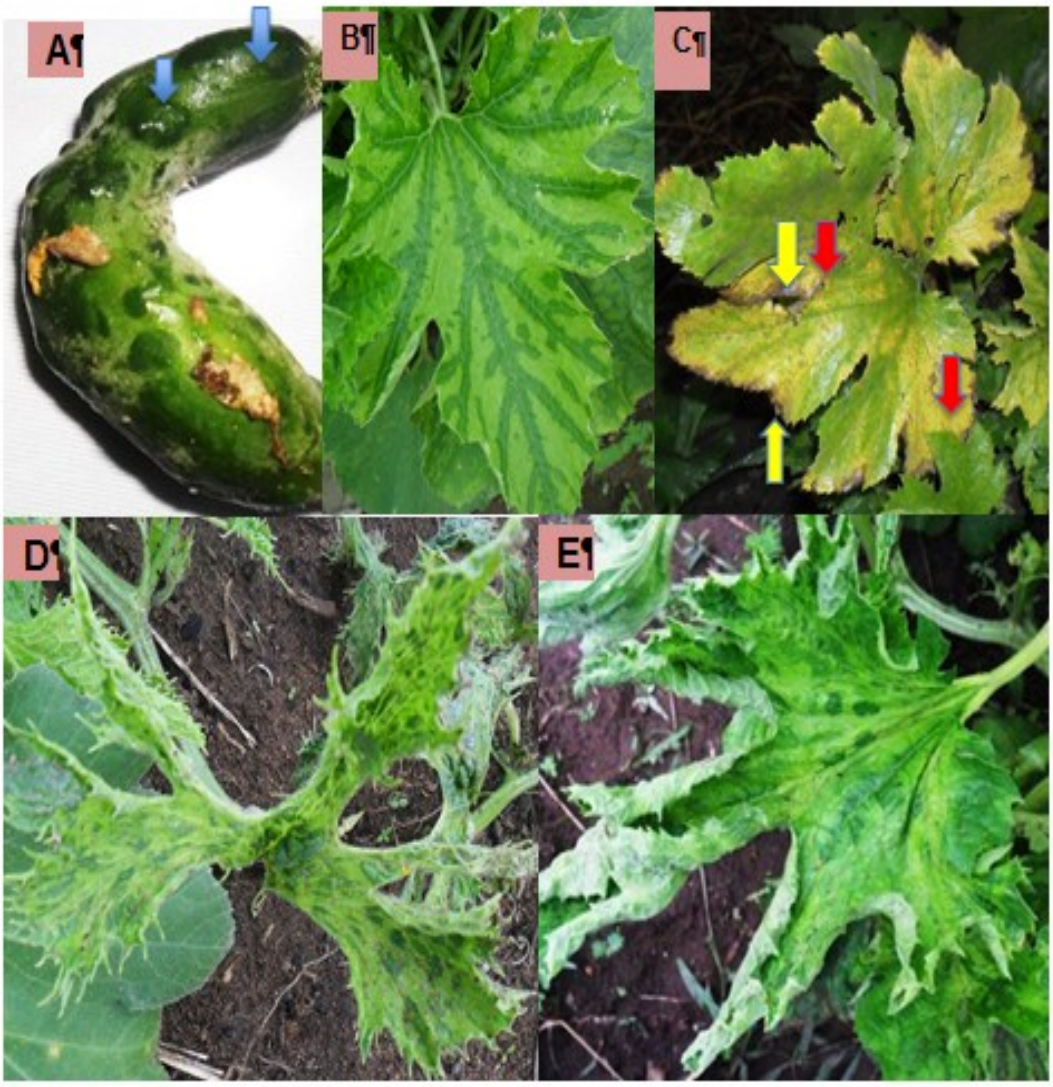

Figure 1 : Symptoms of virus diseases on cucumber fruit and zucchini leaves.

Impact of climate change on virus diseases

According to Adger, Moran, & Wreford, (2010), Climate change is defined as: « Statistically significant variation in the average condition of the climate or in its variability, a variation persisting over a long period of time (decades or more) ». Plant disease risk is greatly influenced by the environmental conditions (De Wolf & Isard, 2007). Thus, it was reported that the dynamics of plant virus epidemics and the losses they cause are likely to be influenced greatly by (i) the direct consequences of climate change such as altered rainfall patterns, increased temperature and greater wind speeds, and (ii) indirectly by factors such as regional alterations in the areas cropped and the ranges of crops grown, and changes in the distribution, abundance and activity of vectors (Jones, 2009). Also, it was reported that the changing climatic conditions by the combined influences of climate change and humankinds' activities, influence viruses by increasing their incidence on the crops and consequently, pose a threat to plant biodiversity and the likelihood of mass species extinctions (Brooks, Mittermeier, & Mittermeier, 2002; Tilman, Gassman, Matson, Naylor, & Polasky., 2002; Norse & Gommes, 2003; Anderson et al., 2004; Cline, 2007; Stern, 2007; Cooper & Jones, 2006; Canto, Aranda, & Fereres, 2009; Jones, 2009; Jones & Barbetti, 2012). Elena et al, (2011), reported also that the emergence of virus is generally associated with ecological change or with intensive agronomical practices and range the causes in four clusters as following: (i) changes in the host plant and/or virus ecology, (ii) changes in the genetic composition of the host populations, (iii) changes in the genetic composition of the virus population, and (iv) in the case

of vectored viruses, changes in the ecology and/or genetic composition of the vector.

Methods used to estimate sample size in survey research

Sample size is one of the four inter-related features of a study design that can influence the detection of significant differences, relationships or interactions (Peers, 1996). Within a quantitative survey design, determining sample size and dealing with nonresponse bias is essential (Holton & Burnett, 1997). To achieve this task it recommended defining the margin error, the level of confidence, and the current estimate of the proportion of the variable of interest. The sample size can be determined using a general biostatistics formula depending weather study is a survey designed to find out the proportion of something, or is designed to find a sample mean. The sample size to estimate the proportion is the following:

$m = \sqrt[t]{p\ (1-p)/n}$ → $n = p\ (1\text{-}p)\ t^2 / m^2$ where,

- m is the is the desired margin of error at 5% level of probability
- t is the t-score, e.g. 1.645 for a 90% confidence interval, 1.96 for a 95% confidence interval, 2.58 for a 99% confidence interval
- p is our prior judgment of the correct value of p.
- n is the sample size (to be determined).

Methods used to identify cucurbits viruses

Serological procedures

Detection of virus by DAS-ELISA

The correct identification of causative agents is crucial in the effective management of virus diseases (Rubio, Soong, Kao, & Falk, 1999). It can hardly be overemphasized since it is fundamental to control (Strange, 2005). Enzyme-linked immunosorbent assay (ELISA) is a successfully established method in routine virus detection (Jones, 2014). The serological method performed was double antibody sandwich Enzyme-linked immunosorbent assay (DAS-ELISA) according to Clark & Adams (1977). Specific antibodies against *zucchini yellow mosaic virus* (ZYMV), cucumber mosaic virus (CMV), *watermelon mosaic virus* (WMV), *Morocco watermelon mosaic virus* (MWMV), and *papaya ringspot virus* (PRSV) were obtained from DSMZ (Braunschweig. Germany). The microplate 96 wells were coated with the antibody IgG diluted in the coating buffer 20 µL in 20 mL buffer at a recommended dilution of 1:1000. Hundred microliter of the dilution is distributed in the wells and incubated for 2-4h at 37˚C. The microplates were washed with PBST. The samples tests homogenized (1/50 g / v) are added and incubated overnight after covering to realize the adhesion of virus particles to the antibody. Virus-specific antibodies were conjugated with alkaline Phosphatase diluted at 1:1000 for the adhesion of antibody to virus particles after washing the microplate the following day. The incubation was performed at the same period at 37˚C. After washing, the microplate, the P-nitrophenyl pNPP dissolved in substrate buffer with a ratio of 10 mg in 10

mL and distributed in the wells then incubated for 30 minutes to 2 hours. The intensity of the virus is determined spectrophotometrically at 405 nm, using an ELISA reader. Each sample was measured in duplicate, and mean values exceeding twice those of negative controls (healthy plant material) were considered virus-positive.

Detection of virus by ACP-ELISA

Antibody coated plate-ELISA (ACP-ELISA) is a rapid serological detection of virus. The antigen is obtained after homogenization of leaf samples in the extraction buffer (binding buffer + 0.05 M DIECA) after diluting at 1:40 (g/v) for 5 min. The samples were then ground and 100 µL of supernatant were distributed in the wells of the microplate and incubated at 37°C for 16 hours. The plates were washed with wash buffer (PBST). Two hundred microliter of the homogenate including 2% skimmed milk dissolved in PBST were added in the wells and incubated at 37°C for 30 min. The following step was the addition of the blocking solution in the plates and the drying using absorbent paper. The MAb was diluted (1: 1000) in the conjugate pad and 200 µL of the mixture was removed and placed in the wells. The plates were then incubated at 37°C for 4 h and washed with PBST. The conjugate of the antibody (RaM-AP) was diluted (1: 1000) in the conjugate and buffer; 200 µL of this mixture was distributed in the wells. The plates were incubated at 37°C for 2 h, and followed by the washing with washed with PBST. The Tablets of P-nitrophenyl phosphate (PNPP) were dissolved in the substrate buffer and 200 µL were added in the wells. The

plates were then incubated at 37°C and the optical density (OD) was read in the spectrophotometer at 460 nm for 30 min, or 2 h.

Molecular procedures

Reverse transcription polymerase chain reaction (RT–PCR) and PCR are popular techniques for detection and identification of RNA and DNA plant viruses respectively. As the viruses studies were RNA viruses, a cDNA strand complementary to the virus is synthetized with reverse transcriptase (RT). Oligonucleotide primers, flanking part of the genome of the virus, are extended by a thermostable DNA polymerase in a series of denaturation and extension steps that exponentially increase the target DNA. PCR-based methods can be adapted to high-throughput applications.

The amplicon or DNA fragments obtained with PCR are visualized on an agarose gel. They are cut and purified from gel for sequencing to provide further data about strain types. Comparisons of genome sequences through phylogenetic studies and systematic searches for recombination events make it possible to assess the relationships between strains, and to formulate hypotheses on their origin and evolution. The information provided can help to identify the pathogenic determinants of the viruses being studied. So far may contribute to the development of breeding programs (Thresh & Fargette, 2003).

Management of virus diseases

Management of these virus diseases is very important in order to improve yields of cucurbit crops. This is a matter of vital importance and concern to the farmer, horticulturist, forester and gardener (Sastry & Zitter,

2014). Various methods employed in the management of cucurbit viral diseases include early monitoring of crops (Zitter, Hopkins, & Thomas., 1996) or monitoring vectors (Sastry & Zitter, 2014); removal of weeds and volunteer cucurbit crops (Bos, 1981; Thresh, 1982); the use of plastic mulch super-reflective (Stapleton & Summers 2002; Barbercheck, 2014); the use of floating row covers (FRC) which delayed the appearance of severe symptoms for over two weeks, (El-Zammar, Abou-Jawdah, & Sobh, 2001).

Other methods have been used to control virus diseases. These include genetic manipulation by introducing resistant genes in the crops (Zitter & Murphy, 2009); use of biological control using micro-organisms antagonist against another one. For instance use of *Bacillus subtilis* and fungus *Sporothrix flocculosa* (syn. *Pseudozyma flocculosa)* against powdery mildew (Nunez-Palenius, Hopkins, & Cantliffe, 2012); use of peutrolum spray oils with effectivity of cinnamon oil (Nunez-Palenius et al. 2012). Control measures can eliminate virus from infected plants by heat or meristem-tip therapy, or by the use of chemicals (Faccioli & Marani, 1998; Mink, Wample, & Howell, 1998). The intercropping using grain sorghum also was applied as virus control method (Damicone & Edelson, 2007) as well as cross protection; the insecticides control (Hull, 2009). Thresh, in (2003) reported that cultural practices can reduce virus diseases when it is applied before, during and after planting time can minimize virus infection. The monitoring of the vectors may contribute to virus diseases management. Thus, the use of non-host "trap plants" may be also considered to attract vectors to reduce the number of

individuals feeding on the crop of interest and thus, the transmission of the disease (Bragard et al., 2013).

The modelling of plants virus epidemics was also used for the management of viral diseases (Nutter, 1997). However each of the methods used presented the limitations.

CHAPTER THREE

DISEASE PREVALENCE AND SEVERITY IDENTIFICATION AND DISTRIBUTION OF VIRUSES INFECTING CUCURBITS IN COTE D'IVOIRE

Introduction

Cucurbits are well-known vegetables in West Africa because of their nutritive value and the incomes they generation Indeed, cucurbits are rich in ingredients such as vitamins (A, B C) and other dietary substances including protein, lipid, carbohydrates, mineral salts (Ca, Fe, P) and lycopene having an antioxidant activity (Ozaslan et al., 2006). Cultivation of cucurbits ensures a substantial income to the farmers (Janssen et al., 2002-2003; Bi et al., 2011). Pests and diseases are major biotic constraints to the production of cucurbits worldwide (Loebenstein & Thottappilly, 2009). Among these, plant viral diseases are the most important constraint to cucurbit production (Lecoq & Katis, 2014; Ayo-John et al., 2014), causing physiological disorders and enormous losses all over the world in terms of quantity and/or quality of products (Nicaise, 2014). It is reported that up to 39 well characterized viruses including *Begomovirus, Crinivirus, Polerovirus, Cucumovirus, Ipomovirus, Tobamovirus, Tospovirus* and *Potyvirus* are known to naturally infect cucurbits (Antignus et al., 2001; Brown, Idris, Alteri, & Stenger., 2002; Salem, El-Gamal, & Sadik 2007; Gholamalizadeh, Vahdat, Keshavarz, Elahima, & Bananej 2008; Knierim, et al., 2010). There are reports of viral infection of cucurbits in several countries including India (Mantri, Kitkaru,

Misal & Ravi, 2005; Madhubala et al 2005), in Serbia (Dukic, Krstic, Vico, Berenji, & Duduk, 2006), Birmania (Kim, Mizutani, Soe, Lee, & Natsuaki, 2010), France (Lecoq, 1990), Japan (Yamashita et al., 1979), Netherlands (Van Dorst, Huijberts. & Bos. 1983), and southern Spain (Esteva, Nunez, & Cuartero., 1988), indicating that virus diseases are spread over the world.

In West Africa, cucurbits infecting diseases are reported in Nigeria, (Owlabi et al., 2008, Ayo-John et al., 2014), Mali (Tsai, Abdourhamane. Knierim, Wang, & Kenyon 2010) and Côte d'Ivoire (Thouvenel & Fauquet, 1987; Koné et al., 2010; Agneroh et al., 2012). Thouvenel & Fauquet in 1987 reported the presence of CMV and PRSV on cucumber and cucurbita, then their geographical distribution. The study conducted by Koné et al. (2010) was in one rainy season in the southern part of Côte d'Ivoire where ZYMV has been reported for the first time in cucurbit. The study done by Agneroh et al. (2012) was conducted in three districts of the country and involved only *Lagenaria siceraria (Molina*) Standl & *Citrullus* sp. Despite these studies, little is known about viral disease in the different and the other agro-ecological zones, and cucurbit crops. These limitations in the previous studies led to the recent studies which were carried out to identify viruses infecting different crops and weeds in both rainy and dry seasons in the six agro-ecological zones of Côte d'Ivoire.

Material and methods

Study areas

The study was conducted in 28 districts areas across six agro-ecological zones in Côte d'Ivoire (Figure 2). The features of the various districts are shown in Table 1.

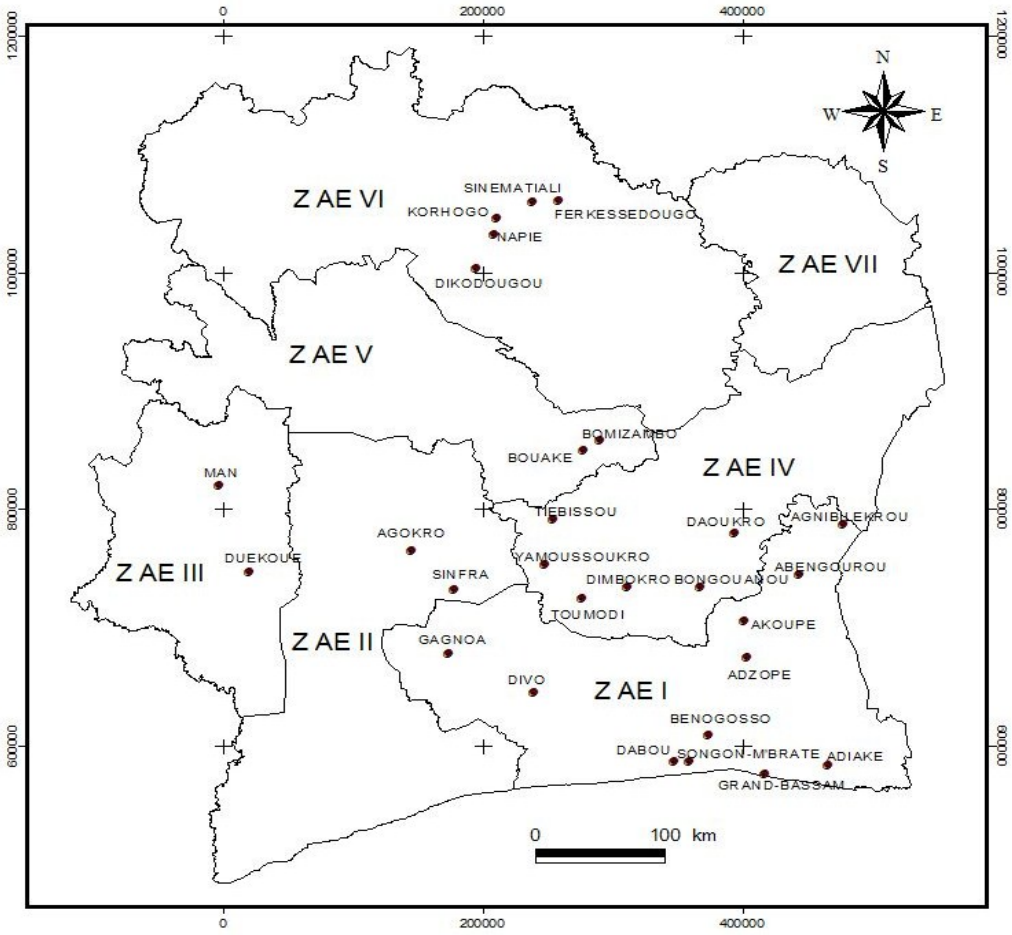

Figure 2: Map of Côte d'Ivoire showing the six agro-ecological zones surveyed with sampling sites.

ZAE: Zone agro-ecologic numbered form I to VI

Table 1: *Features of the agro-ecological zones surveyed in Cote d'Ivoire in 2014*

Districts	AASL (m)	M.AT (°C) ecart-type	MARF (mm)	Vegetation/ seasons
Songon Mb., Dabou, Bassam, Adzope, Divo, Akoupe, Abengourou, Agnibilekro	9-225	29 (5.6)	1400-2500	Humid dense forest of south / 2 DS+2 RS
Agokro, Gagnoa, Sinfra	215-240	23.5(13.4)	1300-1750	Semi-deciduous forest / 2DS+ 2RS
Man, Duekoue	241-340	24.5 (7.7)	1300-2300	Humid green forest of West / 1DS+1 RS
Toumodi, Dimbokro, Daoukro, Yamoussoukro, Tiebissou, Bongouanou	87-193	23.5(13.4)	1300-1750	Transitional forest-savanna zone / 2DS+ 2RS
Bouake, Bomizambo	243-322	23.5 (13.4)	1300-1750 (unimodal)	Transitional Savannah zone / 2DS+2RS
Napie, Korhogo, Dikodougou, Sinematiali, Ferkessedougou	235-378	26.7(1.1)	1150-1350 (unimodal)	Savannah / 1DS +RS

DS: dry season; RS: rainy season

Field survey, data collection, sampling

The survey was conducted on several cucurbits crops including cucumber, squashes (zucchini and pumpkin), watermelon, melon and gourd between February and August 2014. The dry season survey was done between February and March 2014, while that of rainy season was carried out between June and August 2014. Twenty eight districts diverse in their climate and the conditions under which cucurbits crops are grown were surveyed, and fifty eight fields selected at intervals of 5 km were assessed. In a given district, the fields were surveyed in a vicinity of 10 to 30 km.

The locations of the various fields surveyed were taken using the global positioning (GPS) system device.

Plants were randomly evaluated for virus-like symptoms such as vein clearing, vein-banding, mosaic, and mottling, puckering, leaf and fruit malformation. About 10 plants per field were sampled from which 3 to 5 young leaves were taken. The weeds within the fields as alternative sources of virus were also collected. The plants were sampled following an « X» transect. A total of 757 samples were collected. The samples include weeds (appendix A), cucurbits leaves and based on virus symptoms in order to identify viruses associated with them. Field observations were done in order to examine the fields' conditions and the management of cucurbit crops (Table 4).

Estimation of disease prevalence and severity

Disease prevalence was estimated for each farm based on plants found along a 10 m diagonal transect set at the middle of the farm by determining the rate of diseased, plants expressed as a percentage of the total number of plants along the diagonal transect in the delimited area.

$\mathbf{DP}(\%) = \frac{\boldsymbol{n}}{\boldsymbol{N}} \times 100$ Where, n is the number of plants showing symptoms; N is the total number of plants assessed

Severity of viral disease was determined based on the calculation of disease severity index according to the formula:

$$\text{Disease Severity (DSI)} = \frac{\mathbf{0*p0+1*p1+2*p2+3*p3+4*p4+5*p5}}{\mathbf{N\,(G-1)}}\ \mathbf{X}\ 100$$

where, P0 - P5 is the total number of observed plants in each disease

symptoms grading per farm in each state within the agro ecological zone surveyed.

G = Number of grading = 6N = Total number of observations. A modified visual scale (from 0 to 5) of (Merritt et al., 1999) and Steel and Torrie, (1980) based on disease symptoms, was used to score the disease plants as following:

0. No disease symptoms

1. Mild symptoms on 10-25% of leaf surface

2. Mottling symptoms on 50%/ onset of mosaic

3. Chlorosis/leaf rolling/ onset of downward and up cupping

4. Severe mosaic/ severe leaf distortion, deformation/ yellowing

5. Very severe leaf distortion/necrosis/abnormal growth/ death of plant.

Samples collection for viral disease indexing

Cucurbit leaf samples were collected from 58 fields randomly selected from 28 districts across the agro-ecological zones. The samples were then placed in a plastic bag labeled and conserved in the cool box. The samples were transferred to the laboratory for virus identification using polyclonal antisera against five viruses. Once in the laboratory, the leaf samples were cut in small pieces and dried over the calcium chloride contained in the tin labeled then conserved at 4°C.

Identification of viruses isolates using DAS-ELISA

Five kits including polyclonal antisera and positive controls of *Zucchini yellow mosaic virus* (ZYMV), *Water melon mosaic virus* (WMV), *Papaya ringspot virus* (PRSV), *Moroccan watermelon mosaic virus* (MWMV), and *Cucumber mosaic virus* (CMV) were used for virus

identification by DAS-ELISA as described by Clark and Adams (1977). Dried samples were homogenized (dilution 1:50 g / v) in extraction buffer (8.0 g NaCl, 0.2 g KH_2PO_4, 1.1 g Na_2HPO_4, 0.2 g KCl /L, pH 7.4) containing 0.05% v/v Tween 20, and 2% w/v polyvinyl-pyrolidone.

Each microplate was coated with one of MWMV, CMV, WMV, PRSV, and ZYMV. The antibody IgG diluted in the coating buffer 20 µL in 20 mL buffer at a recommended dilution of 1:1000 according to the manufacturer's instructions. Hundred microlitre of the dilution is distributed in the wells and incubated for 2-4h at 37˚C. The microplates were washed with the phosphate buffer saline-Tween (PBST). The samples tests homogenized (1/50 g / v) are added and incubated overnight after covering the microplate, to realize the adhesion of virus particles to the antibody. The microplates were washed with washing buffer PBST, the following day. The washing is done three times at 3 minutes interval.

Virus-specific antibodies were conjugated with alkaline Phosphatase (PBST, 2% PVP and 0.2% albumen) diluted at 1:1000 for the adhesion of antibody to virus particles after washing the microplate the following day. The incubation was performed at the same period at 37˚C. After rinsing off the microplate, the p-nitrophenyl pNPP diluted 10 mg for 10 mL was added to the substrate buffer and distributed in the wells then incubated for 30 minutes or 1 hour. The intensity of the virus is determined spectrophotometrically at 405 nm, using an ELISA reader. Each sample was measured in duplicate, and mean values exceeding twice those of negative controls (healthy plant material) were considered virus-positive.

Identification of viruses isolates using TAS-ELISA

The triple antibody sandwich ELISA (TAS-ELISA) procedure was used to test the presence of other virus in the leaf samples like *Cucumber vein yellowing virus* (CVYV) according to DSMZ instructions. The antigen was obtained after homogenization of leaf samples in the extraction buffer (PBST + 2% PVP) after diluting at 1:50 (g/v) for 5 min.

The antibody IgG diluted in the coating buffer 20 µL in 20 mL buffer at a recommended dilution of 1:1000 according to the manufacturer's instructions. Hundred microliter of the dilution is distributed in the wells and incubated for 2-4h at 37˚C. The microplates were washed 3 times with PBST following by the blotting of the microplate by tapping upside down on tissue paper. One hundred microliter of the blocking solution including 2% skimmed milk dissolved in PBST were added in the wells and incubated at 37°C for 30 min. The blocking was removed tapping dry. The samples were then ground and 100 µL of supernatant were distributed in the wells of the microplate and incubated overnight at 4°C. The plates were washed with wash buffer (PBST). The following step was to add the MAb diluted (1: 1000) in the conjugate buffer and 100 µL of the mixture was added in the wells. The plates were then incubated at 37°C for 4 h and washed with PBST. The conjugate of the antibody (RaM-AP) was diluted (1: 1000) in the conjugate and buffer; 100 µL of this mixture was distributed in the wells. The plates were incubated at 37°C for 2 h, and followed by the washing with washed with PBST. The Tablets of P-nitrophenyl phosphate (PNPP) were dissolved in the substrate buffer and 200 µL were added in the wells. The

plates were then incubated at 37°C and the optical density (OD) was read in the spectrophotometer t 405 nm for 30 min, or 2 h. Any sample was positive to this antibody.

Data analysis

The GPS coordinates were used TO map study areas surveyed. using ENVI 4.7.01. Disease prevalence and severity were analyzed for non-parametric data using Fischer exact test. The means among the agro-ecological zones were compared with Least Significant Difference (LSD) at 5% level of probability using GenStat Discovery. The incidences of CMV, ZYMV, and PRSV were compared by chi-square test among the agro-ecological zones and between the two seasons using Epidemiologic measures (Alexander, et al., 2009).

Results

General observations

Farming practices assessed were cropping patterns, the presence of weeds, seeds supply, roguing practice, field sanitation, pesticides application, and application of irrigation water. The frequencies estimated represent the means of fields assessed. Two categories of agro-ecological zones were observed for this farming practice. The mono-cropping appeared as the predominant cropping patterns practiced by the majority of farmers. Mixed cropping was only observed in AEZ II, where the cucurbit crops were associated to the rice, maize or legumes as tomato, pepper. The weeds play an important role as foci of infection from which there is spread into or within

crops (Bos, 1981; Thresh, 1982). Four levels of weeds were defined, i.e. zero level which was clean field, level 1 equal to low, level 2 corresponding to the average and then level 3 which was thickness of weeds. The general analysis showed a moderated level of weeds in the agro-ecological zones which indicated a sustained maintenance of the fields. Despite, that which Third groups were distinguished. The first ones were those with a high number of cleans fields, observed in AEZ V & VI respectively at 55.5 % and 75% which means; the second group with a few number of clean fields identified in AEZ IV & AEZ I where it was estimated to 23% and 18%, and the latter group where we did not identified clean fields, met in the AEZ II & III suggesting the lack of care and a sign of abandon of the crops. The supply of seeds indicated the farmers' use of the AEZ I used the available seeds in the shop since cucurbits growth represent a professional job for them and provide remunerative incomes.

The other ways to get seeds remaining the local market and the harvest products showed high proportions in general because of the cheaper price and the fact that farmers are heavily depending on the harvest products from the previous works. The overall result showed high means of non-application of roguing in the fields, because the lack of lack of knowledge, the rejection of the practice and especially, because rogueing is generally unpopular with farmers, who are seldom prepared to allocate the time and effort required to inspect crops with the thoroughness and frequency required to identify and remove diseased plants (Thresh, 2003). The application of pesticides by the farmers was inappropriate. The dominant application frequency was in

majority twice suggesting the intensification of the gardening. Crops sanitation was practiced mainly in all agro-ecological zones, suggesting an agricultural practice well-known by the farmers.

The application of irrigation water by the farmers showed only one mode. Out of a total of 50 fields assessed the irrigation none irrigation system was observed suggesting that the irrigation of cucurbit crops was done by aspersion or spray. This may be one of the factors of occurrence of virus disease everywhere, since the practice is involved in the onset of infection on the plant (Afouda et al., 2013).

Disease symptoms observed on cucurbit crops leaves

Symptoms observed in the cucurbits crops during field survey are presented in Table 2 and Figure 3. The survey was conducted on five species of cucurbits namely cucumber, zucchini, pumpkin, watermelon and melon, and watermelon. A total of 345 samples were collected in the dry season comprising 170 cucumbers, 135 zucchini, 2 pumpkins, 2 melons, and 36 watermelons .In the rainy season 306 samples included 183 cucumbers, 92 zucchini, 19 pumpkins, and 12 gourd calabashes (*Lagenaria* sp.). Virus-infected plant may show a wide range of developmental abnormalities. The most commonly observed symptoms on all cucurbit crops were vein banding, (a, b, f, h ,j ,l, o, p, w), downward leaves (m,r), shoe-string and color breaking, of leaf (c, d, I, t, u), leaf rolling (q, v, w). The other symptoms encountered were mottling, stunting, narrowing, leaf rolling and upward cupping (f, g, p, t,), yellowing (a ,e ,h, l), ringspots and blistering of fruit, (g, l, s, x).

Table 2: *Summary of frequent symptoms observed on cucurbit infected plants in the agro-ecological zones*

AEZ	Symptoms developed on the cucurbits plants leaves
I	Vein banding associated to white or yellow, colour on cucumber leaves, vein clearing, upward and downward cupping, shoe-string, stripe leaves on zucchini.
II	Stunted plants, downward cupping, blister, thickening leaves, vein banding.
III	Vein banding, shrink leaves associated to yellowing, blistered leaves, stripe leaves of zucchini plants
IV	Vein banding, crinkled leaves, thickening leaves, upward cupping
V	Mottling, leaf rolling, vein banding
VI	Leaves rolling, Jagged, leaves, shoe-string, yellow ringspot

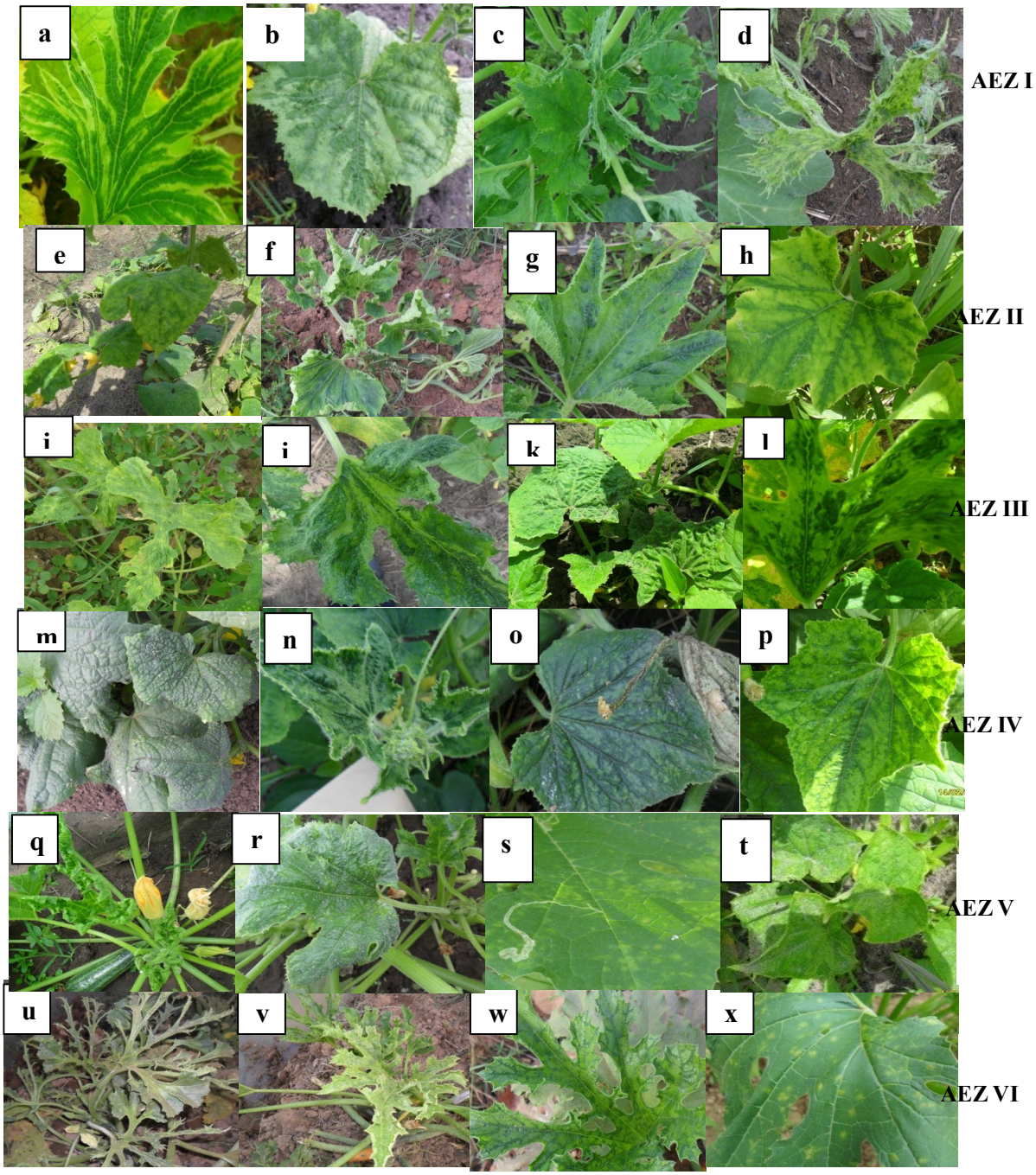

Figure 3: Various symptoms of virus diseases occurring on cucurbit crops.

Prevalence and severity of viral diseases

The mean prevalence of viral diseases on cucurbit crops across the six agro-ecological zones during 2014 in the major and minor cropping seasons are showed in Table 3. The ANOVA did not show significant difference ($p > 0.05$) in mean disease prevalence among the agro-ecological zones in both dry and rainy seasons. However, AEZ II had the highest prevalence of 79.67% whereas AEZ IV had the lowest (19.7%). In the rainy season AEZ III had the highest prevalence of 59.8% whereas AEZ V had the lowest (1.4%).

An ANOVA on mean severity indices revealed significant difference ($p < 0.05$) among the agro-ecological zones. AEZ III had the highest severity indices of 51.7% whereas AEZ VI had the lowest (0.6%).

An independent sample t-test analysis revealed that the mean prevalence of virus diseases in the dry season (53.6%) was significantly higher ($t = 1.835$; $p = 0.04$) than that of the rainy season (29.35%). The mean severity index revealed in the dry season (47.39%) was also significantly higher ($t = 2.159$; $p = 0.003$) than in the rainy season (28.17%) as shown in the Table 4

Table 3: *Mean prevalence and severity index of viral disease in the dry and the rainy seasons in the six agro-ecological zones in Côte d'Ivoire*

Agro-ecological zones	Prevalence (%)		Severity (%)	
	Dry season	Rainy season	Dry season	Rainy season
AEZ I	51.5 ns	37.3 ns	53 ns	43.1 a
AEZ II	79.6	37.8	52.1	27.7 abc
AEZ III	78.7	59.8	56.8	51.7a
AEZ IV	19.7	33.3	26.3	34 ab
AEZ V	36.8	6.5	47.8	11.9 bc
AEZ VI	55.3	1.4	49.5	0.6 c
Mean	53.6	29.3	47.6	28.2
LSD ($p \leq 0.05$)	-	-	-	28.17

AEZ: Agro-ecological zone; Means having the same letters are not significant different by LSD at 5 % level of probability; ns means non-significant difference between the means

Table 4: *Comparison of mean disease prevalence and severity index in both dry and rainy seasons*

Season	Prevalence (%)	Disease Severity index (%)
Dry	53.6	47.39
Rainy	29.35	28.17
t-test	1.835	2.159
p-value	0.04	0.03

Distribution of the viruses on individual cucurbit crop sampled in dry season

Out of the 345 samples collected, 269 (78%) were positive at least to one virus identified (Table 5). The infection rate of virus per crop indicated the highest incidence of CMV in cucumber (57.2%), in zucchini (40.8%), and in

melon (50%). The lowest incidence was recorded in watermelon (5.55%). Moderate incidence of ZYMV was recorded in cucumber (34.5%) zucchini (31.85%) and watermelon (11.1%) but not in melon (0%) and pumpkin (0%). PRSV was detected in cucumber in cucumber (8.3%) and zucchini (14.097%) only but, not in watermelon, melon and pumpkins. The overall infection rate per virus showed that CMV was more prevalent (52%), followed by ZYMV (36.4%), whereas PRSV had the lowest infection rate (11.5%).

Table 5: *Distribution of viruses on individual cucurbit crops collected in the dry season*

	Infection rate per crop (%)							Infection rate per virus (%)
Virus	mel	pum	cuc	zuc	wat	mel	pum	
CMV	0	0	57.2	40.8	5.55	50	0	52
ZYMV	1	0	34.5	31.85	11.1	0	0	36.4
PRSV	0	0	8.3	14.07	0	0	0	11.5
positive	1	0	85.3	86.7	16.7	50	0	78

DS = dry season; cuc= cucumber; zucc= zucchini; lag= lagenaria or calabash gourd; pum= pumpkin; wat = watermelon; mel= melon. *Cucumber mosaic virus; (*CMV), *Zucchini yellow mosaic virus* (ZYMV); *Papaya ringspot virus* (PRSV).

Distribution of the viruses on individual cucurbit crops sampled in the rainy season

The infection rates of CMV, ZYMV and PRSV in each of the 4 cucurbits are shown in Tables 6. A total of 207 (67.7%) samples were positive out of 306. CMV was detected in all four crops, ZYMV was detected in cucumber, zucchini and pumpkin but it was not detected in

lagenaria whereas PRSV was detected in cucumber only. With the respect to virus infection rates per crops, CMV had 100% infection of lagenaria, followed by zucchini (42.7%), cucumber (30%) and pumpkin (25%). ZYMV was more prevalent in pumpkin (75%) followed by cucumber (63%) and zucchini (42.4%). Infection rate of PRSV was only 7 2%. In respect of the overall infection rate per virus, ZYMV was the prevalent (61%), followed by CMV (35%), whereas PRSV was the lowest (4.3%).

Table 6: *Distribution of viruses on individual cucurbit crops collected in the rainy season*

	Infection rate per crop (%)				Infection rate per virus (%)
Virus	cuc	zucc	lag	pum	
CMV	30	42.7	100	25	35
ZYMV	63	42.4	0	75	61
PRSV	7.2	0	0	0	4.3
positive	68	64.8	25	63.1	67.7

RS = Rainy season; cuc= cucumber; zucc= zucchini; lag= lagenaria; pum= pumpkin; RS = rainy season. *Cucumber mosaic virus (*CMV); *Zucchini yellow mosaic virus (*ZYMV*)*; *Papaya ringspot virus (*PRSV*)*

Distribution of the viruses in agro-ecological zones

The infection rate of CMV was higher during the dry season than in the rainy season in the AEZI (26.5% vs 15.3%), AEZII (54.5% vs 20%) and AEZ IV (44% vs 18%). In contrast, it was lower during the dry season in the AEZIII (24% vs 60%), AEZV (2% vs 30%) and AEZVI (22.44% vs 20%) (Table 7).

The infection rate of ZYMV was higher in the dry season than the rainy season in the AEZIII (62% vs 53.5%), and AEZIV (28% vs 23.2%). It was however lower in the dry season than the rainy season in the AEZI (27% vs 35%), AEZII (27% vs 28%) and AEZV (0% vs 20%) and AEZ VI (12.2% vs 13.3%). Chi square test however did not show significant difference ($p > 0.05$) in the ZYMV infection rate between the two seasons for all the six agro-ecological zones (Table 8).

There was no significant difference ($p > 0.05$) in the CMV infection rate between the rainy and dry seasons in the ecological zones V and VI. However, the infection rate in the dry and rainy seasons in the other ecological zones was significantly different among them ($p < 0.05$).

Table 7: *CMV infection rate per agro-ecological zone in both dry and rainy seasons*

zones	Dry season	Rainy season	χ^2	p-value
	IR (%)	IR (%)		
AEZ I	(39/149)26.5	(26/170)15.3	5.14	0.023*
AEZ II	(12/22) 54.5	(5/25) 20	4.65	0.031*
AEZ III	(6/21) 24	(18/30) 60	3.72	0.05
AEZ IV	(10/56) 18	(42/94) 44.7	10.00	0.001***
AEZ V	(2/10) 20	(3/10) 30	0.00	0.5^{ns}
AEZ VI	(11/49) 22.44	(3/15) 20	0.02	0.87^{ns}

AEZ: Agro-ecological zone; IR: Infection rate *: ns: non significance

Table 8: *ZYMV infection rate in both dry and rainy seasons in the six agro-ecological zones*

Zones	Dry season	rainy season	$\chi 2$	p-value
	IR (%)	IR (%)		
AEZ I	(42/149) 28.2	(64/170) 35	2.79	0.09^{ns}
AEZ II	(6/22) 27.3	(7/25) 28	0.07	0.78^{ns}
AEZ III	(13/21) 62	(16/30) 53.5	0.1	0.74^{ns}
AEZ IV	(26/94) 28	(13/56) 23.2	0.17	0.68^{ns}
AEZ V	(0/10) 0	(2/10) 20	0.56	0.45^{ns}
AEZ VI	(6/49) 12.2	(2/15) 13.3	0.11	0.73^{ns}

AEZ: Agro-ecological zones; IR: Infection rate; ns: non-significance

PRSV infection rate in the rainy season were significantly different ($P<0.05$) from the dry seasons in agro-ecological zones I, II, IV and VI (Table 6). In the agro-ecological zones II, III and VI, the virus was detected only in the dry season and absent in the rainy season. PRSV was not detected in AEZ V in both the dry and rainy seasons. However it was detected in both seasons in AEZ I and IV. Chi square test revealed significant difference ($p < 0.05$) in the infection rate in the rainy and dry season at the two agro-ecological zones (Table 9).

Table 9: *PRSV infection rate in both dry and rainy seasons in the six agro-ecological zones*

Zones	Dry season IR (%)	rainy season IR (%)	$\chi 2$	p-value
AEZ I	(13/149) 9	(4/170) 2.3	5.19	0.022*
AEZ II	(6/22) 27	(0/25) 0	5.56	0.01*
AEZ III	(1/21) 5	(0/30) 0	0.03	0.856ns
AEZ IV	(5/94) 5.3	(5/56) 9	3.61	0.05*
AEZ V	(0/10) 0	(0/10) 0	NA	NA
AEZ VI	(8/49) 16.3	(0/30) 0	3.8	0.05*

AEZ = Agro-ecological zones; IR = Infection rate; ns = non-significance;

NA = Non applied

Distribution of viruses within the weeds

Weeds samples were collected from locations that had weeds during the survey. Twenty seven weeds samples were tested with polyclonal antibodies raised against CMV, PRSV, and ZYMV. The occurrence of CMV in both annual and perennial herbs was 15/27(55.5%) while ZYMV occurrence was 7/27 (26%), followed the low occurrence of PRSV of 1/27 (4%) as shown in Table 10. Taking account the annual and the perennial plants infection, CMV occurrence in the annual plants was 9/15 (60%) and was estimated in the perennial to 6/15 (40%). ZYMV was the most virus detected in the annual plants with 6/7 (85.8%) while it occurred in one perennial plant 1/7 (14.3%). PRSV was the less infecting virus detected only in one annual plant 1/1 (100%) as shown in Table 11.

Table 10: *Detection of virus in weeds collected in both dry and rainy seasons in 2014 using DAS-ELISA*

Family	symptoms	Morphology	CMV	ZYMV	PRSV
Asteraceae	downward cup.	Ann.Herb	0.907(+)	0.053 (-)	0.045 (-)
Fabaceae	mosaic	Ann.Herb	0.063 (-)	0.037 (-)	0.043 (-)
Euphorbiaceae	mosaic	Ann.Herb	0.533 (+)	0.577 (+)	0.043 (-)
Asteraceae	downward	Ann.Herb	1.437 (+)	0.295 (+)	0.043 (-)
Asteraceae	none	Ann.Herb	1.027 (+)	0.922 (+)	0.048 (-)
Poaceae	none	Ann.Herb	0.436 (+)	0.552 (+)	0.289 (+)
Commelinaceae	none	peren. Herb	1.094 (+)	0.04 (-)	0.075 (-)
Capparidacea	necrosis	peren. Herb	0.710 (+)	0.028 (-)	0.085 (-)
Portulaceae	narrowing	peren. Herb	0.033 (-)	0.066 (-)	0.085 (-)
Asteraceae	leaf curl	peren. shrub	0.061(-)	0.049 (-)	0.05(-)
Portulaceae	none	peren. Herb	0.08 (-)	0.043 (-)	0.07 (-)
Euphorbiaeae	mosaic	peren. Herb	0.77 (+)	0.021 (-)	0.107 (-)
Solanaceae	none	peren. Herb	0.063 (-)	0.023 (-)	0.092 (-)
Poaceae	brownish	peren. Herb	0.07 (-)	1.471 (+)	0.166 (-)
Euphorbiaceae	none	peren. Herb	0.626 (+)	0.027 (-)	0.078 (-)
Amaranthaceae	leaf rolling	Ann. Herb	1.593 (+)	0.062 (-)	0.062 (-)
Asteraceae	leaf curl	Ann. Herb	1.65 (+)	0.098 (-)	0.108 (-)
Malvaceae	mosaic	peren. Herb	0.533 (+)	0.043 (-)	0.074 (-)
Fabaceae	mosaic	Ann. Herb	ND	0.29 (+)	0.098 (-)
Euphorbiaceae	none	Ann. Herb	1.21 (+)	0.840 (+)	0.059 (-)
Asteraceae	mosaic	Ann. Herb	0.654 (+)	0.023 (-)	0.113 (-)
Asteraceae	none	peren. shrub	0.223 (-)	0.051 (-)	0.012 (-)
Poaceae	none	peren. Herb	0.032 (-)	0.027 (-)	0.119 (-)
Verbenaceae	none	peren. shrub	0.517 (+)	0.271 (-)	0.055 (-)
Poaceae	none	Ann. Herb	ND	ND	ND
Poaceae	brownish	Ann. Herb	ND	ND	ND
Poaceae	none	Ann. Herb	ND	ND	ND
Rubiaceae	none	Ann. Herb	0.052 (-)	0.026 (-)	0.127 (-)

(+): Sample infected; (-): non infected sample TP: Threshold of positivity < 2TP < DO < 3 TP;

ND: samples non-tested.

Table 11: *Mean ELISA optical density of viruses in determined in both annual and perennial weed samples*

Weed type	CMV (%)	ZYMV	PRSV
Annual herb	9/15 (60.00)	6/7(85.80)	1/1(100)
Perennial herb	6/15 (40.00)	1/7 (14.30)	0
Total sample infected	15/27 (55.5)	7/27 (26)	1/27 (3.7)

Cucumber mosaic virus (CMV); *Zucchini yellow mosaic virus* (ZYMV); *Papaya ringspot virus* (PRSV).

Mixed viral infections of cucurbit crops

Apart from AEZ V mixed viral infections of cucurbit crops were in all agro-ecological zones (Table 12). There were double and triple infections of cucurbits by CMV, ZYMV and PRSV. About 6.95% of samples were co-infected by CMV and ZYMV, 1.73% was co-infected by ZYMV and PRSV, 1.44% was co-infected by PRSV and CMV, whereas 3.18% were co-infected by CMV, ZYMV, and PRSV.

Table 12: *Mixed infections of viruses observed in cucurbit crops surveyed during the growing seasons 2014 in Cote d'Ivoire*

zone /crops	samples collected	Double infection			Triple infection
		CMV+ ZYMV	ZYMV+ PRSV	PRSV+ CMV	CMV+ZYM+ PRSV
AEZ I					
cucumber	3/48	1	0	0	2
zucchini	9/63	3	2	1	3
watermelon	1/36	1	0	0	0
melon	1/2	1	0	0	0
Total	14/149	6	2	1	5
AEZ II					
cucumber	3/9	3	0	0	0
zucchini	5/13	0	2	3	0
Total	8/22	3	2	3	0
AEZ III					
cucumber	3/9	3	0	0	0
zucchini	1/12	1	0	0	0
Total	4/21	4	0	0	0
AEZ IV					
cucumber	14/92	10	1	1	2
Total	15/92	10	1	1	2
AEZ V					
cucumber	0/10	0	0	0	0
Total	0/12	0	0	0	0
AEZ VI					
cucumber	0/6	0	0	0	0
zucchini	5/41	1	0	1	3
Total	5/50	1	0	1	3
Global total	45/345	24	5	6	10
Proportion (%)	13.04	6.95	1.44	1.73	3.18

AEZ: agro-ecological zone; *Cucumber mosaic virus* (CMV); *Zucchini yellow mosaic virus* (ZYMV); *Papaya ringspot virus* (PRSV)

Discussion

In the world agriculture context, intensification of cultural practices, climate alterations, and extensive exchanges affecting global markets are associated with an increased incidence of plant viral diseases (Nicaise, 2014). This causes major losses in the quantity and quality of cucurbit crops worldwide and they represent one of the most important limiting factors for growers (Provvidenti, 1996).

The study revealed the presence of CMV, ZYMV, PRSV, which are among the most frequent and economic significance (Yuki et al., 2000; Massumi et al., 2007; Yardmici & Ozgonen, 2007) infecting cucurbits worldwide. The identification of these three (3) viruses in all studies conducted until now indicates that there is lack of resistance in cultivated varieties used by the farmers (Ibaba et al., 2015). These viruses also present a real threat to cucurbits production worldwide causing up to 100% yield losses and up to 95% reduction in market value (Lecoq & Desbiez, 2012). In all agro-ecological zones surveyed, the prevalence of these viruses were observed, suggesting that viral infection of cucurbits is widespread in all agro-ecological zones of Côte d'Ivoire. There was no significant difference in the mean disease in both dry and rainy seasons indicates that virus diseases are prevalent in all seasons. However the highest prevalence was recorded in the AEZ II (79.6%) and AEZ III (78.7%). Mean disease severity indices were not also statistically different among the agro-ecological zones in dry season but showed always a peak in AEZ II and AEZ III. Also, the study showed, based

on mean disease severity recorded in rainy season two groups of agro-ecological zones where the first group including AEZ I, II, III, & IV with the highest means of disease severity, and the second group comprising AEZ V & VI with the lowest disease severity. It was reported that seasonal changes can affect hosts, pathogens and vectors in ways that alter components of the basic reproductive number that determines the rate at which infected hosts are produced (Altizer et al., 2006). And then, these mechanisms include those that influence parasite transmission, in part by altering the behaviour of hosts, the biology of vectors or parasite infectious stages in the environment (Altizer et al., 2006; Jones, 2009). Thus, the variations observed in disease prevalence and severity among the agro-ecological zones could be a change in the transmission of the virus to the host plants or the change of geographical variation in the timing and severity of epidemics with latitudinal and altitudinal gradients in the onset and persistence of infections (Cook, Glass, LeBaron, & Ho, 1990; Randolph et al., 2000; Elena et al., 2011). In addition, the increased temperature might increase the susceptibility of host plants to the infection and may accelerate the fitness of viruses, as reported by Porter, Fitt, Spero, Rogers, & White. (1989); Harvell, Kim, Quirolo, Smith, & Weir, (2001-2002); Mitchell, Rogers, Little, & Read. (2005). These may account for the highest values of prevalence and severity of viral virus diseases in dry season than the rainy season.

Apart from the seasonality and the ecological conditions influencing viral disease dynamic, the structure of soil, virus species and field conditions could influence the course of infection (Plantegenest Le May, & Fabre, 2007;

Hull, 2009). Thus, the high prevalence and severity of disease in AEZ II and AEZ III may be due to poor soil fertility of the fields surveyed (Horst, 1990). It could be consequences of inadequate conditions of cucurbit crops growth observed during the survey, no pesticides application, and thickness of the weeds in the fields and especially the high level of sources of virus because of the overlapping of fields (Thresh, 2003). At least one of the cucurbit crops was infected by a virus that suggests that all the cucurbits constitute a potential and alternative virus reservoir (Ali, Mohammad, & Khattab, 2012). This distribution occurred at different rates depending on the crops. Hence highest infection rates were reported on pumpkin in rainy season (100%), as well as cucumber samples collected in dry season (57.2%) and (78.1%) in (Tables 5).

In dry season, CMV was more frequent on samples (52 % vs 36.4%) whereas, ZYMV was frequent in rainy season (61% vs 35%) shown in Table 6. This variation in virus incidence in the crops could be due to intense activity of aphid population colonizing the host plants, crop species and the weeds (Castle, Perring, Farrar, & Kishaba, 1992; Plantegenest, et al., 2007; Elena et al., 2011). In addition, the lacks of virus-free seeds represent the sources of inoculum and the low level of field sanitary may be some of the factors influencing the infection rate of CMV (Cradock, Graça, & Laing, 2001; Hull, 2009-2013). The study also revealed the effective presence of viruses in weeds hosts. CMV was more infectious virus infecting 15 weeds samples (55.5%) out of 27, followed by ZYMV infecting weeds 7 (26%) and PRSV infecting only 1 sample (4%). The high incidence of CM in weeds is not new. A relative study carried out in Nigeria indicated 78.6% incidence of CMV in weeds

(Ayo-John et al., 2014). It was reported that *Bromoviridae* is one of the most important widespread viruses in the world infecting the largest number (approximately 1000) of plant species (Van Regenmortel et al., 2000). Also, CMV was reported to be seed-borne virus with a transmission estimated between 1% and 50% (Palukaitis & García-Arenal, 2003; Palukaitis et al., 1992). Thus, the high incidence of CMV observed may be linked to these factors.

Considering the infection rates per individual viruses between the seasons, it appears there is generally unequal distribution of the viruses at the various ecological zones. The infection of CMV in the AEZ I, II, and IV at rainy season was significantly different in the dry season. The difference in infection rate between dry and rainy seasons could be dependent on the ecology. For instance in AEZI, AEZ II, and AEZ IV, the infection rate was highest in rainy season than the dry season. This observation could be due to the agricultural practices within the agro-ecological zones which can be continuous cropping, mono-cropping, and pest management methods (Hull 2009, Rybicki, & Pietersen, 1999). In addition, it could be due to the use of unimproved seeds used by the farmers, the abusive used of pesticides which causes the resistance of the pathogens to the chemical and the lack of knowledge on viral diseases by the farmers (Defrancq 1989, Hansen, 1990; Mwaule 1995; Thresh, 1998; Afouda et al, 2013; Ayo-John et al, 2014;).

ZYMV infection rate between the dry and the rainy season in the agro-ecological zones was not significantly different. Infection rate of PRSV in AEZ I, AEZ IV, and AEZ VI in the dry season were significantly different

from that of the rainy season. Apart from the AEZ I, AEZ IV, the virus was not detected in the other ecological zones during the rainy season. This suggests that rainy season is not favourable for the replication and development of this virus. There was an increase in infection rate in AEZ IV from 5.35% in the dry season to 9% in the rainy season whereas in AEZ I there was a decrease in the infection rate from 9% in the dry season to 2.3% in the rainy season. These variations find their explanation, through the effect of seasons and the changing ecological conditions which in turn change the transmission rate of the virus, the behavior of the host plants and the biology of the vectors (Randolph, Green, Peacey, & Rogers, 2000; Hull, 2009; Fadjinmi, 2010).

MWMV and WMV were neither detected serologically nor by molecular analysis of the samples, which suggests for now that these two viruses are not present in Côte d'Ivoire.

Conclusion

CMV, ZYMV and PRSV are widespread in all cucurbits growing areas in Côte d'Ivoire. CMV appears to be the most prevalent virus infecting cucurbits crops as well as the weed hosts in Côte d'Ivoire. However, PRSV was not detected in AEZ V. In dry season CMV and PRSV were more prevalent in the crops while ZYMV was prevalent in rainy season. The weeds are the efficient reservoirs for virus transmission, with CMV infecting 15 weeds belonging to 8 families of weeds hosts compared to the ZYMV which infected seven (7) weeds belonging to 4 families and PRSV infecting only one weed of *gramineous* family.

CHAPTER FOUR: MOLECULAR CHARACTERIZATION OF *PEPO APHID-BORNE YELLOWS VIRUS* (PABVY)

Introduction

Five species of cucurbits are grown in Côte d'Ivoire which include *Citrullus lanatus*, , *Cucumeropsis mannii, Cucumis melo, Cucurbita pepo* and *Lagenaria siceraria* among which the most cultivated is *Citrullus lanatus*, *(Thunberg) Matsumara et Nakai (Cucurbitaceae)* (Bi, Koffi, Djè, Malice, & Baudoin, 2006). Virus infection on these cucurbit crops is often substantial, and constitutes a limiting factor of their production worldwide (Provvidenti, 1996). Mosaic and necrosis, that generally affect fruit quality, may result in more severe economic loses compared to yellowing diseases which only affect fruit production (Lecoq & Desbiez, 2012; Tzanetakis, Martin, & Wintermantel, 2013; Wisler, Duffus, Liu, & Li, 1998). Viruses from the genera *Carlavirus* (Nagata et al., 2010), Polerovirus (Knierim et al., 2010) and *Crinivirus* (Abrahamian & Abou-Jawdah, 2014; Wisler et al., 1998) have been associated with yellowing diseases on cucurbits. At least 39 different virus species are known to naturally infect cucurbit plants worldwide (Lecoq, 2003). Among them, *Pepo aphid-borne yellows virus* (PABYV) first observed in 2008 in Mali (Knierim, Tsai, Maiss, & Kenyon., 2014), has now been reported in Taiwan (Knierim et al., 2010) and more recently in South Africa and Côte d'Ivoire (Ibaba et al., 2015; Koné et al., 2015), Tanzania (Desbiez et al., 2016) and Greece (Lotos, Maliogka, & Katis, 2016).

This virus is a member of the genus *Polerovirus*, family *Luteoviridae* which include officially 13 internationally accepted viruses' species (Knierim

et al., 2013) infecting cucurbits worldwide. As a polerovirus, it causes yellowing symptoms in cucurbits and significantly affects yields. Other poleroviruses reported elsewhere to infect cucurbits include *Cucurbit aphid-borne yellows virus* (CABYV) reported to be causing yellowing disorders of melon and cucumber in France (Lecoq et al., 1992), *Melon aphid-borne yellows virus* (MABYV) reported in Thailand, L*uffa aphid-borne yellows viru*s (LABYV) and *Suakwa aphid-borne yellows virus* (SABYV) reported in Phillipines (Knierim et al., 2010). Even though some viruses detected in cucurbits in Côte d'Ivoire, no poleroviruse has been reported to be infecting cucurbit crops. The detection of a virus belonging to this genus has significant on agronomic importance and especially on the development of management strategies against virus diseases in cucurbits. The objective of this study was to identify and characterize the polerovirus (es) infecting cucurbits in Côte d'Ivoire.

Material and methods

To check the presence of cucurbit-infecting poleroviruses, that are reported to occur frequently in mixed infections with other aphid-borne viruses, RT-PCR screening was performed (Knierim, Tsai, & Kenyon, 2013; Knierim et al., 2014) on dried leaves samples using the universal polerovirus primer pair Gen1 and Gen2 in combination with an internal control (Knierim et al., 2013).

Collection of leaves samples

A survey was conducted from February to August 2014 in 58 fields from 28 districts of Côte d'Ivoire for the collection of leaves samples for virus detection. A total of 757 leaf samples with virus like symptoms were collected from cucumber, zucchini, pumpkin, watermelon, melon and calabash gourd. Samples were dried over calcium chloride and kept at 4°C for later analysis.

Detection of CMV, ZYMV and PRSV by DAS-ELISA

The samples were tested by double antibody sandwich enzyme linked-immunosorbent assay (DAS-ELISA) for the presence of other viruses as described in chapter 3. The samples were then submitted to RT-PCR for polerovirus checking.

Detection of polerovirus by RT-PCR and sequencing

Total RNA extraction

Total RNA was extracted using RNeasy Plant Mini kit (QIAGEN RNeasy kit) from the dried leaf material according to manufacturer's instructions. The RNA was eluted from the columns with 50 µL of RNASE-free water.

Reverse Transcription or cDNA synthesis and Polymerase chain reaction

Reverse transcription of total RNA was done using cDNA synthesis kit (Invitrogen). The first strand cDNA was synthesized using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT). One microliter (1µL) of the first strand cDNA was added to 24 µL PCR reaction mixture containing 5 µL of 10X PCR Buffer (Invitrogen), 1.25µL MgCL2 (50 mM) magnesium

chloride, 0.25 µL dNTP Mix (10 mM), 0.2 µL Taq DNA Polymerase (Invitrogen) (5U/µl), 1µL of each polerovirus universal primer Gen1 and Gen 2 (10µM), and 17.8 µL sterile distilled water for a final volume of 25 µL.

The PCR reaction mixture was incubated in pre-warmed thermal cycler under the following conditions: 94°C for 3 min denaturation; 35 cycles for 30 sec at 94°C (denaturation); 55°C for 30 sec (annealing), and 72°C for 1 min 30 second (extension). The final extension was carried out for 7 min at 72°C and holding at 4°C. The RT-PCR products were separated by 1% agarose gel at 100 V for 1.5 hour. DNA components were then visualized on a UV trans-illuminator. The expected 325 bp dsDNA band indicative of polerovirus presence was amplified from 13 samples (Table 14) and subsequently sequenced.

Cloning of PCR products

Using DNA amplified in PCR experiments, cloning which is the multiplication of DNA into a plasmid vector (Lewin, 1997) was done to purify DNA prior to sequencing. The PCR amplicons containing two or several components were ligated into a plasmid and transformed into *Escherichia coli* (*E. coli*) using pJET1.2/blunt cloning kit (Thermo Scientific, #K1231). The following mixture including 10 µL 2× rapid ligation buffer, 3 µL (0.15 pmol ends) of purified PCR amplicon were prepared and completed to 17 µL with nuclease free water. The total volume was briefly vortexed and centrifuge for 3 to 5 min and then Incubated for 5 min at room temperature at 70°C then add to the mixture, 1 µL T4 DNA ligase and 1 µL PJET 1.2 / blunt vector was added to the mixture can be used directly for transformation or stored to -

20°C. Fifty microlitres (50 µL) of E. coli cells suspension were added to 2 µL ligated plasmid, pipetted to mix and incubated on ice for 15 min. It was then transfer to water at exactly 42 °C for exactly 45 s and then transferred to ice bath for 1-2 min. Bacterial cells of *E. coli* were transformed by exposure to ice cold (4 ºC) temperatures for 30 min; heat shock for 30 seconds at 42 ºC, and ice cooling for 2 min. The mixture was incubated at 37 ºC on a horizontal rotor for 30 min. LB medium was prepared (appendix B) with Ampicillin and X-Gal coating, in plastic Petri dishes on which clones were spread. About 200 µL transformed E. coli cells were plated on LB agar plates containing 100 mg/ml ampicillin. The inoculated plates were incubated overnight at 37ºC (Maniatis Fritsch, & Sanbrook, 1982; Nakhla, Mazyad, & Maxwell, 1993).

Screening for recombinant plasmid and sequencing

The colonies on the agar plate were screened for recombinant plasmids using PCR. During the insertion of the PCR linear DNA fragment in the plasmid, the literature revealed that the gene responsible for breaking down X-Gal (lacZgene) is destroyed. This gene is responsible for synthesis of β-galactosidase, which digests X-Gal leading to formation of blue colonies. Cells were marked and picked up from individual colonies and introduced to 20 µL of clean water. The white colonies were picked and individually sub-cultured in new tubes with 2xYT liquid microbial growth medium, plus ampicillin at 37 ºC overnight.

DNA was extracted from cloned cultures and used for restriction enzyme digestion. Restriction enzyme digestion products were run on a gel, 1% agarose, and in TAE buffer. The desired band was cut carefully from the

stained agarose gel under UV light and put in a clean Eppendorf tube. Agarose gel extraction protocol (QIAEX II, 1997) was used to purify DNA from the agar. Subsequently, restriction enzyme digestion was done in order to determine whether transformation had been successful and the desired DNA fragment had been cloned. The reaction mixture was incubated in a water bath for 4 hours overnight at 37 ºC. The digestion product was run on 1% agarose gel, in TAE buffer. Clones that had clear separation of plasmid DNA were sub-cultured in LB medium with ampicillin and incubated overnight at 37 ºC.

Plasmid DNA was purified from positive recombinant clones using the QlAprep spin miniprep kit (Qiagen). Three independent clones per isolate were sequenced in both orientations in order to ensure consistent and reliable sequence data.

Nucleotides sequences and phylogenetic analyses

The RT-PCR products were extracted from the gel and cleaned with DNA clean kit and then transferred in 1.5mL micro-centrifuge tube labeled with the primer pairs Gen 1/2. Cloned sequences were sequenced in both orientations and in duplicate. Nucleotide sequences identities were obtained using the bioinformatics software programs– Vector NTI Advance II (Invitrogen Corp., Carlsbad, CA, USA) and BioEdit v7.0.5 (Hall, 2005). A consensus sequence was obtained for each isolate, and the sequences were verified by BLASTN searches (http://blast.ncbi.nlm.nih.gov/Blast. cgi). The 'Translate DNA Sequence' option of BioEdit was used to ascertain that the sequences were in the correct translation frame prior to using them for further analyses. Additional sequences published by Knierim et al. (2014) and Ibada

et al. (2015) were retrieved from GenBank, checked and added to the dataset. Multiple nucleotide sequence alignments were performed by using Clustal W (Thompson, Higgins & Gibson, 1994). Alignments were also adjusted manually to guarantee correct reading frames. Nucleotide and the amino acid sequence identities were determined for the P3 and P4 genes.

The sequences were evaluated for the best-fit model of evolution in MEGA6 (Tamura et al., 2013) using the Akaike information Criterion (Akaike, 1974), Bayesian Information Criterion (Scwarz, 1978) and the hierarchical likelihood ratio test (Pol, 2004). The best fit nucleotides substitution model i.e. Jules and Cantor model (Jukes & Cantor, 1969) was used for the phylogenetic analyses based on the Neighbor-joining method implemented in MEGA6.

The phylogenetic study was carried out to analyze the relationships among the isolates of virus collected from different agro-ecological zones of Côte d'Ivoire and compare them with other nucleotides sequences available in Genbank data base. Some details on the collection are shown in the Table 13.

Table 13: *Virus isolates of Pepo aphid-borne yellows virus and associated viruses collected during the dry and the rainy seasons in the six agro-ecological zones surveyed in Côte d'Ivoire*

Samples	Ecozones (locality)	Season (date)	Host
KGOCGT258	AEZ VI (Korhogo)	Dry (Feb 2014)	zucchini
MACGT81	AEZ III (Man)	Dry (Feb 2014)	Zucchini
DACGT369	AEZ I (Dabou)	Rainy (June 2014)	Zucchini
KGOCGT243	AEZ VI (Korhogo)	Dry (Feb 2014)	Zucchini
KGOCGT261	AEZ VI (Korhogo)	Dry Feb (2014)	Zucchini
KGOCGT252	AEZ VI (Korhogo)	Dry (Feb 2014)	Zucchini
DKCGT421	AEZ III (Duekoue)	Rainy (June 2014)	Zucchini
DACGT166	AEZ IV (Daoukro)	Dry (Feb 2014)	Zucchini
DVCGT38	AEZI (Divo)	Dry (Feb 2014)	Zucchini
STCGT367	AEZ I (Songon-Te)	Rainy (June 2014)	Zucchini
AGCGT60	AEZ II (Agokro)	Dry (Feb 2014)	Zucchini
TOCGT577	AEZV (Tombokaha)	Rainy (July 2014)	Zucchini
KKRO561	AEZV(Kpokhankro)	Rainy (July 2014)	zucchini

Genetic diversity

The diversity of nucleotides sequences of PABYV isolates was examined using the software DnaSP 5.10 (Librado & Rozas, 2009). The indices including number of polymrphics sites (s), total mutation (Eta), nucleotides diversity (л, Pi), haplotype diversity (Hd), and number of haplotype were measured.

Neutrality test

To test The hypothesis that patterns of variation within PABYV isolates are consistent with the neutral theory of molecular evolution (Kimura, 1983),

Tajima's D (Tajima, 1989) and Fu & Li' D and F statistics (Fu & Li, 1993) were estimated. Significance of each test statistic was estimated by 10,000 permutations.

Analysis of genetic differenciation and gene flow among the population of PABYV isolates

The tests for genetic differentiation and gene flow among PABYV population at the various ecological zones and different cropping seasons were executed using DnaSP version 5.10 (Librado & Rosa, 2009). The genetic differentiation was determined by the calculation of likelihood ratio ($\chi 2$) (Workman & Niswander, 1970) and permutation based statistic tests, Ks*, Z, Hs and Snn (nearest neighbor statistic) (Hudson, 2000; Hudson et al., 1992). The extent of genetic differentiation or the level of gene flow between populations was estimated by the statistic tests, Nst (estimate of gene flow between populations) (Lynch & Crease, 1990) and Fst (the interpopulational component of genetic variation (Hudson et al, 1992). The absolute values of Fst ranges between 0 and 1 for undifferentiated to fully differentiated populations. Fst < 0.33 assumes frequent gene flow whereas Fst > 0.33 assumes non-frequent gene flow between populations (Rozas et al., 2003, Wei et al, 2009).

Determination of genetic distance and selection pressure analysis among PABYV sequences

The selection is one of the two major evolutionary processes which determines the frequency distribution in the virus population of the genetic variants generated by mutation or genetic exchange (Garcia-Arenal et al., 2003). The selection effect on virus population may decrease the diversity in the population (negaive selection) as it may increase diversity between populations if these are under different selection pressures (positive selction) (Garcia-Arenal et al., 2003). The estimmation of overall genetic distance was estimated using Maximum likelihood method implememted in MEGA7(Kumar et al., 2016). The number of nucleotide diversities at non-synonymous position is d_N, and that one of the nucleotide diversities at synonymous positions is d_S. The statistic test estimating the ratio (d_N/d_S) was used to detect codons that have submitted to positive selection, $d_N / d_S > 1$, negative selection, $d_N / d_S < 1$, and neutral selection, $d_N / d_S = 1$

Recombination analysis

The recombination analysis within the sequences was performed with four different methods available in the Recomnination Detection Program v4.72 (RDPv4.7.2) i.e RDP, GENECONV, MAXCHI and 3SEQ (Martin et al., 2010).

Results

Sixty-eight (68) out of 399 leaf samples (that were positive to CMV, ZYMV and PRSV, in single and mixed infection.) showing symptoms of yellowing, and thickening (Figure 4) characteristic of pepo aphid-borne

yellows virus infection (Lecoq et al, 1992). These symptoms were also combined to other mosaic, blistering and downward cupping. In order to verify the presence of other viruses, the samples were tested by RT-PCR for the presence of polerovirus.

Figure 4: Zucchini plants showing yellowing symptoms in the field.

PCR detection of a polerovirus

In the Polymerase chain reaction (PCR), there was successful amplification of the PABYV fragment (325 kbp) using the nucleic acid molecule from PABYV infected leaf samples and the universal polerovirus primers Gen 1/ Gen 2.. Thus the extraction protocol could be considered to be appropriate for the PCR. (Figure 5). The expected 325 bp dsDNA of genes (gene P3 & P4) band indicate polerovirus presence (Figure 5).

Phylogenetic analyses of different isolates

The sequences of thirteen polerovirus isolates from Côte d'Ivoire, three from Mali, seven from South Africa, and one from Greece were submitted to Genbank. The phylogenetic tree showed nucleotides identity ranged from 95.9 % to 99.6% within the Ivorian isolates. The comparison

of the 13 sequences with those from Mali was between 95% and 97% of nucleotides identity; followed by those of South Africa and the thirteen Ivorian isolates sharing 93% to 97%. Further, the isolate from Greece and those from Côte d'Ivoire shared an identity of 94.1% to 98.1%.

The deduced amino acid of the thirteen field isolates shared identity of 81.3-100% among them. The published isolates from Mali shared 87.9-100% amino acid identity. The South African isolates shared 87.9-97.8% identity. Further, the isolate from Greece shared 87.9-100% amino acid identity with the 13 Ivorian isolates

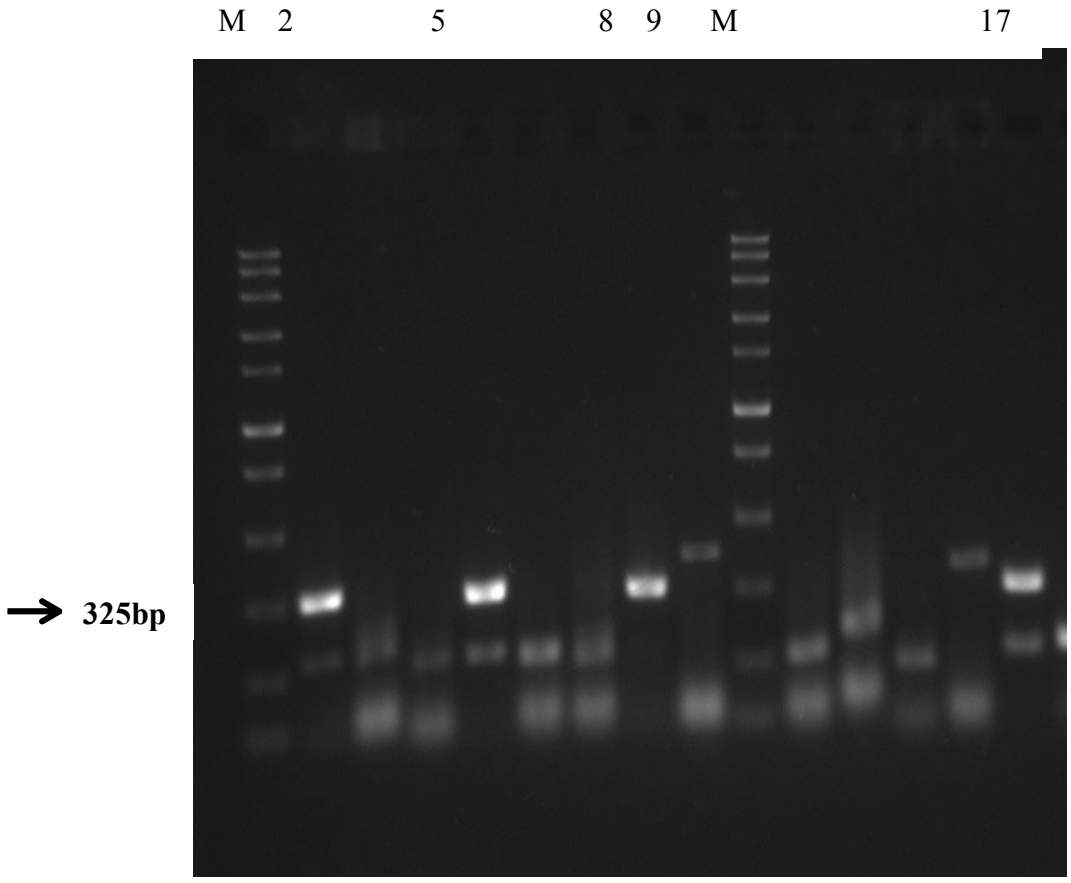

Figure 5: Resolution of PCR products of Pepo aphid borne yellows virus (PABYV) amplified using polerovirus primer pairs Gen1 and Gen2 in one percent agarose gel.

Note: Lane M, 325 kbp DNA size standards [Promega, Madison, USA]. Lanes 2, 5 and 8, infected zucchini plants; Lane 17, positive control.

Table 14: *Nucleotides and amino acid sequences identities of PABYV isolates from Cote d'Ivoire and other published isolates from Genbank*

Sequences	Sequences identity (%)	
	Nucleotides (%)	Amino acid (%)
Between sequences Ivorian isolates	95.8-99.6	81.3-100
Between Ivorian isolates and Mali published isolates	95-97	87.9-100
Between Ivorian isolates and South Africa published isolates	93-97	87.9-97.8
Between Ivorian isolates and Greece published isolates	94.8-98.1	87.9-100

Phylogenetic analysis of nucleotides published sequences of 24 Pepo aphid borne yellows virus (PABYV) isolates from GenBank

The phylogeny analysis of the nucleotides sequences clustered the sequenced field isolates into three clades (I, II and III) supported by of bootstrap value greater thn 80%. The clade I contains four sequences while the clade II containing the majority of sequences (Figure 6). The random distribution of the sequences isolates into the clades was observed. Thus, the isolates collected in both dry and rainy seasons were found in clade I and in clade II. The same observation was also noted in terms of agro-ecological zones. However there is one isolate (PABYV-AGCT60) closed to South Africans isolates.

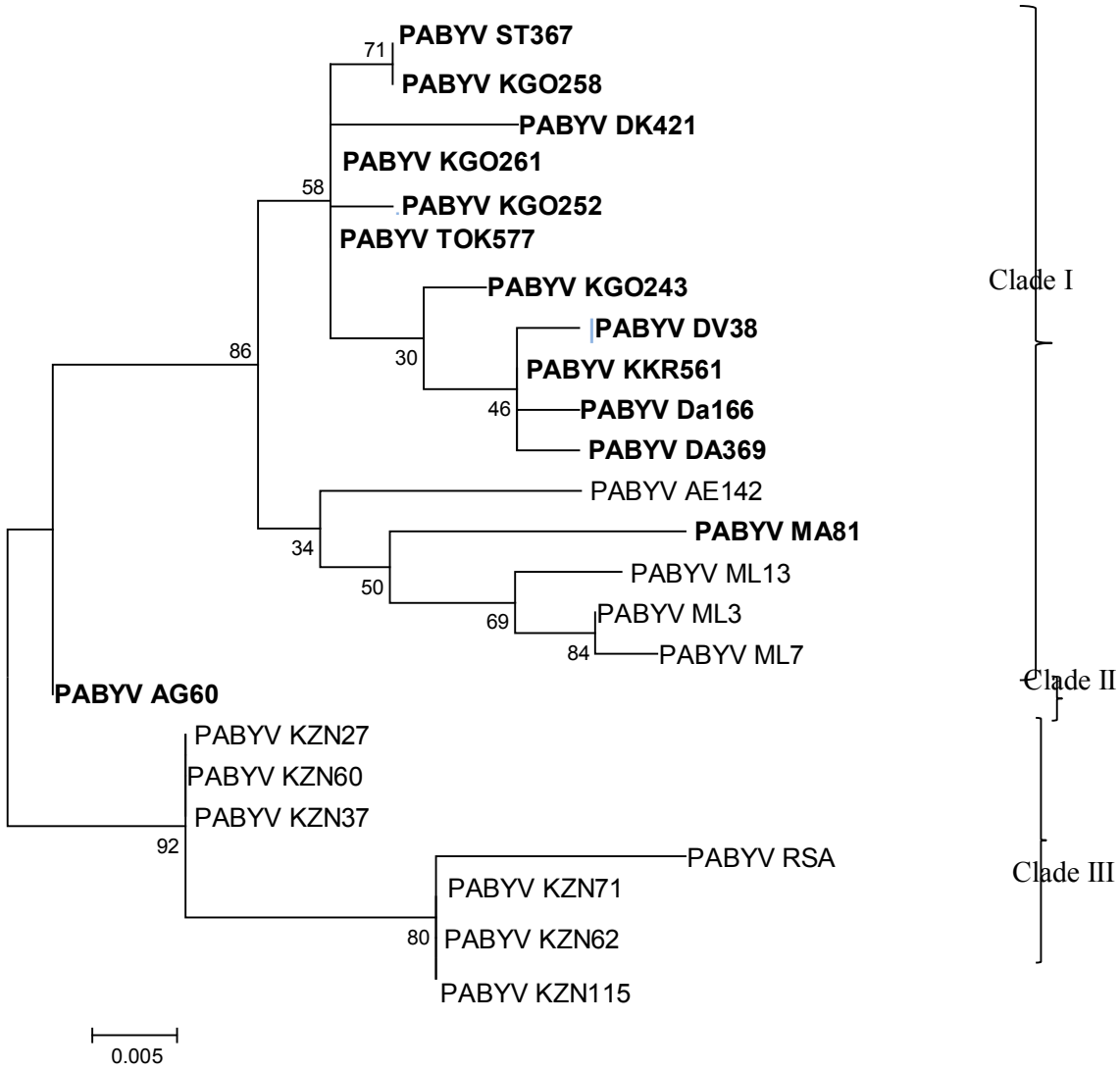

Figure 6: Unrooted Maximum likelihood phylogenetic tree of nucleotides sequences of *Pepo aphid borne yellows virus* (PABYV) isolates from Cote d'Ivoire, Mali, South Africa and Greece.

Note: The scale bar signifies a genetic distance of 0.005 nucleotide substitution per site. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Phylogenetic analysis of amino acid published sequences of *24 Pepo aphid borne yellows virus* (PABYV) isolates from GenBank

The phylogenetic analyses of the deduced amino acid sequences clustered the PABYV isolates into three clades (genetic groups 1, 2 and 3) supported by bootstrap value of greater than 80% (Figure 8). Twelve out 13 Ivorian field isolates clustered with the published Malian and Greece isolates in clade I, sharing amino acid identities ranging from 87.9% and 100%. The other Ivorian sequenced isolate (ACGT60) clustered alone in clade II. All the South African isolates clustered in clade III with amino acid identities of 100% (Figure 7). The amino acid identities between clade I and clade II isolates is 81.3-89%. That of clade II and clade III is 87.9%, whereas the identities between clade I and III is between 90.1% and 97.8%.

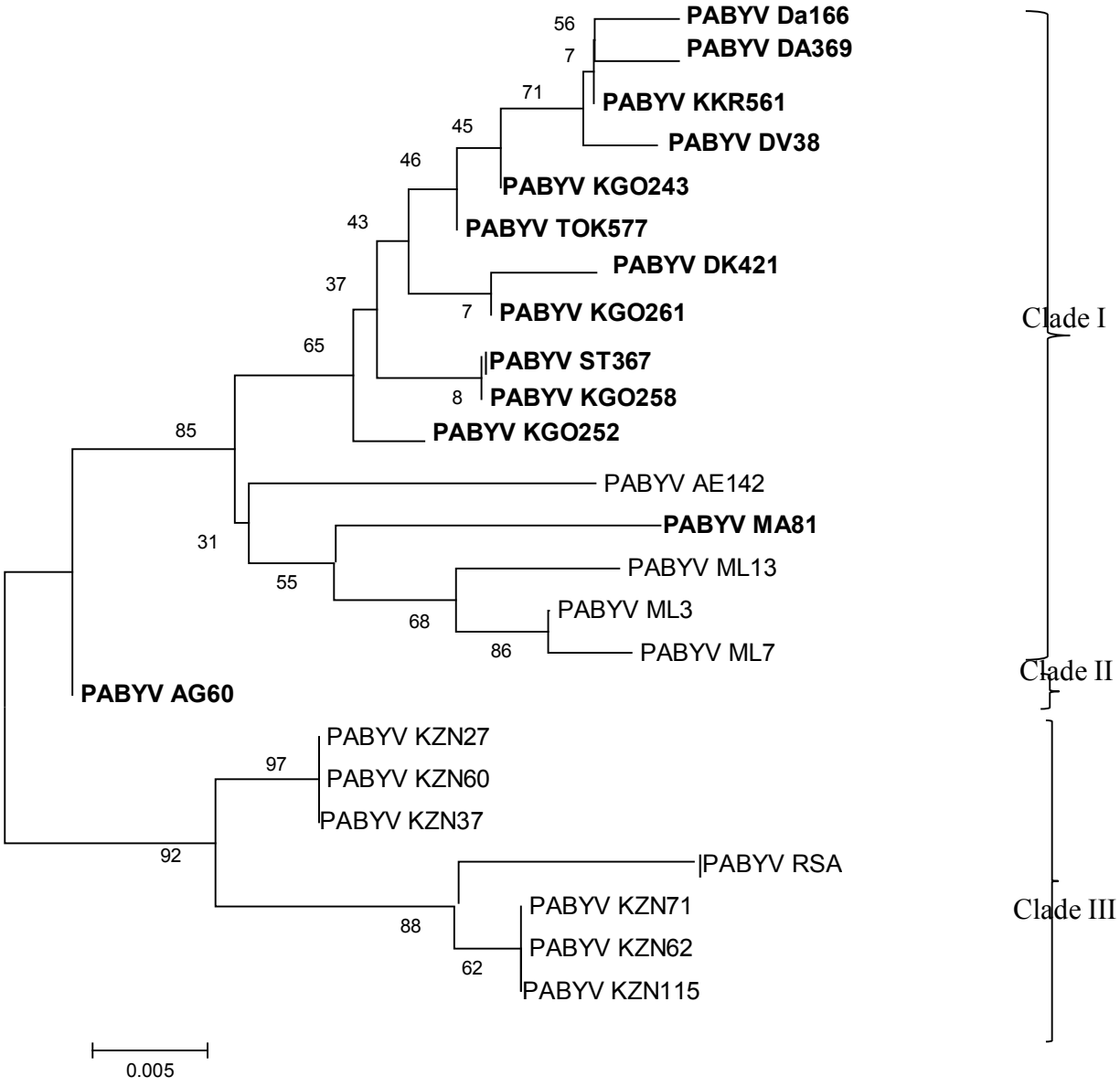

Figure 7: Unrooted Maximum likelihood phylogenetic tree of amino acid sequences of *Pepo aphid borne yellows virus* (PABYV) isolates from Cote d'Ivoire, Mali, South Africa, and Greece.

Note: The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (Jules and Cantor, 1969). The scale bar signifies a genetic distance of 0.005 nucleotide substitution per site. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Table 15: *Sequences identity matrix using clustalW by multiple alignment of nucleotides sequences of the genome of PABYV isolates [n =13] samples*

	RNA-06-	RNA-10-	RNA-14-	RNA-17-	RNA-22-	RNA-24-	RNA-31-	RNA-36-	RNA-49-	RNA-51-	RNA-54-	RNA-62-	RNA-67-
RNA-06-	100												
RNA-10-	95.2	100											
RNA-14-	97.4	96.3	100										
RNA-17-	98.5	95.9	98.5	100									
RNA-22-	98.1	96.7	98.1	98.5	100								
RNA-24-	97.4	94.8	96.7	97.4	97.8	100							
RNA-31-	97.4	95.9	98.1	98.1	98.5	97.8	100						
RNA-36-	97.4	96.3	99.2	98.5	98.1	96.7	98.1	100					
RNA-49-	97.4	96.3	99.2	98.5	98.1	96.7	98.1	99.2	100				
RNA-51-	98.1	96.7	98.1	98.5	99.2	97.4	98.5	98.1	98.1	100			
RNA-54-	94.8	94.8	95.2	94.8	95.9	94.5	94.8	95.2	95.2	95.6	100		
RNA-62-	98.1	94.8	97	98.1	97.4	96.3	97	97	97.4	97.4	93.7	100	
RNA-67-	97.8	96.7	99.6	98.9	98.5	97	98.5	99.6	99.6	98.5	95.6	97.4	100

Genetic diversity analysis

The results of the genetic diversity analysis within the P3 and P4 genes sequenced of PABYV (Table 16), showed low nucleotide diversity (Pi = 0.0196) despite high number of mutations (Eta =30), high number of polymorphism sites (s = 30), high haplotype diversity (Hd = 0.966) suggesting high variation in haplotype, but less variability within individual sequence.

Table 16: *Genetic diversity within Pepo aphid borne yellows virus (PABYV) isolates*

Number of sequences (n)	Number of polymor phic sites (s)	Number of mutation (Eta)	Nucleotide diversity (Pi)	Haplotype diversity (Hd)	Number of haplo-type	Haplo type proba bility
13	30	30	0.01967	0.966± 0.09028	8	0.005

Neutrality of Tajima's D and Fu and Li's D and F statistical tests

Nucleotide polymorphisms in the PABVY populations were evaluated using Tajima's D (Tajima, 1989) and Fu and Li's D and F (Fu & Li, 1993) statistical tests to evaluate the influence of demographic forces on the population (Hey & Harris, 1999; Tajima, 1989; Tsompana et al., 2005). The results for the various neutrality tests are shown in Table17.

None of the statistical tests Tajima's D and Fu and Li's D and F for the P3 and P4 genes sequences detected neutrality deviation for PABYV populations (P>0.10). Therefore the neutral hypothesis is accepted.

Table 17: *Tajima's neutrality test*

Tajima'D	p-value	Fu and Li'D	p-value	Fu and Li'F	p-value
-1.17661	P > 0.10	- 0.79633	P > 0.1	-1.07515	P > 0.10

Significant values that reject the null hypothesis of selective neutrality is P<0.10.

Significant values that accept the null hypothesis of selective neutrality is P>0.10

Determination of genetic distance and selection pressure analysis between the sequences of PABYV

The analysis of genetic distance and selection pressure among the PABYV isolates sequences and the parameters estimated are shown in Table 18. The sequences encoded RNA6-RNA67 of the 13 sequences of PABYV isolates. The parameters dN is the rate of no- synonymous substitution per site, Ds, the rate of synonymous substitution per site. The ratio of non-synonymous (0.0205) and synonymous (0.0195) substitution per site was less than 1. The synonymous and non-synonymous changes were observed in a codon. Seventy (70) codons were counted and the change was done at position 2 as shown in the table below.

Table 18: *Determination of selection pressure and genetic distance between PABYV isolates*

Sequences	Mean genetic distance	d_N	d_S	d_N / d_S	Total number of codon	Codon position under selection pressure
13	0.012	0.0205	0.019	0.1954	70	2

Recombination in PABYV virus isolates

Four methods (RDP, GENECONV, MAXCHI, & 3SEQ) were used to detect the recombination in the genes of PABYV isolates. The results provided showed zero events suggesting that recombinant was not observed during this study in the P3 and P4 genes as shown in Table 19.

Table 19: *Determination of the recombination events in PABYV*

Methods	Recombination events
RDP	0
GENECONV	0
MAXCHI	0
3SEQ	0
TOTAL	0

Genetic differentiation and gene flow based on the seasons

This genetic differentiation of the population according to crop season was assessed using five statistical tests [Snn, Ks, $\chi 2$ (df = 11), Hs, Z] (Table 20). None of these tests showed a significant genetic differentiation, suggesting that there is no genetic diversity in PABYV population assessed in this study. However, the gene flow estimates gave low values of Nst (0.03194) indicating that there is frequent movement of PABYV populations between seasons.

Table 20: *Genetic differentiation and gene flow between PABYV isolates based on the seasons*

Gene differentiation			Gene flow	
Test statistics	Estimates	p-value	Test statistics	Estimates
Snn	0.32051	0.8300 ns	Nst	0.03194
Ks	5.93407	0.6530 ns		
χ^2 (df = 11)	10.888	0.4527 ns		
Hs	1	1.0000 ns		
Z	40.3381	0.8830 ns		

PM test; Probability obtained by permutation test with 1000 replicates.

ns, not significant, *, 0.01 P < 0.05; ** 0.001 P < 0.01; ***, P < 0.001

Snn: Nearnest neighbor statistics (Hudson, 2000).

Hs, Ks, and Z: sequence based statistics of Hudson et al., 1992 for genetic differentiation detection of sub-population.

Nst: Gene flow statistic (Lynch and Crease, 1990)

Genetic differentiation and gene flow based on the agro-ecological zones

This genetic differentiation of the population according to agro-ecological zones was assessed using five statistical tests Snn, Ks, χ2 (df = 11), Hs, Z. All the tests showed non-significant genetic differentiation among the PABYV populations from the different agro-ecological zones (Table 21). This suggests that there is no genetic diversity amongst the PABYV population assessed in this study.

However, the gene flow estimates gave low values of Nst (-0.02053) test indicating that there is frequent movement of PABYV populations between agro-ecological zones (Table 21).

Table 21: *Genetic differentiation between PABYV populations based on the agro-ecological zones*

Genetic differentiation			Gene flow	
Test statistics	Estimates	p-value	Statistic	Estimate
Snn	0.09091	0.7980 ns	Fst	-0.02053
Ks	4.84848	0.5450 ns		
χ^2 (df = 24)	27.5	0.2817 ns		
Hs	0.88889	0.4090 ns		
Z	26.5	0.4350 ns		

PM test; Probability obtained by permutation test with 1000 replicates. ns, not significant, *, 0.01 P < 0.05; ** 0.001 P < 0.01; ***, P < 0.001 Hs and Ks: nucleotides sequence based statistics of Hudson et al., (1992) for genetic differentiation detection of sub-population.

Fst: Gene flow statistics (Hudson, Statkin &Maddison, 1992).

Gene flow between PABYV populations at different agro-ecological zones

Pairwise comparisons of the genetics distances using the Fst and Nst statistics showed that the most frequent movement of PABYV populations occurred between AEZ 1 and AEZ 5 (Nst = -1.2617; Fst = -0.12500), and the least frequent movement occurred between AEZ 3 and AEZ 5 (Nst = 0.06844, Fst = 0.07143) shown in Table 22.

Table 22*: Genetic differentiation and gene flow based on the agro-ecological zones*

Population 1	Population 2	Nst	Fst
AEZ 1	AEZ 3	-0.04971	-0.04651
AEZ 1	AEZ 5	-0.12617	-0.125
AEZ 1	AEZ 6	0.04057	0.04082
AEZ 3	AEZ 5	0.06844	0.97143
AEZ 3	AEZ 6	-0.0599	-0.05952
AEZ 5	AEZ 6	-0.0597	-0.06061

Hs and Ks: nucleotides sequence based statistics of Hudson et al., (1992) for genetic differentiation detection of sub-population.

Nst: Gene flow statistic (Lynch and Crease, 1990)

Fst: Gene flow statistics (Hudson, Statkin & Maddison 1992).

Detection of mixed infection of PABYV with other viruses

Results from DAS-ELISA indicated that in addition to the PABYV all the 13 zucchini samples that were sequenced, were also infected by 1 to 3 other virus species namely CMV, ZYMV, and PRSV (Table 23) Samples KGOCGT258 and KGOCGT243 were each co-infected by four virus species (PABYV, CMV, ZYMV, and PRSV). Six samples namely (MACGT81, DACGT369, KGOCGT261, KGOCGT252, DACGT166 and STCGT367) were co-infected by three viruses (PABYV, CMV-ZYMV,and CMV-PRSV). Five samples (DKCGT421, DVCGT38, AGCGT60, TOCGT577, and KKROCGT561) were co-infected by only two viruses which are PABYV and one of the following virus, CMV, ZYMV and PRSV.

Table 23: *Detection of positive samples of PABYV associated with other viruses*

Samples	Other viruses detected
KGOCGT258	CMV, ZYMV, PRSV
MACGT81	CMV, ZYMV
DACGT369	CMV, ZYMV
KGO243	CMV, ZYMV, PRSV
KGOCGT261	CMV, PRSV
KGOCGT252	CMV, ZYMV
DKCGT421	ZYMV
DACGT166	CMV, ZYMV
DVCGT38	ZYMV
STCGT367	CMV, ZYMV
AGCGT60	PRSV
TOCGT577	CMV
KKRO561	CMV

Discussion

Viral species detected

The study has revealed the presence of four viruses (CMV, ZYMV, PRPV and PBYV) infecting cucurbits in all the six agro-ecological zones in Côte d'Ivoire. The finding of this work therefore confirms that of Thouvenel & Fauquet, (1987), and Koné et al., (2010) who reported on CMV, ZYMV and PRSV in cucurbits in Côte d'Ivoire. However, this is the first report of PABYV (appendix C), a polerovirus infecting cucurbits in Côte d'Ivoire.

This information is quite valuable for developing effective strategies for mitigating the impact of these viruses on sustainable cucurbit production in Côte d'Ivoire.

The yellowing symptom caused on the plants host by the PABYV is associated with the thickening, shoe-string and vein banding symptoms hiding its presence. The presence of PABYV in all the six agro-ecological zones could be an evidence of its existence in Côte d'Ivoire for a long time but has probably been overlooked due to its moderate and non-specific yellowing symptoms and the lack of specific diagnostic tools (Desbiez et al., 2016) or because the symptom was attributed to the ageing, nutrients deficiencies or infection by other pathogens (Lecoq et al., 1992).

Sequence and phylogenetic comparisons of PABY isolates

Blast results of the 13 sequenced isolates from GenBank indicate that the isolates were sharing both nucleotides and amino acid identity of 95%-99.6% and 81.3%-100% respectively, indicating that these isolates are PABYV. This is the first report of pepo infecting cucurbits in Côte d'Ivoire. It should therefore be considered in developing of management strategies against virus diseases of cucurbits in Côte d'Ivoire.

The sequences of Ivorian isolates were more identical (amino acid sequence identity of 95-97%) to the Malian isolates than South Africa isolates (amino acid sequence identity of 93-97%) (Table14). Twelve of the 13 Ivorian isolates were in the same genetic group with Malian and Greece isolates, with nucleotide and amino acid identities respectively of 94.8- 98.1% and 89-100%. This suggests that theses isolates are belonging to the same group and are more closed in response of evolutionary relationship. The single isolate AGCGT60 in clade II of both nucleotides and amino acid phylogenetic trees indicate that it is unique from the other Ivorian isolates (amino acid identity of

81.3-89%) as well as the published Malian, Greene and South African isolates. It should therefore be considered as different species judging from the 10% amino acid difference species demarcating criterion for poleroviruses. The isolates from Cote d'Ivoire are also different in relationship from all the South African isolates since they clustered in different clades or (genetic group) sharing amino acid sequence identities of 87.9-97.8%. The Ivorian PABYV isolates and those from South Africa are clustered in two different clades could be due to the diverse climatic conditions prevailing in the two countries. Mali and Côte d'Ivoire are both Sub-Saharan African countries and are sharing the same border. The introduction of plant materials from one country to another may explain the movement of genes and so far the belonging to the same clade.

The clustering of all the Ivorian isolates except AGCGT60 into one genetic group (clade I) suggests homogeneity and low genetic diversity among them. This is further supported by the low nucleotide diversity despite its high haplotype diversity, high polymorphic sites and high number of mutations observed in the phylogenetic analyses. It was reported that recombination and mutations are two main events that occur during replication of viruses resulting in high degree of variability (Garcia-Arenal,, Fraile, & Malpica, 2003). The high genetic diversity between the random sequences could be the resultant of this fact. But our study revealed low nucleotide diversity sign of the absence of recombination event as described by Desbiez et al., (2016). The low genetic diversity and the high mutation rates among the Ivorian isolates irrespective of the high number of mutations, suggests that the rate of mutation

had no effect on the genetic diversity of the PABYV isolates. This could be due to the fact that the viral isolates were under negative selection (Table 19). Selection is a directional process by which the frequencies of variants that are the fittest in a given environment will increase in the population (positive selection) whereas those of less fit variants will decrease (negative or purifying selection) (Garcia-Arenal et al, 2003). It has been reported that high mutation rates are not necessarily adaptive, as a large fraction of the mutations are deleterious or lethal (Garcia-Arenal et al, 2003).

PABYV population structure

The non-significant neutrality deviation observed from the neutrality tests was an indication that the PABYV populations were not sub-structuring or differentiating. All the statistics tests did not indicate significant genetic differentiation among the isolates based on both the seasons and ecological zones (Table 20, 21, & 22). The results rather indicated frequent gene flow of viral populations between different season and among different agro-ecological zones. This suggests that the PABYV population obtained at different ecological zones and different seasons (dry and rainy) were not genetically different among them. This could account for the clustering of all but one of the sequenced Ivorian isolates with the Malian isolates at the same genetic group (clade 1; Figure 6 & 7) whereas the published South African isolates were clustered in different genetic group (clade III). The lack of genetic differentiation among the PABYV population could be due to the frequent gene flow observed at the two seasons and among the six agro-ecological zones. The exchange of plant materials between the different

seasons, ecological zones and different countries is a powerful factor for virus dissemination. But also the diversification, intensification and extensification, and the domestication, which are the easiest ways for viruses spread in the agro-ecological zones as reported by (Anderson et al., 2004, Jones, 2009) and could partly account for the observed significant gene flow.

Mixed infections

The results from field surveys confirmed the existence of mixed infections with PABYV and the three other viruses in the zucchini plants under natural conditions and the expression of the leaf yellowing symptoms on this crop species was clearly associated with the synergistic effect between all these viruses from different genera i.e. *potyvirus, Cucucmovirus, polerovirus*. Mixed infection of virus from the polerovirus and those from other genera typically produce synergism in plants. The eventual synergistic effect of PABYV is of agronomical importance and has important practical implications in virus disease epidemiology (Zhang et al., 2012). In this study, the polerovirus was found associated to CMV, ZYMV, and PRSV compared to other study with the similar method of DAS-ELISA used where the samples reacted negatively to the antisera (Desbiez et al., 2016). This difference may be due to the climatic conditions and may be the study design as reported by Wintermantel et al., (2009). The detection of PABYV by RT-PCR, rather by DAS-ELISA is a proof of using the appropriate techniques for virus detection to the question being addressed (Hull, 2013). The infected plants of the co-infection of PABYV with one or than one of the three viruses induced yellowing associated to significant reductions in plant height, wilt

and shoe string symptoms and subsequently yield reduction. It was reported that the severe symptoms on cucurbits plants in response to synergistic interaction, indicating the replication of more than one virus in plant cells (Hull, 2009; Gil-Salas et al., 2011; Taiwo et al., 2006). Regarding the damage caused on cucurbits, mixed infection should be considered in developing strategies for mitigating the effect of virus diseases in cucurbits in Côte d'Ivoire.

Conclusion

The study confirmed the presence of CMV, ZYMV, and PRSV infecting cucurbits in Cote d'Ivoire, and in addition reported for the first time the presence of PABYV in the country, indicating a shift in the catalogue of cucurbit-infecting viruses to four. Other cucurbit infecting polerovirus species were not detected during this study. The partial nucleotide sequences analysis of a few number of isolates available showed that PABYV is not genetically diverse and are not differentiated according to the crop season and agro-ecological zones. Thus PABYV is widespread in all the ecological zones and at both wet and dry seasons.

The PABYV occur in mixed infection with one or more of CMV, ZYMV and PRSV, and should therefore be considered in developing effective strategy for the management of viral diseases in cucurbits especially in zucchini.

CHAPTER FIVE

EFFECT OF TEMPERATURE ON CMV AND ZYMV REPLICATION IN SINGLE AND MIXED INFECTIONS AND ON HOST PLANT (CUCUMBER)

Introduction

Plant virus replication and spread require specific interactions between the obligate organism (virus) and its host, the plant. Both virus and plant factors, whether they are involved in virus accumulation or in defense against virus accumulation, require specific environmental conditions to maximize their activity (Liu, Richerson & Nelson, 2009). When such a system is created, it results in varying consequences ranging from symptom amelioration to synergistic exacerbation (Hammond, Lecoq, & Raccah, 1999).

According to Lwoff (1959), viruses are complex organized infectious entities which are strict intracellular parasites. The essence of viral infection is the introduction into a cell or an organism of a genetic material of a virus. In order to develop in its host, the virus has to come in contact with sensitive cells. An organism is sensitive only to a virus if some cells at least are able to adsorb the virus and allow its reproduction. CMV and ZYMV are two viruses transmitted non-persistently by many aphids' species (Ali et al., 2012). They are the most frequently encountered and economically important viruses amongst the 39 that naturally infect cucurbit crops worldwide (Milne et al., 1988; Provvidenti, 1996; Yuki *et al.,* 2000; Fattouh, 2003; Choi *et al*., 2007; Massumi *et al*., 2007; Yardimci and Ozgonen, 2007). They infect a wide range of plants and cause significant losses in agricultural production.

CMV, is a virus with a worldwide distribution, has the widest host range than any known plant virus, and infects more than 1,000 plant species in 100 families (Hobbs *et al.*, 2000). Interactions between *potyviruses* and CMV (*cucumovirus*) in co-infected plants co-infected of WMV and CMV in Cucurbits (Wang *et al.*, 2002) commonly induced synergistic symptoms, and in infected plant CMV titer, increased while the potyvirus titer decreased or remain the same compared with singly infected hosts (Taiwo *et al.*, 2007; Mascia *et al.*, 2010). The infected plants exhibit severe leaf mosaic, systemic mosaic, yellowing and eventually "shoestring" symptoms in the leaves. The fruits are stunted, twisted and deformed, resulting in reduced yield making the plants unmarketable (Desbiez & Lecoq, 1997). These symptoms depend on the cultivar, plant age, crop season and environmental conditions (Allen et al., 1991; Peters et al., 1991).

The environmental conditions under which plants are grown at the time of inoculation and during the development of the disease can have profound effects on the course of infection (Jones, 2009; Hull, 2009). It was also reported that temperature is one of the major environmental factors influencing virus' accumulation in its host (Matthews, 1991; Llamas-Llamas et al., 1998; Liu et al, (2009). Even though CMV, ZYMV, and PRSV were detected in cultivated crops in Côte d'Ivoire, little is known about interactions between the virus, its host and the environment. Information concerning the effect of temperature on incidence, symptom expression, and replication, of these two viruses in single and mixed infection is needed for the development of strategies for virus diseases management. Therefore the purpose of this

study was to compare the effect of temperature on symptoms caused by CMV, ZYMV, severity, and virus particles accumulation in cucumber in single and co-infection at 20˚C, 25˚C, and 30˚C.

Material and methods

Viruses isolates, plant material

The isolate of *Cucumber mosaic virus* used for the study was the CMV isolate encoded (PV-0475) obtained from DSMZ. *Zucchini yellow mosaic virus* isolate encoded DKCGT422 was collected from infected leaves of zucchini in Côte d'Ivoire. The cucumber used for the study was obtained from Julius-Kühn Institute, Braunschweig, Germany.

Plant cultivation

Pre-germination of seeds

` Cucumber seeds (likely treated with gibberellic acid to improve germination) were moistened in a porous container. The second container with water was placed under the first and covered together. The seeds were kept for 3 days in growth chamber at 22-24° C temperature required for germination.

Transplanting

After 3 days, germinated seeds were transplanted individually into sterilized plastic pots filled with sterilized planting substrate (sand: soil; moss = 1:1:1) then watered immediately. One week after planting, the plantlets were ready for inoculation as shown in figure 8. For each type of inoculation, ten plants were inoculated.

Figure 8: One week old cucumber seedling ready for inoculation

Inoculation procedures

Young cucumber, leaves showing severe symptoms were ground in 0.05 M phosphate buffer pH 7.0 and the sap was used for mechanical inoculation of the test plants. Ten plantlets of cucumber plants aged for one week were inoculated with CMV and ZYMV in single and in co-infection respectively. The mock inoculation was performed with the phosphate buffer only. The plantlets were inoculated mechanically by sap inoculation by rubbing cotyledons after dusting with carborumdum and celite. For the purpose, four indicator plants namely, *Nicotiana benthamiana, Nicotiana tabacum*, Zucchini squash and cucumber were selected. Two lots were made, one to inoculate CMV and the other for ZYMV inoculation. After 21 days, 5g of young leaves were collected from source plants and ground in phosphate buffer (50 ml) (pH 8.5). The co-infection was made by preparing extracts from the equal fresh weights of 2.5g of young leaves from the source plants and ground to get the mixture. Mock inoculations (three healthy plants per treatment) were employed as controls. Mock inoculated plants were obtained by grinding 5g of healthy leaves in phosphate buffer.

Experimental design and layout

Three trials were conducted under three temperature regime of 20 °C, 25 °C and 30 °C. Each trial included ten inoculated plants and ten control plants (mock-inoculated). The cucumber plants were inoculated with single virus (CMV or ZYMV) and in co-infection. After inoculation, plants were kept for 4 days before subjecting them under different conditions of temperature (20 °C, 25 °C, 30 °C) in randomized complete block design with three replications. The photoperiod was 14 hours light and 10 hours darkness, with illumination provided by cool-white fluorescent tubes, at intensities ranging from 170 to 370 $mEm^{1}2s^{1}1$. Relative humidity varied from 65 to 75%.

Data collection symptoms severity determination

Symptoms development was assessed and the severity was scored using a modification of visual scale scale 1-5 (Hassan and Al-masri, 1991) where,

1 – No symptom,

2 – Up to 10% of leaf showing disease symptoms

3 –10-30% of leaf showing disease symptoms,

4 - 30-50% of leaf with severe symptom

5 – 75% of leaf showing distortion, yellowing and death

Viral particle quantification

The leaves were harvested and analyzed at 7, 14, 21, and 28 days post-inoculation (dpi) by DAS-ELISA for relative virus accumulation at various temperatures. The leaves were harvested by discs in order to get the same weight of plant material. The experimentation was repeated 3 times from

February 2014 to April 2014. The serological analysis of the leaves were done using (DAS-ELISA) (Clark and Adams 1977) and using commercial polyclonal antiserum (AS-0234 and AS-0929; DSMZ, Braunschweig, Germany) as already described. The dilution used for samples analysis was 1/100 (0.025 g of leaf 2.5 mL of buffer). The replications of the virus isolates in single and co-infection were checked for each temperature treatment, by the measurement of the absorbance of the extract using the spectrophotometer as described.

Determination of mean plant height

Plant height was determined by measuring the height in centimeters from soil level to the tip of the stem using the tape measure. The measurement was carried out at the end of the evaluation (28 dpi). Data was collected from five plants and the means height of the host plant under each of the three temperatures was determined.

Statistical analysis

The analysis of data was submitted to ANOVA using GenStat Discovery version 4 (VSN International) Significant differences among treatment means were separated using Least Significant Difference test at 5% level of probability.

Results

Symptoms development on cucumber plants

At 7 days post-inoculation (dpi) no symptom was observed on plant leaves. The symptoms were visible at 14 dpi (approximately 10-12 dpi)

marked by the systemic infection. Symptoms severity scores were done using a modified visual scale from 1 to 5 of Hassan & Al-masri, (1991), where, 1 represents no symptom; and 5 very severe symptoms.

Spotty symptoms were observed on CMV inoculated-plants at 20°C) and 25°C (SS = 2) (A). However, the symptoms were masked at 30°C (SS = 1). ZYMV inoculated plants showed swollen leaves and vein banding symptoms respectively at 20°C and 25°C (SS = 4) (B,D). But at 30° stunted plants associated to blistered leaves and severe leaf surface reduction were observed (SS = 4). The co-infected plants showed stunted plants with severe mosaic and yellowing under all temperature treatment (C, F) (Figures 9). The figure 10 shows the comparison of the symptoms for each treatment of temperature and type of inoculation.

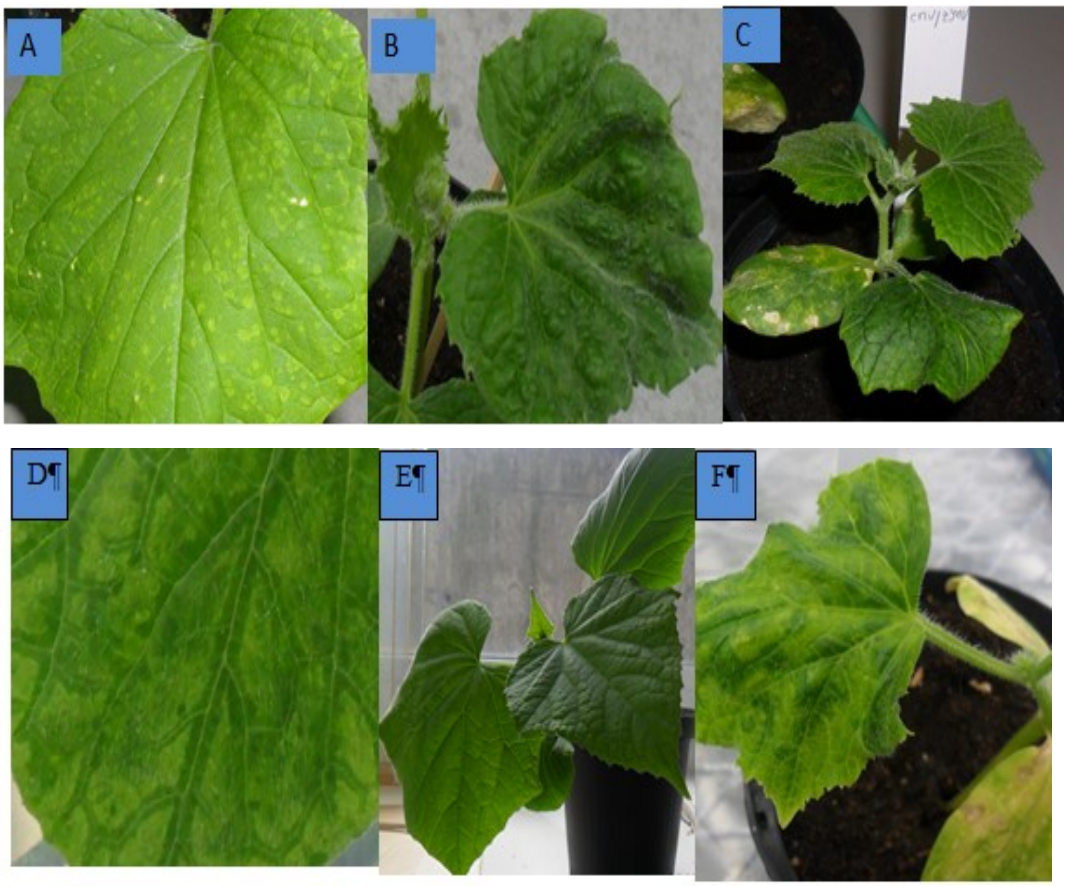

Figure 9: Cucumber plant showing diverse symptoms developed after 21 days post-inoculation.

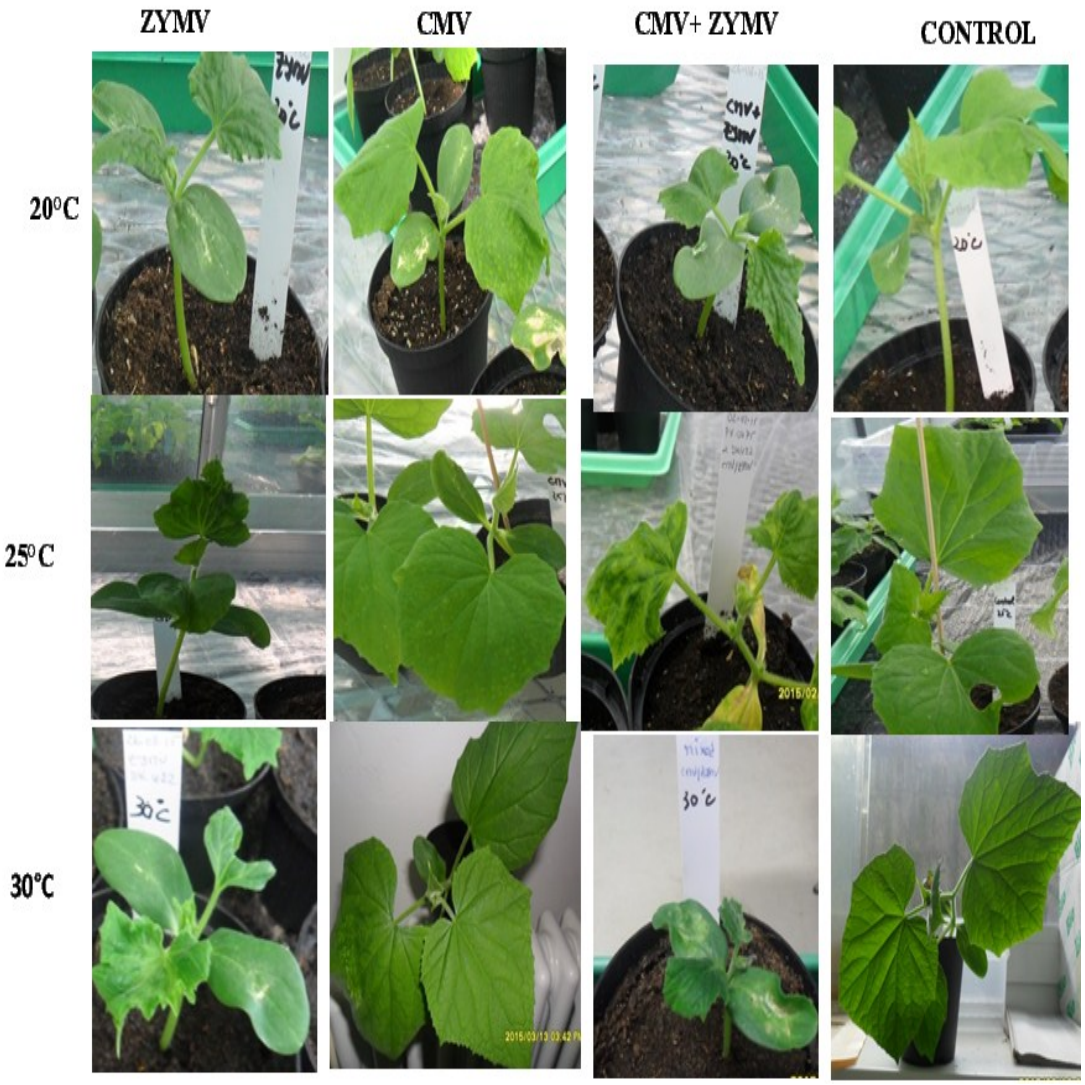

Figure 10: Development of symptoms on cucumber plants inoculated with CMV and ZYMV in single and mixed infections under three temperature treatments.

Virus replication in single infection

ANOVA on virus concentration at 7 dpi showed significant differences between viruses (F=13.67; df= 3 P<0.001), and temperature-virus interaction effect (F = 7.61; df = 6; P < 0.001). But there was no significant difference among the temperatures (F = 1.73; df = 2; P = 0.2) (Table 24).

ANOVA on virus concentration 14 dpi showed significant difference between viruses (F = 9.73; df = 3; P < 0.001) but was not significantly different in the interaction (F = 0.69; df = 6; P = 0.662) and temperature effect (F = 0.11; df = 2; P = 0.662) (Table 25).

The ANOVA showed a significant difference between virus effect (F = 7.64; df = 3; P = 0.001). However, there was no significant difference in virus-temperature interaction effect (F = 0.22; df = 6; P = 0.967) and between temperatures (F = 2.93; df = 2; P = 0.075) (Table 26).

ANOVA showed a significant difference between virus effect (F = 33.58; df = 3; P < 0.001). However, there was no significant difference in virus-temperature interaction (F = 2.22; df = 6; P = 0.080) and between temperatures (F = 2.27; df = 2; P = 0.127) (Table 27).

Table 24*: Mean virus-temperature interaction effect in cucumber inoculated plants with CMV and ZYMV in single and co-infection under varying temperature at 7 days post-inoculation (dpi)*

Virus (7 dpi)	Temperatures		
	20°C	25°C	30°C
CMV	0.54 d	0.608 d	0.934 d
ZYMV	1.749 b	1.487 bc	1.07 cd
CMV + ZYMV	1.08 cd	1.275 bc	2.417 a
ZYMV + CMV	1.748 b	1.136 c	1.039
LSD (P < 0.05)		0.5388	

Means within a column, followed by the same letter(s) are not significantly different (P < 0.05) based on (LSD) test at 5% level.

Table 25: *Mean virus-temperature interaction effect in cucumber inoculated plants with CMV and ZYMV in single and co-infection under varying temperature at 14 days post-inoculation (dpi)*

Virus (14 dpi)	Temperatures		
	20°C	25°C	30°C
CMV	0.808 a	0.765 ab	0.722 bc
ZYMV	1.296 a	0.823 a	1.114 b
CMV + ZYMV	1.551a	1.622 a	1.942 a
ZYMV + CMV	1.109 a	1.043 a	1.001 b
LSD (p < 0.05)		0.6453	

Means within a column, followed by the same letter(s) are not significantly different (P < 0.05) based on (LSD) test at 5% level

Table 26: *Mean virus-temperature interaction effect in cucumber inoculated plants with CMV and ZYMV in single and co-infection under varying temperature at 21 days post-inoculation (dpi)*

Virus (21 dpi)	Temperatures		
Virus	20℃	25℃	30℃
CMV	0.772 a	0.556 ab	0.421 bc
ZYMV	1.16 a	0.704 ab	0.683 b
CMV + ZYMV	1.53 a	1.366 a	1.387 a
ZYMV + CMV	1.03 a	0.800 a	0.502 b
LSD (P < 0.05)		0.6681	

Means within a column, followed by the same letter(s) are not significantly different ($P < 0.05$) based on (LSD) test at 5% level

Table 27: *Mean virus-temperature interaction effect in cucumber inoculated plants with CMV and ZYMV in single and co-infection under varying temperature at 28 days post-inoculation (dpi)*

Virus (28 dpi)	Temperature		
Virus	20℃	25℃	30℃
CMV	0.319 b	0.19 3 bc	0.254 cd
ZYMV	0.511 b	0.552 b	0.633 b
CMV + ZYMV	1.188 a	1.873 a	1.065 a
ZYMV + CMV	0.877 a	0.764 b	0.439 b
LSD (P< 0.05)		0.2855	

Means within a column, followed by the same letter(s) are not significantly different ($P < 0.05$) based on (LSD) test at 5% level

Effect of temperature on infected plant height

Symptoms expression on the host plant at different temperature

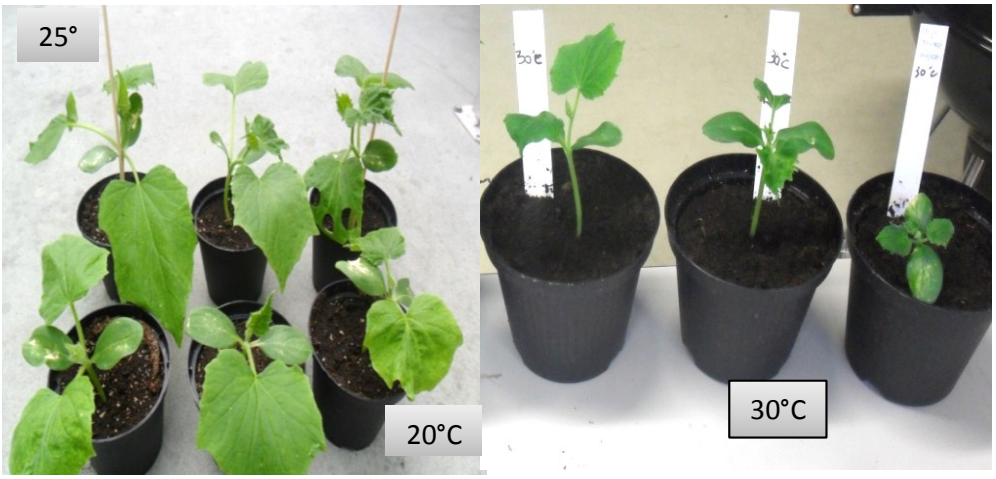

Figure 11: Effect of different temperatures on the height of inoculated cucumber plants at 28 days post-inoculation (28 dpi).

Virus particles accumulation in the host plant

Mean height of cucumber plants challenged with different viruses and grown under different temperatures are shown in Table 31 ANOVA showed significant difference among viruses ($F = 22.43$; $df = 3$; $P < 0.001$) and temperatures effects ($F = 40.32$; $df = 2$; $P < 0.001$) but no significant difference was observe in temperature –virus interaction effect ($F = 1.49$; $df = 6$; $P = 0.226$). In general mock-inoculated plants had significantly highest mean plant height (46.2 cm) compared to those challenged with CMV (28.8 cm), ZYMV (25.2 cm), and CMV+ZYMV (18.9 cm). The mean plant height of plants challenged with both CMV and ZYMV was not significantly different from those challenged with ZYMV only but significantly lower than those challenged with CMV only and mock-inoculated plants.

Table 28: *Mean height of plants inoculated with CMV and ZYMV under varying temperatures at 28 dpi*

Virus (28 dpi)	Temperature		
	20°C	25°C	30°C
CMV	13.9	45.6	27.1
ZYMV	16.4	40.1	19.1
CMV + ZYMV	12.6	28.8	15.5
MOCK-INOCULATION	27.4	63.1	48.1
LSD (P< 0.05)		7.24	

Means within a column, followed by the same letter(s) are not significantly different (P < 0.05) based on (LSD) test at 5% level

Discussion

Mixed infections of viruses from the family *Potyviridae* and those from other genera typically produce synergism in plants (Gil-Salas et al., 2012). Seven days (7) dpi, there was no symptoms on plant leaf but the serological analysis revealed the replication of virus in the plant. Thus, the events of virus infection take place early in the host plant. The absence of symptoms can be justified by the fact that the concentration of virus was not sufficient to induce symptoms and the threshold of positivity required for the sample was not reached. It could also be linked to the physiological state of the host, or the cultivar as reported by others (Peters et al., 1991; German et al., 1992; Hull, 2002). The symptoms were visible after 10 dpi indicating it was at the period of latency. The present work has shown that the accumulation of virus is also affected by the host and the temperature regime to which the plants are exposed. Similar observations were reported by

Llamas-Llamas et al., (1998) when assessing the effect of temperature on symptom expression and accumulation of tomato spotted wilt virus in different host species. The study revealed that in single infection at 20˚C CMV inoculated plants developed severe symptoms of mottling associated with stunting symptoms, whereas at 30°C the symptoms on the leaf were masked accompanied by low concentration of the virus. These results are similar to those found with other studies (Szittya et al., 2003). This fact is explained by a system of defense in the plants regulating their protection against pathogens, referred to as RNA mediated silencing. This RNA silencing mediated defense pathway synthesizes the small RNA, called small interfering RNA (SiRNAs) which interferes with viral particles. This system may be activated in the plant at 30°C impeding the development of the disease.

There was no significant difference in the ZYMV concentration under the three temperature treatments however; at 30° C the symptoms expressed were severe compared to those of 20˚C and 25˚C suggesting a stimulation of disease development by ZYMV at 30˚C. Also, apart from 20˚C the accumulation of ZYMV at 25˚C and 30˚C in single infection was not significantly different from those of the co-infection. Based on this observation, ZYMV might be more pathogenic than CMV which caused fewer symptoms on plants. It was also observed that the accumulation of virus particles was two times higher in the co-infection of the two isolates than in single infection. This indicates the synergistic effect between the CMV and ZYMV. Consequently, serious damage on cucumber plant were

observed which were stunting, yellowing, vein banding and the reduction of internodes from the host. These symptoms were similar to those observed in the fields. The results indicate also that in the co-infection of these two isolates, CMV *Cucumovirus*, and ZYMV, *Potyvirus*, showed a decrease of ZYMV concentration whereas, CMV concentration was enhanced. Indeed, the RNA silencing mediated defense pathway was resulting a suppression by the other associated virus (Pruss et al, 1997). This suppression could induce the subversion of host defense, facilitating, infection by other viruses.

During the study the height of the inoculated plants were affected. This was recorded at 20˚C and 30˚C. The heights of inoculated plant were however not significantly affected at 25˚C. It was reported that there is a mechanism displaying in the plant which regulates the growth of plant by synthesizing the micro RNA (Grishok et al, 2001; Hutvagner et al, 2001; Llave et., 2002; Reinhart et al., 2002). It could be possible that this mechanism was activated at 25˚C and inhibited at 20˚C and 30˚C. The two viral isolates tested are transmitted in non-persistent manner by aphids.. ZYMV induced the waffled leaves, blistered and the reduction of leaf surface at 20°C, vein banding at 25°C and severe reduction of leaf in bract. These symptoms are consistent with those described elsewhere (Lis, Boccardo, D'Agostino, Dellavalle, & D'Aquilio., 1981, Providdenti *et al*., 1984).

Conclusion

This study showed that temperature has an influence on virus replication, disease development and growth of cucumber plants. A temperature of 20°C was more favourable for virus replication and disease

development in CMV inoculated-plants whereas higher temperature of 30°C induced disease development of ZYMV-inoculated plants.

Temperature of 25°C was optimum for the growth of cucumber plants and to cope with viral infection. These results provide valuable information for the development of effective strategies for the management of viral diseases of cucurbits in Côte d'Ivoire.

CHAPTER SIX

ASSESSMENT OF GROWING SEASONS ON VIRUS DISEASES ON CUCURBITS IN FIELD

Introduction

Viral diseases are the major problem of cucurbits production in Côte d'Ivoire. The *Cucumber mosaic virus* (CMV) and *Zucchini yellow mosaic virus* (ZYMV) have been reported as the most prevalent viruses in these crops (Fauquet & Thouvenel 1987, Koné et al., 2010; Agneroh et al., 2012). They are non-persistent aphid transmitted viruses. The viruses cause mosaic, mottling, enation, and puckering of foliage; mosaic and distortion of fruit; and plant stunting (Sherf & MacNab, 1983). It has been reported elsewhere that yields losses due to ZYMV infection ranged between 50% and 94% (Blua & Perring 1989, Müller et al., 2006). Management of these virus diseases is very important in order to improve yields of cucurbit crops. Various methods employed in the management of cucurbit viral diseases include removal of weeds and volunteers cucurbit crops plants (Sharma et al., 2016), use of super-reflective plastic mulch (Stapleton & Summers, 2002; Barbercheck, 2014); the use of natural enemies against aphids (Kos et al. 2008), and the insecticides control (Hull, 2009). However, these methods have not been very effective in the management of viral diseases due partly to the development of resistance by insects against insecticides or because aphids transmit virus before the insecticides act to kill them (Jayasena & Randles 1985, Maelzer 1986, Simmons, 1957; Webb & Linda, 1993); A limitation of reflective films in

cucurbits has been that plant growth rapidly covers the mulch and thereby lessens reflectivity (Damicone & Edelson, 2007).

The most effective and simplest method of controlling viral diseases is growing of resistant varieties. Breeding for host resistance is difficult due to the incompatibility among different cucurbits species (Zitter & Murphy, 2009). Therefore, there is the need to evaluate other cultural methods such as growing season (Hull, 2009). The growing season is important in tropical situations (Hull, 2013). Growing seasons have been used in the avoidance or management of tungro virus disease in rice (Manwan et al., 1987). Information on the use of growing seasons in managing viral diseases in cucurbits in Côte d'Ivoire is limiting. It has also been reported that viral diseases can be confused with many other diseases or sometimes nutrient deficiency symptoms or physiological disorders, hence identification of viral causal agent is very important for its management. The study was therefore conducted to assess the effect of growing seasons on virus disease incidence and severity.

Material and methods

Study site

The field study was conducted in Dabou, situated 45 km west of Abidjan. This location (04° 16 W; 05° 16N) experiences two rainy seasons interrupted by two dry seasons. The major rainy season spans from May to July, followed by the minor dry season in August which can be extended to September. The minor rainy season covers the months of September and October, followed by the major dry season which starts from November to February. The annual mean rainfall is between 1500 and 1600 mm distributed

through the year. The mean annual temperature is estimated at 28℃ with mean relative humidity varying from 80% to 90%. The vegetation is coastal type dominated by the small mangrove (Avenard, 1971; Comoe, 2009).The climatic data collected during the three experiments conducted from May 2014 to April 2015 are shown in Table 29.

Table 29: *Microclimate of the experimentation site*

Trials	Months	Temp (°)	RH (%)	Precipitation (mm)
Trial 1: 2014	May	26.3	83.7	20.57
	June	27.7	80.57	39.83
	July	26.13	81	93.33
Mean		27.09	81.75	51.24
Trial 2: 2014	September	26.4	86.6	102.3
	October	25.4	88.8	174.1
	November	27.1	87.2	260.6
Mean		26.3	87.5	179
Trial 3: 2015	February	27.1	84	16
	March	28	78	0
	April	25.6	90.6	3.2
Mean		26.9	84.2	6.4

Temp: temperature; RH: relative humidity

Plant material

Two varieties of cucumber (Tokyo hybrid F1, and Poinsett) obtained from an agro-input shop and a farmers' preferred variety of Zucchini (Bolle) obtained from the local market was used for the study.

Experimental design and field layout

Randomized complete block design with three replications was used. The treatment comprised three growing seasons (May, 2014, September, 2014 and February, 2015) and two cucurbits (cucumber and zucchini). The field was divided into three (3) blocks with three plots of 20 plants of cultivar each. Plots were 1m x 10 m and each contained two 5-m-long rows of cucumber or zucchini spaced 60 cm apart. Sowing was done at 2 seeds per hill at intra-row spacing of 40 cm and inter-rows spacing of 60cm, and later thinned to one plant per hill when seedlings reached two leaves stage. The organic manure was incorporated into the soil prior to planting.

Cultural practices

Granular fertilizer at 12-22-22 kg/ha N-P-K was incorporated two weeks after sowing. Compost was applied 28 days after germination at a rate of 10 L per 10 m^2 plot. The plots were watered when necessary. Fungicide Mancozan, Ivory 80 WP (content: 800 g/kg), Callicuivre (content: 50%) and the nematicide, Diafuran 5G (Carbofuran: 5%) were applied with the doses recommended by the manufacturer on the plants to prevent fungi and nematode infections. The insecticides K-optimal 35 EC (Content: Lambda-cyhalothrine: 15 g/l, Acétamipride : 20 g/l) , and Decis 12 EC (active molecule: Deltaméthrine : 12,5 g/l) were applied twice per week, starting by the moment the insect were observed on the plants (one week after sowing).

Data collection in the field

Data was collected on disease incidence and severity, the occurrence and the first symptom occurrence. Disease incidence was determined based on the symptoms on diseased plants.

The proportion of diseased plants was estimated by: $\mathbf{IC} = \frac{\boldsymbol{n}}{\boldsymbol{N}} \times \mathbf{100}$ Where, IC = Incidence; n = diseased plants; N = total number of plant assessed.

The severity index of the disease described the damage caused by the diseases on plants leaves. The index was expressed by using a scale varying from 0 to 5 where 0 represents apparent healthy plants and 5, very severely infected plants. A modified 0-5 visual scale of Merritt et al., (1999) and Steel and Torrie, (1980) based on disease symptoms, was used to score the disease plants as following: 0: No disease symptoms; 1. Mild mottling on 10% of leaf; 2. Mottling on 50% of leaf/ light downward cupping; 3. Pronounced downward or up cupping of leaf / Chlorosis/ 75-100% leaf mottling; 4. Severe mosaic/ severe distortion of leaf / crinkled leaf /stunting plant / leaf bunching; 5. Severe leaf distortion/ necrosis/ narrowed or shoes-string leaf.

Disease severity index was then determined for each treatment using the formula Merritt et al., (1999) and Steel & Torrie, (1980):

Disease Severity Index (DSI) $= \frac{\mathbf{0*p0+1*p1+2*p2+3*p3+4*p4+5*p5}}{\mathbf{N\,(G-1)}}\,\mathbf{x}$

100, where, P1 P2 to P5 = Total number of observed plants in each disease symptoms grading per farm site in each state within the agro ecological zone surveyed.

G = Number of grading = 6 and N = Total number of observations.

Areas under disease Progress Curve

The areas of disease progress on the cultivars or varieties were calculated using the incidence and disease severity index. Thus, the Area under severity index progress curve (AUSIPC) for disease severity was calculated using the modified formula described by Shaner & Finney (1977) as below:

AUSIPC = $\sum_{n=1}^{n-1}(\mathrm{DS1} + \mathrm{DS2}/2)$ x $(\mathrm{t2} - \mathrm{t1})$ where, DS1 is disease severity recorded in time 1 and DS2 the disease severity recorded in time 2

Area under disease progress curve (AUDPC) for disease incidence was calculated using the formula described by Muengula-Manyi, Mukwa & Kalonji-Mbuyi, (2013).

AUDPC $\sum_{i=1}^{n}[(\mathrm{Xi} + \mathrm{X(i+1)}/2)\ ]$ x (t) where, Xi is the incidence of disease at time i, Xi+1 is disease incidence recorded at the time i+1, n, the number of observation on the incidence, and t, days between the observations of Xi and Xi+1.

Data analysis

Data on mean disease incidence and severity index were arcsine transformed (Legendre, 1998) before subjecting to analysis of analysis of variance (ANOVA). The means were separated using least significant differences (LSD) test at 5% level of probability. All data were performed in GenStat Discovery version 4 (VSN International).

Results

Incidence of disease

The field evaluation on the effect of growing season and cultivar on the mean incidence of virus disease at different growth stages showed interesting results (Table 30). ANOVA showed significant difference among the growing seasons in terms of mean disease incidence at 14 days after sowing (DAS) ($F= 4.12$; $df = 2$; $P = 0.036$); 28 DAS ($F= 7.37$; $df = 2$; $P = 0.005$); 42 DAS ($F = 11.58$; $df = 2$; $P < 0.001$) and 56 DAS ($F = 13.53$; $df = 2$; $P < 0.001$).

Mean disease incidence on cucurbit varieties increased with the growth stage. The mean incidence recorded for growing seasons 2 and 3 (90% each) at 56 DAS, was not significantly different ($p>0.05$) from each other but significantly higher than the growing season 1 (73%). ANOVA also showed significant difference ($P < 0.05$) in incidences among the cultivars at all growth stages (Table 30). The observation of the final growth stage at 56 DAS indicated that mean incidence recorded for Bo (85%) was not significantly different from that one of P7 (84.3%) but significantly higher than that recorded for TF1 (54.2%).

Disease severity

In Table 31, an ANOVA on the effect of growing season mean disease severity index showed significant difference among them at 14 DAS ($F = 3.99$; $df = 2$; $P < 0.039$); 28 DAS ($F = 29.97$: $df = 2$; $P < 0.001$); and 56 DAS ($F = 72.06$; $df = 2$; $P < 0.001$) but was not significant at 42 DAS ($F = 0.01$; $df = 2$; $P =0.98$) as shown in Table 34. At 56 DAS, the mean severity index

recorded for growing season 2 (82%) was significantly higher ($P < 0.05$) than that of the growing season 1 (51.5%) and growing season 3 (52.1%).

The mean disease severity recorded for the different cultivars were not significantly different at 14 DAS, 42 DAS, and 56 DAS but was significantly different at 28 DAS. In general, disease severity increased steadily from 19.7% at 14 DAS to 62% at 56 DAS, indicating that disease severity increased with growth stages.

Mean disease incidence at 56 DAS among the cultivars was not significantly different between the cultivar TF1 (61.8%), P7 (64.1%), and Bo (59.6%). With respect to the mean disease severity among the growing seasons a highest significant difference of 82% was recorded for the growing season 2. However, they were not significantly different in the growing season 1 (51.5%) and season 2 (52.1%).

Areas under disease Progress Curve using incidence

An ANOVA for the AUDPC values based on disease incidence showed no significant difference ($P > 0.05$) among the growing seasons as well as the cultivars (Figure 13). This suggests that the disease spread at all growing seasons and all the cultivars were also affected by the virus. However, with respect to Bolle cultivar, growing season 1 had the highest AUDPC value whilst growing season 2 had the lowest. For Poinsett cultivar, growing season 2 had the highest AUDPC value whilst growing season 1 had the lowest. For Tokyo F1 cultivar, growing season 2, no significantly different from the growing season 3, had the highest AUDPC value whilst growing season1 had the lowest.

Areas under severity index Progress Curve

The total amount of disease that occurred on each variety at the different growing season was calculated and expressed as the Area Under Disease Progress Curve (AUSIPC) as shown in Figure 13. ANOVA showed a significant difference among the growing seasons ($F = 9.30$, $df = 2$; $P = 0.002$) and the growing season - variety interaction effects ($F = 7.32$ $df = 4$; $P = 0.002$), but there were no significant difference among the varieties ($F = 1.76$ $df = 2$; $P = 0.205$).

For Bolle cultivar, AUSIPC value for the growing season 1 was significantly higher ($P < 0.05$) than growing seasons 3 which was significantly higher than growing season 2.

In Poinsett cultivar growing season 2 had significantly higher value of AUDSIC than growing seasons 1 & 3. Similarly, AUDSIPC values recorded for Tokyo F1 cultivar at growing season 2 was significantly higher ($P<0.005$) than that of growing season 1 , which was also significantly higher than growing season 3 (Figure 13).

Table 30: *Mean disease incidence of virus disease on three cucurbit varieties at different growing stages (%)*

Growing season	14 DAS				28 DAS				42 DAS				56 DAS			
	TF1	P7	Bo	Mean	TF1	P7	Bo	Mean	TF1	P7	Bo	Mean	TF1	P7	Bo	Mean
1	13.7	1.6	36.2	22.2 a	35.3	40.7	54	43.3 b	46	58.7	67	57.4 b	72.5	72.8	74.6	73.3 b
2	18.9	0	19.9	12.9b	69	90	34.9	64.7a	77.3	90	37.1	68.2 b	90	90	90	90.0 a
3	19.3	8.6	15	14.3 b	45.6	46.2	34	41.9 b	73.9	90	90	84.2 a	90	90	90	90.0 a
Mean	17.3a	8.4c	23.7a	16.46	50b	59a	41.0b	50	65.7ab	79.6a	64ab	70	54.2b	84.3a	85a	84.43

Means in the same row within a growth stage bearing the same letters are not significantly different by LSD. at 5% level of probability. Means in the same column within a growth stage, bearing identical letters are not significantly different by LSD at 5% level of probability

TF1: Tokyo F1 hybrid; P7: Poinsett; Bo: Bolle

DAS: Days after sowing

Table 31: *Mean disease severity index in three cucurbit varieties at different growing stages (%)*

Growing season	14 DAS				28 DAS				42 DAS				56 DAS			
	TF1	P7	Bo	Mean	TF1	P7	Bo	Mean	TF1	P7	Bo	Mean	TF1	P7	Bo	Mean
1	26.6	30.1	29.4	28.7 a	39	38.7	41.3	39.7b	44.6	42.7	46.9	44.7a	52.2	49.8	52.5	51.5b
2	21.1	10.0	15.0	15.4 b	46.9	49.2	28.6	41.6a	51.6	54.3	27.3	44.4a	90.0	90.0	66	82.0a
3	16.9	12.3	15.7	15 b	24.4	24.7	21.6	23.6c	34.4	47.1	52.9	44.8a	43.1	52.6	60.5	52.1b
Mean	21.5a	17.5a	20.0a	19.7	36.8a	37.5a	30.5b	34.96	43.5b	48.0a	42.4c	44.63	61.8a	64.1a	59.6a	62

Means in the same row within a growth stage bearing the same letters are not significantly different by LSD. at 5% level of probability. Means in the same column within a growth stage, bearing identical letters are not significantly different by LSD at 5% level of probability TF1: Tokyo F1 hybrid; P7: Poinsett; Bo: Bolle

DAS: Days after sowing; Data on the severity index was arcsine transformed before ANOVA was done

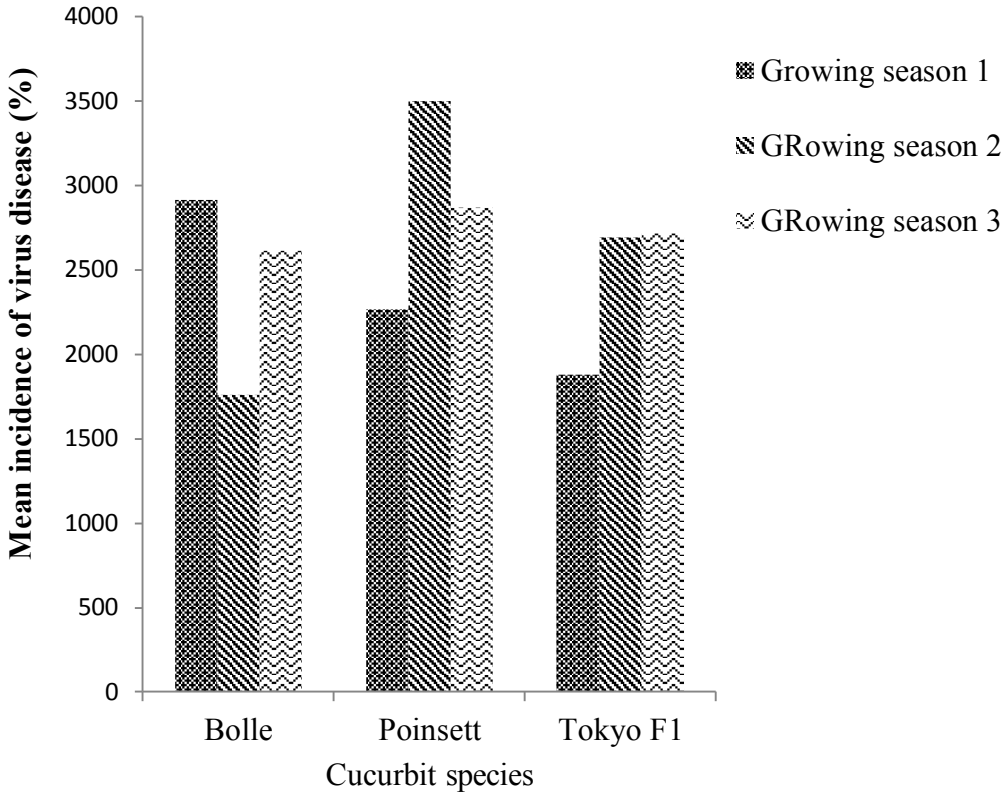

Figure 12: Area under disease progress curve using disease incidence on cucumber varieties, Tokyo F1 and Poinsett, and zucchini variety, Bolle during three growing seasons.

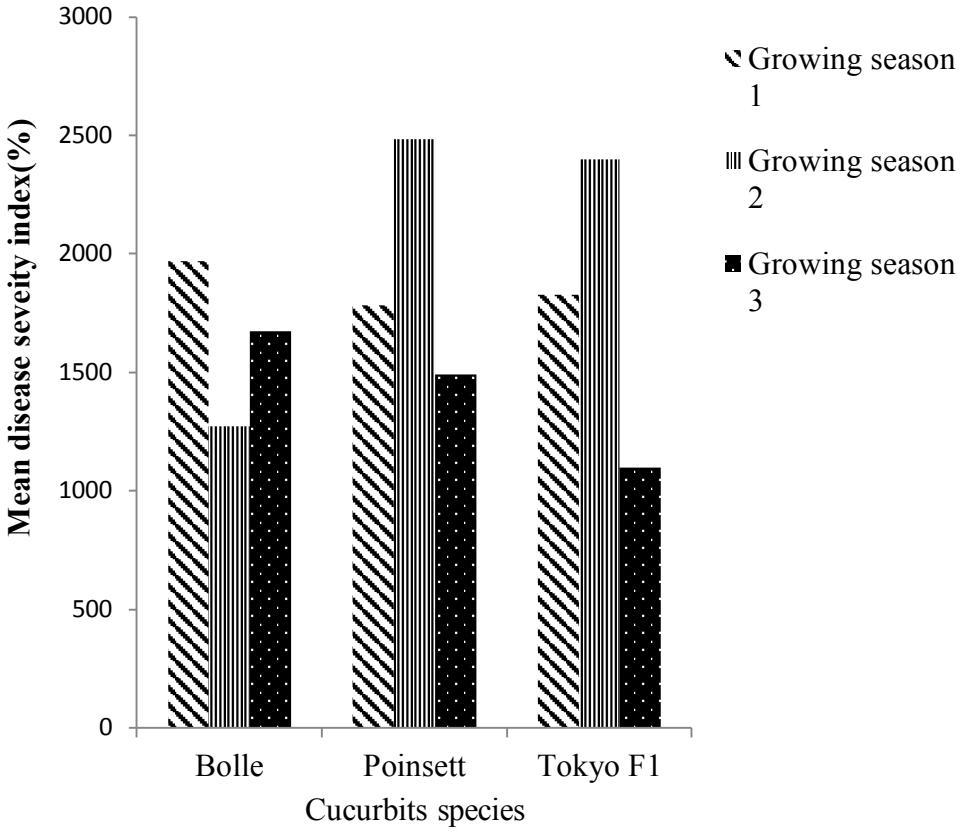

Figure 13: Area under disease progress using disease severity index on cucumber varieties, Tokyo F1, Poinsett, and zucchini variety, Bolle, during three growing seasons.

Discussion

Planting date or the growing season is important since it controls the stage of growth of the crop at the time that aerial vectors arrive thus influencing the attractiveness of the plants on host searching flights and also the susceptibility of the plant with younger plants tending to be more susceptible (Hull, 2013). It can be a means to find the best time to grow in order to avoid virus disease. The field experimentation conducted in three successive growing seasons indicated visual assessment that the first symptoms occurrence was approximately between 10-14 days after sowing

(DAS). All the three cucurbits cultivars were expressed virus disease symptoms started by the zucchini cultivar which was local variety. This suggests the susceptibility to virus infection. At 10 DAS the symptoms were observed indicates the infection of the seedlings was made earlier. It was reported that the early infection of the plant influence disease incidence and severity by an increasing (Hull, 2009-2013). Therefore, as measures to reduce the infection early monitoring of the vectors and the plants is needed

The results revealed that mean disease incidence was not significantly different in the growing seasons 2 and 3 ($P > 0.05$) but a difference was observed between these 2 seasons and the growing season 1 (corresponding to the major rainy season) where the incidence was reduced. This reduction of disease incidence in the growing season 1 could be due to the influence of the rain and the wind which decreasing the number of eggs laid or influence the number of aphids' colonies during this period as reported by Weisser, Volkl, & Hassell, (1997). The reduction of disease incidence may be also due to the reduction of aphids' attractiveness for the plants or change of feeding behavior (Colvin et al, 2006). It was reported that many aphids' species are attracted to the color and the distribution of plants during host-finding flights. And then the yellow colour reflective spectrum attracts many aphids' species (Düring & Chittka, 2007). It could be also a modification in viruses' transmission rates because of the alteration of hosts' biology and their distribution (Randolph, et al. 2000; Anderson et al, 2004, Jones, 2009). Disease incidence increased with the increase in growth stage. This suggests that the susceptibility of the plants to the virus infection was enhanced when the plants were becoming old. This

observation is at variance with the finding of Fargette, Muniyappa, Fauquet, N'Guessan, & Thouvenel, (1993), on *African Cassava Mosaic Virus* (ACMV) interaction. They reported that, the susceptibility of senescent cassava plants to ACMV infection was reduced with growth stage because as the plants mature, they develop the strategies of resistance or tolerance. It was reported elsewhere (Gibbs & Harrison., 1974; Matthews, 1991, Silhavy et al., 2002) that the increase of virus infection may depend on aphids' preference. In fact, the concentration of solutes in the plant sap has an attractive effect on aphid due to the volatiles metabolites released by the infected plants. And that can change the feeding behavior of the aphids.

In terms of disease severity, the highest mean value was recorded in minor rainy season equivalent to growing season 2, suggesting that this period may be favourable for vectors multiplication due to the reduction of humidity and the amount of rainfall which are the means to ensure the persistence of the alternative host and weeds known as green bridge for viruses multiplication in one hand (Fadjinmi, 2010). In other hand, the conditions of the site in growing season 2 may be suitable for the abundance of the aphids' population, or otherwise the higher severity could be linked to virus species transmission which was ZYMV since this study revealed that ZYMV was more prevalent in rainy season. Consequently, the abundance of aphids' population during the growing seasons 2 may be a factor contributing to the increase in disease development.

Otherwise, the increase in disease severity may be due to the field conditions including the inadequate watering of plants which are favorable to

virus disease development (Afouda *et al*., 2013)., the presence of source of virus in the proximity of the field, and the exposition of the field to upwind.

Conclusions

The variation in disease incidence and severity with the changing weather conditions showed evidence that the physical factors through the growing seasons; have an influence on disease incidence and severity. The susceptibility of the two species of cucurbits commonly grown in Côte d'Ivoire, to virus infection indicates the need to look for improved varieties to minimize the risk of infection. The study has revealed that the growing season is an important factor which should be taken into account in developing strategies to manage viral diseases of cucurbits in Côte d'Ivoire.

CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

General discussion

The purpose of this study initiated on cucurbits virus diseases was to provide some information which can help in the development of some control strategies. Based on the results, it can be concluded that viral disease is widespread in Côte d'Ivoire with high mean prevalence and severity. This high disease prevalence and the severity varied in space and time, and influenced by the seasons and the diversity of ecology, but also influenced by the effect of crops management and land use as described by Hull, (2009).

Four virus species were identified in the study using the serological and the molecular methods. The serological assay confirmed the presence of the three common viruses i.e. CMV, ZYMV, PRSV detected in cucurbit crops leaf and weeds samples using double antibody sandwich enzyme-linked immunosorbent assay. These viruses have previously been detected in cucurbit crops in Cote d'Ivoire by Koné et al., 2010; Agneroh et al., (2012). The seasonal distribution of these viruses revealed CMV more prevalent in the dry season, and the ZYMV the dominant virus in the rainy season. The spatial distribution of these viruses also indicated a variation in the frequencies of occurrence in the agro-ecological zones and linked to the variable ecological conditions, diversification and the intensification of crops, and the alteration of temperature, rainfall patterns and wind speed (Anderson, 2004; Elena et al, 2011). These viruses were also detected in both perennial and annual the weeds samples.

RT-PCR, followed by gene sequencing, was used to detect the fourth virus Pepo aphids-borne yellows virus (PABYV), reported for the first time during the study. PABYV was associated in co-infection with other viruses (CMV, ZYMV, and PRSV). The symptoms expressed by the diseased plants indicated a reduction of yields observed over the years. However, the study revealed that virus disease symptoms were not specific to a particular virus. Thus, the association of these viruses in co-infection should be considered in the development of strategies since it represents the major factor of yield losses in cucurbits. The symptoms were dominated by the mosaic, the vein banding, up and down cupping, and shoe-string symptoms on plant leaf. The CP- based phylogenetic analyses of thirteen sequence isolates of PABYV revealed that no genetic diversity among them and no recombination events. The population genetic analysis also revealed that the PABYV isolates did not differentiate according to the seasons and the agro-ecological zones. This suggests that the PABYV populations were not genetically diverse despite the high mutation, the high number of polymorphic sites and the high number of haplotypes. According to Garcia-Arenal et al (2003), high mutation rates are not necessarily adaptive, as a large fraction of the mutations are deleterious or lethal. This suggests that the PABYV isolates were under negative selection as was observed in this current study. This information has an agronomic significance and an advantage in respect of the development of control measures for the virus. The study revealed a significant gene flow between seasons, and agro-ecological zones. This could be due to the exchange of infected plant materials between the farmers, the transfer of plants materials

from one growing area to another through the diversification and the intensification of the crops (Anderson et al., 2004; Fargette et al., 2006; Jones, 2009).

The study on the effect of temperature regime of 20˚C, 25˚C, and 30˚C, on virus replication and the and on the height of inoculated plant development revealed that CMV and ZYMV respond differently to the temperature in single and in co-infection. The infection of the two viral species on the host plant induced a systemic infection and the synergism effect between them. It was observed that the temperature of 20˚C induced the development of disease in CMV inoculated plants whereas; the symptoms were masked on the leaves at the temperature of 30˚C. The ZYMV inoculated plants rather, developed less disease symptoms at 20˚C and developed severe disease symptoms at 30˚C. The co-infection of the two viruses caused serious damage of the plants associated with the high concentration of virus particles than in single infection. Compared to the other two, 25°C was more favorable temperature for plant growth with an acceptable low virus concentration reduction in the plants (chapter 5).

The field studies on the effect of growing seasons on disease incidence and severity indicated an overall high mean disease severity and prevalence in all growing seasons despite in respect of the sowing density. The growth of cucurbit species together indicated the first occurrence of symptoms from the local variety of zucchini although all cultivar showed disease development. The study showed that the severity of the viral disease was hash during the minor season. Planting during the minor season can therefore be avoided in

order to reduce the effect of viral infection. Based on that, the growing season 2 corresponding to minor rainy season should be avoided to reduce virus disease severity in the plant.

Conclusions and recommendations

These results are a contribution in the management of virus disease in Côte d'Ivoire through the different approaches used and developed in light of climate variability. Consequently, our findings concur with hypotheses formulated at the onset of the study, and the following is the summary of the conclusions and recommendations:

Virus diseases are wide spread in all agro-ecological zones at all seasons with high disease prevalence and severity. It is recommended that plants virologists and breeders should develop strategies to minimize viral disease incidence and severity. This Agricultural Extension staff should train farmers on the awareness of these diseases and their control measures. Certified seeds and technologies should be provided by the international organizations like AVRDC, USAID.

Four (CMV, ZYMV, PRSV, and PABYV) viruses were associated with viral disease occurrences with PABYV reported for the first time. Mixed infections of these viruses were detected in the study was therefore important to consider this when developing strategies for their control.

The variability of climate at different seasons and at different agro-ecological zones influences the distribution of the viruses, and the occurrence and the development of the diseases. This affects the phenology of the host,

the virus transmission and the biology of the vectors or parasite infectious stage in environment.

Virus species respond differently to the different temperature regimes. It was also observed that each virus isolate required an optimum temperature to induce the development of disease. Under the temperatures 20°C & 30°C, virus infection resulted in stunted in cucumber plants.

Viruses were detected in some weeds collected near the cucurbit crops within and in the borders of the fields. These are probably sources of virus inoculum. In this way, farmers should adopt good agronomics practices to minimize spread and severity of viral disease. This includes monitoring of pests and disease occurrence, good phytosanitary approaches (farms hygiene), crop and weed-free periods.

Perspectives

Further studies should be conducted to characterize the CMV, ZYMV and PRSV.

Cucurbit germplasms including both wild and cultivated species should be screen against virus infection in order to identify source of resistance.

Study should be conducted to identify the species and biotype of aphid vectors.

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APPENDICES

Appendix A: *Weed adjacent to the cucurbit plants*

Appendix B: *How to prepare LB-ampicillin plates*

1. Prepare LB-agar Medium (1 liter), weigh out:
   - Bacto Tryptone 10 g,
   - Bacto Yeast extract 5 g,
   - NaCl 5 g.
2. Dissolve in 800 mL of water, adjust pH to 7.0 with NaOH and add water to 1000 mL.
3. Add 15 g of agar and autoclave.
4. Allow the medium to cool to 55 °C.
5. Add 2 mL of ampicillin stock solution (50 mg/mL) to a final concentration of 100 μg/mL.
6. Mix gently and pour plates.

Appendix C: *Paper published*

Kone, N, Coulibaly A, Koita O, Koné D, Bediako EA, Knierim D, Menzel W, Winter S. (2015). First report of *Pepo aphid-borne yellows virus* on zucchini in Cote d'Ivoire. *New Disease Reports* **31**, 27.

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