CHARACTERISATION OF *Phytophthora* POD ROT OF COCOA (*Theobroma cacao* L.) AND MANAGEMENT WITH HOST PLANT RESISTANCE IN SOUTHWESTERN NIGERIA

BY

ENOCH BODUNDE ROTIMI

B. Agric. (Hons) (O.A.U Ife), M.Sc. Agric. (Ibadan)

Matric. No. 160891

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ABSTRACT

The productivity of cocoa (*Theobroma cacao* L.), an important economic tree in Nigeria, is limited by *Phytophthora* Pod Rot (PPR). Efficient management of PPR requires identification and characterisation of the Causal Pathogen (CP). The use of Host Plant Resistant Varieties (HPRV) was successful in the management of fungal diseases of some crops with minimal environmental effect. However, there is dearth of information on the management of PPR with HPRV. Therefore, morphological and molecular characterisation of CP and management of PPR with HPRV were investigated in southwestern Nigeria.

Five States: Ekiti, Ogun, Ondo, Osun and Oyo which are the major cocoa growing areas in southwestern Nigeria were purposively selected. Nine cocoa plantations were randomly selected and five PPR-infected pod samples collected per plantation in each of the states. Fungal isolates were obtained from cocoa pods and pure cultures assessed for pathogenicity following standard procedures. Morphological characteristics of 45 isolates (nine/state) were determined using Mycelial Growth (MG), Colony Pattern (ClP), Sporangial Shape (SS), Sporangial Pedicel Length-SPL to Sporangial Breadth-SB (SPL:SB) were used for CP identification. Deoxyribonucleic acid was extracted from 10-day old V8 cultures of the 45 isolates, followed by Polymerase Chain Reaction (PCR) amplification of the Internal Transcribed Spacer (ITS) gene following standard procedures. Amplicons were sequenced to show similarities among PPR isolates and compared with CP genomes within the *Phytophthora* data base. In the laboratory, three-month old pods and two-month old seedlings' Leaf Discs-LD (1.5 cm) of each of the six cocoa varieties (TC-1, TC-3, TC-4, TC-5, TC-6, TC-7) with different levels of resistance and N38 (susceptible control) were inoculated with pure cultures of CP $2x10^5$ spore/mL at 10µL/pod and 10µL/LD in a completely randomised design replicated thrice to screen for resistance. Seven days after inoculation, Infection Growth (IG) on pods were measured, while a rating scale of 0 (highly resistant) to 5 (highly susceptible) was used to assess IG on LD. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05.}$

A total of 135 fungal isolates were identified in all the states and 45 were pathogenic. The MG of all isolates was cottony with slightly papillate ClP. The SPL ranged from 29.9±7.9 to 38.8±8.7, while SB ranged from 24.0±5.4 to 30.8±5.4. The SPL:SB was 1.2:1.4 and the SS was ellipsoid in all isolates. The PCR amplification and sequencing of the CP characterised cacao PPR as

Phytophthora megakarya. The pathogen yielded an estimated 550-bp ITS product with ≥99.9% isolate similarities. The gene accessions of *Phytophthora megakarya* isolates showed evolutionary relationship to strains in Cameroon and Ghana. The IG on pods was least on TC-4 (±9.3mm) and was similar to TC-6 (±23.3mm), TC-3 (±27.8mm), but was significantly lower than TC-5 (±52.5mm), TC-1 (±64.0mm), TC-7 (±68.1mm) and N38 (±73.1mm), Leaf discs of TC-4 had the least IG of (1.2) and were similar to TC-3 (1.5), TC-6 (1.8), TC-5 (2.6) and TC-1 (2.8) but significantly lower than TC-7 (3.4) and N38 (4.2).

Phytophthora megakarya predominantly caused *Phytophthora* pod rot on cocoa in the study site. The variety TC-4 was resistant to *Phytophthora* pod rot of cocoa.

Keywords: *Theobroma cacao*, *Phytophthora megakarya*, *Phytophthora* sporangial morphology

Word count: 500

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CERTIFICATION

I certify that this work was carried out by Enoch Bodunde **Rotimi** in the Department of Crop Protection and Environmental Biology, Faculty of Agriculture, University of Ibadan.

…… ……

Supervisor Date

Professor Babatunde Ikotun
B.Sc. (Ibadan), Ph.D (London) DIC.,
FNFL., M.I. Biol.,FNIOB
Department of Crop Protection and Environmental
Biology, Faculty of Agriculture, University of Ibadan, Ibadan.

…… ……

Co-Supervisor Date

Dr. Sunday Oluremi Agbeniyi
B.Sc. (Ibadan), M.Sc (Ibadan), Ph.D (FUNAAB)
Director, Department of Crop Protection,
Cocoa Research Institute of Nigeria, Ibadan.

DEDICATION

This work is dedicated to the glory of God, and all my loved ones whose support, advice and encouragement kept me going during my studies.

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CHAPTER ONE

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is a major economic tropical rainforest tree native to the Amazon region of South America (Bartley, 2005). The tree is commonly referred to as cacao, while the products made from fermented and dried seeds are referred to as cocoa (Zhang and Motilal, 2016). There are over 20 species in the *Theobroma* family, but the *T. cacao* tree is the only one commonly grown. International cocoa output is largely concentrated in regions between 10° North and 10° South, while western Africa accounts for 65 percent of world production (Zhang and Motilal, 2016).

Traditionally, cacao is cultivated in producing countries and marketed for export in the form of dried beans (Ajayi Adedamola *et al.* 2012). Importing countries then process beans and convert the raw goods into finished or semi-finished products (cocoa butter, cocoa liquor, cocoa powder, chocolates, etc.), not only for their aromatic qualities, but also for potential health benefits (Kris-Etherton and Keen, 2002).

Cacao is a significant crop with more than 20 million Nigerians solely dependent on it for their economic security (World Cocoa Foundation, 2014). The explanation for its popularity around the world could be attributed to its value as a commercial crop generating foreign exchange for producing countries.

Cocoa bean, a partly fermented and dried seed is a major component of chocolate and other beverages (Opeke, 2003). Cocoa is believed to be produced principally in developing countries, having West Africa as major producers, particularly in the forest zone (Marius and Quist-Wessel, 2015). A significant population of Nigerians makes their

livelihood from the production of cocoa, thereby creating explicit or implicit job opportunity (FAOSTAT, 2017).

Nigeria is the fourth-largest producer of cocoa beans in the world, behind Côte d'Ivoire, Ghana and Indonesia (FAOSTAT, 2018). After petroleum, cacao is the country's most important export. Before independence, cocoa generated 90% of Nigeria's foreign exchange earnings. Though, eclipsed these days by crude oil that became the country's major export, Nigeria still produces 300,000-350,000 tonnes of cocoa per year (Ajayi Adedamola *et al.* 2012). This is mostly destined for consumption abroad as the country exports about 98% of its cocoa crop. Cocoa exports for October 2009-March 2010 were up 31% of the previous year, helped by good weather conditions and improved quality in stock in the growing region (FAOSTAT, 2017).

Theobroma cacao is produced in 14 States of Nigeria, where southwestern States are considered Nigeria's cocoa belt (FAOSTAT, 2017). In 2008, the region accounted for over 160,000 metric ton of Nigeria's 242,000 metric ton annual cocoa production (Ajayi *et al.,* 2012). Cocoa production is constrained by major factors which are major constraints to its production. The devastating effect of disease epidemic on cocoa crop yield cannot be over-emphasized among which *Phytophthora* pod rot is of great concern (Akrofi Yaw Andrews *et al.,* 2015). *Phytophthora* pod rot of cocoa is a well-established disease in all the growing agro-ecological zones of Nigeria (Ristaino Jean *et al.,* 2016).

Phytophthora Pod Rot (PPR) of cacao is caused by numerous factors, both environmental and genetic, are involved in disease expression by cocoa in the field, i.e. affect the rotten pod rate. Disease severity therefore varies depending on such different factors as: environmental conditions in the plantations; pathogen conservation and its transmission; the pathogen species and strain involved and the genetic nature of the host (Evans, 2012; Akrofi *et al.,* 2015). Many conditions that are necessary for *Phytophthora* Pod Rot (PPR) of cocoa disease development result from combinations of these different factors, which might interact with each other to promote the disease development. Though this disease affects various parts of the crop plant, but is more profound on fruits (Evans, 2012).

Therefore, identification and screening for the causal pathogen of *Phytophthora* pod rot in the cocoa-growing regions within Southwestern Nigeria will be useful for the implementation of schemes for the management of the cocoa agrosystem (Evans, 2012). Important questions such as which of the *Phytophthora* species is/are discovered in the area? Which one is commonly recognised in the proposed growing area? Which is more virulent among the observed organisms ' pathogenicity, and their molecular diversity?

Control of cocoa PPR disease was primarily based on four management approaches that were broadly categorised as cultural, chemical, biological, and use of resistant / tolerant cocoa cultivars (Akrofi *et al.,* 2015). Nonetheless, research on *Phytophthora* spp's pathogenic heterogeneity, cocoa pod and plant isolates have focused exclusively on the qualitative variations in virulence between two main species of *Phytophthora* (Evans, 2012).

There is a dearth of information on the characterisation of pathogen diversity for species of *Phytophthora* infecting cocoa in Southwestern Nigeria. This has persistently negated the creation of successful black pod disease control interventions and poses a serious threat to all potential control strategies that are being put in place.

A clear report of the genetic characteristics of the pathogens within the environment or region is an important requirement in the creation of a systematic and effective method for handling PPR disease. Hence this research work is targeted at achieving the following objectives:

1 To assess the occurrence of *Phytophthora* Pod Rot of cacao in the growing regions of Southwest Nigeria.

2. To investigate variations among isolates of *Phytophthora* species from the different cocoa growing regions in South western Nigeria using morphological variability study techniques and molecular approaches (tools).

3. To determine the reaction/response of newly released cocoa hybrids available in Cocoa Research Institute of Nigeria to *Phytophthora* species.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Historical Background of Cacao

Theobroma cacao was historically classified in the family Sterculiaceae but has now been accepted in the family Malvaceae in the genus *Theobroma* (Acebo-Guerrero *et al.,* 2012). Cacao is the most economically important crop among its species (Bartley, 2005). *Theobroma* means "lord meat" and cacao comes from the Olmec and Mayan (kawkaw) language (ICCO, 2005). Cacao fruits when processed give chocolate which also serves as source of both local and foreign earnings for producing countries. It provides employment to the populace in developing countries (World Cocoa Foundation, 2018).

This crop is the only cultivated species among the 22 species in the *Theobroma* group. It is grown mainly in tropics, expanding up to 700m above sea (Oyekale, 2012).The partially fermented and dried cocoa seed is commonly sold as beans, used in the manufacture of beverages. In West Africa, Nigeria with an export of 240,000 metric tons in 2017 is number three, behind Cote d'Ivoire, and Ghana (Afolayan, 2016). But, globally behind Indonesia as number five while Cameroon, Brazil, Ecuador, Peru (EPU), and the Dominican Republic take after Nigeria all contributing an outsize share of world cocoa production in that order (FAOSTAT., 2018).

In western Nigeria, the spread of cocoa cultivation was propelled by the efforts of migrant farmers (Opeke, 2002). They were required to create cocoa farms many of whom depended on traditional, non-economic institutions such as the lineage or ethnic communities to mobilize their economic resources (Ajayi *et al.,* 2012). From an evaluation of the practices of migrant farmers in Oyo, Osun, and Ondo States, cocoa

farming revenues improved the educational standard of cultivating farmers and brought significant changes in the size, composition and geographic distribution of rural economic activity in western Nigeria at the time (Opeke, 2003, Ajayi *et al.,* 2012).).

Five states: Ekiti, Ogun, Ondo, Osun and Oyo States in southwestern Nigeria are known to be Nigeria's major cocoa producers that account for 70 percent of the country's total production of cocoa. While, Abia, Adamawa, Akwa Ibom, Delta, Kogi, Kwara, Taraba and Adamawa account for 30% of the total annual production (Ajayi *et al.,* 2012). Wasimi Village in Ondo State is ranked highest producing area followed by Etomi Village in Cross River State, according to Nigeria's production ranking (FAOSTAT, 2017). The cocoa growing region of southwest Nigeria is considered to be Nigeria's cocoa belts (Zhang and Motilal, 2016). This is as indicated in Figure 2:1. The production states in Nigeria which include Ogun, Ondo, Osun and Cross Rivers as major producers; followed by Edo and Ekiti, while the rest are considered low-producing regions (Ajayi *et al.,* 2012).

2.2 Impact of *Phytophthora* Pod Rot on Production of Cocoa in Nigeria

This essential crop is confronted with both abiotic and biotic influences that reduce farmers ' income in Nigeria (Agbeniyi *et al.*, 2014). *Phytophthora* pod rot disease is well spread in all the cocoa growing areas of Nigeria. The invasion and survival of *Phytophthora* species pathogens have been of concern over the years in the production of the crop (Akrofi *et al.,* 2015).

Acebo-Guerrero *et al.* (2012) claimed that production losses due to *Phytophthora* diseases are difficult to determine, more so the species can induce a variety of symptoms under enabling environment that can have a significant effect on the frequency and severity of the disease. Other studies and reports have given a clear production loss due to PPR of cacao (Evans, 2012, Agbeniyi *et al.*, 2014 and Bailey *et al.*, 2016). The most severe economic challenges for all cacao growing areas is the *Phytophthora* pod rot. The causal organism is referred to as "plant-destroyer"(Agbeniyi *et al.*, 2014). While Ali *et al.* (2016) gives the annual loss between 30 and 90 percent due to black pod rot, the pathogen could as well cause a total loss in plantations where none of the control or preventive measure is observed.

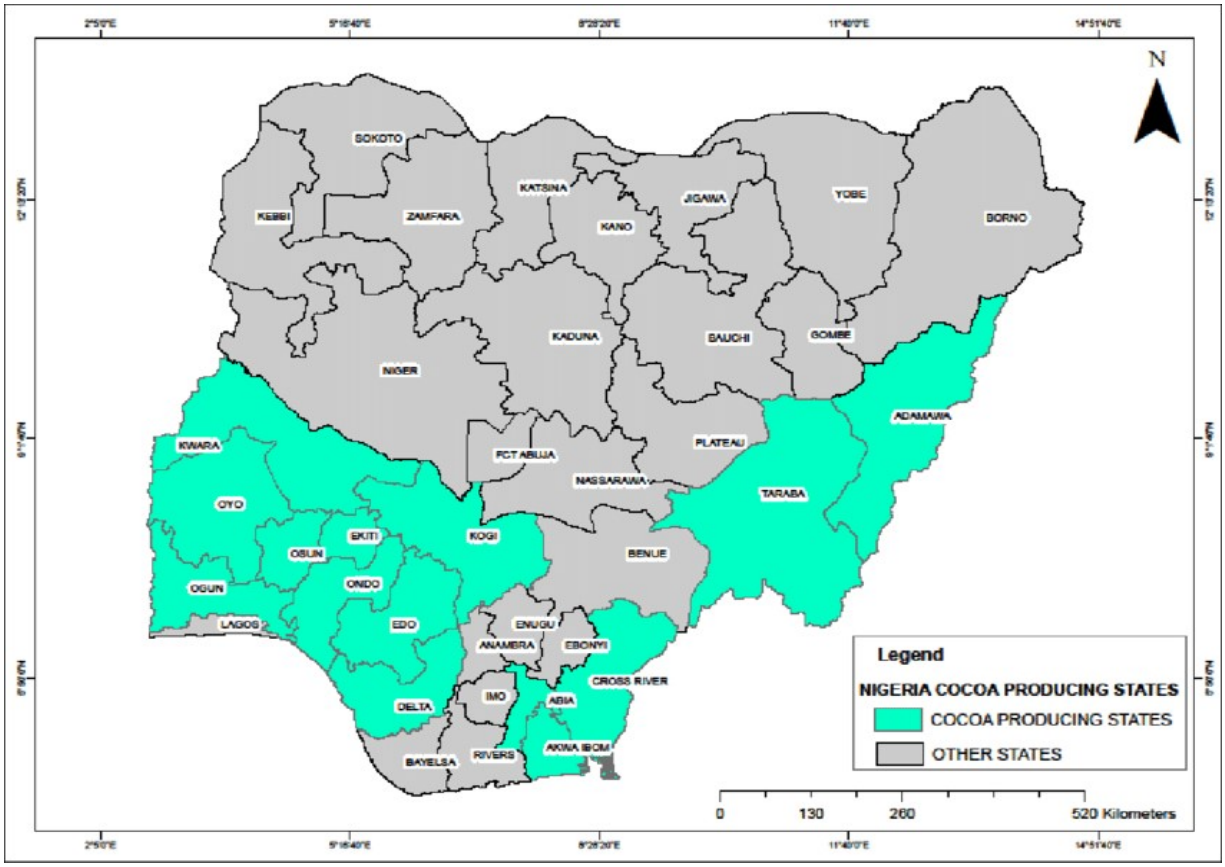


Figure 2.1: Cocoa Producing States in Nigeria
Source: Afolayan *et al.* (2016)

There are many pathogens of cocoa (*Theobroma cacao* L.) affecting its production in which *Phytophthora* species are notably to have greater damage on the crop (Agbeniyi *et al.*, 2014). *Phytophthora palmivora* affects the crop globally while *Phytophthora megakarya* is limited to West Africa but accounts for severe losses much greater than other species of the same genus. The disease causing pathogens of cacao can be classified into those that have spread with the seeds or seedlings of the crop from its centre-of-origin in the Amazon region. In addition, others must have been transferred from plants in locations to which cacao has been established (Opoku *et al.,* 2002). However, a study of the genetic diversity of isolates collected from different regions around the world suggests that at least some of the distribution of *P. palmivora* on cocoa outside its centre of origin has been clonal, which suggests that it has spread with its host (Akrofi *et al*., 2003).

2.3 *Phytophthora* Species Diagnosis from Infected Pods in Africa

Phytophthora is an oomycete genus (Stramenopilous fungi) that exists worldwide and has been responsible for serious crop diseases, industrial forestry and natural ecosystems degradation (Drenth and Barbara, 2001). Two species of *Phytophthora* causing *Phytophthora* pod rot of cocoa were reportedly common in all the growing regions of West and central Africa (Maseko *et al.*, 2007). Despite the high probability of the presence of numerous undescribed species of *Phytophthora*, Africa has recorded low diversity on *Phytophthora* pod rot species diversity (Bailey *et al.*, 2016). Yet, comprehensive studies focusing on the diversity of species of *Phytophthora*, particularly in native ecosystems, are very significant for effective management of the pathogens. This is as highlighted by Akrofi *et al.* (2015). Boughalleb *et al.* (2006) established that most of the global history of the genus of *Phytophthora* still remains in occurrence and spread of *Phytophthora* species from other African countries. Thus, suggesting that African countries could as well be the source of some species of *Phytophthora* affecting other crops (Akrofi *et al.* (2015).

Kolawole (2016) studied *Phytophthora megakarya* Brasier and Griffin's population genomics in Nigeria. All the isolates from infected samples were found to be pathogenic in the test pods. According to the study, it was established that the entire population collected is of type A1 mating. None of them was of type A2.

Ali *et al*. (2016) found out that accurate diagnosis of the organisms that cause *Phytophthora* pod rot of cacao and identifying the factors that enable the pathogen to cause the disease are all important in the development of environmental management strategies. The study developed a basic species recognition approach focused on Polymerase Chain Reaction (PCR) while the predominance of *Phytophthora megakarya* over *Phytophthora palmivora* in Ghana was established.

Appiah *et al.* (2003) carried out their studies in West Africa and discovered the distinct types of *Phytophthora* species causing invasion and inducement of seed of *Theobroma cacao* in the region. *P.palmivora* was isolated from infected pods of cacao and classified as separate species by three morphological forms namely MF-1, MF-3and MF4. Thus, established that *P. palmivora* was simply and exclusively MF-1.

Akrofi *et al.* (2015) demonstrated that variety of cocoa and environment are necessary for successful implementation of disease control. It was observed that *Phytophthora* species are present as isolated from both seemingly non-host and cocoa.

Agbeniyi *et al.* (2014) conducted their studies in Nigeria's main cocoa-growing regions and revalidated the use of historically ignored biological control used to contain the disease. The highest percentage of *Phytophthora* pod rot disease was reported in non-spraying experiment. Thus, confirming the effectiveness of biological control method for PPR of cocoa.

Park *et al.* (2013) focused on the *Phytophthora* Database (PD 2.0) online community tool, which was built to facilitate correct and rapid detection of *Phytophthora* so as to better identify and index variation and evolutionary ties within the genus. Thus, gave the most recently identified and tentative organisms the key characteristics to assist users and address the future direction of the *Phytophthora* Database.

2.4 *Phytophthora megakarya* Distribution in West Africa

P. megakarya pathogen tends to be limited to West Africa and endemic in Nigeria, where the causal pathogen of *Phytophthora* pod rot predominate in the growing region as the most common disease of cocoa (Maseko *et al.*, 2007). This confirms the Akrofi (2015)

study that *P. megakarya* Brasier and Griffin (1979) is one of the recognised oomycetous pathogens of cocoa and most virulent.

P. megakarya was first identified as the causative pathogen of PPR of cacao disease in 1979 after the reclassification of the genus *Phytophthora* retrieved from contaminated cocoa tissues (Appiah *et al.,* 2003). The pathogen is responsible for pod rot disease also known as *Phytophthora* pod rot is present only in West and Central Africa. *Phytophthora megakarya* has advanced west from Cameroon to Nigeria and Togo, the main cocoa-producing regions of Ghana and Côte d'Ivoire, and south to Gabon and Equatorial Guinea (Akrofi *et al.,* 2015; Ali *et al.,* 2016).

Phytophthora megakarya has become the biggest limiting factor in the production of cocoa in the sub-region, easily surpassing *P. palmivora* (Ali *et al.,* 2016). The potential danger of *P. megakarya* on cocoa is of great concern to cocoa farmers and scientists, while the factors that triggered the presence of *P. megakarya* on cocoa need to be considered. There is therefore the need for basic knowledge on the nature and epidemiology of *P. megakarya* to establish efficient and reliable control methods (Abad *et al.,* 2012).

Akrofi (2015) presented evidence of participation of a number of invertebrates, the tent-building ant species, including *Crematogaster striatula, Camponotus acvapimensis* and *Pheidole megacephala*. These insects were considered to contribute to the spread of pathogen through their tunnels from the base of the tree crop to the top within the cocoa tree. Wind scattered spores or droplets of water and flying insects and other fauna are potential agents for the spread of *Phytophthora* pod rot (Opoku *et al.*, 2007). In West Africa, zoospores were discovered above infected pods, suggesting the dispersion of some spores by the wind (Opoku *et al.,* 2007).

2.4.1 Detection and Survey of Symptoms of Black Pod Disease

The infection sign and symptoms are noticed to express the brown colour to black spreading spot on the pod which eventually cover the entire pod (Opeke, 2003). This is advanced by white bloom of the fungal mycelia and sporangia on the infected pod. These further invade the seeds and the entire pod is destroyed.

Awuah and Frimpong (2007) discovered *Phytophthora* pathogens in soils of rhizosphere, roots, stem bases, undetached cotyledons, testas, branches, and from those seedlings ' seeds. Similar results were reported on naturally contaminated plants by (Abad *et al.,* 2012). The fungus had not been detected in the farmer's uninoculated seedlings or in seedlings. It was assumed that *Phytophthora* is difficult to spread effectively from cocoa seed to leaves of seedlings.

2.5 Evolutionary Grouping of *Phytophthora* species

Phytophthora is a member of Kingdom of Stramenopila, Phylum Oomycota, Oomycetes and Peronosporales Table 2.1 Drenth and Barbara, 2001). The class Oomycetes consists of four orders, the Saprolegniales and the Peronosporales, two of which contain important plant pathogens. The other two orders contain small classes of basically marine fungal-like species namely Lagenidiales and Leptomitales. Within the Peronosporales, the Pythiaceae family includes a number of genera, the best known being *Phytophthora* and its sister group, Pythium, a genus of about 120 species (Drenth and Barbara, 2001).

Further groupings confirmed by Drenth and Barbara (2001) are as highlighted in Table 2.2. This gave a distinct group, sporangia shape and *Phytophthora species*. *P. capsici*; P. *palmivora*; *P. megakarya* and *P. citrophthora* were found to be in the same group. Susbsequent studies by Ristaino *et al*. (2012) further confirmed the grouping and in addition, that both *P. palmivora* and *P. megakarya* are of the same characteristic genome grouped in clade 4. They exhibit different morphological and molecular characteristics.

Table 2.1: Classification of the Kingdom Stramenopila

Kingdom	Class	Order	Family	Genus
Stramenopila	Oomycetes	Lagenidiales		
		Leptomitales		
		Saprolegniales	Saprolegniaceae	
Achlya				
Saprolegnia			Peronosporales	Pythiaceae
				Pythium
				Phytophthora
		Peronosporaceae	*Bremia*	
		Peronospora		
			Albuginaceae	*Albugo*

Source: Drenth and Barbara, (2001).

Table 2. 2: Classification of *Phytophthora* into Six groups by Waterhouse (1963)

Group	Sporangia	Antheridial attachment	Examples
I	Papillate	Paragynous	*P. cactorum*, *P. clandestine*
II	Papillate	Amphigynous	*P. capsici*, *P. palmivora* *P. megakarya, P. citrophthora*
III	semi-papillate	Paragynous	*P. inflata*, *P. multivesiculata*
IV	semi-papillate	Amphigynous	*P. infestans*, *P. ilicis*
V	Non-papillate	Paragynous	*P. megasperma*, *P. sojae*
VI	Non-papillate	Amphigynous	*P. cinnamomi*, *P. drechsleri*

Source: Drenth and Barbara, (2001).

2.6 Biology of Oomycetes

The Oomycetes share with fungi many aspects of ecology and life history due essentially to their filamentous growth habit. However, modern molecular and biochemical analyses as well asmorphological features suggest that oomycetes share little taxonomic affinity with filamentous fungi but are more closely related to brown algae (phylum Phaeophyta) in the kingdom Protista (Ristaino *et al*. (2012).

The cell walls of oomycetes are composed mainly of glucans and cellulose and, unlike fungal cell walls, contain little or no chitin. The zoospores display two flagella, with an ultra-structure similar to that of the flagella of the motile spores of heterokont algae. The oomycetes also contain the energy storage chemical mycolaminarin, a molecule that is also found in kelps and diatoms. However, their genetics and reproductive mechanisms clearly distinguish them from the Basidiomycetes and Ascomycetes (Drenth and Barbara, 2001). Their role in the Chromista Kingdom is confirmed by a wide range development of mobile heterokont zoospores, and lifecycle diploid predominance. Others are aerially scattered, especially as caducous (deciduous) sporangia such as *P. palmivora* (Drenth and Barbara, 2001).

2.7 *Phytophthora* Media Growth Resources and inhibitors for separation

Most *Phytophthora* organisms grow very gradually. Therefore, bacterial communities must be kept low because they can inhibit *Phytophthora'*s development by direct competition, antibiotic antagonism, or direct parasitism (Rotimi and Ikotun, 2017). Such problems are usually solved by the use of targeted press. Inhibitors are added to the isolating media to stop the bacterial growth (Ristaino *et al.* (2016).

2.8 Media and antibiotics for isolation of *Phytophthora* from diseased plant tissue and soil

The Oomycetes are not true fungi and therefore special techniques are required for their isolation (Drenth and Barbara, 2001). Most species of *Phytophthora* grow rather slowly *in*

vitro compared with saprophytic fungi and bacteria. In addition, bacterial populations need to be kept low because they may suppress the growth of *Phytophthora* by direct competition, by antagonism caused by antibiotic production, or by direct parasitism. The use of selective media usually overcomes these problems. Antibiotics are added to isolation media in order to suppress the growth of bacteria (Rotimi and Ikotun, 2017). Also, because *Phytophthora* spp. are out-competed by many fungi, it is desirable to choose media which are "weak" in nutritional terms. This reduces the growth rate of fungal contaminants, allowing colonies of *Phytophthora* to become established. Synthetic cornmeal agar Difco is the most frequently used basic medium for isolation of *Phytophthora* from infected plant tissue (Nwaogu *et al.,* 2015).

However, other desirable basal media include: water agar, or 2% or 4% (v/v) V8 juice agar. Suitable antibiotics that are effective against bacteria include streptomycin, ampicillin, penicillin, rifampicin, and vancomycin, alone or in combination. Suitable antibiotics with antifungal activity include nystatin and pimaricin. Nystatin is usually less inexpensive and more readily available than pimaricin (Nwaogu *et al.,* 2015).

Nwaogu *et al.* (2015) showed the effect of seven non-synthetic media assessed on the growth response of the three fungal propagules (*P. megakarya, C. ignotum, F. decemcelluare*) isolated from cocoa pods was tested *in vitro*. It was indicated that V8 extract medium, potato/carrot medium, potato extract medium and pear extract medium significantly supported the mycelial growth of the test fungi. While, pear extracts and V8 local extracts were comparable with the Potato control extract. Listed among the tested media for isolation of *Phytophthora* species from diseased tissue include; V8 juice, Diluted V8 juice agar, Cornmeal agar, Water agar, 3-P, 3-P 10VP, 4-P 10-ARP and Hymexazol-amended media (Drenth and Barbara, 2001; Rotimi and Ikotun, 2017).

2.9 Molecular Tools for Detection of Plant Pathogenic fungi

Plant pathogenic fungi are the causal agents of the most detrimental diseases in plants, including economically important crops, provoking considerable yield losses worldwide.

Fungal pathogens can infect a wide range of plant species or be restricted to one or few host species (Appiah *et al.* (2003). Some of them are obligate parasites requiring the presence of the living host to grow and reproduce, but most of them are saprophytic and can survive without the presence of the living plant, in the soil, water or air (Capote *et al*., 2012). Isolates of a fungal species can be differentiated by morphological characteristics, host range (*formae speciales*), pathogenic aggressiveness (pathotypes or races) or their ability to form stable vegetative heterokaryons by fusion between genetically different strains (belonging to the same vegetative compatibility group, VCG) (Capote *et al*., 2012).

The **s**tarting material for molecular analysis is important for scientific diagnosis of Phytophthora pod rot of cocoa causal organism and other plant pathogenic fungi. When the deep internal tissues of the fungal pathogen have been infected, it may be necessary to cut the plant material and extract the core tissues. Simple fungal colonies must be collected by isolating individual or group hyphae tips (Gezahegn *et al.*, 2017).

2.9.1 DNA Extraction Methods for Fungi

Nucleic acid recovery methods for bacteria, contaminated plant material or soil are not widely accepted. To maintain an effective and reproducible process for extracting DNA from plants and soil, several established protocols are available. This is followed closely by purification of nucleic acids (Gezahegn *et al.,* 2017). This is followed by the selection of target DNA for replication of the causal organism.

Typically, retained recognized genes with appropriate sequence variance are chosen for the design and phylogenetic analysis of PCR diagnostic assays. The most common area used for these purposes was the ribosome RNA expression region which is done through molecular recognition of plant pathogenic fungi (Duran *et al.,* 2010).

2.9.2 Polymerase Chain Reaction (PCR)

High sensitivity, accuracy and reliability are main advantages of PCR techniques (Gezahegn *et al.*, 2017). This is considered to be most important in molecular diagnosis of the causal organism. Specific genes widely involved in diagnosis, closely related pathogens frequently vary in one or a few base pairs. The capacity to distinguish in any

clinical trial must be identified. The DNA nucleotide sequence, a PNS technique was developed to identify and distinguish between isolates (Kroon *et al.,* 2004). This is achieved through single nucleotide polymorphisms (SNPs).

2.9.3 Design of Primers and Probes

The first phase, fit blastn device sequences by utilizing GenBank EMBL-DDBJ registry sequences (Duran *et al.,* 2010). PCR methods are focused on the use of specific oligonucleotides or primers that hybridize directly with the target DNA and are required to start a new DNA chain synthesis. Numerous specific oligonucleotides are used in many real-time PCR processes called probes that hybridize with target DNA between the two primers. The architecture of primers and probes is important for PCR to be accurate and efficient. The primary specificity depends on its quantity, length and consistency of GC, which defines its melting temperature (Tm, the temperature at which 50% of the primary target duplex is hybridized) (Gezahegn *et al.*, 2017).

The National Biotechnology Information Center (NCBI) (http:/www.ncbi.nlm.nih.gov / Genbank/) (Bethesda, MD, USA) contains partial or complete nucleotide sequences of many fungal genes.

2.9.4 Sequencing of Fungal Species

As discussed above, it is not always sufficient to identify a pathogen with morphological characteristics. One of the most direct approaches to this is the PCR amplification of the target gene with universal primers, followed by sequencing and comparison with the available database (Kang *et al.,* 2010).

This then will promote the use of knowledge in *Phytophthora* diversity, similarity of pathogens and the pathogenicity between communities of *Phytophthora* in southwestern Nigeria which then called for in this research.

2.10 Control of Diseases Caused by *Phytophthora* Species

2.10.1 Prevention and Control of *Phytophthora megakarya*

There are three major ways of prevention and control of *P. megakarya.* These include: cultural methods, chemical control and disease resistance. At present, prevention and control relies upon cultural methods (Akrofi *et al.* (2015). Cultural Control of *Phytophthora megakarya* is carried out from selection of appropriate site for the crop establishment to the management of the cacao while fruiting from one season to the other. The target is to make sure that possible means of infection are reduced to the barest minimum. Inoculum levels of *P. megakarya* are rapidly reduced in the absence of the host, and cocoa is the only known host. This affords opportunities to limit the spread through ensuring that disease-free nursery material is planted when clonal material is used for propagation. Spread can also be restricted by surface disinfestation of harvesting implements before moving from one tree to another (Evans, 2012). Improved control is also obtained by avoiding bare earth (thus reducing spore splash) within the plantation and ant tunnel. Management of the amount of light entering the canopy is also critical, to ensure improved aeration and to promote the drying of the pod surface (Opeke, 2003).

Secondly, the use of chemical control of *Phytophthora megakarya* is common in all the cocoa growing regions of Africa.
Agbeniyi and Oni (2014) highlighted that copper-based fungicides are the only pesticides currently registered for use on cocoa for controlling *Phytophthora* pod rot in Nigeria. The other fungicides tested also reduced infection but to a lesser extent when compared with the performance of Ridomil Gold.

Thorold (2008) considered the supplementary methods of controlling black-pod disease (*Phytophthora palmivora*) of cocoa in Nigeria, because fungicide application is not always economical. It was observed that a satisfactory control of black-pod with copper fungicides can be obtained when applied with either hand-sprayers or a power-sprayer. It was recommended that spraying should be practised when the potential yield is twelve or more pods per tree.

Aikpokpodion *et al.* (2012) obtained results which revealed that all the studied cocoa plantations are contaminated with copper while the rest heavy metals are most likely to be

from natural sources. This is due to the fact that to combat the ravages caused by *Phytophtora megakarya* and *P. palmivora*, application of copper- based fungicide is generally the most reliable and popular with Nigerian farmers. The PLI (Pollution load index) values obtained confirmed that the quality of the cocoa soils studied is deteriorating and this may have severe impact on soil biodiversity and ground water.

2.10.2 Host-Plant Resistance of *Phytophthora* species

The use of Host Plant Resistant Varieties (HPRV) was successful in the management of fungal diseases of some crops with minimal environmental effect. This must have informed the production of resistant varieties to diseases in crop production through effective exploitation of breeding programmes.

Nyadanu *et al.* (2012) studied on the role of epicuticular wax on pod and leaf surfaces in the host plant resistance to *Phytophthora* Pod Rot in Cacao (*Theobroma cacao* L.). The results showed that cacao varieties that contain more wax were highly impervious to *Phytophthora* infection compared with those of low wax. This study established that epicuticular wax layer affords cacao pod greater protection against PPR.

2.10.3 Biocontrol and Natural Plant Extracts

Bolanle, 2017, highlighted that medicinal plant metabolites and plant developed pesticides seems to be among the best options because of the little or no environmental effects and eco-friendly attributes compare to synthetic chemical fungicides. Antifungal strength of the 3 medicinal plants was screened and showed effective potentiality to inhibit the growth of *Phytophtora megakarya*.

Nyasse *et al.* (2007) observed that among the methods making up integrated disease management (IDM), the creation of resistant cultivars has been identified as a priority in cocoa breeding research programmes. while the potential use of the leaf disc test as breeding tool and its impact on the genetic improvement of black pod resistance is of importance.

2.10.4 Selecting and Breeding for Resistance

Barreto *et al.* (2015) showed that the most efficient way to control black pod disease is to use resistant crop varieties. The study verified that resistance to the various species that cause black pod disease in cocoa is associated with genetic variability.

Iwaro *et al.* (2005) Evaluated and identified promising resistant genotypes that could be exploited in cacao breeding programmes. This further compared the levels of resistance to *Phytophthora* pod rot among two cacao types (wild and cultivated types), three major groups (Forastero, Trinitario and Refractario) and 11 accession groups. The distribution of scores showed high skewness towards the susceptibility to *Phytophthora* pod rot and low in resistance.

Elisa *et al.* (2011) studied and characterised the gradient of resistance to black pod disease experimentally. The parameters; time interval, number of leaves per genotype and distribution of experimental treatments were evaluated. The resistance gradient observed indicates variability enough for mapping and cocoa breeding in the population.

Diny *et al.* (2015) highlighted that Indonesia was the third largest cocoa-producing country in the world and that limited access of the farmers to diverse planting materials or the strong preference for large pods and large bean size by local farmers, may have affected the selection outcome. Diverse sources of resistance, harbored in different cacao germplasm groups, need to be effectively incorporated to broaden the on-farm diversity and ensure sustainable cacao production in Sulawesi.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Research Locations

The field research was conducted on selected cocoa plantations in the south-western states of Nigeria and laboratory screening at the Cocoa Research Institute of Nigeria (CRIN), Ibadan. (CRIN), Idi Ayunre is on Longitude 3.53^{o}E and Latitude 7.22^{o}N in southwestern Nigeria. Laboratory studies were carried out in the Crop Protection and Environmental Biology (CPEB) Plant Pathology Laboratory, University of Ibadan. The molecular analysis was conducted by the Bioscience Department of the International Institute of Tropical Agriculture (IITA), Ibadan.

3.2 Sample Collection

The research work was accomplished by mapping out the major local governments in each of the states and selections were made before setting out for collection of samples. Thereafter, a systematic and geo-referenced survey was conducted during the rainy season Between July and August when the humidity was high (70% -80%) across the cocoa-growing zone in Southwestern Nigeria so as to identify the *Phytophthora* pod rot disease prevalent areas. Group and individual contacts were made with farmers through the Agricultural Development Programme (ADP) personnels in all the local governments that were covered and CRIN staff to elicit useful information about PPR disease from the farmers.

The study was conducted on 45 smallholder cacao plantations (0.5 – 3.5 ha) in five states which were major cacao production areas in Southwestern Nigeria (Table 3.1). The states were Ekiti, Ogun, Ondo, Oyo and Osun states. Three local governments each were selected per state based on the production of cacao.

3.3 GPS Coordinates and Area Measurement for Sample Collection

The Geographical Positional System (GPS) instrument Garmin Etrex 20 GPS Device was used to estimate locations of the coverage areas where infected cocoa pods were collected. This was accomplished actively using satellite signals in orbit. See the appendices 1 and 2 for details.

Infected cocoa pods were collected from farm locations in all the States within the Southwestern region of Nigeria. These were aseptically packed at 2-infected pods per bag inside polythene bags at 80% humidity, labeled and transported to Cocoa Research Institute of Nigeria for disease diagnosis, isolation, culturing, identification and further pathogenicity tests using Koch's postulate.

Infected pods but not yet mummified were collected and immediately transported to CRIN laboratory (Mycology Department) for symptom expression and morphological assessment of the sample pods infected with *Phytophthora* pod rot disease. The collected pods which were selected among the naturally infected pods in fields/plantations were surface sterilized with cotton swab dipped in 70% ethanol to remove secondary invaders and contaminants

Table 3.1: Geographical Coordinates and Area Measurements of the Five States and Local Governments for the Study in Southwestern Nigeria Between 2014-2017.

State	City/Town	Local Govt.	Field	Plot	Area (m^2)		GPS
EKITI	Aramoko	Ekiti west	EK1	1	1,225.0	Longitude	**5.03E**
						Latitude	**7.43N**
	Ikere	Ikere	EK2	2	1,345.5	Longitude	**5.16E**
						Latitude	**7.40N**
	Ilawe	Ekiti South – west	EK3	3	2,558.7	Longitude	**5.05E**
						Latitude	**7.35N**
OGUN	Owode	Obafemi Owode	OG1	4	1,225.0	Longitude	**3.41E**
						Latitude	**7.14N**
	Odeda	Odeda	OG2	5	1,135.8	Longitude	**3.54E**
						Latitude	**7.04N**
	Mamu	Ijebu North	OG3	7	2,455.8	Longitude	**3.51E**
						Latitude	**7.22N**
ONDO	Idanre	Idanre	ON1	3	1,345.5	Longitude	**4.55E**
						Latitude	**7.05N**
	Owena	Akure South	ON2	4	1,235.7	Longitude	**5.08E**
						Latitude	**7.14N**
	Wasimi	Ondo East	ON3	5	2,558.7	Longitude	**5.01E**
						Latitude	**7.10N**
OSUN	Ile-Ife	Ife Central	OS1	2	5,675.7	Longitude	**4.30E**
						Latitude	**7.32N**
	Gbongan	Ayedaade	OS2	3	2,558.7	Longitude	**4.10E**
						Latitude	**7.20N**
	Ilesa	Atakunmosa	OS3	5	2,455.8	Longitude	**4.37E**
						Latitude	**7.34N**
OYO	Akanran	Ona-Ara	OY1	3	2,445.8	Longitude	**4.06E**
						Latitude	**7.17N**
	Arulogun	Akinyele	OY2	4	1,225.0	Longitude	**4.00E**
						Latitude	**7.35N**
	Idi-Ayunre	Oluyole	OY3	1	9,000.8	Longitude	**3.53E**
						Latitude	**7.22N**

3.4 Apparatus and Materials

Laboratory materials used during the research work include among others (a) Hardware including: autoclave, conical flask, water bath, spirit lamp, scalpel, Petri dishes, aluminum foil, cotton wool, weighing balance, inoculating loops, masking tape, incubator, oven, blender, McCartney bottles, toothpicks / inoculating needles, filter sheet, electronic microscope, slides and cover slips, spatula. while (b) the chemicals includes: 70% ethanol, Streptomycin, Carrot Agar, Potato Dextrose Agar, V8 juice Agar, fructose, sodium hypochlorite, lacto-phenol blue dye, etc.

3.5 Sterilisation of Glassware and other Equipments

All glassware were washed with teepol liquid detergent dissolve in water at 10gm per 500ml, rinsed with tap water and air-dried. Glass Petri dishes were arranged inside canisters. The cannisters were arranged inside an oven (Model Gallenkamp Hotbox oven, Gallenkamp, UK). Sterile Petri dishes were used within two days of sterilisation to avoid recontamination.

3.6 Preparation of Potato Dextrose Agar

Thirty-nine grams of Potato Dextrose Agar (PDA) was weighed into 1000 ml Erlenmeyer flask and distilled water was added to make 1 litre. The PDA suspension was heated to melt the agar which was dispensed into four 500 ml Erlenmeyer flask conical flask in 250 ml amounts and corked with non-absorbent cotton wool. The tops of the conical flasks were wrapped with aluminum foil to prevent wetting the cotton wool. The PDA medium was autoclaved for 15 minutes at a pressure of 1.05 kg/1cm^2 (121^{o}C). The prepared agar medium was allowed to cool to 45^{o}C and 15 ml dispensed into each glass sterile Petri dish in a sterile inoculation chamber. Meanwhile, Two milliliters (2 ml) of streptomycin was added to each Petri dish medium to suppress bacterial growth as contaminant before it solidified. The Petri dishes were then left in the inoculating chamber to let the agar cool to 42^{o}C and solidify.

Where the prepared medium was not used immediately or exhausted after preparation, it was stored in a refrigerator at 4^{o}C until required. When required, Erlenmeyer flasks

containing the sterile medium were autoclaved for 15 minutes at a pressure of 1.05 kg/1cm^2 (121^{o}C) (Rotimi and Ikotun, 2017).

3.6.1 Preparation of Carrot Agar and V8 juice Agar

Two hundred grams of carrot were washed with distilled water, cut and weighed into 2000 ml Erlenmeyer flask and added 1 liter of distilled water. This was cooked to paste and 15 g of Dextrose and 15 g of agar was applied and then dispensed with non-absorbent cotton wool in 250 ml amounts into four 500 ml Erlenmeyer flask conical flask. At a pressure of 1.05 kg/1cm2 (121^{o}C), the Carrot Dextrose Agar (CDA) medium was autoclaved for 15 minutes.

In a sterile inoculation chamber, the prepared agar medium was allowed to cool down to 45^o and 15 ml dispensed into each glass sterile Petri dish. Then the Petri dishes were kept in the inoculating chamber to allow the agar to cool to 42^{o}C for pouring into Petri dishes. For V8 Agar, (Vegetable Juice Agar / V8 (VJA 8) for each medium (made from 200 g V8 juice, 15 g Agar and 1liter sterile distilled water) prepared and sterilisation was done as obtained for Carrot Agar. as a routine procedure. When required, Erlenmeyer flasks containing the sterile medium were autoclaved for 15 minutes at a pressure of 1.05 kg/1cm^2 (121^{o}C). While the same routine procedures were followed for V8 juice Agar (Rotimi and Ikotun, 2017).

3.7 Isolation of Pathogen from infected Cocoa Pod Samples

Phytophthora species isolation was performed using Carrot Dextrose agar and V8 juice Agar for growth, morphological and possible classification of morphological diversity among the isolates from the five states being studied.

Symptomatic or infected cocoa pod was sliced in 0.5 x 0.5 cm sizes, the areas taken were those between the healthy and infected parts. The sliced piece was dipped into 70% ethanol inside Petri dishes, and then rinsed with sterile distilled water for 30 seconds, twice in a row. It was then wind dried at room temperature (30^{o}C) and placed in a Petri dish that already contained filter paper moistened with sterile distilled water. This was

incubated at 25^{O}C for 3-5 days. The growth of the microorganism was observed in terms of colony morphology, color of the cell, hyphae, and spores conidia. Pure cultures of the different isolates collected were stored at 4^{o}C to 0^{o}C in the laboratory refrigerator until required.

3.8 Samples Collection from Various Growing Sites in Southwestern Nigeria

Seventy-five samples were obtained from selected sampling sites in Nigeria's southwest region; these were screened on the pods for infection by *Phytophthora.* Pods harvested were about a month before their usual harvest age at the semi-matured stage (Abad *et al.* (2012). They were taken to the laboratory and in three consecutive cycles of sterile distilled water, the undamaged stable pods were rinsed (Drenth and Barbara, 2001). The pieces of cut tissue from the infected cocoa pods from each location were then checked for the presence of the pathogen using the internal infestation direct positioning technique (Pitt and Hocking, 1997)

Cocoa pods were surface sterilised in 2.5 percent $NaOCl_3$ and then rinsed in five changes of sterile distilled water. In order to suppress bacterial contamination, samples are placed directly on the surface of Carrot Agar and V8 Agar media modified with 2mI / L lactic acid. The agar media of PDA and V8 juice, which had been autoclaved for 15 minutes at 121^{o}C before being taken to a sterile laminar flow hood, were allowed to cool to 42^{o}C before inoculation. The culture media were then kept at 22^{o}C for 3-7 days (Opoku *et al.,* 2004) after which fungal growths were observed.

3.9 Pure Culture Production from Isolates

The final stage in identifying fungal pathogens is the creation of pure cultures. Only a single

spore or hyphal tip was transferred to ensure a pure culture is produced while the organism growth on the media was given a close watch to produce pure culture. The marked plates were opened aseptically to avoid contamination. Each of the fungal isolates was gently picked up using an inoculating needle and gently placed on the prepared

solidified agar and incubated at 22^{o}C. This was consistently done for all the isolates for their molecular diagnosis.

3.10 Preparation and Storage of Stock Cultures

Carrot agar was used for stock cultures. The Carrot agar was prepared inside graduated Erlenmeyer flask was transferred into sterile McCartney bottles. These bottles were tightly closed and sterilised using the autoclave at 121^{o}C for 15 minutes. After sterilisation were placed in a slanting position and were then allowed to cool and solidify. The McCartney bottles were labeled and preserved in the laboratory in a refrigerator at 4^{o}C until required.

3.11 Morphological identification of fungal species

Morphological characteristics of 45 isolates from the states were determined using Mycelial Growth (MG), Colony Pattern (ClP), Sporangial Shape (SS), Sporangial Pedicel Length-SPL to Sporangial Breadth-SB (SPL:SB) were used for CP identification. Slides were prepared and examined under the digital microscope for each isolate using lacto-phenol cotton blue stain. Isolates were classified on the basis of its mycelial morphology, growth and mycelial production.

3.12 Experiment 1: Occurrence and Distribution of PPR in the Southwestern Nigeria

3.12.1 Experiment 1a: Identification of the Cacao Main Growing Local Governments and the States in Southwestern Nigeria

This study was carried out through contact with Cocoa Research Institute of Nigeria (CRIN), Agricultural Development Programme officers and mapping out of the targetted area.

During the survey of black pod prevalence/sample collection exercise, the location of each cocoa growing region from which samples are collected was noted and the logistic movement schematically represented. Following pathogen isolation and characterization, each *Phytophthora* species was matched with the corresponding isolate collection site,

from which a pictorial representation was illustrated on a map to enhance the study of the distribution of the different *Phytophthora* species existing in Southwestern Nigeria.

3.12.2 Experiment 1b: Survey for *Phytophthora* Pod Rot Identification in Southwestern Nigeria.

This was done to identify the symptom, occurrence and incidence of PPR in Southwestern Nigeria cacao plantations, the tropical rain forest that was considered during July to September each year for the study between 2014-2017. When the relative humidity was above 70%.

Locations among the states surveyed for the presence of *PPR* in southwestern Nigeria (Table 3.1) were as follows:

Ekiti: Ilawe; Ikere; Aramoko/ Ekiti West

Ogun: Obafemi-Owode; Odeda; Mamu

Ondo: Idanre; Owena /CRIN; Wasimi/Ondo East

Osun: Ile- Ife/ Ife Central LGA; Gbongan /Ayedaade LGA; Ilesa/Ikoromaja village-Osu LGA

Oyo: Ona-Ara/ Akanran; Akinyele; CRIN farm- Oluyole

Samples were collected from nine plantations in each of the five states, bringing the total number of locations to 45. Samplings were conducted during the months of July through September, which the usual wet (Rainy months) season of the calendar year in order to maximize the chances of detecting PPR positive samples. Both symptomatic and asymptomatic pods were collected from all the locations surveyed. They were properly labelled and collected into sealed sterilized plastic buckets.

A Geo-Positioning System (GPS) device was used in each study site to get the exact locations of each plantation (Table 3.1) (Gezahegn *et al*. 2017). This was used to develop a PPR distribution and population map in Southwestern Nigeria.

3.13. Experiment 2: Morphological Variability Study

3.13.1 Experiment on Morphological Characteristics of the Isolates of PPR from Southwestern Nigeria

Morphological characteristics of the isolates were determined by the use of digital microscopic examination of the three most reliable and fairly consistent distinguishing features of the different species of *Phytophthora* isolates namely sporangial pedicel length, colony pattern and radial growth rate. Each of these features was studied in depth and measured between July-August annually between 2014-2017 using the methods of Rotimi and Ikotun (2017).

Variations within both growth rate and colony pattern were used as yardsticks to determine some level of intra-specific variations as described by Ristaino *et al.* (2012). Pedicel (stalk) length of each sporangium, which is regarded as the most stable character as suggested by Kaosiri *et al.* (1980) from different isolates were examined and used for determining morphological variation in the isolates include colony margin, sporangial length to breadth ratio, and shape.

3.14 Experiment 3: Molecular Diagnosis of *Phytophthora* Pod Rot Isolates from Southwestern Nigeria

Under the two specific subheadings below, the inter-specific and intra-specific variations within *Phytophthora* isolates were considered through molecular diagnosis of their pure culture.

3.14.1 Molecular Diagnosis for Inter-specific Variation in PPR Isolates from Southwestern Nigeria

(i) **DNA extraction:** This process, which relies on the identification of unique patterns or sequences found in DNA. This was used to elucidate variations in the numerous *Phytophthora* isolates in southwestern Nigeria.

(ii) **Polymerase chain reaction (PCR):** PCR was performed in a small plastic pipe containing all the biochemicals required for the synthesis of new DNAs. When amplification was introduced, sequences specific to each species of *Phytophthora* were used to delineate the various isolates as defined by Duncan and Cooke (2002) between two specially designed "primers." (Gezahegn *et al.,* 2017).

3.15 Experiment 4: Screening PPR Resistance on six Varieties of CRIN Genotypes

3.15.1 Experimental Location

The study was conducted at the Nigerian Cocoa Research Institute (CRIN), Idi Ayunre is on Longitude 3.53^{o}E and Latitude 7.22^{o}N in southwestern Nigeria. The plots from which the breeders at the Research Institute picked the six genotypes of cocoa were used in this study.

3.15.2 Cocoa Genotypes from CRIN

The six cocoa varieties from CRIN were used to reflect the characteristics of the genotype as: resistant, moderately resistant and susceptible genotypes stated by Nyadanu *et al.* (2009). The hybrids were global replication trials sponsored by Nigeria's assessment of the CFC / ICCO / Biodiversity campaign. The genotypes are: TC-1=CRIN TC-1; TC-3=CRIN TC-3; TC-4=CRINTC-4; TC-55=CRINTC-5; TC-6=CRIN TC-6; TC-7=CRINTC-7 and N38 was the local varieties used in the study as control.

3.15.3 Isolation of *Phytophthora* Pathogens from PPR-Infected Pods

Isolation of *Phytophthora* species was done from naturally infected pods collected from the cocoa plantations in Southwestern Nigeria. The infected pods were surface sterilised with 70% ethanol and washed thrice in sterile distilled water. The sterilized diseased portion of pods with slightly uninfected parts was cut into 5 mm pieces. The segments were then plated on V8 juice Agar. All the inoculated plates were kept on the laboratory bench for 3 days at a temperature of 25^{o}C-27^{o}C to observe the fungal growth from tissue segments daily.

To obtain pure culture of the isolated fungi, fungal growths from tissue segments were transferred onto another V8 agar medium. The *Phytophthora* species are identified by looking at emerging colonies under a light microscope. The isolates were classified based on the infected pod's characteristic odour, growth on V8 agar medium, sporangial shape and size, and pedicel weight. The isolates were grown on V8 agar medium, and a

zoospore suspension was made by inundating each culture plate (9 cm diameter) with 10 mL sterile distilled water after a ten-day culture.

The spore suspension was then chilled for 25 minutes at 25°C before being moved to a 22°C incubator for another 25 minutes. Regular inoculation of green mature cocoa pods accompanied by re-isolation on V8 agar medium preserved the isolate's pathogenicity in the laboratory.

3.15.4 Pods Inoculation and Infection Lesions Evaluation

Multiple-point inoculation method was used to determine pod tolerance at the penetration stage. On the pod surface, a multiple point inoculation was conducted in which 10 µL drops of inoculum were applied with a micropipette at 3 points along the ridges.To stop the fusion of neighbouring lesions, a gap of about 3 cm was preserved between inoculated sites. A concentration of 2.0x105 zoospore per mL was used. In specific inoculations carried out by Tahi *et al.,* (2007), this concentration of inocula was stated to be optimum. For control experiment, sterile distilled water was used to inoculate a pod of each variety instead of zoospore suspension.

The six varieties were replicated three times with adequate controls and organised in a randomised complete block design. The pods were incubated at 25°C in a 40x60 cm transparent polyethene bag. With the mouth of the bag closed, a beaker of water was held in the pocket. After 6 days, the amount of lesions produced in the pods was measured. Penetration resistance was measured by the number of lesions on the pods created. The experiment was carried out twice more.

3.15.5 Statistical Analysis

The number of lesions and their sizes were subjected to ANOVA using Genstat 10.0 technology, and residual plots were tested to ensure that the data was standard. For the analysis of correlation and regression, MINITAB's statistical method was used. Using the Duncan Multiple Range Test (DMRT), the mean genotypic variance was calculated at α0.05 (Iwaro et al., 1997).

3.16 Screening Test for Resistance to *Phytophthora* Pod Rot on six varieties from CRIN

3.16.1 Rapid Screening Method for *Phytophthora* Pod Rot Resistance by Detached Pod Inoculation of six CRIN Cocoa Genotypes

Spray inoculation of different unwound pods provides an opportunity to quickly check the susceptibility of pods to *Phytophthora* infection. This offers susceptibility information based on the extent and scale of lesions in the penetration and post-penetration phases of infection. The technique is particularly useful in the evaluation of germplasm collections where direct inoculation of attached pods is limited (Tahi *et al.*, 2007).

3.16.2 Collection and Preparation of Pods for Inoculation

Fully grown of four to five months old, unripe pods of the same size were collected between 7.00 and 10.00 a.m () from CRIN Headquarters in Ibadan breeder plots. These were carefully collected and stored in sterile labeled plastic bags. Two pods were kept in one bag to prevent damage to the surface that could occur when many pods are kept in close contact with each other. At the CRIN laboratory, harvested pods were rinsed in sterile distilled water twice and filled with wet paper towels in plastic trays. This was then filled as a lid with another inverted tray and sealed to maintain 80% humidity in a polythene bag. A temperature 25^{o}C was maintain for 12 hours / night until the inoculation was carried out.

3.16.3 Sample Size

The hybrids pod and leaf samples used in the experiment were selected through the supervision of breeders at the CRIN station and as two pods were taken in each of two trials. Two pods were collected for test in each of the trials being; TC-1, TC-3, TC-4, TC-5, TC-6, and TC-7. Meanwhile, mature pods were taken from the variety N38 used in the experiment as control. The design used in the experiment was completely randomized.

3.16.4 Inoculum Preparation

Moderately aggressive *Phytophthora megakarya* isolates were used. Isolate was grown on a 20% V8 juice-calcium carbonate substrate and from a 10-day crop, zoospore suspension

was obtained by inoculating each pod (9 cm in diameter) with 10 mL of sterile distilled water (cooled to 10^{o}C) and cooled for 25 minutes (5^{o}C) to incubate for 30 minutes in the dark at 25^{o}C.The suspension zoospore density was measured using a modified with haemocytometer to 1.0x105 ml-1 (Nyasse *et al.,* 2007).

3.16.5 Inoculation of Pods

In order to obtain a dry surface at room temperature, prepared pods were uncovered for inoculation. The inocula quantity was kept on the pod surface. Too much spray on the pod surface to direct against run-off was avoided. Inoculated pods were lined with moist paper towels in the plastic trays, covered as a lid with another inverted tray and shut in a polythene bag. At 25^{o}C, the covered trays containing the inoculated pods were incubated for four days.

3.16.6 Evaluation of Pod Response to Inoculation

Inoculated pods were evaluated for their reaction to inoculation after 4 days of incubation. The assessment was focused on the occurrence and scale of the lesions that were created. Infection severity has been rated on an 8-point scale with the following as highlighted in Table 3.2.

Table 3.2: Assessment Scale for 3-7 Days Pod Infection Severity on Detached Pod Inoculation of six CRIN Cocoa Genotypes

Disease rating	Infection level	Susceptibility classification
1	No symptom	Highly resistant to penetration
2	1-5 localised lesions	Resistant
3	6-15 localised lesions	Moderately resistant
4	>15 localised lesions	Partly resistant/resistant to spread of lesion alone
5	1-5 expanding lesions	Partially resistant/resistant to penetration alone
6	6-15 expanding lesions	Moderately susceptible
7	>15 expanding lesions	Susceptible
8	Coalesced lesions	Highly susceptible

Source: Tahi *et al.*, 2007.

3.16.8 Data Analysis

The collected data were subjected to analysis of variance in order to evaluate significant differences between the checked accessions.

3.17 Leaf Disc Inoculation Test (LDT)

In the preparation and inoculation of the leaf discs, the leaf flushes were carefully selected from the cocoa genotypes. During the development of young bud split flushes in the area, the age of leaves for each procedure was established. Leaves considered for the experiment were those of similar age (young leaves) that were not exposed to sunlight and void of insect infestation. Thirty-1.5 cm diameter leaf disks are made with a cork borer of each leaf and replicated three times. Leaf discs were placed on wet plastic foam in three trayswith an abaxial layer upwards.

Cocoa genotyping discs were arranged randomly in groups of 7x10=70 discs per tray. At room temperature (27°C), the discs were inoculated and incubated in plastic trays and coated in the laboratory covered with tray to protect it from sunlight while observations were made.

Observed fungal growth were recorded 7 days after inoculation on a scale of 0-5 points depending on the size of the necrosis (0: absence of symptoms, 1: very small necrotic spots, 2: larger number and size of necrotic spots, 3: medium sized brown spots, 4: broad circular brown lesions and 5: very large brown lesions, sometimes stretched out of the inoculous region (Tahi *et al.*(2007).

3.17.1 Statistical Analysis

Data were evaluated using GenStat ® 11th version of variance (ANOVA) (GenStat, 2008). The F-test was used to determine the significance of genotyping differences for each phenotype. When the ANOVA revealed substantial genotypic differences, the Least Significant Difference (LSD) at.0.05 was used to assess the significance of the differences between the genotypic measures.

CHAPTER FOUR

4.0 RESULTS

4.1 The Incidence of *Phytophthora* Pod Rot of Cacao in Southwestern Agro Ecological Zone of Nigeria.

Figure 4.1 shows the area of 15 local governments and 45 cocoa plantations around Ekiti, Ogun, Ondo, Oyo and Osun states all of which are mainly cocoa producing areas in Nigeria. The survey showed that cocoa was successfully grown between Longitude 3.43 - 5.0^{o}E and Latitude 7.00-7.43^{o}N, which comes between 10^{o}E and 10^{o}N of the Equator for cultivation of the crop. The outcome of this research confirmed the existence of *Phytophthora megakarya* in the area through the initial sampling and analysis of specific sites from 2014-2017 where samples were collected.

All surveyed local government areas and all cacao plantation clusters visited had the infection (Table 4.1). The compilation of samples further confirmed the fact that the crop is still considered lucrative, taking into account the area covered by the farm land and the development of new plantations. It was also observed during verbal interactions that most farmers across the state are getting older (±60years) and polygamous although inadequacies in good farming practices introduced in some of the state-wide plantations were the major causes of substantial or total loss to the region's *Phytophthora* pod rot.

There was no *Phytophthora* black pod disease-free plantation during the survey, but there was a 10%-20% percentage infection in most of the surveyed plantations. The attack on the disease was easily recognized. It has been discovered that most cacao growers have less than one hectare thus creating a cluster of plantations of 3-5 plots each with clear boundaries per site. This would have encouraged the seasonal and constant dissemination of *Phytophthora* black pod disease in places where, given their epidemiology, circumstances are conducive to the causal agent.

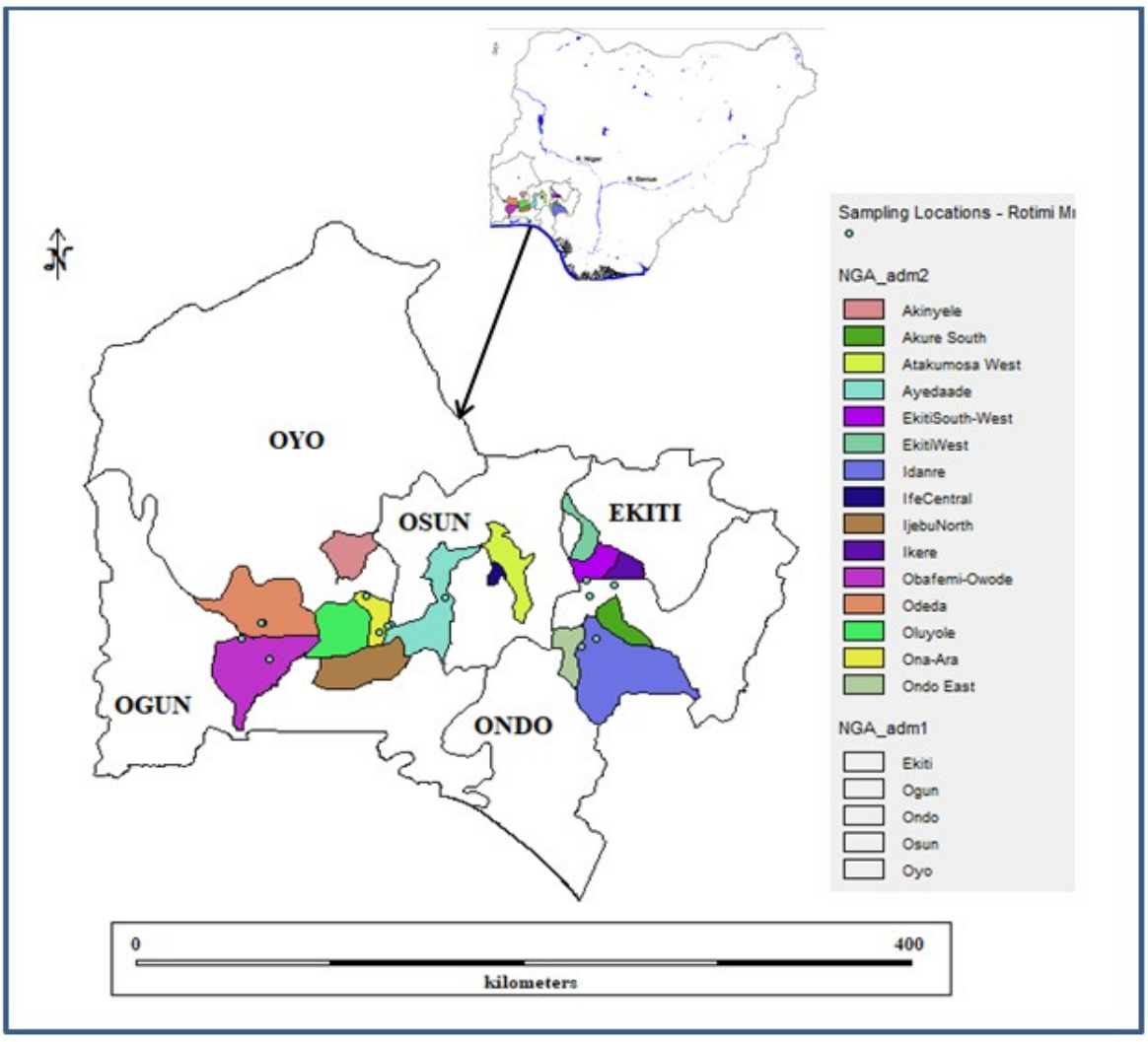


Figure 4.1: Five States in Southwestern Nigeria that are major producers of cocoa (*Theobroma cacao*) and the fifteen local governments where study was carried out across the States.

4.2: Symptomatic and Asymptomatic Comparison of Matured Cocoa Pod from Growing Region in Southwestern States of Nigeria

Plate 4.1P shows the healthy cocoa pods on the tree, the heap of infected pods Plate 4.1Q and the infection spots on pods with *Phytophthora megakarya* as indicated on Plate 4.1R. It provides a clear description of the propagation of the fungus after the inoculum may have found its way to the cell. Plates 4.1Q and 4.1R contrasted the typical symptom of the causal organism on infected pods and other tree crop sections well. The expansion of lesions on the pods from point of infection as seen in Plate 4.1Q and R indicates the coalescing of lesions with excessive sporangial development.

Infection can start at different parts of the pod or tree crop depending on the pathogen's entry point. Plate 4.1R provides the starting points with the distribution patterns on each of the infected pods with PPR on the tree crop from the distal end of the pod, proximal and lateral though canker lesions during the investigation were not visible.

Infection growth on pods that are unique to *Phythophthora megakarya* was reported as shown in Plate 4.1R above indicating distal and lateral infection, pod filled with sporangial to further assess the similarity of the causal organism's infection symptoms. This was developed by collecting samples from all the selected cacao plantations that provided the same symptom of infection on all infected pods.

4.3 Colony Morphology of *Phytophthora megakarya* on Growth Media.

This was observed from day 3 to day 7 after the infected tissue was removed from the Carrot agar medium. On Carrot agar, all *P. megakarya* isolates examined produced cottony mycelial patterns of finely radiating growth. When grown on V8 agar, there was slight variations in growth rates and trends among isolates. On V8, all isolates developed most abundantly. On V8, all *P. megakarya* isolates from across-the-board cocoa samples developed identical thick hyphal rosettes that were very close to those produced by *P. megakarya* isolates.

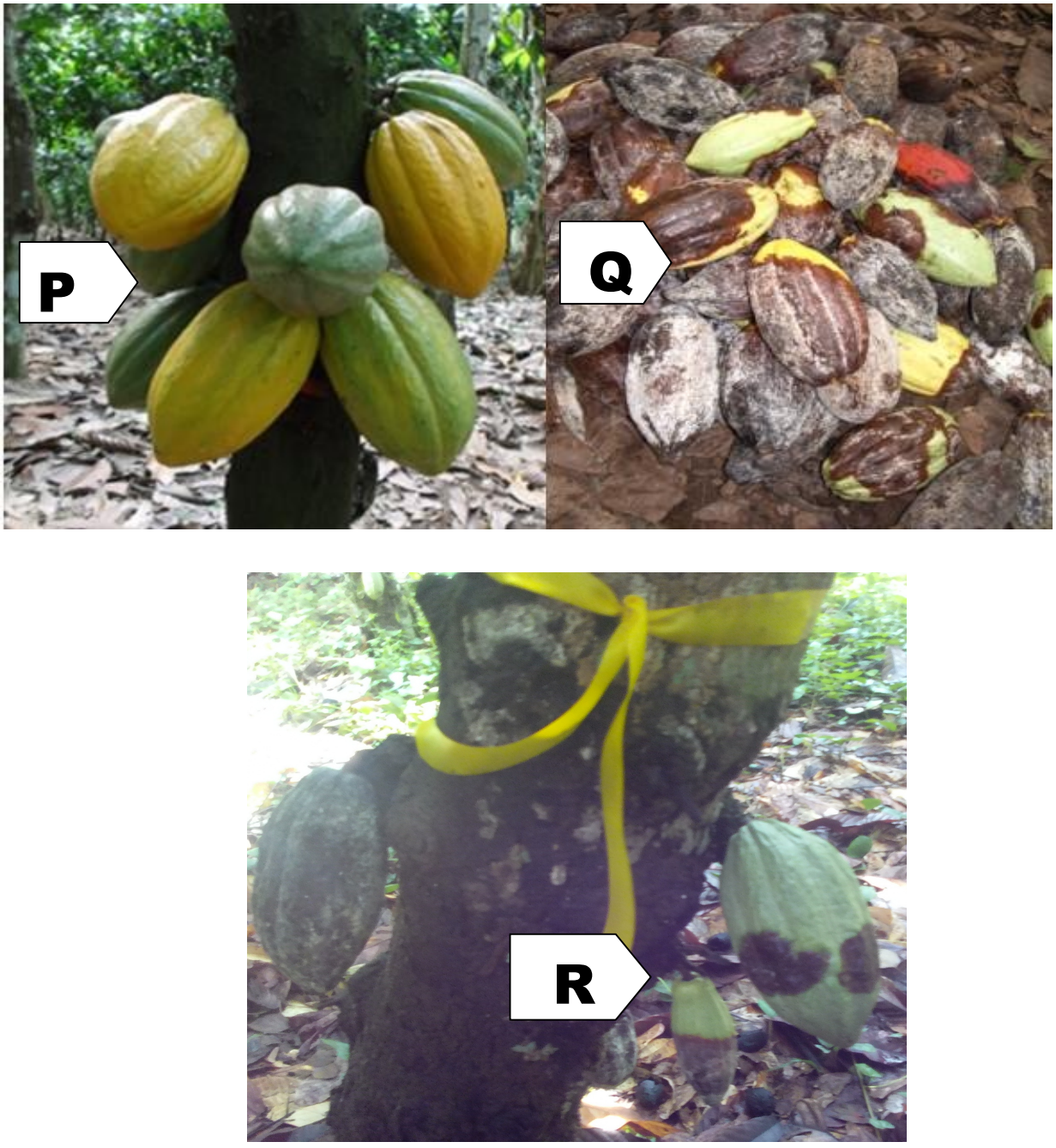


Plate 4.1: **(P) Healthy ; (Q-R) infected cocoa (*Theobroma cacao* L.) during sample collection in Southwestern Nigeria 2016. Q-R.** Infected samples from Southwestern Nigeria, 2016).

4.4 Colony Growth Rates of *Phytophthora megakarya* on Media

P. megakarya cultivation on V8 and Carrot agar media showed a growth of 10mm at 22^{o}C while isolates from infected cocoa pods growth on media was retarded at 25^{o}C and 18^{o}C. The growth of isolates on both the Carrot agar and V8 juice on the 3rd day was observed to be 5 mm, on the 5th day 7 mm and on the 7th day 10 mm respectively.

4.5 Colony Morphology of *Phytophthora megakarya* Isolates on Media

P. megakarya developed very well after seven days on V8 and Carrot agar media with strong sporulation (Plate 4.2a, b, c, d, e, f). Pure isolates from the five States were classified as: (a) 4.2a comprising of Ab2= Ekiti; Ta2= Ogun and Ca2=Oyo consists of C3=Oyo pure culture on Carrot medium; (c) 4.2c consists of g1=Ogun and g2=Ogun consists of pure crops on Carrot agar; (d) 4.2d consists of g1=Ogun; Op2=Osun; C2=Ekiti and Ca3=Oyo consists of pure crops on Carrot agar medium respectively. It was shown that all isolates from the five southwestern states of Nigeria have similar media growth expressing a cotton appearance on Carrot and V8 agar with aerial mycelia and coralloid hyphae.

Plate 4.3 revealed the distinctive features of *P. megakarya* isolates on slides as seen under the optical x40 mm microscope. The hyphae (Plate 4.3I) was full of sporangia that was about to burst open for spores to be released. Slightly papillate and ellipsoid in form were the sporangial colony pattern (Plate 4.3J, 4.3K, 4.3L and 4.3M). All of them share common sporangial characteristics in Southwest Nigeria across the five States.

4.6 Oospore Production on Selected Medium

The use of V8 agar material, as described in Plate 4.3, created an excess of oospores that were identified through the optical compound microscope on slides. Hyphae with *P. megakarya* sporangial was shown in Plate 4.3I.Plate 4.3J, K, L, and M are the complexes of spores on the slide of the causal organism. Compared to Carrot agar and PDA, the production rate of oospores was very high in V8 agar media.

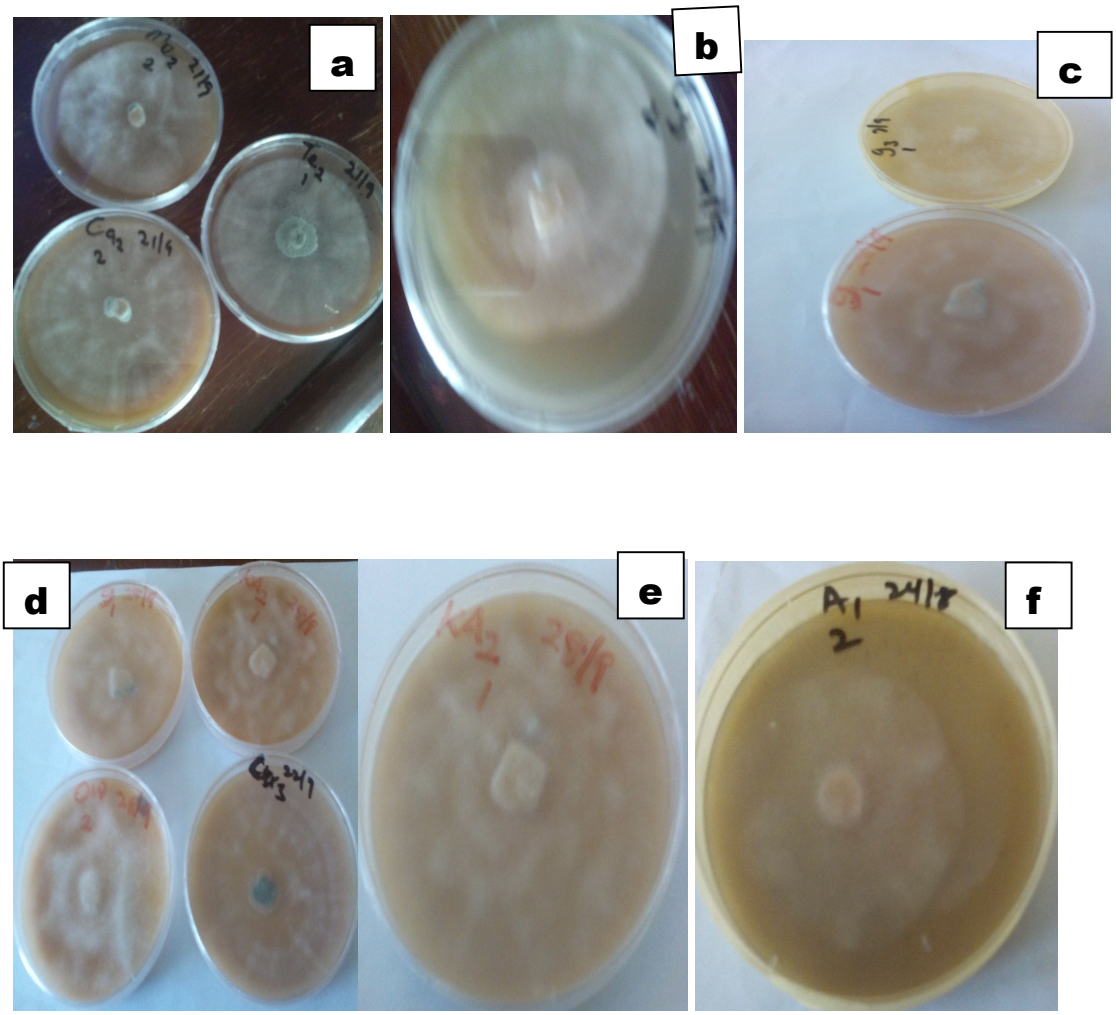


Plate 4.2: Colony morphology of *Phytophthora megakarya* isolate, 10 days growth at 20°C-25^{0}C on Carrot Agar and V8-Agar from infected matured fruits of cocoa plantations across the Western States of Nigeria: (a) 4.3a consists of Ab_2= Ekiti; Ta_2= Ogun and Ca_2=Oyo, (b) 4.3b consist of C_3=Oyo, (c) 4.3c consists of g_1=Ogun; g_2=Ogun, (d) 4.3d consists of g_1=Ogun; Op_2=Osun; C_2=Ekiti and Ca_3=Oyo, (e) 4.3e consisit of Ka_2 =Ondo and (f) 4.3f which is A_1=Ondo plate isolates.

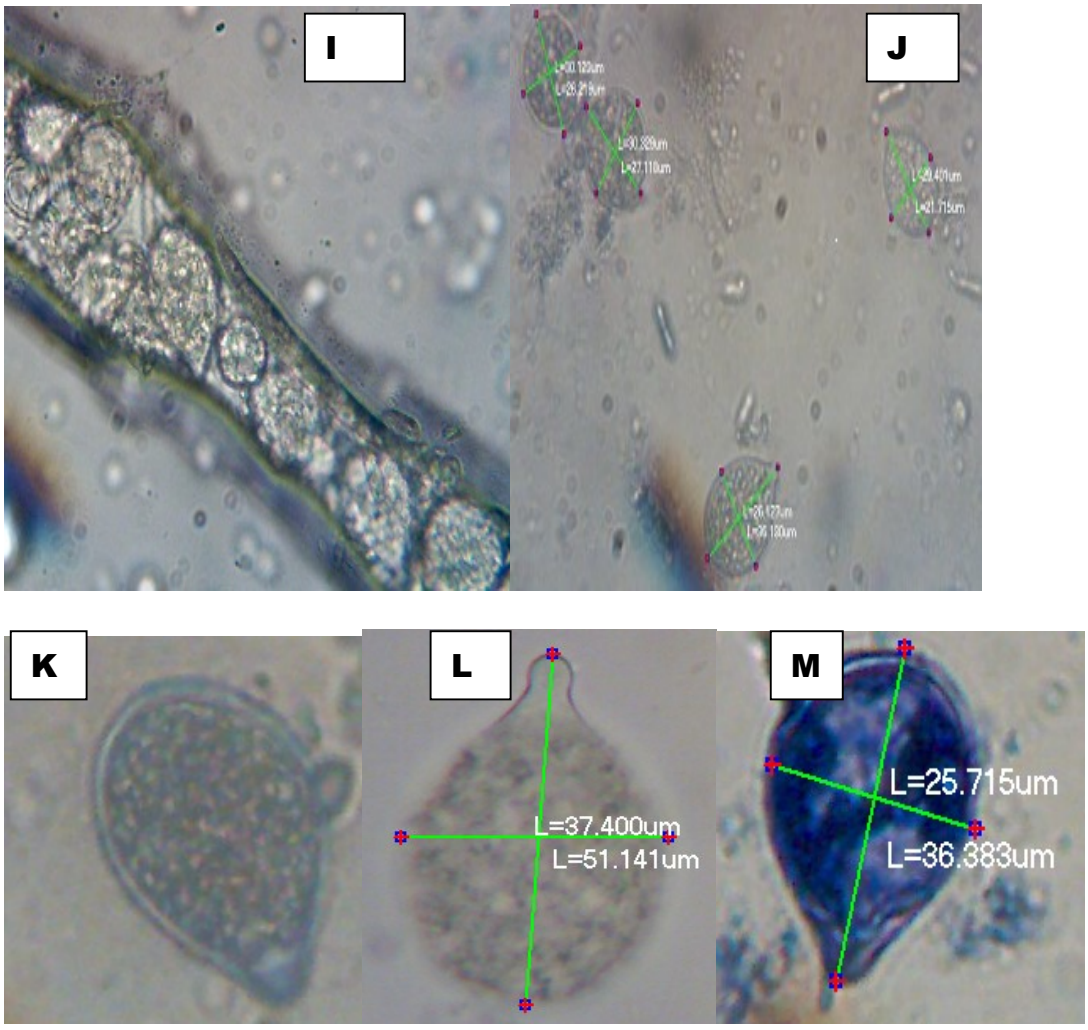


Plate 4.3: ***Phytophthora megakarya*** **Sporangial morphology through digital compound microscope. Structures were produced after 10 days of growth on V8 and Carrot agar.**

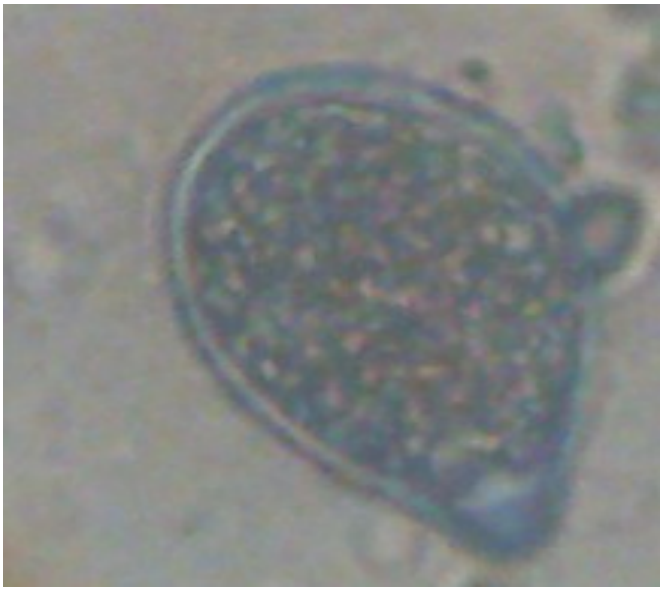
4.7 Characteristics of Colony Morphology of *Phytophthora megakaya* on Media

Plate 4.4 provides microscopic sporangial structural distinction as seen under the optical compound microscope of *Phytophthora megakarya*. Ellipsoid sporangium was observed in *P. megakaya* isolates. The feature was found in all the sample isolates obtained from Southwest Nigeria in all the five States.

4.8 Physiological and Morphological Characteristics of *Phytophthora megakarya*

The result of digital microscopic view of *P.megakarya* morphological characteristic pattern was shown in Table 4.1. The morphological growth pattern on V8 agar and Carrot agar was significant at $p \geq 0.05$. The colony pattern in all the states (slightly papillate) were similar while the mean length to breadth (l: b) was between 1.24-1.36. The mean pedicel length also fall within 4.01-5.13µm.The colony growth rate for the isolates ranges between 4.5mm day-^{1} and 6.2 mm day-^{1}. The sporangial shape was ellipsoid all through the States.

Table 4.2 showed the sporangial length (µm) in the five states where the study was carried out. The colony pattern across the five States was slightly papillate, sporangial shape was ellipsoid. While the mean l/b ratio was lowest in Ogun state isaolates with 1.2±0.13 and highest in isolates from Osun state with 1.36±0.09. The level of significance was much lower in sporangial breadth and pedicel length across the states. The result also showed that the character parameter within the states were not significantly different.



P. megakarya

Plate 4.4: ***Phytophthora megakarya*** sporangial morphology through digital compound microscope X 40mm.

Source: Pure culture isolate from Southwestern Nigeria, 2015.

Table 4.1: Morphological Properties of *Phytophthora megakarya* Isolates from the Five State Studied in Southwestern Nigeria.

State	*Colony pattern*	*Growth rate mm day^{-1}*	*Sporangial shape*	*Mean l/b ratio*	*Mean pedicel length*
Ekiti	Slightly papillate	5.5 ±0.8	Ellipsoid	1.26±0.18^{a}	5.13±0.89^{a}
Ogun	Slightly papillate	4.5±0.8	Ellipsoid	1.24±0.13^{a}	4.01±0.90^{b}
Ondo	Slightly papillate	5.8±0.5	Ellipsoid	1.25±0.10^{a}	4.37±0.70^{ab}
Osun	Slightly papillate	5.2±0.8	Ellipsoid	1.36±0.09^{a}	4.35±0.59^{ab}
Oyo	Slightly papillate	6.2±0.6	Ellipsoid	1.29±0.12^{a}	4.59±0.87^{ab}

Note: the mean ± SD across the column of various superscripts is significant at a 5% with a > ab > b. Mean differentiation achieved by the Duncan Multiple Range Test.

Table 4.2: Mean ± SD Sporangial Character and Variation of *Pyhtophthora megakarya* Disease Isolates Across the Cocoa (*Theobroma cacao* L.) Growing States of Southwestern Nigeria.

	MEAN ± STANDARD DEVIATION			
State	**Sporangial Length l (µm)**	**Sporangial Breath b (µm)**	**l:b ratio**	**Pedicel Lengthpl (µm)**
Ekiti	38.82±8.65[a]	30.8±5.35[a]	1.26±0.18[a]	5.13±0.89[a]
Ogun	29.97±7.89[a]	24.09±5.42[b]	1.24±0.13[a]	4.01±0.90[b]
Ondo	32.99±6.26[a]	26.21±4.21[ab]	1.25±0.10[a]	4.37±0.70[ab]
Osun	35.41±4.98[a]	26.12±3.57[ab]	1.36±0.09[a]	4.35±0.59[ab]
Oyo	35.58±8.23[a]	27.55±5.21[ab]	1.29±0.12[a]	4.59±0.87[ab]

Note: Mean ± SD across the column with different superscript is significant at 5% level with a>ab>b. Mean separation done by Duncan Multiple Range Test

4.9 Morphological Characteristics of Sporangial Features of Isolates from the Five Cocoa Growing States in Southwestern Nigeria.

Figure 4.2 shows that for sporangial / spore length and sporangial / breadth, the spore properties are significant at P>0.05. The mean sporangial / spore length (μm) suggested a slight difference that was the largest in the state of Ekiti, followed by those of Osun, Oyo, Ondo, and Ogun states respectively. The mean sporangia / spore length, from Ekiti state was the strongest preceded respectively by the state of Oyo, Osun, Ondo and Ogun. There was no significant difference in the state-wide ratio of length to width (l: b). likewise, there was no significant difference in pedicel length (μm) from all of the state-wide isolates.

Table 4.3: Descriptive Morphological Characteristics of Sporangia Across the Five Cocoa Growing States in Southwestern Nigeria.

	Description of statistics		
		Mean	Std. Deviation
Sporangial Length	30	34.5552	7.44120
Sporangial Breath	30	26.9544	5.00170
lb ratio	30	1.2803	.12744
Pedicel length	30	4.4924	.83362

Table 4.4: Morphological characteristics of *Phytophthora megakarya* on cocoa across the growing states in Southwestern Nigeria.

Description of Statistics				
State		N	Mean	Std. Deviation
Ekiti	Spore Length	6	38.82	8.65
	Spore Breath	6	30.8	5.35
	lb ratio	6	1.26	0.18
	Pedicel length	6	5.13	0.89
Ogun	Spore Length	6	29.97	7.89
	Spore Breath	6	24.09	5.42
	lb ratio	6	1.24	0.13
	Pedicel length	6	4.01	0.9
Ondo	Spore Length	6	32.99	6.26
	Spore Breath	6	26.21	4.21
	lb ratio	6	1.25	0.1
	Pedicel length	6	4.37	0.7
Osun	Spore Length	6	35.41	4.98
	Spore Breath	6	26.12	3.57
	lb ratio	6	1.36	0.09
	Pedicel length	6	4.35	0.59
Oyo	Spore Length	6	35.58	8.23
	Spore Breath	6	27.55	5.21
	lb ratio	6	1.29	0.12
	Pedicel length	6	4.59	0.87

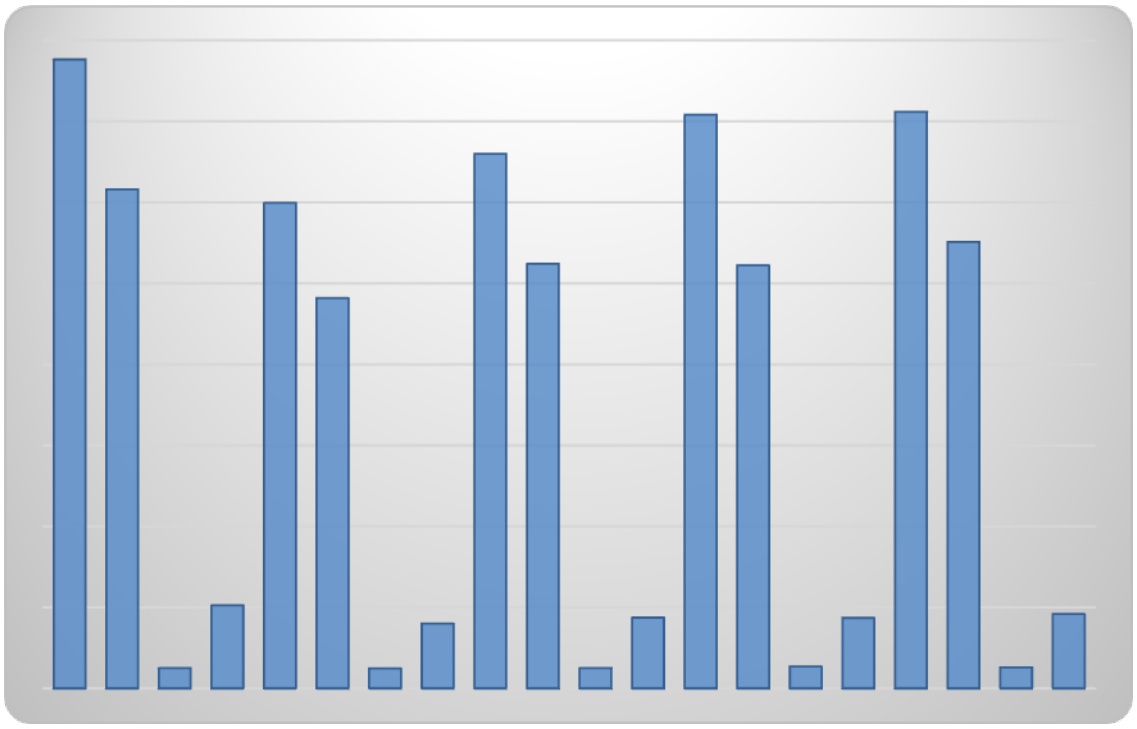


Figure 4.2: Morphological characteristics of sporangia features of isolates from the five cocoa (*Theobroma cacao* L.) growing states in Southwestern Nigeria viewed under digital microscope. X40mm.

4.10 DNA and PCR Analysis of Isolates from the Five Sstates of Nigeria

4.10.1 Integrity Test for Extracted DNA Samples with PCR Amplification

P.megakarya derived DNA has been amplified with ITS1 and ITS 4 main pairs. (Plates 4.5 and 4.6) *Phytophthora* oomycete, *P.megakarya* ITS 1 and ITS 4 PCR amplification yielded an estimated 500-bp and 550-bp sample respectively. The isolates developed a substance of the same size (Plate 4.6).

From this study, pure fungal cultures developed a similar peak as the photo gel of DNA and PCR (Plate 4.5 and Plate 4.6). This displays the genomes of the area similar to those shown in the plates. The test revealed consensus traces of the presumed fungal DNA. The Agarose gel electrophoresis of the internal spacer area base pair (bp) isolate products from five (5) states where the samples were obtained was shown in Plate 4.5. The PCR The extension of the ITS regions with the ITS1 and ITS4 primers culminated in an estimated 500 bp (Figure 4.2). The *Phytophthora megakarya* isolates collected from five Southwestern Nigerian states using nBLAST on Gene Bank was shown in Table 4.5. It was observed that all the isolates are pathogen linked to evolution. In this analysis, the number of nucleotides sequenced from isolates of *Phytophthora* differed. See details in Appendix 1.

Isolate' OY' had the highest number of sequenced nucleotides (849nt) while Osun ‘OS’ had the lowest number of nucleotides (761nt). Using BLAST on NCBI, all isolates had an identity of 99-100 percent with isolates in the sample. In addition, the highest possible query coverage (100 percent) for isolate' OG' was registered, providing a perfect match with Cameroon origin Accession number KR818206. For isolate' OY' at 96 percent coverage with isolate JX315261, also sequenced from Cameroon, the lowest percentage coverage was reported. See the details as presented in Appendix 2. Figure 4.3 indicates pairwise sequence alignment (PSA) showing structural and evolutionary relationship of molecular diagnosed samples’ pathogen between one state and the other. While Ekiti State samples’ pathogen compared well with that of Oyo and Ogun State which is applicable

when Oyo State samples PSA was done with Ekiti and Ogun State and Ogun State samples' pathogen PSA gave similar evolutionary relationship. There was pairwise sequence alignment between Ondo State samples and that of Osun state.

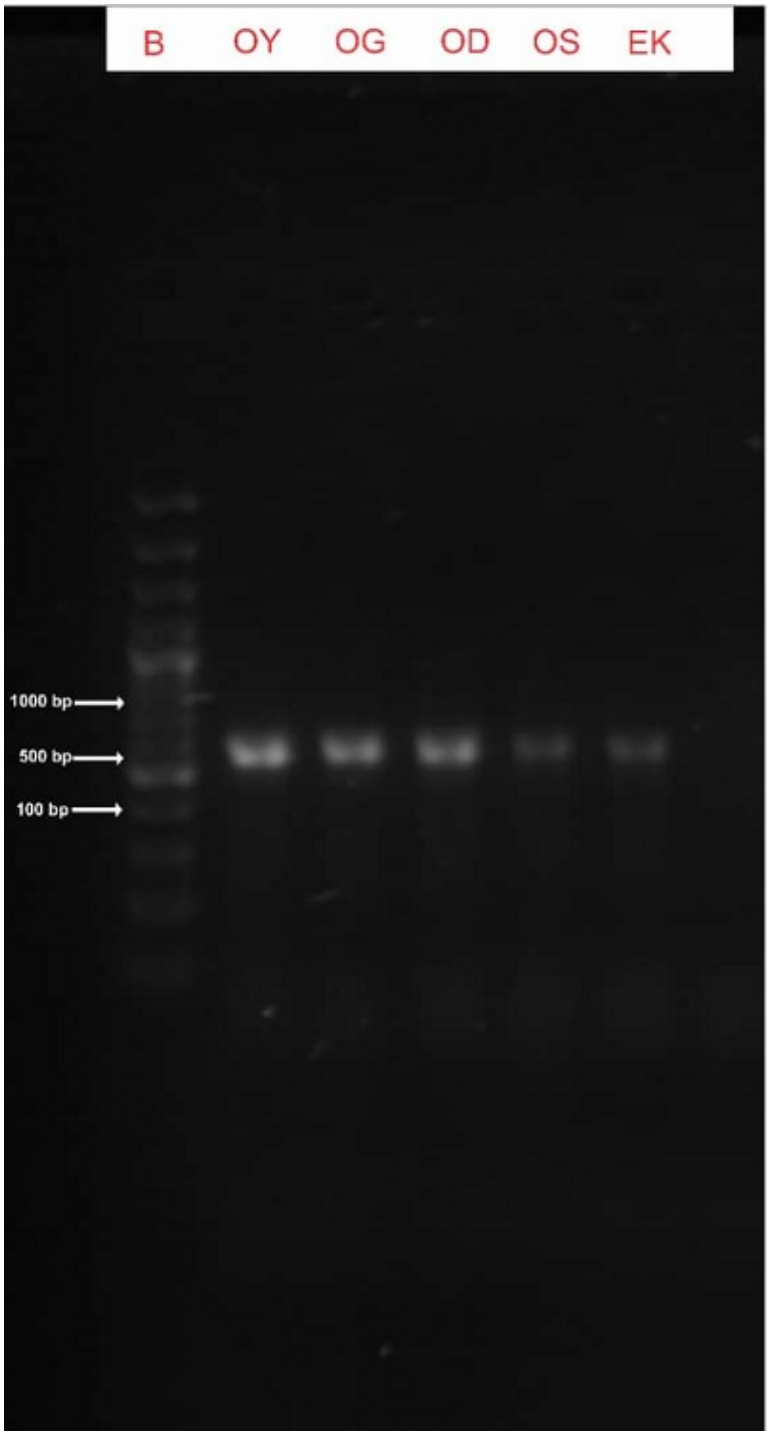


Plate 4.5: Extracted DNA of *P.megakarya* amplified with primer pairs ITS1 and ITS 4. An oomycete in the genus *Phytophthora,* PCR amplification of *P.megakarya* ITS1and ITS 4 yielded an estimated 550-bp product for all the states. OY (State); OS (Osun State), EK (Ekiti State); OD (Ondo State) and OG (Ogun State) B= Buffer respectively.

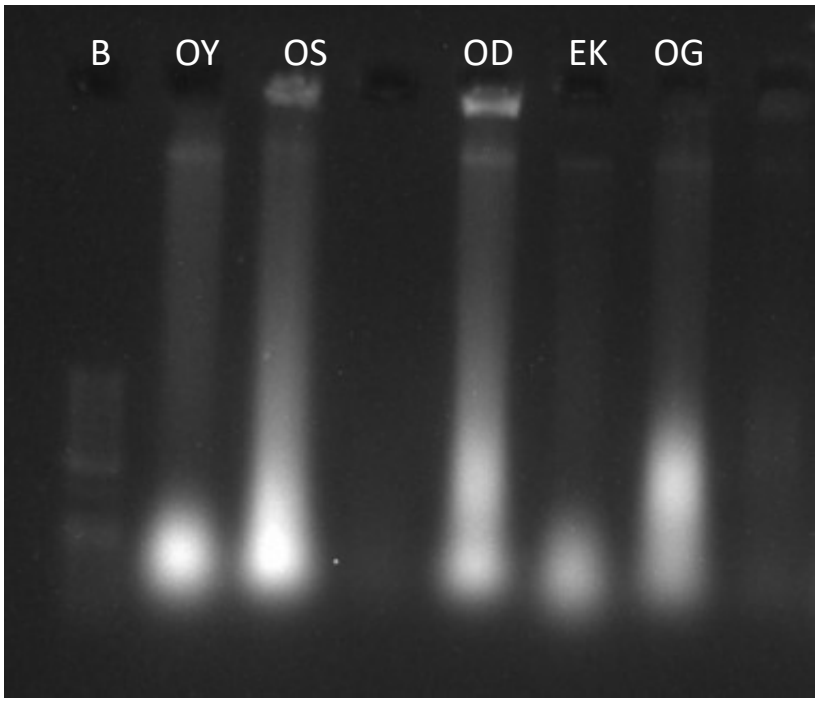


Plate 4.6: Integrity test for extracted DNA samples.

P.megakarya isolated DNA was amplified with ITS and ITS 4 primer pairs. OY (Oyo State); OS (Osun State); EK (Ekiti State); OD (Ondo State); and OG (Ogun State) respectively.

Table 4.5: Characteristics of Partial Internal Transcribed Spacer Region of *Phytophthora* five States in Southwestern Nigeria using nBLAST on Gene Bank

Isolate	Number of nucleotides	Highest nBLAST identity (%)	E value	Alignment score	Highest query coverage (%)
OY	849	99	0.0	> 200	96 (JX315261)
OG	765	99	0.0	> 200	100 (KR818206)
OD	777	100	0.0	> 200	99 (KR818142)
OS	761	99	0.0	> 200	99 (MG865534)
EK	781	99	0.0	> 200	99 (MH620121)

OY= Oyo state; OG= Ogun State, OD= Ondo State, OS= Osun State, EK= Ekiti State

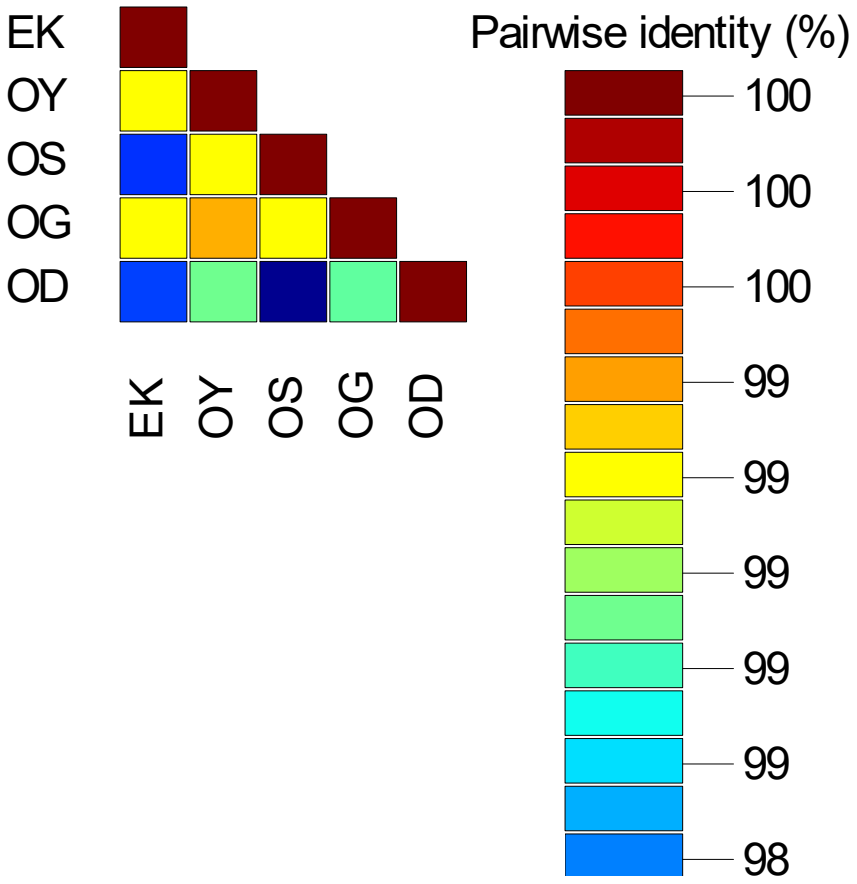


Figure 4.3: Pairwise sequence identities of partial internal transcribed spacer region from *Phytophthora megakarya* isolates obtained from five States in Southwestern Nigeria. OY= Oyo state; OG= Ogun State, OD= Ondo State, OS= Osun State, EK= Ekiti State

Figure 4.4 showed the phylogenetic tree available through the sequence of samples that were molecularly diagnosised. It highlighted the evolutionary relatedness of *P. megakarya* confirmed through the study and compared among the *T. cacao* growing nations in West Africa and Central Africa. The figure further indicates that *P. megakarya* was traced to *P. palmivora* evolutionally as the origin, thus establishing the possible genetical variation/ mutation that might cause the evolution and establishment of the pathogen. While *T. cacao* in Ghana and Nigeria were mostly affected by *P. megakarya* in West Africa, *T. cacao* grown in Cameroon and Togo were not left out from this same *P. megakarya* devastation. Samples from Ekiti, Oyo and Ogun States were found to be on the same lane or main trunk of the tree while Ondo and Osun were on sub lane or sub branch from the main trunk of the phylogenetic tree still farther from origin compared with Ekiti, Ondo and Ogun States as seen on the evolutionary tree.

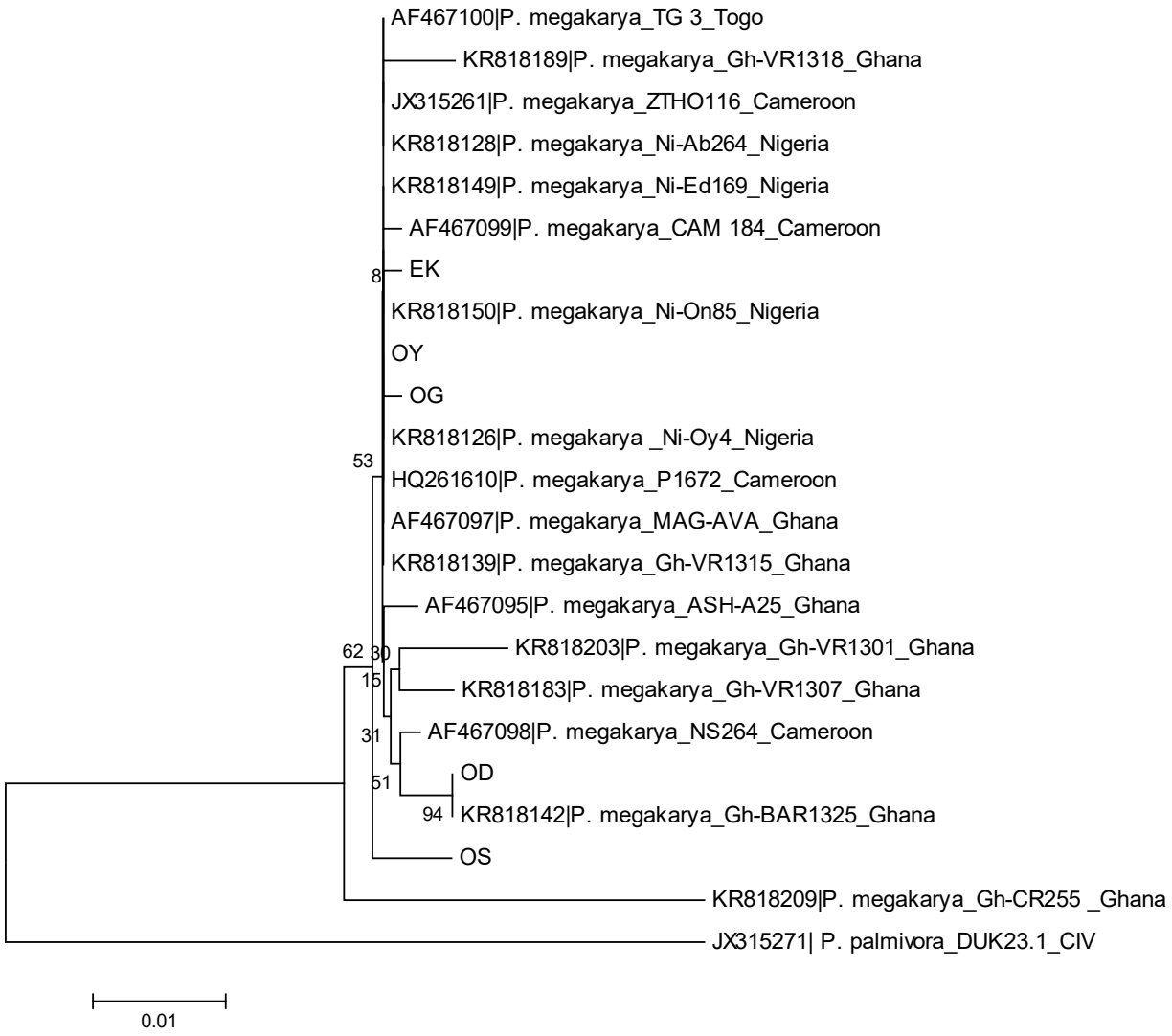


Figure 4.4: Phylogenetic tree showing relationships among partial internal transcribed spacer region of five *Phytophthora megakarya* isolates in Southwestern Nigeria with others across West and Central Africa.

4.11 Screening for Resistant Progenies

4.11.1 Detached Pod Test on the Causal Organism

The detached pod infection test result on Table 4.6 revealed that TC-4 displayed a localised lesion of 9.28±1.75cm after 7days of inoculation and was categorised as moderately resistant to penetration. TC-6 had small localized lesions and was thus classified as partially resistant. TC-3 on the other side had a background of enlarged lesions and was graded as partly resistant. TC-5, TC-1 and TC-7, displayed extensive lesions and were marked as susceptible. The variety N38 displayed lesions in combination and was considered highly susceptible. The mean ± Standard deviation across the column was important at p $\alpha \leq 0.5$. with TC-4 progeny exhibiting the highest level of causal organism tolerance.

This was followed by TC-6, TC-3, TC-5, TC-1 and TC-7 in that order of resistance. Variety N38 used as the control experiment was highest in sensitivity to the *P.megakarya*. The progeny descriptions of the six CRIN progenies used in the resistance test were shown in Table 4.7. The progenies were of mixed charaters, which included tolerance to PPR, competitive vigour (numbers of beans / seeds per pod) and mature bean size.

Table 4.6: Mean Lesion Diameter of *Phytophthora megakarya* on Detached Pod Resistance Test on six CRIN cocoa (*Theobroma cacao* L.) Progenies for day 3, 5 and 7 after Inoculations Respectively.

	Detached Pod: MEAN ± STANDARD DEVIATION		
Genotypes	**Day 3**	**Day 5**	**Day 7**
TC-1	15.5±10.5[a]	24.13±7.63[b]	64.00±6.50[a]
TC-3	5.00±0.00[b]	7.50±0.50[c]	27.75±22.75[b]
TC-4	5.00±0.00[b]	9.25±1.75[c]	9.28±1.75[b]
TC-5	5.00±0.00[b]	12.75±2.75[c]	52.50±7.50[a]
TC-6	5.00±0.00[b]	8.25±2.00[c]	23.33±16.25[b]
TC-7	5.00±0.00[b]	25.25±5.25[b]	68.13±3.88[a]
N38	13.75±1.25[a]	41.25±3.75[a]	73.13±5.38[a]

Note: Mean ± SD across the column with a specific superscript, 5% with a > b > c was significant. Mean separation done by Duncan Multiple Range Test.

Table 4.7: CRIN Progeny Details Screened for Resistant to *Phytophthora* Pod Rot (*Theobroma cacao* L.)

Hybrids for screening	CRIN Nomenclature	Pedigree	Genetic Base
TC-1	CRIN Tc-1	T65/7xN38	Amazon (PA/IMC)/Amelonado
TC-3	CRIN Tc-3	Pound 7xPA150	Amazon hybrids
TC-4	CRIN Tc-4	T65/7xT57/22	Amazon (PA/IMC)/ Trinitario
TC-5	CRIN Tc-5	T82/27xT12/11	Amazon (NA/PA/SCA) Hybrid
TC-6	CRIN Tc-6	PA150xT60/887	Amazon (PA/NA)/Hybrid
TC-7	CRIN Tc-7	T82/27xT16/17	Amazon (NA/PA/IMC)/Hybrid
N38*	Local	Amazon	Amazon/Trinitario

***N.B: N38 was used as control source for the experiment.**

4.11.2 Leaf Disc Resistance Test (LDT)

Table 4.8 showed a mean ± SD with specific superscription, which was significant at p≥0.05 on leaf disc resistance test. T4 was observed to produce a necrotic spot with lesion size 1.23±0.43 after 7days of inoculation and was categorised as moderately resistant to penetration. TC-6 had larger size necrotic spots was thus classified as partially resistant. TC-3 produced medium sized brown spot and was graded as partly resistant. TC-5, TC-1 and TC-7, displayed large brown lesions and were marked as susceptible. The variety N38 displayed lesions in combination and was considered highly susceptible. The mean ± Standard deviation across the column was important at p α≤0.5 with TC-4 progeny exhibiting the highest level of causal organism resistance. Variety N38 used as the control experiment was highest in susceptibility to the *P.megakarya*.

4.11.3 Detached Pod Inoculated with Pure Culture of *Phytophthora megakarya* Isolates for Resistance to *Phytophthora* Pod Test.

Plate 4.10 revealed the fungal growth of *P.megakarya* inoculation on the 3rd day showing lesions diameter on the six CRIN progenies and the variety control pods for the pod resistance test. It was observed that T4 had no lesion on day 3 after inoculation, while T3 showed no localized lesion. A simple extended infection lesion presented on T6 followed in that order by T5, T1 and T7. N38 had a joint lesion suggesting its susceptibility to black pod disease with *Phytophthora*. The 7th day lesion expansion pattern showed that T4 had the lowest expression of localized lesion infection (±9.0 mm), followed by T6 with extended lesion (±23.0 mm), then T3 (±27.0 mm), T5(±52.0 mm), T1(±64.0 mm), T7 (±68.0mm) and N38 (±73.0mm).

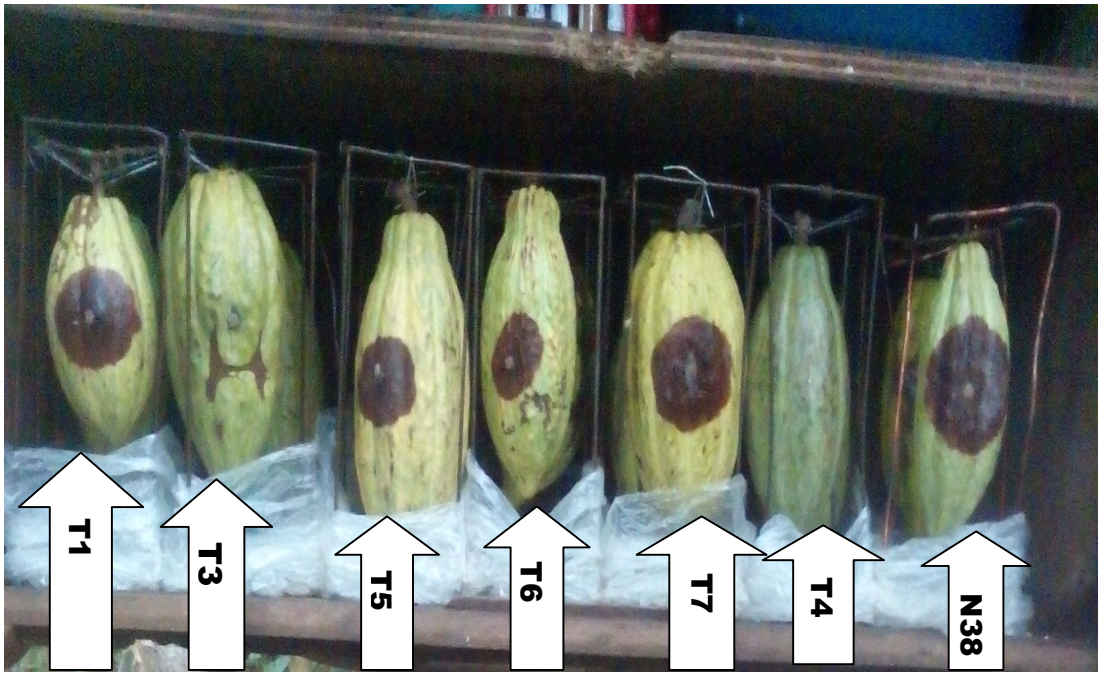


Plate 4.7: Detached pod inoculated with pure culture of *Phytophthora megakarya* Isolates for resistance to *Phytophthora* pod test. Where T1= CRIN TC-1; T3= CRIN TC-3; T4= CRIN TC-4; T5= CRIN TC-5; T6= CRIN TC-6; T7= CRIN TC-6; T7= CRIN TC-7 and N38 is the local variety used as control in the experiment.

Table 4.8: Mean Lesion Diameter of *Phytophthora megakarya* on Leaf Disc Resistance Test on six CRIN Cocoa Progenies at 7th day after Inoculation.

Genotypes	Leaf Disc: MEAN ± STANDARD DEVIATION
TC-1	2.83 ± 1.53^{bc}
TC-3	1.5 ± 0.82^{de}
TC-4	$1.23 \pm 0.43^{e;}$
TC-5	2.57 ± 1.28^{c}
TC-6	1.87 ± 1.22^{d}
TC-7	3.4 ± 1.38^{b}
N38	4.23 ± 1.01^{a}

Note: The mean ± SD for various superscripts was significant at the 5% with a > b > bc. Mean separation done by Duncan Multiple Range Test

4.11.4 Detached Pods Test Lesion Expansion (infection) Pattern

Figure 4.5 showed the mean bar fungal growth of inoculum infection on the six progenies of CRIN and the N38 variety used as control experiment. TC-4, TC-6, TC-3, TC-5 TC-7 at 3rd day revealed ±5cm infection lesion while TC-1 and N38 expressed the infection growth above 10cm. Figure 4.6 showed the observed fungal growth pattern on the progenies. The lesion expression was not conclusive at this stage because of similar growth rate on TC-3, TC-4 and TC-6 though TC-1 and TC-7 showed lesion diameter that is ±25cm with N38 above 45cm lesion diameter. Figure 4.7 showed that after 7 days of inoculation, TC-4 had the least expression of lesion followed by TC-6, TC-3, TC-5, TC-1 and TC-7. The highest level of contamination was shown by N38 used as control.

4.11.5 Leaf Disc Test Lesion Expansion (infection) Pattern

Figure 4.8 is a mean bar chart showed the fungal growth (necrotic spots/infection) after seven days of inoculation. The lesion expression showed different degrees of lesion on each of the six CRIN progenies on the leaf disks. The figures showed that after 7 days of inoculation, TC-4 had necrotic spot of 1.23±0.43cm followed by TC-3 1.5±0.82cm, TC-6 1.87±1.22cm, TC-5 2.57±1.28cm, TC-1 2.83±1.53 and TC-7 3.4±1.3cm. The highest level of infectious disease was shown by N38 4.23±1.01cm used as control.

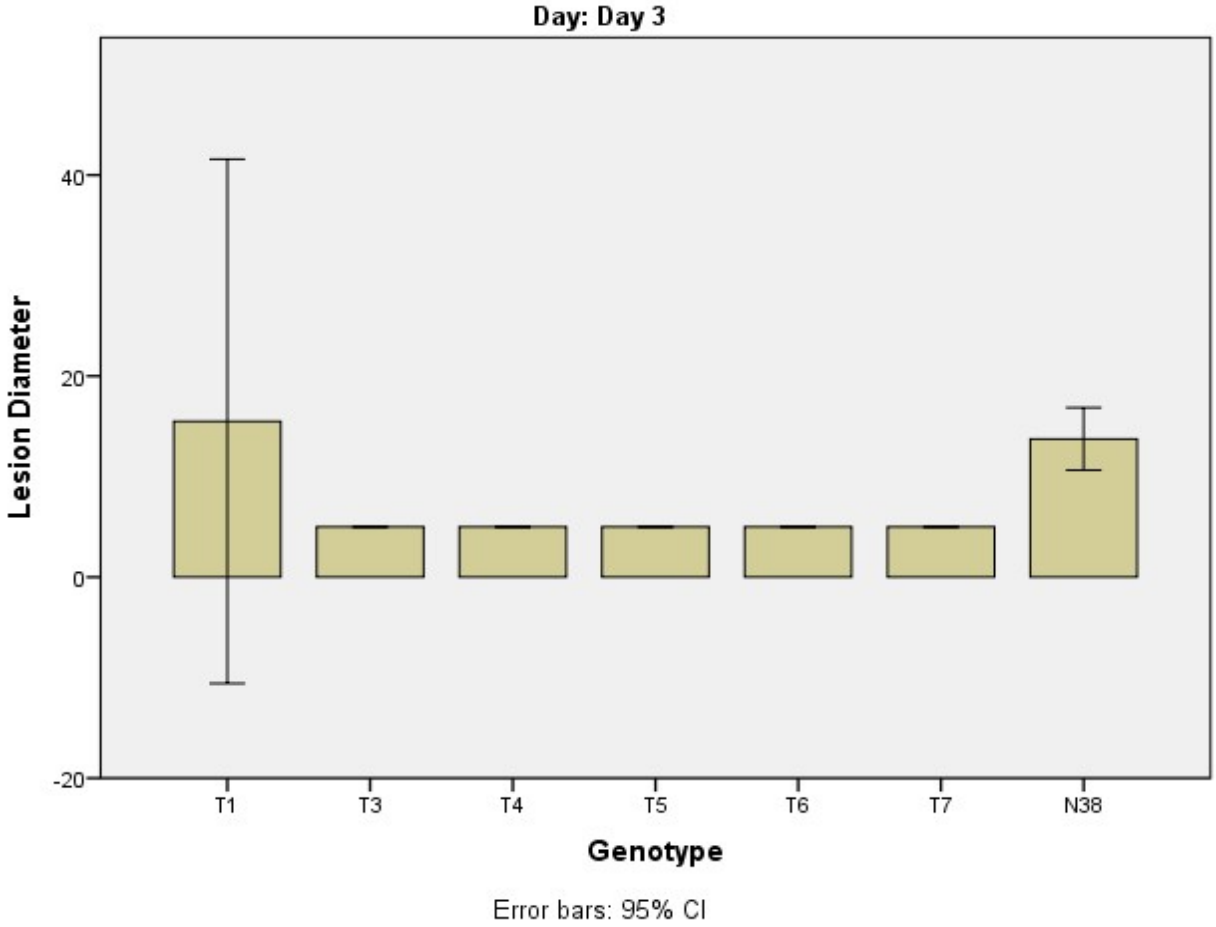


Figure 4.5: Detached Pods Test Columns Showing Fungal Growth (lesion) Pattern in Centimeters as at 3rd day on six Genotypes from CRIN Under a Controlled Temperature of 20°C-24°C and 80% Relative humidity.

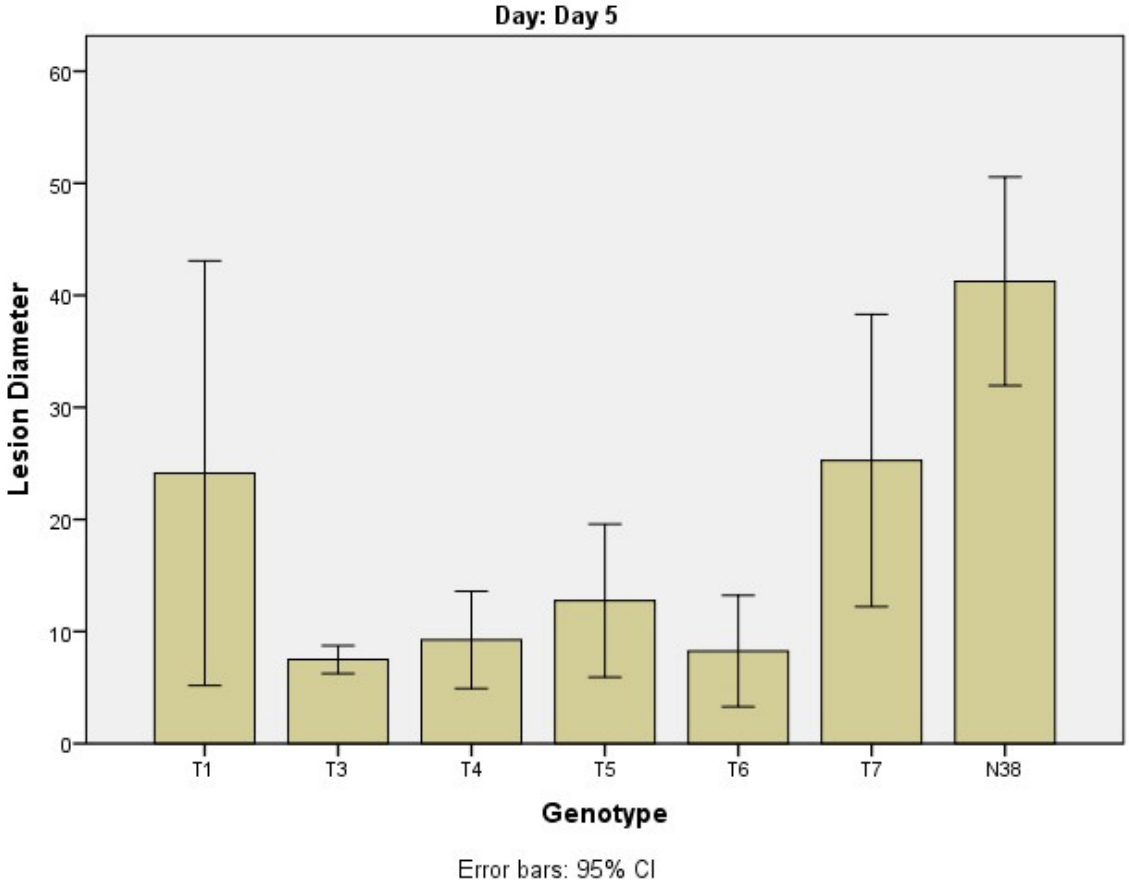


Figure 4.6: Detached Pods Test Columns Showing Fungal Growth (lesion) Pattern in Centimeters at 5th day on six Genotypes from CRIN Under a Controlled Temperature of 20^{o}C-24^{o}C and 80% Relative Humidity.

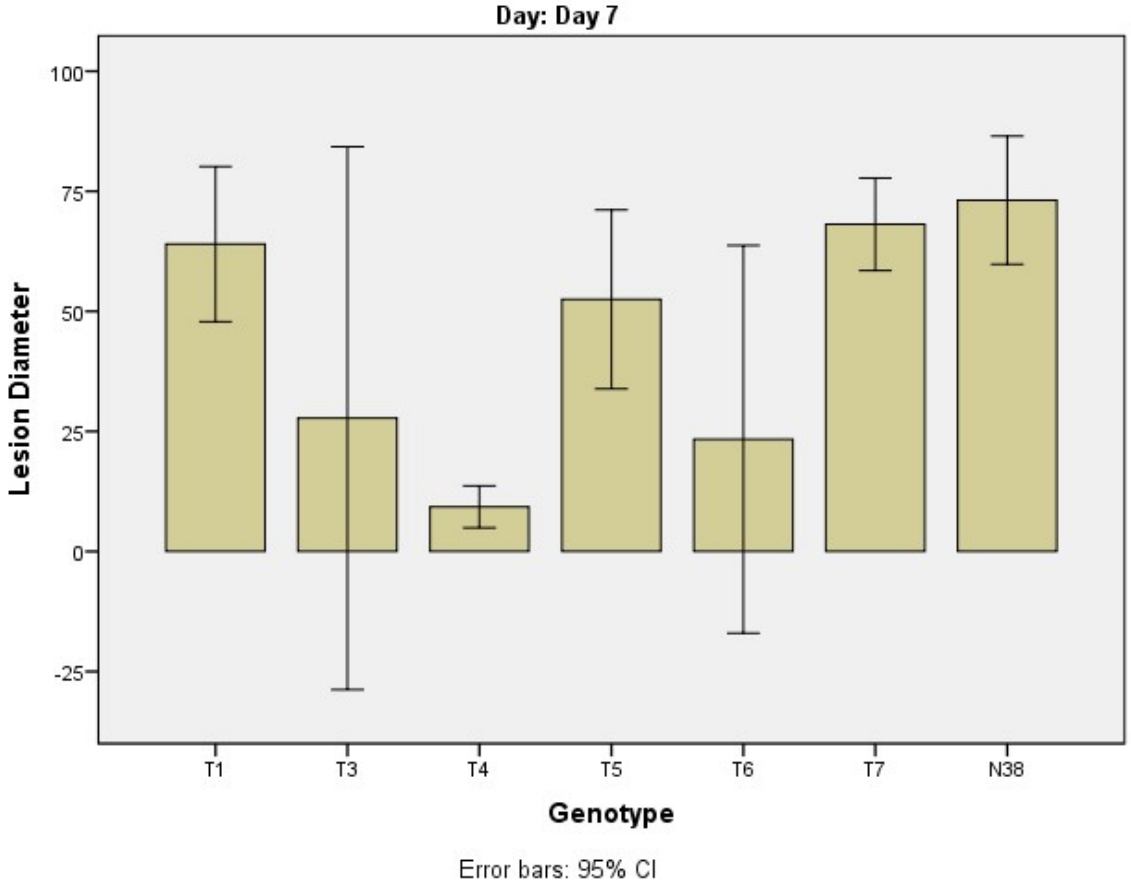


Figure 4.7: Detached pods test columns showing fungal growth (lesion) pattern in centimeters at 7th day on six genotypes from CRIN under a controlled temperature of 20^{o}C-24^{o}C and 80% relative humidity.

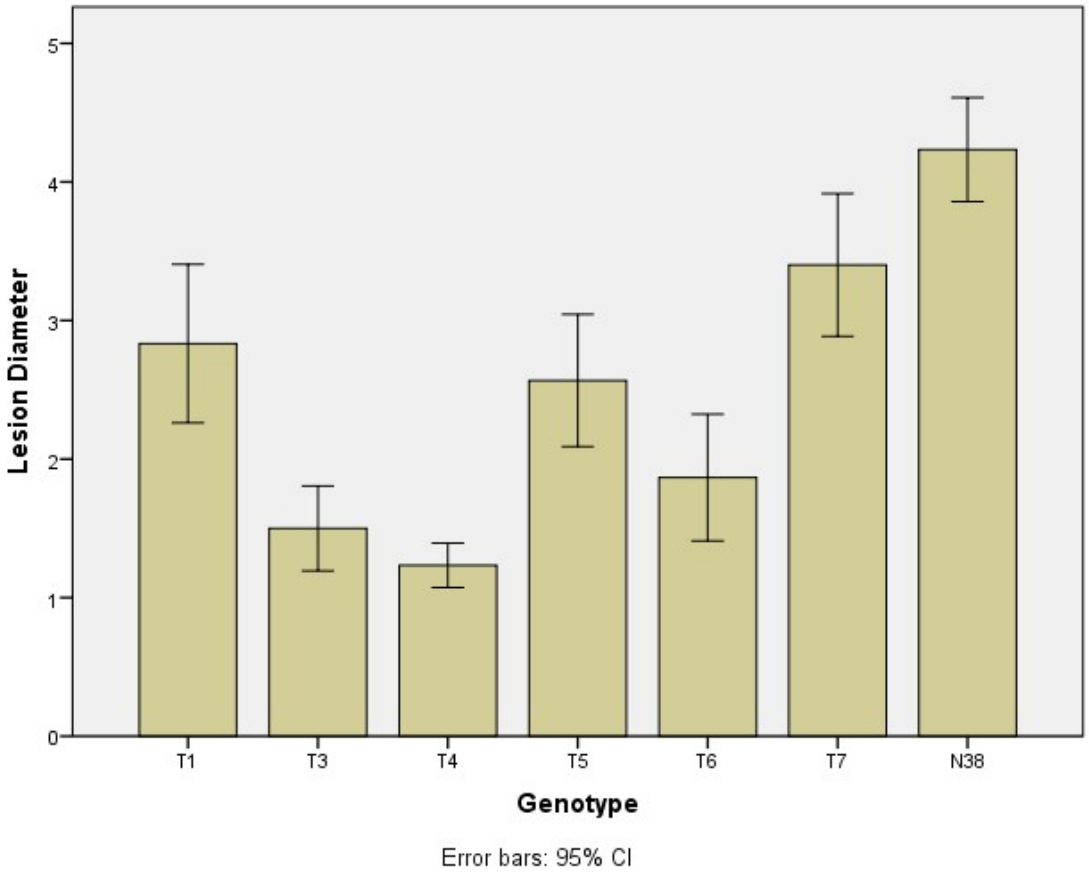


Figure 4.8 : Leaf Discs Test Columns Showing Fungal Growth/Necrotic Spot Pattern in Centimeters at 7th day on six Genotypes from CRIN Under a Controlled Temperature of 20^{o}C-24^{o}C and 80% Relative Humidity.

CHAPTER FIVE

DISCUSSIONS

This study provides morphological and molecular data to identify *Phytophthora megakarya* as a distinct mycological genus that infects *Theobroma cacao* in Southwestern Nigeria. This classification is based on a three-year analysis of a group of isolates from 15 LGA that grow cocoa in Ekiti, Ogun, Ondo, Osun, and Oyo States. Based on analyses of the DNA sequence, *P. megakarya* was found to be in the same clade (clade 4) with *P. palmivora* that morphologically identified papillate sporangia with topology of clad relationships.

This has been reported earlier by Opeke, (2003), Appiah *et al.* (2003) and Agbeniyi *et al.* (2014) that *Phytophthora palmivora* was the causal organism of PPR of cocoa in Africa. While recent studies carried out by Akrofi *et al.*(2015) and Afolayan *et al.*(2020) showed PPR of cocoa is caused by *Phytophthora megakarya* in West Africa though Akrofi et al. (2015) indicated that both *Phytophthora palmivora* and *Phytophthora megakarya* are discovered in the growing regions of Ghana. This research established that it is *Phytophthora megakarya* that is responsible for PPR of cocoa in the study site.

While *P. palmivora* is similar to *P. megakarya* on classification, a distinction is seen in the arrangement of sporangia. Similarly, *P. palmivora* infects multiple hosts while *P. megakarya* infects only cacao (Ristaino *et al.*, 2016).Therefore, it was necessary to utilise taxonomic keys and information of the pathogen's host range according to Ristaino *et al.* (2016). This was primarily recognized in the morphological and molecular study of the *Phytophthora* Pod Rot (PPR) in southwestern Nigeria. The time required to identify and evaluate the potential risk of the isolated pathogen requires frequent use of sequence-based DNA analysis to improve and supplement morphological data.

The results of this study reveal that the media used to grow the causal organism, namely; V8 agar and carrot agar which are favorable for organism growth due to the preferential growth of *Phytophthora* species on media.The relatively large sporangia with a number of spores supported the name of the "super" genus, meaning big/large “karyote”- nucleus *P. megakarya* which could also inform of its virulent nature. This is also consistent with previous research by Afolayan *et al.* (2020).

This study showed that there is a characteristic colony difference between *Phytophthora palmivora* and *Phytophthora megakarya* pure colonies on V8. *P. palmivora* displays a stellate mycelial growth appearance where it occurs as a causal organism; *P. megakaya* shows a cottony mycelial growth in all isolates from the five Southwest Nigerian states thus confirms the earlier reports of Agbeniyi *et al.* (2014) and Afolayan *et al.* (2020). This is a clear difference in the production of the same medium as standard laboratory testing has been observed in each of the experiments. *P. palmivora* showed an ovoid sporangium while *P. megakaya* showed an ellipsoid sporangium. In all the sample isolates obtained in southwestern Nigeria in the five states, the ellipsoid sporangial feature was found. This also clarified the fact that the causal organism is *P. megakaya* in the area being investigated.

The research on causal pathogen of PPR disease of cocoa in Southwestern Nigeria showed that the pathogen was of the same morphological type across the growing region. There was no record of *Phytophthora palmivora* in any of the five states in which the study was conducted.This further showed that the causative organism is *Phytophthora megakarya* with the help of a coherent key for identifying species of *Phytophthora* as predicted earlier by Agbeniyi *et al.* (2014) and Afolayan *et al.* (2020) with Ristaino *et al.,* (2016a) and Ristaino *et al.,* (2016b). The DNA and PCR methods used in this work established the testing tool for plant disease. DNA derived from *P.megakarya* augmented by the primary ITS1 and ITS 4 pairs. *P.megakarya* ITS 1 and ITS 4 PCR amplification produced an average product of 500-bp and 550-bp respectively. This is widely used for oomycete in the *Phytophthora* genus. This was established by Gezahegn *et al.* (2017) for fungi DNA, PCR and sequence in the laboratory.

The isolates of the causal pathogen produced similar pair wise identity compared with Ristaino *et al. (*2016) on key identification of *Phytophthora* species and *Phytophthora* Data gene bank. This further enhances the similarity of the pathogen across the region in Ekiti, Ogun, Ondo, Osun and Oyo States where the research is being conducted in southwestern Nigeria. Pure fungal cultures created a similar peak from this study as shown in the DNA and PCR photo gel. This shows the area's genomes similar to those shown on the plates. The result shows the suspected fungal DNA's consensus remains. This further clarifies the fact that the fungus is widespread across the states of South West Nigeria in all places where the samples were obtained as initially proposed by Agbeniyi *et al.* (2014). *P. megakarya*, however, may have shared a common ancestor with *P. palmivora* or as a result of interspecific hybridisation. Earlier, taxonomic guide by Stamps *et al.,* (1990) was based on Waterhouse earlier work as the typical reference for identifying the pathogen causing *Phytophthora* pod rot disease in cacao. The tabular key breaks down the genus into six morphological categories based on the features of sporangia, gametangia, temperature-specific growth and culture. For all isolate samples from each of the six states of Southwest Nigeria, the studied restriction fragment patterns of amplified ITS DNA were performed.

This *Phytophthora megakarya* fungus has been known as the most commonly found virulent and pathogenic fungus affecting the cocoa plant in Nigeria and other Western African countries, which is the world's largest growing area of cocoa (Evans, 2012 and Afolayan *et al.*, 2020) and regarded as the most commonly found virulent and pathogenic fungus.The research conducted was both on pods and leaf discs to confirm progeny immunity and susceptibility to cacao black pod pods disease from *Phytophthora*. The findings were on both ends experimentally identical.

The symptoms of the causal pathogen was identified in the initial survey and mapping of sample collection sites in cocoa plantations (*Theobroma cacao*). There are plantations where all the pods on the tree crops were infected, resulting in a loss of 100%. Studies from other cocoa-producing countries around the world, such as Ghana and the Ivory Coast, indicate that black pod disease or *Phytophtora* pod rot caused both *P. megakarya*

and *P. palmivora*. as reported by Baah and Anchirinah, 2011 and Evans, 2012). Nevertheless, PPR was primarily caused during this study by *P.megakarya* based on identification of sporangium type in Southwest Nigeria.

In the growing areas, heavy rain (±300 mm / month) combined with high humidity at 80% and weak planting materials caused these attacks was discovered while field studies conducted in 2013-2017. Personal conversations with growers in the five states surveyed during this study showed that neglect in the initial signs of the disease and insufficient cultivation management were the cause of the disease outbreak in all plantations, which was also agreed with previous studies in growing regions around the world as indicated by Nasir and Riazuddin, 2008 and Afriyemi *et al.,* (2013).

Sporangia from all isolates in this study were ellipsoid with rounded bases, ranging from 29.97-38.82 to 24.09-30.8 µm, which is correlated with the earlier study by Erwin and Ribeiro (1996) that provides a direct comparison of the morphological features of each of the studied species of *Phytophthora.* These species can be distinguished from the other three through having persistent sporangia. Sporangial pedicels of *P. palmivora* are short (2-5 µm), those of *P. megakarya* intermediate (10-30 µm).Thus, establishing the morphological features of the isolated pathogen to be *Phytophthora megakarya* with a length-to-breadth ratio of 1.24:1.36, confirming the pathogen. Sporangia are caducous, with pedicels that are 4-01-5.13 µm long. *P. megakarya* is, as described above, a member of the *Phytophthora* Group II. It grew caducous papillate sporangia heterothallic and amphigynous. This validated the earlier study of Ristaino *et al.* (2016).

Molecular analysis of the pure culture obtained from the isolates. A Basic Local Alignment Search Tool (BLAST) showed that ITS PCR products from five (5) states in which *P.megakarya.* The BLAST exhibited 96%-100% homology with the associated sequences in the genebank. A NCBI genebank search reveals that Oyo State samples with gene accession number (GAN)-JX3I5261 and GAN-KR818206 Ogun State has evolutionary relationship with strain from Cameroon. Ondo State with strain from Ghana that has the gene accession number GAN-KR818142. While Osun state samples with

GAN-MG865534 associated with Abad *et al.* (2012) findings predicting their evolutionary relationship with strain from Cameroon. Ekiti state samples GAN-MH620121 accession number with Cameroon strain based on sequence analysis of Yang and Hong (2018). Although the number of nucleotides from all five isolates varies, nBLAST was used to report similar percentages of identity.

This indicates high accuracy and matching already contained in *Phytophthora* isolates ' regional repository in NCBI gene bank. The e-values of zero and very high alignment scores indicate just high match performance. The strong query coverage recorded in the matches also confirms the reliability of the *Phytophthora* sequences, especially as a sequenced match with *P. megakarya* isolates from Cameroon and Ghana.

The pair sequence alignment (PSA) showing the structural and developmental relationship of pathogenic molecular samples from one state and the other. It revealed that pathogenic samples from Ekiti State were well associated with pathogenic samples from Oyo and Ogun State, which is valid because Oyo State PSA samples were produced from pathogenic PSA samples from Ekiti and Ogun State. There is a pair sequence similarity between samples from Ondo State and the one showing Osun State's evolutionary association. In addition, there is a significant Multiple Sequence Alignment (MSA) of all samples from the Five Southwestern States of Nigeria under study in the experiment with sequences that had similar length as earlier studied by Ristaino *et al.* (2016). The consequence of the sequence correlation suggested homological and developmental associations between the observed sequences (Abad *et al.* (2012).

While previous studies have shown that *T. cacao* in Ghana and Nigeria were mostly affected by *P. megakarya*. *T. cacao* grown in Cameroon and Togo were not left out from this same *P. megakarya* devastation (Kolawole *et al.,* 2016). A close look at the phylogenetic tree also confirmed the evolutionary relationship between the samples across the southwestern states of Nigeria and the growing nations especially in West and Central Africa as indicated by Agbeniyi *et al.* (2014).

Samples from Ekiti, Oyo and Ogun States were found to be on the same lane or main trunk of the tree while Ondo and Osun are on sub lane or sub branch from the main trunk

of the phylogenetic tree still farther from origin compared with Ekiti, Ondo and Ogun States as seen on the evolutionary tree (Ristaino *et al.* (2014).

The evolutionary tree further suggests that the pathogen invasion was from Cameroon and Ghana where the pathogen have also been established in their *T. Cacao* growing plantations. This further established the possibility of genetic variability /mutation as the pathogen is distant from point of entrance to new area. This has been confirmed in Pair wise sequence comparison in the nBLAST through the NCBI gene bank. It has been proposed in early studies by Ali *et al.* (2017) that *P. megakarya* appears to be confined to West Africa where it is the most common species of *Phytophthora* causing black pod disease and this has been morphologically confirmed in this study.

Another fungus was discovered from the samples' isolate pure cultures which was *Fusarium spp* in this analysis but never a major pathogen suspected in cocoa plantations, rather a secondary fungus. The causal organism for PPR in southwestern Nigeria is therefore *P. megakarya.*

The unusual aspect of cacao tolerance for *Phytophthora* is that multiple studies correlated sensitivity to one species with susceptibility as earlier reported by Bailey e*t al.* (2016). *Phytophthora megakarya* is the most destructive species on cacao and that varieties of cacao varied in their responses to *P. megakarya* and other species of *Phytophthora* supported by Ali *et al.* (2017). This then means that, irrespective of other factors; environment, time and relative humidity at 70% and above there is varied defence mechanism of varieties of cocoa against *Phytophthora* pod rot. More experiments like that of Lanaud *et al.* (2009) and Ali *et al.* (2017) provides support for the general theory that there is structural resistance to PPR and several genetic causes of resistance in cacao varieties.

The presence of unique R-genes that mediate neural tolerance has not been indicated. If it is accurate that tolerance to one species of *Phytophthora* coincides with resistance to all species of *Phytophthora*, these disparities may be due to non-interaction-based differences between species of *Phytophthora* or to the ability to kill or bypass mechanisms of cacao

protection. For example, the ability to form appressoria and sporangia varies from *P. megakarya to P. palmivora* was indicated earlier by Ali *et al.* (2017).

The six cocoa progenies/ hybrids had both resistant and susceptible interactions with *P. megakarya* while susceptibility / resistance is comparative and incomplete, plant defense mechanism aspects could still be identified in these interactions and therefore recommended for further scientific research.

This study has corroborated initial findings by co-applying *P.megakarya* and *P. palmivora* to an unwounded pod sheet, restoring primarily *P. megakarya* sporangia irrespective of the order of operation. It has been shown that *P. megakarya* produces appressoria more often than *P. palmivora* allowing direct penetration, whereas *P. palmivora* penetrated more frequently through stomata (Ali *et al.,* 2016). This alone may explain for *P. megakarya'*s ability to destroy the court of infection when it is introduced at the same time. Ali *et al.* (2012) showed that *P. megakarya* also led in the development of secondary inoculums, even when *P.palmivora* was added to the infection court 24 hours before *P. megakarya*.

Such results confirm that *P. megakarya* is more aggressive than *P. palmivora* in causing disease in unwounded cocoa tissues (Ali *et al.*, 2016) and has consequences as to why *P. megakarya* replaced *P. palmivora* from cocoa in West African countries such as Nigeria and Cameroon (Afolayan et al. (2020).

The sequence of resistance from the sample was T4>T6>T3 while the T5>T6>T7 progenies displayed a poor level of susceptibility tolerance and N38 was used as a highly susceptible control device. It indicates that T4 is more effective for farmers and is suggested. Nevertheless, farmers need to follow conventional management practices to ensure high yield. During the analysis, the leaf disk test showed a similar correlation with detached pod research, which was also consistent with previous tests (Tahi *et al.* (2007). For TC-4>TC-6>TC-3, the progeny differs significantly from p>0.05.

The results of this research has shown the importance of the inoculation tests for separated pods and leaf discs to determine the resistance six CRIN progenies to PPR caused by *P.*

megakarya. The analysis under uniform conditions found that 60 to 90% of the variability in field resistance to *Phytophthora* spp, which was also accepted with previous studies, was clarified by the leaf disc study (Tahi *et al.* (2007).

Thus, these parameters informed the collection time, preservation and laboratory procedures observed for the detached pods and leaf discs used to produce the experiment with scientifically reliable results during the study (Tahi *et al.* 2006). It is not well known the underlying mechanism of enhanced sensitivity of afternoon leaves and leaves subjected to higher light conditions. Exposure of leaves to high light intensity or shorter light cycles in a day that increase the amount of assimilates within the leaf, making it a more desirable substratum for pathogen development (Tahi *et al.* (2007). Nonetheless, the results suggest that more favorable pathogen growth conditions will reduce the precision of the measurement of variations in genetic resistance between clones (Afolayan *et al.* (2020).

This is applicable when dealing with older, more robust plant leaves the tolerance of very young plants that are more vulnerable.This therefore showed that conditions that are particularly beneficial to pathogen development tend to reduce the ability to differentiate between pathogens in resistance.

The correlations found with the detached pod study for leaf discs obtained in the morning were attributed to the sampling time and age of the samples used in the experiment. This was predicted because for both experiments, these are the same factors that make the most of the correlations with the field results (Akrofi *et al.* 2015).

The predictive ability of a different pod test utilizing spray inoculation that guided the technique used in the analysis has recently been demonstrated. The latter method has the benefit that, in order to obtain significant differences between clones, obviously less pods are required than in wound inoculations (Tahi *et al.,* 2012) and Afolayan *et al.,* 2020). The current results show that it is essential to standardize the conditions under which these tests are performed in order to achieve more reliable results.

It was also observed that *P.megakarya* spores more quickly on infected pods within the

growing necrosis zone than *P.palmivora*, giving the result a further benefit (Appiah *et al.,* 2003). These results supported observations that *P.megakarya* is more aggressive than *P.palmivora* in causing disease in unwounded cocoa tissues and has implications for countries like Nigeria and Cameroon where *P.megakarya* has displaced *P.palmivora* from cocoa plantations (Agbeniyi *et al.,* 2014).

This study showed that differential transcript aggregation does not show that a gene is directly involved in resistance or sensitivity to *P.megakarya* and *P.palmivora*; many of the transcriptional variations observed were due to plant defensive mechanisms and processes (Tahi *et al.,* 2006).

In young leaves such as those used here, stomata development is incomplete and information on stomata development on leaves should be considered for further studies. Positive results linking resistance in cacao pods to resistance in cacao leaves that focus on components potentially shared by leaves and pods should also be considered for further studies. While phenol reactions, lignification processes, and wax layers aspects of resistance / tolerance that could be expected to react consistently are for consideration in further studies as shared by (Barreto *et al.*, 2015).

CHAPTER SIX

SUMMARY, CONCLUSIONAND RECOMMENDATION

Cacao infestation of *Phytophthora megakarya* is a threat to Nigeria's economy. It spreads rapidly in the sub-region; it has displaced the less severe *P. palmivora*'s original populations. This research partly identified the current molecular strategies and common analytical methods used to study fungal species in a specific environment. The clear trend in implementing both morphological and molecular method is to explain the observations and making them scientifically valid.

Through the study's molecular diagnosis, the evolutionary tree further confirmed that the pathogen invasion came probably from Cameroon or Ghana where the pathogen was also found. This was established with the evolutionary relationship linked to Cameroon and Ghana.

Phytophthora megakarya was found to be the common pathogen in the area under study based on surveys. The results indicate that the pathogen has severity potential on cocoa fruits and more work is needed to determine its distribution in the rest of the country where the crop is grown. The research indicated that *Phytophthora* pod rot cocoa caused by *Phytophthora megakarya* is pathogenic to the survival of the crop. While the use of chemical has become detrimental to the soil, through the use of resistant varieties of cacao to control the disease, the study has provided environmentally friendly approach.

It is recommended through the initial findings of this study that the integrated disease management (IDM) should be observed. These will combine the effective cultural management with planting of resistant variety in the establishment cacao plantation.

The study by screening six CRIN progenies found that T4 progeny is the preferred variety most resistant to *P. megakarya*. This is recommended to be grown by farmers in replacement of older plantations. The establishment of new plantations should be observed with the variety under integrated disease management.

The trend in the application of molecular methods will also help in the maintenance of resistant varieties, while the pathogen's ecological and epidemiology must be considered in order to check the causal organism's possible mutation. The research is based on the premise that there is a need for faster methods to diagnose fungi in this part of the world and for economic crops, particularly with the severe disease and devastation caused by fungi.

CONTRIBUTIONS TO KNOWLEDGE

1. *Phytophthora megakarya* is the causal organism of *Phytophthora* Pod Rot of cocoa in southwestern Nigeria. The initial causal species *Phytophthora palmivora* was never found in any of the isolates from all the plantations across the states.
2. *Phytophthora megakarya* was diagnosed morphologically and molecularly. The organism was discovered to have evolutionary relationship with causal pathogen from Cameroon and Ghana.
3. The most resistant of the six hybrids screened is T4 hybrid from Cocoa Research Institute of Nigeria.

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Appendix 1

Internal transcribed spacer (ITS) and complete sequence of DNA/RNA of samples from Southwestern Nigeria. (OY= Oyo State; OG= Ogun State; OD= Ondo State; OS= Osun State and EK= Ekiti State repectivy).

>**OY** 99% identical to *Phytophthora megakarya* isolate ZTHO116 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

ATTAAAACTTTCCACGTGAACCGTATCAACCTTTAGTTGGGGGTCTTTTTCGGC
GGCGGCTGCTGGTTTAATTACTGGCGGTTGCTGCTGGGAGAGCCCTATCATGG
CGAGCGTTTGGGCTTCGGTCTGAACTAGTAGCTTTTTTAAACCCATTCATTATT
ACTGATTATACTGTGGGGACGAAAGTCTCTGCTTTTAACTAGATAGCAACTTT
CAGCAGTGGATGTCTAGGCTCGCACCTCGATGAAGAACGCTGCGAACTGCGA
TACGTAATGCGAATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATAT
TGCACTTCCGGGTTAGTCCTGGAAGTATGCCTGTATCAGTGTCCGTACATCAA
ACTTGGTTTTCTTCCTTCCGTGTAGTCGGTGGAGGATATGCCAGATGTGAGGT
GTCTTGCGGCTGGCCTTCGGGTCGGCTGTGAGTCCCTTGAAATGTACTGAACT
GTACTTCTCTTTGCTCGAAAAGTAAAGCTTGCTTGTTGTGGAGGCTGCTTGTGT
AACCAGTCGGCGACTAGTTTGTCTGCTGTGGCGTTAATGGAGGAGTGTTCGAT
TCGCGGTATGATTGGCTTCGGCTGAACAGAAGCTTATTGGCGTTTTTCCTGCT
ATGGCGGTATGAAGTAGTGAACCGTAGTTATGTGGGCTTGGCTTTTGAATGTG
CTCGCTGTGCGAAGTAGAGTGGCGACTTTGGTTGTCGAGGGTCGATCCATTTG
GGAAATTGTGTGTACTTCGGTATGCATCTCAATTTGGACCCTGATATCAGGCA
AGATTACCCCGCTGAAACTTAAGCATATCAATAAAGGCGCGGGGAGGGGAAA

>**OG** 99% identical >KR818206.1 *Phytophthora megakarya* isolate Ca-ZTHO144 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

CCTAAACTTTCCACGTGAACCGTTCAACCTTTAGTTGGGGTTCTTTTTCGGCGG
CGGCTGCTGGTTTATTACTGGCGGTTGCTGCTGGGAGAGCCCTATCATGGCGA
GCGTTTGGGCTTCGGTCTGAACTAGTAGCTTTTTTAAACCCATTCATTATTACT
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GGCGGTATGAAGTAGTGAACCGTAGTTATGTGGGCTTGGCTTTTGAATGTGCT
CGCTGTGCGAAGTAGAGTGGCGACTTTGGTTGTCGAGGGTCGATCCATTTGGG
AAATTGTGTGTACTTCGGTATG

>OD 99% identical to >KR818142.1 *Phytophthora megakarya* isolate Gh-BAR1325 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

CCCCCTAAAAACTTTCCACGTGAACCGTATCAACCTTTAGTTGGGGGTCTTTTT
CGGCGGCGGCTGCTGGTTTAATTACTGGCGGTTGCTGCTGGGAGAGCCCTATC
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>OS identical to *Phytophthora megakarya* clone WPC1672B723 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence
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TGAGTCATCGAAATTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGGAAGT
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CTTTGGTTGTCGAGGGTCGATCCATTTGGGGAAATTTGTGTACTTCGGTATGC
ATCTAA

>EK identical to *Phytophthora megakarya* isolate 61J5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence
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Appendix 2

Phytophthora megakarya isolate ZTHO116 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequ*ence* 818 bp genomic DNA.

Isolate: ZTHO116.Host: *Theobroma cacao*.Country: Cameroon: CIRAD Bios.Collected_by: M. ten Hoopen.

Accession: **JX315261**.1GI: 498763027

GenBank FASTA Graphics

Phytophthora megakarya isolate Ca-ZTHO144 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequ*ence*

779 bp genomic DNA.

Isolate: Ca-ZTHO144.Host: *Theobroma cacao*.Country: Cameroon.Isolation_source: black pod disease infected pod.Collection_date: 2011.

Accession: **KR818206**.1GI: 942540272

GenBank FASTA Graphics

Phytophthora megakarya isolate Gh-BAR1325 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence 783 bp genomic DNA.

Isolate: Gh-BAR1325.Host: *Theobroma cacao*.Country: Ghana.Isolation_source: black pod disease infected pod. Collection_date: 2013.

Accession: **KR818142.**1GI: 942540208

GenBank FASTA Graphics

Phytophthora megakarya strain CPHST BL 73 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: MG865534.1

FASTA Graphics PopSet

LOCUS MG865534 818 bp DNA linear PLN 10-SEP-2018

DEFINITION *Phytophthora megakarya* strain CPHST BL 73 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.

ACCESSION **MG865534**

VERSION MG865534.1

KEYWORDS .

SOURCE Phytophthora megakarya

ORGANISM *Phytophthora megakarya*

Eukaryota; Stramenopiles; Oomycetes; Peronosporales; Peronosporaceae; Phytophthora.

REFERENCE 1 (bases 1 to 818)

AUTHORS Abad,Z.G., Bienapfl,J.C., Burgess,T.I., Coffey,M.D. and Redford,A.J.

TITLE IDphy: Phytophthora identification based on the Types (Online Resource)

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 818)

AUTHORS Abad,Z.G., Bienapfl,J.C., Knight,L.M., Jennings,K.C. and Srivastava,S.K.

TITLE Direct Submission

JOURNAL Submitted (19-JAN-2018) Center for Plant Health Science and Technology, Contact Z. Gloria Abad, USDA-APHIS-PPQ-S&T, Powder Mill Rd, Beltsville, MD 20705, USA

COMMENT ##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

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/organism="Phytophthora megakarya"

/mol_type="genomic DNA"

/strain="CPHST BL 73"

/db_xref="taxon:4795"

/note="co-identical strain = P1672 WPC (G. Abad selected specimen #2)"

misc_RNA <1..>818

/note="contains internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and large subunit ribosomal RNA"

ORIGIN

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61 ggcggctgct ggtttaatta ctggcggttg ctgctgggag agccctatca tggcgagcgt

121 ttgggcttcg gtctgaacta gtagcttttt taaacccatt cattattact gattatactg

181 tggggacgaa agtctctgct tttaactaga tagcaacttt cagcagtgga tgtctaggct

241 cgcacctcga tgaagaacgc tgcgaactgc gatacgtaat gcgaattgca ggattcagtg

301 agtcatcgaa attttgaacg catattgcac ttccgggtta gtcctggaag tatgcctgta

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541 ccagtcggcg actagtttgt ctgctgtggc gttaatggag gagtgttcga ttcgcggtat

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