KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY,

KUMASI

COLLEGE OF SCIENCE

FACULTY OF BIOSCIENCES

DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

CHARACTERIZATION OF *BOTRYODIPLODIA THEOBROMAE* ISOLATES AFFECTING COCOA, MANGO, BANANA AND YAM IN GHANA

BY

OHENE-MENSAH, GODFRIED

NOVEMBER, 2012

CHARACTERIZATION OF *BOTRYODIPLODIA THEOBROMAE* ISOLATES AFFECTING COCOA, MANGO, BANANA AND YAM IN GHANA

THIS DISSERTATION IS PRESENTED TO THE DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY, KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, IN PARTIAL FULFILLMENT OF REQUIREMENT OF THE M.Sc. (HONS) DEGREE IN BIOTECHNOLOGY

BY

OHENE-MENSAH, GODFRIED

NOVEMBER, 2012

DECLARATION

I hereby declare that this submission is my own work towards the M.Sc and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

…… ……. ……..
Student Name Signature Date
ID: PG 2824008

Certified by:

1. ……	……	…….
Supervisor(s) Name	Signature	Date
2. ……	……...	…….
Supervisor(s) Name	Signature	Date

Certified by:

…….	……..	……...
Head of Dept. Name	Signature	Date

DEDICATION

I dedicate this piece of work to my Dad, Mr. Joseph Ohene-Mensah, on his 61th Birthday (12th December, 2012) and my Mum, Mrs. Christiana Ohene-Mensah and the entire Ohene-Mensah Family.

ABSTRACT

Botryodiplodia theobromae is a very important pathogenic species on many food and tree crops causing significant yield losses. The fungus has been reported to have a wide host range. This study was conducted primarily to establish some of the host range characteristics of the species in Ghana. Twenty-five isolates of *B. theobromae* obtained from cocoa, mango, banana and yam from some agro-ecologies in Ghana were maintained on potato dextrose agar (PDA) and used to

inoculate healthy matured cocoa pods and mango fruits. Symptoms of rot characteristics of the species resulted from inoculation with mycelia of the isolates. *B. theobromae* isolates from cocoa, mango, banana and yam used in the inoculation experiments were not different in their infections on the two crops inoculated. Morphological characteristics of single-spore cultures of the 25 isolates were compared on PDA at 28°C. All the isolates produced black pigments in culture 48 hrs after inoculation. Based on growth rates on PDA, the isolates were separated into four groups. The growth rate ranges were 0.93 to 0.94 mm/hr, 0.91 to 0.92 mm/hr, 0.82 to 0.90 mm/hr, and 0.72 to 0.76 mm/hr. The relatedness of the 25 isolates was investigated at the molecular level using SSR and RAPD primers. Two isolates, B(Kt) and B(Ef), both from banana fruits shared the highest genetic similarity of 83% with all others measured below 75%. The isolates studied were not host specific and ecologically non-specific. Molecular analysis of the isolates indicates that several strains of *B. theobromae* exist in Ghana.

ACKNOWLEDGEMENT

Praise and glory be to the most-high God for the journey thus far to this wonderful finish. My success in this work piece is by the grace and mercies of God. I am most grateful to you my lord.

My deepest and sincere gratitude goes to my supervisor, Dr. Ir. Peter Twumasi of the Department of Biochemistry and Biotechnology, KNUST, and co-supervisor, Dr. Emmanuel Moses, senior research scientist of the Pathology Division of the Crops Research Institute of the Council for Scientific and Industrial Research (CSIR-CRI). Dr. E. Moses, I so much appreciate and value the patience, advice, mentorship and dedication you put into my work to see to its successful completion. I cannot thank you enough on this earth. God will reward you exceedingly and abundantly. It was an honour working under you. Thank you, Sirs.

This piece of work has come this far by the help and assistance given me by the following persons, Mrs. Ziporah Appiah-Kubi of the Pathology Division, Mr. David Appiah-Kubi, Miss Elsie S. Addo and Miss Esther Agyemang, all of the Biotechnology Division of CSIR-CRI particularly Mrs. Marian D. Quain for allowing me use her Biotechnology Laboratory and Mrs Linda Abrokwa, for guiding me throughout the DNA work.

Sincere thanks also go to Mr. Eric Brenyah of KIRKHOUSE Trust Lab., Cocoa Research Institute of Ghana (CRIG), Tafo, for his time, devotion and encouragement in guiding me through the polymerase chain reaction processes and electrophoresis analysis. You are God sent.

I also show my respect to Dr. Fenning of the Entomology Division of CSIR-CRI for helping me to undertake a good statistical analysis of my data.

Thumbs up to my colleagues at Dr. Emmanuel Moses' laboratory, in the persons of Mr. Andrew Nyamfo, Atta Kwasi Aidoo Snr. and Miss Abigail Addo (National Service Personnel) for the countless support they made unconditionally available to me when I needed them the most. Thanks guys.

My heart also goes out to the entire staff of CSIR-CRI, Fumesua and the entire staff and colleague Municipal Development officers of Ministry of Food and Agriculture (MOFA)-Municipal Agricultural Development Unit (MADU), Berekum, especially Mr. Kyei-Baffour Owusu-Achaw (MOFA Director, Berekum), Mr. Emmanuel Bampoe and Mr. Frederick Danso.

I cannot forget the constant encouragement and motivation given me by mentors such as Dr. Michael Owusu-Akyaw and Dr. Bradford Mochiah, of the Entomology Division of CSIR-CRI, Kwadaso, Kumasi.

I am once again indebted to my Dad, Mr. Joseph Ohene-Mensah, formally of the Entomology Division of CSIR-CRI, Kwadaso, Kumasi, for his financial support, encouragement, motivation, praise, prayers and personal support to put me this far. Daddy, you are a true Father. To my mum and my sibling, you were the perfect family I needed when the journey sometimes looked so bleak. I wouldn't have achieved this without you. God bless you all. To all who helped me in one way or the other to get this work done I share my joy with you.

TABLE OF CONTENTS

LIST OF TABLES

PAGE

LIST OF FIGURES

PAGE

LIST OF PLATES

PAGE

LIST OF ABBREVIATIONS

CLA- Carnation Leaf-Piece Agar

CMA- Corn Meal Agar

CRI- Crops Research Institute

CRIG-Cocoa Research Institute of Ghana

CSIR-Council for Scientific and Industrial Research

DAP- Di-Ammonium phosphate

DNA- Deoxyribonucleic Acid

NTYSYS- Numerical Taxonomy and multivariate Analysis System

PCR- Polymerase Chain Reaction

PDA- Potato Dextrose Agar

PSA- Potato Sucrose Agar

RAPD- Random Amplified Polymorphic DNA

SAHN- Sequential Agglomerative Hierarchical and Nested

SSR- Simple Sequence Repeats

WA- Water Agar

YEMA- Yeast Extract Manitol Agar

CHAPTER ONE

1.0 **INTRODUCTION**

Botryodiplodia theobromae (Pat.) Griff. and Maubl. (Syn: *Lasiodiplidia theobromae* Pat.), is a fungal pathogen, and the asexual state of *Botryosphaeria rhodina* (Berk and M.A. Curtis) Arx. It is among opportunistic plant pathogens that cause different types of plant diseases with worldwide distribution within the tropics and subtropical regions (Faber *et al.*, 2007).

Botryodiplodia theobromae has a very wide host range estimated to be more than 280 species (Domsch *et al.,* 2007; Khanzada *et al.,* 2006; Sutton, 1980).

In the tropics, *B. theobromae* is an economically important fungus that causes major losses to farmers who cultivate mango, cocoa, banana, cassava and yam (Rieger, 2006; Amusa *et al.*, 2003). The fungus causes tuber rots in yam and sweet potatoes and root rot in cassava. It also causes collar rot of peanuts, crown rot of banana, stem end rot of mango fruits, stem rot of pawpaw and leaf spot on citrus (Sangeetha *et al*., 2011; Rossel *et al.*, 2008; Khanzada *et al*., 2004; Jiskani, 2002; Arjunan, 1999; Sangchote, 1988). The fungus is associated with die-back on mango (Khanzada *et al.,* 2004a, b) and pod rot of cocoa (Phillips, 2007). Onyenka *et al.* (2005) has reported that the fungus is present in more than 70% of farms surveyed in Nigeria and it is linked to yield losses of about 80%. Jiskani (2002) and Sangchote (1988), respectively, have identified *B. theobromae* to be a virulent fungus and a common isolate found on diseased mango fruits in Pakistan.

1.1 **PROBLEM STATEMENT**

Rots caused by the fungus, particularly in the root and tuber crops often occur underground and so diagnosis of the disease is usually delayed. Moreover, the wider host range (Crammer, 1979) and the host non-specificity (Mohali *et al.*, 2005) make control of *B. theobromae* and management of its diseases difficult. Unfortunately, there is limited information about various strains of the fungus on the host crops in Ghana.

B. theobromae infects mango, cocoa, banana and yam and cause extensive damage to these crops (French, 2006). In Ghana, farmers use some of these crops as intercrops or boundary crops on their farms. These farming practices may facilitate cross infections if strains have broad host range (Mohali *et al.*, 2005). Information on host range of *Botryodiplodia theobromae* based on research work in Ghana is hardly available. It is important therefore, that the actual host range of such an important pathogenic species is defined with respect to some of the important crops in Ghana. This will help in the development of appropriate intercropping systems that can effectively contribute to the reduction in damage caused by *B. theobromae*.

1.2 **GENERAL OBJECTIVE**

This study was conducted to gather information on host range characteristics of *Botryodiplodia theobromae* in Ghana. In addition, morphological and molecular characteristics of isolates from cocoa, mango, banana and yam were studied to determine whether similarities exist among them.

1.3 **SPECIFIC OBJECTIVES**

The objectives of the study were:

a) To inoculate healthy cocoa pods and mango fruits with inoculum of *B. theobromae* isolates from cocoa, mango, banana and yam, to establish their cross infectivity and host range characteristics.

b) To establish morphological similarities or difference among *B. theobromae* isolates from diseased cocoa, mango and banana fruits and yam tubers from some agro-ecological zones in Ghana.

c) To establish similarities or difference among isolates of *B. theobromae* from cocoa, mango, banana and yam at the molecular level.

CHAPTER TWO

2.0 **LITERATURE REVIEW**

2.1 **THE GENUS *BOTRYODIPLODIA***

Fungi belonging to this genus are often considered among the infamous ascomycetes known as parasitic and pathogenic (Taylor *et al*., 2006). As ascomycetes and heterotrops, they obtain

nutrients from dead or living organisms (Griffin, 1994; Carroll and Wicklow, 1992). Some members of the genus *Botryodiplodia* are found to be pathogenic on most food and tree crops (Taylor *et al.*, 2006).

It has been indicated that there are approximately 86 species in the genus *Botryodiplodia* (Anonymous, 2008). Notable ones that are pathogenic on food and tree crops or other plants include *B. acerina* on cassava, *B. acacigena* on acacia, *B. acicola* on pine, *B. acinosa* on longleaf pine (*Tilia platyphylla*), *B. ailanthi* on tree of heaven (*Ailanthus altissima*), *B. digitata* on baobab, *B. solani-tuberosi* on potato, *B. ulmicola* on Dutch elm, and *B. theobramae* on cocoa, mango, banana, yam etc.

2.2 **THE SPECIES *Botryodiplodia theobromae* (Pat.) (Griffon and Maublanc, 1909)**

The species *Botryodiplodia theobromae* is a soil-borne fungus (Domsch *et al.*, 2007) that causes devastating diseases on many crops including cocoa, banana, mango, avocado, okro, guava, pawpaw, cassava and yam (Pitt and Hocking, 2009; French, 2006). It is also a common fungus associated with rots during storage of tubers and fruits (Punithalingam, 1976). The fungus is a common secondary disease organism which means that they only attack plants when damaged by injury (Faber *et al*., 2007), or weakened by nematodes or often following pathogenic fungi such as *Phytophthora* spp. (Opoku *et al.*, 2007). Records indicate that the fungus has a wide host range and is found on over 500 different species of plants (French, 2006; Khanzada *et al.*, 2004).

B. theobromae often spreads by spores through wind (Faber *et al*., 2007; Solomon *et al*., 1987; Punithalingam, 1976). Spores of the pathogen can also be spread through flowing water or rain particularly in splashes from the rain water (Faber *et al*., 2007). Pathogenic spores of *B. theobromae* have also been found to be transmitted through infected seeds and planting materials (Punithalingam, 1976). It can also be spread by insects (Solomon *et al*., 1987), birds or through soil (Punithalingam, 1976). The soil-borne conidia or spore may remain in the soil and serve as the primary source of inoculum from season to season (González-Fernández *et al.*, 2010). The fungus may survive on the bark of its host plant (Arjunan, 1999). Seed-borne conidia and mycelium can live for four months (French, 2006) and one year (Punithalingam, 1976), respectively.

Botryodiplodia theobromae Pat. is the asexual stage of the pathogen *Botryosphaeria rhodina* (Berk and M.A. Curtis) Arx. with the sexual stage being *Physalospora rhodina* Berk. & Curt. apud Cooke. The name *Lasiodiplodia theobromae* has been recognized as a synonym of the fungus *Botryodiplodia theobromae* by a number of authors (Pitt and Hocking, 2009; Khanzada *et al.*, 2004).

2.2.1 **Geographical distribution of *B. theobromae***

The fungus is commonly found in the tropics and subtropics where temperatures are relatively high, between 25 °C and 30 °C (Hseu *et al.*, 2008; French, 2006; Khanzada *et al.*, 2004; Mascarenhas *et al.*, 1996; Alvarez and Nishijima, 1987). *B. theobromae* can grow from temperatures of 20°C to 45°C and has optimum growth at 30°C to 40°C but fails to grow at temperatures below 15°C (Khanzada *et al.*, 2006). The fungus is confined approximately to an area 40° north to 40° south of the equator (Pitt and Hocking, 2009; Punithalingam, 1976).

2.2.2 **Colony characteristics of *B. theobromae***

According to Philips (2007), *B. theobromae* colonies are often greyish sepia to mouse grey to black, fluffy with abundant aerial mycelium. Matured cultures have black pigmentation.

2.2.3 **Morphology of *B. theobromae***

Mature cultures of *B. theobromae* can develop pycnidia and further sporulate. In culturing media, the pycnidia may be found scattered, grouped or centered and visible (Plate 1). Pycnidia can also be found underneath or within the mycelium and may often be covered by bristles. Matured pycnidia can be up to 5mm wide (Pitt and Hocking, 2009).

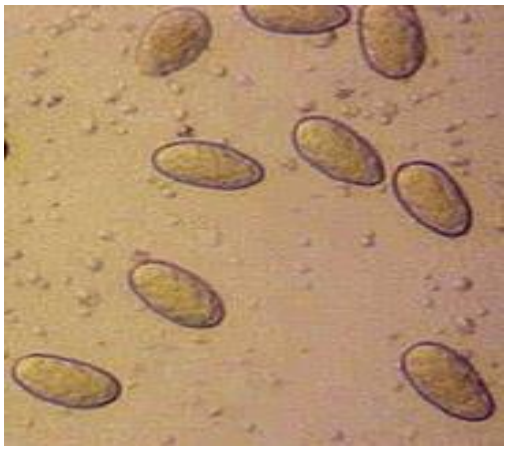
Sporulation is best achieved at a temperature of 28°C (Meah *et al*., 1991). However, Khanzada *et al.* (2006) also found out that maximum numbers of pycnidia are produced at a temperature range of 35°C to 40°C. The process of sporulation is influenced by several environmental factors and culture media composition (Saha *et al.*, 2008; Khanzada *et al.*, 2006). Factors such as irradiation by fluorescent light (Saha *et al.*, 2008) and exposure to ultraviolet radiation (Ekundayo and Haskins, 1969) enhance abundant pycnidia production and sporulation respectively. A study carried out by Khanzada *et al.* (2006) suggests that there is no influence of

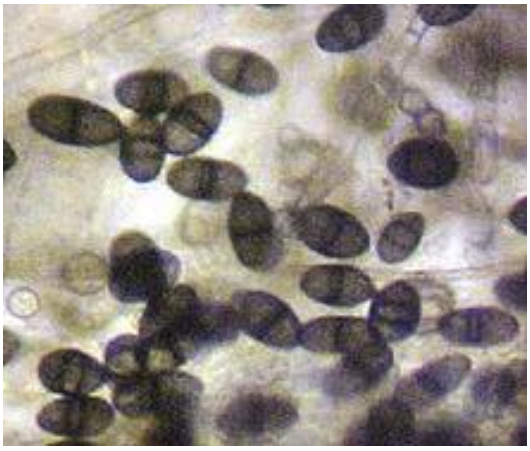
different light regimes on both mycelium growth and pycnidia production. The process of sporulation can be enhanced by incorporation into growth media substances such as ripe mango extract (Meah *et al*., 1991), dextrose or sucrose (Saha *et al*., 2008; Meah *et al*., 1991) and yeast extract and manitol (Khanzada *et al.*, 2006).

B. theobromae forms its spores endogenously within pycnidia (Mascarenhas *et al.*, 1996) from the inner layers of cells lining the pycnidial cavity and exudes spore or conidiophores as pink pigmented fluid (Meah *et al.*, 1991) or blackish fluid into the culture. The exudates are visible to the naked eye. The conidiophores are rarely branched, cylindrical, translucent, simple and sometimes having septations. Conidiogenous cells are translucent, simple, and cylindrical to ovoid, one celled and segmented (Mascarenhas *et al.*, 1996).

Conidia are also noted to be initially unicellular and translucent (Phipps *et al.* 1998; Alvarez and Nishijima, 1987), granulose, sub-ovoid to ellipsoid oblong, thick-walled and base-truncate (Alvarez and Nishijima, 1987) (Plate 2a). Matured conidia are one-septate (two-celled) and cinnamon to dark brown (Phipps *et al.*, 1998; Alvarez and Nishijima, 1987), thick walled, ellipsoidal and often longitudinally striated and 20-30x 10-15µm in size (Plate 2b). When paraphysis are present they are often translucent, cylindrical and sometimes septate and up to 50µm long (Alvarez and Nishijima, 1987).

Plate 1. Mature colony of *B. theobromae* showing protruding pycnidia full of spores.





(x 40) **(x 40)**

(a) **(b)**

Plate 2. Microscopic images showing (a) immature, translucent, unicellular conidia and (b) mature, dark brown, bi-celled, thick walled conidia of *B. theobromae* spores.
Courtesy: Khanzada *et al*. (2004).

2.3 DISEASES CAUSED BY *B. THEOBROMAE*

In Nigeria, Cameroon and Benin, *B. theobromae* has been identified as one of the fungus that causes rot disease of economic importance (Hillocks and Wydra, 2002). They explained that susceptibility of plant tissues to *B. theobromae* is dependent on the amount of sucrose and reducing sugars present. The higher the sucrose content, the higher the susceptibility (Tongdee *et al*. 1980).

B. theobromae has been noted to be primarily a wound parasite (Opoku *et al.* 2007; Ekundayo, 1973) and that diseases caused by the pathogen are often common among cassava tubers under water stress and/or cocoa pods wounded by insects, birds, rodents and man. Water stress has been noted as a predisposing factor to infected plants and enhancement of *B. theobromae* disease severity (Arjunan, 1999; Opoku *et al.*, 2007). Opoku *et al.* (2007) and Ekundayo (1973) again explained that the disease is more prevalent in the dry season and common in tubers with bruises or natural openings.

Typical examples of *B. theobromae* disease include, tuber rots in yam, sweet potatoes and cassava during storage (Ekundayo, 1973), collar rot of peanuts (Phipps and Porter, 1998), stem end rot of banana fruit (Jones *et al.* 2000), stem end rot of mango (Meah *et al.*, 1991), stem rot of

pawpaw and leaf spot on citrus (Alvarez and Nishijima, 1987). It is also associated with die-back and pod rot of cocoa (Phillips, 2007).

2.3.1 **"Charcoal rot" on Cocoa**

Opoku *et al*. (2007) describes the first symptom as a brown spot on the infected pod that latter turns black. The spot further enlarges until the whole pod blackens, a situation referred to as "charcoal rot" because of the thick black sooty spores of resemblance to charcoal (Opoku *et al.*, 2007) (Plate 3b). The spread of the brown necrosis can sometimes be so rapid that it masks the symptoms produced by the primary pod rotting fungus, *Phytophthora* sp. The tissues also show grayish-black mycelia on the surface (Opoku *et al*., 2007).

When young pods are attacked by *B. theobromae* they shrivel and hang as mummies (Plate.3a). A recognized distinction of "Charcoal rot" by *B. theobromae* is the drying out of infected pods with the production of large numbers of black spores having the appearance of soot on the surface of the pods, a situation very different to the white mycelium of the black pod rot by *Phytophthora* sp (Opoku *et al.*, 2007). Another distinction is that, where as the husk of pods with "Charcoal rot" are soft that of the black pod disease is hard and firm. A remarkable symptom is that affected pods show rotten internal tissues and the beans turn black (Plate 3b)

(a) **(b)**

Plate 3. (a) A cocoa tree showing mummified pods (Red Curly brackets) and (b) Charcoal rot disease of a cocoa pod showing rotten and blackened internal tissues and beans caused by *B. theobromae.*

2.3.2. **Stem-end rot of Mango**

The rot starts as a dark-brown firm decay at the epicarp around the base of the pedicel of the mango fruits in the initial stages of infection (Plate 4b) (Faber *et al.*, 2007; Arjunan, 1999). This results in blackish discoloured regions of decay in the disease parts (Plate 4a). The affected area then enlarges to form a circular black patch and, under humid conditions, extends rapidly to engulf the whole fruit and turns it completely black within few days. This makes the fruit dark and shriveled with the pulp becoming brown, softer and watery (Faber *et al*., 2007; Arjunan, 1999). The rot is most often initiated during harvest when fruits are injured around the stem-end and wounded parts through poor handling. Upon infection of the fruit, the rot does not develop until the fruit starts ripening (Faber *et al.*, 2007; Sangchote, 1988). Stem-end rot caused by *B. theobromae* has been rated as the next most important disease to anthracnose caused by *Colletotrichum gloeosporioides* and also the predominant rot of mango fruits (Sangchote, 1988).

In 1988, Sangchote studied stem-end rot disease of mango caused by *B. theobromae* and observed that isolates of the fungus from different sources caused different levels of disease severity. Isolates from diseased mango fruits were found to be most virulent.

2.3.3 **Die-bark of Mango**

The symptoms of die-back of mango caused by *B. theobromae* include discolouration and darkening of the bark of the stem (Arjunan, 1999). The darkened area advances to other parts of the tree, young green twigs wither and affected leaves turn brown with margins rolling upwards (Khanzada *et al.*, 2004). The twigs and branches then die, shrivel and fall sometimes with exudation of gum (Khanzada *et al*., 2006; Arjunan, 1999). In severe conditions drying starts from the branches one after another in a sequence resulting in death of the whole tree. Infected twigs also show brown streaks of their vascular tissues when twig is split open lengthwise (Plate 5) (Khanzada *et al.* 2006; Khanzada *et al.*, 2004; Arjunan, 1999). Jiskani (2002) also reported that the fungus was the most frequent isolate identified on diseased mango and appeared to be the primary cause of die-back in Pakistan. Mascarenhas *et al.* (1996) also identified the fungus to be the major post-harvest pathogen of mango.

As a remedy to the situation Sangchote (1988) has proposed that, to reduce the sources of inoculum, inter planting of host plants must be avoided. Also diseased twigs and diseased fruits must be eliminated.

(a) **(b)**

Plate 4. Diseased mango fruits showing (a) blackish regions of decay and (b) Stem-End rot with discolouration at the stem end caused by *B. theobromae*.

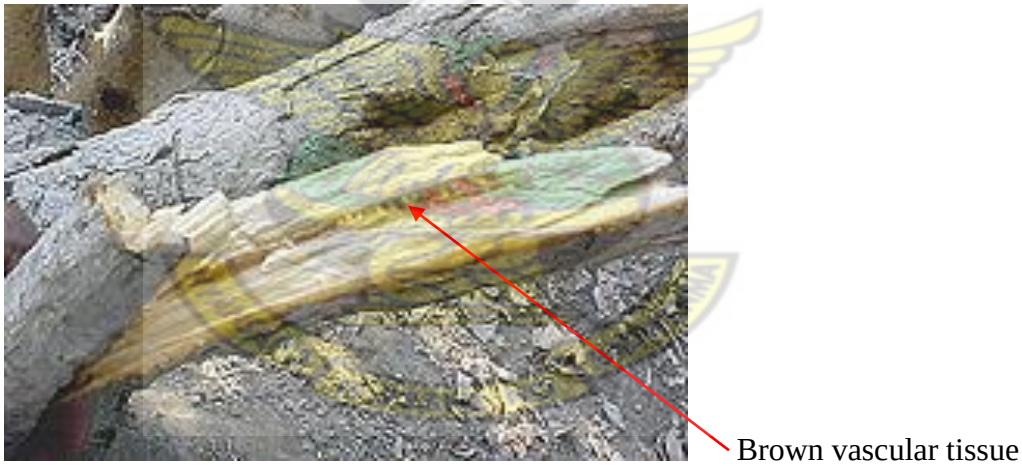


Plate 5. Image of a *B. theobromae* infected mango twig showing brown vascular tissues.

2.3.4 **Crown rot disease of banana**

The major disease caused by the fungus on banana is the crown rot of banana fruits (Plate 6). This is a rot that is characterized by blackening of the crown and the extension of the rot towards and into the fingers. In 1966, Williamson and Tandon conducted some pathological studies on *B. theobromae* in India and found that *B. theobromae* rot of banana was a very common market disease with high magnitude of losses being incurred. The pathogen was observed to be one of

the commonest pathogens responsible for post-harvest decay of bananas. They indicated that the percentage of rot of the banana fruit was highest at 25 °C and 30 °C with the lowest rot occurring at 7 °C.

Plate 6. Crowns of banana fruits with crown rot disease caused by *B. theobromae*.

2.3.5 **Rot disease of Yam**

The disease incidence involves stem rot and silvering of yam leaves. It has also been noted to cause yams that are not growing well to die off more quickly. The fungus also causes soft rots (Plate 7a and b) of yam tubers after harvest (Rossel *et al.*, 2008).

(a) **(b)**

Plate 7. Yam tubers showing soft rot disease caused by *B. theobromae*, on (a) cut surface and (b) uncut tuber.

2.4 FACTORS THAT PROMOTE THE SPREAD OF *BOTRYODIPLODIA THEOBROMAE* DISEASE IN CROPPING SYSTEMS IN GHANA

2.4.1 **Intercropping susceptible crops**

The major concern in intercropping *B. theobromae* susceptible crops is the possibility of alternative hosts occurring. In most situations, if one or more of the crops is infected with *B. theobromae*, it may breed spores. This can serve as a source for inoculation onto other healthy crops by vectors or man (Lichtfouse *et al*., 2009). This makes control of the fungal disease very difficult and expensive.

2.4.1.1 Plantain or banana intercropped with maize

This is the practice where the maize precedes the plantain or banana often in the first season. The intercropping of maize often continues until leaves of the plantain or banana do not allow space and penetration of sunlight for the healthy growth of the maize (Banful, 1998). An advantage of the system is the provision of mulch to the soil and the pseudo-stem of the plantain or banana by the stover of the maize crop after cobs have been harvested (Banful, 1998). French (2006) recognizes a disadvantage of the system as maize (corn) is susceptible to *B. theobromae* and also favours the increase in population of the pathogen.

2.4.1.2 Plantain or banana intercropped with tree crops

Usually, with the exception of cocoa, tree crops such as oil palm and citrus are put in plantations before plantain or banana (Banful, 1998). In the case of cocoa farms, plantains or bananas are planted first to provide a source of shade for young cocoa seedlings. This is a practice that is recommended in the agronomy of cocoa cultivation. The disadvantage of such a system is that some of the tree crops serve as alternative host to *B. theobromae* (Opoku *et al.* 2007; Phillips, 2007; Jones *et al.*, 2000).

2.4.1.3 Intercropping system in cocoa cultivation

Most often than not, when farmlands are cleared indigenous fruit crops, medicinal and timber trees are deliberately retained. Such crops or trees are deliberately left within cocoa farms to serve as a source of shade for both young cocoa seedlings and the farmer. Food crops such as maize, plantain and banana, and cassava are cultivated within three to five years while the cocoa trees mature. This practice is very common within the cocoa growing areas in Ghana. The system is sometimes intensified with the planting of other multi-purpose fruit trees such as mango, pear, guava and citrus species and many more (Anonymous, 2010). But as noted by French (2006), crops such as pear or avocado, citrus and mango are very susceptible to *B. theobromae* attack and its associated disease, hence, may serve as alternative hosts.

2.4.1.4 Intercropping system in mango cultivation.

Intercropping of cereals, legumes and tuber crops has been identified to be a common practice in certain parts of Ghana where mango plantations are common. The intercropping is often done in the first few years when the mango trees have not started fruiting. This is, however, discontinued at the onset of fruiting (ADRA, 2010). The disadvantage is that the plantation becomes a haven of the pathogen through seasonal cultivation of *B. theobromae* susceptible crops. Increase population of vectors of the fungus becomes eminent in such a system. This then eventually leads to total infestation of the plantation.

2.4.1.5 Intercropping system in yam cultivation.

A notable practice of intercropping in yam farms in Ghana is the use of intercrops such as cocoyam, cassava, egg plant, pepper and other vegetables. Since tubers of yam are often underground, injury or damage to the tubers from farm implements due to intercropping of other crops and the subsequent infestation of the tubers by *B. theobromae* may occur unnoticed (FAO, 1998).

2.5 **CONTROL AND MANAGEMENT OF *BOTRYODIPLODIA THEOBROMAE* DISEASES**

2.5.1 **Improved Sanitation**

The rot disease caused by *B. theobromae* can be minimized or prevented by adoption of good sanitation practices as well as optimal cultural practices that ensures minimization of fruit rots (Faber *et al.*, 2007). Practices such as pruning of dead branches and twigs, removal and disposal

of dead wood and infected fruits or pods away from trees by burying or burning controls the spread of rot disease.

2.5.2 **Cultural Practices that minimize *B. theobromae* infections**

Harvesting during dry conditions and ensuring proper handling such as those that prevent shaking off of pedicels of fruits and injury to fruits and tubers have also been noted to minimize the fungal disease (French, 2006). Careful handling avoids damage that serves as the entry point for the fungus as *B. theobromae* attack can only occur through wounds (Opoku *et al*., 2007).

Losses from the attack are reduced by the control of pests which attack pods, fruits and tubers (Opoku *et al.*, 2007). Faber *et al.* (2007) emphasize measures such as the availability of sufficient irrigation, correction of environmental and nutritional stresses as well as maintenance of thick layers of mulch under trees as a safe way to eliminate some of the pre-disposing factors. Storage of fruits at temperatures below 10°C has also been indicated by French (2006) to be normally sufficient to reduce the damage from the storage rot fungus.

Known control of the fungus identified by French (2006) involves techniques such as proper rotation of susceptible crops to avoid nematode damage. Putting such crops first in rotation helps to escape the increasing population of the fungus which is favoured by crops such as maize and peanuts.

2.5.3 **Chemical control of *B. theobromae* disease or rot**

Management of rot disease includes cases where mango fruits are dipped in 6% borax solution at 43°C for three minutes (Arjunan,1999) or in benomyl at 500ppm concentration at 52°C for five minutes (Sangchote, 1988) to destroy the fungus. However, spraying of chemicals such as Carbendazim or Topsin-M (0.1%) or Chlorothalonil (0.2%) in the field before harvesting of mango fruits (Arjunan, 1999) as well as the use of a paste of Bordeaux mixture or Carbendazim on storage tubers (Jiskani, 2002; Narasimhudu and Reddy, 1992) provides a very effective control against the disease.

Jiskani (2002) further recommend a treatment of 1% Bordeaux mixture followed by 0.8% Bordeaux mixture and Carbendazim for yam tubers. Fungicides, such as Dithane M45, applied at a rate of 50g in 20L of water are also used as a preventive measure against the fungus on yam

tubers (Rossel *et al*., 2008). On cocoa, control can be provided by spraying of 1% Bordeaux mixture.

The die-back disease is favoured by relative humidity above 80% and temperature of 25-31°C and rain. Therefore pruning and destruction of infected twigs as well as spraying Carbendazim or Thiophanate Methyl (0.1%) or Chlorathalonil (0.2%) on trees every fortnight, especially during the rainy season provides a control of the disease (Khanzada *et al*., 2005).

2.6 **FUNGAL ISOLATION**

Isolation of target fungi from plant material is influenced or affected by factors including the method of surface sterilization, the plating procedures, the culturing medium, the incubation conditions and, most importantly, the nature of the diseased host tissue (Waller *et al*., 2002). Sample diseased plant tissues with typical symptoms of the disease or signs of the pathogen being investigated are used for plating and isolation of target fungus.

Soon after sterilization or prior to plating of surface sterilized samples, drying under a filtered air flow, often in a sterile hood, on sterile paper tissues is very much recommended (Waller *et al*., 2002). This prevents the growth of bacteria from the tissues.

2.6.1 **Medium for growth of fungi**

The choice of medium for plating and subsequent culturing of target fungi depends largely on the nature of the tissue involved and the nutritional requirement of the fungi. Low nutrient media such as water agar (WA) media, quarter-strength potato dextrose agar media (PDA) and Carnation Leaf-Piece Agar (CLA) media are often used for isolation of fungi from larger tissues and larger roots (Ritchie, 2002). Amendments, such as inclusion of antibiotics, are also recommended in instances where bacteria growth interferes with the recovery of target fungi. However, with the exception of Chloramphenicol antibiotic, some antibiotics, such as Penicillins, Neomycin and Streptomycin in excess dosages, are fatal to the growth of most fungi and are also selective to either Gram-positive or Gram-negative bacteria. Chloramphenicol, however, is active against both Gram-negative and Gram-positive bacteria (Plant pathologist's Pocket book, 1968).

Saha *et al.* (2008) found that among several carbon sources tested on *B. theobromae*, glucose and sucrose were superior for growth. Carbohydrate-rich media favours the growth of fast-growing saprophytic fungi such as *Trichoderma* spp. and most mucoraceous fungi. Also Khanzada *et al.* (2006), upon evaluating the effect of some growth conditions on the *in vitro* growth and sporulation of *Lasiodiplodia theobromae* isolates from mango, established that Potato-sucrose agar (PSA), Corn meal agar (CMA) and Yeast-extract manitol agar (YEMA) were most suitable for mycelial growth. PDA has also been identified by Kausar *et al*. (2009) to give best mycelium growth of *B. theobromae* and it is strongly recommended for the isolation of slow-growing fungi or isolation from very fine roots.

Saha *et al.* (2008) also observed that potassium nitrate supplemented media, amongst the inorganic nitrogen sources, showed maximum growth of *B. theobromae* with peptone-supplemented media producing maximum growth amongst the organic nitrogen sources tested. Increasing concentration of urea and Di-Ammonium phosphate (DAP) reduces the growth of mycelium and production of pycnidia of *B. theobromae* (Khanzada *et al.*, 2006).
Whereas YEMA (Khanzada *et al.*, 2006) was identified to be the best medium for pycnidia formation, Saha *et al.* (2008) identified tea root extract supplemented potato-dextrose agar media to be most suitable for both mycelial growth and sporulation.

pH of growth media has also been noted to have drastic influence on growth of *B. theobromae.* Saha *et al.* (2008) found pH range of 3.0 to 8.0 and optimum pH of 6.0 to be suitable for the growth of *B. theobromae*. He further recommended tea root extract supplemented potato dextrose agar medium with pH 6.0 to be most suitable for conidia production by *B. theobromae* at a temperature of 28°C.

2.7 **INVESTIGATIONS ON HOST RANGE**

Studies of fungal pathogens and their interaction with plants have been performed using a number of approaches, from classical genetics, cell biology and biochemistry to modern, holistic and high-throughput techniques (González-Fernández *et al.*, 2010). One of the very prominent and commonly used techniques is the "Koch's postulate" test established by German bacteriologist, Robert Koch, 1883. This is an accepted scientific method for identifying the correct causal agent of a disease (Cohen, 1994) including plant diseases. The postulate involves

four basic steps. It starts with the finding of the same pathogen in every case of the disease based on clear symptoms. Then the suspected pathogen isolated from the diseased organism, cultured in an appropriate medium, and maintained in pure culture. The pathogenic ability of the pathogen is established by infecting a healthy susceptible test host with the pure form of the pathogen and a careful observation made to identify the disease as same to that of the original disease. The final stage of the postulate is the re-isolation of the pathogen from the test host into its pure culture and comparison made with the pure culture inoculum to confirm it as same. These rules of proof are referred to as "Koch's Postulates."

2.8 **CHARACTERIZATION OF FUNGAL SPECIES AND STRAINS**

2.8.1 **Morphological Characterization**

The characterization of fungal strains on the basis of their morphological characters is a very common approach to understanding and knowing the true nature of most fungi, particularly, pathogenic fungi. It often starts with the isolation of the fungus understudy on an appropriate media and developing of pure cultures from them. This is then followed by studies on growth behaviours of the fungal strains often on Potato Dextrose Agar (PDA) media in sterilized Petri plates as perfomed by Kokub *et al.* (2007), Iotti *et al.* (2005) and Photita *et al.* (2005). Morphological characteristics such as growth rates, mycelium condition, radial or diameter of colony, development of fruiting bodies and size, shape, colour, type and location of fruiting bodies for each strain are observed. Normally, the observations are recorded at regular intervals of hours or days and over required period of time that can run into several months.

Another notable study on morphological characterisation of fungal strains is mycelial compatibility or incompatibility reaction often done on Potato Dextrose Agar media on Petri plates as described by Punja and Sun (2001). This is done by marking PDA plates into portions depending on the number of combinations desired. Mycelial disc of respective strains are inoculated on each portion of PDA plate and incubated at required temperature for a number of days. Plates are normally replicated. This is then examined daily for the development of clearing zones in the region of mycelial contact, an indication of antagonism. In most research work, morphological characterisation is often related to the molecular characterization and completed with phylogenic studies.

2.8.2 **Molecular Characterization**

Molecular characterization provides a great potential for fungal diversity assessment (Liew *et al*., 1998). It is not restricted by the limits imposed by morphological characteristics. This is often done after morphological studies have been carried out on fungal strains under study. It basically supports morphological variations or similarities observed, with genetic proof of variability or similarity with ultimate clustering or separation of strains into groups of close similarities (Kokub *et al.*, 2007; Iotti *et al.*, 2005; Photita *et al.*, 2005).

The process of morphological characterisation of fungal strains, as seen in most studies, begins with the extraction of fungal genomic DNA. The DNA is often obtained from spores or young mycelia grown in appropriate media or broth, such as the Potato Dextrose Broth or V8 juice medium (Photita *et al.*, 2005), incubated for few days under specified conditions of agitation or without shaking or gradual swinging, and illumination or under dark condition. This results in production of sufficient spores or young mycelia rich in genomic DNA.

Harvested mycelium or spores are rinsed with sterile water, frozen with liquid nitrogen and ground using a sterile mortar and pestle. Appropriate methods and protocols are then used to extract the total genomic DNA from the spores or mycelium. Some of the known methods and procedures used by some authors include the CTAB method (Photita *et al.*, 2005), the method of Punja and Sun (2001), and DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) (Iotti *et al.*, 2005). Concentrations of total genomic DNA obtained from strains under study are also estimated by absorbance at 260 nm (Photita *et al.*, 2005).

Molecular techniques such as polymerase chain reaction (PCR) and the use of various primers including Random Amplified Polymorphic DNA (RAPD) primers (Kokub *et al*., 2007), Simple Sequence Repeat (SSR) primer and Internal Transcribed Spacer (ITS) primers (Photita *et al.*, 2005; Iotti *et al.*, 2005) among others, are used to amplify various or desired regions or sequences of the genomic DNA to be studied.

The process of PCR provides a simplified and ingenious way of exponentially amplifying specific DNA sequences through *in vitro* DNA synthesis (Henson and French, 1993). The process involves three main steps. It starts with an initial melting of target DNA, which is

followed by the annealing of two oligonucleotide primers that binds to the denatured DNA strands. The process ends with the final primer extension step by a thermostable DNA polymerase, primarily *Taq* DNA polymerase. Several cycles of the three steps are often undertaken to achieve the desired amplification (Henson and French, 1993; Mullis and Faloona, 1987; Liew *et al*., 1998)).

The PCR products obtained are further resolved on agarose gel through electrophoresis and DNA bands produced are visualized under UV illumination. Amplified regions of genomic DNA are then studied and compared with that of other strains (Kokub *et al*., 2007; Iotti *et al.*, 2005) or further sequenced (Photita *et al.*, 2005) and the data documented.

Genetic data often obtained from bands of DNA from electrophoresis are fed into various computer clustering programs such as DNAPAR and DNAML program (Iotti *et al.*, 2005), Clustal V (Higgins, Bleasby and Fushs, 1992), PAUP (Swofford, 1993), NTYSYS program with UPGMA method or SAHN program (Kokub *et al*., 2007), PHYLIP (Felsenstein, 1993), and Neighbour-joining (NJ) method with Jukes-Cantor (one-parameter) or K2P (two-parameter) method (Photita *et al.*, 2005). A final dendrogram or phylogenetic tree is produced by the use of distance matrix value (NJ method), evolutionary distance matrix (DNAPAR and DNAML program) and similarity coefficient (UPGMA method). The matrices basically compare the presence or absence of specific fragment or band.

The basic principle behind the various methods of linking, joining or relating fungi or their strains, based on their genetic similarity or distance matrices, is the presence of sequences from which phylogenetic information could be inferred. Such sequences are present in genomes either as single copy or several copies that have evolved together. The sequence or sequences exhibit similar functionality within the taxa of the fungi. They also evolve at a constant rate.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 STUDY SITES

The pathological studies and DNA extractions of the work were carried out at the Pathology and Molecular Biology laboratories, respectively, at the Crops Research Institute of the Council for Scientific and Industrial Research (CSIR-CRI) at Fumesua in Kumasi. The PCR work was done at that Molecular Biology laboratory of the Department of Biochemistry and Biotechnology of Kwame Nkrumah University of Science and Technology (KNUST) with assistance from the KIRKHOUSE Trust laboratory of the Cocoa Research Institute of Ghana (CRIG), Akim Tafo.

3.2 COLLECTION AND STORAGE OF DISEASED FRUITS, PODS AND TUBERS

Cocoa, mango and banana fruits, and yam tubers with symptoms of diseases caused by *Botryodiplodia theobromae* were collected from four agro-ecological zones in Ghana (Plate. 8). These were the forest transitional zone in the Brong-Ahafo Region, forest zone in the Ashanti Region, savanna zone in Northern Region and the coastal savanna zone in the Central Region.

The collected diseased samples were stored in a refrigerator at 4°C in the laboratory until required.

3.3 **PREPARATION OF MEDIA**

3.3.1 **Preparation of Potato Dextrose Agar (PDA)**

Composition and preparation of the PDA medium for the isolation of the fungus followed the procedure described. Unpeeled Irish potatoes were washed clean, cut into smaller pieces and 200 g weighed and boiled in 300 ml of distilled water for 30 minutes to cook. The pulp was obtained by grinding cooked potatoes with pestle and the slurry squeezed through a fine sieve into a pyrex bottle. Seven hundred milliliters (700 ml) of distilled water was then added to make 1 litre. With the aid of a magnetic stirrer 20 g each of agar and glucose were added to complete the medium. The medium was next sterilized in an autoclave at 121°C and 15 p.s.i pressure for 25 minutes, allowed to cool and then stored in a refrigerator at 4°C until required.

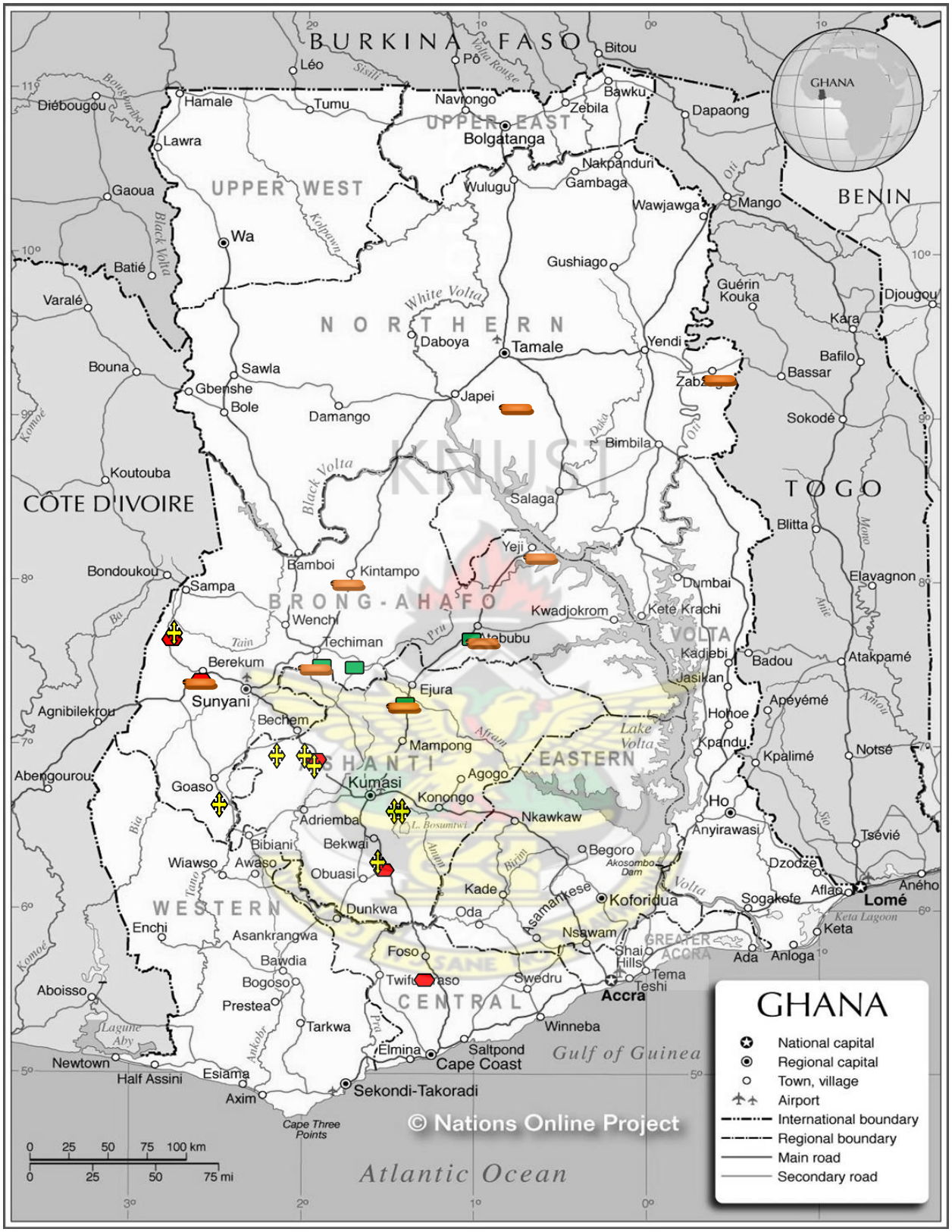


Plate 8. Map of Ghana showing the sources/location (Agro-ecological zones) from which sample diseased crops were collected.

- Location of collection of sample disease mango fruits
- Location of collection of sample disease yam tubers
- Location of collection of sample disease cocoa pods
- Location of collection of sample disease banana fruits

3.3.2 **Preparation of Water Agar Media (WA)**

Two percent (2%) water agar medium was prepared by dissolving 10 g of agar in 500 ml of sterile distilled water and autoclaved at 121°C and a pressure of 15 p.s.i for 25 minutes. This was allowed to cool and stored at 4°C in a refrigerator until required.

3.3.3 **Preparation of V8 juice media**

V8 juice liquid medium was prepared by dissolving 45.5 g of Czapek Diox media, 1 g of mycological peptone, 1 g of acid casein hydroxylate and 1 g of yeast extract in 1 litre of distilled water. Two hundred milliliters (200 ml) of V8 juice was added and the pH maintained at 7. The solution was sterilized in an autoclave at 121°C and a pressure of 15 p.s.i for 25 minutes. The solution was stored after cooling, in a refrigerator at 4°C until required.

3.4 **ISOLATION OF THE FUNGUS**

Pieces of diseased tissues from the collected crop samples were obtained from diseased lesions. The cut pieces from each crop were surface sterilized in 5 % Sodium hypochlorite solution for 5 minutes. The surface sterilized tissues were washed three times in sterile distilled water and air dried in a sterile Lamina Flow Chamber. The dried pieces were plated on potato dextrose agar (PDA) in Petri dishes. The plated diseased tissues were then incubated at 28°C. Fine hyphae that grew from the margins of the diseased tissues were sub-cultured on fresh PDA. Sub-culturing was carried out finally on PDA amended with Chloramphenicol. The pure isolates of *Botryodiplodia theobromae* obtained were maintained on PDA.

3.5 **PRODUCTION OF SINGLE-SPORE CULTURES OF ISOLATES**

Pure culture plates of the *Botryodiplodia theobromae* isolates were incubated at 28°C under flourescent light for 35 days to enhance sporulation. With the aid of an inoculation needle, matured pycnidia produced after 35 days of incubation were transferred into sterile distilled water on a sterilized glass slide and then teased to diffuse spores. The solution on the slide was then observed under a compound microscope to confirm the presence and even distribution of spores. Slides with *B. theobromae* spores identified were then inverted and pressed onto the surface of solidified water agar (WA) media to obtain their growth.

Germinated single spores of each *B. theobromae* isolate from the WA media was transferred onto PDA media amended with chloramphenicol and then incubated at 30 ºC for seven days as recommended by Shah *et al.* (2010). Single-spore pure cultures obtained were then maintained on PDA and preserved at 4ºC in a refrigerator. Single-spores were used to generate all cultures used in the morphological and molecular studies.

3.6. **PATHOGENICITY STUDIES**

The pathogenicity of the *B. theobromae* isolates was studied by inoculating healthy mango fruits and cocoa pods at their physiological maturity. Fruits of Kent mango and Akokra Bedi cocoa varieties were used in these experiments. Fifteen (15) fruits of each crop were used. Four representative *B. theobromae* isolates were used for the inoculation. They were isolates from banana from Goaso (B(Go)), mango from Atebubu (M(At)), cocoa from Drobo (C(Db)) and yam from Kintampo (Y(Kp)) (Table 2) . The isolates were randomly selected. The experiment was conducted in a complete randomized design with 3 replicates. Each replicate consisted of five fruits; four of them inoculated with only one specific *B. theobromae* isolate. In the control, the mango fruits and cocoa pods were not inoculated with the pathogen.

3.6.1 **Inocula preparation for pathogenicity test**

With the aid of a 5 mm-diameter cork borer, discs of mycelia were cut from the margins of actively growing pure cultures of isolates of *B. theobromae* from the representative samples. The discs of mycelia were then transferred onto PDA media and incubated at 28°C for five days. The inocula from five day old actively growing pure cultures were used for the inoculations.

3.6.2 **Inoculation of healthy fruits with *B. theobromae* isolates.**

Fifteen healthy mango fruits and 15 healthy cocoa pods at their near ripen stage, of similar sizes and same physiological maturity were obtained from one Kent mango tree and one Akokora Bedi cocoa tree, respectively. The fruits were washed and surface sterilized in 5% sodium hypochlorite solution for 5 minutes. The fruits were then washed three times with sterile distilled water. The disinfected fruits were then allowed to dry in a sterile hood before inoculating with the fungal isolates.

A disc (0.5 cm depth) of mango or cocoa fruit tissue was removed from two directly opposite sides of each fruit to be inoculated, using a 5 mm diameter cork borer (Plate 9a). Mycelia plugs from five day old representative *B. theobromae* isolates were cut with a sterile 5 mm diameter cork borer. The mycelia plugs were inserted into holes made in the healthy mango or cocoa fruits to be inoculated (one plug per hole) (Plate 9b). After insertion of the mycelia plugs, the removed fruit discs were repositioned. The edges of the wounds were sealed with melted wax (Plate 10a). Controls were set up, in which fruits were not inoculated with mycelia plugs. The inoculated fruits and controls were incubated at 28°C (Plate 10b). The fruits were examined daily and disease development recorded.

(a) **(b)**

Plate 9. (a) Puncturing of a mango fruit with a 5 mm diameter cork borer and (b) inoculation of fruit with inverted mycelia plug.

(a) **(b)**

Plate 10. (a) Sealing of inoculated area of fruit with melted candle wax and (b) subsequent ripening of inoculated fruits in the incubation room.

Daily measurements of the longest radial spread of the lesions resulting from the inoculations were recorded. Koch's postulate was completed by re-isolating *B. theobromae* from the fruits and pods that developed symptoms characteristic of *B. theobromae* infections in mango and cocoa, respectively.

3.7 MORPHOLOGICAL CHARACTERIZATION OF ISOLATES

3.7.1 Growth of isolates on PDA

Single-spore cultures of the various *B. theobromae* isolates were grown on PDA in 90 mm-diameter Petri dishes for comparison of growth and morphological characters. Five-millimeter discs from margins of actively growing 7-day-old fungal cultures of all 25 isolates of *B. theobromae* were centrally placed on PDA in Petri dishes. Each isolate was in triplicate. Growth of colonies was measured at 24 hr intervals for six days of incubation at 28°C. Pigmentation produced by each isolate was documented. Colony texture, pycnidia and spore production were also documented over time.

3.8 **MOLECULAR CHARACTERIZATION OF ISOLATES**

3.8.1 **Genomic DNA isolation**

Four-day old mycelia from single-spore cultures of the 25 *B. theobromae* isolates from mango, cocoa, banana and yam were used to inoculate the V8 juice medium. The inoculated media were incubated at 28°C on a rotary shaker for 48 hrs. Mycelia of each isolate were harvested, 48 hrs after incubation, washed with sterile distilled water and ground in liquid nitrogen. The BioTeke Plant genomic DNA fast isolation Kit (Spin Column) and its accompanying protocol (Appendix IV) were used to extract DNA from the 25 *B. theobromae* isolates. The integrity of the DNA was checked by resolving on a 0.8% agarose gel.

3.8.2 **Storage of extracted DNA of isolates**

As per the BioTeke plant genomic DNA extraction protocol (Appendix IV), 50µl of Elution buffer was added to the extracted DNA contained in a 1.5 ml micro-centrifuge tube. The individual tubes containing the DNA of each isolate were labeled to indicate the source of isolate, i.e., place of collection of sample and the type of crop. The tubes with DNA were stored in a refrigerator at 4°C for later use. Frozen DNA was thawed on ice before use.

3.8.3 **PCR Amplification**

3.8.3.1 Primer selection

The SSR and RAPD primers used for amplification of DNA of the isolates are shown in Tables 1a and 1b. After test runs, RAPD primer MH-S1118; SSR primers, MH-LAS_15 and_16, and MH-BOT_19 and_20 produced the best amplifications. These primers were therefore used for the amplification of the genomic DNA of all the 25 *B. theobromae* isolates.

3.8.3.2 Amplification of DNA

DNA of the isolates was amplified using RAPD primer MH-S1118 and SSR primers, MH-LAS_15 and_16, and MH-BOT_19 and_20. The PCR was performed in 0.2 ml PCR tubes with each tube containing a reaction volume of 10 µl. The reaction volume comprised of 8 µl of the AccuPower PCR premix (5.5 µl sterile milli Q water, 1 µl 10X buffer, 1 µl $MgCl_2$ (25 mM), 0.4 µl dNTP mix (10 mM), 0.1 µl Taq polymerase (5 µg/l)), 1 µl primer i.e. 0.5 µl each of forward

and reverse primer of SSR, and a 1 µl DNA template. The 1 µl volume of DNA was used as a template in each PCR tube to ensure sufficiency of parent DNA.

Eight micro-litres (8 µl) of PCR premix was pipetted into 25 labeled microfuge tubes. In addition, 1 µl of specific primer (1 µl of RAPD primer or 0.5 µl of each forward and reverse primer of SSR primer) were pippetted into the Accupower PCR premix. This was vortexed for a short time and then centrifuged for a short time. The primer-PCR premix mixture (9 µl) was pippetted into labeled 0.2 ml PCR reaction tubes. Genomic DNA (1 µl) was pippetted into their specific labeled 0.2 ml PCR reaction tubes. The final composition was then centrifuged in an Eppendorf centrifuge for a short time.

Amplification of the final composition for SSR primer and RAPD primer was conducted in an Eppendorf Mastercycler. The amplification programme for SSR primer consisted of a denaturation phase at 95°C for 2 minutes followed by 35 cycles at 95°C for 30 seconds, annealing of primers at 52-66°C (specified for each primer in Table 1a) for 40 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 10 minutes.

The RAPD primer-composition was amplified in a programme of a denaturation phase at 94°C for 1 minute followed by 34 °C for 1 minute for annealing of primer to DNA template, extension at 72°C for 2 minutes and a final extension at 72°C for 5 minutes.

3.8.4 **Electrophoresis of PCR products**

The following processes were repeated and conducted separately for PCR products of SSR and RAPD primers. All amplified PCR products were allowed to cool for a while on ice after which 2 µl of Bromophenol blue was added and centrifuged for a short time. By mixing each time with a pipette, 8 µl of each PCR product was loaded into separate wells in a 2.5% agarose gel in 1 X Tris borate EDTA buffer (0.5 M Tris, 0.05 M boric acid and 1 mM EDTA, pH 8.0) and stained with 4 µl ethidium bromide (Plate 11). Five micro-litres of PCR ranger 50bp DNA ladder (50bp – 1000bp) was also loaded into a separate well to serve as a marker. Each set of PCR products i.e. that of SSR primers and that of RAPD primers were resolved separately through electrophoresis in an electrophoretic chamber using the electrophoresis equipment (Consort power system). PCR products were run at 120 V 100 mA 50 W for 1 h.

Plate 11. Loading of PCR products onto 2.5% agarose gel for electrophoresis.

3.8.5 **Visualization and image production of DNA bands**

All DNA bands were visualized under UV light by transferring the agarose gel containing the fractionated PCR products unto a High Performance Ultraviolet Transilluminator. The image produced was captured with a 12.1 Mega pixels camera (Fujifilm).

3.9 **DATA ANALYSIS**

Statistical analysis: Data on the length of the longest radius of lesions caused by the isolates on the mango fruits and cocoa pods were analyzed using the PROC GLM analysis of variance (ANOVA) procedure of Statistical Analysis System (SAS). Where ANOVA showed significant difference between means ($P<0.05$), post-hoc mean separation was done using the Student-Newman-Keul test (SAS Institute Inc., 2003).

Data generated with the SSR and RAPD primers were converted into binary matrices. Analysis was made based on the number of specific DNA bands that isolates share or do not have. Using the SAHN method with the NTSYS 2.0 pc software a final dendrogram was constructed.

Table 1a. Information of Simple Sequence Repeat primers.

Oligo/Primer Name	Sequence	Molecular Weight	Annealing Temperature (°C)	%GC
SSR				
MH-LAS _13 and_14 F	GAG-TTG-TTA-GTG-CGG-GCG-CC	6205.1	66	65
MH-LAS _13 and_14 R	GCA-GCC-CCA-CAA-TTC-ACC-AG	6015.9	64	60
MH-LAS_15 and_16 F	GCC-AGA-TCC-GTG-CCC-ACT-G	5749.8	64	68.4
MH-LAS_15 and_16 R	CAT-GCA-GAG-GTC-GCA-AAG-TG	6191.1	62	55
MH-BOT_11 and_12 F	CGG-CAT-GGT-CTG-CCG-CTC-C	5756.7	66	73.7
MH-BOT_11 and_12 R	GCA-TCT-CCG-GCT-ACC-AAC-CG	6022.9	66	65
MH-BOT_19 and_20 F	GGC-GGT-CGC-AGA-TGC-GGT-C	5885.8	66	73.7
MH-BOT_19 and_20 R	GCC-CTA-TTC-TGC-GTG-CCT-CC	5995.9	66	65
MH-BOT_35 and_36 F	CTC-CAT-CCT-GAT-CCA-GGG-TCC	6318.1	53.1	61.9
MH-BOT_35 and_36 R	GAC-GAA-TCA-AGC-GGG-CTG-CCC	6441.2	55.1	66.7

Table 1b. Information of Random Amplified Polymorphic DNA primers.

Oligo/Primer Name	Sequence	Molecular Weight	Annealing Temperature (°C)	%GC
RAPD				
MH-S111	CTT-TCC-GCA-GT	3283.2	34	54.5
MH-S1110	CAG-ACC-GAC-C	2982	34	70
MH-S1118	ACG-GGA-CTC-T	3028	32	60
MH-S1120	ACC-AAC-CAG-G	3006	32	60
MH-S116	TCT-CAG-CTG-G	3019	32	60

CHAPTER FOUR

4.0 RESULTS

4.1 ISOLATES FROM DISEASED TISSUES OF CROPS

Out of forty diseased sample crop tissues collected from 19 different locations across the agro-ecological zones of Ghana, 32 isolates of *B. theobromae* were obtained. However, only 25 of the isolates produced pycnidia after 35 days of incubation and hence single-spore cultures obtained from these isolates were selected for the morphological and molecular studies. The isolates are B(Ef), B(Kt), B(Eu), B(Yf), B(Ab), B(Kf), B(Db) and B(Go) obtained from banana from Effiduase, Kuntanase, Ejisu, Yamfo, Abesewa, Kyirefaso, Drobo and Goaso, respectively; isolates C(Bk), C(As), C(Af), C(Db) and C(Ab) obtained from cocoa from Berekum, Asumanya, Assin Fosu, Drobo and Abesewa, respectively; isolates M(Ej), M(Nk), M(At) and M(Th) isolated from mango from Ejura, Nkoransa, Atebubu and Techiman, respectively; and isolates Y(Yj), Y(Th), Y(Ch), Y(At), Y(Bk), Y(Zu), Y(Kp), and Y(Ej) from yam from Yeji, Techiman, Chama, Atebubu, Berekum, Zabzugu, Kintampo and Ejura, respectively (Table 2).

4.2 HOST RANGE STUDIES OF *B. THEOBROMAE* ISOLATES

In the inoculation experiments conducted, it was observed that *B. theobromae* isolates, M(At) obtained from mango, C(Db) from cocoa, B(Go) from banana and Y(Kp) from yam were able to cause infections on the mango fruits and cocoa pods inoculated (Plate 12 and Plate 15). Statistical analysis of the daily measurements of the lesions produced by the four isolates at the end of the experiment indicated no significant ($P>0.05$) difference among the isolates on the mango fruits and cocoa pods inoculated (Table 3 and Table 4).

However, with respect to Fig. 1, isolate M(At) produced the longest lesion radius of 51.83 mm, at four days after inoculation, on the mango fruits inoculated. Isolate Y(Kp) produced the least lesion radius of 38.83 mm. Longitudinal sections of the infected mango fruits indicated a watery softening of the pulp (Plate 13) and visible mycelia growth (Plate 14).

Table 2. Isolate label, Ecology and crop variety from which isolates were obtained.

Ecological Zone	Isolate Label	Place of collection	Crop (Cultivar)
Forest Ecological Zone	B(Ef)	Effiduase	Local
	B(Kt)	Kuntanase	Local
	B(Eu)	Ejisu	Local (Asante)
	B(Kf)	Kyirefaso	Local
	C(As)	Asumanya	Hybrid
	M(Ej)	Ejura	Palmar
	Y(Ej)	Ejura	Water Yam
Forest Transitional	B(Ab)	Abesewa	Local (Asante)
Ecological Zone	B(Db)	Drobo	Exotic
	B(Go)	Goaso	Local
	B(Yf)	Yamfo	Local
	C(Bk)	Berekum	Hybrid
	C(Db)	Drobo	Akokra Bedi
	C(Ab)	Abesewa	Akokra Bedi
	M(Nk)	Nkoransa	Local
	M(Th)	Techiman	Local
	M(At)	Atebubu	Keit
	Y(At)	Atebubu	Matches
	Y(Th)	Techiman	Pona
	Y(Bk)	Berekum	Matches
	Y(Kp)	Kintampo	Water Yam
Savanna Ecological Zone	Y(Yj)	Yeji	Pona
	Y(Ch)	Chama	Dente
	Y(Zu)	Zabzugu	Pona
Coastal Savanna			
Ecological Zone	C(Af)	Assin Fosu	Hybrid

C(Ab) =Cocoa isolate from Abesewa; **C(Db)** = Cocoa isolate from Drobo; **C(Bk)** = Cocoa isolate from Berekum; **C(As)** = Cocoa isolate from Asumanya; **C(Af)** = Cocoa isolate from Assin Fosu; **M(At)** = Mango isolate from Atebubu; **M(Ej)** = Mango isolate from Ejura; **M(Th)** = Mango isolate from Techiman; **M(Nk)** = Mango isolate from Nkoransa; **B(Db)** = Banana isolate from Drobo; **B(Kt)** = Banana isolate from Kuntanse; **B(Eu)** = Banana isolate from Ejisu; **B(Yf)** = Banana isolate from Yamfo; **B(Go)** = Banana isolate form Goaso; **B(Ab)** = Banana isolate from Abesewa; **B(Ef)** = Banana isolate from Effiduase; **B(Kf)** = Banana isolate from Kyirefaso; **Y(Bk)** = Yam isolate from Berekum; **Y(Th)** = Yam isolate from Techiman; **Y(Ej)** = Yam isolate from Ejura; **Y(Yj)** = Yam isolate from Yeji; **Y(Ch)** = Yam isolate from Chama; **Y(Zu)** = Yam isolate from Zabzugu; **Y(At)** = Yam isolate from Atebubu; **Y(Kp)** = Yam isolate from Kintampo.

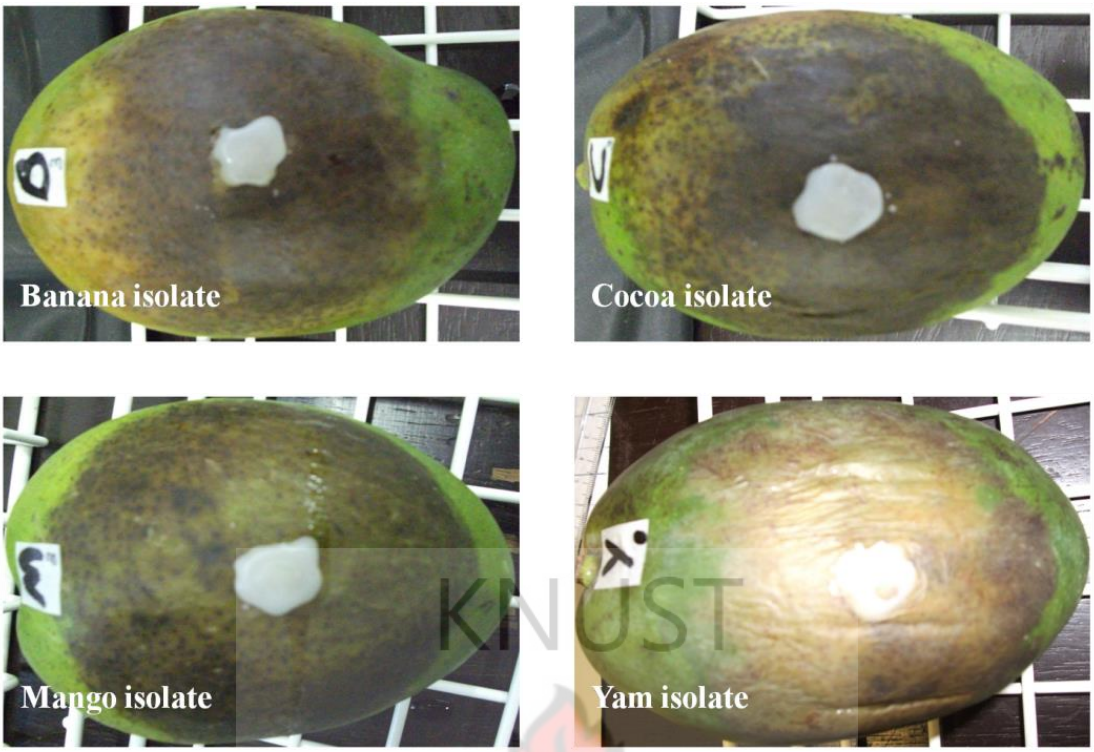


Plate 12. Lesions of infections on Kent mango fruits at 4 days after inoculation with *B. theobromae* isolates from cocoa (C(Db)), mango (M(At)), banana (B(Go)) and yam (Y(Kp).

Table 3. Mean daily lesion size of infection produced by 4 representative *B. theobromae* isolates on Kent mango variety.

Isolates	Radius of infection* (mm)			
	DAY 1	DAY 2	DAY 3	DAY 4
C(Db)	0.00 ± 0.00	4.17 ± 2.31c	26.67 ± 1.59a	40.67 ± 0.33a
M(At)	0.00 ± 0.00	27.17 ± 1.88a	39.67 ± 2.92a	51.83 ± 1.59a
B(Go)	0.00 ± 0.00	17.83 ± 0.67b	30.83 ± 2.89a	44.17 ± 6.01a
Y(Kp)	0.00 ± 0.00	20.67 ± 3.49a,b	27.50 ± 8.89a	38.83 ± 12.32a
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
F value	-	30.49	11.21	10.76
P value	-	<0.0001	0.0010	0.0012
P value	- 0.3579	0.5566	0.1158	0.0117 0.0186

*Values are means of three replications. **C(Db)** = Cocoa isolate from Drobo; **M(At)** = Mango isolate from Atebubu ; **B(Go)** = Banana isolate from Goaso; **Y(Kp)** = Yam isolate from Kintampo.

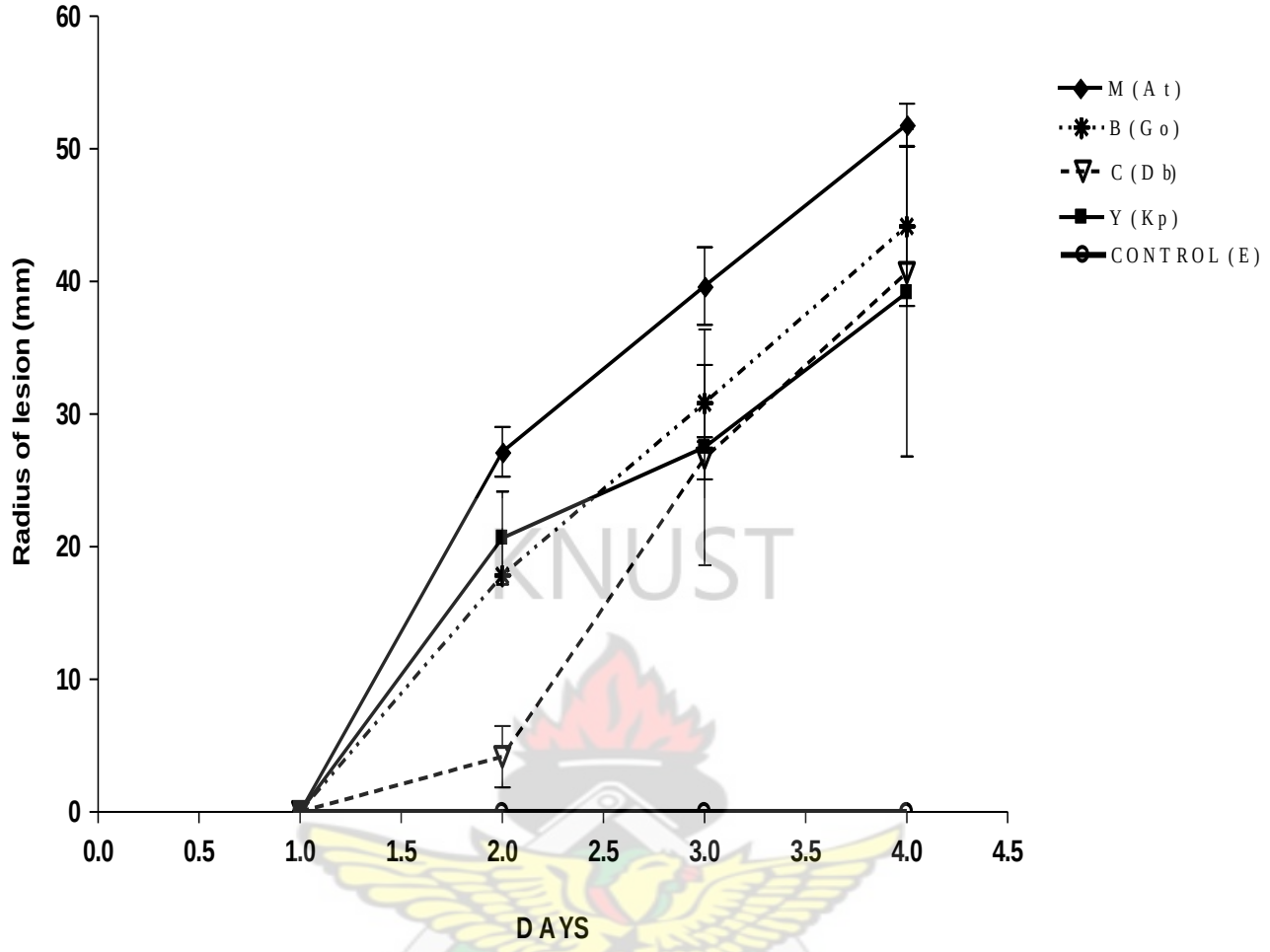


Figure 1. Mean growth of lesions produced by four *B. theobromae* isolates (M(At), B(Go), C(Db) and Y(Kp)) on Kent mango fruits.

M(At) = Mango isolate from Atebubu ; **B(Go)** = Banana isolate from Goaso; **C(Db)** = Cocoa isolate from Drobo; **Y(Kp)** = Yam isolate from Kintampo. (Bar = SEM, N = 15)

Plate 13. Longitudinal sections of infected mango fruits showing watery softened internal tissues (pulp) at 4 days after inoculation with *B. theobromae* isolates from cocoa (C(Db)), mango (M(At)), banana (B(Go)) and yam (Y(Kp).

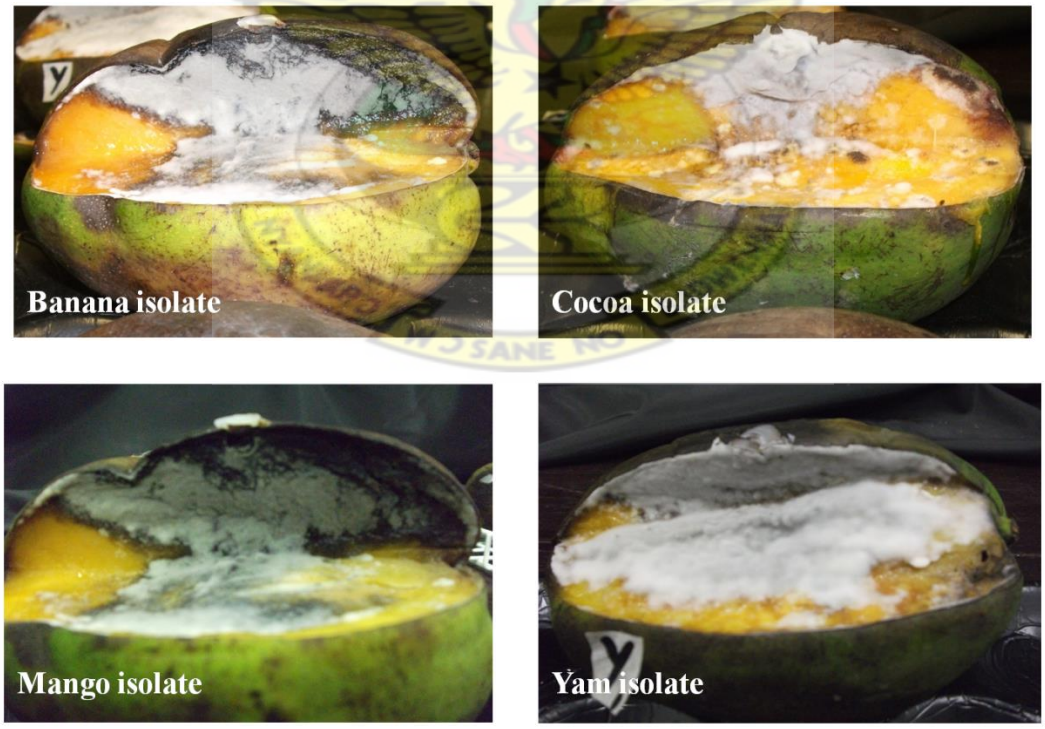


Plate 14. Mycelia growth from pulp of infected mango fruits at 4 days after inoculation with *B. theobromae* isolates from cocoa (C(Db)), mango (M(At)), banana (B(Go)) and yam (Y(Kp)) and after 3 days of exposure of cut surface.

With respect to Fig. 2, isolate B(Go) produced the longest lesion radius of 53.50 mm, at six days after inoculation, on the cocoa pods inoculated. Isolate C(Db) produced the least lesion radius of 33.83 mm. However, most of the pods inoculated with one of the two isolates were totally engulfed by the lesions generated, at 10 days after inoculation, showing visible grayish black mycelia growth and black soot (matured spores) of *B. theobromae* (Plate 16).

(a)

(b)

(c)

Plate 15. Lesions of infections on Akokora Bedi cocoa pods at 6 days after inoculation with *B. theobromae* isolates from cocoa (C(Db)), mango (M(At)), banana (B(Go)) and yam (Y(Kp). (a) Cocoa pods in replication 1; (b) cocoa pods in replication 2; and (c) cocoa pods in replication 3.

Table 4. Mean daily lesion size of infection of 4 representative *B. theobromae* isolates following the cross inoculation technique on Akokora Bedi cocoa variety

Isolates	Radius of infection* (mm)					
	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6
C(Db)	0.00 ± 0.00	1.50 ± 0.76a	3.33 ± 1.69a	11.00 ± 5.50a	21.83 ± 6.00a,b	33.83 ± 10.42a,b
M(At)	0.00 ± 0.00	3.17 ± 2.05a	7.50 ± 5.20a	12.83 ± 11.13a	30.00 ± 7.42a	42.50 ± 8.26a
B(Go)	0.00 ± 0.00	3.50 ± 2.56a	8.50 ± 6.60	27.83 ± 6.84a	38.33 ± 7.84a	53.50 ± 8.04a
Y(Kp)	0.00 ± 0.00	0.00 ± 0.00a	2.50 ± 2.50a	5.83 ± 4.64a	17.17 ± 5.51a,b	34.17 ± 12.82a,b
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
F value	-	1.23	0.79	2.43	5.72	4.93
P value	-	0.3579	0.5566	0.1158	0.0117	0.0186

*Values are means of three replications. **C(Db)** = Cocoa isolate from Drobo; **M(At)** = Mango isolate from Atebubu ; **B(Go)** = Banana isolate from Goaso; **Y(Kp)** = Yam isolate from Kintampo.

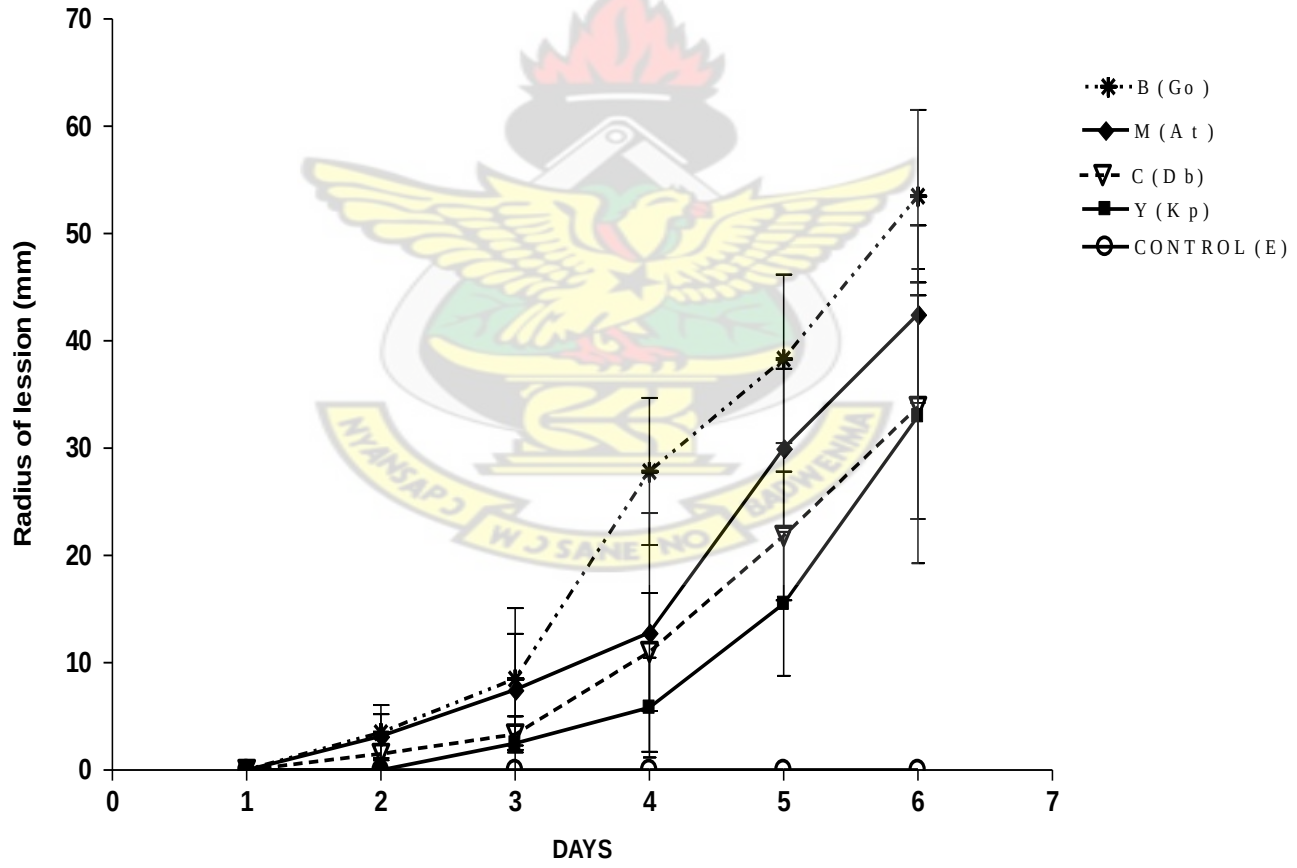


Figure 2. Mean growth of lesions produced by four *B. theobromae* isolates (M(At), B(Go), C(Db) and Y(kp)) on Akokora Bedi cocoa pods.

M(At) = Mango isolate from Atebubu ; **B(Go)** = Banana isolate from Goaso; **C(Db)** = Cocoa isolate from Drobo; **Y(Kp)** = Yam isolate from Kintampo. (Bar = SEM, N = 15)

Plate 16. Akokora Bedi cocoa pods at 10 days after inoculation with *B. theobromae* isolates from cocoa (C(Db)), mango (M(At)), banana (B(Go)) and yam (Y(Kp).

Longitudinal section through the inoculated cocoa pods revealed that the infections reached the beans (Plate 17). The beans turned black with grayish black mycelia, characteristic of *B. theobromae*, highly visible.

Plate 17. Longitudinal section of cocoa pods showing blackened internal tissues and beans at 10 days after inoculation with *B. theobromae* isolates from cocoa (C(Db)), mango (M(At)), banana (B(Go)) and yam (Y(Kp).

4.3 MORPHOLOGICAL CHARACTERISTICS OF ISOLATES

Isolates C(Bk), C(Af), M(Th), B(Db), B(Yf), B(Go), B(Ef), Y(At) and B(Ab), produced growth rates in the range of 0.93 - 0.94mm/hr (Table 5 and Fig. 3). These isolates fully covered the entire 90 mm diameter-Petri dishes in 48hrs. Isolates C(As), B(Kt), B(Eu), Y(Bk), Y(Th), Y(Ej), Y(Yj), Y(Zu), Y(Kp) and M(Nk), produced growth rates in the range of 0.91 - 0.92 mm/hr on PDA. Isolates M(Ej), C(Ab), Y(Ch) and M(At), produced growth rates in the range of 0.82 - 0.90 mm/hr. While B(Kf) and C(Db) produced growth rates of 0.72 and 0.76 mm/hr, respectively.

Isolates (C(Ab), C(Db), C(As), C(Af), M(Th), B(Db), B(Kt), B(Yf), B(Go), B(Ef), B(Kf), Y(Th), Y(Ej), Y(Yj), Y(Ch) and Y(Kp) produced uniformly appressed colonies (Table 5). Whereas M(Nk), B(Eu), Y(Bk), Y(Zu), and Y(At) produced uniformly fluffy colonies. Fluffy colonies

with appressed margins were produced by isolates C(Bk), M(At) and M(Ej). Isolate B(Ab) produced an appressed but irregular colony growth.

Isolates C(Ab), C(Db), M(Th), B(Db), B(Kt), B(Eu), B(Go), B(Ab), B(Ef), B(Kf), Y(Th), Y(Ch) and Y(Zu) produced grayish-black colonies on PDA (Table 5). Isolates C(Bk), C(As), C(Af), M(At), M(Ej), Y(Bk), Y(Yj), Y(At) and Y(Kp) produced grayish black colonies but with whitish margins. Isolates B(Yf) and Y(Ej) produced grayish-white colonies. Isolate M(Nk) produced a dull white colony. All twenty-five isolates studied on PDA showed signs of black pigmentation in the growth medium, 48 hrs after incubation.

The isolates varied in pycnidia production. Isolates C(Bk), M(Ej), M(Th), B(Db), B(Eu), B(Yf), B(Ab), B(Ef), B(Kf), Y(Bk), Y(Ej), Y(Zu), Y(At), and Y(Kp) did not produce spores at 35 days after incubation on PDA (Table 5).

4.4 **GENOMIC DNA ISOLATION**

Bands of DNA of single-spore cultures of all 25 *B. theobromae* isolates observed after resolving the undigested Genomic DNA on a 0.8% agarose gel is shown in Plate 18.

Table 5. Morphological characteristics of 25 *B. theobromae* isolates based on growth on PDA at 28°C.

Isolates	Growth rate of colony** (mm/hr)	Sporulation[#]	Colony texture and colour
C(Ab)	0.88	+	Appressed, uniform, grayish black
C(Db)	0.76	+	Appressed, uniform, grayish black
C(Bk)	0.94	-	Fluffy, grayish black with appressed whitish margin
C(As)	0.92	+	Appressed, uniform, grayish black with whitish margin
C(Af)	0.94	+	Appressed, uniform, grayish black with whitish margin
M(At)	0.82	+	Fluffy, grayish black with appressed whitish margin
M(Ej)	0.90	-	Fluffy, grayish black with appressed whitish margin
M(Th)	0.94	-	Appressed, uniform, grayish black
M(Nk)	0.91	+	Fluffy, uniform, dull white
B(Db)	0.94	-	Appressed, uniform grayish black
B(Kt)	0.92	+	Appressed, uniform, grayish black
B(Eu)	0.92	-	Fluffy, uniform, grayish black
B(Yf)	0.94	-	Appressed, uniform, grayish white
B(Go)	0.94	+	Appressed, uniform, grayish black
B(Ab)	0.93	-	Appressed, irregular, grayish black
B(Ef)	0.94	-	Appressed, uniform, grayish black
B(Kf)	0.72	-	Appressed, uniform, grayish black
Y(Bk)	0.92	-	Fluffy, uniform, grayish black with whitish margin
Y(Th)	0.92	+	Appressed, uniform, grayish black
Y(Ej)	0.92	-	Appressed, uniform, grayish white
Y(Yj)	0.92	+	Appressed, uniform, grayish black with whitish margin
Y(Ch)	0.85	+	Appressed, uniform, grayish black
Y(Zu)	0.92	-	Fluffy, uniform, grayish black
Y(At)	0.94	-	Fluffy, uniform, grayish black with whitish margin
Y(Kp)	0.92	-	Appressed, uniform, grayish black with whitish margin
CD (P = 0.05)	0.01		

Mean of three replications based on growth at 48h; [#] Sporulation at 35 days of incubation (+ = present; - = absent). **C(Ab) =Cocoa isolate from Abesewa; **C(Db)** = Cocoa isolate from Drobo; **C(Bk)** = Cocoa isolate from Berekum; **C(As)** = Cocoa isolate from Asumanya; **C(Af)** = Cocoa isolate from Assin Fosu; **M(At)** = Mango isolate from Atebubu; **M(Ej)** = Mango isolate from Ejura; **M(Th)** = Mango isolate from Techiman; **M(Nk)** = Mango isolate from Nkoransa; **B(Db)** = Banana isolate from Drobo; **B(Kt)** = Banana isolate from Kuntanse; **B(Eu)** = Banana isolate from Ejisu; **B(Yf)** = Banana isolate from Yamfo; **B(Go)** = Banana isolate form Goaso; **B(Ab)** = Banana isolate from Abesewa; **B(Ef)** = Banana isolate from Effiduase; **B(Kf)** = Banana isolate from Kyirefaso; **Y(Bk)** = Yam isolate from Berekum; **Y(Th)** = Yam isolate from Techiman; **Y(Ej)** = Yam isolate from Ejura; **Y(Yj)** = Yam isolate from Yeji; **Y(Ch)** = Yam isolate from Chama; **Y(Zu)** = Yam isolate from Zabzugu; **Y(At)** = Yam isolate from Atebubu; **Y(Kp)** = Yam isolate from Kintampo. CD = Critical Difference.

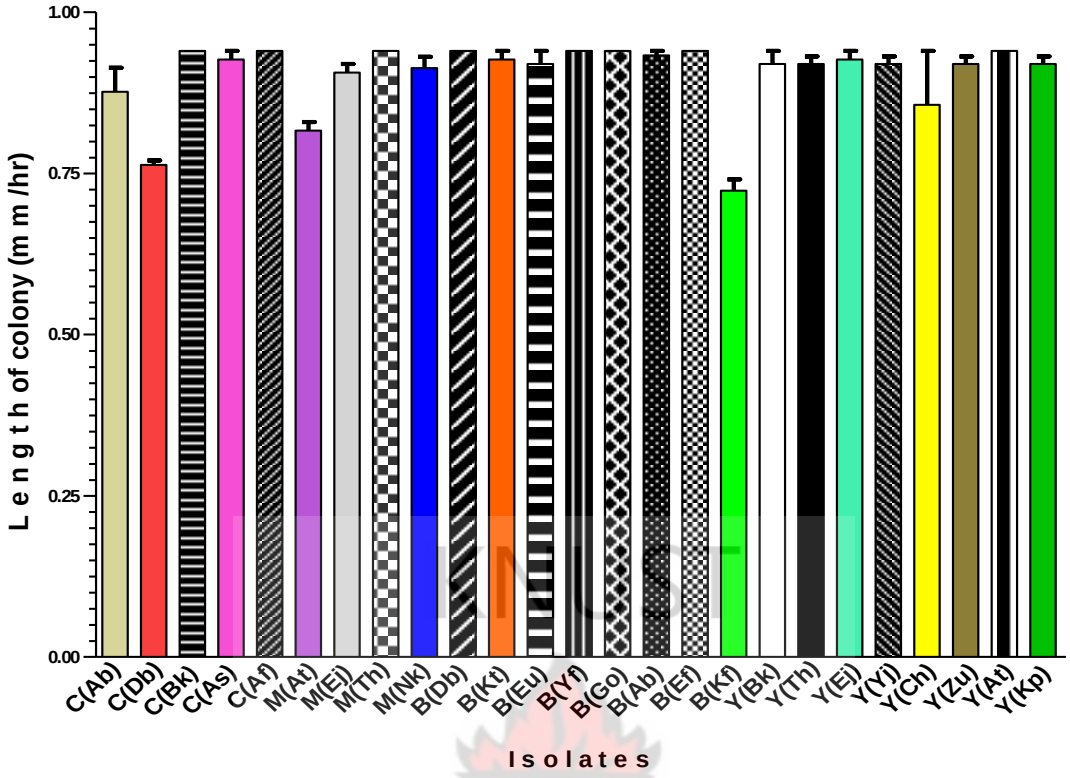


Figure 3. Mean growth rates of *B. theobromae* isolates at 48hrs of incubation on PDA.

C(Ab) = Cocoa isolate from Abesewa; **C(Db)** = Cocoa isolate from Drobo; **C(Bk)** = Cocoa isolate from Berekum; **C(As)** = Cocoa isolate from Asumanya; **C(Af)** = Cocoa isolate from Assin Fosu; **M(At)** = Mango isolate from Atebubu; **M(Ej)** = Mango isolate from Ejura; **M(Th)** = Mango isolate from Techiman; **M(Nk)** = Mango isolate from Nkoransa; **B(Db)** = Banana isolate from Drobo; **B(Kt)** = Banana isolate from Kuntanse; **B(Eu)** = Banana isolate from Ejisu; **B(Yf)** = Banana isolate from Yamfo; **B(Go)** = Banana isolate form Goaso; **B(Ab)** = Banana isolate from Abesewa; **B(Ef)** = Banana isolate from Effiduase; **B(Kf)** = Banana isolate from Kyirefaso; **Y(Bk)** = Yam isolate from Berekum; **Y(Th)** = Yam isolate from Techiman; **Y(Ej)** = Yam isolate from Ejura; **Y(Yj)** = Yam isolate from Yeji; **Y(Ch)** = Yam isolate from Chama; **Y(Zu)** = Yam isolate from Zabzugu; **Y(At)** = Yam isolate from Atebubu; **Y(Kp)** = Yam isolate from Kintampo.

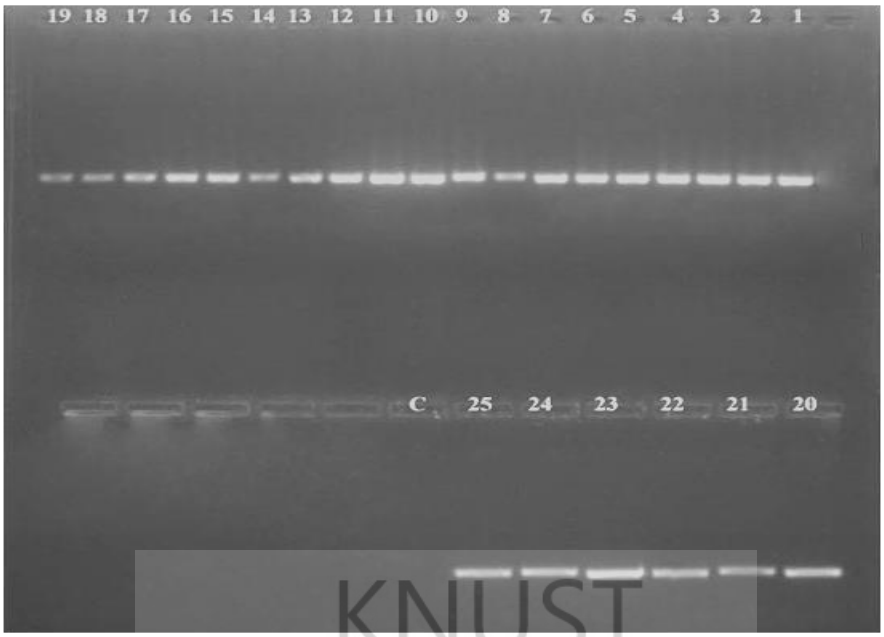


Plate 18. Agarose gel electrogram showing bands of undigested Genomic DNA of all 25 *Botryodiplodia theobromae* isolates.

Lane 1 = C(Ab); Lane 2 = C(Db); Lane 3 = C(Bkm); Lane 4 = C(As); Lane 5 = (Af); Lane 6 = M(At); Lane 7 = M(Ej); Lane 8 = M(Th); Lane 9 = M(Nk); Lane 10 = B(Db); Lane 11 = B(Kn); Lane 12 = B(Eu); Lane 13 = B(Yf) Lane 14 = B(Go); Lane 15 = B(Ab); Lane 16 = B(Ef); lane 17 = B(Kf); Lane 18 = Y(Bk); Lane 19 = Y(Th); Lane 20 = Y(Ej); Lane 21 = Y(Yj); Lane 22 = Y(Ch); Lane 23 = Y(Zu); Lane 24 = Y(At); Lane 25 = Y(Kp) Lane C = Negative control.

4.5 GENETIC VARIABILITY AMONG ISOLATES

4.5.1 DNA fingerprinting of all 25 *Botryodiplodia theobromae* isolates using SSR and RAPD primers

A range of 1 to 8 bands with a total of 19 bands of sizes 0.05 to 1.2 kb were obtained with SSR primer MH- BOT_19 and _20; and MH-LAS_15 and_16 (Plate 19 and Plate 20). RAPD primer MH-S1118 produced banding ranging from 1 to 7 with a total of 22 bands of sizes 0.2 to 1.4 kb (Plate 21).

Isolates C(Ab) and B(Db) shared five bands together with sizes 0.5, 0.633, 1.027, 1.243 and1.351 kb (Appendix V). Isolates B(Kt) and B(Ef) shared six bands with sizes 0.05, 0.1, 0.2, 0.3, 0.4 and 1.297 kb. Isolates B(Kf) and Y(Bk) produced only two bands each and shared a band with size 1.25 kb (Appendix V). Individual bands with sizes 1.055, 0.55, 0.271, and 0.243 kb were present only in isolates Y(Bk), B(Ab), Y(Kp), and C(Af), respectively. Three bands with sizes 1.111, 1.108 and 1.00 kb were present only in isolate M(Ej). Two bands with sizes 1.367 and 1.267 kb were present only in isolate Y(Ej). Isolates C(Db), C(Bk), C(As), M(Th), M(At),

M(Nk), B(Go), B(Eu), B(Yf), Y(Yj), Y(Ch), Y(At), and Y(Th) shared one or more bands of varied sizes with one or more isolates (Appendix V).

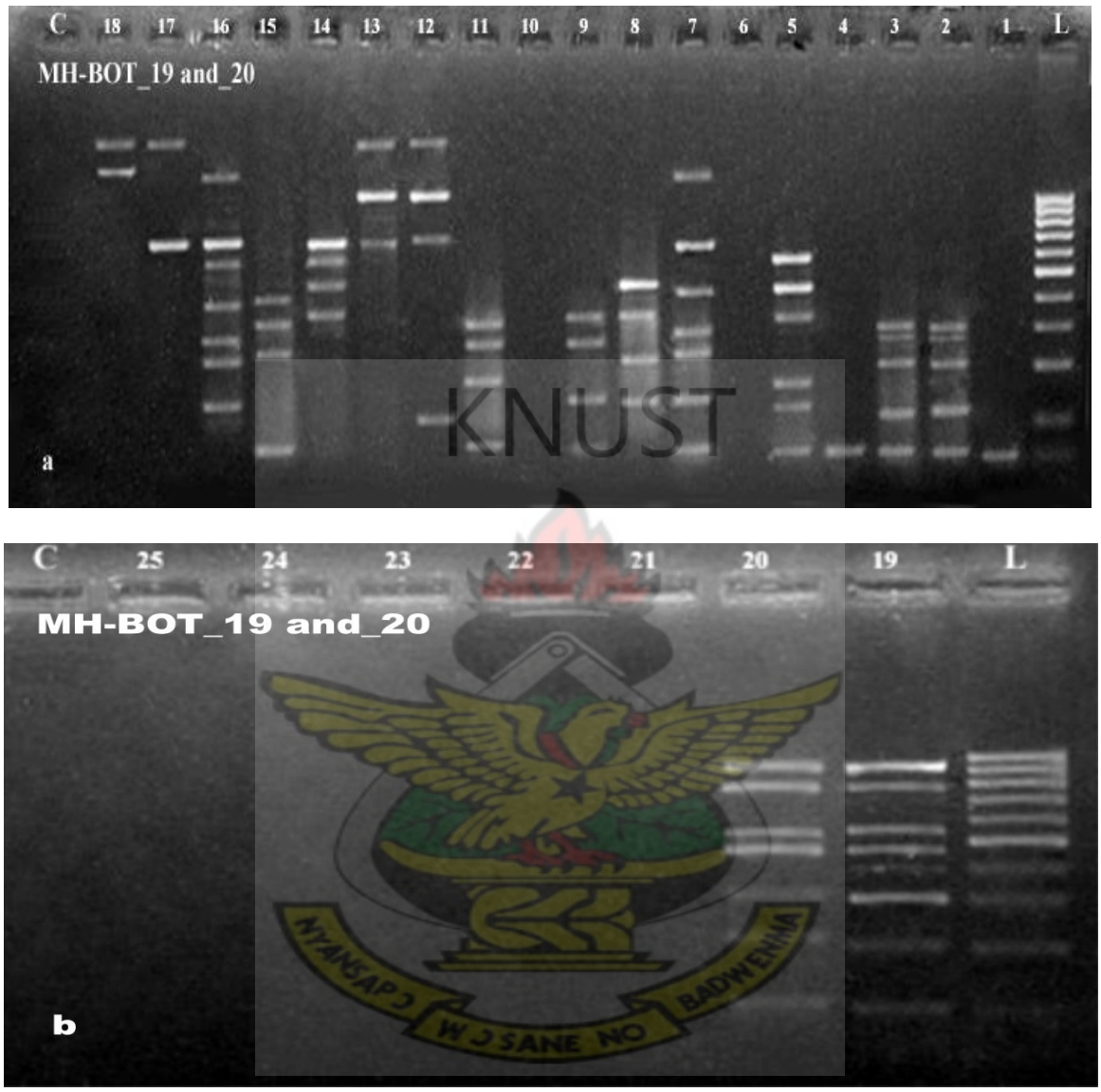


Plate 19. Agarose gel electrogram showing bands obtained from PCR products using SSR primer MH-BOT_19 and_20 on 25 *Botryodiplodia theobromae* isolates.

Lane L = 1000bp DNA ladder; Lane C= Negative control; *lane 1*= C(Ab); *lane 2*= C(Db); *lane 3*= C(Bk); *lane 4*= C(As); *lane 5*= C(Af); *lane 6*= M(At); *lane 7*= M(Ej); *lane 8*= M(Th); *lane 9*= M(Nk); *lane 10*= B(Db); *lane 11*= B(Kt); *lane 12*= B(Eu); *lane 13*= B(Yf); *lane 14*= B(Go); *lane 15*= B(Ab); *lane 16*= B(Ef); *lane 17*= B(Kf); *lane 18*= Y(Bk); *lane 19*= Y(Th); *lane 20*= Y(Ej); *lane 21*= Y(Yj); *lane 22*= Y(Ch); *lane 23*= Y(Zu); *lane 24*= Y(At); *lane 25*= Y(Kp). **C(Ab)** =Cocoa isolate from Abesewa; **C(Db)** = Cocoa isolate from Drobo; **C(Bk)** = Cocoa isolate from Berekum; **C(As)** = Cocoa isolate from Asumanya; **C(Af)** = Cocoa isolate from Assin Fosu; **M(At)** = Mango isolate from Atebubu; **M(Ej)** = Mango isolate from Ejura; **M(Th)** = Mango isolate from Techiman; **M(Nk)** = Mango isolate from Nkoransa; **B(Db)** = Banana isolate from Drobo; **B(Kt)** = Banana isolate from Kuntanse; **B(Eu)** = Banana isolate from Ejisu; **B(Yf)** = Banana isolate from Yamfo; **B(Go)** = Banana isolate form Goaso; **B(Ab)** = Banana isolate from Abesewa; **B(Ef)** = Banana isolate from Effiduase; **B(Kf)** = Banana isolate from Kyirefaso; **Y(Bk)** = Yam isolate from Berekum; **Y(Th)** = Yam isolate from Techiman; **Y(Ej)** = Yam isolate from Ejura; **Y(Yj)** = Yam isolate from Yeji; **Y(Ch)** = Yam isolate from Chama; **Y(Zu)** = Yam isolate from Zabzugu; **Y(At)** = Yam isolate from Atebubu; **Y(Kp)** = Yam isolate from Kintampo.

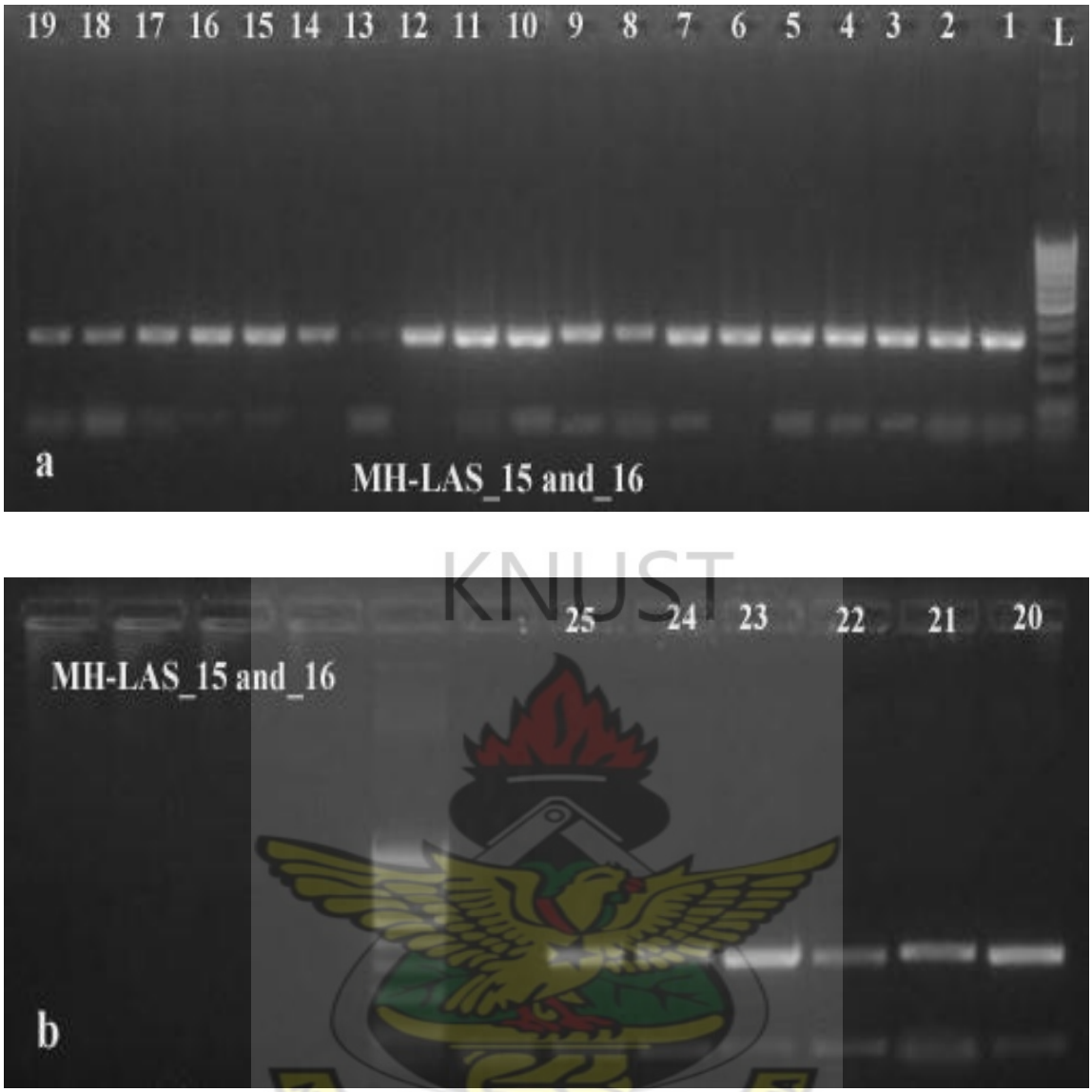


Plate 20. Agarose gel electrogram showing bands obtained from PCR products using SSR primer MH-LAS_15 and_16 on 25 *Botryodiplodia theobromae* isolates.

Lane L = 1000bp DNA ladder; Lane C= Negative control; *lane 1*= C(Ab); *lane 2*= C(Db); *lane 3*= C(Bk); *lane 4*= C(As); *lane 5*= C(Af); *lane 6*= M(At); *lane 7*= M(Ej); *lane 8*= M(Th); *lane 9*= M(Nk); *lane 10*= B(Db); *lane 11*= B(Kt); *lane 12*= B(Eu); *lane 13*= B(Yf); *lane 14*= B(Go); *lane 15*= B(Ab); *lane 16*= B MH-LAS_15 (Ef); *lane 17*= B(Kf); *lane 18*= Y(Bk); *lane 19*= Y(Th); *lane 20*= Y(Ej); *lane 21*= Y(Yj); *lane 22*= Y(Ch); *lane 23*= Y(Zu); *lane 24*= Y(At); *lane 25*= Y(Kp). **C(Ab)** =Cocoa isolate from Abesewa; **C(Db)** = Cocoa isolate from Drobo; **C(Bk)** = Cocoa isolate from Berekum; **C(As)** = Cocoa isolate from Asumanya; **C(Af)** = Cocoa isolate from Assin Fosu; **M(At)** = Mango isolate from Atebubu; **M(Ej)** = Mango isolate from Ejura; **M(Th)** = Mango isolate from Techiman; **M(Nk)** = Mango isolate from Nkoransa; **B(Db)** = Banana isolate from Drobo; **B(Kt)** = Banana isolate from Kuntanse; **B(Eu)** = Banana isolate from Ejisu; **B(Yf)** = Banana isolate from Yamfo; **B(Go)** = Banana isolate form Goaso; **B(Ab)** = Banana isolate from Abesewa; **B(Ef)** = Banana isolate from Effiduase; **B(Kf)** = Banana isolate from Kyirefaso; **Y(Bk)** = Yam isolate from Berekum; **Y(Th)** = Yam isolate from Techiman; **Y(Ej)** = Yam isolate from Ejura; **Y(Yj)** = Yam isolate from Yeji; **Y(Ch)** = Yam isolate from Chama; **Y(Zu)** = Yam isolate from Zabzugu; **Y(At)** = Yam isolate from Atebubu; **Y(Kp)** = Yam isolate from Kintampo.

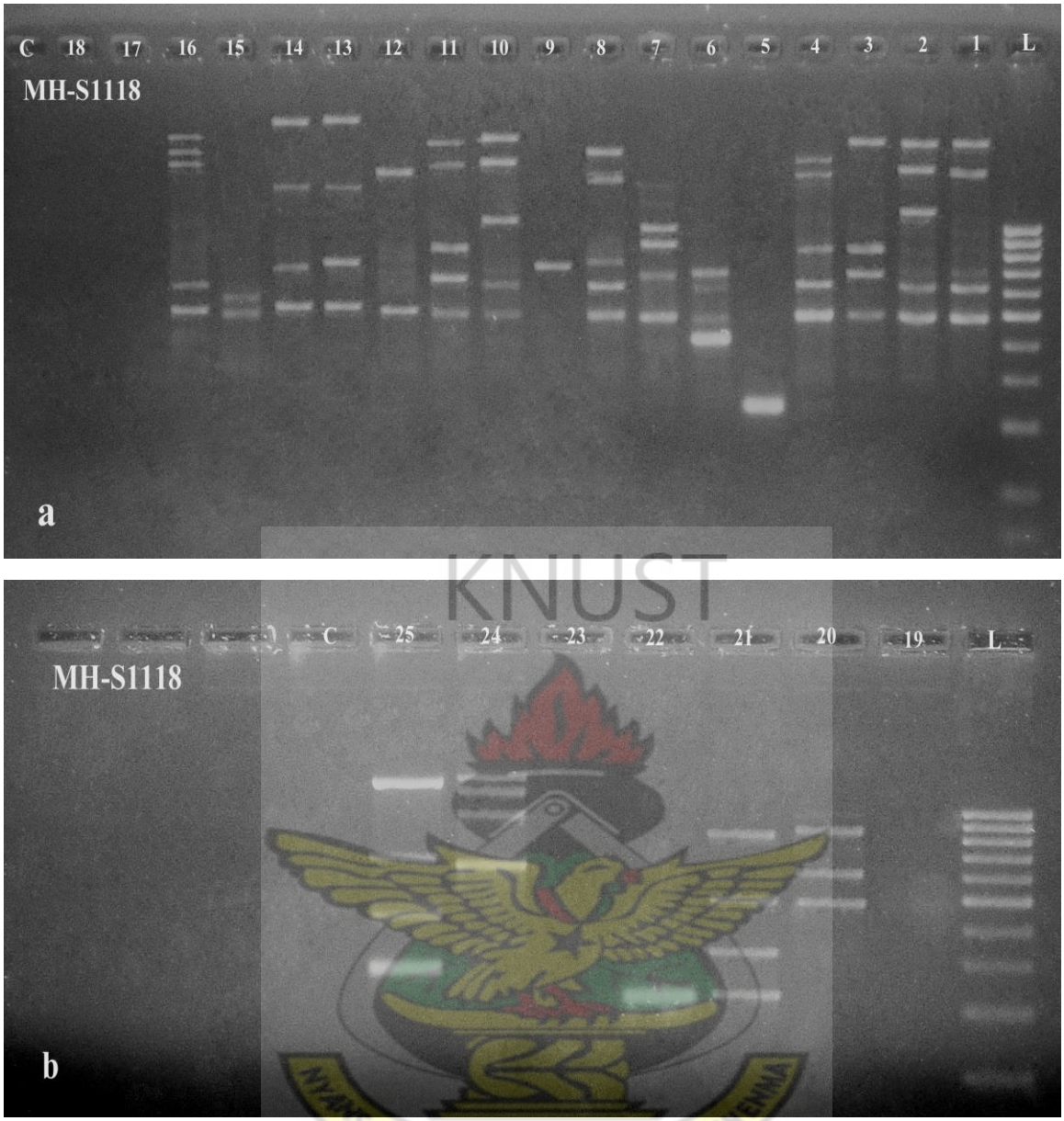


Plate 21. Agarose gel electrogram showing bands obtained from PCR products using RAPD primer MH-S1118 on 25 *Botryodiplodia theobromae* isolates.

Lane L = 1000bp DNA ladder; Lane C= Negative control; *lane 1*= C(Ab); *lane 2*= C(Db); *lane 3*= C(Bk); *lane 4*= C(As); *lane 5*= C(Af); *lane 6*= M(At); *lane 7*= M(Ej); *lane 8*= M(Th); *lane 9*= M(Nk); *lane 10*= B(Db); *lane 11*= B(Kt); *lane 12*= B(Eu); *lane 13*= B(Yf); *lane 14*= B(Go); *lane 15*= B(Ab); *lane 16*= B(Ef); *lane 17*= B(Kf); *lane 18*= Y(Bk); *lane 19*= Y(Th); *lane 20*= Y(Ej); *lane 21*= Y(Yj); *lane 22*= Y(Ch); *lane 23*= Y(Zu); *lane 24*= Y(At); *lane 25*= Y(Kp). **C(Ab)** =Cocoa isolate from Abesewa; **C(Db)** = Cocoa isolate from Drobo; **C(Bk)** = Cocoa isolate from Berekum; **C(As)** = Cocoa isolate from Asumanya; **C(Af)** = Cocoa isolate from Assin Fosu; **M(At)** = Mango isolate from Atebubu; **M(Ej)** = Mango isolate from Ejura; **M(Th)** = Mango isolate from Techiman; **M(Nk)** = Mango isolate from Nkoransa; **B(Db)** = Banana isolate from Drobo; **B(Kt)** = Banana isolate from Kuntanse; **B(Eu)** = Banana isolate from Ejisu; **B(Yf)** = Banana isolate from Yamfo; **B(Go)** = Banana isolate form Goaso; **B(Ab)** = Banana isolate from Abesewa; **B(Ef)** = Banana isolate from Effiduase; **B(Kf)** = Banana isolate from Kyirefaso; **Y(Bk)** = Yam isolate from Berekum; **Y(Th)** = Yam isolate from Techiman; **Y(Ej)** = Yam isolate from Ejura; **Y(Yj)** = Yam isolate from Yeji; **Y(Ch)** = Yam isolate from Chama; **Y(Zu)** = Yam isolate from Zabzugu; **Y(At)** = Yam isolate from Atebubu; **Y(Kp)** = Yam isolate from Kintampo.

4.5.2 **Phylogenetic relationships among the *Botryodiplodia theobrommae* isolates**

Figure 4 is a dendrogram constructed from the combined data sets of DNA markers of all the 25 isolates using SSR and RAPD primers, and analyzed with the SAHN method by the NTSYS 2.0 software. A percent similarity matrix table was also produced (Table 6).

The isolates clustered into 5 major groups. Group 1 comprised of 14 isolates (C(Ab), B(Db), C(Db), C(As), C(Bk), M(Th), B(Kt), B(Ef), B(Ab), C(Af), M(Ej), B(Go), Y(Th) and Y(Ej)) at 23% genetic similarity. Group 2 consisted of 3 isolates (M(At), Y(Kp), and M(Nk)) at 29% genetic similarity. Group 3 was made up of 4 isolates (B(Eu), B(Yf), B(Kf), and Y(Bk)) at genetic similarity of 21%. Group 4 comprised of 3 isolates (Y(Yj), Y(Ch), and Y(At)) and exhibited genetic similarity of 20%. Group 5 was made up of only one isolate (Y(Zu)) which share no genetic similarity (0%) with the rest of the isolates (Fig. 4).

Group 1 further separated into subgroup 1a with isolates C(Ab), B(Db), C(Db), and C(As) at 34% genetic similarity; 1b with isolates M(Th), B(Kt), B(Ef), and B(Ab) at 56% genetic similarity; 1c with isolates M(Ej) and B(Go) at 54% genetic similarity; 1d with isolates Y(Th) and Y(Ej) at 50% genetic similarity. Group 1 also produced individual lineages S1 and S2 at genetic similarity of 44% and 38% with isolates C(Bk) and C(Af) respectively. Group 3 separated into 2 subgroups i.e. 3a with isolates B(Eu) and B(Yf) and 3b with isolates B(Kf) and Y(Bk) at genetic similarity of 55% and 34% respectively. Isolate B(Kt) and B(Ef) of subgroup 1b were most similar, with 83% genetic similarity.

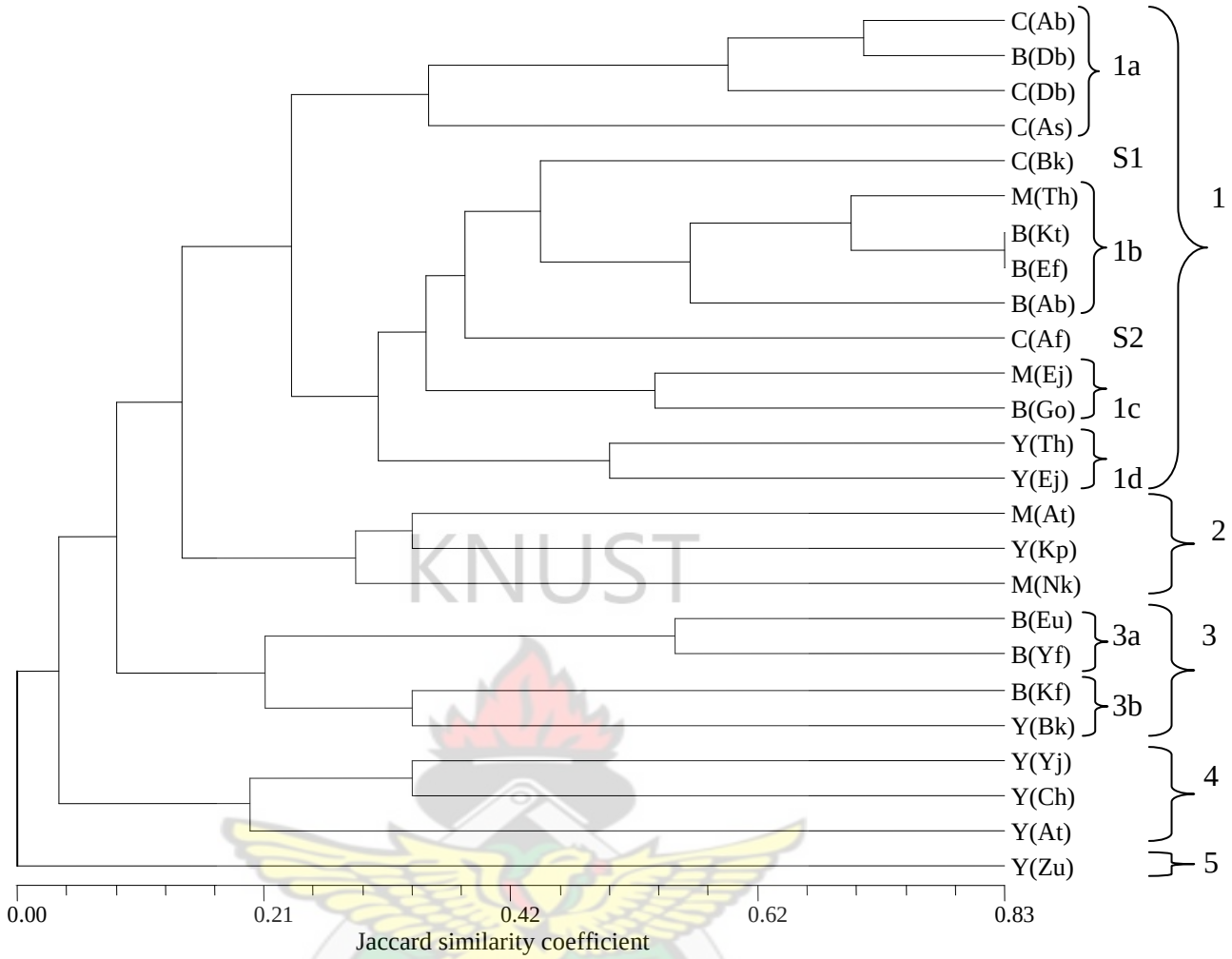


Figure 4. Dendrogram derived from the combined data sets of 25 *Botryodiplodia theobromae* isolates using SSR and RAPD markers.

C(Ab) =Cocoa isolate from Abesewa; **C(Db)** = Cocoa isolate from Drobo; **C(Bk)** = Cocoa isolate from Berekum; **C(As)** = Cocoa isolate from Asumanya; **C(Af)** = Cocoa isolate from Assin Fosu; **M(At)** = Mango isolate from Atebubu; **M(Ej)** = Mango isolate from Ejura; **M(Th)** = Mango isolate from Techiman; **M(Nk)** = Mango isolate from Nkoransa; **B(Db)** = Banana isolate from Drobo; **B(Kt)** = Banana isolate from Kuntanse; **B(Eu)** = Banana isolate from Ejisu; **B(Yf)** = Banana isolate from Yamfo; **B(Go)** = Banana isolate form Goaso; **B(Ab)** = Banana isolate from Abesewa; **B(Ef)** = Banana isolate from Effiduase; **B(Kf)** = Banana isolate from Kyirefaso; **Y(Bk)** = Yam isolate from Berekum; **Y(Th)** = Yam isolate from Techiman; **Y(Ej)** = Yam isolate from Ejura; **Y(Yj)** = Yam isolate from Yeji; **Y(Ch)** = Yam isolate from Chama; **Y(Zu)** = Yam isolate from Zabzugu; **Y(At)** = Yam isolate from Atebubu; **Y(Kp)** = Yam isolate from Kintampo.

CHAPTER FIVE

5.0 DISCUSSION

Botryodiplodia theobromae rots and diseases are among the major problems affecting the production of important food and tree crops, particularly those put into intercrops. Yield losses of over 80% have been linked to the diseases and rots in some food crops. Total crop failure due to *B. theobromae* infections of root and tuber crops in Nigeria have been reported (Onyenka *et al.*, 2005). The wide host range, distribution and the obliviousness of the virulence of *B. theobromae* has made the pathogen and its diseases prevalent in the tropics and subtropics. Results from sampling and isolation of the pathogen in this study are in support of this. Eighty percent (80%) of the sampled diseased crops were infected with *B. theobromae*.

In the inoculation experiments carried out for example, mango isolates from Atebubu, cocoa isolates from Drobo, banana isolates from Goaso and yam isolates from Kintampo were able to cause lesions on both mango fruits and cocoa pods. This result confirms the nature of *Botryodiplodia theobromae* as an unspecific pathogen with a wide host range as has been reported in several studies (Pitt and Hocking, 2009; Opoku *et al.*, 2007; Domsch *et al.*, 2007; French, 2006; Khanzada *et al.*, 2004; Sutton, 1980). Shanthi *et al.* (2008) in a study to identify the host specificity of *B. theobromae* isolates from mango, pawpaw and rambutan in Sri Lanka observed that the isolates infected other crops beside their host crops. Mohali *et al.* (2005) also identified such non-specificity of *B. theobromae* among isolates studied on some tropical trees in Venezuela. These results suggest that intercropping cocoa, mango, banana, yam and other susceptible food crops and tree crops may promote diseases caused by *B. theobromae* among such crops and trees.

Some similarities existed among some isolates with respect to the morphological characteristics studied. Based on growth rates produced on PDA, cocoa isolates from Berekum and Assin Fosu were very similar to mango isolates from Techiman and banana isolates from Drobo, Yamfo, Goaso, Effiduase, Atebubu and Abesewa. Cocoa isolates from Asumanya, mango isolates from Nkoransa and banana isolates from Kuntanase and Ejisu were also found to be very similar to yam isolates from Berekum, Techiman, Ejura, Yeji, Zabzugu and Kintampo. Again, mango isolates from Ejura and Atebubu were found to be similar to cocoa isolates from Abesewa and yam isolates from Chama.

With respect to the texture and colour of the colonies produced on PDA cocoa isolates from Abesewa and Drobo were very similar to banana isolates from Drobo, Kuntanase, Goaso, Effiduase and Kyirefaso, and yam isolates from Techiman and Chama as well as mango isolates from Techiman. Cocoa isolates from Asumanya and Assin Fosu were found to be very similar to two yam isolates from Yeji and Kintampo. Another two yam isolates from Berekum and Atebubu were very similar to each other. Banana isolates from Ejisu and yam isolates from Zabzugu were also very similar to each other. Cocoa isolates from Berekum was also similar to mango isolates from Atebubu and Ejura. However, mango isolates from Nkoransa were very distinct and very different from the rest of the isolates studied.

There was no clear relatedness among the isolates with regards to pycnidia and spore production as the production of the pycnidia was unstable and irregular among the isolates studied. However, single-spore cultures of cocoa isolates from Abesewa, Drobo, Asumanya and Assin Fosu, and mango isolates from Atebubu and Nkoransa, and banana isolates from Kuntanase and Goaso, and yam isolates from Techiman, Yeji and Chama produced pycnidia with matured spores at the end of the 35 days of incubation.

From the morphological characteristics studied, there were similarities among some isolates from different agro-ecologies. Particular examples are isolates from cocoa from Asumanya in the Bekwai Municipality of the forest ecological zone and isolates from yam from Yeji in Pru District of the savanna ecological zone. Isolates from banana from Effiduase in the Sekyere East District of the forest ecological zone was very similar to another banana isolate from Drobo in the Jaman South District and to a mango isolate from Techiman in Techiman Municipality, of the forest transitional ecological zone. Another similarity was found in banana isolates from Kuntanase in the Bosomtwe District of the forest ecological zone and a yam isolate from Techiman. This gives an indication that the morphological attributes of the isolates of *B. theobromae* studied are not location or ecologically specific. This situation was also observed by Shah *et al.* (2010) among *B. theobromae* isolates from avocado varieties from different districts of India. However, Mohali, *et al.* (2005) on the other hand found isolates of *B. theobromae* from different continents to be specific to their geographical origin.

Isolates that were similar morphologically were found to be different at the DNA level. It was observed that *B. theobromae* isolates from bananas from Kuntanase and Effiduase which were

very similar at the morphological level were clustered into a common subgroup (Subgroup 1b) at the highest genetic similarity of 83% (Fig. 4). Such high genetic similarity (above 80%) among banana isolates has also been described (Sangeetha *et al.*, 2011) in India. However, isolates from cocoa from Asumanya and isolates from yam from Yeji; isolates from banana from Kuntanase and isolates from yam from Techiman; and two isolates from bananas from Drobo and Effiduase, which were also very similar at the morphological level, differed markedly at the molecular level. The most closely related isolates among them, i.e., isolates from banana from Kuntanase and isolates from yam from Techiman separated into different subgroups at a low genetic similarity of 32%. This suggests that the morphological observations should be backed by molecular data to confirm or deny genetic diversity, an observation also made by Shah *et al.*, (2010).

Though the molecular tools used in this study could not comprehensively show relatedness among isolates from the different crops, it generally showed that isolates from the same crop were more related to each other. This gives an indication that isolates from the same crop may genetically be closer to each other than to isolates from other crops.

This study indicated clearly that *Botryodiplodia theobromae* from cocoa, mango, banana and yam are non-specific. It is possible that several of the food crops cultivated in Ghana could be infected by *B. theobromae* isolates from different sources. A situation of this nature could promote *B. theobromae* diseases in our cropping systems with possible high yield losses. Intercropping of cocoa, mango, banana and yam could possibly enhance the persistence of *B. theobromae* infections and result in yield losses. A careful look at intercrops involving these four crops, potentially, can reduce yield losses due to infections by *B. theobromae*.

Establishing the host range of *B. theobromae* species on the different major food crops is necessary if losses caused by this important pathogen are to be controlled effectively.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

Botryodiplodia theobromae isolates studied were not host specific and as such can cross-infect susceptible crops. It is also possible that the pathogen may be causing yield losses in other crops that have not been covered in this particular study. Hence, cropping systems that bring such crops together or intercrop them can facilitate and strengthen *B. theobromae* infections and make its control difficult.

The *B. theobromae* isolates studied were also found to be ecologically non-specific. Hence, this makes it possible for any of the isolates to cause diseases or rots in susceptible crops in any agro-ecology of Ghana.

Most of the isolates exhibited similarity in colony characteristics and growth rates. However, using the RAPDs and SSRs to show the degree of relatedness, similarities were found to be weak at the molecular level. This indicates that several strains of the pathogen exist in Ghana.

6.2 RECOMMENDATIONS

- From the cross-inoculation experiments carried out, the possibility of *Botryodiplodia theobromae* isolates from mango, cocoa, banana or yam causing diseases in each of the four crops is high. It is, therefore, suggested that intercrops involving these crops must be reviewed in Ghana's agricultural system.

- It is suggested that host range characteristics of *B. theobromae* among other important food and tree crops must be studied. This will help gather information to control *B. theobromae* diseases in the wider cropping systems.

- It is suggested that the genetic relatedness of *B. theobromae* isolates from cocoa, mango, banana and yam and other major food crops be established using more sensitive molecular tools such as DNA sequencing.

REFERENCES

ADRA International. (2010). Sustainable Rural Livelihood Enhancement and Poverty Reduction Through the Establishment of Perennial Tree Crops; Best Practices Award for Agroforestry, Extension Services/Farmer Training and Horticulture Production. Best Practices and Innovations (BPI) Initiative, December 2010. Available from : http//www.adraghana.org.

Alam, M.S., Begum, M.F., Sarkar, M.A., Islam, M.R. and Alam, M.S. (2001). Effect of temperature, light and media on growth, sporulation, formation of pigments and pycnidia of *Botryodiplodia theobromae* Pat. *Pak. J. Bio. Sci.*, 4(**10**): 1224-1227.

Alvarez, A.M. and Nishijima, W.T. (1987). Postharvest diseases of papaya. Plant Disease pathogen. **71**. pp 681-686.

Amusa, N. A., Adegbite, A. A., Muhammed, S. and Baiyewu, R. A. (2003). Yam disease and Its management in Nigeria. Minireview. African Journal of Biotechnology. 2(**12**) Pp 497-502. Available online at http://www.academicjournals.org/AJB.

Anonymous (2010). Investing in intercropping. New Agriculturist on-line report for the 21st Century. Available from: www.new-ag.info/99-2/focuson/focuson2.html#wa. Accessed 20th May, 2010.

Anonymous (2008). Species of Botryodiplodia**.** GBIF Data Portal May 05, 2008. Available from: http://zipcodezoo.com/fungi/b/botryodiplodia_theobramae/#SimilarSpecies.

Arjunan, G. Karthikeyan, G. Dinakaran, D. Raguchander, T. (1999). Diseases of Horticultural Crops, AE Publications, Coimbatore. pp 56-92.

Banful, B. (1998). Production of plantain, an economic prospect for food security in Ghana. In: Bananas and Food Security. (C. Picq, E. Foure and E.A. Frison, eds). International Symposium,10-11 November, 1998, Douala, Cameroon. pp 151-160.

Carroll, G.C. and Wicklow, D. T. (1992). The Fungal Community: Its Organization and Role in the Ecosystem. Marcel Dekker, Inc., New York. pp 327-354.

Cohen, J. (1994). Fulfilling Koch's Postulates. Science. **266**. pp 1647.

Crammer, J. (1979). *Botryodiplodia theobromae* . Commonwealth Mycological Institute, Kew, Surrey. pp 123.

Domsch, K. H., Gams, W. and Anderson, T. H. 2007. Compendium of Soil Fungi. 2nd Ed. Cornell University. IHW-Verlang. England. ISBN 3930167697, 9783930167692.

Ekundayo, J. A. (1973). Nutrient Requirements for Production of Pycnidia of *Botryodiplodia theobromae* Pat. Journal of General Microbiology, **77**. Great Britain. pp 227-228.

Ekundayo, J. A. and Haskins, R. H. (1969). Pycnidium production by *Botryodiplodia theobromae* : The relation of light to the induction of pycnidia. Canadian Journal of Botany. **47**. pp 1153-1156.

Faber, G. A., Bender, G. S. and Ohr, H. D. (2007). Diseases. UC IPM Pest management Guidelines. UC ANR publication 3436.

FAO. (1998). Storage and processing of roots and tubers in the tropics. Corporate document repository. Available from: http//www.fao.org/docrep/X5415E/X5415E00.htm

Felsenstein, l. (1993). PHYLIP (phylogenetic Inference Package),Version 3.5c. Department of Genetics, University of Washington, Seattle.

French, B. R. (2006). Diseases of Food plants in Papua New Guinea- A compendium, pdf book. 38 West St. Burnie Tasmania 7320, Australia. pp 4, 41, 235-265.

Griffin, D. H. (1994). Fungal Physiology (**2nd Ed**.). Wiley-Liss. New York. pp 140- 149.

Griffon, W. M., and Maublanc, A. (1909). Sur une maladie du cacaoyer. Bulletin de la Société Mycologique de France. **25**. pp 51-58.

González-Fernández, R., Prats, E., and Jorrín-Novol, J. V. (2010). Proteomics of Plant Pathogenic Fungi. Journal of Biomedicine and Biotechnology. **2010**. Hindawi Publishing Corporation. Córdoba, Spain. pp 36. DOI: 10.1155/2010/932527.

Henson, J. M. and French, R. (1993). The Polymerase Chain Reaction and Plant Disease Diagnosis. Annual Review of Phytopathology. **31**. pp 81- 109. DOI: 10.1146/annurev.py.31.090193.000501.

Higgins, D.G., Bleasby, A.J. and Fuchs, R. (1992). CLUSTAL V: Improved software for multiple sequence alignment. Computer and Applied Bioscience. **8**. pp 189-191.

Hillocks, R. J. and Wydra, K. (2002). Bacterial, Fungal and Nematode Diseases In: Cassava: Biology, Production and Utilization. CABI publishing. New York. USA. pp 261. ISBN 0 85199 524 1.

Hseu, S. H., Zeng, W. F., Lai, W. C., Pan, Y. P. and Lin, C. Y. (2008). Fruit rot disease of pineapple caused by *Burkholderia gladioli***.** Plant Pathology. Bull. **17**: 157-167.

Iotti, M., Barbieri, E., Stocchi, V. and Zambonelli, A. (2005). Morphological and molecular characterisation of mycelia of ectomycorrhizal fungi in pure culture. Fungal Diversity. **19**. pp 51-68.

Jiskani, M. M. (2002). Dying of mango orchards needs special attention, Sindh Agriculture University, Tandojam, Pakistan.

Jones, D.R. and Stove, R. H. (2000). Fungal diseases of banana fruit, preharvest disease. In Diseases of Banana, Abacá and Enset, D. R. Jones(ed.). CABI Publishing. Wallingford, UK. pp 173-190.

Kausar, P., Chohan, S. and Parveen, R. (2009). Physiological studies of *Lasiodiplodia theobromae* and *Fusarium solani*, the cause of Shesham deline. Mycopath. Bahauddin Zakariya University, Multan. **7** (1). pp 35-38.

Khanzada, M. A., Rajput, Q. A and Shahzad, S. (2006). Effect of Medium, Temperature, Light and Inorganic Fertilizers on *In Vitro* Growth and sporulation of *Lasiodiplodia theobromae* isolated from Mango. Journal of Botany, 38 (3). Pakistan. pp 885-889.

Khanzada, M. A., Lodhi, M. A and Shahzad, S. (2005). Chemical control of *Lasiodiplodia theobromae*, the causal agent of mango decline in Sindh. Journal of Botany, 38 (4). Pakistan. pp 1023-1030.

Khanzada, M. A., Lodhi, A. M. and Shahzad, S. (2004). Mango dieback and gummosis in Sindh, Pakistan caused by *Lasiodiplodia theobromae*. Online. Plant Health Progress doi: 10. 1094/ PHP- 2004- 0302- 01- DG.

Khanzada, M.A., Lodhi, A.M. and Shahzad, S. (2004a). Decline and gummosis diseases of mango in Sindh caused by *Lasiodiplodia theobromae. Online. Plant Health Progress* doi: 10.1094/PHP- 204-0302-01-DG.
Available from: http://www.plantmanagementnetwork.org

Khanzada, M.A., Lodhi, A.M. and Shahzad, S. (2004b). Pathogenicity of *Lasiodiplodia theobromae* and *Fusatium solani* on mango. *Pak. J. Bot*., **36** (1): 181-189.

Kokub, D., Azam, F., Hassan, A., Ansar, M., Asaid, M. J. and Khanum, A. (2007). Comparative growth, morphological and molecular characterization of indigenous *Sclerotium Rolfsii* strains isolated from different location of Pakistan. Pakistan Journal of Botany. **39**. pp 1849-1866.

Liew, E.C.Y., Guo, L.D., Ranghoo, V.M., Goh, T.K. and Hyde, K.D. (1998). Molecular approaches to assessing fungal diversity in the natural environment. Fungal Diversity. **1**. pp 1-17.

Lichtfouse, E., Navarrete, M., Debaeke, P., Souchère, V. and Alberola, C. (2009). Sustainable Agriculture. Springer. Dordrecht Heidelberg. London. pp 455.

Meah, M. B., Plumbley, R. A. and Jeger, M. J. (1991). Growth and infectivity of *Botryodiplodia theobromae* causing stem-end rot of mango. Mycological Research. **95** (4). pp 405-408. DOI: 10.1016/S0953-7562(09)80837-X.

Mascarenhas, P., Behere, A., Sharma, A. and Padwal-Desai, S. R. (1996). Post-harvest Spoilage of mango (*Mangifera indica*) by *Botryodiplodia theobromae*. Mycologiacal Research. **100** (1). pp 27-30. DOI: 10.1016/S0953-7562(96)80096-7.

Mohali, S., Burgess, T. I. and Wingfield, M.J. (2005). Diversity and host association of the tropical Tree endophyte *Lasiodiplodia theobromae* revealing using simple sequence repeat markers. Forest Pathology. **35**. Blackwell Verlag. Berlin. pp 385-396. DOI:10.1111/j.1439-0329.2005.00418.x. Available from: http://src.fabinet.up.ac.za/Botryosphaeriaceae/pdf/Mohali_et_al_2005_Diversity_host_association_Lasiodiplodia_theobromae_SSR_markers_For_Path.pdf

Mullis, K. B. and Faloona, F. A. (1987). Specific synthesis of DNA *in vitro* via a polymerase catalysed chain reaction. Methods in Enzymology. **155**. pp 335-350.

Narasimhudu, Y. and P.S.N. Reddy. (1992). A note on gummosis of mango. *Indian Phytopathology*.45(2): 261-262.

Onyenka, T. J., Dixon, A. G. O. and Ekpo, E. J. A. (2005). Identification of levels of resistance to cassava root rot disease (*Botryodiplodia theobromae*) in Africa landrace and improved germplasm using *in vitro* inoculation methods. Euphytica **1457**(3), Springer, Netherlands. ISSN 1573-5060 (Online). pp 281-288.

Opoku, I. Y., Assuah, M.K. and Domfeh, O. (2007). Manual for the identification and control of diseases of cocoa. Cocoa Research Institute of Ghana-Ghana Cocoa Board, Ghana. Technical Bulletin **No. 16**. pp 18-19.

Phillips, A.J.L. (2007). Lasiodiplodia theobromae.Version 2, Centro de Recursos Microbiológicos, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Portugal.

Phipps, P. M. and Porter, D. M. (1998). Collar rot of peanut caused by *Lasiodiplodia theobromae.* Plant Disease. **82**. pp 1205-1209.

Photita, W., Taylor, P. W. J., Ford, R., Hyde, K. D. and Lumyong, S. (2005). Morphological and molecular characterization of *Colletotricum* species from herbaceous plants in Thailand. Fungal Diversity. **18**. pp 117-133.

Pitt, J. I. and Hocking, A. D. (2009). Fungi and Food Spoilage. **3rd Ed**. Spinger. New York. pp 126. ISBN 978-0-387-92206-5 DOI: 10.1007/978-0-387-92207-2.

Plant pathologist's Pocket book. (1968). 2nd Ed. Common wealth mycological institute, Kew. Surrey, England. pp 398.

Punithalingam, E. (1976). *Botryodiplodia theobromae*. Descriptions of Fungi and Bacteria. **52**. Sheet 519.

Punja, Z. K. and Sun, L. J. (2001). Genetic diversity among mycelial compatibility groups of *Sclerotium rolfsii* (telemorph *Athelia rolfsii*) and *S. delphenii*. Mycol. Res. **105**. pp 537-546.

Rieger, M. (2006). Introduction to fruit crops. The Haworth Press.10 Alice Street, Binghamton, NewYork, United States of America. pp 87.

Ritchie, B. J. (2002). Mycological Media and Methods. Plant pathologist's pocketbook. (3). CABI publishing. CABI Bioscience UK center, Bakeham Lane, Egham, Surrey TW20 9TY, UK. pp 410.

Rossel, G., Espinoza, C., Javier, M. and Tay, D. (2008). Regeneration guidelines: sweet

potato and yam. In: Dulloo M.E., Thormann I., Jorge M.A. and Hanson J., editors. Crop specific regeneration guidelines [CD-ROM]. CGIAR System-wide Genetic Resource Programme, Rome, Italy. pp 9.

Saha, A., Mandal, P., Dasguspta, S. and Saha, D. (2008).Influence of culture media and environmental factors on mycelial growth and sporulation of *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl. Journal of Environmental Biology, **29** (3). Triveni Enterprise, Lucknow. India. pp 407-410.

Sangchote, S. (1988). Botryodiplodia Stem End Rot of Mango and Its Control In: Kasetsart, J. 1988. National Science Supply. **22**. pp 67-70.

Sangeetha, G., Anandan, A. and Rani, S. U. (2011). Morphological and Molecular characterization of *Lasiodiplodia theobromae* from various banana cultivars causing crown rot disease in fruits. 1st article. Archives of Phytopathology and Plant protection. pp 1-12. DOI:10.1080/03235408.2011.587986. Available from: http://www.tandfonline.com/doi/abs/10.1080/03235408.2011.587986#preview

Saghai-Maroof, M. A., Soliman, K. M., Jorgensen, R. A. and Allard R.W. (1984). PNAS 81:8014-8018.

SAS Institute Inc. (2003). SAS/STAT Users' Guide, version 8 (6th Ed.). **2**. Cary, NC, USA.

Shah, M. D., Verma, K. S., Singh, K. and Kaur, R. (2010). Morphological, pathological and molecular variability in *Botryodiplodia theobromae* (Botryosphaeriaceae) isolates associated with die-back and bark canker of pear tree in Punjab, India. Gent. Mol. Res. **9** (2). pp 1217-1228.

Shanthi, W. W., Yasodha, D. and Deepthi, W. (2008). Host Specificity of *Colletotrichum gloeosporiodes* and *Botryodiplodia theobromae* isolates from Mango, Papaya and Rambutan and their Response to *Trichoderma harzianum*. Tropentag. Conference on International Research on Food Security, Natural Resource Management and

Rural Development. University of Hohenheim, October 7-9. Available from: http://www.tropentag.de/2008/abstracts/full/417.pdf.

Solomon, J. D., McCracken, F. I., Anderson, R. L., Lewis, Jr. R., Oliveria, F. L., Filer, T. H. and Barry, P. J. (1987). A Guide to major Insects, Disease, Air pollution, and chemical Injury. Protection report R8-PR7. USDA Forest Service, Southern Forest Experiment Station. pp 69.

Sutton, B. C. (1980). The Coelomycetes. Commonwealth Mycological Inst., Kew, Surrey.

Swofford, D.L. (1993). PAUP: Phylogenetic Analysis Using Parsimony, version 3.1. Illinois Natural History Survey, Champaign, Illinois.

Taylor, J. W., Spatafora, J. and Berbee, M. (2006). Ascomycota. Sac Fungi. Available from: http://tolweb.org/Ascomycota/20521/2006.10.09. Also *in* The Tree of Life Web Project, http://tolweb.org/

Tongdee, S.C., Kanlayanarat, S. and Poonsawud, S. (1980). Varietal susceptibilities of mango to *Botryodiplodia theobromae* Pat, and chemical changes during pathogenesis. Thailand Institute of Scientific and Technological research, Research Project no. 24-01, Bangkok. pp 20.

Waller, J. M., Lenné, J. M. and Waller, S. J. (2002). Detection and isolation of fungal and bacterial pathogens. Plant pathologist's pocketbook. (3). CABI publishing. CABI Bioscience UK center, Bakeham Lane, Egham, Surrey TW20 9TY, UK. pp 208.

Williamson, D. and Tandon, R. N. (1966). Some pathological studies on Botryodiplodia theobromae pat. causing banana rot. Mycopathologia. **28.** Springer publisher, Netherlands, ISSN 0301- 486X. pp 245-253.

APPENDICES

Appendix I

PREPARATION OF SHELF POTATO DEXTROSE AGAR MEDIA

Thirty-nine grams (39 g) of the PDA was weighed into 1 litre of distilled water stirred with a magnetic stirrer till well dissolved and sterilized in an autoclave at 121°C and a pressure of 15 p.s.i for 25 minutes**.**

Appendix II

COMPOSITION OF V8 JUICE MEDIA

Czapek Diox Media	45.5 g
V8 juice	200.0 ml
Mycological Peptone	1.0 g
Acid Casein Hydroxylate	1.0 g
Yeast Extract	1.0 g
Distilled water	1.0 L

Appendix III

COMPOSITION OF CZAPEK DIOX MEDIA (Fluka Biochemika)

Sodium nitrate	2.0 g/L
Potassium chloride	0.5 g/L
Magnesium sulphate	0.5 g/L
Iron (II) sulfate	0.01 g/L
Potassium sulfate	1.0 g/L
Sucrose	30.0 g/L

Final pH: 7.3 ± 0.2 at 37 °C

Appendix IV

BioTeke PLANT GENOMIC DNA EXTRACTION PROTOCOL

Procedure:

NB: Add 60 µl of β-mecarptoethanol to Buffer P1 in a fume hood and pre-warm at 65°C.

1. Weigh 100 mg fresh mycelium in 2 ml tubes.
2. Grind in liquid nitrogen into fine powder and add 550 µl of pre warmed Buffer P1.
3. Add 4 µl RNase A and vortex.
4. Allow it to sit at room temperature (25 °C) for 20 minutes.
5. Add 130 µl Buffer P2, mix thoroughly and centrifuge at 13,000 rpm for 5 minutes.
6. Carefully transfer supernatant to a separation column and centrifuge at 12,000 rpm for 2 minutes and collect the flow through.
7. Add 1.5 volumes (1000 µl = 1ml) of Buffer P3 to the flow through and mix thoroughly.
8. Place a spin-column AC to a collection tube. Transfer the mixture (including precipitant) to the spin column AC. Centrifuge at 12,000 rpm for 1 minute. Discard flow through repeat with remaining sample.
9. Add 700 µl Buffer WB (Washing Buffer + ethanol).Centrifuge for 1 minute at 13,000 rpm. Discard the flow-through.
10. Add 500 µl Buffer WB. Centrifuge for 1 minute at 13,000 rpm. Discard the flow through.
11. Centrifuge the empty-spin column AC at 13,000 rpm for 3-5 minutes.
12. Transfer the spin-column AC to a clear 1.5 ml micro centrifuge tube and add 50 µl Buffer EB (Elution Buffer, warmed in 65-70°C before use) onto the silicified membrane.
13. Incubate 3-5 minutes at room temperature and centrifuge at 13,000 rpm for 1 minute.
14. Keep DNA at 2-8°C (-20 °C for long time storage)

Table 6

PERCENT (%) SIMILARITY MATRIX OF ALL 25 *Botryodiplodia theobromae* ISOLATES

SAMPLE	C(Ab)	C(Db)	C(Bk)	C(As)	C(Af)	M(At)	M(Ej)	M(Th)	M(Nk)	B(Db)	B(Kt)	B(Eu)	B(Yf)	B(Go)	B(Ab)	B(Ef)	B(Kf)	Y(Bk)	Y(Th)	Y(Ej)	Y(Yj)	Y(Ch)	Y(Zu)	Y(At)	Y(Kp)
C(Ab)	1.00																								
C(Db)	0.70	1.00																							
C(Bk)	0.31	0.54	1.00																						
C(As)	0.40	0.31	0.31	1.00																					
C(Af)	0.08	0.23	0.23	0.83	1.00																				
M(At)	0.38	0.27	0.17	0.22	0.00	1.00																			
M(Ej)	0.18	0.35	0.35	0.11	0.36	0.13	1.00																		
M(Th)	0.27	0.47	0.38	0.46	0.38	0.14	0.47	1.00																	
M(Nk)	0.09	0.36	0.36	0.00	0.22	0.29	0.29	0.21	1.00																
B(Db)	0.71	0.50	0.15	0.33	0.00	0.29	0.06	0.21	0.00	1.00															
B(Kt)	0.42	0.67	0.54	0.42	0.33	0.17	0.35	0.69	0.25	0.36	1.00														
B(Eu)	0.36	0.29	0.13	0.36	0.00	0.33	0.11	0.25	0.08	0.30	0.20	1.00													
B(Yf)	0.18	0.14	0.14	0.08	0.00	0.25	0.19	0.06	0.10	0.10	0.07	0.56	1.00												
B(Go)	0.17	0.21	0.21	0.08	0.30	0.22	0.54	0.36	0.20	0.09	0.21	0.15	0.30	1.00											
B(Ab)	0.15	0.38	0.38	0.15	0.56	0.09	0.50	0.54	0.30	0.08	0.50	0.14	0.17	0.36	1.00										
B(Ef)	0.36	0.57	0.47	0.36	0.38	0.14	0.39	0.71	0.21	0.31	0.83	0.25	0.13	0.27	0.67	1.00									
B(Kf)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.33	0.00	0.11	0.08	1.00								
Y(Bk)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.14	0.00	0.00	0.00	0.33	1.00							
Y(Th)	0.00	0.20	0.20	0.00	0.40	0.00	0.31	0.33	0.30	0.00	0.29	0.07	0.08	0.25	0.45	0.33	0.00	0.00	1.00						
Y(Ej)	0.10	0.24	0.30	0.10	0.22	0.11	0.38	0.27	0.24	0.05	0.24	0.14	0.16	0.28	0.33	0.27	0.00	0.00	0.50	1.00					
Y(Yj)	0.09	0.07	0.15	0.20	0.00	0.13	0.06	0.06	0.00	0.11	0.07	0.08	0.10	0.09	0.08	0.06	0.00	0.00	0.00	0.24	1.00				
Y(Ch)	0.00	0.00	0.08	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.33	1.00			
Y(Zu)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00		
Y(At)	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.25	0.14	0.00	1.00	
Y(Kp)	0.10	0.08	0.08	0.00	0.00	0.33	0.06	0.00	0.29	0.00	0.00	0.09	0.11	0.10	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.13	1.00

Appendix V

Combined data (base pair) of all isolates produced by RAPD primer MH-S1118 and SSR, MH-BOT_19 and_20, and MH-LAS_15 and_16**.**

SAMPLE/ ISOLATE	TOTAL NUMBER OF BANDS	TOTAL BASE PAIR													
C(Ab)	7	1351	1243	1027	700	633	500	50							
C(Db)	10	1351	1243	1027	700	633	500	300	200	100	50				
C(Bk)	10	1432	1351	1297	800	700	500	300	200	100	50				
C(As)	7	1297	1243	800	767	633	500	50							
C(Af)	6	500	400	300	243	100	50								
M(At)	4	700	633	500	400										
M(Ej)	13	1162	1111	1108	1000	900	700	600	500	400	300	200	100	50	
M(Th)	11	1297	1243	767	633	600	500	400	300	200	100	50			
M(Nk)	5	700	400	300	200	100									
B(Db)	5	1351	1243	1027	633	500									
B(Kt)	10	1351	1297	1243	633	500	400	300	200	100	50				
B(Eu)	8	1250	1243	900	767	700	633	562	500						
B(Yf)	6	1250	1162	900	700	562	500								
B(Go)	6	1162	700	600	500	400	300								
B(Ab)	8	562	550	500	400	300	200	100	50						
B(Ef)	11	1351	1297	1243	633	562	500	400	300	200	100	50			
B(Kf)	2	1250	562												
Y(Bk)	2	1250	1055												
Y(Th)	8	900	783	533	500	400	300	200	100						
Y(Ej)	14	1367	1267	900	800	783	700	600	533	500	400	333	300	200	100
Y(Yj)	5	800	600	500	333	299									
Y(Ch)	3	1133	800	229											
Y(Zu)	0														
Y(At)	5	1133	1066	900	600	333									
Y(Kp)	4	1066	700	400	271										

Appendix VI

Matrix of number of differences in DNA bands among 24 *Botryodiplodia theobromae* isolates using RAPD primer S1118 and SSR, MH-BOT_19 and_20, and MH-LAS_15 and_16.

	C(Ab)	C(Db)	C(Bk)	C(As)	C(Af)	M(At)	M(Ej)	M(Th)	M(Nk)	B(Db)	B(Kt)	B(Eu)	B(Yf)	B(Go)	B(Ab)	B(Ef)	B(Kf)	Y(Bk)	Y(Th)	Y(Ej)	Y(Yj)	Y(Ch)	Y(At)	Y(Kp)
C(Ab)																								
C(Db)	3																							
C(Bk)	6	6																						
C(As)	6	9	9																					
C(Af)	9	9	8	9																				
M(At)	5	5	10	7	6																			
M(Ej)	14	14	11	16	9	11																		
M(Th)	10	7	9	6	7	9	10																	
M(Nk)	10	7	7	12	5	5	8	8																
B(Db)	2	5	11	6	9	5	16	10	10															
B(Kt)	7	4	6	7	6	8	11	3	7	7														
B(Eu)	7	10	14	7	12	6	15	11	11	7	12													
B(Yf)	9	12	12	11	10	6	11	15	9	9	14	4												
B(Go)	9	10	10	11	6	4	7	9	5	9	10	10	6											
B(Ab)	11	8	8	11	4	8	9	7	5	11	6	12	10	8										
B(Ef)	8	5	7	8	7	9	12	4	8	8	1	11	13	11	5									
B(Kf)	9	12	12	9	8	6	15	13	7	7	12	6	4	8	8	11								
Y(Bk)	9	12	12	9	8	6	15	13	7	7	12	8	6	8	10	13	2							
Y(Th)	13	10	10	13	6	8	9	9	5	11	8	12	10	8	6	9	10	10						
Y(Ej)	17	14	12	17	12	12	11	13	9	17	14	16	14	10	12	15	16	16	6					
Y(Yj)	10	13	11	8	9	7	14	12	10	8	13	11	9	7	11	14	7	7	11	11				
Y(Ch)	10	13	11	8	9	7	16	14	8	8	13	11	9	9	11	14	5	5	11	15	4			
Y(At)	12	15	15	12	11	9	14	14	10	10	15	11	9	9	13	16	7	7	11	13	6	6		
Y(Kp)	10	12	12	11	8	4	13	13	5	9	12	10	8	6	10	13	6	6	10	14	9	7	7	

Appendix VII

PROC GLM ANALYSED DATA

Analyzed data cross inoculation test on Kent Mango variety

Class Level Information

Class	Levels	Values
trt	5	BGo CDb MAt Ykp Contrl

Number of observations 15

Dependent Variable: day1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0	0	.	.
Error	10	0	0		
Corrected Total	14	0			

R-Square	Coeff Var	Root MSE	day1 Mean
0.000000	.	0	0

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	4	0	0	.	.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	4	0	0	.	.

Dependent Variable: day2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	1575.566667	393.891667	30.49	<.0001
Error	10	129.166667	12.916667		
Corrected Total	14	1704.733333			

R-Square	Coeff Var	Root MSE	day2 Mean
0.924231	25.73253	3.593976	13.96667

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	4	1575.566667	393.891667	30.49	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	4	1575.566667	393.891667	30.49	<.0001

Dependent Variable: day3

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	2649.433333	662.358333	11.21	0.0010
Error	10	591.000000	59.100000		
Corrected Total	14	3240.433333			

R-Square	Coeff Var	Root MSE	day3 Mean
0.817617	30.83283	7.687652	24.93333

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	4	2649.433333	662.358333	11.21	0.0010

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	4	2649.433333	662.358333	11.21	0.0010

Dependent Variable: day4

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	4917.433333	1229.358333	10.76	0.0012
Error	10	1142.666667	114.266667		
Corrected Total	14	6060.100000			

R-Square	Coeff Var	Root MSE	day4 Mean
0.811444	30.45458	10.68956	35.10000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	4	4917.433333	1229.358333	10.76	0.0012

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	4	4917.433333	1229.358333	10.76	0.0012

Test of Means
Student-Newman-Keul test

Test for day1

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0

Number of Means	2	3	4	5
Critical Range	0	0	0	0

Means with the same letter are not significantly different.

SNK Grouping	Mean	N	trt
A	0	3	BGo
A	0	3	CDb
A	0	3	Contrl
A	0	3	MAt
A	0	3	Ykp

Test for day2

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	12.91667

Number of Means	2	3	4	5
Critical Range	6.5383881	8.0442193	8.9775759	9.6575796

Means with the same letter are not significantly different.

SNK Grouping	Mean	N	trt
A	27.167	3	MAt
B A	20.667	3	Ykp
B	17.833	3	BGo
C	4.167	3	CDb
C	0.000	3	Contrl

Test for day3

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	59.1

Number of Means	2	3	4	5
Critical Range	13.985861	17.206891	19.203377	20.657931

Means with the same letter are not significantly different.

SNK Grouping	Mean	N	trt
A	39.667	3	MAt
A	30.833	3	BGo
A	27.500	3	Ykp
A	26.667	3	CDb
B	0.000	3	Contrl

Test for day4

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	114.2667

Number of Means	2	3	4	5
Critical Range	19.447118	23.925909	26.701991	28.724525

Means with the same letter are not significantly different.

SNK Grouping	Mean	N	trt
A	51.833	3	MAt
A	44.167	3	BGo
A	40.667	3	CDb
A	38.833	3	Ykp
B	0.000	3	Contrl

MEANS AND STANDARD ERRORS

---------- trt=BGo ----------

Variable	Mean	Std Error	Coeff of Variation
day1	0	0	.
day2	17.8333333	0.6666667	6.4749563
day3	30.8333333	2.8915586	16.2432208
day4	44.1666667	6.0092521	23.5660302

---------- trt=CDb ----------

Variable	Mean	Std Error	Coeff of Variation
day1	0	0	.
day2	4.1666667	2.3154073	96.2496753
day3	26.6666667	1.5898987	10.3266948
day4	40.6666667	0.3333333	1.4197138

---------- trt=Contrl ----------

Variable	Mean	Std Error	Coeff of Variation
day1	0	0	.
day2	0	0	.
day3	0	0	.
day4	0	0	.

---------- trt=MAt ----------

Variable	Mean	Std Error	Coeff of Variation
day1	0	0	.
day2	27.1666667	1.8782379	11.9749824
day3	39.6666667	2.9202359	12.7512529
day4	51.8333333	1.5898987	5.3127690

---------- trt=Ykp ----------

Variable	Mean	Std Error	Coeff of Variation
day1	0	0	.
day2	20.6666667	3.4920545	29.2665279
day3	27.5000000	8.8928810	56.0106247
day4	38.8333333	12.3164299	54.9339458

Analyzed Data on cross inoculation test on Akokra Badi cocoa variety
PROC GLM procedure

Class Level Information

Class	Levels	Values
trt	5	BGo CDb Contrl MAt Ykp

Number of observations 15

Dependent Variable: day1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0	0	.	.
Error	10	0	0		
Corrected Total	14	0			

R-Square	Coeff Var	Root MSE	day1 Mean
0.000000	.	0	0

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	4	0	0	.	.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	4	0	0	.	.

Dependent Variable: day2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	33.5666667	8.3916667	1.23	0.3579
Error	10	68.1666667	6.8166667		
Corrected Total	14	101.7333333			

R-Square	Coeff Var	Root MSE	day2 Mean
0.329948	159.8495	2.610875	1.633333

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	4	33.56666667	8.39166667	1.23	0.3579

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	4	33.56666667	8.39166667	1.23	0.3579

Dependent Variable: day3

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	151.5666667	37.8916667	0.79	0.5566
Error	10	478.6666667	47.8666667		
Corrected Total	14	630.2333333			

R-Square	Coeff Var	Root MSE	day3 Mean
0.240493	158.4406	6.918574	4.366667

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	4	151.5666667	37.8916667	0.79	0.5566

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	4	151.5666667	37.8916667	0.79	0.5566

Dependent Variable: day4

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	1299.500000	324.875000	2.43	0.1158
Error	10	1335.000000	133.500000		
Corrected Total	14	2634.500000			

R-Square	Coeff Var	Root MSE	day4 Mean
0.493262	100.4715	11.55422	11.50000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	4	1299.500000	324.875000	2.43	0.1158

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	4	1299.500000	324.875000	2.43	0.1158

Dependent Variable: day5

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	2510.233333	627.558333	5.72	0.0117
Error	10	1097.500000	109.750000		
Corrected Total	14	3607.733333			

R-Square	Coeff Var	Root MSE	day5 Mean
0.695792	48.80200	10.47616	21.46667

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	4	2510.233333	627.558333	5.72	0.0117

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	4	2510.233333	627.558333	5.72	0.0117

Dependent Variable: day6

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	4804.066667	1201.016667	4.93	0.0186
Error	10	2435.333333	243.533333		
Corrected Total	14	7239.400000			

R-Square	Coeff Var	Root MSE	day6 Mean
0.663600	47.57791	15.60555	32.80000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	4	4804.066667	1201.016667	4.93	0.0186

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	4	4804.066667	1201.016667	4.93	0.0186

Test of Means
Student-Newman-Keul test

Test for day1

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0

Number of Means	2	3	4	5
Critical Range	0	0	0	0

Means with the same letter are not significantly different.

SNK Grouping	Mean	N	trt
A	0	3	BGo
A	0	3	CDb
A	0	3	Contrl
A	0	3	MAt
A	0	3	Ykp

Test for day2

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	6.816667

Number of Means	2	3	4	5
Critical Range	4.7498675	5.8437914	6.5218362	7.0158307

Means with the same letter are not significantly different.

SNK Grouping	Mean	N	trt
A	3.500	3	BGo
A	3.167	3	MAt
A	1.500	3	CDb
A	0.000	3	Contrl
A	0.000	3	Ykp

Test for day3

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	47.86667

Number of Means	2	3	4	5
Critical Range	12.586705	15.485501	17.282257	18.591296

Means with the same letter are not significantly different.

SNK Grouping	Mean	N	trt
A	8.500	3	BGo
A	7.500	3	MAt
A	3.333	3	CDb
A	2.500	3	Ykp
A	0.000	3	Contrl

Test for day4

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	133.5

Number of Means	2	3	4	5
Critical Range	21.020164	25.861238	28.861872	31.048005

Means with the same letter are not significantly different.

SNK Grouping	Mean	N	trt
A	27.833	3	BGo
A	12.833	3	MAt
A	11.000	3	CDb
A	5.833	3	Ykp
A	0.000	3	Contrl

Test for day5

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	109.75

Number of Means	2	3	4	5
Critical Range	19.058896	23.448277	26.16894	28.151098

Means with the same letter are not significantly different.

SNK Grouping		Mean	N	trt
	A	38.333	3	BGo
	A	30.000	3	MAt
B	A	21.833	3	CDb
B	A	17.167	3	Ykp
	B	0.000	3	Contrl

Test for day6

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	243.5333

Number of Means	2	3	4	5
Critical Range	28.390607	34.929139	38.981906	41.934578

Means with the same letter are not significantly different.

SNK Grouping		Mean	N	trt
	A	53.50	3	BGo
	A	42.50	3	MAt
B	A	34.17	3	Ykp
B	A	33.83	3	CDb
B		0.00	3	Contrl

MEANS AND STANDAR ERRORS

---------- trt=BGo ----------

Variable	Mean	Std Error	Coeff of Variation
day1	0	0	.
day2	3.5000000	2.5658007	126.9742060
day3	8.5000000	6.6017674	134.5246662
day4	27.8333333	6.8455176	42.5992249
day5	38.3333333	7.8386507	35.4181076
day6	53.5000000	8.0363756	26.0175905

---------- trt=CDb ----------

Variable	Mean	Std Error	Coeff of Variation
day1	0	0	.
day2	1.5000000	0.7637626	88.1917104
day3	3.3333333	1.6914819	87.8919792
day4	11.0000000	5.5000000	86.6025404
day5	21.8333333	6.0023144	47.6167028
day6	33.8333333	10.4256628	53.3727418

---------- trt=MAt ----------

Variable	Mean	Std Error	Coeff of Variation
day1	0	0	.
day2	3.1666667	2.0480343	112.0199824
day3	7.5000000	5.2041650	120.1850425
day4	12.8333333	11.1292907	150.2064695
day5	30.0000000	7.4218147	42.8498671
day6	42.5000000	8.2613558	33.6684424

---------- trt=Ykp ----------

Variable	Mean	Std Error	Coeff of Variation
day1	0	0	.
day2	0	0	.
day3	2.5000000	2.5000000	173.2050808
day4	5.8333333	4.6398036	137.7664394
day5	17.1666667	5.5100918	55.5947119
day6	34.1666667	12.8203397	64.9916482

---------- trt=Contrl ----------

Variable	Mean	Std Error	Coeff of Variation
day1	0	0	.
day2	0	0	.
day3	0	0	.
day4	0	0	.
day5	0	0	.
day6	0	0	.