Identification and characterization of *colletotrichum* species associated with mango and citrus diseases in the Ashanti region of Ghana

BY

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IDENTIFICATION AND CHARACTERIZATION OF *COLLETOTRICHUM* SPECIES ASSOCIATED WITH MANGO AND CITRUS DISEASES IN THE ASHANTI REGION OF GHANA

A Thesis submitted to the Department of Crop and Soil Sciences,

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of

DOCTOR OF PHILOSOPHY

in Plant Pathology

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DECLARATION

I, hereby, declare that this submission is my own work towards the PhD degree 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.

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ABSTRACT

The purposes of the study were to identify *Colletotrichum* species, detect their variability, to investigate their cross infectivity potential and determine farmers' awareness about the diseases that are associated with mango and citrus. Culture characteristics, size and shape of conidia and mycelial growth rates were used to characterize the isolates into different morphological groups. Those grouped isolates were further studied for their genetic similarity and differences, using multigene loci primers and restriction enzymes. ITS region, β- tub gene and GPDH primers were also used to characterize the isolates.

Farmers were aware of mango anthracnose but did not know any such disease on citrus. *Colletotrichum* species isolated from mango and citrus had overlap in their cultural characteristics. All of the isolates were assigned into ten morphogroups. Seven of the morphogroups were characterized by their cylindrical conidia and three of the morphogroups (G1, G3 and G6) had curved conidia. Apart from the conidial shape, most of the morphogroups had unique characters to stand as a group on their own. However, G2, G5 and G9 showed overlap in many of their characters.

The results from molecular analysis were different, depending on the gene/region targeted. The ITS region amplification showed high genetic similarity among the ten morphogroups, irrespective of their conidial or other cultural characteristic variations. Further investigation on the same ITS region, using *Colletotrichum* species-specific primer, CgInt, confirmed the similarity for six of the morphogroups such as (G1, G2, G4, G5, G8 and G9) but the other four morphogroups were not picked. The overall results of the ITS region were very

helpful in understanding the close relationship of the different morphogroups. Further, analysis of the same group of isolates, using β-tubulin primer, clearly showed existence of genetic variability in between the groups. The variability expressed on β-tub gene analysis was in line with the characterization made, based on conidial shape. It gave two distinct groups; one group having cylindrical conidia and the other having curved conidia. This result is an indicator that conidial shape is governed by a genetic factor. The third gene, GPDH analysis gave high genetic variability results. It amplified nine distinct groups from the ten morphogroups studied. Eight of them were in line with eight of the morphogroups. Therefore, GPDH results were more detailed than the other studied regions of *Colletotrichum* species in showing genetic variability. However, the combined results of the morphological characterization and the multigene analyses played their own role for a better understanding of species variability.

The pathogenicity tests on mango and citrus fruits confirmed most of the morphogroups as pathogenic. However, all the curved morphogroups isolated from diseased mango tested negative on mango. The cross-infectivity potential of citrus and mango isolates of *Colletotrichum* were confirmed. The cross tests on papaya also showed that most of the morphogroups were pathogenic.

From the study, it is understood that critical investigation of *Colletotrichum* spp., using the traditional techniques of morphological characterization, can assist in determining the genetic variability among isolates but it is tedious, time consuming and wrong conclusions could be drawn if it is first time study. Once the organisms are sequenced and named, the conventional investigation could play a great role to detect the variability. Therefore, it is recommended that the

identified groups of isolates be sequenced for the purpose of accurately naming them to the species level.

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ABBREVIATIONS

ANOVA	Analysis of Variance
bp	base pair
cm	centi meter
^{o}C	Celsius degrees
CSIR- CRI	Council for Scientific and Industrial Research-Crops Research Institute
cv.	Cultivar
DNA	Deoxyribonucleic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
EDTA	Ethylene diamine tetra acetic acid
EDAIF	Export Development and Agricultural Investment Fund
FAOSTAT	Food and Agriculture Organisation Statistics
GAPDH	Partial glyceraldehyde-3- phosphate dehydrogenase
h	hour
ITS	Internal Transcribed Spacers
kb	kilobase
LSD	Least Significant Difference
min	minute
mm	millimetre
NGOs	Non- governmental organisations
PCR	polymerase chain reaction
PDA	Potato dextrose agar
PRA	Participatory rural appraisal
RAPD	Random amplified polymorphic DNA
rDNA	ribosomal DNA
TAE	Tris-acetate ethylene diamine tetra acetic acid
TUB2	partial β- tubulin

USA	United States of America
Uv	Ultraviolet

Symbol

µM	micromolar
mM	millimolar
M	molar
$MgCl_2$	magnesium chloride
NaClO	sodium hypochlorite

Nucleotide Bases

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

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DEDICATION

This thesis dedicated to my beloved uncle, Mr Niguse Mekonen.

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Background of the study

Ghana's economy depends largely on agriculture, and it is the key to overall economic growth and development. It contributes about 40% of national economy, three quarters of export earnings, and employs 60% of the work-force (McKay and Aryeetey, 2004). In the last two decades, the sector has served as the main driver for economic growth (World Bank, 2007).

The government of Ghana has supporteded the development of non-traditional crops industries to diversify the country's export commodity. Horticulture has been a central and a major sector of these efforts. The major horticultural crops that are mainly produced in the country include pineapple, mango, papaya, banana, citrus, chilli, tomatoes, and plantain. Growth in the sector of mango production and export has shown significant improvement (USAID, 2009). There is also a growing worldwide demand for both mango and citrus fruits.

In Ghana, there are many farmers engaged in commercial mango and citrus production with the interest of getting into the world market. Government also has made a great move to extend the sector, especially, mango. Therefore, the Ministry of Food and Agriculture, in collaboration with NGOs, supports extension services and training to improve upon the traditional fruit growing system. Towards this end, fruit farmers are doing their best by coming together in forming association or farmer-based organizations (http://www.freshplaza.com/article/125941, accessed on

29 August 2014). They share their knowledge, challenges and work together for quality and high production that will help them to succeed, certifying their farms to penetrate the world market. However, diseases and pests still remain a big challenge for fruit growers.

Disease and pest attacks, coupled with the lower shelf-life of fruits, pose a serious threat to the mango industry locally as well as in the world. Recent news indicated that Ghana mango export was banned from Europian market because of disease and pest-relate dissues (http:/www.ghanaweb.com/Ghana Home page/business/artikel?ID=278405).

Although the citrus and mango crops are distantly related, there are many diseases and pests that they share in common. Anthracnose is one of the commonest serious diseases that challenge both crops (Cannon *et al*., 2012). *Colletotrichum* is one of the most important plant pathogens causing anthracnose in a wide range of hosts (Bailey and Jeger, 1992). It causes significant economic damage to crops in tropical, subtropical, and temperate regions. Disease outbreaks can be severe, especially under prolonged warm and wet weather conditions (Biggs and Miller, 2001). Two distinct types of anthracnose disease can occur at different stages, in the developing fruit in the field (pre-harvest) and during storage. Due to its ability to cause latent or quiescent infections, *Colletotrichum* species are considered to be very important postharvest pathogens. They cause disease on leaves, stems and fruits of host plants. In addition, infection can occur at any developmental stages of the plant, however most eonomic losses recorded is by post harvest fruit infection.

The climate suitable for mango production has been reported to favour anthracnose disease and it is the most important field and post harvest disease of the crop in the world (Sangeetha and Rawal, 2009; Chowdhury *et al*., 2008). The same disease, caused by *Colletotrichum gloeosporioides*, is also reported as ubiquitous on citrus species and relatives (Huang *et al*., 2013, Lijuan *et al*., 2012).

In general, the anthracnose disease has a wide host range and geographical distribution. Although many crops could be attacked by anthracnose disease, the causal agent, *Colletotrichum* spp., might be the same or different. This situation can vary, based on crop types, geographical location, the growth stage or agronomic practices in farming. Post harvest losses of mango fruits caused by *Colletotrichum* spp. could be as high as 100% on fruits produced in wet or high humid conditions (Arauz, 2000). The disease has also been reported as a major postharvest disease, causing huge losses and threatening mango export and consumption in Ghana (Kumah *et al*., 2011).

Worldwide information on pathogenic or genetic diversity study, infection process and the disease cycle of *Colletotrichum* are required to battle anthracnose disease and breeding for resistance (Than *et al*., 2008). The current status of anthracnose in Ghana still requires improvement to answer many questions. In particular, *Colletotrichum* species associated with different types of crops grown in the country are not well known. There is also little information concerning the complex group of species that might be involved in anthracnose. *Colletotrichum gloeosporioides* is the most mentioned causal agent of anthracnose diseases on different crops, including mango and citrus. However, Honger (2014) indicated that *C.gloeosporioides* is no more the cause of anthracnose disease of mango in Ghana but rather *Colletotrichum asianum* Prihastuti, L. Cai & K.D. Hyde.

Even with the recent change in the understanding of the species concepts in *Colletotrichum*, more understanding is still required to identify the exact species that cause the disease in citrus and mango. This is because, diverse species of *Colletotrichum* may be present on a single host and at the same time the same *Colletotrichum* species can infect more than one host. In addition, *Colletotrichum* species recovered from one host plant can vary, depending on the host growth stage. In Ghana, it is not yet known whether a single species of *Colletotrichum* is infecting or there are more than one species associated with mango and citrus crops. Correct and accurate identification of the species involved in anthracnose will help in effective management (Whitelaw-Weckert *et al*., 2007). For example, *C. acutatum* J.H. Simmonds, was found to be moderately susceptible to the fungicide, benzimidazole, while *C. gloeosporioides* was highly susceptible (Peres *et al*., 2004). Also, implementing a spray programme where mixed population exists without accounting for their differential sensitivity may result in a shift in population ratio and that might affect the pathogen's ability to evolve as a result of resistance or response to the control measure.

Therefore, it was worth enough to identify and characterize *Colletotricum* species associated with anthracnose diseases of mango and citrus, and as well, to find out how farmers knowledge of the disease.

1.2 Objectives of the Study

The main objective of the study was to identify and characterize *Colletotrichum* species associated with anthrachnose disease of mango and citrus and to assess farmers' perception/knowledge, prevalence and distribution of anthracnose in major production areas in Ashanti Region of Ghana.

The specific objectives were to:

i) determine farmers' perception/knowledge and prevalence of anthracnose disease on mango and citrus caused by *Colletotrichum* species, ii) characterize *Colletotrichum* isolates from different growing areas, using morphological characteristics, iii) characterize the genetic diversity of *Colletotrichum* isolates, using molecular markers, and iv) confirm the pathogenicity of identified isolates on its host (citrus or mango) and check their cross infectivity potential to the opposite crops and additionally to pawpaw (*Carica papaya* L.).

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Mango and citrus

Mangoes have been reported to be very good source of vitamin C and A, minerals such as copper and potassium with traces of magnesium and manganese, also citrus has good source of vitamin C and A with minerals such as calcium, iron, copper, phosphorus, potassium, magnesium and sulphur with trace of chlorine (https://www.organicfacts. net/nutrition-facts/ fruits/nutritional-value-oforange-andmango.html. Accessed in August 2015). They are important fruit crops in most tropical regions of the world and predominantly eaten in the developed countries (Diedhiou *et al*., 2007). In Nigeria, although both fruits are eaten as dessert, mango ranks first to citrus and other tropical fruits such as pineapple. Furthermore, fresh fruit and fruit juice consumption in Ghana is very common (Brentu *et al*., 2012). This is mainly fuelled by a growing health-conscious middle class. According to Ministry of Trade and Industry, Ghana imported over 54,000 tonnes of fruit juices in 2012; and it had shown 500% increase from 2005 import.

It has also been reported that the fruits can be processed into dry mango, mango pickle, mango jelly, or can be eaten cooked and also used as a raw material for juice processing companies (Match Maker Associates, 2008, Crane *et al*., 2006). Mango and citrus are specialty/cash crops in most of the international markets and hence, an important source of foreign exchange for most developing countries, including Ghana.

2.1.1 Climatic and ecological requirements of mango and citrus

Citrus is grown throughout the world where rainfall or irrigation water is sufficient to support plant growth. Mangoes are grown at elevations from sea level to 1200 m in

the tropics, but they do best below 600 m and in climates with strongly marked seasons and dry weather for flowering and fruiting (Samson, 1992). Heavy rain during flowering causes a marked reduction in pollination, fruit set and maturing fruits. The optimum growth temperature is 24 - 27 ^{o}C (Kochhar, 1986). They are grown in areas with an annual rainfall of 750- 1900 mm, provided there is an adequate dry season (Kochhar, 1986). They will thrive on a wide variety of soils, provided they are not waterlogged, too alkaline or too rocky. A pH of 6 - 7.2 is preferred (NAD, 2000).

2.1.2 Production, export and market potential

India is the world's largest producer of mango, producing about 70% of total world production. Kenya is the largest producer in Africa with 553, 710 MT, followed by Egypt with 505,741 MT (FAOSTAT, 2010a). Mexico is the world's largest exporter of the crop followed by India and Brazil. In Africa, none of the producer countries is considered as one of the top ten exporting countries (FAOSTAT, 2010b). In West Africa, the current information about countries that produce the crop in commercial quantities includes Cote d'ivoire and Ghana (Honger, 2014). Ghana is a commercial producer of tropical fruits, with a lot of its citrus cultivated in the Ashanti Region. Predominantly the production is by small and medium scale holders' farmers mostly as monoculture capacity (Okorley *et al*., 2014).

The demand for mango in Ghana far exceeds the supply. In terms of export earnings, currently the income per acre for mango and citrus stands higher than Cocoa (http://www.ghanaweb.com/GhanaHomePage/NewsArchive/artikel.php?ID=143520. Mango export earnings have shown that mango has enormous potential that could transform Ghana's economy much better than cocoa and other traditional export products. Currently, high investment has been made by EDAIF of Ghana to plant

mango to make five districts a mango production hub (http:// the chronicle co.mango-marketing-deal).

Demand of South Africa for Ghana's mango fruit is growing, especially for winter months which incidentally coincide with the harvest season in Ghana (http://www.ghanaweb.com/GhanaHomePage/NewsArchive/artikel.php?ID=143520 There is also an emerging demand for local varieties in UK market because of the increasing preference for smaller fruit sizes, which do not need to be sliced for distribution for students in school (Market brief, 2005). The small sized mangoes and bananas are currently supplied mainly from South America. Therefore, even in addition to improved variety, Ghana's local mango variety has future export potential.

2.1.3 Challenges of citrus and mango production

Despite the high nutritional value and their economic importance to the country, mango and citrus development in Ghana has lagged far behind the opportunities. Both citrus and mango also can be attacked by the same diseases and insect pests. In Ghana, the production is constrained by many factors such as unmanaged orchard, cost of production, pests and diseases. Particularly, diseases and insect pests are the major stumbling block of mango and citrus production in the country. However, Nelson (2008) indicated that mango is an important export crop in countries or localities where quarantine pests and diseases can be controlled satisfactorily. Mango is affected by a number of diseases at all stages of its development, from seedlings in the nursery, to the fruits in storage or transit (Prakash, 2004; Ploetz, 2003). According to Awa *et al.* (2012), as a result of mango anthracnose disease outbreak in south western Nigeria,

mango production is no longer attractive to farmers in the area. Recent news indicated that Blue Sky Processing Company of Ghana has started importing fresh mango fruits from Brazil to feed its processing company as consequence of less production of local farmers due to flower abortion (http://www.ghanaweb.com/GhanaHomePage/business/Local-mango-farmers-countrainfall-cost-338232 accessed, December 2014). On the other hand, report of fungal diseases cause serious losses to citrus in Ghana. According to Megan and Timmer (2009), Black spot and *Pseudocercospora* leaf and fruit spot are the most important and severe problems.

2.2 Agricultural science and farmers' role

2.2.1 Farmers' indigenous knowledge and success of farming

Traditional knowledge of farmers is indigenous or local forms of knowledge. It has been also transmitted over numerous generations (Rist and Dahdouh-Guebas, 2006). Indigenous knowledge has often been dismissed as unsystematic and incapable of meeting the rapid economic growth needs of the modern world. Formal science is believed to be a universal, autonomous and value-free knowledge system. However, according to Rist and Dahdouh-Guebas (2006), scientific information and its imposition without proper attention to local knowledge and wisdom has led to considerable disappointment.

Traditional agricultural knowledge addresses the cultural and technical knowledge that farmers, in a specific area, possess. The richness of this knowledge is often enormous (Morales and Perfecto, 2000). The concept of local knowledge should not be seen as acumulated information but is also a practice that unfolds through actual engagement

and performance of environmentally situated actions (Krause *et al* 2010). Mukanga *et al*. (2011) stated that, farmers' knowledge on maize ear rot is more detailed than scientists suspected. Scientists should recognize farmers' constraints and their existing technical knowledge (Morse and Buhler, 1997; Kenmore, 1991). Soleri *et al*. (2000) added, that the acceptability of agricultural technologies by farmers depends on how well farmers' constraints and preferences have been identified. Odendo *et al*. (2002) researched using participatory rural appraisals (PRA) on variety selection helped to know the most important traits for farmers. Nkongolo *et al.* (2008) also used farmer participatory tools to access farmers' indigenous knowledge of the major characteristics of sorghum landraces and was allowed to select already existing outperformed sorghum varieties.

2.2.2 Farmers' perception and its importance for plant protection

In the area of plant diseases and pests, there is already some understanding of the limits of farmers' knowledge (Manu-Aduening *et al*., 2007; Bentley and Thiele, 1999). According to Trutmann *et al*. (1996), farmers were unaware of the causes of various bean diseases, confusing fungal and viral diseases with some kinds of insect damage. However, they know essentially the same things that scientists know about plant diseases (Mukanga *et al.*, 2011; Trutmann *et al.*, 1996; Sherwood and Bentley, 1995). For instance, farmers hypothesize about possible control measures of maize ear rot such as improving soil fertility and testing new varieties in similar way to scientific (Mukanga *et al*., 2011). Farmers have traditional disease prediction systems based on the on-set of dew, when they begin chemical management (Bentley and Thiele, 1999).

Ntow *et al*. (2006) indicated that while pesticides are generally considered a panacea for farmers' pest concerns, farmers' perceptions and use of the chemicals have not received much attention. Sherwood and Bentley (1995) stated that without clear understanding of the cause of disease problems or related issues that aggravate the disease (such as what is the source? how it reproduces, disseminate, and survive?), farming may be a mixture of useful, useless, and even harmful practices. Therefore, understanding the fruit production system of Ghana and how farmers do activities in their fields will be helpful to get a clue in designing integrated disease management of anthracnose disease and create awareness in farmers.

2.3 Anthracnose disease

Of all the diseases that occur in mango, anthracnose has been reported as the most important fungal disease affecting the crop worldwide (Arauz, 2000). Anthracnose disease caused by different *Colletotrichum* species, are also reported by many authors as a common challenging disease on citrus production in many parts of the world (Huang *et al*., 2013; Lijuan *et al*., 2012; Brown and Eckert, 2000). In India, losses due to anthracnose of mango have been estimated to be 2–39 % (Prakash, 2004). In addition, the pathogen causes substantial pre and post-harvest losses in both citrus and mango (Diedhiou *et al*., 2007; Brown *et al*., 1996; Brown, 1975). The postharvest loss on both mango and citrus fruits is the most economically significant throughout the world (Arauz, 2000; Brown and Eckert, 2000; Brown *et al*., 1996). In addition, other fresh fruit crops such as avocado, banana, guava, papaya, strawberry and passion fruit are also affected by the disease (Cannon *et al*.,

2012; Phoulivong *et al*., 2010; Hyde *et al*., 2009). The damage caused by *Colletotrichum* species extends to important staple food crops including cassava, yam and sorghum, grown by small scale farmers in developing countries throughout the tropics and subtropics (Dean *et al*., 2012; Chala *et al*., 2010a; Chala *et al*., 2010b; Peters, 2009) . Plant diseases add costs in the form of institution of control measures to the farmers (Arauz, 2000). Moreover, the world's fresh fruit markets demand very high quality fruit.

2.3.1 Anthracnose disease cycle

In perennial fruit crops, *Colletotrichum* pathogen grows in the deadwood of the tree canopy. Initial infection of the deadwood in perennial crops occurs from airborne spores produced on debris located in the soil. In the dead wood tree canopy, the spores of the fungus are produced in specialized structures called acervuli (Plate .2.1).The produced spores are carried in water to the surface of leaves and immature fruits during the growing season.

During periods of high moisture, such as after rainfall, heavy dew or overhead irrigation, the spores germinate to form microscopic appressoria. A small number of the appressoria germinate, form germ tubes that penetrate the healthy fruit, and then remain quiescent and triggered at ripening stage (Prusky, 1996). The disease cycle of anthracnose disease is shown in Plate .2.1.

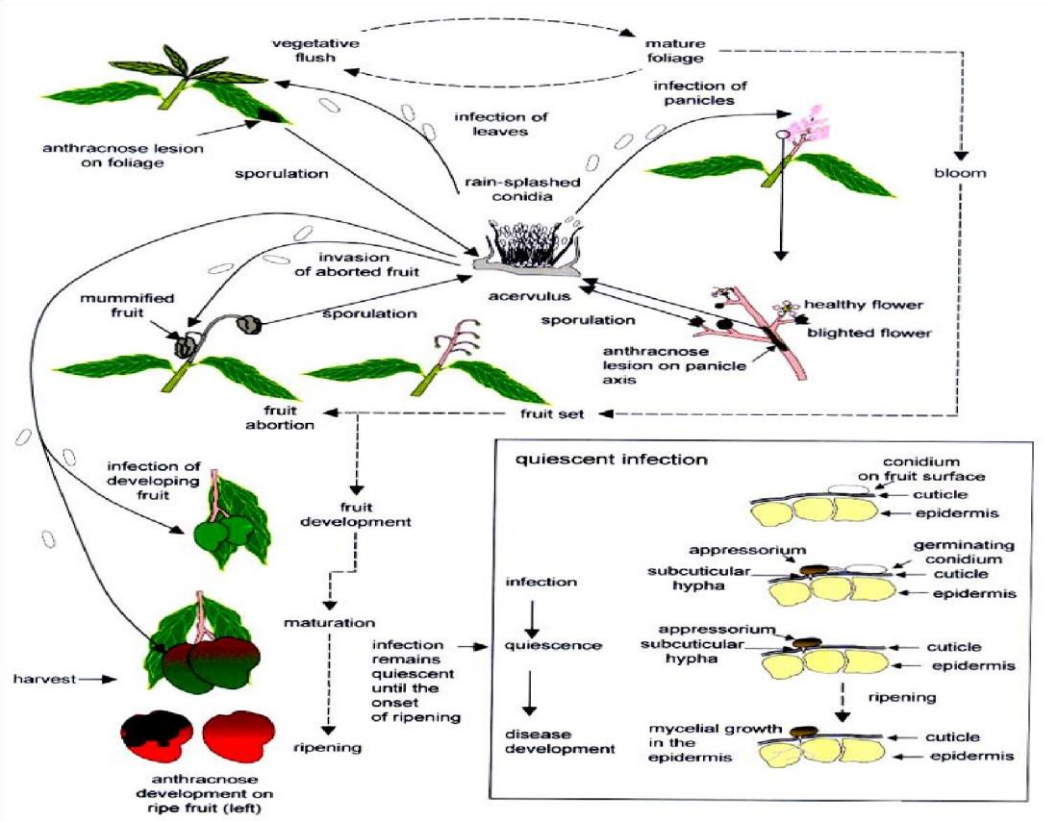

Plate 2. 1: Anthracnose disease cycle (Source: Arauz, 2000)

2.3.2 Anthracnose disease symptoms

The symptoms of anthracnose disease on many crops, including citrus and mango, show sunken necrotic lesions on leaves, stems and fruit, as well as seedling blight (Cannon *et al.*, 2012; Nelson, 2008; Agrios, 2005). The symptoms also include blossom blight of flowers and result in poor fruits set (Estrada *et al.*, 2000). In mango, fruit smaller than pea-size can be infected and aborted.

Anthracnose causes premature fruit drop, reduces quality of ripe fruit and shortens storage life time (Dodd *et al*., 1992). Infection of blossom or young fruit can result in total crop failure (Estrada *et al*., 2000 ; Ploetz, 1994). Bigger size fruit that aborted because of normal self-thinning or other physiological causes are invaded by *Colletotrichum*, and the fungus sporulates abundantly on them. Moreover, the pathogen also causes latent infection on developing fruit in the field (Prusky, 1996).

According to Souza *et al*. (2013), Nelson (2008) and Freeman *et al*. (1998), the postharvest phase of fruit anthracnose is the most economically significant throughout the world. Anthracnose symptom also can develop on fruit in transit or storage and reduce their marketability (Freeman *et al*., 1998). According to Arauz (2000), lesions are usually restricted to the peel, but in severe cases, the fungus can invade the pulp. The other type of symptom is commonly referred to as tearstain symptom in which are linear necrotic region on the fruit that may or may not be associated with superficial cracking of the fruit epidermis, leading to an alligator skin effect on fruit surface (Nelson, 2008).

Anthracnose disease also affects the leaf of both citrus and mango. According to Nelson (2008), the pathogen causes irregularly shaped, black necrotic spots on both sides of mango leaves. The symptom on leaves initially occurs as small angular, brown to black spot that can coalesce to form large extensive lesions on the leaf. This is particularly common around the edges of the leaves and the advanced level lead to leaf blight. Under favourable conditions, the fungus can invade the twigs and cause dieback (Nelson, 2008). Panicle anthracnose or blossom blight can affect both the inflorescence stalk and the individual flowers (Souza *et al*., 2013). In areas where rain is prevalent during flowering and fruit set, anthracnose can cause destruction of the inflorescence, infection and drop of young fruits (Pitkethley and Conde, 2007).

2.3.3 Causal agent (s) of anthracnose disease, *Colletotrichum* species

Colletotrichum is an asexual genus, classified within the imperfect fungi. It belongs to the *Coelomycetes*, producing its conidia in acervuli (Dean *et al*., 2012). Despite its significance, the taxonomy and nomenclature in this genus are confusing. According to von Arx (1957), depending on taxonomic interpretation criteria, the number of

species can range from 29 to over 700. Others also indicated that there are 802 records in mycobank, but only 66 species were listed as being in current use (Hyde *et al*., 2009). The identity of many species is questionable, while large species complexes are assumed to contain various species (Sreenivasaprasad and Talhinhas, 2005; Johnston and Jones, 1997). Colletotrichum species as causal agents of mango and citrus anthracnose are inconsistent from place to place. *Colletotrichum* species that have been reported from many countries are shown in Table 2.1 below. Recent studies have discovered that what were previously thought to be a single species, comprises multiple distinct lineages. For instance, *C. gloeosporioides* (*sensu stricto*) has recently been epitypified with a living strain (Cai *et al*., 2009). This has resulted in the description of about 22 different species in the *C. gloeosporioides* complex (Cannon *et al*., 2012; Noireung *et al*., 2012; Weir *et al*., 2012; Wikee *et al*., 2011; Damm *et al*., 2010; Phoulivong *et al*., 2010; Cai *et al*., 2009; Prihastuti *et al*., 2009; Yang *et al*., 2009).

Table 2.1: *Colletotrichum* species from different countries of the world reported as causal agents of anthracnose disease on citrus and mango

Specific species	Species complex where it belongs	Host reported	References
C. truncatum	*C. capsicum complex*	*Citrus Capsicum annum,*	Huang *et al*.(2013) and Ellison *et al*. (2015)

		Jatropha curcas	
C. gloeosporioides	*C. gloeosporioides*	*Mangifera indica* *Citrus sinensis*	Chowdappa and Kumar (2013); Awa *et al*. (2012); Cannon *et al*. (2012) and Phoulivong *et al*. (2012)
C. godetiae	*C.acutatum*	*Citrus aurantium*	Damm *et al*. (2012b)
C. simmondsii	*C.acutatum complex*	*Citrus reticulata* , *Mangifera indica* and *Carica papaya*	Giblin *et al*. (2010); Lijuan *et al*. (2012); Phoulivong *et al*. (2010) and Weir *et al*.(2012)
C. cordylinicola	*C. gloeosporioides*	*Mangifera indica*	Phoulivong *et al*. (2010b)
C. boninense	*Colletotrichum boninense*	*Mangifera indica*, *Carica papaya*, *Citrus* spp.	Lijuan *et al*.(2012)
C. tropicicola	*C. gloeosporioides*	*Citrus maxima*	Noireung *et al*. (2012) and Lima *et al*. (2013)
C. fructicola	*C. gloeosporioides*	*Mangifera indica*, *Citrus* and *Carica papaya*	Lijuan *et al*.(2012); Lima *et al*. (2013) and Phoulivong *et al*.(2010)
C. karstii	*Colletotrichum boninense*	*Citrus* leaf, *Mangifera indica*	Lijuan *et al*. (2012) and Lima *et al*. (2013)
C. brevisporum		*Citrus maxima*	Lijuan *et al*. (2012) and Noireung *et al*. (2012)
C. murrayae		*Citrus* leaf	Lijuan *et al*.(2012)
C. asianum	*C. gloeosporioides*	*Mangifera indica*, *Carica papaya*	Phoulivong *et al*. (2010); Honger *et al*. (2014); Krishnapillai and Wijeratnam (2014)
C. siamense	*C. gloeosporioides*	*Mangifera indica*, *Carica papaya*	Phoulivong *et al*. (2010) and Yang *et al*. (2012)
C. thailandicum		*Citrus maxima*	Noireung *et al*. (2012)
Colletotrichum acutatum	*C.acutatum*	*Mangifera indica*, *Citrus* spp, *Carica papaya*	Peres *et al*. (2002); Tarnowski and Ploetz (2008) and (Peres, 2008)

The different species included under *C. gloeosporioides* species complex are *C. musae*, *C. kahawae*, *C. asianum C. xanthorrhoeae, C. nupharicola, C. tropicale* and *C. gloeosporioides Sensu stricto, C. siamense, C. fructicola, C. tropicale* and several

other undescribed species (Weir *et al*., 2012). Weir and Johnston (2010) were able to characterise some other more species such as *C. horii, C. theobromicola* and *C. ignotum* on the same *C. gloesporioides* complex. The *boninense* clade (*Colletotrichum boninense* species complex) now comprises about 18 species (Damm *et al*., 2012a), while the *acutatum* clade (*C. acutatum* species complex) now comprises 31 species (Damm *et al*., 2012b).

Such understanding of *Colletotrchum* species complex demands a revisit of the species reported before, where traditional techniques were used for proper taxonomic grouping and its effective management. Further more, Phoulivong *et al*. (2010) reported that some isolates which have wrongly been assumed to be variants of *C. gloeosporioides,* especially on tropical fruit crops, are different *Colletotrichum* isolates whose species status has not been ascertained yet.

2.4 Identification of *Colletotrichum* species

According to McCartney *et al*. (2003), the ability to identify the organism(s) responsible for specific crop diseases is the cornerstone of plant pathology, for without this ability, we can neither understand the disease nor, in many cases, control it. However, there is no single technique perfect for the identification of *Colletotrichum* genus. *Colletotrichum* species identification is challenging because of many reasons. Some of the causes are insufficient information in the original descriptions, similar and highly variable morphological traits (Ko Ko *et al*., 2011), low resolution of species within species complexes by using ITS sequences (Weir *et al*., 2012) and different degrees of host specificity (Souza *et al*., 2013).

2.4.1 Conventional identification techniques

Traditional plant pathology studies have gone through many phases where numerous conventional markers were used for detection, identification, quantification of pathogen species and for evaluation of the genetic variation at either individual, population or species level (Bridge *et al*., 2003; Vunch *et al*., 1999; McLaughlin *et al*., 1981). The conidia formed by *Colletotrichum* species can easily be seen by the compound microscope and it is used as one of the means of identification. However, because of many overlapping characteristics within the species complex, using morphological techniques is insufficient (Phoulivong *et al.*, 2010a). Pathogenicity, virulence, and pesticide sensitivity are also part of the conventional techniques for identification of plant pathogen. Smith and Black (1990), successfully used traditional features in differentiation between species of *C. fragariae*, *C. acutatum* and *C.gloeosporioides* associated with strawberry diseases. The accuracy of conventional identification method and its reliability depend largely on the experience and skill of the person making the diagnosis (Chakrabarty *et al*., 2007;
McCartney *et al*., 2003).

Conventional identification has limitation to differentiate among species within the *Colletotrichum* species complex. It is claimed by many scientists as the cause of the taxonomic confusion (Adaskaveg *et al*., 1997; Freeman *et al*., 1996; Bernstein *et al*., 1995). Existence of diverse subspecies and strains in each *Colletotrichum* species complex, having morphological similarity and its instability with varying environment flexibility, makes the traditional techniques unreliable. Ko Ko *et al*. (2011) also added that, taxonomic relationships within the genus *Colletotrichum* are unlikely to be resolved solely by the use of traditional morphological characters. Conventional

identifications are costly, time consuming and can be impractical when rapid results are required (McCartney *et al.*, 2003). Accordingly, Lima *et al*. (2013), Parinn Noireunga *et al*. (2012) and Ko Ko *et al*. (2011) suggested the use of multi locus sequence analysis to resolve the taxonomic confusion.

2.4.2 Advanced techniques

Molecular pathology is DNA based pathology, an advanced science that is used to identify and quantify plant pathogen inocula (McCartney *et al*., 2003; White *et al*., 1990). Molecular techniques are rapid, highly specific and can be used to detect minute quantities of DNA. It has played a great role for a better understanding of viral pathogens (Shimura *et al*., 2015). Furthermore, its role for fungal taxonomy/identification is so vast and contributes a lot for plant disease control measures decisions.

Effective and successful identification, using molecular techniques, is determined by the selection of specific nucleic acid sequences/region to be used to identify the pathogen, extraction of quality DNA from the sample and using the method used for identifying the presence of the target sequence(s) in the sample (McCartney *et al*., 2003). According to Abang *et al*. (2004), molecular approaches have helped to understand *Colletotrichum* strains associated with yam anthracnose in a better way, and they have helped to resolve *Colletotrichum* species identification confusion.

Molecular techniques have several potential advantages in diagnosis. Due to the sensitivity of molecular techniques, the pathogen can be detected before the beginning of the symptom. McCartney *et al*. (2003) indicated that the ability to diagnose pre-symptomatic disease is potentially of great benefit in disease management and also

improve accuracy of timing of fungicide application and its effectiveness. Molecular techniques, if not alone, can be used in conjunction with classical methods where the latter approaches can, at least narrow pathogen diagnosis to genus level. Once genus is narrowed by morphology, symptomatology and hostspecificity, then Polymerase chain reaction (PCR) based techniques can be used to differentiate species (Chakrabarty *et al*., 2007).

2.4.2.1 PCR- based identification techniques

Molecular approaches mainly, the polymerase chain reaction have been used widely as the tool for detection of fungal pathogens (Schaad and Frederick, 2002; Martin *et al*. 2000). PCR assays based on amplification of sequence of internal transcribed spacer (ITS) region of rDNA or pathogenicity genes have been developed and used for detection of several plant pathogens (Henson and French, 1993). ITS divergence information is also used successfully for designing species-specific primers (Nieto and Rosselló, 2007). Bowman *et al*. (2007) worked on ITS region to detect the soilborne pathogens of citrus, *Phytophtora nicotinae* and *P. palmivora*. Garzón *et al*. (2005) also used the ITS region for assessment of variation within *Pythium irregulare*. The ITS sequences were also used to quickly identify species in the *Fusarium* genus (Abd-Esalem, 2003). Furthermore, the use of ITS region by plant pathologists dominates in systematic study of *Colletotrichum* species (Chowdappa and Kumar, 2013; Sharma *et al*., 2013; Benali *et al*., 2011; Crouch *et al*., 2009).

The ITS regions are particularly attractive loci for PCR-based detection, since they are readily accessible, using universal primers and typically present in high copy

(Benali *et al*., 2011; White *et al*., 1990). According to Freeman (2009), Ford *et al*. (2004) and Freeman *et al*. (2000), due to lower conservation of ITS regions between the small and large nuclear rDNA subunits than in the coding regions, these have been used to detect evolutionary difference within *Colletotrichum* species. The other frequently used gene regions, together with ITS, include the intron of glyceraldehyde-3-phosphate dehydrogenase gene, the intron of the beta tubulin gene and the actin gene (Phoulivong *et al*., 2010; Prihastuti *et al*., 2009; Liu *et al*, 2007).

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CHAPTER THREE 3.0 FARMERS' PERCEPTION AND PREVALENCE OF ANTHRACNOSE DISEASE ON MANGO AND CITRUS IN THE ASHANTI REGION OF GHANA

3.1 Summary

Farmers are the major role players in the agricultural field. However, their understanding of agricultural system has not received much attention but their awareness can make a great change to the sector. Mostly farmers have a limited knowledge about plant diseases and insect pests that interrupt the effectiveness of the management. Understanding farmers' knowledge of plant disease is, therefore, a very important element for the success of plant protection. Therefore, the objective of this study was to investigate farmers' knowledge and perception about anthracnose disease of citrus and mango. Mango farmers (90.3 %) were aware of anthracnose disease as a challenge. They were able to explain and show the different types of symptoms of the disease. In contrast, citrus farmers had no idea about anthracnose disease or any disease caused by *Colletotrichum* species. Instead, citrus farmers explained in detail about two common fungal diseases such as angular leaf spot and black spot that were highly challenging in the fields. Explanation of farmers and our observation on the two citrus

diseases mentioned above suggest possibly anthracnose and other diseases of citrus caused by *Colletotrichum* species. This needs to be further investigeted.

Commercial mango and citrus fruit farmers mostly depended on chemical management for their disease and insect pest problems. Use of cultural practices on the farms as disease management option was minimal. In addition, farmers' knowledge on the ecology of disease and pest was a limiting factor. For instance, seriously infected weeds in farmers' fields were not much an issue for them.

It is important to improve farmers' knowledge of plant diseases, to be able to understand how disease could be favoured and its relationship with cultural practices such as sanitation, weeding and pruning. Farmers also need awareness training to enable them to be good observant of their farm.

3.2 Introduction

Mango and citrus are being promoted as cash crops and important income source for farmers (Arauz, 2000). They also serve as an input for juice processing company. In addition, it is a source of foreign currency. However, in Ghana, farmers as well as the country are not benefiting as expected because of multidiverse problems. One of the main problems is poor quality fruits because of diseases and insect pests. Consequently, it makes it difficult to compete for international market; it is cheap in local market and causes higher postharvest losses.

Farmers have a great contribution on agricultural productivity of crops. Hence, it is very important to know how they understand the problem, the cause and the solution. The success of agricultural sector, in part, depends on traditional people's knowledge. Scientists have to work with farmers to improve crop production as well as protection

(Trutmann *et al*., 1996; Sherwood and Bentley, 1995). The major constraints upon establishing a sustainable disease and pest management programme is inadequate information about farmers' knowledge, perceptions and practices in plant protection. Agricultural scientists, in general, are becoming aware of the potential contribution of farmers in developing integrated management of crop diseases (Sherwood and Bentley, 1995). Farmers inherit part of their knowledge from their ancestors and build it up constantly, based on available information and their own experiment and experience (Morales and Perfecto, 2000). On the other hand, van Huis and Meerman (1997) indicated that knowledge of diseases and pests can vary between farmers working in similar or different agro-ecosystems. In some cases, disease recognition is a major problem, while in others, knowledge about its ecology is the major constraint. On the other hand, farmers are highly influenced by extension workers or easily available management technique pesticide, even though they are not getting any positive result for their problems (Abudulai *et al*., 2006). According to Sherwood and Bentley (1995), teaching missing information in a farmer useful way enable people to overcome knowledge barriers and improve their farming.

Most farmers have good knowledge about easily observable diseases and pests that are causing obvious symptoms in the field (Abudulai *et al*., 2006, Sherwood and Bentley, 1995). However, farmers and scientists may differ in their opinion about the importance of a particular crop production problem (Trutmann *et al*., 1996). Therefore, working with the farmers will help to fill this gap of understanding on the importance of a particular disease problem or its management (Fajardo *et al.*, 2000). In some cases, farmers' idea may give a clue for the scientist.

The success of sustainable disease management highly depends on biology, as well as ecology of the disease causal organism. The ecology of a pathogen is highly influenced by farmers' agronomic practices in the field. Therefore, there is the need to seek farmers' knowledge about the disease or understanding of what the farmers know, what they do not know, and what they misunderstand about the specific disease.

Anthracnose disease is number one limiting factor of mango production in Ghana (Honger *et al*., 2014). In addition, the disease can causes damage on most commonly cultivated crops in the country such as yam (Peters, 2009), cassava (Manu-Aduening *et al.*, 2007), tomato (Živković *et al*., 2010) and others that make the situation more complex. In particular, fresh fruit and vegetable farmers suffer a lot because of anthracnose diseases in the field as well as in storage. Although farmers do management, anthracnose disease is persisting from season to season. Information on traditional/farmer's knowledge about fruit crop disease and pest is very limited.

The purpose of this specific study was to investigate farmers' knowledge and perception about anthracnose disease of citrus and mango, and also determine its prevalence and the different types of symptoms in farmers' field in the Ashanti Region of Ghana.

3.3 Materials and methods

3.3.1 Field surveys of mango and citrus farms

3.3.1.1 Description of the study areas

The field surveys were conducted in 2013 growing season in four major mango and citrus growing areas in Ashanti Region. The selected areas are located in the humid Forest and Transitional agro-ecological zones. Ashanti Region is located between longitude 0 15 – 2 25 W and latitude 5 50 – 7 40 N (http://mofa.gov.gh/site/?page_id=642. accessed in June, 2015). It is bordered by the BrongAhafo Region to the North,

Western Region to the West, Central Region to the South and Eastern and Volta Regions to the East.

The region experiences double maxima rainfall in a year, with peaks in May/June and October. Mean annual rainfall is between 1100 mm and 1800mm. The mean annual temperature ranges between 25.5 and 32 ^{0}C. Humidity is high, averaging about 85% in the southern districts and 65% in the northern part of the region (http://mofa.gov.gh/site/?page_id=642. accessed in June, 2015)

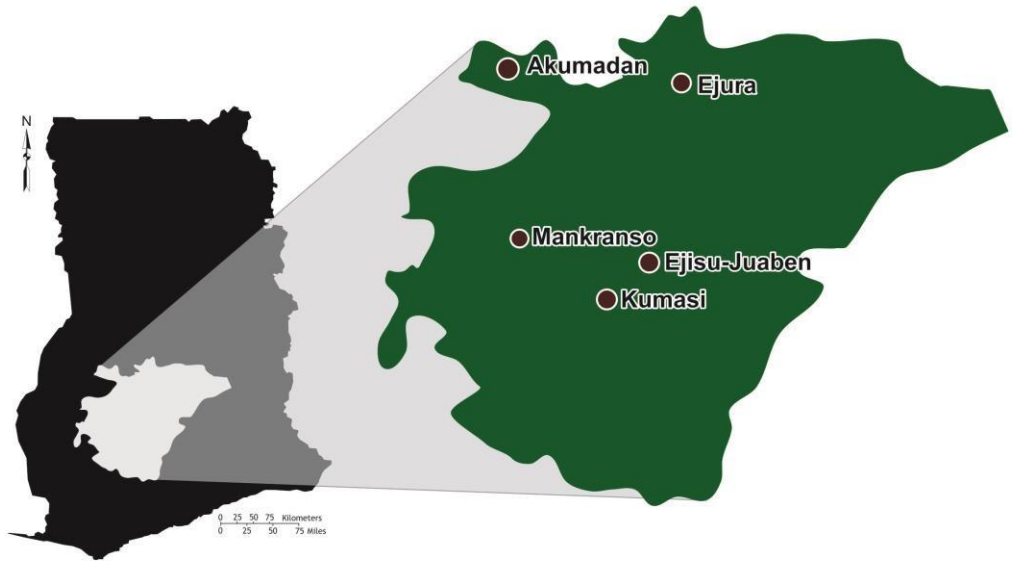

Plate 3. 1: Ashanti Region map showing the surveyed areas (dots) for both citrus and mango

Agriculture is the dominant sector in the region's economic activities and it is endowed with abundant arable lands which support the production of cash crops such as cocoa, coffee, oil palm, citrus, cashew, and mango and food crops such as cassava, plantain,

rice, yam, cocoyam, maize, and vegetables. The farming systems are mixed cropping, mono-cropping and shifting cultivation (short fallow periods usually less than five years are practiced) (http://mofa.gov.gh/site/?page_id=642. accessed in June, 2015). Tree crops such as cocoa, oil palm, citrus and mango are grown as mono-crops in plantations. However, these plantation crops are inter-cropped with food crops during the early periods of establishment. Mechanized farming is practiced mainly at the Ejura Sekyedumasi District, Mampong Municipality and
other districts in the transitional zones.

The selected areas for the survey of mango were Kumasi, Ejisu, Ejura and Akumadan, and those for citrus were Kumasi, Ejisu, Akumadan and Mankranso (Plate 3.1). Ejura and Mankranso are, respectively, the major production areas of mango and citrus in the region.

3.3.1.2 Criteria and strategy used to select Farmers'

In general, 48 mango fields and 53 citrus orchards were visited for disease sample collections. In each area, a minimum of seven farmers were interviewed. Selection of the farm localities were based on the accessibility of the road and availability of the crop. On other hand, interviewees' selection was based on their willingness and citrus or mango growing experience.

The Ministry of Food and Agriculture (MoFA) offices in the study areas were contacted to get contact information of farmers and to get some highlight about the districts' farming system. The contact addresses from Kumasi MoFA office were used to meet the Agriculture officers, Coordinators and chairpersons of associations from

different areas of fruit growers. Based on the discussion with the responsible bodies, farmers in each district were selected. In addition, while visiting the selected farmers; more information was obtained from farmers them selves to locate more farmers in their visinity. Those who were willing and had time to be on their field were used for the interview.

3.3.2 Methods of data collection

Data were collected by interviewing farmers individually and also by group discussion in the major growing areas of the region. Interview questionnaire (Appendix 1) was developed on the interview topics such as variety type and source of planting material, agronomic practices, production constraints, insect pest and disease problems, awareness about anthracnose disease and plant protection techniques.

In addition, observation data were taken to confirm what farmers said was the cause or otherwise of the infection/damage. Diagnostic surveys on plant disease and insect pest damage were made to observe if they had some problems different from than what they had mentioned. In every orchard visited, 10-20 random trees were selected, depending on farm size in a zigzag manner and surveyed specifically for anthracnose disease symptoms and to determine the health status of the mango and citrus trees.

In all the farms visited, records on different types of diseases, insect pests, weeds and different types of symptoms caused by anthracnose disease were taken. Anthracnose disease incidence on a farm was determined by observing the 10-20 randomly selected trees in the farm. In addition, for each selected tree, health status was determined, using

a scale of 1-3 below by Kilalo *et al*. (2011) to determine the health status of selected mango and citrus trees;

1= healthy looking plant (<5% diseased/infected),
2= moderately healthy plant (5-20% diseased/ infected), and 3
= unhealthy plant (>20% diseased/infected).

This information was used for the calculation of anthracnose disease incidence and the overall health status of the trees in the field. Anthracnose disease incidence (DI) for each field was calculated, using the following formula (Meer *et al*., 2013).

$$DI = \frac{Number\ of\ anthracnose\ infected\ plant}{Total\ observed\ plant} \times 100$$

Anthracnose diseased plant samples from the fields visited were collected. The diseased plant sample collections were considered from a range of anthracnose symptoms observed on the surveyed fields from different organs (leaf, fruit and panicle). The samples were placed in polybags, labelled and taken to the KNUST, Department of Crop and Soil Sciences Plant Pathology laboratory for isolations and further investigations on the identity of the causal agents, *Colletiotrichum* spp. The data were analyzed, using descriptive statistics.

3.4 Results

3.4.1 Farmers' backgrounds

Age of the plantation visited in the mango cultivation areas was 1-15 years old (avarage of 8.2 yr) and citrus plantation ranged from 5-22 years (avarage of 14 yr). However, most of the mango plantations were active and young, and the farmers did

a lot to improve upon and replace old and unproductive trees. Therefore, it was common to see mixed ages of trees in the plantation. State Farm owned mango plantation managed by CSIR- Crops Reasearch Institute- Ejura, called mango museum, which was 54 years old was also visited.

The mean range of mango farm size visited was 0.5-50 ha and (avarage of 5.2 ha) and farm size for citrus ranged from 0.5 - 25 ha (avarage of 6.97 ha.). However, most farmers had less than 2 ha size of farm. Majority of the farmers had 0.5 -2 ha of farm sizes for both mango and citrus (Table 3.1).

Table 3. 1: Size of of mango and citrus farms visited

Farm size (ha)	% Farmers	
	Mango	Citrus
0.5- 2	59.00	50.00
2.5- 5	13.60	11.11
6 -12	22.70	16.67
> 12	4.50	22.22

3.4.2 Varieties of mango and citrus planted by farmers

Farmers in the studied areas grew Keitt, Palmer, Jaffna, Haden, local variety and kent mango (Table 3.2). However, most farmers, especially, the commercial growers, planted Keitt variety as a result of market preference and their interest to export. Kumasi and Ejisu areas cultivated more of the local variety because of very few

commercial growers encountered. Most farmers did a mixture of mango varieties planting. However, some explained that the mixture happened as a result of wrong planting materials bought.

At Ejura, mango farmers obtained their planting material from the Ejura scion museum, and at Akomadan, most farmers mentioned seed company as a source of their planting material. Local yellow mango (turpentine) and Jaffna variety were the commonly used mango root-stock in Ejura and Akomadan, respectively. It was observed at Akumadan where some of the sprouts from the root stock of Jaffna mango had serious anthracnose infection. Jaffna variety found in some mix plantings also showed serious anthracnose attack. On the other hand, farmers at Ejura explained that they were trying to eliminate all Kent variety from their field to replace with other varieties as a result of its susceptibility to anthracnose disease. In case of citrus, sweet orange, mandarin, blood orange and ortanique were grown in study areas (Table 3.2). Sweet orange dominated the citrus production, specifically,

Valencia variety. Citrus farmers stated that they used rough lemon as a root stock.

Table 3. 2: Mango varieties and citrus relatives grown by farmers in Ashanti Region

Mango type	**% Mango farmers**	**Citrus types**	**% Citrus farmers**
Local mango variety	29.00	Sweet orange	77.78
Keitt and some local	25.80	Blood orange	11.11
Keitt only	12.90	Mandarin	5.55
Keitt and Palmer	19.35	Ortanique	5.55
Keitt, Palmer and Kent	6.45		
Palmer, Keitt and Jaffna	6.45		
Total	100		100

3.4.3 Challenges of mango and citrus farmers

Farmers raised many issues as challenges in their production. In case of citrus, diseases and insect pests were ranked as major challenge than all others; and in mango production, disease and insect pest were ranked as the second major challenge following unmanaged bushy mango trees or farms (Table 3.3).

In case of citrus, geting reliable planting materials was mentioned as a concern. Mostly, farmers got their planting materials from local nursery-men or from reputable farmers' fields. Farmers also mentioned finance as a major problem and mostly they related it to most of their limitation in their farming, including disease and insect pests management.

Table 3. 3: Constraints to citrus and mango production reported by farmers and their rank in the study areas

Issues raised as a challenge	% Mango farmers	% Citrus farmers
Diseases and insect pests	38.70	77.80
Unmanaged trees	41.90	0.00
Financial constraint	9.70	16.70
Availability of planting materials	12.90	33.33
Access to market	3.22	5.55
Old age trees	9.7	27.78

3.4.2 Farmers' perception about specific diseases and insects of citrus and mango

Fungal diseases and several insects were mentioned by the farmers in all study locations of citrus and mango. About 90.3 % of the mango farmers reported anthracnose disease as a challenge (Table 3.4). However, in citrus, anthracnose disease was not reported by farmers. Instead, other two fungal diseases such as angular leaf spot and black spot diseases were mentioned as major pressing recent challenges (Table 3.4).

Table 3. 4: Specific diseases and insect pests reported by farmers and their importance

Disease and insect pests	% Farmers reporting	
	Mango	Citrus
Diseases		
Anthracnose	90.30	0.00
Gummosis	6.45	16.70
Black sooty mould	6.45	0.00
Angular leaf spot	0.00	83.33
Black spot	0.00	77.80
Tree death	0.00	22.22
Insects		
Fruit fly	22.60	16.70
Stone weevil	12.90	0.00
Scale insect	12.90	0.00
Ants	9.68	0.00
Aphids	6.45	11.11
Leaf miner	3.23	11.11
White fly	0.00	5.60

At Mankranso, citrus farmers also complained about a new emerging disease by an unknown causal agent that causes death of citrus trees. Furthermore, farmers were also faced with insect pest attack in both crops. For instance, fruit fly was recorded as second most important concern reported by 22.6 % mango farmers and fourth important pest for citrus reported by 16.7 % of farmers in the studied areas (Table 3.4). Commercial mango farmers reported mango stone weevil as upcoming concern of mango production; since it is a quarantine important insect, they were very particular about it. They do diagnosing of their field to minimize the risk. Aphids and white fly were also mentioned as a concern. Most of the challenges mentioned by the farmers were confirmed during the visit to citrus and mango fields. Some of the observed insect pest problems are shown on Plate 3.2.

Plate 3. 2: Some insect pests observed in citrus farms. Aphid infestation (a); Aphids and white flies (shown with red arrow) together (b).

3.4.2 Farmers' perception of symptoms of mango anthracnose and citrus diseases

Farmers' description of mangos anthracnose symptom was detailed. The most obvious symptoms (Plate 3.3) of anthracnose disease described by farmers included, black leaf spot, advanced leaf blight, blackening, rain streaks and cracks on the fruit.

However, some farmers' confused fruit crack anthracnose symptoms of mango with insect pest damage because some insects were observed around the diseased fruit.

Plate 3.3: Different types of mango anthracnose symptoms mentioned by farmers and observed in the fields. Leaf spot (a); Black leaf spots that lead to side blight on old leaf (b); Leaf blight on young leaves (c); Rain streaks on fruit (d); Fruit crack (shown with red arrow) (e); Branch splitting/cracking (shown with red arrow)(f).

Mango farmers explained shifting of symptom types from season to season and also introduction of new type of symptom of anthracnose in to their locality. For instance, most farmers in Ejura study areas said for the last two years, they had observed fruit and branch spliting/cracking in addition to fruit spliting as a problem. According to their explanation, it was a new type of symptom they were experiencing. Other farmers in the same study areas (Ejura) complained about flower abortion. However, some of them thought it was caused by the harmattan wind.

In citrus, no farmer reported anthracnose disease as a problem. Instead, they gave very detailed description of symptoms of two major fungal diseases, black spot caused by

Guignardia citricarpa and angular leaf spot caused by *Pseudocercospora angolensis*, of recent concern. In most of the fields visited, the two diseases existed together. In some farms, incidence of angular leaf spot was of higher incidence and in others' black spot incidence was high. Farmers said when citrus fruits get ripen/yellow, they become more susceptible to black spot. However, angular leaf spot could happen at any stage of fruit development. They mentioned early fruit drop as a symptom of angular leaf spot disease. The way they differentiated between the two diseases was based on the size of the black spot on fruits. They said when the spots are smaller, it is black spot and if they are large, circular or irregular spot it is angular leaf spot (Plate 3.4).

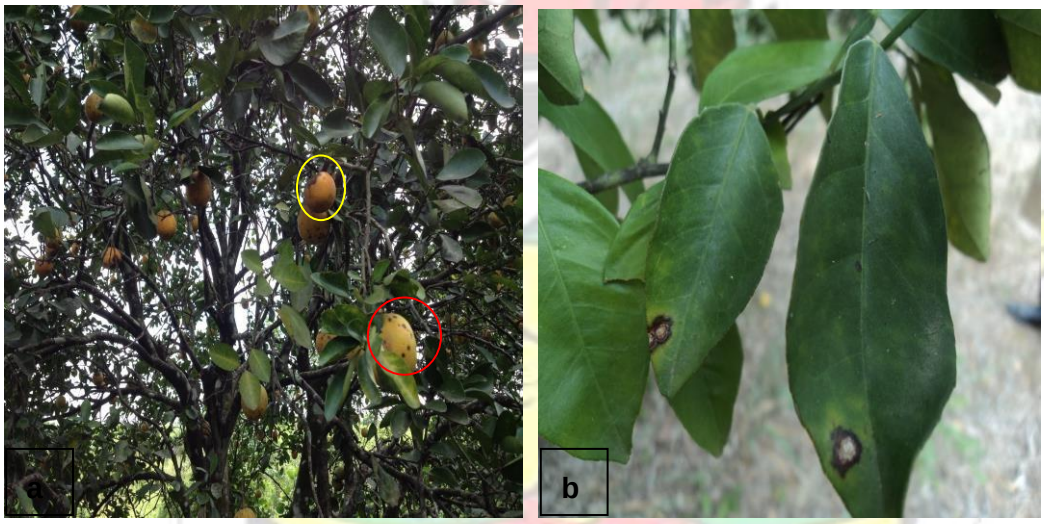

Plate 3.4: Citrus disease symptoms observed in farmers' fields. Black spot (encircled with red) and angular leaf spot on orange fruits (encircled with yellow) (a) ; Angular leaf spot symptom on leaves (b).

However, some farmers explained that, although the diseases have different names, they start as a black spot and then advance to severe blackening. From the explanation and field observations, the citrus diseases mentioned by the farmers could be the same disease. Citrus farmers also explained that they experienced fruit crack and tear-staining of fruit. Some of them explained that the fruit crack was caused by potassium deficiency. Most of the symptoms (Plate 3.4) were confirmed

during the field visits.

Most of those symptoms were repeatedly observed on sweet orange. However, in some farmers' fields the circular black spots were observed on mandarin and blood orange fruits. Although farmers did not mention, it was also observed on alternative crops and weeds around citrus plantation. The alternative hosts that were showing the same symptoms as on the citrus tree leaves included jatropha, cassava, cocoyam, cocoa, *Euphorbia atoto* and *Centrosema pubuscens* (Plate 3.5).

Plate 3. 5: Some of the volunteer/alternative hosts observed in farmer's fields showing the same symptom (shown with red arrow) as found on citrus tree a. Jatropha b. Cassava

The citrus farmers reported the symptom of recent unknown disease which causes citrus leaves to change to yellow, followed by leaf shedding, fruit drop, and then finally killing the tree. They added that it was very rare at first but now it is spreading and common to see one/two trees in a farmer's field. From our observation, the disease kills trees in nearby distance.

3.4.4 Occurrence of mango anthracnose and the health status of mango and citrus

Assesment of anthracnose disease occurrence in the studied areas of mango indicated anthracnose disease was widespread. The highest incidence (78.00 %) of anthracnose disease was observed at Akumadan and lowest disease incidence (48.33 %) was at Ejura (Table 3.5).

Table 3. 5: Occurrence of anthracnose disease on mango and health status of both mango and citrus in various studied areas of Ashanti Region during 2013/2014 sampling period

	mango (%)	**Mango**	**Citrus**
Kumasi	71.43 ± 15.74*	2.29 ± 0.30	2.11 ± 0.75
Ejisu	52.30 ± 13.01	1.92 ± 0.31	2.09 ± 0.34
Akumadan	78.00 ± 10.95	2.28 ± 0.11	1.99 ± 0.67
Ejura	48.33 ± 18.35	1.90 ± 0.53	No citrus
Mankranso	No mango	No mango	2.11 ± 0.93

Study areas	**Incidence of anthracnose on**	**% Health Status/ Crop (Scale 1-3)[X]**	

*Standard deviation. [x]Scale used for health status, where 1= Healthy looking (<5% diseased/damaged, 2= Moderately healty plant (5-20 % diseased/damaged), and 3= Unhealthy plant (> 20% diseased/ infected)

In citrus, the health status records for Mankranso and Kumasi were higher (2.11) than those of the other studied localites (Table 3.5). This implies that most of the trees were generally unhealthy, eventhough farmers did intensive chemical spraying. The lowest record (1.99) was obtained at Akumadan (Table 3.5).

In the case of mango, the lowest scale value or health status was obtained from Ejura (1.90) and it was followed by Ejisu (1.92). The other study localities such as Kumasi and Akumadan had 2.29 and 2.28 record, respectively (Table 3.5).

3.4.3 Farmers' disease management and related agronomic practices of mango and citrus farms

The commercial mango and citrus fruit farmers in the studied areas were very dependent on chemical management for plant protection than any other disease and insect pest management techniques. Those who were not using chemicals mentioned financial problem as the reason. Most of the farmers thought that chemical use was the best management option.

Plate 3. 6: Chemical containers observed around farmers' fields in the study areas

They commonly used cocktail/ mix application in their chemical management practice. Citrus farmers, especially, explained that they mixed chemicals, either insecticide or

fungicide together, before application. Farmers think the cocktail application increases the efficacy of the chemicals, resulting in fast remedial action to disease and pest problems in their fields.

Mango farmers at Akumadan said they sprayed copper-based fungicides such as Kocide (Copper hydroxide) and Ridomil Plus (Metalaxil-M) as fungal protectants during flowering and Bendazin (Carbendazim 50 %) against anthracnose. At Ejura and its environs, most of the farmers used Ridomil Plus and Kocide, while others used Shavit F (triadimenol) for fungal diseases management. In addition, most farmers sprayed insecticides to alleviate insect problems and other chemicals for fungi (Table 3.6).

Table 3. 6: Chemicals used by farmers in the study areas

Pesticide type	Chemical name	Purpose	Crop(s) used on
Fungicide			
	Ridomil Plus	Fungal protection	Mango
	Kocide	Fungal protection	Mango
	Bendazin	Antrachnose control	Mango
	Carbendazim	Angular leaf spot and black spot control	Citrus
	Mancozeb	Angular leaf spot and black spot control	Citrus
	Shavit F	for fungal protection	Mango and citrus
	Diatom	Fungal protection	Citrus and mango
Insecticide			
	Karate	Insect control	Citrus and mango
	Cimetrol	Insect control	Citrus

Farmers also used pheromone traps to manage fruit flies in both crops. In all the studied areas, farmers were aware of necessary cultural/agronomic practices needed for mango

and citrus such as sanitation, weeding, and pruning. However, from discussions, many of them rarely understood their role in disease and insect pest management.

Mostly, the cultural practices were ignored or they did them very late. For instance, farmers did weeding when they wanted to spray or harvest. Pruning was mostly ignored and sanitation of farms was not properly done. A farmer at Akumadan explained that for better production of mango, it is important to do both major and minor pruning, to improve the quality of fruits. However, farmers lack the techniques and are also less aware about the importance of its timing. The same challenge was raised by citrus farmers during the group discussion.

Timely weeding and its cost were also issues raised by farmers. They preferred to use herbicides to manual weeding because of the labour cost**.** The farmers overlooked volunteer crops growing in their field. For instance, cassava was commonly seen in the fruit farmers' fields visited. Farmers were relatively ignorant of the implications of weeds, as compared to diseases and insect pests. It was very common to see plenty of dropped and mumified citrus and mango fruits on the ground as well as on trees (Plate 3.7a).

Plate 3. 7: a. Dropped mango fruits on farmers' field b. Collected dropped mango fruits as part of farm sanitation

The situation makes a lot of pathogens and insects to thrive and serve as inoculum sources for diseases and insects. Farmers who practiced pruning and dropped-fruits collection in their farms, observed obvious differences in disease and insect pests pressure than the others who did not practice any sanitation. Farmers at Akumadan used black poly-bags to collect dropped mango fruits from their farms then left them in the farm to be heated by sun to kill any pathogen or insect pests, and allowed to rot (Plate 3.7b). Finally, it is used as compost on the same farm. However, farmers complained about the high cost of labour associated with the collection of the fruits.

It was observed that, the citrus harvesting system was very traditional (Plate 3.8a). The harvest practice of inversion of fruits on not cleaned ground, with many mummified diseased and dropped fruits (Plate 3.8 a and b), may increase contamination with pathogenic fungi. The harvesting technique also could increase the mechanical damage to fruits and increase pathogen infection. These could also contribute to more losses after harvest.

Plate 3. 8: a. Traditional harvesting system b. Harvested and mummified fruits together on the ground

3.5 Discussion

3.5.1 Disease occurrence, symptoms overlap and chemical management

Citrus disease caused by *Colletotrichum* species have been reported to cause the following symptoms; dark-brown irregular lesions and become sunken on the rind tissues (http://www.appsnet.org/publications/potm/pdf/Feb11.pdf), postbloom fruit drop (Agostini *et al*., 1992), silvery grey, advanced darker decay, tear staining and hard spot in the rind (Peter, 2003). All the above-mentioned symptoms were observed in the citrus fields visited in this study. Although, there is the possibility of different pathogens causing the same symptoms on the crop, the symptom similarity, coupled with the non-satisfactory results in their management and attack on other surrounding plants on farmers' fields, made us to question the identity of the causal agent(s). Therefore, information and sample of the diseased citrus plant and the alternative host/weed that showed the same symptoms were collected for further investigation. In general, citrus health status at Mankranso and its environs was found to be poor because of the unnecessery frequent spraying of chemicals and poor cultural practices done by farmers.

Oduro (2000) and Offei *et al*. (2008) reported mango anthracnose disease in Ghana. According to Honger (2014) higher anthracnose disease incidence has been reported, in Ashanti Region, as compared to other mango growing regions in Ghana. In Kumasi Metro, incidence of anthracnose on mango was as high as 100%, and similarly high incidence results were found at Akumadan and Kumasi. Such high disease incidences could be attributed to the little disease management in Kumasi area, coupled with very favourable microclimate created by unmanaged bushy trees.

At Akumadan, although mango farmers practiced many cultural practices and chemicals spraying, it recorded high anthracnose incidence. The root stock (Jaffna)

mango used in the area was found to be susceptible in the field, and the delay in harvesting of fruits also could be the reason for the highest anthracnose disease incidence. Additionally, composting, using the dropped fruits on mango farms, could also have played a role in increasing the inoculum source of the pathogen in the field. However, Ejura recorded relatively low disease anthracnose incidence. It could be as a result of erratic rain fall reported by farmers on the visiting season and their chemical application might have helped to minimise the disease incidence.

According to Peter (2003), the fungus that causes anthracnose is a weak pathogen and requires susceptible rind, such as injured or aging fruit. Therefore, improving the harvesting techniques of farmers and timely harvesting can contribute to minimize anthracnose disease in farmers' fields. All the descriptions of mango anthracnose disease given by the farmers, as well as observed symptoms in their fields, are in line with literature (Nelson, 2008; Brown and Eckert, 2000).

According to Honger (2014), farmers in Ghana complained about effectiveness of fungicide for mango anthracnose. In the present study, most farmers used chemical management and the chemicals used were in line with literature recommended for anthracnose. However, the regular chemical spraying and mixing with other insecticides, without considering the ecological situation, could be the reason for the non-effective result. It could contribute to the susceptibility of fruit trees and impacted the ecology of the pathogen to develop resistance. Peter (2003) indicated that hard dry spot symptom of anthracnose on citrus rind, was mostly associated with chemical injury or weakness in the rind. Others also added that when fruits with many quiescent

infections are subjected to different types of stress, anthracnose symptom can develop on the fruit surface, resulting in serious postharvest losses (Brown and Barmore, 1977).

Solely depending on chemical management on such perennial crop highly impacts negatively on the environment and can be the reason for the complex disease and pest occurrence in farmers' fields. For instance, serious infestation of citrus with aphids and white flies can occur (Plate 3.2). Intensive use of chemicals may be harming bees and other wild life, and the quality of water and soils are a real threat to food security (http://www.bbc.com/news/uk-29699449). This implies that the intensive chemical application in fruit production could also interrupt the pollination system to hamper productivity.

In this present study, citrus farmers blamed their less frequent application for the non-effective result of chemical management of angular leaf spot and black spot diseases. They were advised to spray seven times each season (from flowering to fruit set) However, the maximum that the farmers could afford to apply was three times, some two times and others even only once. Therefore, such regular chemical spray without having much impact on the targeted disease, highly affects the ecology to bring ecological backlash such as resistance and resurgence. Chemical usage must be the last resort in disease management, especially, if the environment of such perennial crops production is very suitable to implement integrated disease/pest management.

3.5.2 Potential of proper cultural/ agronomic practices as alternative disease management techniques

According to Trutmann *et al*. (1993), farmers placed more value on avoiding conditions that lead to disease than on diseases themselves. In contrast, during the visits to fruit farmers' fields, disease management strategies were found to be based on

curative, rather than preventive measures, although the farmers had little understanding of the role of cultural practices preventing diseases and pests. In perennial crops mango and citrus, preventive rather than curative disease management approaches would be more appropriate. Such approach is also environmentally more sustainable and cost effective.

In the study areas, the ecology of diseases was not understood by most farmers. For instance, in citrus fields, dead citrus trees due to disease were found but did not understand how the disease spreads to the field. On the other hand, when chemicals were applied to manage disease, farmers paid no attention to infected weeds and volunteer plants in the farm. It was also very common to see seriously diseased or insect infested plant parts in the field but farmers did not remove it, even though this could help to prevent the disease and insects from spreading. A study indicated that mango anthracnose caused by *C.gloeosporioides* can be reduced by removal of diseased parts from the trees and its destruction by burning (http://www.mangifera.org/disease.php accessed, August 2015),.

According to Arauz (2000), tree pruning prevents build up of high relative humidity in the tree canopy which eventually contributes to reduced incidence of mango anthracnose. Furthermore, branch terminals, mummified inflorescences, dead wood, flower bracts and diseased leaves are potential sources of inocula (Diedhiou *et al*., 2007; Dodd *et al*., 1991). Pruning, selecting resistant variety and sanitation are recommended as potential cultural techniques for anthracnose disease management (Diedhiou *et al*., 2007). In addition, it is not only practising the cultural techniques, but understanding and doing them at the appropriate time and in proper ways that also matters.

3.6 Conclusion

Mango farmers in the studied areas had good understanding about anthracnose disease symptoms on their fields. However, in case of citrus, although, the farmers mentioned some diseases and their management as challenges, the disease problem was huge and increased from time to time. However none of the citrus farmers interviewed talked about anthracnose disease on citrus. Farmers were aware of the cultural practices that mango and citrus needed for sustainable production. However, they rarely implemented them properly. Farmers in the surveyed areas understood little about the conditions that favoured disease and pest problems, but thought chemical management was the panacea for all diseases and pests problems.

3.7 Recommendation

Awareness must be created to the farmers on how cultural practices can help to manage the disease and pest challenges. More investigations must be done on other alternative hosts, including weeds that can harbour pathogens and insect pests in farmers' fields for better management decisions. The different types of diseases on citrus need further investigations on their causal agent(s). Especially, the citrus disease that kills the trees needs attention before it spreads all over. In general, teaching farmers about the ecology that favours diseases and pests and how they are related with cultural practices will be very useful to regularize many of the cultural practices in fruit farmers' fields. There must also be a plan to establish integrated disease and pest management for fruit farmers. The Research Institutes and other stakeholders should have model site that incorporates all the necessary agronomic practices to demonstrate to farmers during training.

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CHAPTER FOUR 4.0 MORPHOLOGICAL CHARACTERIZATION OF *COLLETOTRICHUM*

ISOLATES OF MANGO (*MANGIFERA INDICA*), CITRUS (*RUTACEAE*) AND SOME ASSOCIATED PLANTS

4.1 Summary

Colletotrichum species are extremely important fungal plant pathogens. They cause anthracnose disease on both citrus and mango fruit crops. However, little is known

about the existence of diversified species that cause the disease in Ghana. This study sought to find out the existence of various *Colletotrichum* species on citrus and mango. Different colony characters, conidial shape, conidial size and growth rates were used as preliminary identifications. The *Colletotrichum* isolates collected from both citrus and mango were grouped into 10 morphological groups (G1 to G10). Seven of the morphogroups were isolated from both diseased citrus and mango diseased plants. This implies that there was morphological overlap of isolates that were found in mango and citrus. However, in case of citrus, most of the isolates (44.44 %) were grouped under G2. In mango, higher numbers of isolates (25.0 %) were allocated to G5. *Colletotrichum* isolates obtained from weeds and other alternative hosts matched the morphogroups G1, G2, G5 and G9. The result suggests that *Colletotrichum* isolates from citrus and mango found in this study showed high variability based on cultural and morphological characteristics.

4.2 Introduction

The genus *Colletotrichum* contains many morphologically similar taxa comprising endophytic, saprobic and plant pathogenic fungi (Photita *et al*., 2005). *C. gloeosporioides* is a cosmopolitan species infecting a wide range of plant hosts, including tropical fruits (Parinn Noireunga *et al*., 2012; Freeman *et al*., 2000; Oduro, 2000; Alahakoon *et al*., 1994; Alahakoon and Brown, 1994). On the other hand, studies indicate that *C. gloeosporioides* complex has genetically different groups of species under it (Damm *et al*., 2010). However, the inadequate understanding of the differences among the *C. gloeosporioides* complex causes a tendency of calling any of the species falling under this species complex *C. gloeosporioides* (Sreenivasaprad *et al*., 1996). Phoulivong *et al*. (2010) and Lima *et al*. (2013) clarified that *C.*

gloeosporioides (*sensu stricto)*, is not a causal agent of most tropical fruits including mango. All such confused results were attributed to the morphological similarity of the species grouped under the same species complexes. The instability of *Colletotrichum* taxonomy is a general problem all over the world. Hyde *et al*. (2009) reported that identity of many important *Colletotrichum* species still requires proper revision. Studies recommended that, combination of different types of identification techniques should be used to explore the differences among *Colletitrichum* species. Consequently, recent studies have focused on phylogenetic reassessments of species complexes (Damm *et al.*, 2012a; Damm *et al.*, 2012b; Weir *et al*., 2012).

According to Lima *et al*. (2013), the causal agents of mango anthracnose are five species of *Colletotrichum*, namely *C. asianum*, *C. fructicola* Prihastuti, L. Cai & K.D. Hyde, *C. tropicale* Rojas, Rehner & Samuels, *C. karstii* Y.L. Yang, Zuo Y. Liu, K.D. Hyde & L.Cai and *C. dianesei* Lima, Nelson B. was thought of as single species of *C. gloeosporioides* in Brazil (Serra *et al.*, 2011). Reaserchers in many countries are revising the species of *Colletorichum* present on fruit crops, including Nigeria (Awa *et al*., 2012), Thailand (Prihastuti *et al*., 2009; Photita *et al*., 2005) and India (Sharma *et al*., 2013; Chowdappa and Kumar, 2012). Since this pathogen is very important all over the world on the fresh fruit production sector, its proper identification and knowledge of the species diversity especially, in the case of

Ghana, is also very important.

The cause of mango anthracnose in Ghana has commonly been reported as *C. gloeosporioides* but *Colletotrichum* as the cause of antrachnose disease of citrus has not been investigated. Therefore, it is very important and interesting to revisit fruit-

attacking *Colletotrichum* species in Ghana where both mango and citrus are commercialized crops.

Accurate classification of *Colletotrichum* species demands multiphase observations, using different techniques. Fungal identification, including species belonging to the genus *Colletotrichum*, has primarily relied on differences in morphological features such as colony colour, size and shape of conidia and appressoria, presence or absence of setae teleomorph, growth rate and optimal temperature for growth (Agrios, 2005). Although many literature show that these traditional techniques alone have some limitations for the identification of the *Colletotrichum* species, they constitute the first step in studies of the genus *Colletotrichum* and other plant pathogens. Moreover, as these traditional techniques are the regular means of identifying many fungal plant pathogens in Africa, in general, these investigations will help to understand how we can improve upon conventional techniques to target the existing *Colletotrichum* species in mango and citrus. Therefore, the objective of this study was to differentiate and group the *Colletotrichum* isolates of mango and citrus based on their morphological characteristics.

4.3 Materials and Methods

Isolation, identification and morphological characterization of *Colletotrichum* species were carried out at the Plant Pathology Laboratory of the Department of Crop and Soil Sciences, KNUST, Kumasi, Ghana. Diseased plant samples, collected from farmers' fields were used for *Colletotrichum* species isolation. The samples were anthracnose-infected leaf, fruit, panicle and shoot/twig of mango and citrus. Samples from weeds and crops present in/arround mango and citrus plantations showing the same anthracnose like symptoms also were included. The other plants included were cocoa

(*Theobroma cacao* L.*)*, cassava (*Manihot esculenta* Crantz), cocoyam (*Xanthosomas sagittifolium* L.), jatropha (*Jatropha curcas* L.) and two types of weeds, namely *Euphorbia atoto* G. Forst and *Centrosema pubescens* Benth.

4.3.1 Preparation of Chloramphenicol-Amended Potato Dextrose Agar (CPDA)

Forty grammes of dehydrated Potato dextrose agar (PDA) powder and 500 mg Chloramphenicol were added to 500 ml of distilled water in a 1 litre conical flask. The content in flask was then mixed, using a magnetic stirrer, on a hotplate. The chloramphenicol-amended PDA (CPDA) mixture was topped with water to I litre. The opening of the flask was stoppered with cotton wool and then wrapped with aluminium foil. The CPDA in the flask was sterilized in an autoclave at 121ºC, 0.98kg/cm^2 pressure for 15 min. The sterilized CPDA was allowed to cool to about 45ºC and then poured into sterile Petri plates in the lamina flow (20 ml/ plate) and allowed to solidify. Each plate was sealed with paper tape and then stored for 48 h before being used to isolate *Colletotrichum* spp.

4.3.2 Isolation of *Colletotrichum* species

All the diseased plant samples were collected from mango and citrus farms in Ashanti Region during the surveys. Four pieces of diseased tissue of a size of 3x3mm were taken from the margins of infected lession plant parts and surface the infected sterilized by dipping in 1% sodium hypochlorite solution for 3 min and rinsed two times with sterile-distilled water. They were then blotted dry with sterile tissue paper and plated on the CPDA plates and incubated at room temperature (28 to 31°C) with a 12-h photoperiod. Sub cultures from the margines of fungal colonies from the plated tissue were made on to new CPDA plates to obtain pure cultures. After seven days,

microscopic observation was used to identify isolates of *Colletotrichum* species. Typical cultures of *Colletotrichum* species were maintained in a refrigerator at 4°C for single spore culturing and used for the morphological studie such as colour, margin, conidial shape and appearance.

4.3.3 Single spore culture preparation

Fungal suspension of each isolate was prepared from the pure culture plates by washing with 10 ml of water, using a soft sterile brush. The suspension was filtered through a double-layer cheese cloth. Then the suspension was further diluted to 1% by distilled-sterile water and an aliquot spread on 2 day old, 2% agar plates and kept overnight. Germinated single conidia were picked with a sharp-sterilized inoculating needle, using a stereo microscope and transferred onto fresh CPDA plates. All activities were done aseptically under lamina flow in the transfer chamber. Each isolate was replicated three times and incubated at room temperature (28 to 31^{o}C) for morphological data collection.

4.3.4 Cultural and morphological data collection

Eighty eight (88) representative isolates of *Colletotrichum* obtained were used for the morphological characterization. Data collected on culture characteristics were culture colour, colony margin, appearance, conidial shape, conidial length, conidial breadth, and growth rate/day.

Subculturing was done, using inocula of 3 mm-diameter mycelial plugs, from the growing margin of seven-day-old single spore cultures made, using sterilized cork borer on CPDA plates from each of the isolates. Each of the isolates was replicated three times and incubated at room temperature (28-31^{o}C). Two-dimension-colony

diameter (cm) measurements, using a ruler, were made on each day for seven days for every replicate. The two diagonals were made at the rear of each petri plate for consistent measurements of the diameter. From each measurement, the initial diameter of inoculum (3mm) that was used was deducted from the final measurements. The mean of the two diagonal diameters in each replication was recorded. The means were used to calculate and determine growth rate per day. Colony colour, margin, texture and appearance were observed on the seventh day. After seven days, data on conidial shape, conidial length and width were recorded. The length and width of 20 conidia from each culture plate were measured at 400x magnification under a compound microscope (Breukhoven Microscope system,

USA) and camera with micrometer software (www.azure micro.net, Accessed in 15 January 2012).

4.3.5 Experimental design and data analyses

The experiment was arranged in a completely randomized design with three replicates per treatment (isolate), representing each morphogroup. Differences in conidial size, and growth rate by the different morphogroups of *Colletotrichum* species were determined by one-way ANOVA, using GenStat 12th edition, VSN International, UK. Means were compared, using LSD at 5 % significance level. The other qualitative data were taken visually and presented with descriptive statistics.

4.4 Results

4.4.1 Characterization of *Colletotrichum* isolates from mango and citrus plants having anthracnose disease

Most of the diseased sample collections of mango and citrus ended up with morphologically different types of *Colletotrichum* species. A total of 88 *Colletotrichum* isolates were obtained from typical anthracnose symptomatic plants. Of these isolates, 44 were obtained from mango and 36 from citrus. Eight isolates were from surrounding plants/weeds. The isolates were classified into 10 morphogroups (group 1 (G1) to group 10 (G10). In this experiment, most of the morphogroups found on mango were also isolated from citrus. In addition, the isolates obtained from the other anthracnose symptomatic crops /weeds surrounding the mango and citrus farms also fell in the characterised morphogroups from mango and citrus.

4.4.1.1 Description of each morphogroup culture characteristics

Group 1: The cultures had dense aerial mycelium, were buff cottony fluffy growth (Plate 4.1a). Most of the isolates in this group produced honey-coloured exudates that started from the centre of the back side of the plate (Plate 4.1 b).

With age, some of the isolates in this group totally changed to honeycolour colony as a result of the honey-coloured exudates. The group was also recognized by their sickleshape conidia (Plate 4.1 c). They were isolated from both citrus and mango. This morphogroup comprises five isolates from mango, three from citrus and one from diseased cocoa plants around citrus orchards.

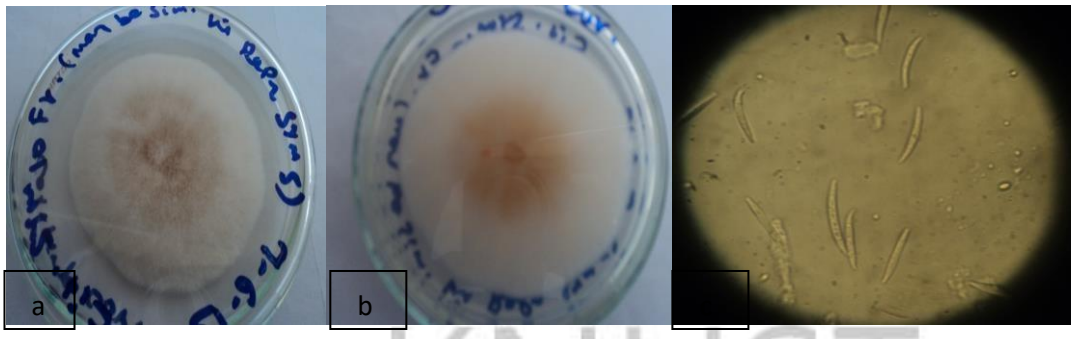

Plate 4.1: Representative colonies that signify the first morphogroup (G1) isolated from mango and citrus anthracnose-diseased plants from Ashanti Region of Ghana. a. Upper side of the colony b. Reverse side of the colony c. Conidial shape under compound microscope

Group 2: This morphogroup comprised of 22 isolates; three from mango, 16 from all the *Citrus* spp. (sweet orange, lime, blood orange and mandarin) and three isolates were obtained from jatropha and weeds such as *E. atoto* and *C. pubuscens* that were growing in citrus plantations. The group is characterized by its numerous bright orange conidial masses produced in concentric rings on the colonies (Plate 4.2 a and b).

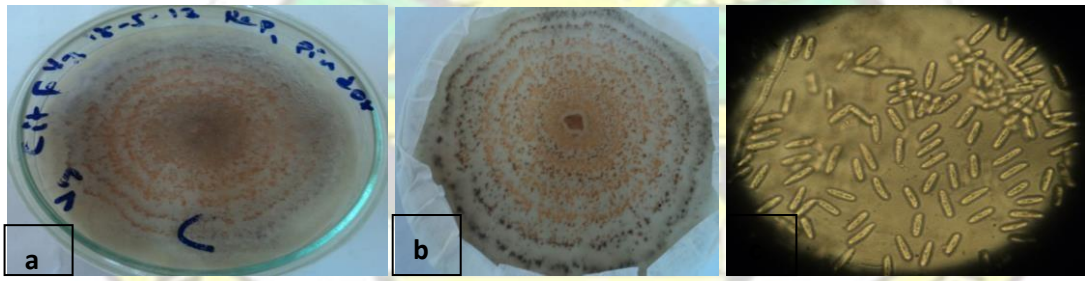

Plate 4.2: Colonies representative of the second morphogroup (G2) isolated from mango and citrus anthracnose-diseased plants from Ashanti Region of Ghana. a. Upper side of the colony b. Reverse side of the colony c. Conidial shape under compound microscope

In some of the isolates within the group, the orange conidial masses were embedded in the cottony mycelium. Some had dense, white mycelium with few orange conidial masses near the inocula points. Morphogroup G2 produced numerous and the same

type of cylindrical-shaped conidia (Plate 4.2 c). Most of the isolates were grouped under this category. Therefore, variation within them were relatively high. The detailed information on each isolate is presented in Appendix 2.

Group 3: This group is deep pink-pigmented with suppressed mycelial growth (Plate 4.3 a and b). However, some of the isolates within this group, produced sparse whitish mycelia. They have long and curved shape conidia (Plate 4.3 c). This group was isolated from only citrus. However, within the citrus group it was found from sweet orange and lime. There was zonation in some of the isolates in the group.

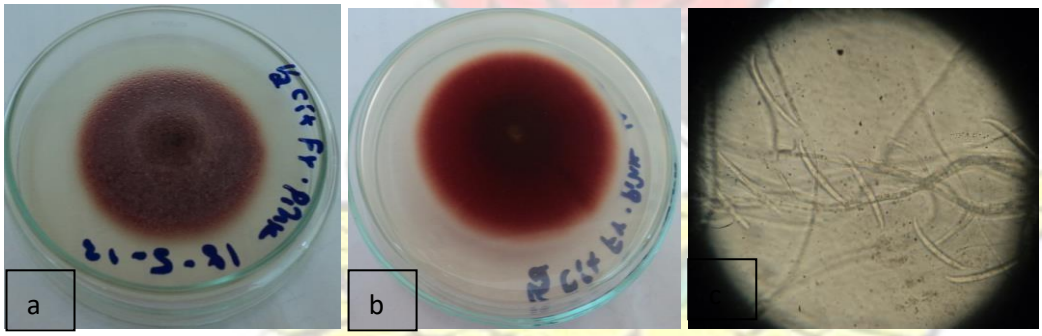

Plate 4.3: Colonies representative of the third morphogroup (G3) isolated from citrus anthracnose-diseased plants from Ashanti Region of Ghana. a. Upper side of the colony b. Reverse side of the colony c. Conidia shape under compound microscope

Group 4: This group highly resembles group G3 above in its colour and appearance (Plate 4.4 a and b). However, they were clearly different with regard to conidial shape under the compound microscope (Plate 4.4 c). Group 4 had cylindrical-shaped conidia, and they were obtained only from mango. Within G4, some isolates formed ascospores on the plate in the laboratory.

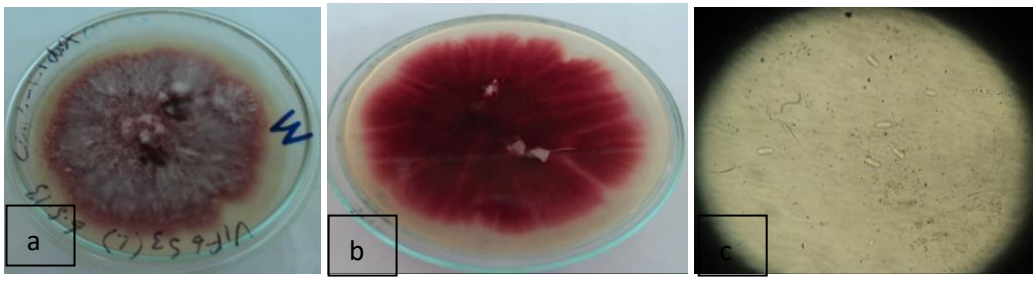

Plate 4.4: Colonies representative of the fourth morphogroup (G4) isolated from mango anthracnose-diseased plants from Ashanti Region of Ghana. a. Upper side of the colony b. Reverse side of the colony c. Conidial shape under compound microscope

Group 5: The group has grey with white periphery coloured colony (Plate 4.5 a). The conidial masses are mostly concentrated near the centre. Most of the isolates in the group did not have much cottony growth. Rather they were crystalline in appearance and coarse when touched with inoculation loop around the centre.

Generally, they produced few conidia. The shapes of their conidia were cylindrical and slightly pointed at one end (Plate 4.5 c).

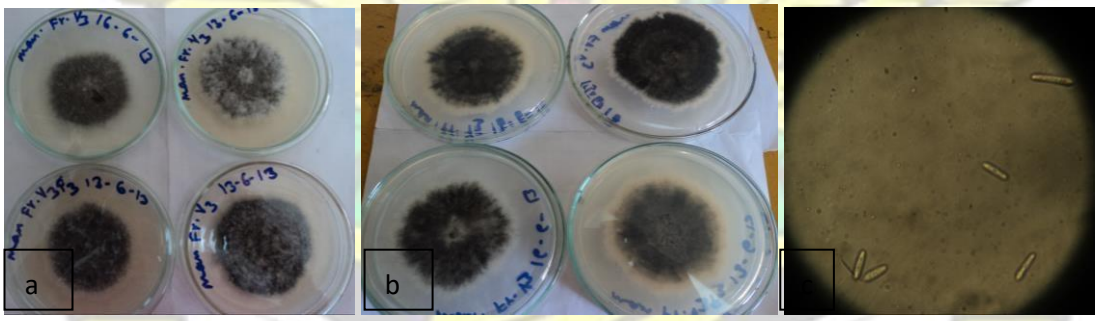

Plate 4.5: Colonies of the fifth morphogroup (G5) isolated from anthracnose-diseased mango and citrus plants from Ashanti Region of Ghana. a. Upper side of the colony b. Reverse side of the colony c. Conidial shape under compound microscope

This group of isolates was found in both mango and citrus, but was dominant in mango. In addition, this group was isolated from other crops (cocoa and cocoyam) that were grown around citrus farms or as intercrop.

Group 6: Most of the isolates of this morphogroup were from the blackening symptom on the panicle and leaf of mango. One isolate was isolated from sweet orange fruit

from Mankranso. The colony colour is orange and had veiny-like or rhizoid growth culture (Plate 4.6. a and b). This isolate produced plenty of mixed micro and macro conidia having curved shape (Plate 4.6. c).

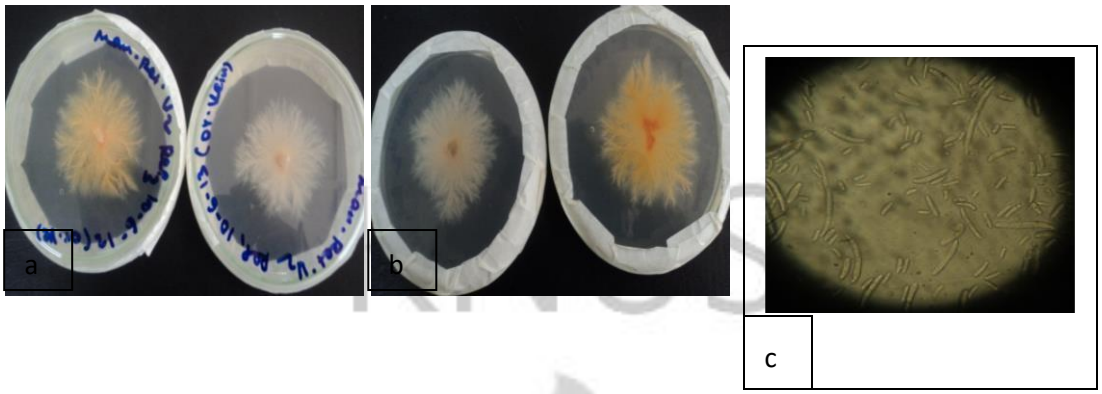

Plate 4. 6: Colonies representative of the sixth morphogroup (G6) isolated from mango and citrus anthracnose-diseased plants from Ashanti Region of Ghana. a. Upper side of the colony b. Reverse side of the colony c. Conidial shape under compound microscope

Group 7: This group of isolates is brown in colour and has no cottony growth. The cultures become very hard like rubber with age (Plate 4.7. a and b). At the centre of the culture, it was deeper brown because of some numerous circular visible structures such as perithecia /fruiting bodies produced by the fungus. The isolates gave very few, short and cylindrical conidia (Plate 4.7 c). The group had only three isolates and all of them were isolated from mango of the different study areas (Appendix 2).

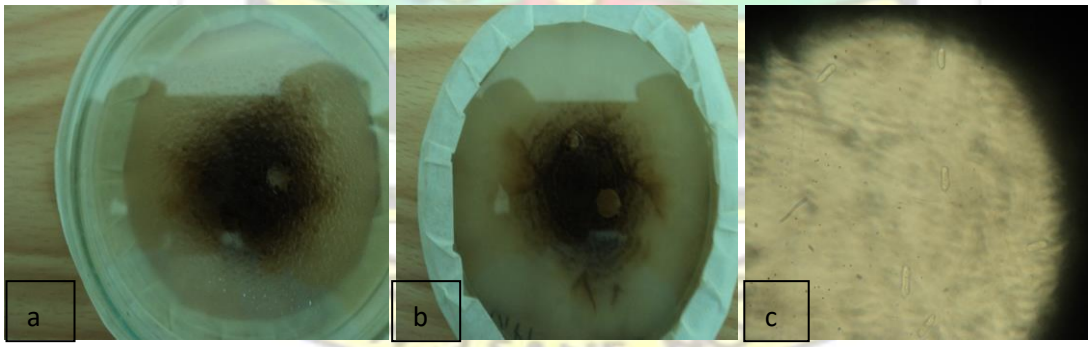

Plate 4.7: Colonies of representative of the seventh morphogroup (G7) isolated from mango anthracnose-diseased plants from Ashanti Region of Ghana. a. Upper side of the colony b. Reverse side of the colony c. Conidial shape under compound microscope

Group 8: The colony of this group looks bacteria-like. The group was isolated from both citrus and mango. The colony of the isolates was sticky and there were no visible aerial mycelial growth (Plate 4.8 a).

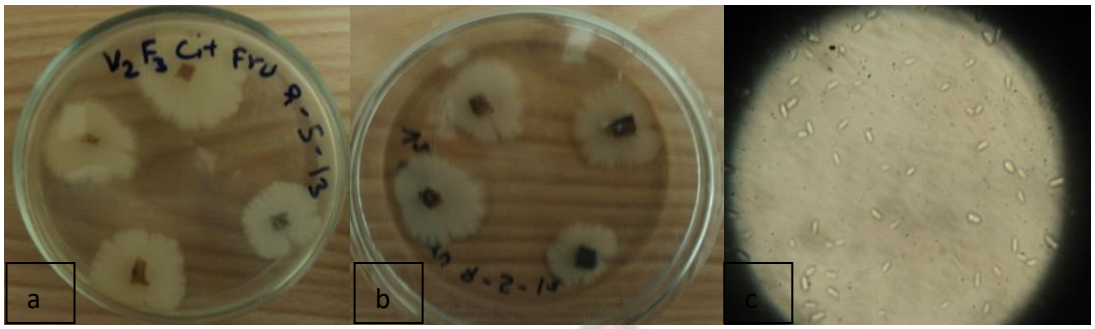

Plate 4.8: Colonies representative of the eighth morphogroup (G8) isolated from mango and citrus anthracnose-diseased plants from Ashanti Region of Ghana. a. Upper side of the colony b. Reverse side of the colony c. Conidial shape under compound microscope

However, conidiogenous hyphae were observed under the microscope. The isolates had restricted growth and a wavy margin. The group produced numerous short cylindrical/globose shape conidia. In addition, one of the citrus isolates from blood orange obtained from Mankranso was produced a mixture of micro and macro cylindrical conidia.

Group 9: This group was characterized by their aerial mycelia from white to pale grey and cottony in appearance (Plate 4.9 a and b). They were isolated from both mango and citrus, but most of the isolates in this group were obtained from mango. One isolate was obtained from cassava grown in mango field. The isolates in this group produced very few conidia (Plate 4.9 c) and some of them had no conidia.

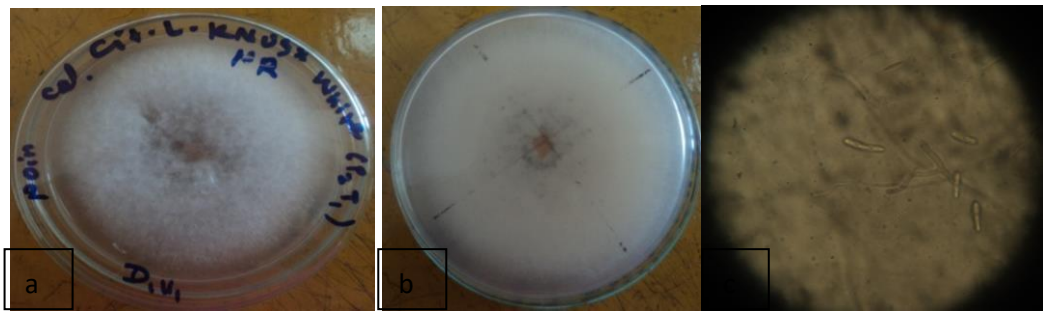

Plate 4.9: Colonies of the ninth morphogroup (G9) isolated from mango and citrus anthracnose-diseased plants from Ashanti Region of Ghana. a. Upper side of the colony b. Reverse side of the colony c. Conidial shape under compound microscope

Most of them had cylinderical shaped conidia and some of the isolates had conidia pointed towards one end (Plate 4.9 c). With the exception of the colour and the dense cottony appearance, most of the other characterstics of G9 morphogroup were similar to that of morphogroup G5.

Group 10: This group was characterized by their light pink, sparse and cottony surface growth (Plate 4.10 a). The reverse side of the colony showed circular zonation and produced plenty of cylindrical conidia (Plate 4.10 b and c). The group comprised of only two isolates, one from mango and the other from citrus.

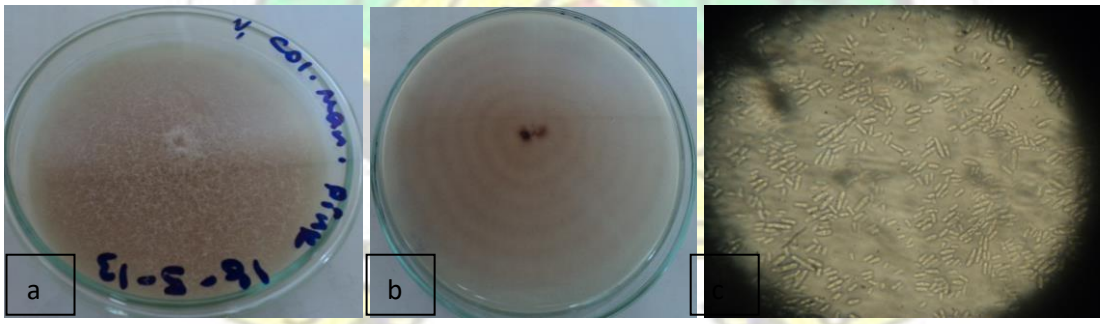

Plate 4. 10: Colonies of representative of the tenth morphogroup (G10) isolated from mango and citrus anthracnose-diseased plants from Ashanti Region of Ghana. a. Upper side of the colony b. Reverse side of the colony c. Conidial shape under compound microscope

Depending on the number of isolates represented in each morphogroup, slight differences were observed within the group. For instance, G2 showed more variation

in culture character as compared to the other morphogroups because G2 encompasses about 22 isolates obtained from different fruit crops and weeds. Therefore, to understand the slight variation observed within the group and to support the representative plates given above, detailed description has been presented in Appendix 2.

4.4.1.2 Conidial measurement for each *Colletotrichum* species morphogroup of mango and citrus

Although there were cultural characterstic overlaps of mango and citrus isolates, the measurments of conidia and growth rates had differences. Therefore, conidial and growth rate measurements for each fruit crop, mango and citrus, were given separately.

The mean length and width of conidia for morphogroups of G2, G5 and G9 of mango were 16.34 x 4.82 µm, 15.45 x 5.03 µm and 14.89 x 5.11µm, respectively.
There was no significant diffrence ($P > 0.05$) between their conidial length and width (Table 4.1).

Mango morphogroup G6 had the longest mean conidial length of 20.74 µm while G8 was the shortest with 9.11 µm. There was no significant difference ($P > 0.05$) between G4 and G8 morphogroups (Table 4.1).

Table 4.1: Conidial size and shape on CPDA for each morphogroup of mango *Colletotrichum* isolates at room temperature

Morphogroups	Conidia of *Colletotrichum* isolates from mango		
	Length (µm)	Width (µm)	Shape
G1	16.27* (9.31 - 21.60)	2.85 (2.21 - 3.53)	Curved

G2	16.34 (12.26 - 18.58)	4.82 (3.16 - 6.00)	Cylindrical
G4	9.28 (5.86 - 12.43)	3.27 (1.89 - 5.05)	Cylindrical
G5	15.45 (12.86 - 19.88)	5.03 (3.31 - 7.43)	Cylindrical
G6	20.74 (20.04 - 30.86)	3.98 (2.38 - 5.71)	Curved
G7	14.27 (13.06 - 15.63)	3.88 (3.70 - 4.00)	Cylindrical
G8	9.11 (8.99 - 9.22)	3.48 (3.43 - 3.51)	Cylindrical
G9	14.89 (10.30 - 17.57)	5.11 (3.16 - 7.81)	Cylindrical
G10	16.21 (15.61 - 16.60)	2.24 (2.12 - 2.73)	Cylindrical
LSD (P =0.05)	**1.95**	**0.34**	
CV (%)	**7.70**	**5.10**	

* Mean value; the values in parenthesis are the minimum and maximum conidial measurements

Morphogroups G1 and G10 did not show significant differences (p> 0.05) in their conidial length. However, from their conidial shape, they were very distinct. Morphogroup G1 produced curved conidia, and G10 had cylindrical conidiacylinderical in shape (Table 4.1).

In case of citrus, the mean conidial length and width mesurements for the morphogroups G2, G5 and G9 were 14.90 x 4.38 µm, 15.27x4.49 µm and 15.40 x 4.83 µm length and width, respectively (Table 4.2). All the three morphogroups had significantly the same conidial mesurments (p > 0.05), except G2 conidial width. The longest conidia were obtained in morphogroup G3 (34.55 µm) and shortest in G8 (10.19 µm) (Table 4.2).

Table 4.2: Conidial size and shape on PDA for each morphogroup of Citrus *Colletotrichum* isolates at room temprature

Colletotrichum	**Conidia of *Colletotrichum* isolates from citrus**		
	Length (µm)	**Width (µm)**	**Shape**

Morphogroups	12.20* (8.61-16.98)	2.70 (1.89-3.53)	Curved
G1			
G2	14.90 (7.68-34.65)	4.38 (2.73-6.55)	Cylindrical
G3	34.55 (26.43-44.24)	3.02 (2.27-3.83)	Cylindrical
G5	15.27 (13.97-16.30)	4.49 (3.51-5.40)	Cylindrical
G6	14.49 (12.28-17.38)	3.28 (3.07-3.58)	Curved
G8	10.19 (9.91-10.54)	3.76 (3.57-4.04)	Cylindrical
G9	15.40 (13.59-17.29)	4.83 (4.54-5.08)	Cylindrical
G10	16.11 (15.99 - 16.33)	2.50 (2.40 - 2.60)	Cylindrical
LSD (P= 0.05)	**1.90**	**0.39**	
CV (%)	**6.60**	**6.20**	

* Mean value; the values in parenthesis ( ) are the minimum and maximum conidial measurements

All the morphogroups of mango and citrus have cylindrical conidial shape, except morphogroups G1 and G6 (Table 4.1 and 4.2). Morphogroups G1 and G6 of both crops were characterized by their curved conidial shape. Morphogroups G2, G5 and G9 of both mango and citrus showed similarity in both their conidial measurments and shape.

4.4.1.3 Growth rate of *Colletotrichum* isolates from citrus and mango morphogroups

The growth rate measurements of mango *Colletotrichum* morphogroups were significantly different ($p < 0.05$). The highest growth rate/day was obtained in G9 (8.74 mm) and followed by G10 (8.06 mm) and the lowest measurement was recorded by isolate of G5 with 2.69 mm (Table 4.3).

Table 4.3: Growth rate of *Colletotrichum* isolates from mango and citrus

***Colletotrichum* morpho- groups**	***Colletotrichum* isolates growth rate (mm/day)**	
	Mango	**Citrus**
G1	6.61	8.68
G2	3.92	7.69
G3	No G3	3.71
G4	4.84	No G4
G5	2.69	9.06
G6	4.74	4.80
G7	3.36	No G7
G8	3.93	2.14
G9	8.74	10.77
G10	8.06	7.94
LSD (P =0.05)	**0.36**	**0.33**
CV (%)	**4.00**	**2.80**

The growth rate measurements for G2, G7 and G8 were 3.92 mm, 3.36 mm and 3.93 mm, respectively. However, three of them were significantly the same ($p > 0.05$) in their growth rate/day measurement. On the other hand, G4 and G6 morphogroups of mango were also significantly the same ($p > 0.05$) in their growth rate measurements, which gave 4.48mm and 4.74 mm, respectively. Morphogroup G1's growth rate was 6.61 mm/ day and showed significant difference ($P < 0.05$) from all morphogroups of mango (Table 4.3).

In the same way, citrus *Colletotrichum* morphogroups showed significant differences ($p < 0.05$) in their growth rate measurements. The highest growth rate/day was obtained by G9 (10.77 mm) and followed by G5 with 9.06 mm. The lowest growth rate in citrus morphogroup was obtained in G8 (2.14 mm) (Table 4.3). In the same crop, morphogroups G2 and G10 were not significantly different ($p > 0.05$) in growth rate

measurements of 7.69 mm and 7.94 mm, respectively. The remaining morphogroups, G1, G3 and G6, had 8.68 mm, 3.71 mm and 4.80 mm growth rates/ day, respectively and they were significantly different ($p < 0.05$) from all morphogroups of citrus isolates (Table 4.3).

4.4.2 *Collettotrichum* morphogroups isolated from mango and citrus

Of the 88 isolates, 50.00 % was isolated from mango, 41.90% from citrus and 9.09 % from other crops/ weeds (Table 4.4). The percentage in each crop was contributed by a group of different isolates. For instance, among the 10 *Colletotrichum* isolate morphogroups, nine of them were obtained from mango and eight from citrus. Morprhogroups G4 and G7 were isolated from only mango and G_3 was isolated from only citrus. However, all the remaining groups were found on both crops.

In case of mango, morphogroups G1, G4, G5, G6 and G9 were dominant and contributed about 77.27 % of the total mango *Colletotrichum* isolates. Each of the groups above contributed about 11.36, 11.36, 25.00, 13.64 and 15.90 % , respectively. In citrus, the single morphogroup G2 contributed about 44.44 % of the total isolates isolated, followed by G3 which contributed about 13.89 %.

Table 4. 4: Distribution of each *Colletotrichum* morphogroup in mango and citrus farmers' field surveyed

Morph group	Morphogroup/ Crop			sub total	% Contribution	Suspected *Colletotrichum* spp.
	Mango	Citrus	Others			
G1	5	3	1	9	10.23	*C. truncatum*
G2	3	16	4	23	26.14	*C. gloeosporioides complex*
G3	0	5	0	5	5.68	*C. capsicum*
G4	5	0	0	5	5.68	*C. acutatum complex*

G5	11	3	2	16	18.18	*C. fructicola*
G6	6	1	0	7	7.95	*C. lini*
G7	3	0	0	3	3.41	*C. gloeosporioides complex*
G8	3	3	0	6	6.82	*C. gloeosporioides complex*
G9	7	4	1	12	13.64	*C. gloeosporioides complex*
G10	1	1	0	2	2.27	Unknown
Total	**44**	**36**	**8**	**88**	100	

All the important morphogroups of mango isolates were also found in citrus. The very important morphogroup of citrus isolate (G2) was also isolated from mango but was rare and contributed about 6.81%. Morphogroup G2 of citrus was isolated from sweet orange, mandarin, blood orange and lime. In general, although most of the morphogroups of *Colletotrichum* were isolated from both crops, their importance/their percentage contribution to each crop anthracnose disease was not the same (Table 4.4). Interestingly, those isolates that were found in both crops such as G1,G2,G5 and G9 were also isolated from other crops and some weeds found around the citrus plantations.

4.4.3 Distribution of *Colletotrichum* isolates isolated from anthracnoseinfected organs of mango, citrus and other hosts

In general, *Colletotrichum* spp. were isolated from every part of the infected-plant samples collected. However, the contribution of each group from diffrent organs varied from crop to crop. In mango, most of the isolates were isolated from leaves (59.09%), followed by fruits (25.00%) and then panicles which contributed about (15.91%). However, in citrus, the reverse was true; most of the isolates (50.00%) were isolated from fruits followed by leaves (47.22 %) and then 2.78% from panicles (Table 4.5).

In addition, the morphotypes isolated from leaves were diverse. In the case of mango, seven of the different morphogroups were isolated from leaves, five from fruits and four from panicles.

In citrus, the first three morphogroups and G5 were isolated from both leaves and fruits and the remaining morphogroups were isolated from either leaves or fruits. Morphogroup G2 in citrus was isolated from all antrachnose-symptomatic organs of citrus (leaves, fruit, and panicle). In case of mango, morphogroup G5 was isolated from leaf, fruit and panicle (Table 4.5).

Table 4. 5: Occurrence of *Colletotrichum* morphogroups on different plant organs

***Colletotrichum* Morphogroups**	**No. of isolates on mango**			**No. of isolates on citrus**			**No. of isolates /others organ**
	Leaf	**Fruit**	**panicle**	**Leaf**	**Fruit**	**panicle**	**leaf**
G1	3	0	2	1	2	0	1
G2	3	0	0	6	9	1	4
G3	0	0	0	2	3	0	0
G4	4	0	1	0	0	0	0
G5	8	2	1	2	1	0	2
G6	3	0	3	1	0	0	0
G7	1	2	0	0	0	0	0
G8	0	3	0	0	3	0	0
G9	4	3	0	4	0	0	1
G10	0	1	0	1	0	0	0
Total	26	11	7	17	18	1	8
Contribution of organ/ crop (%)	**59.09**	**25.00**	**15.91**	**47.22**	**50.00**	**2.78**	**100**

* Total mango isolates were 44, total citrus isolates = 36 and 8 isolates from alternative crops/weeds and grand total of 88 isolates

4.4.4 *Colletotrichum* isolates mophogroup distribution in the study area

Isolates of morphogroups G1, G2, G3, G5, G6 and G9 were found in all the studied localities except morphogroup G3 which was not observed at Ejura (Table 4.6).

Morphogroup G4 was observed in only Kumasi and Ejisu. However, the slight differences that occurred within G4 cultural characteristics, the ascospore were seen in only G4 isolates from Ejisu. Morphogroup G8 was obtained from Ejisu and Mankranso however, most of the isolates were found at Ejisu. In the same way, morphogroup G2 was found in all the studied areas, but most of them were obtained from citrus at Mankranso.

Table 4. 6: Distribution of *Colletotrichum* morphogroups isolated from mango and citrus in the study areas

	No. of *Colletotrichum* isolates identified/ location				
Morphogroups	**Kumasi**	**Ejisu**	**Akumadan**	**Ejura***	**Mankranso[x]**
G1	3	1	2	1	2
G2	2	4	4	2	11
G3 (Citrus only)	1	1	1	0	2
G4 (Mango only)	3	2	0	0	0
G-5	1	4	5	3	3
G-6	1	3	1	1	1
G-7 (Mango only)	1	1	0	1	0
G-8	0	5	0	0	1
G-9	3	5	2	1	1
G-10	1	0	1	0	0
Total	**16**	**26**	**16**	**9**	**21**

* Major growing areas of mango in Ashanti Region, [x] Major growing areas of citrus in Ashanti Region

4.5 Discussion

4.5.1 Mango and citrus anthracnose in Ashanti Region, Ghana

Although piece of information on citrus anthracnose was limited from farmers, existence of morphological variation in *Colletotrichum* isolates was confirmed in case of mango and citrus diseased plants in this study. Moreover, most of the isolates obtained in mango had morphological overlap with that of citrus isolates. For instance, isolate groups G1, G2, G5, G6, G8, G9 and G10 were encomposed isolates from both crops and had a high cultural similarity. According to Lijuan *et al*. (2012) and Noireung *et al.* (2012), *C. boninense* , *C. fructicola*, *C. gloeosporioides*, *C. karstii*, *C. simmondsii* and *C. tropicale* infected citrus leaves, while Lima *et al*. (2013) reported *C. fructicola, C. tropicale* and *C. karstii* as causal agent of anthracnose disease on mango. Therefore, those morphologically similar isolates obtained from citrus and mango in this study could be the same *Colletototrichum* species that were able to cause disease in both fruit crops.

In addition, the types of *Colletotrichum* isolates found in citrus and mango had morphological similarity with most of *Colletotrichum* species reported. For instance, morphogroup 1 (G1) corresponds with *C. truncatum* reported by Photita *et al*. (2005) , G2 with *C.gloeosporioides* reported by Photita *et al*. (2005), G5 with *C. fructicola* and G3 with *C. capsicum*, G9 corresponds with *C.gloeosporioides* (Chowdappa and Kumar, 2012). Therefore, the different forms of *Colletotrichum* isolates from citrus imply that, most of *the* diseased samples collected from citrus field were anthracnose disease symptoms. However, the disease in citrus seems wrongly identified by the farmers in the visited growing areas. Many farmers mentioned angular leaf spot and black leaf spot as the cause of the problem in citrus production. They could not indicate anthrachnose.

The confusion of wrong identification of citrus anthracnose might have arisen as a result of overlap of literature describing its symptoms with the other diseases mentioned such as black leaf spot (Mengan and Timmer, 2009). In addition, Seif and Kungu (1990) reported that wherever there is angular leaf spot disease, *Colletotrichum* can occur as a secondary pathogen to cause anthracnose. Such a report about angular leaf spot disease might be the cause why *Colletotrichum* on citrus in Ghana has been overlooked and not considered as a problem on its own. It might be also a new occurrence on citrus in Ghana. Moreover, anthracnose caused by *Colletotrichum* spp. is a serious problem in citrus production of many other places. Therefore, pathogencity tests on citrus with Colletotrichum spp are very necessary to determine whether *Colletotrichum* spp. isolated from citrus are primary causal agents or not.

Colletotrichum morphogroup G2 of citrus were also isolated from weeds and non-targeted crops around citrus farms. This is an evidence that *Colletotrichum* spp. were playing the role in infection of many plants in the same/vicinity of farms.

It is not common to read a literature of curved spores of *Colletotrichum* spp. as causal agents of mango anthracnose. However, in this study two types of curved morphogroups of (G1 and G6) were obtained from mango plants with anthracnose diseased. It agrees with Afanador-Kafuri *et al*. (2003) who observed curved conidia of *Colletotrichum* species such as *C. dematium* and *C. graminicola*, which had the ability to infect a wide range of host plants. The same morphogroups (G1 and G6) were also isolated from diseased citrus plants. In addition, morphogroup G1 was isolated from cocoa leaf around citrus plantations. From our morphological characterisation results, morphogroup G1 isolates corresponded with *C. truncatum* also reported as a

causal agent of citrus and jatropha anthracnose (Ellison *et al*., 2015; Huang *et al*., 2013). The other curved group G3 was obtained from only citrus plants and was distributed all over the study areas of citrus.

4.5.2 Usefulness of characters used in *Colletotrichum* identification

In this study, combined cultural characters such as colour/pigmentation and exudates produced and conidial shape *in vitro* were important characteristics for distinguishing and characterising the ten *Colletotrichum* morphogroups. Especially in the case of morphogroups G1, G3, G4, G6, G7, G8 and G10, their unique and stable culture characters were helpful to distinct them into different groups.

The culture colour in G2 was distinict from that of G5 and G9. However, the three morphogroups had similarity in their conidial characteristics (shape and size). This is in line with McKay *et al*. (2009) who reported that, several morphological types have been observed within a population of *C. gloeosporioides* from avocado grown in culture. Conidial characteristics mostly help to differentiate taxa into species complexes (Phoulivong *et al*., 2010).

In some of the isolates of G5 and G9, there was instability of colour that was changing from white to grey and *vice versa*. In addition, within the groups G1, G2 and G5 there were variation in growth rate (restricted and spreading). It is reported that, isolates of *C. acutatum* grew at a significantly slower rate than isolates of *C. gloeosporioides*. Sreenivasaprasad and Talhinhas (2005) also reported the difference of *C.acutatum* from other *Colletotrichum* spp. which had predominantly elipsoidal, fusiform conidia produced in bright orange masses and had slow growth rate in culture. In contrary to the above, re-examination of *C.acutatum* revealed that the grey type isolates showed

more variable spore morphology than those of pink to red types (Vinnere, 2004). On the other hand, McKay *et al*. (2009) observed that growth rate of *C. gloeosporioides* isolates from almond in Israel were slow and did not differ significantly from that of *C. acutatum* from strawberry. According to AfanadorKafuri *et al*. (2003), comparison between isolates of *C. gloeosporioides* from almond and avocado showed that avocado isolates had an average of 6.4 mm/day growth rate and almond isolate 2.2 mm/day. Therefore, according to Afanador-Kafuri *et al*. (2003) it implies that the host from which the isolate is found also determines the growth rate measurement. According to Photita *et al*. (2005), three subgroups of *C.gloesporoides* found in their study had similar conidia shape. However, their conidial sizes were significantly different ($P < 0.05$) from each other. Therefore, the variation that happened in morphological characterstic measurments, either growth rate or conidial size, might not lead to species variation.

On the other hand, very few isolates within some of the groups such as G1, G2 and G5 rarely produced colourful hyphae and piece of colourful structure. Although the form of the colourful structure was variable, the colour was the same, irrespective of the culture colour (Plate 4.11).

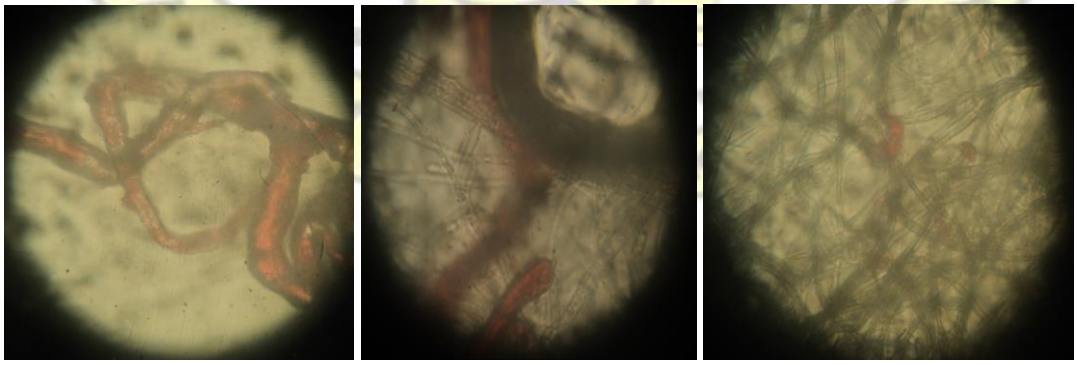

Plate 4.11: Different colourful structures observed under compound microscope from a. Morphogroup G1 b. Morphogroup G2 c. Morphogroup G5

The morphogroup G7 obtained from only mango was additionally characterised by its unique identity in producing plenty of perithecia/fruiting bodies (Plate 4.12).

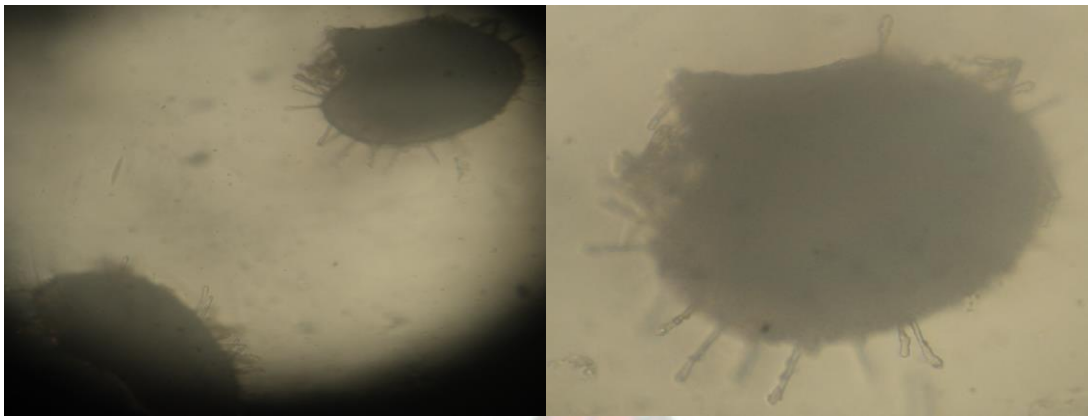

Plate 4.12: Perithecia produced by G7 morphogroups under compound microscope

In line with this finding, Abang *et al*. (2004) reported the production of abundant perithecia in culture by *Colleotrichum* pathogen of yam in Nigeria. The presence of these fruiting structures suggests that isolates of G7 may be able to reproduce sexually in mango fields. However, failure to observe the sexual state by the other morphogroups does not necessarily imply their inability to reproduce sexually, but may simply reflect the absence of conditions optimal for the production of the teleomorph. According to Sutton (1992), many morphological traits of the genus *Colletotrichum* are variable and change with environmental conditions. Thus, in general, while traditional methods for species delineation within *Colletotrichum* and for discrimination of sub-populations within species rely primarily on morphological characteristics, the strong influence of the environment on these traits has made their use unsatisfactory.

4.5.2 Morphological similarity of *Colletotrichum* isolates in mango and citrus field and its implication

Similarity of *Colletotrichum* isolates of mango and citrus implies that the pathogen was expanding its host range. In addition to the main host in this study, morphogroups G1, G2, G5 and G9 isolates were obtained from surounding alternative hosts. This result was in line with report by Sreenivasaprasad and Talhinhas (2005) that *C.acutatum* has complex epidemiology, exhibiting pathogenic and non-pathogenic lifestyles on target hosts, non-target crops and weeds. Freeman *et al*. (1998) and Sanders and Korsten (2003) also stated that, many hosts are susceptible to *C.gloeosporoides* and the loss could be huge when many susceptible hosts are grown in close proximity. Although the similarity in morphotype of isolates from both target crops as well as some surrounding plants other crops/weeds has been confirmed, the genetical similarity of those isolates is yet to be determined.

4.6 Conclusions

Colletotrichum isolates of mango, citrus as well as the surrounding plants shown morphological similarity. All the 88 *Colletotrichum* isolates studied were classified into 10 morphogroups. Isolates within the same morphogroup had high similarity in many of the morphological characters used. Some of the morphogroups encompassed isolates from citrus, mango and other alternative hosts. Among the techniques that were used in this classification, the cultural characteristics played a great role to trace the morphological similarity and differences. Conidial shape variability was also helpful as an indicator for the probable existence of genetic variability between the morphogroups. The overall characterization and identification of *Colletotrichum*

reveal the existence of morphologically diverse *Colletotrichum* species on both mango and citrus crops.

4.7 Recommendations

Although some of the morphological differences observed could be linked to the genetic variability of the studied isolates; to conclude on existence of genetic variability within or between the morphogroups, it is necessary to further investigate the characteristics of the isolates using molecular techniques.

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CHAPTER FIVE 5.0 MOLECULAR ANALYSIS OF *COLLETOTRICHUM* ISOLATES USING

GENUSSPECIFIC UNIVERSAL PRIMERS, TAXONSPECIFIC PRIMERS AND RESTRICTION ENZYMES

5.1 Summary

Identification of *Colletotrichum* species solely by morphological techniques is becoming challenging and unreliable because of highly variable morphological traits and its inconsistency with environment. Molecular techniques are helpful to resolve the difficulty in delimiting the relationships within the genus *Colletotrichum*. In this study, the multi-gene loci gene sequence detection, using primers ITS1/ITS4, Bt2a/Bt2b and GDF1/GDR1, was performed to check the existence of genetically variable *Colletotrichum* species in citrus and mango. The results confirmed the existence of diverse *Colletotrichum* species as causal agents of anthracnose disease on both crops. Especially, the GPDH gene test gave high diverse results than the ITS region and β - tubulin gene and also it was in line with most of the morphological groupings obtained in chapter four. However, further investigation made on the ITS region, using four different restriction enzymes, revealed the presence of isolate variability even on the ITS region. Most of the clusters formed by the combined results of the different enzyme digests on ITS region were in line with some of the

morphogroups. In general, the overall results of the molecular analyses imply the existence of genetically distinct groups of *Colletotrichum* species associated with diseased mango and citrus plant. Therefore, the pathogen in Ghana should be managed accordingly.

5.2 Introduction

Application of molecular techniques in plant pathology are increasingly becoming resourceful tools for quickly and sometimes cheaply assessing diverse aspects of plant pathogen genomes (Benali *et al*., 2011). These include genetic variation characterization, genome fingerprinting, population genetic diversity, taxonomy and phylogeny of plant pathogen taxa.

Identification of *Colletotrichum* species mostly was based on morphology and host association (Hyde *et al*., 2009b). However, presence of morphologically indistinguishable species of *Colletotrichum* on a single host makes the traditional technique complicated and non-consistent (Damm *et al*., 2012a, Damm *et al.*, 2012b, Weir *et al*., 2012). Molecular techniques are important tools in solving the problems of species delimitation (Du *et al*., 2005, Photita *et al*., 2005). Several authors have illustrated the need for resurvey of *Colletotrichum* species with modern approach because the previous morphological-based data might be wrongly named (Damm *et al.*, 2012a, Weir *et al*., 2012, Ko Ko *et al*., 2011). In addition, morphological identification techniques are highly affected by environmental factors. On the other hand, DNA characters are not directly influenced by environmental factors. Therefore, nucleic acid analyses are a reliable framework in classification of *Colletotrichum* species (Cai *et al*., 2009, Hyde *et al*., 2009b). As a result, there is a great move towards

using molecular techniques to analyze the taxonomic complexities and to measure the variability among individual *Colletotrichum* species

(Freeman *et al*., 2001, Lardner *et al*., 1999, Sreenivasaprad *et al*., 1996).

Molecular identification from other countries revealed different *Colletotrichum* species together, rather than as a single species, as causal agents of mango and citrus anthracnose disease (Lima *et al*., 2013, Lijuan *et al*., 2012).

Molecular identification of most fungal phylogenetic studies utilized sequences from the ribosomal gene cluster, since they were present in large numbers as tandem repeats and evolved as a single unit (Mitchell *et al*., 1995). In particular, sequence analysis of the internal transcribed spacer (ITS) regions which lie between the 18S and 5.8S genes and the 5.8S and 28S genes, has proven useful in studying phylogenetic relationships of *Colletotrichum* species because of their comparative variability (Photita *et al*., 2005). Molecular markers such as Random Amplified

Polymorphic DNA (RAPD), microsatellites, Arbitrarily-Primed Polymerase Chain Reaction (apPCR) and Amplified Fragment Length Polymorphism (AFLP) have also been used to demonstrate variation among *Colletotrichum* species populations (Nguyen, 2010, Abang *et al*., 2004, Afanador-Kafuri *et al*., 2003). For accurate species identification in the genus, a polyphasic approach, using combined sequence analysis of multiple loci, coupled with morphological data, is recommended (Cai *et al*., 2009, Chakrabarty *et al*., 2007).

Since there is a great interest to combat fruit anthracnose in Ghana, it is necessary to establish baseline information on different *Colletotrichum* species that exist on both fruit crops. According to Peres *et al.* (2002), for accurate diagnosis of a disease suspected to be caused by members of the genus *Colletotrichum*, it is necessary to

apply a combination of both morphological and molecular data. Molecular techniques are important to improve *Colletotrichum* delimitation of species that are hard to distinguish, based on morphology alone (Cai *et al*., 2009, Crouch *et al*., 2009, Damm *et al*., 2009).

In Ghana, the information on *Colletotrichum* species types present on mango is very limited and in citrus, no information has been reported. Morphological characterization result obtained indicates variability of *Colletotrichum* isolates of both crops. However, it is not yet known whether there is genetic variation or not. The objective of this study was to confirm or otherwise the morphological variability of *Colletotrichum* species result, using molecular techniques based on multi-gene loci primers and restriction enzymes.

5.3 Materials and methods

The study was carried out at Council for Scientific and Industrial Research-Crops Research Institute (CSIR-CRI) Biotechnology laboratory, Fumesua, Kumasi, Ghana. From each morphogroup, a minimum of one isolates and maximum of 16 representatives were selected. Most of the morphogroups represented by isolates from mango and citrus, except for the three morphogroups of isolates found only either of the two. For the repetitively isolated morphogroups, more isolates were considered, based on the localities and organ type from where the isolates were found. Finally, *Colletotrichum* isolates used in the molecular study consisted of 51 representatives, from 10 different morphogroups of mango and citrus (Table 5.1).

Among the 51 samples, five isolates were from weeds, volunteer plants and some intercrops showing the same symptoms as on the main citrus and mango plants.

Details of the isolates and morphogroups used for molecular analysis are shown in Table 5.1

Table 5. 1: Description of representative *Colletotrichum* morphogroups used for the molecular characterization

Morph-groups	Lab code for isolates	Host plant			Total no. of isolates	Representative isolates for the molecular analysis
		Mango	Citrus	Others		
G1	L1-L7	L1-L4	L5-L6	L7	9	7
G2	L8-L23	L8-L10	L11-L21	L22-L23	22	16
G3 (Citrus only)	L37-40	-	L37-L40	-	5	4
G4 (Mango only)	L24-L25	L24-L25	-	-	5	2
G5	L41-L46	L41-L45	-	L46	16	6
G6	L47-L48	L47	L48	-	7	2
G7 (Mango only)	L49	L49	-	-	3	1
G8	L26-L27	L26	L27	-	6	2
G9	L28-L36	L28-L32	L33-L36	-	13	9
G10	L50-L51	L50	L51	-	2	2
Total samples					**88**	**51**

*Note the label name for L4 and L12 are interchanged in all the amplification plates of the result

5.3.1 DNA extraction

Total genomic DNA was extracted from mycelia obtained from eight days old pure single spore cultures of the *Colletotrichum* isolates grown on PDA at room temperature (28-31^{o}C). Aerial mycelia were scooped from each colony surface, using a sterile transfer pipette. ZR Fungal/Bacterial DNA MiniPrep™ Kit (Epigenetics, USA) was used for DNA extraction following the manufacturer's instructions. The DNA quality was checked and DNA concentrations were estimated visually on agarose gel by comparing band intensity with a DNA ladder of 1kb. To support the quality

visualization, DNA viability was tested using RAPD primers, Rp4 and Rp85. Finally, the quality of working primers were tested for each morphological group (10 different groups), using pool DNA within the group.

5.3.2 PCR amplification, using multi-gene loci and species specific primers

The selected *Colletotrichum* isolates were amplified by polymerase chain reaction (PCR) for the following three gene regions; Partial glyceraldehyde-3- phosphate dehydrogenase gene (GPDH), partial β- tubulin gene (TUB2) and the gene with the two flanking internal transcribed spacers rDNA-ITS (ITS). PCR amplification of GAPDH, β- tubulin (TUB2) and ITS regions were carried out, using the following primers GDF1/GDR1 (Guerber *et al.*, 2003), Bt2a/Bt2b (Glass and Donaldson, 1995) and ITS1/ITS4 (White *et al*., 1990), respectively. In addition, to confirm the presence or absence of the two majorly suspected *Colletotrichum* species, *C.gloeosporioides* and *C.accutatum,* forward species specific primer CgInt (Freeman *et al*., 2000) and CaInt2 (Freeman *et al*., 2000), respectively, from the ITS1 region of the ribosomal DNA gene were used in combination with the conserved primer ITS4. The primers sequences are presented on Table 5.2

The total PCR for each *Colletotrichum* isolate was 10- µl. Each 10- µl PCR mixture included 3 µl of PCR-grade water, 1 µl of DNA template, 0.5 µM of each primer (forward and reverse), and 5 µl of dream Taq PCR Master Mix (2×) (Is supplied in 2X Dream Taq buffer,dATP, dCTP, dGTP and dTTP, 0.4 mM each, and 4mM $MgCl_2$. Thermo ScintificApplied Biosystems, Foster City, California, USA). The PCR reactions were carried out in the thermal cycler (GeneAmp® PCR System 9700; Applied Biosystems, Singapore).

The cycling parameters used for GPDH consisted of a denaturation step at 94 °C for 2 min, followed by 35 cycles at 94 °C for 45 s, 60 °C for 45 s, 72 °C for 1 min and a final extension at 72 °C for 10 min. The cycling parameters for partial TUB2 and ITS regions consisted of a 2 min denaturation step at 94 °C, followed by 34 cycles at 94 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min and a final extension of 10 min at 72 °C (Lima *et al*., 2013). After the reaction, 2-µl of orange blue dye (6x) was added to each PCR product.

Table 5. 2: Primers used in this study to target the working region and gene

Locus	**Direction & primer name**	**Sequence (5'-3')**	**Reference**
Partial beta tubulin gene (TUB2)	Forward (Bt2a)	GGTAACCAATCGGTG CTGCTTTC	Glass and Donaldson. (1995)
	Reverse (Bt2b)	ACC CTC AGT GTA GTG ACC CTT GGC	
Partial glyceraldehydehydrogenase 3- phosphate gene (GPDH)	Forward (GDF1)	GCC GTC AAC GAC CCC TTC ATT GA	Guerber *et al*. (2003)
	Reverse (GDR1)	GGG TGG AGT CGT ACT TGA GCA TGT	
Gene with the two flanking internal transcribed spacers (ITS)	Forward (ITS1)	TCCGTAGGTGAACCT GCGG	White *et al.* (1990)
	Reverse (ITS-4)	TCC TCC GCT TAT TGA TAT GC	
Species specific C. gloeosporioides	Forward (CgInt)	GGCCTCCCGCCTCCG GGCGG	Freeman *et al*. (2000)
species specific *C.acutatum*	Forward (CaInt2)	GGGGAAGCCTCTCGC GG	Freeman *et al*. (2000)

5.3.3 Electrophoresis

The PCR products were separated by electrophoresis on 1.5 % agarose gel in 1.0× Tris-acetate acid EDTA (TAE) buffer and were photographed under UV light after staining in ethidium bromide (0.5 µg ml−1) for 1 min (Lima *et al*., 2013).

5.3.4 Restriction enzyme digestion of amplified ITS region of rDNA

Fifty-one samples of amplified ITS region of PCR product were digested with four different types of four-base cutter restriction enzymes; such as Fast digest *Hha*I (GCGC), *Mbo*I(GATC), *Msp*I(CCGG) and *Tas*I (*Tsp*509I)(AATT).

Each digest was performed in 12 µl reaction mixture which contained 5 µl PCR product, 0.6 units enzyme, 2µl 10x enzyme buffer and remaining nuclease free water. The digestion was carried out at 37 ℃ (except *Tas*I at 65 °C) in a water bath for 15 min. Restricted products were analyzed with 1% Agarose gel with ethidium bromide staining and were observed with UV Transluminator. The sizes of the restriction fragments were estimated by comparison with known DNA marker (100bp and 20 bp molecular DNA ladder).

5.4 Results

5.4.1 Polymerase chain reaction, using ITS region primers

All extracted DNA from the 51 samples of morphologically characterized *Colletotrichum* isolates were amplified by ITS region primers. Although the morphological classification was so diverse as indicated on (Table 5.1), the ITS amplification result appeared uniform, on approximately the same looking range

except that of G8 (L26 & 27) and G7 (L49). Groups G7 and G8 showed a sharp move down than the other groups of isolates (Plate 5.1). Eventhough the amplicons look the same, the scoring result of the ITS ranged from 500-630bp (Table 5.3). Therefore, the detailed scoring result of ITS region was implied, showing the existence of slight dissimilarities.

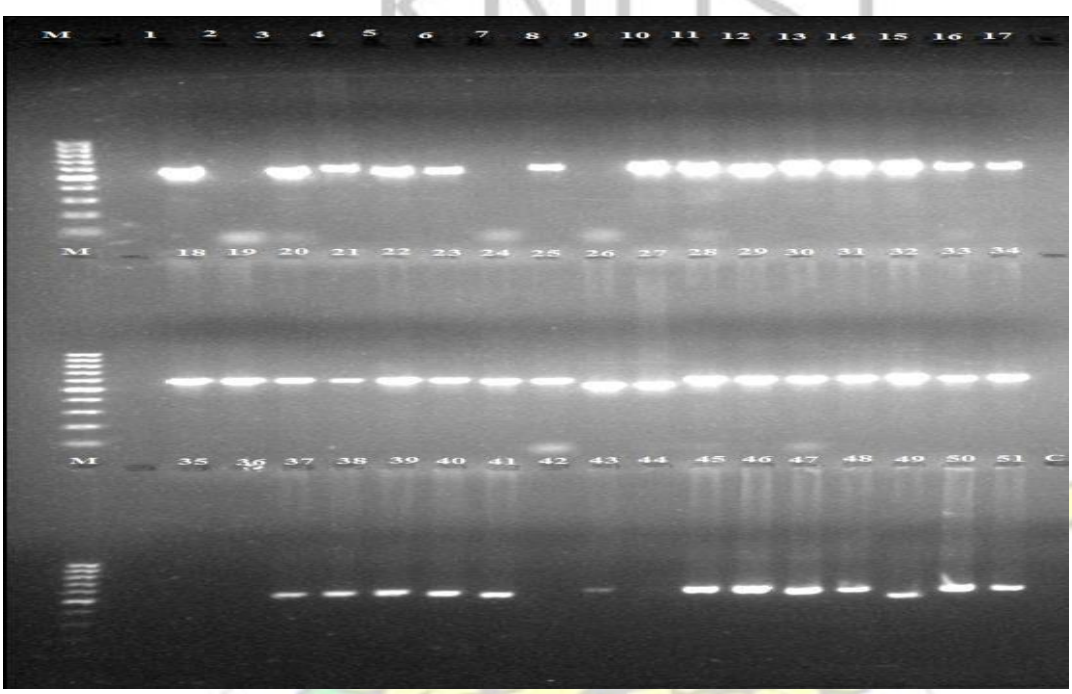

Plate 5.1: Amplification results of 51 citrus and mango representative isolates, M=100bp, sp, 1-17up, M= 100bp 18-34mid, M=100bp 35-51, C using ITS1-ITS4 primers

The differences between isolates within the same morpho-group were much closer than isolates of different groups. However, the scoring result within G1, G2 and G9 isolates showed clustering of two sub-division in each morphogroup. Half of the isolates of G2 were amplified at 535 - 550 bp and the remaining isolates of the same group amplified at 570 - 600 bp. In the case of group G5, amplification happened at 560 bp and the remaining isolates at 600 - 607 bp. The first cluster of G5 encompassed only isolate 41. The remaining isolates of G5 were included in the second cluster. On the other hand, three isolates of G9 amplified at 550bp while the remaining isolates of the same group amplified at 570-590 bp (Table 5.3).

Table 5. 3: ITS region amplicons scored in the morphogroups /for isolates 112

Morph-group	Amplification at base pairs(bp)/isolates in mporphogroup(s)											
	500	**535**	**550**	**557**	**560**	**563**	**570**	**580**	**590**	**600**	**607**	**630**
G1	1,3	12, 5	-	-	-	6	-	-	-	-	-	-
G2	-	10, 11, 13, 14,15	18, 19, 22	-	-	-	4, 20	23	21	16, 17, 8	-	-
G3	-	-	-	-	37	-	38, 39, 40	-	-	-	-	-
G4	-	-	24	25	-	-	-	-	-	-	-	-
G5	-	-	-	-	41	-	-	-	-	45, 46	43	-
G6	-	-	-	-	-	-	-	-	47	-	48	-
G7	49	-	-	-	-	-	-	-	-	-	-	-
G8	26, 27	-	-	-	-	-	-	-	-	-	-	-
G9	-	-	28,29,32	-	-	-	30, 31	-	33, 34	-	-	-
G10	-	-	-	-	-	-	-	-	-	-	50	51

5.4.2 Species specific primer analyses on ITS region

The test on tracing the commonly reported species of *C.gloeosporioides* and *C.accutatum* showed that *C. acutatum* specific primer was not able to pick any of the isolates studied in this work. On the other hand, *C.gloeosporioides* species specific primer (*CgInt*) amplified almost all isolates in morphogroups G1, G2, G4, G5, G8 and G9 (Plate 5.2).

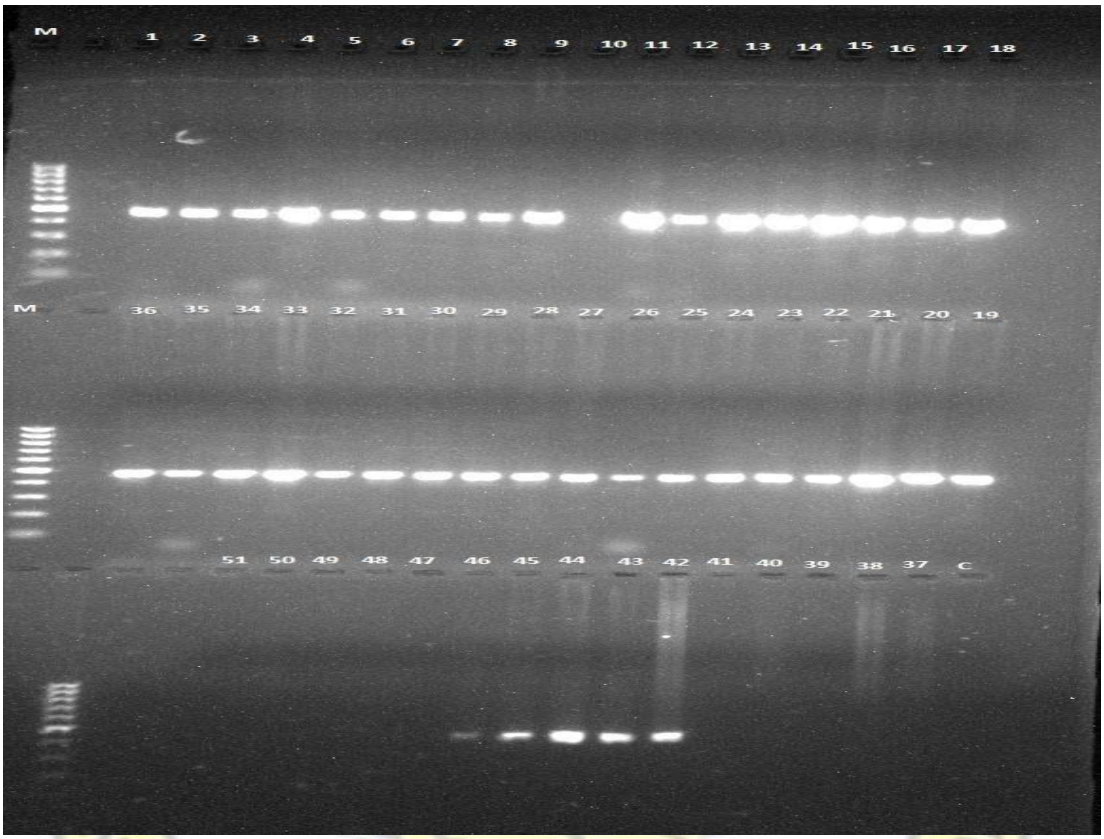

Plate 5. 2: Amplification result of the 51 representative isolates, M= 100bp, sp, 1-18up, M= 36-19mid, M= sp, sp, 51-37, C using *Colletotrichum* species specific primer CgInt-ITS4 amplified approximately at 460bp

The amplification for each of the six morphogroups happened on the same range, irrespective of their cultural characterstics differences (Plate 5.2). On the other hand, the same primer CgInt did not amplify any fragment from morphogroups G3, G6, G7 and G10 isolates (Plate 5.2).

5.4.3 Polymerase chain reaction using partial β-tubulin and GPDH gene primers

Unlike that of the ITS region primers, β-tubulin and GPDH primers picked only some of the isolates within the 51 tested isolates (Plate 5.3). For instance, β-tubulin primers were able to amplify 29 of the 51 representative isolates.

The amplification of the isolates, using β-tubulin primer, ranged from 313 - 553 bp (Table 5.4). Within those amplified isolates, some of the morphological variability were clearer than the ITS region result (compare Plate 5. 1 and Plate 5.3).

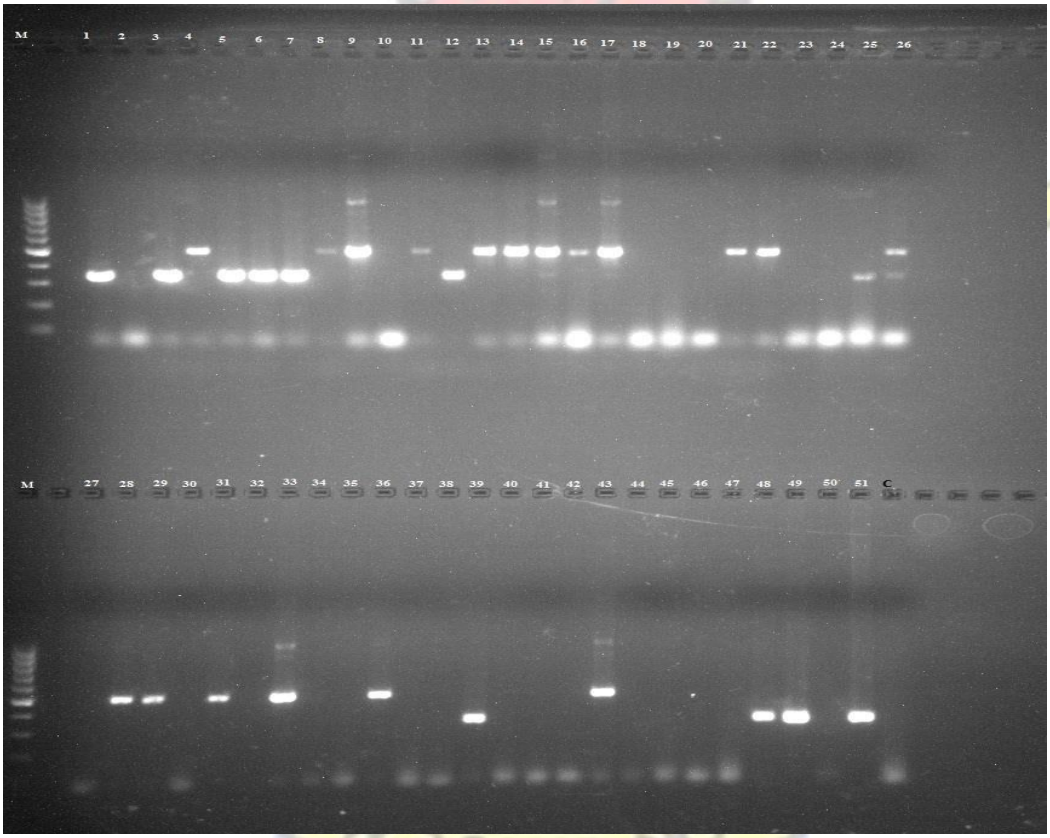

Plate 5. 3: The result on β-tubulin gene amplification of the 51 representative isolates M= 100bp, sp, 1-26 up, M=100bp, sp 27-51, C using Bt2a-Bt2b primers

For instance, the β-tubulin amplification result clearly showed the distinction between morphogroups (G2, G5, G9) that had cylindrical conidia and those that had curved

conidia (G1, G3 and G6) (Plate 5.3**).** There was also a high similarity or closeness of amplicon size of those that share the same shape of conidia (Plate 5.3). Unlike the other cylindrical conidia bearing groups, G10 and G7, G4 amplified closer to groups that were had curved conidia morphgroups when tested for the same β - tubulin primers (Plate 5.3). Furthermore, some isolates of morphogroup G2, G3, G5 and G9 were not amplified by β- tubulin primers. However most of G2 and G9 isolates and some of G5 isolates were picked by the primers (Plate 5.3).

Table 5.4: Amplified β - tubulin fragment scored in the morphogroup/ for isolates

Morphogroup	Amplification at base pairs (bp) / isolates in mporphogroup (s)															
	313	**317**	**325**	**330**	**377**	**381**	**385**	**390**	**468**	**485**	**500**	**517**	**526**	**535**	**544**	**553**
G1	5, 6, 7	1, 3	12	-	-	-	-	-	-	-	-	-	-	-	-	-
G2	-	-	-	-	-	-	-	-	9, 14, 15, 17	13, 22	8, 11, 16, 21, 4	-	-	-	-	-
G3	-	-	-	-	-	39	-	-	-	-	-	-	-	-	-	-
G4	-	-	-	25	-	-	-	-	-	-	26	-	-	-	-	-
G5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	43	-
G6	-	-	-	-	-	-	-	48	-	-	-	-	-	-	-	-
G7	-	-	-	-	49	-	-	-	-	-	-	-	-	-	-	-
G8	-	-	-	-	-	-	-	-	-	-	-	27	-	-	-	-
G9	-	-	-	-	-	-	-	-	-	-	-	28	33	31	-	36
G10	-	-	-	-	-	-	51	-	-	-	-	-	-	-	-	-

GPDH gene primer was able to amplify more samples (33 isolates) than β-tubulin primer. The amplification of GPDH gene primer ranged from 200 to 450 bp (Table 5.5). The GPDH gene amplification of the isolates gave more detailed information to show the diversity of the isolates than the other studied regions. The result of GPDH showed nine very distinct groups of isolates (Plate 5.4).

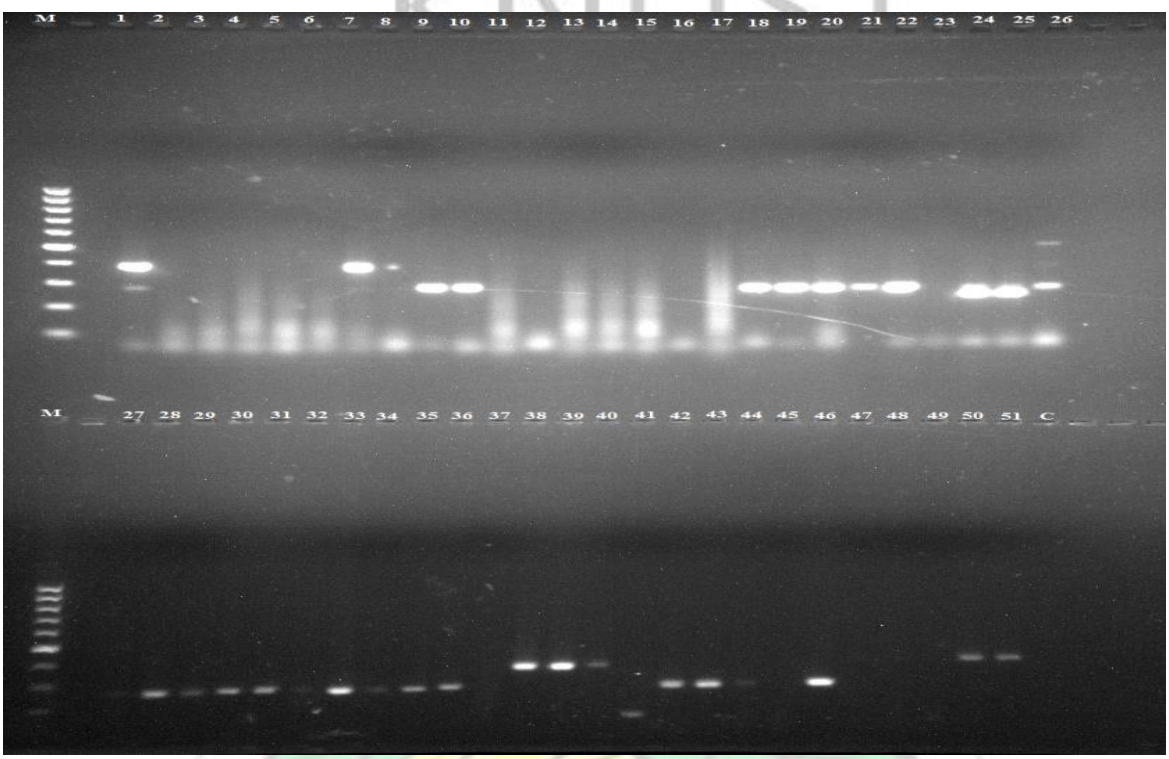

Plate 5. 4: GPDH amplification result of 51 isolate samples, M=100bp, sp 1-26 up, M=100bp, sp, 27-51, C using GDF1 - GDR1 primers

Among the nine distinct groups formed in GPDH, eight of them were in line with the morphogroups G1, G2, G3, G4, G5, G8, G9 and G10. The remaining two morphogroups (G6 and G7) were not amplified by the GPDH primers. One of the new groups by GPDH amplification created as a result of isolate 41 unique amplification, than the other isolates within the same group (G5). There was a high similarity of isolates within the groups amplified by GPDH primer, except the newly created group that was represented by single isolate 41 from G5 (Plate 5.4).

Table 5. 5: GPDH amplicon scored in the morphogroup/isolates

Morpho-group	Amplification at base pairs (bp) / isolates in mporphogroup (s)														
	200	**230**	**265**	**273**	**275**	**280**	**285**	**290**	**300**	**315**	**325**	**350**	**400**	**412**	**450**
G1	-	-	-	-	-	-	-	-	-	-	-	1, 7	-	-	-
G2	-	-	9, 10, 18, 19, 20, 22	-	21	-	-	-	-	-	-	-	-	-	-
G3	-	-	-	-	-	-	-	-	-	-	-	-	38, 39	40	-
G4	-	24, 25	-	-	-	-	-	-	-	-	-	-	-	-	-
G5	41	-	-	-	-	-	-	-	-	42, 43	44	-	-	-	-
G6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G8	-	-	-	-	-	27	-	26	-	-	-	-	-	-	-
G9	-	-	-	28, 29	-	-	30, 31, 32, 33	34	35, 36	-	-	-	-	-	-
G10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	50, 51

Although the GPDH primer amplified isolates of G1 and G2 partially, those amplified isolates range were very similar within both G1 and G2.Those that were not amplified in G1 and G2 might be different *Colletotrichum* species from those amplified within the same group (Plate 5.4).

5.4.4 Restriction enzyme digestion profile on r DNA-ITS region

Restriction endonuclease digest patterns of PCR amplified ITS region using *Hha*I, *Mbo*I, *Msp*I and *Tas*I (*Tsp*509I) were able to give further clarity on the existence of genetic variability of isolates based on the ITS region and gave more detailed information within the morhogroups.

5.4.4.1 Digest using *Hha*I

The restriction digestion of the rDNA-ITS amplified product, using *Hha*I, resulted in different band fragments between different morphogroups. Although most of the digest within the morphogroup showed similarity, some isolates were unique. For instance, all isolates in G1 (Lane 2, 3, 5, 6, 7 and 12) generated three fragments of 80, 178 and 235 base pairs from the amplified ITS product (Plate 5.5).

One of the isolates from morphogroup G2 (isolate 10) had the same pattern of digest as that of G1. However the remaining G2 morphogroup isolates had different restriction sites from the G1 isolates. The fragments generated from the ITS region digest for most G2 isolates (4, 9, 11, 13, 14, 17, 18, 19, 21, 22, 23) were 122, 178 and 235 base pairs and isolates 15 and 20 appeared in a very unique way than the dominant isolates of G2 (Table 5.6).

Table 5.6: Information of enzyme digests, using *Hha*I on ITS region

Morphogroup	Lab Isolate number/code	Point of enzyme cut	No of generated fragments	Size of the fragment in base pair
G1	2,3,5,6,7,12	two	three	80, 178,235
G2	10	two	three	80, 178, 235
	4,9,11,13,14,17 18,19,21,22,23	two	three	122, 178, 235
	15	four	five	40, 122, 178,235, 560
	20	three	four	122, 178, 235, 560
G3	37,39,40	one	two	100, 175
G4	24,25	one	two	100, 165
G5	42,44,45,46	two	three	158,200, 266
	41	three	four	100, 175,300, 400
	43	three	four	158, 200, 266,585
G6	47,48	no cut	one	266
G7	49	no cut	one	235
G8	26	one	two	176, 500
	27	two	three	167, 270, 500
G9	28,35	three	four	123, 153, 240, 561
	29,30,34	three	four	40, 123, 153, 240
	31,36	two	three	123, 153, 240
	32	two	three	40, 123, 175
	33	four	five	40, 123, 153,240, 561
G10	50	no cut	one	266
	51	no cut	one	251

There was similarity within G3 isolates (37, 39 and 40) which resulted in one cut and produced two fragments of 100 bp and 175 base pairs (Table 5.6). Fragments of 100 bp and 146 base pair were generated by G4. Isolates 42, 44, 45 and 46 of G5 were digested in the same manner by *Hha*I. However, two isolates such as 41 and 43 appear uniquely and those dominant isolates in G5 had a similarity with the dominant isolates of G2 (Plate 5.5). The largest variability was detected within G9 using *HhaI*, more than variability within other morphogroups (Plate 5.5). G9 was divided into five distinct groups. For instance, both isolates 32 and 33 appeared in a unique/different pattern of digest, isolate 29, 30 and 34 were digested in the same manner and 28 and

35 were appearing the same, again 31 and 36 also looked alike (Plate 5.5). Finally the isolates within G8 and G10 were unique (note- both group were represented by two isolates each).

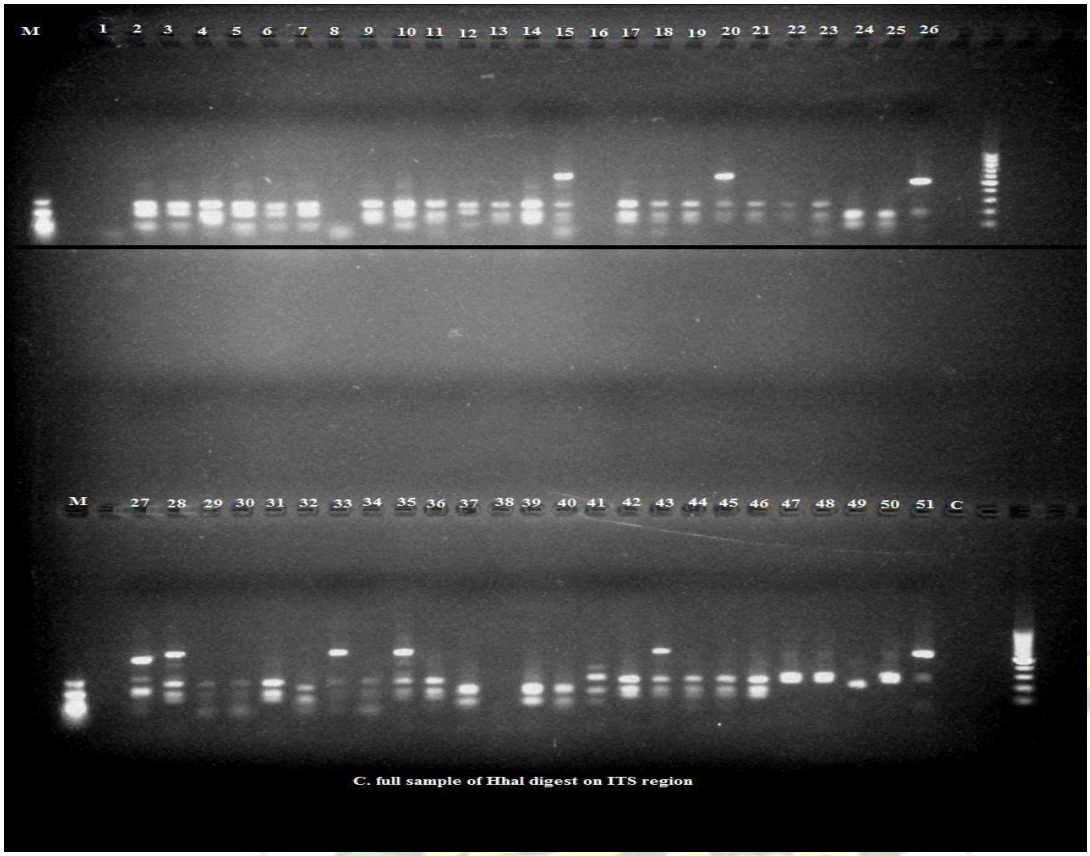

Plate 5. 5: The fragment generated and amplified from 51 isolate samples using *Hha*I enzyme on ITS region M= 20 bp (Left) , sp, 1- 3, 5 - 7, 12 (G1), 4, 8-11, 13-23 (G2), 2425 (G4), 26-27 (G8), 28-36 (G9), 37-40 (G3), 41-46 (G5), 47-48 (G6), 49 (G7), 5051(G10), C= control, sp, M=100 bp (right)

5.4.4.2 r DNA-ITS region digest, using *Mbo*I

Unlike *HhaI*, restriction digest, using *MboI*, showed that some variation existed within the G1 isolates. Isolates 3 and 7 had unique characters, compared to the remaining G1 (2, 5, 6 and 12) isolates. Interestingly, isolate 7, isolated from cocoa plant around citrus plantation, showed its uniqueness with *MboI* digest than the other isolates of G1 (Plate 5.6). As usual, isolate 10 of G2 was similar to that of the dominant group of G1 isolates.

All the remaining G2 isolates (9, 11, 13, 14, 15, 17, 18, 19, 21, 22, 23), except isolates 16 and 20, appeared the same. The two isolates, 16 and 20, were similar. Isolate 15 that showed unique character with *Hha I* digest appeared the same with the dominant isolate of G2 when *Mbo I* enzyme was used (Plate 5.6).

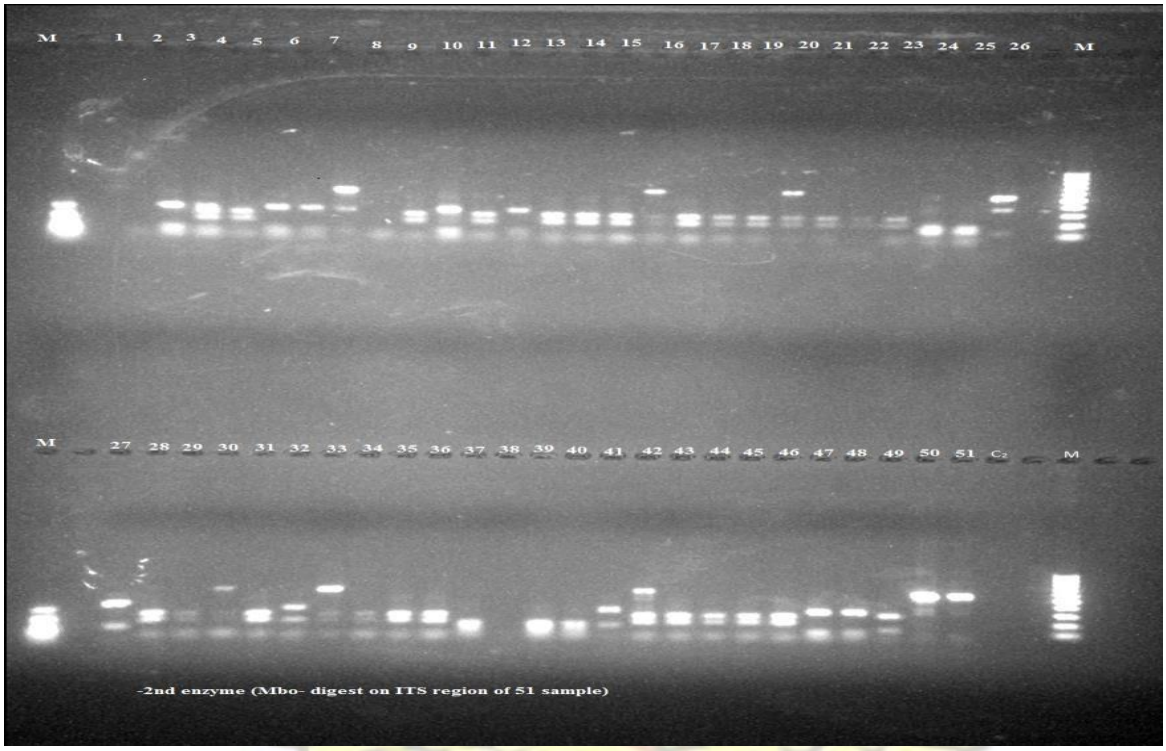

Plate 5. 6: The fragment generated and amplified from each isolates using *MboI* enzyme on ITS region M= 20 bp, sp,1- 3, 5 - 7, 12 (G1), 4, 8-11, 13- 23 (G2), 24-25 (G4), 26-27 (G8), 28 - 36 (G9), 37- 40 (G3), 41-46 (G5), 47-48 (G6), 49 (G7), 50 - 51(G10), C= control, sp, M= 100bp

Like that of *Hha*I, isolates within morphogroup G3, G4 and G6 were similar and the isolates within G8 and G10 were different from each other. Isolates (41 and 42) in G5 were unique, but the remaining isolates (43, 44, 45 and 46) of G5 were similar (Table 5.7). Isolate 41 had two restriction sites and gave three fragments of sizes 100, 230 and 400 bp. Isolate 42 had three restriction sites and gave four fragments of 100, 240, 300 and 670 bp size (Table 5.7). As it had been seen in *Hha*I digest, again *Mbo*I digest showed variability of isolates within the G9 isolates. In this case the G9 was subdivided into three groups. Isolate 28, 29, 31, 34, 35 and 36 were the dominant group, they had the same

number of fragments and shared the same restriction sites (Plate 5.6). Isolates 30 and 33 gave the same four fragment sizes at the same restriction sites. Isolate 32 was unique to all the isolates of G9 (Plate 5.6).

Table 5.7: Enzyme digests information by *Mbo*I

Morpho-group	Lab isolate no. / code	Point of enzyme cuts	Number of generated fragments	Fragment size in base pair
G1	2,5,6,12	one	two	100, 380
	3	two	three	100, 300, 375
	7	one	two	380, 600
G2	10	one	two	100, 380
	4,9,11,13,14,15,17 ,18,19,21,22,23	two	three	100, 220, 273
	16,20	three	four	100, 220, 273, 380
G3	37,39,40	no cut	one	128
G4	24,25	one cut	two	60, 147
G5	41	two	three	100,230,400
	42	three	four	100,240,300, 670
	43,44,45, 46	two	three	100,246,300
G6	47, 48	one	two	100, 340
G7	49	one	two	160, 300
G8	26	two	three	147,380, 500
	27	one	two	200, 500
G9	28, 29,31,34,35,36	two	three	100,270,338
	32	two	three	100, 270,400
	30, 33	three	four	100,270,338,680
G10	50	one	two	346, 500
	51	no cut	one	600

5.4.4.3 r DNA-ITS region digest, using *Msp*I

*Msp*I enzyme did not digest G1 isolates (2, 3, 5, 6, 7 and 12) and isolate 1 was not amplified at all. Unlike the above two enzymes results, *Msp*I results showed that isolate 10 of G2 was different from that of other G1 isolates. Furthermore, in line with

that of *Mbo*I, *Msp*I results showed similarity of isolates 16 and 20 (Plate 5.7) and the remaining isolates in G2 (9,11,13,14, 15, 17, 18, 19, 22 and 23) appeared the same to each other and different from the above mentioned isolates 16 and 20 (Plate 5.7). The *Msp*I digest showed differences in isolates within the following G3, G4, G5 and G6 (Plate 5.7). Isolates variability in G5 happened in the following ways: Isolates 43, 44 and 45 gave the same size of four amplicons of 100, 300, 486, 600 base pairs (Table 5.8). The remaining G5 isolates 41, 42 and 46 were different from the others. Isolates 41 and 42 had two points of cut but the cutting points were not the same and isolate 46 had only restriction site (Plate 5.7).

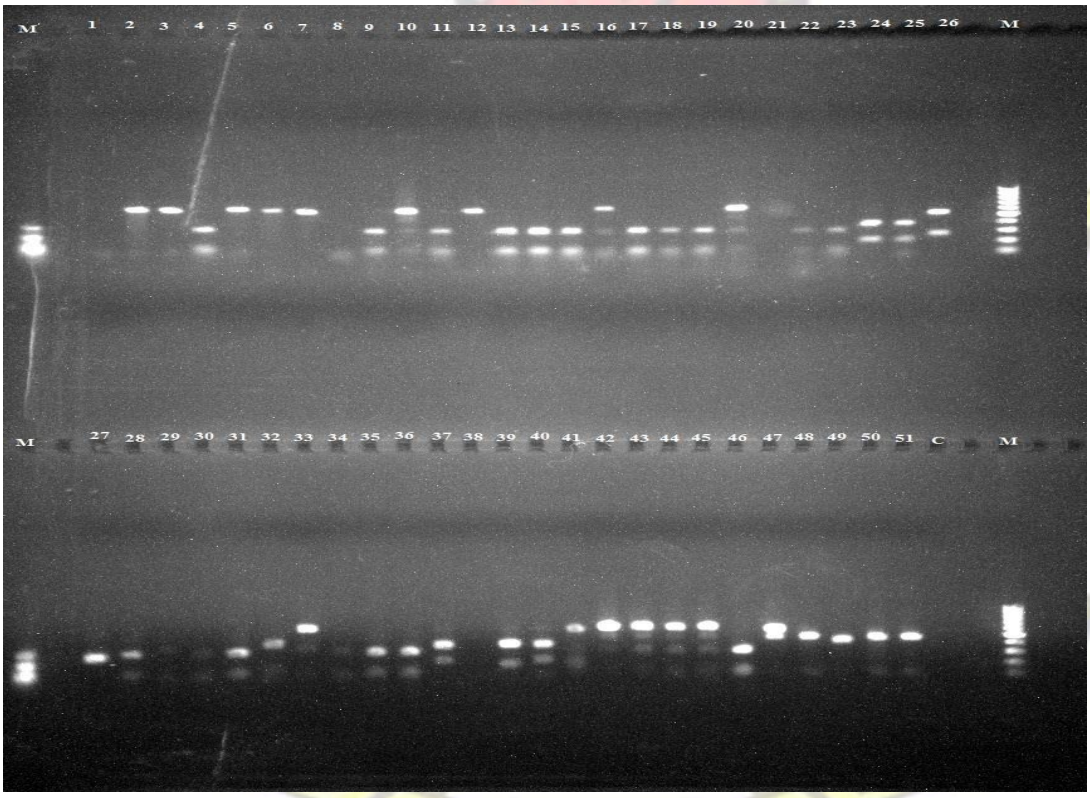

Plate Plate 5.7: The fragment generated and amplified from each isolate using *MspI* enzyme on ITS region M= 20 bp, sp, 1- 3, 5 - 7, 12 (G1), 4, 8-11, 13 - 23 (G2), 24-25 (G4), 26-27 (G8), 28 - 36 (G9), 37- 40 (G3), 41-46 (G5), 47-48 (G6), 49 (G7), 50-51(G10), C=control, sp, M=100 bp

Although the variability of isolates within G9 was observed by *Msp*I digest, unlike the two above enzymes, *Msp*I digest results in most isolates in G9 were encompassed in

one sub group. For instance, isolates 28, 30, 31, 34, 35 and 36 were digested in the same manner (Plate 5.7). The remaining isolates of G9 (29, 32 and 33) were unique on their own and appeared different (Plate 5.7). In addition, unlike the above two enzymes results' *Msp*I results showed the similarity of isolates in G10 (Table 5.8).

Table 5. 8: Information of the third restriction digestion using *Msp*I enzyme

Morph group	Lab isolate number/code	Point of Enzyme cut	Number of generated fragments	Size of the fragment in base pair
G1	2,3,5,6,7,12	no cut	one	550
G2	10	two	three	100,287,550
	20,16	two	three	100,287,588
	4,9,11,13,14,15 ,17,18,19,22,23	one	two	287,100
G3	38, 40	one	two	200, 364
	39	one	two	169,364
G4	24	one	two	206, 377
	25	two	three	100, 206,377
G5	41	two	three	168, 300,600
	42	two	three	300,486,600
	43,44,45	three	four	100,300,486,600
	46	one	two	100,300
G6	47	one	two	486,600
	48	one	two	100,475
G7	49	no cut	one	414
G8	26	one	two	273,533
G9	27	no cut	one	229
	28,30, 31, 34,35,36	one	two	100,280
	29	one	two	100,300
	32	one	two	168, 358
	33	three	four	100,300,600
G10	50,51	one	two	100,475

5.4.4.4 r DNA-ITS region digest using *TasI*

There was no cut in G1 isolates by *Tas*I restriction enzyme. As usual, isolate 10 of

G2 appeared the same (no digest), like G1 isolates. The remaining G2 isolates (9, 11, 13, 14, 15, 16, 17, 18, 19, 20, 21, 23), except 22, showed the same pattern of digest and gave the same size of three amplicons of 100, 226 and 268 bp (Table 5.9).

However, isolate 22 of the same group, isolated from *Centrosema pubuscens* showed its uniqueness and had three amplicons, but at different cutting points of 40, 226 and 268 base pair size than the other isolates of the same group (Table 5.9).

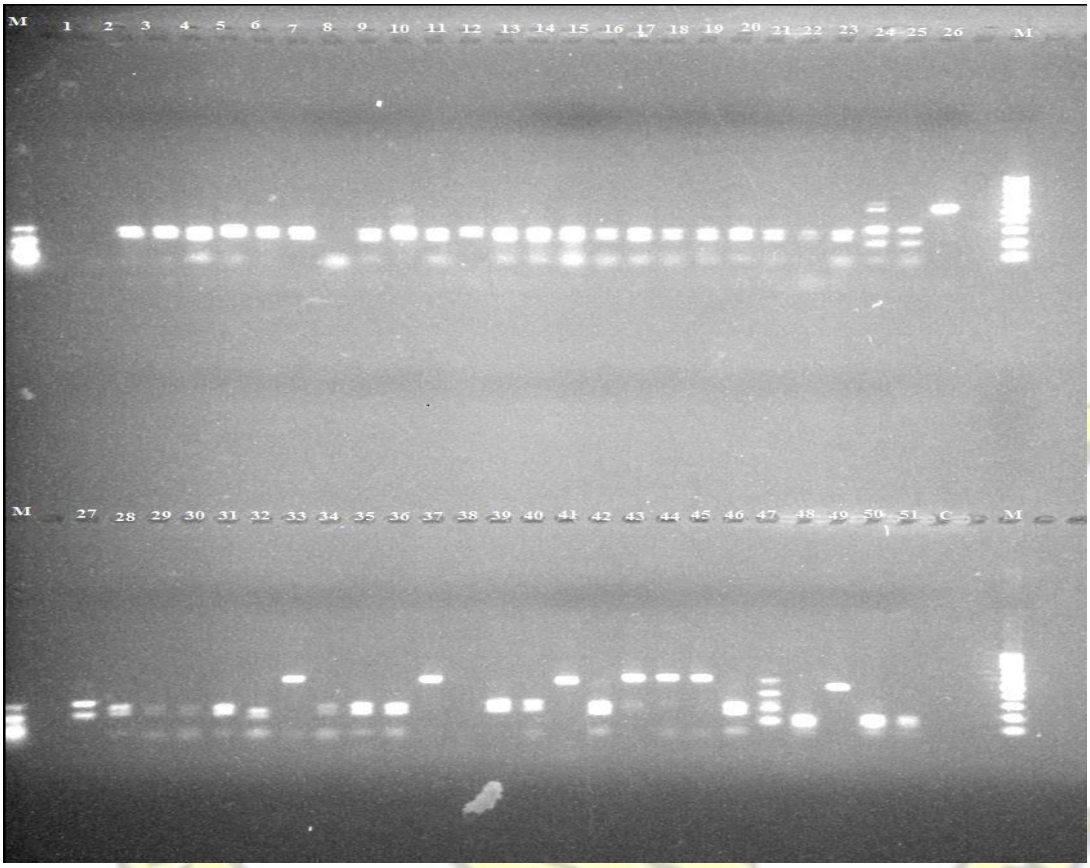

Plate 5. 8: The fragment generated and amplified from each isolates using enzyme *Tas* I on ITS region M= 20 bp, sp, 1-3, 5 - 7, 12 (G1), 4, 8 -11, 13 - 23 (G2), 24-25 (G4), 26 - 27 (G8), 28 - 36 (G9), 37- 40 (G3), 41-46 (G5), 47-48 (G6), 49 (G7), 50-51(G10), C= control, sp, M= 100 bp

Isolates within G3, G4, G6 and G8 showed high distinction when *Tas*I enzyme was used. All isolates of G9 appeared the same except isolate 33 (Plate 5.8).

The digested result of the dominant isolates in group G9 gave band sizes of 100, 250 and 300 bp. However, isolate 33 had only two bands size of 100 and 550 base pairs (Table 5.9).

Table 5. 9: Information of the fourth restriction digestion using *Tas*I enzyme

Morph group	Lab isolate number/cod	Point of Enzyme cuts	Number of generated fragment	Size of the fragment
G1	2,3,5,6,7,12	one cut	two	226,268
G2	10	one cut	two	226,268
	4,9,11,13,14,15,16, 17,18,19,20,21, ,23	two	three	100,226,268
	22	two	three	40,226, 268
G3	37	no cut	one	550
	39	one	two	280,354
	40	two	three	100,280, 354
G4	24	four	five	100,200,300,518,600
	25	two	three	100,200,300
G5	41	no cut	one	550
	43	no cut	one	600
	42,46	two	three	100,250,325
	44,45	one	two	100,600
G6	47	three	four	175,300,450,550
	48	no cut	one	160
G7	49	no cut	one	500
G8	26	no cut	one	500
	27	one cut	two	233,325
G9	28, 29, 30, 31, 32, 34, 35, 36	two	three	100, 250,300
	33	one	two	100,550
G10	50, 51	no cut	one	163

In general, the combined enzyme digest results on ITS regions were able to give detailed information on the *Colletotrichum* isolates relationship/ similarity than the ITS amplification. The genetic relationship (similarity or differences) based on the four above enzyme digest results on ITS region was shown, using the dendrogram generated (Figure 5.1).

On the dendrogram at a distance of 0.79, the isolates were grouped into two clusters from the total of 51 working isolate samples. The first cluster encompassed most of the isolates (forty nine of them) and the second cluster composed of only two isolates such as MKG41 and MEG44 (Figure 5.1), and both isolates are the representative samples of morphogroup G4.

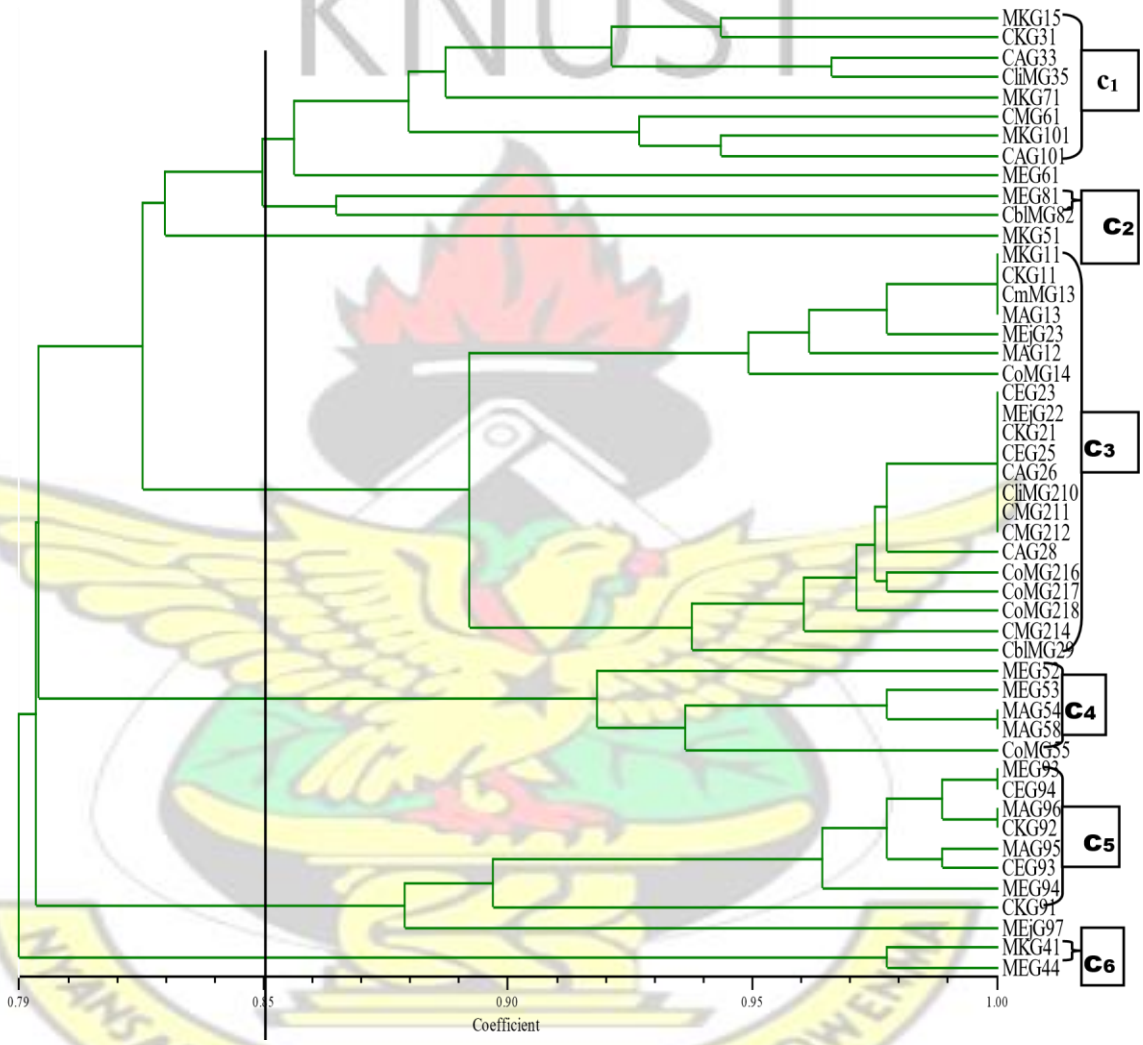

Figure 5.1: Dendrogram showing relationship among different mango and citrus *Colletotrichum* morphogroups (51 isolates) generated, based on the ITS region enzymes digest using (*Hha*I, *Mbo*I, *Msp*I and *Tas*I) and the six clusters formed (C1-C6) at 0.85 distance

Additional look at 0.85 distance of the dendrogram showed more variability between isolates, and one can see six clusters (C_1 to C_6) as six branches occur at 85 % level of

similarity (Figure 5.1). At the same distance, 0.85 MG51 and MEG61 appeared as unique isolate than the other clusters formed at the same point (Figure 5.1). The same isolate, MG51, in morphogroup G5, showed uniqueness on GPDH gene amplification (Plate 5.4).

The six clusters obtained at 0.85 level of similarity (Figure 5.1) from ITS digest implies that, almost all the isolates within each cluster had genetic similarity. Most of the clusters formed by the combined enzyme digest on ITS region were in line with that of the previous morphological groupings except, some of the clusters such as C1 and C3, which hold more than one morphogroup at 0.85 distance. For instance, C1 holds three morphogroups (G3, G6 and G10) and cluster three (C3) also composed of two morphogroups (G1 and G2) at 0.85 distance. On the same distance, C3 was the largest cluster group among the six clusters and comprised 21 isolates from two morphogroups. However, at the highest similarity level of 0.93, cluster 3(C3) further divided in two groups each of the new cluster, comprised of morphogroup G1 and G2 separately (Figure 5.1). In general, the other cluster groups such as C2, C4, C5 and C6 hold isolates from single morphogroup each of from morphogroup G8, G5, G9 and from G4, respectively at 0.85 level of similarity.

5.5 Discussion

5.5.1 Identification of *Colletotrichum* species based on ITS region, using universal and species-specific primers

The amplicon sizes of ITS region in this study were in line with the results of other authors who worked on different species of *Colletotrichum*. For instance, according to Lima *et al.* (2013), the sequences of the ITS region of *Colletotrichum* isolates ranged from 484 to 598 bp. On the other hand, Photita *et al*. (2005) reported that the ITS

region of *Colletotrichum* which they studied varied from 581 to 620 bp. Since most of the isolates studied by Lima *et al*. (2013) belonged to *C. gloeosporioides* and *C. boninense* and they are known by their cylindrical conidia, it could be the reason why the Lima range was narrower than the ITS range found in this study. However, the combined range of Lima *et al*. (2013) and Photita *et al*. (2005) encompassed almost all of the studied isolates. In addition, most of the morphological characteristics reported by Photita *et al*. (2005) and Lima *et al*. (2013) resembled the isolates found in this study.

Ford *et al*. (2004) and Freeman *et al*. (2000) stated that due to lower conservation of nucleotide sequences in the non-transcribed and internal transcribed spacer (ITS) regions, these have been used to detect recent evolutionary divergence within *Colletotrichum* species. However, in this study, although the scoring result of ITS region between groups of isolates showed slight difference, the big variation that was observed in morphological characterization was not noticed in the ITS amplification result. Rather, the ITS primer amplified most isolates uniformly, except three isolates out of the total 51 analysed.

Species-specific PCR, using ITS region of rDNA, has been widely advocated for rapid identification of *Colletotrichum* species and for differentiating closely related fungal species (Schiller *et al.*, 2006; Freeman *et al.*, 2000). According to Serra *et al*. (2011), study in the analysis of the ITS sequence of ribosomal DNA for *Colletotrichum* species, all isolates amplified with the CgInt and ITS4, confirming that they pertained to *C.gloeosporioides.* Therefore, further investigation made using *C. gloeosporioides* species-specific primer (CgInt) and *C.acutatum* primer (CaInt2) and IT4 as reverse primer implied that most of the isolates from mango and citrus in this study belonged to *C. gloeosporioides* (Figure 5.2). The same pathogen from mango and citrus has been

reported by many authors in different countries (Awa *et al*., 2012; Cannon *et al*., 2012; Chowdappa and Kumar, 2012; Phoulivong *et al*., 2012). In contrast, recent reports from many countries, including Ghana, indicated that *C.gloeosporioides sensu stricto* was no more a causal agent of fruit crops anthracnose (Honger *et al*., 2014; Lima *et al*., 2013). Again, according to RiveraVargas *et al*. (2006), perithecia containing asci and ascospores in culture plates explained as a characteristic feature of *C.gloeosporioides* telemorph (*Glomerella cingulata*), although the same characteristics were seen by G7 isolates in this study from mango, it was not picked by *C.gloeosporioides* specific primer. Isolate 41 in morphogroup G5 was also not picked by CgInt primer.

Sreenivasaprasad and Talhinhas (2005) reported that *C. acutatum* causes antrachnose on diverse range of hosts, including citrus. In this study, group of pink culture of isolates from mango such as G4, having one end-pointed conidia were observed. According to Guerber and Correll (2001), pink to red chromomeric colony morphology was frequently noted as *C. acutatum*. *C. acutatum* has also been reported as a major pathogen in various disease complexes where more than one *Colletotrichum* species is associated with a single host (Sreenivasaprasad and Talhinhas, 2005). However, this study did not amplify even a single isolate either from mango or citrus with the use of *C. acutatum* species-specific primer (CaInt2). This result implies that *C.acutatum* was not involved as a causal agent of anthracnose disease on both crops in Ashanti Region, Ghana. These results agree with recent report of Honger *et al*. (2014) in Ghana that, the clade containing the mango isolates was formed far away from the clade containing the *C. acutatum* species, indicating that the mango isolates were not *C. acutatum*.

Furthermore, detailed observation of the morphological history (Table 5.1**)** of the amplified isolates by *CgInt* indicated that the *C.gloeosporioides* result composed of six different morpho-groups (Plate 5.2). These indicated the complexity of the species as shown in the amplified result. According to Peres *et al*. (2002) and Freeman *et al*. (1998), *C. gloeosporioides* is considered as a cumulative species composed of diverse subpopulations. Others also pointed out that *C. gloeosporioides sensu lato* is a species complex with broad genetic and biological diversity grouped together by similar conidial morphology and ITS sequences (Damm *et al*., 2010). Therefore, most of *CgInt* amplified isolates of this study might point to different *Colletotrichum* species within the *Colletotrichum gloeosporioides* complex. However, morphogroup G1 amplification still remains questionable with the given explanation of its the morphological characteristics, such as the curved conidial shape of the group, do not match with the description of *C. gloeosporioides.* In addition, the other amplified morphogroups G4 and G8 were also unique in their cultural characteristics than the description given about *C. gloeosporioides.* Some of the unique characteristics about G4 was production of pink pigmentation, and the cultural characteristic of G8 totally resembled bacteria but all of them were amplified by the *C. gloesporioides* specific primer. Regarding G4 and G8, although they were unique, they had cylindrical conidia; therefore, they might have a probability to be under *C. gloeosporioides* species complex but may be different species. In G8, a clear difference has been observed even in ITS region amplification with the universal primer. However, the species-specific primer was able to pick the isolates as the same species.

According to Cunnington *et al*. (2004) and Abang *et al*. (2004), ITS region represents a small portion of the total genome, hence it is very important to integrate other regions to get better understanding. Despite the usefulness of the ITS region in

resolving systematic issues, studies showed its limitation in studying *Colletotrichum* species (Crouch *et al*., 2009). Others also indicated that even though ITS sequence data may help in *Colletotrichum* species identification; it cannot alone be used to adequately address species delimitation for closely related species. According to Crous *et al*. (1999), caution should be exercised when relying on ribosomal ITS sequence data to discriminate related taxa due to the limited number of informative sites identified.

5.5.2 Identification of *Colletotrichum* species, using β-tubulin and GPDH primers

Further investigation that was made on β-tubulin and glyceraldhyde-3-phosphate gene gave a better understanding on species diversity of the isolates studied than the ITS region. Especially, GPDH gene amplification gave a high diversity result than the other two regions studied (Plate 5.4). This result agrees with the findings of Lima *et al*. (2013) who stated that, *Colletotrichum* isolates from mango showed high variability based on GPDH gene.

Most of the distinction made by GPDH primer was in line with the morpho groupings in this study. Generally, the β tubublin amplification gave two clear distinct groups of isolates studied and the result was in line with conidial shape (Plate 5.3). Therefore, this implies that the β-tubulin gene played a role in conidial variability of *Colletotrichum* species than the ITS region. However, for all the citrus and mango isolates, the expected band size for β-tubulin reported by Lima *et al*. (2013) were totally missed. Since the ITS results confirmed all of the isolates were from *Colletotrichum* genus, it could be as a result of genetic recombination. Genetic

recombination is the production of offspring with combinations of traits that differ from those found in either parent. According to Diao *et al*. (2014) that, high genetic diversities in *C. truncatum* populations on chili peppers in China suggested substantial sexual recombination occurred in *C. truncatum* populations. In case of GPDH, the isolates that were matched with the expected range of Lima *et al*. (2013) were all morphogroups G2, G4 and some of G9 isolates. The remaining morphogroups G1, G3, G5, G8, G10 and most of G9 isolates amplified out of the given range, however, G1, G3, G5, G8 and G9 were confirmed as *Colletotrichum* species by the ITS result. It could be as a result of mutations in the pathogen.

In general, the combined results of β-tubulin and GPDH gene amplification were also interesting, showing some detailed differences existing within and between different morphogroups. Example is case of G2 isolate amplification (Table 5.10).

Table 5. 10: Multi-gene loci test and Species-specific identification of isolates from mango (M), citrus (C) weeds and other infected plants around citrus and mango plantations (O), using primers ITS1/ITS4, CgInt/ITS4, CaInt2/ITS4, Bt2a/Bt2b and GDF1/GDR1*

Morphogroup	Host plant	Lab code	Primer reaction				
			ITS	CgInt	CaInt2	β-tub	GPDH
G1	M	L1	+	+	-	+	+
G1	M	L2	+	+	-	-	-
G1	M	L3	+	+	-	+	-
G2	C	L4	+	+	-	+	-
G1	C	L5	+	+	-	+	-
G1	C	L6	+	+	-	+	-
G1	O	L7	+	+	-	+	+
G2	M	L8	+	+	-	+	-
G2	M	L9	+	+	-	+	+
G2	M	L10	+	-	-	-	+
G2	C	L11	+	+	-	+	-
G1	M	L12	+	+	-	+	-

G2	C	L13	+	+	-	+	-
G2	C	L14	+	+	-	+	-
G2	C	L15	+	+	-	+	-
G2	C	L16	+	+	-	+	-
G2	C	L17	+	+	-	+	-
G2	C	L18	+	+	-	-	-
G2	C	L19	+	+	-	-	+
G2	C	L20	+	+	-	-	+
G2	C	L21	+	+	-	+	+
G2	O	L22	+	+	-	+	+
G2	O	L23	+	+	-	-	-
G4	M	L24	+	+	-	-	+
G4	M	L25	+	+	-	+	+
G8	M	L26	+	+	-	+	+
G8	C	L27	+	+	-	-	+
G9	M	L28	+	+	-	+	+
G9	M	L29	+	+	-	+	+
G9	M	L30	+	+	-	-	+
Morphogroup	**Host plant**	**Lab code**	**Primer reaction**				
			ITS	**CgInt**	**CaInt2**	**β-tub**	**GPDH**
G9	M	L31	+	+	-	+	+
G9	M	L32	+	+	-	-	+
G9	C	L33	+	+	-	+	+
G9	C	L34	+	+	-	-	+
G9	C	L35	+	+	-	-	+
G9	C	L36	+	+	-	+	+
G3	C	L37	+	-	-	-	-
G3	C	L38	+	-	-	-	+
G3	C	L39	+	-	-	+	+
G3	C	L40	+	-	-	-	+
G5	M	L41	+	-	-	-	+
G5	M	L42	+	+	-	-	+

G5	M	L43	+	+	-	+	+
G5	M	L44	+	+	-	-	+
G5	M	L45	+	+	-	-	-
G5	O	L46	+	+	-	-	+
G6	M	L47	+	-	-	-	-
G6	C	L48	+	-	-	+	-
G7	O	L49	+	-	-	+	-
G10	M	L50	+	-	-	-	+
G10	C	L51	+	-	-	+	+

*Universal primers ITS1/ITS4 to amplify the ITS region, β-tub to amplify tubulin gene and GDF1 and GDR to amplify GPDH gene of *Colletotrichum* species and Taxon-specific primers CgInt (*C.gloeosporioides*) and CaInt2 (*C.acutatum*), were coupled with ITS4 M= mango, C= citrus and O= other crops/weed

Most of the isolates of G2 amplified by β-tubulin primers were not by GPDH primers, and the same happened in G1 isolates. Therefore, the situation implied that the changing point in each isolate of the various morphogroups was not only at one point. Rather, some of the isolates changed in their β-tubulin gene and the others changed on their GPDH gene. That was the probable reason that those β-tubulin amplified isolates were not picked by GPDH primer and vice versa, however all of them were confirmed as *Colletotrichum* species by the ITS region test.

For most of the morphogroups amplified by CgInt, their genetic differences were confirmed by the β-tublin and GPDH results. However, the species specific primer used was the most popularly used primer to target *C.gloeosporioides*. This implies that the primer was no more specific. According to Nieto and Rosselló (2007), mostly species-specific primers are designed based on divergence in ITS region. Since the ITS region result amplified most of the group uniformly, that might be the reason that

the species-specific primer also did the same, irrespective of the genetic variability that exists on the other genes.

Therefore, the combination of the multi-gene loci sequence analysis, using different primers, gave more detailed information than the single ITS region result. This result is in agreement with recent studies on *Colletotrichum* species identification (Honger, 2014, Huang *et al*., 2013 Lima *et al*., 2013) that recommended the use of multi-gene loci in *Colletotrichum* identification.

The overall enzyme digest on ITS region showed the existence of unique isolates and high variation within the dominant group isolates (G2, G5 and G9). Even isolates that were confirmed as genetically the same showed difference when tested with restriction enzyme. These situations indicate the existence of point mutation that might lead to a new speciation or resistance to chemicals. However, the other morphogroup isolates digestion results using different restriction enzymes, were consistently the same for isolates within the group.

5.6 Conclusion

ITS region primer was able to amplify all morphogroups and amplified them uniformly. This is a confirmation that all the groups are related organisms belonging to the same genus *Colletotrichum.* Investigation made using species-specific primer on ITS region, detected six morphogroups among the 10 as *C.gloeosporioides*. The four-base-cutter restriction enzyme digest used on the same ITS region to explore details indicated that, genetic variability existed between the morphogroups amplified by the CgInt species specific primer. In some of the dominant morphogroups (G2, G9 and G5) isolates, unique pattern of digest were obtained even within the group. Therefore, *C. gloeosporioides* species-specific primers used in this study amplified

fragments from genetically variable isolates. In general, the ITS amplification results were very limited in revealing the existence of genetic variability of the studied *Colletotrichum* morphogroup isolates but the detailed restriction digest revealed that genetic variability existed within the ITS region.

The other genes, β-tub and GPDH, studied on the same isolates, were very helpful in understanding the variability more clearly than the ITS region. In particular the GPDH results revealed that the morphogroups that were amplified by *C.gloeosporioides* species- specific primer rather belonged to different clusters groups of GPDH gene amplification. The overall results of the molecular identification of *Colletotrichum* species from mango and citrus showed that there are genetically variable species rather than a single species. Although molecular identification is recommended for taxonomic classification, identification of

Colletotrichum genus with limited genes or regions, could also lead to misleading result. Therefore, working on multiple target regions and genes is helpful to understand the taxonomic complexity in the genus *Colletotrichum.*

3.7 Recommendations

Different *Colletotrichum* species respond differently to chemical management. Therefore, fungicide sensitivity tests of the different cluster groups obtained by GPDH primer amplification will be helpful to understand more on Colletotrichum spp. Furthermore, it is recommended that sequencing of the different clusters obtained in the molecular identification of this study will be very helpful in providing basic information on mango and citrus infecting *Colletotrichum* species studies in other parts of Ghana.

5.8 References

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CHAPTER SIX

6.0 PATHOGENICITY AND CROSS INFECTIVITY STUDIES

6.1 Summary

Colletotrichum gloeosporioides is considered to be the causal agent of anthracnose disease of mango in Ghana. In the present study of anthracnose symptomatic mango and citrus plant samples, morphologically and molecularly diverse *Colletotrichum* species have been isolated. Most groups of *Colletotrichum* were obtained from both crops. The objectives of this chapter were to test pathogenicity of the diverse *Colletotrichum* isolates found in each target crop (original host) and to check the cross infectivity potential between the two crops, and additionally on papaya. Pathogenicity tests were made for all isolates on the original hosts, but for cross infectivity test representative isolates for each morpho-group of mango and sweet orange were used. Mango (cv. Kett), sweet orange (cv. Valentia), mandarin and papaya were used for the

tests. All isolates, except *Colletotrichum* group G6, were able to infect the original host citrus. In the case of mango, all the mango morphogroups except G1, G6 and G10, were pathogenic. These three morphogroups (G1, G6 and G10), isolated from the same host, mango, were non-pathogenic. Crossinoculation experiments of the *Colletotrichum* species, belonging to different morphogroups of both crops tested, showed the potential to cross-infect other tested fruit crops. It also demonstrated variation in the level of host preference/virulence among the *Colletotrichum* species tested. Especially, isolates from morphogroups G2, G5 and G9 of both crops showed their pathogeniciy as well as cross-infectivity potential on all the tested fruit crops. In addition, *Colletotrichum* isolates that were found from non-target crops/weeds around citrus and mango plantations, belonging to the same morphogroups above tested positive on mango or citrus. This implies that *Colletotrichum* pathogen has adapted to other plants including weeds in farmers' fields. On the other hand, the repetitively isolated curved conidia from G1 and G6 of mango tested negative on mango fruit. The entire situation could also be the reason for the pathogen speciation or change in form. Therefore, such information is very important to be communicated to farmers and will be useful for the design of integrated disease management.

6.2 Introduction

Colletotrichum is one of known broad-range pathogens with cases of multiple species on a single host and also single species on diverse hosts. Some of the crops infected by genus *Colletotrichum* include avocado, banana, coffee, mango, citrus, guava, papaya, strawberry, passion fruit and chilli (Phoulivong *et al*., 2010a; Hyde *et al*., 2009b). However, not all the species are pathogenic. Some of them are endophytes from healthy plants, and others have been identified as saprobes on dead plants

(Photita *et al*., 2001). Depending on the geographical location, management or agronomic practices and the life style of the same species can vary. Therefore, it is always good to confirm the pathogenicity of *Colletotrichum* species whenever one finds a new type that is not supported by literature. Koch's postulates are an essential tool that should be used to confirm the pathogenicity of *Colletotrichum* isolates (Nguyen, 2010; Cai *et al*., 2009; Peres *et al*., 2002). Cross-infection potential, among different species of *Colletotrichum* from one plant to the other, also has been reported by many authors (Phoulivong *et al*., 2012; Lakshmi *et al.*, 2011; Sanders and Korsten, 2003; Alahakoon *et al*., 1994). Freeman *et al*. (1998) stated that yield loss by anthracnose disease is especially significant in the tropics, where multiple hosts such as mango, avocado, coffee, papaya, banana, and citrus are grown in close proximity.

Assessment of fruit growing farmers' fields showed that the existence of cross-infectivity to the nearby crops and weeds was common and the level of the farmers' understanding about cross-infectivity potential of a pathogen was very low. The taxonomy of *Colletotrichum* species is in a state of constant flux and remains confusing. The situation is more complicated because of some species' ability to attack several hosts. In addition, the cross-infectivity potentials of citrus *Colletotrichum* isolates on mango and *vice versa* have been reported by many authors from many countries (Phoulivong *et al*., 2012; Lakshmi *et al*., 2011). Souza *et al*. (2013) also reported that *C. gloeosporioides* and *C. acutatum* isolates from either mango or citrus can cause anthracnose symptoms on leaves of mango and blossom blight symptoms in citrus flowers. Furthermore, the morphogrouping in this study showed similarity among *Colletotrichum* isolates of mango and citrus.

Therefore, it is very important to determine genus *Colletotrichum* complexity related to different host range or its specificity for each host at every given location (Freeman *et al*., 1998). Cross-inoculation experiments also demonstrates variation in the level of host preference among species isolates and also variation in the susceptibility of the hosts (Alahakoon *et al*., 1994). Moreover, establishing such information may provide useful data for further classification of *Colletotrichum,* useful information about the evolutionary potential of *Colletotrichum* isolates as well as aid in the development of strategies for pathogen control (Cai *et al*., 2009).

Therefore, the objectives of this study were to confirm the pathogenicity of each morphogroup on the original host plant and to understand the host range through cross infection tests.

6.3 Materials and methods

Pathogenicity and cross infectivity tests were carried out at the Plant Pathology Laboratory of the Department of Crop and Soil Sciences, KNUST, Kumasi, Ghana, using single spore isolates that were grouped into different morphogroups and were stored in a refrigerator at 4^0C.

6.3.1 Pathogenicity tests

The single-spore culture representatives of each morphological grouping of mango and citrus were sub-cultured on CPDA and grown for seven days. 10 ml of sterilized distilled water was used to obtain fungal spore suspension from each isolate by washing the surface of the plates using a sterile soft brush. Each suspension was filtered through double-layer cheesecloth. The filtered fungal suspension was kept in sterilized capped test tube as a stock suspension. From the stock suspension, 1 ml was

measured into a new test tube and the suspension topped up with 9ml of sterilized-distilled water. This was done for each representative isolates. About 1µl sample from the diluted suspension of each isolate was used to determine conidia count/ml, using a Neubauer Haemacytometer following manual procedure and the data were recorded (Verma, 2013). The conidial count was done two times, using the two grids. The counts were made from five squares out of the 25. The conidial count for each isolates was determined, using formula (1), and then it was multiplied by five to obtain conidial count for each isolate/ml. The conidial counts were used to estimate the concentration of diluted and the stock suspension, using formula (2 and

3).

$$X = \frac{a + b}{2} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where, a and b= total for each grid, x= mean count of five squares.

$$\text{Concentration of spores per ml (c)} = \text{X x 5 x } 10^4 \ldots\ldots\ldots\ldots\ldots . (2)$$

Then the concentration of spores in the original solutions were calculated, using

$$C = c\ x\ 10^n \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots . (3)$$

Where, n is the number of dilutions and c is the conidial concentration in the diluted suspension.

The tests were conducted on symptomless detached mango and citrus fruits. Freshly harvested, physiologically mature but unripe mango and citrus fruits were used for the tests. The detached fruits were washed individually under running tap water to remove debris. This was followed by surface sterilization by immersing the fruits in 70% ethanol for 1 min, 1% sodium hypochlorite (NaClO) solution for 3 min and then rinsed

three times in sterile-distilled water and blotted dry with tissue paper and air dried (Phoulivong *et al*., 2012). The fruits were wounded with sterile inoculating needle. The above calculated stock spore concentration (C) was adjusted to $1x10^6$ spore concentration and 20µl of conidial suspension was dispensed on the wound site on the mango (cv. Keitt), sweet orange (cv. Valencia), mandarin, blood orange, and lime. Wounded fruits treated with sterile-distilled water served as the control.

Inoculated fruits were placed in polyethylene bag and placed in large plastic containers. The bottom of each container was lined with four-paper-layers moistened with distilled water to maintain humidity. Each fruit was put on a sterilized Petri dish to avoid direct contact with water. The plastic containers were partially sealed with plastic bags and incubated at room temperature (28-31 ^oC) in the dark. The plastic bags and paper towels were removed after 24h and fruits were kept at the same room temperature.

The artificially inoculated fruits were monitored for the onset of symptoms for nine days. Measurement of the lesion diameter (mm) at nine days after inoculation in two perpendicular directions on each fruit was made. The diameter data of sweet orange and mango isolates were used to determine the variability of the isolates' virulence.

The lesion area (mm^2) was calculated using the formula π x l x w, where, l and w are half length and width of the lesion, respectively (Fagan, 1988). Finally after nine days of data record on the diseased fruits, diseased tissue segments were plated on PDA to confirm Koch's postulates (Agrios, 2005).

6.3.2 Cross infectivity tests

Cross infectivity was tested in citrus and mango fruits by inoculating respective *Colletotrichum* isolates to the reverse plants. That means citrus *Colletotrichum* isolates

inculated to mango and mango *Colletotrichum* isolates inoculated to citrus. In these tests, only the morphogroups isolated from both crops were used**.** The same procedure for pathogenicity tests was followed and the same spore suspension prepared for pathogenicity test was used. The only difference was that the artificial inoculation was not made to the original crops but rather to the opposite crop i.e either mango or citrus. In addition, cross infectivity was tested on papaya.

6.3.3 Experimental design and data analyses

The experiment was arranged in a completely randomized design with three fruits tested for reaction to the selected isolates. Differences in virulence caused by *Colletotrichum* species were determined by one-way ANOVA and means were compared by LSD at 5 % significance level, using GenStat 12th edition statistical package.

6.4 Results

6.4.1 Pathogenicity and cross infectivity tests

This study was carried out to check pathogenicity of the *Colletotrichum* isolates on mango and citrus and to evaluate whether there will be cross-infection between sweet orange, mango, mandarin and papaya fruits. The results confirmed that all the *Colletotrichum* isolates from citrus were pathogenic to the original crop (i.e citrus), except isolates in morphogroup G6 (Table 6.1). Whereas in mango morphogroups G1, G6 and G10 were unable to cause infection on mango fruit. In general, G6 tested negative for all hostsfrom which it was of originally isolated as well as other crops used in cross infectivity studies. Although G1 and G6 were commonly isolated from

diseased mango plant, they were non-infective. The remaining six morphogroups G2, G4, G5, G7, G8 and G9 of mango were pathogenic to the original host, mango.

Cross-inoculation experiments of the *Colletotrichum* species belonging to different morphogroups of sweet orange and mango tests showed the potential to cross-infect each other, and also other tested fruit crops, mandarin and papaya (Table 6.1).

Table 6. 1: Pathogenicity and cross infectivity tests on mango, sweet orange, mandarin and papaya

	Mango	Sweet orange	Mandarin	papaya
Mango				
G1	-	+	+	+
G2	++	+	+	+
G4	+	+	+	-
G5	++	-	+	+
G7	+	-	-	-
G8	+	+	+	-
G9	+	-	-	++
G10	-	-	++	-
Sweet orange				
G1	-	+	+	+
G2	++	++	++	+
G3	-	+	-	+
G5	++	+	-	+
G8	-	+	+	-
G9	+	+	++	++
G10	-	+	+	-

Origin of isolate morpho-group	Pathogenicity and cross infectivity test crops and

* The symbol - = avirulent , + = virulent and ++ = highly virulent

In general, morphogroups G1, G2, G5 and G9 isolates were found to be very important *Colletotrichum* species in their ability to cause disease and their cross infectivity potential. The species of *Colletotrichum* isolates in G1, G2, G5 and G9 isolated from mango and citrus were able to cross infect mandarin, papaya and either mango or sweet orange (the non-hosts). The other groups of *Colletotrichum* isolates were also able to infect at least one alternative host in addition to the original (Table 6.1). However, the control of each tested fruit remained healthy and symptomless. The symptoms developed on artificial inoculation of citrus and mango are shown in Plate 6.1.

Plate 6.1: Laboratory infectivity test on citrus and mango fruits and symptoms developed a. symptom on citrus fruits b. closer look at the symptom on sweet orange c. pinkish colour observed inside sweet orange symptom caused by G3 isolates c. Inside symptom on mandarin fruit e. symptoms developed on mango fruits.

In addition, all the *Colletotrichum* isolates isolated from the surrounding alternative hosts, including weeds, tested pathogenic to sweet orange and mango. The few isolates found from lime, mandarin and blood orange tested positive on their host fruits. The re-isolation results from the artificial inoculation resulted in the same isolate types that were used to imitate the symptoms and some of the morpho groups given on (Plate 6.2 b and c).

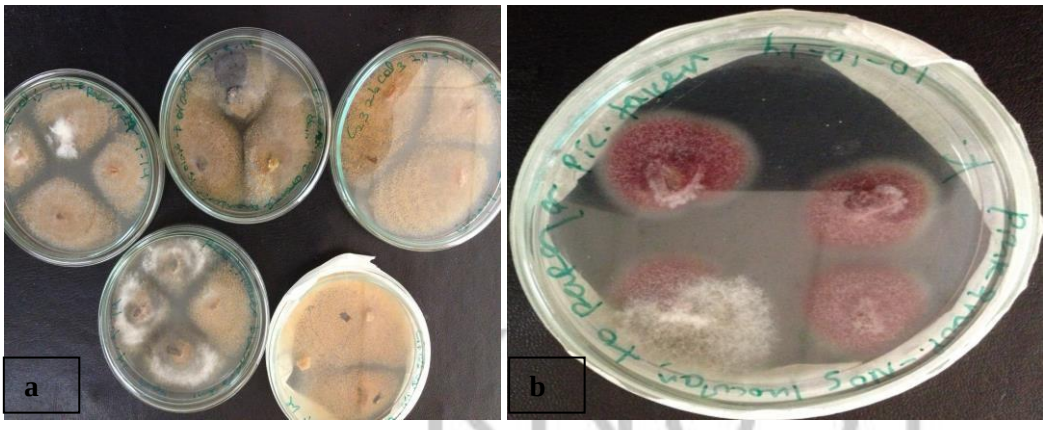

Plate 6. 2: Some of re-isolated cultures (a and b) of *Colletotrichum* pathogen from the laboratory infectivity and cross infectivity tests on citrus, mango and papaya fruits

6.4.2 Virulence of the *Colletotrichum* isolates on test fruits

Differential virulence of *Colletotrichum* species isolated from mango and sweet orange hosts was observed when inoculated into original hosts. In addition, the different groups of isolates found in both crops also showed differential virulence when inoculated on the other cross-infectivity-tested hosts. The different mean comparisons on virulence level is based on the diseased-lesion area measurement obtained from tested mango, sweet orange, mandarin and papaya fruits are shown in Table 6.2 and Table 6.3. In some of the morphogroups, isolates were much more pathogenic on alternative hosts, mandarin or papaya fruits, than that of the original hosts sweet orange or mango fruits.

Lesions caused by G2 isolates on citrus (sweet orange and mandarin) were characterized by rounded black spots, larger than those caused by isolates of G5 and G9. However, in all the three (G2, G5 and G9) morphogroups, progressive blackening symptoms were observed (Plate 6.1 b, and d). The progressive blackening lesion

caused by isolates of morphogroup G9 on mandarin was larger than on sweet orange. The pink citrus *Colletotrichum* isolate of morphogroup G3 also showed progresssive soft lesion symptom on sweet orange and the inside lesion had pink pigmentation (metabolite) was observed (Plate 6.1 c). *Colletotrichum* isolate, G6 tested negative for all the fruit crops tested including the original hosts, sweet orange and mango.

6.4.2.1 Inoculation of sweet orange *Colletotrichum* isolates on mango, mandarin, papaya and sweet orange (original hosts)

Lesion area or size caused by the different groups of sweet orange *Colletotrichums* isolates to sweet orange (itself), mango, mandarin and papaya ranged from 63.3 to 1632.5 mm^2 (Table 6.2). *Colletotrichum* isolate, G2, was the most virulent on its own sweet orange fruit and produced a lesion size of 236.2 mm^2. It was followed by G8 isolate with lession size of 230.3 mm^2. There was no significant difference ($P > 0.05$) between them. Isolate of morphogroup G5 showed more restricted symptom (63.3 mm2) than the other tested isolates on own host, sweet orange (Table 6.2). G2 isolates' cross-infectivity test on mandarin was able to produce larger lesion size (1632.5 mm^2) than on original host, sweet orange (236.2 mm^2). In sweet orange, G2 isolate of sweet orange was also the most virulent isolate on mandarin than all the isolates, followed by G10 and G9, causing 1100.3 mm^2 and 392 mm^2 lesion sizes, respectively (Table 6.2).

In case of papaya, cross-infectivity test by sweet orange isolates, of the same morphogroups of sweet orange, G9, was the most virulent isolate which caused largest lesion area or size (323.2 mm^2) than those found to be pathogenic on the same fruit, and was followed by G3 (320.8 mm^2). There was no significant difference ($P > 0.05$)

in lesion area caused by G9 and G3. However, the lesion size produced by both G9 and G3 isolates on papaya was larger than on sweet orange (original host) (Table 6.2).

Table 6. 2: Virulence tests of sweet orange isolates on the original hosts (sweet orange fruit) and others (mandarin, papaya and mango fruits)

***Colletotrichum* isolates from sweet orange host**	**Lesion area (mm^2) on fruit of**			
	Sweet orange	**Mandarin**	**Papaya**	**Mango**
Gr 1	117.20	93.70	80.60	-
Gr2	236.20	1632.50	154.60	134.00
Gr3	66.70	-	320.80	-
Gr5	63.30	100.90	151.80	79.00
Gr8	230.30	213.90	-	-
Gr9	142.30	392.50	323.20	47.10
Gr10	71.40	1100.30	-	-
LSD (P= 0.05)	**19.20**	**11.81**	**24.65**	**15.50**
CV %	**8.30**	**11.30**	**6.60**	**8.90**

The symbol - = no disease

Cross infectivity test of sweet orange morphogroups on mango showed that only G2, G5 and G9 were infective. Tested G2 isolate on mango fruit recorded largest lesion size of 134 mm^2, followed by G5 isolate (79 mm^2) and the smallest lesion size of 47.1 mm^2 was observed in G9 isolate. There was significant difference ($p < 0.05$) between them.

6.4.2.2 Inoculation of mango isolates on sweet orange, mandarin, papaya and mango fruit (original hosts)

G2 isolate from mango was the most virulent on sweet orange, causing lesion area of 318.2 mm2, compared to other positive-tested mango isolates. This was followed by the G1 isolate that recorded 45.8 mm^2 lesion size. The other positive-tested mango isolates on sweet orange, G4 and G8, caused lesion sizes of 29 and 22.2 mm^2, respectively (Table 6.3). There was significant difference ($p < 0.05$) between G2 isolates and the others. However, there were no significant differences ($p > 0.05$) among G1, G4 and G8 (Table 6.3).

Table 6. 3: Virulence test of mango isolates on the original hosts (mango fruit) and others (mandarin, papaya and sweet orange fruits)

***Colletotrichum* isolates from host mango**	**Lesion area (mm^2) on fruit of**			
	Sweet orange	**Mandarin**	**Papaya**	**Mango**
G1	45.80	80.10	48.40	-
G2	318.20	211.90	165.10	246.80
G4	29.00	267.90	-	119.30
G5	-	92.90	132.90	153.30
G7	-	-	-	73.50
G8	22.20	263.20	-	64.40
G9	-	-	765.40	69.60
G10	-	278.90	-	-
LSD (P= 0.05)	**28.94**	**32.12**	**72.60**	**17.12**
CV%	**14.80**	**9.10**	**13.90**	**7.90**

Note : - = not infective.

On mandarin cross infectivity test by mango isolates, the results showed G10 as the most virulent isolate, causing lesion size of 278.9 mm^2, but the same isolate was nonpathogenic

on the original host, mango. The other morphogroups of mango, G4 and G8 isolates, caused 267.9 and 263.2 mm^2 lesion sizes, respectively, on mandarin fruit. However, the above three groups of mango isolates lesion areas on mandarin there was no significant difference ($p > 0.05$) (Table 6.3). The cross-infectivity tests of mango isolates on papaya showed that G9 was the most virulent species, causing 765.4 mm^2 lesion size, and it was followed by isolate G2 which recorded 165.1 mm^2 lesion size. The lesion sizes obtained by the two morphogroups on papaya were significantly different ($p < 0.05$).

The virulence tests of mango morphogroups on its own fruit showed variability on the pathogen-infectivity potential. G2 was the morphogroup that gave the largest lesion size of 246 mm^2 followed by G5 (153.3 mm^2), and then by G4 with 119.3 mm^2 lesion size. The smallest lesion size recorded by mango isolates tested on mango was obtained by morphogroup G8 with 64.4 mm^2. There was significant difference ($p < 0.05$) between isolates (Table 6.3).

6.5 Discussion

In these pathogenicity tests, most of the isolates were pathogenic to their original hosts. However, some of the morphogroups failed to infect the original hosts. Isolates of morphogroups G1 and G10 were found to be non pathogenic on origional host (mango). However, the same isolates from mango, G1, was able to cross-infect sweet orange, mandarin and papaya, and G10 was able to cross infect mandarin. In addition, G6 isolates from mango and citrus also tested negative in all tested fruits. According to Freeman *et al*. (1998) and Simmonds (1965), infection of fruits may be dependent on factors such as variety and condition of the fruit, humidity, temperature and

concentration of inocula. Therefore, the situation could be possibly be as a result of the fruit maturity status. The conditions created in the laboratory might also not be conducive enough to enable mango G1 and G10 isolates', to cause infection. It might also be possible that the isolates colonised mango plant as opportunistic fungi after infection caused by the other group of isolates or after damage caused by insect and chemical spray. Organ specificity could also be a factor, for instance, most of G6 isolates were obtained from panicle, but our pathogenicity test was only on fruits. Therefore, it is very important to test those isolates on other organ, such as panicles, leaves and seedling before any concrete conclusions on those morphogroups could be made.

Rojas-Martinez *et al*. (2008) reported that *C. gloeosporioides* isolates from mango caused anthracnose symptoms on mango leaves with varying intensity. In this present study, variability of virulence by multiple groups of isolates was observed when citrus and mango isolates were inoculated to their own hosts. These differences in the virulence of the *Colletotrichum* isolates also indicate the existence of multiple species of *Colletotrichum* on the same crop. This result is in line with that of Lima *et al*. (2013) who reported that mango anthracnose in Brazil was caused by five different *Colletotrichum* species.

Among the infective morphogroups, the lesion sizes obtained, especially by G2 isolates in citrus and mango fruits, were the more largest. This implies that G2 was more virulent than the other isolates of both crops. G2 of citrus isolates was also infective on mango, papaya as well as mandarin. G2 of mango also gave similar results. This implies that G2 isolates have a wide host range. Others also reported that

Colletotrichum isolates from avocado, banana and guava could cross infect detached fruits of mango in the laboratory (Lakshmi and Prasad, 2011; Sanders and

Korsten, 2003; Peres *et al.*, 2002; Hayden *et al.*,1994)

Plate 6.3: Similar disease symptoms on citrus fruits in the field (Left) and in the laboratory (Right)

In citrus farmers' fields, G2 was the dominant group of isolates found for the diseases that mentioned. This pathogenicity test was confirmed that the citrus disease in farmer's fields caused *Colletotrichum* species pre dominantly by G2 which belong to *Colletotrichum gloeosporioides.* There are other groups of *Colletotrichum* found from the diseased citrus plant. However, for the common disease problems that farmers are facing, the morphogroup G2 isolate is the major contributor. Furthermore, in this study, the morphogroups isolates G2, G5 and G9 showed progressive infection beyond the peel of citrus and causing inside blackening. In the same way, Arauz (2000) reported that in very severe infections of mango

anthracnose, the lesions were found to have penetrated the pericarp, thereby infecting the pulp. The thin peel and the soft nature of mandarin could have contributed to the more advanced inside blackening, as compared to sweet orange, because sweet orange

have relatively thicker peel than mandarin. Additionally, the same group of isolates, G2, G5 and G9, were isolated from mango and were infective. Recently, *C. gloeosporioides* was reported as a new causal agent of citrus post-bloom fruit drop in Brazil (Lima, 2011). Souza *et al*. (2013) also indicated that *C. gloeosporioides* and *C. acutatum* obtained from mango can cause post-bloom fruit drop in citrus.

Anthracnose was not reported before as a challenge on citrus in Ghana.The cross infectivity potential and similarity of the morphogroups indicated the possibility of host migration of the *Colletotrichum* species. The host migration clearly revealed in citrus fields on non-target crops and also weeds. Therefore, the citrus disease outbreak could be caused by the same group of *Colletotrichum* like that of mango.

In general, most of the morphogroups were able to cross-infect the other cross tested fruits. This result is in line with those reported by other reaserchers (Noireung *et al*. 2012; Yang *et al*. 2012b; Phoulivong *et al*. 2010b). Additionally, Cannon *et al*. (2012), Hyde *et al*. (2009b) andGuerber *et al*.(2003) reported that the genus *Colletotrichum* contains several plant pathogens of major importance capable of causing economical significant disease on wide variety of plants. According to Sreenivasaprasad and Talhinhas (2005), the pathogen has a complex epidemiology, exhibiting pathogenic style on non-target hosts and weeds and this is in line with the field study. Photitia *et al*. (2004) and Sanders and Korsten (2003) also indicated that *Colletotrichum* species ability to infect many hosts and adapt to new environments lead to serious cross infection problems in crop production. Thus, this information should be thought-out in management strategy design, and needs to be communicated to farmers.

The pink colour observed inside orange fruit caused by G3 implies that the metabolites produced by G3 could play a role in its pathogenicity (Figure, 6.1 e).

However, the same group of isolates cross-tested negative on mango. This could be due to lack of pathogenicity factor that could recognize mango fruit cells for infection.

6.6 Conclusions

Most of the morphogroups isolated from citrus and mango tested pathogenic on their hosts but the morphogroups G1, G6 of having curved conidia and G10 of cylindrical conidia shape tested negative. In the same way in citrus, G6 of citrus isolates tested non-pathogenic but all others tested positive on their own hosts. In addition, most of those tested positive to their own host showed their potential for cross infectivity to the opposite crop citrus/ mango and also papaya.

Isolates from citrus farm weeds tested positive on sweet orange fruits. This result implies that the *Colletotrichum* species of citrus has volunteer host/ weed and other crops as alternative hosts in farmers' fields, making the disease more complex. *Centrosema pubuscens* (weed) and *Jatropha carcas* have been found as potential alternative hosts around citrus plantations.

The pathogenicity and cross-infectivity tests showed that different types of *Colletotrichum* species were involved in citrus diseases and mango anthracnose of the studied areas of Ghana. Especially, G2 isolate was found to be very important and virulent in sweet orange and mandarin, mango and also able to cross infect papaya. Also, G2 isolates from mango were the most pathogenic of all the isolates. In general, the citrus diseased plant collected from farmers' field was caused by

Colletotrichum pathogen.

6.7 Recommendations

For some of the morphogroups isolated from diseased citrus and mango plants that couldn't cause disease on fruits in the laboratory, further testing are recommended on other parts of the plant such as leaves, panicle and seedlings because those isolates could be organ specific. Furthermore, it is important to inform farmers about the outbreak of *Colletotrichum* causing disease on citrus to implement the appropriate management for the disease. Assessment should be made for the other alternative hosts of *Colletorichum*. Because the pathogen of citrus showed its migration to other alternative hosts such as cocoa, cassava and cocoyam.

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CHAPTER SEVEN

7.0 GENERAL DISCUSSION

Even though it is recommended to avoid anthracnose-prone areas for establishment of orchards, sometimes farmers could be attracted by certain benefits such as availability of land, rainfall and ability of trees to flower at certain desirable periods to site orchards in such areas. In Ashanti Region of Ghana, although different types of diseases and insect pests exist as challenges to fruit production, there are many farmers engaged in commercial citrus and mango production. Arauz (2000) observed that in anthracnose-prone area, it is almost impossible to produce profitable mango without fungicides. This implies that in a situation like Ashanti Region, where humid weather is prevalent and mostly the flowering season overlap with rainy period, farmers may need to integrate fungicide to manage anthracnose disease. Commercial fruit farmers in the study areas are trying to stick to the recommended calendar/regular spray schedule of

chemicalds in every flowering period without considering the other factors that might have control over the pathogen. According to Nelson (2008), anthracnose disease is favoured by wet, humid and warm weather. If flowering and early fruits-set overlap with dry weather, anthracnose disease incidence and severity can be close to zero, because the pathogen will be relatively inactive in such dry weather (Arauz, 2000). Regular chemical spray and mixing of different kinds of chemicals without considering the weather situation might make the plant susceptible to more infection and could lead to resistance development. Accordingly, Sief and Hillocks (1997) recommended spraying after rainfall, rather than on a fixed schedule, since rainfall is the factor that stimulates spore production and favours infection. Moreover, studies show that the mixture of several chemicals together may amplify their effects even at the low concentrations to be a risk for health, often that found in fruits and vegetables. Therefore, fruit growers in Ashanti region need awareness training on their understanding of disease management techniques specifically, in fresh fruit production system. On the other hand, in addition to farmers' effort, understanding and knowledge, the sector needs strong attention. It requires continuous follow-up to update their knowledge and to inform them about changing situation and to resolve the complex challenges. Since, the sector is desired/ targeted to diversify export basis of the country. Moreover, information delivered to them on disease causal agent, management techniques and its effectiveness must be monitored with the responsible agricultural organization. The extension system on plant protection also needs to be strengthened. Penetrating the world market and succeeding in a sustainable way demand high effort of disease management, using proper techniques.

Anthracnose is a challenging disease, especially in fresh fruit and vegetable sectors (Bailey and Jeger, 1992). The disease has been reported on both citrus and mango in

many parts of the world (Lijuan *et al*., 2012; Trkulja and Oro, 2012; Gupta *et al*., 2010; Afanador-Kafuri, *et al*., 2003). In Ghana, anthracnose disease is a limiting factor in the mango sector. Many reports from different countries indicate that mango anthracnose is caused by *C. gloeosporioides* (Kamle *et al*., 2013; Chowdappa and Kumar, 2012; Dinh and Sangchote, 2002). *Colletotrichum* pathogen causes diseases on a wide host range and it is also reported to cause cross infectivity on alternative hosts (Phoulivong *et al*., 2012; Lakshmi *et al*., 2011; Sanders and Korsten, 2003; Alahakoon *et al*., 1994).

The present studies also have shown that most of the *Colletotrichum* morphogroups from mango grown in Ashanti Region was able to infect citrus fruits and vice versa under artificial inoculation conditions in the laboratory. Although artificial host inoculation is usually not reliable enough for assessing host specificity, it indicates the potential for infection (Freeman *et al*., 1998). In addition to the laboratory artificial inoculation, visit to farmers' fields revealed infected surrounding plants/weeds (alternative hosts) which provide additional evidence that crossinfection also occur naturally. This agrees with Sanders and Korsten (2003) who reported that single *Colletotrichum* species can attack multiple hosts.

Morphological identification of *Colletotrichum* isolates obtained from both citrus and mango in the study areas showed high variability within the 10 morphogroups (G1G10). Seven of the morphogroups were isolated from both Mango and Citrus, and the other three, either from only mango or citrus. The morpohgrouping was a good confirmation for the existence of multiple *Colletotrichum* species as a causal agent of citrus and mango diseases in Ashanti Region. Furthermore, dendrogram generated, using cultural characteristics of 51 *Colletotrichum* isolates (those that were used on

molecular analysis) was helpful to understand more on the relatedness of the morphogroups that were found on citrus and mango.

At 61% level of similarity, the isolates were divided into eight clusters C_1-C_8 (Figure 7.1). Isolates MKG41, MAG12 and MKG71 appeared as an outgroup or unique isolates than that of the eight clusters at distance of 0.61. The result obtained in C1, C3, C4, C5, C6 and C8 was in line with the morphogroups G5, G9, G6, G8, G3 and G1, respectively (Figure 7.1). One of G4 isolate clustered with G3 in cluster C7 and the other one appeared unique than others and also the single representative of G7 isolate appear as outlier than the eight clusters obtained at 0.61 distance of dendogram (Figure 7.1).

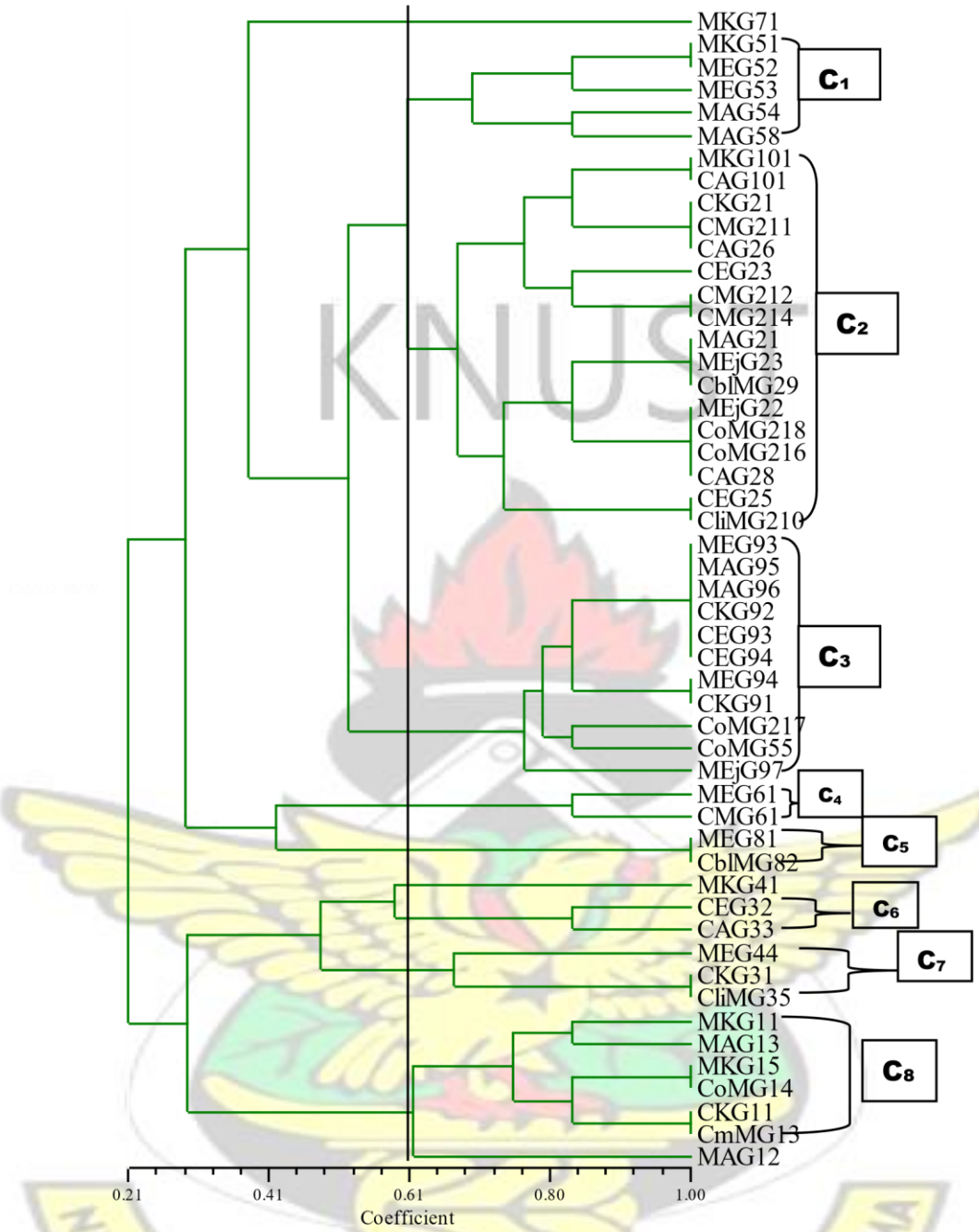

Figure 7.1: Dendrogram showing relationship among selected 51 *Colletotrichum* isolates of mango and citrus and generated based on cultural characteristics and the eight cluster obtained C1-C8 at 0.61 level of similarity.

The other missed morphogroup G10 in this analysis at the same 0.61 level of similarity was clustered in C2 together with G2 isolates. C2 was the biggest cluster than that of the other clusters on the same 0.61 distance. Out of the total 51 samples used to draw

the dendrogram, C2 encompassesd 19 isolates (17 of G2 and 2 of G10). This clustering was in line with most of the molecular characterisation (Figure 7.1).

C. gloeosporioides and *C. acutatum* are known *as* cosmopolitan pathogens because of their ability to cause diseases on many crops (Peres *et al*., 2005). In this study, the cultural and morphological features of some of the morphogroups such as G4, G5 and some of G9 fitted the original description of *C.acutatum* species (Alahakoon *et al*., 1994; Smith and Black, 1990; Simmonds, 1965), especially, in their slow growth rate in culture and their conidial shape. However, the present study of identification, using species specific primers (CaInt2) of *C. acutatum*, indicated absence of *C. acutatum* as causal agent of citrus and mango disease in the studied areas of Ashanti, Ghana. Freeman *et al*. (1998) Indicated that conidial shape of *C. acutatum* are elliptic-fusiform and whereas *C. gloeosporioides* are oblong with obtuse ends however, as a result of its inconsistency conidia shape as well as size criterion is not enough for discerning species.

Detection of *C.gloeosporioides*, using species specific primer (CgInt), was able to detect most (six) of the morphogroups as *C. gloeosporioides* and those amplified by *C. gloeosporioides* primer (CgInt), were very diverse in their cultural and morphological characteristics. Interestingly, the two unexpected groups G1 that is known by its curved conidia, and G8, that was unique in its culture characteristics, were also picked by *C. gloesporioides* species-specific primer. From these result, it is understood that the morphogroups that were picked by CgInt could be diverse species. Chowdappa and Kuma (2012) clarified about the existence of diverse sub group in *C. gloeosporioides* population associated with anthracnose on mango.

Further investigation made on the other gene region, using β-tub and GPDH genes, showed the existence of genetic variability within the isolates that were amplified by *C. gloeosporioides* species specific primer. Sutton (1992) regarded *C. gloeosporioides* as a heterogeneous species complex that hold many species together and also discussed problems surrounding identification of *Colletotrichum* species in general. Johnston and Jones (1997) reported that the most commonly referred *C.gloeosporioides* has extreme morphological and biological variations making it essentially meaningless for relaying information about the specific organism being studied. In this study, the information obtained on *Colletotrichum* species variability of ITS rDNA and β-tub gene was not detailed enough, as compared to the GPDH gene. The GPDH amplification was detailed and most of the clustering was in line with the morphological characterization. This association between morphological grouping and molecular-based clustering of GPDH confirmed the genetic

relationships among the isolates. It is in agreement with the statement that, different *Colletotrichum* species in a group could be involved in single host as a disease causal agent (Sanders and Korsten, 2003). In general, the result implies that, although molecular techniques recommended for resolving the limitation of the conventional techniques, working on very limited gene/ region will give limited information to mislead identification result. According to the finding in this study, the *Colletotrichum* species variability information detected, using the morphological and cultural characteristics, was very detailed and in line with the result of GPDH. The role of traditional techniques is very tremendous and should not be ignored, and it is in agreement with the report by Johnston and Jones (1997). Since both techniques have their own limitation, combination of the traditional and modern techniques is recommendable.

The molecular identification indicated that genetic similarity exists between mango and citrus isolates. In line with the present study, involvement of multiple *Colletotrichum* species as causal agents of anthracnose disease has been also reported on many fruit crops, including mango and citrus (Huang *et al*., 2013; Lima *et al*., 2013; Lijuan *et al*., 2012). Such a situation could be accounted for the unnecessarily intensive chemical management, and also with the farming system. Sanders and Korsten (2003) and Johnston and Jones (1997) reported that, farming system such as intercropping and presence of alternative hosts of *Colletotrichum* pathogen around orchards could also make anthracnose disease more challenging.

Uncommon *Colletotrichum* species, having curved conidia (G1 and G6), were isolated from mango. However, both groups tested negative for pathogenicity on mango fruit. According to Higgins (1926), species that cause a distinctive disease on one particular host may be found as an opportunistic secondary invader on other hosts. Such information implies that occurrence of multiple *Colletotrichum* species in the farmers' fields can happen as a result of other alternative hosts in the vicinity. Since G1 isolate was isolated from many farms of different localities from diseased mango and citrus plants; the pathogen may take advantage of the other species to be involved on the disease. On the other hand, although the G1 and G6 were not pathogenic, their association with diseased organs of mango has its own implication for diseased management. For instance, sexual recombination is reported as a likely mechanism contributing to the high genetic diversity of *Colletotrichum* spp. (Diao *et al*., 2014; Abang *et al*., 2004). Such result has great implication on anthracnose disease

management decision and it requires doing more assessment on some of the other potential alternative crops to deal with the complexity of the disease.

Furthermore, similar *Colletotrichum* morphogroups such as that of citrus and mango isolates also were isolated from the alternative hosts cocoa, jatropha, cocoyam and cassava grown around orchards and showing anthracnose symptoms. Although, there is no much information of cocoa anthracnose in Ghana, it is possible the disease could emerge because of presence of the pathogen in the study areas on other crops. According to Sanders and Korsten (2003) and Photita *et al*. (2004), *Colletotrichum* species can infect many other hosts and may adapt to new environments leading to serious cross-infection problems in plant production. *C. tropicale* and *C. fructicola* have been reported from cocoa in Panamá (Weir *et al*. 2012; Rojas *et al*. 2010). Koranteng and Awuah (2013) also reported *C.gloesporioides* as a challenging contaminant of *Phytophthora palmivora* that was isolated from cacao in Ghana in the laboratory. *C. tropicale* and *C. fructicola* have also been reported to cause mango anthracnose in Brazil (Lima *et al*., 2013) and *C. fructicola* from citrus anthracnose (Lijuan *et al*., 2012). Furthermore, *Colletotrichum* species which isolated was from diseased cocoa in this study was allocated to two morphogroups (G1 and G5), both groups were amplified by *C. gloesporioides* species-specific primers. *Colletotrichum* species of cocoa, reported such as *C. fructicola*, *C. tropicale* and *C. gloeosporioides*, also belong to *Colletotrichum gloeosporioides* complex. Therefore, it is very important/necessary to give attention and assess cocoa for any possibility of anthracnose or other *Colletotrichum*-caused disease. On the other hand, *Jatropha curcas* and *Centrosema pubuscens* (weed) were the other potential alternative hosts seen around diseased citrus fields. Recently, *Colletotrichum* caused a disease on *Jatropha curcas* in Burkina Faso (Ellison *et al*., 2015).

In general, proper identification of *Colletotrichum* species is essential for understanding the epidemiology for effective management strategies (Rivera-Vargas *et al*.,2006); Freeman *et al*., 1998). For example, citrus infection by *C. acutatum* will cause postbloom drop and key lime anthrachnose (Agostini *et al*., 1992); whereas *C. gloeosporioides* mostly causes postharvest fruit decay, shoot die back and leaf spot of citrus species (Zulfiqa *et al*., 1996). Therefore, it is necessary to give attention to the complexity of *Colletotrichum* species type which exists and its interaction with the other potential alternative hosts in the surrounding agricultural system. Abang *et al*. (2004) indicated that anthracnose resistance breeding is hampered by the dearth of knowledge on *Colletotrichum* identity and diversity. According to McDonald and Linde (2002), the greater the genetic diversity of a pathogen population, the greater the evolutionary potential and, consequently, the more likely to adapt to changing environmental conditions.

Therefore, the findings of this study have a great implication to understanding what is going on and could be used as standing information for more investigation to manage *Colletotrichum* causing mango and citrus diseases in the country.

7.1 Future perspectives

- Further studies are required to clarify the *Colletotrichum* identification and distribution all over the country, Ghana. Including more genes/ regions are important for detailed investigation of the molecular identification of *Colletotrichum* species. To come up with specific name of *Colletotrichum* species, it is sugested to sequence them to enable us to compare with recently sequenced and *Colletotrichum* species in the gene bank.

- Awareness must be created for citrus growers in Ashanti Region on citrus anthracnose disease outbreak and information about its management must be consulted accordingly. Assessment on distribution and severity of *Colletotrichum* on citrus in all

citrus growing localities is necessary. Alternative hosts infection on farmers' fields needs to be communicated as a management option and for further information, investigation on any symptomatic surrounding plants, weeds and wild trees as hosts of *Colletotrichum* species.

- Farmers must be trained on the importance of cultural practices and their roles in plant protection. Additionally, farmers must be aware about sole chemical disease management side effects; one in terms of its sustainability and its hindrances to achieve their goal to the export market. There must be a plan for implementing integrated disease management.

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APPENDICES

Appendix 1: Questionnaire to assess mango and citrus farmers' fields for anthracnose disease and other constraints and Farmers perception to plant protection.

1. What is your name (optional) _____

2. Name of the study area (locality) _____

3. Which Crop are you growing? _____

4. What is the average size of your citrus/ mango farm? _____ 5. What type of cultivars/varieties do you grow? If you know it specify_____

6. What reason (s) did you consider for your choice? _____

7. What is the source of planting material?

8. What are the main problem encountered in your production? Rank the problem

a._____

b. _____

c._____

d._____

9. Do you experience pests attack on your farm or any damage to the plant? A. [ ] Yes, B [ ] No

10. If yes, can you explain how it starts, progresses and disseminates?

_____

_____

_____

11. In which part of the plant? _____

12. Do you know the causes of these infections/ damage? Yes [ ], No [ ] specify_____

13. Is there any sample of diseased crop you can show us in the field? _____ 14. Do you have anthracnose disease in your field? (If the farmers do not mention it in questions (13)_____

15. What are the situations that favour the disease?_____

16. Did you try any management for it? Yes [ ], No [ ]

17. If yes, which method(s) do you use to control the pests? _____

18. If it is chemical, can you mention the name? _____

19. Was your management effective? Yes [ ], No [ ]

20. If no, do you know the reason why? Yes [ ], No [ ]

21. If yes, explain it_____ 23. If you want to say anything (message) about how the situation is your field or disease challenge in your production? _____

Appendix 3 - Laboratory code and corresponding field code that used for dendrogram

Isolate code used in laboratory	Corresponding field code	Isolate code used in laboratory	Corresponding field code
L1	MKG15	L26	MEG81
L2	MKG11	L27	CblMG82
L3	MAG12	L28	MEG93
L4	CEG23	L29	MEG94
L5	CKG11	L30	MAG95
L6	CmMG13	L31	MAG96
L7	CoMG14	L33	MEjG97
L8	MAG21	L33	CKG91
L9	MEjG22	L34	CEG93
L10	MEjG23	L35	CEG94
L11	CKG21	L36	CKG92
L12	MAG13	L37	CKG31
L13	CEG25	L38	CEG32
L14	CAG26	L39	CAG33
L15	CAG28	L40	CliMG35
L16	CblMG29	L41	MKG51
L17	CliMG210	L42	MEG52
L18	CMG211	L43	MEG53
L19	CMG212	L44	MAG54
L20	CMG214	L45	MAG58
L21	CoMG216	L46	CoMG55
L22	CoMG217	L47	MEG61
L23	CoMG218	L48	CMG61
L24	MKG41	L49	MKG71
L25	MEG44	L50	MKG101

		L51	CAG101

Apendix 4: ANOVA **tables of conidial size of mango and citrus isolates**

A. ANOVA for conidial length of mango *Colletotrichum* isolate morphogroups

Source of variation	**DF**	**Sum of square**	**Mean square**	**V. ratio**	**F pr**
Treatments	8	316.008	39.501	30.65	< 0.001
Residual	18	23.2	1.289		
Total	26	339.208			

B. ANOVA for conidial width of mango *Colletotrichum* isolates morphogroups

Source of variation	**DF**	**Sum of square**	**Mean square**	**V. ratio**	**F pr**
Treatments	8	24.04385	3.00548	78.33	<.001
Residual	18	0.69065	0.03837		
Total	26	24.7345			

C. ANOVA for conidial length of citrus *Colletotrichum* isolates morphogroups

Source of variation	**DF**	**Sum of square**	**Mean square**	**V. ratio**	**F pr**
Treatments	7	1180.267	168.61	139.31	< .001
Residual	16	19.365	1.21		
Total	23	1199.631			

D. ANOVA for conidial width of citrus *Colletotrichum* isolates morphogroups

Source of variation	**DF**	**Sum of square**	**Mean square**	**V. ratio**	**F pr**
Treatments	7	16.19473	2.31353	46.65	<. 001

Residual	16	0.79356	0.0496
Total	23	16.98829	

APENDIX 4 ANOVA tables on growth rate/day of mango and citrus isolates

A. ANOVA for growth rate of mango *Colletotrichum* isolates morphogroups

Source of variation	**DF**	**Sum of square**	**Mean square**	**V. ratio**	**F pr**
Treatments	8	107.8687	13.48359	308.76	<.001
Residual	18	0.78607	0.04367		
Total	26	108.65477			

B. ANOVA for growth rate of citrus *Colletotrichum* isolates morphogroups

Source of variation	**DF**	**Sum of square**	**Mean square**	**V. ratio**	**F pr**
Treatments	7	185.18528	26.45504	712.4	<.001
Residual	16	0.59417	0.03714		
Total	23	185.77944			

Apendix 5 ANOVA **tables on virulence of sweet orange and mango isolates on different fruit crops**

A. ANOVA on virulence of sweet orange *Colletotrichum* isolate to sweet orange fruit

Source of variation	**DF**	**Sum of square**	**Mean square**	**V. Ratio**	**F pr.**
Treatment	6	100420.3	16736.7	139.23	<.001
Residual	14	1682.9	120.2		
Total	20	102103.2			

B. ANOVA on virulence of sweet orange isolate to mandarin

Source of variation	DF	Sum of square	Mean square	V. Ratio	F pr.
Treatment	5	6039877	1207975	273.98	<.001
Residual	12	52908	4409		
Total	17	6092785			

C. ANOVA on virulence of sweet orange isolate to papaya

Source of variation	DF	Sum of square	Mean square	V. Ratio	F pr.
Treatment	4	144653.6	36163.4	196.96	<.001
Residual	10	1836.1	183.6		
Total	14	146489.7			

D. ANOVA on man

go isolates virulence to sweet orange

Source of variation	DF	Sum of square	Mean square	V. Ratio	F pr.
Treatment	3	184730.7	61576.9	260.64	<.001
Residual	8	1890	236.3		
Total	11	186620.7			

E. ANOVA on mango isolate virulence to mandarin

Source of variation	DF	Sum of square	Mean square	V. Ratio	F pr.
Treatment	5	122522.1	24504.4	75.19	<.001
Residual	12	3910.8	325.9		
Total	17	126432.9			

F. ANOVA on mango isolates virulence on papaya

Source of variation	DF	Sum of square	Mean square	V. Ratio	F pr.
Treatment	3	972110	324037	217.99	<.001
Residual	8	11892	1487		

Total	11	984002

G. ANOVA on mango isolates virulence on original host mango it self

Source of variation	DF	Sum of square	Mean square	V. Ratio	F pr.
Treatment	5	74878.69	14975.74	161.76	<.001
Residual	12	1110.98	92.58		
Total	17	75989.68			

H. ANOVA table on virulence test of sweet orange isolates on mango

Source of variation	DF	Sum of square	Mean square	V. Ratio	F pr.
Treatment	2	11585.58	5792.79	96.27	<.001
Residual	6	361.02	60.17		
Total	8	11946.6			