ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MICROBIAL, CELLULAR AND MOLECULAR BIOLOGY

BIORATIONAL MANAGEMENT OF POSTHARVEST ANTHRACNOSE ON TROPICAL FRUITS AND GUMMY STEM BLIGHT ON CUCURBITS

BY

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ABSTRACT

Anthracnose caused by *Colletotrichum gloeosporioides* and *Colletotrichum acutatum* and Gummy stem blight caused by *Didymella bryoniae* (anamorph *Phoma cucurbitacearum*) are among the most important diseases of fruit and cucurbits. Currently, the control of fruit and vegetable diseases relies mainly on the use of synthetic fungicides. Elucidating non-chemical control methods to reduce postharvest decay is becoming more important. This study investigated biological based approaches to controling these diseases. The first objectives of this study were to analyze morphological, physiological and molecular methods in the differentiation of *Colletotrichum* isolates obtained from banana, mango and papaya fruits and to evaluate the biocontrol potential of antagonistic bacteria, yeasts and fungal isolates to manage anthracnose disease of banana, mango and papaya during storage. In the first phase, a phenotypic analysis of *Colletotrichum* isolated from banana, mango and papaya were carried out to identify the species responsible for anthracnose disease on these hosts. A total of 45 isolates from three hosts were used. The overall similarity among different isolates of *Colletotrichum* was determined using cultural characteristics. According to the results fungal isolates could be divided in to 12 distinct groups based on morphological similarity. Subsequent identification based on ITS sequence lead to the identification of *Colletotrichum* isolates as *C. acutatum* and *C. gloeosporioides*. Isolates from mango were *C. gloeosporioides* while isolates from banana and papaya was *C. acutatum*. Further, the study confirmed the cross infection potential of *Colletotrichum* isolates and absence of host specificity. Effect of different temperature, pH level, culture media, light intensity, carbon and nitrogen sources were tested against the growth of *C. acutatum* and *C. gloeosporioides*. Results indicated that the growth of these isolates varied with the different environmental and nutritional conditions tested.

The second phase of this study was conducted to isolate, screen and identify indigenous microorganisms found on fruit surfaces in order to find a suitable biocontrol agent against postharvest fruit anthracnose caused by *C. gloeosporioides* and *C. acutatum*. Bacteria, yeast and fungal isolates recovered from leaf and fruit surfaces of banana, mango and papaya were tested *In vitro* and *In vivo* against *C. gloeosporioides* and *C. acutatum*. The microbial antagonists inhibited mycelial growth in the dual culture assay and conidial germination of *C. acutatum* and

C. gloeosporioides isolates in vitro. Studies were carried out to analyze the ability of the antagonists to produce extracellular enzymes on an amended solid media. Fourteen (14) isolates used produced cellulose and chitinase on amended media but only four isolates showed glucanase pectinase and protease activity on solid media. Additional experiments were conducted to extract and determine the nature of antifungal substances produced by antagonists that were inhibitory towards *Colletotrichum* isolates. Microbial antagonist isolates differ in their preference of culture media. The results of *In vivo* experiments under artificial infection conditions showed that suspensions containing unwashed cells of antagonists provided the highest levels of inhibition of anthracnose, while the washed cell suspension and autoclaved culture filtrates provided less protection against the disease after 30 d. Nineteen (19) different antagonists were evaluated on their own and in combination with fungicide and hot water for their ability to reduce postharvest fruit anthracnose diseases in vivo under natural infection conditions. Integrating fungicides or hot water with antagonists controlled anthracnose more effectively than fungicide control. Nine antagonists were more effective than other isolates in the control of postharvest anthracnose when fruit were treated under natural infection conditions. After phenotypic and molecular analysis, the bacterial isolates were identified as six *Pseudomonas,* three *Bacillus* and a *Paenibacillus* species. The *Pseudomonas* and *Bacillus* strains can neither be sufficiently re-solved by MALDI-TOF MS nor by 16S rRNA gene sequence analysis. The most effective yeast (M-23-L-1) and filamentous fungal (M-30-F-2) isolates were identified as *Candida rogousa* and *Trichoderma longibrachiatum*, respectively. This experiment identified six *Pseudomonas* and three *Bacilli* antagonist isolates as novel strains to be used as biological control agents against anthracnose of tropical fruits.

The second objectives of this study were to characterize populations of *Didymella bryoniae* from commercial watermelon and other cucurbit hosts from different parts of the USA on the basis of their biological and molecular diversity and to evaluate the effect of tiadinil and two thymol-based formulations against *D. bryoniae* and Gummy Stem Blight (GSB) development. In the first phase, morphological characteristics and rDNA Internal Transcribed Spacer (ITS) sequences were analyzed to identify the causal organism of this disease. Thirty five isolates of *Didymella* spp. and *Phoma* spp. associated with GSB on watermelon, canary melon, muskmelon and squash from Florida and Georgia were characterized based on morphology on culture media, pathogenicity assays and genetic characterization using ITS sequence analysis. All the isolates were pathogenic on watermelon cv. Melody, but to a varying degree. RAPD and ITS sequence analysis indicated genetic variation between the isolates. The ITS region analysis showed the presence of two isolates, DB-05 and DB-33, which showed a higher similarity to ***D. bryoniae*** isolates from China. This is the first description of an isolate with this unique sequence in Florida and Georgia. The present study brings insights into the current genetic profile of ***D. bryoniae*** isolates in Florida and Georgia, and its similarity with international isolates.

During the second phase, direct antifungal activity of tiadinil and the effect of two-thymol formulations on ***D. bryoniae*** were evaluated in vitro. All test materials used in the study affected fungal growth, with tiadinil at 10 ppm and thymol-based formulations at 0.1 ppm significantly ($P \leq 0.05$) reducing mycelial growth, conidia germination and germ tube elongation. Foliar application of tiadinil (before and after inoculation) on artificially infected watermelon seedlings at ≥ 10 ppm significantly reduced the disease severity compared to the untreated controls ($P \leq 0.05$). The disease severity on seedlings treated with tiadinil at $\geq$ 3000 ppm was statistically comparable to chlorothalonil control ($P \leq 0.05$). Plants with foliar applications of tiadinil at $\geq$ 1000 ppm (before pathogen inoculation) had significantly lower disease severity than plants with drench application ($P \leq 0.05$). Foliar application of tiadinil was affected by chemical concentration and frequency of application. Thus, based on this study, it is prudent to say that tiadinil and thymol-based formulations are potential materials for use in watermelon production for effective GSB disease suppression.

This study represents a novel report dealing with the biocontrol of anthracnose in banana, mango and papaya fruit by the application of possibly new antagonist bacterial strains and a yeast isolate. It strongly recommends the use of a combination of biological control agents with commercial treatments as a safe and effective disease management option against the postharvest anthracnose of tropical fruits. The study also highlighted the possible utilization of tiadinil and thymol-based formulations against GSB as a management strategy.

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APPROVAL

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LIST OF ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of Variance
AUDPC	Area Under Disease Progress Curve
cm	Centimeter
cm^2	Square centimeter
CFU/cfu	Colony Forming Unit
CRD	Completely Randomized Design
°C	Degree Celsius
d	Days
DNA	Deoxyribonucleic Acid
DI	Disease Incidence
DS	Disease Severity
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
ELISA	Enzyme-Linked Immunosorbent Assay
g	Gram
GRAS	Generally Regarded as Safe
GSB	Gummy Stem Blight
h	Hour
HWT	Hot Water Treatment
µg	Microgram
µL	Microliter
µm	Micrometer
mg	Milligram
Min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
nm	Nanometer
NA	Nutrient Agar
NB	Nutrient Broth
%	Percent
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
RAPD	Random Amplified Polymorphic DNA
RH	Relative Humidity
rpm	Rotation Per minute
SCAR	Sequence Characterized Amplified Region
Sec	Second
UV	Ultra Violet
v/v	Volume per volume

CHAPTER 1 GENERAL INTRODUCTION

1.1. Background

Fruits and vegetables play a major role in enhancing food security, reducing malnutrition and under nutrition particularly in developing countries (Panhwar, 2006). Fruits and vegetables contain essential minerals and vitamins which are needed for a healthy life. Deficiency in some important nutrients from fruits can result in adverse health effects (Paliyath *et al.*, 2008). World production of fruits and vegetables reached up to 392 and 486 million tons, respectively (Panhwar, 2006). Fruits and vegetables are highly perishable products and their quality can be affected by both pre and postharvest practices such as handling, transportation, storage and marketing (Janisiewicz and Korsten, 2002). Minimizing pre and postharvest losses can, therefore, reduce cost of production, improve trade and distribution, and enhance food security. In developing countries, it lowers the price for consumers and increases farmer's income (Panhwar, 2006).

In Ethiopia, fruit and vegetable crops are part of the dite of the population, and help generate foreign currency earnings for the country (Bekele_Wolde, 1991) and maintaining ecological balance since horticultural crop species are so diverse (Bekele_Wolde, 1991; Yohanese_Agonafir, 1991). According to recent information obtained from the Central Statistics Authority, the total area under fruits & vegetables is about 12,576 hectares in 2011 (CSA, 2011). Of the total land area under cultivation in the country during the same year, the area under fruits and vegetables is less than one per cent (i.e. 0.11%), which is insignificant as compared to food crops (CSA, 2011). In Ethiopia agriculture is the main stay of the economy and postharvest diseases and poor postharvest handling practices are one of the major causes of crop losses (Yohanese_Agonafir, 1991; Janisiewicz and Korsten, 2002).

This is one of the serious challenges facing the country that is struggling with developmental issues, most importantly in the agricultural sector. According to Yohanese_Agonafir (1991) Banana (*Musa sapientum* L.), Mango (*Mangifera indica*), papaya (*Carica papaya*) fruits and cucurbits are among those that are affected by postharvest diseases and results in substantial losses. This does not only lead to economic loss but also has a direct negative impact on food security and nutrition of individuals.

Major losses in fruit and vegetables are caused by postharvest pathogens (Chan and Tian, 2005; Zhang *et al.*, 2008). The filamentous fungal pathogens *Glomerella cingulate* (Stoneman) Spauld. & H. Schrenk (anamorph *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz.) and *C. acutatum* Simmonds cause anthracnose diseases on various crops (Prusky, 1996; Timmer and Brown, 2000). Postharvest anthracnose disease on tropical and subtropical fruits such as mango, avocado, banana and papaya are coused by single or multiple species of *Colletotrichum*. They also cause considerable damage in a large number of crops such as cereals, coffee and legumes (Bailey *et al.*, 1992).

Didymella bryoniae (Auersw.) Rehm (anamorph: *Phoma cucurbitacearum* (Fr.:Fr.) Sacc.) is the most important postharvest pathogen on vegetables (Keinath, 1995; Keinath *et al.*, 2001). *Didymella bryoniae* causes Gummy stem blight, which is one of the most destructive diseases of cucurbits (*Citrullus lanatus* (Thunb.) Matsum. and Nakai) (Keinath *et al.*, 2001). *Didymella bryoniae* is also an important pathogen of other cultivated *Cucurbitaceae* in the genus *Cucumis* (melon, cucumber, cantaloupe) and *Cucurbita* (squash, pumpkin) (Sitterly and Keinath, 1996). It is widely distributed in all cucurbit growing areas of the world, reducing both yield and fruit quality (Sitterly and Keinath, 1996; Somai *et al.*, 2002b). Symptoms include a stem and foliar phase and a fruit rot phase (Keinath *et al.*, 2001).

Ethiopia loses about 30 to 50 % of fruits and vegetables annually, which ageravates food insecurity (Yohanese_Agonafir, 1991). It is reported that, 33% of African populations suffering from chronic malnutrition, food insecurity and getting less than the recommended calorie intake of 2100 kcal (UNSSCN, 2004). In order to reduce food insecurity, eliminate hunger and malnutrition in developing countries like Ethiopia, alternative control measures to reduce both losses of fruits and other sources of nutritious food needs to be identified.

1.2. Statement of the Problem

Diseases limit the yield, storage period and marketing life of fruits and vegetables. Currently, the control of fruit and vegetable diseases relies mainly on the use of synthetic fungicides (Sansone *et al.*, 2005). Elucidating non-chemical control methods to reduce postharvest decay is becoming more important. Consumers are demanding less chemical residue on fresh produce, and many fungi are developing resistance to widely used synthetic fungicides (Conway *et al.*, 2004). The use of fungicides is restricted due to health concerns (Ragsdale and Sisler, 1994). Therefore, it is necessary to develop alternative control methods to reduce environmental risks and raise consumer confidence.

Bacteria, yeast and other microorganisms on the surface of fruits and vegetables have been shown to protect fruits and vegetables against postharvest pathogens (Viñas *et al.*, 1998; Janisiewicz and Korsten, 2002; Cota *et al.*, 2008). Microbial biocontrol agents have shown a great potential as an alternative to synthetic fungicides for the control of postharvest decay of fruits and vegetables (Wisniewski and Wilson, 1992).

The mode of action of antagonists generally involves antibiotics (Bull *et al.*, 1998), nutrient competition and site exclusion (Bencheqroun *et al.*, 2007), induced host resistance (El-Ghaouth *et al.*, 1998), and direct interactions between the antagonist and the pathogen (Castoria *et al.*, 1997). Additional modes of action, including the production of lytic enzymes viz., b-1,3-glucanase and chitinase were also reported (Ippolito *et al.*, 2000; Saligkarias *et al.*, 2002; Yu *et al.*, 2008). Two commercial products, Aspire (based on *Candida oleophila* Montrocher) and Yield Plus [based on *Cryptococcus albidus* (Saito) Skinner] have been registered in the United States or South Africa (Janisiewicz and Korsten, 2002; Fravel, 2005) to control postharvest diseases on fruits.

In order to better understand the epidemiology and thus reduce the financial impact of *Colletotrichum* species and *Didymella bryoniae* on the fruit and vegetable industry, it is important to know as much as possible about the pathogens themself (Keinath, 1995; Adaskaveg and Forster, 2000). Effective, lasting disease control requires a sound understanding of pathogen ecology, both species level identification and diversity. Differences between isolates of *Colletotrichum* and *Didymella bryoniae* from various sources have been widely reported. Techniques ranging from inoculation studies in their host (Agostini *et al.*, 1992; Afanador-Kafuri *et al.*, 2003) to molecular techniques such as electro phoretic patterns from protein extracts, dsRNA patterns and restriction fragment length polymorphisms (RFLP) (Bernstein *et al.*, 1995; Keinath, 2001) were used for this purpose. .

The current study was, therefore, initiated to evaluate biological based approaches in managing anthracnose on fruits and Gummy Stem Blight on watermelon diseases. The morphological, physiological and molecular characteristics of the causal organisms of the two diseases were lso evaluated.

1.3. Research Objectives

1.3.1. General objective

The overall objective of the study was to develop biological based disease management options for anthracnose and gummy stem blight diseases.

1.3.2. Specific objectives

Chapter 3

- To characterize *Colletotrichum* species from mango, banana and papaya fruits in Ethiopia based on morphology, physiological and molecular charcterstics
- To determine the pathogenic variability and cross-infection potential of *Colletotrichum* species

Chapter 4

- To isolate (screen) antagonistic bacteria, yeast and filamentous fungi from the tissues of banana, mango and papaya against *Colletotrichum* species causing fruit anthrachnose
- To determine the inhibitory effect of the selected microbial antagonists and to estimate the inhibition effects against anthracnose for determination of antifungal spectra
- To evaluate methods for application of microbial antagonists and application of combinations of antagonists and other management options for the control of anthrachnose under storage conditions

Chapter 5

- To characterize *Didymella bryoniae* and related *Phoma* isolates isolates from watermelon and other cucurbit hosts, collected from different locations in Florida and Georgia by conducting a morphological examination
- To address and clarify the genetic association between *Didymella bryoniae* and related *Phoma* isolates using phylogenetic relationships based on RAPD markers and sequence information from the internal transcribed spacer (ITS) region of rDNA

Chapter 6

- To evaluate the effect of tiadinil and thymol formulations on *D. bryoniae In vitro*
- To assess the effect on GSB development on watermelon seedlings and determine the optimum concentration, application method, and frequency of application of tiadinil and essential oils required for effective GSB management.

CHAPTER 2 LITERATURE REVIEW

2.1 The Fruit and Vegetable Industry

Tropical fruits and vegetables are products with excellent global market prospects (Bogers *et al.*, 2007; Paliyath *et al.*, 2008). In spite of the considerably rich collection of varieties of fruits and vegetables, most of the African countries have not exploited the potential economic benefits that the fresh produce can attract from the world market (Edney, 1983). Fruit and vegetable production is often in excess of the local demand and the surplus could be meaningfully utilized by careful postharvest management of the produce (Edney, 1983). A substantial portion of the regional harvest is wasted annually due to improper harvesting and postharvest practices, disease and lack of facilities and technology to extend their storage life (Paliyath *et al.*, 2008). This continues to cause heavy losses in revenue to the grower, wholesaler, retailers It also leads to exporter and inconvenience to the consumer and lowers export potential of these commodities (Edney, 1983).

2.1.1 Importance of fruit and vegetable production and consumption

The production of fruits and vegetables has been an important sector in the total world agricultural output (Edney, 1983). It is estimated that the contribution from fruits and vegetables could be over US$600 billion (Paliyath *et al.*, 2008). The weather patterns in the Northern Hemisphere are not suited for the routine production of tropical produce, and therefore fruits such as banana, pineapple, mango and citrus are exported to countries in the Northern Hemisphere (FAO, 2005; Guthrie *et al.*, 2005; Paliyath *et al.*, 2008).

Several international and governmental agencies have kept track of fruits and vegetables available for consumption on a per capita basis. In the United States, the amount of fresh and processed fruits available for consumption increased from 1976 to 2003, registering a 0.2% growth. Similarly, the amount of vegetables available for consumption increased from 1976 to 2003, registering a 0.5% growth (Paliyath *et al.*, 2008).

Several countries produce fruits and vegetables mostly, according to the needs of domestic consumption and export. Apples, bananas, citrus fruits, grapes, tomatoes, and watermelons are the largest fruit commodities produced (FAO, 2005; Paliyath *et al.*, 2008). Asia is the largest producer of fruits and vegetables, with China being the primary producer in the world. On a per capita basis, Israel produces more fruits than any other country in the world. African countries are also major players in fruit production. The world produces greater than 600 million metric tons of fruits and the world vegetable production exceeds 650 million metric tons (FAO, 2005; Guthrie *et al.*, 2005).

Human evolution was potentially linked initially to the consumption of naturally available fruits and vegetables, which later might have resulted in the selection of preferred plants and varieties for agriculture. Fruits and vegetables are major sources of several essential nutrients that include vitamins A and C and folic acid (Serdula *et al.*, 2004). In addition, fruits and vegetables are rich in antioxidants such as carotenoids, polyphenols, and anthocyanins that help combat free radicals produced within the body and the excess production of which has been related to the development of cardiovascular diseases, alzheimer's, macular degeneration, and cancers (Paliyath *et al.*, 2008). Fruits and vegetables are integral components of food in all societies. Fruits are considered as high-value items and not readily accessible to economically challenged segments of population around the world (Bogers *et al.*, 2007). The results from a number of epidemiological studies, spanning several countries, continents and population groups, show a relation between increased fruit and vegetable consumption and a reduced risk of developing maladies and diseases (Serdula *et al.*, 2004; Paliyath *et al.*, 2008).

The recommendations for vegetables include the intake of 2.5 cups equivalent (250 milliliter per cup) per day or more, comprising dark green vegetables such as broccoli and spinach; orange vegetables such as carrots and sweet potatoes; and dry beans and lentils. The recommendations of fruits are to eat a minimum of two cups equivalent every day comprising a variety of fruits and processed products, excluding juice (Serdula *et al.*, 2004). Vegetable consumption is relatively higher with 3.7 servings per day (below the recommended 5 servings or 2.5 cups per day). However, promotions at several levels (media, organizations, schools, fast food chains, etc.) are resulting in an increased understanding of the importance of fruit and vegetable consumption (Paliyath *et al.*, 2008; USDA-NASS, 2013).

2.1.2 Postharvest loss of fruits and vegetables and strategies to improve quality

By feature of their physiological properties, most fruits and vegetables are highly perishable commodities (Panhwar, 2006). Postharvest losses can occur at any point in the production and marketing chain, and may range anywhere from >10 % in advanced countries to >30 % in tropical areas and where storage facilities are limited (Prusky and Plumbley, 1992). Developing countries experiences much more loss (35-50%) due to poor postharvest handling (Paliyath *et al.*, 2008). Since a large proportion of fruits and vegetables produced are immediately consumed, the loss from long-term storage is considerably reduced. As well, processing can also reduce postharvest losses. Cold storage facilities may be available for 10–15% of the fruit crops in several countries (Singh and Pal, 2008). Several fruits from the cucumber family are ripened, after which they can be stored for months without specialized storage facilities. Countries in tropical and subtropical areas, fruits and vegetables characteristic to these climates are produced. As well, the production of many of such commodities is seasonal, and this reduces the necessity of long-term storage. Because of this, it is common to have peaks of availability for fruits such as apple, orange, pear, banana, mango, and guava spread throughout the year. Even then, postharvest loss of 50% or greater is common as the storage facilities at the local vendors are limited and good quality products are hard to find (Paliyath *et al.*, 2008).

The quality of a produce cannot be enhanced through adopting postharvest storage technologies (Timmer and Brown, 2000). The quality of a produce is determined by the growing conditions, nutritional regimes, and the genetic potential of the particular variety. Increased attention is being given to these attributes (Swinburne, 1983; Prusky and Plumbley, 1992). Several novel postharvest technologies developed have the potential to maintain the high quality of produce during subsequent storage at optimal conditions. These include active modified atmosphere and dynamic controlled atmosphere. In addition, there is growing concern about food safety, which is also being addressed in the postharvest area (Paliyath *et al.*, 2008).

Biotechnological approaches are useful for enhancing the shelf life and quality of fruits and vegetables, but the public acceptance of this technology is limited. Several information sites describing the optimal storage procedures have increased the importance and understanding of postharvest storage (Paliyath *et al.*, 2008). Over the last two decades, biological control of plant pathogens has emerged as a viable disease control strategy (Harman, 2000; Elad and Stewart, 2004). Numerous factors are responsible for increasing interest in biological control including the negative effects of fungicides on human health, increased regulatory restrictions, traceability protocols for crop protection practices, nil residue tolerance in some export markets, continued interest in organics, pathogen resistance to commonly used fungicides, and a lack of replacement products (Droby and Chalutz, 1994; Anjaiah, 2004; Sharma *et al.*, 2009).

2.1.3 Fruit and vegetable production in Ethiopia

Ethiopia has a comparative advantage in a number of horticultural commodities due to its favorable climate, proximity to European and Middle Eastern markets and cheap labor (Yohanese_Agonafir, 1991, 1992). However, the production of horticultural crops is much less developed than the production of food grains in the country. On average more than 2,399,566 tons of vegetables and fruits are produced. The total area under fruits and vegetables was about 12,576 hectares in 2011 (CSA, 2011). Of the total land area under cultivation in the country during 2011, the area under fruits and vegetables was less than 1 %, which is insignificant as compared to other food crops.

Both state owned and private farms are operating in the fruit and vegetable production, export and processing sub-sector in Ethiopia (Bekele_Wolde, 1991; Yohanese_Agonafir, 1992). The average yield of fruit and vegetable in the country is low as compared to other countries. One of the major reasons for low yield of these crops is poor management (Yohanese_Agonafir, 1991). At present, many private commercial farms and private farmers are producing fruits and vegetables both for domestic and export markets in the country.

Fresh and processed Fruits and vegetables have a large domestic market in Ethiopia, significantly higher than the exported volumes. There is a strong indication of the existence of large potential demand for fresh fruit and vegetable crops in the country (Fekadu_Tadesse, 1991). The other customer of Ethiopian fresh fruits and vegetables is processing plants, i.e., wineries, tomato processing plants and vegetable canning factories which require grapevine, tomato and various types of vegetables for processing. In Ethiopia, fruit processing is limited mainly to extraction of fresh juice which is sold on the local market. The Merti processing factory is the only plant producing fruit juice for the local market (Fekadu_Tadesse, 1991). A range of fruit juices are imported into the country. The demand for fruit juices on the local market is high as indicated by the volume of imports.

Ethiopia exports fresh fruits and vegetables to the international markets. The major markets for Ethiopian fresh fruits and vegetables are the European Union, the Arab countries and the regional markets. There is a reliable demand for these Ethiopian products during a particular period and a great volume is re-exported (Fekadu_Tadesse, 1991; Yohanese_Agonafir, 1992).

2.2 Anthracnose Disease on Fruits

Anthracnose disease caused by several *Colletotrichum* species is a significant economic constraint on various hosts. *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc and *Colletotrichum acutatum* J.H. Simmonds are two main species of *Colletotrichum* that cause anthracnose on various fruit in most fruit producing areas of the world (Sutton, 1992; Freeman *et al.*, 1998). The fungus is heterogeneous and especially in culture, the characteristics vary greatly (Sutton, 1992) (Sutton, 1992).

Colletotrichum acutatum J. H. Simmonds, is characterized by orange-brown lesions on petals of citrus flowers and results in the abscission of fruits (Sutton, 1992). The characteristics of *C. gloeosporioides* are described by its colonies in greyish white to dark grey, aerial mycelium even and felted or in tufts associated with conidiomata, reverse unevenly white to grey or darker especially with age; setae present or absent; scletoria absent but immature ascomata may be mistaken for sclerotia. Appressoria are clavate, ovate, obvate, sometimes lobed, sepia brown, 6-20 × 4-12µm in size. Coniadia are formed in pale salmon masses, straight, cylindrical, apex obtuse base truncate, 12-17 × 3.5-6µm in size (Sutton, 1992; Freeman *et al.*, 1998). Conidia are produced over wide range of environmental conditions but infection is more dependent on temperature and duration of surface wetness. The teleomorph, *G. cingulata*, has ascomata in black, thin-walled and without stromatic material (Fitzell and Peak, 1984).

Anthracnose caused by *C. gloeosporioides* is the general name implicated for the necrotic lesions caused by *Colletotrichum* spp. It damages pre- and postharvest, causing losses of many high value crops and commonly encountered in subtropical and tropical regions. Anthracnose attacks a very wide range of plants including cereals, grasses, legumes, fruits, and vegetables. Studies on anthracnose have been carried out on various tropical fruits, such as mango, banana, avocado, citrus, blueberry, papaya and guava (Prusky *et al.*, 1983; Fitzell and Peak, 1984; Xiao *et al.*, 2004; Amarjitsingh *et al.*, 2006; Rampersad, 2011; Thangamani *et al.*, 2011).

2.2.1 Symptoms

Blossom blight occurs as a result of the colonization of the blossom by the pathogen. The pathogen colonizes the tissue in favourable conditions causing twig die-back and extensive cankers of stems and trunks. The fungus stays dormant on the fruit until fruit begins to ripen (Forster and Adaskaveg, 1999; Adaskaveg and Forster, 2000; Govender *et al.*, 2005). At the peduncle and pedicel tissues, rot takes place and in severe cases covers the entire body of the fruit. Brown soft decomposition starts at the stem end of the mango and quickly spreads throughout the entire fruit body. A straw coloured fluid from the stem-end and steel-grey mycelium may cover the surface of the fruit (Korsten *et al.*, 1998).

In extreme cases, the decay manifests itself in a similar way only that it does not start at the peduncle and pedicel tissues but rather covers the whole body of the mango fruit. In the orchard, the fungus also passes on the disease to the fruit but remains dormant until fruit begins to ripen after harvest when it recommences activity and colonizes the fruit body giving rise to stem-end rot (Prusky and Plumbley, 1992). Affected fruits may split open as they collapse and straw-coloured fluid comes out of the splits in the side of the fruits. The pathogen easily spreads from affected to healthy neighboring fruit bringing about considerable and substantial losses due to decay at the end of the export chain (Ploetz, 1994). In some instances, *Colletotrichum gloeosporioides* are associated with stem-end rot (Freeman and Shabi, 1996; Freeman *et al.*, 1998).

2.2.2 Disease cycle

Colletotrichum stays alive throughout the winter season in the form of black pycnidia and perithecia in wart-like stroma on living, dead cankered limbs of trees or mummified fruits. Injuries and breaks in the cuticle of the fruits necessitates infection. Ascopores are freed by the perithecia during spring rains and the conidia produce within the pycnidia comes out. They carried by rain splash to other parts of the trees and the dispersion of the conidia may go on throughout the rainy season. Fruit becomes fairly infected early, but rotting does not develop until the fruit reaches its maturity. Symptoms of postharvest development that takes place in this way is termed as stem rot or soft brown rot (Fitzell and Peak, 1984). However, both symptoms have been found to be caused by the same fungal pathogens (Prusky and Plumbley, 1992). Blossoms blight symptoms can also occur on the inflorescence (Waller, 1992; Ploetz, 1994). The spectrum of pathogens that causes fruit rot disease is influenced and favoured by any condition that decreases tree vigour, drought, high temperatures, winter injury, nutrition level of host, sunscald and poor pruning practices as well as low or unbalanced nutrition (Bailey *et al.*, 1992; Prusky *et al.*, 2000).

2.2.3 Pathogenic variability of *Colletotrichum* species

Many inoculation studies have been conducted with *Colletotrichum* species, demonstrating that most isolates are relatively non-host-specific (Freeman *et al.*, 1998). Numerous cases have been reported in which several *Colletotrichum* species or biotypes are associated with a single host. For example, avocado and mango anthracnose caused by *C. acutatum* and *C. gloeosporioides*, affect fruit predominantly as postharvest diseases (Prusky, 1996). Strawberry may be infected by three *Colletotrichum* species, *C. fragariae*, *C. acutatum*, and *C. gloeosporioides*, causing anthracnose of fruit and other plant parts (Howard *et al.*, 1992). Citrus can be affected by four different *Colletotrichum* diseases postbloom fruit drop and key lime anthracnose, both caused by *C. acutatum*, and shoot dieback and leaf spot, and postharvest fruit decay, both caused by *C. gloeosporioides* (Howard *et al.*, 1992).

It is common to find that a single species of *Colletotrichum* infects multiple hosts. For example, *C. gloeosporioides*, which is considered a cumulative species and forms the sexual stage in some instances, is found on a wide variety of fruits, including almond, avocado, apple, and strawberry (Freeman *et al.*, 1998). Similarly, *C. acutatum* has been reported to infect a large number of fruit crops, including avocado, strawberry, almond, apple, and peach (Binyamini and Schiffmann-Nadel, 1972).

2.2.4 Characterization of *Colletotrichum* species by cultural methods

Characterization of *Colletotrichum* species has relied on a number of morphological and cultural criteria (including morphology, optimal growth temperature, vegetative compatibility, benomyl sensitivity and molecular methods (Freeman *et al.*, 1998; Adaskaveg and Forster, 2000).

Morphological and cultural methods: Primarily, identification and characterization of *Colletotrichum* species has been based on morphological characters, such as size and shape of conidia and appressoria; existence of setae; the teleomorph state and cultural characters such as colony color, growth rate and texture (Freeman *et al.*, 1998). Moreover, pathogencity, cross infection potential and benomyl sensitivity were successfully used to differentiate *Colletotrichum* species (Freeman *et al.*, 1998). Conidial shape provides a reliable means of discriminating certain species and has been used to elucidate the identity of *Colletotrichum* species pathogenic to fruits (Freeman *et al.*, 1998; Adaskaveg and Forster, 2000). .

Growth rate and optimal temperature have been useful for differentiating between subpopulations of *C. gloeosporioides* from avocado and almond crops (Shabi *et al.*, 1996). Numerous reports suggest that isolates of *C. acutatum* grew at a significantly slower rate than isolates of *C. gloeosporioides* (Bernstein *et al.*, 1995; Shabi *et al.*, 1996; Freeman *et al.*, 1998).

Colony shape and color of cultures may vary considerably within and between species. Several morphological types have been observed within a population of *C. gloeosporioides* from avocado grown in culture (Adaskaveg and Hartin, 1997; Freeman *et al.*, 1998): (i) conidial type, with masses of conidia and no aerial mycelium; (ii) flat mycelial type, with very little aerial mycelium or conidia; (iii) mycelial type, with dense aerial mycelium; (iv) conidial-mycelial type, with dense aerial mycelium and masses of conidia; (v) protoperithecial type, similar to type (iv) but also producing dark protoperithecia- like hyphal aggregates; and (vi) perithecial type, with simple or complex perithecia. The existence of the teleomorph *Glomerella* may sometimes help in differentiating between species (Shabi *et al.*, 1996; Adaskaveg and Hartin, 1997; Freeman *et al.*, 1998).

It was found that in some cases, fungicide sensitivity can also be used for species and sub specific grouping in *Colletotrichum gloeosporioides* isolates are considered highly sensitive to benomyl; whereas *C. acutatum* isolates are relatively insensitive (Freeman *et al.*, 1998). *Colletotrichum gloeosporioides* from avocado and almond differ in their sensitivity to benzimidazole fungicides: almond isolates are insensitive to benzimidazoles compared with complete growth inhibition of isolates from avocado at 5 µg/ml (Shabi *et al.*, 1996). This simple method can therefore be utilized for additional characterization of local populations of *Colletotrichum* associated with particular anthracnose diseases (Adaskaveg and Forster, 2000).

Cross-infection potential has been reported among different species of *Colletotrichum* and genotypes of *C. gloeosporioides* on a variety of tropical, subtropical, and temperate fruits under artificial inoculation conditions (Bernstein *et al.*, 1995). *Colletotrichum gloeosporioides* isolates from seven tropical fruit crops were also shown to cross-infect alternate detached leaves and fruits (Freeman *et al.*, 1998). Similarly, other crops, such as apple, mango, and strawberry, are known to be susceptible to *Colletotrichum* in Israel (Arauz, 2000).

Identification of *Colletotrichum* species using molecular methods: DNA sequence analyses have been used to characterize and analyze the taxonomic complexity of *Colletotrichum* (Du *et al.*, 2005). Cannon *et al.* (2000) stated that data derived from nucleic acid analyses should provide the most reliable framework to build a classification of *Colletotrichum*, as DNA characters were not directly influenced by environmental factors. 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. In particular, sequence analysis of the Internal Transcribed Spacer (ITS) regions, which lie between the 18S and 5.8S or 5.8S and 28S genes, have proved useful in studying phylogenetic relationships of species of *Colletotrichum* because of their comparative variability (Photita *et al.*, 2004). Apart from rDNA, sequence analysis of protein coding genes such as partial β-tubulin gene; have also been applied to resolve phylogenetic relationships among *C. acutatum* species complexes. (Guerber *et al.*, 2003). Du *et al.* (2005) confirmed that MAT1-2 mating type was useful in differentiating the groups of isolates from the species complexes (*C. graminicola*, *C. gloeosporioides* and *C. acutatum*). Ratanacherdchai *et al.* (2007) separate two species of *Colletotrichum* from chili anthracnose observed in Thailand that is *C. capsici* and *C. gloeosporioides* by RAPD makers. A combined application of molecular diagnostic tools along with traditional morphological characterization is an appropriate and reliable approach for studying *Colletotrichum* species complexes (Cannon *et al.*, 2000).

2.2.6 Management anthracnose disease

Effective control of *Colletotrichum* diseases usually involves the use of one or a combination of the following: (1) resistant cultivars, (2) cultural control, (3) chemical control, and (4) biological control using antagonistic organisms (Arauz, 2000). The applicability of control strategies depends as much on the characteristics of the crop on which they are being used as on the disease at which they are targeted (Koomen, 1990).

Resistant cultivars: Resistance to disease is perhaps the most significant aspect of disease control in agricultural crops. Cultivar resistance in fruit crops is complicated by the ability of most *Colletotrichum* fruit pathogens to form quiescent infections (Prusky *et al.*, 2000). Resistance in fruit to postharvest pathogens has been described as a "dynamic incompatibility" (Prusky, 1998; Prusky *et al.*, 2000). Postharvest differences in resistance among cultivars may be due as much to the conditions under which the fruit is stored as to the occurrence of defense compounds (Prusky *et al.*, 2000). Therefore it is probably more useful to define resistance and susceptibility of fruit cultivars to postharvest disease in terms of the incubation period after fruit ripening, with resistant cultivars having a longer storability and shelf life than susceptible ones (Smith *et al.*, 1996). Host plant resistance would seem to be a logical and efficient way to control anthracnose disease. However, aside from the costs associated with replacing an established crop with a resistant or more tolerant cultivar, most growers tend to select cultivars based on criteria other than disease resistance (Prusky *et al.*, 2000).

Cultural control: This usually refers to the range of methods used to control diseases, mostly using tactics aimed at disease avoidance through phytosanitation, manipulation of cropping patterns or by enhancing resistance and avoiding predisposition (Prusky *et al.*, 2000). However, in relation to fruit crops it also involves the use of proper sanitation techniques during processing of the harvested fruit, transportation, packaging and storage, to avoid exposure of fruit to the pathogen. It also involves proper handling to avoid abiotic factors such as mechanical injury, temperature extremes, and anoxia, which can predispose the fruit to infection by the pathogen. The ubiquitous nature of inoculum sources of *Colletotrichum* diseases and their often-rapid epidemic development under suitable conditions reduces the effectiveness of many pre-harvest general phytosanitary practices. However, general orchard hygiene has a place in integrated disease control, as removal of obvious inoculum sources such as diseased leaves and fruit can increase the efficiency of chemical control (Waller, 1992).

The prerequisite for wet conditions to coincide with susceptible crop stages for development of *Colletotrichum* epidemics also offers an opportunity for disease control through the manipulation of cropping patterns and or irrigation (Fitzell and Peak, 1984; Waller, 1992). This can be achieved to some extent by pruning techniques such as the fine pruning of old, fruited and dead twigs that remain in the bush from year to year.

Chemical control: *Colletotrichum* diseases can be controlled by a wide range of chemicals such as copper compounds, dithiocarbamates, benzimidazole and triazole compounds, and other fungicides such as chlorothalonil, imazalil and prochloraz (Waller, 1992). Newer classes of fungicides such as the strobilurins (e.g. azoxystrobin and pyraclostrobin) are also proving highly effective against *Colletotrichum* species that infect fruits (Prusky *et al.*, 2000). However, the problem of fungicide tolerance may arise quickly if a single compound is relied upon too heavily (Jeger and Plumbley, 1988).

The use and effectiveness of these fungicides may be limited by various factors (Adaskaveg and Forster, 2000; Timmer and Brown, 2000). For example, benomyl has been withdrawn by the manufacturer and only limited stocks remain. The use of fosetyl-Al tends to be costly, and chlorothalonil cannot be used after petal fall in fruits because of phytotoxicity to the fruit. In addition, Captan is currently considered a B-2 carcinogen and its use is restricted by some fruit processors. Ziram has a minimum of14-day pre-harvest interval, whereas the number or applications of azoxystrobin and pyraclostrobin allowed per season is limited as part of a fungicide resistance management protocol. This means that growers have to utilize their fungicide options wisely to attain effective control. Although treatment with fungicides can significantly reduce the incidence and severity of disease, eradication cannot normally be achieved (Adaskaveg and Forster, 2000).

Biological control: Biological control methods for *Colletotrichum* diseases have not received much attention even though the potential of biological control through the effect of phyllosphere antagonists has been realized for some time (Arauz, 2000). The possibilities for biological control of postharvest fruit diseases have been reviewed by Janisiewicz and Korsten

(2002), and the effects of surface microflora on the incidence of anthracnose diseases such as coffee berry disease, avocado anthracnose, and mango anthracnose, are now being clarified with a view to enhancing naturally occurring biocontrol mechanisms (Masaba and Waller, 1992; Janisiewicz and Korsten, 2002). Although most of the technology is still at the research stage, progress has resulted in a number of commercial products, including Aspire™, BioSave™, Trichodex™, AQ10™, and Avogreen™ (Wilson *et al.*, 1997; Janisiewicz, 1998; Korsten *et al.*, 1998). Most of these products have been developed for post harvest applications as this situation offers more advantages for biocontrol strategies (Korsten *et al.*, 1998). For example, environmental conditions during fruit transportation and storage are generally more uniform than in the field and can often be manipulated (Prusky *et al.*, 2000).

2.3 Gummy Stem Blight (GSB) of Cucurbits

Gummy stem blight (GSB) is a major disease of many cucurbits, including watermelon, cantaloupe, cucumber, pumpkin, squash, muskmelon, and other melons (Keinath, 1995; Keinath *et al.*, 1995; Kothera *et al.*, 2003). Infection on watermelon and cantaloupe is commonly seen through the world, and the disease can cause significant production losses when conditions are ideal for the spread of the causal pathogen. The disease is also known as black rot due to its characteristic appearance on infected fruits (Somai *et al.*, 2002a). On fruit, this disease is caused by *D. bryoniae* (anamorph *P. cucurbitacearum*), is known as black rot whereas the foliage disease is known as Gummy Stem Blight (Keinath, 1995; Keinath *et al.*, 1995; Sitterly and Keinath, 1996). Small, water-soaked spots develop on watermelon fruit, enlarge, and exude gummy material. As the symptoms develop, fruiting bodies of the fungus may appear as black specks on the lesions. In the tropics, fruit infection can occur through blossom scars and begin to decay inner fruit tissue, with no visible symptoms on the surface of the host. The decay eventually will progress to the stem end and reach the surface of the fruit (Sitterly and Keinath, 1996).

2.3.1 Epidemiology of GSB

GSB can affect all above-ground parts of the watermelon plant. In the seedling stage, symptoms of GSB are characterized by the appearance of water-soaked lesions on cotyledons. Fungal hyphae from these lesions later can invade the hypocotyl and crown resulting in total girdling of the seedling (Schenck, 1968). On a mature watermelon plant, symptoms include lesions on the leaves and petioles, crown blight, stem cankers and extensive defoliation. GSB also can cause lesions on fruits. But fruit infection is not commonly observed. *Didymella bryoniae* produces pycnidia, the asexual fruiting body, on infected leaves and stems. The conidia are hyaline, cylindrical with rounded ends, non- or monoseptate, and 6-13 µm long (Sitterly and Keinath, 1996; Keinath *et al.*, 2001). The fungus can overwinter on plant debris and produce pseudothecia, the sexual fruiting body. Ascospores produced in pseudothecia are 14-18 × 4-6 µm in size, hyaline, monosepatate with a constriction at the septum and have round ends. The upper cell is usually wider than the lower one (Keinath *et al.*, 1995; Keinath, 2002). GSB can occur both in the transplant production houses and in the fields.

It has been shown that the fungus can overwinter as dormant mycelia on host tissue as long as the debris is not decomposed and can produce both pycnidia and pseudothecia on the debris (Keinath and Duthie, 1998; Keinath, 2001, 2002). Wind-borne ascospores originating from the pseudothecia that form on watermelon debris on the soil may be responsible for the primary infection of the crop. The conidia produced on watermelon debris left in the watermelon field may also have a role as the primary source of infection (vanSteekelenburg, 1983). The fungus can also survive as dormant mycelia in the seed and this infested seed can act as a potential source for primary inoculum (Brown *et al.*, 1970; Lee, 1982). *Didymella bryoniae* has been isolated from testa, perisperm and cotyledons of cucumber, pumpkin and watermelon seeds (Lee, 1982). Seed transmission of *D. bryoniae* has been reported in case of cantaloupe and watermelon seeds. Presence of pathogen in the seed can easily go undetected, especially if the fungus is deep-seated, and can serve as an important source of inoculum (Rankin, 1954; Keinath, 1995).

Sources of inoculum for GSB in the transplant production houses could be seed-borne or ascospores arising from the crop debris left outside or inside the greenhouse (vanSteekelenburg, 1983). Warm, humid conditions inside the transplant production houses favor disease development and overhead irrigation in transplant production houses facilitates rapid spread of this disease (Keinath, 2002).

2.3.2 Identification and characterization of *Didymella* species

Didymella bryoniae can be differentiated from related *Phoma* species by carefully examining colony morphology and the shape of conidia, measuring colony growth rate and conidial dimensions, inducing production of pseudothecia, and conducting pathogenicity assays on seedlings (Keinath, 1995). However, some isolates of *D. byroniae* do not produce pycnidia or pseudothecia in culture (Keinath, 1995; Keinath *et al.*, 1995). Disease symptoms included stem necrosis with an exudation of gummy material, angular water-soaked lesions on the leaves and rotten fruits (Keinath *et al.*, 1995). The presence of black pycnidia on the stems leaves and fruits also help the identification. The fungus can be isolated from diseased stems, leaves and seeds, by plating surface-sterilized plant tissues onto potato dextrose agar (PDA) medium. On PDA, the mycelium is olivaceous green and few pycnidia are observed. White aerial mycelium was also produced. Conidia are hyaline, cylindrical with rounded ends, mostly nonseptate, but a few single-septate, and 6–11 × 3–5 µm in size. Based on the symptoms and the morphological characteristics, the fungus can be identified (Keinath, 1995; Keinath *et al.*, 1995; Keinath, 1996; Somai *et al.*, 2002a).

Random Amplified Polymorphic DNA (RAPD) fingerprinting (Keinath, 1995), and a Polymerase Chain Reaction (PCR) based diagnostic technique (Somai *et al.*, 1997; Keinath, 2001) have been used for more reliable identification of the GSB pathogen. Using RAPD profiles and PCR primers, two genotypes of *D. bryoniae* have been differentiated from each other and from nonpathogenic or weakly virulent *Phoma* spp. (Keinath, 1995; Keinath *et al.*, 1995).

PCR with specific primers has been the most accurate and reliable method currently available to identify pathogens. However, visualization of PCR products is usually performed by electrophoresis in agarose gels followed by staining with ethidium bromide or other fluorescent dyes. The drawback of electrophoretic techniques is that the procedure becomes labor-intensive and limits the number of samples that can be processed simultaneously. Polymerase Chain Reaction with Enzyme-Linked Immunosorbent Assay (PCR-ELISA) is a technique that has been used successfully to detect GSB pathogen (Keinath, 1996; Somai *et al.*, 2002a). This method combines the specificity of the PCR reaction with the increased sensitivity of the ELISA reaction. The entire procedure does not require any sophisticated equipment other than a thermal cycler and an ELISA reader, which already may be available in many laboratories (Somai *et al.*, 2002b).

2.3.3 Management strategies for GSB

Management of GSB requires an integration of both cultural practices and chemical methods in both the transplant production house and the field. Sources for genetic resistance against GSB have been identified but no resistant variety has been released yet (Gusmini *et al.*, 2005; Keinath, 2009). Another method to manage GSB is by reducing the primary sources of inoculum. Deep-turning of infected debris from the previous season will promote the rapid breakdown of debris and thereby the amount of primary inoculum (Keinath and Duthie, 1998). Crop rotation was found to be effective in reducing disease severity in the subsequent season (Keinath, 1996). Incorporation of cabbage residue followed by soil solarization can also be effective in reducing the development of disease in the following season (Keinath *et al.*, 2001). Seed treatment with 1600 μg/ml of peroxyacetic acid for 30 min was found to be effective in preventing seed transmission of GSB to watermelon seedlings (Sitterly and Keinath, 1996). Proper ventilation in the greenhouse will reduce the leaf wetness duration and will help reduce disease development to some extent. Management of irrigation to avoid splash dispersal of the pathogen and periods of prolonged leaf wetness can also help to reduce disease severity (Levi *et al.*, 2001; Keinath, 2009).

Cultural practices can reduce the incidence and severity of GSB only to some extent (Levi *et al.*, 2001). The most effective option for the management of GSB is the application of protectant and curative fungicides. Protectant fungicides have a multisite mode of action and are associated with low risk for resistance development. The most commonly used protectant fungicides are Bravo (chlorothalonil), belonging to chloronitrile group and Dithane (mancozeb), belonging to ethylenebisdithiocarbamate (EBDC) group (Keinath, 2009). Among these two fungicides, chlorothalonil has been found to be more effective against GSB owing to its better retention capacity on the foliage (Keinath, 1995, 1996; Keinath *et al.*, 2001) but this fungicide can cause phytotoxicity to mature watermelon rinds, if applied late in the season. Because of the potential for the explosive spread of GSB under conducive environmental conditions, use of systemic fungicides is usually necessary to manage the disease in the field (Sitterly and Keinath, 1996; Keinath, 2001).

Didymella bryoniae has shown a remarkable ability to adapt and become resistant to most of the effective fungicides developed to control it. Azoxystrobin provided excellent control of gummy stem blight and was granted a section 18 Emergency Exemption in the 1998 growing season in Georgia to control GSB (Keinath and Duthie, 1998; Keinath, 2009). However, *D. bryoniae* isolates that were insensitive to azoxystrobin were found in Georgia, Delaware and Maryland within 2 years of its first commercial use (Stevenson *et al.*, 2008). After development of resistance to azoxystrobin, a new fungicide, Pristine, a formulated mixture of pyraclostrobin and boscalid, showed good efficacy against GSB in the field (Keinath, 2002).

2.4 Eco-Friendly Management of Plant Diseases with Natural Compounds

Increases in food production in the past 50 years have resulted from increasing the intensity of production on agricultural land, with increased use of machinery, fertilisers and pesticides. Indeed, the use of pesticides in agriculture has increased hugely, amounting to some 2.56 billion kg/yr (Pretty, 2008). However, the inefficient use of these inputs has resulted in considerable damage to the environment, with increased agricultural area contributing greatly to the loss of habitats and biodiversity (Walters, 2009). Concerns about sustainability revolve around the need to develop agricultural technologies and practices that: (a) have no adverse effects on the environment, (b) are effective and can be easily accessed by farmers, and (c) lead to increased food productivity, while yielding positive effects on environmental goods and services (Pretty, 2008). The key principles for sustainability are to integrate biological and ecological processes into food production processes and minimise use of non-renewable inputs that harm the environment (Pretty, 2008). A number of different terms have been used to imply greater sustainability in some agricultural systems than others, including ecoagriculture, ecological agriculture, and low input agriculture (Pretty, 2008; Walters, 2009). Many of these approaches involve minimising or even eliminating the use of pesticides in favour of biologically based approaches to crop protection (Pretty, 2008; Walters, 2009).

2.4.1 Botanical products in agricultural disease management

Attention has been paid towards the exploitation of higher plant products as novel chemotherapeutics in plant protection. Because most of them are not phytotoxic, are easily biodegradable and sometimes stimulatory to the host metabolism, plant products possess a high potential for pest management (Dubey *et al.*, 2009). Higher plants contain a wide spectrum of secondary metabolites such as phenolics, flavonoids, quinones, tannins, essential oils, alkaloids, saponins and sterols. Such plant-derived chemicals may be exploited for their different biological properties (Dubey *et al.*, 2009). Many of these are thought to defend the plants producing them against pathogens (Dubey *et al.*, 2009). Therefore, higher plants can be exploited for the discovery of new pesticides or novel structures that could serve as lead compounds in pesticide development. Such structures produced by plants are thought to have novel modes-of-action as natural pesticides (Regnault-Roger *et al.*, 2005). Natural pesticides were partly displaced by synthetic pesticides that at the time seemed easier to handle and longer lasting in 1940s. Because of greater consumer awareness and concerns regarding synthetic chemicals, food preservation against pests with natural additives is becoming more popular (Dubey *et al.*, 2009). Such plant products have also been formulated for large scale application in crop protection (Dubey *et al.*, 2009).

Among the different plant products, essential oils are a very attractive method for crop protection (Dubey *et al.*, 2009). Production of essential oils by plants is believed to be predominantly a defense mechanism against pathogens and pests (Dubey *et al.*, 2009). Essential oils and their components are gaining increasing interest because of their relatively safe use and potential for multi-purpose functional use. Essential oils are mostly a mixture of many different volatile compounds, their composition often varies between species and exert differential effects depending on both the mode of action and the target pest (Liu *et al.*, 2006). They have been tested against a wide range of pests such as food spoiling organisms, food poisoning organisms and mycotoxigenic filamentous fungi and pathogenic (Ghannoum, 1988). Essential oils of many edible and medicinal plants are also used as food preservatives and in different pharmaceutical preparations (Holley and Patel, 2005). They are in many cases endowed with antimicrobial, allelopathic, antioxidant and bioregulatory properties (Holley and Patel, 2005). The volatility, ephemeral nature and biodegradability of flavour compounds of angiosperm may be especially advantageous for treatment of food commodities because only low residues can be expected (Dubey *et al.*, 2009).

Dubey *et al.* (1983) demonstrated the efficacy of essential oils of *Ocimum canum* and *Citrus medica* as volatile fungitoxicants for the protection of some spices against post harvest fungal deterioration. Essential oils of *Cymbopogon citratus*, *Caesulia axillaris* and *Mentha arvensis* have shown in vivo fumigant activity for the control of storage fungi of some cereals (Mishra *et al.*, 1992; Shakarami *et al.*, 2003). Numerous studies have documented the antifungal effects of plant essential oils (Fiori *et al.*, 2000; Adebayo *et al.*, 2013).

Major constituents from aromatic plants, mainly monoterpenes, are of special interest to industrial markets because of their insecticidal, anti-bacterial, anti-fungal and anti-inflammatory activities (Shakarami *et al.*, 2003; Dubey *et al.*, 2009). Perhaps the most attractive aspect of using essential oils and/or their constituents for pest management is their favourable mammalian toxicity (Dubey *et al.*, 2009). Mycotech Corporation produces Cinnamite^{TM}, as an aphidicide/ miticide/ fungicide for glasshouse and horticultural crops, and Valero^{TM}, as a miticide/fungicide for use in grapes, berry crops, citrus and nuts. Both products are based on cinnamon oil, with cinnamaldehyde (30% EC formulation) as the active ingredient (Dubey *et al.*, 2009). In general essential oils have received increased attention due to a growing interest in the need for alternative techniques to assure quality and safety of perishable food (Dubey *et al.*, 2009).

2.4.2 Biological control agents in plant disease control

Over the last 50 years, disease control has relied heavily on the use of chemical fungicides, bacteriocides and soil fumigants (Whipps and McQuilken, 2009). However, there are many problems associated with their continued deployment including increasing pressure to reduce chemical use in the environment in general, development of pesticide resistance in many pathogens, and decreasing availability of active ingredients through stricter registration and difficulty in finding novel active compounds (Rosado and Seldin, 1993). Consequently, the search for alternative non-chemical methods of disease control continues to gain significance. For organic growers where chemical control measures are not permitted, these considerations have been paramount for many years (Whipps and McQuilken, 2009).

Alternative, biologically based disease control measures have been used for many years (Jeger and Jeffries, 1988; Chan and Tian, 2005). These include plant breeding for resistance, crop rotations, tillage systems and fertilizer practices that affect pathogens directly or alter microbial populations to inhibit pathogens, exploitation of disease suppressive soils and growing media, as well as environmental controls, particularly in the glasshouse. However, the greatest interest has been in the development of biological control agents (BCAs) used as microbial inoculants, mimicking the use of chemical pesticides (Bakker *et al.*, 2007; Whipps and McQuilken, 2009).

The greatest advances in biological disease control have been concerned with understanding modes of action (Sharma *et al.*, 2009). This reflects the huge developments in molecular biology of bacteria, fungi and plants providing the tools to dissect the many types of interactions that can occur, particularly through the use of mutants and genetically marked strains of microorganism as well as gene expression studies (Janisiewicz and Korsten, 2002; Sharma *et al.*, 2009). General modes of action include competition, antibiosis, parasitism, induced resistance and plant-growth promotion along with highly specialized mechanisms such as that associated with hypo-virulence. Commonly, biological disease control by a single BCA can involve a number of modes of action and no one mode of action is necessarily mutually exclusive to another (Janisiewicz and Korsten, 2002; Whipps and McQuilken, 2009).

Competition for space and nutrients: One of the classic demonstrations of competition for space, infection sites or nutrients as a mode of action concerns the control of fire blight caused by the pathogenic bacterium, *Erwinia amylovora* by the nonpathogenic bacterium *Pseudomonas fluorescens* A506 (Lindow and Leveau, 2002). By spraying flowers of apple and pear with *P. fluorescens* A506 just as they open, the BCA colonises the flowers, utilizes the available nutrients, and prevents multiplication of small numbers of *Erwiniya amylovora* that might then encounter the flowers, thereby preventing infection by preemptive exclusion. Preemptive colonisation of necrotic leaf tissues by the fungus *Ulocladium atrum* to control the fungal pathogen, *Botrytis cinerea*, is another case of this type of mode of action which may involve both competition for infection sites and nutrients, resulting in reduced pathogen sporulation (Whipps and McQuilken, 2009).

Production of antibiotics: Production of antibiotics and inhibitory metabolites by microorganisms has been well established as a mode of action (Arras, 1996; Sharma *et al.*, 2009). Microorganisms commonly produce such metabolites during the course of their growth and only if production at the site of biocontrol is confirmed, or activity implied by use of either non-producing or over-producing mutants, or reporter strains. Compounds such as amphisin, 2,4-diacetylphloroglucinol, hydrogen cyanide, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, tropolone and cyclic lipopolysaccharides produced by *Pseudomonas* spp. (Whipps and McQuilken, 2009) and gramicidin S, oligomycin A, kanosamine, iturin, zwittermycin A and xanthobaccin produced by *Bacillus*, *Streptomyces* and *Stenotrophomonas* spp. have been identified to have a role in disease biocontrol (Romero *et al.*, 2007; Whipps and McQuilken, 2009). The regulation of many of these bacterial antibiotics has been explored and involvement of regulatory genes, and sigma factors, and key signal molecules has been found. Production is also influenced by nutrient availability, plant type and age, environmental conditions, microorganisms present including other BCAs and the pathogen itself, which all involve complex signaling pathways and so the spectrum of antibiotic production by any of the strains of BCA may differ depending on the situation under consideration.

Parasitism and production of extracellular lytic enzymes: Parasitism and associated production of extracellular lytic enzymes has been thoroughly explored as a mode of action in biocontrol (Markovich and Kononova, 2003). This is a relatively simple phenomenon for bacteria where degradation of target cell walls is generally considered to reflect parasitism, and may range from simple attachment of bacterial cells to hyphae with minimal degradation, through biofilm formation to complete lysis and cell wall breakdown. Lists of extracellular enzymes produced by bacterial BCAs have been produced. These include a number of chitinases, proteases and β-1,3-glucanases (Palumbo *et al.*, 2005; Whipps and McQuilken, 2009). With fungal BCAs the process of parasitism of fungal plant pathogens, or mycoparasitism, is more complex than that for bacteria and a series of interlinked phases of hyphal–hyphal interactions have been recorded especially for *Trichoderma* spp. including: sensing, directed growth, contact and binding, sometimes involving production of appressoria, coiling or alignment of hyphae of the mycoparasite around the host, penetration and then degradation (Lu *et al.*, 2004).

2.4.3 Application of biologically based disease control in crop protection practice

Effective disease control requires a multifaceted approach, using a number of different methods. Control of certain crop diseases requires biologically based methods to be integrated into disease control programs, along with other approaches. For other diseases, those for which no adequate control exists, biologically based methods might offer the only hope of reducing disease to acceptable levels (Kudsk, 2007; Walters, 2009). Sustainable approaches to agriculture, including many biologically based methods of disease control, might be particularly appropriate for fragile and low yielding farming systems located.

In many developing countries, integrated management practices are used to control important pathogens and pests (Phiri *et al.*, 2007). Bean Common Mosaic Virus (BCMV) and Bean Common Mosaic Necrotic Virus (BCMNV) are controlled using virus-free seed, intercropping with non-host crops, and use of resistant varieties, while loose or head smut, caused by *Sphacelotheca reiliana*, is managed through rotation, deep plowing and destruction of plant debris, and use of resistant varieties (Phiri *et al.*, 2007; Walters, 2009). The approach for ‘ecological’ control involves: (a) improving cultivar resistance, (b) changing cultural practices, (c) eradicating volunteer seedlings, (d) regulating planting date and (e) returning land to forestry and pastures (Chen *et al.*, 2007). Irrespective of the system into which biologically based disease control methods are slotted, their use in crop protection programs will first require a number of issues to be resolved and barriers to be overcome (Walters, 2009).

CHAPTER 3 CHARACTERIZATION OF SPECIES RESPONSIBLE FOR ANTHRACNOSE DISEASES ON MAJOR TROPICAL FRUITS

3.1. Introduction

The genus *Colletotrichum* comprises some of the most economically important fungi causing disease on several crops (Bailey *et al.*, 1992; Buddie *et al.*, 1999). *Colletotrichum* species cause anthracnose diseases on various temperate, subtropical and tropical fruits worldwide and are a particularly severe problem in tropical areas (Bailey *et al.*, 1992). Although many cultivated fruit crops are infected by *Colletotrichum* species, the most significant economic losses occur when fruits are affected after harvest (Freeman *et al.*, 1998).

At least two species have been reported as causal agents of fruit anthracnose, *Colletotrichum acutatum*, and *C. gloeosporioides* (teleomorph *Glomerella cingulate*) (Freeman *et al.*, 1998). All these species can be found on all parts of the plant; however fruit rot is the most economically damaging. The late onset of fruit rot can cause serious and sudden losses both pre- and postharvest (Freeman *et al.*, 1998). Conidia germinate on the fruit surface and produce appressoria and quiescent infections which develop only after the fruit begins to ripen (Prusky and Lichter, 2007). The typical disease symptom known as anthracnose is characterized by sunken necrotic tissue where orange conidial masses are produced (Bailey *et al.*, 1992; Bailey, 1997).

Colletotrichum gloeosporioides has been associated with quiescent infections and postharvest diseases on several fruits such as avocado, mango, papaya, guava, passion fruit, citrus, apple and grapes (Simmonds, 1965; Freeman *et al.*, 1998). *Colletotrichum acutatum* has been reported on several subtropical, tropical and temperate fruit hosts including apple, grapes, peach, almond and banana (Bernstein *et al.*, 1995; Adaskaveg and Hartin, 1997).

Numerous cases have been reported in which several *Colletotrichum* species or biotypes are associated with a single host. For example, strawberry may be infected by *C. acutatum, C.*

fragariae and *C. gloeosporioides* (Smith and Black, 1990) and citrus can be affected by *C. gloeosporioides* and *C. acutatum* causing three different diseases (Brown *et al.*, 1996). Several papers have described differences between *C. gloeosporioides* and *C. acutatum* infecting tropical fruits (Bernstein *et al.*, 1995; Freeman and Shabi, 1996; Smith *et al.*, 1996; Adaskaveg and Hartin, 1997).

Accurate pathogen identification and definition of the species is of utmost importance for the successful implementation of any disease management program (Freeman *et al.*, 1998). This is because of the existence of *Colletotrichum* species pathogenic to different fruits (*C. acutatum*, *C. fragariae* and *C. gloeosporioides*) that exhibit differing cultural and physiological characteristics (Gunnell and Gubler, 1992). Some of these species cause similar disease symptoms on other hosts, including crown rot, fruit rot and stolon lesions which further exemplifies the importance for identification of the causal agent of disease (Howard *et al.*, 1992).

Characterization and identification of *Colletotrichum* species are primarily based on shape and size of morphological characters such as conidia and appressoria, and differences in host range and pathogenicity (Freeman and Shabi, 1996; Kuramae-Izioka *et al.*, 1997; Schiller *et al.*, 2006; Sharma-Abishek and Verma, 2007). To this end, identification of *Colletotrichum* species based on cultural, morphological features, fungicide sensitivity and cross infection experiments were successfully used to establish the identity of the causal organism on banana, mango, almond and avocado (Reddy, 2000; Sharma-Abishek and Verma, 2007; Thangamani *et al.*, 2011). Several molecular methods were developed and widely implemented for more accurate characterization of *Colletotrichum* species (Buddie *et al.*, 1999; Freeman *et al.*, 2000; Latunde-dada, 2001).

In Ethiopia the existence of *Colletotrichum* species have been reported among isolates from haricot bean (Bekele_Tesfaye, 1997). Diversity of *Colletotrichum* is also well reported from sorghum anthracnose of Ethiopia. Alemayehu_Chala *et al.* (2011) reported the presence of geographically isolated and diverse populations of *Colletotrichum sublineolum* in Ethiopia, which are not affected by gene flow. They also suggested that *C. sublineolum* might be a species complex made up of several cryptic species. A molecular analysis carried out by Alemayehu_Chala (2013) to study the genetic diversity of *C. sublineolum* isolates collected from a sorghum field in southern Ethiopia, revealed the presence of genetic variation among the tested isolates (Alemayehu_Chala, 2013).

However, studies on the isolation and identification of *Colletotrichum* species areresponsible for anthracnose disease of fruits and vegetables in different parts of Ethiopia has was limited, except for citrus (Sysay_Bekele, 2006). A clear picture about the patho-types of postharvest fruit anthracnose and efficiency of different methods to detect the different species are also not well known.

This work is aimed to establish which species are currently causing anthracnose disease in fruit crops in Ethiopia and to identify *Colletotrichum* species from banana, mango and papaya fruit, from Ethiopia based on their pathogenicity, morphological, cultural, environmental and molecular characterstics.

3.2. Materials and Methods

3.2.1 Sample collection and isolation of pathogen

Forty-five banana, mango and papaya fruits showing typical symptoms of anthracnose were randomly collected from mango, banana and papaya farms in eastern, western and southern Ethiopia in 2010/11 (Table 3.1). The fruits collected from a farm were considered as one sample and labeled. During the survey, anthracnose samples were also collected from five different market places of major towns. The collected *Colletotrichum* isolates were named using the combination of the host and the sample order. Samples were separately put in paper bags and transported to the Mycology laboratory of Addis Ababa University for isolation, identification and characterization of the isolates associated with the diseased fruits.

Using a sharp blade, diseased tissues were cut and put on a glass slide having a drop of water and covered with a cover slip and examined under the binocular research microscope (10X) to confirm the presence of fungal spores. The infected tissue of fruits which showed typical symptoms were cut into small bits measuring about 2 mm and surface sterilized in 0.1% sodium hypochlorite solution for 5 min and washed twice in sterile distilled water (Freeman *et al.*, 2000). Then surface sterilized tissues were transferred to sterile petriplates containing Potato Dextrose Agar (PDA) medium (potato 250 g, dextrose 20 g and agar 20 g) under aseptic conditions. The inoculated petriplates were incubated at room temperature (26°C) for 10 – 14 d.

Table 3. 1 Source of *Colletotrichum* isolates used in this study

Gr [a]	No	Reg [b]	Location [c]	Host [d]	Code [e]	Spp [f]
1	1	Or	36.43 / 7.41	M	Ma-Pa-01	G
1	2	So	38.17 / 6.20	M	Ma-Pa-09	G
1	3	Or	40.73 / 9.08	M	Ma-Pa-11	G
1	4	Or	38.71 / 7.95	M	Ma-Pa-13	G
2	5	Or	35.53 / 8.31	M	Ma-Pa-02	G
2	6	Ga	34.25 / 8.12	M	Ma-Pa-05	G
2	7	Or	34.35 / 8.97	M	Ma-Pa-06	G
2	8	Or	35.51 / 9.09	M	Ma-Pa-08	G
2	9	Or	38.73 / 7.90	M	Ma-Pa-12	G
2	10	Or	41.72 / 9.63	M	Ma-Pa-14	G
3	11	Ga	34.49 / 8.11	M	Ma-Pa-03	G
3	12	Ga	34.27 / 8.13	M	Ma-Pa-04	G
4	13	Or	34.74 / 8.49	M	Ma-Pa-07	G
4	14	So	38.58 / 7.15	M	Ma-Pa-10	G
4	15	Be	34.55 / 10.01	M	Ma-Pa-15	G
5	16	So	37.26 / 7.56	B	Ba-Pa-01	G
5	17	Or	36.45 / 7.44	B	Ba-Pa-03	G
5	18	Ga	34.35 / 8.10	B	Ba-Pa-06	G
5	19	Or	35.01 / 8.44	B	Ba-Pa-09	G
6	20	Or	36.30 / 9.09	B	Ba-Pa-10	A
6	21	So	38.23 / 6.29	B	Ba-Pa-11	A
6	22	Af	39.57 / 8.51	B	Ba-Pa-12	A
7	23	Or	37.35 / 7.46	B	Ba-Pa-02	A
7		So	35.24 / 7.08	B	Ba-Pa-04	A
7		Or	35.06 / 8.15	B	Ba-Pa-05	A
8		Ga	34.24 / 8.15	B	Ba-Pa-07	A
8		Or	34.36 / 8.36	B	Ba-Pa-08	A
8		Af	39.88 / 8.76	B	Ba-Pa-13	A
8		Be	34.55/ 10.01	B	Ba-Pa-16	A
9		Or	41.38 / 9.58	B	Ba-Pa-14	A
9		Dr	41.86 / 9.59	B	Ba-Pa-15	A
10		So	37.54 / 8.25	P	Pa-Pa-01	G
10		So	36.12 / 7.16	P	Pa-Pa-02	G
10		Ga	35.31 / 8.08	P	Pa-Pa-03	G
10		Ga	34.33 / 7.51	P	Pa-Pa-04	G
10		Or	36.39 / 9.01	P	Pa-Pa-07	G
10		So	38.58 / 7.15	P	Pa-Pa-10	G
10		Af	39.78 / 8.67	P	Pa-Pa-12	G
11		Ga	34.24 / 8.15	P	Pa-Pa-05	A
11		So	38.36 / 7.03	P	Pa-Pa-09	A
11		Or	38.71 / 7.95	P	Pa-Pa-14	A
12		Or	34.36 / 8.36	P	Pa-Pa-06	G
12		So	38.23 / 6.29	P	Pa-Pa-08	G
12		Af	39.72 / 8.62	P	Pa-Pa-11	G
12		Or	38.73 / 7.90	P	Pa-Pa-13	G

[a] Morphology group of *Colletotrichum* species in which the isolates were categorized based on colony morphology similarity. Isolates in same group were morphologicaly similar

[b] Geographic regions in Ethiopia from where samples were collected: Or (Oromia), So (SNNP), Ga (Gambella), Af (Afar), Be (Benshangule)

[c] Shows the specific location [longitude (°E) / latitude (°N)] of sampling sites

[d] Host crops from which isolates were recovered: M= Mango B=Banana P= Papaya

[e] Code given to the isolates based on their original host and sequence of sampling

[f] Species of *Colletotrichum* of species: G (*C. gloeosporioides*) and A (*C. acutatum*)

3.2.2 Preservation of cultures

Pure cultures of each isolates were prepared from 12 d old cultures of the pathogen on PDA. Conidial masses were touched with a sterilized wire loop and streaked onto the surface of water agar (WA; 15 g/l agars) and then incubated overnight at 26°C. A single conidium was picked out of the WA under a microscope with a sterilized needle and transferred to a new PDA plate. The isolated single conidium was incubated at 26 °C for 7 d under alternative light at 12 hr interval (Freeman *et al.*, 2000). Monoconidial cultures were maintained on PDA slants at 4°C for short-term storage. Conidia were kept in glycerol for long-term storage. Further experiments were carried out from these pure cultures.

3.2.3. Morphological characterization and identification of isolates

A 4-mm^3 agar block taken from the advancing edge of a 5 d old actively growing culture was transferred to PDA and incubated at 25°C with a 12:12 alternating dark and light photoperiod as described by Sutton (1992). Cultures were examined visually and under light in order to identify suitable characters with variations in character status, for the phenotypic analysis of all isolates. This was repeated two times in order to overcome any deviations.

Colony characteristics: The increase in colony diameter was assessed by measuring them every day using a ruler (Thangamani *et al.*, 2011). Colony color was described using the degree of pigmentation of the colonies. The appearance of colonies, occurrence of sectors, colony margion, elevation, the vegetative and reproductive structures and several conidial and aspersoria characters were described after 14 d of incubation by multi scale category adopted from Munaut et al. (2001) with little modification (Appendix Table 1).

Conidial characters: Suspensions of conidia of each isolate were prepared and the concentration was adjusted to 1×10^7 conidia/ml using a haemocytometer. The conidia were examined under light microscope and the length and width of 100 conidia per isolate were measured using an eye piece graticule at x100 magnification. In addition, the shape of conidia and presence or absence of visible conidial masses was also recorded (Johnston and Jones, 1997).

Appressoria characters: Suspensions of conidia (1 × 10^5 conidia/ml) were prepared and 10 µl of the suspensions were placed on sterile glass slides and the slides were incubated in a moisture chamber for 24-48 hr. Growth of appressoria was monitored and at the end of the incubation period a drop of lactophenol cotton blue was added to arrest further development of the germinating conidia and stain the fungal structures. Finally the appressoria were examined and the length, width and shape of 50 appressoria per slide were measured using an eye piece graticule at 100X magnification (Johnston and Jones, 1997).

The isolates were identified based on morphological characterization and pathogensity according to Sutton (1992). Finally, *Colletotrichum* isolates were grouped based on their morphological resemblance in to distinct similarity groups, irrespective of the origin of host and species. From each group, a representative highly pathogenic isolate was selected for further characterization based on growth characteristics on different media, growth phase, fungicide sensitivity and cross infection potential.

3.2.4 Pathogenicity studies

Pathogenicity tests were conducted to determine the ability of the isolates to produce symptoms on fruits of their original host under controlled conditions. Conidia were harvested by flushing the surface of a 14 d old culture grown on PDA with 10 ml of sterile distilled water. The surface of the plate was scraped with a sterile scalpel blade, and the resulting suspension was filtered through two layers of cotton cloth. The concentration of conidia was adjusted to a final working concentration of 2 × 10^4 conidia/ml using a haemocytometer (Agostini *et al.*, 1992).

Fully matured, green, unripe mango, banana and papaya fruits were collected from a local retailer and washed thoroughly under running tap water. The fruits were blot dried and surface sterilized with 1% sodium hypochlorite for 5 min. The fruits were air dried and the spore suspension of the pathogen was sprayed over the fruits. The fruits inoculated with sterile distilled water served as control. The inoculated fruits surfaces were covered with moist cotton and kept inside the moist chamber. Three replications, each containing five fruits per replication, were inoculated and maintained for each fungal isolate. Type of lesion development and time for lesion development on fruits (latent period) were recorded after 20 d of inoculation. The inoculated isolates re isolated from the artificially inoculated fruits showing typical anthracnose symptoms and were characterized for the same morphology and colony features.

Disease severity on fruits were recorded using a five point rating scale which was described by Lakshmi *et al.* (2011) based on the percentage of fruit area affected by the disease: 0 = No infection, 1= Up to 5 % infection, 2 = 6 - 10 % infection, 3 = 11 - 20 % infection, 4 = 21 - 50 % infection and 5 = more than 50 % infection. Based on the numerical ratings given above a 'Percent Disease iIdex (PDI)' for fruit rot was calculated using the formula described by Lakshmi *et al.* (2011):

$$\text{Percent Disease Index (PDI)} = \frac{\text{Sum of numerical ratings} \times 100}{\text{No. of units (fruits) examined} \times \text{maximum grade}}$$

The numerical values of Percent Disease Index and latent period were used to calculate the Virulence Index (VI) using the following formula (Lakshmi *et al.*, 2011).

$$\text{Virulence Index (VI)} = \text{PDI} \times \text{Latent period}$$

3.2.5 Cross infection studies

For this experiment twelve representative isolates (four mango, five banana and three papaya isolates) from each group were inoculated in fruits of banana, mango and papaya following the cross schematic patern. Conidia of the isolates were cultured and prepared as described before and the concentration was adjusted to a final working concentration of 2×10^4 conidia/ml using a haemocytometer. Non-infected fruits of mango, banana and papaya, obtained from a local retailer were surface-sterilized with 1% sodium hypochlorite for 5 min and washed twice with distilled water (Lakshmi *et al.*, 2011).

Surface sterilized fruits were spray inoculated with pathogen spore suspensions (2×10^4 conidia/ ml) as before and fruits sprayed with sterile distilled water served as controls. Each set of fruits was incubated separately in moist chambers and maintained at 26°C.

Three replications each containing five fruits per replication were inoculated and maintained for each fungal isolate. Observations on type of lesion development and number of days taken for lesion development on fruits were recorded after inoculation. Finally, Percent Disease Index, Latent Period and Virulence Index were assessed as described for pathogencity test in section 3.2.4.

3.2.6 Fungicide sensitivity assays

Molten PDA was amended with benomyl (active ingredient [a.i.] 50% Benlate) to achieve seven different final concentrations of: 0.1, 1, 5, 10, 100., 500 and 1000 µg/ml. Benomyl was prepared in distilled water as a 0.2 g/ml stock solution according to Kuo (2001). Various volumes of this stock were then added to molten PDA to obtain the required concentrations of benomyl. The amended agar was swirled vigorously to ensure that the contents were thoroughly mixed. A 4-mm^3 agar block was removed from the advancing edge of an actively growing colony and placed in the center of the amended PDA plate. Plates were incubated at 25°C for 14 d, and the radial diameter of each colony was measured (mm). Seven *C. gloeosporioides* and five *C. acutatum* isolates, selected from previous experiments, were tested in three replications, and the experiment was conducted twice. Mean values of experiments were presented as percent of radial growth inhibition of the isolates to the control plates (Rampersad, 2011).

3.2.7 Effect of environmental factors on pathogen growth

The pathogen was subjected to a range of culture media, temperature, pH levels and light conditions in order to study suitable conditions for growth and sporulation of the *Colletotrichum* isolates. Twelve *Colletotrichum* isolates (four mango, five banana and three papaya isolates) were evaluated.

Different solid media were used for assessing the growth of isolates of *Colletotrichum*. The medias were prepared in the laboratory by mixing the composition of each media as described by Ainsworth (1961). The media were Potato Dextrose Agar (PDA) medium (potato 250 g, dextrose 20 g and agar 20 g), Martin's Rose Bengal agar medium (magnesium sulphate 0.2 g, dipotassium hydrogen phosphate 0.9 g, ammonium nitrate1.0 g, potassium chloride 0.15 g, glucose 3.0 g, pentachloro nitrobenzene 0.2 g, rose bengal 0.20 g, chlorothallonil 0.20 g and agar 20 g), Waksman's Agar medium (glucose 10 g, peptone 5 g, potassium dihydrogen phosphate 1 g, magnesium sulphate 0.5 g and agar 26 g), Malt Extract Agar (glucose 20.0 g, malt extract 20.0 g, peptone 1.0 g and agar 20.0 g), Sabouraud's Agar (dextrose 20 g, peptone 10 g, agar 20 g), Czapek dox Agar medium (sodium nitrate 2 g, potassium nitrate 1 g, magnesium sulphate 0.5 g, potassium chloride 0.5 g, ferrous sulphate 3 g, sucrose 30 g and agar 20 g), Water agar (agar 20 g), Richards agar (sucrose 50 g, potassium nitrate 10 g, magnesium sulphate 2.5 g, ferric chloride 10 ml and agar 20 g), Oat meal agar (oat meal 40 g and agar 20 g) and Starch Agar (soluble starch 10.0 g, beef extract 3.0 g and agar 12.0 g).

Eight mm disc of pathogen was placed on the center of the Petri plates and plates were incubated 26°C for ten days. Three media replications were used for each test (Thangamani *et al.*, 2011).

The effect of pH on the growth of the pathogen was studied as per the method followed by Kuberan *et al.* (2012) using PDA medium. The medium was adjusted to pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 using 0.1 N Hydrochloric acid and 0.1 N Sodium hydroxide. The media with different pH levels were sterilized, cooled and poured (20 ml) in the sterilized petriplates and allowed to solidify. Eight mm disc of pathogen was placed on the center of the Petri plates. The plates were incubated at 26°C for ten days (Thangamani ***et al.***, 2011).

The effect of temperature on growth of the isolates was studied on PDA at temperatures of 10, 15, 20, 25, 28, 30 and 35°C. Mycelia disc of 8 mm was used to inoculate Petri plates and incubated for ten days.

The effect of light on the growth and sporulation of isolates was studied by inuculating isolates (8 mm) on PDA medium. Cultures were then exposed to four different light conditions (24 h dark light, fluorescent light, UV light and day light). The plates were incubated 26°C for ten days (Thangamani ***et al.***, 2011).

The mycelia growth was recorded on the tenth day after inoculation for each experiment. Sporulation was graded according to the number of conidia: - = no sporulation, + = slight sporulation (51-100 conidia), ++ = moderate sporulation (101-150 conidia) and +++ = abundant sporulation (>150 conidia) per microscopic field as described by Deshmukh ***et al.*** (2012). All experiments were laid out in factorial arrangement in a Completely Randomized Design (CRD) with three replicates.

3.2.8 Effect of carbon and nitrogen on growth of isolates

The best growth of isolates were found on PDA and Richards agar medium in the previous test. Therefore, Richards agar (sucrose 50 g, potassium nitrate 10 g, magnesium sulphate 2.5 g, ferric chloride 10 ml and agar 20 g) was used as basal medium to study carbon and nitrogen utilization by isolates. The utilization of carbon and nitrogen nutrition was studied by replacing the sucrose and potassium nitrate in the basal medium with various nitrogen and carbon compounds as carbon and nitrogen sources.

The carbon sources; sucrose, lactose, starch, glucose, mannose, xylose, fructose and galactose were prepared by mixing 21.053 g carbon /litre medium. The different carbon sources were dissolved separately in the medium. 20 mL of sterilized medium was poured into sterilized petriplates. Plates without carbon source served as control. Each treatment was replicated three times. Each treatment was replicated three times. These petriplates were inoculated aseptically with 8 mm diameter culture block of mycelium obtained from the 10 d old actively growing pure culture with the help of sterilized cork borer and incubated at 26°C (Deshmukh *et al.*, 2012).

The ability of isolates to utilize nitrogen sources were studied on same medium by replacing potassium nitrate from the basal medium with various inorganic and organic sources of nitrogen (ammonium oxalate, ammonium phosphate, ammonium sulphate, calcium nitrate, peptone and urea). Nitrogen sources were added singly to furnish 1.38 g nitrogen/ litre of basal medium. Richards' agar medium without nitrogen source served as control. Each treatment was replicated three times. These petriplates were inoculated aseptically with 8 mm diameter culture block of mycelium obtained from the 10 d old actively growing pure culture as described before and incubated at 26°C (Deshmukh *et al.*, 2012).

The mycelia growth was recorded on the tenth day after inoculation for each experiment. Sporulation was graded according to the number of conidia: - = no sporulation, + = slight sporulation (51-100 conidia), ++ = moderate sporulation (101-150 conidia) and +++ = abundant sporulation (>150 conidia) per microscopic field as described by Deshmukh *et al.* (2012). All experiments were laid out in factorial arrangement in a Completely Randomized Design (CRD) with three replicates.

3.2.9 Sequencing and molecular identification of *Colletotrichum* isolates

Molecular identification through sequencing of the ITS region was conducted to confirm the identity of isolates. The representative isolates of *C. acutatum* and *C. gloeosporioides* were chosen for sequencing based on their conserved ITS regions. *Colletotrichum* isolates were cultivated in 150 ml of PDA at 25°C, with mild agitations twice per day for six days. The DNA from the isolates was extracted from 250 mg of mycelia using NORGEN kit. The extraction was conducted twice and kept at -4°C. The DNA was visually quantified in a 0.8% agar gel stained in an ethidium bromide solution at 0.05 mg/l.

The pure DNA was spotted on FTA card and sent to University of Florida for sequencing. The 5.8S-ITS region was amplified by PCR using the universal primers ITS-1 and ITS-4 (White *et al.*, 1990). PCR products were cleaned with the Gene-Clean II Purification Kit (Bio 101, La Jolla, CA, USA) and directly sequenced in an Applied Biosystems automatic DNA sequencer at the University of Florida. The specificity of each ITS region sequence was tested against all DNA sequences available in the EMBL database with the Blast program (available online at http://www.ebi.ac.uk/blast2/index.html).

The sequences were analyzed using Lasergene® 2001, Version 5 software (DNASTAR Inc., USA) (DNASTAR, 2001), and aligned with the GenBank database of the National Center for Biotechnology Information (NCBI, USA). A phylogenetic tree was obtained using the Neighbor–Joining method, Kimura–2–parameter, with 5000 bootstrap replicates with the MEGA 3.1 program (Kumar *et al.*, 2004). GenBank sequence of *Colletotrichum gloeosporioides* isolates (HQ414623.1, XM007284925.1, AF264028.1, XM007282560.1, DQ084518.1, XM007276420.1 and DQ084502.1) and *Colletotrichum acutatum* (AJ301964.1, AJ301950.1, AF275542.1, U79700.1 and U79695.1) isolates were used to compare the sequence.

3.2.10 Statistical analysis

Range, mean, standard deviation of means was determined for all measurements regarding morphological and cultural characterstics. Percent data and scale units were subjected to Arc sin and square root transformation respectively before analysis. The data from the repeated experimental runs were pooled and means were estimated through analysis of variance using SPSS software V20. A comparison among treatment mean and means of appropriate control treatments was made with Duncan's Multiple Range Test (DMRT), ($P \leq 0.05$ and $P \leq 0.001$ level).

3.3. Result

A total of 45 isolates of *Colletotrichum* were identified based on morphological attributes and pathogenecity (Table 3.2, 3.3, 3.4 and 3.5). All isolates from mango were tentatively identified as *C. gloeosporioides* based on morphological characterstics. From banana fruits, 25% of the isolates were tentatively identified as *C. gloeosporioides* and 75% as *C. acutatum*. On the papaya isolates tested, 78.6 % of the isolates obtained were identified as *C. gloeosporioides* and 21.4 % as *C. acutatum*. The study did not identify any isolates of *Glomerella cingulata*, the teleomorphic stage of *C. gloeosporioides*.

Accordingly, isolates of *C. gloeosporioides* were grouped into seven distinct morphological types, and *C. acutatum* showed five morpho-types based on colony attributes (Table 3.2). *Colletotrichum* isolates showed variation in colony color, sporulation, margin mycela patern and reproductive structure as shown on Table 3.2.

Table 3. 2 Morphological characters of *Colletotrichum* isolates from Ethiopia

Group[a]	Host[b]	%[c]	Colony appearance	Growth rate[d]	Reproductive structure[e] (x/y)[f]					Representative Isolate[g]	Tentative taxonomic position
					i	ii	iii	iv	v		
1	Mango	8.89	Cottony, white to smoke-gray, with small black or peach-colored dots of sporulation	8.50	4/2	4/0	4/2	4/3	4/0	Ma-Pa-01	*C. gloeosporioides*
2	Mango	13.32	Olive-colored colony with dark gray-colored conidial mass in center	8.52	6/1	6/3	6/1	6/0	6/0	Ma-Pa-05	*C. gloeosporioides*
3	Mango	4.44	Cream to light salmon colored colony with no visible conidial mass in center	8.85	2/1	2/1	2/1	2/0	2/0	Ma-Pa-03	*C. gloeosporioides*
4	Mango	6.67	Cream to pink-colored colony with grayish-colored conidial mass in center	8.71	3/2	3/2	3/3	3/0	3/0	Ma-Pa-07	*C. gloeosporioides*
5	Banana	8.89	White floccules colony, no visible conidial mass in center	8.35	4/0	4/0	4/2	4/0	4/0	Ba-Pa-01	*C. gloeosporioides*
6	Banana	6.67	Circular, raised, at first orange-white, sometimes grey and becoming pale orange	2.45	3/0	3/0	3/0	3/0	3/0	Ba-Pa-10	*C. acutatum*
7	Banana	6.67	White mycelium turning gray and powdery with pink spore masses, salmon, produced outward in circles from the center of the culture	2.25	3/0	3/0	3/0	3/0	3/0	Ba-Pa-02	*C. acutatum*
8	Banana	8.89	Greyish white mycelium, no raised growth, center of the colony has salmon pink colored spore masses	2.51	4/0	4/0	4/2	4/0	4/0	Ba-Pa-07	*C. acutatum*
9	Banana	4.44	Dark grayish white mycelium with a flat growth, concentric growth pattern of the mycelium	2.41	2/0	2/0	2/2	2/0	2/0	Ba-Pa-14	*C. acutatum*
10	Papaya	15.6	Salmon pink colored mycelium, scattered spore masses of salmon pink all over the colony, scattered black spots	7.95	7/1	7/0	7/1	7/0	7/0	Pa-Pa-01	*C. gloeosporioides*
11	Papaya	6.67	Greyish white mycelium, cottony texture, middle is black, salmon pink spore masses in the middle	2.32	3/0	3/0	3/0	3/0	3/0	Pa-Pa-05	*C. acutatum*
12	Papaya	8.89	White mycelium becoming gray and aerial with pink spore masses, salmon in color at the center of the colony	7.61	4/2	4/1	4/2	4/1	4/0	Pa-Pa-06	*C. gloeosporioides*

[a] Characters were described and grouped after 14 d of incubation by multi scale category adopted from Munaut et al. (2001)

[b] Original host of isolates recovered from: M= mango, B= banana and P= papaya

[c] Percent of isolates belonging to a group as compared to the total isolates

[d] Growth rate was calculated as cross diameter of growth/ day (mm) for 12 d. The average growth rate for each group displayed. Means of three replications

[e] Reproductive/ vegetative structures: i (Setae), ii (Acervuli), iii (conidiomata), iv (Peritecia) and v (Sclerotia)

[f] (x/y): y; The number of isolates for which a characteristics was recorded for a minimum of two replication plates in each group; x; The number of isolates, out of the y isolates, for which the colonies describe with this characteristics presented the same state

[g] A representative isolate from each group was selected for further study based on aggressiveness among isolates in each group after pathogenicity study

Isolates belonged to morpho-type 10 (8.89 %) showed greyish white mycelium, did not have a raised growth and centre of the colony showed salmon pink colored spore masses. Morpho-type 11 constituted 4.44 % of the isolates and were characterized by dark grayish white mycelium with a flat growth with concentric growth pattern. Morpho-type 12 represented 6.67 % of the isolates and had grayish white mycelium, cottony texture, middle of the colony is black in color, salmon pink spore masses were black in color, salmon pink spore mases in the middle region.

An important comparative character was the growth rate of the colonies in culture. There was no significant difference in growth rate among isolates of the same species, i.e. among isolates of *C. acutatum* belonging to morpho-type 6, 7, 8, 9 and 11 ($P \leq 0.05$) or among isolates of *C. gloeosporioides* belonging to morpho-type 1, 2, 3, 4, 5, 10 and 12 ($P \leq 0.05$) (Table 3.2). Isolates of *C. gloeosporioides* grew faster (7.65 – 8.85 mm/ day) than isolates of *C. acutatum* (2.25 – 2.51 mm/ day).

Colletotrichum isolates varied with respect to conidial shape and size (Table 3.3). There were two types of conidia, (cylindrical and fusiform), observed in the two species of *Colletotrichum* (Table 3.3). Isolates of *C. acutatum* produced predominantly fusiform conidia, tapering at one end. *Colletotrichum gloeosporioides* isolates produced cylindrical conidia with obtuse ends. As showen in Table 3.3, there was a variation in conidia size of *Colletotrichum* isolates. The length and width of the isolates from *C. gloeosporioides* from 12.00 - 17.72 by 3.40 - 4.50 μm respectively and that the conidia length and width of *C. acutatum* isolates were 8.60 to 9.39 by 3.00 - 3.37 μm respectively. *Colletotrichum gloeosporioides* conidia longer than *C. acutatum*

Table 3. 3 Conidial characteristics of different *Colletotrichum* isolates

			Conidial morphology [a]				
Isolate	**Species**	**Host**	χ^{b}	**Length (µm)**	χ^{b}	**Width (µm)**	**Shape**
Ma-Pa-01	*C. gloeosporioides*	Mango	13.2b	(9.9 - 18.1)	3.7b	(3.3 - 4.9)	Cylindrical, obtuse ends
Ma-Pa-05	*C. gloeosporioides*	Mango	16.4bc	(11.5 - 23.1)	3.5b	(3.2 - 4.9)	Cylindrical, obtuse ends
Ma-Pa-03	*C. gloeosporioides*	Mango	15.9bc	(13.2 - 19.8)	3.4b	(3.3 - 4.9)	Cylindrical, obtuse ends
Ma-Pa-07	*C. gloeosporioides*	Mango	15.8bc	(13.2 - 18.1)	4.0b	(3.3 - 6.6)	Cylindrical, obtuse ends
Ba-Pa-01	*C. gloeosporioides*	Banana	12.0b	(11.5 - 16.5)	4.5b	(3.4 - 4.9)	Cylindrical, obtuse ends
Ba-Pa-10	*C. acutatum*	Banana	8.7 a	(5.3 - 16.9)	3.2a	(2.1 - 4.2)	Fusiform, tapering one end
Ba-Pa-02	*C. acutatum*	Banana	9.0 a	(4.2 - 14.8)	3.0a	(2.2 - 4.2)	Fusiform, tapering one end
Ba-Pa-07	*C. acutatum*	Banana	8.6 a	(5.3 - 16.9)	3.2a	(3.2 - 4.2)	Fusiform, tapering one end
Ba-Pa-14	*C. acutatum*	Banana	9.2 a	(5.3 - 21.2)	3.2a	(3.1 - 4.2)	Fusiform, tapering one end
Pa-Pa-01	*C. gloeosporioides*	Papaya	14.5	(12.7 - 17.4)	3.9b	(2.4 - 4.8)	Cylindrical, obtuse ends
Pa-Pa-05	*C. acutatum*	Papaya	9.4 a	(6.4 - 14.8)	3.4a	(2.1 - 4.2)	Fusiform, tapering one end
Pa-Pa-06	*C. gloeosporioides*	Papaya	17.7c	(12.7 - 19.0)	3.7b	(3.2 - 4.7)	Cylindrical, obtuse ends

[a] Means of three replications (100 conidia per replication). Ranges are in brackets (minimum – maximum)

[b] Means with same letters were not significantly ($P \leq 0.05$) different within each column according to DMRT

There were few differences in appersoria shape and size between groups of *Colletotrichum* isolates (Table 3. 4). Most of the isolates formed appersoria in slide cultures. *Colletotrichum gloeosporioides* isolates from groups 1, 10 and 12 showed irregular to ovate appersoria whereas group 2, 3 and 4 showed ovate appersoria. Irregular appersoria were observed in *C. gloeosporioides* group 5. All the *C. acutatum* isolates showed clavate appersoria (Table 3.4). The length and width of appersoria of *C. gloeosporioides* isolates ranged from 8.6 - 12.18 by 6.63 - 8.38 µm, respectively and that of *C. acutatum* isolates appersoria length and width was 7.13 - 9.18 by 5.44 - 6.71 µm, respectively.

Table 3. 4 Characteristics of *Colletotrichum* Appressorial

			Appersoria morphology [a]				
Isolate	**Species**	**Host**	χ [b]	**Length (µm)**	χ [b]	**Width (µm)**	**Shape**
Ma-Pa-01	*C. gloeosporioides*	Mango	9.0b	(8.0 - 12.0)	6.6b	(6.0 - 8.0)	Irregular and Ovate
Ma-Pa-05	*C. gloeosporioides*	Mango	10.1b	(9.2 - 11.9)	7.7bc	(6.4 - 8.3)	Ovate
Ma-Pa-03	*C. gloeosporioides*	Mango	10.9b	(10.0 - 13.0)	8.4c	(7.0 - 9.0)	Ovate
Ma-Pa-07	*C. gloeosporioides*	Mango	10.1b	(8.0 - 12.0)	7.3bc	(7.0 - 8.0)	Ovate
Ba-Pa-01	*C. gloeosporioides*	Banana	10.3b	(8.0 - 12.0)	7.2bc	(7.0 - 8.0)	Irregular
Ba-Pa-10	*C. acutatum*	Banana	8.0a	(6.3 - 9.5)	5.7a	(5.5 - 6.3)	Clavate
Ba-Pa-02	*C. acutatum*	Banana	8.4a	(7.9 - 9.5)	5.4a	(4.7 - 6.4)	Clavate
Ba-Pa-07	*C. acutatum*	Banana	7.2a	(6.3 - 9.5)	6.1a	(5.5 - 6.3)	Clavate
Ba-Pa-14	*C. acutatum*	Banana	7.1a	(6.3 - 9.5)	6.1a	(5.5 - 6.4)	Clavate
Pa-Pa-01	*C. gloeosporioides*	Papaya	8.6ab	(7.9 - 10.3)	6.6b	(5.5 - 7.3)	Irregular and Ovate
Pa-Pa-05	*C. acutatum*	Papaya	8.4a	(7.9 - 9.5)	5.4a	(4.8 - 6.3)	Clavate
Pa-Pa-06	*C. gloeosporioides*	Papaya	12.2	(9.5 - 14.3)	7.8b	(6.3 - 8.7)	Irregular and Ovate

[a] Means of three replications (100 conidia per replication). Ranges are in brackets (minimum – maximum)
[b] Means with same letters were not significantly ($P \leq 0.05$) different within each column according to DMRT

Detached mango, banana and papaya fruits were spray inoculated with conidia from 15 mango,16 banana and 14 papaya isolates. Artificially inoculated banana, mango and papaya fruit showed typical decay symptoms with their original pathogen isolates. A significant difference in Percent Disease Index, Virulence Index and Incubation Period caused by mango, banana and papaya isolates as shown on Table 3.5.

The isolates of banana (Ba-Pa-10), mango (Ma-Pa-03) and papaya (Pa-Pa-01) were more virulent on fruits of banana (74.7, PDI), mango (69.5, PDI) and papaya (66.0, PDI) with an incubation period of 7.0, 6.3 and 4.0 days, respectively than the other isolates (Table 3.5). The mean maximum Virulence Index of 3.9, 5.1 and 2.9 was recorded on banana, mango and papaya fruits and least virulence of 2.0, 2.1 and 1.2 was recorded on banana, mango and papaya fruits which were different with each other in their respective group (Table 3.5). Koch's postulates were fulfilled with the reisolation of the pathogens from symptomatic fruit. No colony was grown on negative control fruits. Colonies that were originated from fruit tissue cultures were differenciated as *C. gloeosporioides* and *C. acutatum*.

Table 3. 5 Pathogenicity of *Colletotrichum* isolates on their original host

Mango	**Pathogenicity on Mango**							**Banana**	**Pathogenicity on Banana**							**Papaya**	**Pathogenicity on Papaya**						
Isolates	**PDI [a]**			**DI [b]**		**VI [c]**		**Isolates**	**PDI**			**DI**		**VI**		**Isolates**	**PDI 1**			**DI**		**VI**	
Ma-Pa-01	61.5	(51.6)[d]	i[e]	5.0	b	3.1	c	Ba-Pa-01	56.3	(48.6)	k	6.0	g	3.4	g	Pa-Pa-01	66.0	(54.4)	i	4.0	c	2.6	fg
Ma-Pa-02	56.3	(48.6)	g	7.0	e	3.9	f	Ba-Pa-02	58.3	(49.8)	m	4.0	b	2.3	cd	Pa-Pa-02	54.2	(47.4)	ef	4.7	e	2.5	f
Ma-Pa-03	69.5	(56.5)	j	6.3	c	4.0	f	Ba-Pa-03	53.3	(46.9)	i	4.7	c	2.5	d	Pa-Pa-03	56.3	(48.6)	fg	4.3	d	2.5	ef
Ma-Pa-04	52.1	(46.2)	d	8.3	g	4.3	h	Ba-Pa-04	50.8	(45.5)	g	5.0	d	2.5	de	Pa-Pa-04	33.3	(35.3)	c	4.0	c	1.3	b
Ma-Pa-05	62.5	(52.2)	i	6.3	c	4.0	f	Ba-Pa-05	51.9	(46.1)	h	5.3	e	2.8	e	Pa-Pa-05	60.4	(51.0)	h	4.3	d	2.6	fg
Ma-Pa-06	54.2	(47.4)	ef	7.3	f	3.9	f	Ba-Pa-06	39.6	(38.9)	e	5.3	e	2.1	b	Pa-Pa-06	62.5	(52.2)	h	4.0	c	2.5	f
Ma-Pa-07	62.5	(52.2)	i	6.7	d	4.2	g	Ba-Pa-07	57.3	(49.2)	l	5.3	e	3.1	f	Pa-Pa-07	29.2	(32.7)	b	4.0	c	1.2	b
Ma-Pa-08	59.4	(50.4)	h	7.3	f	4.3	h	Ba-Pa-08	35.4	(36.5)	c	5.7	f	2.1	b	Pa-Pa-08	57.3	(49.2)	g	4.0	c	2.3	def
Ma-Pa-09	52.5	(46.4)	de	7.0	e	3.7	e	Ba-Pa-09	37.5	(37.8)	d	6.7	i	2.5	d	Pa-Pa-09	56.3	(48.6)	fg	4.7	e	2.6	fg
Ma-Pa-10	30.4	(33.8)	b	6.7	d	2.1	b	Ba-Pa-10	74.2	(59.5)	n	7.0	j	3.9	h	Pa-Pa-10	50.0	(45.0)	d	4.0	c	2.0	cd
Ma-Pa-11	57.3	(49.2)	g	9.0	h	5.2	i	Ba-Pa-11	31.3	(33.9)	b	6.3	h	2.0	b	Pa-Pa-11	56.3	(48.6)	fg	3.7	b	2.1	cd
Ma-Pa-12	52.1	(46.2)	d	7.0	e	3.7	e	Ba-Pa-12	47.9	(43.8)	f	6.3	h	3.0	f	Pa-Pa-12	54.2	(47.4)	ef	5.3	f	2.9	g
Ma-Pa-13	57.5	(49.3)	g	6.7	d	3.8	f	Ba-Pa-13	50.0	(45.0)	g	8.0	k	3.9	h	Pa-Pa-13	52.8	(46.6)	e	4.0	c	2.1	de
Ma-Pa-14	43.8	(41.4)	c	7.3	f	3.2	d	Ba-Pa-14	56.5	(48.7)	kl	5.3	e	3.0	f	Pa-Pa-14	50.0	(45.0)	d	3.7	b	1.7	c
Ma-Pa-15	55.6	(48.2)	fg	6.7	d	3.7	e	Ba-Pa-15	54.9	(47.8)	j	7.0	j	3.8	h	Control	0.0	(0.0)	a	0.0	a	0.0	a
Control	0.0	(0.0)	a	0.0	a	0.0	a	Ba-Pa-16	38.9	(38.6)	e	5.7	f	2.2	bc	Mean	49.2	(43.5)		3.9		2.1	
Mean	51.7	(44.9)		6.5		3.6		Control	0.0	(0.0)	a	0.0	a	0.0	a								
								Mean	46.7	(42.2)		5.5		2.7									

[a] PDI=Percent disease index, Sum of numerical ratings X100) / (No. of units (fruits) examined X maximum grade)

[b] DAI= Incubation period: latent period (days) required for *Colletotrichum* isolates to show symptom

[c] VI= Virulence Index The numerical values of Percent disease index and latent period were used to calculate the VI using the following formula VI = Percent disease reaction (PDI) X Latent period.

[d] Figures in parentheses are arc sin transformed values.

[e] Means with same letters were not significantly ($P \leq 0.05$) different within each column according to DMRT

Cross infection potential of isolates from *C. gloeosporioides* and *C. acutatum* on banana, mango and papaya were observed irrespective of origion of host (Table 3.6). The results revealed that isolate and fruit crops had effect on percent disease index, virulence index and incubation period. The data showed that, the highest PDI was recorded from isolates Ba-Pa--10 (65.6%), Ma-Pa-07 (64.6%) and Pa-Pa-01(78.50%), respectively. The PDI ranged from 22.9 - 65.6 on mango, 14.4 - 64.6% on banana and 22.7 - 78.5% on papaya (Table 3.6). Isolate Ma-Pa-01, Ba-Pa-02 and Pa-Pa-06 on mango Ba-Pa-07 on banana and Pa-Pa-02 on papaya was most aggressive isolates resulting in highest virulence index of 3.9, 5.3 and 3.9, respectively (Table 3.6). As shown on Table 3.6, the least incubation days required to cause disease symptom was recorded from isolate Ma-Pa-07 on mango (2.3 d), isolate Ba-Pa-10 on banana (1.3 d) and isolate Pa-Pa-05 on papaya (2.7 d).

Colony growth inhibition by the fungicide varied significantly among the different *Colletotrichum* isolates ($P \leq 0.05$). Table 3.7 shows the percentage colony growth of the 12 different *Colletotrichum* isolates, when subjected to a concentration of 0.1, 1, 5, 10, 100, 500 or 1,000 µg a.i./ml of benomyl, 12 d after incubation at 26°C. All mango isolates (Ma-Pa-01, Ma-Pa-05, Ma-Pa-03 and Ma-Pa-07) were completely inhibited by benomyl at a concentration starting from 100 µg /ml.

On the other hand isolates of *C. gloeosporioides* from banana (Ba-Pa-01) and papaya (Pa-Pa-01 and Pa-Pa-06) were completely inhibited at 10 and 5 µg /ml concentration respectively. Isolates of *C. acutatum* from banana (Ba-Pa-10, Ba-Pa-02, Ba-Pa-07 and Ba-Pa-14) were completely inhibited at maximum concentration (1000 µg /ml). *Colletotrichum acutatum* isolates from papaya were relatively susceptible at relatively higher concentration (500 µg /ml) tested (Table 3.7). In general, isolates Ba-Pa-10, Ba-Pa-02, Ba-Pa-07 and Ba-Pa-14 were relatively resistant while isolates Pa-Pa-01 and Pa-Pa-06 were relatively susceptible to benomyl.

Table 3. 6 Cross infection potential of *Colletotrichum* isolates on alternate fruit crops

		PDI [a]					**DI** [b]					**VI** [c]				
Isolate	**Host**	**Ban**	**Man**	**Pap**	**Mean**		**Ban**	**Man**	**Pap**	**Mean** [h]		**Ban**	**Man**	**Pap**	**Mean**	
Ma-Pa-01	Mango	51.9 (46.1) [d]	56.3 (48.6)	53.1 (46.8)	53.8 (47.2)	g [e]	5.3	9.3	5.3	6.67	e	2.7	5.3	2.9	3.6	g
Ma-Pa-05	Mango	57.3 (49.2)	51.9 (46.1)	28.1 (32.0)	45.8 (42.4)	d	5.3	5.3	6.3	5.67	c	3.2	2.8	1.8	2.5	bc
Ma-Pa-03	Mango	56.5 (48.7)	52.1 (46.2)	37.7 (37.9)	48.8 (44.3)	e	5.3	9.3	6.3	7.0	g	3.0	4.9	2.4	3.4	f
Ma-Pa-07	Mango	64.6 (53.5)	53.8 (47.2)	50 (45.0)	56.1 (48.6)	h	4.0	6.3	4.0	4.78	b	2.6	3.4	2.0	2.7	c
Ba-Pa-01	Banana	56.3 (48.6)	51.5 (45.8)	22.7 (28.5)	43.5 (41.0)	c	9.3	7.3	7.0	7.89	j	3.3	3.8	1.6	3.5	fg
Ba-Pa-10	Banana	38.2 (38.2)	65.6 (54.1)	54.2 (47.4)	52.7 (46.6)	f	9.0	7.0	5.3	7.11	h	3.4	4.6	2.9	3.6	g
Ba-Pa-02	Banana	22.6 (28.4)	58.3 (49.8)	50 (45.0)	43.7 (41.1)	c	9.0	9.0	9.3	9.11	k	2.0	5.3	4.7	3.9	h
Ba-Pa-07	Banana	38.3 (38.3)	57.3 (49.2)	51.9 (46.1)	49.2 (44.5)	e	10	5.3	5.3	6.89	f	3.9	3.1	2.8	3.2	e
Ba-Pa-14	Banana	36.8 (37.3)	56.9 (49.0)	35 (36.3)	42.9 (40.9)	c	10.3	4.0	6.7	7.0	g	3.8	2.3	2.3	2.8	d
Pa-Pa-01	Papaya	22.1 (28.0)	56.5 (48.7)	78.5 (62.4)	52.3 (46.4)	f	8.7	5.3	3.3	5.78	d	1.9	3.0	2.6	2.5	b
Pa-Pa-05	Papaya	40.3 (39.4)	22.9 (28.6)	58.3 (49.8)	40.5 (39.3)	b	9.0	9.0	2.7	6.89	f	3.6	2.1	1.6	2.4	b
Pa-Pa-06	Papaya	14.4 (22.3)	56.3 (48.6)	66.3 (54.5)	45.6 (41.8)	d	9.0	9.3	4.7	7.67	i	1.3	5.3	2.9	3.2	e
Control		0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	a	0.0	0.0	0.0	0.0	a	0.0	0.0	0.0	0.0	a
Mean		38.4 (36.8) a	49.2 (43.2) c	45.1 (40.9) b			7.26 c	6.7 b	5.10 a			2.82 b	3.51 c	2.34 a		

[a] PDI=Percent disease index

[b] DAI= Incubation period:

[c] VI= Virulence Index

[d] Figures in parentheses are arc sin transformed values.

[e] Means with same letters were not significantly (P≤0.05) different within each column according to DMRT

Table 3. 7 Mycelial growth of *Colletotrichum* isolates in benomyl amended agar assays

			Benomyl/a.i. µg/ml								
No	**Isolate**	**Host**	**0.1**	**1**	**5**	**10**	**100**	**500**	**1000**	**Mean [a]**	
1	Ma-Pa-01	Mango	81.8	52.1	24.5	6.1	-	-	-	23.5	h
2	Ma-Pa-05	Mango	82.8	51.1	22.5	4.1	-	-	-	22.9	h
3	Ma-Pa-03	Mango	75.7	40.9	18.4	3.1	-	-	-	19.7	g
4	Ma-Pa-07	Mango	73.6	39.9	17.4	4.1	-	-	-	19.3	g
5	Ba-Pa-01	Banana	66.4	37.8	15.3	-	-	-	-	17.1	f
6	Ba-Pa-10	Banana	32.7	31.7	28.6	24.5	18.4	9.2	-	20.7	g
7	Ba-Pa-02	Banana	28.6	27.6	22.5	20.4	12.3	6.1	-	16.8	f
8	Ba-Pa-07	Banana	25.6	23.5	21.5	18.4	11.2	5.1	-	15.0	de
9	Ba-Pa-14	Banana	23.5	21.5	19.4	16.4	9.2	5.1	-	13.6	cd
10	Pa-Pa-01	Papaya	72.6	35.8	-	-	-	-	-	15.5	ef
11	Pa-Pa-05	Papaya	20.4	19.4	15.3	12.3	7.2	-	-	10.7	b
12	Pa-Pa-06	Papaya	62.4	30.7	-	-	-	-	-	13.3	c
13	Control		-	-	-	-	-	-	-	-	a
	Mean [a]		49.7 g	31.7 f	15.8 e	8.4 d	4.5 c	2.0 b	-		

[a] In a column, means same letters are not significantly ($P \leq 0.05$) different according to DMRT

[a] - = No growth

The growth characters of *Colletotrichum* species were studied on ten different solid media. The results showed that all 10 media supported the mycelial growth of all the isolates of *Colletotrichum*. Potato Dextrose Agar showed higher mean values (50.6 mm) of mycelial growth as compared to other media followed by Richards agar medium (46.1 mm). Water agar media exhibited the least mycelial diameter (23.5 mm). Maximum (61.1 mm) growth was noticed with Ma-Pa-01 isolate in over all media while the least growth was recorded in Ba-Pa-14 (11.5 mm) (Table 3.8). Isolate Ba-Pa-14 showed comparatively slow growth (11.5 mm) in all the media. As far as the effects of isolate-media interaction, maximum (78.7 mm) growth was recorded with isolate Ma-Pa-01 in PDA media where as minimum growth of 21.1 mm were from isolates Pa-Pa-05 and Ba-Pa-14 (Table 3.8). Abundent sporulation was found on PDA and Richards agar medium and no sporulation was observed on water agar medium.

Table 3. 8 Effect of different solid media on the growth of *Colletotrichum* isolates

	Media										
Isolate	Potato Dextros	Waksman's Agar	Rose Bengal	Malt Extract	Starch Agar	Oat Meal	Water agar	Richard agar	Sabraud Agar	Czapek dox	Mean [a]	
	Mean mycelial growth (mm)											
Ma-Pa-01	78.7	49.9	53.8	72	48	69.1	43.2	75.8	57.6	62.4	61.1	k
Ma-Pa-05	76.8	46.1	52.8	68.2	42.2	65.3	39.4	72.0	57.6	61.4	58.2	j
Ma-Pa-03	73.0	48.0	49.9	66.2	45.1	64.3	38.4	70.1	56.6	58.6	57	i
Ma-Pa-07	70.1	48.0	51.8	65.3	43.2	61.4	36.5	68.2	55.7	58.6	55.9	h
Ba-Pa-01	57.6	36.5	38.4	48.0	33.6	46.1	26.9	52.8	41.3	43.2	42.4	e
Ba-Pa-10	28.8	14.4	17.3	25.0	11.5	23.0	8.6	26.9	19.2	21.1	19.6	d
Ba-Pa-02	26.9	10.6	11.5	19.2	9.6	18.2	6.7	23.0	13.4	16.3	15.6	c
Ba-Pa-07	24.0	11.5	14.4	19.2	9.6	17.3	5.8	22.1	15.4	17.3	15.6	c
Ba-Pa-14	21.1	9.6	10.6	18.2	7.7	15.4	4.8	1.9	12.5	13.4	11.5	a
Pa-Pa-01	68.2	48	49.9	60.5	42.2	59.5	36.5	63.4	52.8	57.6	53.9	g
Pa-Pa-05	21.1	10.6	12.5	17.3	9.6	17.3	6.7	19.2	13.4	15.4	14.3	b
Pa-Pa-06	61.4	36.5	38.4	51.8	33.6	49.0	28.8	57.6	40.3	43.2	44.1	f
Mean e	50.6 j	30.8 c	33.4 d	44.2 h	28.0 b	42.2 g	23.5 a	46.1 i	36.3 e	39.0 f	37.4	
	Extent of sporulation [b]											
Ma-Pa-01	+++	+	++	++	+	++	-	+++	++	+		
Ma-Pa-05	+++	+	+	++	-	++	-	+++	++	++		
Ma-Pa-03	+++	+	++	+++	+	+++	-	+++	++	+		
Ma-Pa-07	+++	+	+	+++	+	+++	-	+++	++	++		
Ba-Pa-01	+++	+	+	++	+	++	-	+++	++	++		
Ba-Pa-10	+++	+	+	+++	+	+	-	+++	+	+		
Ba-Pa-02	+++	+	+	++	-	+++	-	+++	++	++		
Ba-Pa-07	+++	+	+	++	-	+++	-	+++	++	+		
Ba-Pa-14	+++	+	+	++	-	+++	-	+++	++	+		
Pa-Pa-01	+++	+	++	+++	+	++	-	+++	++	++		
Pa-Pa-05	+++	+	+	+++	+	++	-	+++	++	++		
Pa-Pa-06	+++	+	++	+++	+	++	-	+++				

[a] In a column, means same letters are not significantly ($P \leq 0.05$) different according to DMRT

[b] Extent of sporulation: - = no spore, + = slight, ++ = moderate and +++ = abundant.

The mycelial growth was different among isolates and different pH levels ($P \leq 0.05$). The interaction between isolates and pH levels was also significant ($P \leq 0.05$). *Colletotrichum* isolates grew significantly better on medium with pH of 5.0 to 6.0 (Table 3.9). Growth at pH 4.5 and 8 were significantly less than the other pH levels and no growth were recorde at pH 8.5. At all pH levels tested, Ma-Pa-01, Ma-Pa-05, Ma-Pa-03 and Ma-Pa-07 grew significantly better than the other isolates. Isolates Ba-Pa-10, Ba-Pa-02, Ba-Pa-07, Ba-Pa-14 and Pa-Pa-05 showed maximum growth at pH 5.5 while Pa-Pa-01 and Pa-Pa-06 isolates were found to grew beter at pH 6.0. Isolate Ba-Pa-01 grew better at pH 5.0 (Table 3.9). All the isolates preferred pH 6 for better sporulation followed by pH 5.5 and 6.5 (Table 3.9). No isolate was shown at pH 8 and pH 4.5 sporulated except Ba-Pa-01 at pH 4.5 and Ma-Pa-05 at pH 8.0 with poor sporulation.

There were significant variations in temperature tolerance of the isolates (Table 3.10). All isolates were shown best at 28°C. Isolate Ma-Pa-03 also showed better growth at 30°C. The temperature range from 28°C to 30°C was found to be favorable for the spore production of the different isolates of *Colletotrichum* (Table 3.10). There was no spore production at 15 and 10°C. Extent of sporulation was high in most isolates at 28°C (Table 3.10).

Table 3. 9 Effect of pH on the growth and sporulation of *Colletotrichum* isolates

	pH level										
Isolate	**4.5**	**5.0**	**5.5**	**6.0**	**6.5**	**7.0**	**7.5**	**8.0**	**8.5**	**Mean [d]**	
	Mean mycelial growth (mm)										
Ma-Pa-01	23.7	79.5	74.7	71.7	68.7	61.5	31.0	16.0	0.0	47.4	g
Ma-Pa-05	23.4	82.6	76.8	74.9	70.6	62.2	29.9	15.8	0.0	48.5	g
Ma-Pa-03	21.4	83.7	78.5	73.5	69.2	64.4	29.1	14.5	0.0	48.3	g
Ma-Pa-07	23.8	78.5	76.8	73.1	67.6	64.0	32.7	16.0	0.0	48.0	g
Ba-Pa-01	21.6	73.2	67.9	64.2	62.4	60.0	30.8	14.6	0.0	43.9	f
Ba-Pa-10	5.4	25.2	27.2	21.7	19.2	19.2	8.0	3.6	0.0	14.4	ab
Ba-Pa-02	6.3	28.2	32.4	23.5	22.2	19.9	8.4	4.3	0.0	16.1	bc
Ba-Pa-07	5.8	29.4	31.4	27.1	24.5	22.3	9.2	3.9	0.0	17.1	c
Ba-Pa-14	5.5	22.0	24.1	19.7	19.5	17.2	7.4	3.7	0.0	13.2	a
Pa-Pa-01	17.4	51.2	57.3	62.8	48.3	44.7	23.3	11.7	0.0	35.2	d
Pa-Pa-05	6.5	26.2	28.2	25.1	21.4	19.1	9.4	4.4	0.0	15.6	bc
Pa-Pa-06	19.2	59.6	67.0	71.1	55.5	48.3	25.6	13.0	0.0	39.9	e
Mean e	15.0c	53.3h	53.5 h	50.7 g	45.8 f	41.9 e	20.4 d	10.1 b	0.0 a		
	Extent of sporulation [b]										
Ma-Pa-01	-	+	+	+	+	+	+	-	-		
Ma-Pa-05	-	+	++	++	++	++	+	+	-		
Ma-Pa-03	-	+	+	+	+	-	-	-	-		
Ma-Pa-07	-	+	+	++	+	-	-	-	-		
Ba-Pa-01	+	++	++	+++	++	++	+	-	-		
Ba-Pa-10	-	++	++	+++	++	++	+	-	-		
Ba-Pa-02	-	-	+	+	++	++	+	-	-		
Ba-Pa-07	-	-	+	++	+	+	-	-	-		
Ba-Pa-14	-	+	+	+	+	+	-	-	-		
Pa-Pa-01	-	+	+	++	+	++	+	-	-		
Pa-Pa-05	-	+	+	+	+	-	-	-	-		
Pa-Pa-06	-	+	+	++	+	-	-	-	-		

[a] In a column, means same letters are not significantly ($P \leq 0.05$) different according DMRT

[b] Extent of sporulation: - = no spore, + = slight, ++ = moderate and +++ = abundant.

Table 3. 10 Effect of temperature on the growth and sporulation of *Colletotrichum* isolates

	Temperature level (°C)							
Isolate	**10°C**	**15°C**	**20°C**	**25°C**	**28°C**	**30°C**	**35°C**	**Mean [a]**
	Mean mycelial growth (mm)							
Ma-Pa-01	7.5	19.4	37.7	80.9	88.6	86.2	0.0	45.8 f
Ma-Pa-05	7.5	18.3	34.5	76.5	84.1	80.9	0.0	43.1 e
Ma-Pa-03	6.5	15.1	32.3	75.5	81.9	81.9	0.0	41.9 e
Ma-Pa-07	5.4	14.0	30.2	69.0	77.6	75.5	0.0	38.8 d
Ba-Pa-01	4.3	12.9	27.0	57.1	65.8	63.6	0.0	33.0 c
Ba-Pa-10	3.2	6.5	11.9	23.7	32.3	30.2	0.0	15.4 b
Ba-Pa-02	2.2	6.5	10.8	24.8	31.3	30.2	0.0	15.1 b
Ba-Pa-07	3.2	5.4	9.7	20.5	27.0	24.8	0.0	12.9 a
Ba-Pa-14	4.3	7.5	11.9	21.6	27.0	25.9	0.0	14.0 ab
Pa-Pa-01	7.5	17.2	31.3	66.8	77.6	74.4	0.0	39.3 d
Pa-Pa-05	2.2	5.4	10.8	19.4	24.8	22.6	0.0	12.2 a
Pa-Pa-06	6.5	16.2	30.2	64.7	74.4	70.1	0.0	37.4 d
Mean	5.0 b	12.0 c	23.2 d	50.0 e	57.9 g	55.5 f	0.0 a	
	Extent of sporulation [b]							
Ma-Pa-01	-	-	+	++	++	++	-	
Ma-Pa-05	-	-	-	+	++	+	-	
Ma-Pa-03	-	-	-	-	+++	++	-	
Ma-Pa-07	-	-	-	+	+++	++	-	
Ba-Pa-01	-	-	+	-	++	++	-	
Ba-Pa-10	-	-	+	-	++	++	-	
Ba-Pa-02	-	-	+	++	+++	++	-	
Ba-Pa-07	-	-	-	++	++	+	-	
Ba-Pa-14	-	-	+	++	++	++	-	
Pa-Pa-01	-	-	-	-	++	+	-	
Pa-Pa-05	-	-	-	-	+++	++	-	
Pa-Pa-06	-	-	-	+	+++	++	-	

[a] In a column, means same letters are not significantly ($P \leq 0.05$) different according to DMRT

[b] Extent of sporulation: - = no spore, + = slight, ++ = moderate and +++ = abundant.

The different isolates responded differently to different light sources ($P \leq 0.05$). The interaction between isolates and light intensity was also significant ($P \leq 0.05$). *Colletotrichum gloeosporioides* grew significantly better on medium in day light except Ma-Pa-07 and Pa-Pa-01, followed by UV light sources of incubation. (Table 3.11). Growth under fluorescent light was lower than the other light sources. Isolates Ma-Pa-01, Ma-Pa-05, Ma-Pa-03 and Pa-Pa-01 grew significantly better than the other isolates.

The interaction between isolates and light sources was also significant ($P \leq 0.05$). Isolates Ma-Pa-01, Ma-Pa-03, Ba-Pa-10, Ba-Pa-02, Ba-Pa-07, Ba-Pa-14, Pa-Pa-05 and Pa-Pa-06 recorded maximum growth in day light. On the other hand, isolates Ma-Pa-05 and Ba-Pa-01 grew better in UV light and isolates Ma-Pa-07 and Pa-Pa-01 grew better in dark light. Spore production were more in day light followed by growth under UV radiation. However, Ma-Pa-01 and Ma-Pa-05 also showed the best sporulation even under dark light. Spore development was observed much less under fluorescent light conditions (Table 3.11).

Table 3. 11 Effect of light source on the growth and sporulation of *Colletotrichum* isolates

Isolate	**Light source**					
	Day light	**Dark light**	**UV light**	**Fluor light**	**Mean** [a]	
	Mean mycelial growth (mm)					
Ma-Pa-01	73.7	65.5	71.6	68.6	69.9	e
Ma-Pa-05	69.6	66.5	70.6	65.5	68.1	e
Ma-Pa-03	71.6	67.5	68.6	69.6	69.3	e
Ma-Pa-07	69.6	71.6	70.6	57.3	67.3	de
Ba-Pa-01	64.5	61.4	66.5	62.4	63.7	c
Ba-Pa-10	28.7	20.5	25.6	23.5	24.6	ab
Ba-Pa-02	30.7	21.5	26.6	22.5	25.3	ab
Ba-Pa-07	31.7	22.5	28.7	24.6	26.9	b
Ba-Pa-14	29.7	17.4	25.6	20.5	23.3	a
Pa-Pa-01	67.5	74.7	67.5	63.5	68.3	e
Pa-Pa-05	72.7	57.3	66.5	62.4	64.7	cd
Pa-Pa-06	25.6	19.4	23.5	21.5	22.5	a
Mean	53.0 c	47.2 a	51.0 b	46.8 a		
	Extent of sporulation [b]					
Ma-Pa-01	+++	+++	+++	+		
Ma-Pa-05	+++	+++	+++	+		
Ma-Pa-03	+++	++	+++	++		
Ma-Pa-07	+++	++	++	+		
Ba-Pa-01	+++	+	+++	++		
Ba-Pa-10	+++	++	+++	+		
Ba-Pa-02	+++	++	+++	+		
Ba-Pa-07	++	+	++	+		
Ba-Pa-14	+++	++	+++	+		
Pa-Pa-01	+++	++	++	+		
Pa-Pa-05	++	+	++	+		
Pa-Pa-06	+++	++	+++	+		

[a] In a column, means same letters are not significantly ($P \leq 0.05$) different according to DMRT

[b] Extent of sporulation: - = no spore, + = slight, ++ = moderate and +++ = abundant.

The growth of the isolates on different carbon sources was different among one another (P≤0.05). Galactose and lactose were the least utilized carbon compounds by these isolates (Table 3.12). The isolates better grew on fructose recor followed by media containing starch, xylose and gucose. Isolates Ma-Pa-01 and Ma-Pa-05 showed significantly higher mycelial growth on the carbon sourses tested followed by Ma-Pa-03 and Ma-Pa-07. Isolate Pa-Pa-05 showed the the least mycelial growth (17.1 mm). All isolates sporulated when fructose and starch were used as sole carbon sources where as heavy sporulation was shown by Ma-Pa-01, Ma-Pa-05, Ma-Pa-03, Ma-Pa-07 and Ba-Pa-01isolates (Table 3.12).

Table 3. 12 Growth and sporulation of *Colletotrichum* isolates on different carbon sources

	Carbon source										
Isolate	Fructose	Starch	Xylose	Glucose	Sucrose	Mananose	Galactose	Lactose	Control	Mean [a]	
	Mean mycelial growth (mm)										
Ma-Pa-01	83.5	79.8	75.0	71.3	62.8	56.7	51.9	47.0	44.6	63.6	g
Ma-Pa-05	84.5	78.5	73.6	70.0	65.1	60.2	54.2	45.6	40.8	63.6	g
Ma-Pa-03	80.3	76.7	71.8	69.4	62.1	59.6	51.1	48.7	45.1	62.8	g
Ma-Pa-07	79.3	76.8	73.2	68.3	64.7	61.0	56.2	51.3	45.2	64.0	g
Ba-Pa-01	63.4	57.3	51.2	48.8	45.2	42.7	39.1	36.7	33.0	46.4	d
Ba-Pa-10	31.7	29.4	27.1	24.8	22.5	20.2	17.9	14.4	11.0	22.1	c
Ba-Pa-02	32.8	28.1	23.5	22.4	20.1	16.6	14.3	13.2	12.0	20.3	bc
Ba-Pa-07	27.5	25.2	21.7	19.4	19.4	17.1	16.0	12.5	10.2	18.8	ab
Ba-Pa-14	28.5	26.2	25.1	21.6	19.3	18.2	15.9	14.7	12.4	20.2	b
Pa-Pa-01	74.0	67.9	64.2	63.0	60.6	54.5	50.9	48.4	41.2	58.3	f
Pa-Pa-05	24.3	22.0	19.7	19.7	17.4	15.1	13.9	11.6	10.5	17.1	a
Pa-Pa-06	71.9	67.0	59.7	56.1	48.8	45.1	42.7	40.3	36.6	52.0	e
Mean	56.8 i	52.9 h	48.8 g	46.2 f	42.3 e	38.9 d	35.3 c	32.0 b	28.5 a		
	Extent of sporulation [b]										
Ma-Pa-01	+++	+++	++	+	++	+	++	+	-		
Ma-Pa-05	+++	+++	++	+	++	+	+	+	-		
Ma-Pa-03	+++	+++	++	+	+	+	+	+	-		
Ma-Pa-07	+++	+++	++	+	++	+	+	+	-		
Ba-Pa-01	+++	++	++	+	++	+	+	+	-		
Ba-Pa-10	++	++	++	+	+	+	+	+	-		
Ba-Pa-02	++	++	++	+	++	+	+	+	-		
Ba-Pa-07	++	++	+	-	+	-+	+	+	-		
Ba-Pa-14	++	++	++	+	++	-	+	+	-		
Pa-Pa-01	++	++	++	+	++	+	+	+	-		
Pa-Pa-05	++	++	++	+	++	+	+	+	-		
Pa-Pa-06	++	++	++	+	++	+	+	+	-		

[a] In a column, means same letters are not significantly (P≤0.05) different according to DMRT

[b] Extent of sporulation: - = no spore, + = slight, ++ = moderate and +++ = abundant

Mycelial growth of the fungus was also influenced by the nitrogen sources used (Table 3.13). Potacium nirate (54.6 mm) supported the maximum growth followed by ammonium sulphate (50.9) and calcium niteate (47.0) (Table 3.13). Ammonium oxalate was the one that was utilized by the least number of isolates (Table 3.13). Isolate Ma-Pa-05 showed growth with different nitrogen sources more than any of the isolates followed by Ma-Pa-01. Isolate Pa-Pa-05 was the most fastidious isolate on different nitrogen sources tested. All the nitrogen sources supported sporulation of all isolates, with heavy sporulation on potassium nitrate and ammonium sulphate (Table 3.13).

Table 3. 13 Growth and sporulation of *Colletotrichum* isolates in different nitrogen sources

	Nitrogen source								
Isolate	**Potas. Nitrate**	**Ammo. Sulphate**	**Calc. Nitrate**	**Peptone**	**Amon. Phosphate**	**Urea**	**Ammo. Oxalate**	**Control**	**Mean [a]**
	Mean mycelial growth (mm)								
Ma-Pa-01	84.5	78.7	74.1	70.6	66.0	61.3	55.6	47.5	67.3 h
Ma-Pa-05	82.5	80.1	76.7	72.1	68.6	65.1	60.5	55.9	70.2 i
Ma-Pa-03	78.4	75.0	70.3	68.0	61.1	58.8	50.7	48.4	63.8 g
Ma-Pa-07	74.4	68.6	62.9	60.5	57.1	54.8	51.3	49.0	59.8 f
Ba-Pa-01	67.4	61.6	58.1	57.0	54.7	48.9	45.4	43.1	54.5 e
Ba-Pa-10	30.2	28.0	24.7	22.5	22.5	20.3	19.2	15.9	22.9 c
Ba-Pa-02	29.2	27.0	24.8	22.6	20.4	18.2	16.0	12.7	21.4 c
Ba-Pa-07	27.2	25.0	22.8	22.8	20.6	18.4	17.3	15.1	21.1 c
Ba-Pa-14	24.1	21.9	20.8	17.6	15.4	14.3	12.1	11.0	17.1 b
Pa-Pa-01	69.4	65.9	61.3	57.8	49.7	43.9	39.3	34.7	52.8 e
Pa-Pa-05	22.1	17.7	13.4	12.3	10.1	6.8	4.6	3.5	11.3 a
Pa-Pa-06	65.4	60.7	53.8	50.3	43.4	39.9	37.6	35.3	48.3 d
Mean	54.6 h	50.9 g	47.0 f	44.5 e	40.8d	37.6 c	34.1 b	31.0 a	
	Extent of sporulation [b]								
Ma-Pa-01	+++	+++	+++	+++	++	++	+	-	
Ma-Pa-05	+++	+++	+++	++	++	-	+	-	
Ma-Pa-03	+++	+++	+++	++	++	-	+	-	
Ma-Pa-07	+++	+++	++	++	++	++	+	-	
Ba-Pa-01	+++	+++	+++	++	+	-	+	-	
Ba-Pa-10	+++	+++	+++	++	++	++	+	-	
Ba-Pa-02	+++	+++	+++	++	++	-	+	-	
Ba-Pa-07	+++	+++	++	++	+	+	+	-	
Ba-Pa-14	+++	+++	+++	+++	++	-	+	-	
Pa-Pa-01	+++	+++	+++	+++	++	-	+	-	
Pa-Pa-05	+++	+++	++	++	+	+	+	-	
Pa-Pa-06	+++	+++	+++	+++	++	-	+	-	

[a] In a column, means same letters are not significantly ($P \leq 0.05$) different according to DMRT

[b] Extent of sporulation: - = no spore, + = slight, ++ = moderate and +++ = abundant

The DNA from the *Colletotrichum* isolates was amplified with the ITS primers. All the isolates amplified with the ITS-1 and ITS-4 primers, indicated that the isolates represented *C. gloeosporioides* and *C. acutatum* (Table 3.14).

Phylogenetic analysis was performed using the ITS 1 and ITS 4 fragment. Comparative analysis of ITS 1 sequences produced a phylogenetic tree (data not shown) that supported the previous analysis published by Sreenivasaprasad *et al.* (1996). This analysis confirmed grouping of the Ethiopian banana isolate Ba-Pa-02, Ba-Pa-07, Ba-Pa-10 and Ba-Pa-14 in a clade with *C. acutatum* isolates (AJ301964.1, AJ301950.1, AF275542.1 and U79700.1). The papaya isolate (Pa-Pa-05) closely related with *C. acutatum* isolates (U79695.1). Isolate ALM-US-4 was found to be closely related to isolate 179. Isolate form banana (Ba-Pa-01), four mango isolates (Ma-Pa-01, Ma-Pa-03, Ma-Pa-05 and Ma-Pa-07) and two papaya isolates (Pa-Pa-01 and Pa-Pa-06) were closely related with *C. gloeosporioides* isolates of HQ414623.1, XM_007284925.1, AF264028.1, XM_007282560.1, DQ084518.1, XM_007276420.1 and DQ084502.1, respectively (Table 3.14).

Table 3. 14 ITS sequence resultscomparison of *Colletotrichum* isolates used in this study based on BLAST

No	**Isolate code** [a]	**Description** [b]	**Identity** [c]	**Accession Number** [d]
1	Ba-Pa-01	*Colletotrichum gloeosporioides*	99.98 %	HQ414623.1
2	Ba-Pa-02	*Colletotrichum acutatum*	99.99 %	AJ301964.1
3	Ba-Pa-07	*Colletotrichum acutatum*	100.00 %	AJ301950.1
4	Ba-Pa-10	*Colletotrichum acutatum*	100.00 %	AF275542.1
5	Ba-Pa-14	*Colletotrichum acutatum*	99.97 %	U79700.1
6	Ma-Pa-01	*Colletotrichum gloeosporioides*	99.99 %	XM_007284925.1
7	Ma-Pa-03	*Colletotrichum gloeosporioides*	100.00 %	AF264028.1
8	Ma-Pa-05	*Colletotrichum gloeosporioides*	100.00 %	XM_007282560.1
9	Ma-Pa-07	*Colletotrichum gloeosporioides*	99.99 %	DQ084518.1
10	Pa-Pa-01	*Colletotrichum gloeosporioides*	100.00 %	XM_007276420.1
11	Pa-Pa-05	*Colletotrichum acutatum*	100.00 %	U79695.1
12	Pa-Pa-06	*Colletotrichum gloeosporioides*	99.99 %	DQ084502.1

[a] Code given to isolates during the study

[b] Description of species which showed highest similarity with isolates of the current study

[c] Percent similarity of isolates the current study with those of *Colletotrichum* isolates in gene bank

[d] Gene bank accession number of *Colletotrichum* isolates which showed highest similarity with the current study isolates

3.4. Discussion

Morphological identification of *Colletotrichum* isolates was based on colony appearance, and character of vegetative and reproductive structures and ITS sequence combination. The colour of cultures varied considerably within and between isolates of *C. acutatum* and *C. gloeosporioides* (Table 3.2). All the isolates varied with respect to sporulation, pigmentation, margin and topography. Similar observations were made by (Kuramae-Izioka *et al.*, 1997; Freeman *et al.*, 1998; Forster and Adaskaveg, 1999; Manjunath, 2009).

The conidial shape of most *C. acutatum* isolates was fusiform, but conidia of *C. gloeosporioides* isolates were cylindrical with tapering ends (Table 3.3). The conidia of *C. gloeosporioides* isolates measured from 12.00 - 17.72 µm and that of *C. acutatum* isolates measured 8.60 - 12.53 µm These results were consistent with original descriptions given by Sutton (1980) and Smith and Black (1990). There was a variation in the spore size between *C. gloeosporioides* and *C. acutatum* isolates. Das-Gupta (1986) also reported the variation in the spore size (17.36 - 21.8 µm x 2.66 - 2.88 µm) among the isolates of *Colletotrichum* causing anthracnose of betelvine. The average size of the spores however, did not vary among the isolates of *C. gloeosporioides* and *C. acutatum*. Chakrabarty *et al.* (1988) reported that in *C. lindemuthianum* the average size of the spores did not vary much among the isolates. Bernstein *et al.* (1995), reported that *C. gloeosporioides* isolates obtained from apple, peach, pecan and other hosts varied greatly in their growth, virulence and conidial size.

The shape and size of appressoria have also been used for taxonomy of the genus *Colletotrichum*. Isolates of *C. acutatum* showed slightly smaller appressoria than isolates of *C. gloeosporioides* (Table 3.4). These results correspond to the descriptions of Sutton (1992). The appressoria shapes of C. acutatum isolates were clavate or ovate, and appressoria of *C. gloeosporioides* isolates were variable, irregular or ovate. Sanders and Korsten (2003) also reported that appressoria of isolates of *C. acutatum* and *C. gloeosporioides* differed in shape and size.

In the present study, the isolates of *Colletotrichum* from different fruit crops showed that *Colletotrichum* is the pathogen responsible for anthracnose in fruit crops (Table 3.5). The symptoms produced by the pathogen after artificial inoculation on the fruits were similar to the symptoms observed under natural infection. The symptoms appeared as black, sunken lesions, distributed all over the outer part of the fruit. The fungus started developing acervuli, sometimes with concentric rings, sporulating with masses of pinkish conidia under favorable moist conditions. Similar symptoms of anthracnose were also noticed on banana (Thangamani *et al.*, 2011), mango (Afanador-Kafuri *et al.*, 2003) and papaya (Rampersad, 2011) fruits with sunken lesions, covered with salmon colored acervuli.

The current study showed that all isolates were pathogenic when tested on alternate hosts (banana, mango and papaya fruits), inducing symptoms of anthracnose (Table 3.6). Cross and specific pathogenicity are parameters used in diverse studies for the characterization of *Colletotrichum* isolates. Findings of the present study revealed the existence of different isolates of *Colletotrichum* infecting different fruits. In evaluating the cross infection of 290 *C. gloeosporioides* isolates from avocado and mango, Swart (1999) demonstrated the occurrence of variation in the virulent of the isolates, and that avocado isolates inoculated in avocado induce greater lesions than mango isolates in avocado. In the present study, the isolates from mango desplayed high pathogencity than those from banana and papaya. These differences may be attributed to the adaptation of the pathogen to a less susceptible host (Swart, 1999).

Fungicide sensitivity assays was used as a primary method to estimate the potential of such compounds for chemical control (Freeman *et al.*, 1998). Furthermore, was used as a tool for grouping of species and subspecies of *Colletotrichum* (Freeman *et al.*, 1998; Adaskaveg and Forster, 2000; Kuo, 2001). . The results also showed that the effectiveness of benomyl differed for different *Colletotrichum* isolates (Table 3.7). Other studies showed that, differential response towards a range of fungicides was found by different *Collototrichum* species and different isolates of a particular *Colletotrichum* species (Adaskaveg and Forster, 2000; Kuo, 2001).

Fungi secure food and energy from the substrate upon which they live in nature. In order to culture the fungus in the laboratory it is necessary to furnish those essential elements and compounds in the medium for their growth and other life processes. Not all media are not equally good for all fungi, nor there is a universal substrate or artificial medium upon which all fungi can grow. So, different media were tried for *Colletotrichum* isolates. Among the media used for growth and sporulation of *Colletotrichum* isolates, maximum growth (50.6 mm) and good sporulation of the fungus was recorded on PDA followed by Rechards agar, which recorded growth of 84.90 mm. The least growth (23.5 mm) and poor sporulation was observed on water agar media. Present studies are in accordance to the better performance of *Colletotrichum* isolates on PDA which may be attributed to inherent complex nature of material supporting good fungal growth owing to provision of some additional nutrients as reported by (Ekbote *et al.*, 1997 ; Akthar, 2000; Sudhakar, 2000; Prashanth, 2007).

Hydrogen ion concentration is one of the most important factors influencing the growth of fungi. In the present study, maximum mycelial growth was observed at pH 5 to 6 (Table 3.9) which was similar with the results of Kuberan *et al.* (2012) where *C. gloeosporioides* was better grown on pH 6. Similar observations were also reported by some other authors with different species of *Colletotrichum* (Ramakrishnan, 1941; Naik *et al.*, 1988; Banik *et al.*, 1998; Kuberan *et al.*, 2012). Excellent growth and sporulation of *Colletotrichum* isolates were recorded at pH 5.0 to 6.0 which was in line with that of Patel (2004) for *Colletotrichum* isolates from turmeric and chili.

Among the external factors, temperature is an important factor governing distribution, growth, reproduction and survival of the fungus. Temperature affects almost every function of fungi including the growth and sporulation. Temperature has profound effect on the vegetative and reproductive activity of the fungi. Effect of temperature on mycelial radial growth revealed that maximum growth (57.9 mm) was at 28°C. Further increase in temperature level to 35°C decreased mycelial growth and least mycelial growth (5.0 mm) was observed at 10°C. However the temperature at 28-30°C was found to be optimum for the growth and sporulation of *Colletotrichum* isolates. This is in agreement with the observation made on *Glomeralla cingulata*, *C. capsici* and *Colletotrichum gloeosporioides* by various workers who noticed best growth at 25-29°C. (Ekbote ***et al.***, 1996; Prasanna-Kumar, 2001; Venkataravanappa, 2002; Prashanth, 2007; Vinaya, 2008). Excellent sporulation of fungus was at 28-30°C and least sporulation was found at temperature 10o C, which is an important pathogenic character. The present results are also in accordance with (Estrada ***et al.***, 1993; Estrada ***et al.***, 2000)

Light has a profound effect on growth and sporulation of fungus. The preliminary studies carried out in the present investigation with *Colletotrichum isolates* indicated a maximum growth (53.0 mm) and excellent sporulation when it was exposed to day light condition followed by UV light (51.0 mm). Least radial growth (47.2 mm and 46.8 mm) and poor sporulation were recorded when *Colletotrichum* isolates was exposed to dark light and florecent light and both were on par with each other. When exposed to day light it attained maximum radial growth which might be due to induction of certain metabolic process necessary for growth and sporulation of the fungus, which usually occur in continuous light. Similarly Venkataravanappa and Nargund (2002) and Prashanth (2007) observed in their studies that the exposure of day light and UV light favoured good growth and sporulation compared to continuous florecent and continuous dark.

There were differences in the utilization of carbon sources for the growth and sporulation of *Colletotrichum* isolates tested. In general, fructose and starch was found to be the best carbon source in the current study, both for the growth and sporulation of the *Colletotrichum* species (Table 3.12). Sangeetha (2003), observed fructose and sucrose are the best carbon sources for the growth of *C. gloeosporioides* of mango. Apart from these, several other reports showed that fructose was a better carbon source for various species of *Colletotrichum* (Hegde ***et al.***, 1990; Kuberan ***et al.***, 2012). Carbon source also affects the sporulation of *C. gloeosporioides.* Sangeetha (2003), observed *C. gloeosporioides* isolated from mango, heavy sporulation of the fungus when maltose, fructose and sucrose were used as a sole carbon source.

The current study found that there was variation in utilizing nitrogen sources for the vegetative growth and sporulation of *Colletotrichum* isolates. In the cases with potassium nitrate was found as the best source for growth and sporulation of *Colletotrichum* isolates. indicating that nitrate compounds are excellent nitrogen sources for imperfect fungi and for the ascomycetes (Bilgrami and Verma, 1978). Ekbote (1994) reported *C. gloeosporioides* of mango utilized potassium nitrate more efficiently for growth and sporulation. Saxena (2002) also reported potassium nitrate as the best source for growth and sporulation of *C. gloeosporioides* isolated from pomegranate (Deshmukh *et al.*, 2012). Certain sources of nitrogen favor the sporulation of some fungi, which are not necessarily the same as those which are favorable for growth (Lilly and Barnett, 1951). Mishra and Mahmood (1960), reported abundant sporulation of *Colletotrichum capsici* was found on medium containing peptone as a nitrogen source.

All the isolates amplified with the ITS-1 and ITS-4 primers, confirming that the isolates are *C. gloeosporioides* and *C. acutatum* (Table 3.14). Afanador-Kafuri *et al.* (2003), carried out studies demonstrating that the ITS primers were efficient in differentiating *Colletotrichum* isolates obtained from tamarind and mango at the species level in *C. acutatum* and *C. gloeosporioides*. In the case of this study, it was useful for determining the presence of *C. acutatum* and *C. gloeosporioides* as a member of the *Colletotrichum* mango, banana and papaya population.

3.5. Conclusion and Recommendation

This study has revealed that *Colletotrichum gloesporioides* and *C. acutatum* were the causative agents of anthracnose of mango, banana and papaya fruits in Ethiopia. The occurrence of *C. gloeosporioides* isolates producing cylindrical conidia and of *C. acutatum* isolates producing elliptic to fusiform conidia showed a variation in *Colletotrichum* isolates present on banana, mango and papaya fruits. Moreover the identity of the two *Colletotrichum* isolates established in this study will help to facilitate management strateges such as fungicide and cultural metods as well as biological based management strategies to control the disease in Ethiopia.

Moreover, the findings in this study have demonstrated the likelihood of cross infection of some pathogens from one host to another. Mango banana and papaya fruit trees exist together in the same environment in the farms of Ethiopia. Furthermore the isolation of *Colletotrichum* from mango trees could also emanate from the banana and papaya since they are both found in proximity to each other in most fruit orchards.

More work is needed to be done to explain the relationship between *Colletotrichum* pathogens from banana, mango and papaya with other fruit tree. Also further studies are required to verify the gene flow, whether the fungi are moving from these host into the other crops or vice versa. Additional studies of the epidemiology of anthracnose are also needed to define further the disease management strategies.

CHAPTER 4 SCREENING OF ANTAGONISTIC MICROORGANISMS FOR BIOCONTROL ACTIVITY AGAINST *COLLETOTRICHUM* SPECIES ON TROPICAL FRUITS

4.1. Introduction

Tropical fruit such as mango, banana and papaya are some of the most important fruits produced in Ethiopia (Kebede_Semeret, 1992). Postharvest losses caused by fungal diseases are the major factor limiting the storage life of tropical fruits (Singh and Pal, 2008). Anthracnose caused by *C. acutatum* and *C. gloeosporioides*, is the most important postharvest diseases of tropical fruits (Koomen, 1990), limiting the storage period and marketing life of fruits.

Currently, the control of postharvest diseases relies mainly on the use of synthetic fungicides (Korsten *et al.*, 1993; Kuo, 2001; Rahman *et al.*, 2007). The use of fungicides immediately before or after harvest to prevent rots is being increasingly limited because of environmental, toxicological and technical risks (Janisiewicz and Korsten, 2002). Moreover, the increase in resistance towards the few authorized fungicides is a frequent phenomenon in the population of fungal pathogens (Janisiewicz and Korsten, 2002; Govender *et al.*, 2005). Fungicides used alone are therefore ineffective against such strains (Staub, 1991). Furthermore, the use of chemicals is also limited by low or zero-residue tolerance for some export and processing markets (Janisiewicz, 1998). In the absence of fully effective postharvest fungicides, alternative or integrative measures are becoming increasingly important for controlling losses (Janisiewicz and Korsten, 2002).

Several alternatives showed promise, but none alone was as effective as fungicides. A strategy must be developed that combines several of these alternatives to enhance their effectiveness (Jeger and Jeffries, 1988). Pre-storage heat treatment is one of the methods reduce spoilage of various commodities (Jeger and Jeffries, 1988; Conway *et al.*, 2004). It has shown potential as a method to reduce postharvest decay as well (DeVilliers and Korsten, 1994). Heat treatment (55°C for 15 min) was effective in eradicating anthracnose on mango and exhibited no residual activity (Govender *et al.*, 2005). The same heat treatment also reduced decay caused by *C. acutatum* (Janisiewicz *et al.*, 2003).

Another alternative that continues to show promise is that of biological control of postharvest diseases (Cook, 1985; DeVilliers and Korsten, 1994; Janisiewicz and Korsten, 2002; Sharma *et al.*, 2009). Bacteria, yeast, fungi and other microorganisms on the surface of fruits and vegetables have been shown to protect fruits and vegetables against postharvest pathogens (Wisniewski and Wilson, 1992; Janisiewicz, 1998; Janisiewicz and Korsten, 2002; Spadaro and Gullino, 2004). Microbial biocontrol agents have shown a great potential as an alternative to synthetic fungicides for the control of postharvest decay of fruits and vegetables such as green mold on citrus and scabe on apples (Wilson and Wisniewski, 1989; Wisniewski *et al.*, 1991). Two commercial products, Aspire (based on *Candida oleophila* Montrocher) and Yield Plus (based on *Cryptococcus albidus* (Saito) Skinner) have been registered in the United States and South Africa (Janisiewicz *et al.*, 2000; Janisiewicz and Korsten, 2002; Fravel, 2005).

However, biocontrol agents when applied alone under commercial conditions are sometimes not sufficient to control postharvest decay satisfactorily (Droby and Chalutz, 1994; Janisiewicz and Korsten, 2002; Sharma *et al.*, 2009). Integrating biocontrol agents with other means of control in order to make their activity more reliable may be the best option for a large-scale application of an antagonist, with a consequent significant reduction of the fungicide amount used (Conway *et al.*, 2004). Attempts to improve biocontrol activity by combining biocontrol agents with physical treatments (Conway *et al.*, 2004; Droby *et al.*, 2009) or small quantities of fungicides (Droby *et al.*, 1998; Conway *et al.*, 2004) has proven to be extremely useful. However, studies on the combination of biocontrol agents with small quantities of chemicals, were limited because of the presence of fungicide-sensitive and resistant strains of fungal pathogens (Janisiewicz and Korsten, 2002; Sharma *et al.*, 2009).

Due to the devastating losses of fruits, farmers are suffering from anthracnose in major fruit growing areas of Ethiopia (Kebede_Semeret, 1992). For sustainable production, the pathogen needs to be controlled in order to ensure reduction of both pre and postharvest losses. The management of anthracnose disease of fruits has been carried out bwith fungicides in some parts of the country (Eshetu_Bekele, 2006; Sysay_Bekele, 2006). Mohammed_Amin *et al.* (2014) studied on integrated disease management of anthracnose on haricot beans by including soil solarization, bioagents seed treatment and techniques of timing of fungicide applications. Additional studies were undertaken on mango (Yonas_Kefialew and Amare_Ayalew, 2008), papaya (Antenehe_Ademe *et al.*, 2013), with regard to biological control of anthracnose diseases of major fruits in Ethiopia by using antagonistic microorganisms in combination with other methods.

The ultimate goal of this study was to devise a strategy that combines several alternatives that will equal the effectiveness of chemical control. The specific objective of this study was to determine the effect of heat, antagonist, and chemical treatments, alone and in combination, on postharvest decay caused by *C. gloeosporioides* and *C. acutatum* on mango, banana and papaya.

4.2. Materials and Methods

4.2.1 Sampling site and isolation of microorganisms

Specimens of mango, banana and papaya consisting of leaves and fruits were collected from different ago-ecological zones in eastern, southern and western parts of Ethiopia. Geographical and climatic data of the sampling sites are summarized in Appendix Table 2. Trees at different phenological stages were sampled in two rounds of surveys. Samples were collected from relatively healthy-looking trees from the last mature flush of leaves and fruits already ripened and not showing any disease symptoms. Samples were separately put in paper bags and transported to the Mycology Laboratory at Addis Ababa University for isolation and testing of microorganisms.

4.2.2 Isolation of antagonistic microorganisms

4. 2.2.1 Isolation of antagonists

A total of 105 specimens compromising 36 bananas, 35 mangos, and 34 papayas were collected. The collected samples were aseptically processed under laboratory conditions and subjected to serial dilution followed by plating on culture media yielded 1566 microorganisms (653 bacteria, 594 yeast and 319 filamentous fungi) (Appendix Table 3).

Microorganisms associated with the plant samples were isolated as follows. Ten gram of leaves was ground in 50 ml of sterile distilled water to make a suspension. The surfaces of three fruits per sample were rubbed with sterile cotton swab which were then placed in 10 ml of sterile distilled water and agitated on a shaker for 10 min.

Dilutions of 10^{-1}, 10^{-2}, and 10^{-3} were made from the suspensions and wash solutions. After thoroughly mixing on a vortex shaker, aliquots of 0.1 ml each were spread on 9 cm diameter agar plates. Nutrient Agar (NA) plates (3 g of beef extract, 5 g peptone, and 15 g of Agar in 1000 ml distilled water) were used for isolation of bacteria, Malt Extract Agar (MEA) (30 g

Malt extract agar, 5 g peptone, and 15 g Agar in 1000 ml distilled water) and Potato Dextrose Agar (PDA) (200 g potato, 20.0 g glucose, and 15.0 g agar in 1000 ml distilled water) containing antibiotic (50 mg/l streptomycin sulphate) was employed for isolation of yeasts and filamentous fungi respectively. Three plates of the respective medium were used for each dilution. The NA and MEA plates were examined for bacterial or yeast growth after incubation at 25°C for 48 hr. Potato Dextrose Agar plates were incubated at 25°C for 6 to 8 d till fungal colonies developed.

Visible colonies of bacteria, yeasts and filaments fungi from each sample were grouped by their colony morphologies, such as color, consistency, and shape, and representative colonies were aseptically transferred to fresh plates of the respective medium. The density (colony forming units per gram) of each group and frequency of isolation were recorded. Bacteria, yeast and fungi isolates were transferred to fresh NA, MEA and PDA plates respectively. All subcultures were incubated at 25°C while the development of pure culture checked. Isolates of bacteria yeast and filamentous fungi grown in pure culture were transferred to screw-capped culture bottles containing the respective medium. Two slants were inoculated with each isolate, coded and kept in the refrigerator at 4°C until they were used.

4.2.2.2 Test pathogens

Two representative isolates, one each of *C. acutatum* (Ba-Pa-01) and *C. gloeosporioides* (Ma-Pa-03), were previously isolated from anthracnose lesions on banana and mango fruits they were selected for this study. Stock cultures of each isolate were maintained on PDA at 4°C. Working cultures were established by transferring a stock agar plug containing the mycelium of each isolate onto PDA in Petri dishes and incubating for 12 to 15 d in darkness at 25°C (Koomen and Jeffries, 1993).

Conidial suspension of *C. gloeosporioides* and *C. acutatum* was prepared by washing one- to two-week old MEA cultures with 5 ml sterile water containing 0.01% Tween 80. Spores were suspended by gently scraping the colony surface with sterile loop. The suspension was transferred to a sterile test tube and spores were counted using a hemacytometer and the concentration of spores was adjusted to required concentration.

4.2.2.3 Inoculum preparation

The antagonist bacterial suspension was prepared by culturing a loopful of bacteria grown on NA slant in 250 ml Erlenmeyer flasks containing 100 ml Nutrient Broth (NB) (3 g of beef extract and 5 g peptone). After 2 d of incubation with continuous shaking at 110 rpm at 28°C, the cells were harvested by centrifugation for 10 min at 10,000 rpm. The resulting pellet was dissolved in 10 ml of sterile distilled water. The cell concentration was adjusted to the required concentration using a hemacytometer.

Inoculums of the yeasts isolates were produced on MEA media for 5 d at 25°C (He *et al.*, 2003). The mature cells were washed from the medium with 10 ml sterile distilled water and diluted into the suspension and adjusted to the required concentration using a hemacytometer. The cultures of fungal isolates were grown on PDA, and incubated for 10 to 15 d at 25°C. Spores were harvested by flooding the plates with 10 ml sterile distilled water and 0.01% Tween 20, scraping with a loop, and then filtering the suspension through double layers of cotton sheet to remove the mycelial fragments. The concentration of spores in the suspensions were determined with a hemacytometer and adjusted to required concentration.

4.2.3 Testing of antagonism in vitro

Prilimnary screening of all bacteria, yeast and fungal isolates was condacted to determine their posible biological activity. Each isolate was tested against *C. gloeosporioides* or *C. acutatum*, as potential antagonists and pathogen were inoculated in the same culture plate (9 cm diameter petriplate) containing MEA. Five mm diameter mycelial plugs of either pathogen (*C. gloeosporioides* or *C. acutatum*) isolate was placed at the center of the plate. The test isolates (bacteria, yeast or fungal isolates) were inoculated at three nearly equidistant points, 3 cm away, from the mycelial plug. Plates inoculated only with the pathogen served as control. Culture plates were incubated at 25°C and colony diameter of *C. gloeosporioides* or *C.*

acutatum was measured every day (as the average of two cross diameters) for 12 day. The biological activity was recorded as follows (Yonas_Kefialew and Amare_Ayalew, 2008):

1. ***Antibiosis activity*** = The antagonist and the pathogen didn't make contact, rather a distinct inhibition zone formed
2. ***Lytic activity*** = The antagonist and the pathogen made contact and the antagonist started to lyse back the pathogen mycelium
3. ***Overlap/ mycoparastic activity*** = The antagonist and the pathogen made contact and the antagonist started to grow over the pathogen mycelium
4. ***Checked the growth of pathogen activity*** = The antagonist and the pathogen made contact at less than 50% growth of the pathogen and the antagonist surrounded the pathogen colony, while the pathogen stopped growing after 50% growth
5. ***No activity*** = The antagonist and the pathogen made contact and the pathogen started growing over the antagonist colony

When a clear inhibition zone was observed, the circumference of the pathogen colony in each plate was marked on the underside and the plate was further incubated for two days to confirm the presence of the inhibition zone. Isolates showing such inhibitory effects were maintained for further tests and identification.

Observations were also made for antagonists that did not yield inhibition zones, but which could attack hyphae of *C. gloeosporioides* or *C. acutatum*. The colony margin of the pathogen in each plate was marked upon contact with the test colony. Plates were then incubated till the colony of the pathogen in the control plate reached the edge of the plate and were examined to check if hyphae of the pathogen were lysed back or parasitized by the test colony. Test organisms with lytic or mycoparasitic effects were selected for further test as described below. Isolates which checked the growth of the pathogen or were covered by pathogen were discarded. Subsequent screening was conducted to confirm their biological activity as described bellow (Yonas_Kefialew and Amare_Ayalew, 2008).

4.2.3.1 Screening of antagonists for antibiotic production

A dual culture test was carried out following the method described by Aghighi *et al.* (2004). Five mm diameter mycelial plugs of either *C. gloeosporioides* or *C. acutatum* were placed at the one corner of the plate. A test antagonist isolate (5 mm), grown on solid culture (NA for bacteria, MEA for yeast and PDA for filaments fungi) was inoculated at the opposite corner (50 mm) from the mycelial plug of the pathogen. Control assays included fungal mycelial plugs in center of non-antagonist inoculated MEA plates. Plates were incubated at 27°C for 10 d and radial growth as well as the zone of inhibition was measured when maximum growth occurred in the control plates. Each antagonist against each pathogen was tested in three replicated pates and the experiment was laid in Complite Randemized Design (CRD). The efficacy of antagonists was expressed as Percent of Growth Inhibition (PGI) over the control which was calculated by using the formula (Koomen and Jeffries, 1993):

$$\text{PGI (\%)} = \frac{\text{KR-R1}}{\text{KR}} \times 100$$

Where; KR represents the distance (measured in mm) from the point of inoculation to the colony margin on the control dishes and R1 the distance of pathogen growth from the point of inoculation to the colony margin on the treated dishes in the direction of the antagonist. The PGI was categorized on a growth inhibition category (GIC) scale from 0 to 4, where 0 = no growth inhibition; 1 = 1-25% growth inhibition; 2 = 26-50% growth inhibition; 3 = 51-75% growth inhibition; 4 = 76-100% growth inhibition. Isolates which inhibited the growth of the pathogen by >50%, representing PGI 3 and PGI 4 was selected for further experiment.

4.2.3.2 Screening of antagonists for lytic activity

Antagonists selected for their preliminary lytic effects during primary mass screening for biological activity were tested specifically for their lytic activity in dual culture (Pusely and Wilson, 1985; Jeyarajan and Nakkeeran, 2000). Conidial suspensions of *C. gloeosporioides* and *C. acutatum* were prepared as described in the previous section and the concentration of spores was adjusted to about 10^4 conidia/ml. Molten MEA medium kept at about 45°C in a water bath was inoculated with either *C. gloeosporioides* or *C. acutatum* spore suspension (5 ml suspension pipetted into 250 ml medium) and the medium was poured into sterile culture plates. Antagonistic bacteria and yeast were cultured on NA (2 d) and MEA (5 d) respectively and their colonies were removed using a 6 mm diameter cork borer to inoculate on the pathogen mycelium. When the medium was covered by the mycelium, the selected antagonistic organism was spotted at three points over the test pathogen colony. After incubation at 25°C for 14 d, the width of the pathogen mycelium lysed around the colony of the antagonist was measured as cross diameter. Antagonists that showed marked lysis on the pathogen mycelium, as compared to others, were selected and maintained for further evaluation. Each antagonist against each pathogen was tested in three replicated pates and the experiment was laid in CRD.

4.2.3.3 In vitro colonization of biocontrol agents on the pathogen

This experiment was conducted to further confirm the mycoparasitic effect of antagonists and colonization percentage on the pathogen using dual cultures. An agar disk of the pathogens (6 mm) was placed at one side of a new petri dish, and the disk with the potential antagonist was placed at the opposite side of the plate. Each antagonist was tested to *C. gloeosporioides* and *C. acutatum* in three replications. Petri dishes were incubated at room temperature for 15 d. Plates without antagonist and pathogen were included as controls.

Afterwards, the colonization percentage of antagonist over each pathogen colony was estimated according to the following equation (Riddell, 1950):

$$C = \frac{\text{DCAP}}{\text{DSP}} \times 100$$

Where: C=colonization percentage; DCAP=distance covered by the antagonist on the pathogen colony over the axis which separates the antagonist and the pathogen, DSP=distance between sowing points (6.5 cm). For this experiment, a colonization of the antagonist isolates significantly higher than others were considered as an effective colonization over both pathogens. A completely randomized experimental design was set for each antagonist isolate.

4.2.4 Inhibition of conidial germination of *C. acutatum* and *C. gloeosporioides*

Parasitism of spores of *C. gloeosporioides* and *C. acutatum* by bacteria, yeasts and fungal isolates were tested using depression slides. The antagonist bacteria and yeast cell suspension and spore suspension of fungal isolates and the test pathogen isolates were prepared as described in sections 4.2.2.3 and 4.2.2.2. Concentration of cells/ spores of both the pathogen and antagonists in the suspensions were adjusted to 10^5 cell or spores/ml (Rahman *et al.*, 2007).

Fifty (50) µl of the standardized suspensions of antagonists and pathogen isolates were mixed and transferred to sterile microscope slides. The controls consisted of suspensions of pathogen conidia in sterile distilled water only. The slides were then incubated in moist chambers for 48 h using three replicate slides for each antagonist against each test pathogen. At the end of the incubation period, a drop of lactophenole cotton blue was added to each slide to arrest germination. Spore germination was defined as a germ tube that had developed longer than half of the conidial length (Rahman *et al.*, 2007). The percent inhibition of germination and germ tube elongation was determined by counting 100 conidia from each test pathogen under a light microscope to determe the proportion of germination. Antagonistic isolates that significantly inhibited spore germination, as compared to others, were selected for further experiment. Each antagonistic isolates was tested in three replications of slides against each pathogen in CRD. The percentage inhibition of conidial germination of the pathogen isolates were calculated by using the formula (Rahman *et al.*, 2007);

$$\text{Percentage Inhibition (PI)} = \frac{\text{RC-RT}}{\text{RC}} \times 100$$

Where RT was the mean of spore germination on the tested treatment and RC was the mean of spore germination on the control treatment.

4.2.5 Extracellular enzyme activity of antagonists on solid media

Plate assays were performed in order to test the ability of antagonistic isolates to degrade the substrate of cellulose, chitin, glucan, pectin and proteo enzymes. Bacterial, yeast and fungal isolates were grown on NA, MEA and PDA for 2 to 5 d and from the actively growing edge of the colonies disks of antagonist isolates with diameter of 8 mm were inoculated on the medium and enzyme activity was observed after incubation as described bellow. Three petri dishes were used for each of the isolates and enzymes. The ratio of the diameter of the clear zone to colony diameter was measured in order to select the highest enzyme producing isolate. The largest ratio was assumed to contain the highest activity (Hsu and Lockwood, 1975; Hankin and Anagnostakis, 1977; Sazci *et al.*, 1986; Cote *et al.*, 1989):

Cellulase assay:

The test medium consisted of mineral solution ($(NH_4)_2SO_4$ 1 g/l, urea 0.3 g/l, KH_2PO_4 2 g/l, $CaCl_2$ 0.3 g/l, $MgCl_2$ x 6 H_2O 0.3 g/l, $FeSO_4$ x 7 H_2O 0.005 g/l, $MnSO_4$ x H_2O 0.014 g/l, $CaCl_2$ x 2 H_2O 0.002 g/l,) 20 ml/l, carboxymethyl-cellulose (CMC) 10 g/l, agar 15 g/l and 0.1% Triton X-100 1 ml/l in distilled water and the medium was sterilized at 120 °C for 15 minutes. Inoculation was carried out as described above, and cellulase activity was visualized after 7 d of incubation at 26 °C by flooding the plates with 5 ml aqueous solution of Congo red (1 g/l in distilled water) and then the reagent was discarded and the diameter of the colony and the degradation halo was measured (Sazci *et al.*, 1986).

Chitinase assay:

Colloidal chitin was prepared according to the method of Hsu and Lockwood (1975). Agar (20 g) was sterilized in 1 l of distilled water at 120 °C for 15 minutes and then 1 g/l colloidal chitin was added to the medium. Inoculation was performed as described above and colony and solubilization zone diameters were measured following incubation at 26 °C for 2-3 d.

Glucanase assay:

The glucanase medium contained agar 20 g/l and laminarin 0.5 g/l in distilled water, and sterilizated at 120 °C for 15 minutes. Inoculation was performed as described above and the plates were incubated at 28 °C for 2 d. Plates were flooded with 3 ml aqueous solution of 0.1 % Methyl Blue (Fluka) and shaken at 100 rpm for 30 minutes on a rotary shaker, then the reagent was discarded and degradation zones were visualized by UV illumination using a UVP white/UV transilluminator (Cote ***et al.***, 1989).

Pectinase assay:

Component A of the medium contained 5 ml/l mineral solution ($(NH_4)_2SO_4$ 1 g/l, KH_2PO_4 2 g/l, Na_2HPO_4 3 g/l, $FeSO_4 \times 7\ H_2O$ 0.1 g/l, $CaCl_2$ 0.5 g/l, H_3BO_3 0.005 mg/l, $MnSO_4$ 0.005 mg/l, $ZnSO_4$ 0.035 mg/l, $CuSO_4$ 0.025 mg/l, MoO_3 0.005 mg/l in distilled water), yeast extract 2 g/l and pectin10 g/l, while component B consisted of agar 30 g/l in distilled water. An equal amount of them were mixed following sterilization at 120 °C for 15 minutes. ~~P~~ectinase activity was observed after 2 d of incubation at 26 °C by flooding the plates with 5 ml 1 % (10 g/l in distilled water) hexadecyltrimethylammonium bromide (C-TAB), previously sterilized at 120 °C for 15 minutes. After 2 hours of incubation the reagent was discarded and the diameter of the colony and the degradation halo was measured (Hankin and Anagnostakis, 1975).

Protease assay:

Test medium was prepared by adding nutrient agar (OXOID) 28 g/l in distilled water. The medium was sterilized at 120 °C for 15 minutes, and then 50 ml gelatin solution (80 g/l, sterilized at 120 °C for 15 minutes) was added. A cellophane membrane (sterilized twice at 120 °C for 20 minutes) was placed on the solidified medium and inoculation was carried out as described after the membrane was dried. Plates were incubated at 26 °C for 3-4 d then colony diameters were measured and cellophane membranes containing the colonies were removed. If the degradation halos were not visible, plates were flooded with saturated (0.67 g/l) $(NH_4)_2SO_4$ and halos were measured after the removal of the reagent (Hankin and Anagnostakis, 1975)

4.2.6 Primary fermentation and screening for antifungal activity

Based on the activity of antagonists in the previous experiments, extraction and antifungal activity screening of promising isolates were done. Isolates were cultured and extracts were tested by agar well diffusion assay. Each isolate was grown in 250 mL Erlenmeyer flasks containing 100 mL Malt Extract Broth (MEB) medium. Each medium was inoculated with 2 mL of a 48 h culture of antagonists. Inoculated flasks were incubated at 26°C on a rotary shaker at 110 rpm for five days for bacteria/ yeast and twelve days for fungi (El-Banna and Winkelmann, 1998).

After completion of the incubation period, each liquid culture was centrifuged at 10,000 rpm for 20 min and filtered with Whatman filter paper and supernatant was maintained. Supernatants (100 ml) were extracted with equal volume of four different solvents (ethyl acetate, methanol, chloroform and petroleum ether) on rotary shaker at 120 rpm for 24 hr. The organic phase was separated from the aqueous phase using a funnel. The extracts were concentrated using rotavapour at 60°C to near dryness. The extracts were allowed to dry in rotavapour once again and the mass of the residue for each solvent extract was calculated. The dried crude extracts were assayed for antifungal activity (Nonoh *et al.*, 2010).

The solvent extracts of antagonists were tested against both pathogens. Spore suspension of the test pathogens were prepared as described earlier and adjusted to 10^4 conidia/ ml. Ten ml of the spore suspension was added to molten and cooled media. The MEA medium was poured in to the sterile petriplates and allowed to solidify. Wells (6 mm) (5 holes: replications) were made in the medium using sterile cork borer. Then 20µl of each extract was transferred in to the separate wells. The plates were incubated at 26°C for 3 - 5 d. After the incubation the plates were observed for formation of clear zones around the well, which indicated the presence of antifungal activity (Janisiewicz *et al.*, 2008).

4.2.7 Evaluation of media for production of antifungal compound(s)

Five different liquid culture media were used to study the level of production of inhibitory substances by antagonist isolates. The media were Potato Dextrose Broth (PDB), Malt Extract broth (MEB), Yeast Extract Malt Extract broth (YEMEB), Starch Nitrate broth (SNB) and

Sabroud broth (SB). Antagonists were inoculated to these medium and incubated at 26°C on a rotary shaker at 110 rpm for five days for bacteria and yeast and twelve days for fungi. When maximum growth was achieved, the cultures were centrifuged at 10,000 rpm for 20 min and filtered with Whatman filter paper and the supernatant was maintained. The supernatant was extracted with the best solvent determine in the previous section. The best culture medium was selected according to the size of the growth inhibition zone of the pathogen in the agar well method as described earlier.

4.2.8 In vivo efficiency of antagonists against test pathogens

4.2.8.1 Efficacy of antagonists to inhibit the pathogens on hosts

The effectiveness of the antagonists was tested *in vivo* on artificially inoculated banana, mango and papaya fruits. Fruits were harvested at commercial maturity, and selected for uniformity of size and ripeness. Fruit were selected at random, superficially disinfected by immersion for 5 min in 0.1% sodium hypochlorite, rinsed with fresh water, and allowed to air-dry at room temperature (25°C) (Govender *et al.*, 2005).

Ten bacterial, seven yeast and two filamentous fungal isolates with strong inhibitory activity were, selected based on *in vitro* and cultured in nutrient broth, yeast extract malt extract broth and potato dextrose broth, respectively. Flasks consisting 100 ml of each media were incubated in a shaker incubator (150 rpm) for 48 h (for bacteria and yeast isolates) and five days (for fugal isolates). Bacteria and yeast cells were then collected by centrifugation at 3000 rpm for 20 min. The spore suspensions of filamentous fungal isolates were prepared by scraping off the spore masses from 12 - 14 d old cultures of filamentous fungal isolates. To get washed cell/ spore suspensions, the bacterial and yeast cells as well as fungal spores were washed twice with distilled water and resuspended in distilled water. Approximately 50µL of cell/ spore suspension of each isolate at a concentration of 1×10^8 cells/ml was used to treat fruits. Unwashed cells/ spores of bacteria, yeast and fungi were also prepared similarly with out washing and resuspending in distilled water (Wang *et al.*, 2008b).

Culture filtrates of the bacterial, yeast and filamentous fungal isolates were also prepared as described earlier using appropriate media. Culture filtrates were extracted with most effective solvent determined as before. Culture filtrates were used with or without autoclaving. Autoclaved cultures were obtained by sterilizing yeast cultures at 121°C for 20 min (Wang *et al.*, 2008b).

The spore suspension of *C. gloeosporioides* was prepared by scraping off the spore masses from 12- to 14-day-old cultures. They were washed twice with sterile distilled water and the concentration of the suspension was measured by hemocytometer.

Fruits were wounded (5 mm diameter, 3 mm deep and at the equator of each fruit) using a sterile borer for each treatment and 10 µl of 5×10^4 spores/ml suspension of *C. gloeosporioides* were pipetted into the wounds. After 24 hours fruits were treated with 20 µl of on of the treatment mixtures as follows: (i) autoclaved culture; (ii) culture filtrate; (iii) unwashed cell culture mixture at 1×10^8 CFU/ml; or (iv) washed cell suspension at 1×10^8 CFU/ml. Control fruits were dipped in sterile distilled water only. Fruits without any treatment were kept to check whether the developing disease resulted from artificial inoculation or pre-harvest quiescent infections (Wang *et al.*, 2008b).

The fruits were put into plastic boxes at 25°C. The boxes were enclosed with polyethylene bags to maintain high relative humidity. Disease incidence and severity was determined 20 d after inoculation. Each treatment, containing five fruits (mango, banana or papaya), was replicated three times and the experiment was repeated once. Disease severity was scored according to Koomen and Jeffries (1993) as follows: 1 = no spots, 2 = 1 - 3 spots, 3 = 4 - 6 spots, 4 = 7 - 12 spots and 5 = >12 spots per fruit or 30% of the fruit surface affected.

4.2.8.2 Combination of antagonists with other treatments in vivo

Physiologically matured, uninfected fruits of banana, mango and papaya were used for this experiment. Healthy, 3 to 4 week old green, unripe fruit were obtained from a local market. The fruits were surface sterilized by submerging in 1% sodium hypochlorite for 5 min, rinsed twice with sterile water, and dried in a laminar flow hood before inoculation (Koomen and Jeffries, 1993). Prior to inoculation with antagonists, pre infection of the fruit was verified by plating surface-sterilized fruits on PDA medium.

Flasks containing the respective medium were inoculated with a 2 - 5 d pre culture of antagonists and incubated on a shaker at 150 rpm for 2 - 4 d. The spore suspensions of filamentous fungal isolates were prepared by scraping off the spore masses from 12 to 14 d old cultures. Antagonist cell/ spore suspensions of each isolate at a concentration of 1×10^8 cell/ spore per ml were used as treatment.

Surface sterilized Banana, mango and papaya fruits were subjected to three treatments: (i) antagonist only (1×10^8 cells/ml), (ii) antagonist (1×10^8 cells/ml) + prochloraz (180 ml/ L by dipping for 20 s) and (iii) antagonist (1×10^8 cells/ml) + hot water (55°C for 15 min) were applied. For all the trials, an untreated control and a commercial treatment, hot water bath (and prochloraz, were included as a reference standard (Govender ***et al.***, 2005).

After treatment, the fruit were commercially packed (5 fruits per box) into three boxes per treatment. The experiment was set up in CRD. Fruit were monitored regularly for symptom development, which was characterized by black sunken lesions on fruit after 20 d of incubation at room temperature. Disease incidence and severity for anthracnose was done visually using a 0 - 5 scale with zero indicating healthy fruit and five indicating fruit showing total decay as described in the previous section (section 4.2.8.1) (Koomen and Jeffries, 1993).

4.2.9. Identification and confirmation of the identity of antagonists

4.2.9.1 Identification of bacterial isolates

Phenotypic characterizations of effective bacterial isolates were conducted for their colony characteristics (color, form, elevation, margin, diameter, surface, opacity, and texture). Motility, morphology, and size were also evaluated by microscopy. The Gram reaction was performed by using Gram stain method.

The bacterial isolates were further identified by sequencing of the 16S rRNA gene region at the University of Florida (Tallahasse, FL, USA). DNA was extracted and purified with the MOBIO Nucleic Acid Extraction kit. The entire 16S rRNA gene was amplified by PCR, using universal primers. The amplified product was sequenced by Genome Express (University of Florida, USA) from the conserved regions of 16S rRNA. The sequences of selected strains were compared and aligned with those of other strains obtained from the data deposited in GenBank using BLAST (NCBI).

Further identification of bacterial isolates was conducted at Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. Identification by Matrix-Assisted Laser Desorption Ionization-Time-Off Light Mass Spectrometry (MALDI-TOF MS) was conducted as described by Toth *et al.* (2008), and Identification by RiboPrinting using the restriction enzyme EcoRI was applied by the method of Schumann and Pukall (2013). Identification by 16S rRNA gene sequencing of selected strains was also conducted. Sequence similarities were assesed from a search by using the EzTaxon-e Database. Finally, isolates were deposited at DSMZ after DSM numbers were added to those isolates.

4.2.9.2 Identification of yeast and fungal isolates

Morphological characteristics of yeast and fungal antagonists were examined by observing cell/ spore and colony patterns according to the methods described by Kurtzman and Fell (1998).

Identification of antagonistic yeasts and fungal isolates were done using the rDNA sequence comparison technique. DNA was extracted and purified with the NORGEN Nucleic Acid Extraction kit. The extracted DNA was sent to University of Florida and CABI, UK center for sequencing. Primers of ITS region: ITS-1 (**5’**-TCCGTAGGTGAACCTGCGG-**3’**) and ITS-4 (**5**’-TC CTCCGCTTATTGATATG-**3**’), were used to amplify the intervening gene. The 26S and 5.8S genes were amplified by the PCR technique at the University of Florida and CABI, UK center. Then the products were purified and sequencing was carried out using a automate DNA sequencer.

The sequences of selected strains were compared and aligned with those of the other strains obtained from the data deposited in GenBank using BLAST (NCBI) and module MegAlign. Phylogenetic trees were generated using the software Neighbor-joining (Saitou and Nei, 1987). Neighborjoining trees were constructed using PAUP version 4.0b10 with Jukes–Cantor distance correction (http://paup.csit.fsu.edu/). Trees were drawn using TreeView version 1.5 (http://taxonomy. zoology.gla.ac.uk/rod/treeview.html). Robustness was tested by bootstrap analysis with 1.000 replicates.

4.2.10 Statistical analysis

Analysis of variance (ANOVA) was carried out with the statistical software SPSS V. 20 (SPSS Inc., Chicago, USA). To assess the effect of the repitation of experiments, a three-way ANOVA model was used by using repetitions as main plot and treatment combinations as sub effect. Percent data and scale units were subjected to Arc sign and square root transformation, respectively before analysis. The data from the repeated experimental runs were pooled and means were estimated through analysis of variance. A comparison among treatment mean was made with Duncan's Multiple Range Test (DMRT), ($P \leq 0.05$ and 0.001 levels).

4.3. Result

Out of 1566 isolates (653 bacteria, 594 yeast and 319 filamentous fungi isolates) tested, 139 isolates (65 bacteria, 40 yeast and 34 filamentous fungi isolates) showed antagonistic activity against *C. gloeosporioides* and *C. acutatum*. Antagonism through formation of distinct inhibition zones between the pathogen and antagonist isolates were detected from 35 bacteria, 28 yeast and 20 filamentous fungi isolates (Table 4.1 – 3). The bacterial antagonists inhibited the growth of *C. gloeosporioides* and *C. acutatum* with inhibition ranging from 18.7-92 and 11.8-91.3%, respectively (Table 4.1). Similarly, yeast and fungal isolates inhibited the growth of *C. gloeosporioides* / *C. acutatum* with percent inhibition of 16.2/ 18.0 to 82.0/ 64.0% and 6.2/ 19.3 to 82.3/ 62.5%, respectively (Table 4.2 and 3). Among the antagonists, 17 bacteria, 16 yeast and nine filamentous fungal isolates exhibited the strongest antagonism against the isolates of *C. acutatum* and *C. gloeosporioides* respectively; with a moderate (PGI 3) and high (PGI 4) value greater than 50% and they were selected for further experiments (Table 4.1 - 3).

Thirty bacterial and 12 yeast isolates made contact with the pathogens and started to lyse back the pathogen mycelium. These bacterial and yeast isolates significantly ($P \leq 0.05$) lysed the *C. gloeosporioides* and *C. acutatum* mycelia as compared to the untreated control (Table 4.4). Mean lysis diameter by isolates ranged from 2.75 - 33.00 and 1.75 - 30.72 mm against *C. gloeosporioides* and *C. acutatum*, respectively. The highest diameter of lysed mycelium against both pathogens (*C. gloeosporioides* and *C. acutatum*) was found from isolate M-21-F-3 (30.75 and 33.00 mm). Isolate P-11-L-1, B-22-F-6, B-13-L-5, M-03-F-4, M-01-L-4 showed similar effects with isolate M-21-F-3, but superior to the rest of isolates against both pathogens (*C. gloeosporioides* and *C. acutatum*) and they were also selected for further experiments (Table 4.4).

Table 4. 1 Growth inhibition of *C. gloeosporioides* and *C. acutatum* by antagonistic bacteria isolates in dual culture

Isolate code	Growth inhibition (%) on test pathogens [1]					
	C. gleosporides			*C. acutatum*		
B-01-F-2	18.67	(25.60) [2]	b [3]	13.83	(21.83) [2]	b [3]
B-02-F-5	42.00	(40.40)	ef	43.67	(41.36)	fg
B-03-F-2	92.00	(73.57)	q	91.33	(72.88)	o
B-04-L-1	64.00	(53.13)	jk	60.50	(51.06)	ijk
B-06-L-4	72.00	(58.05)	mn	74.17	(59.45)	m
B-07-F-5	60.67	(51.16)	hij	56.67	(48.83)	ij
B-07-F-7	67.33	(55.14)	kl	64.33	(53.33)	kl
B-08-F-2	42.00	(40.40)	ef	33.33	(35.26)	d
B-09-L-5	44.67	(41.94)	fg	41.33	(40.01)	ef
B-11-L-6	61.33	(51.55)	ij	60.83	(51.26)	jkl
B-14-F-1	60.00	(50.77)	hij	57.33	(49.22)	ij
B-16-F-2	39.33	(38.84)	e	36.83	(37.37)	de
M-01-L-2	46.67	(43.09)	fg	44.00	(41.55)	fg
M-02-F-3	46.67	(43.09)	fg	45.33	(42.32)	fg
M-03-F-6	24.67	(29.78)	c	15.33	(23.05)	b
M-04-L-3	70.67	(57.21)	lm	67.33	(55.14)	l
M-05-F-4	88.67	(70.33)	p	75.33	(60.22)	m
M-06-L-3	75.33	(60.22)	no	74.50	(59.67)	m
M-07-F-2	22.00	(27.97)	bc	11.83	(20.12)	b
M-10-F-3	66.00	(54.33)	k	63.17	(52.63)	jkl
M-11-F-5	22.67	(28.43)	c	21.83	(27.86)	c
M-12-L-2	66.67	(54.74)	kl	63.17	(52.63)	jkl
M-13-F-3	45.33	(42.32)	fg	42.83	(40.88)	efg
M-15-F-1	22.67	(28.43)	c	14.67	(22.52)	b
M-16-L-5	30.00	(33.21)	d	23.33	(28.88)	c
M-19-L-6	66.67	(54.74)	kl	64.50	(53.43)	kl
P-02-L-2	77.33	(61.57)	o	87.33	(69.15)	n
P-03-F-3	56.00	(48.45)	h	56.67	(48.83)	ij
P-04-F-1	32.00	(34.45)	d	22.00	(27.97)	c
P-05-F-2	48.67	(44.24)	g	48.83	(44.33)	gh
P-06-F-2	31.33	(34.04)	d	26.00	(30.66)	c
P-08-L-5	58.67	(49.99)	hi	53.83	(47.20)	hi
P-09-L-6	32.67	(34.86)	d	25.83	(30.55)	c
P-10-F-7	90.67	(72.21)	pq	88.67	(70.33)	no
P-16-F-3	48.00	(43.85)	g	47.17	(43.38)	fg
Control	0.00	(0.00)	a	0.00	(0.00)	a

[1] Percent growth inhibition (mean of three replications)

[2] Mean percent growth inhibition mycelia subjected to arcsin transformation (mean of three replications)

[3] Means within a column followed by the same letters were not statistically significant ($P \leq 0.05$), DMRT

Table 4. 2 Growth inhibition of *C. gloeosporioides* and *C. acutatum* by antagonistic yeast isolates in dual culture

	Growth inhibition (%) on test pathogens [1]					
Isolate code	*C. gleosporides*			*C. acutatum*		
B-15-L-2	40.67	(39.62)[2]	E[3]	44.17	(41.65)[2]	Fg[3]
B-18-L-4	69.33	(56.37)	k	65.67	(54.13)	klmn
B-19-F-1	16.00	(23.58)	b	10.67	(19.06)	bc
B-21-L-2	52.00	(46.15)	g	51.67	(45.96)	ghi
B-22-F-1	76.67	(61.12)	m	74.00	(59.34)	no
B-26-F-2	60.67	(51.16)	hi	58.00	(49.60)	hijk
M-18-F-4	64.67	(53.53)	j	62.17	(52.04)	jklm
M-20-F-3	44.00	(41.55)	ef	49.17	(44.52)	fgh
M-23-L-1	82.00	(64.90)	n	78.67	(62.49)	op
M-24-L-5	31.33	(34.04)	d	40.17	(39.33)	f
M-25-L-6	64.67	(53.53)	j	60.67	(51.16)	ijkl
M-27-F-4	80.00	(63.43)	n	82.33	(65.15)	p
M-30-F-3	16.00	(23.58)	b	10.67	(19.06)	bc
M-31-F-2	63.33	(52.73)	ij	58.17	(49.70)	hijk
P-01-F-4	58.67	(49.99)	h	53.33	(46.91)	ghij
P-02-L-5	75.33	(60.22)	lm	65.33	(53.93)	klmn
P-05-F-2	64.67	(53.53)	j	62.83	(52.44)	jklm
P-10-F-4	72.67	(58.48)	l	68.67	(55.96)	lmn
P-13-L-2	18.00	(25.10)	b	6.17	(14.38)	b
P-14-L-1	46.00	(42.71)	f	45.33	(42.32)	fg
P-18-L-5	64.67	(53.53)	j	65.17	(53.83)	klmn
P-19-L-4	75.33	(60.22)	lm	71.00	(57.42)	mno
P-21-L-4	18.67	(25.60)	b	15.67	(23.32)	cd
P-25-F-2	22.67	(28.43)	c	21.67	(27.74)	de
P-26-F-3	40.67	(39.62)	e	25.17	(30.11)	e
P-28-F-2	24.00	(29.33)	c	26.67	(31.09)	e
P-29-F-5	30.00	(33.21)	d	24.67	(29.78)	e
P-31-F-3	58.00	(49.60)	h	57.17	(49.12)	hijk
control	0.00	(0.00)	a	0.00	(0.00)	a

[1] Percent growth inhibition (mean of three replications)

[2] Mean percent growth inhibition mycelia subjected to arcsin transformation (mean of three replications)

[3] Means within a column followed by the same letters were not statistically significant (P≤ 0.05), DMRT

Table 4. 3 Growth inhibition of *C. gloeosporioides* and *C. acutatum* by antagonistic fungal isolates in dual culture

	Growth inhibition (%) on test pathogens 1					
Isolate code	C. gleosporides			C. acutatum		
B-21-L-1	63.33	(52.73)2	mn3	59.83	(50.67)2	h3
B-25-L-1	24.00	(29.33)	d	30.83	(33.73)	de
B-29-F-3	44.00	(41.55)	h	47.50	(43.57)	fg
B-30-F-1	20.67	(27.04)	c	25.83	(30.55)	cde
B-31-L-3	28.00	(31.95)	e	32.33	(34.65)	e
M-26-F-1	64.00	(53.13)	n	69.50	(56.48)	i
M-26-L-2	54.67	(47.68)	j	54.33	(47.49)	gh
M-30-F-1	54.67	(47.68)	j	54.00	(47.29)	gh
M-32-F-2	18.67	(25.60)	b	24.83	(29.89)	bcd
M-32-L-1	18.67	(25.60)	b	22.33	(28.20)	bc
M-33-F-2	23.33	(28.88)	d	30.00	(33.21)	de
M-35-F-1	50.67	(45.38)	i	53.83	(47.20)	gh
M-35-F-3	18.00	(25.10)	b	19.33	(26.08)	b
P-01-F-1	59.33	(50.38)	kl	59.33	(50.38)	h
P-06-F-2	57.33	(49.22)	k	57.50	(49.31)	h
P-07-L-1	61.33	(51.55)	lm	59.67	(50.57)	h
P-10-F-1	31.33	(34.04)	f	32.33	(34.65)	e
P-11-L-2	42.00	(40.40)	gh	47.33	(43.47)	fg
P-22-F-1	41.33	(40.01)	g	46.33	(42.90)	f
P-22-F-2	54.67	(47.68)	j	54.50	(47.58)	gh
Control	0.00	(0.00	a	0.00	(0.00)	a

[1] Percent growth inhibition (mean of three replications)

[2] Mean percent growth inhibition mycelia subjected to arcsin transformation (mean of three replications)

[3] Means within a column followed by the same letters were not statistically significant (P≤ 0.05), DMRT

Table 4. 4 Lytic effect of bacterial and yeast antagonists against *Colletotrichum isolates*

		Lysis diam. (mm) on test pathogens[1]					
Isolate code	Organism	*C. gleosporides*			*C. acutatum*		
B-02-F-3	Bacteria	5.00	(2.35)[2]	Cd[3]	4.00	(4.00)[2]	Bcde[3]
B-04-L-1	Bacteria	13.00	(3.67)	hij	8.75	(8.75)	ghijk
B-05-L-3	Bacteria	13.00	(3.67)	hij	7.00	(7.00)	defghi
B-06-L-6	Bacteria	5.00	(2.35)	cd	4.00	(4.00)	bcde
B-08-F-4	Bacteria	11.00	(3.39)	ghi	4.00	(4.00)	bc
B-09-L-1	Bacteria	4.75	(2.29)	cd	4.00	(4.00)	bcde
B-13-L-1	Bacteria	9.75	(3.20)	fghi	6.00	(6.00)	cdefgh
B-13-L-5	Bacteria	26.50	(5.20)	k	23.00	(23.00)	m
B-14-F-3	Bacteria	6.00	(2.55)	cde	4.75	(4.75)	bcdef
B-16-F-4	Bacteria	16.25	(4.09)	j	12.75	(12.75)	kl
B-17-F-3	Bacteria	4.00	(2.12)	c	4.00	(4.00)	bcde
B-20-L-1	Bacteria	12.50	(3.61)	hij	13.00	(13.00)	kl
M-01-L-4	Bacteria	24.75	(5.02)	k	23.75	(23.75)	m
M-02-F-5	Bacteria	4.25	(2.18)	c	5.75	(5.75)	bcdefgh
M-03-F-4	Bacteria	26.00	(5.15)	k	21.00	(21.00)	m
M-05-F-2	Bacteria	4.00	(2.12)	c	5.75	(5.75)	bcdefgh
M-07-F-4	Bacteria	12.25	(3.57)	hij	10.50	(10.50)	ijkl
M-08-F-2	Bacteria	5.00	(2.35)	cd	5.25	(5.25)	bcdefgh
M-09-L-5	Bacteria	5.00	(2.35)	cd	5.00	(5.00)	bcdefg
M-10-F-6	Bacteria	1.75	(1.50)	b	8.00	(8.00)	fghij
M-11-F-4	Bacteria	13.00	(3.67)	hij	7.67	(7.67)	fghi
M-14-L-1	Bacteria	14.00	(3.81)	ij	13.00	(13.00)	kl
M-15-F-3	Bacteria	5.00	(2.35)	cd	4.00	(4.00)	bcde
M-20-F-4	Bacteria	13.25	(3.71)	ij	9.25	(9.25)	hijk
P-03-F-5	Bacteria	7.75	(2.87)	defg	4.00	(4.00)	bcd
P-06-F-3	Bacteria	14.50	(3.87)	ij	13.75	(13.75)	l
P-11-L-1	Bacteria	27.75	(5.32)	k	24.75	(24.75)	m
P-14-L-2	Bacteria	7.00	(2.74)	cdef	4.00	(4.00)	bcd
P-15-L-3	Bacteria	14.25	(3.84)	ij	12.25	(12.25)	jkl
P-19-L-2	Bacteria	8.00	(2.92)	defg	4.00	(4.00)	bcd
B-22-F-6	Yeast	27.00	(5.24)	k	33.00	(33.00)	n
B-26-F-4	Yeast	5.75	(2.50)	cde	5.00	(5.00)	bcdefg
B-27-F-2	Yeast	8.75	(3.04)	efgh	9.25	(9.25)	hijk
M-17-L-5	Yeast	6.00	(2.55)	cde	5.00	(5.00)	bcdefg
M-21-F-3	Yeast	30.75	(5.59)	k	33.00	(33.00)	n
M-27-F-5	Yeast	4.00	(2.12)	c	4.00	(4.00)	bcde
P-16-F-2	Yeast	4.00	(2.12)	c	2.75	(2.75)	b
P-17-L-3	Yeast	6.00	(2.55)	cde	7.50	(7.50)	efghi
P-17-L-5	Yeast	4.75	(2.29)	cd	5.25	(5.25)	bcdefgh
P-22-F-1	Yeast	5.50	(2.45)	cde	3.75	(3.75)	bcd
P-22-F-4	Yeast	5.00	(2.35)	cd	6.25	(6.25)	cdefgh
P-26-F-4	Yeast	8.00	(2.92)	defg	5.00	(5.00)	bcdefg
Control		0.00	(0.71)	a	0.00	(0.00)	a

[1] Diameter of lysed mycelium (mean of three replications)

[2] Mean lysed mycelia subjected to square root ($\sqrt{X+0.5}$) transformation (mean of three replications)

[3] Means within a column followed by the same letters were not statistically significant ($P \leq 0.05$), DMRT

Fourteen filamentous fungal antagonist isolates made contact with the pathogens in dual culture and the antagonists started to grow over the pathogen mycelium (Figure 4.1). An antagonist isolate (M-30-F-2) showed the highest effect for invading the mycelium of *C. gloeosporioides* and *C. acutatum*, by which the isolate were selected for the next experimental stages (Figure 4.1).

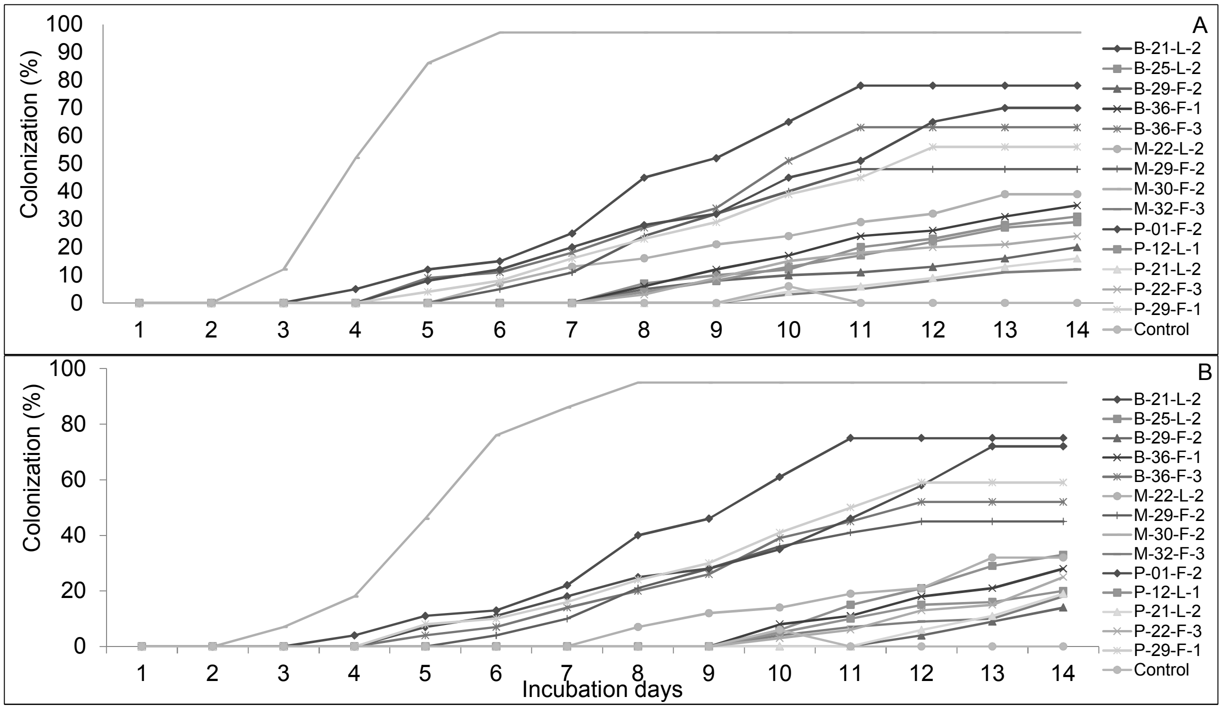


Figure 4. 1 Colonization percent during the mycoparasitism process of antagonist fungal isolates on colonies of *C. gloeosporioides* (A) and *C. acutatum* (B)

** Control treatments were inoculated with water. Data were analyzed for significance after arcsine transformation with analysis of variance, followed by DMRT test. Each value plotted represents the average of three replicates. Experiments against *C. gloeosporioides* and *C. acutatum* were conducted and analyzed separately.

The conidia of *Colletotrichum* isolates that were incubated in water (control) at 25°C, swelled and after 8 h started to germinate, producing one or two germ tubes. After 12 h about 50% of the conidia were germinated. However, conidial germination was strongly limited in co-cultivation with the antagonists. They surrounded the spores of *C. acutatum* and *C. gloeosporioides* and inhibited their germination. After 24 h of co-cultivation, there was significant inhibition of the germination in all mixtures with the antagonists (Table 4.5).

All antagonist isolates significantly inhibited spore germination of *C. gloeosporioides* and *C. acutatum* as compared to the controls (Table 4.5). Mean percent inhibition ranged from 9.6 to 96.0% and 10.7 to 95.1% against *C. gloeosporioides* and *C. acutatum,* respectively. The highest inhibition of conidial germination of *C. gloeosporioides* and *C. acutatum* was found from isolate B-03-F-2 (96.0%) and M-05-F-4 (95.1%), respectively (Table 4.5).

Germination and germ tube elongation were significantly inhibited by the antagonist, and the maximum inhibition of 90.2 and 85.3% was recorded from isolate B-03-F-2 on *C. gloeosporioides* and *C. acutatum* respectively (Table 4.5). Among the antagonist isolates, ten bacterial isolates (B-03-F-2, P-10-F-7, M-05-F-4, P-02-L-2, M-06-L-3, B-06-L-4, M-04-L-3, B-07-F-7, P-11-L-1 and B-13-L-5) seven yeast isolates (M-23-L-1, M-27-F-4, B-22-F-1, P-19-L-4, P-02-L-5, P-10-F-4 M-21-F-3) and two fungal isolates (M-26-F-1 and M-30-F-2) affected spore germination significantly higher than the rest of antagonist isolates against both pathogens and were selected for further experimentation (Table 4.5).

Table 4. 5 Effect of cell/ spore suspension of antagonistic bacteria, yeast and fungal isolates on the spore germination and germ tube elongation of *C. gloeosporioide* and *C. acutatum*

Isolate	*C. gleosporides*					*C. acutatum*				
	Spore germination inhibition (%) 1			**Hyphal length (µm) 2**		**Spore germination inhibition (%)1**			**Hyphal length (µm) 2**	
B-03-F-2	96.0	(78.5) 3	r 4	12.7	a 4	93.1	(74.8) 3	st 4	19.3	a 4
B-04-L-1	23.3	(28.9)	cd	95.3	uvwx	18.7	(25.6)	cdefg	96.7	stuv
B-06-L-4	78.1	(62.1)	mn	64.7	klmnop	78.8	(62.6)	opq	41.0	ghi
B-07-F-5	22.0	(27.9)	cd	93.7	uvw	18.8	(25.7)	cdefg	99.0	tuvw
B-07-F-7	71.6	(57.8)	m	34.7	efgh	74.6	(59.8)	op	51.3	j
B-11-L-6	22.8	(28.5)	cd	96.0	uvwx	15.7	(23.4)	bcdef	97.0	stuv
B-13-L-5	81.5	(64.6)	nop	32.7	defg	79.7	(63.2)	opq	39.3	fgh
B-14-F-1	22.4	(28.3)	cd	93.3	uvw	15.6	(23.3)	bcde	101.0	uvwx
B-18-L-4	60.8	(51.2)	l	63.3	klmn	42.3	(40.6)	mn	31.3	cdef
B-21-L-1	45.2	(42.2)	jk	74.7	nopqr	31.9	(34.4)	jklm	70.0	klm
B-21-L-2	36.4	(37.1)	c	60.7	rstu	31.4	(34.1)	cdefg	71.0	vwx
B-21-L-2	20.2	(26.7)	hij	83.7	klm	19.2	(26.0)	jkl	104.3	lmn
B-22-F-1	82.5	(65.2)	nop	28.0	cdef	88.2	(69.9)	rs	32.3	cdefg
B-22-F-6	20.3	(26.8)	c	106.0	x	19.9	(26.5)	defgh	82.3	opq
B-26-F-2	22.5	(28.3)	cd	99.0	vwx	19.7	(26.4)	defgh	91.7	rst
B-36-F-3	36.4	(37.1)	hij	67.3	lmnop	29.6	(33.0)	hijkl	80.0	op
M-01-L-4	25.0	(30.0)	cdef	87.7	tuv	19.7	(26.4)	defghi	95.7	stuv
M-03-F-4	9.6	(18.0)	b	96.7	vwx	10.7	(19.0)	b	102.7	uvwx
M-04-L-3	77.7	(61.8)	mn	35.0	efgh	72.6	(58.4)	o	49.3	ij
M-05-F-4	90.1	(71.7)	q	19.0	abc	95.1	(77.2)	t	21.0	ab
M-06-L-3	86.4	(68.4)	opq	30.7	cdef	85.7	(67.7)	qr	30.3	cde
M-10-F-3	24.3	(29.5)	cde	91.7	tuvw	21.3	(27.5)	defghij	94.0	stu
M-12-L-2	25.1	(30.0)	cdefg	91.7	tuvw	22.1	(28.0)	defghij	94.7	stu
M-18-F-4	33.6	(35.4)	fghi	70.0	mnopq	30.8	(33.7)	jkl	80.3	op
M-19-L-6	31.7	(34.3)	efghi	88.7	tuv	19.8	(26.4)	defghi	96.0	stuv
M-21-F-3	74.8	(59.9)	mn	39.0	fgh	74.8	(59.9)	op	42.7	hij
M-23-L-1	88.0	(69.8)	pq	30.3	cdef	89.2	(70.8)	rst	37.0	defgh
M-25-L-6	45.9	(42.7)	k	75.0	nopqr	42.9	(40.9)	n	44.3	hij
M-26-F-1	76.4	(60.9)	mn	53.3	ijk	70.3	(56.9)	o	50.3	j
M-26-L-2	32.1	(34.5)	efghi	88.0	tuv	25.7	(30.4)	fghijk	80.3	op
M-27-F-4	90.0	(71.6)	q	26.7	bcde	90.6	(72.1)	rst	31.7	cdef
M-30-F-1	29.4	(32.8)	defgh	76.0	pqrs	27.1	(31.3)	ghijk	85.0	opqr
M-30-F-2	76.6	(61.1)	mn	45.7	hij	72.6	(58.4)	o	62.3	k
M-31-F-2	26.2	(30.8)	cdefg	93.7	uvw	24.6	(29.8)	efghijk	81.7	opq
M-35-F-1	20.0	(26.6)	c	70.7	mnopq	22.7	(28.5)	efghijk	88.3	pqrs

Table 4.5 continued…

Isolate	***C. gleosporides***					***C. acutatum***				
	Spore germination inhibition (%) 1			**Hyphal length (µm) 2**		**Spore germination inhibition (%)1**			**Hyphal length (µm) 2**	
P-01-F-1	37.6	(37.8)	hijk	60.3	klm	38.8	(38.5)	lmn	76.3	mno
P-01-F-2	19.5	(26.2)	c	91.3	tuvw	19.7	(26.3)	defghi	101.3	uvwx
P-01-F-4	29.6	(32.9)	defgh	102.0	wx	30.2	(33.3)	ijkl	81.7	opq
P-02-L-2	87.6	(69.4)	pq	21.7	abcd	90.6	(72.1)	rst	28.0	bc
P-02-L-5	81.9	(64.8)	nop	32.7	defg	82.9	(65.6)	pqr	37.3	defgh
P-03-F-3	12.9	(21.0)	b	96.0	uvwx	11.5	(19.8)	bc	98.0	tuv
P-05-F-2	32.0	(34.5)	efghi	80.3	qrst	31.5	(34.1)	jkl	101.7	uvwx
P-06-F-2	35.8	(36.8)	hi	87.0	stuv	29.3	(32.8)	hijkl	79.0	no
P-07-L-1	40.8	(39.7)	ijk	56.3	jkl	33.1	(35.1)	klmn	66.0	kl
P-08-L-5	10.7	(19.1)	b	102.0	wx	13.2	(21.3)	bcd	107.0	wx
P-10-F-4	77.9	(61.9)	mn	44.0	ghi	75.8	(60.6)	op	38.3	efgh
P-10-F-7	91.9	(73.4)	q	16.3	ab	89.6	(71.1)	rst	29.3	cd
P-11-L-1	82.2	(65.0)	nop	23.7	abcde	83.7	(66.2)	pqr	45.0	hij
P-18-L-5	33.7	(35.5)	ghi	75.3	opqr	27.9	(31.9)	ghijkl	108.7	x
P-19-L-4	79.8	(63.3)	no	32.0	def	78.1	(62.1)	opq	39.0	efgh
P-22-F-2	32.3	(34.6)	efghi	63.7	klmno	23.1	(28.8)	efghijk	90.3	qrst
P-31-F-3	22.8	(28.5)	cd	97.3	vwx	20.9	(27.2)	cdefg	103.0	uvwx
Control	0.0	(0.0)	a	129.0	y	0.0	(0.0)	a	132.0	y

[1] Percentage of inhibition of spores germination at 48 hr (mean of three replications);

[2] Average of five measurements from germinated spores (mean of three replications)

[3] values in parenthesis are arcsine transformed.

[4] Not significant at 0.05% level of significance

The cellulolytic, chitinolytic, glucanolytic, proteolytic and pectinolytic activities of the different antagonist isolates are summarized in Figure 4.5. The isolates B-13-L-5, M-21-F-3, M-30-F-2 and P-11-L-1 produced cellulolytic, chitinolytic, glucanolytic, proteolytic and pectinolytic activities revealed by the formation of clear zones of 31.7, 32.3, 27.0 and 30.0 cellulolytic activity; 28.0, 31.0, 46.0 and 34.0 mm diameter for chitinolytic activity; 23.0, 28.7, 33.0 and 30.7 mm diameter for glucanolytic activity; 28.0, 26.0, 18.7 and 28.3 mm diameter for proteolytic activity and 13.7, 15.0, 17.7 and 14.7 mm diameter for pectinolytic activity, respectively (Figure 4.2).

Hydrolytic activity of M-01-L-4 was detected in medium supplemented with CMC, chitin and skimmed milk with diameters of clear zones of 27.7, 28.0 and 19.7 mm, respectively. No glucanolytic or pectinolytic activity was observed for this isolate. The other isolates B-21-L-2, B-22-F-6, B-36-F-3, M-03-F-4 and P-01-F-2 also produced cellulolytic and chitinolytic activity with the formation of clear zones of 20.0, 34.7, 21.0, 18.0 and 8.0 mm diameter cellulolytic activity and 32.0, 14.0, 15.0, 12.7 and 17.0 for chitinolytic activity, respectively. No glucanolytic, proteolytic and pectinolytic activities were observed for these five isolates (Figure 4.2).

Four isolates (B-13-L-5, M-21-F-3, M-30-F-2 and P-11-L-1) that showed cellulolytic, chitinolytic, glucanolytic, proteolytic and pectinolytic activities were selected for further experiments.

Results from the agar well assay showed that all antagonistic microorganisms inhibited the mycelial growth of *C. acutatum* and *C. gloeosporioides*, with varying efficiencies (Figure 4.3) The effect of antagonist isolates, to inhibit the mycelial growth of *C. acutatum* and *C. gloeosporioides* is shown on Figure 4.3.

Mean separation showed that isolate M-30-F-2 had the highest inhibition zone (24.2 mm and 21.8 mm) in inhibiting the mycelial growth of both pathogens (*C. gloeosporioides* and *C. acutatum*) and significantly higher than the rest of the isolates. The smallest inhibition zone against *C. gloeosporioides* and *C. acutatum* by antagonists were recorded from isolate M-26-F-1 (2.0 mm) (Figure 4.3). Comparisons between the kind of solvent revealed that chloroform (17.3 mm and 14.5 mm) and ethyl Acetate (17.1 mm and 15.6 mm) were statistically different in comparison with other solvents against both pathogens (*C. gloeosporioides* and *C. acutatum*). The methanol extracts (9.4 mm and 7.6) showed significantly higher inhibition than petroleum ether extracts, which showed the least activity (5.6 mm and 5.0 mm) against the test pathogens (Figure 4.3).

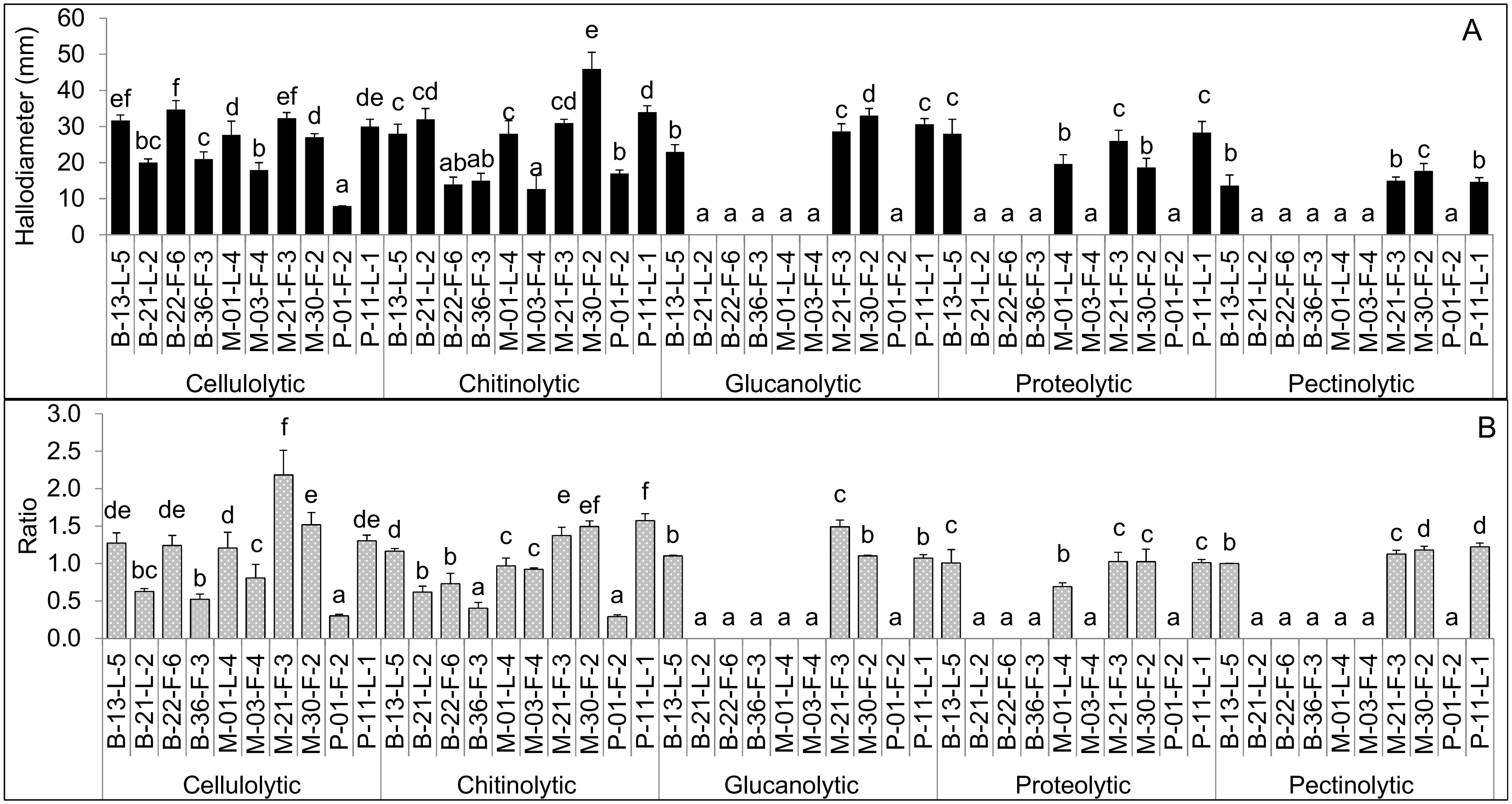


Figure 4. 2 Extracellular enzyme activity of antagonist isolates on solid media

** (A) Colony diameter and (B) ratio of hallow/ Colony area. Six petri dishes were used for each of the isolates and enzymes; three with medium amended with the enzymes inducing substrates and three without the inducing substrate (controls). Cultures were examined on a daily basis and data was collected on the diameters of the antagonist colonies and clear zone (halo) and the proportion of the area of the antagonist colonies and clear zone and the antagonist colony calculated to determine the levels of enzyme production (Ration). Each value plotted represents the average of three replicates. Points with same letters are not significantly ($P \leq 0.05$) different according to Duncan's multiple range test. Vertical bars are standard deviation of the mean. Experiments on each specific enzyme substrate were conducted and analyzed separately

Distinct inhibition zones were observed when bacterial, yeast and fungal isolates were extracted with chloroform and ethyl acetate against *C. gloeosporioides* and *C. acutatum*. However, no distinct inhibition zones were observed in methanol extracts and petroleum ether extracts of some of the test antagonist isolates. All bacterial isolates gave very strong inhibition zones when extracted with ethyl acetate. On the other hand, chloroform extracts of yeast and fungi isolates gave very strong inhibition zones (Figure 4.3).

The inhibitory effect of metabolites extracts was significantly affected by the kind of culture media. As shown in Figure 4.4, all antagonistic microorganisms significantly inhibited the mycelial growth of *C. gloeosporioides*, with varying efficiencies (Figure 4.4).

The maximum mean inhibition was found from PDB (19.4 mm) followed by ME broth (15.7 mm) and the least was found from Sabroud broth 5 (4.6 mm). The effect of MEYE broth (13.1 mm) and starch nitrate broth (13.0 mm) was statistically similar but significantly higher than Sabroud broth (4.6 mm) (Figure 4.4). The highest zone of inhibition was achieved by metabolites of M-23-L-1 (22.4 mm) followed by M-27-F-4 (21.2 mm) and M-22-F-1 (19.6 mm). The lowest zone of inhibition of *C. gloeosporioides* was recorded from metabolites of M-26-F-1 (3.16 mm).

Similar trends were observed on the test against *C. acutatum* (Figure 4.4. The maximum mean inhibition was found from PDB (21.3 mm) followed by ME broth (18.5 mm) and the least was found from Sabroud broth 5 (6.4 mm). The highest zone of inhibition was achieved by metabolites of M-26-F-1 (24.3 mm) followed by B-03-F-2 (21.3 mm) and M-30-F-2 (21.2 mm). The lowest zone of inhibition of *C. acutatum* was recorded from metabolites of M-26-F-1 (3.16 mm).

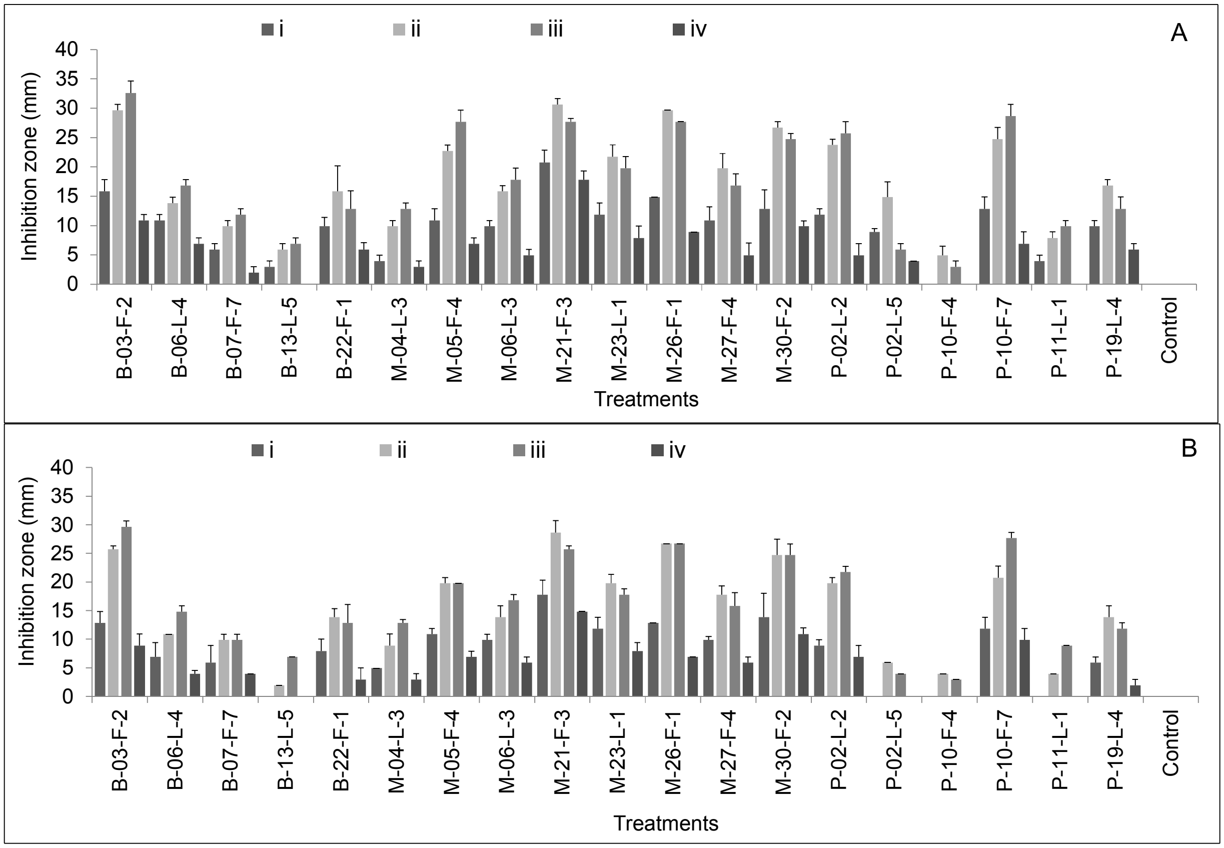


Figure 4. 3 Inhibition of growth of *C. gloeosporioides* (A) and *C. acutatum* (B) by antifungal substances of antagonists

**Each antagonist were grown in MEB media and extracted with: methanol (i), chloroform (ii), ethyl acetate (iii) and petroleum ether (iv). Spore suspension of the test pathogens were adjusted to 10^4 conidia/ ml and 10 ml of the spore suspension was added to molten and cooled media. Wells (6 mm) were made in the medium and 20µl of each extracts was transferred in to the separate wells. The plates were incubated at 26°C for 3 to 5 d. Diameter (millimeter) of the inhibition zone around holes was determined. Each value plotted represents the average of five replicates. Vertical bars are standard deviation of the mean.

All 19 antagonist isolates showed inhibition zones when cultivated with PD broth and ME broth. Most isolates, except two (P-11-L-1 and B-13-L-5), showed inhibition zones when cultivated with YEME broth. Thirteen isolates showed inhibition zones when cultivated with Sabroud medium. Three isolates (M-30-F-2, M-21-F-3 and M-26-F-1) did not show inhibition zones when cultured in starch nitrate broth. All bacterial isolates showed maximum effect when they were grown in Starch nitrate media. On the other hand, yeast and fungal isolates gave maximum effect when they were cultured in YEME broth and by PD broth, respectively.

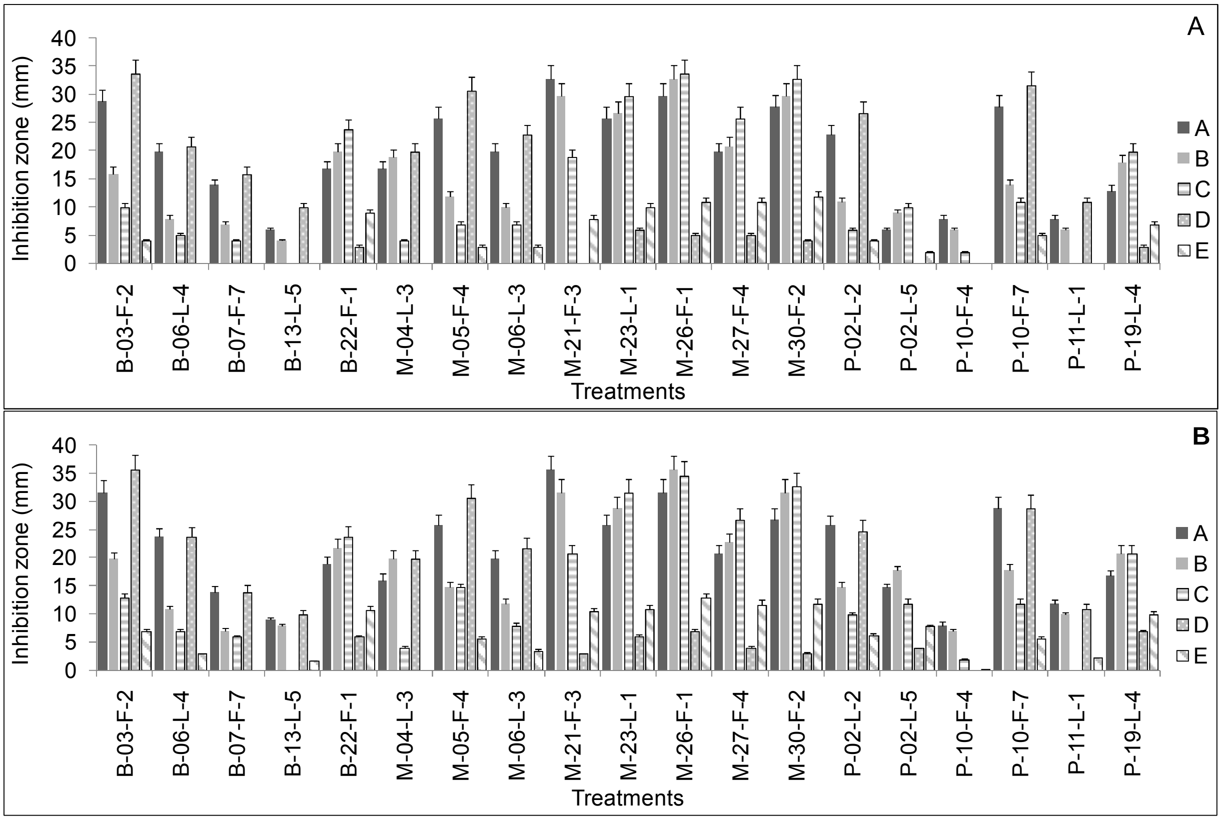


Figure 4. 4 Inhibition of radial growth of *C. gloeosporioides* (A) and *C. acutatum* (B) by antifungal substances produced by antagonists in different liquid culture media

**Inhibition zone of *C. gloeosporioides* treated with culture filtrates of 19 antagonist isolates obtained from five growth media. The media used include: PDB (A), MEA (B), YEMEB (C), SNB (D), and SB (E). Spore suspension of the test pathogens (10^4 conidia/ ml) was added to molten and cooled media. Twenty (20) µl of each extracts was transferred in to the separate wells. The plates were incubated at 26°C for 3 to 5 d and diameter (millimeter) of the inhibition zone around holes was determined. Each value plotted represents the average of five replicates. Vertical bars are standard deviation of mean.

A total of 19 antagonist isolates, which showed the highest antagonistic activity during *in vitro* experiments, were chosen in order to study their effectiveness in the biocontrol of anthracnose in mango, banana and papaya fruit under artificial infection conditions (Figure 4.5, 6, 7). Significant differences ($P \leq 0.05$) were obtained in anthracnose incidence and severity on fruit of banana (Figure 4.5), mango (Figure 4.6), and papaya (Figure 4.7) artificially infected with *C. gloeosporioides* when they were treated with the different application methods of the 19 antagonists and compared with the controls (treatment with sterile water).

Antagonist cell suspensions, applied at 10^8 cells/ spore per ml, on banana reduced the incidence and severity of anthracnose. The major reduction of incidence (92%) and severity

(93%) was due to the application of the isolates B-03-F-2, P-10-F-7 and P-02-L-2 (Figure 4.5). Culture filtrates also caused reduction of the incidence and severity but significantly lower than cell suspensions. Washed cell suspensions and autoclaved cell suspensions of all isolates were least effective.

Similar trends were observed on mango and papaya. Cell suspensions (10^8 cells per ml) of the isolates significantly reduced incidence and severity of anthracnose on mango. B-03-F-2, P-10-F-7 and M-05-F-4 cell suspensions resulted in a remarkable reduction in the incidence and severity of anthracnose by 93 and 95%, respectively (Figure 4.6). Addition of cell suspensions of any of the 19 antagonistic isolates significantly reduced papaya anthracnose after 20 d of storage at 26°C. The cell suspensions of the four isolates (B-03-F-2, P-10-F-7, M-05-F-4 and P-02-L-2) applied on papayas was highly effective against the pathogen after 20 d of storage. Reduction of anthracnose incidence and severity ranged between 92-93% and 93-95%, respectively (Figure 4.7).

The anthracnose controlling ability of antagonists was enhanced, to varying degrees, by the different antagonist application methods. In particular, application of unwashed cell/ spore suspensions of antagonists enhanced the anthracnose controlling ability of all antagonists against the pathogen on the three hosts (banana, mango and papaya) (Figure 4.5, 6, 7).

In all fruit, the unwashed cell/ spore suspension of antagonists, when applied after inoculation with *C. gloeosporioides* was significantly more effective in controlling anthracnose than antagonists applied as culture filtrate and autoclaved culture filtrates. The application of washed cell suspensions resulted in the lowest efficacy of the ability of antagonists against the pathogen on the three hosts (banana, mango and papaya). Banana, mango and papaya fruit inoculated with the pathogen and then treated with the unwashed cell/ spore suspension of antagonists, showed symptoms of infection only after 10, 8 and 7 d of storage at 26°C while, among control fruit, lesions were visible by the 4th day of storage (Figure 4.5, 6, 7).

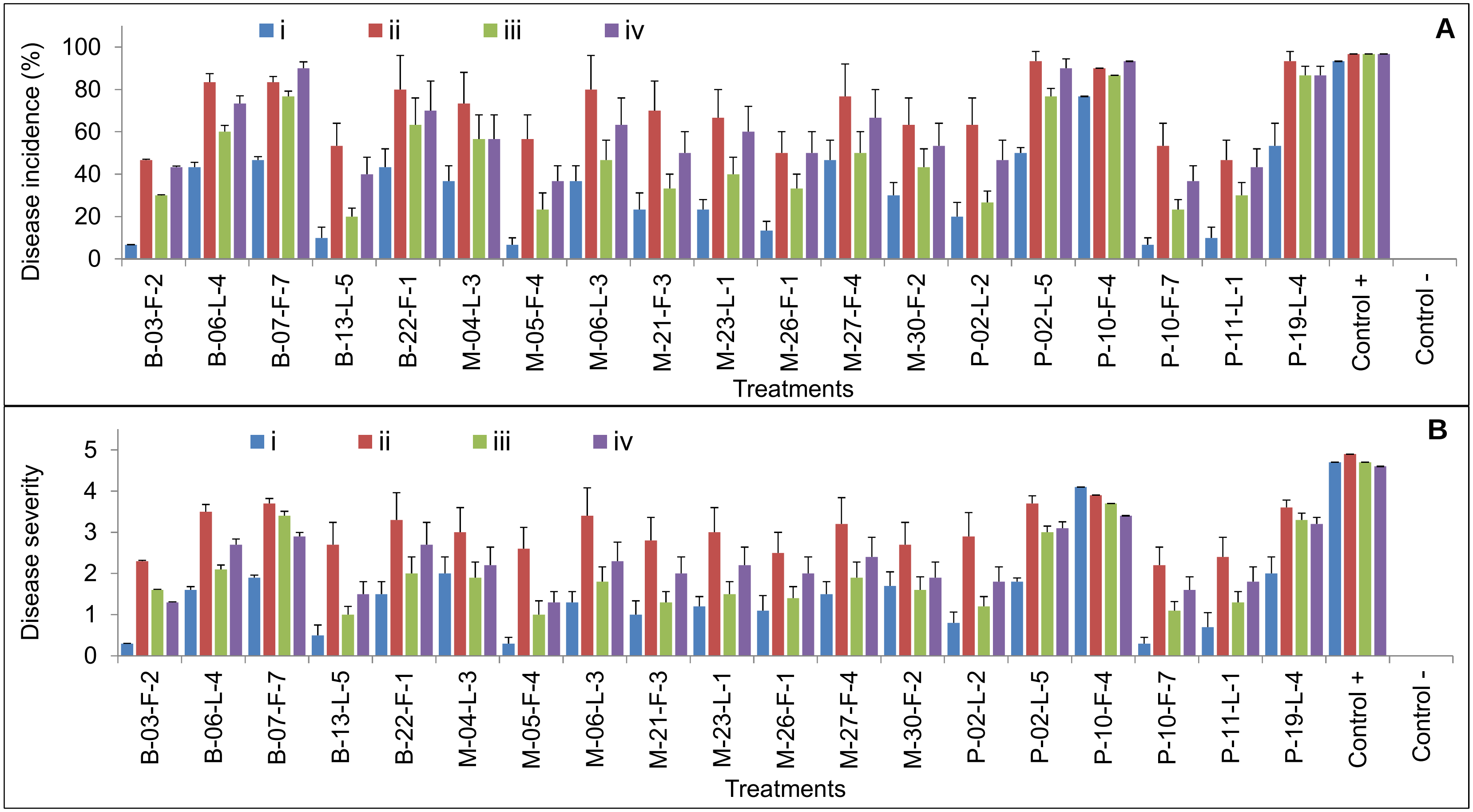


Figure 4. 5 Effect of antagonists on control of anthracnose in artificially inoculated banana fruits

** Treatments applied were (i) 1 x 10^8 CFU/ml unwashed cell/ spore mixture; (ii) 1 x 10^8 CFU/ml washed cell/ spore suspension; (iii); culture filtrate and (iv) autoclaved culture filtrate. The control is treated with pathogen (Control +) and without any treatment (Control -). Fruits were assessed for anthracnose incidence (A), and anthracnose severity (B) after 20 d of incubation after treatment. Five fruits per treatment replicated three times. Error bars represent the standard deviation of three replications. The results from two independent experiments are not significantly different; therefore, data from one representative experiment are presented.

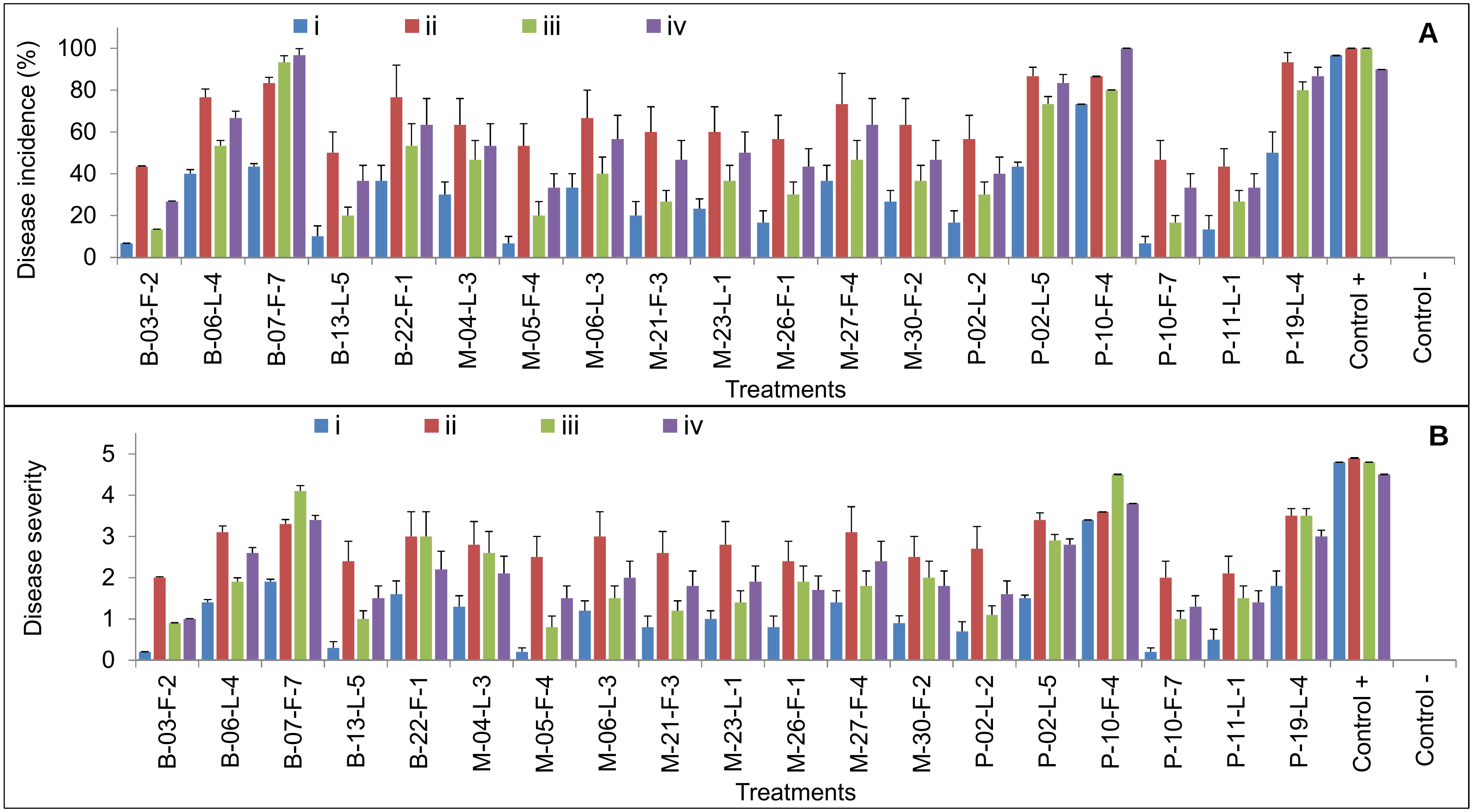


Figure 4. 6 Effect of antagonists on control of anthracnose in artificially inoculated mango fruits

** Treatments applied were (i) 1 x 10^8 CFU/ml unwashed cell/ spore mixture; (ii) 1 x 10^8 CFU/ml washed cell/ spore suspension; (iii); culture filtrate and (iv) autoclaved culture filtrate. The control is treated with pathogen (Control +) and without any treatment (Control -). Fruits were assessed for anthracnose incidence (A), and anthracnose severity (B) after 20 d of incubation after treatment. Five fruits per treatment replicated three times. Error bars represent the standard deviation of three replications. The results from two independent experiments are not significantly different; therefore, data from one representative experiment are presented.

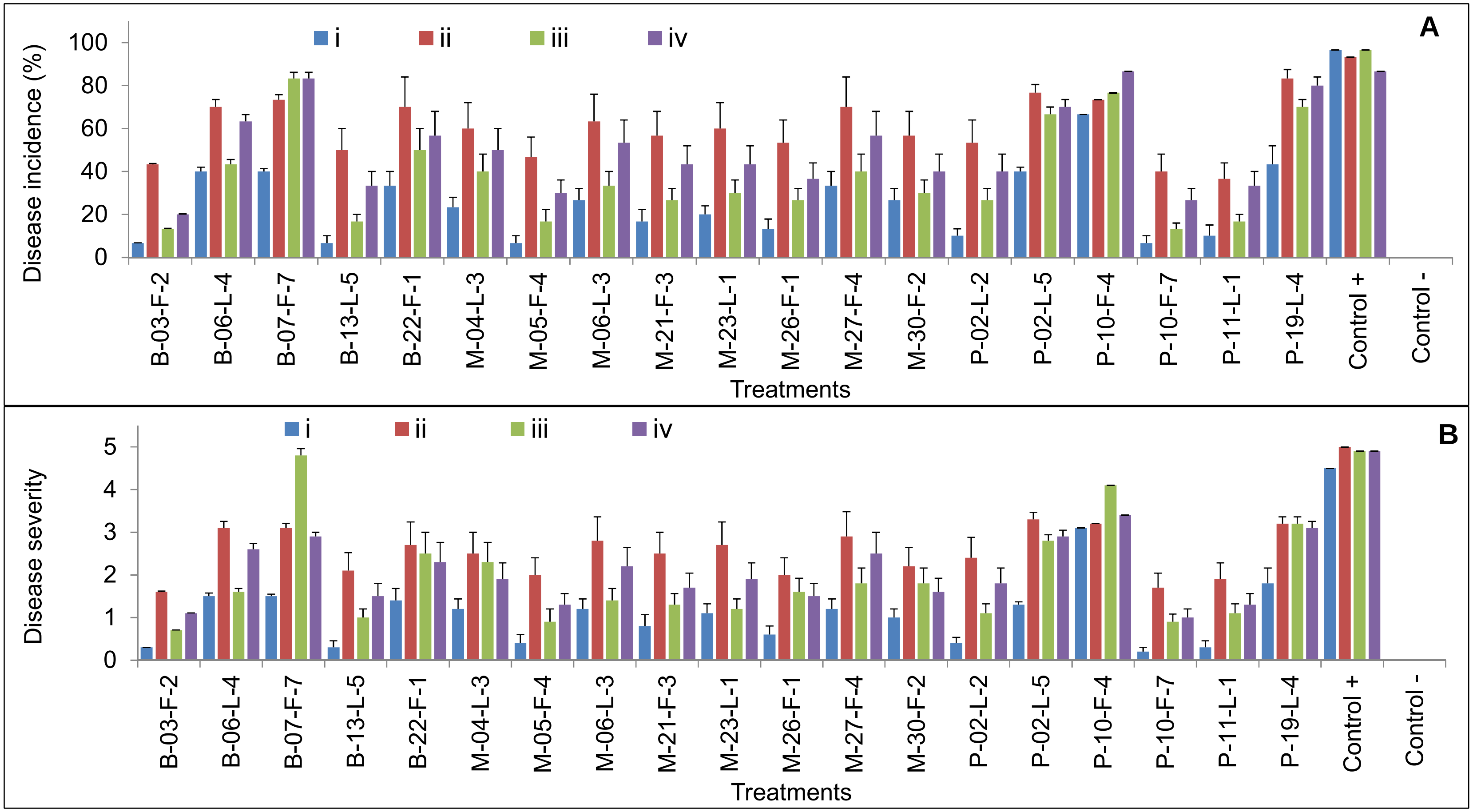


Figure 4. 7 Effect of antagonists on control of anthracnose in artificially inoculated papaya fruits

** Treatments applied were (i) 1 x 10^8 CFU/ml unwashed cell/ spore mixture; (ii) 1 x 10^8 CFU/ml washed cell/ spore suspension; (iii); culture filtrate and (iv) autoclaved culture filtrate. The control is treated with pathogen (Control +) and without any treatment (Control -). Fruits were assessed for anthracnose incidence (A), and anthracnose severity (B) after 20 d of incubation after treatment. Five fruits per treatment replicated three times. Error bars represent the standard deviation of three replications. The results from two independent experiments are not significantly different; therefore, data from one representative experiment are presented.

The most effective hot water treatment (55°C for 5 min.), fungicide (Procloraz 75% a.i. at 1 g/l) and the antagonistic concentration (10^8 cells / ml) were used in different combinations and results obtained are shown in Figure 4.8, 9, 10. The different treatment combinations showed significant ($P \leq 0.05$) variation in their ability to control the anthracnose disease. Out of the three application methods, the lowest disease incidence and severity was found when antagonists were combined with fungicides followed by combination of antagonists with hot water (Figure 4.8, 9, 10).

All treatments tested showed more than 50% reduction of anthracnose on naturally infected banana fruits. Treatments of antagonist isolates in combination with fungicide, and antagonist isolates in combination with hot water, gave more than 95% control against the disease on banana (Figure 4.8). The highest reduction in all disease parameters on banana was achieved when B-03-F-2, P-10-F-7 and P-11-L-1 were applied. There was no significant difference among the three isolates. After 20 d of incubation, the reduction in disease was found to be 88.3, 87.2, and 86.1 %, respectively (Figure 4.8). Isolate M-05-F-4 came in second place with regard to the control of anthracnose, in that it reduced the decay percentage to 84.5 % at the end of the incubation period (Figure 4.8). Three isolates (P-02-L-2, YA06, and M-06-L-3) occupied third place in terms of the control of the disease, and there was no significant difference between their respective effects except after 20 d of incubation. Reduction in the disease as a result of their application was 82.6, 82.1 and 81.3%, respectively. (Figure 4.8). There was no significant difference among these seven isolates and fungicide control.

Isolates B-13-L-5, M-30-F-2 and M-06-L-4 occupied fourth place in terms of the control of the pathogens, and there was no significant difference between their respective effects after 20 d of incubation. Reduction in the disease as a result of their application was 76.6, 73.5, 73.1 %, respectively and it was comparable with hot water control (Figure 4.8).

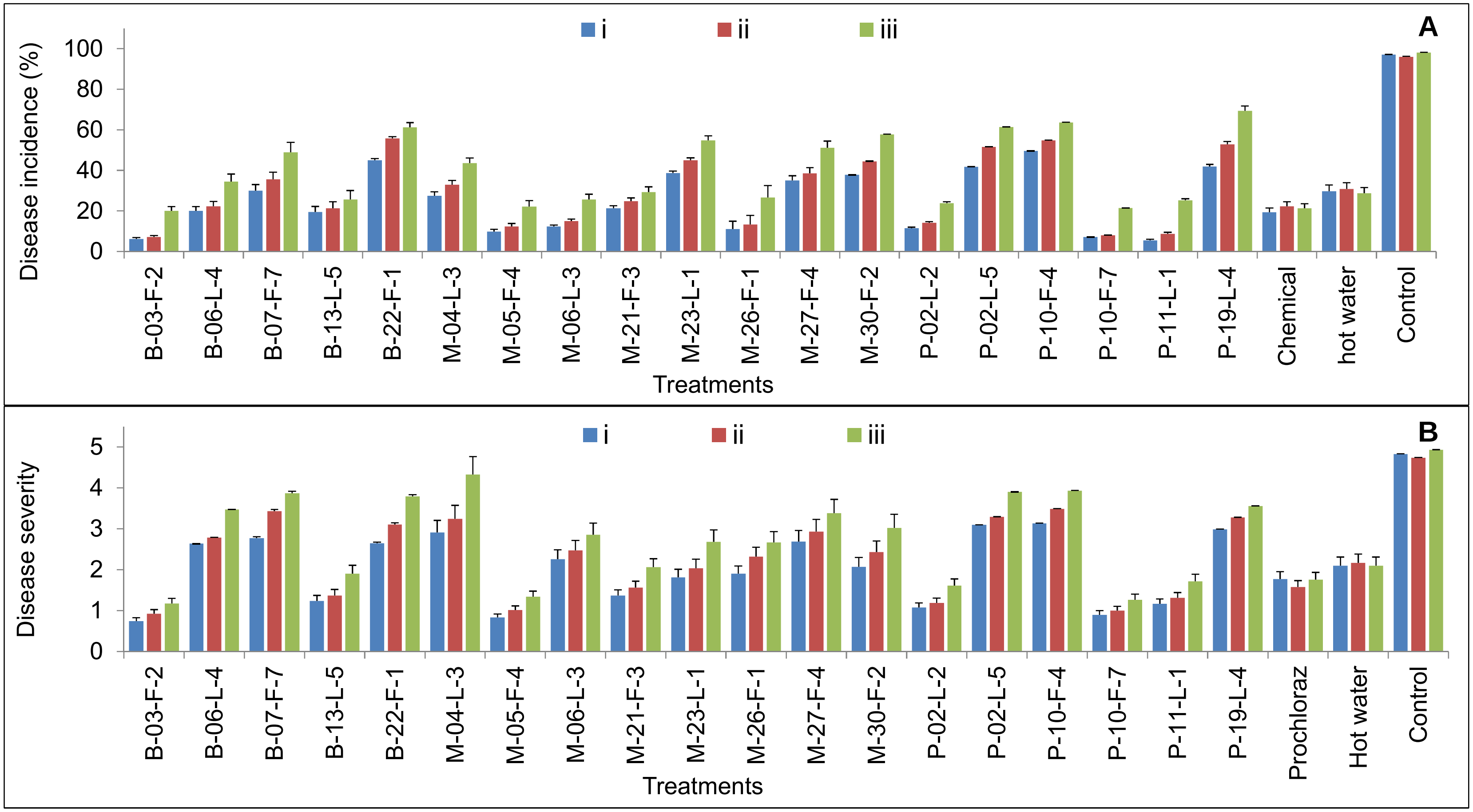


Figure 4. 8 Effect of antagonists and their combinations with hot water and fungicide on anthracnose in naturally infected banana.

**Anthracnose disease incidence (A) and severity (B) on fruits were recorded after stored at 26°C for 3 weeks. Application of antagonist isolates in combination with fungicide (i), with hot water (ii) or on their own (iii) was assessed. Data represent the means of three replicates (Five fruits per treatment). Antagonist isolates (10^8 cells / ml); Prochloraz = (180 ml/ L by dipping for 20 s); and, hot water treatment (55°C for 15 min). Commercial reference standaerd treatments of hot water (55°C for 15 min), and fungicide (Procloraz at 180 ml/ L by dipping for 20 s) and control treatment with sterile water only were maintained.

Figure 4.9 shows the effect of integrated control methods on mango fruit. The antagonist isolates in combination with fungicide and antagonist isolates in combination with hot water gave the highest control of the disease. As shown in Figure 4.9, the highest reduction in all disease parameters on mango was achieved when B-03-F-2, P-10-F-7, P-11-L-1, M-05-F-4, P-02-L-2 and M-23-L-01 were applied. There was no significant difference between results coming from the six isolates, reflectedby disease reduction of 87.3, 86.8, 85.5, 83.8, 82.4 and 80.3 %, respectively after 20 days of incubation (Figure 4.9). The reduction in disease of anthracnose by these six isolates was comparable to fungicide control. Three isolates (M-06-L-3, B-13-L-5 and M-30-F-2) occupied second place in terms of the control of the pathogen. Reduction in the disease as a result of their application was 78.9, 75.7 and 73.6 %, respectively which was comparable to hot water control (Figure 4.9).

Figure 4.10 shows the effect of integrated control methods on papaya fruit naturally infected with anthracnose. The disease development was lowest in papaya treated with antagonist isolates in combination with fungicide and antagonist isolates in combination with hot water. The highest reduction of 88.2% in all disease parameters on papaya was achieved when isolate B-03-F-2 was applied (Figure 4.10). Isolate P-10-F-7, P-11-L-1 and M-05-F-4 came in second place with regard to the control of anthracnose, in that it reduced the decay percentage to 85.9, 85.0 and 84.5%, respectively at the end of the incubation period (Figure 4.10). Isolates P-02-L-2 occupied third place in terms of the control of the pathogen, and disease deduction as a result of its application was 81.1% (Figure 4.10). Isolates M-23-L-01, M-06-L-3 and B-13-L-5 occupied fourth place in terms of the control of the pathogen, and there was no significant difference between their respective effects after 20 d of incubation. Reduction in the disease as a result of their application was 78.9, 77.6 and 77.4%, respectively. There was no significant difference among these eight isolates and fungicide control in disease reduction effect (Figure 4.10).

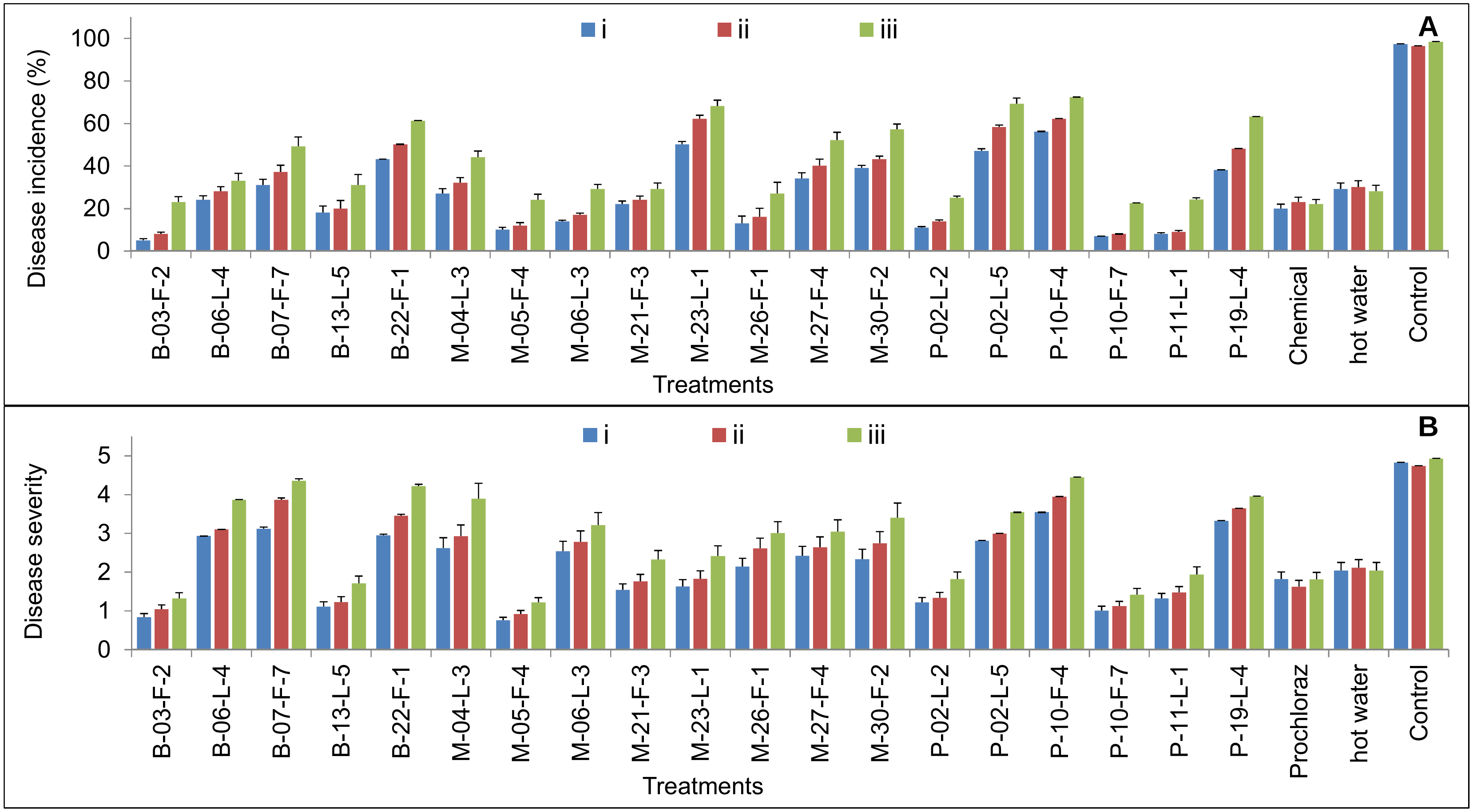


Figure 4. 9 Effect of antagonists and their combinations with hot water and fungicide on anthracnose in naturally infected mango.

**Anthracnose disease incidence (A) and severity (B) on fruits were recorded after stored at 26°C for 3 weeks. Application of antagonist isolates in combination with fungicide (i), with hot water (ii) or on their own (iii) was assessed. Data represent the means of three replicates (Five fruits per treatment). Antagonist isolates (10^8 cells / ml); Prochloraz = (180 ml/ L by dipping for 20 s); and, hot water treatment (55°C for 15 min). Commercial reference standaerd treatments of hot water (55°C for 15 min), and fungicide (Procloraz at 180 ml/ L by dipping for 20 s) and control treatment with sterile water only were maintained.

Isolate M-30-F-2 occupied fifth place and reduction in disease of anthracnose was 71.7 %, after 20 d as a result of the application of this isolate which was comparable to hot water treatment (Figure 4.10). Since *C. acutatum* and *C. gloeosporioides* are responsible for postharvest anthracnose, this information provides confirmatory results on the effectiveness of antagonist isolates in combination with fungicide or in combination with hot water treatments.

Complete suppression of anthracnose development was not observed in any of the antagonist treatments or the commercial control treatments. The anthracnose controlling ability of antagonists was enhanced, to varying degrees, by the different application methods. In particular, different application methods enhanced the anthracnose controlling ability of nine antagonists (B-03-F-2, P-10-F-7, M-05-F-4, P-02-L-2, M-06-L-3, P-11-L-1, B-13-L-5, M-21-F-3 and M-26-F-1) resulting higher disease reduction than the chemical and hot water control treatment. Consistent disease reduction on the three fruits (banana, mango and papaya) evaluated was found by the use of these nine isolates (Figure 4.8, 9 & 10).

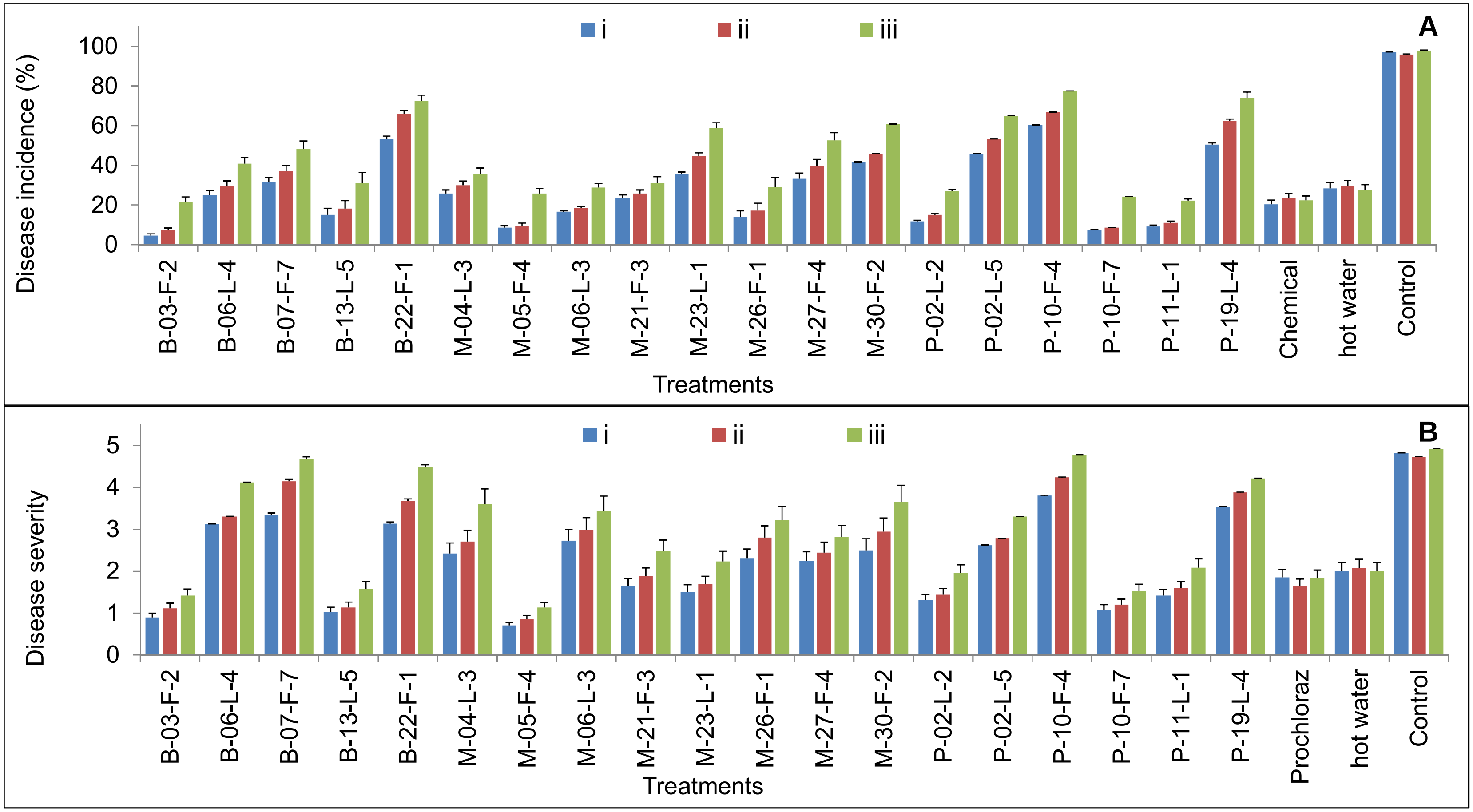
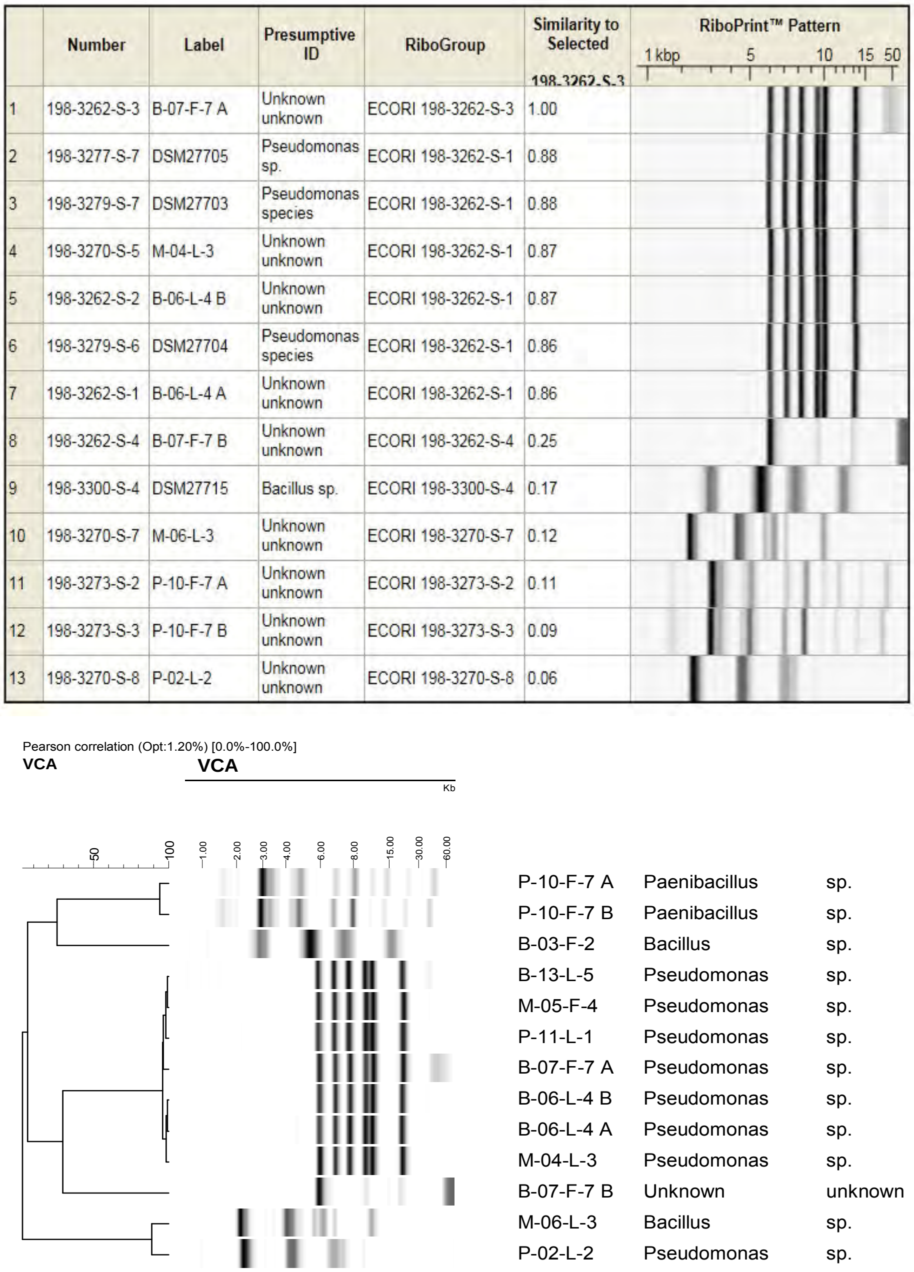


Figure 4. 10 Effect of antagonists and their combinations with hot water and fungicide on anthracnose in naturally infected papaya. **Anthracnose disease incidence (A) and severity (B) on fruits were recorded after stored at 26°C for 3 weeks. Application of antagonist isolates in combination with fungicide (i), with hot water (ii) or on their own (iii) was assessed. Data represent the means of three replicates (Five fruits per treatment). Antagonist isolates (10^8 cells / ml); Prochloraz = (180 ml/ L by dipping for 20 s); and, hot water treatment (55°C for 15 min). Commercial reference standaerd treatments of hot water (55°C for 15 min), and fungicide (Procloraz at 180 ml/ L by dipping for 20 s) and control treatment with sterile water only were maintained.

Biochemical tests and molecular techniques, including RiboPrinter, MALDI-TOF MS and 16S rRNA, were performed at DSZ culture collection in order to identify the promising antagonistic bacterial isolates. Results of the RiboPrinter system pattern from DSMZ revealed that patterns of all bacterial isolates, except P-10-F-7, are too dissimilar to those of the Identification Library. Strain P-10-F-7 is the only isolate for which the RiboPrinter system provides a proposal for identification: ***Paenibacillus graminis***. The RiboPrint patterns of strains P-11-L-1 (DSM 27705), B-13-L-5 (DSM 27703), M-05-F-4 (DSM 27704), M-04-L-3 and B-06-L-4 were affiliated to the RiboGroup ECORI 198-3262-S-1 (Figure 4.14). Patterns sharing the same RiboGroup cannot be differentiated by the RiboPrinter System and the respective strains should be considered identical (Figure 4.11). List of bacterial species obtained from gen bank used to compare antagonist isolates based on riboprint, MALDI-TOF MS and gene sequence are listed in Appendix Table 6.

The MALDI-TOF MS Identification Report is shown in Figure 4.12 and Table 4.6. The bacterial strains: B-06-L-4, B-13-L-5 (DSM 27703), M-05-F-4 (DSM 27704), P-11-L-1 (DSM 27705), M-04-L-3 and B-07-F-7 belong to the *Pseudomonas fluorescens* group. According to MALDI-TOF MS, all six strains show the highest similarity to *Pseudomonas synxantha* within this group. Strain P-10-F-7 was tentatively identified as *Paenibacillus brasilensis* or *Paenibacillus peoriae* by MALDI-TOF MS. The *Bacillus* strains B-03-F-2 (DSM 27715), M-06-L-3 (DSM 27716) and P-02-L-2 (DSM 27717) belong to the *Bacillus subtilis* group. Within this group, strains M-06-L-3 (DSM 27716) and P02-L-2 (DSM 27717) were tentatively identified by MALDI-TOF MS as *Bacillus amyloliquefaciens* or *Bacillus atrophaeus*. Strain B-03-F-2 (DSM 27715) was tentatively identified as *Bacillus mojavensis* or *Bacillus subtilis*.



	Number	Label	Presumptive ID	RiboGroup	Similarity to Selected 198-3262-S-3	RiboPrint™ Pattern 1 kbp 5 10 15 50
1	198-3262-S-3	B-07-F-7 A	Unknown unknown	ECORI 198-3262-S-3	1.00	
2	198-3277-S-7	DSM27705	Pseudomonas sp.	ECORI 198-3262-S-1	0.88	
3	198-3279-S-7	DSM27703	Pseudomonas species	ECORI 198-3262-S-1	0.88	
4	198-3270-S-5	M-04-L-3	Unknown unknown	ECORI 198-3262-S-1	0.87	
5	198-3262-S-2	B-06-L-4 B	Unknown unknown	ECORI 198-3262-S-1	0.87	
6	198-3279-S-6	DSM27704	Pseudomonas species	ECORI 198-3262-S-1	0.86	
7	198-3262-S-1	B-06-L-4 A	Unknown unknown	ECORI 198-3262-S-1	0.86	
8	198-3262-S-4	B-07-F-7 B	Unknown unknown	ECORI 198-3262-S-4	0.25	
9	198-3300-S-4	DSM27715	Bacillus sp.	ECORI 198-3300-S-4	0.17	
10	198-3270-S-7	M-06-L-3	Unknown unknown	ECORI 198-3270-S-7	0.12	
11	198-3273-S-2	P-10-F-7 A	Unknown unknown	ECORI 198-3273-S-2	0.11	
12	198-3273-S-3	P-10-F-7 B	Unknown unknown	ECORI 198-3273-S-3	0.09	
13	198-3270-S-8	P-02-L-2	Unknown unknown	ECORI 198-3270-S-8	0.06	

Figure 4. 11 Riboprint patterns of EcoRI restricted DNA of the antagonist bacterial isolates studied

**Identification was conducted at DSMZ-German Collection of Microorganisms and Cell Cultures, Germany. RiboPrinting using the restriction enzyme EcoRI was applied by the method of Schumann and Pukall (2013). RiboPrint patterns of ten bacterial isolates. Cluster analysis was performed by UPGMA based on the Pearson correlation coefficient (opt. 1.20 %) [0.0–100.0%]. VCA indicates a standard EcoRI batch. Patterns sharing the same RiboGroup cannot be differentiated by the RiboPrinter System and the respective strains should be considered identical. List of bacterial species obtained from gen bank used to compare antagonist isolates based on riboprint, MALDI-TOF MS and gene sequence are listed in Appendix Table 6.

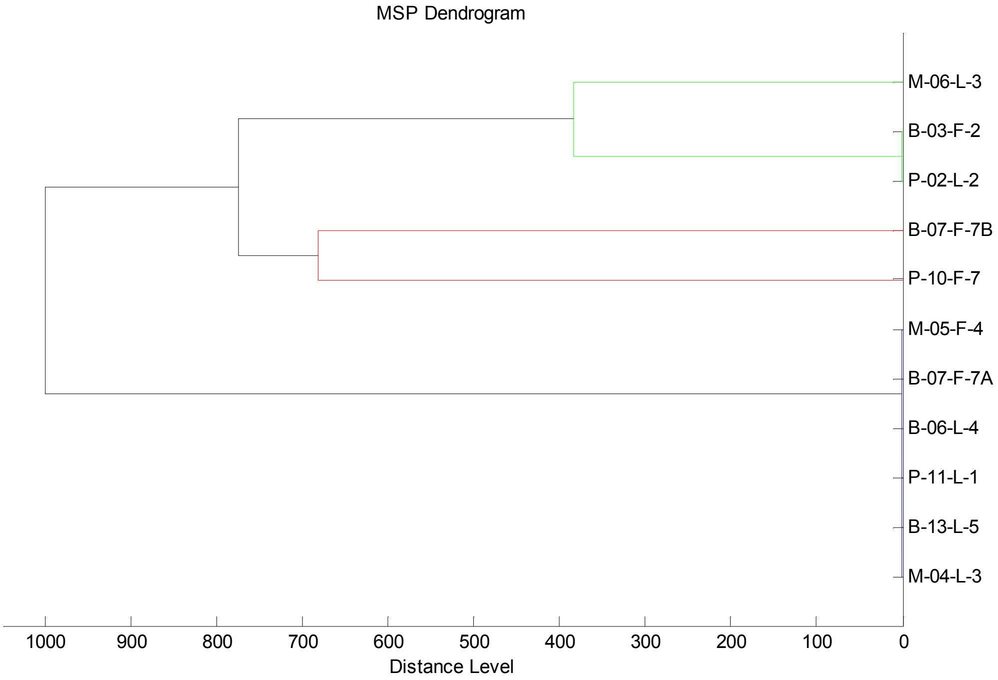


Figure 4. 12 Dendrogram showing the diversity of MALDI-TOF MS of 10 antagonist bacterial strains (*Pseudomonas, Bacillus* and *Paenibacillus*).
** Identification was conducted at Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. MALDI-TOF MS was conducted as described by Toth *et al.* (2008). List of bacterial species obtained from gen bank used to compare antagonist isolates based on riboprint, MALDI-TOF MS and gene sequence are listed in Appendix Table 6.

A stretch of 900 bases of the 16S rRNA gene of selected strains was sequenced at DSMZ and the sequence similarities resulted from a search by using the EzTaxon-e Database is presented in Table 4.7. Isolate P-10-F-7 (DSM 27718) belong to the genus *Paenibacillus* and showed high sequence similarity with *P. polymyxa* (99.88%), *P. jamilae* (99.77%), *P. peoriae* (99.64%) and *P. brasiliensis* (99.3%). All *Bacillus* strains [B-03-F-2 (DSM 27715), M-06-L-3 (DSM 27716) and P-02-L-2 (DSM 27717)] are related to the *B. subtilis* group. The *Pseudomonas* strains (B-06-L-4, B-13-L-5 (DSM 27703), M-05-F-4 (DSM 27704), P-11-L-1 (DSM 27705), M-04-L-3 and B-07-F-7) could not be sufficiently resolved by 16S rRNA gene sequence analysis. In general, Ethiopian *Pseudomonas* and *Bacillus* groups could neither be sufficiently re-solved by MALDI-TOF MS nor by 16S rRNA gene sequence analysis.

The three *Pseudomonas* strains were deposited at DSMZ with the accession numbers DSM 27703 (strain B-13-L-5), DSM 27704 (strain M-05-F-4) and DSM 27705 (strain P-11-L-1.). The three strains were not distinguishable from each other by Riboprinting (Qualicon), that is, at the species or subspecies level. DSMZ could not assign the strains properly to a species. The members of the species cluster *P. fluorescens* (biochemical identification) including *P. synxantha* or *P. libanensis* (next similar MALDI-TOF spectra) and *P. azotoformans* or *P. libanensis* (highest similarity of the 16S rRNA sequence according to EzbioCloud) were highly related. The corresponding positions of the nine identified bacteria (*Pseudomonas* and *Bacillus* strains) in their phylogenetic trees further indicated that they were new bacterial species. The *Bacillus* strains: B-03-F-2 (DSM 27715), M-06-L-3 (DSM 27716) and P-02-L-2 (DSM 27717), and a *Paenibacillus* strain: P-10-F-7 (DSM 27718) were also given DSM numbers and added to those isolates that were deposited in the DSMZ culture collection.

Yeast strains M-23-L-1, M-27-F-4, B-22-F-1, P-19-L-4, P-02-L-5, P-10-F-4 and M-21-F-3 were found to be different in colony characteristics and cell morphologies. The results of physiological characteristics of yeast strains according to fermentation and assimilation tests indicated that M-23-L-1, M-27-F-4, B-22-F-1, P-19-L-4, P-02-L-5 and P-10-F-4 showed high similarity with species of *Candida*, while M-21-F-3 showed high similarity with those of *Rhodotorula* (Table 4.8).

In order to verify species identification of the most effective yeast (M-23-L-1) and fungal (M-30-F-2) isolates, ITS-1 and ITS-4 sequences of these isolates were analyzed with previously reported sequences of other yeast and fungal species in the GenBank of NCBI. Analysis of the ITS-1 sequence grouped the fungal isolate M-30-F-2 as *Trichoderma longibrachiatum (100%)* while the yeast isolate, M-23-L-1, showed high identity (99%) with those of *Candida rugosa* (Table 4.8).

Table 4. 6 The MALDI-TOF MS Identification Report of bacterial isolates from DSMZ

Isolate-code	Proposed species compared with isolate	Similarity	Proposed species compared with isolate	Similarity
P-11-L-1 (+++) B	*Pseudomonas synxantha*	2.435	*Pseudomonas libanensis*	2.352
P-10-F-7 (+) B	*Paenibacillus brasilensis*	1.976	*Paenibacillus peoriae*	1.958
P-02-L-2 (+) B	*Bacillus amyloliquefaciens*	1.739	*Bacillus atrophaeus*	1.704
M-06-L-3 (+) B	*Bacillus amyloliquefaciens*	1.704	*unzuverlassige Identifikation*	1.523
M-05-F-4 (+++) B	*Pseudomonas synxantha*	2.464	*Pseudomonas libanensis*	2.281
M-04-L-3 (+++) B	*Pseudomonas synxantha*	2.383	*Pseudomonas rhodesiae*	2.255
B-13-L-5 (++) B	*Pseudomonas synxantha*	2.309	*Pseudomonas rhodesiae*	2.185
B-06-L-4 (+++) B	*Pseudomonas synxantha*	2.417	*Pseudomonas rhodesiae*	2.31
B-03-F-2 (+) B	*Bacillus mojavensis*	1.749	*Bacillus subtilis*	1.727
B-07-F-7A (++) B	*Pseudomonas synxantha*	2.139	*Pseudomonas tolaasii*	2.056

Table 4. 7 Comparison of sequences of Ethiopian bacterial isolates with other bacterial isolates

	Isolate	Comparison isolates from DSMZ		
No	**Code**	**Genus**	**Species**	**Similarity (%)** [a]
1	M-06-L-3	*Bacillus*	*Bacillus siamenis*	100%
			B. amyloliquefaciens	99.89%
			B. atrophaeus	99.77%
			B. subtilis subsp. *subtilis*	99.66%
2	M-05-F-4	*Pseudomonas*	*Pseudomonas azotoformans*	99.80%
			P. libanensis	99.60%
			P. gessardii	99.50%
			P. cedrina	99.30%
			P. synxantha	99.30%
3	B-03-F-2	*Bacillus*	*Bacillus siamenis*	100%
			B. subtilis subsp. *Subtilis*	99.77%
			B. amyloliquefaciens	99.77%
			B. mojavensis	99.31%
4	P-02-L-2	*Bacillus*	*B. siamenis*	100%
			B. amyloliquefaciens	99.88%
			B. atrophaeus	99.77%
			B. subtilis subsp. *subtilis*	99.65%
5	P-10-F-7	*Paenibacillus*	*Paenibacillus polymyxa*	99.88%
			Pa. jamilae	99.77%
			Pa. peoriae	99.64%
			Pa. brasiliensis	99.30%

[a] Comparison was done at DSMZ from a search of 900 bases of the 16S rRNA gene of selected strains by using the EzTaxon-e Database

Table 4. 8 Comparison of nucleotide sequences and biochemical reactions of fungal and yeast isolate with those of other fungal isolates from BLAST searches of GenBank

		Nucleotide sequences comparison [a]			**Biochemical reactions** [b]
Isolate [c]	**Description**	**Max score**	**Identity** [d]	**Accession** [e]	
M-30-F-2	*T. longibrachiatum* 12711	1083	100%	KC254099.1	ND [f]
	T. longibrachiatum 13448	1083	99%	KC254100.1	ND
	T. longibrachiatum LIPIMC0570	1081	99%	KC847179.1	ND
	T. longibrachiatum TP14	1081	99%	JN039071.1	ND
	T.longibrachiatum TP13	1081	99%	JN039070.1	ND
	T.longibrachiatum TL2	1079	99%	JN039076.1	ND
	T.longibrachiatum TL3	1077	99%		ND
M-26-F-1	*Alternaria tenuissimia*	1077	100%		
M-23-L-1	*C. rugosa* strain ONF19B	632	99%	KF479404.1	ND
	C. rugosa subtype B	625	99%	HM641832.1	ND
	C. rugosa strain ATCC 10571	588	94%	GU144663.1	ND
	C. sp. 10-1 genes	573	94%	AB727605.1	ND
M-27-F-4	*Candida laurentii*	ND	ND	ND	Fermentation & assimilation
B-22-F-1	*Candida saitoana*	ND	ND	ND	"
P-19-L-4	*Candida quillermondil*	ND	ND	ND	"
P-02-L-5	*Candida musae*	ND	ND	ND	"
P-10-F-4	*Candida altosa*	ND	ND	ND	"
m-21-F-2	*Rhodotorula glutinis*	ND	ND	ND	"

[a] The percentage identity among DNA fragments was calculated with BLAST program and the sequences were compared with those from NCBI database.

[b] Comparison of biochemical reactions (using fermentation and assimilation tests) of the yeast isolates were conducted

[c] isolates M-30-F-2 and M-26-F-1 are filamentus fungi while isolates M-23-L-1, M-27-F-4, B-22-F-1, P-19-L-4, P-02-L-5, P-10-F-4 and m-21-F-2 are yeast.

[d] Percentage similarity of DNA with other isolates from NCBI database.

[e] Acession number of isolates at NCBI to which the current isolates were compared

[f] ND=Not determined

4.4. Discussion

In the present study, naturally occurring bacteria, yeast and fungal strains were isolated from the tissues of banana, mango and papaya and tested for their potentials as antagonists against *C. gloeosporioides* and *C. acutatum*.

A total of 653 bacterial, 594 yeast and 319 mold isolates were isolated from the tissues of healthy banana, mango and papaya in Ethiopia and screened *in vitro* against *C. gloeosporioides* and *C. acutatum*. Out of these, 19 isolates (ten bacterial, seven yeast and two filaments fungi) showed antagonistic activity against isolates of *C. gloeosporioides* and *C. acutatum* and selected for further screening. Preliminary results showed that, antibiotics, direct parasitism and production of lytic enzymes was the mechanisms exhibited by microbial antagonists. Similar to these finding, a significant amount of research on such modes of action of microbial antagonists has been reported by previous workers (Wisniewski *et al.*, 1991; Wilson *et al.*, 1993; Droby *et al.*, 2002).

In the present study, bacterial, yeast and filamentous fungi isolates inhibited radial growth by establishing a clear inhibition zone in dual culture tests. Bacterial antagonists such as *B. subtilis* produced several kinds of antimicrobial peptide substances including subtilin, bacilysin, mycobacillisyn, and iturin (Yoshida *et al.*, 2001). Several yeast isolates including *Debaryomyces melissophilus*, *Rhodotorula glutinis* and *Cryptococcus laurentii* are known to produce antibiotics (Arras, 1996; Ragaert *et al.*, 2006). The fungal antagonists such as *Trichoderma*, are known to produce a number of antibiotics, including trichodermin, trichodermol, trichotoxin, harzianum A and harzianolide (Dennis and Webster, 1971). Such compounds could be responsible for most of the inhibition of *Colletotrichum* isolates by antagonists in this study. The inhibition of radial growth by the forming of an inhibition zone against *C. acutatum* and *C. gloeosporoides* is considered as antibiosis, whereby the antibiotic metabolites may penetrate the pathogen cell and inhibit its activity by chemical toxicity (Compant *et al.*, 2005).

Lyses of mycelia of the pathogen by bacterial and yeast isolates markedly affected growth of the pathogen. The challenged mycelia of the pathogen showed a disintegrated appearance when examined microscopically. In experiments with *Aureobasidium pullulans*, a yeast-like antagonist of postharvest pathogens, exo-b-1,3-glucanase activity was detected both in vitro and in apple wounds (Castoria *et al.*, 2001). Microbial antagonists produce lytic enzymes such as gluconase, chitinase, and proteinases that help in the cell wall degradation of the pathogenic fungi (Castoria *et al.*, 2001).

The *in vitro* assay confirmed the effectiveness of antagonist isolates for parasitizing the mycelia of *C. acutatum* and *C. gloeosporoides*. Similar results have been reported in dual cultures (Aryantha and Guest, 2006; Begum *et al.*, 2008). The mycoparasitism of the antagonist isolates varied among each other and with combination with the pathogen species. All antagonist isolates were capable of growing on the pathogen mycelium, although not all mycelium were fully parasitized. This result agrees with results from Rey *et al.* (2005) in which some fungal antagonist species are able to parasite mycelium, and their effectiveness depended on the antagonist isolate and on the target pathogen. The colonization and subsequent growth inhibition observed on the pathogen colony of both pathogens by the antagonists may be attributed to the release of hydrolytic enzymes such as α 1-3-glucanase, chitinase, protease, and cellulase, which are a key step for initiating the pathogen cell wall degradation during mycoparasitism (Rey *et al.*, 2005). Begum *et al.* (2008), also found that the fungal isolates coiled around the hyphae of *Colletotrichum truncatum*. Mycoparasitism is the most common modes of antagonism of antagonists toward fungal plant pathogens (Sutton, 1992).

Inhibition of spore germination was used as a bioassay in this experiment to evaluate the level of antifungal activity of microbial antagonists against *C. acutatum* and *C. gloeosporioides*. The inhibitory effect on conidial germination of *Colletotrichum* species were observed by all of the microbial antagonists. The antagonistic isolates exhibited varying degrees of inhibition on the conidial germination of *C. acutatum* and *C. gloeosporioides*. Similar observations have been reported by Korsten and DeJager (1995) who reported that the effectivnes of several antagonists against *C. gloeosporioides* was variable in vitro. This finding complies with other reports which asserted that antagonist isolates are very effective at inhibiting conidia germination of fungal pathogens ***In vitro*** (Haran ***et al.***, 1996; Tronsmo and Hjeljord, 1997). The current study showed that antagonist isolates which had antibiosis, lysis and mycoparasitic activity, affected spore germination and germ tube elongation of *C. acutatum* and *C. gloeosporiodes.*

The results of this study showed that all isolates indused cellulase and chitinase, whereas glucanase, protease and pecinase were produced by four isolates only. This result supported by the findings of El-Katatny *et al.* (2001) Who showed that chitinase and endo-b-13-glucanase production from *Trichoderma harzianum* differ among isolates. The finding that extracellular enzymes (cellulase and chitinase) were produced during the antagonist's growth suggests that they have a role in breaking down complex polysaccharide polymers into small subunits, which can then be consumed as a carbon source by the antagonists. This finding may have important implications regarding the possible involvement of these enzymes in the biocontrol activity of the antagonist against *C. acutatum* and *C. gloeosporioides*. In addition, production of these lytic enzymes may have a direct effect on the pathogens (*C. acutatum* and *C. gloeosporioides*) mycelium.

The cellulolytic and chitinolytic activity of the antagonists could help the antagonistic activity of the current sturdy isolates through affecting the structural integrity of the walls of *C. gloesporioides* and *C. acutatum* leading to the release of molecules which may be responsible for deregulation of membrane-bound enzymes. Such a mechanism has been proposed to explain cell wall abnormalities induced by chitosan in *Fussarium oxysporum* and *Pythium aphanidermatum* (Benhamou, 1992; El-Gaouth *et al.*, 1994) or by the fungicide cyproconazole in *Sclerotium rolfsii* (Fuller *et al.*, 1990). Glucanases are another group of key enzymes involved in the lytic activity of antagonist isolates. These enzymes hydrolyze ß-1,6-glucans, the minor structural polymers of the fungal cell wall (Viterbo *et al.*, 2002).

The present study revealed that antifungal activities of the antagonists were affected by the type of extraction solvent. The current study revealed that chloroform and ethyl acetate extracts were more effective than methanol and petroleum ether extracts. The chloroform extracts of fungal and yeast antagonists were effective against the test pathogen; while, the ethyl acetate extract of bacterial isolates was effective against the test pathogen. Antifungal activity depends on both, microorganism and the efficiency of the extraction method finding (DelVal *et al.*, 2001; Yoshida *et al.*, 2001; Ilic *et al.*, 2007; Martin *et al.*, 2008).

This result reflects the presence of bioactive metabolites of the microbial isolates which are soluble in these solvents. There have been reports on significant antimicrobial activities of microorganisms by several workers which confirms the current finding (DelVal *et al.*, 2001; Yoshida *et al.*, 2001; Ilic *et al.*, 2007; Martin *et al.*, 2008). Some studies concerning the effectiveness of extraction methods highlight that methanol extraction yields higher antimicrobial activity than ethyl acetate (Sastry and Rao, 1994), whereas others reported that chloroform is better than methanol and benzene (Febles *et al.*, 1995; Ilic *et al.*, 2007).

In this present study it was found that the antifungal metabolite production of antagonists was affected by types of culture medium. Bacterial isolates incubated in Starch Nitrate Broth (SNB) medium gave highest inhibition of the test pathogens, whereas, yeast and fungal isolates showed maximum inhibition in YEME broth and PD broth media respectively. This result is in agreement with Hwang *et al.* (2002) who observed that the maximum cell concentration and antibiotic production of the bacterial antagonists were found in SNB medium.

In the *in vivo* tests under artificial infection conditions, anthracnose incidence and severity control was obtained with unwashed cell suspensions of antagonists at levels significantly better than other treatments. Moreover, culture filtrate also reduced disease incidence, but the efficacy was lower than unwashed cell suspensions. This was a similar phenomenon from some other antagonists such as grey mold and blue mold on apple by *Cryptococcus albidus* (Fan and Tian, 2001) and gray mold decay of strawberry with *Rhodotorula glutinis* (Zhang *et al.*, 2007). Such results may be due to some other mechanism of the antagonists, other than secretion of active metabolites as reported by some authors (Chet, 1987; Arras, 1996; Castoria *et al.*, 2001; Droby *et al.*, 2002; Stockwell and Stack, 2007). On the other hand, autoclaved cultures and washed suspensions of antagonists also reduced disease incidence, but the efficacy was significantly lower than unwashed cell suspensions and culture filtrates. Spadaro *et al.* (2002) reported that the autoclaved cells of *Metschnikowia pulcherrima* were ineffective on the control of *B. cinerea*.

In this study antagonist isolates strongly reduced anthracnose on banana, mango and papaya when applied 24 h after the pathogen. This result is very interesting for postharvest treatments as pathogens may infect fruit before antagonist applications during harvest and postharvest handling operations (Jarvis, 1994; Janisiewicz, 1998). As antagonists were isolated from these hosts, the results strengthen the opinion that biocontrol agents should be more competitive in the same environment and on the same host from where they were isolated (Wilson *et al.*, 1993; Lima *et al.*, 1997; Rahman *et al.*, 2007; Begum *et al.*, 2008).

Integrated control has always been studied as a viable option to bring about a more complete disease control strategy (Guetsky *et al.*, 2002; Buck, 2004; Spadaro *et al.*, 2004). This approach was also chosen in this study and antagonist combined with chemicals brought about control that was comparable to the standard treatment used in the store house. In all the fruits, the chemical control provided effective control. This is comparable with results obtained previously by Roux and Boshoff (1999). In semicommercial studies they found that anthracnose was effectively controlled when prochloraz was included in the treatment. The antagonist combined with both the hot water and the prochloraz used in the storage house generally indicated a lower disease incidence than the antagonist on its own as was previously found on *Bacillus* species (Korsten *et al.*, 1992; DeVilliers and Korsten, 1994).

When the hot water was combined with antagonists for control of anthracnose in naturally inoculated fruit, the efficacy was higher than antagonistic treatment alone. Similar results were obtained by Zong *et al.* (2010) This suggested that hot water could enhance the biocontrol efficacy of antagonists on fruit. Similar results showed that the combination of *Metschnikowia pulcherrima* and hot water treatment was more effective to control *Pencillium expansum* and *B. cinerea* on apple fruit than individual treatment (Spadaro *et al.*, 2004). The synergistic effect may be a result of several different interactions taking place.

Sequencing of 16S rRNA provided consistent results in identifying a set of 10 bacterial strains. They emerged as strains belonging to the ***P. fluorescens*** group (designed B-06-L-4, B-13-L-5, M-05-F-4, P-11-L-1, M-04-L-3 and B-07-F-7), ***B. subtilis*** group (B-03-F-2, M-06-L-3 and P-02-L-2) and ***Paenibacillus*** group (P-10-F-7). The RiboPrint patterns, MALDI-TOF MS analysis and 16S rRNA homology provided the necessary information for phylogenetic position of the six different ***Pseudomonas*** strains, indicating that they form new and distinct bacterial strains in ***P. fluorescens*** species for biocontrol purpose. These strains were found to be highly related to ***P. fluorescens*** on the basis of comparisons of the 16S rRNA gene sequence, indicating a clear affiliation to the species ***P. fluorescens*** according to taxonomic rules (Wayne ***et al.***, 1987). However, when compared to other strains of ***P. fluorescens***, the antagonists showed differences in the Ribotyping pattern that is normally used to distinguish species.

Another powerful fingerprint technique that enables differentiation at the genus, species and sometimes even strain level is MALDI-TOF MS (Conway ***et al.***, 2004). MALDI-TOF MS analysis of the novel antagonist isolates and their close phylogenetic relatives analysis was performed. The antagonist isolates formed a cluster that was separated from the related ***Pseudomonas fluorescens*** species in the DSMZ library by clear differences in spectral patterns. Comparable to the Ribotyping results, the spectra of ***P. fluorescens*** were closer to those of the novel antagonist isolates of the current study. These results supported the assumption that the novel strains formed a separate group within the species ***P. fluorescens***.

Bacterial strain P-10-F-7 was identified as ***Paenibacillus polymyxa***. This species was formerly known as ***Bacillus polymyxa*** and reclassified by Ash ***et al.*** (1993). Strains of this species were isolated previously from white clover, perennial ryegrass, crested wheatgrass (Holl ***et al.***, 1988), and wheat (Heulin ***et al.***, 1994) as a biological control agent aginst fungal plant pathogens.Some strains of ***P. polymyxa*** exhibit plant growth-promoting properties, while others produce various peptide antibiotics (Holl ***et al.***, 1988; Rosado and Seldin, 1993; Heulin ***et al.***, 1994). In addition, there are many reports in the literature of antimicrobial and antifungal properties of ***P. polymyxa*** isolates against ***Fusarium*** species where the nature of the inhibitory agent is undefined (Rosado and Seldin, 1993; Seldin *et al.*, 1999). Therefore, antibiotic production is a frequently encountered but non uniform characteristic of *P. polymyxa* strains.

Three bacterial isolates from Ethiopia were determined to be in the *B. subtilis* group based on its ribotyping, MALDI-TOF MS patterns and 16S rRNA sequence similarity. Within this group, strains M-06-L-3 (DSM 27716) and P02-L-2 (DSM 27717) were tentatively identified by MALDI-TOF MS as *Bacillus amyloliquefaciens* or *Bacillus atrophaeus*. Strain B-03-F-2 (DSM 27715) was tentatively identified as *Bacillus mojavensis* or *Bacillus subtilis*. However, isolates in this group could not be sufficiently re-solved by MALDI-TOF MS nor by 16S rRNA gene sequence analysis.

Over the last 20 years, several species very closely related to *B. subtilis* have been described. These species include *Bacillus amyloliquefaciens*, *B. atrophaeus*, *B. axarquiensis*, *B. malacitensis*, *B. mojavensis B. sonorensis*, *B. tequilensis, B. vallismortis* and *B. velezensis* (Wang et al., 2008a). Most of these above species share a remarkably high level of 16S rRNA gene sequence similarity to *B. subtilis* (often 99% or greater). Moreover, very few phenotypic or biochemical characteristics can be found that differentiate these species from *B. subtilis*. This high degree of phenotypic and biochemical similarity prompted Govender *et al.* (2005) to characterize species closely related to *B. subtilis* as members of the 'subtilis-group' or 'subtilis spectrum'. Therefore, we propose that the three *Bacillus* isolates from Ethiopia should be recognized as representing novel taxa in the *B. subtilis* group. However, detailed biochemical and molecular characterization is still required.

The most effective yeast strain from Ethiopia M-23-L-1 was successfully identified as *Candida rugosa* by using the smaller subunit, and ITS-1/ ITS-4. Natural epiphytic yeasts including *Candida sake*, *C. pulcherrina* and *Aurobasidium pullulans* demonstrated promising biocontrol activity against several postharvest pathogens including *Botrytis cinerea* and *Penicillium digitatum* (Arras, 1996; Droby *et al.*, 2002; Saligkarias *et al.*, 2002). One of the antagonist yeast isolates were identified as *Rhodotorula* species. *Rhodotorula* species including, *Rhodotorula glutinis* (Fresenins) Harrison has been proposed for the postharvest biological control of blue mold decay of pears (Zhang *et al.*, 2008), green mold decay of oranges (Zhang *et al.*, 2007) and gray mold decay of apple (Sansone *et al.*, 2005). However, there is no information concerning the effect of Rhodotorula on control of the postharvest decay of fruits in Ethiopia.

Molecular analysis of the fungal isolates showed that isolate M-30-F-2 and M-26-F1 likely represents as *T. longibrachiatum* and *A. tenuissima*, respectively. The fungus *Trichoderma* is one of the biological control agents most widely used in agriculture as an alternative to synthetic chemical products (Chet, 1987; Kindermann *et al.*, 1999). Isolates of this fungus have been employed against a wide spectrum of phytopathogenic fungi, including *Fusarium oxysporum*, responsible for Fusarium wilt in melon plants (Bernal-Vicente *et al.*, 2009). However, *Trichoderma* species have been reported to cause respiratory problems due to volatile organic compounds they produce (Larsen *et al.*, 1998), but more importantly, *T. longibrachiatum* can infect immune-compromised patients after transplantations or suffering from leukemia or HIV. Most intriguingly, *T. longibrachiatum* not only causes disease; at the same time, it seems to be a source for potential antifungal drugs efficient against *Candida* and *Aspergillus* species (Vicente *et al.*, 2001).

Most of *Alternaria* species including *A. alternata, A. mali, A. tenuis, A. tenuissima, A. gossipi* and *A. consorcialae* were reported as pathogenic to most of the plants (Patil and Shastri, 1982; Anand, 2002; Manjunath, 2009). Moreover Suheri and Latin (1991) reported that *Alternaria* species obtained from apple was highly pathogenic to vegetables and young wheat seedlings. A. tenuissima is also known to the secretion of several toxic products. One of these products was a cell wall degrading enzyme, polygalacturonase (Patil and Shastri, 1982). The antagonistic potential of *Alternaria tenuissima* was reported by Ragazzi and Turco (1997) in that they found greatest antagonistic activity was recorded for *Alternaria tenuissima* and *Acremonium strictum,* that effectively inhibited *Colletotrichum* growth, both *in vitro* and on banana fruits.

Due to its health associated and plant pathogenic risk, the study can not recommend *T. longibrachiatum* and *A. tenuissima* for practical application unless biotechnological approaches are evaluated to explore its beneficial antagonistic effects.

4.5. Conclusion and Recommendation

In this study, 653 bacterial, 594 yeast and 319 filamentous fungal isolates were isolated from the tissues of healthy banana, mango and papaya located in an area naturally affected by anthracnose. Nineteen (ten bacterial, seven yeast and two fungal) isolates with high biocontrol activity against *C. acutatum* and *C. gloeosporoides* were selected by using *in vitro* and *in vivo* tests.

Analysis of the ITS-1 and ITS-4 sequences of yeast and fungal isolates confirmed the identity of the seven yeast and two fungal isolates. Six yeast isolates (M-23-L-1, M-27-F-4, B-22-F-1, P-19-L-4, P-02-L-5, P-10-F-4 and M-21-F-3) showed high similarity with species of *Candida*, while M-21-F-3 showed high similarity with those of *Rhodotorula.* The fungal isolate (M-30-F-2 and M-26-F-1) were identified as *Trichoderma longibrachiatum* (100%) and *A. tenuissimia* (100%), respectively.

Sequencing of 16S rRNA provided consistent results in identifying a set of ten bacterial strains. Isolate P-10-F-7 (DSM 27718) belong to *Paenibacillus* species and showed high sequence similarity with *P. polymyxa* (99.88%). Nine bacterial isolates emerged as distinct strains belonging to *Pseudomonas fluorescens* [designed B-06-L-4, B-13-L-5 (DSM 27703), M-05-F-4 (DSM 27704), P-11-L-1 (DSM 27705), M-04-L-3 and B-07-F-7) and *B. subtilis* [B-03-F-2 (DSM 27715), M-06-L-3 (DSM 27716) and P-02-L-2 (DSM 27717)]. The Riboprint patterns and MALDI-TOF MS characteristics and 16S rRNA homology provided the necessary information for phylogenetic position of the different strains, indicating that they form new and distinct bacterial strains for biocontrol purpose. This study is the first to identify and describe bacteria from the tissues of banana, mango and papaya that confer an efficient protection against *C. acutatum* and *C. gloeosporoides* in Ethiopia.

Both fungal isolates are potentially pathogenic to humans or at least to patients whose immune system is compromised. In view of the genotypic and phenotypic diversity reported in fungal species possible utilization of the isolates based on evaluation of their safety cannot be ruled out. The bacteria and yeast isolates have no known toxicity to humans or plant pathogenic effects and could be applied as postharvest biocontrol agents.

In conclusion, the current study demonstrated that some banana, mango and papaya associated non-pathogenic bacteria belonging to *P. fluorescens* and *B. subtilis* species and yeast (*Candida* and *Rhodotorula* spp.) are identified as prospective new biocontrol agents against *C. acutatum* and *C. gloeosporoides*. The antagonistic isolates exhibited dual antifungal mechanism through direct antagonism. Assuming suitability of the yeast and seven bacteria, further evaluations would still be required towards optimizing their multiplication, formulation and application. Furthermore, although these isolates were isolated from banana, mango and papaya surfaces, a comprehensive study of their exact mode of action, and any non-target effects, will have to be carried out before they can be recommended for commercial use.

CHAPTER 5 MORPHOLOGICAL AND GENETIC CHARACTERIZATION OF *DIDYMELLA BRYONIAE* INFECTING WATERMELON AND OTHER CUCURBITS

5.1. Introduction

Didymella bryoniae (Auersw.) Rehm (anamorph: *Phoma cucurbitacearum* (Fr.:Fr.) Sacc.) is a phytopathogenic fungus that causes Gummy Stem Blight (GSB), a destructive foliar disease of cucurbits. Gummy Stem Blight has been reported throughout the world, but the disease is more devastating in warm and humid climates (Sitterly and Keinath, 1996). *Didymella bryoniae* infects a wide range of plants in the *Cucurbitaceae* family including watermelon (*Citrullus lanatus* (Thunb.) Matsum. and Nakai), muskmelon (*Cucumis melo* L.), cucumber (*Cucumis sativus* L.), squash and pumpkin (*Cucurbita* spp.) (Norton and Cosper, 1989; Sitterly and Keinath, 1996); limiting their production. Among the cucurbits, watermelon is the most affected in the southeastern U.S. leading to reduced yield (Sitterly and Keinath, 1996; Keinath and Duthie, 1998). The fungus infects all above-ground parts of the plant causing leaf necrosis, stem canker and fruit lesions known as black rot (Sitterly and Keinath, 1996).

Florida and Georgia are the two major producers of watermelon in the U.S. with a combined area of 50,300 acres (20,120 hectares) and with a $219 million farm value (USDA-NASS, 2013). This represents 39.5 % of the U.S. area under production and 42.1% of the total farm value. The U.S. imported 322, 000 lbs (144,900 kg) of watermelon seed in 2011 of which 79% were from China, Egypt, Chile, Peru, Israel, and Thailand (USDA-NASS, 2013). Clearly there is a huge influx of seed into USA watermelon industry from international locations. The increased global trade of seeds significantly increases the risk of introduction of exotic isolates of *D. bryoniae*. However, international movement of *D. bryoniae* is poorly understood even though the pathogen is known to be seed-borne (Brown *et al.*, 1970; Lee, 1982).

The first step in understanding the potential movement of *D. bryoniae* into the USA through seed import is to understand the current genetic profile of *D. bryoniae* isolates present in the USA. Advances in molecular techniques such as Polymerase Chain Reaction (PCR), Amplified Fragment Length Polymorphism (AFLP) and Random Amplified Polymorphic DNA (RAPD) techniques have been well utilised for characterization of *D. bryoniae* (Keinath, 1995; Somai *et al.*, 2002a; Somai *et al.*, 2002b). The RAPD technique along with the PCR amplification of ITS region and its subsequent sequence information facilitates the genetic fingerprinting of isolates in a geographical location (Keinath *et al.*, 2001; Somai *et al.*, 2002a).

Previous studies using RAPD analysis differentiated *D. bryoniae* isolates into three genotypes; RG-I, RG-II and RG-IV, and *Phoma* spp. (RG-III) (Keinath, 1995; Somai *et al.*, 2002b). Sequence Characterized Amplified Region (SCAR) marker based on RAPD profiles, have been used for the development of specific primers, for the PCR based differentiation of the four *D. bryoniae* groups (Keinath *et al.*, 2001; Somai *et al.*, 2002a; Somai *et al.*, 2002b). Among the groups, RG-I was found to be the most predominant one in the U.S. compared to the RG-II group (Keinath *et al.*, 2001).

The aggressiveness of the *D. bryoniae* isolates differ with isolates from RG-I group being more aggressive on watermelon than other groups (Somai *et al.*, 2002b). PCR analysis of the ITS-1 and ITS-2, which are considered to be the universal barcode for fungal species differentiation, clustered the *D. bryoniae* isolates from selected geographical locations in the USA also into four RG groups (Somai *et al.*, 2002b). Analysis grouped together the RG-I and RG-II groups into a common phylogenetic cluster along with the closely related RG-IV; while the *Phoma* spp. (RG-III) was clustered separately. Kothera *et al.* (2003), reported the presence of several clusters within the RG-I and RG-II group, as well as among the isolates from northern and southern U.S. using AFLP analysis (Kothera *et al.*, 2003).

All of these previous studies on the genetic characterization of *D. bryoniae* isolates from the USA were carried out mostly on isolates from New York, Wisconsin and South Carolina (Keinath, 1995; Somai *et al.*, 2002b). These studies included two Florida isolates, one each from watermelon and muskmelon. Another genetic characterization study included 6 Florida and 3 Georgia isolates using AFLP analysis (Kothera *et al.*, 2003). Besides these there has been no any new studies on *D. bryoniae* isolates from Florida and Georgia. Considering the importance of Florida and Georgia as the major watermelon producers in the USA, an extensive genetic characterization of the isolates is highly essential for future detection of exotic isolates into U.S.

The objectives of this study were to address and clarify the genetic association between ***D. bryoniae*** and related ***Phoma*** isolates, by conducting a morphological examination and using phylogenetic relationships based on RAPD markers and sequence information from the internal transcribed spacer (ITS) region of rDNA

5.2. Materials and Methods

5.2.1 Sample collection and pathogen isolation

Watermelon, squash, canary melon and muskmelon showing typical symptoms of GSB were obtained from infected stems or leaf tissues in 2011-2012, from several locations in Florida and Georgia. One square cm of the infected tissue from symptomatic plants were surface sterilized in 1% sodium hypochlorite for 30 s. The tissue was then rinsed twice in sterile deionized water and placed on to Petri dishes containing Quarter-Strength Potato-Dextrose Agar (QPDA) consisting of granulated agar (5 g), Potato Dextrose Agar (7.6 g) and distilled water (1 L). The plates were then incubated at 24°C with a 12 h photoperiod for 10-14 d under white fluorescent light (Keinath, 1995). The isolates were purified and characterized based on colony morphology and spore characteristics (Keinath, 1995).

5.2.2 Maintenance of pathogen isolates

A total of 35 isolates were collected from Florida and Georgia: 30 cultures were isolated from watermelon, muskmelon, 3 from canary melon, 1 from muskmelon and 1 from squash. The origin and host plants for each isolate are listed in Table 5.1. Cultures were maintained on Quarter-Strength Potato Dextrose Agar at 4°C for further study.

5.2.3 Morphological and cultural characteristics

The morphological and cultural characteristics of the isolates were evaluated using four different media. Each isolate was grown on QPDA, Malt extract agar [Malt extract (10.0 g), Peptone (2.5 g) and Agar (15.0 g)]; Yeast Extract Malt Extract Agar (YEMEA) [Glucose (10 g), Peptone (5 g), Malt extract (3 g), Yeast extract (3 g) Agar (20 g) and Distilled water (1 L)]; and V8 Agar [V8 juice (200 mL), $CaCO_3$ (2 g), agar (15 g) and distilled water (800 mL)]. Five mm diameter of three days old pre-culture of each isolate on PDA was inoculated in 9 cm diameter Petri plate containing the respective medium. Each media for each isolate was replicated three times.

Cultures were incubated at 25°C for 5 d in the dark and 10 d more with 12 hour light. Daily growth of mycelium was recorded as average of cross diameters. Colony characteristics were also recorded on colony color, elevation, margin, and conidia formation (Keinath *et al.*, 1995).

Table 5. 1 Source of *D. bryoniae* and *Phoma* Isolates

No	**Acce. No.**[a]	**Isolate**[b]	**Host**[c]	**Origin**[d]	**No**	**Acce. No.**[a]	**Isolate**[b]	**Host**[c]	**Origin**[d]
1	KF990378	DB-01	WM	Georgia	19	KF990396	DB-24	WM	Florida
2	KF990379	DB-02	WM	Georgia	20	KF990397	DB-25	WM	Florida
3	KF990380	DB-04	WM	Georgia	21	KF990409	DB-27	WM	Florida
4	KF990381	DB-05	WM	Georgia	22	KF990398	DB-30	WM	Florida
5	KF990382	DB-06	WM	Georgia	23	KF990399	DB-32	WM	Florida
6	KF990383	DB-07	WM	Georgia	24	KF990400	DB-33	WM	Florida
7	KF990384	DB-08	WM	Georgia	25	KF990401	DB-34	WM	Florida
8	KF990385	DB-09	WM	Florida	26	KF990411	DB-35	WM	Florida
9	KF990386	DB-10	WM	Florida	27	KF990402	DB-36	WM	Florida
10	KF990387	DB-11	WM	Florida	28	KF990403	DB-37	WM	Florida
11	KF990388	DB-12	WM	Florida	29	KF990404	DB-38	WM	Florida
12	KF990389	DB-13	WM	Florida	30	KF990405	DB-39	WM	Florida
13	KF990390	DB-15	CM	Florida	31	KF990406	DB-40	WM	Florida
14	KF990391	DB-18	CM	Florida	32	KF990407	DB-42	WM	Florida
15	KF990392	DB-19	CM	Florida	33	KF990410	DB-43	WM	Florida
16	KF990393	DB-20	WM	Florida	34	KF990408	DB-44	SQ	Florida
17	KF990394	DB-21	WM	Florida	35	KF990412	DB-45	WM	Florida
18	KF990395	DB-23	WM	Florida					

a Accession number given to the isolates

[b] Code given to isolates in this study

c Host of origion of isolates: WM Watermelon, CM= Canary Melon and SQ= Squash

d Geographic origin from where they were collected

Slides were prepared to make detailed observations of the morphological features of fungal structures (i.e., mycelium, conidia, Pycnidia and Psudothesia) with a light microscope (Nicon 80i; Nikon, Tokyo, Japan). Fungal structures were taken from actively growing colonies on PDA and mounted in lactophenol cotton blue, and examined for presence, size and shape of conidia, Pycnidia, Chlamydospores and swollen cells. The length and width of 50 conidia from each isolate was measured at 100 × magnification and was microscopically examined for the presence of septae. The morphological data were compared with previously described data for *D. bryoniae* and its anamorph *Phoma* (Keinath *et al.*, 1995).

5.2.4 Pathogenicity studies

Watermelon (*Citrullus lanatus* cv. 'Melody ') was seeded in 60% vermiculite -40% peat potting mix. After 1 wk, three seedlings were transplanted per 10-cm plastic pot. All isolates of *D. bryoniae* and *Phoma* were tested for their pathogncity. Isolates were grown on QPDA plates for 2–3 wk at 26 ℃ and a 12 h photoperiod. Cultures were flooded with sterile de-ionized water and gently scraped. In this test, a suspension of $2x10^5$ conidia/ ml was used. Two week old plants were inoculated by spraying approximately 2 ml conidial suspension per pot. Control (non-inoculated) plants were sprayed with sterile water. Plants were held in a mist chamber at 90-100% RH for 3 d to promote infection and lesion expansion (Keinath, 1995). The individual tests were Complete Block Designs with five replicate pots for each isolate arranged with blocks in mist chambers. The experiment was performed two times. Plants were examined every other day for disease symptoms beginning at the second day after inoculation.

Disease severity was assessed visually and rated on a 0 to 11 Horsfall-Barratt scale, (Horsfall and Barratt, 1945). The Horsfall-Barratt scales were converted to midpoint averages of disease severity. Isolates were categorized based on their final percentage disease severity as += low (1-25 %), ++ = medium (25-50 %), +++ = high (50-75 %), and ++++ = extreme (75-100 %). Diseased leaf tissue from selected plants was cultured on QPDA to re-isolate *D. bryoniae* and *Phoma*.

5.2.5 Molecular characterization

5.2.5.1 DNA extraction

Purified isolates were re-cultured on fresh QPDA medium by removing a 1 cm^2 agar plug from the culture and placing it on to the center of a Petri plate containing the medium, and incubated at 28°C with a 12 h photoperiod for 10-14 d. Mycelia of 100-120 mg was scraped from the culture plate under aseptic conditions, and homogenized using liquid nitrogen in a pestle and mortar. The tissue was ground into a fine powder and the genomic DNA was extracted using Plant/Fungi DNA Isolation Kit (NORGEN-Biotek, Ontario, Canada). The DNA was eluted in sterile nuclease free water and stored at -20°C. The DNA was quantified in 1% agarose gels, stained with ethidium bromide.

5.2.5.2 Diversity analysis of *Didymella bryoniae* based on RG-specific primers

The ***D. bryoniae*** and ***Phoma*** were first tested with RG-specific primers designed by Somai *et al.* (2002a). Primers were synthesized and purified by Integrated DNA Technologies (Coralville, IA). The sequence of the ***D. bryoniae*** RG I-specific forward primer was TGTCGTTGAC ATCATTCCAG C and the sequence of the reverse primer was ACCACTCTGC TTAGTATCTG C (Keinath, 2001). The sequence of the ***D. bryoniae*** RG II forward primer was GCTAAGCCTT AATCTAGCTG C and sequence of the reverse primer was GAGAGTAAGC TAACCTAAAG G (Keinath, 2001). The sequence of the ***Phoma***-specific forward primer was GTCAGAGTTC AGAATGCTTC G and sequence of the reverse primer was ACGGATGAAT AACACGGAAG A (Keinath, 2001).

The identity of all isolates of ***D. bryoniae*** and ***Phoma*** species collections was confirmed with PCR using these specific primers and it was performed in 20 µL reactions, according to procedures described by Keinath (2001). The reaction mixture contained 40 ng DNA, 2.0 µL of 10 × reaction buffer (50 mM Tris-HCl, 1.5 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$ and 0.1 % Triton® ×-100), 200 µM dNTPs, 2.5 U of Taq DNA polymerase (5U/ µL), 0.25 µM of each primer. Each reaction was repeated four times. Amplification was carried out in Eppendorf

Master cycler gradient ES, but with a modification, with the drop down in annealing temperature from 64°C (Somai ***et al.***, 2002a) to 62°C. The thermal cycling programs were: 94°C for 2 min, and 35 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 2 min, followed by 72°C for 10 min.(Somai ***et al.***, 2002a).

In order to verify if amplification of rDNA occurred in the PCR, electrophoresis was carried out which separated the fragments. A 1% agarose gel stained with ethidium bromide with wells made in it was formed. The gel was placed in the electrophoresis machine containing 0.5% TAE (Tri/ Acetate/ EDTA) - buffer and the DNAs loaded with buffer (5 µL for each isolates) were carefully pipetted into the wells in the slab gel. A 100-bp DNA ladder Mix was also pipetted into ends of the wells in the slab gel. The machine was turned on at 100V for 20 minutes after which slab gel was then viewed under the UV- trans illuminator and photographed and photographed under UV-gel doc system (Alpha imager, Protein Simple, CA, USA).

5.2.5.3 Diversity analysis of *Didymella bryoniae* based on RAPD

The genetic diversity of ***D. bryoniae*** isolates were analyzed using RAPD genetic fingerprinting. A set of RAPD 10-mer oligonucleotide primers (Operon Technologies, Alameda, CA): OPA1-06, OPAA-04, OPAA-15, OPAA-17, OPK-01, OPK-04, OPK-08, OPL-03, OPL-05, OPL-15, OPL-19, OPN-09, OPT-07, OPT-13 and OPT-14 was initially screened with the 35 isolates. The assay was performed using the GoTaq Flexi DNA polymerase (Promega, Madison, WI) in a 20 µL reaction mixture containing 40 ng DNA, 4.0 µL of 5× reaction buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 2.5 U of GoTaq Flexi DNA Polymerase (5U/ µL), 0.25 µM of each primer.

The PCR was performed in Eppendorf Master cycler gradient ES, with the following cycling conditions: 95°C for 2 min, and 35 cycles of 95°C for 1 min, 35°C for 1 min and 72°C for 1 min, followed by 72°C for 10 min (Keinath, 1995). The whole reaction volume was electrophoresed in a 1.5% agarose gel in 1• TBE buffer, at 100 V for 3 h. For each DNA sample, reactions were repeated two times to ensure consistency and reproducibility of each

RAPD band. A negative control (sample without template DNA) was included in all tests. Selected primers that yielded clear, consistent and polymorphic bands were selected for further analysis.

5.2.5.4 PCR amplification of the isolates using ITS-1 and ITS-4 primers

The genomic DNA of the isolates were subjected to PCR amplification using the universal primers (Somai *et al.*, 2002b) ITS-1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3'), which spans the partial 3' end of 16S gene, ITS-1, 5.8S gene, ITS-2 and partial 5' end of the 28S gene (White *et al.*, 1990). The PCR reactions were performed in a 20 µL reaction mixture containing 40 ng DNA, 2.0 µL of 10× reaction buffer (50 mM Tris-HCl, 1.5 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$ and 0.1 % Triton® X-100), 200 µM dNTPs, 2.5 U of Taq DNA Polymerase (5U/ µL), 0.25 µM of each primer.

The PCR was performed in Eppendorf Master cycler gradient ES, with the following cycling conditions: 95°C for 4 min, and 35 cycles of 95°C for 1 min, 62°C for 1 min and 72°C for 2 min, followed by 72°C for 8 min. The amplified product was analyzed on 1% agarose gel, stained with ethidium bromide and photographed under UV-gel doc system (Alpha imager, ProteinSimple, CA, USA).

5.2.6 Statistical analysis

Analysis of Variance (ANOVA) was used to analyze the difference between treatments for each experiment using SPSS V20. The percentage data were Arcsine-transformed prior to analysis and means were back-transformed to percentage values after analysis. Measurement data were subjected to square root transformation before statistical analyses. Means for different treatments in each experiment were compared using Duncan's Multiple Range Test (DMRT) and Dunnett's T test. Computer-assisted evaluation of the RAPD-generated fingerprints was made using the GelCompare program (version 6.6.8; Applied Math, Kortrijk, Belgium). For each primer, the RAPD markers that were polymorphic across the set of isolates were considered for analysis. The presence or absence of a marker allele was scored as 1 or 0, respectively. The relative size of each band was determined by comparison with 1-kb ladder (Promega, Madison, WI). After processing the gel images for individual primers, a composite data was constructed for the selected set of primers; and all pair wise similarity values were calculated with a similarity coefficient.

5.3. Result

The 35 isolates from USA evaluated in this study were grouped into four distinct groups based on morphology characteristics on QPDA after 12 d (Table 5.2). Isolates in group 1 produced Medium gray or dark gray colonies with white or olive areal mycelium on the top and reverse color was gray to dark. Twelve isolates showed Pycnidia and Psedotesia with relatively uniform or irregular distribution. Group 2 isolates produced white colonies on the top and black or light white substrate color. Pycnidia and *Psedotesia* with relatively uniform distribution was observed in four isolates of this group. Isolates in group 3 produced light brown to olive drab color on the top and black or dark brown substrate color. Three isolates showed Pycnidia and *Psedotesia* with irregular distribution.

Isolates in group 4 produced light green or brown to silver color on the top and reverse color was black or brown. No isolate showed Pycnidia and *Psedotesia* in this group. Culture characteristics on MEA and V8 medium were more or less similar to QPDA but faster growth and high sporultion was found from MEA (Figure 5.1). However, most isolates showed different color, predominantly white, on YEMEA and it was not consistently observed among the 4 groups (Table 5.2). There was a difference in the rate of daily growth among isolates. Rate of growth per day after 10 d on QPDA was higher for group 1 (4.2 to 20.1mm), followed by group 2 (3.5 to 15.7mm), and group 3 (4.8 to 8.7mm). The lowest growth rate per day was found from group 4 (5.4 to 7.8mm) (Table 5.2).

The length and width of conidia ranged from (7.3 - 9.9 µm x 2.7 - 4.8µm) group 1, (7.2 - 9.4 µm x 2.4 - 3.78µm) group 2, (7.9 - 8.6 µm x 3.42 - 3.55µm) group 3 and (6.9 - 7.3 µm x 2.9 - 3.01µm) group 4. Septated condia was observed in groups 1, 2 and 3 ranging from 5 - 21 % but no septation was observed from group 4 isolates (Table 5.3). Based on colony morphology, growth rate and conidia size and presence of septa isolates in group 1, 2 and 3 were considered as *D. bryoniae* and group 4 isolates were considered to be *Phoma* species (Table 5.3, Figure 5.2).

Table 5. 2 Morphology of *D. bryoniae* and *Phoma* Isolates on PDA media

No	**Code**	**Colony color upper** [a]	**Colony color lower**	**Pycnidia (P/A)** [b]	**Conidia** [c]	**Growth rate (mm)** [d]
1	Db-01	Dark khaki	Olive	P	**	6.3
2	Db-02	Ghost white - light gray	Dark gray - light olive	P	**	4.3
3	Db-04	light gray	Black	P	**	4.3
4	Db-05	Dark gray - black	Black	P	**	5.0
5	Db-06	Medium gray - white	Black	A	-	4.7
6	Db-07	Dark gray - black	Black	P	**	4.5
7	Db-08	Olive Drab	Dark gray	P	**	5.3
8	Db-09	light brown- olive drab	Dark gray to black	A	-	5.0
9	Db-10	Light gray	Dark gray - black	P	**	6.7
10	Db-11	light gray - dark gray	Black - light gray	P	**	5.7
11	Db-12	Light olive drab	Black	P	**	8.7
12	Db-13	light gray	Black	P	**	8.0
13	Db-15	Dark brown - olive	Black	P	**	4.8
14	Db-18	Medium gray - Olive	Black	P	**	5.2
15	Db-19	White	black	P	**	6.0
16	Db-20	Dark gray to brown	Brown	P	**	4.8
17	Db-21	Dark gray	Black	P	**	8.3
18	Db-23	Dark gray - black	Black	P	**	7.3
19	Db-24	Dark gray - olive	Black	P	**	9.7
20	Db-25	Medium gray	Silver	P	**	6.7
21	Db-27	Silver	Medium gray	P	**	7.5
22	Db-30	Gray	Black	P	**	6.8
23	Db-32	White	White	P	**	15.7
24	Db-33	Silver	Medium gray	A	-	20.2
25	Db-34	Light brown	Black	P	**	4.3
26	Db-35	Light gray	Black	A	-	7.0
27	Db-36	Dark gray - white	Black	A	-	5.7
28	Db-37	Gray to black	Gray to black	P	***	7.0
29	Db-38	White	black	P	**	3.5
30	Db-39	White	black	P	**	5.2
31	Db-40	White	black	A	-	5.3
32	Db-42	White	black	P	**	9.2
33	Db-43	light gray	brown	A	-	6.0
34	Db-44	grayish white	black	A	-	9.9
35	Db-45	gray	black	A	-	12.3

[a] Detailed observations of the morphological features of fungal structures (i.e., mycelium, conidia, Pycnidia and Psedotesia) were observed with a light microscope (Nicon 80i; Nikon, Tokyo, Japan).

[b] P= Present, A= Absent

[c] * = low sporulation, **= medium sporulation, ***= high sporulation, and - = no sporulation

[d] Growth rate was calculated as cross diameter of growth/ day (mm) for 12 d. The average growth rate for each group displayed. Means of three replications

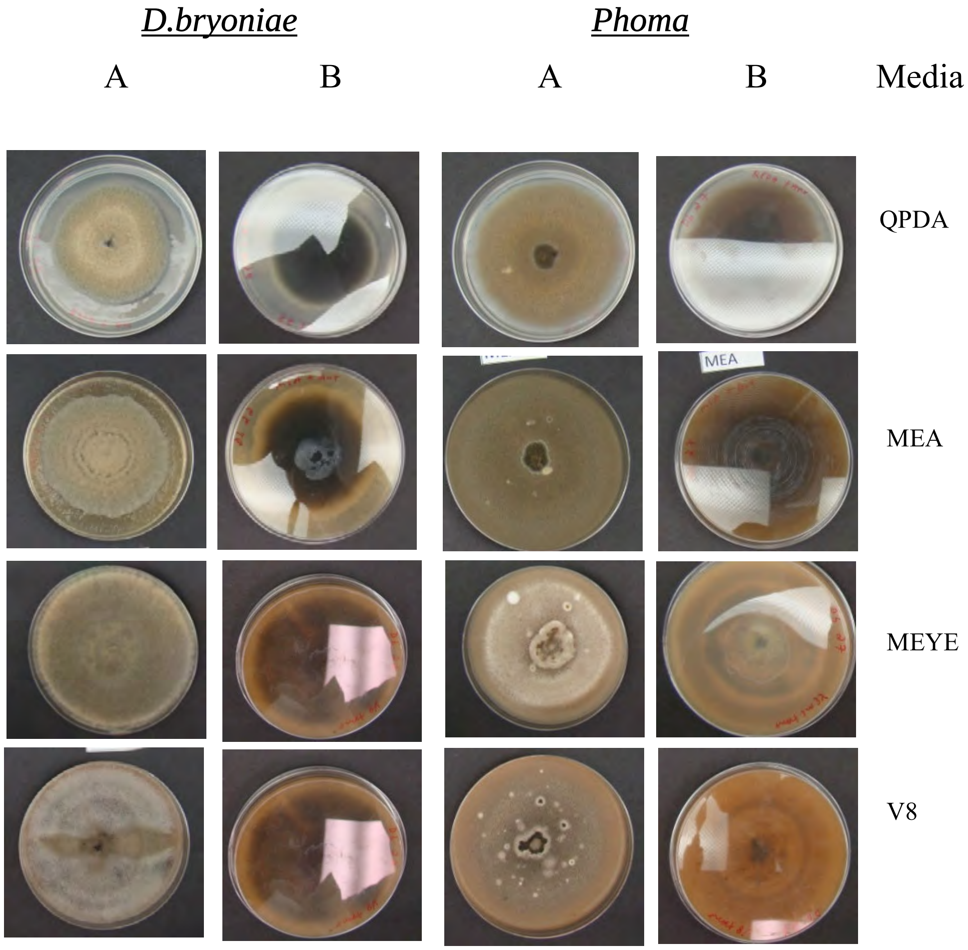


Figure 5. 1 Morphology of isolates of *D. bryoniae* and *Phoma* on different media

**The medias used were Quarter strength Potato dextrose Agar (QPDA), Malt extract Agar (MEA), Yeast Extract Malt Extract Agar (YEMEA) and V8 Agar medias. Colony characteristics: Plates in column A, aerial view; and B, reverse view.

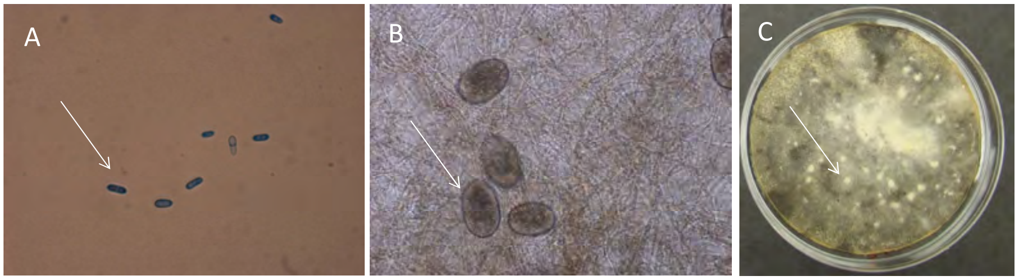


Figure 5. 2 Culture and morphological characteristics of pathogenic *D. bryoniae*

**Conidia and Picnidia of *D. bryoniae* cultured on QPDA after exposure to alternate light for 12 hr (A and B). Colony formed on potato dextrose agar after 10 d of incubation (C). Note the white conidial mass in the center of the colony on QPDA after 14 d of incubation

Table 5. 3 Reproductive structures of *D. bryoniae* and *Phoma* Isolates on PDA media

No	**Isol code**	**Pseudothecia distribution** [a]	**Pycnidia formation**	**septa** [b]	**Length** [c]	**Width**
1	Db-01	Uniformly through	uniformly	5	8.11	2.95
2	Db-02	Concentrated in the center	Concentrated	6	8.54	3.95
3	Db-04			8	9.27	3.83
4	Db-05	Concentrated in the center	Concentrated	10	7.58	3.21
5	Db-06			ND [d]	ND	ND
6	Db-07			0	8.3	3.11
7	Db-08	Uniformly through	uniformly	12	8.6	3.42
8	Db-09			ND	ND	ND
9	Db-10	Concentrated in the center	Concentrated	20	9.45	3.45
10	Db-11			21	9.32	4.16
11	Db-12	Irregularly distributed	irregular	0	8.51	3.46
12	Db-13	Concentrated in the center	Concentrated	18	9.57	4.83
13	Db-15	Irregularly distributed	irregular	19	7.94	3.55
14	Db-18	Uniformly through	uniformly	12	7.2	2.77
15	Db-19			9	8.9	2.89
16	Db-20	Irregularly distributed	irregular	8	9.3	3.56
17	Db-21	Irregularly distributed	irregular	4	9.6	4.19
18	Db-23			8	8.45	3.44
19	Db-24	Concentrated in the center	Concentrated	6	9.84	3.73
20	Db-25	Irregularly distributed	irregular	0	9.24	4.65
21	Db-27			0	6.99	2.89
22	Db-30	Irregularly distributed	irregular	0	9.89	4.77
23	Db-32	Irregularly distributed	irregular	0	7.75	3.22
24	Db-33			ND	ND	ND
25	Db-34	Concentrated in the center	Concentrated	9	8.67	3.56
26	Db-35			ND	ND	ND
27	Db-36			ND	ND	ND
28	Db-37	Uniformly through	irregular	4	9.9	4.12
29	Db-38	Concentrated in the center	Concentrated	0	9.35	3.46
30	Db-39	Uniformly through	uniformly	0	7.2	2.97
31	Db-40			ND	ND	ND
32	Db-42	Uniformly through	uniformly	0	9.45	2.45
33	Db-43			ND	ND	ND
34	Db-44			ND	ND	ND
35	Db-45			ND	ND	ND

[a] Detailed observations of the morphological features of fungal structures (i.e., mycelium, conidia, Pycnidia and Psedotesia) were observed with a light microscope.

[b] Number of isolates with conidial septa

[c] The length and width of the 50 conidia from each pathogen was measured at 400× magnification and was microscopically examined for the presence of septae

[d] ND= Not Determined

All of the *D. bryoniae* and *Phoma* spp. isolates were pathogenic on watermelon cv. Melody, irrespective of their original host of origin. The control plants did not develop any symptoms of GSB. The isolates were found to have varying degrees of pathogenicity (Table 5.4). Among the isolates tested, 40% of the isolates were extremely pathogenic (75-100% disease severity), 26% were highly pathogenic (50-75% disease severity), 14.3% were moderately pathogenic (25-50% disease severity) and the remaining 20% had low levels of pathogenicity (1-25% disease severity).

Among the *Phoma* spp. one of the isolates was highly pathogenic (DB-27) and one isolate (DB-43) was extremely pathogenic, while the isolates DB-35 and DB-45 were found to have low and moderate pathogenicity, respectively. There was no clear link between isolate pathogenicity on watermelon and host of origin. For example, two canary melon isolates from Florida (DB-15 and DB-18) were found to be highly pathogenic on watermelon, while the isolate DB-19 from canary melon was found to have low pathogenicity on watermelon. The pathogen was reisolated from the inoculated plants and recultured on QPDA plates, thus confirming Kochs postulates (Figure 5.3).

PCR analysis of the 35 isolates using the RG specific primers RG-I-F and RG-I-R resulted in an amplification of 735 bp amplicon in 25 isolates (Table 5.5; Figure 5.4). Sequence analysis of the amplicon showed 99% identity with the *S. cucurbitacearum* isolate Db-23 (Accession no: GQ872463) from the NCBI database, and the isolates shared 96-99% identity among themselves. The RG-II primers did not give amplification with any of the isolates under study. However the *Phoma* specific primers RG-III gave an amplification with one of the watermelon isolates from Florida (DB-27) producing an amplicon of 704 bp. Analysis of the *D. bryoniae* isolates using the RG specific primers showed that 71% of the isolates belong to the RG-I group, one isolate from RG-III *Phoma* group, while the remaining 26% of the isolates tested (9 out of 35) did not gave any positive amplification by any of the primers for the RG groups (Table 5.4, Figure 5.4).

Table 5. 4 Molecular characterization and Pathogenicity of *D. bryoniae* and *Phoma* Isolates

				Molecular characterization					**Pathogenicity**	
No	**Accession** [a]	**No.Isolate code** [b]	**Orgin Host** [c]	**RG** [d]	**IRG-II**	**RG-III**	**ITS-1/ ITS-4**	**Molecular ID** [e]	**AUDPC** [f]	**Rank** [g]
1	KF990378	DB-01	WM	+	-	-	+	*Septoria cucurbitacearum*	1060.67	*****
2	KF990379	DB-02	WM	+	-	-	+	*S. cucurbitacearum*	26.50	**
3	KF990380	DB-04	WM	+	-	-	+	*S. cucurbitacearum*	379.17	***
4	KF990381	DB-05	WM	+	-	-	+	*S. cucurbitacearum*	884.00	*****
5	KF990382	DB-06	WM	-	-	-	+	*S. cucurbitacearum*	870.83	****
6	KF990383	DB-07	WM	+	-	-	+	*S. cucurbitacearum*	276.50	****
7	KF990384	DB-08	WM	+	-	-	+	*S. cucurbitacearum*	1539.33	*****
8	KF990385	DB-09	WM	-	-	-	+	*S. cucurbitacearum*	22.50	**
9	KF990386	DB-10	WM	+	-	-	+	*S. cucurbitacearum*	1906.67	*****
10	KF990387	DB-11	WM	+	-	-	+	*S. cucurbitacearum*	284.83	***
11	KF990388	DB-12	WM	+	-	-	+	*S. cucurbitacearum*	1542.33	*****
12	KF990389	DB-13	WM	+	-	-	+	*S. cucurbitacearum*	706.17	****
13	KF990390	DB-15	CM	+	-	-	+	*S. cucurbitacearum*	684.83	****
14	KF990391	DB-18	CM	+	-	-	+	*S. cucurbitacearum*	975.50	****
15	KF990392	DB-19	CM	+	-	-	+	*S. cucurbitacearum*	119.50	**
16	KF990393	DB-20	WM	+	-	-	+	*S. cucurbitacearum*	439.50	****
17	KF990394	DB-21	WM	+	-	-	+	*S. cucurbitacearum*	2005.33	*****
18	KF990395	DB-23	WM	+	-	-	+	*S. cucurbitacearum*	502.83	****
19	KF990396	DB-24	WM	+	-	-	+	*S. cucurbitacearum*	2048.33	*****
20	KF990397	DB-25	WM	+	-	-	+	*S. cucurbitacearum*	963.17	****
21	KF990409	DB-27	WM	-	-	+	+	*Phoma* spp.	449.83	****
22	KF990398	DB-30	WM	+	-	-	+	*S. cucurbitacearum*	2060.33	*****
23	KF990399	DB-32	WM	+	-	-	+	*S. cucurbitacearum*	176.50	***
24	KF990400	DB-33	WM	-	-	-	+	*S. cucurbitacearum*	58.50	**
25	KF990401	DB-34	WM	+	-	-	+	*S. cucurbitacearum*	1908.00	*****
26	KF990411	DB-35	WM	-	-	-	+	*Phoma* spp.	95.17	**
27	KF990402	DB-36	WM	-	-	-	+	*S. cucurbitacearum*	31.50	**
28	KF990403	DB-37	WM	+	-	-	+	*S. cucurbitacearum*	1816.33	*****
29	KF990404	DB-38	MM	+	-	-	+	*S. cucurbitacearum*	1611.67	*****
30	KF990405	DB-39	WM	+	-	-	+	*S. cucurbitacearum*	1619.00	*****
31	KF990406	DB-40	WM	-	-	-	+	*S. cucurbitacearum*	461.50	***
32	KF990407	DB-42	WM	+	-	-	+	*S. cucurbitacearum*	1798.33	*****
33	KF990410	DB-43	WM	-	-	-	+	*Phoma* spp.	826.67	*****
34	KF990408	DB-44	SQ	-	-	-	+	*S. cucurbitacearum*	45.50	**
35	KF990412	DB-45	WM	-	-	-	+	*Phoma* spp.	259.33	***

[a] Accsesion number of isolates from NCBI

[b] Code given to isolates in this study

[c] Host of origion of isolates: WM Watermelon, CM= Canary Melon and SQ= Squash

[d] Method adopted frmKeinath et al., 2001 and Somai et al., 2002b,

[e] Identity based on nucleotide sequence obtained using ITS-1 and ITS-4 primers.

[f] Area under disease progress curve (AUDPC) value after 3 weeks

[g] =low pathogenic (1-25 %), ** = medium pathogenic (25-50 %), *** = highly pathogenic (50-75 %) and **** = extremely pathogenic (75-100 %).

Figure 5. 3 Pathogenicity study of GSB pathogens in the green house

**Plates showing seedling preparation on tray and setup in the green house (A and B), symptoms of gummy stem blight on watermelon plants caused by *D. bryoniae* infected leaf in early stage and advanced stage (C and D).

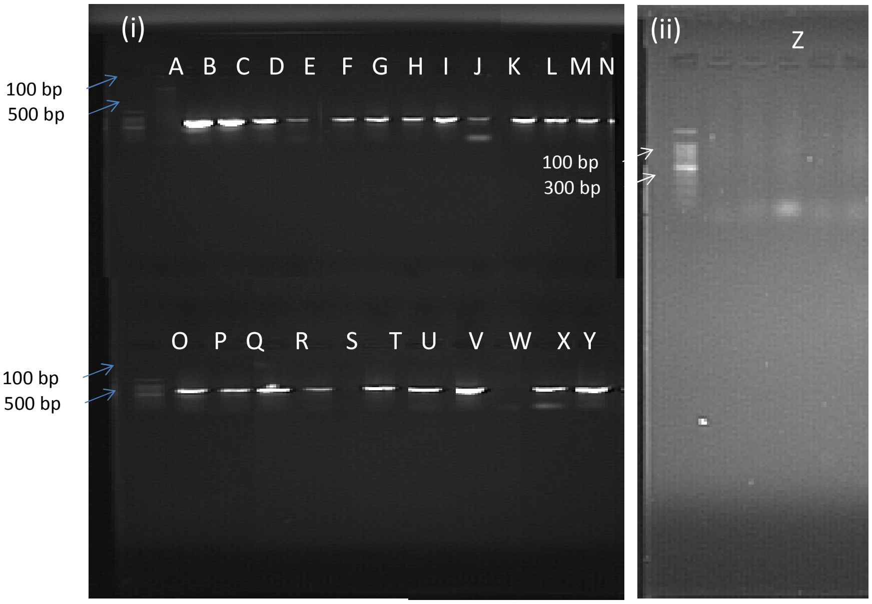


Figure 5. 4 DNA of 25 *D. bryoniae* RG I isolates (i), and a *Phoma* isolates (ii) amplified with the *D. bryoniae*- specific primers: RG I and Phoma RG III, Lanes: 1. 100bp DNA ladder,for both plates

**Isolates designated by letter A-Y are *D. bryoniae* and "Z" is a *Phoma* isolate: A (DB-01), B (DB-02), C (DB-04), D (DB-05), E (DB-07), F (DB-08), G (DB-10), H (DB-11), I (DB-12), J (DB-13), K (DB-15), L (DB-18), M (DB-19), N (DB-20), O (DB-21), P (DB-23), Q (DB-24), R (DB-25), S (DB-30), T (DB-32), U (DB-34), V (DB-37), W (DB-38), X (DB-39), Y (DB-42) and Z (DB-27)

RAPD analysis was performed to evaluate 15 random primers that could reveal genomic DNA polymorphisms in the *D. bryoniae* isolates collected from Georgia and Florida. Operon primers of A, K, L, N and T series (OPA1-06, OPAA-04, OPAA-15, OPAA-17, OPK-01, OPK-04, OPK-08, OPL-03, OPL-05, OPL-15, OPL-19, OPN-09, OPT-07, OPT-13 and OPT-14) were selected and tested on this collection of isolates. Among the RAPD primers, majority of them failed to amplify one or the other isolates. From the set of 15 primers evaluated, two RAPD primers OPAA-04 and OPAA-15 were selected for the final analysis (Figure 5.5).

The DNA fragments generated using the two primers were used as markers to estimate pairwise genetic similarity between the isolates; and on an average these primers produced 20-25 polymorphic and reproducible markers (Figure 5.5). On the basis of genetic similarity and UPGMA cluster analyses, the isolates were clustered in a dendrogram and two discrete clusters were observed. Phylogenetic analysis showed that the majority of the isolates were clustered in RAPD group 1 (28 isolates), irrespective of the isolates from Georgia or Florida and from the host of origin.

The second RAPD group was the clustering of the *Phoma* spp. with a 60% identity (bootstrap consistency of 94%). However three isolates (DB-06, DB-33 and DB-01) shared a lower identity level (< 50%) with the group 1 and group 2. RAPD phylogenetic analysis of the whole genome indicated that the DB-06 (watermelon isolate from Georgia) was the most distinct followed by DB-33 (watermelon, Florida) and DB-01 (watermelon, Gerogia) respectively. However, the isolates in group 1 were also found to exhibit higher variability within the group. The isolates in group 1 were further sub-clustered into group 1a, which contained only the Florida isolates from both watermelon and canary melon. The subgroup 2a consists of both the Georgia and Florida isolates, but includes isolates from watermelon only. The remaining isolates DB-02, DB-38, DB-09, DB-32, DB-36, Db-11, DB-37 and DB-44 shared a lower identity of (55%-72%) with the other subgroup isolates; the most distant of them was the DB-44, a squash isolate from Florida.

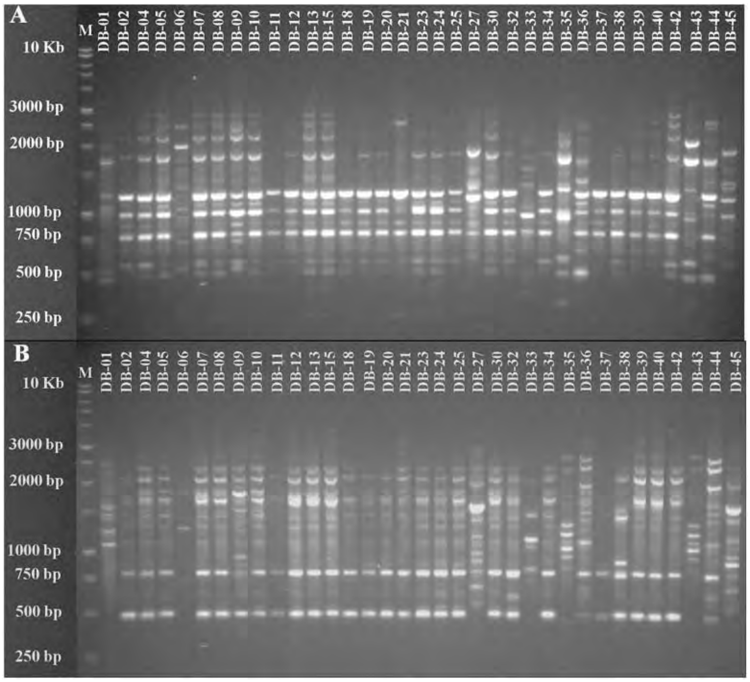


Figure 5. 5 Random amplified polymorphic DNA (RAPD) fingerprints of 35 isolates of *Didymella bryoniae* from Florida and Georgia amplified with Operon primer (A) OPAA-04 (B) OPAA-15. Lane 1 (M) is a 100-bp marker

PCR analysis using ITS-1 and ITS-4 primers produced an amplicon of 545 bp in all the isolates (Table 5.4; Figure 5.4). Sequence analysis of the amplified product showed 98-99% identity with that of the *D. bryoniae* (*Septoria cucurbitacearum*) isolates, expect with the four watermelon isolates from Florida (DB-27, DB-35, DB-43, DB-45) which showed a high identity of 99% with that of the *Phoma* spp. in the NCBI database. Comparative analysis among the isolates showed that *Phoma* spp. exhibited only 96-97% identity with that of the *D. bryoniae* isolates. Most of the *D. bryoniae* isolates had an adenine residue at position 131 of the ITS-1 region (alignment position 140), whereas the two watermelon isolates, one each from Georgia and Florida (DB-05 and DB-33) had a guanine substitution (Table 5.4). Multiple sequence alignment of the amplified regions showed the presence of 9 substitutions and 2 deletions in the ITS-1 region, and 3 substitutions and 2 deletions in the ITS-2 region of the *Phoma* spp. compared to the *D. bryoniae* isolates. ITS-1 region of the *D. bryoniae* isolates were found to be more varying with an identity ranging between 92-100%. Among the isolates, one of the watermelon isolate from Florida, DB-24 was found to be highly variable with an identity ranging from 92-94%, followed by DB-21 (96-97%). However the ITS-1 region of the four *Phoma* spp. showed only 84-91% identity with that of the other *D. bryoniae* isolates, clearly exhibiting the demarcation between the two. There was considerable variation in ITS-1 region of the *Phoma* spp. Among the *Phoma* isolates, DB-27 shared an identity of 98% with that of DB-45, while exhibiting a lower identity of 96% with that of DB-35 and DB-43. The *Phoma* isolates DB-35 and DB-43 shared a higher identity of 99%. The ITS-2 region among the isolates was found to be highly conserved with an identity of 99-100%, with the exception of the two *Phoma* spp. DB-27 and DB-45 which exhibited an identity of 96-97% with other isolates, while sharing a higher identity of 99% among them (Table 5.4).

5.4 Discussion

In this study, variation in morphology was found among *D. bryoniae* and *Phoma* isolates. Morphological variation among *D. bryoniae* isolates and between *D. bryoniae* and *Phoma* isolates from different sources were reported previously by Keinath (1995) and its use to distinguish *D. bryoniae* from related *Phoma* species was justified. In this experiment, the morphological variation of *D. bryoniae* and *Phoma* isolates was observed.

The variation in conidia characteristics evidenced in this experiment and it was also reported by several authors (Keinath, 1995; Choi *et al.*, 2010). Choi *et al.* (2010) used characteristics such as the size of the conidia and the percentage of septa to study the taxonomy of the genus *D. bryoniae* and *Phoma* spp. The size of the conidia and the percentage of monosepta in *D. bryoniae* were the same as described previously. Choi *et al.* (2010) suggest that the presence of septae may not be a useful characteristic by which to distinguish the anamorph of *D. bryoniae* from *Phoma* spp.

All 35 isolates from USA studied were pathogenic to watermelon, but there were clear distinctions in the pattern of aggressiveness to host plants. The susceptibility of some cultivars of watermelon to GSB earlier described by Dos-Santos *et al.* (2006) has been confirmed. Fungal isolates from melon and *Cucurbita* hosts infected watermelon in this study, and the isolates from watermelon were generally more aggressive on their original host. The information on the cross infection and variation among gummy stem blight pathogen isolates is certainly useful for resistance breeding programmes. Although the disease is considered of great importance for commercial watermelon production, cultivars with high levels of genetic resistance are not currently available, and there is an ongoing need for increasing watermelon resistance against gummy stem blight (Keinath, 1996; Sitterly and Keinath, 1996; Levi *et al.*, 2001; Dos-Santos *et al.*, 2006).

Symptom development by *D. bryoniae* isolates irrespective of origin and host was previously reported. Dos-Santos *et al.* (2009) reported that, irrespective of original host or geographic origin, 40 isolates tested incited lesions on stems of watermelon in their pathogenicity assay.

Keinath (1995) also reported that eight *Phoma* isolates and two *D. bryoniae* isolates from watermelon were not pathogenic when compared to the control. In this experiment, all isolates studied were pathogenic to watermelon, but there were clear distinctions in the pattern of severity to host plant. The variation among *D. bryoniae* isolates in causing disease on watermelon is conformed as described earlier by Keinath (1995).

The use of RG-specific primers allowed for the classification of the majority of the isolates of *D. bryoniae* in the current study into the RG I group and one isolate into RG III (Figure 5.4). However, about 25.7 % of the isolates could not be classified in either group (RG not determined). None of the isolates was classified as RG II using the RG-specific primers. Similar analysis by Keinath (2001) revealed RG I as the most prevalent group of *D. bryoniae* isolates in America. Moreover, Kothera *et al.* (2003) found 69% of a worldwide collection of *D. bryoniae* isolates, belonged to RG I, and 17.6% not classified in RG groups. This is more or less similar to the finding of this study using the same set of primers. Similarly, the degree of molecular variability found in *D. bryoniae* populations in the current study is compatible with the previously described variation found in the worldwide collection studied by Kothera *et al.* (2003).

The use of a new set of random primers described in this study allowed for separation of isolates into two distinct groups, based on RAPD fingerprinting profiles. The first group, RAPD group 1, included about 80 % of the isolates and is clearly related to RG I. The RAPD group 2 cluster included 3 isolates that represent RG III (Db-06, Db-33 and Db-01). RAPD profiling clustered the *D. bryoniae* isolates into a single phylogenetic group with several subgroupings. The RAPD markers could not give elaborate information on the diversity of the isolates under study; but clearly differentiated the *Phoma* isolates from the *D. bryoniae*.

The RAPD analysis is based on multiple loci in the entire genome (Welsh and McClelland, 1990; Williams *et al.*, 1990). Even though entire fungal genome is subjected to faster evolution (Bruns *et al.*, 1991), which could provide insights in to the diversity of the organism through marker analysis, our RAPD assays produced less information. This could possibly be due to the homothallic nature of the *D. bryoniae* isolates, producing fewer variations in the entire genome. However advanced genetic fingerprinting of the isolates is in progress to have a better understanding of the isolates. Similar result was reported by Santos ***et al.*** (2009), who reported that ***D. bryoniae*** and ***Phoma*** isolates were grouped into three categories based on RAPD profile.

Analysis of the ITS region of the isolates indicated that the ITS-1 region was highly efficient in differentiating the variability within the ***D. bryoniae*** isolates, as well as with the ***Phoma*** spp., whereas the ITS-2 region was efficient in only producing a clear demarcation between the ***D. bryoniae*** and ***Phoma*** spp. These data correlates with the previous reports of rapid evolution and higher variability of the ITS-1 region in fungal communities (Hershkovitz and Lewis, 1996; Nilsson ***et al.***, 2008).

Phylogenetic analysis derived from the ITS sequence data grouped 33 ***D. bryoniae*** isolates into a single, common phylogenetic cluster (Clade 1), while the two isolates DB-05 and DB-33 formed a separate group. The clade 1 cluster comprised of both RG-I positive and negative isolates, and hence it was not possible to group them according to the system of previous RG classification. Moreover the isolates in the clade 2 also differed, such that the DB-05 was found to be positive with the RG-I specific primers, while the DB-33 was found to be negative.

In the current study, the pathogen isolate was identified as ***D. bryoniae*** and ***Phoma*** on the basis of comparison of rDNA ITS sequence data with other related data from GenBank and previous studies. Other authors also conducted ITS sequence analyses to distinguish ***D. bryoniae*** from ***Phoma*** spp. isolated from cucurbits. (Keinath, 1995; Somai ***et al.***, 2002a; Somai ***et al.***, 2002b).

5.5. Conclusion and Recommendation

On the evidence presented using morphology, pathogenicity and molecular techniques, this study isolated *D. bryoniae* and *Phoma* species from cucurbits, *D. bryoniae* being predominant over *Phoma*. The *D. bryoniae* isolates collected from cucurbit species in USA therefore show a level of genetic diversity which is higher than has been previously reported. The data, however, indicate that the majority of *D. bryoniae* isolates are classified as RG I. The pattern of variability described here is analogous to earlier observations of independent studies. Part of the uniformity observed in this collection could be explained by dissemination of genetically similar isolates by seeds from the same lot, since most of them originated from plants obtained in commercial fields. The biological and molecular analyses performed in this study were suitable for revealing previously undescribed genetic variation in populations of *D. bryoniae* and *Phoma* in the USA.

Even though the use of molecular tools such as ITS, RAPD and RG specific primers allowed us to differentiate among *D. bryoniae* and *Phoma* isolates, future studies using DNA analysis of other genetic regions will be important in order to resolve relationships among these taxa. The present study also emphasizes the potential of introduction of several new exotic isolates such as DB-05 and DB-33. The potential entry of such new exotic isolates can bring new problems, including the presence of unique fungicide resistance patterns. *Didymella bryoniae* isolates in the countries listed above may potentially have unique fungicide resistance patterns, which if introduced poses a major risk to U.S. watermelon production. The study also stresses the need for the advanced quarantine measures to prevent the possibilities of introduction of new isolates.

CHAPTER 6 EFFECT OF TIADINIL AND THYMOL-BASED FORMULATIONS ON GROWTH OF *DIDYMELLA BRYONIAE* AND GUMMY STEM BLIGHT DEVELOPMENT

6.1. Introduction

Gummy stem blight (GSB) caused by *Didymella bryoniae* (anamorph; *Phoma cucurbitacearum*) is a major foliar fungal disease of watermelon (*Citrullus lanatus*) throughout the world limiting the production by 30-50% during ideal conditions for pathogen spread and disease development (Keinath, 1995). GSB was reported for the first time in the U.S. affecting watermelon fruit in 1917 in Florida (Sherbakoff, 1917) and now is present throughout the world (Keinath and Duthie, 1998).

Losses of marketable fruit because of the disease in untreated experimental plots averaged 43% of the maximum yields (Keinath and Duthie, 1998). The disease also infects other cucurbits including cantaloupe (*Cucumis melo* L.), cucumber (*Cucumis sativus* L.), and pumpkin (*Cucurbita* spp.) (Zitter, 1996).The GSB causal agent, *D. bryoniae*, can survive on seeds, weeds and plant debris from previously infected cucurbits (Sitterly and Keinath, 1996). The fungus infects all above ground parts of the plant and. symptoms of GSB fruit infection (known as black rot) may be evident in the field or develop later in storage (Zitter, 1996; Keinath, 2002).

Long-term crop rotation with non-cucurbits (>2 year) has the potential to reduce *D. bryoniae* inoculum levels in soil/plant debris. However, in most cases this is not practical due to limitations in finding new fields of the cultivable land and thus, not economically not feasible for growers. Most growers practice a short-term rotation of one year (Keinath, 1996), which may not be sufficient in reducing soil inoculum levels. Bio-control agents have been previously tested against GSB, but they are not effective as a stand-alone product (Utkhede and Koch, 2002).

Chemical control has been the most effective method for GSB management for many decades including use of fungicides with Chlorothalonil, Mancozeb, Maneb, Difenoconazole, Tebuconazole, and Thiophanate-Methyl as active components. However, isolates of *D. bryoniae* resistant to benomyl and thiophanate-methyl were found in all major watermelon producing areas of U.S. and the rest of the world (Lee, 1982; Johnson *et al.*, 1995; Wehner and Shetty, 2000). Subsequently, resistance to strobilurin (QoI) fungicides (e.g., azoxystrobin, famoxadone, kresoxim-methyl, pyraclostrobin, trifloxystrobin), benzimidazoles (e.g., thiophanate-methyl, thiabendazole), boscalid and penthiopyrad was reported in the USA and Brazil (Dos-Santos *et al.*, 2006; Keinath, 2009).

Current recommendations emphasize use of protectant fungicides with multi-site activity against the fungal organism, such as chlorothalonil and in rotation with chemicals having relatively newer chemistries. However, the ability of *D. bryoniae* isolates continually to develop resistance to different classes of fungicides, used for its control, emphasizes the need for screening of additional and eco-friendly protective methods for watermelon growers long term and effective control of GSB (Dos-Santos *et al.*, 2006).

Host plant defense inducers are a potential option for disease management, but currently no effective inducers against GSB has been reported. A relatively newer material is Tiadinil, which was developed in Japan against *Magnaporthe grisea*, the rice blast fungus. The chemistry in tiadinil is characterized by a unique heterocyclic moiety, 1,2,3-thiadiazole, and is described as a novel host plant defense inducer (Figure 6.6). It is registered in Japan and South Korea and is used in combination with other fungicides against rice blast (Tsubata *et al.*, 2006).

Foliar treatment with natural compounds with having anti-microbial properties is another biorational possibility in effectively managing GSB. A previous study has shown that various plant extracts and essential oils inhibited mycelial growth and conidia germination of plant pathogens including *D. bryoniae* (Pattnaik *et al.*, 1996; Fiori *et al.*, 2000; Adebayo *et al.*, 2013). Thymol is an active ingredient in many plant essential oils with antimicrobial properties and has been shown to be an effective bio-fumigant in treating *Ralstonia* *solanacearum*, the causal agent for bacterial wilt in tomato and anti-fungal properties of thymol could control against various plant pathogens (Ji *et al.*, 2005; Ji *et al.*, 2007; Adebayo *et al.*, 2013).

The objectives of this study were to evaluate the effect of tiadinil and thymol-based formulations on *D. bryoniae* growth *in vitro* and to assess the effect on GSB development on watermelon seedlings under different concentration, application method, and frequency of applications.

6.2. Materials and Methods

6.2.1 Test pathogen

The experiments were conducted at the University of Florida, North Florida Research and Education Center, Quincy, FL between November 2012 and May 2013. A highly pathogenic isolate of *D. bryoniae* (DB-32) isolated from watermelon was used throughout the experiment. The pathogen was confirmed as *D. bryoniae*, based on morphology, and PCR using RG-specific primers and ITS sequencing (Somai *et al.*, 2002a).

6.2.2 Effect of formulations against growth of the pathogen

The poison food method was used for evaluating effect of test compounds on fungal growth inhibition tests. Stock solutions (360 g/ L) of tiadinil (Nichino America, Inc., Wilmington, DE) and thymol-based formulations I (MPMB919121, AgriMor Int'l Co., Aventura, FL) and II (MPMB919122, AgriMor Int'l Co) were mixed with sterile Quarter-strength Potato Dextrose Agar (QPDA) consisting of 5 g granulated agar and 7.6 g potato dextrose agar in 1 liter of sterile deionized water and poured (20 mL) on to 80 mm petriplates to get final concentrations of 0.1, 1, 10, 100, 1000, 2000, 3000, 4000, and 5000 ppm active ingredient.

The control QPDA medium was amended with equal amounts of sterilized deionized water instead of the chemical suspension. The culture medium was inoculated at the center with a 5 mm agar disc, cut from the margin of actively growing culture of *D. bryoniae*, DB32 isolate. Three replicates were maintained in each case and inoculated petri plates were kept incubated at 24°C for 21 d. The radial growth (an average cross diameter starting from inoculation point) of the colonies was measured on every other day for 14 d as average cross diameter starting from inoculation for three weeks. The measurements in the control and treatment lines were transformed into percent inhibition of radial growth using the following formula:

$$\text{Growth inhibition (\%)} = \frac{\text{C-T}}{\text{C}} \times 100$$

where C= growth in the control and T= growth in the treatment.

6.2.3 Effect on conidia germination

Didymella bryoniae isolate Db32 was grown at 24℃ for 2-15 d on Malt Extract Agar (MEA; Maltose 12.75 g, dextrin 2.75 g, glycerol 2.35 g, peptone 0.78 g, Agar 15.0 g in 1 liter sterile deionized water) under 12 hour light period in 8-cm diameter petriplates. Sterile distilled water (20 mL) containing 0.05% Tween 20 was added to each plate, and the surface was scraped gently with a sterile loop to release the conidia. The resulting conidial suspension was filtered through cotton sheath cheese cloth to remove any mycelial fragments. The conidial suspension was diluted with sterile deionized water and adjusted to the final concentration of 10^4 conidia/ mL using a hemacytometer. Conidial suspensions were made separately against different concentrations (0.1, 1, 10, 100, 1000, 2000, 3000, 4000, and 5000 parts per million (ppm) of tiadinil and thymol-based formulations. Control consisted of sterile deionized water only.

Depression slides (Fisher scientific 25.4 x 76.2 mm) received 90 µl of the chemicals and 10 µl of the conidia suspension and each chemical concentration were replicated three times, mixed, covered with cover slip and incubated at 25°C for 48 h. To determine the number of germinated conidia, the slides were observed under a compound microscope (Nikon Eclipse 80i, Nikon, Japan) fixed fitted with a micrometer, at 48 hours h. The percent germinated conidia and germ tube lengths were determined by examination of 100 conidia for evidence of germ tube emergence (Schmit *et al.*, 1975). Percent inhibition of conidia germination was calculated and compared to the control. The experiment was laid in a 3 × 10 Complete Factorial Design in CRD, including all combinations of three levels of chemical formulation (tiadinil, thymol I and thymol II) and ten levels of concentration (0.1, 1, 10, 100, 1000, 2000, 3000, 4000, and 5000 ppm).

6.2.4 Evaluation of chemicals on watermelon seedlings

To evaluate the effect of tiadinil and thymol-based formulations on GSB disease development, seeds of watermelon variety “Melody” (Syngenta Seeds Inc., Minnetonka, MN) were planted in a seedling tray (size of the tray; cell size consisting of 128 holes with individual hole size of 3.5 × 3.5 × 4.5 cm (L × W × H). The green house temperature was set at 28°C and relative humidity was maintained at 75-80%. After 3 weeks, four-leaf stage seedlings were transplanted in 10 cm plastic pots with one seedling per pot. Pathogen conidia were prepared as described under conidia germination test and the concentration was adjusted to 10^5 conidia/ ml.

The disease development was evaluated at different concentrations (0.1, 1, 10, 100, 1000, 2000, 3000, 4000 and 5000 ppm) of each test compound (tiadinil and the two thymol-based formulations) with two different sets of application timings. In the first set, pathogen conidia were inoculated (suspension was sprayed on each seedling until run-off), and then each concentration of the test materials (20 mL/ plant) was treated after 48 h of inoculation. In the second set, seedlings were first treated with the test materials followed by pathogen inoculation after 48 h. Control treatments consisted of water and a commercial standard chlorothalonil (Bravo Weatherstik®; 3,000 ppm).

Plants were examined every day for disease symptoms beginning at the second day after treatment. GSB severity was assessed visually and rated on a 0 to 11 Horsfall-Barratt scale, (Horsfall and Barratt, 1945). The scales were converted to midpoint averages of disease severity and the disease progression was calculated using Area Under Disease Progress Curves (AUDPC); (Madden ***et al.***, 2007), after 3 wk post pathogen inoculation.

The treatments were replicated five times (one plant/replication) and the experiment was repeated. A factorial split-plot model was adopted, in which the factorial crossing design (3 × 11) was subordinated to the application time. Thus, the application time was the main factor, and three chemical formulations (tiadinil, thymol I and thymol II) were sub factor and eleven concentration of chemicals (0, 0.1, 1, 10, 100, 1000, 2000, 3000, 4000, 5000 ppm and Bravo instead of chemicals) were used as sub-sub plot factors of variation.

6.2.5 Effect of application method (foliar and drench) on efficacy of Tiadinil

To evaluate the effect of application method, tiadinil was prepared at three concentrations (1000, 3000 and 5000 ppm). Four-leaf stage seedlings of watermelon variety “Melody” were planted at one plant per pot. Pathogen conidial suspension of the pathogen was prepared as described previously and concentration was adjusted to 1×10^5 conidia/ml and the suspension was sprayed on each seedling until run-off. After 48 h of pathogen inoculation, plants were treated either as drench or foliar applications with 20 mL volume of each concentration of the formulations. There were five replications per treatment. Control plants were treated with sterile deionized water and chlorothalonil was used as a commercial standard (control). Plants were examined every other day for disease symptoms beginning of second day after inoculation. GSB severity was assessed with Horsfall-Barratt scale and AUDPC was calculated as described above and the experiment was repeated once.

6.2.6 Effect of application interval and concentration on efficacy of Tiadinil

Pathogen conidia conidial suspensions were prepared as described previously and concentrations adjusted to 10^5conidia/ ml. Seedlings of the watermelon cv. “Melody” at four-leaf stage were transplanted to pots with one plant per pot. Tiadinil was prepared at three concentrations (1000, 3000 and 5000 ppm) and each concentration was applied under five different timing scenarios which are as follows: (i) 2 d before pathogen inoculation (DBPI), (ii) 2 d after pathogen inoculation (DAPI), (iii) 2 DBPI and 2 DAPI, (iv) 2 DBPI, 2 DAPI and 6 DAPI and (v) 2 DAPI and 6 DAPI. The experimental design was a two factor (treatment concentration and frequency of application) arranged in CRD with five replications and the experiment was repeated. Seedlings were kept under greenhouse conditions for 3 weeks and disease severity was examined every other day as described above.

6.2.7 Statistical analysis

Statistical analyses of the data were performed with the SPSS software, version 20 for Windows. To assess the effect of the replication of experiments, a three-way ANOVA model was used by using repetitions as main plot and treatment combinations as sub effect. Because number of trial in all experiment had neither a significant ($P \leq 0.05$) main effect nor significant ($P \leq 0.05$) interactions with factors, the data were merged for the 2 trials. Percentage data was transformed ASIN prior to data analysis to normalize variance. Repeated measures ANOVA was performed on mycelia growth inhibition experiment at different sampling time. Two-way ANOVA was performed to determine the effect of Effect of application method and Effect of application interval and concentration. Three-way ANOVA was used to determine the influence of chemical formulation at different concentration and two application timing. Means were separated by S-K-N least significant ($P \leq 0.05$) differences at $P \leq 0.05$.

6.3. Result

The growth of *D. bryoniae*, as determined by the increase in mycelia growth, was significantly reduced by the presence of tiadinil and two thymol-based formulations at 0.1 to 5000 ppm a.i. (Figure 6.1). Analysis of variance revealed that the kind of chemical formulation, the concentration applied and the interaction were significant ($P \leq 0.05$) in terms of reducing the mycelial growth of the pathogen over the 14 observation days. Among the chemical formulations tested, the highest growth inhibition percent was recorded for the thymol-based formulation I (82.03 %) followed by thymol-based formulation II (80.23 %) and the least was found from tiadinil formulation (79.83 %) (Figure 6.1).

All the concentrations tested significantly ($P \leq 0.05$) affected the mycelia growth of the pathogen as compared to the control. Complete suppression of the mycelial growth of the pathogen throughout the incubation period was found from all formulations (tiadinil, thymol-based formulation I and II) starting from 100 ppm. The combination of *D. bryoniae* conidia and tiadinil and two thymol-based formulations at 10 ppm resulted in complete suppression of mycelia growth up to the 4th and 8th day from the start of trials, respectively. Although there was no complete inhibition, statistically significant ($P \leq 0.05$) inhibition was found at concentrations of 0.1 and 1 ppm of all formulations. As the incubation day increase progressive growth of the pathogen was observed at concentration between 0.1 and 10 ppm of all formulations (Figure 6.1).

Most *D. bryoniae* spores germinated in the untreated control (99 to 100% germination). The tiadinil and two thymol-based formulations, when applied separately, significantly inhibited spore germination at most of the concentrations tested. ANOVA revealed that the kind of chemical formulation, the concentration applied and the interaction were significant ($P \leq 0.05$) in terms of reducing the spore germination and germ tube elongation of the pathogen (Figure 6.2). There was a significant ($P \leq 0.05$) difference among the 3 chemical formulations. The highest mean percent inhibition of spore germination was found from thymol I (68.53 %) followed by thymol II (68.3 %) and there was no significant ($P \leq 0.05$) difference between these two formulations (Figure 6.2). On the other hand tiadinil gave the least mean percent inhibition of spore germination (59.97 %) and statistically lower than thymol-based formulations.

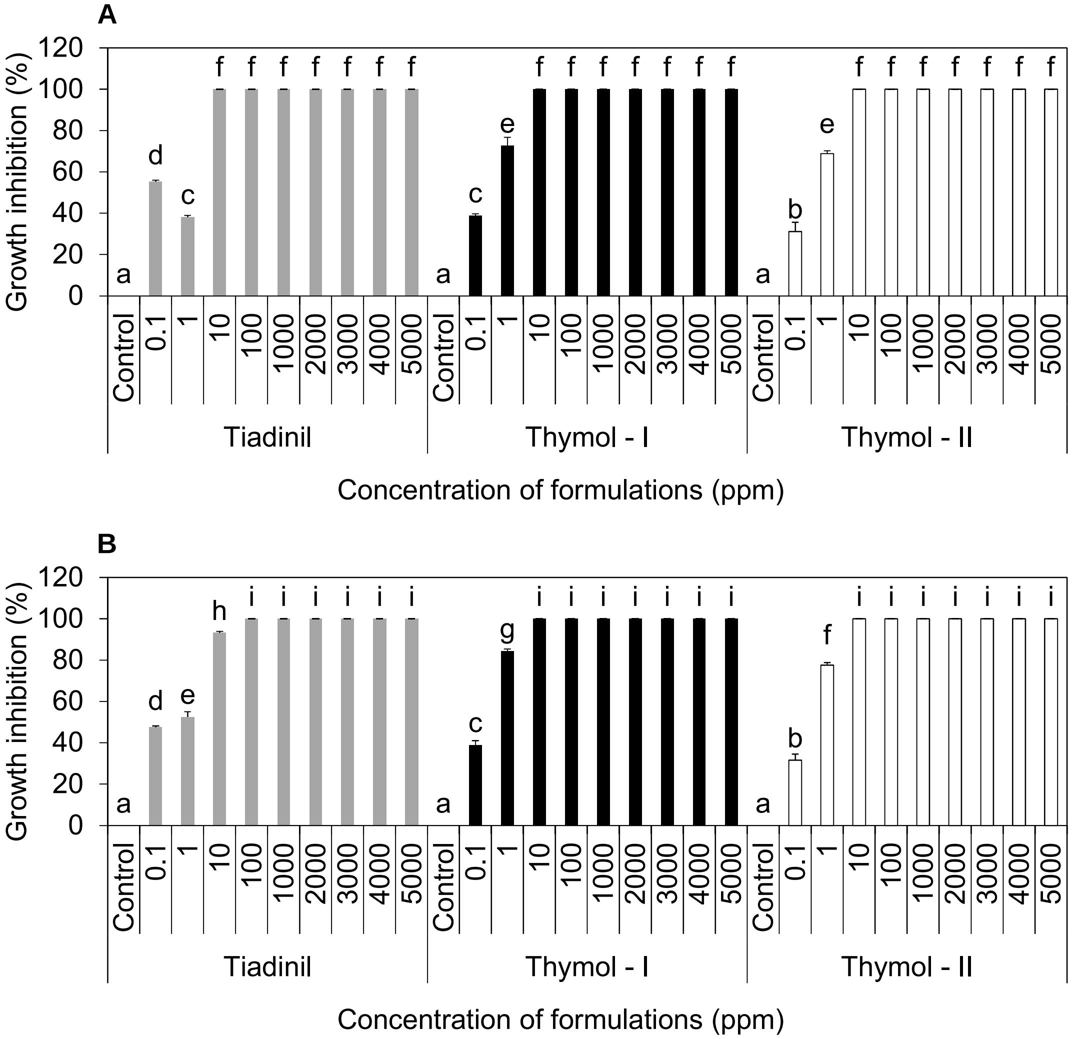


Figure 6. 1 Mycelial growth of *D. bryoniae* as affected by different concentrations of tiadinil, thymol-based formulation I and II

**Tiadinil and thymol-based formulations I and II were mixed with QPDA medium at concentrations of 0.1, 1, 10, 100, 1000, 2000, 3000, 4000, and 5000 ppm a.i. Plates were inoculated with *D. bryoniae* isolate or water (control). Percent mycelial growth inhibition of *D. bryoniae* recorded at (A) 4th and (B) 8th days after inoculation. Mean of three replications for each treatment. Means followed by same letter are not significantly different by Student-Newman-Keuls test ($P \leq 0.05$).

Germ tube elongations were significantly inhibited by the addition of tiadinil and two thymol-based formulations to the slide (Figure 6.2). There was a significant ($P \leq 0.05$) difference among the three chemical formulations. The lowest mean germ tube elongation was recorded from thymol II (19.05 μm) followed by thymol I (6 19.68 μm) and there was no significant

($P \leq 0.05$) difference between these two formulations. On the other hand tiadinil gave the highest mean germ tube elongation (28.15 μm) and statistically different than thymol-based formulations. All concentrations tested, significantly ($P \leq 0.05$) affected spore germination and germ tube elongation as compared to the control. Complete suppression of spore germination and germ tube elongation was found at concentrations starting from 2000 ppm and the list was found at 0.1 ppm (57.78 μm).

Tiadinil and two thymol-based formulations effectively reduced GSB development on watermelon seedlings. The antifungal efficacy of tiadinil was without noticeable phytotoxicity, but the two thymol-based formulations were toxic at 3000 ppm. Most tiadinil and two thymol-based formulations preparations applied at 24 h before spore inoculation on the plants significantly reduced both diseases compared with the water control ($P \leq 0.05$). The pre-inoculation applications of tiadinil (3000 ppm) and thymol I and II (2000 ppm) were found to be the most effective, allowing less than 1.5% AUDPC by ***D. bryoniae*** under highly disease conducive environmental conditions, which caused over 800 AUDPC on water treated control plants (Figure 6.3). Some pre-inoculation treatments of tiadinil (4000 ppm) and thymol I and II (3000 ppm) completely reduced disease, and they still were as effective as Bravo (chlorothalonil) control at 3000 ppm. (Figure 6.3). In contrast, delayed applications of tiadinil and two thymol-based formulations at 24 h as post inoculation treatments, effectively reduce both diseases, however causing >7.5% AUDPC (Figure 6.3).

Complete disease suppression was not recorded for any of the chemicals at any concentrations tested when they were applied 48 hour after the pathogen. Significant ($P \leq 0.05$) disease suppression as compared to the control was found from thymol at 100 ppm and thymol formulations at 10 ppm. In general, the effect of tiadinil and thymol was statistically comparable with bravo when they were applied at concentrations 48 hour before pathogen starting from 1000 and 100 ppm respectively. However the effect of tiadinil and thymol when they were applied at concentrations 48 hour after pathogen was comparable with bravo starting from 2000 and 1000 ppm respectively.

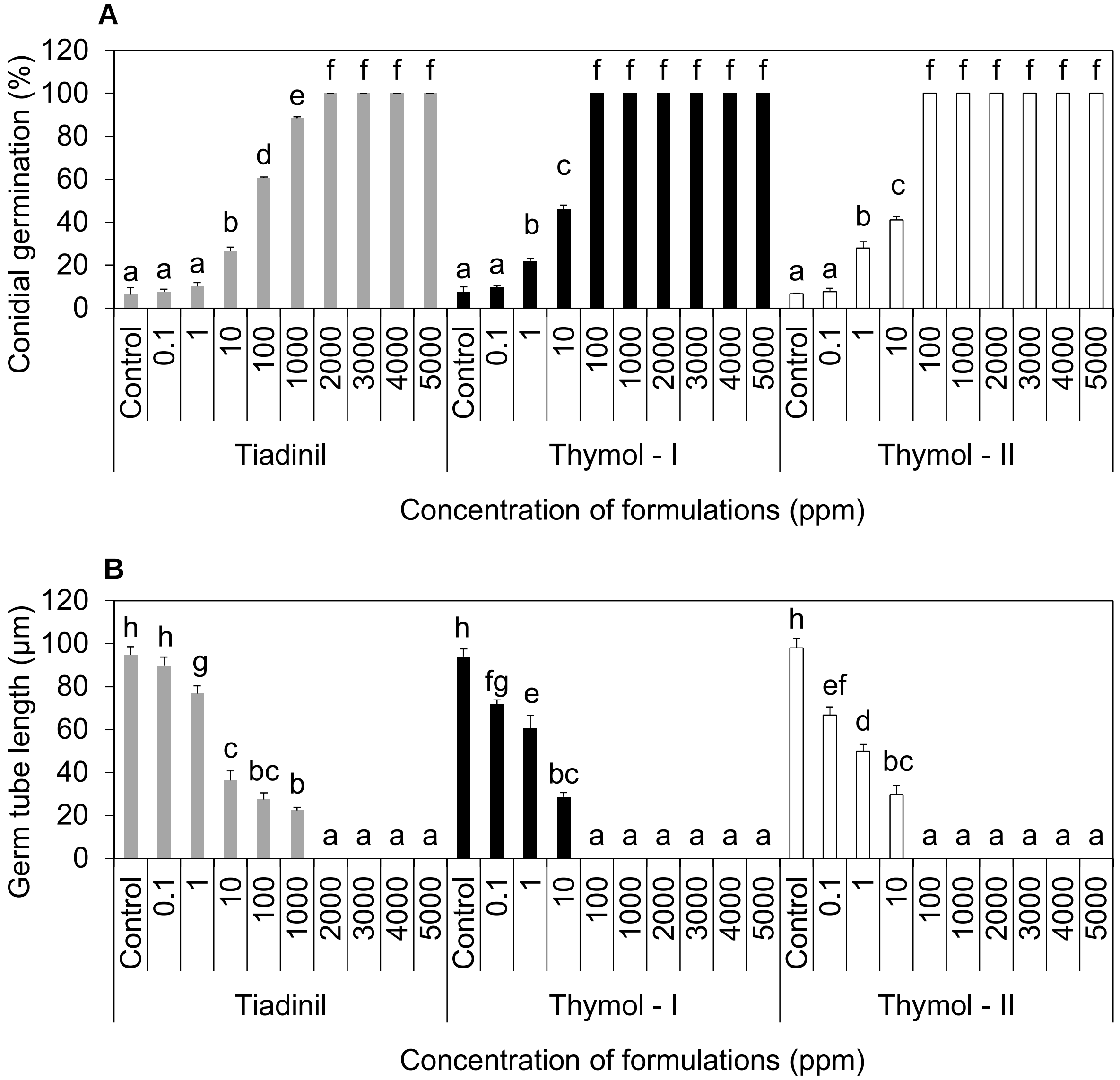


Figure 6. 2 Effect of different concentrations of tiadinil and thymol-based formulations on (A) *D. bryoniae* conidia germination and (B) germ tube elongation

**Tiadinil and thymol-based formulations I and II were mixed with conidia of *D. bryoniae* (10^4 conidia/ mL)at concentrations of 0.1, 1, 10, 100, 1000, 2000, 3000, 4000, and 5000 ppm a.i or water (control). Percent conidial germination and germ tube elongation of *D. bryoniae* was calculated from 100 random conidia. Mean of three replications for each treatment. Means followed by same letter are not significantly different by Student-Newman-Keuls test (P≤0.05) and are shown separately for all the three formulations. Error bars represent the standard error of mean.

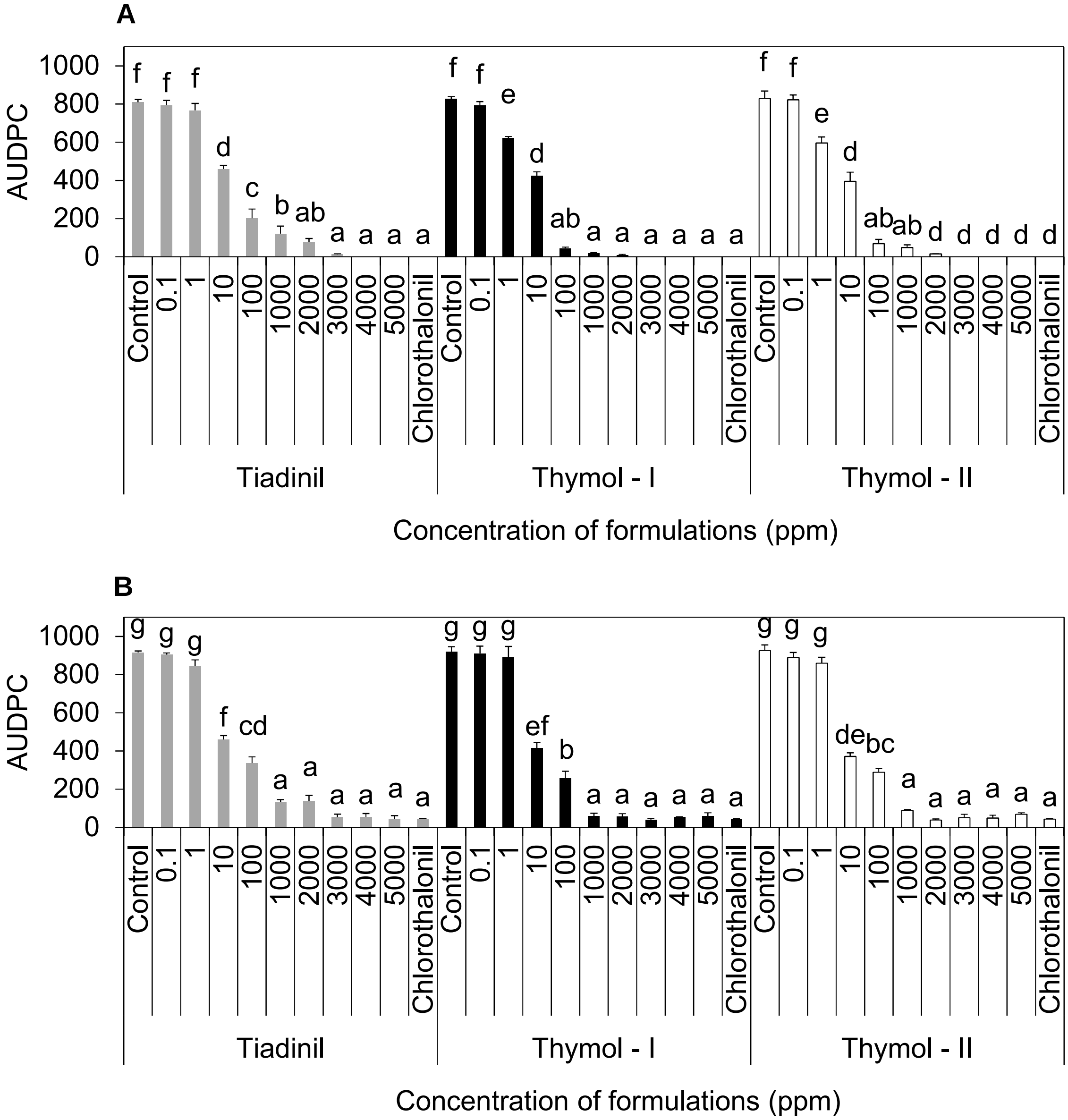


Figure 6. 3 Gummy stem blight, as measured by Area Under Disease Progress Curve (AUDPC) on watermelon seedlings treated with different concentrations of tiadinil and two thymol-based formulations

**Treatments were spray-applied 2 days before inoculation (DBI) (A) and 2 days after inoculation (DAI) (B). Chlorothalonil was applied at 3000-ppm active concentration (negative control) and water was applied as a positivecontrol. Plants were examined every other day for disease symptoms beginning at the 2nd day after treatment. GSB severity was assessed visually and rated on a 0 to 11 using Horsfall-Barratt scale. The scales were converted to midpoint averages of disease severity and the disease progression was calculated using AUDPC after three weeks. Mean of three replications for each treatment. Means followed by same letter are not significantly different by Student-Newman-Keuls test (P≤0.05). Error bars represent the standard error of mean

Differences in tiadinil concentrations occurred within the watermelon seedlings inoculated with ***D. bryoniae*** in terms of total AUDPC. Total AUDPC on watermelon seedlings were highest in the 1000 ppm, and lowest in the 5000 ppm (Figure 6.4). Disease development was significantly affected at 5000 ppm and 3000 ppm which showed comparable result. Spray application showed significantly lower AUDPC at all levels of thidanil on the watermelon seedlings than did drench treatments (Figure 6.4). There were significant interactions between application methods and tiadinil concentration. After 2 wk of treatment, disease levels decreased from 1000 to 5000 ppm with all application methods, but with drench, the values were similar for the 100, 3000 to 5000 ppm (Figure 6.4).

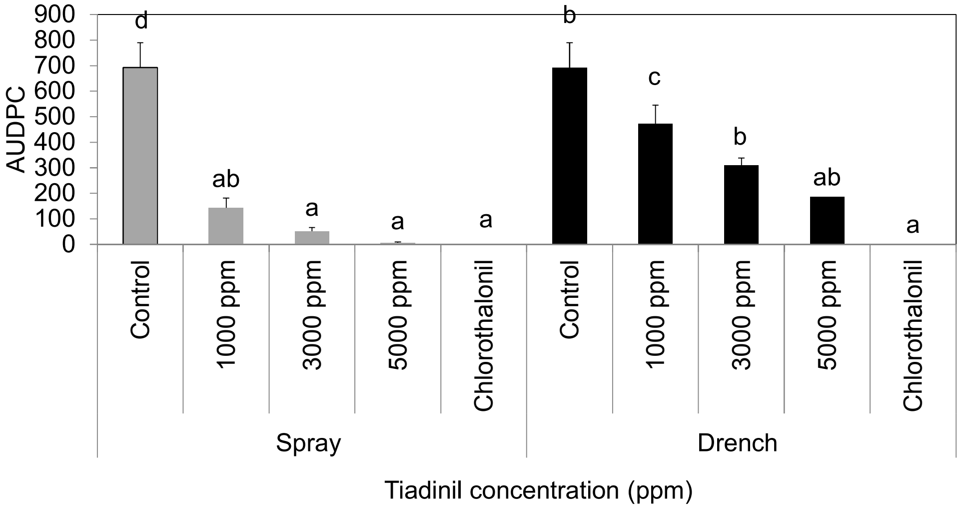


Figure 6. 4 Effect of application method (foliar and drench) and concentration (1000, 3000, and 5000 ppm) of tiadinil on gummy stem blight applied 2 days after inoculation
**Chlorothalonil was applied at 3000-ppmactive concentration. Mean of three replications for each treatment. Plants were examined every other day for disease symptoms beginning at the 2nd day after treatment. GSB severity was assessed visually and rated on a 0 to 11 using Horsfall-Barratt scale. The scales were converted to midpoint averages of disease severity and the disease progression was calculated using AUDPC after three weeks. Means followed by same letter are not significantly different by Student-Newman-Keuls test ($P \leq 0.05$). Error bars represent the standard error of mean

Both times of application and tiadinil concentrations treatments under conditions favorable for disease development in greenhouse experiments contributed to large differences in disease development on watermelon seedlings. All tiadinil concentrations reduced overall disease (AUDPC) for all application frequency treatments, compared with the non-sprayed treatment. The reduction in disease by 5000 and 3000 ppm was statistically comparable but not significantly higher than 1000 ppm. The two-spray (2 DAPI and DAPI and 2 DBPI and DAPI) and three-spray (2 DBPI, 2 DAPI and 6 DAPI) programs, provided significantly better control of disease (AUDPC) and comparable to bravo control. Spray treatment 2 DAPI and 6 DAPI provided the lowest disease (AUDPC = 88.10) of all treatments. Three application timings (2 DBPI, 2 DAPI and 6 DAPI and 2 DBPI and 2 DAPI) also had the lowest disease with AUDPC of 95.00 and 143.80, respectively. The remaining treatments had infection rates significantly lower than that of the control treatment. However no interaction between time of application and tiadinil concentrations treatments was found (Figure 6.5).

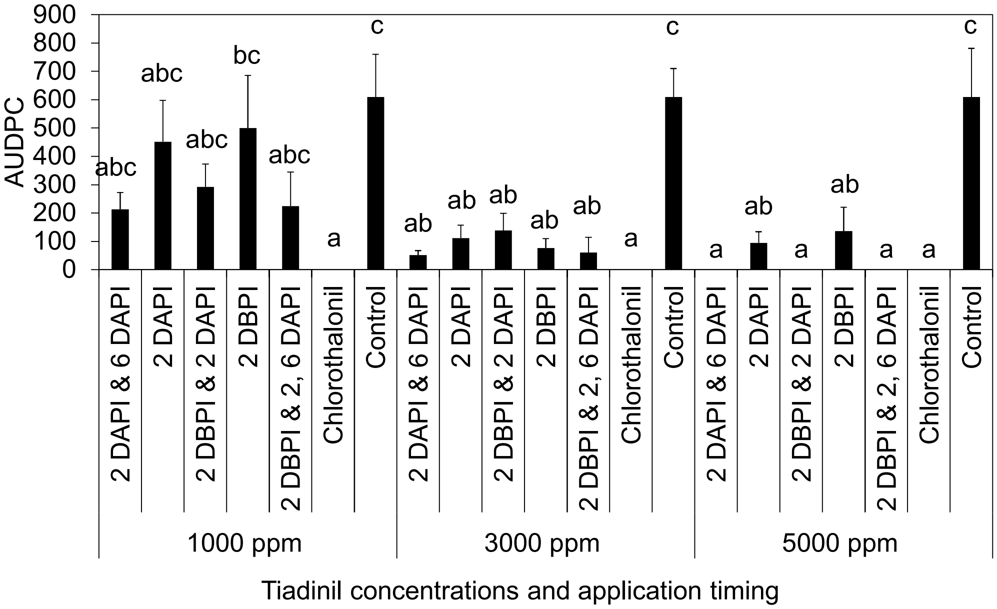


Figure 6. 5 Effect of application timing and concentration of foliar spray application of tiadinil on gummy stem blight disease development on watermelon seedlings

**The repeated numbers of applications are expressed as days before pathogen inoculation (DBI) and days after pathogen inoculation (DAI). Chlorothalonil was applied at 3000-ppm active concentration. Plants were examined every other day for disease symptoms beginning at the 2nd day after treatment. GSB severity was assessed visually and rated on a 0 to 11 using Horsfall-Barratt scale. The scales were converted to midpoint averages of disease severity and the disease progression was calculated using AUDPC after three weeks. Means followed by same letter are not significantly different by Student-Newman-Keuls test ($P \leq 0.05$). Error bars represent the standard error of mean

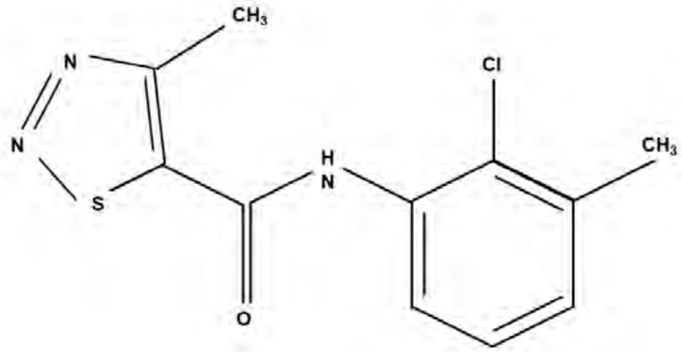


Figure 6. 6 Chemical structure of tiadinil, adapted from Yasuda *et al.* (2006)

6.4. Discussion

Tiadinil and the two thymol based formulations significantly inhibited the mycelial growth of *D. bryoniae* at all concentrations tested (0.1 to 5000 ppm a.i). The antifungal activity of thyme oils is well established against fungi such as *Rhizopus stolonifer* (Reddy *et al.*, 1998), *Rhizoctonia solani, Pythium ultimum, Fusarium solani*, and *Colletotrichum lindemuthianum* (Zambonelli *et al.*, 1996), and thymol appears to be one of the predominant active components of these oils (Shaaya *et al.*, 1991; Zambonelli *et al.*, 1996; Reddy *et al.*, 1998).

The three formulations completely inhibited the mycelial growth of *D. bryoniae* at the low concentration tested (10 ppm). Pattnaik *et al.* (1996) showed that three essential oils (*Cymbopogon citrates, Eucalyptus citriodora* and *Ageratum conyzoides*) completely inhibited the mycelial growth of *D. bryoniae* even at the lowest concentration (20 µl/ml). The decrease in percentage inhibition of mycelial growth found in higher concentrations of tiadinil as the incubation days increased may be attributed to the increase of some growth factor, which in high concentrations of tiadinil turned into a substrate stimulating the pathogen development as reported by Fiori *et al.* (2000) who reported that the efficacy of mycelial growth inhibition by *E. citriodora* and *Cymbopogon citratus* against *D. bryoniae* decreased in higher concentrations.

Tiadinil was reported to have Systemic Acquired Resistance (SAR) inducer activity (Yasuda *et al.*, 2006) and now we report that it has direct antifungal activity for the first time. Concentration of extract used was significant and mycelial inhibition was significantly lower with the lower concentrations for the three formulations. Tiadinil and both of the thymol based formulations resulted in 100% mycelial inhibition of *D. bryoniae*. D'Aulerio *et al.* (1996), reported the significance of concentration effect on control of some plant pathogens using aqueous extracts of garlic.

In the present study, tiadinil and the two thymol based formulations provided significant inhibition of spore germination and germ tube elongation at all concentrations tested. Similarly, Singh *et al.* (1988) showed that some phenolic compounds of *Echinops echinatus* proved to be efficient in inhibiting conidial germination of ***Alternaria tenuissima*** in concentrations of 25 to 150 µl/ml. Upadhyaya and Gupta (1990), reported the inhibition of conidial germination and germ tube elongation of ***Curvularia lunata*** with extracts of ***Ocimum sanctum***. Lakshmanan (1990) demonstrated the high antifungal properties of ***A. sativum***.

The tiadinil and the two thymol based formulations showed varying degree of inhibiting spore germination. Such variation in inhibition of spore germination by extracts of ***C. citrates, E. citriodora, A. conyzoides*** and ***Achillea millefolium*** was also verified by Fiori ***et al.*** (2000). It was observed that the two thymol based formulations completely inhibited germination of ***D. bryoniae*** spores in the presence of the aliquots of 100 ppm a.i. However, an aliquot of 1000 ppm a.i of tiadinil was not enough to completely inhibit spore germination.

The strong inhibitory effect of the thymol based formulations presented here supports other experiments with high dilutions of essential oils in order to obtain curves capable of showing minimum values of fungitoxicity. The results obtained are in accordance with those of Zambonelli ***et al.*** (1996) who verified that essential oils of ***Thymus vulgaris***, ***Lavandula*** and ***Mentha piperita*** in concentrations of 200, 400 and 800 ppm significantly affected spore germination and caused degeneration of hyphae as well as the cytoplasmatic emptying of ***Colletotrichum lindemuthianum*** and ***Pythium ultimum***.

Application time was the most important component of the three chemicals examined in these experiments. It was more important than type of of chemical or their active ingredient for improving disease control. Application of formulations 48 hour before pathogen inoculations were more effective overall than application 48 hours after pathogen inoculation, although both intervals reduced AUDPC. Berger (1977), suggested that fungicide applications early in the season were more effective because the amount of inoculum is lower than later in the season. In a previous study, delaying chlorothalonil applications did not reduce gummy stem blight compared with the non-sprayed control (Johnson ***et al.***, 1995). Johnson ***et al.*** (1995), found the delayed applications were less effective than early applications. Epidemics of downy mildew and GSB progressed more slowly on watermelon sprayed with chlorothalonil before symptoms appeared than on plants sprayed after symptoms appeared (Keinath, 1995, 1996).

There was a difference between the three formulations used in these experiments. Thymol based formulations always provided better disease control than tiadinil, although all formulations were applied at the same rate. This difference has been observed previously on other fungicides with GSB on watermelon and Alternaria leaf blight of muskmelon (Suheri and Latin, 1991; Keinath, 1995).

The results showed foliar application of tiadinil to be most effective in reducing the severity of GSB on watermelon. Other studies have shown foliar application of chemical formulations to be ineffective against certain fungal pathogens. Homma *et al.* (1981), reported that foliar applied sodium bicarbonate was shown to inhibit the germination of powdery mildew *Podosphaera xanthii* (syn. *Sphaerotheca fuliginea*) on cucumbers and cause abnormal conidial formation with reduced pathogenicity. Cook *et al.* (1993), showed foliar-applied potassium chloride to be effective against powdery mildew (*Erysiphe graminis*) in a field experiment. Foliar-applied tiadinil was more effective in controlling GSB than appears to be the case for drench application. Therefore, an osmotic mode of action was possible. Foliar-applied tiadinil may adversely affect both conidial germination of *D. bryoniae* on the leaf surface and leaf colonization by means of a depression in the water potential of the plant (Kettlewell *et al.*, 2000). Applying tiadinil to the foliage rather than the soil at a time when the plant requires high amounts may reduce the potential for leaching and provide fungicidal benefits.

Tiadinil applications at different concentrations and frequent intervals to control GSB during the green house experiment were important to reduce GSB development. In the current study, the most important component of the formulation application was the application frequency. More frequent applications also reduced GSB severity more than single applications. The benefits of frequent fungicide applications also have been demonstrated with other diseases. For example, melanose control on grapefruit was significantly better with four applications of copper hydroxide than with one, two, or three applications, although equal amounts of metallic copper were applied in all treatments Timmer ***et al.*** (1998).

6.5. Conclusion and Recommendation

In this study, both tiadinil and thymol-based formulations inhibited growth of *D. bryoniae in vitro* and reduced GSB severity on watermelon seedlings compared to untreated controls. Tiadinil inhibited the mycelial growth of *D. bryoniae* and EC_{50} value was found to be between 100 and 1000 ppm. It was also observed that tiadinil completely inhibited germination of *D. bryoniae* conidia at concentrations of ≥2000 ppm. Germ tube elongation was also affected by all concentrations of tiadinil, which indicated to the direct antifungal activity.

Conidial germination and germ tube elongation are critical steps in the infection process of fungal pathogens, and results from the current study indicate that tiadinil has the potential to suppress *D. bryoniae*. However, the effect of tiadinil on inhibition of conidial germination on the host tissue has to be further investigated. Foliar applications of tiadinil at ≥ 3000ppm were effective in reducing GSB disease severity compared to the untreated control; and the disease severity was comparable to the commercial control. Tiadinil is classified under the FRAC code “P 3” which indicates to the host plant defense induction mode of action. This study also proposed that the SAR activity noticed apparent SAR activity might be due to triggering of a signaling pathway at the same level or downstream of the salicylic acid pathway.

In conclusion, tiadinil and thymol-based formulations are potential low-risk approaches for GSB management. This is the first study on the effect of tiadinil on GSB and the description of a material described as a host plant defense inducer in one crop to have direct antifungal activity against another pathogen on another crop. The result also highlighted the posible utilization of the formulations against GSB after evaluation on isolates of GSB causal pathogen.

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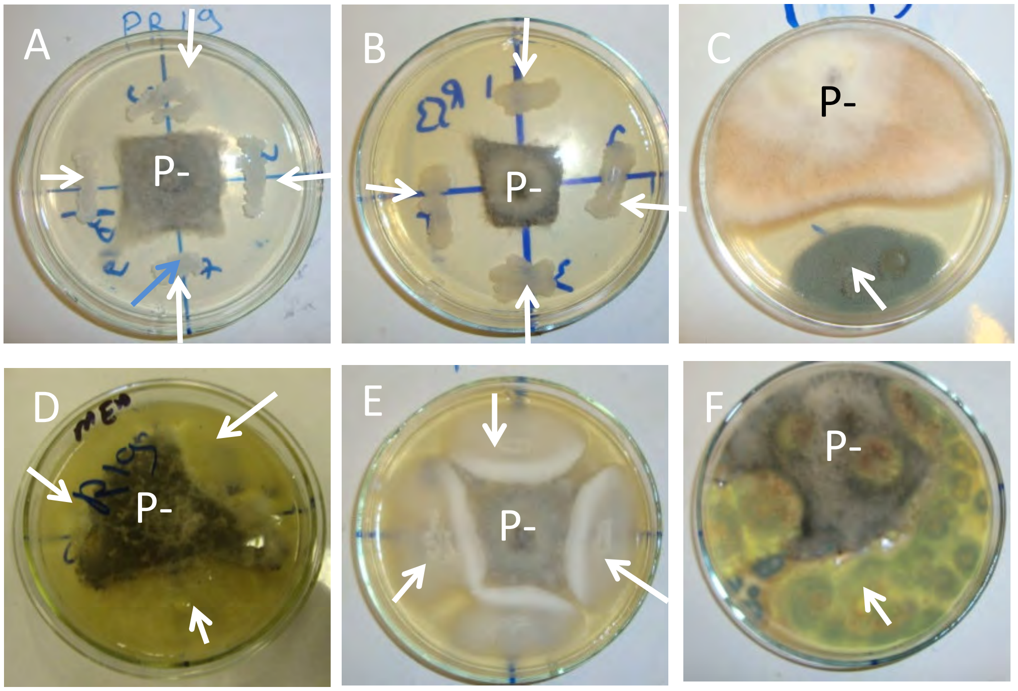
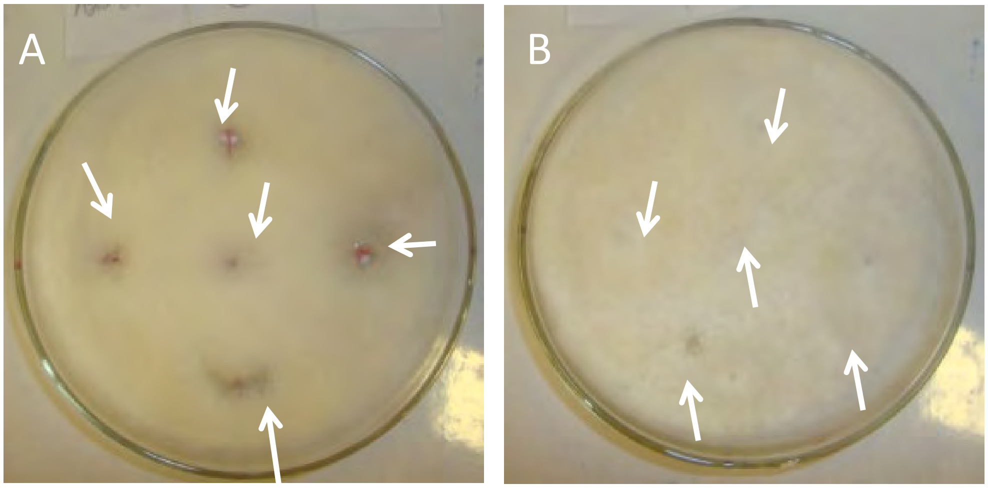
APPENDICES



Appendix Figure 1 Screening of antagonistic activity against *C. gloeosporioides* on MEA agar resulting in inhibitory effect of bacterial (a), yeast (b) fungal isolates (c), lytic activity by bacterial (d) yeast (e) and mycoparasitic activity of fungal (f) isolates. P- indicates the pathogen inoculation point and arrows indicate antagonist inoculation points



Appendix Figure 2 Plates showing clear zones of inhibition against *C. gloeosporioides* (By agar well diffusion method). (a) treated with antagonist metabolite and (b) control: arrows indicate inoculation holes

Appendix Table 1 Category of morphological characteristics used to compare 45 *Colletotrichum* isolates [*]

Category	Description
1 Overall appearance	
1.1 Colony development	Uniform, Concentric ring, Sector, Irregular, Irregular and Sector, Concentric ring and Sector, Spiral, Concentric ring - Spiral, Concentric ring - Sulcate, Uniform Sulcate, Other
1.2 Colony color	White, Orange, Yellow, Green, Brown, Black, Cream, Other
1.3 sector development	Uniform, Concentric ring, Irregular
1.4 sector color	White, Orange, Yellow, Green, Brown, Black, Cream, Other,
1.5 Colony diameter	,25mm, 26 - 35 mm, 36 - 45 mm, 46 - 55 mm, > 55 mm
2 Mycelium	
2.1 Mycelium form and elevation	
2.2 Mycelium color	
2.3 Mycelium appearance of sector	
2.4 Mycelium color of sector	
2.5 Sclerotia body	
2.5.1 Structure	Present, Absent
2.5.2 Distribution	Sparse, On plag, Not applicable
2.5.3 Number per dish	<10, 10 t0 50, >50, Not applicable
3 Reproductive structure	
3.1 acervuli,	
3.1.1 Structure,	Present, Absent
3.1.2 Setea	Present, Absent
3.1.3 Distribution	Sparse, Concentric ring, On plag, Not applicable
3.1.4 Number per dish	<10, 10 t0 50, >50, Not applicable
3.1.5 Spore mass color	Whitish, Orange, Blue, Not applicable
3.2 Complex conidiomata	
3.2.1 Structure	Present, Absent,
3.2.2 Setea	Present Absent
3.2.3 Distribution	Sparse, Concentric ring, On plag, Not applicable
3.2.4 Number per dish	<10, 10 t0 50, >50, Not applicable
3.2.5 Spore mass color	Whitish, Orange, Not applicable
3.3 Peritecia	
3.3.1 Structure	Present, Absent
3.3.2 Distribution	Sparse, Concentric ring, On plag, Not applicable
3.3.3 Number per dish	<10, 10 t0 50, >50,
3.1.4 Spore mass color	Whitish, Orange, Blue, Not applicable
3.4 Conidia	
3.4.1 Structure	Present, Absent
3.4.2 Shape	
3.4.3 Edge	<25, 25 to 50, 50 to 75, >75, Not applicable
3.4.5 length and width	<10, 10 to 15, 15 to 20, >20
3.5 Appersoria	
3.5.1 Structure	Present, Absent
3.5.2 Shape	
3.5.3 Edge	Ovoid to slight road, Crenate or deeply lobed, Not applicable
3.5.5 length and width	

[*] The appearance of colonies, occurrence of sectors, the vegetative and reproductive structures and several conidial and aspersoria characters of *Colletotrichum* isolates were described after 14 d of incubation by this multi scale category developed by Munaut et al. (2001)

Appendix Table 2 Sample collection and isolation of antagonist microorganisms

Farm Code		Spp. Location [b]	Altitude (m.a.s.l)	Mean Temp (°C)	Number of samples collected by host and plant part									
					Banana			Mango			Papaya			
	Re[a]				Le[c]	Fr	Sum	Le	Fr	Sum	Le	Fr	Sum	total
F001	So	37.54/ 8.25	1340	22.11	0	0	0	1	0	1	0	0	0	1
F002	So	37.26/ 7.56	1880	19.14	0	1	1	0	1	1	0	1	1	3
F003	Or	37.35/ 7.46	1660	16.53	0	1	1	0	1	1	1	0	1	3
F004	Or	36.43/ 7.41	1420	19.63	0	1	1	1	0	1	0	1	1	3
F005	Or	36.45/ 7.44	1420	19.63	1	0	1	0	1	1	0	1	1	3
F006	So	36.12/ 7.16	1940	18.29	1	0	1	1	0	1	0	1	1	3
F007	So	35.24/ 7.08	1200	21.30	1	0	1	0	1	1	0	1	1	3
F008	Ga	35.31/ 8.08	1500	18.12	0	1	1	0	1	1	1	0	1	3
F009	Or	35.53/ 8.31	1500	19.10	0	1	1	1	0	1	1	0	1	3
F010	Or	35.06/ 8.15	100	20.10	1	0	1	0	1	1	1	0	1	3
F011	Ga	34.49/ 8.11	520	27.43	0	1	1	0	0	0	0	1	1	2
F012	Ga	34.33/ 7.51	400	25.48	1	0	1	0	1	1	1	0	1	3
F013	Ga	34.27/ 8.13	480	26.54	0	1	1	1	0	1	1	0	1	3
F014	Ga	34.35/ 8.10	420	26.54	1	0	1	0	1	1	1	0	1	3
F015	Ga	34.25/ 8.12	440	26.54	0	1	1	1	0	1	1	0	1	3
F016	Ga	34.24/ 8.15	462	26.54	1	0	1	0	1	1	1	0	1	3
F017	Or	34.35/ 8.97	1280	19.80	0	1	1	1	0	1	0	1	1	3
F018	Or	34.36/ 8.36	1280	19.80	0	1	1	1	0	1	1	0	1	3
F019	Or	34.74/ 8.49	1920	20.71	1	0	1	0	1	1	1	0	1	3
F020	Or	35.01/ 8.44	1920	20.71	0	1	1	1	0	1	1	0	1	3
F021	Or	35.51/ 9.09	1520	20.08	1	0	1	0	1	1	0	1	1	3
F022	Or	36.39/ 9.01	1720	17.72	1	0	1	0	1	1	1	0	1	3
F023	Or	36.30/ 9.09	1400	17.72	0	1	1	1	0	1	0	1	1	3
F024	So	38.17/ 6.20	1820	17.51	0	1	1	1	0	1	1	0	1	3
F025	So	38.23/ 6.29	1800	17.51	0	1	1	1	0	1	1	0	1	3
F026	So	38.36/ 7.03	1840	19.14	1	0	1	1	0	1	0	1	1	3
F027	So	38.58/ 7.15	1800	18.52	0	1	1	1	0	1	0	1	1	3
F028	Or	40.73/ 9.08	1560	26.28	0	1	1	0	1	1	1	0	1	3
F029	af	39.72/ 8.62	1200	24.58	1	0	1	1	0	1	0	1	1	3
F030	af	39.57/ 8.51	1480	18.01	0	1	1	0	1	1	0	1	1	3
F031	af	39.78/ 8.67	1300	24.58	0	1	1	0	1	1	1	0	1	3
F032	af	39.88/ 8.76	980	24.24	1	0	1	0	1	1	0	1	1	3
F033	Or	38.73/ 7.90	1680	18.89	0	1	1	0	1	1	0	1	1	3
F034	Or	41.38/ 9.58	940	27.63	0	1	1	0	1	1	0	0	0	2
F035	Or	38.71/ 7.95	1680	18.89	0	1	1	0	1	1	0	1	1	3
F036	Dr	41.86/ 9.59	1100	26.31	0	1	1	0	0	0	0	0	0	1
F037	Or	41.72/ 9.63	1100	26.25	0	0	0	0	1	1	0	1	1	2
F038	be	34.55/ 10.01	1000	26.29	0	1	1	0	0	0	0	0	0	1
		Total			**13**	**23**	**36**	**15**	**20**	**35**	**17**	**17**	**34**	**105**

[a] Geographic regions in Ethiopia from where samples were collected: Or (Oromia), So(SNNP), Ga (Gambella), Af (Afar), Be (Benshangule)

[b] Shows the longitude (°E) / latitude (°N) of sampling sites

[c] Tissu of host crops from which isolates were recovered: Le = Leaf, Fr = Fruit, Sum = Sum of Leaf and Fruit

Appendix Table 3 Number of isolated microorganisms from collected plant tissues

Host		No of microorganisms collected [a]				Percent of microorganisms collected [b]			
crop	Tissue	Bac[c]	Yea	Fun	Total	Bac	Yea	Fun	Total
Banana	Leaf	82	72	33	187	5.24	4.60	2.11	11.94
	Fruit	150	144	78	372	9.58	9.20	4.98	23.75
	Total	232	216	111	559	14.81	13.79	7.09	35.70
Papaya	Leaf	92	71	49	212	5.87	4.53	3.13	13.54
	Fruit	83	77	46	206	5.30	4.92	2.94	13.15
	Total	175	148	95	418	11.17	9.45	6.07	26.69
Mango	Leaf	106	99	50	255	6.77	6.32	3.19	16.28
	Fruit	140	131	63	334	8.94	8.37	4.02	21.33
	Total	246	230	113	589	15.71	14.69	7.22	37.61
Total	Leaf	280	242	132	654	17.88	15.45	8.43	41.76
	Fruit	373	352	187	912	23.82	22.48	11.94	58.24
	Total	**653**	**594**	**319**	**1566**	**41.70**	**37.93**	**20.37**	**100.00**

[a] Number of microorganisms isolated from each host and tissue

[b] Percentage of of microorganisms from each host and tissue as compared to the total microrganisms isolated

[c] Category of microorganisms: Bac = Bacteria, Yea = Yeast, Fun = Fungi

Appendix Table 4 Summury of Primary screening for potential antagonistic effect of microbial isolates

Group	host	tisue	Total	No of isolates with activity [a]					% of isolates with activity [b]					
			isolates	Ant [1]	Lyt	MP	Chk	NA	Ant	Lyt	MP	Chk	NA	Total
Bacteria	Banana	Leaf	82	5	8	0	8	61	0.8	1.2	0.0	1.2	9.3	12.6
		Fruit	150	14	14	0	16	106	2.1	2.1	0.0	2.5	16.2	23.0
	Mango	Leaf	106	3	4	0	12	87	0.5	0.6	0.0	1.8	13.3	16.2
		Fruit	140	10	5	0	15	110	1.5	0.8	0.0	2.3	16.8	21.4
	Papaya	Leaf	92	5	3	0	5	79	0.8	0.5	0.0	0.8	12.1	14.1
		Fruit	83	3	1	0	6	73	0.5	0.2	0.0	0.9	11.2	12.7
Yeast	Banana	Leaf	72	11	1	0	32	28	1.9	0.2	0.0	5.4	4.7	12.1
		Fruit	144	18	1	0	60	65	3.0	0.2	0.0	10.1	10.9	24.2
	Mango	Leaf	99	1	2	0	46	50	0.2	0.3	0.0	7.7	8.4	16.7
		Fruit	131	3	4	0	57	67	0.5	0.7	0.0	9.6	11.3	22.1
	Papaya	Leaf	71	0	3	0	0	68	0.0	0.5	0.0	0.0	11.4	12.0
		Fruit	77	0	1	0	0	76	0.0	0.2	0.0	0.0	12.8	13.0
Fungi	Banana	Leaf	33	1	0	6	7	19	0.3	0.0	1.9	2.2	6.0	10.3
		Fruit	78	9	0	8	27	34	2.8	0.0	2.5	8.5	10.7	24.5
	Mango	Leaf	50	3	0	0	14	33	0.9	0.0	0.0	4.4	10.3	15.7
		Fruit	63	6	0	0	9	48	1.9	0.0	0.0	2.8	15.0	19.7
	Papaya	Leaf	49	4	0	0	30	15	1.3	0.0	0.0	9.4	4.7	15.4
		Fruit	46	2	0	0	24	20	0.6	0.0	0.0	7.5	6.3	14.4
	Total		**1566**	**98**	**47**	**14**	**368**	**1039**	**30.7**	**14.7**	**4.4**	**115.4**	**325.7**	**490.9**

[a] Number of microorganisms with biological activity by organism group, host and tissue

[b] Percentage of of microorganisms with biological activity as compared to the total microrganisms tested

[c] biological activity of microorganisms: Ant=Antibiosis, Lyt=Lytic, MP= Mycoparacitic, Chk= checke the pathogene growt before 50% of its growth and NA= isolates without any biological activity

Appendix Table 5 Alignment of the gene sequences of the 16rRNA, ITS-1 and ITS-4 domains from different bacteria, yeast and fungal antagonist isolates in this study

B-03-F-2
AGCAAAACGG CAGATACACA TGCAGTCGAG CGGTAGAGAG AAGCTTGCTT CTCTTGAGAG CGGCGGACGG GTGAGTAATG CCTAGGAATC TGCCTGGTAG
TGGGGGATAA CGTTCGGAAA CGGACGCTAA TACCGCATAC GTCCTACGGG AGAAAGCAGG GGACCTTCGG GCCTTGCGCT ATCAGATGAG CCTAGGTCGG
ATTACCTAGT TGGTGGGGTA ATGGCTCACC AAGGCGACGA TCCGTAACTG GTCTGAGAGG ATGATCAGTC ACACTGGAAC TGAGACACGG TCCAAACTCC
TACGGGAGGC AGCAGTGGGG AATATTGGAC AATGGGCGAA AGCCTGATCC AGCCATGCCG CGTGTGTGAA GAAGGTCTTC GGATTGTAAA GCACTTTAAG
TTGGGAGGAA GGGTTGTAAA TTAATACTCT GCAATTTTGA CGTTACCGAC AGAATAAGCA CCGGCTAACT CTGTGCCATC

P-10-F-7
GGGCCCCTTA ATACTCCGTG GTAGCGTGCT CCCGAAGGTT AGACTAAGCC TACTTCTGGA GCAACCCACT CCCATGGTGG GACGGGCGGT GTGTACAAGG
CCCGGGAACG TATTCACCGT GACGTTCTGA TTCACGATTA CTAGCGATTC CGACTTCACG CAGTCGAGTT GCAGACTGCG ATCCGGACTA CGATCGGTTT
TATGGGATTA GCTCCACCTC GCGGCTTGGC AACCCTTTGT ACCGACCATT GTAGCACGTG TGTAGCCCTG GCCGTAAGGG CCATGATGAC TTGACGTCAT
CCCCACCTTC CTCCGGTTTG TCACCGGCAA TCTCCTTAAA TGGGCCACCC TCTGGTGCTG CCATGCCACG AGTGTGAAGA AGGTCCTCAC GGCACTTAAA

M-05-F-4
GGGGAAGTGG GCGGCAGATA ACACATGCAG TCGAGCGGTA GAGAGAAGCT TGCTTCTCTT GAGAGCGGCG GACGGGTGAG TAATGCCTAG GAATCTGCCT
GGTAGTGGGG GATAACGTTC GGAAACGGAC GCTAATACCG CATACGTCCT ACGGGAGAAA GCAGGGGACC TTCGGGCCTT GCGCTATCAG ATGAGCCTAG
GTCGGATTAG CTAGTTGGTG GGGTAATGGC TCACCAAGGC GACGATCCGT AACTGGTCTG AGAGGATGAT CAGTCACACT GGAACTGAGA CACGGTCCAG
ACTCCTACGG GAGGCAGCAG TGGGGAATAT TGGACAATGG GCGAAAGCCT GATCCAGCCA TGCCGCGTGT GTGAAGAAGG TCTTCGGATT GTAAAGCACT

P-02-L-2
GGGGAAGTGG GCGGCAGATA ACACATGCAG TCGAGCGGTA GAGAGAAGCT TGCTTCTCTT GAGAGCGGCG GACGGGTGAG TAATGCCTAG GAATCTGCCT
GGTAGTGGGG GATAACGTTC GGAAACGGAC GCTAATACCG CATACGTCCT ACGGGAGAAA GCAGGGGACC TTCGGGCCTT GCGCTATCAG ATGAGCCTAG
GTCGGATTAG CTAGTTGGTG GGGTAATGGC TCACCAAGGC GACGATCCGT AACTGGTCTG AGAGGATGAT CAGTCACACT GGAACTGAGA CACGGTCCAG
ACTCCTACGG GAGGCAGCAG TGGGGAATAT TGGACAATGG GCGAAAGCCT GATCCAGCCA TGCCGCGTGT GTGAAGAAGG TCTTCGGATT GTAAAGCACT

P-11-L-1
CGGCTTTTCC CGGTTTACAC ATGCAGTCGA GCAGGTAGAG AGAAGCTTGC TTCTCTTGAG AGCGGCGGAC GGGTGAGTAA TGCCTAGGAA TCTGCCTGGT
AGTGGGGGAT AACGTTCGGA AACGGACGCT AATACCGCAT ACGTCCTACG GGAGAAAGCA GGGGACCTTC GGGCCTTGCG CTATCAGATG AGCCTAGGTC
GGATTAGCTA GTTGGTGGGG TAATGGCTCA CCAAGGCGAC GATCCGTAAC TGGTCTGAGA GGATGATCAG TCACACTGGA ACTGAGACAC GGTCCAGACT
CCTACGGGAG GCAGCAGTGG GGAATATTGG ACAATGGGCG AAAGCCTGAT CCAGCCATGC CGCGTGTGTG AAGAAGGTCT TCGGATTGTA AAGCACTTTA

M-23-L-1
GTGGGTCTCA ATATGATGCG GGCTGGACCT CTCGGGGTTA CAGCCTTGCT GAATTATTCA CCCTTGTCTT TTGCGTACTT CTTGTTTCCT TGGTGGGTTC
GCCCACCACT AGGACAAACA TAAACCTTTT GTAATTGCAA TCAGCGTCAG TAACAAATTA ATAATTACAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAGTGTGAAT TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT GCGCCCTTTG GTATTCCAAA
GGGCATGCCT GTTCGAGCGT CATTTGTACC CTCAAGCTTT GCTTGGTGTT GGGCGTCTTG TCTCTAGCTT TGCTGGAGAC TCGCCTTAAA GTAATTGGCA

M-30-F-2
CGGGTAGTCC TACCTGATTT GAGAATAAGA TCAGAGTCTG TAACAAGCTT AACTGTTTTA GACAATTTCG TTTCTGGCAG ACGCCTGCCG GCGTGGTGCC
CAACACCTTG CGAGAGAGAA ATATTGCTCA AACAGGCATG CTGTGTGGAA TGCCACACAG CGCAATGTGC GTTCAAAGAT TCGATGATTC ACGACTTGCG
TTTCGTACTA CGTATCGCAT TTCGCTGCGT TCTTCATCGA TGCGAGAACC TAGAGATCCG TTGTTGAAAG TTTTGAATTA ATTAATTGAT GATCGGTTTT
AGATGTTATT GCTCTGACAC AATGTTGAAT TATTGTTGTA ATGATCCTTC CGCAGGTTCA CCTACGGAAA CCTTGTTACG ACTTCACCTT CA

*DNA was extracted and purified with the MOBIO and NORGEN Nucleic Acid Extraction kit. Isolates coded as B-03-F-2, P-10-F-7, M-05-F-4, P-02-L-2 and P-11-L-1 were bacterial antagonists while isolate M-23-L-1 and M-30-F-2 are yeast and fungal isolates, respectively. The entire 16S rRNA gene of bacterial isolates was amplified by PCR, using the universal primers. The fungal and yeast isolates were amplified with universal primers of ITS region (Internal Transcribed Spacer region), ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5,-TC CTCCGCTTATTGATATG-3'). The amplified product was sequenced at University of Florida, USA and CABI, UK center).

Appendix Table 6 Bacterial species from gen bank used to compare antagonist isolates

Species	**Isolate code**	**NCBI code**
Aeromonas media	CECT 4232T DSM	651
Aeromonas veronii	0807M090438 IBS	654
Aeromonas veronii	0807M090438 IBS	654
Bacillus amyloliquefaciens	CIP 103265T CIP	1390
Bacillus atrophaeus	DSM 2277 DSM	1452
Bacillus atrophaeus	DSM 5551 DSM	1452
Bacillus atrophaeus	DSM 675 DSM	1452
Bacillus mojavensis	DSM 9205T DSM	72360
Bacillus subtilis	DSM 5552 DSM	1423
Bacillus subtilis subsp. *Spizizenii*	DSM 10T DSM	135461
Bacillus subtilis subsp. *Spizizenii*	DSM 15029T DSM	96241
Bacillus subtilis subsp. *Subtilis*	DSM 10T DSM	135461
Bacillus subtilis subsp. *Subtilis*	DSM 5660 DSM	135461
Bacillus vallismortis	DSM 11031T DSM	72361
Lactobacillus paralimentarius	DSM 13238T DSM	83526
Lactobacillus plantarum	DSM 20205 DSM	1590
Paenibacillus brasilensis	DSM 14914T DSM	128574
Paenibacillus jamilae	DSM 13815T DSM	114136
Paenibacillus mendelii	DSM 19248T DSM	206163
Paenibacillus peoriae	DSM 8320T DSM	59893
Paenibacillus polymyxa	DSM 292 DSM	1406
Paenibacillus polymyxa	DSM 742 DSM	1406
Phoma exigua ssp *exigua*	CBS 431_74 CBS	79605
Pseudomonas antarctica	DSM 15318T HAM	219572
Pseudomonas azotoformans	CIP 106744T HAM	47878
Pseudomonas extremorientalis	CIP 15824T HAM	169669
Pseudomonas extremorientalis	DSM 15824T HAM	169669
Pseudomonas fluorescens	DSM 50090T HAM	294
Pseudomonas gessardii	CIP 105469T HAM	78544
Pseudomonas libanensis	CIP 105460T HAM	75588
Pseudomonas orientalis	CIP 105540T HAM	76758
Pseudomonas pictorum	LMG 981T HAM	86184
Pseudomonas rhodesiae	DSM 14020T HAM	76760
Pseudomonas stutzeri	B367 UFL	316
Pseudomonas synxantha	DSM 18928T DSM	47883
Pseudomonas thivervalensis	DSM 13194T HAM	86265
Pseudomonas tolaasii	LMG 2342T HAM	20442
Pseudomonas veronii	B559 UFL	76761
Pseudomonas veronii	B560 UFL	76761
Pseudomonas veronii	B561 UFL	76761
Stenotrophomonas acidaminiphila	DSM 13117T HAM	128780
Stenotrophomonas maltophilia	(PX) 23086229 MLD	40324
Stenotrophomonas maltophilia	10942 CHB	40324
Stenotrophomonas maltophilia (Pseudomonas beteli)	LMG 978T HAM	40324
Stenotrophomonas maltophilia (Pseudomonas geniculata)	LMG 2195T HAM	86188
Stenotrophomonas maltophilia (Pseudomonas hibiscicola)	LMG 980T HAM	86189
Stenotrophomonas nitritireducens	DSM 12575T HAM	83617