CHARACTERISATION OF *Colletotrichum gloeosporioides* AND EFFECTIVENESS OF MANAGEMENT STRATEGIES OF ANTHRACNOSE DISEASE OF AVOCADO IN MURANG'A COUNTY, KENYA

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DECLARATION

This thesis is my original work and has not been presented for the award of a degree in any other University or any other award.

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I dedicate this thesis to my family, relatives and friends who were source of motivation and encouragement.

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ACRONYMS AND ABBREVIATIONS

a.s.l	Above sea level
AEZs	Agro-ecological zones
ANOVA	Analysis of variance
DNA	Deoxyribonucleic acid
FAO	Food and Agricultural Organisation
FVO	Food and Veterinary Office
HCDA	Horticultural Crop Development Authority
KALRO	Kenya Agricultural and Livestock Research Organisation
KEPHIS	Kenya Plant Health Inspectorate Service
SNV	Stichting Nederlandse Vrijwilligers (Netherlands Development Organisation)
PCPB	Pest Control Products Board
PCR	Polymerase Chain Reaction
rRNA	Ribosomal ribonucleic acid

ABSTRACT

Avocado (*Persea americana* Mill) is a fruit commercially grown worldwide. Its production is affected by anthracnose disease which causes huge loss of avocado fruits both at farm level and in storage. Management of anthracnose disease worldwide has been mainly through use of fungicides. In Kenya, farmers use fungicides such as bayleton, milraz and Copper oxychloride which are not registered for controlling anthracnose in avocado. Despite this, the disease has continued to cause significant fruit losses of up to 40% as a post-harvest disease. This study was conducted to evaluate avocado production and anthracnose disease management practice; determine the incidences of anthracnose disease in avocado in Murang'a County; determine the morphological and genetic characteristics of the *Colletotrichum gloeosporioides*; determine the pathogenicity of the representative isolates of the fungus on avocado varieties (Fuerte and Hass) and determine the inhibitory effect of selected fungicides on *C. gloeosporioides* isolates *in vitro*. A completely randomized block design was used in the laboratory tests while questionnaires were used during surveillance. The data on incidence, latent and surveillance was analysed descriptively into histograms and percentages using IBM SPSS version 21 software. Cultural and morphological data was analysed through ANOVA and Fisher's LSD to compare means using Genstart version 6 while molecular data was analyzed through PCR, sequencing (sanger sequencing), alignment (Bio edit software version 7.2.1 and phylogentics relationship of sequences using MEGA 7.0.18. Surveillance on anthracnose disease incidence and the control strategies adopted by farmers was done in the three agro-ecological zones upper midland (UM) 2, 3 and 4 where avocado is grown. The survey revealed that agro- ecological zone 2 had the highest disease incidence of 24.3% followed by zone 3 with 15% and lastly zone 4 with 9.6%. Farmers controlled the disease by pruning, sanitation and use of fungicides not registered for use in avocado. Eight percent of the farmers used fungicides while 30.2% used pruning strategy. Morphological and molecular studies identified *C. gloeosporioides*, *C. boninense* and *Pestalotiopsis microspora* as the causal agents of anthracnose. Further studies on *C. gloeosporiodes* showed a signicant difference ($P \leq 0.05$) among isolates in mycelia diameter and size of conidia. Both Hass and Fuerte fruit varieties were susceptible to all *C. gloeosporioides* isolates. Diameter of lesions on unripe Fuerte varieties four days after inoculation were not significantly different ($P \geq 0.05$) ranged from 7.33 -10.00 cm while in unripe Hass it was 6.33-7.33cm. However, lesions on ripe, Fuerte variety 2 days after inoculation ranged 10-17cm while ripe Hass was 9.67cm - 12.33cm. Hass variety was more tolerant to the disease as compared to Fuerte variety. The fungicides Bayleton, Milraz and Copper oxychloride significantly ($P \leq 0.05$) inhibited mycelial growth and sporulation of *C. gloeosporioides invitro*. Farmers should be encouraged to use cultural methods and field sanitation as management strategies to reduce loses and should be trained on strategies in handling, harvesting and storage. They should also be encouraged to grow Hass variety which is tolerant to anthracnose as compared to Fuerte variety. Field studies on the effectiveness of fungicides Bayleton, Milraz and copper oxychloride on anthracnose disease of avocado is recommended.

CHAPTER 1: INTRODUCTION

1.1 Background of the study

Avocado (*Persea americana* Mill.) is believed to have originated from Mexico and is currently cultivated all over the world including tropical areas of Africa (Shaffer *et al*., 2013). In Kenya, avocado is one of the most economically important fruits grown by both small and large scale farmers (HCDA, 2016). Avocado production in 2014 was 225,808 Metric tons accounting for Ksh 3.83 billion which was 5% of the total value of the fruits sub sector (HCDA, 2014). The avocado fruit has a high nutritional value since it contains vitamins (E, B and C), minerals (potassium, iron and phosphorus) and a great amount of oil (Shaffer *et al*., 2013). The fruit is mainly grown for fresh market but there is increasing demand from pharmaceutical, cosmetics and vegetable oil industries (HCDA, 2014).

In Kenya avocado is grown in various Counties including; Murang'a, Bungoma, Migori, Kiambu, Kisii, Nyamira, Tharaka Nithi and Embu. Avocado production in Murang'a County is growing tremendously due to increased demand for local consumption and export market accounting to 50% of total production in Kenya (HortiNews, 2015).

The most common avocado varieties grown in Murang'a County include Hass, and Fuerte. These varieties are preferred due to their high productivity and acceptance by the consumer both in local and export market. Further, farmers are

increasingly planting the two varieties replacing coffee and tea plantations in the region. As such, to maintain high productivity of the two varieties, good agricultural practice and appropriate management of diseases and pest is paramount. Other varieties grown though not common include Pinkerton, Hardy and Local.

Avocado production in the County however, is faced by constraints such as diseases, pests and poor agronomic practices. Of great economic importance is anthracnose disease which is caused by the fungus, *Colletotrichum gloeosporioides.* The anthracnose disease causes severe losses of avocado fruits while in the field and after harvest (Pernezny *et al.,* 2000). Over 60% of the Kenyan avocado production cannot be marketed because of damage due to anthracnose and their low quality, emanating from poor production procedures (Chege *et al*., 2006).

Management of anthracnose disease of avocado has been through the use of fungicides (Smith *et al.,* 2011). In Kenya, however use of fungicides to control the disease is limited due to lack of registered fungicide for use (PCPB, 2016). This has resulted to avocado farmers using fungicides registered for use in other crops to control the disease. Lack of knowledge on chemical use, the high cost associated to chemicals and inappropriate spray equipments compounds the problem further (Ippolito and Nigro, 2000; Agrios, 2005). Use of biological control method which is considered to be environmentally safe, has not been popular due to lack of knock down effect (Janisiewicz and Cornway, 2010).

Moreover, few biological products are available for use in disease management and little information is available for their effective use (Agrios, 2005; Janisiewicz and Cornway, 2010).

Use of integrated disease management involving use of appropriate fungicides, cultural practices such as pruning, field sanitation through collection and disposing of infected fruits, leaves and twigs could offer an effective strategy in the management of the disease. However, this is not practised by majority of avocado farmers in Murang'a County.

1.2 Statement of the problem

The economic significance of avocado production in Kenya is quite considerable since it represents one of the major fruit crops grown for both domestic and export market (HCDA, 2016). Its production is affected by diseases such as anthracnose, *Armillaria* root rot, *Cercospora* spot, *Phytophthora* root rot, *Dothiorella* fruit rot, Stem end rot, *Verticillium* wilt and bacterial canker which cause yield reduction (HCDA, 2015).

Among the diseases, anthracnose is the most devastating since it affects the avocado fruits while in the field and after harvest (Menge and Ploetz, 2003). This is due to its mode of infection, genetic diversity and wide host range (Agrios, 2005). Thus cross-infection from other hosts causes serious implications in the epidemiology and control of the disease (Sideney and Dirlane 2014).

Infested fruits, leaves, plant debris and soils are potential sources of primary inocula (Agrios, 2005). The disease is widespread in most countries where avocado is grown including Kenya (Darvas and Kotze, 1987; Snowden, 1990) and often causes severe damage, which affects yield, quality, taste and marketability of the avocado fruits (Freeman *et al.,* 1998; Pernezny *et al.,* 2000; HCDA, 2015). Significant disease development and yield losses occur if wet conditions favourable for *C. gloeosporioides* occur in the field, during fruit harvesting, storage and transportation. The management of primary inoculum sources and good post harvesting handling of fruits is a key strategy in controlling anthracnose. Such strategy includes sanitation where dead branches, fallen rotten fruits and twigs are removed from the field since they harbour the fungus. Application of copper-based fungicides at pre-flowering, fruit formation, and after the harvest are also effective (Scot, 2008; Willis and Mavuso, 2009). However, farmers are not aware of the best fungicide to control the disease.

1.3 Justification of the study

Avocado production has spread worldwide due to its wide range of use as source of nutrients and a raw material in the pharmaceutical and cosmetic industries. In Kenya, the economic significance of avocado production is quite considerable since it represents one of the major fruit crops grown for both domestic and export market (HCDA, 2015).

Despite the huge losses associated with the anthracnose disease of avocado in Kenya, no molecular studies have been done to establish whether the causal agent is various strains of the fungus, *C. gloeosporioides*, or in combination with other *Colletotrichum species* such as *C. acutatum*, *C. bovinense* and *C. karstii* reported to be pathogenic to avocado (Boesewinkle, 1982; Peres *et al*., 2002; Cannon *et al.,* 2012; Silva-Rojers and Avila Quezeda, 2011 and Velázquez-del Valle *et al*., 2016). The knowledge of the causal agent of the disease is useful in deploying disease management strategies by targeting a specific stage(s) of its life cycle. In other countries, anthracnose disease management has involved use of fungicides such as copper hydroxide 77%, Metalaxyl 32%, Fosetyl-Al 80%, Carbonic acid and monopotassium salt 85% and Prochloraz (Scot, 2008 and Agrios, 2005). In Kenya however, use of chemicals is limited by lack of registered fungicides for control of anthracnose of avocado (PCPB, 2016). The inhibition trials of fungicides on the fungal isolates in this study could form a base for development of fungicides to be used commercially for management of the disease.

Further the need to identify the *Colletotrichum* species affecting avocado is of great importance due to the current innovations where *C. gloeosporioides* strains have been developed as bio-control agents for weed management (Butt, 2001; Yakoby *et al*., 2002). Such agents include *C. gloeosporioides* f. Sp *cuscutae, C. gloeosporioides* f.sp *malvae* and *C. gloeosporioides* f.sp *aeschynomere* registered in China, Canada and USA, respectively.

Pathogenicity studies of fungal isolates causing anthracnose disease in avocado varieties Fuerte and Hass have not been done yet. This study would establish the variety which is more tolerant to the disease than the other. The information obtained would advise on the variety to be grown where effect of the disease is minimal.

The study therefore evaluated the disease incidences in avocado growing region, Murang'a County in Kenya, determined the morphological and genetic diversity of the *Colletotrichum gloeosporioide*s, compared the pathogenicity of the isolates of the fungus to avocado varieties, Fuerte and Hass and finally validated the inhibitory effect of the fungicides used by farmers, Bayleton 25 WP (triadimefon 250g/Kg), Milraz 76 WP (Propineb 70% and cymoxaxil 6%) and Greencop 500WP (copper Oxychloride 500g/Kg) on *C. gloeosporioides* isolates.

1. 4 Hypotheses

i. Avocado production in Murang'a County is affected by farmers' practices.

ii. Avocadoes in Murang'a County are affected by anthracnose caused by *Colletotrichum gloeosporioides.*

iii. Isolates of *Colletotrichum gloeosporioides* from diseased plant material are morphologically and genetically similar.

iv. Isolates of *Colletotrichum gloeosporioides* from avocado plants growing in Murang'a County are pathogenic to avocado variety, Fuerte and Hass.

v. Fungicides used by the farmers to manage anthracnose disease of avocado in Murang'a County are effective in controlling the disease.

1.5 Objectives

1.5.1 Broad objective

To investigate the effectiveness of management strategies of anthracnose disease in avocado and characterisation of *C. gloeosporioides* isolates in Murang'a County, Kenya.

1.5.2 Specific objectives

i. To evaluate avocado production practices by farmers in Murang'a County.

ii. To determine incidence of anthracnose disease in avocado fruit grown in Murang'a County.

iii. To determine morphological and genetic characteristics among *Colletotrichum gloeosporioides* isolated from infected avocado fruits in Murang'a County.

iv. To determine pathogenicity of selected *C. gloeosporioides* isolates on the avocado varieties, Fuerte and Hass grown in Murang'a County.

v. To evaluate the *in vitro* inhibitory effect of the selected fungicides used by farmers on *C. gloeosporioides*.

CHAPTER 2: LITERATURE REVIEW

2.1 Avocado Origin and Spread

Avocado (*Persea americana* Mill.), which belongs to the family of the Lauraceae, originated in Central America and Southern Mexico (Chen *et al*., 2008). It is divided into 3 sub-species, i.e. Mexican (sub-tropical), Guatemalan (semi-tropical) and West Indian (tropical) (Wasilwa *et al*., 2006). Avocado popularity has grown tremendously over the years and the fruit is currently cultivated worldwide, including tropical and subtropical areas of Africa (Schaffer *et al.*, 2013; Zentmyer, 1994). Avocado plants were first introduced in Kenya in the 1930s by the Portuguese (Griesbach, 1985; 2005) to be used for subsistence, whereas commercial cultivation of avocado in Kenya started in the early 1960s (Griesbach, 1985). Currently, avocado is grown in several agro-ecological zones of Kenya by both small-scale growers (85%) and large-scale growers for subsistence, local and export markets (Cooper *et al.*, 2003, Wasilwa *et al*., 2006, HCDA, 2014).

2.2 Avocado Production Worldwide

Avocado production has grown gradually over the years among the 21 top avocado producing countries, for example from 4,183,157 tons in 2011 to 4,509,325 tons in 2013 (FAOSTAT, 2013). Among the top producing countries, Mexico is the leading producer, while Kenya has been ranked number six worldwide in the same period from 2011 to 2013 (Fig. 1). In 2013, 4.5 million tons were harvested commercially worldwide, 25% of which was traded

internationally (SNV, 2013; FAOSTAT, 2013). In 2014, avocado production in Kenya was 225,808 tons accounting for KES 3.83 billion, which was 5% of the total value of the fruits sub-sector (HCDA, 2014).

Avocado plays an important role in Mexico in terms of food security, as it is a staple food for Mexican households (Hernandez, 2011). Further, avocado contributes to the economy of the country as more than 13% of the total production is traded internationally (FAO, 2013). In Chile, South Africa, Israel and Spain, the avocado production is mainly intended for the overseas export market.

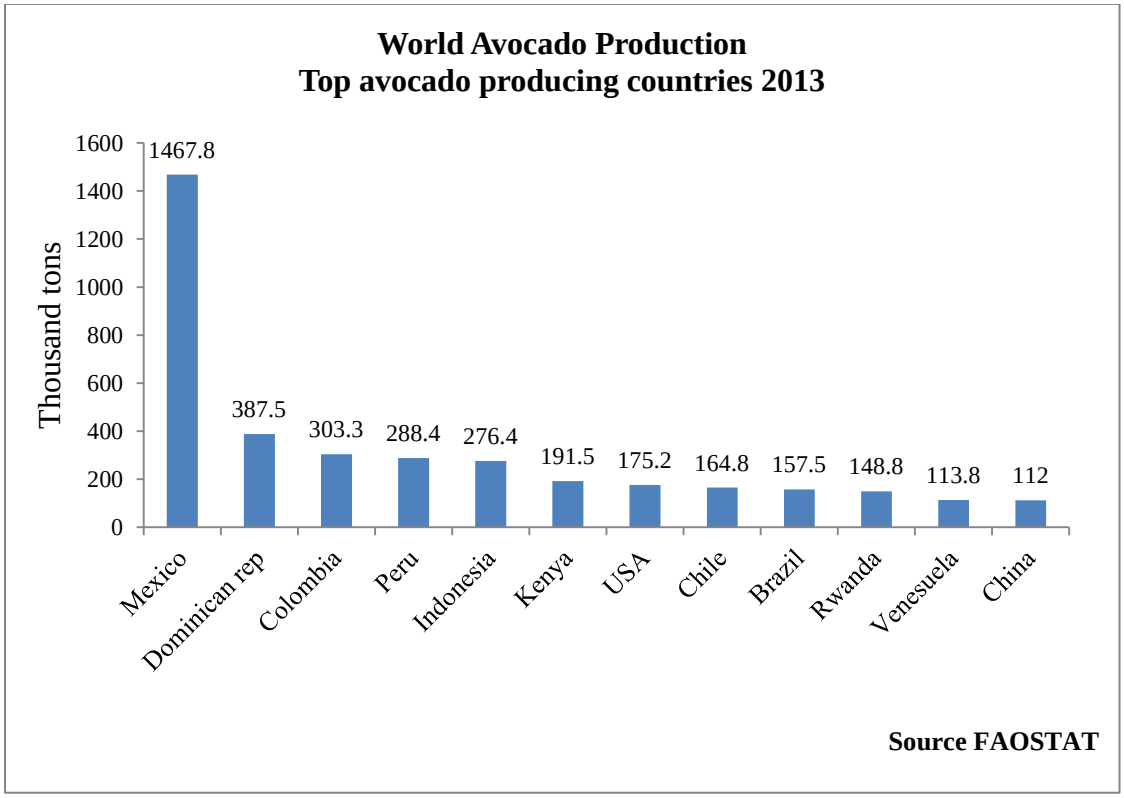


Figure 1: An overview of the avocado production by the 12 top avocado producing countries of the world. Source: FAOSTAT 2014.

The major avocado markets are the European Union and the United States (FAO, 2014). Kenya is ranked eighth after Mexico among avocado exporters globally (FAOSTAT, 2014). Avocados are produced mainly on a small scale in the tropics and sub tropics, though its large scale production is gaining popularity. In Kenya, avocado is the most important fruit at 62%, followed by mango at 26%, in terms of foreign exchange earnings in the mid 2000's upto the year 2015 (HCDA 2014). Peru, South Africa, Kenya and Israel are the major avocado exporters to the European markets, where The Netherlands, France and the United Kingdom are the major avocado importing countries (FAO, 2014). Other importers include USA, Canada and Japan. The most commonly traded variety is "Hass", which is as a result of general customer acceptance due to its longer shelf-life and its rich nut-like flavor (FAO, 2010 and USAID-TAPP, 2011). The demand for the variety "Fuerte" however, though admirable to the customer due to its green color after ripening, is trending downwards due to its short shelf life and susceptibility to post-harvest diseases (Malick Bill *et al*., 2014).

In Kenya, avocado is mainly grown in Murang'a, Bungoma, Migori, Kiambu, Kisii, Nyamira, Tharaka Nithi and Embu Counties, where like other crops it is susceptible to various pests and diseases. Pests and diseases of significant economic importance to avocado production in Kenya are fruit flies and anthracnose (HCDA, 2014). Murang'a County produces 50% of the total avocado fruits shipped to the export market (Hortnews, 2015).

2.3. Uses of avocado fruit

Avocado fruit is used for both its nutritive and medicinal value. The fruits are known not only for their nutritive value but also for use in pharmaceutical and cosmetic industries.

2.3.1. The nutritive use of avocado fruits

Avocado fruits are pressed for oil, blended into dessert drinks, used to make guacamole and in sushi, given as wedding gifts, eaten with ice cream, added to salads, or eaten alone (IFAS, 2006). In Kenya, the domestic consumption of avocado is about 1-2 kg /person/year and is on the increase as the population becomes aware of its nutritional aspects (Chege *et al*., 2006). The chemical composition of the edible portion of the fruit as reported by FAO (2000) is as shown in Table 1. This edible portion of the fruit is rich in oleic, palmitic, linoleic and palmitoleic acids, and is also high in lipids (Lu *et al*., 2009). The mesocarp of the avocado fruit contains fatty acids, like oleic acid (86 mg g−1 oil), palmitic acid (32 mg g−1 oil), linolenic acid (19 mg g−1 oil) and palmitoleic acid (14 mg g−1 oil) (Ozdemir and Topuz 2004, Lu *et al*., 2009).

This fruit contains one to two times more protein than any other fruit, is rich in minerals such as manganese, phosphorous, iron and potassium, but is low in sodium, and also contains the vitamins niacin, vitamin E, vitamin C, ß-carotene, thiamin, riboflavin, nicotinic acid and folate (Schafer *et al*., 2013). Its nutritive composition contributes positively to the health of those who consume it

including the maintenance of good cholesterol levels (Naveh *et al*., 2002; Griesbach, 2005; Njuguna, 2005). In cosmetics, avocado oil is considered to be superior due to its higher penetration ability as compared to other vegetable oils and its nourishment to the glands beneath the skin with vitamins. Oil is rich in vitamins A, B, D and E and has excellent keeping quality. Avocado oil has healing, regenerative and moisturizing properties therefore beneficial in reducing age spots and the healing of scars (Eyres *et al.*, 2001). The oil is used in hairdressing and is used in making hand lotions, facial creams and fine soap. The oil is known to filter out the tanning rays of the sun and is similar to lanolin in its penetrating and skin softening action. The pulp residue after oil extraction can be used as stockfeed.

Table1: Chemical composition of avocado fruit (per 100g of edible portion)

Chemical compositions of avocado fruit (per 100 g of edible portion)*a*			
Energy value (cal)	245.00	K (mg)	368.00
Protein (g)	1.70	P (mg)	38.00
Fat (g) Mg	26.40	(mg)	35.00
Total carbohydrate (g)	5.10	S (mg)	28.50
Crude fiber (g)	1.80	Chlorine	11.00
β-carotene (mg)	0.17	Ca (mg)	10.00
Ascorbic acid (mg)	16.00	Mn (mg)	4.21
Niacin (mg)	1.01	Na (mg)	368.00
Riboflavin (mg)	0.13	Fe (mg)	0.60
Thiamine (mg)	0.06		

a Source FAO (2000)

2.3.2 Medicinal uses

Avocado plant is used as a source of medicine in different cultures where it is grown. The leaf juice has been used as an antibiotic to cure a bacteria disease such as dysentery (Dermarderosian and Beutler, 2002). The leaves are heated and applied on the forehead to relieve neuralgia. Further, the leaf decoction is used to cure ailment like diarrhoea, sore throat and haemorrhage. In Cuba, a decoction of the new shoots is a cough remedy (Anderson, 2003). The roasted seed is used to cure diarrhoea and dysentery while powdered seed is used to cure hypertension (Ortiz *et al*., 2004; Ozolua *et al*., 2009). Further, seed decoction, is used to relieve toothache due to tooth cavity.

2.4. Avocado tree (*Persea americana* Mill.)

The Avocado tree which develops many branches is usually green and it grows to a height in the range of 9 M to 12 M. The shape of the leaves is either elliptic or oval and is 7 cm to 25 cm in length. The Flowers have both female and male parts and are green in colour. The avocado fruit shape varies from pear shaped, round or ovoid. The fruit exhibits various colours ranging from green-yellow, reddish-purple, purple to black and the texture of the skin being smooth or rough. The fruit has a flesh that is greenish- yellow to bright yellow when ripe. Further the flesh may appear fibrous or buttery. The avocado fruit has one seed which weight ranges between 0.1kg to 1.3kg. The weight of the seed accounts for between 10 and 25 % of the total fruit weight. Further, the oil content in avocado varies among different avocado varieties.

Pollination in avocado plant is by insects especially honeybees. The avocado plant exhibits two flowering types A and B where each flower opens twice, functionally female opens first and male opens second. Normally the Type A flower opens first in the morning, closes at midday, and reopens in the afternoon of the following day while Type B opens first in the afternoon, closes in the evening, and reopens the following morning. The presence of both types of trees is important in orchards to improve production by adequate pollination.

Avocado trees can be propagated either through seed or grafting. Grafting is the most common method of propagation in avocado and grafted plants produce fruits after two years while seeded trees produce fruit after about eight years. The longer juvenile period of the trees propagated from seed is associated with higher risk of losses in fruit yield and quality (Brecht *et al*., 2009).

Avocado is a climacteric fruit (Hofman *et al*., 2013). It reaches maturity when it is attached to the tree but the ripening phase of the fruit starts when the fruit is harvested (Brecht *et al*., 2009). To delay the ripening process it is of importance to lower the temperature, eliminate mechanical damage and reduce ethylene production (Yahia, 2011). The ripening phase is essential for the eating quality but the post-harvest life of the fruit is shortened as soon as the ripening starts. This is partly due to emergence of post- harvest diseases. Ripening of climacteric fruits can be started artificially by several external means (Gamage and Rehman, 1999).

2.5. Avocado varieties grown in Kenya

In Kenya, several cultivars have been introduced (Puebla, Pinkerton, Hass and Fuerte) both for local and export market. Other cultivars which have been introduced into Kenya include Booth 7 and Booth 8 whose susceptibility to anthracnose is unknown (SNV, 2013). However, only Fuerte, Hass and Pinkerton are in production together with the local varieties in Murang'a County. Below are various characteristics of different avocado varieties as described in the book "The Avocado: Botany production and uses" by Bruce *et al*. (2013).

2.5.1 Fuerte

The fruit is Pear shaped, medium to large in size, the skin is slightly rough to touch, with many small yellow dots. The flesh is green near skin and contains 12 to 17% oil. The seed is generally small. This variety is an early mid-season bearer and its fruits have flavor. However, this variety is susceptible to fungal diseases which include anthracnose. This variety has good attributes which favours its production worldwide. Such includes, extended picking season and good shelf life, green pear-shaped fruit having good quality and cold-tolerance however, its disadvantages includes, its susceptibility to anthracnose.

2.5.2 Hass

The fruit is Pear shaped to ovoid. The skin is tough, leathery, dark purple or nearly black when ripe. The flesh contains 18 to 22% oil and their seed are generally small. This variety is a mid-late season, medium sized fruit with good

shipping qualities but its shelf life is shortened due to anthracnose, a post- harvest disease. Their taste, high fat content has increased its popularity especially in the European market.

2.5. 3 Pinkerton

This variety has pear- shaped with neck fruits of medium size. The skin is leathery and pliable. The flesh is thick and up to 10% more than in Hass or Fuerte. The flesh has good flavor with high oil content of good quality but inferior to Hass and Fuerte. The fruits darken upon maturity. The seed is generally small, separates readily from the flesh with the coat adhering to the seed. It is high yielding and occasionally affected anthracnose during storage.

2.5.4 Puebla

This variety originated from Atlixco, Mexico in 1911. It is considered as pure Mexican, but some suggest it may be a Mexican × Guatemalan hybrid belonging to the flower type A. The medium-sized, mid-season cultivar is of ovate shape. Its thin skin is smooth, glossy and purplish-red at maturity. The light green flesh is juicy, melting and of good flavour with an oil content of nearly 20%. The ovate seed is medium to large and tightly fixed in its cavity. The tree is a very rapid grower, erect with drooping branches but does not set fruits regularly. In Kenya, Puebla was mainly planted as a pollinator for Fuerte. The recommended tree spacing is 7 m x 8 m. This variety has various advantages ranging from good

spreading of the tree growth and therefore to easy harvest, flesh peels readily from the skin, has few fibres and good eating quality.

2.5.5 Booth 7

This variety originated from Florida in 1920. It has been under commercial production since 1935. Its parentage is not well known but it is thought to be a Guatemalan ×West Indian hybrid and it produces type B flowers. It has a medium-sized dark-green fruit which is round or ovate in shape. It has a slightly rough/ grainy, thick and brittle skin. The fruit has a yellow flesh of good quality and almost free of fibre. The average oil content ranges between 10 – 14%. The big ovate seed separates cleanly from the flesh at maturity. Fruits normally weigh 250 – 380 g and mature at mid-season. The vigorous, spreading tree is a good cropper but shows a tendency towards alternate bearing. Recommended spacing is 7 m × 8 m. The variety has some advantages such as the spreading tree-growth and consequently easy harvesting, the firm, leathery fruit-skin prevents damage during handling and the flesh peels readily from the skin. But it is disadvantageous since it has an alternate bearing, susceptible to infestation by false codling moth and has large seed cavity.

2.5.6 Booth 8

This variety originated from a chance seedling of unknown parentage, which was planted in Florida in 1920, but is assumed to be a Guatemalan × West Indian hybrid and produces type B flowers. It is the most commonly grown cultivars in Florida. The medium to large, oblong ovate fruit weighs around 350 – 600 g. It

has a green skin which is slightly rough, fairly thick and brittle. The light-green/yellow fruit flesh is of good quality and contains 8 – 15% oil. The medium large seed is tightly set, and the fruit ripens at late mid-season. It is a good producer but shows a tendency to biennial bearing. Recommended spacing is 7 m ×7 m. It is suitable for lower altitudes, easy to pick, good fruit quality and withstand transports challenges. However, it has tendency to alternate bearing and susceptible to infestation by false codling moth.

2.6 The pathogen: *Colletotrichum gloeosporioides*

Colletotrichum species filamentous fungi are cosmopolitan with either multiple species occurring on a single host or a single species on multiple hosts causing anthracnose disease worldwide (Alahakoon *et al*., 1994; Cai *et al*., 2009; Crouch and Beirn 2009; Hyde, *et al.*, 2009b and Dean *et al*., 2012). There are difficulties of recognizing *Colletotrichum* species as a result of few and variable morphological characters, an extensive host range and variability in pathogenicity (Latunde-Dada, 2001).

Molecular approaches are being employed to discriminate among *Colletotrichum* species since morphotaxonomic criteria are not accurate. An A1T-rich DNA have been used to group various isolates of *C. gloeosporioides* from strawberries (Freeman *et al*., 1993**)**. Further, a repetitive sequence comprising human minisatellite DNA has been used to separate isolates of *C. gloeosporioides* from *Stylosanthes* spp which could not be distinguished morphologically. In addition,

arbitrarily primed-PCR (ap-PCR) analysis has been used to differentiate isolates of *C. gloeosporioides* from strawberries, mangoes, avocado and papayas (Mills, *et al*., 1992; Freeman and Rodriguez, 1995). Similarly, polymorphisms in mitochondrial DNA (mtDNA and DNA encoding ribosomal RNA (rDNA) have been used to assess variability within populations of *C. gloeosporioides* that infect tropical fruit (Hodson *et al*., 1993 and Alahakoon, 1994). Further, *C. gloeosporioides* isolates have variance in rDNA and mt DNA in the banding patterns (Hodson *et al*., 1993).

However, *C. gloeosporioides* the causal agent of anthracnose is classified as follows:

Kingdom: Fungi

Division: Ascomycota

Class: Sordariomycetes

Order: Phyllachorales

Family: Phyllachoraceae

Genus: *Colletotrichum*

Species: *gloeosporioides*

Scientific Name: *C. gloeosporioides* (Penz.) Penz. and Sacc.

Teleomorph: *Glomerella cingulata* (Stoneman) Spauld. and H.Schrenk.

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. is an asexual facultative (anamorph imperfect) parasite which belongs to the family Phyllachoraceae of the division Ascomycota. *Glomerella cingulata* however is the sexual (perfect) teleomorph state. The pathogen *C. gloeosporioides*, which is the asexual stage of

the fungus, is one of the most widely distributed pathogen in the world (Cannon *et al*., 2012). The fungus thrives best in warm humid environmental conditions (Farr *et al*., 2006 and Ajaykumar, 2014). The fungus/host relationship is broad, imprecise and often overlapping. It infects many hosts and is adaptable to new environments (Sanders and Korsten, 2003a), leading to serious cross infection problems in plant production.

The fungus causes anthracnose disease on various fruits such as avocado, apple, citrus, papaya, passion fruit mango, guava and grapes (Alahakoon *et al.,* 1994). Due to this, differentiation of *Colletotrichum* species based on the host is not an accurate method to identify fungi in this genus, because species such as *C. gloeosporioides*, *C. dermatium*, *C. acutatum*, *C. graminicola* infect a wide range of plants (Freeman *et al*., 1998). Dianese *et al*. (1984) showed that isolates of *C. gloeosporioides* from *Eucalyptus pellita* infected guava, mango, papaya, red pepper and *E. grandis.* The fungus produces abundant conidia, acervuli, setae and appressoria on infected leaves and fruit. Fungal conidia are spread by splashing rain or overhead irrigation and cause new infections on leaves, blossoms and fruit (Agrios, 2005 and Farr *et al*., 2006).

The fungus primarily invades plants tissues where it produces various specialized structures during infection process. The penetration into host tissues relies on formation of specialized infection structures known as appressoria. These appressoria allow the fungus to penetrate the host cuticle and epidermal cell wall

directly by a narrow penetration peg that emerges from the base of appressorium (Agrios, 2005). The fungus may cause infection forming pepper spots (Giblin *et al*., 2010) or remain dormant after infecting fruit in the field until ripening starts when it becomes active and cause lesion formation. The fungus produces acervuli, the asexual bodies during the infection process in the tissue of infected host. The acervulus is a small, flask shaped structure with a small cushion at the bottom, of which short crowded conidophores are formed and can be observed on the surface of diseased plants (Ajaykumar, 2014).

The fungus produces hyaline, one- celled, ovoid to oblong, slightly curved conidia. The spore measures about 10-15 μm in length and 5-7 μm in width. The conidial masses appear pink or salmon in colour. There exist variation in size and shape among conidia of *C. gloeosporioides* depending upon the host from which the pathogen is isolated and its origin. Usually, conidia are oblong with obtuse ends (Freeman *et al*., 1998).

The fungus produces waxy acervuli at the sub-epidermal layer of the infected tissue. The arcervuli has setae, and simple, short, erect conidiophores. The morphological features of the fungus especially colony colour however changes under different conditions of growth, the type of media, temperature, light regime and repeated sub culturing (Johnston, 2000; Vidyalakshini and Divya, 2013). The fungus thrives best at temperatures around 28^{o}C and humid conditions (Agrios, 2005).

Spores require water to germinate and they rarely germinate at relative humidity below 97%. When the moisture content is high, the spores are released from acervuli and are spread mainly by rain splash. The severity of disease is related to weather conditions and the fungus is relatively inactive in dry weather. Extreme conditions of low humidity and temperatures below 18^{o}C or greater than 28^{o}C rapidly inactivate spores (Scot, 2008).

2.6.1 Infection of avocado by *Colletotrichum gloeosporioides*

Anthracnose disease caused by *C. gloeosporioides* has been reported on a wide variety of crops which includes avocado, almond, coffee, guava, apple, dragon fruit, cassava, mango, sorghum and strawberry (Amusa *et al*., 2005; Masyahit *et al*., 2009; Owolade *et al*., 2009; Erpelding, 2010). One species of *Colletorichum* infects multiple hosts by use of diverse strategies for invading host tissue. Such strategies range from intracellular hemibiotrophy to subcuticular intramural necrotrophy. Research on anthracnose disease of avocado has reported other species of *Colletotrichum* being causal agents. Such studies in Mexico reported species, *C. acutatum, C. boninense* being causal agents of anthracnose (Silva-Rojas *et al*., 2011). The genus, *Colletotrichum* is ranked as the eighth most important group of plant pathogenic fungi in the world, based on the economic importance (Dean *et al*., 2012).

The infection occurs when a conidium lands on the surface of the fruit and germinates to produce a germ tube, which develops a terminal appressorium for

initial penetration (Agrios, 2005). The appressorium development is triggered by chemical signals from the host wax (Gopi *et al*., 1993). From the appressorium, the infection peg emerges and penetrates through the fruit wax and the cuticle of the fruit skin. This infection either results to disease spread forming pepper spots (Giblin *et al*., 2010), premature fruit ripening and eventual fruit abscission (Fitzell, 1987) or the fungus then ceases growth and remains quiescent close to the cell wall until fruit ripening (Jeffries *et al.*, 1990; Giblin and Coates, 2007). There are four mechanisms proposed to explain the resistance of unripe avocado fruit to the fungal attack. These includes; (i) lack of nutritional requirements for the pathogen, (ii) presence of preformed antifungal compounds, (iii) presence of inducible antifungal compounds, and (iv) lack of activation of fungal pathogenicity factors (Beno-Moualem and Prusky, 2000).

The fungus remains dormant, due to the presence of fungitoxic concentrations of preformed antifungal compounds in the exocarp of unripe fruits known as dienes, 1,2,4-trihydroxyheptadec-16-yne, 1,2,4-trihydroxyheptadec-16-ene and 1-acetoxy-2,4-dihydroxyheptadec-16-ene (Prusky *et al.*, 1993). There is a significant physiological change which occurs in the host fruit that enable activation of the dormant pathogen. The diene levels decrease when the fruit ripening begins and this correlate with the resumption of fungal growth, and subsequent disease development. Further, epicatechin found in the peel of unripe avocado was found to affect the activity of endopolygalacturonase enzyme of *C. gloeosporiodes* (Prusky *et al*., 1993). During ripening, infection pegs penetrate

into the cells through the epidermal walls resulting to cell death. After penetration, the fungus initiates subcuticular intramural colonization (Perfect *et al.*, 1999) spreading rapidly throughout the tissue killing cells as they advance. The initial infecting hyphae, biotrophic (Kramer-Haimovich *et al*., 2006) subsequently giving rise to secondary necrotic hyphae as the infection progresses killing more cells (Bailey and Jeger, 1992; Coates *et al*., 1993; Latunde-Dada *et al*., 1996; Mendgen and Hahn, 2001).

Further, the fungus, *C. gloeosporioides* produces pectin lyase A (pnlA) (Templeton *et al*. 1994 and Bowen *et al.*, 1995), endopolygalacturonase (Prusky *et al*., 1989 and Yakoby *et al*., 2000b), pectate lyase B (pelB) (Wattad *et al.*, 1997) and pectin methyl esterase (Ortega, 1996) during the colonization of infected host tissue. These enzymes aid in degradation of plant cell wall (Coates, *et al.*, 1993). Such cell wall-degrading enzymes (CWDEs) play a role in the pathogenesis of the fungi on their plant hosts (Collmer, 1988; Annis and Goodwin, 1997). The whole process of infection, including the germination of conidia, formation of appressoria, setae, acervuli and spores results into tissue necrosis.

Fruit infection occurs from fruit set until harvesting as such the disease is associated to fruit abortion. The main sources of fungal inoculums are diseased dead wood, leaves entangled in the tree canopy, branch terminals, mummified fruits and flower bracts (Dodd *et al.*, 1992). In the field the conidia are spread by

means of rainfall, irrigation and heavy dew, thus wet conditions being conducive to conidia production, dispersal and infection (Prusky, 1994).

Lesions of various sizes, dark in colour, can occur on avocado fruits and expand rapidly in size and becoming slightly sunken in their centres and their colour turns to dark- brown to tan or black (Scot, 2008) while affecting the skin and pulp. However, great variation in the symptoms produced by *C. gloeosporioides* has been recorded from host to host. Symptoms appears as black spots, pepper spot on fruit while still on tree and after fruit harvest during ripening as darkly coloured, rounded lesions on fruit skins. In some cases, skin symptoms are more difficult to detect on avocado cultivars with dark coloured skins and some symptoms may be related to fruit injury or openings created during harvesting (Hofman *et al.,* 2013). *C. gloeosporioides* is also known to cause infection through wounds created by some other means, in order to penetrate the fruit and subsequently cause disease (Pernezny *et al*., 2000). Anthracnose is characterized by sunken spots of various colours on stems, leaves and fruits. These spots enlarge leading to the death of the infected plant tissues (Farr *et al*., 2006). *Colletotrichum gloeosporioides* may also develop on avocado fruit as pre-harvest disease named as pepper spot or speckle (Willingham *et al.,* 2000). *Colletotrichum gloeosporioides* causing anthracnose is a significant economic constraints to avocado production worldwide (Gautamm, 2014).

2.7 Management of anthracnose

Anthracnose disease needs to be controlled in order to maintain the quality and abundance of food, feed, and fibre from avocado produced by growers around the world. The postharvest decay of the fruits due to infection by the fungus during the supply chain is a major factor causing postharvest loss which results in serious economic loss, especially in the fruit marketing chain (Prusky, 2011). The disease occurs at different stages of the postharvest chain; during harvesting, field handling, packing operations, transportation and storage. The pathogen, *C. gloeosporioides* infects the fruit while the fruit is still attached to the mother plant in the field or after harvesting (wound infections). However, anthracnose caused by *C. gloeosporioides* (mango, papaya and avocado) or *C. musae* (banana) show symptoms after ripening and these types of infections are known as quiescent infections (Snowden, 1990). Since *C. gloeosporioides* remains quiescent in the cuticle of unripe fruit, different approaches may be used to prevent, mitigate or control plant diseases.

2.7.1. Cultural practices

Control of anthracnose is initiated in the field, through implementation of good orchard sanitation procedures involving collection and disposal of diseased fruits (Agrios, 2005). Further, adequate postharvest handling practices must be applied to prevent cuts and bruises to fruit surfaces (Wasilwa 2006). Those fruits showing symptoms of postharvest disease are not packed into cartons containing healthy fruits (Sarkhosh *et al*., 2017).

2.7.2 Chemical control

Chemical control is the main strategy used to reduce the incidences of postharvest diseases in fruits including anthracnose caused by *C. gloeosporioides* (Barrera-Necha *et al*., 2008). Control of anthracnose disease in avocados has been through application of fungicides such as prochloraz fungicide at commercial level in South Africa, New Zealand and Australia (Everett *et al*., 2005, Scheepers *et al*., 2007, Smith *et al.*, 2011). Prochloraz, a non-systemic fungicide, is used as a first-defense mechanism in the packing line to control postharvest diseases such as anthracnose and stem-end rot in avocados. It affects the mycelial growth of the pathogens and acts as a sterol inhibitor impeding the ergesetrol (fatty acid) synthesis, which is an important component of the fungal cell wall (Malick *et al*., 2014).

Other fungicides used to control the disease with good results includes, copper oxychloride, Mancozeb, Metiram, Propineb, Thiabendazole (Agrios, 2005). Use of fungicides however, is not sustainable due to the development of fungicidal resistant strains (Ippolito and Nigro, 2000). For example, isolates of *C. gloeosporioides* from mango and avocado fruit exhibited considerable variability in their sensitivity towards prochloraz (Arauz, 2000) and benzimindazole, respectively (Gina *et al*., 2000). Benzimidazole fungicides mode of action is by inhibition of β- tubulin biosynthesis (Davidse, 1973). Development of resistance to this fungicide by fungi is due to mutations in the β-tubulin gene which has been

related to specific amino acid substitutions at several distinct regions within the β-tubulin molecule (Fujimura *et al*., 1992). Further, the fungus has a wide host range which ensures re-infection after application of control strategy like use of chemicals (Sideney and Dirlane 2014). In addition, fungicides are source of environmental contamination due to the disposal of large volumes of fungicidal dipping solutions, especially soil and water resources.

Due to the risks associated with the use of postharvest fungicides, the avocado farmers needs to find an alternative solution to fungicide applications. These alternative methods include; controlled and modified atmosphere storage (Kader, 1994), biocontrol agents (Janisiewicz *et al.,* 2001), heat treatments (Barkai-Golan and Phillips, 1991; Lurie, 1998; Fallik *et al.,* 1999), microwaves (Karabulut and Baykal, 2002), chitosan and natural products such as essential oils (Kalemba and Kunicka, 2003; Burt, 2004).

Sodium bicarbonate and antagonist *C. oleophila* have been used to control anthracnose in papaya as an alternative to chemicals. Further, essential oils are known to have fungicidal properties and therefore being used as safer alternative to the environment than synthetic chemicals (Pitarokili *et al*., 1999).

Generally chemical control methods have raised concerns worldwide due to adverse effect of pesticide residues on human and environment (FVO, 2013). For example, the set maximum residue level (MRL) of prochloraz a fungicide used to control anthracnose in avocado in South Africa is is 2 mg/ kg for the EU countries

and Japan (National Department of Agriculture and Fisheries, 2012; Njombolwana *et al*., 2013).

In Kenya, the use of fungicides in management of anthracnose disease in avocado has been a challenge due to lack of registered fungicides for use (PCPB, 2016). This has resulted to use of fungicides by farmers registered for the management of other diseases in other crops other than avocado. Such fungicides being used include bayleton 25WP (Triadimefon 250g/Kg), milraz 76WP (Propineb 70% and Cymoxaxil6%) and Green cop 500WP (copper oxychloride 500g/Kg). Use of fungicide not registered for use in management of anthracnose has also been reported in Europe where fungicide, guazatine, is used as a pre-storage drench treatment to control sour rot (*Geotrichum candidum*) in citrus fruit during the wet seasons, but it is not registered in the EU (European Food Safety Authority EFSA, 2013). Further, lack of motorized spray equipments to spray during the production as the avocado tree grows tall limits the use of fungicides.

2.7.3 Biological control

Biological control of the disease can be achieved through use of antagonistic microorganisms such as *Bacillus subtilis* which inhibits the growth of *C. gloeosporioides, in-vitro* (Piteira and Rodrigues, 1999). *Bacillus subtilis*, applied as a wax formulation removes nutrients from the immediate surroundings of the appresorium of *C. gleosporioides* thus maintaining its dormancy (Korsten *et al*., 2000).

Use of bio-control in post-harvest management of anthracnose has been reported (Korsten *et al*., 1995). *Bacillus* spp. on their own or combined with a fungicide could be used to control postharvest diseases of avocados (Korsten *et al*., 1993). The *Bacillus* spp. isolated from avocado leaves were found to be more effective in controlling anthracnose of avocados compared to prochloraz when applied as a postharvest dip (Korsten *et al*., 1993; El Ghaouth *et al*., 2002). Use of biocontrol agents on their own or in combination with a reduced concentration of synthetic fungicides is more effective method of controlling the disease. This strategy aids in reducing synthetic fungicide use known to be environmentally hazardous (Janisiewicz and Cornway, 2010). For example, the combination of *B. subtilis* and prochloraz was more effective than when they were applied separately (Korsten *et al.*, 1989). The use of antagonistic microrganisms as a biocontrol is a more viable disease management strategy other than chemical control (Korsten *et al.*, 1989). To manage anthracnose disease of avocado in South Africa, a biocontrol agent (*Bacillus subtilis* B246; Avogreen®) (Korsten *et al.*, 1991) has been registered for use commercially by avocado growers and is more popular to organic avocado growers. Though bio control are proving to be better alternative to Chemical fungicides, they have a challenge of not having a knock down effect when used and also little is known on how to handle them (Mangue, 2001).

Due to this among other challenges the use of biocontrol agent was found to be less competitive compared to chemical fungicides (Mangue, 2001). Post- harvest

decay of the avocado due to anthracnose is a major challenge in avocado production and supply chain. The use of fungicides such as prochloraz and copper oxychloride is the main strategy used to manage these diseases. However, the risks associated with chemicals and consumer concerns regarding food safety an alternate and safer method of disease management are needed. The use of biocontrol agents, application of essential oils, plant extracts, edible coatings, and heat treatment have been advocated as possible alternatives to fungicide use. But most effective currently is the combination of a biocontrol agent with lower concentrations of fungicides or GRAS (Generally Regarded as Safe) compounds to reduce the postharvest losses due to postharvest diseases during the supply chain. The alternative treatments to chemical however, must be evaluated on different cultivars at different seasons before being introduced commercially (Malick *et al*., 2014).

In India, integrated pest management is practised involving, removal of infected plant parts to avoid further dispersal of the fungus inoculum, avoiding fruit injury during transport, packaging and storage process, avoidance of pathogen infection by the use of some registered pesticides during pre and post- harvest processes to control anthracnose (Gutam, 2014).

Due to the toxic and harmful effects of chemical pesticides, the use of bio-pesticides is becoming popular in controlling anthracnose. Some studies on control of anthracnose on mango (Sohi *et al*., 1973; Sundravadanam *et al*., 2007; Chowdury and Rahim, 2009; Pandey *et al*., 2010a).

The use of botanical fungicides for example, *Ocimum basilicum* L. and *Allium sativum* L for the control of the pathogen may be less expensive, safer for the applier and the ecosystem and could serve as a good alternative to synthetic fungicides (Ogbebor *et al*., 2007). Among these, use of essential oils (EOs) which shows antimicrobial property activities, low mammalian toxicity, biodegradable, eco-friendly, economical and less environmental effects (Kalemba and Kunicka, 2003; Isman, 2000; Burt, 2004,) is the most accepted alternative methods by the consumer because they are widely used in general culinary practices. Further essential oils are considered as Generally Recognised as Safe (GRAS) which favours their application as biopesticides to control disease to provide safe food (FDA, 2014).

Thyme oil was reported to exhibit 100% control of *C. gloeosporioides* during *in vitro* tests (Sellamuthu *et al*., 2013a). Further fumigation of avocado with thymol vapour was effective in control of anthracnose (*C. gloeosporiodes*) in avocados (Sellamuthu *et al*., 2013a).

Applications of Carnuba wax containing *Lippia scaberrima* essential oil improved the protection of cv. 'Fuerte' against anthracnose (Lopez-Reyes *et al*., 2010). The length of the postharvest storage time can also influence the efficacy of the antifungal activity in essential oils treatments (Lopez-Reyes *et al*., 2010). Although a combination of essential oils with a commercial coating of Midseason 865® (MS865) has been recommended as an acceptable postharvest treatment for

controlling anthracnose in avocados, seven organic niche markets, specifically in the developed countries, however, prefer fruit that is free from fruit coatings (Regnier *et al*., 2010).

Dipping in latex has delayed decay in avocados stored at room temperature. Removal of ethylene from controlled atmospheric storage of 2% oxygen and 10% carbon dioxide prolongs the marketable life of avocados. Reduction of atmospheric pressure to sub-atmospheric 60 mm Hg in the refrigerated storage unit at 6 °C retards ripening of avocados by reducing respiration and ethylene production. When removed after 70 days, avocados have ripened normally at atmospheric pressure and 14 °C. Experimental calcium treatments have delayed ripening and reduced internal chilling injury in storage but made the avocados externally less attractive and they are, therefore, considered commercially undesirable. Hass fruit dipped in fungicide 24 hours after harvest and sealed in polyethylene bags containing an ethylene absorbent (potassium permanganate on vermiculite or on aluminum silicate), has been successfully stored for 40 or 50 days at 10 °C. Waxed Fuerte avocados stored for 2 weeks at 5°C and ripened at 20 °C ripened only 1 day later than non-waxed fruit, however, waxing does reduce weight loss.

Use of biocontrol agents to control anthracnose in avocado has been a challenge due to the quiescent infection caused by pathogen. This fungus causing infection is therefore protected from biocontrol agents by the plant cuticle (Prusky *et al*., 2000).

2.8 Characterization of *C. gloeosporioides*.

2.8.1 Cultural and morphological Characteristics

In cultural media, the fungus generally produces circular, woolly or cottony colonies with characteristic colour, pale brown or grayish white (Prabakar *et al*., 2005 and Vidyalakshni and Divya, 2013). It produces hyaline, one- celled, ovoid to oblong, slightly curved conidia. The spore measures about 10-15 µm in length and 5-7 µm in width. The conidial masses appear pink or salmon in colour.The fungus also produces sclerotia, which are dark brown, occasionally setose. The setae are long, brown and septate (Vidyalakshni and Divya, 2013). It has been demonstrated that nutritional components of culture media, the growth parameters like temperature, moisture and pH affects the growth and sporulation of *C. gloeosporioides* (Sangeetha and Rawal, 2009; Hubballia *et al*., 2011; Pandey *et al*., 2012). *C. gloeosporioides* showed maximum growth in pH range of 6-7 and temperature 25-30^{o}C. Further, the exposure of the fungus to alternate cycles of 12 hours light and 12 hours darkness resulted in the maximum mycelial growth of *C. gloeosporioides* in comparison to 24 hours exposure to continuous light and 24 hours exposure to continuous dark (Hubballia *et al*., 2011).

2.8.2 Molecular Characterization of *Colletotrichum gloeosporioides*

Several molecular techniques have been developed to identify and characterize *Colletotrichum* spp. (Garrido *et al*., 2008; Crouch *et al*., 2009). Arbitrarily primed polymerase chain reaction and limited restriction digest analyses of PCR-amplified ribosomal RNA (rRNA) were employed to differentiate between *C.*

gloeosporioides and *C. acutatum* isolates from a diverse host range (Freeman *et al*., 2000; Tapia-Tussell *et al*., 2008).

Species-specific primers have also been designed based on dissimilarities in the sequence of the internal transcribe spacer (ITS) regions of representative isolates of *Colletotrichum spp*. This approach has been demonstrated to be a reliable technique to identify and differentiate *C. acutatum* from *C. gloeosporioides* (Sreenivasaprasad *et al.*, 1992; 1994; Harp *et al*., 2008). In addition, diagnostic tools were developed based on β-tubulin 2 sequences which facilitate quick and reliable detection in *C. acutatum* groups (Talhinhas *et al*., 2005). Further epitypical characterization of *C. gloeosporioides* based on morphological and molecular methods has improved the differentiation between *C. acutatum* and *C. gloeosporioides* (Cannon *et al*., 2008; Poulivong *et al*., 2010). More recently, a single copy intron of the glutamine synthetase (GS) gene was used for classification and differentiation of *Colletotrichum* species. The intron sequences of the GS gene (885 to 915 bases) were shown to be phylogenetically informative (Guerber *et al*., 2003; Liu *et al*., 2011). Phylogenetic analysis based on intron sequence alignment showed that *Colletotrichum* spp. possess higher intron sequence variation and higher bootstrap value support than the sequences based on ITS regions and the beta-tubulin gene (González *et al*., 2006; Than *et al*., 2008; Crouch *et al*., 2009).

Freeman *et al*. 1996 demonstrated pathogenic and genotypic diversity among *C. gloeosporioides* avocado isolates but also he did not observe phenotypic

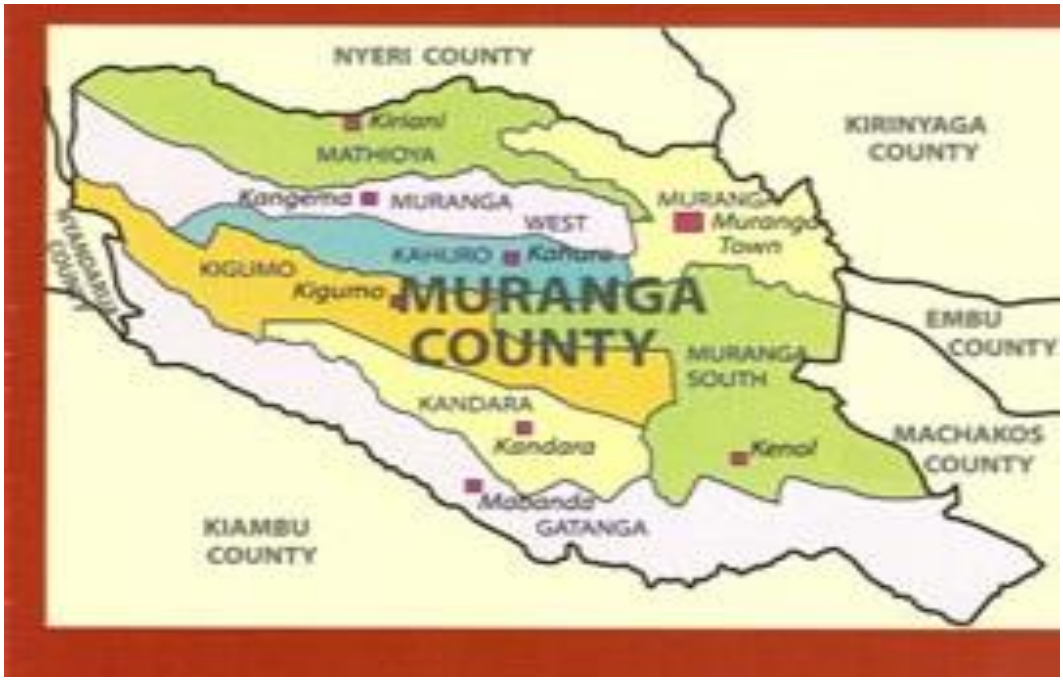
correlations between isolates from Israel and USA. In his study the avocado isolates were identified as *C. gloeosporioides* by morphotaxonomic criteria, but there were possibility of other species such as *C. acutatum* being involved in pathogenesis, as has been reported in New Zealand (Cannon *et al.*, 2012).

The avocado isolates from Israel and the United States were diverse, with numerous arbitrarily primed-PCR phenotypes being observed. Southern hybridization of the repetitive nuclear-DNA element GcpR1 to *Pst*I-digested genomic DNA of avocado isolates revealed polymorphic fragments among the avocado isolates (Freeman, 1996).

CHAPTER 3: MATERIALS AND METHODS

3.1 Study area

The study was carried out in Murang'a County which lies between latitude 0^{o} 34' south and 1^{o}7' South and longitudes of 36^{o} East and 37^{o}27' East. The County is bordered by Nyeri in the North, Nyandarua in the west, Kiambu in the South and Kiranyaga, Embu and Machakos Counties in the East. It occupies a total area of 2,558.8Km^2 (Murang'a County Government, 2017).



Map: Study area (Adopted from MOA)

The county lies between 914m in the East and 3,353m above sea level (a.s.l) along the slopes of the Aberdare Mountains in the West. The county is divided into six agro ecological zones. The agro ecological zone 1 is the highest zone, where forestry, tea and tourism industry are economic activities. Zones 2 is suitable for Tea, Dairy and avocado while zone 3 has coffee, dairy, avocado,

maize and beans as major economic activities. The AEZ 4 has avocado, maize and beans while 5 and 6 are semi-arid areas where coffee and pineapple plantations thrive by irrigation (Appendix 1; Murang'a County Government, 2017). The County is divided into three climatic regions: The western region with an equatorial type of climate, the central region with a sub-tropical climate and the eastern part with semi-arid conditions (Murang'a County Government, 2017).

The County receive rains in two seasons, long rains in the months of March, April and May while short rains are received during the months of October and November. The western region of the County, Kangema, Gatanga, and higher parts of Kigumo and Kandara, are wet and humid due to the influence of the Aberdares ranges. However, the eastern region, lower parts of Kigumo, Kandara, Kiharu and Maragwa constituencies are relatively drier and receive less rain (Murang'a County Government, 2017).

The County was selected due to its high production of avocado both for export and local market accounting to 50% of total production in Kenya (HortiNews, 2015), cultivation of various avocado varieties and high fruit losses associated with the disease. The County has three agro- ecological zones, 2, 3, and 4 where avocado is grown (Ralph *et al*., 2006). These zones show variations in temperatures and rainfall which affect anthracnose disease development in avocado. Further these zones present a good representation of various avocado growing regions in Kenya in terms of environmental conditions. The cultural and

morphological studies, pathogenicity test were carried out at mycology laboratory at Kenya Agricultural and Livestock Research Organisation (KALRO), Kandara Centre (former KARI Thika) while molecular studies were done at Plant Quarantine Station, KEPHIS Muguga, Kenyatta University and Inqaba Biotech South Africa.

Surveillance was carried out in Murang'a County between June and October 2015. This was done with an aid of four hundred (400) questionnaire administered in Kandara, Kigumo, Kahuro and Gatang'a sub-counties, 100 questionnaires per Sub county. The questionnaires (Appendix 2) were administered through face to face interview and focused group interview. Sampling of farmers was by stratified random sampling where transect across the sub-county was drawn along the road from low altitude to the highest. Farmers were sampled at equidistant along the transect on both sides. The questionnaires were administered with the assistance of the staff from the Ministry of Agriculture.

3.2 Determination of the anthracnose disease incidence on avocado in Murang'a County

3.2.1 Field visits and sample size determination

Reconnaissance field visit was made to establish the administrative network and authority to conduct research, study the growth and distribution of avocado in Murang'a County specifically in Kandara, Gatanga, Kigumo and Kiharu sub-counties. Further studies were conducted to establish the nature and management

of the anthracnose disease of avocadoes in the field. This was done through interaction with 400 farmers through administration of the questionnaire through face-face interviews and focused group discussion (Appendix 2). The number of farmers was determined by use of sample size table at 95% confidence and margin error of 5% (Research advisor, 2006). The visit was also used to identify farms that formed part of the sample used in the study of disease incidence.

3.2.2 Determination of the incidence of anthracnose disease of avocado in Murang'a County

The survey to determine disease incidence of anthracnose disease of avocado in the field was carried out during the avocado fruiting seasons, June to October, 2015. The survey was carried out in three agro- ecological zones (2, 3 and 4). Six hundred fruits were randomly selected from 3600 fruits harvested in the three zones. Sixty avocado farms with more than 10 avocado trees were selected at random along a transect drawn across the three agro-ecological zones in each subcounty. A total of twenty farms were sampled in each zone. A total 1200 fruits per zone were harvested, 60 fruits per farm from 10 trees having 6 fruits randomly selected per tree and pooled. The sample size (n), the number of fruits was determined by use of the formula; (Kothari, 2004);

$n = \frac{z2pq}{e2}$,

Where, Z = Standard normal deviation at the required confidence level, Z= 1.96 at 95% confidence level. P=0.5 and q=0.5 and e= margin error = 4% = 0.04.

Therefore, n= 1.96^2 x0.5x0.5/0.04^2 = 600

Whereas, the percentage of fruits inspected showing the disease symptom was calculated using the equation (Hossain *et al*., 2010):

$$\%\ infestation = \frac{Number\ of\ fruits\ showing\ disease\ symptoms}{Number\ of\ fruits\ inspected} \times 100$$

3.2.3 Determination of latent infections of avocado anthracnose disease on mature fruits

Four hundred mature avocado fruits showing no symptoms of the disease were harvested in each zone from 20 farms. The fruits were bulked together and 200 fruits randomly sampled. The fruits were put in crates and taken to mycology laboratory, in KALRO, Kandara. The fruits were washed using 0.5% sodium hypochlorite and thoroughly rinsed 3 times with water and divided into lots of 10. Fruits were packed in sterilised plastic baskets, covered with a sterile cotton material and left on benches in the laboratory at temperature range 22-25°C for one weeks. The fruits were then inspected for symptoms of anthracnose disease. The incidence of the disease was calculated as stated in the Section 3.2.2.

3.3 Morphological characterization of *C. gloeosporioides* isolates

3.3.1 Fungal isolation and culture

One hundred and fifty samples of infected fruits both Hass and Fuerte varieties were collected from each sub-county, Murang'a County and brought to the laboratory for fungal isolation. The samples were washed clean using tap water and blotted to remove excess water. The fruits were surface sterilized using 0.5% sodium hypochlorite for 30 seconds. Small sections of the diseased area from the

margins of the lesions were cut aseptically and placed on hardened potato dextrose agar (PDA) in petri dishes for fungal growth. The emerging two fungi were sub -cultured to obtain pure cultures. The fungi, *C. gloeosporioides* and *Pestalotiopsis microspora* were morphologically identified based on cultural and microscopical characteristics using published fungal key (Nagamani *et al*., 2006, Freeman *et al*., 1998 and Domsch *et al*., 1980). A co-infection by *C. gloeosporioides* and *P. microspora* was observed. Single spore pure cultures of the isolates were preserved on PDA slant universal bottle and stored in the fridge for later use at 4^{o}C.

3.3.1.1 Single spore isolation technique.

For single-spore isolation, conidia were scraped off the plate and suspended in 1 ml of sterile distilled water. The conidial suspension (50 µl) was spread on 1.5% (wt/vol) water agar and incubated at 24°C overnight in a sterilized lamina flow. The germinating conidia were observed under microscope. Using a sterile carpel, a section of the media containing a germinated conidium was cut and asceptically transferred onto PDA slant in a universal bottle and incubated at 25°C for 24 hours for mycelia growth. Each isolate was then stored in the fridge at 4^{o}C for later use (Chen *et al*., 2016).

3.3.2 Mycelial growth and sporulation of *C. gloeosporioides* isolates

Five- millimeter mycelia plugs of pure isolates of *C. gloeosporioides* cut using five millimeter -diameter cork borer, were placed at the centre of hardened PDA

culture in 9cm-diameter petri dishes. The cultures were placed on the laboratory bench to grow at room temperatures (22-25^{o}C). Colony diameters of the isolates were measured at day 2, 4, 6, 8 and 10 after inoculation. On the eleventh day, cultures were flooded with distilled water to bring the spores into suspension. The spores were scraped with a sterilized wire loop. The suspension was filtered through double layer cheese cloth to remove mycelia. Using a Gilson pipette, spore suspension was drawn and filled gently into the haemocytometer by resting the end of the Gilson tip at the edge of the chambers. The sample was drawn out of the pipette by capillary action and the fluid run to the edges of the grooves only. The haemocytometer was placed on the stage of light compound microscope and spores counted per millilitres. The total number of spores per millilitre was estimated using haemocytometer as described by the formula below (Louis and Siegel, 2011),

$S = \frac{NV}{v}$, where,

S = Number of spores per/ml

N = Mean number of spores counted in 5 large squares

V = 1ml = 1000mm^3

v = volume of spore suspension under cover glass = 0.0004 mm^3

3.3.3 Determination of conidial morphology

To harvest conidia, pure cultures of fungal isolate grown on PDA for 10 days at room temperature were flooded with sterile distilled water and filtered through double layer cheese cloth to remove mycelia. The filtrate was collected in clean

universal bottles and then diluted serially. Using a pipette, a drop of the filtrate was placed in a slide, covered with a cover slip and then placed on microscope stage. Lactophenol cotton blue stain was added to stain the spores to improve visibility. The shape of the spores was noted and the size (length and width) of the 3 spores per isolate was measured using calibrated ocular slide and stage micrometer.

3.4 Determination of genetic characteristics of *C. gloeosporioides, C. boninense* and *P.microspora* isolates

3.4.1 DNA extraction

Cultures derived from a single spore from the original isolate were inoculated in Potato Dextrose Agar. These Cultures were incubated at room temperature for 5 days. Mycelia were harvested by vacuum filtration onto a Watman No.1 filter paper disk. The mycelia were dried between sterile filter paper and approximately 0.5g of mycelium for each sample ground using a sterile mortar and pestle under liquid nitrogen. An improved fungal extraction protocol as described by Liu *et al* (2012) was used. Fourty milligram (40 mg) of mycelia was ground to a fine powder in 2 ml of extraction buffer (Tris-HCl, 100 mM; EDTA, 10 mM; NaCl, 1M; SDS, 1%; proteinase K, 0.05 mg ml-1; pH 8.0) and 10% (v/v) glass beads. Samples were vortexed and incubated at 65°C for 30 minutes. After incubation, samples were centrifuged at 10,000 × g for 15 min and supernatant transferred to a fresh tube. To the supernatant, 150 µl of 3 M guanidine hydrochloride was

added and incubated at - 20°C for 10 minutes. Samples were centrifuged at 10,000 × g for 10 minutes.

After centrifugation supernatant was transferred to a fresh tube, and an equal volume of isopropanol was added. Samples were incubated at -20°C for 3 hours. The samples were then centrifuged for 10 min at 10,000 × g and 70% ethanol was added and centrifuged once more for 10 min at 10,000 × g. The pellet obtained was air dried and dissolved in 50 µl of TE buffer (Tris-HCl, 10 mM, pH 8; EDTA, 1 mM). The nucleic acid dissolved in TE buffer was treated with 3 µl of RNase A (10 mg $ml^{-}1$), incubated at 37°C, and stored at -20°C for later use. The quality of DNA was determined by loading 5µl of DNA on 0.8% agarosegel before running it for 45 volts for 30 minutes. Presence and quality of the bands was noted by visualization under the UV gel imager. Where the DNA was found to be of poor quality, back up samples were used for the extraction of the DNA.

3.4.2 Polymerase chain reactions and gel electrophoresis

The DNA extracted from isolates of *C. gloeosporioides, C. boninense and P. microspora* was used as templates in polymerase chain reaction. The reactions were carried out in the Molecular laboratory, Kenya Plant Health Inspectorate Services (KEPHIS). The internal transcribed spacer region of r DNA of fungal isolates was amplified by two sets of primers. The first set contained the universal primers ITS1 (5'-TCCGTAGGTGA ACCTGCGG-3') and ITS4 (5-'TCCTCCGCTTATTG ATAT GC-3') while the other set was species specific

primer CgInt; (5'-GGGGAAGCCTCTCGCGG-3') specific to *Colletotrichum gloeosporioides* combined with the universal primer ITS4; (TCCTCCGCTTATTGATATGC) sourced from Inqaba Africa Genomic Platform, South Africa . The PCR reaction was carried out in 25 µl reaction volumes containing 50 ng of DNA, 1× PCR buffer (Invitrogen), 0.25 mM of each dNTP (Invitrogen), 2 mM $MgCl_2$, 0.8 µM primers and 1 U Taq polymerase (Invitrogen). DNA amplification was performed in a GeneAmp 9700 DNA Thermal Cycler (Perkin-Elmer), and consisted of an initial denaturing step at 94°C for 5 min, followed by 34 cycles including: denaturing at 94 °C for 60 s, annealing for 60s at 55 °C extension for 90s at 72 °C; and a final extension step of 5 min at 72 °C (Borneman and Hartin, 2000).

The amplified PCR products were run on agarose gel to confirm amplification of the DNA fragments.

3.4.2.1 Agarose gel electrophoresis

A 1.5% agarose gel was prepared using 1x TBE- buffer and stained with 5µl of syber safe and poured into casting tray having a comb to solidify. The first well of the solid gel was loaded with 1.5µl 1Kb marker, followed by 2µl of each amplified product and a control at the end. The gel was connected to electric voltage of 100volts for 45 minutes to allow migration of amplified PCR products. The DNA bands formed were visualised under UV light and images photographed using a camera connected to a computer.

3.4.3 DNA cleaning and Sequencing

The amplified products of the target fragments in section 3.4.2 were cleaned using the Qiagen PCR cleaning kit according manufacturer instructions. The cleaned fragments were submitted for Sanger sequencing to a service provider Inqaba Africa Genomic platform in South Africa together with the primers used for amplification (ITS1&4 and ITS4 & CgInt).

3.4.4 Bioinformatics analysis

The sequences obtained from Inqaba Africa Genomic platform at South Africa were trimmed before subjecting them to alignment with other Gene bank sequences. Gene alignment was done using Bio edit software version 7.2.1 while phylogenetic analysis was done by MEGA Molecular Evolutionary Genetic Analysis version 7.1.8. Sequences of *Colletotrichum* and *Pestalotiopsis* isolates from different regions were aligned in Mega 7.1.8 (Tamura *et al*., 2016) and optimized manually to assure positional homology. Gaps were treated as missing data. Phylogenetic analyses were performed using Mega 7.18 (Tamura *et al*., 2016). Maximum parsimony (MP) trees were obtained using the Close-Neighbor-Interchange algorithm with search level 1, in which the initial trees were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset. Clade stability of the tree resulting from maximum parsimony analysis was assessed by bootstrap analysis with 1000 replicates. The minimum evolution (ME) trees were also created and evaluated with 1,000 bootstrap replicates. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*.,

2016) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset.

3.5 Determination of pathogenicity of *C. gloeosporioides* isolates on avocado fruits

3.5.1 Avocado fruit

A total of three hundred and sixty healthy avocado fruits (Fuerte and Hass) were collected from 60 farms selected randomly in the study area (Murang'a County) and brought to the laboratory. The avocado fruits were surface sterilized by use of 0.5% sodium hypochlorite and rinsed thoroughly with clean water. The excess water was bloated. Three inoculation sites, holes of about 10-mm in depth were made along the longitudinal axis of a fruit using 5mm-diameter cork borer. Spot inoculations of fruits was carried out by placing 5mm-diameter mycelia plugs on each hole. The mycelia plugs were obtained from actively growing mycelia in petri-dishes. *C. gloeosporioides* isolates from the three agro-ecological zones where avocado is grown were used for the pathogenicity assay where three fruits were inoculated per isolate. The fruits were placed in sterilized basin lined with moistened sterile tissue paper to maintain high-humid conditions for 48 hrs at room temperature. Lesion development of decaying avocado fruit was assessed by measuring the diameter of lesion 4 days and 2days after inoculation for unripe and ripe fruits, respectively.

3.6 Determination of *in vitro* inhibitory effect of selected fungicides on *Colletotrichum gloeosporioides* the causal agent of anthracnose in avocado

The effect of the fungicides used by farmers to control anthracnose in avocado was evaluated. Such fungicides included, Bayleton 25 WP (triadimefon 250g/Kg), Milraz 76 WP (Propineb 70% & cymoxaxil 6%) and Greencop 500WP (copper Oxychloride 500g/Kg). Although these fungicides are used by the farmers, their inhibitory effect on the *Colletotrichum gloeosporioides* has not been tested, therefore not registered for use in management of anthracnose in avocado in Kenya. These fungicides were identified during the survey using questionnaire.

Different concentrations of these fungicides were used to assess their inhibitory effects on *C. gloeosporioides* mycelial growth and sporulation. The concentrations used were determined based on the manufacturers recommended dose rate on the fungicide label. Three concentrations of each fungicide were formulated for use as follows; twice the recommended rate, the recommended rate and half the recommended rate (Table: 3.1). Each concentration of the fungicide was replicated three times and used to amend PDA in petridishes. The amended petridishes were inoculated with mycelia plugs and arranged in a completely randomized block design on the sterilized hood/chamber at room temperatures of 22-24^{o}C in Mycology laboratory, KALRO Kandara. Petridishes containing PDA not amended with fungicides acted as controls.

Table 3.1: Various fungicide concentrations used in the inhibitory trials on *C. gloeosporioides* isolates

Fungicide	**Manufacturer recommended dose**	**Formulated concentration for inhibition test**
Bayleton 25WP (Triadimefon 250g/Kg)	0.25g/l	0.500g/l 0.250g/l 0.125g/l
Milraz 76WP (Propineb 70% and cymoxaxil 6%)	0.50g/l	1.00g/l 0.50g/l 0.25g/l
GreenCop 500WP (Copper oxychloride 500g/Kg)	1.75g/l	3.50g/l 1.75g/l 0.87g/l

3.6.1 Effects of fungicides on mycelia growth and sporulation of *C. gloeosporioides*

Fungicide suspensions of different concentrations were prepared by dissolving requisite quantities of each fungicide in warm PDA medium in flask after autoclaving at 121^{o}C for 15 minutes. The fungicides were thoroughly mixed with the medium in the sterile flask by shaking with hands gently. About 15 ml of sterilized molten fungicide amended PDA medium was poured into sterile petridishes, 9- cm in diameter. Each fungicide concentration was replicated three times. Petri dishes, filled with molten non-amended PDA served as controls.

Once solidified, 5-mm disks of PDA with actively growing mycelium of 7- day old colony of the isolate of *C. gloeosporioides* were cut out with sterile cork borer

and placed in the centre of each plate. Each isolate was inoculated in the three replicated plates of each fungicide concentration. The inoculated plates were incubated at room temperature of between 22-24°C on the sterilized hood/chamber until the growth of the control plates are covered with mycelia. After every two days, two diagonal measurements of mycelium growth were taken on each plate and recorded. Final measurements were taken when fungal growth in the control fully covered the plates. Inhibition of radial growth due to various fungicidal treatments was computed based on colony diameter on control plate using the following formula as stated by Sundar *et al.* (1995);

$$\%\ Inhibition = \frac{x - y}{x} \times 100$$

Where, x= Growth in control plate and y= Growth in fungicide treated plate

Further, the plates were flooded with distilled water to bring the spores into suspension. Sporulation capacity was determined by counting the number of spores using a haemocytometer as described in section 3.3.2.

3.7. Data analysis

The data obtained from the questionnaire was analysed using student t- test, Pearson Chi-square and cross tabulation for percentages and frequencies in IBM SPSS version 21. Analysis of variance (ANOVA) was used for disease parameters (incidence and lesion diameter) and cultural characteristics of the fungus (radial mycelial growth, sporulation) using the Genstart version 6 software. Mean pair wise separation test at P=0.05 was done using Fischers Least Significant Difference (LSD).

Pairwise and multiple sequence alignment of the sample sequences obtained and Internal transcribed spacer (ITS) reference sequences was done using clustalw on molecular evolutionary genetic analysis (MEGA 7.0.18 version) computer program.

Phylogenetic relationship among *C. gloeosporioides, C. boninense* and *P. microspora* isolates was determined on maximum likelihood aligorithms using Mega 7 (Tamura *et al*., 2016). The evolutionary divergence between the isolates was done using maximum composite likelihood model according to Kumar *et al.,* 2016).

CHAPTER 4: RESULTS

4.1: Avocado production and management of anthracnose in Murang'a County

4.1.1: Age and gender of farmers involved in avocado production

The total of four hundred avocado farmers were involved as respondents in this study. Avocado farming in the County involved all age groups from youth aged below thirty years to the old, above 60 years. Two hundred and seven of the respondents were in the category of 60 and above years of age, constituting 51.75% (Figure 4.1). Those farmers below 30 years were the lowest at 9.25% followed by age group 40-49 years at 11.25%, respectively (Figure 4.1). The majority of those in age group below thirty years were young mothers. Overall, the numbers of male and female farmers were 51.7% and 48.3%, respectively (Table 4.1).

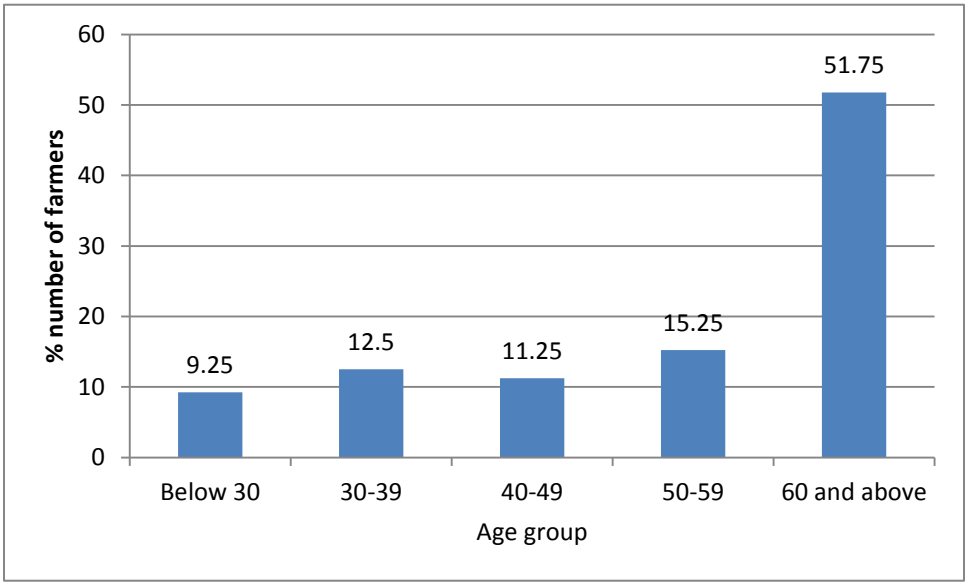


Figure 4.1: Percentage age distribution of farmers' growing avocado in the surveyed sub-counties.

The avocado production by females and males farmers differed significantly ($t=2.344$, $P<0.05$) with a mean fruits production of 21,639.81 per male compared to 10,937.3 fruits per female (Table 4.1).

Table 4.1: Farmers gender and avocado production in the study area

Gender	Number of respondents	% of respondents	Mean number of fruits produced per farmer
Male	207	51.7	21639.81
Female	193	48.3	10937.31
Total	400	100.0	

4.1.2 Avocado farmers' categories and the level of their education

Majority (89.2%) of the farmers growing avocado in the study area were on full time basis, while the rest were involved with other economic activities and formal employment (Table 4.2). Few of the farmers reported their involvement in sales of agricultural produce in the nearby local and urban market centres. Production by farmers on full time basis however, did not differ significantly ($t=1.073$, $P\geq0.05$ from part-time farmers.

Table 4.2: Farmer's categories and their mean avocado production

Farmers category	Number of farmers	Mean %	Mean production of fruits/ farmer
Full- time	356	89.2	15,389.45
Part- time	44	10.8	23,893.08
Total	400	100	

Majority (34.25 %) of the farmers had primary level of education while 28.25% and 20.75% had secondary school and tertiary levels, respectively. However, 16.75% of the farmers did not have any formal education (Figure 4.2).

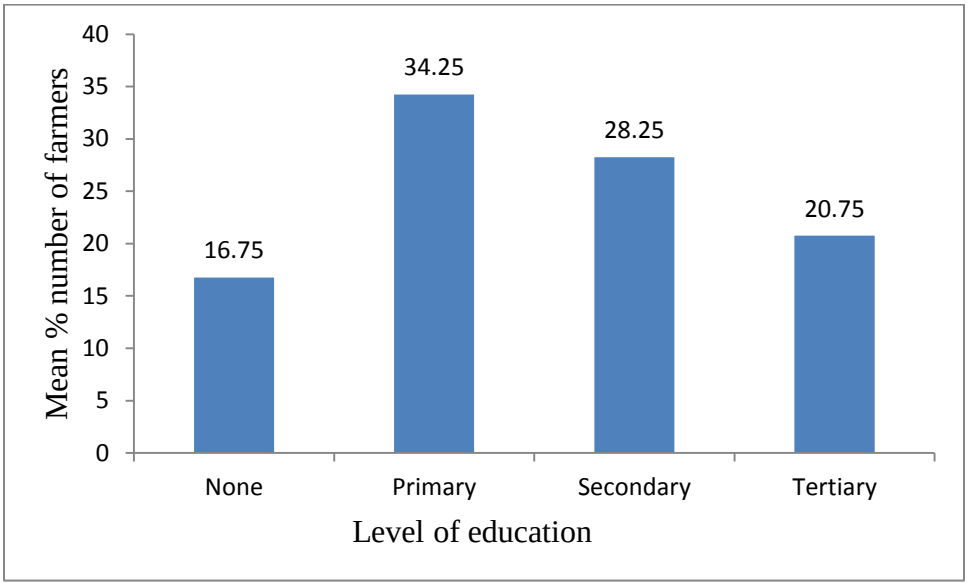


Figure 4.2: Educational level of the avocado farmers in Murang'a County

Farmers with tertiary education were highest at the age of 60 and above, with 62.3 % of the total number who responded (Table 4.3).

Table 4.3: A crosstabulation of Farmers Age and Education Level of avocado farmers'

Farmers Age	Education Level					Total
	No response	None	Primary	Secondary	Tertiary	
No Response	23	7	27	17	13	**87**
Below 30	0	0	7	10	3	**20**
30-39	0	0	20	10	3	**33**
40-49	0	0	14	13	0	**27**
50-59	0	7	29	7	0	**43**
60 and above	14	26	90	26	33	**190**
Total	**37**	**40**	**187**	**83**	**53**	**400**

There was a significant correlation between farmers age and farmers gender $P<0.05$ according to Pearson correlation test (Table 4.4). Further studies showed a significant correlation of -0.387 between farmers age and education level, at

P<0.05 level of significance of 2-tailed test (Table 4.4)

Table 4.4: Correlations among farmers' age, gender, occupation and education level

		Farmers Age	Farmers Gender	Occupation of the Farmer	Education Level
Farmers Age	Pearson Correlation	1	**-.253****	**-.313****	.048
	Sig. (2-tailed)		.005	.000	.601
	N	400	400	400	400
Farmers Gender	Pearson Correlation	**-.253****	1	-.123	**-.387****
	Sig. (2-tailed)	.005		.183	.000
	N	400	400	400	400
Occupation of the Farmer	Pearson Correlation	**-.313****	-.123	1	.161
	Sig. (2-tailed)	.000	.183		.079
	N	400	400	400	400
Education Level	Pearson Correlation	.048	**-.387****	.161	1
	Sig. (2-tailed)	.601	.000	.079	
	N	400	400	400	400

**. Correlation is significant at the 0.01 level (2-tailed).

4.1.3: Language literacy levels of the avocado farmers

A total of 157 farmers interviewed were able to communicate in Kikuyu and Kiswahili while the rest, two hundred and fourty three farmers could communicate in both Kikuyu and English (Table 4.5). Further it was noted that all the farmers were able to communicate in their local language, Kikuyu.

Table 4.5: Language literacy levels of avocado farmers

Language literacy	**Number of farmers**	**Percentage (%)**
Kikuyu and Kiswahili	157	39.2

Kikuyu and English	243	60.8
Total	400	100.0

4.1.4: The Source of avocado seedlings grown by farmers in Murang'a County

The study revealed that farmers obtained the avocado seedling from various sources. Thirty five percent of the farmers used self propagated seedlings while 20% obtained their seedlings from tree nurseries along Thika road. In addition, 10% of the farmers got the seedlings from their neighbouring farmers (Table 4.6). Twenty five percent of farmers obtained seedlings from certified nurseries in KALRO Seed Unit at the Horticultural Research Centre, Kakuzi Company Ltd, and contracted nurseries by the Ministry of Agriculture in Murang'a County (Table 4.6). However, 10.8% did not know the source as they inherited the farm containing the avocado trees from their parents and grand parents.

Table 4.6: Source of avocado seedling grown by farmers in Murang'a County

Source of avocado seedlings	Frequency of farmers	Percent
KALRO, Kandara	57	14.2
Nurseries along Thika Road	80	20.0
Self propagated seedlings	140	35.0
Not aware of the source	43	10.8
Other farmers	40	10.0
Supply from Murang'a County Governoment	17	4.2
Kakuzi	13	3.3
MoA -Murang'a County	10	2.5
Total	400	100.0

4.1.5: Methods used to enhance avocado yields in Murang'a County

It was observed that farmers rarely use fertilizer to enhance growth and

production of the avocado crops. Seventeen percent of the farmers used fertilizer Calcium Amonium Nitrate (CAN) only at the flowering stage (Table 4.7). But, 22.5% of the farmers used farm yard manure at various stages of plant growth, where 4.2 % of this number used it at planting stage. Majority of farmers, 60% of the total however, did not apply any form of fertilizer during the plant growth and development (Table 4.7).

Table 4.7: Proportion of the farmers using organic and inorganic fertilizer in avocado production

Type of fertilizer	Frequency of farmers	Percent
Calcium Ammonium Nitrate	70	17.5
Zero Fertilizer	240	60.0
Farm yard manure	90	22.5
Total	400	100.0

4.1.6: Number of avocado trees grown by individual farmers

Avocado plants were available in all the farms visited. The number of trees in each homestead differed depending on the intended use. It was observed that forty one percent among the homesteads visited, avocado trees were below five in number. Twenty six percent of the farmers had six to ten trees. The number of farmers having more than ten trees progressively reduced significantly to about 0.8 % of those farmers with 46-50 trees. However, the number rose to about 10% for those with a total of 51 trees and above (Table 4.8).

Table 4.8: Number of avocado trees per farmer

Number of trees	Frequency of farmers	Percent
5 trees and below	167	41.7
6-10 trees	106	26.7
11-15 trees	43	10.8
16-20 trees	17	4.2
21-25 trees	7	1.7
26-30 trees	7	1.7
31-35 trees	7	1.7
36-40 trees	3	.8
46-50 trees	3	.8
51 and above trees	40	10.0
Total	400	100.0

4.1.7. Marketing of avocado fruits in Murang'a County

Most of the avocado fruits produced in the Murang'a County are sold in the local market while the rest is sent to the export markets. Five percent of the farmers grow avocado trees for home consumption of fruits only (Table 4.9). Sixty seven percent of the farmers sold their avocado fruits to the brokers while 12.5% and 10.9% of the farmers sold their avocado fruits in the local market and export market, respectively (Table 4.9). But only 4.2% of the farmers sold their fruits to the processors.

Table 4.9: Marketing of avocado fruits in Murang'a County

Marketing of fruits	Frequency of farmers	Percent
Local market	50	12.5
Brokers	269	67.5
Export market	44	10.9
Home consumption only	17	4.2
Processors	17	4.2
No sale	3	.8
Total	400	100.0

Some avocado farmers in the County were engaged in contractual farming system with the exporters. Eighty seven percent of the farmers had no contract agreement

with exporting companies but solely depended on the brokers for marketing (Table 4.10).

Table 4.10: Avocado farming system in Murang'a County

Farming system	Frequency of farmers	Percent
Contract farming	50	12.5
No contract farming	350	87.5
Total	400	100.0

4.1.8. Identification of avocado diseases by farmers in Murang'a County

The disease identification in avocado grown in the study area was mainly through farmers (self), extension officer, family member and exporter. Sixty percent of the farms had the symptoms of avocado diseases being identified by the individual farmer while in 1.7% of the farms it was through the Agricultural Extension Officer. A similar number of farms 1.7% had members of the family identifying the diseases (Table 4.11). Thirty five point eight percent of the farms however, had no one to identify the diseases (Table 4.11).

Table 4.11: Disease and pest identification

Disease/ Pest identification	Frequency	Percent
Self	240	60.0
Extension officer	7	1.7
Family member	7	1.7
No one	143	35.8
Exporter	3	0.8
Total	400	100.0

Among the pre and post-harvest diseases identified in the farms included anthracnose as black spots, yellowing and drying of leaves, blight, brown spot and die back (Table 4.12). Anthracnose accounted for 50.8% of the total while the rest were significantly low with die back accounting for 3.3% (Table 4.12).

Table 4.12: Diseases identified in avocado

Avocado disease	Frequency of farmers	Percent
No diseases identified	170	42.5
Anthracnose in fruits	203	50.8
Yellowing and drying of leaves	7	1.7
Blight and fruit fly	3	0.8
Brown spot	3	0.8
die back	14	3.3
Total	400	100.0

4.1.9. Management of avocado diseases in Murang'a County

4.1.9.1. Chemical Control

Farmers used various chemicals to control anthracnose disease affecting avocado production in the study area. Fungicides used included GreenCop (Copper oxychloride 500/Kg), Milraz (Propineb 70% and Cymoxaxil6%), Bayleton (Triadimefon 250g/Kg). (Table 4.13). Three point three percent of the farmers used Copper Oxychloride, 4.2% used Bayleton while 0.8% used Milraz to manage anthracnose. Majority of the farmers, 86.7% did not use any chemical to manage diseases (Table 4.13). Some farmers used fungicides to manage the anthracnose but could not remember the chemical used since there were no records kept (Table 4.13).

Table 4.13: Type of fungicides used in control of anthracnose disease and other pests

Name of chemical	Frequency of farmers	Percent
Copper based	13	3.3
Milraz	3	0.8
No chemicals	347	86.7
smoking	3	0.8
Boom flower and booster	3	0.8
Bayleton	17	4.2
lime	3	0.8
Cannot remember fungicide used	7	1.7
weedal herbicide	3	0.8
Total	400	100.0

4.1.9.2. Cultural methods of controlling anthracnose diseases

Majority of the farmers, 55.8% did not apply any disease management strategy to their avocado plants (Table 4.14). But 25% of the farmers pruned the avocado trees to improve aeration in the tree canopies and reduce humidity which favoured the establishment and spread of anthracnose disease and also to reduce fruit drop caused by overproduction of fruits in relation to the capability of the individual tree (Table 4.14). Some farmers, 6.6% improved field sanitation by collecting the infected fruits and leaves under the avocado tree and either buried or made compost out of it (Table 4.14). However, insignificant number of avocado farmers, 0.8% hand weeded their avocado crops in the field (Table 4.14).

Table 4.14: Cultural methods for used by farmers to control anthracnose disease in avocado

Cultural method used	Number of farmers	Percent (%)
None	223	55.8
Pruning	126	30.2
Burying diseased fruits/ leaves	35	8.7
Composting diseased fruits/ leaves	13	3.3
Weeding	3	0.8
Total	400	100.0

4.1.10. Harvesting of avocado fruits

Harvesting of avocado fruits by farmers involved various methods such as use of Thirty point eight percent of the farmers used handpicking only to ensure no fruit damage occurs during harvesting (Table 4.15). But 42.5 % of farmers however, used both handpicking and hooks to harvest the fruits (Table 4.15). A significantly small number of farmers, 0.8% shook the tree for fruits to drop as a method of harvesting (Table 4.15). Some farmers (4.2%) waited for the fruits to drop, attached no economic value to them and used them for home consumption including giving them to livestock and dogs.

Table 4.15: Methods of harvesting avocado

Methods of picking	Frequency of farmers	Percent
Using hooks	83	20.8
Hand picking	123	30.8
Waiting for the fruits to drop	17	4.2
Hand picking and using hooks	173	42.5
Shaking the tree/ branches	4	0.8
Total	400	100

4.1.11. Determination of fruit maturity

Farmers determined the maturity of avocado fruits mainly through change of fruit colour from light green to dark green (57.5%) (Table 4.16). Twenty five percent

of the farmers relied on buyers to know whether the fruit are mature or not especially when they visited to buy the produce (4.16). Some (6.7 %) however, waited for mature fruits to drop from the trees. A small percentage of farmers (4.1%) determined fruit maturity by tasting or size of the fruit (Table 4.16).

Table 4.16: Determination of the avocado fruit maturity by farmers

Determination of fruit maturity by farmer	Number of farmers	Percentage (%)
Colour change (green to dark green)	230	57.5
The buyers	100	25.0
Time of the year	23	5.8
Fruit drop	27	6.7
Shaking of fruit	3	0.8
Fruit taste	3	0.8
Size of fruit	14	3.3
Total	400	100.0

4.1.12: Packaging of avocado fruits

Majority of the farmers (54.2%) used sacks to pack their fruits after harvest to the market, mainly local market (Table 4.17). However, avocado fruits meant for export markets were packed in crates, buckets and some time placed in the floor of pick up vehicle as they are transported to the export company. Twenty seven point five percent of the farmers used bucket alone to package their harvest (Table 4.17). However, 6.7% of the farmers used crates only to package avocado fruits (Table 4.17). An insignificant number of farmers, 0.8% did not use any packaging material (Table 4.17).

Table 4.17: Carrier material for the harvested avocado fruits

Packaging material	Number of farmers	Percentage (%)
Crates	27	6.7
Buckets	110	27.5
Sack	217	54.2
Buckets and floor of pick up vehicle	20	5.0
sacks and buckets	23	5.8
Total	397	99.2
No packaging material	3	0.8
	400	100

4.1.13. Farmers' perception of the effect of pesticides to the environment

The majority of farmers, 87.5% interviewed expressed lack of awareness on the effect of pesticides to the environment and more so, on the useful organisms like bees, butterflies and earthworms (Table 4.18). The 12.5% of farmers associated pesticides harmful effect on bees, butterflies, earthworms at rate of 13.3%, 6.7% and 13.3%, respectively (Table 4.18). Nevertheless, 66.7% of those farmers who acknowledged the negative effect of pesticides to the environment did not associate pesticides with environmental risk when used as per manufacturer's label instructions (Table 4.18).

Table 4.18: Risk of pesticides use to the environment * Affected organisms by pesticides Crosstabulation

			Affected organisms by pesticides				Total
			Bees	Butterflies	Earthworms	None	
Risk	yes	Count	7	3	7	33	50
		% Risk	13.3%	6.7%	13.3%	66.7	100.0
	no	Count	0	0	0	143	143
		% within Risk	.0%	.0%	.0%	100.0 %	100.0 %
	Not aware	Count	0	3	0	204	207
		% within Risk	.0%	1.6%	.0%	98.4 %	100.0 %
Total		Count	7	7	7	379	400

	% within Risk	1.7%	1.7%	1.7%	95.0 %	100.0 %

4.1.14. Cultivation of avocado in different agro-ecological zones

Avocado production was spread from the lowest Agro –ecological zone of 1160-1340M above sea level (a.s.l) to the highest, 2130-2430 m above sea level (Figure 4.3). Hass and local varieties were cultivated in all the zones, but variety Fuerte and Pinkerton were not found in the zone, 1160-1340M a.s.l. (Figure 4.3). The agro-ecological zone 1730-2130m had the highest production for Hass, Fuerte, local and Pinkerton at the rate of 37.5%, 51%, 50% and 42.9%, respectively (Figure 4.3).

The Fuerte variety was more popular and productive compared to the other varieties, however, promotion of Hass variety through County Government and Ministry of Agriculture and Livestock had resulted to its being widespread throughout the zones. As such, most Hass trees are still young and have not reached production stage.

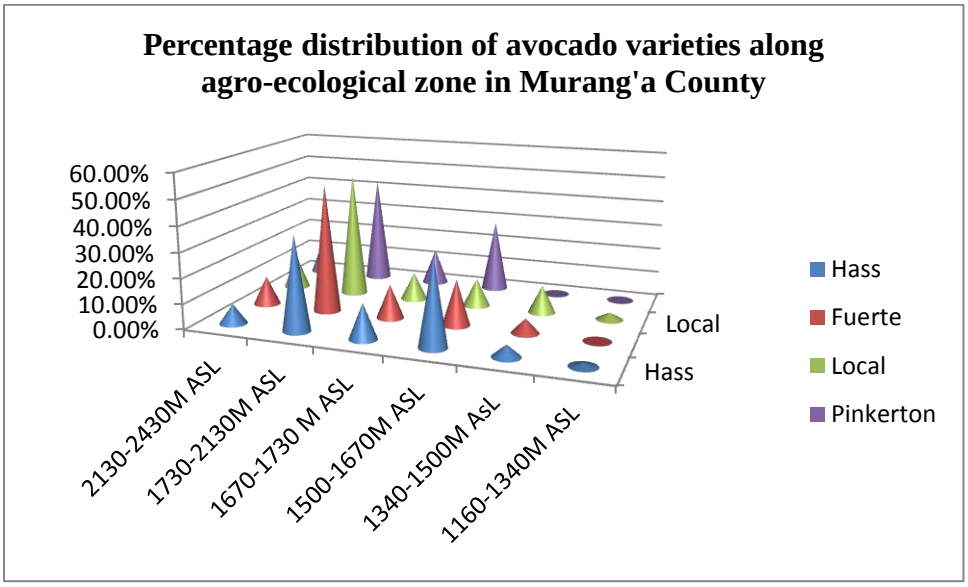


Figure 4.3: Avocado production in different agroecological zone in Murang'a County

4.1.15. Avocado production in Murang'a County

The number of avocado fruits produced in Gatanga, Kandara, Kigumo and Kahuro sub-counties by 100, 100, 98 and 98 farmers in 2015 was 2987414, 2700313, 2449255 and 1049177 fruits, respectively (Table 4.19). Gatanga farmers had the highest production mean number of 29874.14 while Kahuro farmers had the lowest mean of 10708 avocado fruits (Table 4.19). Kandara farmers was the second highest producer, with a mean of 27003 fruits while the farmers from Kigumo was third with a mean number of 24992 fruits (Table 4.19).

Table 4.19: Avocado production by 396 farmers in different sub-counties of Murang'a County in the year 2015

Sub-County	Number of farmers	Mean number of avocado	Total number of fruits
Gatanga	100	29874.14	2987414
Kandara	100	27003.13	2700313
Kigumo	98	24992.4	2449255
Kahuro	98	10705.89	1049177
			9,186,159

4.2. Morphological characteristics of *Colletotrichum gloeosporioides* and *Pestalotiopsis microspora* isolates

4.2.1 Isolation and identification of the pathogens

A total of 80 fungal isolates from diseased avocado fruits showing symptoms of anthracnose collected from the study area were obtained. The isolates were identified based on cultural morphological characteristic on the PDA media and spore characteristics as observed under microscope ((Nagamani *et al*., 2006, Freeman *et al*., 1998 and Domsch *et al*., 1980).

A total of 46 isolates had whitish, greyish or creamish colour and cottony, velvety mycelium on the top side and greyish cream with circular orange-pinkish colour on the reverse side. Their spores were straight with rounded end (Plate 1, i), typical of *C. gloeosporioides* (Mordue, 1971, Sutton, 1992, Silva-Rojas and Avila-Quenzeda, 2011). The remaining 34 isolates had whitish grey mycelium with black fruiting structure on the upper side and greyish black on the lower side. Their spores had 3-4 septa and 2 or 3 appendages at one end characteristics of *Pestalostiopsis microspora* (Kevin *et al*., 2011) (Plate 1,ii).

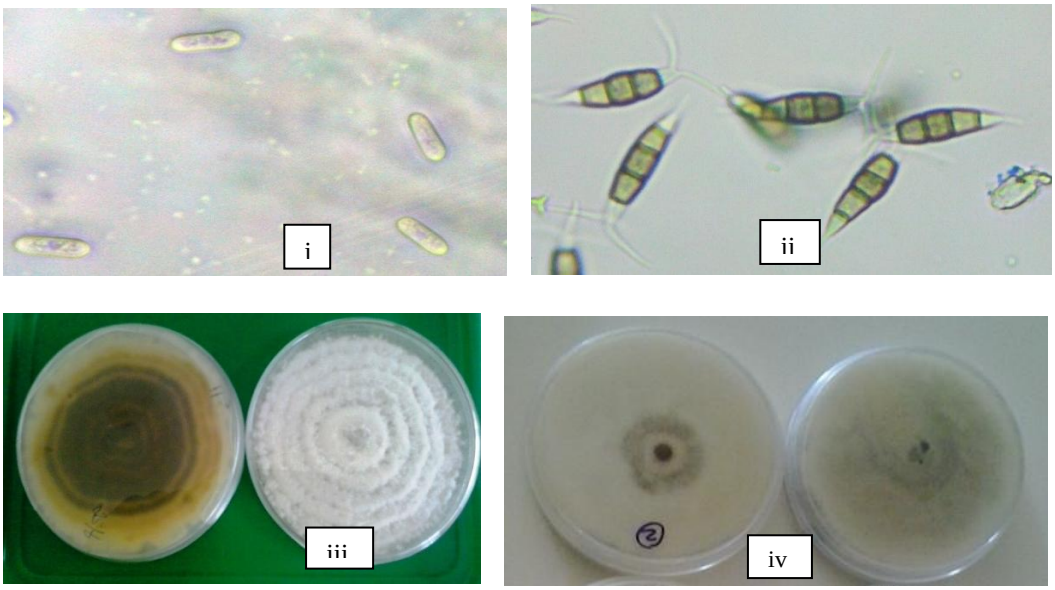


Plate 1: *Colletotrichum gloeosporioide*s spores (i) (x400), *Pestalotiopsis microspora* spores (ii) (x400) and mycelial of *Colletotrichum gloeosporioides* (iii), and mycelial of *Pestalotiopsis microspora* (iv).

The isolates were further confirmed through Koch's postulate where the pure cultures of the isolates growing on PDA were used to inoculate healthy ripe avocado fruit, Fuerte variety. After two days, a characteristic black spot was

formed by both *Colletotrichum* and *Pestalotiopsis* isolates. Each of the re-isolated fungus from the diseased fruit showed similar morphological, cultural and spore characteristics as initial isolates. *Colletotrichum gloeosporiodes* isolates were subjected to more detailed study while the *Pestalotiopsis* spp was identified further at molecular level using universal primers ITS1 and ITS4.

4.2.2 Morphological characterization of *C. gloeosporioides* isolated from Hass and Fuerte varieties

4.2.2.1 The mycelial growth of *Colletotrichum gloeosporioides* isolates

The *Colletotrichum* isolates from Gatanga, Kigumo, Kandara and Kahuro sub-counties grew rapidly on the PDA medium covering the whole surface of the petri dish in 10-12 days after inoculation. The mycelial colour of the isolates varied from whitish grey, whitish cream and greyish pink on the upper side of the culture (Table 4.20). Similarly, the lower side of the cultures had creamish grey, greyish orange and grey (Table 4.20). In terms of mycelia structure, cottony was observed in 24 isolates as compared to velvety observed in 22 isolates (Table 4.20).

Table 4.20: Mycelia characteristics of the *C. gloeosporioides* isolates on PDA from 4-sub counties of Murang'a County

	Upper side		Lower side		
Number of Isolates	**Colony colour**	**Texture**	**Colour**	**Zonation**	**Conidial shape**
24	Whitish grey	Cottony	Creamish grey	Concentric	Cylindrical
12	Whitish cream	Velvety	Greyish orange	Concentric	Cylindrical
10	Greyish pink	Velvety	Grey	Concentric	Cylindrical

Table 4.21a: Daily mycelial growth (cm) of *Colletotrichum gloeosporiodes* isolates from Kigumo (ko), Kandara (k), Kahuro (ka) and Gatanga (G) sub-counties in Murang'a County

Isolate	Day2	Day4	Day6	Day8	Day10
k10b	0.77* bcd	1.10 jklmn	1.33 pq	2.57 l	3.57 m
k10c	0.63 defgh	1.02 mno	1.60 no	3.05 ijk	4.00 efghij
ko11b	0.57 fgh	1.67 d	2.23 def	3.77 ab	4.03 efghi
k10c	0.63 defgh	1.02 mno	1.60 no	3.05 ijk	4.00 efghij
ko11c	0.57 fgh	1.67 d	2.23 def	3.77 ab	4.03 efghi
G12a	0.65 cdefgh	1.43 efg	2.12 efg	3.45 bcdefgh	4.23 bcde
ko12c	0.63 defgh	1.47 ef	2.33 de	3.77 ab	4.17 defg
ko13a	0.53 gh	1.03 lmno	1.73 lmn	3.30defghij	3.90 hijkl
ko13b	0.50 h	1.17 ijklm	1.83 ijklm	3.23 fghij	3.80 ijklm
ko13c	0.63 defgh	1.10 jklmn	1.97 ghijk	3.40 cdefgh	4.07 efgh
ko17b	0.63 defgh	1.10 jklmn	1.97 ghijk	3.40 cdefgh	4.07 efgh
ko14a	0.60 efgh	0.93 no	1.20 q	1.47 n	2.60 n
ko14c	0.60 efgh	0.93 no	1.20 q	1.47 n	2.60 n
ka14b	0.60 efgh	0.87 o	1.73 lmn	3.63 abcd	4.17 defg
ka15b	0.60 efgh	0.87 o	1.73 lmn	3.63 abcd	4.17 defg
k15a	0.50 h	1.43 efg	2.03 fghi	3.67 abc	4.07 efgh
k15b	0.70 cdef	1.27 ghij	1.97 ghijk	3.63 abcd	4.10 efgh
ko15c	0.60 efgh	1.20 hijkl	1.70 lmno	3.70 abc	4.00 efghij
ko16a	0.73 bcde	1.37 fgh	2.03 fghi	3.60 abcde	4.20 cdef
G16b	0.87 ab	1.57 de	2.80 b	3.70 abc	4.03 efghi
ka16c	0.60 efgh	1.10 jklmn	1.65 mno	2.82 kl	4.37 abcd
ko17c	0.60 efgh	1.00 mno	1.60 no	2.97 jk	4.03 efghi
k19b	0.67 cdefg	1.20 hijkl	1.97 ghijk	3.43 bcdefgh	4.13 Defgh
G19c	0.93 a	1.90 c	3.23 a	3.57 bcdef	4.10 Efgh
LSD(0.05)	0.16	0.17	0.23	0.34	0.25
P-value	<0.001	<0.001	<0.001	<0.001	<0.001

*Data are mean radial growth of three replicates. Means on the same column followed by similar letter(s) are not significantly different at P≥ 0.05 according to the Fisher's protected LSD test.

Table 4.21b: Daily mycelial growth (cm) of *Colletotrichum gloeosporioides* isolates from Kigumo (ko), Kandara (k), Kahuro (ka) and Gatanga (G) sub-counties in Murang'a County

Isolate	Day2	Day4	Day6	Day8	Day10
ka1c	0.50* h	1.43 efg	2.03 fghi	3.30 defghij	4.50 a
k20a	0.63 defgh	2.18 b	2.37 cd	3.28 efghij	3.65 Lm
ko20b	0.80 abc	1.13 jklm	1.88 hijkl	3.18 ghij	3.90 Hijkl
k20c	0.80 abc	1.90 c	2.58 bc	3.15 hijk	3.93 Ghijk
ka22b	0.67 cdefg	1.32 fghi	2.08 fgh	3.43 bcdefgh	4.02Efghij
ka24a	0.70 cdef	1.37 fgh	2.10 fgh	3.37 cdefghi	4.43 Abc
ko24b	0.77 bcd	1.23 hijk	1.60 no	3.13 hijk	3.97 Fghij
k24c	0.80 abc	2.63 a	3.40 a	3.93 a	4.13 Defgh
ka25a	0.60 efgh	1.03lmno	1.50 op	2.50 l	3.77 Jklm
ka26c	0.57 fgh	0.93no	1.33 pq	2.03 m	2.37 n
ko27b	0.30 i	0.40 p	0.97 r	1. 43 n	2.60 n
ko29a	0.70 cdef	1.03 lmno	1.70 lmno	3.25 fghij	4.07 Efgh
ko29b	0.70 cdef	1.17 ijklm	1.97 ghijk	3.63 abcd	4.10 Efgh
ko2a	0.63 defgh	1.10 jklmn	1.77 klmn	2.83 kl	4.00 Efghij
ko30a	0.60 efgh	1.13 jklm	1.33 pq	2.53 l	3.80 Ijklm
ka30b	0.57 fgh	1.07 klmn	2.00 ghij	3.50 bcdefg	4.47 Ab
ka31b	0.70 cdef	1.13 jklm	1.77 klmn	3.50 bcdefg	4.47 Ab
ko31c	0.77 bcd	1.07 klmn	1.78 jklmn	3.27 efghij	4.03 Efghi
ko7c	0.77 bcd	1.07 klmn	1.67 lmno	1.87 m	4.03 Efghi
ko7c	0.77 bcd	1.07 klmn	1.67 lmno	1.87 m	4.03 Efghi
G8a	0.77 b-d	1.13 jklm	1.63 mno	2.97 jk	3.70 Klm
G9a	0.70 c-f	1.58 de	2.67 b	3.55 bcdef	4.22 Bcdef
LSD(0.05)	0.16	0.17	0.23	0.34	0.25
P-value	<0.001	<0.001	<0.001	<0.001	<0.001

*Data are mean radial growth of three replicates. Means on the same column followed by similar letter(s) are not significantly different at P≥ 0.05 according to the Fisher's protected LSD test

The mycelial diameter of the isolates showed significant differences (P<0.05) throughout the growth period (Table 4.21a, b). However, they exhibited similar trend in growth with day two having the least and day 10 having the largest diameter per isolate (Table 4.21a, b). The diameter of all the isolates ranged from 0.30- 0.93 cm and 2.37 -4.5cm in day 2 and day 10, (Table 4.21a, b) respectively.

Isolates ka26c, ko27b and ko14a had significantly lower growth rate as compared to the rest with a mean diameter of 2.37, 2.60 and 2.60, respectively at day 10. However, isolate ka1c, had the second lowest mean radial diameter at day 2 and the highest radial diameter at day 10, covering the petri dish fully. This isolate had the highest growth rate as compared to the rest (Table 4.21a, b).

Isolates from Gatanga sub-county did not differ significantly at $P<0.05$ at day 2 except isolate G12a which was significantly different from the rest (Table 4.21a, b). In day 6 the mean radial diameters for the isolate G9a and G16b were not significantly different $P\geq0.05$ but they were different from the rest. In day 10, isolate G22b differed significantly from the rest but isolate G12a, G16b, G19c, G20b and G9a were not significantly different (Table 4.21a,b).

All the isolates from Kahuro sub-county did not differ significantly ($P\geq0.05$) in day 2. In day 4 and 6, the mean diameter of majority of the isolates differed significantly (Table 4.21). In day 8, the radial diameter for the isolate ka26c, was significantly different from the other isolates. At day 10, eight out of the total eleven isolates did not differ significantly $P\geq0.05$ in their mean diameter. In the same day 10, isolate ka26c and ka25a had the lowest and second lowest mean diameter of 2.37 and 3.77, respectively, differed significantly (Table 4.21a,b).

When isolates from Kandara sub-county were considered separately, isolate k20a had the highest mean radial diameter of 0.83cm while the isolate k15a had the

lowest mean of 0.50cm in day 2. The mean diameter of the two isolates differed significantly from each other at P<0.05 (Table 4.21a,b). Majority of the isolate k20a, k20c, k24c, k10b, k15b and k19b from kandara did not differ significantly P≥0.05 in their mean diameter at day 2 (Table 4.21a, b). At day 10, the isolate k15c had the lowest mean diameter of 3.57cm which was significantly different P<0.05 from the rest isolates in the sub-county (Table 4.21a, b).

At day 2, the isolate ko27b, had significantly the lowest mean diameter of 0.30 cm as compared to other isolates which had the highest mean of 0.77cm in kigumo (Table 21a, b). The mean diameter for the isolates ko11b, ko13c, ko17c, ko29a, ko31c and ko7c were not significantly P ≥0.05 different at day 10. The isolate, ko16a had the highest mean diameter of 4.20cm which was significantly different from the rest. (Table 21a, b).

Table: 4.22a: Regression analysis of mycelial radial growth of different *Colletotrichum* isolates against time (days)

Location	**Isolate**	**Equation**	**R^2**
Gatanga	G12a	Y=0.431x-0.476	0.968
	G16b	Y=0.423x+0.053	0.966
	G19c	Y=0.4x+0.346	0.938
	G20b	Y=0.456x-0.286	0.949
	G22b	Y=0.365x-0.003	0.960
	G8a	Y=0.385x-0.27	0.943
	G9a	Y=0.418x-0.07	0.967
Kandara	k10b	Y=0.353x-0.253	0.849
	k10c	Y=0.438x-0.57	0.946
	k15a	Y=0.468x-0.47	0.962
	k15b	Y=0.458x-0.416	0.936
	k19b	Y=0.458x-0.47	0.961
	k20a	Y=0.368x+1.096	0.682
	k20c	Y=0.403x+0.6	0.874
	k24c	Y=0.398x+0.59	0.862

R^2 indicates the proportion (%) of the variance in mycelia radial diameter that is predictable from days.

Table: 4.22b: Regression analysis of mycelial radial growth of different *Colletotrichum* isolates against time (days)

Location	Isolate	Equation	R^2
Kahuro	ka12a	Y=0.486x-0.28	0.981
	ka14b	Y=0.495x-0.77	0.914
	ka16c	Y=0.462x-0.668	0.936
	ka1c	Y=0.493x-0.606	0.959
	ka22b	Y=0.516x-0.68	0.975
	ka24a	Y=0.473x-0.446	0.973
	ka25a	Y=0.39x-0.46	0.940
	ka26c	Y=0.235x+0.036	0.952
	ka30b	Y=0.511x-0.75	0.970
	ka31b	Y=0.49x-0.656	0.946
	ka9a	Y=0.481x-0.243	0.993
Kigumo	ko12c	Y=0.468x-0.336	0.973
	ko12a	Y=0.423x-0.473	0.958
	ko11b	Y=0.451x-0.256	0.964
	k013a	Y=0.45x-0.6	0.959
	ko13b	Y=0.433x-0.493	0.965
	ko13c	Y=0.458x-0.516	0.967
	ko14a	Y=0.226x	0.845
	ko15c	Y=0.465x-0.55	0.926
	ko16a	Y=0.458x-0.363	0.967
	ko17c	Y=0.441x-0.61	0.947
	ko20a	Y=0.345x-0.53	0.884
	ko20b	Y=0.368x-0.303	0.946
	ko20c	Y=0.348x-0.163	0.942
	ko24b	Y=0.415x-0.35	0.933
	ko27b	Y=0.281x-0.55	0.900
	ko29a	Y=0.447x-0.535	0.944
	ko29b	Y=0.463x-0.466	0.954
	ko30a	Y=0.39x-0.46	0.916
	ko31c	Y=0.436x-436	0.947
	ko7c	Y=0.366x-0.32	0.812

R^2 indicates the proportion (%) of the variance in mycelia radial diameter that is predictable from days.

Isolate ka9a from kahuro sub-county had the highest R^2 value of 0.993, followed by isolate ka12a that had R^2 value of 0.981 while isolate ka22b had R^2 value of 0.975 as indicated in (Table 4.22a, b). Isolate G12a had the highest R^2 value of 0.968 compared to other isolates from Gatanga. In Kigumo sub-county, ko12c had

the highest R^2 value of 0.973 as compared to the rest in the sub-county (Table 4.22a, b). Overall, the isolate ko7c had the lowest R^2 value of 0.812 (Table 4.22a, b).

4.2.2.2 Sporulation of *Colletotrichum gloeosporioides* isolates

Colletotrichum gloeosporioides isolates sporulation exhibited a wide range of mean number of spores per isolate from the lowest 0.67×10^6 to the highest 9.00×10^6 spores (Table 4.23). The mean number of spores per ml differed significantly at $P<0.05$ among isolates. Seven isolates, ko20c, ko30a, ko20b, ko12c, ko13a, ko13c and ko14a from Kigumo sub-county (ko) with the least number of spores per ml did not differ significantly at $P\geq0.05$ however, they differed significantly ($P<0.05$) from the rest (Table 4.23). Isolate G16b, from Gatanga sub- county had the highest mean number of 9.0×10^6 per ml which was significantly different from the rest at $P<0.05$ (Table 4.23). Twenty four isolates had mean number of spores lower than the tabulated mean of all the isolates of 3.49×10^6 per ml. A total of 21 isolates had a mean number of spores in the range of $2.33\text{-}4.00 \times 10^6$ per ml which were not significantly different. Similarly, 13 isolates had their mean number of spores in the range of $4.33\text{-}6.00 \times 10^6$ per ml (Table 4.23).

Table 4.23: Mean number of spores of *C. gloeosporioides* produced per ml ten days after inoculation

Isolate	Mean ($\times 10^6$)/ml	Isolate	Mean ($\times 10^6$)/ml
G16b	9.00*a	G9a	3.00 efg
ko29b	6.00b	k19b	3.00 efg
G12a	5.67bc	ka30b	3.00 efg
ka22b	5.33bc	ka31b	3.00 efg
G20b	5.00bc	ko17c	3.00 efg
ka10b	5.00bcd	ka9a	2.67fg
ka14b	5.00bcd	ko2a	2.67fg
ka26c	5.00bcd	k20a	2.33fgh
ko29a	5.00bcd	k24c	2.33 fgh
G19c	4.67bcde	ka 25a	2.33 fgh
ka16c	4.67 bcde	ko20c	2.33 fgh
ka1c	4.67 bcde	ko30a	2.33 fgh
k15a	4.33 bcde	k10c	2.00 gh
ko24b	4.33 bcde	k20c	2.00gh
G8a	4.00cdef	ko20b	2.00gh
ko15c	4.00 cdef	ko12c	1.00h
ko7c	4.00 cdef	ko13a	1.00h
ko11b	3.67def	ko13c	0.67h
k15b	3.33defg	ko14a	0.67h
ka12a	3.33 defg	ka 11a	0.66h
ka24a	3.33 defg	ko26c	0.66h
ko16a	3.17efg	k13a	0.65hi
G22b	3.00 efg	ko 12a	0.65hi
LSD	1.673	LSD	1.673
Pvalue	<0.001	Pvalue	<0.001

*Data are means of three replicates. Means on the same column followed by similar letter(s) are not significantly different at P≥ 0.05 according to the Fisher's protected LSD test.

4.2.2.3 Conidial morphology and size

All the *C. gloeosporioides* spores observed were cylindrical and straight with smooth round end. The spore size varied significantly at P<0.05 among isolates ranging from 3.0-5.0 µm in width and 10.3 – 18.2 µm in length (Table 4.24a, b). The spore width of isolates G16b, ko29b and G12a was highest at 5µm and they differed significantly from the rest at P<0.05 (Table 4.24a, b).Similarly, these

isolates produced the longest spores of 18.2, 18.0 and 18.0 µm for isolate G16b, ko29b and G12a, respectively. Isolate k13a and ko12a however, produced the smallest spores having a mean of 3.0 µm in width and 10.3 µm in length. Thirty one isolates produced spores having widths within the range of 3.1 to 3.5 µm which did not differ significantly at P≥0.05 (Table 4.24a, b). Overall, spore size in terms of width and length differed significantly at P<0.05 among isolates.

Table 4.24a: The mean width and length (µm) of spores produced by 10 day - old *Colletotrichum gloeosporioides* isolates

Isolate	Width (µm)	Length (µm)
G16b	5.0*a	18.2a
ko29b	5.0a	18.0a
G12a	5.0a	18.0a
ka22b	4.7a	17.1ab
G20b	4.7a	17.1ab
ka10b	4.7a	16.2bc
ka14b	3.7b	16.1bc
ka26c	3.7b	16.1bc
ko29a	3.6bc	16.0bc
G19c	3.6bc	15.4cd
ka16c	3.6bc	15.3cd
ka1c	3.6bc	15.2cde
k15a	3.5bcd	15.2cde
ko24b	3.5bcd	15.2cde
G8a	3.5bcd	15.1cde
ko15c	3.5 bcd	15.0cde
ko7c	3.5 bcd	15.0cde
ko11b	3.5 bcd	15.0cde
k15b	3.5 bcd	15.0cde
ka12a	3.5 bcd	14.5def
ka24a	3.4 bcde	14.4defg
ko16a	3.4 bcde	14.2defgh
G22b	3.4 bcde	14.0efgh
G9a	3.4 bcde	14.0efgh
LSD	0.4031	1.2096
P value	<0.001	<0.001

*Data are means of three replicates. Means on the same column followed by similar letter(s) are not significantly different at P≥ 0.05 according to the Fisher's protected LSD test.

Table 4.24b: The mean width and length (µm) of spores produced by 10 day - old *Colletotrichum gloeosporioides* isolates

Isolate	Width (µm)	Length (µm)
k19b	3.3* bcde	13.5fgh
ka30b	3.3 bcde	13.2ghi
ka31b	3.3 bcde	13.2ghi
ko17c	3.3 bcde	13.2ghi
ka9a	3.3 bcde	13.1hi
ko2a	3.2cde	13.1hi
k20a	3.2 cde	13.0hi
k24c	3.2 cde	12.2ij
ka 25a	3.2 cde	12.2ij
ko20c	3.2 cde	12.2ij
ko30a	3.2 cde	12.1ij
k10c	3.2 cde	12.1ij
k20c	3.2 cde	12.1ij
ko20b	3.2 cde	12.1ij
ko12c	3.2 cde	12.0ij
ko13a	3.1de	12.0ij
ko13c	3.1 de	11.1jk
ko14a	3.1 de	11.0jk
ka 11a	3.1 de	11.0jk
ko26c	3.1 de	10.4k
k13a	3.0e	10.3k
ko12a	3.0e	10.3k
LSD	0.4031	1.2096
P value	<0.001	<0.001

*Data are means of three replicates. Means on the same column followed by similar letter(s) are not significantly different at P≥ 0.05 according to the Fisher's protected LSD test.

4.3 Pathogenicity of *C. gloeosporioides* isolates on avocado fruits

4.3.1 Lesion diameter on unripe healthy mature fruit (Fuerte) 4 -days after inoculation

The lesion diameter on healthy mature avocado fruit, Fuerte variety four days after inoculation did not differ significantly at P≥0.05 (Figure 4.4). Fruits inoculated by isolates k24c, ka25a and ka1c had the highest mean lesion diameter

of 10.00cm followed by those inoculated with isolates G12a, ko31c, k15b, k20a, 14b, ka30b, ko11b, ko13a, ko15c, ko16a, ko24b and ko29b with a mean lesion of 9.00cm. A total of 18 isolates formed lesions on healthy avocado fruits after inoculation in the range of 7.67cm to 8.00cm in diameter (Figure 4.4).

Fruits inoculated with isolates ko20c and G20b had the lowest mean lesion diameter of 7.33 cm. Overall, all the isolates were pathogenic to Fuerte variety at unripe stage (Figure 4.4).

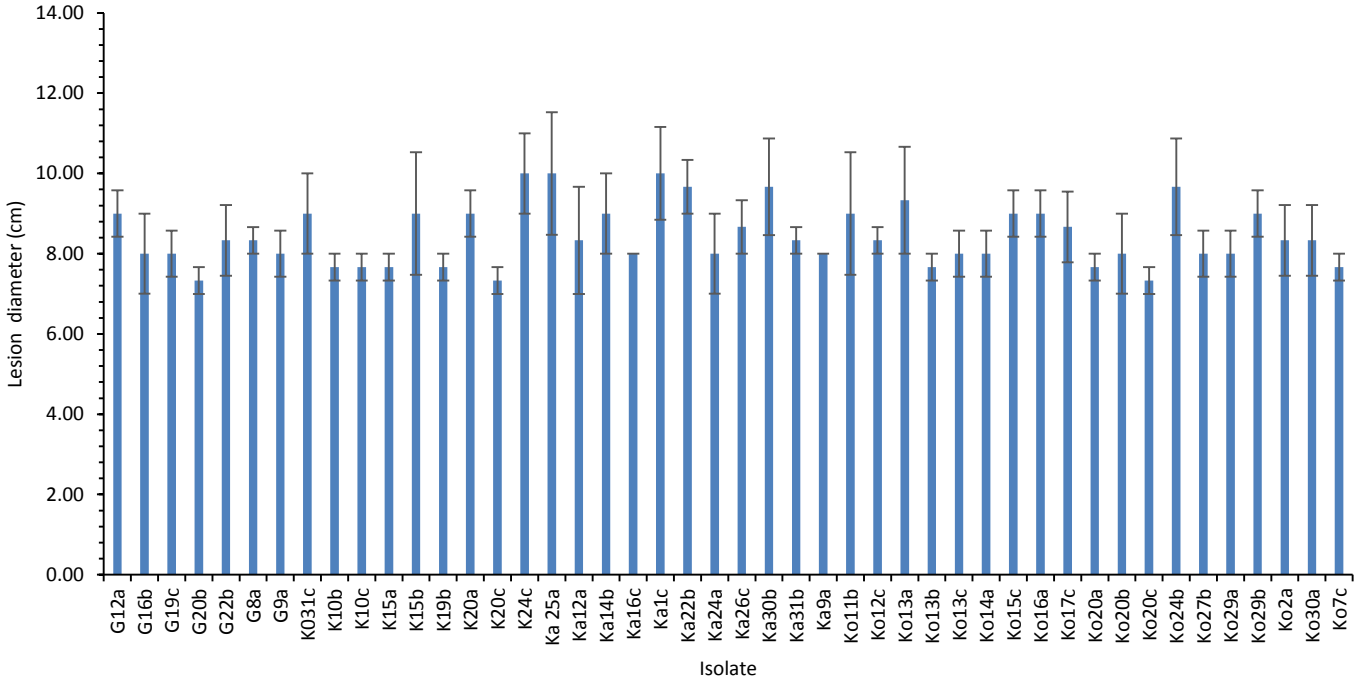


Figure 4.4: Mean lesion diameter (cm) on unripe avocado fruit (Fuerte), four days after inoculation with different *Colletotrichum gloeosporiodes* isolates. Error bars represents standard error (SE) of the mean

4.3.2 Lesion diameter on ripe mature avocado fruit (Fuerte variety) 2 days after inoculation with *Colletotrichum gloeosporioides* isolates

Lesions formed on ripe mature healthy avocado fruits, Fuerte variety inoculated with *Colletotrichum gloeosporioides* isolates from Gatanga, Kandara, Kigumo and Kahuro differed significantly at $P<0.05$ in their mean diameter (Table 4.25). Isolates ka30b and ko29a produced the largest lesions with a mean diameter of 17.00cm on the inoculated fruits, followed by the lesion of 16.00cm in mean diameter associated with isolate ka9a (Table 4.25). Lesions within the range of 13.67-17.00cm in mean diameter associated with a group of twenty two isolates did not differ significantly ($P\geq0.05$) in their mean diameter. The lesions associated with the other group of twenty four isolates in the range of 10.00-13.33cm mean lesion diameter, also were not significantly different ($P\geq0.05$). However, the lesion diameters produced by the isolates in one group differed significantly ($P<0.05$) from the mean lesion diameter of the other group (Table 4.25). Overall, the isolates resulted into two groups based on the mean lesion diameter.

Table 4.25: Mean lesion diameter (cm) on ripe avocado fruit, Fuerte variety 2 days after inoculation with *Colletotrichum gloeosporioides* isolates

Isolates	Mean Lesion diameter (cm)	Isolates	Mean Lesion diameter (cm)
ka30b	17.00*a	k20a	13.00 b-h
ko29a	17.00a	ka12a	13.00 b-h
ka9a	16.00ab	ko13c	13.00 b-h
ka 25a	15.67a-c	ko14a	13.00 b-h
G20b	15.33 a-d	k15b	12.67 b-h
k10c	15.33 a-d	k20c	12.67 b-h
ko13b	15.33 a-d	ko11b	12.67 b-h
k10b	15.00a-e	ko20c	12.67 b-h
ko27b	15.00 a-e	ko2a	12.67 b-h
G19c	14.67 a-f	G12a	12.33 c-h
ko15c	14.67 a-f	k24c	12.33 c-h
G16b	14.33 a-f	ka22b	12.33 c-h
ka31b	14.33 a-f	ko16a	12.33 c-h
k19b	14.00 a-g	ko13a	12.00 d-h
ka16c	14.00 a-g	ko29b	12.00 d-h
ka24a	14.00 a-g	k15a	11.67 e-h
ka26c	14.00 a-g	ka14b	11.33 f-h
ko7c	14.00 a-g	ko12c	11.33 f-h
G22b	13.67 a-g	ko20a	11.33 f-h
k031c	13.67 a-g	ko20b	11.33 f-h
ka1c	13.67 a-g	G9a	10.67 gh
ko16c	13.67 a-g	G8a	10.00 h
ko30a	13.33 b-h	ko24b	10.00 h
LSD	3.644	LSD	3.644
P value	<0.001	P value	<0.001

*Data are means of three replicates. Means on the same column followed by similar letter(s) are not significantly different at P≥ 0.05 according to the Fisher's protected LSD test.

4.3.3 Lesion diameters on unripe mature avocado fruit (Hass) 4 days after inoculation with different *C. gloeosporioides* isolates.

Lesions formed on healthy mature avocado Hass variety four days after inoculation with different *Colletotrichum gloeosporioides* isolates did not differ significantly (P≥ 0.05) in their mean diameter (Figure 4.5). Isolate k20a had the highest mean lesion diameter of 7.33cm followed by isolates ko17c, ka22b and

G8a with mean lesion diameter of 7.00cm each (Figure 4.5). The lesion of mean diameter 5.33cm, associated with isolates k15a, ko13a and ko30a was the lowest. Lesions with a mean diameter of 6.33 cm were associated with *Colletotrichum gloeosporioides* isolates from different sub counties, Gatanga (G12a, G16b and G19c), Kandara (k20c and k24c), Kahuro (ka14b, ka26c, ka30b) and Kigumo (ko 12c, ko13c, ko16a, ko20a and ko29b) (Figure 4.5).

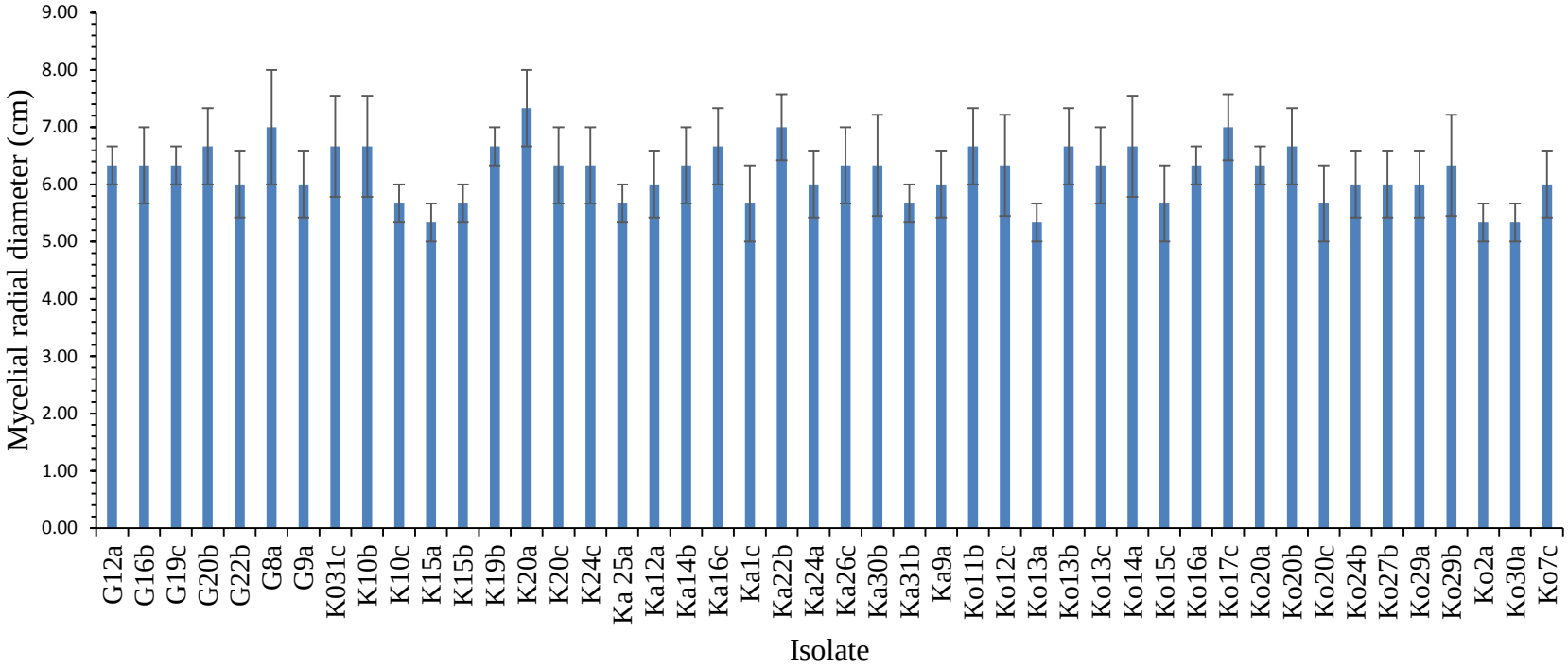


Figure 4.5: Mean lesion diameter (cm) on unripe Hass fruit variety four days after inoculation with *Colletotrichum gloeosporioides* isolates. Error bars represents standard error (SE) of the mean

4.3.4 Lesion diameter on ripe avocado Hass variety, 2 days after inoculation with different *Colletotrichum gloeosporioides* isolates

There was no significant difference ($P \geq 0.05$) in the mean lesion diameter in fruits inoculated with different *C. gloeosporioides* isolates after two days (Figure 4.6). The fruit inoculated with isolate ko29b had the highest lesion mean diameter of 12.33cm while the one inoculated with ko20b had the lowest (Figure 4.6). Isolates k15b and ka16c were associated with the second highest lesion mean diameter of 11.67cm. A total of 5, 6, 7, 11, 4 and 3 isolates were associated with mean lesion diameter of 11.33, 11.00, 10.67, 10.33, 10.00 and 9.67 cm, respectively (Figure 4.6).

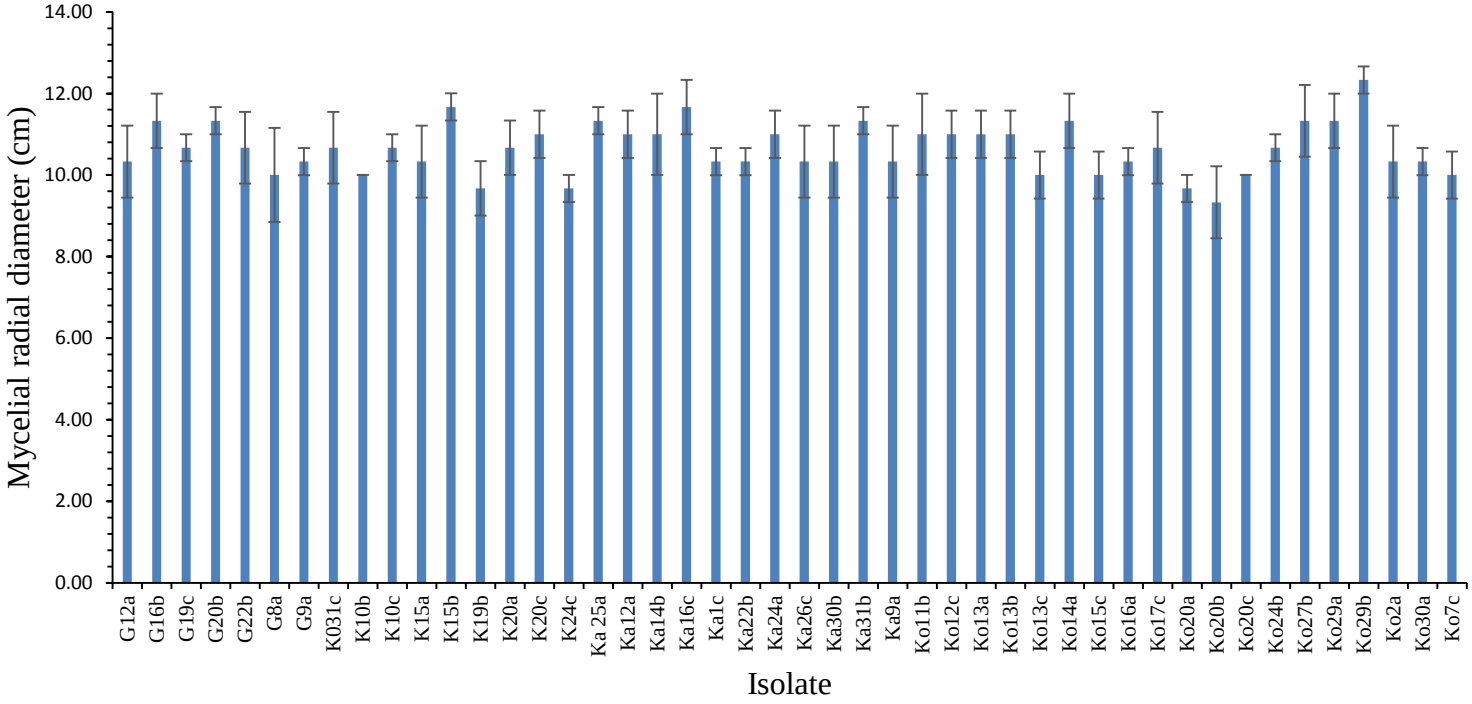


Figure 4.6: Mean lesion diameter (cm) on ripe Hass fruit variety two days after inoculation with *Colletotrichum gloeosporioides* isolates. Error bars represents standard error (SE) of the mean.

4.4 The incidence and latent infection of anthracnose disease in avocado in Murang'a County

4.4.1 Latent infections of anthracnose disease on mature avocado fruits

Healthy fruits from the four different sub-counties were infested by both *C. gloeosporioides* and *P. microspora* as co-infection with an average of 37.8% latent infection (Figure 4.7). However, the latent infection on avocado fruits did not differ significantly (P≥0.05) among the sub-counties (Figure 4.7). The highest latent infection of the fruits was recorded in Gatanga (43.5%), followed by Kigumo (38%) and then Kahuro (36%). Kandara, however recorded the lowest latent infection of 34 percent (Figure 4.7).

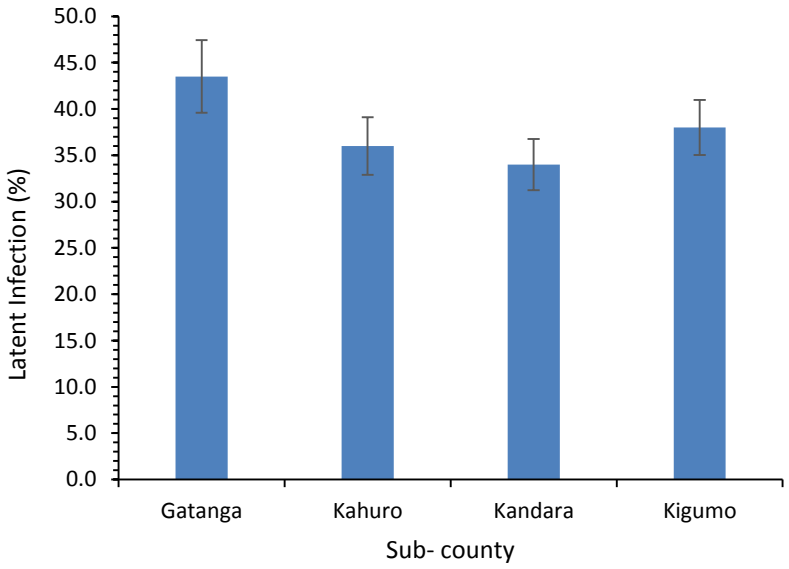


Figure 4.7: Latent infection of anthracnose disease on healthy avocado fruits in four sub -counties of Murang'a County. Error bars represents standard error (SE) of the mean.

4.4.2 Anthracnose disease incidence on avocado fruits in Murang'a County

All the three agro- ecologicalzones recorded incidences of anthracnose disease in the Gatanga, Kigumo, Kandara and Kahuro sub-county (Table 4.26). The disease

incidence was significantly different at P<0.05 between agro-ecological zones in all the sub- counties (Table 4.26). Agro- ecological zone 2 had the highest disease incidence of 25.5% followed by zone 3 with a record of 15.5% and lastly zone 4 with 9.0% in Gatanga sub-County (Table 4.26). A similar trend of the disease incidence was recorded for agro ecological zones 2, 3 and 4 in Kandara, Kigumo and Kahuro sub-counties. Although zone 3 had comparatively higher mean disease incidence than zone 4 in both Kigumo and Kahuro sub-county, they were not significantly different at P≥0.05 in each sub-county (Table 4.26).

Overall, the zones had significantly p<0.05 different mean disease incidence, where zone 2 recorded the highest mean disease incidence of 24.3 % while zone 3 recorded 15.3% and zone 4 had 9.6% which was the lowest mean disease incidence (Table 4.26).

Table 4.26: Mean incidence of anthracnose disease on avocado fruits in Murang'a County

Zone	% Mean disease incidence				
	Gatanga	Kandara	Kigumo	Kahuro	Overall
2	25.5*a	26.5a	23.5a	21.5a	24.3a
3	15.5b	16.0b	15.5b	14.0b	15.3b
4	9.0c	10.0c	10.0b	9.5b	9.6c
LSD	5.36	5.138	5.7	4.775	2.489
P-value	<0.001	<0.001	<0.001	<0.001	<0.001

*Data are means of three replicates. Means on the same column followed by similar letter(s) are not significantly different at P≥ 0.05 according to the Fisher's protected LSD test.

4.5 Molecular identification of *Colletotrichum gloeosporioides, Colletotrichum boninense* and *Pestalotiopsis microspora* isolates of avocado fruit

4.5.1 Amplification of genomic DNA by PCR

The genomic DNA of the fungi amplified through PCR was confirmed by running the PCR products in a gel (Figure 4.8). A single band was obtained after amplification of the genomic DNA, Internal Transcribed spacer (ITS) region using Universal primers (ITS1 & ITS4) for *P. microspora, C. boninense and C. gloeosporioides* and specific primers (CgInt & ITS4) (Figure 4.8) to confirm *C. gloeosporioides.*

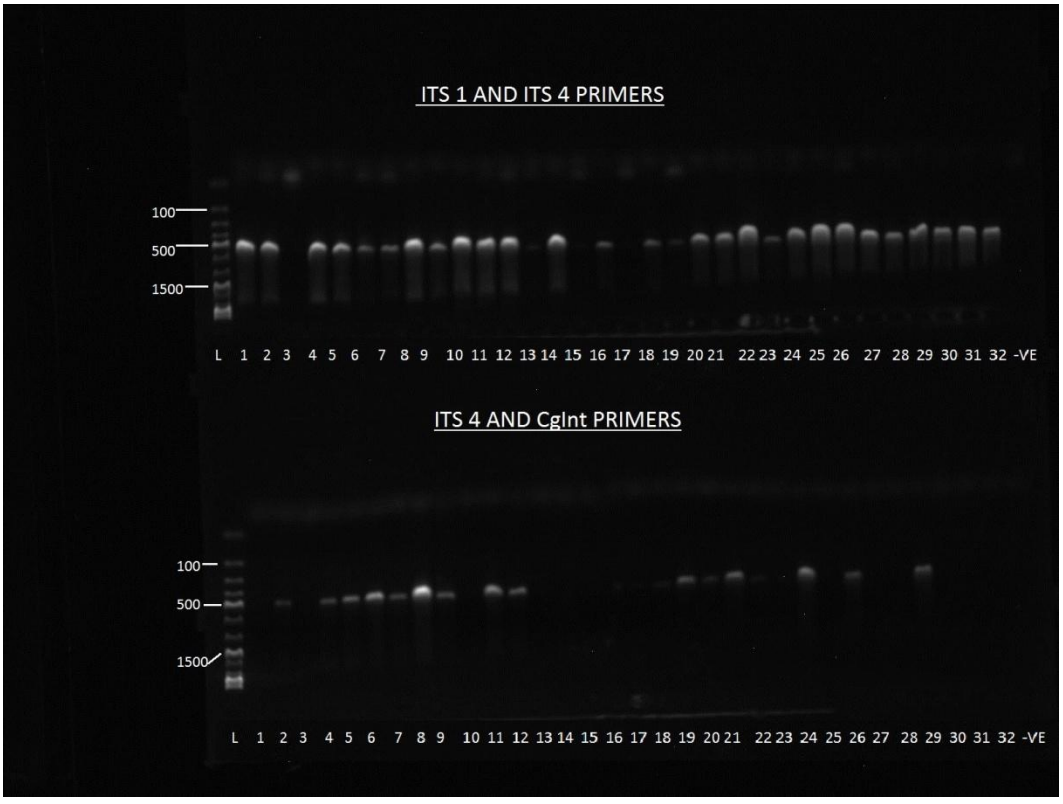


Figure 4.8: Agarose gel amplification of PCR products for ITS region of *C. gloeosporioides, C. boninense* and *P. microspora* isolates. All isolates were amplified by universal primers ITS1 and ITS4 (Upper bands). *C. gloeosporioides* and *C. boninense* (L26) isolates formed bands after amplication with ITS4 and CgInt primers (Lower bands).

4.5.2 Phylogenetic analysis of *Colletotrichum gloeosporioides, Colletotrichum boninense* and *Pestalotiopsis microspora* isolates

Molecular identification of *C. gloeosporioides*, *Colletotrichum boninense* and *P. microspora* was inferred from 12 sequences of *C. gloeosporioides*, 1 sequence of *C. boninense* and 10 sequences *P. microspora* isolates. A phylogenetic tree (Figure 4.9) was made of sequences from C. *gloeosporioides, C. boninense, P. microspora* and reference sequences from the Genebank (Figure 4.9). The identity of the isolates both *Colletotrichum spp* and *Pestalotiopsis* to the GeneBank isolates ranged between 94% and 100%.

The phylogenetic analysis of the isolates and references from the GeneBank resulted into three clades; Clade1; (*Colletotrichum gloeosporioides*), Clade 2; (*Colletotrichum boninense*) and Clade 3; (*Pestalotiopsis microspora*). *Colletotrichum gloeosporioides* clade comprised of *C. gloeosporioides* GeneBank reference (KX437750.1, KX227593.1, KR995714.1, KX197389.1, KU145153.1, KX022503.1, KU662388.1, KF177682.1, KX786433.1, KX262969.1, KX347478.1 and KT342873.1). In this clade, isolates 14a, 39a, 4b, 5b, 6b, 7b, 8b, 9b, 10b, 11b, 12b, 13b clustered together with the above reference sequence from the Genebank with 94% bootrap support.

Colletotrichum boninense clade comprised of *C. boninense* Gene Bank reference numbers (KX 343044.1 and KU356916.1). In this clade one isolate, 26a clustered together with the reference sequence with 98% bootrap value.

Pestalotiopsis microspora clade comprised of GeneBank reference (KT459353.1, DQ0010002.1, DQ000997.1, KT372347.1 and JX436801.1). In this clade, isolate 27a, 30a, 6a, 28a, 2a, 31a, 32a, 4a, 25a and 18a clustered together with the reference sequence with 100% bootrap value.

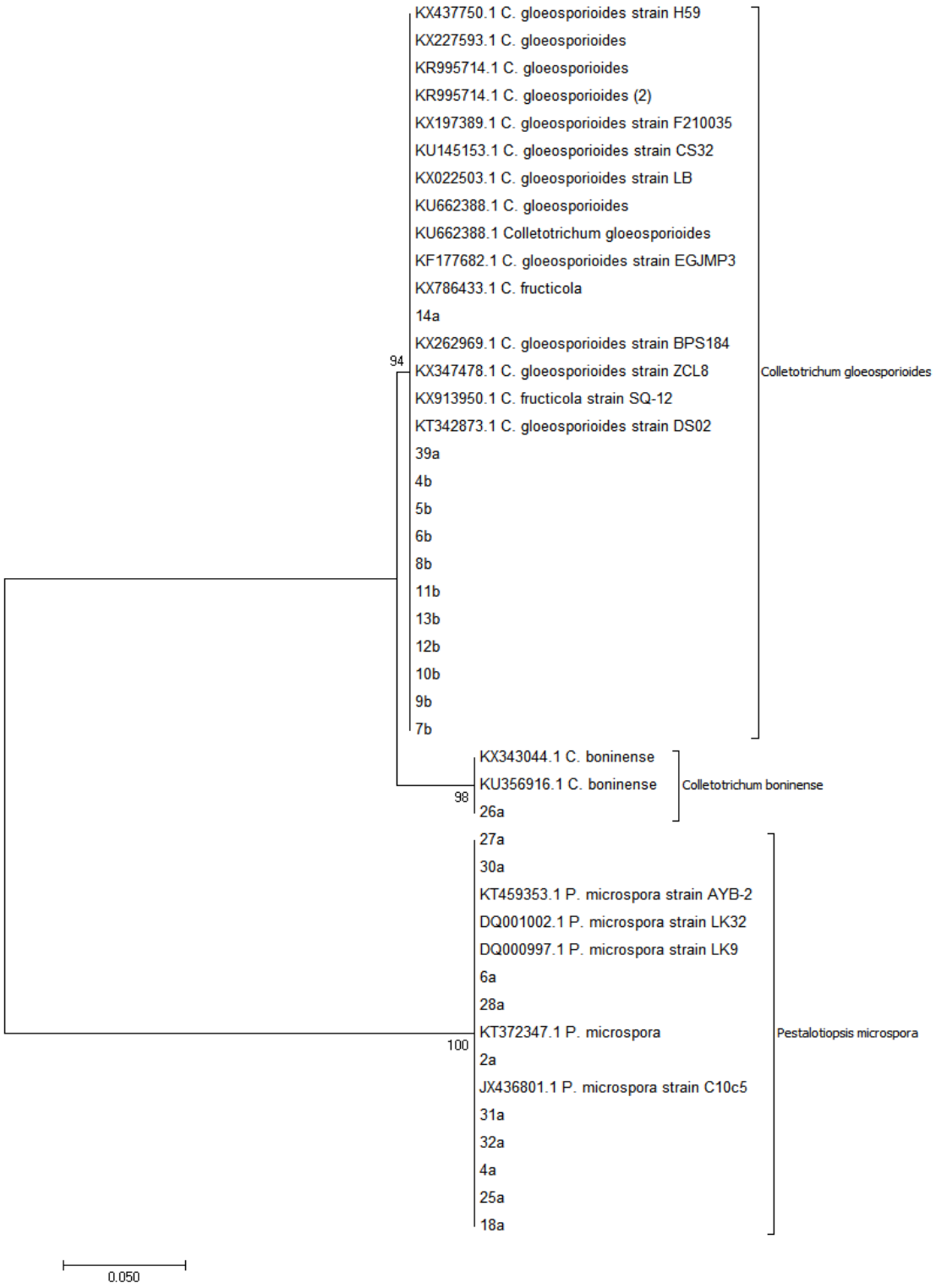


Figure 4.9: Maximum likelihood tree showing the relationship of *Colletotrichum gloeosporioides*, *Colletotrichum boninense* and *Pestalotiopsis microspora* isolates based on ITS region. Sequences 4b, 5b, 6b, 7b, 8b, 10b, 11b, 12b, 13b, 2a, 4a, 6a,

14a, 18a, 25a, 26a, 27a, 28a, 30a, 31a, 32a and 39a were derived from the isolates while the rest were obtained from the NCBI database. The numberss at the nodes refers to the bootrap values. A two set of primers were used ITS1&4 (universal) and ITS4 & CgInt (specific).

4.6. Sensitivity of *C. gloeosporioides* isolates to selected fungicides

4.6.1. Effects of selected fungicides on mycelia growth of *Colletotrichum gloeosporioides*

The mycelial growth of *C. gloeosporioides* isolates was inhibited significantly $P<0.05$ by the three fungicides used, Milraz, Copper oxychloride and Bayleton. The inhibition percentage of the fungicides increased with the increase in the fungicide concentration, for example the concentration of 0.25g/l, 0.50g/l and 1.00g/l of Milraz had mean inhibition percent of 82.1, 84.1 and 85.9, respectively (Table 4.27).

Among the fungicides, Bayleton had comparatively the highest mean inhibition percentage in the range of 84.5 to 87. 6 followed by Milraz in the range of 82.0 to 85.8 and Copper oxychloride lowest in the range 80.8 to 85.1 (Table 4.27). At manufacturer's recommended rates, Bayleton had the highest inhibition percentage of 86.1 while Copper oxychloride had the lowest at 83.3 (Table 4.27).

Table 4.27: Mean inhibition percentage of different concentrations of fungicides on mycelial growth of *Colletotrichum gloeosporioides* isolates

Fungicide	Concentration (g/l)	Overall Mean inhibition (%) on mycelia growth
Milraz	0.25	82.1*c
	0.50 (Recommended)	84.1b
	1.00	85.9a
	LSD	1.5184
	P value	<0.001
Copper Oxychloride	0.88	80.9c
	1.75(Recommended)	83.3b
	3.50	85.1a
	LSD	1.035
	P value	<0.001
Bayleton	0.13	84.5c
	0.25(Recommended)	86.2b
	0.50	87.6a
	LSD	**0.6423**
	Pvalue	<0.001

*Data are means of three replicates. Means on the same column followed by similar letter(s) are not significantly different at P≥ 0.05 according to the Fisher's protected LSD test.

The inhibition trend of fungicide, where the highest concentration resulted to the highest inhibition percentage was observed regardless of the source of the isolate (Figure 4.10). The 1.00g/l of milraz had the highest mean inhibition percent while 0.25g/l had the least. The isolates from Kandara were the most sensitive to Milraz with 86.9, 84.9, and 83.4 mean inhibition percentage at concentration of 1.00g/l, 0.50g/l and 0.25g/l, respectively (Figure 4.10). Isolates from Gatanga however, showed the least sensitivity to Milraz where 1.00g/l, 0.50g/l and 0.25g/l of Milraz recorded 84, 81.8 and 78.9 mean inhibition percentage, respectively (Figure 4.10).

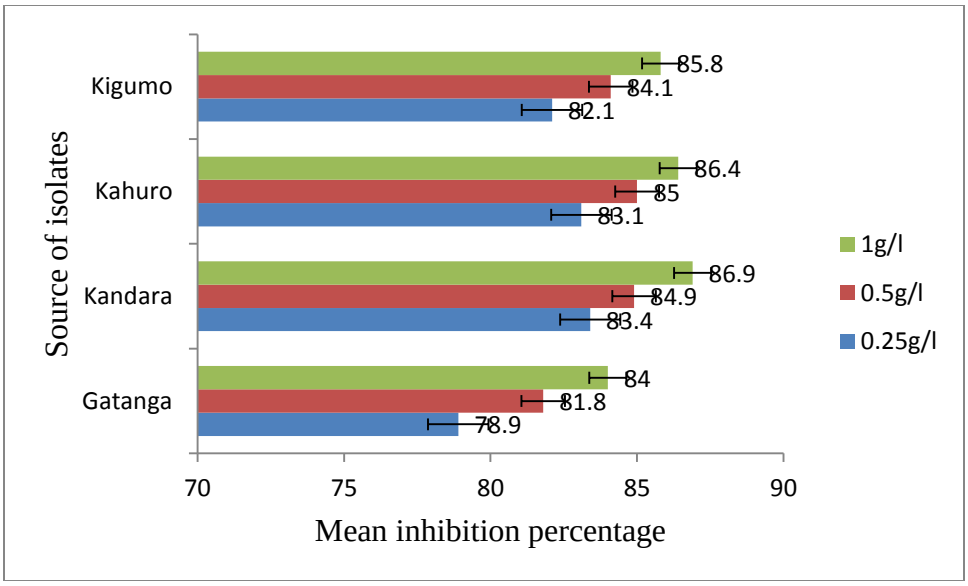


Figure 4.10: Mean inhibition % of different concentrations of milraz on *Colletotrichum gloeosporioides* isolates from Kigumo, Kahuro, Kandara and Gatanga subcounties.

Isolates from Gatanga showed various sensitivity levels to different concentrations of Milraz fungicides (Figure 4.11). Isolate G8a, was the most inhibited by the three different concentrations of Milraz as compared to the rest (Figure 4.11). The isolate 16b however, was the most tolerant to milraz fungicide concentration as the least inhibition percentage was recorded in the three concentrations compared to the other isolates (Figure 4.11). The mean inhibition percent of different concentration was significantly different at $P<0.05$ for isolate G9a and G22b. The rest isolates however, the mean inhibition percent of different concentration per isolate did not differ significantly ($P\geq0.05$).

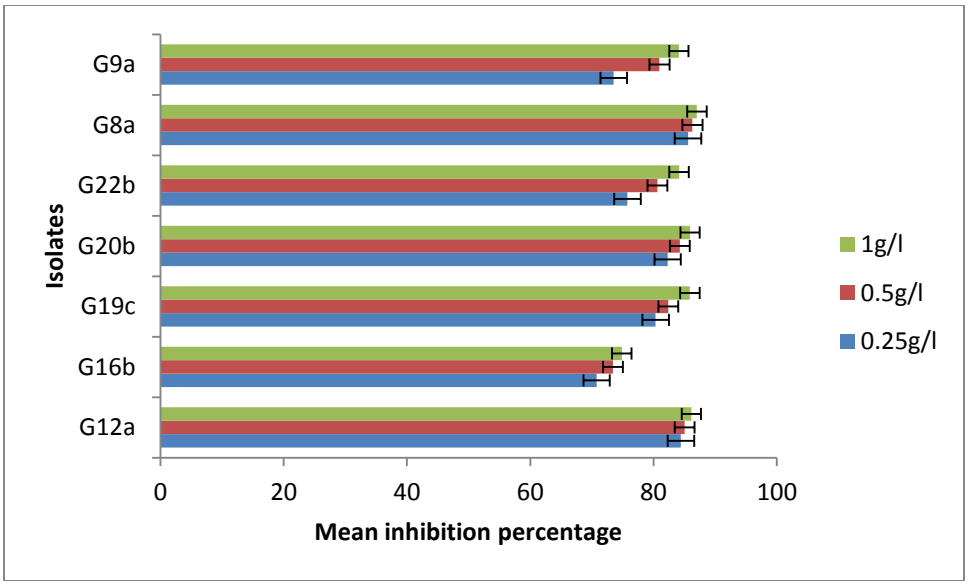


Figure 4.11: Mean inhibition percentage of different concentrations of Milraz on *Colletotrichum gloeosporioides* isolates from Gatanga County.

The inhibition percentage of Copper oxychloride concentrations on *C. gloeosporioides* isolates differed significantly ($P<0.05$) among isolates from Kigumo, Kahuro, Kandara and Gatanga sub – counties (Figure 4.12). Isolates from Gatanga were the least sensitive to different Copper oxychloride concentrations while isolates from Kigumo were the most sensitive (Figure 4.12). The concentration of 3.50g/l of Copper oxychloride had the highest mean inhibition percentage on isolates sourced from different locations, Kigumo, Kahuro, Kandara and Gatanga. These mean inhibition percentages were significantly different ($P<0.05$) from those obtained from 1.75g/l concentration of Copper oxychloride the manufacturer's recommended rate (Figure 4.12).

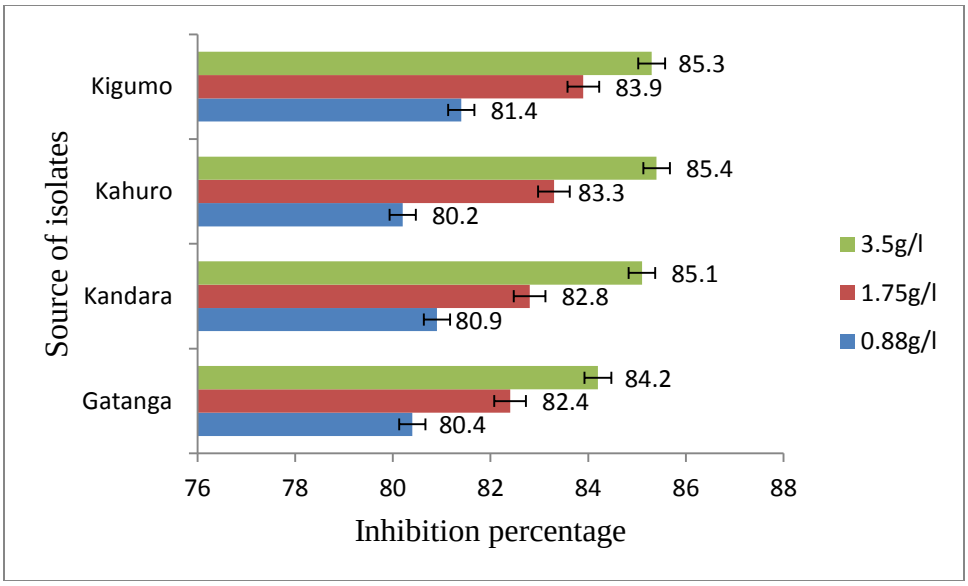


Figure 4.12: Mean inhibition % of different concentrations of Copper oxychloride on *C. gloeosporioides* isolates from Kigumo, Kahuro, Kandara and Gatanga sub-counties.

The different concentrations of Bayleton differed significantly ($P<0.05$) in their levels of inhibiting mycelial growth of different *C. gloeosporioides* isolates sourced from different locations (Figure 4.13). The mean inhibition percentage of the concentration 0.50g/l was significantly the highest among isolates from different locations (Figure 4.13). The mycelial inhibition of the isolates was concentration dependant where the highest concentration of the fungicide (0.50g/l) had the highest mean inhibition percent while the concentration 0.125g/l had the lowest mean inhibition percent for isolates from different locations, Kigumo, Kahuro, Kandara and Gatanga (Figure 4.13). Isolates from Kahuro were least sensitive to the fungicide with mean inhibition percentages of 84.1, 85.8 and 87.3 associated with 0.125, 0.50 and 1.00g/l of Bayleton, respectively (Figure 4.13). However, Bayleton fungicide was most effective on isolates from Gatang'a

where, mean inhibition percentages of 84.8, 86.8 and 87.9 were recorded for the concentration 0.125, 0.50 and 1.00g/l, respectively (Figure 4.13).

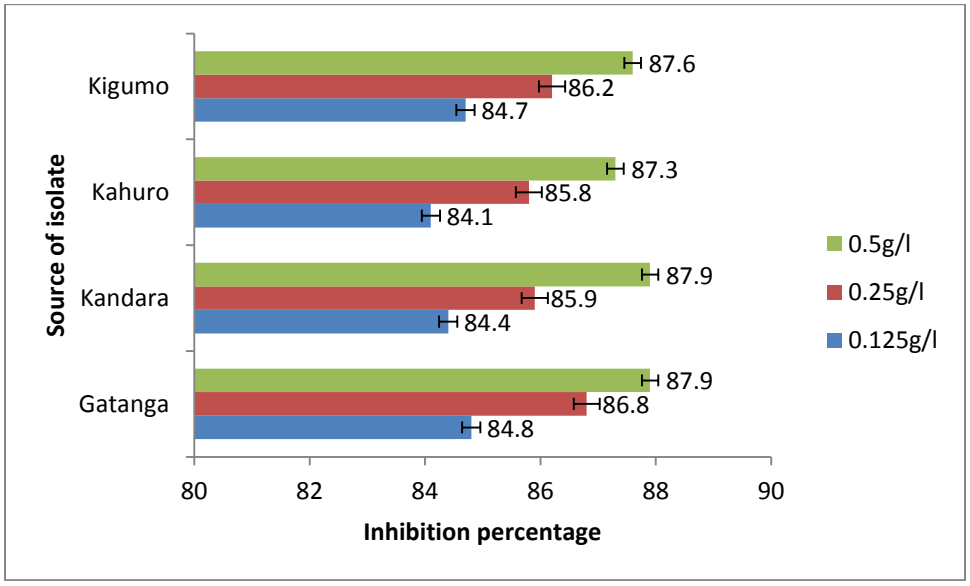


Figure 4.13: Mean mycelial growth inhibition % of different concentrations of Bayleton fungicide on *Colletotrichum gloeosporioides* isolates from Kigumo, Kahuro, Kandara and Gatanga sub-counties.

4.6.2. Effects of selected fungicides on sporulation of *Colletotrichum gloeosporioides* isolates

The mean number of spores recorded from *C. gloeosporioides* isolates treated with different concentrations of fungicides differed significantly ($P<0.05$) (Table 4.28a, b). Isolate Ko2a, had the highest mean number of spores 2.875×10^6 per ml after being treated with Copper oxychloride fungicide while isolate G22b had the lowest mean number of spores, 1×10^6 per ml (Table 4.28). Among isolates treated with Milraz fungicide, isolate G16b had the highest mean number of spores (3.25×10^6/ml while Ko24b had the lowest (1×10^6 spores/ml (Table 4.28a, b). Similarly, Ko20b, had the highest mean number of spores, 4.25×10^6 spores/ml among the isolates treated with Bayleton fungicide. Different isolates

showed varied sensitivity to different fungicides in relation to the mean number of spores produced (Table 4.28a, b). For example, isolate Ko2a produced spores with a mean number of 2.875, 2.0, 3.0 × 10^6/ml when treated with copper oxychloride, Milraz and Bayleton, respectively (Table 4.28a, b). While, isolate k19b produced spores with a mean number of 2.625, 1.625, 2.875 × 10^6/ ml when treated with Copper oxychloride, Milraz and Bayleton, respectively (Table 4.28a, b).

Table 4.28a: Mean number of spores (×10^6/ml from *Colletotrichum gloeosporioides* isolates treated with fungicides

Copper oxychloride		**Milraz**		**Bayleton**	
Isolate	**Mean (×10^6)**	**Isolate**	**Mean(×10^6)**	**Isolate(×10^6)**	**Mean**
ko 2a	2.86* a	G16b	3.25 a	ko20b	4.25 a
k19b	2.63 ab	ka14b	2.50 b	ka30b	3.75 ab
ka1c	2.63 ab	ka30b	2.50 b	ko 12c	3.63 a-c
ko24b	2.63 ab	ko20c	2.50 b	k20c	3.50 a-d
ko31c	2.63 ab	ko30a	2.50 b	ko14a	3.38 b-e
G16b	2.50 a-c	ko20b	2.38 bc	ko29a	3.25 b-f
k24c	2.50 a-c	k15a	2.25 b-d	k10b	3.13 b-g
ka12a	2.50 a-c	G22b	2.13 b-e	ko24b	3.13 b-g
ka30b	2.50 a-c	G8a	2.13 b-e	k20a	3.00 b-h
ko15c	2.38 a-d	ka22b	2.13 b-e	ka25 a	3.00 b-h
G20b	2.25 a-e	ko31c	2.13 b-e	ko 2a	3.00 b-h
k10c	2.25 a-e	G9a	2.00 b-f	ko13b	3.00 b-h
ka24a	2.25 a-e	k20a	2 b-f	ko20c	3.00 b-h
K20c	2.13 b-f	ko 2a	2.00 b-f	k15b	2.88 c-i
ka26 c	2.13 b-f	ko14a	2.00 b-f	k19b	2.88 c-i
ko11b	2.13 b-f	G19c	1.88 c-g	ka14b	2.88 c-i
ko17c	2.13 b-f	G20b	1.88 c-g	Ka 24a	2.88 c-i
G19c	2.00 b-g	k10b	1.88 c-g	ko13a	2.88 c-i
G8a	2.00 b-g	k10c	1.88 c-g	ko16a	2.88 c-i
G9a	2.00 b-g	K20c	1.88 c-g	ko7c	2.88 c-i
ka16 c	2.00 b-g	ka12a	1.88 c-g	k15a	2.75 d-j
ko13b	2.00 b-g	ko11b	1.88 c-g	ko17c	2.75 d-j
k15b	1.88 c-h	ko29b	1.88 c-g	G8a	2.63 e-k
LSD	**0.7251**	LSD	**0.6077**	LSD	**0.7833**
P value	<0.001	P value	<0.001	P value	<0.001

*Data are means of three replicates. Means on the same column followed by similar letter(s) are not significantly different at P≥ 0.05 according to the Fisher's protected LSD test.

Table 4.28b: Mean number of spores (×10^6/ml from *Colletotrichum gloeosporioides* isolates treated with various fungicides

Copper oxychloride		Milraz		Bayleton	
Isolate	**Mean (×10^6)**	**Isolate**	**Mean(×10^6)**	**Isolate(×10^6)**	**Mean**
ko29a	1.88* c-h	G12a	1.75 d-h	ka16 c	2.63 e-k
ko7c	1.88 c-h	Ka 31b	1.75 d-h	ka22b	2.63 e-k
G12a	1.75 d-i	ko16a	1.75 d-h	ko20a	2.63 e-k
k20a	1.75 d-i	ko17c	1.75 d-h	ko30a	2.50 f-l
ka9a	1.75 d-i	ko20a	1.75 d-h	G12a	2.38 g-m
ko13c	1.75 d-i	k19b	1.63 e-i	G16b	2.38 g-m
ko20c	1.75 d-i	ko13b	1.63 e-i	ka1c	2.38 g-m
k10b	1.63 e-j	ko13c	1.63 e-i	ka31b	2.25 h-n
ko13a	1.63 e-j	ko7c	1.63 e-i	ko31c	2.25 h-n
ko14a	1.63 e-j	ka16 c	1.50 f-j	G20b	2.13 i-o
ko27b	1.63 e-j	ka24a	1.50 f-j	G22b	2.13 i-o
ko30a	1.63 e-j	ka25 a	1.50 f-j	ka12a	2.13 i-o
ka25 a	1.50 f-j	ko13a	1.50 f-j	ko13c	2.13 i-o
ko16a	1.50 f-j	ko15c	1.50 f-j	G9a	2.00 j-o
ko20a	1.50 f-j	k15b	1.38 g-j	k10c	2.00 j-o
k15a	1.50 f-j	ka1c	1.38 g-j	ko29b	2.00 j-o
ka14b	1.50 f-j	ko27b	1.38 g-j	k24c	1.88 k-p
ka31b	1.38 g-j	k24c	1.25 h-j	ka9a	1.88 k-p
ko29b	1.38 g-j	ka9a	1.25 h-j	ko15c	1.75 l-p
ko12c	1.25 h-j	ka26 c	1.13 ij	ka26 c	1.63 m-p
ka22b	1.13 ij	ko12c	1.13 ij	ko27b	1.50 n-p
ko20b	1.13 ij	ko29a	1.13 ij	ko11b	1.38 op
G22b	1.00j	ko24b	1.00 j	G19c	1.13 p
LSD	**0.7251**	LSD	**0.6077**	LSD	**0.7833**
P value	<0.001	P value	<0.001	P value	<0.001

*Data are means of three replicates. Means on the same column followed by similar letter(s) are not significantly different at P≥ 0.05 according to the Fisher's protected LSD test.

Overall, the mean number of spores produced by the isolates treated with different fungicides, Milraz, Copper oxychloride and Bayleton differed significantly (P<0.05) from their controls (Table 4.29). *C. gloeosporioides* isolates treated with

different concentrations of Milraz fungicides differed significantly. Those treated with 0.25g/l of Milraz had the highest mean number of spores, 1.902×10^6/ml followed by those treated with 0.50g.l of Milraz with 1.326×10^6/ml while those treated with 1.00g/l of Milraz had the lowest mean number, 0.348×10^6ml (Table 4.29). Comparatively, isolates treated with different concentration of Bayleton produced highest, higher and high number of spores per ml for twice the recommended, recommended and half recommended rate, respectively as compared to when treated with milraz and bayleton (Table 4.29).

Table 4.29: Overall Mean number of spores per ml of 46 *C. gloeosporiodes* isolates

Fungicide	**Concentration (g/l)**	**Mean number of spores ($\times 10^6$)/ml from 10 day old cultures**
Milraz	0.25	1.90* b
	0.50	1.33 c
	1.00	0.35 d
	Control	3.91 a
	LSD	**0.231**
	Pvalue	**<0.001**
Copper Oxychloride	0.88	1.95 b
	1.75	1.22 c
	3.50	0.59d
	Control	3.92a
	LSD	**0.231**
	P value	**<0.001**
Bayleton	0.125	3.01b
	0.25	1.72c
	0.5	0.92d
	Control	3.92a
	LSD	**0.231**
	P value	**<0.001**

*Data are means of three replicates. Means on the same column followed by similar letter(s) are not significantly different at P≥ 0.05 according to the Fisher's protected LSD test.

CHAPTER 5: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Avocado production and management of anthracnose in Murang'a County

The increased avocado production for both commercial and consumption purposes reflects the significance attached to avocado both as a source of income and nutrients (Schafer *et al*., 2013; Menzel and Le Lagadec, 2014; HCDA, 2015). Increased farmers' awareness of the economic and nutritional value of avocado fruit has resulted in cultivation of avocado in every household. This is in agreement with a report in Hortinews (2015) on avocado production in Kenya. Further, avocado production campaigns by the Murang'a County government through distribution of free avocado seedlings to farmers has also resulted in the increment. Both male and female of all age groups are involved in cultivation of the avocado in the region. However, male farmers were more than female farmers probably (Table 4.1) due to the fact that land ownership has mainly been associated with male culturally (Abang *et al*., 2014). This resulted in a significant difference in production by male farmers as compared to female farmers.

Fifty one percent of the farmers in the study area were in the age group of 60 years and above representing most of those who have retired from formal employment. The age group between 30-59 years was relatively few, probably

due to rural –urban migration in search of formal employment and business opportunities in Towns. (Nyamweri and Gorran, 2011).

Farmers who had no formal education and those with basic primary level education were involved in farming throughout the year. The study showed that farmers had challenges in understanding various agronomic, production and pest management practices. Similar observations were reported by Abang *et al.* (2014) when they were assessing vegetable farmers' knowledge of disease and pest management on vegetables. Poor management of diseases and pests in avocado by the farmers may be associated by their failure to read technical information instruction on pest and disease control on pesticides label (Panuwet *et al.,* 2012; Mengistie *et al*., 2015; Nambangia *et al*., 2016).

These farmers have been involved in routine application of fungicides and pesticides to control diseases and pests of avocado, respectively. Mengistie *et al.* (2017) reported similar observation in his study on pesticide use among smallholder vegetable farmers in Ethiopia. Such practices have been associated with increased cost of production and over-use of chemical pesticides and accumulation of pesticide residue which exceed the set maximum residual level at the export market (FVO, 2013; Njombolwana *et al*., 2013, Abang *et al*., 2014).

Although farmers who had secondary and tertiary level of education were aware of the anthracnose disease of avocado they were not able to effectively control the

disease due to lack of fungicides registered to control this disease in Kenya (PCPB 2016). Instead, farmer have been using fungicides registered to control diseases in other crops other than anthracnose in avocado. EFSA (2013) reported similar practice in Europe where farmers were using fungicide guazatine to control sour rot of citrus although not registered in EU.

The source of avocado seedlings varied significantly from nurseries along the roads, from neighbors, KEPHIS certified nurseries to self- propagated. Only about 25% of the farmer sourced avocado seedlings from certified nurseries in the region (Table 4.6). Majority of the farmers therefore started their avocado production with uncertified seedlings. Further, farmers did not apply organic manure/ fertilizer to enhance avocado production. This was attributed to farmers' assumption that avocado tree absorbs nutrients applied to the other crops within the vicinity due to their extensive root system (Schaffer *et al*., 2013). In addition, the number of trees an individual farmer had was limited to about five for most farmers (Table 4.8) due to the size of the farmer's acreage in relation to wide coverage of the avocado tree (Schaffer *et al.,* 2013). Farmers in the study area practiced mixed cropping with crops such as coffee, tea, mangoes, guavas, maize, beans, vegetables and fodder for livestock. This concurs with studies carried out in Kenya by Wasilwa, (2006) revealing mixed cropping system among avocado farmers in Kenya.

Marketing of avocado in the region was mainly through individual farmer selling to brokers and the exporters with the best offer. However, some farmers had formed groups and practised contract farming with the exporters, particularly Kakuzi limited (Table 4.9). Consequently, such farmers were promised of a sustainable market and consistent payment for their produce. Fluctuation in the prizes of the fruit in local and export market however, leads to farmers selling to brokers offering better prices than the contracting farm while on the other side the contracting company collect less produce when the prices are low and also when the supply is so high and demand is low. Due to this unpredictable market trends coupled with improper handling and storage, significant losses were recorded. This is in agreement with findings by Dessalegn, *et al*. (2016) who reported that improper handling, storage and microbial infection were the major cause of post - harvest loss of avocado fruits. Losses in the range of 48.5 to 60% of avocado and other fruits due to post-harvest disease has been documented (Wasilwa, 2006; Dessalegn *et al*., 2016).

A proportion of the avocado farmers in the study area were not able to identify diseases associated with avocado (Table 4.11), probably due to lack of knowledge and visitation by agricultural extension workers (Auwal *et al.*, 2015). However, about 50% of the farmers were able to identify anthracnose due to severe losses of up to 40% during production, harvesting due to quality considerations and after harvest due to rots.

Management of diseases, especially anthracnose was mainly through the use of fungicides such as Bayleton, Milraz and Copper oxychloride as well as cultural practices such as pruning and burying of diseased fruits (Table 4.13). Use of these fungicides was limited due to the fact that they were not registered for use in control of anthracnose of avocado in Kenya. Farmers therefore have been using these fungicides on the basis of their effectiveness in the management of diseases on other crops upon which they are registered for (PCPB, 2016). Use of chemical pesticides to control pests and diseases can be hazardous human, animals and environment due to their toxic inherent nature (Agrios, 2005; Abang *et al*., 2014; Megistie *et al.*, 2017). To ensure safety, efficacy trials for the pesticides are conducted before registration and commercialisation by pesticides regulatory authority in a country, for example PCPB in Kenya (PCPB, 2016). Use of chemical pesticides on other crops other than those in which efficacy trials were done could lead to accumulation of pesticide residue on the produce thereby exposing human and animals to pesticides. Consumer demands for pesticide residue free agricultural produce including avocado fruits has been reported (SNV, 2013).

Pruning of avocado trees by farmers significantly reduced the incidence of anthracnose disease in the field (Table 4.14). This could be attributed to improved aeration in the tree canopies and thereby reduced humidity which favours the establishment and spread of anthracnose disease (Agrios, 2005). Further, fewer fruits drop was observed on avocado trees which were pruned compared to the

unpruned, this could be due to the reduction in overproduction of fruits in relation to the capability of the individual tree.

The harvesting of avocado fruits by farmers involved using of hooks, hand picking, shaking the tree and waiting for the fruits to drop (Table 4.15). A proportion of the farmers used handpicking only. This ensured that no fruit damage occurred during harvesting as a way of reducing post-harvest loss associated to fruit injuries. However, about 42.5 % of farmers used both handpicking technique and hooks to harvest the avocado fruits because the avocado trees were overgrown, tall and had widely spread branches. The farmers used hooks because they could not reach out to the fruits using hands.

A significantly small number of farmers, 0.8% shook the tree for fruits to drop as a method of harvesting. This method led to a lot of fruit injuries and total loss of the fruits due to post -harvest diseases when stored. This is in agreement with findings by Omolo *et al.* 2011 and Dessalegn *et al.* (2016) where 100% avocado fruit rot on storage was recorded on avocado fruits harvested by tree shaking method. Further, total losses of avocado fruit was recorded by some farmers (4.2%) who waited for the fruits to drop after maturity as a method of harvesting. Such fruits were injured and infected by microorganisms causing fruit rot while in the farm. This concurs with Mezgebe *et al.* (2016) findings on post -harvest losses of perishable fruits during harvesting, handling and storage in Ethiopia. Such diseased fruits in the farm might act as the source of inoculum for new infection (Agrios, 2005).

Some farmers, 57.7% determined the maturity of avocado fruit by change of colour from light green to dark green. The rest of the farmers used taste, fruit drop and advice from the buyers (brokers) (Table 4.16). The farmers carried their avocado fruits using sacks, buckets and open pick up to the markets. Similarly, Seid *et al.* (2013) reported use of sack in avocado fruit transport and packaging industry in Ethiopia. Mechanical damage during loading, unloading and the exposure of the avocado fruits to high temperature in the open pick during transport led to physiological changes in the fruit in favour of post-harvest diseases (Dessalegn *et al*., 2016).

Majority of farmers, 87.5% interviewed expressed lack of awareness on the effect of pesticides to the environment and other non- target organisms (Table 4.18). This was in harmony with observation made by Teklu *et al.* (2015) and Megistie *et al.* (2017) in Ethiopia where all farmers interviewed lacked knowledge on the effects of pesticide on human and environment. However, 12.5% of farmers associated the reduction of useful insects such as bees, butterflies, and earthworms on pesticides effect. Nevertheless, 8.3% of farmers aware of the negative effects of pesticides to the environment considered pesticide safe when used as per manufacturer's label instructions.

This study noted that avocado cultivation was practiced in all agro-ecological zones in Murang'a County similar to earlier report by Cooper *et al.* (2003).

Avocadoes were grown in a mixed cropping system with coffee, tea, banana, mangoes, guavas, citrus, maize and beans. Similarly, Tripathi and Karunakaran (2013) in their study in India reported mixed cropping system of avocado and coffee. Fuerte variety, however, was the most common in all the zones as earlier reported by Wasilwa, (2006). Hass variety, the customers' preferred variety at the export market has been gaining popularity among farmers in the study area thereby gradually replacing Fuerte variety. Further, the County government also has been promoting Hass variety through the Murang'a County initiative program of promoting avocado production in the region.

Avocado production by the farmers showed that Gatanga sub-county recorded the highest yields while Kahuro had the lowest (Table 4.19). This could be attributed to presence of well-organized avocado farmers group in Gatanga sub-county who have embraced avocado farming as business as compared to Kahuro where majority of farmers do not attach a lot of economic value to the avocado crop. Further, the proximity of farmers in Gatanga to major urban markets and Jomo Kenyatta International Airport (JKIA), exit port for export market could be another reason for higher avocado production.

5.1.2 Cultural and morphological characteristics of *Colletotrichum gloeosporioides* and *P. microspora* isolates

The 80 fungal isolates from diseased avocado fruits showing symptoms of anthracnose collected from the study area varied in their cultural characteristics

on PDA media in terms of appearance and colour. A total of 46 isolates had whitish, greyish or creamish colour and cottony, velvety mycelium on the top side and greyish cream with circular orange-pinkish colour on the reverse side (Table 4.20). These cultural characteristics were similar to those of *Colletotrichum gloeosporioides* isolates observed by Maziah and Bailey (2000) in avocado fruits. Pallem *et al.* (2012), reported the wide cultural variations among *C. gloeosporioides* isolates. The mycelial growth however, had uniform radial growth characterised by circular ring-like patterns common to *C. gloeosporiodes.* Mila *et al.* (2012) reported similar mycelial growth characteristic of *C. gloeosporioides* in vitro. The cultures produced spores which were straight with rounded end, ranging within 3.0-5.0 micron in width and 10.3 – 18.2 micron in length characteristic of *C. gloeosporiodes* as also reported by Pallem *et al.* (2012).

The remaining 34 isolates had whitish cream mycelia with black fruiting structures, arcevuli on the upper side of the culture and while on the lower side they were light orange to orange in colour. These isolates produced spores having 3-4 septa and 2 or 3 appendages which are characteristics of *P. microspora* as reported by Eman, (2015). Molecular characteristics of these isolates using universal primers ITS1 and ITS4 further confirmed the species to be *P. microspora.*

The isolates were further confirmed through Koch's postulate where the pure cultures of the isolates growing on PDA were used to inoculate healthy ripe avocado fruits, Fuerte variety. After two days, characteristic black spots were

formed on the inoculated fruits. The fungus re-isolated from the inoculated fruits had similar cultural, morphological and spore characteristics as initial isolates as reported by (Misale *et al*., 2016). *Colletotrichum gloeosporioides* and *P. microspora* were isolated from the same lesion from all the diseased fruit showing some levels of co-infection. However, *C. boninense* could not be differented from *C. gloeosporioides* by cultural and morphological characteristic but through molecular characterisation.

All the *C. gloeosporioides* isolates from different locations Gatanga, Kigumo, Kandara and Kahuro sub-counties grew rapidly on the PDA medium covering the whole surface of the petridish in 10 days after inoculation. The mycelial colour of the isolates observed was predominantly whitish grey compared to white cream and grey pink on the upper side of the culture (Table 4.20). Creamish-grey however, was the most common colour on the lower side of the culture among isolates. Culturally, cottony mycelia were the most recorded in 30 isolates as compared to velvety recorded in 15 isolates on the upper side (Table 4.20). There was significant difference (χ 2 =23.455, df=2, $p<0.001$) among the observed and expected frequencies for the colour of various *Colletotrichum* isolates (Table 4.20). The differences observed in cultural and morphological features of the fungus could be associated to their genetic variations and different conditions of growth in terms of temperature, light regime and repeated sub culturing (Johnston, 2000; Vidyalakshini and Divya, 2013).

The mycelial growth rate differed significantly among the isolates from different location in the study area. However, they exhibited similar trend in growth with day two having the least and day 10 having the largest diameter per isolate (Table 4.21). The diameter of all the isolates ranged from 0.3.0- 0.93 cm and 2.37 - 4.5cm in day 2 and day 10, respectively (Table 4.21). Such growth rate among *C. gloeosporioides* isolates was also reported by Maziah and Bailey, (2000). The daily mycelial radial growth ranged from 2.07mm/day to 3.57mm/day. The proportion in percentage (%) of the variance in mycelia radial diameter that was predictable from days for the isolates varied from the lowest (R^2=0.812) for isolate ko7c to highest (R^2 =0.993) for isolate ka9a.

In this study, *Colletotrichum gloeosporiodes* isolates exhibited a wide range of the mean number of spores per isolate from the lowest 0.67×10^6 to the highest 9.00×10^6 spores (Table 4.23). Similar sporulation observation has been made by Maziah and Bailey, (2000). The spores observed in this study were cylindrical, straight with smooth round end. Similar spores of *C. gloeosporioides* were observed by Maziah and Bailey, (2000) and Pallem *et al.* (2012). The spore size varied significantly at $P<0.05$ among the isolates ranging from 3.0-5.0 micron in width and 10.3 – 18.2 micron in length (Table 4.24).

5.1.3 Pathogenicity of *Colletotrichum gloeosporioides* isolates on avocado fruits- Hass and Fuerte varieties.

Pathogenicity test were conducted on two varieties Hass and Fuerte due to their popularity both at farm level and at the export markets. In this study, all *C. gloeosporioides* isolates from different locations caused lesion formation on both Hass and Fuerte fruit varieties after inoculation. This demonstrated the susceptibility of the two varieties to *C. gloeosporioides* as reported by Bruce *et al*. (2013). The size of the lesion formed on unripe mature avocado fruits, both Fuerte and Hass varieties four days after inoculation did not differ significantly at $P \geq 0.05$ (Figure 4.4 and 4.5) in their diameter. Some isolates such as k24c, ka25a and ka1c caused the highest mean lesion diameter of about 10cm on unripe Fuerte variety, four days after inoculation while isolate k20 caused the highest mean lesion diameter of 7 cm in Hass variety (Figure 4.4 and 4.5). This could partly be associated due to the aggressiveness of different isolates of *C. gloeosporioides* (Giblin *et al*., 2010). Another possible reason is the presence of epicatechin found in the thicker peel of unripe Hass variety which is known to affect the activity of endopolygalacturonase enzyme of *C. gloeosporiodes* (Prusky *et al*., 1993).

However, the lesions formed on ripe avocado of both Fuerte and Hass varieties differed significantly $P < 0.005$ in their diameter two days after inoculation (Figure 3). The isolates showed varied levels of aggressiveness in lesion formation in both unripe and ripe fruits (Figure 4.4 and 4.5). Giblin *et al*. (2010) made similar observation after inoculating unripe and ripe avocado with different isolates of *C.*

gloeosporioides. The lesions formed on ripe avocado fruits of Fuerte and Hass varieties were comparatively larger than the lesions formed on the unripe fruits two and four days after inoculation, respectively. This could be associated to physiological changes in the avocado fruit during ripening where ripening ethylene chemicals increases while antifungal compounds such as diene diminishes (Prusky, 1982; Giblin *et al*., 2010) favouring the growth of *C. gloeosporioides*.

5.1.4. Anthracnose disease incidence and latent infection on avocado fruits.

Anthracnose disease caused a lot of damage to the avocado fruits in all the farms sampled in different agro ecological zones in this study. Generally, the zones had significantly $P<0.05$ different mean disease incidence, where zone 2 recorded the highest mean disease incidence of 24.25 % while zone 3 and 4 recorded 15.25% and 9.63%, respectively (Table 4.26). The difference in disease incidence between zone 2 and both zones 3 and 4, respectively could be attributed to the wet and moist environmental conditions in zone 2. Such conditions are favourable for germination, spread and proliferation of *C. gloeosporioides* and *P. microspora* and athracnose disease development (Prusky, 1994; Agrios, 2005; Ajaykumar, 2014). The agro ecological zone 4 had the least mean disease incidence of 9.63% which can be attributed to relatively high temperature and low humidity which have been reported to be unfavourable to both fungi which were associated with co-infection in avocado and disease development (Willis and Mavuso, 2009).

Failure by farmers to prune the avocado tree in Murang'a County might have provided a suitable environment for spread of the causal agent to healthy fruits through rain splash. Thus the main sources of fungal inoculum are diseased mummified fruits, flower bracts, dead wood, leaves entangled in the tree canopy (Dodd *et al.,* 1992; Farr *et al*., 2006). Further, lack of proper field sanitation through removal of fallen rotten fruits and poor post harvesting handling of fruits by farmers in the same County might have contributed to spread of the disease in all the agro ecological zones (Agrios, 2005). Growing of other fruit crops susceptible to *C. gloeosporioides* such as mangoes, guava, pawpaw, passion fruits and citrus in the study area might have acted as reservoir for the causal agent when the avocado fruits are not in season. (Arnaud, 2012).

Healthy fruits from the four different sub- counties in Murang'a County were infested by *C. gloeosporioides* with an average of 37.8% latent infection (Figure 4.7). However, the latent infection did not differ significantly at $P \geq 0.05$) between the sub-counties (Figure 4.7). This latent infection could be partly explained by the mode of infection of the fungus where after penetrating healthy unripe fruit in the field, the fungus may remain quiescent until ripening starts when it becomes active and cause lesion formation (Jeffries *et al.,* 1990; Agrios, 2005; Giblin and Coates, 2007). Four mechanisms have been proposed to explain the resistance of unripe avocado fruit to the fungal attack. Such included; (i) lack of nutritional requirements for the pathogen, (ii) presence of preformed antifungal compounds, (iii) presence of inducible antifungal compounds, and (iv) lack of activation of

fungal pathogenicity factors (Beno-Moualem and Prusky, 2000). However, during ripening the breakdown of antifungal compounds such as dienes resulted to resumption of fungal growth and disease development forming lesions (Bailey and Jeger, 1992; Coates *et al*., 1993; Latunde-Dada *et al*., 1996; Perfect *et al.*, 1999; Mendgen and Hahn, 2001).

The highest latent infection was recorded in Gatanga (43.5%), followed by Kigumo (38%) and Kahuro (36%). Kandara, however recorded the lowest latent infection of 34 percent (Figure 4.7). This could be associated with high avocado population in Gatanga sub-county as compared to the rest.

5.1.5 Phylogenetic studies of *Colletotrichum gloeosporioides* and *Pestalotiopsis microspora*

Phylogenetic results showed that ribosomal internal transcribed spacers (ITS) DNA can be used to infer the relationships within *Colletotrichum* and *Pestalotiopsis* fungal species. *C. gloeosporioides* and *C. boninense* isolates were identified by use of universal primers for fungi (ITS1 & ITS4) and further confirmed through the use of specific primer (ITS4 & CgInt). The study identified *C. gloeosporioides* and *C. boninense* based on 13 sequence of *Colletotrichum* isolates using ITS4 (universal) and CgInt (*C. gloeosporioides* specific) primers yielding single band of 500bp and 100% homology with nucleotide sequence of ITS region of DNA with *C.gloeosporioides* isolates in the genebank. This is in agreement with the nucleotide sequence of ITS region of ribosomal DNA of *C.*

gloeosporiodes isolates from orchids amplified using specific primer CgInt and ITS4 primer as reported by Pallem *et al.* (2012). *Pestalotiopsis microspora* was based on 10 sequences of *Pestalotiopsis* isolate using universal ITS1 and ITS4 primers for DNA sequencing.

Twelve isolates of *C. gloeosporioides*, one isolate of *C. boninense* and thirteen isolates of *P. microspora* from the study area gave identical sequences to published sequences already deposited at Genbank with 94%, 98% and 100% bootrap values respectively (Figure 4.9). *Colletotrichum. gloeosporioides* identified in this study has been reported as the most common and wide spread pathogen in avocado growing region worldwide (Amusa *et al*., 2005; Masyahit *et al*., 2009; Owolade *et al*., 2009; Erpelding, 2010). Further it has been associated with infection of other hosts such as almond, coffee, guava, apple, dragon fruit, cassava, mango, sorghum and strawberry (Agrios, 2005; Erpelding, 2010; Dean *et al*., 2012). In Kenya, It has been associated with losses of up to 60% of avocado fruits for export markets. (Chege, 2006).

Colletotrichum boninense was identified, though with low occurrence/ incidence in the study area. Its sequence was identical to published sequence (KX 343044.1 and KU356916.1) in the Genebank. In addition to *C. boninense* related species like *C. acutatum*, *C. godetiae*, *C. fioriniae, C. aenigma* and *C. gigasporum* have also been reported to cause anthracnose of avocado (Ploetz, 2003; Giblin *et al*., 2010; Silva-Rojers and Avila, 2011; Hunupolagama *et al*.,2015; Herna´ndez-Lauzardo *et al*., 2015; Velázquez-del Valle *et al*., 2016).

The ten isolates of *P. microspora* identified in this study had 100% identity with the published sequences in Genbank hence confirming their identity (Figure 4.9). *Pestalotiopsis* species are wide spread both in tropical and subtropical regions (Ding *et al*., 2009 and Liu *et al*., 2009). Individual species of *Pestalotiopsis* are known to cause infection on a wide range of hosts for instance *Pestalotiopsis clavispora* is known to cause stem end rot of avocado (Valencia *et al*., 2011). *Pestalotiopsis versicola* has been reported as a causal agent of anthracnose in avocado (Darvas and Kotze, 1987). *Pestalotiopsis palmarum* causes leaf spot and fruit canker in avocado (Kamhawy *et al*., 2011). While *P. microspora* is prevalent in both tropics and sub tropics its effects on host plants is not well understood (Keith *et al*., 2006). It has been regarded both as an endophyte and as a pathogen causing post -harvest diseases (Metz *et al*., 2000). It has also been reported to cause scab disease of guava fruits in Hawaii (Keith *et al*., 2006). In this study, the fungus was isolated from diseased avocado fruits showing symptoms associated with anthracnose disease of avocado. Despite the prevalence of *P. microspora* on diseased plants it remained a poorly profiled plant pathogen (Metze *et al*., 2000).

5.1.6 Sensitivity of fungal isolates to selected fungicides, Bayleton, Milraz and Copper oxychloride

The mycelial growth of *C. gloeosporioides* isolates was inhibited significantly $P<0.05$ by the three fungicides used, Milraz (propineb 70% and cymoxaxil 6%), Copper oxychloride and Bayleton (Triadimefon 250g/kg) as compared to the

control (without fungicide). Sander *et al*. (2000) observed similar results where most of the *C. gloeosporioides* isolates obtained from diseased avocado fruit were highly sensitive to prochloraz and thiabendazole, with no growth being observed at all. Mycelial inhibition percentage of the fungicide increased with increase in the fungicide concentration, for example the concentration of 0.25g/l, 0.5g/l and 1g/l of Milraz had mean inhibition percent of 82.1, 84.1 and 85.9, respectively (Table 4.27). Similar observation were made by Sander *et al.* (2000) when they subjected *C. gloeosporiodes* isolates to various concentrations of benomyl fungicide *in vitro*.

Bayleton fungicide had comparatively the highest mean inhibition percentage in the range of 84.5 to 87.6 followed by Milraz in the range of 82.0 to 85.8 and Copper oxychloride lowest in the range 80.8 to 85.1 (Table 4.27). At manufacturer's recommended rates, Bayleton had the highest inhibition percentage of 86.1 while Copper oxychloride had the lowest at 83.3 (Table 4.27). The sensitivity of isolates to each of the fungicide tested showed some variation (Table 4.27), this could be due to differences among *C. gloeosporiodes* isolates in relation to their physiology and genetic composition. (Sreenivasaprasad *et al*., 1993; Freeman *et al*., 1998).

Generally, these fungicides inhibited the mycelial growth of the *C. gloeosporioides* isolates from different locations in the study area. The fungicides probably acted as a sterol inhibitor impeding the ergesterol (fatty acid) synthesis, which is an important component of the fungal cell wall (Malick *et al*., 2014). The

inhibition results in this study were in agreement with observation by Iqbal *et al*. (2010) where fungicides Benlate and Carbendazim inhibited the mycelial growth of fungus *Fusarium mangiferae* 16 days of inoculation. Inhibition of mycelial growth could further be explained by the multisite activity of the fungicide such as Milraz (Propineb and cymoxaxil) which inhibits synthesis of nucleic acids, amino acids and other cellular processes (Joshi, 2003). Such modes of action lead to inhibition of β- tubulin biosynthesis (Davidse, 1973).

However, development of resistance by the fungus to benomyl fungicide has been reported. This has been attributed to mutations in the β-tubulin gene associated to specific amino acid substitutions within the β- tubulin molecule (Fujimura *et al*., 1992). Though farmers have been using these fungicides on avocado, they are not registered as pre-harvest chemical treatments for anthracnose on avocado fruits in Kenya. Similarly, farmers have been using benomyl for the management of anthracnose of avocado in South Africa though registered for pre-harvest *Cercospora* spot control of avocado (Nel *et al*., 1999). Use of fungicides whose efficacy has not been tested might lead to lower dosage which favours the development of resistant strains of the causal agent (Agrios, 2005).

The mean number of spores recorded from *C. gloeosporioides* isolates in this study treated with different concentrations of fungicides differed significantly ($P<0.05$) (Table 4.28). Different isolates showed varied sensitivity to different fungicides in relation to the mean number of spores produced (Table 4.28).

Generally, the mean number of spores produced by the isolates treated with Milraz, Copper oxychloride and Bayleton differed significantly ($P<0.05$) from their controls (Table 4.29). Comparatively, isolates treated with different concentration of Bayleton produced highest, higher and high number of spores per ml for twice the recommended, recommended and half recommended rate, respectively as compared to when treated with milraz and copper oxychloride (Table 4.29).

Although these fungicides were effective in inhibition of mycelial growth and sporulation of *C. gloeosporioides*, there is need to evaluate the reaction of different strains to treatment with fungicides from possible isolate resistance. Resistance of several species of genus, *Colletotrichum* has been reported. Xu *et al*. (2014) reported some *C. gloeosporoides* isolates which were tolerant to fungicide containing tebuconazole and prochloraz and Zhang *et al*. (2013) isolates resistant to carbendazim. Some isolates of *C. cereale* were resistant to thiophanate-methyl (Young *et al*., 2010) and benzimidazole (Wong *et al*., 2008). Further, the fungus has a wide host range which ensures re-infection after application of control strategy like use of chemicals (Abang *et al*., 2002; Sideney and Dirlane, 2014).

5.2 Conclusions

1. Most household in Murang'a County has avocado trees in its farm. Avocado fruits produced are consumed as a source of nutrients and sold as source of income both at local market.
2. Production of avocado in the County is mainly by unskilled farmers who lacked knowledge and awareness on proper crop husbandry.
3. Most farmers did not use any strategy to manage anthracnose of avocado. However, few farmers used fungicides such as Bayleton, Milraz and copper oxychloride to control anthracnose in avocado though not registered for use in avocado by the pesticide regulatory authority in Kenya, PCPB.
4. Cultivation of avocado trees occurs in all the agro-ecological zones in the County. Some farmers were uprooting coffee in favour of avocadoes due to its economic returns. Avocadoes were grown in a mixed cropping system with mangoes, guavas, citrus and pawpaw.
5. Anthracnose disease caused by co-infection of *C. gloeosporiodes* and *P. microspora* in avocado was noted in all the study area.
6. There was higher incidence of anthracnose in agro-ecological zone 2 as compared to zones 3 and 4.
7. Avocado varieties, Hass and Fuerte grown in Murang'a County were affected by anthracnose disease.
8. All *Colletotrichum gloeosporioides* isolates were pathogenic to both Fuerte and Hass varieties.

9. *Colletotrichum gloeosporiodes, Colletotrichum boninense and Pestalotiopsis microspora* were identified through molecular technique as the causal agents of anthracnose of avocado in Kenya for the first time.
10. *Pestalotiopsis microspora* was identified as a causal agent of anthracnose of avocado in Kenya for the first time.
11. *C. gloeosporioides* isolates were inhibited by fungicides Bayleton, Milraz and Copper oxychloride in vitro. Bayleton was the most effective in inhibiting mycelial growth of *C. gloeosporioides* in vitro as compared to the rest.

5.3 Recommendations

1. Farmers should be sensitized on the application of disease management strategies during cultivation of avocado crop to increase the yield and quality of the avocado fruits.
2. Farmers should be trained on proper handling of avocado fruits during harvesting, transport and storage of avocado fruits to reduce post- harvest losses caused by damages/ injury, infection associated improper handling.
3. Mode of infection of *Pestalotiopsis microspora* which was isolated from diseased avocado fruits for the first time in Kenya and its co-infection with *C. gloeosporioides* should be studied.
4. More trials for the fungicides Bayleton, Milraz and copper oxychloride under field conditions on the control of anthracnose in avocado are recommended.

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APPENDICES

Appendix 1: Map of Murang'a County showing various Agro-Ecological Zones

Appendix 2: Questionnaire for collecting data on avocado production and disease management practices in Murang'a County

Name of the farmer. : …… Date: ……

Sheet no: ……

Investigator code: …… Location: …

RESPONDENT IDENTITY

1.1. Age /__/__/ 1.2. Sex M /__/ F /__/ 1.3. Occupation: ……

1.4. Level of education: None /__/ Primary /__/ Secondary /__/ Tertiary

1.5. Literacy language: English /__/ Local language /__/

1.6. Are you doing contract farming? Yes ( ) No ( ) If yes which company?..

2. Avocado varieties, source of seedlings and acreage

2.1. Which avocado varieties do you grow? .....

2.2 Where do you source your seedlings from? .....

2.3 Do you apply fertilizer to your crop? Yes ( ) No ( ), At what growth stage do you apply your fertilizer? At Planting ( ), Flowering ( ), Fruit setting ( ), Harvesting ( )

2.4 What is the acreage of the farm under avocado cultivation/ no. of trees? .....

2.5 a) What is your total avocado production? …….

b) Approximately what Percentage of your total production goes to waste during?

i) Harvesting ……

ii) Storage…….

iii) Transportation…….

iv) Grading stage for export ( ), At the local market ( )

2.6 Where do you sell your avocado fruits? Local Mkt ( ), Export ( ), Broker ( ) other ( )

3 Fungicide used to manage avocado diseases

3.1. Who identify the diseases and pest affecting your crop? Self ( ), Extension officer ( ) Family member ( ) other ( )

3.2. Which are the major diseases and which pesticides do you use? (Please specify names, target disease and their quantities)

Name of pesticide: ***Disease:*** ***quantities***

3.3. If the farmer does not know product names, ask her/him why? .....

3.4. How do you acquire pesticide products you are using?

At the local market /__/ at a licensed retailer /__/ Neighbour---/, Exporter (_)

3.5. Do you spray the pesticides? Yes ( ) No ( ). If not who spray your crop?

a) If yes do you think you incur risks of being exposed to those pesticides?

Yes /__/ No /__/ and if yes, which risks……..

3.6. Do you have spray programs? Yes /__/ No /__/

If yes, do you spray during harvesting season? Yes ( ) No ( ):

And which chemicals do you use? .....

Do you apply chemicals during storage and transport? Yes ( ) No ( ) If yes, which chemicals ……

3.7. If there are unused products left, what do you do with them? ……

3.8. Are you trained on safe use and handling of pesticides? ……. By who and when? ……

3.9. What other disease and pest management practices do you practice other than use of fungicides? …….

4. Harvesting and storage of avocado fruits

4.1. How do you harvest your avocado fruits? .....……

4.2 How do you determine the maturity of the avocado fruits before harvest?

4.2.1 Where do you put your fruits after harvest? Crates /__/ Bucket /__/ Sack / /

4.2.2. Do you treat your avocado fruits before storage? Yes ( ) No ( ). If yes what do you use? .....

4.3. Where do you store your harvested avocado before sale? ……

4.4. What do you do with the diseased fruits before and during storage?

Leave/throw them in farm ( ), Feed them to livestock ( ), bury them ( )

Dispose them along the road ( ) Sell at lower cost ( ) any other ( ).

4.5. Approximately what quantities of the fruits are lost due to decay? ½ ( ) ¼ ( )

1/5 ( ) others ( )

5. Perception of environmental risks

5.1. Is there any water source (well, stream, river, forage,) in the vicinity or in your fields? Yes /__/ No /__/

5.1.1. If yes, specify ……

5.1.2. What is the distance between the water source and the area you are treating?

5.1.3. What is the water source used for? ……..

5.2. Have you noticed the death or disappearance of some insects or animals since you have been using the chemicals? Yes /__/ No /__/

5.2.1. If yes, which ones? .....

5.3. Do you think that those products pose a risk to the environment? Yes /__/ No

5.3.1. If yes, why? ……

5.3.2. If not, why? …….

6. Suggestions and recommendations

6.1. Please provide your suggestions/recommendations concerning the use of pesticides in general.
