UNIVERSITY OF CAPE COAST

SEED-BORNE FUNGI OF CHILLI (*CAPSICUM FRUTESCENS*) AND STUDIES ON THE SEED TRANSMISSION OF *COLLETOTRICHUM DEMATIUM* IN THE COASTAL SAVANNAH ZONE OF CENTRAL REGION OF GHANA

BY

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Thesis submitted to the Department of Crop Science of the School of Agriculture, University of Cape Coast in partial fulfillment of the requirements for award of Master of Philosophy Degree in Seed Science and Technology

JULY, 2012

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original work and that no part of it has been presented for another degree in this university or elsewhere.

Candidate's Signature:…….……………………… Date:.…..……………………….

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Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

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ABSTRACT

The study was carried out to assess the influence of *Colletotrichum dematium* one of the major causes of pepper diseases on the declining yield of pepper in the Ajumako Enyan Essiam district of the Central Region and to find the extent of transmission through different inoculation methods.

Three fruit types (riped, unriped and dropped) were collected from six farms across four locations (Akuamase, Nkodwo, Esikado and Mando) and another sample which was made up of only riped fruits was taken from the Mankessim market after which the seeds were extracted. This gave a total of 19 samples. Seed vigour, germination and health tests were conducted to assess the quality of the extracted seeds. Two media (sand and blotter) were used to assess the percent vigour and germination in a Completely Randomised Design in the laboratory. A transmission study was carried out to assess the extent of transmission of *Colletotrichum dematium* through four methods of inoculation (stem injection, leaf inoculation, flower inoculation and control) in a Randomised Complete Block Design with 4 replications.

There were significant differences for both mean percent vigour (8.18% and 5.29%) and mean percent germination (30.04% and 14.17%), for sand medium and blotter paper respectively. A total of 35 species of fungi belonging to 17 genera were isolated from the host. Only flower inoculation with *Colletotrichum dematium* resulted in seed transmission.

Unriped fruits should not be collected for seed extraction for planting purposes because they have high seed mycoflora infection.

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To my lovely and caring wife Mrs. Stella Kuukua Afutu and my cousin Marcelyn Baaba Sam.

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LIST OF ABBREVIATIONS

AEE	Ajumako Enyan Essiam
ANOVA	Analysis of variance
AOSA	Association of Official Seed Analysts
BP	Between papers
CA	Cluster analysis
cm	Centimetre
CRD	Completely Randomized Design
CRI	Crop Research Institute
CSIR	Council for Scientific and Industrial Research
IRST	International Rules for Seed Testing
ISTA	International Seed Testing Association
Kg	Kilogram
L	Litre
m	Metre
MoFA	Ministry of Food and Agriculture
NUV	Near ultra violet
^{o}C	Degree Celsius
PC	Principal component
PCA	Principal component analysis
PDA	Potato dextrose agar

Psi	Per Square Inch
RCBD	Randomized Complete Block Design
RH	Relative humidity
rpm	Revolutions per minute
SAR	Systemic Acquired Resistance
SEM	Scanning Electron Microscope
SPSS	Statistical package for Social Sciences
SRID	Statistics Research and Information Directorate
TWA	Tap water agar
USDA	United States Department of Agriculture
UV	Ultraviolet

CHAPTER ONE

INTRODUCTION

Background

Hot Pepper (*Capsicum frutescens* L.) is a herbaceous annual dicotyledonous plant which is sometimes also described as being a perennial shrub due to its ability to survive under harsh conditions for years. Hot pepper fruits have been given several names depending upon the locality and type of fruits. Among the names are 'chilli', 'chilli pepper', 'cayenne' and 'bird-eye'. The crop is a native of the tropics of the new world (South America) where the pungent fruits were gathered from wild plants before the development of agriculture. It was subsequently spread to Europe and Africa by the Portuguese and Spanish explorers (Pickersgill, 1997).

According to Sinnadurai (1992) and Rajput and Parulekar (1998), the chilli fruit is a berry which develops from a bicarpellary ovary with axile placentation and with a short thick peduncle, which varies in shape, colour, and pungency. The pericarp is leathery or succulent, and changes in colour from green or purple to red. The placenta carries numerous seeds. The major fruit components are seed, pericarp, placenta, and pedicel. All of these components vary greatly, depending on the variety and climatic conditions (Rajput & Parulekar, 1998).

It takes about 30-35 days from fruit set to complete development of fruit for harvest at the green stage. The fruit starts ripening 80-90 days after

fruit set (Rajput & Parulekar, 1998). Its shape may range from long cylindrical to conical, which points downwards and upwards respectively.

Hot pepper is a warm season crop that requires good sunshine and occasional dry weather for good growth and harvesting. The suitable temperatures for growth ranges from 18°C to 32°C, but it can withstand higher temperatures of 38°C (Sinnadurai, 1992).

The Ministry of Food and Agriculture, MoFA (2006) reported that the average yield of pepper under rain fed conditions was 6.5 mt/ha with achievable yields standing at 18 mt/ha depending on the variety. The volume or quantity of pepper exported as part of Ghana's non-traditional export commodities has ranged from 2,088 mt in 1998, reaching its peak at a total of 5,281 in 2001. However, the value exported has been on the decline since 2001, reaching its barest minimum of 282 mt in 2005 (MoFA, 2006).

The decline in yields of the harvested produce with the subsequent decline in the total export has been attributed to many factors, paramount among them are diseases. Fungi are one of the major causes of diseases of pepper. Within the fungi group, *Colletotrichum* is one of the most important plant pathogens worldwide causing the economically important disease anthracnose in a wide range of hosts including cereals, legumes, vegetables, perennial crops and tree fruits (Bailey & Jeger, 1992). Among these hosts, chilli (*Capsicum* spp.), an important economic crop worldwide (Poulos, 1992), is severely infected by anthracnose which may cause yield losses of up to 50% (Pakdeevaraporn *et al.*, 2005). Within the genus *Colletotrichum*, species such as *acutatum, cocoides, gloeosporioides, graminicola, capsici*, and *nigrum* are the most important species (Sutton, 1980). *Colletotrichum capsici* is the cause

of the pepper anthracnose and *C. nigrum* is the cause of the fruit rot of pepper (Critchley, 1997).

The diseases are mainly problematic on mature pepper fruits, causing severe losses due to both pre- and post-harvest fruit decay (Hadden & Black, 1989; Bosland & Votava, 2003). However, die-back of shoots, leaf spot and green matured fruit damage have also been reported. The two pathogenic fungi have been reported to be seed-borne (Kulshrestha *et al*., 1976; Critchley, 1997).

Anthracnose and Fruit rot are among the major factors limiting pepper production throughout the sub-tropical and tropical regions. For anthracnose, typical symptoms on mature fruits are circular, black sunken spots on both green and red ripe fruits. It produces conidia under moist conditions on the lesion, often showing as concentric circles bearing masses of spores. Once a lesion develops, the fungus produces the conidia within 3-5 days at $30^{o}C$ and 90% relative humidity. The various species of *Colletotrichum* which cause the anthracnose of pepper over-season and survive in infected seeds, plant tissue debris, alternative hosts or in soils as appresoria. Fruit rot caused by *C. nigrum* is characterized by dark sunken spots which develop on green and ripen fruits (Critchley, 1997).

Various control strategies have been employed to reduce losses caused by anthracnose and fruit rot. These include; crop rotation; removal and destruction of infected fruits; and spraying with recommended fungicides (Critchley, 1997; Obeng-Ofori *et al*., 2007). Despite these control measures, there has not been significant improvement in the elimination of the diseases because the pathogens responsible are seed-borne. Due to the seed-borne

nature of the pathogens, though symptoms may not be showing on a particular plant, the pathogens may have caused latent infections of the seeds. There was therefore the need to study the various fungal microflora of the seed present within the location, their effect on some seed quality parameters and finally, their effect on chilli fruit yield.

Problem statement

According to Poonpolgul and Kumphai (2007), anthracnose disease caused by *Colletotrichum* species is one of the most economically important diseases reducing marketable yield from 10% to 80% of the crop in some developing countries, particularly in Thailand.

Despite measures adopted to help reduce the problems posed by *Colletotrichum spp.* to chillies, there has still not been tremendous improvement at reaching this goal. Measures such as crop rotation and early harvesting of fruits at the green unripe stages have been adopted to avoid the fruits being on the field to full maturity and ripening. These have been done in order to avoid the fungal infection but have proven to yield little or no success even though the pathogens which cause fruit rot and anthracnose are mainly problematic on mature pepper fruits (Surianingsih, 1991).

An effective control measure is yet to be developed to contain *Colletotrichum spp.* because it is seed-borne, as well as transmitted through the system of the plant to the seeds in the new fruits produced (Kulshrestha *et al*., 1976). According to Grover and Bansal (1970), *Colletotrichum* can cause death of seedlings and reduce the vigour of infected seedlings. Consequently, early harvesting of the produce at the green unripe stage has not been found to

be effective since the harvested fruits may already have latent infections not yet manifested at the green unripe stage. This then becomes prominent upon ripening of the produce in storage and thereby reducing the value of the harvested produce.

Since *Colletotrichum spp.* are able to thrive well under warm moist tropical conditions, with about 30°C of temperature and 90% relative humidity, there has not been significant improvement towards reaching the ultimate aim of reducing losses caused by *Colletotrichum spp.*

Justification

Chillies have very important uses in the diets of man. The long viability of the seeds and the ease of transportation, as well as dispersal of the fruits and seeds, have been instrumental in the spread of chillies through the Tropics and Sub-tropics of the world (Purseglove, 1984). According to Rajput and Parulekar (1998), it is an important vegetable and condiment crop grown in the Tropical and Sub-tropical regions of the world. It is an indispensible commodity in every home in the Tropics. The authors further added that due to their medicinal properties and vitamin contents the demand for chillies have been increasing all over the world.

The crop is well adapted to our tropical conditions in the country and is able to do well on marginal lands with a rainfall of 600 mm to 1200 mm per annum being adequate for good performance (Purseglove, 1984).

Nutritionally, the crop is a rich source of vitamins A, C and E with 83% moisture, 0.3% fat, 3% protein, 6% carbohydrate, and 7% fibre which are very vital for the body (Cobbley & Steel, 1976). According to Osuna-Garcia *et*

al. (1998) and Marin *et al*. (2004), fresh green chilli peppers contain more vitamin C than citrus fruits and fresh red chilli has more vitamin A than carrots. It is a desirable ingredient in meal preparations due to its pungency. Primarily, the fruits are consumed as fresh vegetable or are dehydrated for use as a spice. When added to the food as a spice it adds flavour and colour to the food. Pepper also enhances the intake of otherwise bland diets in many households. Local foods like kenkey, fried yam, cocoyam and roasted plantain are eaten with pepper sauce "shito", which is mostly used by students. It has powdered pepper as a major ingredient and is a long lasting sauce when well prepared. It is added in various proportions to herbs for herbal medicine, and also incorporated in ginger beer or other beverages as well as in laying mixtures for poultry (Purseglove, 1984). It purges the bowels when eaten in excess and this is due to its laxative effects.

In view of these numerous uses, large portions of land are put in *Capsicum sp* cultivation. However, annual yields are low due to poor management practices, incidence of insect pests and diseases, with fruit drop contributing greatly to the field losses in yield (Opoku-Asiama *et al.*, 1993).

The role of pepper in the economy of Ghana in terms of its contribution to agricultural non-traditional export in 2001 was US$1,938,000. The quantity exported in 2001 was 5,281 metric tons. From 2001, yield continued to decline through the years 2002, 2003, 2004 and 2005, having the values of 4,687, 4,674, 282 and 483 mt respectively. The corresponding figures in monetary terms were from the highest of US$1,938,000 in 2001, to the lowest of US$107,000 in 2004 (MoFA, 2006). These drastic reductions in Ghana's total exports have in no doubt caused a loss of revenue to the country

in terms of its foreign exchange earnings from the non-traditional agricultural export sector. The drastic reductions in the overall export has been as a result of the failure of the fruits to pass the various tests carried on the produce before acceptance on the foreign market. The rejections have been premised on the early rotting of the fruits whiles in storage and on transit to the importing countries.

This therefore has led to a loss in revenue of up to US$1,831,000 and pepper farmers still battle with the problem of yield losses caused by diseases, paramount of which is that caused by *Colletotrichum.* Since anthracnose (*C. capsici*) alone is one of the major limiting factors to pepper production throughout the Subtropical and Tropical regions, known to cause yield losses of over 50% (Surianingsih, 1991), there was therefore the need to conduct a survey of fungi associated with pepper fruit in the agro-ecological zone, find the rate of infection and transmission to seed through different inoculation techniques and the effects of *C. spp.* on some seed quality parameters. This was done to help classify the fungi found within the agro-ecological zone, find the transmission rate and ultimately, their effect on seed quality. It is expected that the information obtained would serve as the basis for further studies.

Objectives

Main objective

The main objective of the research was to obtain data on *Colletotrichum* which would be used to increase the yield of Chilli peppers.

Specific objectives

Specifically, the study sought to:

i. determine the incidence level of *Colletotrichum spp.* on the different categories of fruit samples collected from the study location;

ii. determin the incidence level of rot-causing fungi other than *Colletotrichum spp* as well as the incidence of other fungi on seeds of the different fruit samples collected from the location;

iii. determine the quality of seeds of the different types of pepper fruits in terms of vigour and percentage germination using 2 different media, and seed health using the blotter method;

iv. determine the extent of transmission of *Colletotrichum dematium* through different inoculation methods;

v. assess the yield of chilli inoculated through different methods.

Tests of Hypotheses

a. H_{0a}: No significant differences exist for seed mycoflora infection within each treatment (pretreated and untreated samples).

H_{1a}: Significant differences exist in seed mycoflora infection within treatments.

b. H_{0b}: There is no significant difference ($P \leq 0.05$) in percent seed infection of mycoflora between treatments.

H_{1b}: There is significant difference ($P \leq 0.05$) in percent seed infection of mycoflora between treatments.

CHAPTER TWO

LITERATURE REVIEW

Seed and its development

Biologically, seed is the ripened ovule (Singh & Mathur, 2004). In angiosperms, to which a majority of the crop plants belong, the ovules are borne in the ovary, the basal part of the gynoecium (pistil). The seed formation takes place *in situ* through a series of integrated sequential steps in the life cycle of the flowering plant (Maheshwari, 1950, 1963; Johri & Ambegaokar, 1984). After pollination and fertilization, changes in different parts of the ovule, i.e., the embryo sac (zygote and primary endosperm nucleus), nucellus, chalaza, and integument, lead to the formation of the seed.

The term seed when used *sensu lato*, includes one-seeded dry indehiscent fruits that are the dispersal propagative units in plants of several families such as Poaceae, Asteraceae, Apiaceae, and Chenopodiaceae. In the present treatment, the term seed is used in a loose sense (Singh & Mathur, 2004).

Seed is an autonomous living unit and links successive generations. Structurally, it consists of an embryo (new plantlet), a protective covering, i.e. seed coat, pericarp, or both, and reserved food material, which may be present in the endosperm, perisperm, or embryo. It has the capacity to withstand desiccation and retain viability under unfavourable environments or until it germinates. These properties of seed make it an important commodity for

storage as well as transportation to new areas or countries, for planting or for edible purposes (Singh & Mathur, 2004). The structure of seed is fairly constant in a species, but varies in different plant taxa (Netolitzky, 1926; Corner, 1976).

According to Singh and Mathur (2004), seeds, if infected by microorganism, will act as carriers and if the organism remains viable they will result in development of disease in the new crop. The authors further noted that infected seeds are often responsible for the spread of diseases to new areas. For this reason, seed has become an object of plant quarantine internationally and therefore, countries also use domestic seed certification, including seed health testing, as a method of quality control of seed.

Histopathology

The reports on the number of microorganisms associated with seeds have increased gradually during the latter half of the 20th century (Noble *et al.,*1958); Richardson, 1990). The organisms occur with seed either as contaminants adhering to the seed surface, loosely mixed with seed, or as infections present within the seed tissues (Singh & Mathur, 2004).

Singh and Mathur (2004) have observed that the structure of flowers and the changes in stamen and carpel influence the penetration course, location and effect of micro organisms during seed formation. The authors further observed that the variation in the hilum, micropyle opening nature and thickness of the cuticle and the thickness of seed coat and pericarp directly influenced host pathogen relationships (Singh & Mathur, 2004).

Various histological techniques, e.g., the embryo extraction method initially used by Skvortzov (1937), whole-mount preparations of seed components (Maden *et al.*, 1975; Singh *et al.,* 1977), free hand sections, and microtome sections, and some recent procedures are being used.

Penetration and establishment of fungi in seed

According to Singh and Mathur (2004), fungal infection of seed-borne pathogens may reach the ovule and seed at any stage from the initiation of ovule to mature seed. They further stated that fungi, bacteria, and viruses, differ in their modes of multiplication and attack on the host. For example, fungal propagules germinate, and the hyphae grow while phytopathogenic bacteria multiply and viruses replicate intracellularly. Also, Singh and Mathur (2004) stated that the germination of propagule and initiation of hyphal growth are important factors that determine the entry of fungal pathogens in any tissue, including the fruit and seed.

Penetration and establishment in the ovule

Singh and Mathur (2004) have noted that the environment of the ovule and the seed plays an important role in the penetration and establishment of fungi in seeds. In addition to that, the physiological and biochemical factors inside a fruit in general and the fleshy fruit in particular, may further control the establishment of infection. This is borne out by the fact that seeds of fleshy fruits such as cucumber, squash, melon, and tomato are usually remarkably free from fungi (Singh & Mathur, 2004).

According to Christensen and Kaufman (1969) and Jain *et al.* (1994), since seeds are stored under dry conditions, and no free moisture is available, the chief determinants for the development of fungi in and on seeds during storage are temperature and available moisture (water) of the grain.

Nature of the pathogen

The seed-borne fungi may be parasitic or saprophytic and, according to Dickinson and Lucas (1977), may be biotrophs or necrotrophs. Biotrophs according to Dickinson and Lucas (1977), cause minimal damage to the host, including seed tissues; are in fair harmony with the host; have a narrow host range and are usually obligate parasites. Necrotrophs, on the other hand cause apparent damage to the host cells and have a wide host range; secrete enzymes and bring about the disintegration of cell components, resulting in cell death. The released cell contents are used by such pathogens for their growth. Basically, the mode of nutrition is like that of saprophytes (Singh & Mathur, 2004).

According to Singh and Mathur (2004), the majority of seed-borne fungi are known to be necrotrophs and the obligate parasites that belong to Peronosporaceae, Albuginaceae, Erysiphales, Ustilaginales, and Uredinales are biotrophs.

Many intermediate conditions occur between the true necrotrophs and biotrophs. Maudee (1996) believes that necrotrophs, which degrade tissues as they spread, are rarely transmitted to the embryo through the mother plant.

Neergaard (1979) has listed eight disease cycles for seed-borne pathogens taking into consideration the location of primary inoculum in seed,

course of disease development, and reinfection of ovule and seed. According to Neergaard (1979), the infection may be systemic, local, or organospecific and the systemic infection may follow a vascular or a nonvascular course.

Infection in developing seeds

According to Singh and Mathur (2004), two levels need to be recognized as the passages for the invasion of fungi in seeds since the ovules and developing seeds are present inside the ovary: (1) routes leading to internal ovary infection and (2) ovary to ovule and seed infection.

Routes leading to internal ovary infection

Infection may either reach the pistil directly from the mother plant through the vascular supply or the parenchyma, primarily through the intercellular spaces of the pedicel, or take place indirectly from outside using stigma-style, ovary or fruit wall, and other floral parts, including nectaries, as sites for the receipt of inoculum (Singh & Mathur, 2004).

Investigations carried out using artificial inoculation and histological techniques, including Scanning Electron Microscope (SEM), have improved the understanding of the course of hyphae during penetration and growth in the tissues of the pistil (Marsh & Payne, 1984; Chikuo & Sugimoto, 1989; de Neergaard, 1989; Kobayashi *et al.*, 1990).

According to Lawrence *et al.* (1981), systemic plant infection of most vascular and nonvascular pathogens enter the flower and fruit through the pedicel. They further noted that local infection below the flower, if it becomes systemic, can also cause infection via the pedicel.

Direct infection from mother plant

Direct infection from the mother plant may be by entry through the vascular supply or through nonvascular tissues. Some pathogens transmitted through vascular infection include: *Fusarium* and *Verticillium* spp. (e.g. *F. moniliforme, F. oxysporum* and *F. scirpi*) in cotton (Rudolph & Harrison, 1945); *Verticillium albo-atrum* in sugar beet and spinach (Snyder & Wilhelm, 1962); and *F. moniliforme* and *F. oxysporum* in maize (Kingsland & Wernham, 1962; Lawrence *et al*., 1981).

Examples of the direct infection from the mother plant through nonvascular tissues of the plant include: smuts and downy mildews in cereals and grasses where the mycelium moves through the intercellular spaces and enters the ear from the mother plant leading to malformation and disruption of reproductive structures (Safeeulla, 1976; Mathre, 1978); *Sclerospora graminicola* in pearl millet, *Peronosclerospora sorghi* in sorghum where the inflorescence axis is directly infected from the mother plant in which the mycelium subsequently invades floral primordia and enters anthers and ovaries with secondary infection by conidia occurring through the stigma, style or ovary (Prabhu *et al.,*1983).

Indirect infection from outside

Halfon-Meiri and Rylski (1983) reported fruit infection in pepper by *Alternaria alternata* as a result of stigmatic infection. According to Chau and Alvarez (1983), the conidia of *Colletotrichum gloeosporioides* germinated and penetrated the cuticle and epidermal cells after artificial inoculation of green fruits of *Carica papaya*.

Roberts and Snow (1984) observed that when cotton bolls were artificially inoculated with *Colletotrichum capsici*, the conidia germinated on the fruit wall and penetrated directly or through the stigmata and hairs. They further observed that the hyphae invaded the pericarp, parenchyma, the vascular supply, endocarp and the lint fibres.

From a comparative study conducted by Kabayashi *et al*. (1990), the germination of fungal conidia on the stigma of both susceptible and resistant cultivars was observed and the hyphae grew in their styles for the first 4 days. The authors further observed that growth was retarded in the styles of the resistant cultivar, but continued in those of the susceptible cultivar.

Chikuo and Sugimoto (1989) and Agarwal and Sinclair (1997) have reported that localized infection of the fruit wall causing internal infection of the locule was common. Example is the infection of sugar beet flower and seed balls by *Colletotrichum dematium* f. sp. *Spinaciae*.

According to Singh and Mathur (2004), fungal spores and conidia result in indirect infection when transferred from other infected plants or from a local infection on the same plant to the ovary or fruit, in which case the inoculums may be transferred through various dispersal agencies such as wind, water (rain, irrigation), and insects. It was also observed that the routes for internal infection vary depending on the site of receipt of the inoculums and the route for indirect infection may be through the stigma and style, ovary and fruit wall, and floral parts including nectary.

Microorganisms in seed

According to Neergaard (1979), Maude (1996) and Agarwal and Sinclair (1997), fungi, bacteria, viruses, and nematodes are known to be seed-borne, but fungi are the major group of pathogens that are seed-borne as well as seed-transmitted.

Richardson (1990) has stated that the list of saprophytic and parasitic fungi associated with seeds of different plants is very large and they cut across all fungal classes.

Major fungal disease pathogens of chilli

According to Bosland (1999), fungi comprise one of the largest groups of organisms causing diseases among chilles. The most important fungal diseases are: Anthracnose (*Colletotrichum spp*.); Early blight (*Alternaria solani*); Cercospora leaf spot (*Cercospora capsici*); Damping-off (*Pythium, Rhizoctonia* and *Fusarium*); Gray mould (*Botrytis cineria*); Phytophthora rot (*Phytophthora capsici*); Southern blight (*Sclerotium rolfsii*); Verticillium wilt (*Verticillium dahliae*); and White mould (*Sclerotinia sclerotiorum*).

Seed-borne fungal pathogens of chilli

The following fungal disease pathogens are known to be seed-borne and seed transmitted; *Colletotrichum spp., Alternaria solani, Fusarium, Cercospora capsici, Botrytis cineria*, and *Sclerotinia sclerotiorum* (Neergaard, 1979; Bosland, 1999).

Transmission rates of fruit rot following different inoculation methods

There are some reports on resistance to the fruit rot disease (Ullasa *et al.,* 1981; Rawal *et al.,* 1983; Mah, 1985). Chew (1987) and Kraikruan *et al.* (2008) have suggested that capsaicin content is related to resistance.

According to Hartman and Wang (1992), there are no standard techniques used in inoculation to evaluate resistance, but the pin-prick method used by Barksdale and Koch (1969) for evaluating resistance in tomatoes has been used for pepper. They further stated that other factors like age of pepper fruit and the effect of temperature on assessing resistance have not received much study.

Some studies have been done on the pathogen *Colletotrichum* species where inoculation of plants were done under field, green house and laboratory conditions, at different conidia concentrations, on different crop plants, using different methods (Freeman *et al*., 2001; Lewis-Ivey *et al*., 2004).

Colletotrichum

According to Cannon *et al.* (2000) and Johnston (2000), *Colletotrichum* is a taxonomically confused genus in urgent need of revision, especially as its members are important plant pathogens. Species estimates range from 11 in von Arx (1957), 22 in Sutton (1980), about 40 in Sutton (1992) and 60 in the *Dictionary of the Fungi* (Kirk *et al.*, 2008), while there are 708 names in *Index Fungorum* (2008).

Variability in *Colletotrichum*

The reproduction mode in many *Colletotrichum* populations is mainly or exclusively vegetative. In the absence of a sexual stage, the only means of exchanging genetic material between two strains would be anastomosis and heterokaryosis. Microscope examination reveals that anastomosis occurs between lateral branches, which grow out of neighbouring hyphae and form anastomosis bridges connecting two hyphae. The resultant fused cells are binucleate and appear not to proliferate, but support adjacent uninucleate cells with genes of either nuclei (Katan, 2000). Heterokaryosis and parasexuality are the crucial factors determining the phenotypic heterogeneity within the group and a thorough analysis at the molecular and genetic level is needed.

Around 98% of conidia from a single agar culture of *Colletotrichum* are uninucleate, but a small number is always multinucleate. Nuclear heterogeneity can be increased under different environmental conditions and growth in liquid culture can increase the proportion of binucleate conidia to 17% and tri-nucleate conidia to 3-5% in some species (Jeffries *et al.*, 1990).

Identified species of importance to chilli

Five species including: *Colletotrichum acutatum* Simmonds; *C. capsici* (Sydow) Butler and Bisby; *C. coccodes* (Walk) Hughes; *C. gloeosporioides* (Pent.) Penz. and Sacc.; and *C. graminicola* (Ces.) Wils., have been reported to cause anthracnose of pepper (Verma, 1973; Sutton, 1980; Hadden & Black, 1987). Among these species, *C. gloeosporioides* was the predominant species causing anthracnose on pepper fruits (Oh *et al.*, 1999).

However, no races of the fungus have been reported. Kim *et al.* (1985) found some isolates of *C. gloeosporioides* only infected red fruits and were designated as strain `R', while other isolates infected both green and red fruit were designated as strain 'G'. Geographical location may be a determining factor for infection patterns, because some species seem to predominate by location, but *C. capsici* and *C. gloeosporioides* are the most frequently reported species Kim *et al.,* (1985).

Infection strategies of *Colletotrichum* species

The typical infection process of *Colletotrichum* involves a common sequence of events as described by Dodd *et al.* (1989). The authors stated that the inoculum arrives at the host's surface as water- or splash–borne conidia, which become attached to the plant cuticle. Further, the conidia become septate prior to germination usually within 12-48 hours after inoculation, after which a germ tube emerges and grows for about 10-20 µm before terminating in an appressorium (Dodd *et al.,* 1989).

According to Bailey *et al.* (1992), *Colletotrichum* species are among the most successful plant pathogenic fungi exhibiting three main initial infection strategies, according to which species are loosely categorized. The infection processes of several *Colletotrichum* species such as *C. lindemuthianum* (O'Connell *et al.,* 1993), *C. destructivum* (Latunde-Dada *et al.,* 1996), *C. truncatum* (O'Connell *et al.,* 1993) and to some extent *C. dematium* (Smith *et al.,* 1999) have been well characterized by light and electron microscopy.

There are differences in the duration as well as styles of initial penetration and infection processes of the species. These morphological event variations are listed by O'Connell *et al.* (1985); Jeffries *et al.* (1990); van Dyke and Mims (1991); and Smith *et al.* (1999).

Colletotrichum dematium (Pers. ex Fr.) Grove

According to the ISTA (2003a), *Colletotrichum dematium* (Pers. ex Fr.) is synonymous to the following *Colletotrichum species*: *C. capsici* (Syd.) Butler and Bisby; *C. circinans* Berk.; *C. glycines* Hori; *C. hibsici* Poll.; and *C. truncatum* (Schwein.) Andrus and Moore.

Host range of pathogen

Many *Colletotrichum* species with curved conidia had been synonymised with *C. dematium* by von Arx (1957), who considered this taxon to be a common saprotroph on many herbaceous plants with several host specific forms. Descriptions of *C. dematium* in literature are very variable and confusing (von Arx, 1957; Sutton, 1980; Baxter *et al.,* 1983). Therefore, this taxon was epitypified and compared with other curved spored species from herbaceous hosts based on morphological and molecular data (Damm *et al.*, 2009). The authors confirmed *Colletotrichum dematium* to be plurivorous, probably having pathogenic, saprotrophic and endophytic lifestyles (Damm *et al.*, 2009).

Importance of Colletotrichum in pepper production

Effect on pepper fruit yield

Descriptions of the infection process and symptoms have been reported (Adikaram *et al.,*1983; Mah, 1985). Hartman and Wang (1992) have stated that yield losses on an experimental basis have not been determined, but estimates of losses are as high as 50%. They further stated that symptoms of the disease include pre- and post emergence damping-off, dieback of shoots, leaf spots and fruit rots either in the field or in storage.

According to Hartman and Wang (1992), fruits of hot and sweet peppers *(Capsicum* spp.) are susceptible to several diseases including anthracnose and fruit rot. The authors further noted that the disease limits production and losses occur in the field, in transit, and in storage. Adding that, the primary losses occur on fruit because any infection on the fruit reduces its quality and sale (Hartman & Wang, 1992).

Anthracnose caused by *C. capsici* is considered to be a dry fruit rot (Pearson *et al.*, 1984). *C. capsici* and *C. gloeosporioides* are the two main casual agents of pepper anthracnose in the hot humid tropics of Asia. They are also the most important *Colletotrichum* spp. in reducing marketable fruit yields of pepper (Manandhar *et al.*, 1995).

Anthracnose has been found not only on mature fruit but also on seedlings, leaves and immature green fruits (Lee & Chung, 1995). Recently, Park and Kim reported that five anthracnose fungi, *C. gloeosporioides*, *C. dematium* (Persoon: Fries) Grove, *C. coccodes* (Wallr.) S. Hughes, *C. acutatum* Simmonds, and *Glomerella cingulata* (Stoneman) Spaulding & v. Schrenk, were pathogenic to different tissues of pepper plants. Of these

anthracnose fungi, *C. gloeosporioides* attacks the fruit at all stages of development, but not the leaves and stems of plants (Marvel, 2003). Leaf anthracnose of pepper seedlings caused by *C. coccodes* was first found in pepper-growing fields in Chungnam province of Korea in 1988 (Hong & Byung, 1998). Although all ages of pepper fruits were susceptible to infection by *C. gloeosporioides*, purple and ripe red fruits developed more anthracnose than the immature stages (Oh *et al.*, 1999).

Effect on pepper seed quality

According to Grover and Bansal (1970), *Colletotrichum dematium* is known to be seed-borne and can cause death of seedling and also reduce the vigour of infected seedlings. Anthracnose and fruit rot are both seed borne and air borne and affect seed germination and vigour to a greater extent (Ahmed, 1982; Perane & Joi, 1988; Mesta, 1996; Asalmol *et al.,* 2001).

It has been shown that *C. dematium* readily colonizes the seed coat and peripheral layers of endosperm even in moderately colonized seeds. Heavily colonized seeds had abundant inter- and intra-cellular mycelium and acervuli in seed coat, endosperm and embryo, showing disintegration of parenchymatous layers of the seed coat and depletion of food material in endosperm and embryo (Chitkara *et al.*, 1990).

According to Doijode (2001), Seed infestations with fungi result in loss of viability, reduction in vigour, discolouration, and generation of heat and rotting. Hoffman *et al.* (1998) have noted in the effect of *Sclerotinia sclerotiorum* infection on the yield and seed quality of soybean cultivars that,

disease incidence was negatively correlated ($P < 0.05$) with seed germination for all cultivars.

Disease epidemiology

According to Hong and Byung (1998), *C. coccodes* can infect pepper seeds, seedling leaves and stems, mature leaves, and sometimes green but not red fruits. They further noted that in general, pepper plants seem to acquire resistance to *C. coccodes* as they mature, since the anthracnose caused by *C. coccodes* does not readily occur in mature plants. The authors also stated that the primary inoculum density of *C. coccodes* seems to be important for producing typical anthracnose lesions on pepper plants. However, anthracnose caused by *C. coccodes* does not result in severe epidemics in mature leaves and fruits of pepper (Hong & Byung, 1998).

Conidia of *Colletotrichum* do not function as survival structures as their viability declines rapidly. Mycelium, however, may remain viable for long periods in/on colonized seeds, plant debris, or as latent infections in plants not showing any disease symptoms (Manandhar *et al.*, 1995). Microsclerotia, formed sparsely by species such as *C. gloeosporioides* and *C. coccodes*, play an important role in survival (Baxter *et al.*, 1985). *C. gloeosporioides* for example was recovered from leaf spots on sicklepod (*Senna obtusifolia*), and *Colletotrichum* spp. has been reported on sicklepod (Whiting & Roncadori, 1997).

In particular, researchers have noticed that some growers leave infected fruits on the plant when harvesting thus providing an inoculum source for further infection (Pearson *et al.*, 1984). A separate study clarified that

seed-borne *C. gloeosporioides* was transmitted from endosperm tissue to hypocotyls and radicles in red pepper (Lee & Chung, 1995).

Conidia of *Colletotrichum* germinate on fruit and produce germ tubes with adhesive appressoria (Manandhar *et al.*, 1995). Germination and development of appressoria occurred at 95 to 100% RH and at 20 to 30 ^{o}C; however, abundant surface moisture was only visible on leaf and fruit surfaces at 100% RH (Dodd *et al.*, 1991). The conidia of *C. gloeosporioides* germinated on both green and red fruits within 2 hours after inoculation. Infection of green fruits by the fungus may lead to anthracnose development on immature fruit (Oh *et al.*, 1997). On green fruits of pepper, only one isolate caused dark, brown to black lesions 6 days after inoculation. Later, these lesions slowly increased in size and became sunken. On red fruit of pepper, all isolates produced more severe symptoms (Yu *et al.*, 1987). The colonizing hyphae grow both intracellularly and intercellularly as a lesion develops. It is during the initial phase of colonization that the resistance responses of the plant may be expressed (Jeffries *et al.*, 1990).

Many post-harvest diseases of fruits exhibit the phenomenon of quiescence in which symptoms do not develop until the produce ripens. *Colletotrichum* and *Glomerella* species are by far the most important pathogens that cause this type of infection. Although these genera have been the subject of numerous investigations, there remain many gaps in our knowledge of the disease process and our understanding of the complex relationships between the various fungi involved (Jeffries *et al.*, 1990).

According to Manandhar *et al.* (1995), conidia often do not germinate *in situ* because of the presence of germination inhibitors in the spore matrix,

but will germinate after being washed or rain-splash disseminated. Under normal conditions, conidia dispersed by rainfall may remain on a plant surface and retain potential to cause disease for periods of over 7 days (Estrada *et al.*, 1993). During wet periods, appressoria have been reported to produce secondary conidia, which may be involved in secondary spread to pepper fruits (Manandhar *et al.*, 1995).

Disease control

According to Dodd *et al.* (1991), several management strategies have been developed to control quiescent infections in tropical fruit but they often involve the extensive use of fungicides, which are both expensive for growers in developing countries and potentially damaging to the environment.

Bailey (1987) and Agrios (2005) have recommended the use of integrated management techniques, since no single specific management program could eliminate chilli anthracnose. According to Wharton and Diéguez-Uribeondo (2004), effective control of *Colletotrichum* diseases usually involves the use of a combination of cultural control, biological control, chemical control and intrinsic resistance.

According to Romero *et al.* (2001), Actigard induces Systemic Acquired Resistance (SAR). Kousik and Subramanya, (2001) have noted that Actigard can activate resistance not only in leaves, but also in pepper fruits.

Freeman *et al.,* (1998) have stated that differentiating between *Colletotrichum* species responsible for disease epidemics is vital for developing and implementing effective control strategies.

Seed vigour and germination

According to Dornbos (1995), the vigour of a seed refers to both the ability and strength of the seed to germinate successfully and establish a normal seedling. This is positively related to the ability of a seed population to establish an optimum plant stand, in both optimum and sub-optimum soil environments.

Brown (2008) however, defined vigour as the ability of the seed to germinate and develop into strong seedlings under a wide range of simulated field conditions, e.g., cold and hot.

Germination on the other hand has dual meanings, the specific meaning being dependent upon whether the audience is physiologically or technologically oriented (Dornbos, 1995). First, as defined in the Rules for Testing Seeds of the Association of Official Seed Analysts (AOSA, 1978), to the seed technologist, germination is "the emergence and development from the seed embryo of those essential structures, which for the kind of seed in question, are indicative of the ability to produce a normal plant under favourable conditions." However, to the seed physiologist, germination is described simply as "protrusion of the radicle through the seed coat, indicating whether or not seeds within a population are alive".

Since the technologist requires that a seed produces both a normal root and shoot under the germination conditions defined by the AOSA (1978) for each seed type, attainment of high germination percentage by a seed population is more restrictive than from a physiological perspective. Seed germination according to the seed technologist is more relevant to the grower and field environment, whereas germination to the physiologist pertains more

to investigative study" (Dornbos, 1995). According to Thomson (1979), the germination capacity of a seed lot indicates its ability to establish seedlings in good field conditions; while vigour indicates its ability to do so even under poor conditions.

According to Dourado and Vince-Prue (2002), germination is the term used to describe the physiological and physical changes immediately prior to and including the first visible signs of growth. The authors further noted that the dry seed is inert until sufficient water enters the cells to allow metabolic reactions to begin. Once in the soil, water becomes available and enters the dry seed by the process of imbibition. Dourado and Vince-prue (2002) also added that water is taken up through openings in the seed coat and causes the seed to swell which ruptures the seed coat, allowing the entry of gases and the uptake of more water.

As the cells become hydrated, enzymes are activated and begin to break down the storage tissue and transfer nutrients to the embryo. Some of the metabolic activities that occur during early germination are directed at repairing or replacing cell membranes (which are selectively permeable and control the exchange of materials through the cell) and organelles (sub-cellular structures that are found throughout a cell and are the sites for processes such as respiration and photosynthesis) that are damaged during the drying process. However, most activities are directed towards embryo growth.

CHAPTER THREE

MATERIALS AND METHODS

Study area

The study was carried out in the Ajumako – Enyan – Essiam (AEE) District of the Central region of Ghana. The four major chilli growing areas in the district were selected. They were Akuamase, Nkodwo, Esikado and Mando as shown in Figure 1. The map (Figure 1) was generated using the Arc-GIS software version 9.3.

Chilli variety

The variety of chilli used for the study was the Scotch bonnet, (*Capsicum chinense*) locally known as 'Mako hwam'. This is a late variety and its harvesting starts 3 months after planting out (MoFA, 2005). This variety was used because that is the chilli variety widely cultivated by chilli pepper farmers within the study area.

Sampling and collection of chilli fruits

The purposive sampling technique of Gray *et al.* (2007) was used in selecting the farms from which samples were taken for the research. This was done to ensure fair coverage of the district and also to capture the major pepper farmers within the district. Fruit samples were taken from the two (2)

major chilli farms each within Akuamase and Nkodwo, and one (1) farm each from Esikado and Mando adding up to six farms.

Three different types of fruits were taken from each farm. These were Physiologically Matured Unriped Fruits, Riped Fruits, and Dropped Fruits, thus giving a total of eighteen (18) fruit samples. A final sample (Riped Fruits) was obtained from two (2) different chilli sellers at the Mankessim market and bulked into one sample, thus, making the number of samples nineteen (19).

The last sample from the Mankessim market was taken because all the six (6) farmers visited said they purchased pepper fruits from the Mankessim market to extract their own seeds.

The entire research was carried out in five (5) phases namely; Experiments 1, 2, 3, 4 and 5.

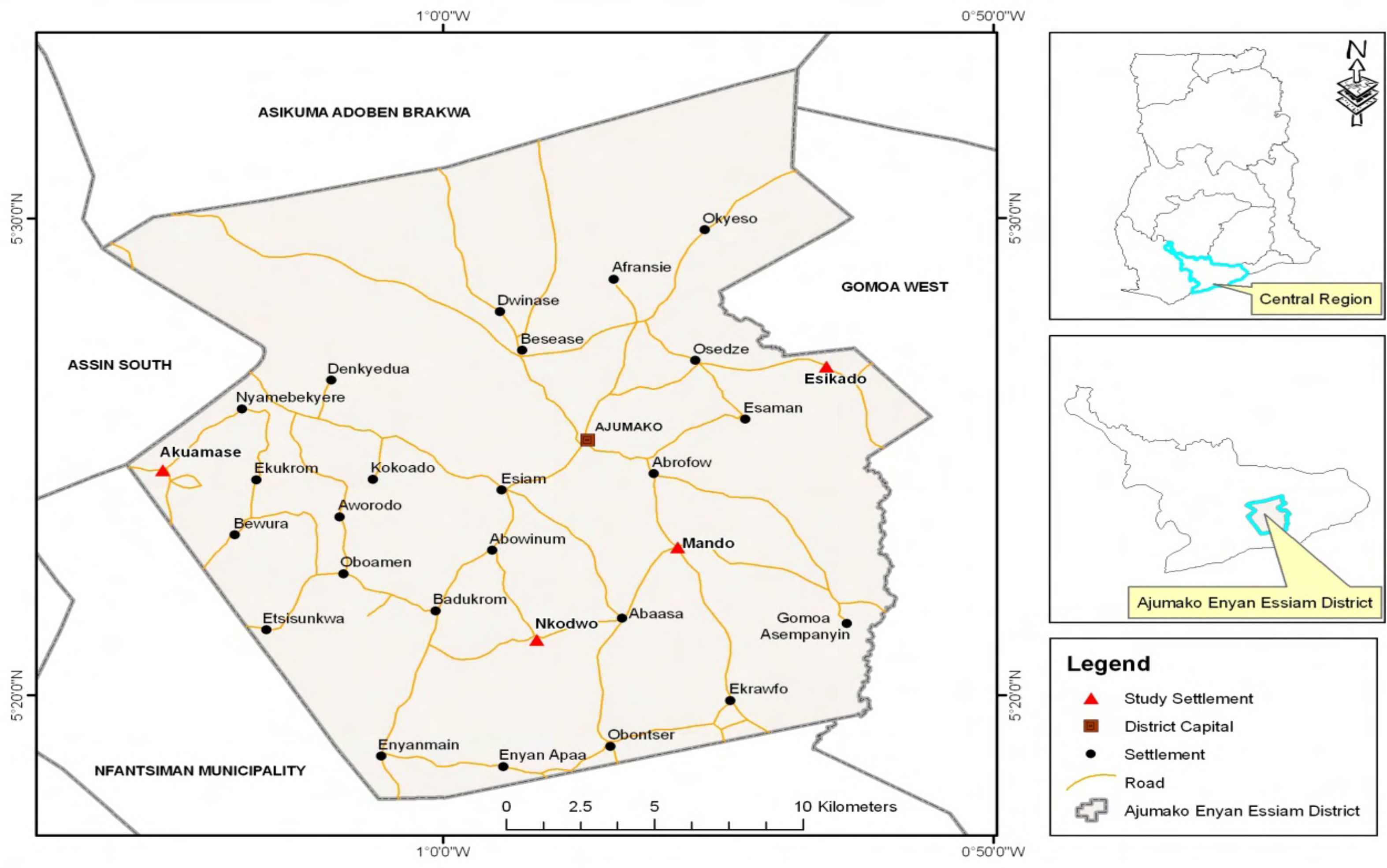

Fig. 1. Map of study area showing sampling sites

EXPERIMENT ONE

Introduction

Seeds were extracted from each of the three types of chilli fruits collected from the study area. The health of the seeds was tested using the standard blotter method (ISTA, 2003a). The seeds were examined for seed mycoflora after 7 days. This was followed by isolation and culture of *Colletotrichum* species after which permanent cultures of *Colletotrichum dematium* was prepared and stored for use in experiment three.

The experiment was conducted in the Seed Pathology Laboratory of Crops Research Institute of the Council for Scientific and Industriial Research, Fumesua.

Seed extraction

Each sample of fruits was cut open with a sharp knife and the seeds were removed into a bowl of water. The seeds were then rinsed off any foreign particles or attachments such as hila or pieces of fruit pericarps and were air-dried as shown in plate 1.

Standard blotter method (ISTA, 2003a)

Preparation of petri dishes

Sixteen petri dishes (90mm diameter) were used for each seed sample. They were placed on a clean working table. Each petri dish was labeled with the name of the sample, treatment and date of examination. Three filter papers were stacked, dipped into tap water, and placed in the petri dish. All petri dishes were first prepared in this way.

Plate 1: Air-drying of extracted seeds (NKO-U means seeds extracted from physiologically matured but unriped fruit samples from a farm in Nkodwo)

Sample size

Each whole sample was put in a tray. Small portions of seeds were randomly taken with a spatula and transferred to the working area for seed counting and plating. 400 seeds were tested for each sample as required by the ISTA (2003a) but with a modification. Out of the 400 seeds taken for health testing, each 400 seed sample was divided into 2 sub-samples; made up of 200 seeds each.

Pre-treatment

One sub-sample was pre-treated with 1% Sodium hypochlorite (NaOCl) solution for 10 minutes to prevent the growth of saprophytic fungi to

enable the free growth of pathogenic fungi (Dhingra & Sinclair, 1995; ISTA, 2003a). It also served as a basis for comparison within each sample of 400 seeds for fungi that will grow on treated and non-treated seeds.

Surface lesions, as on diseased fruit tissues with areas 1 cm^2 excised from the lesion margin on fruits were also surface sterilized for 10 minutes in NaOCl, washed in sterile distilled water and then placed onto agar surface (Dhingra & Sinclair, 1995; Hemannava, 2008).

Plating of seeds

Seeds were plated on the wet blotters placed in petri dishes. Twenty-five (25) seeds were plated in each 9 cm (90 mm) diameter petri dish ensuring that seeds were equidistant from each other to reduce the spread of pathogens from one seed to the other as shown in plate 2 (ISTA, 2003a).

Incubation of seeds

All the dishes of each sample were put in trays (one tray per sample of seeds) and transferred to the incubation room. Care was taken while handling the dishes in the tray and transferring them to the incubation room to prevent the displacement of plated seeds from their original positions.

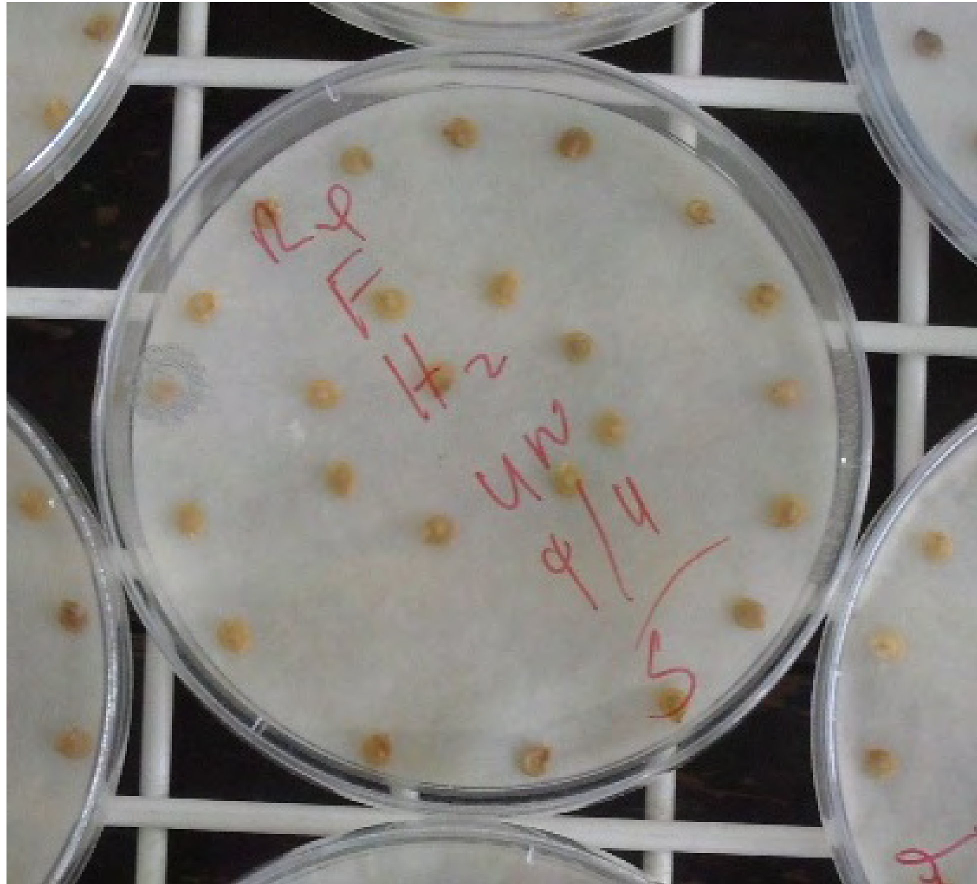

Plate 2: Seeds plated on wet blotters (25 seeds per dish)

The dishes were incubated at 22°C for 7 days in alternating cycles of 12 hours darkness and 12 hours light. The light source used for incubation was near ultra violet (NUV) supplied by two black light tubes (Philip TL-D 36W/08) hanging horizontally, 20 cm apart from each other and the distance between the light tubes and the dishes was 40 cm (plate 3a and b). The NUV light was used in order to induce sporulation since more sporulation helps to identify the fungi (ISTA, 2003a).

The statistical design used was the Completely Randomized Design (CRD).

Plate 3a: Plated chilli seeds incubated under Near Ultra Violet (NUV) light at CRI-CSIR, Fumesua (NUV lights put on)

Examination of incubated seeds

The procedure for examination and identification of fungi described in the first edition of the Common Laboratory Seed Health Testing Methods for Detecting Fungi (ISTA, 2003a) was followed.

After incubation, the Petri dishes were brought to the examination area in the laboratory and each set of 16 Petri dishes (8 dishes each for treated and untreated seeds respectively) representing each sample of 400 seeds were numbered serially, 1, 2 ,3, through to 8.

Plate 3b: Plated chilli seeds incubated under light (NUV) at CRI-CSIR, Fumesua (NUV lights put off).

Each seed was examined in a certain fixed sequence. The habit characters of each fungus were examined under different magnifications of the stereomicroscope. Slides of the fruiting structures of fungi were prepared and examined under a compound microscope whenever identification of a particular fungus was difficult or confusing. The mycoflora were identified by consulting mycological literature (Kulshrestha *et al.*, 1976; ISTA, 2003a).

According to the ISTA (2003a), a seed is counted as infected even if one identifiable fructification is observed, such as a single conidiophore with conidia of *Alternaria* and *Bipolaris*, a Pycnidium of *Ascochyta*, *Botryodiplodia* and *Macrophomina*, an acervulus of *Colletotrichum* and a sporodochium of *Fusarium*.

Once a fungus was identified to species level under the compound microscope, the infected seeds were marked by writing an abbreviation for that fungus on the wet blotter, such as ‘Cd’ for *Colletotrichum dematium*, ‘Pl’ for *Phoma lingam* and ‘Fo’ for *Fusarium oxysporum*.

Recording of infection

The different fungi in each group were counted by crossing the abbreviations, one by one. The crossings of the abbreviations were done using a pencil of a different colour (brown) to facilitate the final checkup. This was done to ensure that all marked abbreviations were counted (ISTA, 2003a). The counts of each fungus from each plate were entered into a Working Recording Sheet immediately after examination of the plate. Recording Sheet number 1 (Appendix III) was used in accordance with ISTA (2003a) standards which required that for a plate tested containing 25 seeds, Recording Sheet No. 1 should be used.

Media preparation and isolation of pathogens

Sterilization and cleaning of glassware

Pyrex petri dishes were sterilized in an oven at 140°C for 3 hours (Ritchie, 2002). However, McCartney bottles and other pyrex bottles and flasks for holding Agar media were sterilized by autoclaving at 121°C for 15 mins (Dhingra & Sinclair, 1995) at a pressure of 1.02 bar (15 p.s.i.).

Potato Dextrose Agar (PDA) Preparation

Industrially manufactured potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) Lewis-Ivey *et al.,* (2004) was obtained. Twenty grams of the PDA was weighed into a 500 ml bottle and distilled water was added to the 500 ml mark and shaken vigourously. The mixture was then autoclaved at 121°C for 20 mins (1.02 bar or 15 p.s.i.) (Ritchie, 2002) after which the bottles were removed from the autoclave and kept in the laminar flow chamber fitted with UV Sterilization lamp.

Addition of antibacterial supplement

An antibiotic stock solution was prepared using penicillin at 50-500 p.p.m. stock solution. For each 500 ml bottle of medium, 5 ml of stock antibiotic solution was added (500 ppm of stock solution). The addition of the antibacterial supplement was done when the autoclaved medium was 'hand-hot' (50°C), since excessive heat denatures most antibiotics (Ritchie, 2002). Again, according to Ritchie (2002), Penicillin is active against Gram-positive bacteria; and should therefore be used in conjunction with streptomycin sulphate.

The stock solution of streptomycin sulphate was prepared by dissolving 1g in 100 ml of sterile distilled water to obtain a 1% solution. This antibacterial supplement was used at 200 ppm and it was added to the medium because it has a broad-spectrum of activity (Ritchie, 2002).

Leaf agar preparation

This medium was prepared by following two main procedures (Dhingra & Sinclair, 1995). First, preparation of water agar (plain agar) and secondly, preparation of Panicum leaves (*Panicum maxima*).

In preparing the plain agar, 1000 ml of distilled water was measured into an Erlenmeyer flask and 15 g of agar solidifying agent (Oxoid agar no. 3) was added. It was shaken vigourously to get the agar to dissolve thoroughly. The mixture was then dispensed into two 500 ml bottles while being agitated to ensure each bottle had a fair proportion of the mixture. The bottles were then kept in an autoclave and sterilized at 121°C for 20 mins (1.02 bar or 15 p.s.i.) (Dhingra & Sinclair, 1995; Ritchie, 2002).

The second part was the preparation of the panicum leaves. Fresh and disease free green leaves of *Panicum maxima* were harvested from the field. The leaf blades were then cut into smaller rectangular pieces with dimensions of 1.5 cm × 1 cm. These pieces were then put in a conical flask containing distilled water. The flask together with its content was autoclaved for 20 mins at 121°C to sterilize the leaves. After sterilization, the flask was kept in a laminar flow chamber to cool.

After the first and second procedures have been followed, the water agar prepared was poured into sterilized petri dishes when the medium was 'hand–hot' (50°C) and the plates were packed in a laminar flow chamber to solidify. After solidification of the plain agar, the autoclaved leaves were removed from the flask using forceps sterilized in an open flame kept in the laminar flow chamber. Excess moisture on the leaves were removed by putting

them between 2 sterile blotters, and then transferred to plain agar plates (Dhingra & Sinclair, 1995; Ritchie, 2002).

Isolation and culturing of pathogen

From the incubation of seeds and pericarps of chilli fruits, the most prevalent species of *Colletotrichum* was *C. dematium*. The sporulating fungus on the surface of the incubated seeds as well as on lesions of fruit samples was used to obtain pure cultures by direct transfer to the PDA prepared. This was done by touching the spores under a low-power stereoscopic microscope with a sterile fine gauge inoculating needle, either dry or moistened, by first stabbing it into the sterile medium, and then streaking on to the plate (Medium).

Isolation from fruits (pericarps) was done by placing small portions of the relevant tissue on agar. To avoid contamination, the excised tissue was surface sterilized before plating out. The isolations from the seed and fruit pericarps were carried out on plain agar (tap-water agar, TWA) (Waller, 2002). According to Waller (2002), this is done to favour the growth of the pathogen, which uses the plant material as a food base, rather than growth of contaminant saprophytic fungi because nutrient agars favour the growth of faster-growing saprophytes. To prevent contamination from airborne pathogens, the isolation and inoculation was carried out in a laminar flow chamber and petri dish lids were carefully lifted and replaced to avoid entry of air borne contaminants.

Single spore isolation of *Colletotrichum dematium*

After the isolation and culture of the pathogen on plain agar, single spore isolation was carried out. The semi-mechanical method of Smith (2002) was used for isolation.

Using a Borrowdale needle for preference, a 2 mm square cut was made in the agar around the selected spores. These squares and the areas immediately around each of them were examined under the low power to ensure that only one (single) spore was picked in each case. Each of the agar blocks and germinating spores were then transferred by means of a sterile needle onto an agar plate.

The single spores isolated were inoculated on full strength PDA in the centre and the plates were incubated at 24°C for 7 days in alternating cycle of 12 hour light and 12 hour darkness. The light source in the incubation room was a Philips TLD 36W/08 which emits near ultra violet light (NUV) (plate 3a).

Preparation of permanent culture

Permanent cultures were prepared for storage to be used for inoculation of chilli plants on the field. To give a better chance of permanent cultures being pure, the cultures were prepared from single spore isolations (Smith, 2002). Spores (conidia) were observed to verify that their shape and size matched those of *C. dematium* (Kulshrestha *et al*., 1976; ISTA, 2003a).

For long period of storage (Dhingra & Sinclair, 1995), the fungus was transferred by using needle to pick the inoculum from plates and inoculating at the centre of the agar slants by stabbing the surface of the slants in the McCartney bottles (Waller, 2002). The bottles were covered using screw stoppers and incubated for 7 days at 24 ^{o}C under 12 hours cycle of alternating NUV light and darkness.

The slants were stored in a refrigerator at 4-7°C to prevent drying out of the cultures as well as gathering of dust if stored on shelves at room temperature. Cold storage was also used in order to extend the interval required for transfer of cultures to fresh media to at least 4-6 months on the average (Smith, 2002). Therefore, the refrigerator storage was done primarily to increase the shelf-life of the pathogen to ensure that the organism remained viable for subsequent experiments.

EXPERIMENT TWO

Introduction

Following seed health testing, two other seed quality parameters were measured i.e. seed vigour and seed germination percentages. Two different media i.e. sand (river sand) and blotters (between papers) were used for the tests to help compare the performance of the seed samples in terms of vigour and germination percentages in the different media.

Secondly, the tests were carried out to identify the best quality seed sample among the 19 samples in terms of vigour and germination percentage to be used for field planting and transmission tests.

The vigour and germination tests were carried out in the Seed Technology Laboratory of the Crop Research Institute of the Council for Scientific and Industrial Research, Kwadaso, Kumasi.

Seed vigour and germination testing

Working sample

400 seeds were counted at random from the well-mixed pure seeds of each of the samples (ISTA, 2003b). Care was taken that there was no selection of seeds thus causing biased results. Replicates of 100 seeds were used, spaced sufficiently on the germination media (Sand and Blotter) used to minimize the effect of adjacent seeds on seedling development.

Test media preparation

Sand substrate

Sand was collected from the ‘Twahel’ river which flows from Edwenase (in the Bosomtwe District of the Ashanti region) and through CRI-CSIR, Kwadaso. The sand was dumped in the open under the sun for six (6) weeks to help sterilize it. This was done in order to kill any pathogen that might be present in the medium and also to get rid of weeds, or other crop seeds that might be present in the medium. The dry heat sterilization method was preferred to the chemical method in order not to have any chemicals deposited in the medium which might suppress or kill seed-borne disease organisms.

The medium was sieved to obtain a reasonably uniform medium free from very small and large particles (ISTA, 2003). The mesh used for sieving had holes of width 0.8 mm. The sterilization and sieving were performed to get the sand medium to be free from seeds, fungi, bacteria or toxic substances which might interfere with the germination of seeds, the growth of seedlings or their evaluation. The pH of the sand was determined using a pH meter and the value obtained was 6.6.

A plastic bowl 5 cm deep and 30 cm in diameter was used for the test as shown in plates 4a, b and c. The bowl was filled to a depth of about 3.5 cm leaving 1.5 cm space above.

This was followed by putting the plastic bowls (medium) in transparent poly bags to help retain the moisture in the medium for the entire duration of the research, thus, reducing evaporation of moisture from the medium. The bowls were then packed on shelves within the growth room (Plate 5).

Plate 4a: Plastic bowl used for Germination test (5 cm deep and 30 cm wide)

Plate 4b: Plastic bowl filled with sand to a depth of 3.5 cm leaving 1.5 cm space above.

Plate 4c: Sand medium prepared for seeding (100 holes punched per plate).

Paper substrate (Blotter)

The between paper method (BP) as described by Mathur *et al.* (2003) was used but with slight modifications. The 9 cm diameter blotters and petri dishes were used for the test.

Three blotters were stack together, dipped in tap water and placed in the base of the petri dishes. To ensure adequate spacing, split replicates of 25 seeds were made, particularly because seed-borne disease was suspected to be

present (ISTA, 2003b). After plating each petri dish, another single layer of blotter was wetted and placed on the plated seeds to increase contact of the seeds to the blotter (medium) and also to increase the amount of moisture surrounding the seeds.

Plate 5: Germination and vigour test set up on shelves in a growth room.

Petri dishes were then covered with transparent poly bags and arranged on the laboratory bench for the test period. The Completely Randomized Design was used for the tests involving the two different media since a uniform condition was expected to prevail in the laboratory.

Recording of vigour and germination results

According to the ISTA (2003b), first count (days) for vigour and final count (days) for germination percentage calculations in *Capsicum species* are 7 and 14 respectively. Any seed which germinated within the first 7 days of the test period were removed, counted and discarded. These recordings represented the vigour by number and were thus converted to percentage values.

The final counting for the computation of the percentage germination was done on the 14th day and the set up was discarded. This was repeated for all 19 seed samples under consideration.

EXPERIMENT THREE

Multiplication of fungal inoculum and preparation of conidia suspension

Introduction

Experiment three involved multiplication of the conidia of *Colletotrichum dematium* isolated and preserved (Dhingra & Sinclair, 1995) from experiment one to be used for inoculation of chilli plants on the field.

Two different methods were used for conidia multiplication in order to obtain more inoculum. The grass leaf method also known as the leaf agar method (Dhingra & Sinclair, 1995) and the Czapek Dox Broth method (Robinson *et al.,* 1998) were used.

Multiplication using the grass-leaf method (Leaf Agar)

The Leaf agar was prepared as described under Experiment one (1) and this was followed by seeding of the medium.

According to Dhingra and Sinclair (1995), seeding of the medium should be done with conidia obtained from 48- to 72-hr-old colonies prepared from the single spore (conidium) cultures. In this procedure, fresh sub-cultures of the pathogen were prepared using conidia obtained from the permanent cultures prepared from Experiment one (1). The fresh culture was done using PDA as medium and the conidia for seeding of the Leaf agar was obtained from the PDA culture on the third day (72 hrs).

The seeding of the Leaf agar was done by using a sterile needle to pick the conidium from the 3-day-old colonies on PDA cultures and streaking on the leaf strips (seeding). This was followed by incubation of the plates in 12 hrs of alternating cycles of NUV light and darkness at 28°C (Hartman & Wang, 1992) for 7 days.

Multiplication using the Czapek Dox Broth method

Czapek Dox Broth is a semi-synthetic medium used for the general cultivation of fungi. It contains Sodium nitrate as the sole source of nitrogen, Sucrose as the sole source of carbon and Dipotassium phosphate buffers the medium. Magnesium sulphate, potassium chloride and Ferrous sulphate serve as sources of essential ions (Robinson *et al.,* 1998).

The medium was prepared according to the formula developed by Thom and Church (1926), which had a defined chemical composition. Czapek Dox Broth is the modification of the original medium Czapek (1903 – 1920) and Dox (1910) as per Thom and Raper (1945).

Thirty grams of sucrose was weighed and added to 1000 ml (1L) of distilled water and the flask was placed on a magnetic stirrer to get the powder to dissolve. While still on the stirrer, 2.0 g, 1.0 g, 0.5 g, 0.5 g and 0.01 g of Sodium nitrate, Dipotassium phosphate, Magnesium Sulphate, Potassium Chloride, and Ferrous sulphate respectively, were weighed and added to the solution one after the other. The mixture was then autoclaved at 121°C for 15 mins at 1.02 bar (15 p.s.i) pressure.

The solution (Czapek Dox Broth) was then poured into four (4) sterilized conical flasks and each of the flasks was seeded (inoculated) with conidia suspensions prepared from 7 day old cultures of *C. dematium*.

The seeded flasks were transferred to a darkroom for incubation at 28°C for 4 days (Dhingra & Sinclair, 1995). The flasks were put on a magnetic shaker (Stuart Scientific Flask Shaker SF1) set to 100 oscillations per minute (osc/min) as shown in Plate 6. The darkroom incubation coupled with the regular shaking of the suspension was done to prevent mycelia formation within the culture and to promote conidia formation within the Czapek Dox Broth.

Plate 6: Incubation of Czapek Dox Broth solution in a dark room after seeding (Cultures held on flask shaker throughout the incubation period to prevent mycelia formation within the culture)

Preparation of conidia (spore) suspension

Preparation from Czapek Dox

After culturing, the conical flasks were removed from the incubation room and kept in a laminar flow chamber. The Czapek Dox Broth solution in each of the flasks was poured into a 1L conical flask by sieving the solution using a sterilized funnel and cheesecloth.

Preparation from leaf agar cultures

The conidia suspension was prepared by washing the conidia from leaf agar cultures with sterile distilled water.

First, a small amount of sterile distilled water was poured onto the leaf agar cultures and a sterilized special spatula was used to rupture the acervuli to release the conidia into solution. The solution was then poured into a conical flask by sieving it using a sterilized funnel and cheese cloth. The washing was repeated a number of times to make sure most of the conidia were washed out as much as possible.

Purification of conidia suspension by washing

Conidia suspensions obtained from leaf agar cultures and Czapek Dox Broth solutions were purified through a repeated number of washings (four times) to obtain pure spore (conidia) suspension (Canale-Parola, 1973; Wyss, 1996).

The suspensions were first poured into sterilized 50 ml centrifuge tubes each filled up to the 40 ml mark and were spun using an electronic Merlin Spectra Scientific Centrifuge at 5,000 revs/min. for 10 mins. After this, the centrifuge tubes were removed and the supernatant poured out leaving 10 ml of the supernatant over the sediment of conidia at the base of the centrifuge. 30 ml of sterile distilled water was then added to top the suspension in the tubes to the 40 ml marks again and the spinning was repeated. The whole process of spinning, decanting and refilling (spore washing) was repeated for three other times.

After the final spore washing, the conidia sediments in the centrifuge tubes were pooled into one centrifuge tube. This was then spun at 5,000 revs/min for 15 mins after which the supernatant was decanted.

Spore count using haemocytometer

Next, the suspension was vigourously shaken to have the conidia sediments mixed thoroughly. The conidia concentration was then determined using the Bürker-Türk counting chamber. According to Waller (2002), the use of the haemocytometer employs the direct counting technique unlike the dilution plating which estimates spore concentration by the cultural technique. The haemocytometer is a counting chamber used for determining the number of cells/spores/conidia per unit volume of a suspension.

The conidia suspension was diluted by a factor of two (2) i.e. $dilution\ factor = \frac{final\ volume}{initial\ volume} = 2$.

The cover slip was placed over the counting surface prior to loading of the conidium suspension. The suspension was introduced into one of the V-shaped wells using a sterilized micro pipette. Enough suspension was introduced so that the mirrored surface was just covered.

The conidia were counted in each of the four large corner squares systematically. A conidium was counted as “in” if it overlaps the top or right ruling and a conidium was counted as “out” if it overlaps the bottom or left ruling (Waller, 2002).

The conidia concentration was calculated using a formula adopted from ISTA (2003b);

$$Conidia\ Concentration = \ Dilution\ \times \frac{C_total}{volume}$$

Where; C_total = total number of conidia found in the four large corner squares.

The volume of suspension within the four large corner squares was obtained as follows;

area of each large corner square = 1 mm^2 (i.e. 1mm × 1mm),

depth of the V-shaped well was 0.1 mm,

therefore, volume of one large corner square = area × depth

= 1 mm^2 × 0.1 mm = 0.1 mm^3,

since counting was done from the four squares, it implies volume of the four squares = 4 × 0.1 mm^3 = 0.4 mm^3.

From the conidia count, C_total was found to be 70, and dilution factor was 2.

Therefore, conidia concentration $= 2 \times \frac{70}{0.4\text{mm}^3}$

⇨ = 2 × 175/ mm^3 = 350 /mm^3

but, 1000 mm^3 = 1 cm^3 [(same as milliliter) (ml)],

therefore, the conidia count was 350/mm^3 × 1000 = 350000/ml

= 3.5×10^5 conidia per ml.

EXPERIMENT FOUR

Field work

Introduction

From the results of experiments 1 and 2, the seeds extracted from matured ripe fruits collected from Esikado were the best in terms of seed health and seed percentage germination. This sample was therefore selected for use in the raising of seedlings for the transmission experiment on the field.

Transplanted seedlings were raised to about 50% flowering and three different methods of inoculation were tested for the transmission and recovery of *Colletotrichum dematium.*

Experimental site

The experiment was carried out at the Teaching and Research Farm of the School of Agriculture, University of Cape Coast, Cape Coast. Cape Coast is a typical coastal savannah low land zone characterized by an annual rainfall range between 800 and 1000 mm and a mean monthly temperature of 26°C.

The soil has been described by Asamoah (1973) as Atabadze Series which is the same as Utisol in the United States Department of Agriculture (USDA) classification. It belongs to the Edina-Benya-Udu Compound Association, developed over the Sekondian Material.

Prior to the planting of the chilli on the field, the area had been cropped for two seasons with maize, followed by another season with cassava.

Field preparation

An area of 400 m^2 (i.e. 20 m × 20 m) was cleared and all debris collected. This was followed by ploughing and harrowing to give a fine tilth. After this, marking out and setting out was done and a nursery bed was prepared with the dimensions 2 m × 1 m.

Soil analysis

Soil analysis was carried out to determine the following parameters: soil pH, total nitrogen (N), available phosphorus (P), and exchangeable K (potassium) and the results are shown in Appendix I. This was done to be better informed on the soil acidity or basicity levels as well as the nutrient status of the soil being used for the research since these parameters have been found to have effect on the resistance or susceptibility of plants to disease pathogens.

Nursing of seeds and transplanting

Seeds were nursed in the beds and allowed to stay in the nursery till the 6th week when transplanting was done. The transplanting was done on the 6th week due to the advantage of 5 and 6 weeks old transplants growing more quickly and yielding more than transplants older than 6weeks (Norman, 1977).

Five weeks after nursing, the main field was pegged and was divided into four main blocks representing the four replications. The breadth of each block was 4 m and the length was 16 m with a distance of 0.5 m allowed between the blocks (replications). Each block was sub-divided into four (4) sub-plots representing the four different treatments applied on the field. This

therefore gave a total of 16 sub-plots (i.e. 4 sub–plots × 4 replications). Each sub–plot had an area of 4 m × 4 m. The planting distance was 1 m × 1 m giving 4 rows and 4 plants within rows and thus, 16 plants within each sub-plot (treatment). Out of the 16 plants within a sub–plot, treatments were applied to the four central plants and the peripheral plants were left to serve as border plants. This was done to eliminate the border effects which were bound to occur, thus, to minimize error.

The Randomized Complete Block Design (RCBD) was used for the experiment.

Treatments (inoculation methods)

There were four (4) treatments in all. These were T1, T2, T3, and T4, where;

T1 was the control (No inoculation),

T2 was Leaf or Foliar inoculation method,

T3 was Flower inoculation, and

T4 was Stem injection method of inoculation.

Leaf or Foliar inoculation method (i.e. T2), involved inoculation of all plant leaves (both matured and young leaves) produced at the time of inoculation without selection. The leaves were inoculated with the conidia suspension using a hand sprayer (atomizer- spray master consolidated plastics) and an average of 5 ml of conidia was applied per plant.

Flower inoculation (i.e. T3), was done by applying the spore suspension (inoculum) in drops to all flowers produced on the plant using a sterile disposable hypodermic needle. 5 ml spore suspension was applied per

plant after which a tag was hanged on the stalks of inoculated flowers (Hartman & Wang, 1992).

The Stem injection method (i.e. T4), involved creating holes on the stem using sterile disposable hypodermic needle to facilitate injection of 5 ml spore suspension. The holes for inoculation were created on the main stem as well as points of branching of the plant.

The quantity of inoculum to apply per treatment was adopted from Vakalounakis and Williams (2010), who did a similar work in which they used a cotyledon double inoculation technique for the evaluation of resistance to anthracnose (*Colletotrichum orbiculare*) and scab (*Cladosporium cucumerinum*) in cucumber. This was however modified by using 350 conidia/µl (3.5×10^5 conidia/ml) instead of 200 spores in 2µl (100 spores/µl).

After inoculation in T2 and T3, each plant was covered with a plain plastic bag for 48 hours to maintain a relative humidity greater than 95% (Kim *et al.,* 2007; Posada *et al.*, 2007). However, the plants in T4, (i.e. Stem injection), had no poly bag coverage as the fungus was injected into the plant, where humidity is naturally high. The treatments (inoculations) were applied 8 weeks after transplanting to the field (i.e. 1 week after the plants started flowering on the field) and the experiment was ran for a total of 10 weeks after inoculation. The recovery of *Colletotrichum dematium* was evaluated by culture methods at three (3), six (6) and ten (10) weeks after inoculation as explained in experiment five (5).

Data analysis

The results were statistically analysed according to the ANOVA procedure described by Little and Hills (1978). The t-Testing of the mean percent vigour and percent germination, principal component analysis (PCA) and cluster analysis (CA) of mycoflora data by location and by specific organisms were determined using SPSS software version 16. Mean fruit weight, mean number of fruits and average fruit weights were computed using Microsoft Excel 2007. GenStat Discovery Edition 3 was used to transform all data in percentage to angular values before analysing.

EXPERIMENT FIVE

Recovery of *Colletotrichum dematium*

Seed extraction and plating

After the inoculation of the plants on the field, fruits were harvested at the scheduled times i.e. 2, 4, 6, and 10 weeks after inoculation and the seeds were extracted. This was followed by plating of the seeds for the recovery of the pathogen following inoculation.

The procedures for the plating, examination of plates, and isolation of the pathogen (*Colletotrichum dematium*) were as described under experiment one (1).

The Completely Randomized Design was used in this experiment in the arrangement of plates in the incubation room.

CHAPTER FOUR

RESULTS

Mean percent vigour of 19 seed samples recorded for different substrates

Figure 2 is a histogram of the mean percent vigour recorded for 19 seed samples tested using different substrates (sand and blotter). The results show a significant difference ($P \leq 0.05$) in the mean percent vigour. Sand recorded a mean percent vigour of 8.18% (16.62) whiles blotter recorded 5.29% (13.30) as test substrates.

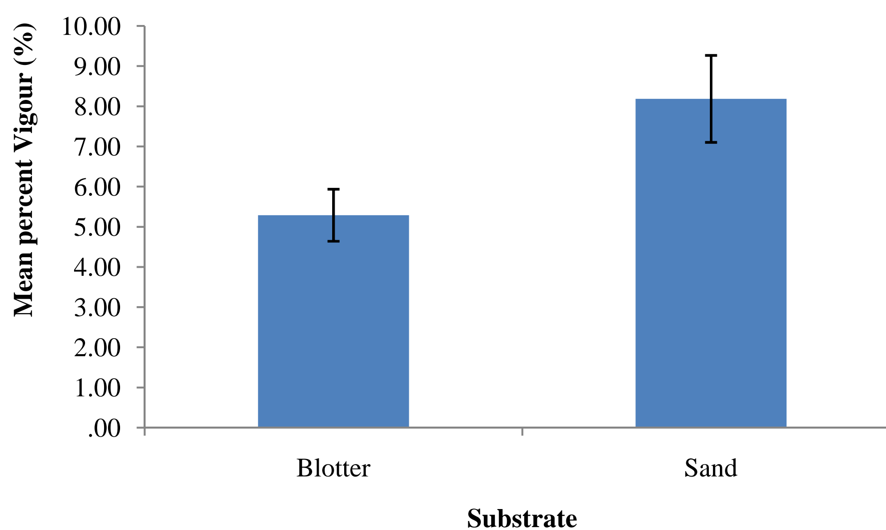

Figure 2: Mean percent vigour for different substrates

Mean percent germination of 19 seed samples recorded for different substrates

Results of the mean percent germination for 19 seed samples are presented in Figure 3. It shows a significant difference ($P \leq 0.05$) in mean

percent germination recorded for sand and blotter as test substrates. Blotter had a mean percent germination of 14.17% (22.11) while sand as a substrate recorded a mean of 30.04% (33.24) for the 19 seed samples tested.

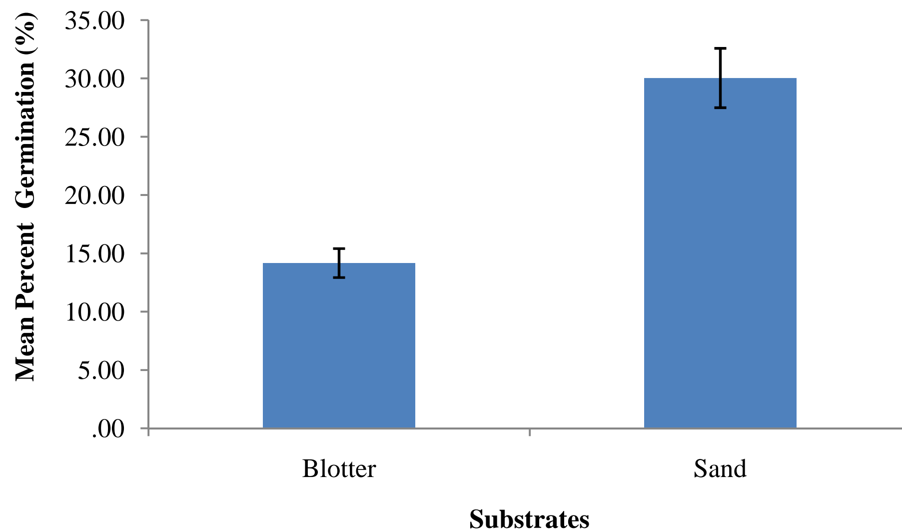

Figure 3: Mean percent germination recorded for different substrates

Mean percent vigour of 19 seed samples collected from 7 locations

Figure 4 shows the mean percent vigour recorded for seed samples collected from 7 locations. Esikado recorded the highest mean percent vigour of 16.79% while Akuamase A recorded the least mean percent vigour of 1.21%. There were no significant differences ($P \leq 0.05$) among the samples collected from Mankessim 1.25% (6.42), Nkodwo B 2.92% (9.32), Akuamase A 1.21% (6.32), and Akuamase B 1.71% (7.51). However, there were significant differences between samples collected from Esikado 16.79% (24.19), Mando 12.13% (20.28), and Nkodwo A 7.50% (15.89). Also, significant differences existed between Esikado, Mando, and Nkodwo A on one side compared with samples taken from Mankessim, Nkodwo B, Akuamase A, and Akuamase B.

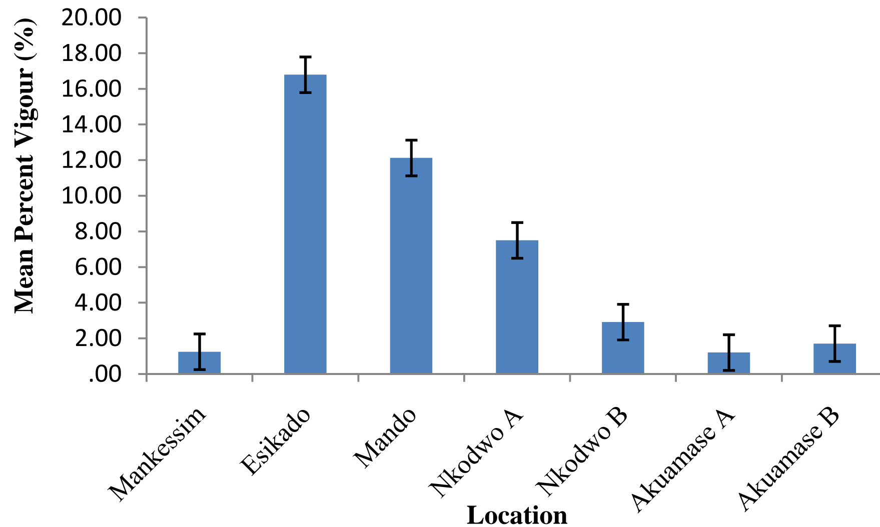

Figure 4: Mean percent vigour recorded for different locations

Mean percent germination of 19 seed samples collected from 7 locations

Figure 5 is a graphical representation of the mean percent germination recorded for seed samples collected from 7 locations. Samples collected from Esikado had the highest mean percent germination (40.75%) while the least was recorded by samples collected from Akuamase A (9.75%). It shows no significant differences ($P \leq 0.05$) among samples collected from Mankessim 17.63% (24.83), Nkodwo B 12.88% (21.03), Akuamase A 9.75% (18.19), and Akuamase B 12.25% (20.49); and between Mando 37.13% (37.54) and Esikado 40.75% (39.67). There were however, significant differences between Esikado and Mando compared to the remaining 5 locations.

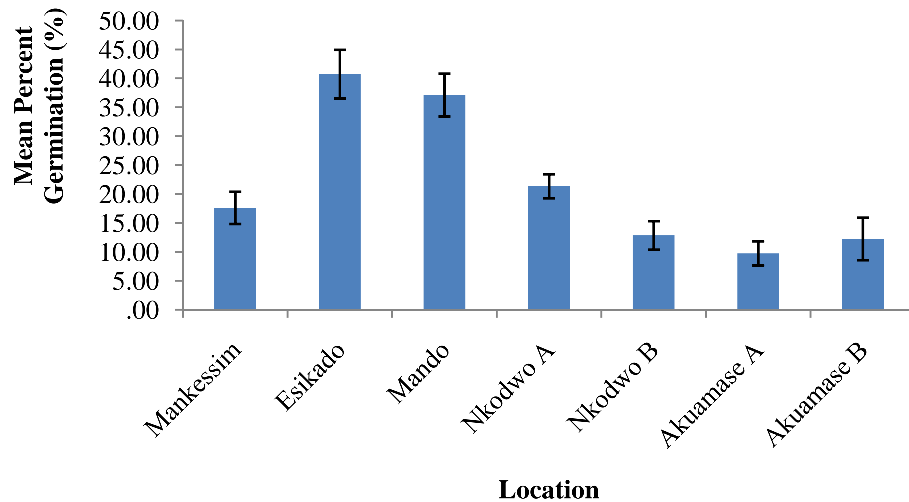

Figure 5: Mean percent germination recorded for different locations

Mean percent vigour recorded for 3 different fruit types

Results of the mean percent vigour recorded for the 3 different fruit types (riped, unriped and dropped fruits) are graphically represented in Figure 6. The Figure shows no significant difference ($P \leq 0.05$) between mean percent vigour of dropped fruits and riped fruits. However, significant differences ($P \leq 0.05$) existed when the mean percent vigour of dropped and riped fruits were compared with unripe fruits.

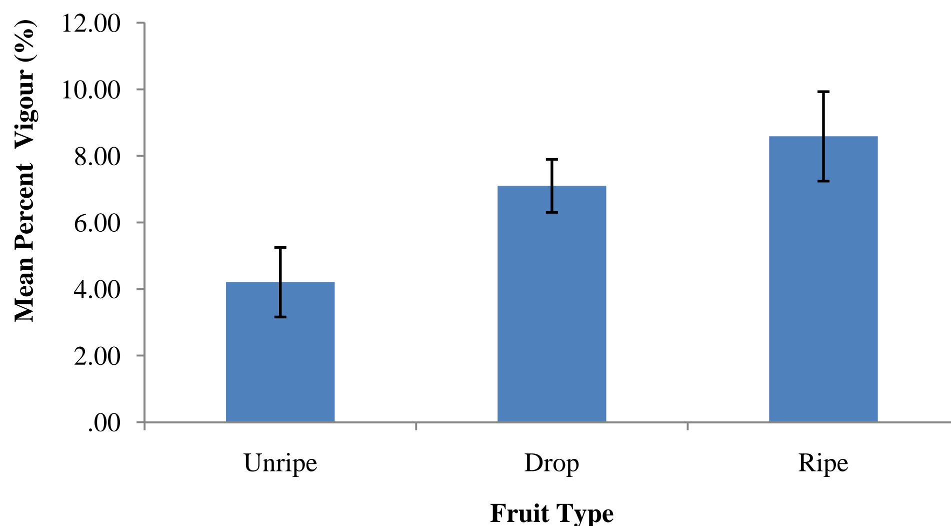

Figure 6: Mean percent vigour recorded for 3 fruit types

Mean percent germination recorded for 3 different fruit types

Figure 7 is a graphical representation of the mean percent germination recorded for 3 fruit types, namely, riped, unripped, and dropped. The graph shows no significant differences ($P \leq 0.05$) in the mean percent germination between unripped and dropped fruits. On the other hand, a comparison of unripped and dropped fruits with riped fruits showed a significant difference ($P \leq 0.05$) in the mean percent germination.

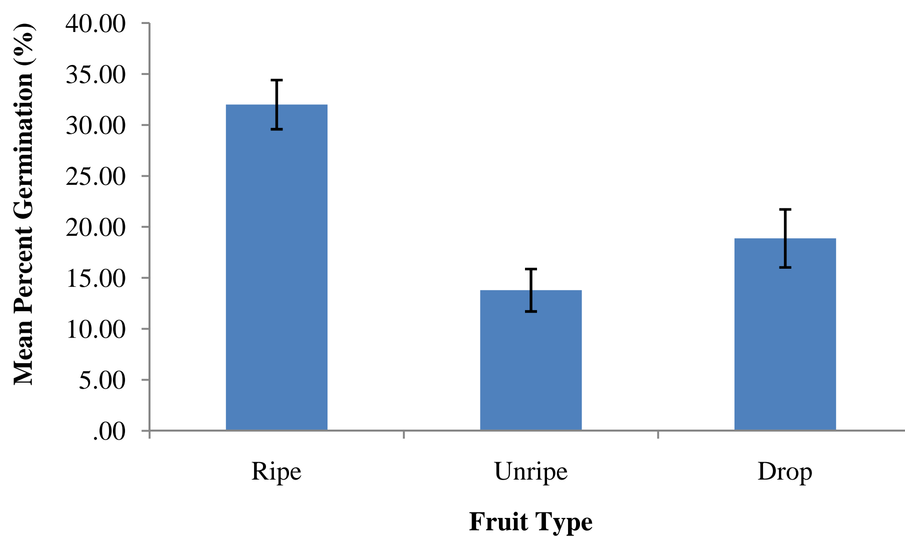

Figure 7: Mean percent germination recorded for 3 fruit types

Mycoflora infection on seeds for different fruit types

Figure 8 shows the results of the mean of percent of each mycoflora infections recorded for the three fruit types considered. Unriped fruits recorded the highest (14.40%) mean of percent infection by each fungi present. This was followed by riped fruits (11.94%) and dropped fruits (11.64%) in that order. The results however, showed no significant difference in the mean of percent infection by each fungi present on the 3 fruit types.

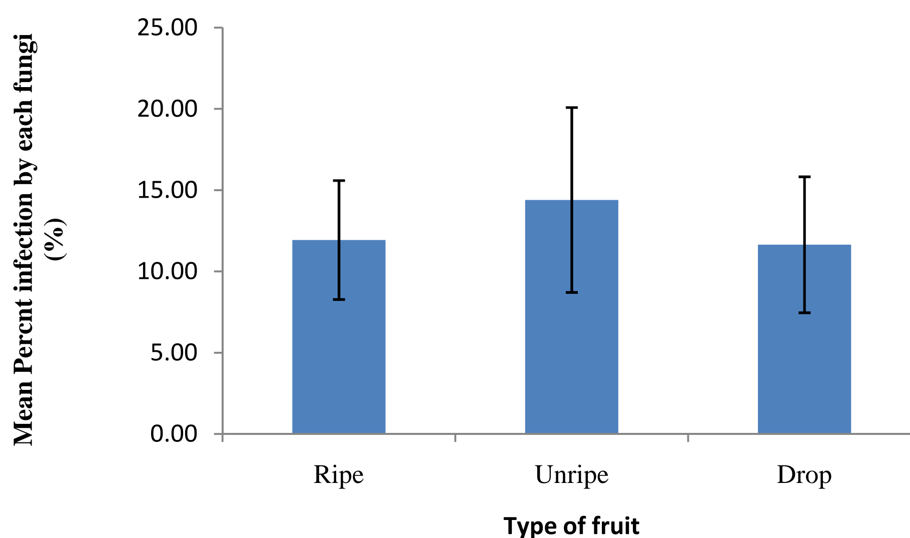

Figure 8: Mean Percent infection by each mycoflora for fruit types

Comparison of Seed mycoflora infection for pre-treated and untreated seed samples

Table 1 shows results of the percent seed infection for pre-treated and untreated seed samples. In all, a total of 35 species of fungi belonging to 17 genera were isolated from the chilli seed samples. The fungi isolated were identified as *Acremonium strictum, Alternaria circinans, A. alternata, A. sesame, A. sesamicola, A. solani, Aspergillus flavus, A. niger, Bipolaris cynodontis, Botrytis cineria, Cercospora sojina, Cladosporium sphaerospermum, Colletotrichum coccodes, C. dematium, C. gloeosporioides, C. lindemuthianum, C. acutatum, Corynespora cassiicola, Curvularia geniculata, C. lunata, C. pallescens,* and *C. trifolii.* The rest were *Exserohilum turcicum, Fusarium avenaceum, F. equiseti, F. moniliforme, F. oxysporium, F. pallidoroseum, F. solani, Glomerella cingulata, Myrothecium leucotrichum, Phoma exigua, P. lingam, Phomopsis vexans* and *Rhizopus spp.*

Chi-square test results of percent seed mycoflora infection gave the following test statistic values ($\chi^2 = 7012.17$, df = 29, and sig. = 0.00) and ($\chi^2 = 10067.26$, df = 33, and sig. = 0.00) for within pretreated and untreated seed samples respectively. From the test statistic values obtained, there are significant differences ($P \leq 0.05$) in percent seed mycoflora infection within treatments. Therefore, H_{0a} is rejected and H_{1a} is accepted.

Also in Table 1, a comparison of the percent seed mycoflora infection between pretreated and untreated seeds showed significant differences ($P \leq 0.05$) in the percent seed infections of *Alternaria solani, Aspergillus niger, Curvularia lunata, Curvularia trifolii* and *Fusarium oxysporum. Alternaria solani* recorded 2.55% (9.19) and 1.08% (5.97) for pretreated and untreated seeds; *Aspergillus niger* 0.50% (4.05) and 1.39% (6.77), *Curvularia lunata* 1.45% (6.92) and 0.68% (4.78), *Curvularia trifolii* 0.03% (0.99) and 0.34% (3.34), and *Fusarium oxsporum* recorded 15.97% (23.55) and 20.47% (26.90) respectively for pretreated and untreated seed samples.

Table 1: Comparison of individual mycoflora infection on pre-treated and untreated seeds

Fungi	Percent Seed Infection (%)	
	Pre-treated	Untreated
Acremoniun strictum	6.21 (14.43)	6.29 (15.22)
Alternaria circinans	2.05 (8.23)	1.37 (6.72)
Alternaria alternata	3.92 (11.42)	4.47 (12.21)
Alternaria sesami	0.42 (3.72)	0.13 (2.07)
Alternaria sesamicola	0.11 (1.90)	0.18 (2.43)
Alternaria solani	2.55 (9.19)*	1.08 (5.97)*
Aspergillus flavus	2.63 (9.33)	3.55 (10.86)
Aspergillus niger	0.50 (4.05)*	1.39 (6.77)*
Bipolaris spp	0.34 (3.34)	0.13 (2.07)
Botrytis cineria	0.32 (3.24)	0.21 (2.63)
Cercospora sojina	0.00 (0.00)	0.03 (0.99)
Cladosporum	1.66 (7.40)	2.37 (8.86)
Colletotrichum coccodes	0.05 (1.28)	0.03 (0.99)
Colletotrichum dematium	0.05 (1.28)	0.03 (0.99)
Colletotrichum gloeosporioides	0.08 (1.62)	0.39 (3.58)
Colletotrichum lindemuthianum	0.00 (0.00)	0.03 (0.99)
Colletotrichum acutatum	0.13 (2.07)	0.05 (1.28)
Corynespora cassiicola	3.71 (11.11)	3.24 (10.37)
Curvularia geniculata	0.05 (1.28)	0.00 (0.00)
Curvularia lunata	1.45 (6.92)*	0.68 (4.73)*
Curvularia pallescens	0.11 (1.90)	0.13 (2.07)
Curvularia trifolii	0.03 (0.99)*	0.34 (3.34)*
Exserohilum turcicum	0.00 (0.00)	0.03 (0.99)
Fusarium avenaceum	0.08 (1.62)	0.24 (2.81)
Fusarium equiseti	0.05 (1.28)	0.11 (1.90)
Fusarium moniliforme	3.24 (10.37)	4.63 (12.43)
Fusarium oxysporium	15.97 (23.55)*	20.47 (26.90)*
Fusarium pallidoroseum	12.87 (21.02)	11.95 (20.22)
Fusarium solani	5.79 (13.92)	6.32 (14.56)
Glomerella	0.00 (0.00)	0.11 (1.90)
Myrothecium leucotrichum	0.03 (0.99)	0.03 (0.99)
Phoma exigua	1.21 (6.32)	1.68 (7.45)
Phoma lingam	0.21 (2.63)	0.03 (0.99)
Phomopsis vexans	0.82 (5.20)	0.87 (5.35)
Rhizopus spp	0.00 (0.00)	0.11 (1.90)
Total	66.63 (54.71)	73.29 (58.89)
Mean	8.37 (16.82)	9.95 (18.38)
Standard Error of Mean	4.31	3.46

Note: Figures in parentheses indicate angular transformed values

* = Figures showed significant difference at $P \leq 0.05$

Infections recorded for pre-treated and untreated seed samples

Figure 9 shows the mean percent infections recorded for pre-treated and untreated seed samples after harvesting. The Figure shows a significant difference ($P \leq 0.05$) in the mean percent infections recorded for the two treatments. Treated seed samples recorded a mean percent infection of 9.94% (18.38) while untreated seed samples recorded a higher mean percent infection of 11.81% (20.10) out of 3200 seeds tested.

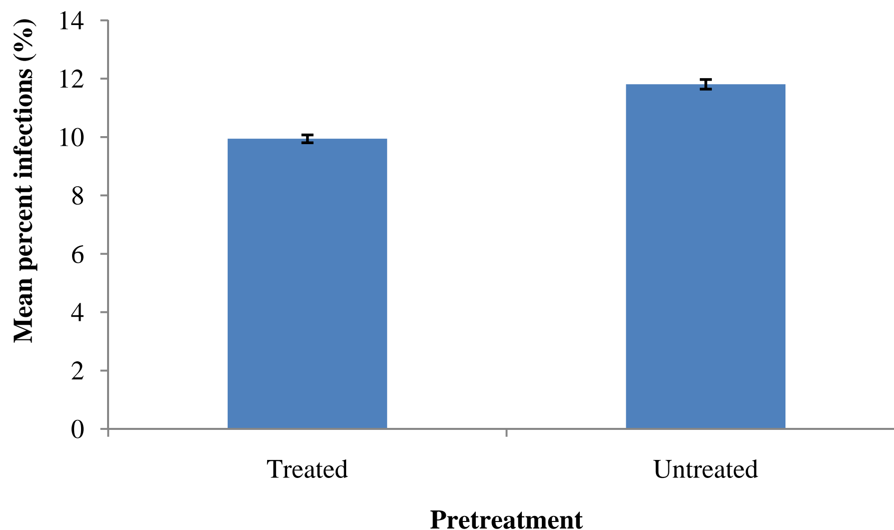

Figure 9: Mean percent infections recorded for pre-treated and untreated seed samples

Mycoflora infection on seeds extracted from 3 fruit types

The comparative percent seed infections of mycoflora on seeds obtained from 3 fruit types [(riped (2800 seeds), unriped (2400 seeds) and dropped fruits (2400 seeds)] are presented in Figure 10.

Alternaria alternata showed no significant difference between unriped 0.46% (3.88) and dropped 0.75% (4.97), but riped fruits 10.36% (18.77) were significantly different from unriped and dropped fruits. *A. sesame* also showed

no significant difference in percent seed infection for unriped 0.33% (3.31) and dropped 0.42% (3.70) however, both unriped and dropped were significantly different from riped fruits 0.11 (1.88).

There was a significant difference in the percent seed infection of riped fruits 7.89% (16.32) from unriped 0.42% 3.70) and dropped fruits 0.17% (2.34) but no significant differences exist between unriped and dropped fruits for *Aspergillus flavus* infection. *Aspergillus niger* infected only unriped 0.58% (4.38) and dropped fruits 2.42% (8.94). The percent seed infections were significantly different. *Botrytis cineria* also infected only riped 0.07% (1.53) and unriped 0.75% (4.97) but not dropped fruits and there was a significant difference in the percent seed infection of riped and unriped fruits. *Cladosporium sphaerospermum* showed no significant difference for unriped 1.58% (7.23) and dropped fruits 1.29% (6.53) but, it showed a significant difference between riped 3.00% (9.97) and dropped fruits.

There was no significant difference in percent seed infection of riped 0.39% (3.59) and unriped 0.25% (2.87) for *Colletotrichum gloeosporioides*; however, riped and unriped were significantly different from dropped fruits 0.04% (1.17). Further, *Corynespora cassiicola* showed no significant difference between riped 2.75% (9.55) and unriped fruits 1.83% (7.78), but, percent seed infection of dropped fruits 5.96% (14.13) were significantly different from riped and unriped fruits.

Curvularia lunata and *C. pallescens* both infected only riped and dropped fruits but not unriped fruits. *C. lunata* showed significant differences in percent seed infection of riped 0.89% (5.4) and dropped fruits 2.33% (8.79).

However, *C. pallescens* unlike *C. lunata* showed no significant difference for riped 0.14% (2.17) and dropped 0.21% (2.62).

Fusarium equiseti infected only riped 0.04% (1.08) and unriped fruits 0.21% (2.62) but there was no significant difference between the percent seed infections. For *F. moniliforme*, no significant differences existed in percent seed infection of riped 3.29% (10.44) compared with unriped 6.54% (14.82) and dropped fruits 2.08% (8.30) notwithstanding, there was significant difference between riped and dropped fruits. *F. oxysporum* on the other hand showed no significant difference in percent seed infection between unriped 28.96% (32.56) and dropped 18.29% (25.32). However, *F. oxysporum* infections in riped fruits 8.96% (17.42) were significantly different from both unriped and dropped fruits.

No significant difference exist between riped 4.50% (12.25) and dropped fruits 2.63% (9.32) but percent seed infection for unriped fruits 11.29% (19.64) was significantly different from riped and dropped for percent seed infection of *F. solani*.

Phoma exigua showed significant differences in infection among all 3 fruit types and was highest in dropped fruits i.e. riped 0.43% (3.75), unriped 1.97% 7.96) and dropped fruits 2.17% (8.46). *P. lingam* on the other hand only infected riped and unriped fruits but not dropped and there was no significant difference in the percent seed infection between riped and unriped fruits.

There was no significant difference in the percent infection of dropped 0.67% (4.68) fruits compared with riped 0.43% (3.75) and unriped fruits

1.50% (7.03). However, unriped fruits were significantly different from riped fruits in percent seed infection of *Phomopsis vexans.*

Alternaria circinans, Bipolaris cynodontis, Colletotrichum lindemuthianum, Curvularia trifolii, and *Glomerella* infected only unriped fruits at percent seed infections of 5.42% (13.46), 0.75% (4.97), 0.04% (1.17), 0.58% (4.38), and 0.17% (2.34) respectively.

Only dropped fruits were found to be infected with *Alternaria solani, Cercospora sojina, Colletotrichum acutatum, Exserohilum turcicum, F. avenaceum,* and *Rhizopus spp*. at rates of 5.75% (13.87), 0.04% (1.17), 0.29% (3.10), 0.04% (1.17), 0.50% (4.05) and 0.17% (2.34), respectively.

Alternaria sesamicola and *Curvularia geniculata* only infected riped fruits at percent seed infection rates of 0.39% (3.59) and 0.07% (1.53), respectively.

Acremonium strictum, and *F. pallidoroseum* showed no significant differences in percent seed infection among the riped, unriped and dropped fruit types .i.e. riped 5.89% (14.05), unriped 7.83% (16.25) and dropped 6.04% (14.23) for *A. strictum*; and riped 9.64% (18.09), unriped 12.54% (20.74) and dropped fruits 15.50% (23.18) for percent seed infection of *F. pallidoroseum. Colletotrichum coccodes* and *C. dematium* both infected only riped 0.04% (1.08) and dropped fruits 0.08% (1.65); riped 0.07% (1.53), dropped fruits 0.04% (1.17), respectively. Both fungi showed no significant differences in their percent seed infections.

Finally, *Myrothecium leucotrichum* was found to have only infected riped 0.04% (1.08) and dropped fruits 0.04% (1.08) which shows no significant difference between the 2 infections.

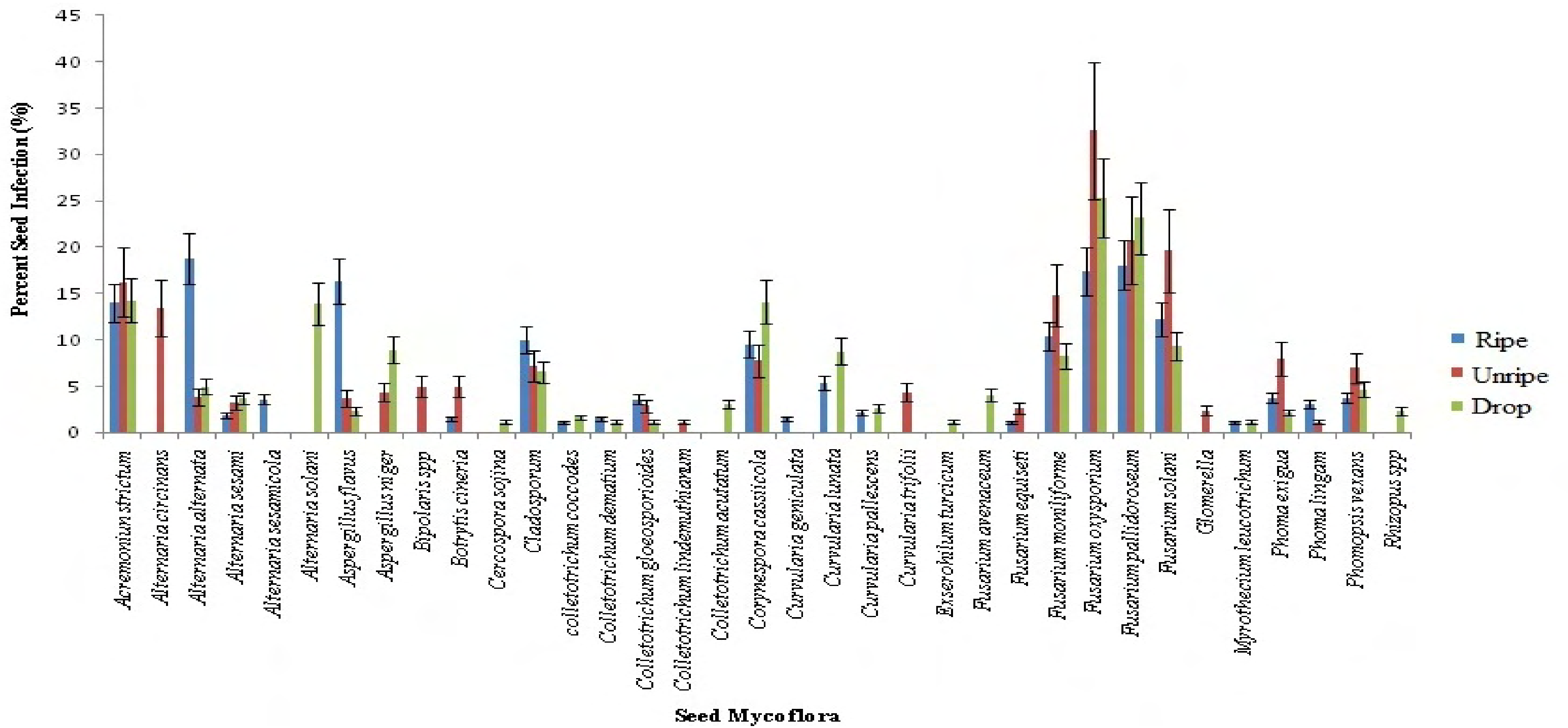

Figure 10: Comparison of percent seed infection of mycoflora on seeds extracted from 3 fruit types

Clustering of Locations and fruit types by mycoflora infection

The results of cluster analysis (CA) performed on fruit type-location data set are presented in Figure 11. The fruit type-location data was normalized using Ward's method of linkage with squared Euclidean distance as a measure of similarity. Cluster analysis performed in the R-mode on all the 19 samples examined revealed 3 distinct groups or clusters.

Cluster 1 consists of Esikado dropped, Akuamase A riped, Akuamase B riped, Mankessim riped, Mando dropped, Mando riped, Akuamase A dropped, Esikado riped, Esikado unriped, Nkodwo B unriped, Akuamase B unriped, Akuamase A unriped, Akuamase B dropped, Nkodwo A dropped, Nkodwo B dropped, Mando unriped, and Nkodwo A unriped. The sample Nkodwo B riped is in cluster 2 whereas Nkodwo A riped belongs to cluster 3. Thus, the R-mode CA grouped all the 19 samples into 3 statistically significant clusters (fig. 11).

The results of the R-mode principal component analysis (PCA) are presented in Table 2 with significant factor loadings in bold typed face. Three principal components were obtained with Eigenvalues greater than 1 (> 1), explaining 90.72% of the total variance in the sample data set.

The first principal component (PC1) was correlated with Esikado riped, Esikado unriped, Mando riped, Nkodwo B unriped, Akuamase A riped, Akuamase A unriped, Akuamase A dropped, Akuamase B riped, Akuamase B unriped and Akuamase B dropped. The second principal component (PC2) was correlated primarily with Mankessim riped, Mando unriped, Mando dropped, Nkodwo A riped, Nkodwo A unriped, Nkodwo A dropped and

Nkodwo B dropped whereas the third principal component (PC3) was weighted on Esikado dropped and Nkodwo B dropped.

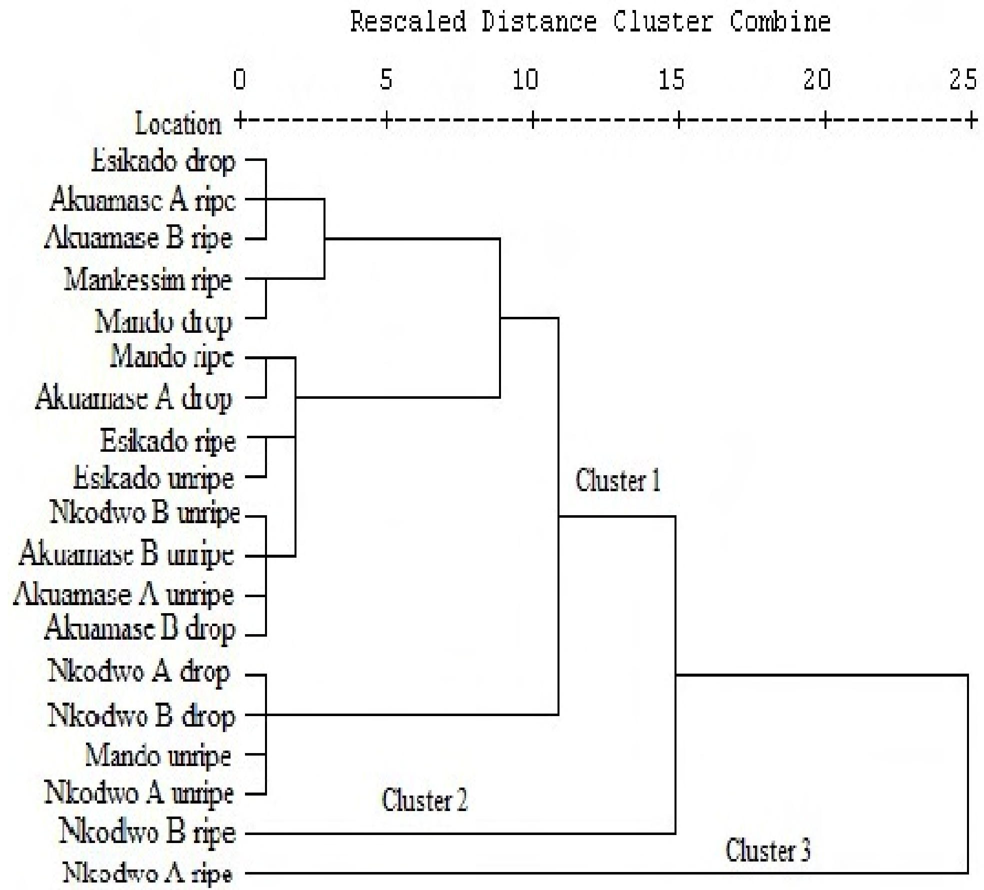

Figure 11: A dendrogram showing clustering of analysed samples of fruits taken from various locations

Table 2: Rotated component matrix of three factor model explaining 90.72% of the total variance for samples

Location and fruit type	PC1	PC2	PC3	Communalities
Mankessim (Ripe)	0.13	**0.98**	0.01	0.99
Essikado (Ripe)	**0.81**	-0.52	-0.05	0.92
Esikado (Unripe)	**0.89**	-0.27	-0.05	0.87
Esikado (Drop)	-0.12	-0.36	**-0.70**	0.63
Mando (Ripe)	**0.73**	0.67	0.03	0.99
Mando (Unripe)	0.44	**0.89**	-0.01	0.98
Mando (Drop)	0.30	**0.93**	-0.07	0.97
Nkodwo A (Ripe)	-0.11	**0.66**	-0.03	0.45
Nkodwo A (Unripe)	0.53	**0.84**	0.02	0.99
Nkodwo A (Drop)	0.45	**0.89**	0.00	1.00
Nkodwo B (Ripe)	-0.15	-0.25	**0.77**	0.67
Nkodwo B (Unripe)	**0.86**	-0.46	-0.01	0.96
Nkodwo B (Drop)	0.60	**0.80**	-0.01	0.99
Akuamase A (Ripe)	**0.79**	-0.51	0.32	0.99
Akuamase A (Unripe)	**0.90**	-0.42	0.01	0.98
Akuamase A (Drop)	**0.92**	0.37	0.04	0.99
Akuamase B (Ripe)	**0.78**	-0.49	-0.25	0.91
Akuamase B (Unripe)	**0.90**	-0.41	0.08	0.99
Akuamase B (Drop)	**0.89**	-0.42	-0.06	0.98
Eigenvalues	8.41	7.56	1.26	
Percentage of total variance	44.27	39.80	6.65	
Cummulative percentage of variance	44.27	84.07	90.72	

Clustering of 35 mycoflora identified on 19 seed samples tested for seed health

Figure 12 shows the results of cluster analysis (CA) performed on seed mycoflora infection data set. The data was normalized using Ward's method of linkage with squared Euclidean distance as a measure of similarity. The R-mode cluster analysis performed on all 35 fungi identified showed 12 distinct groups or statistically significant clusters.

Cluster 1 is made up of *Colletotrichum lindemuthianum, Exserohilum turcicum, Phomopsis vexans, Myrothecium leucotrichum, Curvularia geniculata, Colletotrichum coccodes, Colletotrichum dematium, Glomerella, Fusarium equiseti, Colletotrichum acutatum, Rhizopus spp., Curvularia pallescens,* and *Fusarium avenaceum*.

Other mycoflora in cluster 1 include *Alternaria sesami, A. sesamicola, Phoma lingam, Colletotrichum gloeosporioides, Botrytis cineria, Bipolaris cynodontis, Curvularia trifolii, Aspergillus niger, Phomopsis vexans, Phoma exigua,* and *Curvularia lunata. Alternaria circinans, A. alternata, Acremonium strictum, Alternaria solani, Aspergillus flavus*, and *Cladosporium sp.* are in clusters 2, 3, 4, 5, 6 and 7 respectively. Finally, clusters 8, 9, 10, 11 and 12 are made up of *Corynespora cassiicola, Fusarium moniliforme, F. oxsporum, F. pallidoroseum* and *F. solani,* respectively.

R-mode principal component analysis (PCA) with significant factor loadings in bold typed face is shown in Table 3. Twelve (12) principal components were obtained from the 35 mycoflora with Eigenvalues greater than 1 (1) explaining 92.89% of the total variance in seed mycoflora infection.

The first principal component (PC1) was correlated with *Alternaria sesami, A. solani, Colletotrichum acutatum, Curvularia lunata, C. pallescens, Myrothecium leucotrichum and Rhizopus spp.* PC2 was correlated with *Alternaria sesamicola, Cladosrorum, Colletotrichum dematium, C. gloeoesporioides, Fusarium pallidoroseum and Phoma lingam.* PC3 is weighted on *Botrytis cineria, Colletotrichum lindemuthianum, Fusarium equiseti, F. moniliforme, F. oxysporum and Phoma exigua whiles* PC4 is

weighted on *Aspergillus niger, Curvularia sojina and Fusarium solani. Bipolaris spp., Curvularia trifoli and Glomerella* are correlated with PC5 while *Acremonium strictum, Aspergillus flavus*, and *C. cassiicola* are correlated with PC6. *Alternaria circinans* and *Exserohilum turcicum* were correlated with PC7 while *Fusarium solani, Alternaria alternate and C. coccodes are weighted on* PCs 8, 9 and 10 respectively.

Finally, *Curvularia geniculata* and *Phomopsis vexans* are weighted on PC 11 whiles PC12 is weigthed on *Exserohilum turcicum*.

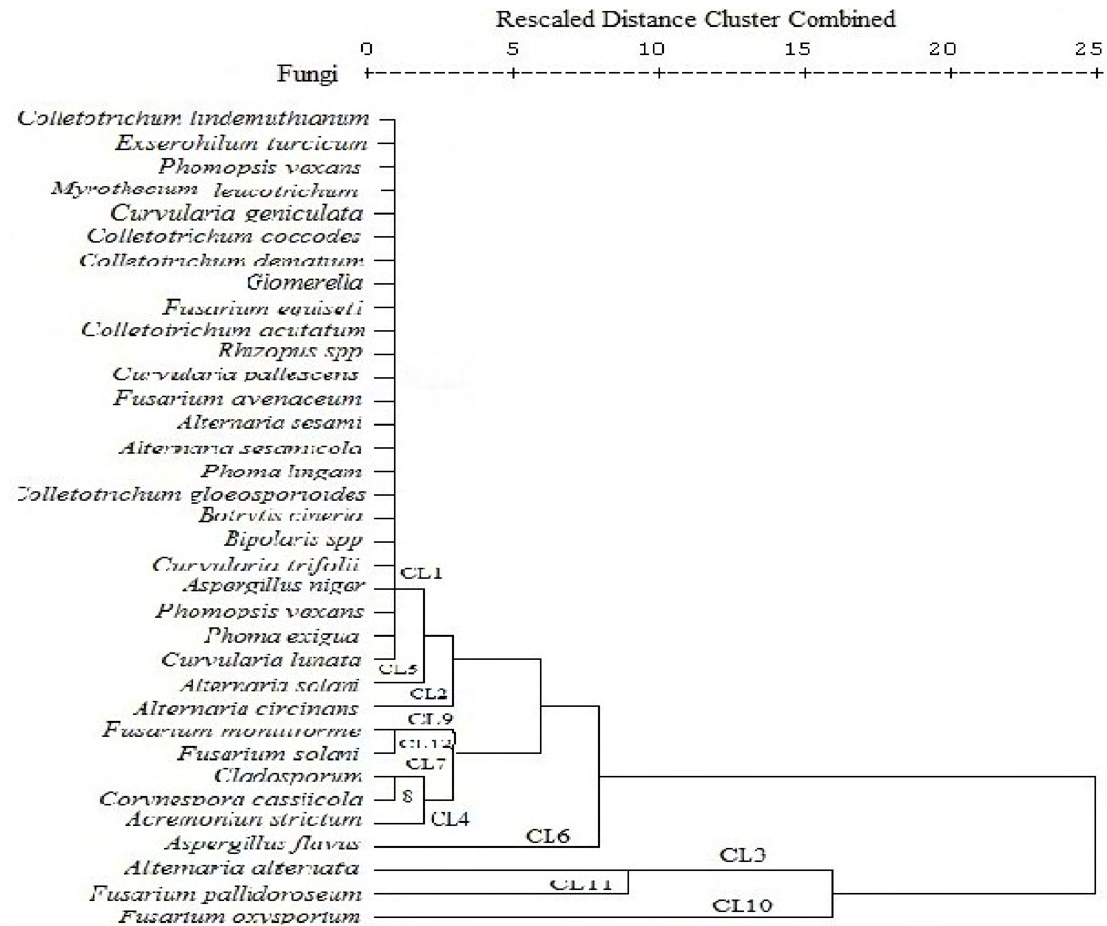

Figure 12: A dendrogram showing clustering of 35 analysed mycoflora observed on samples taken from study area

Table 3: Rotated component matrix of twelve factor model explaining 92.89% of the total variance for mycoflora on samples

Fungi	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12	Communalities
Acremoniun strictum	0.25	-0.38	0.13	-0.01	-0.06	**0.62**	-0.28	-0.24	0.04	-0.03	-0.32	-0.21	0.89
Alternaria circinans	0.12	-0.02	0.03	-0.06	0.04	0.26	**-0.63**	-0.02	-0.62	0.01	0.12	0.08	0.89
Alternaria alternata	0.24	0.12	-0.07	0.04	-0.09	0.07	-0.50	0.22	**0.68**	-0.25	-0.16	0.23	0.99
Alternaria sesami	**0.82**	0.08	0.21	0.12	0.08	0.14	-0.36	-0.10	-0.24	-0.05	0.13	0.06	0.98
Alternaria sesamicola	-0.16	**0.58**	-0.47	0.44	0.24	0.18	0.19	-0.16	-0.08	-0.17	0.03	0.21	1.00
Alternaria solani	**0.83**	0.10	0.21	0.04	0.12	-0.09	0.31	-0.18	0.06	-0.02	0.11	-0.14	0.92
Aspergillus flavus	0.05	0.10	-0.26	-0.03	-0.15	**-0.61**	-0.01	0.01	-0.15	0.49	-0.38	0.24	0.94
Aspergillus niger	0.30	0.05	-0.01	**-0.59**	0.42	0.00	0.37	-0.03	0.10	-0.07	-0.02	-0.17	0.80
Bipolaris cynodontis	-0.16	-0.59	0.03	0.46	**0.61**	-0.15	-0.07	0.03	0.11	0.07	-0.01	-0.02	0.99
Botrytis cineria	-0.37	0.40	**0.79**	0.09	0.00	-0.14	0.03	0.19	0.00	-0.01	-0.02	-0.02	0.98
Cercospora sojina	0.03	0.04	-0.08	**-0.69**	0.53	0.15	0.24	0.27	0.01	0.02	0.09	0.18	0.95
Cladosporium sphaerospermum	-0.09	**-0.61**	-0.12	0.34	-0.13	0.03	0.28	0.41	-0.12	-0.27	0.01	-0.09	0.88
colletotrichum coccodes	-0.12	0.04	-0.06	0.06	-0.19	0.40	-0.02	-0.05	0.26	**0.68**	0.31	-0.32	0.95
Colletotrichum dematium	-0.17	**0.55**	-0.49	0.44	0.15	0.32	0.17	-0.09	0.02	0.14	0.17	0.05	0.97
Colletotrichum gloeosporioides	-0.39	**0.67**	0.20	0.36	0.14	0.15	0.13	-0.05	0.00	0.05	0.01	-0.05	0.82
Colletotrichum lindemuthianum	-0.28	0.45	**0.69**	0.13	0.09	-0.13	0.01	0.37	-0.08	0.00	-0.13	-0.17	0.99
Colletotrichum acutatum	**0.87**	0.09	0.27	0.19	0.10	-0.05	0.22	-0.19	-0.01	0.00	0.12	-0.05	0.98
Corynespora cassiicola	0.17	-0.40	-0.07	0.20	-0.16	**0.57**	0.25	0.48	0.03	0.28	0.00	-0.11	0.96
Curvularia geniculata	-0.06	-0.05	-0.23	-0.02	-0.21	-0.39	0.01	0.20	-0.08	-0.41	**0.46**	-0.35	0.80
Curvularia lunata	**0.67**	-0.20	0.17	0.26	-0.24	0.22	0.45	0.15	-0.13	-0.14	-0.09	0.07	0.97
Curvularia pallescens	**0.74**	0.12	0.06	0.13	-0.02	-0.43	0.17	-0.13	-0.10	0.32	-0.16	0.11	0.96
Curvularia trifolii	-0.16	-0.59	0.03	0.46	**0.61**	-0.15	-0.07	0.03	0.11	0.07	-0.01	-0.02	0.99
Exserohilum turcicum	0.00	-0.28	0.05	0.11	-0.31	0.34	**0.35**	0.26	-0.18	-0.13	-0.29	**0.35**	0.74
Fusaium solani	0.03	0.04	-0.08	**-0.69**	0.53	0.15	0.24	0.27	0.01	0.02	0.09	0.18	0.95
Fusarium equiseti	-0.31	0.56	**0.59**	0.22	0.13	-0.10	0.05	0.34	-0.10	-0.04	-0.12	-0.13	0.99
Fusarium moniliforme	-0.46	-0.09	**0.48**	-0.14	-0.02	0.14	0.21	-0.44	0.21	-0.02	0.11	0.23	0.84
Fusarium oxysporium	-0.13	-0.44	**0.50**	-0.06	0.35	0.27	-0.06	-0.26	-0.32	-0.02	-0.06	0.13	0.86
Fusarium pallidoroseum	0.35	**0.56**	0.26	-0.02	0.29	0.30	-0.39	0.34	-0.02	0.09	0.14	0.09	0.98
Fusarium solani	-0.43	-0.35	0.41	0.00	-0.20	-0.07	0.01	**-0.46**	0.02	-0.09	0.34	0.22	0.91
Glomerella	-0.16	-0.59	0.03	0.46	**0.61**	-0.15	-0.07	0.03	0.11	0.07	-0.01	-0.02	0.99
Myrothecium leucotrichum	**0.78**	0.14	0.13	0.15	0.00	0.01	-0.22	0.05	0.48	-0.18	-0.05	0.12	0.99
Phoma exigua	-0.34	-0.08	**0.55**	0.06	-0.30	0.13	0.27	-0.04	0.25	0.09	0.12	0.24	0.75
Phoma lingam	-0.17	**0.57**	-0.47	0.41	0.24	0.19	0.19	-0.21	-0.08	-0.20	-0.04	0.15	0.99
Rhizopus spp	**0.87**	0.09	0.27	0.19	0.10	-0.05	0.22	-0.19	-0.01	0.00	0.12	-0.05	0.98
Phomopsis vexans	-0.24	0.23	0.02	-0.21	0.14	0.19	0.09	-0.46	0.05	-0.18	**-0.50**	-0.47	0.93
Eigenvalue	6.08	4.69	3.65	3.11	2.75	2.44	2.25	2.08	1.59	1.43	1.26	1.18	
Percentage of total variance	17.36	13.41	10.44	8.88	7.86	6.98	6.41	5.95	4.54	4.09	3.59	3.37	
Cummulative percentage of variance	17.36	30.77	41.20	50.08	57.95	64.93	71.34	77.29	81.84	85.93	89.51	92.89	

Fruit weights recorded for different methods of inoculation

Fruit weights obtained following different methods of inoculating plants on the field are presented in Figure 13. The yield (kg/ha) recorded for the various treatments, from highest to the lowest were, 7284.53, 6491.82, 5422.08 and 5136.20 for Control, Stem, Flower and Leaf inoculation methods respectively. The results showed no significant differences ($P \leq 0.05$) in the fruit weights recorded following the different inoculation methods.

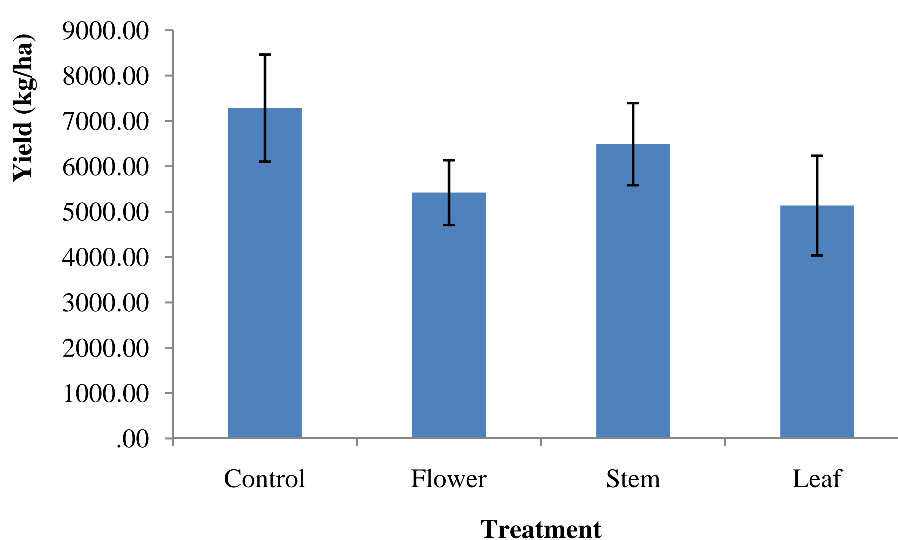

Figure 13: Fruit weights recorded for different inoculation methods

Mean number of fruits recorded for different methods of inoculation

Figure 14 shows the mean number of fruits recorded from the different methods of inoculating plants on the field. The control recorded the highest fruit number (3.25×10^6), followed by stem (2.89×10^6), flower (2.93×10^6) and leaf inoculation methods (2.31×10^6) fruits/ha. The results, as shown below indicate no significant differences ($P \leq 0.05$) in the mean number of fruits obtained following the different methods of inoculation.

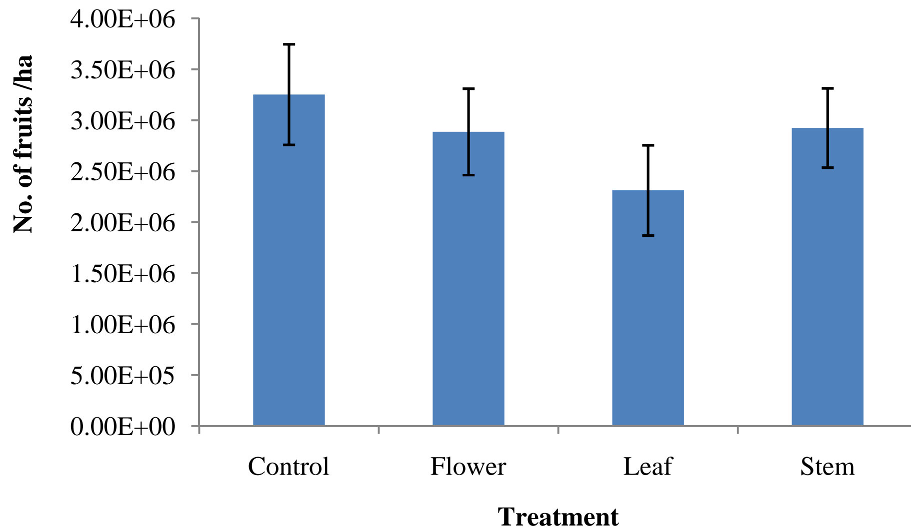

Figure 14: Mean number of fruits recorded for different inoculation methods

Average weight of fruits recorded for different methods of inoculation

The results of the average weight of fruits recorded for the various treatments (different inoculation methods) are presented in Figure 15. There were no significant differences ($P \leq 0.05$) among the flower (0.46 g), leaf (0.45 g) and stem (0.50 g) inoculation methods in the average weight of fruits obtained. There was however, a significant difference ($P \leq 0.05$) between the average fruit weight of the control (0.52 g) and flower (0.46 g) inoculation methods.

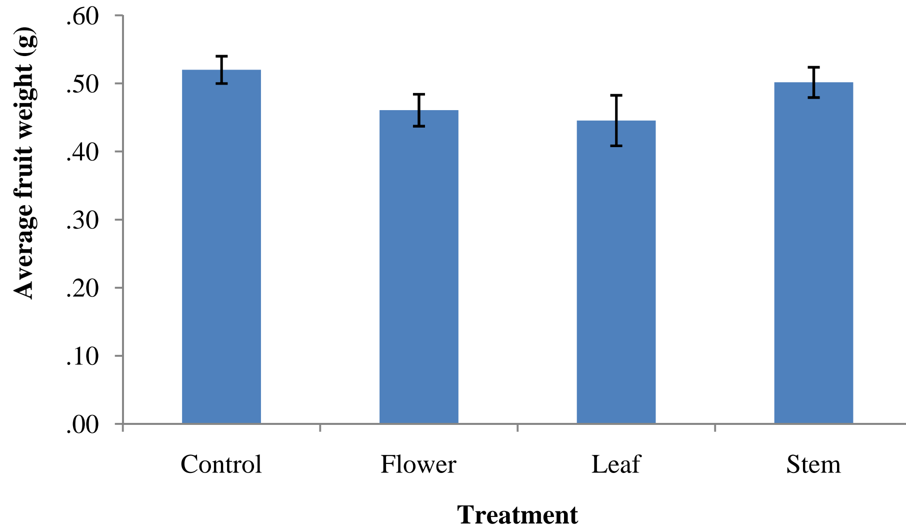

Figure 15: Average fruit weight recorded for different inoculation methods

Results of transmission studies through different methods of inoculation

Out of the three methods of inoculation (stem, flower and leaf inoculations) and control (4 treatments), only flower inoculation with *Colletotrichum dematium* resulted in seed transmission. No seed transmission was observed for the other treatments even after 10 weeks of inoculation.

At first harvesting (3 weeks after flower inoculation), only unriped fruits were produced and it showed only 1% transmission to seeds. Unriped fruits obtained from second harvesting (6 weeks after flower inoculation) recorded a mean percent transmission of 1.75% while riped fruits recorded 4.88% transmission to seeds. No records were obtained for the flower inoculation method after the second harvest (6 weeks post inoculation).

Mycoflora Infection on seeds produced from the School of Agriculture Teaching and Research Farm

The results of percent seed mycoflora infection on seeds extracted from plants inoculated with *C. dematium* at the School of Agriculture Teaching and Research Farm are presented in Figure 16.

From the results, *Aspergillus flavus, Alternaria alternata*, and *Fusarium oxysporum* were the three highest recorded fungi with seed infections of 13.02% (21.15), 12.31% (20.54), and 5.03% (12.95), respectively. However, there was no significant difference between *Aspergillus flavus* and *Alternaria alternata* but both organisms were significantly different from *F. oxysporum* in the percent seed infection.

There was also no significant difference between *Fusarium pallidoroseum* 3.23% (10.35) and *Aspergillus niger* 2.92% (9.83), and among *F. moniliforme* 2.27% (8.67), *Curvularia palliscens* 2.02% (8.17), *Phoma exigua* 1.86% (7.84) and *Curvularia lunata* 1.97% (8.07). Furthermore, no significant differences existed among *Phomopsis vexans* 0.72% (4.87), *Cladosporium sphaerospermum* 0.75% (4.97), *Colletotrichum dematium* 0.86% (5.32) and *Ulocladium consontiale* 1.06% (5.91) in their percent seed infections. *Macrophomina phaseolina* 0.44% (3.80), *Botryodiplodia theobromae* 0.28% (3.03), *Nigrospora oryzae* 0.69% (4.76), *Fusarium equiseti* 0.14% (2.14) and *Cercospora sp*. 0.34% (3.34) showed no significant difference in their percent seed infections. *Verticillium* recorded the least percent seed infection of 0.03% (0.99).

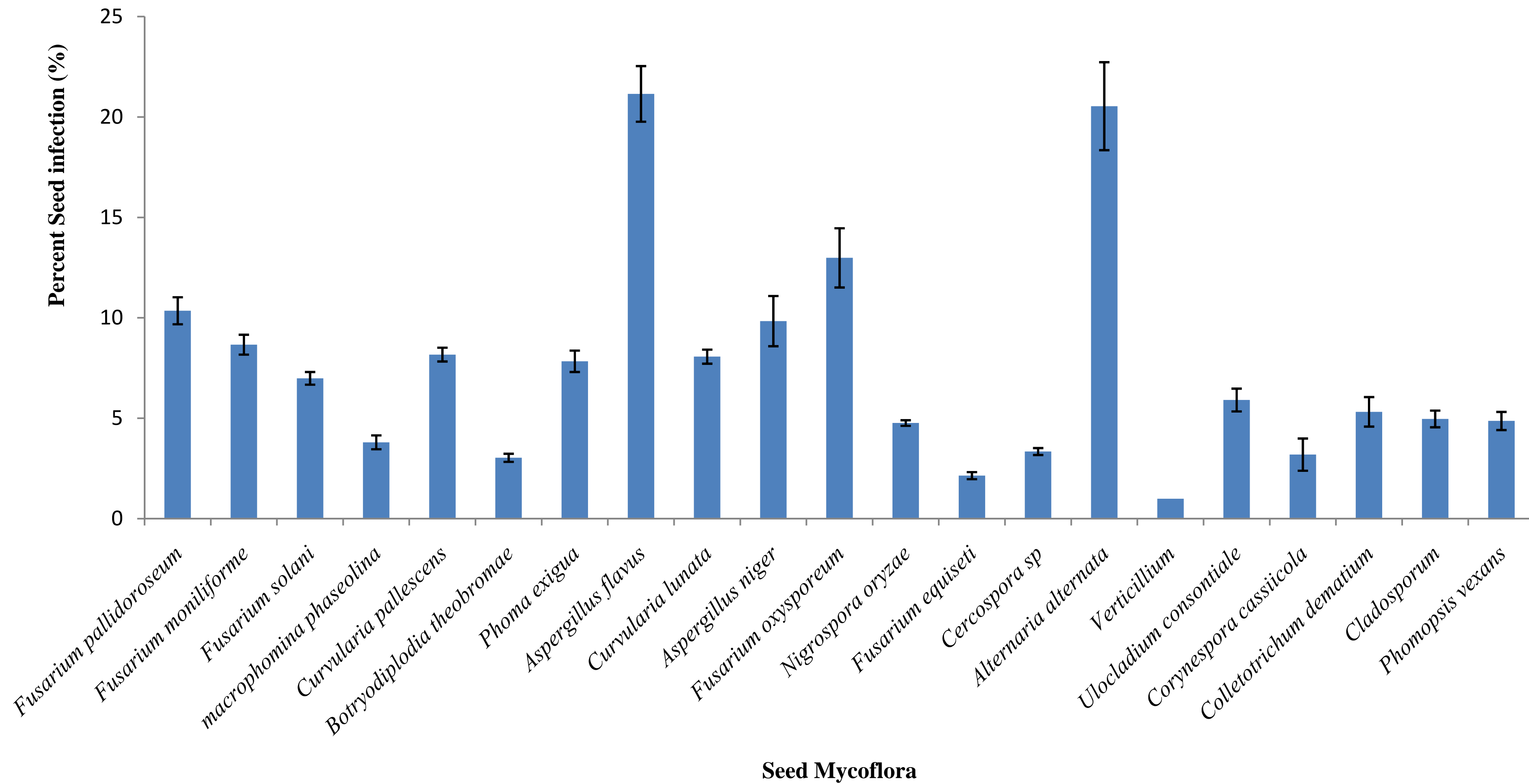

Figure 16: Percent seed infection by mycoflora

CHAPTER FIVE

DISCUSSION

The significant differences observed in the mean percent vigour and the mean percent germination of the samples tested using different media were unexpected and are not in conformity with ISTA standards. This is because both media (i.e. sand and blotter) have been recommended by the ISTA in the International rules for seed testing (ISTA, 2003b) for germination testing of pepper seeds. The lower mean percent vigour and germination recorded for the blotter method could be due to differences in the moisture available to the seeds during the test period and therefore could be more of a physiological reason than genetic (Dornbos, 1995). Since the sand was covered with polybags during the test period while the blotters were only covered with the lids of the petri dishes, the blotters relatively lost more water to the environment more rapidly than the sand. The significant differences could also be attributed to the all round surface area contact in the sand as a medium compared to the blotter method in which the seeds only absorbed moisture from the points of contact with the moist blotter.

The observed significant differences in terms of mean percent vigour and mean percent germination of the seed samples showed the variations in the quality of the seeds from the various locations. The differences in seed

quality were expected because of the differences in the environmental conditions as well as the differences in the relative levels of seed mycoflora infections from one location to another. Ahmed (1982) has stated that seeds heavily infected with mycoflora showed poor germination and vigour. Finch-Savage (1995) has also observed that seed-borne mycoflora adversely affect germination and early seedling emergence. The findings of this study are at variance with the observations of Ahmed (1982) and Finch-Savage (1995) because some seed samples which recorded higher values for vigour and germination also recorded relatively more seed mycoflora infections but with lower inoculum densities. The differences could therefore not solely be due to the presence of seed mycoflora but could also be the result of physical damage to some of the seeds or physiological decline in quality (Dornbos, 1995; Copeland & McDonald, 2001) adverse environmental conditions or provenance effects as noted by Hemannavar (2008).

The observed differences in seed vigour among the three fruit types might be due to the differences in seed physiology (Bennett, 2004). This observation could also be due to the differences in the amounts of assimilate composition in the three fruit types, as well as early breaking of dormancy in seeds obtained from riped and dropped fruits compared to seeds obtained from physiologically matured but unriped fruits.

The non-significant differences observed in mean percent germination for unriped and dropped fruits and the observed significant difference between riped fruits and both unripe and dropped, were expected. Unriped fruits recorded the least mean percent germination and this is supported by Bennett

(2004). He found that, seeds with physiological problems in terms of low food reserves as a result of nutrient deficiencies or less number of days for assimilate accumulation have low germination rates. This finding may also be due to the high percent seed infection observed for unriped fruits, followed by dropped fruits and the least percent seed infection observed for riped fruits (Appendix II). This is in agreement with the findings of Ahmed (1982) and Finch-Savage (1995). The results also compare favourably with the work by Dornbos (1995), Doijode (2001) and Copeland and McDonald (2001) who observed among other things, that, considerable vigour and viability loss, and deterioration may occur through physical damage, physiological decline and mycoflora infection.

The results of this work showed no significant differences in the mean percent mycoflora infection contrary to the general expectation that dropped fruits would have higher mean percent mycoflora infection. Dropped fruits were expected to record higher mean percent infection irrespective of the maturity (whether riped or unriped) probably due to infections from soil-borne mycoflora in addition to infections from air-borne mycoflora or those transmitted from the mother plant. Though no significant differences were observed in mycoflora infections, the higher levels of capsaicin in riped fruits compared to unriped fruits could account for the differences in infection rates. Norman (1992) observed that the level of capsaicin is higher in riped fruits than unriped fruits. The level of capsaicin is thought to have contributed to the differences in mycoflora infection among the three fruit types (riped, unriped and dropped) because high levels of capsaicin have been found to inhibit the growth of certain fungi (Kraikruan *et al.,* 2008).

The numbers of species of fungi and genera isolated in this work were found to be less than the number of species and genera identified by Sharfun-Nahar and Iqbal (2004). The number of species and genera observed however, were far more than the fungal species identified by Mesta (1996) and Hemannavar (2008). Hemannavar (2008) observed that the differences in the number of fungi identified could be due to provenance effects, such as the differences in environmental factors (climatic conditions) within different locations, and differences in cultural practices (watering, weeding, pruning, etc.) from one farm to another. These could influence the fungal genera and species that may be found in an area.

Since different fungi have different potencies or infection capacities (Agrios, 2005), the results of percent mycoflora infections observed within treatments were therefore expected. The growth of most fungi especially pathogenic fungi, are inhibited by the presence and growth of saprophytic fungi (Neergaard, 1979). Therefore, where the two types of fungi are present in a sample, differences in their percent infections or counts are expected. The significant differences in the percent infections of *Curvularia lunata, Alternaria solani* and *Aspergillus* agree with that obtained by Khan *et al*., (1988) who found that rice seeds pre-treated with sodium hypochlorite led to a higher incidence of *Curvularia spp, Fusarium spp, Myrothecium*, among others. The authors also observed that the occurrence of other fungi such as *Cladosporium spp*., *Chaetomium* and *Aspergillus spp*. considerably reduced. The increase in the percent incidence of *Alternaria* agrees with Mesta (1996) who stated that surface sterilization of chilli seeds did not eliminate *Alternaria* and *Cercospora* from some chilli seeds. The results of this work are however

in contrast with the observations of Mesta (1996). This could either be due to differences in the species identified in the two different researches or due to differences in strains of the mycoflora as a result of mutation or development of resistance with time and location.

Though the incidence of *Curvularia lunata* increased with the pre-treatment with NaOCl and is in agreement with Khan *et al.* (1988), the same could not be said of the incidence of *C. trifolii* in chilli. Therefore, the response to NaOCl pre-treatment within the same genus of *Curvularia* could be different in chilli. Hence, the generalization of the response of the pathogen in rice could be true but not applicable in chilli pepper seeds. The variation in response could be attributed to the relative differences in infection (growth) of the two pathogens since variation in growth may even occur within the same species or different strains of the same species.
The incidence of *Fusarium oxysporum* in untreated seed samples was higher than in treated samples. A similar trend in incidence was observed for the other species of *Fusarium* (except for *F. pallidoroseum*) in this study. This observation however, is at variance with the findings of Khan *et al.* (1988) and could be due to the differences in the strains of fungi.

The significant difference observed between pre-treated and untreated samples was expected because the incidence of saprophytic fungi were expected to be reduced by the pre-treatment, and hence, the low mean percent infection recorded for pre-treated samples. This could also be due to the fast or vigourous growing habit of most saprophytic fungi than pathogenic fungi which are slow growing. The significant difference could also be due to the

fact that most of the fungi identified in this study were pathogenic (slow growing) rather than saprophytic (fast growing) Khan *et al*., (1988).

The results of this study therefore confirms the statement made by Danquah (1973) that chlorine treatment does not follow a regular behaviour and its action does not depend on whether an organism is a saprophyte or a pathogen.

Contrary to what was stated by Asalmol *et al*. (2001) that *Aspergillus flavus, Rhizopus, Fusarium moniliforme, Colletotrichum capsici* and *Aspergillus niger* (from highest to lowest) were the predominant seed-borne fungi in chilli seeds, this work proved contrary. This is because the predominant fungi found in this work were *F. oxysporum, F. pallidoroseum, Alternaria alternata, F. solani* and *Aspergillus flavus*. This finding also contradicts that of Hemannavar (2008) who found only 5 fungal species on chilli seeds i.e. *Colletotrichum capsici, Cercospora* sp., A*lternaria* sp., *Penicillium sp.* and *Aspergillus* (from highest to lowest incidence). Hemannavar (2008) further observed that differences in incidences of chilli mycoflora could be due to the differences in the environmental conditions (chief among them being moisture, temperature and edaphic factors) prevailing in different locations.

Considering the infection patterns of fungi in the 3 fruit types examined, the results showed that some fungi infected only certain fruit types. The reasons for the differences in the patterns of infection by some of the fungi could not be fully explained since there were no consistent patterns shown in infection of fruit types by particular fungi. However, this could partly be due to the differences in the tolerance of the various fungi to the levels of capsaicin in the various fruit types since the level of capsaicin varies

in the various fruit types (Norman, 1992). This assertion could hold because Kraikruan *et al.* (2008) have proven that high levels of capsaicin prevented the growth of *Colletotrichum capsici*.

The cluster analysis used all the variance or the information contained in the original or raw data on mycoflora infection on the various fruit types from the various locations. Wards' method was selected for sample classification because it possesses a small space distortion effect, uses more information on cluster contents than other methods (Pallant, 2007) and has been proven to be an extremely powerful grouping mechanism (Boamponsem *et al.,* 2010).

Following sample analysis, 3 statistically different (distinct clusters) could be observed in the sample cluster dendogram at a cut off distance of 15. Clustering of samples were based on three factors; *viz* type of fungi infecting samples, number of seeds infected and number of fungi (number of species) infecting a particular sample. Based on the above mentioned criteria, Esikado dropped, Akuamase A riped, Akuamase B riped, Mankessim riped, Mando dropped, Mando riped, Akuamase A dropped, Esikado riped, Esikado unriped, Nkodwo B unriped, Akuamase B unriped, Akuamase A unriped, Akuamase B dropped, Nkodwo A dropped, Nkodwo B dropped, Mando unriped, and Nkodwo A unriped were clusterd in cluster 1. This means all these samples at a distance of 15 on the rescaled distance cluster shared some similarities in the type(s) of fungi infecting them, the percent infection, as well as the number of species of fungi.

On the other hand, Nkodwo B riped and Nkodwo A riped belonged to two statistically different clusters i.e. clusters 2 and 3. This meant that the two

samples showed significant differences from the remaining 17 samples. This was so because even at a distance of 1 on the rescaled distance cluster, the 2 samples belonged to 2 different clusters from the rest (Figure 11). Nkodwo B riped recorded infections from only 5 species of fungi whiles Nkodwo A riped recorded infections from a total of 11 fungal species.

Principal component analysis (PCA) was used to separate the various samples into clusters because it is a powerful pattern recognition technique that attempts to explain the variance of a data set of intercorrelated variables with a smaller set of independent variables (principal component) (Hussain *et al.,* 2008). The PCA was performed in R-mode. The 3 clusters formed were confirmed by the rotated component matrix because communalities greater than 0.3 were obtained. This therefore gives more information about how much of the variance in each item was explained. According to Pallant (2007), communalities less than 0.3 (< 0.3) could indicate that an item does not fit well with other items in its component.

Cluster analysis of all 35 species of fungi identified from the 19 samples yielded 12 statistically significant clusters based on 2 factors (infection patterns). The factors were; samples infected by particular species of fungi as well as the number of seeds of the sample infected. A distance of 2 was adopted as cut off point on the rescaled distance cluster for grouping fungi since highly significant differences were sought for. This was informed by the fact that different fungi (saprophytic and pathogenic) infect seeds differently and cause different levels of damage to seeds in particular and to plants in general, hence, the need for seed health testing and seed quarantine if some species of fungi are detected (Neergaard, 1979).

Cluster 1 weighed significantly more than any cluster and this was confirmed by the R-mode PCA where it showed cluster 1 alone contributing a significant proportion to the total variance. Again, cluster 1 was the most compacted cluster meaning all the fungi in that cluster were very similar in their infection patterns. This is because, compactness is said to be a measure of how similar to one another the elements of a cluster are (Anonymous, 2002).

Clusters 2 to 12 were each weighted on one fungal organism, and therefore show that they each had different patterns of infection or were unique in the samples infected and the number of seeds infected. Though *Alternaria alternata* and *Fusarium oxysporum* were significantly different from each other, they both showed the same level (highest) of distinctness from a scale of 1-16.5 before a similarity could be drawn from their modes of infection. It has been stated that the measure of distinctness indicate how different the clusters are from their closest neighbours. The statistically significant clusters formed were confirmed by the rotated component matrix which showed each of the fungi recording communalities greater than 0.3 (0.3) and therefore, this indicates that each fungus fitted well either on its own or with the other fungi in its component.

The general performance in terms of the yield obtained was lower than achievable yields given by the SRID of MoFA (2006) which is between 6.5-18 mt/ha depending on the variety. This could be due to poor seed quality as a result of provenance effect (Hemannavar, 2008) or deterioration in the genetic quality of the seeds since the seeds used for this work were extracted from fruits collected from farmers' field (Finch-Savage, 1995).

Comparatively, the leaf inoculation method yielded the lowest followed by the flower inoculation method. This could be due to the effect of the two treatments because the procedures for the two inoculation methods involved covering the plants with transparent poly bags for 48 hrs in order to maintain high relative humidity (95%) for incubation of the inoculums (conidia) inoculated onto the plants (Kim *et al.,* 2007; Posada *et al*., 2007).

The covering of the plants as in the two treatments could have interfered with the process of photosynthesis, thereby causing shock over the period or a period of 'dormancy' in the plants. Hence, the two treatments recording lower yields compared to the control and stem inoculation methods which did not require covering of the plants. The reason why the leaf inoculation method recorded a lower yield compared to the flower inoculation method could be due to the sunken lesions or necrotic spots and mottling observed on the leaves after the incubation period which negatively affected photosynthesis.

The yield of chilli expressed as number of fruits/ha followed a similar trend as the yield on weight basis. That is, the leaf and flower inoculation methods produced the lowest number of fruits per ha. Therefore, the same reasons could be advanced for the observed differences in the number of fruits produced per treatment per ha. However, the non-significant differences could imply that the observed differences were not necessarily as a result of the treatments imposed on the plants, but by some other factor.

In contrast to the trend of the results on yields obtained in terms of the weights of fruits/ha and the number of fruits produced/ha, the average weight of fruits produced/ha according to the treatments imposed showed a significant

difference. This could be attributed to the effect of the treatments in two directions. The first is the direct effect of the covering of the plants with poly bags for 48 hrs thereby affecting the photosynthetic processes (assimilate production and partitioning). This assertion is made because the two treatments which involved covering the plants for 48 hrs both had lower values.

The second reason could be due to the effect of the development of necrotic spots on the leaves thereby reducing the photosynthetic surface area of the leaves because the leaf inoculation method resulted in infection of the leaves by the pathogen (*Colletotrichum dematium*). This second reason could explain why the flower inoculated plants had slightly heavier average fruit weight than the leaf inoculated plants which had no leaf spots (lesions).

From the results of the transmission experiment, no transmission was observed for the inoculation methods (i.e. stem injection and leaf inoculation) except for flower inoculation. According to Kumar *et al*. (2004), the pathogen (i.e. *C. dematium*) was never found to be systemic in transmission trials performed, and that though the pathogen was observed in the embryo of infected seeds to the extent of 31.25%, the pathogen was transmitted to young seedlings through the inoculum developed on infected seeds by local contact.

The zero (0) seed transmission recorded for both stem and leaf inoculation methods agrees and confirms the findings of Kumar *et al.* (2004) that *Colletotrichum dematium* is not systemic. Fungal infection of seed-borne pathogens according to Singh and Mathur (2004), may reach the ovule and seed at any stage from the initiation of ovule to mature seed. The authors

further recognized two passages for the invasion of fungi in seeds, *viz*., routes leading to internal ovary infection and the second being ovary to ovule and seed infection. Singh and Mathur (2004) further stated that among other methods, infection may take place directly from the outside through stigma-style, ovary or fruit wall and other floral parts including nectaries or sites for the receipt of inoculum. Prabhu *et al*. (1983) have described this mode or mechanism of transmission as a secondary infection by the conidia through the stigma, style and ovary. The success of the transmission for the flower inoculation method therefore affirms the findings of Prabhu *et al.* (1983) and Singh and Mathur (2004).

Seed health testing of the chilli seeds obtained from the harvested chilli fruits from the University farms (Cape Coast) where the transmission test was carried out showed *Aspergillus flavus* to be the most predominant fungi. This confirms the findings of Asalmol *et al*. (2001) who observed *Aspergillus flavus* to be the most predominant among 5 predominant fungi associated with chilli seeds. The next 4 predominant fungi observed were *Alternaria alternata, Fusarium oxysporum, Aspergillus niger* and *F. pallidoroseum*. Besides *C. capsici,* Mesta (1996) found also other fungi like *Alternaria alternata, Cercospora, Fusarium* and *Curvularia* as being predominant on chilli seeds. Therefore, *Alternaria* being the second predominant fungi associated with the chilli seeds produced from Cape Coast agrees with the findings of Mesta (1996).

Among the fungi found associated with the seeds obtained from Cape Coast, *Macrophomina phaseolina, Botryodiplodia theobromae, Nigrospora oryzae* and *Verticillium* are new seed-borne record on the host.

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Summary

The findings of this study are summarized as follows:

1. From the seeds used for the study, sand as a medium had higher mean percent vigour and germination than blotter paper as a medium.

2. Seed Samples collected from Esikado performed better than the seeds collected from the remaining six locations both in mean percent vigour and mean percent germination.

3. Seeds extracted from riped fruits recorded the highest mean percent vigour and mean percent germination followed by seeds from dropped fruits and the least was the seeds extracted from unriped fruits.

4. Unriped fruits recorded the highest mean percent infection.

5. In general, within a particular treatment (pre-treated and untreated seeds) different fungi infected seed samples at different rates.

6. Significant differences exist in percent seed infection of *Alternaria solani, Aspergillus niger, Curvularia lunata, Curvularia trifolii* and *Fusarium oxysporum* for pre-treated and untreated samples.

7. On the whole, pre-treated seeds recorded lower seed infection than the untreated.

8. Some fungi were observed to have infected seeds extracted from only unriped fruits or dropped fruits whiles some infected seeds extracted from all

three fruit types. Eg. *Alternaria sesamicola* and *Curvularia geniculata* only infected seeds extracted from riped fruits; *Alternaria circinans, Bipolaris cynodontis, Colletotrichum lindemuthianum, Curvularia trifolii,* and *Glomerella cingulata* infected seeds extracted from only unriped fruits; while *Alternaria solani, Cercospora sojina, Colletotrichum acutatum, Exserohilum turcicum, F. avenaceum,* and *Rhizopus spp* infected only seeds extracted from dropped fruits.

9. The 19 seed samples could be clustered into 3 statistically significant clusters based on the fungal species infecting the samples, the number of seeds infected and the number of species of fungi infecting a particular sample.

10. The 35 mycoflora isolated could be clustered into 12 statistically significant clusters based on the samples infected by the particular species of fungi and the infection rates of the fungi.

11. Leaf and Flower inoculation methods resulted in low fruit yields as well as lower number of fruits though the differences were not statistically significant.

12. Leaf inoculation method recorded the least in terms of the average weight of fruits produced.

13. Only flower inoculation resulted in seed transmission of *Colletotrichum dematium*.

14. Among the fungi found associated with seeds obtained from the School of Agiculture Teaching and Research Farm *Macrophomina phaseolina, Botryodiplodia theobromae, Nigrospora oryzae* and *Verticillium* are the new seed-borne record on the host in Ghana.

Conclusions

The following conclusions were made from the study;

1. On the average, the incidence of *Colletotrichum* spp. observed for ripe, unripe and drop fruits were 6.2%, 4.04% and 7.09% respectively and the incidence of *C. dematium* alone was 1.53% for ripe fruits and 1.17% for drop fruits.
2. Other rot-causing fungus found on the seeds was *Alternaria alternata* and the percent infection for riped, unriped and dropped fruits were 18.77%, 3.85% and 4.97% respectively.
3. The five most prevalent fungi observed on the seeds were *Acremonium strictum, Fusarium solani, F. moniliforme, Corynespora cassiicola* and *Alternaria alternata*.
4. Seeds extracted from the ripe fruits recorded the highest percent vigour and germination whilst the unripe fruits recorded the least.
5. Unripe fruits recorded the highest mean mycoflora infection.
6. Sand as a medium gave the highest percent vigour and germination than the blotter method.
7. Only flower inoculation resulted in transmission of *C. dematium* to seeds.
8. Transmission of *C. dematium* to seeds was observed after 3 weeks post inoculation.
9. Leaf inoculation resulted in lower average fruit weight.
10. No significant differences exist in yield of chilli inoculated through the different methods, though leaf inoculation recorded the least.

Recommendations

1. Sand is better as a medium for seed vigour and germination testing of chilli seeds.

2. Transmission studies should be tried during the major cropping season to compare transmission levels for different seasons.

3. Inoculum concentration and volume could be varied in subsequent trials to compare transmission rates.

4. Pathogenicity tests should be conducted with the new seed-borne record on the host in Ghana.

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APPENDIX

Appendix I:

N, P, and K levels at the research site (School of Agriculture Teaching and Research Farm)

	pH	%N	P(μg/g)	K(cmolckg^{-}1)
	7.60	0.06	0.14	0.70
	7.70	0.06	0.14	0.70
Mean	7.65	0.06	0.14	0.70

Appendix II:

Percent mycoflora infections for different fruit types

Fruit Type	Total no. of mycoflora infections	Percent Infections
Ripe	1671	59.68
Unripe	2016	84.00
Drop	1630	67.92

Appendix III: ISTA working recording sheet No. 1 for the blotter test

WORKING RECORDING SHEET NO. 1 FOR THE BLOTTER METHOD

Accession No. ___________________ Host__________________________

Date of plating __________________ Date of recording ______________

Analyst No. _____________________ Method _______________________

No. of seeds per dish 25 Total No. of seeds tested _______

Dish No. / Fungi	1	2	3	4	5	6	7	8		Remarks*

*Use this space if extra space is needed for writing remarks.

____

Signature of the analyst