ISOLATION AND ANTIFUNGAL SUSCEPTIBILITIES OF PHYTOPATHOGENIC FUNGI FROM YAM LEAVES (*Dioscorea rotundata and cayenesis*) IN ZARIA, NIGERIA.

By

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December, 2012

DECLARATION

I Sa'adatu Mu'azu Jodi hereby declare that the work in this dissertation entitled "Isolation and antifungal susceptibilities of phytopathogenic Fungi from yam leaves (*Dioscorea rotundata*) in Zaria, Nigeria" was carried out by me in the Department of Pharmaceutics and Pharmaceutical Microbiology, ABU, Zaria under the supervision of Professors J. O. Ehinmidu, Stephen Olonitola and Dr. T. S. Allagh. It has not been presented in any previous application for degree at any university. The information derived from literature and the work of other investigators has been duly acknowledged both in the text and reference list accordingly.

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CERTIFICATION

This dissertation entitled "ISOLATION AND ANTIFUNGAL SUSCEPTIBILITIES OF PHYTOPATHOGENIC FUNGI FROM YAM LEAVES (D*ioscorea rotundata*) IN ZARIA, NIGERIA" by Sa'adatu Mu'azu Jodi meets the regulations governing the award of the degree of Doctor of Philosophy (Ph.D) of Ahmadu Bello University, and is approved for its contribution to Knowledge and literary presentation.

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DEDICATION

I dedicate this work to my Family and well wishers

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In the Name of Allah, the Most Beneficent and the Most Merciful. Every new altitude in life should command a heart of gratitude if you must go to a greater altitude. With this, firstly, my profound gratitude goes to Almighty God, the most high, whose mercy and kindness is beyond human knowledge and measure, for God indeed is the author of knowledge. I am truly grateful to Him for standing by and with me all through up to this moment. To Him be all the glory and praises forever.

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ABSTRACT

Fungal attack of yams leaves has been reported to substantially result in low yam tuber yield. This study was designed to isolate phytopathogenic fungi from yam leaves in Zaria, Nigeria and to evaluate their susceptibilities Minimum Inhibitory Concentration (MIC) and Minimun Inhibitory Concentration (MFC) to some antifungal agents (Terbinafine; Fluconazole; Flucytosine; Benomyl and Sodium propionate). The fungistatic activities of these chemical compounds alone and in combinations at varying pH, temperature and different water sources used as diluents were investigated. Fourteen fungi species namely *Aspergillus niger*, *Cladosporium sp, Aspergillus ostianus, Scopolarium sp, Aspergillus flavus, Rhizopus sp, Penicillium citrinum, Aspergillus wentii, Penicillium oxalicum, Aspergillus cervinus, Penicillium sp, Aspergillus fumigatus, Aspergillus oryzae* and *Rhizopus stolonifer* were isolated from infected yam leaves in eleven farms in Zaria, Nigeria. The MIC values varied significantly from one organism to another. Generally, *Aspergillus niger, Penicillium citrinum, Aspergillus cervinus,* and *Rhizopus stolonifer* were the most resistant to the test antifungal agents. The order of fungistatic activity (MIC) of the test antifungal agents against resistant *Rhizopus stolonifer* spores was Flucytosine (200.00µg/ml) > Terbinafine HCl (250.00µg/ml) > Benomyl (500.00µg/ml) > Fluconazole (1000. 00µg/ml) > Sodium propionate (2500. 00µg/ml). However the lethal activity profile was not the same with the fungistatic activity and the order of lethal activity (MFC) was Terbinafine HCl (500.00µg/ml) > Flucytosine (1600.00µg/ml) > Benomyl (2000.00µg/ml) = Fluconazole (2000. 00µg/ml) > Sodium propionate (5000. 00µg/ml). The effect of fungicidal concentration of the test antifungal agent against viable test fungal spores at different time interval showed rapid sporicidal effects. The cmbination of either Terbinafine HCl or Fluconazole or Benomyl with Sodium propionate displayed

synergistic antifungal activity with FICs of 0.098 to 0.140 against resistant *Rhizopus stolonifer* spores. The antifungal effects of the test antifungal agent and their combinations were shown to be more active at temperature of 45^0C. Combinations of the antifungal agents' were effective at neutral pH with the exception of Terbinafine HCl and Sodium propionate that were only active at alkaline pH. The antifungal activities of the combined test antifungal agents were reduced when unboiled water sources were used as diluents. The in-vivo antifungal activity of the potential fungicides combination such as Benomyl/Fluconazole (1000/5,000µg/ml), Benomyl/Terbinafine HCl (1000/200µg/ml) and Benomyl/Sodium propionate (1000/15,000µg/ml) were observed to protect yam leaves from resistant *Aspergillus niger* spores ($2.0x10^8$cfu/ml) on the farm in Zaria, Nigeria.

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LIST OF ABBREVIATION

µg	=	microgram
ml	=	millilitre
%	=	Percent
Min	=	minute(s)
L.G.A	=	Local Government Area
Flu	=	fluconazole
Np or Sop	=	sodium propionate
Ben	=	Benomyl
Ter	=	Terbinafine and sodium propionate
MIC	=	Minimum Inhibitory Concentration
MFC	=	Minimum Fungicidal Concentration
FFC	=	fractional fungicidal concentration
FIC	=	fractional Inhibitory Concentration

CHAPTER ONE

INTRODUCTION

Yams (*Dioscorea* spp) belong to the Family Dioscoreceae, order Liliales, subclass lilidae, class liliopsida (monocotyledons), division magnoliophyta (seed plants), sub-kingdom spermatophyte (flowering plants), sub kingdom tracheobionta (vascular plants) and finally kingdom plantae (plants) (Coursey, 1967a'b; Okigbo and Ikendiugwu, 2000; Okigbo and Nmeka, 2005 and USDA 2009).

The cultivated yam species in Nigeria are *D. rotundata* (white yam), *D. cayenesis* (yellow yam), and *D. alata*, (water yam). There are also species of wild yam growing in Nigeria whose tubers are collected for eating in times of food shortage such as *D. dumtorum* (Cluster, or bitter yam), *D. esculenta* (*Loir bark Chinese yam*) and *D. bulbifera* L. (aeria yam) (Adeniji, 1970; Okigbo, 2004).

Yams are valuable source of carbohydrate, minerals (calcium, phosphorus, iron) and vitamins (riboflavin, thiamine and vitamin B and C) to the people of tropical and sub-tropical Africa, central and South America, parts of Asia including parts of China, Malaysia, Japan and Oceania and the Caribbean Islands (Coursey, 1967a'b; Okigbo and Ikediugwu, 2000; Okigbo and Nmeka, 2005; Panneerselvam *et al.,* 2007). Some species of yam are used as pharmaceutical bases and the toxic bye products have been used for hunting and as insecticides

(Degras, 1993; Panneerselvam *et al.,* 2007; Jaleel *et al.,* 2007). In addition, there are numerous medicinal values from yams, which have been used medically to treat diseases such as diabetes mellitus, to increase coronary flow and prevent hypercholesterolemia. Some yam bye products have also been used in dermatology, gastroenterology and traditional human and veterinary gynaecology in Asia, Africa and America (Coursey, 1967a).

Yam tuber can be cooked in various ways by boiling and mashing, but roasting and frying are widely used. Yam in Nigeria is processed in various staple forms and yam bye-products have been used for direct consumption by animals, and as the basic ingredients for snacks or flour used for making instant puree (Coursey, 1983; Okaka and Okechukwu, 1987; Okaka and Anajekwu, 1990; Okaka *et al,* 1991).

Nigeria alone has been reported to produce three quarters of the worlds total output of yams. For instance, in 1998, Nigeria produced about 70% of all yam tubers. The Food and Agricultural Organization (FAO, 1998) estimated that out of the world production of over 30 million tonnes per year, Nigeria alone produces 22 million tonnes. Losses in yams in storage mostly due to rot are considered to be heavy in Nigeria. These losses are attributed by many workers to be due to microbial (fungi, bacteria and nematode) rots (FAO, 1998). Post-harvest handling and storage of yams is an essential aspect of economic development in Nigeria. It was estimated that economic loss due yam rots was in the tune of two hundred million naira (=N=200m) or two million dollars ($2m), (Coursey, 1967b; Ayensu and coursey, 1972). Despite this, demand for Yam tubers in Nigeria has always exceeded its supply. This is because yam tuber is an

important socio-cultural crop that is prominent in the cultural and religious festivals of the people of West Africa (FAO, 1994). Farming and production of this tuber is a major source of income for farmers and traders. Yam has the potential to contribute significantly to the alleviation of food security concerns in West Africa (Bancroft, 2000) and foreign exchange earnings as an export crop (Degras, 1993).

1.1 Statement of research problems

In spite of its importance, post harvest deterioration of yam has been a major problem facing farmers and traders (Osagie, 1992; Knoth, 1993; Amusa *et al.,* 2003). It has been estimated that an average of over 25% of the yield is lost annually to diseases and pest (Arene, 1987; FAO, 1998) with estimated losses in storage of over three hundrend and twenty million naira (=N=320m) or two million dollars ($2m) (Coursey, 1967b). These losses are attributed by many workers mostly to rot caused by bacteria, fungi and nematodes. The disease causing organisms not only reduce the quantity of yam produced, but also the quality by making them unappealing to the consumers. Yam is exposed to infections right from the seedling stage through to harvesting and even after harvesting, in storage (Adeniji, 1970; Enyi, 1970; Ekundayo and Naqvi, 1972; Ferguson, and Gumbs, 1977; coursey and Ferber, 1979; Amusa *et al*., 2003). Yams are susceptible to a variety of pest and diseases during growth as well as postharvest, and these infections are devastating. The major postharvest disease of yam tubers is mostly caused by fungi (Opera, 1999). Most of the pathogens of yam tuber are soil-borne, but manifestations of the tuber disease are observed mostly during storage (Jone, 1985). While these fungal infections originate in

the fields, they often are not fully evident at the time of harvest and may carry on and spread during storage after harvest (Demand Media, 2010).

The use of synthetic chemicals such as sodium orthiophenylphenate, borax, captan, thiobendazole, benomyl, bleach (sodium hypochloride) has helped in increasing yields and significantly reduces storage rot in yam (Brooth, 1974; Noon, 1978). Also treatment of yam tubers with fungicides such as benlate and captan is reported to be effective in reducing fungal yam rot (Ogundana, 1971; Ogundana *et al.,* 1981; Barnard *et al.,* 1997). This notwithstanding, there is a major problem of resistance emergence of the target organisms, chemicals potential accumulation in the ecosystem, chronic toxicity to humans and animals (Balasubramaniam *et al.,* 1993; Okigbo and Ikediugwu, 2000; Harris *et al.,* 2001; Okigbo, 2004; Mosley *et al.,* 2011).

1.2 Significance of the Study

Phytopathogenic fungi can be serious competitors with yams tuber production in the field. Fungal diseases, which produce black necrotic lesions on yam leaves and stems, can, kill the plant by attacking the terminal bud, and leaf spot. Control involve yam leaves diseases removal of infected crop debris, and fungicide treatment, such as benomyl (Methyl [1-[(butylamino)carbonyl]-1H-benzimidazol-2-yl]carbamate), benomyl (Methyl [1-[(butylamino)carbonyl]-1H-benzimidazol-2-yl]carbamate)+ propineb (polymeric ainc propylenebis [dithiocarbamate]), zineb (zinc ethylenebis [dithiocarbamate]) and mancozeb (Dithiocarbamate) have all been reported to give reasonably good control measure in the yam yam field.

1.3 Aim and Objectives

1.3.1 Aim

This study was designed to isolate phytopathogenic fungi from yam leaves and determine their susceptibilities to single and combination of conventional chemical agents at varying environmental conditions such as different water sources, pH and temperature.

1.3.2 Specific Objectives

To achieve the aim of this study, therefore the following specific objectives were examined:

1. Isolation and identification of phytopathogenic fungi from white yam leaves (*D. rotundata)* from eleven yam farms in Zaria.
2. Determination of predominant pathogenic fungi associated with white yam (*D. rotundata)* spoilage right from the field in Zaria.
3. Assessment of the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of the test conventional chemicals against the test phytophatogenic fungi isolated alone and in combination.
4. Determination of combined antifungal properties of the carious antifungal agents against resistant fungal spores.
5. Determination of the rate of kill of resistant *A. niger* spores isolated with one and admixtures of test antifungal agents.
6. Determination of the effect of the antifungal agents on the DNA release from cytoplasmic constituents of resistant *A. niger* spores isolated.
7. Determination of the effects of different water used as diluent on the antifungal activities of test fungicides and their combinations.

8. Determination of the presence of organic and inorganic matter in the different water sources used in these investigations.
9. Determination of the effect of different pH and temperatures on the antifungal activities of antifungal agents.
10. Determination of the *in-vivo* effect of the potential combined fungicides on yam leaves infected with phytopathogenic fungi spores.

1.3 Null Hypothesis:

1. *Dioscorea rotundata and D. cayenensi* leaves in Zaria are not infected by Phytopathogenic fungi.
2. When Phytopathogenic fungi are present, they are not sensitive to antifungal agents.

CHAPTER TWO

LITERETURE REVIEW

2.1 MICROORGANISMS OF PLANTS

All plants surfaces have natural flora of microorganisms which may be sufficiently specialized to be referred to as the phylloplane flora, for the leaf surface and the rhizoplane flora for the surface of the roots. The number of organisms on the surfaces of healthy young plant leaves may be quite low, but the species which do occur are well adapted for this highly specialized environment (Almeida *et al.,* 2002; Saunders *et al.,* 2010).

Plants have evolved several mechanisms for resisting microbial infection, but there are many species of bacteria and fungi which overcome this resistance and cause disease in plants and some of these may also cause spoilage problems after harvesting and storage (Royle, 1976; Dodman, 1979; Mount, 1978; Durbin, 1979; Kollattukudy, 1985; Agarwal and Sinclair, 1997; Saunders *et al.,* 2010).

2.1.1 History of Plant Pathology

Plant pathology (Phytopathology) is that branch of Agricultural science which deals with the cause, etiology, resulting losses, and management of plant diseases. In 1858 Julius Kuhn, in Germany, had defined plant disease as abnormal changes in physiological processes which disturb the normal activity

of plant organs. According to American Phytopathological Society, plant disease is a deviation from normal functioning of plant physiological processes of sufficient duration or intensity that can cause disturbance or cessation of vital activities. The British Mycological Society defines disease as a harmful deviation from the normal functioning of plant physiological processes. Plant diseases mainly occur when phytopathogens alter one or more of a series of phytophysiological processes within the host plant, (Saunders *et al.,* 2010). This eventually manifest as morphological changes which are commonly referred to as symptoms. Sometimes, the presence of the phytopathogen is more obvious than any morphological change in the plant. Visible growths of the pathogen are often called the signs of the disease as seen in rusts and powdery mildews (Balasubramaniam, 2007).

Like human beings and other animals, plants are also subject to diseases caused by micro-organisms. In order to maintain a sufficient food supply for the world's population, it is necessary for those involved in plant growth and management to find ways to combat plant diseases that are capable of destroying crops on a large scale (Science Encyclopedia, 2008). Crops are any organisms that humans utilize as a source of food, materials, or energy. Crops may be utilized for subsistence purposes, to barter for other goods, or to sell for cash profit (Harlan 1971; Saunders *et al.,* 2010 and Yeni, 2011).

The literature of human civilization from the ancient time speaks of the truth that ever since man started depending on plants for his needs, the enemies of plants have been harming the organized agriculture and creating unfavorable

conditions of food storage and consequent famine. The learned men in the Vedic period were aware of the fact that plant diseases can be caused by microogarnisms. This fact was not believed by a vast majority of scientist in Europe until about 125 years ago. Vraksha Ayurveda's book is the first book eclusively devoted to diseases of plants in ancient India. The foundation of modern experimental plant pathology was laid by the German scientist Anton de Bary (1831-1888). The credit for a detailed study of the late blight fungus, its nomenclature, and proof of organisms being plant pathogens goes to the work of de Bary. He studied other diseases also which included rusts, smuts, downy mildews, and rots (Kwon-Chung and Bennett, 1992).

2.1.2 General Symptoms of Plant Diseases

Plant disease development depends on the interactions between the pathogen, host plant and the environment (Saunders *et al.,* 2010). When a plant is infected by a pathogen, there are often a number of symptoms which form a characteristic symptom-picture to which the specific disease name is given (Balasubramaniam, 2007). Symptoms are seen on plant either due to character and appearance of the visible pathogen or due to its destructive effect on/in the plant (Hansen and Wick, 1993; Moorman, 2008 and Johnson, 2008).

2.1.3 Isolation and identification of plant disease causative agent

Isolation of fungi usually requires that pieces of infected plant tissue be placed on various nutrient media (Shutleff and Averre, 1997).The organism that grows

out of this tissue is then isolated in pure culture (Putnam, 1995; Agrios, 1997; Waller *et al.,* 1998).

Microorganisms are often isolated by chopping up infected tissue in a small amount of sterile water aseptically. The microbial suspension is then streaked onto a selective microbiological medium and incubated appropricitely. Koch's postulates (Agrios, 1997), which involves the inoculation of healthy plants with the isolated organisms, may be necessary to conclusively its disease causative profential.

Once an infectious plant pathogen is successfully isolated, the organism must be identified (Kendrick, 2000).

2.2 FUNGI

Fungi are extraordinary organisms which are plant-like organisms with thallus body. Fungi are eukaryotic with true nucleus; nuclear membrane and mitochondria. Unlike plant, fungi lack chlorophyll (Schlegel, 1995; Fawole and Oso, 1995) and most of them absorb nutrient from the environment (Kwon-Chung and Bennett 1992; Heritage *et al.,* 1996; Mahon *et al.,* 2007).

2.2.1 The Importance of fungi

Fungi are extremely beneficial microorganisms. They act as decomposers of complex organic compounds and inorganic molecules. By this recycling method, carbon; nitrogen; phosphorus and other critical constituents of dead organisms are released and made available for living organisms in the soil (Prescott *et al.,* 2005).

Additionally, fungi have been reported to absorb water and dissolved minerals which enhance plant growth. Humans use certain fungi for food (mushrooms, puffballs and truffles), religious ceremonies (because of their hallucinogenic properties), as aphridiacs and in the manufacture of foods and beverages (Kvesitadze and Kvesitadze 1999; Wippat *et al.,* 2002), such as bread, alcoholic beverages, citric acid (the basis of soft drink industry), soy sauce, and some cheeses as fermenters. Fungi have also been reported to produce antibiotics such as penicillin, griseofulvin, cephalosporins and non-antibiotic immunosuppressive drug such as cyclosporine, which makes organ transplants possible, and mevinic acid, which are cholesterol- reducing agents (Singleton and Sainsbury, 1978; British Mycological Society, 2011).

In addition, fungi are important research tools in the study of fundamental biological processes. Cytologists, geneticists, biochemists, biophysicists, and microbiologists regularly use fungi in their research (Prescott *et al.,* 2005). But not all fungi are beneficial – about 30% of known fungal species produce mycoses, which are fungal diseases of plants, animals, and humans (Bauman, 2006).

2.2.2 Distribution

Fungi are found worldwide, distributed throughout the environment in soil, plants and water. Fungi are primarily terrestrial organisms, although a few are freshwater or marine (Prescott *et al.,* 2005).

2.2.3 Dispersal of fungi

The two methods of reproduction in fungi sexual and asexual are intended to accomplish more or less two distinct acts in the life history of the fungus. The asexual spores are primarily meant for rapid dissemination while the sexual fruits mainly help the fungus to survive through adverse conditions. Dispersal of fungal has three distinct stages: liberation or take off (spore release), transport or flight (dispersal) and finally deposition or landing on the new site (Kwon-Chung and Bennett 1992; Science Encyclopedia, 2008; Moorman, 2008 and Johnson, 2008).

2.2.3.1 Active discharge of spores

In active discharge the spore are provided with some structural mechanism or physiological responses which enable them to be thrown out with some force. E.g Liberation due to bursting of turgid cells: very common in Ascomycotina for the release of ascospores from asci (Kwon-Chung and Bennett 1992; Science Encyclopedia, 2008; Moorman, 2008 and Johnson, 2008).

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2.2.3.2 Passive discharge of spore

In passive spore liberation the spores is mainly force from some outside source such as strong air current, raindrops falling on the spore mass, and insects' visiting the surface containing the spores (Kwon-Chung and Bennett 1992; Science Encyclopedia, 2008; Moorman, 2008 and Johnson, 2008).

2.2.3.3 Dispersal of spores

Spore dispersal is essentially a passive process determined by physical agencies such as wind or water or living organisms such as insects and higher animals including man. Modes of disparsal vary with the habitat of fungi and their morphological characteristics. These include mycelial growth (mostly in soil fungi), dispersal by soil movement, dispersal by water, dispersal by air, dispersal by seed, animals and raindrop splashes.

2.2.4 Phenomenon of infection

A parasitic fungus must establish an intimate relationship with the host tissues to absorb the desired nutrient. Successful establishment of this relationship is called infection. During penetration, the infective hyphae or rhizoid tips enter the host by any of the following method:

1) Entry through natural openings likes stomata, lenticels, etc.
2) Entry through rupture of the host surface due to development of organs like prop roots.
3) Entry through wounds due to mechanical or insect injury.
4) Direct penetration:

a) Mechanical pressure by the infective rhizoid of the growing mycelia.
b) Chemical action ie. Enzymic activities of the mycelia hyphae or rhizods (Kwon-Chung and Bennett 1992; Heritage *et al.,* 1996; Casida, 2009).

The entry of the infective fungi thread into the host is dependent upon several environment factors. After entry, if physiological conditions of the host are favorable, the infective fungi thread will invade the nearest cell. This will result

in the absorption of food from the host protoplasm. In addition to absorption of food, the fungus may interfere with the host activity in various ways such excretion of destructive enzymes to dissolve the cell-wall or toxins may be produced which may cause various types of damage to plant tissues. Due to these interferences the host may exhibits symptoms of disease conditions. The fungus will continue growth, produce spores or reproductive unit, which will find an exit through the host surface and spread to repeat the same process as above in similar plant in the surrounding environment (British Mycological Society, 2011).

2.2.5 Classification

The classification of fungi is based primarily on the characteristics of the sexual spores and fruiting bodies during stages of their life cycles (Pelczar *et al*., 1999; Kazlev, 2002). Mycelial fungi or moulds are classified according to both their macro and micro-morphology. Yeasts are structurally simpler and therefore display a limited range of morphology which creates difficulties in their classification; therefore their group is subdivided partly on the basis of their reaction in biochemical tests (Kwon-Chung and Bennett 1992; Heritage *et al.,* 1996; Buzzle, 2010). The following groups and families are of pharmaceutical interest (Kazlev, 2002; Collins *et al.,* 2004; Grady, 2005; Evans, 2005; Casida, 2009).

Table 2.1: Classification of Fungal of Pharmaceutical interest

	Order	Families
Phycomycetes	Mucorales	Mucoraceae
Ascomycetes	Protoascales	Saccharomycetaceae
	Plecatscales	Aspergillaceae
	Sphaeriales	Hypocreaceae
	Clavicipitales	Clavicipitaceae
Basidiomycetes	Polyporinales	Polyporaceae
	Agaricales	Tricholometaceae
		Amanitaceae
		Agaricaeae
	Phallinales	Phallinaceae
Fungi imperfecti	Moniliales	Dematiaceae

(Kazlev, 2002; Collins *et al.,* 2004; Grady, 2005; Evans, 2005; Casida, 2009).

2.2.5.1 Phylum Phycomycetes: Order Mucorales

The fungi Family Mucoraceae have an aseptate mycelium; members include *phytophthora infestans,* which causes potato blight. In the mucoraceae we have mucor (40 spp) and *Rhizopus* (8 spp), which are among the moulds associated with badly stored food products. Some *Rhizopus* species are used industrially for the saccharification of starchy material and for producing D-lactic acid from glucose; they are important in the microbiological conversions of steroids (Evans, 2005).

2.2.5.2 Phyllum Ascomycetes:

Order Protoascales

The Family Saccharomycetaceae of the order Protoascales includes the yeasts, some 30 species of *Saccharomyces*. Dried yeast is prepared from a strain of *S. cerevisia*. A member of the related family cryptococcaceae is *Candida utilis*, which produces torula yeast, a rich source of single cell proteins and vitamins (Evans, 2005).

Order Plectascales

The family Aspergillaceae belongs to the order plectascales, in this order the conidial stage is more prominent than the ascale stage *penicillum* (over 100 spp). They Yields important antibiotics such as penicillin and griseofulvin: also the immunosuppressant mycophenolic acid. *P. islandicum* forms emodin. Among the 60- species of *Aspergillus* may be noted *A. oryzae*, used in the manufacture of soya sauce; *A. fungigatus*, producing the antibiotic fumagillin; and *A. flavus* producing aflatoxin in poorly stored feeding materials (Kazlev, 2002; Collins *et al.,* 2004; Grady, 2005; Evans, 2005; Casida, 2009).

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Order Sphaeriales

The family Hypocreaceae consist of *Gibberella* (10 spp) which produces the plant growth regulators known as gibberellins, first isolated from *G. fugikuro* (Evans, 2005; Casida, 2009).

Order Clavicipitales

The family Clavicipitaceae Like other Ascomyeetes, the ascospores are produced in a sac or ascus genera of the clavicipitaceae include claviciptaceae include *clavieps* (10 spp) and *cordyceps* (100 spp).

2.2.5.3 Phylum Basidiomycetes

Order Polyporinales

In the family Polyporaceae the basidiomycetes produce basidiospores, bome externally on the spore mothercell or basidium. They have septate mycelia which produce elaborate fruiting bodies (e.g. mushrooms). Some members are edible, others poisonous.

The polyporaceae includes *polyporus, polystichus, fomes, ganoderma* and *Boletu*s (200 spp*), polyporus officinalist* (white agaric) and *P. fomentarius* were formerly used in Chinese medicine and its biologically active triterpenoids have attracted recent attention. *Boletus edulis* is edible (Collins *et al.,* 2004; Grady, 2005; Evans, 2005).

Order Agaricales

Tricholometaceae is afamily from an order of several families, of which the tricholometaceae contains *Clitocybe* (80 spp), species of which produce muscarine. Other families of the order contain *stropharia, psilocube* and *conocybe*, which produce hallucinogenic substances such as psilosin and

psilocybin (Kazlev, 2002; Collins *et al.,* 2004; Grady, 2005; Evans, 2005; Casida, 2009).

Order Agaricales

The Agaricaceae family includes the common edible mushroom, *Agaricus campestris*, the genus *Agaricus (psaliota)* contains about 30 species.

Order Phallinales

This Phallinaceae is one of the two families of the order Phallinales. The phallinaceae takes its name from *phallus* (10 spp), *phallus impudicus* is the stinkhorn known for it activities with antibiotic production (Casida, 2009).

2.2.5.4 Fungi Imperfect: Moniliales

The Family Dematiaceae belongs the fungi imperfecti and it is a group in which sexual spores have not been deomonstrated. Some members may be ascomycetes which have completely lost the ascus stage. The family dematiaceae contains *Helminthosporium* (150-200 spp), a species of which, *H, graminium*, produces helminthosporin another family of the group, the tuberculariaceae, contains *Fusarium* (65 spp). *Fusarium lini* will transform digitoxigenin into digoxigenin (Evans, 2005; Mahon *et al.,* 2007).

2.2.6 Pathogenesis

The vast majority of the known fungal species are strict saprophytes, although there are a few capable of causing disease in plants animals or humans. However, there are several fungal genera containing species that cause disease (e.g., infections, allergies, toxicity) in plants, animals and man. These fungi can be categorized into two groups in regards to infection: (1) saprophytic fungi which can be opportunistic pathogens that enter via wounds or due to a weakened state of the host and (2) true pathogens that may depend on living plant or human tissues for nutrients but can also survive outside of the hosts. The fungal species that cause not only disease in plants and but also in humans include *Aspergillus* and *Fusarium* as well as other phytopathogenic fungi now considered members of the "emerging pathogen" group in humans (MCH, 2008; Mahon *et al.,* 2007). It has been widely reported that *Aspergellus* sp and *Fusarium* sp are common Phytopathogenic fungi isolated from infected yam tubers (Ogundana, 1971; Ogundana *et al.,* 1981; Okezie *et al.,* 1981; Bancroft, 2000; Koirala *et al.,* 2005 and De Lucca, 2007).

2.3 PHYTOPATHOGENIC FUNGI OF INTEREST IN AGRICULTURE

Most fungi are saprophytic however; a relatively few fungal species are phytopathogenic, and produce toxins which affect plants, animals and humans. Such fungi include *Aspergillus* sp*, Fusarium* sp*, Alternaria* sp*, Curvularia* sp*, Cladosporum* sp*, Colletotrichum* sp and *Mucorales.* These fungi present a

common threat to both agricultural production and the health of healthy and immunocompromised human beings. These relatively few fungi have been reported to cause huge economic losses to agriculture production and serious but often fatal diseases in humans and animals (Ainsworth and Austwick, 1973; De Lucca, 2007).

2.3.1 *Aspergillus*

The *Aspergillus* genus are saprophytes found worldwide in soil, forage products, food products, dust, organic debris and decomposing matter, although they are considered weak plant pathogens (Gugrani, 2003).

2.3.1.1 Aspergillus infection in Plant

Plants have several levels of resistance (structural, innate, induced) to fungal infection. However, certain factors, such as heat stress, insect damage and drought, weaken crop resistance to these fungi, and so play an important role in the fungal invasion of these crops and subsequent toxin production (Leitao *et al.,* 1988; Abbas *et al.,* 2002; Cotty *et al.,* 1994; Windstom, 1996; Mac million *et al.,* 1978; Pitt, 2000; Koirala *et al.,* 2005). Two species of *Aspergillus* such as *Aspergillus flavus* and *Aspergillus parasiticus* have been reported to produce potent aflatoxins on infected crops and food stuff (Kollattukudy, 1985; Versonder *et al.,* 1991; Severns *et al*., 2003).

2.3.2 *Fusarium*

The genus *Fusarium* consists of septate filamentous fungi commonly reported found in soil, marine or river environments, and some on plants throughout the world. Some are soil saprophytes while others are true plant pathogens (Guy St-Germain and Summerbell, 1999).

2.3.2.1 Fusarium infection in Plant

The phytopathogens of this genus are the most important toxigenic fungi on crops in the northern temperate regions (Gutleb *et al*., 2002; Schollenberger *et al*., 2004). *Fusarium* head blight (FHB) of small grain cereals and ear rot in maize cause significant loss of crops worldwide (Loos *et al*., 2004; Choo *et al.,* 2004; Roháãik and Hudec, 2005). In wheat, this disease is caused by several *Fusarium*, species including *Fusarium avenaceum, Fusarium graminearum, Fusarium culmorum* and *Fusarium poae* (Edwards, 2004). Infected grains and tuber are also contaminated by potent toxic secondary metabolites (described below) produced by these fungi (Choo *et al*., 2004). *Fusarium solani* is considered the major plant pathogen of its genus though it can also cause disease in humans (Boutati and Anaissie, 1997; Abbas *et al.,* 1998; Anaissie *et al.,* 2001; Abramson *et al.,* 2001; Escola *et al.,* 2001; Sampathkumar and Paya, 2001; Nucci and Anaissie, 2002; Shono, 2003; Verma *et al.,* 2003; Doczi *et al.,* 2004; Jensen *et al.,* 2004; Bretz *et al.,* 2005; Zhang *et al*., 2006).

2.3.3 Mucorales

2.3.3.1 Mucorales infection in plants

Mucor and *Rhizopus* are members of the Mucorales group containing pathogens of both plants and humans. They are found throughout the world and in many climatic conditions and terrain. These fungi cause rot in fruits both in the field and post harvest. Mechanical injury during harvesting and post harvest handling provide a site of infection by these fungi (Bruton, 1994). *Mucor piriformis* causes decay of cherries on the tree as well as rot of apples stored under humid conditions (Børve and Stensvand 2003; Michailides and Spots, 1990). *Rhizopus arrhizus* causes a wet, juicy rot of grapes in warm, wet climates. In humid weather the fungus can spread to other berries in the grape cluster causing bunch rot (Pearson and Goheen, 1998).

2.3.4 *Colletotrichum*

Colletotrichum is distributed worldwide and is considered one of the most serious phytopathogenic fungal genera (Cano, *et al*., 2004).

2.3.4.1 Colletotrichum infection in plants

Genus members cause field anthracnose, resulting in major economic losses of many crops such as cereals, legumes, coffee and rubber (Latunde-Dada, 2001). Two of the phytopathogenic species are also human pathogens (O'Quinn *et al.,*

2001). One of these, *Colletotrichum gloeosporioides*, affects many crops of agricultural importance throughout the world. It is the etiological agent of anthracnose, resulting in serious economic and crop losses of strawberry, yams (Abano, *et al.*, 2004) and mango (Dodd *et al*., 1991). The other phytopathogen of this group affecting human health is *Colletotrichum coccodes*, an increasingly important potato pathogen. This fungus causes tuber blemish (black dot disease) and foliar disease (Lees and Hilton, 2003). Losses from this disease occur in potatoes destined for the growing market for fresh, prepackaged potatoes, which demands tubers with a high quality appearance (Lees and Hilton, 2003). The majority of the disease reports originate in North America, Europe and Australia (Harrison, 1963; Mordue, 1967). This fungus is also pathogenic on several plants in the *Solanaceae* and *Curcurbitaceae* groups (Mordue, 1967; Dillard, 1992).

2.3.5. *Cladosporium*

Members of the *Cladosporium* genus are ubiquitous in the environment and can be isolated from many sources, including dead organic matter, woody plants, soil, straw, paint and textiles. One species, *Cladosporium cladosporioides*, can be pathogenic in both plants and humans (Drabick *et al.,* 1990; Pearson and Goheen, 1998; Tournas, 2005).

2.3.5.1 Cladosporium infection in plants

Cladosporium species cause spoilage of freshly harvested vegetables, resulting in great economic losses (Tournas, 2005). They enter plant tissue after mechanical

or chilling injuries or after the plant skin barrier has been compromised by other organisms (Tournas, 2005). *Cladosporium cladosporioides* causes decay in tubers, fruits, vegetables and other agricultural products, especially after harvesting (Pearson and Goheen, 1998).

2.3.6 *Alternaria*

2.3.6.1 Alternaria infection in plants

Alternaria alternata is a seed pathogen of cereals, such as sorghum and wheat, where fungal growth causes surface discoloration initially, followed by breakdown of the seed components (Li and Yoshizawa, 2000; Waniska *et al*., 2001). This reduces the crop yield, viability, and quality as well as nutritional and economic values (Lillehol *et al* 1987; Ansari and Shrivastava, 1990; Polis and Kovacs, 1997; Rodriguez-Herrera *et al*., 1999; Li *et al.,* 2001). *Alternaria tenuis*, *Alternaria brassicola* and *Alternaria oleracea* are recognized as spoilage organisms causing rot of vegetables such as tomatoes, peppers, cucurbits and leafy crucifers (Tournas, 2005).

These toxins are known to block synthesis of sphingolipid by inhibiting the rate-limiting enzyme, ceramide synthase though they are not as active as fumonisin B1 in this regard (Van der Westhuizen *et al*., 1998).

The *A. alternata* group also produces plant specific phytotoxins (Otani *et al*., 1996) such as AAL-toxin produced by *Alternaria alternate* f. sp. *lycopersici*. This

toxin, structurally similar to fumonisin B1, causes necrotic lesions in genetically susceptible tomato lines (Gilchrist *et al*., 1992; Mirocha *et al*., 1992).

2.4 YAMS (*Dioscorea* species)

The genus *Dioscorea* includes some 600 species, of which 50 – 60 are cultivated or gathered for food or pharmaceutical purposes. However, there are only about 12 species of economic significance as food plants of these, *D. rotundata*, grown in Africa, and *D. alata*, grown large in Asia, are by far the most important, making up about 90% of world production of food yams (Coursey, 1967a; Coursey, 1967b; Coursey, 1976a; Tobih and Emosairue, 2008; Tobih, 2011).

The appearance of the yellow yam is very similar to that of the white yam (Onwueme, 1978; Passam, 1977). The yellow yam has a longer period of vegetation and shorter dormancy than the white yam. The yam is thought to have been cultivated in Asia and Africa as many as 8000 years ago.

Yams are a valuable source of carbohydrates, fibres, and low level fats, which makes them a good dietary source (Panneerselvam *et al.* 2007). Yams also have medicinal properties. Several species of *Dioscorea* are amongst the principle sources of diosgenin, which can be converted to medicinally important steroids (Jaleel *et al.,* 2007).

2.4.1 Taxonomy

Yams belong to the family Dioscoreacea, and grown most often on a tropical soil. The word "yam" is African for the phrase, "to eat" (HungryMonster, 2011).

For Scientific classification, Yams make up the genus Dioscorea of the family Dioscoreaceae. The water yam is classified as *Dioscorea alata*, the Chinese yam, or Chinese potato, as *Dioscorea batatas*, the air potato as *Dioscorea bulbifera*, the elephants foot as *Dioscorea elephantipes*, and cush cush, or yampi, as *Dioscorea trifida* (United State Department of Agriculture, 1999; International Starch Institute, 2010).

Kingdom:	Plantae – Plants
Subkingdom:	Tracheobionta – Vascular plants
Superdivision:	Spermatophyta – Seed plants
Division:	Magnoliophyta – Flowering plants
Class:	Liliopsida – Monocotyledons
Subclass:	Liliidae
Order:	Liliales
Family:	Dioscoreaceae
Genus:	Dioscorea L. – yam

2.4.2 Origin, evolution and dispersal

The family Diocoreaceae is probably one of the oldest groups of angiosperms, known to be first reported in Southeast Asia. *D. alata* and *D. rotundata* are not known in the wild state. *D. alata* is likely to have developed, perhaps through hybridization, from wild species ‘ in the north – central parts of the southeast Asian peninsula’ (Coursey, 1976a and Coursey, 1976b). The date of domestication is unknown, but was certainly before 3500 BC, since cultivated

Asian yams spread across the pacific in the Polynesian migrations of that period, although *D. alata* reached the east coast of Africa 1500 BC (Norman *et al.,* 1995).

It has been shown that *Dioscorea cayenensis* and *D. rotundata* are distinct species with intermediate hybrid forms. This study contributes to an increased understanding of the genetic organization of the core germplasm (Hamon and Toure, 1990; Obidiegwu *et al.,* 2009).

Dioscorea cayenensis and *D. rotundata* (also known as Guinea yams) are the most popular and economically important yams in West and Central Africa where they are indigenous and represent the largest depository of biodiversity, as a result of centuries of large domestication, production, trade and consumption (Degras, 1993; HungryMonster, 2011). The name Guinea yam does not only signify their intensive cultivation and great importance in the socio-cultural life of the people of this region, but also suggests similarities in various aspects of their botany and agriculture. Asiatic and later, African yams were used extensively for their vitamin C content. During the slave trade both *D. alata* and *D. rotundata* were taken from West Africa to the Caribbean (Akoroda and Chheda, 1983).

2.4.3 Health Benefits of Yams

Recent research studies on yams focus on two areas of unique health benefit (HealthAliciousNess, 2011). First, yam tuber stores proteins that have been observed to have significant antioxidant capacities. In one study, these proteins

had about one-third of the antioxidant activity of glutathione one of the body's most impressive internally produced antioxidants (Institute of Medicine, Food and Nutrition Board, 2000; Corradaa *et al.,* 2005).

Second is the recent classification of yams as an "antidiabetic" food. The yam has been given this label, because of some recent animal studies in which the yam helped to stabilize blood sugar levels and lowered insulin resistance. Insulin resistance is a problem caused when cells don't respond to the hormone insulin, which is supposed to act as a key and unlock the cell in order to allow sugar to pass from the blood into the cell. Some of its blood sugar regulatory properties may come from the fact that yams are concentrated in carotenoids. Research has suggested that physiological levels, as well as dietary intake, of carotenoids may be inversely associated with insulin resistance and high blood sugar levels. Although, more research is needed in this area, but the stage is set for the yam to show unique healing properties in the area of blood sugar control (Giovannucci *et al.,* 2002; Doshi *et al.,* 2002; Corradaa *et al.,* 2005).

The purple yam is a good source of vitamins C and E as well as dietary fiber, potassium, and iron, and they are low in fat and cholesterol (see the Table below for more detail).

In general the health benefits of yams include:

- Increased Immune Function
- Protection Against Heart Disease
- Slowing Aging
- DNA Repair and Protection

- Alleviation of Cardiovascular Disease
- Alleviation of Hypertension (High Blood Pressure)
- Alzheimer's Prevention
- Osteoporosis Prevention
- Stroke Prevention
- Antioxidant Protection
- Prevention of Epileptic Seizures
- Prevention of Alopecia [Spot Baldness] (Hajjar *et al.,* 2001; Fleming *et al.,* 2002; Deicher and Hörl 2003; Corradaa *et al.,* 2005; Hemilä *et al.,* 2007; HealthAliciousNess, 2011).

Yams are high in potassium which is good for blood pressure; they have also been used as a diuretic and to relieve coughing. Some of these health benefits are due to the nutrients highly concentrated in Yams. Natural vitmains, minerals, and nutrients found in Yams are Carbohydrates; Vitamin B1 (Thiamin); Vitamin B6; Vitamin B9 (Folate, Folic Acid); Vitamin C; Dietary Fiber; Manganese; Potassium; Copper (Institute of Medicine, Food and Nutrition Board, 2000; HealthAliciousNess, 2011; Doshi *et al.,* 2002; Giovannucci *et al.,* 1998; Corradaa *et al.,* 2005; Bazzano *et al.,* 2001; Barri and Wingo, 1997; Hajjar *et al.,* 2001; Hemilä *et al.,* 2007; Fleming *et al.,* 2002; Deicher and Hörl 2003).

Table 2.2 Nutritional content of yam tuber (*Dioscorea rotundata*)

Nutrition Facts				
Yam Raw				
Serving Size 100g				
Calories 118				
% Daily Value*				
Total Fat 0.17g				0%
Saturated Fat 0.037g				0%
Cholesterol 0mg				0%
Sodium 9mg				0%
Total Carbohydrate 27.88g				9%
Dietary Fiber 4.1g				16%
Sugar 0.5g				.
Protein 1.53g				.
Vitamin A	3%	•	Vitamin C	29%
Calcium	2%	•	Iron	3%
*Percent Daily Values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs.				

Vitamins	%DV
Vitamin A 138IU	3%
Retinol equivalents 7µg	.
Retinol 0µg	.
Alpha-carotene 0µg	.
Beta-carotene 83µg	.
Beta-cryptoxanthin 0µg	.
Vitamin C 17.1mg	29%
Vitamin E 0.35mg	1%
Vitamin K 2.3µg	3%
Vitamin B12 0µg	0%
Thiamin 0.112mg	7%
Riboflavin 0.032mg	2%
Niacin 0.552mg	3%
Pantothenic acid 0.314mg	3%
Vitamin B6 0.293mg	15%
Folate 23µg	6%
Folic Acid 0µg	.
Food Folate 23µg	.
Dietary Folate Equivalents 23µg	.
Choline 16.5mg	.
Lycopene 0µg	.
Lutein+zeazanthin 0µg	.

Minerals	%DV
Calcium 17mg	2%
Iron 0.54mg	3%
Magnesium 21mg	5%
Phosphorus 55mg	6%
Sodium 9mg	0%
Potassium 816mg	23%
Zinc 0.24mg	2%
Copper 0.178mg	9%
Manganese 0.397mg	20%
Selenium 0.7µg	1%
Water 69.6g	.
Ash 0.82g	.

Useful Stats	
Percent of Daily Calorie Target (2000 calories)	5.9%
Percent Water Composition	69.6%
Protein to Carb	

Institute of Medicine, Food and Nutrition Board (2000) and HealthAliciousNess (2011).

2.4.4 Microbial Infection of Yam leaves and Tubers

The yam leaves and tubers are subjected to several diseases caused by fungi, bacteria and viruses (Yeni, 2011).

It has been reported that microbial infection of yam leaves resulted to low yam tuber productivity Garrido *et al*. (2003). Moura (1997), stated that leaf spot caused by fungus called *Curvularia eragrostides* (Henn.) was the major yam leaf disease in Brazil, with symptoms characterized by the formation of necrotic brown leaf lesions, frequently surrounded by a yellowish halo. *Colletotrichum gloeosporioides* (Penz.) and *Curvularia eragrostides* has been resported to be secondary pathogen to yam leaves, which caused dark brown leaf lesions with irregular borders and yellowish halo (Garrido *et al*., 2003; Moura, 1997).

Fungi have been reported to significantly responsible for low yam tuber productivity. The fungi infection yam tuber render unfit for human consumption by reducing their nutritive value and often by producing mycotoxins (Diekman and Green, 1992; Kuiper-Goodman, 1994; Janardhana *et al*., 1998; Marin *et al*., 1999 and Krysinka-Traczyk *et al.,* 2007; Sargent *et al.,* 1961; Scott, 1987; Scott and Stoltz, 1980; Lacey, 1988; Lacey and Dukiewiez, 1994; Van der Westhuizen *et al.,* 1998; Yau *et al.,* 1994; Massey, 1996; Chandra and Sarbhoy, 1997; Desjardins *et al.,* 2000; Meky *et al.,* 2001; Bogdan, 2001; Berek *et al.,* 2001; Akan *et al.,* 2002; Dorner, 2002; Okigbo, 2002; Yao *et al.,* 2003; Navi *et al.,* 2005; Minervini *et al.,* 2005; Pestika and Smolinski, 2005). The reduction of fungi infestation of yam leaves which reduces yam tuber productivity will be a right

step to improve or enhance food security and improvement on the economy of the yam growers and farmers worldwide. The need for effective and cheap antifungal agents will be the right step in this direction (Majumder *et al.,* 1997; Janardhana *et al*., 1999; Chandra and Sarbhoy, 1997; Devi *et al.*, 2001; Marasas *et al.,* 2004).

2.5 ANTIFUNGAL AGENTS

Antifungal agents are chemical compounds that selectively inhibit or kill the growth of fungi cells/spores with little or no toxicity on the host organism. Several classes of antifungal agent are used today to treat fungal infections in plants and animals (Kwon-Chung and Bennett 1992).

Table 2.2: Antifungal agents: activities against principal fungal pathogens, mode of actions and resistance mechanisms.

	Antifungal	Spectrum/comments	Mode of action	Mechanism of resistance observed in clinical isolates
1	Polyenes - Amphotericin B	Broad activity against *Candida* spp., (except *C. lusitaniae*), *Cryptococcus neoformans* and filamentous fungi (except in the *Aspergillus* spp. *A. terreus* and *A. flavus*).	Binding to ergosterol and destabilization of cell membrane functions	Alteration in specific steps of ergosterol biosynthesis.
2	Pyrimidines analogues - 5-fluorocytosine (5-FC)	Active against *Candida* spp and *Cryptococcus* spp., however rapid emergence of resistance can appear when 5-FC is used as monotherapy.	Impairment of nucleic acid biosynthesis by formation of toxic fluorinated pyrimidine antimetabolites	- Decreased uptake of 5-FC. - Decreased formation of toxic antimetabolites.
3	Azoles - Fluconazole - Itraconazole - Voriconazole Clotrimazole Miconazole	Active against *Candida* spp. and *Cryptococcus* spp., less active against *C. glabrata* and no activity against *C. krusei*. No activity against filamentous fungi. Like fluconazole, but enhanced activity against filamentous fungi. Like fluconazole, but enhanced activity against filamentous fungi, including *Aspergillus* and *Fusarium* spp.	Inhibition of cytochrome P450 14α-lanosterol demethylase..	- Enhanced efflux by upregulation of multidrug transporter genes. - Target alterations by occurrence of mutations. - Alteration of specific steps in the ergosterol biosynthetic pathway.
4	Allylamines - Terbinafine	Active against most dermatophytes, poor activity against *Candida* spp..	Inhibition of squalene epoxidase	Unknown
5	Morpholines - Amorolfine	Active against most dermatophytes, poor activity against *Candida* spp..	Inhibition of sterol _14 reductase and _7-8 isomerase	Unknown

2.5.1 Antifungal Treatment

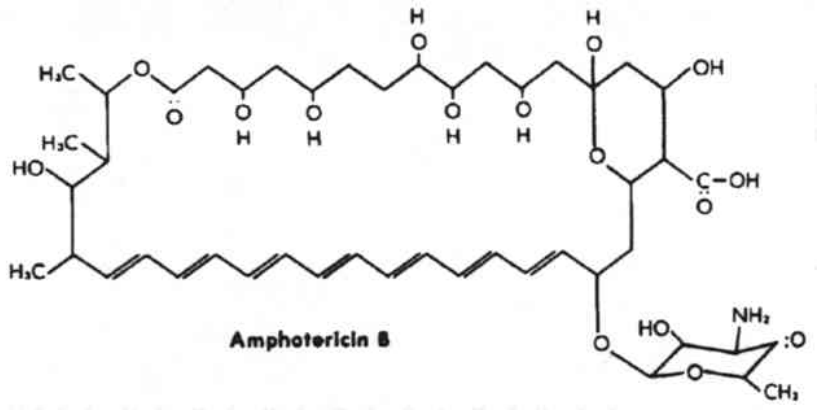
There are relatively few useful antifungal agents compared to the large number of antibacterial agents that are available in the market to arrest the growth of these phytopathogenic fungi. The different classes of fungicides commoly employed to limit the phytopathogenic activity of fungi include:

2.5.1.1 Polyene antifungal agents

Polyene antifungal agents are characterized by having the alternating conjugated double bounds that form part of their macrolide ring structure. They are originally produced by *streptomyces* species (Schlegel, 1995; Fogel, 2000; Denyer *et al.,* 2005 and Kuiper *et al.,* 2011).

.

The principal polyenes are amphotericin B and nystatin. Amphotericin B is produced by the bacterium *Streptomyces nodosus* and its activity is due ′ to its ability to bind ergosterol, which is the dominant sterol in fungal cell membranes, and consequently increases membrane permeability by the formation of extensive leaking pores through fungal cell membrane (Schlegel, 1995; Fogel, 2000; Denyer *et al.,* 2005 and Kuiper *et al.,* 2011).



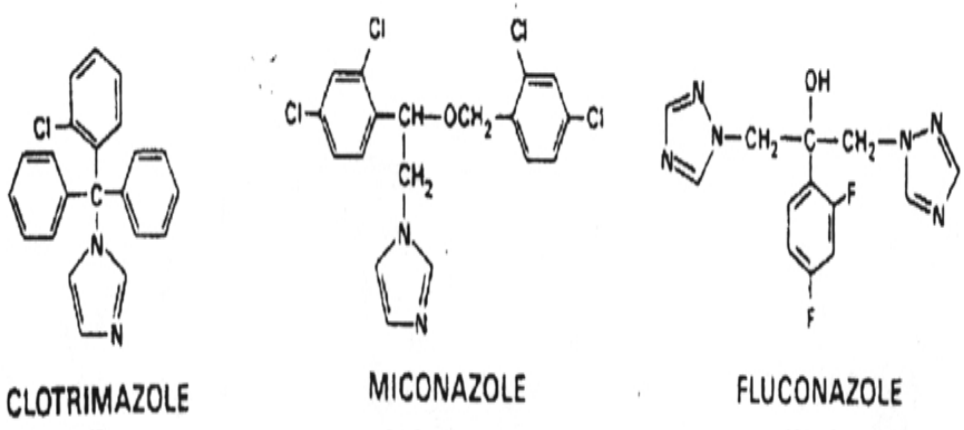
The action of amphotericin B seems to rely on the formation of pores through which actively vital intracellular contents can escape from the cell i.e Amphotericin B - binds to ergosterol moiety in the plasma membrane causing derangement of the membrane integrity and leakage of cytoplasmic contents. Amphotericin B is active against a broad range of fungal pathogens and is considered the 'gold standard' against which the activity of other antifungal agents is measured. Due to its renal toxicity amphotericin B tends to be reserved for severe cases of systemic fungal disease, but recent formulations in which the drug is encapsulated within liposomes have been shown to reduce it toxicity (Denyer *et al.,* 2005).

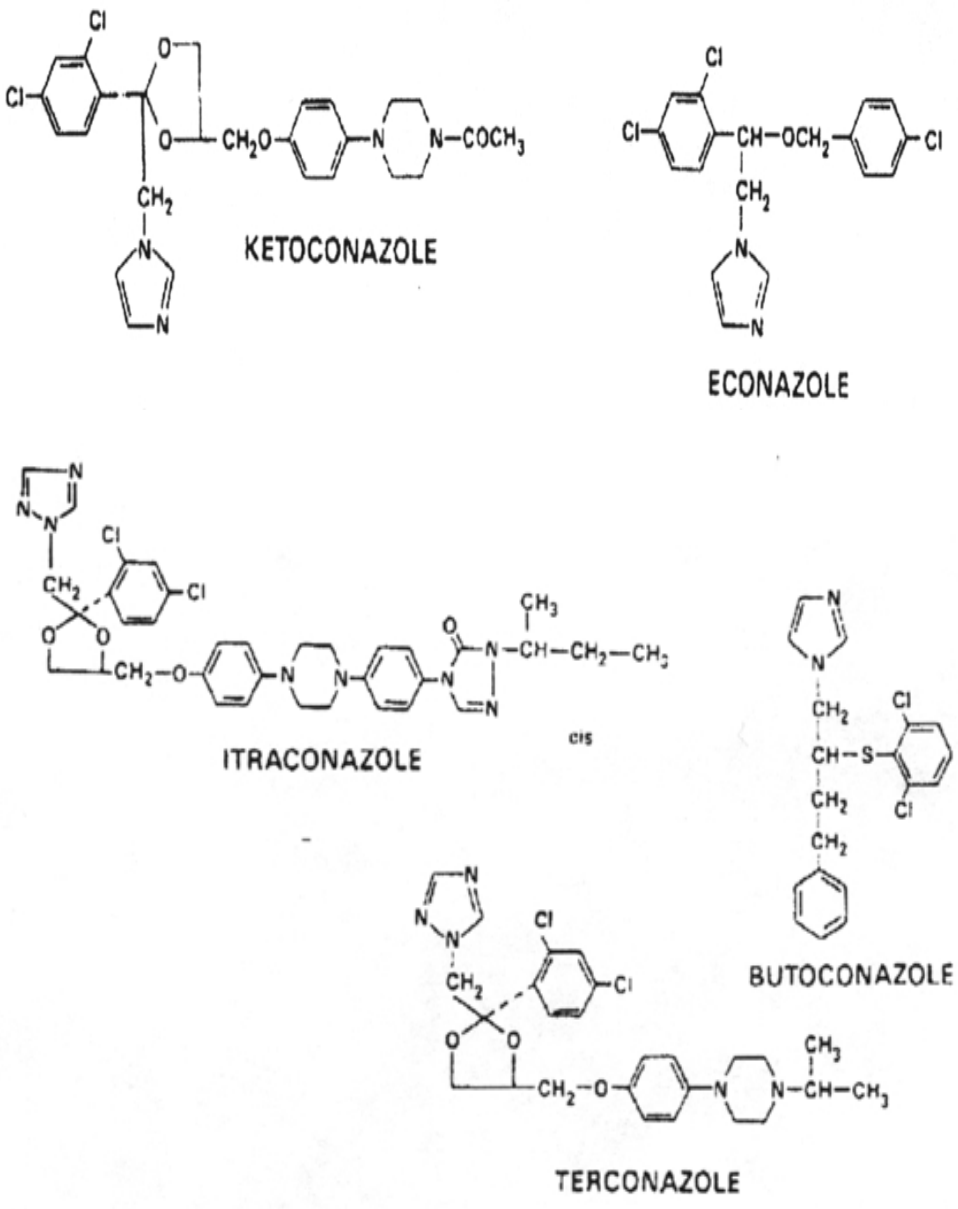
Polyene antibiotics may be microbiostatic or microbicidal depending on concentration used (Singleton and Sainsbury, 1978; Pelczar *et al.,* 1999). Nystatin discovered in 1950 exhibits the same mode of antifungal action as elicited by amphotericin B. Nystatin has been reported to be effective for the treatment of pathogenic conditions such as oral and vaginal candidosis however; its use has been over-taken by the introduction of azole antifungal drugs (Denyer *et al.,* 2005). Azole antifungals' have been reported to be active against a wide range of fungi and

against certain pathogenic protozoans. It has been used to combat systemic mycoses (Denyer *et al.,* 2005).

2.5.1.2 Azole antifungal agents

The first generation of azole antifungal agents' revolutionized the treatment of mucosal and invasive fungal infections and azoles are still the most widely used group of antifungal agents (Sheehan *et al.,* 1999). The azole derivatives are classified as imidazoles or triazoles on the basis of whether they have two or three nitrogen atoms in the five-membered azole ring. The development of the triazole antifungal agents in the 1980s and 1990s greatly enhanced physicians' ability to treat fungal infections due to the lower toxicity of these agents compared with previous antifungal therapies (Kale and Johnson, 2005). The azoles in current and long-standing clinical use are clotrimazole, miconazole, econazole and, ketoconazole, while newer drugs such as itraconazole, fluconazole and voriconazole have important applications in the treatment of systemic infections (Sheehan *et al.,* 1999; Denyer *et al.,* 2005; Kale and Johnson, 2005).





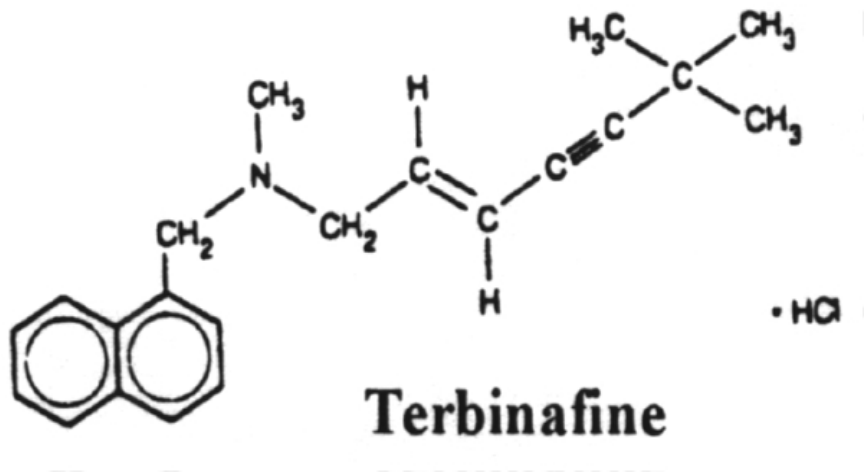
Azoles function by interfering with ergosterol biosynthesis (which replaces ergosterol in the plasma membrane causing selective leakage and increased osmotic sensitivity). They also disrupt chitin synthesis. All effects are due to by binding to the cytochrome P-450- mediated enzyme known as 14-α-demethylase ($P\text{-}450_{DM}$). This blocks the formation of ergosterol by preventing the methylation of lanosterol (a precursor of ergosterol), resulting in a reduction in the amount of ergosterol in the fungal cell membrane, which leads to membrane instability, growth inhibition and

cell death in some cases. An additional consequence of the block in ergosterol biosynthesis is the build-up of toxic intermediates, which can prove fatal to the cell (Denyer *et al.,* 2005).

Newer triazoles such as fluconazole and itraconazole have increased the options for dealing with fungal pathogenesis. Fluconazole has proved highly effective in the treatment of infections caused by C. a*lbicans,* but shows limited activity against *Aspergillus.* Itraconazole became available for clinical use in the late 1980s and was the first azole with proven efficacy against *Aspergillus.* Itraconazole is effective in treating severe *Aspergillus* infections and exhibits both fungicidal and fungistatic effects (Kale and Johnson, 2005). Upon ingestion itraconazole undergoes extensive hepatic metabolism which yields up to 30 metabolites, a number of which retain antifungal activity. Itraconazole is currently available as an intravenous formulation and is widely used for the treatment of severe *Aspergillus* infection in this form. Fluconazole and itraconazole demonstrate significantly reduced side-effects compared with ketoconazole. Novel azole drugs with increased ability to inhibit the fungal 14-α-demethylase are also becoming available. These agents, which include voriconazole, posaconazole and ravuconazole, have a wider spectrum of activity than fluconazole and it has been suggested that some of them show fungicidal effects with some species (e.g. *Aspergillus* spp). Voriconazole is one of the newest second generation triazole antifungal drugs and it shows good activity against pulmonary aspergillosis and cerebral aspergillosis (Sheehan *et al.,* 1999; Kale and Johnson 2005; Denyer *et al.,* 2005).

2.5.1.3 Allylamine and morpholine group

This includes terbinafine, naftifine and amorolfine. These antifungal agents bind and thereby inhibit squalene epoxidase which blocks ergosterol synthesis e.g terbinafine (Schuster and Ryder, 1990).



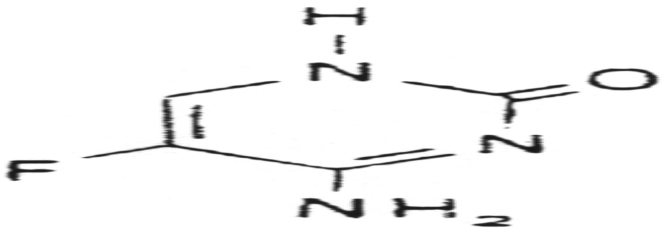
Terbinafine

Terbinafine hydrochloride chemically called (E)-N-(6,6-dimethyl-2-hepten-4-ynyl)-N-methyl-1-naphthalene methanamine hydrochloride, has a molecular formula: $C_{21}H_{25}ClN$, with a molecular weight of 327.90. It generally appears as white to off-white finely crystalline powder with solubility of 45 mg/ml in ethanol and 3 mg/ml in water. Terbinafine hydrochloride is a new potent antifungal agent of the allylamine class with a broad-spectrum activity against yeast, fungi, moulds, and dermatophytes and is indicated for both oral and topical treatment of mycoses (El-Saharty, *et al.,* 2002; Fuller *et al.,* 2001; Mikuš, *et al.,* 2005).

Terbinafine is well absorbed from the gastro intestinal tracet, and metabolized in the liver with an elimination half-life of 17 hours (Mukherjee *et al.,* 2003)

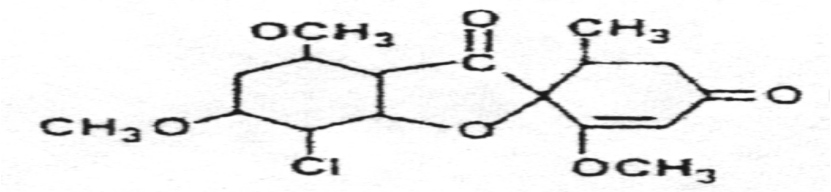
2.5.1.4 Antimetabolite group (Pyrimidine analogues)

This group include (5-Fluorocytosine) flucytosine and griseoflulvin. Flucytosine has been reported to be deaminated to fluracil which and either incorporated into RNA in place of uracil and inhibits protein synthesis or metabolized to 5-fluorodeoxy-uridylic acid which inhibits thymidylate synthetase, thus blocking DNA synthesis.



FLUCYTOCINE

Griseofulvin, has been reported to cause disruption of the mitotic spindle by interacting with polymerized microtubules proteins (Wildfeuer *et al.,* 1998; Montero-Gel 1998; Jessup *et al.,* 2000a'b).



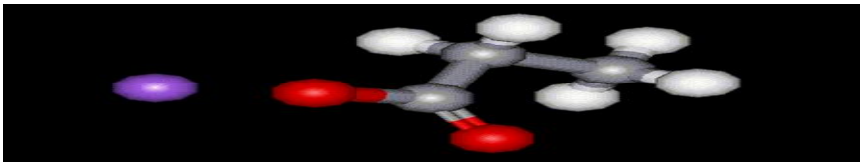
GRISEOFULVIN

2.5.2 Chemical antifungal agents

There are some widely celebrated chemical compounds to which may be natural or synthetic that have been reported effective against phytopathogenic fungi such as sodium propionate, Benomyl, Carbendazim and Thiophanate-methyl as briefly discussed below (The Lancet, 2007).

2.5.2.1 Sodium propionate

Sodium propionate is the sodium salt of propionic acid. It is used as an antimicrobial agent to prevent germination of some types of bacteria which causes sticky yellow patches to occur in foods. Typical products include bakery products and dairy products.



Physical and Chemical Properties of sodium propionate

Other names: propionic acid, sodium salt.

Molecular formula: CH3CH2COONa

Physical appearance: white powder

Melting point: 285^0C (545F)

Solubility: Appreciable (> 10%)

Stability: Stable under ordinary conditions of use and storage. Other information: May irritate the eyes and skin, readily absorbed through the skin (The Lancet 2007; Richard and Pressinger, 1997; Shephard and Sue, 2006).

2.5.2.2 *Benlate Fungicide (DuPont's Benlate® Fungicide)*

Benlate is the brand name of a fungicide produced by the DuPont chemical company between 1970 and 2001 to kill fungus growing on commercial crops. Benomyl is the active ingredient in the brand name product Benlate®, produced exclusively by DuPont. This fungicide had long been one of DuPont's most successful products and was registered worldwide for many crops. Benomyl, carbendazim, and thiophanate-methyl are evaluated together because benomyl and thiophanate-methyl are rapidly converted mainly to carbendazim in the environment (Enviromental Toxins, 2010).

Benomyl

Benomyl was first reported as a fungicide in 1968 and introduced in the UK market in 1971 by the US Company Du Pont (Tomlin, 1994). It is a systemic benzimidazole fungicide that is selectively toxic to microorganisms and to invertebrates, especially earthworms (Extoxnet, 1994). Benomyl and its main metabolite carbendazim bind to microtubules (an essential structure of all cells) and therefore interfere with cell functions such as cell division and intracellular transportation. The selective toxicity of benomyl as a fungicide is possibly due to its heightened effect on fungal rather than mammalian microtubules (WHO, 1993; WHO/PCS, 1994; Phillips, 2001; Agrow, 1992; Extoxnet, 1994; Office of Pesticide Programs, 1996; Kontoyiannis *et al.,* 2002; MAFF, 1992; Environmental Toxins, 2010). Benomyl is used as a pre-harvest systemic fungicide, and as a post-harvest dip or dust. It combats a wide range of fungal diseases of arable and vegetable crops, apples, soft fruit, nuts, ornamentals,

mushrooms, lettuce, tomatoes and turf. It is also available widely for amenity and amateur garden use (Whitehead, 1996).

Benomyl has an aerobic half-life of 2 h in water and 19 h in soil; thiophanate-methyl has an aerobic half-life of less than one day in soil and two days in water.

Classification of Benomyl activity

Primary use: Systemic fungicide

Secondary use: Acaricide, mite ovicide

Chemical group: Benzimidazole carbamate

General information on Benomyl activity

Common name: Benomyl (ISO)

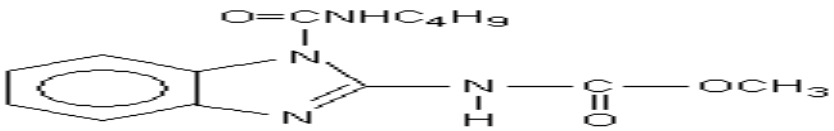
IUPAC chemical name: Methyl1-[(butylamino) carbonyl]-1H benzimidazol-2-ylcarbamate

CAS chemical name: Carbamic acid, [1-(butylamino) carbonyl]-1H-benzimidazol-2-yl]-, methyl ester.

Molecular formula: $C_{14}H_{18}N_4O_3$

Relative molecular mass: 290.3

Structural formula:



Trade names and synonyms: Benlate[R]; Tersan[R]; Fungicide 1991; methyl 1 (butylcarbamoyl)-2-benzimidazolecarbamate.

Synopsis: Benomyl is a systemic, broad spectrum benzimidazole carbamate fungicide.

Selected physical properties of Benomyl compounds

Physical characteristics: A tan-coloured odourless crystalline solid which decomposes at 140 °C just after melting. Technical benomyl is greater than 98% (w/w) pure.

Solubility: In water at 25 °C and pH 5 its solubility is 3.6 mg/L. Soluble in several organic solvents, especially heptane and chloroform (40 and 9.4 g/100 g solvent at 25 °C respectively).

Stability: Rapidly hydrolysed in dilute aqueous solutions and in soil to butyl isocyanate and the fungicide methyl-2-benzimidazole carbamate (carbendazim), decomposed by strong acids and alkalis stable to light.

Vapour pressure: Negligible (less than 5 x 10^{-6} Pa).

Carbendazim

Carbendazim is an odourless, white crystalline solid

General information on Carbendazim

Chemical names:

i) Methyl-2-benzimidazole carbamate

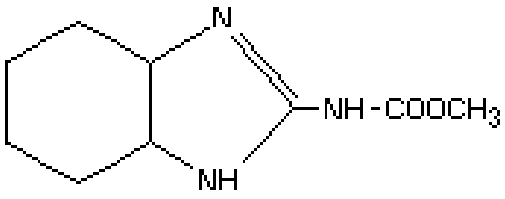
ii) Methyl benzimidazole-2-ylcarbamate

iii) 2-(methoxy-carbamoyl)-benzimidazole

iv) 2-(methoxycarbonylamino)benzimidazole

v) Carbomethoxyaminobenzimidazole

Synonyms: Bavisitin$^{(R)}$, MBC, MCAB, BCM, Hoe 17411, Drosal



$C_9H_9N_3O_2$

Structural formula:

Selected physical properties of Carbendazim

Molecular weight: 191.2

State: light grey powder

Melting point: 307-312°C (decomposition)

Vapour pressure: 0.1 × 10-6 mmHg (20°C)

Solubility: Soluble in acetic acid and dimethyl-formamide

Water, pH 4: 28 ppm

Water, pH 7: 8 ppm

Water, pH 8: 7 ppm

Hexane: 0.5 ppm

Benzene: 36 ppm

Methylenechloride: 68 ppm

Ethanol: 300 ppm

Acetone: 300 ppm

Chloroform: 100 ppm

Stability: Stable in acidic and alkaline solution at room temperature and when exposed to light. Stable in storage at 35-50°C

Purity of Technical product: Better than 98% (WHO/PCS, 1994; Tomlin, 1994 and Enviromental Toxins, 2010).

Carbendazim has been produced commercially since 1970. It is a systemic fungicide with a broad spectrum of activity on many economically important phytopathogens of the Ascomycetes, Deuteromycetes, and Basidiomycetes groups. It is used on agricultural crops (e.g. cereals, rice, grapes, cucurbits, tomatoes, pome fruits, stone fruits, and strawberries) and horticultural plants and in forestry and home gardening. Pest resistance has been seen, e.g. apple scab, eyespot, and *Botrytis*. In order to combat resistance, carbendazim is combined with other fungicides with different modes of action (WHO/PCS, 1994; Tomlin, 1994 and Enviromental Toxins, 2010).

General Information on Thiophanate-methyl

ISO common name: thiophanate-methyl

Chemical name: dimethyl 4,4'-*(o*-phenylene)bis(3-thioallophanate)

(IUPAC), dimethyl [1,2phenylenebis (iminocarbonothioyl)] bis [carbamate] (CAS)

Molecular formula: C12H14N4O4S2

Molecular weight: 342.4

Physical and chemical properties of Thiophanate-methyl

Appearance: white powder

Vapour pressure: <1.3 x 10-2 mPa at 25°C (OECD No104) 1134 thiophanate-methyl

Melting point: decomposition at 165.0°C (CIPAC MT-2) Octanol/water

Partitition coefficient: Pow 25.3 (log Pow 1.40)

Solubility: water 24.6 mg/l at 25°C 40 mg/l at 25°C Organic solvents (g/100 ml solvent at 25°C) acetone 2.9, methanol 0.6, acetonitrile 1.3 g/100 ml, toluene 0.04, n-Hexane 0.6 x 10-4.

Specific gravity: 1.45 at 20°C (CIPAC MT-3.2.1 iii)

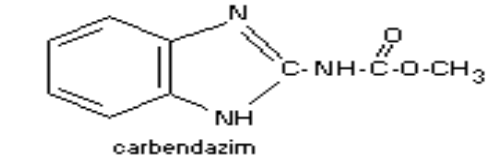
Hydrolysis: half-life at 25°C 867 days (pH 5), 36 days (pH 7), 0.7 days (pH 9).

Dissociation constant, pKa: 7.28 at 25°C

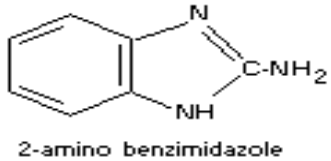
Melting point: decomposition 162.6 °C (CIPAC MT-2).

Stability: stable for 14 days at 54°C; for 3 years at room temperature

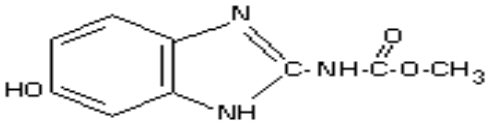
Benomyl and thiophanate-methyl degrade primarily to methyl-2-benzimidazole carbamate (carbendazim), which is also fungicidal.



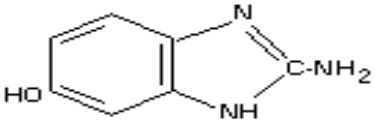
carbendazim



2-amino benzimidazole



5-hydroxy carbendazim



5-hydroxy amine

Hammerschlag (1973) indicated an effect of carbendazim on mitosis, DNA synthesis and cytokinesis in two fungi species. Certain differences in effects of benomyl and carbendazim were attributed to the occurrence of butylisocyanate, a potential metabolite of benomyl.

The mode of action of carbendazim involves interference in the biosynthesis of DNA during fungal cell division. Benomyl and thiophanate-methyl have been reported with a highly specific mode of action. The application rates and the

number of treatments allowed per crop depend on the chemical in-use. Overall, 0.18-0.60 kg/ha per application for carbendazim, 0.14-1.12 kg/ha for benomyl, and 0.22-2.13 kg/ha for thiophanate-methyl has been reported (Richard and Pressinger; 1997; Shephard and Sue, 2006).

Carbendazim, thiophanate-methyl, and benomyl are absorbed by sprouts, leaves, and roots and are translocated within the transpiration system in the plants xylem. They inhibit the development of fungal germ tubes, the formation of appressoria, and the growth of mycelia. Their fungitoxic action is based on blockage of nuclear division during mitosis and destabilization of fungal cell structures. Carbendazim, thiophanate-methyl, and benomyl persist on leaf surfaces and in leaf litter. The conversion of benomyl to carbendazim on leaf surfaces varies with the leaf surface, light intensity, and other environmental factors.

Environment

Benomyl binds strongly to organic matters in soil and does not dissolve in water largely. When applied to turf, it has a half-life of three to six months, and when applied to bare soil the half-life is six to 12 months (Extoxnet, 1994).

2.5.3 Mechanisms of antifungal drug resistance

Microbial resistance to antimicrobials agents is a major threat to public health and food production and these have been reported to be due to numerous

"smart" mechanisms which microorganisms have evolved with the passing years to escape the lethal effect of antimicrobial agents. Whether inherent or acquired, resistance can be ascribed to one of several mechanisms such as:

1. Production of a drug-inactivating enzyme:
2. Target alterations
3. Impermeability to the drug
4. Efflux action etc.

Fungal pathogens have been reported to involve several mechanisms to circumvent the inhibitory actions of antifungal drugs (Cosgrove, 2006). The variety of these mechanisms was revealed in recent years by several laboratories with molecular approaches. Besides the identification of genes involved in antifungal drug resistance and the discovery of alternative pathways of resistance (i.e. biofilm formation), novel concepts in the understanding of the survival of fungal pathogen in the presence of antifungal drug (antifungal tolerance) have emerged (Kohn *et al.,* 1963; Walsh and Pizzo, 1988; Bauersachs *et al.,* 1997; Waniska *et al.,* 2001; Kumatowski and Kilipiak, 2001; Giovanncci *et al.,* 2002; Kumar *et al.,* 2002; Sanglard & Odds, 2002; Walsh *et al.,* 2004; Casalinuovo *et al.,* 2004).

One of the most important mechanisms of resistance is the over expression of efflux proteins which act by pumping the drug out of the cell at a rate faster than the drug enters the cell. Therefore the intracellular concentration of the drug does not reach a level high enough to inhibit the target enzyme (Sanglard *et al.,* 1997; Denyer *et al.,* 2005; Yamanoi *et al.,* 2003).

Another resistance mechanism that has been identified is mutation within the gene encoding the cytochrome P-450. These amino acid substitutions do not affect the activity of the enzyme, but the conformation of the enzyme is sufficiently altered to decrease the affinity of the enzyme for the drug, thus the levels of membrane ergosterol are not altered significantly (Bart-Delabesse and Latge, 2004).

An alternative resistance mechanism is the over expression of the gene encoding the target cytochrome enzyme, this results in increased levels of the cytochrome inside the cell, thus countering the effects of the drug. Finally, another resistance mechanism is mutation in genes that encode other proteins involved in the biosynthesis of ergosterol. These mutations prevent the build-up of toxic byproducts, thus the inhibitory effects of the azoles are decreased. Very often in azole-resistant clinical isolates of C. *albicans* multiple resistance mechanisms have been identified as contributing to the resistance phenotype, thus it is difficult to determine which are the most important mechanisms (Sanglard *et al.,* 1997; Denyer *et al.,* 2005; Mahon *et al.,* 2007).

To solve the problems associated with antifungal resistance a number of novel azole drugs (e.g. voriconazole) and novel classes of drug (e.g echinocandins) have been developed (Denyer *et al.,* 2005; Mahon *et al.,* 2007).

Resistance to azole antifungal agents in *Candida albicans* can be mediated by multidrug efflux transporters. In a previous study, it has been identified that at least two such transporters, Cdr1p and Benp, which belong to the class of ATP-binding cassette (ABC) transporters and of major facilitators, respectively (Sanglard *et al.,* 1997; Mahon *et al.,* 2007).

2.5.4 The Rate of Kill

The viability of the resistant test organism (*A. niger*) against the test antifungal agents were determined through the process of viable spores counts at different times or varying concentrations at fixed time.

Generally, when microorganisms were exposed to biocidal antimicrobial concentation, the populations of microorganisms lose their viability in an `exponential fashion, independent of the initial number of organisms. This can be represented graphically with a 'survivor curve' drawn from a plot of the logarithm of the fraction of survivors against the exposure time or vary concentration which fall on a descending straight line. The typical curves obtained, all have a linear portion which may be continuous (plot A), or may be modified by an initial shoulder (B) or by a reduced rate of kill at low survivor (C). This is also referred to as the logarithmic order of death or first order of kinetics (Fig 2.1).

The resistance of an organism to an antimicrobial agent can be described by means of the D-value. This is defined as the time taken at fixed concentration required to achieve a 90% reduction in viable cell (i.e a log cycle reduction in survivors Fig 2.2). The calculation of D-value assumes a linear type A survivor curve (Fig 2.1 A) and must be corrected to allow for any deviation from linearity with type B and C curves.

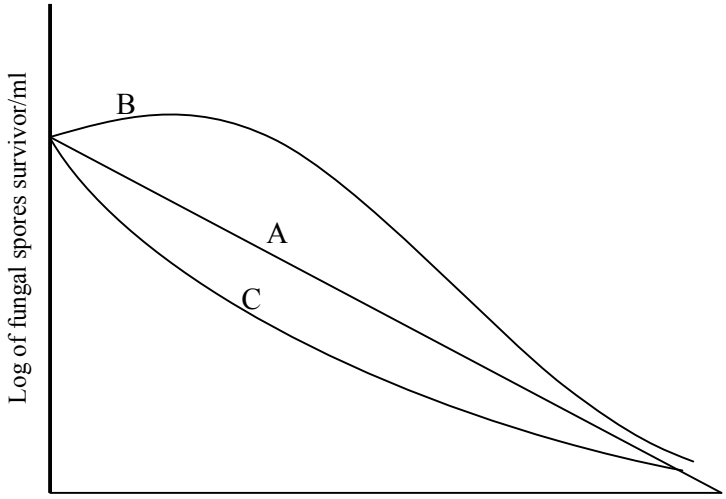


Fig 2.1: Typical survivor curve

A = Type of antimicrobial obeying first order of kinetics.
B = Sigmodal curves common with many biological resposes to mirobiological agents at low microbiocidal concentration with subsection
C = the initial rate of kill is very fast, but subsequently decrease with time.

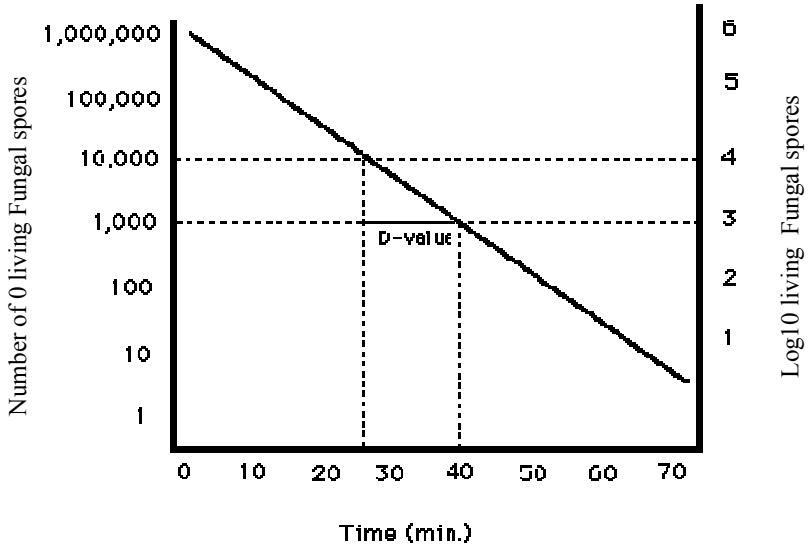


Fig 2.2: Calculations of D-value

2.5.5 Antimicrobial combination

Combinations of antimicrobials are sometimes needed to overcome severe fungi resistance emerging in the contemporary world. Combinations are also important for certain infections in which the fungus rapidly develops resistance to a single antifungal agent (Hessen and Kaye, 2004). Phytopathogenic

infections caused by many fungi which each fungus is susceptible to a different antifungal require a combination of antifungal agents.

Combinations of antifungal agents may be chosen not only for broad of spectrum, but for favorable effects, such as synergistic killing, in which two agents demonstrate greater cidal effect than additive activity (Gupta *et al.,* 2001; Hessen and Kaye 2004).

2.5.6 Mechanisms of Synergistic Interaction of Antifungal Agents

Several mechanisms of antifungal synergisms have been proposed:

1. Inhibition of different stages the same biochemical pathway e.g combination of Terbinatine and azole (Barchiesi et *al.,* 1998; Perea *et al.,* 2002).
2. One antifungal agent may increase the penetration or permeability of the other one antifungal agent as a result of cell wall or cell membrane antifungal activity from the other agent e.g amphotericin B or Fluconazole with antibacterial such as rifambicin (Beggs *et al.,* 1976; Medoff, 1983; Garret and Brown, 1964) and quinolones (Sugar *et al.,* 1997; Sugar and Liu, 2000), this allows antifungal agent to penetrate easily ino the fungal cell membrane and reach their target of fungal DNA synthesis. Another example is the combination of azole antifungal agent with amphotericin B (Medoff *et al.,* 1971; Barchiest *et al.,* 2001) or azol with Flucytosine, in each case the azole or polyne damages the fungal cell membrane allowing the increased uptake of flucytosine

3. Simultaneous inhibition of different fungal cell targets e.g cell membrane and cell wall at the same time such as combination of echinocandins (cell active) with amphotericin B (Arikan *et al.,* 2001).
4. Reduce fungal load with a rapid fungal agent whom will then allows one to subsequently work well consolidation or clearance therapy on a reduced number of fungal cell (Polak, 1982).
5. Transport interactions e.g amphotericin B with Flucytosine (Beggs *et al.,* 1981; Beggs and Sarosi 1982; Beggs, 1986).

Resistant phytopathogenic fungi

Fungi often infect crops in the field before harvesting resulting in considerable economic losses to farmers and producers worldwide. When food stuff produced in the farms are contaminated with fungi, the toxins they may produced, are ingested by humans and/or animals, a wide variety of debilitating diseases can occur with some leading to death. Some of the major phytopathogens affecting food production also cause serious, and very often lethal, infections in man. This rather small group of resistant fungi affect the world food production and the well being of millions of persons worldwide. Famine, death and enormous economic losses are the high probability with the emergent resistant phytopathogenic fungi which result to agricultural losses and medical care costs for the populace. Research like this is needed to develop a series of novel antifungal targeted specifically for particular life-threatening food supply like yams. Successful development of combined antifungal compounds could prove eventually useful in combating phytopatogenic infectious and toxin-producing fungi in the food

production in the fields and storage. The development of good combined antifungal agents from known conventional antifungal agents may be a good source of novel fungicide to slow down the rate of resistant phytopathogenic fungi among yam farmers world wide.

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Yam Leaves Sample

The phytopathogenic fungi infected yam leaves samples (five) from the centre of each of the twelve randomly selected farms of *Dioscoria* species were taken aseptically. Four farms from each of the three selected local Government areas viz: Kauyen Ali Dakaci, Zaria L.G.A; Wanka, Soba L.G.A. and Rafinyashi, Giwa L.G.A. of Kaduna state, Nigeria in 'Latitude $10^0$57"E and $11^0$12"N, Longititude $7^0$45"E and $8^0$6"N. These yams species were confirmed to be *D. Rotundata* and *Dioscorea cayenesis* (yellow yam) in the Department of Botany, Ahmadu Bello Univesity, and Zaria.

3.1.2 Test Organisms

The following fungi isolated and identified from field infected yam leaves were used: *Penicillium citrinum*, *Cladosporium* sp, *Aspergillus wentii*, *Aspergillusfumigates*, *Penicillium oxalicum*, *Aspergillus flavus*, *Aspergillus cervinus*, *Rhizopus* sp, *Penicillium sp*, *Aspergillus ostianus*, *Aspergillus niger*, *Aspergillus oryzae*, *Scopolarium* sp and *Rhizopus stolonifer*.

3.1.3 Culture Media and Suspending Media

Sabouraud dextrose agar (SDA) (Fluka Biochemika 84085 India)

Sabouraud dextrose liquid media (Oxoid CM0147 England)

Havesting Diluent (Nomal slime + 0.05% Tween 80)

3.1.4 Test Antifungal Agents

Fluconazole (Drugfield Nigeria)

Terbinafine (Somex pharma, UK)

Flucytosine (5-Fluorocytosine) Valeant Pharmaceuticals International 3300 Hyland Ave., Costa Mesa, CA 92626 U.S.A.

Benomyl (DuPont's Benlate® Fungicide, US)

Sodium propionate (India)

3.1.5 Instruments

Microscope (Wild M11 Heelbrugg Switzerland)

Autoclave (Adelphi MFG Co L^{TD} England)

Hot air oven (Gallen kamp Hot box England)

Incubator (QL Qoincylab, inc model 12-140 E) digital microprocessor 140 series

Centrifuge (Gallenkamp England and 5417R eppendorf Germany)

Water bath (Gallenkamp England)

Biospectrophotometer (eppendorf AG, 22331 Humburg Germany)

3.1.6 Other Materials

70% Ethanol- (BDH) Laboratory reagent

Tween 80- (Riedel-deHaen 63161)

Penicillin- (NCITC China)

Streptomycin- Septocin (Shijrazhuang China) co. Ltd

Petridishes and tubes (Pyrex England)

Chromatographic tank

3.1.7 Preparation of combined test antifungal agents' solutions

The appropriates concentration of each test-antifungal agents Viz: (A) Benomyl / Fluconazole (1,000 µg/ml /15,000µg/ml); (B) Benomyl /Terbinafine (1,000 µg/ml /200µg/ml); (C) Benomyl / Sodium Propionate (1,000 µg/ml /15,000µg/ml); (D) Benomyl /Sodium Propionate (1,000 µg/ml / 20,000µg/ml); (E) Fluconazole /Sodium Propionate (1000µg/ml /10,000µg/ml ml) and (F) Terbinafine / Sodium Propionate (200µg/ml /10,000µg/ml) were weighed and dissolved in 25%v/v ethanol aseptically. These preparations were stored in amber bottles in refrigerator (4^0C) until required for use.

3.2 METHODS

3.2.1 ISOLATION OF PHYTOPATHOGENIC FUNGI FROM INFECTED LEAVES OF YAM IN THE FIELD IN ZARIA, NIGERIA.

Infected yam cultivars, were first identified through careful physical examination of the entire plant for symptoms of infection such as necrotic

lesions on the leaves, petioles and stems as observed in the attached yam leaves plate 3.1; 3.2 and 3.3.



Plate 3.1: *Dioscorea rotundata* (White yam) healthy leaves



Plate 3.1: *Dioscorea rotundata* (White yam) infected leaves



Plate 3.2: *Diocorea rotundata* (White yam) whole plant

3.2.1.1 Isolation of Phytopathogenic Fungi from Infected Yam Leaves from Selected Field.

The fungi infected yam leaves were surface sterilized with 70% ethanol and were cut from advancing edge of a rot infected tissue. These samples were swabbed aseptically with sterile cotton swabed stick and plated out on sabouraud dextrose agar (SDA) supplemented with penicillin (0.1%) and streptomycin (0.1%). The plates were incubated at 30^0C for 5 days. The fungal growths observed were identified using, morphological and cultural characteristics. The frequency of fungi isolates occurrence in diffent farms were also documented (Green, *et al.,* 1995, Amusa and Baiyewu, 1999; Okigbo and Ikediugwu, 2000; Okigbo and Nmeka 2005). The isolated and identified fungi were cultured on SDA slants in triplicates and kept until required for further investigations.

3.2.1.2 Stock culture preparation

Stock cultures of fungi spores were prepared using SDA slants incubated at 30^0C for five days and stored in a refrigerator at 4^0C (Okigbo and Nmeka 2005; Olurinola *et al.,* 1992) until required for use or further studies/investigations.

3.2.1.3 Spores preparation

Fungal spores' suspensions were prepared from SDA slant culture. The spores were harvested using sterile glass beads and normal saline containing 0.05% Tween 80 to obtain homogenous spores suspension. The harvested fungal spores' suspensions were standardized to approximately 10^8 spores per ml (Olurinola *et al.,* 1992).

3.2.2 Culture Preparation

3.2.2.1 Media preparation

Sabouraud Dextrose Agar (SDA) and Sabouraund Dextrose liquid medium (SDLM) were prepared according to the manufacturer's specification and sterilized at 121^0C for 15 minutes.

3.2.2.2 Preparation of inactivation agent

Sodium chloride (0.90 g) was weighed and dissolved in 95 ml of distilled water; plus 5 ml Tween 80 was added to make 100 ml volume giving a concentration of 0.9 %w/v and 5 % v/v Tween 80.

3.2.2.3 Preparation of Harvesting medium

Sodium chloride (0.90 g) was weighed and dissolved in 99.9 5ml of distilled water plus 0.05ml Tween 80 to make up 0.9%w/v and 0.05% v/v Tween 80. Heat was applied to enhanced solubility.

3.2.3 Antifungal Studies

3.2.3.1 Determination of Minimum Inhibitory Concentration (MIC) of Test Antifungal Agents

The graded concentrations of each test-antifungal agents Viz: Benomyl (0.50-2000 µg/ml), Flucytosine (0.20-800 µg/ml), fluconazole (0.5-2000 µg/ml),

Terbinafine (0.5-2000 µg/ml), and Sodium propionate (2.0-10,000 µg/ml) in 5ml volume were prepared aseptically. These were mixed with melted double strength (5ml) SDA at 45^0C and poured aseptically in to sterile petri dishes and allowed to set (Shettima *et al.,* 2005).

Three pairs of sterilized filter discs were equidistantly place on the set SDA-plates aseptically. An aliquot of 20 µl of each spectrophotometrically standardized test fungal spore suspension (10^8 cfu or10^8 spores/ml) was inoculated on each pair of filter paper discs on the test compound in SDA plates. The plates were allowed to stand for one hour (1hr) and then incubated at 30^0C for 72 hours (Shettima *et al.,* 2005). Control was also set up i.e SDA plate without test chemical agent but inoculated with the test fungi spores (Olurinola *et al.,* 1992).

The lowest concentration of each test antifungal agent and in admixture that inhibited the growth of the test fungal spores was regarded as MIC (Olurinola *et al.,* 1992; Shettima *et al.,* 2000; Ehinmidu *et al.,* 2003; Shettima *et al.,* 2005).

3.2.3.2 Determination of Minimum Fungicidal Concentration (MFC) of test antifungal agents

The MFC of each test compounds was determined by inoculating the discs that showed no visible growth during MIC determinations into 5ml Sabouraud Dextrose Liquid Medium, supplemented with 5% $^{v}/_{v}$ of Tween 80 as inactivator aseptically (Olurinola *et al.,* 1991; Shettima *et al.,* 2000; Shettima *et al.,*

2005).This medium with filter paper discs was incubated at 30^0C for 5 days (Olurinola *et al.,* 1991; Shettima *et al.,* 2000; Shettima *et al.,* 2005). The lowest concentration that showed no growth was regarded as MFC of the test Antifungal agent against test phytopathogenic fungi spore.

3.2.3.3 Determination of Combined MIC of Test Antifungal Agents.

Graded concentrations of each test compound e.g Sodium propionate (5 ml volume) ranging from 200 to 1000 µg/ml was mixed with fixed concentrations of each of Benomyl, Fluconazole and Terbinafine HCl (5 ml volume) concentrations ranging from 0.1 to 50µg/ml and with melted Double strength Sabouraud dextrose agar (SDA 10 ml) at 45^0C in sterile petri dishes and allowed to set.

The admixtures of Fluconazole with Sodium propionate, Benomyl with Sodium propionate and Terbinafine with Sodium propionate were similarly prepared aseptically. Sterile discs were aseptically placed on the set plate at equidistance and inoculated with 20µl of spectrophotometrically standardized spores of test fungi (10^8 cfu/ml) in triplicate. These were incubated at 30^0C for 5 days. Control was also set up, i.e SDA plate without test antifungal agents, but inoculated with the test fungi spores. The susceptibilities of the test fungi spores to each combination test agents were recorded as minimum inhibitory concentration (MIC). The MICs for each antifungal-pair was calculated in terms of fractional inhibitory concentration (FIC) according to Elion and co-worker (1954).

3.2.3.4 Determination of Combined MFC of Test Antifungal Agents.

The MFC of each combination was determined by inoculating the discs that showed no visible growth during combined MICs determinations. These discs were inoculated aseptically into 5ml Sabouraud Dextrose Liquid Medium, supplemented with 5 $\%^{v}/_{v}$ of Tween 80 as inactivator (Olurinola *et al.,* 1991; Shettima *et al.,* 2000; Shettima *et al.,* 2005).The inoculated medium was incubated at 30^{0}C for 5 days. The lowest concentration that showed no growth was regarded as MFC of the combined test antifungal agents.

3.2.3.5 Determination of Biocidal Activities of the Test Antifungal Agents

The biocidal activities of the test antifungal agent alone and in admixture on the viability of the spores of test fungi were studied. Ten mililiter (10 ml) volume solutions of the desired single and combined concentrations of the test antifungal agents were prepared aseptically. These were inoculated with 0.1ml of standardized spores of test fungi.

At different specified time interval, 1.0 ml was taken from the reaction mixtures and ten-fold dilutions was made using sterile normal saline (9 ml) supplemented with 5 %v/v Tween 80. One milliliter of each dilution was mixed with 19 ml of melted SDA at 45 ^{0}C and plated in duplicates, aseptically.

The viability of the test fungal spores and the sterility of the SDA medium used were also determined as control. The fungal spores' survival was documented and a graph plotted.

3.2.3.6 Determination of Effects of Test Compounds on Cytoplasmic Constituent of Resistant A. niger Spores.

The effect of fixed concentration of Terbinafine HCl, Benomyl, Fluconazole and Sodium propionate on the leakage of cytoplasmic constituents' such as DNA content of test fungal isolate spores was determined at varying times.

The concentrations of Terbinafine HCl (20 µg/ml); Benomyl (2,000 µg/ml); Fluconazole (5,000 µg/ml) and Sodium propionate (40,000 µg/ml) were prepared aseptically with normal saline in 9ml volume. These preparations were inoculated with 0.1ml 10^8 cfu /ml of test fungal spores at ambient temperature (25±2 ^{0}C).

At different time interval of 0, 5, 10, 20, 40, 60, 120, 180 and 240 minutes contact time, the reaction mixtures 1ml was centrifuge at 5,000 g for 5 minutes and the DNA present in the supernatant was determined using Biospectrophotometric at a wavelength of 260nm (Wilson and Walker, 1995; CBRT, 2009, 2011). The mixtures without the spores i.e containing only the diluents and the same antifugal agents concentrations were centrifuged as blanks for each preparation. The observed DNA released from the test fungal spores was documented.

3.2.3.7 Determination of the effects of different water sources water on antifungal activities of combined test antifungal agents.

The combined test antifungal agents were diluted using both boiled and unboiled well water, tap water, river water, pond water and Distilled water. The standardized culture of the test fungi spores (1.0 ml) was used to flood the prepared sabourand dextrose agar plates. The plates were allowed to dry at 37 ^{0}C in a sterile incubator. A hole was bored on each of the plate using no. 4 sterile cork borer (6 mm diameter). The bottom of the hole/well was sealed with a drop of molten sterile SDA.

Using a micropipette, 0.1 ml of the diluted combined test antifungal was dispensed into the holes while sterile distilled water without combined test antifungal agents served as control. The inoculated combined test antifungal agents (0.1 ml) were allowed to diffuse into the agar at room temperature for one hour before incubation at 30 ^{0}C for 48 hours. The zones of inhibition of test phytopathogenic fungi spores were measured to the nearest millimeter using calibrated meter ruler. This experiment was carried out in duplicates.

3.2.3.8 Determination of the presence of organic and inorganic matter in the different water sources used in this investigations.

Each water source was dispensed (100 ml) into cleaned, labeled; weighed beakers. The different cleaned, labeled beaker with 100ml water was heated in a water bath at 100 ^{0}C until all the water content in the beaker evaporated. The beakers were then transferred to the hot air oven for 30 minutes at 60 ^{0}C and

later weighed again. The initial weight of each beaker was subtracted from the final weight of the same beaker, hence the resulting weight differences represent the organic and inorganic matters present in that particular water sample. The results were documented.

3.2.3.9 Determination of Effects of varying pH values on the Antifungal activity of the combined test antifungal agents.

Three different pH values viz: pH 4, 7 and 9 were employed. These were prepared with the different antifungal agents' combination according to manufacturer's instruction and pH confirmed with pH meter. The whole content was allowed to stand for 30 minutes.

Sabourand dextrose agar was flooded with standardized cultures of the test fungi spores (1.0 ml). The plates were allowed to dry at 37 ^{0}C in a sterile incubator for 30 minutes. A sterile cork borer no. 4 (6mm diameter) was used to bore a hole in each test fungal spore's flooded Sabourand dextrose agar plates. The bottom of each well was sealed with a drop of molten sterile SDA.

Using a micropipette, 0.1 ml of the test combined antifungal agents at different pH solutions was dispensed into each of the hole, Sterile distilled water was used as control. These were allowed to diffuse into the agar at room temperature (25 ± 2 ^{0}C) for one hour, before incubation at 30 ^{0}C for 48 hours. The zones of inhibition of test organisms were measured to the nearest millimeter. This experiment was carried out in duplicates.

3.2.3.10. Determination of Effects of varying temperature on the Antifungal activity of combined test antifungal test agents.

The combined test antifungal agents were dissolved in 10mls of sterile distilled water. The set up was maintained at different temperatures (37^0C, 45^0C, 70^0C and 100^0C) in the water bath for thirty minutes. 0.1ml of the combined antifungal agents' solution was aseptically dispensed into the hole made on the agar plate and incubated as in pH study. Sterile water was used as the negative control. The zones of inhibition of the test organisms were measured using calibrated meter ruler and documented.

3.2.3.11. Determination of Physical and chemical interaction between the test antifungal agent alone and in admixtures.

The TLC plates were cut to the required size of 10cmx5cm. Different solvent systems (chloroform and methanl in a ratio of 6:4; Hexane and Ethyl acetate ratio 3:7; Hexane and Ethyl acetate ratio 7:3; Butanol, Acetic acid and water in a ratio of 8:1:1) were developed in a chromatographic tank leaving it to saturate. Samples were dissolved in 95% methanol, a drop of each was then spotted on prepared TLC plates and allowing it to dry. The plates were then immersed in to the chromatographic tank. The lid was replaced and allowed running until the solvent reached about ¾ of plate. The plates were removed and the solvent front was marked immediately. The plates were air dried, viewed in daylight, after spraying with visualizing reagent (10% sulphoric acid in absolute methanol) and

after heating in hot air oven. Different spots were displayed on the plates and R_f was calculated for each spot.

3.2.3.12. In vivo effect of the combined test antifungal agents' solution on yam leaves infected with phytopathogenic fungi spores.

The experimental *D. rotundata* yam farms were divided into three equal blocks (for example A7, A8 and A9). The yam leaves in Blocks A8 and A9 were sprayed with 10^8cfu/ml of phytopathogenic *Aspergillus niger* spores suspensions, while sterile distilled water was sprayed on the yam leaves in the blocks A7. *D. rotundata* leaves in Block A9 were counter sprayed with known concentrations of the combined antifungal agents prepared with sterile distilled water as diluent.

The infected and untreated yam leaves in block A8 and uninfected but sprayed with sterile distilled water A7 serve as positive and negative contrls. The fungal growth or change in colour of yam leaves observed was documented. These observations were made for two weeks.

CHAPTER FOUR

RESULTS

4.1 PHYTOPATHOGENIC FUNGI ISOLATED FROM YAM LEAVES FROM THE FIELD INCLUDES:

Many different fungal species were isolated from twelve randomly selected farms of *Dioscoria* species (four farms) in Kauyen Ali Dakaci, Zaria L.G.A; Wanka, Soba L.G.A. and Rafinyashi, Giwa L.G.A. of Kaduna state, Nigeria in 'Latitude $10^0$57"E and $11^0$12"N, Longitude $7^0$45"E and $8^0$6"N. Fourteen different fungi species were isolated and identified from the twelve farms in Zaria as shown in the Table 4.1 and 4.2.



Plate 4.1: One of the Yam (*Dioscoria* species) Farm kauyen Ali Dakaci, Zaria, Nigeria.

Aspergillus niger occurred nine times from the twelve farms. The order of occurance of isolated phytopathogenic fungi spores was *Aspergillus niger* (10) > *Cladosporium sp* (7) = *Aspergillus ostianus* (7) > *Scopolarium* sp (5) = *Aspergillus flavus* (5) = *Rhizopus sp* sp (5) = *Penicillium citrinum* (5) > *Aspergillus wentii* (4) > *Penicillium oxalicum* (3) = *Aspergillus cervinus* (3) = *Penicillium* sp (3) = *Aspergillus fumigatus* (3) > *Aspergillus oryzae* (2) = *Rhizopus stolonifer* (2) (Table 4.1).

All the observation from the twelve yam farms investigated showed that all the farms displayed mixed fungal infection of the yam leaves. However, the degree of mixed infections varied. The order of fungal infections from the farms studied are as follows: Farm G (9) > B (8) = D (8) > A (7) = H (7) > C (5) = F (5) = J (5) > E (4) > I (3) > K (2) = L (2) (Table 4.2).

Further more, when the four farms in each of the three towns were considered, the order of phytopathogenic fungal infections observed was kauyen Ali Dakaci (27 fungal species)> wanka (25 fungal species)> Rafin yashi (12 fungal species). Statistically there is a significant difference in the fungal infection in these investigated yam leaves in the three towns in Kaduna state, Nigeria.

Table 4.1 Percentage of occurance of phytopathogenic fungi isolates from leaves of yams in 12 farms in three towns in Kaduna state, Nigeria.

S/N	Organisms	% of occurance
1	*Penicillium citrinum* (PC)	7.813
2	*Penicillium oxalicum* (Po)	4.688
3	*Penicillium sp* (Pen sp)	4.688
4	*Aspergillus cervinus* (Ac)	4.688
5	*Aspergillus fumigatus* (Afm)	4.688
6	*Aspergillus niger* (An)	15.625
7	*Aspergillus wentii* (Aw)	6.250
8	*Aspergillus ostianus* (Ast)	10.938
9	*Aspergillus oryzae* (Aory)	3.125
10	*Aspergillus flavus* (Afv)	7.813
11	*Scopolarium* sp (Sco)	7.813
12	*Cladosporium* sp (Clod)	10.938
13	*Rhizopus* sp sp (R. sp)	7.813
14	*Rhizopus stolonifer* (Rs)	3.125

Table 4.2: Distribution of fungi isolates from leaves of *D. rotundata* in 12 farms in three towns in Kaduna state, Nigeria.

Farms Studied	Number of species	Type of Organisms Isolated
A	7	Pc, Pen. sp, Sco, Clo, Afv, Rs, A. ost
B	8	Pc, Ac, Pen. sp, An, Clo, Afm, Afv, R. sp,
C	5	Po, An, Sco, R. sp, A. ost
D	8	Aw, Po, Pen. sp, An, Afm, Afv, A.ost, R.sto,
E	4	An, Sco, Clo, R. Sp
F	5	Pc, An, Sco, Clo, A. ory
G	9	Pc, Aw, Po, Ac, An, Clo, Afv, A.ost, A. ory
H	7	Pw, Ac, An, Clo, Afv, A.ost, R. sto,
I	3	An, Afv, R. Sp
J	5	Pen. sp, An, Sco, Clo, A. ost
K	2	Aw, A. ost
L	2	An, Pc

Key:

1. *Penicillium citrinum*	(PC)	8. *Cladosporium* sp	(Clo)
2. *Aspergillus wentii*	(Aw)	9.*Aspergillus fumigates*	(Afm)
3. *Penicillium oxalicum*	(Po)	10. *Aspergillus flavus*	(Afv)
4. *Aspergillus cervinus*	(Ac)	11. *Rhizopus* sp	(R.sp)
5. *Penicillium sp*	(pen. sp)	12.*Aspergillus ostianus*	(A.ost)
6. *Aspergillus niger*	(An)	13. *Aspergillus oryzae*	(A.ory)
7. *Scopolarium* sp	(Sco)	14. *Rhizopus stolonifer*	(R.sto)

Table 4.3: Distribution of fungi isolates from leaves of *D. rotundata* in 12 farms in three towns in Kaduna state, Nigeria.

	Isolates number				
Town studied	As	Rs	Ps	Scs	Cls
Zaria	12	4	7	2	2
Soba	13	2	4	2	4
Giwa	7	1	2	1	1
Total isolates	32	7	13	5	7

Key:

Ps = *Penicillium* sp
As = *Aspergillus* sp
Cls = *Cladosporium* sp
Scs = *Scoppolarium* sp
Rs = *Rhizopus* sp

4.2 MINIMUM INHIBITORY CONCENTRATION (MIC) OF TEST ANTIFUNGAL COMPOUNDS AGAINST PHYTOPATHOGENIC YAM FUNGAL ISOLATE SPORES

The result of minimum inhibitory concentration of Terbinafine, Sodium propionate, Fluconazole, Flucytosine and Benomyl against the isolated phytopathogenic yam fungi shows that: benomyl has a higher antifungal inhibitory activity with a lower range of 0.98-15.25 µg/ml; followed by terbinafine with a range of 0.98-31.25 µg/ml; flucytosine with range of 0.78-200 µg/ml; fluconazole with a range of 1.95-500 µg/ml while sodium propionate has the lowest inhibitory activity with a higher range value of 19.53-10,000 µg/ml (Table 4.4 and 4.5).

The sensitivity of these test isolated fungi spores varies from one organism to the other. Observation from this work showed that *Rhizopus* sp and Aspergillus *ostianus* were the most sensitive test fungi spores followed by *Aspergillus oryzae* and *Scoppolarium* sp., *Rhizopus stolonifer* and *Penicillium citrinum*. *Aspergilus niger* was observed to be the most resistant test fungal spores to the test chemical agents (Table 4.5).

Table 4.4: The Range of Minimum Inhibitory Concentration (MIC) values of test antifungal compounds against phytopathogenic yam fungal isolate spores at 30^0C after 48hours

	MIC(µg/ml)				
Test Organisms	**Terbinafine**	**Fluconazole**	**Sodium Propionate**	**Flucytosine**	**Benomyl**
Ps	7.81-31.25	62.50-125.00	1250.00-2500.00	6.25-100.00	0.49-0.98
As	0.98-15.25	1.95-500.00	78.25-10000.00	0.78-200.00	0.98-15.25
Scs	1.85-3.91	31.25-62.50	625.00-1250.00	3.125-6.25	0.98-1.95
Cls	0.49-0.98	1.95-3.91	62.25-312.50	50.00-100.00	7.81-15.25
Rs	1.95-250.00	3.91-1000.00	19.53-2500.00	0.78-200.00	0.98-500.00

Key:

Ps	*Penicillium* sp	(11)
As	*Aspergillus* sp	(34)
Cls	*Cladosporium* sp	(5)
Scs	*Scoppolarium* sp	(7)
Rs	*Rhizopus* sp	(7)

Table 4.5: The Minimum Inhibitory Concentration (MIC) values of test antifungal compounds against resistant phytopathogenic yam fungal isolate spores 10^8 cfu/ml at 30^0C after 48hours

	MIC(µg/ml)				
Test Organisms	**Terbinafine**	**Fluconazole**	**Sodium Propionate**	**Flucytosine**	**Benomyl**
1	31.25	125.00	2500.00	100.00	0.98
2	15.25	250.00	2500.00	50.00	15.25
3	15.63	500.00	1250.00	25.00	7.81
4	0.97	125.00	2500.00	100.00	0.98
5	7.81	62.25	2500.00	6.25	0.98
6	3.91	500.00	10000.00	0.78	1.95
7	3.91	62.50	1250.00	6.25	1.95
8	0.98	3.91	312.50	100.00	15.25
9	1.95	1.95	1250.00	200.00	7.81
10	1.95	1.95	1250.00	0.78	0.98
11	1.95	3.91	19.53	0.78	0.98
12	1.95	3.91	78.25	0.78	0.98
13	0.98	1.95	312.50	50.00	0.98
14	250.00	1000.00	2500.00	200.00	500.00

Key:

1.	*Penicillium citrinum* (PC)	8.	*Cladosporium* sp (Clod)
2.	*Aspergillus wentii* (Aw)	9.	*Aspergillus fumigatus* (Afm)
3.	*Penicilium oxalicum* (Po)	10.	*Aspergillus flavus* (Afv)
4.	*Aspergillus cervinus* (Ac)	11.	*Rhizopus* sp (2)Rs
5.	*Penicillium sp* (pen sp)	12.	*Aspergillus ostianus* (Ast)
6.	*Aspergilus niger* (An)	13.	*Aspergillus oryzae* (Aory)
7.	*Scoppolarium* Sp (Sco)	14.	*Rhizopus stolonifer* (1)Rs

4.3 MINIMUM FUNGICIDAL CONCENTRATION (MFC) VALUES OF TEST ANTIFUNGAL COMPOUNDS AGAINST PHYTOPATHOGENIC YAM FUNGAL ISOLATES

The result of minimum fungicidal concentration of terbinafine, Sodium propionate, fluconazole, flucytosine and Benomyl against the isolated phytopathogenic yam fungi spores (Table 4.7 and 4.8) showed the following order of antifungal activity Viz: terbinafine (1.95-2000µg/ml); fluconazole (3.91-4000µg/ml); benomyl (15.25-4000µg/ml); flucytosine (200-1600µg/ml) and sodium propionate (156.25-20,000µg/ml).

The sporicidal activities of the test antifungal agents depend on the test phytopathogenic fungal spores involved. Generally, observations from this research have shown that, the order of test fungal spores susceptivbility to test antifungal agents was *Cladosporium* sp > *Scoppolarium* sp > *Penicillium* sp > *Rhizopus* sp > *Aspergillus* sp (Table 4.6).

Table 4.6: The Range of Minimum Fungicidal Concentration (MFC) values of test antifungal compounds against phytopathogenic yam fungal isolate spores at 30^0C after 48hours

	MFC(µglml)				
Test Organisms	**Terbinafine**	**Fluconazole**	**Sodium Propionate**	**Flucytosine**	**Benomyl**
Ps	15.63-62.50	125.00-1000.00	2500.00-5000.00	400.00-800.00	62.50-1000.00
As	1.95-7.81	3.91-4000.00	156.00-20000.00	200.00-800.00	15.25-2000.00
Scs	3.91-7.81	62.50-125.00	1250.00-2500.00	400.00-800.00	500.00-1000.00
Cls	1000.00-2000.00	3.91-7.81	10000.00->10000.00	200.00-400.00	31.25-62.50
Rs	3.91-500.00	7.81-2000.00	312.00-5000.00	400.00-1600.00	250.00-2000.00

Key:

Ps	*Penicillium* sp	(11)
As	*Aspergillus* sp	(34)
Cls	*Cladosporium* sp	(5)
Scs	*Scoppolarium* sp	(7)
Rs	*Rhizopus* sp	(7)

Table 4.7: The Minimum Fungicidal Concentration (MFC) values of test antifungal compounds against phytopathogenic yam fungal isolate spores at 30^0c after 48hours

	MFC (µg/ml)				
Test Organisms	**Terbinafine**	**Fluconazole**	**Sodium Propionate**	**Flucytosine**	**Benomyl**
1	62.50	250.00	5000.00	400.00	62.50
2	31.25	500.00	5000.00	200.00	500.00
3	31.25	1000.00	2500.00	>800.00	1000.00
4	1.95	250.00	5000.00	400.00	15.25
5	15.63	125.00	5000.00	800.00	1000.00
6	7.81	4000.00	20000.00	1600.00	1000.00
7	7.81	125.00	2500.00	800.00	1000.00
8	2000.00	7.81	>10000.00	400.00	62.50
9	3.91	3.91	>10000.00	400.00	>2000.00
10	3.91	3.91	>10000.00	800.00	250.00
11	3.91	7.81	312.50	400.00	250.00
12	3.91	7.81	156.25	800.00	500.00
13	1.95	3.91	625.00	400.00	62.50
14	500.00	2000.00	5000.00	1600.00	2000.00

Key:

1. *Penicillium citrinum* (PC)
2. *Aspergillus wentii* (Aw)
3. *Penicilium oxalicum* (Po)
4. *Aspergillus cervinus* (Ac)
5. *Penicillium sp* (Pen)
6. *Aspergilus niger* (An)
7. *Scoppolarium* sp (Sco)
8. *Cladosporium* sp (Clad)
9. *Aspergillus fumigatus* (Afm)
10. *Aspergillus flavus* (Afv)
11. *Rhizopus* sp (2)Rs
12. *Aspergillus ostianus* (Ast)
13. *Aspergillus oryzae* (Aory)
14. *Rhizopus stolonifer* (1)Rs

4.4 COMBINED MINIMUM INHIBITORY CONCENTRATION (MICs) OF TEST ANTIFUNGAL AGENTS AGAINST ISOLATED FUNGI SPORES.

Combined MICs determination enables one to calculate fractional inhibitory concentration (FICs) which display the type of microbial response to test combination of antimicrobial agents. This involves the calculation of FICs of the single and combined MICs data of test chemical agents against a test organism. The result obtained from the calculation exhibits additive, synergistic or antagonistic action against the test organism.

Table 4.8 shows the FIC of benomyl with sodium propionate; Table 4.9 shows the FIC of Terbinafine in admixture with sodium propionate that gives complete inhibition of *R. stolonifer* spores suspension. Table 4.11 shows the summary of FICs of Terbinafine HCl, Benomyl and Fluconazole each in admixture with sodium propionate that gives complete inhibition of test fungal spores' suspension.

Table 4.8: FIC of benomyl (BEN) in admixture with sodium propionate (NP) that gives complete inhibition of *R. stolonifer* spores suspension (10^8 cfu/ml)

MIC of Ben 500µg/ml		MIC of Np 2500 µg/ml		
Conc. of Ben in admixture	FIC	Conc. of Np in admixture	FIC	∑FIC
50.00	0.10	200.00	0.08	0.18
40.00	0.08	200.00	0.08	0.16
30.00	0.06	200.00	0.08	0.14
20.00	0.04	200.00	0.08	0.12
10.00	0.02	200.00	0.08	0.10

Mean of FIC =0.14

FIC = A/B

A = the concentration of antifungal agent present in the combination that is inhibitory

B = the concentration of the antifungal agent which would produce the same effect as the combination when acting alone (Elion, *et al*., 1954).

FIC =Fractional inhibitory concentrations of the combined antifungal agents.

Table 4.9: FIC of terbinafine (TER) in admixture with sodium propionate (NP) that gives complete inhibition of *A. niger* spores suspension (10^8 cfu/ml)

MIC of Ter 3.9 µg/ml		MIC of Np 10,000 µg/ml		
Conc. of Ter in admixture	FIC	Conc. of Np in admixture	FIC	∑FIC
1.00	0.26	200.00	0.02	0.28
0.70	0.18	200.00	0.02	0.20
0.50	0.13	200.00	0.02	0.15
0.25	0.06	200.00	0.02	0.08
0.10	0.04	400.00	0.04	0.07

Mean of FIC = 0.156
FIC = A/B
A = the concentration of antifungal agent present in the combination that is inhibitory
B = the concentration of the antifungal agent which would produce the same effect as the combination when acting alone (Elion, *et al*., 1954).
FIC = Fractional inhibitory concentrations of the combined antimicrobial agents.

Table 4.10: Summary of Fractional Inhibitory Concentrations of combined test antifungal compounds against resistant phytopathogenic yam fungal spores at 30^0c after 48hours.

	FIC of combined Antifungal agents		
Combination of fungal agents	Pc	An	Rs
Fluc/Np	0.117	0.112	0.126
Ben/NP	0.590	0.444	0.140
Ter/Np	0.128	0.154	0.098

Key:

Pc- *Penicillium citrinum*
An - *Aspergillus niger*
Rs - *Rhizopus stolonifer*

Interpretation:

FIC Value less than (□) 1.0	=	Synergistic
FIC Value between 1- 4.0	=	Additive or indifference
FIC Value greater than (□) 4.0	=	Antagonistic

4.5 DETERMINATION OF THE FRACTIONAL FUNGICIDAL CONCENTRATION (FFC) VALUES OF TEST ANTIFUNGAL COMPOUNDS AGAINST PHYTOPATHOGENIC YAM FUNGAL ISOLATE SPORES

The fractional fungicidal concentration is a quantitative representation of microbial lethal response to the combined antifungal activities of antimicrobial agents. The data obtained from the calculated antimicrobial response may be additive, synergistic or antagonistic against the test organism.

Table 4.11 shows the FFCs of terbinafine in admixture with sodium propionate that gives complete inhibition of *A. niger* spores suspension. Similarly Table 4.12 also showed FFCs of benomyl in admixture with sodium propionate that gives complete inhibition of *R. stolonifer* spores suspension.

Table 4.11: FFC of terbinafine (Ter) in admixture with sodium propionate (Np) that gives complete fungicide of *A. niger* spores suspension

MFC of Ter 7.81 µg/ml		MFC of Np 20,000 µg/ml		
Conc. of Ter in admixture	FFC	Conc. of Np in admixture	FFC	∑FFC
1.00	0.128	200.00	0.010	0.138
0.70	0.090	200.00	0.010	0.100
0.50	0.064	200.00	0.010	0.074
0.25	0.032	400.00	0.020	0.052
0.10	0.012	600.00	0.030	0.043

Mean of FFC = 0.081
FFC = A/B
A = the concentration of antifungal agent present in the combination that is fungicidal
B = the concentration of the antifungal agent which would produce the same effect as the combination when acting alone (Elion, *et al*., 1954).
FFC = Fractional fungicidal concentrations of the combined antimicrobial agents.

Table 4.12: FFC of benomyl (Ben) in admixture with sodium propionate (Np) that gives complete fungicide of *R. stolonifer* spores suspension

MFC of Ben2000 µg/ml		MFC of Np 5000 µg/ml		
Conc. of Ben in admixture	FFC	Conc. of Np in admixture	FFC	∑FFC
50.00	0.025	400.00	0.080	0.105
40.00	0.020	800.00	0.160	0.180
30.00	0.015	800.00	0.160	0.175
20.00	0.010	800.00	0.160	0.170
10.00	0.005	1000.00	0.200	0.205

Mean of FFC = 0.167

FFC = A/B

A = the concentration of antifungal agent present in the combination that is fungicidal

B = the concentration of the antifungal agent which would produce the same effect as the combination when acting alone (Elion, *et al.*, 1954).

FFC = Fractional fungicidal concentrations of the combined antimicrobial agents

Table 4.13 Summary of Fractional Fungicidal Concentrations Of Combined Test Antifungal Compounds against Resistant Phytopathogenic Yam Fungal Spores At 30^0C After 48hours

FFC of combined Antifungal agents			
Combination of fungal agents	Pc	An	Rs
Fluc/Np	0.336	0.063	0.230
Ben/NP	0.528	0.031	0.167
Ter/Np	0.056	0.081	0.410

Key:
Pc - *Penicillium citrinum*
An - *Aspergillus niger*
Rs - *Rhizopus stolonifer*

Interpretation:

FFC Value less than (□) 1.0	=	Synergistic
FFC Value between 1- 4.0	=	Additive or indifferece
FFC Value greater than (□) 4.0	=	Antagonistic

4.6 THE EFFECT OF TEST COMPOUNDS ON THE VIABILITY OF THE TEST FUNGAL SPORES

The viability of the resistant test organism (*A. niger*) against the test antifungal agents were determined through the process of viable spores counts at different times or varying concentrations at fixed time.

At a fixed concentration of 550.00, 8,000.00, 55,000.00 and 65, 000.00µg/ml for Terbinafine HCl, Benomyl, Fluconazole and Sodium propionate respectively, rapid fungicidal activities were observed. The rate of kill of test fungal spores was observed to be fungicide dependent. Figures 4.3, 4.4, 4.5, and 4.6 depict the test fungal spores' death curves at fixed fungicidal concentration and varying contact time. From these graphs it can be seen that the rate of kill of *A. niger* spores by the fixed fungicidal concentrations of test antifungal agents viz Terbinafine HCl [550µg/ml], Benomyl [8,000µgml], Fluconazole [55,000.00µg/ml] and Sodium propionate [65,000.00µg/ml] progressively diminishes with time. For instance, the rate of kill by 55,000µg/ml of Fluconazole after initial five minutes contact was 3.83 log cycles reduction and that of the next fifteen minutes was 0.48 log cycle reduction (figure 4.3). This pattern of rate of fungicidal activity was the same for the other test antifungal agents (figure 4.4, 4.5, and 4.6).

Reduction of spores population range between 3.5 to 7.56 log cycles within 20 minutes contact time for *A. niger* spores and 4.31 to 8.80 log cycles reduction within 60 minutes contact time.

The slope of the death curves for all test antifungal agents against *A. niger* spores were similar, presenting the C type curve figure 4.1 in which there was an initial very rapid rate of kill, which diminishes with time. The concave shape is however not maintained throughout the exposure time, it come inverted or reversed of the usual type B curve in figure 2.1.

There were some differences in the effect of individual test antifungal agents on the resistant *A. niger* spores, Terbinafine HCl appeared generally to be more fungicidal than all the other test antifungal agents.

Table 4.14 Rate of kill of 10^8cfu/ml of *A. niger* spores suspension after 20 minutes contact time with test antifungal agents.

Antifungal Agent	K- value (min^{-1})
Fluconazole	0.100
Terbinafine	0.378
Benomyl	0.107
Sodium propionate	0.108

At 95 % confident limit, there was no significant difference of the antifungal activities between fluconazole, benomyl and sodium propionate on the test *A. niger*'s spores. But, there was a significant difference of the antifungal activities between the test fungicides and terbinafine on the *A. niger*'s spores investigated in this studies.

The death rate, (K-value) of 10^8cfu/ml of *A. niger* spores were calculated from the slopes obtained from the plot of log survivors versus time according to equation in K = 1/t [log No/Nt] applying to unimolecular reaction.

The higher the value of K, the faster the efficiency of the biocidal activity of the test antifungal agent, which is concentration depenent. The rate of the test antifungal agents activities on the spores generally was observe to be in the order of Terbinafine HCl (0.378) □ Sodium propionate (0.108) □ Benomyl (0.107) □ Fluconazole (0.100). The rate of kill of *A. niger* spores decreases rapidly till after 20 minutes of contact time. Generally Fluconazole and Sodium propionate appear to display a slow rate of kill for the two test antifungal agents (figure 4.3 and 4.5) after 20 minutes contact.

Figure 4.7, 4.8, 4.9, and 4.10 illustrate the effect of varying fungicidal concentrations of the test antifungal agents at 60 minutes fixed contact time. Figure 4.7 displayed an initial slow rate of fungicidal activity which increases with higher concentration of test antifungal agents. Generally, the four test antifungal agents were obviously fungicidal against *A. niger*.

It was observed that increase in sporicidal concentration also increases the rate of *A. niger* spores kill. It was also observed that each test antifungal agent has specific threshold concentration that effect maximum sporicidal activity against the test *A. niger* spores. The threshold maximum rate of sporicidal activities for Terbinafine HCl, Benomyl, Sodium propionate and Fluconazole were observed to be 250.00, 1000.00, 45,000.00 and 35,000.00µg/ml respectively.

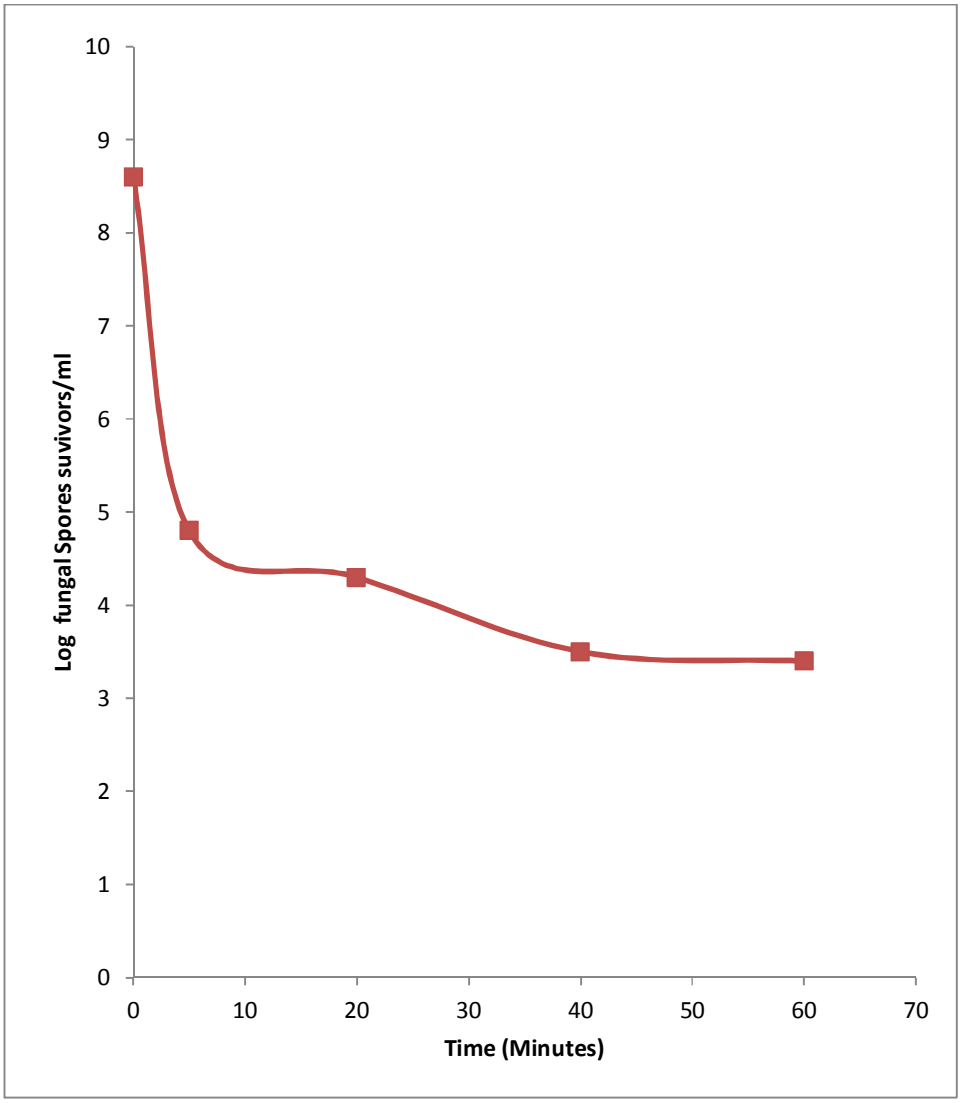


Fig 4.3 Fungicidal effect of 55,000µg/ml of fluconazole on resistant *Aspergillus niger* spores suspension at 25 ±2^{0}C.

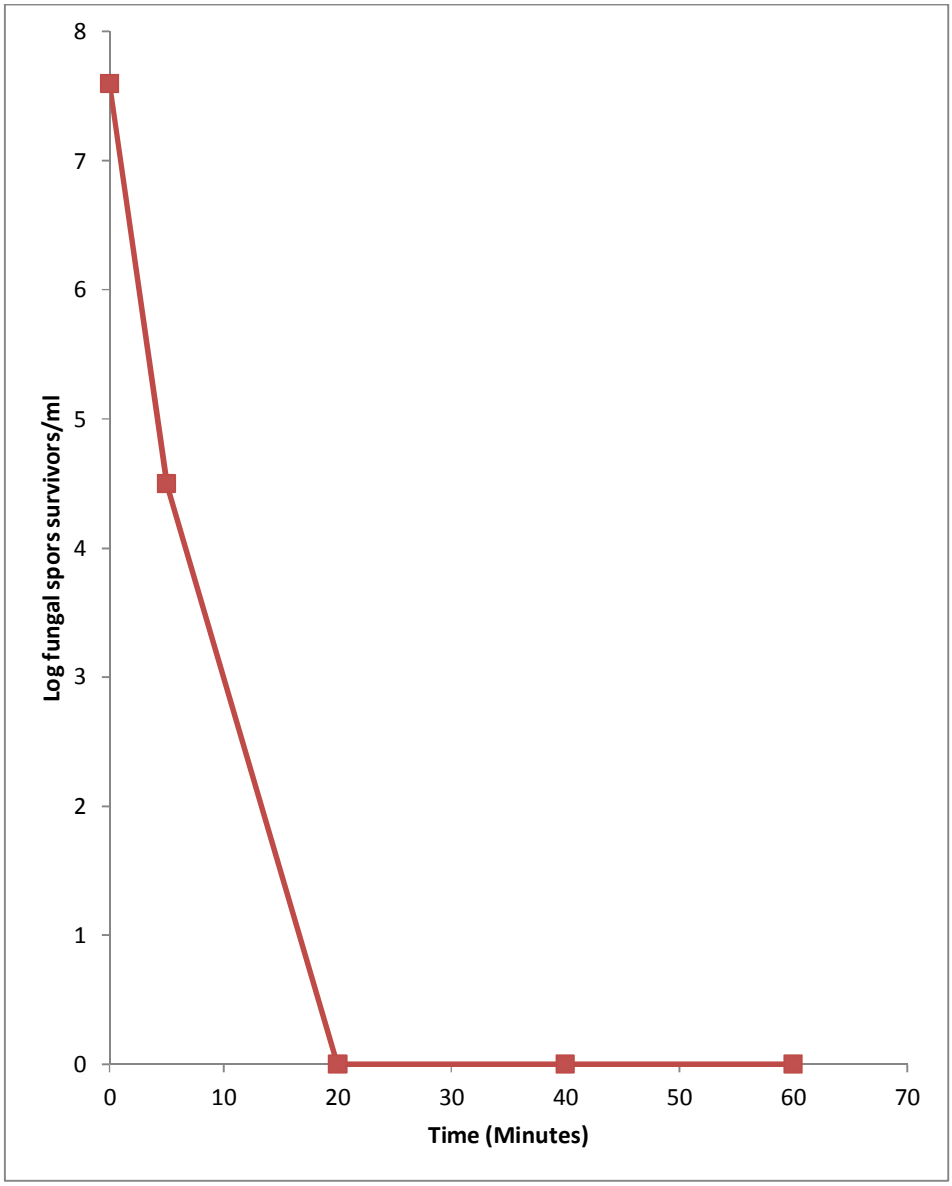


Fig 4.4: Fungicidal effect of 550µg/ml of Terbinafine HCl on resistant *Aspergillus niger* spores suspension

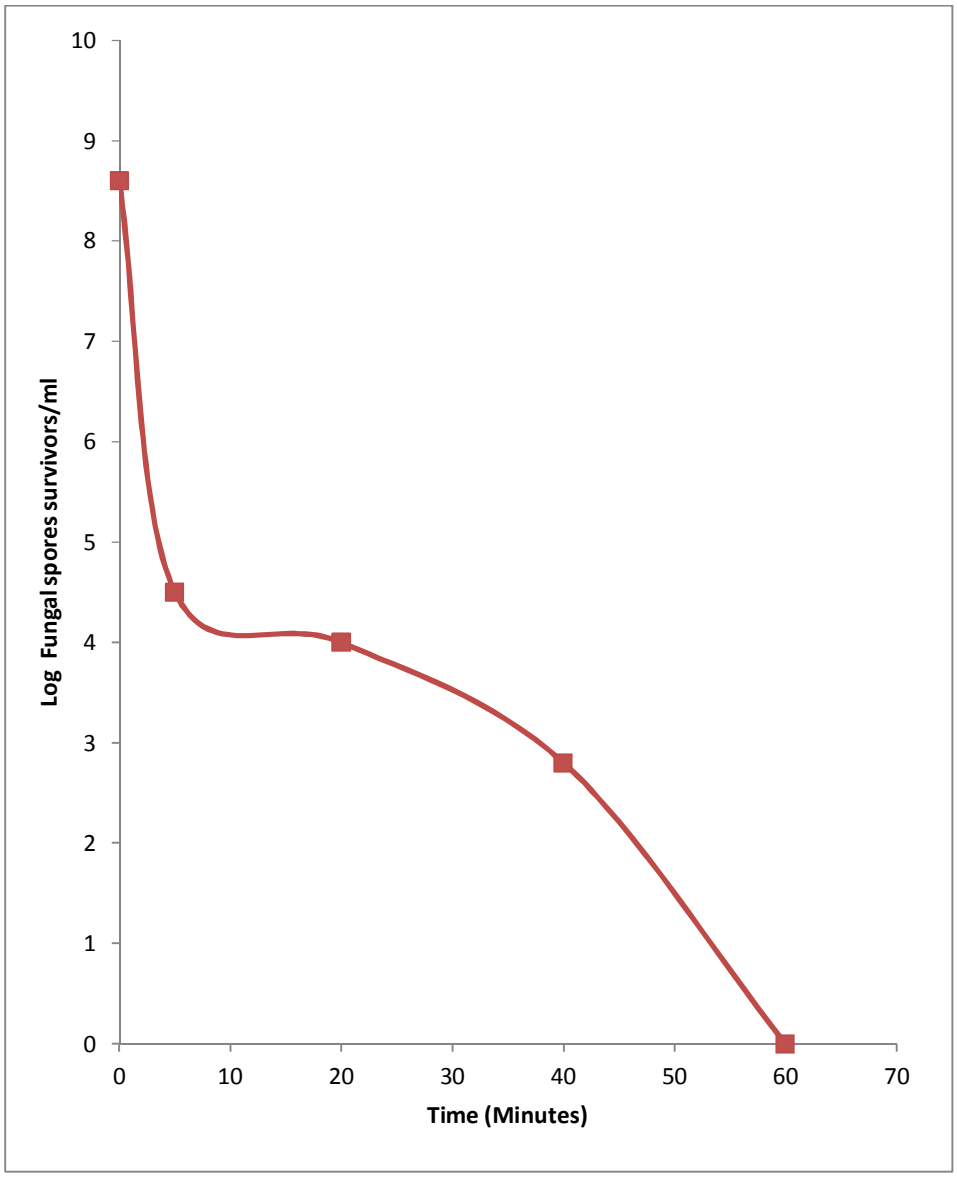


Fig 4.5: Fungicidal effect of 65000 µg/ml of Sodium propionate on resistant *Aspergillus niger* spores suspension

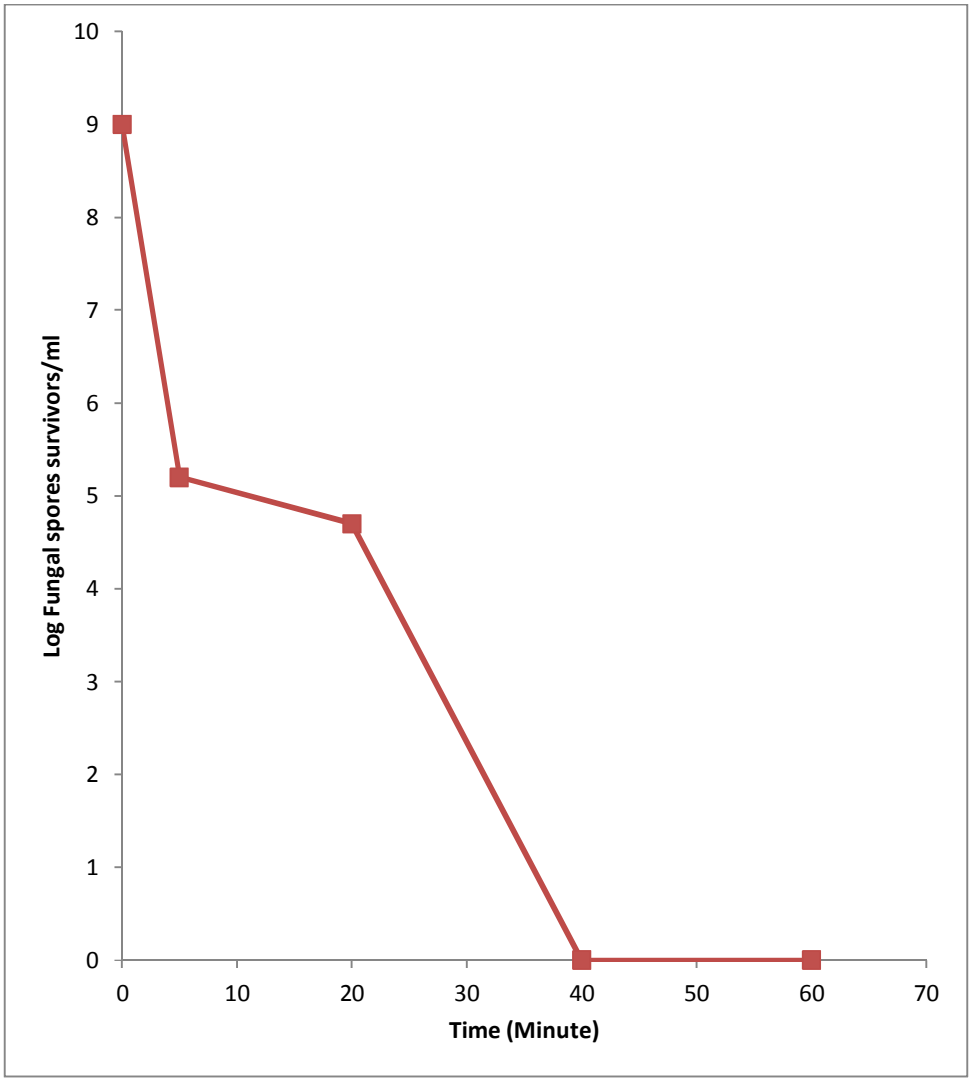


Fig 4.6: Fungicidal effect of 8000 µg/ml of Benomyl on resistant *Aspergillus niger* sporessuspension.

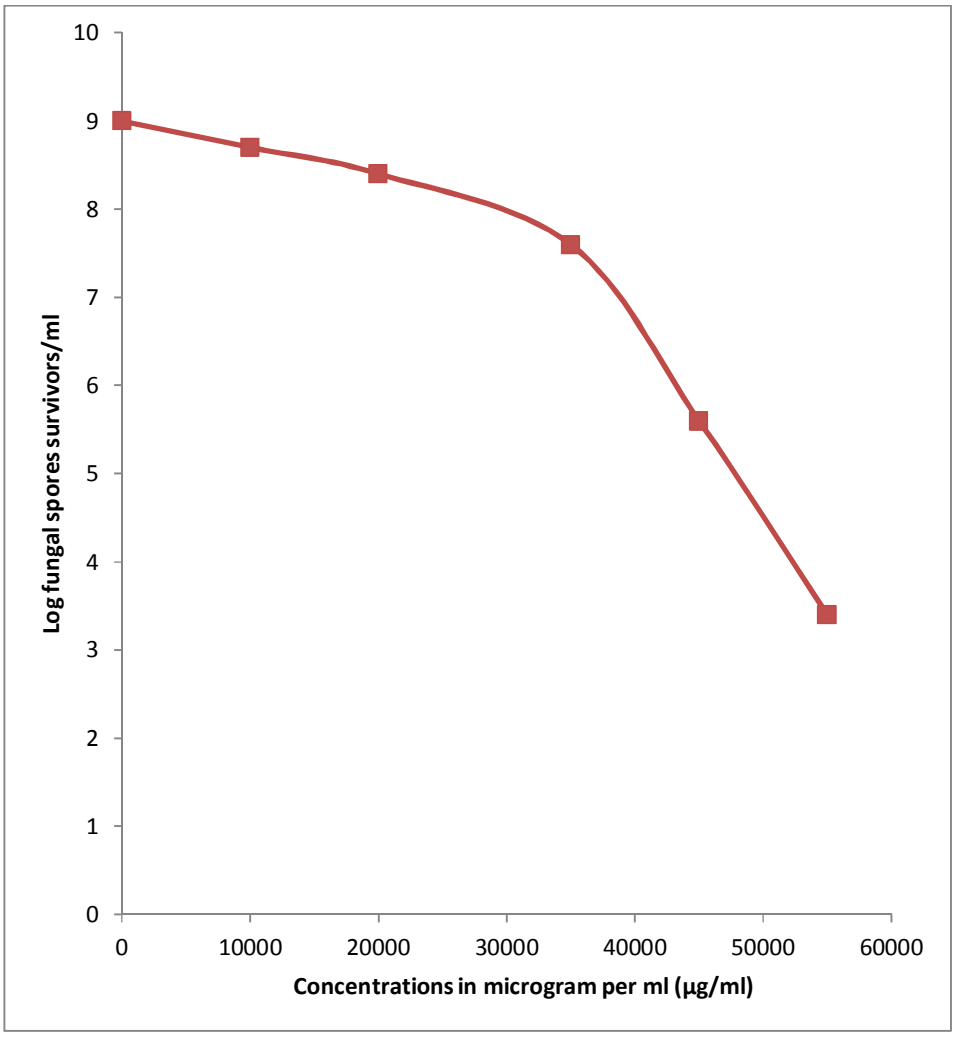


Fig 4.7: Fungicidal effect of varying concentrations of fluconazole on resistant *Aspergillus niger* spores suspension at fixed contact time of 60minute.

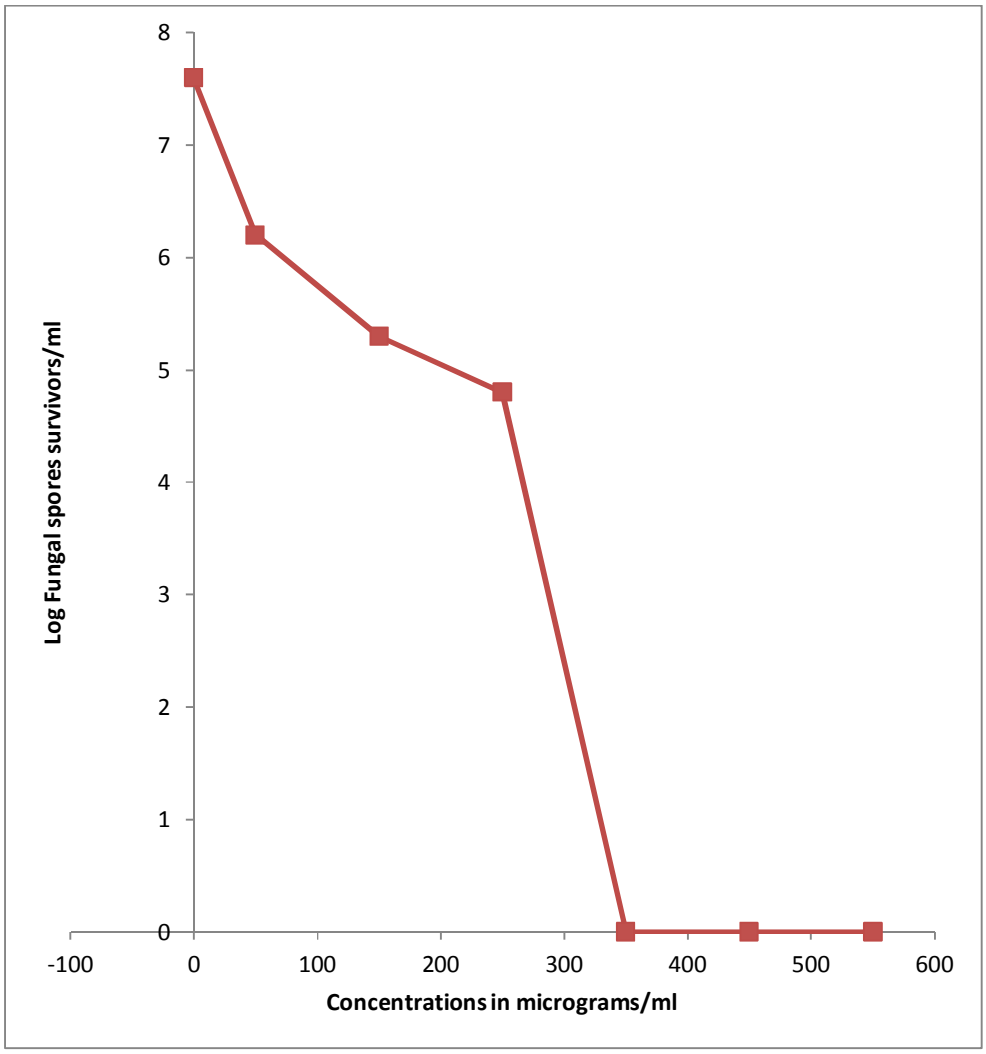


Fig 4.8: Fungicidal effect of varying concentrations of Terbinafine HCl on resistant *Aspergillus niger* spores suspension at fixed contact time of 60minute.

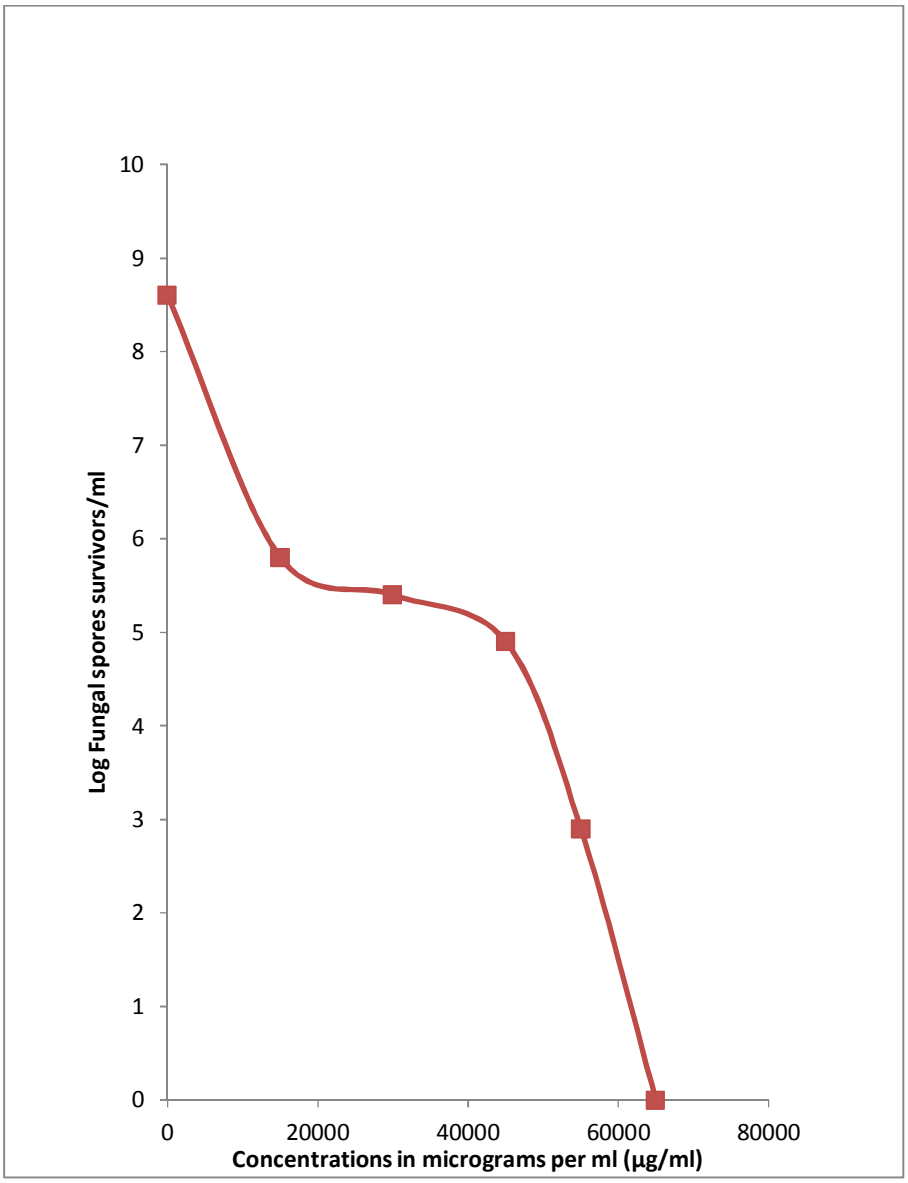


Fig 4.9: Fungicidal effect of varying concentrations of Sodium propionate on resistant *Aspergillus niger* spores suspension at fixed contact time of 60 minute.

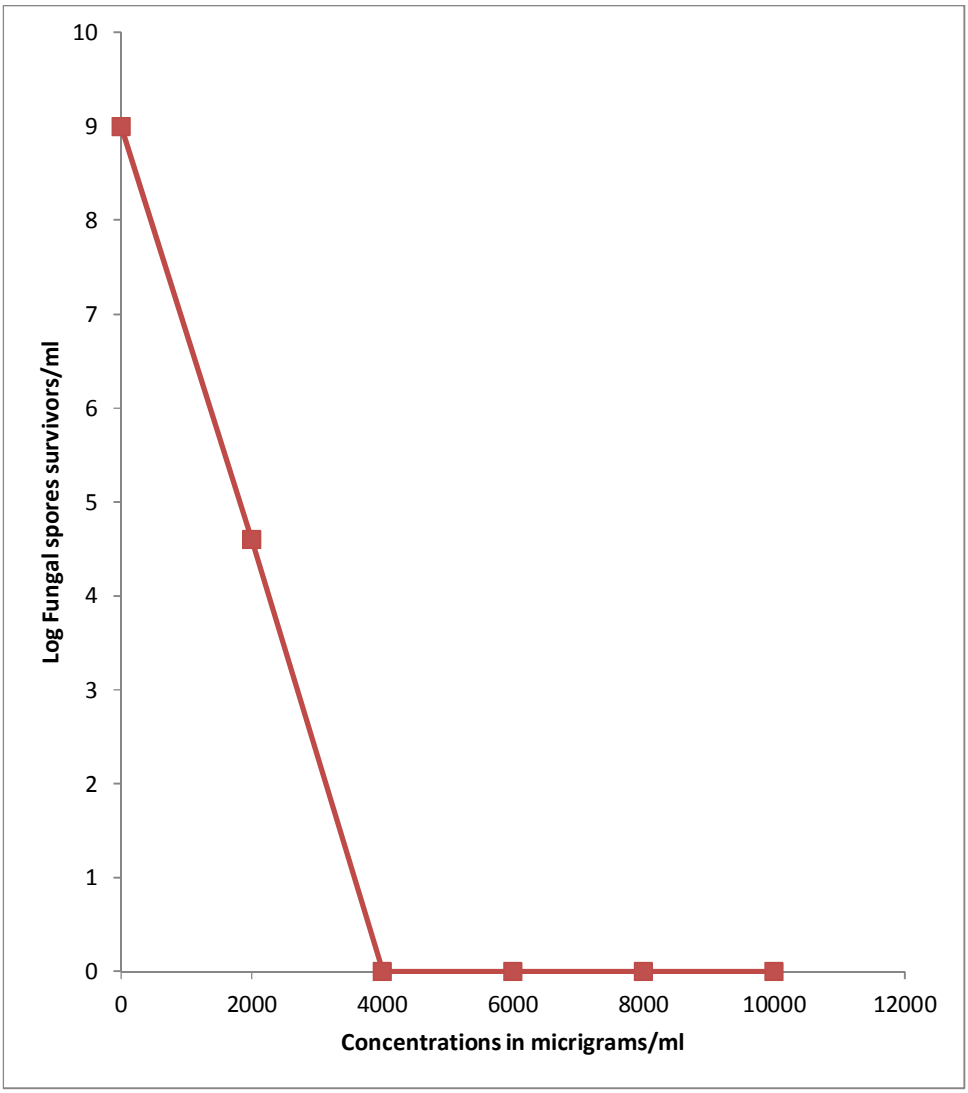


Fig 4.10: Fungicidal effects of varying concentrations of Benomyl on resistant *Aspergillus niger* at of spores suspension fixed contact time of 60 minute.

4.7 FUNGICIDAL ACTIVITY OF COMBINED ANTIFUNGAL AGENTS

When the approach of Jawezt (1967) was used for the interpretation of the combined fungicidal responses of the test antifungal agents admixtures, the results showed that the enhanced sporicidal activity produced by the combined test antifungal agents at lesser fungicidal concentration was more than the one produced by each antifungal agents in double concentration. This was an indication of either additive or synergistic responses of the combined test antifungal agents (Figures 4.11 and 4.12).

Generally, the death curves of the test *A. niger* spores by these admixtures were of 'C' type (Fig 2.1) while the duration of exponential death phase for the test fungal spores was not more than 5 to 15 minutes. This was followed by drastic reduction in the rate of sporicidal activities. For example 30,000.00 µg/ml of Fluconazole effected a one log cycle kill of *A. niger* spores ($4.0x10^7$cfu/ml) within the first five minutes, while not more than 0.20 log cycle kill was effected within the next fifteen minutes. Similarly, 2,000.00µg/ml of Benomyl, after 5 minutes and 15 minutes contact time effected 1.20 log cycles kill of *A. niger* spores (Figure 4.11), an indication of decrease in death rate. It is interesting to note that lower concentration of test antifungal agents in combination effected 2.90 log cycles kill of *A. niger* spores after 5 minute. 4.7 log cycles kill of the same test fungal spores was achieved within the next fifteen minutes contact time (Figure 4.11). This combination response probably indicates a synergistic effect and possible way of reducing the cost of effective fungicides production and probable toxicity. Similar observation was displayed with the response of

benomyl and Terbinafine HCl combination. However, Benomyl/Sodium propionate combination displayed death curve that probably indicate additive effect (Fig 4.13).

Table 4.15: Time taken to effect 1 log cycle (90%) reduction in 10^8cfu/ml of *A. niger* spores suspension with test antifungal agents

Test antifungal agents	D - values (minutes)	Ratio of D- values for Test antifungal agents in combinations/single activity
Benomyl (2,000µg/ml)	9.09	------
Fluconazole (30,000 µg/ml)	18.18	------
Terbinafine (200 µg/ml)	10.00	------
Sodium propionate (15,000 µg/ml)	10.53	------
Benomyl / Fluconazole (1000/15,000µg/ml)	2.63	3.46/6.91
Benomyl / Terbinafine (1000/100µg/ml)	3.77	2.41/2.65
Benomyl / Sodium propionate (1000/7,500µg/ml)	5.40	1.68/1.95

The result obtained in this study showed that the D_{10}-values of the entire test antifungal agent at fungicidal concentrations investigated range from 9.09 to 18.18 minutes when acting alone on spores of the test *A. niger*. However, when the Fluconazole, Terbinafine HCl and Sodium propionate were combined with Benomyl at half concentration each, the observed D_{10}-values drastically reduced to 2.63, 3.77 and 5.40 minutes respectively, (Table 4.16). This observation showed that the combined test antifungal agents possessed enhanced antifungal activity than when acting alone. The statistical analysis of these observed results showed that at 95% confident limit a significant difference exist between the antifungal agents (Benomyl with Fluconazole; Benomyl with Terbinafine; Benomyl with Sodium propionate single and their combinations in antifungal activity) and contact time. Lesser concentrations of the two combined agents were observed to be more potent than the higher concentration of the each single agent.

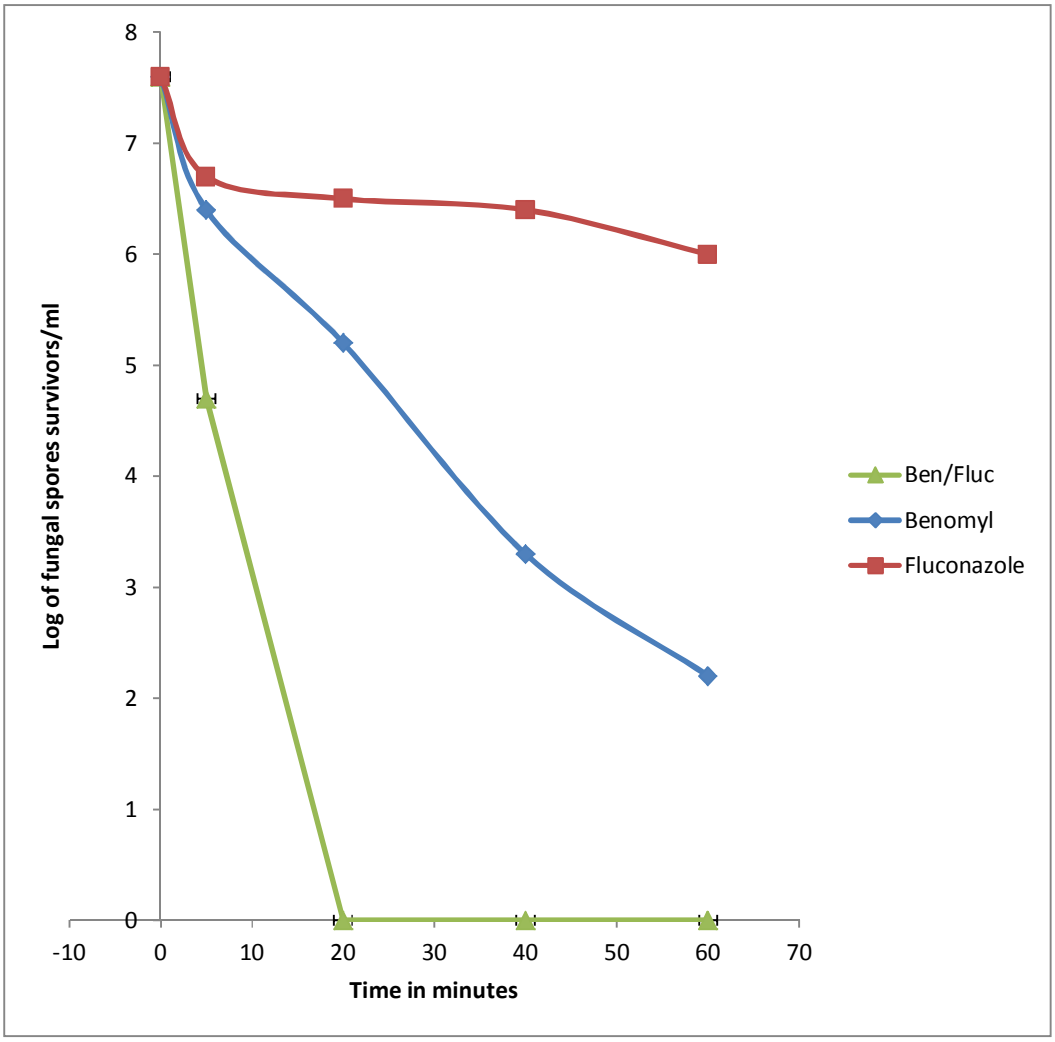


Fig 4.11: Graphical representation of the survival of resistant *Aspergillus niger* spore suspension against Benomyl (2000 µg/ml), Fluconazole (30,000µg/ml) and Benomyl (1000 µg/ml)+ Fluconazole (15,000µg/ml) at different time (minutes).

		Benomyl	Fluconazole	Ben/Flu
D value	=	8.33.0 minute	18.18 minutes	2.63 minutes

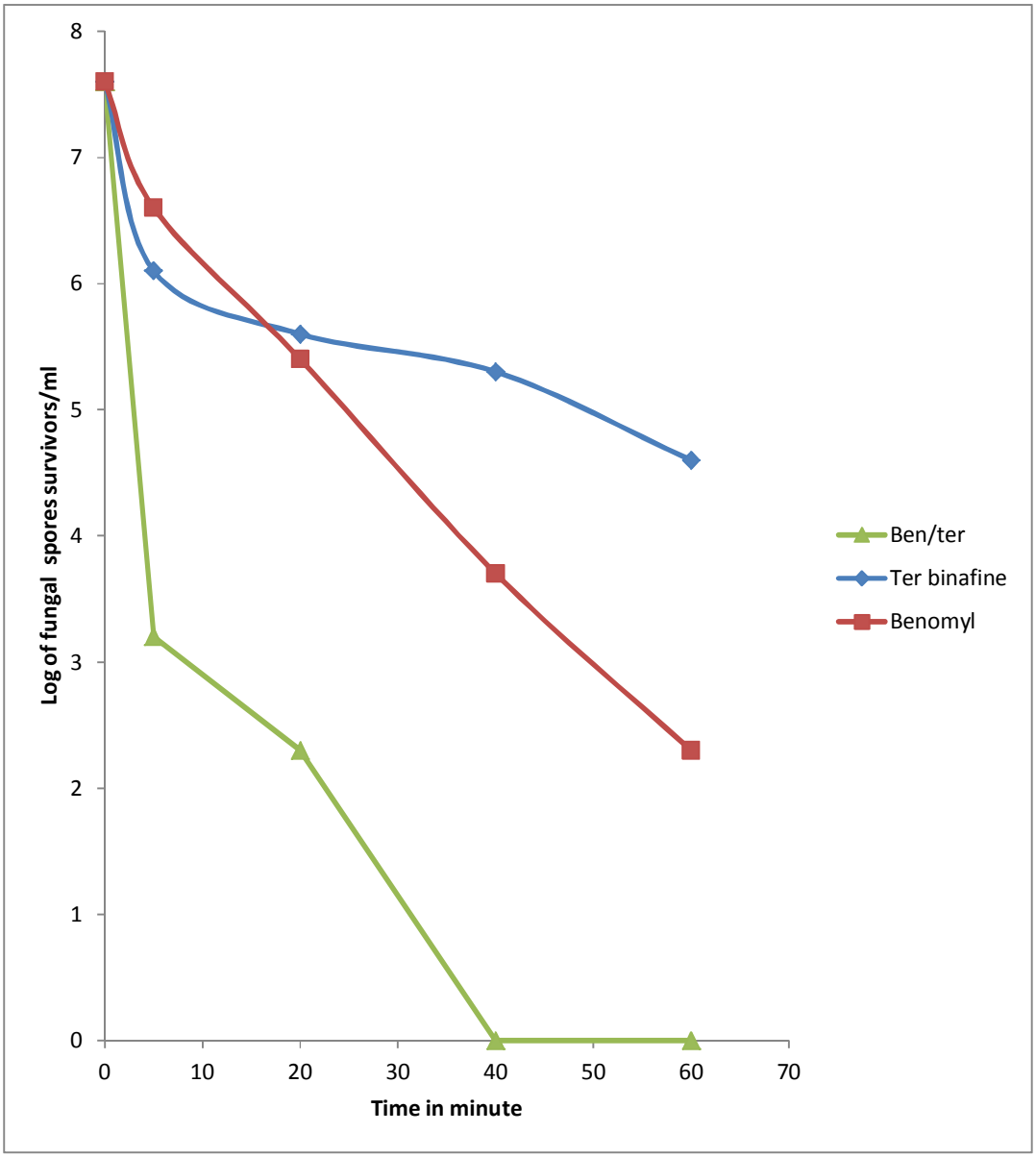


Fig 4.12: Fungicidal activity of Benomyl (2000 µg/ml), Terbinafine HCl (200µg/ml) and Benomyl (1000 µg/ml) + Terbinafine HCl (100µg/ml) against resistant *Aspergillus niger* spores suspension.

		Benomyl	Terbinafine	Ben/Ter
D value	=	9.09 minute	10.00 minutes	3.77 minutes

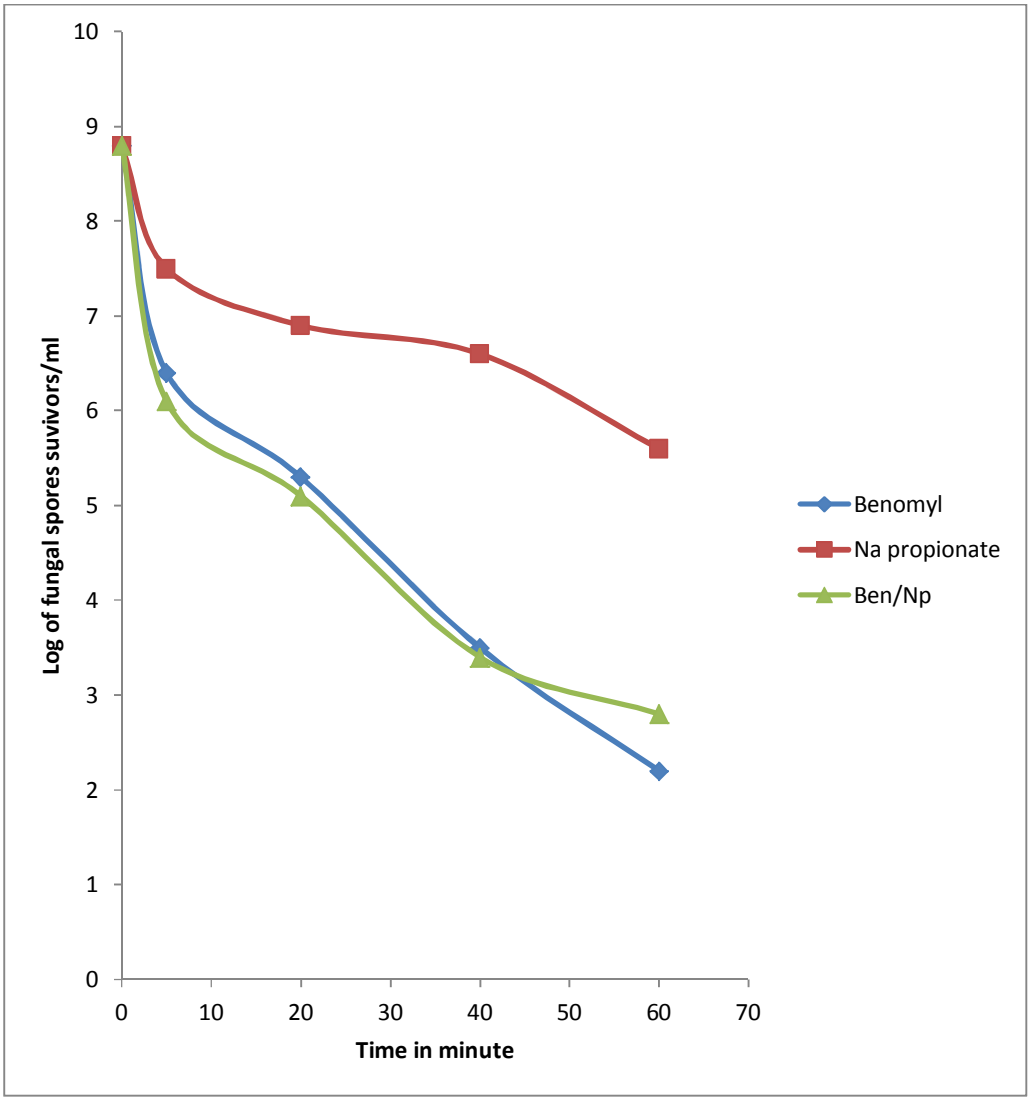


Fig 4.13: Graphical representation of the survival of resistant *Aspergillus niger* spores suspension against Benomyl (2000 µg/ml), Sodium propionate (15000µg/ml) and Benomyl (1000 µg/ml)+ Sodium propionate (7500µg/ml) at different time (minutes)

		Benomyl	Sodium propionate	Ben/Sod
D value	=	5.71 minute	10.53 minutes	5.40 minutes

4.8 DNA RELEASE FROM CYTOPLASMIC CONSTITUENT OF TEST *A. niger* SPORES.

The effect of chemical compounds such as Fluconazole (5,000µg/ml); Terbinafine (20µg/ml); Sodium propionate (40,000µg/ml) and Benomyl (2,000µg/ml) were studied for their influence on cytoplasmic leakage from *A. niger* spores. The leakage may lead to the release of the cytoplasmic constituents such DNA which can be detected using biospecrophotometer at a wavelenght of 260nm. The obtained results showed that with fixed concentration of each compound at varying contact time (0, 5, 10, 20, 40, 60, 120, 180 and 240 minutes), Fluconazole displayed a higher DNA release followed by Terbinafine HCl. The order of DNA release was Fluconazole > Terbinafine > Sodium propionate > benomyl (Fig 4.14).

The amount of DNA released by the Fluconazole increased with increased contact time till the DNA becomes constant as from contact time of 180 to 240 minutes. Terbinafine HCl DNA release was gradual with increased contact time till 40 minutes contact time and subsequently fell drastically without further increase. The DNA released when benomyl was test antifungal agent followed a similar pattern but with very low activity. Sodium propionate displayed the lowest DNA release from *A. niger* sporec suspensions. Statistical analysis showed a significant difference between the antifungal agent at $p<0.05$. However, for fixed concentration of Terbinafine, Sodium propionate and Benomyl antifungal agents, there was no significant increase in DNA release with increased time.

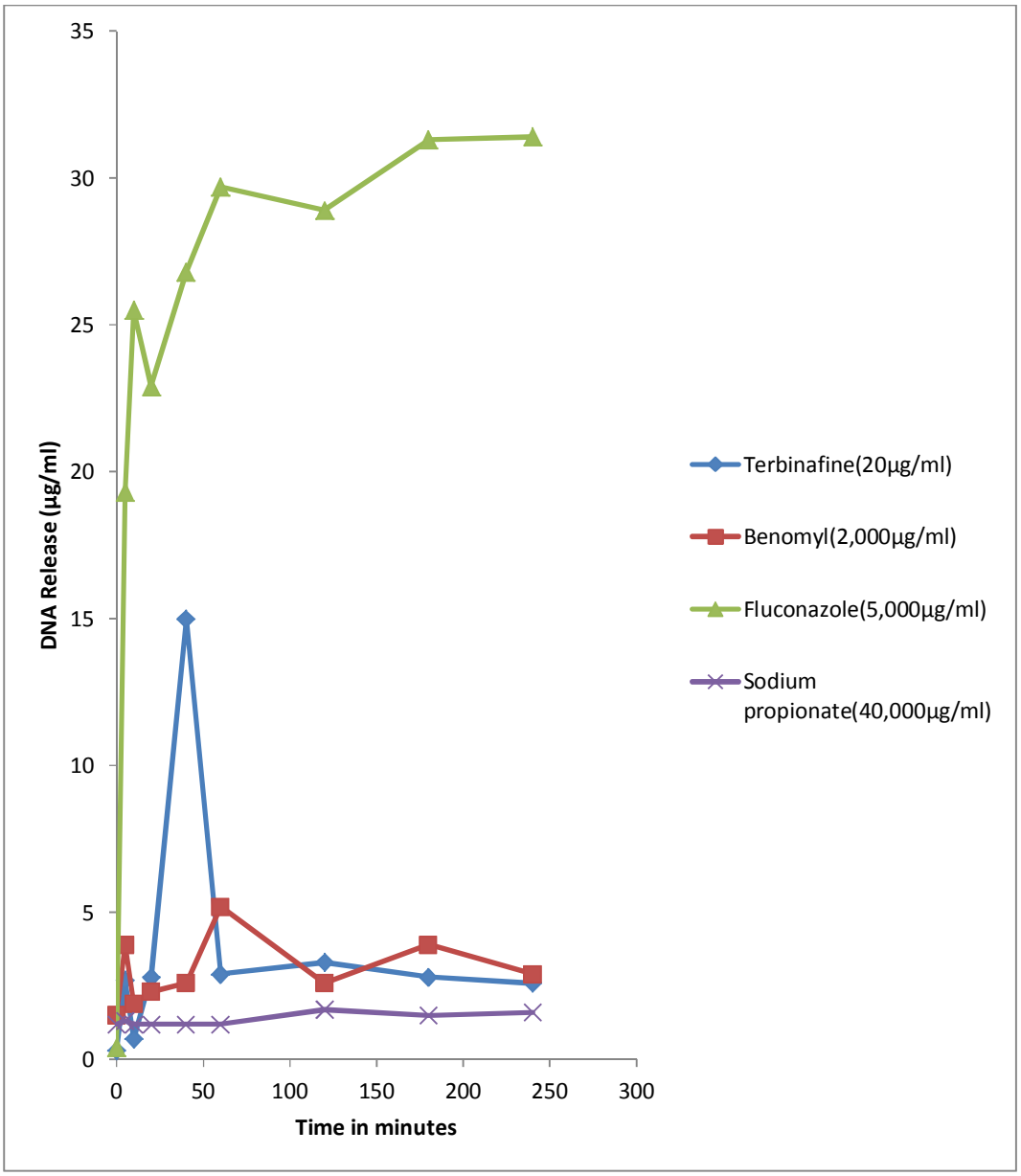


Fig 4.14: Leakage of 260nm-absorbing materials from *Aspergillus niger* with fixed fungicidal concentration of test compounds at different time contact time (minutes).

4.9 EFFECTS OF DIFFERENT WATER SOURCES ON THE TEST FUNGICIDES COMBINATION.

The effect of boiled and unboiled Well, Tap, River, Pond and Distilled waters used to dissolve the fungicides combination such as Benomyl (1000µg/ml) + fluconazole (15,000ug/ml); Benomyl (1000µg/ml) + Terbinafine (200µg/ml) and Benomyl (1000µg/ml) + Sodium propionate (15,000ug/ml) are shown in Figures 15; 16 and 17 respectively and statistical analysis showed that at 95% confident limit $p<0.05$ for the effect of different sources of water but $p>0.05$ for the boiled/unboiled and antifungal combination (Table 4.16).

The results of the effect of boiled and unboiled Well, Tap, River, pond and Distilled water used to dissolve the test fungicides combination of Benomyl (1000µg/ml) and fluconazole (15,000ug/ml) concentration is as presented in Fig 15. These results indicate that the test fungicides preparations displayed a better, antifungal activity when diluted with boiled water source than the unboiled waters with a significant difference in their zone of inhibition (mm). However the activities of fungicides when dissolved in distilled water produced highest zone of inhibition (mm). The zone of inhibition produced by diluting the test combined fungicides with unboiled water sources was in the following order: Distilled water (mm) >Tap water (mm) > Well water (mm) > River water (mm) > Pond water (mm) Fig 15. The statistical analysis of the observed data showed that, there is no significant difference at 95% confident limit between the boiled and unboiled water sources with the same antifungal combination, but a

significant difference exist between the different water sources used as diluents at 95% confident limit.

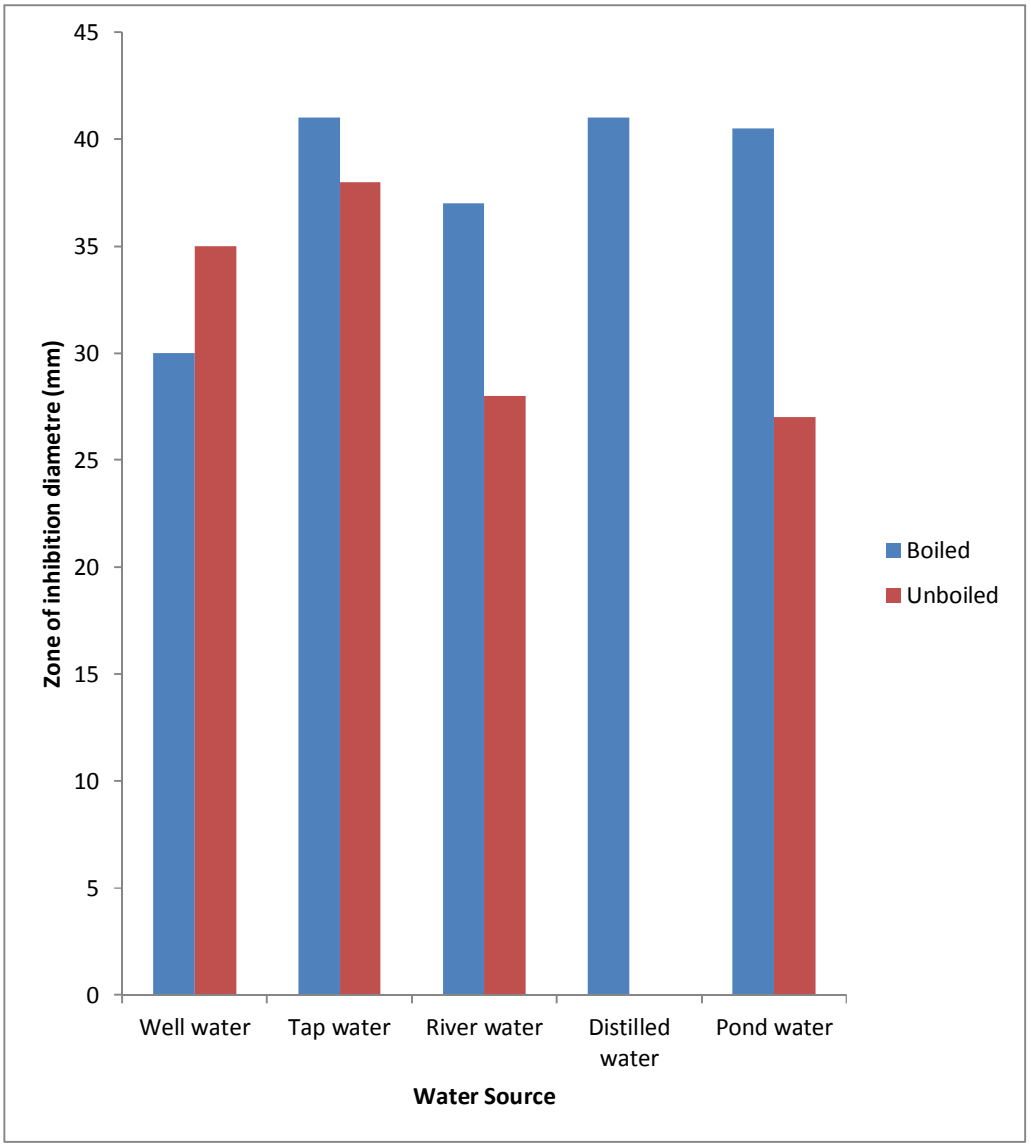


Fig 4.15: Zone of inhibition of Benomyl / Fluconazole (1000µg/ml / 15,000ug/ml) concentration diluted with boiled and unboiled well; tap; River, pond and distilled water after 48hrs incubation at 30^0C against 10^8 cfu *A. niger*.

The result of boiled and unboiled water sources viz, Tap, Well, River, Pond water showed that the antifungal activities of Benomyl / Terbinafine (1000µg/ml / 200µgml) was more effective when the boiled water was used as diluents (Fig 16). The order of the antifungal activities of the diluted fungicides Benomyl / Terbinafine with boiled water sources was as follows: Distilled water (mm) > Tap water (mm) > Pond water (mm) > River water (mm) > Well water (mm). However the pattern of antifungal activities of the test fungicides preparations Benomyl / Terbinafine diluted with the unboiled water sources showed the following order of zone of inhibition that Distilled water (mm) > Pond water (mm) > Tap water (mm) > Well water (mm) > River water (mm).

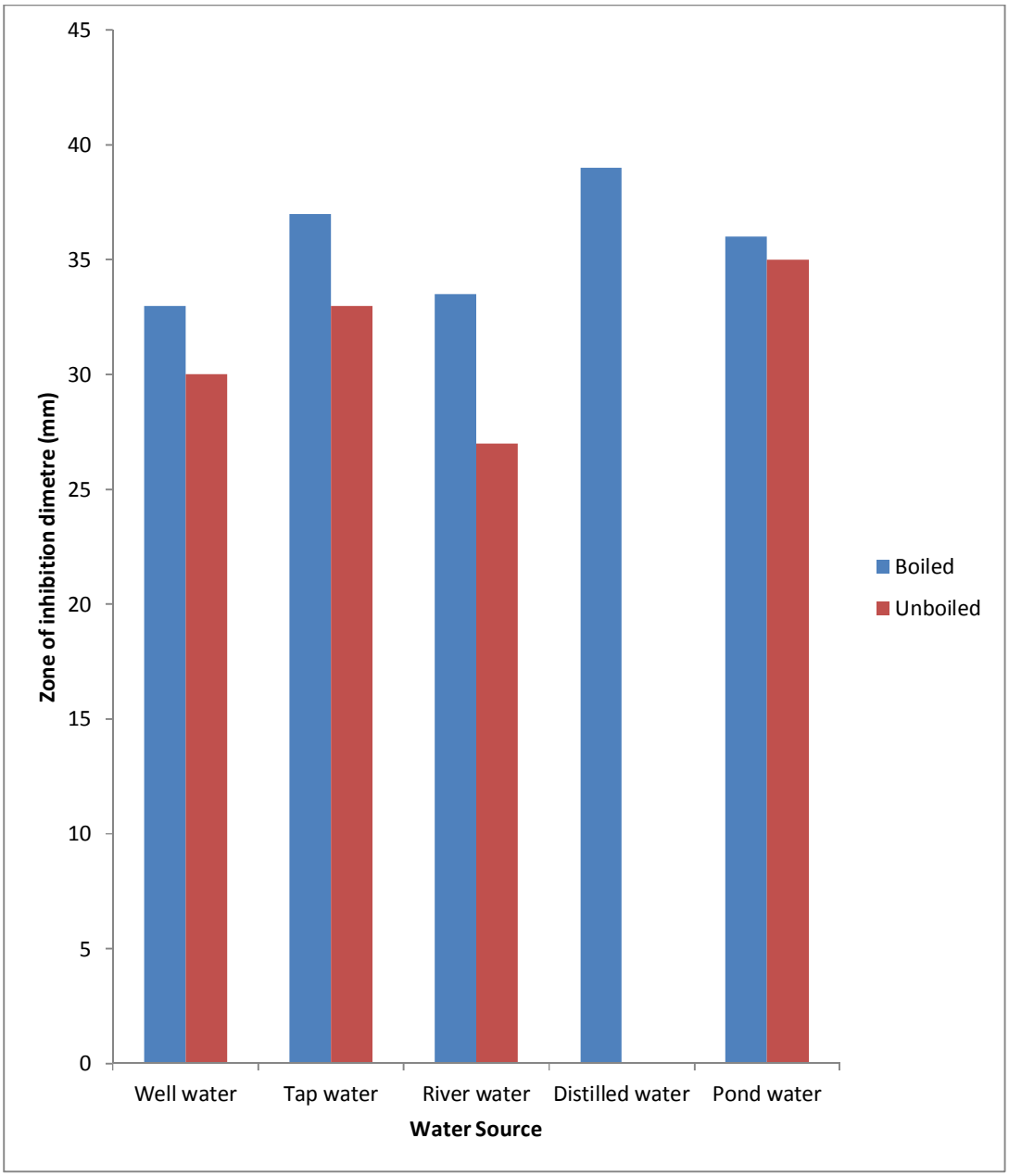


Fig 4.16: Zone of inhibition effected by Benomyl / Terbinafine (1000µgml /200µgml) diluted with boiled and unboiled water sources against 10^8 cfu of *A. niger* after 48hrs incubation \at 30^0C

The result of boiled and unboiled water sources viz, Tap, Well, River, Pond and Distilled water showed that the antifungal activities of Benomyl / Sodium propionate (1000µg/ml / 15,000ug/ml) was more effective when the boiled water was used as diluents (Fig 17). The order of the antifungal activities of the diluted fungicides Benomyl / Sodium propionate with boiled water sources was as follows: Distilled water (mm) > Tap water (mm) > Pond water (mm) > River water (mm) > Well water (mm). However the pattern of antifungal activities of the test fungicides preparations Benomyl / Sodium propionate diluted with unboiled water sources showed the following pattern Distilled water (mm) > Pond water (mm) > Tap water (mm) > River water (mm) > Well water (mm).

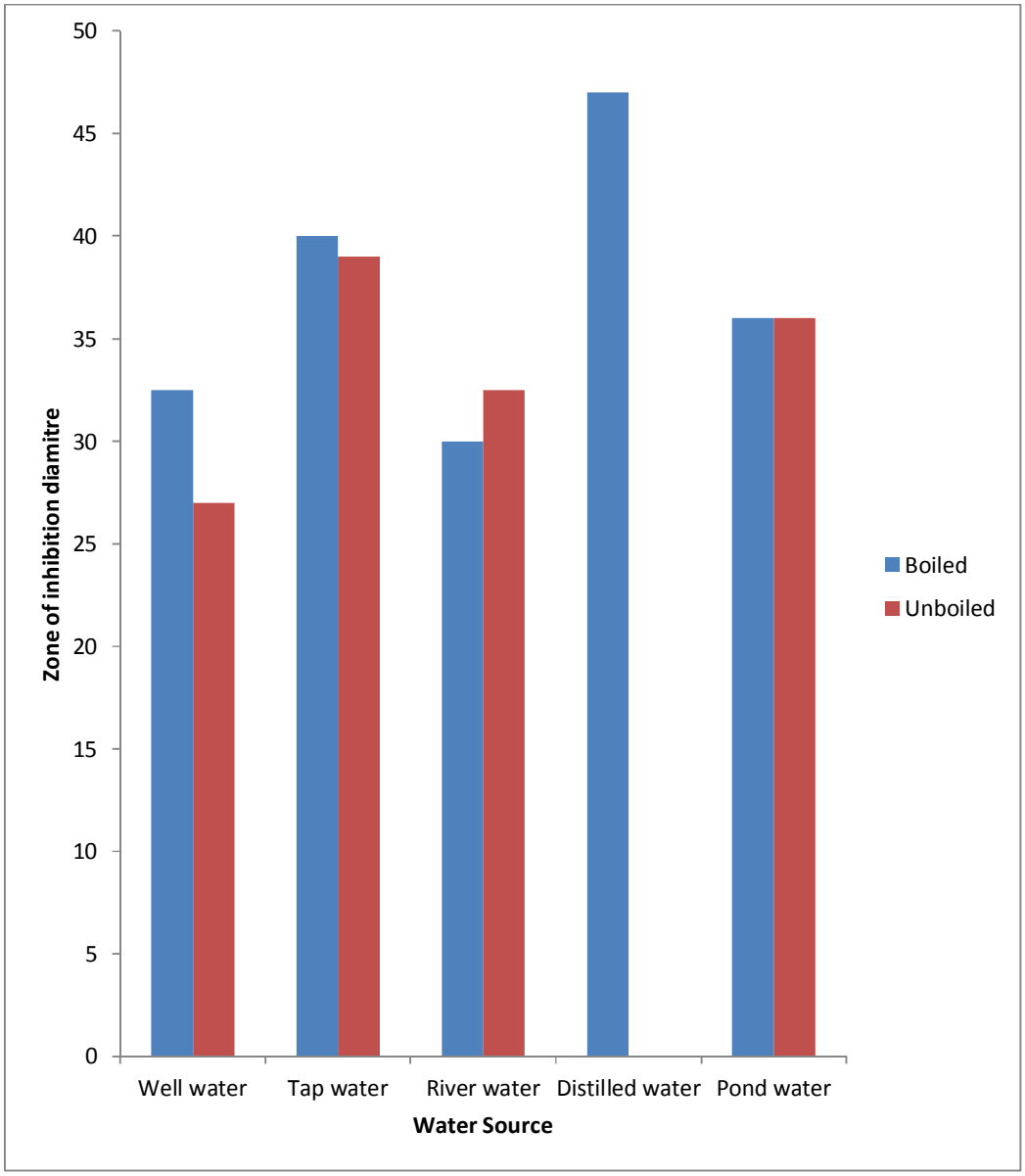


Fig 4.17: Zone of inhibition effected by Benomyl / Na propionate (1000µg/ml / 15,000ug/ml) diluted with boiled and unboiled water sources against 10^8 cfu of *A. niger* after 48hrs incubation \at 30^0C

4.10 PERCENTAGE OF ORGANIC AND INORGANIC MATTER PRESENT IN THE DIFFERENT WATER SOURCES USED IN FUNGICIDE DILUTIONS

The percentage of organic and inorganic matter observed in different water sources used in this investigation are as shown in Table 4.16. This result shows that the water from the well source contains the highest organic matters (0.050%). Other water sources used such as Tap Water, Pond water and Distilled water contained 0.045, 0.030 and 0.022% w/v organic matter respectively.

Table 4.16: The percentage of organic and inorganic matter in different water sources used as diluents.

s/n	Water	% of organic/inorganic matter
1.	Well water	0.050
2.	Pond water	0.030
3.	Tap water	0.022
4.	River water	0.045
5.	Distilled water	-----
6.	Distilled water+25% ethanol	-----

4.11 EFFECT OF VARYING pH ON ANTIFUNGAL FUNGICIDE PREPARATIONS

The effect of varying pHs of 4, 7 and 9 on antifungal activities of the combined, Sodium propionate with Terbinafine (Ter/Np – 1µg/ml/1000µg/ml); Sodium propionate, with Fluconazole (Flu/Np – 50µg/ml /1000µg/ml); Sodium propionate with Benomyl (Ben/Npa – 50µg/ml /1000µg/ml); Sodium propionate with Benomyl (Ben/Npb – 100µg/ml /200 µg/ml); Benomyl with Terbinafine (Ter/Ben – 2µg/ml/100µg/ml); Benomyl with Fluconazole (Ben/Flu – 100µg/ml/1500µg/ml) and distilled water as control against 10^8cfu/ml of *A. niger* spores was determined. The result showed there was a significant difference on the antifungal activities of the test- fungicides. Sodium propionate with Fluconazole; Sodium propionate with Benomyl; Benomyl with Terbinafine; Benomyl with Fluconazole were observed to be more active at neutral pH (pH7). However, Sodium propionate with Terbinafine was observed to be effective in the alkaline pH (pH9). Generally antifungal compounds displayed low zones of inhibition (mm) at acidic pH (pH4) against the test fungal spores of *A. niger* (Figure 4.18 and Table 4.17). In spite of this, statistical analysis showed no significant difference between the effects of pH groups with $P>0.05$ but there was a significant difference between the combinations with $P<0.05$ at 95% confident limit (Table 4.17).

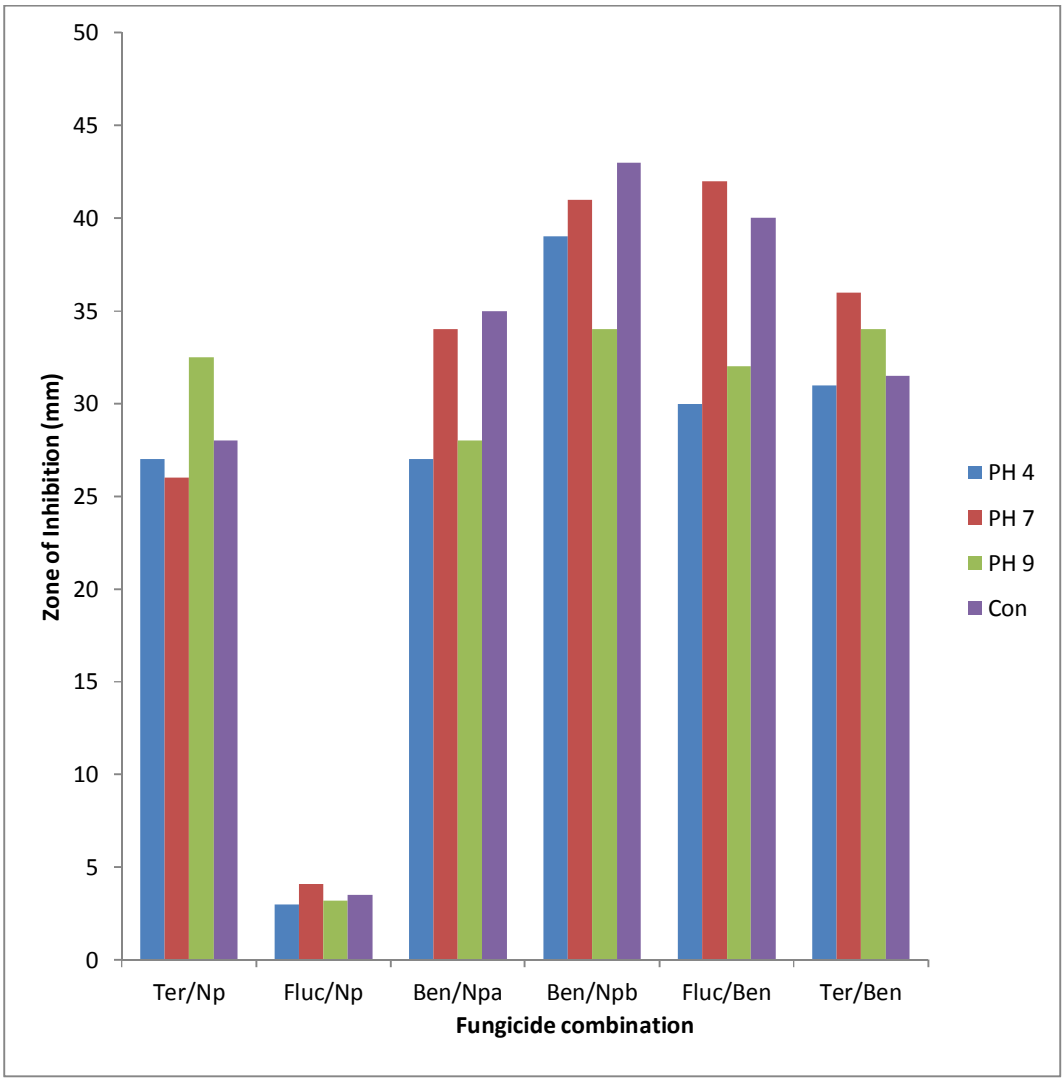


Fig 4.18: Zone of inhibition effected by combined antifungal agents at varying pH against 10^8 cfu/ml of *A. niger* spores after 48hrs incubation at 30^0C.

Key:
Terbinafine HCl/ Sodium propionate (Ter/Np-1µg/ml/1000 µg/ml) suspension
Fluconazole/ Sodium propionate (Fluc/Ben-50µg/ml /1000µg/ml) suspension
Benomyl/Sodium propionate (Ben/Npa – 50µg/ml /1000µg/ml) suspension
Benomyl/Sodium propionate (Ben/Npb – 100µg/ml /200 µg/ml µg/ml) suspension
Fluconazole/Benomyl (Ben/Flu – 100µg/ml/1500µg/m) suspension
Terbinafine HCl/ Benomyl (Ter/Ben – 2µg/ml/100µg/ml µg/ml) suspension
Con = Control with pH 6.8

4.12 EFFECT OF HEAT/TEMPERATURE ON POTENTIAL ANTIFUNGAL FORMULATION

The effect of varying temperatures of 37^0C, 45^0C, 70^0C and 100^0C on the activities of the combined antifungal agents such as, Sodium propionate (1000µg/ml) with Terbinafine (1µg/ml); Sodium propionate (1000µg/ml) with Fluconazole (50 µg/ml); Sodium propionate 'a' (1000µg/ml) with Benomyl (Ben 50 µg/ml); Sodium propionate 'b' (2000 µg/ml) with Benomyl (Ben100 µg/ml); Benomyl (100 µg/ml) with Terbinafine (20µg/ml); Benomyl (100µg/ml) with Fluconazole (500µg/ml) are shown in figure 4.19 and Table 4.17.

The combination of Benomyl with either Sodium propionate or Fluconazole produced the highest zones of inhibition at different temperature ranges investigated. However, there is no significant difference for the antifungal activities of all the combined antifungal agents at 37^0,45^0,70^0 and 100^0C. Generally the combined test antifungal agents displayed highest antifungal activities at 45^0C. The orders of antifungal activities at 45^0C for the combined test antifungal agents are as follows:

Fluconazole/Benomyl (54.50±0.710mm) > Benomyl/Sodium propionate (54.50±1.410mm) > Terbinafine HCl/Benomyl (38.50±3.180mm) > Terbinafine HCl/ Sodium propionate (37.50±0.710mm). Hence statistical analysis showed no significant difference between the temperature groups but there was a significant difference between the test antifungal conbinations at 95% confident limit.

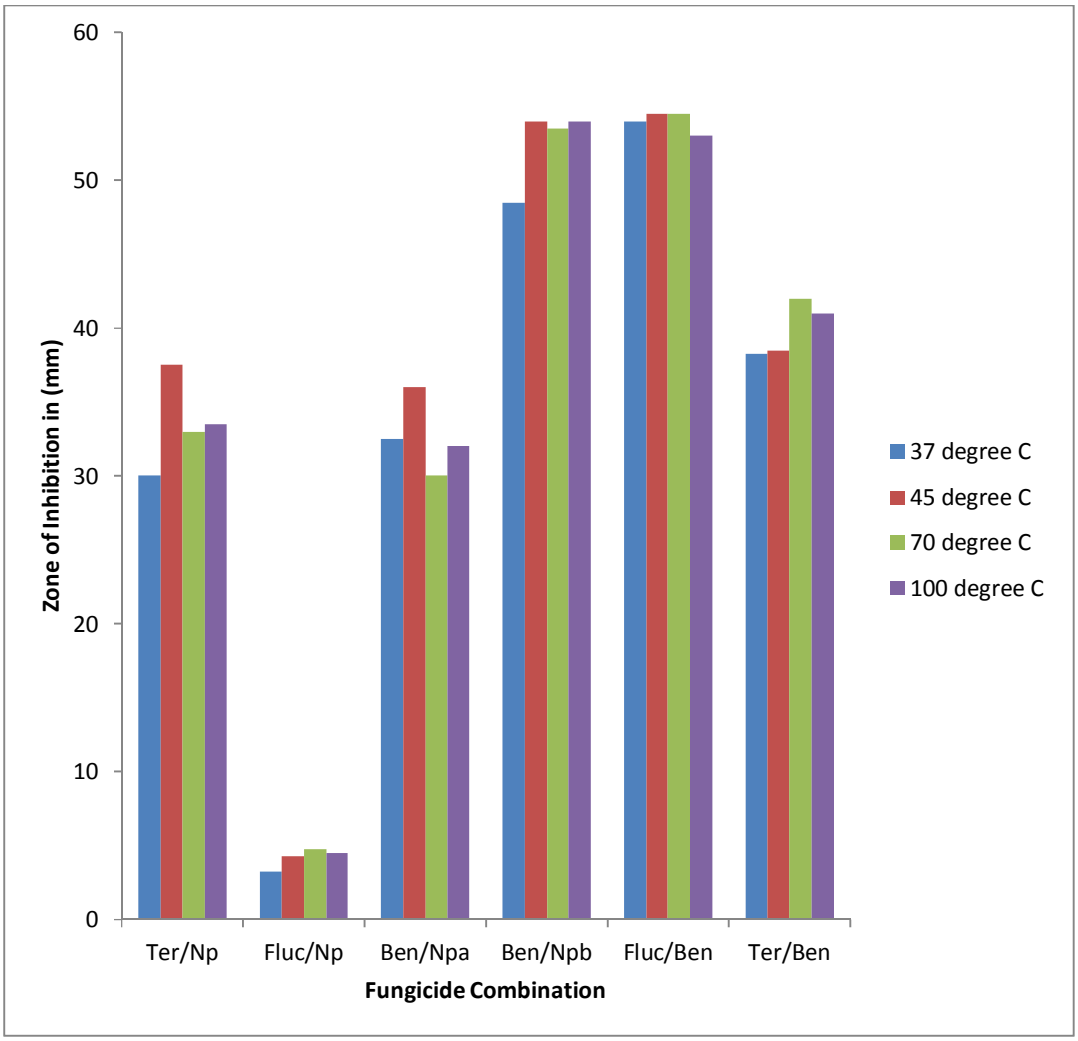


Fig 4.19: Zone of inhibition effected by some combined antifungal agents at different temperatures against 10^8 cfu/ml of *A. niger* spores suspension after 48hrs incubation at 30^0C.

Key:

Terbinafine HCl/ Sodium propionate (Ter/Np-1µg/ml/1000 µg/ml) suspension
Fluconazole/ Sodium propionate (Fluc/Ben-50µg/ml /1000µg/ml) suspension
Benomyl/Sodium propionate (Ben/Npa – 50µg/ml /1000µg/ml) suspension
Benomyl/Sodium propionate (Ben/Npb – 100µg/ml /200 µg/ml µg/ml) suspension
Fluconazole/Benomyl (Ben/Flu – 100µg/ml/1500µg/m) suspension
Terbinafine HCl/ Benomyl (Ter/Ben – 2µg/ml/100µg/ml µg/ml) suspension

4.13 PHYSICAL AND CHEMICAL INTRACTION STUDIES

The test chemical compounds alone and in admixture were chromatographically seperated and the R_f values were calculated using, distance moved by test compound divided by distanced moved by solvent. The results obtained showes that there were no interactions in all the combinations 5 - 9 i.e they all shows complete separations. The appearance of one spot in the combination 5, 6 and 7 were due to the fact that sodium propionate was non soluble in the solvent used as it does not show any spot movement in the single test chemical chromatography of sodium propionate (Table 4.17).

Table 4.17: Result for the R_f values of the test antifungal agents

S/N	Samples	Number of spots	R_f values
1	T	1	0.74
2	F	1	0.13
3	B	1	0.18
4	N	-	-
5	TN	1	0.71
6	FN	1	0.13
7	BN	1	0.17
8	TB	2	0.74 / 0.18
9	BF	2	0.18 / 0.11

Key:

T = Terbinafine
F = Fluconazole
B = Benomyl
N = Sodium propionate
TN = Terbinafine/ Sodium propionate
FN = Fluconazole/ Sodium propionate
BN = Benomyl/ Sodium propionate
TB = Terbinafine/Benomyl
BF = Benomyl/Fluconazole

4.16 EFFECT OF ANTIFUNGAL COMBINATION OF ANTIFUNGAL AGENTS PREPARATIONS AGAINST INFECTED YAM LEAVES

There were no signs of infection on the inoculated yam leaves treated with fungicides A-F, (A) Benomyl / Fluconazole (1,000 µg/ml /15,000µg/ml); (B) Benomyl /Terbinafine (1,000 µg/ml /200µg/ml); (C) Benomyl / Sodium Propionate (1,000 µg/ml /15,000µg/ml); (D) Benomyl /Sodium Propionate (1,000 µg/ml / 20,000µg/ml); (E) Fluconazole /Sodium Propionate (1000µg/ml /10,000µg/ml ml) and (F) Terbinafine / Sodium Propionate (200µg/ml /10,000µg/ml) and the yam leaves without fungal spores inoculation as shown in A1; A2; A3 in plate (4.2 and 4.18), B1; B2; B3 in plate (4.3 and 4.9), C1; C2; C3 in plate (4.4 and 4.10), D1; D2; D3 in plate (4.5 and 4.11), E1; E2; E3 in plate (4.6 and 4.12) and F1; F2; F3 in plate (4.7 and 4.13) respectively.

On the seventh to thirteen days after the applications of prepared fungicides (A), there were no signs of infection or changes in the physical appearance of the test yam leaves on plate 4.2. Picture A3, A6, A9. This observation is comparable to yam leaves without inoculation of test fungal spores but sprayed with sterile distilled water A1; A; A7 (plate 4.2 and 4.8). However there was a sign of fungal infection or deteriorating changes in the physical appearance in the yam leaves inoculated with test fungal spores without prepared fungicides treatment on the yam leaves as demonstrated in plates 4.2-4.13: 2; 5; 8.

Observation from pictures 7, 8 and 9 in plate 4.2-13 which corresponds to the 13th day after the prepared fungicide treatments showed that the new formulated

antifungal agents (A-F) maintain the physical appearance of the yams leaves with no sign of infection and healthy looks in pictures A9; B9; C9; D9; E9 and F9 in plate 4.2-4.13. This observation compared favourably with negative control of non inoculated yam leaves in picture 1, 4 and 7, plates of 4.2-4.13. There were losses or deterioration of the yam leaves in infected untreated group (picture 2,5 and 8 plates 4.2-4.13), some of the yam leaves developed yellow pigments while some were with dry brown spots.

Similar observations were obtained when the green leaves *D. rotundata* and *D. cayenesis* were inoculated with spores of *A. niger* and treated with prepared fungicides system (Plate 4.8-4.13.). The prepared fungicides were observed to prevent the deterioration of the green leaves of the *D. rotundata* (white yam) and *D. cayenesis* (yellow) yam by *A. niger* when treated with 10-folds dilutions of the prepared fungicides.

Fungicides A; B; C; D; E and F were observed to be effective in combating the inoculated *A. niger* spores from destroying the green leaves of the test *D. Rotundata* (white yam) and *D. Caeyenesis* (yellow) yams in this study.

D*ioscorea rotundata* (White yam)

Non-infected control	Infected	Infected and treated with A'
		
A1	**A2**	**A3**
	Day 1	
		
A4	**A5**	**A6**
	After 7 days	
		
A7	**A8**	**A9**
	After 13 days	

Plate 4.2: Comparative pictures of *Dioscorea rotundata* (white yam) leave with infected but treated (A) Benomyl/Fluconazole (1,000 µg/ml /15,000 µg/ml) uninfected and infected without treatment with (A).

Dioscorea rotundata (White yam)



Plate 4.3: Comparative pictures of *Dioscorea rotundata* (white yam) leave with infected but treated (B) Benomyl /Terbinafine (1,000 µg/ml /200µg/ml); uninfected and infected without treatment with (B).

Dioscorea rotundata (White yam)

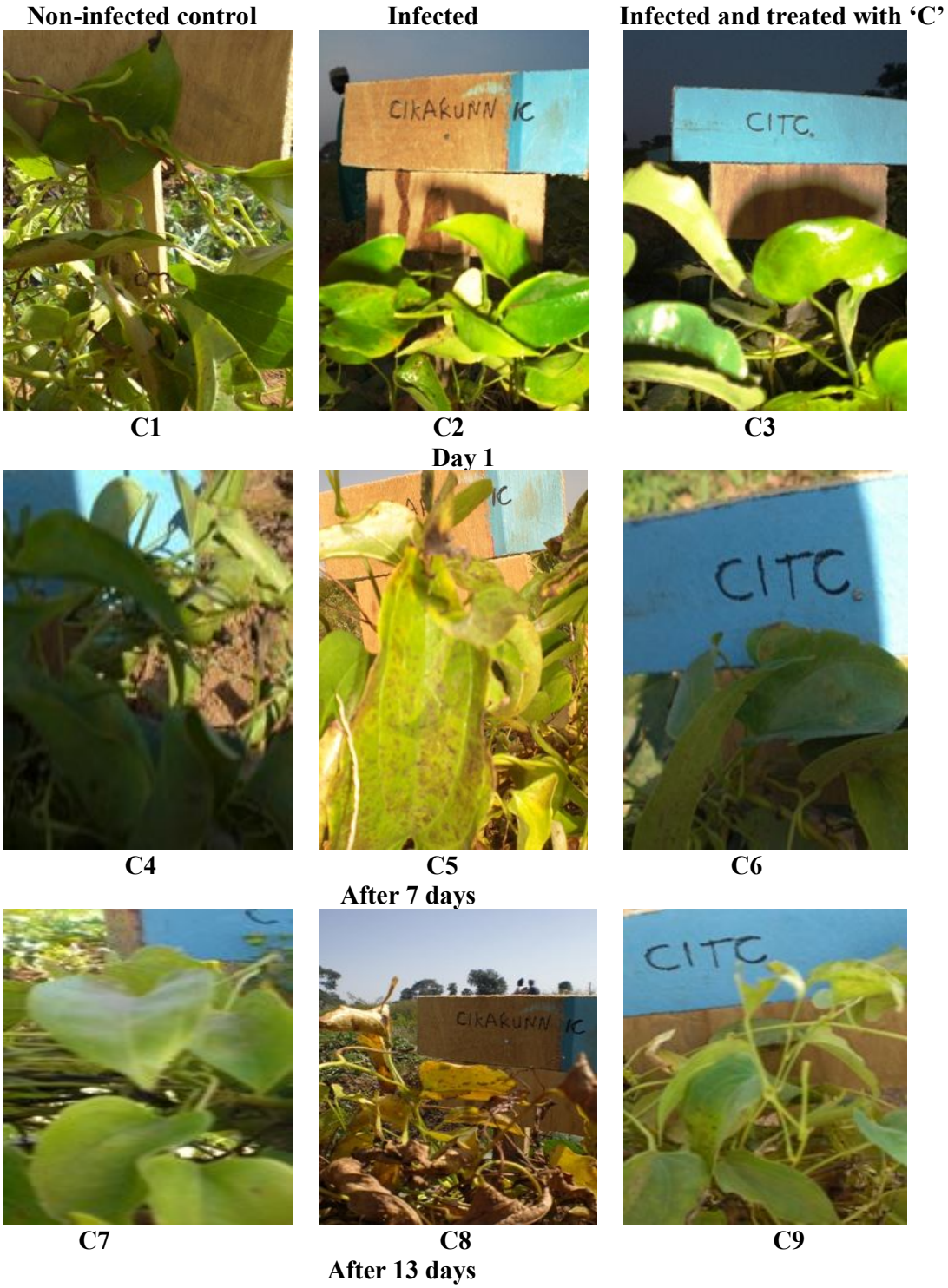


Plate 4.4: Comparative pictures of *Dioscorea rotundata* (white yam) leave with infected but treated (C) Benomyl / Sodium Propionate (1,000 µg/ml /15,000µg/ml); uninfected and infected without treatment with (C).

Dioscorea rotundata (White yam)

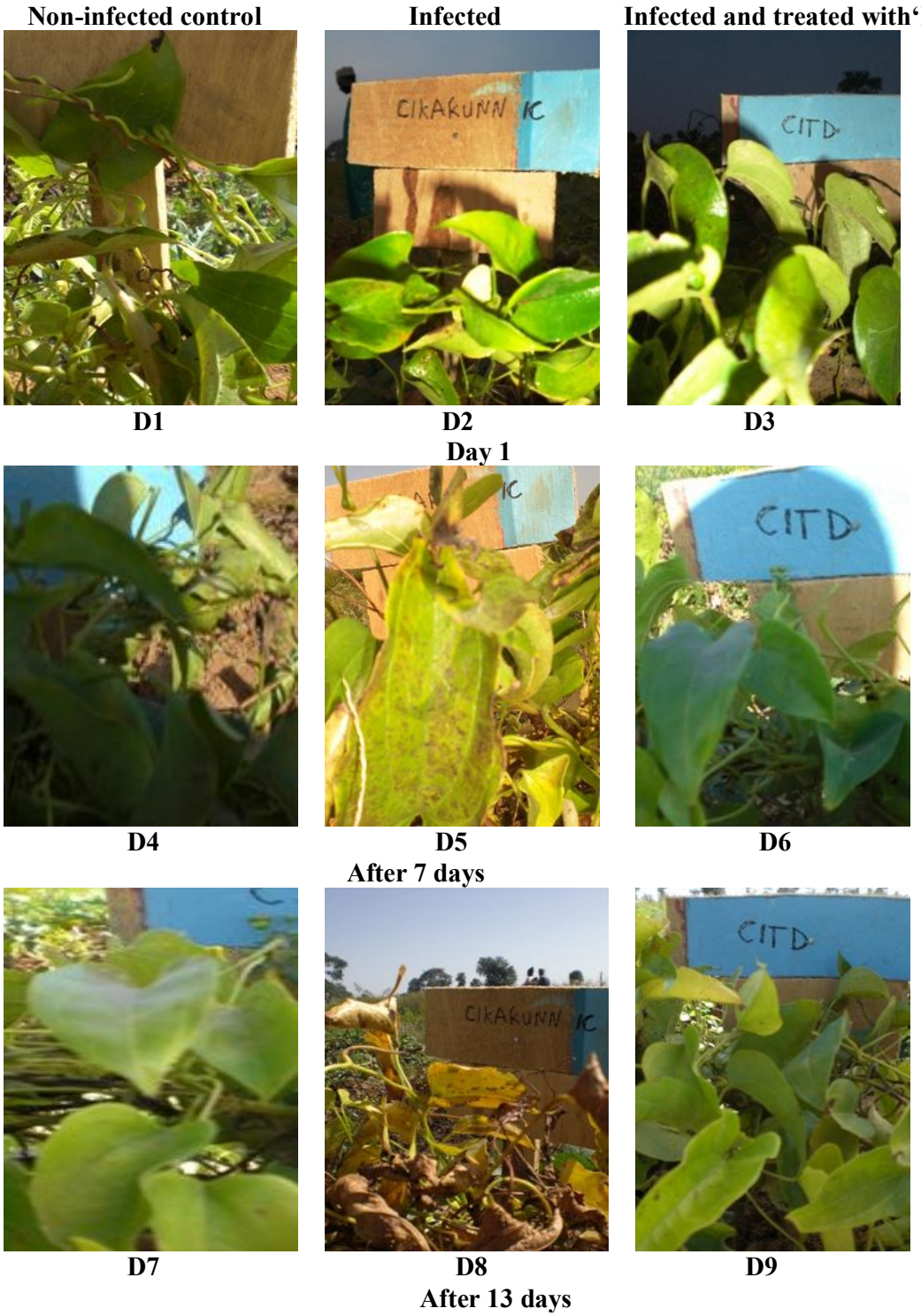


Plate 4.5: Comparative pictures of *Dioscorea rotundata* (white yam) leave with infected but treated (D) Benomyl /Sodium Propionate (1,000 µg/ml / 20,000µg/ml); uninfected and infected without treatment with (D).

D*ioscorea rotundata* (White yam)

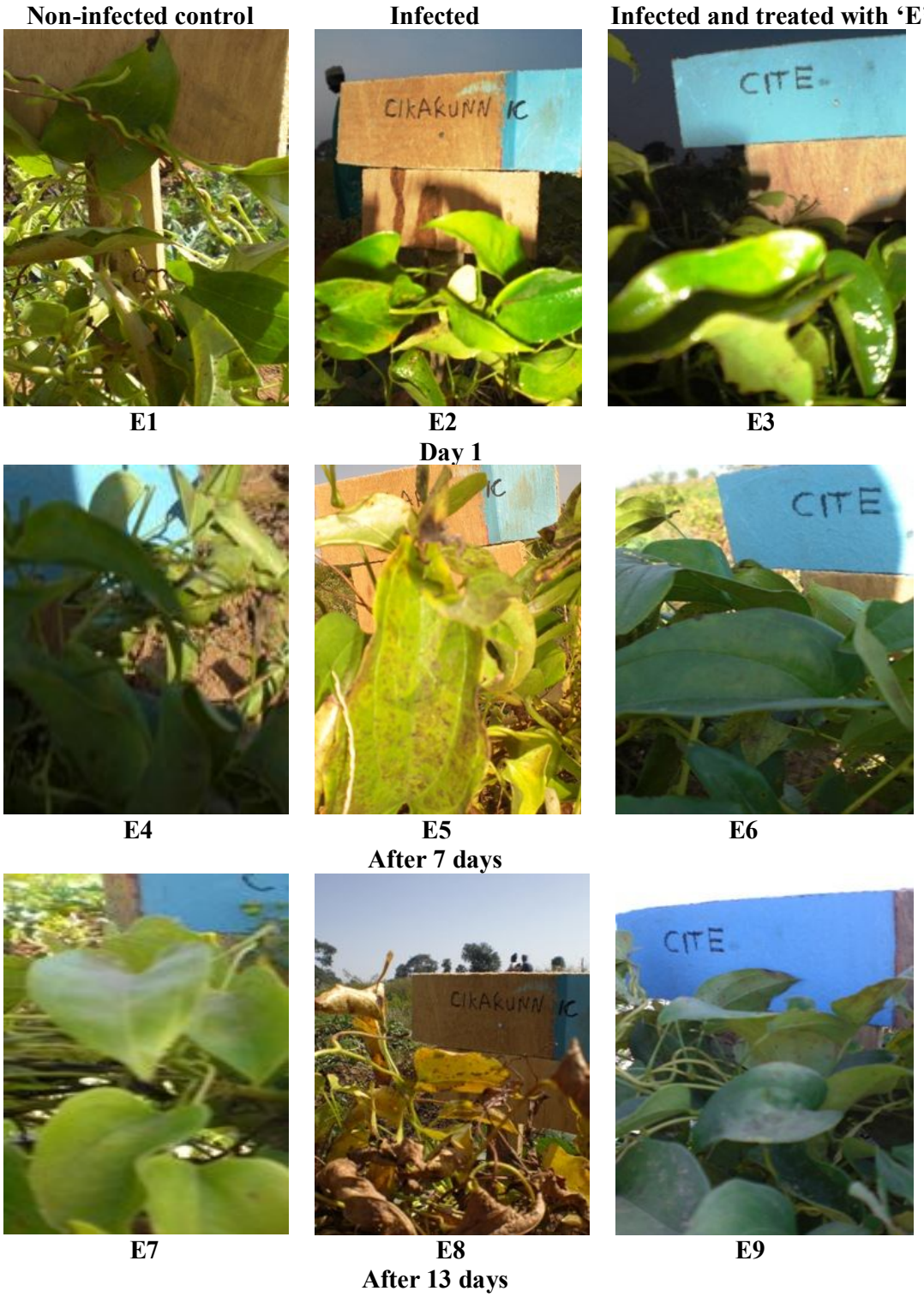


Plate 4.6: Comparative pictures of *Dioscorea rotundata* (white yam) leave with infected but treated (E) Fluconazole /Sodium Propionate (1000µg/ml /10,000µg/ml ml); uninfected and infected without treatment with (E).

Dioscorea rotundata (White yam)

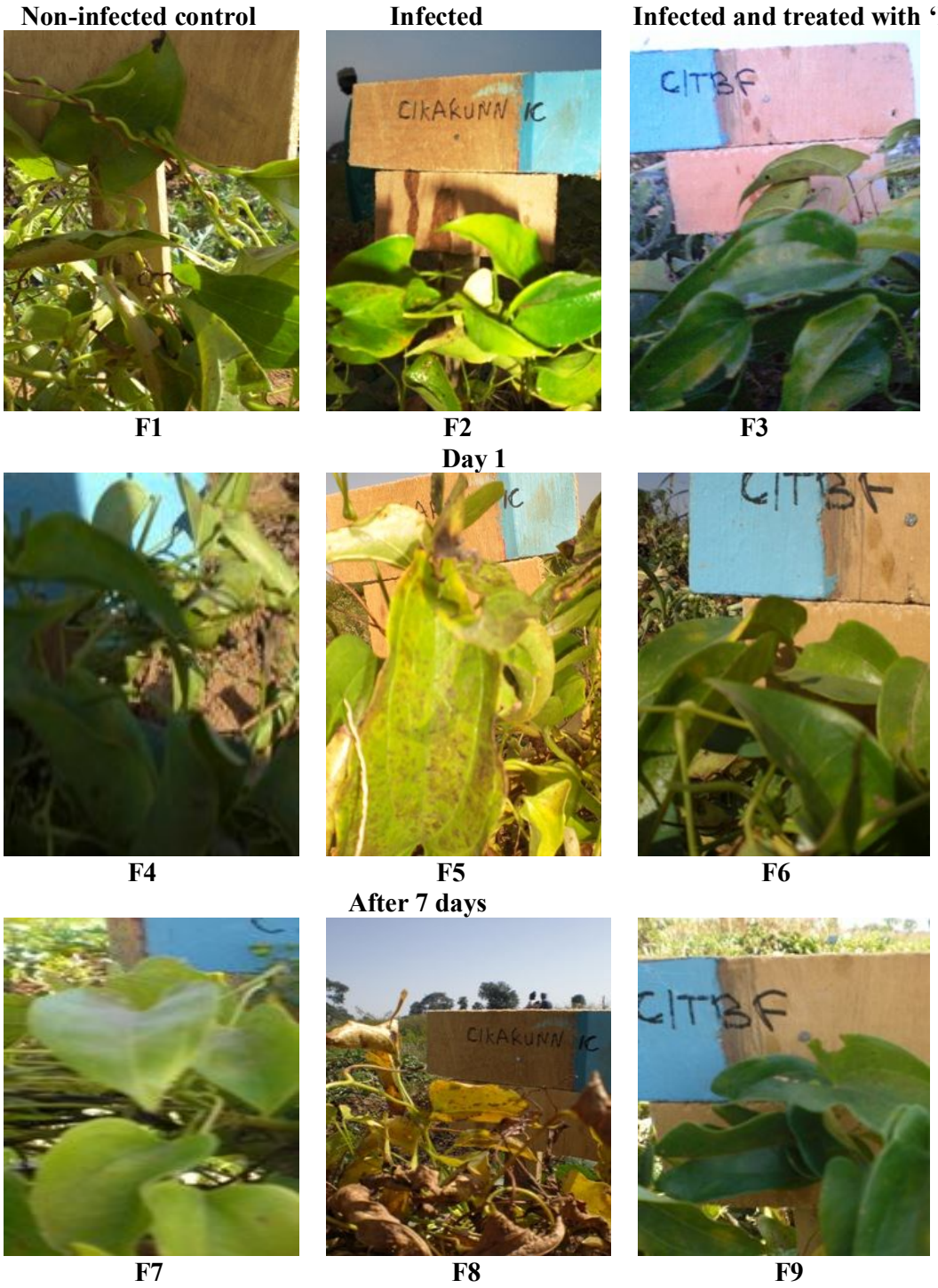


Plate 4.7: Comparative pictures of *Dioscorea rotundata* (white yam) leave with infected but treated (F) Terbinafine / Sodium Propionate (200µg/ml /10,000µg/ml); uninfected and infected without treatment with (F).

Dioscorea cayenesis (yellow yam)



Plate 4.8: Comparative pictures of *Dioscorea cayenesis* (yellow yam) leave with infected but treated (A) Benomyl/Fluconazole (1,000 µg/ml /15,000 µg/ml); uninfected and infected without treatment with (A).

Dioscorea cayenesis (yellow yam)



Plate 4.9: Comparative pictures of *Dioscorea cayenesis* (yellow yam) leave with infected but treated (B) Benomyl /Terbinafine (1,000 µg/ml /200µg/ml); uninfected and infected without treatment with (B).

Dioscorea cayenesis (yellow yam)



Plate 4.10: Comparative pictures of *Dioscorea cayenesis* (yellow yam) leave with infected but treated (C) Benomyl / Sodium Propionate (1,000 µg/ml /15,000µg/ml); uninfected and infected without treatment with (C).

Dioscorea cayenesis (yellow yam)

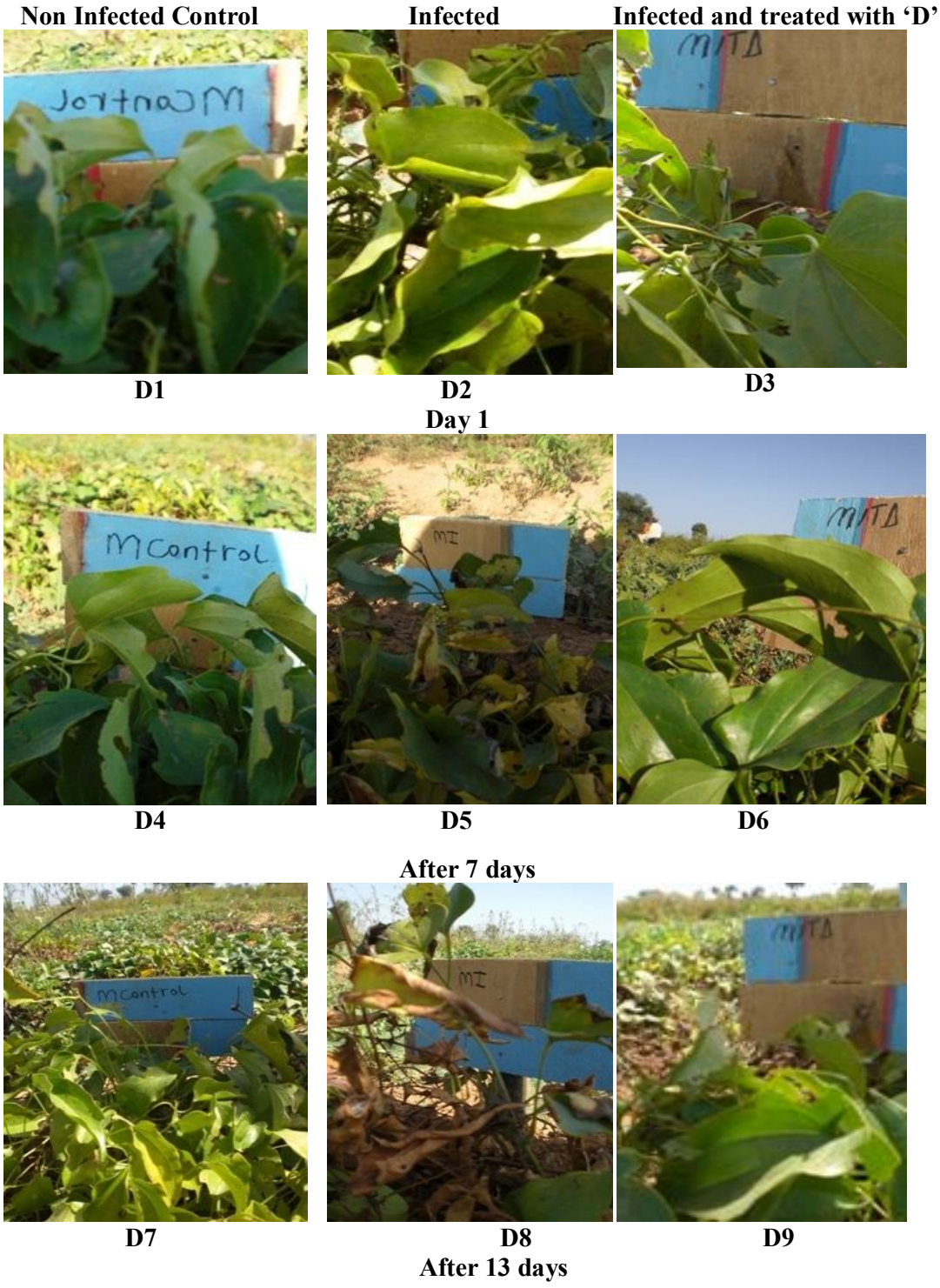


Plate 4.11: Comparative pictures of *Dioscorea cayenesis* (yellow yam) leave with infected but treated (D) Benomyl /Sodium Propionate (1,000 µg/ml / 20,000µg/ml); uninfected and infected without treatment with (D).

***Dioscorea cayenesis* (yellow yam)**

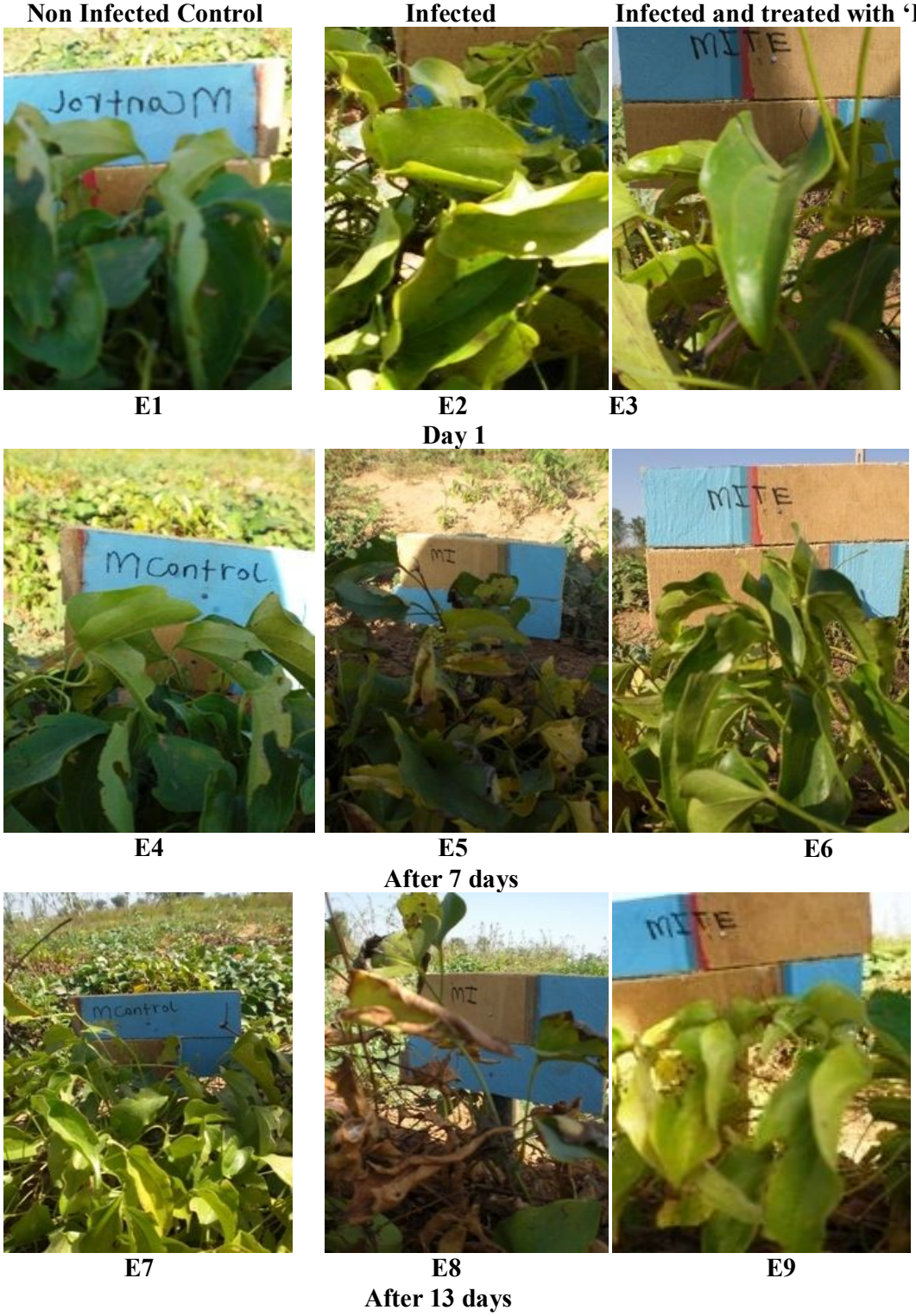


Plate 4.12: Comparative pictures of *Dioscorea cayenesis* (yellow yam) leave with infected but treated (E) Fluconazole /Sodium Propionate (1000µg/ml /10,000µg/ml ml); uninfected and infected without treatment with (E).

***Dioscorea cayenesis* (yellow yam)**

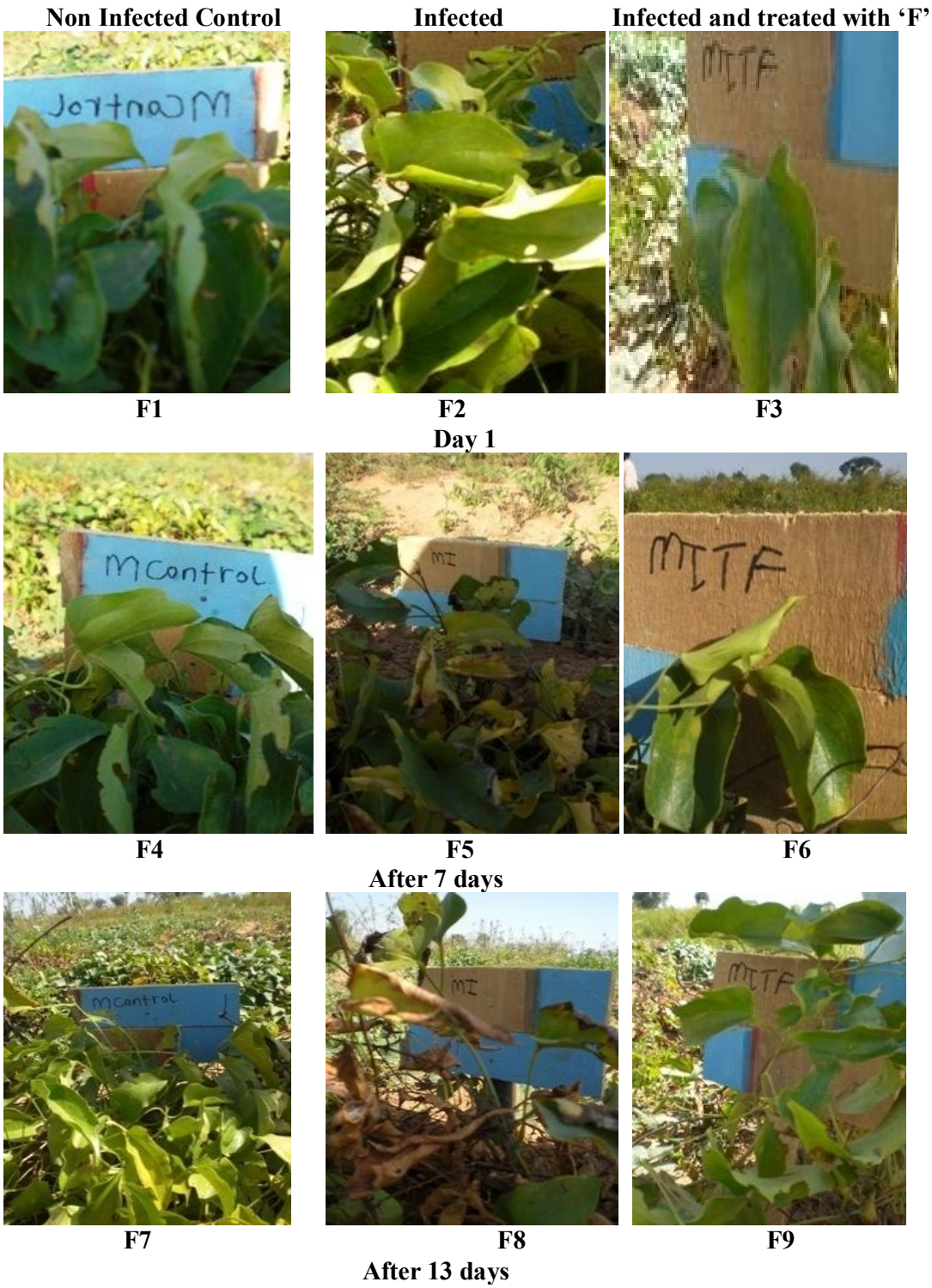


Plate 4.13: Comparative pictures of *Dioscorea cayenesis* (yellow yam) leave with infected but treated (F) Terbinafine / Sodium Propionate (200µg/ml /10,000µg/ml); uninfected and infected without treatment with (F).

CHAPTER FIVE

5.0 GENERAL DISCUSSION

Fungal infection of yam leaves is a major factor leading to reduction in yam tuber growth and spoilage in storage (Aboagye-Nuamah *et al.,* 2005). Many of these fungi attack the yam leaves right in the field. The post-havest infections of yam tuber which are later transported to store have been implicated in most yam tuber spoilage. Rot of yam tubers may be caused by a wide variety of fungi, including those isolated in this study from yam leaves in the field and have also been found associated with post harvest rot and store yam rot (Adeniji, 1970; Ogundana *et al.,* 1970; Okigbo and Ikediugwu, 2001).

The yam phytopathogenic fungi isolated and identified in the field from white yam infected leaves in this work include: *Aspergillus niger; Aspergillus fumigatus; Aspergillus flavus; Aspergillus wentii; Aspergillus ostianus; Aspergillus oryzae; Aspergillus cervinus; Rhizopus stolonifer; Rhizopus* sp; *Penicillium citrinum; Penicilium oxalicum*; *Penicillium* sp*; Clodosporium* sp and *Scoppolarius* sp.This coresponds with the works of several researchers in the area of yam tuber rot and in the field (Ogundana *et al.,* 1970; Adeniji, 1970; Okigbo and Ikediugwu, 2000; Okigbo, 2002; Okigbo, 2004; Okigbo and Nmeka, 2005; Aboagye-Nuamah et al., 2005; Okigbo,2005).

Aspergillus niger was the most frequently isolated phytopathogenic fungi (9 times) from the yam leaves in the eleven farms investigated in this study in Zaria, Nigeria.). *Aspergillus oryzae* and *Rhizopus stolonifer* were observed to be

the least occuring infective fungus (2 times) from the eleven farms. The fungal infection of yam leaves in the farms has been reported to drastically affect yam tuber production (Amusa *et al.,* 1996; Amusa, 1999). Hence, the observed myriads of infective fungi from the leaves of yams in the eleven farms potend a serious danger for high yam tuber production and subsequent low income to farmers and the subsequent food insecurity to the nation that depend on this food stuff to feed their populace. This low yield of the yam tuber could result in instability in the society due to lack of food and loss of livelyhood in need of food.

The need to reduce this high level of fungal infection of yam leaves in farms with effective fungicides cannot be over-emphasized.

5.1 Susceptibility Test

The susceptibility of spores of fungi isolated from the yam leaves which were also implicated in yam postharvest rot to test chemical compounds, such as Benomyl, Terbinafine, Fluconazole, Flucytosine and sodium propionate showed that Benomyl had the best inhibitory activity with MICs range of 0.98-15.25µg/ml, This result is consistent with values previously obtained with wild-type *A. nidulans* strains (Oakley, 1981; Van Tuyl,1977; Yulia *et al.,* 1999); Terbinafine had a range of 0.98-31.25µg/ml; Flucytsine had a range of 0.78-200µg/ml; Fluconazole with a range of 1.95-500µg/ml while Sodium propionate had the lowest inhibitory activity with a higher range value of 19.53-10,000µg/ml. From this result the order of fungistatic activity of the test

compounds was Benomyl > Terbinafine > Fluconazole > Flucytosine > Sodium propionate. The sensitivity of the test isolated fungi spore varied. Observation from this study showed that *Rhizopus stolonifer*; *Aspergilus niger* and *Penicillium citrinum* were the most resistant to the test chemical agents which is in line with previous researchies, (Moore *et al.,* 2000; NCCLS, 2002; AFST 2008; Qiao *et al.,* 2008 and Rodrinuez-Tudela, 2008) while *Rhizopus* sp and *Aspergillus ostianus* were the most sensitive. Similarly, the order of susceptibliity of the isolated test fungi spores to the test compounds was *Rhizopus* sp > *Aspergillus ostianus* > *Aspergillus oryzae* > *Scoppolarium* sp. > *Penicillium citrinum* > *Aspergilus niger* > *Rhizopus stolonifer*; respectively. A similar pattern of the antifungal activity was observed in Minimum Fungicidal Concentration of the same test agents determined in this study. These test compounds show different degree of fungicidal activities against test fungal isolates spores. Terbinafine HCL (1.95-2000µg/ml); fluconazole (3.91-4000µg/ml); benomyl (15.25-4000µg/ml); flucytosine (200-1600µg/ml) and sodium propionate (156.25-20,000µg/ml) and the order of this lethal activity was, Terbinafine HCL > fluconazole > benomyl > flucytosine > sodium propionate.

Generally, Terbinafine HCl was the most active in terms of inhibitory and lethal activities (i.e MIC and MFC) in this study while sodium propionate was the least active followed by fluconazole. Other workers have reported high MICs values of azole against *Aspergillus* species; *Rhizopus stolonifer* and *Penicillium citrinum* as observed in this study. (Moore *et al.,* 2000; Walsh *et al.*, 2004; Qiao *et al.,* 2008; Donald *et al.*, 2009; Susan *et al.,* 2009).

This work shows that Terbinafine could be used to reduce fungal growth. It has been reported that indiscriminate use of any of these antifungal agents singly may affect their effectiveness due to resistance development (Falade *et al.,* 1983 and Liu *et al.,* 2004). Thus a combination study of terbinafine HCl with other fungicides could be a possible way out of the challenges of resistance development. Therefore the effectiveness of terbinafine as an antifungal agent could be maximized with two antifungal compounds with one potentiating the other with lesser toxicity and cost effectiveness with possible delay in resistance development.

5.2 Combination of fluconazole and sodium propionate

The combination of fluconazole100µg/ml with 200µg/ml of sodium propionate was found to completely inhibit the growth of *Rhizopus stolonifer* isolate. Their MICs against the isolate was 1000 µg/ml and 2500 µg/ml, respectively. This implies that a complete inhibition of growth is possible with lower concentrations in combination than with individual agents (Beggs *et al.,* 1976; Medoff, 1983; Sugar *et al.,* 1997; Barchiest *et al.,* 2001; Gupta *et al.,* 2001; Hessen and Kaye, 2004). This approach applied to other combinations of fluconazole and other chemical compounds as well as other test organisms (Table 4.4). Several combinations of fluconazole resulted in a clear synergistic antifungal effect against the isolated yam phytopathogenic *Rhizopus stolonifer* with mean FICs of 0.126 which is highly synergistic.

The combined effect of fluconazole 100µg/ml and Sodium propionate 200 µg/ml completely inhibited the growth of *Aspergilus niger* spores. Their individual MICs against *Aspergilus niger* spores were 500 µg/ml and 10,000 µg/ml, respectively (Table 4.4). This result proves a highly marked synergistic antifungal effect against the test phytopathogenic *Aspergilus niger* spores with mean FICs of 0.112.

The combined effect of fluconazole and sodium propionate against the growth of *Penicillium citrinum* spores was similar to the observation with *Aspergilus niger* and *Rhizopus stolonifer* spores with marked synergistic antifungal activitity (FICs 0.117). So this work has shown that a combination of an azole plus sodium propionate has high potential as an antifungal against certain phytopathogenic fungi that infect yam leaves.

5.3 Combination of Benomyl and sodium propionate

The growth of *Rhizopus stolonifer* spores was completely inhibited with 10-50µg of Benomyl per ml in combination with 200µg of sodium propionate per ml. Their MICs against the *Rhizopus stolonifer* spores were 500µg/ml and 2500µg/ml, respectively (Table 4.4) with FIC 0.14 (Table 4.7). The combined effect of Benomyl and sodium propionate against the growth of *Aspergilus niger* and *Penicillium citrinum* spores were observed to be completely inhibited growth at low concentration. These combination produced synergistic activity and the order of Benomyl / Sodium propionate synergictic activity against the test fungal spores was *Rhizopus stolonifer* 0.14 > *Aspergilus niger* (0.44) >

Penicillium citrinum (0.59). Benomyl has been reported to prevent mitotic-spindle formation and germlings are blocked. This blockage has been reported to inhibit spindle formation or other essential mitotic event (WHO, 1993 and Yulia *et al.,* 1999).

5.4 Combination of Terbinafine and sodium propionate

The combined effect of Terbinafine and Sodium propionate against the growth of *Rhizopus stolonifer* spores was complete inhibition of the growth of the spores with 0.10-1.00µg of Terbinafine per ml in combination with 200-400µg of sodium propionate per ml, whereas their individual MICs against *Rhizopus stolonifer* spores was 250 µg/ml and 2500 µg/ml, respectively with FIC 0.098 (Table 4.9). The combined effect of Terbinafine and sodium propionate against the growth of *Aspergilus niger* and *Penicillium citrinum* spores were observed to be synergistic at low concentration with FICs ranging from 0.128 to 0.150 respectively.

5.5 Fractional fungicidal concentration (FFC)of the combined agents

The fractional fungicidal concentration (FFC) of two combinations of the test antifungal agents against the test organisms as investigated also showed synergistic effect. The observed FFC (0.23) lethal effect of fluconazole and sodium propionate against *Rhizopus stolonifer* spores was drastic at 10-100µg of fluconazole per ml in combination with fixed concentration 1000µg/ml of sodium propionate. Whereas the MFCs of Fluconazole and Sodium propionate

against these test fungi spores was 2000 µg/ml and 5000 µg/ml, respectively. This combination showed a complete lethal effect on test fungi spores at a lower concentrations than could be extrapolated from the single MFCs.

Similar synergistic lethal effect were observed for Fuconazole and Sodium propionate against *Aspergilus niger* and *Penicillium citrinum* spores with FFCs 0.063 and 0.336 respectively (Table 4.12).

Other combinations such as Benomyl / Sodium propionate displayed marked lethal synergistic effects lower combined concentration against the test spores of *Rhizopus stolonifer* (FIC = 0.167), *Aspergilus niger* (FIC = 0.031) and *Penicillium citrinum* (FIC = 0.528).

Similarly the combination low concentrations of Terbinafine / sodium propionate showed marked lethal synergistic effects against test spores of *Rhizopus stolonifer* (FFC 0.410), *Aspergilus niger* (FFC 0.0814) and *Penicillium citrinum* (FFC 0.056) Table 4.10 and 4.12.

In this study, the low concentration combinations of sodium propionate with Benomyl; terbinafine and fluconazole antifungal agents displayed enhanced antifungal activities. This study clearly showed a substantial cooperative antifungal effect of sodium propionate with Benomyl; terbinafine and fluconazole at low concentration below either MICs or MFCs. The combination of sodium propionate with terbinafine or sodium propionate with fluconazole appeared to be the most potential combination particularly when compared with

effective single MICs or MFCs. Walsh and Pizzo (1988) reported that synergistic phenomenon often enable reduction of effective doses of chemotherapeutic agents, which is a desirable to reduce many of the advanced side effects of these test antifungal agents.

There are three generally accepted mechanisms of synergistic activity namely, increase by one agent of the permeability of the cell wall and membranes to the second agent; inhibition of enzymes that degrade the second antifungal agent by the first agent; and double blocking steps in the metabolic sequences(Klastersky, 1989). Terbinafine HCl has been reported to inhibit squalene 2, 3 epoxidase enzymes which convert squalene to lanosterol, a precursor needed for the synthesis of ergosterol with 14-α-demethylase enzyme which is the target site for imidazole group of antifungal agents (Balfour and Frauds, 1992). The sequential actions of terbinafine and the fluconazole in the absence of multi-drug efflux transpoters (Albertson *et al.,* 1996; Niimi, 2004), probably accounted for the synergistc effect observed in this study.

The mechanisms of action of Propionic acids and its salts have been reported to inhibit molds and certain bacteria based in its ability to stop the test organism from metabolising the three carbon chain (Budavari, 1996).

These differences in the mechanisms of action of terbinafine, Sodium propionate, fluconazole, and Benomyl probably explain the observed synergistic activity. In this study the significant importace of this finding is the potential antifungal agents' combinations that will reduce phytopathogenic fungal

infection of yam leaves. This inhibition or fungicidal effects will have a great impact on the improvement of yam tuber production in Nigeria.

5.6 Influence of Time on sporicidal Activity of test Antifungal Agents

The rate of kill of test phytopathogenic fungal spores displayed by Terbinafine, Sodium propionate, Fluconazole and Benomy in this investigation is probably being reported for the first time. This useful observation probably opens a new field of study as to how fast antifungal agents act after applications for combating fungal spores on yam leaves. The results obtained from this study showed that the test compounds possess significant fungicidal activity against spores of resistant *A. niger* spores with $P < 0.05$. The fungicidal activity was rapid and generally concentration dependent. This agrees with the work of Garrett and Brown (1964) who reported that there was no single concentration of the antimicrobial agents at which all cells in a suspension would be killed instantly. Killing of cells occur chiefly as a function of time within a range of concentrations (Denyer and Hugo, 1991; Sheehan *et al.,* 1999; Kale and Johnson, 2005) and this probably explains the increased lethal activities of higher concentrations of these test agents above minimum effective fungicidal concentration.

The shape of the death curves eventually varies, some present the C type curve as shown in fig 4.1 in which there is a rapid rate of kill but the rate at which population decreases diminishes with time and this shape was maintained in some graphs throughout the exposure (fig 4.3). In some the concave shape

eventually became reversed or inverted as usual curve of type B (fig 4.5 and 4.6). This indicated that the test antifungal compounds possess a sporicidal antifungal activity. The linear or straight line graph (fig 4.4) is used to evaluate the fungi-toxic characteristics of chemical compounds. When data of antifungal agents' concentrations are plotted against logarithm of survivor spores, an extended sigma curve is the usual response (Bliss, 1957). This observation is a common microorganism antimicrobial phenomenon.

This work agrees with findings of the earlier works that, though death have always been assumed to follow the first order of kinetics, covering a straight line on semi-log coordinates but this however, can no longer be sustained as reported, and that careful experimentation has shown deviation from linear thermal death curve for spores through the development of ''tails'' to the curves at low proportion of survivors (Burton, 1977; Bentley, 1977; Denyer and Hugo, 1991; Sheehan *et al.,* 1999; Kale and Johnson, 2005). This means that there is occurrence of a small number of test spores that are highly resistant within the surviving spores in a normal population. There is a slow initial reduction of the susceptible members of the fungal population and this was later followed by fast linear rate of kill showing a similar pattern to the first order of kinetics which is followed by a slower death rate of the resistant test fungal spores.

Each of the test antifungal agents showed a similar pattern of sporicidal activity against the most resistant test *A. niger* spores within the range of biocidal concentration examined. For any particular test antifungal agent, as the sporicidal concentration increased, the susceptibility becomes more pronounced as indicated in the death curves in figure 4.7, 4.8, 4.9, and 4.10. This similar

pattern of sporicidal death curve may suggest a common mode of sporicidal action against the yam leaves phytopathogenic *A. niger* spores. Terbinafine and Fluconazole have been reported to interfere with fungal cell membrane synthesis (Schuster and Ryder, 1990; Denyer and Hugo, 1991; El-Saharty *et al.,* 2002; Kale and Johnson, 2005; Mikus *et al.,* 2005). Similarly Benomyl and Sodium propionate have been reported to block fungal cell membrane enzymes commonly engaged in energy production (Richard and Pressinger, 1997; Shephard and Sue, 2006). Hence, the observed similar pattern of death curves with increase sporicidal concentrations of the test antifungal agents.

The D value is the time in minutes at a given concentration of the sporicidal agent required to destroy 1 log cycle (90%) of the target test fungal spores. For example D value of sporicidal activity of fluconazole (55mg/ml); Sodium propionate 65mg/ml and Benomyl 8mg/ml were observed to be 1 minute. This means after 1 minute contact of the above mentioned concentrations of Fluconazole, Sodium propionate and Benomyl, the fungal population of $1.5x10^8$ spores/ml *A. niger* spores suspension was reduced by 90% (fig 4.4). While Terbinafine (0.55mg/ml) achieved the same one log cycle reduction after 1minute 30 seconds.

At a fixed contact time (figure 4.5) time of 60 minutes test fungicide viz fluconazole; terbinafine; sodium propionate and benomyl test antifungal agents reduction of *A. Niger* spores suspension was concentration dependent (fig 4.4; 4.6; 4.8 and 4.9). This means, as the concentrations of these test antifungal agents increased, the log cycle reduction of *A. niger* spores increases at logarithmic order or first order of kinetics. Example, varying sporicidal

concentrations of 28.0, 38.0, 43.0, 47.0 and 52.0mg/ml of killed a 1.0. 2.0, 3.0, 4.0 and 5.0 l0g cycle reduction of $1.5x10^8$ spores/ml of *A. niger* spores suspension after 60minutes contact time respectively (figure 4.7).

Similar rapid sporicidal activities at varying concentrations after 60minutes contact time was observed with terbinafine, Sodium propionate and Benomyl (figure 4.8, 4.9 and 4.10)

There was rapid biocidal activity of test fungal spores of *A. niger* with the combinations of Benomyl / Fluconazole; Benomyl / Terbinafine and Benomyl / Sodium propionate as clearly demonstrated in figures 4.11; 4.12 and 4.13. A quick glance on the viability of *A. niger* spores curves indicated that there was a synergistic sporicidal effect on the test fungal spores and the degree of synergism of the test antifungal agents against the test fungal spores varied. The observed sporicidal activity was observed to be time dependent, meaning, as the time increases the sporicidal activity increases (Hugo and Russel, 1988).

One of the objectives of this investigation was to establish probable combination advantage of Benomyl/Fluconazole, Terbinafine/Benomyl, Benomyl/ Sodium propionate, Terbinafine/ Sodium propionate and Fluconazole/ Sodium propionate as fungicides against yam rot fungi, in view of the inherently potent activity of these test antifungal agents when acting alone. Results from thse studies have shown that Benomyl/Fluconazole, Terbinafine/Benomyl, Benomyl/ Sodium propionate, Terbinafine/ Sodium propionate and Fluconazole/ Sodium propionate (Table 4.12 and figure 4.11) combinations produced synergistic effect. The most desirable effects resulting from simultaneous application of two

antifungals responses as observed in this study. The combination of 2.0mg/ml of Benomyl with 15.0mg/ml of Fluconazole for example achieved 100% kill of $1.5x10^8$ spores/ml within 20 minutes.

Fluconazole and other Azole compounds have been reported to interfere with ergosterol biosynthesis thereby effecting selective leakage of cell membrane and increased osmotic sensitivity. It has also been reported that azole disrupt chitin synthesis. All these effects are known to be due to the azole binding to the cytochrome P-450- mediated enzyme known as 14-α-demethylase ($P\text{-}450_{DM}$) (Sheehan *et al.,* 1999; Kale and Johnson, 2005; Denyer *et al.,* 2005; Greewood *et al.,* 2006). This phenomenon of the fluconazole action may have increased easy penetration of benomyl through the cell membrane and cell wall (Sugar *et al.,* 1997; Sugar and Liu, 2000). This enhanced penetration of benomyl potentiate activity on target DNA synthesis, cytokinesis and some other closely related processes such as nuclear or cell division in test fungi spores germination. Benomyl and its main metabolite carbendazim have been reported to bind to microtubules and therefore interfere with fungal cell nuclear division during mitosis thereby destabilized the fungal cell structures and its intracellular transportation (Barchiest *et al.,* 2001).

Furthermore, Terbinafine hydrochloride has been reported as potent antifungal agent which selectively binds to and inhibits fungal squalene epoxidase in ergosterol synthesis (Mikuš, *et al.,* 2005; El-Saharty, *et al.,* 2002; Milan *et al.,* 2010).

The reported activity of Terbinafne hydrochloride on fungal cell membrane certainty explained potentiating effects observed in combination with Sodium propionate or Benomyl test antifungal agents. Generally all the test agents combination with reduced viability of test fungal spores by a factor of 10^4 within 60minutes with resistant *A. niger* (Hugo and Russell's, 2005).

The observed synergistic activity of Sodium propionate in combination with Terbinafine; Fluconazole and Benomyl against *A. niger; R. stolonifer* and *P. citrinum* showed the potential utility of these combinations. It is widely known that there is no single ideal antifungal agent (Kohn *et al.,* 1963). Therefore compatible antifungal combinations with synergistic activities as observed in this study are warranted for logical further development (Brumfitt, 1981).

5.7 DNA Release from cytoplasmic constituent of test *A. niger* spores

The activity of the fixed concentration of the test compounds viz: Fluconazole, Terbinafine, Benomyl and Sodium propionate at varying time on test fungal spores membrane intergrity showed a significant difference between the test antifungal agents with a P value less than 0.05.

The study showed that Fuconazole has a rapid break down of the *A. niger* spore membranes intergrity which corresponds with (Milan *et al.,* 2010). Terbinafine hydrochloride has been reported to selectively inhibit fungal squalene epoxidase which drastically affect spore membrane ergosterol synthesis (Wilson and Walker, 1995; El-Saharty *et al.,* 2002 Mikuš *et al.,* 2005). This makes the fungal

spore to lose its membrane integrity and probably lead to the swelling up of the spores which finally leaks out the spores' constituent. The extent at which these test chemical compounds caused DNA release were in the order of fluconazole > terbinafine > benomyl > sodium propionate. The leaked cytoplasmic constituents of the test fungal spores probably accounted for the sporicidal activities observed in this study.

5.8 Effect of different water sources on fungicides combination

The results from the study of the effect of boiled and unboiled well, tap, river, pond and Distilled water used to dissolve the fungicides combination of Benomyl; fluconazole; terbinafine and sodium propionate (Fig 12; 13 and 14) showed asignificant difference between the different water sources with a P value less than 0.05. The activities of fungicides when dissolved in distilled water produced highest zone of inhibition than other water sources. The better activity of distilled could be due to the absence of organic matter and other impurities that may possibly interfere with the compounds active sides and reduce their activity and effectiveness as seen in the non-distilled water sources. Hugo and Russells (2005) stated that it has long been known that the cidal activity of many antimicrobial compounds is seriously impaired under dirty conditions. In addition, divalent cations (e.g. Mg^{2+}, Ca^{2+}) presence in hard water may also interact with the microbial cell surface and block antimicrobial adsorption sites necessary for activity. However, the observed differences in the zone of inhibition of diluted fungicides preparations that, displayed better antifungal activity when diluted with boiled water source than the unboiled may

be due to the reduction of organic matter in the boiled waters. Moreover, there was indication that between 30min to an hour many of the organic matter present had been sedmented. Reduction of these impurities enhanced the antimicrobial activities.

5.9 Effect of different pH values on fungicides combination

The result for the effect of varying pH of test fungicide formulation on the *A. niger,* generally shows significant difference on their antifungal activity with $P<0.05$. Sodium propionate with Fluconazole; Sodium propionate with Benomyl; Benomyl with Terbinafine; Benomyl with Fluconazole were observed to be more active at neutral pH (pH7) and distilled water medium. However, Sodium propionate with Terbinafine was observed to be more effective in alkaline pH of 9.

Effects of pH on antimicrobial activity can be complex. As well as influencing the survival and rate of growth of the microorganism under test, change in pH may affect the potency of the agent and its ability to combine with cell surface active sites (Denyer and Hugo, 1991; Hugo and Russell's, 2005). If the agent is acidic or basic, its degree of ionization will depend on the pH, the non ionized molecule is the active state and the alkaline pH which favour the formation of ions of such compounds will decrease the activity. Others reveal increased cidal activity as the pH rises and are best used under alkaline conditions

5.10 Effect of Heat/Temperature on Potential Antifungal Formulation

From the result of this research on the effect of varying temperature 37^0C; 45^0C; 70^0C and 100^0C on potential antifungal formulations, Sodium propionate with Terbinafine; Sodium propionate with Fluconazole; Sodium propionate with Benomyl; Sodium propionate with Benomyl; Benomyl with Terbinafine; Benomyl with Fluconazole. This showed a slight increased effect on their antifungal activities with differences in zones of inhibition (mm) at different temperatures the higher differencewas with Sodium propionate/Terbinafine and Sodium propionate /Benomyl at 45^0C. This suggests that heating to 45^0C may increase the activity of that combination contrary to the others. Decreased activity of some test compounds formulated may be due to chemical destruction at higher temperature after heating, while the increased activity of the test compound formulated may due to increased in solubility at higher temperature before destruction occur.

5.11 Physical and chemical interactions of the test compounds

Examination of the combined antifungal agents using chromatographic methods showed that the activities of the combination fungicides contains only the individual components of the test antifungal agent without forming any complex with each other which was in line with Wilson and Walker (1995).

5.12 Field Trial of the Combined Antifungal Agents

Observations from the application of test antifungal agents

(A) Benomyl / Fluconazole (1,000 µg/ml /15,000µg/ml);

(B) Benomyl /Terbinafine (1,000 µg/ml /200µg/ml);

(C) Benomyl / Sodium Propionate (1,000 µg/ml /15,000µg/ml);

(D) Benomyl /Sodium Propionate (1,000 µg/ml / 20,000µg/ml);

(E) Fluconazole /Sodium Propionate (1000µg/ml /10,000µg/ml ml) and

(F) Terbinafine / Sodium Propionate (200µg/ml /10,000µg/ml)

Showed effective control of test fungal infection of the test yam leaves after or at the end of thirteen days of application compared to untreated infected yam leaves in this study. This study has shown that any of these combined test antifungal agents could be used to control all the tested (14 isolates) fungal infection of yam leaves.

The overall impact of these formulations on yam yield and money accruable to the farmer cannot be over emphasized. This study has shown the possibility of enhancing food security for the nation by limiting yam foliage deterioration with the use of any of the potential combination in this work.

CHAPTER SIX

6.0 SUMMARY, CONCLUTIONC AND RECOMENDATIONS

6.1 SUMMARY

a) This study has shown that *Aspergillus niger; Aspergillus fumigatus; Aspergillus flavus; Aspergillus wentii; Aspergillus ostianus; Aspergillus oryzae; Aspergillus cervinus; Rhizopus stolonifer; Rhizopus* sp; *Penicillium citrinum; Penicilium oxalicum*; *Penicillium* sp*; Clodosporium* sp and *Scoppolarius* sp.were found associated with fungal infections of yam leaves in the farm in Zaria, Nigeria.

b) The fourteen phytopathogenic fungi isolated and identified in the investigated yam leaves in Zaria were found susceptible to the test antifungal agents.

c) Observation from this study has also revealed that test antifungal agents Fluconazole, Terbinafine HCl and Flucytosine were effective in arresting phytopathogenic fungi spores at a dose MIC of 3.91µg/ml of Terbinafine against *A. niger*).

d) Antifungal activities of Terbinafine/Fluconazole; Fluconazole/Sodium propionate; Benomyl/Sodium propionate; Fluconazole/Benomyl and Terbinafine/Benomyl were found to be synergistic. An effect highly desired in any antimicrobial combination

   Therefore, additions of Sodium propionate enable a significant reduction in dose of the other component of the preparation and yet maintain effective antifungal activities. Further more, the combination of these test agents displayed synergistic activities which will reduce the effective concentration when used in

the eventual formulation of these potential fungicides. Reduction in the concentration also reduces the toxicity of the test agents

e) The potential antifungal agents combinations developed in this study were found to be effective at neutral pH (7) except Terbinafine/Sodium propionate combination that was observed to be most effective at alkaline pH (9).

f) There was reduction in antifungal activities of potential combined antifungal agents when diluted with unboiled River, Tap, Well and pond water. However using distilled water maintained a high level of antifungal activity.

g) The new antifungal agents' combinations developed also showed increased activity at 45^0C.

h) Further more this study showed that there were no chemical interactions between the combined antifungal agents in the chromatographic result obtained.

i) Finally, field work of this study showed that any of these combined test antifungal agents could be used to control fungal infection of yam leaves by above test fungi.

6.2 CONCLUSION

The new combinations developed were found to arrest the growth of infective test fungi spores on the yam leaves in Zaria, Nigeria. This is a probable solution to yam leaves fungal infections increase in yam tuber production in Nigeria.

6.3 RECOMENDATION

Further work about their stability in the soil and their impact on the environment will need verification before practical application in the yam farm.

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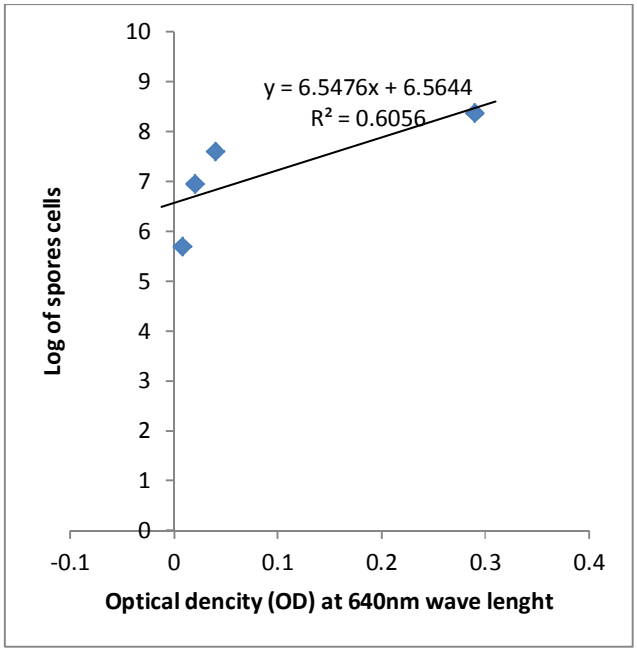
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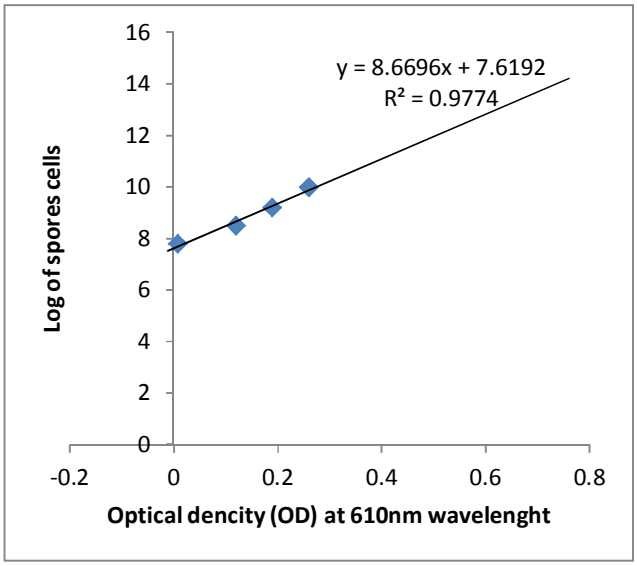
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APPENDICES

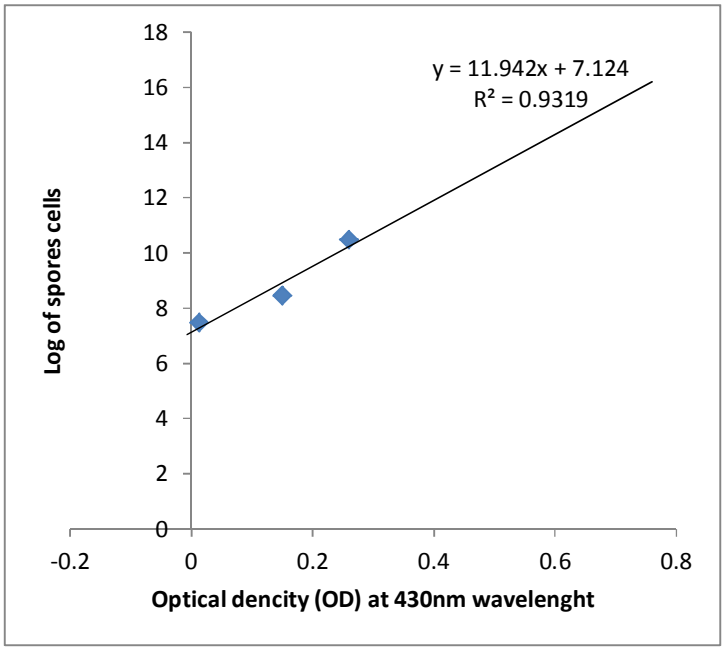
Appendix I: Spectrophotometric Standardization of test fungal spores suspention (108 cfu or 108 spores/ml).



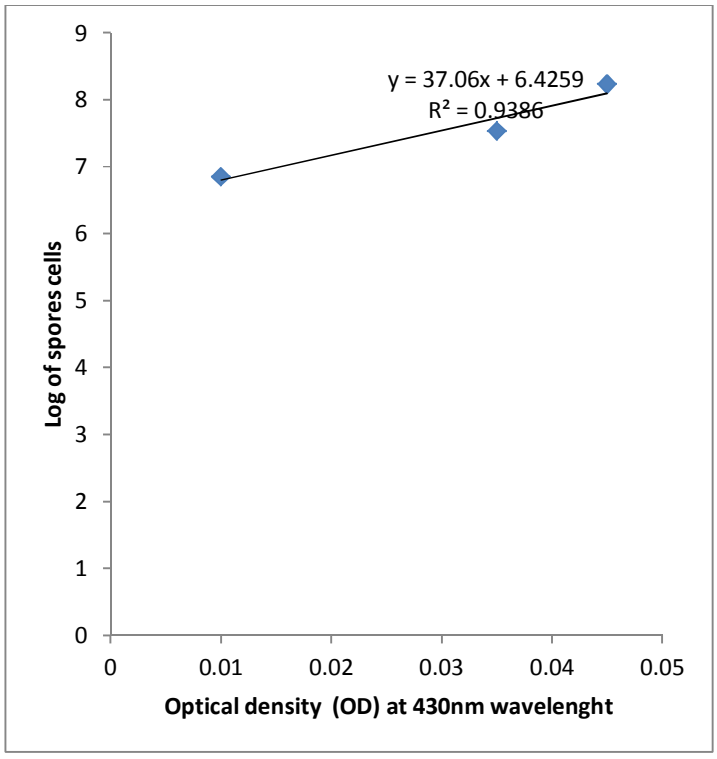
Spectrophotometric Standardization of *Penicillium citrinum* fungal spores suspention (10^8 cfu or 10^8 spores/ml) at wave length of 460 nm.



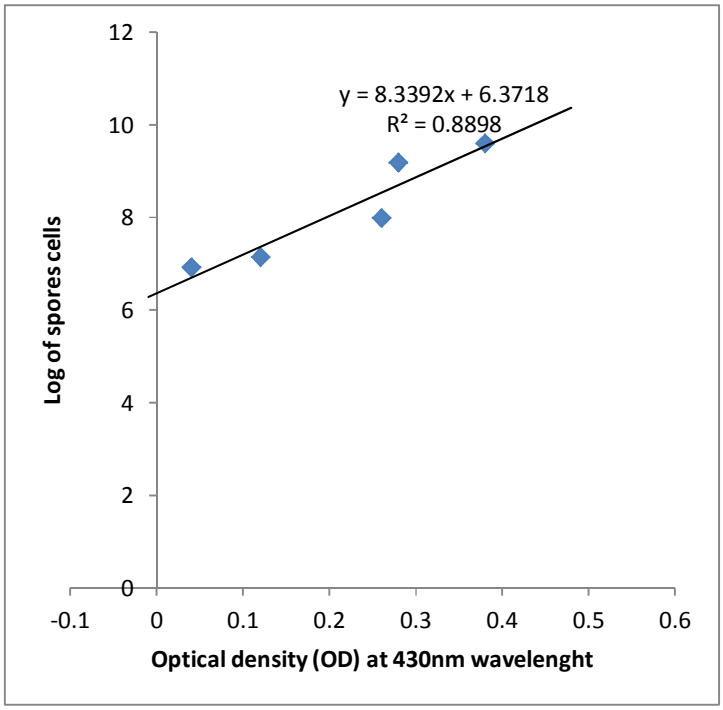
Spectrophotometric Standardization of *Aspergillus wentii* fungal spores suspention (10^8 cfu or 10^8 spores/ml) at wave length of 460 nm.



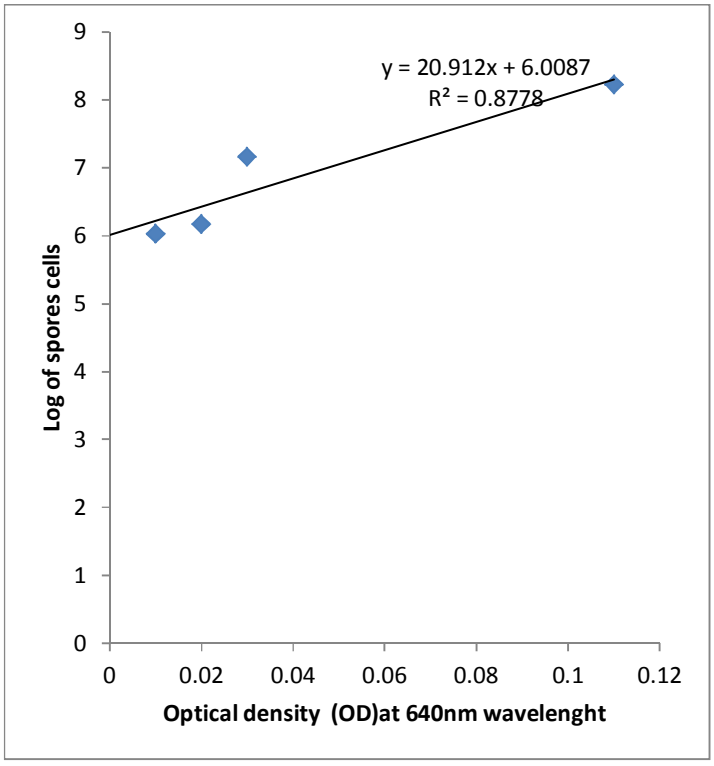
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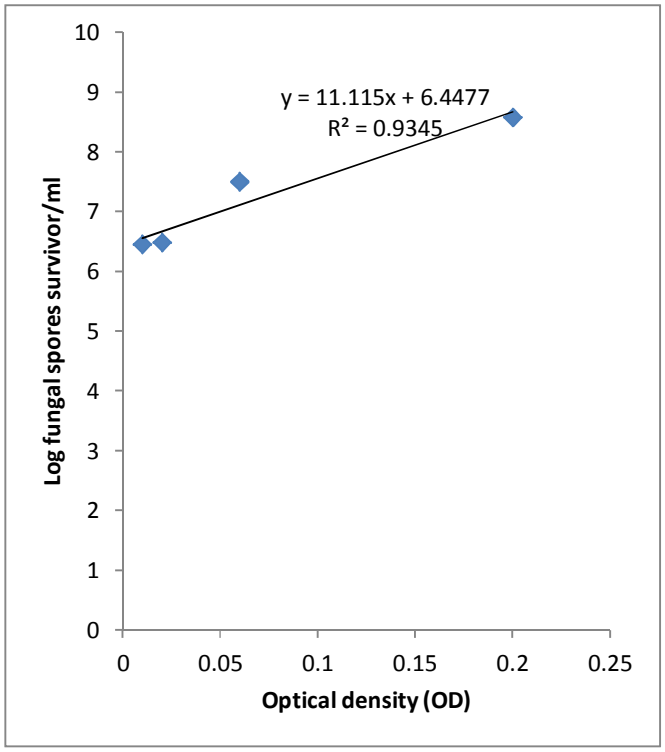
Spectrophotometric Standardization of *Aspergillus cervinus* fungal spores suspention (10^8 cfu or 10^8 spores/ml) at wave length of 460 nm.



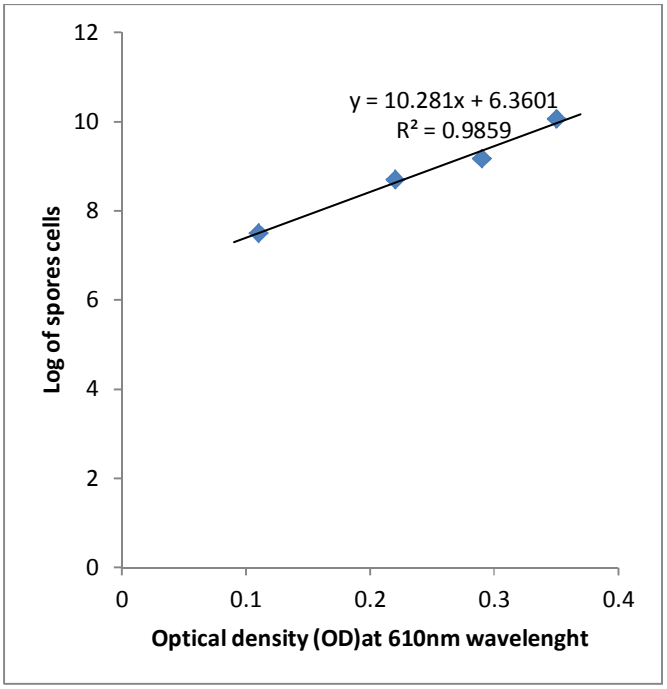
Spectrophotometric Standardization of *Peenicilium* spfungal spores suspention (10^8 cfu or 10^8 spores/ml) at wave length of 460 nm.



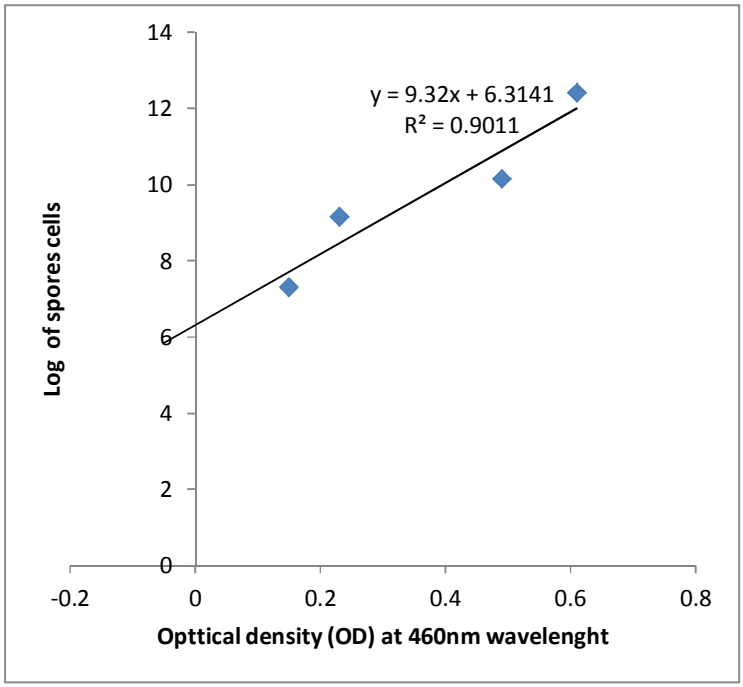
Spectrophotometric Standardization of *Scopolarius* sp fungal spores suspention (10^8 cfu or 10^8 spores/ml) at wave length of 460 nm.



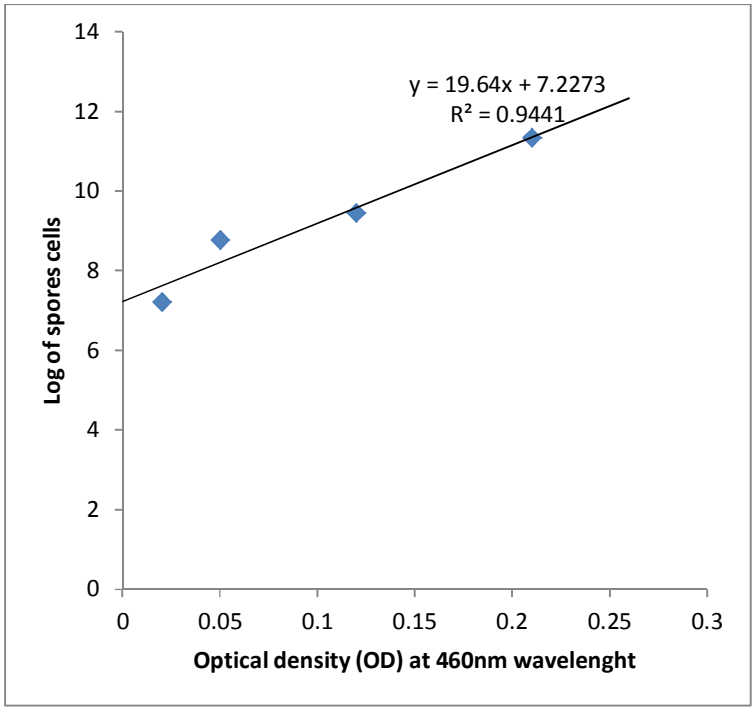
Spectrophotometric Standardization of *A. niger* fungal spores suspention (10^8 cfu or 10^8 spores/ml) at wave length of 460 nm.



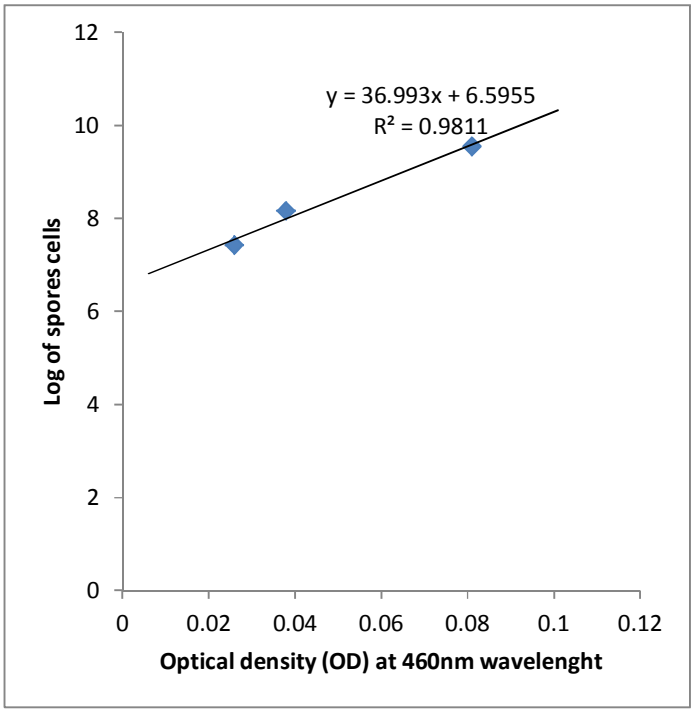
Spectrophotometric Standardization of *Clodosporium* sp fungal spores suspention (10^8 cfu or 10^8 spores/ml) at wave length of 460 nm.



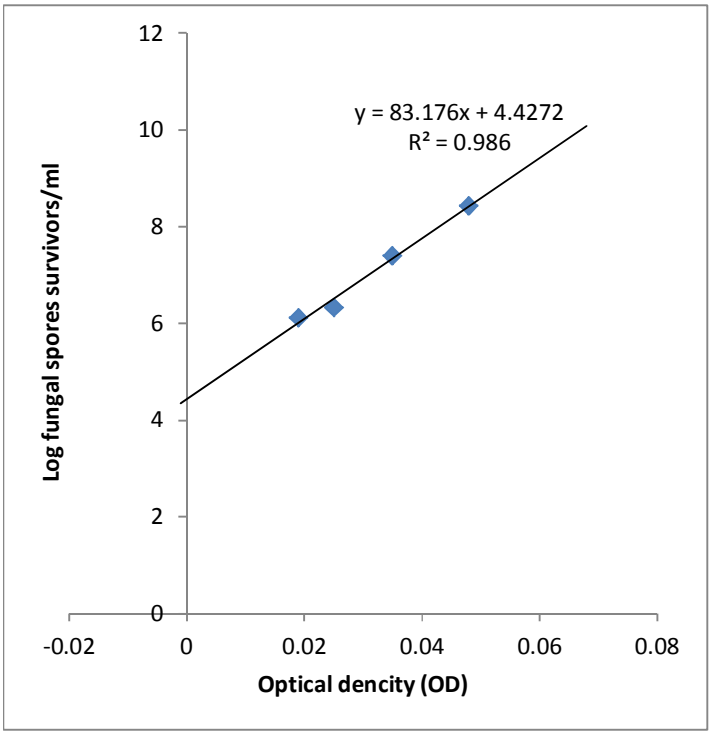
Spectrophotometric Standardization of *Aspergillus fumigates* fungal spores suspention (10^8 cfu or 10^8 spores/ml) at wave length of 460 nm.



Spectrophotometric Standardization of *Aspergillus flavus* fungal spores suspention (10^8 cfu or 10^8 spores/ml) at wave length of 460 nm.



Spectrophotometric Standardization of *Rhizopus* sp fungal spores suspention (10^8 cfu or 10^8 spores/ml) at wave length of 460 nm.



Spectrophotometric Standardization of *R. stolonifer* fungal spores suspension (10^8 cfu or 10^8 spores/ml) at 460nm wave length.

Appendix II: MIC of The combined antifungal agents in Admixture with Sodium propionate that gives Complete Inhibition of test *A. niger* Spores Suspension

MIC of Benomyl in Admixture with Sodium propionate that gives Complete Inhibition of test fungal Spores Suspension

µg/ml

	Combined MIC	*Penicillium citrinum*	*Aspergillus niger*	*Rhizopus stolonifer*
A	Benomyl/Sodium propionate	1.50/400	1.50/400	1.50/800
B	Benomyl/Sodium propionate	1.25/400	1.25/400	1.25/800
C	Benomyl/Sodium propionate	1.00/400	1.00/400	1.00/800
D	Benomyl/Sodium propionate	0.70/400	0.70/400	0.70/800
E	Benomyl/Sodium propionate	0.50/400	0.50/750	0.50/800
F	Benomyl/Sodium propionate	0.25/400	0.25/750	0.25/800
G	Benomyl/Sodium propionate	0.10/750	0.10/800	0.10/800

MIC of Fluconazolein Admixture with Sodium propionate that gives Complete Inhibition of test fungal Spores Suspension

µg/ml

	Combined MIC	*Penicillium citrinum*	*Aspergillus niger*	*Rhizopus stolonifer*
A	Fluconazole/Sodium propionate	1.50/400	1.50/400	1.50/800
B	Fluconazole/Sodium propionate	1.25/400	1.25/400	1.25/800
C	Fluconazole/Sodium propionate	1.00/400	1.00/400	1.00/800
D	Fluconazole/Sodium propionate	0.70/400	0.70/400	0.70/800
E	Fluconazole/Sodium propionate	0.50/400	0.50/400	0.50/800
F	Fluconazole/Sodium propionate	0.25/750	0.25/400	0.25/800
G	Fluconazole/Sodium propionate	0.10/800	0.10/800	0.10/800

MIC of Terbinafine in Admixture with Sodium propionate that gives Complete Inhibition of test fungal Spores Suspension

µg/ml

	Combined MIC	*Penicillium citrinum*	*Aspergillus niger*	*Rhizopus stolonifer*
A	Terbinafine/Sodium propionate	1.00/200	1.00/200	1.00/200
B	Terbinafine/Sodium propionate	0.70/200	0.70/200	0.70/200
C	Terbinafine/Sodium propionate	0.50/200	0.50/200	0.50/200
D	Terbinafine/Sodium propionate	0.25/200	0.25/200	0.25/200
E	Terbinafine/Sodium propionate	0.10/600	0.10/400	0.10/400

Appendix III: MFC of The combined antifungal agents in Admixture with Sodium propionate that gives complete fungicidal activity of test *A. niger* Spores Suspension

MFC of Benomyl in Admixture with Sodium propionate that gives Complete fungicidal activity of test fungal Spores Suspension

µg/ml

	Combined MIC	*Penicillium citrinum*	*Aspergillus niger*	*Rhizopus stolonifer*
A	Benomyl/Sodium propionate	50/200	50/400	50/400
B	Benomyl/Sodium propionate	40/200	40/600	40/800
C	Benomyl/Sodium propionate	30/200	30/600	30/800
D	Benomyl/Sodium propionate	20/400	20/600	20/800
E	Benomyl/Sodium propionate	10/400	10/600	10/1000

MFC of Fluconazole in Admixture with Sodium propionate that gives Complete fungicidal activity of test fungal Spores Suspension

µg/ml

	Combined MIC	*Penicillium citrinum*	*Aspergillus niger*	*Rhizopus stolonifer*
A	Fluconazole/Sodium propionate	100/400	100/800	100/1000
B	Fluconazole/Sodium propionate	60/400	60/1000	60/1000
C	Fluconazole/Sodium propionate	40/400	40/1000	40/1000
D	Fluconazole/Sodium propionate	20/400		
E	Fluconazole/Sodium propionate	10/400		

MFC of Terbinafine in Admixture with Sodium propionate that gives Complete fungicidal activity of test fungal Spores Suspension

µg/ml

	Combined MIC	*Penicillium citrinum*	*Aspergillus niger*	*Rhizopus stolonifer*
A	Terbinafine/Sodium propionate	1.00/200	1.00/200	1.00/200
B	Terbinafine/Sodium propionate	0.70/200	0.70/200	0.70/200
C	Terbinafine/Sodium propionate	0.50/200	0.50/200	0.50/200
D	Terbinafine/Sodium propionate	0.25/200	0.25/200	0.25/200
E	Terbinafine/Sodium propionate	0.10/600	0.10/400	0.10/400

Appendix IV: Data on the Rate of Kills

Fungicidal effect of 55,000µg/ml of fluconazole on the viability of resistant *Aspergillus niger* spores suspension at 25 ±2^{0}C.

55,000µg/ml OF FLUCONAZOLE

Time in minutes	Mean Spores cell count	Mean log ±SD
0	4.10×10^8	8.61±1.41
5	6.00×10^4	4.78±1.41
20	2.00×10^4	4.30±1.41
40	2.80×10^3	3.45±5.65
60	2.40×10^3	3.38±4.95

Fungicidal effect of varying concentrations of fluconazole on the viability of resistant *Aspergillus niger* spores suspension at fixed contact time of 60minute.

FLUCONAZOLE AT FIXED TIME 60 MINUTES

Concentration in µg/ml	Mean Spores cell count	Mean log ±SD
10,000	5.05×10^8	8.70±3.54
20,000	2.55×10^8	8.41±7.78
35,000	3.55×10^7	7.55±6.36
45,000	5.65×10^5	5.75±6.36
55,000	2.80×10^3	3.45±4.24

Effect of 550µg/ml of Terbinafine HCl on the viability of resistant *Aspergillus niger* spores suspension.

550µg/ml OF TERBINAFINE

Time in minutes	Mean Spores cell count	Mean log ±SD
0	3.65×10^{7}	7.56±6.36
5	3.50×10^{4}	4.54±0.71
20	0.00×10^{0}	0.00±0.00
40	0.00×10^{0}	0.00±0.00
60	0.00×10^{0}	0.00±0.00

Fungicidal effect of varying concentrations of Terbinafine HCl on the viability of resistant *Aspergillus niger* spores suspension at fixed contact time of 60minute.

Fig4 TERBINAFINE AT FIXED TIME 60 MINUTES

Concentration in µg/ml	Mean Spores cell count	Mean log ±SD
50	1.70×10^{6}	6.23±1.41
150	1.95×10^{5}	5.29±9.19
250	6.50×10^{4}	4.81±2.12
350	0.00×10^{0}	0.00±0.00
450	0.00×10^{0}	0.00±0.00
550	0.00×10^{0}	0.00±0.00

Effect of 65000µg/ml of Sodium propoinate on the viability of resistant *Aspergillus niger* spores suspension

65,000µg/ml OF SODIUM PROPIONATE

Time in minutes	Mean Spores cell count	Mean log ±SD
0	$3.75x\ 10^8$	8.60±2.12
5	$3.00x\ 10^4$	4.50±1.41
20	$1.00x\ 10^4$	4.00±1.41
40	$7.00x\ 10^2$	2.80±1.41
60	$0.00x\ 10^0$	0.00±0.00

Effect of varying concentrations of Sodium propionate on the viability of resistant *Aspergillus niger* spores suspension at fixed contact time of 60minute.

Fig6: SODIUM PROPIONATE AT FIXED TIME 60 MINUTES

Concentration in µg/ml	Mean Spores cell count	Mean log ±SD
15,000	$6.25x\ 10^5$	5.80±17.68
30,000	$2.45x\ 10^5$	5.39±4.95
45,000	$8.50x\ 10^4$	4.90±2.80
55,000	$7.50x\ 10^2$	2.88±0.71
65,000	$0.00x\ 10^0$	0.00±0.00

Effects of varying concentrations of Benomyl on the viability of resistant *Aspergillus niger* at of spores suspension fixed contact time of 60 minute.

BENOMYL AT FIXED TIME 60 MINUTES

Concentration in µg/ml	Mean Spores cell count	Mean log ±SD
2,000	$1.00x\ 10^4$	4.0±1.41
4,000	$0.00x\ 10^0$	0.0±0.00
6,000	$0.00x\ 10^0$	0.0±0.00
8,000	$0.00x\ 10^0$	0.0±0.00
10,000	$0.00x\ 10^0$	0.0±0.00

Effect of 8000µg/ml of Benomyl on the viability of resistant *Aspergillus niger* sporessuspension.

8000µg/ml OF TERBINAFINE

Time in minutes	Mean Spores cell count	Mean log ±SD
0	$10.00x\ 10^{10}$	10.10±6.36
5	$1.50x\ 10^5$	5.18±1.41
20	$5.00x\ 10^4$	4.70±2.82
40	$1.00x\ 10^0$	0.00±0.00
60	$0.00x\ 10^0$	0.00±0.00

Effect of Benomyl (2000 µg/ml), Sodium propionate (15000µg/ml) and Benomyl (1000 µg/ml)+ Sodium propionate (7500µg/ml) at different time (minutes) on the viability of resistant *Aspergillus niger* sporessuspension.

a: 2,000µg/ml OF BENOMYL

Time in minutes	Mean Spores cell count	Mean log ±SD
0	6.70×10^{8}	8.80±21.21
5	2.25×10^{6}	6.40±7.78
20	1.85×10^{5}	5.30±3.54
40	2.95×10^{3}	3.50±2.12
60	1.45×10^{2}	2.20±0.70

b: 15,000µg/ml OF SODIUM PROPIONATE

Time in minutes	Mean Spores cell count	Mean log ±SD
0	6.70×10^{8}	8.80±12.21
5	3.25×10^{7}	7.50±0.71
20	8.95×10^{6}	6.90±24.70
40	3.75×10^{6}	6.60±7.78
60	4.00×10^{5}	5.60±11.31

c: 1,000µg/ml OF BENOMYL+: 7,500µg/ml OF SODIUM PROPIONATE

Time in minutes	Mean Spores cell count	Mean log ±SD
0	6.70×10^{8}	8.80±21.21
5	1.30×10^{6}	610±1.41
20	1.25×10^{5}	5.10±2.12
40	2.60×10^{3}	3.40±1.41
60	6.80×10^{2}	2.80±9.90

Effect of Benomyl (2000 µg/ml), Fluconazole (30,000µg/ml) and Benomyl (1000 µg/ml)+ Fluconazole (15,000µg/ml) at different time (minutes) on the viability of resistant *Aspergillus niger* sporessuspension.

a: 2,000µg/ml OF BENOMYL

Time in minutes	Mean Spores cell count	Mean log ±SD
0	4.10×10^{7}	7.60±8.49
5	2.55×10^{6}	6.40±7.78
20	1.75×10^{5}	5.20±0.71
40	2.15×10^{3}	3.30±4.95
60	1.55×10^{2}	2.20±3.54

b: 30,000µg/ml OF FLUCONAZOLE

Time in minutes	Mean Spores cell count	Mean log ±SD
0	4.10×10^{7}	7.60±8.41
5	4.50×10^{6}	6.70±8.49
20	3.30×10^{6}	6.50±2.83
40	2.25×10^{6}	6.40±3.54
60	1.02×10^{6}	6.00±1.41

c: 1,000µg/ml OF BENOMYL + 15,000µg/ml OF FLUCONAZOLE

Time in minutes	Mean Spores cell count	Mean log ±SD
0	4.10×10^{7}	7.60±8.49
5	4.50×10^{4}	4.70±1.41
20	0.00×10^{0}	0.00±0.00
40	0.00×10^{0}	0.00±0.00
60	0.00×10^{0}	0.00±0.00

Fungacidal activity of Benomyl (2000 µg/ml), Terbinafine HCl (200µg/ml) and Benomyl (1000 µg/ml)+ Terbinafine HCl (100µg/ml) against resistant *Aspergillus niger* spores suspension.

a: 200µg/ml OF TERBINAFINE HCL

Time in minutes	Mean Spores cell count	Mean log ±SD
0	3.95×10^{7}	7.60±2.12
5	1.40×10^{6}	6.10±1.41
20	4.20×10^{5}	5.60±9.90
40	2.00×10^{5}	5.30±5.66
60	4.45×10^{4}	4.60±9.19

b: 2,000µg/ml OF BENOMYL

Time in minutes	Mean Spores cell count	Mean log ±SD
0	4.10×10^{7}	7.60±2.12
5	2.55×10^{6}	6.60±5.66
20	1.75×10^{5}	5.40±7.07
40	2.15×10^{3}	3.70±5.66
60	1.55×10^{2}	2.30±2.12

c: 100µg/ml OF TERBINAFINE + 1,000µg/ml OF BENOMYL

Time in minutes	Mean Spores cell count	Mean log ±SD
0	3.95×10^{7}	7.60±2.12
5	1.55×10^{3}	3.20±2.12
20	2.00×10^{2}	2.30±1.41
40	0.00×10^{0}	0.00±0.00
60	0.00×10^{0}	0.00±0.00

Appendix V: Leakage of 260nm-Absorbing Materials From *Aspergillus niger* with Fixed Fungicidal Concentration of Test Compounds at Different Contact Time.

DNA Release Trom Cytiplasmic Leakage				
Time in minutes	Ter (20µg/ml)	Ben (2,000µg/ml)	Fluc (5,000µg/ml)	Np (40,000µg/ml)
0	0.30	1.50	0.40	1.20
5	2.70	3.90	19.30	1.30
10	0.70	1.90	25.50	1.20
20	2.80	2.30	22.90	1.20
40	15.00	2.60	26.80	1.20
60	2.90	5.20	29.70	1.20
120	3.30	2.60	28.90	1.70
180	2.80	3.90	31.30	1.50
240	2.60	2.90	31.40	1.60

Key:

Ter = Terbinafine HCL
Ben = Benomyl
Fluc = Fluconazole
Np = Sodium propionate

Appendix VI: Effects of Different Water Sources Viz: Well Water, Tap Water, River Water, Pond Water And Distilled Water on the Drugs Combination.

Effect of Ben 1000µg/ml+Fluc 15,000µg/ml on boiled and unboiled water

Didluent	Mean Zone of inbition with Boiled (mm) ± SD	Mean Zone of inhibition with Unboiled (mm) ± SD
Well water	30.00±2.82	35.00±4.24
Tap water	4.001±0.00	38.00±0.00
River water	37.00±4.24	28.00±1.41
Distilled water	41.00±1.41	--------
25% ethanol in Distilled water	43.00±0.00	--------
Pound water	40.50±0.71	27.00±9.89

Effect of Ben 1000µg/ml+Ter 200µg/ml on boiled and unboiled water

Didluent	Mean Zone of inbition with Boiled (mm) ± SD	Mean Zone of inhibition with Unboiled (mm) ± SD
Well water	33.00±4.24	30.00±0.00
Tap water	37.00±0.00	30.30±4.24
River water	33.50±2.12	27.00±8.49
Distilled water	39.00±2.83	-------
25% ethanol in Distilled water	39.00±1.41	-------
Pound water	36.00±1.41	35.00±3.54

Effect of Ben 1000µg/ml+ Np 15,000µg/ml on boiled and unboiled water

Didluent	Mean Zone of inbition with Boiled (mm) ± SD	Mean Zone of inhibition with Unboiled (mm) ± SD
Well water	32.50±3.54	27.50±3.54
Tap water	40.00±0.00	39.00±2.83
River water	36.00±1.41	32.50±3.54
Distilled water	47.00±2.82	--------
25% ethanol in Distilled water	45.00±2.82	--------
Pound water	38.00±2.82	36.00±1.41

Appendix VII: Zone of Inhibition on the Effects of Some Selected Antifungal Combinations against *A. niger* Spores Suspension at Different pH.

Antifungal combination	PH 4(mm)	PH 7 (mm)	PH 9 (mm)	Control(mm)
Ter/Np	27.00±1.41	26.00±2.83	32.50±1.77	28.00±2.83
Fluc/Np	3.00±0.50	4.10±0.10	3.20±0.30	3.10±0.30
Ben/Npa	27.00±2.00	34.00±1.00	28.00±3.00	35±1.00
Ben/Np	39.00±3.53	41.00±7.07	34.00±4.24	43.00±1.41
Fluc/Ben	30.00±1.41	42.00±4.24	32.00±2.83	40.00±2.83
Ter/Ben	31.00±1.41	36.00±1.41	34.00±2.82	31.50±0.71

Key:

Ter = Terbinafine
Ben = Benomyul
Fluc = Fluconazole
Np = Sodium propionate

Appendix VIII: Zone Of Inhibition On The Effects Of Some Selected Antifungal Combinations Against *A. niger* Spores Suspension At Different Temperature.

	37 ^{0}C (mm)	45 ^{0}C (mm)	70 ^{0}C (mm)	100 ^{0}C(mm)
Ter/Np	30±5.66	37.5±0.71	33±5.66	33.5±3.53
Fluc/Np	3.25±1.06	4.25±1.06	4.75±0.35	4.5±0.71
Ben/Npa	32.5±2.12	36±1.41	30±1.41	32±2.83
Ben/Npb	48.5±4.95	54±8.49	53.5±3.54	54±5.60
Fluc/Ben	54±2.82	54.5±0.71	54.5±1.41	53±2.82
Ter/Ben	38.25±3.18	38.5±3.53	42±0.71	41±0.71

Key:

Ter = Terbinafine
Ben = Benomyul
Fluc = Fluconazole
Np = Sodium propionate

Appendix IX: Statistical Analysis

Univariate Analysis of Variance on the level of Fungal infections between the three Local Governments Areas Studied, in Kaduna satate, Nigeria.

Tests of Between-Subjects Effects

Dependent Variable:IsolatedOrganisms

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
LocalGovenment	Hypothesis	36.167	2	18.083	6.084	.021
	Error	26.750	9	2.972[b]		

At 95 % confident limit, P value is less than 0.05, so we reject the Ho. Since a significant difference exist between the subjects. Meaning there is a significant difference in the level of fungal infection of *D. rotundata* leaves in the three local Govenments Areas of Kaduna state Nigeria.

Univariate Analysis of Variance on the Rate of Kill of Benomyl, Tebinafine and in Combination

Tests of Between-Subjects Effects

Dependent Variable: Benomyl, Terbinafine and their Combination

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Antifungal	Hypothesis	28.561	2	14.281	11.001	.005
	Error	10.385	8	1.298		
Time	Hypothesis	52.023	4	13.006	10.018	.003
	Error	10.385	8	1.298		

At 95 % confident limit, P value is less than 0.05 for both the antifungal agent and contact time, so we reject the Ho. Thus, there is a significant difference exist between the fungal activities of the test fungicides and contact time investigated in this studies

Univariate Analysis of Variance on the on the rate of kill of benomyl, flconazole and in combination

Tests of Between-Subjects Effects

Dependent Variable: Benomyl, Fluconazole and their Combination

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Antifungal	Hypothesis	44.188	2	22.094	8.534	.010
	Error	20.712	8	2.589		
Time	Hypothesis	49.764	4	12.441	4.805	.029
	Error	20.712	8	2.589		

At 95 % confident limit P value is less than 0.05 for both the antifungal agent and contact time, so we reject the Ho. There is a significant difference that exists between the fungicidal activities of each antifungal chemical compeared to their combinations. The studied showed that these sporicidal activities was time dependent in varies with the different fungicides investigated.

Univariate Analysis of Variance on the rate of kill of benomyl, sodium propionate and in combination

Tests of Between-Subjects Effects

Dependent Variable:BenNp

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Antifungal	Hypothesis	44.188	2	22.094	8.534	.010
	Error	20.712	8	2.589		
Time	Hypothesis	49.764	4	12.441	4.805	.029
	Error	20.712	8	2.589		

At 95 % confident limit, P value is less than 0.05 for both the antifungal agent and contact time, so we reject the Ho. There is a significant difference, between the sporicidal effects of the test agent when studied singly than when in combination. The sporicidal activities were observed to time and test agents dependents which varied at different concentration.

Univariate Analysis of Variance on the effect of test antimicrobial agents on cytoplasmic constituent

Tests of Between-Subjects Effects

Dependent Variable:Amount of DNA release test *A. niger* spores

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Time	Hypothesis	324.576	8	40.572	1.573	.185
	Error	618.920	24	25.788		
Antifungals	Hypothesis	3201.060	3	1067.020	41.376	.0001
	Error	618.920	24	25.788		

There was no significant difference between DNA release by terbinafine HCl, Benomyl and Sodium propionate at 95% confident limit because the P value is less than 0.05. Hence Ho is rejected. However when the amount of DNA released from spores of *A. niger* was considered at different time intervals Null hypothesis at 95% confident limit cannot be sustained because the P value was observed to be greater than 0.05. Hence Ho of significant difference in DNA releasaed at different time interval was sustained. The DNA releasesd by the fungicides were observed to be time dependent and varies.

Univariate Analysis of Variance on the effect of different water sources on test antimicrobial combination

Tests of Between-Subjects Effects

Dependent Variable: Zone of Inhibition of antifungal combination

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Sources	Hypothesis	6.856	5	1.371	11.946	.027
	Error	.377	3.280	.115		
Boiled/unboiled	Hypothesis	.601	1	.601	11.983	.251
	Error	.036	.719	.050		
Combinations	Hypothesis	.845	2	.423	24.853	.587
	Error	.003	.191	.017		

At 95 % confident limit, P value is less than 0.05 therefore the effect of different waters sources on the combined antifungal activity was significant. However, there was no significant difference at 95 % confident limit in the antifungal activity in the boiled and unboiled water sources as observed statistically in this study.

Univariate Analysis of Variance on the effect of different pH on test antimicrobial combination

Tests of Between-Subjects Effects

Dependent Variable:pH

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Combinations	Hypothesis	3334.208	5	666.842	60.735	.0001
	Error	164.692	15	10.979		
Groups	Hypothesis	77.453	3	25.818	2.351	.113
	Error	164.692	15	10.979		

At 95% confident limit, P value is less than 0.05 for the antifungal combinations, thus there was a significant difference between the fungicidal activities of different test antifungal combinations. However there was no significant difference between the effects of different pH on the test antifungal combinations activity at different pH investigated.

Univariate Analysis of Variance on the effect of different Temperature on test antimicrobial combination

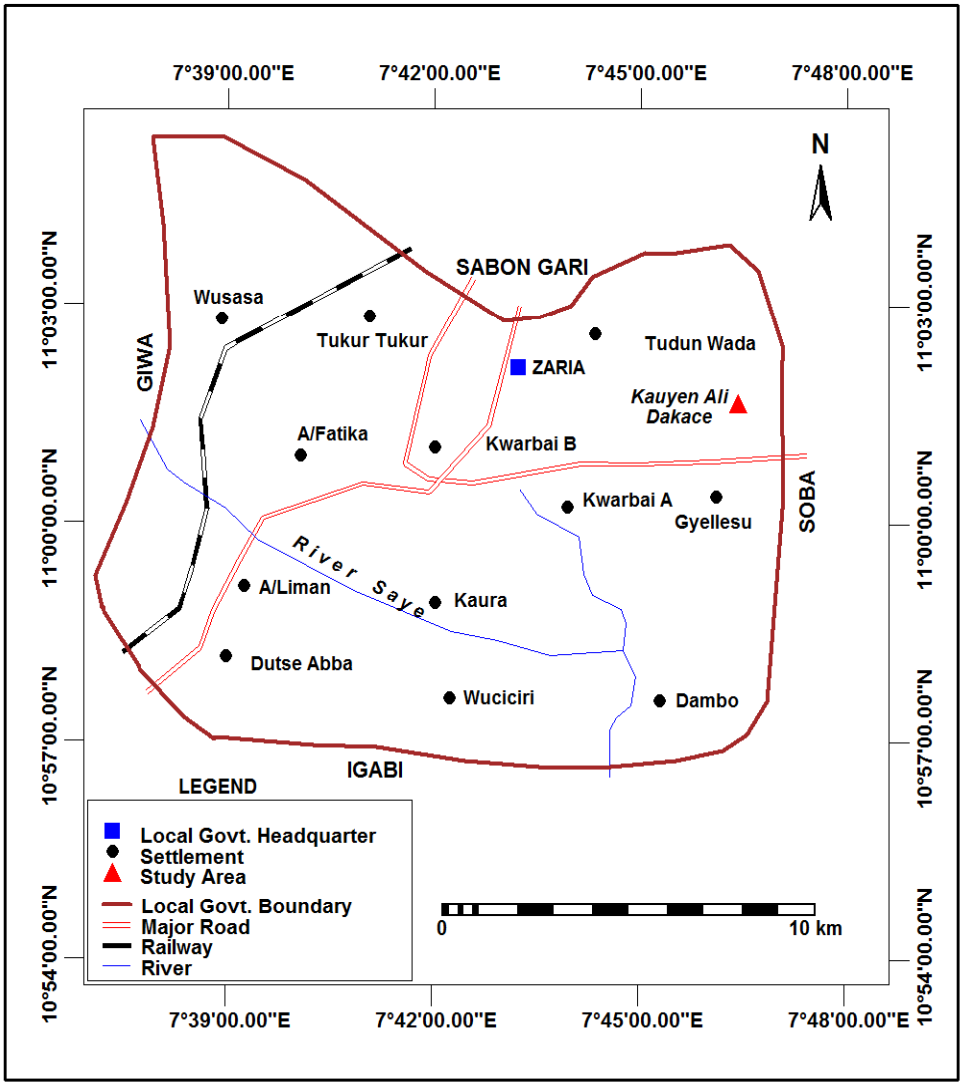
Tests of Between-Subjects Effects

Dependent Variable:Teperature

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Group	Hypothesis	29.466	3	9.822	2.769	.078
	Error	53.206	15	3.547		
Combination	Hypothesis	6563.451	5	1312.690	370.080	.0001
	Error	53.206	15	3.547		

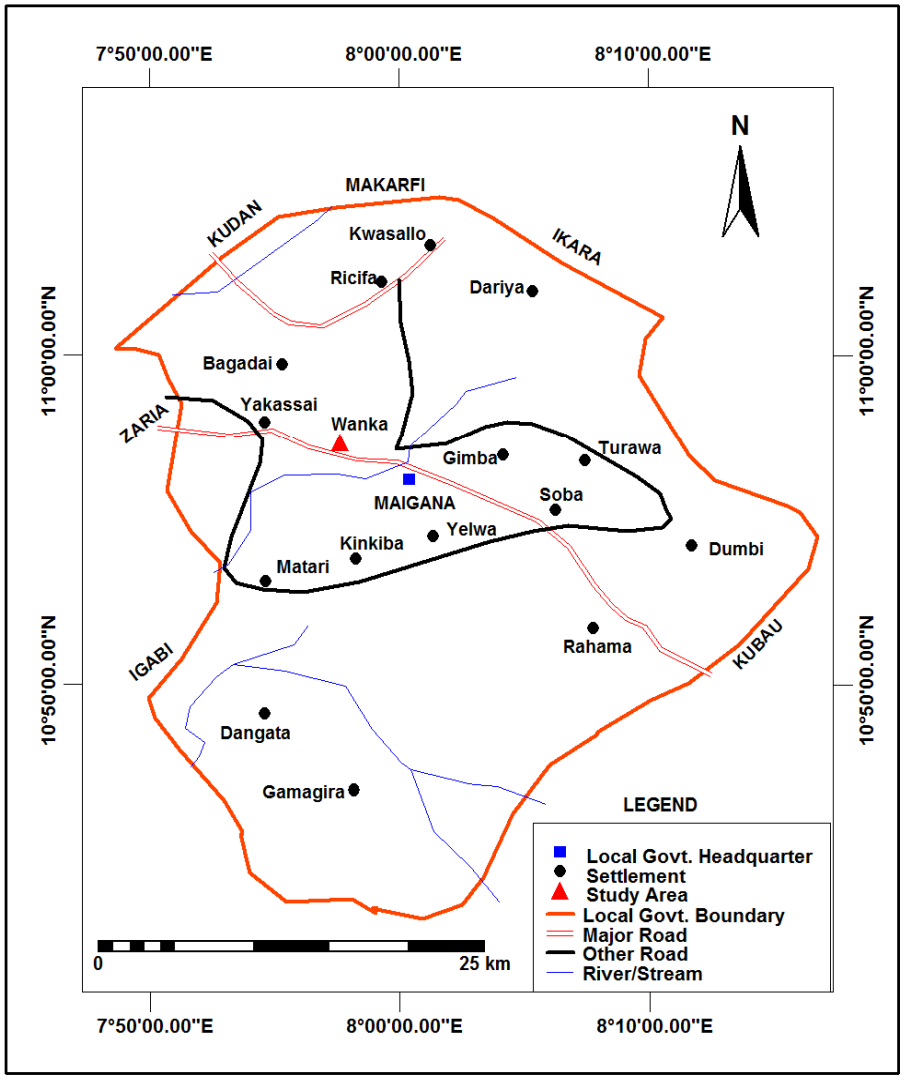
At 95% confident limit, P value is less than 0.05 for the different antifungal combinations, therefore there was a significant difference between the fungicidal activities of different test antifungal combinations. However at 95% confident limit, the variation in the antifungal activity of the test combined agents were not significant at the temperatures range examined.

Appendix X : Maps of The Study



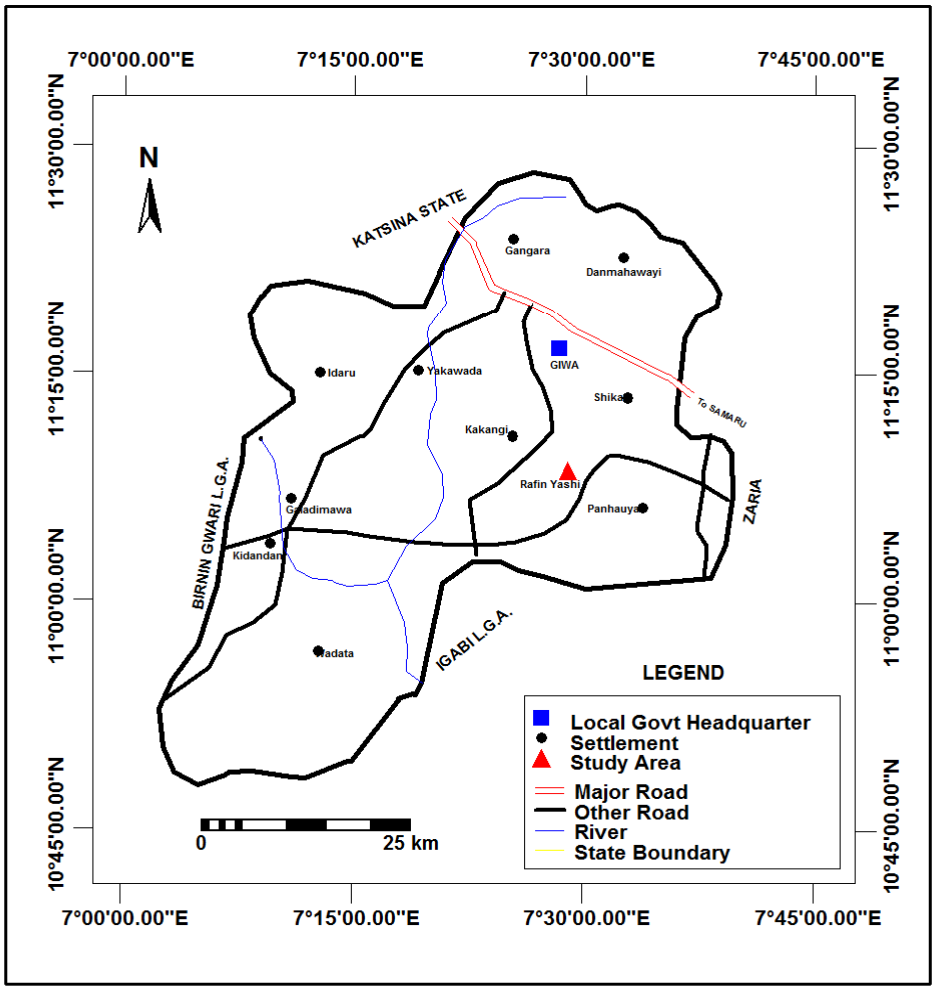
Map of Zaria Local Govt. Area Showing The Study Area

Source : Adapted and modified from Administative map of Kaduna State



Map of Soba Local Govt. Area Showing The Study Area

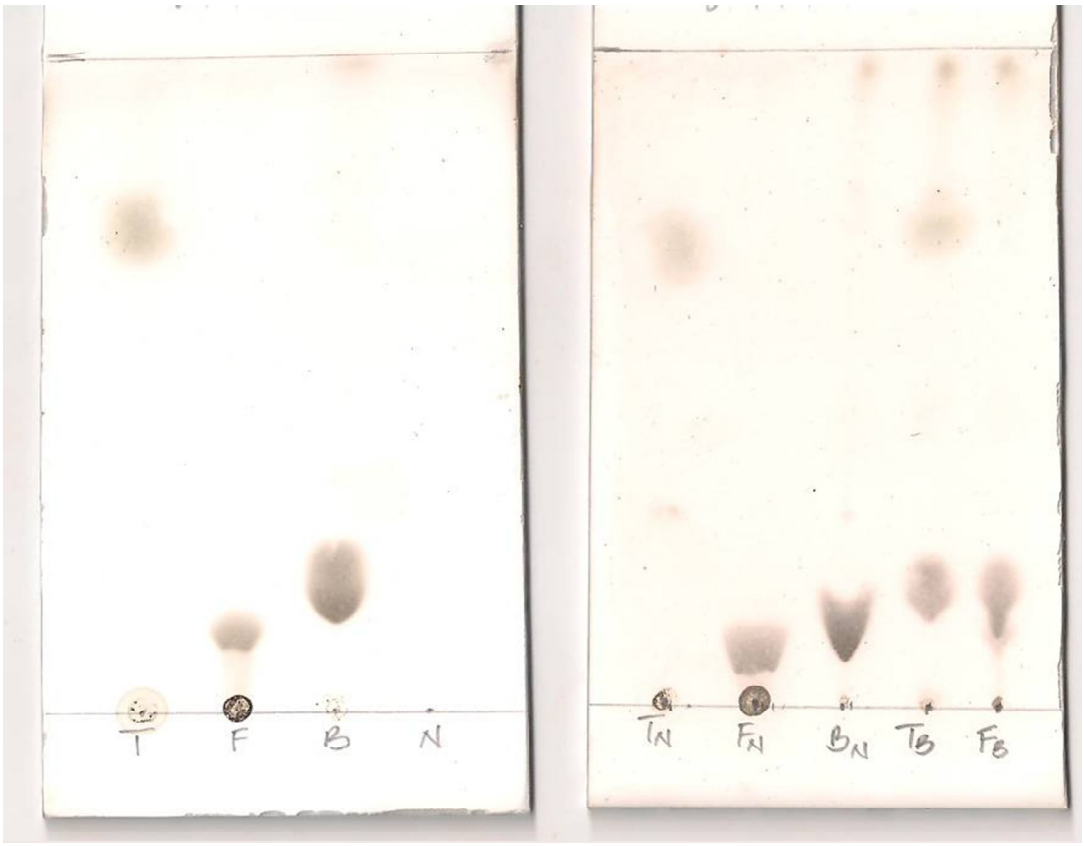
Source :Adapted and modified from Administative Map of Kaduna State



Map of Giwa Local Govt. Area Showing The Study Area

Source : Adapted and modified from Administrative Map of Kaduna State

Appendix XI: A Scan of Thin Layer Chromatograpy of R_f



Rf = x/y where
Y = Sf = distance moved by the solvent system or mobile phase on stationary phase
X = Distance moved by individual compound on the stationary phase
And Rf is always less than one
T = Terbinafin HCl
F = Fluconazole
B = Benomyl
N = Sodium propionate

Appendix XII: Written Consent from the Farms Owners

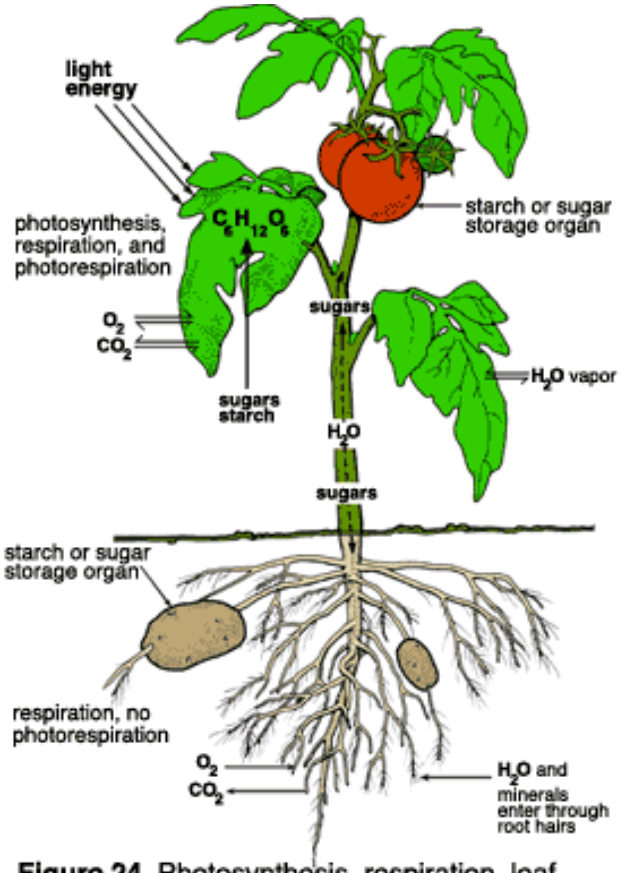


Figure 24. Photosynthesis, respiration, leaf water exchange, and translocation of sugar (photosynthate) in a plant.