CHARACTERIZATION OF ALLERGENIC FUNGAL SPORES FROM SELECTED LOCATIONS IN LAGOS AND IBADAN, NIGERIA

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DEDICATION

This work is dedicated to God Almighty, my ever present help in times of need who answer prayers and also to my wonderful parents Reverend Professor and Mrs A.C Odebode.

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ABSTRACT

Airborne fungal spores (Air mycoflora) are ubiquitous both indoors and outdoors due to their sizes, predominance and aerodynamic properties which enhance their distribution. Fungal spores are associated with dysfunction of multiple system and organs such as respiratory, nervous, immune, haematological and skin. Hence, this study investigated a two-year comparative survey of airborne fungal spores in parts of Lagos and Ibadan, Nigeria. Sedimentation plate technique using Dichloran Glycerol-18 (DG-18) and Potato Dextrose Agar (PDA) (culture dependent) was employed for fungi collection. Fungi samples were subjected to polymerase chain reaction while the most abundant were screened by quantitative PCR analysis. Histology, total protein content, blood parameters and biochemical profile of oxidation stress markers on fungi inoculated balb/c mice were determined to compare the immune response and effects of different fungi dosage inoculated intranasally. Data obtained were analysed (ANOVA) using SAS (version 9.1). Means were separated using the Duncan Multiple Range Test at $p \leq 0.05$. The results revealed species diversity in all the sampling locations. A total of 39 fungal species and strains were isolated from all locations sampled with *Aspergillus* and *Penicillium* identified as the most abundant and frequent fungal species in all the surveyed environments while *Absidia*, *Curvularia* and *Mucor* had very low values of spore count in all locations. It was observed that spore count in the dry season was significantly less than that of the wet season, with DG- 18 agar having higher collection (53.8%) than Potato dextrose agar (46.2%). Lagos state also gave more pooled abundant fungi collection than Ibadan. Molecular characterization result of the isolated fungi revealed that no DNA sequence data was 100% homologous with those in the Gene bank. The mean relative gene expression values ranged from 18.95 – 31.28 for Actin, 17.38 – 26.77 for β tubulin, 19.74 – 30.63 for *P. oxalicum* and 30.22 – 37.56 for *P. citrinum* allergenic genes. All genes were significantly correlated to the Bestkeeper index ($p < 0.001$). Histopathology result for all inoculated organisms on mice lung appear to be similar although with varying degrees of severity. Almost all of them had intra-lesional unstained fungal hyphae. Pathologies include thickening of alveolar sepatae, which causes impairment in vascular exchange and/or respiratory movements. Hyperplasia of the bronchiolar epithelium was observed which was caused by the fungal hyphae. The contribution of principal component analysis (PCA) showed variations in the Eigen proportion of fungi inoculum at different concentrations on extent of infection recorded on the lungs of balb/c albino mice inoculated with different fungi treatments. The first principal component analysis accounted for the highest variation with the highest Eigen value for *A. flavus* at proportion of 38.13%. The fifth component showed positive and more relatedness for control treatment and *A. penicilloides*. *P. citrinum*, *P. chrysogenum*, *A. flavus* and *A. penicilloides* produced significant ($p<0.05$) effect on Glutathione, Superoxide dismutase, Nitric oxide, Hydrogen peroxide and Protein. Fungal treatment is positive and highly significantly ($p<0.01$) correlated with MPO and Hydrogen peroxide with $r=0.66$ and 0.38. Blood parameters showed that eosinophils, basophils, monocytes, neutrophils and leucocytes are significantly different from one another for each inoculated organism. Statistical analysis show there was negative but significant relationship between relative humidity and temperature and also a negative relationship exist between temperature and fungi count. Positive and significant correlation occur between asthma and wheezing cough situations.

KEYWORDS: Air-mycoflora, Allergenic, Asthma, Characterization.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Fungi are eukaryotic unicellular or multicellular organisms that lack chlorophyll and are dependent upon external food sources therefore live as saprophytes, parasites or symbionts of animals and plants under nearly all environmental conditions. Fungi grow best in moist habitats because they require water to live. Optimum pH for fungal growth is 5.6, but many of them can tolerate values in the range 2-9 (Bogacka, 2008). Low sensitivity to changes in osmotic pressure causes the ability of fungi to grow in solutions with high salt or sugar. Fungi are ubiquitous in all environments and play a vital role in the Earth's ecology by decomposing organic matter. Fungi, due to their requirements of life, occupy the microenvironment in which people live and they have an influence on quality of life (Lipiec, 2002). Familiar fungi include yeasts, rusts, smuts, mushrooms, puffballs and bracket fungi. Many species of fungi live as commensal organisms in or on the surface of the human body. Out of over 100,000 fungal species reported, a few hundred occur as opportunists and about 100 are known to elicit mycoses in man and animals (De Hoog *et al*., 2000). For decades, fungal spores and mycelial cells have been known to be a major health risk. In contrast to airborne pollen, fungal spores are not only primarily associated with IgE-mediated type I allergies but also with a broad panel of other diseases, e.g. life-threatening primary and secondary infections in immunocompromised patients. Also fungi have been described to cause allergic bronchopulmonary mycosis (ABPM), hypersensitivity pneumonitis, fungal sinusitis and toxic pneumonia. In contrast to other allergenic sources, fungi are very common in the environment and exposure to airborne spores is almost constant throughout the year. A major difference to other sources, e.g. house dust mite or pollen is that fungi may colonize the human body and they may damage airways by the production of toxins, proteases,

enzymes (Kurup, 2003) and volatile organic compounds (Kauffman *et al*., 2000). Thus, fungi have a far greater impact on the patients' immune system than pollen or other allergenic sources. Symptoms of the respiratory system of an organism caused by the response to the fungi allergens are much stronger than to other allergens commonly encountered in the environment. The reason for this is probably that fungi have the ability to reproduce and infect the skin and are able to colonize the respiratory system (Papuas *et al*., 2000). Moreover, they can synthesize secondary metabolites such as toxins, hypo-allergenic enzymes and volatile organic compounds and functional non-protein components of the cell wall (glucans and chitin) (Fogelmark *et al*., 2001). Another aspect are the cross autoimmune reactions caused by high homology to fungal antigens of some human proteins which occur especially in patients with chronic asthma (Beijer *et al*., 2003).

1.1.1 Biology of Fungi

Fungi are eukaryotic, filamentous and mostly sporebearing organisms representing a separate entity within living organisms. In general, a sexual generation is followed by an asexual generation during a life cycle. Each of these generations may propagate independently, exhibiting different morphologies (pleomorphism). The broad majority of allergy-causing fungi belong to the divisions of ascomycotina or basidiomycotina. Ascomycotina produce their ascospores in the course of sexual reproduction in the ascus, whereas basidiomycotina produce their meiospores or basidiospores respectively on the basidium. About 30,000 species of ascomycotina and 25,000 species of basidiomycotina have been described. The size of fungal spores ranges from 2–3µm (*Cladosporium, Aspergillus* and *Penicillium)* up to 160 µm (*Helminthosporium*). The average size lies between 2 and 10 µm but spores of 500 µm *(Alternaria longissima)* have also been found (Bush *et al*. 2006).

Although optimal growth conditions vary among fungi, their optimal growth temperature ranges from 18 to 32°C. For growth, they require oxygen, water and a carbohydrate source.

Fungi occur in outdoor and indoor environments and they grow on virtually any substrate, including glass and plastic surfaces. Atmospheric fungal spore concentration exceeds mean pollen concentration 100–1,000 times (Black *et al*., 2000). Spore concentration in the air varies substantially depending on climatic factors such as temperature, wind and moisture. Indoor fungi are a mixture of those growing indoors and those that have entered from outdoors (Kauffman *et al*., 2000). Their incidence is influenced by humidity, ventilation, the content of biologically degradable materials and the presence of pets, plants and carpets. In general, indoor spore concentration is less than half of the outdoor count (unless there is indoor fungi growth) varying from 100 to 1,000 spores/m^3 (Dales *et al*., 2003) while the outdoor spore concentration ranges from 230 to 10^6 spores/m^3 (Denning *et al*., 2006).

1.2 Human Health and Fungal Spores

The term allergy was coined in 1906 by von Pirquet to describe an altered reactivity in living beings (i.e., an IgE mediated hypersensitivity) caused by a foreign substance (Horner *et al*., 2000). Allergens, therefore, are the subset of antigens that stimulate an IgE-mediated response. Genetic factors are known to influence the ability to mount an IgE-mediated reaction and those individuals with sustained elevated IgE levels are referred to as atopic. Type I allergic disease to fungal spores and pollen allergens is typically manifested either as rhinitis (hay fever), conjunctivitis, dermatitis, asthma etc. Allergic reactions, including respiratory allergy, may occur in two phases. The early-phase reaction occurs within minutes as a result of the release of preformed mediators. Late-phase responses occur 3 to 4 h after allergen exposure as a result of cellular infiltrates responding to early-phase mediators. A dual reaction involves both early- and late-phase reactions. Emerging evidence indicates that a significant, persistent inflammatory component in addition to IgE-triggered effects underlies the etiology of asthma. Fungi spores and pollen allergen belong to type one (1) hypersensitivities, their protein are immunomodulatory substances which play crucial roles in

the sensitization and/or exacerbation of allergies such as seasonal rhinitis, conjunctivitis, asthma, bronchial constriction and obstruction, pollinosis and atopic dermatitis etc. (D'Amato *et al*., 2002, Kamijo *et al.*, 2013). On an immunological response to allergen, the immune system makes immunoglobulin E antibodies which attach to immune cells, these undergo degranulation and trigger the cells to release histamine and other inflammatory chemical mediators such as cytokines, interleukins and prostaglandins. These chemical mediators elicit symptoms of allergy including wheezing, runny nose, itching, rashes, sneezing etc. These symptoms range from mild to life threatening symptoms and could either be localized or systemic (Singh and Kumar, 2004).

Allergic reactions from fungi spores normally occur at the site of allergen deposition. Most inhaled particles greater than 10µm are deposited in the nasopharynx and are associated with nasal and or ocular symptoms known as hayfever while particles less than 10µm especially those of 5µm can penetrate the lower airways. Fungi spores differ in sizes and are associated with both upper and lower respiratory systems (Horner and Levetin, 2004). Additionally, there is now evidence that secondary dispersal of allergens, i.e., on other smaller particles, possibly spore fragments, may serve as a vehicle for allergens. This would permit the deposition of allergens even from large spores into the lower airways (Horner and Levetin, 2004). Fungi spores allergy are more difficult to diagnose and treat than other allergies because they are far more numerous and antigenically variable than other allergies and are exceedingly difficult to avoid (Garijo, 1996).

1.2.1 Fungal Type I Allergy

Type I allergy is induced by a large number of fungal genera. The majority of them are members of the Ascomycotina or the basidiomycotina. The most important allergy- causing fungal genera belonging to the Ascomycotina are *Alternaria*, *Aspergillus*, *Bipolaris*, *Candida*, *Cladosporium*, *Epicoccum* and *Phoma*, whereas *Calvatia*, *Coprinus*, *Ganoderma*, *Pleurotus* and *Psilocybe* are the most prominent genera of the basidiomycotina.

1.2.2 Clinical Manifestations of Fungal Type I Allergy

1.2.3 Allergic Rhinitis

Allergic rhinitis is characterized by sneezing, rhinorrhea and nasal obstructions. It is induced by a large number of fungal species with *Alternaria*, *Aspergillus*, *Bipolaris*, *Cladosporium*, *Curvularia* and *Penicillium* being the most prominent.

1.2.4 Allergic Asthma

Comparing the size of pollen grains and fungal spores, it is obvious that fungal spores are generally smaller therefore, they may reach the alveolar surface of the lung inducing chronic inflammation of the lung tissue (Denning *et al*., 2006). In children, fungal allergy was shown to be associated with increased bronchial reactivity (Zureik *et al*., 2002), whereas in adults severe asthma, intensive care unit admission and even death was observed (Black *et al*., 2000). In a Canadian study dealing with 'thunderstorm asthma'. High spore fungi counts in the course of thunderstorms were strongly correlated with asthma exacerbations (O'Driscoll *et al*., 2005). Additionally, a strong association between fungal sensitivity, exposure to fungal spores and life-threatening asthmatic episodes was described by Burge (2002).

1.2.5 Atopic Dermatitis (AD)

AD is a chronic inflammatory disease of the skin that is associated with high levels of total and allergen-specific IgE (Leung, 2000). Recently, *Malassezia furfur* has been implicated in the pathogenesis of AD whereas 40–65% of AD patients either have a positive skin test, atopy patch test or radioallergosorbent test (RAST) with *M. furfur* extract (Mari *et al*., 2003). Sensitization to *Malassezia* allergens may be favored by impaired epidermal barriers, increased T-cell reactivity and distinctive features of antigen-presenting cells (Black *et al*., 2000). *Saccharomyces cerevisiae* is another yeast species showing a significant correlation between a positive skin prick test (SPT) and AD (Mari *et al*., 2003)

1.2.6 Fungal Type II, III and IV Allergies

The immunological mechanisms underlying fungi allergies are hypersensitivity reactions of types I, II, III and IV. The spectrum of allergic symptoms caused by these hypersensitivity reactions is very broad, including rhinitis, asthma, atopic dermatitis (AD) and allergic bronchopulmonary mycosis (ABPM).

1.2.7 Clinical Manifestations of Fungal Type II, III and IV Allergies

1.2.7.1 Allergic Bronchopulmonary Mycoses

ABPM is caused most frequently by *Aspergillus fumigatus* which may grow in the bronchial lumen leading to a persistent bronchial inflammation inducing bronchiectasis in asthmatic patients. Seven to 22 % of asthmatic patients suffer from allergic bronchopulmonary aspergillosis (ABPA). Besides *A. fumigatus,* ABPM is also induced by *Candida albicans, Curvularia*, *Geotrichum* and *Helminthosporium* (Kurup, 2003).

1.2.7.2 Allergic Sinusitis

Fungi (e.g. *Aspergillus, Curvularia*, *Alternaria* and *Bipolaris*) may cause allergic sinusitis and fungal ball production in the patients' sinuses (Denning *et al.,* 2006). In the patients

mucus, fungal hyphae are detectable. Additionally, patients may show a cutaneous hypersensitivity to specific allergens along with specific IgE and IgG antibodies and an elevated total IgE level (Kurup, 2003). Immunologically, allergic sinusitis is a type I, III and IV-mediated allergic reaction.

1.2.7.3 Hypersensitivity Pneumonitis

Hypersensitivity pneumonitis (also known as extrinsic allergic alveolitis) is based on type III/IV allergic reactions to repeated inhalation of allergens and may lead to a chronic disease with irreversible lung damage. The following fungi have been associated with hypersensitivity pneumonitis: *Aspergillus* and *Penicillium* species and the basidiomycetes *Lentinus edodes*, *Merulius lacrymans* and *Pleurotus ostreatus* (Bhatnagar *et al*., 2002).

1.2.8 Fungal Allergens

Allergens from the Ascomycotina

1.2.8.1 *Alternaria alternata*

Among fungi associated with allergic disorders, *A. alternata* is one of the most frequently encountered species predominantly occurring in the outdoor environment. Most of the *A. alternata* allergens cloned so far are minor allergens except for Alt a 1 which is recognized by up to 98 % of *A. alternata* -sensitized patients (Asturias *et al*. 2005). Alt a 1 can be found as a predominant component in mycelial and culture filtrate extracts (Simon-Nobbe *et al*. 2000). Alt a 1, the major allergen of *A. alternata*, was analyzed in respect to its B-cell epitopes.

1.2.8.2 *Cladosporium herbarum*

Airborne spores of *C. herbarum* are prominent causes of fungal allergy and can be found indoors as well as outdoors. In a study by Tariq *et al*. (1996), they found out that 2.9 % of 981 4-year-old children reacted to *C. herbarum*. In their study, *C. herbarum* together with *A.*

alternata were the third most common causes of sensitization after house dust mite and grass pollen. Monosensitization to *C. herbarum* is rather seldom within fungi-allergic patients. So far, 14 allergens have been identified from *C. herbarum* whereas seven of them have been cloned as recombinant proteins. All of these allergens are minor allergens with a prevalence of about 20 %. The only major allergen, Cla h 8, an NADPdependent mannitol dehydrogenase is recognized by 57 % of the *C. herbarum* -allergic patients and represents a predominant component of the crude extract (Breitenbach and Simon-Nobbe, 2002).

1.2.8.3 *Aspergillus Species*

The saprophytic genus *Aspergillus* includes over 500 different species. It is distributed ubiquitously in our natural environment and represents a dominant indoor pathogen (Kurup and Shen, 2000). *Aspergillus* grows outdoors on decaying vegetation or indoors (e.g. in air conditioning systems) and has the ability to release large quantities of small conidiospores of 2–3 µm. In case of inhalation, they either reach terminal airways or are deposited in large clusters in the upper respiratory tract (Kurup ad Banerjee, 2000; Vijay and Kurup, 2004). Human disorders caused by *Aspergillus* range from colonization of the respiratory tract, hypersensitivity pneumonitis (extrinsic allergic alveolitis), allergic rhinitis, sinusitis and asthma to life-threatening systemic invasive aspergillosis and Aspergillus BronchioPulmonary Aspergilliosis (Terr, 2004). Very often aspergillosis is favored by an impaired immune status of the patient either caused by immunosuppressive treatment after transplantation surgery, HIV infection or being under intensive care. The biological characteristics of *Aspergillus* are its small spore size, its thermo-tolerance allowing growth at human body temperature, its resistance to oxidative killing and its ability to produce small metabolites and enzymes with proteolytic or even immunosuppressive activity (Banerjee *et al*. 1997).

1.2.8.4 *Penicillium Species*

More than 500 *Penicillium* species exist. *Penicillium* can cause atopic asthma in sensitive persons after inhalation of their spores (Shen *et al*., 2003). In a study conducted inTaiwan, 22 % of the asthmatic children showed a positive reaction in intracutaneous skin tests for *Penicillium* species (Wei *et al*., 2003). Shen *et al*. (1999) showed that IgE antibodies against components of *P. citrinum, P. notatum, P. oxalicum* and *P. brevicompactum* could be detected in the sera of 16–24% of asthmatic patients. In 100 patients, *P. chrysogenum* had the highest positive intradermal skin test reactivity (68 %). Therefore, *P. chrysogenum* is the most frequent *Penicillium* species used for the clinical diagnosis of fungal allergy.

1.2.8.5 *Candida albicans*

Although six *C. albicans* allergens have been described so far, it is unclear whether the inhalation of this fungus is causative for its allergenicity (Martinez *et al*., 1998).

1.2.9 Allergens from the Basidiomycotina

Among fungi, the basidiomycotina are a very large phylum comprising approximately 20,000 species including puffballs, bracket fungi, toad stools, jelly fungi, plant rusts, smuts and mushrooms like the edible *Boletus*, *Cantharellus* and *Coprinus*. Of the large number of basidiomycete species, about 25 species have been shown to be allergenic (Tang *et al*., 2005). Horner *et al*. (1995) observed that basidiospores contribute most of all to the airborne fungal spore load ranging from 5 to 30%. They particularly occur outdoors, but can also be found indoors, e.g. on wet decaying wood or as infiltrates from outdoors. In temperate zones, seasonal peaks of basidiospores are observed in spring and autumn (Leventin, 1990). The diameter of basidiospores ranges from 3 to 15 µm enabling them to reach the lower respiratory tract (Luo, 1991). In contrast to ascomycotina, basidiomycotina do not have vegetative spore production. Since not only the spores but also the fruiting bodies of

Ganoderma, *Coprinus* and *Pleurotus* contain allergens, they may induce food allergy in sensitized patients upon consumption of these mushrooms (Roncarolo *et al*., 1998). Hence, basidiomycotina as well as Ascomycotina are known to cause atopic asthma in susceptible persons (Epton *et al*., 1997). According to Brander *et al*. (1999), the incidence of basidiomycota-caused allergy ranges from 3.5 to 25.4 %.

1.2.9.1 *Malassezia furfur*

M. furfur previously also known as *Pityrosporum ovale* or *Pityrosporum orbiculare*, is a member of the normal cutaneous mycoflora preferentially colonizing the skin of the head-neck-face region as single-cell yeast and mostly being non-pathogenic. Nevertheless, this yeast can act as a pathogen causing pityriasis versicolor and seborrheic dermatitis (Kieffer *et al*., 1990). IgE reactivity to *M. furfur*, as shown in skin tests and radioallergosorbent tests, has frequently been observed in patients with Atopic dermatitis (Lintu *et al*., 1997). *M. furfur* contains several IgE reactive proteins ranging from 14 to 94 kDa (Johansson and Karlstrom, 1991).

1.2.9.2 *Psilocybe cubensis*

Skin test reactivity to *P. cubensis* spore extract is the highest (13.7 %) among basidiomycetes in Europe and the USA (Lehrer *et al*., 1994). More than ten allergens have been identified by SDS-PAGE immunoblots. Psi c 2 as reported by Horner *et al*. (1995) is the first recombinant basidiomycete allergen (molecular weight: 16 kDa) shows high homology to cyclophilins and is recognized by 82 %, representing a major allergen.

1.2.9.3 *Rhodotorula mucilaginosa*

Rhodotorula mucilaginosa also known as *R. rubr*, is one of the most frequently encountered yeast species in the environment. Chang *et al*. (2002) reported the isolation of an enolase (Rho m 1) which shows high sequence identity with other fungal IgE-reactive enolases. Rho

m 1 is recognized by 21.4 % of *R. mucilaginosa* -sensitized patients and cross-reacts with several fungal enolases. Rho m 2, a vacuolar serine protease is the second cloned allergen which also cross- reacts with other fungal vacuolar serine proteases (Chou *et al*., 2005).

Fungi may adversely affect human health through three processes: 1) allergy; 2) infection; and 3) toxicity. Furthermore, outdoor fungi are generally more abundant and important in airway allergic disease than indoor fungi, leaving the latter with an important but minor overall role in allergic airway disease. Most fungi generally are not pathogenic to healthy humans but quite a number of fungi commonly cause superficial infections involving the feet (*Tinea pedis*), groin (*Tinea cruris*), dry body skin (*Tinea corporus*), or nails (*Tinea onchomycosis*). A very limited number of pathogenic fungi, such as *Blastomyces*, *Coccidioides*, *Cryptococcus*, and *Histoplasma*, infect nonimmunocompromised individuals. Individuals with severely impaired immune function, for example, cancer patients receiving chemotherapy, organ transplant patients receiving immunosuppressive drugs, AIDS patients, and patients with uncontrolled diabetes are at significant risk for more severe opportunistic fungal infection.

High-throughput sequencing methods suggest that up to 5.1 million fungal species exist inhabiting practically every niche on Earth (O'Brien *et al*., 2005; Ovaskainen *et al*., 2013) They form a significant portion of the Earth's biomass (25 %) and thus exposure to fungi and their products is substantial and ubiquitous. Because of an elaborate collection of human defensive mechanisms, fungi rarely cause infectious diseases in healthy immunocompetent hosts (Sorenson, 1999).

1.3 The Relationship of Fungi to Allergy and Asthma

It is estimated that approximately 10 % of the population have IgE antibodies to common inhalant fungi. About half of these individuals (5 % of the population) are predicted to have at some time allergic symptoms as a consequence of exposure to fungal allergens. Although indoor fungal allergen exposure occurs, outdoor exposure is generally more relevant in terms of sensitization and disease expression. Sensitization to fungi particularly *Alternaria alternata*, has been linked to the presence, persistence and severity of asthma. Exposure to atmospheric fungal spores has been related to asthma symptoms. Several report confirm that children living in damp houses, homes with visible fungi growth or both were more likely to experience lower respiratory tract symptoms of cough and wheeze than children who do not (Robbins *et al*., 2004).

1.4 Toxic Effects of Fungi Exposure

1.4.1 Ingestion

Ingestion of mycotoxins in large doses (generally on the order of a milligram or more per kilogram of body weight) from spoiled or contaminated foods can cause severe human illness (Belanger *et al*., 2003) Toxicity from ingested mycotoxins is primarily a concern in animal husbandry, although human outbreaks do occur occasionally when starvation forces subjects to eat severely contaminated food. Specific adverse effects from a given toxin generally occur in a narrower and better-defined dose range than for immunologic or allergic effects that might vary across much broader dose ranges. Some mycotoxins, such as ochratoxins and aflatoxins are commonly found in food stuffs including grain products and wines and peanut products respectively, such that there are governmental regulations as to the amounts of allowable aflatoxin in foods (Monaci and Palmisano, 2004) Acute high-intensity occupational

exposures to mixed bioaerosols have given rise to a clinical picture called ‘‘toxic dust syndrome.’’

1.5 Irritant Effect of Fungi Exposure

An irritant is a material causing ‘‘a reversible inflammatory effect on living tissue by chemical action at the site of contact.’’ Irritant effects are dose related and the effects are transient, disappearing when the exposure has decreased or ceased. Fungi produce a number of potentially irritating substances that can be divided into volatile organic compounds (VOCs) and particulates (eg, spores, hyphae fragments, and their components). The threshold level of irritant response depends on the intrinsic properties of the specific material involved, the level plus length of exposure and the innate sensitivity of the exposed tissues (eg, the skin versus nasal mucosa). VOCs made by fungi (MVOCs) are responsible for their musty odor. MVOCs include a wide range of alcohols, ketones, aldehydes, esters, carboxylic acids, lactones, terpenes, sulfur and nitrogen compounds and aliphatic and aromatic hydrocarbons (Pasanen *et al*. 1998). Although levels causing irritant effects have been established for many VOCs, MVOC levels measured in damp buildings are usually at a level so low (on the order of nanograms to micrograms per cubic meter) that exposure would not be expected to cause complaints of irritation in human subjects (Campbell *et al*., 2004). Because there are other sources of VOCs indoors, measurement of indoor airborne concentrations of MVOCs is rarely done. Fungi particles (spores, hyphal fragments and their structural components) are not volatile. These structural fungi compounds (particulates) have been suggested to cause inflammation through deposition on mucus membranes of their attached glucans and mannans. However, whether such effects occur clinically remains unproved. Therefore, Fungi particulates generally found indoors, even in damp buildings are not likely to be irritating (Bush and Prochnau, 2004). It should be emphasized that irritant effects involve the mucus membranes of the eyes, upper and lower respiratory tracts and are transient so that symptoms

or signs persisting weeks after exposure and those accompanied by neurologic, cognitive, or systemic complaints (e.g chronic fatigue) should not be ascribed to irritant exposure.

1.6 Statement of Problem

The most common form of hypersensitivity to fungi is immediate type one hypersensitivity or IgE-mediated "allergy" to fungal proteins. This reactivity can lead to allergic asthma or allergic rhinitis that is triggered by breathing in fungi spores or hyphal fragments. Residential or office fungal exposures may be a substantial factor in an individual's allergic airway disease depending on the subject's profile of allergic sensitivity and the levels of indoor exposures. Individuals with this type of fungi allergy are "atopic" individuals that is they have allergic asthma, allergic rhinitis, or atopic dermatitis and manifest allergic (IgE) antibodies to a wide range of environmental proteins among which fungi are only one participant. These individuals generally will have allergic reactivity against other important indoor and outdoor allergens such as animal dander, dust mites and weed, tree and grass pollens. Among fungi, the most important indoor allergenic are *Penicillium* and *Aspergillus* species. Airborne fungi are being proposed as a cause of adverse health effects. They may adversely affect human health through infection, toxicities and allergy. Moreover, they have a great influence on urban air quality therefore the detection and characterization of fungi in atmospheric aerosol will help to elucidate the regional and global spread and diversity of fungi. Fungi spores can also be the origin of respiratory problems which necessitated this study.

1.7 Aim and Objectives

The aim of this research work is to isolate, identify and characterize allergenic fungal spores in different locations using culture dependent method.

1.7.1 The specific objectives are to:

1. isolate fungal spores from different locations using culture plate method.
2. measure the density and pattern of occurrence of airborne spores in the study areas.
3. evaluate Immunoglobulin E antibody levels and immune cells response elicited by some selected fungi spores in mice.
4. compare the effects of fungal spores on inoculated mice at different concentrations.
5. to correlate the seasonal variations of atmospheric fungal spores prevalence with patients complaint of respiratory problem in Lagos University Teaching Hospital.

1.8 Significance of Study

Information about the diversity and abundance of airborne fungi is relevant for many areas of research such as climate and ecology, human and veterinary medicine, industrial and environmental hygiene and agriculture. The information on the detection and prevalence of fungal spores as well as their allergenic potential will be used to assist health practitioners develop counter measures on the various adverse health effects of fungal spores therefore helping individuals live healthier and more productive lives by avoiding places of high fungi load. This study will also provide relevant data on seasonal variations of air mycoflora of locations of Lagos and Ibadan, examining the impact of weather and determine allergenic potentials using mice model.

1.9 Operational Definition of Terms

Allergens: Antigens that elicit an IgE-mediated type I allergic reaction are called allergens. They are usually non-harmful substances except in those individuals predisposed to generating an IgE response to them. In general, allergens are low molecular weight proteins or glycoproteins with no distinguishing physical characteristics when compared with other antigens.

Antibody: A protein produced by the body's immune system when it detects harmful substances called antigen.

Eosinophils: The eosinophil leukocyte is produced in the bone marrow from myeloid progenitors. It is a pro-inflammatory cell which can be identified by its bi-lobed nucleus and cytoplasmic granules which stain bright red with eosin.

Histamine: Chemical found in some body cells which causes symptoms of allergy

Intranasal: Within the nose

IgE: producing plasma cells are distributed primarily in the mucosal lymphoid tissues adjacent to the respiratory and gastrointestinal tracts; antibody is synthesised on allergen exposure and subsequently distributed throughout the body where it promptly binds to its high affinity receptor which is densely expressed on the surface of mast cells. IgE is normally present in the blood at a very low concentration.

Mast cells: Mast cells are found in most tissues, commonly close to blood and lymphatic vessels. They are found in especially large numbers subjacent to mucosal surfaces of the respiratory and gastrointestinal tracts and in the dermis of the skin.

1.9.2 Abbreviations/Acronyms

ABPA	Allergic bronchopulmonary aspergillosis
ABPM	Allergic bronchopulmonary mycosis
AR	Allergic rhinitis
AS	Asthma
a_w	water activity
BLAST	Basic Local Alignment Search Tool

cDNA	Complementary Deoxyribonucleic Acid
ddH_20	double distilled water
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide triphosphates
ELISA	Enzyme Linked Immunosorbent Assay.
gDNA	Genomic DNA
IgE	Immunoglobulin E
kDa	kiloDalton
MVOC	microbial volatile organic compound
NOS	nitric oxide synthase
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
ROS	reactive oxygen species
SDS-PAGE	Sodium Dodecyl Sulphate –Polyacrylamide Gel Electrophoresis
SAFS	Severe asthma with fungal sensitivity
SPT	Skin prick test
UPGMA	Unweighted Pair-group Method with Arithmetic averages.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Aerobiology

Detailed information about fungal identity and concentration in the air is meager. Only a small fraction of the different fungal spore types are recognizable. Available literatures on studies carried out in Nigeria which include published works by Agwu and Osibe (1992); Adekunle, (2000); Agwu (2001); Agwu *et al*. (2004), Njokuocha and Osayi 2005; Ezike *et al*., 2017). Fungal spores are cosmopolitan in distribution and constitute a large proportion of the airborne palynomorphs trapped in most aeropalynological studies (Njokuocha and Ukeje, 2006).

Aerobiology studies small particles from biological origin that are airborne. They fly passively due to wind transport. These particles are mainly spores from fungi and pollen grains from plants. Aerobiology studies not only their liberation from their sources, transport and deposition, but also their effects on humans, animals, plants and even over food, building, works of art, etc. Aerobiology is not only concerned about outdoors (extramural environment) but also indoors (intramural ones).

The air we breathe contains inorganic and organic particles airborne transported. Inorganic particles come from fires (combustion particles, fumes), dust or mist originated by a mechanical process. Particles from biological origin include viruses, bacteria, spores and pollen grains. Time that airborne particles spend to settle depends on their size. Smallest airborne particles can be suspended in the air some hours until they settle (landing). They are named: submicronic aerosols and paucimicronic spores. The biggest airborne particles remain in air only a few minutes or seconds. Nevertheless wind can transport these particles long

distances, depending on their speed (Adeonipekun, 2012). The smaller these particles are the further they are transported.

2.1.1 Dispersal of allergens

Air transports allergens mainly as fungi spores and pollen grains. They cause different allergic disorder depending on their size such as rhinitis, asthma, alveolitis because they reach different part in the respiratory system

- Head airways (nasopharyngeal region) - rhinitis
- Lung airways (tracheobronchial region) – asthma: This is mainly caused by fungi spores and not pollen grains because of their small size.
- Alveolar/pulmonary region – alveolitis (Caused mainly by fungi spores)

2.1.2 Indoor air quality

Many fungi can appear indoors (inside homes) when circumstances allow their growth (either wet or warm environment). Apart for the damage they do in the home such as defacing buildings, their spores can be the origin of respiratory problems. They may also transport toxins (mycotoxins) that are breathed in or even deposited over food.

2.1.3 Crop disease epidemiology

Air is the medium fungi use to transport their spores (propagules) to other places. Many fungi are crop pathogens and cause important economic losses, hazards or even famines. One of the most important famines was the Highland Potato Famine, causing over 1.7 million people to leave Scotland during the mid-1840s, similar to the Great Irish Famine. Potato crops were widely spread and they were the main food source. In 1846 potato crops were blighted by fungi; *Phythophthora infestans* that spread its propagules by wind. By knowing the airborne spore concentration and environmental conditions which favours its growth and development, it is possible to forecast their spread and regulate the use of fungicides.

2.1.4 Monitoring climate change

The onset of flowering (when flowers blossom) is directly affected by weather parameters. It is well known that the world is in a process of global climate change, apparently with warmer conditions. It seems that the onset of many plants, including crops is happening earlier than before because the various seasons are warmer. This means that plants seem to flower earlier and pollen is shed in advance. This show the pollen season is beginning earlier now than before (Chakraborty and Gupta-Bhattacharya, 2001).Therefore, aerobiology is a tool to monitor climate change and evaluate its effects over plants. Also because cities constitute a "heat-island", and produce many of the gases (e.g., carbon dioxide, ozone) that are responsible for environmental change at regional and global levels, it has been suggested that cities may provide analogy for studying ecological responses to global change. Data do suggest that the higher carbon dioxide concentrations and increased air temperatures associated with urbanization may be a harbinger of what could be expected with respect to pollen production and allergic rhinitis / asthma with global climate change.

2.1.5 Biodeterioration over works of art

Biodeterioration can be defined as the irreversible loss of value and/or information of an object of art following the attack by living organisms. Historical cultural legacy is often supported over materials that can be damaged by organisms (biodeterioration). This includes organic materials as books, cloth, wood, etc., but even inorganic substrate can be biodeteriorated too. Most of the organisms responsible for biodeterioration are fungi, although algae, bacteria, lichens, mosses and arthropods (insects) can also cause important damage. For example foxing on book is a series of brown dots that appear on many old books, maps, drawings, etc. that have been stored in damp, humid places or storerooms over a long period of time. Foxing is a fungal growth on old paper and books if the relative humidity is greater than 65 % over a long period of time and can ultimately destroy the cellulose fiber

in the paper if left untreated. Fungi presence can be detected using aerobiology methods and so their effect reduced or avoided.

Aerobiology can thus provide very useful information for conservation of works of art by being able to identify sources, access flow and areas of major accumulation of airborne microorganisms, including their time variations (daily, seasonal).

2.1.6 Factors affecting particles caught

To take a representative sample in aerobiology, factors considered must include

- time of the day
- day of the year (season)
- weather (wind, temperature, rain, relative humidity)
- location of a sampling site
- duration of sampling (length of time)

2.1.7 Size of fungi spores and its effects on health

There is a strong relationship between fungi spore diameter and the illness they can provoke in respiratory system. The biggest spores include that of *Alternaria*, *Fusarium*, *Curvularia* which affects mainly the upper parts. Medium size spores include *Cladosporium*, *Memnoniella*, *Stachybotrys* and mostly affect the middle respiratory part while the smallest spores *Aspergillus, Penicillium* and *Cladosporium* are the most dangerous because they can reach the alveoli and thus cause great damage.

Only a few fungi make do without spores, surviving solely by means of mycelium and sclerotia. Spores may be organs of sexual or asexual reproduction, and they are involved in dispersal and survival. Some spores are violently discharged from the organs which bear them, energy for dispersal being provided by the spore itself or the structure producing it (Ingold, 1971). However, many spores are dispersed passively by the action of gravity, air or

water currents, rain splash or by animals especially insects. Dispersal may also occur by human traffic. Spores may be present in the outdoor air at such high concentrations that they can cause allergic respiratory diseases when inhaled (Okten, 2005). The morphology and structure of fungal spores show great variability, from unicellular to multicellular, branched or unbranched or sometimes spirally coiled, thin- or thick-walled with hyaline or pigmented walls, dry or sticky, smooth or ornamented by mucilaginous extensions, spines, folds or reticulations.

2.1.7.1 Morphology of Spores:

Single-celled spores are termed amerospores, two-celled spores are called didymospores, and spores with more than one transverse septum are termed phragmospores while spores with transverse and longitudinal septa are called dictyospores. Special terms have also been used to refer to spore shape e.g Scolecospores are worm-shaped, helicospores are spores with a two- or three-dimensional spiral shape, while staurospores have arms radiating from a central point or axis (Levetin and Van de Water, 2001).

2.1.8 Types of Fungal Spores

A. **Zoospores:** These are spores which are self-propelled by means of flagella. Propulsion is often coupled with chemotactic movement, zoospores having the ability to sense chemicals diffusing from suitable substrata and to move towards them. In some cases oxygen or light are also stimuli for tactic movement. The fungal groups which possess flagella are mostly aquatic or if terrestrial rely on water for dispersal or infection. Zoospores with the two different kinds of flagellum are heterokont.

B. **Sporangiospores:** In the Zygomycotina, and especially in the Mucorales, the asexual spores are contained in globose sporangia or cylindrical merosporangia. Because they are non-motile, the spores are sometimes termed aplanospores. The spores may be

uni- or multinucleate and are unicellular. They generally have thin, smooth walls and are almost always globose or ellipsoid in shape. They are formed by cleavage of the sporangial cytoplasm. They vary in colour from hyaline (colourless) to yellow, due to carotenoid pigments in the cytoplasm. When mature, they may be surrounded by mucilage, in which case they are usually dispersed by rain splash or insects, or they may be dry and dispersed by wind currents. In some genera, e.g. *Pilobolus*, entire sporangia become detached. The number of sporangiospores per sporangium may vary from several thousand to only one.

C. Ascospores: Ascospores are the characteristic spores of the largest group of fungi, the ascomycetes. They are meiospores and are formed in the developing ascus as a result of nuclear fusion immediately followed by meiosis. The four haploid daughter nuclei then divide mitotically to give eight haploid nuclei around which the ascospores are cut out. Ascospores vary greatly in size, shape and colour. The shape of ascospores varies from globose to oval, elliptical, lemon-shaped, sausage-shaped, cylindrical, or needle-shaped. Ascospores are often asymmetric in form with a wider, anterior part and a narrower posterior. This shape increases their acceleration as they are squeezed out through the opening of the ascus. Ascospores may be uninucleate or multinucleate, unicellular or multicellular, divided up by transverse or by transverse and longitudinal septa. The ascospore wall may be thin or thick, hyaline or coloured, smooth or rough. Because the formation of ascospores involves meiosis, they are important not only as a means of dispersal and survival but also in genetic recombination.

D. Basidiospore: Basidiospores are the sexual spores which characterize a large group of fungi, the basidiomycetes. In comparison with the morphological diversity of ascospores, basidiospores are more uniform. They also show a smaller size range,

from about 3 to 20 µm, which is possibly related to their unique method of discharge. They are normally found in groups of four attached by tapering sterigmata to the cell which bears them, the basidium. At the time of their discharge all basidiospores, are unicellular, but they may become septate after release in some members of the Heterobasidiomycetes. In shape, basidiospores are asymmetric and vary from sub-globose, sausage-shaped, fusoid to almondshaped (i.e. flattened) and the wall may be smooth or ornamented with spines, ridges or folds. The colour of basidiospores is important for identification. They may be colourless, white, cream, yellowish, brown, pink, purple or black. The spore colour may be due to pigments in the spore cytoplasm or in the spore wall. The appearance of pigments in the wall occurs relatively late in spore development. Most basidiospores have a flatter adaxial face and a more curved abaxial face. The point of attachment of the spore to the sterigma is the hilum, which persists as a scar at the base of a discharged spore. Close to the hilum is a small projection, the hilar appendix. This is involved in the unique mechanism of basidiospore discharge. Violently projected spores are termed ballistospores. A histochemical feature of the walls of some basidiospores is that they are amyloid, i.e. they include starch-like material which stains bluish-purple with iodinecontaining stains.

E. **Zygospore:** Zygospores are sexually produced resting structures formed as a result of plasmogamy between gametangia which are usually equal in size. Nuclear fusion may occur early or may be delayed until shortly before meiosis and zygospore germination. They are often large, thick-walled, warty structures with abundant lipid reserves and are unsuitable for longdistance dispersal, usually remaining in the position in which they were formed and awaiting suitable conditions for further development. The gametangia which fuse to form the zygospore may be uninucleate

or multinucleate and correspondingly the zygospore may have one, two or many nuclei within it. Zygospore germination may be by a germ tube or by the formation of a germ sporangium.

F. **Oospore:** An oospore is a sexually produced spore which develops from unequal gametangial copulation or markedly unequal (oogamous) gametic fusion. It is the characteristic sexually produced spore of the Oomycotina. In the Oomycotina, oospore development begins with the formation of one or more oospheres within the larger gametangium, the oogonium. After fertilization, i.e. the receipt of an antheridial nucleus by the oosphere, this lays down a thick wall and becomes the oospore. The number of oospores per oogonium may vary, and this is an important taxonomic criterion. Meiotic nuclear divisions precede oosphere and antheridial maturation in the Oomycota and nuclear fusion follows fertilization, so that the oospore is diploid. The oospore develops a thick outer wall and lays down food reserves, usually in the form of lipids. Oospores are sedentary (memnospores) and are important in survival rather than dispersal. They often require a period of maturation before germination can occur and may remain dormant for long periods (Hood, 2002).

G. **Chlamydospores:** In most group of fungi, the terminal or intercalary segments of the mycelium may become packed with lipid reserves which later develop thick walls within the original hyphal wall. The new walls may be colourless or pigmented and are often hydrophobic. Structures of this type have been termed chlamydospores. They are formed asexually. Generally there is no mechanism for detachment and dispersal of chlamydospores, but they may become separated from each other by the collapse of the hyphae producing them. They are therefore typical memnospores, forming important organs of asexual survival, especially in soil fungi. The Glomales, which are fungal partners in symbiotic mycorrhizal associations with many vascular

plants, reproduce primarily by large, thick-walled chlamydospores. These develop singly or in clusters (sporocarps) on coarse hyphae attached to their host plants.

H. Conidiospores: Commonly known as conidia are asexual reproductive structures. The word is derived from the Greek word 'konidion'. Conidia are found in many different groups of fungi, but especially within Ascomycotina and Basidiomycotina. In many fungi conidia represent a means of rapid spread and colonization from an initial focus of infection. In general, conidia are dispersed passively, but in a few cases discharge is violent. Cells which produce conidia are conidiogenous cells. In most conidia, development is blastic, i.e. there is enlargement of the conidium initial before it is delimited by a septum. Two main kinds of blastic development have been distinguished:

1. Holoblastic: in which both the inner and outer wall layers of the conidiogenous cell contribute to conidium formation e.g of this kind of development is shown by the conidia of *Sclerotinia fructigena*.
2. Enteroblastic: in which only the inner wall layers of the conidiogenous cell are involved in conidium formation. Where the inner wall layer balloons out through a narrow pore or channel in the outer wall layer. Development is described as tretic e.g of enteroblastic tretic development are found in *Helminthosporium velutinum.*

Fungi can cause internal and external infections and also can be allergenic factors in humans and animals. Recently, allergies in humans caused by fungi spores present in the air are a very serious problem. They can manifest in the form of food allergy, contact allergy, allergies to antibiotics etc. Fungi can be found throughout the world. They may live as saprophytes, parasites or symbionts of animals and plants in indoor as well as outdoor environment.

2.2 The Immune System: Innate and Adaptive Immunity

The immune system refers to a collection of cells and proteins that function to protect the skin, respiratory passages, intestinal tract and other areas from foreign antigens, such as microbes (organisms such as bacteria, fungi, and parasites), viruses, cancer cells, and toxins (Boitano *et al.,* 2011). The immune system can be assessed as having two "lines of defense": innate immunity and adaptive immunity. Innate immunity represents the first line of defense to an intruding pathogen. It is an antigen-independent (non-specific) defense mechanism that is used by the host immediately or within hours of encountering an antigen. The innate immune response has no immunologic memory and therefore it is unable to recognize or "memorize" the same pathogen should the body be exposed to it in the future. Adaptive immunity on the other hand is antigen-dependent and antigen-specific and therefore involves a lag time between exposure to the antigen and maximal response. The hallmark of adaptive immunity is the capacity for memory which enables the host to mount a more rapid and efficient immune response upon subsequent exposure to the antigen. Innate and adaptive immunity are not mutually exclusive mechanisms of host defense but rather are complementary with defects in either system resulting in host vulnerability.

2.2.1 Innate immunity

The primary function of innate immunity is the recruitment of immune cells to sites of infection and inflammation through the production of cytokines (small proteins involved in cell-cell communication). Cytokine production leads to the release of antibodies and other proteins and glycoproteins which activate the complement system, a biochemical cascade that functions to identify and opsonize (coat) foreign antigens rendering them susceptible to phagocytosis (process by which cells engulf microbes and remove cell debris). The innate immune response also promotes clearance of dead cells or antibody complexes and removes foreign substances present in organs, tissues, blood and lymph. It can also activate the

adaptive immune response through a process known as antigen presentation. Numerous cells are involved in the innate immune response such as phagocytes (macrophages and neutrophils), dendritic cells, mast cells, basophils, eosinophils, natural killer (NK) cells and lymphocytes (T cells). Phagocytes are sub-divided into two main cell types: neutrophils and macrophages. Both of these cells share a similar function: to engulf (phagocytose) microbes. In addition to their phagocytic properties, neutrophils contain granules that when released assist in the elimination of pathogenic microbes. Unlike neutrophils (which are short-lived cells), macrophages are long-lived cells that not only play a role in phagocytosis but are also involved in antigen presentation to T cells. Macrophages are named according to the tissue in which they reside. For example, macrophages present in the liver are called Kupffer cells while those present in the connective tissue are termed histiocytes (Williams, 2005).

Dendritic cells also phagocytose and function as antigen- presenting cells (APCs) and act as important messengers between innate and adaptive immunity. Mast cells and basophils share many salient features with each other and both are instrumental in the initiation of acute inflammatory responses such as those seen in allergy and asthma. Unlike mast cells which generally reside in the connective tissue surrounding blood vessels, basophils reside in the circulation. Eosinophils are granulocytes that possess phagocytic properties and play an important role in the destruction of parasites that are too large to be phagocytosed. Along with mast cells and basophils they also control mechanisms associated with allergy and asthma. NK cells (also known as large granular lymphocytes [LGLs]) play a major role in the rejection of tumours and the destruction of cells infected by viruses. Destruction of infected cells is achieved through the release of perforins and granzymes from NK-cell granules which induce apoptosis (programmed cell death) (Van Dyken *et al*. 2011).

Innate immunity can be viewed as comprising four types of defensive barriers: anatomic (skin and mucous membrane), physiologic (temperature, low pH and chemical mediators), endocytic and phagocytic and inflammatory.

2.3 Allergies

Allergic diseases are the result of an interaction between the immune system and the interplay between genetics and the environment, primarily at the mucosal surfaces, and include rhinitis, conjunctivitis, sinusitis, asthma, urticaria, atopic and contact dermatitis, and gastrointestinal disorders such as food allergy, but also insect sting hypersensitivity

2.3.1 Fungi Involved in Allergy

The fungal kingdom could contain as many as 1.5 – 3 million species (Seedat *et al*. 2006). Any fungi can be allergenic; however, the most common fungal allergens are those present in high levels either outdoors or in an occupational or residential setting. Most are mesophilic (unable to grow at body temperature, with optimum growth occurring at 18–22 ^{0}C) and thrive in temperate climates. Thermophilic fungi can grow at body temperature, but are unable to grow below 20 ^{0}C, so are not present in the environment and are rarely associated with human infections. Thermotolerant fungi grow in the environment and at body temperature and are thus associated with human disease including AFAD (Allergic fungal airway disease).

Allergy is one form of human disease which affects about 20 % of the population. A number of allergens associated with various forms of allergy have been reported from all over the world. The concentration of allergens in the environment varies depending on various factors including climate, vegetation, and air quality. The outdoor allergens are predominantly constituted by plant pollens and fungal spores. The indoor allergens on the other hand, are represented by allergens from dust mites, cockroaches and pets (Blumenthal and Rosenberg,

1999). Fungal spores also have been reported from the indoor environment. The concentration and prevalence of the indoor allergens vary substantially and are dependent on moisture content, ventilation, and the presence or absence of pets, carpets, and houseplants. Fungi from human environment or growing in human body may cause allergic reactions. They are associated with number of allergic diseases in humans including allergic rhinitis, conjunctivitis, bronchial asthma and allergic broncho pulmonary mycoses resulting from exposure to spores. The most common fungal allergens are *Alternaria, Cladosporium, Asperigillus, Penicillium* and yeasts. Clinically the presenting symptoms associated with allergy are sneezing, nasal discharge, coughing, wheezing and shortness of breath. Reversible pulmonary airway obstruction, angiodema, urticaria, and even anaphylaxis may manifest in the patients (Graveen, 1985).

2.4 Air Pollution

The massive increase in emissions of air pollutants due to economic and industrial growth in the last century has made air quality an environmental problem which is of utmost concern. The enormous world-wide increase in the number of motor vehicles has resulted in a tremendous increase in energy consumption and in air-polluting emissions from cars, in particular those with diesel engines. Several air pollutants are on the list of greenhouse gases involved in global warming. In urban areas with high levels of vehicular traffic, the most abundant air pollutants are respirable particulate matter (PM), nitrogen dioxide (NO_2) and ozone (O_3). Other than NO_2 and precursors of O_3, diesel fuel combustion results in the production of diesel exhaust particles (DEPs), which consist of an elemental carbon core with a large surface area to which hundreds of chemicals and transition metals are attached. The effects of air pollutants on lung function depend on the environmental concentration of the pollutant, the duration of pollutant exposure and the total ventilation of the exposed persons.

It is important to note that an individual's response to pollution exposure depends on the source and components of air pollution, as well as meteorological conditions.

2.5 Asthma Prevalence is influenced by Gender

Abundant epidemiological data available show that asthma incidence, prevalence and severity differs according to gender (Tomita, 2012). Asthma prevalence, severity, exacerbation rate, hospitalizations and mortality are higher among women than men overall; however, asthma related office and emergency room visits and hospitalizations are higher among boys than girls 0 to 14 years of age (Patel, 2014). The reasons for the gender difference are unknown but have been linked to immunological and hormonal factors, and/or to differences in gender-specific responses to environmental or occupational exposures (Becklake and Kauffmann, 1999; Vink, 2010). For example, children living on farms have a lower incidence of asthma (Fuch, 2012), which has been related to modulation of the immune system by early life exposures, differences in physical activity levels or eating habits (Takai and Ikeda, 2011). However gender influences the impact of this exposure, i.e. the cumulative asthma incidence is lower in girls as compared to boys raised on a farm. For instance, Wang (2014) reported that asthma in early childhood is only associated with obesity in young girls, not in young boys in two large cross-sectional series from China and the Netherlands and in two longitudinal cohorts from the United Kingdom and Taiwan. The U.K. study followed children longitudinally until the age of 8 years and found that a higher body mass index (BMI) was associated with increased wheezing in girls but not in boys. Interestingly, the lifetime diagnosis of asthma and current asthma was recently reported to be higher among same-sex partnered men and women. Such increased risk may be mediated by a higher prevalence of obesity among same-sex partnered women and by the higher prevalence of smoking among same-sex partnered men (Blosnich, 2013).

2.6 Climate Change and Allergy

The effects of climate change on respiratory allergy is still unclear and current knowledge is provided by epidemiological and experimental studies on the relationship between asthma and environmental factors, such as meteorological variables, airborne allergens and air pollution. Data about the influence of weather on asthma are poor and inconclusive (Atkinson and Strachan, 2004). Weather affects asthma directly acting on airways or indirectly influencing airborne allergens and pollutant levels. Indoor and outdoor allergen exposure is a well-known aggravating factor for asthmatic patients as reported by GINA (2006) even if its role in asthma development is not fully understood. Pollen grains are also responsible for seasonal exacerbations of allergic asthma and rhinitis and they disperse according to the flowering period of the plant of origin. Knowledge of a plant's geographical distribution and its flowering period (D'Amato, 2007) and possible variations induced by climate change scenarios is of great importance.Studies have shown that air pollution is consistently associated with adverse health effects as reported by Dominici *et al*. (2006) and it has a quantifiable impact on respiratory diseases, on cardiovascular diseases and stroke (Dockery and Stone, 2006).

2.7 Sex Hormones and Asthma

Asthma is more prevalent and severe in young boys (Wijga, 2011), but there is a gender-switch at puberty, which has been related to increase of sex hormones (Leynaert, 2012). The transition from childhood to adulthood is characterized by a higher odds ratio of persistence of wheezing in females (Sears, 2003), and by asthma, improvement in males but asthma worsening in females (Kjellman and Gustafsson, 2000). Likewise, in a series of 1,261 children and adolescent with moderate to severe asthma, enrolled in The Epidemiology and Natural History of Asthma: Outcomes and Treatment Regimens (TENOR) cohort, IgE levels were higher among boys 6 to 17 years old compared to girls, but girls had higher IgE levels

during puberty (12–14 years). A higher IgE level was associated with more symptoms triggered by dust, pollen and animals, and was associated with a lower FEV1/FVC ratio even after adjustment for age, gender and race (Haselkorn, 2010).

2.8 Occupational Asthma

With changes in the global market, particularly with the rise of biotechnology, new occupational hazards have emerged (Peden and Reed, 2010). Approximately, 200 biotic (organisms or particles of viral, prokaryote, or eukaryote origin) and an even greater number of abiotic (physical and chemical) agents have been associated with adverse health outcomes. In certain occupational settings, particularly those engaged in handling purified microbial proteins in baking and manufacturing sectors, workers are at increased risk of becoming sensitized and developing respiratory disease (Lachowsky and Lopez, 2001). Occupational asthma (OA) is the most common respiratory disease reported in the workplace. Occupational asthma has been defined as either irritant induced or immunemediated (Baur, 2005). Immunologically mediated OA accounts for approximately 90 % of cases (Mapp, 2005), but the severity of disease is dependent on the concentration, route, agent of exposure, and the latency period (Lachowsky and Lopez, 2001). Both high- and low-molecular-weight antigens can induce Occupational asthma but the immunological mechanisms are distinctly different. High-molecular-weight allergens are generally proteins that are greater than 5 kDa, and production of immunoglobulin E (IgE) results in the release of mediators from mast cells and eosinophils. More than 250 high-molecular-weight allergens that induce OA have been identified (Vanhanen, 2001). Many are derived from animals or plants, and exposure usually involves mixtures of many proteins (Baur, 2005). Occupations where high-molecular-weight allergens have been characterized include seafood processing (tropomysin), dairy, poultry, citrus, greenhouse, baking, healthcare (latex), pharmaceutical (drugs), and detergent manufacturing (fungal enzymes) (Elms, *et al*., 2003). Some of the best examples of high-molecular-weight occupational allergens are the fungal enzymes. They are particularly suited

for study because they are often used as purified preparations in baking, food, detergent, textile, and pharmaceutical industries. The most widely used enzymes of occupational importance are derived from the genus *Aspergillus* and include *α*-amylase, xylanase, and cellulase. It is uncommon for individuals in the general population to be exposed and sensitized to these antigens. In fact, in the general population, the prevalence of sensitization to fungal enzymes has been reported to be as low as 1 %and as high as 15 % (Biagini *et al*., 2004). However, in the occupational environment, workers that handle purified fungal enzymes are at an increased risk of becoming sensitized to enzymes (van Rooy *et al*., 2009). This is especially the case for workers whose occupation requires debagging, sieving, weighing, dispensing and mixing enzymes (Smith and Lumley, 1996). Adverse health effects associated with enzyme exposure are well characterized in the baking industry. In some countries, bakery exposures to enzymes are one of the leading causes of occupational allergy. Fungal enzymes are commonly used as baking additives to improve the dough, increase shelf life, and decrease production time (Elm *et al*., 2006). Airborne concentrations ranging from 5.3 ngm^{-3} to 200 ngm^{-3} have been reported in occupational environments (Houba *et al*., 1997).

The cycle of climate and allergic disease. The generation of greenhouse gases, whether due to the nature's own cycle or in combination with man-made sources, generates conditions that promote changes in the climate that affect the flora and fauna associated with allergic disorders. These include alterations in habitats and allergen content of stinging insects to the impact on flora seasonal variations, geographic distribution, and allergen burden (concentration of allergen per grain of pollen or the actual amount of pollen produced per plant), as well as the potential changes in genetics of plants that may lead to the uncovering of novel allergens. Clinically, the exposure would lead to increased allergen sensitization with subsequent increases in frequency, prevalence, incidence, and severity of allergic disorders, including asthma. The end of the cycle is the impact that this would have on cost of

illness (economics) through the various participants, including the health care providers and facilities, and pharmaceutical and insurance industries that would also leave a footprint in generating additional greenhouse gases.

2.9 Weather and Allergy

Data about the influence of weather conditions on asthma are poor and debated. Weather conditions affect asthma directly acting on airways or indirectly influencing airborne allergens and pollutant levels. A decrease in air temperature is an aggravating factor for asthmatic symptoms, regardless of the geo-climatic areas. While results of the effects of cold air on asthma are consistent, the role of humidity, wind and rainfall is still unclear and studies including these variables showed inconclusive and inconsistent results maybe because their impact on the diffusion of pollen and pollutants is higher than that of air temperature (Jones and Harrinson, 2004). Humidity indirectly affects respiratory allergic diseases, influencing atmospheric levels of aeroallergens. There is evidence that low humidity allows the release of pollen from anthers favouring dispersion and transport phases while high humidity is associated with lower airborne pollen concentration. Conversely, spore release is generally favoured by high levels of humidity even if mechanisms of release show differences among fungi species. Several asthma outbreaks during thunderstorms were described in the United Kingdom, Australia and Italy (D'Amato *et al*., 2007). Despite some uncertainties, the mechanism underlying asthma epidemics might be pollen grain rupture by osmotic shock and release of part of their content, including respirable, allergen-carrying starch granules (0.5–2.5mm) into the atmosphere. However, recent findings also suggest an impairment of natural immunity mechanisms of airways induced by breathing cold air (Johnston *et al*., 2006).

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Sample Collection

3.1.1 Atmospheric Fungi Collection

Aero spores were sampled monthly for the period of 24 months between May, 2014 and April, 2016 at various locations in Lagos and Ibadan, Nigeria (Table 3.1). Open plate method was used for sampling by opening plates containing agar (Dichloran glycerol 18 and Potato Dextrose Agar) which was prepared under aseptic condition in the laboratory. Plates were opened for 10 minutes at human height and thereafter covered. Samples were collected in triplicates and later transferred to the Mycology Laboratory of the Department of Botany, University of Lagos for further observation and studies.

3.1.2 Identification of Fungi

Once there is presence of growth, the topography, texture and pigmentation of each specific type of colony is noted in order to identify the fungi accurately. A little portion of the growth colony of each pure plate was teased with an inoculating needle and mount on the slide with a drop of Lactophenol cottonblue, covered with a cover slip. The preparation was examined under a light microscope with an attached camera (Motic Mc Camera {2000} 2.0 megapixel digital colour camera) connected to a computer for the microscopic photography of the fungi. The identities of these fungi were certified using cultural, morphological as well as comparing them with confirmed representatives of different species in relevant texts such as Alexopolous *et al*. (2007) and Ellis *et al*. (2007).

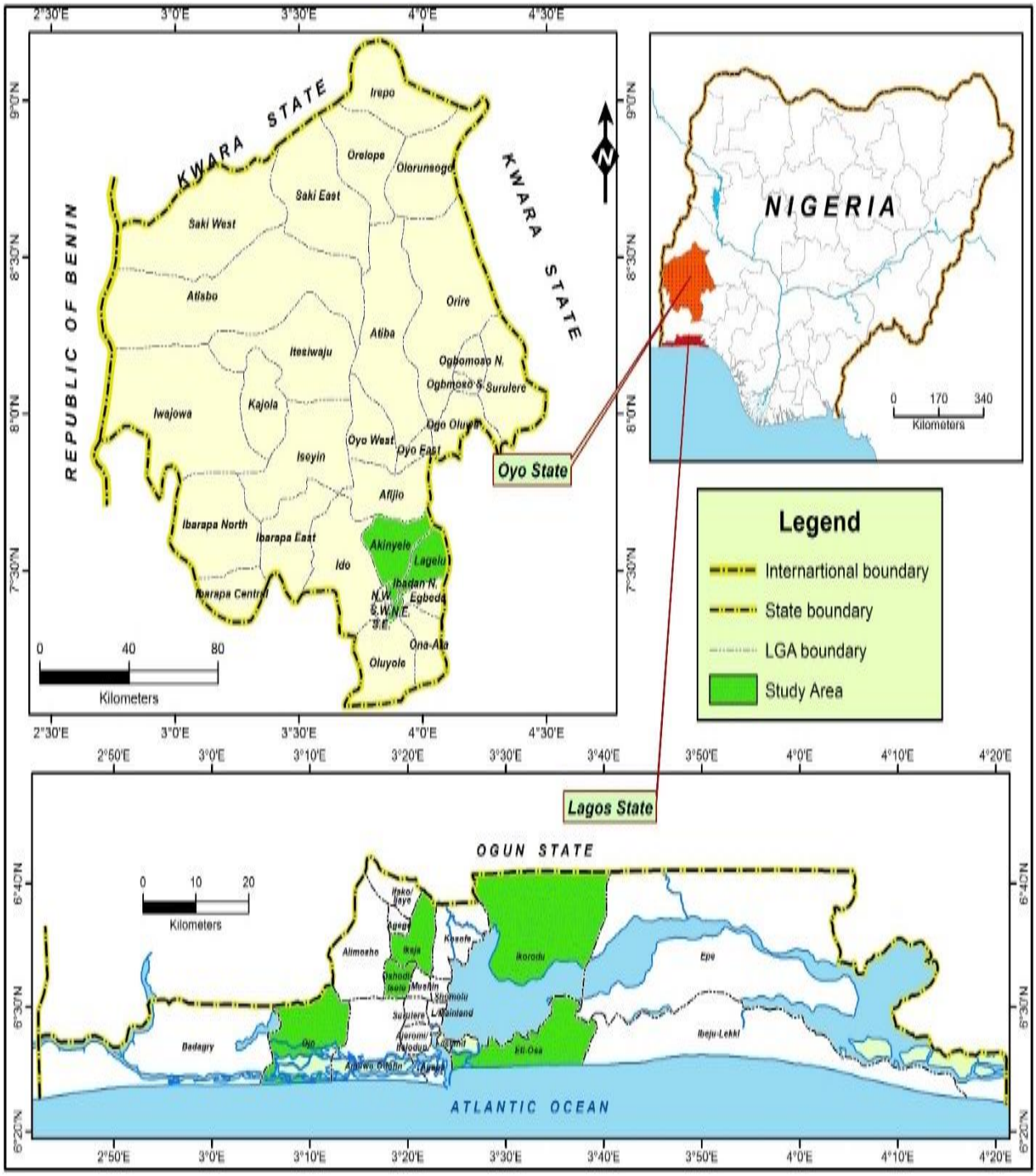


Figure 3.1: Map showing sampling locations of air spores from different locations in Lagos and Ibadan, Nigeria.

Table 3.1: Coordinate descriptions of different sampling locations.

LOCATION	Description	G.P.S
Iba	The location is a farm settlement where different different fruits and vegetables are grown.	$N6^{0}27'38$, $E3^{0}12'20$
Oshodi	This is a densely populated area which serves as motor park, garage, filing station with few buildings around.	$N6^{0}33'18$, $E3^{0}20'75$
Ikorodu	The location is a cosmopolitan area with diverse activities like trading, motor park, housing, bus stop.	$N6^{0}38'51$, $E3^{0}25'25$
Ikeja	The sampled area is an hospital environment where patients are attended to.	$N6^{0}60'18$, $E3^{0}35'15$
Vic.Island	The location is an open space which serve as fun spot, residential buildings close to it.	$N6^{0}42'81$, $E3^{0}42'19$
Moniya	It is a residential place where street trading goes on with few activities.	$N7^{0}52'49$, $E3^{0}91'52$
Iyana Church	This is a commercial place with heavy presence of mechanic workshop and business activities.	$N7^{0}42'47$, $E3^{0}97'6$
Beere	The location is strictly for various commercial activities. Farm products are sold here.	$N7^{0}40'19$, $E3^{0}93'94$
Bodija	The area is densely populated with various activities like trading, motor park et.c.	$N7^{0}43'59$, $E3^{0}91'93$
Mokola	This is a school environment with students and teachers.	$N7^{0}40'63$, $E3^{0}88'94$

3.2 Molecular Characterization of Isolated Fungi

3.2.1 DNA Ctab Protocol Extraction Method

Tissues were harvested carefully by cutting out all agar and disposing them using a sterile spatula to separate mycelium and immediately tissues were flash freezed in liquid nitrogen. Tissue was ground into fine powder using a mortar and pestle. This was done under liquid nitrogen, the mortar and pestle was pre chilled same with spatula, tube, and any other tools that came in contact with the tissue. Using a sterile spatula the tissue powder was transferred

into labeled 1.5ml microcentrifuge tubes, filling tubes to 100 µL mark. Five hundred microliters (500 µL) CTAB buffer was added to each tube to fully suspend tissue in solution. Tubes were incubated at 65 °C for 1 hour, occasionally vortexing or inverting to mix. In fume hood equal volumes (500µL) Phenol/Chloroform/Isoamyl Alcohol (25:24:1) were added to each tube and vortexed briefly to fully mix solution. Tubes were centrifuged at max speed for 15 minutes. After centrifugation, the solution was clearly separated into three layers: a lower organic layer, an interphase layer and an upper aqueous layer. Upper aqueous layer was carefully removed by pipetting (without disturbing the interphase), and transferred to the appropriately labelled new 1.5ml tube. The lower layer and interphase was properly discarded in the fume hood. Ten microliters (10µl) of RNase A (10mg/ml) was added to the aqueous layer and spinned down to remove any droplets on tube walls. Thereafter tubes were incubated at 37°C for 1 hour. Volumes of each sample was brought back up to 500µl by adding nuclease free water. In fume hood equal volumes (500µl) Phenol/Chloroform/Isoamyl Alcohol (25:24:1) was added to each tube and vortexed briefly to fully mix solution. Tubes were centrifuged at max speed for 15 minutes. Samples were separated into two phases, the lower organic phase and the upper aqueous phase. Carefully the upper aqueous layer was removed and transferred into appropriately labeled new 1.5ml tube. Volumes of samples was brought up to 500µl by addition of nuclease free water. Equal volumes (500µl) of Chloroform/Isoamyl Alcohol (24:1) was added to each sample and vortexed briefly to fully mix solution. Samples were centrifuged at maximum speed for 10 minutes. Upper aqueous layer was carefully transferred to a new appropriately marked tube. 1/10 volumes of 3M Sodium Acetate was added to each sample and also 2.5 volumes of 100 % ethanol. Samples were incubated overnight at -20°C and later centrifuged at 12,000g for 10 minutes. The supernatant was thereafter transferred to a new tube. DNA appeared as a white to translucent pellet at the bottom of the tube. Pellet was washed by adding 1mL 70% EtOH to each tube

and inverted several times to mix before centrifuging samples at 7,000g for 5 minutes. Supernatant was carefully poured off and wash step repeated. Pellet was allowed to dry by placing open tubes upside down on a paper towel for several minutes. DNA pellet was resuspended in preheated 100µl TE buffer and heated to 65^0 C. The pellet was allowed to dissolve on its own and the quality of DNA extraction was checked by electrophoresis using a large cast 1 % agarose gel. The 1kb ladder for size reference was used and it was run at 120v for 80 minutes.

3.2.2 Gel Extraction Protocol

The DNA fragment was excised from agarose gel with a clean, sharp scalpel. The size of the gel was minimized by removing extra agarose, 300µl of buffer QG was added to each 100mg of gel and incubated at 50 ^{o}C for 10 minutes. To bind DNA, NucleoSpin gel Column were placed into a centrifuge tube (2ml) and 700 µl sample were loaded, which were centrifuged for 1 minute at 13,000 rpm. The flow-through were discarded, then the column was placed back into collecting tube. For washing of the DNA, 0.70 ml of buffer PE was added to QIAquick column and centrifuge for 1 minute at 13,000 rpm. The flow-through was discarded and QIAquick column was centrifuged for additional 1 minute at 13,000 rpm. The QIAquick column was placed into clean 1.5 ml microcentrifuge tube. The DNA was eluted by adding 35µl of buffer EB to the center of the membrane and the columns were allowed to stand for 1minute before centrifuging for 1 minute at 13,000 rpm, then the DNA purity were checked on NanoDrop and the DNA were stored at -20 ^{0}C.

3.2.3 Sequencing

Molecular assays were carried out on the samples using nucleic acid as a template. A proprietary formulation [microLYSIS®-PLUS (MLP), Microzone, UK] was subjected to the rapid heating and cooling of a thermal cycler, to lyse cells and release deoxyribonucleic acid

(DNA). Following DNA extraction, Polymerase Chain Reaction (PCR) was employed to amplify copies of the partial ITS fragment of rDNA in vitro. The quality of the PCR product was assessed by undertaking gel electrophoresis. PCR purification step was carried out to remove unutilized dNTPs, primers, polymerase and other PCR compounds in order to obtain a highly purified DNA template for sequencing. This procedure also allowed concentration of low yield amplicons.

Sequencing reactions were undertaken using BigDye® Terminator v3.1 kit from Applied Biosystem (Life Technologies, UK) which utilises fluorescent labelling of the chain terminator ddNTPs, to permit sequencing. Removal of excess unincorporated dye terminators were carried out to ensure a problem-free electrophoresis of fluorescently labelled sequencing reaction products on the capillary array AB 3130 Genetic Analyzer (DSI) DyeEX^{TM} 2.0 (Qiagen, UK) modules containing prehydrated gel-filtration resin were optimized for cleanup of sequencing reactions containing BigDye® terminators. Dye removal was followed by suspension of the purified products in highly deionized formamide Hi-Di^{TM} (life Technologies, UK) to prevent rapid sample evaporation and secondary structure formation. Sequence identity was confirmed using the standard nucleotide BLAST function on the NCBI website.

3.3 qPCR Reaction

3.3.1 RNA Extraction Procedure

It was ensured that only RNA of high purity (no contaminants) and high integrity (not degraded) were used for the experiment. One hundred milligram of fungus was scraped into a sterile mortar and pestle and liquid nitrogen was added. Sample was crushed till a paste was obtained and lysate transferred to a centrifuge tube where 450 µl of buffer RLT was added to it and vortexed rigorously. The lysate was transferred to a QIA shredder spin column placed

in a 2 ml collection tube and centrifuged for 2 mins at full speed. Supernatant of flow through was transferred to a new micro centrifuge tube without disturbing the cell debris pellet. Ethanol (96-100 %) (0.5 vol) was then added to the cleared lysate and mixed immediately by pipetting before transferring the sample to an RNeasy mini spin colmn in a 2ml collection tube and later centrifuged for 15 seconds at 8000 x g. Flow through was discarded. Seven hundred microliter (700 µl) Buffer RW1 was added to the RNeasy spin column, centrifuged for 15 s at 10, 000 rpm and thereafter flow through discarded. To the same RNeasy spin column, 500 µl Buffer RPE was added, centrifuged for 15s at 10, 000 rpm and flow through discarded. Rneasy spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 min to dry the membrane and later spin column was transferred to a new 1.5 ml collection tube and 30 µl RNase free water was added directly to the spin column membrane and centrifuged for 1 min at 10,000 rpm to elute the RNA. This step was repeated to get high amount of RNA.

3.3.2 Reverse Transcription

Given the prevalence of Rnase in the environment, reverse transcription of total RNA samples to cDNA was done immediately after RNA extraction to avoid degradation of samples.

3.3.3 Primer and amplicon Design

To make 100 µm concentration stock solution of Primers, the number of nmoles in the tube was multiplied by 10 to give the amount of Elution buffer used to dilute after which 10 µl was pipetted out and mixed with 90 µl of elution buffer to give a working solution. The target sequence selected was between 75- 100 bp long with a GC content between 50 and 60 % which did not contain secondary structures. The primers had a melting temperature of between 55- 65 ^{0}C. Oligonucleotides were designed using Primer-Blast, a program developed

by NCBI that uses the algorithm Primer 3. Primer sequences were compared (blasted) to the user-selected databases to ensure they are specific for the gene of interest. The program MFOLD was then used to analyse the amplicons for potential secondary structures that may prevent efficient amplification.

3.3.4 Preparation of RNA Sample Prior to Reverse Transcriptase-PCR

Duplicate tubes were prepared for positive and negative reverse transcriptase (RNA) samples used for the amplification reaction. The following were added RNase-free (0.5-ml) microcentrifuge tube on ice, 1µg of RNA sample plus 1µl 10X DNase 1 Reaction Buffer plus 1 µl DNase 1, Amp Grade, 1U/ µl plus DEPC-treated water to make up 10 µl, which was incubated for 15 minutes at room temperature. The DNase 1 was inactivated by the addition of 1µl of 25 mM EDTA solution to the reaction mixture, which was heated for 10 minutes at 65^0C, the RNA samples were now ready to be used for reverse transcription.

3.3.5 Quantitative Polymerase Chain Reaction Reaction Set Up

The BioRad SYBR Green Supermix was used because it contains an antibody that keeps the polymerase inactive until the reaction is heated. This permits the reaction to be set up at room temperature and to remain stable prior to quantitative PCR. The reaction mix contained 12.5 µl of SYBR Green Supermix (Biorad), 0.25 µl of forward primer, 0.25 µl of reverse primer and 10 µl of water and 2 µl of cDNA which was added to a 96 well plate. It was pipetted carefully to minimize errors after which plate was sealed with BioRad optical sealer (MSB 1001) and spinned briefly at 50 x g to eliminate air bubbles and thereafter put into the qPCR machine (iQ5) for the reaction. The polymerase activation was at 95^0C for 3 mins followed by denaturation at 95^0C for another 15 secs. Amplification (annealing/extension) was at 55^0C for 30 secs which was done for 30 cycles. The melt curve analysis was at 55^0C for 5 sec/step.

Table 3.2: List of Primers for fungi characterization and identification using culture-dependent molecular techniques.

Locus	Primer name	Direction	Sequence	Target region
Internal Transcribed Spacer (ITS)	ITS 1	Forward	5'TCCGTAGGTGAACCTGCGG3'	18S rDNA
	ITS 4	Reverse	5'TCCTCCGCTTATTGATATGC3'	
Large Ribosomal Unit	LRO5	Forward	5'TCCTGAGGGAAACTTCG3'	LSU
	LROR	Reverse	5'ACCCGCTGAACTTAAGC 3'	
Beta tubulin	Bt 2a	Forward	5'TTCCTACGGTGACCTGAACC3'	control gene
	Bt 2B	Reverse	5'CCATGAAGAAGTGCAGACGA3'	
Actin	Ac 2a	Forward	5'TCCAGCCTCGAGAAGTCCTA3'	control gene
	Ac 2b	Reverse	5'CTCGAGGCCAAGAACGTTAG3'	
RNA polymerase gene	P. cit	Forward	5'GAGCGTGCTTACTTCTCCAAC3'	vacuolar serine
	P. cit	Reverse	5'AGCGTCCTTGATCTCGCTGTAG3'	
	P. ox	Forward	5'GGTCTCTCTCGCATCTCT3'	vacuolar serine
	P. ox	Reverse	5'AGTTGGTGAAGGAAGCAC3'	

3.3.6 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS- PAGE was carried out according to the protocol described by Laemmli *et al*. (1970). Resolving gel (10 %) contained 1.5 M Tris-HCL (pH 8.8.), 30 % bisacrylamide/ acrylamide, 10 % SDS, ammonium per sulphate (APS) / tetra methyl ethyl anediamine (TEMED) IN 10:1. Stacking gel (4 %) contained 1.5 M Trsi- HCL (pH 6.8), 30 % bisacrylamide/ acrylamide, 10 % SDS, APS /TEMED in 10:1.

The Bio-Rad MiniProtean-II apparatus was assembled followed by preparation of the resolving gel. The resolving gel was poured immediately into the glass plate sandwich (0.4 mm spacing) avoiding air bubbles. Water was added on top of the resolving gel to prevent drying. The gel was allowed to polymerize for 45 minutes. After polymerization, water was removed and the prepared stacking gel was loaded on the polymerized resolving gel avoiding bubbles. Combs were carefully inserted to make wells. The stacking gel was allowed to polymerize for 20 minutes. Working electrophoresis buffer, 1 x concentrated was prepared from 10 x stock solution (24 g Tris-HCL, 115.2 g glycine and 8 g SDS in 800 mls of ddH_20)

and poured into the gel electrophoresis tank covering the small inner glass of the glass plate sandwitch.

3.4 Mice Experiment

Fifty four Balb b mice (4- 6 weeks old) were purchased from Veterinary Teaching Hospital of the University of Ibadan and kept in the animal house of the Biochemistry Department. The animals were acclimatized for one week prior to commencement of experiment. They were fed mice pellet purchased from Ladokun factory, Ibadan and water *ad libitum.* After acclimatization, mice were grouped six in separate cages for each concentration of fungal spore making a total of eight groups and one group of control which was without any fungal inoculation.

3.4.1 Selection of fungi and preparation of Inocula

Aspergillus flavus, *A. penicilloides*, *Penicillium citrinum* and *P. chrysogenum* were selected for mice allergen experiment due to their abundance throughout the sampling period and in all the locations sampled. Pure culture plates of organisms devoid of contamination were used. The spore suspensions of the fungi selected were made using sterilized normal saline water of 10ml. The top of the colonies were touched with a loop and seeded into sterilized normal saline water of 10ml in the test tube. Drops of the spore suspensions were dropped on a sterile glass slide using a dropper. The spores were counted using haemocytometre and adjusted to make 2×10^{-5} and 3×10^{-7} spores for each organism.

3.4.2 Inoculation and Lung Excision:

Each mice was inoculated intranasally with fungal spore suspension at the two fungi concentration dose and control group was inoculated with distilled water. They were monitored for 48 hours before they were administered first ketamine to immobilize them and later subjected to cervical dislocation. Lungs were thereafter excised for further analysis.

Blood samples were obtained by retro- orbital bleedings using heparinze capillary tubes. Sera were obtained by centrifugation at 12, 000 rpm for 10 mins and stored at -80^{0}C for late use in detecting immunoglobulin E (IgE) levels. Blood smears were also obtained and fixed with methanol for 2-3 minutes. One in three dilutions of Leishman stain and buffered water was prepared and slides were covered for 7 – 10 minutes. The stain was washed off in a stream of buffered water. Distilled water was added on the slide and left for 2-3 minutes to differentiate the film. The slides were allowed to dry on a rack. Using x100 objective lens with oil immersion, the blood smears were examined and the different immune cells identified and counted.

3.4.3 Histopathology study of the lungs

Processing of tissue samples for histological assessment followed established procedures of Adeline *et al*. (2010). In brief, the tissue samples were rinsed with 0.9% saline solution, fixed in 10% formalin. Then the transverse sections of the lung were obtained and processed as follows: (1) 10 % eutral buffered formalin for 1 hour(h), twice; (2) 70 % alcohol for 1.5 h; (3) 80 % alcohol for 1.5 h; (4) 90 % alcohol for 1.5 h; (5) absolute alcohol for 1.5 h, twice; (6) xylene for 1.5 h, twice; (7) in molten wax at 65°C for 2.5 h two changes. The processed tissues were embedded in paraffin and sectioned at 4 microns thickness, placed on frosted glass slides and dried on a 70°C hot plate for 30 minutes. The tissues were stained using the hematoxylin and eosin (H&E) stains. The sections were dewaxed in two changes of xylene (3 min each), hydrated in two changes of 100 % ethanol, followed by 90 % ethanol and 70 % ethanol, for 3 min each, rinsed with water (3 min) and stained. The stained tissues were dehydrated with 70% ethanol followed by 90 % ethanol, placed in two changes of 100 % ethanol for 3 minutes each and cleaned with two changes of xylene (3 min each). Histopathology changes were observed.

3.5 Protein Profiling Analysis

Protein profiles of *Aspergillus flavus, A. terreus, Penicillium citrinum and P. chrysogenum* extracts were studied by subjecting them to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using vertical slab gel in discontinuous buffer system containing 0.025M Tris, 0.2M glycine and 0.1% SDS (Laemmli, 1970). The stacking and resolving gels contained 5% and 12% polyacrylamide, respectively. Lyophilized extract was reconstituted in sample buffer containing mercaptoethanol followed by boiling at 100^0C for 5min. The sample containing 100 g of protein was applied in each well and electrophoresis was carried out at 80 volts for 30 min followed by 120V for 2 h. The bands were detected by staining with 0.1% Coomassie brilliant blue R-250 stain. Protein standards of 19.8–103 kDa (Bio-Rad, CA, USA) were used to determine the molecular weights of allergenic proteins.

3.6 Biochemical Analysis Procedure

3.6.1 Determination of Protein Concentration

The protein concentrations of the various samples were determined by means of the Biuret method as described by Lowry *et al*. (1951) with a slight modification: potassium iodide was added to the reagent to prevent precipitation of Cu^{2+} ions as cuprous oxide.

Procedure

The lung supernatants were diluted 10 times with distilled water. This was done to reduce the level of protein in the samples to the sensitivity range of Biuret method. One millilitre of the diluted sample was taken and added to 3ml of Biuret reagent in triplicate. The mixture was incubated at room temperature for 30 minutes after which the absorbance was read at 540 nm using distilled water as blank. The protein content of the samples were usually extrapolated from the standard curve prepared using bovine serum albumin and multiplied by 10 to get the actual amount in the fraction.

3.6.2 Determination of Lipid peroxidation (Malondialdehyde-MDA)

Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation. This was carried out by the method of Varshney and Kale (1990).

Procedure

An aliquot of 0.4ml of the sample was mixed with 1.6ml of Tris-KCl buffer to which 0.5ml of 30 % TCA was added. Then 0.5ml of 0.75 % TBA was added and placed in a water bath for 45 minutes at 80^{o}C. This was then cooled in ice and centrifuged at 3000g for 15 minutes. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532 nm. The MDA level was calculated according to the method of Buege and Aust (1978). Lipid peroxidation in units/mg protein or gram tissue was computed with a molar extinction coefficient of 1.56 x 10^5 $M^{-1}Cm^{-1}$.

$$\text{MDA (units/mg protein)} = \frac{\text{Absorbance x volume of mixture}}{E_{532nm} \text{ x volume of sample x mg protein}}$$

3.6.3 Determination of Superoxide Dismutase (SOD) Activity

The level of SOD activity was determined by the method of Magwere (1997).

Procedure

One millilitre of sample was diluted in 9ml of distilled water to make a 1 in 10 dilution. An aliquot of 0.2ml of the diluted sample was added to 2.5ml of 0.05 M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3 ml of freshly prepared 0.3mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5 ml buffer, 0.3ml of substrate (adrenaline) and 0.2ml of water. The increase in absorbance at 480 nm was monitored every 30 seconds for 150 seconds.

Calculation

Increase in absorbance per minute = $\frac{A_3 - A_0}{2.5}$

Where A_0 = absorbance after 30 seconds

A_3 = absorbance after 150 seconds

% inhibition = $100 - \frac{(\text{Increase in absorbance for substrate} \times 100)}{\text{Increase in absorbance for blank}}$

One unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline.

3.6.4 Estimation of Reduced Glutathione (GSH) Level

The method of Jollow *et al*. (1974) was followed in estimating the level of reduced glutathione (GSH).

Proceedure for GSH determination in samples

A 0.2ml of sample was added to 1.8 ml of distilled water and 3ml of the precipitating agent was mixed with the sample. This was centrifuged at 3,000g for 4 minutes. Thereafter, 0.5 ml of the supernatant was added to 4.5 ml of Ellman reagent. A blank was prepared with 0.5 ml of the diluted precipitating agent and 4ml of phosphate buffer and 0.5 ml of Ellman's reagent. The absorbance of the reaction mixture was read within 30 minutes of colour development at 412 nm against a reagent blank.

3.6.5 Hydrogen Peroxide (H_2O_2) Generation

Hydrogen peroxide generation was determined according to the method of Galli *et al.* (2005).

Procedure

The total peroxide concentrations were determined using the FOX method. The FOX test system is based on the oxidation of ferrous iron to ferric iron by the various types of peroxides contained in samples in the presence of xylenol orange, which produces a colored ferric-xylenol orange complex whose absorbance can be measured. FOX reagent was prepared with 100 μmol/L xylenol orange, 100 mmol/l sorbitol, 25 mmol/L sulfuric acid and 250 μmol/L ammonium ferrous sulfate in acetic:water (1:1 v/v) solution. An aliquot of 200 μL of serum was mixed with 2 ml of FOX1 reagent, vortexed and incubated at room temperature for 30 minutes. The tubes were then centrifuged at 2.500 rpm for ten minutes. Absorbance of the supernatant was measured at 560 nM. The total peroxide content of the samples was determined by the difference in absorbance between test and blank samples using a solution of H_2O_2 as standard.

3.6.6 Myeloperoxidase (MPO) activity assay

1. O-dianisidine dihydrochloride (o-dianisidine) solution was prepared by combining 16.7 mg of o-dianisidine dihydrochloride, 90 mL of dH_2O, and 10 mL of potassium phosphate buffer. This solution was prepared fresh for every assay.
2. Fifty microliter of diluted H_2O_2 (4 μL of 30% H_2O_2 diluted in 96 μL of dH_2O) to the o-dianisidine mixture.
3. Seventy microliter of tissue homogenate to 2000 μl of the o-dianisidine mixture containing H_2O_2 was added to each of the wells.
4. Absorbance was measured at 450 nm using a spectrophotometer and three readings were taken at 30 second intervals.
5. MPO activity is measured in units (U) of MPO/mg tissue, where one unit of MPO is defined as the amount needed to degrade 1 μmoL of H_2O_2 per minute at room temperature. Considering that one unit (U) of MPO= 1 μmoL of H_2O_2 split and that 1

μmoL of H_2O_2 gives a change of absorbance of 1.13 x 10-2 nm/min, units of MPO in each sample is determined as change in absorbance [ΔA(t2-t1)]/Δmin x (1.13 x 10-2).

3.6.7 Determination of Total Nitrite (NO)

Nitrite determination was done using the method described by Ignarro *et al*. (1987). The assay relies on a diazotization reaction that was originally described by Griess in (1879). The procedure is based on the chemical reaction which uses sulfanilamide and naphthylethylenediamine dihydrochlorate (NED) under acidic condition. Sulfanilamide and NED compete for nitrite in the Griess reaction.

Procedure

Griess reagent was prepared by mixing equal volume of 0.1% NED and 1 % sulfanilic acid in 5 % phosphoric acid. Fifty microliter of sample was added to a 96 plate well in duplicate and 100 μl of griess reagent was added. The mixture was incubated at room temperature protected from light for 10 minutes to allow for colour development. The absorbance was measured within 30 minutes in a microplate reader at 550 nm.

3.7 Immunoperoxidase Assay for Determination of IgE level of Mice

Immunoperoxidase assay for the determination of IgE level in the mice sera was employed and Mouse IgE kit was used. All manufacturer's instructions on reagent dilution with distilled water were strictly adhered to. One hundred microliter test samples were measured using micro pipette into labelled designated microtitre wells and incubated for for 30 minutes. After removal of unbound protein by washing with Elisa machine, the wells were blotted to remove residual buffer. Anti IgE antibodies conjugated with horseradish peroxidase (HRP) were added and incubated for 30 minutes, this was followed by another washing. The enzyme bound to the immunosorbent was assayed by the use of 3, 3, 5, 5 – tetramethyl benzidine (TMB). Absorbance at 450 nm was determined, this was a measure of the concentration of

IgE in the tested samples. The quantity of IgE in the samples was interpolated from the standard curve constructed from the standards and corrected for sample dilution.

3.8 Statistical Analysis

Data obtained were analysed using multiple analysis of variance (ANOVA) and means were separated using Duncan Multiple Rnge Test (DMTR) with the level of significance at $P<0.05$ (95% confidence interval). Histograms and line graphs were also used for graphical representations.

CHAPTER FOUR

4.0 RESULTS

Table 4.1: Molecular Identification of fungi isolated from different sampling sites

Sample	Organism	Match identity	E-value	Query cover
31 ITS	*Aspergillus aculeatinus*	99%	1e	285
1 ITS	*A. flavus*	94%	3e	345
25 ITS	*A. fumigatus*	97%	3e	305
24LR	*A. niger*	99%	1e	138
34LR	*A. niger strain B*	95%	2e	301
6LR	*A. ochraceus*	98%	3e	175
9 ITS	*A. oryzae*	99%	2e	302
41 ITS	*A. oryzae*	96%	2e	233
30 ITS	*A. penicilloides*	96%	3e	175
14LR	*A. protuberus*	97%	4e	309
4LR	*A. subramanianii*	99%	3e	169
29 ITS	*A. tamari*	96%	3e	238
5LR	*A. tubingensis*	93%	4e	254
36 ITS	*Absidia blakesiana*	96%	1e	197
39 ITS	*Aphaderanum spp*	97%	2e	192
7LR	*Aspergillus terreus*	95%	2e	256
19 ITS	*Cladosporium herbarium*	97%	3e	201
14 ITS	*Curvularia spp*	95%	2e	145
10 ITS	*F. verticilloides*	99%	4e	269
17LR	*Fusarium sublunatum*	99%	4e	239
13 ITS	*Mucor spp*	96%	3e	166
35 ITS	*Neurospora crassa*	97%	4e	234
40 ITS	*P. chrysogenum*	98%	3e	256
16LR	*P. citrinum*	86%	3e	345
20LR	*P. citrinum strain B*	97%	1e	198
25LR	*P. oxalicum*	99%	3e	209
26LR	*P. pinophilum*	96%	1e	354
27LR	*P. simplicissimum*	98%	2e	233
33LR	*Paecilomyces spp*	99%	1e	276
11LR	*Penicillium funiculosum*	100%	2e	147
23LR	*Perenniporia koreana*	97%	2e	324
33 ITS	*Phoma eupyrema*	98%	1e	304
12 ITS	*Rhizopus stolonifer*	97%	2e	197
21 ITS	*Sistotrema brinkmanii*	95%	1e	179
19LR	*T. asperellum*	97%	3e	198
8LR	*T. harzianum*	87%	2e	321
7 ITS	*T. helicum*	97%	2e	230
32LR	*T. viride*	99%	3e	124
18LR	*Trichoderma harzanium*	98%	3e	234

4.1 Fungal spore count and identification of fungal species collected from different locations

The monthly collection for one hundred and four consecutive weeks produced a total of fifteen thousand six hundred and forty two colonies in all (15, 642). These were collected from different locations in Lagos and Ibadan with the following percentage: 1,442 (22.83 %) from Iba, 1,399 (22.15 %) from Ikorodu, 1,333 (21.10 %) from Ikeja, 897 (14.20 %), from Victoria Island and 1,246 (19.72 %) from Oshodi. While for Ibadan, the following were collected: 1,033 (18.51 %) from Bodija, 1,099 (21.48 %) from Beere, 1,129 (20.23 %) from Moniya, 1,126 (20.17 %) from Mokola and 1,095 (19.61 %) from Iyana Church.

4.1.1 Distribution pattern of fungi spores sampled in Lagos, Nigeria

The airborne fungal spores type and abundance did not vary much between the various locations from season to season in the two states.

Thirty six different fungal spore types were recorded from Iba location. The atmosphere was dominated by fungal spores from majorly Ascomycetes and Deuteromycetes. *Aspergillus flavus* had the highest spore abundance of (13.3 %), *A. niger* (9.2 %), *Penicillium funiculosum* (6.1 %). Lowest record of fungal spores were from *Phoma eupyrema* (0.2 %), *Aspergillus subramanianii* (0.8 %), *Aphaderanum sp*. (0.4 %). Other fungal species include: *Penicillium funiculosum* (6.1 %), *Aspergillus protuberus* (1.9%), *Fusarium verticilloides* (5.0 %), *Neurospora crassa* (3.0 %), and *Penicillium citrinum* (3.1 %) (Fig 4.1).

Thirty two fungal spores belonging to different families were isolated for Ikorodu location. The atmosphere was qualitatively and quantitatively dominated with varied fungal species of different fungal spores especially those of Ascomycetes and Deuteromycetes including *Aspergillus niger* (17.61 %), *A. flavus* (10.43 %) and *A. fumigatus* (5.98 %). Lowest record of

fungal spores were observed in *Aphaderanum sp* (0.22 %), *Trichoderma viride* (0.22 %) and *Penicillium pinophilum* (0.33 %) (Fig 4.2).

Twenty eight fungal spores were recorded from the aero flora for the duration of sampling belonging to different families for Ikeja location.

Majority of the fungi spores recorded were found concentrated between the months of June and November, although organisms like *Aspergillus niger* and *A. sydowii* occurred throughout the year. Dominant spores were; *A. niger* (14.47 %), *A. sydowii* (10.37%) *and A. flavus* (7.93 %). Others which were sporadic included those of *Aphaderanum sp*.,(0.22%), *Curvularia* sp., (0.22 %) and *A. oryzae (0.22 %)*. Other fungi spores isolated also include: *Paecilomyces sp.. (0.61%), Mucor* sp., (3.44 %), *Neurospora crassa* (5.32 %), *Penicillium funiculosum* (5.49 %), *P. simplicissimum* (1.83 %), *P. oxalicum* (4.71 %), *P. pinophilum* (2.88 %), *Fusarium verticilloides* (3.05 %), *Rhizopus oryzae* (4.10 %) amongst others. Generally, fungi spores were sporadically distributed throughout the sampling period and were more recorded in the month of May-October (Fig 4.3).

The result provided detailed information about the relative abundance of fungal spores recorded for Victoria Island location (Lagos).

Twenty (20) fungal spores were identified from aeroflora of Victoria Island, Lagos, Nigeria. Dominant fungi include those of *Aspergillus niger* (14.6 %), *A. fumigatus* (6.5 %), *Penicillium notatum* (6.4 %), while spores of *Rhizopus sp*., (1.5 %), *T. helicum* (2.1 %) and *A. oryzae* (1.9 %) recorded lowest abundance of spores. The months of June, September and October were more dominated with more fungal spores than the other months of the year.

Other spore types were identified, among which include *A. aculeatinus* (2.1 %), *P. pinophilum* (6.2 %), *P. citrinum* (6.3 %), *Fusarium sublunatum* (5.2 %), *Trichoderma viride*

(2.6 %), *Mucor sp.*, (4.5 %). There were reductions in fungal spore concentration in the air from the month of January and February (Fig 4.4).

Fungal spores were more dominant in the atmosphere in the months of May through June, July, August, September to October, than in the months of November, December, January and February. Twenty seven (27) different fungal spores were isolated for Oshodi location. Ascomycetes and Deuteromycetes spores were the major contributors of the fungal spores in the rainy months. *Penicillium notatum* (4.57 %), *A. niger* (9.23 %), *A. fumigatus* (8.99 %) were the most occurring fungal spores while *P. simplicissimum* (0.40 %), *Aphaderanum sp.*, (0.48 %), and *Phoma eupyrema* (0.48 %) were the lowest in abundance.

Other fungi spores also included *P. citrinum* (4.57%), *T. harzanium* (4.90%), *A. versicolor* (2.3 %), *A. wentii* (0.64 %), *Absidia* (2.01 %), *Curvularia* (3.77 %), *A. flavus* (1.52 %), *Paecilomyces* (1.12 %), *A. subramanianii* (3.85 %). Generally, fungi spores were sporadically distributed throughout the sampling period and were more recorded in the month of April-October (Fig 4.5).

4.2 Distribution pattern of fungal spores sampled in Ibadan

In Bodija, South-western Nigeria, twenty - seven different fungal spore types were recorded from the aero flora for the period of sampling. The spores of fungi that were most abundant at this period, include those of: *Aspergillus niger* (15.99 %), *A. flavus* (10.18 %), *P. notatum* (7.67 %) fungal spore were present in higher abundance in the atmosphere throughout the sampling period. There was a quantitative fungal spore record which was dominated by spore of *A. niger*. The months of May, June, July and August, September and October were qualitatively and quantitatively dominated by various species of fungi. Spores of *Curvularia lunata* (0.65 %), *Mucor sp.*, (1.05 %), and *Absidia sp.*, (0.89 %) had lower spore abundance. Some fungi spores favourably sporulated throughout the sampling period, Instances are

Trichoderma harzanium (3.07 %), *P. funiculosum* (3.39 %), *Paecilomyces spp,* (4.04 %), *P. citrinum* (6.79 %), *A. aculeatinus* (3.63 %). Others such as *Rhizopus oryzae* (5.87 %), *Fusarium sublunatum* (2.67 %) *F. verticilloides* (4.20 %), also occurred throughout the sampling period (Fig 4.7).

Twenty-four (24) different fungal spore types were identified from the aeroflora samples for Beere location. Dominant fungal spore types include those of *Aspergilus niger* (8.43 %), *Penicillium simplicissimum* (8.07 %), *A. flavus* (7.64 %). The following fungi recorded lower amount of spores during the duration of sampling: *Cladosporium herbarium* (0.29 %), *Rhizopus* sp., (3.17 %) and *Sistotrema brinkmanii* (1.51 %). Higher number of fungal spores was recorded during the rainy season (May- October) than other months. *Trichoderma viride* (3.96 %), *A. versicolor* (4.76 %), *Fusarium verticilloides* (2.74 %), *Penicillium citrinum* (5.69 %), *P. oxalicum* (4.39 %), *A. fumigatus* (5.55 %), *A. tamari* (5.04 %), *A. aculeatinus* (4.03 %) *and T. harzanium* (3.48 %) were also dominant during the period of samping. *Aspergillus niger* occurred throughout the year (Fig 4.8).

Twenty- three (23) different fungal spore types which comprised of were identified from aeroflora of Moniya, SouthWest Nigeria. Dominant spores include those of *Aspergilus niger* (11.94 %), *Penicillium citrinum* (8.26 %), *A. tamari* (7.35 %). *Aspergillus versicolor* (1.30 %), *Penicillium simplicissimum* (1.84 %) *and Phoma eupyrema* (1.61 %) recorded lower spore count throughout the period of study. The months of May, June, July, September and October recorded higher fungal counts than the other months of the year. Other fungal spore types identified include but not limited to *Penicillium oxalicum* (4.13 %), *Aspergillus ochraceus* (4.28 %), *P. notatum* (5.51 %), *Fusarium verticilloides* (3.21 %), *Trichoderma harzanium* (2.22 %), *Paecilomyces variotii* (4.21 %), *T. viride* (1.68 %), *P. funiculosum* (6.43 %) *Neurospora crassa* (1.76 %) (Fig 4.9).

The result provided detailed information about the relative abundance of fungal spores for Iyana Church location. Twenty four (24) different spore types were identified. The atmosphere was qualitatively and quantitatively dominated with varied species of fungal spores. Dominant fungal spore producers during the course of sampling are: *Aspergillus niger* (12.27 %), *A. flavus* (7.08 %) and *A. fumigatus* (9.97 %). Other fungal contributors include: *Penicillium citrinum* (4.04 %), *Trichoderma viride* (3.72 %), *P. funiculosum* (2.83 %), *Fusarium sublunatum* (2.60 %), *Paecilomyces variotii* (6.02 %), *Rhizopus oryzae* (2.3 %), *P. notatum* (4.07 %), *Mucor sp.*, (2.83 %) *P. oxalicum* (6.90 %). There were reductions in fungal spore concentration of the air from the month of December, January through February. Lower fungal spore were recorded from the following species: *Phoma* sp., (0.77 %), *Neurospora crassa* (1.59 %) and *Aspergillus aculeatinus* (4.48 %) (Fig 4.10).

Twenty five different fungal spore types were recorded for the period of sampling in Mokola, Ibadan. Dominant fungal spore species were *Aspergillus niger* (12.10 %), *Aspergillus fumigatus* (7.80 %), *Paecilomyces variotii* (6.90 %). *A. niger* was recorded throughout the sampling period. Majority of the fungi spores recorded were found concentrated between the months of April, May, June, July, September and October. Others fungal spores which were found also include those of *A. aculeatinus* (6.59 %), *Trichoderma viride* (4.60 %), *A. flavus* (5.14 %), *A. tamari* (3.57 %), *P. citrinum* (5.08 %), *Fusarium sublunatum* (4.60 %), *Aspergillus japonicus* (2.24 %), *Pereniporia koreana* (2.60 %), *Neurospora crassa* (5.14 %), *F. verticilloides* (3.27 %). Other fungi spores recorded but in low frequency also include: *Curvularia lunata* (0.91 %), *Rhizopus* sp., (1.57 %), *Trichoderma harzanium* (1.94 %) amongst others. Generally, fungi spores were sporadically distributed throughout the sampling period and were more recorded in the month of April – October (Fig. 4.11).

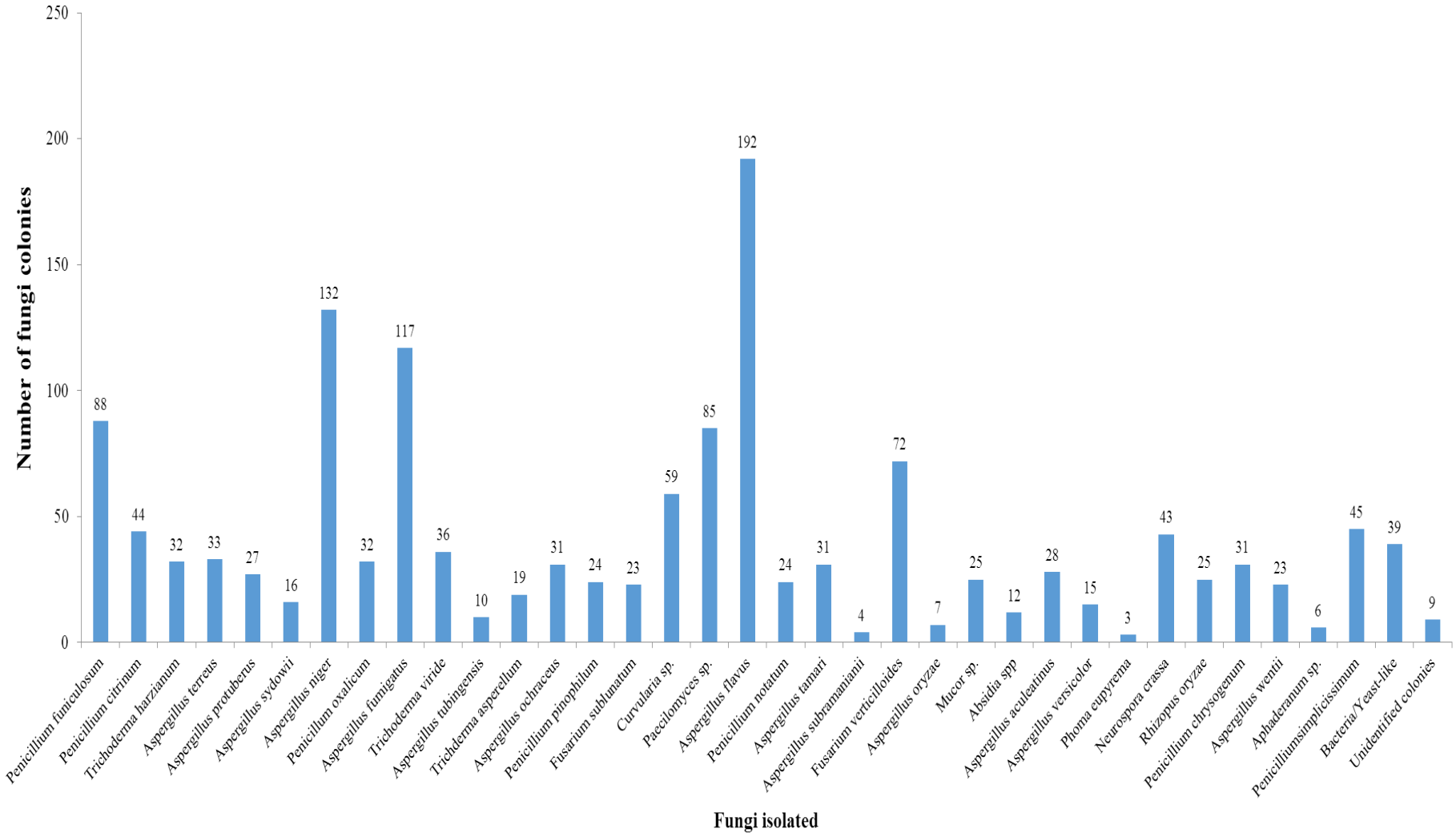


Figure 4.1: Frequency of fungi isolated in Iba, Lagos state

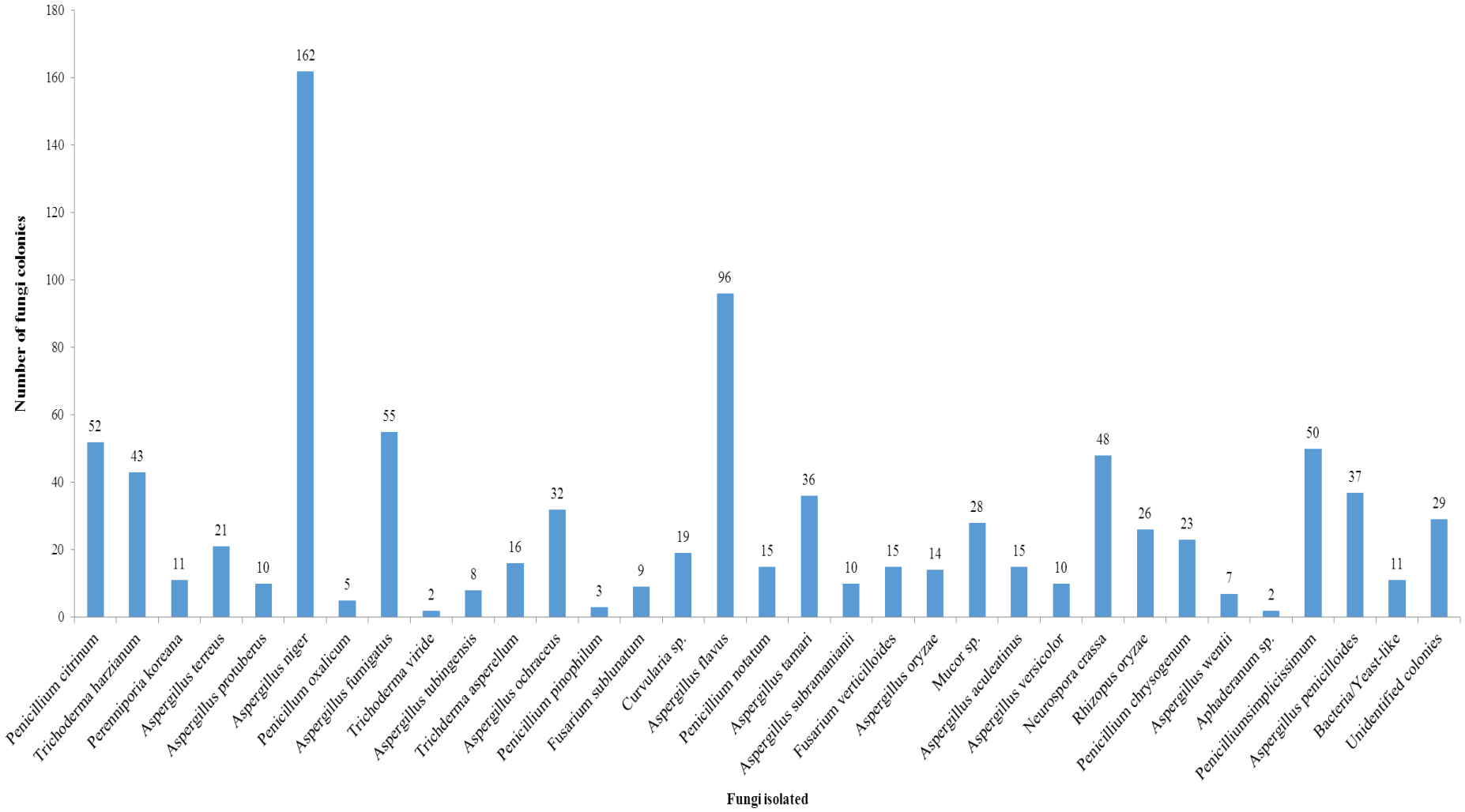


Figure 4.2: Frequency of fungi isolated in Ikorodu, Lagos State.

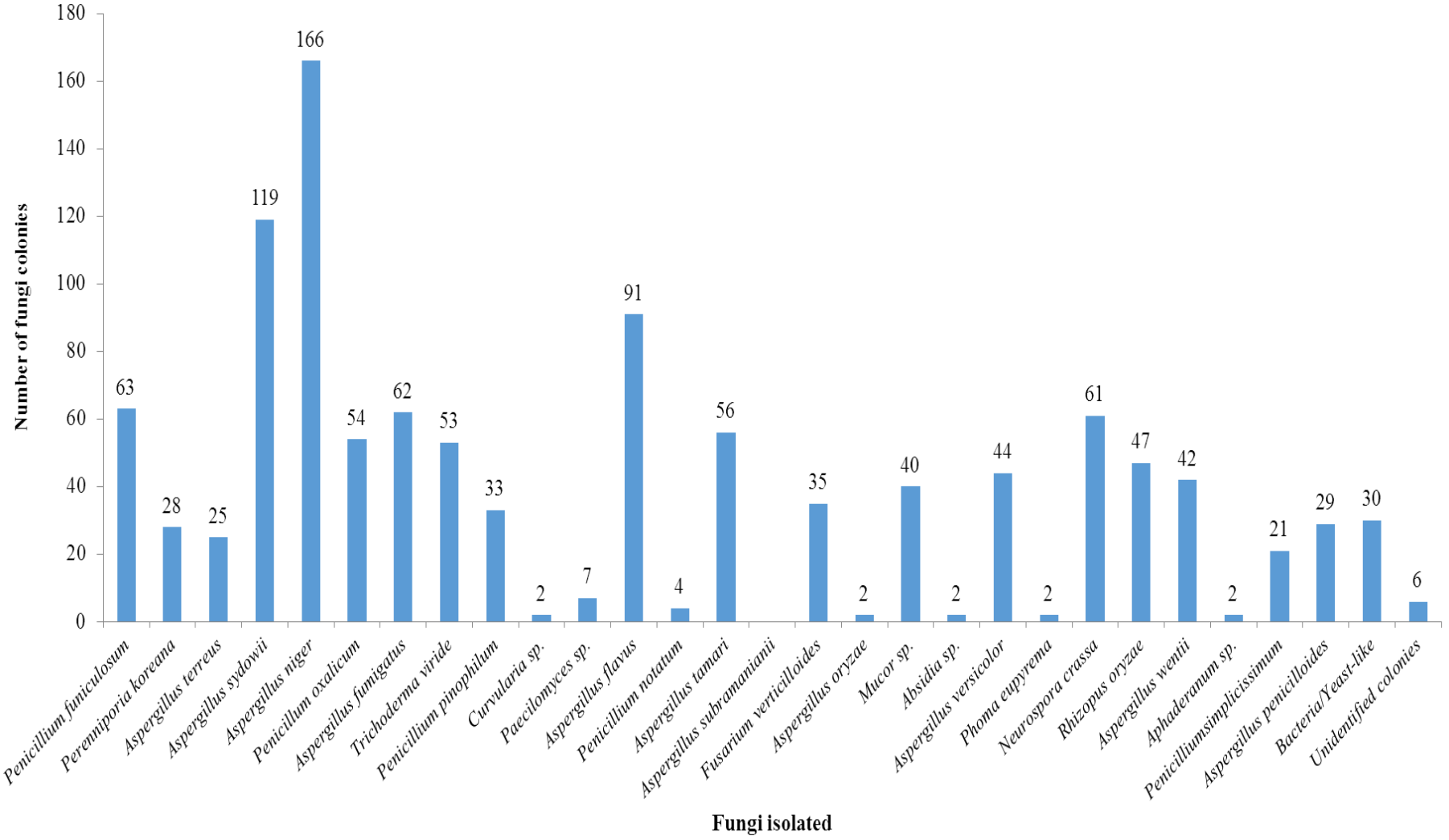


Figure 4.3: Frequency of fungi isolated in Ikeja, Lagos State.

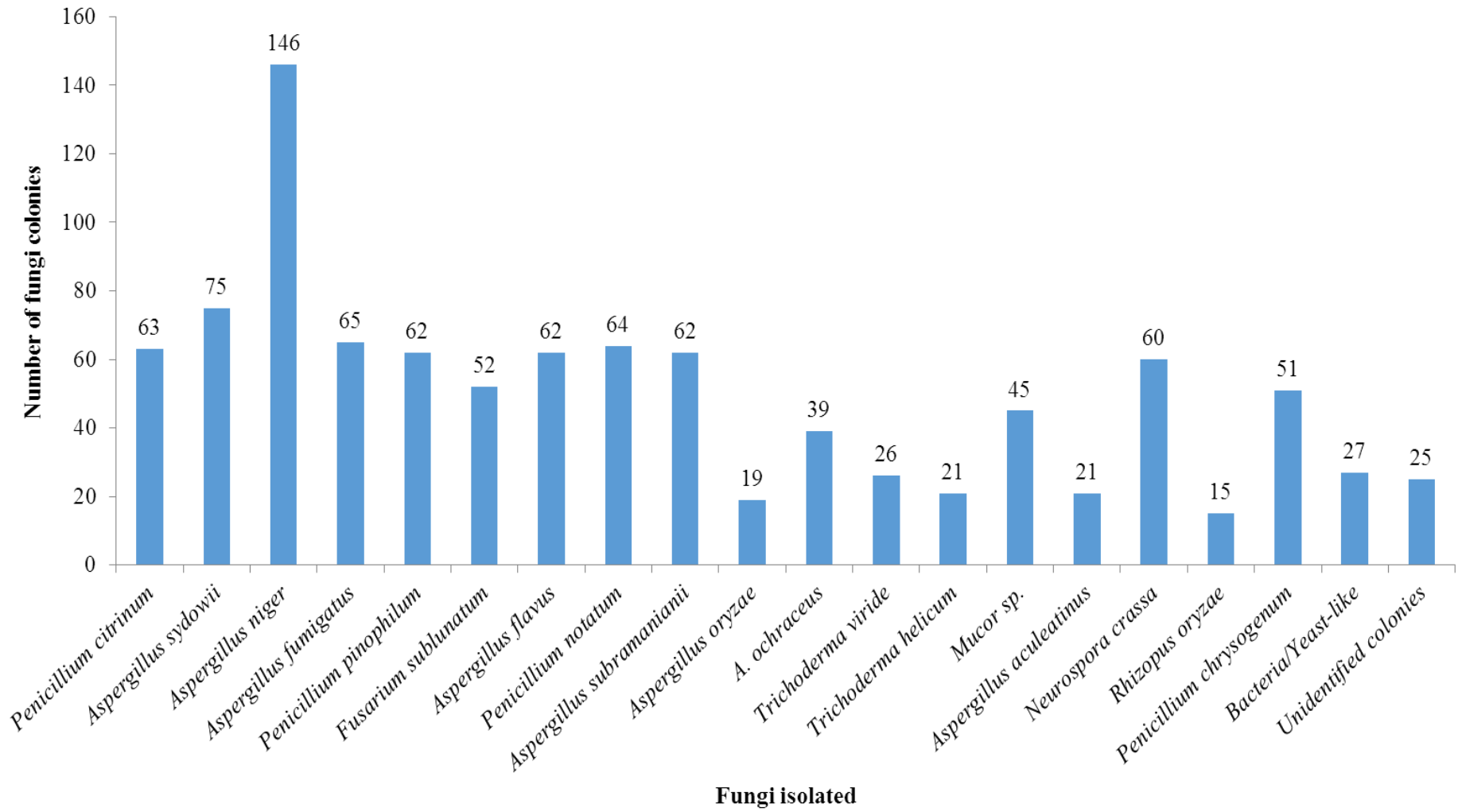


Figure 4.4: Frequency of fungi isolated in Victoria Island, Lagos State.

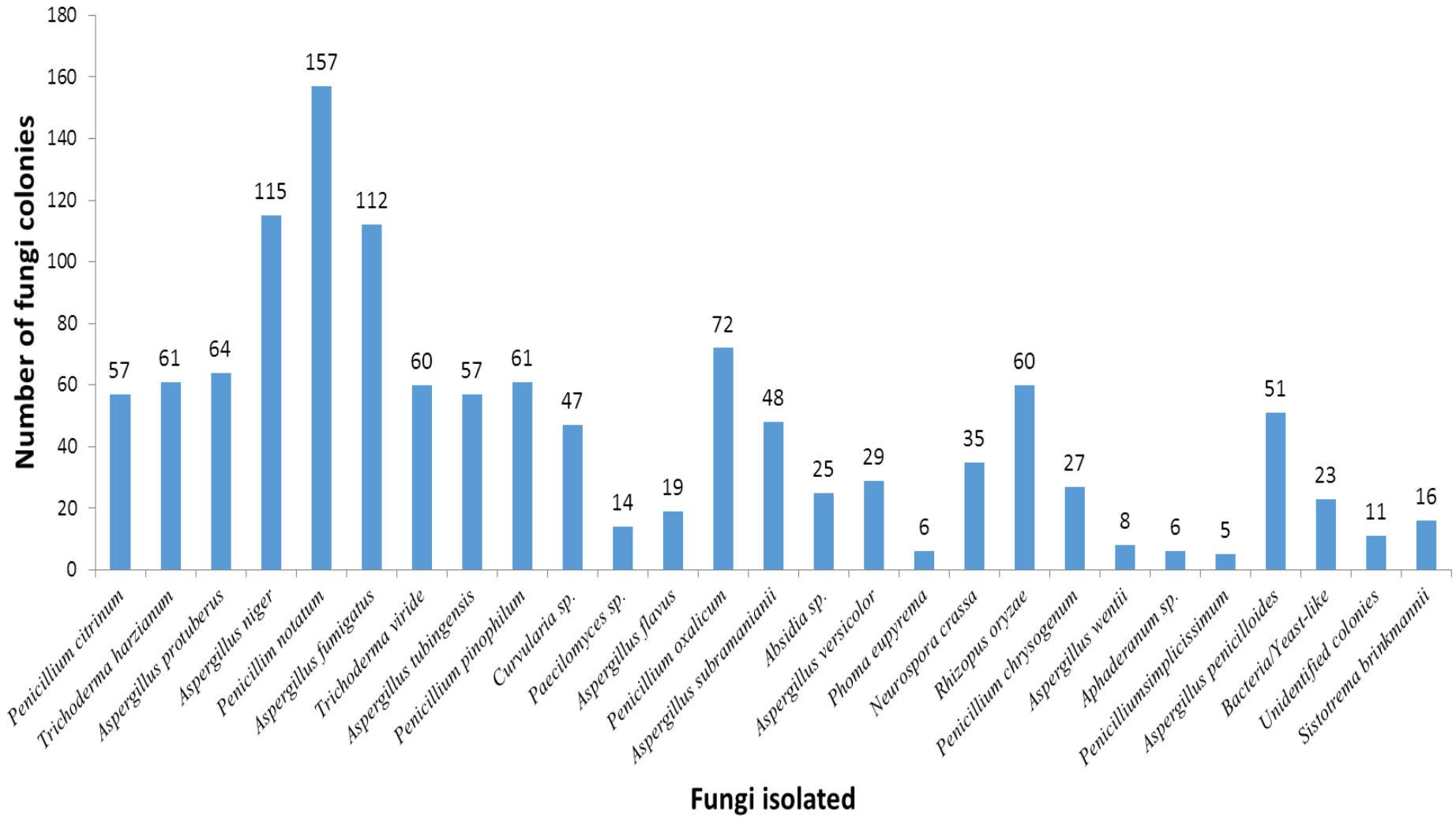


Figure 4.5: Frequency of fungi isolated in Oshodi, Lagos State.

4.3 Location and fungi distribution

The various locations showed differences in monthly abundance/collection of fungal spores. Iba had a total of (25 %) fungal collection in May, (27 %) in June which dropped in July, August and slightly increased in September and October (28 %). The second year collection showed similar pattern/frequency with highest fungal peak in May (29 %) and lowest frequency in December and January of the second year.

For the different locations in Lagos, the following were obtained (fig 4.6):

For Ikorodu, frequency of collection increased in August (20 %) of the first year while the month of June of the second year (21 %) and August (20 %) had similar results of highest fungal collection. Least fungal collection was obtained in March of the second year (13 %).

In Ikeja, highest collection was in October (21 %) followed by May (22 %) and March of the second year with (21 %).

Victoria Island also had varying monthly fungal frequency collection. September had (18 %), December (19 %), January and February (18 %) and also December with (19 %).

In Oshodi, July and September collection was (22%) during the first year while August had (20 %) and December (19 %). For the second year July had (21 %), September (22 %), October (21 %) and March (24 %).

For the different Locations in Ibadan, the following were obtained (fig 12):

In Bodija, June had (16 %), August (16 %) and December (14 %) for the first year while for the second year, May (18 %), September and October (17 %), December (14 %).

At Beere, the month of May had (18 %), June (19 %), January and February (17 %). The second year collection had May, July, August, September with (17 %), June (18 %) and January (16 %).

In Moniya, the months of May, June, July, September recorded (20 %) of fungal collection during the first year while for the second year June had (22 %), August (18 %) and January (18 %).

Iyana Church had for the month of May (21 %), June (22 %), July (23 %). Similar results was obatained for the second year of collection.

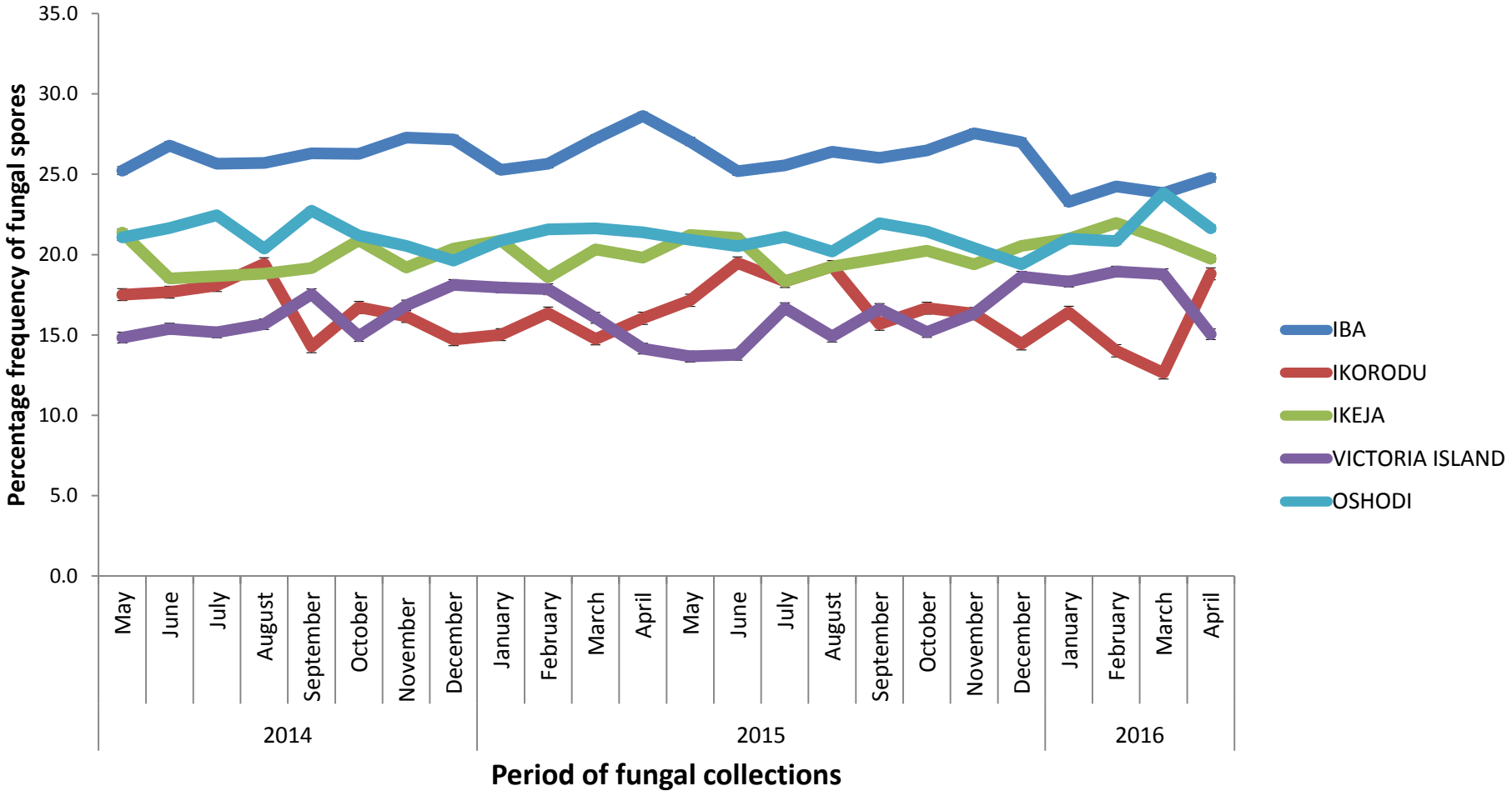


Figure 4.6: Frequency of fungi occurrence in Lagos, State.

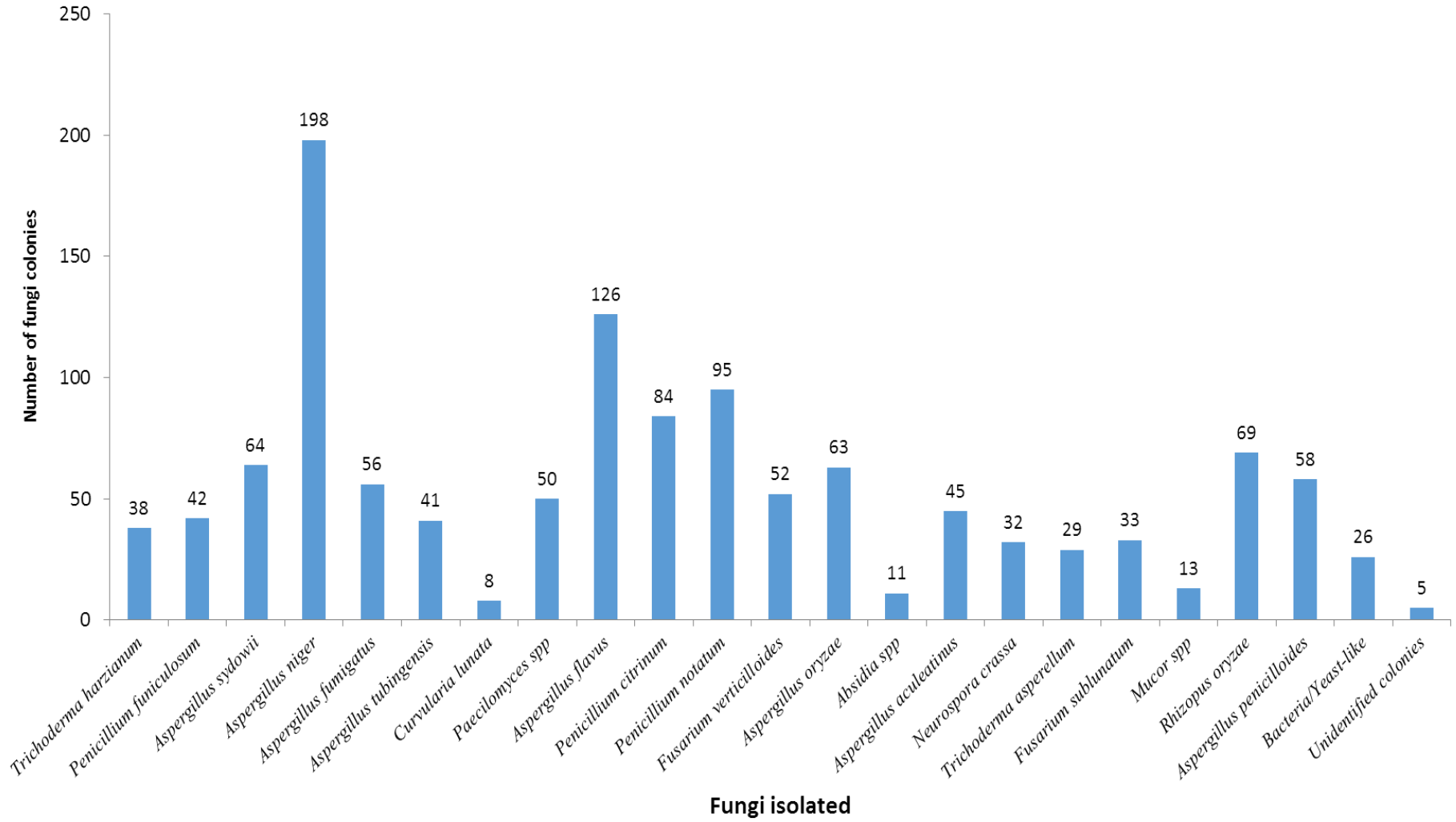


Figure 4.7: Frequency of fungi isolated in Bodija, Ibadan.

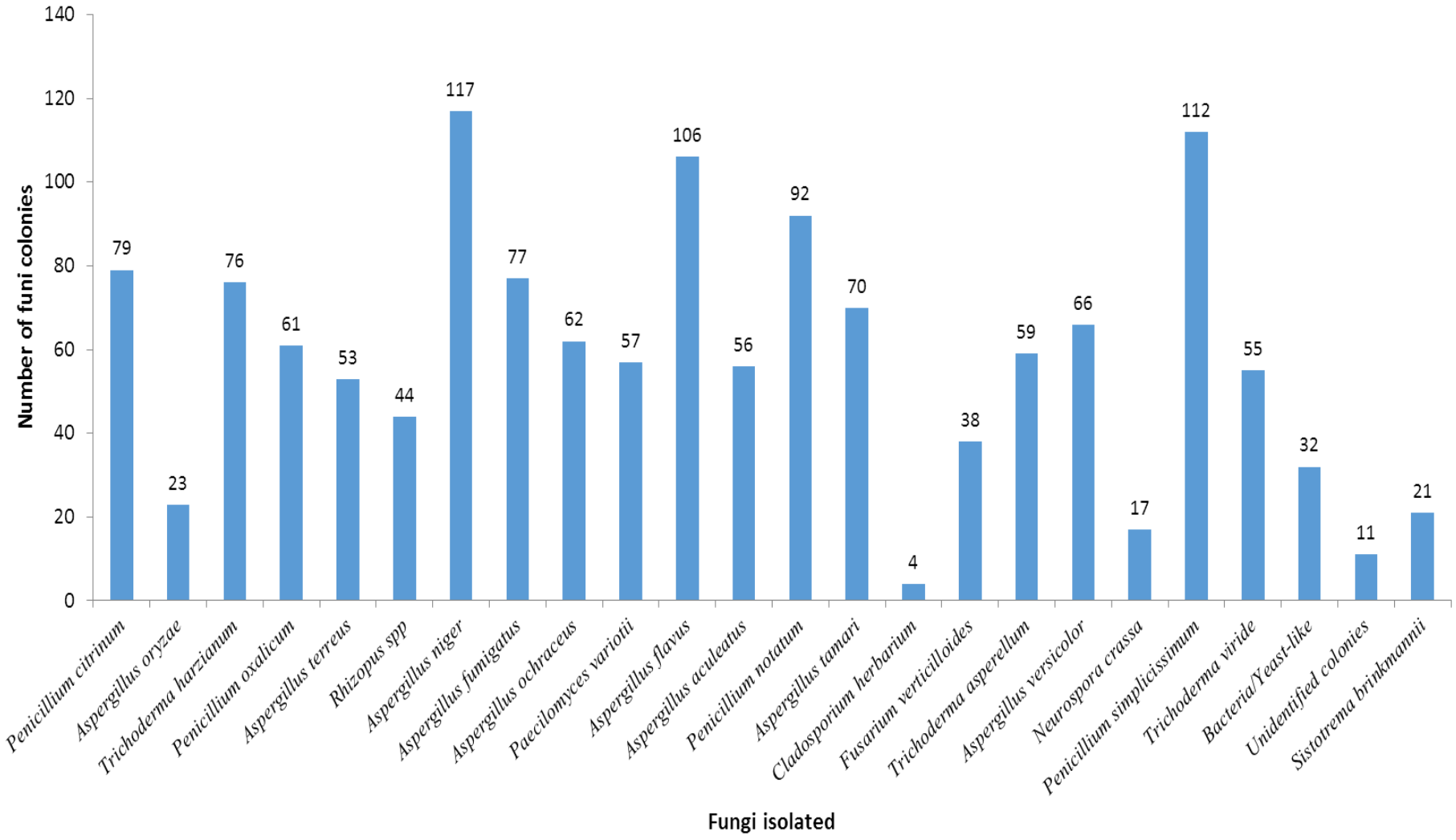


Figure 4.8: Frequency of fungi isolated in Beere, Ibadan.

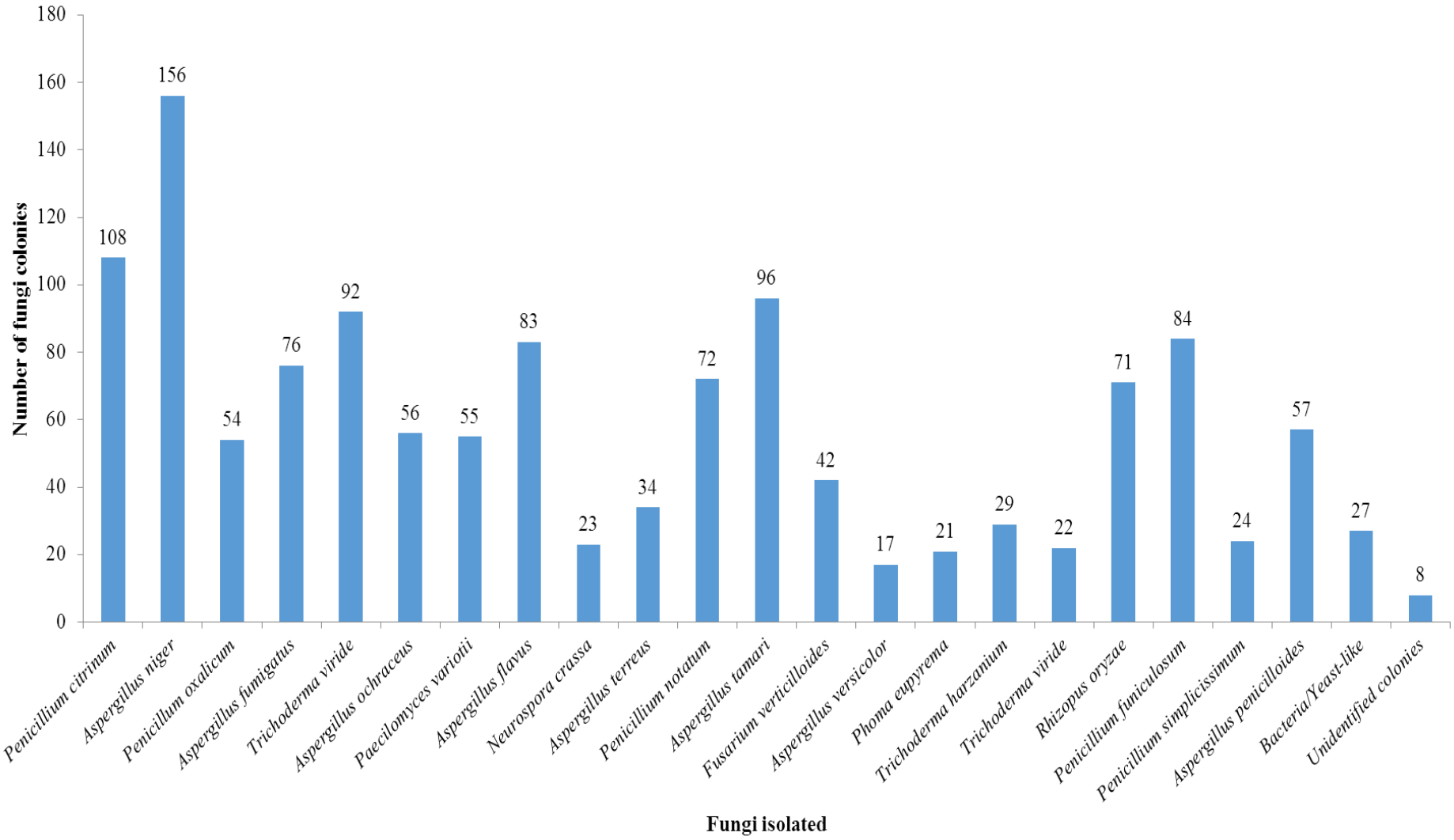


Figure 4.9: Frequency of fungi isolated in Moniya, Ibadan.

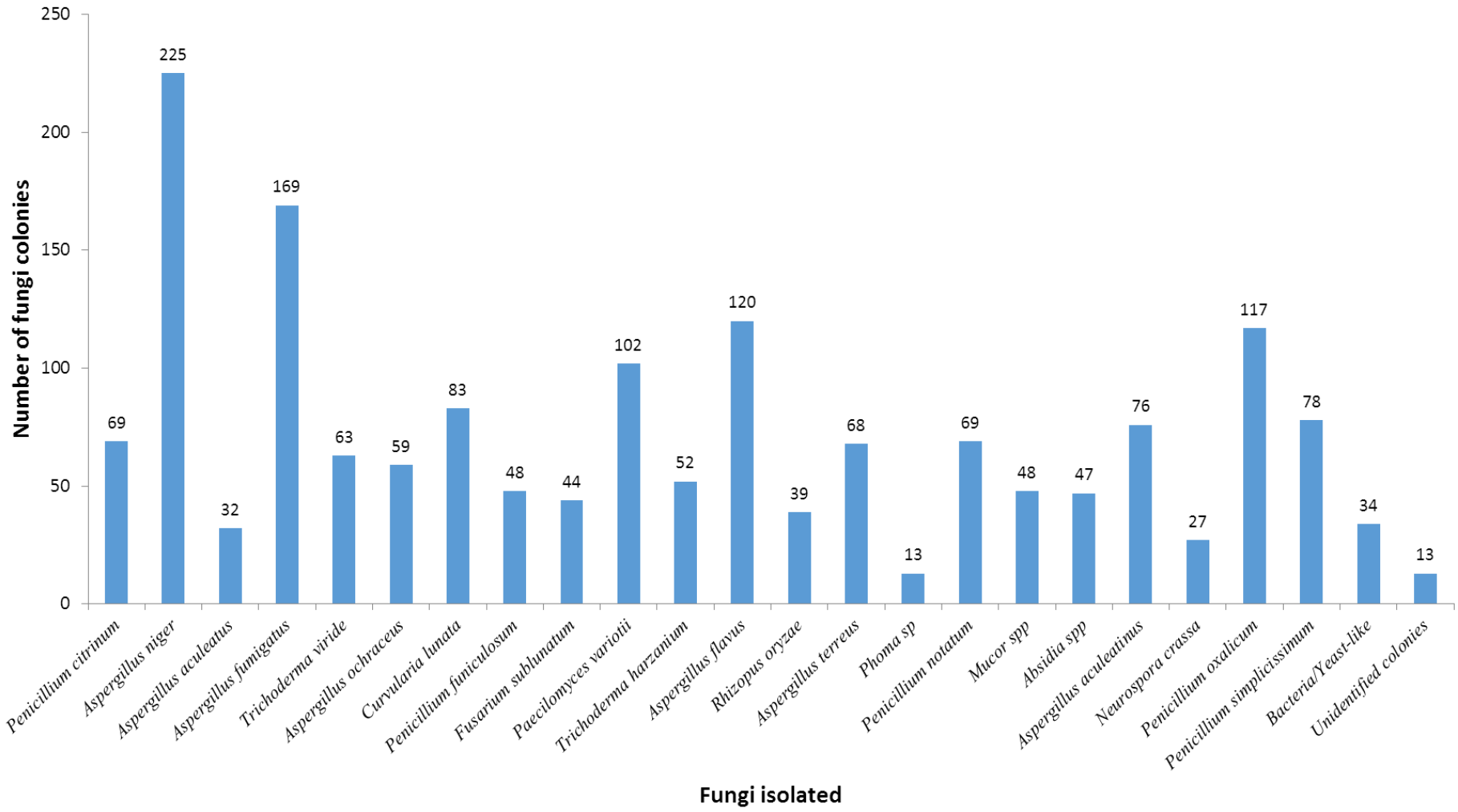


Figure 4.10: Frequency of fungi isolated in Iyana church, Ibadan

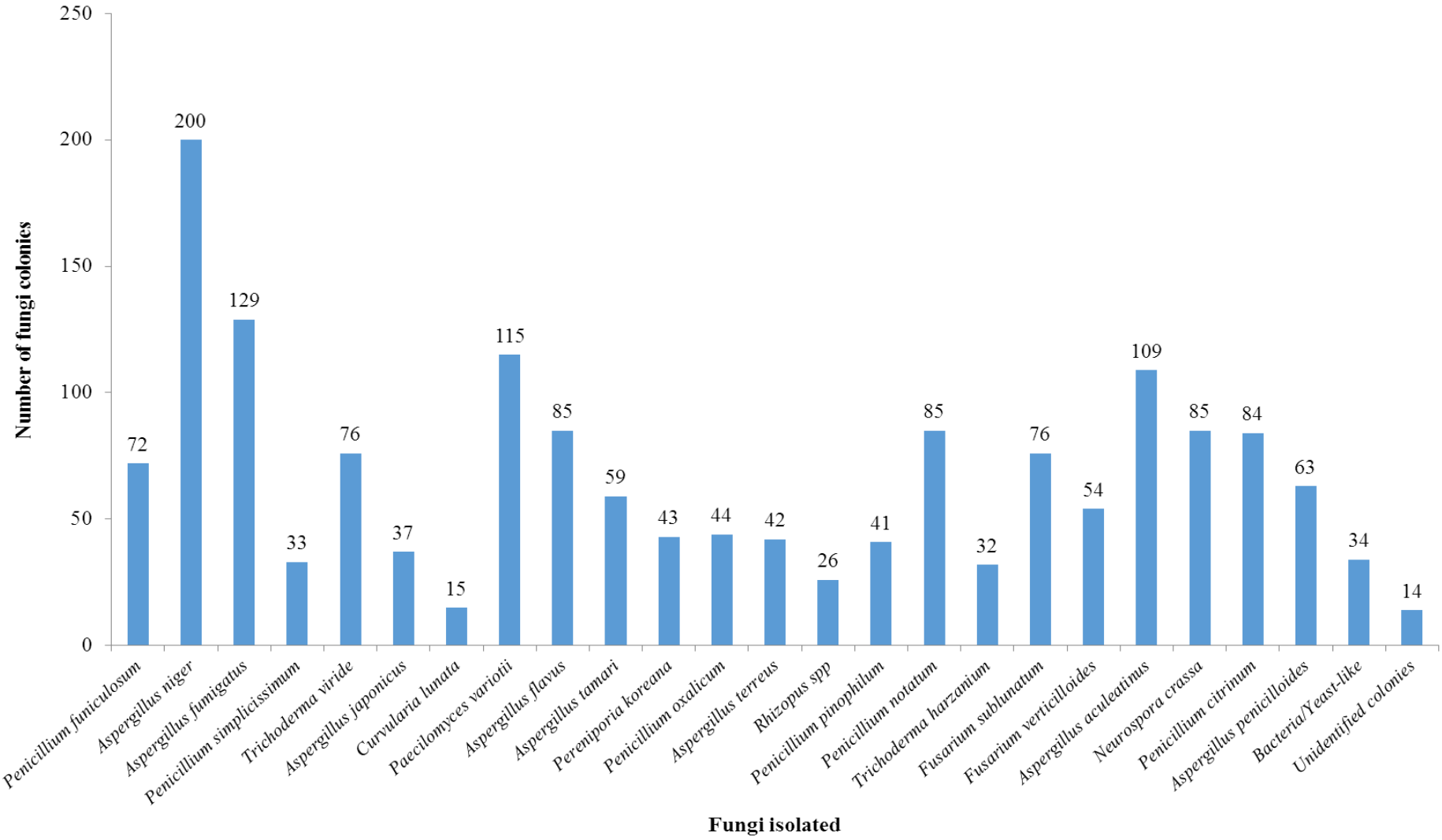


Figure 4.11: Frequency of fungi isolated in Mokola, Ibadan.

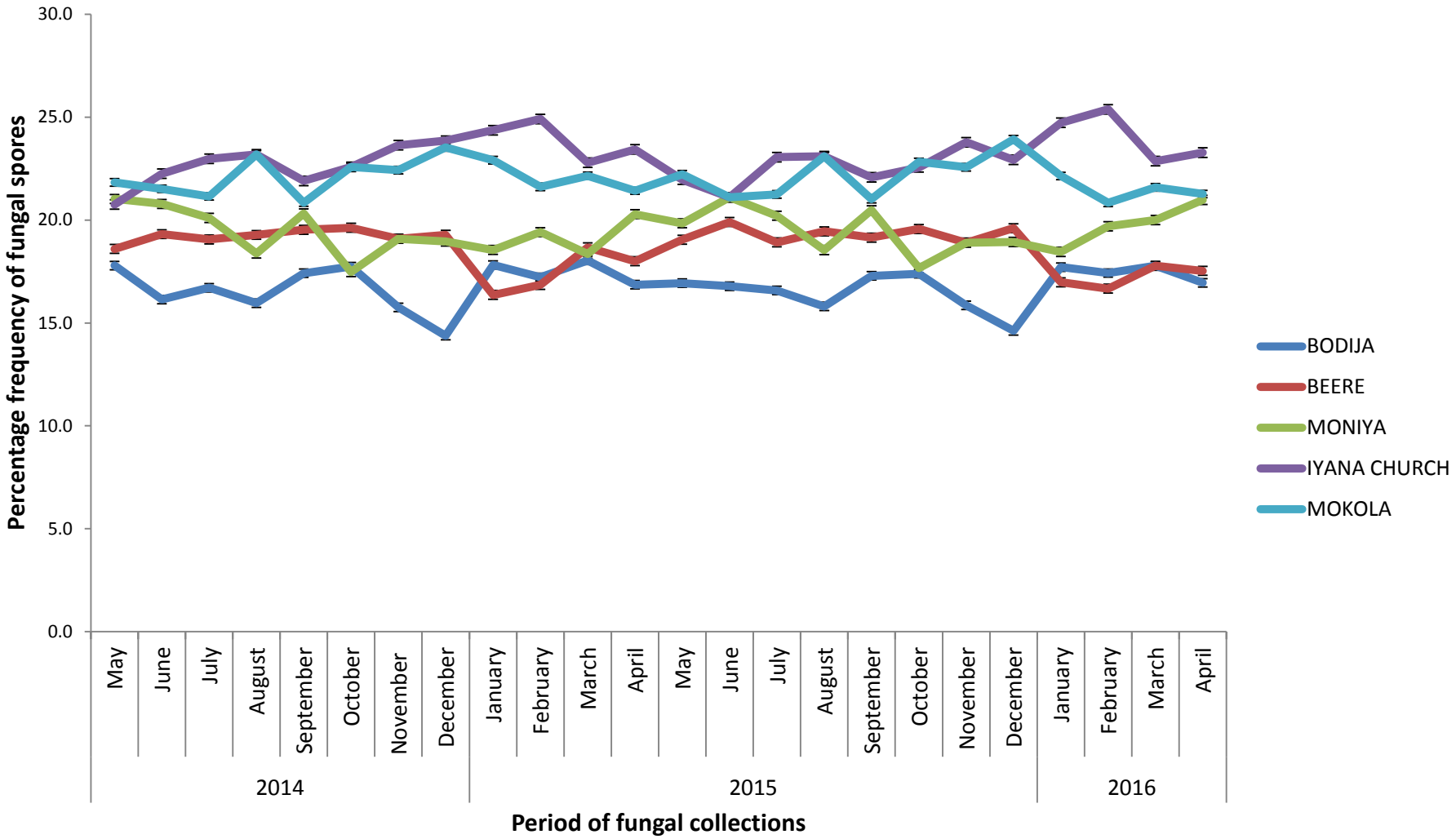


Figure 4.12: Frequency of fungi occurrence in Ibadan

4.4 Variations in the monthly distribution of fungal spores

The fitted model of the analysis of variance for locations and monthly abundance of fungi spores produced a highly significant ($p<0.01$) effect on the abundance of fungi in the two locations investigated. A significant ($p<0.01$) number of fungi were collected at different locations with respect to time using both media, but DG-18 showed no significant ($p>0.05$) difference on the different location of spore collection in Ibadan (Table 4.2).

Monthly abundance of fungal spores collected for twenty four months spanning May, 2014 – April, 2016 for both states showed that the month of June 2014 for Ibadan was significantly different ($p<0.05$) from other months with the highest value of (31.00) for DG-18 and (29.20) for Lagos State while lowest abundance was in December, 2014 with (14.80). In the second year of collection, Lagos state (31.40) had highest abundance of spores collected also in the month of June with respect to DG- 18 agar medium while December same year in Lagos with PDA as medium had least fungal spores collected (15.20) (Table 4.3).

Abundance of fungal spores in different locations with respect to media showed that organisms collected at Beere with PDA and DG- 18 agar had significant difference ($p<0.05$) from all other locations in Ibadan while Moniya had the lowest number of spores collected on PDA and Mokola had lowest number of fungi spores for DG- 18 agar. For Lagos state, Iba produced significant difference ($p<0.05$) from other locations for both PDA and DG- 18 agar followed by Ikeja (20.71 and 23.95) while Ikorodu had the lowest value (19.33) for Lagos location with respect to both media (Table 4.4).

Results for pooled abundance percentage of fungal spores collected during the twenty four months of sampling months showed that Ibadan had slightly lower mean value (49.50 %) than Lagos State (50.50 %) (Fig 4.14).

The results of pooled percentage of total fungal collection with respect to media showed that DG- 18 agar with (53.8) had higher percentage of fungal spores collected during the period of sampling than PDA (46.2 %). Fungi spores sporulated more and had more number of colonies in DG- 18 agar than PDA media (Fig 4.15).

Results for pooled percentage of fungal spores collected monthly for the period of two years showed that December, 2014 had the lowest collection of (3.1 %) followed by December, 2015 (3.2 %). The highest collection was in the month of June (5.2 %). Results also showed that the second year collection had higher monthly percentage fungi spores collected than the first year. There was gradual increase in fungal spore collection from the months of March which was (4.0) to 4.3 in April, 4.6 in May and highest collection in June (Fig 4.13).

4.5 Media comparison in relation to fungi abundance

For Potato Dextrose Agar, June 2014 had the highest value followed by June 2015 while December 2015 had the lowest value in Ibadan. In Lagos, the highest value for PDA treatment was observed in the month of June, 2015 which is not significantly different $p<0.05$ from the value (31.00) observed during June 2014. Dichloran-Glycerol 18 had the highest value in June 2014 at Ibadan which is not significantly different from the value in June 2015 in Lagos. The lowest values were observed in December for years 2014 and 2015 in both states.

Towards the middle of the year, both PDA and DG-18 were not significantly different ($P \leq 0.05$) from each other for both Lagos and Ibadan. Higher values were observed during the periods of May –August which are significantly different ($P \leq 0.05$) from the values observed early and late in the year for both 2014 and 2015, states and treatments (Table 4.3).

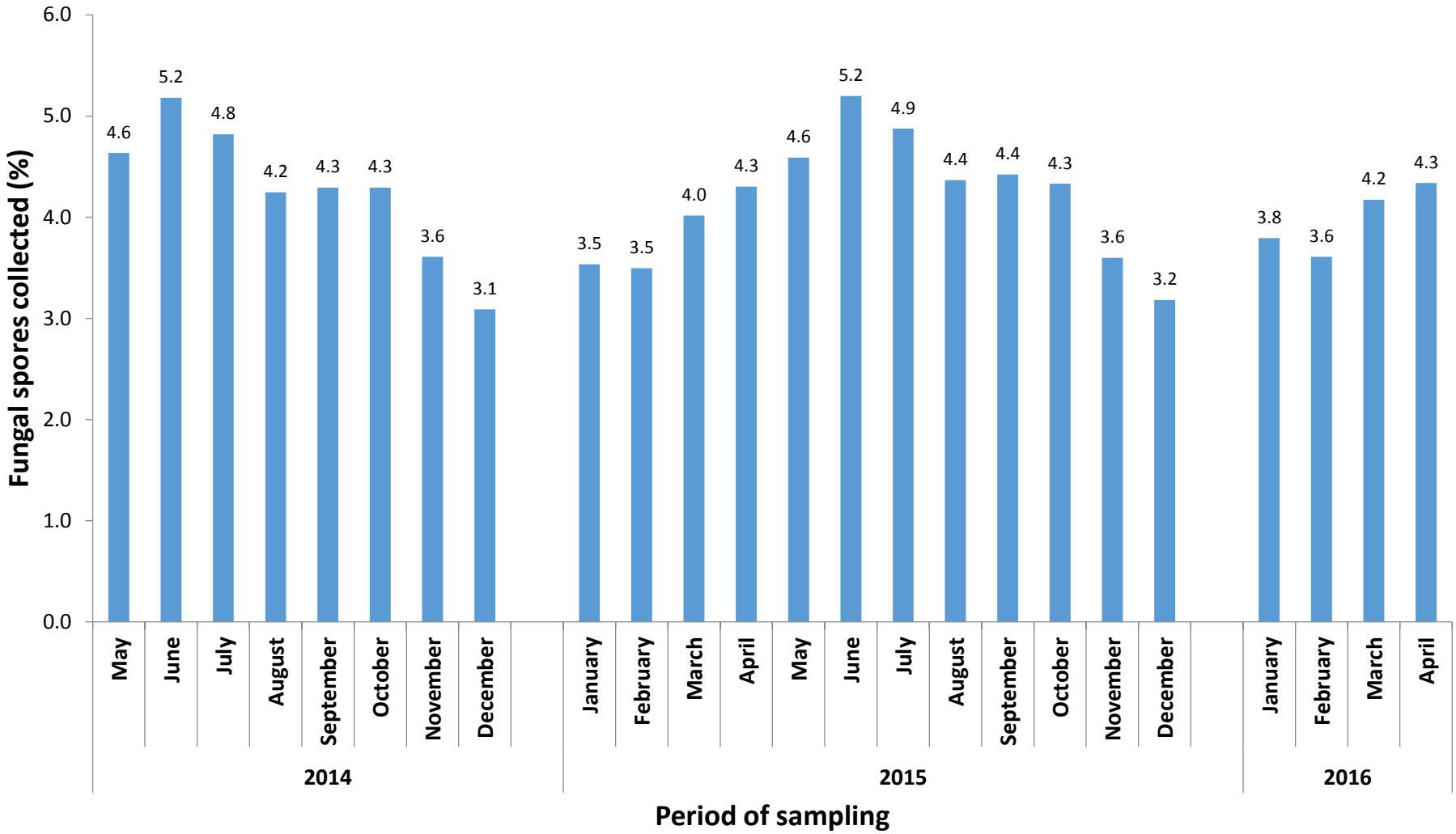


Figure 4.13: Pooled percentage fungal spores collected monthly during the period of sampling.

Table 4.2: ANOVA of media comparison of locations with monthly abundance of fungi spores.

Source	df	PDA		DG-18	
		IBADAN	LAGOS	IBADAN	LAGOS
Model	27	38.21**	50.13**	50.23**	53.74**
Time (Months)	23	42.73**	48.24**	57.68*	53.92**
Location	4	12.19**	60.97**	7.41	53.72**
Error	92	116.07	160.52	127.16	245.1
Corrected total	119	1147.7	1513.92	1483.47	1696.32

Note: ** = Highly significant ($p<0.01$), * = Significant ($p<0.05$), ns = not significant.

Table 4.3: Monthly comparison of abundance of fungal spores isolated

Year	Months	PDA		DG-18	
		IBADAN	LAGOS	IBADAN	LAGOS
2014	May	23.00c	23.40cd	26.60c	27.20bc
	June	26.60a	25.20ab	31.00a	29.20b
	July	25.40ab	23.40cd	29.00b	26.40cde
	August	21.60cde	21.20fg	24.60de	24.40ef
	September	21.60cde	21.20fg	25.20cd	24.80def
	October	21.40def	21.60efg	25.60cd	24.20f
	November	18.60hij	17.20h	22.20fg	20.00ghi
	December	15.80k	14.80i	17.80i	18.40i
2015	January	17.20jk	17.40h	20.60h	21.20g
	February	17.20jk	17.20h	20.40h	20.80g
	March	20.00fgh	20.00g	23.00f	23.80f
	April	22.00cde	21.40fg	25.20cd	24.40ef
	May	22.80cd	23.20cde	26.40c	26.80cd
	June	25.00b	26.00a	30.00ab	31.40a
	July	25.20ab	24.00bc	29.00b	27.20bc
	August	22.00cde	21.80def	25.60cd	25.00def
	September	22.20cde	22.00def	25.80cd	25.60cdef
	October	21.40def	22.00def	25.60cd	24.60ef
	November	18.80ij	17.40h	22.20fg	20.20ghi
	December	16.40k	15.20i	18.60i	18.60hi
2016	January	19.80gh	17.40h	23.20ef	21.60g
	February	18.80hi	17.20h	21.40gh	20.60gh
	March	21.00efg	20.60fg	24.60de	24.00f
	April	22.20cde	21.80def	26.00cd	23.80f
	LSD	1.41	1.65	1.47	2.05
	EMS	1.26	1.74	1.38	2.66

Note: Means with different letters across the column are significantly ($p < 0.05$) different from one another with respect to each parameter (One way Anova).

LSD = Least Significant Difference

EMS= Error Mean Square

Table 4.4: Abundance of fungal spores in different locations

STATE	LOCATION	MEDIA	
		PDA	DG-18
IBADAN	Beere	22.29a	25.46a
	Bodija	20.96b	24.62b
	Iyana church	20.83b	24.54b
	Mokola	20.58b	24.17b
	Moniya	20.58b	24.04b
	LSD	0.64	0.67
	EMS	1.26	1.38
LAGOS	Iba	23.21a	26.50a
	Ikeja	20.71b	23.95b
	Ikorodu	19.33c	23.08bc
	Oshodi	19.92c	23.25bc
	Victoria Island	19.46c	23.83c
	LSD	0.75	0.94
	EMS	1.74	2.66

Mean with the different letter across the column are significantly ($p < 0.05$) different from one another with respect to each parameter
LSD = Least Significant Difference

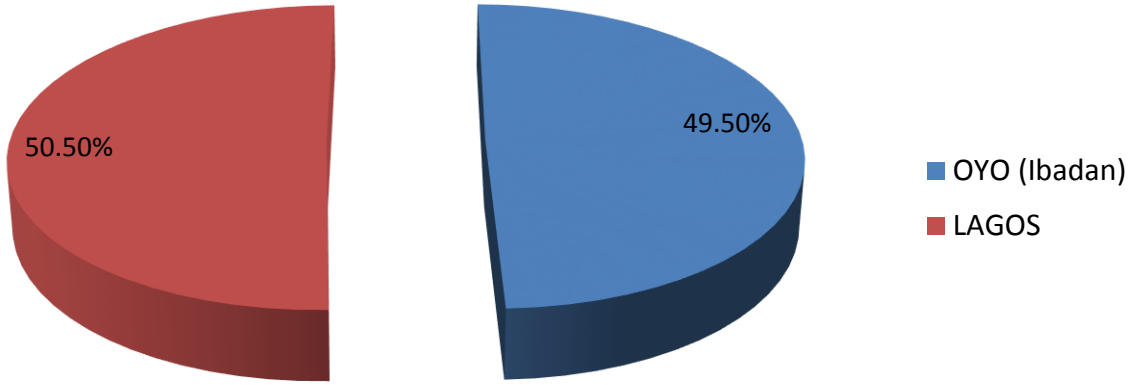


Figure 4.14: Pooled abundance of fungi spores in Lagos and Ibadan.

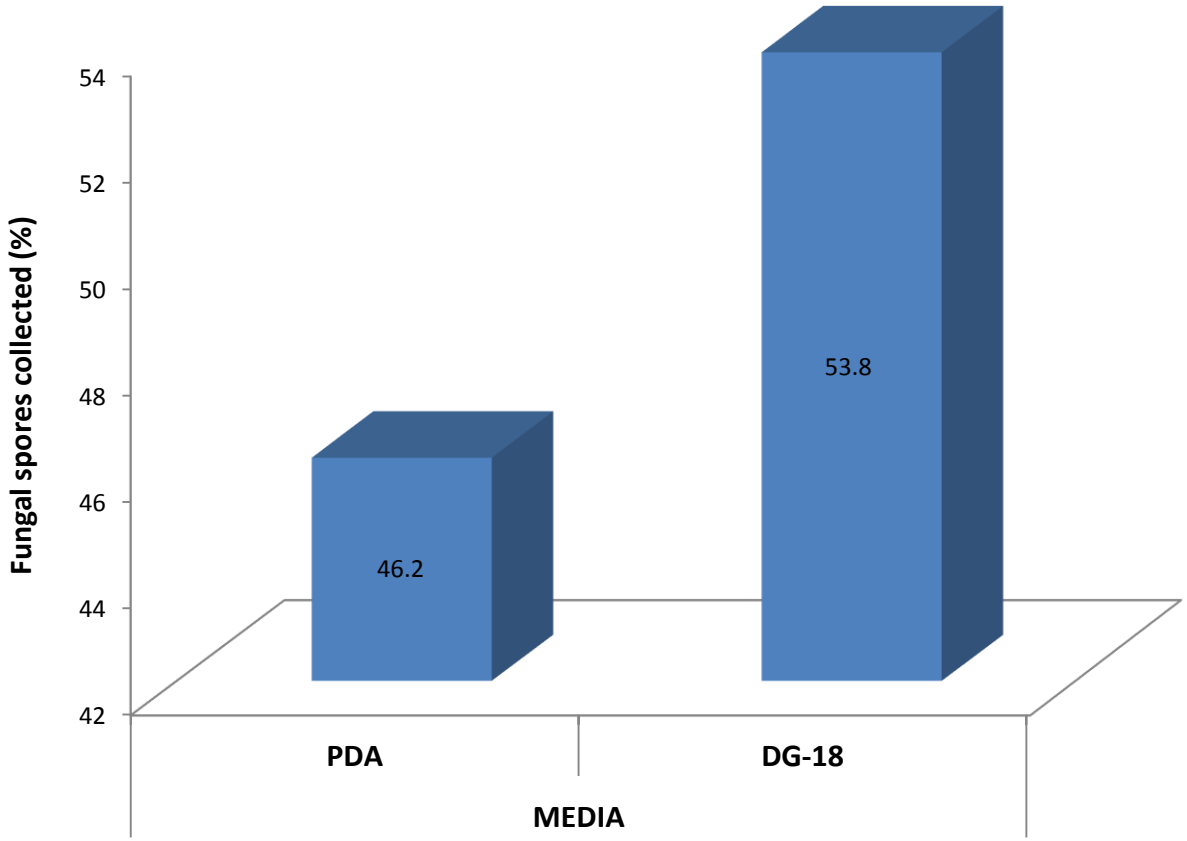


Figure 4.15: Pooled percentage fungal spores collected based on media comparison

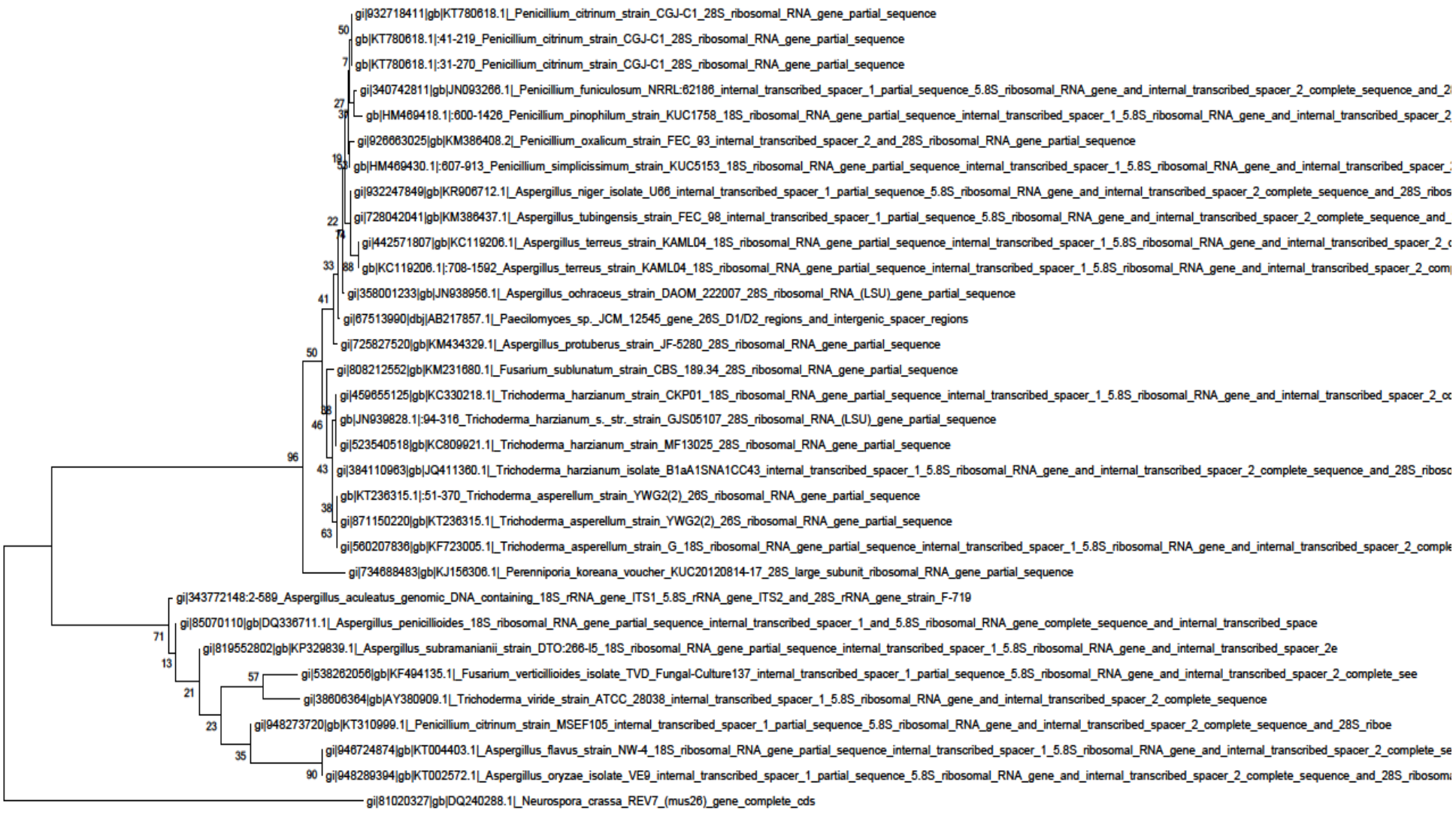


Figure 4.16: Phylogenetic tree of isolates showing relationship of the isolates using Neighbour Joining Method.

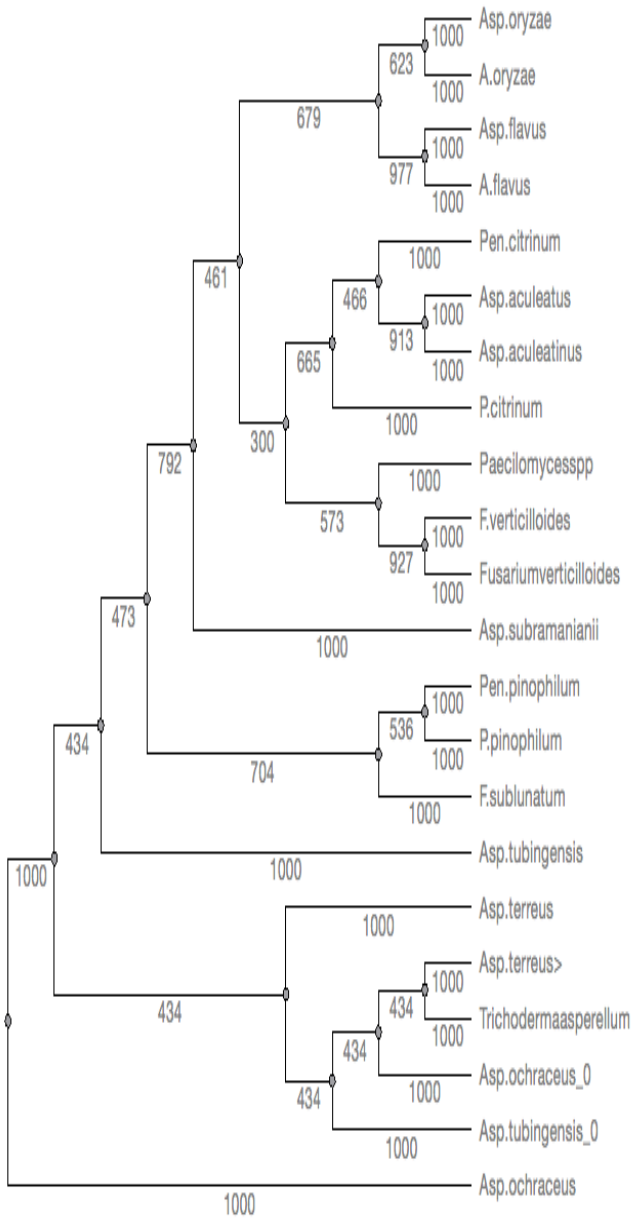


Figure 4.17: Phylogenetic analysis showing relationship of the isolates using Jalview

4.6 Meteorological data for the period of sampling

The meteorological data covering the months of May, 2014 to April 2016 for Lagos State showed a fairly steady total rainfall except for the months of January and February, 2015 and 2016 while the rainfall rose in June (55 mm) – July (51 mm), 2014, May (57 mm) to July (65 mm), 2015 and March (56 mm) – April (59mm), 2016 but a decline was recorded in the months of January (25 mm) and February (20 mm), 2015 (Fig 4.18). For Ibadan, highest rainfall was recorded in the months of May (170.1 mm), August (174.4 mm) and October (221.4 mm) 2014 recorded the highest during the period of sampling. April (132.2 mm), May (159.9 mm), June (183.6 mm), September (180.9 mm) and October (184.3 mm), 2015 while February (17.7 mm), 2015 recorded the least rainfall during the period of sampling.

Atmospheric temperature was between 25 ^{0}C – 28 ^{0}C throughout the period of sampling in Lagos State with highest temperature recorded in February (29.3^{0}C) and March (29.2^{0}C) 2016. The months of Feb (35.4 ^{0}C), Mar (35.4 ^{0}C) 2015 same with March (35.6 ^{0}C) and April (36.1 ^{0}C) 2016 recorded the highest months of atmospheric temperature for Ibadan while the month of August (27.6 ^{0}C) 2014 recorded the lowest atmospheric temperature.

Relative humidity was constant at 88 % for the months of June, August, September, and peaked in July (89 %) in 2014 while the month of July (90 %) recorded the highest relative humidity in 2015. The month of January (76 %) 2015 recorded the month with lowest relative humidity throughout the period of sampling for Lagos State. For year 2014, the month of July (89 %) had the highest relative humidity, while July (90 %) also recorded highest month of rainfall in 2015. In Ibadan, August 2015 (88 %) had highest relative humidity in 2015 while the month of July (91 %) 2015 recorded highest rainfall for the period of sampling. December (67 %) recorded lowest relative humidity throughout the period of sampling (Fig 4.17).

The wind speed which was high in July (8) 2014 to October (8) gradually reduced in November, December, January till May (6) 2015. There was gradual increase in July (9) 2015. Wind speed in Ibadan did not differ much from what was obtained in Lagos. Highest wind speed was recorded in July 2015 (9.2), while lowest was in December 2014 (5.1) (fig 4.17).

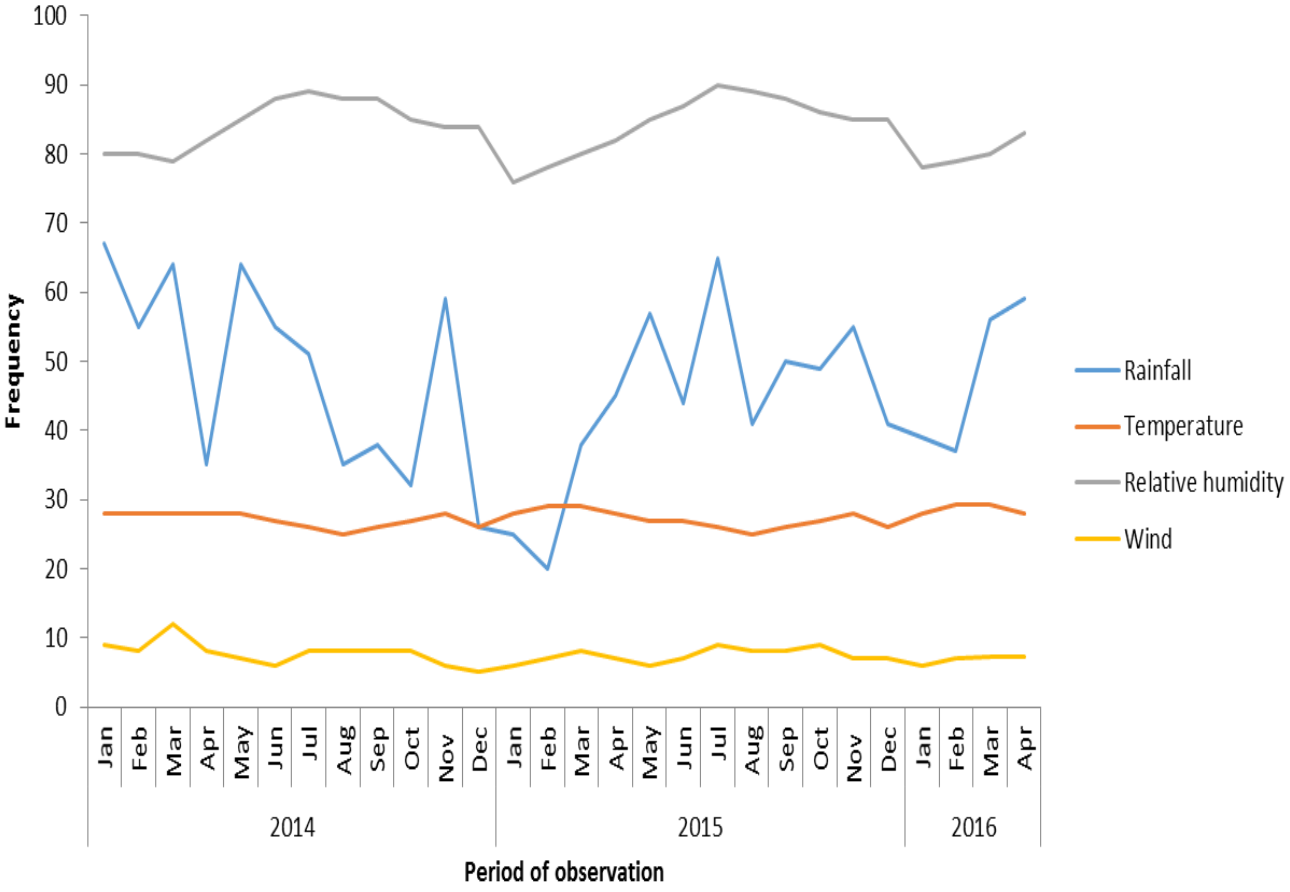


Fig 4.17: Meteorological data for Lagos during the period of fungi collection.

Source: Nigerian Meteorological Agency

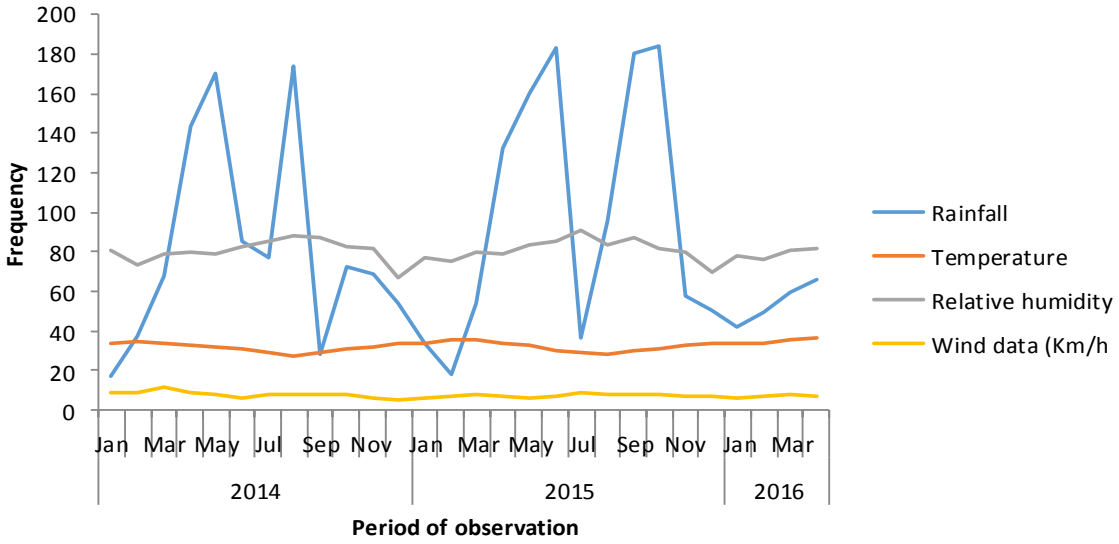


Fig 4.18: Meteorological data for Ibadan during the months of fungi collection.

4.7 Molecular characterization

Plates 4.1 and 4.2 show the gel picture of DNA extracted with modified CTAB protocol while Plate 4.3 showed the electrophorogram of DNA extracted with Qiagen plant kit.

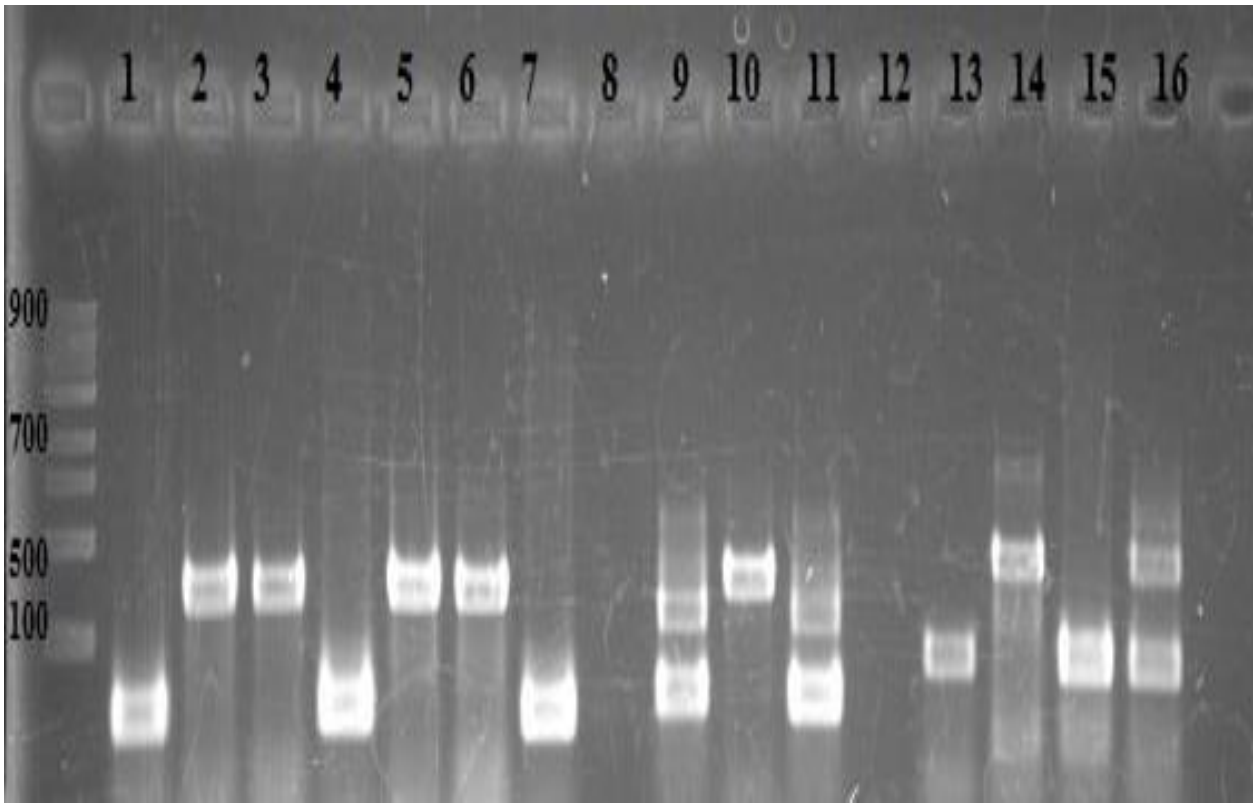


Plate 4.1A: Gel picture of extracted DNA samples using CTAB protocol. The first lane contains the marker. 1. *A. niger*, 2. *A. tubingensis*, 3. *A. flavus*, 4 *Neurospora crassa*, 5. *A. aculeatinus*, 6. *A.fumigatus*, 7. *A. niger* stran B, 8. *A. ochraceus* 9. *A. oryzae*, 10. *A. penicilloides*, 11. *A. protuberus*, 12. *A. subramanianii* 13. *A. tamari* 14. *A. tubingensis* 15. *Absidia blakesina* 16. *Aphaderanum sp*.

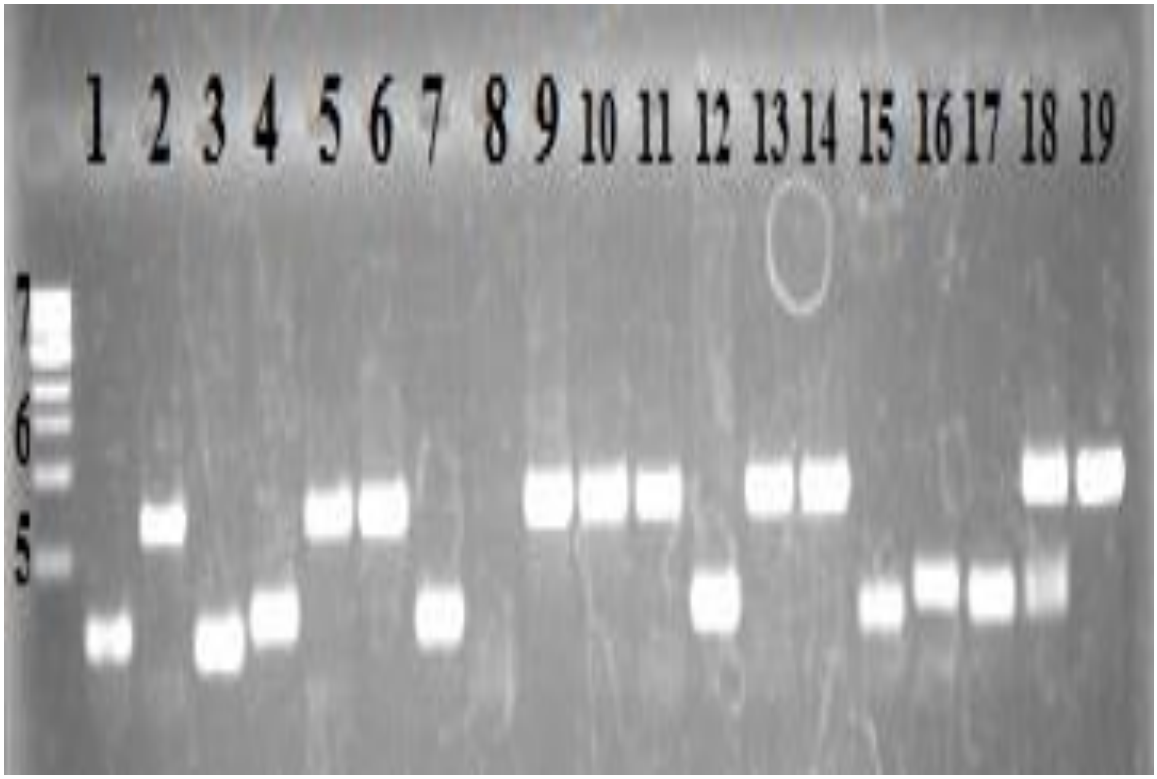


Plate 4.1B: Gel picture of extracted DNA samples using CTAB protocol. The first lane contains the marker. 1. *Aspergillus terreus*. 2. *Cladosporium herbarum*. 3. *Curvularia sp*. 4. *Fusarium verticilloides*. 5. *F. sublunatum*. 6. *Mucor* sp. 7. *Neurospora crassa*. 8. *Penicillium chrysogenum*. 9. *P. citrinum*. 10. *P. citrinum* strain B. 11. *P. oxalicum*. 12. *P. pinophilum*. 13. *Paecilomyces sp*. 14. *P. funiculosum*. 15. *P. simplicissimum*. 15. *Pereniporia koreana*. 16. *Phoma eupyrema*. 17. *P. citrinum*. 18. *Trichoderma helicum*. 19. *T. asperellum*

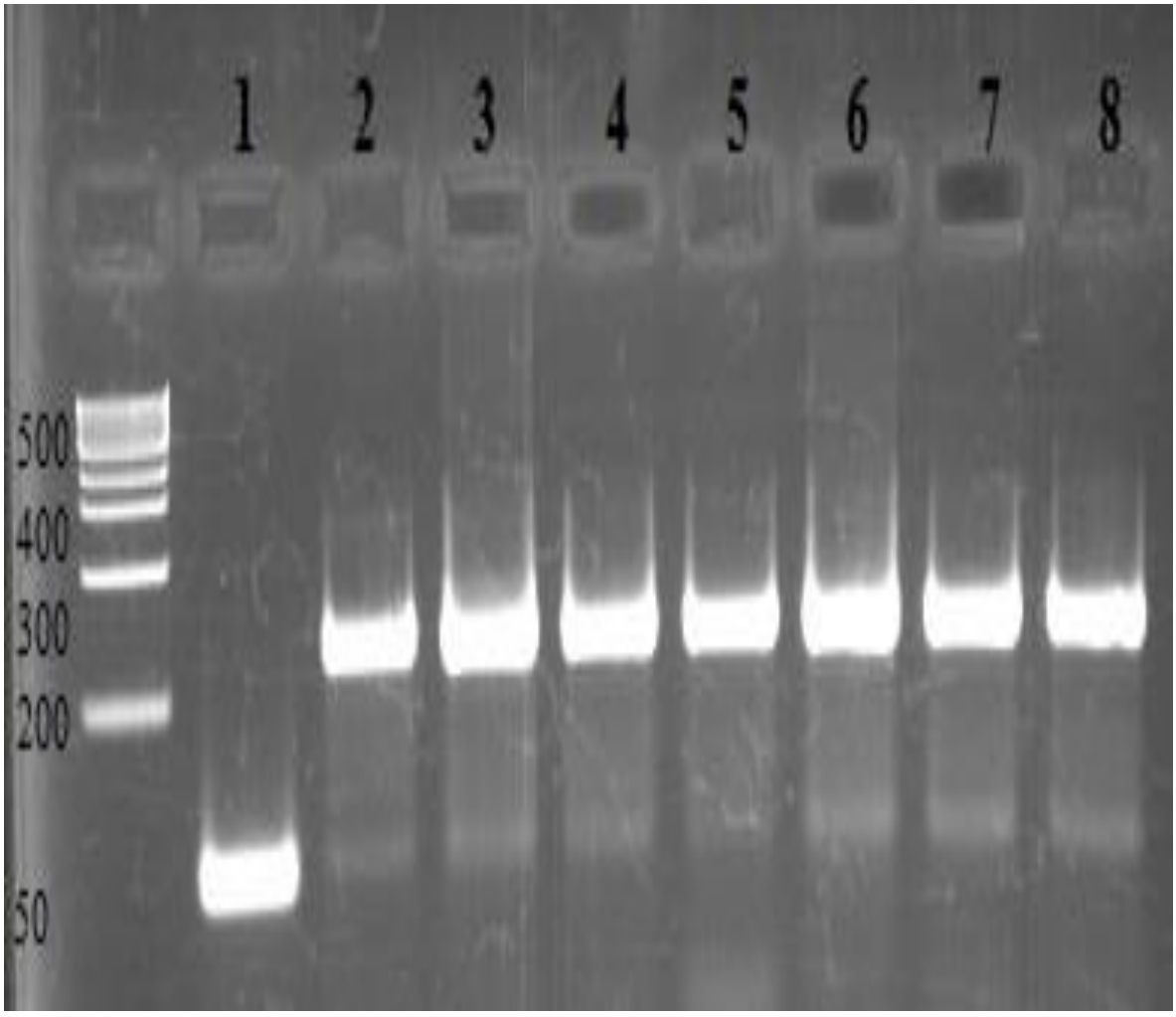


Plate 4.1C: Gel picture of extracted DNA samples using qiagen plant mini kit protocol.
The first lane contains the marker. 1. *Rhizopus stolonifer*. 2. *Sistotrema brinkmanii*. 3. *Trichoderma viride*. 4. *T. harzanium*. 5. *Curvularia sp*. 6. *Mucor sp*. 7. *A. tubingensis*

4.8 Multivariate linear regression Analysis showing relationship between fungal spore and weather parameters

For rainfall, the r value for Lagos was 0.20, p- value of 0.33 while slope was 5.72 (fig 4.19) while for Ibadan, slope was 37.07, p-value of 0.08 and r-value was 0.36.

Relative humidity had slope of 3.74 for Lagos, p-vaue of 0.01 and r-value of 0.62 while for Ibadan, the slope was 6.71, r value of 0.73 and p-value of 0.005.

For Lagos, temperature had slope of -0.58, r-value of -0.31 and p-value of 0.004 while Ibadan had -2.10 for slope, r-value of -0.56 and p-vaue of 0.004.

For Wind, Lagos had slope of 0.82, p- value of 0.074 and r-value of 0.37 while for Ibadan, slope was 0.54, r-value of 0.37 and p- value of 0.077 (fig 4.26).

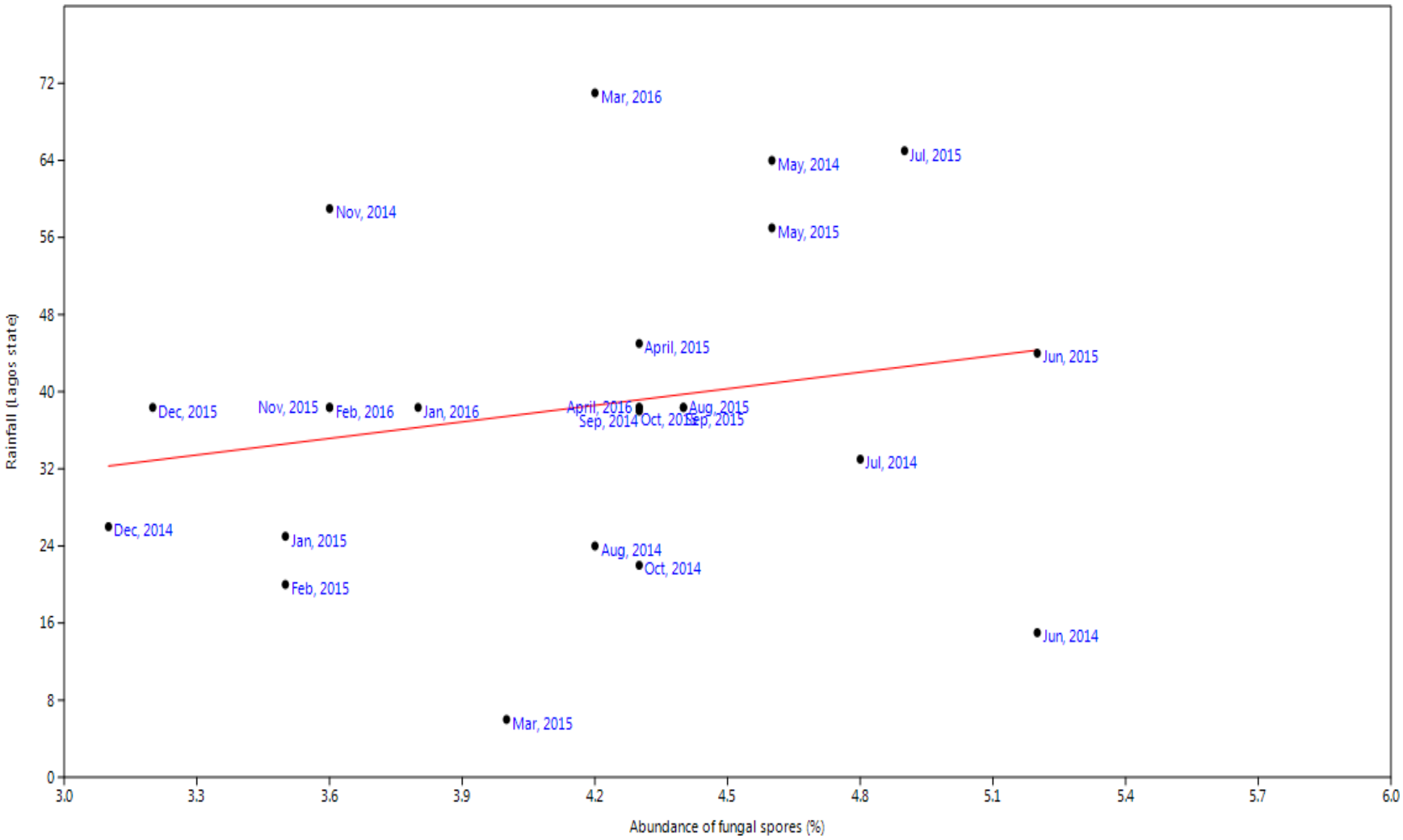


Fig 4.19: Multivariate linear regression between fungal spore abundance and rainfall in Lagos.

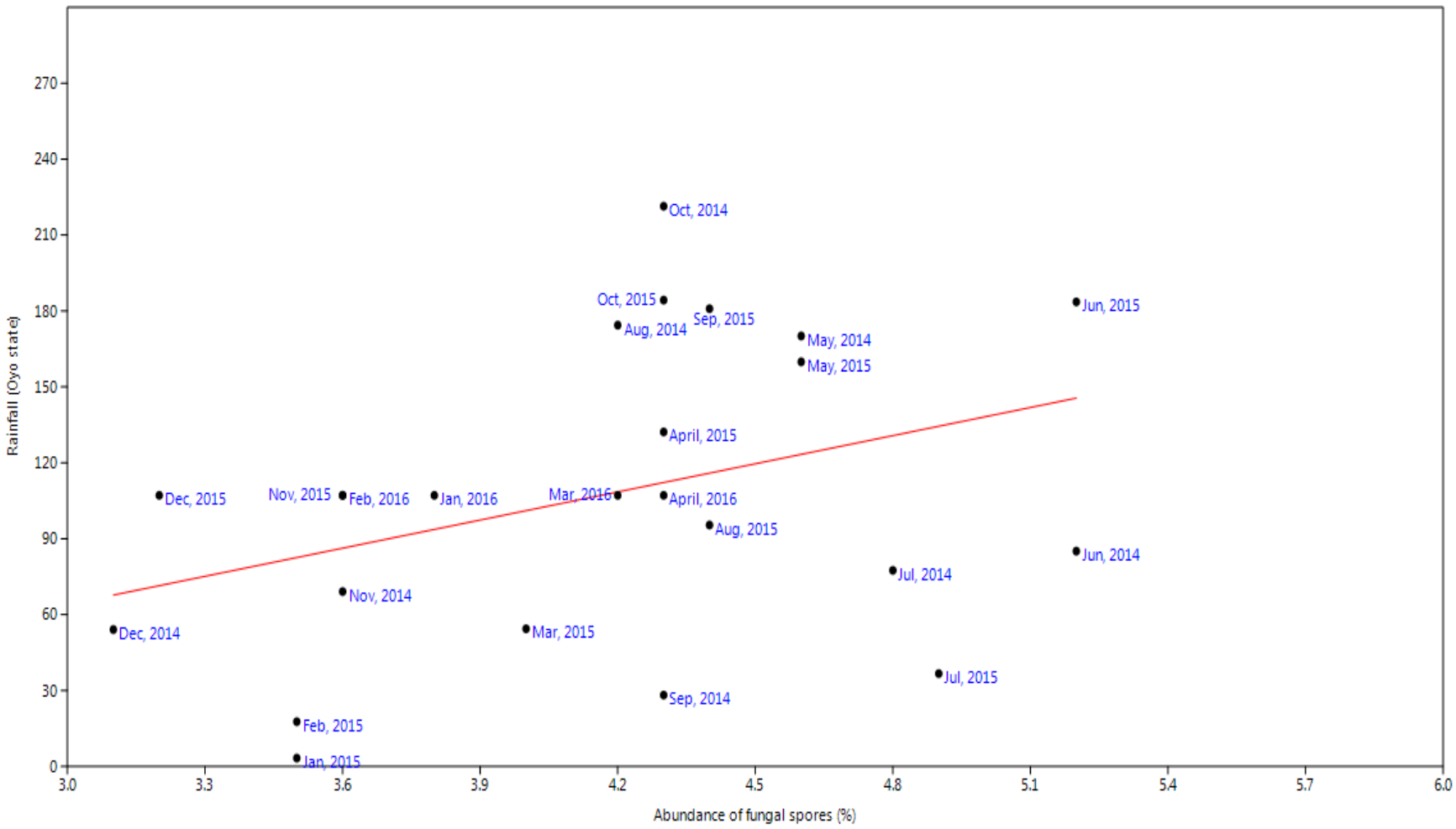


Fig 4.20: Multivariate linear regression between fungal spore abundance and rainfall in Ibadan.

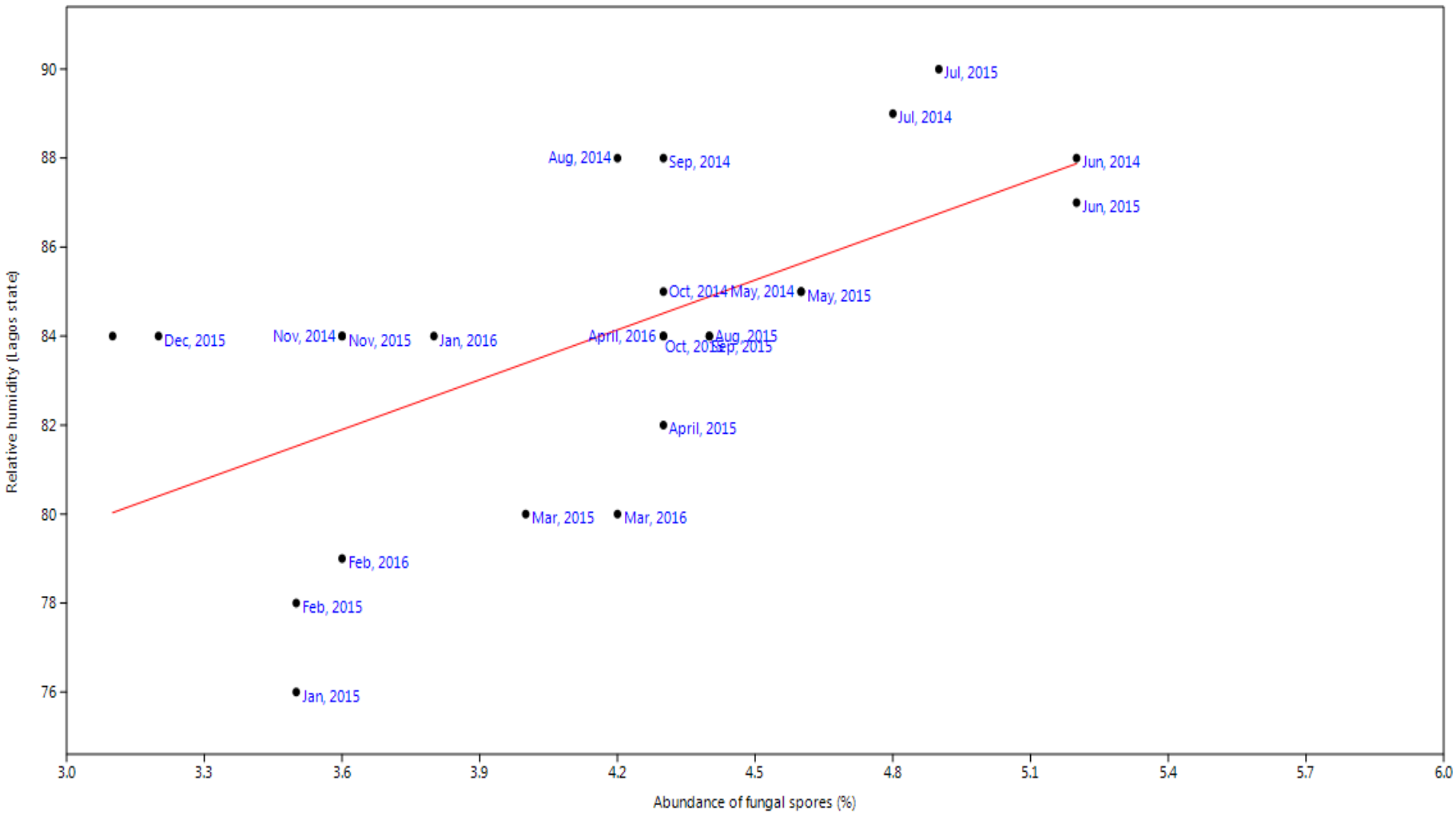


Fig 4.21: Multivariate linear regression between fungal spore abundance and relative humidity in Lagos.

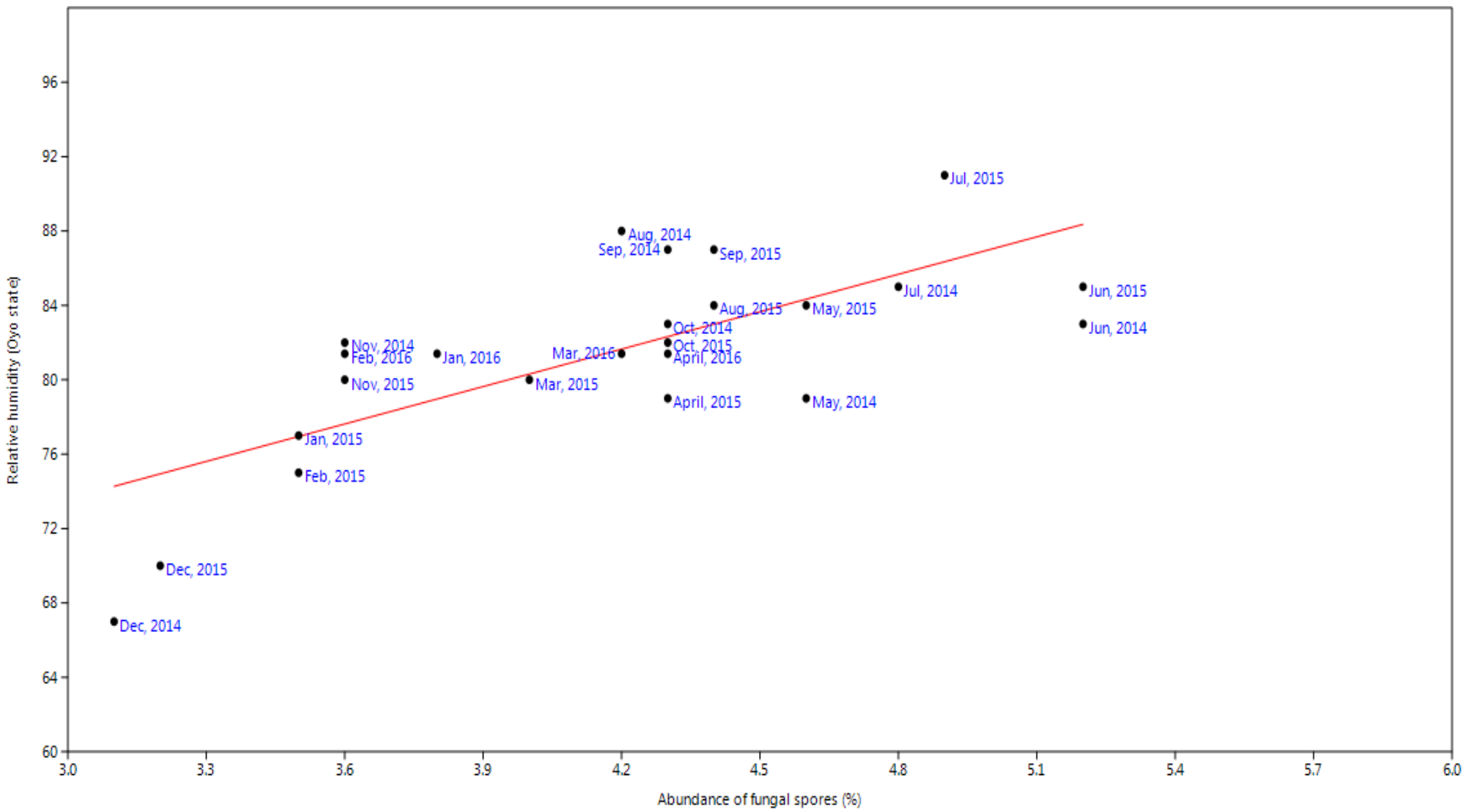


Fig 4.22: Multivariate linear regression between fungal spore abundance and relative humidity in Ibadan.

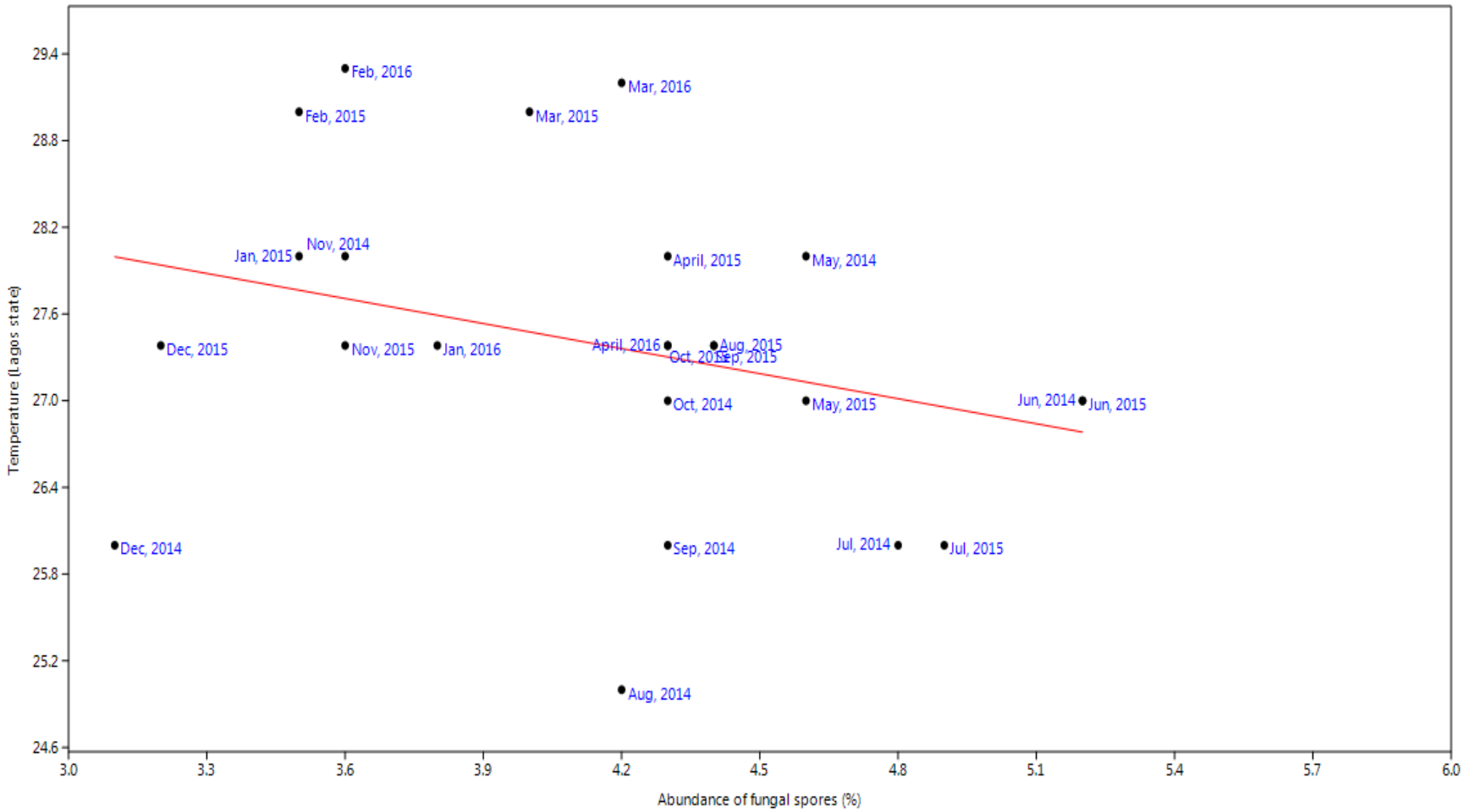


Fig 4.23: Multivariate linear regression between of fungal spore abundance and temperature in Lagos.

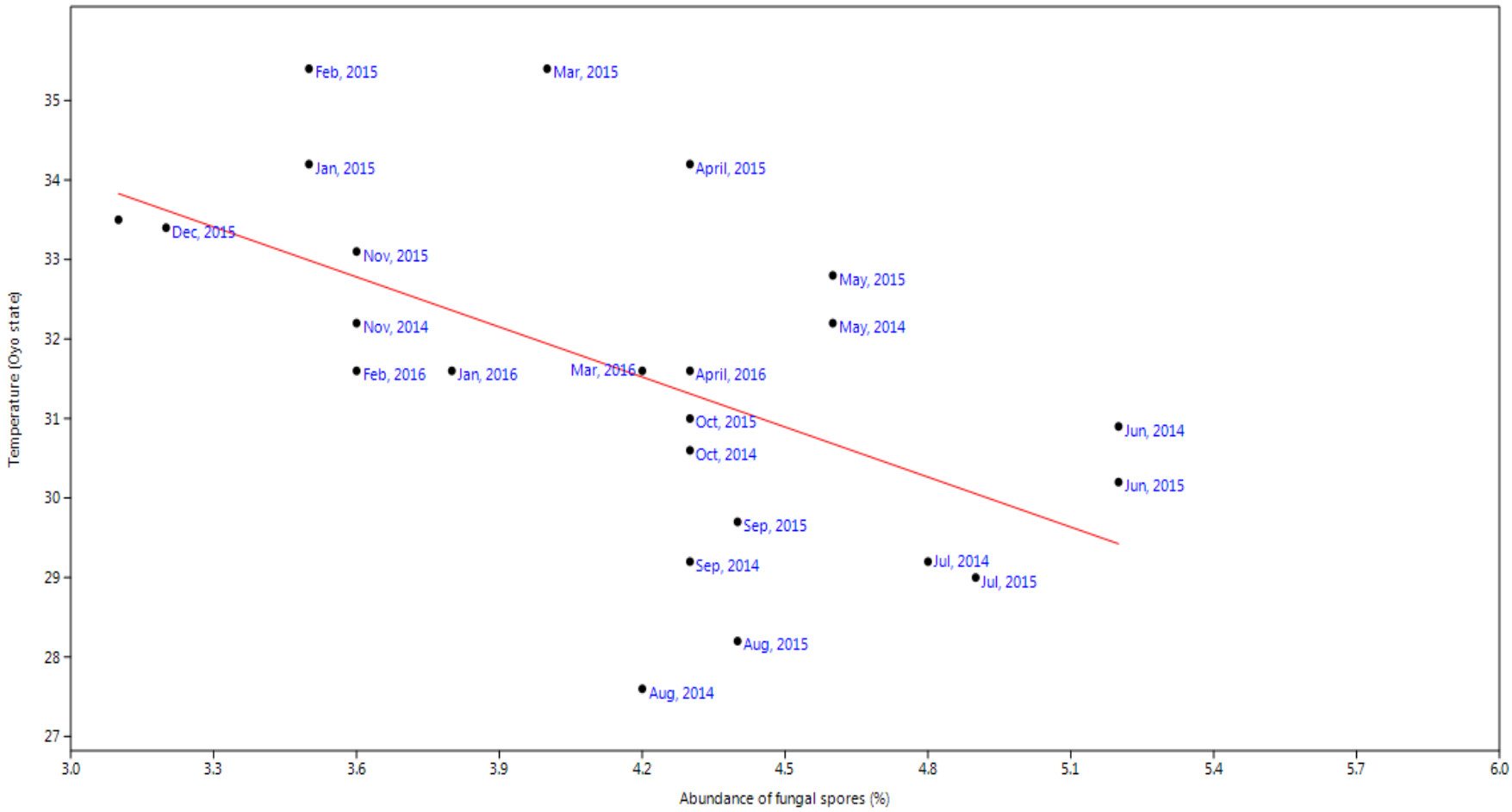


Fig 4.24: Multivariate linear regression between fungal spore abundance and temperature in Ibadan.

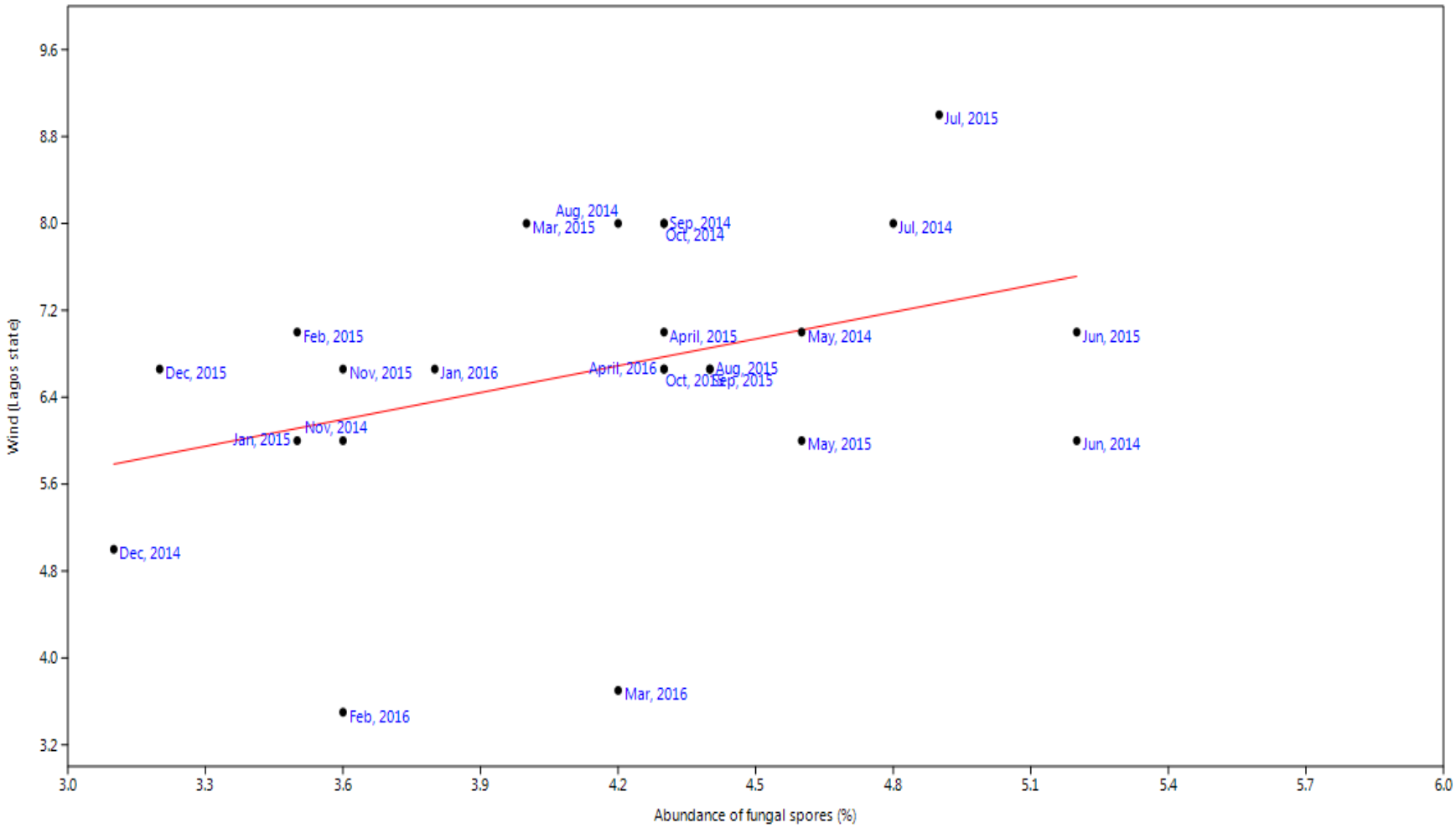


Fig 4.25: Multivariate linear regression between fungal spore abundance and wind speed in Lagos.

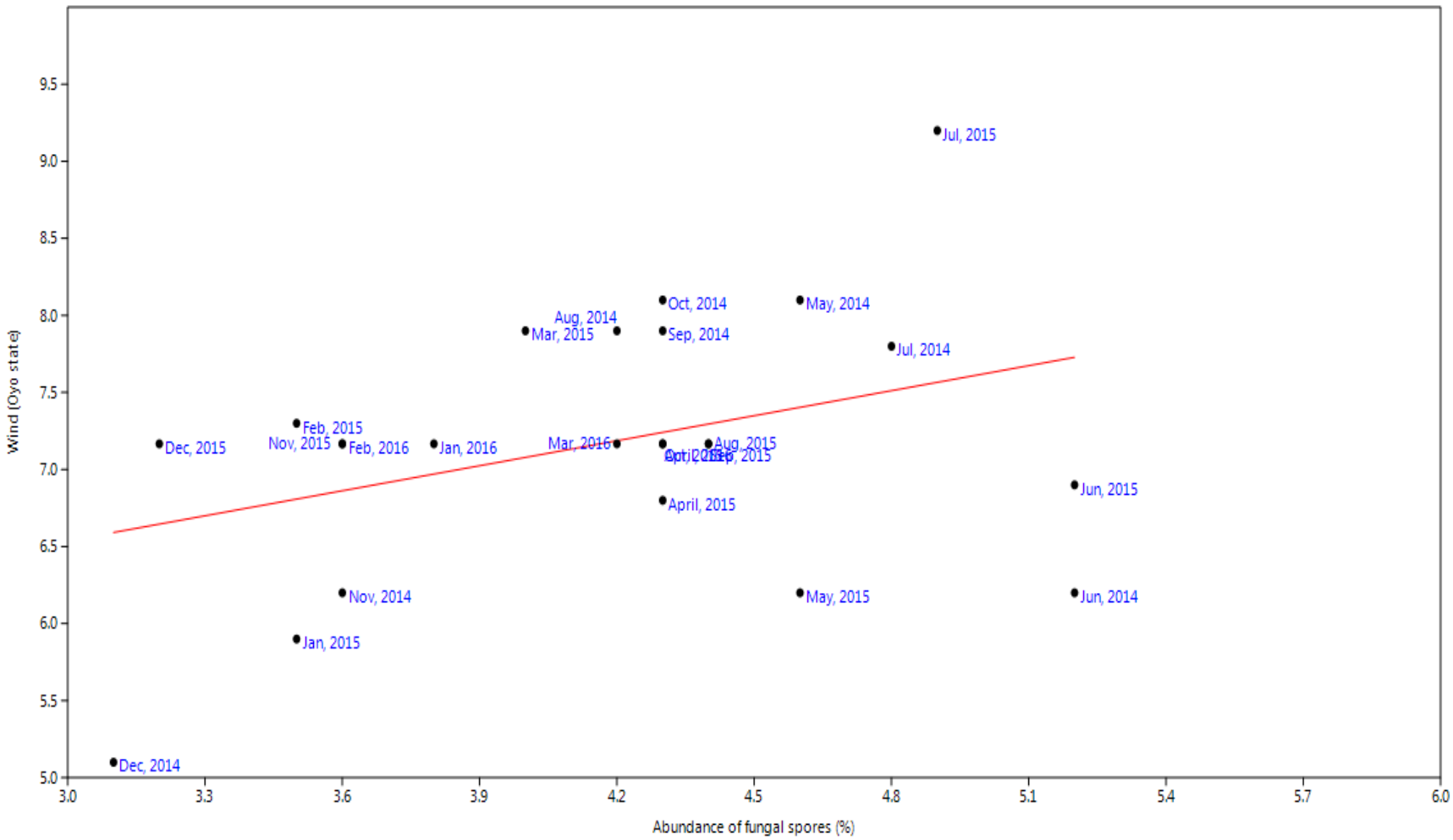
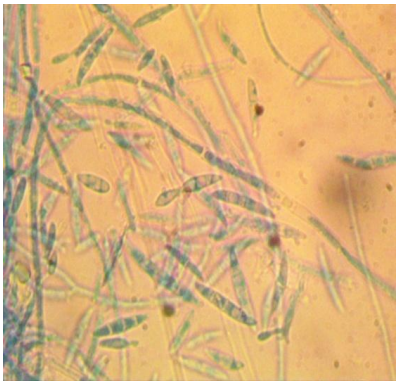
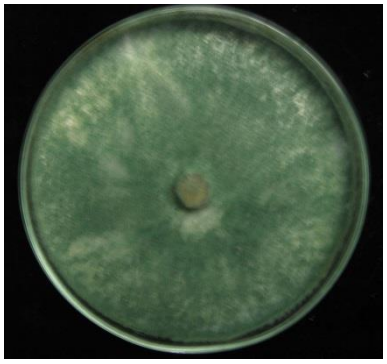


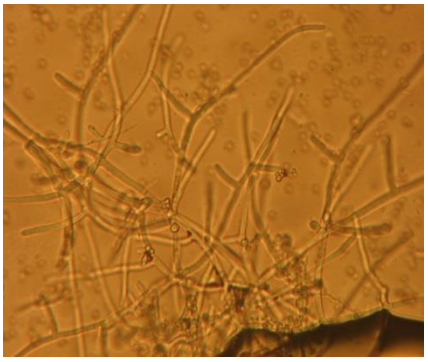
Fig 4.26: Multivariate linear regression between fungal spore abundance and wind speed in Ibadan.



Ai aii.

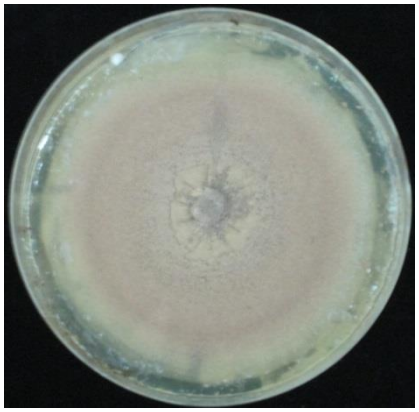
Plate 4.4 a: Culture plate and photomicrograph of *Fusarium verticilloides*





b bii.

Plate 4.4b: Culture plate and photomicrograph of *Trichoderma harzanium*



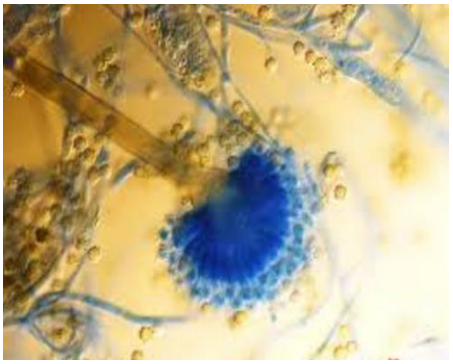
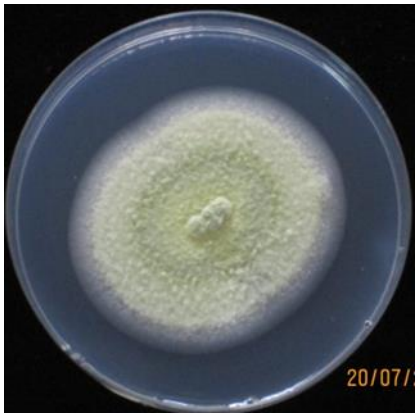
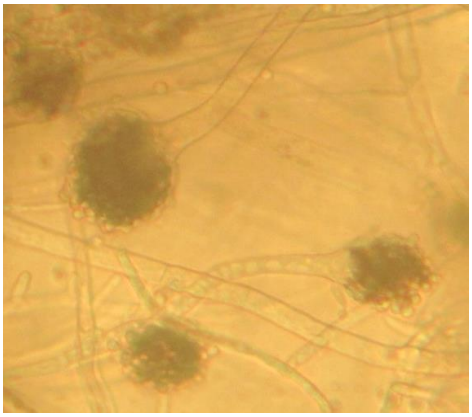


Plate 4.4c: Culture plate and photomicrograph of *Aspergillus tamari*

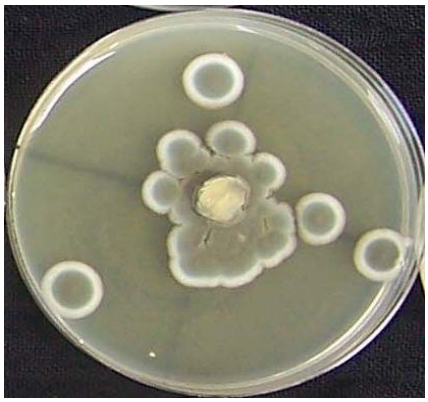
Plates 4.4 a-c: Culture plate and photomicrographs of some selected isolates.

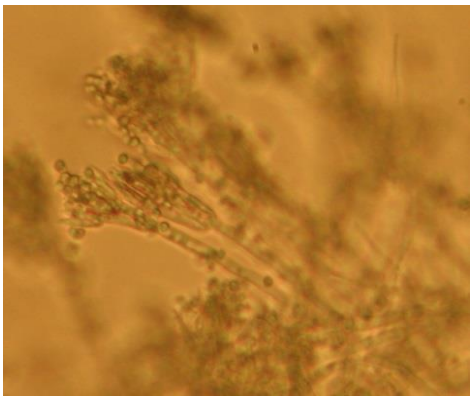




d(i) dii.

Plate 4.4 di-dii: Culture plate and photomicrograph of *Aspergillus flavus*

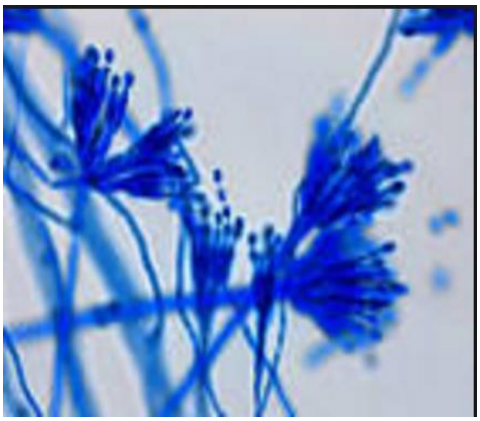




e(i) e(ii).

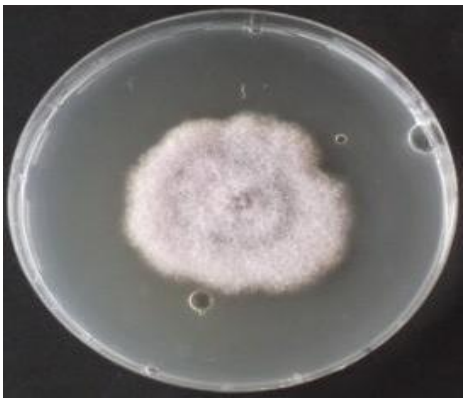
Plate 4.8: Culture plate and photomicrograph of *Penicillium chrysogenum*

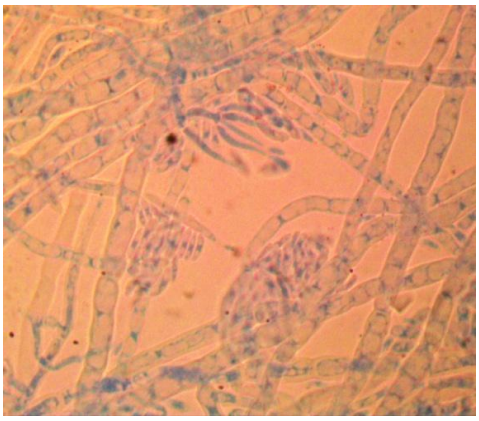
Plates 4.4 d-e: Culture plate and photomicrographs of some selected isolates.



F(i) f(ii).

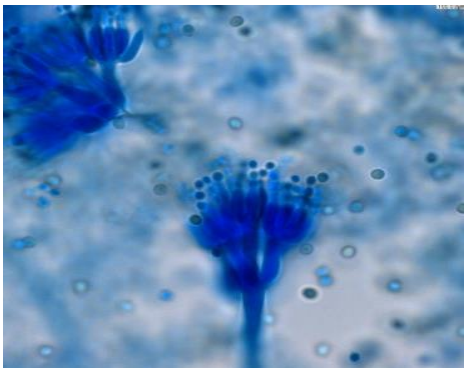
Plate 4.4f i-ii: Culture plate and Photomicrograph of *Paecilomyces sp*





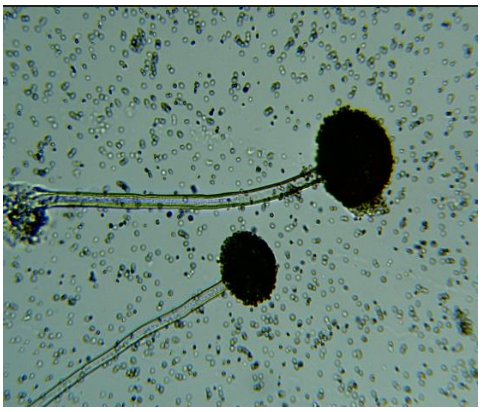
G(i) G(ii)

Plate 4.4G i-ii: Culture plate and photomicrograph of *Fusarium sublunatum*



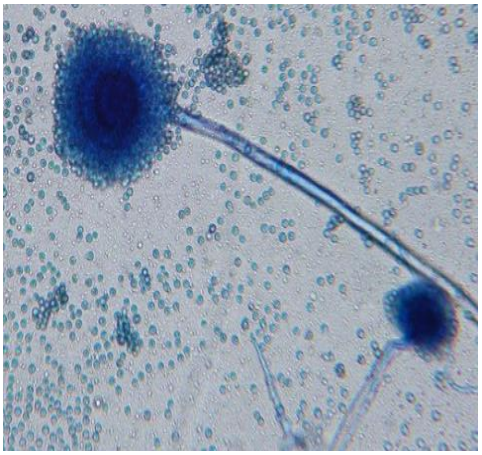
H(i) H(ii).

Plate 4.4H i-ii: Culture plate and photomicrograph of *Penicillium citrnum*



I(i) I(ii).

Plate 4.4 Ii-ii: Culture plate and photomicrograph of *Rhizopus spp.*



J(i) J(ii).

Plate 4.4Ji-ii: Culture plate and photomicrograph of *Aspergillus terreus*

Plates 4.4 I-J: Culture plate and photomicrographs of some selected isolates

4.9 Electron microscopy of some selected isolated fungi

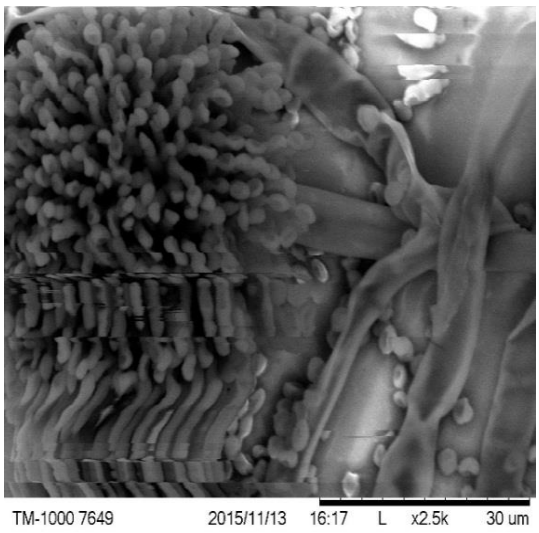


Fig 4.27: *Aspergillus ochraceus*

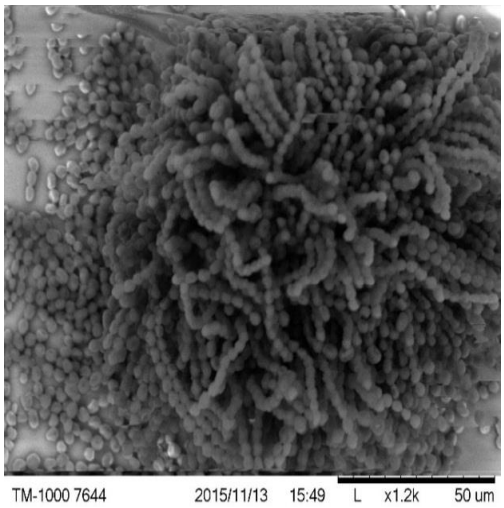


Fig 4.28: *Aspergillus terreus*

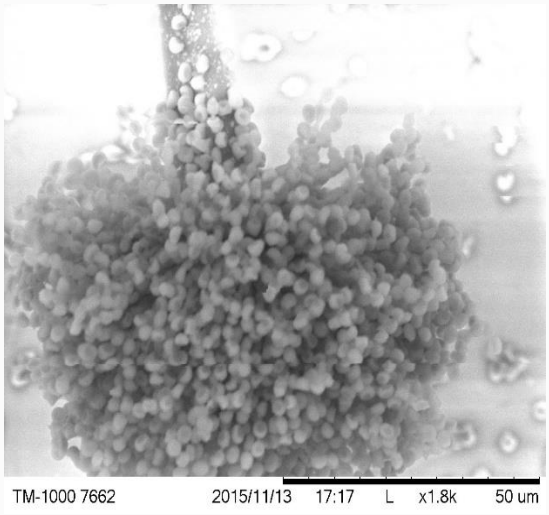


Fig 4.29: *Aspergillus oryzae*

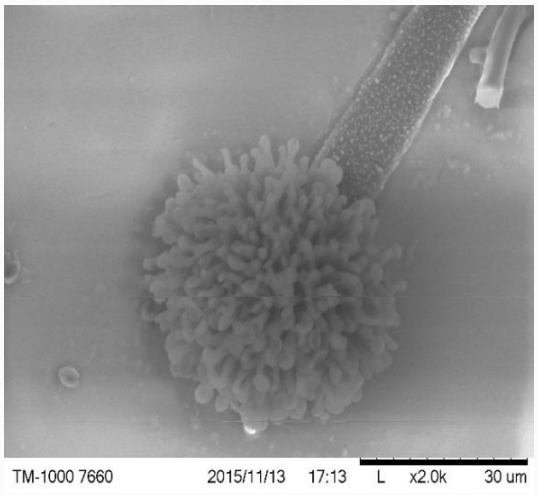


Fig 4.30: *Aspergillus protuberus*

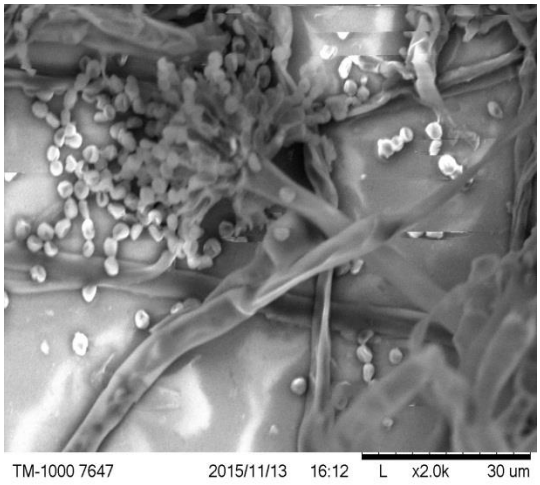


Fig 4.29B: *Aspergillus tubingensis*

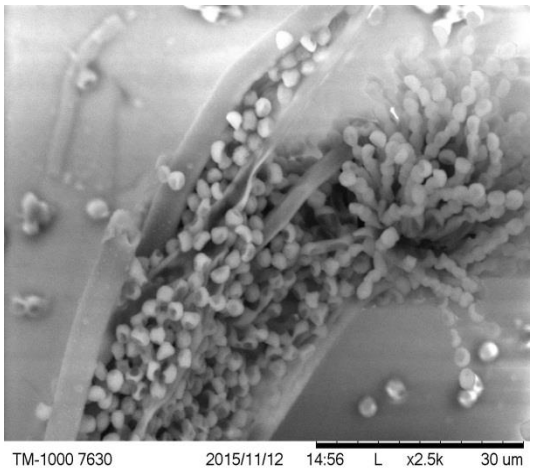


Fig 4.30B: *Aspergillus tamari*

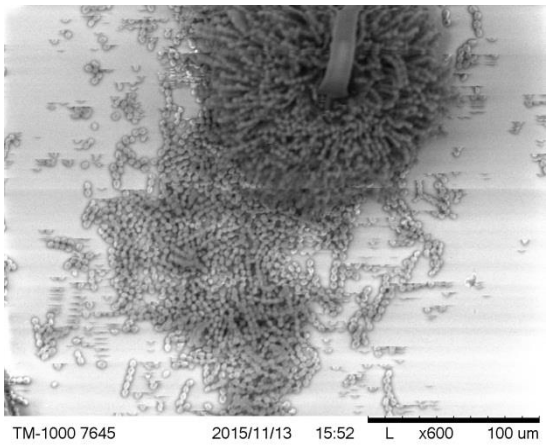


Fig 4.29C: *Aspergillus penicilloides*

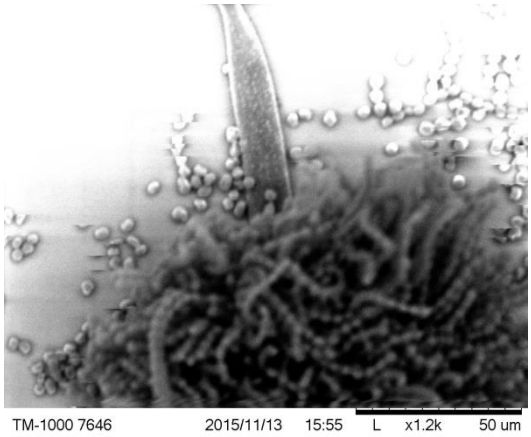


Fig 4.30C: *Aspergillus aculeatinus*

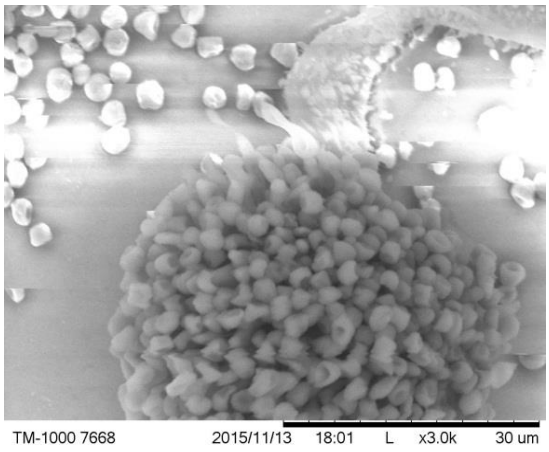


Fig 4.29D: *Aspergillus flavus*

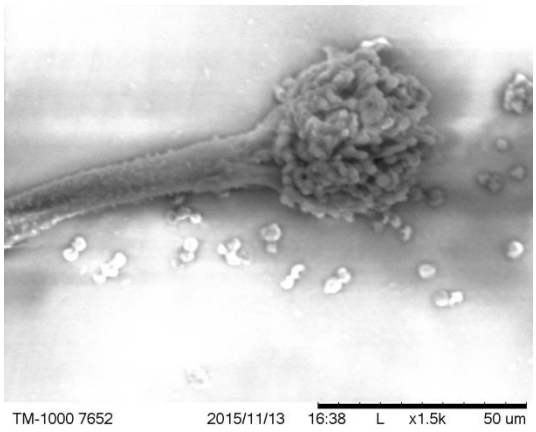


Fig 4.30D: *Aspergillus protuberus*

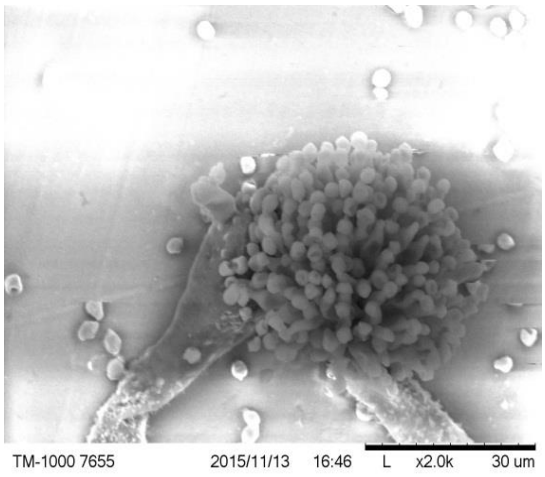


Fig 4.29E: *Aspergillus niger*

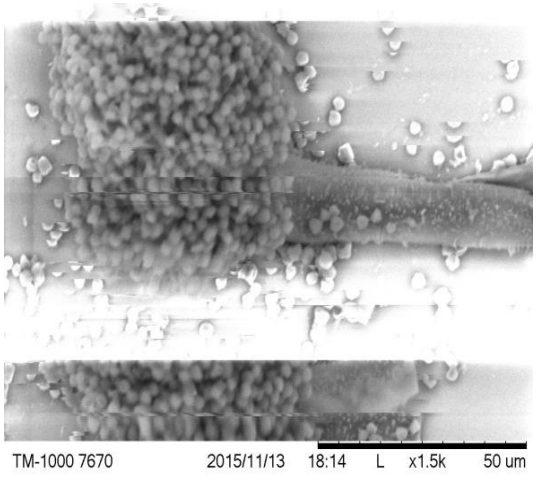


Fig 4.30F: *Aspergillus subramanianii*

Table 4.5: Characterization of the Fungi primers by the Number of Amplified RNA regions, Allele frequency, Gene diversity and Polymorphic Information Content.

Plate ID	Fungi Barcoding Primers	Primer Sequence	Major Allele Frequency	Number of Isolates	Number of Amplified RNA Regions	Gene Diversity	PIC
1	**ITS 1F and ITS 4R**	5' TCCGTAGGTGAACCTGCGG 3' 3' TCCTCCGCTTATTGATATGC 5'	0.5000		2	0.5000	0.3750
2	**ITS 2 and ITS 5**	5' GCTGCGTTCTTCATCGATGC 3' 3'GGAAGTAAAAGTCGTAACAA 5'	0.3333		3	0.6667	0.5926
3	**LROR and LR5**	5' ACCCGCTGAACTTAAGC 3' 5' TCCTGAGGGAAACTTCG 3'	0.2500		4	0.7500	0.7031
4	**LROR and LR5**	5' ACCCGCTGAACTTAAGC 3' 5' TCCTGAGGGAAACTTCG 3'	0.5000		2	0.5000	0.3750
	Mean		**0.3958**		**2.75**	**0.6042**	**0.5114**

The PIC shows the usefulness of fungal barcode primers used. LROR and LRO5 had highest PIC Value while ITS 1F and ITS 4R and LROR and LR05 had the least and same PIC value.

4.10 Gene Expression Analysis

4.10.1 Quantitative relative gene expression analysis for allergenic gene

The greatest variability among the seven isolates was observed as evidenced by both the range in standard deviation (SD) values and CT values observed as calculated with Bestkeeper (Table 4). All the genes were stable, as the S.D was less than one. In general most genes displayed overall stability with respect to allergen expression. Six isolates (*A. terreus, P. funiculosum, P.citrinum, P. oxalicum, P. pinophilum* and *P. simplicissimum*) displayed the greatest expression stability for tubulin gene (S.D = 0.0157, 0.0711, 0.0520, 0.8913, 0.0793 and 0.0806) respectively, while *A. flavus* was the least stable (S.D = 3.8680). Isolate displaying the lowest S.D and thus the greatest stability was *A. terreus* which has S.D of 0.0157. All genes were significantly correlated to the Bestkeeper index ($P < 0.001$). *P. pinophilum* and *P. simplicissimum* recording almost identical stability values (0. 0793 and 0.0806) respectively.

For Actin gene, all isolates were stable as S.D were all less than one (Table 4.5.4). *Penicillium pinophilum* displayed the lowest S.D (0.0006) and thus the greatest stability. All the organisms displayed overall stability with respect to actin reference gene expression. *Penicillium funiculosum* and *P. citrinum* gave almost similar stability values (S.D 0.0516 and 0.0507).

All genes of interest were stably expressed as reflected in the S.D values for both Pen ox and Pen cit allergenic gene. For Pen ox, *Aspergillus terreus, Penicillium funiculosum, P. citrinum, P. simplicissimum* and *A. flavus* (S.D = 0.0457, 0.0046, 0.0592, 0.8814 and 0.9053) exhibited the greatest stability as shown in Table 4.5.3 while for pen cit, *A. terreus, P. funiculosum, P. pinophilum, P. simplicissimum* and *A. flavus* exhibited the greatest stability as S.D values were less than one as shown in Table 4.5.2. *P. oxalicum* was the least stable (SD 1.0386)

while *P. funiculosum* (SD = 0.0046) displayed the greatest expression stability for pen ox allergenic gene whereas for pen cit gene, *P. simplicissimum* gave the greatest expression stability (SD =0.0098) while *P. citrinum* was the least stable (SD = 1.4736) as shown in Table 4.5.1. *A. terreus* and *P. citrinum* had similar values (SD = 0.0457 and 0.0592) for pen ox gene while *P. simplicissimum* and A. *flavus* recorded almost identical stability values (SD = 0.0098 and 0.1066) for pen cit gene expression.

Table 4.5.1: Descriptive statistics and stability values of candidate gene of interest

Isolate	Standard dev.	Mean	Primer
A.terreus	**0.0457**	**28.43**	**Pen ox**
P. funiculosum	0.0046.	30.63	Pen ox
P. citrinum	0.0592	21.23	Pen ox
P. oxalicum	1.0385	20.74	Pen ox
P. pinophilum	1.0248	19.74	Pen ox
P. simplicissimum	0.8814	27.22	Pen ox
A.flavus	0.9053	30.22	Pen ox

Table 4.5.2: Descriptive statistics and stability values of candidate gene of interest

Isolate	Standard dev.	Mean	Primer
A.terreus	**0.0180**	**37.56**	**Pen cit**
P. funiculosum	0.0375	34.10	Pen cit
P. citrinum	1.4736	32.24	Pen cit
P. oxalicum	1.0386	36.02	Pen cit
P. pinophilum	0.5151	33.49	Pen cit
P. simplicissimum	0.0098	30.22	Pen cit
A.flavus	0.1066	34.03	Pen cit

Table 4.5.3: Descriptive statistics and stability values of reference gene

Isolate	Standard dev.	Mean	Primer
A.terreus	**0.0179**	**28.84**	**Actin**
P. funiculosum	0.0516	30.24	Actin
P. citrinum	0.0507	30.81	Actin
P. oxalicum	0.2817	29.83	Actin
P. pinophilum	0.0006	1.28	Actin
P. simplicissimum	0.0422	18.95	Actin
A.flavus	0.0309	30.05	Actin

Table 4.5.4: Descriptive statistics and stability values of reference gene

Isolate	Standard dev.	Mean	Primer βtubulin
A.terreus	**0.0157**	**23.16**	
P. funiculosum	0.0711	18.67	βtubulin
P. citrinum	0.0520	26.77	βtubulin
P. oxalicum	0.8913	23.64	βtubulin
P. pinophilum	0.0793	17.38	βtubulin
P. simplicissimum	0.0806	20.96	βtubulin
A.flavus	3.8680	20.71	βtubulin

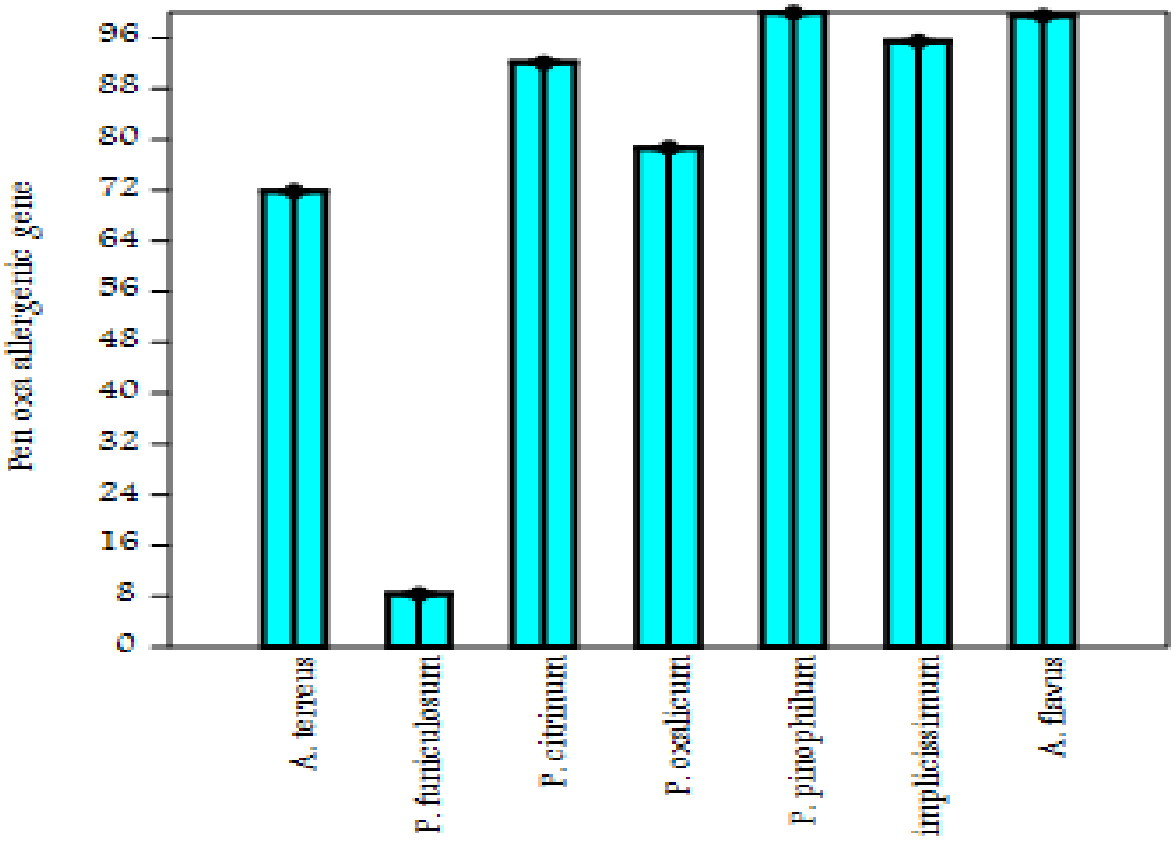


Fig 4.31: Box plot analysis showing mean values of relative gene expression for *Pen oxalicum* allergenic gene. Mean bars with different alphabets are significantly different (P< 0.05).

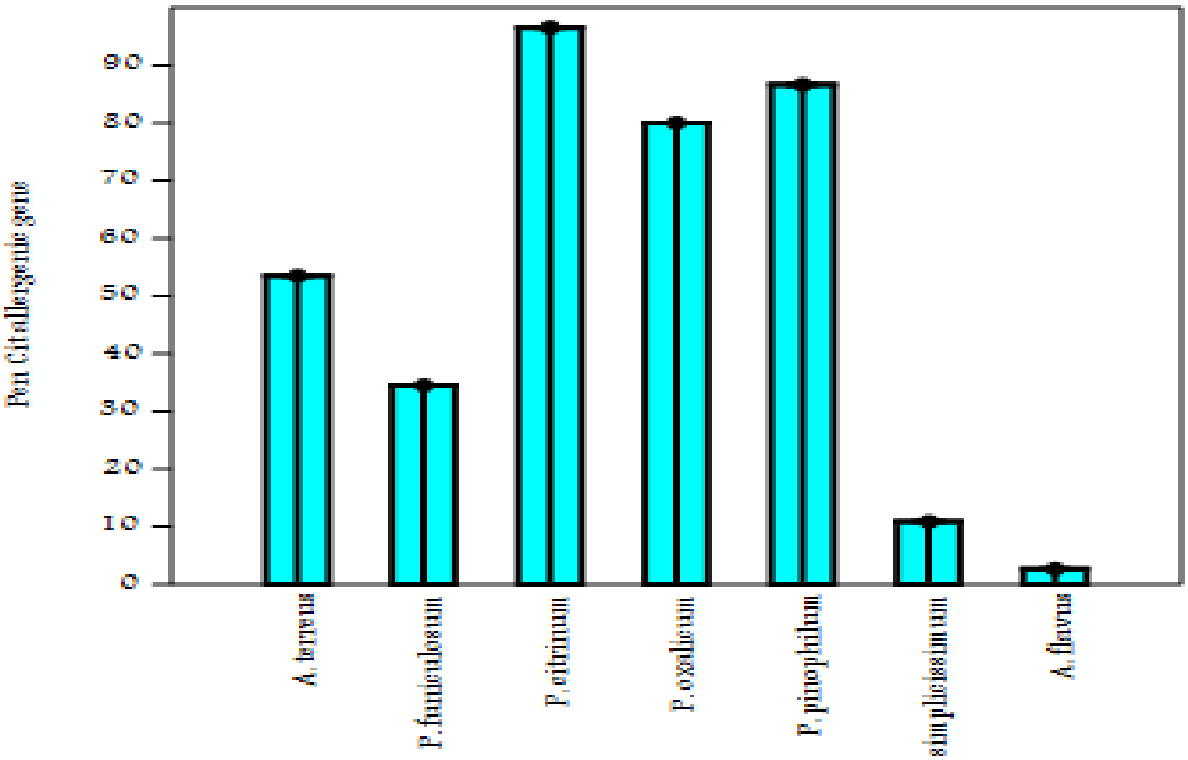


Fig 4.32: Box plot analysis showing mean values of relative gene expression profile for *Pen citrinum* allergenic gene. Mean bars with different alphabets are significantly different (P< 0.05).

4.11 SDS-PAGE analysis of fungal isolates

4.11.1 Protein variations by SDS-PAGE

In the present study, results from the SDS-PAGE method indicated that extracts obtained from various *Aspergillus* and *Penicillium* species had 25 protein bands, which ranged from 5 to 100 kDa. Most bands were concentrated between 14 and 66 kDa in all of the species. Among these species, *Aspergillus terreus* and *Penicillium chrysogenum* had the maximum number of protein bands (6 bands) ranging from 14 to 66 kDa and *P. oxalicum* had the minimum protein bands (3 bands) ranging from 30 to 100 kDa. Among the different bands, protein bands with a molecular weight of 30 kDa were present in all species of samples except for *P. citrinum* (Table 4.6).

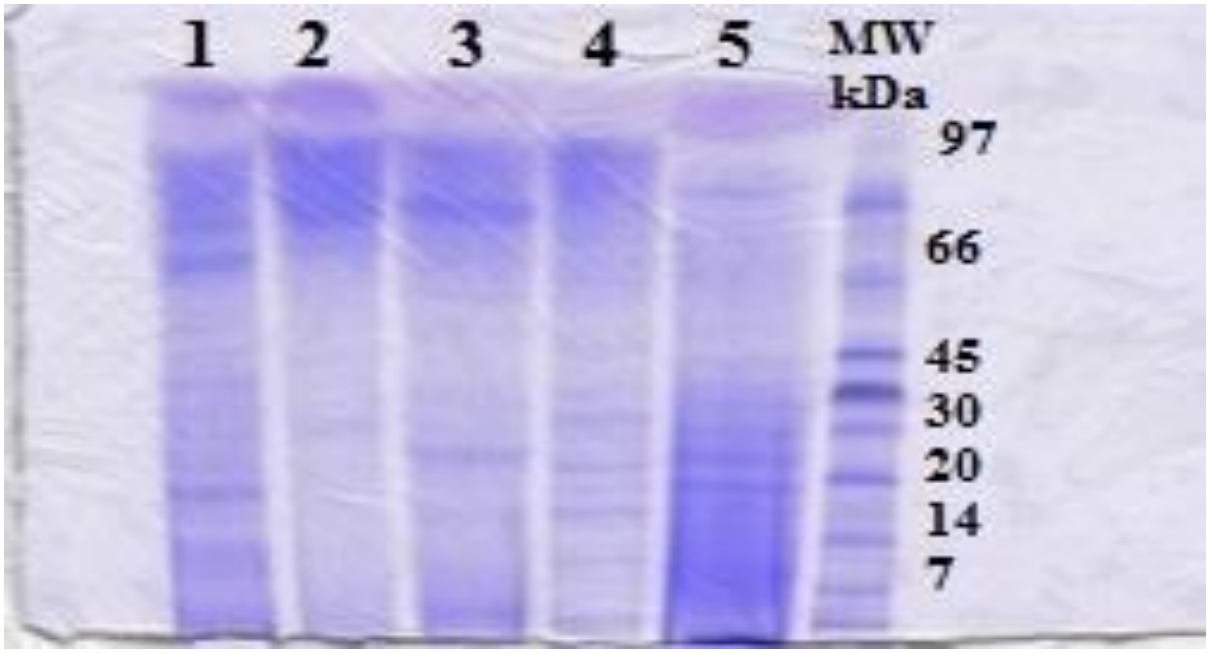


Fig. 4.33: SDS-PAGE of purified vacuolar serine proteases from 1). *P. citrinum*, 2). *P. oxalicum*, and 3). *P. chrysogenum*, *4). A. flavus* and 5) *A. terreus*

Table 4.6: Number of Protein Bands Observed by the SDS-PAGE Technique in each of the Species isolates

Organisms	Protein bands, kDa	Total bands
P. citrinum	80, 65, 25, 15, 7	5
P. oxalicum	100, 95, 30	3
P. chrysogenum	60, 40, 30, 25, 15, 5	6
A. flavus	40, 30, 25, 15,5	5
A. terreus	90, 40, 30, 25, 20, 15	6

4.12 Allergy and Fungal spore abundance

Negative and highly significant ($p<0.01$) correlative effect exists between the temperature with relative humidity ($r = -0.799$), while the association between relative humidity with Fungi count ($r =0.517$) was significant at $p<0.05$ level. Also, strong and significant ($p<0.01$) relationship was recorded between asthma and wheezing cough ($r =1.000$) while rainfall as well showed negative correlation ($p<0.05$) with temperature. (Table 4.7).

Table 4.7: Correlation of weather and fungi count on occurrence of asthma and wheezing cough

Correlation	Rainfall	Temp	Rel. humidity	Asthma	Wheezing cough	Fungi count
Rainfall	1					
Temperature	-0.119	1				
Rel. Humidity	0.303	-0.799**	1			
Asthma	0.19	0.398	-0.103	1		
Wheezing cough	0.19	0.388	-0.103	1.000**	1	
Fungi Count	0.298	-0.205	.517*	0.458	0.458	1

**. Correlation is significant at the 0.01 level; *. Correlation is significant at the 0.05 level

4.13 Mice response to fungi inoculation

Table 4.11 shows the effect of time and fungi treatment at different inocula load on the lungs of balb/b albino mice. At 48 hrs, the concentration 1 of *A. flavus* x 10^{-3}, both concentrations of *P.citrinum* x 10^{-3} and *P.chrysogenum* x 10^{-3} do not differ significantly from the control experiment. The concentration 2 of *A. flavus* x $10^{-3}$and both concentrations in *A. penicillioides* x10^{-3} do not differ significantly but are significantly lower than the control experiment at ($P \leq 0.05$). At 72 hrs, there were no significant differences between the concentration of the control experiment and the concentrations of *A. flavus* x 10^{-3}, *A. penicillioides* x10^{-3}, *P.citrinum* x 10^{-3}, and *P.chrysogenum* x 10^{-3} at ($P \leq 0.05$).

There were significant differences ($P \leq 0.05$) between the concentration 2 of *A. flavus* x 10^{-3} at 48 hrs and at 72hrs. There are significant differences ($P \leq 0.05$) between the concentration 1 of *A. penicillioides* x10^{-3} at 48 hrs and 72 hrs. There were significant differences $P \leq 0.05$) between the concentration 2 of *A. penicillioides* x10^{-3} at 48 hrs and 72 hrs (Table 4.11).

The fitted model for the effect of fungi treatments at different inoculum load on the lungs of balb/c albino mice produced a significant ($p<0.01$) effect. Similarly, time showed significant effect on the lungs of *Aspergillus flavus* inoculated mice at concentration 2 and *Aspergillus penicilloides* at both concentrations. The period of fungi inoculation showed no significant effect on *Penicillium citrinum* and *P. chrysogenum* inoculated lungs for the period of study while replicate recorded no significant difference for all inoculated organisms at both concentrations (Table 4.10).

Weight of lungs showed that *A. penicilloides* had the heaviest lungs compared with the rest which was followed by *Penicillium chrysogenum* (22.7) while the lowest weight was found in *Penicillium citrinum* inoculated lungs (17.7). Control lungs was (14.6) (Fig. 4.34).

Table 4.8: ANOVA effect of fungi treatment at different inoculum load on the lungs of balb/c albino mice

Source	Df	Control x 10^{-3}	*A. flavus* x 10^{-3}		*A. penicillioides* x10^{-3}		*P.citrinum* x 10^{-3}		*P.chrysogenum*x 10^{-3}	
			Conc 1	Conc 2	Conc 1	Conc 2	Conc 1	Conc 2	Conc 1	Conc 2
Model	7	0.265 *	0.891	2.679 *	4.469 *	3.28	0.794	0.378	0.0437	0.759
Time (hours)	1	0.283	2.901	14.25 **	28.50 **	19.687 *	0.622	0.272	55.1	0.005
Replicate	6	0.283 *	0.555	0.751	0.46	0.546	0.822	0.397	41.78	0.008
Error	6	2.33	3.496	3.771	5.584	10.875	8.8	5.046	249.51	8.113
Corrected total	13	2.09	9.731	2.253	3.687	33.838	14.36	7.698	555.33	13.43

*significant at $p<0.05$, **significant at $p<0.01$

Conc 1 = 2×10^{-5}

Conc 2 = 3×10^{-7}

Table 4.9: Effect of time and fungi treatments at different inoculum load on the lungs of balb/c albino mice

Parameter	Treatments	Control x 10^{-3}	*A. flavus* x 10^{-3}		*A. penicillioides* x10^{-3}		*P.citrinum* x 10^{-3}		*P.chrysogenum*x 10^{-3}	
			Conc 1	Conc 2	Conc 1	Conc 2	Conc 1	Conc 2	Conc 1	Conc 2
Time	**48 hours**	0.11 a	0.15 a	0.14 b	0.13 b	0.14 b	0.14 a	0.12 a	0.13 a	0.14 a
	72 hours	0.10 a	0.12 a	0.20 a	0.22 a	0.22 a	0.12 a	0.13 a	0.25 a	0.14 a
	LSD value	8.2	31.6	32.8	39.9	55.7	50.1	37.9	26.67	48.1
	EMS	0.039	0.583	0.63	0.93	1.81	1.47	0.84	41.59	1.35

Means with the same letter are not significantly ($p<0.05$) different across the column

LSD = Least Significant Difference

EMS = Error Mean Square

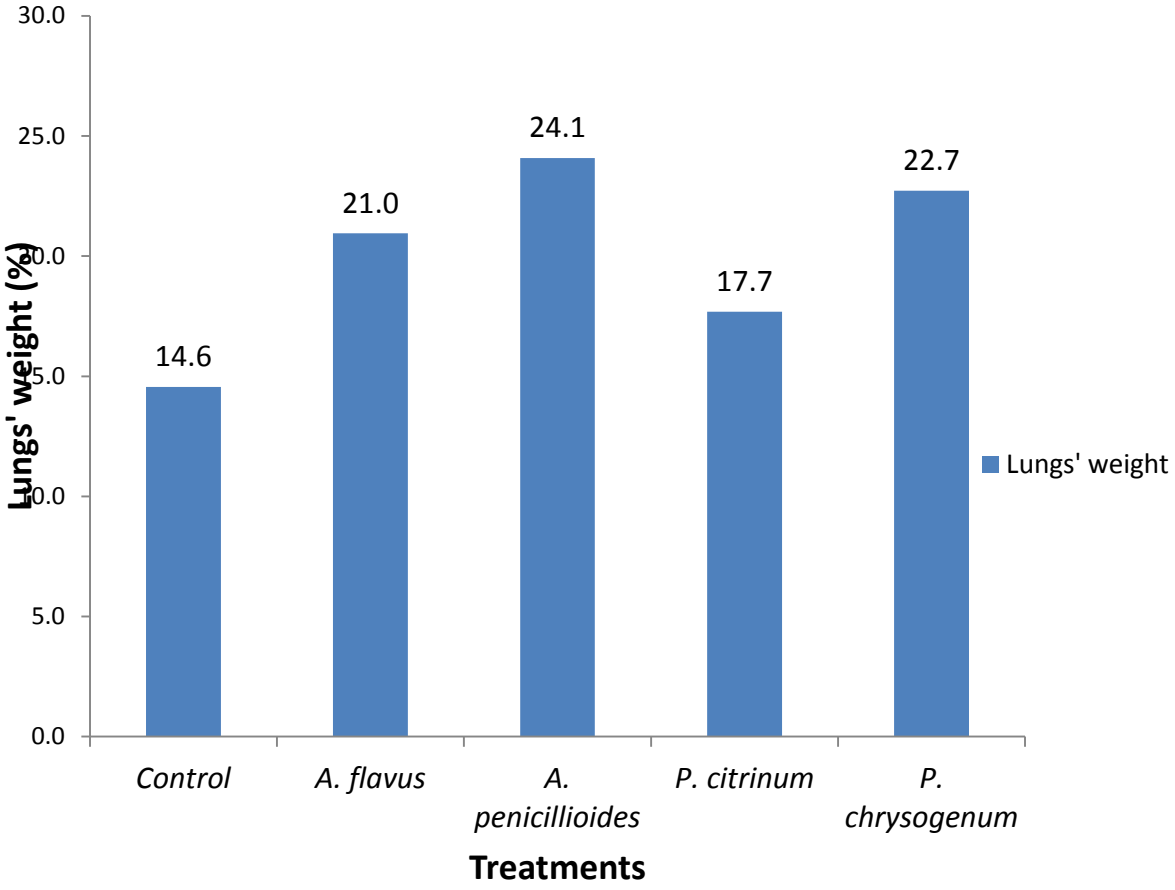


Figure 4.34: Percentage pooled effect of lung weight of control and fungi inoculated balb/c mice

4.14 Relationship between fungal inoculation and time of response in mice

Positive and highly significant ($p<0.01$) correlative effect exists between the time with *Aspergillus flavus* at concentration 2 (r =0.80), *A. penicilloides* at concentration 1 (r =0.88) and concentration 2 (r = 0.77) with time, while the association with *A. flavus* at concentration 1 (r =- 0.54) was negative but significant at $p<0.05$ level. Also, strong and significant ($p<0.01$) relationship was recorded between *A. flavus* and *A. penicilloides* (r =0.77), while *P. citrinum* at concentration 2 as well showed significant ($p<0.05$) but negative increase with *P. chrysogenum*. However, *A. penicilloides* at concentration 2 was only found significant ($p<0.05$) and negatively correlated with *A. flavus* at concentration 1 (r = -0.64) (Table 4.12).

Evaluations of the Principal Component Axis (PCA) to the variation in the extent of infection recorded on the lungs of balb/b albino mice inoculated with different fungi treatments experiment showed that; Prin 1 with Eigen value of 3.43 contributed 38.13 % of the total variation and it decreases with all the four inoculated organisms. Prin 2 contributed 26.87% of the variation, although showed negative association with *Aspergillus flavus*, *Aspergillus penicilloides* and *Penicillium citrinum* but significantly increases with increase in *P. chrysogenum* at concentration 1. While Prin 3 also showed positive relationship with *A. flavus* at concentration 1, it however decreases with increase in inoculum load of *P. chrysogenum* at concentration 2. Prin 4 contributed 9.73% of the total variation. It also increased with increase in the *P. citrinum* at concentration 1 but decrease with *P. chrysogenum* at concentration 2. Prin 5 accounted for 4.67% of the total variation and it showed positive association with control and negative association with *A. flavus*, *P. citrinum* and *P. chrysogenum* (Table 4.13).

Table 4.10: Association between fungi, inoculum load, time and their effect on the lungs of balb/c albino mice

Correlation	Conc	Time (hours)	Rep	Control	*A. flavus*x10^{-3}		*A. penicillioides* x10^{-3}		*P. citrinum* x10^{-3}		*P. chrysogenum* x10^{-3}	
					Conc 1	Conc 2	Conc 1	Conc 2	Conc 1	Conc 2	Conc 1	Conc 2
Time	-	1										
Replicate	-	0.00	1									
Control	-	-0.29	0.23	1								
A. flavus	1	-0.54*	0.15	0.47	1							
	2	0.80**	0.02	0.04	-0.37	1						
A. penicillioides	1	0.88**	0.20	-0.22	-0.35	0.77**	1					
	2	0.77**	-0.13	-0.47	-0.61*	0.41	0.49	1				
P. citrinum	1	-0.20	-0.15	-0.39	-0.47	-0.39	-0.29	-0.06	1			
	2	0.18	-0.38	-0.09	-0.23	-0.12	-0.28	0.51	0.19	1		
P. chrysogenum	1	0.32	0.36	-0.42	-0.30	-0.03	0.52	0.23	0.20	-0.29	1	
	2	0.02	-0.14	-0.11	-0.15	0.36	0.15	-0.12	-0.12	-0.57*	-0.24	1

*Correlation is significant at p<0.05 , **Correlation is significant at p<0.01, Con = Concentration, Rep = Replicate

- Resistance

+ Susceptibility

Table 4.11: Contribution of Principal Component Analysis (PCA) to the variation in the extent of infection observed in lungs of balb/c albino mice inoculated with different fungi treatments

PCA	Inoculum load	Prin 1	Prin 2	Prin 3	Prin 4	Prin 5	Prin 6	Prin 7	Prin 8	Prin 9
Control	0	0.24	-0.43	0.04	0.41	0.57	0.48	0.01	0.13	-0.02
A.flavus	1	0.25	-0.26	0.56	-0.19	-0.45	0.15	0.25	0.46	-0.08
	2	0.43	-0.14	-0.37	0.10	0.12	-0.65	0.3	0.35	0.01
A. penicilloides	1	0.37	0.40	0.28	0.01	0.15	0.06	0.43	-0.32	0.56
	2	-0.4	-0.07	-0.09	-0.06	0.42	0.1	0.5	0.13	-0.07
P.citrinum	1	-0.31	0.31	-0.27	0.52	-0.29	0.27	0.48	0.26	-0.06
	2	-0.41	-0.40	0.01	0.10	-0.09	-0.14	-0.1	0.17	0.77
P.chrysogenum	1	-0.15	0.51	0.36	0.09	0.38	-0.17	-0.3	0.56	0.02
	2	0.33	0.20	-0.50	-0.36	-0.19	0.44	-0.27	0.34	0.29
Eigen Value	-	3.43	2.42	1.44	0.87	0.42	0.22	0.11	0.07	0.00
Proportion	-	38.13	26.87	16.01	9.73	4.67	2.45	1.29	0.83	0.00

4.15 Cluster analysis relationship

The Cluster diagram showing the relationship in the different fungi inoculated in mice showd that there were two main clusters with control standing alone and all the four inoculated also clusterd in two distinct clusters. *Aspergillus penicilloides* and *Penicillium citrinum* were on same sub-cluster while *Aspergillus flavus* and *Penicillium chrysogenum* also sub clustered together. Control was alone on a separate cluster (Fig 4.35).

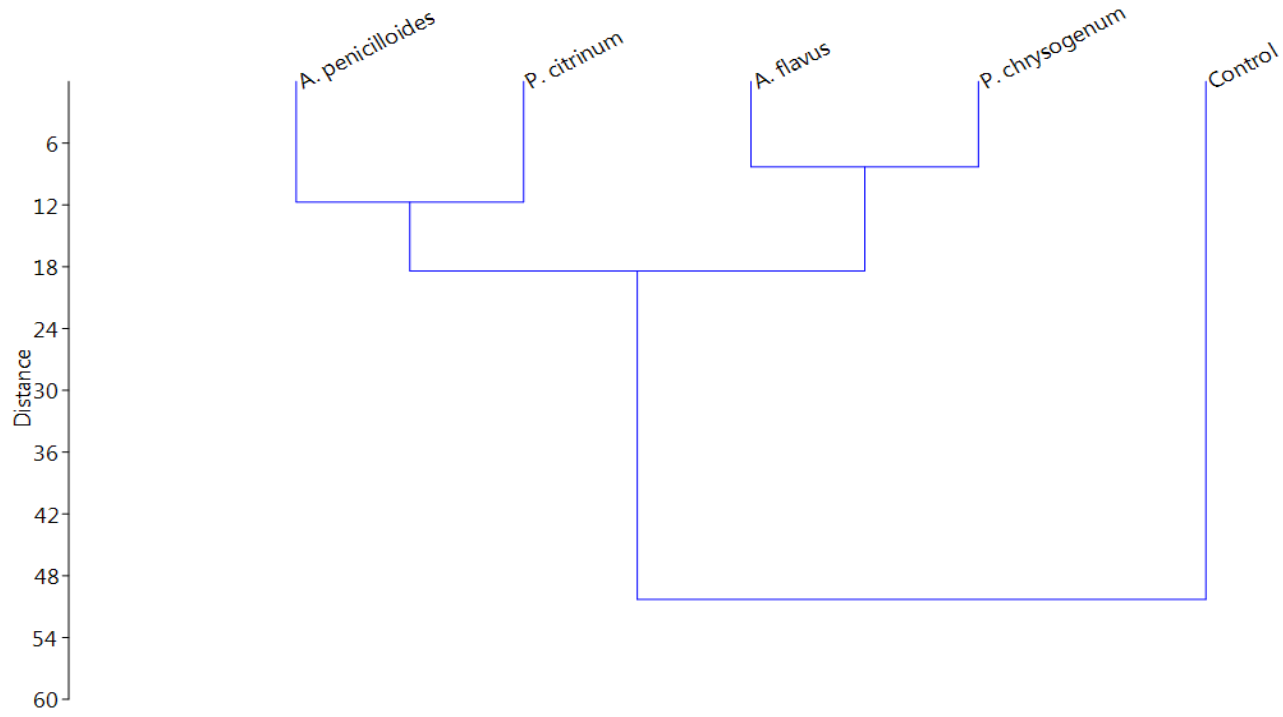


Figure 4.35: Cluster diagram showing the relationship in the biochemical performances of fungi inoculated in mice.

Table 4.12: ANOVA effect of fungal treatments at different inoculum load on the biochemical properties of lungs of balb/c albino mice

Source	df	Protein	MDA	MPO	H_2O_2	SOD	NO	GSH
Model	9	0.003*	0.313**	135.7**	3.54	2585.05**	127.40*	1.378**
Treatments	4	0.007**	0.64**	246.78**	6.06	5533.38**	234.67*	2.43**
Concentration	1	0.0009	0.05	197.00*	5.04	1072.55*	96.07	1.00*
Replicate	4	0.0003	0.04	9.29	0.65	14.85	27.95	0.417*
Error	40	0.05	3.25	812	108.15	149.01	2152.56	5.87
Corrected total	49	0.085	6.07	2033.3	140.08	29226.3	3299.2	18.277

Note: **= Highly significant ($p<0.01$), *= Significant ($p<0.05$), ns = not significant.

MDA - malondialdehyde

MPO - myeloperoxidase

H_2O_2 – hydrogen peroxide

SOD - sodium dismutase

NO - nitric oxide

GSH - gluthathione

Table 4.13: Effect of inoculated fungi on the biochemical properties of lungs of balb/c albino mice

Parameters	Variables	Protein	MDA	MPO	H_2O_2	SOD	NO	GSH
	Control	0.516a	3.384b	8.977c	11.647ab	88.36a	34.00a	4.08a
	A. penicilloides	0.480b	3.573b	8.334c	11.354b	43.08bc	24.03b	3.41bc
Treatments	*P. citrinum*	0.457b	3.868a	15.321b	12.695ab	52.15b	26.13b	3.59b
	A. flavus	0.461b	3.320b	15.795b	12.71ab	28.72d	25.00b	2.74d
	P. chrysogenum	0.445b	3.846a	20.071a	13.18a	34.55cd	21.00b	3.23c
	LSD	0.033	0.28	4.07	1.49	11.03	6.63	0.35
Concentration	1	0.48a	3.56a	11.72b	12.00a	54.00a	27.42a	3.55a
	2	0.47a	3.63a	15.68a	12.63a	44.74b	24.64a	3.27b
	LSD	0.02	0.16	2.58	0.94	6.98	4.19	0.22
	1	0.47a	3.60a	14.54a	11.92a	49.88a	27.28a	3.35b
	2	0.46a	3.60a	13.68a	12.30a	49.80a	25.38a	3.19b
Replicate	3	0.47a	3.52a	12.58a	12.58a	50.13a	24.07a	3.74a
	4	0.47a	3.70a	14.77a	12.52a	47.20a	25.24a	3.35b
	5	0.46a	3.56a	12.91a	12.29a	49.84a	28.20a	3.45ab
	LSD	0.03	0.26	4.07	1.49	11.03	6.63	0.35
	EMS	0.001	0.08	20.3	2.7	149.02	53.81	0.15

Mean with the different letter across the column are significantly ($p < 0.05$) different from one another with respect to each parameter.

LSD = Least Significant Difference

MDA- malondialdehyde

MPO- myeloperoxidase

H_2O_2 – hydrogen peroxide

SOD- sodium dismutase

NO- nitric oxide

GSH- gluthathione

4.16 Effect of Fungal treatments and biochemical properties in mice

The ANOVA table shows the effect of fungal treatments and concentration on the biochemical properties of lungs of balb/b albino mice. The effects of the fungal treatments on the protein, malondialdehyde, (MDA), myeloperoxidase, (MPO), (SOD) and glutathione (GSH) of the lungs of the albino mice were highly significant at ($P \leq 0.05$) and ($P \leq 0.01$). The effect of the fungal treatments on nitric oxide was significant at ($P \leq 0.05$) and there was no significant effect of the treatments and concentration on H_20_2 $P \leq 0.05$) and ($P \leq 0.01$). The effect of fungal concentrations on MPO, SOD and GSH were significant at ($P \leq 0.05$) and there were no significant effect of the fungi concentration on the protein, MDA, hydrogen peroxide (H_20_2) and NO concentration in the lungs of the balb/b albino mice (Table 4.14).

The treatments recorded positive and significant ($p<0.05$) relationship with protein ($r= 0.55$), No ($r= 0.59$) and GSH ($r = 0.56$) while it was significantly ($p<0.01$) related with MPO ($r = 0.66$) and SOD ($r= 0.71$). Positive and significant ($p<0.05$) association exists between protein and MDA ($r = 0.65$) while the significance was at $p<0.05$ with MPO ($r = 0.55$) and NO ($r =0.52$) (Table 4.16).

Table 4.14: Association between the treatments, inocula load and biochemical properties of the lungs of balb/c albino mice

Correlation	Treatments	Concentration	Replicate	Protein	MDA	MPO	H_2O_2	SOD	NO	GSH
Treatment	1									
Concentration	0.00	1								
Replicate	0.00	0	1							
Protein	-0.55 **	-0.11	-0.03	1						
MDA	0.27	0.09	0.01	-0.65**	1					
MPO	0.66**	0.31*	-0.05	-0.55**	0.34*	1				
H_2O_2	0.38 **	0.19	0.08	-0.48**	0.25	0.47**	1			
SOD	-0.71 **	-0.19	-0.02	0.46**	-0.12	-0.34**	-0.21	1		
NO	-0.59 **	-0.15	-0.05	0.52**	-0.21	-0.27**	-0.11	0.79	1	
NO„	-0.44 **	-0.17	0.03	0.46**	-0.23	-0.19**	-0.03	0.62	0.87**	
GSH	-0.56 **	-0.24	0.09	0.38**	-0.04	-0.48**	-0.21	0.65	0.47**	1

*significant at $p<0.05$, **significant at $p<0.01$

MDA- malondialdehyde

MPO- myeloperoxidase

H_2O_2 – hydrogen peroxide

SOD- sodium dismutase

NO- nitric oxide

GSH- gluthathione

4.17 Scatter plot and biochemical parameters

The scatter plot showed variations of the Principal Component Axis in the activities of the inoculated organisms on the biochemical parameters of mice lungs (Table 4.17). The PC1 which explained 92.85 % of the total variations recorded positive association with SOD, glutathione and control with *P. chrysogenum*, *P. citrinum* and *A. penicilloides* while Nitric oxide and protein content with *A. flavus* showed negative relationship with Prin 1 with nitric oxide expressing stronger association. The PC 2 explained 6.73 % of the total variation and showed positive association with control and SOD experiment while all other biochemical parameters were negatively associated with PC 2 (Figure 4.36)

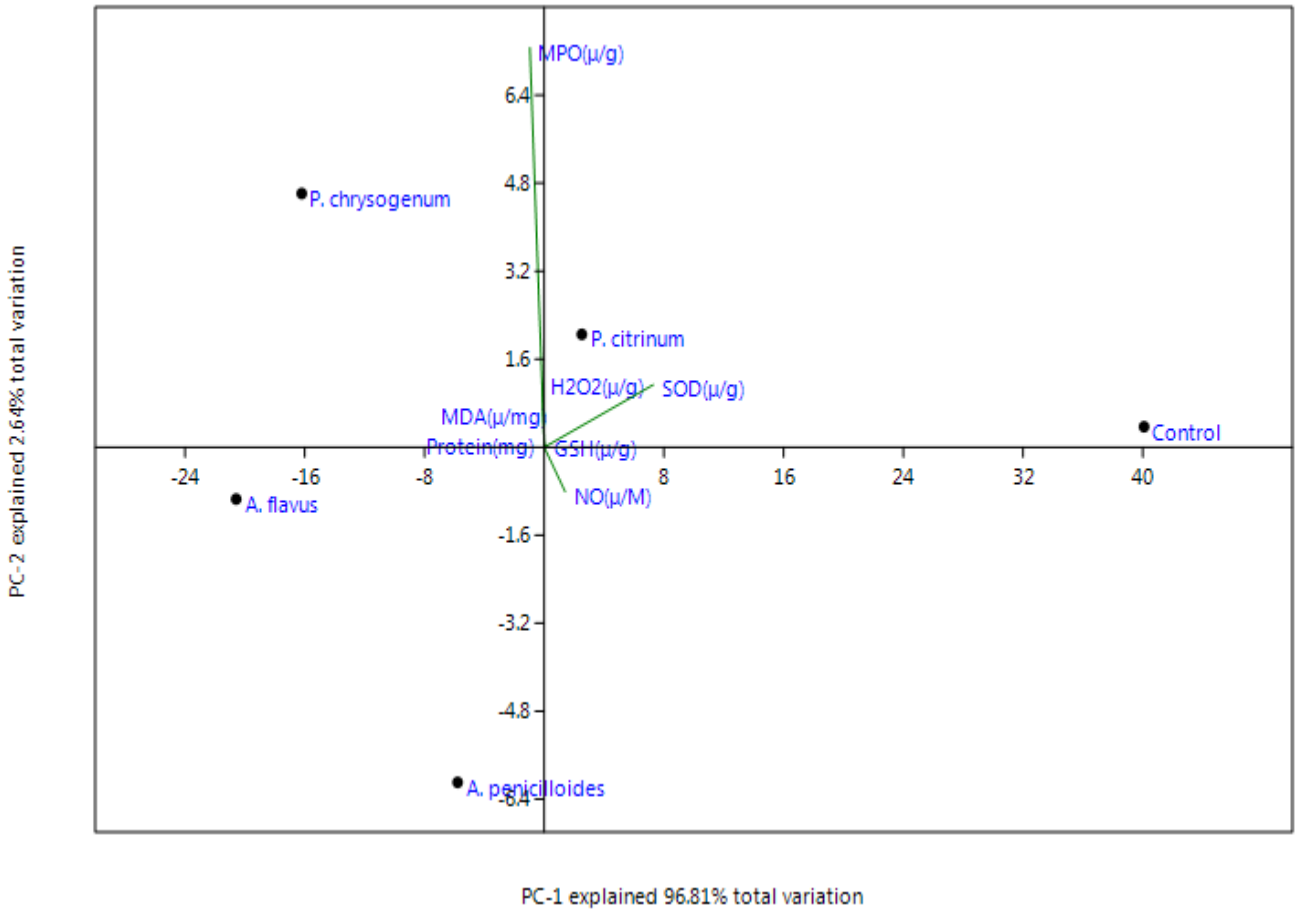


Figure 4.36: Contribution of PC 1 and PC 2 to the variation in the biochemical properties of the lungs caused by the fungi spores

Table 4.15: Contribution of Principal Component Analysis (PCA) to the variation in the biochemical properties of balb/c albino mice lungs inoculated with different fungi treatments

PCA	Prin 1	Prin 2	Prin 3	Prin 4	Prin 5	Prin 6	Prin 7	Prin 8
Protein	0.10	-0.64	0.08	0.01	0.13	0.11	0.74	0.03
MDA	0.03	0.56	0.28	-0.25	-0.50	0.09	0.53	0.02
MPO	0.30	0.33	-0.33	-0.41	0.50	0.40	0.11	-0.02
H_2O^2	0.21	0.34	-0.25	0.82	0.13	-0.08	0.28	-0.01
SOD	0.50	0.06	0.25	-0.19	0.22	-0.73	0.02	0.24
NO g	0.55	-0.13	-0.05	-0.02	-0.28	0.02	-0.11	-0.76
NO	0.50	-0.17	-0.22	0.04	-0.42	0.30	-0.19	0.6
GSH	0.19	0.03	0.80	0.24	0.24	0.42	-0.19	0.02
Eigen Value	2.69	1.98	1.18	0.80	0.63	0.33	0.26	0.13
Proportion	33.6	24.74	14.8	10.03	7.89	4.13	3.21	1.00

4.18 Box plot analysis of Immunoglobulin E (IgE)

The mean IgE induced by *Penicillium chrysogenum* fungal protein in mice was highest and followed by that of *Penicillium citrinum*, *Aspergillus flavus*, *Aspergillus fumigatus, Penicillium funiculosum, Aspergillus terreus* while *Aspergillus penicilloides* protein was the least induced and were all significantly different from control samples. (Fig. 4.37).

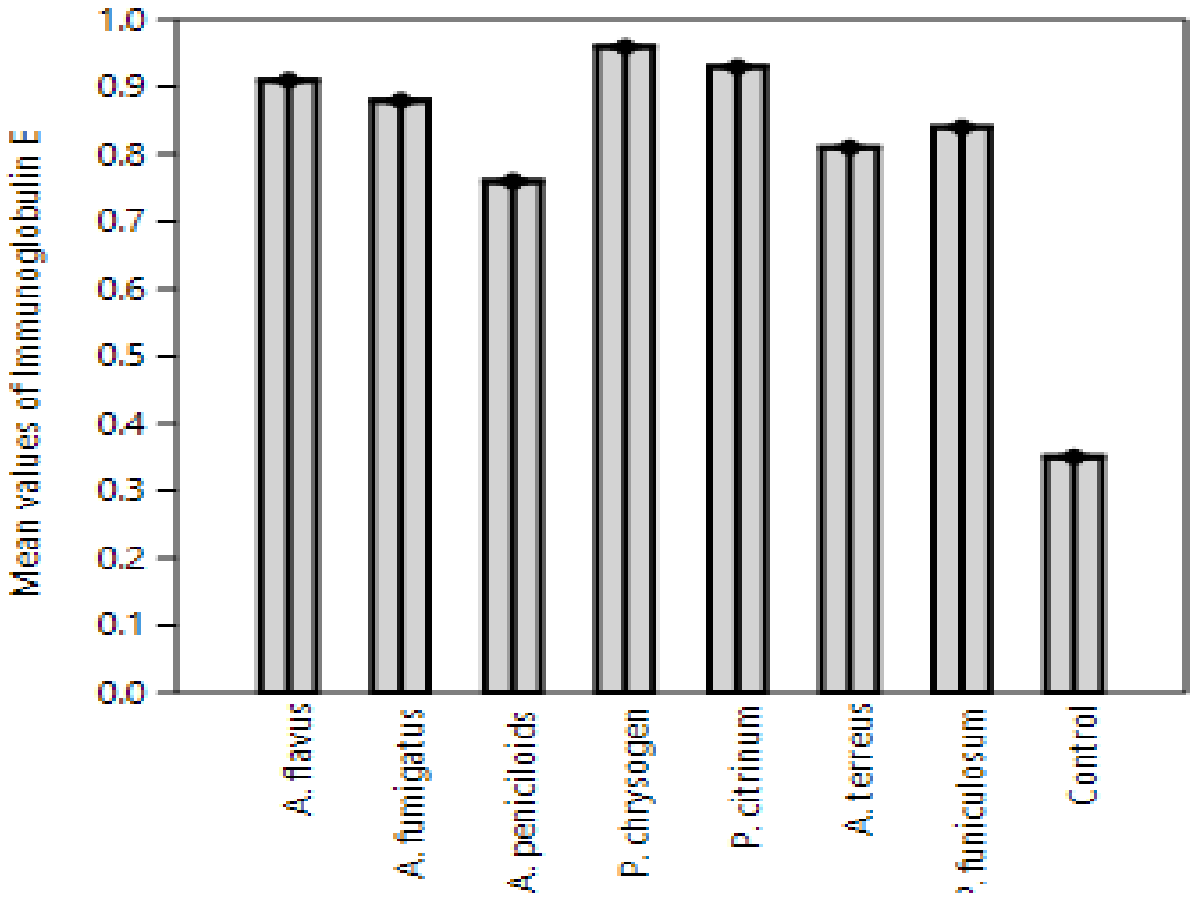
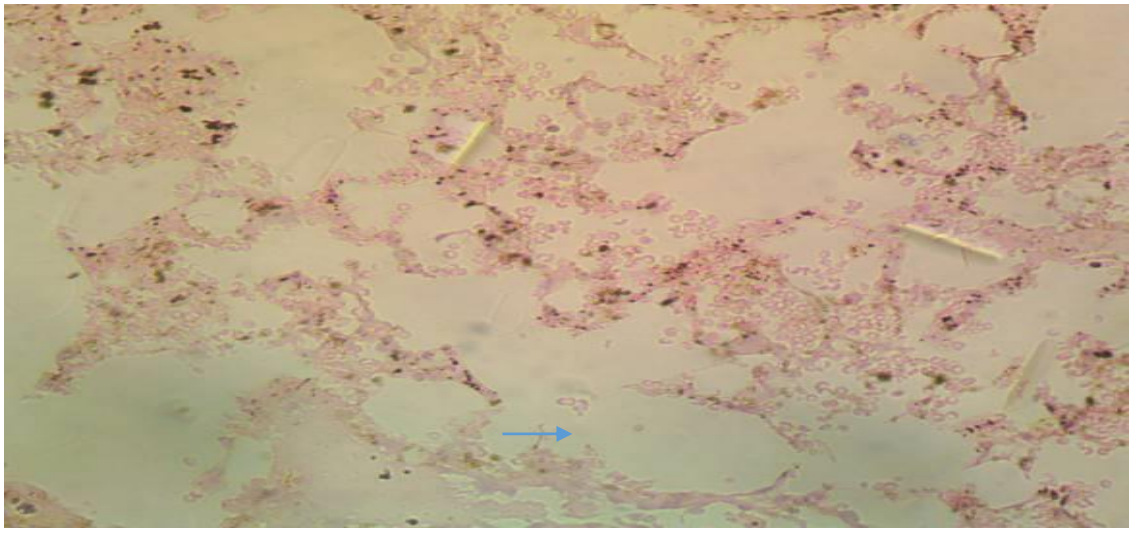


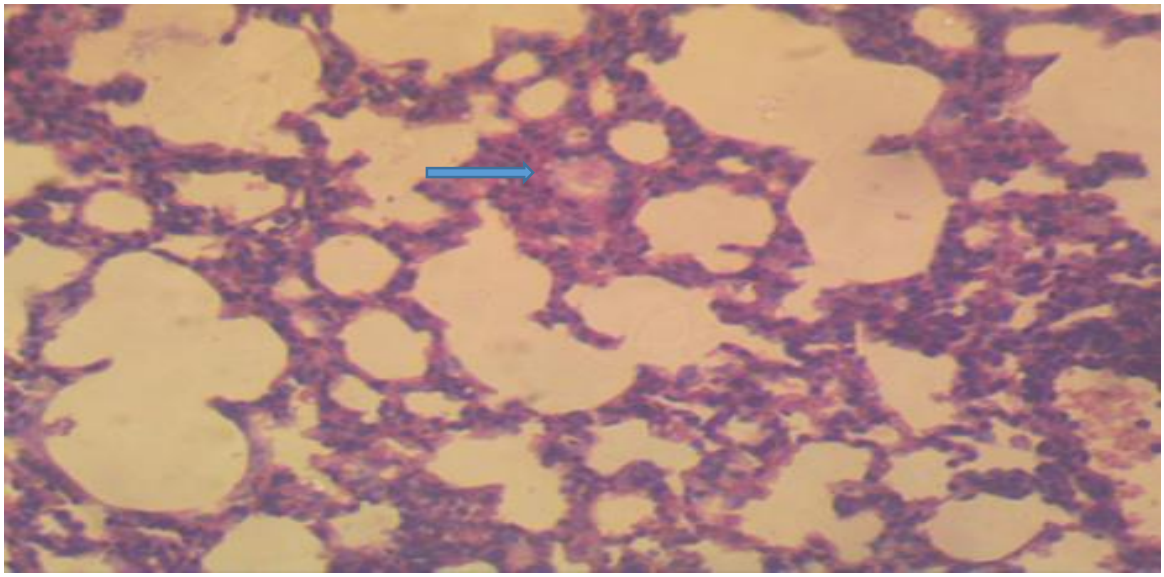
Fig. 4.37: Mean values of Immunoglobulin E elicited by fungal protein in mice

4.19 Histopathological studies of the organs of balb/c albino mice compared with the control

Plates 4.16 – 4.20 shows the photomicrograph of the lungs of different fungal spore concentrations on balb/c inoculated mice compared with the control.

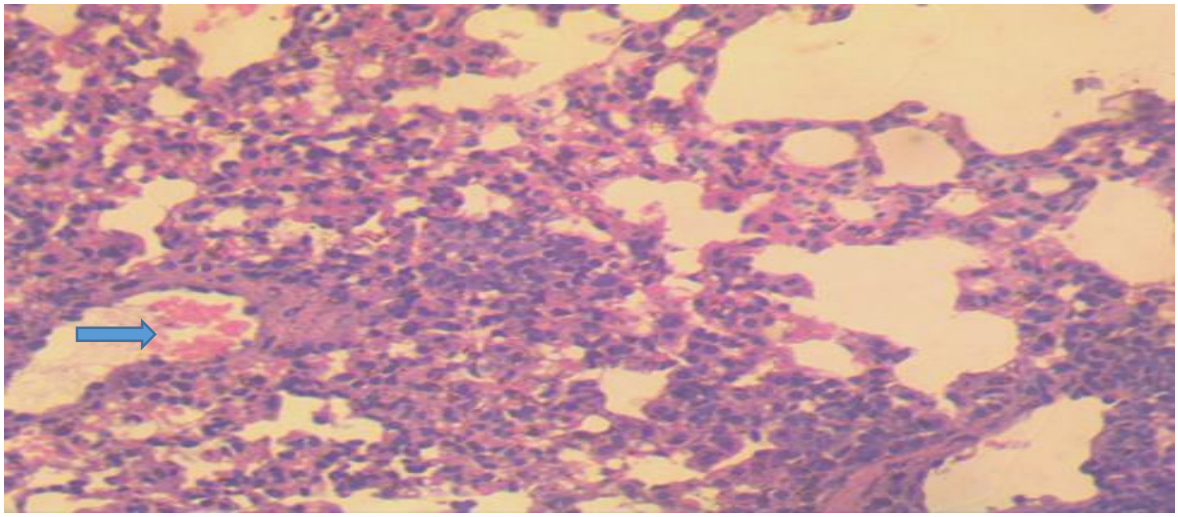


a.After 48 Hour

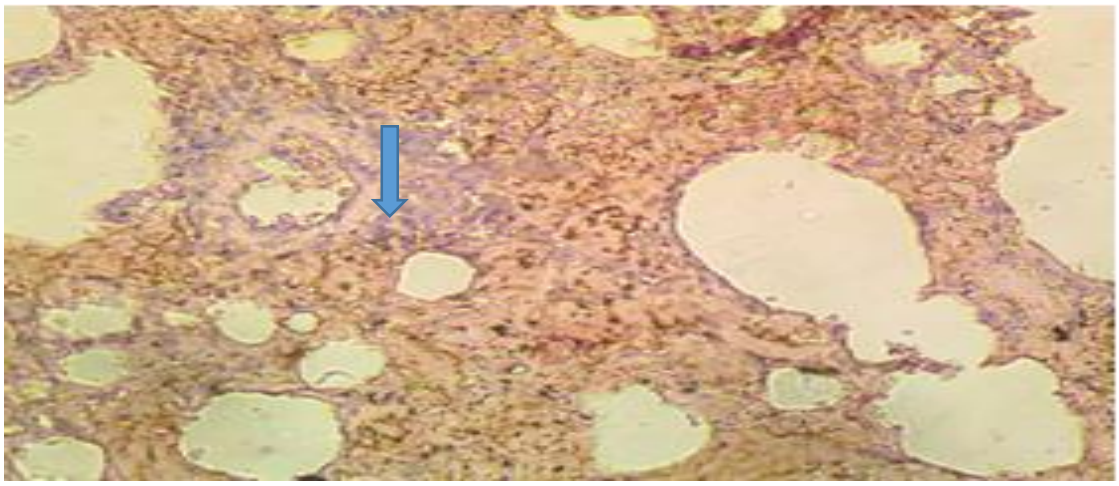


b. After 72 Hours

Plate 4.16 a&b*:* *Aspergillus penicilloides* (Conc 1): Lung. The airways (alveoli and bronchioles) are clear. There is however moderate haemorrhages and multiple foci of alveolar macrophages laden with dark pigment materials.

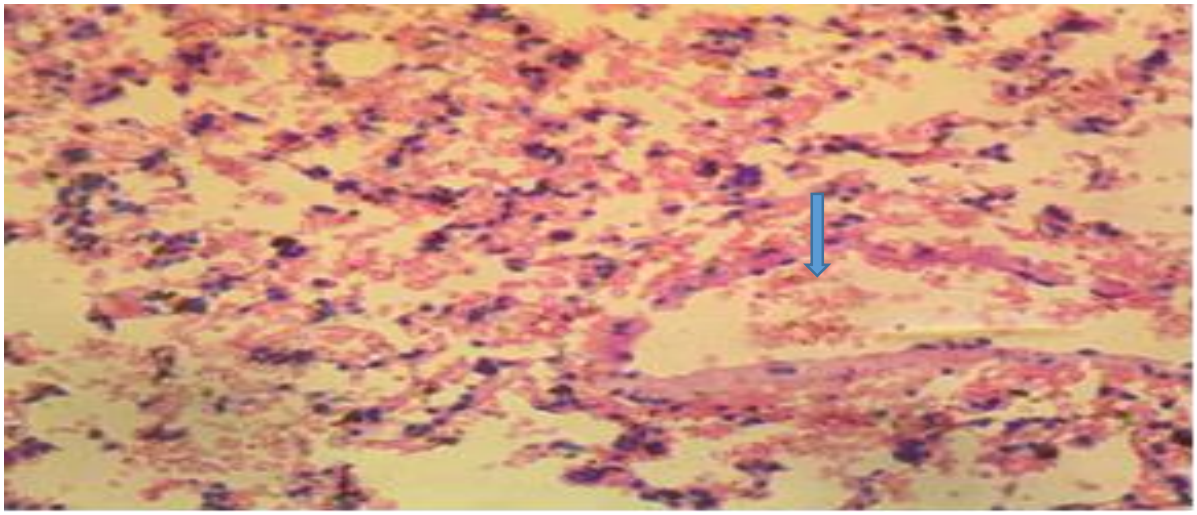


c.After 72 Hours

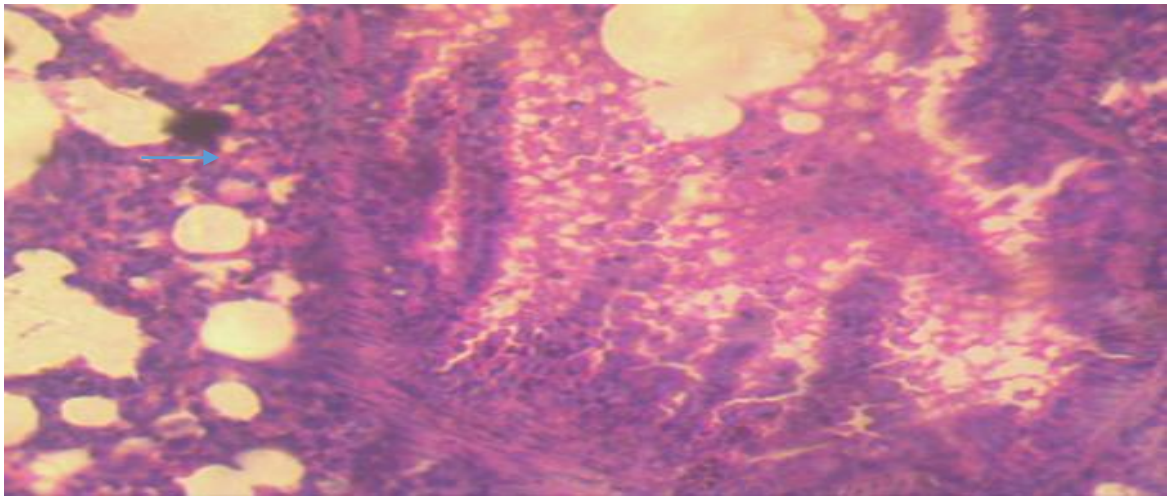


d.After 48 Hours

Plate 4.17 c&d: *Aspergillus penicilloides* (Conc 2): Lung. There is extensive severe thickening of alveolar interstitium. There is accumulation of inflammatory cells around blood vessels suggestive of vasculitis. Numerous unstained clear fungal hyphae are trapped in the necrotic debris. Mg x 100.

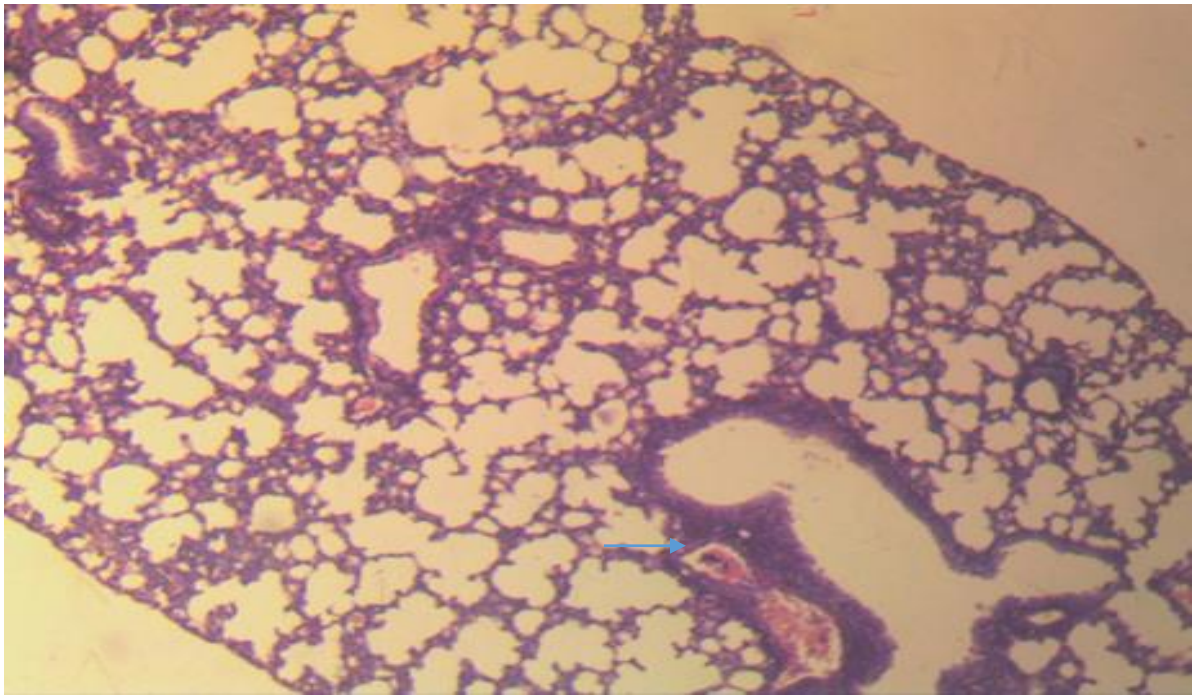


a. After 48 hours

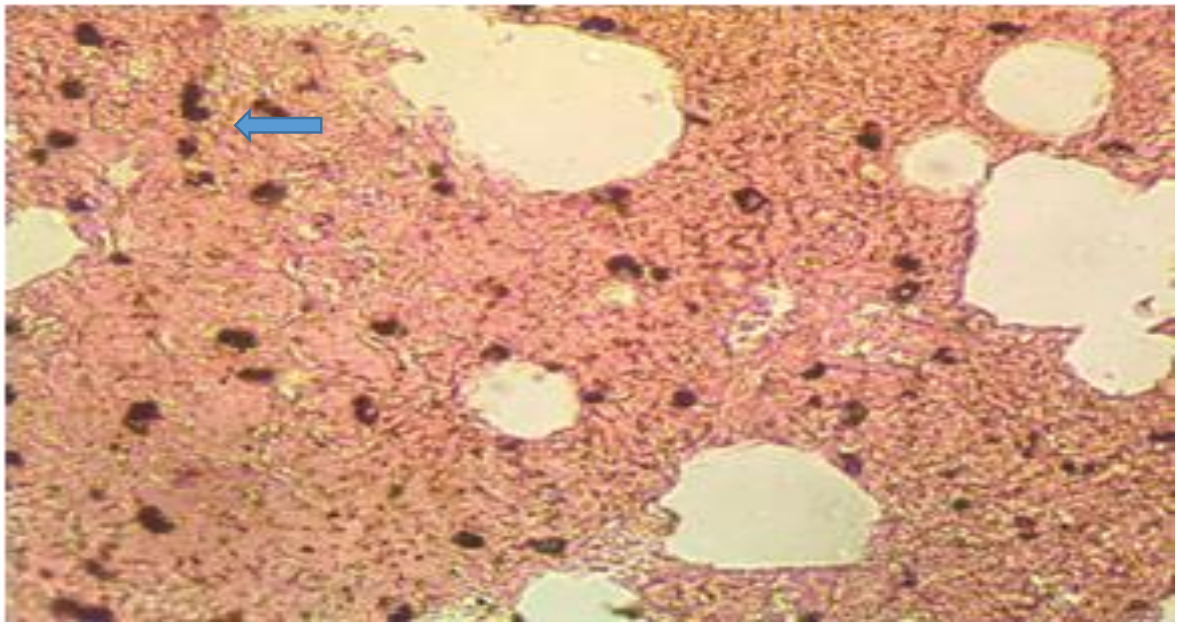


b. After 72 hours

Plate 4.18 *a&b:* *Penicillium chrysogenum* (Conc 1): Lung. There are severe widespread haemorrhages into the alveoli. There are a few foci of formation of giant cells.

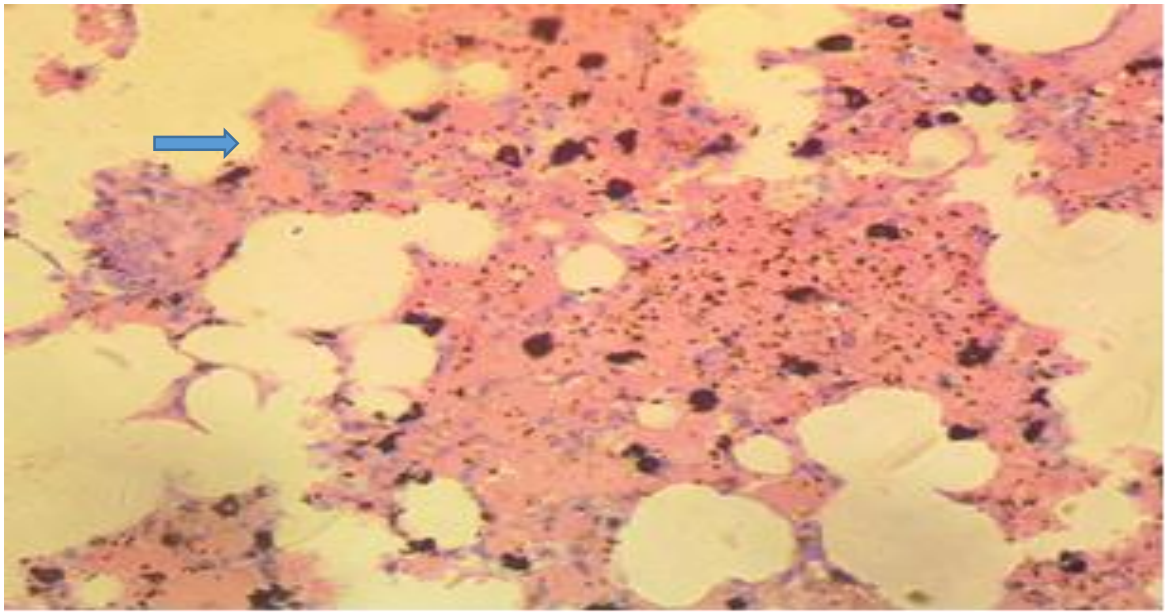


b. After 72 hours

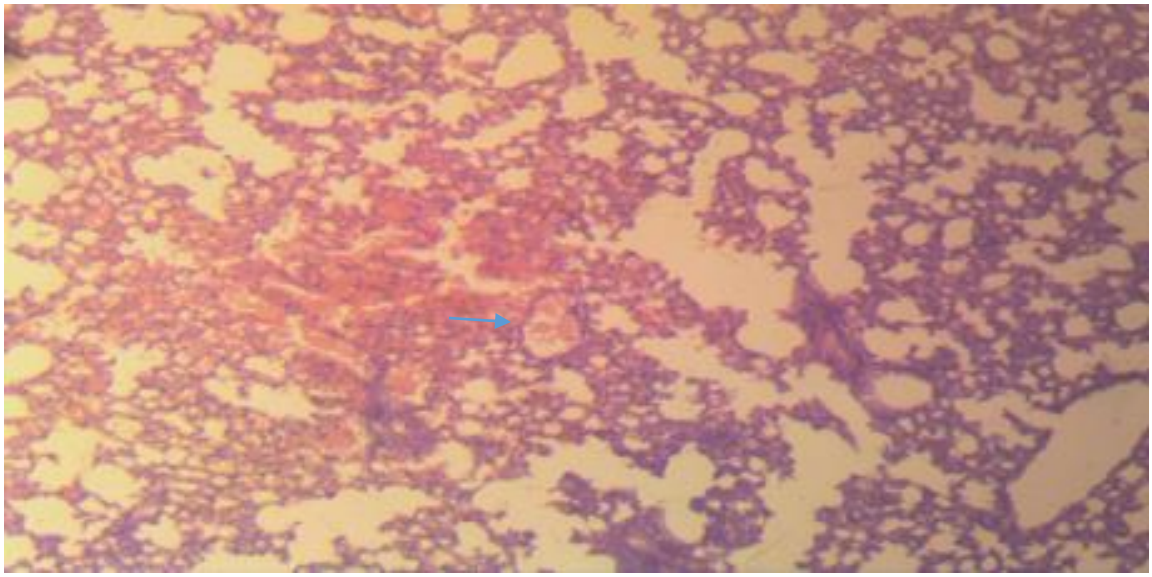


d. After 48 hours

Plate 4.18 b&d: *Penicillium Chrysogenum* (Conc 2): Lung. There is extensive severe thickening of alveolar interstitium. There are numerous foci of macrophages laden with dark pigments. Numerous unstained clear fungal hyphae are trapped in the necrotic debris. There is moderate congestion of blood vessels.

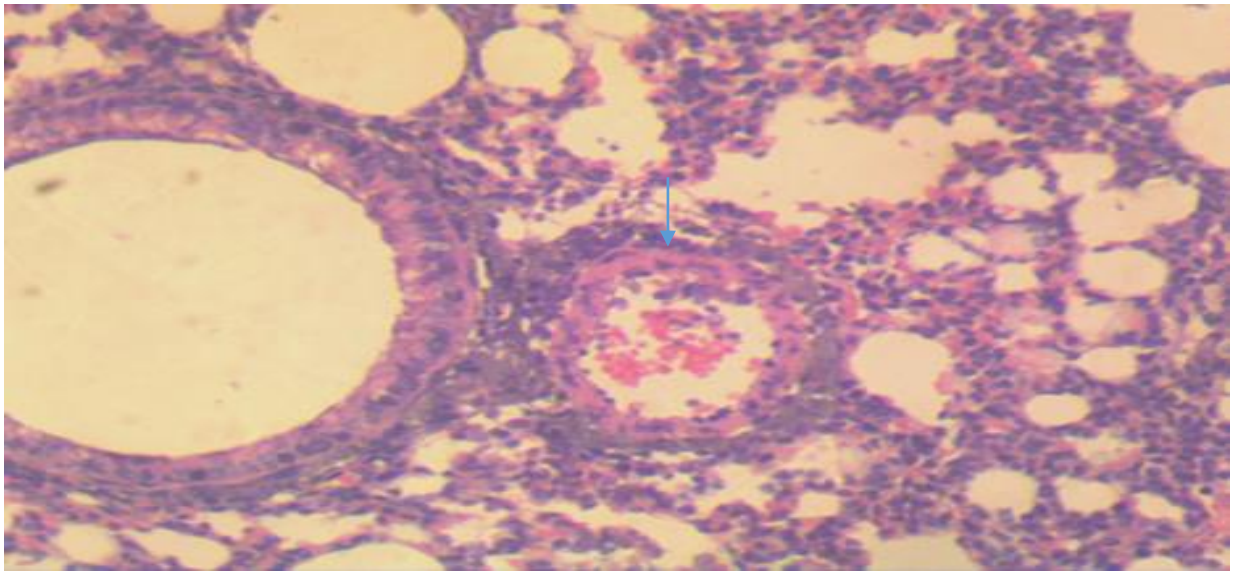


a. After 48 hours

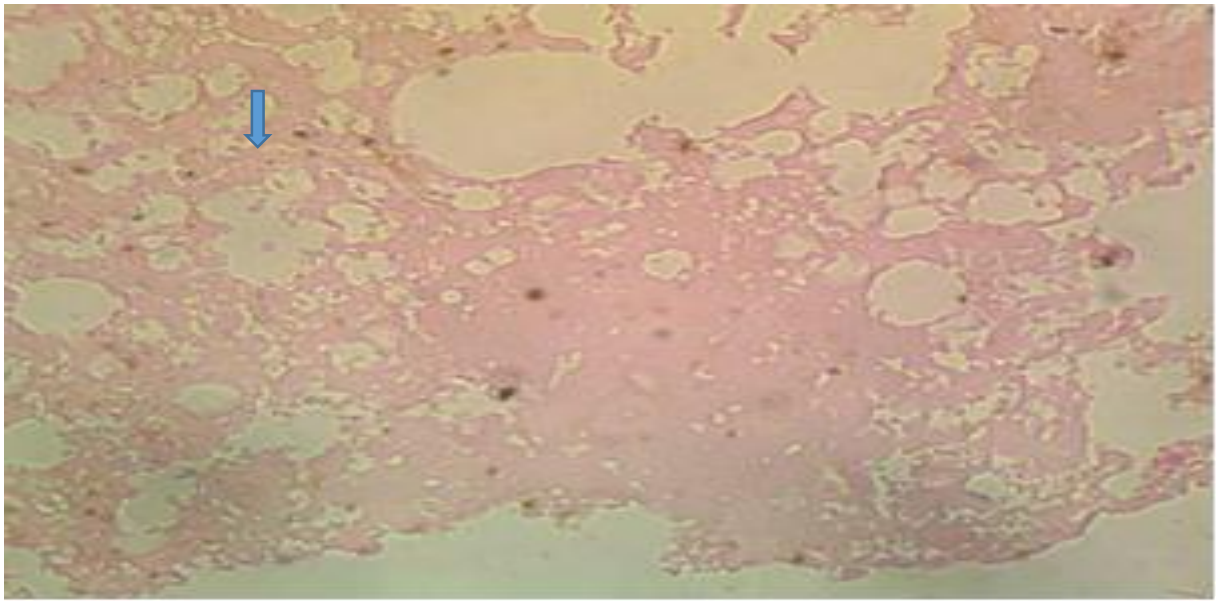


d. After 72 hours

Plate 4.19 *a-d:* *P. citrinum* (Conc 1): Lung. There are multiple foci of necrosis and accumulation of necrotic debris. Embedded within the debris are clear unstained fungal hyphae. There are multiple foci of alveolar macrophages laden with dense aggregates of black pigments.

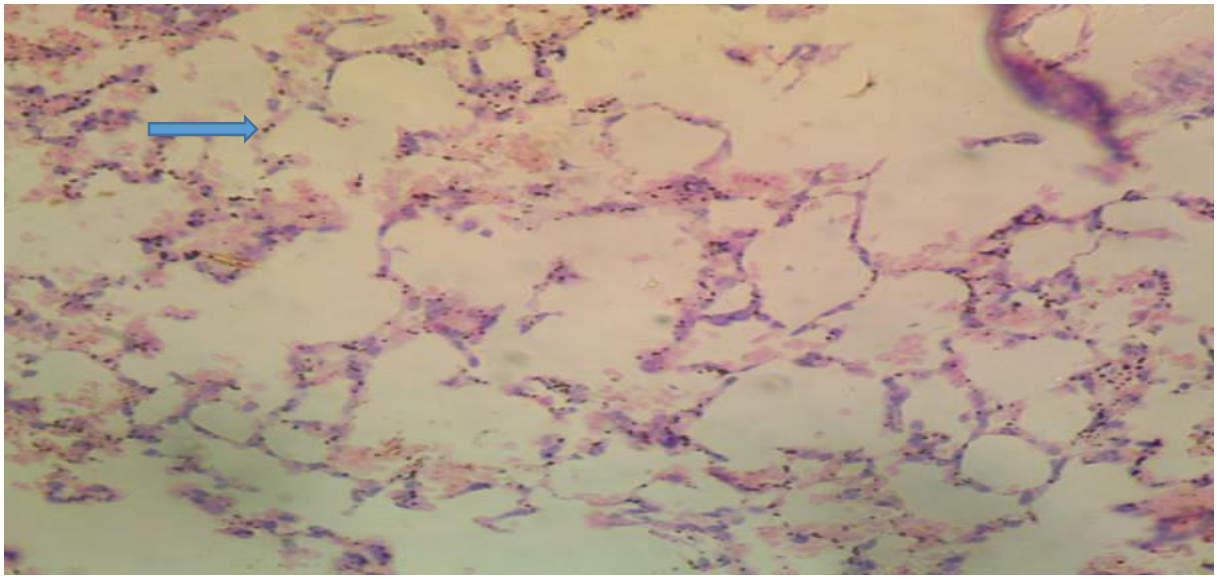


b. After 72 hours

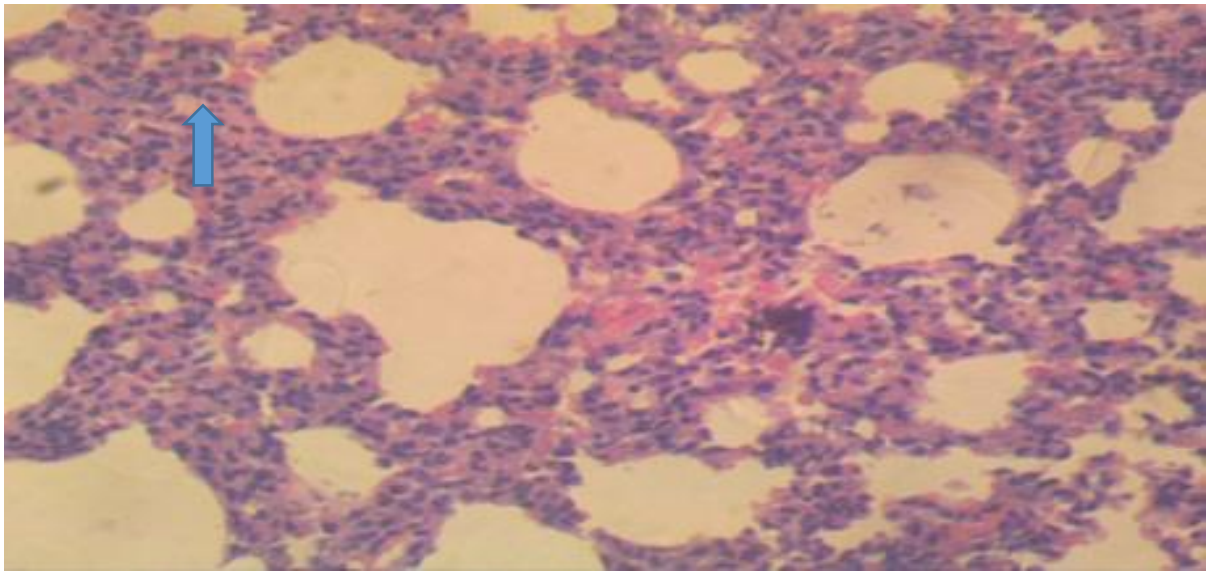


c. After 48 hours

Plate 4.19 b&c*: P. citrinum* (Conc 2): Lung. There are multiple foci of necrosis and accumulation of necrotic debris. There are extensive foci of alveolar macrophages laden with mild aggregates of black pigment. Mg x 100.

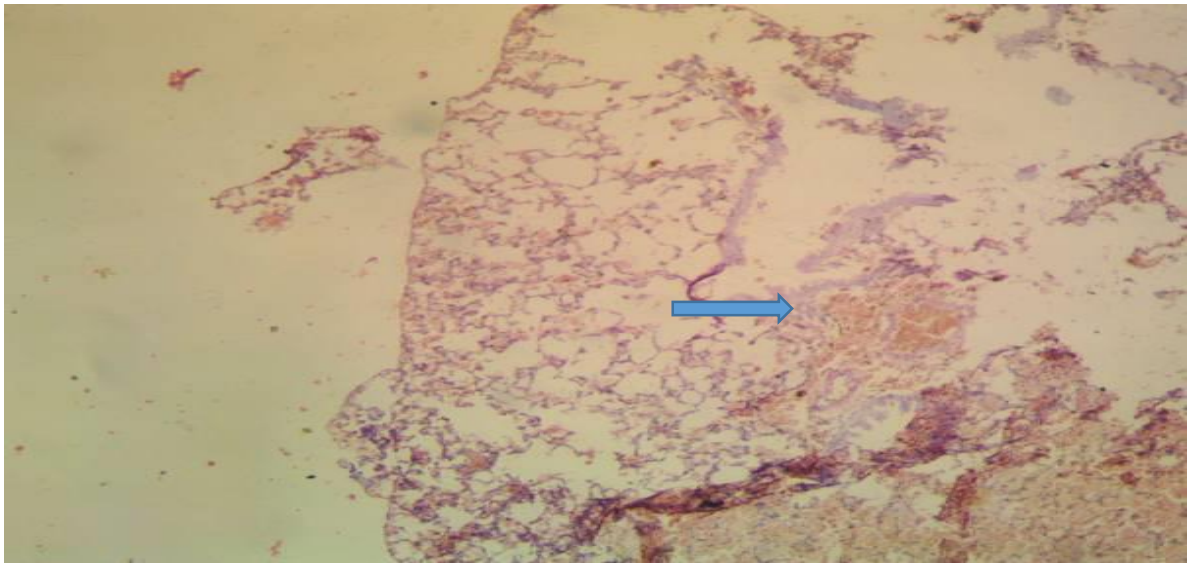


a. After 48 hours

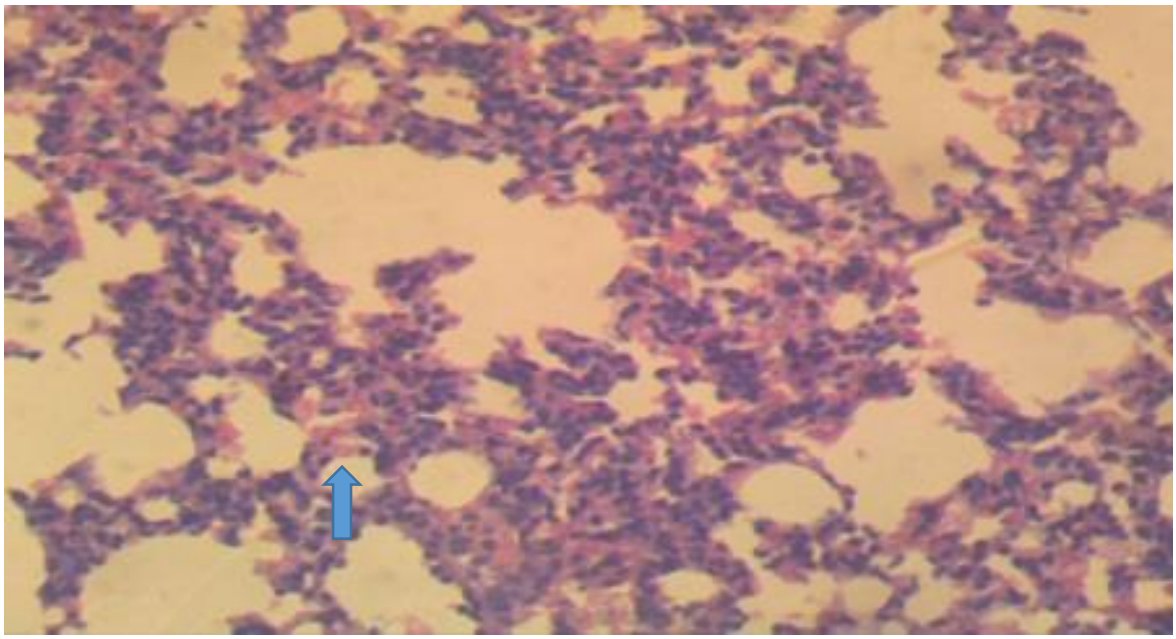


b. After 72 hours

Plate 4.20 *a-b**:* *Aspergillus flavus*** (Conc 1): There are foci of thickened alveolar septae and alternate foci of over-distended alveoli [emphysema]. Mg x 100.

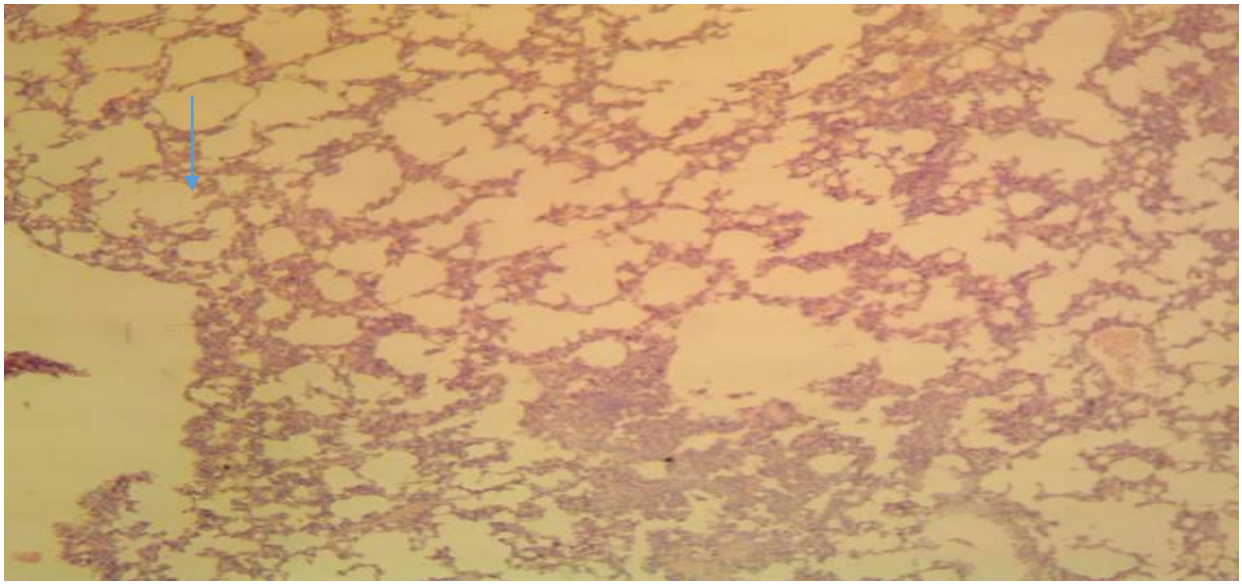


c. After 48 hours

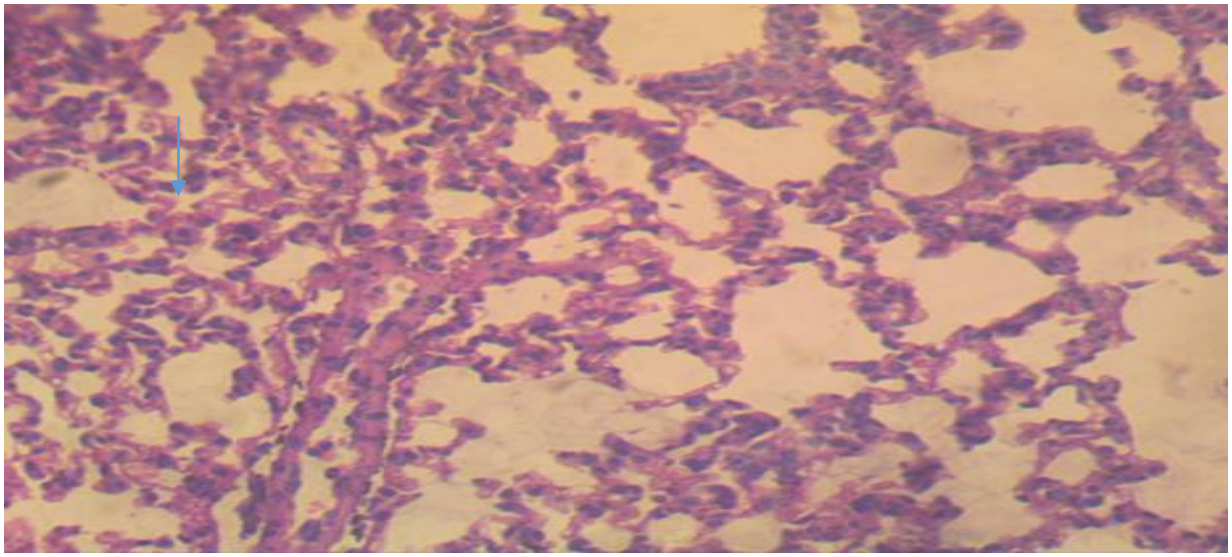


d. After 72 hours

Plate 4.20 c&d*; A. flavus* (Conc 2): Lung. There is widespread haemorrhage and over-distension of the alveoli indicative of pulmonary emphysema. Mg x 100.



a. Control after 48 hours



b. Control after 72 hours

Plate 4.21: Control: Alveoli are clear and devoid of exudates. There is no congestion. Mag: 400X.

(Conc 1 = 2.3 x 10^7 Conc 2 = 3.2 x 10^5).

Essentially, the pathology shown by the tissues appear to be similar although with varying degrees of severity. All of them had intra-lesional unstained fungal hyphae. Pathologies include thickening of alveolar sepatae, which causes impairment in vascular exchange and/or respiratory movements. This in turn results in forced expansion of adjacent alveoli which is manifested morphologically as the over-distension of alveoli [pulmonary emphysema] (Plates 4a – 8).

4.20 Immune cells of fungi inoculated mice

All the fungi inoculated resulted in significant ($p<0.05$) increase in the monocyte and basophils contents compared to the control. *P. citrinum* significantly increased the eosinophils as *A. terreus* increased the lymphocytes contents, while white blood cell was significantly raised by *P. funiculosum, A. terreus* and *P. chrysogenum.* The neutrophils content was higher in the *A. penicillioides, P. chrysogenum* and *P. citrinum* incoculated treatments. Both haemoglobin and red blood cell contents recorded were increased in the samples inoculated with *A. funiculosum, P. chrysogenum* and *A. flavus* compared to the control experiment (Table 4.18).

Table 4.16: Effects of different fungi treatments on Blood parameters in balb/c albino mice

Fungi	WBC	Neutrophils	Lymphocytes	Monocyte	Eosinophils	Basophils	Haemoglobin	RBC
P. funiculosum	6.90a	33.20d	60.10d	6.30c	0.10e	0.30c	15.70b	10.56c
A. terreus	5.38b	25.30h	66.70a	6.30c	0.40c	0.40b	13.50f	9.70f
A. penicilloides	2.67h	38.50b	56.60g	4.70f	0.01f	0.40b	14.00d	9.45h
P. chrysogenum	5.22c	36.30c	58.00e	5.20e	0.30d	0.20d	16.30a	10.70b
A. fumigatus	3.04g	25.50g	64.80c	8.60b	0.40c	0.70a	13.90e	9.68g
A. flavus	3.60f	32.70e	56.90f	10.80a	0.30d	0.10e	15.70b	11.70a
P. citrinum	3.61e	39.50a	54.30h	5.30d	0.60a	0.30c	13.40g	10.52d
Control	3.63d	29.20f	65.60b	4.50g	0.50b	0.00f	14.30c	9.91e

Mean with the different letter across the column are significantly ($p < 0.05$) different from one another with respect to each parameter.

CHAPTER FIVE

5.0 Discussion

In spite of several studies that have associated respiratory symptoms with air quality in different work environments, only limited information exists on biological exposure of allergenic fungi and associated health effects from different locations in Sub-Saharan Africa. In particular, the molecular studies on the genes encoding fungal allergic proteins are generally limited and consequently there are few analyses conducted on allergen proteins especially considering specific or closely related fungal taxa (Jousson *et al*., 2004; Hong *et al*., 2005). In this study, allergenic gene of fungi mainly of the genera *Aspergillus* and *Penicillium* were investigated at quantitative Polymerase chain reaction level since these fungi have been identified as important causative agents of allergy (Gupta, 2004; Xu *et al*., 2005). In addition, the potential allergenicity of many fungal species has not been widely studied (Westwood, 2005). Shen *et al*. (2006) have shown that two major allergens of *P. chrysogenum* are the vacuolar serine proteases (i.e., Pen ch 18) and the alkaline serine proteases (i.e., Pen ch 13 and Pen n 13). Similarly, the current molecular investigations in this work are focused strictly on the genes encoding the alkaline serine proteases from the sequence of the alkaline serine protease from selected *Aspergillus* and *Penicilium* species sampled from the air in various locations. The phylogenetic analysis conducted using Neighbour Joining method revealed the same tree topology showing different lineages of the ancestors of the isolates characterized.

The qRT-PCR data in this study showed that the expression of Pen citri allergenic gene was more expressed in *P. citrinum, P. pinophilum* and *P. oxalicum* species than in *P. funiculosum* and *P. simplicissimum*. However, quantitative real time PCR further revealed that for Pen oxa gene, *Aspergillus flavus, P. pinophilum, P. simplicissimum, P. citrinum* and *P. oxalicum* expressed the allergenic gene than *P. funiculosum* and *A. terreus* isolates. Ettenauer *et al*.

(2014) also reported in their work, a qPCR method targeting the β-actin gene which was developed for the quantitative assessment of fungi on different insulation materials. The advantage of qPCR as used in this study relies in the quantification of a gene. In their work, the β-actin gene has been proved to appear as a single actin gene copy per haploid genome in fungi. This fact enables a more precise quantification of the actual amount of fungal cells in an environmental sample than when using universal rRNA primers, due to the great variation of the number of rRNA gene clusters in a genome and among species. The obtained β-actin gene numbers were generally low and close to levels below the limit of detection of the assay.

This study also provide detailed information on the relative abundance of fungi from ten different locations in two South-western locations of Nigeria. The similarities in the distribution pattern of fungi spore can be said to be modulated by vegetation and close meteorological data.

Although, several works have been published on the composition and distribution of airborne fungi spores in indoor and outdoor environments in developed countries, only a few studies have been carried out in Nigeria. Makut *et al*. (2014) used plate sedimentation method during their investigation of microflora of outdoor air in Nasarawa State, Nigeria. Their monitoring of fungal and bacterial spore revealed that six bacterial species belonging to 6 genera and nine fungal species belonging to 7 genera were isolated at varying frequency of distribution. The results in this present study is in agreement with the report of Ekhaise *et al*. (2008) who also reported *Aspergillus* species as the most common genus of fungi in the air in the hospital environment. The findings in this study also showed *Aspergillus* and *Penicillium* species as the most dominant fungal isolates in the various locations sampled. Aerosolized *Aspergillus* spores are found nearly everywhere so we are routinely and almost constantly exposed to them. They have been proposed to cause human and animal diseases through the production

of mycotoxins, by the induction of allergenic responses and through localized or systemic infections (Bush *et al*. 2006). Farmer's lung, one of the best known of these, is an occupational mycosis correlated with inhalation of high concentrations of *Aspergillus* spores from contaminated agricultural products (Salvaggio, 1997).

Epidemiological studies have shown that a few hundred million people around the world are exposed to biological agents such as fungi. Unfortunately, there are no quantitative health-based guideline values or thresholds for acceptable levels of microbial contamination. Among the most common fungal species were those from genera *Aspergillus* and *Penicillium*, which are broadly present in nature including soil, cereal grains, hay and other plant materials or foodstuff. Exposure to these fungi has been associated with a variety of adverse health outcomes including respiratory, hematological, immunological, and neurological system disorders and/or diseases (Dutkiewicz and Górny, 2002; Samson *et al*., 2004). Tsai and Macher (2005) in their study found *Aspergillus fumigatus* and *Aspergillus flavus* as the most dominant fungal species in their sampling of US homes and therefore classified them as risk group 2 i.e., as possibly responsible for allergic effects and potentially hazardous to workers health. This is also similar to results obtained in this present study which showed *Aspergillus* and *Penicillium* as the most dominant fungal genera obtained in all the sampling locations.

In this study, a total of thirty-nine different fungal species were identified from the various location. From the *Aspergillus* genus, 12 species and 2 strains were identified of which; *A. flavus, A. fumigatus, A. niger* and *A. terreus* were the most common species sampled while for *Penicillium*, 6 species and 1 strain were identified. Also, in this present study, Lagos location had higher fungi spore collection than Ibadan. This could be attributed to the fact that Lagos is more populated and because of its population more dirtier than Ibadan because of the enormous number of people living in the city.

According to Ogunlana (1975), the wet and dry seasons (indicated by the temperature, relative humidity, and rainfall data) caused seasonal periodicity in colony numbers of fungal spores sampled in the city of Ibadan which is also similar to the results obtained in this study where months with high relative humidity and rainfall witnessed significant increase in fungal spore collection. Ogunlana also obtained different types of fungal spores such as: *Penicillium citrinum*, *Penicillium herquei*, *Penicillium clavigerum*, *Fusarium semitectum*, *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus fumigatus* and *Aspergillus flavus* amongst others. This is also in agreement with the types of fungal spores isolated in this study from various locations where species such as *Fusarium verticilloides*, *Aspergillus niger*, *Penicillium chrysogenum* amongst others were found to be abundant. The lowest total number of colonies was obtained in the month of December for both Potato dextrose agar and DG- 18 agar. This is also what was obtained in this research work. In his submission, Ogunlana 1975 observed that fungal spore population of the atmosphere was high at the beginning of the dry season, i.e., October; thereafter there was a gradual decrease to the middle of the dry season, i.e., December. This is also similar to the findings obtained in this work.

Ezike (2016) also conducted a survey of pollen and fungal spores constituents of the atmosphere of Garki, Abuja and reported that numerous fungal spores were trapped throughout the sampling periods among which *Alternaria* spp., *Fusarium* spp., *Cladosporium* spp. and *Curvularia* spp. were dominant. This however slightly differ with with the findings in this study. The occurrence of some of the dominant fungal spores could be an indicator of pathogen development in the area and could warn the farmers and agriculturists to protect their crops from diseases. According to Njokuocha (2006), most of the fungi species identified in the air spora have also been associated with diseases of many agricultural crops and wild plants in Nsukka. Among the diseases are loose smut of maize, leaf blight and spots,

damping-off and scab caused by species of *Alternaria* sp, *Dreschelia* sp; and cassava blight caused by *Alternaria* sp among others. Most of these fungi pathogens show multiple and whole plant host ranges, while some others are saprophytic on agricultural produce. Njokuocha and Ukeje (2006) also opined that fungal spores are cosmopolitan in distribution and constitute a large proportion of the airborne palynomorphs trapped in most aeropalynological studies. Some of the spores identified in their work are also among the invasive airborne fungal spores that have been implicated in nosocomial (hospital) infection of patients with solid organ transplants (Sanchez and Bush, 2001; Cashel *et al.*, (2004).

An accurate, rapid, cost-effective, and universally accessible identification system is needed for fungi. Recent estimates suggest that 1.5 million species of fungi exist, but <10% are formally described. The frequent lack of distinctive morphological characters, the preponderance of microscopic species, and the considerable socioeconomic importance of this kingdom reinforce the need for a DNA-based identification system. The 28S nuclear ribosomal large subunit rRNA gene (LSU) sometimes discriminates species on its own or combined with ITS. The 18S nuclear ribosomal small subunit rRNA gene (SSU) is commonly used in phylogenetics, and although its homolog (16S) is often used as a species diagnostic for bacteria (Geml *et al*., 2008), it has fewer hypervariable domains in fungi. Among the regions of the ribosomal cistron, the internal transcribed spacer (ITS) region has the highest probability of successful identification for the broadest range of fungi. ITS is also used in some fungi for providing an indication of delimitation by a measure of the genetic distances (Schindell, 2005). However, phylogenetic approaches are also being used to identify taxonomic units in environmental sampling of fungi and are often more effective in comparison (Stackebrandt and Goebel, 1994). However, across the fungal kingdom, ITS is generally superior to LSU in species discrimination. Given the fungal kingdom's age and genetic diversity, it is unlikely that a single-marker barcode system will be capable of

identifying every specimen or culture to species level. Data from this study indicate that ITS and LSU perform very similarly as barcodes and that differences in these sequences correlate well with current species concepts. Combinations of both ITS and LSU sequences are also applied in environmental sampling (Gorfer *et al*., 2010), where tandem amplification can allow simultaneous species identification with ITS and phylogenetic analysis with LSU. Despite these challenges, ITS combines the highest resolving power for discriminating closely related species with a high PCR and sequencing success rate across a broad range of Fungi.

There is ample evidence that allergic disorders, such as asthma, rhinitis, and atopic dermatitis, are mediated by oxidative stress. Excessive exposure to reactive oxygen and nitrogen species is the hallmark of oxidative stress and leads to damage of proteins, lipids, and DNA. All organisms have evolved elaborate cellular defenses that are collectively termed *antioxidants* to overcome this toxicity. The imbalance between reactive oxygen species and antioxidants is termed *oxidative stress*. Oxidative stress occurs in many allergic and immunologic disorders. Exposure to reactive oxygen species damage can be through various routes which may include for instance, cigarette smoke inhalation results in increased exposure to both superoxide and hydrogen peroxide. Other sources of environmental oxidants include air pollution, which contains ozone. Measurement of products damaged by reactive oxygen species is the most common technique to indirectly measure reactive oxygen species. The primary defense against reactive oxygen species is endogenous antioxidants, which can be subdivided into enzymatic and nonenzymatic categories. The enzymatic antioxidants include the families of superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione S-transferase, and thioredoxin. The nonenzymatic category of antioxidant defenses includes low-molecular-weight compounds, such as glutathione, ascorbate, urate, α-tocopherol, bilirubin and lipoic acid. Concentrations of these antioxidants vary depending on

both subcellular and anatomic location. For instance, glutathione is 100-fold more concentrated in the airway epithelial lining fluid compared with plasma. Other high-molecular-weight molecules that might be considered antioxidants include proteins that have oxidizable thiol groups, such as albumin and proteins that bind free metals, such as transferrin. Thus the composition of airways antioxidant defenses and reactive oxygen species depends on both subcellular and anatomic localization. However, in this present study, there was a decrease in protein content in the lungs of all the fungi inoculated mice compared with control. *Penicillium chrysogenum* inoculated mice produced the least protein when compared with the protein produced by other fungi inoculated mice. Wu *et al*. (2000) reported that elevated nitrotyrosine and chlorotyrosine levels from airway lavage samples suggest that proteins are also damaged. Although, the consequences of oxidative modifications to proteins are not well studied (Kelly *et al*., 1999).

Although airway glutathione is increased in asthmatic patients, the ratio of oxidized to reduced glutathione also increases (Rubbo *et al*., 1994). This increase in reduced glutathione suggests an adaptive response. In this study, superoxide dismutase was produced more in *Penicillium citrinum* inoculated mice while *Aspergillus flavus* inoculated mice had the least superoxide dismutase produced compared with control. Smith, (1997) and Hanazawa *et al*., (2000) showed in their work that superoxide dismutase activity is diminished in cells from lavage and brushing samples of patients with asthma. This is also similar to what is obtained in this work where levels of superoxide dismutase reduced in all inoculated lungs compared with control which had higher levels of SOD. The increase in reactive oxygen species during an asthma exacerbation might overwhelm endogenous antioxidant defenses (Sadeghi-Hashjin, 1996). Kaminsky *et al*. (1999) explained that asthmatic patients have increased levels of exhaled nitric oxide that can be suppressed by corticosteroids. They also explained that the role of nitric oxide in the lung is complicated because there are 3 distinct sources of

nitric oxide synthases (NOSs). NOS I (nNOS, or neuronal NOS) is found at non-adrenergic nerve terminals of smooth muscle and might cause nitric oxide–mediated bronchodilation. NOS III (extracellular NOS) is found primarily on endothelium and mediates vasodilation. NOS II (inducible NOS) can be found on a wide variety of inflammatory and epithelial cell types. Rubbo *et al*. (1994) however revealed that it is primarily inducible NOS that is induced in asthma and responsible for increased levels of exhaled nitric oxide. This is also in line with what was obtained in this study where nitric oxide level was reduced in all fungi inoculated samples. Lowest level was recorded in *Penicillium chrysogenum* inoculated mice whereas *Aspergillus flavus* and *Aspergillus penicilloides* inoculated lungs produced similar levels of nitric oxide. The impairment of nitric oxide signaling as reported by van der Vliet *et al*., (2000); Kang *et al*. (2000) might be mediated by nitric oxide's reaction with other reactive oxygen species. For instance, nitric oxide rapidly reacts with superoxide to form peroxynitrite. Peroxynitrite formation increases during inflammation and is toxic to microbes; however, peroxynitrite can also cause airway hyperresponsivenes (MacPherson *et al*., 2000; Arner and Holmgren, 2000). The glutathione antioxidant and detoxification systems play a major role in the antioxidant function of cells. Exposure to mycotoxins in humans requires the production of glutathione on an "as needed" basis (Guilford and Hope, 2014). Gluthathione is the main non-protein thiol responsible for cellular homeostasis and maintenance of the cellular redox balance (Wu *et al*., 2004; Forman *et al*., 2009). Also it has been shown that glutathione is decreased due to an inability to produce glutathione in the extracellular lung fluid of children with chronic asthma (Fitzpatrick *et al*., 2011) and in the macrophages of adults with human immunodeficiency virus (HIV) (Morris *et al*., 2013). In this present study, glutathione followed similar trend, there was reduction in the level of all fungi inoculated mice compared with control. *Penicillium citrinum* inoculated mice had higher levels of glutathione in relation to *Penicillium chrysogenum, Aspergillus flavus* and *A.*

penicilloides inoculated mice. Control mice produced elevated levels of glutathione. In humans, deficiency of glutathione can lead to chronic conditions (Ballatori *et al*., 2009) including chronic asthma (Fitzpatrick *et al*., 2011).

The degree of lipid peroxidation is often used as an indicator of reactive oxygen species (ROS) mediated damages (Kühn and Borchert, 2002) and the concentrations of malondialdehyde (MDA) in blood and tissues are generally used as biomarkers of radical-induced damage and the endogenous lipid peroxidation (Sehirli *et al*., 2008; Yousef *et al*., 2009). In the present study, the influence of four different fungi inoculum on the oxidative injury and immunity status in the lungs of mice were investigated and it was found out that all inoculated mice had increased MDA levels except for *Aspergillus flavus* inoculated mice. The increase in the activities of the MDA enzyme result in the involvement of deleterious oxidative changes which led to the increase in the oxidant level of the lungs of the mice. Hydrogen peroxide transfer stimulating signals as a critical intracellular second messenger, resulting in the modulation of immune responses (Stone and Yang, 2006). They also reported that many types of cellular stimuli, including antigens, infections, various chemical mediators, and growth factors, induce a transient increase in intracellular ROS—in particular H_2O_2—mainly through activation of NADPH oxidase immediately after the exposure of the stimuli to cells (Brown and Griendling, 2009). Oxidative stress such as Hydrogen peroxide (H_2O_2) can initiate various intracellular signaling pathways that lead to a break in immune tolerance and exaggerated allergic inflammation. In this work, H_2O_2 levels was increased as a result of intranasal inoculation of fungi spores into the nose of Balb/c mice. The four different fungi inoculated gave higher levels of hydrogen peroxidase in the lungs of the mice. This probably may lead to degenerative diseases by inducing unscheduled death and cell cycle arrests, neoplasia (triggered by insensitivity to stress) and allergic or autoimmune disorders caused by immune cell overstimulation and a break in immune tolerance as reported by

Matsue *et al*., (2003); Williams and Kwon, (2004) in their studies . According to Hood (1999), Myeloperoxidase (MPO) levels have also been found to be elevated in the BAL fluid of asthma patients compared to controls as observed. Higher levels of MPO have been found in induced sputum and BAL fluid in patients with asthma than in control subjects, demonstrating that degranulation of primary granules takes place in asthma. In another study by Carlson *et al*. (1991), a greater release of MPO was observed in allergic patients than in controls when neutrophils were stimulated with particles of Sephadex opsonized with serum. Carlson *et al*. (1992) reported that MPO release has been seen to be greater in pollen-atopic patients at the end of spring than at times when these patients are asymptomatic. This is in agreement with this present study which also confirmed the increase in Myeloperoxidase level in fungi inoculated mice compared to control samples. Only *Aspergillus penicilloides* gave a slightly lower MPO level compared to control sample. *Penicillium chrysogenum* lungs produced the highest MPO level in all the four inoculated fungal spore lungs.

Neutrophils are the first cells recruited to the site of the allergic reaction. Rabier *et al.* (1991) reported that only around 50% of asthma cases were associated with eosinophilic inflammation, and that in most other cases asthma was accompanied by an increase in airway neutrophils and interleukin 8 (IL-8). Neutrophils are polymorphonuclear leukocytes that play an essential role in the immune system, acting as the first line of defense against bacterial and fungal infections. Kanazawa *et al*. (1993) reported that neutrophils from the BAL fluid of asthmatic patients have been seen to produce greater amounts of toxic oxygen radicals than those from a reference group of healthy controls. In this present study, neutrophils cells were produced in higher numbers in all fungi spore inoculated mice when compared with control mice. In this work, only mice inoculated with *Aspergillus terreus* spores produced lesser number of neutrophils but all other mice had higher neutrophilic cells. Neutrophil factors

involved in the initiation and maintenance of late allergic reactions are released at 18 hours after the challenge of these cells (Monteseirín *et al*., 2002).

Hoselton *et al*. (2010) and Amin (2012) reported that repeated exposure to allergens like *A. fumigatus* results in the accumulation of neutrophils, basophils, and mast cells which are characteristic features associated with the early phase inflammatory reaction while the late phase reaction is characterized by the accumulation of Th2 lymphocytes (Horwitz and Busse, 1995), B lymphocytes (Horwitz and Busse, 1995; Ghosh *et al*., 2012), neutrophils (Hogaboam *et al*., 2003), macrophages (Hogaboam *et al*., 2003; Samarasinghe *et al*., 2011b), basophils (Horwitz and Busse, 1995; Smit and Lukacs, 2006), and eosinophils (Campos and Pereira, 2009; Samarasinghe *et al*., 2011b). The result from the blood analysis in this thesis revealed that monocytes were produced in large numbers in all the fungal spores inoculated mice compared to control. Similar result was also obtained for basophils which were produced in higher quantities in all the fungal spores inoculated mice. *Aspergillus fumigatus* inoculated mice produced the highest basophil cells whereas for eosinophils, *Penicillium citrinum* inoculated mice recruited most eosinophils to the centre of infection. Eosinophils have been considered end-stage cells in immunopathology of fungal allergic asthma. Observations from experimental animals and asthmatic patients as reported by Ghosh *et al*. (2013) suggest a direct participation of eosinophils in mediating the pathophysiology associated with allergic/fungal asthma, although the mechanisms by which eosinophils contribute to the pathogenesis are rather complicated.

The results from the SDS-PAGE analysis indicated that extracts obtained from various *Aspergillus* species contained 25 protein bands with molecular weights between 5 and 100 kDa. The highest protein bands were associated with *A. terreus* and *P. chrysogenum* (6 bands) in a range from 5 to 90 kDa, while the lowest ones were detected in *P. oxalicum* (3 bands) in a range from 30 to 100 kDa. Protein bands of 30 and 25 kDa had 80 % frequencies

in the five species of *Aspergillus* and *Penicillium* respectively. In a study by Saeednejad *et al*. (2010), 69 protein bands with molecular weights between 11.5 and 178 kDa were identified. Among these , protein bands with molecular weights of 15, 23.5, 27, 33.5 and 61 kDa were found in two species, *A. fumigatus* and *A. flavus*, protein bands 28.5, 40 and 47 kDa were found in two species of *A. flavus* and *A. niger*, and a band with 120 kDa was found in *A. fumigatus* and *A. niger*. The whole-cell proteins profile obtained by the SDS-PAGE analysis can provide valuable criteria for serological and immunological studies of people exposed to aeroallergens in different locations.

In this study, vascular changes observed in the histology of the lungs ranged from congestion to pulmonary haemorrhages to vasculitis (inflammation of the pulmonary blood vessels).Gupta *et al*. (2008) attributed the vasculities found in a similar work to be mediated by mononuclear inflammatory cells which manifested as thickening of the walls of blood vessels. Hyperplasia of the bronchiolar epithelium was observed in one of the sections. This is likely to be due to localization of the fungal hyphae in the lumen of the bronchioles. Fokkens *et al*. (2012) also identified hypeplasia as the major symptoms of fungal allergy in their experiment. The hyperplasia is a response to the irritiation caused by the fungal hyphae as well as an attempt to replace necrotic epithelial cells of the bronchioles.

5.1 Summary of Findings

Objectives	Summary of Findings
1. Isolate and identify fungi from various environment such as Farm land, schools, residential estates, Market, Hospital in Lagos and Ibadan monthly for twenty four months (24 months) and characterize the fungi found.	✓ Forty species of fungi were isolated and characterized, in which majority are of *Aspergillus* and *Penicillium* species/strains while others include *Trichoderma* spp, *Fusarium* spp, *Cladosporium herbarium*, *Phoma eupyrema*, *Mucor* sp, *Absidia blakelsiana*, *Paecilomyces* sp, *Curvularia lunata*, *Neurospora crassa*, *Rhizopus oryzae*, *Perenniporia koreana* and *Sistotrema brinkmannii*. ✓ This study revealed that there is significantly high distribution of fungi from farm settlement (Iba), Lagos which had higher number of colonies than other locations sampled.
2. measure the abundance/distribution of these fungal spores in the environment in relation to weather.	✓ Monthly spore distribution showed that January, February and December had lowest spore count while June had the highest fungal spore count. Also, weather parameters such as higher rainfall, lower relative humidity and temperature favoured the abundance and higher distribution of fungi found in the various environments.
3. characterize fungal gene expression encoding allergen and determine allergen expression in some fungal isolates.	✓ RT-QPCR (gene expression) analysis showed that for *P. citrinum* allergenic gene, it was more expressed in *some Penicillium* spp. than *Aspergillus* spp. while *P. oxalicum* allergrnic gene was also expressed in certain *Aspergillus* spp. than *Penicillium* isolates.
4. compare the immune response and systemic effect induced by selected fungi spores at different doses in mice.	✓ Both dose responses and time-courses studied showed various degree of inflammation, structural and tissue damage. ✓ Antioxidant and oxidative stress parameters which are markers of allergy were significantly different in fungi inoculated mice from control and showed initiation of inflammation in the cells.
5. correlate the monthly abundance of pores in the atmosphere with patient data in the hospital.	✓ Hospital record show that patients population complaining of respiratory problems and asthma are higher during rainy season while *Aspergillus* and *Penicillium* species have significant positive correlation with asthma and respiratory problems.

5.2 Conclusion

Fungi are ubiquitous component of the atmosphere. Their presence, abundance and concentrations are influenced by weather parameters, vegetation and time. As a result of these variables, their atmospheric count vary from one season to another resulting in more exposure to fungal spores during the rainy season and pollens during the harmattan period. Sampling method and medium used determines and influences the abundance and number of colonies to be collected. The allergenic gene expression and systemic effect varies from organism to organism.

This work will form the basis for a forecast to inform and sensitize allergy sufferers about the Fungi distribution in the two states. Thus, bioaerosols present in sampled locations, which penetrate the human respiratory tract, may be deposited in the oral and nasal cavities, secondary bronchi and bronchioles and be responsible for a wide variety of adverse health effects from nose and eye irritation to asthmatic reactions to allergic inflammation. The study has also provided useful baseline information and data which could be used predictively in forecasting season which medical practitioners can use to advice for safety of those suffering from allergies such as hay fever (pollinosis) and to monitor the frequency and intensity of fungal allergies and various disease conditions of plants, animals and man in the surrounding environment.

5.3 Contributions to Knowledge

1. This work established that Penicillium cit allergenic gene was highly expressed in certain *Penicillium* species than other *Penicillium* species while Pen oxa gene was more expressed in *Aspergillus* species than *Penicillium* isolates.
2. This research work also shows that intranasal inhalation of fungi spores produces oxidative stress responses in mice which are destructive especially the spores of *Penicillium chrysogenum*.
3. This work established that *Aspergillus penicilloides* spores in addition to *A. flavus* had highly significant lethality on lungs of mice than those of *P. citrinum* and *P. chrysogenum*.
4. This study was able to isolate, identify, characterize and document 39 different fungi species and strains found in various locations in Lagos and Ibadan, Nigeria. This however showed that the dominant fungi found outdoors belong to subdivisions of *Ascomycotina* and *Deuteromycotina.* This study was able to report new species of fungi like *P. simplicissimum*, *Pereniporia koreana*, *Penicillium pinophilum* and *Aspergillus protuberus* which has not been reported in literature before in Nigeria to the best of my knowledge.

5.4 Recommendations

1. It is important to isolate and identify important airborne fungi that cause allergic disorders.
2. Knowledge of life history of these fungi is essential in identifying their source and environmental conditions that help in getting airborne in significant concentration.
3. Prevention is better than cure, therefore it is suggested that substrate on which microorganism build up their numbers should be removed from the environment.
4. Air quality can be enhanced by periodic maintenance of air treatment plant, fumigation, application of antifungal agents etc.

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Table 4.17: Fungi Morphology and Microscopic Description

Fungi	Characteristics
Aspergillus terreus	This fungus colony is typically suede-like and Cinnamon-buff to sand brown in colouration. Conidial heads are short columnar and biseriate, with short, brownish and smooth-walled stipes.
Mucor racemosus	Colonies are very fast growing, cottony to fluffy, white to yellow colouration. The culture consists of spherical, non-apophysate sporangia with pronounced columellae and conspicuous collarette at the base of the columella following sporangiospore dispersal.
Penicillium citrinum	Colony is fast growing in shades of green and white. Cultures consisting of chains of single-celled conidia are produced in basipetal succession from a specialized conidiogenous cell called phialide. The phialides are produced singly in groups or from branched metulae giving a brush-like appearance.
Paecilomyces species	Conidiophores hyaline, erect, branched apically bearing conidia on terminal phialides. Phialide opposite with cylindrical base. Conidia are terminal, slightly rough on the surface.
Aspergillus fumigatus	Conidiophores hyaline, inflated clavately at the apex forming nodded vesicles, bearing conidia heads composed of uniserate phialides on pale brown vesicles. Conidial head is dark bluish green.
Aspergillus niger	Conidiophores hyaline or pale brown, erect, simple, thick walled inflated at the apex forming globose vesicles bearing conidial head split into over four loose conidial borne on uniserate or biserate phialides on pale brown globose vesicles and phialides tapered at apex.
Trichoderma hamatum	Conidiophores developed on cushion shaped structures, hyaline erect, branched bearing spore masses on alternate phialides together with setae-like sterile hyphae. Phialides are short and thick, densely arranged.
Trichoderma harzianum	Conidiophores hyaline, erect, branched, bearing spore masses apically at verticillate Phialides. Phialides are short and thick.
Curvularia affinis	Conidiophores pale brown, erect, simple or branched bearing conidia apically and laterally. Conidia long, ellipsoidal, almost uncurved, mainly 5-celled, large and more pigmented in 1 central cell.
Aspergillus sydowii	This species form velvety colonies of deep bluish green or greenish blue colour with revers side usually deep red. Dwarfed heads with clusters of phialides are found in nearly all isolates.
Aspergillus versicolor	As the name implies, cultures show considerable range in colour from pale green to yellow green, grey green, or even pink in small areas. Conidia globose and delicately roughened.
Aspergillus tamari	Mature colony is brownish greenish to brown but often starting as a deep yellowish-green brown. Head is large with single chains of conidia. Vesicles are thin walled and conidia dark, cylindrical when young and globose with age.
Aspergillus oryzae	Colonies seldom show true green but are yellow becoming brownish green. Conidiophores long, thin walled, usually rough, biserate. Conidia are globose to elliptical usually finely roughened.

Aspergillus flavus	Colonies are yellow at first later turning yellow-green. Conidiophores coarsely roughened, heads varying in size, biserate but having some heads with phialides borne directly on the vesicle. Conidia usually globose occasionally elliptical.
Aspergillus ochraceus	Colonies often developing reddish purple colour in reverse; conidiophores distinctly rough and long. Vesicles globose with a close range of metulae; conidia are small and roughened. Sclerotia are often produced.
Aspergillus fumigatus	Conidia smaller, globose.
Aspergillus penicilloides	Spore heads are radiate rather than columnar. Conidia at first elliptical becoming globose. Phialides and conidia are often swollen.
Penicillium citrinum	Colonies are dull grey-green with narrow margin Conidia globose, smooth packed in divergent Columns one to each metula.

APPENDIX I

CORRELATIONS

/VARIABLES=Rainfall Temp RHumidity Asthma Wheezing FungicountIkeja

/PRINT=TWOTAIL NOSIG

/MISSING=PAIRWISE.

Correlations

Notes

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Correlations

		Rainfall	Temp	RHumidity	Asthma	Wheezing
Rainfall	Pearson Correlation	1	-.119	.303	.190	.190
	Sig. (2-tailed)		.627	.207	.450	.450
	N	19	19	19	18	18
Temp	Pearson Correlation	-.119	1	-.799**	.388	.388
	Sig. (2-tailed)	.627		.000	.112	.112
	N	19	19	19	18	18
RHumidity	Pearson Correlation	.303	-.799**	1	-.103	-.103
	Sig. (2-tailed)	.207	.000		.685	.685
	N	19	19	19	18	18
Asthma	Pearson Correlation	.190	.388	-.103	1	1.000**
	Sig. (2-tailed)	.450	.112	.685		.000
	N	18	18	18	23	23
Wheezing	Pearson Correlation	.190	.388	-.103	1.000**	1
	Sig. (2-tailed)	.450	.112	.685	.000	
	N	18	18	18	23	23
FungicountIkeja	Pearson Correlation	.298	-.205	.517*	.371	.371
	Sig. (2-tailed)	.216	.400	.023	.082	.082
	N	19	19	19	23	23

Correlations

		FungicountIkeja
Rainfall	Pearson Correlation	.298
	Sig. (2-tailed)	.216
	N	19
Temp	Pearson Correlation	-.205
	Sig. (2-tailed)	.400
	N	19
RHumidity	Pearson Correlation	.517
	Sig. (2-tailed)	.023
	N	19
Asthma	Pearson Correlation	.371
	Sig. (2-tailed)	.082
	N	23
Wheezing	Pearson Correlation	.371
	Sig. (2-tailed)	.082
	N	23
FungicountIkeja	Pearson Correlation	1
	Sig. (2-tailed)	
	N	24

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

*. Correlation is significant at the 0.05 level (2-tailed).

APPENDIX II

The PRINCOMP Procedure

Observations	**15**
Variables	**6**
Partial Variables	**2**

Simple Statistics

	Feb	Mar	June	July
Mean	**4.133333333**	**3.933333333**	**4.733333333**	**4.533333333**
StD	**1.726543478**	**0.883715102**	**1.533747356**	**1.884776101**

Simple Statistics

	Aug	Total_spores	FUNGI_ Locations
Mean	**2.266666667**	**19.60000000**	**2.666666667** **4.333333333**
StD	**0.961150105**	**3.73783742**	**2.794552524** **2.768874621**

Correlation Matrix

		Feb	Mar	June	July	Aug	Total_ spores	FUNGI_ Locations
Feb	Feb	**1.0000**	**0.5680**	**-.3093**	**-.3307**	**-.2812**	**0.2302**	**-.2270** **0.7371**
Mar	Mar	**0.5680**	**1.0000**	**0.0913**	**0.0658**	**-.2299**	**0.5103**	**-.1253** **0.3308**
June	June	**-.3093**	**0.0913**	**1.0000**	**0.6952**	**0.3909**	**0.7401**	**-.1889** **-.6167**
July	July	**-.3307**	**0.0658**	**0.6952**	**1.0000**	**0.3102**	**0.7320**	**0.2260** **-.6524**
Aug	Aug	**-.2812**	**-.2299**	**0.3909**	**0.3102**	**1.0000**	**0.3897**	**0.0355** **-.5189**
Total_spores	Total spores	**0.2302**	**0.5103**	**0.7401**	**0.7320**	**0.3897**	**1.0000**	**-.0889** **-.2968**
FUNGI_	FUNGI	**-.2270**	**-.1253**	**-.1889**	**0.2260**	**0.0355**	**-.0889**	**1.0000** **-.0769**
Locations	Locations	**0.7371**	**0.3308**	**-.6167**	**-.6524**	**-.5189**	**-.2968**	**-.0769** **1.0000**

Regression Statistics

	Feb	Mar	June	July	Aug	Total_spores
R-Square	0.5725018329	0.119489336	0.4365163052	0.4567461585	0.2692778674	0.100627965
RMSE	1.2193209691	0.895680216	1.2435633059	1.5004950401	0.8874435623	3.8288071472

Standardized Regression Coefficients

	Feb	Mar	June	July	Aug	Total_spores
FUNGI_	-.1713069244	-.1004786913	-.2377188054	0.1768797732	-.0044862555	-.1123904354
Locations	0.7239308151	0.3231075294	-.6350043480	-.6388079121	-.5192457376	-.3054135486

The PRINCOMP Procedure

Partial Correlation Matrix

		Feb	Mar	June	July	Aug	Total_spores
Feb	Feb	1.0000	0.5005	0.2135	0.3742	0.1798	0.6932
Mar	Mar	0.5005	1.0000	0.3856	0.4327	-.0731	0.6712
June	June	0.2135	0.3856	1.0000	0.6048	0.1088	0.7452
July	July	0.3742	0.4327	0.6048	1.0000	-.0438	0.7985
Aug	Aug	0.1798	-.0731	0.1088	-.0438	1.0000	0.2901
Total_spores	Total spores	0.6932	0.6712	0.7452	0.7985	0.2901	1.0000

Eigenvalues of the Partial Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
1	3.24135038	2.14599981	0.5402	0.5402
2	1.09535058	0.22146122	0.1826	0.7228
3	0.87388936	0.39763998	0.1456	0.8684
4	0.47624938	0.16308909	0.0794	0.9478
5	0.31316030	0.31316030	0.0522	1.0000
6	0.00000000		0.0000	1.0000

		Eigenvectors					
		Prin1	Prin2	Prin3	Prin4	Prin5	Prin6
Feb	Feb	**0.383658**	**0.205939**	**-.621125**	**-.413974**	**-.429166**	**0.262756**
Mar	Mar	**0.405654**	**-.230106**	**-.415077**	**0.694369**	**0.301337**	**0.193014**
June	June	**0.415633**	**-.075135**	**0.579146**	**0.259624**	**-.589047**	**0.267980**
July	July	**0.448946**	**-.233992**	**0.286055**	**-.499482**	**0.554825**	**0.323348**
Aug	Aug	**0.100308**	**0.912556**	**0.151786**	**0.166765**	**0.264117**	**0.191239**
Total_spores	Total spores	**0.551259**	**0.107163**	**0.040483**	**-.042167**	**0.021154**	**-.825085**

CORRELATIONS

/VARIABLES=TREATMENT REPLICATE PROTEIN1 PROTEIN2 MDA1 MDA2 MPO1 MPO2 H2O21 H2O22 SOD1 SOD2 NOum1 NOum2 NOut1 Nout2 GSH1 GSH2

/PRINT=TWOTAIL NOSIG

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Correlations

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Correlations

		TREATMENT	REPLICATE	PROTEIN1	PROTEIN2	MDA1
TREATMENT	Pearson Correlation	1	.000	-.778**	-.254	.510**
	Sig. (2-tailed)		1.000	.000	.220	.009
	N	25	25	25	25	25
REPLICATE	Pearson Correlation	.000	1	-.043	-.004	-.062
	Sig. (2-tailed)	1.000		.837	.984	.769
	N	25	25	25	25	25
PROTEIN1	Pearson Correlation	-.778**	-.043	1	.038	-.599**
	Sig. (2-tailed)	.000	.837		.857	.002
	N	25	25	25	25	25
PROTEIN2	Pearson Correlation	-.254	-.004	.038	1	-.057
	Sig. (2-tailed)	.220	.984	.857		.787
	N	25	25	25	25	25
MDA1	Pearson Correlation	.510**	-.062	-.599**	-.057	1
	Sig. (2-tailed)	.009	.769	.002	.787	
	N	25	25	25	25	25
MDA2	Pearson Correlation	.007	.087	.116	-.725**	.113
	Sig. (2-tailed)	.973	.678	.580	.000	.592
	N	25	25	25	25	25
MPO1	Pearson Correlation	.708**	.121	-.690**	.163	.298
	Sig. (2-tailed)	.000	.564	.000	.438	.148
	N	25	25	25	25	25
MPO2	Pearson Correlation	.686**	-.190	-.475*	-.429*	.529**
	Sig. (2-tailed)	.000	.364	.016	.032	.007
	N	25	25	25	25	25

	Pearson Correlation	.351	-.022	-.593**	.083	.079
H2O21	Sig. (2-tailed)	.085	.918	.002	.693	.708
	N	25	25	25	25	25
	Pearson Correlation	.414*	.189	-.368	-.302	.419*
H2O22	Sig. (2-tailed)	.040	.366	.071	.143	.037
	N	25	25	25	25	25
	Pearson Correlation	-.852**	-.084	.483*	.478*	-.222
SOD1	Sig. (2-tailed)	.000	.689	.014	.016	.287
	N	25	25	25	25	25

Correlations

		MDA2	MPO1	MPO2	H2O21	H2O22	SOD1
	Pearson Correlation	.007	.708	.686**	.351	.414**	-.852
TREATMENT	Sig. (2-tailed)	.973	.000	.000	.085	.040	.000
	N	25	25	25	25	25	25
	Pearson Correlation	.087	.121	-.190	-.022	.189	-.084
REPLICATE	Sig. (2-tailed)	.678	.564	.364	.918	.366	.689
	N	25	25	25	25	25	25
	Pearson Correlation	.116**	-.690	-.475	-.593	-.368**	.483
PROTEIN1	Sig. (2-tailed)	.580	.000	.016	.002	.071	.014
	N	25	25	25	25	25	25
	Pearson Correlation	-.725	.163	-.429	.083	-.302	.478**
PROTEIN2	Sig. (2-tailed)	.000	.438	.032	.693	.143	.016
	N	25	25	25	25	25	25
	Pearson Correlation	.113**	.298	.529**	.079	.419	-.222
MDA1	Sig. (2-tailed)	.592	.148	.007	.708	.037	.287

	N	25	25	25	25	25	25
	Pearson Correlation	1	-.254	.364	-.182**	.423	-.127
MDA2	Sig. (2-tailed)		.221	.074	.385	.035	.544
	N	25	25	25	25	25	25
	Pearson Correlation	-.254**	1	.358**	.464	.221	-.433
MPO1	Sig. (2-tailed)	.221		.079	.019	.288	.031
	N	25	25	25	25	25	25
	Pearson Correlation	.364**	.358	1*	.115*	.423**	-.601
MPO2	Sig. (2-tailed)	.074	.079		.585	.035	.002
	N	25	25	25	25	25	25
	Pearson Correlation	-.182	.464	.115**	1	.106	-.234
H2O21	Sig. (2-tailed)	.385	.019	.585		.613	.261
	N	25	25	25	25	25	25
	Pearson Correlation	.423*	.221	.423	.106	1*	-.225*
H2O22	Sig. (2-tailed)	.035	.288	.035	.613		.280
	N	25	25	25	25	25	25
	Pearson Correlation	-.127**	-.433	-.601*	-.234*	-.225	1
SOD1	Sig. (2-tailed)	.544	.031	.002	.261	.280	
	N	25	25	25	25	25	25

Correlations

		SOD2	NOum1	NOum2	NOut1	Nout2	GSH1
	Pearson Correlation	-.635	-.822	-.415**	-.637	-.274**	-.582
TREATMENT	Sig. (2-tailed)	.001	.000	.039	.001	.185	.002
	N	25	25	25	25	25	25
REPLICATE	Pearson Correlation	.039	-.008	-.081	-.039	.092	.068

	Sig. (2-tailed)	.855	.972	.700	.852	.663	.748
	N	25	25	25	25	25	25
	Pearson Correlation	.462**	.582	.160	.563	.169**	.474
PROTEIN1	Sig. (2-tailed)	.020	.002	.444	.003	.419	.017
	N	25	25	25	25	25	25
	Pearson Correlation	.445	.357	.473	.113	.335	.087**
PROTEIN2	Sig. (2-tailed)	.026	.080	.017	.590	.102	.678
	N	25	25	25	25	25	25
	Pearson Correlation	-.194**	-.366	-.041**	-.515	-.049	-.152
MDA1	Sig. (2-tailed)	.354	.072	.846	.008	.816	.468
	N	25	25	25	25	25	25
	Pearson Correlation	-.009	-.315	-.051	-.310**	.080	.193
MDA2	Sig. (2-tailed)	.965	.126	.807	.132	.705	.356
	N	25	25	25	25	25	25
	Pearson Correlation	-.319**	-.439	-.053**	-.314	-.015	-.681
MPO1	Sig. (2-tailed)	.120	.028	.801	.126	.943	.000
	N	25	25	25	25	25	25
	Pearson Correlation	-.222**	-.661	-.104*	-.614*	-.020**	-.354
MPO2	Sig. (2-tailed)	.286	.000	.621	.001	.923	.083
	N	25	25	25	25	25	25
	Pearson Correlation	-.291	-.219	-.144**	-.180	-.314	-.354
H2O21	Sig. (2-tailed)	.157	.292	.492	.389	.126	.082
	N	25	25	25	25	25	25
	Pearson Correlation	-.130*	-.544	.020	-.730	.172*	.203*
H2O22	Sig. (2-tailed)	.537	.005	.925	.000	.412	.330
	N	25	25	25	25	25	25

SOD1	Pearson Correlation	.672**	.780	.623*	.460*	.421	.507
	Sig. (2-tailed)	.000	.000	.001	.021	.036	.010
	N	25	25	25	25	25	25

Correlations

		GSH2
TREATMENT	Pearson Correlation	-.566
	Sig. (2-tailed)	.003
	N	25
REPLICATE	Pearson Correlation	.108
	Sig. (2-tailed)	.609
	N	25
PROTEIN1	Pearson Correlation	.366**
	Sig. (2-tailed)	.072
	N	25
PROTEIN2	Pearson Correlation	.257
	Sig. (2-tailed)	.215
	N	25
MDA1	Pearson Correlation	-.102**
	Sig. (2-tailed)	.628
	N	25
MDA2	Pearson Correlation	.123
	Sig. (2-tailed)	.559
	N	25
MPO1	Pearson Correlation	-.465**
	Sig. (2-tailed)	.019

	N	25
	Pearson Correlation	-.264**
MPO2	Sig. (2-tailed)	.202
	N	25
	Pearson Correlation	-.238
H2O21	Sig. (2-tailed)	.252
	N	25
	Pearson Correlation	.006*
H2O22	Sig. (2-tailed)	.976
	N	25
	Pearson Correlation	.543**
SOD1	Sig. (2-tailed)	.005
	N	25

Correlations

		TREATMENT	REPLICATE	PROTEIN1	PROTEIN2	MDA1
	Pearson Correlation	-.635	.039	.462**	.445	-.194**
SOD2	Sig. (2-tailed)	.001	.855	.020	.026	.354
	N	25	25	25	25	25
	Pearson Correlation	-.822	-.008	.582	.357	-.366
NOum1	Sig. (2-tailed)	.000	.972	.002	.080	.072
	N	25	25	25	25	25
	Pearson Correlation	-.415**	-.081	.160	.473	-.041**
NOum2	Sig. (2-tailed)	.039	.700	.444	.017	.846
	N	25	25	25	25	25
NOut1	Pearson Correlation	-.637	-.039	.563	.113	-.515

	Sig. (2-tailed)	.001	.852	.003	.590	.008
	N	25	25	25	25	25
	Pearson Correlation	-.274**	.092	.169**	.335	-.049
Nout2	Sig. (2-tailed)	.185	.663	.419	.102	.816
	N	25	25	25	25	25
	Pearson Correlation	-.582	.068	.474	.087**	-.152
GSH1	Sig. (2-tailed)	.002	.748	.017	.678	.468
	N	25	25	25	25	25
	Pearson Correlation	-.566**	.108	.366**	.257	-.102
GSH2	Sig. (2-tailed)	.003	.609	.072	.215	.628
	N	25	25	25	25	25

Correlations

		MDA2	MPO1	MPO2	H2O21	H2O22	SOD1
	Pearson Correlation	-.009	-.319	-.222**	-.291	-.130**	.672
SOD2	Sig. (2-tailed)	.965	.120	.286	.157	.537	.000
	N	25	25	25	25	25	25
	Pearson Correlation	-.315	-.439	-.661	-.219	-.544	.780
NOum1	Sig. (2-tailed)	.126	.028	.000	.292	.005	.000
	N	25	25	25	25	25	25
	Pearson Correlation	-.051**	-.053	-.104	-.144	.020**	.623
NOum2	Sig. (2-tailed)	.807	.801	.621	.492	.925	.001
	N	25	25	25	25	25	25
	Pearson Correlation	-.310	-.314	-.614	-.180	-.730	.460**
NOut1	Sig. (2-tailed)	.132	.126	.001	.389	.000	.021
	N	25	25	25	25	25	25

	Pearson Correlation	.080**	-.015	-.020**	-.314	.172	.421
Nout2	Sig. (2-tailed)	.705	.943	.923	.126	.412	.036
	N	25	25	25	25	25	25
	Pearson Correlation	.193	-.681	-.354	-.354**	.203	.507
GSH1	Sig. (2-tailed)	.356	.000	.083	.082	.330	.010
	N	25	25	25	25	25	25
	Pearson Correlation	.123**	-.465	-.264**	-.238	.006	.543
GSH2	Sig. (2-tailed)	.559	.019	.202	.252	.976	.005
	N	25	25	25	25	25	25

Correlations

		SOD2	NOum1	NOum2	NOut1	Nout2	GSH1
	Pearson Correlation	1	.517	.797**	.184	.717**	.518
SOD2	Sig. (2-tailed)		.008	.000	.379	.000	.008
	N	25	25	25	25	25	25
	Pearson Correlation	.517	1	.344	.816	.148	.324
NOum1	Sig. (2-tailed)	.008		.092	.000	.481	.115
	N	25	25	25	25	25	25
	Pearson Correlation	.797**	.344	1	.001	.897**	.323
NOum2	Sig. (2-tailed)	.000	.092		.998	.000	.115
	N	25	25	25	25	25	25
	Pearson Correlation	.184	.816	.001	1	-.134	.051**
NOut1	Sig. (2-tailed)	.379	.000	.998		.524	.808
	N	25	25	25	25	25	25
	Pearson Correlation	.717**	.148	.897**	-.134	1	.324
Nout2	Sig. (2-tailed)	.000	.481	.000	.524		.114

	N	25	25	25	25	25	25
	Pearson Correlation	.518	.324	.323	.051**	.324	1
GSH1	Sig. (2-tailed)	.008	.115	.115	.808	.114	
	N	25	25	25	25	25	25
	Pearson Correlation	.733**	.413	.556**	.146	.471	.680
GSH2	Sig. (2-tailed)	.000	.040	.004	.486	.017	.000
	N	25	25	25	25	25	25

Correlations

		GSH2
	Pearson Correlation	.733
SOD2	Sig. (2-tailed)	.000
	N	25
	Pearson Correlation	.413
NOum1	Sig. (2-tailed)	.040
	N	25
	Pearson Correlation	.556**
NOum2	Sig. (2-tailed)	.004
	N	25
	Pearson Correlation	.146
NOut1	Sig. (2-tailed)	.486
	N	25
	Pearson Correlation	.471**
Nout2	Sig. (2-tailed)	.017
	N	25
GSH1	Pearson Correlation	.680

	Sig. (2-tailed)	.000
	N	25
	Pearson Correlation	1**
GSH2	Sig. (2-tailed)	
	N	25

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

*. Correlation is significant at the 0.05 level (2-tailed).

The GLM Procedure

Class Level Information

Class	Levels	Values
Organisms	8	1 2 3 4 5 6 7 8

Number of Observations Read 24
Number of Observations Used 24

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The GLM Procedure

Dependent Variable: White_blood_cells White blood cells

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	44.37420000	6.33917143	Infty	<.0001
Error	16	0.00000000	0.00000000		
Corrected Total	23	44.37420000			

R-Square	Coeff Var	Root MSE	White_blood_cells Mean

1.000000 0 0
4.265000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Organisms	7	44.37420000	6.33917143	Infty	<.0001

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The GLM Procedure

Dependent Variable: Neutrophils Neutrophils

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	635.0850000	90.7264286	Infty	<.0001
Error	16	0.0000000	0.0000000		
Corrected Total	23	635.0850000			

R-Square	Coeff Var	Root MSE	Neutrophils Mean
1.000000	0	0	32.52500

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Organisms	7	635.0850000	90.7264286	Infty	<.0001

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The GLM Procedure

Dependent Variable: Lymphocytes Lymphocytes

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	467.5050000	66.7864286	Infty	<.0001
Error	16	0.0000000	0.0000000		
Corrected Total	23	467.5050000			

R-Square	Coeff Var	Root MSE	Lymphocytes Mean
1.000000	0	0	60.37500

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Organisms	7	467.5050000	66.7864286	Infty	<.0001

The GLM Procedure

Dependent Variable: Monocytes Monocytes

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	100.0162500	14.2880357	Infty	<.0001
Error	16	0.0000000	0.0000000		
Corrected Total	23	100.0162500			

R-Square	Coeff Var	Root MSE	Monocytes Mean
1.000000	0	0	6.462500

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Organisms	7	100.0162500	14.2880357	Infty	<.0001

The GLM Procedure

Dependent Variable: Eosinophils Eosinophils

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.80576250	0.11510893	Infty	<.0001
Error	16	0.00000000	0.00000000		
Corrected Total	23	0.80576250			

R-Square	Coeff Var	Root MSE	Eosinophils Mean
1.000000	0	0	0.326250

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Organisms	7	0.80576250	0.11510893	Infty	<.0001

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The GLM Procedure

Dependent Variable: Basophils Basophils

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.96000000	0.13714286	Infty	<.0001
Error	16	0.00000000	0.00000000		
Corrected Total	23	0.96000000			

R-Square	Coeff Var	Root MSE	Basophils Mean
1.000000	0	0	0.300000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Organisms	7	0.96000000	0.13714286	Infty	<.0001

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The GLM Procedure

Dependent Variable: Haemoglobin Haemoglobin

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	26.70000000	3.81428571	Infty	<.0001
Error	16	0.00000000	0.00000000		
Corrected Total	23	26.70000000			

R-Square	Coeff Var	Root MSE	Haemoglobin Mean
1.000000	0	0	14.60000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Organisms	7	26.70000000	3.81428571	Infty	<.0001

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The GLM Procedure

Dependent Variable: Red_blod_cell Red blod cell

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	11.55285000	1.65040714	Infty	<.0001
Error	16	0.00000000	0.00000000		
Corrected Total	23	11.55285000			

R-Square	Coeff Var	Root MSE	Red_blod_cell Mean
1.000000	0	0	10.27750

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Organisms	7	11.55285000	1.65040714	Infty	<.0001

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The GLM Procedure

Duncan's Multiple Range Test for White_blood_cells

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0

Number of Means	2	3	4	5	6	7	8
Critical Range	0	0	0	0	0	0	0

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	Organisms
A	6.970	3	1
B	5.380	3	2
C	5.220	3	4
D	3.630	3	8
E	3.610	3	7
F	3.600	3	6
G	3.040	3	5
H	2.670	3	3

The GLM Procedure

Duncan's Multiple Range Test for Neutrophils

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0

Number of Means	2	3	4	5	6	7	8
Critical Range	0	0	0	0	0	0	0

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	Organisms
A	39.50	3	7
B	38.50	3	3
C	36.30	3	4

D	33.20	3	1
E	32.70	3	6
F	29.20	3	8
G	25.50	3	5
H	25.30	3	2

The GLM Procedure

Duncan's Multiple Range Test for Lymphocytes

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0

Number of Means	2	3	4	5	6	7	8
Critical Range	0	0	0	0	0	0	0

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	Organisms
A	66.70	3	2
B	65.60	3	8
C	64.80	3	5
D	60.10	3	1
E	58.00	3	4
F	56.90	3	6
G	56.60	3	3
H	54.30	3	7

The GLM Procedure

Duncan's Multiple Range Test for Monocytes

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0

Number of Means	2	3	4	5	6	7	8
Critical Range	0	0	0	0	0	0	0

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	Organisms
A	10.80	3	6
B	8.60	3	5
C	6.30	3	1
C			
C	6.30	3	2
D	5.30	3	7
E	5.20	3	4
F	4.70	3	3
G	4.50	3	8

The GLM Procedure

Duncan's Multiple Range Test for Eosinophils

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0

Number of Means	2	3	4	5	6	7	8
Critical Range	0	0	0	0	0	0	0

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	Organisms
A	0.6000	3	7
B	0.5000	3	8
C	0.4000	3	5
C			
C	0.4000	3	2
D	0.3000	3	6
D			
D	0.3000	3	4
E	0.1000	3	1
F	0.0100	3	3

The GLM Procedure

Duncan's Multiple Range Test for Basophils

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0

Number of Means	2	3	4	5	6	7	8
Critical Range	0	0	0	0	0	0	0

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	Organisms
A	0.7000	3	5
B	0.4000	3	2
B			
B	0.4000	3	3
C	0.3000	3	1
C			
C	0.3000	3	7
D	0.2000	3	4
E	0.1000	3	6
F	0.0000	3	8

The GLM Procedure

Duncan's Multiple Range Test for Haemoglobin

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0

Number of Means	2	3	4	5	6	7	8
Critical Range	0	0	0	0	0	0	0

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	Organisms
A	16.30	3	4
B	15.70	3	1
B			
B	15.70	3	6
C	14.30	3	8
D	14.00	3	3
E	13.90	3	5
F	13.50	3	2
G	13.40	3	7

The GLM Procedure

Duncan's Multiple Range Test for Red_blod_cell

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0

Number of Means	2	3	4	5	6	7	8

Critical Range 0 0 0 0 0 0 0

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	Organisms
A	11.70	3	6
B	10.70	3	4
C	10.56	3	1
D	10.52	3	7
E	9.91	3	8
F	9.70	3	2
G	9.68	3	5
H	9.45	3	3

The GLM Procedure

Class Level Information

Class	Levels	Values
TIME__Months_	5	1 2 3 4 5
LOCATION	10	1 2 3 4 5 6 7 8 9 10

Data for Analysis of MEDIA

Number of Observations Read	100
Number of Observations Used	100

Data for Analysis of POOLED_NUMBER_OF_COLONIES_

Number of Observations Read	100
Number of Observations Used	99

NOTE: Variables in each group are consistent with respect to the presence or absence of missing values.

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The GLM Procedure

Dependent Variable: MEDIA MEDIA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	0.00000000	0.00000000	0.00	1.0000
Error	86	25.00000000	0.29069767		
Corrected Total	99	25.00000000			

R-Square	Coeff Var	Root MSE	MEDIA Mean
0.000000	35.94426	0.539164	1.500000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TIME__Months_	4	0	0	0.00	1.0000
LOCATION	9	0	0	0.00	1.0000

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The GLM Procedure

Duncan's Multiple Range Test for MEDIA

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	0.290698

Number of Means	2	3	4	5
Critical Range	.3389	.3567	.3684	.3770

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	TIME__ Months_
A	1.5000	20	1
A			
A	1.5000	20	2
A			
A	1.5000	20	3
A			
A	1.5000	20	4
A			
A	1.5000	20	5

The GLM Procedure

Duncan's Multiple Range Test for MEDIA

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	**0.05**
Error Degrees of Freedom	**86**
Error Mean Square	**0.290698**

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Critical Range	**.4793**	**.5044**	**.5210**	**.5331**	**.5426**	**.5501**	**.5564**	**.5617**	**.5662**

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	LOCATION
A	**1.5000**	**10**	**1**
A			
A	**1.5000**	**10**	**2**
A			
A	**1.5000**	**10**	**3**
A			
A	**1.5000**	**10**	**4**
A			
A	**1.5000**	**10**	**5**
A			
A	**1.5000**	**10**	**6**
A			
A	**1.5000**	**10**	**7**
A			
A	**1.5000**	**10**	**8**
A			
A	**1.5000**	**10**	**9**
A			
A	**1.5000**	**10**	**10**

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The GLM Procedure

Dependent Variable: POOLED_NUMBER_OF_COLONIES_ POOLED NUMBER OF COLONIES

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	280.5577637	21.5813664	2.75	0.0027
Error	85	666.6139535	7.8425171		
Corrected Total	98	947.1717172			

R-Square	Coeff Var	Root MSE	POOLED_NUMBER_OF_COLONIES_ Mean
0.296206	33.89297	2.800449	8.262626

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TIME__Months_	4	177.2082687	44.3020672	5.65	0.0004
LOCATION	9	103.4439412	11.4937712	1.47	0.1740

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The GLM Procedure

Duncan's Multiple Range Test for POOLED_NUMBER_OF_COLONIES_

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	7.842517
Harmonic Mean of Cell Sizes	19.79167

NOTE: Cell sizes are not equal.

Number of Means	2	3	4	5
Critical Range	1.770	1.863	1.924	1.969

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	TIME__ Months_
A	10.8000	20	3
B	8.2105	19	4
B			
B	7.7000	20	2
B			
B	7.6500	20	5
B			
B	6.9500	20	1

The GLM Procedure

Duncan's Multiple Range Test for POOLED_NUMBER_OF_COLONIES_

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	7.842517
Harmonic Mean of Cell Sizes	9.89011

NOTE: Cell sizes are not equal.

Number of Means	2	3	4	5	6	7	8	9	10
Critical Range	2.504	2.635	2.722	2.785	2.834	2.874	2.906	2.934	2.958

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	LOCATION
A	10.500	10	3
A			
B A	9.600	10	5
B A			
B A	8.600	10	2
B A			
B A	8.300	10	1
B A			
B A	8.200	10	8
B A			
B A	7.800	10	10
B			
B	7.556	9	4
B			
B	7.500	10	9

B			
B	7.500	10	6
B			
B	7.000	10	7

Correlation and PCA

The PRINCOMP Procedure

Observations	108
Variables	6
Partial Variables	3

Simple Statistics

	Control_ plate	Conc_5_	Conc_10_	Conc_15_	Conc_20_
Mean	3.200000000	2.440740741	2.545833333	2.543981481	2.619907407
StD	0.944239792	1.122312952	1.053390720	1.080211073	1.072923768

Simple Statistics

	Conc_25_	Extracts	Days_ interval	Replicates
Mean	2.505555556	2.000000000	4.500000000	2.000000000
StD	1.078232660	0.820303112	1.715787060	0.820303112

Correlation Matrix

		Control_ plate	Conc_5_	Conc_10_	Conc_15_
Control_plate	Control plate	1.0000	0.9136	0.9435	0.9553
Conc_5_	Conc 5%	0.9136	1.0000	0.9709	0.9750
Conc_10_	Conc 10%	0.9435	0.9709	1.0000	0.9804
Conc_15_	Conc 15%	0.9553	0.9750	0.9804	1.0000
Conc_20_	Conc 20%	0.9606	0.9547	0.9683	0.9847
Conc 25	Conc 25%	0.9529	0.9696	0.9807	0.9856
Extracts	Extracts	0.0000	-.2264	-.1044	-.1187
Days_interval	Days interval	0.9501	0.9345	0.9617	0.9711
Replicates	Replicates	0.0000	-.0274	0.0416	-.0090

Correlation Matrix

	Conc_20_	Conc_25_	Extracts	Days_ interval	Replicates
Control_plate	0.9606	0.9529	0.0000	0.9501	0.0000
Conc 5	0.9547	0.9696	-.2264	0.9345	-.0274
Conc_10_	0.9683	0.9807	-.1044	0.9617	0.0416
Conc_15_	0.9847	0.9856	-.1187	0.9711	-.0090
Conc_20_	1.0000	0.9812	-.0802	0.9698	-.0027
Conc_25_	0.9812	1.0000	-.0708	0.9699	-.0042
Extracts	-.0802	-.0708	1.0000	0.0000	0.0000
Days interval	0.9698	0.9699	0.0000	1.0000	0.0000
Replicates	-.0027	-.0042	0.0000	0.0000	1.0000

The PRINCOMP Procedure

Regression Statistics

	Control_ plate	Conc_5_	Conc_10_	Conc_15_	Conc_20_	Conc_25_
R-Square	0.9026684636	0.9253005237	0.9374016663	0.9571084226	0.9469118916	0.9457991844
RMSE	0.2988025553	0.3111343583	0.2673290123	0.2269184733	0.2507510214	0.2546188855

Standardized Regression Coefficients

	Control_ plate	Conc_5_	Conc_10_
Extracts	0.0000000000	-.2263772350	-.1043709624
Days_interval	0.9500886609	0.9345066202	0.9616519432
Replicates	0.0000000000	-.0274089029	0.0416402285

Standardized Regression Coefficients

	Conc_15_	Conc_20_	Conc_25_
Extracts	-.1186549227	-.0801714888	-.0707952603
Days_interval	0.9710556421	0.9697821285	0.9699326531
Replicates	-.0089650386	-.0026546851	-.0042265827

Partial Correlation Matrix

		Control_ plate	Conc_5_	Conc_10_	Conc_15_	Conc_20_	Conc_25_
Control_plate	Control plate	1.0000	0.3024	0.3830	0.5057	0.5462	0.4318
Conc 5	Conc 5%	0.3024	1.0000	0.7272	0.7143	0.4803	0.7400
Conc_10_	Conc 10%	0.3830	0.7272	1.0000	0.6664	0.4759	0.6992
Conc_15_	Conc 15%	0.5057	0.7143	0.6664	1.0000	0.7013	0.7315
Conc_20_	Conc 20%	0.5462	0.4803	0.4759	0.7013	1.0000	0.6513
Conc_25_	Conc 25%	0.4318	0.7400	0.6992	0.7315	0.6513	1.0000

Eigenvalues of the Partial Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
1	3.96141088	3.10771552	0.6602	0.6602
2	0.85369536	0.39211305	0.1423	0.8025
3	0.46158231	0.18795980	0.0769	0.8794
4	0.27362251	0.01518180	0.0456	0.9251
5	0.25844070	0.06719246	0.0431	0.9681
6	0.19124824		0.0319	1.0000

The PRINCOMP Procedure

Eigenvectors

		Prin1	Prin2	Prin3	Prin4	Prin5	Prin6
Control_plate	Control plate	0.311003	0.730007	0.570346	-.148369	0.139905	0.059041
Conc_5_	Conc 5%	0.417432	-.435749	0.105167	-.517221	0.121532	0.585256
Conc_10_	Conc 10%	0.412976	-.327832	0.443201	0.637070	-.345572	0.016492
Conc 15	Conc 15%	0.450588	0.011981	-.186312	-.428332	-.527629	-.547955
Conc_20_	Conc 20%	0.396118	0.383375	-.640395	0.312730	-.115143	0.418270
Conc_25_	Conc 25%	0.445578	-.150393	-.149667	0.152782	0.744706	-.422508

CORRELATIONS

/VARIABLES=TREATMENT REPLICATE CONTROL APX1 APX3 PNX1 PNX3 AFX1 AFX3 PCX1 PCX3

/PRINT=TWOTAIL NOSIG

/MISSING=PAIRWISE.

Correlations

Notes

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Missing Value Handling	Definition of Missing	User-defined missing values are treated as missing.
	Cases Used	Statistics for each pair of variables are based on all the cases with valid data for that pair.

Syntax		CORRELATIONS /VARIABLES=TREATMENT REPLICATE CONTROL APX1 APX3 PNX1 PNX3 AFX1 AFX3 PCX1 PCX3 /PRINT=TWOTAIL NOSIG /MISSING=PAIRWISE.
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[DataSet0]

Correlations

		TREATMENT	REPLICATE	CONTROL	APX1	APX3
TREATMENT	Pearson Correlation	1	.000	.315*	.326*	.289
	Sig. (2-tailed)		1.000	.048	.040	.071
	N	40	40	40	40	40
REPLICATE	Pearson Correlation	.000	1	.006	-.020	.079
	Sig. (2-tailed)	1.000		.972	.905	.627
	N	40	40	40	40	40
CONTROL	Pearson Correlation	.315*	.006	1	.981**	.870**
	Sig. (2-tailed)	.048	.972		.000	.000
	N	40	40	40	40	40

	Pearson Correlation	.326*	-.020	.981**	1	.909**
APX1	Sig. (2-tailed)	.040	.905	.000		.000
	N	40	40	40	40	40
	Pearson Correlation	.289	.079	.870**	.909**	1
APX3	Sig. (2-tailed)	.071	.627	.000	.000	
	N	40	40	40	40	40
	Pearson Correlation	.315*	-.058	.963**	.969**	.913**
PNX1	Sig. (2-tailed)	.048	.723	.000	.000	.000
	N	40	40	40	40	40
	Pearson Correlation	.307	-.020	.975**	.983**	.838**
PNX3	Sig. (2-tailed)	.054	.904	.000	.000	.000
	N	40	40	40	40	40
	Pearson Correlation	.316*	.015	.982**	.982**	.901**
AFX1	Sig. (2-tailed)	.047	.926	.000	.000	.000
	N	40	40	40	40	40
	Pearson Correlation	.299	.023	.940**	.965**	.858**
AFX3	Sig. (2-tailed)	.061	.890	.000	.000	.000
	N	40	40	40	40	40
	Pearson Correlation	.296	.042	.935**	.967**	.946**
PCX1	Sig. (2-tailed)	.064	.797	.000	.000	.000
	N	40	40	40	40	40
	Pearson Correlation	.276	-.113	.915**	.919**	.729**
PCX3	Sig. (2-tailed)	.085	.488	.000	.000	.000
	N	40	40	40	40	40

Correlations

		PNX1	PNX3	AFX1	AFX3	PCX1	PCX3
	Pearson Correlation	.315	.307	.316*	.299*	.296	.276*
TREATMENT	Sig. (2-tailed)	.048	.054	.047	.061	.064	.085
	N	40	40	40	40	40	40
	Pearson Correlation	-.058	-.020	.015	.023	.042	-.113
REPLICATE	Sig. (2-tailed)	.723	.904	.926	.890	.797	.488
	N	40	40	40	40	40	40
	Pearson Correlation	.963*	.975	.982	.940**	.935**	.915**
CONTROL	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000
	N	40	40	40	40	40	40
	Pearson Correlation	.969*	.983	.982**	.965	.967**	.919**
APX1	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000
	N	40	40	40	40	40	40
	Pearson Correlation	.913	.838	.901**	.858**	.946	.729**
APX3	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000
	N	40	40	40	40	40	40
	Pearson Correlation	1*	.939	.966**	.889**	.933**	.897
PNX1	Sig. (2-tailed)		.000	.000	.000	.000	.000
	N	40	40	40	40	40	40
	Pearson Correlation	.939	1	.968**	.967**	.931**	.948**
PNX3	Sig. (2-tailed)	.000		.000	.000	.000	.000
	N	40	40	40	40	40	40
	Pearson Correlation	.966*	.968	1**	.958**	.955**	.928**
AFX1	Sig. (2-tailed)	.000	.000		.000	.000	.000
	N	40	40	40	40	40	40
AFX3	Pearson Correlation	.889	.967	.958**	1**	.963**	.917**

	Sig. (2-tailed)	.000	.000	.000		.000	.000
	N	40	40	40	40	40	40
	Pearson Correlation	.933	.931	.955**	.963**	1**	.857**
PCX1	Sig. (2-tailed)	.000	.000	.000	.000		.000
	N	40	40	40	40	40	40
	Pearson Correlation	.897	.948	.928**	.917**	.857**	1**
PCX3	Sig. (2-tailed)	.000	.000	.000	.000	.000	
	N	40	40	40	40	40	40

*. Correlation is significant at the 0.05 level (2-tailed).

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

HYDROSTATIC LUNG TEST

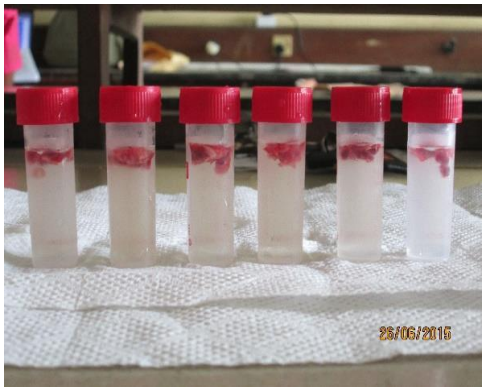
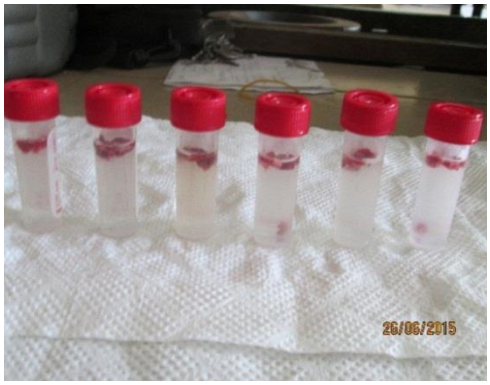


Plate 5 a and b: *Penicillium citrinum* spores infected lungs at two different concentrations ($2x10^5$ and $3.5x\ 10^7$ml).

Plate 6a and b: *Penicillium chrysogenum* infected lungs of mice at two different concentrations ($2x10^5$ and $3.5x\ 10^7$ml) floating.

Plate 7a and b: *A. flavus* innoculated mice infected lungs at two different concentrations ($2x10^5$ and $3.5x\ 10^7$ml) floating.



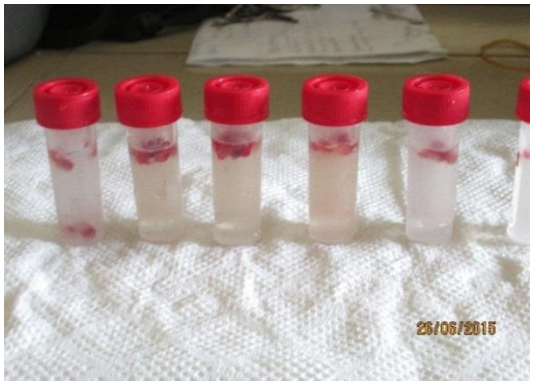


Plate 8a and b: *A. penicilloides* inoculated mice infected lungs at two different concentrations ($2x10^5$ and $3.5x\ 10^7$ml) floating.

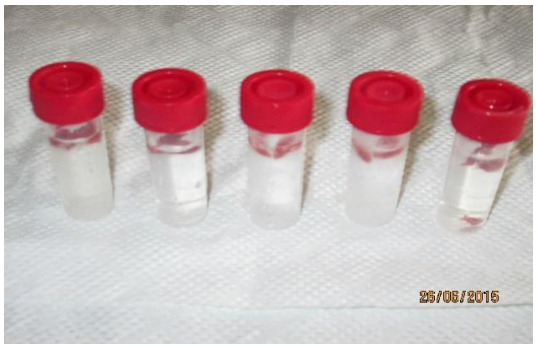


Plate 9a and b: *A. tamari* infected lungs at two different concentrations ($2x10^5$ and $3.5x\ 10^7$ml) floating.

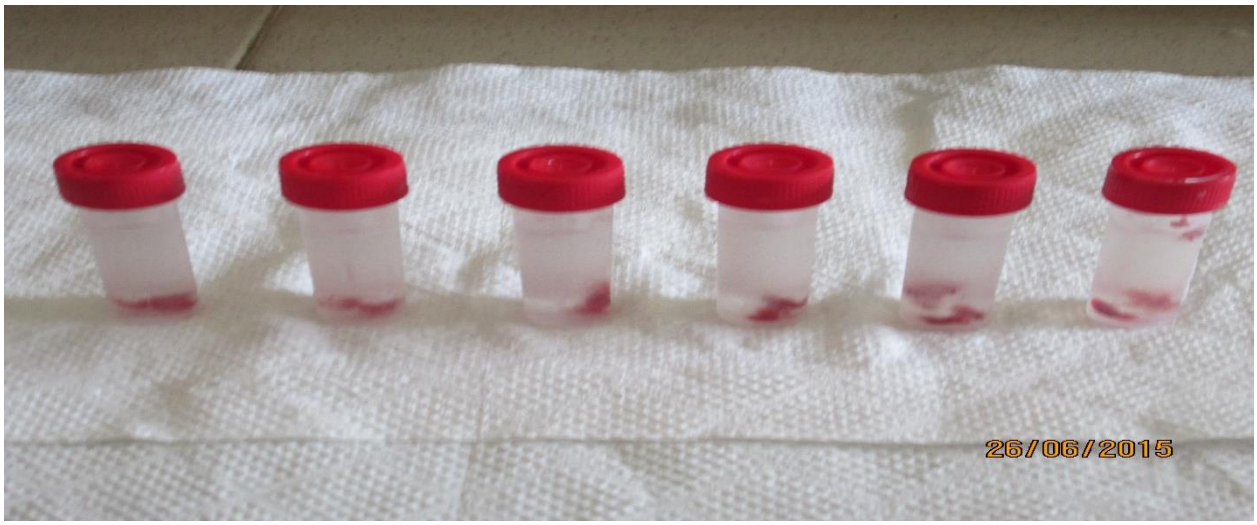
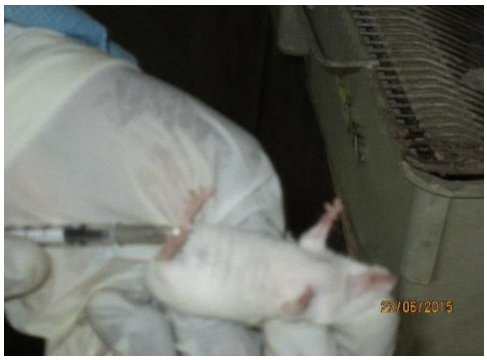


Plate 10: Control sample which was not infected with any fungus sinking to the bottom due to its weight.

4.21 Mice in Cage

Plate 11: Mice kept in cages (six per group).



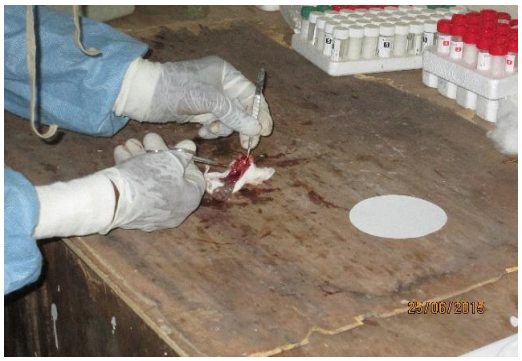


Plate 12a: Injection of fungal spores into mice b) Dissection to bring out lungs.

SEQUENCE ALIGNMENT OF BLASTED FUNGAL ISOLATES