DEVELOPMENT OF CHITOSAN-BASED BIO-FUNGICIDES FOR THE CONTROL OF FIELD BLAST PATHOGEN (*Magnaporthe oryzae*) AND SELECTED POSTHARVEST MYCOTOXIN PRODUCING FUNGAL PATHOGENS OF RICE

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PhD/SLS/2018/9295

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NIGERIA

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A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL. FEDETRAL UNIVERSITY OF TECHNOLOGY, MINNA, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF DOCTOR OF PHILOSOPHY (PhD) IN BOTANY (APPLIED MYCOLOGY)

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DECLARATION

I hereby declare that this thesis titled **"Development of Chitosan-based Biofungicides for the control of Field Blast Pathogen (*Magnaporthe oryzae*) and Selected Postharvest Mycotoxin Producing Fungal Pathogens of Rice"** is a collection of my original work and it has not been presented for any other qualification anywhere. Information from other source (published and unpublished) has been duly acknowledged

…… ……..

AREMU, Mariam Bukola DATE

(PhD/SLS/2018/9295)

CERTIFICATION

The thesis titled **"Development of Chitosan-based Bio fungicides for the control of Field Blast Pathogen (*Magnaporthe oryzae*) and Selected Postharvest Mycotoxin Producing Fungal Pathogens of Rice"** by AREMU, Mariam Bukola (PhD/SLS/2018/9295) meets the regulations governing the award of the degree of PhD of The Federal University of Technology, Minna and it is approved for its contribution to scientific knowledge and literary presentation.

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ABSTRACT

Rice (*Oryzae sativa* L.) production is constrained by several factors. Among these, Fungal pathogens one of (biotic factors) causing rice diseases is a major constraint. The use of synthetic chemicals to control these pathogens presents frightening health problems, leading to increase demand for the development of biofungicide for rice protection. Therefore, the use of chitosan-based fungicides, as a new approach is important. This research aimed at developing and evaluating the antifungal efficacy of chitosan in the control of Blast Pathogen (*Magnaporthe oryzae*) and postharvest seed borne mycotoxin producing fungal pathogens of rice. Fifty (50) rice samples were collected from rice farmers across nine (9) local government area of Niger State. Fungal isolation, identification and frequency of occurrence were carried out. Chitosan was synthesize from crab shell, and three other different molecular eight chitosan were purchased. *In vitro* inhibition of *M. oryzae* and selected mycotoxin producing fungal pathogens in all the four chitosan was carried out. Chitosan nanoparticle was synthesized from the three chitosan purchased and inhibitory test was carried out. Two purchased chitosan (HMWC and MMWC) were used to coat the rice samples collected from farmers and stored for period of six months and fungi were re-isolated and compared to the original rice samples. Blast infected rice plant were also treated with all the chitosan on the farm. All data generated were subject to analysis of variance (ANOVA), means were separated using both Duncan multiple range test and pairwise test at $P \leq 0.05\%$.The results of the fungal isolation and incidence from all the samples showed that local rice accession rice had the highest mean fungal incidence of 10.00±0.00. Eight (8) postharvest fungal pathogens were isolated and identified, although three (3) were mycotoxin producing fungal pathogens. *F. moniliforme* has the highest percentage frequency of occurrence (38.5%), followed by *R. stolonifer* (37%) while *Aspergillus niger, Aspergillus flavus* and *Cladosporium* sp were low with 6.5 %, 6.2 % and 4.0 %, respectively. However *Aspergillus fumigatus* and *Rhizoctonia solani* has the lowest percentage frequency of occurrence. The degree of deacetylation of the Chitosan synthesize from crab shell was 98.6%. *In vitro* percentage mycelia radial growth inhibition reveals that most of all the chitosan treatment inhibited above 50 % of the mycelia radial length of *M. oryzae, A. flavus* and *A. fumigatus.* However, 100% inhibition of *F. monilforme* was observed. The synthesized chitosan nanoparticles particle size range from 468.3 nm to 711.7 nm. Percentage fungal mycelia radial length inhibition of chitosan nanoparticle showed that, the highest percentage inhibition of 87.18% was recorded in chitosan nanoparticle with 711.7nm particle size for *A. flavus, A. fumigatus* had 87.5 % inhibition, while 100% inhibition of *F. moniliforme* irrespective of the chitosan nanoparticle size was observed. The result of the fungal counts and percentage frequency of occurrence of identified organism from rice samples after coating with two chitosan (HMWC &MMWC) and stored for six months reveals that HMWC inhibit the growth of fungi in almost all the samples as there were no growth in almost all the samples, however, highest fungal incidence was recorded in samples treated with MMWC with the highest count (8.00±00) recorded in local rice accession. The highest percentage frequency of occurrence (38.8%) *of A. fumigatus* was recorded in MMWC while *A. flavus* has the highest percentage frequency of occurrence (40.25%) in HMWC. *F. monilfrome* record the least percentage, however there were no occurrence of other fungal earlier isolated before coating and storage. The result of the blast pathogen infected field treated with chitosan shows that HMWC 2.0% reduced the severity and percentage incidence of blast from 5(Susceptible) and 20% to 1 (Resistance) and 2.3% respectively in FARO 52, while 6(Susceptible) and 23.3% to 3(Resistance) and 0.7% respectively in FARO 66. It is

therefore concluded that HMWC in its free form and nanoparticle size is effective and recommended for the control of *M. oryzae* and postharvest seedborne mycotoxin producing fungal pathogens of rice

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

Abbreviations	Meaning
ANOVA	Analysis of Variance
Cs	Chitosan
CSCS	Chitosan Synthesis from Crab Shell
DDA	Degree of Deacetylation
DLS	Dynamic Light Scattering
FTIR	Fourier Transform Infrared
HMWC	High Molecular Weight Chitosan
KDa	KiloDalton
LMWC	Low Molecular Weight Chitosan
MMWC	Medium Molecular Weight Chitosan
NP	Nanoparticles
TPP	Sodium Tripolyphosphate
UV	Ultra Violet

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Rice (*Oryzae sativa* L.) is one of the world's primary food crops mostly grown in tropical and sub-tropical climates. It is the agricultural commodity with the third highest worldwide production after sugarcane and maize (FAO, 2014). It is one of the main staple foods in Nigeria as the demand for rice is growing faster than any other staple food such as maize, sorghum and millet with consumption broadening across all socio-economic classes (Iwuagwu *et al.,* 2018). Nigeria has been identified to consume about 5.4 million metric tonnes of rice annually (valued at over N1.4 trillion), while local production amounts to 2.3 million metric tonnes per year and the balance of 3.1 million metric tonnes is imported (Ora *et al.,* 2011).

Though, measures have been put in place to halt importation and encourage local production of rice, Nigeria has not been able to meet up with rice demand through local production. Rice productivity and production at the farmers' level are constrained by several abiotic and biotic factors. Among these, biotic factors which include, weeds, insect pests and diseases caused by fungi pathogen, constitute the main constraints. Fungal pathogens causing diseases constitute the major biological constraint, infecting rice crop from the field to storage and new and potentially destructive species are being discovered every day (Islam and Ahmed, 2017). These inpacts reduces total output and availability of rice seed and grain quality for processing as well as storability for next planting season. Rice is contaminated in the field with numerous fungi which include rice blast pathogens (*Magnaporthe oryzae*), rice sheath blight pathogens (*Rhizoctonia solani*) and brown spot pathogens (*Cochliobolus miyabeanus*) (Suleiman and Akaajime, 2010).

Stored rice seeds are continuously threatened by stored grain pests like rodents, insects and fungal pathogens which also results in economic losses (Islam *et al.,* 2016, 2017), because they are the best carrier of several pathogens which are responsible for most plant diseases leading to considerable loss of crop yield (Islam and Ahmed, 2017). Rice crop is known to be attacked by many seed-borne fungal diseases of major and minor importance that are grouped into different categories as field and storage fungi. The diseases caused by these fungi are disastrous as they reduce seed vigor and weaken the plant at its initial growth stages (Islam *et al*., 2016).

The yield of rice varieties in Nigeria is comparatively low and seems impossible to improve under the prevailing situation due to diseases caused by the seed-borne fungi. Plant disease control of seed-borne pathogenic fungi of rice are very important as they are capable of causing the following; (i) introduction of new pathogens (ii) quantitative and qualitative crop losses and (iii) permanent contamination of the soil (Ora *et al.,* 2011). Rice suffers from more than 60 different diseases, 43 diseases are known to occur on the rice crop in the field, and from this number, 27 are seed borne while 14 are of major importance (Monajjem *et al.,* 2014). The extremely seed-borne fungi pathogens of rice are brown spot (*Bipolaris oryzae*), bakanae (*Fusarium moniliforme*), blast (*Magnaporthe oryzae*), sheath blight (*Rhizoctonia solani*), sheath rot (*Sarocladium oryzae*), stem rot (*Sclerotium oryzae),* and flag smut (*Urocystis tritici*). They cause yield reduction, quality seed deterioration and germination failure (Butt *et al.,* 2011; Ora *et al.,* 2011, Ahmed *et al.,* 2013). Among the storage fungi are *Aspergillus flavus, Aspergillus niger* and *Penicillium* sp (Suleiman and Akaajime, 2010). If seeds infected or contaminated by these pathogens (that is also soil-borne), are sown in non-infested soil, they may be established and continue to transfer them into subsequent non-infected seeds.

However, while the seeds of maize, wheat and other cereal crops have received adequate attention of their post-harvest and storage, comparatively little has been done for rice, a staple food for thirty percent of the world's population (Habib *et al.,* 2012). This neglect is more glaring in the context of African countries especially Nigeria. Some of the means of storage are simply outmodelled, therefore, poor and practically unserviceable. During storage, the stocks are exposed to fungi, insects, and mites which attack and destroy them in varying degrees. Among these, fungi play a major role in degrading the seeds during poor storage rendering them unfit for sowing and for human consumption or domestic animals (Habib *et al.,* 2012).

The word "fungicide" originated from two Latin words, viz., "fungus" and "caedo". The word "caedo" means "to kill". Thus, fungicide is any agents/chemical which has the ability to kill the fungus. However, in common usage, the meaning is restricted to chemicals only. Hence, fungicide is a chemical which is capable of killing fungi (Kumar *et al.,* 2013). Some of the fungicides used for rice seed storage include Antracal, Mancozeb (Diathane M-45), Cordate, Derosal, Carbendazim, Redomil and Topsin-M.

To many in the public arena, the use of synthetic or manufactured chemicals on crops presents a frightening image of immediate and long-term health problems. Other disadvantages of synthetic chemicals include contamination of crop products with harmful chemical residues, contamination of soils and groundwater as well as development of crop pest populations that are resistant to the synthetic chemical treatment. Concerns about chemical residues on food products has led to a whole new approach in which crop products are chemical residue free. However, when the disadvantages of synthetic fungicides outweigh the advantages, farmers look to alternative methods of control such as selection of crop, using resistant varieties and

monitoring physical condition among others, and the most common being biological control methods (Biofungicides).

Biofungicides means fungicides of biological origin. It may be from microorganisms such as bacteria, fungi and animal or plant-based products like secondary metabolites (bacteriocin). Unlike synthetic chemical, biofungicides use natural methods against fungi. The most obvious advantage of this method over the synthetic fungicides is that the natural balance in the ecosystem remains fairly undisturbed.

Biofungicides are biological agents for the control of fungi and one of the solutions for sustaining agricultural output and environmental quality. In order to implement these environmentally friendly biofungicides on the disease-causing fungi of crop plant effectively. It is important to pay attention to their synthesis, formulation and application.

Biofungicides have many advantages over chemical fungicides like being biodegradable, cheaper and harmful residues are not detected (Choudhary *et al.,* 2014). Plant extract and other microorganisms have been used as biofungicides but little has been done on animal-based products. Therefore, conversion of shell waste to chitosan which is a commercially valuable product with a myriad of uses among which is biofungicides, could serve as an effective mode of shell remediation as well as plant protection.

The production of shell wastes from crab by the seafood industry is among other environmental problems, one of noteworthy concern, contributing to environmental and health hazards (Divya *et al.,* 2014). Indeed, a great amount of crab and shrimp shells are produced as waste by worldwide seafood companies (Teli and Sheikh, 2012). Unfortunately, about 45 % of waste resulting from processed seafood is

disposed as landfill, consequently leading to environmental pollution producing bad odour and causing aesthetic damage to the environment (Ahing and Wid, 2016). To dispose off this waste, burning is proposed; however, this solution is costly due to the low burning capacity of shells (Divya *et al.*, 2014). However, it is worth mentioning that fishery by-products have economical value for chitin and chitosan production (Ahing and Wid, 2016). Thus, the conversion of shell waste to commercial products such as chitin and chitosan could be considered an effective approach for shell wastes remediation as well as integrated pest management (Divya *et al.,* 2014, Premasudha *et al.,* 2017).

Chitosan is a natural polymer derived from chitin by a chemical deacetylation process using a strong alkali such as sodium hydroxide at high temperature; it is the main component of the shell of crustaceans and the feather of pota (giant squid; *Dosidicus gigas*). It is a natural, biodegradable polymer with a wide range of uses in cosmetology, food industry, biotechnology, medicine and agriculture (El –Hadrami *et al.,* 2010). Another important attribute of this natural compound is associated with its fungistatic or fungicidal properties against pathogens of various crops (Madushani *et al.,* 2012). Chitin and its derivative chitosan are of commercial interest due to their excellent biocompatibility, biodegradability, non-toxicity, and chelating and adsorption power (Anand *et al.,* 2018).

1.2 Statement of the Research Problem

Postharvest loss due to seed-borne diseases caused by fungi is an important threat to rice yield and quality on the field as well as during storage. Rice blast pathogen (*Magnaporthe oryzae*) remains the most devastating agent causing serious damage to rice on the field in Nigeria. Mycotoxins are secondary metabolites produced by filamentous fungi which contaminate a large fraction of the world's food, mainly

staple foods in which rice is not an exception. The adverse effects of mycotoxins on human health can be acute and chronic, which include provoking problems such as liver cancer which thus increased health care costs and premature deaths.

The use of synthetic fungicides, constitute the primary means of controlling both field and postharvest seed-borne mycotoxin producing fungi overtime (Gilberto *et al*., 2017). However, despite their popularity and extensive use, serious concerns have been raised about health risks from the exposure of farmers working with these fungicides and the residues on food when the population is in the period of food scarcity Therefore, there is an increasing demand for the development of alternative strategies for crop protection. The use of microorganisms, plant extract and resistance varieties have failed to yield the desired results due to the breakdown in the efficacy potential as biocontrol as well as development of mutant's overtime.

The use of chitosan-based as a new approach is important. Chitosan is a natural nontoxic biopolymer, commercially produced by the partial deacetylation of chitin, which is obtained from the exoskeleton of crustaceans and cell wall of fungi. As a polycationic and nontoxic polymer, chitosan has found numerous applications in food, agriculture, pharmaceutical, and biotechnology. The use of chitosan as an edible film to extend the shelf life of foods has been studied by many researchers. The shelf life of hot smoked fillets with a shelf life of 14–16 days, vacuum-packed and stored at +4 °C was extended to 24 days for fillets treated with chitosan. Chitosan has excellent features that enable it to be used as wound dressing/healing in the medical area,

1.3 Aim and Objectives of the Study

The aim of this study was to evaluate the antifungal efficacy of chitosan in the control of blast pathogen (*Magnaporthe oryzae*) and postharvest mycotoxin producing fungal pathogens of rice.

The objectives of the study were to:

i. determine seed-borne fungal pathogens and frequency of occurrence in stored rice collected from nine local government area of Niger State

ii. synthesize and characterize chitosan and chitosan nanoparticle from crab shell

iii. determine the *in vitro* effects of chitosan against *Magnaporthe oryzae* and selected mycotoxin producing fungal pathogens of stored rice

iv. determine the *in vitro* effects of chitosan nanoparticles against selected mycotoxin producing fungal pathogens of stored rice

v. determine the inhibition potential of chitosan on the seed-borne fungal pathogens of stored rice using post harvest coating.

vii. determine the efficacy of chitosan against blast fungal pathogen (*M. oryzae*) growing in rice field

viii. determine the Lowest inhibitory Concentration Dose at 50% (LCD_{50}) of synthesized chitosan biofungicide.

1.4 Justification for the Study

The problem caused by fungi and other microorganisms is the decrease in quality and quantity of rice, which has led to the use of synthetic fungicides used for their control. However, rising public awareness about the toxicological effects of fungicide to human health, as well as the environmental impact, necessitate the development of non-toxic biofungicide. Over time, in the search for ecofriendly Integrated Pest Management (IPM), the use of biocontrol agents and induced systemic resistance is a welcome strategy of disease management. The application of plant extracts and other biological agents as biofungicides has been in use overtime but the limitation of not

totally been killed or able to surpress the growth of fungi has called for other alternative biocontrol methods.

The introduction of new biofungicides (Chitosan synthesized from shellwaste) is an alternative solution to solve this problem as it has been used in crop production and protection of various fruits and vegetables such as pea, tomato, strawberry, celery, pepper, cucumber, pumpkin, chili, cabbage, papaya, mango, watermelon, potato, and other crops like maize, peanut, soybean among others. For example, Madushani *et al.* (2012) reported the effectiveness of five chemically-modified chitosan derivatives in restricting the growth of *Saprolegnia parasitica*. Results indicated that methylpyrrolidinone chitosan, *N*-phosphonomethyl chitosan, and *N*-carboxymethyl chitosan, did not allow the fungus to grow normally. It has also been reported to enhanced germination index, reduced the mean germination time, and increased shoot height, root length, and shoot and root dry weights in two tested maize lines.

Many studies reported the antimicrobial properties of Chitosan and its derivatives as well as its inhibitory effect on fungi (Zahid, 2014; Iriti and Varoni, 2015).

Therefore, if industries processed crab and shrimp shellwaste into an abundant by-product of chitosan, this will add value to the nation's economy as well as support sustainable organic agriculture. The role of chitosan, not only as the coating but also, as plants defense booster will increase long time storage as well as yield. Chitosan has a potential role to be developed as alternative biopesticide for postharvest. Hence, evaluating its efficacy against pre- and post-harvest fungi of rice is important towards achieving integrated pest management practices.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Morphology of Rice

Rice belongs to the family Poaceae (Gramineae) tribe oryzeae. This tribe has 11 genera of which *Oryza* is the only one with cultivated species. *Oryza* has two cultivated (*Oryza sativa* and *Oryza glaberrima)* and 22 wild species. From the two cultivated species, *Oryza sativa* (2n = 24, AA) the Asian rice is grown worldwide while *Oryza glaberrima* is most common in Africa. Rice (*Oryzae sativa* L.) is sown in the irrigated areas of the world as the crop requires higher irrigation percentage than the other cereal crops. It is also grown under rainfall ecology (Islam and Ahmed, 2017).

Rice (*Oryza sativa or Oryza glaberrima*) is a cereal grain most widely consumed staple food for a large part of the world's population especially in Asia. In Africa, rice consumption is growing at even faster rate, and replacing more traditional crops due to urbanization and population growth (Nwilene *et al.,* 2011). It is one of the common staple foods widely consumed in Nigeria, which produces vast number of grains consumed by man. It has been under intensive cultivation originating in Asia for over 4,000 years and has since spread across the world, where almost a third of the population depends on for vital nutrition (Islam and Ahmed, 2017).

Rice is the most widely grown cereal cultivated in more than 147 million hectares worldwide. It is an extremely important food and cash crop in Nigeria with a total annual production at about 2 million metric tonnes (MT) (Nwilene *et al.*, 2011). As a self-pollinated crop, it has now moved from a ceremonial, to a staple food for both rural and urban dwellers in Nigeria. It ranked the fourth most important cereal crop in

terms of production (after sorghum, maize and millet). Although Nigeria is the largest rice importer in West Africa despite having about 5.0 million hectares of land suitable for rice cultivation (Nwilene *et al.,* 2011).

Rice has become an important strategic and daily staple food crop in Nigeria. The potential land area for rice production in Nigeria is between 4.6million and 4.9million ha. Out of this, only about 1.7million ha or 35 percent of the available land area is presently cropped to rice. Small-scale farmers with farm holdings of less than 1 ha cultivate most of the rice produced in Nigeria (Nwilene *et al.,* 2011). However, rice productivity and production at the farmer's level are constrained by several factors. These constraints include biotic factors, insufficient appropriate technologies, poor supply of inputs, ineffective farmer organizations and groups, low yield and poor milling quality of local rice varieties, poor marketing arrangements, inconsistent agricultural input and rice trade policies, poor extension systems and environmental constraints (Nwilene *et al.,* 2011).

The major biotic factors include weeds, insects (African rice gallmidge (AfRGM), Stem borers) and diseases (Blast, Rice yellow mottle virus (RYMV), Sheath rot, Smut). Habib *et al.* (2012) reported that fungal pathogens constitute the major biological constraints in rice production, and new and potentially destructive fungal species are being discovered every day. Rice seed is known to be colonized by various types of fungi, among which many are identified as plant pathogens. The infected seed not only affects the quality of the grain but also spreads the seed-borne pathogens to different regions. Rice is known to be attacked by 56 fungal pathogens (Gopalakrishnan *et al*., 2010), of which 41 are seed-borne (Habib *et al.,* 2012), and a number of fungal species on rice responsible for grain discolouration.

Some of these are widely distributed causing significant yield as well as economic loss particularly blast, brown spot, bakane diseases. The seed-borne fungi have been grouped into different categories by many workers as field and storage fungi, obligate parasites, facultative saprophytes and facultative parasites or saprophytes and parasites (Butt *et al*., 2011). The emphasis was given to fungi associated with rice seed because most fungi identified on rice are either pathogenic fungi (causing characteristic disease symptoms) or deleterious fungi (normally do not cause any disease but affect rice quality in storage, seed germination and cause seed rot).

2.1.1 Importance of rice seeds

Rice Seed is the primary basis of crop production and is the most important available input factor for smallholder farmers (Biemond *et al.,* 2012). In most parts of the world, smallholder farmers use their produced seed for next year planting. Consequently, they attempt to stock their own produced seed for several months or several years. These seeds are often of poor quality, impure and contaminated with pathogens (Monajjem *et al.,* 2014). Seed contamination of pathogens during storage could reduce seed vigour, germination, and cause negative effect on appearance and chemical composition of seeds in addition to accelerate seed deterioration, it can also inhibit germination, transmission of the pathogen from seed to seedling or main plant leading to reduction in crop yield and threatened food security (Monajjem *et al.,* 2014). Food security of the world depends on sufficient production of small seed cereals as the cheapest source that provided about 70 of absorbable energy (Raj Paroda *et al.,* 2013). Based on seed production, rice is the second small seed cereals, with area under cultivation about 154 million hectares, with a global production of 800 million tons of paddy (FAO, 2020).

Studies have shown that in poor storage conditions, pathogens are the most important factors causing seed aging and deterioration as well as seed and seedling rotting or abnormal production of seedlings in nursery (Monajjem *et al.,* 2014). Several reports indicated that the majority of these pathogens that lead to the production of abnormal seedlings are seed-borne fungi (Ahmed *et al.,* 2013). The rate of damage of these fungi depends on their genus and species, density, fungi invaded, environmental conditions, cultivar susceptibility and interaction of these factors on cultural practice (Monajjem *et al.,* 2014). More than 100 species of fungi have been identified on rice seeds so far. However, their severity depends on the time of sampling, location and varieties (Monajjem *et al.,* 2014).

2.1.2 Effects of poor storage on rice seed

Seed is a common carrier of plant pathogens. It acts as the primary source of many diseases. Most of the major diseases of rice are seed-borne. Many high-yielding varieties have shown susceptibility to different diseases and many of these diseases are seed-borne. The seed primordium or the maturing seed may be infected either (i) directly from the infected plant through the flower or fruit stalk and the seed stalk or directly from the seed surface, or (ii) infection from outside may be introduced through stigma or ovary wall or pericarp, and the lower or fruit stalk, and later through the seed coat. A pathogen may penetrate several of these parts of the seed and in turn infect them. The infestation/contamination of the seed may occur during harvesting, threshing and processing (Butt *et al.,* 2011).The seed-borne pathogens may result in (i) poor germination (ii) discolouration and shrivelling (iii) development of plant diseases (iv) distribution of pathogen to new areas (v) introduction of new strains or physiologic races of the pathogen along with new germplasm from other countries (vi) toxin production in infected seed etc.

While the seed of maize, wheat and other cereal crops have received adequate attention of the post harvest and storage (Madushani *et al.,* 2012), comparatively little has been done for rice, a staple food for thirty percent of the world's population (Madushani *et al.,* 2012). This neglect seems rather glaring in the context of African countries especially Nigeria. The available stock is stored to adjust the supply to demands, both for seed and food. However the storage facilities in these countries are far from satisfactory. Some of the means of storage are simply outmoded, poor and practically unserviceable. Although the storage period is not long, the paddy due to unavailability of proper drying and storage facilities are prone to damage and deterioration (Asha and Pushpalatha, 2013). During storage, the stocks are exposed to a number of pests such as insects, mites and fungi which attack and destroy them in varying degrees. Of these, fungi play a major role in degrading the seeds during poor storage rendering them unfit for sowing and human consumption or domestic animals (Madushani *et al.,* 2012). Most of the diseases of rice are carried through seed and cause enormous losses to the crop.

2.1.3 Fungal pathogens causing disease of rice

Fungal disease is considered as the principal disease of rice because of its wide distribution and its destructiveness under favourable conditions for yield loss. The pathogens can infect the crop at any time from seed germination to harvest. The biochemical changes in seed leading to seed deterioration generally takes place when the seed moisture level is favourable for the growth of storage moulds. Colonization of storage fungi led to decrease in carbohydrate content in most cases (Asha and Pushpalatha, 2013).

These pathogens are known to cause damage at different stages like storage, seed germination, seedling establishment, vegetative growth and reproductive phase. Fungi

including *Alternaria alternata, A. padwickii, A. longissima, Aspergillus niger, A. flavus, A. fumigatus Curvularia oryzae, C. lunata, Drechslera oryzae, Fusarium miniliforme, F. semitectum, F. oxysporum, F.solani, Magnaporthe oryzae,* and species of *Phoma, Cercospora, Chaetomium, Sclerotium, Penicillium, Myrothecium* and *Colletotrichum* have been isolated from seeds of different rice varieties. These fungi may decrease seed germinability, cause seed discolouration; produce toxins that may be injurious to man and domestic animals, and may reduce seed weight (Butt *et al.,* 2011)

The term disease applies to a plant expressing abnormal state of the whole or a part of the plant due to the influence of the environment. Parasitic diseases are caused by the aggression of an organism such as parasite and pathogen, while physiological diseases which are caused by physical factors (Habib *et al*., 2012). The tropical environment, when it's hot and humid seasons, which may be more or less prolonged depending on the region, is favourable for the development of pathogen, especially fungi. These organisms go into the resting stage in various resistant forms (chlamydospores, oospores, cysts, etc.) during dry periods. When the conditions become favourable again, the parasite resume their activity and sporulate profusely (Asha and Pushpalatha, 2013).

Food safety remains a crucial issue, which have impact on human life, has now become of great importance. Therefore, many efforts are been made to improve food safety, especially for cereals, fruits and vegetables. However, making these agricultural products safe for human consumption by elimination of these fungal pathogens in storage which decrease the quality and food safety of these crops has proven difficult. The interaction between the needs for agriculture, environmental protection and concerns for human health is complex. (Habib *et al*., 2012).

2.1.4 Rice blast pathogen

Blast disease (caused by teleomorph *Magnaporthe oryzae* (Hebert) Barr), Anamorph (*Pyricularia oryzae* Cavara (synonym *P. grisea* Sacc), is one of the most destructive diseases of rice (*Oryza sativa* L.) worldwide (Pagliaccia *et al.,* 2018, Sing *et al.,* 2021). *M. oryzae* is one of the most serious disease due to its rapid growing on a large scale (Pham *et al.,* 2018). The pathogen is most common on leaves, causing leaf blast during the vegetative stage of growth, or on neck nodes and panicle branches during the reproductive stage, causing neck blast (Pagliaccia *et al.,* 2018). Leaf blast lesions reduce the net photosynthetic rate of individual leaves to an extent far beyond the visible diseased leaf fraction (Pagliaccia *et al.,* 2018). Neck blast is considered the most destructive phase of the disease and can occur without being preceded by severe leaf blast. Moreover, spores of *M. oryzae* are exceedingly tiny and slight, and are easily spread through the air, this can cause crop losses up more than 80% of total production. Rice blast disease, caused by *Magnaporthe oryzae* (Ascomycota), occurs in about 80 countries on all continents where rice is grown, in both lowland paddy fields and upland cultivation (Boddy, 2016). The extent of damage caused depends on environmental factors, but worldwide it is one of the most devastating cereal diseases, resulting in losses of 10–30% of the global yield of rice (Boddy, 2016). In rice growing areas of Nigeria, a blast disease outbreak could cause the loss of of about 35-50% of rice yield, while 100% loss could be recorded in a serious outbreak of the disease (WARDA, 2004). According to Hadiza *et al.* (2022), blast disease symptoms appeared on more than 40% of the rice cultivated field surved in selected local government area of Jigawa State, Nigeria

At present, the methods of disease resistant variety breeding and chemical control are mainly used for the prevention and control of *M. oryzae* in Nigeria. As a result of the

shortcomings of traditional breeding of disease-resistant varieties, such as long breeding cycles, easy-to-lose resistance, and the high cost of research and development in the field of chemical pesticides, pesticide residues, and drug resistance (Song *et al.*, 2021). Despite being the main treatment to control blast disease, fungicides can cause many serious of environmental problems and human health. In pursuing consistently high yielding crops, farmers usually use high doses of fungicide (much higher than the permitted level), which could destroy beneficial insects and create pathogen resistance. It is necessary to minimize the toxicity of chemicals by other promising products that are healthier for people and safer for environment, hence, a move away from the traditional excessive dependence on chemical pesticides during crop management became necessary. Under the premise of stabilizing production, intensive efforts were needed to develop biological pesticide technology with low toxicity and low residue characteristics. In this sense, chitosan (CS) as an active material has become of great interest for antimicrobial activity owing to its combining ability with other substances and biological properties (Pham *et al.,* 2018). It has been shown to have antifungal activity against the grey mould Botrytis cinerea Pers, in pre- or post-harvest treatments and, in addition, its derivatives exhibited a highly improved antifungal effect against the rice leaf blast *Pyricularia grisea*.

2.1.5 Origin of mycotoxin

Mycotoxins are chemically diverse, toxic secondary metabolites produced by certain fungi, mostly those from the genera *Aspergillus, Fusarium,* and *Penicillium*. They contaminate food sources and have several toxicological effects on humans and animals when consumed directly or indirectly (Wawrzyniak and Waskiewiez, 2014). The earliest report on the impact of mycotoxins on human health was related to ergot-poisoning in Europe, which resulted in thousands of people suffering from severe

symptoms, and also leading to death. The disease was named “Holy fire” or “St. Anthony’s fire” but is known today as ergotism and is caused by the consumption of ergot-infected cereals (Mannaa and Kim, 2016). The toxicity of the ergot-infected products is related to ergot alkaloids. These ergot alkaloids were originally named after their first known source, the sclerotia (ergot) of the fungus Claviceps purpurea. The ergot alkaloids have various toxic effects, such as painful spasms, diarrhea, nausea, headache, or gangrenous symptoms in the fingers and toes. However, the alkaloids have potential, biological functions, such as anti-herbivory defense and pharmaceutical applications, due to their structural similarities with three neurotransmitters, which can bind serotonin, dopamine, and adrenergic receptors (Gerhards *et al.,* 2014). Another famous early outbreak of mycotoxicosis was associated with the consumption of contaminated, discolored yellow rice in Japan in the early 19th century, which was later attributed to a toxigenic entity (Mannaa and Kim, 2016). Rice, along with other cereal crops, is susceptible to contamination by a wide range of mycotoxin-producing filamentous fungi, which usually infect crops in the field and continue in storage facilities. In addition, rice is a good substrate for mycotoxin biosynthesis by grain fungi such as *P. verrucosum* (Wawrzyniak and Waśkiewicz, 2014) and *Fusarium subglutinans* (Mannaa and Kim 2016). For example, P. verrucosum was known to be related with ochratoxin-A contamination in rice, which was reported as the most commonly detected mycotoxin with levels above the tolerated limits in Korean rice. Several mycotoxins, including aflatoxins (Oh *et al.,* 2010), citrinin, cyclopiazonic acid, fumonisins, fusarin C, gliotoxin, moniliformin, ochratoxin A, patulin, sterigmatocystin, trichothecenes (Mannaa and Kim 2016) and zearalenone (Lee *et al.,* 2011), have been found in rice grains. Rice-associated mycotoxins and their related fungi reported in previous studies are summarized in Table 2.1.

2.1.6 Mycotoxigenic fungi in rice

Rice is not spared from natural mycotoxin contamination as bulk of it is grown in the wet season. During the wet season, sun drying practiced by most farmers may not adequately reduce the moisture content in grains. Thus, rice grains with moisture content higher than the desired level enter the storage system. As a result, invasion by both field and storage fungi takes place. Therefore, mycotoxin-producing moulds could contaminate the grain and produce important quantities of mycotoxins during storage (Reddy, 2022).

Climate and storage conditions have been registered to have influence on the formation of the intended mycotoxins. Crops in cool climate are less contaminated than those in warm areas due to the nature of mycotoxigenic fungi. However, mycotoxigenic fungi are varying even geographically. There are two possible phases of infection, first the infection in the crop development and secondly the contamination of the matured crops. This phase occurs by the exposure of the crops to warm, moist conditions either on field or in the storage facility, where even initially dry seeds are susceptible to fungal contamination. Furthermore, various insects have been associated to distribute mycotoxigenic fungi (Laut *et al.*, 2023).

The major mycotoxigenic fungi in rice are *Aspergillu*s sp., *Fusarium* sp. and *Penicillium* sp. The harmful effects of such fungal invasion are glume or grain discoloration, loss in viability, quality and toxin contamination. Aflatoxins, fumonisins, trichothecenes, ochratoxin A, cyclopiazonic acid, zearalenone, deoxynivalenol (DON), citrinin, gliotoxin and sterigmatocystin are the mycotoxins reported in rice (Reddy *et al.*, 2008).

Mycotoxin contamination often occurs in the field prior to harvest. Post-harvest contamination can occur if the drying is delayed and during storage of the crop if moisture is allowed to exceed critical values for mold growth. Delayed harvest in rainy weather frequently leads to grain's sprouting on the panicle, particularly for non-dormant japonica rice. The fungi, *A. flavus, A. parasiticus, A. niger*, and *A. ochraceus*, have been reported earlier by Reddy *et al.* (2008), of which *A. flavus* have been identified as the primary quality deterrent, producing aflatoxincontaminated seeds when in storage (Reddy *et al.* 2008) explored the incidence of *Aspergillus* sp. in 1,200 rice samples consisting of paddy (675) and milled rice (525) collected from 43 locations in 20 rice-growing states across India. The seeds collected were either from areas exposed to different weather conditions or stored at various storage conditions, namely seeds from the crop exposed to heavy rains and floods, seeds from submerged or damp conditions, seeds stored in the warehouse for 1 to 4 years, or seeds from the grain market.

Rice, like other cereals, can be subject to contamination by mycotoxins, both in field and during storage. Several studies have reported mycotoxigenic fungi and mycotoxin occurrence in rice; in particular *Aspergillus, Fusarium* and *Penicillium* Genera (Bertuzzi *et al.*, 2019). The safety of rice and rice products is essential for food security and human health. However, rice is easily contaminated by toxigenic fungi that might have resulted into fungal secondary metabolite accumulation. In particular, mycotoxins produced by toxigenic fungi carry significant health risks and cause toxic carcinogenic, mutagenic teratogenic, and estrogenic effects. Rice grains have evolved with diverse fungal communities during seed development, maturation, and harvest periods, including some harmful toxigenic fungi. The composition and distribution of

grain-associated fungi can affect mycotoxin production, thereby resulting in food safety issues (Ok *et al.*, 2014; Mannaa and Kim, 2017).

According to FAO estimates, more than 25% of global food crops are contaminated by mycotoxins (Eskola *et al.*, 2020), the major mycotoxin contaminants in rice grain are aflatoxins (AFTs), ochratoxins, citrinin, and deoxynivalenol (DON), which are primarily produced by *Aspergillus* species, *Penicillium* species, and *Fusarium* species, respectively (Ok *et al.*, 2014; Reddy, 2022). Among these, the AFTs produced by *Aspergillus* species are the most harmful and carcinogenic toxins found in rice grains and can provoke tumor formation or even rapid death (Ding *et al.*, 2015). The substantial climatic differences and different geographic locations could result in varied compositions and distributions of toxigenic fungi. For example, *A. flavus* and other fungal species favor high temperatures and high relative humidity conditions in tropical and subtropical regions. A report by Onyenma (2020) on Isolation And Identification of Fungi Producing Mycotoxins From Rice Grains Sold In Umuahia Markets revealed that *Aspergillus niger, Aspergillus flavus, Penicillium* sp., *Rhizopus* sp., *Fusarium* sp. and *Yeast* sp are the major fungi isolated, however *Aspergillus flavus* and *A.niger* produced Aflatoxin, *Fusarium* sp., *Rhizopus* sp., *Penicillium* sp., and *Yeast* sp., did not produce any toxin.

Table 2.1: Worldwide Contamination of Mycotoxins observed in Rice and Rice based cereals / products

Mycotoxin: **Fungal species**	**Disease/ Syndrome**	**Country**	**Levels (Range)**
Aflatoxins:	Liver lesions, Cirrhosis, Primary hepatocellular	China	0.99 - 3.87 µg/kg
Aspergillus flavus	carcinoma	India	0.1 - 308 µg/kg
A. parasiticus.	Kwashiorkor, Reye's syndrome.	Korea	1.8 - 7.3 ng/g
		Philippines	0.27 - 11 µg/kg
		Vietnam	3.31 - 29.8 ng/g
Fumonisins:	Esophageal Carcinoma.	Canada	0 - 10 ng/g
Fusarium verticillioides,		India	0.01 - 65 mg/kg
F. prolifratum		Korea	48.2 - 66.6 ng/g
		US	2.2 - 5.2 mg/kg
Ochratoxin A	Endemic nephropathy	Canada	0.3 - 2.4 ng/g
A. ochraceus,	Urothelial tumours.	Korea	0.2 - 1 ng/g
A. carbonarius		Morocco	0.15 - 47 ng/g
Penicillium verrucosum		Nigeria	24 - 1164 µg/k
		Vietnam	0.75 - 2.78 ng/g
Deoxynivlenol	Nausea, Vomitting, Headache,	Germany	0 - 0.058 mg/kg
F. graminearum,	Abdominal pain, Diarrhea, Dizziness		
F. culmorum			
Zearalenone:	Premature puberty in girls,	Canada	0.1 - 1 ng/g
F. graminearum,	Cervical Cancer	Korea	21.7 - 47 ng/g
F. culmorum		Nigeria	24 - 116 µg/kg

Source: Reddy, 2022 (http://www.rkmp.co.in)

2.2 Fungicides and Human Health

Nigeria economy is dependent upon agriculture and agriculture has one of the major problems of fungal diseases. Fortunately, agricultural production has made tremendous progress during the last century, and part of this progress has been the development of modern means of plant disease control using synthetic chemicals. Unfortunately, the constant and regular use of these chemicals has resulted in detrimental effects to the environment and human health (Dhiraj *et al.,* 2014). About 0.1 % of agrochemicals used for crop protection reach the target pest leaving the remaining 99.0% to enter the environment and cause hazards to non-target organisms including human.

Risk assessment of fungicide impact on human health is not easy to determine, particularly the accurate process because of differences in the periods and the levels of exposure, type of pesticides (regarding toxicity), mixtures or cocktails used in the field, and the geographic and meteorological characteristics of the agricultural areas where pesticides are applied (Damalas, 2009). Such differences refer mainly to the people who prepare the mixtures in the field, the pesticide sprayers, and also the population that lives near the sprayed areas, pesticide storage facilities, greenhouses, or open fields. Therefore, considering that human health risk is a function of pesticide toxicity and exposure, a greater risk is expected to arise from high exposure to a moderately toxic pesticide than from little exposure to a highly toxic pesticide (Zyoud *et al.,* 2010).

Regardless of the difficulties in assessing risks of pesticide use on human health, the authorization for pesticide commercialization globally, currently requires data of potential negative effects of the active substances on human health. These data are

usually obtained from several tests focused on metabolism patterns, acute toxicity, sub-chronic or sub-acute toxicity, chronic toxicity, carcinogenicity, genotoxicity, teratogenicity, generation study, and also irritancy trials using rat as a model mammal or in some cases dogs and rabbits (WHO, 2010; FAO, 2020).

The respective toxicity tests for human health risk assessments required by Enviromental Protection Agency (2009) are (1) the acute toxicity test, which assesses the effects of short-term exposure to a single dose of pesticide (oral, dermal, and inhalation exposure, eye irritation, skin irritation, skin sensitization, neurotoxicity), (2) the sub-chronic toxicity test, which assesses the effects of intermediate repeated exposure (oral, dermal, inhalation, nerve system damage) over a longer period of time (30–90 days), (3) the chronic toxicity test, which assesses the effects of long-term repeated exposure lasting for most of the test animal's life span and intended to determine the effects of a pesticide product after prolonged and repeated exposures (e.g., chronic non-cancer and cancer effects), (4) the developmental and reproductive tests, which assess any potential effects in the fetus of an exposed pregnant female (i.e., birth defects) and how pesticide exposure may influence the ability of a test animal to reproduce successfully, (5) the mutagenicity test which assesses the potential of a pesticide to affect the genetic components of the cell, and (6) the hormone disruption test, which measures the pesticide potential to disrupt the endocrine system (consists of a set of glands and the hormones they produce that regulate the development, growth, reproduction, and behavior of animals including humans). Therefore, alternative methods are necessary to solve these problems of reducing the human health risk and environmental impact of the activity without affecting agricultural productivity and with economic benefits for farmers.

2.2.1 Biofungicides as alternative strategies for crop protection

Synthetic fungicides constitute the primary means of controlling postharvest diseases in cereals, rice inclusive (Gilberto *et al*., 2017). However, increasing concerns of health hazards and accumulation of toxic residues in the ecosystem has demanded the development of alternative strategies for crop protection (Faoro *et al.,* 2008). In addition, over usage of synthetic fungicides has facilitated the development of fungicide resistance among some pathogenic populations. Biofungicide are biologically pest (fungi) control agent and one of the solutions for sustaining agricultural output and environmental quality.

Biofungicide is the general name given to microorganisms (microbial pesticides) and naturally occurring compounds that possess the ability to control plant diseases (biochemical pesticides) Plant extracts and other plant-based compounds such as essential oils have been tested for their bioefficacy in the management of wide range of fungal diseases in plants (Abbey *et al.,* 2018). Biofungicide have many advantages over chemical fungicide like it is biodegradable, cheaper and harmful residues are not detected

In recent years, a number of essential oils have been reported to have high antifungal activities and is gaining popularity in the agricultural sector due to their volatility, ecofriendly and biodegradable properties. With more research efforts being channelled into realising the full potential of biofungicides; it is likely that their use in agriculture for disease management will continue to rise.

Although some commercial products are available, the single application of some of these biofungicides does not always give the desired disease control on the field. Therefore, integrated biological control technique, in which different biological

control agents are combined for disease control (Abbey *et al.,* 2018), can be a promising disease control strategy. The adoption and widespread use of biofungicide will make it possible to produce food with no or minimal fungicide residue if any. Therefore, in the absence of resistant cultivars, plant extract and oil extract biopesticides; chitosan can serve as biofungicides for sustainable approach in the control of postharvest diseases in cereals (Ali *et al.,* 2010; Gilberto *et al*., 2017).

2.2.2 Shellfish as a new source of biofungicides

Shellfish is a culinary and fisheries term for exoskeleton-bearing aquatic invertebrates mostly used as food. They include various species of molluscs, crustaceans, and echinoderms. Taxonomically, shellfishes belong to the phylum Arthropoda and phylum Mollusca. Familiar marine molluscs used as food source by humans include many species of clams, mussels, oysters, wrinkles and scallops.

Some crustaceans such as shrimps, prawns, lobsters, crayfish, and crabs are a feature of almost all the cuisines in the world (Ojiako *et al.,* 2018).In Nigeria, South East(Anambra, Imo), South South (Akwa Ibom, Delta, Rivers) and South West (Lagos) zone, where water covers the largest land mass are abundant of the shellfish. However, it would be quite profitable to recover the by-products released from shellfish processing because of its richness in compounds of high value added such as chitin products. Chitin and its derivatives have high economic value owing to their versatile biological activities and agrochemical applications (Biofungicides).

2.2.3 Crabs as one of the shellfish

The marine species of true crabs (brachyurians) are divisible into three (3) groups. These are the terrestrial (land-based) crabs found on the fringes of estuaries, and are mainly members of the family gecarcinidae, grapsidae and ocypopidae e.g. Ocypode africana; the swimming species in shore waters and even in estuaries, all belonging to

the family portunidae, and typical examples of these species in Nigerian waters include Callinectes pallidus (blue crab), Callinectes amnicola (big-fisted swim crab) and Cardiosoma armatum. The third group is the deep-sea crabs (geryonidae) and is part of the benthic ecosystem on the continental slope (Ojiako *et al.*, 2018).

2.2.4 Problems of shell fish waste management

Unscientific dumping of shellfishery waste is a major environmental concern worldwide and a serious threat to the coastal area (Suryawanshi *et al.,* 2019). With growing population, waste generation is also increasing and major proportion of by-products generated by contemporary food remains underutilized which may often contain high-value substances. Crucial problem faced by industries and society during food processing is disposal of food waste. Habitually, seafood waste is burned, land filled, dumped at sea or left to get spoiled (Xu *et al.*, 2013). If not processed properly, it may have a negative impact on human health, biodiversity and environment. Decaying crab wastes have been reported to release ammonia and nitrates that evaporate in air and seep through soil; which in large concentrations can pollute the slowing moving ground waters below and freshwater wells that provide drinking water and shallow aquifers that is eventually to feed to the populace.

However, around 10^{12}–10^{14} tonnes of chitin is produced annually by living organisms in ocean (Dhillon *et al.,* 2013), out of which 2.8×10^{10} kg is generated by arthropods in freshwater and 1.3×10^{12} kg in marine environment. This huge quantity of chitin would provide enough raw materials, if commercial procedures were developed for extraction of commercially competent polymers.

2.3 Chitosan as alternative biofungicide

Chitosan is a natural polymer derived from chitin by a chemical deacetylation process using a strong alkali such as sodium hydroxide at high temperature [poly-β- (1 → 4) -N-acetyl-Dglucosamine], it is the main component of the shell of crustaceans and the feather of pota (giant squid; *Dosidicus gigas*). It is also found in the cell wall of the fungi and in the exoskeleton of some insects, and is the second most abundant natural biopolymer after cellulose (Malerba and Cerana, 2016). Chitosan is a natural, biodegradable, high molecular weight polymer with a wide range of uses in cosmetology, the food industry, biotechnology, medicine and agriculture (Zahid, 2014). Another important attribute of this natural compound is associated with its fungistatic or fungicidal properties against pathogens of various crops (Zahid, 2014; Gilberto *et al*., 2017).

Growth of important postharvest fungi such as *Alternaria alternata* (Fries) Keissler, *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc., *Fusarium oxysporum* Schlecht., *Rhizopus stolonifer* (Ehrenb. Fr.) Lind and *Penicillium* spp. was inhibited on nutrient media amended with various concentrations of chitosan (Zahid, 2014; Gilberto *et al*., 2017). For some years, it has been receiving a lot of attention due to its industrial applications, in the bimedical, agricultural, food, chemical industry, etc. (El-Hadrami *et al.,* 2010). Chitosan is being commercially produced using the exoskeletons of crabs, shrimps and prawns as well as the feather of the pota with different degrees of deacetylation and molecular weights, for different uses in some countries such as Peru.

In nature, there are three types of chitin, which have different configurations and properties, which include form α with a conformation of chains in antiparallel, the

form β, with a conformation of chains in parallel, while the form γ, with a conformation of chains alternating parallel and antiparallel; Chitin α (↑↓↑↓), Chitin β(↑↑↑↑), and Chitin γ (↑↑↓↑↑)

The antiparallel form of chitin α allows the formation of a greater number of intermolecular hydrogen bonds, which are responsible for its insolubility in aqueous solvents and a large amount of organic solvents (Jose *et al.,* 2017). On the other hand, the configuration in parallel form of the chitin β provides a weaker intermolecular force, which allows the hydration of the molecule.

2.3.1 Structure of chitosan (chitin derivative)

Chitin is a natural polysaccharide synthesized by a great number of living organisms and functions as a structural polysaccharide. Chitin is a chain polymer containing two of N-acetylglucosamine units, an amide derivates of glucose ($C_8H_{13}O_5N$)n (Figure 2.1), while Chitosan consists of three functional groups, including an amino group and primary and secondary hydroxyl groups (Figure 2.2). According to BioPesticides manual (Keith *et al.,* 2019), chitosan is described as a 'crustacean-derived plant defence booster. It is a polysaccharide consisting of 2-Amino-2-deoxy-beta-D-glucosasmine. Chitosan is a modified natural carbohydrate polymer derived from chitin, which occurs principally in animals of the phylum Arthropoda. The primary unit in the chitin polymer is 2-deoxy-2-(acetylamino) glucose. The units are combined by 1, 4 glycosidic linkages, forming a long chain linear polymer. Removal of most of the acetyl groups of chitin by treatment with strong alkalis yields chitosan.

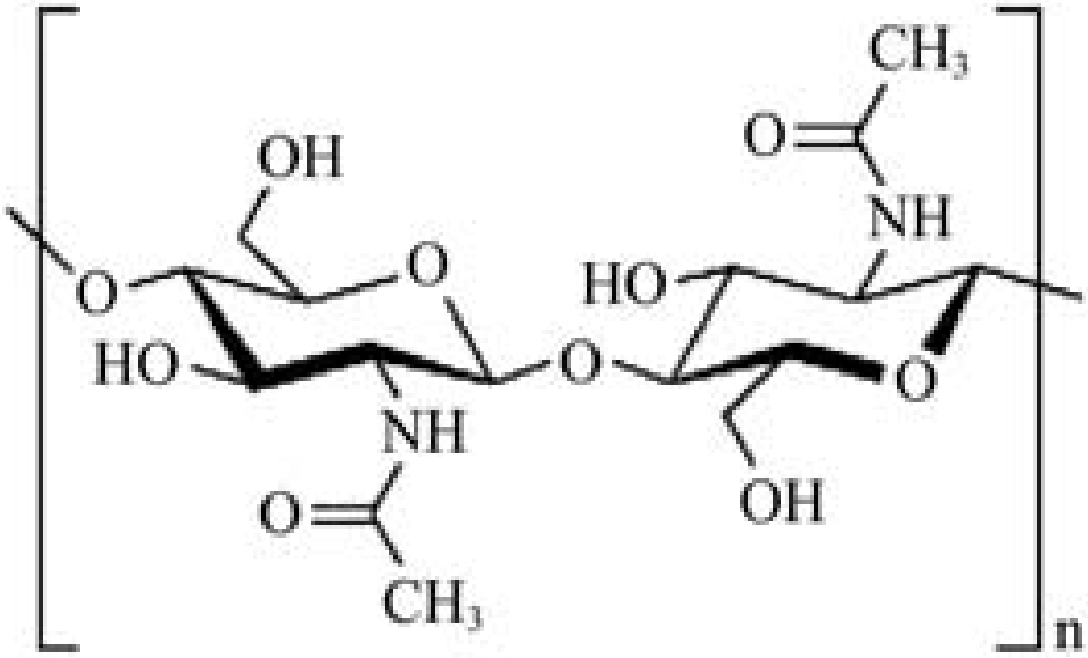

Figure 2.1: Structure of Chitin

Source: El-Mohamedy *et al*., 2019

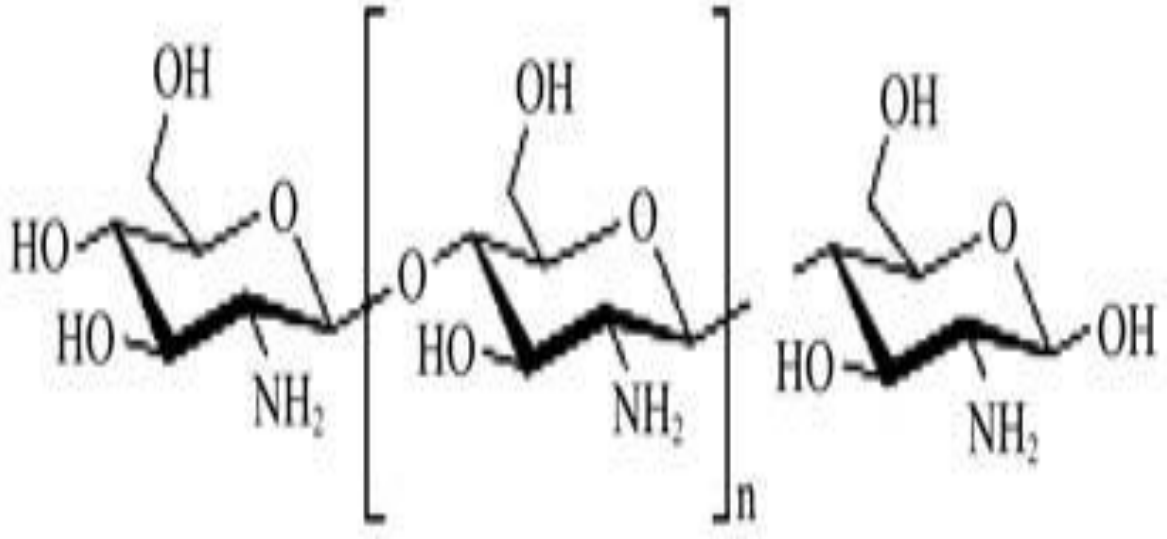

Figure 2.2: Structure of Chitosan

Source: El-Mohamedy *et al*., 2019

2.3.2 Physico-chemical properties of chitosan

In the chitosan, the content of N-acetyl groups and their distribution along the chain, depend on the conditions of the deacetylation applied, and the origin of the chitin. The degree of deacetylation is one of the most important factors influencing the properties of the biopolymer due to the role that it plays in its solubility. The molecular weight of the chitin is greater than 1000×10^3 g mol^{-1} and the commercial chitosan is between 100-500 $\times$ 10^3g mol^{-1}, depend on the origin of the chitin. Different factors during the chitin extraction process and chitosan preparation may influence the molecular weight of the biopolymer. High temperatures, acid and alkali concentrations as well as reaction times that can degrade and produce depolymerization of the polymer chains (Jose *et al.,* 2017) is also a factor. In addition, there is evidence that the molecular weight of chitosan influences its biological activity (El-Hadrami *et al.*, 2010; Jose *et al.*, 2017).

2.3.3 Biological properties and antimicrobial activity of chitosan

Chitosan and chitin are non-toxic and biodegradable biopolymers. Their bioactivity includes stimulation in healing processes, potentiate the immune system and antimicrobial activity. Chitosan oligomers also exhibit physiological functions, including induction of phytoalexins, antimicrobial activity and immune activity (Jose *et al.,* 2017). Chitosan and its derivatives have antimicrobial activity against bacteria and fungi (Jose *et al.,* 2017). The mechanism of action is being elucidated. There are different hypotheses, which are related to their cationic nature, allowing them to react with molecules and negatively charged surfaces such as microbial walls (Jose *et al.,* 2017). Chitosan acts as a soluble chelating agent and behaves as an activator of the defense mechanisms of several plants (Jose *et al.*, 2017). It also induces the release of

protein and intracellular components, due to the interaction between positively charged chitosan molecules and negatively charged microbial cell membranes (Jose *et al.,* 2017)

2.3.4 Agricultural, medical and industrial applications of chitosan

Due to its bactericidal and fungicidal properties, chitosan is being used in the agricultural field. It is being used in the coating of fruits to control the postharvest deterioration, thus increasing the storage time. Another of its uses is as a matrix for controlled release of fertilizers and chemicals to combat pests (Jose *et al.,* 2017). In the biomedical area, chitosan has interesting uses such as, in the treatment of burns, as a cover of drugs for controlled release and in the retention of fats for obesity control. It is also an immune system enhancer. In the industrial area, it has important applications such as wine clarifier, in the process of treatment of water and effluents contaminated by mining activity, and also as a fixative for dyes in the textile industry (Jose *et al.*, 2017).

2.3.5 Antimicrobial mechanism of action of chitosan

The mechanism of antimicrobial activity of chitosan is the charge interaction, which is based on the interaction between the charges (+) of the amino group of the biopolymer and the charges (-) of the cellular components (phospholipids, proteins, amino acids) present in microbial cell membranes. This alters cellular integrity and permeability (Madushani *et al.,* 2012; Jose *et al.,* 2017), resulting in the release of intracellular components such as glucose and lactate dehydrogenase (Correa-Pacheco, 2016).

In fungi, the effect is independent of the phospholipid and ergosterol composition of the plasma membrane; however, the fungal sensitivity to the polymer depends on the

content of unsaturated fatty acids (Jose *et al.*, 2017). Another important mechanism is the interference caused by protein synthesis, which can be a consequence of membrane damage. This interference involves the ability of chitosan to cross the microbial cell membrane and subsequently interact with DNA, interfering with the synthesis of proteins and RNA messenger, which occurs when very low molecular weight molecules penetrate into the cell (Jose *et al.*, 2017). However, this theory has not yet been fully accepted.

The first study on antipathogen activity of Chitosan was published by Allan and Hadwiger (Iriti and Varoni, 2015). This study reported the fungicidal effect of Chitosan on fungi of different cell wall composition. This attracted the attention of agricultural industry on Chitosan since pathogens can cause severe diseases and significant losses in crop yield and quality worldwide. Many pathogens can also produce toxins and metabolites, which can greatly affect the safety of agricultural products. The report of many studies on the antimicrobial properties of Chitosan and its derivatives on many plant pathogens resulted in positive inhibition (Iriti and Varoni, 2015). In fact, during both pre- and postharvest processes, infection of pathogenic fungi results in major losses of cereals, fruits and vegetables. At present, synthetic chemical fungicides are the primary choice to manage these pathogens. However, synthetic fungicides are potentially harmful on human health and their indiscriminate use induces the emergence of resistant strains.

Chitosan is a promising alternative to control these diseases. It has been shown to possess a broad-spectrum fungicidal activity against several phytopathogenic fungi, effectively inhibiting their development at different life-cycle stages. For example, in pear (*Pyrus pyrifolia* L.) fruit, chitosan completely prevented germination and growth of *Alternaria kikuchiana* and *Physalospora piricola* (Meng *et al.*, 2010). Chitosan

inhibited growth of *Botrytis cinerea* in liquid culture and suppressed grey mold disease caused by the fungus on detached grapevine leaves and bunch rot in Chardonnay and Sauvignon blanc wine grapes (Reglinski *et al.,* 2015). In rice (*Oryza sativa*), chitosan showed marked antifungal activity against *Rhizoctonia solani*, the rice sheath blight pathogen (Liu *et al.,* 2012). Cowpea (*Vigna unguiculata* L.) plants were protected by chitosan against *Fusarium oxysporum* infection (Berger *et al.,* 2016).

Chitosan combined with clove oil or *Bacillus subtilis* endospores protected mature satsuma mandarin (*Citrus unshiu* Marc. cv. Miyagawawase) and Shogun mandarin oranges (*Citrus reticulate* Blanco cv. Shogun) fruits against *Penicillium digitatum*, the causal agent of citrus green mold (Shao *et al.,* 2015; Waewthiongraka *et al.,* 2015). Dragon fruit (*Hylocereus polyrhizus*) plants were protected by chitosan against *Colletotrichum gloeosporioides* (Zahid *et al.,* 2015). Chitosan enriched with lemongrass oil was very effective *in vivo* and *in vitro* control of anthracnose caused by *Colletotrichum capsici* in bell pepper (*Capsicum annuum* L.). Additionally, chitosan and glycol chitosan have affected the cell development and physiology of *Ustilago maydis*, the dimorphic fungus causing corn smut disease (Olicon-Hernandez *et al.,* 2015). Scots pine (*Pinus sylvestris* L.) seedlings sprayed with chitosan were effectively protected against parasitic damping-off and *Lophodermium* needle cast (Aleksandrowisz-Trzcinska *et al.,* 2015) and chitosan induced cell death in spores of *Fusarium eumartii*, a fungal pathogen of potato and tomato (Terrile *et al.,* 2015). Chitosan also prevented the growth of several pathogenic bacteria including *Xanthomonas, Pseudomonas syringae* (Mansilla *et al.,* 2013), *Acidovorax citrulli* (Li *et al.,* 2013), *Agrobacterium tumefaciens,* and *Erwinia*

carotovora (Badawy *et al.*, 2014). Recently, a protective action of chitosan against other affecting plants pathogenic organisms has been also reported.

2.3.6. Antimicrobial properties of chitosan

Chitosan exhibits a variety of antimicrobial activities (Badawy *et al.*, 2014), which depend on the type of chitosan (native or modified), its degree of polymerization, the host, the chemical and/or nutrient composition of the substrates, and environmental conditions. In some studies, oligomeric chitosans (pentamers and heptamers) have been reported to exhibit a better antifungal activity than larger units (El-mohamedy e*t al.*, 2014). In others, the antimicrobial activity increased with the increase in chitosan molecular weight (Malerba and Cerana, 2016), and seems to be faster on fungi and algae than on bacteria (El-Hadrami *et al.*, 2010).

2.3.7 Chitosan inhibitory potentials against fungi and *Oomycetes*

Fungicidal activities of chitosan has been documented against various species of fungi and oomycetes (El-Hadrami *et al.*, 2010; Madushani *et al*., 2012). The minimal growth-inhibiting concentrations varied between 10 and 5,000 ppm (El-Hadrami *et al.*, 2010; Madushani *et al.*, 2012; Malerba and Cerana 2016). The maximum antigungal activity of chitosan is often observed around its *p*Ka (pH 6.0)

A report on the fungicidal activity of 24 new derivatives of chitosan (*i.e.*, *N*-alkyl, *N*-benzylchitosans) on radial hyphal growth bioassay of *B. cinerea* and *P. grisea*, showed that the derivatives have a higher fungicidal action (El- Mohamedy *et al*., 2014) than the native chitosan. *N*-dodecylchitosan, *N*-(*p*-isopropylbenzyl) chitosan and *N*-(2, 6-dichlorobenzyl) chitosan were the most active against *B. cinerea*, with EC_{50} values of 0.57, 0.57 and 0.52 $g.L^{-1}$, respectively. While *N*-(*m*-nitrobenzyl) chitosan was the most active against *P. grisea* with 77% inhibition at 5 $g.L^{-1}$. *O*-

(decanoyl) chitosan at mol ratio of 1:2 (chitosan to decanoic acid) was the most active compound against *B. cinerea* (EC_{50} = 1.02 $g.L^{-1}$) and *O*-(hexanoyl) chitosan displayed the highest inhibition against *P. grisea* (EC_{50} = 1.11 $g.L^{-1}$). Some of the derivatives also repressed spore formation at rather high concentrations (1.0, 2.0 and 5.0 $g.L^{-1}$). Recently, Palma-Guerrero *et al.* (2010) demonstrated that chitosan is able to penetrate the plasma memebrane of *Neurospora crassa* and kills the cells in an energy-dependent manner

In general, chitosan applied at a rate of 1 mg/mL, is able to reduce the *in vitro* growth of a number of fungi and oomycetes except Zygomycetes, which have chitosan as a component of their cell walls (Madushani *et al*., 2012; Hadwiger, 2015). Another category of fungi that seems to be resilient to the antifungal effect of chitosan are the nemato-/entomo-pathogenic fungi that possess extracellular chitosanolytic activity (Geopheagan and Gurr, 2017)

2.4 Nanotechnology

Nanotechnology is based on the ability to characterize, manipulate, and organize materials on a nanoscale, which gives the products characteristics and behaviors different to those found at the larger scale (Wijayadi and Rusli, 2019). Nowadays, nanotechnology has emerged as the third approach, which has opened opportunities for skin drug delivery via nanosystem-like particles, dendrimers, etc. The size of nanosystems for topical and transdermal delivery generally ranges from 1 to 1000nm. They increase skin permeation by enhancing drug solubilization, partitioning of drug into the skin layers, and fluidizing the stratum corneum lipids (Wijayadi and Rusli, 2019).

2.4.1 Chitosan nanoparticles

Nanoparticles are defined as particulate dispersions or solid particles with a size in the range of 1–1000 nm. Methods, such as the emulsion method, ionic gelation method, reverse micellar method, self-assembling method etc., have been used to prepare chitosan nanoparticles (Zhao *et al*., 2011).

2.4.2 Ionic gelation method

Chitosan nanoparticles can be prepared by the interaction of oppositely charged macromolecules. Tripolyphosphate (TPP) has often been used to prepare chitosan nanoparticles because TPP is nontoxic, multivalent and able to form gels through ionic interactions. The interaction can be controlled by the charge density of TPP and chitosan, which is dependent on the pH of the solution. Studies have reveal the influence of a number of factors, such as pH, concentration, ratios of components, and method of mixing, on the preparation of chitosan/TPP nanoparticles. Zhao *et al.* (2011) investigated the relationship between free amino groups on the surface and the characteristics of chitosan nanoparticles prepared by the ionic gelation method. These factors were unaffected by TPP concentration in these references

Applications of chitosan nanoparticles in agriculture are synthesized because their higher mobility and surface area for the efficient delivery of agrochemicals and micronutrients. They have been used in crop production and protection in fruits and vegetables such as: pea, tomato, strawberry, celery, pepper, maize, cucumber, pumpkin, chili, cabbage, peanut, soybean, potato, papaya, mango, rice, watermelon, among others. It applications such as antifungal, antibacterial, hormone delivery, enhancement in respiration rate, increment in the seed germination rate and enhancement in growth as well as yield has been reported (Kashyap *et al.*, 2015). The

in vitro antifungal efficacy of oleoyl-chitosan nanoparticles dispersion system against plant pathogenic fungi was investigated by Xing *et al*. (2016). For the tested fungi, mycelium growth experiment showed that *Nigrospora sphaerica*, *Botryosphaeria dothidea*, *Nigrospora oryzae* and *Alternaria tenuissima* were chitosan nanoparticle-sensitive. The best results were obtained for the carvacrol based nanoemulsion which was incorporated into modified chitosan to form a bioactive coating being effective during storage period of the green beans (Severino *et al.,* 2015).

The filamentous plant pathogen *Colletotrichum gloeosporioides* is responsible for anthracnose on a large scale in various tropical regions. Nanoparticles encapsulated with chitosan were prepared in order to control the anthracnose of dragon fruit plants. The average size of the nanoemulsion droplet was within 1000 nm. Chitosan and emulsifiers were subjected to ultrasonication and to achieve smallest droplet (600 nm). The *in vitro* antifungal activities of 600 nm droplet size along with conventional form of chitosan were investigated against anthracnose caused by the fungus *C. gloeosporioides* isolated from dragon fruit. Results showed that 600 nm sizes at 1.0% chitosan concentration was more effective in controlling mycelial growth (79.57 %) and also altered the spore and mycelia structure of the fungus. The results of the study suggest that instead of applying chitosan in the conventional form, chitosan in the form of nanoparticle emulsions could be more effective as a biofungicide for controlling anthracnose of dragon fruit plants (Zahid, 2014)

2.4.3 Synthesis of chitosan nanoparticles (NPs)

Chitosan substance can be converted into chitosan Nanoparticles with many advantages. Chitosan NPs can be used as a delivery agent for drugs, proteins and antigens. They also have antifungal and antimicrobial activity. Besides, chitosan

Nanoparticles have been applied for the removal of metal ions and could serve as a promising adsorbent in wastewater treatment (Amri *et al.,* 2020)

Nanoparticles can be synthesized through two approaches; top down and bottom-up approaches (Zulfajiri *et al.,* 2020). The top-down approach reduces the size of bulk materials to nanoscale by grinding or etching while the bottom-up approach is the synthesis of nanoparticles involving chemical reactions from small (bio)molecules (Sanap *et al.,* 2020) Based on previous studies, there were several methods involving chemical reactions in the preparation of the NPs. They are sonochemical method (Chen *et al.,* 2020. Dheyab *et al.,* 2020) ionic gelation method

The report of Amri *et al.* (2020) on the effect of different volumes of tripolyphosphate (TPP) on the synthesis of chitosan nanoparticles (NPs) was evaluated. The synthesis was performed using the ionic gelation method with a 0.5% chitosan solution and 1% TPP. A particle size analyser (PSA) was used to obtain the size of the chitosan NPs while a Fourier Transform Infrared (FTIR) spectrophotometer was utilized to explore the functional groups of the NPs. The results showed that the average size of chitosan NPs were 648.2 nm (20:4), 515.7 nm, 648.2 nm, 515.7 nm, and 340.3 nm for different volume ratios of 20:4, 20:3, and 20:2, respectively. The lesser the TPP volume added, the smaller the particle size produced. The chitosan NPs have several functional groups including –OH, amines, -CH alkanes, -C=O groups, amides, P-O groups, and P=O groups, indicating that the synthesis product corresponded to the chitosan-TPP compound.

2.5 Application of Modified Chitosan in Plant Disease Control

Chitosan used to control plant pathogens has been extensively explored with more or less success depending on the pathosystem, the use of derivatives, concentration,

degree of deacylation, viscosity, and the applied formulation (*i.e.*, soil amendment, foliar application; chitosan alone or in association with other treatments)..

Substratum amendment with chitosan was reported to enhance plant growth and suppress some of the notorious soil-borne diseases. For example, in soilless tomato, root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* was suppressed using chitosan amendments (El-Mohamedy *et al.*, 2014). Similarly, in order to control post-harvest diseases, addition of chitosan stimulated microbial degradation of pathogens in a way resembling the application of a hyper-parasite (Gilberto *et al.*, 2017). This area of application is important because it suggests alternatives to the use of pesticides on fresh produce in storage. Recent investigations on coating tomatoes with chitosan have shown that it delayed ripening by modifying the internal atmosphere, which reduced decays due to pathogens (Povero *et al.*, 2011; Madushani *et al.*, 2012). Various methods of application of chitosan are practiced to control or prevent the development of plant diseases or trigger plant innate defenses against pathogen

2.5.1 Application of chitosan as seed coating agents

Guan *et al*. (2009) examined the use of chitosan to prime maize seeds. Although chitosan had no significant effect on germination under low temperatures, it enhanced germination index, reduced the mean germination time, and increased shoot height, root length, and shoot and root dry weights in two tested maize lines. In both tested lines, chitosan induced a decline in malonyldialdehyde content, altered the relative permeability of the plasma membrane and increased the concentrations of soluble sugars and proline, and of peroxidase and catalase activities

In other studies, seed priming with chitosan improved the vigor of maize seedlings (El Hadrami *et al.,* 2010). It was also reported to increase wheat seed resistance to certain diseases and improve their quality and/or their ability to germinate (Gilberto *et al.,* 2017). Similarly, peanut seeds soaked in chitosan were reported to exhibit an increased rate of germination and energy, lipase activity, and gibberellic acid and indole acetic acid levels (El Hadrami *et al.,* 2010) showed that rice seed coating with chitosan may accelerate their germination and improve their tolerance to stress conditions. In carrot, seed coating helps restrain further development of Sclerotinia rot (El Hadrami *et al.,* 2010).

2.6 Mechanism of Action of Chitosan in Reducing Plant Disease

Although the exact mechanisms of action of chitosan in reducing plant disease are currently not fully understood, there is growing evidence showing its action through direct toxicity or chelation of nutrients and minerals from pathogens. Because of its biopolymer properties, this compound can also form physical barriers around the penetration sites of pathogens, preventing them from spreading to healthy tissues (Malerba and Cerana, 2016). This and bioactive derivatives can activate H^+-ATPases, depolarizing biological membranes and inducing other series of events. Chitosan is known to induce reactions locally and systemically that involve signaling cascades, and the activation and the activation and accumulation of defense-related antimicrobial compounds and proteins.

Chitosan, when applied to plant tissues, often agglutinate around the penetration sites and has two major effects. The first one is the demercation of the penetration site through the formation of a physical barrier preventing the pathogen from spreading and invading other healthy tissues. This phenomenon resembles the abscission zones

often observed on leaves preventing several necrotrophic pathogens from spreading further. It is widely observed on potato tubers for example (El Hadrami *et al.*, 2010). Around the isolated zones, often an elicitation of a hypersensitive response occur with the accumulation of H_2O_2 that helps in cells wall fortification and serves as an alert signal for other healthy parts of the plant. The second effect is due to the chitosan's ability to bind various materials and initiate fast the wound healing process (El-Hadrami *et al.*, 2010)

2.6.1. Direct activity of chitosan against pathogen

Chitosan is often used in plant disease control as a powerful elicitor rather than a direct antimicrobial or toxic agent. Its direct toxicity remains dependent on properties such as the concentration applied, the molecular weight, degree of acetylation, solvent, pH and viscosity (El Hadrami *et al.*, 2009; El-Hadrami *et al.*, 2010). The degree of acetylation defines the sites with which nucleophilic groups could react and viscosity provides an environment that could extend the duration and intensity of reactions

Chitosan against fungi, oomycetes and other pests are likely to operate indirectly *via* other means such as the enhancement of host resistance. However, a number of studies have shown that chitosan, at defined concentrations, presents antimicrobial properties (El Hadrami *et al.*, 2010; Zahid, 2014; Malerba and Cerana, 2016). For instance, chitosan was reported to exert an inhibitory action on the hyphal growth of numerous pathogenic fungi, including root and necrotrophic pathogens, such as *Fusarium oxysporum*, *Botrytis cinerea*, *Monilina laxa*, *Alternaria alternata* and *Pythium aphanidermatum* (Li *et al.*, 2013; Mansilla *et al.*, 2013;

Badawy *et al.*, 2014; Goni *et al.*, 2014) besides inhibiting spore germination in some of them

2.7. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR offers quantitative and qualitative analysis for organic and inorganic samples. Fourier Transform Infrared Spectroscopy (FTIR) identifies chemical bonds in a molecule by producing an infrared absorption spectrum. The spectra produce a profile of the sample, a distinctive molecular fingerprint that can be used to screen and scan samples for many different components. FTIR is an effective analytical instrument for detecting functional groups and characterizing covalent bonding information (Rohman *et al.*, 2020).

FTIR testing is often used in fields such as chemistry and pharmaceuticals, where it can be used to identify unknown compounds or to confirm the identity of known compounds. It can also be used to determine the purity of a compound, as well as its physical and chemical properties. FTIR analysis is used to identify molecular compounds. It works by measuring the absorbance of infrared radiation by a sample. The resulting spectrum can then be used to identify the functional groups present in the compound (Lawson *et al.*, 2018).

Essentially, by applying infrared radiation (IR) to samples of materials, FTIR analysis measures a sample's absorbance of infrared light at various wavelengths to determine the material's molecular composition and structure. The Fourier transform spectrometer works to convert the raw data from the broad-band light source to actually obtain the absorbance level at each wavelength. FTIR spectroscopy has been widely used to quantitatively study the parameters of the chitin deacetylation. A research on a Canadian chitin has shown that a degree of deacetylation (DD) of 90% has been reached

with a base concentration of 12.5 M, a reaction time of 120 min, and a temperature of 110°C.

2.7.1 Degree of deacetyaltion (DD)

The degree of deacetylation (DD, %) is defined as the molar fraction of the average number D-glucosamine unit (GlcN) in the copolymers (chitosan) composed of average number of N-acetylglucosamine units (GlcNAc) and GlcN (Jiang *et al.*, 2017). The DD value of a chitosan sample is one of the most important factors in assessing its applications in the medical, nutritional, sewage treatment, pharmaceutical, agriculture and biotechnological fields. It can also be related back to the specific biological and structural properties and functions of chitin or chitosan, and it should be clear that chitosan is the deacetylated form of chitin and it must be characterized by a degree of acetylation when a degree of deacetylation is valid for chitin, the initial form of the polymer nearly fully acetylates (Hussain *et al.*, 2013). Several methods for determining the degree of deacetylation have been elaborated, from simple, such as pH-metric titration, UV-Visible spectroscopy, infrared spectroscopy, elemental analysis, to complex ones, which require complicated and expensive equipment, such as 1H NMR spectroscopy and 13C NMR spectroscopy (Jiang *et al.*, 2017).

The degree of deacetylation (DDA) influences the physical, chemical and biological properties of chitosan, such as acid base and electrostatic characteristics, biodegradability, self-aggregation, sorption properties, and the ability to chelate metal ions. In addition, the degree of deacetylation, which determines the content of free amino groups in the polysaccharide, can be employed to differentiate between chitin and chitosan. The process of deacetylation involves the removal of acetyl group from the molecular chain of chitin, leaving behind a complete amino group (-NH2) and

chitosan versatility depends mainly on this high degree of chemical reactive amino groups (Jiang *et al.*, 2017). There are various methods available to increase or decrease the degree of deacetylation. An increase in either temperature or strength of sodium hydroxide solution can enhance the removal of acetyl groups from chitin, resulting in a range of chitosan molecules with different properties and hence its applications (Hussain *et al.*, 2013). Since the degree of deacetylation (DDA) depends mainly on the method of purification and reaction conditions, it is, therefore, essential to characterize chitosan by determining its DDA prior to its utilization.

The degree of deacetylation (DDA) allows one to define the terms chitin and chitosan, that is, chitosan is usually defined as the derivative that is soluble in dilute acidic solutions. The lowest DDA corresponding to chitosan varies in literature and ranges from 40% to 60%. The majority of the commercial chitosan samples have average DDAs of 70-90%. For some special biological applications, chitosan of higher DDA (> 95%) may be prepared via further deacetylation steps which not only increases the cost of the preparation but also often results in partial depolymerization (Czechowska-Biskup *et al.*, 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was conducted at the Department of Plant Biology Laboratory, Federal University of Technology, Minna, Niger State, Nigeria on Latitude 9^{o} 39' 12.38"N and Longitude 6^{o} 31'24.79"E. The State is situtated between Latitude 8^{o} 12'N and 11^{o} 30'N and Longitude 3^{o} 30'E and 7^{o} 20'E which is Southern Guinea Savanna ecological zone of Nigeria

3.2 Collection of Samples

A total of fifty (50) rice seed samples (5 kg each) of both known varieties and unknown (local accession) stored between six months to one year by farmers and in different storage facilities (bags, open floor in stores and silos) were collected from farmers during the month of December, 2019 and May, 2020 across nine (9) Local Government Areas (LGAs) of Niger States. The representative rice seed samples were collected from different sacks of a stack/pile and from different points in a sack or open floor in order to get complete sample. The samples collected were then separated to either improved release varieties (known) and local accession (unnamed varieties). The samples collected were stored at 20^{o} C in the fridge before being used for further study. Table (3.1) shows the rice varieties/cultivars collected from different villages across the nine Local Government Areas (LGAs) of Niger States. A working sample of approximately 20 g was taken out from each sample using a standard divider.

Two rice varieties (FARO 52 and FARO 66) for the field trial were also collected from the breeding unit of the National Cereals Research Institute (NCRI) Badeggi, Niger

State, Nigeria. Blast infested leaves of rice were collected into labelled sterile polythene bags from farmer's field and transported to the Plant Biology Laboratory of Federal University of Technology, Minna, Niger State. All samples collected were stored at temp of 4^{o}C until further use.

Table 3.1: Rice Seeds Samples collected and Location

Location/ Village	Local Government Area	Rice Variety/cultivar	Number of farmers samples
Agaie	Agaie	Faro 44, Alhaji Baba*	3
Bussu	Bargu	Faro 44, 52, Kuddo*	3
Doko	Lavun	Faro 44,52	4
Eddo	Lapai	Faro 52	4
Edozhigi	Gbako	Faro 52	3
Faka	Munya	Faro 44	3
Goyedangi	Lavun	Faro 44, 52	5
Jima	Lavun	Faro 44, 52	3
Kodo	Wushishi	Faro 44, 52	3
Kpatsuwa	Mokwa	Faro 44, 52, 61, kuddo*, Alhaji Baba*	5
Loguma	Agaie	Faro 44, 61	2
Tungaruwa	Rijau	Faro 44, 52, Kuddo*, Sakpefu*	5
Wushishi	Wushishi	Faro 52, 44, Alhaji Sule*, Ekangi*	4
Yelwa	Mokwa	Faro 52, Rabach*, Dgwagwa*,	3
Total		_	50

*Local name used by the farmers

3.2.1 Collection of chitosan

Three different molecular weight chitosans viz; Low molecular weight chitosan (LMWC) (MW 50 kDa; 75–85 % deacetylated), Medium molecular weight chitosan (MMWC) (MW 400 kDa; 75–85 % deacetylated) and High molecular weight chitosan (HMWC) (MW 760 kDa; ≥ 85 %)) were purchased from Chitin-Chitosan BioChemika and Sigma- Aldrich Company, USA. One kilogram (1 kg) of Crab shells were also obatined from sea shores in Warri, Delta State Nigeria. The growth medium, Potato Dextrose Agar (PDA) was also sourced from Bristol Scientific, Lagos, Nigeria.

3.2.2 Preparation of media

3.2.2.1 *Potato dextrose agar*

Thirty-nine (39) gram of PDA was suspended in 1000 ml distilled water and homogenized using magnetic stirrer to dissolve the powder completely. The medium was sterilized by autoclaving at 121^{o}C for 15 minutes.

3.2.2.2 *Potato dextrose broth*

Three hundred gram (300 g) scrubbed, peelded and diced Irish potatoes were boiled in 900 ml water for 1hour. The composition was made to pass through a fine sieve and sterilized in autoclave for 15 min at 121^{o}C. Two hundred and thirty (230) ml potato filtrate was mixed with 20 g dextrose. The composition were made up to 1 litre with distilled water and homogenized until dissolved completely. The media was then re-sterilized at 121 ^{o}C for 15 mins (Adebola and Amadi, 2012)

3.3 Isolation of Fungal Strains and Determination of Percentage Frequency of Occurrence

Fungal strains were isolated from stored rice seeds using agar plate methods and identified using fungal family of the world mycological monograph (Al-Hetar *et al*., 2010; Iwuagwu *et al.,* 2018). The percentage frequency of occurrence was calculated using the formular $\% = \frac{F}{N} \times 100$, Where F is total number of times the fungal is isolated across samples and N is total number of all the fungi isolated

3.3.1. Agar plated method

Fourty (40) rice seeds of each sample were surface sterilized in 15 ml of 1 % Sodium hypochlorite and 10 seeds were inoculated on PDA in 4 different Petri dishes. Two plates were incubated in a light room, while the other 2 plates were placed in a dark room at 28±2 ^{o}C for 7 days (Madushani *et al.,* 2012).

3.3.2 Identification of the seed-borne fungi

Fungal isolates were subcultured to obtain pure cultures of the isolates. The isolates were identified on the basis of their morphological and cultural characteristics (Zahid *et al.,* 2015; Iwuagwu *et al.,* 2018). The identified cultures of the isolates were preserved in PDA slants for further use. The selected potential seed-borne fungi mycotoxin secreting isolates from all the rice samples collected were used for further study.

3.3.3 Pathogenicity test

Pathogenicity test was carried out according to the method described by Ali *et al.* (2012) and Zahid (2014) using healthy rice collected from National Cereal Research Institute (NCRI) Baddegi rice seed banks. Isolated fungal pathogens were taken using

cork borer of 4mm disc cultured into 100ml potato dextrose broth (PDB) and incubated at 28±2°C for 7 days on a rotary shaker at 150 rpm. Mycelia were harvested by filtering the broth through four layers of cheesecloth. Sterile water was used to adjust the conidial concentration to 1×10^6 conidia ml^{-1} using a haemocytometer. Already sterilized rice seeds (FARO 52) collected from NCRI Seed bank were mixed with 20 ml of conidial suspension using a mixer, air dried on a tray in the laboratory and Symptom developments such as discolouration and viability were observed regularly after 14 days of inoculation (Maqbool *et al*., 2012).

3.4 Extraction of Chitosan from Crab Shell

Two-hundred-gram (200 g) of crab (*Callinectes amnicola*) shells waste were washed and dried in hot air oven at 60 °C for 24 hrs. Dried shell waste were packed in polyethylene bag and stored at -4 °C. Dried shells were pulverized manually using mortar and pestle. The modified extraction procedure of Gaikwad *et al*. (2015) was followed which included basic steps of deproteinization, demineralization, decolouration and deacetylation.

Pulverized shells were deproteinized by treating with 3.5 % (w/w) NaOH solution for 2 hrs at 65 °C with constant stirring at a solid to solvent ratio of 1:10 (w/v), demineralized with 1N HCL for 30 min at ambient temperature in a solid to solvent ratio of 1:15 (w/v) for 15 min and decolourized with acetone for 10 min and dried for 2 hrs under hood, followed by bleaching with 0.32 % (v/v) solution of sodium hypochloride (containing 5.25 % available chlorine). After each step, the chitin was filtered, washed with distilled water to neutral pH. Chitin deacetylation was carried out at 15 psi/121 °C using 50 % sodium hydroxide (NaOH) solution for 15 min. The

samples were filtered off using Whatman No. 1 filter paper, washed with distilled water to neutral pH and dried in an oven at 60 ^{o}C for 24 hrs.

3.4.1 Determination of the degree of deacetylation

To determine the degree of deacetylation of the synthesised chitosan, Fourier transform infrared (FTIR) analysis was done. Chitosan solution was prepared in potassium bromide (KBr) as a pellet under 1:99 ratio of chitosan sample to KBr. The sample mixture was then subjected to the infrared (IR) radiation spectroscopy machine (Model-ABB FTLA 2000-100 Quebec, Canada) at a resolution limit of 16 cm^{-1} (Sneha *et al.,* 2014). The degree of deacetylation of the chitosan was determined from IR results based on the ratio between peak areas at wavelength 1655 cm^{-1} and 3450 cm^{-1}

$$DDA = 100 - \frac{A_{1655`}}{A_{3450}} \times 1.15 \quad (3.1)$$

$$But,\ A_{1655} = -\log\left(\frac{Transmittance}{100}\right) \quad (3.2)$$

$$A_{3450} = -\log\left(\frac{Transmittance}{100}\right) \quad (3.3)$$

3.4.2 Preparation of chitosan solution

Four types of chitosan were used for the preparation of the solution, i.e., High molecular weight chitosan (HMWC), Medium molecular weight chitosan (MMWC), low molecular weight chitosan (LMWC), and Chitosan Synthesize from Crab Shell (CSCS).

Chitosan solutions were prepared by weighing 0.5, 1.0, 1.5, and 2.0g of all the four chitosans and dissolved in 100 ml sterile water containing 0.5 ml (v/v) glacial acetic acid. The mixture were dissolved using an overhead stirrer. The pH of the solution was

adjusted to 5.6 by adding either 1N NaOH or 1N HCl depending on the pH reading, using a digital pH meter (Madushani *et al.*, 2012; Zahid *et al.*, 2015).

3.5 *In vitro* Antifungal Assay of Chitosan against the Selected Fungal Isolates

The following solutions were made:

T1: 0.0 % chitosan (Control. Distilled water and acetic acid only) Negative control

T2: 0.5 % chitosan

T3: 1.0 % chitosan

T4: 1.5 % chitosan

T5: 2.0 % chitosan

3.5.1 Inhibition of radial mycelial length of fungal isolates

The *in vitro* antifungal activities of the three purchased conventional chitosan (HMWC, MMWC and LMWC) and chitosan synthesize from Crab Shell (CSCS) were determined using food poisoning method. A disc (6 mm diameter) each was taken from the pure cultures of the selected fungal isolates (*M. oryzae*, *A. flavus, A. fumigatus* and *F. moniliforme*) using 6mm diameter cork borer and inoculated at the centre of each petri dish containing PDA and chitosan solutions at 0.5, 1.0, 1.5 and 2.0 %. Petri dish containing PDA with sterile distilled water and glacial acetic acid was used as controls. The plates were in triplicates and incubated at laboratory temperature (28±2 °C). Daily mycelial radial length was measured for 5 days (Zahid, 2014). The percentage inhibition of mycelial radial length was calculated using the formula described by Al-Hetar *et al*. (2010).

$$\%\text{Inhibition} = \frac{R1-R2}{R1} \times 100 \qquad (3.4)$$

Where R1 = mycelial growth in control plates, R2 = mycelial growth in treated plates

3.5.2. Determination of dry weight of fungal mycelium in chitosan medium

Potato Dextrose Broth (PDB) and chitosan solutions were separately autoclaved at 121 ^{o}C for 15min and 100 ml of chitosan (0.5, 1.0, 1.5 and 2.0 % treatment) was separately added to the PDB (100 ml) in 250 ml Erlenmeyer flasks. Three mycelial discs (5 mm) from a 7-day-old culture of the selected fungal isolates were added to each flask and incubated at laboratory temperature (28±2 °C) for 14 days. Pre-weighed Whatman no. 1 filter paper was used to filter mycelium and dried in an oven at 60 °C for 24 h, and then weighed. The dry weight of mycelium was calculated by modified method of Al-Hetar *et al*. (2010)

$$\%\text{Dry weight of mycelium} = \frac{\text{Biomas of control sample} - \text{Biomass of treated sample}}{\text{Biomass of control sample}} \qquad (3.5)$$

3.5.3 Synthesis of chitosan nanoparticles

Chitosan nanoparticle was synthesized using ionic gelation methods (Kashyap *et al.,* 2015). Different molecular weight Chitosan (LMWC, MMWC and HMWC) solutions (0.1M) were prepared by dissolving 16 g of chitosan in 1000 ml of 2 % glacial acetic acid with stirring for 6hours at temperature of 60 ^{o}C. Thirty seven gram of Sodium Tripolyphospate was also dissolved in 1000 ml of double distilled water to prepare 0.1M solution. Chitosan nanoparticle of different degrees of cross-linking and diameters were prepared by adding the chitosans solution drop wise to Sodium Tripolyphospate solution in ratio (1:1, 1:3 and 1:5 v/v). The resultant chitosan nanoparticles were filtered, wash several times with double distilled water and oven dried at 60 ^{O}C. The chitosan nanoparticle formation was characterized using UV-Vis

Spectrophotomer (UV-visible19002 PC China) and the droplet size was measured using a dynamic light scattering (DLS) technique (Zahid *et al.*, 2015).

3.5.4 Characterisation of chitosan nanoparticles

3.5.4.1 *UV-Visible spectra*

Ultra Violet-visible spectra was recorded using a UV-visible1800 Spectrophotometer (manufactured in china) for the confirmation of nanoparticle formation (Zulfajiri *et al.,* 2020)

3.5.4.2 *Dynamic light scattering (DLS) measurements of chitosan nanoparticle*

Dynamic light scattering (DLS) was used for the measurement of average particle size, and polydispersity index (PDI) on a high-performance particle Zetasizer HPPS-5001 (Malvern, UK). Each sample was analyzed in triplicates at 25 ^{o}C at a scattering angle of 90 ^{o}C. Sterile water was used as a reference for dispersing medium. The results were given as the average particle size obtained from the analysis of three different batches in triplicate (Zulfajiri *et al.,* 2020)

3.6 Inhibition of Fungal Mycelial Radial Growth by Chitosan Nanoparticle (Cs NPs)

Two concentrations (0.25 mg/ml and 0.50 mg/ml) of chitosan Nanoparticle (Cs NPs) were prepared using all chitosan nanoparticles droplet sizes. *In vitro* antifungal activities of chitosan nanoparticles on mycelia radial growth inhibition were determined using food poisoning method. A disc of 6 mm diameter each was taken from the pure cultures of the selected fungal isolates and inoculated at the centre of each petri dish containing PDA and chitosan nanoparticle at 0.25 % and 0.50 %. Petri dish containing PDA with sterile water and glacial acetic acid was used as control.

All the Petri dishes were incubated at room temperature (28±2 °C). Daily mycelial radial length measurements were taken for 5 days. The percentage inhibition of mycelia radial length was calculated using the formula described by Al-Hetar *et al*. (2010).

$$\%\text{Inhibition} = \frac{R1-R2}{R1} \times 100 \quad (3.6)$$

Where R1 = mycelial growth in control plates, R2 = mycelial growth in treated plates

3.6.1. Determination of dry weight of fungal mycelium in chitosan nanoparticle

Potato Dextrose Broth (PDB) and chitosan nanoparticles were separately autoclaved at 121 °C for 15 min. Five (5) ml each of two different concentrations (0.25 mg/ml and 0.50 mg/ml) of chitosan nanoparticles was added to the PDB (50 ml) (ratio 1:5) in 250 ml Erlenmeyer flasks. Two mycelial discs (6 mm) from a 5days old culture was added to each flask and incubated at room temperature for 14 days. Pre-weighed Whatman No. 1 filter paper was used to filter mycelium and dried in an oven at 60 ºC for 24 h, and then weighed (Zahid *et al.*, 2015). The dry weight of mycelium was calculated by the following formula

$$\%\ \text{Dry Weight of Mycelium}\ \frac{Biomass\ of control\ sample - Biomass\ of\ treated\ sample}{Biomass\ of\ control\ sample} \quad (3.7)$$

3.7 Antifungal Activity of Chitosans on Storage of Rice Seeds

Two molecular weight chitosan HMWC and MMWC effects were determined on seed-borne mycotoxin producing mycoflora of rice after storage for 6 months.

Two kg each of the samples were dried in the sun and moisture content (MC) was taken at interval of 6 hours until the MC was 10%. One percent (1 %) each of the two chitosan was then used to spray the rice seeds, allow to dry to safe MC before

bagging and stored at room temperature. After six (6) month of storage, the samples were then re-subjected to the same screening for fungal contamination using Agar plate method earlier described under section 3.2.1. The fungal incidence and percentage frequency of occurence of fungal isolate were analysed and compared to the original samples collected from farmers (Butt *et al.,* 2011).

3.8 Chitosan Efficacy against Rice Blast Pathogen Fungi (*Magnaporthe oryzae*) on the Field.

3.8.1 Study area

The study was conducted at hydromorphic field (latitude N9º.04'02.05 and longitudes E6º.01'30.31) of the National Cereals Research Institute, Badeggi, Niger State, Nigeria in year 2021 cropping season.

3.8.2 Experimental design

Factorial experiment (Different chitosan and Concentrations) was set out in a Randomized Complete Block Design (RCBD), with three replicates using chitosan with four different molecular weight at four different concentrations (0.5, 1.0, 1.5 and 2.0 %). Two rice varieties were used for the experiment (Table 3.2). The same plant population was maintained throughout the plot, with the spacing of 20cm by 20cm between rows and between plants. Scoring of the disease incidence was done four times using International Rice Research Institute (IRRI) standard evaluation system (SES) at the appearance of symptoms, 42, 63 and 90 days after transplanting (DAT) to monitor the progress of the disease and efficacy of the chitosans (Quazi *et al.,* 2021).

3.8.3 Parameters determined

The inoculum of pathegen ($\times 10^6$/L) was used to spray the rice field at 14days after plant transplanting. After 7days of the introduction of the inoculum, the rice field was examined for blast symptoms. Five plants role from each plot was used for data collection after symptoms appearance. The plant were then treated at the appearance of symptoms with all the different molecular weights of the chitosan solution applied as spray until run off. Development of foliar symptoms with blast necrotic lesions after the treatment were assessed at intervals of 21 days for three months. Disease incidence was expressed as the percentage of leaves showing blast necrotic symptoms out of the total number of inoculated plants in each treatment and the following data were collected from the experiment;

- ✓ Disease incidence at the appearance of symptoms (21days);
- ✓ Disease incidence after the application of chitosan at 42 days after transplanting
- ✓ Disease incidence after the application of chitosan at 63 days after transplanting
- ✓ Disease incidence after the application of chitosan at 90 days after transplanting

Fertilizer application was at 80, 40, 40kg per Ha of N, P_2O_5 and K_2O, with N applied in two splits at 21 and 42 days after planting. Weeds were controlled using propanol and 2-4-D formulation at 4 litres per hectare of Orizo plus with supplementary hand weeding.

Table 3.2: Experimental Plot design (Randomized Complete Block Design (RCBD)

REPLICATION NO. 1		REPLICATION NO. 2		REPLICATION NO. 3	
PLOT NO.	TREATMENT ID	PLOT NO.	TREATMENT ID	PLOT NO.	TREATMENT ID
1	VR1 DC4 MW4	1	VR1 DC3 MW1	1	VR2 DC4 MW1
2	VR1 DC1 MW4	2	VR2 DC2 MW1	2	VR1 DC1 MW3
3	VR1 DC4 MW3	3	VR1 DC1 MW4	3	VR1 DC1 MW4
4	VR1 DC4 MW1	4	VR2 DC3 MW4	4	VR1 DC3 MW1
5	VR2 DC1 MW4	5	VR2 DC1 MW1	5	VR2 DC1 MW1
6	VR1 DC2 MW3	6	VR1 DC4 MW4	6	VR1 DC4 MW2
7	VR1 DC2 MW4	7	VR1 DC1 MW2	7	VR2 DC4 MW3
8	VR2 DC2 MW4	8	VR2 DC4 MW3	8	VR2 DC2 MW2
9	VR2 DC1 MW2	9	VR1 DC4 MW2	9	VR1 DC1 MW1
10	VR1 DC1 MW1	10	VR1 DC2 MW4	10	VR2 DC3 MW3
11	VR2 DC1 MW1	11	VR2 DC1 MW4	11	VR2 DC1 MW4
12	VR1 DC3 MW4	12	VR1 DC1 MW1	12	VR1 DC2 MW2
13	VR1 DC4 MW2	13	VR1 DC2 MW1	13	VR2 DC1 MW2
14	VR1 DC3 MW3	14	VR2 DC2 MW2	14	VR1 DC2 MW3
15	VR2 DC2 MW1	15	VR2 DC4 MW4	15	VR1 DC1 MW2
16	VR1 DC2 MW2	16	VR1 DC3 MW4	16	VR2 DC1 MW3
17	VR1 DC2 MW1	17	VR2 DC3 MW1	17	VR1 DC4 MW3
18	VR2 DC3 MW4	18	VR2 DC2 MW4	18	VR2 DC3 MW1
19	VR2 DC1 MW3	19	VR2 DC2 MW3	19	VR1 DC3 MW3
20	VR1 DC1 MW2	20	VR1 DC4 MW1	20	VR2 DC2 MW1
21	VR2 DC4 MW4	21	VR1 DC3 MW3	21	VR1 DC2 MW4
22	VR2 DC3 MW2	22	VR2 DC1 MW3	22	VR2 DC2 MW4
23	VR1 DC1 MW3	23	VR1 DC2 MW2	23	VR2 DC4 MW4
24	VR2 DC4 MW2	24	VR2 DC4 MW1	24	VR1 DC3 MW4
25	VR2 DC3 MW3	25	VR2 DC3 MW2	25	VR2 DC3 MW4
26	VR1 DC3 MW1	26	VR1 DC1 MW3	26	VR1 DC4 MW1
27	VR2 DC4 MW3	27	VR1 DC3 MW2	27	VR1 DC2 MW1
28	VR2 DC2 MW3	28	VR1 DC4 MW3	28	VR1 DC4 MW4
29	VR1 DC3 MW2	29	VR1 DC2 MW3	29	VR2 DC4 MW2
30	VR2 DC4 MW1	30	VR2 DC1 MW2	30	VR1 DC3 MW2
31	VR2 DC3 MW1	31	VR2 DC3 MW3	31	VR2 DC2 MW3
32	VR2 DC2 MW2	32	VR2 DC4 MW2	32	VR2 DC3 MW2

Replications = 3; Treatments = 2 X 4 X 4. FACTOR(S): VarietieS (VR) = 2 levels Variety (1) = VR1 (FARO 52). Variety (2) = VR2 (FARO 66), Molecular Weight (MW) = 4 levels: MW1, MW2, MW3, MW4. Concentration (C) = 4 levels: C1 (0.5%), C2 (1.0%), C3 (1.5%), C4 (2.0%)

3.8.4 Determination of the Lowest Inhibitory Concentration Dose at 50 % (LCD_{50}) of Chitosan

The LCD_{50} was determined using the food poisoning method earlier describe. The concentration of the chitosan solution at which the growth of the selected fungi were inhibited by 50 % was expressed as the LCD_{50}

3.9 Data Analysis

The data collected were subjected to Two-way Analysis of Variance (ANOVA) using Statistical Tools for Agricultural Research (STAR) and mean separation using LSD at 5 % (0.05) level of probability. Graphs were plotted using 2013 version of Microsoft excel and significant difference were determined using error bar.

CHAPTER FOUR

4.0 RESULTS

4.1. Occurrence of Fungi in Stored Samples of Rice

During sample collection, it was observed that most rice farmers in Niger State cultivates improved released rice varieties such as are Faro 44, Faro 52 and Faro 61 while some farmers are cultivating their local accessions such as Alhaji Baba, Kuddo, Allhaji Sule, Sakpefu, Ekangi, Rabach and Dgwagwa (Table 1a &1b).Therefore, all the samples were grouped into either improved release varieties or local acccession.

The results of the mean fungal colony count in improved varieties of rice samples (Table 1a), showed that fungal incidence was high in rice seed samples R6, R25, R26, R27, R29 and R30 with mean fungal incidence of 8.00±0.71, 6.75±1.38, 8.75±0.75, 7.25±0.75. 7.00±0.91, 7.75±0.48, respectively which were highly significant from other samples, while samples R14, R15, R19 and R21) have little or no fungal.

The results of the mean fungal incidence in local accession of rice samples (Table 1b) showed that fungal imcidence was high in almost all the samples collected with highest mean incidence in accessions R36, R37, R44, R46 and R47 with highest mean incidence of 9.50±0.50, 9.50±0.50, 9.50±0.50, 10.00±0.00 and 10.00±0.00 respectively in all samples collected which were significantly ($P<0.05$) higher than other samples. It was observed that local accession were more susceptible to fungal infection than the improved varieties of rice samples collected from Niger State.

Table 4.1a: Occurrence of Fungi in Stored Rice Varieties samples

Rice Tags	Abbreviated name of Varieties	Fungal Incidence(cfu)
Rice1	F44	3.00 ± 0.00^{ef}
Rice2	F44	6.75 ± 0.95^{jk}
Rice3	F44	6.00 ± 0.58^{j}
Rice4	F44	5.00 ± 0.71^{jk}
Rice5	F52	5.50 ± 1.85^{jk}
Rice6	F61	8.00 ± 0.71^{mn}
Rice7	F44	4.25 ± 1.11^{ij}
Rice8	F44	4.75 ± 1.11^{ij}
Rice9	F61	4.00 ± 0.41^{i}
Rice10	F52	2.25 ± 0.48^{de}
Rice11	F52	4.50 ± 1.84^{ij}
Rice12	F52	4.00 ± 1.65^{ij}
Rice13	F44	3.75 ± 1.65^{gh}
Rice14	F44	0.75 ± 0.75^{abc}
Rice15	F52	0.50 ± 0.28^{ab}
Rice16	F52	3.00 ± 0.91^{ef}
Rice17	F44	3.75 ± 1.55^{gh}
Rice18	F44	1.40 ± 0.93^{cd}
Rice19	F52	0.00 ± 0.00^{a}
Rice20	F52	3.25 ± 1.97^{de}
Rice21	F52	0.50 ± 0.50^{ab}
Rice22	F44	7.25 ± 1.38^{kl}
Rice23	F52	7.50 ± 0.65^{kl}
Rice24	F52	5.00 ± 0.41^{jk}
Rice25	F44	6.75 ± 1.38^{jk}
Rice26	F44	8.75 ± 0.75^{mn}
Rice27	F52	7.25 ± 0.75^{kl}
Rice28	F44	4.25 ± 0.25^{ij}
Rice29	F44	7.00 ± 0.91^{kl}
Rice30	F52	7.75 ± 0.48^{kl}

Means having common letter within the same column are not significantly different at the 5% level of significance, F44-Faro44, F52-Faro 52, F61-Faro 61

Table 4.1b: Occurrence of Fungi in Stored Local accessions Rice Samples

Rice Tags	Abbreviated name of Accessions	Fungal Incidence (cfu)
Rice31	DG	8.00 ± 2.00^{mn}
Rice32	KD	8.75 ± 0.75^{mn}
Rice33	KD	9.25 ± 0.48^{n}
Rice34	F44	9.00 ± 1.47^{mn}
Rice35	F52	9.25 ± 0.48^{n}
Rice36	KD	9.50 ± 0.50^{n}
Rice37	AB	9.50 ± 0.50^{n}
Rice38	KD	9.00 ± 0.71^{mn}
Rice39	RB	7.50 ± 0.96^{kl}
Rice40	AB	8.75 ± 0.63^{mn}
Rice41	F52	8.00 ± 1.15^{mn}
Rice42	SK	7.25 ± 0.85^{kl}
Rice43	KD	7.25 ± 1.60^{kl}
Rice44	F52	9.50 ± 0.50^{n}
Rice45	KD	9.00 ± 1.00^{mn}
Rice46	AS	10.00 ± 0.00^{n}
Rice47	AB	10.00 ± 0.00^{n}
Rice48	KD	4.25 ± 0.95^{ij}
Rice49	EK	8.75 ± 0.95^{mn}
Rice50	KD	8.00 ± 0.91^{mn}

Means having common letter within the same column are not significantly different at the 5% level of significance

DG-Dgwagwa, KD- Kuddo, AB- Alhaji Baba, RB-Rabach, SK-Sakpefu, AS-Alhaji Sule, EK-Ekangi, F52- Faro 52

The fungal isolates have different types of colour ranging from yellow, light green, dark green, black, white, and pink with different texture ranging from velvety, powdery and floppy on potato dextrose agar plates. Most of the isolates have septate hyphae with branched conidiophores bearing conidia with conidiospores except for a few with aseptate hyphae bearing unbranched sporangiospore arising from the rhizoid. From the morphological and microscopic identification of the isolated fungi, eight species of fungi belonging to five genera were isolated and identified from all the samples collected from farmers in Niger State Nigeria. The fungal pathogens population varies from one rice variety to another, as well as the frequency of occurrence. The pathogens isolated include: *Fusarium moniliforme, Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus, Cladosporium cladosporoides, Rhizopus stolonifer, Paeciliomyces lilacinus* and *Rhizoctonia solani*

Overall percentage frequency of occurrence (Figure 4.1) shows that *Fusarium moniliforme* has the highest prevalence of 38.5 % followed by *Rhizopus stolonifer* (37 %) and were significantly different from other isolates. *Aspergillus niger, Aspergillus flavus* and *Cladosporium cladosporoides* were also high with 6.5 %, 6.2 % and 4.0 %, respectively, however *Aspergillus fumigatus* and *Rhizoctonia solani* have the lowest percentage.

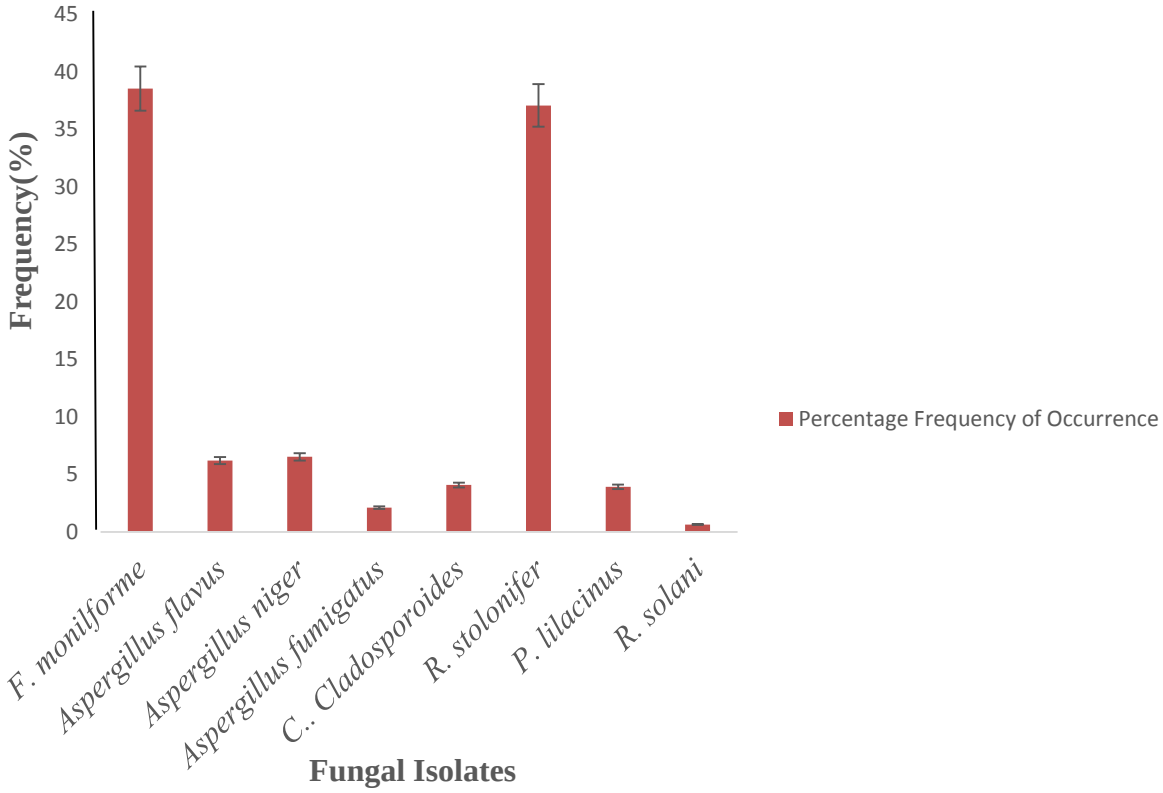

Figure 4.1: Percentage Frequency of Occurrence of Fungal Isolates in Stored Rice Samples

4.1.1 Pathogenicity of fungal pathogen isolated from stored rice samples

The characteristics symptoms of the pathogenicity result confirmed that isolated fungal pathogens were the causative organism of grain discolouration and reduction in germinability of the rice seeds. The results illustrated in the Plate I (A&B) confirmed that the isolated fungi has characteristic pathogenic effect on rice showing the maximum symptoms *invitro*.

A: rice seeds before fungal inoculation

B: Rice seed after fungal inoculation

Plate I: Pathogenicity Test Results

4.1.2 Chemical structure and bonding pattern of chitosan synthesized from crab shells (CSCS)

The Fourier Transfrom Infra Red (FTIR) spectrum of the Chitosan showed major absorption bands ranges from 3444.72, 2966.17, 2512.60, 2144.84, 1429.74, 1258.12, 1160.05, 1025.2, 869.92, 710.50, 608.40 to 559.36 (Figure 4.2).

4.1.3 Degree of deacetylation of synthesised chitosan

The degree of Deacetylation was determined using a standard formular (Gaikwad *et al*., 2015).

DDA = $100\text{-}\ \frac{A_{1429}}{A_{3444}} \times 1.15$, where Area of peak of 1439.7=21.628T and Area of peak of 3444=34.188T

$A3444 = \frac{-\log T}{100} = -\frac{\log 21.628}{100} = 0.66498$

$A_{1439.5} \equiv \frac{-\log T}{100} = -\frac{\log 34.188}{100} = 0.8080$

$\therefore DDA \equiv 100 - \frac{0.8080}{0.66498} \times 1.15$ DDA= 98.6%. The degree of deactylation (DDA) of the chitosan synthesize from crab shells (CSCS) was 98.6 %.

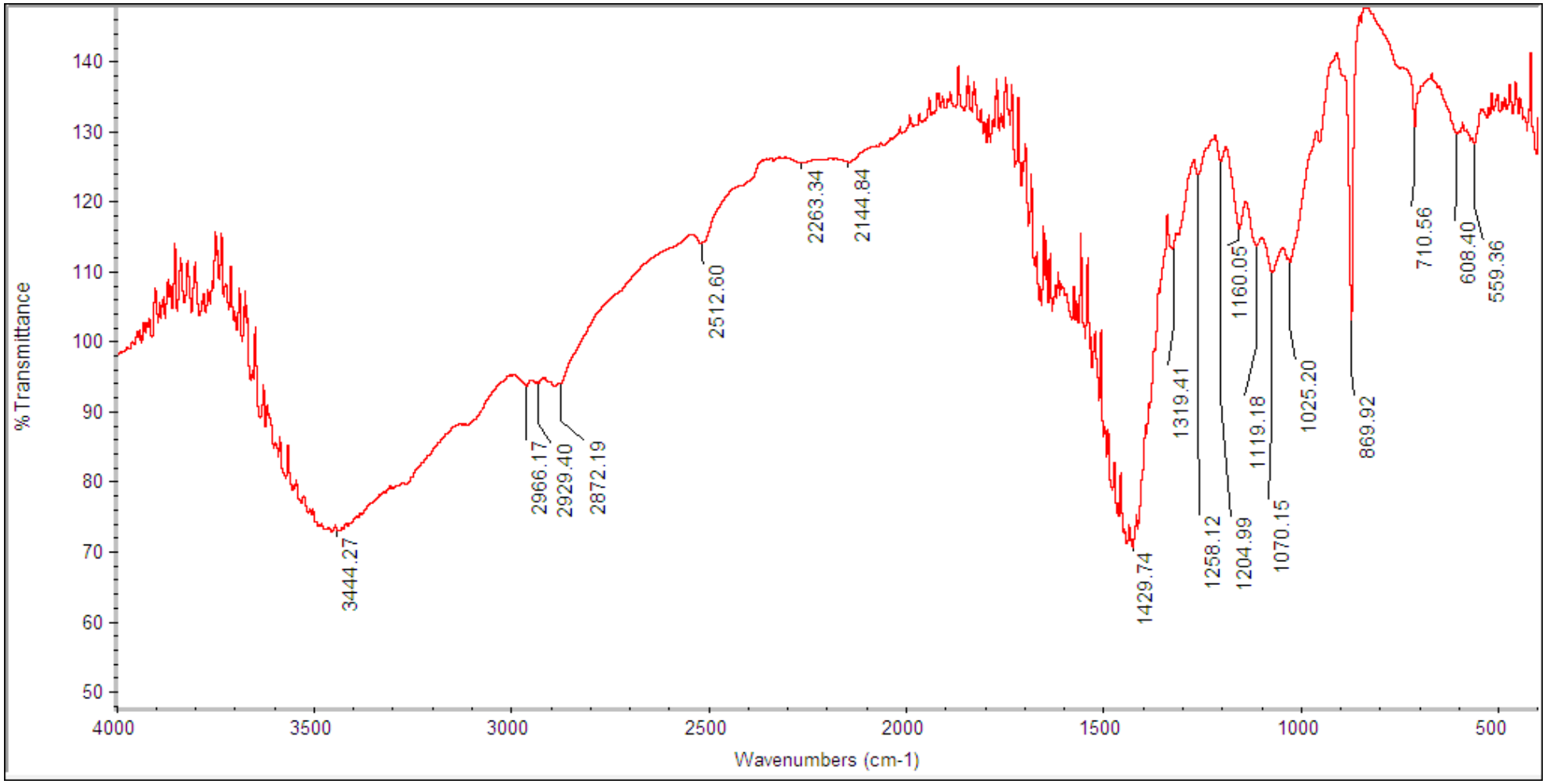

Figure 4.2: Fourier Transforms Infra Red (FTIR) spectra graph of Synthesized Chitosan for DDA determination

4.1.4 Mycelia radial growth in length of *Magnaporthe oryzae* (rice blast) in chitosan

The results of the *in vitro* mycelia radial growth in length of *M. oryzae* in chitosan treatments and control at the end of the 5th incubation day (Table 4.2) showed that there were significant difference ($P<0.05$) in growth of both the chitosan treatment and control. The lowest mycelia growth of 0.60±.00 was recorded in the highest concentration (2.0 %) of HMWC while the highest growth of 4.0±.05 was recorded in the lowest concentration (0.5%). However, the growth was significantly different ($P<0.05$) from growth 6.05±.54 observed in controlled. The lowest growth of 1.8±.00 was recorded in 1.5 % concentration of MMWC which was significantly different ($P<0.05$) from control. The lowest mycelia growth of 3.0±.00 and 3.5±.00 were recorded in 2.0 % concentration of LMWC and CSCS, respectively. However, they were significantly different ($P<0.05$) from the growth of 6.05±.54 in the control (0%)

Table 4.2: Mycelia Radial Growth (cm) of *M. oryaze* in Different Concentration of Chitosan at the End of Incubation Days

Concentration	HMWC	MMWC	LMWC	CSCS
0	6.05±.54^{c}	6.05±.54^{c}	6.05±.54^{c}	6.05±.54^{c}
0.5	4.0±.05^{bc}	4.5±.10^{bc}	4.5±.00^{bc}	4.5±.05^{bc}
1.0	3.2±.50^{b}	3.0±.50^{b}	4.0±.10^{bc}	4.5±.00^{bc}
1.5	2.4±.00^{b}	1.8±.00^{b}	3.5±.05^{b}	4.0±.00^{bc}
2.0	0.60±.00^{a}	2.0±.00^{b}	3.0±.00^{b}	3.5±.00^{b}

Means having common letter within the same column and across rows are not significantly different at the 5%level of significance

4.1.4.1 *Percentage chitosan inhibition of mycelia radial growth of M. oryzae*

The results of the percentage chitosan inhibition of mycelia radial growth of *M. oryzae* (Fig 4.3) showed that there was significant difference ($p<0.05$) in the percentage growth inhibition in all concentrations of HMW chitosan. The result of HMWC showed that 2.0 % concentration has the highest percentage inhibition of 90 % which was significantly different from other concentration. 1.5 % concentration of HMWC also inhibit the organism by 60 % which was significantly different ($p<0.05$) from the two lowest concentration.

Concentration of 1.5 % of MMWC inhibit *M. oryzae* by 70 %, being the highest, although not significantly different ($p>0.05$) from the 1.0 % and 2.0 % concentrations (50 % and 66 % Percentage inhibition) but was significantly different ($p<.005$) from the inhibition in 0.5 % concentration.

For LMWC, highest percentage inhibition of 50 % was recorded in 2.0 % concentration of the chitosan which was significantly different ($p<0.05$) from other lower concentrations at the end of 5^{th} day of incubation. Highest percentage inhibition of 41.6 % was recorded in 2.0 % concentration of Chitosan synthesised from Crab Shell (CSCS) which was significantly different ($p<0.05$) from other concentration inhibition. at the end of the 5^{th} day of incubation.

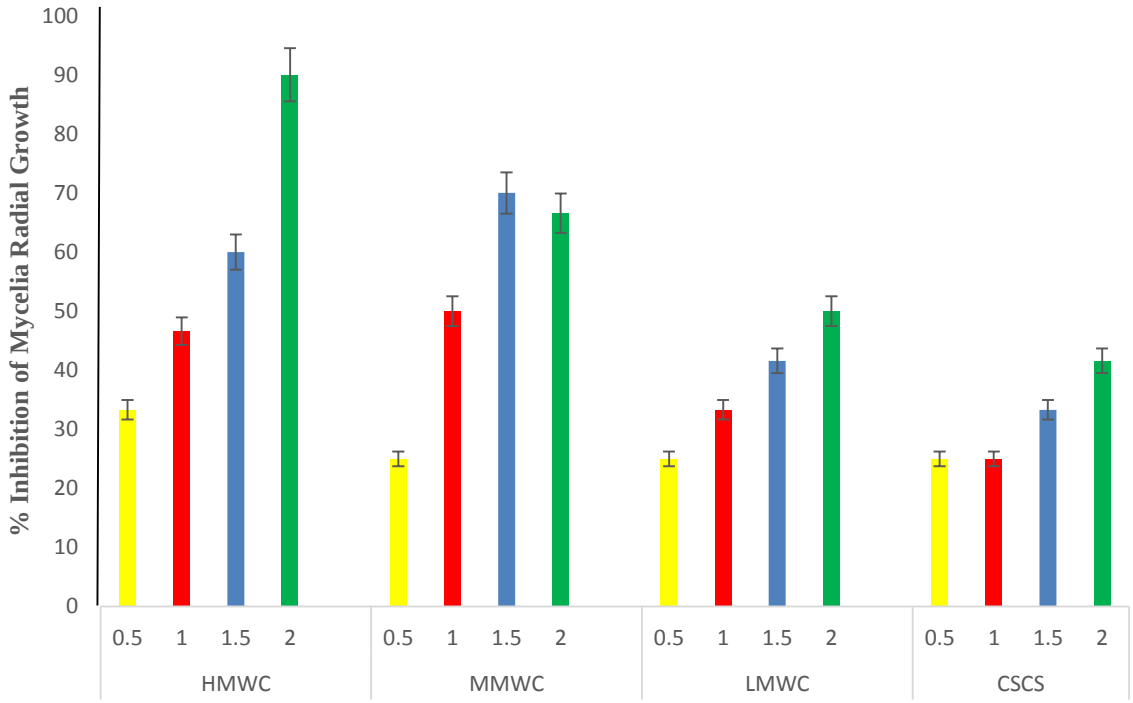

Figure 4.3: Percentage Inhibition of Mycelia Radial Growth in Length of *M. oryzae* in Different Concentrations of Chitosan

The growth mass of *Magnaporthe oryzae* in different concentrations of the four chitosans (HMWC, MMWC, LMWC and CSCS) (Plate II) showed that control plates has the highest growth mass compared to the treated plates. However, there was no difference in the growth mass of all plates treated with CSCS.

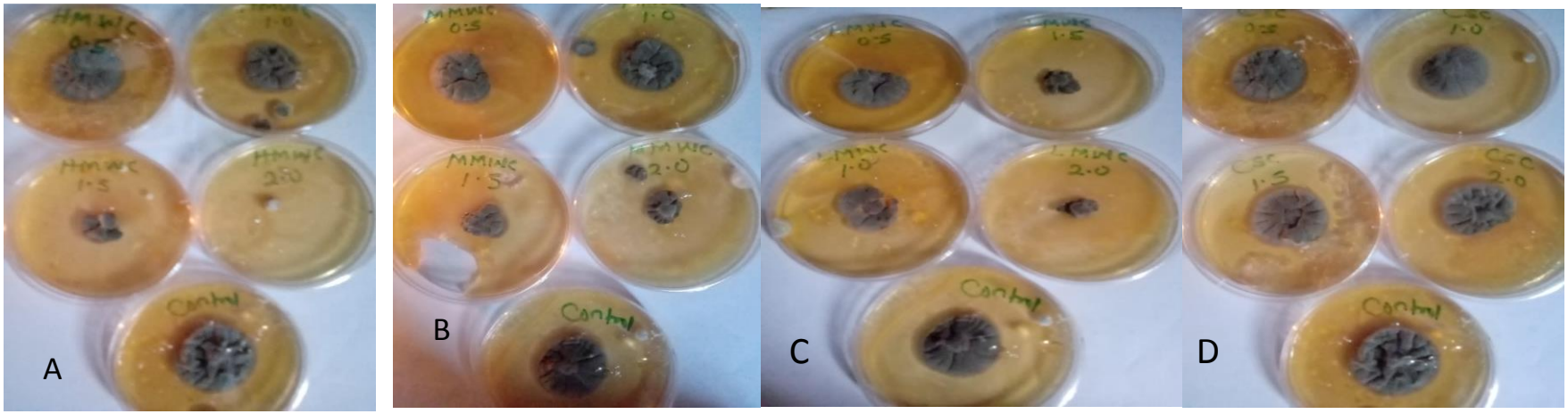

Plate II: Growth Inhibition of *Magnaporthe oryzae* in Different Molecular Weight (MW) Chitosan

A: High (MW) Chitosan B: Medium (MW) Chitosan C: Low (MW) Chitosan D: Chitosan synthesis from crab shell

4.1.5 Mycelia radial growth in length of rice mycotoxin producing fungi in chitosan

The results of the *in vitro* mean mycelia radial growth in length of selected mycotoxin producing fungi (*A. flavus, A. fumigatus and F. moniliforme*) in chitosan treatments and control (Table 4.3) showed that there were significant difference ($p<0.05$) in growth. For *A. flavus* in HMWC treatment, the highest mycelia radial growth in length of 8.00±.06 was recorded in control while the high mycelia radial growth of 1.80±1.04 was recorded in 0.5 % chitosan treatment (Table 4.3). However, there was no growth at 1.5 and 2.0 % treatment.

For MMWC, mycelia radial growth in length of *A. flavus* was recorded in all the treatment as well as control, although, there was significant difference ($p<0.05$). The highest growth of 8.00±00 was recorded in control which is significacntly different ($p<0.05$) from that of 0.5 % concentration treatment with mycelia growth of 5.20±.40 been the highest in treatments. The least growth of 2.10±.81 was recorded in 2.0 % concentration which is significantly different ($p<0.05$) from the lowest concentration treatment and the control.

For LMWC, mycelia radial growth in length of *A. flavus* was recorded in all the plates with highest growth of 8.00±.06 in control plate which was significantly different ($p<0.05$) from that of 0.5 % concentration with mycelia radial growth of 3.43±1.16 (Table 4.3). The least mycelia growth of 1.07±.71 was recorded in 2.0% concentration of the chitosan.

For CSCS, mycelia radial growth in length of *A. flavus* was also recoreded in all treatments as well as control, although there was significant difference ($p<0.05$) in the growth (Table 4.3). The highest growth of 8.00±.00 was recorded in control. The least

growth of 1.27±.57 was recorded in 2.0 % concentration which is significantly different ($p<0.05$) from both the control and the lowest concentration treatment.

For *A. fumigatus* in HMWC treatment, the highest mycelia radial growth in length of 5.00±.06 was recorded in control, while the high mycelia radial growth of 2.00_a±.00 was recorded in 2.0 % chitosan treatment (Table 4.3), there was no growth at 0.5 and 1.5% treatment.

For MMWC, mycelia radial growth of *A. fumigatus* was recorded in all the treatment except for 2.0 % concentration, although there was significant difference ($p<0.05$) in the growth (Table 4.3). The highest growth of 8.00±06 was recorded in control which is significacntly different ($p<0.05$) from that of 0.5 % concentration with mycelia radial growth of 4.57±.07 being the highest in all the concentrations.

For LMWC, mycelia radial growth of *A. fumigatus* was recorded in all the treatments with highest growth of 8.00±.06 in control which is significantly different ($p<0.05$) from that of 0.5% concentration with mycelia radial growth of 5.80±.12 (Table 4.3).

For CSCS, mycelia radial growth of *A. fumigatus* was also recorded in all treatment as well as control although, there was significant difference ($p<0.05$) in the growth (Table 4.3). The highest growth of 8.00±.06 was recorded in control plate while the highest growth of 6.00±.06 was recoreded in 0.5 % concentration. However, the least growth of 1.33±.17 was recorded in 2.0 % concentration which was significantly different ($p<0.05$) from both the control and the lowest concentration.

For *F. moniliforme* in HMWC treatment, the highest mycelia radial growth of 4.50±.06 was recorded in control. However, there was no growth in all treatment regardless of the concentration (Table 4.3).

For MMWC, the highest mycelia radial growth of *F. moniliforme* was recorded in control (6.50±.06), while the least growth of 1.67±.03 was recorded in 0.5 % treatment. There was no growth in other treatment (Table 4.3)

For LMWC, the highest myclia radial growth of *F. moniliforme* was recorded in control (4.50±.06) however; there was no growth in all the treatment irrespective of the concentration (Table 4.3)

For CSCS, the highest mycelia radial growth of *F. moniliforme* was recorded in control (6.50±.06), however, there was no growth in all the treatment (Table 4.3).

The results shows that the higher the concentration of the chitosan treatment, the lower the fungal mycelia radial growth. The result also shows that there was no significant different ($p>0.05$) in mycelia radial growth of all the selected fungal in 1.5 % and 2.0 % treatment concentration.

Table 4.3: Mycelial Radial Growth (cm) of Rice Mycotoxin Producing Fungi in Different Concentrations of Chitosan

Chitosan concn	Fungi Isolates											
	A Flavus				***A. A. fumigatus***				***F. moniliforme***			
	HMWC	MMWC	LMWC	CSCS	HMWC	MMWC	LMWC	CSCS	HMWC	MMWC	LMWC	CSCS
0	8.00_{c}± .06	8.00_{c}± .06	8.00_{c}± .06	8.00_{c}±.00	5.00_{b}±.06	8.00_{c}±.06	8.00_{e}± .06	8.00_{e}± .06	4.50_{b}±.06	6.50_{b}±.06	4.50_{b}± .06	4.50_{b}± .06
0.5	1.80_{a} ±1.0	5.20_{b} ±.40	3.43_{b}±1.16	4.53_{b}±.72	0.00_{a}±.00	4.60_{b}±.06	5.80_{b}± .12	6.00_{b}± .06	0.00_{a}±.00	1.37_{a}±.03	0.00_{a}± .00	0.00_{a}± .00
1	0.67_{a}± .67	4.53_{b} ±.29	1.73_{a}± .42	1.73_{a}±.15	1.83_{a}±.17	3.93_{b}±.07	4.70_{b}± .06	5.40_{b}± .06	0.00_{a}±.00	0.00_{a}±.00	3.17_{b}± .17	3.17_{b}± .17
1.5	0.00_{a} ±.00	4.90_{b} ±.10	1.50_{a}± .29	2.33_{a}±.35	0.00_{a}±.00	1.63_{a}±.03	4.83_{b}± .17	3.50_{b}± .20	0.00_{a}±.00	0.00_{a}±.00	0.00_{a}± .00	0.00_{a}± .00
2	0.00_{a} ± 00	2.10_{a} ±.81	1.07_{a}± .71	1.27_{a}±.57	2.00_{a}±.00	0.00_{a}±.00	5.23_{b}±.19	1.33_{a}± .17	0.00_{a}±.00	0.00_{a}±.00	0.00_{a}± .00	0.00_{a}± .00

Note: Values in the same row and subtable not sharing the same subscript are significantly different at p≤.05 in the two-sided test of equality for column means.

4.1.5.1 *Percentage chitosan inhibition of rice mycelia radial growth of mycotoxin-producing fungi*

The results of the percentage chitosan inhibition of mycelia radial growth of fungi (Figure 4.4a-4.4c) shows that there was no significant difference ($p>0.05$) in the percentage growth inhibition in concentration of 1.5 % and 2.0 % of all the chitosan concentration. Most of all the chitosan concentration inhibited above 50 % of the fungi mycelia growth except for 0.5 % concentration of MMWC (35 % PIRG), LMWC (38.9 % PIRG) and CSC (43.8 %) in *A. flavus* (Figure 4.4a) and MMWC (42.5 %), LMWC (30 %) and CSCS (26.7 %) in *A. fumigatus* (Figure 4.4b), while higher inhibition was observed in higher concentration except for MMWC which inhibit below 50 % average across all chitosan

All the chitosan concentration inhibits *Fusarium moniliforme* by 100 % except 0.5 % LMWC with lowest inhibition of 27.5 % and 0.5 %MMWC with moderate to high inhibition of 80.1 % (Figure 4.4c).

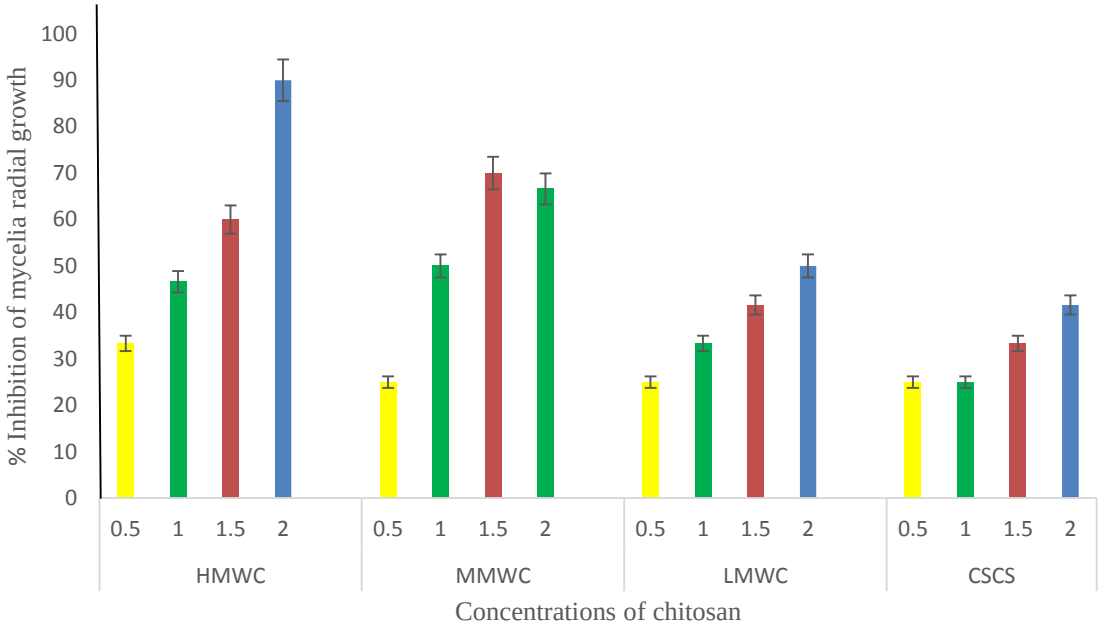

Figure 4.4a: Percentage Mycelia Radial Growth in Length of *A. flavus* in different concentrations of Chitosan

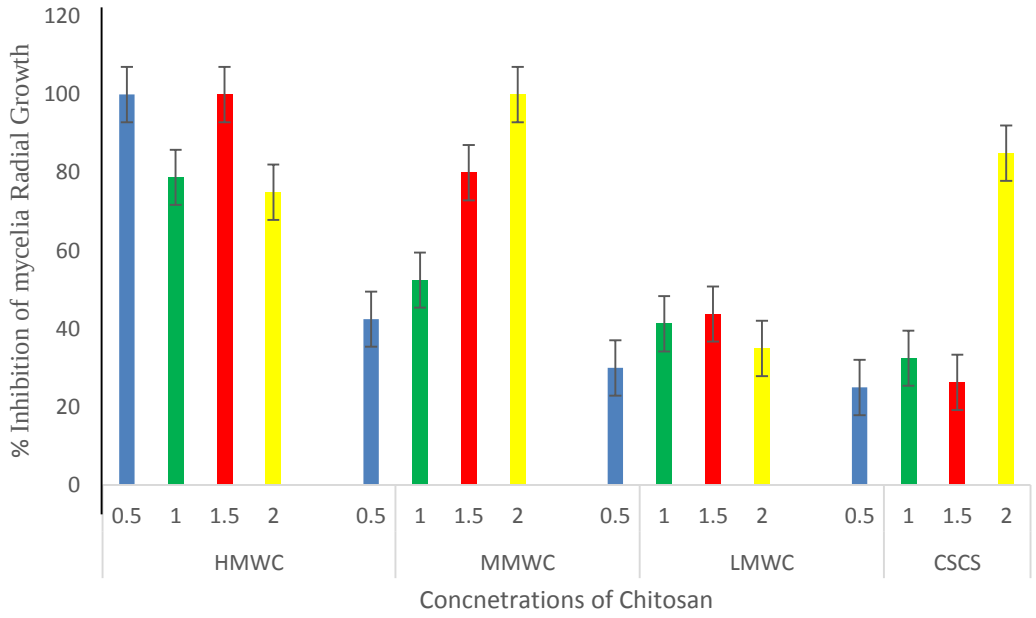

Figure 4.4b: Percentage Inhibition of Mycelia Radial Growth in Length of *A. fumigatus* in different concentrations of Chitosan

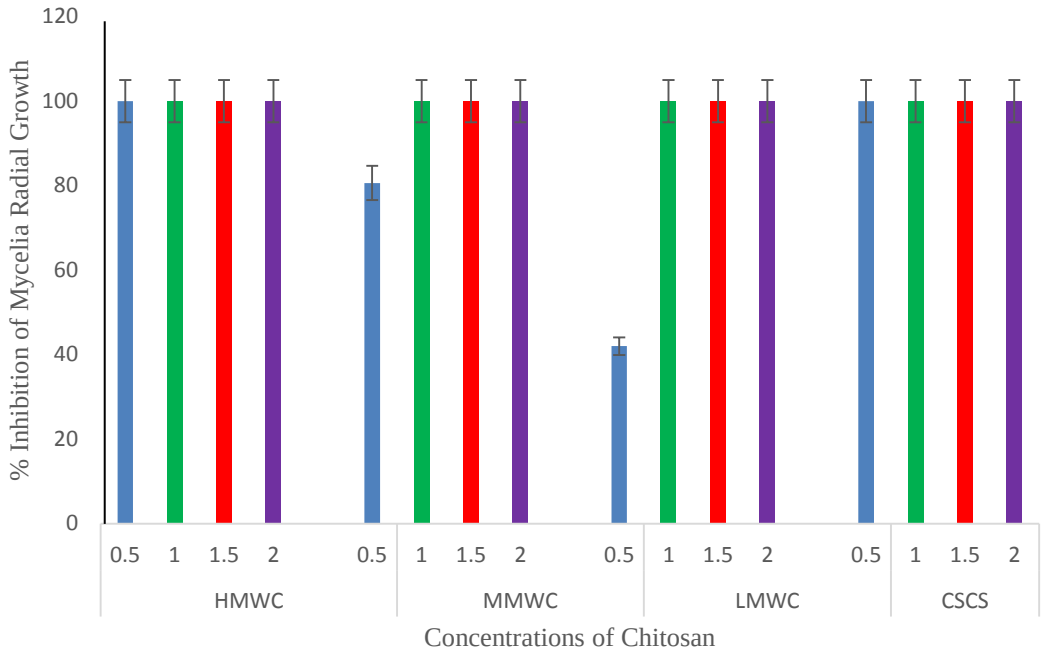

Figure 4.4c: Percentage Inhibition of Mycelia Radial Growth in Length of *F. moniliforme* in different concentrations of Chitosan

The growth mass of *A. flavus* in different concentrations of the four chitosans (HMWC, MMWC, LMWC and CSCS) (Plate III) showed that control has highest growth mass compared to the treated plates.

The growth mass of *A. fumigatus* in different concentration of the chitosans (HMWC, MMWC, LMWC and CSCS) (Plate IV) showed that the control plate has the highest growth mass. Growth decrease with increase in concentration of the chitosan in treated plates.The growth mass of *F. moniliforme* in the different concentration of the chitosans (HMWC, MMWC, LMWC and CSCS) (Plate V) showed that no growth was observed in almost all the treated plates except for 0.5 % of MMWC and LMWC respectively. The control plate has the highest growth mass.

Plate III: Mycelial Radial Growth Inhibition of *Aspergillus flavus* in Different Molecular Weight (MW) Chitosan

A: Highr (MW) Chitosan B: Medium (MW) Chitosan C: Low (MW) Chitosan D: Locally Produced Chitosan

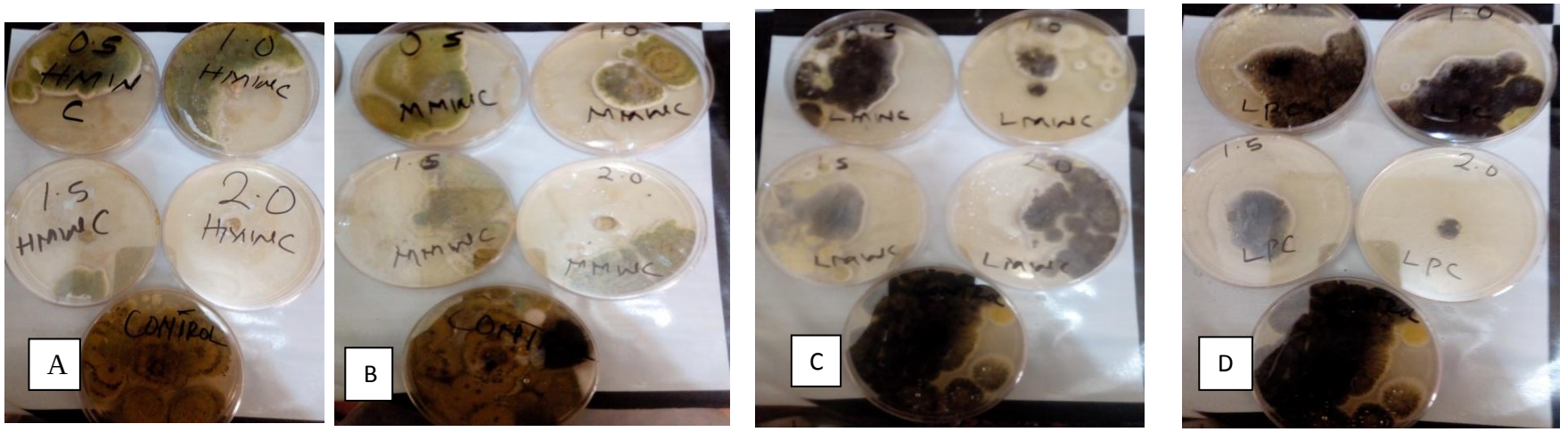

Plate IV: Mycelial Radial Growth Inhibition of *Aspergillus fumigatus* in Different Molecular Weight (MW) Chitosan

A: Highr (MW) Chitosan B: Medium (MW) Chitosan C: Low (MW) Chitosan D: Locally Produced Chitosan

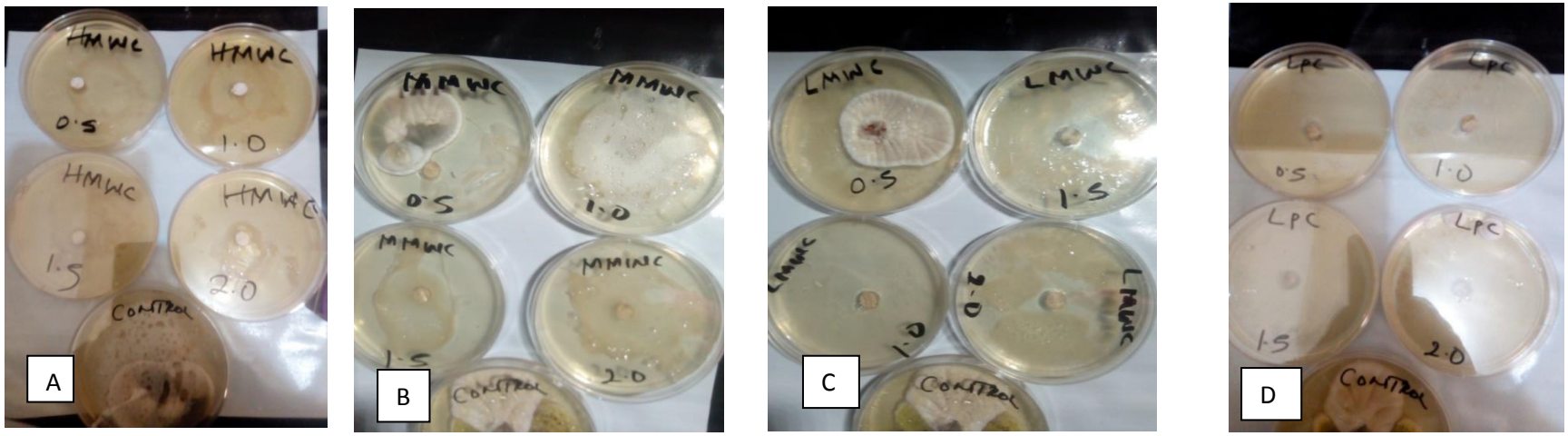

Plate V: Mycelial Radial Growth Inhibition of *Fusarium moniliforme* in Different Molecular Weight (MW) Chitosan

A: High (MW) Chitosan B: Medium (MW) Chitosan C: Low (MW) Chitosan D: Locally Produced Chitosan

4.1.5.2 *Percentage dry mycelia weight of rice mycotoxin producing fungi in chitosan treatments*

The result of the percentage dry mycelia weight of *A. flavus* in all the chitosan treatments (Figure 4.5a) showed that LMWC, MMWC and HMWC were not significantly different ($P>0.05$). However, CSCS 0.5 and CSCS 1.0 % showed lower percentage dry mycelial weight of 15.5 % and 17.0 %, respectively and were significantly ($P<0.05$) lower than other chitosan concentrations. The results showed that the higher the concentration of the chitosan treatment, the higher the percentage dry mycelia weight inhibition.

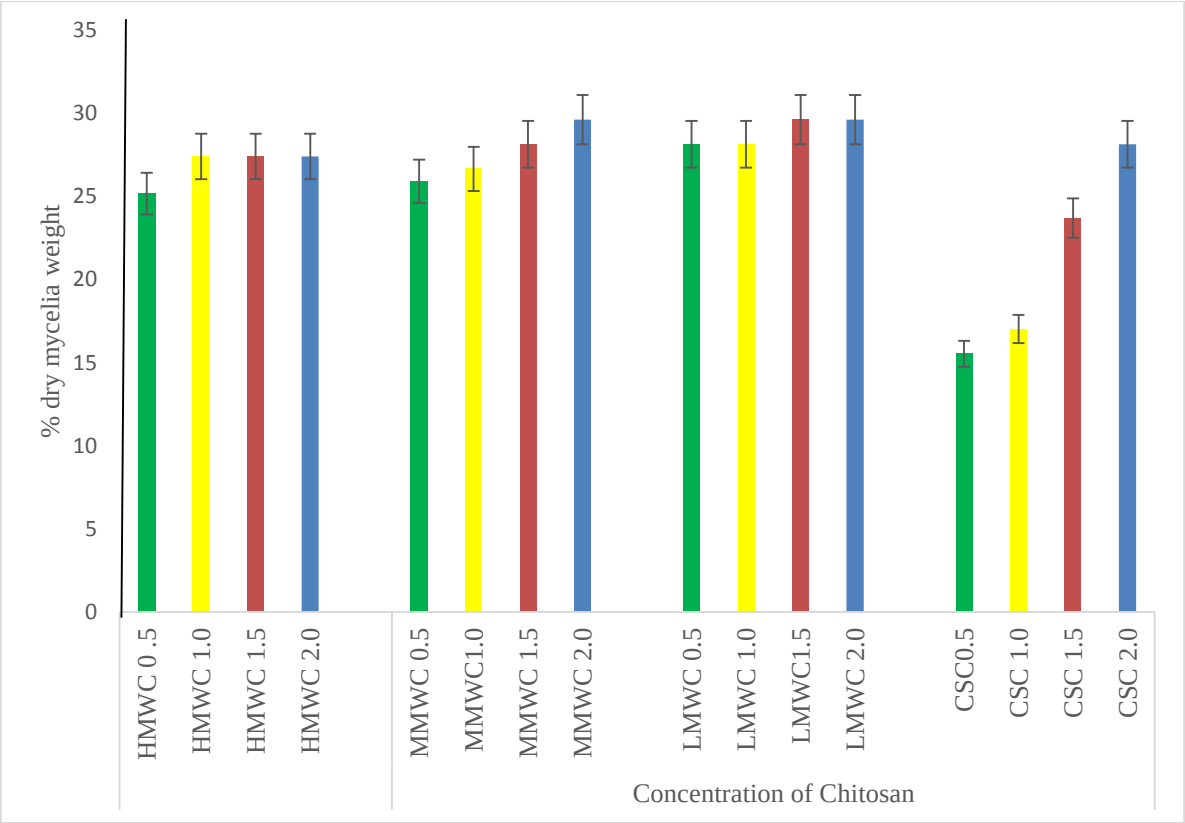

Figure 4.5a: Percentage Dry Mycelia Weight of *Aspergillus flavus* in Different Concentrations of the Chitosan.

The result of the percentage dry mycelia weight of *A. fumigatus* in all the different concentrations of chitosans (Fig 4.5b) shows that HMWC, MMWC and LMWC were not significantly different ($P>0.05$) in their percentage inhibition while CSCS 0.5 % concentration has the least percentage inhibition of 32.9% though not significantly different ($P>0.05$) from other chitosan concentration. There was no significant difference ($P>0.05$) in the percentage dry mycelia weight in 1.0, 1.5 and 2.0 % concentrations of all the chitosan.

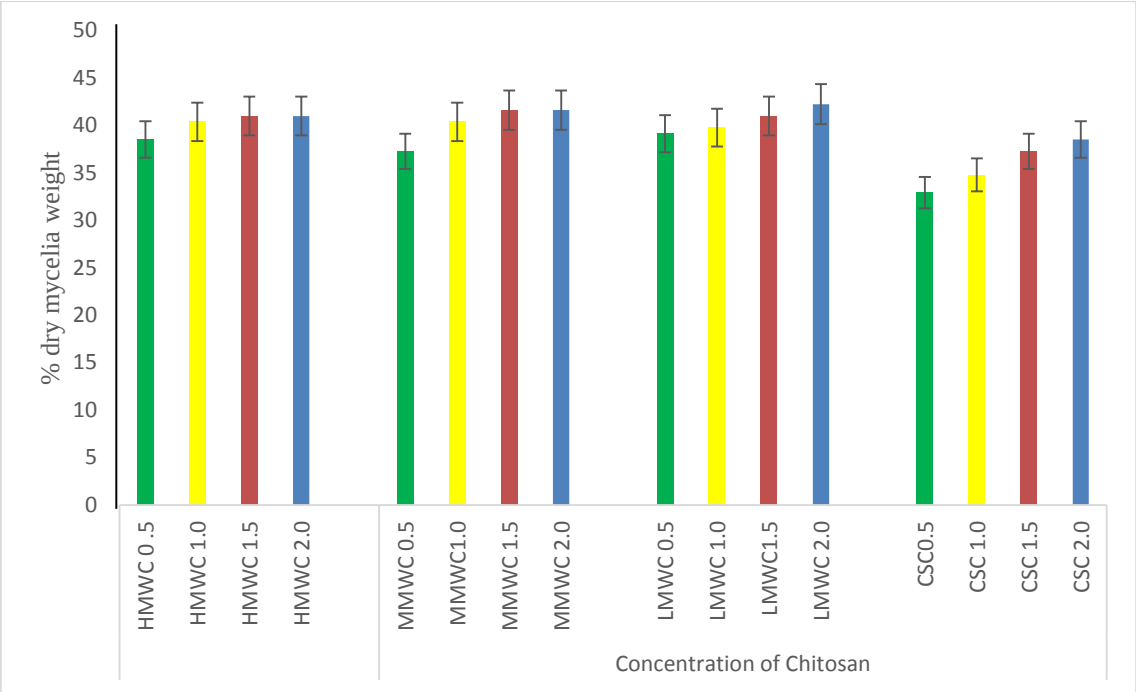

Figure 4.5b: Percentage Dry Mycelia Weight Inhibition of *Aspergillus fumigatus* in the Different Concentrations of the Chitosan

The result of the percentage dry mycelia weight of *F. moniliforme* (Figure 4.5c) in the different concentrations of chitosan shows that the higher the concentration of the chitosan, the higher the percentage inhibition. Although concentration of HMWC 0.5, 1.0 and 1.5 % records percentage inhibitions of 2.8 %, 8.9 % and 14.6 % which were significantly lower ($P<0.05$) compare to other chitosan concentration. MMWC 2.0 % has the highest percentage inhibition of 43.8 % followed by LMWC 2.0 (40.8 %) inhibition and CSC 2.0 with 38.5 % which were not all significantly different ($P>0.5$).

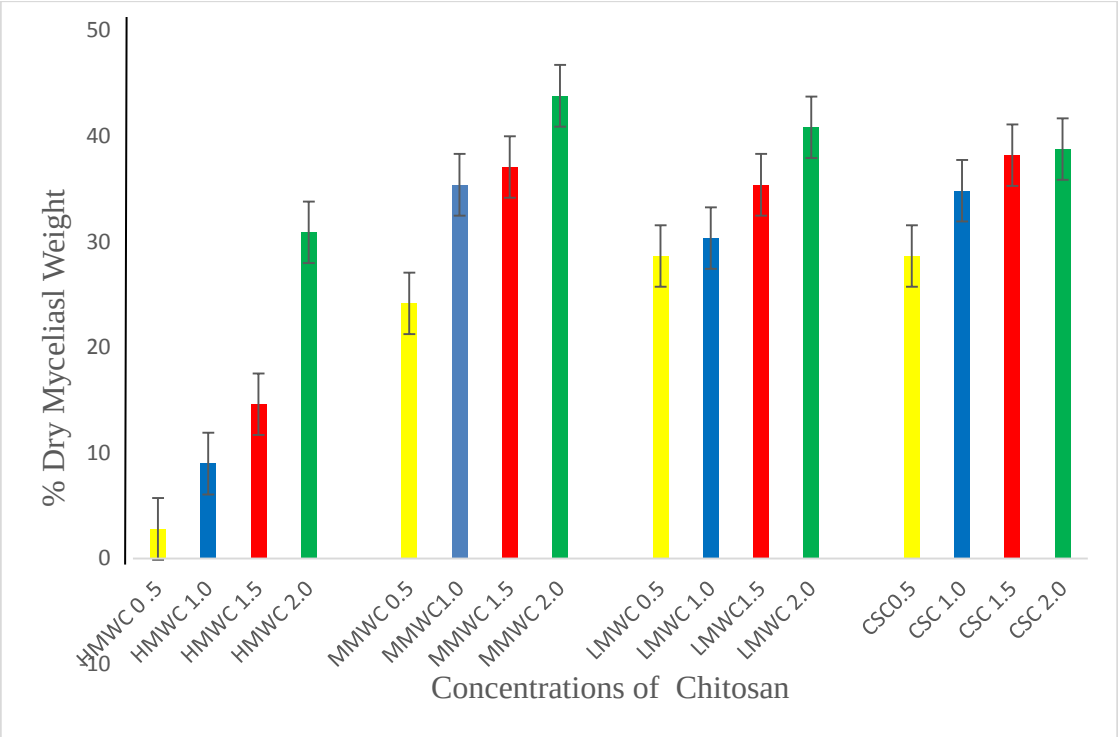

Figure 4.5c: Percentage Dry Mycelia Weight Inhibition of *Fusarium moniliforme* in the Different Concentrations of the Chitosan

4.1.6 Synthesised and characterised chitosan nanoparticles

The results revealed that the chitosan nanoparticle size was affected by the molecular weight and the concentration of sodium trypolyphosphate and the ratio of chitosan (Cs) concentration to sodium trypolyphosphate (Tpp). The size of chitosan nanoparticle at selected ratio of chitosan and sodium trypolyphosphate ranges from 468.3 nm to 711.7 nm. It was also observed that the higher the particle sizes of the nanoparticle, the higher the poly disparity index (Table 4.4). Cs/Tpp 1:1 and 1:5 produce higher nanochitosan particle sizes while 1:3 produce lowest particle size. 1:3 of LMWC: Tpp and HMWC: Tpp produce the lowest nanoparticle size of 488.7nm and 468.5nm respectively. While 1:5 of LMWC: Tpp and HMWC: Tpp produce the highest nanoparticle size of 643.4 nm and 711.7 nm respectively.

Table 4. 4: Chitosan Nanoparicle Synthesised

Chitosan	Cs: Tpp	UV Spectroscopy Wavelength(nm)	Absorbance peak (A)	Nanoparticle size Synthesis(nm)	Poly disparity index (PdI)
LMWC	1:1	270	-0.611	612.2	0.570
LMWC	1:3	232	-1.276	488.7	0.536
LMWC	1:5	231	-2.214	643.4	0.587
MMWC	1:1	638	0.148	NS	-
MMWC	1:3	560	0.127	NS	-
MMWC	1:5	233	-1.420	556.5	0.570
HMWC	1:1	231	-2.652	525.4	0.483
HMWC	1:3	233	-0.425	468.3	0.433
HMWC	1:5	233	-1.420	711.7	0.631

4.1.7 Mycelial radial growth of rice mycotoxin producing fungi in chitosan nanoparticles

The result of the *A. flavus* mycelial radial growth in chitosan nanoparticle treatment (Table 4.5) shows that the higher the concentration of chitosan nanoparticle, the lower the mycelia radial growth, thus the higher the inhibition. The highest mycelia radial growth of 3.10^{a}±0.12 was recorded in 0.25 $mgml^{-1}$ concentration of (1:1) HMWC: TPP nanopaticle with particle size 524.4 nm. However, the least mycelia radial growth of 1.00^{b}±0.00 were recorded in 0.25 $mgml^{-1}$ of (1:1) LMWC: TPP, 0.25 $mgml^{-1}$ of (1:5) LMWC: TPP and 0.50mgml-1 of (1:5) LMWC: TPP which were all significantly different (P<0.05) from the control with 8.00^{i}± .06 mycelia radial growth.

The result of the *A. fumigatus* mycelia radial growth in chitosan nanoparticle (Table 4.5) shows that the higher the concentration of chitosan nanoparticle, the lower the mycelia radial growth. The mycelia radial growth of *A. fumigatus* increased with increase in the number of days of incubation but not with the concentration althought it was significantly different (P<0.05) from the control.

The highest mycelia radial growth of 4.43±0.23 were recorded in 0.25 mg/ml concentration of (1:1 LMWC: TPP) nanoparticle with particle size 643.4 nm and 4.37^{f}± .09 in 0.50 $mgml^{-1}$ of (1:5) MMWC: TPP nanoparticle with particle size 556.5 nm. However, the least mycelia radial growth of 1.00±.00 and 1.17±.12 were recorded in 0.5 $mgml^{-1}$ concentration of (1:5 HMWC:TPP) Nanoparticle with particle size 711.7 nm, and 0.5 $mgml^{-1}$ concentration of (1:3LMWC:TPP) Nanoparticle with particle size 488.7 nm respectively, which were significantly different(P<0.05) from control with mycelia radial growth of 7.80 ± .06.

The result of *F. moniliforme* mycelia radial growth in chitosan nanoparticle (Table 4.5) shows that no growth was recorded in all the concentration of chitosan nanoparticles and across all the 5 days of incubation while the control has the highest mycelia radial growth of 6.00±0 .06 at day 5 of incubation which is highly significantly different ($P<0.05$).

Table 4.5: Mycelia Radial Growth of Rice Mycotoxin Producing Fungi in Chitosan Nanoparticles

Chitosan nanoparticle treatments	Mycelial growth of Mycotoxin-producing Fungi`		
	A. flavus	*A. fumigatus*	F. *moniliforme*
	Mean ± SEM	Mean ± SEM	Mean ± SEM
HMWC1:1TPP0.25	3.10^{a}± .12	$3.03^{a,e,j}$± .09	0.00^{a}±.00
HMWC1:1TPP0.50	$2.87^{a,e}$± .09	$2.27^{a,b}$± .24	0.00^{a}±.00
HMWC1:3TPP0.25	$2.73^{a,c,g}$± .37	$1.80b^{,c,g,h}$± .29	0.00^{a}±.00
HMWC1:3TPP0.50	1.13^{b}± .13	$1.23^{c,d}$± .09	0.00^{a}±.00
HMWC1:5TPP0.25	$2.07^{c,f,g,h}$± .07	$2.60^{a,e,j}$± .10	0.00^{a}±.00
HMWC1:5TPP0.50	1.07^{b}± .07	1.00^{d}± .00	0.00^{a}±.00
MMWC1:5TPP0.25	$2.13^{c,d,f,g,h}$± .09	$3.07^{e,j}$± .07	0.00^{a}±.00
MMWC1:5TPP0.50	$2.20^{c,e}$± .17	4.37^{f}± .09	0.00^{a}±.00
LMWC1:1TPP0.25	$1.47^{b.f}$± .03	$2.33^{a,e,g}$± .18	0.00^{a}±.00
LMWC1:1TPP0.50	1.00^{b}± .00	$2.53^{a,e,h,j}$± .13	0.00^{a}±.00
LMWC1:3TPP0.25	$2.33^{e,g}$± .17	$3.00^{a,e,j}$± .06	0.00^{a}±.00
LMWC1:3TPP0.50	$1.47^{b,h}$± .07	$1.17^{c,d,I}$ ± .12	0.00^{a}±.00
LMWC1:5TPP0.25	1.00^{b}± .00	4.43^{f}± .23	0.00^{a}±.00
LMWC1:5TPP0.50	1.00^{b}± .00	3.13^{j}± .09	0.00^{a}±.00
Control	8.00^{i}± .06	7.80^{k}± .06	6.00^{b}± .06

Note: Values in the same column and subtable not sharing the same subscript are significantly different at p< .05 in the two-sided test of equality, LMWC- Low Molecular Weight Chitosan, MMWC-Medium Molecular Weight Chitosan, HMWC-High MolecularWeght Chitosan, Cs-Chitosan, Tpp-Sodium Tri-PolyPhosphate

4.1.7.1 *Percentage mycelia radial growth inhibition of rice mycotoxin producing fungi in chitosan nanoparticle*

The result of the chitosan nanoparticle percentage fungi inhibition (Figure 4.6a-4.6c) shows that the range of inhibition was affected by the concentration of the chitosan nanoparticle as well as the number of the days of incubation. For all the chitosan nanoparticle, irrespective of the molecular weight of the chitosan, the highest percentage inhibition of *Aspergillus flavus* was 87.18 % while the lowest was 57.69 % which were significantly (Figure 4.6a) different ($P<0.05$). The concentration has significant effect on the inhibition. As the concentration increase, the percentage inhibition increase across the incubation days. Highest percentage inhibition of 87.18 % was recorded in HMWC:TPP chitosan nanoparticle with 711.7 nm particle size while lowest inhibition was recorded in LMWC Nanoparticle.

The highest percentage inhibition of *Aspergillus fumigatus* was 87.5 % observed in 0.5 % concentration of almost all the nanoparticles except 1:3MMWC and 1:1 HMWC nanoparticle and lowest was 61.25 % in 1:1 HMWC nanoparticle which were significantly different ($P\leq0.05$) (Figure 4.6b). The concentration had no significant effect on the inhibition. Highest percentage inhibition of 87.5 % was recorded in LMWC with 643.4nm nanoparticle size and HMWC with 711.7 nanoparicles while the lowest percentage inhibition was also recorded in HMWC with 525.4nm particle size.

The result of the chitosan nanoparticle percentage fungal inhibition shows that there was 100% inhibition of mycelia radial growth of *F. moniliforme* irrespective of the particle size of the chitosan nanoparticle size as well as the concentration. (Figure 4.6c).

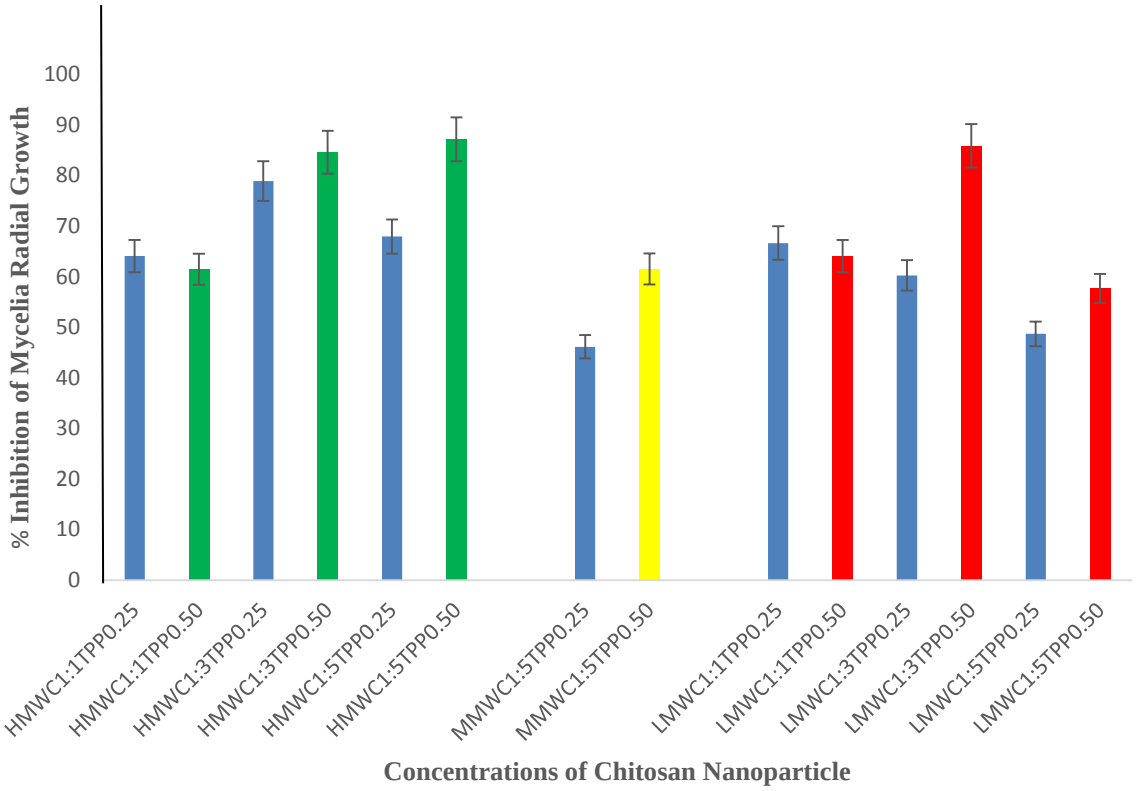

Figure 4.6a: Percentage Mycelia Radial Growth inhibition of *A. flavus* in two Different Concentrations of Chitosan Nanoparticle

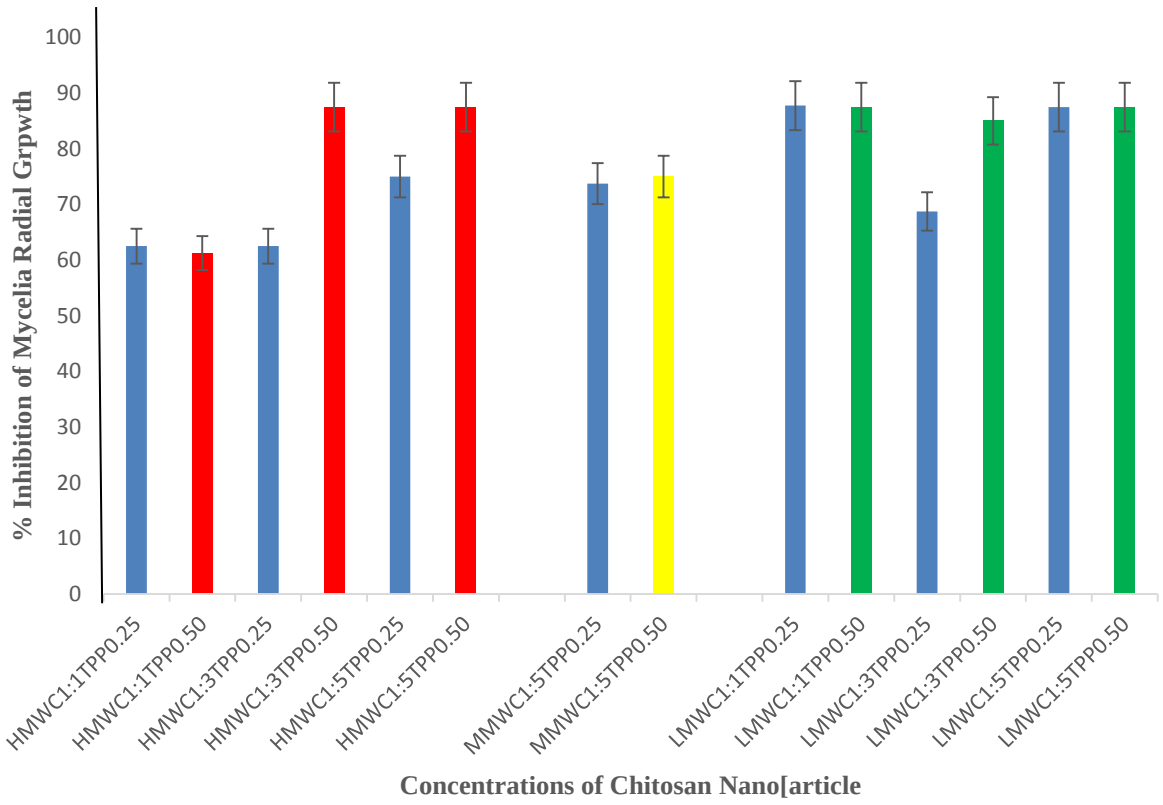

Figure 4.6b: Percentage Inhibition of Mycelia Radial Growth of *A. fumigatus* in Different Concentrations of Chitosan Nanoparticle

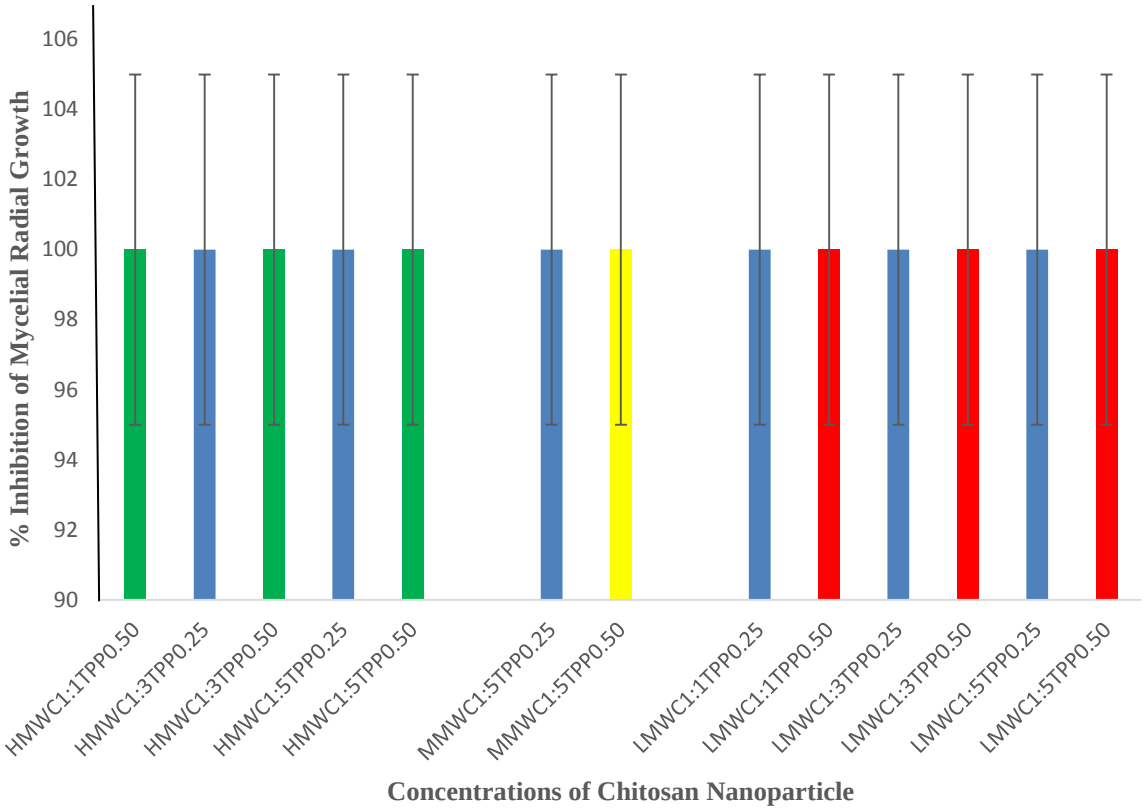

Figure 4.6c: Percentage Inhibition of Mycelia Radial Growth of *F. moniliforme* in Different Concentrations of Chitosan Nanoparticle

The growth rate of *A. flavus* in two concentrations (0.25 $mgml^{-1}$ and 0.50 $mgml^{-1}$) of the entire petri dishes containing PDA amended with chitosan nanoparticles (Plate VI-VII) showed that the chiosan nanoparticle affects the growth of the organism, however the lowest concentration of 0.25 $mgml^{-1}$ inhibit the growth of the fungi very well.

The growth of *A. fumigatus* in the two concentrations (0.25 $mgml^{-1}$ and 0.50 $mgml^{-1}$) of all petri dishes containing PDA amended with chitosan nanoparticles (Plate VIII-IX) showed that all the chitosan nanoparticle inhibits the growth of the organism, however there was no significant difference in growth in both concentration tested although it was different from the control. The growth of *F. moniliforme* in the two concentrations ((0.25 $mgml^{-1}$ and 0.50 $mgml^{-1}$) of all chitosan nanoparticle (Plate X-XI) showed that all the concentration inhibits the organism 100 %

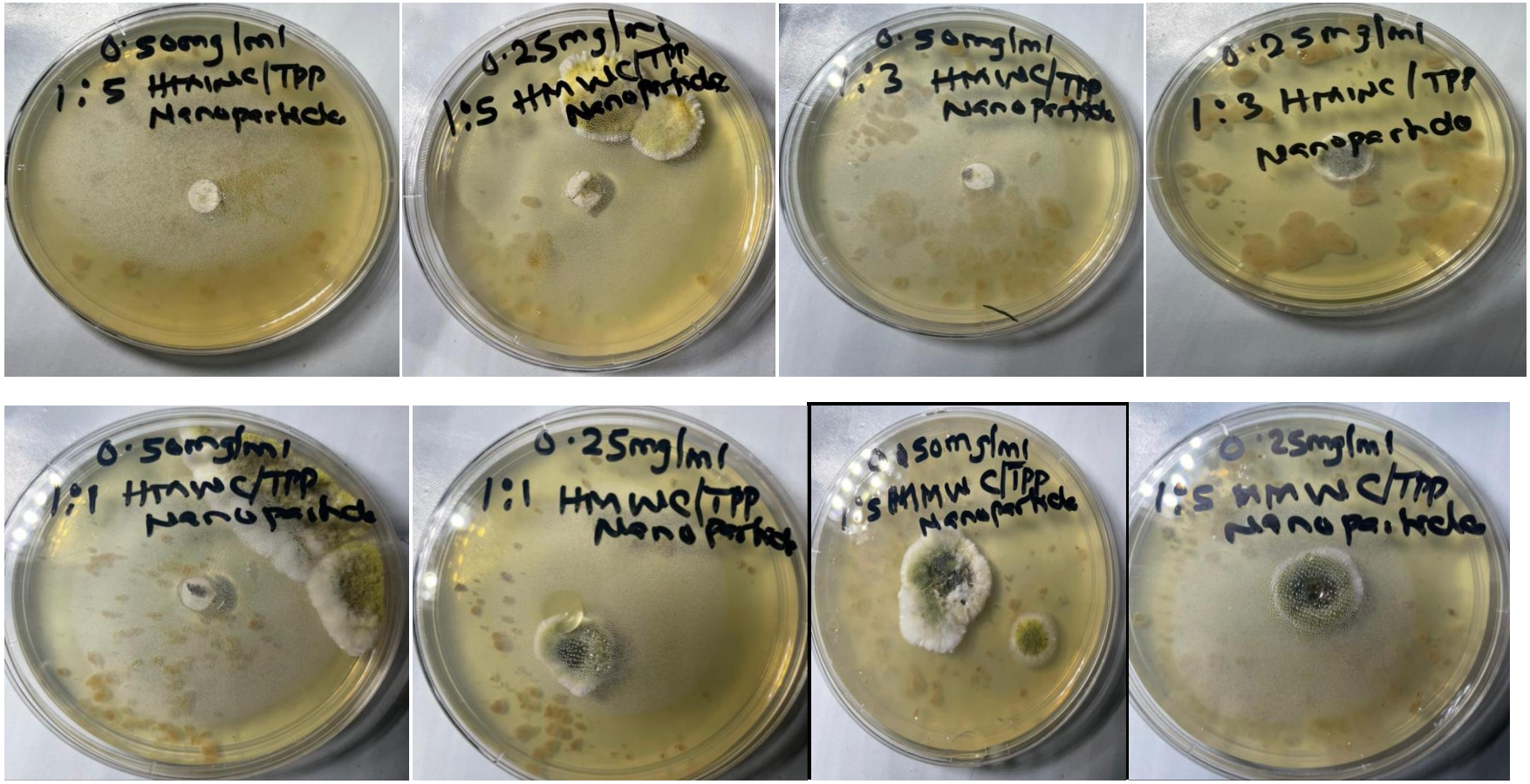

Plate VI: *In vitro* growth Inhibition of *Aspergillus flavus* in Different Concentration HMWC and MMWC Nanoparticle at the End of Incubation Days

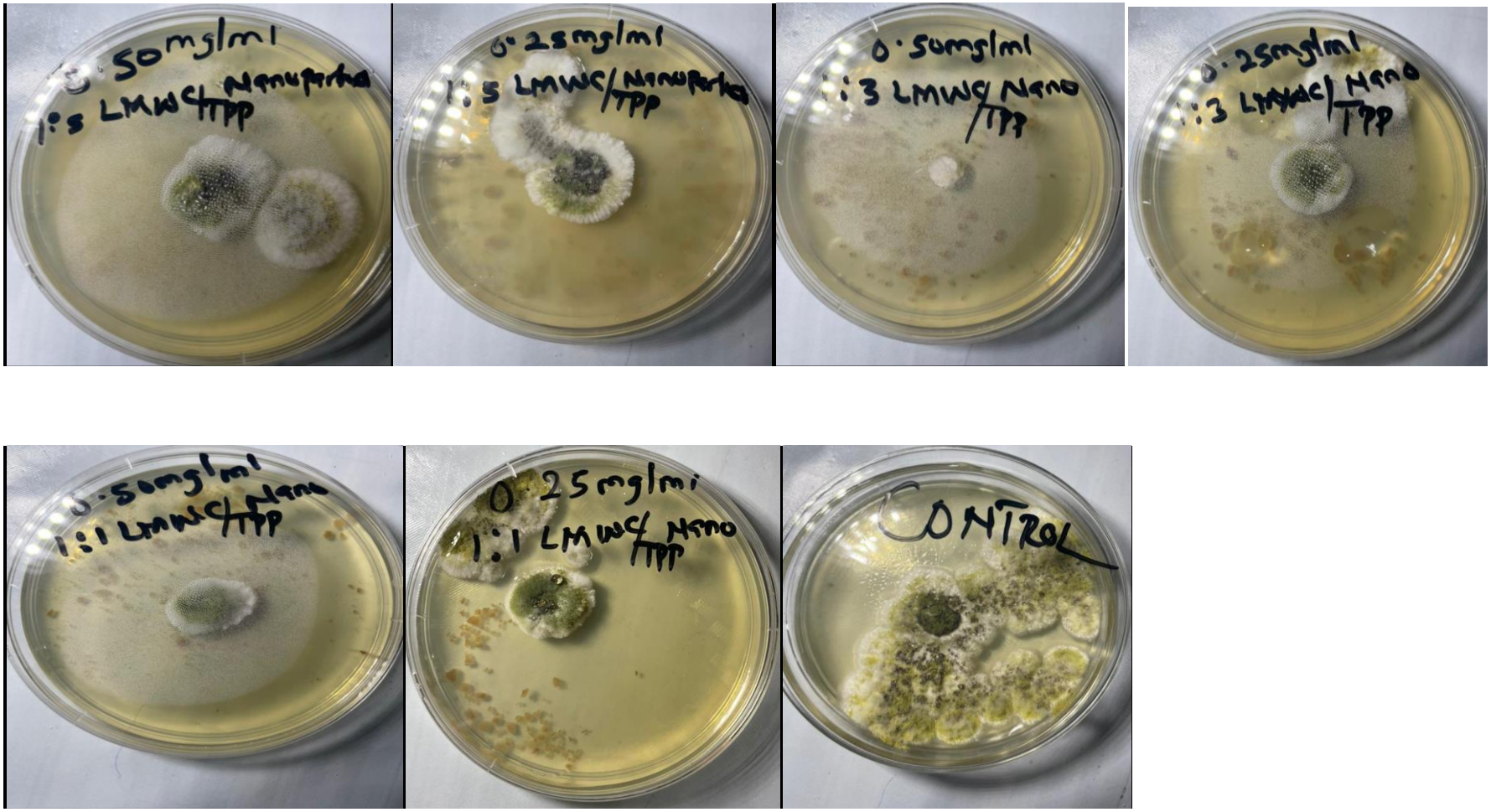

Plate VII: *Invitro* growth Inhibition of *Aspergillus flavus* in Different Concentration LMWC Nanoparticle at the End of Incubation Days

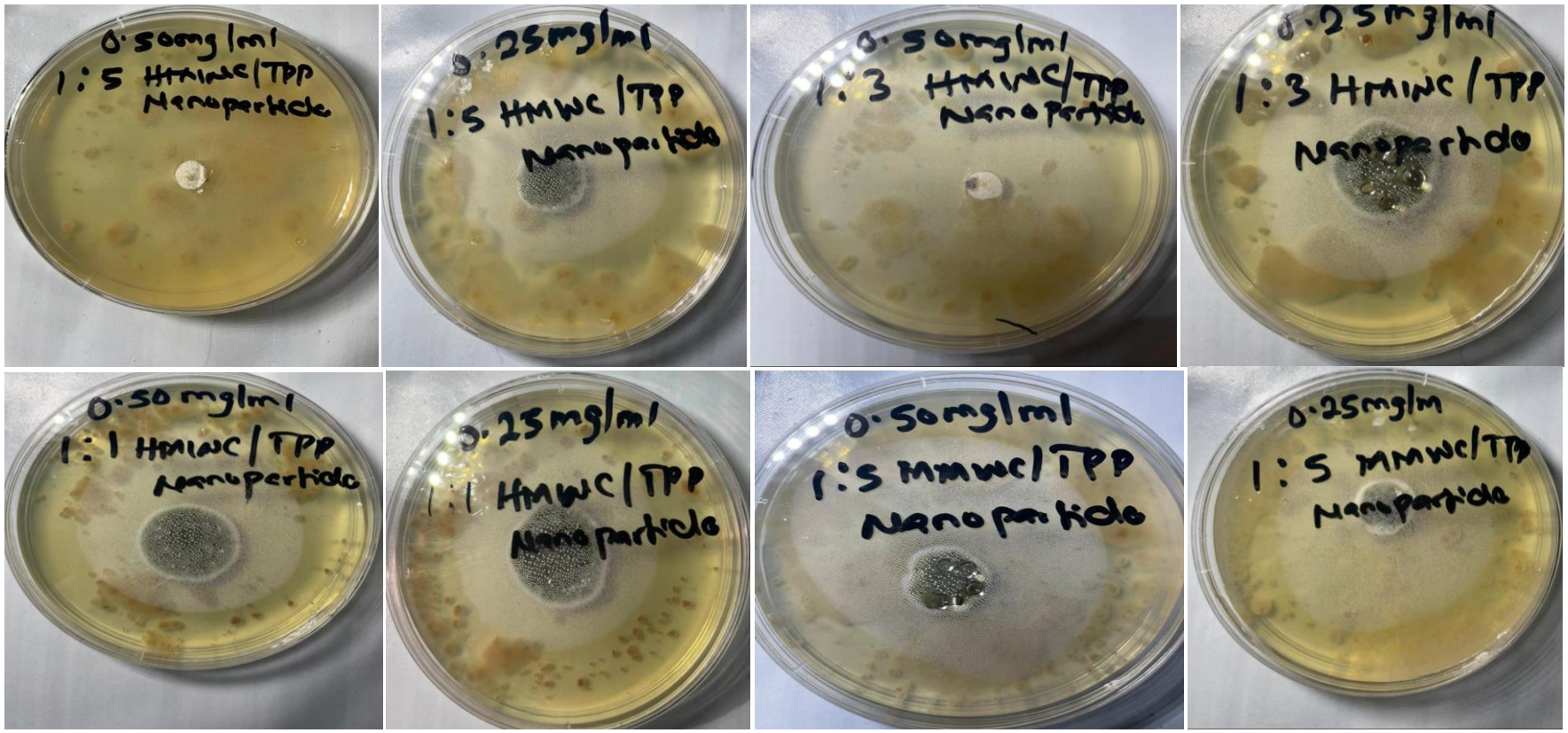

Plate VIII: *Invitro* growth Inhibition of *Aspergillus fumigatus* in Different Concentration of HMWC and MMWC Nanoparticle at the End of Incubation Days

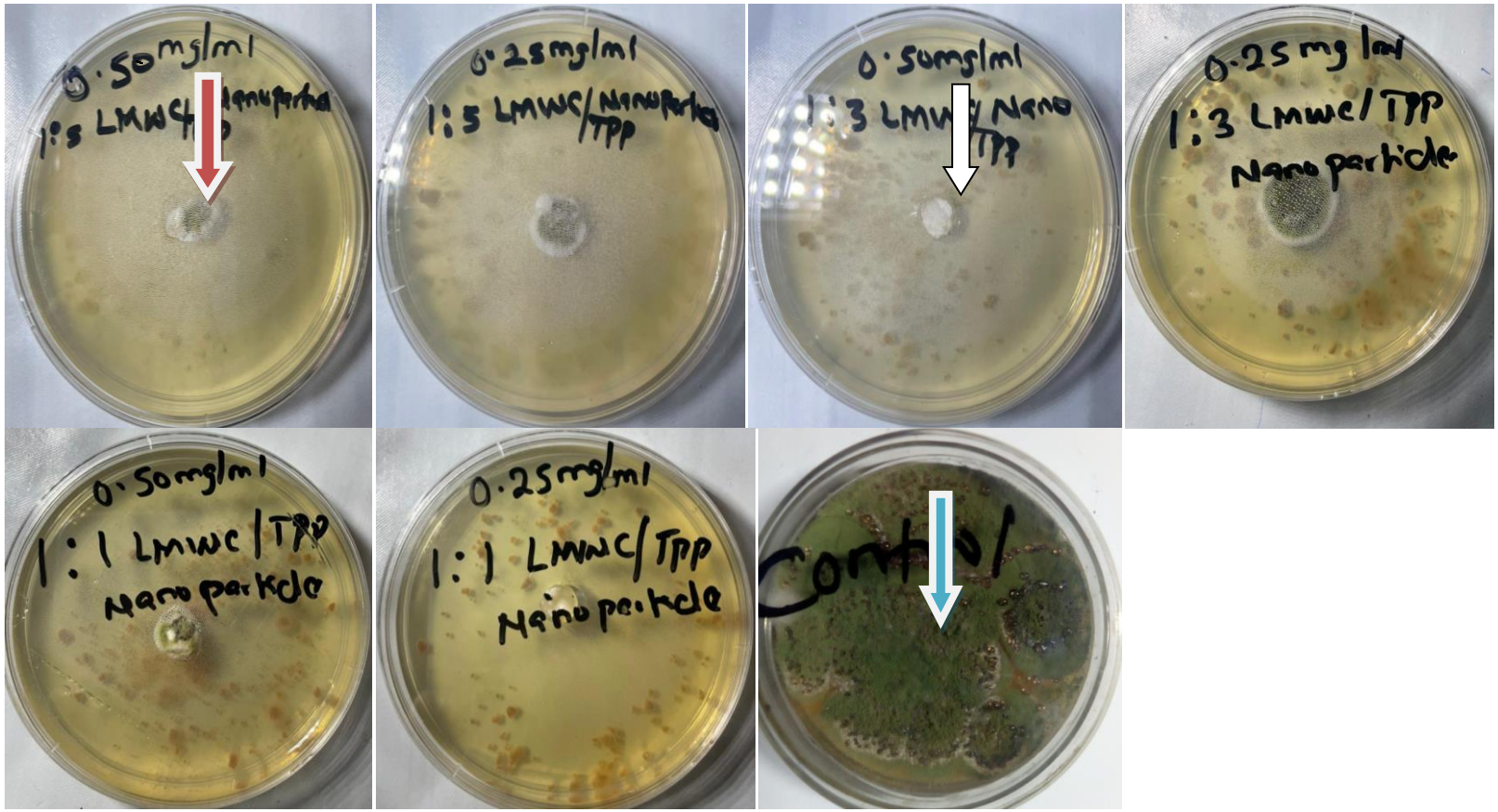

Plate IX: *Invitro* growth Inhibition of *Aspergillus fumigatus* in Different Concentration`of `LMWC Nanoparticle at the End of Incubation Days

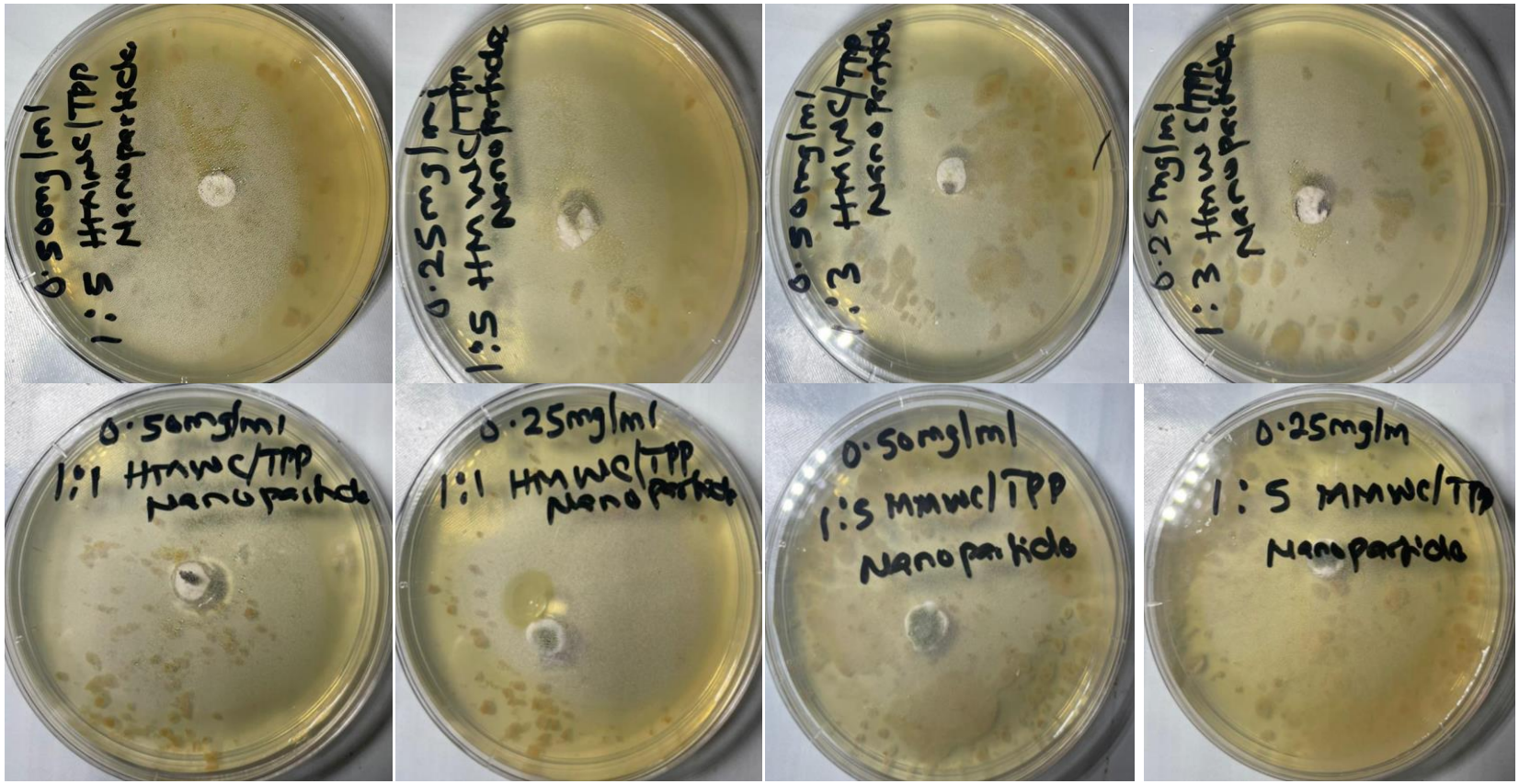

Plate X: *Invitro* growth Inhibition of *Fusarium moniliforme* in Different Concentration HMWC and MMWC Nanoparticle at the End of Incubation Days

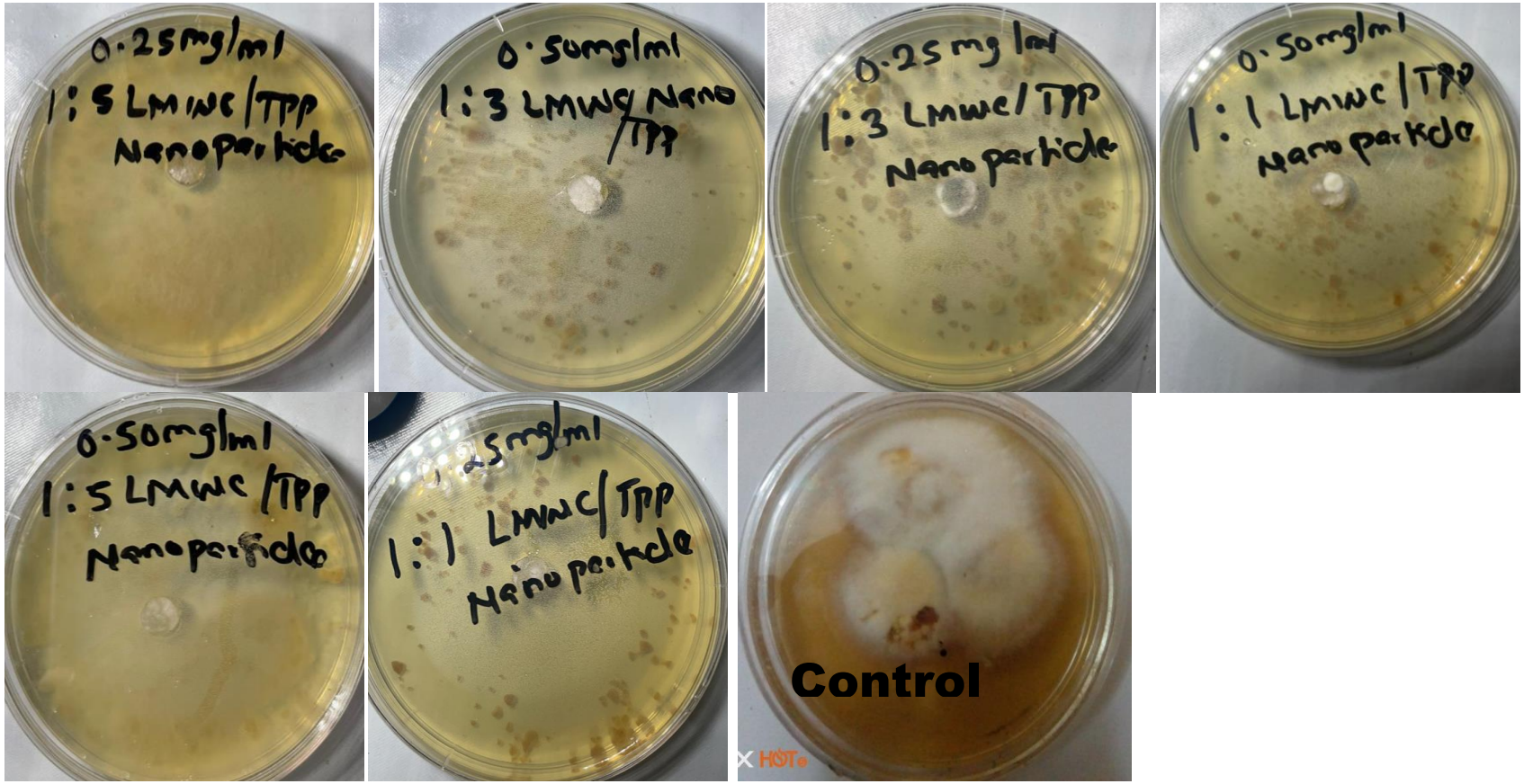

Plate XI: *Invitro* growth Inhibition of *Fusarium moniliforme* in Different Concentration LMWC Nanoparticle at the End of Incubation Days

4.1.7.2 *Percentage dry mycelia weight of rice mycotoxin producing fungi in chitosan nanoparticle treatment*

The results of the percentage dry mycelia weight of *A. flavus* in different concentrations of chitosan nanoparticle (Figure 4.7a) shows that the inhibition was affected by the concentration of the nanoparticle irrespective of the particle size. The higher the concentration of the chitosan nanoparticle, the higher the percentage mycelia weight inhibition. The highest percentage inhibition of 64.75 % was recorded in 0.50 mg/ml of HMWC1:5 nanoparticle with particle 711.7 nm while the lowest percentage inhibition of 40.54 % was recorded in 0.25mg/ml of LMWC 1:1 nanoparticle with particle size 612.2 nm. However 0.50 mg/ml of LMWC 1:5 nanoparticle with particle size 643.4 nm also has high percentage inhibition of 60.5 %.

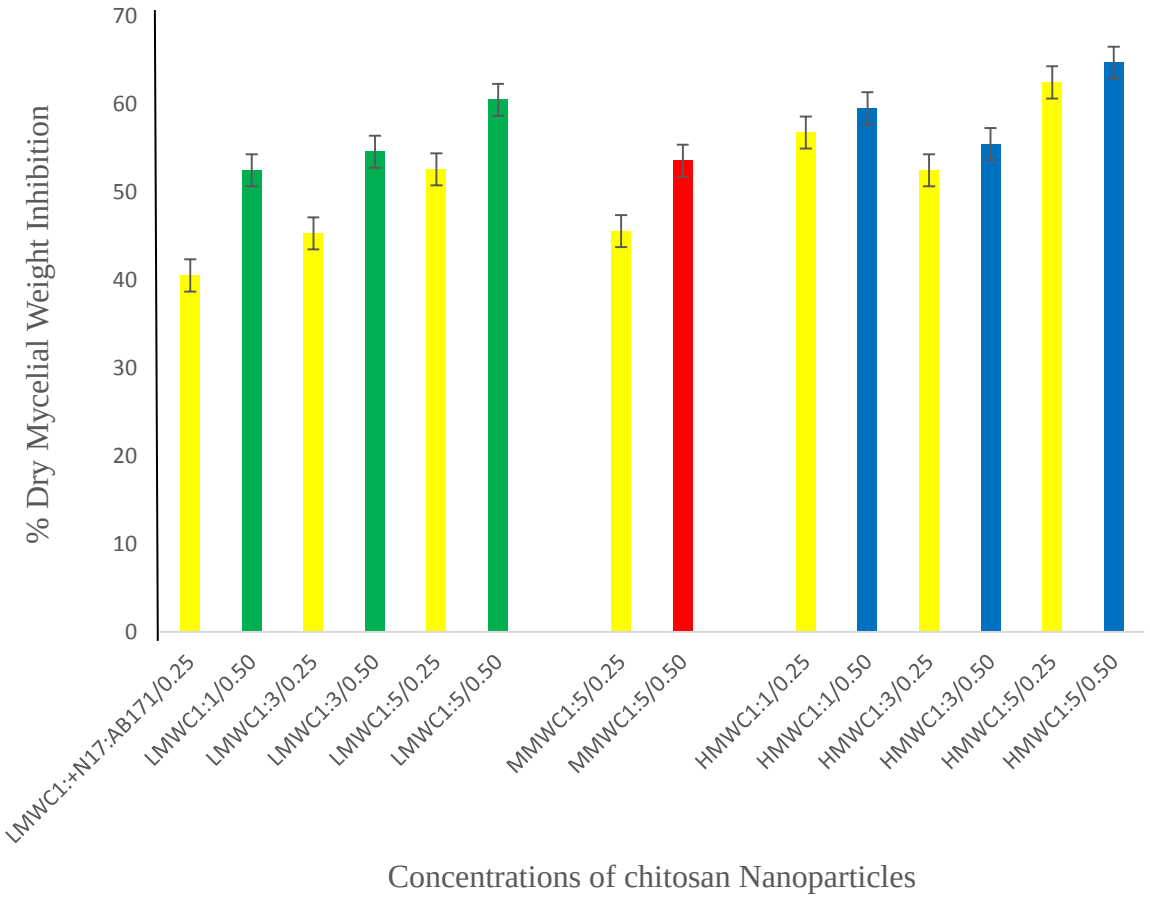

Figure 4.7a: Percentage Dry Mycelia Weight Inhibition of *A. flavus* in Chitosan Nanoparticles

The results of the percentage dry mycelia weight inhibition of *A. fumigatus* in different concentration of chitosan nanoparticle (Figure 4.7b) shows that the inhibition is affected by the concentration of the nanoparticle irrespective of the particle size. The higher the concentrations of the chitosan nanoparticle, the lower the percentage mycelia weight. The highest percentage inhibition of 62.16 % and 62 % was recorded in 0.50 mg/ml of LMWC 1:5 nanoparticle with particle size 643.4 nm and 0.50 mg/ml of HMWC 1:1 nanoparticle with particle size 525.4 nm respectively, while the lowest percentage inhibition of 15.54 % was recorded in 0.25 mg/ml of LMWC 1:3 nanoparticle with particle size 488.7 nm.

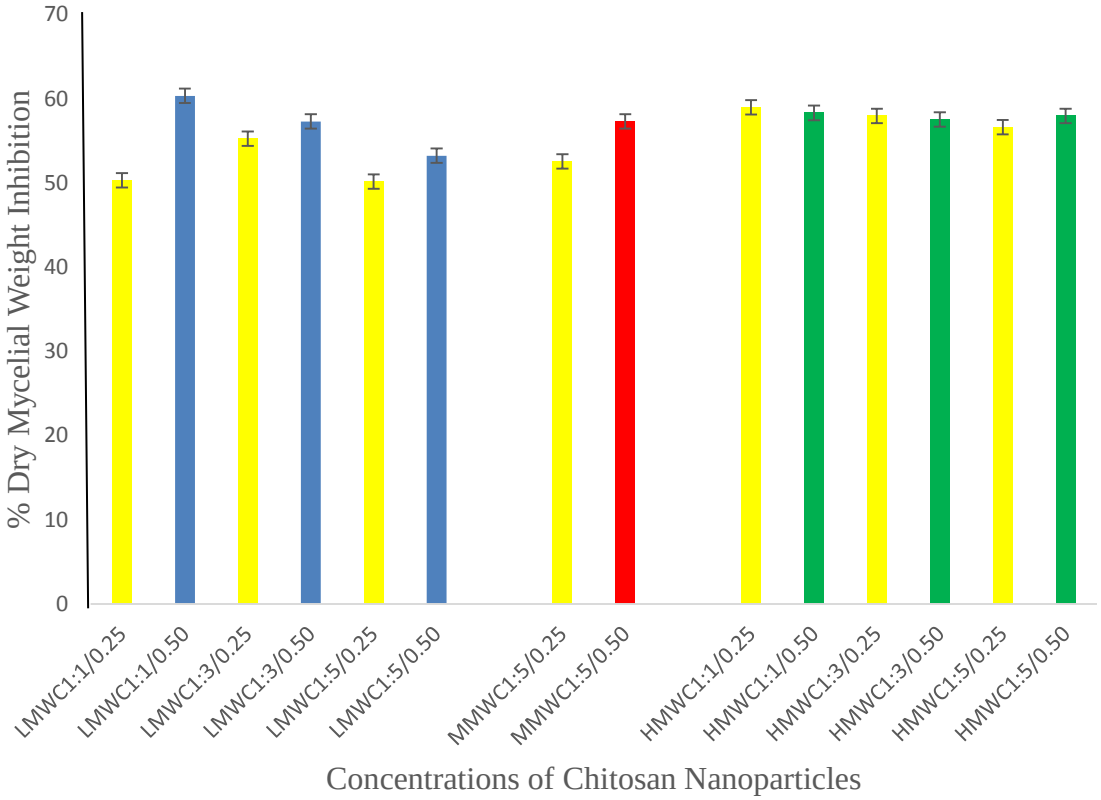

Figure 4.7b: Percentage Dry Mycelia Weight of *A. fumigatus* in Chitosan Nanoparticles

The results of the percentage dry mycelia weight of *F. moniliforme* in different concentration of chitosan nanoparticle (Figure 4.7c) shows that the inhibition was not affected by the concentration of the nanoparticle as well as the particle size. There was no significant difference ($P>0.05$) in the percentage inhibition of *F. moniliforme* across all the concentration of chitosan nanoparticle. The highest percentage inhibition of 60.34 % was recorded in 0.50 mg/ml of LMWC 1:1TPP nanoparticle with particle size 612.2 nm while the lowest percentage inhibition of 50.3 % was also recorded in 0.25 mg/ml of LMWC1:1 nano-synthsis, although, not significantly different ($P>0.05$).

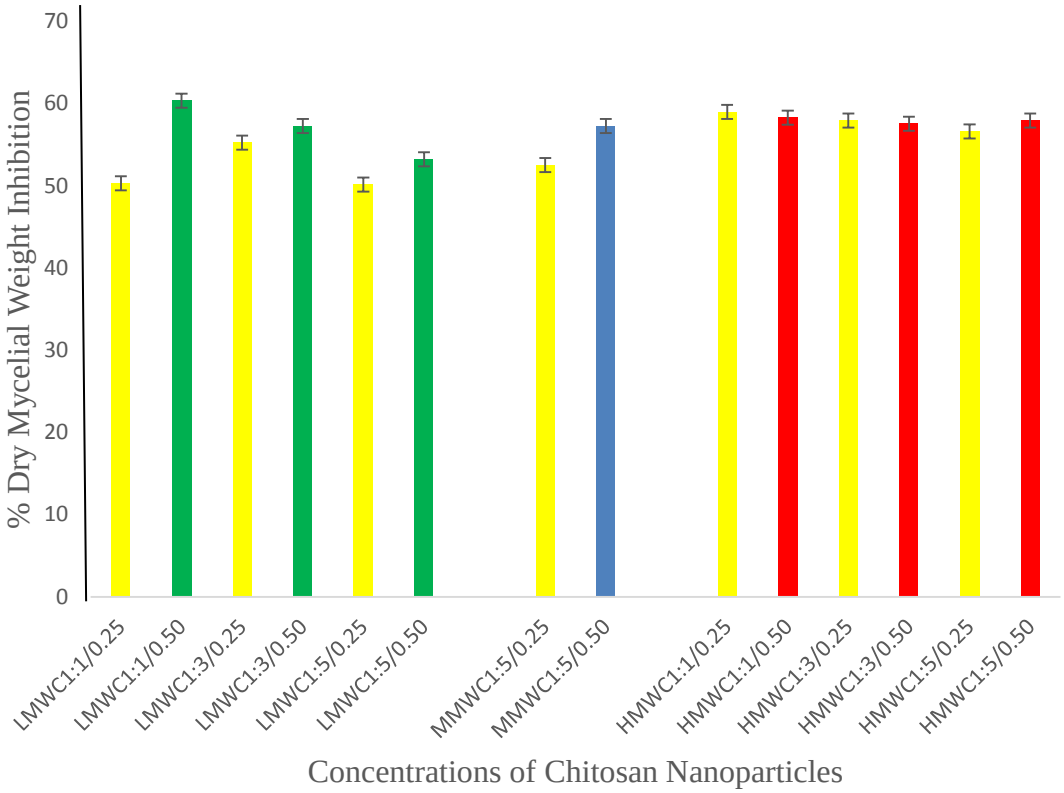

Figure 4.7c: Percentage Dry Mycelia Weight of *F. moniliforme* in Chitosan Nanoparticles

4.1.8 Effects of chitosan seed coating on storage fungi occurence

The result of the two chitosan (HMWC and MMWC) as seed coating of the rice samples collected from farmers showed that the fungal occurence in seed has been reduced or totally eliminated fron some of the samples (Tables 4.6a & 4.6b).

For improved rice variety genotypes using HMWC as coating, the highest fungal occurrence of 3.00±0.08 was recorded tag R1. However, no occurence was recorded in almost all the rice tags after six month of storage with the chitosan coating. Also in improved rice varieties using MMWC as coating, the highest fungal occurrence of 8.00±.16 was recorded rice tag R23. No occurence was recorded in rice tags R7, R8, R10 and R19 (Table 4.6a)

For local accession rice using HMWC as coating, the highest fungal occurrence 2.00±0.58 was recorded rice tag R45. Although, no occurence was recorded in rice tags R35, R36, R47, R48, R49 and R50. Also in local accession rice using MMWC as coating, the highest fungal occurrence 6.67±1.45 was also recorded in rice tag R45. However, no occurrence of disease was recorded in rice tag R34 (Table 4.6b).

Table 4.6a: Fungal Occurrence from Improved Rice Varieties after Coating with Chitosan (HMWC and MMWC)

Rice Tags	Abbreviated names of Varieties	Fungal Incidence	
		1.0% HMWC	1.0% MMWC
		Mean±SE_Mean(cfu)	Mean±SE_Mean (cfu)
Rice1	F44	3.00±0.58	1.00±1.00
Rice 2	F44	1.33±0.88	6.00±0.58
Rice 3	F44	0.00±0.00	2.00±1.16
Rice 4	F44	1.67±0.67	2.33±1.20
Rice 5	F52	0.00±0.00	0.00±0.00
Rice 6	F61	0.00±0.00	0.67±0.33
Rice 7	F44	0.00±0.00	0.00±0.00
Rice 8	F44	0.00±0.00	0.00±0.00
Rice 9	F61	0.33±0.33	1.00±0.58
Rice 10	F52	0.00±0.00	0.00±0.00
Rice 11	F52	0.00±0.00	0.67±0.33
Rice 12	F52	0.67±0.67	0.00±0.00
Rice 13	F44	0.00±0.00	1.00±0.00
Rice 14	F44	0.00±0.00	2.33±0.33
Rice 15	F52	0.00±0.00	2.67±0.88
Rice 16	F52	0.00±0.00	1.33±0.33
Rice 17	F44	0.00±0.00	1.00±0.58
Rice 18	F44	0.00±0.00	2.67±0.88
Rice 19	F52	0.00±0.00	0.00±0.00
Rice 20	F52	0.00±0.00	7.67±0.88
Rice 21	F52	0.00±0.00	1.33±0.33
Rice 22	F44	0.00±0.00	3.00±0.58
Rice 23	F52	0.00±0.00	8.00±1.16
Rice 24	F52	0.00±0.00	0.67±0.33
Rice 25	F44	0.00±0.00	1.00±0.58
Rice 26	F44	1.33±0.88	5.67±0.67
Rice 27	F52	0.00±0.00	4.67±0.88
Rice 28	F44	0.33±0.33	1.33±0.67
Rice 29	F44	0.67±0.67	4.00±0.58
Rice 30	F52	0.00±0.00	1.33±0.67
Mean		**0.53±0.07**	**2.26±0.18**
HSD		**2**	**4.04**

Table 4.6b: Fungal Occurence from Local accession Rice after Coating with Chitosans (HMWC and MMWC)

		Fungal Incidence	
Rice Tags	**Abbreviated names Varieties**	**1.0% HMWC Mean±SE_Mean (cfu)**	**1.0% MMWC Mean±SE_Mean (cfu)**
Rice 31	DG	0.00±0.00	2.67±0.88
Rice 32	KD	1.00±0.58	1.00±1.00
Rice 33	KD	1.33±0.33	0.67±0.67
Rice 34	F44	2.00±0.58	0.00±0.00
Rice 35	F52	1.00±0.58	1.67±0.88
Rice 36	KD	0.00±0.00	2.33±0.88
Rice 37	AB	0.00±0.00	2.00±0.00
Rice 38	KD	1.00±0.58	2.00±0.00
Rice 39	RB	1.00±0.58	1.33±0.88
Rice 40	AB	1.33±0.67	2.33±0.67
Rice 41	F52	1.00±0.58	3.00±1.00
Rice 42	SK	1.33±0.33	2.00±0.58
Rice 43	KD	1.00±0.58	1.33±0.33
Rice 44	F52	1.33±0.33	4.00±1.00
Rice 45	KD	2.00±0.58	6.67±1.45
Rice 46	AS	1.67±0.88	3.67±0.88
Rice 47	AB	0.00±0.00	3.67±0.88
Rice 48	KD	0.00±0.00	4.33±0.67
Rice 49	EK	0.00±0.00	2.67±0.33
Rice 50	KD	0.00±0.00	2.33±0.33
Mean		**0.53±0.07**	**2.26±0.18**
HSD		**2**	**4.04**

Overall percentage frequency of occurrence of fungi in rice samples coated with HMWC (Figure 4.8) shows that *A. flavus* has the highest prevalence of 40.25 % followed by *A. fumigatus* (28.5 %) and the least prevalence was observed in *F. moniliforme* (7.5 %). *A fumigatus* was the most prevalent (38.8 %) in MMWC and the least also in *F. moniliforme*. However, the two chitosan suppressed the growth of *Cladosporium cladosporoides, Rhizopus stolonifer, Paecileomyces lilacinus and Rhizoctonia solani* completely as the fungi were not isolated in all the samples coated after the six month of storage.

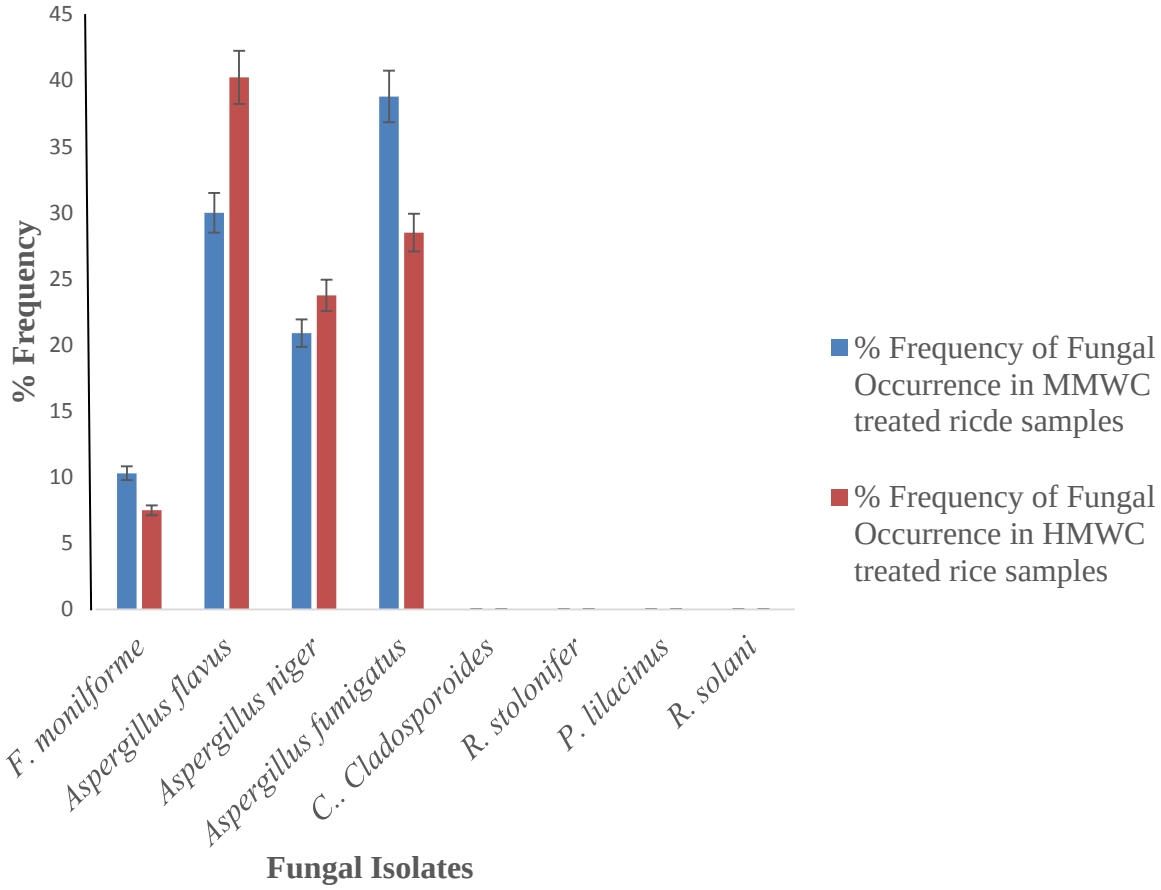

Figure 4.8: Percentage Frequency of Occurrence of Fungal Isolates across all Samples Coated with 1.0% HMWC and MMWC

4.1.9 Efficacy of chitosan against blast pathogenic fungi (*Magnaporthe oryzae*) on rice field.

The result of the blast screening and other growth and yield parameters of rice varieties with chitosan treatments (Table 4.7a and 4.7b) showed that the severity and incidence of blast decrease with application of the chitosan as foliar spray. For rice variety 1 (FARO 52), the highest blast severity score of 6 and incidence of 28.3 % were observed in rice plot treated with MMWC 1.5 %, followed by HMWC 2.0 % with severity score of 5 and incidence of 20 %, LMWC 2.0 % with severity of 5 and incidence of 20 % and CSCS 2.0 % with severity score of 4 and incidence of 24.3 %. However, the incidence reduced at ninety (90) days after treatment to 9.3 %. 2.3 %, 1.0 % and 2.0 %, respectively (Table 4.7a).

There was no significant difference ($P>0.05$) in all other growth and yield parameters such as days to 50 % flowering, panicle count, panicle length, tiller count, plant height, and 1000 seed weight except for grain yield per plot with highest yield of 717 gram in HMWC 2.0 % treated plot while the lowest yield of 190 gram was observed in MMWC 0.5 % which was significantly different ($P<0.05$).

For rice variety 2 (FARO 66), the highest blast disease severity score of 6 and incidence of 23.3 % were observed in rice plot treated with HMWC 2.0 %, followed by LMWC 1.0% with severity score of 5 and incidence of 26.7 %, MMWC 2.0 % with severity of 4 and incidence of 21.7 % and LMWC 1.5 % with severity score of 4 and incidence of 23.3 %. However, the incidence reduced at ninety (90) days after treatment to 0.7 %. 2.0 %, 3.7 % and 0.7 %, respectively (Table 4.7b).

There was no significant difference ($p>0.05$) in all other growth and yield parameters such as days to 50% flowering, panicle count, panicle length, tiller count, plant

height, and 1000 seed weight except for grain yield per plot with highest yield of 783 gram in CSCS 2.0 % treated plot, and 777 gram in HMWC 2.0 % while the lowest yield of 411 gram was observed in LMWC 0.5 % which is significantly different ($P<0.05$).

Table 4.7a: Blast Severity, Percentage Incidence and Agronomic Parameters of Faro 52 Varieties Treated with Chitosan

Chitosan Conc	BSSA	BIAS	BI@42	BI@63	BI@90	BSSAT	Days 50%FLW	Panicle Count	Panicle length	Tiller Count	Plant height	1000Seed Weight	Grain yield/plot
HMWC 0.5	2^{b}	8.0^{b}	2.0^{b}	3.3^{b}	2.0^{b}	1^{ab}	76^{a}	5.80^{b}	24.9^{a}	9.6^{b}	94.9^{a}	20.4^{a}	363^{b}
HMWC 1.0	3^{ab}	14.3^{ab}	3.3^{b}	7.0^{ab}	2.7^{b}	2^{ab}	77.0^{a}	7.93^{a}	23.5^{a}	11.5^{ab}	91.5^{a}	19.9^{a}	443^{ab}
HMWC 1.5	3^{ab}	11.7^{b}	2.0^{b}	2.7^{b}	3.3^{b}	1^{ab}	79.7^{a}	8.53^{a}	25.4^{a}	10.8^{ab}	88.3^{a}	21.1^{a}	621^{a}
HMWC 2.0	5^{a}	20.0^{a}	9.0^{a}	2.7^{b}	2.3^{b}	1^{ab}	76.3^{a}	6.60^{ab}	23.1^{a}	10.5^{ab}	82.3^{a}	20.4^{a}	717^{a}
MMWC0.5	2^{b}	6.7^{b}	2.0^{b}	1.7^{b}	1.0^{b}	1^{ab}	78^{a}	7.27^{a}	24.3^{a}	13.4^{a}	83.6^{a}	19.0^{a}	190^{c}
MMWC1.0	3^{ab}	8.3^{b}	5.0^{ab}	1.3^{b}	1.7^{b}	1^{ab}	76.7^{a}	7.53^{a}	24.5^{a}	9.7^{b}	87.5^{a}	20.2^{a}	454^{ab}
MMWC1.5	6^{a}	28.3^{a}	10.3^{a}	15.0^{a}	9.3^{a}	3^{a}	79.7^{a}	7.93^{a}	25.1^{a}	12.3^{a}	86.7^{a}	20.7^{a}	471^{ab}
MMWC2.0	5^{a}	13.3^{ab}	5.0^{ab}	4.3^{b}	4.0^{b}	2^{ab}	74.7^{a}	7.60^{a}	25.1^{a}	10.5^{ab}	94.2^{a}	20.6^{a}	522^{ab}
LMWC 0.5	3^{ab}	8.3^{b}	4.3^{ab}	3.0^{b}	3.3^{b}	1^{ab}	77.7^{a}	8.33^{a}	24.2^{a}	10.4^{ab}	82.6^{a}	19.8^{a}	298^{b}
LMWC 1.0	4^{a}	10.0^{b}	2.7^{b}	6.3^{ab}	2.3^{b}	1^{ab}	76.3^{a}	7.47^{a}	24.6^{a}	15.4^{a}	85.1^{a}	20.0^{a}	333^{b}
LMWC 1.5	3^{ab}	8.3^{b}	2.7^{b}	4.7^{b}	2.3^{b}	1^{ab}	77.0^{a}	7.93^{a}	23.2^{a}	10.5^{ab}	85.5^{a}	19.0^{a}	432^{ab}
LMWC 2.0	5^{a}	20.0^{a}	7.3^{ab}	4.0^{b}	1.0^{b}	1^{ab}	75.3^{a}	6.40^{ab}	24.8^{a}	12.5^{a}	83.9^{a}	20.3^{a}	512^{ab}
CSCS 0.5	4^{a}	16.7^{ab}	5.0^{ab}	1.3^{b}	2.7^{b}	1^{ab}	78^{a}	6.73^{ab}	25.6^{a}	10.8^{b}	82.5^{a}	20.4^{a}	373^{b}
CSCS 1.0	2^{b}	9.3^{b}	3.0^{b}	3.0^{b}	3.3^{b}	1^{ab}	74.7^{a}	7.40^{a}	24.6^{a}	12.9^{a}	87.1^{a}	19.8^{a}	344^{b}
CSCS 1.5	3^{ab}	10.0^{b}	2.3^{b}	6.3^{ab}	4.7^{b}	2^{ab}	75.7^{a}	7.60^{a}	23.9^{a}	11.7^{ab}	85.4^{a}	19.8^{a}	479^{ab}
CSCS 2.0	4^{a}	24.3^{a}	11.0^{a}	6.3^{ab}	2.0^{b}	1^{ab}	78.3^{a}	8.07^{a}	24.9^{a}	12.7^{a}	81.7^{a}	19.7^{a}	520^{ab}

Means with the same letter along the rows are not significantly different at 0.05 percent probability level

Keys: BSSA-Blast score at appearance of symptoms, BIAS-Blast incidence at appearance of symptoms, BI@42- Blast incidence at 42days after transplanting, BI@63- Blast incidence at 63days after transplanting, BI@90- Blast incidence at 90days after transplanting, 50%FLW-Days to 50% flowering, BSSAT- Blast severity score after Treatment, HMWC- High molecular weight chitosan, MMWC-Medium molecular weight chitosan, LWMC-Low molecular weight chitosan, CSCS-Chitosan Synthesis from Crab Shell

Table 4.7b: Blast Severity, Percentage Incidence and Agronomic Parameters of Faro 66 Varieties Treated with Chitosan

Chitosan Conc	BSSA	BIAS	BI@42	BI@63	BI@90	BSSAT	50% FLW	Panicle Count	Panicle length	Tiller Count	Plant height	1000 Seed Weight	Yield /plot
HMWC 0.5	2^{b}	12.7^{b}	3.0^{c}	3.7^{c}	3.0^{b}	1^{b}	69.3^{a}	9.00^{a}	24.9^{a}	10.6^{a}	91.0^{a}	23.0^{a}	645^{a}
HMWC 1.0	3^{ab}	11.0^{b}	7.7^{b}	2.7^{c}	2.7^{b}	1^{b}	70.0^{a}	7.60^{a}	24.6^{a}	11.3^{a}	86.1^{a}	22.6^{a}	524^{ab}
HMWC 1.5	4^{a}	23.3^{a}	6.7^{b}	14.7^{a}	0.7^{c}	2^{ab}	72.7^{a}	7.27^{a}	24.3^{a}	10.3^{a}	88.5^{a}	22.3^{a}	440^{b}
HMWC 2.0	6^{a}	23.3^{a}	15.0^{a}	10.7^{a}	0.7^{c}	3^{a}	69.3^{a}	8.53^{a}	24.5^{a}	11.2^{a}	90.1^{a}	23.2^{a}	777^{a}
MMWC 0.5	2^{b}	9.0^{bc}	4.0^{c}	2.3^{c}	3.7^{b}	1^{b}	69.3^{a}	8.27^{a}	25.1^{a}	11.8^{a}	86.5^{a}	23.0^{a}	552^{ab}
MMWC 1.0	5^{a}	18.3^{a}	6.7^{b}	7.7^{b}	2.7^{b}	2^{b}	71.3^{a}	7.60^{a}	25.0^{a}	10.4^{a}	93.2^{a}	23.6^{a}	720^{a}
MMWC1.5	4^{a}	11.0^{b}	6.7^{b}	3.7^{c}	2.3^{b}	1^{b}	72.3^{a}	8.53^{a}	25.2^{a}	13.2^{a}	85.5^{a}	22.3^{a}	629^{a}
MMWC2.0	4^{a}	21.7^{a}	9.3^{ab}	3.0^{c}	3.7^{b}	2^{b}	70.0^{a}	7.47^{a}	25.1^{a}	12.4^{a}	89.2^{a}	23.0^{a}	723^{a}
LMWC 0.5	3^{ab}	18.3^{a}	10.0^{ab}	2.7^{c}	0.0^{c}	0^{b}	72.3^{a}	7.40^{a}	24.5^{a}	13.0^{a}	84.1^{a}	21.3^{a}	411^{b}
LMWC 1.0	5^{a}	26.7^{a}	2.3^{c}	14.0^{a}	2.0^{b}	3^{a}	71.3^{a}	7.60^{a}	24.8^{a}	12.4^{a}	85.9^{a}	22.3^{a}	587^{ab}
LMWC 1.5	4^{a}	20.3^{a}	9.3^{b}	7.7^{b}	9.0^{a}	2^{ab}	68.7^{a}	7.67^{a}	23.7^{a}	12.1^{a}	93.5^{a}	23.5^{a}	605^{ab}
LMWC 2.0	2^{b}	7.3^{c}	2.7^{c}	3.3^{c}	1.0^{bc}	1^{b}	70.0^{a}	8.93^{a}	23.9^{a}	11.9^{a}	89.7^{a}	22.8^{a}	622^{a}
CSCS 0.5	2^{b}	11.7^{b}	3.3^{c}	3.0^{c}	1.3^{bc}	1^{b}	70.0^{a}	7.33^{a}	24.8^{a}	12.2^{a}	92.6^{a}	23.3^{a}	442^{b}
CSCS 1.0	2^{b}	10.0^{b}	4.7^{c}	3.3^{c}	1.3^{bc}	1^{b}	69.7^{a}	7.00^{a}	23.5^{a}	10.5^{a}	87.9^{a}	21.3^{a}	449^{b}
CSCS 1.5	2^{b}	10.0^{b}	4.0^{c}	1.7^{c}	2.0^{b}	1^{b}	72.3^{a}	7.20^{a}	22.8^{a}	11.1^{a}	83.8^{a}	21.5^{a}	464^{b}
CSCS 2.0	2^{b}	7.0^{c}	2.0^{c}	2.0^{c}	4.0^{b}	1^{b}	74.3^{a}	7.80^{a}	23.3^{a}	11.9^{a}	85.7^{a}	21.9^{a}	783^{a}

Means with the same letter are not significantly different at 0.05 percent probability level

Keys:BSSA-Blast score at appearance of symptoms, BIAS-Blast incidence at appearance of symptoms, BI@42- Blast incidence at 42days after transplanting, BI@63- Blast incidence at 63days after transplanting, BI@90- Blast incidence at 90days after transplanting, 50%FLW-Days to 50% flowering, BSSAT- Blast severity score after Treatment, HMWC- High molecular weight chitosan, MMWC-Medium molecular weight chitosan, LWMC-Low molecular weight chitosan, CSCS-Chitosan Synthesis from Crab Shell

4.1.9.1 *Lowest inhibitory concentration dose at 50 % (LCD_{50}) of chitosan and chitosan nanoparticles*.

The results of the lowest concentration Dose at 50 % (LCD_{50}) of chitosan in its free form and nanoparticle form (Table 4.8) shows that chitosan solution in its free form has less inhibitory effect with high inhibitory concentration than the chitosan nanoparticle with high inhibitory effects and less inhibitory concentration. For *A. flavus*, LCD_{50} of 0.25 $mgml^{-1}$ was observed in HMWC nanoparticle (612 nm), LMWC nanoparticle (525 nm and 468 nm) while LCD_{50} of 2.0 $mgml^{-1}$ was observed in MMWC solution in its free form. For *A. fumigatus*, LCD_{50} of 0.25 $mgml^{-1}$ was observed in HMWC nanopartcle (612 nm, 643 nm), MMWC nanoparticle (556 nm) and LMWC nanoparitcle (525 nm) while high LCD_{50} of 2.0 mgml-1 was recorded in MMWC and LPC solution in its free form. For *F. moniliforme*, LCD_{50} of 0.25 $mgml^{-1}$ was recorded in all nanoparticle size irrespective of the molecular weight of the chitosan used for the synthesis of the nanoparticle while high LCD_{50} of 1.0 $mgml^{-1}$ was observed in MMWC and LMWC; however. LPC also has average LCD_{50} of 0.50 $mgml^{-1}$. The nanoparticle formulation reduces the concentration of the chitosan for maximum percentage mycelia inhibition

Table 4.8: Lowest Concentration Dose at 50% (LCD_{50}) ($mgml^{-1}$) of Chitosan Solution and Chitosan Nanoparticles against *M. oryzae* and Selected Rice Mycotoxin producing Fungi. (*A. flavus, A. fumigatus and F. moniliforme*)

					CS/TPP for Nanoparticle ($mgml^{-1}$)						
	Chitosan solution ($mgml^{-1}$)				HMWC/TPP(v/v)			MMWC /TPP (v/v)	LMWC/TPP(v/v)		
Fungus	HMWC	MMWC	LMWC	CSCS	1:1	1:3	1:5	1:5	1:1	1:3	1:5
					612nm	488nm	643nm	556nm	525nm	468nm	711nm
	LCD_{50}	LCD_{50}	LCD_{50}	LCD_{50}	LCD_{50}	LCD_{50}	LCD_{50}	LCD_{50}	LCD_{50}	LCD_{50}	LCD_{50}
M. oryaxe	1.5	1.5	1.5	>2.0							
A.flavus	1.5	2.0	1.5	1.0	0.25	0.50	0.50	0.50	0.25	0.25	0.50
A.fumigatus	1.5	2.0	1.5	2.0	0.25	0.50	0.25	0.25	0.25	0.50	0.50
F. moniliforme	0.5	0.5	0.5	0.5	0.25	0.25	0.25	0.25	0.25	0.25	0.25

LMWC- Low Molecular Weight Chitosan, MMWC-Medium Molecular Weight Chitosan, HMWC-High MolecularWeght Chitosan, CSCS-Chitosan synthesized from Crab Shell, Cs-Chitosan, Tpp-Sodium Tri-PolyPhosphate, LCD50- Lowest Concentration Dose at which 50% fungal inhibition was recorded

4.2 Discussion

4.2.1 Fungal occurrence on rice seeds

The variation in fungal occurence on rice seeds from both the improved rice varieties and local accession sampled showed that the local accession rice samples were highly infected with fungal pathogens than improved rice varieties. The lower occurrence of fungal in improved varieties might be due to the fact that the varieties were breed for disease resistances as well as other features, hence their resistance to fungal infection and susceptibility. The result is in line with the work of Monajjem *et al.* (2014), that there was a significant difference in fungus severity among the cultivars.

4.2.2 Fungal species isolated from the stored rice seed samples

Eight fungal species belonging to five genera (*Fusarium moniliforme*, *Aspergillus flavus, Aspergillus niger*, *Aspergillus fumigatus, Cladosporium cladosporoides, Rhizopus stolonifer, Paeciliomyces lilacinus,* and *Rhizoctonia solani*) were isolated from all the rice seeds samples collected. The presence of these fungal pathogens may be due to their ubiquitous nature (cosmopolitan distribution) as well as their ability to be in anamorphic state for a long period of time. However, some are xerophillic (low water content a_w at or below 0.85), or thermophilic (temperatures as high as 50 °C and possibly 60 °C) thus they are major crop spoilage organisms, growing on stored cereals, therefore colonization of food and feedstuff can result in its contamination with serious toxin.

The isolates were earlier reported by Ora *et al*. (2011), Ahmed *et al*. (2013), and Monajjem *et al.* (2014) to be fungal species associated with seed-borne diseases of rice and other cereal crops. Makun *et al*. (2007) also reported the same mycotoxin fungal species from rice samples in some parts of Nigeria.

The result is in agreement with earlier findings of Isalm and Ahmed (2017) which revealed that *Fusarium* sp and *Aspergillus* species isolated from rice are associated with grain discolourization diseases complex of rice. Iwuagwu *et al.* (2018) also reported that *Fusarium moniliforme, Aspergillus* spp, among others are mostly isolated from rice seeds.

4.2.3 Percentage frequency of fungal species iolated from the stored rice seed samples

The percentage frequency of fungal species showed that *F. moniliforme* has the highest percentage frequency (38.5 %) followed by *Rhizopus stolonifer* (37 %), *A. niger* (6.5 %) and *A.flavus* (6.2 %). The variation may be due to the fact that most of these fungi are widely distributed in all rice growing environments and mostly prevalent in temperate subtropical climates. However, the high frequency of *F. moniliforme* may be due to the fact that the fungus may be isolated from healthy looking seeds collected from either long-time stored seeds or an infected field harvested seed. The result is in agreement with the work of Butt *et al*. (2011), Karami *et al*. (2012), Uma and Wesely (2013), Iwuagwu *et al.* (2018) whose results also showed that *F.moniliforme* and *Aspergillus* species were the most occurring fungi.

4.2.4. Extraction of chitosan and determination of the degree of deacetylation

The result of the FTIR spectra determines biomolecules that bound specifically on the given chitosan which was between the peaks (3444 to 559 cm^{-1}). The intense broad peak at 3444 cm^{-1} was the characteristic of the hyrogen-bonded O H stretch band. A peak at 3444 was observed for the main functional group of stretching vibrations. The presences of`5 absorption peaks of 1429 cm^{-1} are due to the N-H bonding vibration of protonated amino(-NH) group and C-H bonding vibration of the alkyl group. The

absorption peak at 559 is recognized due to the anti-symmetric stretching vibration of C-O-C bridges and assigned to glucopyranose ring in chitosan matrix. The result of the banding pattern was supported by the report of Anand *et al.* (2018) whose FTIR spectrum band is between 3430 to 599 cm^{-1}

The protocol used for the extraction of chitosan that yield chitosan with 98.6% degree of deactylation was similar to the work of Gaikwad *et al*. (2015), Yong *et al*. (2015) and Ali *et al.* (2018) who reported different protocol for the synthesis of chitosan. The result showed that the best method that produces the highest degree of deacetylation is deproteinization, demineralization, decolourization and deacetylation (DPMCA).

4.2.5 Chitosan inhibition potential

The results of chitosan inhibition potential showed that chitosan solution in free form at concentration of 1.5 % and 2.0 % has Fungistatic properties against *M. oryzae, A. flavus* and *A. fumigatus* while it has fungicidal property against *F. moniliforme.* In the two concentrations, *M oryzae* and the two *Aspergillus* species still grows in the medium although, the growth was inhibited in varying degrees and mostly inhibited by 50% while *Fusarium* did not grow in any of the chitosan irrespective of the molecular weight of the chitosan or the concentration. In the same vein, there is no significant difference in the percentage of inhibition between the two concentrations (1.5 % and 2.0 %). The result also revealed that the inhibition is concentration dependent and is in agreement with the report of Contreras-Cortés *et al*. (2019), that the higher the concentration, the higher the percentage inhibition and vice versa.

The results of the chitosan inhibition against *M. oryzae* showed that the chitosan impairs growth of the blast fungus *Magnaporthe oryzae* and has a pronounced effect

on appressorium-mediated plant infection of the pathogen. The result is in line with the result of Parthasarathy *et al*. (2022) who reported that Nickel-Chitosan Nanoparticle inhibited *M.oryzae* by 64 %. Result is also in conformity with the report of Pham *et al.* (2018) who reported that the higher the concentration of the chitosan and Ag-Nanochitosan, the lower the mycelial growth of *Pyricularia oryzae* (*M.oryaze* Anamorph), hence the higher the percentage inhibition. Lopez-Moya *et al.* (2021) also reported that chitosan is effective for the control of rice blast disease.

It was also observed that the percentage inhibition of the entire test organisms in chitosan synthesised from crab shell (CSCS) was not significantly different from that of MMWC and LMWC, hence chitosan can be synthesised by individual, stakeholders and companies in the Country instead of importing.The result is in agreement with the earlier findings of Zahid (2014), whose report revealed that the dry mycelium weight of *C. gloeosporioides* was significantly (P <0.05) reduced by all types and concentrations of chitosan compared to the control.

4.2.6 Synthesise and characterization of chitosan nanoparticles

The result of the Nanoparticle of the three purchased chitosan (HMWC, MMWC and LMWC) showed that the molecular weight as well as the ratio of chitosan to Sodiun trypolyphosphate (Cs/Tpp) affects the particle size of the chitosan nanoparticle. It was observed that the ratio of 1:1 and 1:5 Cs/Tpp gave a higher nanoparticle size while 1:3 Cs/Tpp produce smaller particle size. The variation in the particle size was affected by the molecular weight of the chitosan as well as the concentration of the sodium trypolyphospate. Particle size and zeta potential are the important properties which may influence the antifungal activity of nanoparticles. Nanoparticles with different particle size or zeta potential may have different mechanisms of inhibition

against fungi The result is in agreement with the work of Anand Raj *et al*. (2015) who reported that the size of chitosan nanopartcle synthesise is affected by the molecular size of the chitosan as well as the ratio of chitosan and Sodium Trypolyphosphate used.

When CS is added to the TPP solution, cross-linking of the negative charge of TPP with the positive charge of CS occurs. This interaction result in the formation of a nano-sized polyelectrolyte complex with the help of stirring. Based on the research of Chen *et al*. (2020), it shows that the concentration of each solution also affect the particle size of the complex. The increasing concentration of CS will increase the size of the particles formed. This also occurs in the increasing concentration of TPP. The results obtained proved that the magnitude of particle positive charge increased progressively with the increasing concentration or molecular weight

The result of characterization by UV Spectroscopy and Dynamic Light Scattering (DLS) showed absorption peaks of the formed CSNPs ranges from 200 to 300nm (Table 6) However, any absorption peak above the range shows that the nanoparticle was not synthesis from the formulation. DLS showed that the particle size ranges from 400 to 700 nm which is supported by the work of Vaezifar *et al*. (2013), that the higher the ratio of TPP to chitosan, the higher the particle size.

4.2.7 Chitosan nanoparticle fungi inhibition potential

The result of the percentage inhibition of the chitosan nanoparticle of the entire test organism showed that at low concentration of 0.25 $mgml^{-1}$ of all the chitosan nanoparticle, the organisms had 50 % inhibition while at a higher concentration of 0.50 $mgml^{-1}$, the organisms (*Aspergillus* species) had 75% inhibition while *Fusarium moniliforme* had 100 % inhibition in both lower and higher concentration.

Nanoparticles have emerged as novel antifungal agents owing to their high surface area to volume ratio and the unique chemical and physical properties, which increases their contact with fungi and their ability to permeate cells. The cationic chitosan nanoparticles (ChNPs) interact with the anionic surfaces of the microbial cell membrane, thereby promoting its antifungal activity. The report shows that there was no significant differences in the rate of fungal inhibition across the different molecular weight of chitosan Nanoporticle, but there was significant difference in the concentrations. This might be due to ratios of Tpp used in the synthesis. The report in inline with the findings of Kheiri *et al.* (2017) and El-Mohamedy *et al.* (2019), they both reported that low molecular weight (LMW) CS and its NPs had high potential of antifungal activity on suppression of fungus growth than high molecular weight (HMW) chitosan nanoparticles

The result is in agreement with the earlier findings of Abdel-Aliem *et al.* (2019) who reported that Chitosan Nanoparticles showed an excellent antifungal activity against some selected fungi in dose dependent manne

In the same vein, the percentage inhibition of the dry mycelia weight could be said to be affected by the ratio of chitosan/Sodium Trypolyphosphate (Cs: Tpp) during the nano-synthesis

4.2.7.1 *Lowest inhibitory concentration dose (LCD_{50}) of free chitosan and chitosan nanoparticle*

The Lowest inhibitory concentration Dose (LCD_{50}) of chitosan in free form was 1.5 % (1.5 $mgml^{-1}$) LCD_{50} while chitosan nanoparticle was 0.5 $mgml^{-1}$ LCD_{50}. At 0.25 $mgml^{-1}$, chitosan nanoparticle was fungicidal against *F. moniliforme* (100 %

inhibition) and Fungistatic against *A.flavus* and *A. fumigatus* (Above 50 % inhibition).

According to Saharan *et al.* (2013), fungi have tendency to produce different level of acids during growth resulting in acidic pH which induces the protonation of amino groups of chitosan leading to damage biomolecules (Brunel *et al.,* 2013). The effect of chitosan as defense enzyme inducer may be another mechanism of the higher antifungal activity of chitosan nanoparticles. The different tolerance of fungi to chitosan nanoparticles may be due to the difference in unsaturated fatty acid composition which is the key part of phospholipids in the cell membrane's lipid bilayer which represent the important factor influence the membrane stability and fluidity. Generally, the antifungal activity of chitosan nanoparticles contributes to larger surface area which enables chitosan nanoparticles to adsorb more tightly onto the surface of fungal cells and disrupt the membrane integrity (Abdel-Aliem *et al.*, 2019)

The different tolerance of the tested fungi to Chitosan nanoparticles may be due to the difference in fatty acid composition. However, the plasma membranes of Chitosan sensitive fungi were shown to have more polyunsaturated fatty acids than Chitosan resistant fungi (Palma-Guerrero *et al.,* 2010; Abdel-Aliem *et al.,* 2019;)

The result is in agreement with the work of Ing *et al.* (2012) and Singh *et al*. (2021), whose result revealed that chitosan in its free form has less inhibitory effect than chitosan nanoparticle.The results is also in agreement with the reports of Bangun *et al*. (2018) whose results reveal that variation of CS and TPP concentrations affect the particle size of nanoparticles suspension.The result is also in agreement with the report of El-Mohamedy *et al*. (2019) whose result reveal that CH-HMW-NPs and

CH-LMW-NPs at lowest concentrations shows complete inhibition (100 %) of the mycelial growth of all tested pathogens.This study therefore claimed that chitosan NPs could be a new development for the generation of chitosan based biofungicides against fungal diseases exploited for delivery

4.2.8: Fungal occurence from chitosan coated and stored rice samples

The result of the fungal occurence from rice samples after coating with two different chitosan (1 % HMWC and MMWC) and stored for period of six months shows that the fungal occurence reduced drastically in some samples while there are no occurence in other samples. The result shows that HMWC reduce the population of the fungal in the samples compared to MMWC (Table 8a and 8b). However, the occurence was significantly reduced or totally eliminated compared to the occurence in the original samples as collected from the farmers (Table 1a and 1b)

4.2.8.1 *Percentage frequency of fungal species isolated from the chitosan coated and stored rice samples*

The percentage frequency of the fungal isolates shows that *Aspergillus species* has higher frequency while *F.moniliforme* had the least frequency in all the samples. However, four fungi genera (*C cladosporoides, R.stolonifer, Paecilomyces lilacinus, and R. stolonifer*) ealier isolated before chitosan coating were not re-isolated.

4.2.9 C**hitosan efficacy on blast pathogenic fungi (*Magnaporthe oryzae*) and other agro-morphological parameters**

The result of the field evaluation of chitosan treatment against blast pathogenic fungi (*M. oryzae*) and other agronomic parameters of two susceptible rice varieties shows that the higher the concentration of chitosan treatment, the reduction in the severity

and incidence of blast pathogen to bearest minimal. However, the treatment did not affect other agronomic parameters such as days to 50 % flowering, panicle count, panicle length, tiller count, plant height, and 1000 seed weight except for grain yield per plot. It was observed that 1.5 % and 2.0 % concentration of HMWC increase the grain yield of both varieties significantly ($P<0.05$). The result further validates the hypothesis that chitosan is known to act as elicitor with plant showing high content of chitin enzyme had a good chance of disease resistance to pathogen. The result is in line with the work Boonlertnirun *et al*. (2008), who reported that the foliar spray of chitosan decreases disease incidence but do not affect plant height, tiller per plant, panicle number, 1000 seed weight but affect the average yield per plot. The report also corroborates the report of FNCA 2016 that chitosan control rice blast within 71.41 to 92.0 %, Therefore, chitosan does not only affect pathogenic fungi but also exhibit growth promoting effect.

CHAPTER FIVE

5.0 CONCLUSION, RECOMMENDATION AND CONTRIBUTION TO KNOWLEDGE

5.1 Conclusion

The research revealed that local accession rice samples were highly infected with fungal pathogens than improved rice varieties. Eight fungal species belonging to five genera (*Fusarium moniliforme, Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus, Cladosporium cladosporoides, Rhizopus stolonifer, Paeciliomyces lalicnus,* and *Rhizoctonia solani*) were isolated from rice seeds samples collected from Niger State.

Fusarium Moniliforme and *Rhizoctonia solani* has the highest and lowest frequency of 38.56% and 0.65% respectively.

The protocol used for the extraction of chitosan, yield chitosan with 98.6 % degree of deactylation. Chitosan solution in free form at concentration of 1.5 % and 2.0 % has Fungistatic properties against *A. flavus* and *A. fumigatus* while it has fungicidal property against *F. moniliforme.*The ratio of 1:1 and 1:5 Cs/Tpp gave a higher chitosan nanoparticle size while 1:3 Cs/Tpp produce smaller particle size

Concentration of 0.25 $mgml^{-1}$ of the all the chitosan nanoparticle, recorded 50% inhibition while at a higher concentration of 0.50 $mgml^{-1}$, the organisms (*Aspergillus* species) had 75 % inhibition while *Fusarium moniliforme* had 100 % inhibition. Chitosan in free form has 1.5 % (1.5 $mgml^{-1}$) LCD_{50} while chitosan nanoparticle has 0.5 $mgml^{-1}$ LCD_{50}.

The chitosan coated and stored rice saamples showed that HMWC reduced the fungal population in the samples compared to MMWC. However, they both reduced the fungal population compared to the original samples as it was collected from the farmers.

The efficacy of chitosan on the field showed that higher concentration of chitosan treatment reduced the severity and incidence of blast disease. However, the treatment did not affect other growth and yield parameters, except for increase in grain yield per plot

5.2 Recommendations

i. Farmers are encouraged to cultivate rice varieties that have been improved for disease resistance
ii. Industries, government, farmers association and non governmental organistion should encourage chitosan production from fisheries shell in Nigeria
iii. Farmers should be encouraged to practice the use of biological control methods such as use chitosan for the treatment of their infected fields and application into their stored produce
iv. More research should be carried out on the method of chitosan and nanochitosan synthesis.

5.3 Contributions of the Research to knowledge

The following are the established knowledge from the research findings

i. Chitosan can be synthesized by individuals or companies
ii. Chitosan in its free form can be used for the suppression of rice fungal infestation during storage

iii. Chitosan can be used for the control of blast fungal pathogen severity and also improve yield of rice on the field.

iv. Chitosan Nanoparticle synthesised reduced the concentration of the chitosan to be used by 75%

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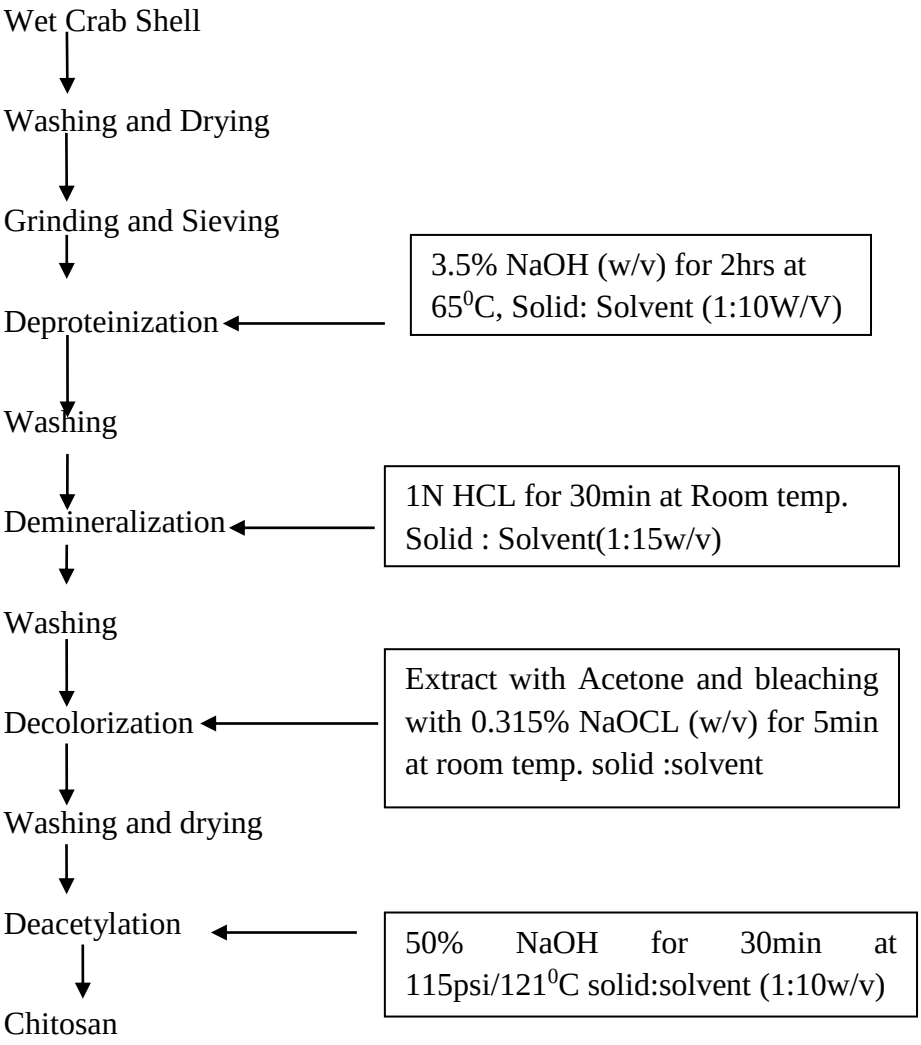
APPENDIX A

Extraction of Chitosan from Crab (*Callinectes amnicola*) Shell



Flow chart of Chitosan Extraction steps

The flow chart above is a stepwise procedure followed for the synthesis of chitosan from crab shell (*Callinectes amnicola*).

APPENDIX B

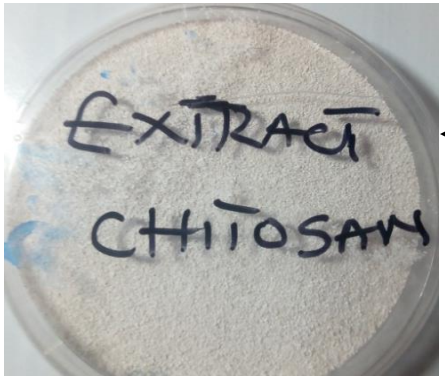
Demineralized crab shell

Decolourization with NaOCL

Deproteinization process

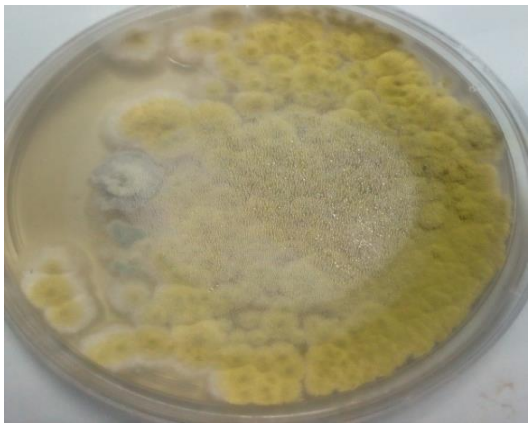
Decolorization process with Acetone

Deacetylation process carried out inside Autoclave at 115psi and 121^0C for 1hrs

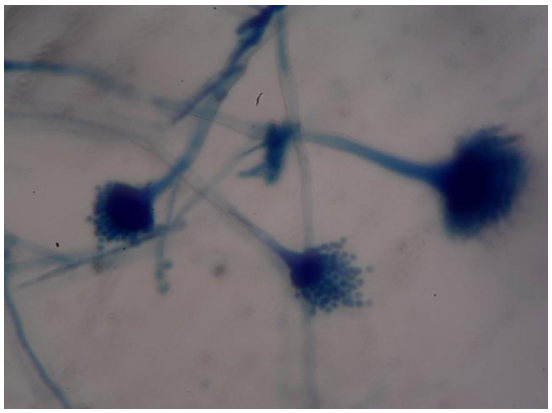


Pictorial Respresentation of Chitosan Extraction Protocol

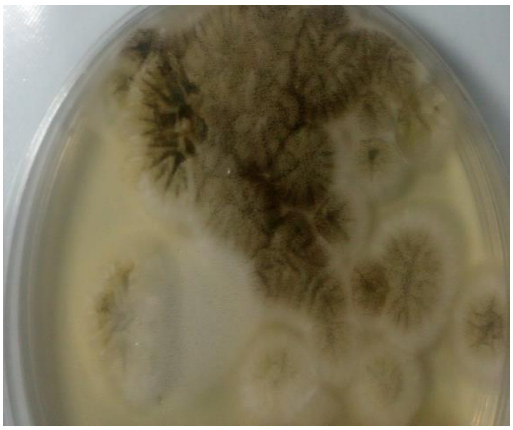
APPENDIX C



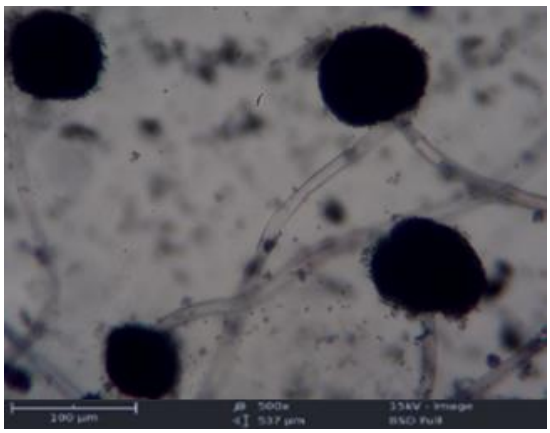
A: Pure culture *Aspergillus flavus*



B*:* Photomicrograph of *Aspergillus flavus*

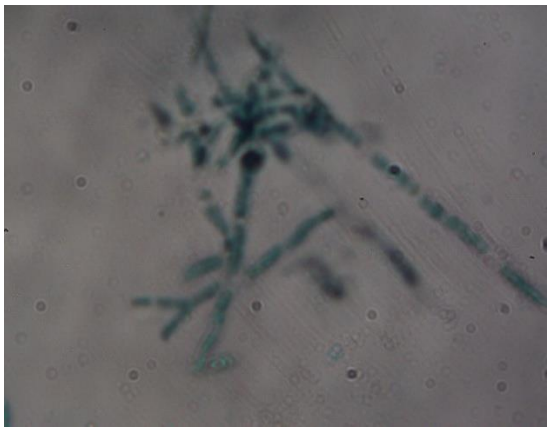


A: Pure culture of *Aspergillus niger*

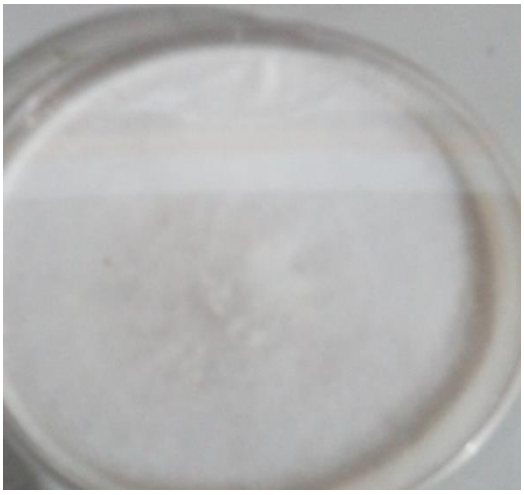


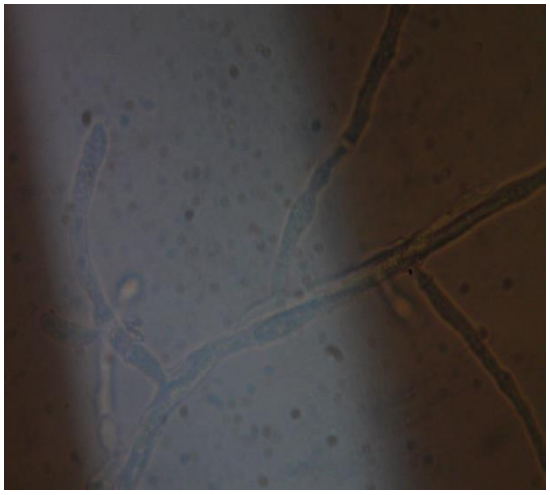
B*:* Photomicrograph of *Aspergillus niger*

A: Pure culture of *Fusarium moniliforme*



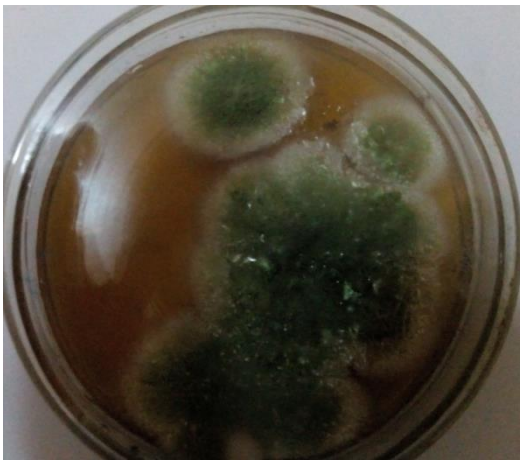
B: Photomicrograph of *F. moniliforme*

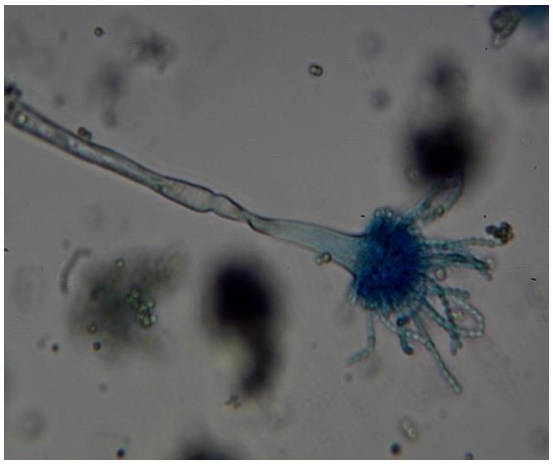




A: Pure culture of *Rhizoctonia solani*

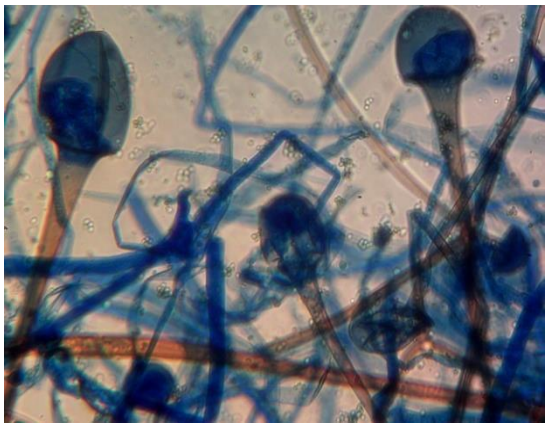
B: Photomicrograph of *Rhizoctonia solani*





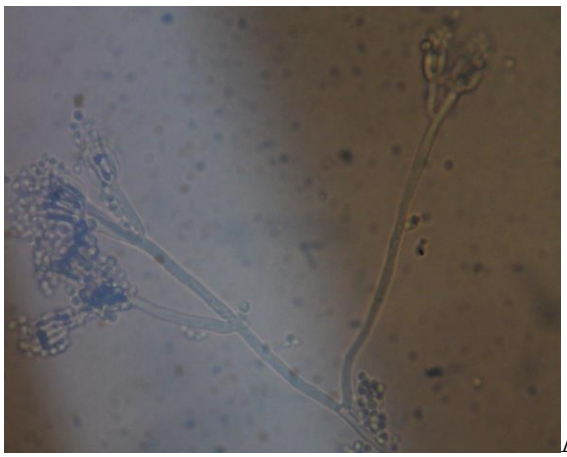
A: Pure culture of *Aspergillus fumigatus*

B: Photomicrograph of *A. fumigatus*

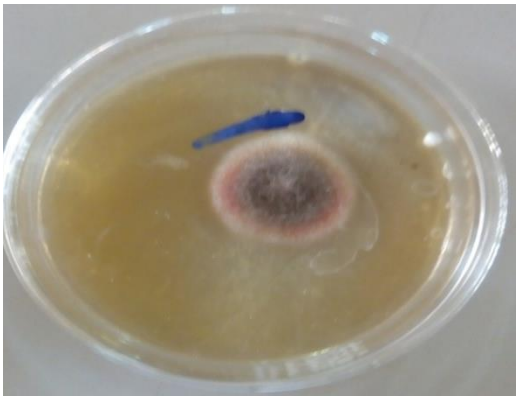
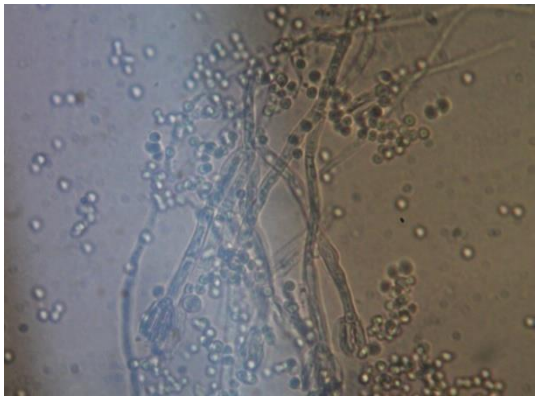


A: Pure culture of *Rhizopus stolonifer*

B: Photomicrograph of *Rhizopus stolonifer*



A:

Pure culture of *Cladosporium cladosporoides* B: Photomicrograph of *Cladosporium cladosporoides sp*

A: Pure culture of *Paeciliomyces lilacinus* B: Photomicrograph of *Paeciliomyces lilacinus*

Morphological and Microscopy Structure of the fungal isolates

APPENDIX D

Morphological and Microscopic Characteristics of Fungal Pathogens Isolated from Rice

Texture	Morphological characteristics of Isolates	Microscopic characteristics	Identified Fungal Isolates
Floppy	White thick cotton-like colony and orange in the reversed	Sepate hyphae, side shaped macroconidia, conidiophores bears conidia containing conidiospores	*Fusarium moniliforme*
Velvety	Light green/yellowish colony with white to cream edge	Sepate hyphae, unbranched conidiophores scanty sterigmata	*Aspergillus flavus*
Powdery	Dark green colony with whitening powdery that grows moderately. The reverse side is cream	Septate hyphae, unbranched conidiophores with secondary branches metulas. Sterigmata bears round conidia in chain	*Aspergillus fumigatus*
Velvety	Whitish to black pigment that latertum, with conidial production, brownish red on the reversed side	Septate hyphae, unbranched conidiophores from the foot of species	*Aspergillus niger*
Powdery	Whitish to Creamy colony that later turns black	Aseptate hyphae, unbranched, sporangiophores arose from foot of rhizoid. Scattered spores which submerged in agar	*Rhizopus stolonifer*
Powdery	White colony that turns brown when its old	Hyphae are partitioned unto individual cells by septum, and mycelia form a right-angled branching	*Rhizoctonia solani*
Velvety	Dark greenish-black colony	Septate hyphae with erect and pigmented conidiophores and conidia. Conidia are elliptical and cylindrical in shapes and are in branching chains.	*Cladosporium sp*
Powdery	Early stage colonies were white, later changing to wine red; reverse mostly dark brown,	Vegetative hyphae smooth-walled, hyaline, septate .Conidiophores borne on thin-walled hyalin and smooth-walled stalks. Conidia are produced in connected chain	*Paeciliomyce sp*

APPENDIX E

Table of International Rice Research Institute (IRRI) Standarad blast severity score scale

Scale	Disease severity	Host Response
0	Lesion are not present	Resistance (R)
1	Small brown specks of pin points size os larger brown speck without sporulating centre	Resistance (R)
2	Small roundish to slightly elongated, nectrotic grey spots , about 1-2mm in diameter, with a distinct brown margin. Lesions are mostly found in the lower lesion	Resistance (R)
3	Lesions type is same as in scale 2 but a significant number of lesion on upper leaf area	Resistance (R)
4	Typical susceptible blast lesions, 3mm or longer infecting less than 4% of leaf area	Moderately Resistance (MR)
5	Typical susceptible blas lesions infecting 4-10% of the leaf area	Moderately Resistance (MR)
6	Typical susceptible blast lesions infecting 11-25% of the leaf area	Moderately Susceptible (MS)
7	Typical susceptible blast lesions infecting 26-50% of the leaf area	Susceptible(S)
8	Typical susceptible blast lesions infecting 51-75% of the leaf area aand many leaf are dead	Susceptible (S)
9	More than 75% leaf area affected	Susceptible(S)

APPENDIX I

Size Distribution Report by Volume

v2.2

Malvern

Sample Details

Sample Name: LMWC 1:1
SOP Name: D S L.sop
General Notes: Average result created from record number(s): 942 943 944

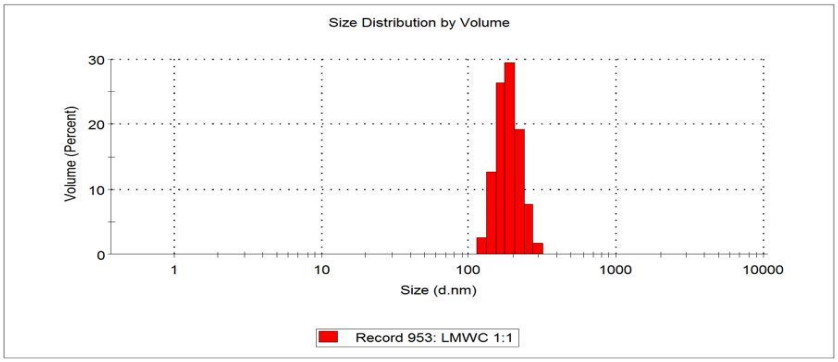
File Name:	D L S.dts	**Dispersant Name:**	Water
Record Number:	953	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	23 March 2021 10:59:44

System

Temperature (°C):	25.0	**Duration Used (s):**	70
Count Rate (kcps):	235.3	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	9

Results

			Size (d.n...	% Volume:	St Dev (d.n...
Z-Average (d.nm):	612.2	**Peak 1:**	188.0	100.0	35.78
PdI:	0.570	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.770	**Peak 3:**	0.000	0.0	0.000
Result quality	Refer to quality report				



APPENDIX II

Size Distribution Report by Volume

v2.2

Malvern

Sample Details

Sample Name: LMWC 1:3

SOP Name: D S L.sop

General Notes: Average result created from record number(s): 933 934 935

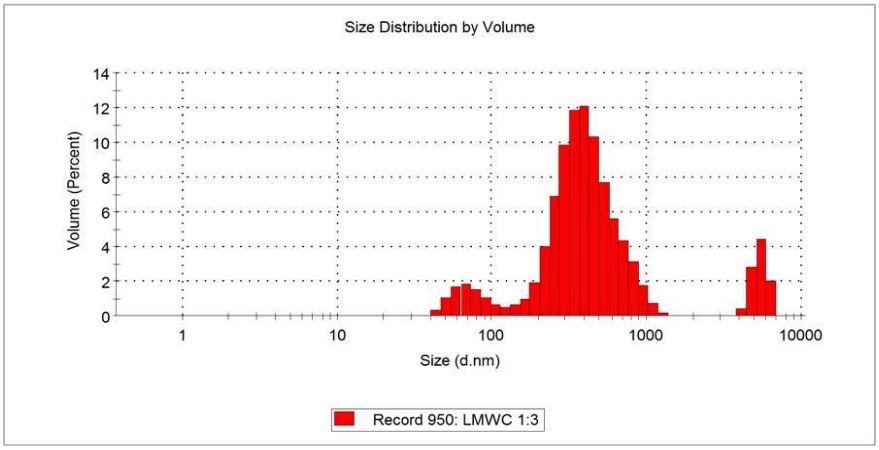
File Name:	D L S.dts	**Dispersant Name:**	Water
Record Number:	950	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	23 March 2021 10:58:42

System

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	527.9	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	10

Results

			Size (d.n...	% Volume:	St Dev (d.n...
Z-Average (d.nm):	488.7	**Peak 1:**	433.4	82.0	191.1
PdI:	0.536	**Peak 2:**	5462	9.6	639.7
Intercept:	0.558	**Peak 3:**	74.03	8.4	20.12
Result quality	Refer to quality report				



APPENDIX III

Size Distribution Report by Volume

v2.2

Malvern

Sample Details

Sample Name: LMWC 1:5

SOP Name: D S L.sop

General Notes: Average result created from record number(s): 945 946 947

File Name:	D L S.dts	**Dispersant Name:**	Water
Record Number:	954	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	23 March 2021 11:00:02

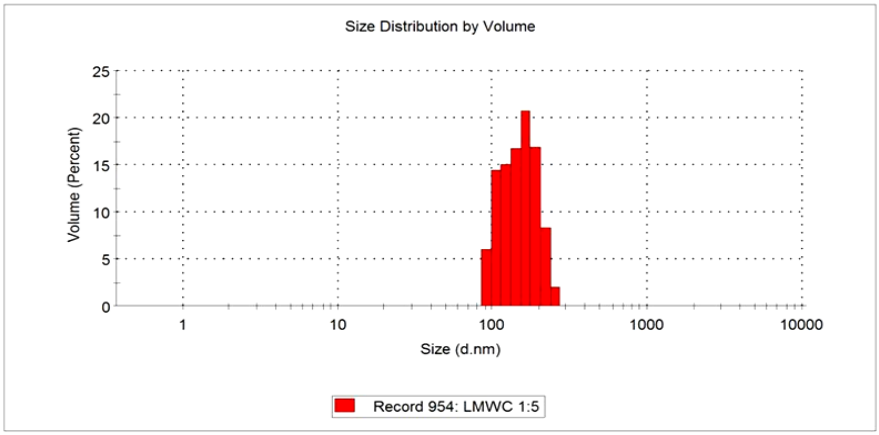
System

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	470.7	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	10

Results

			Size (d.n...	% Volume:	St Dev (d.n...
Z-Average (d.nm):	643.4	**Peak 1:**	152.1	100.0	39.35
PdI:	0.587	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.963	**Peak 3:**	0.000	0.0	0.000

Result quality Refer to quality report



APPENDIX IV

Size Distribution Report by Volume

v2.2

Malvern

Sample Details

Sample Name: MMWC 1:5

SOP Name: D S L.sop

General Notes: Average result created from record number(s): 927 928 929

File Name:	D L S.dts	**Dispersant Name:**	Water
Record Number:	949	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	23 March 2021 10:58:14

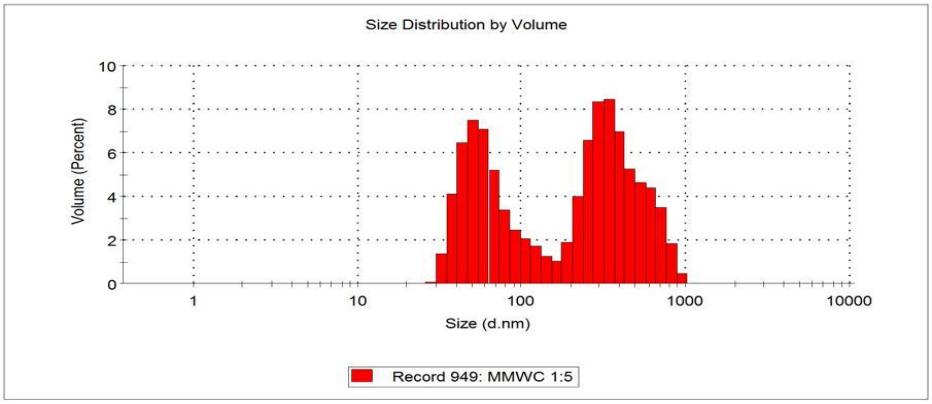
System

Temperature (°C):	25.0	**Duration Used (s):**	70
Count Rate (kcps):	233.3	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	10

Results

			Size (d.n...	% Volume:	St Dev (d.n...
Z-Average (d.nm):	556.5	**Peak 1:**	66.47	43.2	29.62
PdI:	0.570	**Peak 2:**	404.8	56.8	169.0
Intercept:	0.788	**Peak 3:**	0.000	0.0	0.000

Result quality Refer to quality report



APPENDIX V

Size Distribution Report by Volume

v2.2

Malvern

Sample Details

Sample Name: HMWC 1:1

SOP Name: D S L.sop

General Notes: Average result created from record number(s): 936 937 938

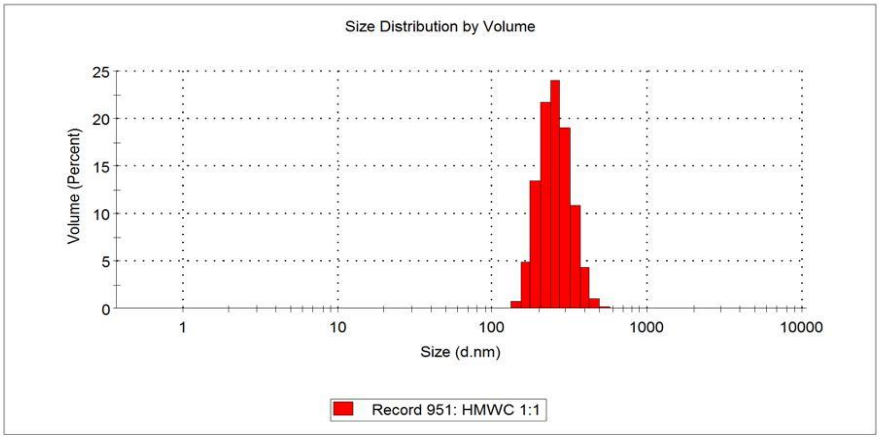
File Name:	D L S.dts	**Dispersant Name:**	Water
Record Number:	951	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	23 March 2021 10:59:08

System

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	382.2	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	9

Results

			Size (d.n...	% Volume:	St Dev (d.n...
Z-Average (d.nm):	525.4	**Peak 1:**	258.9	100.0	61.48
PdI:	0.483	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.647	**Peak 3:**	0.000	0.0	0.000
Result quality	Refer to quality report				



APPENDIX VI

Size Distribution Report by Volume

v2.2

Malvern

Sample Details

Sample Name: HMWC 1:3

SOP Name: D S L.sop

General Notes: Average result created from record number(s): 921 922 923

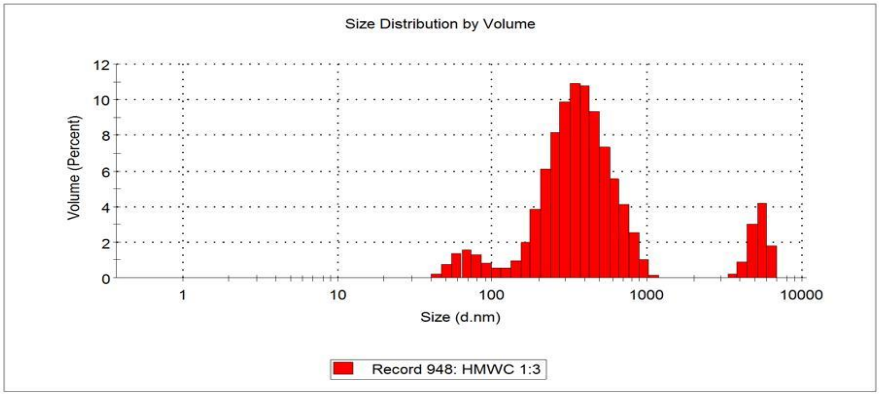
File Name:	D L S.dts	**Dispersant Name:**	Water
Record Number:	948	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	23 March 2021 10:57:55

System

Temperature (°C):	25.0	**Duration Used (s):**	70
Count Rate (kcps):	237.9	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	9

Results

			Size (d.n...	% Volume:	St Dev (d.n...
Z-Average (d.nm):	468.3	**Peak 1:**	401.0	82.8	175.8
PdI:	0.433	**Peak 2:**	5316	10.1	724.7
Intercept:	0.637	**Peak 3:**	75.76	7.1	20.90
Result quality	Refer to quality report				



APPENDIX VII

Size Distribution Report by Volume

v2.2

Malvern

Sample Details

Sample Name: HMWC 1:5

SOP Name: D S L.sop

General Notes: Average result created from record number(s): 939 940 941

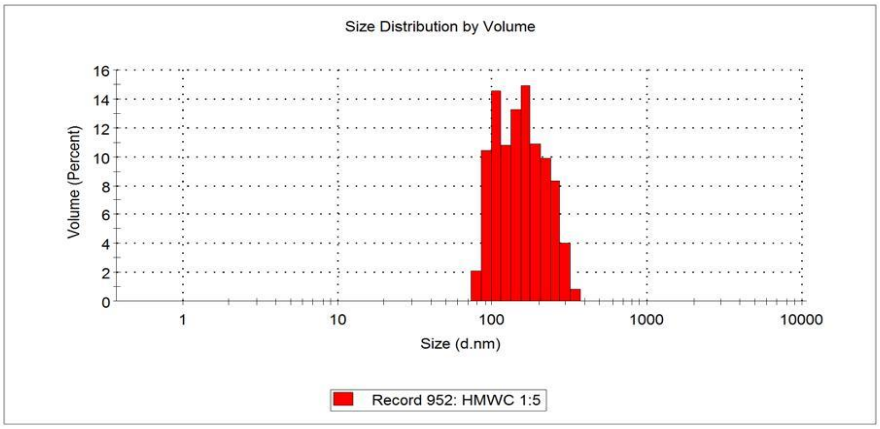
File Name:	D L S.dts	**Dispersant Name:**	Water
Record Number:	952	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	23 March 2021 10:59:29

System

Temperature (°C):	25.0	**Duration Used (s):**	80
Count Rate (kcps):	191.1	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	9

Results

			Size (d.n...	% Volume:	St Dev (d.n...
Z-Average (d.nm):	711.7	**Peak 1:**	105.0	34.2	13.29
PdI:	0.631	**Peak 2:**	185.0	65.8	51.87
Intercept:	1.13	**Peak 3:**	0.000	0.0	0.000
Result quality	Refer to quality report				



APPENDIX A

ANOVA Tables for Blast severity at the Appearance of symptoms

Source	DF	SS	MS	F-value	Pr(>F)
Block	2	57.000	28.500	18.490	0.051
Varieties	1	1.260	1.260	0.820	0.461
Error(a)	2	3.083	1.542		
Conc	3	28.031	9.344	2.380	0.121
Varieties:Conc	3	11.948	3.983	1.020	0.420
Error(b)	12	47.083	3.924		
MW Chitosan	3	16.865	5.622	2.590	0.064
Varieties:MW Chitosan	3	10.115	3.372	1.550	0.213
Subplot:SubSubplot	**9**	**45.260**	**5.029**	**2.320**	**0.030**
Varieties:Conc:MW Chitosan	9	28.844	3.205	1.480	0.184
Error(c)	48	104.167	2.170		
Total	95	353.656			

APPENDIX B

ANOVA Table for Blast severity after treatment

Source	DF	SS	MS	F-value	Pr(>F)
Block	2	5.083	2.542	1.970	0.337
Varieties	1	0.167	0.167	0.130	0.754
Error(a)	2	2.583	1.292		
Subplot	3	12.583	4.194	2.400	0.119
Varieties:Conc	3	1.417	0.472	0.270	0.846
Error(b)	12	21.000	1.750		
MW Chitosan	3	3.417	1.139	0.840	0.480
Varieties:MW Chitosan	3	5.250	1.750	1.290	0.290
Conc:MW Chitosan	9	16.833	1.870	1.370	0.226
Varieties:Contosnc:MW Chitosan	**9**	**30.167**	**3.352**	**2.460**	**0.021**
Error(c)	48	65.333	1.361		
Total	95	163.833			

APPENDIX C

ANOVA Table for Blast Incidence at Appearance of Symptoms

Source	DF	SS	MS	F-value	Pr(>F)
Block	2	2976.521	1488.26	16.6	0.0568
Varieties	1	54	54	0.6	0.5189
Error(a)	2	179.3125	89.6562		
Conc	3	433.875	144.625	1.3	0.3209
Varieties:Conc	3	357.0833	119.0278	1.07	0.3998
Error(b)	12	1339.667	111.6389		
MW Chitosan	3	136.7083	45.5694	0.54	0.6563
Varieties:MW Chitosan	3	479.75	159.9167	1.9	0.1422
Conc:MW Chitosan	9	774.0417	86.0046	1.02	0.4367
Varieties:Conc:MW Chitosan	**9**	**1604.5**	**178.2778**	**2.12**	**0.0461**
Error(c)	48	4040.5	84.1771		
Total	95	12375.96			

APPENDIX D

ANOVA Table for Blast Incidence at 42 days after Transplanting

Source	DF	SS	MS	F-value	Pr(>F)
Block	2	696.5833	348.2917	10.23	0.089
Varieties	1	38.7604	38.7604	1.14	0.3977
Error(a)	2	68.0833	34.0417		
Conc	3	180.6146	60.2049	1.28	0.3249
Varieties:Conc	3	36.6146	12.2049	0.26	0.8528
Error(b)	12	563.3333	46.9444		
MW Chiotsan	3	48.1146	16.0382	0.48	0.6988
Varieties:MW Chitosan	3	104.6146	34.8715	1.04	0.3834
Conc:MW Chitosan	9	335.1771	37.2419	1.11	0.3735
Varieties:Conc:MW Chitosan	9	276.5104	30.7234	0.92	0.5193
Error(c)	48	1609.333	33.5278		
Total	95	3957.74			

APPENDIX E

ANOVA Table for Blast Incidence at 63 days after Transplanting

Source	DF	SS	MS	F-value	Pr(>F)
Block	2	129.1458	64.5729	3.44	0.2251
Varieties	1	38.7604	38.7604	2.07	0.2872
Error(a)	2	37.5208	18.7604		
Conc	3	79.3646	26.4549	1.76	0.2087
Varieties:Conc	3	11.0312	3.6771	0.24	0.8638
Error(b)	12	180.6667	15.0556		
MW Chitosan	3	9.6979	3.2326	0.18	0.9112
Varieties:MW Chitosan	3	22.8646	7.6215	0.42	0.7407
Conc:MW Chitosan	9	198.6771	22.0752	1.21	0.3103
Varieties: Conc:MW Chitosan	9	132.8437	14.7604	0.81	0.6093
Error(c)	48	874.6667	18.2222		
Total	95	1715.24			

APPENDIX F

ANOVA Table for Blast Incidence at 90days after Transplanting

Source	DF	SS	MS	F-value	Pr(>F)
Block	2	77.1458	38.5729	0.76	0.5679
Varieties	1	60.1667	60.1667	1.19	0.3898
Error(a)	2	101.3958	50.6979		
Conc	3	216.0833	72.0278	2.42	0.1168
Varieties:Conc	3	31.25	10.4167	0.35	0.79
Error(b)	12	357.2917	29.7743		
MW Chitosan	3	103.5833	34.5278	1.17	0.3304
Varieties:MW Chitosan	3	173.5833	57.8611	1.96	0.1321
Conc:MW Chitosan	9	341.3333	37.9259	1.29	0.2687
Varieties: Conc:MW Chitosan	9	432.6667	48.0741	1.63	0.1333
Error(c)	48	1414.833	29.4757		
Total	95	3309.333			

APPENDIX G

ANOVA Table for Days to 50% flowering

Source	DF	SS	MS	F-value	Pr(>F)
Block	2	223.3125	111.6562	18.51	0.0512
Varieties	1	900.375	900.375	149.28	0.0066
Error(a)	2	12.0625	6.0312		
Conc	3	27.0833	9.0278	1.34	0.3081
Varieties:Conc	3	13.7083	4.5694	0.68	0.5825
Error(b)	12	80.9583	6.7465		
MW Chitosan	3	4.0833	1.3611	0.24	0.8672
Varieties:MW Chitosan	3	11.2083	3.7361	0.66	0.5796
Conc:MW Chitosan	**9**	**135**	**15**	**2.66**	**0.0138**
Varieties: Conc:MW Chitosan	9	31.7083	3.5231	0.62	0.7707
Error(c)	48	271	5.6458		
Total	95	1710.5			

APPENDIX H

ANOVA Table Average Panicle Count

Source	DF	SS	MS	F-value	Pr(>F)
Block	2	12.2408	6.1204	1.21	0.4522
Varieties	1	3.4504	3.4504	0.68	0.4955
Error(a)	2	10.1058	5.0529		
Conc	3	1.6546	0.5515	0.22	0.884
Varieties:Conc	3	9.1312	3.0437	1.19	0.3559
Error(b)	12	30.7667	2.5639		
MW Chitosan	3	2.0646	0.6882	0.29	0.8345
Varieties:MW Chitosan	3	3.0013	1.0004	0.42	0.7413
Conc:MW Chitosan	9	4.2338	0.4704	0.2	0.9935
Varieties: Conc:MW Chitosan	9	22.2238	2.4693	1.03	0.4303
Error(c)	48	115.0467	2.3968		
Total	95	213.9196			

APPENDIX I

ANOVA Table for Average Panicle Length

Source	DF	SS	MS	F-value	Pr(>F)
Block	2	12.0506	6.0253	1.9	0.3453
Varieties	1	0.3541	0.3541	0.11	0.7703
Error(a)	2	6.3547	3.1774		
Conc	3	4.6776	1.5592	1.56	0.2495
Varieties:Conc	3	1.3892	0.4631	0.46	0.7125
Error(b)	12	11.9693	0.9974		
MW Chitosan	**3**	**8.618**	**2.8727**	**2.81**	**0.0492**
Varieties:MW Chitosan	**3**	**9.0923**	**3.0308**	**2.97**	**0.0412**
Conc:MW Chitosan	9	17.3879	1.932	1.89	0.0761
Varieties: Conc:MW Chitosan	9	7.0401	0.7822	0.77	0.648
Error(c)	48	49.0345	1.0216		
Total	95	127.9682			

APPENDIX J

ANOVA Table for Average Tiller Count

Source	DF	SS	MS	F-value	Pr(>F)
Block	2	9.9358	4.9679	1.74	0.3653
Varieties	1	0.135	0.135	0.05	0.8481
Error(a)	2	5.7175	2.8587		
Conc	3	1.4883	0.4961	0.2	0.8938
Varieties:Conc	3	14.7283	4.9094	1.99	0.1696
Error(b)	12	29.6333	2.4694		
MW Chitosan	**3**	**30.895**	**10.2983**	**3.24**	**0.0302**
Varieties:MW Chitosan	3	3.9683	1.3228	0.42	0.7424
Conc:MW Chitosan	9	57.3217	6.3691	2	0.0596
Varieties: Conc:MW Chitosan	9	36.3683	4.0409	1.27	0.2773
Error(c)	48	152.6867	3.181		
Total	95	342.8783			

APPENDIX K

ANOVA Table for Average Plant Heights

Source	DF	SS	MS	F-value	Pr(>F)
Block	2	1132.099	566.0495	15.38	0.0611
Varieties	1	87.9751	87.9751	2.39	0.2622
Error(a)	2	73.6315	36.8157		
Conc	3	14.4186	4.8062	0.1	0.9599
Varieties:Conc	3	27.417	9.139	0.19	0.9043
Error(b)	12	591.7813	49.3151		
MW Chitosan	3	175.907	58.6357	1.95	0.134
Varieties:MW Chitosan	3	80.7886	26.9295	0.9	0.4502
Conc:MW Chitosan	9	506.1126	56.2347	1.87	0.0796
Varieties: Conc:MW Chitosan	9	396.4209	44.0468	1.47	0.1882
Error(c)	48	1442.908	30.0606		
Total	95	4529.46			

APPENDIX L

ANOVA Table for 1000 Seed Weight

Source	DF	SS	MS	F-value	Pr(>F)
Block	2	196.628	98.314	86.94	0.0114
Varieties	**1**	**148.5535**	**148.5535**	**131.36**	**0.0075**
Error(a)	2	2.2617	1.1309		
Conc	3	1.0239	0.3413	0.28	0.8383
Varieties:Conc	3	0.5978	0.1993	0.16	0.9186
Error(b)	12	14.5894	1.2158		
MW Chitosan	3	7.5712	2.5237	1.44	0.2424
Varieties:MW Chitosan	3	2.1914	0.7305	0.42	0.7415
Conc:MW Chitosan	9	13.1106	1.4567	0.83	0.5904
Varieties: Conc:MW Chitosan	9	14.7297	1.6366	0.93	0.5044
Error(c)	48	84.0548	1.7511		
Total	95	485.3118			

APPENDIX M

ANOVA Table for Grain Yield per Plot

Source	DF	SS	MS	F-value	Pr(>F)
Block	2	1970447	985223.5	8.24	0.1082
Varieties	1	496512.7	496512.7	4.15	0.1785
Error(a)	2	239178.4	119589.2		
Conc	3	20245.38	6748.458	0.14	0.9325
Varieties:Conc	3	68105.08	22701.69	0.48	0.7026
Error(b)	12	568335.2	47361.26		
MW Chitosan	3	135052.2	45017.4	0.84	0.4774
Varieties:MW Chitosan	3	115916.8	38638.92	0.72	0.5432
Conc:MW Chitosan	9	644665.7	71629.52	1.34	0.2418
Varieties: Conc:MW Chitosan	9	438229.2	48692.13	0.91	0.5235
Error(c)	48	2564900	53435.42		
Total	95	7261588			