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INYUVESI YAKWAZULU-NATALI

Management of *Aspergillus flavus* Link Infections in Groundnut Seeds Using Hot Water Treatment and Biological Control Agents.

By

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DISSERTATION SUMMARY

Groundnut (*Arachis hypogaea* L.) is the 13th most important crop and classified as a cash flow crop. Groundnuts' economic importance includes food and fodder purposes in the agriculture and food industries. The major constraint in the cultivation of groundnuts is *Aspergillus flavus* Link contaminating the kernels, subsequently resulting in aflatoxin contamination. Aflatoxins are highly toxic secondary metabolites produced by fungi of the genus *Aspergillus*. Under favourable conditions, A. *flavus* grows and develop in groundnuts at pre and postharvest. *A. flavus* causes yellow mould disease and can cause severe damage to the kernel. Cultural management practices are used to control yellow mould; however, they are often ineffective. Fungicides are considered the best available method for managing yellow mould, but they are inadequate in achieving *A. flavus* inhibition; therefore, alternative control strategies and integrated strategies are needed to properly manage the disease.

This study was conducted to evaluate the efficacy of using potential yeast and *Bacillus* spp. with hot water treatment (HWT) in controlling *A. flavus* in groundnut seeds at optimum temperature x time combination without affecting seed germination rate and seed vigour. The efficacy of the treatments in minimizing aflatoxin concentration in groundnuts were also evaluated.

In vitro screening trials were conducted to select the best yeast and *Bacillus* spp. antagonists from 169 yeast isolates and 60 *Bacillus* spp. isolated from leaves of different plant species. *In vitro* screening was carried out using the dual culture technique, and data was presented as average percentage inhibition. Both *Bacillus* and yeast isolates were grouped according to their *in vitro* performance, and the percentage inhibition data was subjected to Analysis of Variance (ANOVA) using Statistical Analysis System (SAS Version 9.4.). The best 10 yeast and best 10 *Bacillus* isolates were selected for secondary screening. The best two *Bacillus* spp. and best two yeast isolates were used as potential biological control agents in the *in vivo* experiments. The in *vivo* trial was repeated once. From the screening trials, the best performing isolates were; Isolate CC1y (yeast) with 72.6% inhibition, Isolate PF3y (yeast) with 70.8% inhibition, Isolates LM1b (*Bacillus* spp.) with 70.3% inhibition and Isolate PTP1b (*Bacillus* spp.) with 68.6%. inhibition. Only 1.7% of the 169 yeast isolates provided *A. flavus* mycelial inhibition greater than 70%**.** The best performing yeast antagonists were isolates from the spider plant, *Chlorophytum comusum* (Thunb.), Jacques and protea flower, *Protea cynaroides* (L.) L. Moreover, only 1.6% of the 60 *Bacillus* isolates provided a more significant mycelial inhibition

with average inhibition of 70%. The best performing *Bacillus* species were isolated from citrus, *Citrus x limon* (L.), and pink purslane, *Portulaca pilosa* (L.). For *in vitro* hot water treatment (HWT) experiments, the best temperature x time combination provided the least percentage infection, with a significant reduction in disease intensity over time (AUPDC) and a non-significant reduction in seed germination rate. The best temperature x time combination was at 40ºC for 60 seconds, followed by 40ºC for 20 seconds. The 40ºC for 60 seconds showed the least mycelial growth of *A. flavus* (*in vivo*), with the least disease progress over time and stimulating the best germination rate of treated seeds. The 40ºC for 60 seconds was the best combination of all the 17 treatments with the least/worst treatment at 75ºC for 60 seconds. The number of seed infections was recorded over two weeks. From the *in vitro* screening trials, the best two performing yeast spp. and best two *Bacillus* spp. were sent to Inqaba Biotechnical Industries (Pty) Ltd for molecular characterization and identification to species level. Isolates were identified as follows: Isolate CC1y as *Suhomyces kilbournensis* KU751783, Isolate PF3y as *Rhodotorula mucilaginosa* MK267619.1, KY076610.1, Isolate LM1b as *Bacillus cereus* JX218990.1, and Isolate PTP1b as *Alcaligenes faecalis* MG746621.1. The best performing combination treatment was HWT + *Suhomyces kilbournensis* in the inhibition of *A. flavus* in groundnut seeds. HWT + *S. kilbournensis* achieved 52% control after 12 weeks storage. *Bacillus cereus* alone provided the lowest aflatoxin concentration of 0.00840 $mgkg^{-1}$ in treated seeds. Under storage conditions, the best treatment for disease reduction did not necessarily produced the least concentration of AFB_1 and the lowest percentage of infection.

In conclusion, the tested yeast, *Bacillus* spp., and hot water treatment were effective in reducing *A. flavus* infections in groundnut seeds. Moreover, the combination of these treatments provided enhanced disease control. For the first time, this study reports the application of hot water treatment combined with biological control agents on groundnuts to manage aflatoxin B_1 and *A. flavus* infections in groundnut seeds.

DECLARATION

I, **Phiwokuhle Zasemangweni Phelele Mazibuko** declare that:

The research reported in this thesis except otherwise indicated is my original work;
This dissertation has not been submitted for any degree or examination at any other university
This dissertation does not contain other person's data, pictures or graphs or other information, unless specifically acknowledged as being sourced from other persons
This dissertation does not contain text, graphics or tables copied and pasted from the Internet unless specifically acknowledged and the source being detailed in the thesis and in the References section.
This dissertation does not contain other person's writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, their words have been re-written, but the general information attributed to them has been referenced.

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To my friends, thank you for the laughs, support and for keeping me sane. To everyone who has offered a helping hand and a listening ear, I am truly grateful.

DEDICATION

To my superwoman mother for the unquestionable and unconditional love, spiritual warrior and unending support throughout my studies.

May God bless you abundantly.

DISSERTATION INTRODUCTION

Aflatoxins are heat stable chemical compounds found in several crops produced by *Aspergillus flavus* Link. The occurrence of *A. flavus* in grains and seeds diminishes the crops' economic value and limits the yield as a result of aflatoxin contamination **(Mupunga et al., 2014)**. The aflatoxin B_1 and aflatoxin B_2 contaminated fooder feed when consumed by animals result in a by-product called aflatoxin M_1 and M_2. Aflatoxin M_1 and M_2 were found on eggs and milk produced by animals resulting from the indigestible aflatoxin **(Nayak et al., 2017).** The contaminated crops directly affect food safety and present deleterious effects on human and animal health **(Sarma, 2016).** *A. flavus* contaminate groundnuts (*Arachis hypogaea* L.) at pre and postharvest, which constrains proper cultivation of the kernel. *A. flavus* results in a significant loss in groundnuts due to aflatoxins contamination. Groundnuts are originally from Bolivia, South America, and are now cultivated worldwide. They are essential global food and oil crop that underpins agriculture-dependent livelihood strategies meeting food, nutrition, and income security **(Ojiewo et al., 2020).** Confirming its significance in 2016, South Africa experienced a loss of 58% on export trades, which contributed 2% to the seed loss contribution of the GDP **(Sihlobo, 2019).**

Different approaches have been used to manage *A. flavus* and to achieve some level of disease reduction by using certain fungicides and agronomic practices (**Bhatnagar-Mathur et al., 2015).** However, South Africa has limited effective fungicides that can simultaneously achieve *A. flavus* disease reduction and minimize aflatoxin contamination**.** Cultural practices have achieved only limited control **(Mohamed et al., 2018; Achar et al., 2020)**. No single strategy has achieved effective control of *A. flavus*, **Jadon et al., (2015),** hence the integration of multiple management strategies can reduce *A. flavus* incidence providing enhanced disease control. This study aimed to develop an integrated management strategy for *A. flavus* in groundnut seeds using epiphytic yeasts, *Bacillus* spp. and hot water treatment, individually and in combination.

Research objectives

The specific objectives of this study are to:

1. Isolate and screen yeast and *Bacillus* spp. from leaves of different plant species and evaluate the potential antagonists against *Aspergillus flavus in vitro*.

2. Formulate the best combination of temperature and time for the effective control of *A. flavus* which will not hinder seed germination and seed vigour.
3. Identify yeast and *Bacillus* isolates antagonistic to *A. flavus* and further investigate their efficacy in controlling infection by the pathogen in *in vivo* studies.
4. Identify the best combination treatments that minimize aflatoxin B_1 contamination in groundnut seeds in *in vivo* studies.

The dissertation has been written in the form of four chapters. Each chapter is focused on a specific objective of the research that was conducted. With an exception of Chapter One, "Literature Review", the other three chapters were independent studies and were written in the form of research chapters. Each chapter is following the format of a stand-alone research paper. This format is the standard dissertation model that has been adopted by the University of KwaZulu-Natal because it facilitates the publishing of research out of the dissertation far more readily than the older monograph form of dissertation. As such, there is some unavoidable repetition of references, methods and some introductory information between chapters.

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CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Groundnuts (*Arachis hypogaea L.*) are native to South America (Bolivia and adjoining countries), but are now grown throughout the tropical and warm regions of the world **(Ndung'u et al., 2013)**. Groundnuts are legumes for food or fodder purposes, and the third most crucial vegetable protein **(Guchi et al,. 2014)**. The best soil for growth is in well-drained fertile sandy to sandy-loam soil with a pH of 5.5-7.0 **(Taffouo et al., 2010).** Africa's significant groundnuts producers are Nigeria, Sudan, Chad, Cameroon, and Senegal **(Adetunji et al., 2014).** Commercial use of groundnuts includes animal feed, oil, food spread, and pastes, and baking. Groundnuts serves as staple food and cash flow crop for informal traders (domestic cash flow) in Africa. Groundnuts are susceptible to many bacterial and viral infections, and fungal mould species, including *Aspergillus* species **(Ezekiel et al., 2014).** Fungal infections occur at pre- and postharvest stages of the crop because of inferior harvesting methods (injuries on seeds), poor handling and storage.

Aspergillus flavus L. also infect on other food commodities such as maize (*Zea mays* L), wheat (*Triticum aestium L.*), and barley (*Hordeum vulgare L*) **(Amaike & Keller., 2011).** *Aspergillus flavus* produces poisonous secondary metabolites called aflatoxins. They are toxins that cause harm when consumed **(Adetunji et al., 2014)**, and these are also carcinogenic and mutagenic **(Alshannaq et al., 2018).** *Aspergillus flavus* deteriorate groundnut, which leads to economic losses. A seed oil content and seed quality are also lost, pose health hazards to consumers due to aflatoxins production **(Nyirahakizimana et al., 2013).** The influential groups of aflatoxins produced by *A. flavus* are B1, B2, G1, and G2. **(Guchi et al., 2014).** Africa's warm and humid environmental conditions provide suitable conditions for *A. flavus* infections in groundnuts **(Ashiq, 2015).** An effective control and management strategy of *A. flavus* is essential to minimize the loss of seed quality and health risks associated with seed or food commodities exposure to aflatoxins. **(Illa et al., 2020).**

1.2 The pathogen: *Aspergillus flavus* Link.

Aspergillus flavus was described by Link in 1809 **(Schroeckh, 2001).** It is in the Phylum Ascomycota (class *Eurotiomycetes,* Order *Eurotiales,* Family *Trichocomaceae*). *Aspergillus flavus* is a group of moulds found throughout the world. They are saprophytic soil fungus that infects and contaminates seed crops and is the most common type of fungi in the environment. *A. flavus* has two strains, L and S strain, which are the major fungal pathogens infecting groundnuts **(Gilter, 2018).** The L strains belong to Group I with sclerotia greater than 400 µm in diameter. Group II consists of S strains with sclerotia less than 400 µm in diameter. This strain produces a consistently high content of aflatoxin that affects crops and animals alike. The L strain also has a more acidic homoeostatic point and produces fewer sclerotia than the S strain under more limiting conditions **(Williams et al., 2004)**

Other main morphology features include the stipe, conidia, vesicle, metula, and phialide. Conidiophore stipes are hyaline and coarsely roughened, often more noticeable near the vesicle. Conidia are globose to subglobose (3-6 µm in diameter), pale green, and conspicuously echinulate. Some strains produce brownish sclerotia **(Thathana et al., 2017)**. *A. flavus* produces asexual spores, conidia, and the overwintering asexual fruiting bodies, sclerotia. The sexual stage of *A. flavus* has been reported and classified as *Petromyces flavula* **(Amaike and Keller, 2001).** *A. flavus* best-growing temperature conditions are 36-38℃ at a pH of 5.0-7.0. *Aspergillus* is a saprophytic mould, and in warm climates, they are more active **(Pratiwi et al., 2015).** *A. flavus* is best known for producing the family of potent carcinogenic secondary metabolites called aflatoxins **(Cary et al., 2018).** The food and fodder commodities contaminated with aflatoxins have a health risk if consumed by animals and humans. **(Udomkun et al., 2017).** Human consumption of aflatoxin-contaminated food and fodder can lead to an outbreak of aflatoxicosis **(Williams et al., 2004).**

1.3 Host range

A plant is called a host of the pathogen when a pathogen can infect, grow, and finalize its life cycle on it **(Buttergenhaeuser et al., 2014).** *A. flavus* has a wide range of hosts, which include cereal grains, legumes, and tree nuts. *A. flavus* infects many leguminous plants, more than 31 legumes species in 17 genera, which includes groundnuts (**Kachapulula et al., 2017).**

A. flavus can infect leaf tissues, seed, and grains. The germ tube structure, which is used for elongation and tissue infection, can produce numerous extracellular hydrolases that aid the infection process **(Mellon et al., 2007)**

1.3.1. The Host: Groundnuts

Groundnuts originated in Bolivia, South America. Groundnut discovery was during World War 2 as the staple food source **(Hammons, 1994)**. The Portuguese transported it to West Africa and since then, it has been distributed and grown throughout the world, especially in the tropical and warm temperature regions. It has 69 identified species, and only six species are cultivated **(Stalker, 2017).**

Linnaeus first described the cultivated groundnut in 1753. They are commonly known as peanuts or groundnuts. Groundnut is a self-pollinating, indeterminate, annual herbaceous legume crop. The crop belongs to the family Leguminosae /Fabales, Order Fabaceae, genus *Arachis* and species *hypogae*a.

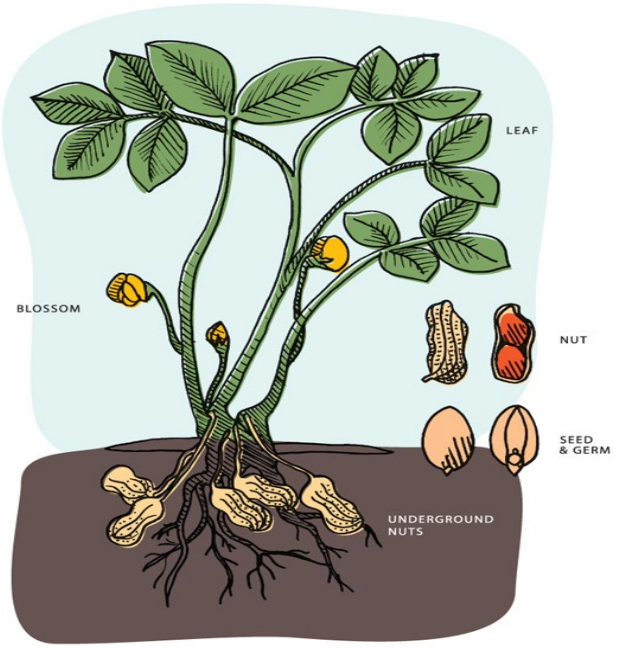

Figure 1.1: Groundnut plant at a full stage of growth above and below the soil **(Boote, 1982)**

1.3.2 Production of groundnuts

The world production of groundnuts has increased over the years. Leading countries in groundnuts production are China, India, Nigeria, and the United States of America. China is leading country in the world with 42.4% of the world's groundnut production, followed by India (14.5%) and Nigeria (7.8%) (Figure 1.2). Nigeria is Africa's leading country in groundnut production and the first to compete at the international trade **(Embaby and Abdel-Galel, 2014).**

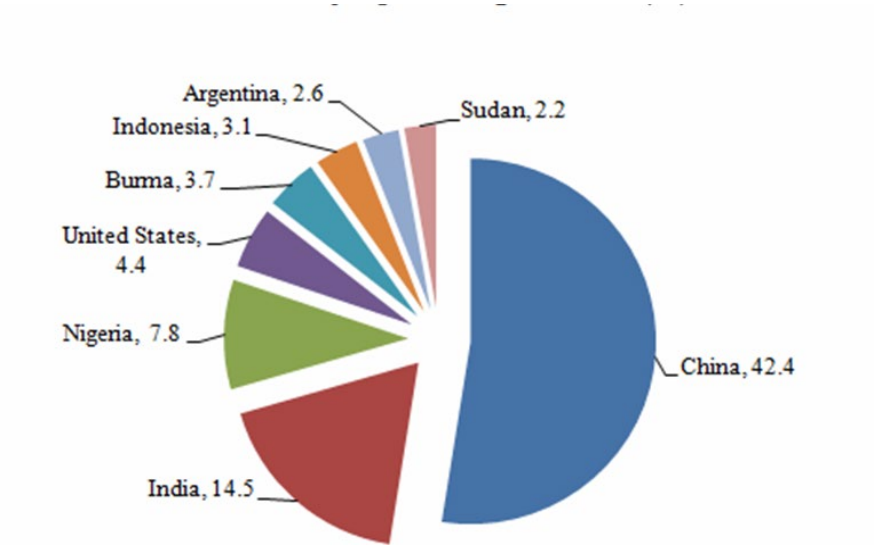

Figure 1.2: World production scale of groundnuts (percentage of division) per country **(Meneses et al., 2014).**

China cultivates over an area of 9.04 million ha **(Yang and Zheng, 2018),** Nigeria 6.7 million ha with a total production of 8.7 million tons (mt). **(Akpo et al., 2020),** the U.S. has 0.7million ha with a total output of 1.6million Mt and India, 4.7 million ha **(Madhusudan, 2018).** More than 63.5% of the world produced groundnuts are used for food purposes, followed by oil production**.**

In Africa, the leading country for groundnut production is Nigeria 30%, followed by Senegal and Sudan, each with about 8%, and Ghana and Chad with about 5% each. They are grown nearly exclusively for domestic use, either for consumption or as a cash crop for small-scale farmers **(Ajeigbe et al., 2015).**

In South Africa, groundnuts are grown in the summer rainfall regions, under irrigated or rainfall conditions **(Mapunga et al., 2017).** Groundnuts are mainly grown for farmer's consumption, especially those with limited resources. Groundnuts production in South Africa occurs in Northern Cape, Free State, Limpopo, and North West province. The percentage production for each province in Figure 1.3. Free State is the leading producing province (37400 tons), North West (34400 tons), Northern Cape (9800 tons), and Limpopo (5250 tons). **(Fletcher and Shi, 2017).**

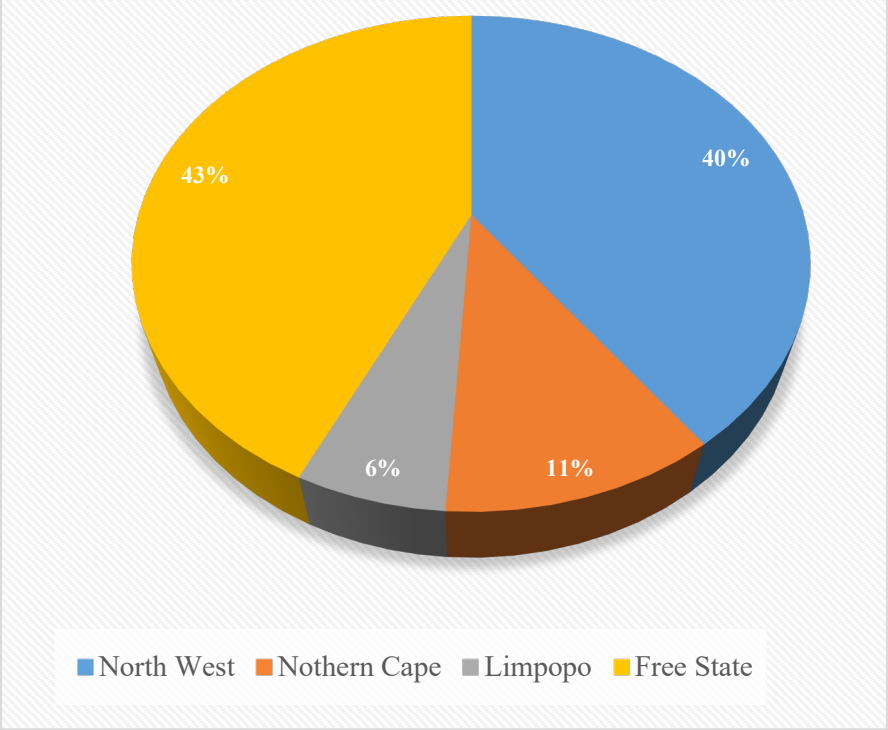

Figure 1.3: South Africa groundnut production by percentage in four major producing provinces **(Ajeigbe at al., 2014)**.

Over the years, production has declined significantly due to drought, which has affected South Africa and globally due to global climate change. To the world production and international trade of groundnuts, South Africa does not produce and contributes significantly to the market.

1.3.3. Consumption of Groundnuts

Groundnuts are grain legumes and oil crop, which are consumed all over the world. A 5% significant increase gradually occurred in the consumption of groundnuts on a global scale, in 2017/18, totalling to 43.1 million metric tons **(Guasch-Ferre et al., 2017).** The top ten countries globally leading in groundnuts consumption are China, India, Nigeria, United States of America, Indonesia, Vietnam, Brazil, Mexico, South Africa, and Canada (Figure 1.4). They were identified based on volumes consumed per country**.** South Africa consumes around 136 168 metric tons per year **(Sihlobo, 2019).**

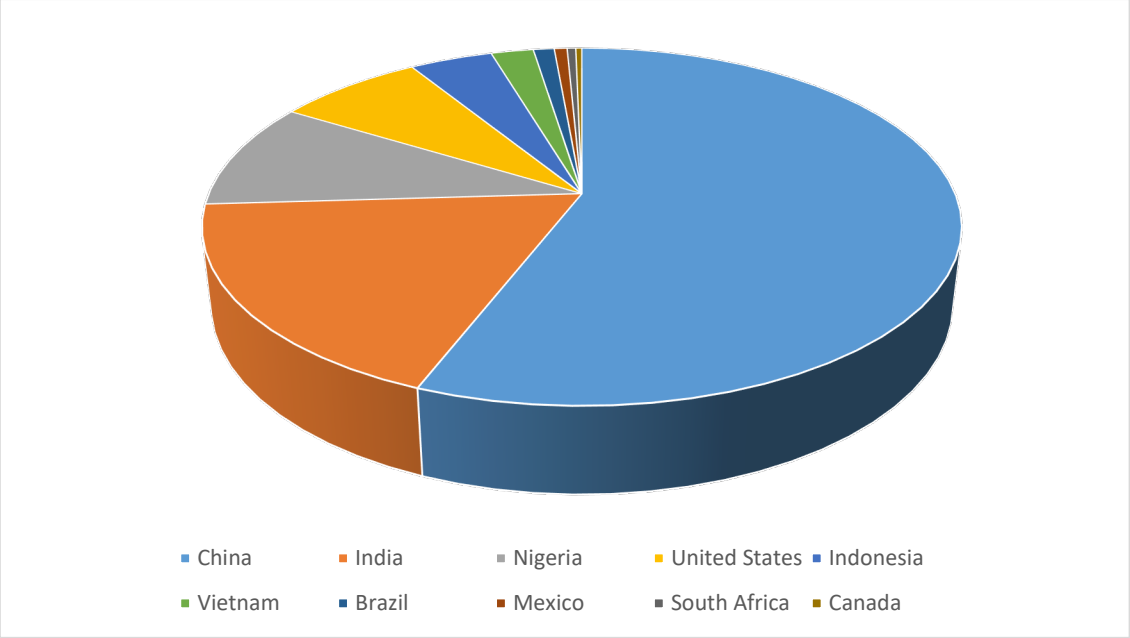

Figure 1.4: Top 10 countries around the world for groundnuts consumption Add Reference

Groundnuts are a good source of vitamin E, omega 3, fiber, antioxidants, and good carbohydrates apart from other health benefits **(Bonku and Yu 2019).** They contain good fats (44–56% fat). It consists of mono- and polyunsaturated fat mostly made up of oleic and linoleic fatty acids**.** They are a good source of protein (30%). Their health benefits include reducing the risk of blood clots rate, lower cholesterol, reduced risk of cardiac arrest, and Alzheimer's disease **(Olatunya et al., 2017).** Table 1.1 shows the details of nutritional facts of groundnuts.

Table 1.1. Nutritional of peanuts per 100 g, adapted from (Arya et al., 2017).

Nutrient	Nutrient Value	Percentage of RDA
Carbohydrates	16.13g	12
Protein	25.80g	46
Total fat	49.24g	165
Dietary Fiber	8.5g	22
Energy	567Kcal	29
Vitamins		
Folates	240µg	60
Niacin	12.066mg	75
Pantothenic acid	1.767m	35
Vitamin E	8.33mg	55.5
Electrolytes		
Sodium	18mg	1
Potassium	705mg	15
Minerals		
Calcium	92mg	9
Copper	1.144mg	127
Iron	4.58mg	57
Magnesium	168mg	42
Manganese	1.934mg	84
Phosphorus	76mg	54

Groundnuts are the best source of edible oil in legumes after green peas and lentil beans **(Guchi et al., 2018)**. In developing African countries, groundnuts are a significant income source. They are an essential cash crop for domestic markets. They significantly contribute to food security and poverty alleviation. Groundnuts uses include food fodder and animal feed. The use of groundnuts in food industries include flavouring products in ice cream, massage oil, and milk. Industrial services include pharmaceuticals like soaps, cold creams, cosmetics, dyes, and emulsions for insect control and fuel for diesel engines **(Mupunga et al. 2014).** In Africa, groundnuts serve as a staple food source. In rural areas of Mpumalanga and KwaZulu-Natal in South Africa, where meat is not affordable, natives of these rural places consume groundnuts

as protein source. South Africa exports groundnut to countries such as Japan (34.7%), Netherlands (13.6%), Switzerland (8.7%), and New Zealand (5.3%) **(SAGIS, 2020).**

1.4 Epidemiology of *Aspergillus flavus*

Aspergillus flavus is a saprophyte in soil (lives on dead and decaying organic matter).The pathogen overwinters (as long as three years) as propagules on decaying matter, plant debris as mycelium, or as sclerotia in the soil **(Bailley et al., 2018).** These serve as primary inoculum. The conidia are spread by wind, insects, and equipment to the new leaf tissue or kernel cause infection. *A. flavus* requires high humidity, low soil moisture 11.5–11.8%, warm to high temperature, 17- 42ºC, the optimum growth and a pH 7.0**. (Alam et al., 2020)**. The spores infect groundnuts while they are still in the soil underground.

1.5 Dispersal and Distribution of *Aspergillus flavus*

A. flavus sclerotia is a survival body formed by mycelia so to survive unfavorable conditions. The sclerotia on the ground is dispersed through wind and insects. The wind/insect-mediated delivery of conidia to above-ground plant parts, such as flowers and developing pegs, occasionally leads to fungal infestation **(Alam et al., 2020).** The wind blows the mycelium/ sclerotia and results in the spores landing on damaged plant surfaces **(Saleem et al., 2017).** Insects damage on plants provide a point of entry into the plant, and so as mechanical injury. The insect damage releases moisture, and that promotes fungal growth within the seed.

During favorable environmental conditions (75 % relative humidity and 38 ºC), infection will occur in the new plants. Spores are transported from field to storage via transportation mechanisms such as trucks, and crop machinery. At the warehouse, the spore's presence and favorable conditions accelerate the infection at postharvest. The storage system with an indoor ventilation system spread spores by forcing in air. Insects damage the seed by piercing mouth during feeding and transmit spores to the wounded sites, encouraging the grain's rapid pathogen colonization **(Agbetiameh et al., 2019).**

1.6 Disease cycle and development of symptoms

The life cycle has two stages: (1) colonization of plant debris in soil and (2) invasion of seeds and grain in actively growing crop plants. *Aspergillus* species infectious cycle is mostly dependent upon host species **(Ojiambo et al., 2018).**

The fungus overwinters either as mycelium in plant debris and sclerotia in the ground. The fungus produces sclerotia, which germinate to produce asexual spores known as conidia and hyphae. Conidia serves as a primary inoculum that is dispersed by the wind and insects (Figure 1.5) **(Islam et al., 2018).** The germination occurs producing foot cells, more branching occurs, and elongation of hyphae creating a mass of hyphae and mycelium. Conidiophores then grow from the foot cells to produce spores on leaf parts, secondary inoculum grow from foot cells to have spores on leaf parts, and *A. flavus* grows on leaves after damage by leaf-feeding insects.

The fungus overwinters either as mycelium in plant debris or as sclerotia in the soil. The contamination rate of aflatoxin depends upon humidity, temperature, storage, and soil conditions. Contamination occurs more when night temperatures are high, and drought stress predisposes plants to aflatoxins. Aflatoxin production occurs at 25℃ and 0.95 water activity (aw) **(Lahouar et al., 2016).**

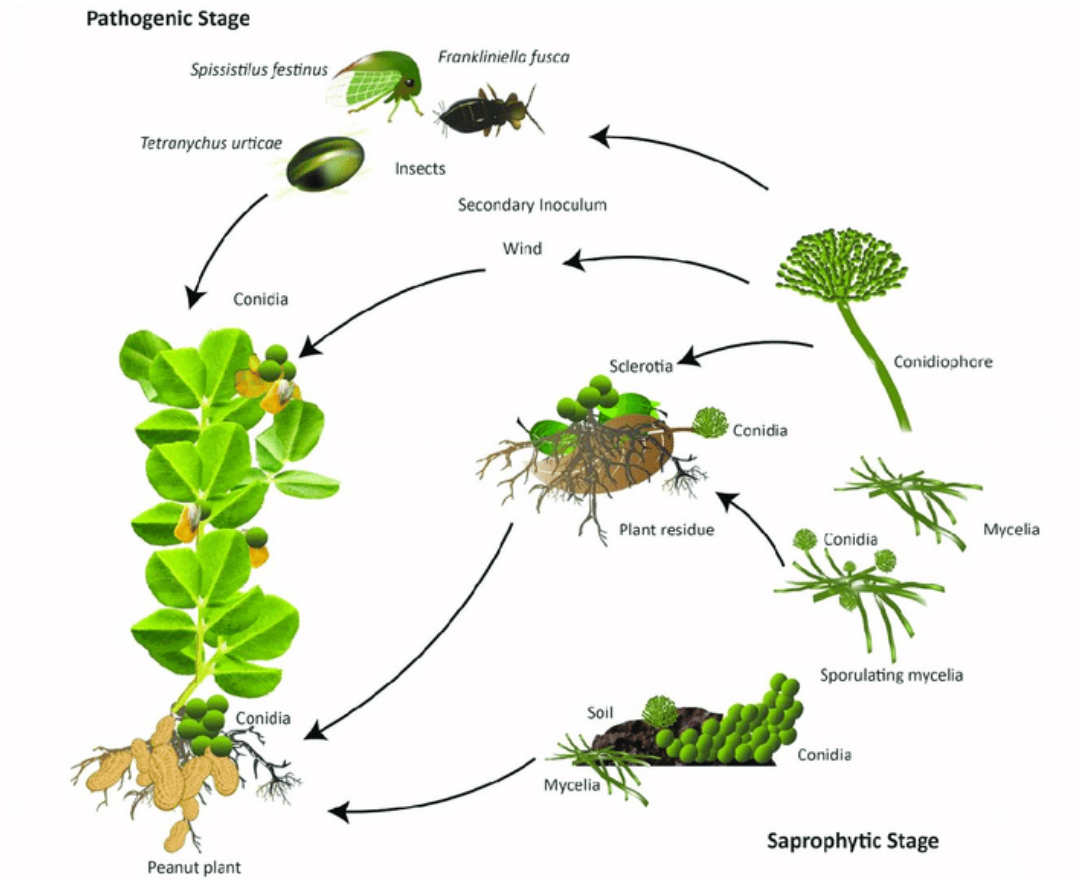

Figure 1.5 The life cycle of *Aspergillus flavus* **(Alam et al., 2020)**

Aspergillus flavus colonies are powdery masses of yellow-green spores (Figure 1.6) on the upper surface and reddish-gold on the lower body. Growth is rapid, and colonies appear downy or powdery in texture. *A. flavus* causes a disease called afla root. Symptoms of afla root disease

include withered and dried groundnut seed. Yellow or greenish spores cover the seed. Cotyledons show necrotic lesions with reddish-brown margins. Seedlings are stunted, leaf size significantly reduced, and pale to light green **(Liang et al., 2006).**

Symptoms of yellow mould disease causes shriveled, and dryness on seed and unemerged seeds. Seedlings already infected by the mold emerge with cotyledons showing necrotic lesions with reddish margins **(Kumari et al., 2017)**. The major problem with *A. flavus* is the effects caused by aflatoxins from the reduction of food quality to animal feed which also has a significant health impact on the consumers.

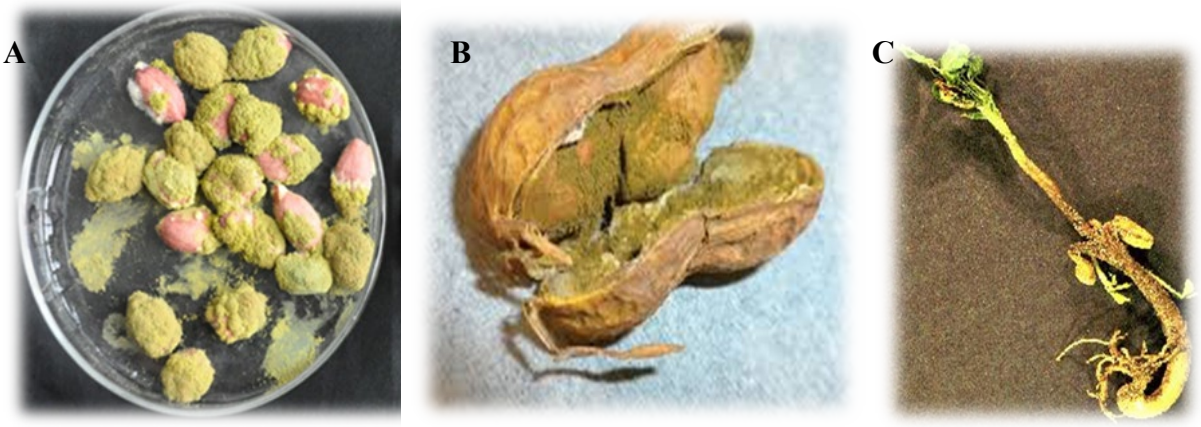

Figure 1.6: Sporulation of *Aspergillus flavus* in *Arachis hypogaea* seed and seedlings.

A: Stored groundnut seeds for seven days at 28℃ incubators, B: shows the infection within the kernel at postharvest and C: shows symptoms of the disease on a young plant in the field **(Guchi et al., 2014).**

1.7 Aflatoxins

Aspergillus flavus produces aflatoxin secondary metabolites that are carcinogenic and toxic to animals and humans. There are four critical toxins regarding food safety and public health aflatoxins: B_1, B_2, G_2, and G_2. **(Norlia et al., 2019)**. The most potent, naturally occurring carcinogen toxin is AFB_1 **(Coppock et al., 2018).** The S strain is called the S strain because of a small and the L strain after the large sclerotia, and produces more aflatoxins. Production of aflatoxins occurs under favourable conditions, which include high temperature (37^{o}C), high

moisture (0.99aw), pH 5-7, and high humidity **(Coppock et al., 2018)**. The pathogen presence in the seed does not guarantee aflatoxins' secretion, but it comprises the seed quality. Resulting in included food/feed quality and safety **(Rajasekaran et al. 2017)**

1.7.1 Effects of aflatoxins on human and animal health

Aflatoxins (AFs) are small molecular weight fungal toxins, which are potent toxic, carcinogenic, immune-suppressive, and teratogenic chemical residues and are common contaminants in foods **(Niyibituronsa et al., 2018).** Aflatoxin binds proteins, minerals, and vitamins so that the body will not absorb nutrients. Aflatoxins cause a health condition called aflatoxicosis. Aflatoxicosis is the poisoning caused by the consumption of substances or foods contaminated with aflatoxin **(Kumari et al., 2017)**. The biotransformation in human bodies cannot digest and get rid of aflatoxins. As a result, aflatoxins are harmful to the liver and kidneys **(Bbosa et al., 2014).** They are also known for causing cancer and liver diseases **(Niyibituronsa et al., 2018).** Acute symptoms of aflatoxicosis include jaundice, lethargy, nausea, haemorrhagic necrosis of liver tissues, bile duct hyperplasia, impaired immune function, malnutrition, stunted growth, and eventually death **(Sirma et al., 2018).** The severity of aflatoxicosis a humans depends on the mycotoxin's toxicity, the extent of exposure, age, and nutritional status of the individual **(Torres et al., 2018).**

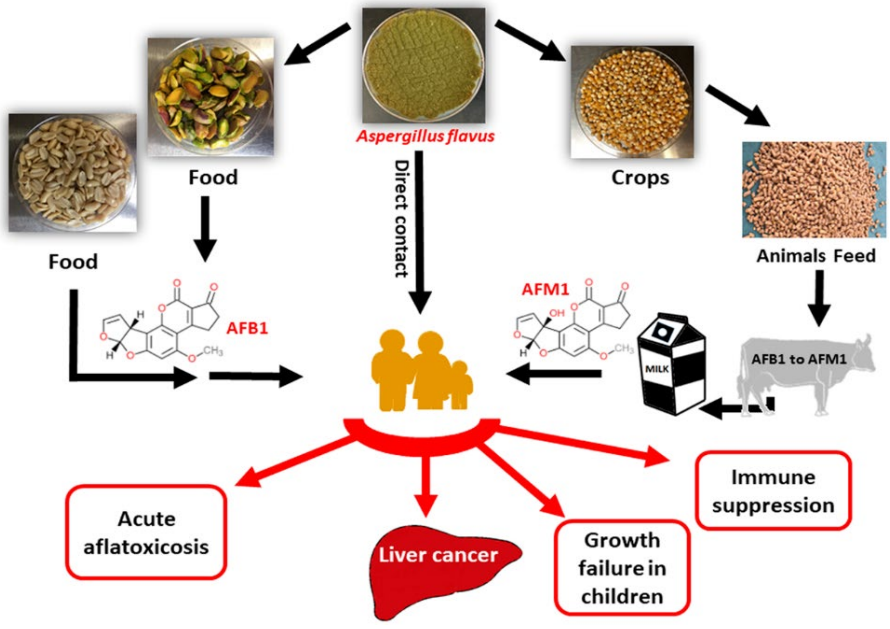

Figure 1.7: A diagram summarising aflatoxins' flow from grains to animals and humans **(Alshannaq et al., 2019).**

Poultry is generally susceptible to AFB_1 and adverse health effects have been reported in turkeys, quail, chickens, and ducks **(Pele et al., 2019).** AFs pose a health risk to livestock; when consumed. They reduce livestock productivity via transfer from feed to the food animals produce (Figure 1.7). AFM1 is aflatoxin derived from AFB1 found in animal products such as milk and eggs **(Sirma et al., 2018)**. Common symptoms of aflatoxins in poultry include reduced feed intake, low growth rate, increased susceptibility to diseases, reduced fertility, and increased mortality **(Peles et al., 2019; Gallo et al., 2015).** The lethal dose in humans was suggested to range from 10 to 20 µg/kg for adults **(Benkerroum.2020)**, Over the years, there have been severe reported cases of aflatoxin poisoining that led to animals and human death in countries like Kenya **(Sirma et al., 2018),** Tanzania (**Kuhamba et al., 2018)**, Uganda **(Lukwango et al., 2019),** and Rwanda **(Niyibituronsa et al., 2018)**.

1.7.2 Aflatoxins regulatory standards

The regulation of aflatoxin content acceptable in commodities was required to set rules and standards that each country has for food security and public health safety reasons. The aim was to limit aflatoxin exposure to the possible lowest level **(Fakruddin et al., 2015).**

The World Health Organization (WHO) has international standards for both animals and humans (Tables1.2 & 1.3), which are in place to regulate aflatoxins exposure. Animals feeding on contaminated commodities pose a risk to animal health as first consumers and to humans that are secondary consumers **(Jawid et al., 2015).** AFM1 is a potent carcinogen, teratogen, and mutagen toxin found in the milk when lactating animals consume feed contaminated with aflatoxin B_1 (AFB_1). A study by **Mulunda et al. (2015)** showed an outbreak in dog feed that killed 40% of the animals exposed and fed on the contaminated supermarket feed. Such incidence affects the economic trade in countries. Animal feed is essential; importing and exporting in the poultry industry also bind to such standards for profit gain and economy reasons, farmers lose a lot of market due to contaminated poultry meat **(Allah et al., 2019).**

Table 1.2: Internationally accepted levels of aflatoxin concentration in different essential crops consumed by human beings in µg/kg (FAO, 2016).

Commodities	Aflatoxin Level	Aflatoxin type
Groundnuts	$5\mu g/kg$	B1B2G1G2
Maize	$15\mu g/kg$	B1B2G1G2
Wheat	$20\mu g/kg$	AFB1,
Wheat cereals	$4\ \mu g/kg$	B1+B2+G1+G4
Wheat cereals (baby infants)	$0.1\mu g/kg$	AFB1
Wheat dietary foods (medicinal)	$0.1\mu g/kg$	AFB1
Sorghum	$20\mu g/kg$	B1B2G1G2
Oats	$20\mu g/kg$	B1B2G1G2
Sugar beans	$15\mu g/kg$	B1B2G1G2
Milk (milk products)	$0.05\mu g/l$	AFM1

Table 1.3: Internationally accepted levels of aflatoxins in different essential commodities consumed by the animals in parts per billion (FAO, 2016).

Commodities (Animal feed)	Aflatoxin Level (ppb)	Aflatoxin type
Corn and Pecan	300	B1,B2,G1,G2
Maize		B1,B2,G1,G2
Wheat	20	AFB1,
Wheat	20	B1+B2+G1+G4
Brazil nut	20	B1,B2,G1,G2
Pistachio	20	B1,B2,G1,G2
Feedlot (fishing)	300	B1,B2,G1,G2
Milk (milk products)	0.5	AFM1
Poultry (slaughter)	100	AFB1, B1+B2+G1+G4
Breeding ruminant	300	B1,B2,G1,G2
Breeding cattle	100	B1,B2,G1,G2

Each country has its standards of Food and Drug Administration (FDA) Regulatory rules of aflatoxin content in commodities. Every country in Africa has different accepted aflatoxin levels on various commodities and they differ from each other (Table 1.4). For economic trade acknowledging these standards is profitable for those countries who are trading (import and export) business **(Kumar et al., 2017).**

Table 1.4: Aflatoxin concentration (µg/kg) in staple agricultural products of the selected countries from Sub-Saharan Africa for aflatoxin (B1, B1, G1, and G2) Lukwango et al., 2019).

Country	Agricultural products				
	Groundnut	**Maize**	**Millet**	**Sorghum**	**Sunflower**
Uganda	7.3 µg/kg	20µg/kg	14µg/kg	11.5 µg/kg	-
Tanzania	5.6 µg/kg	22 µg/kg	-	17.6 µg/kg	4.9 µg/kg
Kenya	5 µg/kg	20 µg/kg	17 µg/kg	13 µg/kg	4.9 µg/kg
Zambia	3.9 µg/kg	18 µg/kg	-	-	-
South Africa	6.0 µg/kg	20 µg/kg	18 µg/kg	10.5 µg/kg	-
Nigeria	20 µg/kg	4 µg/kg	-	10 µg/kg	-
Ghana	4 µg/kg	25 µg/kg	-	14 µg/kg	-
Cameroon	22 µg/kg	30 µg/kg	-	-	-
Mozambique	5 µg/kg	22 µg/kg	-	-	-
Mali	6 µg/kg	-	-	-	-
Benin	7.6 µg/kg	15 µg/kg	-	-	-

(Benkerroum, 2020)

The enforcement rate or application of the standards in Africa is not as strict as European Countries. The staple food source commodities are cultivated at the domestic level and sold at the informal markets, making it hard for governments to enforce the standards**. (Falade, 2019).** Africans who belong in the low-income to middle-income class are more exposed to aflatoxicosis since they target the informal market **(Ahlberg et al., 2018)**. Africa grows a wide range of crops that serve as staple foods (maize, *Oryza sativa* -rice, wheat, groundnuts, sorghum, and many more); these serve as hosts for *A. flavus*, which results in the frequent outbreak **(Falade, 2019).** FDA regulatory levels of mycotoxins in food commodities in South Africa is present in Table 1.5

Table 1.5: FDA regulatory accepted levels of food commodities in South Africa (Daoff, 2019).

Country	Commodity	Mycotoxin accepted level (µg/kg)	Aflatoxin type
South Africa	All foods	10	Aflatoxin type
	Milk, dairy products	0	M1,M2,G1,G2
	Feed components	50	B1, B2,G1,G2
	Mixed feeds for beef cattle, sheep, and goats	50	B1, B2,G1,G2
	Mixed feeds for lactating cows, swine, calves, lambs	20	B1, B2,G1,G2

1.8 Economic and social importance of yellow mould

Aspergillus flavus causes various diseasesincluding yellow mould, afla root in groundnuts **(Arya et al., 2015)** and *Aspergillus* ear rot in maize. Its sclerotia produce harmful chemical toxins called aflatoxins, which cause aflatoxicosis.

The loss in profit on export and import business is high globally and locally due to aflatoxin contamination in seeds, grains, and tree nut. China is leading in groundnuts production, yet the loss due to yellow mould is estimated at1.68 – 52.1 million U.S. dollars **(Pankaj et al., 2018).** Nigeria, the leading groundnut producing country in Africa, recorded losses of 50 million U.S. dollars **(Boboh-Van et al., 2018),** and Sub-Saharan Africa, in general, lost 750 million in U.S. dollars **(Misihairabgwi et al., 2019)** in export and import trade in the period of 2015-2017.

A.flavus contamination, despite aflatoxin occurrence, still results in the losses because it affects seed quality. Contamination in staples such as maize, sorghum, wheat, and groundnuts can directly reduce the availability of food **(Kange et al., 2015).** The contamination of staple food source by yellow mould comes with limited human and animal access to safe food. Food and Agriculture Organization of the United Nations (FAO) estimated 25-35% of food loss in developing countries in Africa **(Shafiee-Jood et al., 2018).** The social importance of yellow

mold is aflatoxicosis and food poisining in humans and animals. The health cost to treat aflatoxins outbreaks in humans in pharmaceutical and health costs estimated 32 million U.S. dollars globally throughout five years **(Benkerroum, 2020).** Worldwide, aflatoxins contribute to new cancer cases by 25% annually **(FOA, 2019; Bobo- Van et al., 2018)**

1.9 Current control strategies

The best way to control aflatoxin contamination in groundnuts is first to prevent infection by *A. flavus*. There are methods available for both pre- and postharvest control, but most do not provide excellent results, and groundnuts free from moulds.

1.9.1 Agronomic practices

Agronomic practices are more effective and efficient when customized to meet specific fields and the compatibility with the socio-economic condition under which one farmer function **(Lavkor et al., 2017).** Effective practices are site selection, land preparation, seed selection, sowing, and crop rotation that farmers afford to practice without a high cost. Table 1.6 elaborates on the use and results achieved by each practice. They are essential since most are preventative practices. **Parimi et al. (2018)** showed that agronomic practices are efficient when practiced methods are practiced simultaneously and demonstrated to be cost-effective and easily adaptable strategy.

Table 1.6: The list of agronomic practices affordable to farmers and their output.

Agronomic Practice	Method	Outputs/Results	Reference
Site selection and land preparation	Characteristics of good land cultivation include deep, well-drained, loose with a soil type of light-textured, well-aerated, and loam/dry loam Spacing row to row can be 30-45cm and plant within a row 10-15cm.	A study by **Phokane et al., 2019** showed the reduction of aflatoxin levels on maize and groundnuts. The technique allows maximum water retention and improves weed and disease control	**Chiafetz et al. ,2015** **Bediako et al. ,2018**
Weed Control	Taking out weed at the early (3-6 weeks after sowing) stages of seed development is essential. During weed control, pulling them out by hand or with the aid of a hoe or the of herbicide (2,4-D amine, AAtrex AAtrex Nine-O 90 WDG)	A study by **Jat et al. (2011)** showed the reduction of *A.flavus* incidence and aflatoxin levels. Elimination of weed reduces insects which use weeds as a hiding place, and they are disease vectors	**Torrez et al. ,2014** **Bediako et al. ,2018**
Crop rotation	Groundnuts rotation with a non-host crop is an effective strategy for breaking the infection cycle to reduce *Aspergillus* inoculum in the field. Groundnuts should not be monocropped. Sweet potato is a suitable rotation crop for use with peanut.	A study by **Abraham et al. (2007)** has shown that crop rotation aids in reducing certain types of weeds, pests, diseases, and reduces competition.	**Kimbll et al. ,2002** **Purwanto et al. , 2020**
Sowing	The planting aisle is linked to rainfall distribution in the area and length of the crop season. Delaying sowing can reduce yield by up to 50% and affects the seed quality. The recommended sowing depth is 5cm; groundnuts must be sown on flatbeds/ ridges/ raised beds separated by furrows.	A correct right spacing, sowing in rows, groundnut seed helps reduce the incidence of disease, maximize yield, better seed quality, and ensure uniformity and maturity.	**ICRISAT, 2016** **Sihlobo, 2019**

The most effective to moderate practices include fertilization, irrigation, pest control kernel moisture, removal of damaged kernels, storage conditions, and irrigation **(Mutengo and Hell, 2011).**

1.9.1.1 Pest control

Insects act as vectors of the fungus. Different insects persist in groundnut fields, but there are frequent ones. Common insects on groundnuts include thrips (*Frankilinella fusca*), three-cornered alfa alfa hopper (*Spissiustiulus festinus* Presch.), and spider mite (*Tetranychus urticae* Acri.). They transfer *A. flavus* spores from infected peg flowers from one plant to the other **(Alam et al., 2011; Gebreselassie e al., 2014).** Insects can manifest at storage hence

1.9.1.2 Fertilizers

A study by **Gebresclassie et al. (2014)** revealed that using or applying gypsum as a calcium source at early flowering stages lowers *Aspergillus flavus* infection. Sulfur(S) application of gypsum (200-400 $kgha^{-1}$) provides adequate sulphur to the crop. Phosphorus and zinc application reduce *A.flavus* effect significantly. Zinc (Zn) applied 10-20 $kgha^{-1}$, zinc sulphate, should be applied to the soil once in three years at land preparation. A combination of phosphorous and zinc increased or promoted nodulation, and zinc enlarged the plant capacity for building metabolites **(Meressa et al., 2020).**

Nitrogen as basal application and calcium (Ca) 200-400 $kgha^{-1}$ is needed at the peak of flowering. The application of boron (B) 3-4 $kgha^{-1}$ borax at the time of land preparation is essential.

1.9.1.3 Irrigation

Proper arrangements for drainage of rainwater should be made to avoid stagnating water in the field **(Janila and Mula, 2015**). Drought or over-irrigation can lead to *A.flavus* infection. A study by **Waliya et al. (2003)** report evidence of irrigation levels and cultivar effect on *A.flavus* infections and influence on aflatoxin contamination. The study showed the directly proportional relationship between overflooding and *A.flavus* incidence. Optimum irrigation is required at 2-3 weeks old crop emergence, pegging, and pod and seed development **(Sezen et al., 2019).**

1.9.1.4 Kernel moisture

Drying is done by lifting the plants and inverting them with the pods uppermost in windows for 2-3 days by small scale farmers. Picking the pods and spreading them out in a thin layer to sun dry for three days is also useful **(Hossane et al., 2011).** Accepted low humidity levels in groundnut kernel are 8-10% at around 82% RH. At 10% and higher levels that is where high aflatoxin levels are found **(Torres et al., 2014)**. The unshelled moisture content at 9% is more acceptable and shelled at 7% with 70% RH at 25-27 ºC to avoid infections. Storage avoiding stockpiling of groundnuts is essential since the heat buildup and moisture accumulation create favourable conditions for *A.flavus* occurrence **(Craufurd et al., 2006)**.

1.9.2 Chemical control

Fungicides or pesticides play a crucial role in crop protection. They play a vital role in controlling pests and reducing disease incidence**. (Lagogianni and Tsitsigiannis, 2018).** Table 1.7 listed fungicides that are Food Drug Administration (FDA) approved and are regarded as safe chemicals (GRAS).

Table 1.7 List of fungicides which control *Aspergillus flavus* and their mode of action.

Fungicide	Comments	Mode of action	Reference
Butylated hydroxyanisole (BHA)	Highly effective against both *Aspergillus flavus* and aflatoxins.	BHA treatment induced the loss of cytoplasm and cellular constituents, as well as distortion of mycelia, but it did not directly degrade the **aflatoxin**	**Li et al . ,2016** **Nesci et al. ,2019** **Sameer and Ibrahim, 2019**
Butylated hydroxytoluene (BHT),	Highly effective against both *Aspergillus flavus* and aflatoxins.	They inhibited conidial germination. Controlled *Aspergillus* elongation rate. Prevent aflatoxin B1 production	**Nesci et al. ,2003** **Nesci et al. , 2016**
Prothioconazole and tebuconazole	Moderate effective on *Aspergillus flavus*	Combination of prothioconazole- and tebuconazole-based fungicide provides the inhibition of conidial germination, constraining germinal potential hence the inhibitory of fungal growth.	**Masiello et al. ,2019** **Ferrigo et al. ,2019**
Propyl paraben (P.P.)	Highly effective against aflatoxins at pre-and postharvest.	Reduce the germ tube elongation rate. Inhibit β –d-glucosidase and α –d-galactosidase enzyme activity	**Passone et al. ,2005** **Nesci et al. ,2011** **Torres et al. ,2019**

1.9.3 Biological control

The purposeful utilization of living organisms, whether introduced or indigenous, other than the disease-resistant host plants, to suppress the activities or populations of one or more plant pathogens is referred to as biocontrol. Biocontrol agents use different mechanisms of function to achieve pathogen suppression and, subsequently, disease suppression. A mechanism of action can be described as the strategy used by a beneficial microorganism against a disease-causing pathogen **(Liu et al., 2019).** Mechanisms of biological control include antibiosis, mycoparasitism, competition for space, and limited resources, and induced systemic resistance of the host plant.

1.9.3.1 Bacteria as a biocontrol agent

Different bacteria species use other mechanisms to achieve disease control. *Bacillus* species have proven to be effective against a broad range of plant pathogens. The primary means of action, such as the excretion of antibiotics, toxins, siderophores, lytic enzymes, and induced systemic resistance, make them efficient biocontrol agents **(Hashem et al., 2019)**. Antibiosis is where biocontrol agent produces antibiotics or low molecular weight compounds that directly affect the growth of plant pathogens; for example, *B. subtilis* AU195 producing bacillomycin D (an antibiotic) that controls *A. flavus*.

Mycolytic enzymes including chitinases, proteases, and glucanases are used to control phytopathogenic fungi. They degrade a pathogens cell wall by chitinase and β -1, 3 glucanases**.** They also utilise competition as a mechanism of action. The competition is for nutrients, space, and environmental resources between a biocontrol agent and plant pathogen.

Bacillus produce siderophores that chelate the Fe (II) ions. The membrane binds protein receptors that being specifically recognize and take up the siderophores-Fe complex resulting in the iron not available for pathogens **(Meena et al. 2017).** Table 1.8 b shows some *Bacillus* species and their mode of action against selected plant pathogens, which effectively control the disease.

Table 1.8: Examples of *Bacillus* species used as antagonists against fungal pathogens.

Biocontrol agent	Mode of action	Targeted organism	Disease Controlled	References
Bacillus subtilis BSCBE4	Induction of defense enzymes and phenolic compounds	*Pythium aphanidermatum*	Damping-off on peppers	**Nakkeeran et al. 2006**
B. subtilis *B.subtillus* strain GB03 *B.amyloliquefaciens*	extracellular cell wall-degrading enzymes such as chitinases and β-1,3-glucanase induced systemic resistance principle eliciting factors (ethylene biosynthesis enzymes) and target pathogens: extracellular cell wall-degrading enzymes such as chitinases and β-1,3-glucanase	*Podosphaera fusca synonym Podosphaera xanthi* *Rhizoctonia, Botrytis cinerea* *Botryosphaeria dothidea*	Powdery mildew of curcubit Gray mold in strawberries, grapes, pears Apple ring rots	**Perez-Garcia et al. 2009** **Hajek and Eilenberg 2018** **Yan et al., 2013**

Previous studies have shown *Bacillus* species alone achieving control of *A. flavus* infection which is 72% **(Gojera, 2018; Yobo et al., 2016).** It has also reduced aflatoxin contamination in seeds.

Table 1.9: List some commercially available *Bacillus* species-based plant growth promoters and biological control agents (Govindasamy et al., 2010).

Biological control agent	Target Organism	Application/activity application	Trade name	Company
Bacillus subtilis F2B24	*Rhizoctonia solani* *Rhizoctonia* *Fusarium*	Biofungicide	Taegro	Earch Biosciences Inc (USA)
B. subtilis GB03	*Pseudomonas* syringae p.v	Seed treatment	Kodiak	Bayer Crop Science (North California)
B. pumilis GB34	*mucullcolo*	Biofunficide, Elicits	Yield Shield	Becker underwood Saskatoon
B. amyloliefaciens	*Rhizoctonia* *Fusarium*	Combination of strong ISR Activity	Bio Yield	Becker underwood Saskatoon (Canada)
B. subtilis Mb1600	*Rhizoctonia* *Aspergillus*	Biofungicide		
B. subtilis Mb1600 +*Rhizobia* strain	*Fusarium* *Aspergillus*		Subtilex	Becker underwood Saskatoon (Canada)
B. subtilis QST713	species	Enhancing growth on beans and groundnuts	VAULT	Bio Stacked
B. pumilus QST 2808	*Plasmodiophaora* *Brassicae* *Leptosphaeria* *muculans*	Biofungicide for prevention, suppression and control of soil-borne plant pathogens	Serenade	Agra Quest Inc. California
B. subtilis	Powdery mildew	Biofungicide	Sonata	Agra Quest Inc. California
	Colletotrichum *Cercospora*	Biofungicide	Avogreen	BASF, South Africa

1.9.3.2 Yeast as a biocontrol agent

Antagonistic yeast (biocontrol agents) refers to yeast and yeast-like structures that inhibit or interfere with growth, development, reproduction, and the activity of phytopathogens **(Zhang et al., 2020).** Yeast became attractive to be used as a biocontrol agent because (1) it is safe to use, (2) possess adequate stress tolerance, and (3) can potentially be genetically improved **(Perez et al., 2016)**. Yeast have a developed system for culturing, fermentation, storage, and handling.

The main mechanisms of action include competition for nutrients and space, mycoparasitism (enzyme secretion), induction of host resistance, production of volatile organic compounds (VOCs), and toxins **(Dukare et al., 2018)**. Once the yeast is in contact with the surface of the injured fruit, limits the germination of fungal spores. Antagonist yeast suppresses postharvest fungal pathogens by competition **(Liu et al., 2013).**

Mycoparasitism refers to the phenomenon of antagonistic yeast feeding on fungal pathogens via attaching to the fungal pathogen hyphae and the secreting cell-wall degrading enzymes to destroy/lyse the fungal structures **(Alvarez et al., 2019).** The phenomenon is demonstrated by *Candida famata* reducing green mould decay caused by *Penicillium digitatum* on *Citrus* L. and increase the phytoalexins scoparane **(Perez et al., 2016).**

Enzymes involved in fungal pathogen cell degradation are β -1, 3 glucanase (GLU), chitinase (CHT), and proteases. In a previous study by **Lui et al. (2013),** *Pichia guilliermondii* degraded *Botrytis cinerea* cell wall by secretion of β -1, 3 glucanases, which resulted in the inhibition of the fungal growth**.**

Production of volatile compounds and killer toxins is another mechanism used by yeast to inhibit the plant pathogen **(Abdel-Kareem et al., 2018).** Volatile compounds (VOCs) are low molecular weight compounds (300DA) and low polarity and high vapour pressure **(Benkerroum .2020)**.

Commercially available yeasts have been developed, which are used as a biocontrol agent in the agricultural industry. Table 1.9 is a summary of the available products, action, and manufacturing company. Antagonistic yeast has provided effective control on *A. flavus* **(Jiabangyang et al., 2020; Tian et al., 2017).** Yeast has also been shown to reduce aflatoxin concentrations **(Sukwmawati et al., 2020).**

Table1.10: Examples of commercial formulations using a variety of antagonistic yeast. (Lui et al., 2013).

Biological control agent	**Target pathogen**	**Product**	**Company Manufacturer**
Candida oleophila	*Botrytis cinerea, Penicillium*	Aspire	Ecogen, USA
Aureobasidium pullulans	*Penicillium Botrytis cinerea*	Blossom protect	Bio-ferm, Austria
C.oleophila	*Botrytis Penicillium* species	Nexy	Le Saffre, Belgium
Saccharomyces cerevisiae	*Erysiphe Botrytis*	Remeo	BASF, France
Cryptococcus albidus	*Penicillium Botrytis*	Yield Plus	Lallem, South Africa
C. Sake	*Penicillium Botrytis*	Candifruit	IRTA/Sipcam Inagra Spain
A. pullulans	*B. cinerea*	Botector	Bio-ferm, Austria
Metschnikowia fructicola	*Aspergillus Penicillium Botrytis Penicillium*	Shemer	Kopper, The Netherlands

1.9.3.3Fungi as a biocontrol agent

Over time there have been discoveries of fungus controlling other fungus, insects, bacteria, and viruses as casual organisms in plant diseases**.** The potential use of atoxigenic *A. flavus* strain to control the toxigenic *A. flavus* introduces a carefully selected atoxigenic strain of *A. flavus.* This harmless strain has a considerable competitive advantage over the toxigenic strain **(Dorner, 2006).** This atoxigenic strain effectively eliminates the toxic relative by competitive exclusion reducing the aflatoxin contamination **(Chang et al., 2005**). The atoxigenic strain cannot produce aflatoxin and cyclopiazonic acid (CPA) due to a mutation in polyketide synthase gene **(Chang et al., 2009).** The US Environmental Protection Agency (USEPA) to be used as aflaguard on maize **(Dorner, 2006).**

A Nigerian atoxigenic strain, *A. flavus* NRRL 21882 had gained provisional registration as aflasafe for groundnuts **(Agbetiameh et al., 2019).** The strain nominated has quality traits including agroecology adaptation, highly competitive with toxigeric strain, clonal with stable atoxigenic genotype and shown molecular analysis to lack of gene to produce aflatoxin**.** Aflasafe reduces aflatoxin concentration in treated crops by 80% compared to untreated crops **(Chang et al., 2020).** The strain usage in Africa has increased over the years; many countries are now registering their use, such as Ghana, Nigeria, and Senegal.

1.9.4 Breeding for resistance

Screening tools have been advanced and used to facilitate corn and groundnut breeding for developing germplasm resistant to fungal growth and or aflatoxin contamination **(Fountain et al. 2015).** Three types of resistance can be achieve: (1) dry seed resistance, (2) aflatoxin production resistance and (3) pre-harvest infection resistance **(Japyaprakash et al., 2019).** Understanding the R gene, a disease resistance gene in the host plant is essential. Achieving a better understanding will be useful in breeding for resistance. Regardless of the worldwide efforts, there is little progress in breeding for aflatoxin resistance. These constraining factors are (i) the low level of resistance to different components of resistance, (ii) lack of a reliable screening protocol and (iii) the limited understanding of genetics to achieve resistance **(Fountain et al., 2015)**

Commercial groundnut lines released for low aflatoxin contamination as an agronomic trait includes: j-11, 55-437, ICG 7633, ICG 4749, ICG 1326, ICG 3863, ICG 9407, ICG 10094, ICG 1859, ICG 9610 genotypes achieved by breeding approaches. **(Konate et al., 2020; Pandey et al., 2019)**

1.9.5 Heat treatment

Heat treatment is dipping crops in hot water at a specific temperature for a particular duration of time. It's a postharvest treatment of fruits developed to control insect infestation, disease control, modify fruit responses to cold stress, and maintain fruit quality during storage **(Usall et al., 2016).** Heat treatment has been investigated over the years. It has been useful to control plant diseases on several fruits including *Persea Americana* Mill, *Citrus sinensis* L, *Prunus persica* L, and *Pyrus communis* L.

The heat shock stimulates proteins involved in plant defense mechanisms including phenylalanine ammonia-lyase (PAL), chitinase, β-1,3-glucanase, and pathogenesis-related proteins in fruit. **Lu et al (2009)** conducted a study that showed that *Solanum lycopersicum* L., heat treated at 36ºC for 12 mins decreased the disease incidence of *B. cinerea* and maintaining the firmness of the fruit.

Hot water treatment (HWT) reduces the pathogens mycelial development on fruit during storage, also assists with ripening and maintain fruit quality during storage **(Sriram and Rao. 2019).** HWT affects seed vigour and seed viability. Hot water seed treatment has beneficial effects of priming seeds, resulting in faster germination **(Musazura and Bertling, 2013).** Heat treatment has been used successfully in *Pyrus communis* L. (pears) heat treatment at 54 °C for 7 min increased total soluble solids and fruit flavor index, but did not affect weight loss, fruit firmness, pH and titrable acidity **(Seo et al., 1997).**

Prunus persica var. persica (L.) (Peach) **(Jitareerat et al., 2018)** and *Persea Americana* Mill. (Avocado) **(Sivankalyani et al., 2015)** to decrease the incidence of fungal pathogens at postharvest in horticultural studies. The rate of success is 60% in fruits and vegetables **(Albuzaudi et al., 2017, Liu et al., 2013).**

1.9.6 Integrated control strategies

The control of *A. flavus* incidence on grains, corn, legumes, and tree beans has evolved over the years. The use of different methods, from agronomic practices to breeding resistant cultivars, has been investigated. The biocontrol agents (BCAs) often fail to consistently control plant pathogens due to the influenced of several factors. No single control strategy can provide complete control of both *A. flavus* and aflatoxins, especially under environmental conditions favourable for disease development **(Medina et al., 2014).** Optimizing and improving BCAs performance by combining those with other control strategies have been attractive to researchers over the years.

The integration of hot water treatment (HWT) and *Bacillus* species have been used on different fruits such as *Fragaria x ananassa* L (strawberries), cherry tomatoes, apples, and citrus **(Wu et al., 2019)**. HWT and antagonist yeast have been also used on fruits for the inhibition of plant pathogens and on other crops (**Zhang et al., 2010).** Integrated strategies involving hot water and yeast **(Zhao et al., 2010)** and hot water and *Bacillus* species are useful on different fruits and vegetables **(Wu et al., 2019).** Table 1.11 summarises some studies that have shown the efficacy of integrated control on different fruits.

Table 1.11: The list of combination treatment integrated to control fungus diseases and their efficacy.

Integrated Control	Crop	Pathogen	Comments	Reference
Hot water treatment and *Bacillus subtilis* CPA	Peaches Nectarines	*Monilia laxa*	HWT at 60°C for 40 s + *B. subtilis* achieved a better inhibition of *M. laxa*	Casals et al., 2010
Hot water treatment and *B. amyloliquefaciens*	Mandarin fruit	*Penicillium digitatum, P. italicum Geotrichum citri-aurantii*	HWT at 45 °C for 2 min + *B. amyloliquefaciens* combined with 2% SBC was as effective as the fungicide treatment and reduced decay	Hong et al, 2014
Hot water treatment and *Rhodotorula glutinis*	Pear fruit	*P. expansum*	HWT at 46 °C for 10 mins + *R. glutinis* achieved a better inhibition of spore germination and mycelial growth	Zhang et al., 2008
Hot water treatment and *Cryptococcus laurentii,*	Peaches	*P.expansum Rhizopus stolonifer*	HWT 37 °C in 2 days + *C. laurentii* decreases the blue mold by 52.2% and reduced *Rhizopus* decay by 62.5%	Zhang et al., 2007 [a]
Hot water treatment, *Candida guilliermondii* and *Pichia membranaefaciens*	Tomato	*Botrytis cinerea*	HWT 20°C at 40 mins + *C. guilliermondii* provided a better reduction of disease incidence than the combination HWT 20 °C at 40 mins + *P. membranaefaciens.* Both combinations reduced the disease by 26.75% and 21%	Zong et al., 2010

1.10 Research gap

Groundnuts are highly cultivated crops in South Africa at any level; their growth is beneficial and has many uses in different. Contamination by *A. flavus* compromises the quality value and food safety and security. The main concern with *A. flavus* producing aflatoxin is the toxic traces it leaves on feed and food. They expose animals and humans to the health risk of diseases, low production in animals and cancer, and other diseases in animals and humans—*A. flavus* occurrence in seed results in aflatoxin contamination. Aflatoxin contaminated commodities to animals and human beings threatens food safety, public health, and constrains economic growth due to profit loss in the agricultural industry.

Each management strategy cannot achieve pathogen-free seed. This research focuses on the use of hot water treatment for the first time in groundnuts to manage *A. flavus* infection. Moreover, information on the use of integrated strategy involving biocontrol agents and hot water treatment (HWT) is lacking. This work will further explore the potential of using BCAs and HWT to manage *A. flavus* in groundnuts.

1.11 Conclusion

Groundnut is susceptible to several pre and postharvest diseases. *Aspergillus flavus* is the most prevalent fungus affecting seed with aflatoxin contaminant as a major threat to food availability, food safety, and the public. Implementation of an integrated disease control program that includes physical treatments and biocontrol agents could minimize *A. flavus* contamination both at pre and postharvest and increase economic returns to the producer.

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CHAPTER TWO

ISOLATION AND *IN VITRO* SCREENING OF *BACILLUS* AND YEAST SPECIES FOR BIOLOGICAL CONTROL OF *ASPERGILLUS FLAVUS* LINK IN GROUNDNUTS.

Abstract

Groundnuts (*Arachis hypogaea* L.) are susceptible to infection by *Aspergillus flavus* Link, the fungi that produce aflatoxins as secondary metabolites. Aflatoxins contaminate groundnuts, causing a decrease in seed quality and health problems to consumers. This work aimed to identify native yeast and *Bacillus* species isolated from the phyllosphere of 52 plant species with an inhibitory effect against *A. flavus.* A total of 60 *Bacillus* and 169 yeast strains were isolated and screened for antagonistic activity against *A. flavus*. There were two screening tests to identify potentially useful *Bacillus* and yeast strains. During the *in vitro* primary screening test, 3.33% of the total *Bacillus* isolates gave an average of 0-39% inhibition, 1.6% had inhibition of 70%, and the rest of the isolates with average inhibition of 40-69%. Yeast isolates with <70% average inhibition constituted 1.7% of the total yeast isolates, 10.6% had inhibition ranging from 0-39%, and 87.5% had average inhibition from 40-69%. For the *in vitro* secondary screening, ten *Bacillus* and ten epiphytic yeast isolates were selected based on their average inhibition and persistence of inhibition over time. *Bacillus* Isolate LM1b was the best *Bacillus* isolate with 70.0 % average inhibition, followed by PTP1b with 68.6%, and SF4 with 67.5%. The plant sample with most *Bacillus* species isolated was cowpeas leaves. Yeast Isolate CC1y was the best yeast isolate with 72.6 % average inhibition, Isolates PF3y with 70.8%, and CF1y with 70.0% inhibition, respectively. The best *Bacillus* isolate LM1b was isolated from citrus, and the best yeast isolate CC1y was isolated from spider plant. The results suggest that the *Bacillus* isolates (LM1b and PTP1b) and epiphytic yeast isolates (CC1y and PF3y) have the potential as biological control agents against *A. flavus* on groundnuts. The potential of these isolates was investigated on groundnut seed during postharvest trials in Chapter Three.

Keywords: *Aspergillus flavus* Link. *Bacillus* spp., Yeast, *Arachis hypogaea* L.

2.1 Introduction

Groundnuts (*Arachis hypogaea* L.) are cash flow crops, which also contribute to poverty alleviation in developing countries in Africa. They are high oil seed content that contains protein and other nutritional values. They have several industrial purposes **(Jaibangyang et al., 2020).** Aflatoxins are known to be toxic cardiogenic secondary metabolites produced by *Aspergillus flavus* Link. during pre-and postharvest conditions. The occurrence of aflatoxins in groundnuts contaminate the seed and affect the quality and reduce commercial production. Several factors also do influence *A. flavus* infection, including drought, temperature, soil pH, and soil humidity **(Bediako et al., 2018).**

Management of *A. flavus* includes chemical, mechanical, and cultural control measures that are significantly not cost-effective. Biological control agents have become attractive over the years for managing plant diseases. It is cost-effective, less harmful to consumers, and environmentally friendly **(Panebianco et al., 2015).** The benefits of biological control include: (1) being environmentally friendly because it causes no pollution and affects only the target pathogen. They are safe, meaning they pose no threat to human health, crop production, or beneficial organisms. They are also selective and only affect the target organism; and (2) they are self-perpetuating or self-sustaining and, therefore, permanent. **(Freimoser et al., 2019; Sharma et al., 2013)**. Biological control agents exhibit several mechanisms that aid in achieving control of plant pathogens. These mechanisms of action include antibiosis, competition, mycoparasitism, production of cell wall degrading enzymes, and induced systemic resistance **(McGuire, 2000, Spaaji et al., 1993).** Some bacteria have become attractive and practical for use as biocontrol agents due to their resistance to adverse environmental conditions and their ability to control a broad range of pathogens **(Mohsen et al., 2015).** *Bacillus* species have become popular as biological control agents due to their ability to produce endospores that withstand adverse environmental conditions and antibiotics, which control a broad range of plant pathogens **(Shafi et al., 2017).** Several commercial products based on *Bacillus* species have been developed to manage various fungal diseases **(Karthick et al., 2017, Munir et al., 2018).** Examples of such commercial products include Taegro (*B. subtilis* E. F2B24), Kodiak (*B. subtilis* E, GB03), Yield Shield (*B. pumilis* E. GB34), and Avogreen (*Bacillus subtilis*). *B. subtilis* effectively controls *A. flavus* by secretion of Bacilliomycin D, an antibiotic that causes severe injury to both cell wall and cell membrane of spores and hyphae **(Gong et al., 2014, Mohsen et al., 2018).** *Bacillus subtilis* NCIB 8872 controlling *Fusarium oxysporum* and *A. flavus* by secretion of antibiotics and inhibit

phospholipase A2 production of the pathogen **(Zaim et al., 2018).** Yeast's potential as a biological control agent is determined by its ability to antagonize pathogens, undemanding cultivation (easily propagated), and no safety concerns. All these make yeast attractive as a biological control agent **(Freimoser et al., 2019).** A previous study by **Perez et al. (2016)** successfully used native killer yeast to control *Penicillium digitatum* P, *P. italicum S*, and *P. citri L.,* which causes postharvest diseases of citrus (*Citrus limon* (L.) Burm. f.). The mechanism of action of the killer yeast include**s** competition for nutrients and secretion of specific enzymes such as chitinases, glucanases, and/or proteases **(Lui et al., 2019; Gong et al., 2014).** Yeast has also been found to exhibit induced systemic resistance and secrete antimicrobial substances (soluble or volatile) as possible modes of action **(El-Tarabily and Sivasithamparam 2006).** This study aimed to isolate, screen, and identify potential antagonistic bacterial and yeast strains against *A. flavus*, the causal agent of yellow mold in groundnut.

2.2 Materials and Methods

2.2.1 Isolation of *Aspergillus flavus* from groundnuts

Two raw groundnut seed samples were acquired from a local spice shop in Pietermaritzburg, KwaZulu-Natal, South Africa. Fifty groundnut kernels per sample were surface sterilized with 3% sodium hypochlorite (v/v) for 3 min and rinsed three times with double sterilized distilled water. The surfaced sterilized seed samples were then separately air-dried on a sterile paper towel under laminar flow before being plated onto potato dextrose agar (PDA) media. There were ten groundnut kernels per plate. The plates were incubated at 25°C for 7 days.

The suspected pure colony of *A. flavus* emanating from the groundnut kernels were sub-cultured onto freshly prepared PDA media incorporated with streptomycin and chloramphenicol. The identification of *A. flavus* was based on the presumptive measure on morphological features, including color or pigmentation, texture, and conidia morphology **(Kifle et al., 2017; Yobo et al., 2017)**, comparing with a reference culture previously isolated by **Chiuraise et al. (2016).** The suspected *A. flavus* isolate was stored on barley seeds and in 70% sterile glycerol.

2.2.2 Sample collection and isolation of biological control agents

The *Bacillus* and yeast strains were isolated from the leaves of 52 plant species collected from the Controlled Environmental Research Unit (CERU) and at Ukulinga Research Farms, University of KwaZulu-Natal Pietermaritzburg. *Bacillus* and yeast strains were isolated using the wash-method technique **(Li et al., 2011).**

Isolation of yeast strains

The plant species' leaf samples were cut into pieces, rinsed with double sterilized tap water to wash off the soil on the leaf surface. Approximately 5g of each sample were weighed and placed in a McCartney bottle containing 50 ml of double sterilized distilled water. The McCartney bottles were shaken at 150 rpm in an orbital shaker incubator at 28°C for 15 mins to create a stock solution.

A serial dilution was prepared from the stock solution, and 100µl of each dilution (10^{-1} to 10^{-4}) was plated on Yeast Malt Agar (YMA) plates supplemented with 10 ml of 40 mg L^{-1} of streptomycin and 5 mgL^{-1} of chloramphenicol. The YMA media plates were incubated at 28 °C for 7 days. Suspected pure yeast colonies were aseptically isolated and purified by sub-culturing onto freshly prepared YMA. According to their macroscopic features (x40 magnification) (texture, surface, margin, elevation, and color) and morphology, yeast colonies were selected using presumptive identification. Yeast isolates were identified by observing microstructures unique to yeasts such as budding. A total of 169 yeast strains were stored in 70% glycerol at -80°C.

Isolation of *Bacillus* species

For *Bacillus* spp. isolation, the same procedure for yeast was followed with some modifications. The McCartney bottles containing the leaf disc samples with double sterilized distilled water were shaken at 150 rpm in a water bath shaker maintained at 80°C for 30 mins before preparing the serial dilutions. Aliquots (100 µl) of the serial dilutions (10^{1}-10^{4}) were plated on Tryptic Soy Agar (TSA) media and incubated at 28ºC for 4 days. Using presumptive identification (colony morphology), suspected *Bacillus* colonies were selected and sub-cultured onto fresh TSA media and incubated at 28 ºC. Gram stain technique was used to confirm the *Bacillus* isolates. Purple gram-positive, rod-shaped colonies were confirmed to be *Bacillu*s spp. The isolates were stored at -80℃ in sterile 70% glycerol. A total of 60 *Bacillus* isolates were stored.

2.2.3 *In vitro* screening of biological control agents against *A. flavus*

The *in vitro* inhibition of mycelial growth of *A. flavus* by potential biological control agents was conducted using a dual culture technique described by **Perez et al. (2016)** and **Girish and Bhavya (2018)**. Yeast isolates were plated onto YMA media plates supplemented with 10 ml of 40 mgL^{-1} of streptomycin and 5 mgL^{-1} of chloramphenicol and incubated at 28°C for 3 days. A single mycelial cube (4x4 mm) was cut from the actively growing edge of a 4-day-old mycelial mat on PDA plate containing the *A. flavus* pathogen and placed at the centre of the YMA plate. The yeast isolate was streaked horizontally on both sides at an equal distance away from the pathogen. The pathogen and yeast isolate were both inoculated at the same time. The same method was carried out for all the 169 yeast isolates. A control plate was without a yeast isolate. There were three replicates per isolate, and the plates were incubated at 28°C for 7 days. The zone of inhibition was measured. The same protocol was followed for the screening of the 60 *Bacillus* isolates except that PDA medium was used for the bioassay. The percentage of inhibition was calculated by using the following formula:

$PI=\frac{C-T}{C} \times 100$; where

PI= percent inhibition

C= diameter of *A. flavus* (control)

T= diameter of *A. flavus* in the dual test plate

After 7 days, the ability of each isolate to continue to inhibit the pathogen was measured. The persistence of each yeast and *Bacillus* isolates on inhibiting the pathogen was recorded on days 8, 11, and 15. The ratings of the persistence of inhibition with incubation time were classified as negative (-) or positive (+) over 7 days after the zone of inhibition was measured on every isolate.

2.2.4 *In vitro* secondary screening of biological control agents against *A. flavus*

Based on the primary screening results, the ten best-performing *Bacillus* isolates and ten best-performing yeast isolates were selected for secondary screening, which was a repetition of the first preliminary screening protocol using the 10 best yeast isolates and 10 best *Bacillus* isolates. The bioassay was repeated once with three replicates per isolate. A scale was developed to group the isolates based on their ranges of inhibition in which Class 1 contained isolates that achieved ≤ 40% inhibition, Class 2 contained isolates that provided between 41-

69% inhibition, and Class 3 contained isolates that achieved ≥ 70% inhibition. The best two performing isolates for yeast and *Bacillus* from the secondary screening were selected for *in vivo* trial (Chapter Four). These isolates were sent to Inqaba Biotech for molecular identification at the species level.

2.2.5 Pathogenicity of *A. flavus* isolate

Fresh certified groundnut kernels (cultivar Akwa) obtained from Vaalharts Groundnuts Marketing CC, Plot 2E5, Hartswater, 8570, South Africa was used for this test. Groundnuts kernels were surface sterilized with 3% Sodium hypochlorite for 3 min and rinsed three times in sterile distilled water. The sterilized kernels were air-dried on sterile paper towel under laminar flow before. The seeds were wounded using a flamed sterile needle. Spores of *A. flavus* were scraped off with 5 ml of double sterilized water from the PDA media plate. Since *A. flavus* spores are hydrophobic, this was to reduce their hydrophobicity and stick in distilled water. Mira cloth (cheesecloth) was used to filter out possible mycelium and to obtain spores only. The inoculum was prepared to a 1×10^4 spores ml^{-1} concentration. This was achieved through a hemocytometer count. Spores were inoculated into the wounds (7 kernels) with a sterile loop by dip with 1×104 spores ml-1 concentration suspension. Inoculated seeds were placed in an empty sterile petri dish and incubated for 7 days at 28°C. After seven days, the seeds developed a characteristic yellow-green mold of *A. flavus* **(Porter et al., 2015).**

2.2.6 Data analysis

All data sets from the secondary screening results were subjected to Analysis of Variance (ANOVA) using the Statistical Analysis System (SAS Version 9.4) (S.A.S. Institute Inc, 2014). Where ANOVA was significant, means were separated using Duncan Multiple Range Test (D.M.R.T.) at a 5% significance level. Standard Error (SE) were also calculated for all primary screening results.

2.3 Results

2.3.1. Isolation of biocontrol agents

A total of 169 yeast and 60 *Bacillus* isolates were successfully isolated from the various plant leaf samples and used during the preliminary screening. The results of primary identification of the yeast and *Bacillus* isolates using the wet mount and Gram stain techniques, respectively,

positively confirmed the presumptive identification of the yeast and *Bacillus* isolates (Figure2.1 and Figure 2.2). The 10 yeasts and 10 *Bacillus* isolates were effective in inhibiting the *A. flavus in vitro* (Table 2.3 and 2.4);

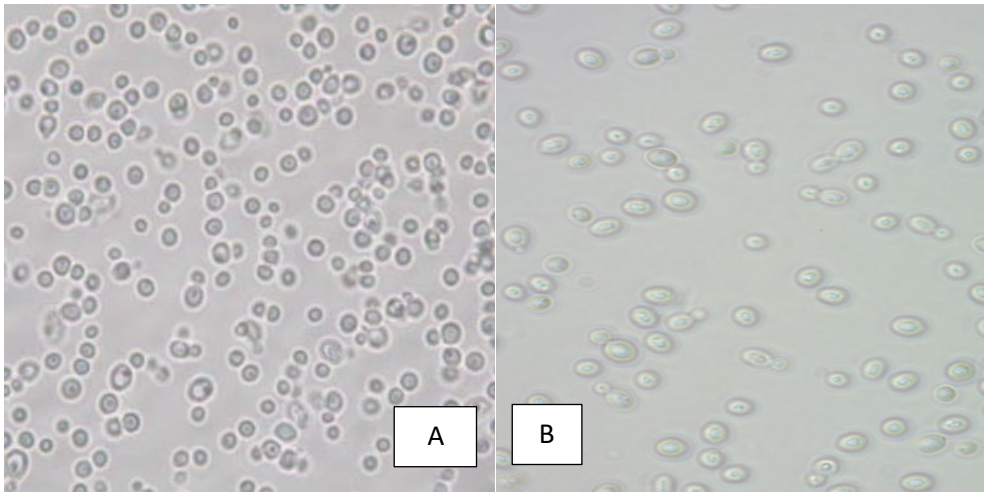

Figure 2.1: (A) Micrograph of yeast isolate observed under a light microscope at X40; (B) Globose yeast cells under a light microscope undergoing budding X40.

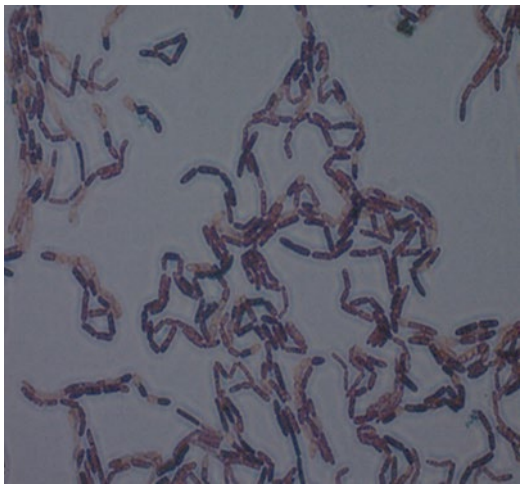

Figure 2.2: Micrograph of gram staining of *Bacillus* species isolate observed under light Carl Zeise microscope at 100X magnification.

The most effective yeast strain was isolated from *Chlorophytum comosum* J. while the crop with the highest number and percentage of yeast isolates in terms of the overall isolates was from *Helianthus annuus* L. All 10 effective yeast isolates were randomly distributed among the various crop samples. Effective *Bacillus* strains were from *Citrus x Limon* L. and *Carica*

papaya L. crops. The highest number of *Bacillus* isolates (six isolates each) were obtained from cowpeas leaves (*Vigna unguiculata* L. (Walp), green beans leaves (*Phaseolus vulgaris* L.), cabbage leaves (*Brassica oleracea vr. Capitate* P.) and sweet potato leaves (*Ipomoea batatas*-Lam.). All 10 effective *Bacillus* isolates were also randomly distributed among the various crop samples.

2.3.2 Pathogenicity test of *A. flavus*

A. flavus was able to grow and infect the inoculated groundnut kernels. Approximately 80% of the seeds showed disease symptoms typical of *A. flavus* (Figure 2.3).

Figure 2.3: *Aspergillus flavus* emerging from the inoculated groundnut kernels showing characteristic yellow-green spores and mycelial growth on a groundnut kernel.

2.3.3 Primary screening of yeast and *Bacillus* isolates against *A. flavus*.

Preliminary screening of yeast isolates

All the 169 yeast isolates were used during the *in vitro* preliminary screening test. Of these, only 1.7% of the yeast isolates were grouped in Class 3 (< 70% average inhibition), 87.5% (40-69% average inhibition) in Class 2, and 10.6% in Class 1 (0-39% inhibition). Isolates in Class 2 made the highest percentage of the total yeasts isolated. The control plates without yeast isolates were covered entirely by the pathogen. The average inhibition of each isolate, the class, and the persistence of inhibition are presented in Table 2.1 (refer to appendix). Isolates from pawpaw and sunflower leave provided moderate inhibition and fair persistence of inhibition on Day 8, Day 11, and Day 15. Yeast isolated from corn head/ maize head had low inhibition

percentages but showed better persistence of inhibition on Day 8, Day 11, and Day 15 better than all other isolates.

Preliminary screening of *Bacillus* isolates

Of the 60 *Bacillus* species used during the *in vitro* preliminary screening test, 3.33% of total collected *Bacillus* isolates were grouped under Class 1 (0-39% inhibition), 1.6% under Class 3 (< 70% average inhibition), and 93% under Class 2 (40-69% inhibition). Table 2.2 (refer to appendix) shows the percentage inhibition and persistence of inhibition of each isolate over 15 days and their respective class groupings. Some *Bacillus* isolates were consistent in inhibiting the pathogen on Days 8, 11, and 15 (Table 2.2 – refer to appendix). In terms of pathogen inhibition, the most effective and persistent isolate was isolated from cowpea leaves compared to other isolates from different sources.

2.3.3 *In vitro* secondary screening of yeast and *Bacillus* isolates against *Aspergillus flavus*

In vitro* secondary screening of yeast isolates against *Aspergillus flavus

Ten yeast isolates were selected based on their ability to inhibit *A. flavus* mycelia growth during the preliminary screening test and efficient inhibition with incubation time. The secondary screening of the selected yeast isolates provided an average inhibition ranging from 64-72% (Table 2.3). The best three isolates with high percentage inhibition were from spider plants, sugar bushes flower, and groundnut leaves, respectively. All the three yeast isolates were from Class 3, which was the group with isolates with percentage inhibition >70%. Sunflower leaves had the highest number of isolates among the best ten.

Table 2.3: Sources of the selected potential yeast antagonists, their percentage of inhibition obtained from secondary screening, and their group ratings.

Source of Isolates	Isolate name	Average inhibition + SE	Class
Chlorophytum comosum	CC1	$72.1^{a} \pm 4.2$	3
Protea cynaroides	PF3	$70.8^{a} \pm 1.8$	3
Arachis hypogaea	CF1	$70.0^{a} \pm 3.5$	3
Arachis hypogaea	CF5	$68.6^{a} \pm 3.1$	2
Carica papaya	PW2	$66.8^{a} \pm 2.4$	2
Carica papaya	PW4	$66.6^{a} \pm 3.1$	2
Chlorophytum comosum	CC5	$64.8^{a} \pm 1.6$	2
Helianthus annuus L	SFL7	$64.8^{a} \pm 3.1$	2
Helianthus annuus L	SFL17	$64.5^{a} \pm 2.7$	2
Helianthus annuus L	SFL15	$64.5^{a} \pm 4.7$	2
F value		**1.42**	
P value		**0.212**	
%CV		**9.50**	

* = Each value is reported as an average of three replicates; means followed by the same letters are not significantly different based on Duncan's Multiple Range Test at 5% significance level (P = 0.05).

In vitro secondary screening of *Bacillus* spp. against *Aspergillus flavus.*

The best 10 *Bacillus* isolates were selected based on their ability to grow on media, average inhibition, and efficient inhibition over the incubation period from the preliminary screening test results. The selected *Bacillus* isolates provided percentage inhibition ranging from 59-70% (Table 2.4). The isolates with high percentage inhibition were isolated from lemon leaves, pigweed, and blueberry leaves. Papaya leaves (PW) had the highest number of isolates among the best ten (two isolates).

Table 2.4: Sources of the selected potential antagonistic *Bacillus* isolates, their percentage of inhibition obtained from secondary screening, and their group ratings.

Source of Isolates	Isolate name	Average inhibition ± SE	Class
Citrus limon	LM1b	70.3^{a} ± 0.5	3
Portulaca pilosa	PTP1b	68.6^{ab} ±2.4	2
Helianthus annuus L	SF4b	67.5^{ab} ±2.0	2
Carica papaya	PW3b	61.6^{ab} ±1.2	2
Carica papaya	PW4b	61.3^{ab} ± 1.8	2
Allium cepa	ON3b	61.3^{ab} ± 1.2	2
Asimina triloba	PW2b	60.1^{bc} ± 0.9	2
Vigna unguiculata	CP5b	60.1^{bc} ± 3.7	2
Phaseolus vulgaris	GB6b	59^{c} ± 2.1	2
Brassica oleracea vr. capitata	BL3b	59^{c} ± 2.8	2
F value		**8.66**	
P value		**<0.001**	
%CV		**7.76**	

*= Each value is reported as an average of three replicates; means followed by the same letters are not significantly different based on Duncan's Multiple Range Test at a 5% significance level (P = 0.05).

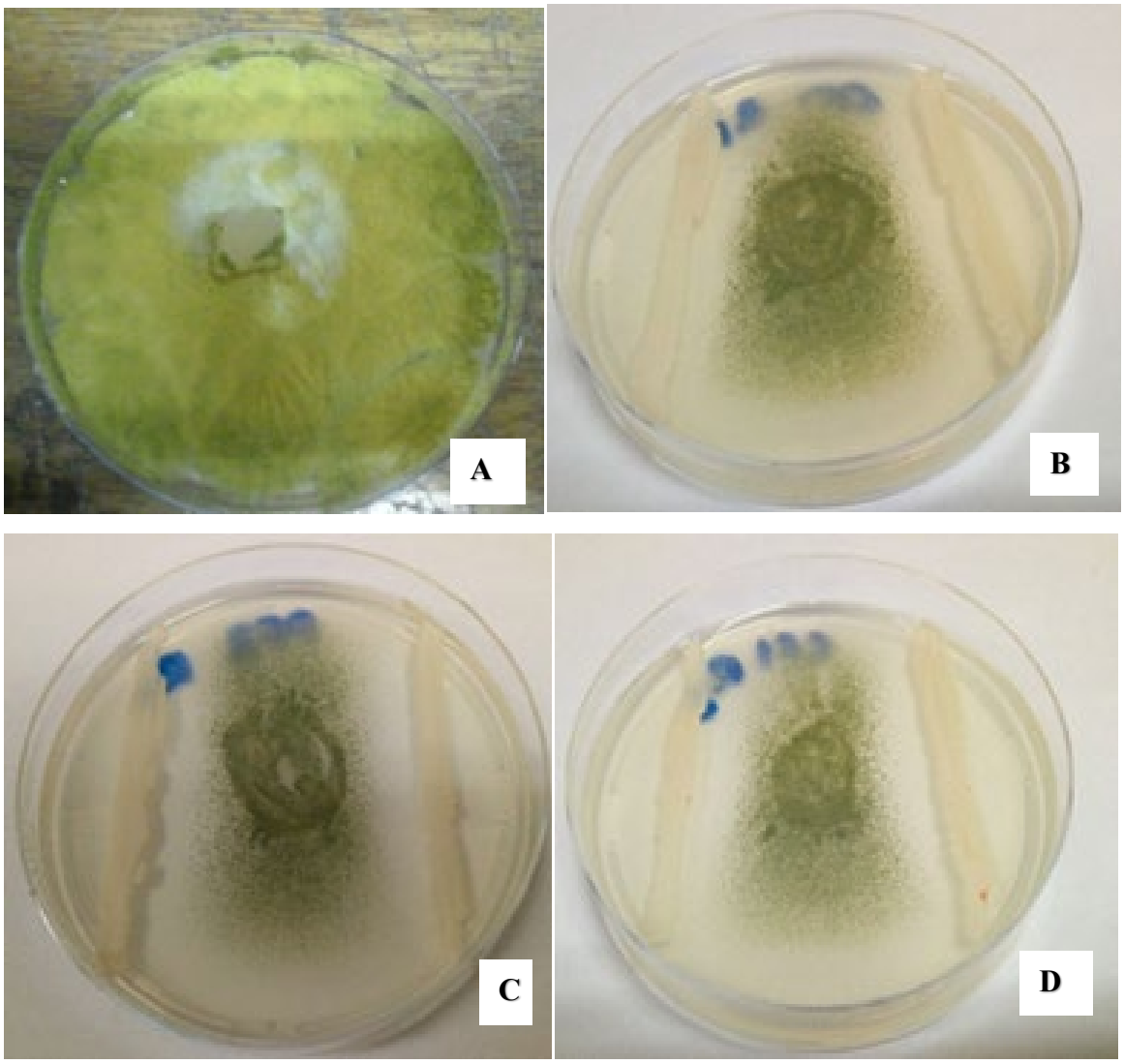

Figure 2.4: *In vitro* inhibitory bioassay of yeast against *A. flavus* on PDA media after 10 days at 25°C. (A) Control plate (B) Isolate CC1y, (C) Isolate PF3y and (D) Isolate CF1y. These are the best three isolates compared to the control.

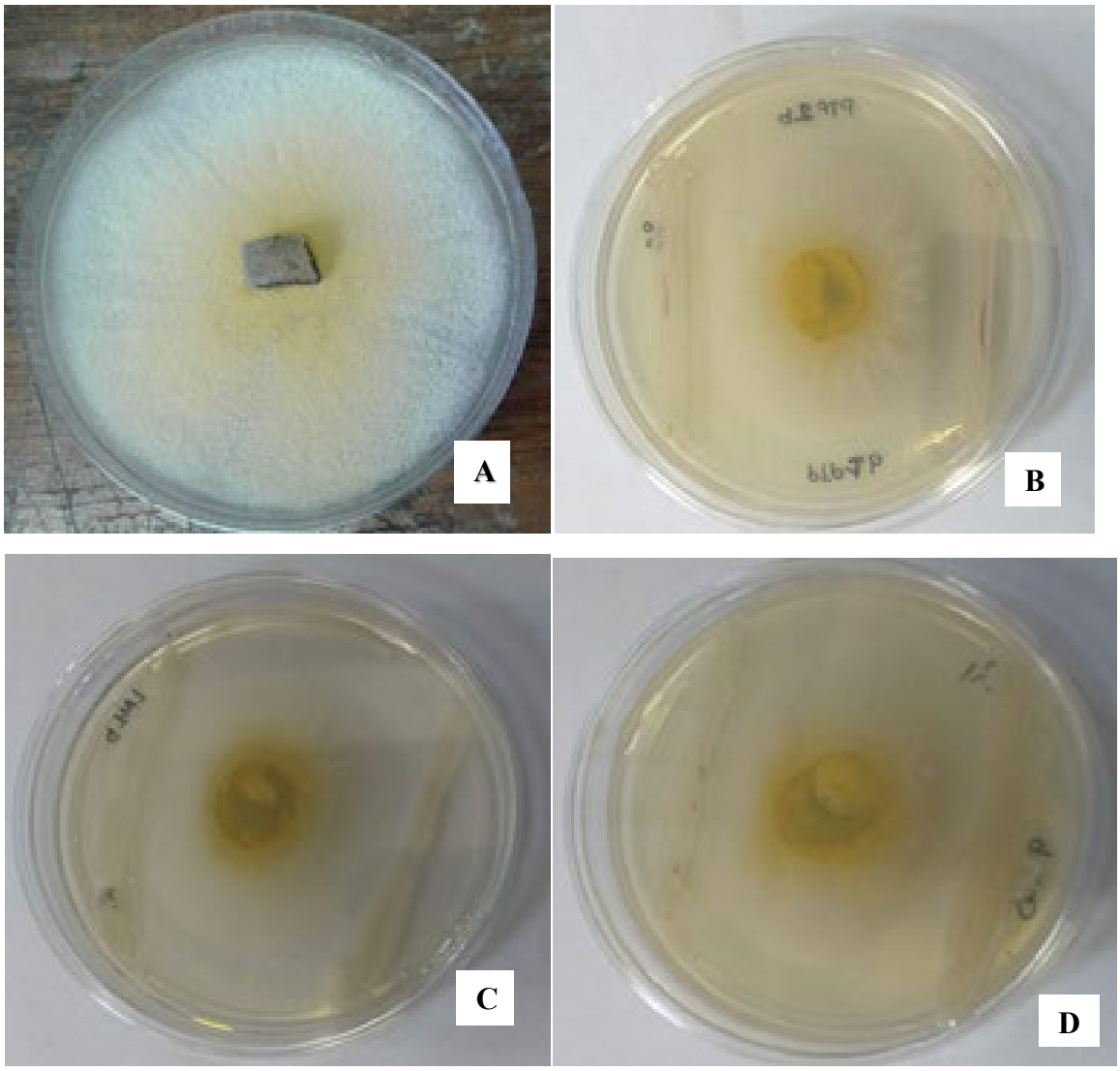

Figure 2.5: *In vitro* inhibitory activity of *Bacillus* isolates against *A. flavus* on PDA media after 10 days at 25℃. The top three isolates that displayed a potential with a high percentage inhibition. (A) Control plate (B) Isolate PTP1b, (C) Isolate LM1b, (D) Isolate SF4b

2.4 Discussion

This study aimed to isolate, screen, and identify bacterial and yeast isolates antagonistic to *A. flavus*. A total of 60 *Bacillus* and 169 yeast isolates were screened for their ability to suppress *A. flavus* in an *in vitro* dual culture bioassay. With the average inhibition, 87.5% of the yeast and 93% of the *Bacillus* isolates exhibited a moderate average percentage inhibition of the pathogen. In the secondary screening test, isolates repeatedly showed potential as biological control agents of *A. flavus* though they did not all maintain the same percentage inhibition. A biological control agent's antagonistic property can be affected by storage method, media, and storage duration. Temperature, pH, and nutritional requirements impact a biocontrol agent's growth and antagonistic potency **(Huang and Dong, 2003; Hartati et al., 2017).**

The papaya leaves had the highest number of *Bacillus* species isolates than any other samples used. The high average inhibition of 58-65% in all *Bacillus* isolates from the papaya leaves (PW) is the single sample that achieved such high inhibition compared to other isolates from all samples. *Bacillus* species were isolated from papaya leaves for various uses, e.g., they were used as a seed protectant on papaya against rot root pathogens **(Krishan et al., 2012; Thomas et al., 2007).** A study by **Ranasinghe et al. (2018)** showed that the rhizobacterial mixture of *Bacillus* spp. and *Pseudomonas* spp. used as a seed treatment, and a root dip reduced the Papaya ringspot virus disease and increased peroxidase and phenylalanine ammonia- lyase PAL enzyme activity **(Bakker et al., 2015).** The bacterial strains were isolated from the papaya leaves. The *Bacillus* species antagonistic potency isolated from the cowpea leaves (C.P.) was effective till day 15. *Bacillus* species isolated from cowpea can reduce and manage bacterial blight disease growth in cowpea **(Kannan et al., 2020).** Both cowpea and groundnuts are legumes and have common physiological and nutritional requirements; hence the probability of *Bacillus* on groundnuts is high **(Samireddypalle et al., 2017).** *Bacillus* Isolate LM1 was isolated from *Citrus limon* L. (Lemon leaves) with the highest percentage average inhibition of 70.0%. **Daungfu et al. (2019)** study showed *Bacillus* spp efficacy in controlling citrus canker in limes. Several authors have reported *Bacillus* spp effectively inhibiting *A. flavus* growth through antifungal metabolites production, including **Zhang et al. (2007), Kumar et al. (2014), Siahmoshteh et al. (2016), and Yobo et al. (2016)**. The inhibition zones are evidence of either antibiotic, toxic metabolites, or lytic enzymes secretion (**Zhao et al., 2013)**. These compounds dissolve the cell wall, inhibit mycelial growth, and minimize the pathogens' tube elongation. Isolates from papaya and cowpea leaves showed potential for efficient

inhibition over time. Yeast Isolate CP was effective in inhibiting *A. flavus* post the 7 days compared to other yeast isolates. Yeast isolated from papaya was effective against fungal pathogens **(Parameswari et al., 2015).** In this study, the yeast isolated from sunflower leaves were effective with 61.1% average inhibition during the secondary screening. Sunflower produces phytoalexin, which can inhibit *Sclerotinia sclerotiorum*; hence, the yeast isolated from its leaves is proposed to have the potency to inhibit fungal pathogens **(Urdangarin et al., 2009).** Sunflower has the highest number of isolates with a high percentage average inhibition compared to other isolates from different samples. In previous work, **Fareed et al. (2019)** and **Li et al. (2019)** have shown that yeast isolated from sunflower has antifungal potency and efficiency against *Alternaria alternate, Fusarium solani*, and *Fusarium oxysporum* **(Moradi et al., 2019).**

The secondary screening results showed, yeast Isolates CC1 (72.1%), PF3 (70.8%), and CF1 (70.0%) achieved high percentage average inhibitions. In the current study, the yeast that achieved the highest inhibition was isolated from the *C. comosum* leaves (spider plant). **Manhert et al. (2018)** research showed a yeast isolated from the *C. comosum* completely inhibited mycelial growth and inhibited sporulation of the plant pathogen *Botrytis cinerea* Pers.:Fr by the production of antifungal volatile organic compounds (VOCs). Yeast isolate PF3 (70.8%) was isolated from *Protea cynaroides* L. (protea flower). In previous studies, yeast strains (*Metschnikowia drakensbergensis* sp. nov., *Metschnikowia Pulcherrima* sp nov., and *Metschnikowia caudata sp*. Nov) isolated from protea flower have been used as a biocontrol agent **(Spaaji et al., 2013).** In a study by **Türkel et al. (2014)**, Yeast *Metschnikowia Pulcherrima* isolated from protea flower showed effective antagonistic against several fungal pathogens. It was further shown that *M. pulcherrima* UMY15 is an effective biocontrol yeast against various species of postharvest pathogens, including *Penicillium, Aspergillus, Fusarium* and *Rhizopus* **(De Vega et al., 2014).** The yeast Isolate CF1 was isolated from groundnut leaves. The strain showed the potential of inhibiting *A. flavus* with a 70% of mycelial growth. It will therefore be interesting to see how this yeast isolate performs during *in vivo* studies. **Afsah-Hejri (2013)** research indicates saprophytic yeast isolated from the *Pistacia vera* L. (pistachio nut) and groundnut leaves reduced the growth of a toxigenic strain of *A. flavus*. The yeast significantly reduced *A. flavus* growth and aflatoxin levels in pistachio nuts **(Moradi et al., 2020, Culliney, 2005).** Yeast isolated from different crop hosts or sources could effectively inhibit fungal pathogens affecting different crop hosts. A study by **Wang et al. (2010)** indicated that yeast sourced from orange leaves was used to control *Botrytis cinerea* on tomatoes

(*Solanum lycopersicum* L). Hence, in this study, the yeast from different sources was able to inhibit *A. flavus.* Different yeast strains use different mechanisms of action to inhibit plant pathogens **(Spadaro and Droby, 2016; Zhang et al., 2007).** The difference in the percentage inhibition observed for the other yeasts in both the primary and secondary screenings could be due to the yeast isolates mechanism employed. The results in this study demonstrate that the *Bacillus* and yeast isolates obtained from the different plant samples have the potential to control *A. flavus* on groundnuts. This study is useful in identifying potential candidates for the biocontrol of *A. flavus* on groundnuts. These isolates will be tested on groundnut seeds for long-term storage of groundnut seeds to ascertain their effectiveness in controlling *A. flavus* in groundnuts.

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CHAPTER THREE

EFFECT OF HOT WATER TREATMENT ON *ASPERGILLUS FLAVUS* LINK INFECTION IN GROUNDNUT SEEDS

Abstract

Infection of *Aspergillus flavus* Link, which can produce aflatoxin, is a significant problem for the safe storage of groundnuts (*Arachis hypogaea* L.). The efficacy of hot water treatment to reduce fungal disease incidence during postharvest storage has been investigated and found to be useful in different crops. The limitation is that the temperature and treatment duration can significantly affect the color, appearance, and quality of fruits. This study aimed to investigate the effect of temperature (Hot Water Treatment – HWT) and time to manage *A. flavus* infections in groundnut seeds. There were 17 treatments from 20ºC to 75ºC for 20 and 60 seconds, respectively, including a control (untreated). Seeds were dipped in hot water at a specific temperature and time period air dried for 30 minutes per treatment then stored in Petri dishes under laboratory conditions. Percentage infection, disease severity, germination rate, and seed vigor were measured over 14 days. Results showed that under *in vitro* conditions, the hot water treated seeds at 40°C for 60 seconds showed zero *A. flavus* infection with maximum seed germination percentage. This was followed by 40ºC at 20 seconds; with the least effective treatment at 75ºC at 60 seconds. Disease severity increased as increase in the temperature and time combination increases. The results obtained in this study show that HWT at 40°C for 60 seconds can be used to reduce *A. flavus* contamination in *A. hypogaea* seed.

Keywords: Hot water treatment, percentage of infection, seed germination, *Aspergillus flavus*, groundnuts

3.1 Introduction

Postharvest spoilage of fruits and vegetables cause losses of about 30% worldwide and 45% in Africa due to the inadequacy of the postharvest storage system and disease manifestation **(Yahaya and Mardiyya, 2019).** Lack of postharvest management skills and techniques such as temperature control to maintain the cold chain, value addition, and packaging have caused several economic and food security setbacks **(Yánez-Mendizábal, and Falconí, 2018).** The use of chemicals become unattractive due to its effects on consumers. Postharvest fungicide treatments have not significantly affected disease incidence or severity after storage **(Yan et al., 2016).**

Hot Water Treatment (HWT) is the physical treatment of fruits and vegetables by applying or immersing commodity in hot water **(Yan et al., 2016),** exposure to vapour heat (**James and Zikankuba 2017)**, and exposure to hot, dry air or treatment with infrared **(Lui et al., 2021)**. Over the years, HWT has been used on different crops and proven to be effective on citrus fruits, avocado (*Persea Americana* Mill.), tomatoes (*Solanum Lycopersicum* L.), mango (*Mangifera indica* L.), and apples (*Malus* domestica Borkh.) in reducing postharvest decay and maintain fruit quality **(Wassermann et al., 2016; Agustí-Brisach et al., 2012).** The treatment effectively reduced fungal diseases, including powdery mildew (*Oidium mangiferae* Berthet), anthracnose (*Colletotrichum gloesporioides* Sacc) on mango **(Angasu et al., 2014)** . Brown rot (*Monilinia fructicola* G.Winter.) on peaches (*Prunus persica (L.) Batsch*) fruit, and green mould (*Penicillium digitatum* (Pers.:Fr.) Sacc), and blue mould (*Penicillium italicum* Wehmer.) on oranges (*Citrus* × *sinensis* (L.) Burm. f.) have been reduced by hot water dipping **(Opio et al., 2017).**

Heat treatment has an impact on the physiology of the fruit. **Lui et al. (2012)** showed that heat treatment on peaches (*Prunus persica* (L.) Batsch) induced defense-related gene expression. It increased the activity of enzymes such as chitinase (CHI), β-1, 3-glucanase (GNS), and phenylalanine ammonia-lyase (PAL). The heat treatment maintained the fruit quality during the long term, fruit firmness, or potentially maximizing it, increasing storage stability. Hot water treatment has been used as seed treatment to minimize seed-borne pathogens, including *Alternaria brassica* (Berk.), *Sclerospora graminicola J.*, *Magnaporthe grisea* (T.T. Hebert) M.E. Barr, and *Helminthosporium oryzae* Breda de Haan **(Sharmal et al., 2015).** Hot water treatment has been used for seeds with surface or deep-seated infections **(Singh et al., 2020).** The principle is to eliminate pathogens as far as possible without decreasing germination of

seeds. The objective of this study was to develop a hot water treatment regime that controls *A. flavus* in groundnut seeds and providing long-term protection of shelled groundnut seeds from postharvest infection.

3.2 Materials and Methods

3.2.1. Sourcing of groundnut seeds

A 10 kg groundnut seeds was obtained from a commercial retailer, Agricole Seed Supplier, in Howick, KwaZulu-Natal, South Africa. The seeds were stored in a cold room at 2ºC for 2 weeks.

3.2.2 Hot Water Treatment (HWT)

A 350 L dipping water tank was filled and heated from the lowest desired temperature to the highest temperature (Table 3.1). The temperature of the water was monitored using a Brannan Immersion glass thermometer (-10 +360°C, Lo-tox Filled Laboratory, London) for each treatment. Seventy-two seeds (72) per replicate were placed in a mesh bag (made of nylon or nylon plus polyester material), submerged in the water bath, and a timer was activated for a specific time interval according to each temperature and time treatment combination. After each treatment combination, seeds were air-dried on a paper towel under a laminar flow for 30 minutes, poured into a petri dish, and stored in boxes at ambient temperature in the laboratory. The *A. flavus* infection levels were evaluated from day 7 to day 14 by counting the number of seeds visibly infected. There were five replicates for each treatment, and the experiment was repeated twice.

Table 3.1: The formulated codes correspondent to the full name for the treatment of all samples used in this research.

Sample Code	Treatment
20C/20s	Dipping at 20°C for 20 seconds
20C/60s	Dipping at 20°C for 20 seconds
40C/20s	Dipping at 40°C for 20seconds
40C/60s	Dipping at 40°C for 20seconds
45C/60s	Dipping at 45°C for 20 seconds
45C/60s	Dipping at 45°C for 60 seconds
50C/20s	Dipping at 50°C for 20 seconds
50C/60s	Dipping at 50°C for 60 seconds
55C/20s	Dipping at 55°C for 20 seconds
55C/60s	Dipping at 55 °C for 60 seconds
60C/20s	Dipping at 60°C for 20 seconds
60C/60s	Dipping at 60°C for 60 seconds
63C/20s	Dipping at 63°C for 20 seconds
63C/20s	Dipping at 63°C for 60 seconds
75C/20s	Dipping at 75°C for 20 seconds
75C/60s	Dipping at 75°C for 60 seconds.
Control	No treatment

3.2.3 Seed assay

Four treatments were selected to evaluate the effect hot water treatment has on seed germination. From the preliminary hot water dipping results, the treatments selected were no treatment (control), best treatment (40ºC at 60 seconds), second best treatment (40ºC at 20 seconds), and 75ºC at 60 seconds. The protocol used for the heat treatment is as described under Section 3.2.2. The difference was in the number of seeds used for the seed assay, which was 60 seeds per replicate. Determine the effect of the various temperatures on seed germination, thirty seeds (30) from each replicate were planted in seedling trays using composted pine bark, and each treatment was labelled accordingly. The trays were placed under

greenhouse at 20-28ºC at a relative humidity ranging from 70-75% to observe percentage seed germination.

To determine the number of seeds infected by *A. flavus*, six seeds were plated on a PDA media and incubated at 28ºC for 7 days with five replicates per treatment (i.e., untreated control, 40ºC at 60 seconds, and 40ºC at 20 seconds and 75ºC at 60 seconds) to observe *A. flavus* infection. After 7 days, the *A. flavus* infection was evaluated by counting the number of seeds that were visibly infected. The experiment was repeated twice with five replicates for each treatment. The seed germination percentage was calculated using the following equation,

Percentage seed germination = $\frac{number\ of\ seeds\ germinated}{number\ of\ seeds\ planted}$ x100

3.2.4 Data analysis

Quantitative, discrete data was recorded. The number of infected seeds was recorded from day 1 till day 14. The disease rating was accessed and recorded based on the infection per seed. Disease evaluation was done from day 0 to day 14 with interval of 2 days. The Area Under the Disease Progress Curve (AUDPC) was calculated using the trapezoidal method. This discretizes the time variable (days) and calculate the average disease intensity between each pair of adjacent time points **(Madden et al., 2007).**

AUDPC = $\frac{Nt-1}{i=1}\sum_{2}^{(yi+y2)}(t1+1+t1)$

n = total number of observations,

yi = injury intensity (usual incidence in crop health data),

i th observation,

t = time at the ith observation.

Data for the hot water and temperature interactions experiment for the control of *A. flavus* were subjected to two-way Analysis of Variance (ANOVA) using the Statistical Analysis System (SAS Version 9.4) (SAS Institute Inc., 2014). Where ANOVA was significant, means were separated using Duncan Multiple Range Test (DMRT) at a 5% significance level.

3.3 Results

3.3.1 Hot Water Treatment

The best temperature range with controlled *A. flavus* infection was 40°C at all exposure times compared to the control (Untreated) (Table 3.2).

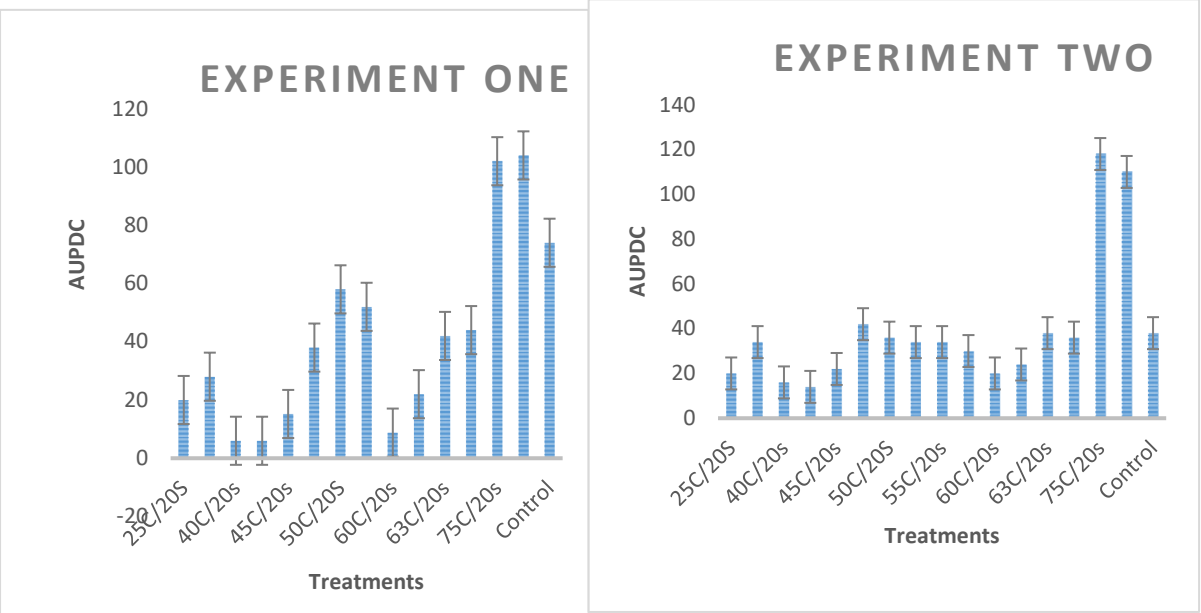

Figure 3.1 AUDPC of each treatment in seed stored over two weeks (14 days).

Figure 3.1 shows the summary of disease intensity over time for Experiments one and two. The best treatment was 40ºC at 60 seconds, followed by 40ºC at 20 seconds. This was indicated by low seed infections compared to the rest of the treatments including the untreated control. The worst treatment was 75ºC at 60 seconds. In both experiments, as the temperature increases, the AUDPC units increased simultaneously. The simultaneous increase in temperature and time severely denatured the seeds. The longer the time of exposure of the seeds to the temperatures, the higher the AUPDC units (Figure 3.1). For example, the 45ºC at 20 seconds treatment had a low disease intensity and AUDPC units compared to 45ºC at 60 seconds, so as in other treatments.

Table 3.2 The percentage seed infection recorded for each treatment for two weeks at storage

	Experiment One		Experiment Two	
Temperature (ºC)	**Time (Secs)**	**Percentage Infection**	**Time (Secs)**	**Percentage Infection**
25	20	64 ^{ef}	20	70^{cd}
25	60	72^{cde}	60	92^{ab}
40	20	48^{f}	20	52^{e}
40	60	48^{f}	60	49^{f}
45	20	62^{ef}	20	86^{abc}
45	60	88^{abc}	60	100^{a}
50	20	80^{bcde}	20	90^{ab}
50	60	98^{ab}	60	100^{a}
55	20	96^{ab}	20	92^{ab}
55	60	72^{cde}	60	76^{bcd}
60	20	66^{edf}	20	84^{abc}
60	60	74^{cde}	60	76^{bcd}
63	20	76^{cde}	20	84^{abc}
63	60	84^{abcd}	60	88^{ab}
75	20	92^{ab}	20	100^{a}
75	60	86^{abcd}	60	100^{a}
Control (No treatment)	-	100^{a}	-	100^{a}
Effects	F- value	P-value	F- value	P-value
Temperature	9.34	<.0001	11.12	<0.001
Time	2.28	0.14	1.92	0.16
Temperature*Time	2.93	0.010	2.36	0.032
%CV		18.67		22.3

*Each value is reported as an average of three replicates; means followed by the same letters are not significantly based on Duncan's Multiple Range Test at 5% significance level (P=0.05).

The best treatment with the lowest disease infection was 40ºC at 60 seconds, followed by 40ºC at 20 seconds. Treatment with the highest disease infection was 75ºC at 60 seconds. There was a significant difference in temperature as a parameter alone for the first experiment (P-value = 0.001), and the second experiment (P-value =0.001). The increase in temperature resulted in the percentage increase in seed infection (Table 3.2). The temperature and time interaction showed a significance difference for both experiments respectively (Experiment One, P-value

= 0.010 and Experiment Two, P-value = 0.032) while time alone showed no significant difference (P-value = 0.14 for Experiment One) and (P-value = 0.16) for Experiment Two. The severity of the disease in terms of the number of seeds infected over time increased as the temperature and time exposure increased.

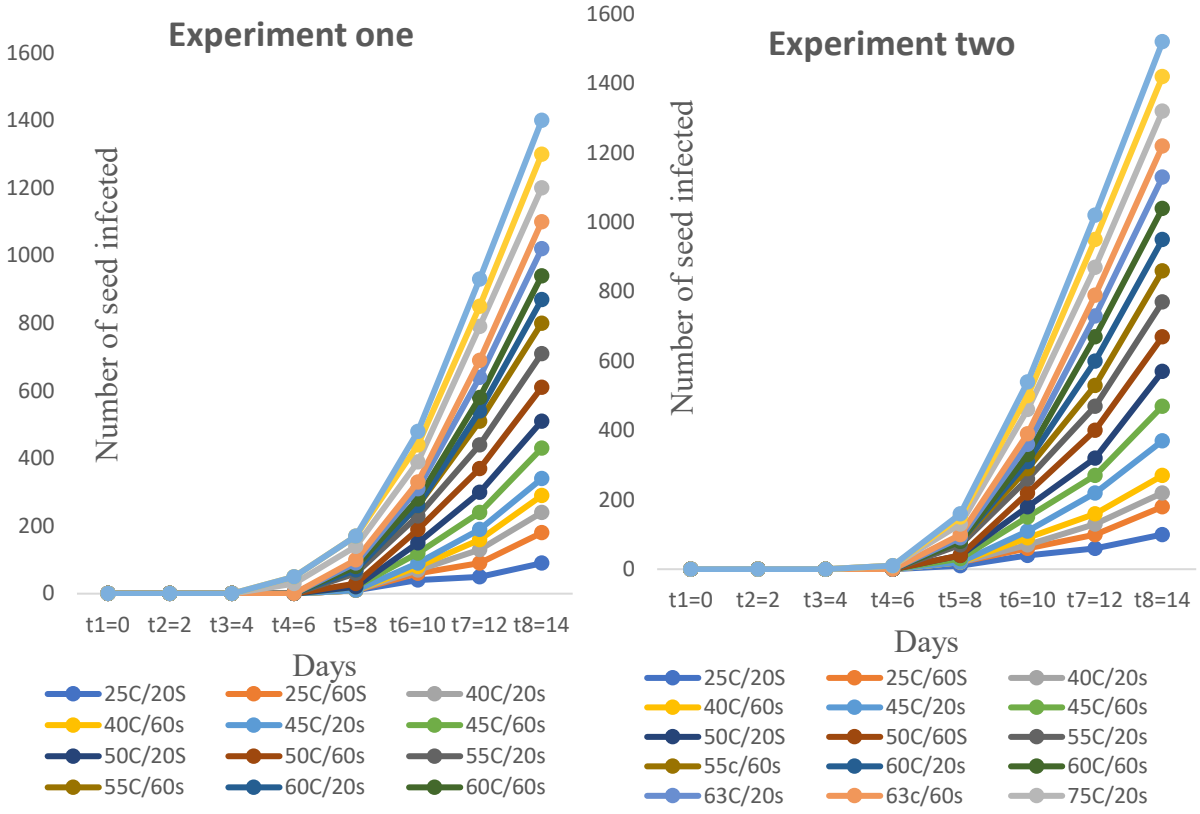

Figure 3.2 Progress curve for disease severity (number of on heat treated seeds over two weeks period stored at ambient temperature in the laboratory.

From Day 1 to Day 14, the disease progress increased exponentially. In both experiments, the increase in disease severity for each treatment increased as the storage days increased (Figure 3.2). The disease progress curve showed a linear increase in disease progression in terms of the percentage seed infection.

3.3.2 Seed ssay

The *in vivo* evaluation of *A. flavus* occurrence on seed treated with 40ºC at 20 seconds, 40ºC at 60 seconds and control. The germination percentage of seeds was evaluated by planting seeds treated at 25ºC for 20 seconds, 40ºC for 20 seconds, 40ºC for 60 seconds, 60 ºC for 20 seconds, 75ºC for 60 seconds and control

Figure 3.3: A seed assay of *A. flavus* incidence on treated seeds, A: Untreated Control; B: HWT at 40ºC for 60 seconds; C: HWT at 40ºC for 20 seconds after 5 days of incubation at 25ºC .

The effect of the selected two temperature-time treatment combinations compared to the untreated control showed that the seeds for the best treatment plated on PDA showed no fungal infection compared to the untreated control, which was completely infected with *A. flavus* (Figure 3.3).

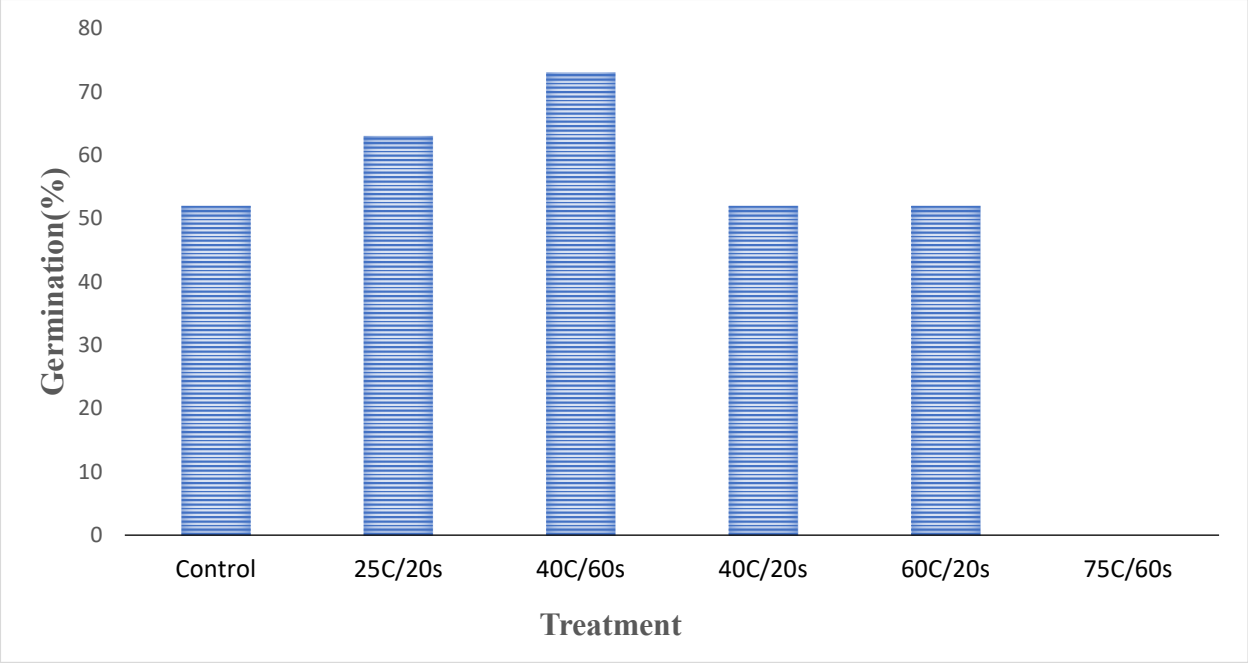

Figure 3.4 Percentage germination of hot water treated groundnut seeds under greenhouse conditions.

The 40ºC at 60 seconds has the best germination percentage (73%), and 75ºC at 60 seconds had no germination (0%) (Figures 3.4 and 3.5). There was a low germination percentage at high temperature-time treatment compared to control as the seeds are killed at higher temperatures.

Figure 3.5: Seed germination from different treatments 14 days after planting. A: Control Treatment (No treatment); B: 40ºC at 60 seconds; C: 40ºC at 20 seconds and D: 75ºC at 60 seconds.

At 75ºC for 60 seconds, there was no germination. This was the worst of all the four treatments evaluated.

3.4 Discussion

Seed treatment is a standard non-chemical alternative for seed-borne diseases. Hot water treatment has been influential in the maintenance of fruit quality, reduce postharvest infection, and maintaining seed health (germination rate, vigour) in different crops, including peaches and oranges. **(Agusti et al., 2019, Singh et al., 2019).** The experiment was designed to formulate the best hot water treatment and time combination to minimize infection from *A. flavus*. The best treatments were 40ºC at 60 seconds, 40ºC at 20 seconds, and the least effective was 75ºC at 60 seconds**.** According to **Gupta and Kumar (2020),** temperature and time treatment reduced the infection of *A. flavus* on groundnuts at 40ºC for 3 minutes. This study showed that the best treatment was 40ºC at 60 seconds, followed by 40ºC at 20 seconds. This concur with **Opio and Photchanachai (2016)** findings that 40ºC treatment has the lowest infection percentage with a high germination percentage on groundnuts compared to other treatments (50ºC, 60ºC, and 70ºC).

The AUPDC percentages (disease intensity over time), disease progress, and disease severity low at 40ºC at 60 seconds hence the low infection percentage. *In vitro* mycelial growth was inhibited entirely using heat treatment at 40°C for 20 and 60 seconds respectively. The disease progress and severity of percentage seeds infection increased as the number of storage days increased in both experiments. At different temperatures and time combinations, the *A. flavus* infection, AUDPC, seed germination percentage, and disease progress vary. The linear trend in both experiments showed the increase in disease intensity over time (AUDPC) is proportional to the increase in temperature **(Kabelitz et al., 2019; Kharel et al., 2019). .** An increase in *A. flavus* infection, hence an increase in disease severity. Hot water treatment at higher temperature damage the seed coat, which may have increased the tissue's susceptibility to *A. flavus* **(Khan et al., 2002).** At high temperatures, enzymes and proteins denature, limiting the seed defense mechanism, affecting the seeds' enzyme activity (**Qu et al., 2019).** High temperature provides moisture at a high rate compared to low temperatures. Hence as temperature increase in treatments so is the moisture content. High moisture content in seed offers favourable conditions for fungal spores (*A. flavus)* to germinate; hence infection increases resulting in increased disease intensity at high temperatures (**Egley, 1990**; **Wan et al., 2020**). The increase in exposure time (from 20 seconds to 60 seconds) favours increased moisture content. A study by **Hassane et al. (2017)** shows a maximum peak growth of A. *flavus* at 40ºC with a moisture content of 5% on wheat grains. The groundnuts seeds were stored at approximately 25ºC in the laboratory for 14 days possibly resulting in humidity changes. The

higher the humidity, the more *A. flavus* infection will increase **(Adefunke and Elizabeth, 2018)**. *A. flavus* infection is enhanced with increasing temperature and relative humidity, regardless of the HWT treatment applied **(Mannaa and Kim, 2018).**

Heat treatment stimuli activate the seeds systemic acquired resistance reactions, resulting in phytoalexins release, which inhibits the growth of *A. flavus* **(Bediako et al., 2019; Sobolev, 2008)**. Phytoalexin is a substance produced by plant tissues in response to contact with a pathogen and specifically inhibits that pathogen's growth **(Ishita et al., 2012).** HWT is associated with the elicitation of defense response in the seeds **(Chen et al., 2015).** A temperature of 40-50ºC was able to reduce *Aspergillus* spp. development on seeds due to activation of systemic reactions, the infection was minimal at 40ºC. The higher disease incidence in this treatment (50, 60, 75ºC at 20 and 60 seconds) indicates that the mycelium of *A. flavus* survived treatment with high water temperature and that hot water treatments at elevated temperatures were ineffective in deactivating the conidia of *A. flavus*. Increase in infection at higher temperatures result from seed denatured enzymes **(Rezvani and Zaefarian, 2017)**. It can be concluded that these heat shock trigger induced resistance, but if the heat level was too high or far too long, the induced resistance mechanism was damaged or deactivated.

The germination percentage significantly decreased when the temperature on treatments increased to 63 °C and 75 °C respectively. Seed germination was considerably reduced as the temperature increased**.** Seed germination is dependent on germination viability over time, depending on the seed's vigour and viability **(Kumah et al., 2011; Hurdle et al., 2020).** Seed vigour decreases over time, so as viability, which reduced the seed germination percentage in this study. At 75ºC at 60 seconds, the germination percentage was low at high temperatures. The means that the seeds were damaged, causing the seed enzymes to denature **(Muntz, 2001, Filho et al., 2015).** The germination percentage was low at high temperatures possibly due to high temperatures, resulting in DNA methylation of ABA catabolism-related and α-amylase gene promoters, delaying germination heat-stressed seeds **(Suriyasak et al., 2020).** The seeds are damaged, which disrupts the functioning of seeds physiology **(Guzman-Oritz et al., 2019)**. Temperature increase provides high relative humidity and increases in moisture content, which are advantageous to the growth of the *A. flavus* in the seed. The best treatment showed (40ºC at 60 seconds) healthy emerged seedlings; they were faster than the control and 75ºC treatment for 20 and 30 seconds respectively.

In conclusion, the efficiency of hot water treatment is dependent on the temperature used x time of exposure. The best treatment was 40ºC at the 60 seconds, based on the percentage of disease incidence, the percentage germination of the seedlings and moreover, the disease intensity was slow. At high temperatures, the seed is exposed to high heat and resulting in damage.

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CHAPTER FOUR

EFFECT OF COMBINATION OF HOT WATER TREATMENT AND BIOLOGICAL CONTROL AGENTS ON *ASPERGILLUS FLAVUS* INFECTION AND AFLATOXIN PRODUCTION IN GROUNDNUT SEEDS UNDER STORAGE.

Abstract

Aflatoxin contamination in groundnut seeds remains a significant challenge in South Africa. This study evaluated the efficacy of combining hot water treatment and biological control agents in managing the incidence of *Aspergillus flavus* Link. infection in groundnut (*Arachis hypogaea* L.) seeds during long term storage and subsequent effect on aflatoxin production. The biocontrol agents were selected from the *in vitro* screening trials (Chapter 2).The best four bacterial and yeast isolates were identified as follows: Isolate LM1b as *Bacillus cereus* JX218990.1, Isolate PTP1b as *Alcaligenes faecalis* MG746621.1, Isolate CC1y as *Suhomyces kilbournensis* KU751783 and Isolate PF3y as *Rhodotorula mucilaginosa* MK267619.1, KY076610.1. A 40ºC for 60 seconds heat treatment trial (*in vivo*) was determined as the best treatment for groundnut kernels (Chapter 3). Using an ELISA test Kit, aflatoxin B_1 concentration was measured after 12 weeks of storage in selected treatments. The best combination treatment was HWT+BCA1 (*Suhomyces kilbournensis* KU751783.1.), which reduced *A. flavus* infections by 52% compared to other treatments after 12 weeks at storage. HWT alone and BCA1 (*Suhomyces kilbournensis*) alone provided inadequate protection against *A. flavus* infection at storage. Combination of yeast biocontrol agent and HWT decreased *A. flavus* occurrence in groundnut seeds. Aflatoxin B_1 was present in all treated kernels with no significant difference (P=1.6) amongst treatments, suggesting no difference in Aflatoxin B_1 concentration in all combined and single treatments. BCA4 (*B. cereus*) treatment alone resulted in the lowest Aflatoxin concentration after 12 weeks of storage. This study shows that using physical control method combined with biocontrol agents provided a significant reduction of *A. flavus* infection in groundnut seeds at storage.

Keywords: Biocontrol; Hot Water Treatment; Groundnuts; Aflatoxins.

4.1 Introduction

Aspergillus flavus Link manifest and infect groundnut seeds from pre to postharvest, and their presence set a limitation on clean and safe food. They cause many groundnut diseases, including yellow mould **(Chalivendra et al., 2018**). The secondary metabolites of *Aspergillus* species called aflatoxins are chemicals that cause the limitation and constraint in the cultivation of groundnuts **(Zhou et al., 2014).**

Aflatoxins are heat stable, which makes it more challenging to eliminate them on commodities. There are four significant aflatoxins (B1, B2, G1, and G2). Aflatoxin B_1 indigestion by cows can be metabolized into carcinogenic aflatoxin M_1 in milk. Aflatoxins pose a health risk to animals and humans. Between 2004 and 2005 in Kenya, the reported number of deaths recorded was 125 as a result of aflatoxin B_1 contamination in food **(Mutegi et al., 2018).** Besides groundnut, aflatoxins contaminate many crops, including wheat (*Triticum* L), maize (*Zea mays* L.) in pre and postharvest. The international accepted levels of aflatoxins in groundnuts are 5 μgkg^{-1} in humans and 60 μgkg^{-1} in animals **(Lien et al., 2019).** Food commodities with high levels of aflatoxins than the standard are usually rejected and destroyed. Aflatoxins affect the economic value of crops resulting in financial losses. In the United States of America, aflatoxin contamination in food causes over $25.8 million in losses per year from 2016-2018 **(Lian et al., 2019).** The management of *A. flavus* and aflatoxin is a challenge even at postharvest. The best approach to reduce aflatoxin contamination in crops is to prevent the fungal growth **(Xia et al., 2017).** Many methods have been used, from biological control to physical control measures over the years. Biological control using microbial antagonists has shown great potential in controlling fungal diseases in fruit and vegetables **(Perez et al., 2014).** Physical treatment, such as hot water, has effectively managed fruit quality and reduced postharvest infections. Combining multiple management strategies to minimize aflatoxin contamination in food commodities has been widespread over the years. The combination of biocontrol agents and hot water treatment has been effective in many crops **(Zong et al., 2010).**

Heat treatment is efficient in enhancing resistance against *Botrytis cinerea* in tomatoes (*Solanum lycopersicum* L) **Zong et al. (2010),** and on lemon (*Citrus* x *Limon L*), reducing *Penicillium digitatum* by rinsing for 2 minutes in 53^{o}C hot water **(Perez et al., 2017).** The *P. digitatum* growth on lemons was inhibited by the prevention of hyphal elongation, which was achieved by upregulated defense proteins stimulated in lemons. Biocontrol agents or their

secreted elicitors and heat treatment can prime plant resistance through abiotic stress**.** Hot water treatment induces the phytoalexins, phenylalanine ammonia-lyse (PAL), chitinase (CHI) and β-1,3-glucanase production in crops. This studys' primary objectives were to evaluate the efficacy of Hot Water Treatment (HWT), two yeast and two *Bacillus* isolates in controlling *A. flavus* infections, and the reduction of aflatoxin B_1 production in groundnut seeds under storage.

4.2 Materials and Methods

4.2.1 Groundnut seeds

A 10 kg groundnut seeds (uncertified) was obtained from a commercial seed retailer, Agricole Seed, Howick, KwaZulu-Natal, South Africa. The seeds were stored in a cold room at 2ºC until use.

4.2.2 Inoculum preparation

The yeast and bacterial isolates used in this study were selected from *in vitro* screening studies for antifungal activity against *A. flavus* (Chapter Two). The best two yeast isolates selected were Isolates CC1y and PF3y and the best two *Bacillus* isolates were LM1b and PTP1b. Two other isolates, one yeast and one *Bacillus* spp. which were not part of the best four isolates were added. This was to evaluate if the isolates below average antifungal activity will control *A. flavus*. Fresh cultures were prepared from frozen stock cultures by sub-culturing the individual yeast and bacterial isolate as follows. The yeast isolates were plated on freshly prepared yeast malt agar and incubated at 28ºC for seven days while the *Bacillus* isolates were plated on freshly prepared tryptic soy agar and incubated at 28ºC for three days. The cultures were each resuspended in 50 ml of sterile distilled water and cell densities were determined using a haemocytometer (for yeast isolates) and a Helber Bacterial Counting Chamber (for *Bacillus* isolates). Cell densities were adjusted to $1x10^7$ cells mL^{-1}.

4.2.3 Molecular characterization

From the *in vitro* screening experiment (Chapter Two), the best two yeasts and best two *Bacillus* isolates were selected and sent to Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) for molecular characterization and identification to species level.

Bacterial isolates were biochemically characterized using a gram stain. For molecular characterization, using the ZR Fungal/Bacterial DNA Kit™ (Zymo Research), DNA was obtained from the cultures. The 16S target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486), and the primers shown in Table 4.1 PCR products were gel extracted (Zymo Research, Zymoclean™ Gel DNA Recovery Kit, D4001), and sequenced in the forward and reverse directions on the ABI PRISM™ 3500xl Genetic Analyser. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, D4050) were analyzed using CLC Main Workbench 7, followed by a BLAST search (NCBI). **(Altschul et al. 1997)**

Table 4.1: 16S Primers sequence used in identifying the bacterial isolates selected from *in vitro* screening studies.

Name Primer	Target	Sequence (5' to 3')	Size (bp)	Reference
16S-27F	16SrDNA sequence	AGAGTTTGATCMTGGCTCAG	24	**Garbeva et al., 2003**
16S-1492R	16SrDNA sequence	CGGTTACCTTGTTACGACTT	24	

For the yeast isolates, genomic DNA was extracted from the cultures using the ZR yeast DNA Kit™ (Zymo Research, Catalogue No. D6005). The ITS target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486). The primers are presented in Table 4.2. The PCR products were run on a gel and gel extracted with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analysed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each sample's reaction. CLC Bio Main Workbench v7.6 was used to analyze the .ab1 files generated by the ABI 3500XL Genetic Analyzer, and results were obtained by a BLAST search (NCBI). **(Gargas and White,1990, Altschul et al. 1997)**

Table 4.2: Primers sequences used in identifying the yeast isolates selected from *in vitro* screening studies.

Name Primer	Target	Sequence (5' to 3')	Size (bp)	Reference
ITS1	Small Sub-Unit	TCCGTAGGTGAACCTGCGG	4,607,442	**White et al., 1990**
ITS4	Large Sub-Unit	TCCTCCGCTTATTGATATGC	1,471	

4.2.4 Hot water treatment (HWT) on groundnut seeds

The best temperature and time (40ºC at 60 seconds) of the hot water treatment experiment identified in Chapter Three was used in this experiment. Groundnut seeds (360 seeds) were placed in a mesh bag and heat treatment applied as described in Chapter Three Section 3.2.2. The temperature of the water bath was monitored continuously with a thermometer and maintained at 40ºC. Groundnut seeds were immersed in the hot water bath for 60 seconds. After treatment, the seeds were air-dried for 30 minutes on a laminar flow bench. The experiment was repeated twice with four replicates. The seeds were not inoculated with *A. flavus* after treatment with HWT.

4.2.5 Effect of hot water treatment combined with yeast and *Bacillus* isolates

To determine the influence of treatment combinations with hot water and potential biocontrol agents on *A. flavus* on groundnut seeds, a mesh bag with 360 seeds was immersed in the water bath, with 40ºC hot water for 60 seconds. After treatments, the seeds were air-dried under the laminar flow on a sterile paper towel for 30 minutes. After drying, the seeds were inoculated with yeast and *Bacillus* isolates respectively, using a spray dispenser containing an inoculum concentration of $1x10^7$ cells mL^{-1}. The seeds were further air-dried for 10 minutes then stored in a mesh bag. They were then placed in a box and kept under laboratory conditions for 12 weeks at ambient laboratory temperature (20 – 25ºC). *A. flavus* infection count of each treatment was done every four weeks beginning from week 0. The experimental design was completely randomized with four replicates, and the experiment was repeated once**.** A fungal commercial biological control product, Eco-T (a.i. *Trichoderma asperellum*) was included in the treatment. There were 15 treatments. These were as follows: HWT+BCA1 (CC1y), HWT+BCA2 (PF3y), HWT+BCA3 (CF1y), HWT+BCA4 (LM1b), HWT+ BCA5 (PTP1b), HWT BCA6 (SF4b), HWT alone, BCA1 (CC1y), BCA2 (PF3y), BCA3 (CF1y), BCA4

(LM1b), BCA5 (PTP1b), BCA6 (SF4b), Eco-T and NoHWT + NoBCA (control). The seeds were not inoculated with *A. flavus* after treatment.

4.2.6 Determination of Aflatoxin B1 concentration in groundnut seeds

Aflatoxin B_1 concentration in the groundnut seed were determined using a competitive enzyme-linked immunosorbent assay (ELISA) using MaxSignal Total Aflatoxin ELISA test kit (PerkinElmer, U.S.A.). A total of 11 samples (HWT+BCA1: CC1y, HWT+BCA2: PF3y, HWT+BCA4:LM1b, HWT+ BCA5:PTP1b, HWT alone, BCA1 (CC1y), BCA2 (PF3y), BCA4 (LM1b), BCA5 (PTP1b), BCA6 (SF4b), Eco-T, NoHWT + NoBCA (control Week 0) and NoHWT + NoBCA (control Week 12), with four replicates were analyzed for Aflatoxin B_1. Aflatoxins were tested on NoHWT + NoBCA (control) sample at week 0, and at week 12 for all the 11 treatments, including NoHWT + NoBCA (control week 12) treated seeds.

Groundnut were ground using the pestle and mortar. 5.0g of the ground sample was mixed in a 50ml conical tube with 25ml of 70% methanol. The sample was vortexed manually for 3 minute at maximum speed. The samples were placed in a centrifuge and centrifuged at 4000x g for 10 minutes at room temperature (25^{o}C). Then 300µl of supernatant was placed into 2ml tube containing 900µl of Solution C. Solution C was prepared as follows: seven volumes of 100% methanol with three volumes of 1X oil extraction buffer solution and mixed well. The mixer was vortexed for 1 minute at maximum speed. A volume of 50µl of the sample was placed in the ELISA well. All eleven samples were prepared using the same protocol.

The procedure was done according to the manufacturer's instructions. The absorbance values were determined at 450nm using the BioTek® 800™ TS spectrophotometer ELISA plate reader (Darmstadt, Germany). Below (Table 4.3) shows the ELISA plate map presentation of the arrangement of treatments on the ELISA plate for testing and their replicates.

Table 4.3 ELISA Plate map for the aflatoxin analysis

	1	2	3	4	5	6	7	8	9	10	11
A	AFB1 0ng/mL	0.6	C R1	C12 R1	HWT R1	BCA1 R1	BCA2 R1	BCA4 R1	BCA5 R1	HBCA1 R1	
B											
C	0.02	1.5	C R2	C12 R2	HWT R2	BCA1 R2	BCA2 R2	BCA4 R2	BCA5 R2	HBCA1 R2	
D											
E	0.06	HBCA2 R2	C R3	C12 R3	HWT R3	BCA1 R3	BCA2 R3	BCA4 R3	BCA5 R3	HBCA1 R3	
F											
G	0.2	HBCA2 R3	HBCA5 R2	HBCA5 R1	HBCA4 R2	HBCA2 R1	HBCA5 R3	HBCA4 R3	HBCA4 R1		
H											

4.2.7 Data analysis

All data sets from the seed infection studies and Aflatoxin results were subjected to two-way Analysis of Variance (ANOVA) using the Statistical Analysis System (SAS Version 9.4) (SAS Institute Inc., 2015). Where ANOVA was significant, means were separated using Duncan Multiple Range Test (DMRT) at a 5% significance level.

4.3 Results

4.3.1 Molecular characterization of the potential biological control agents

The bacterial and yeast isolates were confirmed as follows: Isolates LM1 and PTP1 were gram-positive bacterial strains with 97% similarity to *Bacillus cereus* and *Alcaligenes faecalis,* respectively (Table 4.4). The Isolates CC1 and PF3 were yeast isolates with 92% similarity to *Suhomyces kilbournensis* and *Rhodotorula mucilaginosa* (Table 4.4)

Table 4.4: Blast identification details of the selected bacterial and yeast isolates.

Isolate Name	Identifies species
CC1y (BCA1)	*Suhomyces kilbournensis* KU751783.1
PF3y (BCA2)	*Rhodotorula mucilaginosa* MK267619.1
LM1b (BCA4)	*Bacillus cereus* JX218990.1
PTP1b (BCA3)	*Alcaligenes faecalis* MG746621.1

4.3.2 Combination of *Bacillus* or yeast isolates with hot water treatment for long-term storage of groundnut seeds.

The occurrence of *A. flavus* on each treatment was recorded every four weeks. The number of seeds infected 12 weeks after storage determined the efficacy of treatments.

Table 4.5: Efficacy of combined treatment of *Bacillus* or yeast and hot water treatment on reducing infection by *Aspergillus flavus* on groundnut seeds over 12 weeks at storage.

Treatments	Number of seeds infected with *A. flavus* over time at storage		
	Week 4	**Week 8**	**Week 12**
HWT + BCA 1 (*Suhomyces kilbournensis*)	78 a (21%)	154 bcd (43%)	175 cdefg (48%)
HWT + BCA2 (*Rhodotorula mucilaginosa)*	81 a (22%)	175 bcdefg (48%)	213 kl (59%)
HWT + BCA3 (Yeast spp)	75 a (20.8%)	185 defgh (52%)	256 l (71%)
HWT + BCA4 (*Bacillus cereus*)	63a (17%)	147a (41%)	203ghij (56%)
HWT + BCA5 (*Alcaligenes faecalis*)	74a (20.5%)	169bcdef (46%)	225kj (63%)
HWT + BCA6 (*Bacillus* spp.)	76a (21%)	162bcde (45%)	243kl (68%)
HWT (Hot Water Treatment)	75a (20.5%)	183defgh (51%)	274i (75%)
BCA1 (*Suhomyces kilbournensis*)	74a (20.5%)	150bc (41%)	185defgh (51%)
BCA2 (*Rhodotorula mucilaginosa)*	88a (24.4%)	200 fghij (55%)	242 kl (67%)
BCA3 (Yeast spp)	85a (24%)	168 bcdef (47%)	246kl (68%)
BCA4 (*Bacillus cereus*)	65a (18%)	162 bcde (45%)	178cdefg (49%)
BCA5 (*Alcaligenes faecalis*)	85a (23%)	162 bcde (45%)	245kl (68%)
BCA6 (*Bacillus* spp)	82a (23%)	191efghi (53%)	246kl (69%)
EcoT (*Trichoderma asperellum*)	94a (36%)	212 hij (58%)	2741 (75%)
NoHWT + NoBCA (Control)	78a (21%)	183 defgh (50%)	285i (79%)
Effects	**F-value**	**P-value**	**Significance**
Treatment	9.87	<.001	***
Time	963.71	<.001	***
Treatment*Time	2.46	<.001	***
%CV	1.0		

*** Highly significant; #Values in parentheses (brackets) are calculated average percentage seeds infected for each treatment.

The accumulation of seed infection increased for each treatment over time. The best treatment was the combination of HWT + BCA1 (*Suhomyces kilbournensis*) out of all eleven treatments with a final percentage seed infection of 48% after 12 weeks of storage (Table 4.5). This was followed by BCA4 (*Bacillus cereus*) at 49% seed infection after 12 weeks of storage. There were significant differences between the treatment, time and treatment*time interaction (P = <.001) (Table 4.5) All treatments were better than the control (NoHWT+NoBCA) after 12 weeks of storage with the control having a percentage infection of 79% (Table 4.5).

After 12 weeks of storage, the single BCA4 (*B. cereus*) treatment had percentage seed infection of 49% which was almost equal to the percentage seed infection achieved by the best combined treatment (HWT+ BCA1: *S. kilbournensis*), 48 and 49% respectively. There is a huge difference in the control levels achieved by HWT+BCA1 (*S. kilbournensis*) and HWT + BCA4 (*B. cereus*), which was the second-best treatment among the combination treatments. HWT alone (75% seed infection) had a high percentage seed infection compared to the best treatment HWT+BCA1 (*S. kilbournensis*) (48% seed infection). None of the two unidentified *Bacillus* and yeast isolates which showed below average antifungal activity were better than the best four selected isolates (two *Bacillus* and two yeast isolates).

4.3.3 Determination of aflatoxin B1 concentration in groundnut seeds

On the ELISA plate, a positive reaction was shown by bright yellow colour on the scale, indicating the presence of aflatoxins. Total aflatoxin contents in eleven treatments are presented in Table 4.6.

Table 4.6 Aflatoxin concentration measured in treated groundnuts using ELISA technique.

Sample	Treatment	ELISA Test	Concentration (µg/kg)
1	HWT + BCA 1	+	0.01252
2	HWT + BCA2	+	0.01563
3	HWT + BCA4	+	0.00845
4	HWT + BCA5	+	0.01452
5	HWT	+	0.00849
6	BCA1	+	0.00918
7	BCA2	+	0.00951
8	BCA4	+	0.00840
9	BCA5	+	0.01230
10	NoHWT+NoBCA (Control week 0)	+	0.00135
11	NoHWT+NoBCA (Control week 12)	+	0.01521
	P value		**1.6**
	F value		**0.365**
	%CV		**5.6**

BCA4 (*B. cereus*) had the lowest aflatoxin concentration, best treatment above all 11 treatments. On combination treatments, HWT+BCA4 (*B. cereus*) achieved a low aflatoxin concentration. The control (No HWT+No BCA) at week 12 concentration is high compared to the aflatoxin concentration of all the best three treatments.

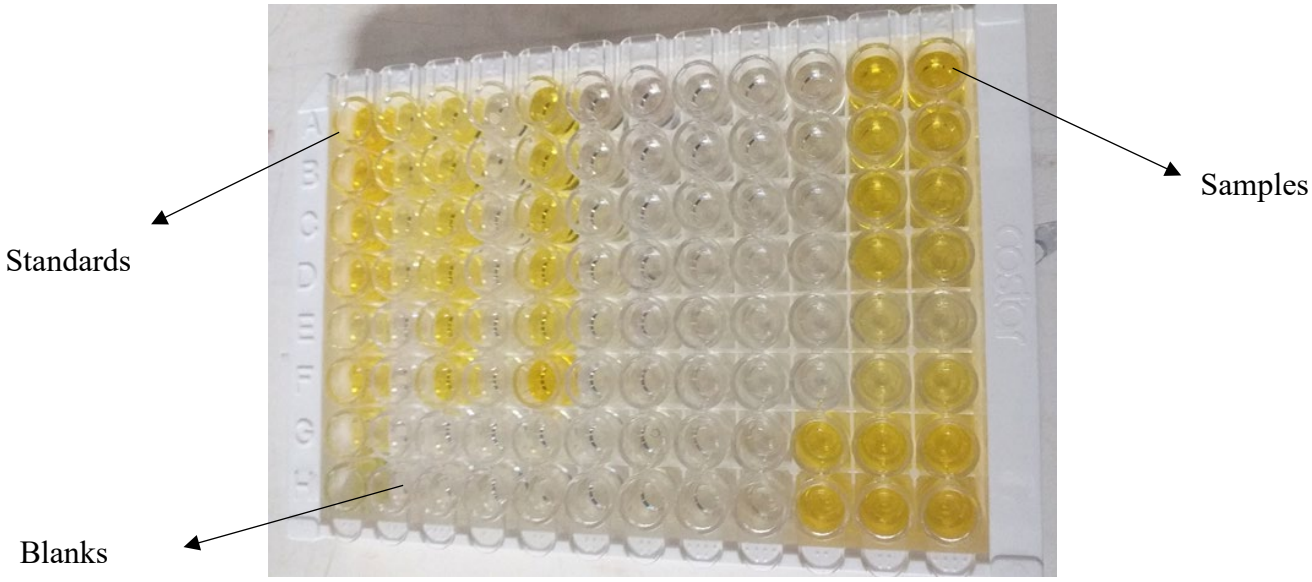

Figure 4.1: ELISA plates map reaction on treatments: The picture shows the reaction of bright yellow colour for the reactions where aflatoxin was detected.

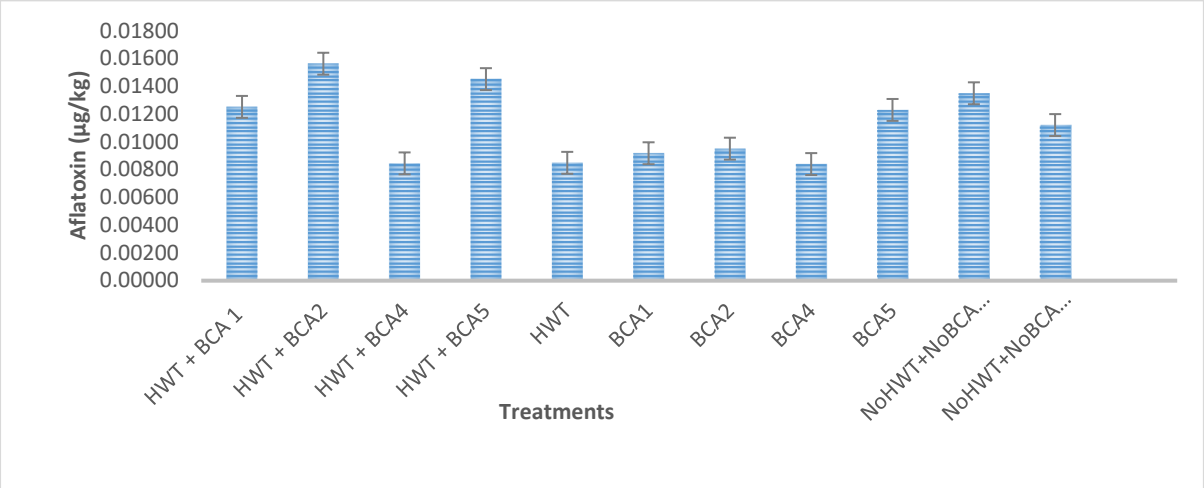

Figure 4.2 Comparison of aflatoxins B_1 concentrations in selected single and combined treatments after 12 weeks of storage.

The aflatoxin B_1 concentration shows no significant difference in all 11 treatments. The highest concentration of aflatoxin B_1 was determined in HWT+BCA1 (*S. kilbournensis*). The lowest concentration of aflatoxin B_1 was determined in HWT+BCA4 (*B. cereus*), followed by BCA4 (*B. cereus*). The combination of HWT+BCA4 (*B. cereus*) reduced aflatoxins concentration and limited the *A. flavus* growth on seeds for this treatment.

4.5 Discussion

Non-chemical control strategies of *A. flavus* on groundnut kernels were evaluated in the study. Molecular characterization to identify isolates showed Isolate LM1b as *Bacillus cereus* JX218990.1, Isolate PTP1b as *Alcaligenes faecalis* MG746621.1, Isolate PF3y as *Rhodotorula mucilaginosa* MK267619.1, KY076610.1, and Isolate CC1y as *Suhomyces kilbournensis* KU751783.1. The results of the study indicated that the combination of heat treatment/non-chemical treatment and biocontrol agents can provide adequate control of *A. flavus* on groundnuts during storage at 26ºC. Hot water alone could not provide a low percentage seed infection, compared to the application of HWT + antagonist treatment. Yeast combined with hot water treatment has been utilized on different crops, including apples, oranges, and tomatoes **(Terao et al., 2017).** *S. kilbournensis* is exclusively found on maize, together with *Candida kruisii* are effective in the inhibition of *A. flavus* and limiting insects on maize in the United States of America **(Kurtzman et al., 2016). Zong et al. (2010)** studied the effects of yeast antagonist combined with hot water treatment on postharvest diseases of tomato. The suggested mechanism of action of HWT is the induction of antifungal-like substances (chitinase, protease, and cellulase) that inhibit fungal development in fruit tissue and the heat treatment kills or inhibits the spore tube elongation of the fungal pathogen. We propose that a similar mechanism might have been used by the HWT+*S. kilbournensis* biological control treatment in the current study. The combination effectively reduced *A. flavus* infections.

In this study, groundnut kernels treated with BCA4 (*B. cereus*) provided with lowest Aflatoxin B_1 concentration. In a study by **Kumar et al. (2014),** *B. cereus* was reported to inhibit *A. flavus* on groundnut kernels by producing an antifungal compound, diketopiperazine (cyclo 4-hydroxy-L-Pro-L-Trp). According to **Zhou et al. (2017)**, a study on *B. cereus* was found to control *Verticillium wilt* on cotton (*Gossypium hirsutum* Tod.), leading to an increase in cotton yield due to the activated basal defense responses. *B. cereus* mode of antagonism include production of chitinase, volatiles, and other antifungal molecules **(Khan et al., 2018; Chauhan et al., 2016)**. It also has the potential to enhance defense-related activities such as PAL, chitinase, β-1,3- glucanase, and PPO **(Shafi et al., 2017).** HWT inhibits spore germination and germ tube elongation of fungi **(Zhao et al., 2013; Vilaplana et al., 2018).** We propose that *B. cereus* was able to use different modes of the proposed mechanism in achieving *A. flavus* minimal infections and reducing the aflatoxins levels. *S. kilbournensis* (BCA1) achieved the third best low percentage infection by *A. flavus*. *S. kilbournensis* (BCA1) and *Candida kruisii* clade are phylogenetic related **(Kurtzman et al., 2016).** The *C. kruisii* clade have potential

properties that exhibits a strong potential in various areas of biotechnology such as biological control, bioremediation, but also in the production of valuable bio-compounds **(Yadav et al., 2012).** The *S. kilbournernsis* research on antagonist properties is so far limited, although in this study we observe its properties to be similar to its close related species, i.e. the potential to be a biocontrol agent. Hence it was able to control effectively *A. flavus* in both *in vitro* and *in vivo* trials **(Defosse et al., 2018).** HWT+*R. mucilaginosa* also provided low percentage infection. *R. mucilaginosa* enhances defense-related activities such as polyphenol oxidase, peroxidase, phenylalanine ammonia-lyase, chitinase, and β-1,3-glucanase activity in apples **(Yang et al., 2015).** A previous study by **Zhang et al. (2016)** indicates that *R. mucilaginosa* has antifungal activity achieved by expressing defense proteins such as glycoside hydrolases, phosphoribosyl pyrophosphate and NADH dehydrogenases. Stimulated defense protein on the seed aid in protecting the groundnut seed from penetration or germination of the fungus. This provided protection minimising the occurrence *A. flavus*. A study by **Yang et al. (2015)** provided the same results, which indicates the efficacy of expression of defense proteins. The yeast obtained during the *in vitro* screening (Chapter Two) was efficient in the inhibition of *A. flavus* mycelial growth.

Aflatoxin B_1 results in this study showed no significant difference (P-value =1.6) for all 11 treatments. However, the aflatoxin B_1 concentration differs from treatment to treatment. *Aspergillus flavus* strain can be pathogenic and virulent with a limit in aflatoxin secretion due to norsolorinic acid reductase NorA (AflE) **(Ren et al., 2018)** a key enzyme responsible for aflatoxin production. HWT+BCA1 (*S. kilbournernsis*) has the highest aflatoxin B_1 content yet have a low percentage infection of *A. flavus*, and the lowest is BCA4 (*B. cereus*) alone. The FAO has a standard concentration of aflatoxin for groundnuts, which is $5mgkg^{-1}$. All the concentrations obtained are lower than the standard concentration. **Watanakij et al. (2020)** showed the extracellular fraction of *Bacillus cereus* BCC 42005 isolated from Iru (African locust bean) potentially possessed aflatoxin B_1-degrading ability. *Bacillus cereus* and *Bacillus subtilis* strains reduced the fungal incidence and number of spores on kernels and low aflatoxin concentration **(Siahmoshteh et al., 2017).** A study by **Shafi et al. (2016)** suggested that *Bacillus* strains could reduce the mycelial growth of the pathogenic fungi, *A. flavus*. They were able to degrade the four aflatoxins (AFG_1 and AFG_2) during the first three days after inoculation. This result concurs with the current study, where *B. cereus* minimised the concentration of aflatoxins in groundnut seed.

In conclusion, the combination of HWT and *Suhomyces kilbournensis* KU751783.1 effectively reduced postharvest yellow mould and minimise the occurrence of *Aspergillus flavus* (the causal agent of yellow mould) in groundnut compared to other single treatments. *B. cereus* reduces aflatoxin B_1 concentration at storage. The combined strategy of biological and physical control may substitute chemical control.

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CHAPTER 5: DISSERTATION OVERVIEW OF THE MAJOR RESEARCH FINDINGS AND THEIR IMPLICATIONS.

Groundnuts (*Arachis hypogaea* L.) is the 13th most essential seed, domestic cash flow legumes. Despite their importance, groundnuts constrained cultivation is due to aflatoxin contamination. The most severe pre-harvest and postharvest diseases worldwide are causing up to 60% losses **(Pandey et al., 2016).** The fungus responsible for aflatoxin production is *Aspergillus flavus* Link, the causal agent of yellow mould disease in groundnuts. The main aflatoxins produced by *A. flavus* are aflatoxin B_1, and B_2. Aflatoxin AFM_1 is a hydroxylated metabolite of aflatoxin B_1, secreted in mammals' milk **(Alshannaq et al., 2018).** Due to their high toxicity and carcinogenicity, over 120 countries have set maximum limits. In South Africa, the accepted total aflatoxin levels in groundnuts is <10 µg/kg, and aflatoxin B_1 is 5 μgkg^{-1} **(FAO, 2016).**

Animals and humans feeding on contaminated seeds lead to the outbreak of aflatoxicosis. Aflatoxicosis is a condition of acute poisoning that can be life-threatening, usually through damage to the liver. Various physical, chemical and biological methods of reducing aflatoxins have been recommended, but only a few have been accepted for practical use **(Hazbavi et al., 2015).** The physical management strategy of heat-treating different crops have been predominantly been used in horticulture as postharvest treatment **(Cheng et al., 2016).** The heat treatment has not been used on groundnut seeds till this current study.

The aim of the present study was (1) to isolate, screen, and identify potential antagonistic bacterial and yeast strains against *Aspergillus flavus*, the causal agent of yellow mould in groundnut; (2) to develop a hot water treatment (HWT) that controls *Aspergillus flavus* in groundnut seed by providing long-term protection of shelled groundnut seed from postharvest infection; (3) to evaluate the efficiency of HWT, two yeast, and two *Bacillus* isolates in controlling *Aspergillus flavus*; and (4) to quantify aflatoxin B_1 in treated seeds after 12 weeks of storage.

5.1 Summary of significant research findings

5.1.1 Isolation and *in vitro* screening of *Bacillus* and yeast isolates for biological control of *Aspergillus flavus* in groundnuts.

- Sixty bacteria biocontrol agents were isolated from the phyllosphere of fifty-two plant species and screened *in vitro* for their antagonistic ability against *A. flavus*. Both yeast and *Bacillus* isolated were efficient in inhibiting mycelial growth of *A. flavus*.

- The effective yeast isolates were no more than 1.7% of the total isolated yeast. The effective *Bacillus* isolates were 5% of the total isolated *Bacillus.*
- The yeast isolate inhibition was moderate to good (60-70% inhibition) of *Aspergillus flavus* as compared to *Bacillus* isolates (50-70% inhibition). Yeast isolates inhibited *A. flavus* mycelial growth at a higher percentage compared to *Bacillus* isolates.

Implications:

The dual test results suggest that *Bacillus* spp and yeast isolates are good biocontrol candidates for further studies because they resulted in the pathogens' greatest suppression in dual-plate assays. With the 169 yeast and 60 *Bacillus* isolates screened, 20 isolates showed potential as biocontrol agents. This indicates the importance of the stringent screening process to select the most promising biocontrol agents.

5.1.2 Effects of Hot Water Treatment (HWT) on *Aspergillus flavus* infection in groundnut seeds.

Major findings:

- The hot water treatment at 40°C for 60 seconds and at 40°C for 20 seconds significantly reduced pathogen development of *A. flavus* and stimulating a good germination percentage.
- The efficiency of hot water treatment is dependent on the temperature used x time of exposure for the inhibition of *A. flavus.*
- Hot water treatment slowed down disease development, decreasing disease intensity, reduce disease severity, and slow down disease progression.
- Hot water treatment stimulated seed germination done at concussive temperature and denatures the seed vigour at high temperatures.
- Hot water treatment did not damage or decrease the seed quality when done at a suitable temperature and time.

Implications:

HWT has the potential to prevent *A. flavus* infection and symptoms at harvest from developing further during storage. HWT generates systemically acquired resistance reactions at a correct temperature-time treatment resulting in phytoalexins' release, inhibiting *A. flavus* development. HWT minimizes postharvest infection and subsequent production of aflatoxins in clean seeds.

For the effective treatment, germination percentage was high, and the *in vitro* test showed no *A. flavus* development from treated groundnut seeds on PDA plates.

5.1.3 Effects of combination of hot water treatment and biological control agents on *Aspergillus flavus* infection and aflatoxin production in groundnut seeds under storage.

Major findings:

- HWT+BCA1 (*Suhomyces kilbournensis* KU751783.1.) significantly reduced the *A. flavus* seed infection but had high aflatoxin levels.
- BCA1 achieved a minimal *A. flavus* infection but did not reduce aflatoxin concentration in seeds
- BCA4 (*Bacillus cereus*) significantly reduced the aflatoxin concentration in treated seeds and achieved low *A. flavus* incidence
- HWT alone reduced aflatoxin concentration despite the high *A. flavus* infection
- All 10 treatments excluding the control were able to achieve aflatoxin levels to be below the minimal accepted threshold in South Africa

Implications

The two antagonists combined with HWT at 40°C for 60 seconds exhibited potential commercial use in controlling postharvest *A. flavus*. All 10 treatments can be used to achieve minimal aflatoxin levels. HWT+BCA1 (*Suhomyces kilbournensis*) has a limitation in achieving low aflatoxin concentration although low *A. flavus* infection was recorded.

5.2 Recommendation and future research

More research is required to optimize the inhibition of *A. flavus* on seed using non-chemical control at an efficient rate while minimizing the aflatoxin levels. The study showed that it is still impossible to achieve zero aflatoxin contamination and 100% inhibition of *A. flavus,* which is the level that commercial store rooms need to succeed.

Some specific recommendations include:

a) The experiment can use more than one type of seed during the investigation, and this provides a better evaluation of the efficiency of the combination of physical and biological control methods.
b) Elucidate the possible mechanisms of action of the yeasts and *Bacillus* spp.
c) Measure seed dry matter and water activity on hot water treated seeds.

d) *In vivo* seed germination test on selected treatments be done on the field and evaluate the possible mechanism activated or elicited on groundnuts by hot water treatment.
e) Aflatoxin B_1 quantification on seeds be done at three different intervals, Week 4, Week 8, and Week 12 for all samples, which more precisely shows aflatoxin accumulation over time.

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Appendix

Table 2.1 Average percentage inhibition of selected potential yeast antagonists, their class ratings, and efficiency over the incubation period

Source of Isolation	Isolate name	Average inhibition ± S.D. error	Class	Efficient inhibition with incubation time
Acacia pennata	AP 1	48.8 ± 2.7	2	-
	AP 2	45.5 ± 3.7	2	-
	AP 3	62.8 ± 2.0	2	-
	AP4	57.1 ± 1.4	2	-
	AP 5	45.6 ± 1.6	2	-
	AP 6	58.6 ± 2.8	2	-
	AP 7	42.8 ± 2.8	2	+
Persea americana	AV1	49.3 ± 1.4	2	+
	AV2	31.1 ± 3.6	2	+
	AV3	45.8 ± 1.5	2	+
	AV4	40.8 ± 4.3	2	+
Phaseolus vulgaris	BG1	50.6 ± 1.5	2	+
	BG2	56.5 ± 2.9	2	+
Brassica oleracea	BL1	49.8 ± 4.0	2	+
vr. capitata	BL2	45.5 ± 0.4	2	+
	BL3	59.5 ± 3.5	2	+
	BL4	52.1 ± 3.0	3	+
	BL5	52.6 ± 3.4	2	+
	BL6	37.6 ± 2.2	1	+
	BL7	51.5 ± 1.8	2	+
Chlorophytum	CC1	72.6 ± 4.2	3	+
comusum	CC2	52.6 ± 1.2	2	+
	CC3	60.6 ± 1.1	2	+
	CC4	46.3 ± 4.7	2	+
	CC5	65.0 ± 1.6	2	+
	CC6	38.6 ± 1.7	1	+
	CC7	33.5 ± 4.6	1	+
	CC8	39.8 ± 3.3	1	+
Arachis hypogaea	CF1	70.0 ± 3.5	3	+
	CF2	60.0 ± 3.5	2	+
	CF3	62.8 ± 1.2	2	+
	CF4	58.8 ± 0.5	2	+
	CF5	68.8 ± 3.1	2	+
	CF6	58.6 ± 4.0	2	+
	CF7	47.6 ± 4.3	2	+
	CF8	54.6 ± 3.6	2	+
	CF9	46.0 ± 3.6	2	+
	CF10	48.8 ± 2.4	2	+
Capsicum annuum	CIP1	52.0 ± 3.8	2	-
	CIP2	49.5 ± 3.8	2	-

Source of Isolation	Isolate name	Average inhibition ± S.D. error	Class	Efficient inhibition with incubation time
Vigna unguiculata	CP1	56.3 ± 2.6	2	-
	CP2	47.6 ± 5.8	2	-
	CP3	43.8 ± 2.9	2	-
Chlandrope tulum	CSP1	47.6 ± 1.7	2	-
sp.	CSP2	43.8 ± 2.2	2	-
Cyperus textilis sp.	CT1	62.3 ± 1.9	2	-
	CT2	54.1 ± 1.5	2	-
Fuchsia	FUC1	50.3 ± 0.9	2	-
magellanica	FUC2	47.6 ± 1.5	2	-
	FUC3	52.8 ± 2.4	2	-
	FUC4	61.6 ± 2.7	2	-
	FUC5	47.6 ± 1.6	2	-
	FUC6	48.6 ± 1.3	2	-
Poaceae	FVL1	24.5 ± 2.0	1	+
	FVL2	49.8 ± 6.1	2	+
Phaseolus vulgaris	GB1	51.5 ± 6.3	2	+
	GB2	53.6 ± 3.7	2	+
	GB3	51.6 ± 1.9	2	+
	GB4	59.6 ± 2.4	2	+
	GB5	57.6 ± 2.0	2	+
	GB6	54.0 ± 3.3	2	+
	GB7	52.5 ± 3.3	2	+
	GB8	54.5 ± 1.5	2	+
	GB9	56.3 ± 4.9	2	+
	GB10	56.0 ± 2.0	2	+
Psidium guajava	GG1	56.8 ± 3.2	2	+
	GG2	53.6 ± 3.6	2	+
	GG3	55.0 ± 3.0	2	+
	GG4	43.8 ± 0.8	2	+
	GG5	47.8 ± 3.3	2	-
	GG6	50.6 ± 0.8	2	-
	GG7	61.3 ± 1.8	2	-
Capsicum annuum	GP1	54.5 ± 2.6	2	-
var. *annuum*	GP2	54.3 ± 4.4	2	-
Pablano				
Oryza sativa	GR1	51.6 ± 2.2	2	+
Prunus persica	GFR1	54.5 ± 2.7	2	+
	GFR2	54.5 ± 2.7	2	+
	GFR3	51.8 ± 4.0	2	+
Lactuca sativa	LT1	55.0 ± 1.6	2	+
	LT2	60.6 ± 3.3	2	+
Mangifera indica	MO1	54.0 4.2±	2	-
Protea cynaroides	PF1	34.8 ± 4.1	1	+

Source of Isolation	Isolate name	Average inhibition ± S.D. error	Class	Efficient inhibition with incubation time
	PF2	59.3 ±1.4	2	+
	PF3	70.8 ± 1.8	3	+
	PF4	61.1 ± 2.0	2	+
	PF5	32.6 ± 4.7	1	+
	PF6	39.1 ± 5.3	1	+
	PF7	51.5 ± 6.3	2	+
	PF8	62.1 ± 2.5	2	+
Zea Mays	MH1	46.0 ± 2.4	2	-
	MH2	54.3 ± 3.2	2	-
	MH3	50.8 ± 3.8	2	-
	MH4	57.1 ± 3.1	2	-
	MH5	43.8 ± 4.3	2	-
	MH6	34.6 ± 2.3	1	-
	MH7	45.0 ± 2.5	2	-
	MH8	51.6 ± 1.3	2	-
	MH9	56.1 ± 3.0	2	-
	MH10	54.0 ± 1.9	2	-
Purslane weed	PNW1	33.0 ± 2.3	1	+
	PNW2	45.4 ± 6.1	2	+
	PNW3	36.5 ± 3.6	1	+
Purtuloca Pilosa	PTP1	48.1 ± 1.7	2	-
	PTP2	56.6 ± 3.4	2	-
	PTP3	65.1 ± 3.3	2	-
	PTP4	48.3 ± 2.3	2	-
	PTP5	55.3 ± 3.2	2	-
Carica papaya	PW1	55.0 ± 2.7	2	+
	PW2	66.3 ± 2.4	2	+
	PW3	34.8 ±4.5	1	+
	PW4	66.6 ± 3.1	2	+
	PW5	54.1 ± 2.2	2	+
	PW6	27.1 ± 2.0	1	+
	PW7	49.3 ± 1.0	2	+
	PW8	54.5 ± 1.9	2	+
	PW9	53.3 ± 1.8	2	+
	PW10	48.1 ± 4.9	2	+
	PW11	49.3 ± 3.3	2	+
	PW12	44.6 ± 3.2	2	+
	PW13	47.6 ± 2.5	2	+
	PW14	33.0 ± 4.7	1	+
	PW15	53.1 ± 1.4	2	+
	PW16	55.1 ± 2.1	2	+
	PW17	41.6 ± 2.3	2	+
	PW18	43.1± 4.8	2	+

Source of Isolation	Isolate name	Average inhibition ±SD error	Class	Efficient inhibition with incubation time
Tulipa	RS1	51.1 ± 2.9	2	-
	RS2	48.3 ± 1.2	2	-
	RS3	48.6 ± 1.3	2	-
	RS4	52.1 ± 1.8	2	-
Cyanococcus	SF1	46.0 ± 4.6	2	-
	SF2	55.5 ± 3.0	2	-
	SF3	63.6 ± 2.4	2	-
	SF4	53.3 ± 4.7	2	-
	SF5	48.5 ± 2.2	2	-
Helianthis annuus	SFL1	52.0 ± 3.3	2	+
L.	SFL2	50.1 ± 3.3	2	+
	SFL3	60.3 ± 2.7	2	+
	SFL4	60.3 ± 2.7	2	+
	SFL5	49.8 ± 3.1	2	+
	SFL6	50.8 ± 4.2	2	+
	SFL7	64.8 ± 3.1	2	+
	SFL8	45.8 ± 2.7	2	+
	SFL9	56.0 ± 4.0	2	+
	SFL10	51.8 ± 3.0	2	+
	SFL11	59.8 ± 3.0	2	+
	SFL12	49.1 ± 5.6	2	+
	SFL13	56.3 ± 2.8	2	+
	SFL14	56.1 ± 1.0	2	+
	SFL15	64.5 ± 4.7	2	+
	SFL16	50.6 ± 2.3	2	+
	SFL17	64.5 ± 2.7	2	+
	SFL18	56.8 ± 3.6	2	+
Sorghum bicolor	SGH1	56.0 ± 2.4	2	-
	SGH2	54.0 ± 6.6	2	-
	SGH3	53.5 ± 3.1	2	-
	SGH4	51.7 ± 4.9	2	-
Ipomoea batatas	SP1	52.1 ± 2.0	2	+
	SP2	57.1 ± 2.7	2	+
	SP3	55.1 ± 3.0	2	+
	WA1	45.1 ± 2.1	2	-
Watsonia angusta	WA2	57.5 ± 2.9	2	-
	WA3	48.6 ± 3.2	2	-
	WA4	39.6 ± 4.1	1	-
	WA5	56.1 ± 2.7	2	-
	WA6	53.6 ± 1.6	2	-
	WA7	39.6 ± 3.1	1	-

*= Each value is reported as an average of three replicates; means followed by the same letters are not significantly different based on Duncan's Multiple Range Test at 5% significance level (P = 0.05).

Table 2.2 Average percentage inhibition of selected potential *Bacillus* species antagonists, their class ratings and efficiency over the incubation period

Source of isolation	Isolate name	Average inhibition ± S.D. error	Class	Efficient inhibition with incubation time
Acacia pennata	AP1	41.6 ± 2.7	2	-
Brassica oleracea	BL1	41.0 ± 3.6	2	+
vr. capitata	BL2	54.6 ± 2.0	2	+
	BL3	58 ± 2.6	2	+
	BL4	52.3 ± 2.6	2	+
	BL5	48.3 ± 1.5	2	+
	BL6	56 ± 2.4	2	+
Solanum tuberosum	CD1	47.1 ± 4.6	2	-
	CD2	46.5 ± 2.6	2	-
Cyperus textilis sp.	CT1	56.8 ± 4.1	2	-
Vigna unguiculata	CP1	50.3 ± 1.0	2	-
	CP2	54.3 ± 2.2	2	-
	CP3	54.1 ± 1.9	2	-
	CP4	48.3 ± 1.8	2	-
	CP5	60.1 ± 3.7	2	-
	CP7	54.3 ± 1.8	2	-
Chlandrope tulum	CSP1	48.0 ± 2.8	2	-
sp.	CSP2	52.3 ± 2.7	2	-
Phaseolus vulgaris	GB1	50.3 ± 0.9	2	+
	GB2	54.1 ± 2.3	2	+
	GB3	55.6 ± 3.0	2	+
	GB4	54.8 ± 2.0	2	+
	GB5	55.5 ± 2.4	2	+
	GB6	58.3 ± 2.1	2	+
Psidium guajava	GG1	35.0 ± 3.5	1	+
	GG2	40.0 ± 3.1	1	+
	GG7	52.8 ± 1.7	2	+
Citrus × limon	LM1	70.3 ± 0.5	3	+
	LM2	49.8 ± 0.7	2	+
Mangifera indica	MO1	45.6 ± 3.4	2	-
Allium cepa	ON1	45.0 ± 2.3	2	-
	ON2	52.0 ± 0.9	2	-
	ON3	61.3 ± 1.2	2	-
	ON4	55.3 ± 3.2	2	-
Purtuloca Pilosa	PTP1	65.6 ± 2.4	2	-
	PTP2	33.3 ± 2.4	1	-
Fragaria ×	PUP4	51.5 ± 1.5	2	+
ananassa	PUP5	44.1 ± 2.0	2	+
Carica papaya	PW1	49.0 ± 1.6	2	+
	PW2	60.1 ± 0.9	2	+
	PW3	61.6 ± 1.2	2	+
	PW4	61.3 ± 1.8	2	+

Source of Isolation	**Isolate name**	**Average inhibition ± S.D. error**	**Class**	**Efficient inhibition with incubation time**
Cyanococcus	SF1	50.2 ± 0.8	2	+
	SF2	57.3 ± 2.0	2	+
	SF3	55.8 ± 1.9	2	+
	SF4	67.5 ± 2.0	2	+
Helianthis annuus	SFL1	46.5 ± 1.8	2	+
L	SFL2	54.1 ± 1.8	2	+
	SFL3	44 ± 1.2	2	+
Ipomoea batatas	SP1	48.1 ± 3.8	2	-
	SP4	52.0 ± 2.4	2	-
	SP5	54.1 ± 2.6	2	-
	SP6	51.6 ± 0.9	2	-
	SP7	46.8 ± 2.8	2	-
	SP8	49.3 ± 2.6	2	-
Spinacia oleracea	SPN1	57.3 ± 4.3	2	+
	SPN2	44.1 ± 2.8	2	+
	SPN3	49.1 ± 2.0	2	+
Watsonia angusta	WA1	46.6 ± 1.8	2	-
	WA2	43.8 ± 2.9	2	-
	WA3	46.0 ± 4.2	2	-

*= Each value is reported as an average of three replicates; means followed by the same letters are not significantly different based on Duncan's Multiple Range Test at 5% significance level (P = 0.05).