MANAGEMENT OF *ASPERGILLUS FLAVUS* LINK INFECTIONS IN GROUNDNUT USING BACTERIAL ENDOPHYTES AS BIOLOGICAL CONTROL AGENTS.

By

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DISSERTATION SUMMARY

Microorganisms deteriorate groundnut seeds pre- and post-harvest. One of these microorganisms includes a fungus known as *Aspergillus flavus* Link. This pathogen reduces seed germination rate, see total oil content, carbohydrates, and proteins. *Aspergillus flavus* is a mycotoxigenic fungus that produces aflatoxins as secondary metabolites. As a result, seeds infected or colonized by *A. flavus* are considered not biologically unsafe for consumption and processing. Current management strategies have only provided temporary relief and increased susceptibility to the pathogen. Therefore, the use of environmentally friendly strategies is important. This study aimed to evaluate the efficacy of potential endophytic bacterial strains isolated from seeds, roots, leaves and stems of beans (*Phaseolus vulgaris* L.), peas (*Pisum sativum* L.), and groundnuts (*Arachis hypogaea* L.) in controlling *A. flavus* during storage and greenhouse trials.

During the *in vitro* studies, 106 endophytic bacterial isolates were isolated and screened against *A. flavus* using the disc diffusion method. The best 10 isolates were selected for secondary screening against *A. flavus* in the dual culture assay. Only 13 (12%) of the isolates inhibited the growth of *A. flavus in vitro* after nine days of incubation. The best isolates from the screening trials include Isolate ALA (55.2%), followed by Isolates KI (40.9%) and KG (40.2%) inhibitions, respectively. Isolate ALA was obtained from *Phaseolus vulgaris* L. (dry bean) leaves, while Isolates KI and KG were isolated from groundnut stems and leaves. From the secondary screening, the best five isolates were sent to Inqaba Biotech Industries (Pty) Ltd for molecular characterization and identification at the species level. Three of the isolates KI, KG, and BB that were gram-positive were identified as either *Bacillus siamensis, Bacillus velezensis* or *Bacillus amyloliquefaciens*. Isolate BB also had similarities to *B. subtilis*. Isolate GNLA and ALA were identified as *Pantoea dispersa* and *Pseudomonas fluorescens,* respectively. Three of these strains (*Bacillus siamensis/ Bacillus velezensis/Bacillus subtilis/Bacillus amyloliquefaciens* strains BB, KG, and KI) were then used in *in vivo* experiments to assess their efficacy in controlling *A. flavus* on two groundnut cultivars (Akwa and Sellie-Plus) during a storage trial. In a short-term trial (7-day period), culture filtrate of *B. amyloliquefaciens/B. siamensis/ B. velezensis* strain KG showed potential as a biological control agent against *A. flavus*. An average percentage infection of 38.2% on Cultivar Akwa and 33.0% and Cultivar Sellie-Plus were observed.

In contrast, the rest of the treatments (bacterial cells and crude lipopeptide extract) provided the least control. In the medium-storage trials (21-day period), the best endophytic bacterial treatment was the acidic cultured filtrate of *B. amyloliquefaciens B. siamensis/ B. velezensis* strain KG, which provided constant control for 21 days. The interaction of these isolates with *A. flavus* was studied using Scanning Electron Microscopy (SEM).

In the greenhouse trials, three treatments were evaluated. These were (i) *B. amyloliquefaciens/B. siamensis/ B. velezensis* strain KG was used as a seed treatment, (ii) *P. fluorescens* strain ALA as a

foliar spray treatment, and (iii) the combination of both seed treatment and foliar spray to control *A. flavus* during the greenhouse trial using the two groundnut cultivar Akwa and Sellie-Plus. Both cultivars performed somewhat differently for each treatment. The highest number of seeds and seed weight was recorded for the seed treatment for Sellie-Plus. In contrast, the combination of seed and foliar spray treatment recorded the highest number of seeds and seed weight for Cultivar Akwa. However, the combination of seed and foliar spray treatment showed potential as the best treatment against *A. flavus* with an average percentage disease incidence of 54.8% and 46.8% for Akwa and Sellie-Plus respectively.

In conclusion, the endophytic bacterial strains demonstrated potential in controlling *A. flavus* infection in groundnut seeds. Adjusting the pH of cultured filtrate provided a constant and enhanced control against the pathogen. Furthermore, the combination of seed and foliar spray treatment using the two bacterial strains showed potential as a biological control agent against *A. flavus*.

DECLARATION

I, **Nokulunga Nompilo Sibisi** declare that:

The research work presented in this thesis was carried out in the School of Agricultural, Earth and Environmental Sciences, University of KwaZulu-Natal, Pietermaritzburg is my original work completed under the supervision of Prof. Kwasi Sackey Yobo and Professor Mark D. Laing.

These studies have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others, including text, images, or tables, it has been duly acknowledged in the text.

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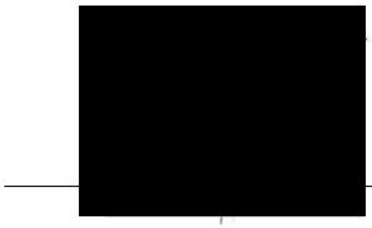
N. N. Sibisi (Candidate)



Prof. K.S. Yobo (Supervisor)

Prof. Mark D. Laing (Co-Supervisor)

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I am very thankful to my family for their support, prayers and patience that enabled me to complete my studies.

Finally, I would like to thank the God of my ancestors for seeing me throughout my studies.

DEDICATION

Madi, you did it.

DISSERTATION INTRODUCTION

Groundnuts (*Arachis hypogaea* L.) are one of the most essential seed crops, ranked 4th oilseed crop and 6th most important source of vegetable protein for human consumption (Ahmed *et al.*, 2016, Aderiye *et al.*, 2021). They are best produced between 20^{o}C and not more than 30^{o}C and grow well in sandy-loam soils (Prasad *et al.*, 2010). Groundnuts are produced in large quantities globally as they are consumed raw, roasted and/or processed to make confectionaries (Bediako *et al.*, 2019). Due to various environmental conditions in which groundnuts are produced, some constraints limit successful growth, harvest, and storage. Drought, heat stress, and plant diseases negatively affect the growth and yield of many seed crops, including groundnuts. With these constraints, *Aspergillus. flavus* Link (yellow mould) is significant in groundnut production (Singh *et al.*, 1992, Pal *et al.*, 2014, Kumar *et al.*, 2016).

Yellow mould is a recognized global disease of groundnuts caused by *A. flavus* that results in enormous economic losses. *A flavus* produces mycotoxins. Aflatoxins are highly important as they are carcinogenic, mutagenic, immunosuppressive and teratogenic to humans and animals (Kebede *et al.*, 2020). Therefore, controlling this pathogen has been the focus for many scientists, including setting the limit for the concentration of aflatoxins for all feed and foodstuff. The use of chemical control has been widely suggested for the quick and thorough eradication of fungal pathogens. Even though there is no registered chemical control for *A. flavus*, fungicides like fludioxonil (Masiello *et al.*, 2019) and other fungicides containing copper hydroxide and carbendazim (Nayak *et al.*, 2018) have been studied for their control against *A. flavus*. Due to the need for environmental safety, the continuous use of chemical control has been minimized. Cultural control is used but can only provide limited control and temporary relief. Biological control has become an alternative for controlling many plant pathogens, including *A. flavus*. Biological products, including Aflaguard and Aflasafe, are currently used to control *A. flavus* (Amaike *et al.*, 2011*)*. This study aimed to find an endophytic bacterial isolate to control *A. flavus* pre- and post-harvest.

Research objectives

The objectives of this study were to:

1. Isolate, screen, and identify endophytic bacteria from different plant parts of groundnuts, beans and pea plants and evaluate their efficacy in inhibiting the growth of *A. flavus in vitro*;
2. Find the best endophytic bacterial strains against *A. flavus* for short and medium-term storage *in vivo* studies using two South African groundnut cultivars, Akwa and Selli-Plus and;
3. Identify the best endophytic bacterial strains and assess their efficacy against *A. flavus* under greenhouse conditions.

The following dissertation has been written in four chapters, each covering a specific aspect of the research conducted on bacterial endophytes as a potential strategy to manage *A. flavus* in groundnuts. With the exception of the literature review and the general overview, each of the chapters were set up independently and prepared in the format of a scientific paper. This format is the standard dissertation model that the University of KwaZulu-Natal has adopted because it facilitates the publishing of research out of the dissertation far more readily than the older monograph form of a dissertation. As such, there is some unavoidable repetition of references, methods and some introductory information between chapters.

This research was undertaken in the Discipline of Plant Pathology at the University of KwaZulu-Natal, Pietermaritzburg Campus, under Prof K.S. Yobo and Professor M.D. Laing.

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CHAPTER 1

Literature Review

1.1 Introduction

In agriculture, seeds play a significant role in producing healthy crops, and 90% of the crops in the world are produced using seeds (Chavan, 2011). Seeds in the field and in harsh storage conditions interact with several microbes, which both qualitatively and quantitatively deteriorate those seeds. One of those pathogenic microbes includes the *Aspergillu*s spp., which are fungi active in both fields and storage. Fungi growing on stored grains reduce the germination rate, carbohydrate, protein, and total oil content, increase moisture content, and also enhance other biochemical changes of grains (Bhattacharya and Raha, 2002). The seeds are then considered not fit for consumption or processing. For instance, developing countries account for approximately 95% of the world's groundnut (*Arachis hypogaea* L.) seed production but cannot sell large quantities of groundnut on the international market because of aflatoxin contamination (van Egmond *et al.*, 2007).

Aspergillus flavus Link and *Aspergillus parasiticus* Spear are the predominant mycotoxigenic fungi associated with significant contamination of groundnuts (Dorner, 2008). As a result, aflatoxin contamination of agricultural commodities poses a considerable risk to human and livestock health and has significant economic implications for the agricultural industry worldwide (Richard *et al.*, 2003). According to Cardwell (2001), aflatoxin contamination of crops, such as groundnut and cereals, causes annual losses of more than $750 million in Africa. Aflatoxin contamination of groundnut could be minimized through agronomic practices, including biological, chemical, and cultural control.

Crop losses constitute a significant threat to food security, especially in developing countries. A variety of control strategies have been employed to control *Aspergillus* spp. (Okello *et al*., 2010, Acharya *et al*., 2021). The use of biological control agents has been described as one of the possible methods to manage both pre- and postharvest diseases. *Bacillus* spp. is one of the organisms investigated as biological control agents (Yobo, 2005, Villarreal-Delgado *et al.*, 2018, Penha *et al.*, 2020). *Bacillus subtilis*, a bacterium isolated from groundnuts, was found to inhibit the growth of *A. flavus* in groundnuts (Guchi, 2015). Various microbial species have been demonstrated as natural antagonists to several plant pathogens. This review will focus on groundnut production and how it is negatively affected by pathogens such as *A. flavus*, thus leading to mycotoxin contamination. Various control measures were also reviewed.

1.2 The Crop: Groundnuts

Groundnut (*Arachis hypogaea L*. (2n = 4x = 40)), also known as peanuts in rural homes of sub-Saharan Africa (Banla *et al.*, 2018), is one of the world's most important oilseed crops which falls under the Leguminosae family. Groundnuts are one of the essential cash crops and components of many household diets in developing countries, including South Africa. They are consumed raw, roasted, and

processed in confectioneries or ground into peanut butter (Bediako *et al.*, 2019). In South Africa, they are a source of nutrition in the northern KwaZulu-Natal and Mpumalanga provinces (Cilliers, 2015), with many produced by smallholder farmers. They were grown in large quantities by the South American natives, specifically Bolivia and adjoining countries, before colonization by Europe. Now, they are distributed in European, African, Asian countries, and the Pacific Island (Alamene, 2015).

Groundnuts are of South American origin but now are grown chiefly in the tropics and warm temperature regions around the world (Prasad *et al.*, 2010). Depending on the variety, environment, and weather, they take 90 to 150 days from planting to harvest (Okello *et al.*, 2013). They grow best in light, sandy loam soils with a pH of around seven and require five months of warm weather (20 – 30 ^{0} C) and an annual rainfall of 500 to 1000 mm (Prasad *et al.*, 2010). However, it can bear up to 50 days of drought, and its reproduction is almost exclusively by self-pollination (Coleman, 2020, Okello *et al.*, 2013). They are suitable for production as they are self-pollinating crops, producing flowers above ground and, after fertilization, moving pegs towards the soil to form seed-containing pods (Prasad *et al.*, 2010). As they are legumes, they also can fix atmospheric nitrogen into the soil to improve soil fertility and thereby increase the productivity of other crops (Ncube and Maphosa, 2020) during inter-cropping.

Abiotic and biotic constraints limit groundnut production all over the world. The major abiotic constraints to groundnut production are drought, temperature extremes, soil factors such as alkalinity, poor soil fertility, and nutrient deficiencies (Prasad *et al.*, 2010, Shifa *et al.*, 2016). Biotic constraints include diseases, pests, and weeds. Diseases including rust (*Puccina arachidis* Speg), early leaf spot (*Cercospora arachidicola* Hori), and leaf spot (*Phaseoisariopsis personata* Berk and Curtis) are said to affect production in African countries (Daudi *et al.*, 2018). Pests such as aphids (*Aphids craccivora* Koch), leaf miner (*Aproarema modicella* Deventa), thrips (*Thripes palmi* Karny), and termites (*Isoptera*) (Abady *et al.*, 2019) have been shown to affect groundnut production. Seed-borne pathogens affect the seed quality and the overall yield. The most important diseases affecting groundnuts are leaf spots, rust, and yellow mould caused by the toxin-producing fungus, *A. flavus*. Starr and Selim (2008) stated that strains of *Aspergillus* spp. are frequently found in farmland topsoil and may serve as the primary source of inoculum for the colonization of crops. The cultivation of suitable and improved cultivars and the use of appropriate agronomic practices should be practised by farmers (Mastewal *et al.*, 2017, Abady *et al.*, 2019). This includes the knowledge of the type of the pathogen associated with a farmer's seed and their effect on seed quality helps in adopting suitable strategies to manage them (Adithya, 2016).

1.3 Production of groundnuts

The leading countries that account for most production of groundnuts are found in Asia (India and China), Africa (Nigeria), and North America (United States of America (USA)). In Asia, China is the

leading producer with 18.20 million metric tons (Mmt) followed by India, Burma, and Indonesia with 6.80, 1.60, and 0.96 Mmt respectively (USDA, 2022). In Africa, the leading producers of groundnuts are Nigeria (4.45 Mmt), Sudan (2.50 Mmt), Senegal (1.70 Mmt), Guinea (0.90 Mmt), Chad (0.90 Mmt), and Cameroon (0.60 Mmt) (USDA, 2022). This makes Nigeria the first to contend with international trade as it leads in production (Samuel, 2019, Ojiego and Nnaji, 2021). According to Prasad *et al.* (2010), in 2007, Africa had a lower production output of groundnuts (964 kg ha^{-1}) when compared to the USA (3500 kg ha^{-1}) and other developed countries. In the year 2020/2021, Nigeria produced two times (4.45 Mmt) the production output of the USA (2.90 Mmt), showing an impeccable increase in the overall production (USDA, 2022). Groundnut production goes far beyond 90 countries, making it a globally produced oilseed crop.

According to DAFF (2010), groundnuts in South Africa are mainly produced in the western regions (Table 1.1) with 40% of production in the western and north-western Free State, 29% in the North West, and 24% in the Northern Cape. Production increased significantly during the 2000/01 season with 200 000 t because of larger planting areas (DAFF, 2010). According to Coleman (2020), South African farmers planted nearly 100 000 ha of groundnuts in the early 2000s, but by 2010 this had decreased to about 57 000 ha. This indicates the decrease in production outputs because of constraints and costs. This can be corrected by using cultivars that are tolerant to most constraints, especially diseases and drought-resistant cultivars.

Table 1.1: Major groundnut production areas in South Africa (DAFF, 2010).

Province	District	Town
North West	Ngaka Modiri Malema	Mafikeng, Delareyville, Lichtenburg, Zeerust, Sannieshof
	Bojanala	Rustenburg
	Dr. Ruth Segomotsi Mompati	Schweizer-Reneke, Vryburg, Christiana
	Dr. Kenneth Kaunda	Potchefstroom, Wolmaransstad
Northern Cape	Francis Baard	Kimberley
Free State	Lejweleputswa	Welkom, Reitz
	Northern Free State	Kroonstad
Limpopo	Waterberg	Bela-Bela, Mookgopong, Vaalwater
	Mopani	Giyani, Letsitele, Mooketsi, Bolebedo, Tzaneen, Phalaborwa
	Vhembe	Messina and vicinity, Malamulele, Thohoyandou, Makhado
Mpumalanga	Nkangala	Middelburg, Witbank, Siyabuswa
	Enhlanzeni	Thulamahashe, Makwitswi, Mkhuhlu, Bushbuckridge

1.4 Economic importance of groundnuts

According to FAOSTAT (2012), groundnuts are ranked the 13th most important food crop globally and the 6th most important source of vegetable protein for human consumption to meet protein requirements for the increasing population (Aderiye *et al.*, 2021). According to Gulluoglu *et al.* (2016), groundnuts are a rich source of oil (45–56%), protein (25–30%), carbohydrates (9.5–19.0%), minerals (P, Ca, Mg, and K), and vitamins (E, K, and B). In addition, the kernels contain many health-enhancing nutrients such as minerals, antioxidants, and vitamins that are rich in mono-unsaturated fatty acids (Pasupuleti *et al.*, 2013). They are an essential source of nutrition in the northern KwaZulu-Natal and Mpumalanga areas. The crop can also contribute to more viable and sustainable cropping systems in other parts of the country.

Global groundnut production has increased slightly in the last decade, as it also contributes to providing nutritious forage (haulms) to livestock (Pasupuleti *et al.*, 2013). But, according to Abady *et al.* (2019), South Africa and Mozambique showed a decline in groundnut yields between 1997 and 2016. This may be due to pests and diseases affecting the growth of groundnuts and their upkeep as they have several requirements for successful growth. Groundnuts are important in small-holder agriculture, grown mostly under subsistence farming conditions, and are a major source of income, especially for women in third-world countries (Coulibaly *et al.*, 2008).

1.5 Economic important diseases of groundnuts

Despite the importance of groundnuts, this crop is prone to various diseases both post- and pre-harvest (Bediako *et al.*, 2019). According to Singh *et al.* (2020), the magnitude of yield losses caused by groundnut diseases is alarming. It ranges from 10% to 70% caused by economically important foliar fungal diseases, as they reduce pod yield and affect the fodder quality of straw. However, disease incidence and severity depend largely upon locations and weather conditions. It is suggested that most pathogen disseminates from season to season on volunteer groundnut plants and infected plant debris, building up an inoculum reservoir for several seasons (Kumar *et al.*, 2016, Jones, 2021).

1.5.1 Bacterial diseases

There are not many bacterial diseases reported that affect groundnut production. For the longest time, the two known diseases were bacterial wilt, caused by *Ralstonia (Pseudomonas) solanacearum* which was first reported in Indonesia (Asia) and later in Georgia (USA) (Singh and Oswalt, 1992), and leaf spot (*Cercospora arachidicola* and *Cercosporidium personatum*). In 2020, an unknown disease resulted in $1.12 million in groundnut production losses. The observed symptoms included seed rot, poor seed vigour, little to no nodule formation, pre and post-emergence damping-off, and death. In a study by Obasa and Haynes (2022), two bacteria were identified using the Basic Local Alignment Search Tool (BLAST) as *Ralstonia* sp. strain B265 and *Pantoea* sp. strain B270. The findings from the study provided evidence for two new bacterial pathogens in groundnuts.

1.5.2 Viral diseases

A few viral pathogens have been reported to be destructive. This includes viruses like Peanut Mottle Virus (PMV), Peanut Stripe Virus (PStV), Peanut Clump Virus (PCV), and Tobacco Streak Virus (TSV) (Pal *et al.*, 2014). Rosette disease, a viral infection transmitted by *Aphis craccivora* can cause stunted growth, loss of vigour, curling of the leaves, slow growth rate, yellowing, browning, wilting, bunchy top head, defoliation of the leaves, poor yield return, and plant death (Richard *et al.*, 2017). Two serologically distinct viruses cause bud necrosis disease; Bud Necrosis Virus (BNV) and Tomato Spotted Wilt Virus (TSWV) (Kumar *et al.*, 2016).

1.5.3 Nematodes

Meloidogyne spp. root-knot nematodes are the most important nematode species causing damage ranging from 2-9% in infested fields of groundnut and resulting in enlarged roots and pegs in various degrees of stunting and chlorosis also wilting under drought conditions (Kumar *et al.*, 2016). Globally, root-knot nematode, *Meloidogyne arenaria* Neal occurs at all growth stages and increases the susceptibility of the crop to other pathogens (Sarkar *et al.*, 2021). In 1977, *Aphelenchoides arachidis* Bos was found first in Northern Nigeria causing shrivelled and discoloured kernels (Steenkamp *et al.*, 2021). In South Africa, *Meloidogyne incognite*, *Meloidogyne javanica* (Treub) Chitwood, *Ditylenchus africanus* Wendt et al., and *A. arachidis* have been found to affect seed quality and overall production (Steenkamp and Fourie, 2018).

1.5.4 Fungal diseases

Fungi are the most economically important group of plant pathogens, causing both quantitative and qualitative yield losses. Fungal diseases destructive to groundnuts include fungal early leaf spot or tikka disease (*Cercospora arachidicola* Hori) (Kumar *et al.*, 2016), late leaf spot (*Phaeoisariopisis personata*), and rust (*Puccinia arachidis* Speg) which can infect the leaf, stems, petioles, pegs causing significant premature leaf deterioration, and yield reduction (Richard *et al.*, 2017). Groundnut kernels are known to harbour several species of seed-borne fungi including *Macrophomina phaseolina* (Tassi) Goid, *Rhizoctonia Solani* Kühn, *Fusarium oxysporum* Schlechtend, and *Fusarium Solani* (Mart.) Sacc. *(*Pal *et al., 2014).* Other fungal pathogens that affect groundnuts include *Pythium ultimum* Trow and *Pythium myriotylum* Drechsler commonly known as damping off, which causes soft rot of the hypocotyl region (Singh and Oswalt, 1992). *Aspergillus* spp. have been known to cause ernomous losses in the seed industry; including *A. niger, A. parasiticus, A. ochraceus*, and *A. flavus* (Mohammed *et al.*, 2018). *A. flavus* and *A. parasiticus* fall under section *Flavi*, this section consist of economically important species which produce aflatoxins (Klick, 2007). In groundnuts, *A. flavus* is known to cause yellow mold on pods and thereafter seeds, both pre- and post-harvest (Guchi, 2015).

1.6 The pathogen of focus: *Aspergillus flavus* Link

A. flavus was first defined by Link in 1809 to be the most virulent saprophytic pathogen (Jayaprakash *et al.*, 2019). This fungus belongs to the phylum Ascomycota, class *Eurotiomycetes*, order *Eurotiales,* and family *Trichocomaceae (Fakruddin et al., 2015).* In culture, it is characterized by the fast-growing yellow-green colonies, usually 65–70 mm in diameter after seven days of growth in the dark at 25 °C (Klich, 2007). *Aspergillus* genus consists of an assemblage of phylogenetically related aflatoxin and non-aflatoxin-producing strains. The toxin production is dependent on the type of isolate. This fungus is divided into S (produces much higher aflatoxins) and L morphotypes (lower aflatoxin production) (Kagot *et al.*, 2019). Environmental factors play a significant role in the inception of aflatoxin as it

infects many crops (Waliyar *et al.*, 2008, Gebreselassie *et al.*, 2014).Soil is the primary source of infection for *A. flavus.* At the beginning of the growing season and the end of winter, the sclerotia are exposed to the soil surface under favourable environmental conditions. It germinates and produces new conidia (asexual spores) that serve as a new source of inoculum that spreads via insects or wind to new plants (Alam *et al.*, 2020). The pathogen continuously colonizes and infects the new crop and produces aflatoxin, as elaborated in Figure 1.1. This pathogen is a saprophytic fungus that survives on dead plant tissue. It sometimes behaves as a weak and opportunistic pathogen with its source of inoculum sclerotia, conidia, and mycelia over-wintering in plant debris (Guchi, 2015). In fields that are repeatedly cropped with groundnut or rotated between groundnut, *Zea mays* L. (maize) and *Gossypium arboretum* L. (cotton), conidia from sclerotia are the primary sources of *A. flavus* inoculum, and if environmental conditions are hot and humid, spores get released on plant residues, and these spores are dispersed by the wind throughout the field (Guchi, 2015). Infection severity increases simultaneously with drought, high temperatures, and high humidity. Like other *Aspergillus* spp, *A. flavus* has a worldwide distribution, possibly resulting from the production of numerous airborne conidia. These conidia easily disperse by air movements and possibly by insects (Hedayati *et al.*, 2007) as conidiospores adhere to insect bodies and are physically moved to plant parts and flowers on groundnut.

Crops that are frequently affected by this pathogen include cereals ((maize, *Sorghum bicolor* L. (sorghum), *Cenchrus americanus* L. (pearl millet), *Oryza sativa* L. (rice), *Triticum aestivum* L. (wheat)), oilseeds (groundnut, *Glycine max* L. (soybean), *Helianthus annuus* L. (sunflower), cotton), spices (*Capsicum annuum* L. (chilli peppers), *Piper nigrum* L. (black pepper), *Coriandrum sativum* L. (coriander), *Curcuma longa* L. (turmeric), *Zingeiber officinale* Roscoe (ginger)), and tree nuts (*Prunus amygdalus* Batsch (almond), *Pistacia vera* L. (pistachio), *Juglans regia* L. (walnut), *Cococ nucifera* L.(coconut) *Betholletia excelsa* Humb. and Bonpl. (brazil nut)) (Abnet, 2007), indicating its wide host range. Infections on crops happen during pre-and postharvest stages after an insect, or mechanical damage, as well as during storage. Diseases of groundnut kernels differ at growth stages, where smaller kernels are easily infected, and mature kernels carry spores for postharvest infestation (Kagot *et al.*, 2019). Symptoms on seeds are usually the same for seeds of different crops. The seed gets covered by white powdery mould, which with time changes to yellow, then lime green when mature.

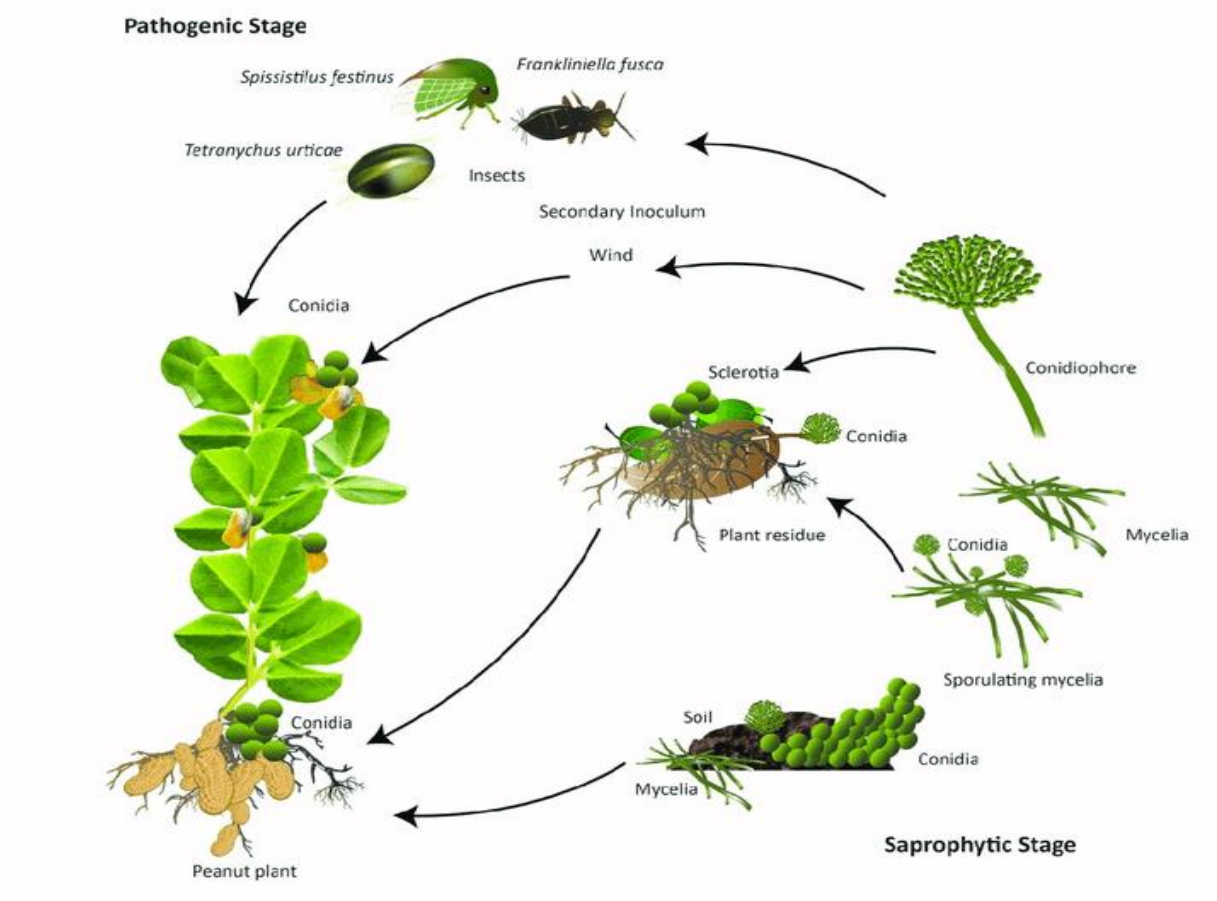

Figure 1.1: The life cycle of *A. flavus* (Alam *et al.*, 2020)

1.6.1 Economic importance of *Aspergillus flavus*

Aflatoxins, produced by strains of *A. flavus* are the most toxic and carcinogenic of the known mycotoxins and imposes an enormous socioeconomic cost on human society (Wu and Khlangwiset, 2010). Aflatoxins are a major constraint of groundnut production in most countries worldwide (Ncube and Maphosa, 2020). Aflatoxin accumulation occurs during poor storage practices, including high humidity and temperature, which is typical in tropical areas (Bediako *et al.*, 2019). In immunocompromised patients, *A. flavus* is one of the main agents of human allergic bronchial aspergillosis and pulmonary infections (Denning and Chakrabarti, 2017). *A. flavus* is also the most common species associated with aflatoxin contamination of crops (Kifle *et al.*, 2017). The most significant positive economic impact of *Aspergillus* spp. has been in the exploitation of the enzymes and acids produced by several species (Khan *et al.*, 2014, Alamene, 2015).

Two of the most important industrial products produced by *Aspergilli* are amylase, an enzyme (Hara *et al.*, 1992), and oxalic and citric acid (Arslan, 2019). Amylase uses range from textile, liquor, bakery, infant feeding, cereals, and starch to chemical uses (Abdel-Azeem *et al.*, 2019). Citric acid has been

used for over a thousand years to produce several Asian foods and beverages, including sake and soy sauce (Hara *et al.*, 1992). Citric and oxalic acids are also leaching agents to dissolve iron oxides (Arslan, 2019).

1.6.2 Aflatoxin contamination in groundnuts

Aflatoxins are mycotoxins that contaminate many economically important crops, including groundnuts. They are a group of structurally related toxic polyketide-derived secondary metabolites produced by certain strains of *A. flavus* and *A. parasiticus* (Misihairabgwi *et al.*, 2019, Bediako *et al.*, 2019). They are mutagenic, carcinogenic, teratogenic, and immunosuppressive agents destructive to humans and livestock. Naturally, *Aspergillus* produces four major aflatoxins known as B1, B2, G1, and G2, where aflatoxins B1 and B2 show a blue fluorescence under UV light, whereas aflatoxins G1 and G2 show a greenish-yellow fluorescence under UV light (Pitt and Hocking, 2009, Waliyar *et al.*, 2015).*A. flavus* produces B1 and B2 while *A. parasitica* produces G_1 and G_2 and the level of carcinogenicity is B1>G1>B2>G2 in that order (Guchi, 2015). The structure (Figure 1.2) of these aflatoxins consists of 5 rings with a furofuran moiety (rings II and III) in all aflatoxin (Jayaprakash *et al.*, 2019).

Humans are exposed to aflatoxins mainly by consumption of food commodities contaminated with aflatoxins producing *A. flavus*. Also, infants may be exposed to aflatoxins by ingesting breastfed contaminated milk. This increases the vulnerability of infants and children who rely on breastfeeding milk. When large quantities of aflatoxin are taken frequently within a short period, they may cause acute poisoning referred to as "acute aflatoxicosis" which results in weariness, vomiting, jaundice, damage of liver tissues, enlargement of the bile duct, and ultimately death (Massomo, 2020). Aflatoxins are currently listed as a Group 1a carcinogen, as they can suppress the immune system and may act synergistically with Hepatitis B virus infections (IARC, 2012). It is important to note that chronic sublethal doses of aflatoxins are also harmful and may result in cancer and reduced life expectancy (Massomo, 2020).

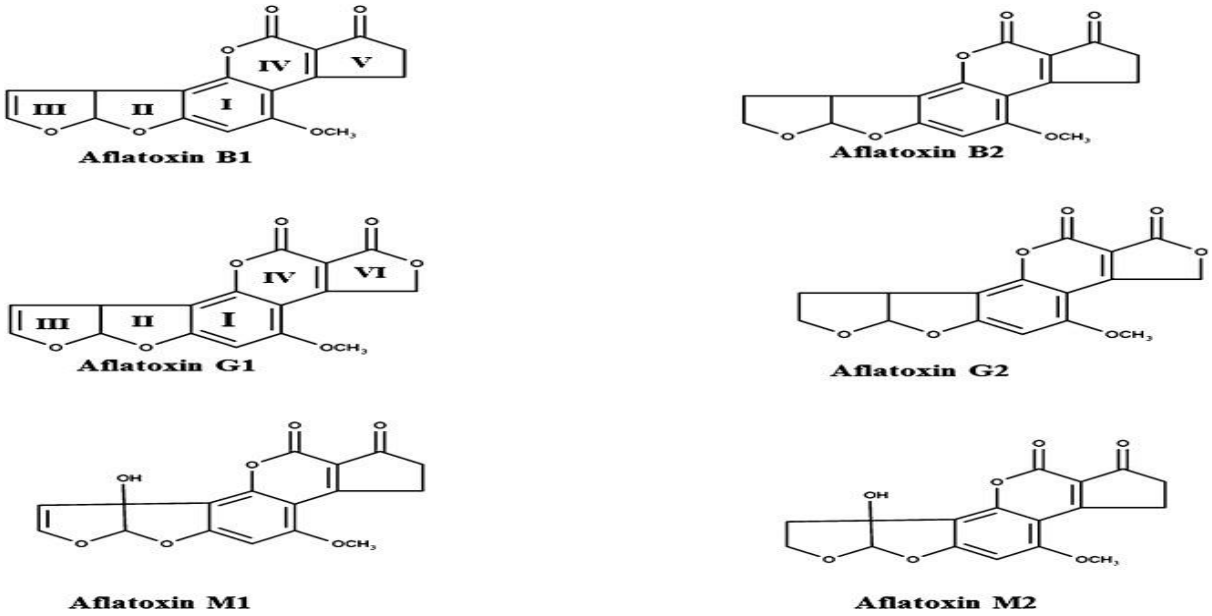

Figure 1.2 :Structures of the predominant Aflatoxins produced by *A. flavus (Jayaprakash et al.,* 2019*)*

1.6.3 Limits of aflatoxin concentration in food and feed

Aflatoxin contamination is demonstrated to be a most critical barrier in linking African farmers to local and international markets as it prevents goods from meeting international, provincial and local regulations and standards governing agricultural trade (Coulibaly *et al.*, 2008). The potential hazard of aflatoxins to human health has led to worldwide monitoring programs for the toxin in various commodities, as well as regulatory actions by nearly all countries

Low dose consumption is said to cause liver cancer, poor nutrition absorption, malnutrition and retarded growth in children, and immune system suppression over an extended period (Bediako *et al.*, 2019). Exposure of children to aflatoxins causes stunting and predisposes them to other diseases such as malaria and Acquired Immunodeficiency Syndrome (AIDS) (Misihairabgwi *et al.*, 2019). Livestock fed with contaminated feed passes on the aflatoxins to their products such as milk, eggs, and meat. Furthermore, the productivity of infected livestock decreases due to disorders (Denning and Chakrabarti, 2017, Senerwa *et al.*, 2016). Globally, countries have different aflatoxin limits (Table 1.2). It is, therefore crucial that food regulatory bodies ensure that food commodities on the market with aflatoxin contamination above the maximum allowable limits are brought to book without any compromise on set standards (Achaglinkame *et al.*, 2017).

Products highly affected by aflatoxins are maize, groundnuts, cotton, millet, sorghum, and other feed grains. In contrast, wheat, oats, millet, barley, rice, cassava (*Minihot esculenta* Crantz), soybeans, beans, and sorghum are less affected (Guchi, 2015). Economic yield losses may be up to 100% if the

aflatoxin levels exceed stipulated levels (Ncube and Maphosa, 2020). Despite extensive research done during the last few decades, which helped authorities around the world establish control measures, aflatoxin contamination in food and agricultural commodities remains one of the most challenging, serious food safety problems (Guchi, 2015).

Table 1.1: The maximum total limit of aflatoxins for food and feed in some African countries

Country	The maximum permitted total aflatoxin concentration (µg kg^{-1})		Reference
	Food	Animal feed	
Cote d' Ivoire	-	10-100	(Grace *et al.*, 2015)
Kenya	10	10	(Sirma *et al.*, 2018)
Morocco	-	5-50	(Grace *et al.*, 2015)
Mozambique	10	10	(Cambaza *et al.*, 2018)
Nigeria	20	50	(FAO, 2004, Vabi *et al.*, 2018)
Peru	15	-	(FAO, 2004)
Senegal	-	50-300	(Grace *et al.*, 2015)
South Africa	10	10	(FAO, 2004)
Tanzania	10	10	(Grace *et al.*, 2015)
Uganda	10	10	(Sirma *et al.*, 2018)
Zimbabwe	5	10	(FAO, 2004)

- no information

1.7 Control strategies for *Aspergillus flavus*

To protect a crop from diseases, it is important to know the causal organism, symptoms, and method of infection. Control of aflatoxin in groundnut is very difficult as they are geocarpic along with the groundnuts; therefore, many approaches have been developed for all the stages of the cropping period, such as the development of resistant lines. Groundnut infection by *Aspergillus* spp. can occur during pre-harvest, harvest, or postharvest stages such as storage, transportation, and processing (Ajeigbe *et al.*, 2015); therefore, it is advised to implement control strategies at either of these stages individually if not all. Several management strategies are currently undertaken to deal with the problem of aflatoxins, including using, an atoxigenic strain to increase soil production and biological protection against toxic strains (Ncube and Maphosa, 2020).

1.7.1 Cultural control

Cultural control aids to a degree in controlling many plant pathogens and have been used longer as a traditional method by smallholder farmers. It includes ensuring the retention of soil moisture content, improved postharvest techniques, and varietal resistance. Prevention of pre-harvest aflatoxin contamination depends on several factors, including timely planting, soil amendments such as fertilizer

and gypsum (calcium) application, moisture conservation practices and irrigation, and good insect and weed management practices (Achaglinkame *et al.*, 2017). Crop rotation also helps to reduce aflatoxin prevalence in crops by breaking the cycles and build-ups of toxin-producing microorganisms, thus reducing the high densities of *Aspergillus* spp. (Commission, 2004).

Provided the ability of *A. flavus* to produce aflatoxins when kernel moisture goes below 28%, the time of harvesting should be planned accordingly and logistics of harvest, drying, and storage systems should be put into consideration to avoid any increase in contamination (Ojiambo *et al.*, 2018). Therefore, harvesting on time can help reduce or limit aflatoxin contamination. Sorting grain before storage and cleaning can improve the benefits of proper storage techniques, reducing contamination. Farm machinery harbours most pathogens; hence it is important to properly sanitize farm utilities and machinery to reduce pathogens from the field to storage or packhouse. It is also important to note that workers must wear personal protective equipment when working with either healthy or infested seeds to protect themselves against cross-contamination.

1.7.2 Chemical control

The use of pesticides to control the growth of *A. flavus* to reduce aflatoxin contamination has created mixed results (Kabak *et al.*, 2006). Essentially, the plant disease management programs focus on pesticide use; therefore, farmers are rarely persuaded by other bio-intensive eco-friendly management strategies (Singh *et al.*, 2020). Using pesticides to control insect damage during plant growth may reduce the risk of fungal invasion and aflatoxin contamination, even though reductions may not be significant relative to the legal limits (Commission, 2004). Along with all the alternatives available in the agronomic industries, fungicides play a valuable role in controlling plant diseases; however, their application can cause serious environmental problems and encourage resistance in some fungi. The problem is that chemical control methods are not sustainable and eco-friendly as they increase environmental and health hazards; hence it is important to avoid such practices.

Aflatoxin contamination is said to be reduced by up to 90% when amending the soil with calcium and manure by thickening the cell walls and fast-tracking pod filling and also promoting the growth of microbial antagonists in soil (Waliyar *et al.*, 2015). There is no registered fungicide for *A. flavus* but there have been studies looking at the effect of a few chemicals in reducing the production of aflatoxins (Lagogianni and Tsitsigiannis, 2018). Wheeler *et al.* (1991) showed that chlobenthiazone, a fungicide, is highly effective in inhibiting aflatoxin biosynthesis by cultures of *A. flavus* but in return stimulated the aflatoxin synthesis by *A. parasiticus*. A study by Nayak *et al.* (2018) indicated that fungicides containing 50% Carbendazim and 77% Copper hydroxide, respectively, reduced the ability of the fungus to produce aflatoxin B1 on first-generation sclerotia, but the second generation showed no reduction. A study done by Masiello *et al.* (2019) showed that *in vitro*, succinate dehydrogenase inhibitors fungicides and fludioxonil were more active against *A. flavus* than *Fusarium* spp., and in

field trials, prothioconazole and boscalid could reduce *A. flavus* contamination at values of 75% and 56%, respectively.

1.7.3 Biological control

Biological control has become a promising tool as it is a possible alternative to chemical methods. Chemical control is known to eliminate beneficial soil organisms and has a negative impact on soil properties (Lagogianni and Tsitsigiannis, 2018, Tahat *et al.,* 2020). The use of biological control, focusing on competitive exclusion, has been developed for groundnuts (Pitt and Hocking, 2009). Most of the existing biocontrol agents for managing soil-borne diseases were isolated from the rhizosphere (Gayathri and Velmurugan, 2020). Table 1.3 below summarises some of the research done on plant endophytic bacteria. There are many more which have not been listed in Table 1.3. Substantial research studies have led to the development of biological control agents over the years for commercial application, based on the ability of atoxigenic strains to reduce toxin contamination in cotton seeds, groundnuts, and maize (Kagot *et al.*, 2019). The application of atoxigenic strains of *A. flavus* before flowering has aided in the reduction of aflatoxin contamination. The USA was the first to apply this technology, followed by Africa and Europe (Dorner and Lamb, 2006, Bandyopadhyay *et al.*, 2016, Mauro *et al.*, 2018). Products including Aflaguard and AF36 registered by the USA Environmental Protection Agency and Aflasafe registered in Nigeria and Kenya are currently used to combat this pathogen (Amaike and Keller, 2011). Aflaguard is registered for use on maize and groundnuts. In contrast, AF36 is registered for use in almonds (*Prunus dulcis* (Mill.) D.A. Webb), cotton, maize, and pistachio *(Pistacia vera* L.) (Ojiambo *et al.*, 2018) with Aflasafe being a commercial product that is based on a mixture of four atoxigenic strains for use in African countries. In biological control formulations, atoxogenic strains are abundant for a year after application but tend to decline after that. Therefore, reapplication is suggested to sustain aflatoxin contamination reduction (Ojiambo *et al.*, 2018).

Lactic acid bacteria have demonstrated the ability to bind with aflatoxins in aqueous solutions. Hathout *et al.* (2011) observed that induced stress in rats due to aflatoxins, which lead to debilitating health and deteriorating liver functions, was restored to health by treatment with lactic acid bacteria. Other laboratory assays have shown that *Pseudomonas* spp. and *Bacillus* strains can inhibit the growth of *A. flavus* and consequent toxin production (Amaike and Keller, 2011). A study done by Anjaiah *et al.* (2006) showed that in greenhouse and field experiments, inoculation of selected antagonistic strains of *Trichoderma*, *Bacillus* and *Pseudomonas* spp. on groundnut resulted in a significant reduction of seed infection by *A. flavus*, and it also reduced >50% of the *A. flavus* populations in the geocarposphere of groundnut. Table 1.3 summarises some of the biological control studies where antagonistic bacteria and fungi were used to inhibit the growth and aflatoxin production of *A. flavus* and *A. parasiticus*.

Table 1.2. Antagonistic fungi and bacteria evaluated for their activity against aflatoxigenic moulds.

Genus	Species	Activity	Reference
Pseudomonas	*P. fluorescens, P. chlororaphis, P. protegens*	Inhibit *A. flavus* growth in grains	(Palumbo *et al.*, 2007, Yang *et al.*, 2017, Mannaa *et al.*, 2017)
Lactobacillus	*L. plantarum, L. rhamnosus, L. casei, L. fermentum*	Bind aflatoxin M1 Inhibit aflatoxin production Inhibit fungal growth	(Sangmanee and Hongpattarakere, 2014, Hauser and Matthes, 2017)
Bacillus	*B. amyloliquefaciens, B. megaterium, B. mojavensis, B. subtilis*	Inhibit the growth of *A. flavus* and *A. parasiticus,* inhibit aflatoxin production	(Kong *et al.*, 2014, Siahmoshteh *et al.*, 2018, Pereyra *et al.*, 2018)
Streptomyces	*S. yanglinensis, S. anulatus, S. alboflavus*	Inhibit *A. flavus* growth Inhibit *A. flavus* growth	(Wang *et al.*, 2013, Mander *et al.*, 2016, Shakeel *et al.*, 2018)
Trichoderma	*T. harzianum, T. viride,*	Biocontrol *A. flavus* growth	(Yobo, 2005, Anjaiah *et al.*, 2006, Mostafa *et al.*, 2013)
Saccharomyces	*S. cerevisiae*	Inhibit mycotoxins production	(Armando *et al.*, 2012)

1.8 Integrated control strategies

Integrated control management is the use of various effective strategies to control pests and diseases. This strategy uses a mixture or combination of cultural, biological, and chemical control methods. Management techniques that lower disease occurrence and aflatoxin contamination during pre-harvest in the field are crucial. These consist of planting on schedule, preserving ideal plant densities, preventing drought stress, and managing various plant diseases, weeds, and insect pests (Bruns, 2003). During soil preparation and planting of groundnuts, it has been shown that aflatoxin contamination can be reduced by chemical fumigation soil amendment by applying lime, poultry manure, and cereal crop residue (Bruns, 2003).

Studies have been done to test the effectiveness of this strategy. Mohammed *et al.* (2018) evaluated the effect of farmyard manure (FYM), the inhibitory efficacy of *Trichoderma* spp., and seed treatment by carbendazim against *Aspergillus* spp. pod colonization and aflatoxin accumulation under field conditions. The results showed that the integrations of *T. harzianum* as biocontrol seed treatment and

soil amendment with FYM were effective in the pre-harvest management of *Aspergillus* spp. and aflatoxin contamination. Studies on plant extracts have also demonstrated a positive effect on the control of aflatoxin contamination by Asper*gillus* spp. A study done by Sadhasivam *et al.* (2019) used pomegranate peel extract (PPE), and azole fungicide prochloraz (PRZ) alone and in combination to control *A. flavus* and *Fusarium proliferatum.* The findings demonstrated a significant delay of conidial germination and hyphal elongation rate in both fungi following PPE treatment in combination with PRZ, blocking aflatoxin B1 production by *A. flavus*.

1.9 Plant endophytes as plant disease management option

Endophytes have recently been coined for in-plant growth improving microbes for their interesting role in easing biotic stresses. Endophytes are micro-organisms living inside plant tissues without causing any damage and/or disease; they can be isolated from inside plant tissues using a strict sterilization technique (Shahzad *et al.*, 2017). Endophytes colonize the roots of seedlings during plant growth (Morales-Cedeno *et al.*, 2021) and thereafter are distributed using the xylem throughout the plant, including the roots, leaves, stems, fruits, seeds, and flowers (Arnold and Lutzoni, 2007) either local or systemic (Rangjaroen *et al.*, 2017). Endophytes can be either fungal or bacterial (Eid *et al.*, 2021). The plant-endophyte interaction is achieved by balanced antagonism and is maintained by avoiding the activation of the host defences (De Silva *et al.*, 2019), and the colonization would seemingly be asymptomatic and avirulent (Kusari *et al.*, 2012). Different methods used to identify endophytes have been used. This includes a conventional method of surface sterilization, cultivating in a nutrient-rich growth medium, and morphologically identifying organisms using microscopy or staining (Kandel *et al.*, 2017, Ahmad *et al.*, 2019, Eid *et al.*, 2021). Recently, molecular identification has been used, using 16S rRNA gene sequencing (Ahmad *et al.*, 2019).

Endophytes are beneficial as they can directly and/or indirectly promote plant growth (Eid *et al.*, 2021). They produce active metabolites such as immune suppressive compounds, plant growth promoters, antimicrobial volatiles, anti-cancer agents, antioxidants, antibiotics, and insecticides (Strobel, 2018). Bacterial endophytes use different mechanisms that restrict the growth of plant pathogens, including competition for space, nutrients, and ferric iron, antibiosis, induced systemic resistance (ISR), and detoxification of virulence factors (Kandel *et al.*, 2017, Shahzad *et al.*, 2017, Morales-Cedeno *et al.*, 2021). Competition for space and nutrients is an indirect method used by plant endophytes to prevent the activity and growth of plant pathogens (van der Lelie *et al.*, 2009). When there is limited iron available for the plant, endophytes produce siderophores to competitively acquire ferric iron (Whipps, 2001, Compant *et al.*, 2005). During antibiosis, endophytes release antibiotics (2,4-diacetylfloroglucinol acid, phenazine-1-carboxylic acid, phenazine-1-carboxamide, oomycin A, viscosinamide, kyanoaminectone, zymicrolactone A, aerugina, rhamnolipids, cepacyamide A, ecomycins, cepafungi that contribute to the suppression of soil-borne diseases (Santoyo *et al.*, 2019). In ISR, endophytes colonize the plant and activate certain signalling pathways that when the plant

receives a stress or pathogenic stimuli, these stimuli activate resistance response (Mazhar *et al.*, 2021). This stimulus is termed an elicitor and is recognized by specific receptors (Köhl *et al.*, 2019). Detoxification of virulence factors by endophytes is achieved by the production of a protein that reversibly binds to the toxin thus detoxifying the pathogen (Compant *et al.*, 2005). Most often, microbes have several signalling molecules used for sensing the presence of other microbes within the vicinity, therefore, these molecules are used to kill nearby microbes to eliminate competition and survive (Mazhar *et al.*, 2021). Table 1.4 shows a list of plant endophytes and their proposed mechanisms to control several pathogens.

The use of bacterial endophytes in agriculture as biological control has immense potential to reduce the environmental impacts caused by harsh chemical fertilizers. Through research, some of the successful Plant growth promotion (PGPR) and biological control agents (BCA) have been commercialized and are currently marketed. There is no doubt that many of these beneficial bacteria and fungi are currently under intense research. After agriculture, endophytes have industrial and pharmaceutical applications. Wu *et al.* (2018) concluded that the endophytic *Pseudomonas aeruginosa* L10, associated with the roots of *Phragmites australis* (reed), efficiently degraded hydrocarbons and produced a biosurfactant (Palwe *et al.*, 2021). With the research already done, it is highlighted that the economic importance of plant endophytes is high, with a great impact on most industries.

1.10 Research gaps

Biological control has shown to be effective in the control of *A. flavus*, but not much research has been done using plant endophytes from leguminous plants to control *A. flavus*. Sourcing bacteria found within plants and using them for protection against pathogens opens a new avenue for research. It is worth noting that more research needs to be done using bacterial endophytes for disease control, such as yellow mould, besides beneficial bacteria and fungi from the rhizosphere. As *A. flavus* is aflatoxic, it contaminates agricultural commodities, threatening economic growth, food safety, and public health on a global scale. This research focuses o using plant bacterial endophytes found in leguminous plants to control *A. flavus* on groundnuts.

1.11 Conclusion

Aspergillus flavus has been known to be a major problem for groundnut, maize, and cotton production. They continue to negatively affect both animals and humans. With chemical control being limited in the past for *A. flavus*, management of these species has advanced over the years. To date, many organisms, including bacteria or fungi, have been investigated for their potential in the reduction of aflatoxin contamination in crops. For A. *flavus*, aflasafe, AF36, and Aflaguard have shown great ability to naturally inhibit the growth of this pathogen in their respective countries. This makes biological control a top-tier choice for a healthy environment compared to other strategies. The introduction of plant endophytes has opened a new avenue for research, which does not limit it only to agricultural

applications. It is also worth noting that in some instances, integrated management strategies work better for pathogen control for farmers. The use of plant endophytes together with other management strategies could heighten the control of *A. flavus* and co-existing species.

Table 1.5: A list of bacterial endophyte species used as biocontrol agents against several pathogens, targeted crops, and their mode of application

Organism(s)	Intended Use	Mechanism	Target crop(s)	Mode of application	Comments	Reference(s)
Lactobacillus plantarum CM-3	Biological control of *Botrytis cinerea*	Competition for nutrients and space	Strawberry	Cell suspensions inoculated on fruit	Should be applied during the postharvest storage	(Chen *et al*., 2020)
Bacillus Subtilis 10− 4 *Bacillus Subtilis* 26D	Biological control of Late blight by *Phytophthora infestans*, *Fusarium* wilt, and dry rot by *Fusarium oxysporum*	Competition for nutrients and space	Potato	Immersing	Applied for postharvest decays of potato during long-term storage	(Lastochkina *et al.*, 2020)
Pseudomonas synxantha DLS65	Biological control of Postharvest brown rot by *Monilinia fructicola* & *Monilinia fructigena*	Competition for nutrients and space, production of diffusible toxic metabolites and VOCs	Stone fruit	Cell suspensions inoculated on peach fruit	It also increases the plant biomass	(Aiello *et al.*, 2019)
Bacillus amyloliquefaciens RS-25, *Bacillus licheniformis* MG-4, and *Bacillus subtilis* Pnf-4	Biological control of *Botrytis cinerea*	Antibiosis, cellulase and protease activities, biofilm production	Tomato, strawberry, and grapefruit	Cell suspensions inoculated on fruit	All the strains showed effective cellulase and protease activities, but no chitinase activity	(Chen *et al*., 2019)
*Bacillus velezensis, Citrobacter freundii, Enterobacter asburiae*_01, *E. asburiae*_02, *E. cloacae, E. xiangfangensis, Pantoea dispersa,*	Biological control of *Anthracnose* by *Colletotrichum musae*	VOCs production and lytic enzymes activity	Banana	Sprayed, fruit immersion, or systemic application	Should be applied postharvest to limit postharvest decay	(Damasceno *et al.*, 2019)
Bacillus amyloliquefaciens NCPSJ7	Biological control of Gray mould by *Botrytis cinerea*	Antibiosis and polyphenol oxidase, chitinase, and β-1,3- glucanase activities	Grapes	Cell suspensions inoculated on grapes	Should be applied during postharvest storage	(Zhou *et al*., 2020)
Pseudomonas sp. 135 *Bacillus sp*. 52	Biological control of *A. flavus*	Antibiosis	Groundnuts	Seed dressing, soil drenching	Worked best when applied at different growing stages of the groundnut	(Anjaiah *et al.*, 2006)
Bacillus amyloliquefaciens BZ6-1	Biological control of *Ralstonia solanacearum*	Production of antimicrobial compounds	Groundnuts	Cell Suspension	72% decrease in the incidence of the disease	(Wang and Liang, 2014)

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Chapter 2

Isolation and *in vitro* screening of plant endophytic bacteria for biological control of *Aspergillus flavus* Link

Abstract

In this study, 106 endophytic bacterial isolates obtained from leguminous plants were screened against *A. flavus*, the causal agent of the yellow mould of groundnut, using *in vitro* disc diffusion method. Of the 106 bacterial endophytes, 32 were obtained from roots, a further 32 from leaves, 29 from stems, and 13 from seeds. During the *in vitro* primary screening, only 13 (12%) of the isolates inhibited *A. flavus* after nine days of incubation. In the secondary screening of the best 10 isolates using the dual culture assay, Isolate ALA had the highest percentage inhibition of 55.2%, followed by Isolates KI (40.9%) and KG (40.2%). Isolate ALA was obtained from *Phaseolus vulgaris* L. (dry bean) leaves, while Isolates KI and KG were isolated from groundnut stems and leaves. Molecular techniques were used to identify the best five isolates from the secondary screening. The isolates were identified as *Bacillus amyloliquefaciens/ B. siamensis/ B. velezensis* (Isolates KG, and KI), *Bacillus amyloliquefaciens/ B. subtilis/ B. velezensis* (Isolate BB), *Pseudomonas fluorescens* (Isolate ALA), and *Pantoea dispersa* (Isolate GNLA). These five isolates were used in a series of *in vivo* studies to assess their antagonistic effect on *A. flavus.*

Keywords: *Arachis hypogaea* L, yellow mould, bacterial endophytes, aflatoxins

2.1 Introduction

Groundnut (*Arachis hypogaea* L.) is an annual legume that is one of the world's most important oilseed crops ranking fourth in the world (Coleman, 2020). A number of constraints impact the market demand for groundnuts in developing and developed countries, one being *Aspergillus flavus* Link infections and contamination (Guchi, 2015). *A. flavus* is one of the fungi responsible for mycotoxin contamination in foodstuff. The fungus produces aflatoxins as its secondary metabolite. *A. flavus* contaminate groundnut seeds and decrease their production value due to aflatoxin contamination (Bediako *et al.*, 2019). Consumption of these contaminated seeds causes aflatoxicosis in humans and animals (Guchi, 2015). Therefore, developing an effective and accurate control strategy is needed.

The interest in biological control of *A. flavus* has increased in recent years, due to the need for environmentally friendly alternatives compared to the frequent use of chemical control strategies (Ongena and Jacques, 2008). Other than being cost-effective (Alamene, 2015), the added benefits of using biological control agents (BCA) are that they are: self-sustaining (permanent/continuous reproduction) (Saharan and Nehra, 2011) and pathogen-specific (targets one species at a time) (Santoyo *et al.*, 2012). Therefore, for effective protection against a specific pathogen, understanding how BCAs work against that pathogen is vital. The series of mechanisms used by BCAs include antibiosis, induced systemic resistance (ISR), competition, siderophore production, and detoxification of virulent factors (Fira *et al.*, 2018, Köhl *et al.*, 2019). Therefore, BCAs use at least one or more of these mechanisms to control plant pathogens. For example, *Pseudomonas chlororaphis* produce antibiotics (phenazines) to control *Fusarium oxysporum* Schlechtendal on tomatoes (*Solanum lycopersicum* L.) (Chin-A-Woeng *et al.*, 2001). In the past, most BCAs were isolated from the rhizosphere, with little attention to plant endophytic BCAs. In the last few years, studies have shown that plant endophytes are effective in controlling plant pathogens, from *in vitro* studies to field environments (Mmbaga *et al.*, 2018). Several of these plant endophytes belonging to the genus *Bacillus*, produce various antimicrobial substances. *B. subtilis* is one of the primary producers of these antimicrobial substances in this genus (Földes *et al.*, 2000). Bacilliomycin D, an antibiotic produced by *B. subtilis* disrupts the cell membrane of *A. flavus*, resulting in pathogen control ((Kaspar *et al.*, 2019)).

In this chapter, 106 bacterial endophytes were isolated from different parts of leguminous plants, screened using the dual culture bioassays technique, and the best isolates were identified using molecular techniques.

2.2 Materials and Methods

2.2.1 Collection of plant samples and sources of endophytic bacterial isolates

Plant samples were collected from three locations around Pietermaritzburg, KwaZulu-Natal, South Africa. The samples include seeds, stems, leaves, and roots of groundnuts, dry and white beans

(*Phaseolus vulgaris* L.), and peas (*Pisum sativum* L.). The crop samples were all collected from home gardens with manure and mulching from the following locations: Groundnut (from Scottsville, Pietermaritzburg, KwaZulu-Natal, South Africa), Beans, and peas (from Scottsville - Pietermaritzburg, Ixopo, and Sweetwaters, KwaZulu-Natal, South Africa). The plant parts were washed with distilled water to remove dust and soil particles. They were then soaked in a 5% sodium hypochlorite solution for 60 seconds and subsequently in 70% ethanol for 30 seconds. After that, the samples were rinsed three times in sterile distilled water. Twenty microlitres (20µ*l*) of the third wash was plated on a Tryptone Soya Agar (TSA) to test the effectiveness of the sterile technique. The plant parts were allowed to air-dry under the laminar flow in sterile Petri dishes.

Ten grams (10g) of each of the fresh plant part was weighed in a sterile weighing boat under sterile conditions and ground using a sterile mortar and pestle. Ten ml (10ml) of sterile distilled water was aseptically added during the grinding process. Aliquots of 10µ*l* of the resulting suspension were plated on TSA and incubated at 28^{o}C. After 72 hours of incubation, morphologically different bacterial colonies were selected and subcultured onto Potato Dextrose Agar (PDA) in 90mm Petri dishes to obtain pure cultures. The bacterial colonies were further purified by repeated subculturing on PDA plates. Pure cultures were appropriately labelled and then stored in Eppendorf tubes with 2ml of 30% glycerol at -80^{o}C for further use.

2.2.2 Source and culturing of *A. flavus*

A pure culture of *A. flavus* (Accession number: PPRI 26007) originally isolated from *A. flavus* infected groundnut plant was used in this study. The *A. flavus* strain was obtained from the Agricultural Research Council – Plant Health Protection (PHP), National Collection of Fungi, in Roodeplaat, Pretoria (Gauteng Province, South Africa).

2.2.2.1 Media preparation

The agar media was prepared by adding 39g of Potato Dextrose Agar (PDA) with 1L of distilled water in a 2L Schott bottle. The flasks' content was mixed thoroughly and autoclaved at 121^{o}C for 15 minutes. Once cooled to about 45^{o}C, the media was poured into 90 mm Petri-dishes under sterile conditions in a laminar flow and left to solidify overnight at an ambient temperature for further use.

2.2.2.2 *Aspergillus flavus* inoculum preparation

Five hundred grams (500g) of barley seeds were weighed into a 2L Erlenmeyer flask, and 1L sterile distilled water was added to it and left overnight. After the period, the water was drained, and the seeds were autoclaved twice at 121^{o}C for 15 minutes at 24 hours intervals. A five-day old culture *A. flavus* on a PDA plate was cut into small squares and was used to inoculate the barley seeds and incubated for nine days at 28^{o}C. The infected seeds were air-dried under the laminar flow for 72 hours. Five (5) samples of *A. flavus* inoculum were stored in McCartney bottles under ambient laboratory conditions and used for the duration of the experiment.

2.2.3 *In vitro* screening of endophytic bacterial isolates against *A. flavus*

The endophytic bacteria suspension was prepared by adding five loopfuls of bacteria into 20ml sterile distilled water in a tube and shaking to create a slury. The suspension was used as is ($1x10^0$). Four antibiotic paper discs were placed at equidistant points along the margins of PDA plates, and a 20µl suspension of three bacterial isolates was placed separately on a paper disc. A 20µl sterile distilled water was placed on the fourth antibiotic paper disc and was used as a control. A 4 x 4 mm^2 agar plug carrying a mycelial of *A. flavus* was placed at the centre of the plate and incubated at 28 °C for nine days (Figure 2.1). This was repeated until all the 106 bacterial isolates were screened. There were three replicates for each PDA plate assay. The antagonistic effect was determined by measuring the size of the inhibition zone formed on the plate around the disc. The experiment was repeated once.

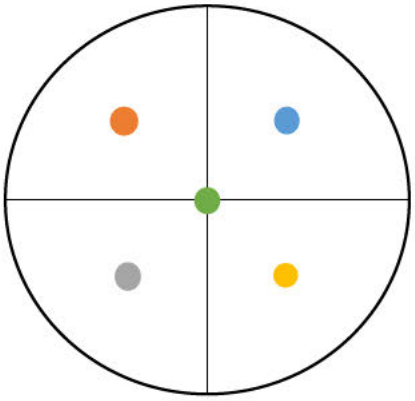

Figure 2.1: Disc diffusion method for screening bacterial isolates. Orange, blue and yellow represent the different isolates. Grey represents the control disc.

2.2.4 *In vitro* secondary screening of endophytic bacterial isolates against *A. flavus*

The *in vitro* inhibition of mycelial growth of *A. flavus* by the endophytic bacterial isolates was done using the dual culture technique described by (Landa *et al.*, 1997). The 10 best performing bacterial endophytes were selected for secondary screening based on the diameter of the zones of inhibition. After that, the selected bacterial antagonists were re-screened to confirm their efficacy. The *in vitro* inhibition of mycelial growth of *A. flavus* by the bacterial isolates was done using the dual culture technique on PDA plates. This was done by drawing two lines parallel to each other 10 mm from the edge of a 90 mm Petri dish PDA plate. The two parallel lines were streaked with loopfuls of the bacterial endophyte. After that, a 4 x 4 mm^2 of agar block carrying the mycelia of an actively growing *A. flavus* culture was placed in the centre of the two parallel lines streaked with the bacterial endophyte.

The control plates had sterile distilled water streaked instead of an isolate. Three replications of each treatment were used. The plates were incubated at 28°C for nine days. The secondary screening bioassay was repeated once. After the incubation period, the results were measured and recorded and used to calculate the percentage inhibition using the formula below.

$$\text{Pi} = \frac{\text{Dc}-\text{Dt}}{\text{Dc}} \text{x } 100 \text{ ;}$$

where;

Pi= Percent inhibition

Dc= diameter of control plate (*A. flavus*)

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

2.2.5 Identification of bacterial isolates using 16S primer sequence

The best five isolates based on the zones of inhibitions from the secondary screening were selected and sent to Inqaba Biotec Laboratories (Pretoria, South Africa) for species-level identification. Genomic DNA was extracted from the cultures using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Pretoria, South Africa. Catalogue No. D6005). The 16S target region was amplified using Onetaq® Quick-Load® 2X Master Mix (New England Biolabs, Pretoria, South Africa. Catalogue No. M0486) with the primers 16S-27F and 16S-1492R (table 2.1). The PCR products were run on a gel and extracted with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Pretoria, South Africa. Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse direction using the Nimagen, Brilliant Dye™ Terminator Cycle Sequencing Kit (V3.1, BRD3100/1000) and purified using the ZR-96 DNA Sequencing Clean-up Kit (Zymo Research, Pretoria, South Africa Catalogue No. D4050). The purified fragments were analysed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific, Gauteng, South Africa) for each reaction for every sample. CLC Bio Main Workbench v7.6 was used to analyze the .abl files generated by the ABI 3500XL Genetic Analyzer, and results were obtained by the Basic Local Alignment Search Tool (BLAST) search (NCBI). The BLAST was done according to Altschul *et al.* (1997).

Table 2.1: Primer sequences used in identifying endophytic bacterial isolates from *in vitro* studies.

Name of Primer	**Target**	**Sequence (5' to 3')**
16S-27F	16S rDNA sequence	AGAGTTTGATCMTCMTGGCTCAG
16S-1492R	16S rDNA sequence	CGGTTACCTTGTTACGACTT

2.2.6 Data analysis

A general linear model (GLM) was used to run an ANOVA on the percentage inhibition data from the primary and secondary screening experiments using SAS software version 9.4 (SAS, 2016). If the

ANOVA was significant (P<0.05), the means were separated using Duncan's multiple range test (DMRT) at a 5% significance level. The data for the repeated experiments were merged before analysis.

2.3 Results

2.3.1 Endophytic isolates

A total of 106 bacterial endophytes were obtained from groundnuts, dry and white beans, and peas. Out of the total, 32 isolates were obtained from leaves, 29 from stems, 32 from roots, and 13 from seeds (Table 2.2). The average zone of inhibition after nine days of each isolate is present in Appendix 2.1. Isolates from groundnuts and beans provided moderate to high percentage inhibition compared to peas. The isolates from bean leaves had the most persistent inhibition compared to the rest of the bacterial isolates from the different sources.

Table 2.2: The number of bacterial endophytes per plant part for each of the three leguminous crops

Sample	Leguminous crop source						
	Groundnuts		Beans			Peas	
	Scottsville	Scottsville*	Ixopo	Sweetwaters	Scottsville*	Sweetwaters	Scottville*
Leaves	7	5	4	3	7	2	4
Stem	2	5	4	5	9	3	1
Roots	7	4	-	5	12	2	2
Seeds	5	-	-	-	5	-	3

Note: * represents samples collected from fertilized soil

2.3.2 *In vitro* primary screening of endophytic bacterial isolates against *A. flavus*

All 106 isolates were screened against *A. flavus* using the dual culture assay. On average, 13 (12%) of the isolates inhibited the growth of *A. flavus in vitro* after nine days of incubation. The best 10 isolates were ALA from dry beans leaves (Ixopo), Isolates BB and BD from bean seeds (Scottsville), Isolate GBSA from bean stem (Scottsville), Isolate GLC from fertilized groundnut leaves (Scottsville), Isolate GNLA from unfertilized groundnut leaves (Scottsville), Isolate GNPA from groundnut seeds, Isolate GRC from fertilized groundnut roots (Scottsville), Isolate KG from groundnut seed (Scottsville), and Isolate KI from groundnut stem (Scottsville).

2.3.3 *In vitro* secondary screening of endophytic bacterial isolates against *A. flavus*

The ten endophytic bacterial isolates were selected based on their diameter of zones of inhibition against *A. flavus* during the primary screening trial. The secondary screening of the selected endophytic

bacterial isolates provided average percentage inhibitions ranging from 7.14-55.24%. Groundnuts accounted for 60% of the isolates selected, and beans accounted for 40%. The best three isolates with the highest average percentage inhibitions were obtained from bean leaves, groundnut stems and leaves (Table 2.3)

Table 2.3. Average inhibition (%) of the best ten endophytic bacterial isolates from different sources obtained from secondary screening against *A. flavus*.

Isolate	Source of Isolate	% Average Inhibition + SE
ALA	*Phaseolus vulgaris* L. (leaves)	55.24 ± 2.39 a
BB	*Phaseolus vulgaris* L. (seeds)	36.43 ± 1.45 b
GBSA	*Phaseolus* vulgaris L. (stem)	17.38 ± 0.55 c
GLC	*Arachis hypogaea* L. (leaves)	10.95 ± 4.39 cd
GNLA	*Arachis hypogaea* L. (leaves)	21.19 ± 2.74 cp
GNPA	*Arachis hypogaea* L. (seeds)	9.52 ± 1.37 cd
GRC	*Arachis hypogaea* L. (roots)	7.14 ± 0.00 cd
KG	*Arachis hypogaea* L. (seeds)	40.24 ± 4.76 b
BD	*Phaseolus vulgaris* L. (seeds)	20.71 ±1.25 c
KI	*Arachis hypogaea* L. (stem)	40.95 ± 2.18 b
Control		0.00 d
F-value		14.3
P-value		0.0001
%CV		47.36

Note: The values are presented as an average of three replicates. Different letters represent significant differences at a 5% significance level, according to DMRT.

Table 2.3 shows the percentage zones of inhibition calculated for all 10 bacterial isolates used for the secondary screening. The best isolate (Isolate ALA) from dry bean leaves showed an average percentage inhibition of 55.24%, followed by KI (40.95%), KG (40.24%), and BB (36.34%). The best bacterial isolate, ALA was significantly different from the rest of the nine isolates selected for the secondary screening (Table 2.2; Figure 2.2). Isolate BB, KG, and KI from groundnut (stem and leaves) and bean (leaves) showed no significant difference from each other (Table 2.2; Figures 2.3 and 2.4). A high percentage co-efficient of variation (47.36%) indicates a high variation within the best ten isolates.

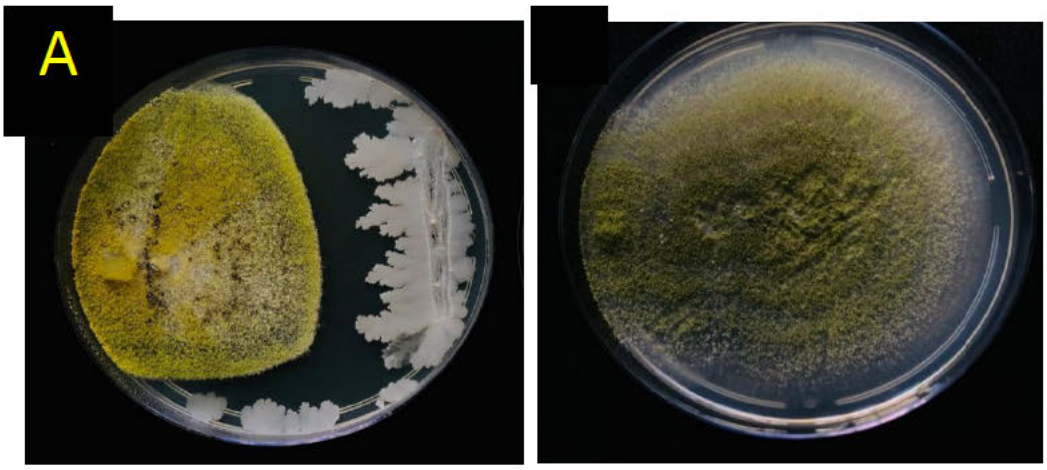

Figure 2.2: The inhibition of *A. flavus* mycelial growth by the best endophytic bacteria isolate ALA (A) compared to the control plate with *A. flavus* only (B).

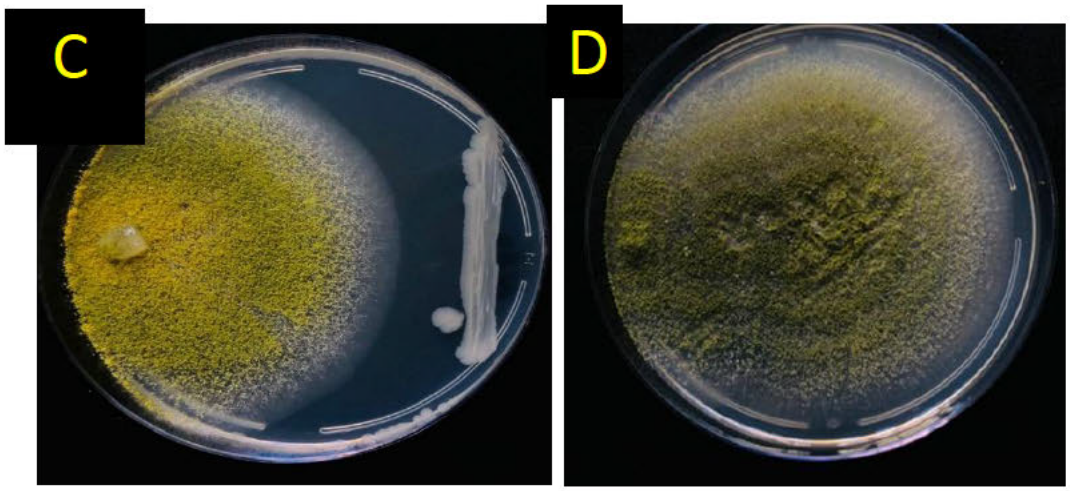

Figure 2.3: The inhibition of *A. flavus* mycelial growth by the second-best endophytic bacteria isolate KG (C) compared to the control plate with *A. flavus* only (D).

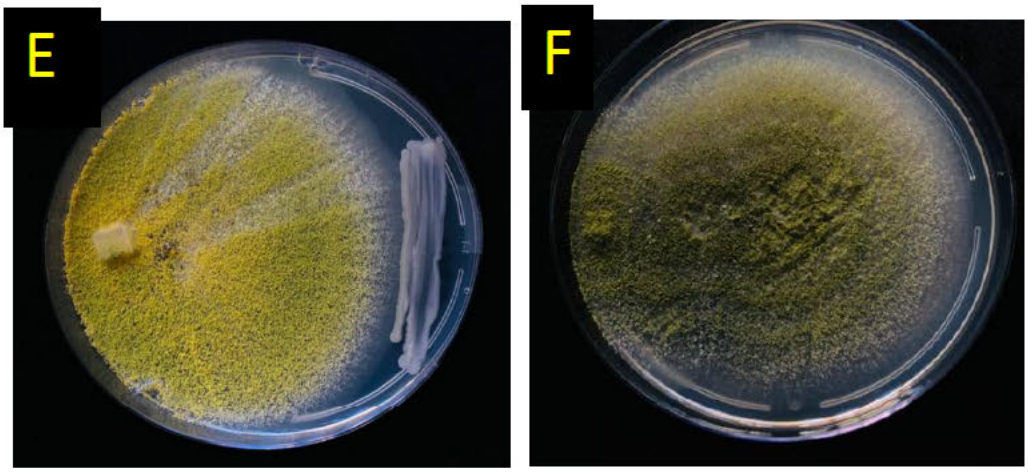

Figure 2.4: The lowest inhibition of *A. flavus* mycelial growth by the endophytic bacteria isolate GRC (E) compared to the control plate with *A. flavus* only (F).

2.3.4 Bacterial identification using 16S primer sequence

The best five isolates were identified using the 16S primer sequence (Table 2.4). Three of the isolates KI and KG that were gram-positive, were identified to be either *Bacillus siamensis, Bacillus velezensis or Bacillus amyloliquefaciens*. Isolate BB had similarities to *Bacillus subtilis, Bacillus amyloliquefaciens and Bacillus valezensis*. Isolate GNLA and ALA were identified as *Pantoea dispersa* and *Pseudomonas fluorescens*, respectively.

Table 2.4: Molecular identification of the best five endophytic bacterial isolates using the 16S primer sequence.

Name of Sample	Predicted organism	Request ID	Sequence	Gram stain (+/-)	Similarity %
KI	*Bacillus siamensis/ Bacillus velezensis/Bacillus amyloliquefaciens*	Y3ZT6WKW013		+	99
KG	*Bacillus siamensis/ Bacillus amyloliquefaciens/ Bacillus velezensis*	Y3ZSESAV016		+	99
BB	*Bacillus velezensis/Bacillus amyloliquefaciens/ Bacillus subtilis*	Y3ZUAUW101R		+	99
GNLA	*Pantoea dispersa*	Y43DNUG6013		-	99
ALA	*Pseudomonas fluorescens*	Y43MBD8X013		-	99

2.4 Discussion

This study aimed to isolate, screen, and identify bacterial plant endophytic isolates from sampled leguminous crops that can inhibit the growth of *A. flavus*. A total of 106 isolates were screened using the dual culture disc diffusion method for their antagonistic ability against *A. flavus*. Of the 106 bacterial endophytes, the best ten were used for secondary screening; subsequently, five demonstrated great potential. The best isolates were selected based on showing the highest average percentage inhibition against *A. flavus*. Using 16S rRNA genes, the best five bacterial isolates were identified to belong to either *Pseudomonas, Bacillus,* and/or *Pantoea* species.

A dual culture assay was used *in vitro* as a screening technique to select the best isolates. The inhibition zones between the fungus and bacteria are suggestions of the presence of either antibiotics, toxic metabolites, or lytic enzyme secretion during the screening process (Köhl *et al.*, 2019, Shaikh *et al.*, 2020). These metabolites can degrade the cell membrane and cell wall, resulting in the inhibition of

pathogens. The BLAST taxonomic identification was used to identify the best five isolates to species level. All the identified isolates corresponded with bacteria genera associated with plants. *Pseudomonas fluoresens, Bacillus siamensis, B. velezensis, B. amyloliquefaciens, B. subtilis* and *Pantoea dispersa* were among the identified isolates. The presence of these isolates in groundnut and bean plants is in accordance with the studies done by Walker *et al.* (1998), Costa *et al.* (2012), Sobolev *et al.* (2013), Wang *et al.* (2013), and Emmer *et al.* (2021) who isolated similar bacterial strains from beans and groundnuts.

The isolate with the highest inhibition percentage was ALA, identified as *Pseudomonas fluoresens,* which was an ideal candidate in the control of *A. flavus*. *Pseudomonas* spp. produce a variety of metabolites such as HCN, siderophore, fluorescein, pyocyanin, and phenazine-1-carboxylic acid. These metabolites have been suggested to control a wide range of pathogens (Shaikh *et al.*, 2020). For example, *P. fluorescens* strain 3JW1 effectively reduced aflatoxin contamination on peanut kernels by suppressing fungal growth and aflatoxin biosynthesis and breaking down the synthesized aflatoxin (Yang *et al.*, 2017). A study done by (Marrez *et al.*, 2019) indicates that *P. fluorescens* FP10 cell-free supernatant and chloroform fragments would be ideal as biocontrol agents against foodborne fungal pathogens such as *A. flavus*. Two second-best isolates (KG and KI) with percentage inhibitions of 40.24% and 40.95%, respectively, were identified as one of three *Bacillus* spp.; *B. siamensis, B. velezensis or B. amyloliquefaciens*. Species from the genus *Bacillus* produce secondary metabolites with strong antifungal activity (Jiang *et al.*, 2018, Li *et al.*, 2022). A study by (Jin *et al.*, 2020) indicated that bacillomycin D isolated from *B. velezensis* HN-2 has more potent antifungal activity against *Colletotrichum gloeosporioides* Penz. than the fungicides prochloraz and mancozeb. In this study, antifungal activity was provided by the identified *Bacillus* isolates. Santoso *et al.* (2021) concluded that *B. siamensis* LDR inhibited the growth of the fungal phytopathogens, *Fusarium sp*., *Ganoderma* sp., and *Chaetomium globosum* strain InaCC F228. Groundnut seeds have been shown to accommodate several species of endophytic bacteria, among which *Bacillus* spp. often dominated (Berg *et al.*, 2005). The ability of this genus to suppress plant pathogens on groundnuts has been suggested by Anjaiah *et al.* (2006), Sobolev *et al.* (2013), Wang and Liang (2014), and Shifa *et al.* (2016).

Even though *Bacillus* species are considered attractive biocontrol agents due to their ability to control a broad range of fungal plant pathogens (Cavaglieri *et al.*, 2005), the presence of *P. dispersa* (GNLA) and *P. fluorescens* is attractive in the biological control of *A. flavus*. The genus *Pantoea* is highly adaptive for colonization in different hosts due to its ability to utilize diverse compounds, including antibiotics pantocins, herbicolins, microcins, and phenazines (Walterson *et al.*, 2014, Walterson and Stavrinides, 2015, Suman *et al.*, 2020). With limited studies as biological control agents, more and more research is now focusing on these bacteria. A study by Cavaglieri *et al.* (2005) suggested that *P. dispersa* strains represent useful biocontrol agents for protecting sweet potatoes from post-harvest

infection by *Ceratocystis fimbriata.* Gohel *et al.* (2007) reported that *P. dispersa* significantly inhibited the growth of *Fusarium udum* on pigeon pea (*Cajanus cajan* L. Millsp) by producing mycolytic enzymes.

Aflatoxins produced by *A. flavus* invade groundnuts from sowing until storage. Aflatoxins are extremely toxic and results in atleast 25% of crop production (including groundnuts) contaminated (Martey *et al*., 2020). As different countries set the maximum concentration of *A. flavus*, this results in the farmers earning less profit because the produce is rejected in the international trade market and thereafter reduced markert value. To control *A. flavus,* various strategies have been employed, including cultural control and chemical control which has resulted in *A. flavus* resistance. However, the inhibition of *A. flavus* has been attributed to several biological control strains (Acharya *et al*., 2021). Biological control is not only tied to pathogen control but also growth promotion and soil improvement. Therefore the use of biological control agents that were isolated and identified in control *A. flavus*, could result in improved plant growth (biofertilizers), plant protection (through different mechanisms) and less resistance (Ghosh *et al*., 2020).

In this study, the five isolates that were identified demonstrated their potential candidacy in inhibiting *A. flavus* growth in *in vitro* studies. These isolates will further be tested to assess their ability to control *A. flavus* under storage and greenhouse conditions to ascertain the effectiveness against *A. flavus* control.

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Chapter 3

The effect of three endophytic *Bacillus amyloliquefaciens/ B. siamensis/ B. velezensis* strains on postharvest management of *A. flavus* in groundnut seeds

Abstract

In this study, the preventative efficacy of three endophytic bacterial strains of *Bacillus amyloliquefaciens/ B. siamensis/ B. velezensis* against *A. flavus* in medium- and short-term storage trials using two groundnut cultivars (Akwa and Sellie Plus) was studied. For the short-term trial, three treatments: crude lipopeptide extract (CE), culture filtrate (CF), and bacterial cells (BC), prepared using Landy medium, were used as seed treatment. The culture filtrate of *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strains KG and KI showed potential as biological control agents against *A. flavus* as they had an average percentage infection of 38.2% and 37.4%, respectively on Cultivar Akwa and 33.0% and 10.8% on Cultivar Sellie Plus for the short term storage trial. The pH of the culture filtrate was adjusted to 2.1 and 7.4 for both strains and used as a treatment for 21 days. The acidic pH (2.1) culture filtrate of *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strain KG showed a constant antifungal activity until after 14 days. After 14 days, and by 21 days after storage, all treatment lost their antagonistic effects on *A. flavus*. The interaction effects of the two *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strains on *A. flavus* were observed using scanning electron microscopy (SEM). The results showed degradation and shrivelling of mycelia, vesicles, and the inability of *A. flavus* to produce conidia during the interaction process.

Keywords: *Bacillus* spp., biological control, culture filtrate, antimicrobial compounds, pH

3.1 Introduction

Contamination of groundnuts (*Arachis hypogaea* L.) and groundnut products by *Aspergillus flavus* Link. is known to occur very widely. The seed quality of groundnuts is primarily reduced by the presence of yellow mould during pre-harvest and post-harvest storage (Wild and Hall, 2000, Guchi, 2015, Lavkor and Var, 2017). Conventionally, food preservatives and chemical fungicides have been used to control postharvest decay as they are effective. However, using these products has led to several adverse effects, such as resistance and chemical residues affecting food safety (Shakeel *et al.*, 2018). The constant use of fungicides has a long-term effect on humans, animals, and the environment (Droby *et al.*, 2016). In order to protect food and feedstuffs (Pócsi *et al.*, 2020), various possible control measures have been used to manage pathogens both pre- and postharvest such as fumigation, hot water treatment, the use of atoxigenic strains, and biological control, to name a few (Kong *et al.*, 2010, Bhatnagar-Mathur *et al.*, 2015, Lopez-Reyes *et al.*, 2016, Siddique *et al.*, 2018, Agbetiameh *et al.*, 2020, Ma and Johnson, 2021).

A. flavus produces harmful substances such as aflatoxin (Chuaysrinule *et al.*, 2020, Khalil *et al.*, 2021). Therefore, finding a control method that causes zero effects or low risks on humans, animals, and the environment is ideal. Research in the past decade has focused on using biological control and cultural practices to manage this pathogen, mostly pre-harvest and post-harvest. Biological control products based on plant growth-promoting bacteria (PGPB) have been considered a possible alternative to synthetic fungicides and/or food preservatives in the control of fungi like *A. flavus* (Lastochkina *et al.*, 2019). These biological control products are beneficial as they, directly and indirectly, promote plant growth, disease resistance, and abiotic stress tolerance (Hashem *et al.*, 2019, Elnahal *et al.*, 2022). Endophytic bacteria have recently been considered suitable biological control agents against pre- and post-harvest diseases, as they produce antimicrobial compounds that inhibit pathogens' growth (Qadri *et al.*, 2020, Morales-Cedeño *et al.*, 2021).

Bacillus spp. are attractive biological control agents (BCAs) and have aided in controlling several pathogens, plant growth promotion and development (Caulier *et al.*, 2018, Lahlali *et al.*, 2022). They are abundant and predominant in the rhizosphere (Kang *et al.*, 2015). The role of *Bacillus* spp in inhibiting a variety of plant diseases post-harvest and their primary mechanisms for regulating fruits/vegetable storage quality remain primarily unknown (Lastochkina *et al.*, 2019). In this chapter, the efficacy of three strains of *Bacillus amyloliquefaciens* was tested against *A.flavus in vivo* using two South African groundnut cultivars, Akwa and Sellie Plus.

3.2 Materials and methods

3.2.1 Sourcing of groundnut seeds

Certified seeds of two groundnut cultivars, Selli Plus and Akwa were used for this study. The seeds were donated by Vaalharts Groundnuts Marketing CC, Plot 2E5, Hartswater, 8570, South Africa.

Cultivar Selli Plus has a growth period of 155 days and shows good resistance to pod worms, blackened pods, Botrytis stem rot, and reasonable resistance to Sclerotinia. Cultivar Akwa has a growth period of 150 days and shows good resistance to pod worms, blackened pods, Sclerotinia, and poor resistance to Botrytis stem rot. The seeds were stored in a cool, and dry room until further use.

3.2.2 Preparation of endophytic biological control agents (BCAs) treatments (Bacterial cells, Culture filtrates, and Crude lipopeptide extract)

The three endophytic bacteria that were each stored in 2µ*l* of 30% glycerol were sub-culture onto potato dextrose agar (PDA) media for 48 hours at 28ºC. The isolates were then cultured in broth Landy medium (containing glucose, 10.0 g; L-monosodium glutamate, 5.0 g; $MgSO_4$, 0.5g; KCl, 0.78g; KH_2PO_4, 1.0g; $FeSO_4$, 0.05mg; $MnSO_4$, 5.0mg; $CuSO_4$, 0.16mg; and distilled water, 1000mL; pH 7.2), prepared according to Landy *et al.* (1948) to promote the production of the crude extract/lipopeptide compounds (McKeen *et al.*, 1986). The medium was dispensed as 180ml aliquots into three 500ml Erlenmeyer flasks, respectively, and autoclaved at 121ºC (100 kPa) for 15 minutes. Starter cultures of *B. amyloliquefaciens* strain BB, *B. amyloliquefaciens* strain KI, and *B. amyloliquefaciens* strain KG, were established by inoculating a 10ml Landy medium with a loop of pure cultures biomass from a 24-hour agar plate and incubating overnight at 30ºC with agitation (150rpm). One millilitre of each culture was used to inoculate the 180ml of Landy medium and incubated for 72 hours under the same conditions described above. The culture medium was centrifuged at 12,000×g for 30 minutes at 4ºC (JA-10 rotor, Avanti J-26XPI, Beckman) to obtain the bacterial cells. Lipopeptide compounds were extracted from the culture filtrates by acid precipitation (Vater *et al.*, 2002, Hsieh *et al.*, 2008). The pH of the cell-free culture filtrates was adjusted to pH 2.0-2.1 with 1M hydrochloric acid (37% HCl) before storing at 4ºC for a minimum of 4 hours. This was followed by a centrifugation step (12,000xg for 30 minutes), after which the culture filtrate was removed, and the precipitate was extracted twice with 2.5ml of methanol. The pH of the culture filtrate was adjusted to pH 7.4 with 0.1M sodium hydroxide (NaOH) before testing. Appropriate amounts of the crude lipopeptide extracts of *B. amyloliquefaciens* strain BB, *B. amyloliquefaciens* strain KI and *B. amyloliquefaciens* strain KG were each dissolved in sterile distilled water to obtain a concentration of 200µ*g*ml^{-1} for each isolate. These were used as treatments for further experiments.

3.2.3 Treatment and storage of groundnut seeds

3.2.3.1 Short-term storage treatment of groundnut seeds

The two groundnut cultivars, Akwa and Sellie Plus were prepared by surface sterilizing the seeds with 5% sodium hypochlorite (commercial JIK) for 60 seconds, ethanol for 60 seconds, and two one-minute washes with sterile distilled water. These were then air-dried under the laminar flow for two hours. The groundnuts were treated with (1) culture/bacterial cells diluted with sterile distilled water to a concentration of $1x10^8$ $CFUml^{-1}$; (2) culture filtrates; and (3) crude lipopeptide extracts to test the *in*

vivo activity against *A. flavus* in short-term storage of 7 days. Each treatment had three replicates per cultivar. The treatment combinations included:

a. 3 cultured filtrate x Akwa

b. 3 crude lipopeptide extract x Akwa

c. 3 bacterial cells x Akwa

d. 3 cultured filtrate x Sellie Plus

e. 3 crude lipopeptide extract x Sellie Plus

f. 3 bacterial cells x Sellie Plus

Fifteen grams (15g) of groundnut kernels (average of 40 groundnut kernels of cultivar Akwa and 48 groundnut kernels for cultivar Sellie Plus) were weighed for each of the three replicates per treatment, per cultivar. A total of 54 clean sterile Petri dishes were used as storage containers, with six controls (three for each cultivar) treated only with sterile distilled water. For each treatment, 2ml suspension of bacterial cells concentration of $1x10^8$ $CFUml^{-1}$, 2 ml of culture filtrate, and 2 ml of crude lipopeptide extract concentration ($200\mu g ml^{-1}$) of each strain were dispensed onto each of the three replicates, shaken, and allowed to completely dry before inoculation with 1ml suspension of *A. flavus* concentration of $1x10^6$ conidia ml^{-1}. The Petri dishes were stored in a 25°C incubator and monitored. The percentage of disease incidence was calculated after seven (7) days. The two best treatments were used for a medium-term storage trial for 21 days. The experiment was repeated once.

3.2.3.2 Medium-term storage treatment of groundnuts seeds

Seeds of the two groundnut cultivars were surface sterilized as described under Section 3.2.3.1. The seeds were then air-dried under the laminar flow for two hours. The culture filtrate treatment was prepared as per Section 3.2.3.2. In this experiment, the culture filtrate for each of the *B. amyloliquefaciens* strains was divided into two equal volumes. The pH of one culture filtrate treatment was adjusted to 2.1 and the other to 7.4 using 1M HCL and 0.1M NaOH, respectively. An appropriate amount of groundnut kernels (15g) were weighed for each of the three replicates per treatment, per cultivar, and per each day of disease incidence measurement. For each treatment (Culture filtrate pH 2.1 and pH 7.4), aliquots of 2ml of the respective pH-adjusted culture filtrate were dispensed onto the three replicates, shaken, and allowed to completely dry before inoculation with 1 ml of *A. flavus* concentration $1x10^6$ conidia ml^{-1}. The Petri plates were stored in a 25°C incubator and monitored for 21 days. A discontinuous measure of disease incidence (%) was calculated after every seven (7) days for a period of 21 days. The experiment was set up so that each seven (7) day assessment had dedicated Petri-dishes; hence, measurements of disease incidence were not done as a repeated measure.

3.2.4 Scanning Electron Microscopy (SEM)

In vitro dual culture bioassay of the three *B. amyloliquefaciens* strains used in the experiments was prepared according to Section 2.2.4 and incubated at 28°C for seven (7) days. Then, sections of the

media colonized by mycelia of *A. flavus* were sampled close to the zone of inhibition of each strain and fixed at 4ºC for 12 h in 2% (w/v) glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.0). The mycelial specimens were next dehydrated in graded ethanol series [10, 20, 50, 70, 80% (v/v)] and twice in 100% (v/v). After drying in a critical point dryer (Model K850, Quorum supplies, East Sussex, United Kingdom) and gold-coating in a sputter coater (Model Q150RS ES, East Sussex, United Kingdom), the mycelial specimens were examined under a Scanning Electron Microscope (Zeiss EVO L515, Oberkochen, Germany). Three samples per *B. amyloliquefaciens*-*A. flavus* interactions were examined.

3.2.5 Data analysis

A general linear model (GLM) was used to run an ANOVA on the percentage infection data. The data for the primary and repeated experiments were merged before the analysis was done. If the ANOVA was significant ($P < 0.05$), the means were separated using Duncan's multiple range test (DMRT) at a 5% significance level using GenStat (Version 20.1).

3.3 Results

3.3.1 Short-term storage control of *A. flavus* using three different strains of *B. amyloliquefaciens*

Tables 3.1 and 3.2 show the summary of percentage infection on groundnut seeds (Akwa and Sellie Plus cultivars) after being treated with the three bacterial strains over time for a short-term storage trial. The overall best treatment for both cultivars was the culture filtrate treatment. The culture filtrate treatment was consistently the best treatment among all the three *B. amyloliquefaciens* strains. However, the crude lipopeptide extract was found to be the worst treatment among the three strains used (Tables 3.1 and 3.2).

Table 3.3: Short-term storage trial of groundnut seeds (Akwa cultivar) treated with *B. amyloliquefaciens, B. subtilis, B. velezensis and/or B. subtilis* bacterial cells, crude lipopeptide extract and cell-free culture filtrate.

Strain	Treatment	%Infection±SE
B. amyloliquefaciens/ B. subtilis/ B. velezensis strain BB	CE	94.5±1.1 a
B. amyloliquefaciens/ B. subtilis/ B. velezensis strain BB	CF	65.9±2.2 b
B. amyloliquefaciens/ B. subtilis/ B. velezensis strain BB	BC	88.7±2.2ab
B. amyloliquefaciens/ B. siamensis/ B. velezensis strain KG	CE	93.9±1.6 a
B. amyloliquefaciens/ B. siamensis/ B. velezensis strain KG	CF	38.2±5.6 c
B. amyloliquefaciens/ B. siamensis/ B. velezensis strain KG	BC	83.8±2.7 ab
B. amyloliquefaciens/ B. siamensis/ B. velezensis strain KI	CE	95.7±0.6 a
B. amyloliquefaciens/ B. siamensis/ B. velezensis strain KI	CF	37.4±6.8 c
B. amyloliquefaciens/ B. siamensis/ B. velezensis strain	BC	82.5±3.6 ab
Control (Distilled water)	Control	100±0 a

Effects	**F-value**	**P-value**	**Significance**	**Description**
Strain	1.66	0.20	NS	Not significant
Treatment	25.13	0.0001	***	Highly significant
Strain*Treatment	1.01	0.41	NS	Not significant
%CV = 28.1				

*Note: CF- culture filtrate, BC- bacterial cells and CE- crude lipopeptides extract

In the short-term trial, the culture filtrate treatment on the Akwa cultivar seeds was the overall best treatment. *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strain KI demonstrated a low percentage infection of 37.4%, followed by *Bacillus amyloliquefaciens/ B. siamensis/ B. velezensis* strain KG and *B. amyloliquefaciens/ B. subtilis/ B. velezensis* strain BB. The crude lipopeptide extract treatment provided little to no protection against *A. flavus* and the results were not significantly different for all

three strains and the control (Table 3.1). No significant differences were found between the three bacterial strains used. However, there was a highly significant difference between the various treatments applied to the groundnut seeds (P = 0.0001). Moreover, no significant interactions were observed between the different bacterial strains and the treatments applied (P = 0.41) (Table 3.1).

Table 3.4: Short-term storage trial of groundnut seeds Sellie Plus cultivar treated *B. amyloliquefaciens, B. siamensis, B. velezensis* and/or *B. subtilis* bacterial cells, crude lipopeptide extract and cell-free culture filtrate.

Strain	Treatment	%Infection±SE
Bs amyloliquefaciens/ B. subtilis/ B. velezensis strain BB	CE	100±0 a
B. amyloliquefaciens/ B. subtilis/ B. velezensis strain BB	CF	62.2±5.8 b
B. amyloliquefaciens/ B. subtilis/ B. velezensis strain BB	BC	93.1±1.3 a
B. amyloliquefaciens/ B. siamensis/ B. velezensis strain KG	CE	80.4±4.2 ab
B. amyloliquefaciens/ B. siamensis/ B. velezensis strain KG	CF	33.0±4.7 c
B. amyloliquefaciens/ B. siamensis/ B. velezensis strain KG	BC	84.7±2.2 ab
B. amyloliquefaciens/ B. siamensis/ B. velezensis strain KI	CE	83.4±4.5 ab
B. amyloliquefaciens/ B. siamensis/ B. velezensis strain KI	CF	10.8±1.4 d
B.amyloliquefaciens/ B. siamensis/ B. velezensis strain	BC	100±0 a
Control (Distilled water)	Control	100±0 a

Effects	**F-value**	**P-value**	**Significance**	**Description**
Strains	5.77	0.006	***	Highly Significant
Treatment	44.93	0.0001	***	Highly Significant
Strains*Treatment	3.29	0.02	**	Significant
		%CV = 25.5		

*Note that: CF- culture filtrate, BC- bacterial cells and CE- crude extract

In the short-term trial, the culture filtrate was the overall best treatment and *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strain KI demonstrated a low percentage infection of 10.8% followed by *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strain KG and *B. amyloliquefaciens/ B. subtilis/ B. velezensis* strain BB. There was a highly significant difference between the bacterial strains used (P = 0.006). A high significant difference was also observed among the different treatments applied (P = 0.0001). As was the case for cultivar Akwa, the crude lipopeptide extract treatment provided little to no protection against *A. flavus* and was not significantly different for all three strains and the control (Table 3.2). The interactions between the three bacterial strains and the treatments applied was significant (P = 0.02) (Table 3.2). All three treatments (CE, CF, and BC) demonstrated the same trend within the two cultivars (Figure 3.1). For both cultivars, the bacterial cells and crude lipopeptide extract treatments for all three bacterial strains showed no significant difference between each other and the control. The percentage infection was recorded to be lowest for all three culture filtrate treatments for all three bacterial strains and highest for all three crude lipopeptide extract treatments for all three bacterial strains (Tables 3.1, 3.2 and Figure 3.1).

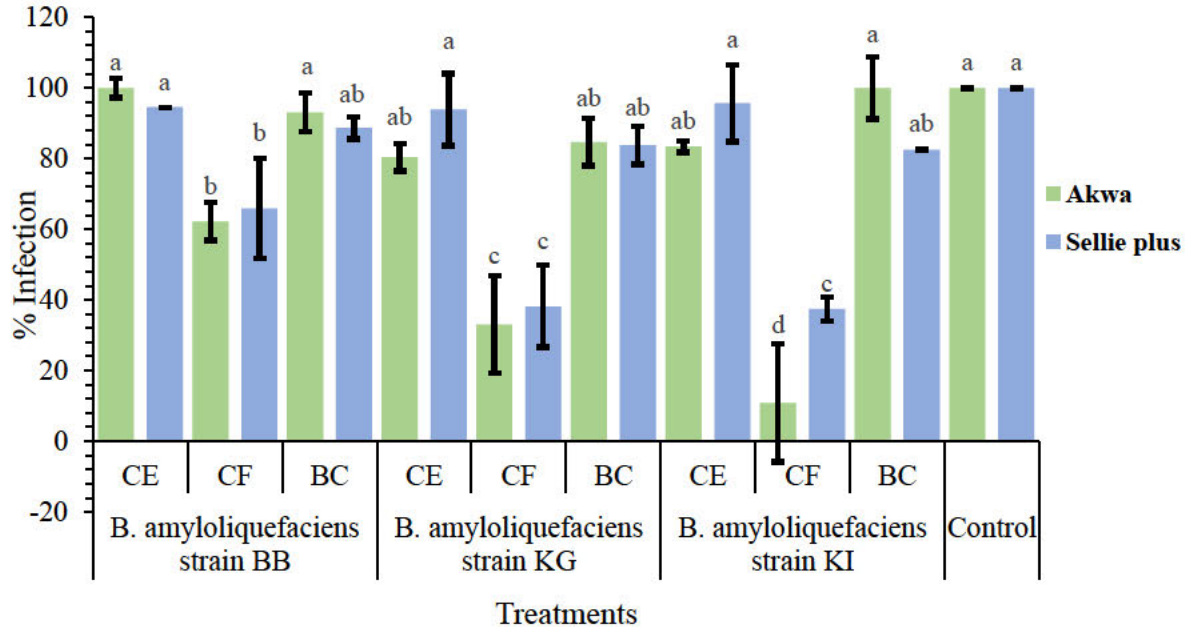

Figure 3.1: Efficacy of three *B. amyloliquefaciens/ B. subtilis/ B. velezensis/B. siamensis* strains treatments (crude lipopeptide extract (CE), cultured filtrate (CF) and bacterial cells (BC) on the control of *A. flavus* under storage conditions.

3.3.2 Medium-term storage control of *A. flavus* using a pH-adjusted *B. amyloliquefaciens/ B. subtilis/ B. velezensis* culture filtrate

The efficacy of the pH adjusted culture filtrate (pH 2.1 and 7.4) of *B. amyloliquefaciens/ B. subtilis/ B. velezensis* strain KI and *B. amyloliquefaciens/ B. subtilis/ B. velezensis* strain KG was evaluated

against *A. flavus* over a period of 21 days on the two groundnut cultivars. Tables 3.3 and 3.4 show the summary of the percentage infection on groundnut seeds (Akwa and Sellie Plus cultivars) after being treated with the culture filtrate adjusted at different pH. After 7 days, the best treatment on the Akwa cultivar was the alkaline (pH 7.4) culture filtrate of *B. amyloliquefaciens/ B. subtilis/ B. velezensis* strain KG.

Table 3.3: Medium-term storage trial of groundnut seeds Akwa cultivar treated with a pH-adjusted culture filtrate of the two *B. amyloliquefaciens/ B. subtilis/ B. velezensis* strains for a period of 21 days.

Treatments	**Culture filtrate pH**	**Time (Days) and Percentage infection±SE**		
		Day 7	**Day 14**	**Day 21**
B. amyloliquefaciens/ B. subtilis/ B. velezensis strain KI	Acidic (pH 2.1)	24.5±2.9 a	73.4±5.1 ab	95.2±1.7 ab
B. amyloliquefaciens/ B. subtilis/ B. velezensis strain KI	Alkaline (pH 7.4)	20.8±3.3 bc	37.7±10.9 b	100±0 a
B. amyloliquefaciens/ B. subtilis/ B. velezensis strain KG	Acidic (pH 2.1)	14.5±1.8 bc	49.3±6.8 ab	91.1±1.4 b
B. amyloliquefaciens/ B. subtilis/ B. velezensis strain KG	Alkaline (pH 7.4)	8.4±1.8 c	64.5±5.5 ab	93.3±2.1 ab
Control		81.6±0.8 a	88.4±2.2 a	100±0 a
F-ratio		54.01	2.91	2.84
P-value		0.0001	0.08	0.08
%CV		23.23	32.29	4.30

After 14 days, the best treatment was the alkaline (pH 7.4) culture filtrate of *B. amyloliquefaciens/ B. subtilis/ B. velezensis* strain KI (37.7%). All treatments of *B. amyloliquefaciens/ B. subtilis/ B. velezensis* strains had percentage infections above 90% after 21 days. The control treatment was completely infected (100%) after 21 days. The infection levels were significant on Day 7 (P = 0.0001) but not significant on Days 14 and 21 respectively (Table 3.3).

Table 3.4: Medium-term storage trial of groundnut seeds Sellie Plus cultivar treated with a pH-adjusted culture filtrate of the two *B. amyloliquefaciens/ B. subtilis/ B. velezensis* strains for a period of 21 days.

Treatments	**Culture filtrate pH**	**Time (Days) and Percentage infection±SE**		
		Day 7	**Day 14**	**Day 21**
B. amyloliquefaciens/ B. subtilis/ B. velezensis strain KI	Acidic (pH 2.1)	13.1±1.5 b	58.9±4.7 b	100±0 a
B. amyloliquefaciens/ B. subtilis/ B. velezensis strain KI	Alkaline (pH 7.4)	4±1.4 b	27.9±3.7 c	92.3±1.8 a
B. amyloliquefaciens/ B. subtilis/ B. velezensis strain KG	Acidic (pH 2.1)	23.6±6.8 b	29.6±4.8 c	77.6±3.7 b
B. amyloliquefaciens/ B. subtilis/ B. velezensis strain KG	Alkaline (pH 7.4)	4±1.4 b	30.3±1.1 c	95.6±0.9 a
Control		83.4±2.9 a	87.7±1 a	100±0 a
F-ratio		29.9	18.7	8.4
P-value		0.0001	0.0001	0.0031
%CV		41.21	22.38	5.94

After 7 days, the best treatment on the Sellie Plus cultivar was the alkaline (pH 7.4) culture filtrate of both *B. amyloliquefaciens* strains KG and KI. After 14 days, all four treatment lost the antifungal activity against *A. flavus*. The acidic (pH 2.1) culture filtrate of *B. amyloliquefaciens/ B. subtilis/ B. velezensis* strain KG showed to have its antifungal activity until Day 21 when compared to the acidic and alkaline culture filtrate of strain *B. amyloliquefaciens/ B. subtilis/ B. velezensis* KI, and alkaline culture filtrate of strain *B. amyloliquefaciens/ B. subtilis/ B. velezensis* KG on both cultivars. All treatments of *B. amyloliquefaciens/ B. subtilis/ B. velezensis* strains had percentage infections above 90% after 21 days except for the acidic culture filtrate of *B. amyloliquefaciens/ B. subtilis/ B. velezensis* strain KG. The infections levels were significant on Day 7 ($P = 0.0001$), Day 14 ($P = 0.0001$) and Day 21 ($P = 0.0031$) for all three bacterial strains and the two pH-adjusted culture filtrates (Table 3.4).

3.3.3 Scanning Electron Microscopy (SEM)

A. flavus was culture on PDA alongside each of the three *B. amyloliquefaciens/ B. siamensis/B. subtilis/ B. velezensis* strains and the effects on mycelial and conidia morphology of *A. flavus* were observed under SEM. In the untreated controls (*A. flavus* only), the development of mycelium and conidiophore was normal with abundant conidia (Figures 3.2B, 3.3D, and 3.4F). While in the dual culture assay plate, the development of *A. flavus* was suppressed. Under SEM, *A. flavus* growth was visibly irregular,

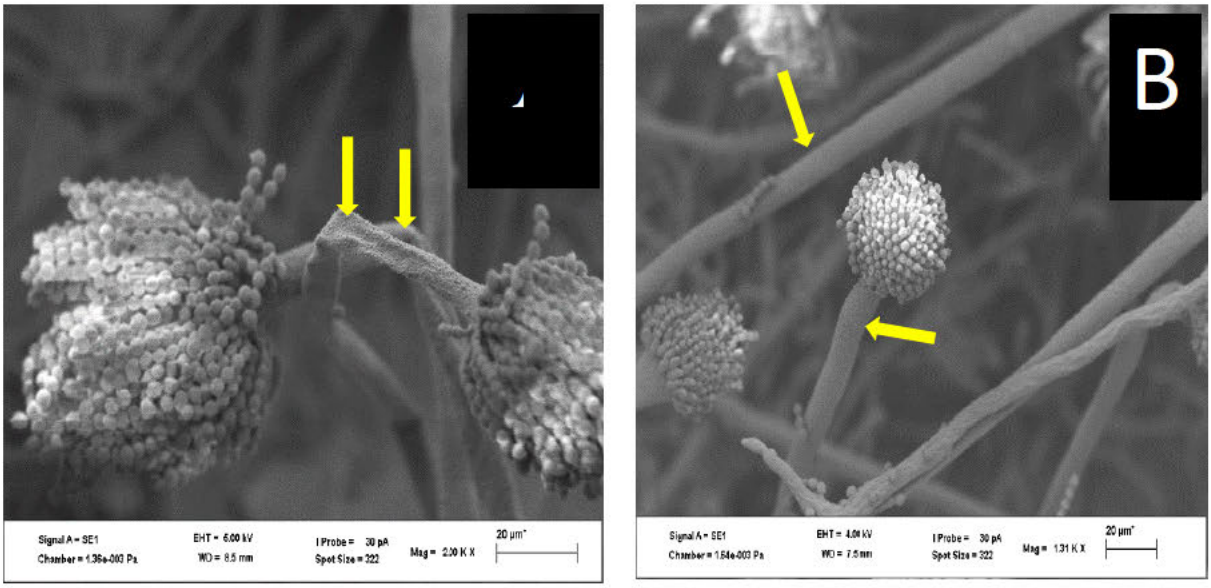

Figure 3.2: Scanning electron micrograph showing a shrivelled hyphae (arrowed) (A) of *A. flavus on* PDA growing alongside *B. amyloliquefaciens/* B. *subtilis/* B. *velezensis* strain BB, sampling site compared to the healthy one (arrowed) (B) that was grown alone on PDA.

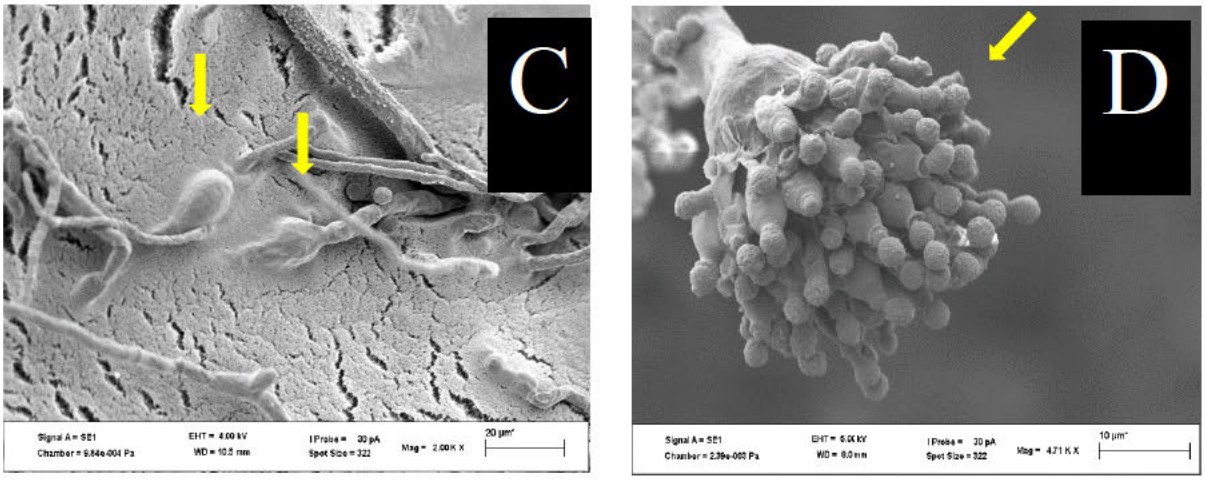

Figure 3.3: Scanning electron micrograph showing vesicle with no conidia formation (arrowed) (C) of *A. flavus* on PDA growing alongside *B. amyloliquefaciens/* B. *siamensis/* B. *velezensis* strain KG, sampling site compared to the healthy conidia forming one (arrowed) (D) that was grown alone on

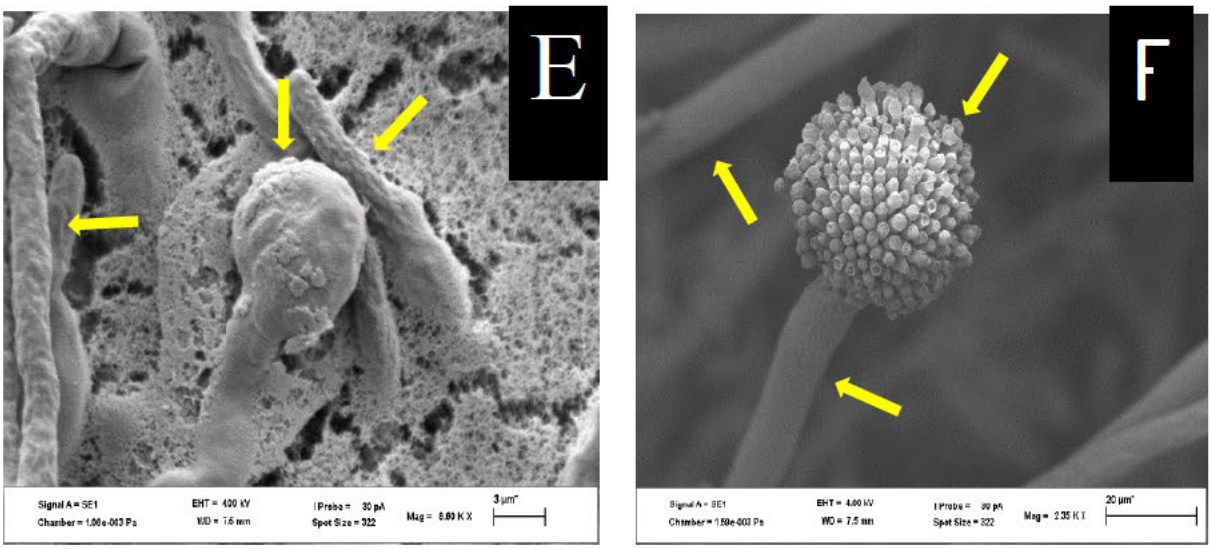

Figure 3.4: Scanning electron micrograph showing a shrivelled vesicle/metulae and hyphae (arrowed) (E) of *A. flavus* on PDA growing alongside *B. amyloliquefaciens*/ B. *siamensis*/ B. *velezensis* strain KI, sampling site compared to the healthy one with fully grown phialides and hyphae (arrowed) (F) that where mycelia and vesicles were shrivelled compared to untreated controls (Figures 3.2A, 3.3C, and 3.4E).

3.4 Discussion

This study aimed to control *A. flavus* infections on two groundnut seed during short-term and medium-term storage trials, using three different strains of *B. amyloliquefaciens*. From the three treatments; bacterial cells, culture filtrate and crude lipopeptide extract, the culture filtrate was the best treatment from all three *B. amyloliquefaciens/ B. siamensis/ B. velezensis/ B. subtilis* strains (BB, KI and KG) in both groundnut cultivars. The culture filtrate of *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strains KI and KG showed great potential as a biological agent *in vivo* and was used for a prolonged 21 day storage trial. The acidic culture filtrate of *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strain KG showed a strong antifungal activity towards *A. flavus*.

Endophytic bacteria have been shown to have antifungal activity against plant pathogens (Kgosi *et al.*, 2022). The effect of each of the three strains to inhibit *A. flavus* growth was studied using scanning electron microscopy. The morphological changes, including degradation and shrivelling of mycelia, vesicles, and the inability of the fungus to produce spores demonstrated the presence of antifungal compounds. *B. amyloliquefaciens* produces antifungal compounds comprising surfactin, iturin, and fengycin, which are considered the main compounds against a list of plant pathogens (Li *et al.*, 2019, Jiao *et al.*, 2021). Surfactin interferes with the colonization process of the pathogen by interacting with the biological membranes to induce structural modifications (Stoll *et al.*, 2021). The mode of action used by iturins and fengycins is still partially understood, but it is believed that their ability to form pores in the plasma membranes of fungal pathogens results in cell death (Zakharova *et al.*, 2019).

Dimkić *et al.* (2013) also concurred that iturins and fengycins from *B. amyloliquefaciens* strains SS-12.6 and SS-13.1 have antifungal abilities against *A. flavus*. In hindsight, the production of antifungal metabolites, and competition for space and nutrition seem to be the possible mechanisms of antagonistic activity of bacterial strains *in vivo* (Kong et al., 2010) (Kumar *et al.*, 2014). In the short-term storage trial, the culture filtrate was the best treatment for all strains, whereas the crude lipopeptide extract showed the least control. Thus, it can be concluded that the cells and crude lipopeptide extract treatments had some inhibitory effect on *A. flavus*, but the culture filtrate were more strongly inhibitory to *A. flavus.* A study by Yoshida *et al.* (2001) also found that *B. amyloliquefaciens* culture filtrate completely inhibited postharvest *Colletotrichum dematium* (Persoon: Fries) Grove symptoms on *Morus nigra* L. (mulberry) leaves. The inability of the crude lipopetide extract treatment to control *A. flavus in vivo* may be due to the solvent used which in this study was methanol. In a study by Jiao *et* al. (2021), where n-butanol was used as precipitating solvent for the crude lipopeptide extract, the crude lipopeptide extract showed to be a good control. A study done by Yokota *et al.* (2012) also compared the butanol precipitation and methanol extraction, where butanol precipitation was able to quantify the amount of iturin from the culture filtrate of *Bacillus* spp. compared to methanol extraction. This is because methanol is much more corrosive than n-butanol, therefore the antimicrobial compound structures of one or more compounds might have been degraded during the precipitation process. It should also be noted that for pathogens to be inhibited, antimicrobial substances do not act alone but act synergistically to produce antimicrobial effects (Carrión *et al.*, 2019, Duan *et al.*, 2021). For example, the combination of surfactin and fengycin control *Phytophthora infestans* (Wang *et al.*, 2020) and *Fusarium oxysporum* f. sp. *iridacearum (*Mihalache *et al., 2018)*. Therefore, the co-production of two antifungal compounds by *Bacillus* spp. improves the antifungal effects on pathogens. The treatments were demonstrated to be cultivar dependent. Even though the culture filtrate was a better control on both cultivars, bacterial cells was a much better treatment on Akwa cultivar compared to Sellie Plus. The culture filtrate proved to be a potential control option in the initial trial as it had several antifungal compounds. However, for an extended period, biological control agent treatments tend to lose efficacy when exposed to different environmental conditions such as pH. Therefore, in the medium-term storage trial, the most desirable pH of the potential culture filtrate was also assessed. This was done by adjusting the pH to either acidic (pH 2.1) or alkaline (pH 7.4). Both the alkaline and acidic culture filtrate treatments demonstrated good antifungal activity against *A. flavus* until day 14 for both cultivars when compared to the controls. This is because strains of *Bacillus* spp. grow at a faster rate than *Aspergillus* spp. during the first step of co-incubation (Siahmoshteh *et al.*, 2017). At day 21, all treatments were unable to control *A. flavus* except the acidic cultured filtrate of *B. amyloliquefaciens* strain KG which was better. The ability of the acidic culture filtrate of strain KG to inhibit *A. flavus* for an extended time, suggests that there are antifungal compounds present that display a preventative effect on the pathogen. These findings were also demonstrated by a study done by Kgosi *et al*. (2022), where the culture filtrate antifungal activity of

B. subtilis strain DL76 increased when the culture filtrate was acidic, and the suppression activity decreased with increased alkalinity.

The groundnuts in this study regardless of the cultivar, treatment, or strain demonstrated a high percentage of infection than normal. This is because a high concentration of conidia per milliliter ($1x10^6$) used is could be potentially more than the average concentration of conidia found in the environment that would cause natural infestation. In this study, the acidic culture filtrate of strain KG demonstrated its potential as a storage biocontrol control option. This validifies that *B. amyloliquefaciens* as a gram positive bacteria has excellent potential as a storage BCA as their spores are resistant to most harsh environmental conditions (heat and desiccation) (Ugoji *et al*., 2006). Therefore, they can be formulated as a dry powder to keep them viable when stored for a long time (Lastochkina *et al*., 2019).

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Chapter 4

Greenhouse testing of two endophytic bacterial isolates as potential biocontrol agents against *Aspergillus flavus* Link in groundnuts

Abstract

In this study, the efficacy of two bacterial isolates viz: *Bacillus amyloliquefaciens/ Bacillus siamensis/ Bacillus velezensis* strain KG and *Pseudomonas fluorescens* strain ALA were evaluated against *A. flavus* infections in groundnuts under greenhouse conditions. Two groundnut cultivars, Akwa and Selli-Plus were used. Treatments were: (i) seed treatment, (ii) foliar spray treatment and (iii) a combination of both seed and foliar spray treatment. All plants were fertilized with Osmocote Exact Mini 5-6M slow-release fertilizer once two weeks after germination. The foliar spray treatment was applied once at four weeks to designated plants. Two *A. flavus* colonized barley seeds were used to inoculate each plant three weeks before harvest. The results indicated that both cultivars responded differently to inoculations with the two bacterial biological control agents. On Sellie-Plus, the highest number of seeds and seed weight was recorded for the seed treatment. In contrast, in Akwa, the combination of seed and foliar spray treatment recorded the highest number of seeds and seed weight. The combination of seed treatment and foliar spray treatment was the best against *A. flavus*, with an average percentage disease incidence of 54.8% and 46.8% on Akwa and Sellie-Plus, respectively. This combination suggests that the best and the most appropriate way to use these two organisms is through a combination of seed treatment and foliar spray to effectively manage *A. flavus* infections in groundnuts.

Keywords: *Pseudomonas fluorescens*, seed treatment, drought, mycotoxin

4.1 Introduction

The exploration and application of plant growth-promoting bacteria has become a focused area of research as an alternative to chemical control. As the soil is rich in beneficial bacteria, including *Bacillus* and *Pseudomonas* species (Xu *et al*., 2021), these species are said to improve plant growth, seed germination, and overall plant biomass. Moreover, they have also been proven to be effective in managing various plant diseases. Plant bacterial endophytes have been used to control various fungal and bacterial diseases. As the host plant strictly limits the growth of the endophytes, they adapt to the new living environment by using many mechanisms that benefit both the plant and the endophytes (Nair and Padmavathy, 2014). This makes it possible for these organisms to safeguard/biologically control newly introduced pathogens. A study by Sahib *et al.* (2020) suggested that a dense and rhizobacterial diversity may improve soil-plant interactions and total crop productivity under constrained environmental conditions. This indicates that using endophytic bacteria for abiotic disease control is possible.

Pre- and post-harvest infection by *A. flavus* during hot and dry environmental conditions has been tagged as an undeniable production problem for groundnuts (*Arachis hypogaea* L.). As *A. flavus* overwinters in the soil and plant matter, seed colonization by the pathogen is most significant since aflatoxins may be synthesized in the seeds (Mickler *et al.*, 1995, Pandey *et al*., 2019). As groundnut production occurs during warm weather, the combination of heat and water stress further increases disease infections. The potential for biological control of pre-harvest aflatoxin contamination of groundnuts has been demonstrated using biological control agents. However, limited information is available on using bacterial endophytes from groundnut-related plants as biological control agents against groundnut-related diseases.

Few research journals have reported on the use of plant endophytes as biological control under controlled greenhouse conditions and field conditions (Ziedan, 2006). This research chapter evaluated two endophytic bacterial isolates (*Bacillus amyloliquefaciens* strain KG and *Pseudomonas fluorescens* strain ALA) for their ability to reduce infections caused by *A. flavus* in groundnuts under greenhouse conditions. Two South African groundnut varieties, Akwa and Selli-Plus were used as test varieties for this study.

4.2 Materials and methods

4.2.1 Groundnut cultivars

Certified seeds of two groundnut cultivars, Selli-Plus and Akwa, were used for this study. The seeds were donated by Vaalharts Groundnuts Marketing CC, Plot 2E5, Hartswater, 8570, South Africa. Cultivar Selli-Plus has a growth period of 155 days and shows good resistance to pod worms, blackened pods, *Botrytis* stem rot, and reasonable resistance to *Sclerotinia*. Cultivar Akwa has a growth

period of 150 days and shows good resistance to pod worms, blackened pods, *Sclerotinia*, and poor resistance to *Botrytis* stem rot. The seeds were stored in a cool and dry room until further use.

4.2.2 *Aspergillus flavus* Link inoculum preparation

A.flavus mycelium of 1x1 cm^2 square plugs was subcultured on fresh potato dextrose agar (PDA) and incubated at 28^{o}C for seven days. The *A. flavus* inoculum for greenhouse trials was prepared by weighing 150g of barley seeds into a 500ml Erlenmeyer flask and soaked in 350ml distilled water to cover the barley seeds. This was left to stand for 24 hours at ambient temperature in the laboratory. Thereafter, the excess water was drained off, and the flask was covered with a cotton plug and aluminium foil. The contents were sterilized in an autoclaved at 121^{o}C for 15 minutes twice at 24-hour intervals. A PDA plate containing actively growing *A. flavus* was cut into 1x1cm^2 blocks, and ten blocks were added into the flask, mixed with the autoclaved barley seeds and incubated at 28^{o}C. The pathogen was allowed to colonize the seeds for a period of 7 days and air-dried under the laminar flow for 72 hours. The colonized seeds were stored in McCartney bottles and maintained at ambient temperature in the laboratory for later use.

4.2.3 Study site

This study was carried out at the experimental greenhouses at the University of KwaZulu-Natal, Agriculture Campus, Pietermaritzburg, South Africa. Daily temperatures and percent humidity were ambient, as no particular temperature and humidity settings were used. A five-minute drip irrigation system was used after groundnut seed germination in two daily irrigation cycles.

4.2.4 Preparation of endophytic biological control agents (BCAs) treatments (Seed treatment and Foliar spray treatment)

4.2.4.1 Seed treatment

Endophytic bacteria, *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strain KG previously stored in 2µ*l* of 30% glycerol solution, was sub-cultured onto potato dextrose agar (PDA) plates and incubated for 48 hours at 28^{o}C. Ten loopfuls of *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strain KG were mixed with 2% (w/v) sterile carboxymethyl cellulose (CMC) sticker suspensions (20 mL) in a 100 mL glass beaker to form a slurry. Sterile distilled water was used for the CMC sticker suspensions. Seeds of the two groundnut cultivars were separately surface sterilised with 5% sodium hypochlorite (commercial JIK) for 60 seconds, then in ethanol for a further 60 seconds, and two one-minute washes with sterile distilled water. These were then air-dried on a sterile paper towel under the laminar flow for two hours. 25 groundnut seeds per cultivar were then added to the slurry suspension, mixed, and allowed to soak for 30 minutes. The treated seeds were placed in sterile 90 mm Petri dishes and air-dried on a laminar flow bench for two hours at ambient temperature before planting.

4.2.4.2 Foliar treatment

Endophytic bacteria, *Pseudomonas fluorescens* strain ALA previously stored in 2µ*l* of 30% glycerol solution, was sub-cultured onto 15 Petri-dishes containing potato dextrose agar (PDA) media and incubated for 48 hours at 28ºC. A 250 ml bacterial suspension was prepared from the culture plates with sterile distilled water. A serial dilution of $1x10^{-1}$ to $1x10^{-8}$ was prepared from the bacterial suspension. The $1x10^{-4}$ to $1x10^{-8}$ dilutions were plated on PDA to determine the colony-forming units (CFUs). The final bacterial suspension was adjusted to 10^8 cells ml^{-1} where necessary.

4.2.5 Greenhouse trial

The first trials were established in mid-August (winter) and were run until early January (summer) 2022. The repeated trial (second trial) was set up two weeks after the first trial. The experiment was arranged in a randomized complete blocks design with three bacterial treatments and control: (i) seed treatment, (ii) foliar spray treatment, and (iii) a combination of seed treatment and foliar spray. The control treatment had groundnut seeds treated with CMC sticker solution only in order to unify all treatments. The treated groundnut seeds were planted into 25 cm diameter pots (approximately 2.3 L volume) filled with commercially prepared composted pine bark growing medium (Organic for Africa, Greytown, South Africa). Three seeds were planted per pot per cultivar, with five replicates per treatment. The trial in the greenhouse was repeated once. Each pot was hand watered with a watering can from the first day of planting until germination. The plants were drip-irrigated twice a day for 5 minutes per cycle.

Two weeks after germination, Osmocote Exact Mini 5-6 M 15-3.9-9.1 + 1.2 Mg + TE [supplied by Greenhouse Products (Pty) Ltd, Helderkruin, Republic of South Africa (RSA)], an ammonium-based slow release fertilizer, was applied in each pot at a rate of 3 gL^{-1} of potting media. The foliar spray treatment was applied on designated plants four weeks after planting (after the development of true leaves), ensuring that the growing media was not drenched with the bacterial spray suspension (Figure 4.1). Three weeks (21 days) before harvest, two *A. flavus* colonized barley seeds were inoculated per plant, and the irrigation was stopped to subject the plants to water stress three weeks before harvest.The plants were harvested 150 days after planting. The following parameters were evaluated: (i) number of pods, (ii) number of seeds, (iii) seed weight, and (iv) disease incidence.

Figure 4.1: A cross section of the greenhouse trial ready for foliar spray treatment. The paper towels were used to absorb any bacterial suspension droplets.

4.2.6 Data analysis

A general linear model (GLM) was used to run an ANOVA on the number of pods, number of seeds, seed weight and disease incidence data. The data for the first and repeated experiments were merged before the analysis. All the data were arcsine transformed before analysis. If the ANOVA was significant ($P < 0.05$), the means were separated using Duncan's multiple range test (DMRT) at a 5% significance level using SAS (Version 9.4).

4.3 Results

4.3.1 Number of pods, number of seeds, seed weight and disease incidence

The plants did not present any visible symptoms of *A. flavus* after inoculation with *A. flavus* and before drought stress. However, during harvest the seeds for both cultivars demonstrated severe infection with yellow mould, more-so in control plants roots and pods (Figure 4.2). Table 4.1 shows the summary of the number of pods, number of seeds, mean seed weight and overall percentage disease incidence from harvested groundnut seeds for both cultivars, Akwa and Sellie-Plus. At harvest, the highest number of pods was recorded for seed treatment for both cultivars. However, Sellie-Plus had the highest mean number of pods per plant (20 pods). On Sellie-Plus, the highest number of seeds (22 seeds) was recorded for the seed treatment. In contrast, in Akwa, the combination of seed and foliar spray treatments recorded the highest number of seeds. The highest seed weight (12.5g) was recorded in the seed treatment for Sellie-Plus, and for the Akwa cultivar, the combination of seed and foliar spray treatment had the highest seed weight (12.0g). The lowest case of percentage disease disease incidence was reported on the combination of both seed treatment and foliar spraying of Sellie-Plus(46.8). In Akwa seeds, both the foliar spraying treatment and the combination of both seed treatment

demonstrated a low percentage incidence. These treatment demonstrated no significant difference. The highest percentage disease incidence was recorded in control treatment. A high percentage co-efficient of variation (45.8-54.8%) demonstrated a high variation within the experinment which resulted in the lack of statical difference.

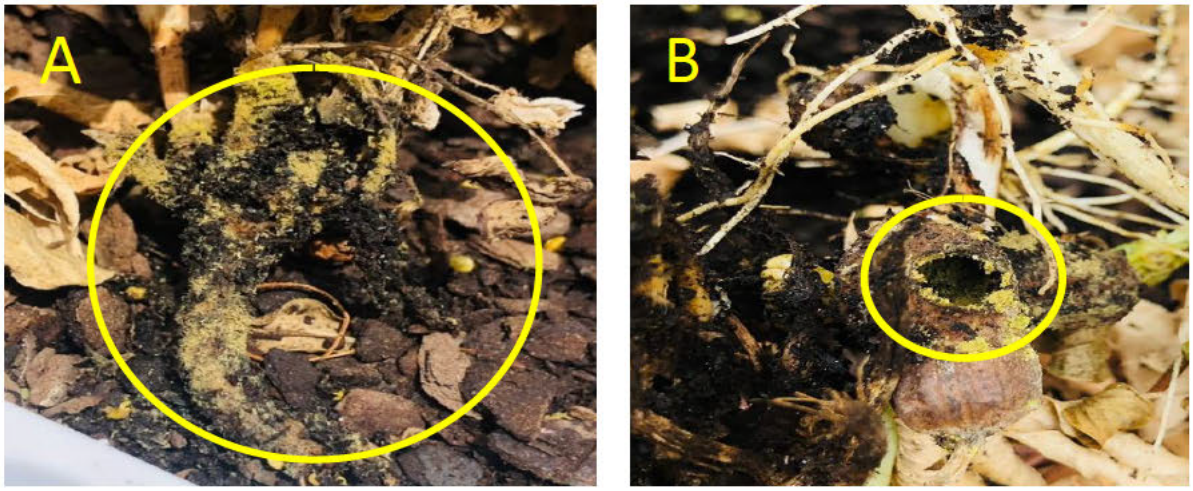

Figure 4.2: The root (A-circled) and seed (B- circled) of the control treatment of Akwa (A) and Sellie-Plus (B) groundnut cultivar at harvest, completely colonized by *A. flavus*.

Table 4.1: Efficacy of *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strain KG as seed treatment and *P. fluorescens* strain ALA as foliar spray treatment, and the combination of both seed treatment and foliar spray in managing *A. flavus* infections under greenhouse conditions.

Treatments	Cultivar	Treatment type	Mean no. of pods	Mean no. of seeds	Mean Seed weight (g)	%Disease Incidence
B. amyloliquefaciens/ B. siamensis/ B. velezensis strain KG	Akwa	Seed treatment	14(8)±0.9 a	15(9)±1.1 a	9.9(4.3)±0.7 a	82.6(93.5)±1.5 ab
P. fluoresens strain ALA	Akwa	Foliar spray	15(8)±0.5 a	17(8)±0.4 a	11.0(3.7)±0.2 a	54.0(60.0)±4.1 bc
B. amyloliquefaciens/ B. siamensis/ B. velezensis strain KG + *P. fluoresens* strain ALA	Akwa	Seed treatment + Foliar spray	16(10)±0.9 a	18(12)±1.1 a	12.0(5.7)±0.7 a	54.8(63.2)±3.4 bc
A. flavus inoculated control	Akwa		6(3)±0.8 b	6(3)±0.9 b	4.2(1.5)±0.6 b	90.0(100)±1.5 $^{-15}$
B. amyloliquefaciens/ B. siamensis/ B. velezensis strain KG	Sellie Plus	Seed treatment	20(13)±0.7 a	22(16)±1.0 a	12.5(5.4)±0.5 a	66.53(73.3)±3.9 abc
P. fluoresens strain ALA	Sellie Plus	Foliar spray	17(10)±0.9 a	18(11)± 1.0a	10.2(4.0)±0.6 a	61.3(69.1)± 4.3abc
B. amyloliquefaciens/ B. siamensis/ B. velezensis strain KG + *P. fluoresens* strain ALA	Sellie Plus	Seed treatment + Foliar spray	14(7)±0.8 a	15(8)±0.8 a	8.7(2.8)±0.5 ab	46.8(53.0)±3.7 c
A. flavus inoculated control	Sellie Plus		15(7)±0.3 a	17(9)±0.5 a	10.9(3.7)±0.3 a	90.0(100)±1.5 $^{-15}$ a
F-value			2.9	2.6	2.3	2.99
P-value			0.01	0.02	0.04	0.01
%CV			52.5	54.5	54.8	45.8

Note: The values presented are arcsine transformed values.
Means followed by the same letter are not significantly different ($p<0.05$) according to DMRT.
The original value are in brackets

4.4 Discussion

This study aimed to control *A. flavus* infections on seeds of two groundnut cultivars (Akwa and Selli-Plus) during greenhouse trials, using two different endophytic bacterial strains as a seed treatment (*B. amyloliquefaciens* strain KG) and foliar spray treatment (*P. flourescens* strain ALA). In all three treatments, seed treatment, foliar spray, and the combination of seed treatment and foliar spray, the evaluated parameters were mostly not significantly different ($p > 0.05$) from each other in both cultivars.

In the greenhouse trial, the plants showed no symptoms of *A. flavus* after inoculation till harvest. This is because plants colonized by this pathogen do not show any visible foliar symptoms except for the

accumulation of green/yellow powdery spores on the pods and/or seeds (Pandey *et al*., 2019). None of the treatments showed an increase in the total yield of pods/seeds. This may be because the concentration of *A. flavus* was high in the geocarposphere. Media inoculation with *A. flavus*-infested barley seeds was done before subjecting the plant to water stress. The moisture in the media allowed rapid growth of the pathogen (Hassane *et al.*, 2017). Abiotic stress in the form of drought also increased the chances of *A. flavus* contamination in the soil before harvest (Pandey *et al*., 2019). This is because drought weakens the plant's defence mechanism and benefits the growth of *A. flavus* and the subsequent production of aflatoxin (Jeyaramraja *et al.*, 2018). The number of seeds per pod follows the two seed in one pod ratio (2:1) for the majority, if not all, groundnuts, including both cultivars Akwa and Sellie-Plus at harvest. This ratio provides the number of seeds expected per pod per cultivar and the overall expected vegetable oil per hectare in a production cycle. However, in this study, most pods in all three treatments did not follow the ratio because of the complete colonization of pods by *A. flavus*. This is because draught also results in pod injury, allowing the fungi to enter and infect the seeds (Okello *et al*., 2010). At harvest, a significantly high concentration of *A. flavus* was seen in the pod zone. The seed and foliar spray treatments independently were less effective in controlling *A. flavus*. A study by Nahdi (2002) also found a high population of *A. flavus* in the pod zone of groundnuts which increased with the maturity of the crop in the soil.

The presence of endophytic bacteria as biological control agents did demonstrate a difference in disease incidence. This is observed when comparing the percentage disease incidence of all three treatments and that of the controls per cultivar. The combination of *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strain KG and *Pseudomonas fluorescens* strain ALA as seed treatment and foliar spray on Sellie-Plus showed potential in managing *A. flavus* infections. *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strain KG was isolated from groundnut seeds and was also used as a seed treatment to enhance the protection against *A. flavus*. The ability of *Bacillus* spp. to biologically control pathogens is due to the biosynthesis of antifungal polypeptides such as proteins, surfactin, iturins and chitinase (Qin *et al.*, 2015, Yan *et al.*, 2018). *B. amyliloquefaciens* does not only inhibit pathogen growth but also promotes plant growth (Wang *et al.*, 2017) as it can survive on plant roots (Reva *et al.*, 2004). *Pseudomonas fluorescens* strain ALA was isolated from dry bean leaves and was used as foliar spray treatment in this study. *P. fluorescens* strains have widely been used as plant growth-promoting rhizobacteria (PGPR), as they are abundant in the rhizosphere (Sivasakthi *et al.*, 2014, David *et al.*, 2018). *Pseudomonas* spp. isolated from peanut root apoplast, and nodules have been reported by Dudeja *et al.* (2012) to enhance plant yield. In this study, *P. fluorescens*, strain ALA in combination with *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strain KG demonstrated the potential to control *A. flavus* on both cultivars. This is because *P. fluorescens* has been reported to produce siderophores and proteases. This could contribute to its antagonistic effect against fungi (Chang *et al.*, 2011). Meena

et al. (2000) found that foliar application of *P. fluorescens* strain Pf1 significantly controlled *Puccinia arachidis* on groundnuts under greenhouse conditions.

In Chapter 2, both bacterial strains demonstrated great potential in controlling *A. flavus in vitro*. Again, the supernatant of *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strain KG demonstrated potential against *A. flavus* on groundnut seeds. During the greenhouse trial *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strain KG as seed treatment was unable to give enhanced protection against the *A. flavus*. This may be because the concentration of the bacterium was too low to withstand a high disease pressure as a result of the high concentration of *A. flavus*. The combination of both strains suggests a synergistic/additive effect for an effective *A. flavus* control.

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DISSERTATION OVERVIEW OF THE MAJOR RESEARCH FINDINGS AND THEIR IMPLICATIONS.

The importance of beneficial microorganisms in promoting plant growth and controlling plant diseases has been focused in academic research (Ali and Xie, 2020, Chakraborty and Akhtar, 2021). *Bacillus*, *Pseudomonas*, and *Pantoea* species are among the most beneficial microorganisms studied for their role in plant growth and disease control (Jiang *et al.*, 2019, Kamble *et al.*, 2020, Eid *et al.*, 2021). These genera have also been studied for their association with plant species, including groundnuts (*Arachis hypogaea* L.) as endophytes (Khan, 2019, Preyanga *et al.*, 2021). Endophytes are microorganisms found or residing within the plant without causing any negative impact on host plants (Kandel *et al.*, 2017). Plant endophytes promote nutrient acquisition on behalf of the host plants, nitrogen fixation, and tolerance to biotic and abiotic factors (Lata *et al.*, 2018, Eid *et al.*, 2021).

Groundnut seeds are essential in South Africa and globally for their contribution to food security, nutrition and economically as an international cash crop (Prasad *et al.*, 2010, Banla *et al.*, 2018, Phokane *et al.*, 2019). Groundnut seeds are negatively affected by plant pathogenic fungi, including *Aspergillus flavus* Link, so finding a potential endophytic bacterium to combat *A. flavus* infections is paramount for South African groundnut growers. This study aimed to isolate, screen, and identify bacterial plant endophytes to manage *A. flavus* in groundnuts, in short, medium-term storage trials and in greenhouse trials.

Summary of significant research findings

(a) Isolation and in vitro screening of endophytic bacterial isolates for biological control of *A. flavus* in groundnut seeds.

Major findings:

- The inhibition provided by the best isolates ranged from 7.1-55.2%
- The best isolates were identified as *B. amyloliquefaciens/ B. siamensis/ B. velezensis/ B. subtilis*, *P. fluorescens*, and *Pantoa dispersa*

Implications:

The results suggest that endophytic bacteria are potentially good biological control agents. Five isolates showed potential as biocontrol agents and confirms the importance and the need to screen many endophytic isolates from different plant parts to obtain the best-performing strain.

(b) The effect of three endophytic *B. amyloliquefaciens/ B. siamensis/ B. velezensis/ B. subtilis* strains on postharvest management of *A. flavus* in groundnut seeds

Major findings:

- Cultured filtrate provided better control against *A. flavus* during the short-term storage trials
- The acidic pH (2.1) cultured filtrate of *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strain KG showed a constant antifungal activity until 21 days.

Implications

The cultured filtrate of *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strains has the potential to control *A. flavus* during storage. In this study, the acidic (pH 2.1) cultured filtrate demonstrated a better control against *A. flavus in vivo*. This suggests the presence of antifungal compounds (such as surfactin, iturin, and fengycin) actively displaying preventative effects on the pathogen at low pH. For the effective storage treatment, the percentage disease incidence was moderately low and constant till 21 days after treatment with the cultured filtrate.

(c) Greenhouse testing of two endophytic bacterial isolates as potential biocontrol agents against *A. flavus* in groundnuts.

Major findings

- *B. amyloliquefaciens/ B. siamensis/ B. velezensis* and *P. fluorescens* demonstrated a positive combined effect in controlling *A. flavus* on both cultivars (Akwa and Sellie-Plus).
- None of the treatments showed an increase in the total yield of pods/seeds.
- The combination of foliar spray and seed treatment reduced *A. flavus* infections resulting in low disease incidence on both cultivars.

Implications

The combination of foliar spray and the seed treatment can potentially reduce *A. flavus* infections pre-harvest. The low disease incidence from the combined treatment of *B. amyloliquefaciens/ B. siamensis/ B. velezensis* strain KG and *P. fluorescens* strain ALA stresses the importance of additive/synergistic effects in disease control.

(d) Recommendations and future research

Environmentally safe control strategies against *A. flavus* should be prioritised by researchers to minimize *A. flavus* infections.

From this study, the specific recommendations include:

i. Exploring the possible compounds present in the acidic cultured filtrate that enabled better control against *A. flavus in vivo*. This will provide information on compounds needed for biocontrol formulation.

ii. In the *in vivo* studies, growth promotion should be evaluated without *A. flavus* inoculation, to better assess the growth promotion capabilities of the strains.

iii. Measurements of key growth enhancement indicators such as leaf chlorophyll content and nitrogen fixation should be considered under greenhouse studies.

iv. Lastly, field trials should be performed to evaluate the potential of the best endophytes.

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Appendix 2.1

Average zone inhibition of screened endophytic bacterial isolate after even days.

No.	Isolate	Average zone of inhibition (mm)
1	KA	0.39
2	KB	0.28
3	KC	0.94
4	KD	0.56
5	KE	3.61
6	KF	1.50
7	KG	7.33
8	KH	1.50
9	KI	5.06
10	PA	0.33
11	PB	0.50
12	BA	1.57
13	BB	4.06
14	BC	1.11
15	BD	1.83
16	BE	0.00
17	WRA	0.28
18	WRB	0.22
19	WRC	0.33
20	WRD	0.11
21	WRE	0.44
22	WRF	1.00
23	WRG	0.61
24	WRH	0.56
25	WRI	0.72
26	WRJ	0.39
27	WLA	1.39
28	WLB	0.17
29	WLC	0.22
30	WLD	1.28
31	WSA	0.22
32	WSB	0.83
33	WSC	0.78
34	WSD	0.44
35	WSE	1.11
36	GBRA	0.56
37	GBRB	0.39
38	GBLA	1.89
39	GBLB	0.00
40	GBLC	0.00
41	GBSA	0.44

No.	Isolate	Avarage zone of inhibition (mm)
42	GBSB	0.00
43	GBSC	6.11
44	GBSD	0.67
45	PSA	1.44
46	PLA	0.00
47	PLB	0.56
48	PLC	0.22
49	PLD	0.72
50	PDA	0.28
51	PRA	0.56
52	PRB	1.28
53	GSA	2.50
54	GSB	0.89
55	GSC	0.67
56	GSD	1.61
57	GSE	0.11
58	GLA	0.44
59	GLB	0.50
60	GLC	11.22
61	GLD	0.56
62	GLE	2.38
63	GRA	1.33
64	GRB	1.22
65	GRC	2.28
66	GRD	0.94
67	GNRA	2.89
68	GNRB	0.28
69	GNRC	1.89
70	GNRD	0
71	GNPA	1.94
72	GNPB	0.94
73	GNPC	0.28
74	GNPD	0.28
75	GNLA	2.78
76	GNLB	0.16
77	GNLC	0
78	GNLD	0.28
79	ALA	3.83
80	ALB	0.33
81	ALC	0.50
82	ALD	0.67
83	XSA	0.61
84	XSB	0.28
85	XSC	0.22

No.	Isolate	Diameter zone of inhibition (mm)
86	XSD	0.11
87	SSA	1.22
88	SSB	0.56
89	SSC	0.39
90	SLA	0.17
91	SLB	0.61
92	SRA	0.44
93	SRB	0.56
94	RSA	0.22
95	RSB	0.17
96	RSC	0.11
97	RSD	0.00
98	RSE	0.00
99	RLA	0.00
100	RLB	0.33
101	RLC	0.78
102	RRA	0.67
103	RRB	0.00
104	RRC	0.33
105	RRD	0.00
106	RRE	0.22