Effects of plant essential oils and biocontrol agents on the growth of and mycotoxin production by *Aspergillus* spp. on groundnut

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Abstract

Groundnut, *Arachis hypogaea* (L.), can be attacked by a range of pathogens, including *Aspergillus* species, which can cause accumulation of the mycotoxin aflatoxin. Although some success in controlling this pathogen has been achieved with application of fungicides, their use is not always feasible in developing nations like Nigeria. The aim of this study was, therefore, to evaluate naturally-occurring plant oils and BCAs with a past history of efficacy as alternatives to fungicides for reduction of *Aspergillus* infection and aflatoxin accumulation in groundnut. *Aspergillus* strains and thirteen different plant essential oils were tested. The oils were derived from clove, camphor, vanilla, garlic, galangal, green oregano, lemon grass, neem, ginger, basil, tea tree, thyme and onion. The biocontrol agents used were fungi *Trichoderma harzianum* strain T-22, *T. asperellum* and *T. viride* from a commercial biocontrol product, TUSAL, and bacteria *Pseudomonas chlororaphis* ssp*. aureofaciens* and *Bacillus amyloliquefaciens* (strains MBI600, 62P, and 66P). The identities of a strain of *A. niger,* isolated from Nigerian groundnut samples, and of *T. asperellum* and *T. viride* were confirmed by PCR amplification of DNA and sequence comparison to reference isolates in the GenBank database. Some of the plant oils (clove, camphor and vanilla) and biocontrol agents (*Trichoderma* strains) tested proved effective in inhibiting the *A. flavus* and *A. niger* strains used in the research, in both *in vitro* and *in planta* experiments. Improved seedling emergence in pathogen-contaminated compost and reduced post-harvest pod infection were observed. Combinations of the most active BCAs and EOs also provided disease suppression. ELISA analysis of aflatoxin B_1 in treated, *A. flavus*-inoculated groundnut pods showed a reduction in toxin concentrations, to a level below that recommended by the European Commission of 15 ppb. Of the control agents tested, the most effective were *T. harzianum* T-22 as a BCA and probably clove oil as a plant extract. Commercial products based on *Trichoderma* are used world-wide. EOs, have, to date, had little use in control of *Aspergillus* infection of groundnut.

It was also demonstrated that detection of asymptomatic *A. flavus* pod infection could be achieved by the traditional method of surface sterilisation and plating out, and by use of a LAMP assay to detect pathogen DNA. The latter could provide a rapid, portable method for *A. flavus* detection in harvested groundnut pods and could have application in both developed and developing nations. Since low resource growers in nations like Nigeria need alternative, low-cost methods for protecting groundnut from *Aspergillus* infection, to produce a nutritionally-valuable, high protein foodstuff low in toxin contamination, such alternative methods of disease control may have a future role to play in global food security. It may prove possible to extract antifungal components from appropriate, locally-sourced plant material in a cost-effective manner. However, whether the level of disease control and suppression of aflatoxin accumulation reported here was adequate for possible commercial application is unclear. Further evaluation, including field experiments, is required.

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Abbreviations

Aflasafe.....Biocontrol product

AFs.....Aflatoxins (B_1, B_2, $G_{1,}$ G_2)

ANOVA.....Analysis of variance

BCAs.....Biocontrol agents

CABI.....Centre for Agricultural Biotechnology Information

$CaCl_2$.....Calcium chloride

CRBD.....Completely Randomized Block Design

DAS.....Days after sowing

DNA.....Deoxyribonucleic acid

DPV.....Direction de la Protection des Végétaux

ECOWAS.....The Economic Community of West African States

ELISA.....Enzyme-Linked Immunoabsorbent Assay

EOs.....Essential oils

EPA.....Environmental Protection Agency

Eurofin MWG.....A certified service provider for exome sequencing

FAO.....Food Agricultural Organization

GGC.....Gambian Groundnut Corporation

g L^{-1}.....gram per liquid

GNARI.....The Gambia's National Agricultural Research Institute

ha.....hectare

h.....Hour

IARI.....Indian Agricultural Research Institute

IITA.....International Institute of Tropical Agriculture

ISR.....Induced Systemic Resistance

ITS.....Internal Transcribed Spacer

LAMP.....Loop-mediated isothermal amplification

LSD.....Least significant difference

MBI600.....Isolate of *Bacillus amyloliquefaciens*

NAICPP.....National Accelerated Industrial Crops Production Program

NaOC.....Sodium hypochlorite

MN.....Macherey-Nagel

NCBI.....National centre for Biotechnology Information

NSPRI.....Nigerian Stored Product Research Institute

MRD.....Maximum recovery diluent

PCR.....Polymerase Chain Reaction

PDA.....Potato dextrose agar

PGPR.....Plant Growth Promoting Rhizobacterium
PVP.....Polyvinylpyrrolidone
RO.....Reverse osmosis
s.....second
SAR.....Acquired Systemic Resistance
SD.....Standard deviation
SDW.....Sterilised distilled water
SE.....Standard error
T.a. .....*Trichoderma asperellum*
TBE.....Tris-Borate-EDTA
TSB.....Tryptic-soy broth
T-22.....*Trichoderma harzianum*
TUSAL®.....Commercial *Trichoderma* product
T.v. .....*Trichoderma viride*
UK.....United Kingdom
µL.....Microliter
µm.....Micrometre
UON.....University of Nottingham
UN.....United Nations
UV.....Ultra violet
USDA.....United States Department of Agriculture
V.....Voltage
VNX.....Vanilla preparation
v/v.....Volume per volume
WFLO.....World Food Logistics Organisation
62P.....Isolate of *Bacillus amyloliquefaciens*
66p.....Isolate of *Bacillus amyloliquefaciens*

General Introduction

1. The groundnut crop

Groundnut, *Arachis hypogaea* (L.), is an annual herbaceous plant in the Fabaceae (legume or bean family) (Encyclopedia of Life). It is also known by different local names such as earthnuts, groundnuts, goober peas, monkey nuts, pygmy nuts and pig nuts, but commonly used ones worldwide are groundnut and peanut. The name groundnut is used in most countries of Asia, Africa, Europe and Australasia, while peanut is commonly used in North and South America. Despite its name and appearance, groundnut is not a true nut, but rather a legume. Groundnut is a herbaceous, self-pollinated annual plant that grows to a height of 20 - 60 cm, depending on the variety. Plants grow erect or creeping, with lateral shoots, having a breadth of 30 – 80 cm, creating branches at the surface of the soil (Booke, 1982).

Groundnut blossoms open up in the early morning, usually after self-pollination has taken place. The blossoming period usually begins 3-4 weeks after sowing, and can last up to 12 months, depending on the type of variety. Groundnut species are known as geocarpic reproducers, i.e. they sink a stalk-like structure called a peg, which penetrates into the soil after fertilisation, for groundnut pod and seed formation.

There are different types of groundnuts that are classified into two major subgroups, which can be cross-bred amongst each other: *A. hypogaea* ssp. *hypogaea* (Virginia variety) and ssp. *fastigiata* (Spanish Valencia variety) (Rundgren, 1998).

1.2 Origins and History

Groundnut originated in South America (Bolivia and adjoining countries) as a cultivated crop about 4000 years ago, and is now propagated throughout the tropical and warm temperate countries of the world. Groundnut was grown in large quantities by the native peoples, before European exploration in the sixteenth century, and was subsequently distributed to countries in Europe, Africa, Asia, and to the Pacific islands. Figure 1.1 shows where groundnut originated and Figure 1.2 illustrates

the centre of origin (solid line), area of intensive cultivation (dotted line) and areas of maximum groundnut cultivation (coloured).

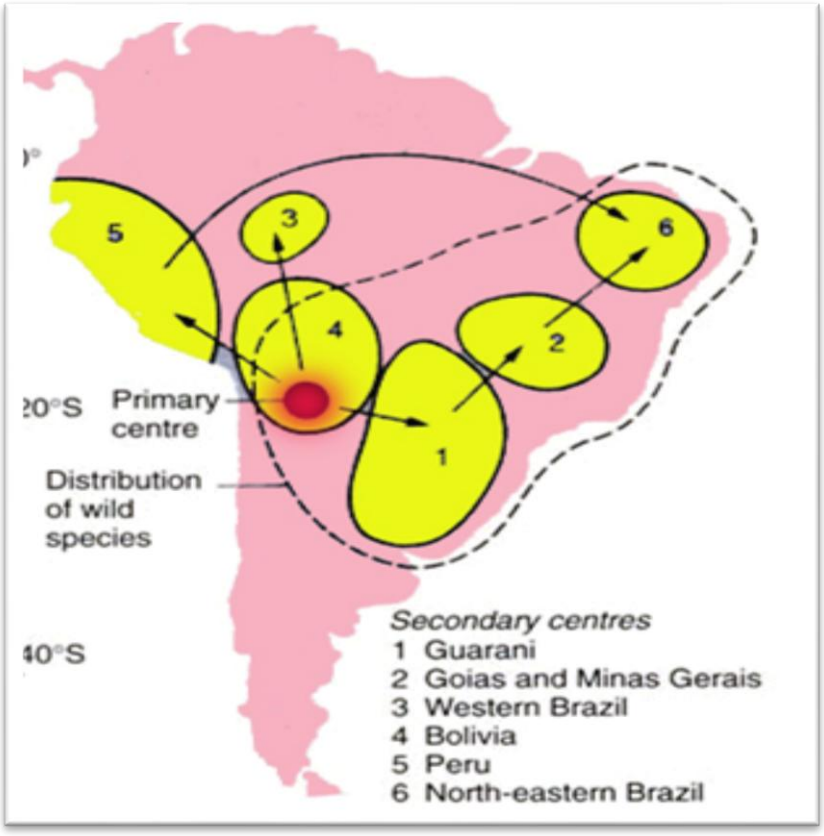


Figure 1.1 Origin of groundnut production, from FAO, (2003).

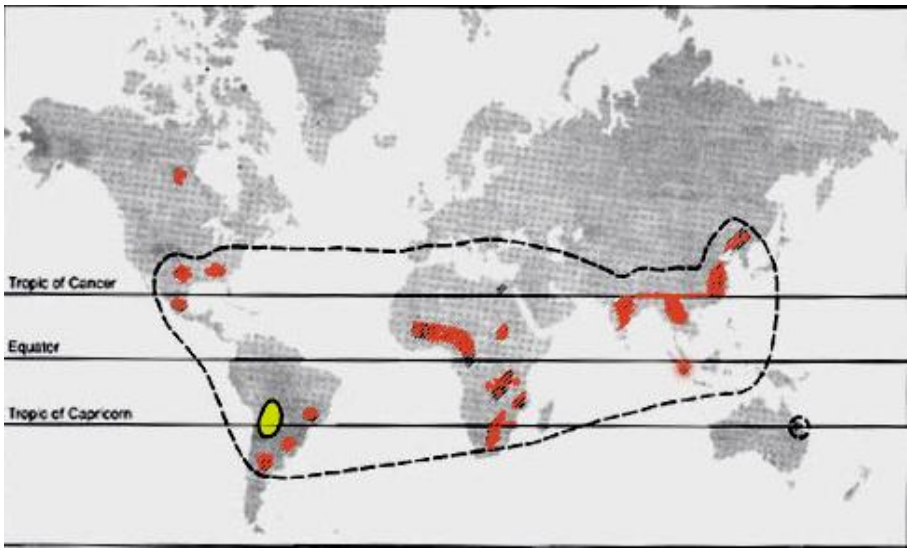


Figure 1.2 Groundnut centre of origin, area of intensive cultivation (Weiss, 2000). Yellow is centre of origin and red areas of cultivation.

Groundnut was introduced to the south eastern United States during the colonial era by the Portuguese, and was grown primarily as a garden vegetable crop until 1870 (FAO Food Outlook, 1990). As a field crop, it was used commonly for hog pasture until about 1930.

In Nigeria, groundnut was first introduced into the country in the early nineteenth century. Today, a wide range of locally adapted varieties are grown. Small-seeded runners and bunch varieties of various seed and pod types are propagated.

1.3. Uses

Globally 50% of groundnut produced is used for oil extraction, 37% for confectionery and 12% for seed and only 1% for direct consumption (FAO, 2004). In India, 80% of the total production of groundnut is used for oil extraction, 11% is used as seed, 8% for direct food consumption and 1% for export (FAO, 2004). Groundnut haulms (vegetative plant part) provide hay for livestock consumption, to enrich meat, milk and egg quality (Ososanya, 2012). Groundnut is ranked the 13^{th} most important food crop of the world, and the 4^{th} most important source of vegetable protein for human consumption, to meet protein requirements for the increasing population (FAO, 2004). Groundnut oil is one of the most important vegetable oils, along with that of soybean, sunflower and palm oil (FAO, 2007).

Groundnut consumption per year in the United States is greater than all other nuts (Putnam and Allshouse, 1999). Groundnut contains heart-healthy nutrients, such as monounsaturated and polyunsaturated fatty acids, potassium, magnesium, copper, niacin, fibre, α-tocopherol, folates, phytosterols, and flavonoids. Its consumption has enriched overall dietary quality and nutrient profile (Kerckhoffs *et al*., 2002; Griel *et al*., 2004). Cardiovascular disease (CVD) is still the number one disease that causes death of Americans (Lloyd-Jones and Adams, 2009). Groundnuts, groundnut oil, and fat free groundnut flour reduced CVD risk factors and development of atherosclerosis in Syrian Golden Hamsters (Stephens *et al*., 2010).

Groundnut seeds contain high quality edible oil (45%), easily digestible protein (25%), carbohydrate (20%), water (5%), raw fibres (3%) and ash (2%), that has significant impact on human and animal nutrition in both tropical and subtropical countries of Asia, Africa, North and South America (FAO, 2004). Seeds are eaten raw, cooked, or roasted, and also processed into groundnut butter, sweets and snacks (FAO, 2003). They are used to make soups and sauces (FAO, 2003). Groundnut cake is used to enrich foodstuffs with protein, for example manioc flour (FAO, 2003). Foliage and pasture are also used as protein-rich feed for livestock consumption. Pods are used for fuel, as fibre in fodder, as raw material for light construction boards, as a source of cellulose and for composting (FAO, 2003). Groundnut is also used for manufacturing of soaps, medicines, cosmetics, lubricants and to increase the nitrogen content of soil, with the help of nitrifying bacteria in root nodules of the crop. Its agronomic role in traditional farming systems as a nitrogen fixer in crop rotation cannot be over emphasized (Ustimenko-Bakumovsky, 1993). Groundnut has encouraged international trade among countries. In Nigeria specifically, it is an important cash crop for small scale farmers to sustain their families.

1.4 Current cultivation

Global production since 2000 and national distribution for 2012 are shown in Figures 1.3 and 1.4 respectively. Groundnut is cultivated in nearly 100 countries, with China, India, Nigeria, USA, Indonesia and Sudan the major producers. Developing countries account for 96% of the global groundnut producing area and 92% of global production. Asia accounts for 58% of the global groundnut area and 67% of production with an annual growth rate of 1.28% for area, and 2.00% for yield. India, China, and the United States are noted for having been the leading producers for over 25 years and propagate 70% of the world crop. Groundnut was ranked ninth in sown area among row crops in the United States in 2004. Production in the United States is now ranked the world's third largest, after China and India (USDA, 2012). Groundnut production increased from 33.736 million tons in 2010, to 35.995 million tons in 2011 (due to increased planting in India), but latterly a decrease in production was observed to 35.367 million tons in 2012 (USDA, 2012). The decrease was as a result of the drought situation in the USA.

Unit 1000 Metric tons

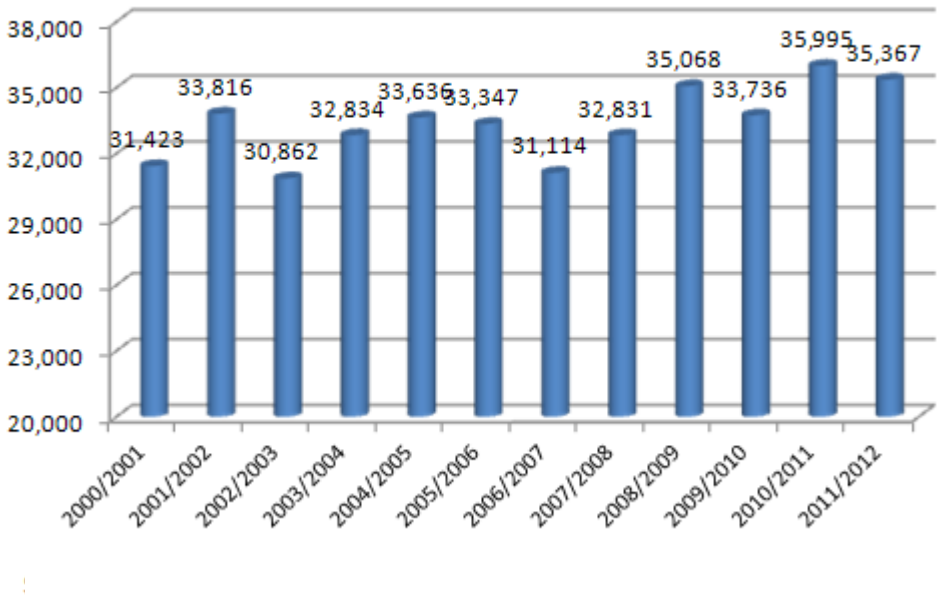


Figure 1.3 Global Groundnut Production from 2000 to 2012. USDA, 2012

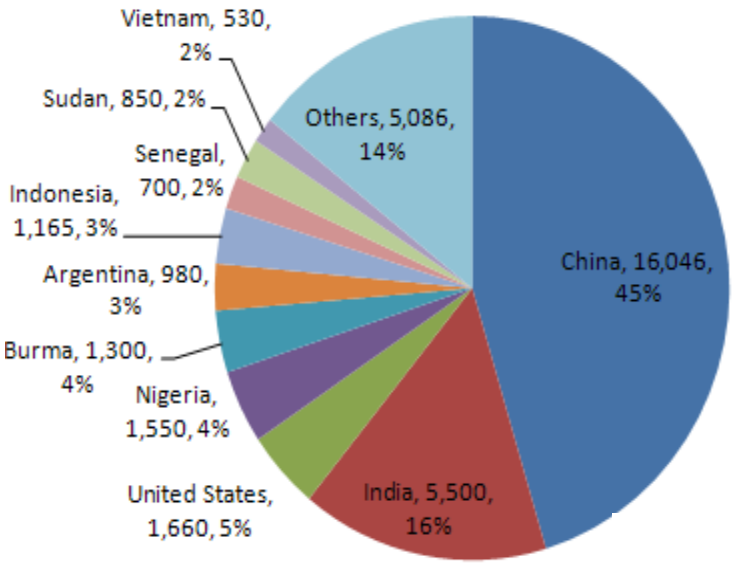


Figure 1.4 Global Groundnut Production Distribution (yield in thousand metric tonnes + percentage of total global production) in 2011/2012. USDA, 2012.

China is the world's largest groundnut producer, accounting for 45% of the total world production (USDA, 2012). The country's share of total groundnut production has been increasing every year. China is only second to India in terms of land area dedicated to the crop. China has one–fifth of the world

area under groundnut production and more than two-fifths of the total world groundnut production. Since the early 1960s, the total groundnut production in China has increased 12 fold.

Production of the groundnut crop in Nigeria in the 1960s, and even up to the early 1970s, was a rewarding and satisfying experience for farmers. Farmers, with adequate technical support and incentives, had demonstrated their ability to cultivate the crop, as evidenced by the famous groundnut pyramids, which were once a symbol of the country's abundance in groundnut production. A rapid decline in production from 1975 up to the mid 1980s was due to several factors, including biotic and abiotic stresses, a general neglect of agriculture (due to over-dependence on oil) and a lack of technical support and price incentives. This made it imperative for the government of Nigeria to intervene, to stop the total collapse of the groundnut sub-sector. These efforts, started in 1987, culminated in the initiation of the National Accelerated Industrial Crops Production Program (NAICPP) aimed at promoting the production of eight prominent industrial crops, of which groundnut was one. These efforts have led to a gradual increase in the production of groundnut, through increased land area put under cultivation and improved yield (Echekwu, 2003).

The success of these rehabilitation efforts depends very heavily on the recognition of the crucial role of improved seed in groundnut production. Good quality seed of improved varieties remains a primary input in groundnut production for the simple reason that fertilizers, pesticides, and other inputs are only able to give high returns when farmers plant seeds of groundnut varieties with high genetic potential.

Commercial production of groundnut in Nigeria is concentrated in the northern parts of the country, particularly in the areas between the Northern Guinea and the Sudan Savanna zones (Misari *et al*., 1988). However, due to the high commercial value and high demand, the crop is now gaining popularity as a cash crop for small scale farmers in the rain forest zone of Nigeria.

Groundnut is usually intercropped with millet or sorghum, and sole cropping in small plots is not encouraged, to avoid crop failure due to disease outbreaks. Farmers prefer to intercrop groundnut with different crop mixtures, because two or more crops grown at different times produce greater yield than sole crops.

In the past Nigeria produced up to 1.2 million metric tons per year and was once an exporter of groundnut, but became an importer (Gibbion and Pain, 1988; Libura *et al.*, 1990). Nigeria is still an importer of groundnut according to the European Union Commission (2013). One of the main reasons for this setback was due to outbreaks of disease during storage (Libura *et al.,* 1990; Mehan *et al.,* 1986; Yayock, 1976). Consequently, farmers preferred practice shifted from groundnut production to other grain crops that would enhance economic growth, and when crude oil was discovered in the early 1950s, its exploration also contributed to setback of groundnut production in the country. Okolo and Utoh (1999) reported that Nigeria's area under groundnut production was about 1.0 to 2.5 million ha, with an annual yield in the range of 500-3000 kg ha^{-1}. Taru *et al*. (2010) reported that seed production yield in northern Nigeria is about 3000 kg ha^{-1}. Total yield as of 2011/2012 was estimated at 1.55 metric tonnes (USDA, 2012).

1.4.1 Routine cultivation requirements

The optimum temperature required for the vegetative growth of groundnut is approximately 34^0C. Lower temperatures, below 20^0C, affect seed germination and the rate of growth and development of groundnut will be rapidly reduced. Higher temperatures, above 34^0C, damage flower formation. The optimum temperature influences the net rate of photosynthesis, flower formation and the growth of the pods, and is therefore important to maximise yields (Franke, 1994). Night temperatures should not sink below 10^0C during the fructification process, because low temperature kills the plant, when it falls below this point.

Light

Groundnut can tolerate shade; it therefore poses no problems when it is cultivated with trees or other crops (intercropping or mixed cropping). When placed in extreme shade, the leaves get bigger, and the number of reproductive organs is reduced, resulting in poor output. *A. hypogaea* is, in a photoperiodic sense, practically neutral, although photoperiod sensitive and insensitive varieties exist.

Water

The optimum time to plant groundnut is the start of the rainy season. The yield declines rapidly when the plants are propagated outside this planting time. The germination process also requires adequate soil aeration. Groundnut plants can withstand a flooding period of up to seven days (Weiss, 1983). In the case of regular heavy rainfall during vegetative growth, the soil must be well drained, or the crop should be propagated on ridged platforms. Interplanting varieties require 500-1000 mm of rainfall (up to 145 days vegetation period), and this is reduced to 300-500 mm for early cultivars. The type of soil and its capacity to retain water before planting also play a very important role in seed emergence. Rainfall at 300 mm is required between the plant's appearance and the main flowering period, in order to ensure sufficient vegetative growth (Keenan and Savage, 1994). Adequate information on the average rainfall to be expected at the site is useful in selecting an appropriate variety, to ensure ripening occurs before the rain. Stress conditions due to drought during the ripening period of the seeds can also lead to infection by *A. flavus* (Keenan and Savage, 1994).

Soil

Optimum soil for groundnut propagation is a well-drained, light, loose, finely grained sandy loam soil, with plenty of lime and sufficient organic matter. Other conditions required for high crop yields are soils which neither harden nor crust over, nor create water-logging. Cotyledon emergence must not be inhibited, and, after flowering, pegs must penetrate the soil in order for the pods to expand for nutrient uptake and pod development. In hard and heavy soils, if not properly managed, it is difficult to harvest pods, which can become malformed and heavily contaminated with soils, thus reducing crop value. Groundnut grows best in weakly acidic soil (pH 6.0 - 6.5) although a pH value of 5.5 - 7.0 is acceptable, and local varieties can adapt themselves to pH values of 7.8. Groundnut plants are sensitive to a high salt content in the soil (ICRISAT, 1992).

1.4.2. Seeds

Good seed production requires great care during harvest to avoid damage. The seeds should be harvested separately, mechanically or preferably manually, and the pods should be removed from the plant by hand to avoid damage. To avoid mould development, and to maintain germination potential in extremely wet regions, it might also be necessary to apply drying agents such as $CaCl_2$ (ICRISAT, 1992).

It is advised that seeds for propagating are removed from the pods shortly before sowing, because once opened, their viability will be reduced. The seed coat should be kept intact when planting, in order to reduce penetration by pathogens.

1.5 *Aspergillus* spp.

Aspergillus species are grouped in the Ascomycota. *Aspergillus* can reproduce both sexually or asexually. In most cases the group reproduces asexually, conidia being released in the air and then carried or dispersed by wind. After landing in a place where there are appropriate conditions, they start to germinate producing foot cells. More branching occurs, with elongation of hyphae creating a mycelium. Soon conidiophores grow from the foot cells (Bennett and Klich, 1992).

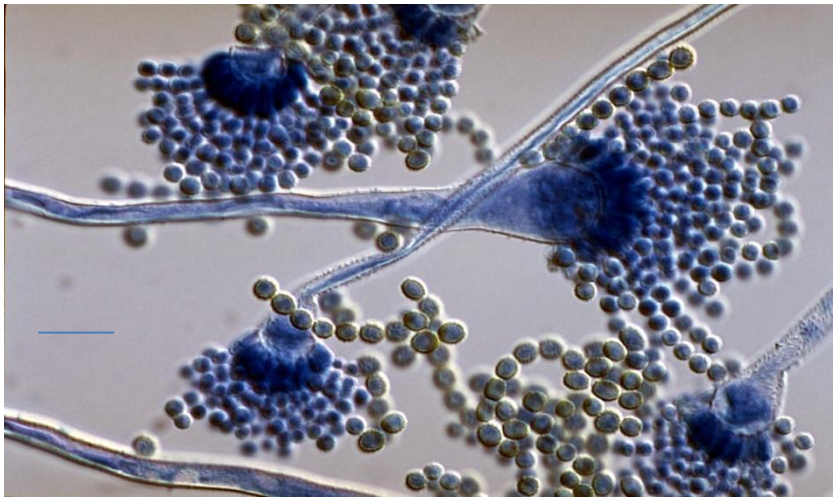


Figure 1.5 Conidial head of *Aspergillus*.
http://www.clt.astate.edu/mhuss/Aspergillus%20flavus%20pict.jpg. Author of the description: Fekete-Kertész Ildikó
Scale bar 20µm

Aspergillus is a genus of anamorphic fungi reproducing by production of phialospores (conidia borne on phialides). This is a large genus with over 180 recognized species (Pitt *et al*., 2000). Some of these species are very uncommon while others are among the most common on earth. *Aspergillus* is classified by its distinctive conidiophores. The conidiophore base usually forms in a T or L shape, where it connects with the vegetative hyphae, as shown in Figure 1.5. It is known as the foot cell, even though it is not a separate cell. The aerial hypha / conidiophore extends from the foot cell and may be quite short (50 µm or less) to several millimetres in length. Some species that produce *Aspergillus* anamorphs (asexual) also produce a sexual state (teleomorphs). The sexual states belong to eight or more teleomorphic genera (Bennett and Klich, 1992). It has been argued that once a teleomorphic stage is found, the anamorphic name should no longer be used. This was not practical with a large genus like *Aspergillus*, with so many economic important species. Currently, those species with teleomorphic states have two legal names, and the *Aspergillus* name should only be used when referring to the asexual, anamorphic state. In naming fungi, mycologists have followed the International Code of Botanical Nomenclature (Greuter *et al*., 1994). If all the rules of nomenclature are not followed, a proposed new species name would not be accepted.

Aspergillus was first illustrated by a Florentine priest-mycologist, P.A. Micheli, in 1729, and was given the name because the spore-bearing feature of the genus was similar to an aspergillum, a tool used by the Catholic Church to sprinkle holy water (Bennett and Klich, 1992). *Aspergillus* was advantageous, since some of the aspergilli have been a blessing to the human race. However, members of the genus have also been a curse, degrading agricultural products from field to storage, producing toxic metabolites and causing diseases that are detrimental to humans and livestock (Bennett and Klich, 1992). The greatest positive economic impact of the aspergilli has been in the exploitation of the enzymes and acids produced by a number of species. Two of the most important industrial products produced by aspergilli are amylase and citric acid, which have been used for more than a thousand years to produce a number of Asian foods and beverages, including sake and soy sauce. Amylases break down starch and contribute to the flavour and colour of the products (Hara *et al*., 1992).

1.5.1 A*. niger* description and significance

A. niger Van Tieghem causes a disease called black mould on some fruits, legumes and vegetables, such as grapes, onions and groundnut, and is a common contaminant of food. Some strains of *A. niger* produce potent mycotoxins called ochratoxins (Abarca *et al.*, 1994). *A. niger*, which causes collar rot disease on groundnut seedlings, was first investigated by Jochem (1926). *A. niger* may cause an average of 5% loss in yield but in some locations it may cause losses as high as 40% in groundnut. Collar rot disease is a serious problem in sandy soil (Gibson, 1953; Chohan, 1965). This disease can be effectively controlled by crop rotation, use of resistant varieties and treatment of seeds or soils with fungicides. Frequently, however, some methods are unsuitable or not effective, mainly due to the generic variability presented by the pathogen, the capacity to survive in soil and in seeds, and physiologic flexibility to infect different hosts. *A. niger* has many applications in biotechnology (Oliveira *et al*., 2008). The significance of *A. niger* is the industrial role that it plays in the production of proteins, enzymes and fermentation. Citric acid production by *A. niger* was developed in 1916, and by the mid 1920s over three quarters of the citric acid used worldwide was produced by fungal fermentation. It is still used today

according to available literature to produce more than 500,000 tons per year globally (Roehr *et al.*, 1992). It is capable of producing heterologous proteins, such as the human cytokine interleukin-6 (Semova *et al.*, 2006). This very useful microbe is even descripted as an ''industrial *workhorse*'' because of the frequent use in many applications in biotechnology (Anderson *et al*., 2008).

A. niger is known to produce asexual spores only, with no known sexual reproduction. This pathogen grows aerobically on organic substrates and it can be found almost everywhere in environments that contain soil. *A. niger* has been noted to survive in freezing temperatures. It can also survive at very high temperatures. Its thermotolerant abilities enable growth in a wide range of temperatures from 6 to 47^0C with a preferred optimum temperature at 35-37^0C. The fungus is capable of growing over a very wide pH range, from 1 to 9.8 (Reiss, 1986).

1.5.2 *A. flavus* description and significance

A. flavus is a soil-inhabiting filamentous fungus that is able to utilise a wide range of organic substrates. This organism is both a saprophyte and an opportunistic pathogen (Mellon *et al*., 2007). It can aggressively destroy agronomically-important oil seed crops such as corn, groundnut, and cotton, especially when they are under biotic or abiotic stress. Populations are genetically diverse and phenotypic variations have been well reported (Geiser *et al*., 2000; Horn and Dorner, 1999; Pildain *et al.*, 2004; Takahashi *et al*., 2004). *A. flavus* grows at temperatures of 25-42^0C, but the optimal temperature for growth is 37^0C. It survives through winter as mycelium or sclerotia, which are resistant structures that can develop into hyphae or conidia. The conidia are scattered into air and soil, by wind and insects. *A. flavus* isolates vary considerably in their abilities to colonize plants in tropical and subtropical soils (Mellon and Cotty, 2004). They generally can be grouped into two sclerotial morphotypes, L strains and S strains (also named *A. flavus* var. *parvisclerotigenus* (Saito and Tsuruta, 1993). L strain isolates produce many conidiospores and sclerotia that are usually larger than 400 µm in diameter (Cotty, 1989; Horn and Dorner, 1999), while S strains produce fewer conidiospores and numerous sclerotia that are usually smaller than 400 µm in diameter. S strains produce a mycotoxin, aflatoxin B, which is carcinogenic, causing liver damage. S strains typically produce a

higher amount of aflatoxin than the L strains on the same media in a controlled environment (Bayman and Cotty, 1993; Novas and Cabral, 2002). The aflatoxigenic trait of the S strain isolates seems very stable. In contrast, from available records, a significant portion of *A. flavus* L strain field isolates do not produce aflatoxins (Horn and Dorner, 1999; Mphande *et al*., 2004; Pildain *et al.*, 2004; Tran-Dinh *et al.*, 1999; Vaamonde *et al*., 2003). The genetic relationship between L and S strains is still not clear. The divergence of L and S strains has been estimated to have occurred between 1 and 3 million years ago (Ehrlich *et al.*, 2005).

1.6 *Aspergillus* infection of groundnut

Groundnut production is hampered by attack from a range of pathogens, including bacteria, fungi, viruses and nematodes (Smith, 1994). Fungi are the most economically important group of plant pathogens, causing both quantitative and qualitative yield losses (Fletcher *et al.*, 2006). Many of these pathogenic microorganisms are transmitted by seed, and with suitable environmental conditions, seed-borne pathogens can adversely affect germination, plant vigour, and cause disease in seedlings and plants, if not properly managed (Agarwal and Sinclair, 1997). Of all the pathogens, *Aspergillus* spp. are considered to be some of the most important, posing a serious threat to groundnut production. Infection of groundnut by *Aspergillus* occurs under both pre-harvest and post-harvest conditions and aflatoxin accumulation is a serious concern in groundnut production in Nigeria. With pre-emergence infection, the symptoms first appear as spots on the cotyledons of the seedlings in droughted soils. Seedlings and ungerminated seeds shrivel to become a dried brown to black mass covered by yellow or green spores, in the case of *A*. *flavus.* Plants that survive germination and emergence appear chlorotic (Agrios, 2005). The roots are stunted and lack a secondary root system, a condition known as *aflaroot.* The leaves are small and pointed with a thick and leathery texture. Infected seedlings may survive infection in optimal growing conditions. Yellow mould of groundnut pods and seeds may occur, especially in dry conditions. During harvest, further infections may develop, with fungal growth covering the seed surface and invading the seed itself.

Seedlings and young plants are more susceptible to *A. niger* in the field and the most obvious symptom is sudden wilting of the young groundnut plant. Diseased areas of the plant are covered in dark fungal growth which causes crown rot of groundnut plant. Infection of seedlings commonly occurs soon after germination. The disease progresses rapidly, and most affected plants will die within 30 days of planting. Post-emergence, wounded shrivelled seeds, subjected to poor storage, transportation, processing facilities and monitoring, result in aflatoxin contamination (FAO, 2007).

1.7 Aflatoxin

Major interest in aflatoxin contamination of groundnut dates back to 1961, with the outbreak of the Turkey X disease in Britain, which led to the death of thousands of turkeys fed with contaminated Brazilian feed (Sargeant *et al*., 1961). This incident laid the foundation for world research on aflatoxin and other mycotoxins in food crops and livestock feeds.

The production of mycotoxins by several fungi has added a new dimension to the problem of fungal diseases. Pathogenic fungi are significant destroyers of foodstuffs during storage, rendering them unfit for human consumption by retarding their nutritive value and sometimes by the production of mycotoxins. According to FAO estimates, 25% of the world food crops are affected by mycotoxins each year (Dubey *et al.*, 2008). Generally, climatic conditions such as high temperature and moisture, unseasonal rain during harvest, and flash flooding lead to mycotoxin accumulation.

Inadequate handling practices and marketing also contribute to the proliferation of mycotoxins. Among the mycotoxins, aflatoxins raise the most concern, posing a great threat to human and livestock health, as well as international trade. Aflatoxins are the most dangerous and about 4.5 billion people in the developing countries are exposed to aflatoxicosis, a deadly disease (Williams *et al.*, 2004; Srivastava *et al.*, 2008). Aflatoxins are potent toxic, carcinogenic, mutagenic, immunosuppressive agents, produced as secondary metabolites by fungi including *A. flavus* and *A. parasiticus* on a variety of food products. In Nigeria there was a serious outbreak of aflatoxin accumulation in 1988, that led to the Ibadan Local Government warning members of the public to desist from eating ''Kulikuli',

as it had caused the death of primary school pupils. Kulikuli is locally produced groundnut cake which is eaten as snacks in most public schools. Results of a survey showed very high levels of aflatoxin B_1 in market samples of Kulikuli. Furthermore, study on the impact of aflatoxin on human reproduction in Nigeria was conducted by the Nigerian Stored Product Research Institute (NSPRI), cited in Ndukka *et al*. (2001). Results in the 1970s from the University of Benin showed that 37% of infertile men had aflatoxin in their blood and semen ranging from 700 to 1392 ng mL^{-1}, 8% of fertile men also had aflatoxin in their semen, ranging from 0.1 to 5 ng mL^{-1}. Therefore aflatoxin might have contributed to infertility in men (Ndukka *et al*., 2001).

Groundnut importing countries have set maximum tolerance values for the presence of aflatoxin in foodstuffs, in order to protect local consumers from being exposed to this metabolite. For consumers in the producing countries, in developing nations, the risks due to the poison are more difficult to ascertain, because a larger part of the products are consumed and sold on local markets, which exposes people to aflatoxin contaminated groundnut. Fungi can penetrate pods during their growth period whilst still in soil, resulting in invisible damage to the pods and invisible infections of undamaged pods. Mechanically damaged or animal bitten pods will quickly become infected by the fungi, which grow primarily on dead and dry tissues. Hot, dry soil conditions encourage termite attack on groundnut, and the insects can act as vectors for the fungi's spores (Self EL-Nasr, 1998). Alternating phases of rain and drought cause pods to break and produce high aflatoxin values in the seeds. Many pods are infected after the pegs have penetrated into the soil. When groundnut plants undergo favourable environmental growing conditions, fungi may remain inactive and no significant amount of aflatoxin is produced. This is because groundnut plants have natural protection mechanisms: the growing plant produces immune substances (phytoalexins), which have anti-microbial and fungal suppressing effects, e.g. arachidin (Keenan and Savage, 1994). The production of phytoalexins declines towards maturity, as well as due to water deficiency. In contrast, the fungus *A. flavus* is still able to proliferate and cause aflatoxin accumulation. In addition a special set of problems arises when groundnuts are shipped in containers, even when an appropriate aflatoxin management test has been conducted in the country of origin. When they are shipped to the importing country, the shipping conditions might still stimulate the production of aflatoxin to such an extent

that the consignment is ruined within a few weeks of being loaded. An aflatoxin test at the port of arrival might reveal entirely different results to the ones performed before loading. If the allowed values are exceeded, the entire consignment must be discarded. Temperature fluctuations inside the container can be quite extreme, especially when the container is shipped on the deck. Groundnut sweats, after the outside temperatures have cooled, and water condenses and trickles down the walls to cause mould formation. When condensation comes in contact with groundnuts this causes an increased infestation of *Aspergillus* at the point of contact, according to Augustat (1998) and Keenan and Savage (1994). They also made some useful recommendation that groundnuts should be dried down properly to a safe moisture content of 6-7% before shipping. Groundnuts should also be shipped in cooled, ventilated containers (this approach is expensive, yet will avoid loss of the entire load) and the walls of the containers all around should be covered with special moisture-absorbing foils, or at least cardboard. An aflatoxin test should be carried out in accordance with Dutch Code of Practice. Permitted levels of aflatoxin, as well as the number of samples required, vary from country to country. Germany: aflatoxin B_1 and B_2, 4 ppb; Switzerland, Austria and Scandinavia; aflatoxin B_1, 1 ppb and aflatoxin total of 5 ppb; UK, groundnuts used in processing 10 ppb and groundnuts for consumption 5 ppb; Netherlands aflatoxin B_1 also 5 ppb; and for the United States of America aflatoxin total 15 ppb (Augustat, 1998).

Food security has become a very important issue globally and the potential effect of climate change on yields and quality of food crops cannot be over emphasized. This can also involve the accumulation of mycotoxins and is now receiving serious attention in the developing nations, particularly in Nigeria where consumers have been exposed to aflatoxin. Many staple food crops (cereals, nuts, fruits) can be colonized and infected by fungi from the genera *Aspergillus, Fusarium* and *Penicillium* which can contaminate the edible parts of, for example, cereals, maize, groundnuts, spices, figs, and Brazil nuts with the toxic secondary metabolites (Pitter, 1998; Lewis *et al*., 2005; Bandyopadhyay *et al*., 2007; Bartine and Tantaoui-Elaraki, 1997; Doster *et al*., 2005) respectively. Many toxins are very heat stable and thus difficult to eliminate during processing.

In African countries, where legislation is often applied to export crops only, consumption of mycotoxin contaminated stable foods is a significant risk, with rural populations being exposed to aflatoxins throughout their lives, with serious impacts on health (Wagacha and Muthomi, 2008). This was illustrated by the latest outbreak of acute aflatoxicosis in Kenya, which killed hundreds of people living in the eastern and central provinces in April 2004, as a result of aflatoxin poisoning from ingestion of contaminated maize (Lewis *et al.*, 2005).

In 2010 the Kenyan authorities reported that about 2.3 million bags of corn harvested were contaminated with aflatoxin $B_{1,}$ the most potent naturally occurring cause of liver cancer. Outbreaks of aflatoxin accumulation have killed hundreds of people in developing nations, whereas toxicity risk of aflatoxin is extremely low in developed nations, as shown in Figure 1.6.

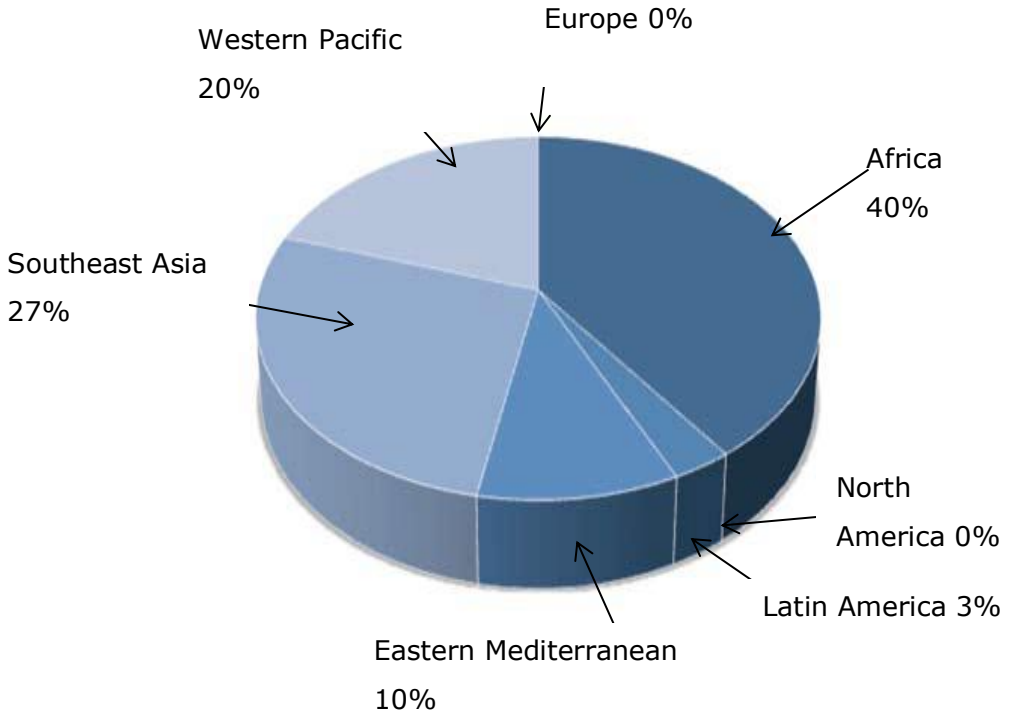


Figure 1.6 Distribution of Hepatocellular Carcinoma Attributable to Aflatoxin (Liu and Wu, 2010).

Williams *et al*. (2004) reported a high positive correlation between exposure of the population to aflatoxin contaminated food and the incidence of liver cancer.

1.7.1 Determination of aflatoxin

Aflatoxin content in food and fodder has been investigated with a number of conventional analytical techniques, such as thin layer, gas, or liquid chromatograpy, spectrofluorometry and spectrophotometry (Barberi *et al*., 1994 and Espinoza *et al.*, 1996). These methods require specialised and expensive devices to work with, as well as being time consuming in preparation of samples connected with the separation of the given component from a mixture.

The immunoabsorbance methods (e.g. ELISA), which use antigen-antibody reactions, are selective and fast techniques, and are also relatively inexpensive. Recently, they have been more frequently used for the determination of aflatoxin contamination of food commodities (Peska *et al.*, 1995).

1.8 Control options for *Aspergillus* infection of groundnut

1.8.1 Fungicides

Fungicides are specific types of pesticide that can control fungal disease by specifically inhibiting or killing the fungus causing the infection. Fungicides are an important part of disease management, because they control many diseases satisfactorily, whereas cultural practices often do not provide adequate disease control and resistant cultivars are not always available. Moreover, certain high value crops have an extremely low tolerance for disease symptoms. Not all diseases caused by fungi can be adequately controlled by fungicides. These include the vascular diseases *Fusarium* and *Verticillium* wilt.

Most fungicides need to be applied before disease occurs or at the first appearance of symptoms to be effective. Unlike many diseases of humans and animals, the damage (including mycotoxin accumulation) caused by diseases on plants often does not go away, even if the pathogen is killed.

Fungicides that have curative properties tend to have a higher risk of pathogens developing resistance to the fungicide, as the compounds have specific, single-site modes of action. A resistant pathogen is less sensitive to the action of the fungicide, which results in the fungicide being less effective or even ineffective. Since these curative fungicides must be able to penetrate into plants and selectively kill the invading fungi, they are designed to target specific enzymes or other proteins made by fungi. Since the mode of action of these fungicides is so specific, small genetic changes in fungi can overcome the effectiveness of these fungicides and pathogen populations can become resistant to future applications. Disease management strategies that rely heavily upon the curative application of fungicides often lead to more resistance problems as (a) the size of the population from which resistant individuals are being selected is larger and

(b) it is difficult to eradicate all of the fungi inside the plant and often, some pathogens escape the fungicide. Fungicide resistance is covered in more detail in a separate section.

Growers often use disease forecasting systems or action thresholds, when these are available, to ensure fungicides are applied when needed and to avoid the expense and possible environmental impact of unnecessary applications. Forecasting systems have been developed for a number of diseases based on an understanding of the environmental conditions favourable for their development. Typically these are based on temperature and relative humidity or leaf wetness in the area where the crop is grown. Threshold-based fungicide programs involve routinely scouting the crop for symptoms, then applying fungicides when the symptom reaches a critical level beyond which the disease cannot be controlled adequately. An example of a critical level is one disease spot per five leaves examined. Knowledge of the disease cycle of the pathogen is important when developing and using forecasting systems and thresholds during the study of the disease triangle in relation to pathogen interactions with host and environment (Agrios, 2005). Important aspects of the disease cycle include whether the disease is monocyclic (one generation per year) or polycyclic (multiple generations) and latent period (time between infection and symptom expression).

Economics often influence the choice of fungicide and application timing. Expensive fungicides and numerous applications are used on valuable plantings that might incur substantial economic loss in the absence of treatment, such as fruit trees and golf courses. Recognizing that with some diseases crop yield is not impacted when severity is low, an economic threshold is used to determine when fungicide treatment is needed. The crop tolerance level, or damage threshold, can vary depending upon the stage of the crop development when attacked, crop management practices, location and climatic conditions.

Jockey (produced by BASF) is a fluquinconazole - based triazole fungicide, which is usually applied for the protection of wheat against the root disease take-all, for protection of canola (oilseed rape) against phoma leaf spot and for other wheat and barley diseases. Application of Jockey as a seed treatment to wheat significantly suppresses the incidence of take-all. Suppression of take-all with Jockey is characterised by reduced root lesions and a reduction in the number of white heads. Jockey also provides season long control of common bunt and loose smut in wheat. In addition Jockey provides early season control of leaf rust and stripe rust and suppression of septoria leaf blotch. Jockey is applied to wheat and canola seed prior to

sowing. The low water solubility of the main active ingredient, allows extended uptake for long lasting protection of the plant, thus reducing the yield decline usually associated with the above-mentioned diseases (Crop Care, 2013).

1.8.2 Fungicide Resistance

Fungicide resistance is a stable, transferable trait that results in reduction of sensitivity to a fungicide by an individual fungus. This trait results from genetic changes in the pathogen and selection pressure put on the population. Fungicides with single-site mode of action are at relatively high risk for resistance development compared to those with multi-side mode of action. Many fungicides developed today have a single-site mode of operation because this is associated with lower risk of negative impact on the environment, and non-target organisms (APS, 2014).

Fungicide resistance is attributed to the change of a single major gene. Pathogen subpopulations are either sensitive or highly resistant to the pesticide. Resistance in this case is seen as complete loss of disease control that cannot be regained by using higher rates or more frequent fungicide applications. This type of resistance is known as qualitative resistance.

Fungicide resistance can also be attributed to change in several interacting genes. Pathogen isolates exhibit a range in sensitivity to the fungicide depending on the number of gene changes. Variation in sensitivity within the population is continuous. Resistance in this case is seen as an erosion of disease control that can be regained by using higher rates or more frequent applications. Long-term selection for resistance in the pathogen by repeated applications may eventually result in the highest labelled rates and/or shortest application intervals not being able to adequately control the disease. This type of fungicide resistance is known as quantitative resistance.

Isolates of fungi that are resistant to a particular fungicide might often be resistant to other related fungicides, even though they have not been exposed to these other fungicides, because they have similar modes of action. This process is called cross resistance. Fungicides that belong to the same Group Code are

likely to exhibit cross resistance. Sometimes negative cross resistance might occur between different fungicides, because the genetic change that causes resistance to one fungicide makes the resistant isolate more sensitive to another fungicide.

Efficient management of fungicide resistance is a vital strategy to prolong the duration that an at-risk fungicide may be functional. The purpose of resistance management is to delay its development rather than to manage resistant fungal strains after they have evolved. Resistance management procedures need to be encouraged when at-risk fungicides are first made available for commercial use. The aim of resistance management is to reduce use of at-risk fungicide without compromising disease control. This is achieved by using the at-risk fungicide with other less risky fungicides and with non-chemical control strategies, e.g. disease resistant cultivars, in an integrated disease management programme.

1.9 Alternatives to Fungicides

Success in controlling fungal pathogens has been recorded from the use of synthetic fungicides. They can, however, have side effects, such as potential toxicity to plants, animals and man, and they may also have a negative environmental impact. Fungicides can also be expensive, and indiscriminate application can result in resistance, which can negate their effectiveness. Based on these constraints, alternative strategies using applications of natural products and/or biocontrol agents (BCAs) may have a role in crop protection.

1.9.1 Use of natural products

Chemical control remains the main measure to reduce the incidence of post-harvest diseases in various foods. Antimicrobial chemicals belonging to the groups of benzimidazoles, aromatic hydrocarbons, and inhibitors of sterol biosynthesis are often used as post-harvest treatments; however, the application of high concentrations increases the risk of toxic residues in the products (Al-Omair and Helaleh, 2004; Baird *et al*., 1991; Šimko, 2005).

Therefore, there has been increased interest in research on using natural antifungal substances, which may replace synthetic fungicides or contribute to the development of new disease control agents. During the past 22 years, some essential oils (EOs) have been shown to possess a broad spectrum of antifungal activity (Thompson, 1989; Tian *et al*., 2011). Screening experiments with 41 aqueous and ethanolic extracts and 22 EOs against *Aspergillus* section Flavi strains have shown boldo, poleo, clove, anise and thyme oils as potential antifungal candidates (Bluma *et al*., 2008). In that study, EOs were screened for antifungal effect by direct addition to and diffusion in the media. However, recent studies have shown that smaller compounds such as monoterpenes are most efficient when used as headspace volatiles (Avila-Sosa *et al*., 2011). This characteristic makes EOs attractive as possible fumigants for the protection of stored products.

The antimicrobial efficacy of clove oil treatment of groundnuts at 50 and 100 µL mL^{-1} caused significant reductions in the *A. flavus* population compared to the control. The maximum reductions of *A. flavus* were reported as 6.7 $\log_{10}$ times on groundnuts treated with 50-100 µL mL^{-1} clove oil. In addition, *A. flavus* recovery after 3 days of storage was not detected. Three constituents, eugenol (89.8%), caryophyllen (4.7%) and vanillin (2.9%) representing 98.4% of the clove oil were identified. Clove oil suspensions can be used to enhance the microbial safety of groundnuts (Narumol and Jantamas, 2014).

Syzygium aromaticum (clove) is widely cultivated in Indonesia, Sri Lanka, Madagascar, Tanzania and Brazil. Previous studies has shown antifungal activity of clove oil and eugenol against yeasts and filamentous fungi, on several food-borne fungal pathogens (Lopez *et al*., 2005) and human pathogenic fungi (Chaieb *et al*., 2007; Gayoso *et al*., 2005*)*. *S. aromaticum* active ingredients of cinnamaldehyde and eugenol are noted as antifungal components against filamentous soil and seed borne fungi (Paranagama, 1991; Jayaratne *et al.*, 2002). Clove oil and eugenol have also been tested as antifungal agents in animal models (Ahmad *et al*., 2005; Chami *et al*., 2004). In order to further clarify the spectrum of antifungal activity and its relationship to chemical composition, some general considerations must be established regarding the study of the antimicrobial activity of EOs and the compounds isolated from them. Of the highest relevance is the definition of common parameters, such as the techniques employed, growth medium

and micro-organisms tested. Standardization of both the methods of analysis of the EO and the assays for *in vitro* testing is required so that research in this area can be systematic and objective and the interpretation of results validated. The limited knowledge concerning antimicrobial activity and the mechanism of action of plant extracts has led to the addressing of such issues, although the main antifungal action of phenolic compounds, such as eugenol, appears to be exerted on the cellular membrane (Carson *et al*., 2006; Cox *et al*., 2001).

Antifungal investigations revealed that garlic extract was effective against oilseed-borne toxigenic *Aspergillus* and *Penicillium* species (Ikeura *et al*., 2011; Tagoe *et al*., 2011). In general, garlic has been found to have potential antifungal properties (Pereira *et al*., 2006; Kanan and Al-Najar, 2008). Moreover, in a study by Muhsin *et al.* (2001) growth of 18 different fungal species was effectively inhibited by crude garlic bulb extract. Antifungal activity of garlic juice could be attributed to its phytochemical properties (Obagwu, 2003). Garlic allicin decomposes into several effective compounds, such as diallylsulphide, diallyldisulphide, diallyltrisulphide, allyl methyl trisulphide, dithiins and E,Z-ajoene, that serve as antimicrobial agents (Jabar and AL-Mossawi, 2007). Inhibitory effects of garlic juice against *Aspergillus* and *Penicillium* fungi suggest the possible use of garlic in controlling food-spoiling fungi. Meanwhile, the use of water-based juice provides an alternative to chemical solvents, which can be toxic at certain concentrations. Garlic juice was capable of inhibiting fungal growth, and it can be used as a source of antifungal compounds to prevent fungal infections of stored groundnuts (Tagoe *et al.*, 2011).

The effectiveness of garlic extract against a range of plant pathogenic organisms was tested *in vitro* and *vivo* in diseased tissues. Allicin in garlic extracts was quantified spectrophotometrically and a rapid bioassay was developed for routine use. Slusarenko and Schlaich (2003) reported on the efficacy of garlic in reducing downy mildew disease of *Arabidopsis thaliana* due to direct action against the pathogen, since no accumulation of salicylic acid (a marker for systemic acquired resistance, or SAR) was observed after application. Slusarenko *et al.* (2008) also reported on the efficacy of garlic in controlling plant disease due to the present of allicin as an active substance for disease control measures, which is compatible with organic farming. Allicin oxidizes thiol groups and appears to act as a redox toxin by disrupting the electrochemical cell potential and driving the cells into

apoptosis or necrosis in a concentration - dependent manner (Slusarenko and Schlaich, 2003).

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a major constituent of vanilla bean, an orchid (*V. planifola*, *V. pompona* or *V. tahitensis*). Vanilla is widely used in flavouring materials worldwide and is the second most expensive spice in the world next to saffron (Lubinsky *et al.*, 2008). Despite its broad utilization it had not been seriously researched for any bioactivity. However, it has been reported by some few researchers that vanilla might possess antimicrobial activity (Beuchat and Golden, 1989). Jay and Rivers (1984) found that vanilla was very active in suppressing moulds and non-lactic Gram positive bacteria. Lopez-Malo *et al*. (1995) investigated with different fruit based agar media containing mango, papaya, pineapple, apple and banana with 2000 µg mL^{-1} vanillin and incubated each with *A. flavus, A. niger, A. ochraceus*, or *A. parasiticus*. Vanillin concentration at 1500 µg mL^{-1} significantly inhibited all the strains of *Aspergillus* in all media. However, vanillin had less effectiveness in banana and mango agars. Vanillin has also been reported to possess anticlastogenic, antimutagenic and antitumour properties and, therefore, it can be considered as a nutraceutical molecule (Shyamala *et al.*, 2007; Sinigaglia *et al*., 2004). The antimicrobial property of vanillin is the effect of a phenolic compound which makes vanillin effective in inhibiting bacteria, yeasts and moulds. It is structurally similar to eugenol (2-methoxy-4-(2-propenyl) phenol) from clove and is known to be antimycotic (Beuchat and Golden, 1989) and bacteriostatic (Fitzgerald *et al*., 2004). At low concentrations, phenols affect enzyme activity, especially those enzymes associated with energy production, while at greater concentrations they cause proteins to denature (Prindle and Wright, 1977). In other work on *Aspergillus* infection of groundnut, Mondali *et al*. (2009) reported the efficacy of aqueous and alcoholic extracts of neem leaf, garlic, ginger and onion against seed-borne *A. flavus*, which showed that treatments were effective in inhibiting the pathogen. Srichana *et al*. (2009) screened the efficacy of betel leaf extract on the growth of *A. flavus*, and it was shown that the extract at 10,000 ppm concentration was highly significant in suppressing the tested pathogen. This, however, is an extremely high concentration and the commercial viability of this material must be questioned.

Over 280 plant varieties have been evaluated in Nigeria for their inhibitory effect on toxigenic *Aspergilli*; about 100 of these plants had antifungal activity on growth or toxin production by fungi (Montes-Belmont and Carvajal, 1998). Plants have the ability to synthesis many secondary metabolites. Components with phenolic structures, like carvacrol, eugenol and thymol, were highly effective in suppressing *Aspergillus* strains. They show an antimicrobial effect and can contribute to the control of pathogenic microorganisms (Paster and Bullerman, 1988). Clove oil and its major component, eugenol, have been screened extensively to control mycotoxigenic fungi and mycotoxins. On rice grains treated with 2.4 mg eugenol g^{-1}, the inoculum of *A. flavus* failed to grow and thus suppressed aflatoxin B_1 biosynthesis (Reddy *et al*., 2007). Jham *et al*. (2005) reported on the antifungal activity of cinnamon bark oil against *A. flavus*. More recently Dambolena *et al*. (2010) reported the constituents and the efficacy against *F. verticillioides* infection and fumonisin production of essential oils of *O. basilicum* L (Saint Joseph's Basil). and *O. gratissimum* L. (Clove Basil, African Basil) from different locations in Kenya. All the oils showed some inhibitory effect on the growth of the evaluated pathogen.

Passone *et al*. (2012) reported on the antifungal activity of five plant essential oils: from boldo *Peumus boldos* Molina, Poleo *Lippia turbinate* var. integrifolia, (Griseb.), clove *S. aromaticum* L., anise *Pimpinella anisum* and thyme *Thymus vulgaris.* These were tested against aflatoxigenic strains of *A. flavus* and *A. parasiticus* on groundnut-based media, conditioned at different water activities (a_W) of 0.98, 0.95, 0.93. The effects of EOs were assessed, when the oils were applied to groundnut meal extract agar, by recording the lag phase, growth rate, and aflatoxin B_1 accumulation of the tested pathogens. The results showed that lowest concentration (500 ppm) had no effect on the pathogens, but higher concentrations (2500 μL^{-1} for boldo and poleo; 1500 μL^{-1} for clove) completely inhibited the growth of *Aspergillus* spp. irrespective of the medium.

1.9.2 Use of Biocontrol Agents (BCAs)

Biological control is an efficient means of reducing the damage caused by plant pathogens through different mechanisms, including antibiosis, competition, suppression, direct parasitism, induced resistance, hypovirulence and predation (Cardwell and Henry, 2004). Such antagonistic activities of beneficiary microorganisms, associated with production of secondary metabolites for inhibition of plant disease causing pathogenic microorganisms, particularly *Aspergillus* species, have been reported (Howell, 2003). Biological control has been reviewed extensively in the past, including a recent review by Heydari and Pessarakli (2010).

The majority of existing biocontrol agents for management of soil-borne diseases were isolated from the rhizosphere. There is, however, a possibility to explore antagonists from other habitats as potent biocontrol agents (Manjula *et al*., 2002). Some biocontrol agents have also been isolated from seed surfaces (Guanlin *et al*., 1997).

Biocontrol preparations of both fungi and bacteria have been applied to seeds, seedlings and planting media in several ways to reduce plant diseases in the field with various degrees of success as reviewed by Alabouvette *et al.* (1996) and Baker (1990).

Trichoderma spp. are filamentous fungi commonly found in the soil community that are facultative saprophytes. They are members of a group of largely asexually reproducing fungi that range from very effective soil colonizers with high biodegradation potential to facultative plant symbionts that colonize the rhizosphere.

Trichoderma has been reported by many researchers as one of the most effective biocontrol agents against soil-borne, foliar and postharvest phytopathogenic fungal pathogens in crops through different mechanisms of interaction, such as colonization, antibiotics, and mycoparasitism (Noronha *et al*., 1996; Grondona *et al*., 1997; Sharma *et al*., 2012). This leads to overall improvement of plant health, enhanced nutrient availability and uptake, and finally encourages induced systemic resistance (ISR), similar to that stimulated by beneficial rhizobacteria (Harman *et al.*, 2004; Howell, 2003; Woo and Lorito, 2006).

Srilakshmi *et al*. (2013) investigated the bioactivity of secondary metabolites or small molecules produced by *Trichoderma* spp. and their efficacy against aflatoxin contamination in groundnut. The results indicated that 48 strains of *Trichoderma* were highly significant in suppressing an *A. flavus* isolate (AF11-4) and subsequently reduced aflatoxin production in groundnut. It is also feasible to apply BCAs in combination, sometimes including bacterial and fungal antagonists.

Thakur *et al*. (2003) stated that *Trichoderma* species were effective BCAs that provided more efficient protection against groundnut seed infection caused by *A. flavus* strain Af 11-4 than others. Seed contamination was reduced because of a significant reduction in the *A. flavus* population in the rhizosphere of groundnut.

Gachomo and Kotchoni (2008) stated that *Trichoderma* species showed a significant inhibitory effect against groundnut infection caused by *A. flavus, A. parasiticus, A. niger, A. ochraceus* and *Fusarium* species. The effectiveness of these microbes was related to extracellular enzymatic activities, such as amylolytic, chitinolytic, pectinolytic, proteolytic, lipolytic and cellulolytic, to eliminate other pathogenic microbes in the media. According to the authors the higher the enzymatic activities in the reaction, the greater the antagonistic effect in reducing infection by the pathogens, with an associated reduction in aflatoxin accumulation in the experimental samples.

Bacillus subtilis is a non-pathogenic Gram positive rod-shaped bacterium that can enhance plant growth, a growth promoting rhizobacterium (PGPR), which is among plant associated microorganisms that can inhibit plant diseases (Joseph *et al.*, 2004). In the 1990s, several PGPR products became commercially available in the United States and most of these contained strains of *bacilli* (Idiris *et al.*, 2002).

B. subtilis is used on plants as a biofungicide. It is also used on agricultural seeds, such as vegetables and soya bean as a fungicide. These bacteria colonize the root zones and compete with disease causing fungal organisms. *B. subtilis* fortunately does not affect humans. Morikawa (2006) stated that *B. subtilis* strains served as biofungicides for benefiting agricultural crops and antibacterial agents.

B. amyloliquefaciens at 10^9 colony forming units mL^{-1} was applied as seed treatment and significantly suppressed *Fusarium verticillioides* infection of root tissues of crop seedlings, according to Pereira *et al*. (2010).

Pseudomonas spp. are Gram negative bacteria which are often efficient root colonizers and biocontrol agents. BCA activity is usually mediated by production of antibiotics and other antifungal metabolites, including hydrogen cyanide and iron-chelating siderophores, active against soil-borne pathogens (O'Sullivan and O'Gara, 1992; Haas and Defago, 2005). A positive relationship was also discovered between the antifungal activity of chitinolytic *P. fluorescens* isolates and their level of chitinase production (Velazhahan *et al.*, 1999).

The suppressive effect of *Bacillus megaterium* was investigated against aflatoxin production and cyclopiazonic acid biosynthetic pathway gene expression in *A. flavus* and was reported by Qing *et al*. (2014). The results indicated that aflatoxin synthesis was reduced in tests undertaken; accumulation in potato dextrose broth and in liquid minimal medium was inhibited by co-inoculation with *B. megaterium*. The growth rate of the pathogen was also significantly reduced, and a gene expression assay indicated that fungal genes were down regulated by co-inoculation with *B. megaterium* across the entire fungal genome, and specifically within the aflatoxin pathway gene cluster (*aflT, aflF, aflS, aflJ, aflL, aflX*). According to the report, modulation of these genes can be used for efficient management of aflatoxin contamination in food crops such as corn, cotton and groundnut.

Although not studied in this research, greatest successes to date in biological control of aflatoxin contamination in both pre- and post-harvest crops have been achieved through application of competitive non-toxigenic strains of *A*. *flavus* and/or *A*. *parasiticus*. In Africa, non-toxigenic strain BN30 was very effective in reducing the amount of toxin produced in maize when co-inoculated with the highly toxigenic S-strain (Cardwell and Henry, 2004). In Australia, application of non-toxigenic strains could reduce aflatoxin formation in groundnuts by 95% (Pitt and Hocking, 2006). China has recently screened one highly competitive strain, AF051, from more than 30 non-toxigenic strains of *A*. *flavus* (Yan-ni *et al*., 2008). Field tests showed that this strain reduced naturally *Aspergillus* populations by up to 99% in the soil of groundnut fields.

Rosada *et al*. (2013) evaluated nonaflatoxingenic strains of *A. flavus* as a potential biocontrol agents, which were effective in their antagonistic effect in reducing the pathogen and also suppressing aflatoxin contamination in agricultural crops, including groundnut and maize.

Zanon *et al*. (2013) reported that BCAs significantly reduced aflatoxin accumulation in Argentinean groundnut by up to 71% in treated plots with different treatment levels. The authors also stated that a nontoxigenic strain of *A. flavus*, AFCHG2, could be applied to reduce aflatoxin contamination in groundnut.

Horn and Dorner (2009) reported on the efficacy of isolates of atoxigenic *A. flavus* and *A. parasiticus* on aflatoxin contamination of wounded groundnut seeds inoculated with agricultural soils containing natural antagonistic fungal populations. According to the researchers, aflatoxin suppression depended on both the density of the aflatoxin-producing pathogens and the fungal isolate used for biological control. Wild type isolate of *A. flavus* NRRL 21882, and its *niaD* mutant, were also effective in significantly suppressing aflatoxin in groundnut, revealing that nitrate-nonutilizing mutants, which are easily monitored in the field, can be used as BCAs.

Dorner and Horn (2007) reported that single and combined application of atoxigenic isolates of *A. flavus* and *A. parasiticus*, for biocontrol of aflatoxin in groundnut, successfully reduced aflatoxin by an average of 91.6%. Regression analysis showed a strong significant correlation between the presence of nontoxigenic strains in groundnuts and aflatoxin suppression. According to the authors, an *A. flavus* strain single application proved more effective than the *A. parasiticus* single isolate, which was as effective as the combination treatment.

The International Institute of Tropical Agriculture (IITA, 2011) reported the importance of biocontrol and Aflasafe products as new strategies for fighting aflatoxin in the field. Aflasafe is a safe and cost effective biocontrol product that reduces aflatoxins in both field and stores. It contains a mixture of four nontoxigenic strains of *A. flavus* of Nigerian origin. The World Bank, funding an agriculture development project, bought 5 tonnes of Aflasafe and distributed it to Nigerian farmers from 2004 - 2007 years. Bandyopadhyay *et al*. (2007) reported that Aflasafe significantly reduced aflatoxin contamination in maize and groundnuts by 80-90% and in some cases by up to 99%. A type of Aflasafe, SN01, has signficantly reduced aflatoxin in

groundnut by 85–95% and has been adopted in Senegal and other African countries. Due to the success achieved, The Gambia's National Agriculture Research Institute (GNARI) and the Gambian Groundnut Corporation (GGC, the largest groundnut exporter) requested IITA to develop a local version of Aflasafe for The Gambia. The Economic Community of West African States (ECOWAS) also reinforced Aflasafe and offered assistance to obtain political support from the Gambian government (The Gambian Government endorses Aflasafe SN01 22nd September 2014).

Combinations of microorganisms may be needed to control different diseases that affect the same crop. BCAs are specific only for a given type of target pathogen (Sivan and Chet, 1993). Although this property represents an advantage from the environmental point of view, it creates great difficulties to the growers who may need to control several plant pathogens in the same crop. Moreover, the combination of two or more antagonists also requires multiple registration processes, with increased costs and difficulties in matching all the studies required by strict legislation.

However, this option could be feasible with products already registered. Biofungicides based on different antagonistic strains may be labelled as compatible with each other and proposed for joint use. Alabouvette *et al*. (1996) demonstrated that a synergistic effect can be obtained in controlling *F. oxysporum* f.sp. *radicis-lycopersici* by combining a fluorescent *Pseudomonas* sp. with a non-pathogenic *F. oxysporum*. The non-pathogenic *Fusarium* competes for carbon sources, while the bacterial antagonist produces a siderophore competing for iron (Lemenceau *et al*., 1993). Moreover it was noted that the antagonistic strain Fo47 was less sensitive to pseudobactin-mediated iron competition than the pathogenic *F. oxysporum* f.sp. *dianthi*. Park *et al*. (1988) also showed that interaction between the bacterium *Pseudomonas putida* and saprophytic strains of *F. oxysporum* could achieve effective control of *F. oxysporum* f. sp. *cucumerinum*. A positive, possibly synergistic, interaction between *Trichoderma* spp. strains and bacterial antagonists such as *Pseudomonas syringae* has been reported for combined applications in the control of plant pathogens (Whipps, 1997). Part of the mechanism could be explained by the positive interaction among the lipodepsipeptides of the bacterial antagonist and the fungal cell wall-degrading enzymes of the fungal biocontrol agent (Fogliano *et al*., 2002). Effective control was also demonstrated for the combination of *T. harzianum*, protecting against

infection by *Pythium ultimum* in the rhizosphere, and *Pythium nunn* reducing inoculum density of the same pathogen in the soil mass (Paulitz *et a*l., 1990). By combining specific strains of microorganisms, multiple traits antagonizing the pathogen can be combined and this may result in a higher level of protection. When *P. putida* strain WCS358, competing for iron through the production of its pseudobactin siderophore, was combined with *P. putida* strain RE8, inducing systemic resistance against *F. oxysporum* f.sp. *raphani*, fusarium wilt suppression was significantly enhanced (de Boer *et al*., 2003). Previously, a mixture of three different plant growth-promoting rhizobacteria (PGRP), applied as a seed treatment, showed intensive plant growth promotion and reduction of multiple cucumber diseases (Raupach and Kloepper, 1998). Another innovative approach for improving soil-borne disease control could be the development of cocktails containing strains that communicate with each other (through quorum sensing) to maximize antibiotic production and disease control (Becker *et al*., 1997; Davelos *et al*., 2004). Hoitink *et al*. (1991) incorporated several antagonists in combination in peat substrates rendering them disease suppressive. A broad-spectrum biological control of *Pythium*, *Phytophthora*, and *R. solani* requires the introduction into or presence of organic nutrients in the soil to maintain several taxa of biocontrol agents (Hoitink and Boehm, 1999). The composition of the microflora active in control changes as the organic matter decomposes, while the microbial carrying capacity of the amendment declines**.** Bagwan (2011) evaluated a combination treatment of five biocontrol agents in aflatoxin B_1 management applied at preharvest to groundnut cultivar GG-20. The results revealed that the combination of *T. viride, B. subtilis* and *P. fluorescens* was highly effective in reducing the *A. flavus* population in the rhizosphere, also eliminate aflatoxin and infection and colonization of seeds.

Waliyar *et al*. (2008) evaluated pre- and post-harvest management of aflatoxin contamination in groundnut. Strategies were formulated at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), with its partners, for an integrated strategy to manage *A. flavus* infestation and aflatoxin contamination. Combining the following steps would be useful to low resource farmers in developing nations: host plant resistance; soil amendments with lime and organic supplements to enhance water holding capacity, plant vigour and healthy seed; timely harvesting and postharvest drying techniques; the use of antagonistic biocontrol agents, such as

Trichoderma and pseudomonads, and awareness campaigns and training modules to disseminate methods to the final users. This approach can successfully successfully suppress aflatoxin contamination in groundnut in West and Central Africa. Becaause this strategy is simple, cost effective and suitable for subsistence agriculture in developing countries.

1.10 Objectives

The aim of this study was to evaluate naturally-occurring plant oils, and BCAs with a past history of efficacy, as alternatives to fungicides for reduction of *Aspergillus* infection and aflatoxin accumulation in groundnut. Control agents were evaluated singly and in combinations, for the control of both pre- and post-harvest infection. Specifically, the objectives were:

1. Confirmation of the identity of *A. niger* from Nigerian groundnut and two isolates derived from a commercial granular formulation of *Trichoderma.*
2. Evaluation of *in vitro* and *in vivo* antifungal activities of plant essential oils against two strains of *A. niger* and *A. flavus.*
3. Determination of *in vitro* and *in vivo* antifungal, antibacterial activities of BCAs against two strains of *A. niger* and *A. flavus.*
4. Evaluation of competitive antifungal and antibacterial activities of EOs against BCAs.
5. Assessment of the efficacy of combination and single treatments tested above as a postharvest treatment for groundnut pods.
6. Evaluation of the treatments as seed dressings to prevent seedling infection, in comparison to a standard fungicide (Jockey; ai fluquinconazole + prochloraz).
7. Quantification of aflatoxin in infected groundnut seeds and pods treated with BCAs and plant oils, in relation to applied post-harvest treatments.
8. Quantification of infection by DNA assessment using a loop-mediated isothermal amplification (LAMP) assay.

Chapter 2 General Methods

2.1 Culture media

All microbiological media were supplied by Sigma (Dorset, UK) or from Oxoid (Basingstoke, UK). All media were prepared according to the manufacturer's instructions and sterilised by autoclaving at 121^0C for 20 min.

2.2 Source of biological materials

2.2.1. Groundnut seeds

Groundnut seeds were initially procured from two locations, Abua and Yenagoa, in the Niger Delta region of Nigeria. Seed supply was inconsistent, so an alternative arrangement was made. Groundnut pods were purchased from Tesco and seed proved viable in the experiments.

2.2.2 *Aspergillus* spp.

Table 2.1 Source of *Aspergillus* isolates

Aspergillus spp.	Source	Isolate code	Origin
A. flavus	Professor David Archer	ATCC204304	Human sputum in Virginia
A. flavus	CABI	AF364493	Peanut in Brazil
A. niger	Self	Internal reference AA	Nigerian infected groundnut pods
A. niger	CABI	AN42054	Peanut in Tanzania

Isolates of *A. niger* were selected and tested for pathogenicity on groundnut pods. Once their pathogenicities was established, they were considered to be appropriate for the work reported in this thesis and their use was continued. Two isolates were used throughout the programme, to ensure consistency of control of the pathogen species. Two *A. niger* isolates were either purchased from CABI (AN42054) or isolated from infected groundnut seed obtained from Nigerian sources. The pathogenicity was again confirmed and use of two isolates provided are indication of the consistency of disease control.

A. *flavus* strains were either donated by Professor David Archer (University of Nottingham) (ATCC204304) or purchased as a stock culture from CABI (AF364493). They were tested for pathogenicity on groundnut pods and their ability to elicit aflatoxin accumulation. It was essential that toxigenic isolates were used. Once these parameters were established it was deemed that both isolates were suitable for this research to evaluate the consistency of efficacy of the control programmes.

2.2.3 Method for confirmation of identity *A. niger* (Nigeria) isolate

One hundred seeds were used from the two Nigerian locations. Seeds were ground with mortar and pestle. (100 mL) was added to the samples, which were vigorously shaken and filtered through sterile muslin to remove debris. Aliquots (20 µL) of the filtrate were spread onto the surface of PDA plates containing 1 mL L^{-1} of streptomycin and 0.5 mL L^{-1} of penicillin (from filter-sterilised stock solutions comprising 300 mg of penicillin and 1330 mg of streptomycin in 10 mL of RO water), to prevent bacterial contamination. Plates were incubated at 30^0C for 7 d. Colonies with black conidia were identified on the PDA plates. Slides were prepared from the conidia and viewed using a compound microscope, to see the morphology of the conidial structure (Frank, 2006). The putative *A. niger* from Nigerian groundnut was then further screened, using PCR and DNA sequence identity analysis to confirm its identification.

2.2.3.1 DNA extractions for PCR identification of *A. niger*

All extractions of genomic DNA from fungi were performed with an extraction kit (DNeasy® Plant Mini Kit (50), QIAGEN, GmbH). DNA extracted from *A. niger* at the first attempt was impure. The extract was thus further purified using a Micro Bio-Spin Chromatography column purification method, where polyvinylpyrrolidone (PVP) was used as a pre-prepared column, to which the extracts were added before elution by centrifugation at 1000 g (Bio-Rad, UK). This purified the DNA, prior to PCR amplification.

2.2.3.2 PCR primers and amplification

Two oligonucleotide fungal primers were used for *A. niger* identification (White *et al.,* 1990). The ITS region primers (ITS 1, 5' TCC GTA GGT GAA CCT GCG-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT G-3') were used on the conserved regions of the 18S (ITS1) and the 28S (ITS4) rRNA genes. The intervening ITS and 5.8S region was used for PCR amplification. The PCR conditions were: denaturing (95^0C), annealing (50^0C) and extension (72^0C) for both *A. niger* and *Trichoderma* spp.

2.2.3.3 Agarose gel preparation and electrophoresis

Agarose gels were prepared by suspending agarose at 1-1.5% in 1X Tris-Borate-EDTA (TBE) and dissolved using a microwave oven. Ethidium bromide (0.5 µg L^{-1}) (Fisher Scientific UK Limited, Loughborough, UK) was dispensed to the solution which was cooled to 60^0C. The solution was thoroughly mixed manually and dispensed into a plastic plate mounted with a comb. Instantly, and before the gel solidified, bubbles seen around the comb tips and on the surface of the gel were removed with a pipette. After the solidification of the gel, the comb was gently removed to allow appropriate loading of dye, DNA or PCR products. Electrophoresis was carried out at 90 V for 60 to 80 min, after which DNA was visualised under UV illumination and photographs taken.

2.2.3.4 Gene sequencing and alignment

PCR fragments were sequenced by Eurofins MWG Operon, Germany. Sequences were aligned and analysed by using BioEdit software (Biological Sequence alignment editor 7.0.9).

2.2.4 Biocontrol agents

The following biocontrol agents (BCAs) were used throughout this research. These were provided as microorganisms with a prior history of efficacy against fungal plant pathogens. The biocontrol agents used in this evaluation are fungi *Trichoderma harzianum*, *T. asperellum* and *T. viride* and bacteria *Pseudomonas chlororaphis* spp*. aureofaciens* and *Bacillus amyloliquefaciens* (MBI600, 62P and 66P). The mean zone of inhibition of *Aspergillus* growth was measured in mm after seven days.

2.2.4.1 *Bacillus amyloliquefaciens*

Stock cultures of 62P and 66P were originally isolated from lettuce leaves by Dr. Rozeita Laboh (School of Biosciences, University of Nottingham), in a programme to evaluate antagonists of *Botrytis cinerea* on that crop. MBI600 (originally identified as *B. subtilis*), was isolated by Dr. Stephen Rossall in a programme to identify seed antagonists of *Botrytis* on *Vicia fabe*.

2.2.4.2 *Pseudomonas chlororaphis*

This strain was donated by Dr. Rupert Fray (School of Biosciences, University of Nottingham). This antagonist has previously been shown to have activity against take-all of wheat and against phoma leaf spot on oilseed rape.

2.2.4.3 *Trichoderma harzianum* (T-22)

This stain has a widely published history as an antagonist against many plant pathogens (Abdel-Kader *et al*., 2013; Mastouri *et al*., 2010; Sharma *et al*., 2012). This was kindly donated by Professor Stephen Woodward (University of Aberdeen).

The commercial preparation of *Trichoderma* TUSAL (shown to comprise *T. asperellum* and *T. viride*) was donated by Certis (UK) Ltd.

2.2.4.4 Application methods of BCAs represented in a tabular form

Table 2.2 Application methods of BCAs

BCAs	Spore suspension	Treatment for i*n vitro* application	Treatment for *in planta* application
T. harzianum *T. asperellum* *T. viride* MBI600 62P 66P *P. chlororaphis*	Conidia of *Aspergillus* strains were harvested and placed at the centre of PDA plate for point inoculation assays.	*Trichoderma* spp. spores were used in point inoculation against the pathogen in PDA plate. Colonies of bacteria were harvested with sterile plastic loop and placed on the PDA plate, to test against the tested pathogen. Compatibility of BCAs with EOs using the point inoculation *in vitro* plate assay was also assessed before combination treatments were evaluated.	Groundnut seeds were dipped into 10^6 mL^{-1} diluted spores of *Trichoderma* spp. in a plastic beaker and manually shaken for 5 min. Treated seeds were air-dried for 24 h inside a laminar flow cabinet. Thereafter planted in pathogen amended compost. 48 h stationary phase cultures of bacteria in TSB were applied as described in the above protocol. *T. harzianum* and essential plant oil combinations were applied as a groundnut seed treatment (T-22 10^6 mL^{-1} + camphor/clove oils at 0.01, 0.1, and 1%).

Plant essential oils

Plant essential oils are composed of volatile aromatic compounds, extracted from plants by steam distillation. The 13 plant essential oils used in this study were obtained from Sigma, except vanillin oil which was supplied as formulated product by Omex Ltd. The oils tested were derived from clove *Syzygium aromaticum*, camphor *Cinnamomum camphora*, vanilla *Vanilla planifolia*, garlic *Allium sativum*, galangal *Alpinia galanga*, green oregano *Origanum vulgare*, lemon grass *Cymbopogon citratus*, neem *Azadirachta indica*, ginger *Zingiber officinale*, basil *Ocimum basilicum*, tea tree *Melaleuca alternifolia*, thyme *Thymus vulgaris* and onion *Allium cepa*. Initially, to evaluate the efficiency of performance of these oils *in vitro* experiments against the two species *A. flavus* and *A. niger* were undertaken, using seeded plate and point inoculation assays and later by using *in planta* tests in pathogen-amended compost.

2.2.6 Plant cultivation

Compost used for groundnut seed cultivation in this study was John Innes No.2, plus silver sand of low nutrient content at a 1:1 ratio.

2.3 Culturing of fungi

2.3.1 *Trichoderma harzianium* (T-22)

The T-22 strain was sub-cultured on to antibiotic-amended PDA and also in slant culture in 25 mL universal tubes. Cultures were incubated at 20^0C until sporulation occurred.

2.3.2 Preparation of a suspension of commercial granule *Trichoderma*

Five granules of the commercial *Trichoderma* product, TUSAL, were suspended in 10 mL, and shaken with a vortex mixer to disrupt the granules. Aliquots (100 µL) of suspension were pipetted and spread onto solidified PDA containing antibiotics, using a sterile plastic inoculation loop. Inoculated PDA plates were incubated at 20^0C for up to 14 d, to permit the

isolation of discrete colonies. Individual colonies were sub-cultured onto fresh PDA plates, to obtain pure isolates of the components in the mixed product.

2.3.3 *Aspergillus* spp

Aspergillus spp. spores were harvested with sterilised scalpel / plastic loop and inoculated onto antibiotic amended PDA plates and incubated at 20^0C until sporulation occurred.

2.4 Culturing of bacteria

2.4.1 Petri dish cultures on solid medium

Three isolates of *Bacillus amyloliquefaciens* previously shown to have biocontrol activity (Pereira *et al*., 2010) in other systems (MBI 600, 66P and 62P), were cultured on unamended PDA plates, by zig-zag inoculation with a sterile plastic loop dipped into thawed glycerol stocks, which had been maintained at -80°C. Plates were incubated at 34^0C for 24 h.

2.4.2 Liquid cultures

Liquid cultures of *B. amyloliquefaciens* isolates were prepared in tryptic-soy broth (TSB). Thirty grams of TSB was dispensed into 1 L of reverse osmosis (RO) water, vigorously shaken and autoclaved at 120^0C for 15 min. Aliquots (100 mL) of solution were dispensed into sterile 250 mL conical flasks, inoculated with a loop of the required bacterial strains harvested from a plate and incubated in a shaking incubator at 30^0C for 48 h. The resultant suspensions were used to treat groundnut seeds and pods. Pods or seeds were dipped into 48 h bacterial liquid cultures for 5 min and air dried for 24 h in a microbiological safety cabinet. Samples were inoculated with 20 µL droplets of *Aspergillus* suspensions as described in section 2.9.2 and incubated at 20^0C for 14 d.

2.5 Fungal spore suspensions

Aspergillus spores were suspended in SDW by scraping Petri dish PDA cultures to dislodge conidia. Suspensions were filtered through sterile muslin to remove debris, then diluted with SDW according to the requirement of the experiment, after enumeration using a haemocytometer (Improved Neubauer, Weber Scientific International, Sussex, UK).

2.6 Bacterial cell suspension

Liquid cultures of *Bacillus* strains (48 h) were serially diluted in maximum recovery diluent (MRD) which was previously used by Auty *et al*. (2001). Aliquots (100 µL) of several dilutions of each of the strains were pipetted and spread onto the surface of nutrient agar plates. Plastic loops were used to gently spread the suspensions uniformly on the surface of the agar plates which were incubated 30^0C for 24 h before colony counting.

2.7 *In vitro* bioassays

Two different temperature 20^0C and 30^0C, were used in plate assays to check the potential effectiveness of groundnut disease management for both temperate and tropical climates.

2.7.1 Biocontrol agents (BCAs) / Essential oils (EOs)

In vitro assays were used in order to ascertain the intrinsic activity of BCAs and EOs against *Aspergillus* spp. These comprised:

2.7.2 Seeded plate assay

In vitro assays were used to assess the activity of BCAs or plant essential oils against the pathogens. Seeded PDA plates were prepared using sterilized medium which was melted in a microwave oven, then cooled in a water bath to 45^0C. Enumerated spore suspensions of the pathogens were dispensed into the molten PDA and the medium poured into Petri dishes. Plates contained 10^6 *Aspergillus* spores mL^{-1} agar.

A flame-sterilized cork-borer was used to create wells in the agar at three different positions, in a triangular form, surrounding the inoculum at the centre. Each replicate plate had wells filled with 10 µL of different concentrations of the oils tested (0.01, 0.1, and 1% (v/v)) EOs were diluted in methanol. A pure methanol control gave no zones of inhibition. The solvent evaporated before fungal growth occurred. Treatments were replicated 4 times in Completely Randomized Block Design (CRBD). The method was also used to assess the activity of EOs against *Trichoderma* spp. to determine the feasibility of combining control strategies. Inoculated plates were sealed with micropore tape and incubated for 7 d at 20 and 30^{o}C before assessment of the size of zones of inhibition of *Aspergillus* growth (mm).

2.7.3 Point inoculation assay

Biocontrol agents were point inoculated onto PDA plates 6 cm from the site of *Aspergillus* to assess antifungal activities by measurement of zones of inhibition between the colonies.

2.8 Viability test

Viability of groundnut seeds was ascertained by placing ten seeds in moistened seed germination test paper in a damp chamber. Each test was replicated four times. Percentage of seed germination was evaluated after 5 d incubation at 30^{0}C.

2.9 *In Planta* assays

2.9.1 Postharvest infection

A. flavus strains (ATCC204304 and AF364493) were used to inoculate groundnut artificially to cause aflatoxin accumulation for treatment evaluation. This involved the following processes:

2.9.2 Groundnut whole pod assay

Groundnut pods were inoculated with 20 µL droplets of *Aspergillus* spp., at spore concentrations of 10^6, 10^5, 10^4 mL^{-1}. Pods were arranged in tissue-lined damp chambers, supported on plastic mesh covering damp tissue paper. Inoculated pods were incubated at 20^0C for 14 d before assessment of pathogen infection. Visible signs of *Aspergillus* infection were assessed on inoculated pods treated with BCAs and EOs. Infection was assessed by determination of visible seed infection in ten seeds derived from five Pods.

2.9.3 Groundnut half seed assay

Aspergillus spore suspensions (20 µL droplets) were pipetted onto each half of 30 groundnut seeds, both wounded and unwounded, in damp chambers as described above. Seeds were wounded with hypodermic needles before spores were dispensed on the wound. Unwounded groundnut seeds were inoculated with spores on the surface of the transverse section of the seeds. Inoculated seeds were incubated at 20^0C for 5 d before assessment.

2.9.4 Preparation of pathogen-amended compost and plant infection assay

Compost was weighed in a 9 cm diameter plastic pot and sufficient compost for 20 pots was added to a cement mixer. The compost was amended with *Aspergillus* conidia to give final pathogen densities of 10^4, 10^3 and 10^2 conidia mL^{-1} by thorough mixing for 10 min, before sowing seed for disease assessment. Three seeds were planted per pot maintained at 27 ^{0}C day temperature and 20^0C night temperature with 16 h photoperiod for 4 weeks before assessment. The temperature used in this assay was selected to be comparable to a tropical climate. Assessment of the number of plants present was made after 14 d to determine treatment effects on emergence, and at 27 d post-sowing to ascertain effects on post-emergence survival.

2.9.5 Treatment of groundnut pods and seeds with EOs and BCAs

Groundnut pods and seeds were treated with water-diluted plant oils at 0.01, 0.1, and 1% (v/v) concentration, by suspending the plant materials in the solutions and manually shaking for 5 min. They were then air-dried for 24 h in a laminar flow cabinet before pathogen inoculation. The near-commercial product based on vanillin (VNX; Omex Ltd) was applied by dipping seeds in a 1.5% aqueous solution of the product, which contained 25% active ingredient. Aqueous suspensions of BCAs were applied by dipping groundnut seed in stationary phase TSB cultures and drying, as described for EOs. For conventional fungicide comparisons, seeds were treated with Jockey produced by BASF (fluquinconazole + prochloraz; 167 + 31 g L^{-1}) at a rate equivalent to 45 g product per 100 kg seed by shaking aliquots of seed for 3 min.

Initially disease development assessment was evaluated with 10^4, 10^3 and 10^2 mL^{-1}, before the best (10^3) concentration for disease development was chosen. Compost was amended with 10^3 spores mL^{-1} of the pathogens suspended in sterile water, before 3 seeds per pot were sown. Comparisons were made between emergence and survival in untreated and pathogen-amended compost. The conventional fungicide seed treatment product Jockey was used for comparative purposes. The efficacy of BCAs and EOs was assessed for four replicates for each treatment.

2.10 Detection of aflatoxin

An ELISA test kit was used to detect aflatoxin in *Aspergillus*-inoculated groundnut pods. Groundnut pods were surface-sterilized in 0.5% sodium hypochlorite for 2 min, rinsed with SDW for 3 min, and air-dried in a microbiological safety cabinet. Seeds were placed in seed germination test boxes (15 seeds per box) and inoculated with 10^4 mL^{-1} spores of *A. flavus*, as described in section 2.9.2. Each treatment was replicated 3 times and the control comprised uninoculated seed. Boxes were incubated at 20^0C for 4, 6, and 8 d.

2.10.1 Extraction of aflatoxin

Aliquots (5 g) of seed were macerated in a mortar and pestle in 25 mL 70% aqueous methanol. The extract was shaken vigorously for 3 min before filtration through Whatman No 1 filter paper. Extracts were diluted at a 1:20 ratio in RO water before assay. Aflatoxin extraction method used was previously applied by Aycicek *et al.* (2005).

2.10.2 Evaluation of aflatoxin accumulation

The procedure used was the R-Biopharm AG RIDASCREEN® protocol. Aliquots (50 µL) of standard or prepared samples were pipetted into 96-well ELISA plates. Enzyme conjugate (50 µL) was added to the bottom of each of the 96 wells. Aliquots (50 µL) of anti aflatoxin antibody solution were also added to each well, mixed gently by shaking the plate manually and incubated for 30 min at room temperature. The liquid was poured out of the wells and the micro-well holder was tapped upside down and vigorously shaken 3 times to remove the liquid. Wells were then filled with 250 µL of washing buffer and shaken dry to remove residual material. This was repeated twice.

Aliquots (100 µL) of the substrate / chromogen were added to the wells, which were mixed gently by shaking the plate manually before incubation for 15 min at room temperature in the dark.

Finally, aliquots (100 µL) of stop solution were added into each well, and mixed gently by shaking. Absorbance to determine aflatoxin concentration was measured within 15 min after the addition of the stop solution using a microtitre plate spectrophotometer. Aflatoxin concentration was then determined by comparison to standards provided, according to the manufacturer's instructions.

2.11 DNA extraction

Aliquots (10 g) of groundnut seed tissues were homogenised in a FastPrep machine as described in section 2.11.1. Extraction of DNA using the Boline kit was then performed with the following stages. Buffer PA1 (400 µL) was pipetted into tubes that contained samples and vortexed to mix thoroughly. *RNase A* solution (10 µL) was also added and mixed thoroughly and incubated at 65^0C for 10 min. The lysate was filtered with ISOLATE II filter into a new 2 mL collection tube, dispensed onto columns and centrifuged for 2 min at 11,000 *g*. Clear liquid was collected and the filter discarded. Thereafter, 450 µL of binding buffer PB was added and mixed thoroughly by pipetting up and down 5 times. An ISOLATE II plant DNA spin column was placed into a new 2 mL collection tube and 700 µL of the extract was added, centrifuged for 1 min at 11,000 *g* and the flow through discarded, to enable the DNA to bind. The silca membrane in the spin column was washed by adding 400 µL washing buffer PAW1 and centrifuged for 1 min at 11,000 *g* and the flow through was also discarded. The process was repeated using 700 µL of washing buffer PAW1. Another 200 µL aliquot of washing buffer PAW2 was finally added and centrifuged for 2 min at 11,000 *g* to remove the wash buffer and to dry the silica membrane completely. Finally, pure DNA was eluted by placing the spin column into a new 1.5 mL micro centrifuge tube. Preheated buffer PG (50 µL at 65^0C) was added to the centre of the silica membrane and incubated 5 min at 65^0C and centrifuged for 1 min at 11,000 *g*. The process was repeated to elute any remaining DNA. The method is based on that described by Niessen and Vogel (2010).

2.12 Detection of *A. flavus* in groundnut seeds in asymptomatic pods

Pods and seeds inoculated with *A. flavus* as described above were routinely scored by assessing visible symptom development. However, asymptomatic infection could occur, ultimately leading to aflatoxin accumulation. Infection was therefore further evaluated by alternative assessment of infection of seeds within previously inoculated pods. This was achieved by surface sterilisation and plating out half seeds to detect *Aspergillus* growth, and DNA extraction from the other half of seeds, before LAMP detection of *Aspergillus* DNA.

2.13 Surface sterilisation and plating out

Half seeds were sterilized with NaOCl (8% available chlorine) for 5 min, then rinsed with SDW, plated onto antibiotic amended PDA then incubated for 5 d at 20^0C before assessment of seed infection by detection of typical *Aspergillus* morphology. This was undertaken to validate the LAMP assay as a method to quantify seed infection. The other half seed was used for DNA extraction, prior to LAMP evaluation.

2.14 Groundnut tissue lysis before DNA extraction

Groundnut tissues were lysed with a FastPrep tissue disruptor. Samples (0.1 g) were added to tubes that contained eight 2 mm glass beads and dipped into liquid nitrogen for 5 min. Thereafter, tubes were inserted in the FastPrep machine, which was run at maximum speed for 40 s. Tubes were again placed in liquid nitrogen for 5 min. The process was repeated as required to achieve necessary tissue disruption.

2.14.1 DNA extraction for LAMP assays

Five different procedures were initially evaluated for DNA extraction. These were:

- Macherey-Nagel (MN), Genomic DNA from plant tissue;
- Nucleo Spin® Plant 11 (December 2010/Rev.05)
- Sigma, Gen Elute™ Plant Genomic DNA Mini Prep Kit
- DNeasy® Plant Mini Kit (50), QIAGEN, GmbH
- Bioline Isolate II Plant DNA Mini Kit

Extracted DNA which gave the best results in the subsequence LAMP assays was obtained using the Bioline kit, which was then routinely used. DNA was quantified using a Nanodrop spectrophotometer.

2.14.2 DNA amplification

A loop-mediated isothermal amplification (LAMP) assay was carried with different primers: fungal primers: (Apara ID153 and Alpha ID58) and plant DNA Primers (Cox primer) were used for the assays and were donated by Prof. Matthew Dickinson, University of Nottingham, UK. These primers increased the concentration of DNA produced during the LAMP assay reaction according to the protocols described in Luo *et al*. (2012), Niessen and Vogel (2010) and Nagamine *et al.* (2002). The sequences of the Cox and fungal primers used are given in Table 2.3.

Solutions for LAMP assay were prepared from the master mix of primers (Luo *et al*., 2012), both forward and reverse primers. Eight reaction samples were prepared thereafter from the master mix, comprising 46 µL water, 23 µL primer mix and 115 µL isothermal enzyme master mix (Optigene Ltd). Aliquots (20 µL) from the mixture were dispensed into eight LAMP assay tubes and 1 µL DNA of *A. flavus* infected half groundnut seeds was added. Samples were placed in the Genie II machine for 30 min to evaluate the level of amplification and annealing of fungal and plant DNA products (Figure 2.1).

Table 2.3 LAMP primers. Forward and backward inner primers (FIP,BIP), outer primers (F3,B3), and loop primers (F3,B3)

Primers Plant DNA	5'-3' oligonucleotide sequence
COXY	TATGGGAGCCGTTTTTGC
COXB3	AACTGCTAAGRGCATTCC
COXFIP	ATGGATTTGRCCTAAAGTTTCAGGGCAGGATTTCACTATTGGGT
COXBIP	TGCATTTCTTAGGGCTTTCGGATCCRGCGTAAGCATCTG
COXFL	ATGTCCGACCAAAGATTTTACC
COXBL	GTATGCCACGTCGCATTCC
Aspergillus	
FIP-Anom ID9	CCG GGT CAC CGT TGA GGA CTT GGC CTG GAT ACA ACA AAG C
BIP-Anom ID9	TGT CCC TAC CAG GAC GTC ATG GGG GTG AGA CTG CAA GAA GAG
F3-Anom ID9	AAC ACG TCC AGA AGG ACT TC
B3-Anom ID9	ACT GGT TTT CAT CCG GCT TG
LoopF-Anom ID9	CCG ATG CAG TAC ACG CCT G
LoopB-Anom ID9	CGG CGT ACT GAA CTA CCC AA
FIP-Afla ID58	TAG ACC TGC TTG AGC ACG CCA TGA GGG AGG CTG GTA TCC
BIP-Afla ID58	ÁGG TCA GCA AGG GCA ACA TCC GGC CCA GGA GTA GTC GAT AG
F3-Afla ID58	ACC GCT GTT GCT AAG AAC AA
B3-Afla ID58	TTACGG ACG AGA CCG AGC
LoopF-Afla ID58	ATG TCC TCA AAG GTC TCG GG
LoopB-Afla	GAG CCT GTT CCC CCT AAG AT
FIP-Apara ID153	CCT GGG TCT GAT CCT CAT AGT CCA GTT CCC AAG ACT ACT TCC
BIP-Apara ID153	TTG AGA ATT GCT GGC TAG GAG ATG TAC CAT TCA TTT TTG ACC TCA TC
F3-Apara ID153	TTA CAG TGT GTT TAA ACC GTT
B3-Apara ID153	GTA GTT CGA TAC CAA TGT TCC
LoopF-ApaID153	TTG AAT GAG ACA GAA CGA GT
LoopB-ApaID153	TTG CCT GAT CTT GAT ACC A

The forward inner primer (FIP) consisted of the F1 complementary sequence and the F2 direct sequence, the backward inner primer (BIP) consisted of the B1 direct sequence and the B2 complementary sequence. F1c, sequence complementary to F1; F2c, sequence complementary to F2; B3c, sequence complementary

Figure 2.1 Genie II machine used for *Aspergillus* DNA.

Optigene's Genie® II is a lightweight and robust compact apparatus which is good for both field and laboratory, because it requires little technological training. It was designed specifically to run any isothermal amplification assay that is employed for targeted pathogen detection by fluorescence evaluation.

2.15 Data analysis

Initial data analysis was undertaken using Microsoft Excel 2010. For general analysis of variance (ANOVA), Genstat version 16 was used. Fisher's least significant difference (LSD) with a significance level of 5% was used to identify significant differences between means.

Chapter 3 Determination of *in vitro* and *in vivo* antifungal activities of plant essential oils against *Aspergillus* spp.

3.1 Introduction

Plant essential oils have been shown to possess antifungal activity and to suppress or inhibit plant pathogenic microorganisms, in both *in vitro* and *vivo* experiments (Passone *et al*., 2012; Burt, 2004). Gullino *et al*. (2000) also reported that plants produce several bioactive components which have antimicrobial properties. As a result of these observations, the feasibility of utilising plant oils to suppress disease and enhance food security is worthy of consideration. Antimicrobial components commonly comprise phenolics and polyphenols, quinones, flavones, flavonoids and flavonols, tannins, coumarins, terpenoids, alkaloids, lectins and polypeptides. Such compounds have been detected from plants screened for different usages. Cowan (1999) reviewed the antimicrobial activity of molecules of plant origin. Secondary metabolites have been discovered and many more are yet to be explored. There is evidence that most of these ingredients are involved in the interaction of plants with other species, primarily for defence of the plant against pests and plant pathogenic microbes (Tripathi *et al*., 2004; Philogene *et al*., 2005; Isman and Akhtar, 2007).

Plant essential oils are commonly volatile oils, which are liquids at room temperature and derived from many plant tissues, including flowers, buds, seeds, leaves, bark, wood, fruits, and roots. They can be harvested by fermentation or extraction, however, steam distillation is most often used for commercial production. About 3000 plant essential oils are known worldwide of these 300 are commercially important in the fragrance market (Van de Braak and Leijten, 1999). The following are some of the essential oils commonly obtained from plant materials; aniseed, calamus, camphor, cedarwood, cinnamon, citronella, clove, eucalyptus, lavender, lemon, lemongrass, lime, mint, nutmeg, orange, palmarosa, rosemary, basil, vetiver, onion, tea tree, vanilla, neem, garlic, ginger, green oregano, galangal, and wintergreen. These have been traditionally used for different purposes in many parts of the globe.

Secondary metabolites represent a large reservoir of phytochemical molecules with bioactivity (Duke *et al*., 2003). Using such secondary metabolites as biopesticides remains largely untapped. Viewing the potential advantages of organic pesticides over synthetic compounds, attempts have been made by researchers to screen plants for antimicrobial activity, and to isolate and characterize the bioactive ingredients from different plant parts. *Alpinia galanga* essential extract has been evaluated to have inhibitory antioxidant and antimicrobial activities against bacteria (Mayachiew and Devahastin, 2008). Benkeblia (2003) reported that high concentrations of essential oils derived from green and yellow onion and garlic showed strong inhibition against *A. niger*. Clove oil significantly suppressed the growth of *Aspergillus* spp., *Rhizopus*, and *Penicillium* as reported by Joseph and Sujatha (2011), in their experiments on the antimicrobial activities of this material against food-borne pathogens. Vanilla is intensively used for its flavouring properties worldwide and is the second most expensive spice in the world next to saffron. Despite its broad usages, it had not been widely reported that vanilla possesses antimicrobial activity that is useful in inhibiting plant pathogenic microorganisms. Jay and Rivers (1984) demonstrated that vanilla was active in suppressing moulds and non-lactic Gram positive bacteria. Lopez-Malo *et al.* (1995) demonstrated antifungal activity, using fruit based agar media containing mango, papaya, pineapple, apple and banana amended with 2000 µg mL^{-1} vanilla, and incubated with *A. flavus, A. niger, A. ochraceus*, or *A. parasiticus*. Vanilla concentrations above 1500 µg mL^{-1} significantly inhibited all the strains of *Aspergillus* in all media assays used. However, vanilla was less effective in banana and mango agars. The antimicrobial property of vanillin is associated with phenolic components, which makes vanillin effective in inhibiting bacteria, yeasts and moulds. Its structure is similar to eugenol (2-methoxy-4-(2-propenyl) phenol) from cloves and is known to be antimycotic (Beuchat and Golden, 1989) and bacteriostatic (Fitzgerald *et al*., 2004). Literature also revealed that garlic (*Allium sativum*) has antioxidant activity and is capable of directly scavenging free radicals, and possesses antimicrobial activity against a wide range of Gram-negative and Gram-positive bacteria, and also showed antifungal activity. This is related to the presence of allin, allicin and ajone (Ankiri and Mirelman, 1999; Prasad and Sharma, 1981; Wei and Lau, 1998). The antibacterial principle of garlic was identified by Cavallito in 1944 as diallythiosulphinate and was given the trivial name allicin (Cavallito and

Bailey, 1944). The objectives of this research were therefore to evaluate the antifungal activities of plant essential oils *in vitro* and in *planta* against *Aspergillus* spp.

3.2 Results

The identification of a strain of *A. niger*, isolated from Nigerian groundnut samples, was confirmed by PCR amplification of DNA and sequence similarly, in comparison to reference isolates in the GenBank database.

Plant essential oils were screened for antifungal activity against *Aspergillus* spp. in PDA plates, using either an agar well point inoculation assay or pathogen-seeded PDA. As the *in vitro* assays used EOs diluted in methanol, this solvent was used as a control treatment in all the bioassays. No antifungal activity was detected in any methanol treated wells (data not shown). This probably reflects the evaporation of the solvent before fungal growth. Plant oils antifungal activity was tested against *A. flavus* ATCC204304 on both seeded and point inoculation at 30^0C. At the higher temperature, fungal growth was totally inhibited possibly reflecting high antifungal activity associated with volatility of the oils (Data not shown).

Oils selected on the basis of *in vitro* activity were then evaluated, singly and in combination, using an *in planta* assay, to test their ability to improve seedling emergence and plant survival in compost amended with *Aspergillus.* Data obtained are given below.

3.2.1 Seeded-plate *in vitro* bioassays to show antifungal activity of essential oils against *Aspergillus flavus*

An example of a seeded plate assay showing inhibition of *A. niger* (Nigeria) is given in Figure 3.2.1

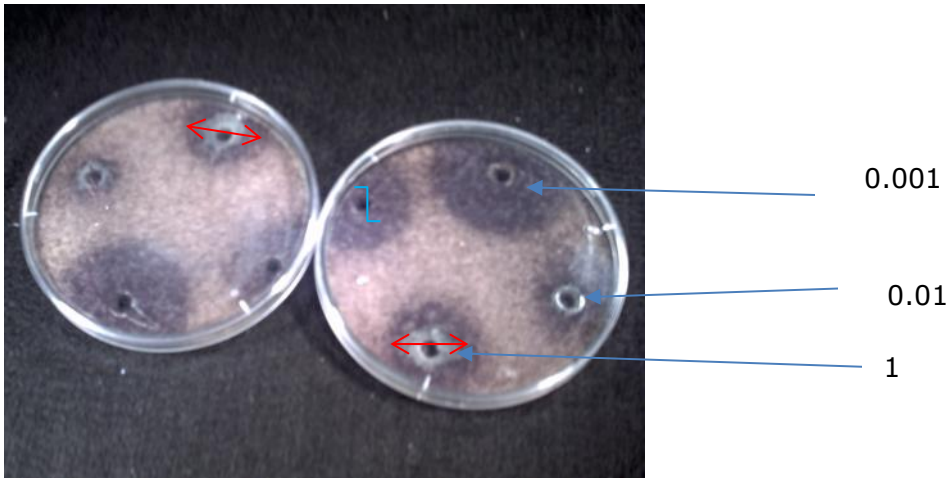
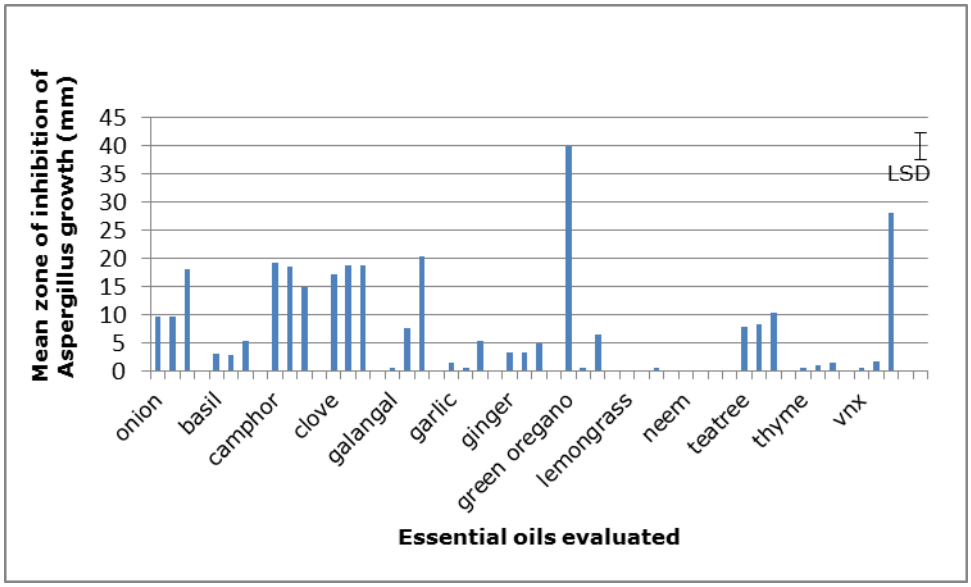


Figure 3.2.1 Antifungal activity of galangal oil at 0.01, 0.1, and 1% on PDA plates seeded with *A. niger* (Nigeria) conidia (10^6 mL^{-1}) and incubated at 20^0C for 7 d. Clear zones of inhibition are indicated with the red arrow. Dark shadows do not indicate fungal inhibition; these reflect diffusion of solvent affecting transparency of agar. Un labelled well is the control (Methanol). ANOVA used the mean values of the three concentrations as replicates.

3.2.1.1 CABI isolate (AF364493) at 20°C

The efficacy of antifungal activity of plant oils at 20°C was tested against the *A. flavus* strain (AF364493) from CABI (Figure 3.2.1.1). ANOVA detected a highly significant treatment effect ($P<0.001$) of associated with use of EOs to inhibit growth of the pathogen. The consistently most active oils were camphor and clove, for which the LSD value indicated a significantly higher activity than shown by basil, garlic, ginger, neem, tea tree and thyme. Onion, galangal and VNX (vanillin oil) inhibited activity at the higher concentrations tested. One spurious result was the apparent high activity of green oregano at the lowest concentration tested, which was not detected at higher concentrations.

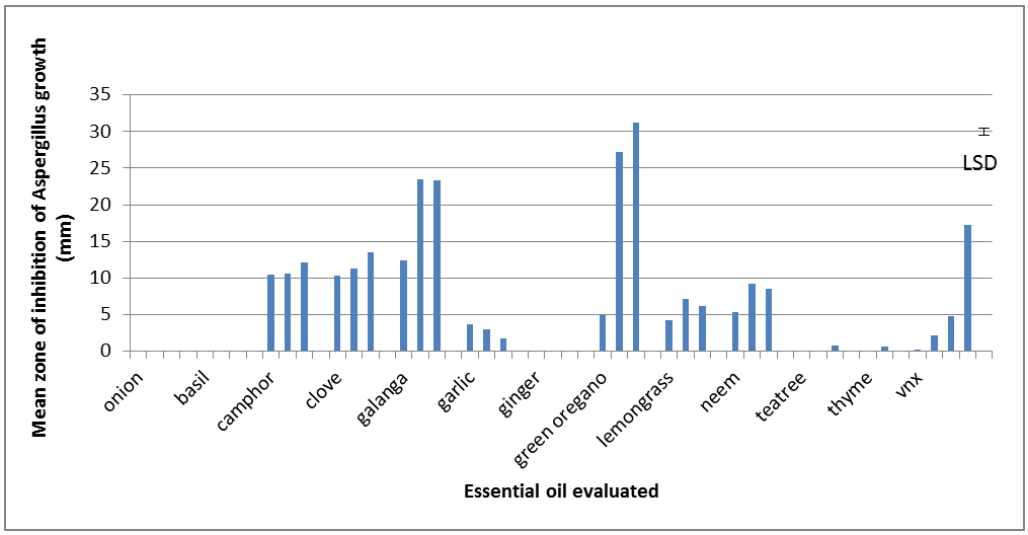


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Plant oils	13	2996.09	230.47	7.42	<0.001
Residual	146	4532.87	31.05		
Total	159	7528.96			

Figure 3.2.1.1 *In vitro* activity of plant essential oils against *A. flavus* (AF364493) at 20^{0}C, using seeded plate assay. Bar represents the LSD. Treatment concentrations in the graph are arranged in ascending order 0.01, 0.1 and 1% from left to right.

3.2.1.2 CABI isolate (AF364493) at 30°C

The antifungal activity at 30°C of plant oils was screened against *A. flavus* (AF364493) from CABI (Figure 3.2.1.2). The experiment was repeated at 30°C, a temperature more commonly associated with the tropics. ANOVA once again indicated a highly significant treatment effect (P<0.001). The LSD value indicated that camphor, clove and VNX again showed significantly more activity than onion, basil, garlic, ginger, lemongrass, neem, tea tree and thyme oils. Galangal and green oregano showed enhanced activity at the higher temperature.

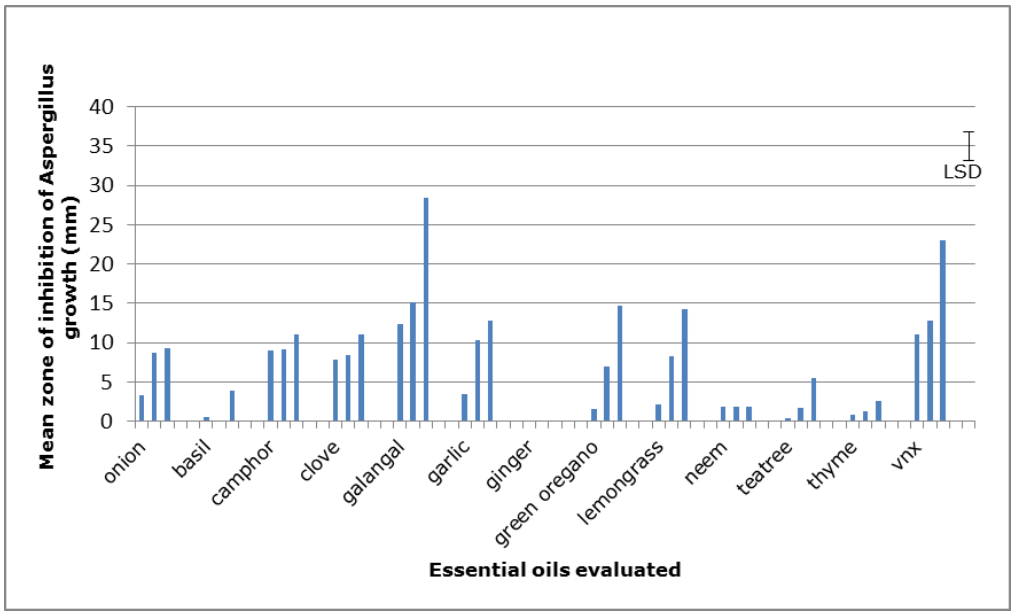


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value.
Plant oils	13	36579.0	2813.8	3.57	<0.001
Residual	146	115021.1	787.8		
Total	159	151600.1			

Figure 3.2.1.2 *In vitro* activity of plant essential oils against *A. flavus* (AF364493) at 30°C, using seeded plate assay. Bar represents the LSD. Treatment concentrations in the graph are arranged in ascending order 0.01, 0.1 and 1%.

3.2.1.3 Nottingham isolate (ATCC204304) at 20°C

Plant oils were evaluated for their antifungal activity at 20°C against the *A. flavus* isolate (ATCC204304) from the University of Nottingham (Figure 3.2.1.3). Anova indicated a highly significant treatment effect (P<0.001). The LSD value again confirmed camphor, clove, galangal and VNX oils had significantly higher activity than basil, ginger, neem, tea tree and thyme. At the higher concentrations tested, onion, garlic, green oregano and lemongrass had statistically similar activity to the four oils which had shown consistent activity (camphor, clove, galangal and VNX).



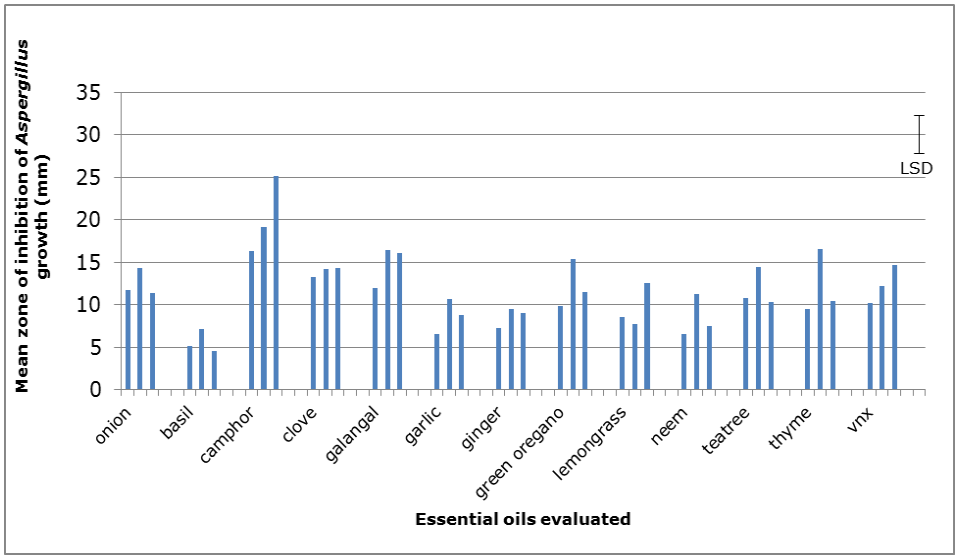
Source of variation	Degree of Freedom	Sum Squares	Mean Square	F-Ratio	P-value
Plant oils	13	4790.01	368.46	18.62	<0.001
Residual	146	2889.41	19.79		
Total	159	7679.42			

Figure 3.2.1.3 *In vitro* activity of plant essential oils against *A. flavus* (ATCC204304) at 20°C, using seeded plate assay. Bar represents the LSD. Treatment concentrations in the graph are arranged in ascending order 0.01, 0.1 and 1%.

3.2.2 Point-inoculation *in vitro* assays to show antifungal activity of essential oils against *Aspergillus* spp. at 30°C

3.2.2.1 *A. flavus* CABI AF364493

Antifungal activity of plant oils was tested against *A. flavus* at 30^{0}C. ANOVA showed no significant treatment effect (P=0.082). The results suggest that there was a trend of inhibition of *A. flavus* (AF364493). The most active oils tested were camphor, clove, galangal, thyme and VNX which had no significant difference when compared to the rest of the treatments (basil, ginger, lemongrass, onion and neem) (Figure 3.2.2.1).

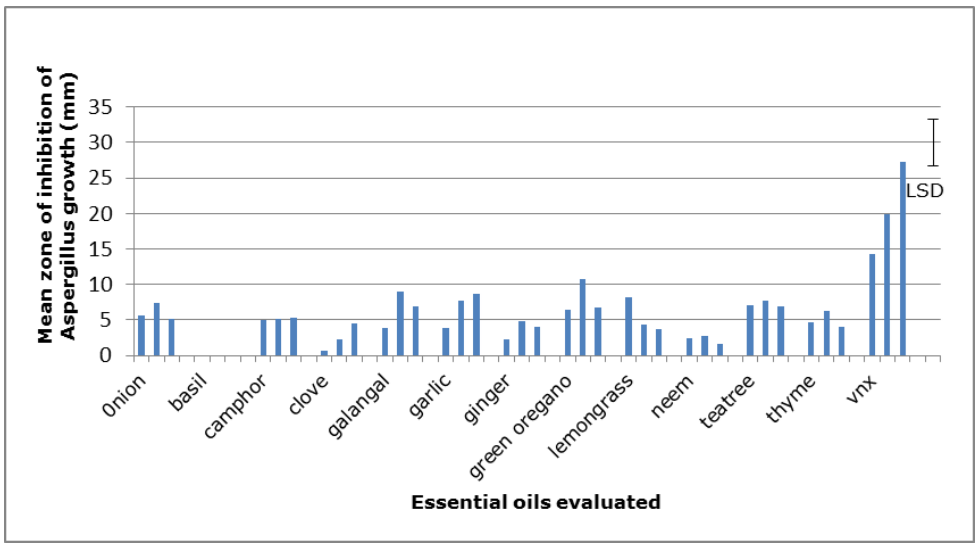


Source of variation	Degree of Freedom	Sum of Squares	Mean Squares	F-Ratio	P-value
Plant oils	13	1527.93	117.53	1.63	0.082
Residual	146	10503.80	71.94		
Total	159	12031.73			

Figure 3.2.2.1 *In vitro* activity of plant essential oils against *A. flavus* (AF364493) at 30^{0}C, using the point inoculation assay. Bar represents the LSD. Treatment concentrations in the graph are arranged in ascending order 0.01, 0.1 and 1%.

3.2.2.2 *A. niger* CABI (AN42054)

ANOVA again indicated a highly significant treatment effect (P<0.001). The LSD value revealed that with the exception of the basil oil, all materials tested had some antifungal activity against the CABI strain (AN42054) of *A. niger* at 30^{0}C, but the highest activity was shown by the vanilla-based material, VNX, significantly different from the rest of the plant oils tested in this assay. The rest of the oils also had the trend of inhibition, but had no significant differences (Figure 3.2.2.2).

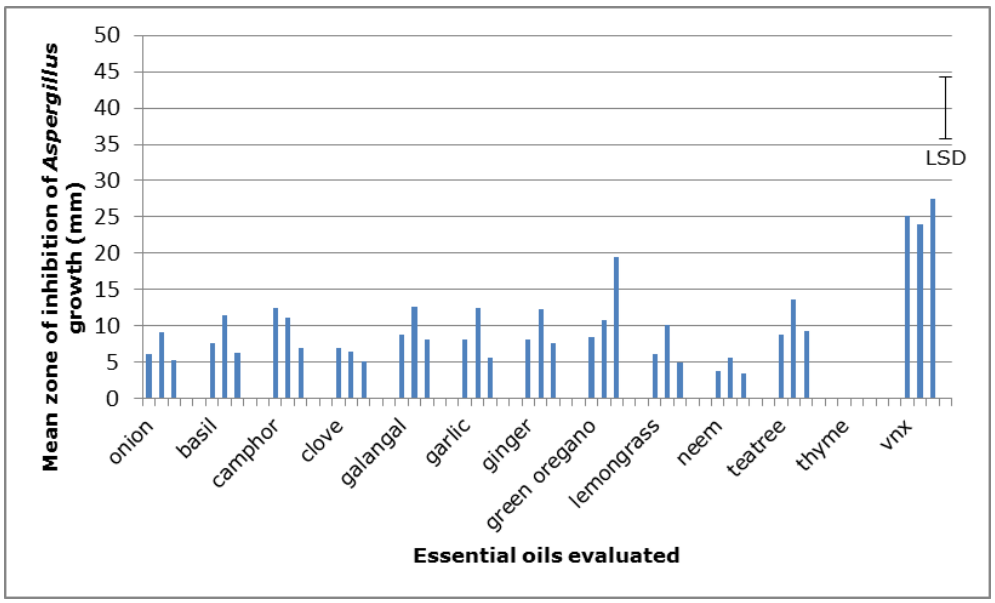


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Plant oils	13	30802.30	2369.41	71.09	<0.001
Residual	146	4866.20	33.33		
Total	159	35668.50			

Figure 3.2.2.2 *In vitro* activity of plant essential oils against *A. niger* (AN42054) at 30^{0}C, using the point inoculation assay. Bar represents the LSD. Treatment concentrations in the graph are arranged in ascending order 0.01, 0.1 and 1%.

3.2.2.3 *A. niger* Nigeria

ANOVA showed a highly significant treatment effect (P<0.001). The LSD value indicated that with the exception of thyme oil, all EOs tested showed activity against the Nigerian strain of *A. niger* at 30^{0}C, but once again, VNX proved to be the most active, significantly different from onion, basil, camphor, clove, galangal, garlic, ginger, green oregano, lemongrass, neem, tea tree and thyme (Figure 3.2.2.3). Green oregano at 1% proved significantly effective in suppressing the pathogen.



Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Plant oils	13	30930.1	2379.2	21.49	<0.001
Residual	146	16167.4	110.7		
Total	159	47097.5			

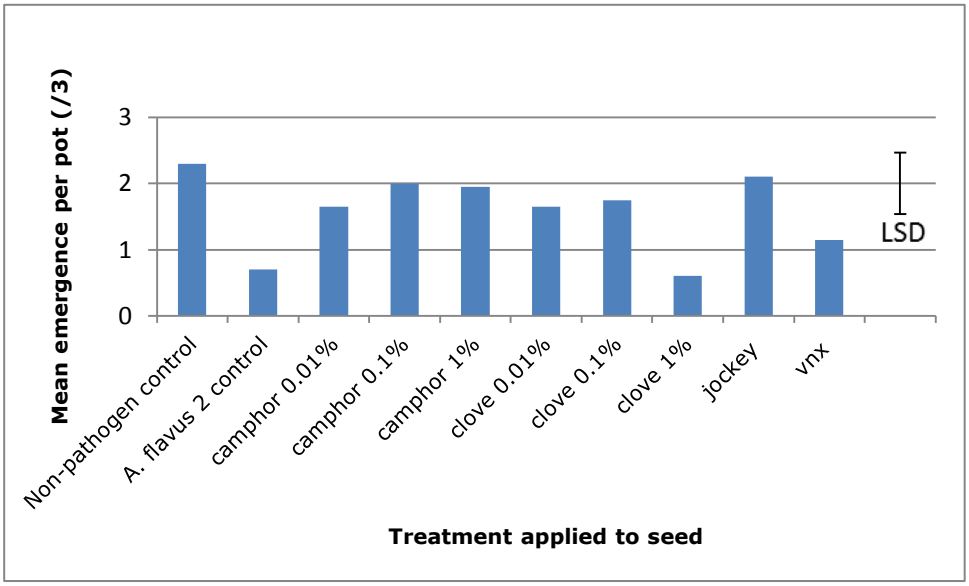
Figure 3.2.2.3 *In vitro* activity of plant essential oils against *A. niger* (Nigeria) at 30^{0}C, using the point inoculation assay. Bar represents the LSD. Treatment concentrations in the graph are arranged in ascending order 0.01, 0.1 and 1%.

- From these *in vitro* results, camphor and clove oils were selected for evaluation of activity when applied as a seed treatment, with seeds sown in *Aspergillus*-amended compost. Comparisons were made with the formulated natural product VNX and with the commercial seed dressing Jockey.

3.3 Pathogen-amended compost *in planta* assays, with single oils applied as seed treatments; assessed 14 d after sowing

3.3.1 *A. flavus* CABI (AF364493)

Antifungal activity of plant oils was evaluated against the isolate (AF364493) from CABI in comparison with Jockey (Figure 3.3.1). ANOVA indicated a significant treatment effect (P=0.006). The LSD value shows that camphor oil at 0.1% and 1% and Jockey had high antifungal activity, with enhanced emergence approaching that obtained in clean compost. The rest of the treatments had no significant effects.

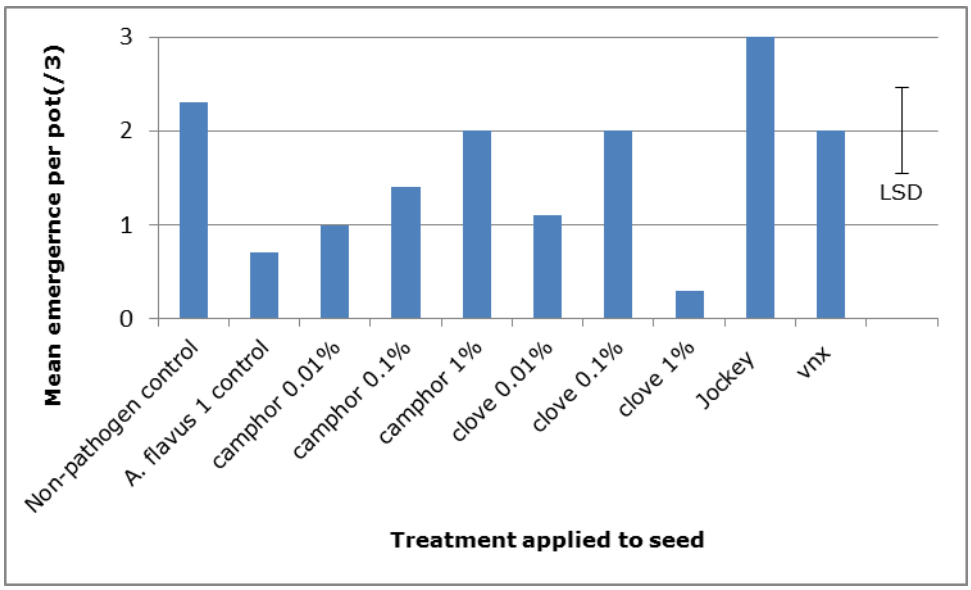


Source of variation	Degree of Freedom	Sum of Squares	Mean of Square	F-Ratio	P-value.
Plant oils/Jockey®	9	12.2410	1.3601	3.30	0.006
Residual	30	12.3500	0.4116		
Total	39	24.5910			

Figure 3.3.1 Activity of plant essential oils applied to groundnut seeds planted in *A. flavus* (AF364493) amended compost at 14 d after planting. Bar represents the LSD.

3.3.2 *A. flavus* Nottingham (ATCC204304)

The efficacy of antifungal activity of plant oils was tested against *A. flavus* (ATCC204304) from Nottingham, in comparison with Jockey (Figure 3.3.2). ANOVA indicated a highly significant treatment effect (P<0.001). The LSD value indicated the highest antifungal activity was obtained in seed treated with camphor oil at 1%, and clove oil at 0.1% or with vanillin (VNX). They had no significant difference from one another, but different from Jockey. Clove oil at 1% again were the lowest plant emergence.

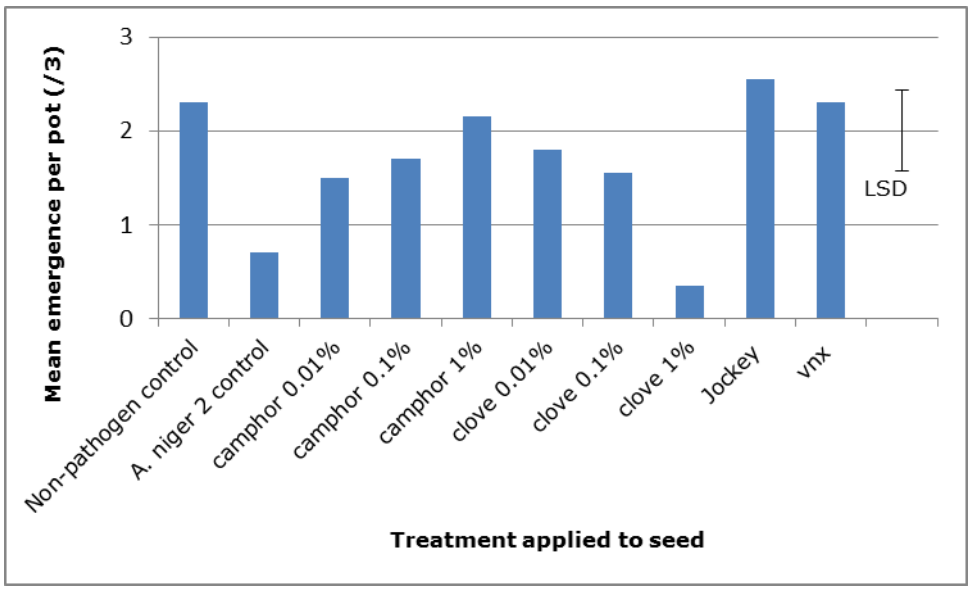


Source of variation	Degree of Freedom.	Sum of Squares	Mean of Square	F-Ratio	P-value.
Plant oils/Jockey®	9	15.7840	1.7538	4.37	<0.001
Residual	30	12.0400	0.4013		
Total	39	27.8240			

Figure 3.3.2 Activity of plant essential oils applied to groundnut seeds planted in *A. flavus* (Nottingham) amended compost at 14 d after planting. Bar represents the LSD.

3.3.3 *A. niger* CABI (AN42054)

The antifungal activity of plant essential oils was investigated against *A. niger* (AN42054) from CABI, to determine the level of plant emergence (Figure 3.3.3). ANOVA indicated a highly significant treatment effect (P<0.001). The LSD value indicated a high antifungal activity of camphor at 1%, clove at 0.01%, Jockey and VNX in suppressing the pathogen in the amended compost and all treatments supported higher plant emergence, except clove oil at 1%.

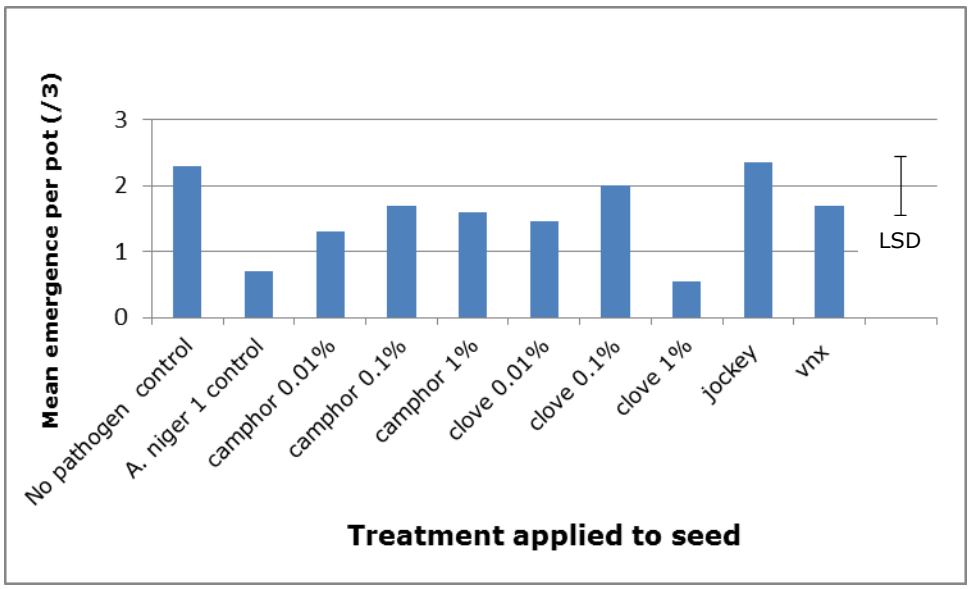


Source of variation	Degree of Freedom	Sum Squares	Mean Square	F-Ratio	P-value
Plant oils /Jockey®	9	18.1560	2.0173	5.69	<0.001
Residual	30	10.6400	0.3547		
Total	39	28.7960			

Figure 3.3.3 Activity of plant essential oils applied to groundnut seeds planted in *A. niger* (AN42054) amended compost at 14 d after planting. Bar represents the LSD.

3.3.4 *A. niger* Nigeria

The antifungal activity of plant oils was evaluated against the *A. niger* isolate from Nigeria, compared with Jockey and non-pathogen control (Figure 3.3.4). ANOVA indicated a significant treatment effect (P=0.003). The LSD value shows clearly that clove oil at 0.1% and Jockey applied to groundnut seeds significantly provided the highest antifungal activity in inhibiting pathogen, and they are not significantly different from each other. Clove at 1% is ineffective; it had the lowest plant emergence.



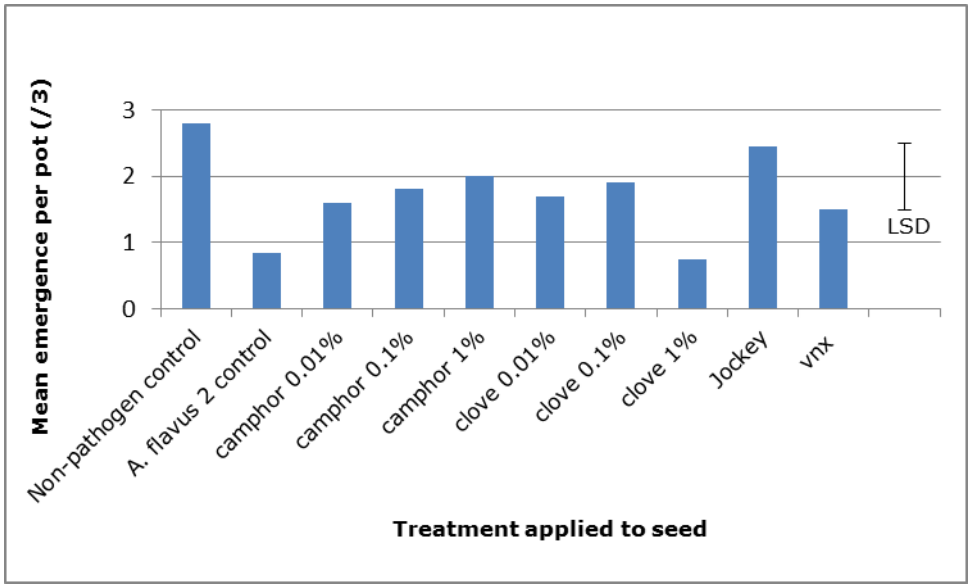
Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Plant oils /Jockey®	9	12.9810	1.4423	3.71	0.003
Residual	30	11.6500	0.3883		
Total	39	24.6310			

Figure 3.3.4 Activity of plant essential oils applied to groundnut seeds planted in *A. niger* (Nigeria) amended compost at 14 d after planting. Bar represents the LSD.

3.4 Pathogen-amended compost *in planta* assays, with single oils applied as seed treatments; assessed 27 d after sowing.

3.4.1 *A. flavus* CABI (AF364493)

Antifungal activities of plant oils were tested against *A. flavus* (AF364493). ANOVA indicated a highly significant treatment effect (P<0.001). The LSD value again indicated antifungal activity of EOs and Jockey suppressing the tested pathogen. Significantly more plants than in the pathogen control survived with camphor oil at 1%, clove at 0.1% and Jockey (Figure 3.4.1). Post-emergence damping off was observed on 0.1% camphor treated plants when compared to plant emergence at 14 d after planting.

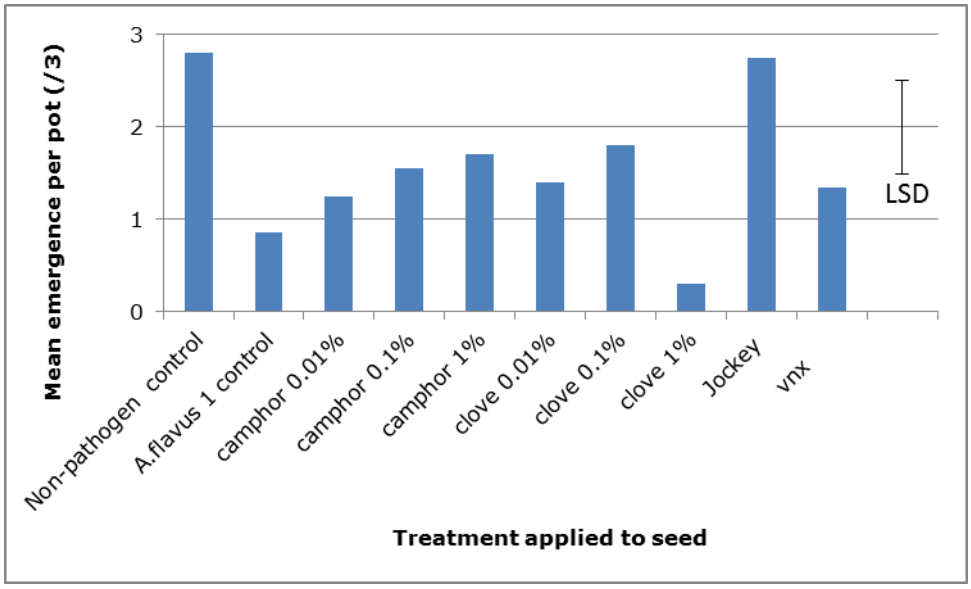


Source of variation	Degree of Freedom	Sum of Squares	Mean of Square	F-Ratio	P-value.
Plant oils/Jockey®	9	14.3010	1.5890	5.59	<0.001
Residual	30	8.5300	0.2843		
Total	39	22.8310			

Figure 3.4.1 Activity of plant essential oils applied to groundnut seeds planted in *A. flavus* (CABI) amended compost at 27 d after planting. Bar represents the LSD.

3.4.2 *A. flavus* Nottingham (ATCC204304)

ANOVA indicated a highly significant treatment effect (P<0.001). The LSD value only indicated that Jockey had high antifungal activity in suppressing the tested pathogen and improved plant survival (Figure 3.4.2). In this experiment post-emergence damping off was observed in a high disease pressure environment on plants treated with camphor at 1% and clove at 0.1%.

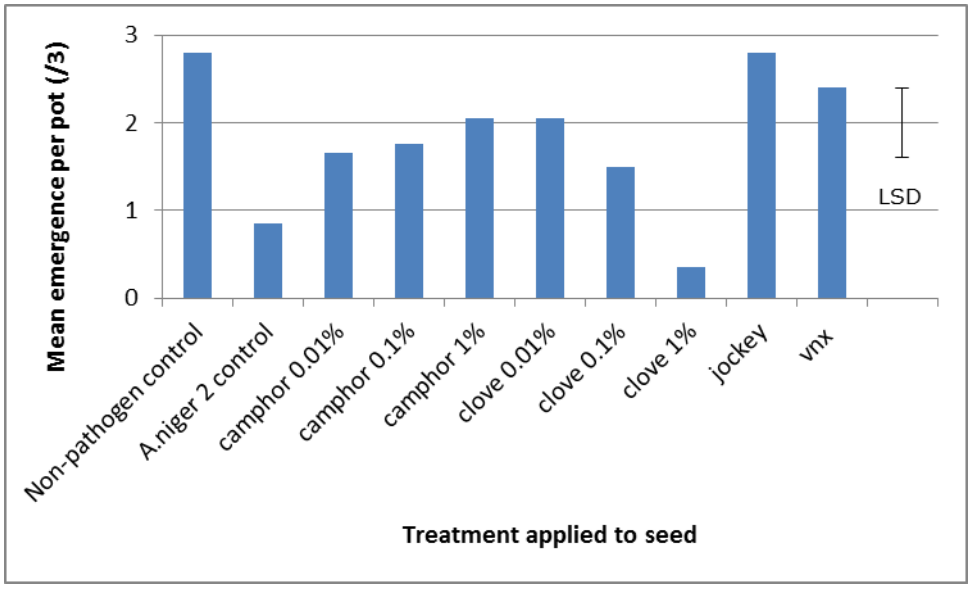


Source of variation	Degree of .Freedom	Sum of Squares	Mean Square	F- Ratio	P value
Plant oils/Jockey®	9	21.1450	2.3494	4.82	<0.001
Residual	30	14.6300	0.4877		
Total	39	35.7750			

Figure 3.4.2 Activity of plant essential oils applied to groundnut seeds planted in *A. flavus* (ATCC204304) amended compost at 27 d after planting. Bar represents the LSD.

3.4.3 *A. niger* CABI (AN42054)

ANOVA also indicated a highly significant treatment effect (P<0.001). The LSD value shows that all the treatments except clove oil at 0.1% and 1% significantly improve plant survival on *A. niger* (AN42054) amended compost at 27 d post sowing (Figure 3.4.3). No post-emergence damping off was observed.

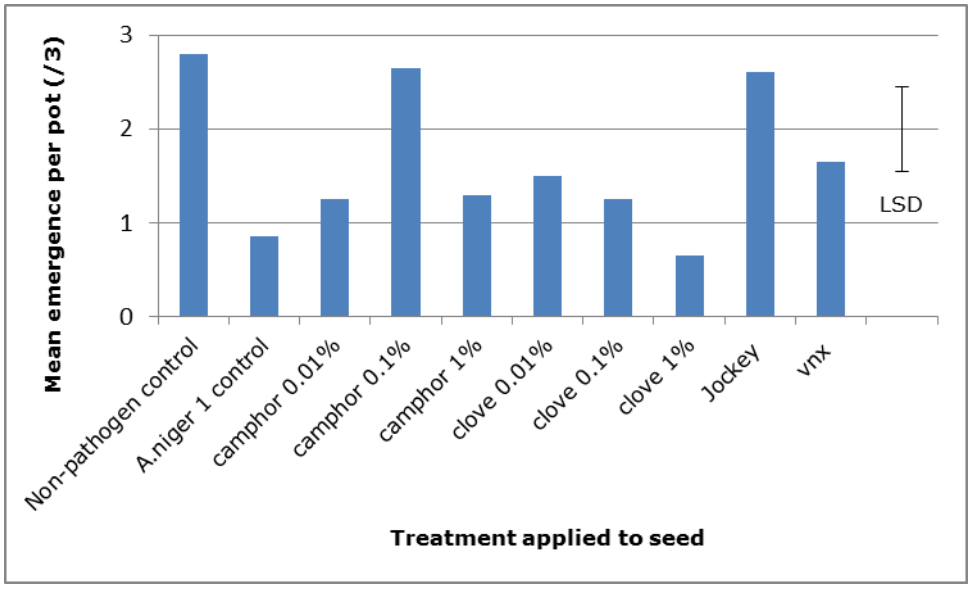


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Plant oils /Jockey®	9	22.4040	2.4893	7.54	<0.001
Residuals	30	9.9000	0.3300		
Total	39	32.3040			

Figure 3.4.3 Activity of plant essential oils applied to groundnut seeds planted in *A. niger* (CABI) amended compost at 27 d after planting. Bar represent the LSD.

3.4.4 *A. niger* Nigeria

ANOVA indicated a highly significant treatment effect (P<0.001). The LSD value indicated that camphor oil at 0.1% significantly exhibited high antifungal activity in suppressing *A. niger* from Nigeria in the pathogen inoculated compost. Camphor oil (0.1%) gave similar efficacy with Jockey, and both differed from other treatments (Figure 3.4.4). Post-emergence damping off was shown for plants treated with 0.1% clove oil.



Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Plant oils/Jockey®	9	21.3200	2.3689	5.99	<0.001
Residual	30	11.8600	0.3953		
Total	39	33.1800			

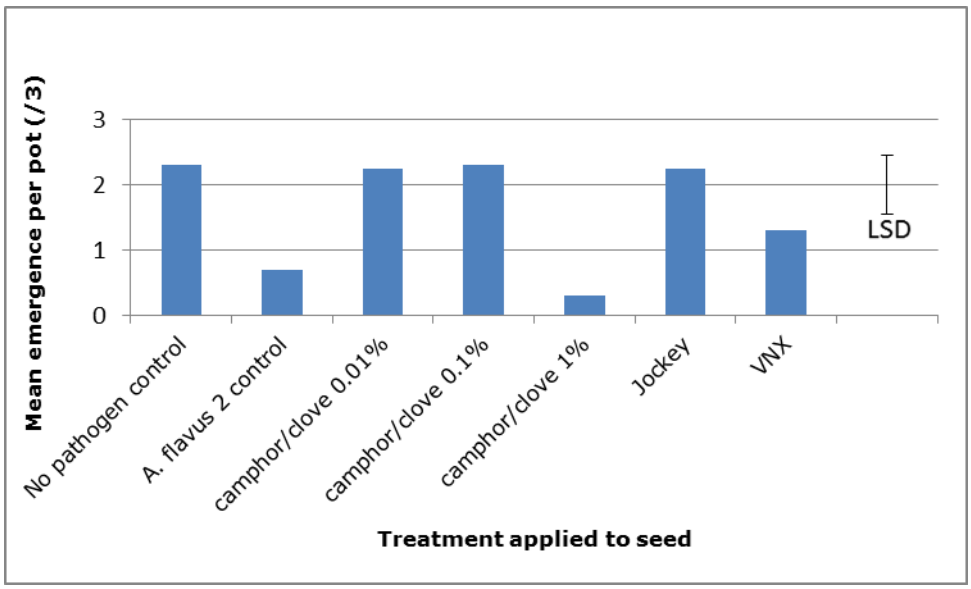
Figure 3.4.4 Activity of plant essential oils applied to groundnut seeds planted in *A. niger* (Nigeria) amended compost at 27 d after planting. Bar represents the LSD.

- All the oils tested showed antifungal activity against the two strains of *A. flavus* and *A. niger*. On the basis of *in vitro* assays, the antifungal activity of plant oils was further evaluated, to assess their efficacy in suppressing *Aspergillus* in amended compost, which revealed disease suppression to be very highly significant (P<0.001). The exception was clove oil at 1% which was ineffective, possibly reflecting post emergence damping off.

3.5 Pathogen-amended compost *in planta* assays, with combined oils applied as seed treatments

3.5.1 *A. flavus* CABI (AF364493) 14 d post planting

The experiment was conducted with combinations of plant oils to improve plant emergence. ANOVA indicated highly significant treatment effect (P<0.001). The LSD value indicated that a combination of camphor and clove oils exhibited high antifungal activity against *A. flavus* (AF364493) 14 d post-sowing, and increased plant emergence more than single application, but had no significant difference from Jockey and little difference from VNX. Exceptionally, camphor plus clove oils at 1% was ineffective in suppressing the pathogen (Figure 3.5.1).

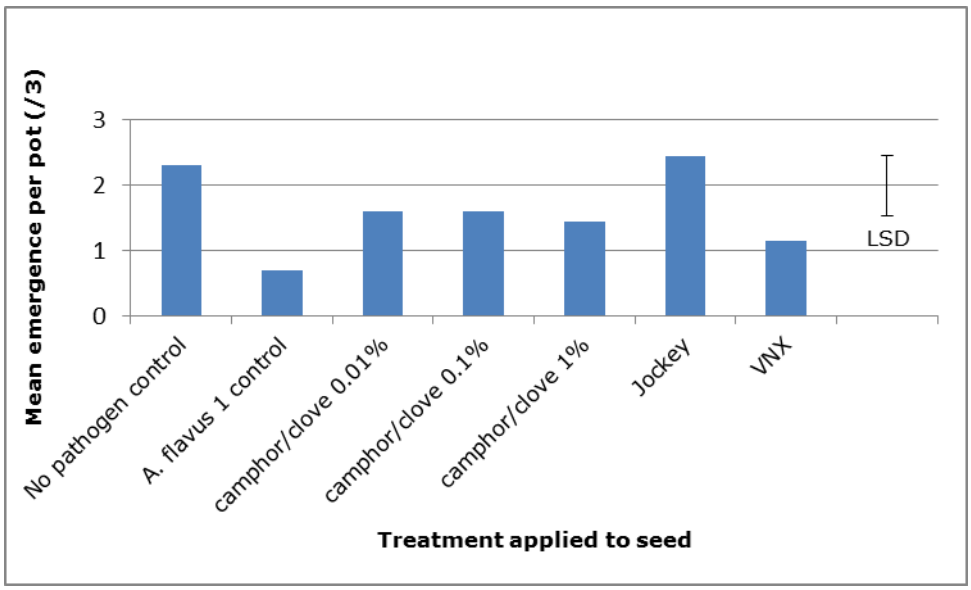


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Plant oils / Jockey	6	17.6371	2.9395	7.19	<0.001
Residual	21	8.5800	0.4086		
Total	27	26.2171			

Figure 3.5.1 Activity of combination treatment of plant essential oils applied to groundnut seeds planted in *A. flavus* (CABI) amended compost at 14 d after planting. Bar represents the LSD.

3.5.2 *A. flavus* Nottingham (ATCC204304)

ANOVA indicated a significant treatment effect (P=0.015). The LSD value shows a trend of pathogen inhibition at 14 d post-sowing and enhanced plant emergence, although the oils were not as effective as Jockey (Figure 3.5.2).

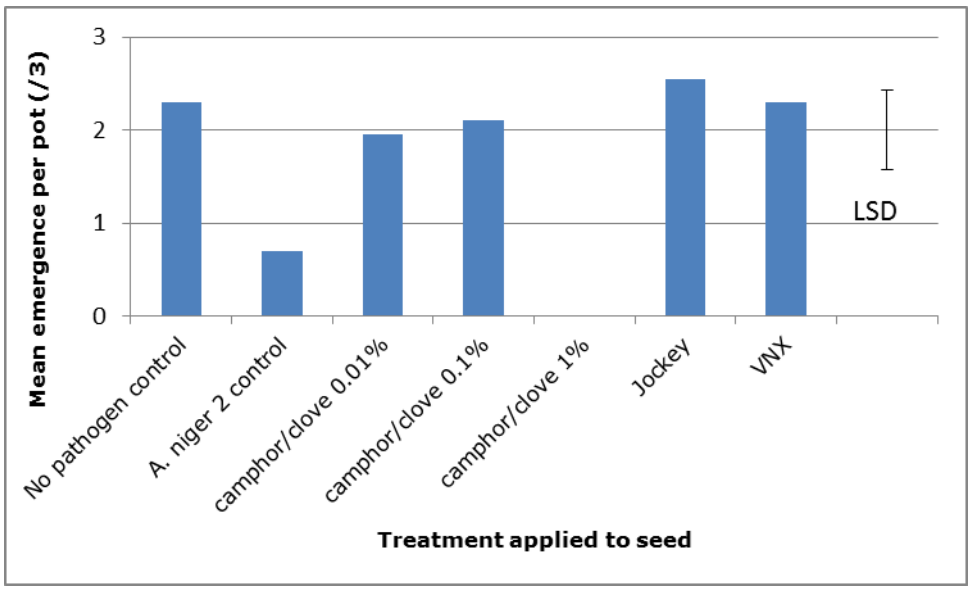


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Plant oils / Jockey	6	8.9886	1.4981	3.51	0.015
Residual	21	8.9700	0.4271		
Total	27	17.9586			

Figure 3.5.2 Activity of combination treatment of plant essential oils applied to groundnut seeds planted in *A.* flavus (ATCC204304) amended compost at 14 d after planting. Bar represents the LSD.

3.5.3 *A. niger* CABI (AN42054)

The antifungal activity of plant oils in combination against *A. niger* from CABI (AN42054) was assessed 14 d after sowing. ANOVA showed a highly significant treatment effect (P<0.001). The LSD value indicated that treatment with camphor and clove oils at 1% gave no seedling emergence. Other treatments had no significant differences and were highly effective in suppressing the pathogen and enhancing plant emergence (Figure 3.5.3).

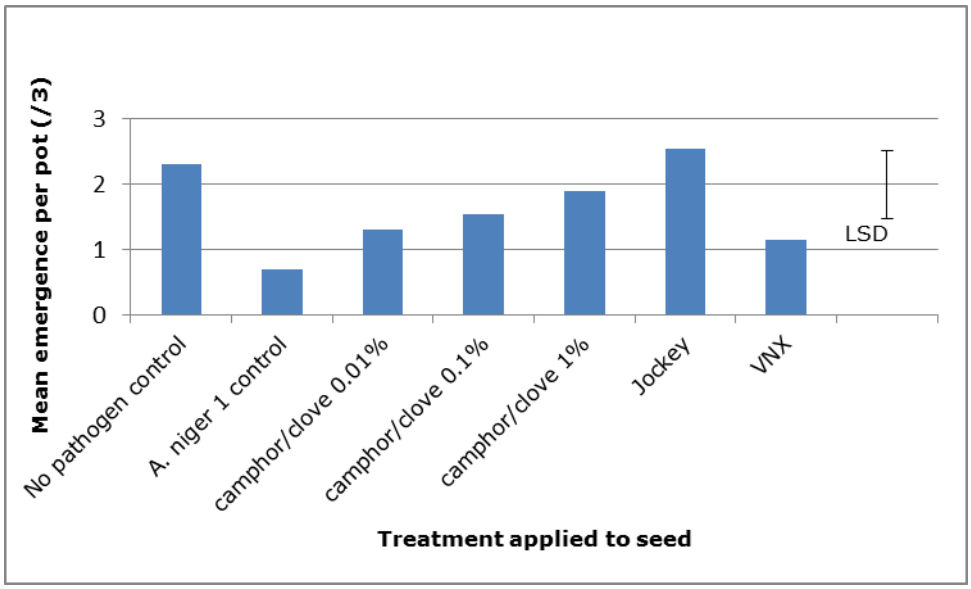


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Plant oils / Jockey	6	22.2200	3.7033	10.37	<0.001
Residual	21	7.5000	0.3571		
Total	27	29.7200			

Figure 3.5.3 Activity of combination treatment of plant essential oils applied to groundnut seeds planted in *A. niger* (AN42054) amended compost at 14 d after planting. Bar represents the LSD.

3.5.4 *A. niger* Nigeria

The efficacy of combination treatments of plant oils against *A. niger* from Nigeria was screened 14 d after sowing. ANOVA indicated a significant treatment effect (P=0.021). The LSD value shows that only 1% camphor/clove and Jockey significantly improved plant emergence (Figure 3.5.4). Other treatments also had the trend of pathogen inhibition.

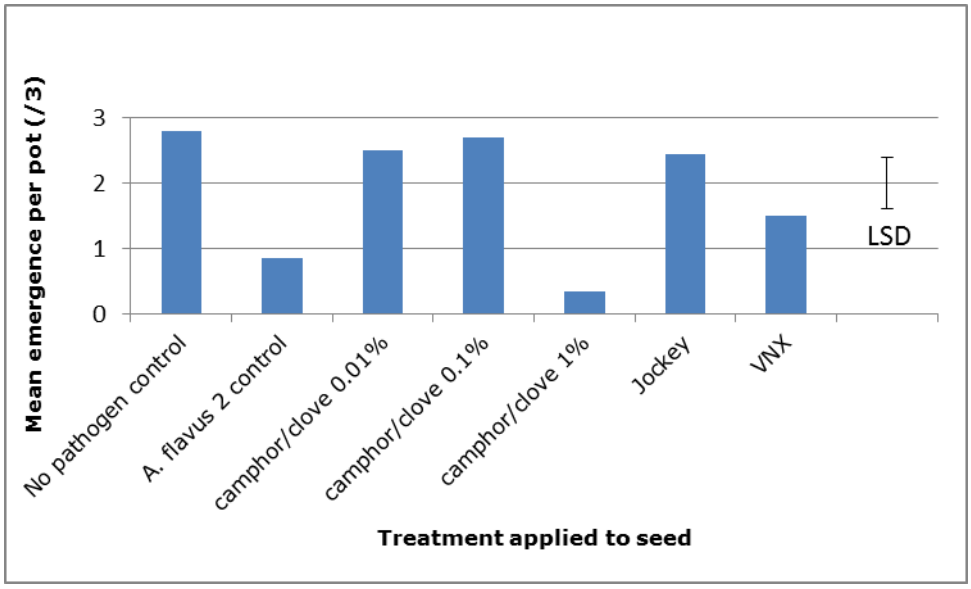


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F- Ratio	P- value
Plant oils / Jockey	6	10.3143	1.7190	3.22	0.021
Residual	21	11.2100	0.5338		
Total	27	21.5243			

Figure 3.5.4 Activity of combination treatment of plant essential oils applied to groundnut seeds planted in *A. niger* (Nigeria) amended compost at 14 d after planting. Bar represents the LSD.

3.6 1 *A. flavus* CABI (AF364493) 27 d post planting

ANOVA indicated a highly significant treatment effect (P<0.001). Assessments were made for a second time 27 d after sowing, to ascertain whether treatment could allow plants to survive in the high disease pressure environment, and the results obtained are given below. The LSD value indicated that camphor and clove oils at 1% were ineffective, whilst the other treatments were highly effective in suppressing *A. flavus* CABI (AF364493) in amended compost. The results presented here had similar results to the 14 d seedling emergence (Figure 3.6.1). However, VNX is not significantly effective.

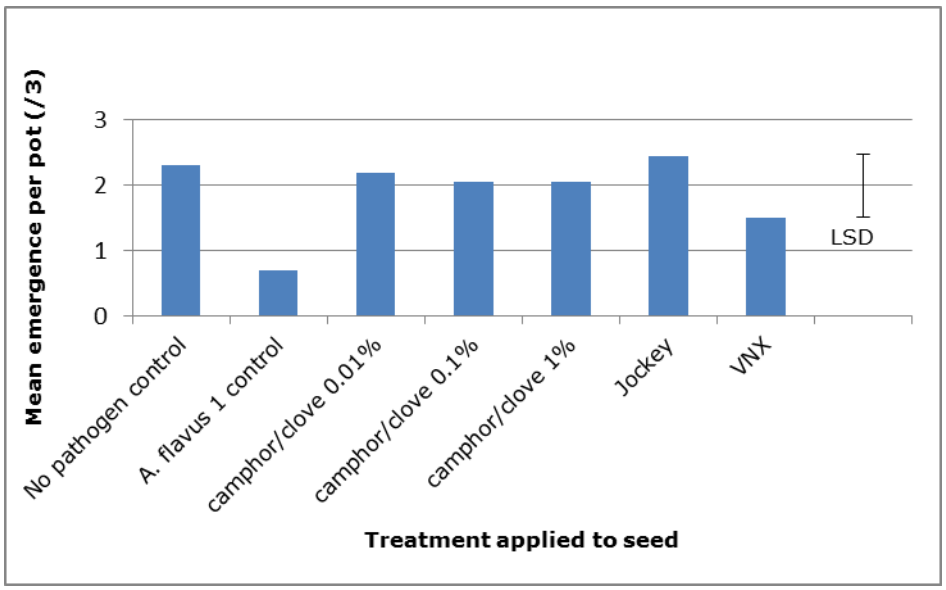


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Plant oils / Jockey	6	23.0971	3.8495	12.69	<0.001
Residual	21	6.3700	0.3033		
Total	27	29.4671			

Figure 3.6.1 Activity of combination treatment of plant essential oils applied to groundnut seeds planted in *A. flavus* (AF364493) amended compost at 27 d after planting. Bar represents the LSD.

3.6.2 *A. flavus* Nottingham (ATCC204304)

ANOVA indicated a significant treatment effect (P=0.024). The LSD value shows that all treatments had high antifungal activity in inhibiting *A. flavus* (ATCC204304) in the amended compost and increased plant survival, except VNX which is different from the others (Figure 3.6.2).

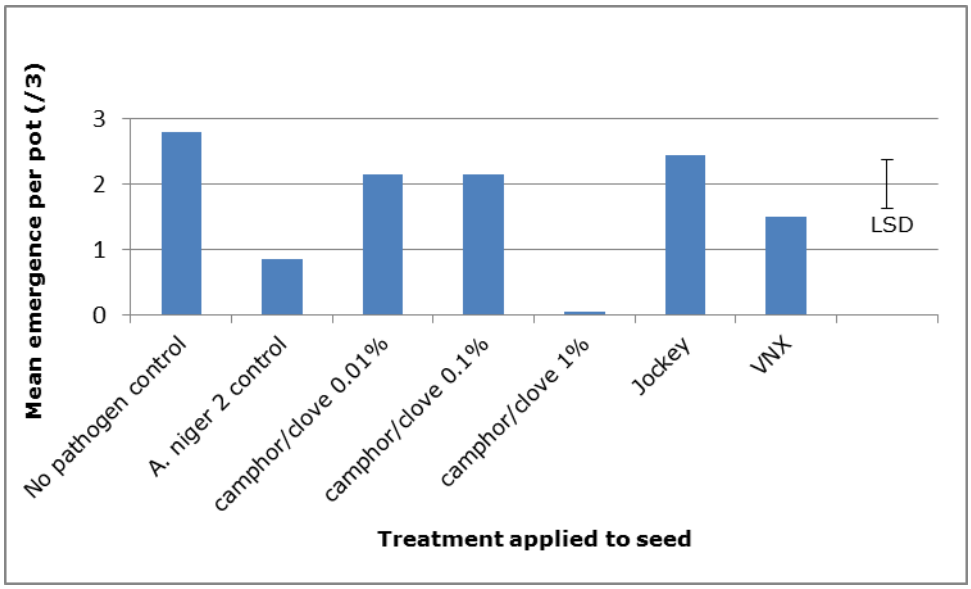


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Plant oils / Jockey	6	8.7886	1.4648	3.14	0.024
Residual	21	9.8100	0.4671		
Total	27	18.5986			

Figure 3.6.2 Activity of combination treatment of plant essential oils applied to groundnut seeds planted in *A. flavus* (ATCC204304) amended compost at 27 d after planting. Bar represents the LSD.

3.6.3 *A. niger* CABI (AN42054)

ANOVA also indicated a highly significant treatment effect (P<0.001). Antifungal activity of camphor and clove oils at 1% was not detected against *A. niger* (AN42054). The LSD value shows that the other combination treatments tested were highly active against the pathogen (Figure 3.6.3), except VNX.

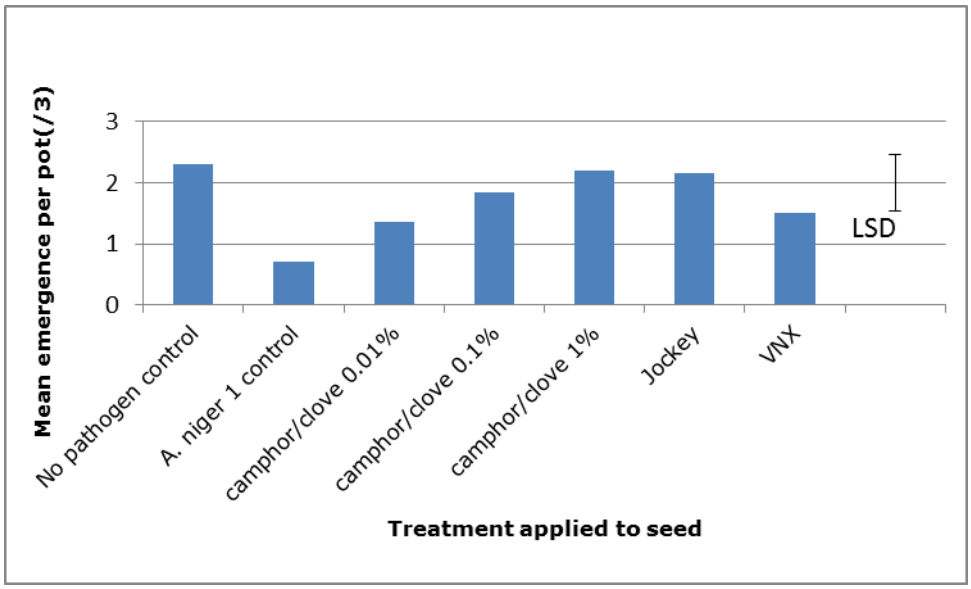


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Plant oils / Jockey	6	22.6486	3.7748	13.69	<0.001
Residual	21	5.7900	0.2757		
Total	27	28.4386			

Figure 3.6.3 Activity of combination treatment of plant essential oils applied to groundnut seeds planted in *A. niger* (AN42054) amended compost at 27 d after planting. Bar represents the LSD.

3.6.4 *A. niger* Nigeria

ANOVA shows a significant treatment effect (P=0.027). The LSD value again shows that all the treatments except 0.01% camphor/clove and VNX suppressed *A. niger* from Nigeria and provided more plant survival on 1% camphor/clove and Jockey when compared to 14 d post-sowing seedling emergence (Figure 3.6.4).



Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Plant oils /Jockey	6	7.9771	1.329	3.04	0.027
Residual	21	9.1700	0.4367		
Total	27	17.1471			

Figure 3.6.4 Activity of combination treatment of plant essential oils applied to groundnut seeds planted in *A. niger* (Nigeria) amended compost at 27 d after planting. Bar represents the LSD.

- The results presented in Chapter 3 showed that camphor, clove, VNX, galangal, green oregano EOs were the most effective in suppressing the pathogen in, an *in vitro* assay
- in the *in planta* assay camphor, clove, VNX were the best EOs, which were equally useful for integrated approach in comparison with Jockey.

3.7 Discussion

Food security is a highly significant issue for both consumers and the food industry across the globe, partly associated with losses caused by fungal infection of crops. Synthetic fungicides that combat phytopathogenic fungi can increase crop yields and provide stability of crop production and market quality. However, rapid increase in the use of fungicides has resulted in the development of fungicide-tolerant pathogen strains (Staub, 1991) and accumulation of fungicide residues in the food chain, above a safe level (Simko, 2005). Based on these associated predicaments, scientists are seeking alternative approaches for fungal disease management. These can include strategies which utilise plant-derived compounds (Kishore and Pande, 2004). Abdel-Kader *et al*. (2013) reported on the inhibitory effect of thyme, rose, and lemongrass oils against the linear growth of *A. niger*. All the treatments applied significantly reduced the linear growth of *A. niger*. The fungal growth was reduced more by increasing concentrations of tested essential oils. This previous finding is in conformity with the results of this present research. This could enhance groundnut production and contribute to meeting the requirement for plant protein of the world population. In this study the *in vitro* and *in vivo* anti-fungal activity of plant essential oils, applied singly and in combinations, was tested against the target pathogens.

In vitro experiments indicated that some of the plant oils screened were highly effective in suppressing the four strains of *Aspergillus* tested; particularly vanilla, camphor, clove, galangal, garlic, green oregano, and lemon grass were active at the lower temperature, while at the higher temperature generally all the oils that were evaluated significantly suppressed the pathogens. The volatile nature of the oils could determine that they were more active at the higher temperature of 30^0C.

In *in planta* experiments, plant oils applied to seeds, both singly and in combination, enhanced seed germination in *Aspergillus*-amended compost, sometimes as effectively as the conventional seed-treatment fungicide Jockey. However, the 1% concentration of clove oil significantly reduced emergence, possibly reflecting post-emergence damping off at the highest concentration tested. Vanilla oil was used for the first time as a groundnut seed dressing. This proved to be one of the best oils to suppress fungal infection and enhance groundnut emergence. This might be attributed to

the cell wall degradation of fungi by the active compound of vanilla oils, known as vanillin, which is a phenolic compound. Previous research showed that vanillin was more effective at low pH values and at higher temperatures (Mourtzinos *et al*., 2008). Some plant–derived products that contain terpenoid compounds and their oxygenated derivatives can effectively suppress pathogenic microorganisms (Wijesekara *et al*., 1997). These oils are known for their broad spectrum antifungal activity against plant pathogens in both *in vitro* and *in planta* experiments (Daferera *et al*., 2003; Isman, 2000). Growth inhibition by essential plant oils might be attributed to the activity of compounds which involves induction of changes in cell wall composition of fungi (Ghfir *et al*., 1997), plasma membrane disruption, mitochondrial structure disorganization (de Billerbeck *et al*., 2001), and interference with enzymatic reactions of mitochondrial membrane, such as respiratory electron transport, proton transport, and coupled phosphorylation pathways (Knobloch *et al.*, 1989). The antifungal activity of plant oils, reducing hyphal growth of *Aspergillus* spp. and causing lysis and cytoplasmic evacuation in fungi, was also reported by Fiori *et al*. (2000).

Lee and Shibamoto (2002) stated that clove oil is used as anti-carcinogenic agent, due to its antioxidant properties, and a potential chemo-preservative agent, because of its active ingredient eugenol (Cai and Wu, 1996; Dorman and Deans, 2000; Ranasinghe *et al.*, 2002 and Rajkumar and Berwal, 2003). Currently clove oil is used in the pharmaceutical, food and cosmetic industries, because of its efficacy in inhibiting the growth of a wide range of pathogenic microorganisms (Joseph and Sujatha, 2011). Camphor exhibits a number of biological properties, such as insecticidal, antimicrobial, antiviral, anticoccidial, anti-nociceptive, anticancer and antitussive activities (Weiyang *et al*., 2013). Deng *et al*. (2004) reported that *C. camphora* ethereal oil was effective for fumigating against maize weevil (*Sitophilus zeamais* L.). Wang (2007) stated that cinnamon ethereal oil inhibited growth of *Botrytis cinerea, Alternaria solani, Cladosporium fulvum* and *Pseudoperonospora cubensis.*

In most instances the combination treatments of clove and camphor oils showed good antifungal activity against the two strains of *A. flavus* and *A. niger*. The antifungal activity of plant oils was evaluated, to assess their efficacy in suppressing *Aspergillus* in inoculated compost, and was revealed to be very highly significant ($P<0.001$). The exception was the combination of camphor and clove oils at 1%, which had no antifungal activity. Generally, the oils performed better in combination at 27 d post-planting on *A. niger* Nigeria, ATTC204304 and *A. flavus* 3464493 amended compost than when applied singly. Single treatment was also effective in suppressing the tested pathogen. Significantly this could help low resource famers to save money for procurement of plant oils for single application, instead of combination treatment. Post-emergence damping off was recorded on plants treated with camphor and clove oils 27 d after planting in AF364493, ATCC204304 and *A. niger* (Nigeria) amended compost. Generally, clove oil at 1% had the lowest surviving plants.

Abdel-Kader *et al*. (2013) reported on efficacy of antifungal activities of plant oils singly and in combination for the control of groundnut crown rot disease. Treatments were effective in suppressing the disease and survival rates of plants were more in combination treatment than singly. This previous report conforms to the present study as stated above.

Thus, from the results reported here, it could be concluded that some essential plant oils have the ability to suppress *Aspergillus* growth *in vitro* and to enhance groundnut seed germination in compost amended with the pathogens.

Chapter 4 Determination of *in vitro* and *in vivo* antifungal activities of BCAs against two strains of *A. niger* and *A. flavus*

4.1 Introduction

Control of plant disease and microbial contamination of agricultural commodities is frequently achieved by the use of synthetic fungicides. However, the incessant and indiscriminate application of such compounds has caused health hazards in animals and humans, due to residual toxicity (Dohroo, 1990). Considering the potentially-hazardous effects and high cost of such pesticides, plant pathologists have researched alternative techniques of plant disease control, which might cause lesser effects on the ecosystem (Ghaffar, 1992). Currently, there has been a worldwide swing towards the use of eco-friendly methods for protecting crops from pests and diseases. Biocontrol (or biological control) involves application of disease-suppressive microorganisms to improve plant health for increased productivity. Biocontrol agents are non-pathogenic microorganisms that suppress disease by interactions involving the plant, the pathogen, the biocontrol agent, the microbial community on and around the plant, and the physical environment.

Biocontrol of soil-borne diseases is difficult to comprehend, because these diseases occur in the dynamic environment at the interface of root and soil known as the rhizosphere, which is defined as the region surrounding a root that is affected by it. The rhizosphere is noted for rapid change, intense microbial activity, and high populations of microorganisms, compared with non-rhizosphere soil (Barea *et al*., 2005).

The first requirement of biological control is the identification and deployment of highly effective strains, to either the ecosystem or to growth media. Fungi within the genus *Trichoderma* have attracted attention because of their multipurpose action against various plant pathogens (Harman *et al.*, 2004).

Although biological control under controlled environmental conditions may be successful, for effective control under commercial agricultural production there are certain constraints that make success very low (Larkin *et al*., 1998). Few microbial BCAs studied in the laboratory are currently sold for commercial purposes. There are certain factors that are responsible for this lack of commercial application, including:

- Inadequate screening or testing processes for the isolation of potential BCAs
- Problems of inoculant stabilization or formulation procedures
- Poor understanding of the ecology of the microbial antagonist
- Inconsistent disease control compared with synthetic chemicals (Wathaneeyawech *et al*., 2014)
- Cost-prohibitive registration processes
- Inadequate patent protection of potential products, formulations or active components
- Unfavourable economics of inoculant production and low market size.

Combinations of multiple antagonistic organisms could play a vital role in improving disease control, compared to the use of single organisms. Multiple organisms might enhance the level and consistency of control by providing multiple disease suppression mechanisms and delivering a more stable rhizosphere community, over a wider range of environmental conditions. Particular combinations of fungi and bacteria might provide protection at different times or under different conditions and occupy different or complementary niches. As well as acting directly on target pathogens, they can also compete effectively for infection sites on the root and can trigger plant defence reactions through systemic resistance (Benhamou *et al.,* 2002).

Trichoderma virens showed biocontrol activity against sugar beet seedling damping-off caused by *Rhizoctonia solani* (Hanson and Howell, 2003). Etebarian *et al*. (2000) reported that *T. harzianum,* in combination with *T. virens*, significantly reduced disease severity in shoots and roots of potatoes 10 weeks after inoculation with the pathogen, *Phytophthora erythroseptica*, that causes a root and stem rot. Bhuiyan *et al*. (2003) reported work with two commercial *Trichoderma* products (Trichopel and Trichoflow), and with two isolates of *Penicillium citrinum*, which inhibited germination of macroconidia of *Claviceps africana*, the cause of ergot sugary disease of sorghum. *Trichoderma viride* and *T. harzianum*, in a pot study, reduced collar rot disease incidence in groundnut caused by *A. niger* (Gajera *et al*., 2011; 2014).

Moreover application of essential oils, integrated with the bio-agent *T. harzianum*, was found to be suitable, safe and cost-effective for controlling the soil-borne disease faba bean root rot (Abdel-Kader *et al*., 2011). Similar observations were recorded with other species of *Trichoderma* for controlling soil-borne diseases. In greenhouse experiments, the application of *T. viride* with pungam cake at 5 g kg^{-1} of soil markedly reduced bean root rot incidence caused by *Macrophomina phaseolina* (Sharma and Dureja, 2004). *T. viride,* applied with *Pseudomonas fluorescens*, increased the biocontrol activity against stem rot of groundnut caused by *Sclerotium rolfsii* (Manjula *et al*., 2004). It has been found by several researchers that *Trichoderma* can reduce disease incidence caused by *A. niger* (Gajera *et al*., 2011; Rajkonda *et al*., 2011) and *A. flavus* (Reddy *et al*., 2009). *Bacillus* spp. and *P. chlororaphis* also play an important role in suppressing fungal pathogens in roots and foliage of crop plants (Paola *et al*., 2009; Velazhahan *et al.*, 1999).

Nigerian groundnut growers suffer huge losses from groundnut pathogen attacks that cause diseases at all growth stages from pre-emergence rotting in seeds, soft rot in emerging seedlings to crown rot in mature plants. *A. niger* is a major problem of groundnut production in the field. Crown rot of groundnut caused by *A. niger* is common in warm and dry climatic zones and its incidence ranges from 2% to 14% (Pande and Narayana Rao, 2000). The level of *A. flavus* infection in groundnut seed may result in pre-emergence rotting of seeds and seedlings, to cause yellow mould development.

The following aims were evaluated in this chapter:

- To use PCR and DNA sequencing to confirm the identification of components of a commercial *Trichoderma* BCA product
- To evaluate the *in vitro* activity of BCAs against *Aspergillus* spp.
- To screen the *in planta* activity of BCAs against *Aspergillus* spp., in pathogen-amended compost.
- To determine the compatibility of BCAs with plant oils using the point inoculation *in vitro* plate assay.

- For comparative purposes, when evaluating efficacy of non-conventional strategies, the seed treatment fungicide Jockey was included in experiments.

4.2 Results

4.2.1 Molecular identification of pathogens

Two different colony colours (green and light yellow) emerged from the cultured plates derived from the commercial product TUSAL, as shown below in Figures 4.2.1.1 and 4.2.1.2. The different coloured isolates were further sub-cultured and used for PCR analysis for molecular identification of the species of *Trichoderma* as described below.

Figure 4.2.1.1 *Trichoderma* spp. isolated from granular commercial *Trichoderma.*

Figure 4.2.1.2 Pure culture of isolate 1 from granular commercial *Trichoderma*

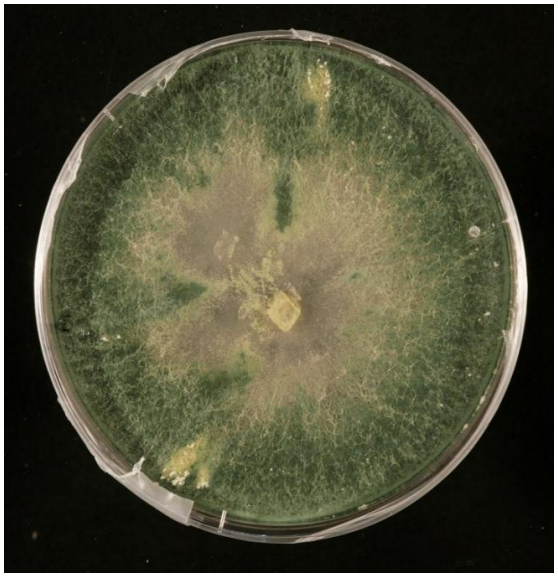


Figure 4.2.1.3 Pure culture of isolate 2 from granular commercial *Trichoderma*

Sequence analysis of the PCR products was used to identify the two species contained within the commercial *Trichoderma*, from pure PDA cultures. Sequencing was undertaken by Eurofin. A Blast search indicated 99% identity of *T. asperellum* and *T. viride*, with accession numbers of gb/HQ293149.1/ and gb/HMO37928 .1/ respectively from the NCBI Database search. A gel photograph is shown in Figure 4.2.1.4.

Figure 4.2.1.4 Detection of DNA of *T. asperellum* and *T. viride.* PCR products were electrophoresed and visualized by staining with ethidium bromide on a 1.5% agarose gel. *M = Molecular marker, 1 = T. asperellum,* 2 = *T. asperellum,* 3 = *T. asperellum,* 4 = *T. viride,* 5= *T. viride,* 6 = *T. viride,* 7= *T. viride,* 8 = *T. asperellum,* 9 = *T. asperellum,* 10 = *T. asperellum,* 11 = *T. asperellum,* 12 = *T. viride,* 13 = *T. viride*, 14 = *T. viride*, 15 = *T. viride*, 16 = *T. viride* and arrow indicates 600 bases. The isolates were extracted separately.

4.2.2 Point-inoculation *in vitro* assays to show antifungal activity of BCAs against *Aspergillus* spp. at 30°C

An example of point-inoculation Petri dish assay, illustrating a zone of inhibition between *A. niger* (Nigeria) and *T. asperellum*, is provided in Figure 4.2.2.

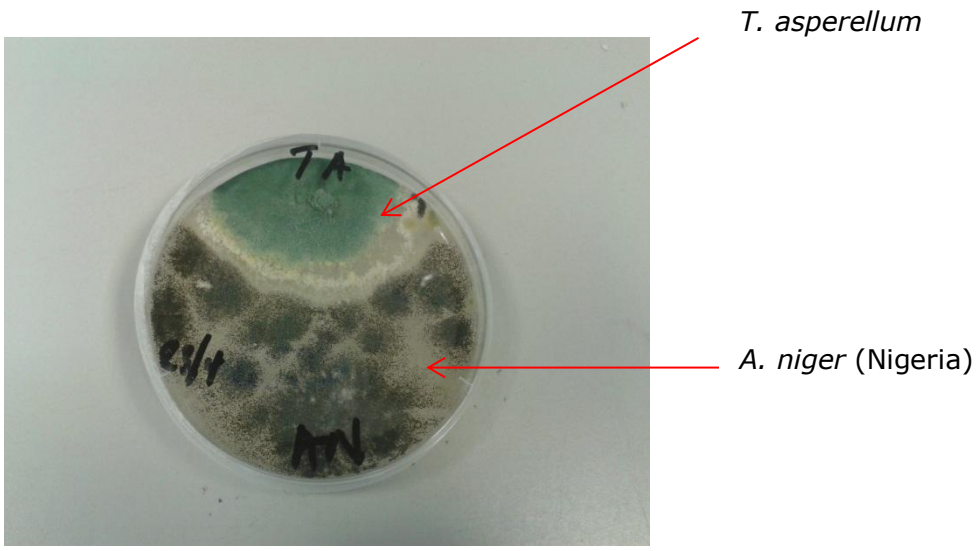
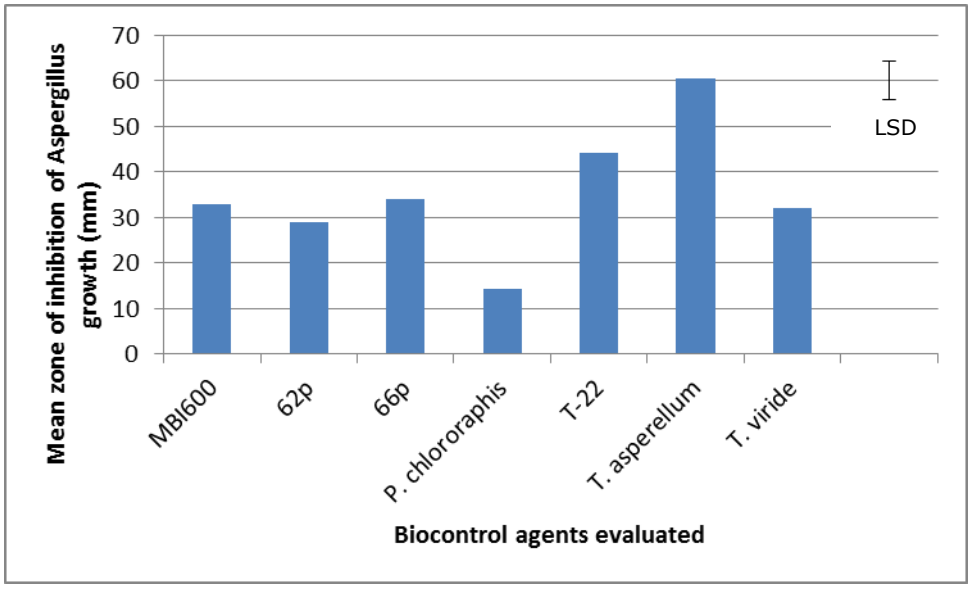


Figure 4.2.2 *T. asperellum* inhibiting *A. niger* by creating zone of inhibition in point inoculation assay. Incubated at 20°C for 7 d.

4.2.2.1 *A. flavus* CABI (AF364493)

Preliminary experiments were done to evaluate the *in vitro* antifungal activity of BCAs. ANOVA indicated a highly significant treatment effect (P<0.001). The LSD value indicated that T-22 and *T. asperellum* had more antimicrobial activity in suppressing *A. flavus* (AF364493) than the *Bacillus* strains, at 30^{0}C. *T. asperellum* gave highly significant inhibition compared to the other BCAs. *P. chlororaphis* had the least activity in inhibiting the pathogen (Figure 4.2.2.1).

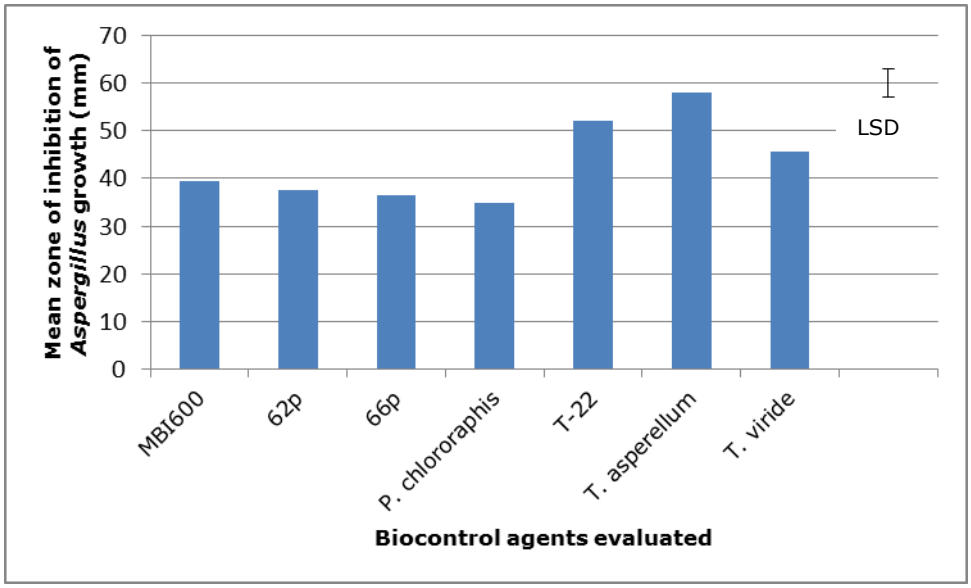


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
BCAs	7	15311.38	2187.34	59.29	<0.001
Residual	24	885.47	36.89		
Total	31	16196.85			

Figure 4.2.2.1 *In vitro* activity of BCAs against *A. flavus* (AF364493) at 30^{0}C, using the point inoculation assay. Bar represents the LSD. MBI600, 62P and 66P are strains of *Bacillus amyloliquefaciens*.

4.2.2.2 *A. flavus* Nottingham (ATCC204304)

The biocontrol agents were also evaluated for inhibition of growth of *A. flavus* (ATCC204304) at 30^0C. ANOVA also indicated a highly significant treatment effect (P<0.001). The LSD value shows that all the tested stains of BCAs were effective in suppressing the University of Nottingham isolate of the pathogen (ATCC204304); once again, *Trichoderma* isolates were the most active (Figure 4.2.2.2). *Trichoderma* isolates, effectiveness did not differ from each other significantly, but were different from the bacterial strains. Although *T. asperellum* is significantly more effective than *T. viride*.

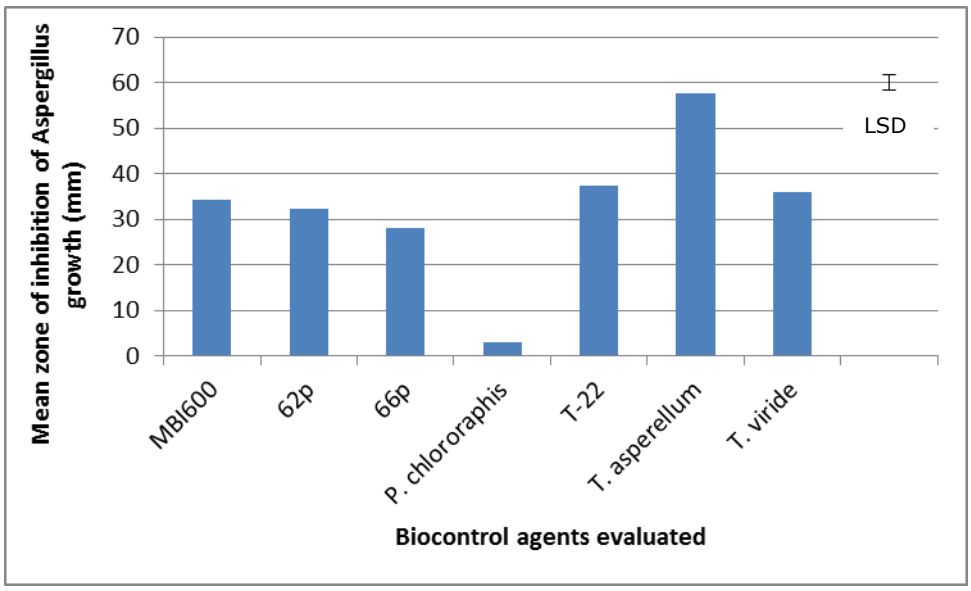


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
BCAs	7	9420.988	1345.855	222.63	<0.001
Residual	24	145.086	6.045		
Total	31	9566.07			

Figure 4.2.2.2 *In vitro* activity of BCAs against *A. flavus* (ATCC204304) at 30^0C, using the point inoculation assay. Bar represents the LSD. MBI600, 62P and 66P are strains of *Bacillus amyloliquefaciens*.

4.2.2.3 *A. niger* CABI (AN42054)

In vitro activity against *A. niger* (AN42054), at 30^{0}C is shown in Figure 4.2.2.3. ANOVA also indicated the treatment effect is highly significant (P<0.001). The LSD value indicated all the evaluated stains of BCAs were significantly effective in suppressing the tested pathogen, except *P. chlororaphis. Trichoderma* isolates were all active, with *T. asperellum* being the most effective. They tended to be more active than *Bacillus* isolates.

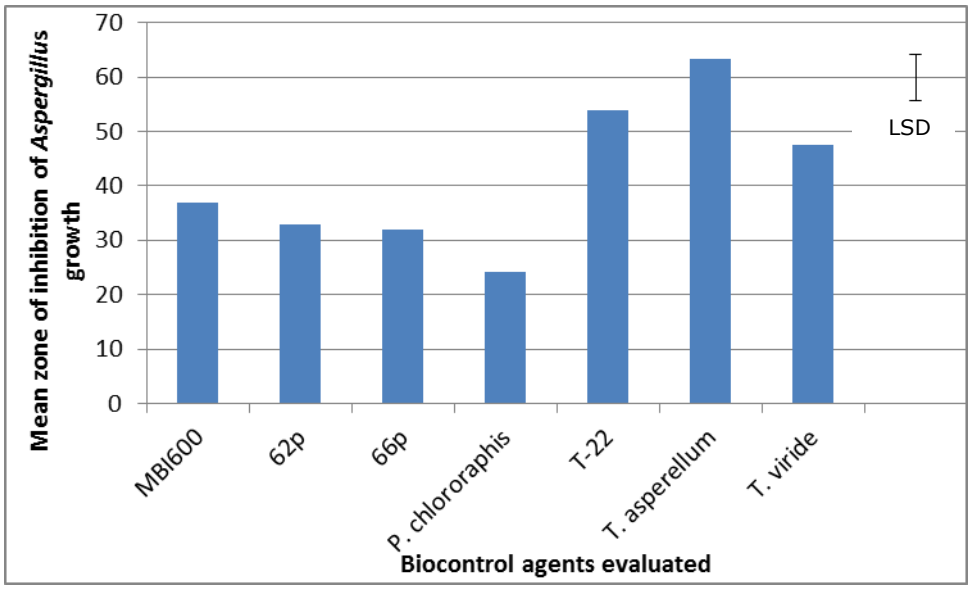


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
BCAs	7	17780.08	2540.01	123.65	<0.001
Residual	24	492.99	20.54		
Total	31	18273.07			

Figure 4.2.2.3 *In vitro* activity of BCAs against *A. niger* (AN42054) at 30^{0}C, using the point inoculation assay. Bar represents the LSD. MBI600, 62P and 66P are strains of *Bacillus amyloliquefaciens*.

4.2.2.4 *A. niger* Nigeria

Antimicrobial activities of BCAs were evaluated against *A. niger* from Nigeria, at 30^0C (Figure 4.2.2.4). ANOVA indicated a highly significant treatment effect (P<0.001). The LSD value shows that all the evaluated stains of BCAs were significantly effective in inhibiting the tested pathogen. *P. chlororaphis* was the least active. *Trichoderma* strains had the highest effect. These isolates did not differ significantly from each other, but again proved more active than the *Bacillus* isolates. However, *T. asperellum* seems to be significantly better than *T. viride.*



Source of variation	Degree of Freedom.	Sum of Squares	Mean Square	F-Ratio	P-value
BCAs	7	12793.70	1827.67	53.45	<0.001
Residual	24	820.67	34.19		
Total	31	13614.37			

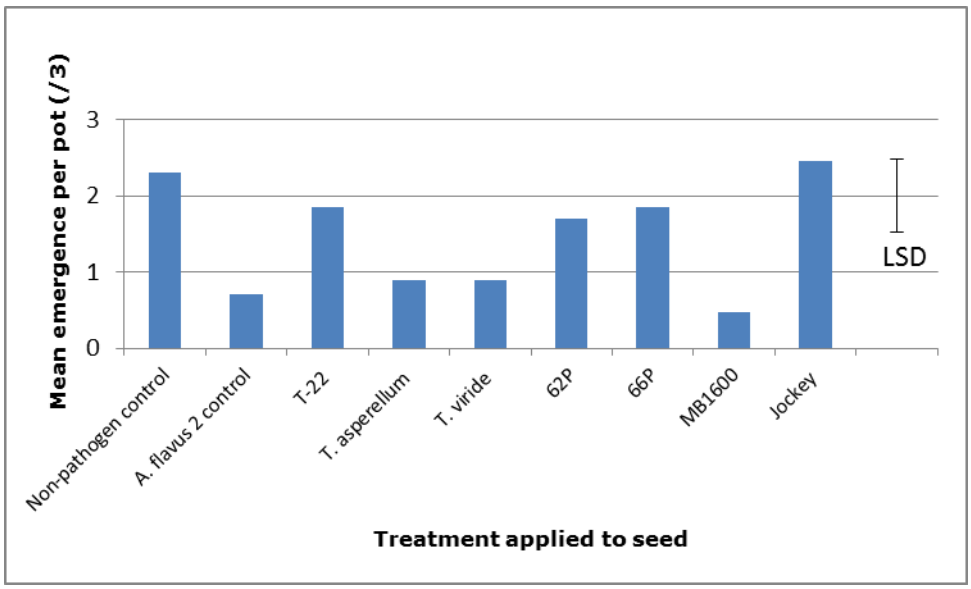
Figure 4.2.2.4 *In vitro* activity of BCAs against *A. niger* (Nigeria) at 30^0 C, using the point inoculation assay. Bar represents the LSD. MBI600, 62P and 66P are strains of *Bacillus amyloliquefaciens.*

- From these *in vitro* results, *Trichoderma* strains proved to be the most-inhibitory putative BCAs tested. These could be used for combination treatment with camphor and clove oils, selected for high activity, when applied as a seed treatment, with seeds sown in *Aspergillus*-amended compost. Comparisons were also made to the commercial seed dressing fungicide, Jockey.

4.3 Pathogen-amended compost *in planta* assays, with single BCAs applied as seed treatments

4.3.1 *A. flavus* CABI (AF364493)14 d after planting

In the first set of experiments, the efficacy of BCAs was compared to the fungicide Jockey. Seedling emergence was assessed 14 d after sowing. The efficacy of the BCAs against *A. flavus* (AF364493) is illustrated in Figure 4.3.1. ANOVA indicated that the treatment effect is highly significant (P=0.002). The LSD value shows that the highest seedling emergence was obtained with seed amended with T-22, 62P, 66P and Jockey, which had no significant difference from each other, but were different from MBI600, *T. asperellum, T. viride* and *A. flavus* 2 controls.

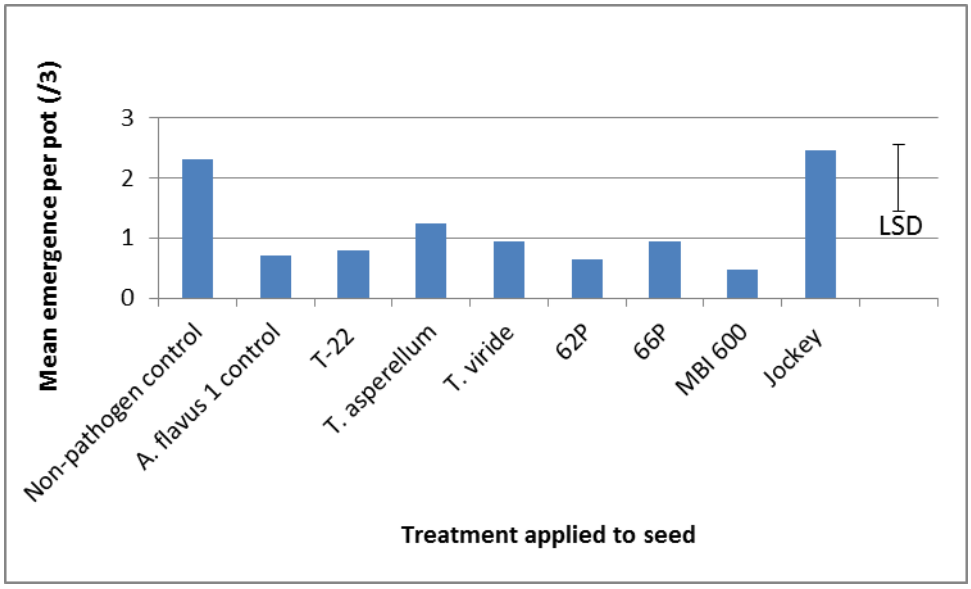


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Treatment	8	16.3508	1.8168	4.01	0.002
Residual	27	13.1467	0.4533		
Total	35	29.4974			

Figure 4.3.1 Activity of biocontrol agents applied to groundnut seeds planted in *A. flavus* (AF364493) amended compost at 14 d after planting. Bar represents the LSD. MBI600, 62P and 66P are strains of *Bacillus amyloliquefaciens*.

4.3.2 *A. flavus* Nottingham (ATCC204304)

Antimicrobial activity of BCAs was investigated against *A. flavus* (ATCC204304) from the University of Nottingham. ANOVA indicated that the treatment effect was significant (P=0.018). In this experiment, however, only Jockey was able to provide emergence comparable with the non-pathogen control. The rest of the treatments had little antifungal activity (Figure 4.3.2).

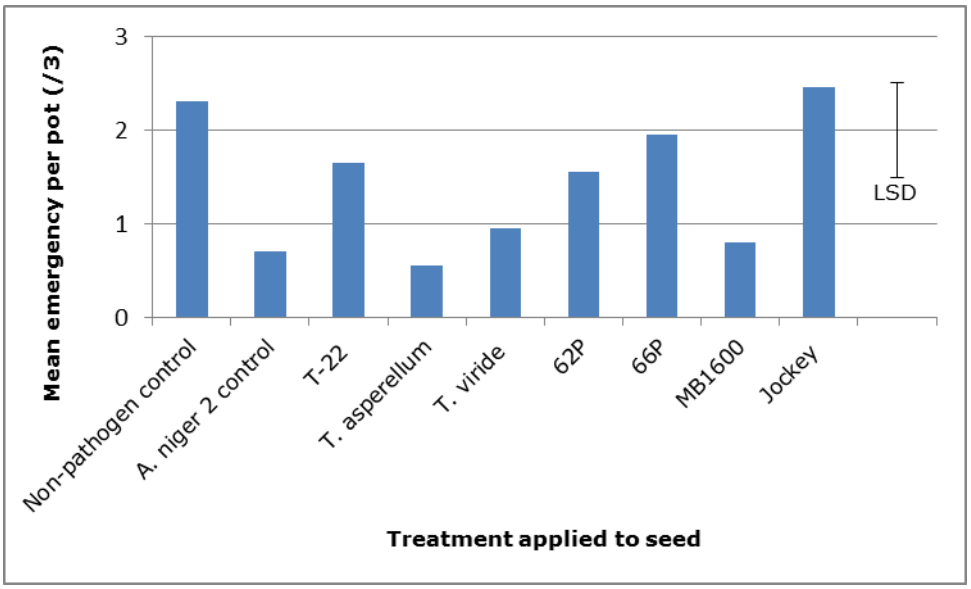


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Treatment	8	14.3889	1.7986	2.90	0.018
Residual	27	16.7400	0.6200		
Total	35	31.1289			

Figure 4.3.2 Activity of biocontrol agents applied to groundnut seeds planted in *A. flavus* (ATC204304) amended compost at 14 d after planting. Bar represent LSD. MBI600, 62P and 66P are strains of *Bacillus amyloliquefaciens*.

4.3.3 *A. niger* CABI (AN42054)

ANOVA indicated a highly significant treatment effect (P=0.002). The LSD value indicated the highest antimicrobial activity against *A. niger* (AN42054) was obtained in seed amended with Jockey, 66P, T-22 and 62P which enhanced more plant emergence (Figure 4.3.3). Statistically they did not differ from each other.

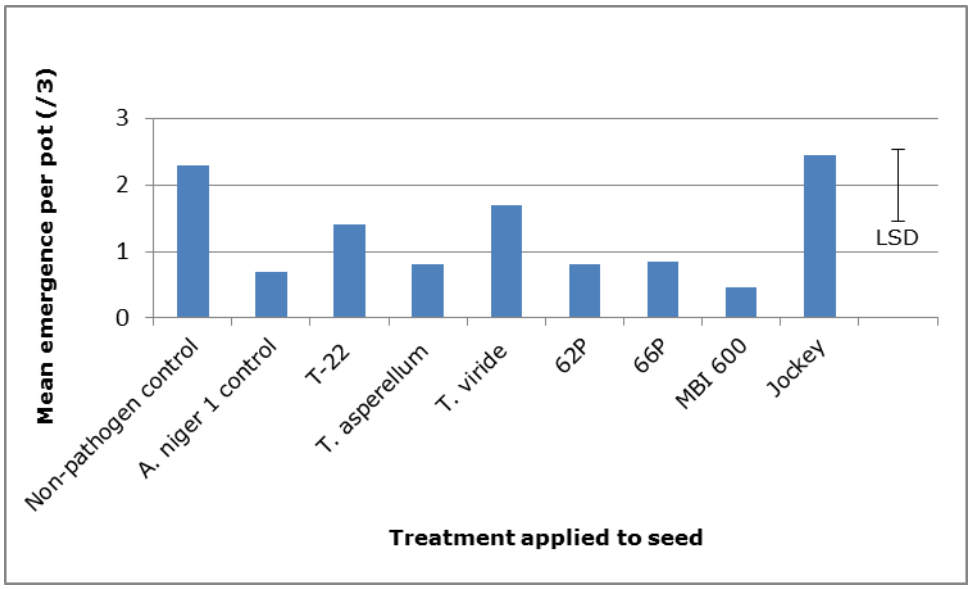


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Treatment	8	16.2600	2.0325	4.20	0.002
Residual	27	13.0600	0.4837		
Total	35	29.3200			

Figure 4.3.3 Activity of biocontrol agents applied to groundnut seeds planted in *A. niger* (AN42054) amended compost at 14 d after planting. Bar represents the LSD. MBI600, 62P and 66P are strains of *Bacillus amyloliquefaciens*.

4.3.4 *A. niger* Nigeria

The efficacy of bio-control agents was evaluated against *A. niger* from Nigeria. ANOVA indicated a highly significant treatment effect (P=0.004). The LSD value showed that Jockey significantly did not differ from *T. viride* and T-22. Other treatments gave little trend of pathogen inhibition (Figure 4.3.4).



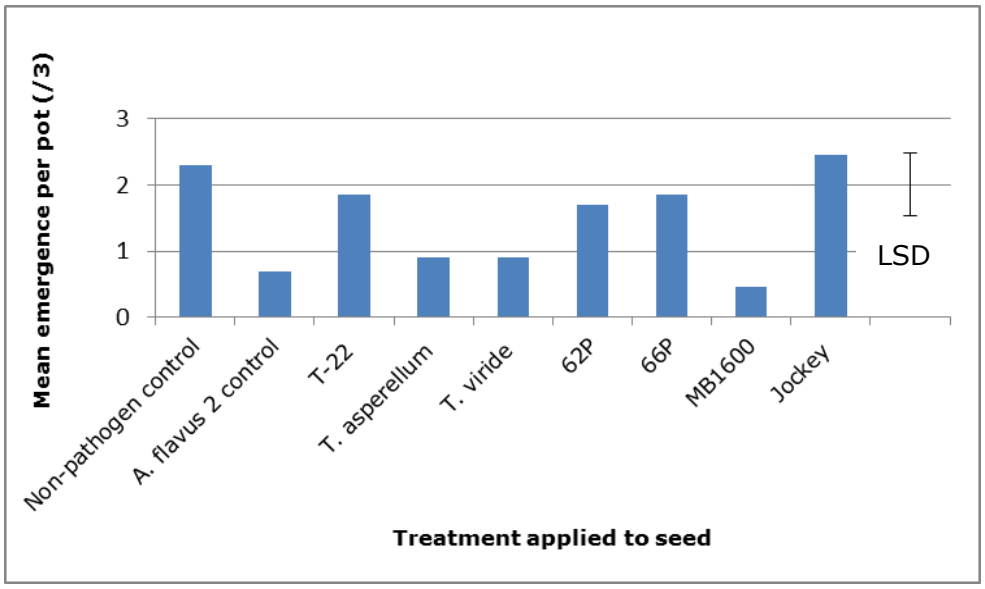
Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Treatment	8	17.7756	2.2219	3.81	0.004
Residual	27	15.7300	0.5826		
Total	35	33.5056			

Figure 4.3.4 Activity of biocontrol agents applied to groundnut seeds planted in *A. niger* (Nigeria) amended compost at 14 d after planting. Bar represents the LSD. MBI600, 62P and 66P are strains of *Bacillus amyloliquefaciens*.

- The experiments were continued to determine whether any of the effects on emergence (14 d after sowing) were extended to plant survival assessed at 27 d after sowing. That is to say, did the efficacy of the BCAs also extend to prevent post-emergence damping-off?

4.4.1 *A. flavus* (AF364493) CABI (27 d post planting)

In planta efficacy against *A. flavus* (AF364493), assessed as plant survival 27 d after sowing, is shown in Figure 4.4.1. ANOVA indicated a highly significant treatment effect (P<0.001). The LSD value shows that T-22, Jockey, 62P, and 66p treated seeds supported the highest antifungal activity, and statistically these treatments have the same significant effect. Jockey gave the higher emergence. However, 62P did not differ significantly from non-pathogen control. Plant survival in a high disease pressure environment was similar to plant emergence at 14 d.

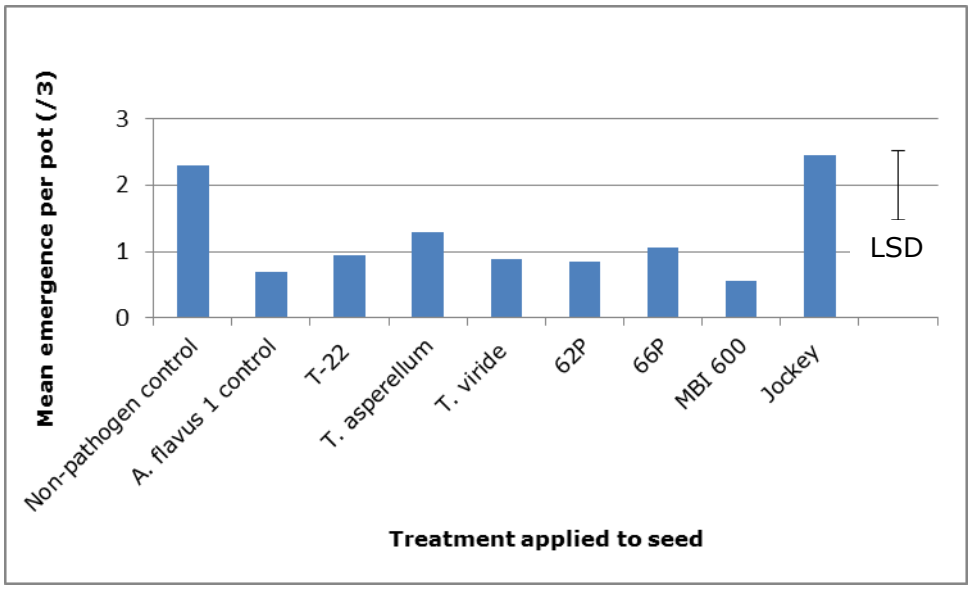


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Treatment	8	15.5622	1.9453	4.99	<0.001
Residual	27	10.5300	0.3900		
Total	35	26.0922			

Figure 4.4.1 Activity of biocontrol agents applied to groundnut seeds planted in *A. flavus* (AF364493) amended compost at 27 d after planting. Bar represents LSD.

4.4.2 *A. flavus* Nottingham (ATCC204304)

The efficacy of biocontrol agents was also evaluated against isolate ATCC204304 from the University of Nottingham. ANOVA indicated a highly significant treatment effect (P=0.009). The LSD value shows that none of the BCA treatments tested were as active as Jockey in suppressing *A. flavus* strain ATCC204304 (Figure 4.4.2).

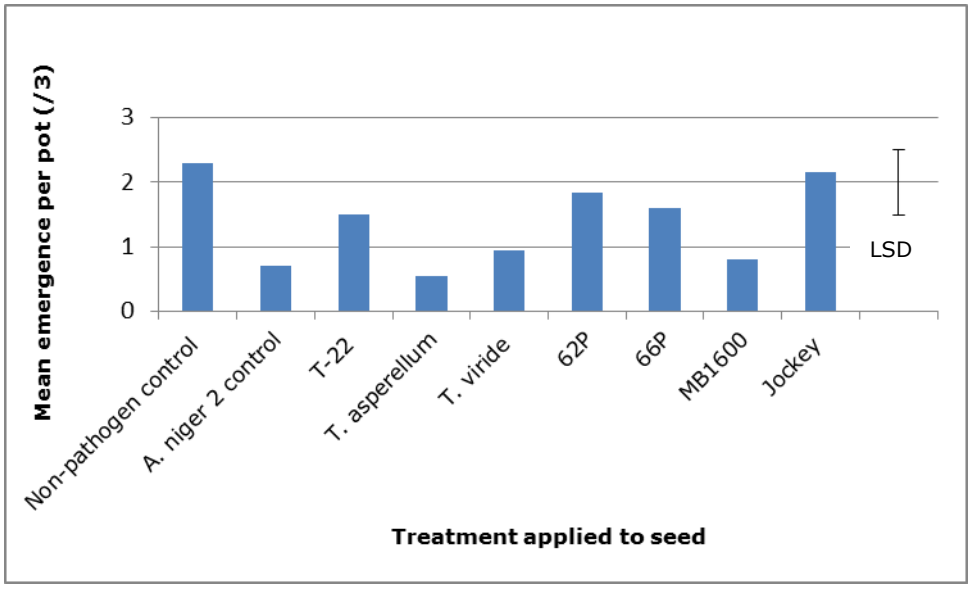


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Treatment	8	15.0500	1.8813	3.36	0.009
Residual	27	15.1375	0.5606		
Total	35	30.1875			

Figure 4.4.2 Activity of biocontrol agents applied to groundnut seeds planted in *A. flavus* (ATCC204304) amended compost at 27 d after planting. Bar represents the LSD. MBI600, 62P and 66P are strains of *Bacillus amyloliquefaciens*.

4.4.3 *A. niger* CABI (AN42054)

The antimicrobial activity of beneficial microorganisms against *A. niger* (AN42054) was evaluated. ANOVA indicated that there was a significant treatment effect (P=0.0014). The LSD value indicated that there was a trend of pathogen inhibition effect. Groundnut seeds treated with Jockey had the highest seedling survival, but T-22, 62P and 66P showed non-significant disease suppression (Figure 4.4.3).

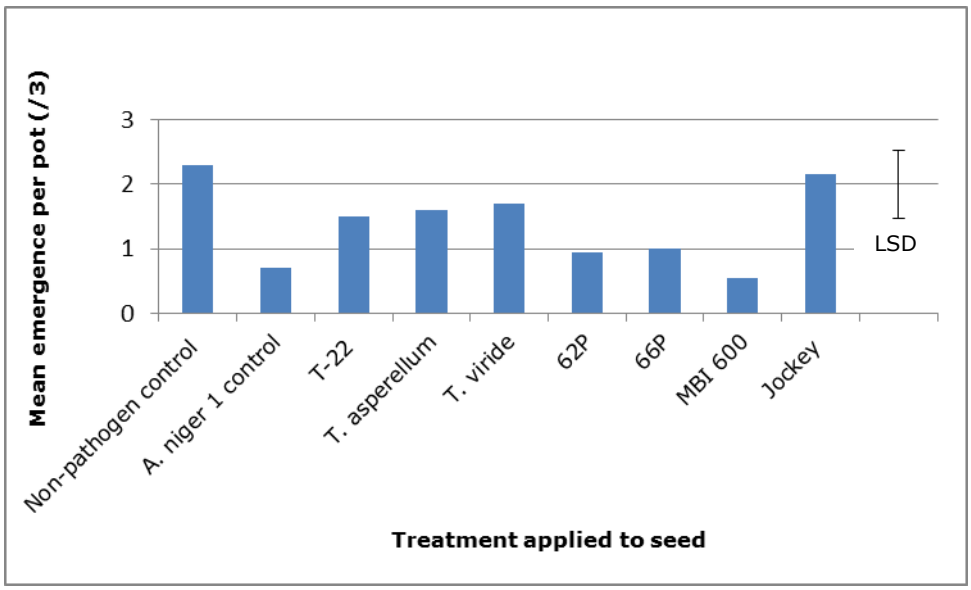


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Treatment	8	13.5822	1.6978	3.06	0.014
Residual	27	14.9600	0.5541		
Total	35	28.5422			

Figure 4.4.3 Activity of plant biocontrol agents applied to groundnut seeds planted in *A. niger* (AN42054) amended compost at 27 d after planting. Bar represents the LSD. MBI600, 62P and 66P are strains of *Bacillus amyloliquefaciens*.

4.4.4 *A. niger* Nigeria

ANOVA indicated that the treatment effect was significant (P=0.022). The *A. niger* isolate from Nigerian groundnut was adequately suppressed by Jockey which enhanced plant survival (Figure 4.4.4). The LSD value also indicated that other treatments showed non-significant pathogen inhibition.



Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Treatment	8	12.340	1.5425	2.78	0.022
Residual	27	14.9700	0.5544		
Total	35	27.3100			

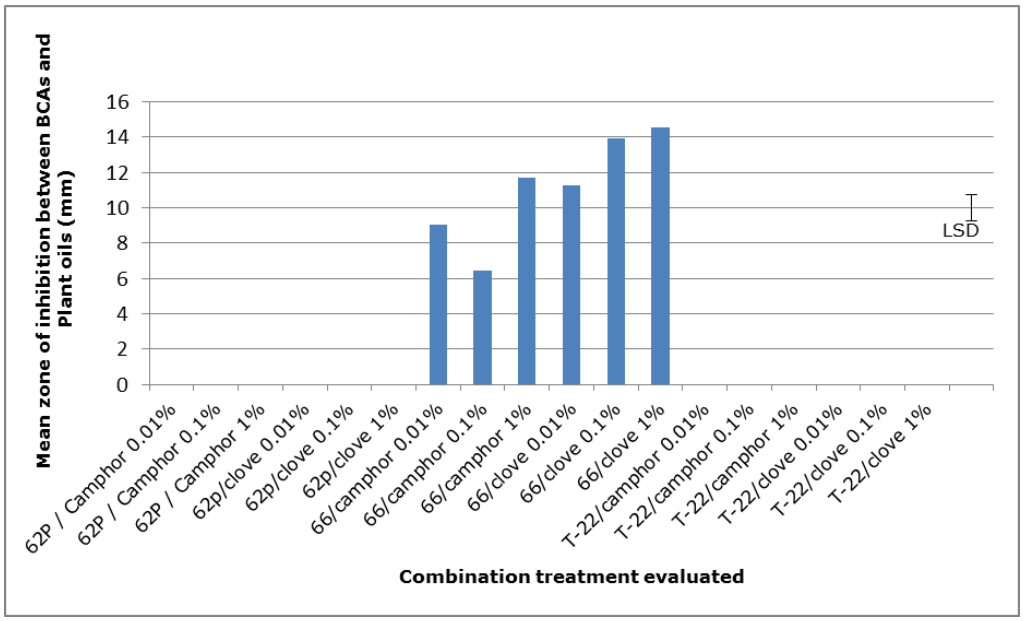
Figure 4.4.4 Activity of biocontrol agents applied to groundnut seeds planted in *A. niger* (Nigeria) amended compost at 27 d after planting. Bar represents the LSD. MBI600, 62P and 66P are strains of *Bacillus amyloliquefaciens*.

- Overall, the *Trichoderma* strains tested showed some antimicrobial activity against the strains of *Aspergillus*, shown to be very highly significant (P<.001). MBI 600, however, had least antimicrobial activity.

- Of all the BCAs screened, in both *in vitro* and *in planta* experiments, T-22 proved the most consistent, and was combined with the best EOs, camphor and clove. They were tested in PDA plate assays for negative interactions, which revealed that they are not sensitive to each other. Hence, they were combined for the combination treatment for *in vivo* experiments.

4.5 Compatibility of BCAs with plant oils using point inoculation *in vitro* assay

Evaluation of the compatibility or incompatibility of the interaction between BCAs and EOs was undertaken to assess whether they could be used for combination treatment against *Aspergillus* strains in amended compost. Anova indicated a highly significant treatment effect (P<0.001). From the data given in Figure 4.5, the LSD indicated that camphor and clove oils were inhibitory towards *B. amyloliquefaciens* strain 66p, but not towards strain 62p or *T. harzianum* T-22. As the latter was the overall most active BCA tested, this was chosen for combination with the oils in an integrated approach to suppress *Aspergillus* infection.



Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value.
BCAs/plant oils	18	2189.022	121.612	101.81	<0.001
Residual	57	68.085	1.194		
Total	75	2257.10			

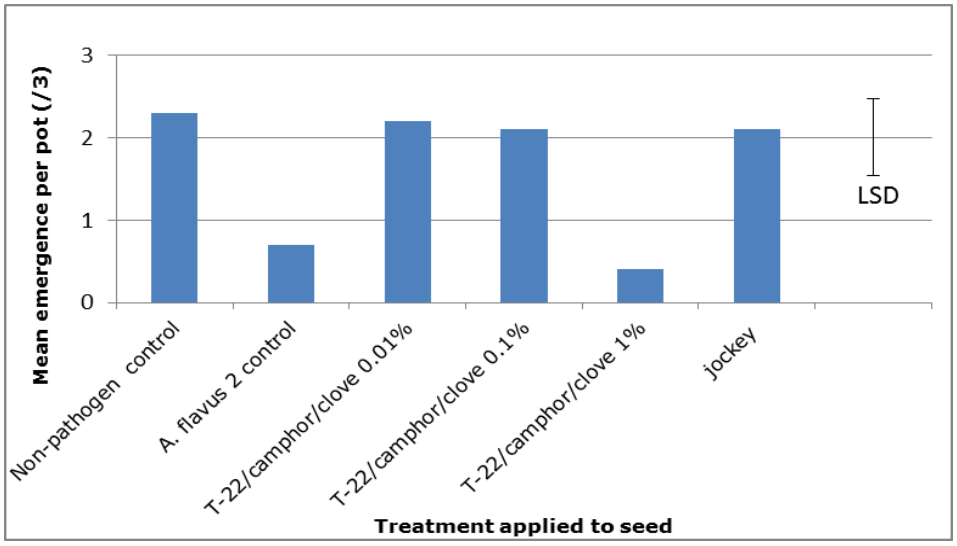
Figure 4.5 *In vitro* antifungal and antibacterial activity of plant oils or BCAs in point inoculated plate assay at 20^{0}C. Bar represents the LSD. Control was not included in the graph, because zone of inhibition is zero. MBI600, 62P and 66P are strains of *Bacillus amyloliquefaciens*.

4.6 Pathogen-amended compost *in planta* assays, with combined oils + T-22 applied as seed treatments

Experiments were conducted to evaluate the efficacy of a combination treatment comprising the most active BCA (T-22) with the two most active EOs (clove and camphor), for seed amendment to enhance emergence and plant survival in *Aspergillus*-amended compost, in comparison to Jockey.

4.6.1 *A. flavus* (AF364493) CABI (14 d post planting)

ANOVA indicated a highly significant treatment effect (P<0.001). The LSD value shows that the combination treatment of T-22 plus camphor and clove oils, at 0.01 and 0.1% dilutions, provided the highest antifungal activity in suppressing *A. flavus* (AF364493). Inclusion of the oils at 1% dilution in this combination treatment suppressed emergence (Figure 4.6.1). Plant emergence was enhanced in this experiment, more than 14 d single application of T-22. The best combination treatments were equivalent to Jockey.

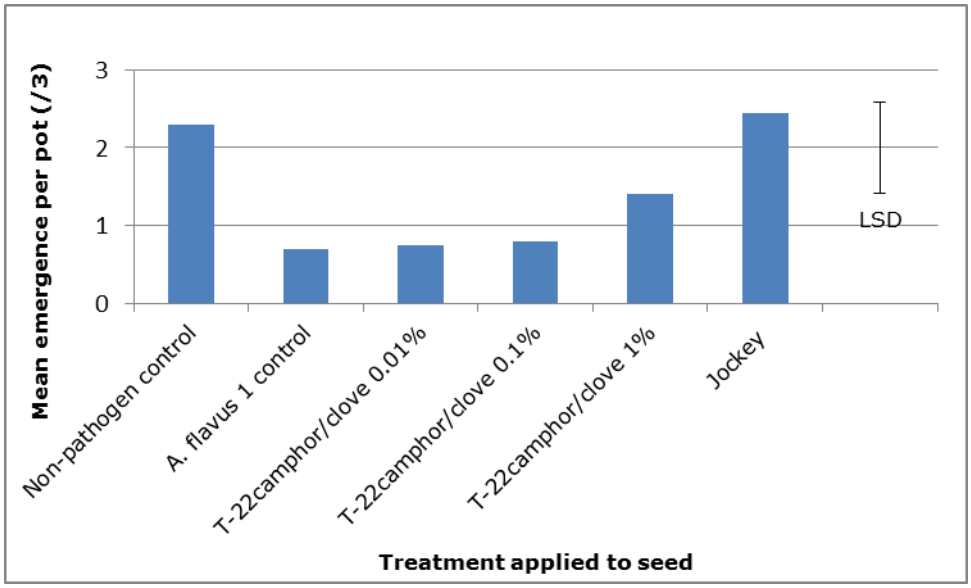


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Combination Treatment	5	15.0083	3.0017	6.97	<0.001
Residual	18	7.7500	0.4306		
Total	23	22.7583			

Figure 4.6.1 Activity of combination treatment of T-22 plus plant essential oils applied to groundnut seeds planted in *A. flavus* (CABI) amended compost at 14 d after planting. Bar represents the LSD.

4.6.2 *A. flavus* Nottingham (ATCC204304)

ANOVA in this experiment shows a significant treatment effect (P=0.017). The LSD value shows little pathogen suppression (Figure 4.6.2); none of the unconventional treatments gave good control when compared to Jockey.

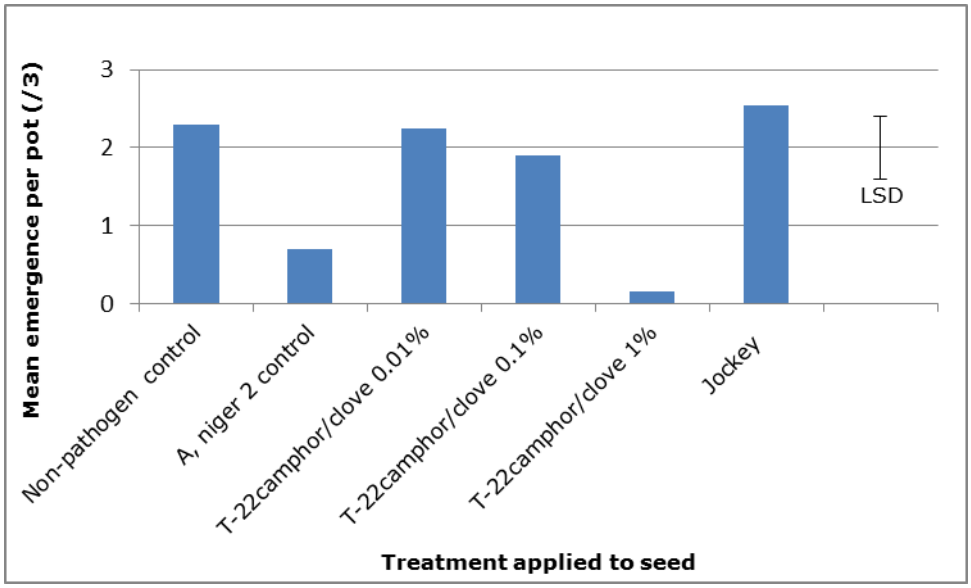


Source of variatio	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value.
Combination Treatment	5	12.7400	2.5480	3.73	0.017
Residual	18	12.3000	0.6833		
Total	23	25.0400			

Figure 4.6.2 Activity of combination treatment of T-22 plus plant essential oils applied to groundnut seeds planted in *A. flavus* (ATCC204304) amended compost at 14 d after planting. Bar represents the LSD.

4.6.3 *A. niger* (AN42054) CABI

The activity of combined EO and BCA treatments, applied as seed amendments, against soil-borne *A niger* (AN42054) was evaluated as emergence assessed 14 days after sowing (Figure 4.6.3). ANOVA shows that the treatment effect was highly significant (P<0.001). The LSD value reveals that T-22 + camphor and clove oils at 0.01 and 0.1% supported groundnut seed emergence similar to the control and Jockey in compost amended with *A. niger* (AN42054). Once again, the combination treatment containing 1% oils was apparently suppressive to seedling emergence.

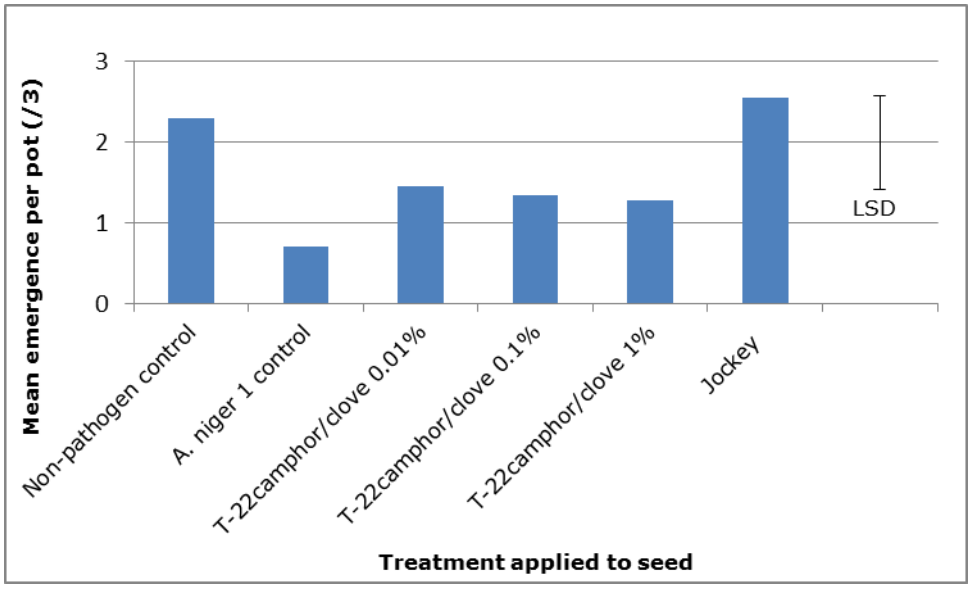


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Combination Treatment	5	19.2283	3.8457	11.59	<0.001
Residual	18	5.9700	0.3317		
Total	23	25.1983			

Figure 4.6.3 Activity of combination treatment of T-22 plus plant essential oils applied to groundnut seeds planted in *A. niger* (AN42054) amended compost at 14 d after planting. Bar represents the LSD.

4.6.4 *A. niger* Nigeria

ANOVA shows that there was less significant treatment effect (P=0.047). The LSD value indicated that all the combination treatments tested provided a non-significant trend in suppression of *A. niger* (Nigeria) in pathogen amended compost as shown in Figure 4.6.4. More plant emergence was obtained with Jockey.

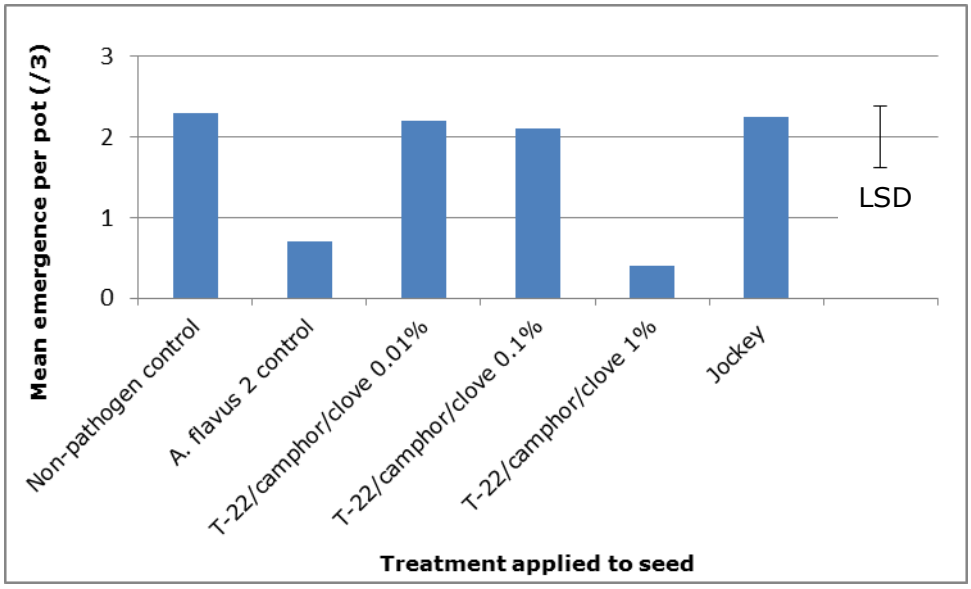


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Combination Treatment	5	9.5721	1.9144	2.83	0.047
Residual	18	12.1575	0.6754		
Total	23	21.7296			

Figure 4.6.4 Activity of combination treatment of T-22 plus plant essential oils applied to groundnut seeds planted in *A. niger* (Nigeria) amended compost at 14 d after planting. Bar represents the LSD.

4.7.1 *A. flavus* (AF364493) CABI 27 d post planting

Again, the experiment was extended to 27 d post sowing to ascertain whether the seed treatments provided enhanced seedling survival (Figure 4.7.1). ANOVA indicated a highly significant treatment effect (P<0.001). The LSD value indicated that the findings were parallel to those reported in Figure 4.6.1, indicating good survival of the plants at 27 d post-sowing, in all treatments except 1% EOs. More plant emergence was obtained when compared to a single application of T-22. It is interesting, however, to note that T-22 when used alone provide better disease suppression (Section 4.3 and Figure 4.3.1).

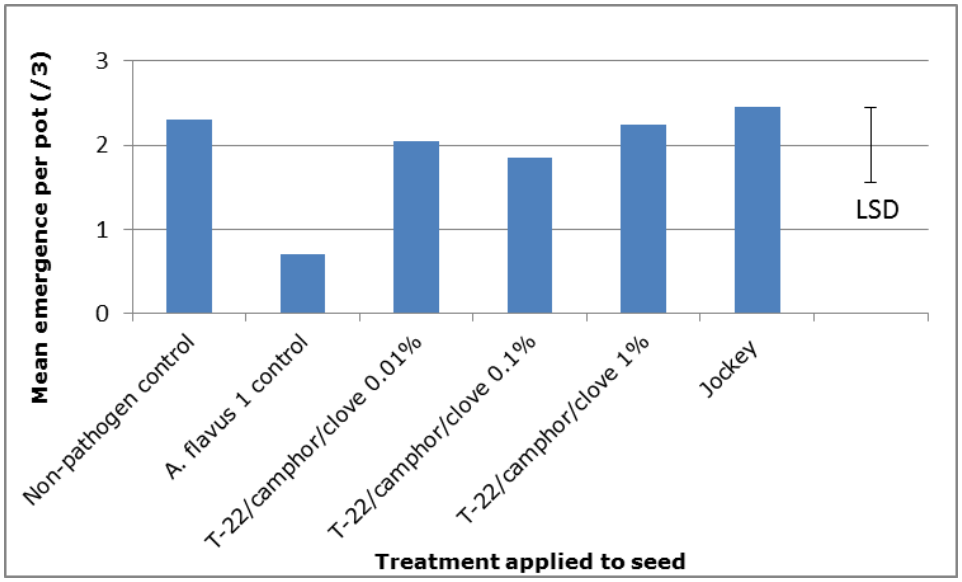


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Combination Treatment	5	20.268	4.0537	13.59	<0.001
Residual	18	5.3700	0.2983		
Total	23	25.6383			

Figure 4.7.1 Activity of combination treatment of T-22 plus plant essential oils applied to groundnut seeds planted in *A. flavus* (AF364493) amended compost at 27 d after planting. Bar represents the LSD.

4.7.2 *A. flavus* Nottingham (ATCC204304)

ANOVA indicated a significant treatment effect (P=0.012). The LSD value shows that all the combination treatments of seeds, planted in *A. flavus* (ATCC204304) amended compost, suppressed infection up to 27 d post sowing (Figure 4.7.2).

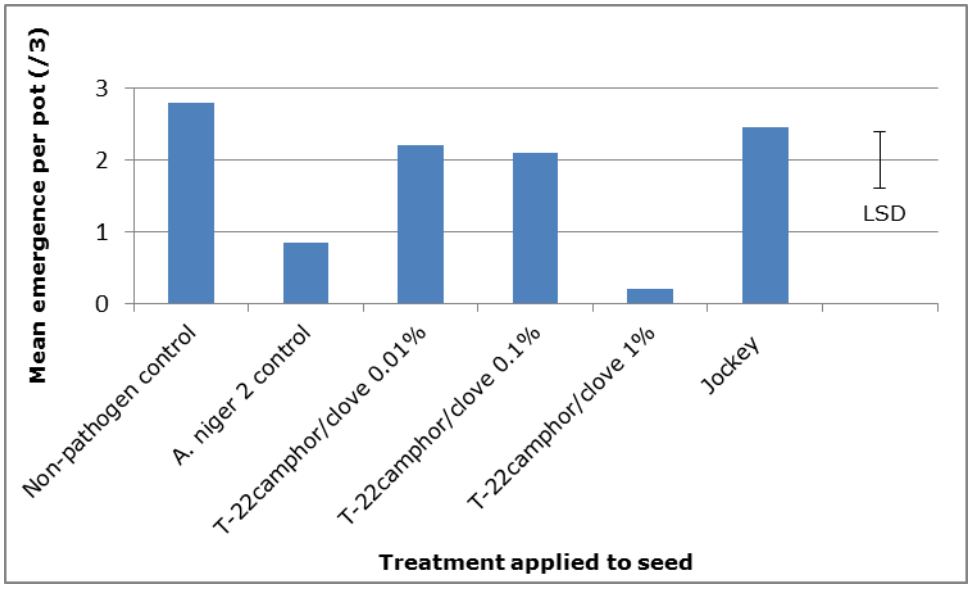


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Combination Treatment	5	8.1733	1.6347	4.11	0.012
Residual	18	7.1600	0.3978		
Total	23	15.3333			

Figure 4.7.2 Activity of combination treatment of T-22 plus plant essential oils applied to groundnut seeds planted in *A. flavus* (Nottingham) amended compost at 27 d after planting. Bar represents the LSD.

4.7.3 *A. niger* (AN42054) CABI

ANOVA indicated a highly significant treatment effect (P<0.001). The LSD value shows that this experiment, using *A. niger* (AN42054), gave similar results to those reported in Figures 4.6.3 and 4.7.1. Where good emergence was detected, the plants survived to the 27 d assessment. Once again the EOs at 1% proved phytotoxic.

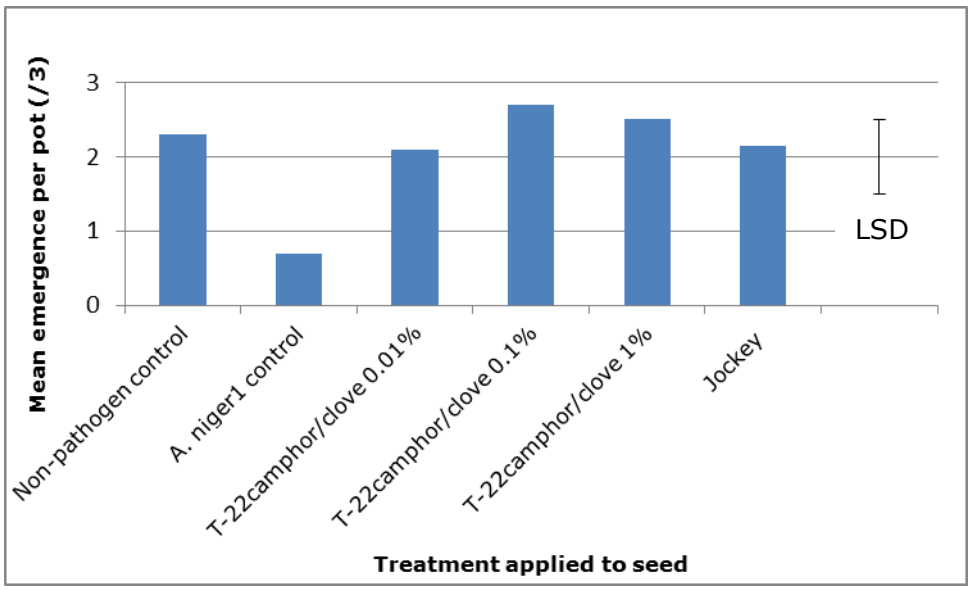


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Combination Treatment	5	19.5533	3.9107	12.71	<0.001
Residual	18	5.5400	0.3078		
Total	23	25.0933			

Figure 4.7.3 Activity of combination treatment of T-22 plus plant essential oils applied to groundnut seeds planted in *A. niger* (AN42054) amended compost at 27 d after planting. Bar represents the LSD.

4.7.4 *A. niger* Nigeria

When assessed against the *A. niger* (Nigeria) strain, all the combination treatments tested provided good survival of the groundnut seedlings up to 27 d post-planting (Figure 4.7.4). ANOVA indicated a significant treatment effect (P=0.012). The LSD value indicated all the treatments had antifungal activity in suppressing the pathogen and enhanced plant survival in the highly pathogen-amended compost.



Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Combination Treatment	5	10.0750	2.0150	4.09	0.012
Residual	18	8.8700	0.4928		
Total	23	18.9450			

Figure 4.7.4 Activity of combination treatment of T-22 plus plant essential oils applied to groundnut seeds planted in *A. niger* (Nigeria) amended compost at 27 d after planting. Bar represent LSD.

- From the results obtained using combination treatments, the treatments proved effective in inhibiting the strains of *Aspergillus* and increased plant growth, except T-22 / camphor / clove oil at 1% on CABI strains.

4.8 Discussion

This project generated some useful results on the effectiveness of biocontrol agents for control of *Aspergillus* on groundnut. *T. harzianum* (T-22 strain) proved more effective than *Bacillus* spp., and *P. chlororaphis*, in suppressing the growth of *A. flavus* and *A. niger*, both *in vitro* and *in planta*. This might reflect production of antibiotics and toxins that inhibited these pathogens, and the release of a chitinase that disrupted the cell wall of the fungi. Such inhibitory effects by *T. harzianum* T-22 have been described by many authors, including Grondona *et al*. (1997) and Howell (2003). This strain was recently used by Mastouri *et al.* (2010) for tomato seed treatment which proved effective in enhancing seed germination and seedling development. Multiple antagonistic effects have played a role for effective biocontrol by different *Trichodema* strains, as alternative measures to chemicals for suppression of a wide spectrum of plant pathogens, which have been described by many researchers including Chet (1987) and Harman and Bjorkman (1998).

The commercial granular formulation of *Trichoderma* (TUSAL) comprised two species, identified from sequence analysis as *T. viride* and *T. asperellum.* These were the second most effective BCAs in the experiments reported here. According to earlier reports, they can maintain their activity for a long period without losing their efficacy, as described by Jin *et al.* (1991, 1992, 1996), and they effectively antagonized *A. flavus* and *A. niger*, suppressing growth both on PDA plates and in *in planta* experiments. This supports published findings that the antagonistic activity served as the basis for effective biological control, using different *Trichoderma* strains, as an alternative to chemicals, for the control of a wide spectrum of plant pathogens. This was reportedly brought about by competition, colonisation, antibiosis and mycoparasitism (Howell, 2003; Chet, 1987; Harman and Bjorkman, 1998).

Bacillus amyloliquefaciens (MBI600, 62P and 66P isolates) also exhibited antifungal activity in inhibiting *Aspergillus* spp, both in *in vitro* and *in planta*. These are Gram positive bacteria that also have the potential to enhance plant growth, acting as growth-promoting rhizobacteria PGPR (Zehnder *et al.*, 2001). These species are among various groups of plant-associated microorganisms that suppress plant diseases (Joseph *et al.*, 2004). The efficacy of *Pseudomonas chlororaphis* spp*. aureofaciens* has also

been attributed to its rapid colonization of plants and production of antibiotics and other fungal metabolites in suppressing the growth of *A. niger* and *A. flavus.* This finding agreed with some earlier reports using *Pseudomonas* spp. to control stem rot disease in groundnut at ICRISAT, India. The strains were effective because they were effective root colonizers and had biocontrol activity associated with production of antibiotics, hydrogen cyanide and siderophores (Manjula *et al.*, 2004; O'Sullivan and O'Gara, 1992).

The BCAs were also significantly effective in improving emergence when applied as a seed amendment to seed sown in *Aspergillus*-inoculated compost, in comparison to the commercial fungicide seed dressing, Jockey. Vanilla oil and *T. harzianum* (T-22 strain) proved to be more effective than *Bacillus amyloliquefaciens* and *Pseudomonas chlororaphis* in suppressing the growth of *Aspergillus,* both *in vitro* and in *in planta* experiments. Compatibility of BCAs and plant oils was also evaluated in PDA plates by point inoculation assay and the results demonstrated the feasibility of using certain combinations.

The principal active ingredient of Jockey, fluquinconazole, caused inhibition of *Aspergillus* spp., and increased emergence of groundnut plants. This is the first report in which Jockey has been used as a groundnut seed dressing. It is mainly used for canola, wheat, and barley disease control. This could help to reduce crown rot and afla root disease in groundnut plants and could be widely recommended for groundnut seed dressing before planting.

The molecular approach used, by sequencing the PCR products, was able to identify *A. niger* from Nigerian groundnut, with greatest identity to an accession number of gi/4092044/AF078895.1/. Identity was confirmed with other strains in the NCBI database. This pathogen causes black mould on some fruits, legumes and vegetables, such as grapes, onions and groundnut, and is a common contaminant of food. Some strains of *A. niger* have been reported to produce potent mycotoxins called ochratoxins (Abarca *et al.*, 1994). *A. niger*, which causes collar rot disease on groundnut seedlings, was first investigated by Jochem (1926). *A. niger* may cause an average of 5% loss in yield, but in some parts it may cause losses as high as 40% in groundnut. Collar rot disease is a serious problem in sandy soil (Gibson, 1953; Chohan, 1965).

Trichoderma species are economically important biological control agents used in plant disease management. They are better rhizosphere colonizers than many plant pathogens, and hence compete with other organisms for food and space in the rhizosphere, thereby reducing the chances of colonization by plant pathogenic fungi. Commercial products based on *Trichoderma* are used world-wide, particularly in the United States, European countries and China, due to their effectiveness in disease management.

Trichoderma asperellum performed better than the other tested *Trichoderma* species in PDA plate assays this might have been a consequence of its fast sporulation in the medium, which inhibited the tested pathogens. *In planta* assay sporulation might have been delayed in the growth medium to colonize and suppress the toxigenic *Aspergillus* strains at the rhizosphere of groundnut plant, this could be responsible for its poor performance when compared to T-22.

Both BCAs in Chapter 4 and EOs in Chapter 3 results were effective in inhibiting the tested pathogens in PDA plate assays, improved plant emergence and also enhanced plant survival in a high disease pressure environment and only small amount of plant damping off was observed.

Chapter 5 Efficacy of post-harvest treatment, detection of aflatoxin using an ELISA test kit and use of a LAMP assay to monitor infection by detection of pathogen DNA

5.1 Introduction

5.1.1 Post harvest losses

Post-harvest preservative treatments are treatments given to prevent losses in agricultural products during storage. These can be caused by both biotic and abiotic agents and can significantly reduce food sustainability, as qualitative and quantitative losses. Such losses contribute to global food insecurity, despite the use of modern storage facilities and techniques in developed nations. Losses are estimated from 10-30% in developing countries, which often lack adequate infrastructure, causing a serious negative impact on food availability (Gustavsson *et al*., 2011). Currently, the global population is predicted to reach 9.6 billion by 2050 (UN June 16, 2013), which contributes to food security problems. This increase is mainly associated with the poorest communities of the world. According to Alexandratos and Bruinsma (2012), food supplies would need to be increased by 60% (estimated at 2005 food production levels) in order to meet the teeming population demand for food by 2050. Food availability and accessibility can be rapidly increased by increasing production, improving distribution, and reducing food losses. However, reduction of post-harvest food losses is a critical aspect of ensuring future food security.

In the past decades, significant resources have been utilised to increase food production. Ninety-five percent of global research investment to tackle food security over the past 30 years is reported to have focused on increasing productivity, and only 5% was channelled towards reducing post-harvest losses. This could be considered inadequate (Kader, 2005; Kader and Roller, 2004; WFLO, 2010). Increasing agricultural productivity is critical for ensuring food security, but this might not be sufficient. Food production is currently being challenged by limited land, water, pests and diseases and increased weather variability due to climate change. To sustainably achieve the goals of food security, food availability needs to be increased, through reductions in the post-harvest losses at farm, retail and consumer levels. Post-harvest food loss is defined as measurable qualitative and quantitative loss along the supply chain, starting at the time of harvest until consumption or other end use (De Lucia and Assennato, 1994; Hodges

et al., 2011). Food losses can be quantitatively measured by decreased weight or volume, or can be qualitative, such as reduced nutrient value and unwanted changes to taste, colour, texture, or cosmetic features of food (Buzby and Hyman, 2012). The United Nations estimates that 1.3 billion tons of food is lost globally every year (Gustavsson *et al.*, 2011). Infection associated with *A. flavus* often causes areas of brown or yellow discolouration, that may also be associated with external sporulation of the fungus. However, considerable invasion of and aflatoxin contamination can commonly occur without visible signs of sporulation. Concealed damage, in which the inner lumen between the cotyledons is occupied with conidial heads, could be detected only by splitting the seeds in half (Horn, 2005). Diener *et al*. (1987) described consequences of the high level of *A. flavus* infection in groundnut seed, which might result in pre-emergence rotting of the seed and seedlings, a condition known as yellow mould. Brown, necrotic lesions with sporulation of *A. flavus* are found on the cotyledons, radicles, and hypocotyls of ungerminated and germinated seeds. Emerged seedlings also had necrotic lesions on the cotyledons, which might result in stunting and chlorotic lesions in plants which developed aflatoxin infected roots (afla roots).

5.1.2 Management practices

Efficient management strategies would assist in minimizing aflatoxin contamination in the field, such as irrigation, which could alleviate drought stress to groundnut plants. If irrigation is not provided, the problem might be reduced by early harvesting during a drought period, before contamination becomes extensive. Control of insects with insecticides also reduces the incidence of damaged seed that contains high levels of aflatoxin, but drought situations limit the use of some insecticides, which require moisture to be effective (Okello *et al*., 2010; Hell *et al*., 2000). Pre-emergence rotting of seed and seedlings caused by *A. flavus* is best avoided by propagating high quality seeds (Okello *et al*., 2010; Hell *et al*., 2000). Appropriate storage in warehouses might prevent further contamination of groundnut seed with aflatoxin (Okello *et al*., 2010). Seed should be adequately protected from rehydration caused by intense insect activity, leaking roofs, or moisture condensation resulting from temperature fluctuations. Good ventilation with adequate roof and wall insulation could limit condensation in warehouses. Aflatoxin is not uniformly distributed in a contaminated seed lot, and early removal of high-risk seed, such as those

that are damaged, immature, or loose (shelled during combining operations), could reduce by more than 95% aflatoxin contamination during the processing period (Okello *et al*., 2010; Rao *et al*., 2010). Procedures for removing high-risk seed include removal of loose seed and small pods with a high-capacity belt screen, separation of small, immature seed after shelling by use of vibratory screens, density separation, in which lighter, aflatoxin-contaminated seed are sorted on gravity tables, electronic colour sorting, which removes seed discoloured by fungal colonization and blanching, in which the outer seed coat, or skin, is exposed to discolouration that could be detected by electronic colour sorters (Whitaker, 1997). Seeds from these high-risk categories are then diverted from the edible market to oil production.

5.1.3 LAMP assays to quantify infection

During the past two decades, molecular methods such as PCR and real-time PCR have been applied to the detection of *Aspergillus* species by amplifying genes relevant to the biosynthesis of aflatoxins (Shapira *et al*., 1996; Geisen, 1996; Medeiros *et al*., 2009). However, the requirement for trained personnel, specialised equipment and reagents would hamper the broad practical application of PCR-based methods (Mullah *et al*., 1998). Preparation of Mass spectra (Frisvad *et al*., 2007) and the use of housekeeping gene sequencing methods (Samson *et al.*, 2006) provide powerful tools for species differentiation and phylogenetic study but their application is time consuming and needs highly sophisticated laboratory equipment. A great variety of methods have been described which use PCR-based assays for identification, detection or quantification of important aflatoxin producing species (Niessen, 2008). However, since PCR-based methods all need some kind of DNA clean up and concentration prior to analysis, screening of a mass of fungal pure cultures is still a very time-consuming and cumbersome job. However, identification of pure cultures is an easy task compared with detection of contaminants directly from infected commodities, where a complex mycobiota is present, often containing closely related species and several compounds which may affect the efficiency and sensitivity of the detection assay used (Rossen *et al*., 1992; Färber *et al*., 1997). In some cases even additional incubation of the food samples for several days is required in order to increase the target organism's biomass prior to analysis (Chen *et al*., 2002). As an alternative to PCR-based analysis, loop-mediated isothermal amplification

(LAMP, Notomi *et al.,* 2000) has been described as an easy and rapid diagnostic tool for the early detection of microbes and viruses (Parida *et al*., 2008). LAMP assays have been developed for rapid detection of fungi in clinical samples (Endo *et al*., 2004; Ohori *et al*., 2006; Uemura *et al*., 2008), and Sun *et al*., 2010), in plants (Tomlinson *et al*., 2008 ; Gadkar and Rillig, 2008) and during the process of brewing (Hayashi *et al*., 2007). Recently, Niessen and Vogel (2010) and also Denschlag *et al*. (2012) investigated the use of LAMP-based methods as an alternative to PCR in the detection of *Fusarium graminearum* and other grain quality relevant species in pure cultures and in contaminated samples of wheat and barley seeds. Using the assay of Niessen and Vogel (2010), Abd-Elsalam *et al*. (2011) developed a simple, rapid, and efficient protocol for isolating LAMP-ready genomic *F. graminearum* DNA from germinated wheat seeds. Based on the data presented in the literature, LAMP may constitute a potentially valuable tool also for the rapid diagnosis of aflatoxigenic fungi in food.

In this study, the development and evaluation of simple and rapid LAMP-based assays for identification and detection of *A. flavus* from pathogen infected groundnut pods is described. Validation of the LAMP assay, based on plating-out and identification of target fungi directly, from treated and untreated pathogen infected groundnut seeds is reported. Toxin quantification was done using an ELISA test kit.

5.1.4 Objectives

This chapter focuses on the following objectives:

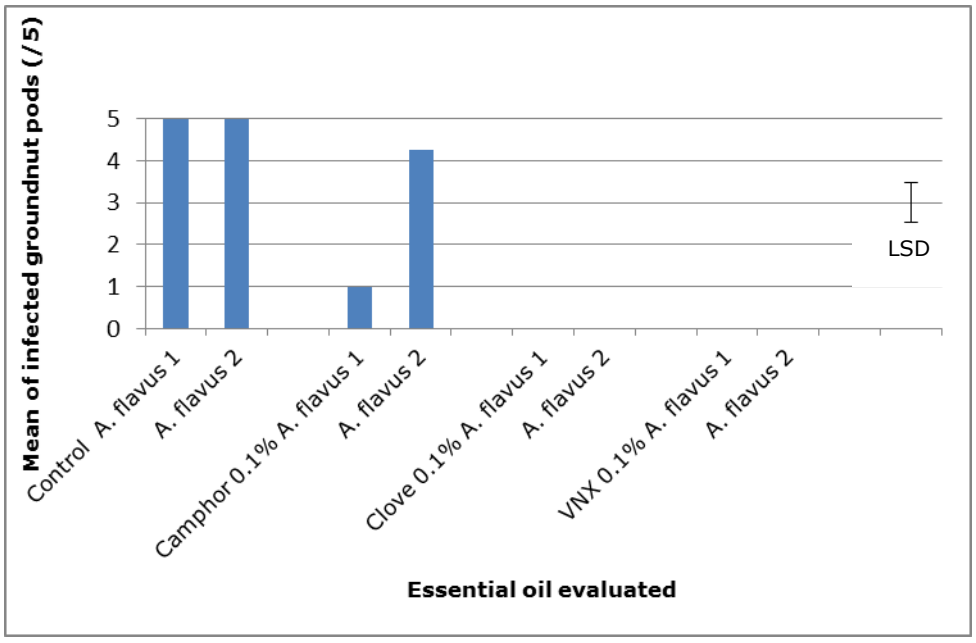
- Evaluation of plant oils and BCAs to suppress post-harvest infection of groundnut pods.
- Determination of the level of aflatoxin in pods inoculated with *A. flavus*, following application of post-harvest treatments.
- Determination of *A. flavus* infection of asymptomatic pods, by comparing a LAMP assay for pathogen DNA detection with conventional plating-out of seeds.

5.2 Results

5.2.1 Inhibition of pod and seed infection by *A. flavus* strains ATCC204304 [1] and AF364493 [2] using essential oils assessed by visible symptoms 14 d after inoculation

5.2.1.1 Groundnut whole pod infection

The ability of plant oils applied to pods inoculated with the two isolates of *A. flavus* to suppress visible post-harvest infection development was determined. ANOVA indicated a highly significant treatment effect (P<0.001). *Aspergillus* isolates were analysed separately. The LSD value shows that the oils based on clove and vanilla were able to markedly reduce infection compared to the untreated control (Figure 5.2.1.1). Camphor oil was less effective. Clove and VNX had no significant difference from each other. Numbers 1 (ATCC204304) from University of Nottingham and 2 (AF364493) from CABI were used in representing *A. flavus* strains.

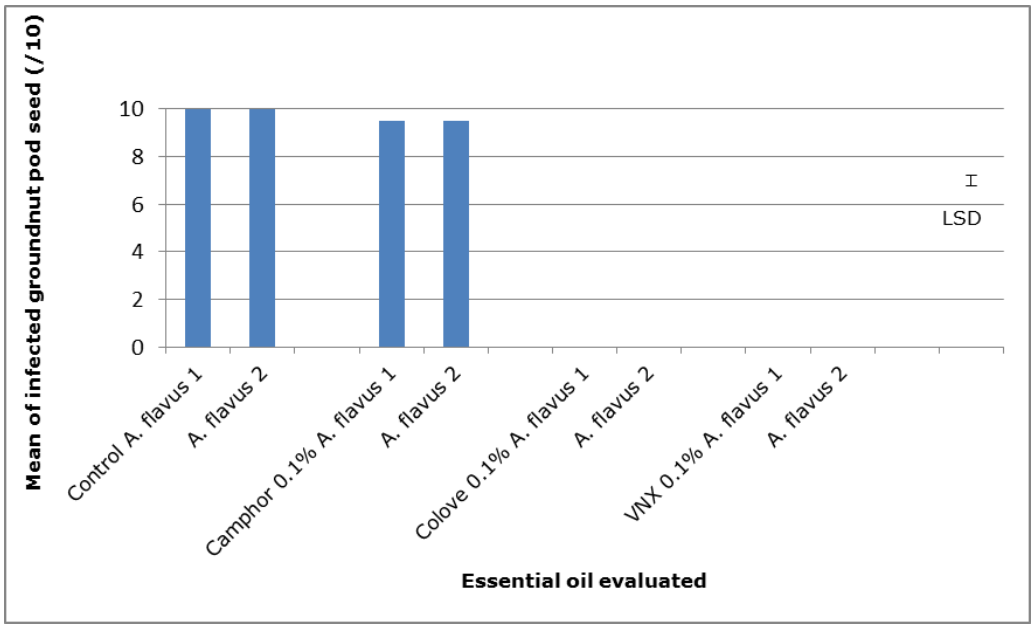


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P value
Plant oils	3	138.8438	46.2812	54.28	<0.001
Residual	28	23.8750	0.8527		
Total	31	162.7188			

Figure 5.2.1.1 Activity of plant EOs in suppression of post-harvest infection of *A. flavus*-inoculated pods 14 d after inoculation. Bar represents the LSD.

5.2.1.2 Groundnut pod – seed contamination

The pods were opened and the number of seeds contained within exhibiting visible signs of *A. flavus* infection was determined. ANOVA indicated a highly significant treatment effect (P<0.001). The LSD value reveals that results obtained (Figure 5.3.1.2) again show high efficacy of clove and vanilla oils, and poor activity of camphor oil.



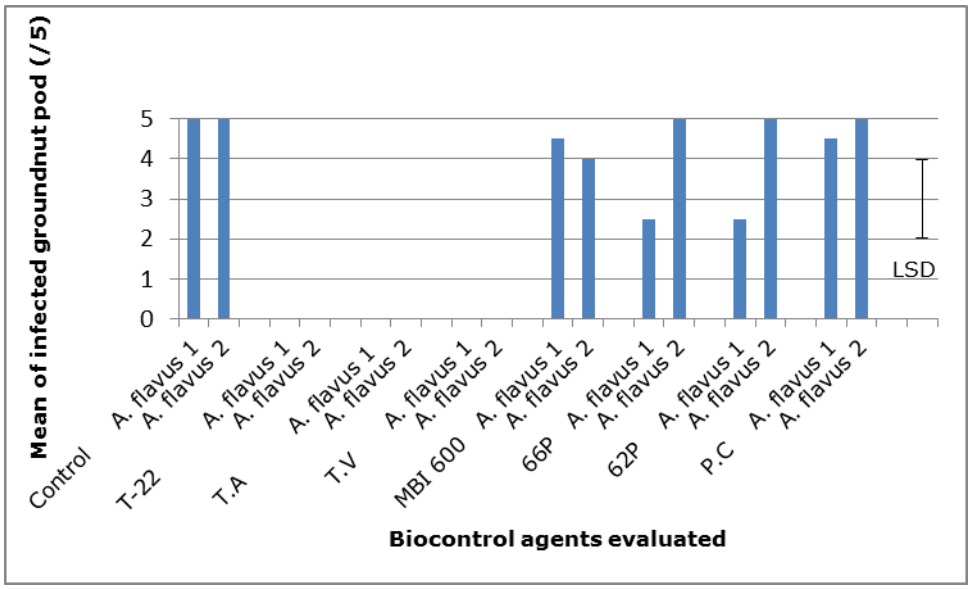
Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P valve
Plant oils	3	761.5000	253.8333	1184.56	<0.001
Residual	28	6.0000	0.2143		
Total	31	767.5000			

Figure 5.2.1.2 Activity of plant EOs in suppression of post-harvest infection of seeds within *A. flavus*-inoculated pods 14 d after inoculation. Each replicate of germination test box contains 5 pods for assessment. Number of seeds per replicate was 10. Assessment on visible infection was based on two seeds per pod. Bar represents the LSD.

5.3.1 Inhibition of pod and seed infection by *A. flavus* strains (ATCC204304 [1] and AF364493[2]) using BCAs assessed by visible symptoms 14 d after inoculation

5.3.1.1 Groundnut whole pod contamination

The ability of BCAs applied to pods inoculated with the two isolates of *A. flavus* to suppress visible post-harvest infection development was evaluated. ANOVA indicated a highly significant treatment effect (P<0.001). The LSD value indicated that the *Trichoderma* strains were the most effective BCAs in inhibiting post-harvest pod infection of *A. flavus* spp. as shown (Figure 5.3.1.1). They are not significantly different from each other, but different from the bacterial BCAs tested.

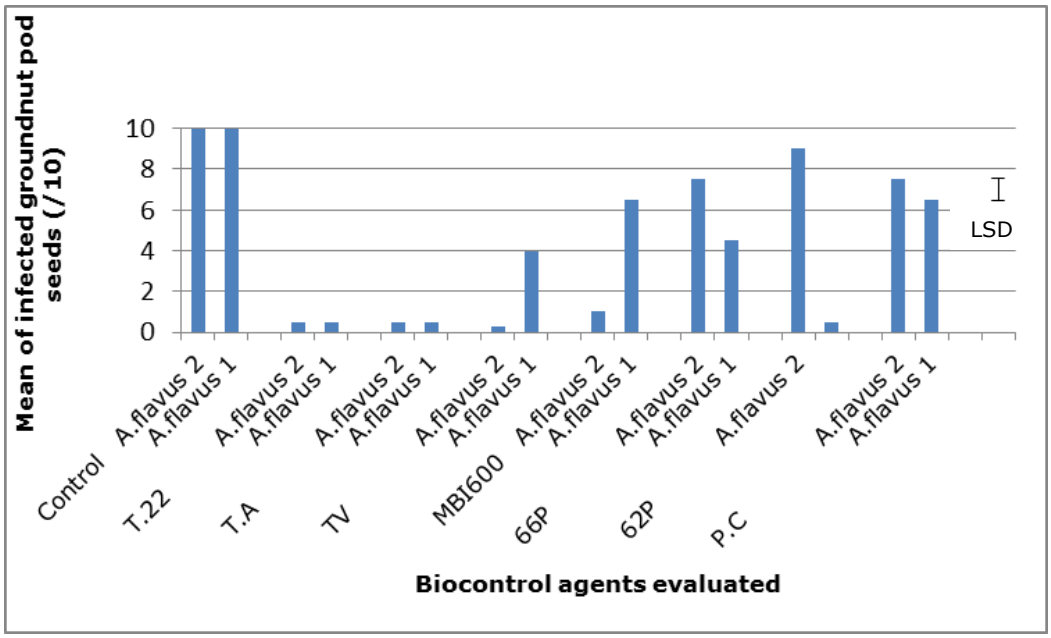


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P value
BCAs treated groundnut pods	7	311.7500	44.5357	46.19	<0.001
Residual	56	54.0000	0.9643		
Total	63	365.7500			

Figure 5.3.1.1 Efficacy of BCAs to suppress *A. flavus* infection of unwounded groundnut pods, 14 d after incubation at 20^{0}C. T-22=*T. harzianium*, T.A=*T. asperellum*, T.V=*T. viride*, MBI600, 66P, 62P are strains of *B. amyloliquefaciens*, P.C=*P. chlororaphis*. Bar represents the LSD.

5.3.1.2 Groundnut pods - seed contamination

The pods from the experiment described immediately above were opened and the number of seeds contained within exhibiting visible signs of *A. flavus* infection was investigated. ANOVA showed a highly significant treatment effect (P<0.001). From the results obtained (Figure 5.3.1.2), the LSD value showed that all the strains of *Trichoderma* significantly suppressed both strains of the pathogen and hence seed contamination. These strains are significantly more effective than the bacterial strains.



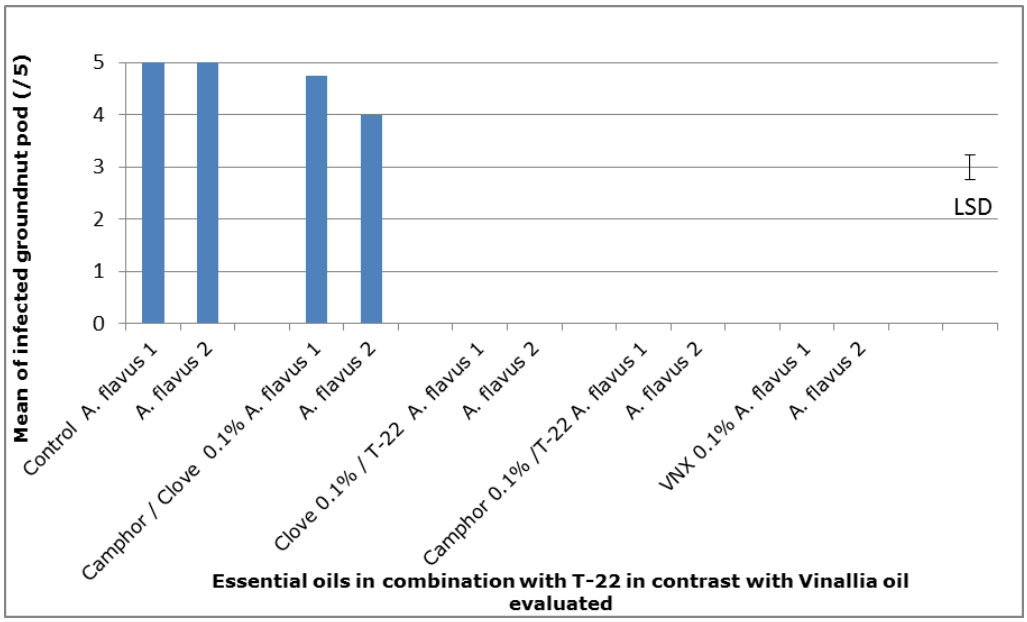
Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P value
BCAs treated groundnut pods	7	614.234	87.748	13.43	<0.001
Residual	56	365.875	6.533		
Total	63	980.109			

Figure 5.3.1.2 Efficacy of BCAs to suppress *A. flavus* infection of seeds within unwounded groundnut pods, 14 d after incubation at 20^{0}C. T-22=*T. harzianium*, T.A=*T. asperellum*, T.V=*T. viride*, MBI600, 66P, 62P are strains of *B. amyloliquefaciens*, P.C=*P. chlororaphis*. Each replicate of germination test box contains 5 pods. Number of seeds per replicate is 10. Assessment on visible infection was based on two seeds per pod. Bar represents the LSD.

5.4.1 Inhibition of pod and seed infection by *A. flavus* strains using combination treatment assessed by visible symptoms 14 d after inoculation

5.4.1.1 Groundnut whole pod contamination

Groundnut pods were treated with mixed plant oils, oils combined with *Trichoderma* T-22 and with vanilla oil alone. Visible signs of *A. flavus* infection were determined. ANOVA showed a highly significant treatment effect (P<0.001). From the results obtained (Figure 5.4.1.1), the LSD value indicated that camphor + clove oils at 0.1% had the least antifungal activity, when compared to vanilla oil with other combination treatments, which totally suppressed visible infection.

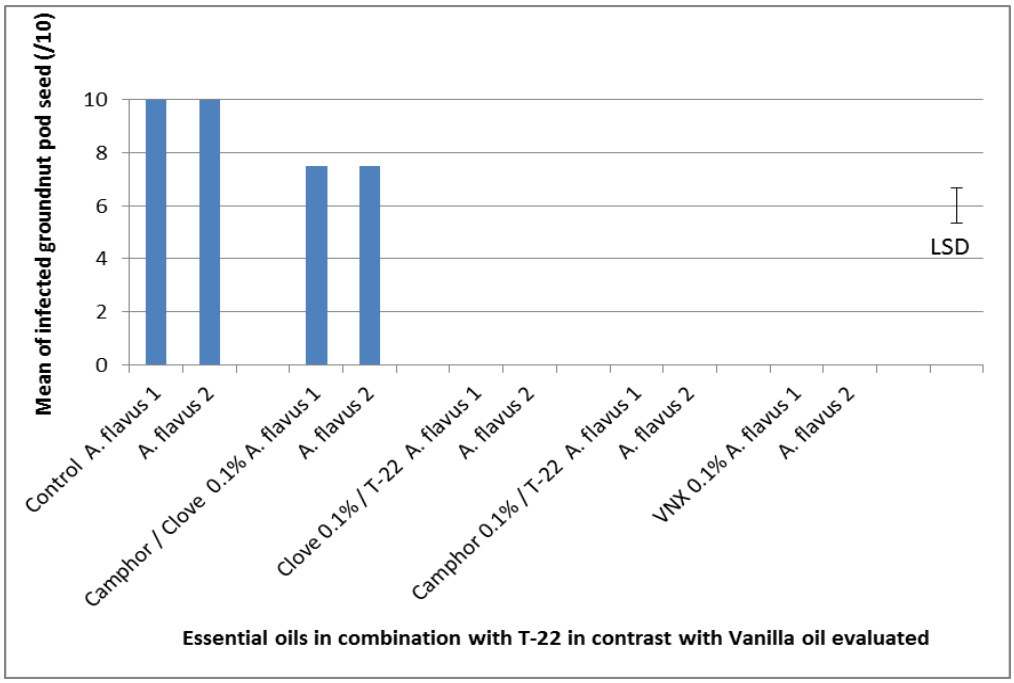


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P value
Combination /vnx treatment	4	212.5000	53.1250	236.11	<0.001
Residual	35	7.8750	0.2250		
Total	39	220.3750			

Figure 5.4.1.1 Efficacy of combination treatments and VNX to suppress *A. flavus* infection of unwounded groundnut pods, 14 d after incubation at 20^{0}C. Bar represents the LSD.

5.4.1.2 Groundnut pods - seed contamination

The pods from the experiment described immediately above were opened and the number of seeds contained within exhibiting visible signs of *A. flavus* infection was evaluated. ANOVA indicated a highly significant treatment effect (P<0.001). From the results obtained (Figure 5.4.1.2), the LSD value showed that except for the camphor oil combination, which had less antifungal activity, all the other treatments significantly suppressed infection by preventing visible seed contamination.

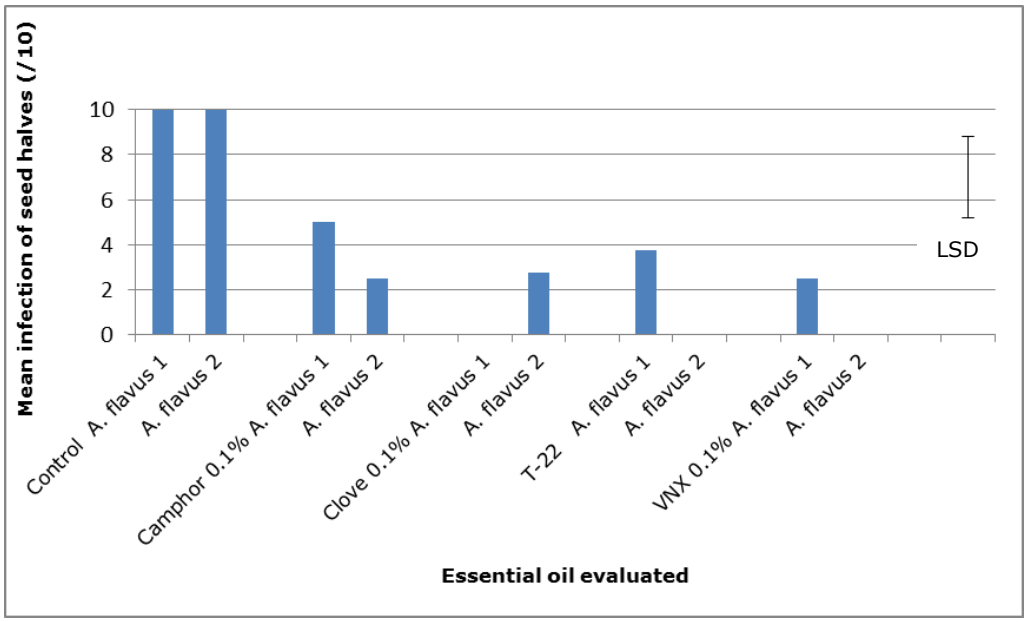


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P value
Combination/ vnx treatment	4	760.000	190.000	107.26	<0.001
Residual	35	62.000	1.771		
Total	39	822.000			

Figure 5.4.1.2 Efficacy of combination treatments and VNX to suppress *A. flavus* infection of seeds within unwounded groundnut pods, 14 d after incubation at 20^{0}C. Each replicate of germination test box contains 5 pods. Number of seeds per replicate is 10. Assessment on visible infection was based on two seeds per pod. Bar represents the LSD.

5.5 Detection of *A. flavus* infection of seeds within inoculated pods (4 weeks after inoculation) by plating-out onto PDA.

To determine whether the pathogen was present in asymptomatic seeds, seed halves removed from pods inoculated with *A. flavus*, following BCA or EO treatment, were surface sterilised and plated onto antibiotic-amended PDA. Results indicated that all the treatments reduced infection, but in some instances the pathogen could be detected by culturing, when no visible symptoms had been recorded earlier (Figure 5.5). A highly significant treatment effect was found (P<0.001). The LSD value indicated that all the treatments were significantly effective suppressing the tested pathogens. Treatments did not significantly differ from each other.



Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P value
Plant oils/T-22	4	435.35	108.84	8.55	<0.001
Residual	35	445.75	12.74		
Total	39	881.10			

Figure 5.5 Detection of *A. flavus* in half seeds evaluated by plating out onto PDA. Bars represents the LSD.

5.6 LAMP assay analysis with fungal primers

Pathogen infection of asymptomatic seed halves could be detected with fungal primers (Alpha and Apara) using a LAMP assay (Table 5.1). The results showed that all the treatments used were highly effective in suppressing the pathogen, except T-22 with the CABI isolate, where infection was detected. Tp results column indicates the time taking for positive amplification of the DNA. Tmelt results column shows the specific values of temperature required for DNA amplification and annealing. In principle, the LAMP pathogen detection assay could be better than plating out, because this protocol detects directly DNA of the pathogen inside infected groundnut tissue. However, direct comparison of the plating out and LAMP assays for detection of *A. flavus* in asymptomatic seeds (Table 5.1) indicates some discrepancies. In five instances plating-out detected the pathogen when LAMP failed to do so. LAMP alone detected *A. flavus* once. As LAMP is an indirect method, in which contamination could interfere with detection, the plate-out method, which indicates viable *A. flavus*, could be deemed more sensitive. The LAMP assay could, however, have some value in rapid, screening of samples, quickly, and without the need for laboratory culturing facilities.

Table 5.1 Detection of pathogen asymptomatic infection of halves seed (Ten per treatment)

Pathogen	DNA	Alpha	Apara	Treatment	No. of positive samples	T_P (min,s)	Tmelt (^{0}C)
A. flavus 1 (UON)	Half seed	Alpha	Apara	Control	10	16.91±4.1	78±2.1
A. flavus 2 (CABI)	Half seed	,,	,,	Control	10	19.5±3.4	87.2 ±1.5
A. flavus 1 (UON)	Half seed	,,	,,	Clove oil	0	0	0
A. flavus 2 (CABI)	Half seed	,,	..	Clove oil	0	0	0
A. flavus 1 9UON)	Half seed	,,	,,	Camphor oil	0	0	0
A. flavus 2 (CABI)	Half seed	,,	,,	Camphor oil	0	0	0
A. flavus 1 (UON)	Half seed	,,	,,	T-22	0	0	0
A. flavus 2 (CABI)	Half seed	,,	,,	T-22	1	12.45	90.85
A. flavus 1 (UON)	Half seed	,,	,,	VNX	0	0	0
A. flavus 2 (CABI)	Half seed	,,	,,	VNX	0	0	0

Tp=Time to positive and T melt=Melting Temperature.

Tp means the positive nature of the reaction is expressed by its time of positivity value i.e. amplification time at which the influrorescence second derivative reaches it peak above the baseline. T melt means values of specific amplification. Tempearture at which the double stranded DNA product dissociated into single strands. Therefore, T melt of a given LAMP amplicon is specific under given reaction conditions and differs between amplicons of tested samples with their nucleotide composition.

The same replication was applied in both procedures for pathogen in symptomless samples. Table 5.2 comparing the two methods, plate out and LAMP assay.

Table 5.2 Comparison of plate out and LAMP assay on pathogen detection

Aspergillus flavus	Treatment	Total No. of Half seeds	Plate out assay	LAMP assay
ATCC204304 (UON) Af364493(CABI)	Control	10	10	10
	Control	10	10	10
ATCC204304 (UON) Af364493(CABI)	Clove	10	0	0
	Clove	10	3	0
ATCC204304 (UON) Af364493(CABI)	VNX	10	3	0
	VNX	10	0	0
ATCC204304 (UON) Af364493(CABI)	Camphor	10	5	0
	Camphor	10	2	0
ATCC204304 (UON) Af364493(CABI)	T-22	10	4	0
	T-22	10	0	1

5.7 Aflatoxin quantification in seed harvested from pods 30 d after inoculation with *A. flavus* strains

The ability of post-harvest treatments, comprising BCAs and EOs, applied singly and in combination, to reduce the accumulation of aflatoxin B_1 in seeds harvested from *A. flavus*-inoculated pods was evaluated using an ELISA kit to quantify the toxin concentrations in nut tissues. In the initial experiment, leading treatments comprising clove, camphor, vanilla, *T. harzianum* T22, 62P and 66P were evaluated. The calibration curve derived as a mean from two evaluations is given in Figure 5.7.

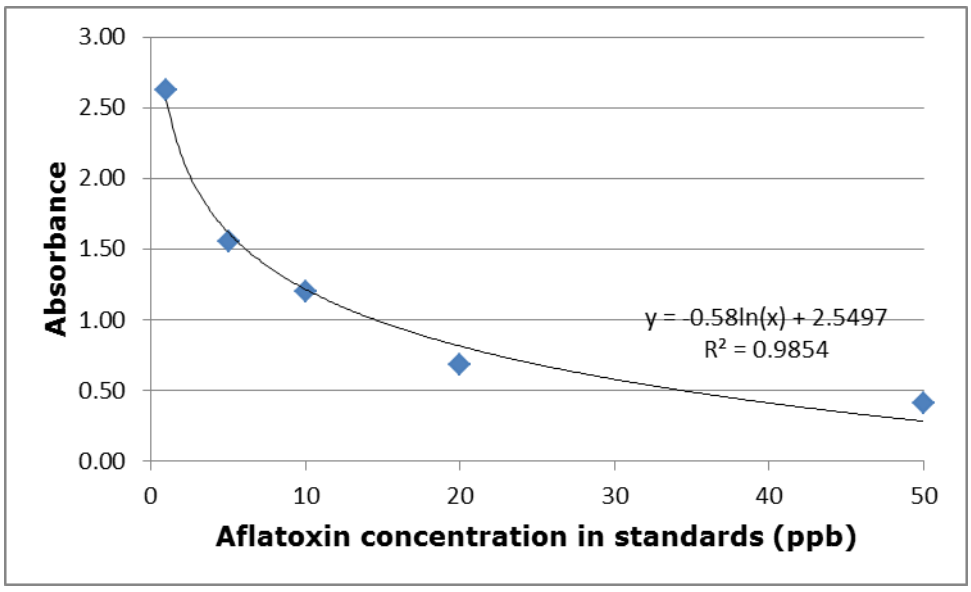
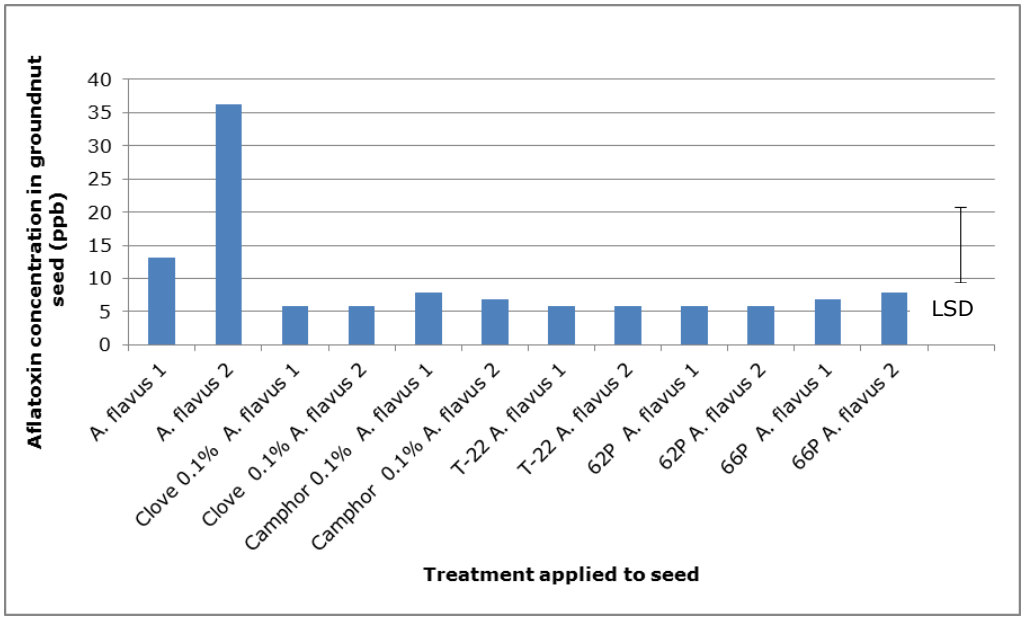


Figure 5.7 Calibration curve of aflatoxin B_1 quantification. The equation y=-0.58In(x)+2.5497 was used to evaluate aflatoxin concentration in experimental samples.

5.7.1 First evaluation; EOs and BCAs used singly

ANOVA indicated a highly significant treatment effect (P<0.001). *A. flavus* 2 (AF364493) was much more toxigenic than *A.flavus* 1 (ATCC204304). The LSD value indicated that all single treatments applied significantly reduced the concentration of the toxin in the seeds exposed to *A. flavus* 2 below 15ppb. It was surprising to note that all seed treatments gave a quite similar reduction in aflatoxin accumulation.

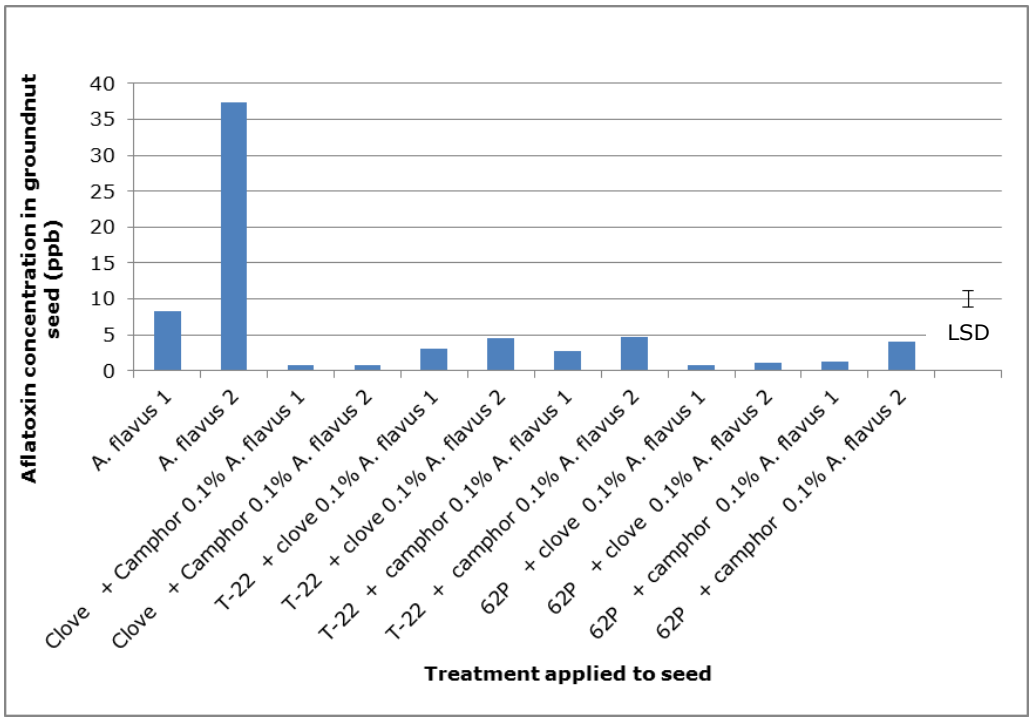


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Plant oils/BCAs	11	2539.81	230.89	4.79	<0.001
Residual	24	1155.83	48.16		
Total	35	3695.65			

Figure 5.7.1 The concentration of aflatoxin B_1 in groundnut seed treated with plant oils (clove and camphor) and BCAs (*T. harzianum* T-22 and *B. amyloliquefaciens*) at 30 d after inoculation with strains of *A. flavus*. Bar represents the LSD.

5.7.2 Second evaluation; EOs and BCAs used in combination

The results obtained when EOs and BCAs were used in combination (Figure 5.7.2) indicate a highly significant treatment effect (P<0.001). The LSD value showed that all the treatments significantly reduced the aflatoxin level so that it was below 15 ppb for both isolates.

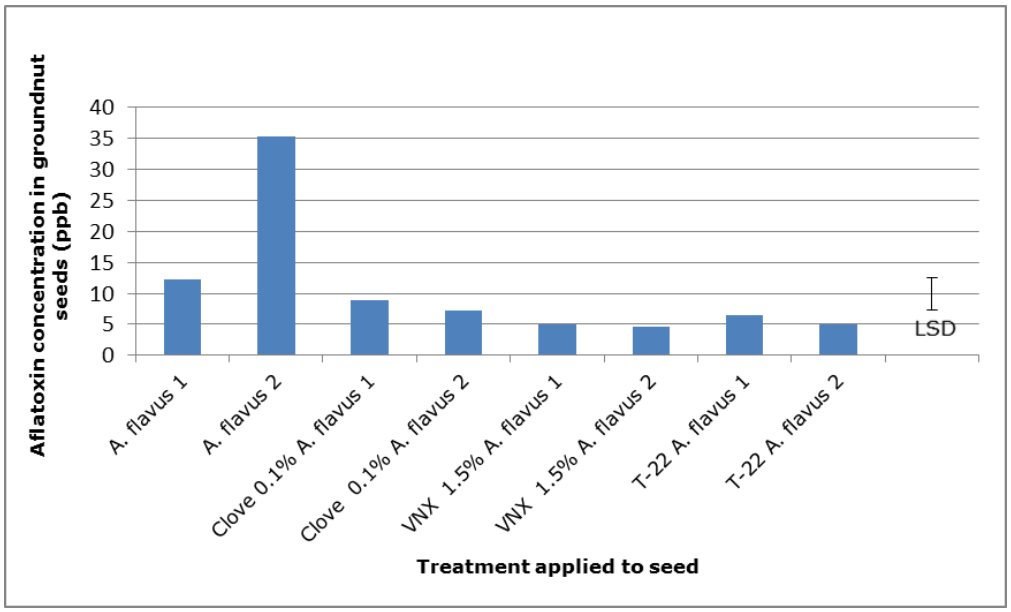


Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Plant oils/BCAs	11	3242.965	294.815	137.96	<0.001
Residual	24	51.285	2.137		
Total	35	3294.251			

Figure 5.7.2 The concentration of aflatoxin B_1 in groundnut seed, treated with combined plant oils/BCAs at 30 d after inoculation with strains of *A. flavus*. Bar represents the LSD.

5.7.3 Third evaluation; EOs and BCAs used singly

To confirm the validity of the reduction in aflatoxin contamination, a second ELISA kit was purchased and the single treatments re-evaluated for their ability to suppress toxin accumulation. The best EO and BCA (clove oil and T-22) were used and compared with vanilla oil. Results are given in Figure 5.7.3. ANOVA indicated a highly significant treatment effect (P<0.001). The LSD value shows that all treatments are highly significant in suppressing aflatoxin for *A. flavus* 2 below 15 ppb. Treatments had similar effects in evaluations using the two separate ELISA kits.



Source of variation	Degree of Freedom	Sum of Squares	Mean Square	F-Ratio	P-value
Plant oils/BCAs	7	2393.48	341.93	33.76	<0.001
Residual	16	162.03	10.13		
Total	23	2555.51			

Figure 5.7.3 The concentration of aflatoxin B_1 in groundnut seed, treated with clove, vanilla and *T. harzianum* T-22 at 30 d after inoculation with strains of *A. flavus*. Bar represents the LSD.

- The results obtained showed that both single and combination treatments significantly reduced *A. flavus* pod infection and aflatoxin accumulation below 15ppb.

5.8 Discussion

Single treatment with plant oils (vanilla and clove), and BCAs (*Trichoderma* strains) significantly reduced post-harvest groundnut pod and seed infection during incubation. Combination treatment with plant oils and T-22 also proved effective in suppressing *A. flavus* strains on groundnut pods and associated seed infection. Analysis of variance and determination of the LSD showed that the treatments also significantly suppressed aflatoxin B_1 concentration to within the approved level recommended by the European Commission, as a safe guide for global food security. Assessment of visible infection was made by plating out the pathogen and by detection using a LAMP assay. The latter could be used to rapidly detect asymptomatic infection, although at times it failed to detect the pathogen.

T. harzianum (T-22) proved to be more effective than *Bacillus* spp. and *P. chlororaphis* in suppressing the growth of *A. flavus* in the post-harvest infected groundnut pods. Growth inhibition of *A. flavus* by essential plant oils might be attributed to the active ingredients which induced damage to the cell wall composition of the pathogenic fungi, as suggested by Ghfir *et al*. (1997), in their work on the effect of the oil derived from *Hyssopus officinalis* on the pathogen *Aspergillus fumigatus.*

These results are in conformity with some previous findings, which showed were similar to the current treatment on post-harvest *A. flavus* groundnut pod and seed infection. Extracts of *Cymbopogon citratus* inhibited the growth of fungi, including the toxigenic species *A. flavus* and *A. fumigatus* (Adegoke and Odesola, 1996). Awuah and Ellis (2002) evaluated the use of powders of leaves of *Ocimum grattisimum* and cloves (*S. aromaticum)*, which also reduced infection of groundnut kernels artificially inoculated with conidia of *A. parasiticus.* Fan and Chen (1999) reported the inhibitory effects of onion extracts on *A. flavus* growth, with an ether extract, thio-propanol-S-oxide, being the active principle. Ito *et al.* (1994) stated pepper extracts reduced aflatoxin production in *A. parasiticus* IFO 30179 and *A. flavus* var *columnaris* S46. Razzaghi-Abyaneh *et al.* (2005) reported that aflatoxin production in fungal mycelia grown for 96 h in culture media containing 50% neem leaf and seed extracts was reduced by up to 65 and 90%, respectively. The findings reported here are consistent with published work.

Detoxification of mycotoxin contaminated food by microorganisms has also been researched extensively (Dorner, 2004; Shetty and Jespersen, 2006). Many strategies, including use of biological control agents such as *Aspergillus niger, Fusarium*, *Trichoderma*, and *Rhizopus*, have been used to suppress aflatoxin production by *A. flavus* (Dharmaputra, 2003). Addition of a non aflatoxigenic strain of *A. flavus* to soil around plants also inhibited aflatoxin contamination (Cotty, 1992; Dorner *et al*., 2003).

Trichoderma viride was found to inhibit the production of aflatoxin B_1 (73.5%) and aflatoxin G_1 (100%) when cultured with *A. flavus* (Bilgrami and Choudhary, 1998). *Bacillus pumilus* is also reported as suppressing growth and aflatoxin production by *A. flavus* by up to 99.2% (Sinha and Choudhary, 2008). *T. harzianum* was reported to be antagonistic towards toxigenic *A. flavus* (Dharmaputra, 2003) and *Lactobacillus casei* was also shown to be an antagonist of this pathogen (Chang and Kim, 2007). The strategy reported here, of using a combination of plant extracts and BCAs, may provide a more robust method of reducing post-harvest infection and mycotoxin accumulation.

Raymond *et al*. (2000) reported that low fungal colonization occurred on the surface of seeds when fungal inoculum was placed on undamaged seeds, and the appearance of the seeds did not change. *A. flavus* could not be recovered from asymptomatic surface-sterilized seeds. Results reported here disagree with that observation. All strains grew when they were injected directly into the seed, although the size of the colonies on the seeds varied in a qualitative manner, but the seeds would be highly toxic to livestock and humans. Culbreath *et al*. (1992) reported that the incidence of asymptomatic infection was as high as disease incidence, based on visible foliar symptoms. Thus alternative methods of detection, like the LAMP assay used here, could be valuable in assessment of *Aspergillus* infection, especially in bulk samples under non-laboratory conditions.

Chapter 6 General Discussion and Conclusions

The aims of this study were to ascertain alternative strategies to fungicide application for the control of groundnut *Aspergillus* diseases and toxin accumulation, which could be useful to low resource farmers in Nigeria and other countries in the developing world. These alternative strategies should be environmentally friendly, biodegradable, easily available, and cost-effective, when compared to synthetic fungicides. Indiscriminate use of fungicides, which can lead to environmental issues and resistance problems, has been reported by many researchers, including Kishore and Pande (2004) and El-Sarkhawy *et al*. (1998). The crop groundnut was selected for this research because it is one of the most lucrative crops in Nigeria, playing an important role in the economy of the nation, for both industrial and domestic usages. Hence, this project was sponsored by the Nigerian Tertiary Education Trust Fund (TETfund) in collaboration with Niger Delta University, Bayelsa State of Nigeria.

6.1 Antifungal activity of plant oils against *Aspergillus* spp.

The EOs were significantly effective, at both temperatures of 20^0C and 30^0C, at suppressing *Aspergillus* infection. Higher temperature efficacy could enhance groundnut disease management in tropical climates during postharvest storage and field conditions. Some of the active plant extracts can easily be obtained from the local environment of Nigeria for the control of *Aspergillus* in groundnut and in other crops like maize. Such extracts should have low toxicity to non-target organisms, unlike some synthetic chemicals. Other researchers have worked with different plant oil concentrations which proved effective in pathogen inhibition, but in this study the minimum dose used was 0.1% of the pure oils, which proved effective for pathogen suppression and enhanced groundnut emergence/survival. The present research findings are in conformity with previous research conducted by several workers. For instance, Benkeblia (2004) reported the antimicrobial activities of different concentrations (50, 100, 200, 300 and 500 mL L^{-1}) of essential oil extracts of three types of onions (green, yellow and red) and garlic against two bacteria, *Staphylococcus aureus* and *Salmomella enteritidis,* and three fungi, *Aspergillus niger*, *Penicillium cyclopium* and *Fusarium oxysporum*. The essential oil (EO) extracts of these *Allium* plants exhibited marked antibacterial activity, with garlic showing the highest inhibition and green onion the lowest. The fungus *F. oxysporum* showed the lowest sensitivity to

EO extracts, whereas *A. niger* and *P. cyclopium* were significantly inhibited, particularly at lower concentrations. However, the work of Benkeblia (2004) used higher concentrations when compared with the present study, but all proved effective. *Allium* extracts had a low effect in pathogen suppression in the current study, but could easily be procured in the local environment of Nigeria.

Juglal *et al*. (2002) reported the effectiveness of nine essential oils to control the growth of mycotoxin-producing moulds. Clove, cinnamon and oregano were able to control the growth of *A. parasiticus* while clove (ground and oil) significantly reduced aflatoxin biosynthesis in infected grains. Likewise in this study clove oil, singly or in combination treatments, was very effective in suppressing *A. flavus* and aflatoxin accumulation.

Matan *et al*. (2006) evaluated a combination of cinnamon and clove oils for inhibitory activity against important spoilage microorganism of intermediate moisture foods. Four fungal species (*A. flavus*, *Penicillium roqueforti, Mucor plumbeus* and *Eurotium* spp.), four yeast species (*Debaryomyces hansenii*, *Pichia membranaefaciens*, *Zygosaccharomyces rouxii* and *Candida lipolytica*), and two bacteria species (*Staphylococcus aureus* and *Pediococcus halophilus*) were inoculated separately on agar plates which were sealed in a barrier pouch and exposed to essential oil volatiles. The oils proved antimicrobial in the vapour phase. Varying ratios of cinnamon and clove oil vapours gave differing responses by the organisms tested. The EOs used in this work include clove oil, one of the best oils in the current study which showed significant inhibition of the four strains of *Aspergillus*.

Kishore and Pande (2006) evaluated clove oil, cinnamon oil, and five essential oil components (citral, eugenol, geraniol, limonene, and linalool) for growth inhibition of 14 phytopathogenic fungi. Citral completely inhibited the growth of *Alternaria alternata, Aspergillus flavus*, *Curvularia lunata, Fusarium moniliforme, F. pallidoroseum,* and *Phoma sorghina* in paper disc agar diffusion assays. Cinnamon oil, citral, and clove oil at 0.01% inhibited the spore germination of *Cercospora arachidicola, Phaeoisariopsis personata*, and *Puccinia arachidis* by >90% *in vitro.* Limonene and linalool were observed to be the least antifungal against the test fungi and were not used in further studies. Clove oil 1% applied as a foliar spray, 10 min before *Phaeoisariopsis personata* inoculation, reduced the severity of late leaf spot of groundnut up to 58% when plants were challenge inoculated with 10^4

conidia mL^{-1}. This treatment was more effective than 0.5% citral, cinnamon oil, or clove oil and 1% eugenol or geraniol. Seed treatment with the test compounds had no effect on the incidence of crown rot in groundnut in *A. niger*-infested soil. However, soil amendment with 0.25% clove oil and cinnamon oil reduced pre-emergence rotting by 71 and 67% and post-emergence wilting by 58 and 55%, respectively, compared with the non-treated control. These two treatments were more effective than geraniol for preventing pre-emergence rotting, and more effective than citral, eugenol, and geraniol for post-emergence wilting. All treatments significantly outperformed the non-treated control but none were as effective as the thiram fungicide. In relation to the current study, it would prove interesting to isolate the active components of pungent oils, like clove and camphor, to ascertain whether they could provide effective disease control in the absence of aroma which could taint food.

Pawar and Thaker (2006) conducted a study with 75 different essential oils for the inhibition of hyphal growth and spore formation in *A. niger*. *Cinnamomum zeylanicum* (bark), *C. zeylanicum* (leaf), *C. cassia*, *Syzygium aromaticum* and *Cymbopogon citratus* were the top five essential oils, which showed a marked inhibitory effect against hyphal growth and spore formation of *A. niger*. These oils, efficacy was also in conformity with this present research but lemon-grass (*C. citratus*) proved to be one of the least effective oils in my work.

Adjou *et al*. (2012) evaluated the antifungal activity of *Ocimum canum* (African basil) essential oil against the toxigenic fungi *A. flavus* and *A. parasiticus* isolated from groundnut. The essential oil was found to be highly fungicidal and aflatoxin production was reduced by the application of the oil, extracted from fresh leaves of the plant. However, in the current study basil oil had low antifungal activity *in vitro.* Soliman and Badea (2002) reported that thyme oil (≤500 ppm) completely inhibited the growth of *A. flavus* and *A. parasiticus*. A similar effect was also observed by Nguefack *et al.* (2004) who reported that thyme oil at 200 ppm reduced the radial growth of *A. flavus* by 81%. These previous reports are also supported by the present study in which thyme oil was effective in inhibiting *A. flavus* at the higher temperature used.

EOs with consistent *in vitro* activity were further tested in *in planta* experiments to evaluate their suppression of the pathogens in amended compost, in comparison with a standard seed treatment fungicide, Jockey. Clove and camphor oils were combined for seed treatment to increase their activity, supported by the previously presented work of Matan *et al*. (2006) in which mixtures of cinnamon and clove oils proved effective against four strains of *Aspergillus*. This was also supported by the findings of Mokhtar *et al*. (2013) in an integrated approach to control *A. niger* using *Trichoderma* and EOs.

It would be interesting in future work to evaluate the efficacy of EOs (or their components) used in combination with low rate fungicides. This could provide effective crop protection with minimal environmental impact.

6.2 Efficacy of bio-control agents against *Aspergillus* spp.

The present study showed that BCAs were effective in suppressing the four strains of *Aspergillus* at 30^0C *in vitro* and 27^0C *in planta*. This suggests that the control agents could work effectively in Nigerian soils, and can withstand tropical climates. The most effective BCAs were *Trichoderma* spp., with *T. harzianum* T-22 proving the most active against the tested pathogen.

Abd-El-Khair *et al.* (2010) tested the antagonistic effect of four *Trichoderma* species, *T. album, T. hamatum, T. harzianum* and *T. viride,* against *F. solani* and *R. solani in vitro*, in greenhouse experiments and in the field in Eygpt. In the *in vitro* tests, all *Trichoderma* spp. reduced mycelial growth of the two pathogenic fungi. In greenhouse experiments, using soil treatments, the antagonists significantly reduced pre- and post-emergence damping off after inoculation with *F. solani* and *R. solani*. The best protection to damping off disease was obtained with *T. hamatum*, followed by *T. viride, T. album* and *T. harzianum,* respectively. The treatments gave higher plant survival and improved the growth and yield parameters (Abd- El-Khair *et al*., 2010). Thus *Trichoderma* can function well in a hot climate.

Application to tropical cropping situations was further explored by Malathi and Sabitha (2003), who reported the influence of temperature on growth, survival and antagonistic performance of various strains of *Trichoderma* against the groundnut dry root-rot pathogen *Macrophomina phaseolina*. Different isolates of *T. viride*, *T. harzianum*, *T. longibrachiatum*, *T. hamatum*, *T. koningii* and *T. pseudokoningii* were employed at various temperatures, ranging from 15 to 45^{0}C when growth and antagonistic behaviour were studied. Minimum growth of the pathogen and growth of the antagonists was observed between 25 and 35^{0}C. Antagonistic activity of *Trichoderma* spp. against *M. phaseolina* decreased with increase in temperatures above 30^{0}C except for *T. pseudokoningii*, which showed maximum inhibition at 35^{0}C. Longer-term survival of *Trichoderma* on the seed coat was maximum at 15^{0}C and declined significantly by 35^{0}C. At all the temperature regimes, *T. harzianum* strain Th-5 showed higher suppression of the root-rot pathogen, better growth and survival than strains of other species. Thus, biocontrol based on *Trichoderma* isolates should be viable in tropical climates.

In other published work on groundnut Anjaiah *et al*. (2006) isolated *Pseudomonas, Bacillus* and *Trichoderma* spp., potentially antagonistic to *A. flavus*, from the geocarposphere (pod zone) of groundnut and used them successfully for the control of pre-harvest groundnut seed infection by this pathogen. In greenhouse and field experiments, inoculation of selected antagonistic strains onto seed resulted in a significant reduction of seed infection by *A. flavus*, and also reduced the *A. flavus* populations more than 50% in the geocarposphere.

One aspect of the work reported here was the evaluation of EOs and BCAs used in combination to suppress *Aspergillus* infection of groundnut. Before the use of combinations of treatments for *in planta* experiments, a bioassay was conducted to assess the sensitivity of BCAs and EOs in Petri dish assays. The results showed clearly that the EOs had no inhibitory effect on T-22. Hence clove and camphor oils were used in combination with T-22, the two components being the most effective control agents in this research. The combination treatment improved plant emergence and plant survival compared to T-22 used as a single application. The efficacy of the combination might be attributable to their different mode of actions that enhanced the effectiveness in suppressing *Aspergillus* strains in the inoculated compost.

In the work reported by Adandonon *et al*. (2006) the efficacy of BCAs alone or combined with *Moringera oleifera* leaf extracts was tested for control of *Sclerotium rolfsii*, a destructive soil-borne pathogen of many crops in the tropics and sub tropics. In the greenhouse and field, *Trichoderma* Kd 63, *Trichoderma* IITA 508 and *Bacillus subtilis* applied as seed treatments, soil drench or by water sprinkler application were evaluated separately or combined with *Moringa* leaf extracts. Percentage disease incidence, severity and control were recorded. Integration of the BCAs with the plant extract gave significantly better disease control than application of BCAs alone. This is the first report of *Moringa* leaf extract combined with *Trichoderma*, as an integrated control programme for *Sclerotium* and stem rot of cowpea in the field.

Abdel-Kader *el al*. (2013) also reported an integrated approach with plant oils and *T. harzianum* T-22. The effect of T-22 and some essential oils, alone or in combination, on groundnut crown rot disease under field conditions was evaluated. Results indicated that all treatments significantly reduced the severity of groundnut crown rot disease. The highest reduction was obtained with combined treatments (compost + *T. harzianum* + thyme, and compost + *T. harzianum* + lemongrass), which reduced disease incidence at both pre- and post-emergence growth stages. In the work reported in this thesis, however, thyme and lemongrass had relatively low activity. Integrated treatments involving combinations of BCAs with EOs may therefore be a viable alternative to fungicide application.

One approach to biological control of *Aspergillus flavus* infection of groundnut, which was not addressed in this programme of work, is the use of non-toxigenic strains of the fungus. Application of competitive, non-toxigenic isolates of *A. flavus* and *A. parasiticus* has been successfully evaluated. In many field experiments, particularly with groundnut and cotton, significant reductions in aflatoxin contamination in the range of 70% to 90% have been observed consistently by the use of non-toxigenic *Aspergillus* strains (Dorner, 2004; Pitt and Hocking, 2006; Dorner, 2008). Two products of non-toxigenic strains have received U.S. Environmental Protection Agency (EPA) registration as biopesticides to control aflatoxin contamination in cotton and groundnuts, in several states of the USA (Dorner, 2004). This strategy is based on the application of non-toxigenic

strains to competitively exclude naturally toxigenic strains in the same niche and compete for crop substrates. Thus, for competitive exclusion to be effective, the biocontrol non-toxigenic strains must be predominant in the agricultural environments when the crops are liable to be infected by the toxigenic strains. In the late 1980s, Cotty (1990) tested non-toxigenic *A*. *flavus* strains for their ability in reducing aflatoxin contamination of cottonseed. Results from greenhouse experiments showed that six of seven non-toxigenic strains significantly reduced the amount of aflatoxin produced by the toxigenic strains in cottonseed when they were co-inoculated with toxigenic strains, and that strain AF36 was the most effective in reducing aflatoxin contamination (Cotty, 1994). This strain has been registered on cotton for control of aflatoxin contamination of cottonseed in Arizona. As well as strain AF36, other non-toxigenic strains of *A*. *flavus* and *A*. *parasiticus* have also given effective reduction of aflatoxin contamination of crops. *A*. *flavus* NRRL1882, a natural strain isolated from groundnut in Georgia, has been tested in fields for more than 10 years. Several field experiments have shown that this strain is very effective in controlling aflatoxin contamination in both pre - and post-harvest groundnuts. Recently, a commercial biopesticide product (called afla-guard) has been developed based on *A. flavus* strain NRRL21882. This strain is the active ingredient in an EPA-registered biopesticide. These results indicate that applications of non-toxigenic strains could be used for the control of aflatoxin contamination. It would be interesting to combine non-toxigenic strains of *A. flavus* with the EOs and BCAs reported here, although the *Bacillus* and *Trichoderma* could antagonise the non-toxigenic isolates.

Currently, more emphasis needs to be laid on combined use of biocontrol agents, with different mechanisms of disease control, for improved efficacy, to overcome the inconsistency in performance of introduced BCAs, and to enhance effectiveness over a wider range of environmental conditions. Particular combinations of fungi and bacteria may provide protection at different times or under different conditions and occupy different or complementary niches. They may also compete for infection sites on the root and trigger plant defence reactions, including systemic resistance (Benhamou *et al.*, 2002). Moreover, there is also the potential to enhance non-fungicidal disease control by combining EOs with BCAs.

6.3 Prevention of post-harvest infection and mycotoxin accumulation

The ability of EOs and BCAs to prevent post-harvest pod infection was examined. Both strategies gave good control of *A. flavus* infection and reduced toxin contamination. Integration of the two methods was most effective. Such non-conventional approaches are especially important as fungicides could not be used in this situation, due to food contamination issues. Previous work by Palumbo *et al*. (2006) reported that in laboratory experiments several bacterial species, including *Bacillus subtilis*, *Lactobacillus* spp., *Pseudomonas* spp., *Ralstonia* spp. and *Burkholderia* spp., were shown to inhibit fungal growth and production of aflatoxins by *Aspergillus* spp. Strains of *B. subtilis* and *P. solanacearum* isolated from the non-rhizophere of maize soil were also able to inhibit aflatoxin accumulation (Nesci *et al*., 2005). In most cases, although these strains were highly effective against aflatoxin production and fungal growth under laboratory conditions, they did not give good efficacies in the field, because it is difficult to bring the bacterial cells to the *Aspergillus* infection sites on commodities under field conditions (Dorner, 2004). In published work cited for reduction of post-harvest infection BCAs were used alone. The integrated approach of using BCAs combined with EOs as a post-harvest application to groundnut pods showed some promise, but efficacy needs to be evaluated under local conditions of storage in Nigeria. The application of non-toxigenic isolates of *A. flavus* for the suppression of aflatoxin also requires evaluation as a post-harvest treatment, but disease development by such strains on the pod may make them non-marketable.

6.3.1 Detection of *A. flavus* infection on asymptomatic seed from inoculated pods

It was also shown that *A. flavus* infection could be detected in asymptomatic seeds removed from inoculated pods. This could have serious consequences for aflatoxin accumulation. Plating-out surface sterilised seed detected the pathogen, but this was labour-intensive and timing consuming. Detection using a LAMP assay was therefore evaluated. This is a rapid method which can also be used under field conditions. Use of this method to detect plant pathogens has been widely reported, including the work of Luo *et al.* (2012 and 2014) who detected *Aspergillus* spp. in groundnut, Brazil nut and coffee beans. However, in the work reported here, LAMP was shown to be less effective than conventional plating-out. As the primers were designed to detect *A. flavus*, this may reflect impurities interfering with the assay or poor DNA extraction. More work is required to optimise this method for detection of *A. flavus* in groundnut.

6.4 Conclusions

Plant oils and biocontrol agents tested proved effective in inhibiting the *A. flavus* and *A. niger* strains used in the research, in both *in vitro* and *in planta* experiments. Improved seedling emergence in pathogen-contaminated compost and reduced post-harvest pod infection were observed. Combination of the most active BCAs and EOs also provided disease suppression. ELISA analysis of aflatoxin $B_{1,}$ in treated *A. flavus*-inoculated groundnut pods showed a reduction in toxin concentrations, to a level below that recommended by the European Commission of 15 ppb.

Of the control agents tested, the most effective were *T. harzianum* as a BCA and probably clove oil as a plant extract. *Trichoderma* species are economically important biological control agents widely used in plant disease management. They are better rhizosphere colonizers than many plant pathogens, and hence can compete with other organisms for food and space in rhizosphere, thereby reducing the chances of colonization by plant pathogenic fungi. Commercial products based on *Trichoderma* are used world-wide. EOs have to date had little use in crop protection.

It was also demonstrated that detection of asymptomatic *A. flavus* pod infection could be achieved by the traditional method of surface sterilisation and plating out, and by use of a LAMP assay to detect pathogen DNA. The latter could provide a rapid, portable method for *A. flavus* detection in harvested groundnut pods and could have application in both developed and developing nations. The method, however, needs to be optimised.

Since low resource growers in nations like Nigeria need alternative, low-cost methods for protecting groundnut from *Aspergillus* infection, to produce a nutritionally-valuable, high protein foodstuff low in toxin contamination such alternative methods of disease control may have a future role to play. It may prove possible to extract antifungal components from appropriate, locally-sourced plant material in a cost-effective manner. However, whether the level of disease control and suppression of aflatoxin accumulation reported here was adequate for possible commercial application is currently unclear. Further evaluation, including field experiments, is required.

Future Work

The research reported here could be extended in the future to address the following questions:

- Do the control agents tested work in Nigerian soils and at local tropical temperatures and conditions?
- What is the minimum dose of the materials tested required to achieve effective disease and toxin suppression?
- Are the materials used compatible with fungicides which may be used in the control of *Aspergillus*?
- Do the EOs and BCAs tested have low toxicity towards non-target organisms?
- Could the application of non-toxigenic strains of *A. flavus* be integrated into an overall control programme using the materials tested here?
- Do the BCAs and EOs function on other crops to suppress *Aspergillus*?
- Can active extracts be obtained from locally-grown plants in Nigeria?
- Does the LAMP assay used to detect *Aspergillus* work in the field in Nigeria?

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