INDUCTION OF PHYTOALEXIN ACCUMULATION IN THE COTYLEDONS OF SELECTED LEGUMES FOLLOWING TREATMENTS WITH BIOTIC ELICITORS

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BY

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DECLARATION

I hereby declare that, except for specific references which have been duly acknowledged, this project is the result of my own research and has not been submitted either in part or whole for other degree elsewhere.

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We declare that we have supervised the student in undertaking the study submitted herein and confirm that he has our permission to submit.

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DEDICATION

This thesis is dedicated to Prof. R. Akromah and Dr. Charles Kwoseh for mentoring me and playing pivotal roles in my pursuit of a higher degree.

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ABSTRACT

The accumulation of phytoalexins at the site of infection is one mechanism by which plants resist diseases. The general objective of this study was to investigate the potential of cowpea, soybean, groundnut and lima bean to synthesize and accumulate phytoalexins upon attack by fungi. Three genotypes each of cowpea, soybean and groundnut and a genotype of lima bean were used in the study. Lesions were produced in all fungus-inoculated cotyledons (elicited) 36 h after incubation whilst sterile distilled water-inoculated cotyledon surfaces which served as control did not produce lesions except cowpea which exhibited some lesions. Radial growth bioassay of concentrated cotyledon extracts obtained from fungus-inoculated cotyledons showed significantly ($p < 0.05$) higher per cent inhibition of *Curvularia lunata* than extracts obtained from sterile distilled water-inoculated cotyledons. Thin layer chromatography bioassay also revealed that extracts obtained from fungus-inoculated cotyledons had more ultra violet (UV) absorbing compounds than extracts from sterile distilled water-inoculated cotyledons which among some genotypes did not show the presence of UV absorbing compounds. Localised fungitoxic activity were detected in the lanes of spotted fungus-inoculated concentrated extracts for a UV absorbing compound designated as L-I with R_f 0.07 in Lima bean and a non-UV absorbing compound in Azivivi, a groundnut genotype with R_f 0.92. The toxicity exhibited by these compounds indicates the defensive function of phytoalexins. The results of the experiment are discussed.

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CHAPTER ONE

1.0 INTRODUCTION

Legumes contribute immensely to food security, income generation and maintenance of the environment for millions of small-scale farmers in sub-Saharan Africa (Tarawalli *et al*., 2002). Grain legumes complement cereals in both human diets and production systems, especially in efficient use of land and water resources and sustainability of agricultural systems globally. They form a major source of proteins of high biological value, energy, minerals and vitamins for many people in Africa where main diets consist mostly of starchy staples and minimal animal protein (Taylor *et al*., 2005). Grain legumes are key sources of nitrogen-rich edible seeds, providing a wide variety of high-protein products and contributing 33 % of the dietary protein nitrogen (Vance *et al*., 2000) in the diets of the poor in most parts of sub-Saharan Africa. Legumes such as soybean (*Glycine max* (L.) Merr) and groundnut (*Arachis hypogeae* L.) provide more than 35 % of the world's processed vegetable oil (Graham and Vance, 2003). However, the overall yield of grain legumes including cowpea (*Vigna unguiculata* (L.) Walp), soybean, groundnut and lima bean (*Phaseolus lunatus* L.) are less than 1 ton per ha in sub-Saharan Africa (FAOSTAT, 2010). This is very low considering the fact that a food secure Africa is to be ensured amidst the ever increasing world population.

Legumes like other plants are exposed to a wide array of pathogenic and non-pathogenic microorganisms. However, they do not easily succumb to diseases because of their physical and biochemical mechanisms that enable them to resist infection (Dangl and Jones, 2001; Neuenschwander *et al*., 1995). The physiological and biochemical basis of plants resistance to pathogens has been associated with both preformed and infection-induced anti-microbial compounds (Felton *et al*., 1999; Osbourn, 1996). The induced accumulation of secondary metabolites such as phytoalexins is one of the most extensively studied plant defense responses to pathogen

infection (Hammerschmidt, 1999). Phytoalexins are a diverse group of low molecular weight anti-microbial compounds that are synthesized and accumulated in appreciable amounts in plants after stimulation by various types of pathogens, and are toxic to pathogens (Mansfield, 2000; Smith, 1996).

Biotic constraints related to diseases are among the factors militating against profitable legume production. Diseases caused by fungi are very widespread in crop plants and the control of most of these diseases has been sought through the use of fungicides. The use and the misuse of these fungicides have posed serious environmental hazards such as the pollution of water bodies, residue in food crops, adverse effect on non-target organisms including beneficial ones and imbalance in the ecosystem, and direct hazard to the user. Also, the high cost of these fungicides has contributed to high production cost thereby reducing the profit margin of resource-poor farmers. The emergence and spread of microbial resistance is growing, thereby necessitating the need for development of new antimicrobials of natural or synthetic origin (Gislene *et al*., 2000). Phytoalexins are natural chemical compounds produced by plants as a consequence of microorganism attack and it is considered as an important defense mechanism in plants (Harborne, 1999). Most research on resistance mechanisms have shown that plants use defenses that are activated after infection to stop pathogen development (Dixon and Harrison, 1990).

Phytoalexins accumulate at the site of infection of pathogen and are thought to restrict ingress of invading pathogen(s). An investigation on the induction and accumulation of phytoalexin in the cotyledons of cowpea, soybean, groundnut and lima bean is therefore needed to elucidate the contribution of phytoalexins to resistance. This research will therefore provide information on the defensive potential of cultivars by delineating the

probable utility of detected phytoalexin as a chemical marker of resistance among legume crops in Ghana.

The general objective of this study was to investigate the potential of cowpea, soybean, groundnut and lima bean to synthesize and accumulate a range of phytoalexins upon attack by a fungus.

The specific objectives were to;

i. ascertain crop species reaction to fungi challenge-inoculation
ii. determine antifungal activity of legume cotyledon extracts
iii. detect the presence of UV absorbing compounds in the cotyledon extracts and
iv. identify localised fungitoxic activity on thin layer chromatography plates.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Elicitation of phytoalexins

The description and characterisation of phytoalexins from legumes of the family Leguminosae have been done (Ingham, 1982). Plants have evolved complex physical and biochemical mechanisms against pathogen infections (Dangl and Jones, 2001; Neuenschwander *et al*., 1995). The physiological and biochemical basis of plants' resistance to pathogens has been associated with both preformed and infection-induced antimicrobial compounds (Felton *et al*., 1999; Osbourn, 1996). The induced response mechanism also involves the trafficking and secretion of antimicrobial compounds to the infection site (Bednarek and Osbourn, 2009). Inducible defense responses are triggered following recognition of a range of chemical factors termed 'elicitors' (Hammond-Kosack and Jones, 2000). Elicitor was used for molecules capable of inducing the production of phytoalexins (Darvill and Albersheim, 1984), but it is now commonly used for compounds stimulating any type of plant defense (Nürnberger, 1999; Hahn, 1996; Ebel and Cosio, 1994, Roby *et al*., 1985). Inducers of other defense reactions have been found to elicit phytoalexin synthesis (Walker-Simmons *et al.*, 1984).

Model experiment systems have also been developed where the defense response is generated by elicitors of biotic or abiotic origin such as cell wall preparations of pathogens, heavy metals or thiol reagents (Robbins *et al.*, 1995; Edwards *et al.*, 1991; Wingate *et al.*, 1988).

According to Radman *et al*. (2003), elicitors are classified as physical or chemical, biotic or abiotic and complex or defined, depending on their origin and molecular structure. After the description of the first biotic elicitors in the early 1970 (Keen, 1975) numerous publications have accumulated evidence for pathogen-derived compounds

that induce defense responses in intact plants or plant cell cultures. Biotic elicitors include complex carbohydrates from fungal and plant cell walls, lipids, microbial enzymes, and polypeptides, whereas abiotic elicitors range from heavy-metal salts to detergents, autoclaved ribonuclease, cold, and UV light (Grisebach and Ebel, 1978). Biotic elicitors often originate from the pathogen (exogenous elicitors) but in some cases are liberated from the attacked plant by the action of enzymes of the pathogen (endogenous elicitors) [Boller, 1995; Ebel and Cosio, 1994]. Albersheim *et al*. (1977) were the first to isolate oligosaccharides that activated a variety of plant defense genes.

2.2 Phytoalexins associated with legumes and some known examples

Phytoalexins are involved in preventing infection by many pathogens and can therefore be a major defense system of plants (Keen, 1986). The phytoalexins in legumes are mostly phenylpropane derivatives synthesized via the shikimate pathway together with the acetate-malonate pathway (Ingham, 1982).

In contrast to preformed antifungal compounds, phytoalexins are not present in healthy plant tissue and are synthesized from remote precursors in response to pathogen attack or stress (VanEtten *et al*., 1994). Phytoalexin accumulation is also linked with *de novo* protein synthesis of enzymes required for their biosynthesis (Grayer and Kokubun, 2001; Dixon and Paiva, 1995; Berenbaum and Zangerl, 1992; Cramer *et al.*, 1985; Dixon *et al.,* 1983). Therefore, the production of phytoalexins requires transcriptional and/or translational activity in the plant once the pathogen has been detected. Genes coding for enzymes predicted to be involved in 3-deoxyanthocyanidin biosynthesis are induced and up-regulated over time in response to infection with *Cochliobolus heterostrophus* (Drechsler) Drechsler in sorghum (*Sorghum bicolor* (L.) Moench) (Farag *et al*., 2010).

Glyceollins are pterocarpans derived from the phenylpropanoid pathway and they occur as a series of isomers I–IV (Paxton, 1995). Daidzein, a dihydroxylated isoflavone, is the immediate precursor of the glyceollins (Paxton, 1995; Bailey and Mansfield, 1982). Genistein (trihydroxylated isoflavone) is another antimicrobial isoflavonoid that is accumulated in soybean tissues during incompatible reactions (Dakora and Phillips, 1996; Dixon *et al*., 1995).

The phytoalexins phaseollidine, kievitone and phaseollin were isolated from tobacco necrosis, virus-infected cowpea hypocotyls (Bailey, 1973). Treatment of seeds with 20 mg ml^{-1} acibenzolar-S-methyl (benzo (1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester, BTH) followed by inoculation of the emerging seedlings with *C. destructivum* resulted in the accumulation of the cowpea phytoalexins; kievitone and phaseollidin (Latunde-Dada and Lucas, 2001).

Phytoalexins known in groundnut include trans-resveratrol, trans-arachidin-1, trans-arachidin-2, transarachidin-3, trans-3-isopentadienyl-4, 3, 5-trihydroxystilbene, and SB-1 (Sobolev, 2008).

2.3 Flavonoids and isoflavonoids

In legumes, flavonoids play an important role in the interactions between plants and microorganisms (Harborne and Williams, 2000; Shirley, 1996).

Isoflavones are a group of diphenolic secondary metabolites produced in a very limited distribution of higher plants, most frequently in the leguminosae (Dewick, 1994). Isoflavonoids represent a different branch of the flavonoid pathway and are characterised by a migration of the phenyl ring and occur principally in legumes. They are involved in the defense response of plants against pathogens (Dixon *et al.*, 1995; Kuc, 1995) as they serve as precursors for the production of phytoalexins during plant

microbe interactions (Dixon and Ferreria, 2002; Aoki *et al.*, 2000). Isoflavonoids, especially the pterocarpans, isoflavans, isoflavones and isoflavanones, are extremely toxic to fungal pathogens. These flavonoids inhibit fungal spore germination, germ tube elongation, and hyphal growth through causing damage to membrane systems (Higgins, 1978; Skipp and Bailey, 1977). Other compounds such as kievitone and phaseollin isoflavonoids lead to mycelial leakage of metabolites and shrinkage of hyphal tip protoplasts (VanEtten and Bateman, 1971).

2.4 Species distribution and variations in the production and accumulation of phytoalexin

During a given interaction, frequently, more than one phytoalexin is synthesized by the plant and in such cases the phytoalexins are related biosynthetically (van der Heijden *et al*., 1989). A major example is provided by cell suspensions of Taber-*Naemontana divaricata* and these elaborate a mixture of 10 distinct but related terpenoid phytoalexins in response to treatment with an elicitor from *Candida albicans* (van der Heijden *et al*., 1989). In eight species and three subspecies of the genus *Vigna*, the phytoalexins dalbergioidin, kievitone and phaseollidin were produced in the hypocotyl, epicotyl and seed (Seneviratne and Harborne, 1992). In the root, however, only dalbergioidin and kievitone were detected (Seneviratne and Harborne, 1992). This provides evidence that variations in the relative proportions of the phytoalexins accumulated by a particular species will often occur, depending upon such factors as the agent employed to elicit the response (biotic or abiotic elicitors) and the type of tissue under investigation (leaves, cotyledons, callus, cell suspension).

The quality and quantity of the phytoalexins synthesized in a particular interaction are determined by the genotype of the plant, though the genetic background of the invading micro- organism might also play a part (Ausubel *et al*., 1995).

Environmental and experimental conditions can influence the relative concentrations of the phytoalexin synthesized (Classen and Ward, 1985). Increased soil salinity, for example, significantly decreased accumulation of the phytoalexin 6, 7-dimethoxycoumarin in various citrus rootstocks challenged with *Phytophthora citrophthora* (Sulistyowati and Keane, 1992). A similar effect of salinity on accumulation of glyceollin, a pterocarpan phytoalexin, has been observed in soybean after inoculation with *Phytophthora megasperma* var. sojae (Murch and Paxton, 1980). Increasing the temperature of citrus tissues inoculated with *P. citrophthora* from 20 to 28 ^{o}C increased the concentration of scoparone accumulated (Afek and Sztejnberg, 1993).

2.5 The role of phytoalexins in disease resistance

The well established fact that phytoalexins are absent in healthy plants and are accumulated at the site of microbial infection and inhibits their growth gives an indication of a defense function of phytoalexins. The speed, magnitude, and site of accumulation of phytoalexins following penetration by the microorganism appear to determine disease resistance in some plant-microbe interactions. The half maximal effective concentration (EC_{50}) for fungi is generally 10^{-3} to 10^{-5} M depending upon the phytoalexin, fungus and bioassay (Kuc, 1995). The speed of the accumulation and localisation of the phytoalexin at the infection site may permit the pathogen to encounter concentrations far in excess of the EC_{50} at early stages in the infection process (Figen, 2002).

Resistance of plants to potential pathogen is associated with the localised production of phytoalexin (Cooper *et al*., 1996). Resistance occurs when one or more phytoalexins reach a concentration sufficient to restrict pathogen development (Lo *et al.*, 1996). A correlation between the accumulation of phytoalexin and the restriction of microbial

growth has been reported in lettuce and groundnuts (Subba Rao *et al*., 1996; Bennett *et al*., 1994).

Latunde-Dada *et al.* (1999) studied the mechanism of resistance to anthracnose in the cowpea assession TVx 3236. In this accession, the initially injected epidermal cells underwent a hypersensitive response restricting the growth of the pathogen. The phytoalexins kievitone and phaseollidin accumulated more rapidly in the stem tissue of TVx 3236 compared to the susceptible variety.

2.6 Establishing the importance of phytoalexins in disease resistance

In order to evaluate the importance of phytoalexin in diseases resistance (Hammerschmidt, 1999), the following criteria must be met;

a. the restriction of the pathogen development must be associated with phytoalexin production,
b. phytoalexins must accumulate to antimicrobial levels at the infection site in resistant plants or cultivars that could result in the cessation of the pathogen growth and
c. there must be strong evidence that the phytoalexins have vital importance in resistance, and absence of these compounds would result in enhanced susceptibility.

2.7 Disease constraint of cowpea, soybean, groundnut and lima bean production

2.7.1 Fusarium wilt of cowpea

Fusarium wilt caused by *Fusarium oxysporum* f.sp. *tracheiphilum* is a major disease of cowpea (Teri, 1981) and with economic importance in Africa (Emechebe and Shoyinka,

1985). *Fusarium* wilt of cowpea has been reported in three African countries, namely Nigeria, Tanzania, and Uganda (Teri, 1981; Oyekan, 1975; Booth, 1971; Holliday, 1970). Studies revealed *Fusarium* wilt incidence of 21, 15 and 55 %, respectively in Ife Brown, Tvu 4557 and Prima cultivars in Ibadan, Nigeria (Oyekan, 1975). *Fusarium oxysporum*, although considered to be soil-borne is also seed-transmitted. Fawole *et al*. (2006) reported *F. oxysporum* to cause seed rot and stunted growth of cowpea at Ilorin, Nigeria. According to Hare and Thompson (1990), no adequate chemical control measures are known for *Fusarium* diseases of cowpea.

2.7.2 Anthracnose of soybean

The incidence of soybean anthracnose was first reported in Korea in 1917 (Gupta and Paul, 2006) and now is known to occur wherever soybeans are grown (Gupta and Paul, 2006). The disease causes serious economic consequences especially in wet, warm and humid areas. It is a serious disease in almost all soybean growing areas of the world including Ghana and causes losses of 30 to 70 % (Chandrasekaran and Rajappan, 2002; Lenne, 1992; Backman *et al*., 1982).

Colletotrichum truncatum (Schw.) Andrus & Moore is the most common pathogen associated with soybean anthracnose (Sinclair, 1988). However, the disease can also be caused by other species of *Colletotrichum* such as *C. destructivum* O'Gara [teleomorph *Glomerella glycines* (Hori) Lehman and Wolf], *C. gloeosporioides* (penz.) Sacc. (teIeomorph *G. cingulata* (Ston.) Spauld. and Scherenk), and *C. graminicola* (Ces.) Wilson (teleomorph unknown) (Sinclair, 1988).

Soybean plants can be infected at any stage of development. Germinating seeds are killed in the ground when infected seeds are planted. The seedlings from such seeds that emerge from the soil often have brown, sunken cankers on the cotyledons. The fungus may grow from them into the young stem. Pre-emergence and post-emergence seedling

losses are probably more serious phases of soybean anthracnose than are the more evident symptoms on older plants (www.soydiseases.uiuc.edu). The continuous and indiscriminate use of chemicals to manage the anthracnose disease results in accumulation of harmful chemical residues in the soil, water and grains. Activation of the plants' natural means of resistance such as phytoalexin accumulation can be a more viable option for the control of the anthracnose disease.

2.7.3 Crown rot of groundnut

Crown rot disease of groundnut (*Arachis hypogaea* L.) caused by the highly virulent strain of *Aspergillus niger* Teigh is a very important plant disease (Carina *et al*., 2006, Elwakil, 2003; Phipps, 2000). Germinating seeds are covered with masses of black conidia followed by rapid drying of plants. Later, whole collar region becomes shaded and dark brown (Subrahmanyam and Ravindranath, 1988). Groundnut seeds infected by *Aspergillus niger* (seed-borne fungus) have been reported to produce seed abortion, shrunken seeds, reduce seed size, seed rot, seed necrosis, seed discolouration, reduction of germination capacity and physiological alteration of seed (Elwakil, 2003). The disease is seed-borne and survives on infected groundnut seeds (Carina *et al*., 2006). Owing to environmental concerns posed by the use of chemicals such as pentachloronitrobenzene (PCNB) there is considerable interest in finding alternatives to the use of chemical fungicides. Biological control such as resistance induction can be an attractive alternative.

2.7.4 Stem anthracnose of lima bean

Stem anthracnose is the most common disease of lima beans. The causal agent is *Colletotrichum lindemuthianum* (Sacc. and Magn.) Br. and Cav. The first stages of infection appear on pods as small, brick-red blotches. These blotches may spread over

the entire surface of the pods. Later, the diseased areas become brownish to grayish and may have many tiny black specks which are fruiting bodies of the fungus. Occasionally, diseased pods fall from the plant. A brick-red streaking may occur along the veins on the underside of leaves and on young stems. Reddish spots occur on the lower leaf surface and enlarge and become noticeable on the upper leaf surface. Occasionally, leaves are killed and fall from the plant. Severely diseased plants are yellow and stunted (Hagedorn and Inglis, 1986). The worldwide demand for food free of fungicides as a result of the negative consequences of these chemicals has stimulated research on alternative methods to control anthracnose diseases. Investigations into the induction of phytoalexin accumulation are therefore needed to avert the problems associated with the use of chemicals since phytoalexins are natural defense responses of plants.

2.8 Means of resistance of plants to pathogens

In most plant-pathogen interactions, a number of inducible defense mechanisms are expressed at the same time, normally in association with the hypersentive response (HR), a phenomenon that is characterized by the rapid death of cells at the site of invasion and cessation of growth of the pathogen (Fritig *et al*., 1987). The occurrence of cell death obviously adds a further complication to analysis of localised resistance but whatever their respective roles, phytoalexin accumulation is only one of a group of active defense mechanisms that includes deposition of hydroxyproline-rich proteins (Showalter and Varner, 1987) and lignin (Bruce and West, 1989) in the cell wall, production of pathogenesis-related proteins (Bol *et al*., 1991), lipid peroxidation reactions (Slusarenko *et al*., 1991), production of chitinases and glucanases (Kombrink *et al*., 1991), generation of active oxygen species (Tenhaken *et al*., 1995) and synthesis of inhibitors of proteases (Ryan, 1990) and poly- galacturonases (Degra *et al*., 1988). Not all of these will occur in a single plant-pathogen interaction nor will they

necessarily be expressed with identical intensities. Nevertheless, there are problems in resolving the effects of any one defense response upon the invading micro-organism from the effects of any other that is operating simultaneously. Likewise, identifying the signalling events associated with individual mechanisms is difficult.

2.9 Plant-induced resistance

An approach to establishing a correlation between phytoalexin accumulation and resistance has been to raise the phytoalexin content of tissues and assess the change in resistance.

Plant induced resistance is the process of activating the natural defense system present in plants that responds to one set of biotic (Strobel *et al*., 1996) or abiotic (Kawahara *et al*., 2006; Sato *et al*., 2003) factors. Systemic acquired resistance (SAR) is a phenomenon whereby localised inoculation with a necrotising pathogen or pre-treatment with a chemical inducer, primes a plant to be more resistant to any subsequent pathogen infection.

During SAR reactions, several defense mechanisms are known to be induced in plants following challenge inoculation with a pathogen. These mechanisms include lignification (Park *et al*., 2002), deposition of callose (Ishii *et al*., 2000; Jeun *et al*., 2000) on the plant cell wall where the pathogen tended to penetrate, and the accumulation of pathogenesis-related (PR) proteins (Cools and Ishii, 2002; Sundar *et al*., 2001; Narusaka *et al*., 1999) and production of phytoalexins (Daayf *et al*., 2000; Daayf *et al.*, 1997; Fawe *et al.*, 1998).

2.10 Effects of phytoalexins on plants and invading pathogens

Phytoalexins have fairly low specificity as a group. They have been shown to be effective against bacteria (Fagboun *et al*., 1987), fungi (Smith &Banks, 1986), animal cells (Alphey *et al*., 1988), viruses (Sun *et al.*, 1988), and plant cells (Kurosaki *et al*., 1984). Some phytoalexins are biocidal and some show biostatic effects, at least *in vitro*, but the actual effect might depend upon the precise conditions of the bioassay and the micro-organism concerned (Smith and Banks, 1986).

2.11 Toxicity and detoxification of phytoalexins

In general, phytoalexins are not particularly potent antibiotics and, in keeping with their structural variety, toxicity covers a range of 10^{-6} to 10^{1} M. Various factors can influence toxicity, most evidently in bioassays but presumably within the plant as well. These include pH, the growth stage of the micro-organism used or the timing of the growth measurements (Smith, 1982). The races of many microorganisms, particularly fungi, show a wide variation in their sensitivities to a particular phytoalexin, with the pathogenic race often being more tolerant than the non-pathogen (Smith, 1982). Frequently, the difference results from the greater capacity of the virulent strain to detoxify the phytoalexin and there are examples where tolerance to a phytoalexin correlates closely with this ability (VanEtten *et al*., 1989).

Fungi metabolise phytoalexins to less toxic derivatives through demethylation of the methoxyl groups and hydroxylation of the nucleus (Delserone *et al*., 1999) and this is a common phenomenon in fungi. Hydroxylation and demethylation reduce lipid solubility and increase water solubility (Harborne, 1993). The products of such reactions are immediately more susceptible to oxidative cleavage of the aromatic rings and undoubtedly the ultimate fate of the phytoalexins is to be broken down by well-

described aromatic-cleavage pathways to eventually yield carbon dioxide. According to Del Sorbo *et al*. (1997), pathogens may have phytoalexin tolerance mechanisms that are specific membrane pumps that remove the phytoalexin from the cell. Another common mechanism of tolerance involves a change in the cellular component affected by the toxin (Bolhuis *et al*., 1997). Where other factors are almost equal, detoxification has been used to explain the pathogenicity or non-pathogenicity of the micro- organisms involved (VanEtten *et al*., 1989). There are cases where a pathogen shows quite a low tolerance to a phytoalexin *in vitro* but develops in the host despite the presence of a substantial concentration of phytoalexin (Mace *et al*., 1990; Smith, 1982). These examples serve to indicate that *in vivo* there are parameters other than those determined by bioassay which must be considered when the results of bioassays are extrapolated to the effects of phytoalexins *in vivo*.

2.12 Sample preparation, thin layer chromatography (TLC) bioassay and solvent systems

The aim of sample preparation is that the components of interest should be extracted from complex matrices with the least time and energy consumption but with highest efficiency and reproducibility. Conditions should be mild enough to avoid oxidation, thermal degradation, and other chemical and biochemical changes. TLC requires an absolute minimum of sample preparation (Smith, 2003).

The method of choice for simple and inexpensive analytical runs is TLC. The advantages of this technique are short separation times, amenability to detection reagents and the possibility of running several samples at the same time. TLC is also ideally suited for the preliminary screening of plant extracts before high-performance liquid chromatography (HPLC) analysis (Jork *et al*. 1994). TLC has been of notable

success in the detection of anti-fungal compounds in plants (Rahalison *et al.*, 1991; Homans and Fuchs, 1970).

Most TLC for the isolation of isoflavonoid phytoalexin have been done on silica gel and different solvent systems have been employed for the separation of flavonoids using TLC but the solvent of choice for the separation of isoflavonoids is Chloroform-methanol ($CHCl_3$–MeOH, 92:8 or 3:1) (Markham, 1975). Gnanamanickan (1978) however, used chloroform-methanol ($CHCl_3$–MeOH, 25:1), Chloroform-Ethanol ($CHCl_3$–CH_3CH_2OH, 100:3) and benzene-ethyl acetate-methanol (C_6H_6-$C_4H_8O_2$ - MeOH, 25:8:4) to separate isoflavonoid phytoalexins from seeds of *Phaseolus vulgaris* L.

For the purposes of visualisation after development, brief exposure of the TLC plates to iodine vapour produces yellow-brown spots against a white background or observation under UV light will produce UV spots. Flavonoids appear as dark spots against a fluorescent green background when observed in UV light (254 nm) on plates containing a UV-fluorescent indicator such as silica gel F254. In 365 nm UV light, depending on the structural type, flavonoids show dark yellow, green, or blue fluorescence, which is intensified and changed by the use of spray reagents (Markham, 1975).

2.13 TLC direct bioautography

Although other techniques exist, bioautography is the method of choice when searching for antifungal compounds from plants. This is because it allows the combination of bioassay *in situ* and, at the same time, localisation of active constituents on the TLC plate employed for the assay.

Spore-producing fungi, such as *Aspergillus, Penicillium* and *Cladosporium* spp. can all be employed as target organisms in direct bioautographic procedures **(**Homans and Fuchs, 1970**).** After migration of an extract or sample on a TLC plate, the plate is

sprayed with the microorganism and incubated in a humid atmosphere. Zones of inhibition appear where spore or mycelial growth is prevented or reduced by the active constituents of the plant extract.

Induction of plant resistance mechanisms can be achieved by the application of elicitors which sensitize or condition plants for protection upon challenge. According to Kuc **(**2001), the use of beneficial microorganisms or treatment with microbial components can induce systemic resistance (ISR) in plants. An important aspect of ISR is the priming effect such as pre-immunisation with a non-pathogen which accelerates defense response by the plant upon pathogen attack, resulting in enhanced resistance to the pathogen (van Hulten *et al*., 2006). The expected differential responses of the crop species to induced accumulation of antimicrobial compounds as a result of pathogen attack can be used to predict the performance of genotypes on the field. The screening procedure can also be considered as a quick laboratory technique of screening for resistance to fungal infections.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Location of experiment

The experiments were conducted in the Plant Pathology Laboratory, Department of Crop and Soil Sciences and the Project Laboratory, Department of Pharmacognosy, KNUST, Kumasi.

3.2 Source of experimental materials and their characteristics

Three genotypes of cowpea, soybean and groundnut, and one genotype of lima bean were used in the study.

3.2.1 Cowpea genotypes

Asontem, Nhyira and Bengpla were the cowpea genotypes used for the cowpea phytoalexin studies. They were obtained from the Council for Scientific and Industrial Research-Crop Research Institute (CSIR-CRI), Fumesua, Kumasi.

Asontem is an early maturing variety (65–70 days) with a semi-erect growth habit and narrow leaves. It has purple pigmentation on the joints connecting the petiole with the main stem as well as on the standard and wing petals. It has red colour and medium size seed with a smooth seed coat. Asontem is resistant to major diseases such as anthracnose caused by *Colletotrichum lindemuthianum*, web blight caused by *Rhizoctonia solani* Kühn, brown blotch caused by *C. capsici* Syd., Cercospora leaf spots caused by *Cerscospora cruenta* Sacc. and *Cercospora canescens* Ellis and Martin, Septoria leaf spot caused by *Septoria vignae* P. Henn, scab caused by *Elsinoe phaseoli* Jenkins and bacterial blight caused by *Xanthomonas campestris* pv. *vignicola*

Burkholder, as well as Cowpea yellow mosaic virus and Cowpea aphid-borne mosaic virus (Asafo-Adjei *et al*., 2005).

Nhyira is known to be early maturing (65-68 days), high yielding (2.3 tons per hectare), it has bold white seed with brown eye, moderately resistant to virus, resistant to anthracnose and Cercospora leaf spot, high in iron, energy and phosphorus contents, protein, tolerant to leaf hoppers, and drought tolerant.

Bengpla is an early maturing (65days) variety. It is resistant to pod shattering and lodging. It has medium sized seeds with smooth white shiny testa and black hilum. Resistant to most diseases such as anthracnose caused by *Colletotrichum lindemuthianum*, web blight caused by *Rhizoctonia solani*, brown blotch caused by *Colletotrichum capsici*, Septoria leaf spot caused by *Septoria vigna*, bacterial blight caused by *Xanthomonas campestris* pv. *Vignicola* as well as cowpea mosaic virus, southern bean mosaic virus, and cowpea aphid-borne mosaic virus.

3.2.2 Soybean genotypes

Varieties of soybean namely Anidaso (TGx813-6D), Jenguma (TGx1830) and CRI-Nangbaare (TGX 1830-20E) were used for phytoalexin response study in soybean. Anidaso and Nangbaare were obtained from the CSIR-CRI, Fumesua, Kumasi whilst Jenguma was obtained from a farmer at Navrongo in the Upper East Region of Ghana. Anidaso is high yielding. Jenguma is high yielding and shattering resistant (Baijukya *et al*., 2010).

CRI-Nangbaare is moderately resistant to shattering, tolerant to lodging, moderately resistant to root-knot nematode, high yielding, bold seed, high protein content, good soy milk, high phosphorus, calcium and iron, moderately resistant to virus and anthracnose (WASNET News 16, 2006).

3.2.3 Groundnut genotypes

The groundnut genotypes were obtained from the CSIR-CRI, Fumesua, Kumasi. Varieties of groundnut CRI-Adepa (M576-79), CRI-Azivivi (RMP12) and CRI-Nkosuor (M578-79) were used for phytoalexin studies in groundnut.

CRI-Adepa is early maturing, high fat content, high energy, high oil content, tolerant to virus, anthracnose and leaf hoppers, and it also has promiscuous nodulation (WASNET News 16, 2006).

CRI-Azivivi is high yielding, resistant to rosette virus and Cercospora leaf spot, bold seed (suitable for confectionary) drought resistant, high iron content and high in protein (WASNET News 16, 2006).

CRI-Nkosuor is drought resistant, high in calcium, high in protein, medium fat content, medium iron, high yielding and resistant to rosette virus and Cercospora leaf spot (WASNET News16, 2006).

3.2.4 Lima bean genotype

Lima beans were obtained from the Ayigya Market, near KNUST, Kumasi. The commonest local Lima beans which have bold and white seeds were obtained. Information on disease resistance of lima bean is scanty in Ghana but according to FAO (1984), lima bean is a hardy crop and may be advantageous in adverse conditions where other leguminous vegetables do not grow well.

3.3 Preparation of potato dextrose agar (PDA) and pouring into plates

A growth medium was prepared by weighing 39 g of potato dextrose agar and re-suspending in 1 litre of sterile distilled water contained in a 2 litre glass beaker. To suppress the growth of bacteria, 250 mg chloramphenicol was added to the suspension.

The suspension was stirred with a magnetic stirrer to dissolve completely. This was followed by pouring 500 ml each of the dissolved mixture into 750 ml volumetric flask. The volumetric flask containing the mixture was corked with a non-absorbent cotton wool and sterilisation was done by autoclaving at 121 ºC at 15 psi for 20 min.

Glass Petri dishes with a diameter of 8 cm were sterilized in an oven at a temperature of 160 ºC for 1 h. Autoclaved PDA was poured under a laminar flow into the sterilized glass Petri dishes and allowed to solidify before used.

3.4 Sources of fungal cultures

The fungal cultures used in this study were *Fusarium* sp., *Colletotrichum* sp. *Aspergillus niger* and *Curvularia lunata.* Pure cultures of *Curvularia lunata* isolated from infected *Colocasia* leaves were obtained from the Plant pathology laboratory of the Department of Crop and Soil Sciences, KNUST. The pure cultures were sub-cultured by culturing a plug of mycelia of *Curvularia lunata* on potato dextrose agar (PDA) amended with 250 mg chloramphenicol in Petri dishes. *Fusarium* sp., *Colletotrichum* sp. and *Aspergillus niger* were not readily available and so were isolated from infected seeds of cowpea (Asontem), soybean (Nangbaare) and groundnut (Adepa), respectively.

3.4.1 Isolation and identification of *Fusarium* sp., *Colletotrichum* sp. and *Aspergillus niger* isolates

Fusarium sp. was isolated from infected Asontem (cowpea) seeds, *Colletotrichum* sp. from infected Nangbaare (soybean) seeds and *Aspergillus niger* from infected Adepa (groundnut) seeds.

Seeds were surface sterilized in 10 % sodium hypochlorite (1 % chlorine) for 1 min, rinsed with sterile distilled water and dried on blotter paper before plating on chloramphenicol amended potato dextrose agar (PDA). Depending on the size of seeds, 10 seeds each of cowpea and soybean, and six seeds of groundnut were plated on the amended PDA in a laminar flow cabinet. This was replicated five times for each legume crop.

Identification of the fungal isolates was done after seven days by visual observation of the mycelium and with the aid of a compound microscope, and identification manual of genera of imperfect fungi by Barnett and Hunter (1972).

After isolation and identification, the preferred fungal isolates pure cultures of *Fusarium* sp., *Colletotrichum* sp. and *Aspergillus niger* were stored for subsequent experiments.

3.5 Preparation of the cowpea, groundnut, soybean and lima bean cotyledons for challenge inoculation

One hundred seeds of each genotype above were surface sterilized in 10 % sodium hypochlorite (1 % chlorine) for 1 min, rinsed with sterile distilled water followed by soaking the sterile seeds in 300 ml sterile distilled water in a 500 ml glass beaker for 24 h.

The testa of each seed was removed with a pair of sterilised forceps and the two cotyledons were carefully separated without causing any visible damage. One set of cotyledons, adaxial surface uppermost was placed in one Petri dish and the other set of cotyledon placed in a seperate Petri dish lined with a sterile-distilled-water-absorbed sterile filter paper (Whatman No. 1). Depending on the size of the seeds 15 of one set of adaxial cotyledons of cowpea and soybean, 10 for groundnut and seven for lima bean

were placed in each Petri dish seperately. This was replicated five times for each genotype.

3.6 Preparation of *Fusarium* sp., *Colletorichum* sp. and *Aspergillus niger* spore inoculum for inoculation of adaxial cotyledon surface

Spore inoculum was prepared from 10-day-old cultures of *Fusarium* sp., *Colletotrichum* sp. and *Aspergillus niger* by adding 10 ml sterile-distilled-water separately in each of the plates. The surface of the culture was rubbed with a sterile glass rod to displace mycelia and spores from the surface of the medium into the sterile-distilled-water in each plate separately. Each suspension was filtered through four layers of muslin into a sterilised glass beaker. The spore concentration was measured with a haemocytometer and the concentration adjusted to $1x10^5$ spores/ml as described by Hargreaves *et al*. (1977). The host-pathogen combinations used were cowpea-*Fusarium* sp., soybean-*Colletotrichum* sp., groundnut-*Aspergillus niger* and Lima bean-*Colletotrichum* sp. One set of adaxial cotyledon surfaces of the legumes (cowpea, soybean, groundnut and lima bean) were inoculated with 30 µl drops ($1x10^5$ spores/ml) of the spore inoculum of their respective fungus whilst the other set of adaxial cotyledons were inoculated with sterile distilled water using a micropippette. The inoculated cotyledons in the Petri dishes were covered and incubated at an ambient temperature in a sterile transfer room for 36 h. Inoculated adaxial cotyledons surfaces were assessed for the presence and absence of lesions and colour of lesions recorded with the aid of Munsell plant tissue colour chart.

3.7 Extraction of antimicrobial compounds from cotyledon tissues

Thirty-six hours after inoculation with the biotic elicitors and sterile distilled water for each set of adaxial cotyledon surfaces, cotyledon tissues were collected by slicing upper layers (sites inoculated with conidial suspension and sterile distilled water) not

more than 1 mm thick for phytoalexin extraction. Cotyledon tissues collected from those inoculated with sterile distilled water served as control.

One gram fresh weight of tissues excised from the fungus-inoculated (elicited) and sterile-distilled-water-inoculated (control) were collected and homogenized in 10 ml of 100 % methanol in a mortar until a fine suspension was obtained. The homogenate was centrifuged at 4000 g for 10 min and the supernatant poured into clean plastic vials. The pellet was re-extracted with a further 5 ml of 100% methanol and both supernatants were combined and concentrated *in vacuo* at 25^{o}C to half the initial volume.

3.8 Radial growth bioassay

The method of Fiddaman and Rossall (1994) was followed with modifications. After pouring 5 ml of melted autoclaved PDA medium into 8 cm Petri dishes, 50µl each of the concentrated extracts obtained from fungus-inoculated cotyledons, sterile-distilled water-inoculated cotyledons and for control, 50 µl of methanol (solvent used in extraction) were added before the medium congealed. After the amended PDA had solidified, 1 cm diameter disc was taken with a sterile cork borer from a 10-day-old culture of *Curvularia lunata*. The disc was placed at the centre of the amended medium in each Petri dish. The dishes were sealed with a masking tape and incubated at an ambient temperature in the sterile transfer room. Fungal radial mycelia growth was measured on two preset diametral lines at two, four, six and eight days of growth. Percentage mycelial growth inhibition was calculated from the formula [(DC-DT)/DC]*100 (Pandey *et al*., 1982), where DC and DT are average diameters of fungal colony of control (PDA amended with methanol) and the other treatment (PDA amended with legume cotyledon extract), respectively. The per cent inhibition was calculated based on the radial growth on the sixth day because maximum growth was observed in the control.

3.9 Analysis of cotyledon extracts by thin layer chromatography

With the aid of a micropipette, 20 µl of the concentrated cotyledon extract for both the control (extracts obtained from sterile-distilled-water-inoculated cotyledons) and elicited (extracts obtained from fungus-inoculated cotyledons) of each legume genotype were spotted on a pre-coated 10 cm origin thin layer chromatography plates (Merck Kieselgel 60 F254 Silica gel). The spotting was done with different pipette tips for each extract.

The TLC plates were then developed in tanks pre-equilibrated with 25 parts of chloroform: one of methanol (25:1v/v), air dried and observed visually and under ultra violet (UV) light. Spots were detected on TLC plates by their characteristic blue appearance under UV light. The phytoalexins were detected on TLC plates by their fluorescence under UV and rate of flow (R_f) values recorded. R_f value is the ratio of the distance travelled by a compound during chromatography and the distance travelled by the solvent from the origin (Jork *et al*., 1994). The process was replicated three times and means of R_f values recorded for the separated compounds.

3.10 TLC direct bioautography

TLC plate bioassays were used to detect antimicrobial compounds in the extracts (Bennett *et al*., 1994). Concentrated methanolic extracts of fungus-inoculated and sterile-distilled-water inoculated cotyledon tissue were spotted on pre-coated 10 cm origin TLC plates.

Following development of chromatograms, plates were air-dried and sprayed with concentrated suspensions (5×10^8 spores/ml) of conidia of *Aspergillus niger* in Potato Dextrose Broth. The sprayed plates were incubated at an ambient temperature in darkness in a moist chamber for four days. Inhibition was observed as reduced or lack of growth, visible as a clear zone against a darker background of mycelial growth.

3.11 Parameters determined

Radial growth (cm) of *Curvularia lunata.*

Per cent inhibition of *C. lunata.*

Rate of flow (Rf) values of UV absorbing compounds.

Rate of flow (Rf) values of zones of inhibition.

Diameter of zones of inhibition (cm).

Area of zone of inhibition (cm^2) calculated from πr^2, where π is 3.14 and r is the radius of the circular zone of inhibition.

3.12 Statistical analysis

The GenStat statistical package edition nine (2007) was used for the statistical analysis. The per cent inhibition obtained for each of the cotyledon extracts (control and elicited) for the three genotypes of cowpea, soybean and groundnut, each giving a total of six treatments were arranged in Completely Randomised Design with three replications and analysed separately using ANOVA. The significance ($p < 0.05$) of differences between treatments was determined, using the least significant difference (lsd) at 5%. Only one genotype of Lima bean was used therefore the per cent inhibition by the control (extracts obtained from sterile-distilled-water-inoculated) and elicited (extracts obtained from fungus-inoculated) cotyledons were subjected to t-test analysis. This was also replicated three times.

Correlation between the number of UV absorbing compound(s) and the per cent inhibition of *Curvularia lunata* for the cotyledon extracts of each legume genotype was determined.

CHAPTER FOUR

4.0 RESULTS

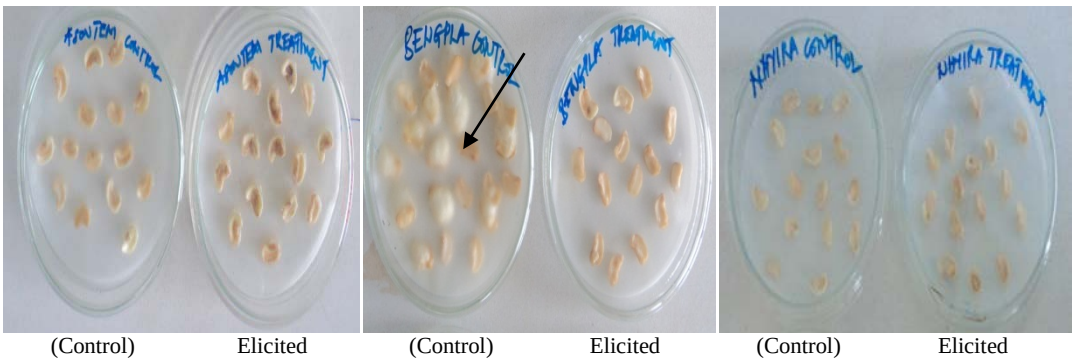
4.1 Crop species reactions to fungi

Table 1: Response of adaxial cotyledon surfaces to challenge inoculation

Legume cotyledon	Challenge inoculation	Lesion expression	Colour reaction of adaxial cotyledon surface
Cowpea	*Fusarium oxysporium*	+	Brown
	Sterile distilled water	+	Brown
Soybean	*Colletotrichum* sp	+	Dark brown
	Sterile distilled water	-	No colour
Groundnut	*Aspergillus niger*	+	Brown
	Sterile distilled water	-	No colour
Lima bean	*Colletotrichum* sp	+	Brown
	Sterile distilled water	-	No colour

+ (Present) ; -(Absent)

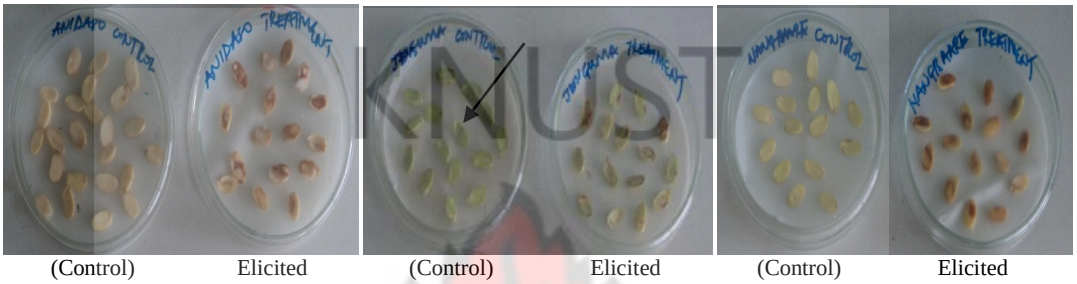
The three genotypes each of cowpea, soybean, groundnut and the genotype of lima bean reacted similarly to the fungus and sterile-distilled-water inoculation. The results presented in Table 1 are therefore summaries of lesion expression and colour reaction of the cotyledons of the crop species investigated. Fungus-inoculated adaxial surfaces of cotyledons of cowpea, soybean, groundnut and lima bean resulted in the formation of lesions and browning of tissues (Table 1). The colour reaction ranged from brown in



(Control) Elicited (Control) Elicited (Control) Elicited

Asontem Bengpla Nhyira

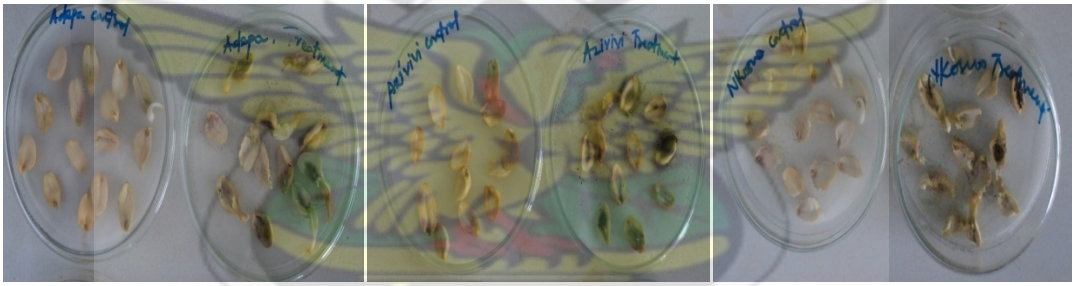
a.Cowpea genotypes



(Control) Elicited (Control) Elicited (Control) Elicited

Anidaso Jenguma Nangbaare

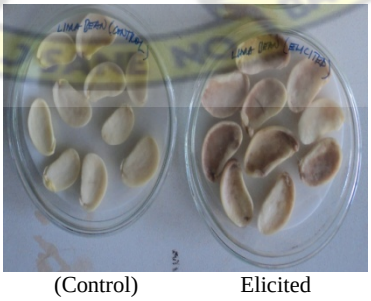
b. Soybean genotypes



(Control) Elicited (Control) Elicited (Control) Elicited

Adepa Azivivi Nkosuor

c. Groundnut genotypes



(Control) Elicited

Lima bean

d. Lima bean genotype

Plate 1: Symptoms on sterile distilled water inoculated (Control) and fungus inoculated (Elicited) cotyledons of legumes.

cowpea, groundnut and lima bean to dark brown in the soybean genotypes (Plate 1). Lesions formed were confined to the inoculum droplets. There were some fungal growth on *Colletotrichum* sp.-inoculated soybean cotyledons and *A. niger*-inoculated groundnut cotyledons. However, there were no fungal growth on the *Fusarium* sp.-inoculated cowpea cotyledons and the *Colletotrichum* sp.-inoculated lima bean cotyledons. The adaxial cotyledon surfaces of soybean, groundnut and lima bean genotype(s) inoculated with sterile-distilled water did not show lesions except cowpea which had some lesions. Cotyledons of Jenguma, a soybean genotype turned green at the sterile-distilled water-inoculated site (Plate 1b) and some cowpea cotyledons showed signs of rot especially, adaxial cotyledons of Bengpla inoculated with sterile-distilled water (Plate 1a).

4.1.2 Antifungal activity of the selected legume cotyledon extracts

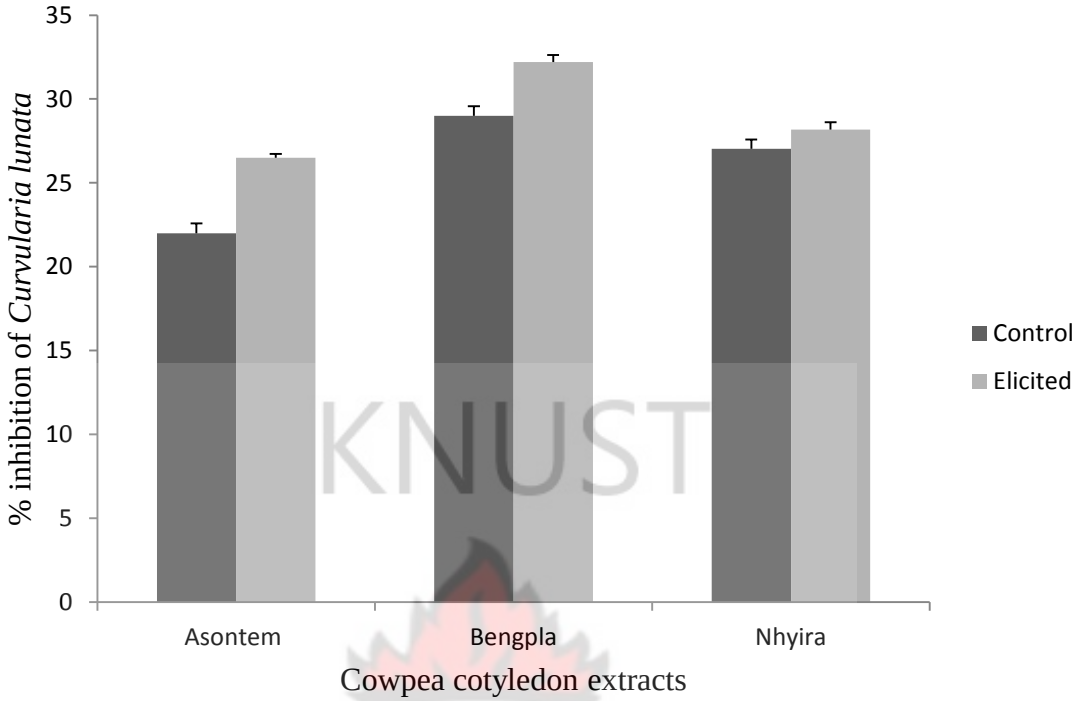


Fig 1: Effect of cowpea cotyledon extracts on the growth of *Curvularia lunata*

The per cent inhibitions of *C. lunata* were significantly different ($p < 0.05$) among the cowpea cotyledon extracts (Fig 1 and Appendix 5). Control cotyledon extracts (obtained from sterile-distilled-water-inoculated adaxial cotyledon surfaces) had significantly ($p < 0.05$) lower per cent inhibitions than the elicited cotyledon extracts (obtained from *Fusarium* sp.-inoculated adaxial cotyledon surfaces) except Nhyira control and Nhyira elicited (Fig. 1). Among the three genotypes of cowpea used in the study, Bengpla had the highest per cent inhibition of 29.0 % and 32.2 % for both the control and elicited cotyledon extracts, respectively. This was followed by Nhyira with the control having 27.0 % and elicited having 28.2 %. Asontem had the lowest per cent inhibition for both the control (22.0 %) and the elicited (28.2 %). However, there was no significant difference ($p > 0.05$) between per cent inhibition of Asontem elicited (26.5 %) and Nhyira Control (27.0 %).

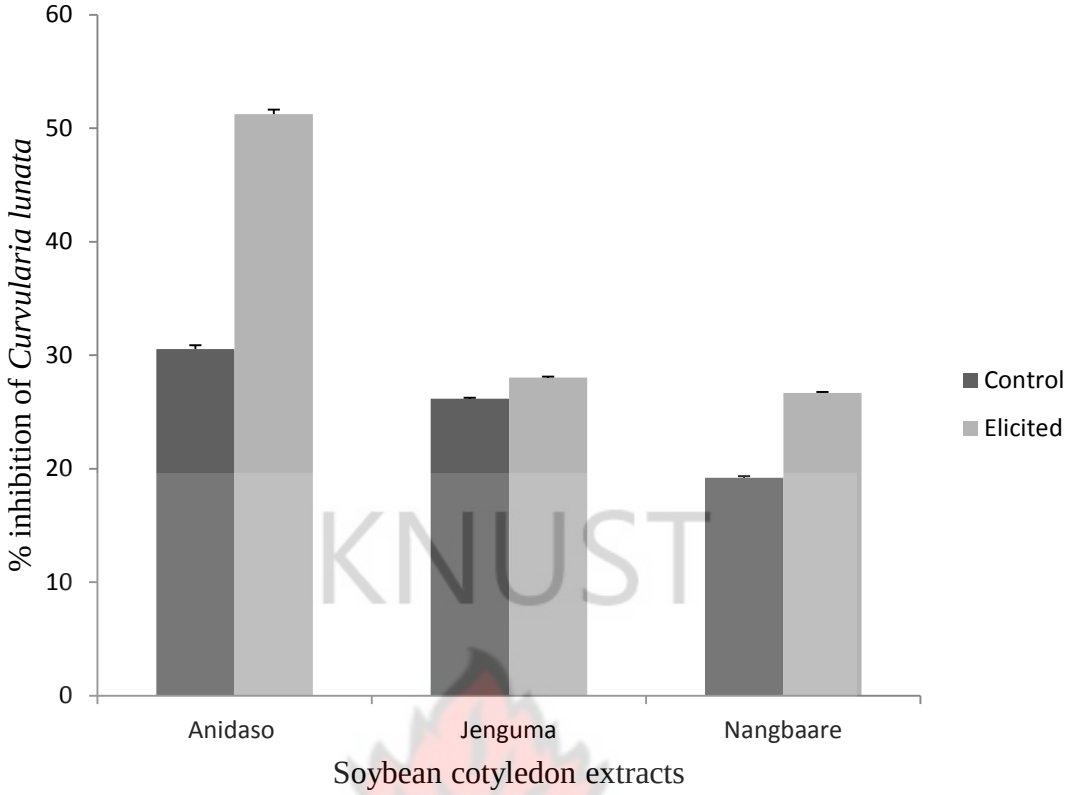


Fig 2: Effect of soybean cotyledon extracts on the growth of *C. lunata*

There were significant differences ($p < 0.05$) between the soybean cotyledon extracts in relation to the inhibition of *C. lunata* (Fig. 2 and Appendix 6). Generally, elicited-cotyledon extracts (adaxial cotyledon surfaces inoculated with *Colletotrichum* sp.) had higher inhibitions than the control cotyledon extracts (adaxial cotyledon surfaces inoculated with sterile distilled water). The lowest inhibitions were recorded for Nangbaare control (19.2 %) and Nangbaare elicited (26.7 %). However, there was no significant difference ($p > 0.05$) between the inhibitions of Jenguma control (26.2 %) and Nangbaare elicited (26.7 %). Jenguma elicited had an inhibition of 28.0 % with Anidaso control and Anidaso elicited recording the highest inhibitions of 30.5 % and 51.2 %, respectively (Fig. 2 and Plate 2b).

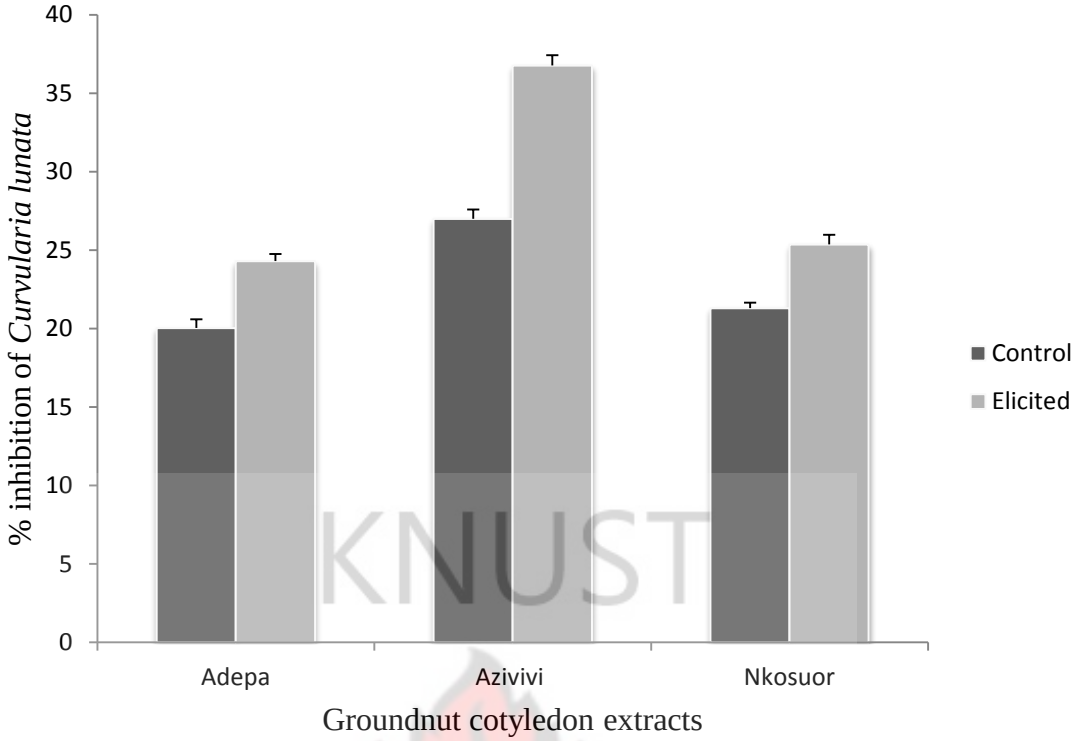


Fig 3: Effect of groundnut cotyledon extracts on the growth of *C. lunata*

The mean per cent inhibition of *C. lunata* was significantly different ($p < 0.05$) from each of the groundnut cotyledon extracts (Appendix 7). The elicited cotyledon extracts (adaxial cotyledon surfaces inoculated with *Aspergillus niger*) had significantly ($p < 0.05$) higher per cent inhibitions than control cotyledon extracts (adaxial cotyledon surfaces inoculated with sterile distilled water) in Fig. 3. Azivivi had the highest per cent inhibition for both the control (27.0%) and the elicited (36.8 %) among the three groundnut varieties. Although, Adepa control recorded the lowest per cent inhibition, there were no significant difference ($p > 0.05$) between Adepa control (20.0 %) and Nkosuor control (21.3 %). There was no significant difference ($p > 0.05$) between the per cent inhibition of Adepa elicited (24.3 %) and Nkosuor elicited (25.4) (Fig. 3 and Plate 2c).

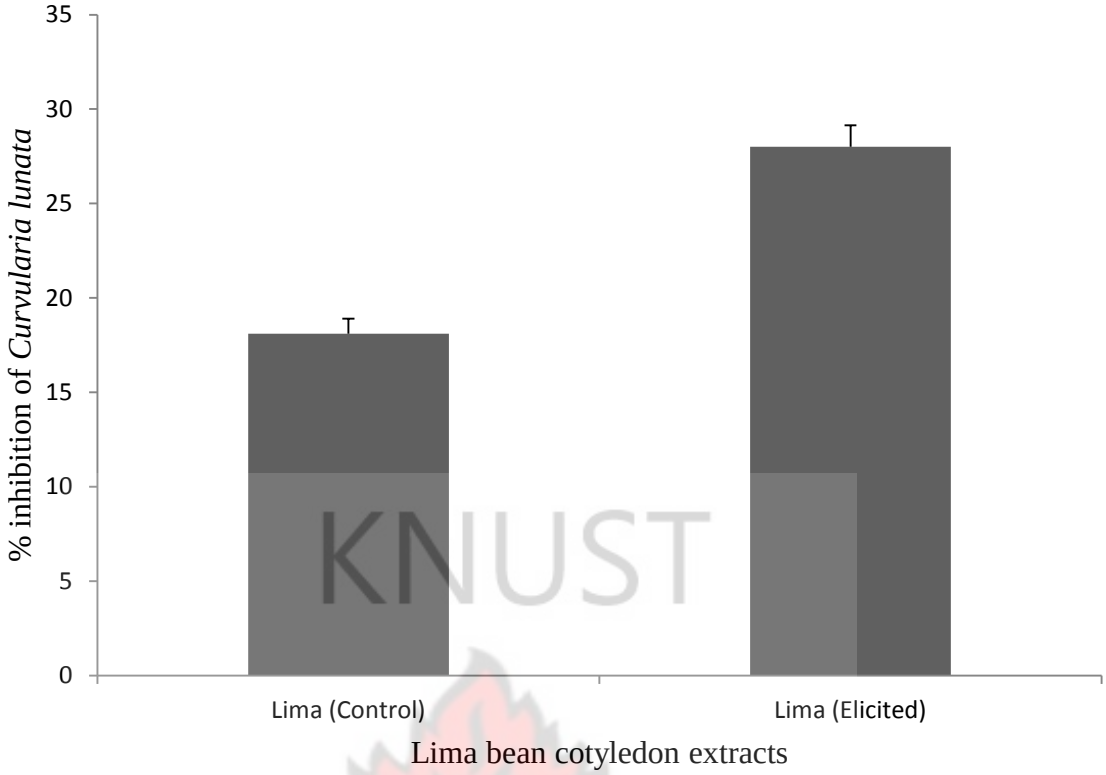
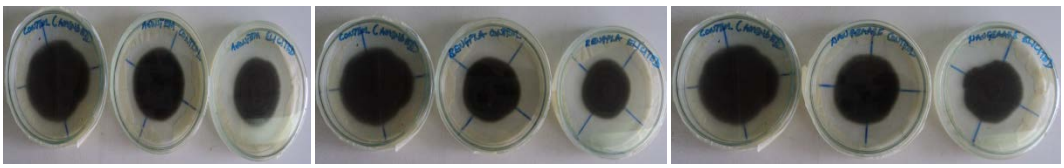


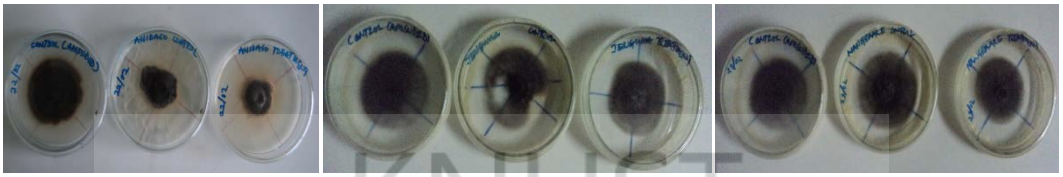
Fig 4: Effect of lima bean cotyledon extracts on the growth of *C. lunata*

Elicited lima bean cotyledon extracts (adaxial cotyledon surfaces inoculated with *Colletotrichum* sp.) had a significantly ($p < 0.05$) higher inhibition (28.0 %) of *C. lunata* (Appendix 8) than control lima bean cotyledon extract (adaxial cotyledon surfaces inoculated with sterile distilled water) (Fig 4 and Plate 2d) which had an inhibition of 18.1 %.



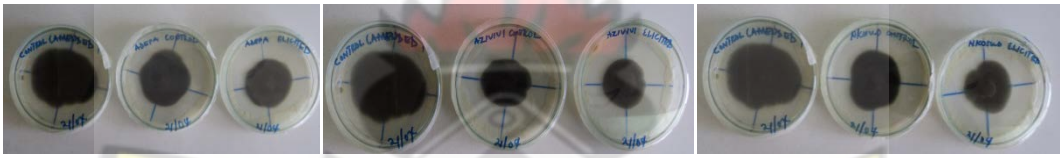
Asontem Bengpla Nhyira

a. Cowpea



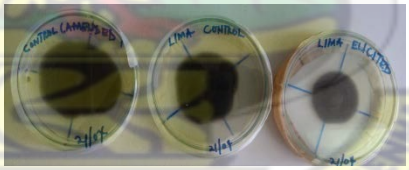
Anidaso Jenguma Nangbaare

b. Soybean



Adepa Azivivi Nkosuor

c. Groundnut



d. Lima bean

Plate 2. Radial growth of *Curvularia* sp. on PDA amended with methanol (left) and legume cotyledon extracts control (centre) and elicited (right)

4.1.3 Thin layer chromatography (TLC) and detection of compounds under UV light

Table 2: Rate of flow (R_f) values of separated compounds of cowpea cotyledon extracts using chloroform-methanol (25:1) solvent system

Cowpea cotyledon extracts	Rate of flow (R_f) of UV absorbing compounds				
	Compounds				
	C-I	C-II	C-III	C-IV	C-V
Asontem (Control)	0.06	0.09	Nil	Nil	Nil
Asontem (Elicited)	0.06	0.09	Nil	Nil	0.35
Bengpla (Control)	0.06	0.09	Nil	Nil	Nil
Bengpla (Elicited)	0.06	0.09	0.16	0.24	Nil
Nhyira (Control)	0.06	0.09	Nil	Nil	Nil
Nhyira (Elicited)	0.06	0.09	0.16	Nil	Nil

Values are means of three replicates

Five different compounds were recovered from the cowpea cotyledon extracts (Table 2). Compounds C-I (R_f 0.06), C-IV (R_f 0.24) and C-V (R_f 0.35) were seen as UV quenching spots whilst compounds C-II (R_f 0.09) and C-III (R_f 0.16) were blue flourescing spots (Plate 3). Compounds C-I (R_f 0.06) and C-II (R_f 0.09) appeared in both the control and elicited cotyledons of all the cowpea cotyledon extracts (Table 2). Compound C-III (R_f 0.16) occurred in elicited cotyledons of Bengpla and Nhyira whereas compounds C-IV (R_f 0.24) and C-V (R_f 0.35) occurred in elicited cotyledons of Bengpla and Asontem, respectively. Generally, elicited cotyledons produced more and different compounds based on their R_f values than control cotyledons (sterile-distilled-

water-inoculated). Bengpla elicited produced the highest number of compounds (four) followed by elicited Asontem and Nhyira elicited with three compounds, each (Table 2). The controls for each of the cowpea genotypes yielded only two compounds.

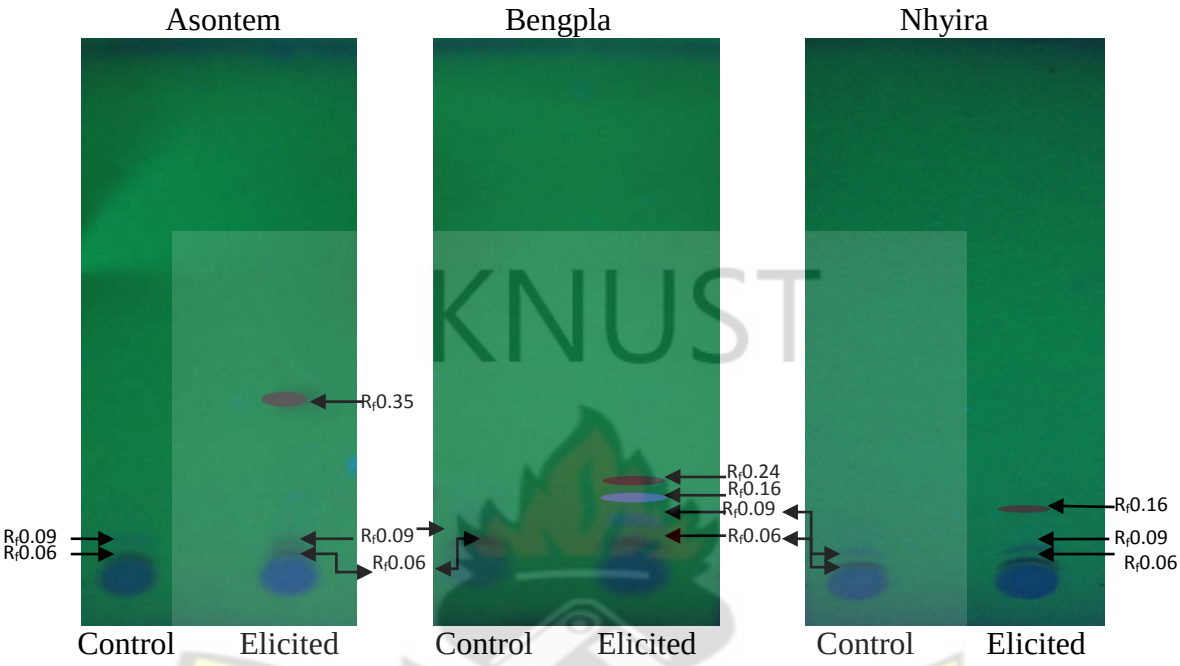


Plate 3. UV absorbing compounds of cowpea cotyledon extracts on TLC plates

Table 3: Rate of flow (R_f) values of separated compounds of soybean cotyledon extracts using chloroform-methanol (25:1) solvent system

Soybean cotyledon extracts	Rate of flow (R_f) of UV absorbing Compounds					
	Compounds					
	S-I	S-II	S-III	S-IV	S-V	S-VI
Anidaso (Control)	Nil	Nil	Nil	Nil	0.78	Nil
Anidaso (Elicited)	0.19	0.36	0.40	Nil	Nil	0.90
Jenguma (Control)	Nil	Nil	Nil	Nil	Nil	Nil
Jenguma (Elicited)	0.19	0.36	Nil	0.51	Nil	Nil
Nangbaare (Control)	Nil	Nil	Nil	Nil	Nil	Nil
Nangbaare (Elicited)	0.19	0.36	0.40	Nil	Nil	Nil

Values are means of three replicates

Six different compounds were separated from soybean cotyledon extracts (Table 3). Compounds S-I (R_f 0.19) and S-II (R_f 0.36) were identified in all the fungus-challenged cotyledons (elicited) of the three varieties of soybean used. Compounds S-IV (R_f 0.51) and S-VI (R_f 0.9) occurred in only elicited Jenguma and Anidaso cotyledon extracts, respectively. Compound S-V (R_f 0.78) occurred in only sterile distilled water-treated cotyledon of Anidaso (control). A total of four compounds namely S-I, S-II, S-III and S-VI were separated from elicited Anidaso and Compounds S-III and S-IV appeared as UV quenching spots (Table 3 and Plate 4). Compound S-II in Jenguma was seen as a yellowish green spot. Compounds S-I and S-II in Anidaso and Nangbaare, Compound S-V and S-VI were blue fluorescing. Compound S-I in Jenguma was quenching (Plate 4).

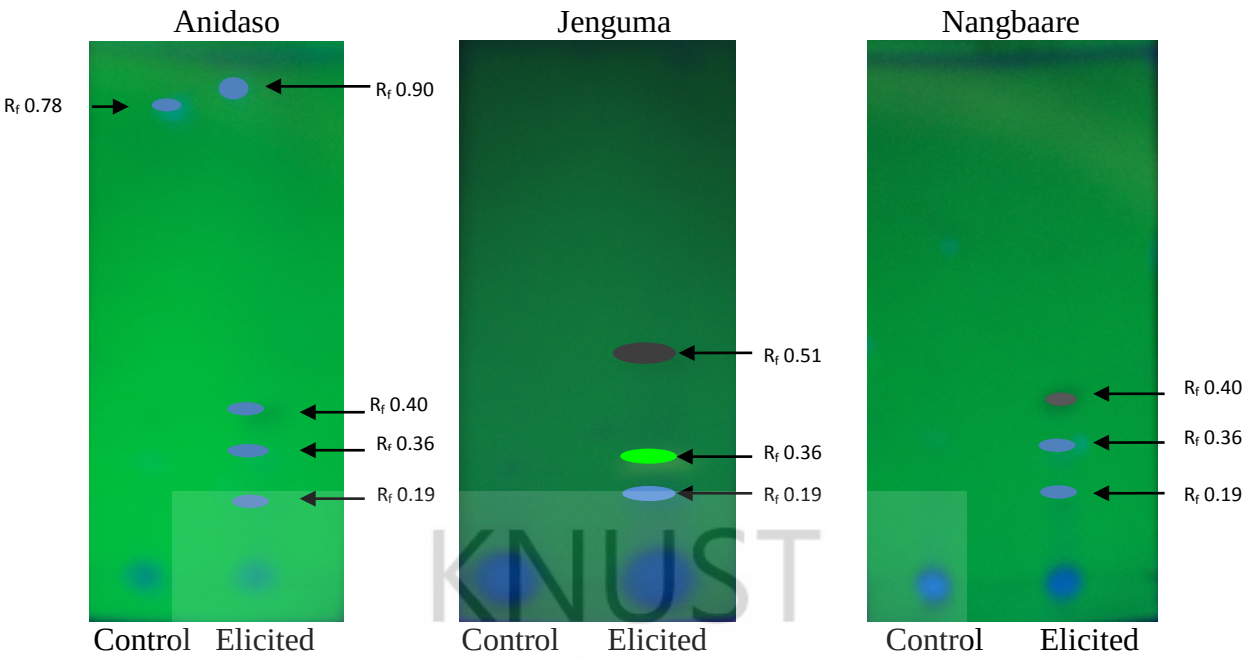


Plate 4. UV absorbing of soybean cotyledon extracts on TLC plates

Table 4: Rate of flow (R_f) values of separated compounds of groundnut cotyledon extracts using chloroform-methanol (25:1) solvent system

Groundnut cotyledon extracts	Rate flow (R_f) of UV absorbing Compounds				
	Compounds				
	G-I	G-II	G-III	G-IV	G-V
Adepa (Control)	Nil	Nil	Nil	Nil	Nil
Adepa (Elicited)	0.09	Nil	Nil	Nil	Nil
Azivivi (Control)	0.09	Nil	Nil	0.29	Nil
Azivivi (Elicited)	0.09	Nil	0.20	0.29	Nil
Nkosuor (Control)	Nil	0.14	Nil	Nil	Nil
Nkosuor (Elicited)	Nil	0.14	Nil	Nil	0.40

Values are means of three replicates

Five different compounds were produced by groundnut cotyledons after challenge with *Aspergillus niger* (Table 6). Compounds G-I (R_f 0.09) and G-IV (R_f 0.29) were found in control and elicited cotyledon extracts of Azivi. Compound G-II (R_f 0.14) also occurred in Nkosuor for control and elicited extracts (Table 4). Compound G-III (R_f 0.20) was seen under UV light in only Azivivi elicited. Compound G-V (R_f 0.40) occurred only in Nkosuor elicited. For the *A. niger*-inoculated cotyledons (elicited), two different compounds were recovered from Nkosuor, three from Azivivi and one compound was from Adepa (Table 4). No compound was detected in Adepa control but Azivivi and Nkosuor controls yielded two compounds and one compound, respectively (Table 4). All the compounds detected were all blue fluorescing under UV light (Plate 5).

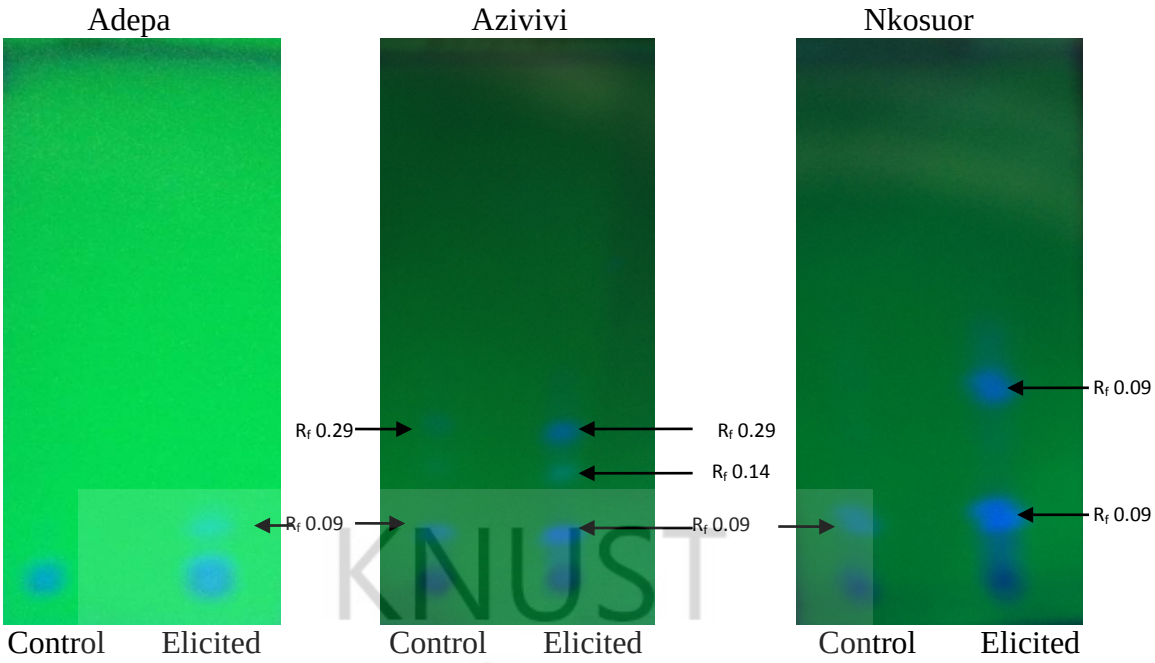


Plate 5. UV absorbing compounds of groundnut cotyledon extracts on TLC plates

Table 5: Rate of flow (R_f) values of separated compounds of Lima bean cotyledon extracts using chloroform-methanol (25:1) solvent system

Lima bean cotyledon extracts	Rate of flow (R_f) of UV absorbing Compounds			
	Compounds			
	L-I	L-II	L-III	L-IV
Lima (Control)	0.07	Nil	0.17	Nil
Lima (Elicited)	0.07	0.13	0.17	0.75

Values are means of three replicates

Four different compounds were produced by Lima bean cotyledons after treating cotyledons with sterile distilled water and *Colletotrichum* sp. spore-inoculum (Table 5). Compounds L-I (R_f 0.07) and L-III (R_f 0.17) were found in both control and elicited cotyledons. Compounds L-II (R_f 0.13) and L-IV (R_f 0.75) were found in only elicited Lima bean cotyledons. Compounds L-I (R_f 0.07) for both the control and elicited, and

compound L-IV (R_f 0.75) in elicited were quenching. Compounds L-II (R_f 0.13) and L-III (R_f 0.17) were blue fluorescing in elicited but compound L-III (R_f 0.17) in control was quenching (Plate 6).

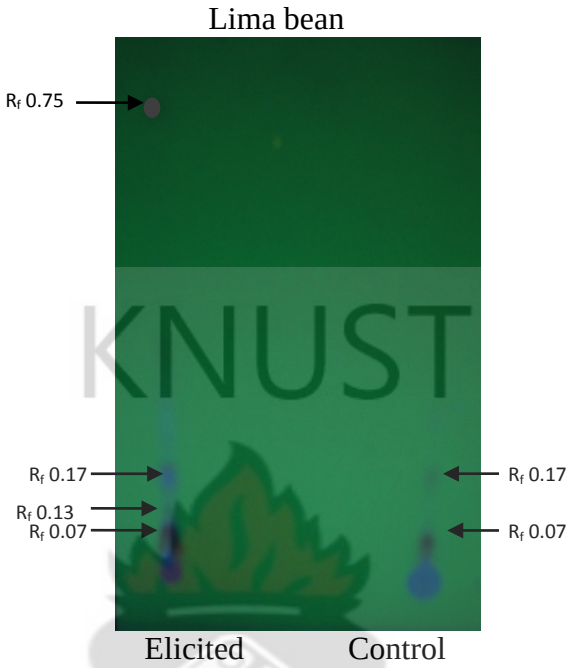


Plate 6. UV absorbing compounds of lima bean cotyledon extracts on TLC plates

4.1.4 Thin layer chromatography direct bioautography

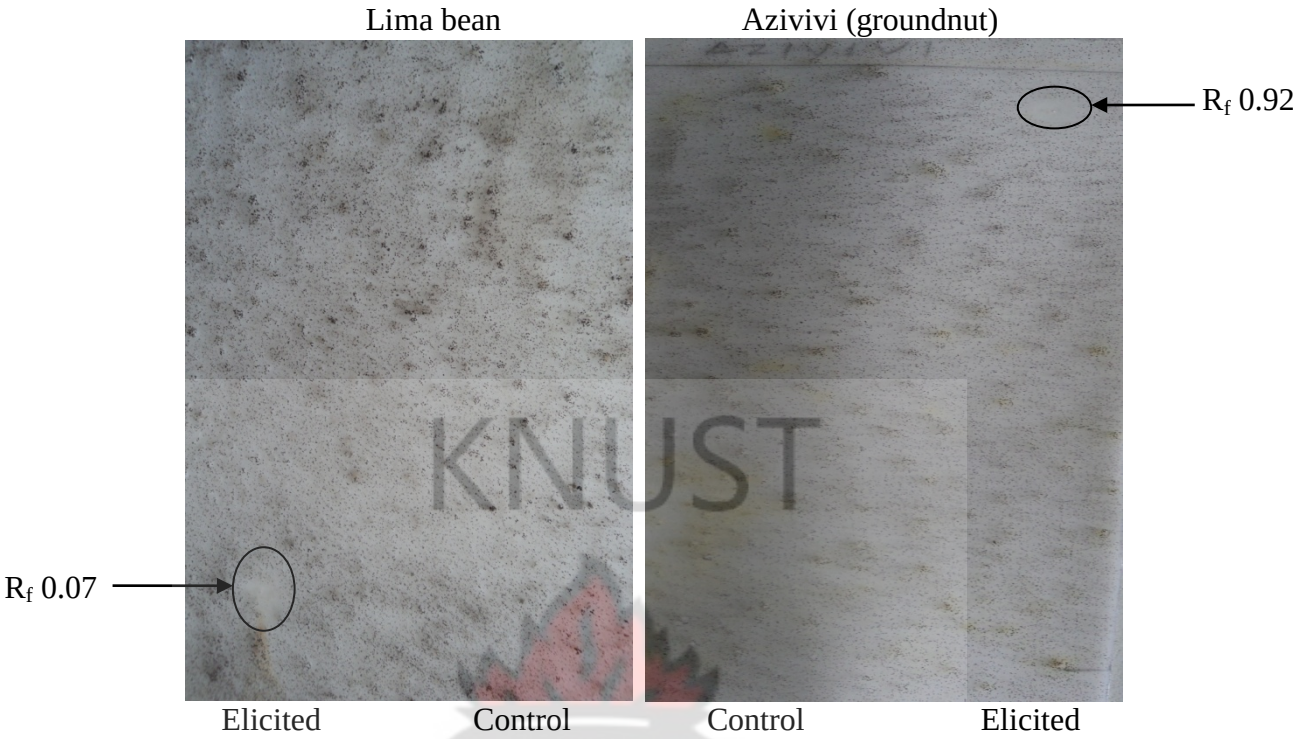


Plate 7. .*Aspergillus niger* TLC bioassay of lima bean and groundnut (Azivivi) cotyledon extracts

Only Lima bean and Azivivi variety of groundnut showed localised fungitoxic activity on the TLC plates among all the legume genotypes studied (Plate 7). Compound L-I (R_f 0.07) produced a corresponding zone of inhibition in an area of 0.13 cm^2. A non-UV absorbing compound with R_f 0.92 inhibited the growth of *A. niger* on TLC in an area of inhibition of 0.07cm^2. All the inhibitions were seen in the lane where the concentrated extracts from fungus-inoculated cotyledons were spotted.

4.1.5 Genotype response to accumulation of antimicrobial compounds

Table 6: Correlation between the number of UV absorbing compounds and the per cent inhibition of *C. lunata*

Crop species	Correlation coeffiecient (r)
Cowpea	0.7
Soybean	0.7
Groundnut	0.9
Lima bean	1.0

The number of UV absorbing compounds in the cotyledon extracts of each crop species was positively correlated (r = 0.7, 0.7, 0.9 and 1.0) with the per cent inhibition of *C. lunata* of the same cotyledon extracts (Table 6) for cowpea, soybean, groundnut and lima bean, respectively.

CHAPTER FIVE

5.0 DISCUSSIONS

5.1 Crop species reactions to fungi

Lesions confined to the inoculum droplets were found in all the fungus-challenged cotyledons among the different crop species. This is in agreement with Soylu *et al.* (2002) who reported the presence of lesions in pathogen-challenged cotyledon tissues of *Vicia faba* L. According to Cooper *et al*. (1996), these lesions can be considered as localised defense response by plant tissue to prevent further spread of fungi. The variation in lesion colour conforms to the findings by Soylu *et al.* (2002) who observed the presence of different colours of lesions when cotyledons of *V. faba* were exposed to biotic and abiotic elicitors. Dark brown necrotic tissue of the soybean cotyledons restricted to the area of contact with the *Colletotrichum* inoculum droplet is consistent with Stephen *et al*. (2000) who also observed dark brown lesions in cotyledons of soybean inoculated with *Aspergillus*. The colouration in cotyledon tissues may have resulted from the accumulation of certain phytoalexin precursors following exposure to the elicitor as reported by Ingham *et al*. (1981). The presence of dark brown colouration in cotyledons of soybean has been correlated with the presence of the phytoalexin glyceollin (Ayers *et al*., 1976). Studies have shown that the major components of resistance in soybeans to pathogens included production of the isoflavonoid phytoalexin, glyceollin (Graham, 1995; Graham *et al*., 1990; Ward *et al*., 1979).

Lesions produced in the cowpea cotyledons inoculated with sterile distilled water may be attributed, partly, to the small nature of the seeds and therefore have limited reserves than the soybean, groundnut and lima bean seeds hence, predisposing it to minimal amount of abiotic stress such mechanical injury which caused reactions in the tissues.

Soylu *et al.* (2002) reported the presence of lesions in injured cotyledon tissues of *V. faba*.

5.2 Antifungal activity of legume cotyledon extracts

Radial growth bioassay established that extracts from fungus-inoculated cotyledons and sterile-distilled-water-inoculated cotyledons had some level of antifungal activity. However, extracts from fungus-inoculated cotyledons showed significantly higher per cent inhibition.

The higher percentage inhibitions recorded may be due to the rapid accumulation of phytoalexin at the site of infection. This phenomenon is a manifestation of induced resistance, in which an elicitor will magnify, in time and amount, the defense response of the plant to fend off a pathogen (Benhamou, 1996). Harborne (1999) reported that phytoalexins are produced by plants as a consequence of microorganism attack and can be considered as an important defense mechanism in plants. The observation also agrees with Ingham (1973) who found that phytoalexin biosynthesis is confined to infected cells and their immediate vicinity in most plant species and this further elucidates higher per cent inhibition. The higher inhibition due to extracts from the fungus-inoculated cotyledons gives an indication of the chemical toxicity of the phytoalexins which repressed the fungal growth. Due to the toxicity level in phytoalexins, plants produce them when fungi begin their attempt to take over (Ayers, 2008).

5.3 Thin Layer Chromatography (TLC) and detection of separated compounds under ultra violet (UV) light

The concentrated legume cotyledon extracts spotted on the TLC plates revealed a mixture of extracts. The presence of UV absorbing compounds in extracts obtained

from fungus-inoculated cotyledons and their absence in extracts obtained from sterile distilled water-inoculated cotyledons is an indication that infection-induced antimicrobial compounds have been produced. This agrees with the fact that phytoalexins are produced after infection by a pathogen (Manfield, 2000) and are virtually absent in healthy host tissues. The presence of these compounds in only extracts obtained from fungus-inoculated cotyledons gives an indication of a defense function for the compounds. These compounds can therefore be considered as phytoalexins since they were produced *de novo* in response to infection (Smith, 1996).

The presence of UV absorbing compounds in the controls (extracts obtained from sterile-distilled-water-inoculated cotyledons) supports the fact that there exist constitutive antimicrobial compounds in intact healthy tissues. Kraus *et al*. (1995) reported that non-elicitor-treated soybean seeds contained trace levels of glyceollin and were detected at high concentrations during stress. Also, the presence of UV absorbing compounds in control treatment could be attributed to an abiotic stress. Soylu *et al.* (2002) observed that abiotic agents including injury and UV radiation trigger the accumulation of phytoalexins.

5.4 Thin layer chromatography (TLC) direct bioautography

Majority of the separated compounds did not show localised fungitoxic activity against *A. niger* on TLC plates. However, compound L-I (R_f 0.07) produced in Lima bean inhibited the growth of *A. niger* in an area of inhibition of 0.13 cm^2. Also, a non- UV absorbing compound with R_f 0.92 inhibited the growth of *A. niger* on TLC plates in an area of inhibition of 0.07 cm^2. These compounds, if isolated and identified, can be considered as chemical markers for resistance. The fact that these compounds inhibited growth of *A. niger* in the lane of TLC plate spotted with the concentrated extracts from fungus-challenged cotyledons, gives a strong indication that they are phytoalexins since

they were produced *de novo* in response to infection and inhibited the growth of the pathogen. VanEtten *et al*. (1995) made a similar observation. This also proves the chemical toxicity of phytoalexins and supports the fact that plants produce phytoalexins when attacked by fungi (Ayers, 2008).

All the concentrated cotyledon extracts tested by radial growth bioassay showed some level of inhibition but generally did not produce any zone of inhibition in the *A. niger* TLC bioassay. This is probably due to the synergistic action between the compounds present in the cotyledon extract that got separated through the TLC, a reduced level or some chemical alteration of the active compound. Fungi metabolise phytoalexins to less toxic derivatives through demethylation of the methoxyl groups and hydroxylation of the nucleus (Delserone *et al*., 1999).

The absence of inhibition at corresponding UV absorbing spots may also be as a result of the possible detoxification of the phytoalexin by the fungi (Miao and VanEtten, 1992 a, b). Numerous pathogens have been shown to degrade their host's phytoalexins to non-toxic products (Morrissey and Osbourn, 1999; VanEtten *et al*., 1995) making the plants susceptible to attack (Eisberg, 2009).

If the phytoalexins were detoxified by the *A. niger*, it does not mean they do not have a role in resistance. According to Kuc (1995), phytoalexin degradation could be important in determining compatibility. Degradation of host phytoalexin have been shown to determine the virulence of the pathogen, therefore phytoalexin accumulation is needed to limit the virulence of pathogens. Bailey and Mansfield (1982) have postulated that such pattern of accumulation and degradation is typical of phytoalexins in plant tissues.

5.5 Genotype response to accumulation of antimicrobial compounds

The strong positive correlation between number of UV absorbing compounds and per cent inhibition can be used to explain how the genotypes responded to fungal elicitation by producing antimicrobial compounds. As the number of UV absorbing compounds increase, the per cent inhibition of *Curvularia lunata* increases. The response of the selected legumes to the accumulation of antimicrobial compounds after infection can therefore be grouped as Bengpla > Nhyira > Asontem for cowpea; Anidaso > Nangbaare > Jenguma for soybean; Azivivi > Nkosuor > Adepa for groundnut. Hence, Bengpla, Anidaso and Azivivi can be said to have produced more of the antimicrobial compounds in response to infection among the genotypes of cowpea, soybean and groundnut studied, respectively. They will therefore be more resistant to fungal infections.

The distinctive response of the three genotypes each of cowpea, soybean, groundnut and the genotype of Lima bean to their biotic elicitor is indicative of a differential capacity of these genotypes to respond to microbes in the field, since the production of phytoalexins can be triggered by a large number of biotic and abiotic inducers. The procedure described in this study can therefore be effectively used by Plant breeders to efficiently screen for resistance to fungal infections.

5.6 Significance of the study in legume improvement

Resistance can be induced by controlled elicitation which stimulates natural disease resistance without the use of environmentally damaging compounds. The plants' immune response to disease is improved as disease response mechanisms such as the accumulation of phytoalexins is activated or invigorated by the elicitor.

Spraying of plants and planting materials with phytoalexin elicitors or pre-immunisation through a non-pathogen inoculation (Mastouri *et al*., 2010) can result in the production of plants that express a higher quantity of phytoalexins.

Also, genes controlling phytoalexin synthesis are worthy targets for genetic manipulation with potential for crop improvement. Breeding plants that super-produce phytoalexins in response to biotic elicitors will help curb crop losses due to disease-causing-pathogens. Transfer of genes involved in the synthesis of phytoalexins to yield more resistant crops has been found to be successful in the case of tobacco plants in which transformation with stilbene synthase, involved in resveratrol synthesis, provided plant resistance to *Botrytis cinerea* infection (Hain *et al*., 1993).

The study and synthesis of phytoalexins also open up tremendous prospects for the discovery of new natural products with antimicrobial activity whose structures can serve as models for the chemical synthesis of natural pesticides.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

It can be concluded from the study that adaxial cotyledon surfaces reacted to fungal elicitation by producing lesions. Extracts obtained from fungus-inoculated cotyledons (elicited) had significantly higher per cent inhibition of *C. lunata* than extracts obtained from sterile-distilled-water-inoculated cotyledons (control). Subsequently, thin layer chromatography (TLC) also revealed that the extracts obtained from fungus-inoculated cotyledons (elicited) produced more ultra violet (UV) absorbing compounds than the extracts obtained from sterile distilled water-inoculated cotyledons (control). However, some cotyledon extracts did not produce any UV absorbing compound.

Majority of the separated compounds did not show localised antifungal activity on the TLC plates when the plates were sprayed with *A. niger* spores in potato dextrose broth. However, localised fungitoxic activity were recognised for a UV absorbing compound of Lima bean and a non-UV absorbing compound of Azivivi, a genotype of groundnut.

Based on the per cent inhibition of *C. lunata* and the number of UV absorbing compound(s) produced by the cotyledon extracts, Bengpla (cowpea), Anidaso (soybean) Azivivi (groundnut) and Lima bean showed the best response to induced accumulation of phytoalexins.

The procedure described in this study can therefore be effectively used by Plant breeders to efficiently screen for resistance to fungal infections.

6.2 Recommendations

The compounds that showed localised antifungal activity should be isolated and identified.

Pre-immunisation of seeds through a non-pathogen such as *Neurospora crassa* and *Trichoderma viride* inoculation can induce systemic resistance because of the priming effect which accelerates defense responses by the plant upon pathogen attack. Pre-immunisation can be achieved by soaking seeds in *Trichoderma viride* or *Neurospora crassa* inoculum before sowing.

Further studies should be conducted to determine gene(s) controlling phytoalexin synthesis.

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APPENDICES

Appendix 1a: Effect of cowpea cotyledon extracts on the radial growth of *C. lunata*

Cowpea cotyledon extracts	Radial growth of *C. lunata* (cm)
Asontem (Control)	5.7
Asontem (Elicited)	5.4
Bengpla (Control)	5.2
Bengpla (Elicited)	5.0
Nhyira (Control)	5.4
Nhyira (Elicited)	5.3
Control (Amended)	7.3
Lsd(5%)	0.2
cv(%)	2.2

Values are means of 3 replicates

Appendix 1b: Analysis of variance for the effect of Lima bean cotyledon extract on the radial growth of *C. lunata*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatments	6	10.62952	1.77159	112.74	<.001
Residual	14	0.22000	0.01571		
Total	20	10.84952			

Appendix 2a: Effect of soybean cotyledon extracts on the radial growth of *C. lunata*

Soybean cotyledon extracts	Radial growth *C. lunata* (cm)
Anidaso (Control)	5.0
Anidaso (Elicited)	3.5
Jenguma (Control)	5.3
Jenguma (Elicited)	5.2
Nangbaare (Control)	5.8
Nangbaare (Elicited)	5.3
Control (Amended)	7.2
Lsd(5%)	0.7
cv(%)	7.9

Values are means of 3 replicates

Appendix 2b: Analysis of variance for the effect of soybean cotyledon extract on the radial growth of *C. lunata*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatments	6	20.8857	3.4810	19.92	<.001
Residual	14	2.4467	0.1748		
Total	20	23.3324			

Appendix 3a: Effect of groundnut cotyledon extracts on the radial growth of *C. lunata*

Groundnut cotyledon extracts	Radial growth *C. lunata* (cm)
Adepa (Control)	6.0
Adepa (Elicited)	5.5
Azivivi (Control)	5.3
Azivivi (Elicited)	4.6
Nkosuor (Control)	5.7
Nkosuor (Elicited)	5.4
Control (Amended)	7.2
Lsd(5%)	0.4
cv(%)	4.4

Values are means of 3 replicates

Appendix 3b: Analysis of variance for the effect of groundnut cotyledon extract on the radial growth of *C. lunata*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatments	6	10.88000	1.81333	29.29	<.001
Residual	14	0.86667	0.06190		
Total	20	11.74667			

Appendix 4a: Effect of lima bean cotyledon extracts on the radial growth of *C. lunata*

Lima bean cotyledon extracts	Radial growth *C. lunata* (cm)
Control	6.0
Elicited	5.3
Control (Amended)	7.2
Lsd(5%)	0.5
cv(%)	3.8

Values are means of 3 replicates

Appendix 4b: Analysis of variance for the effect of Lima bean cotyledon extract on the radial growth of *C. lunata*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	2	5.36889	2.68444	48.32	<.001
Residual	6	0.33333	0.05556		
Total	8	5.70222			

Appendix 5: Analysis of variance for per cent inhibition of *C. lunata* by cowpea cotyledon extracts

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatments	5	168.7517	33.7503	48.60	<.001
Residual	12	8.3333	0.6944		
Total	17	177.0850			

Appendix 6: Analysis of variance for per cent inhibition of *C. lunata* by soybean cotyledon extracts

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	5	1790.6761	358.1352	2294.10	<.001
Residual	12	1.8733	0.1561		
Total	17	1792.5494			

Appendix 7: Analysis of variance for per cent inhibition of *C. lunata* by groundnut cotyledon extracts

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatments	5	532.9494	106.5899	121.82	<.001
Residual	12	10.5000	0.8750		
Total	17	543.4494			

Appendix 8: t-test analyses for % inhibition of *C. lunata* by Lima bean cotyledon extracts

Sample	Size	Mean	Variance	deviation	mean
Lima_Control-Lima_Elicited	3	-9.900	0.3600	0.6000	0.3464

95% confidence interval for mean: (-11.39, -8.410)

Test of null hypothesis that mean of Lima_Control-Lima_Elicited is equal to 0

Test statistic t = -28.58 on 2 d.f.

Probability = 0.001

Appendix 9: No. of UV absorbing compounds and % inhibition of *C. lunata* for cowpea cotyledon extracts

Cowpea cotyledon extracts	% inhibition of *C. lunata*	No. of UV absorbing compounds
Asontem (Control)	22.0	2
Asontem (Elicited)	26.5	3
Bengpla (Control)	29.0	2
Bengpla (Elicited)	32.2	4
Nhyira (Control)	27.0	2
Nhyira (Elicited)	28.2	3

Appendix 10: No. of UV absorbing compounds and % inhibition of *C. lunata* for groundnut cotyledon extracts

Soybean cotyledon extracts	% inhibition of *C. lunata*	No. of UV absorbing compounds
Anidaso (Control)	30.5	1
Anidaso (Elicited)	51.2	4
Jenguma (Control)	26.2	0
Jenguma (Elicited)	28.0	3
Nangbaare (Control)	19.2	0
Nangbaare (Elicited)	26.7	3

Appendix 11: No. of UV absorbing compounds and % inhibition of *C. lunata* for groundnut cotyledon extracts

Groundnut cotyledon extracts	% inhibition of *C. lunata*	No. of UV absorbing compounds
Adepa (Control)	20.0	0
Adepa (Elicited)	24.3	1
Azivivi (Control)	27.0	2
Azivivi (Elicited)	36.8	3
Nkosuor (Control)	21.3	1
Nkosuor (Elicited)	25.4	2

Appendix 12: No. of UV absorbing compounds and % inhibition of *C. lunata* for Lima bean cotyledon extracts

Lima bean cotyledon extracts	% inhibition of *C. lunata*	No. of UV absorbing compounds
Lima (Control)	18.1	2
Lima (Elicited)	28.0	4