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Evidence for the Production and Accumulation of Phytoalexins in the Cotyledons of Selected Legumes Following Treatments with Biotic Elicitors

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ABSTRACT

The accumulation of phytoalexins at the site of infection is one mechanism by which plants resist diseases. The present study sought to investigate the potential of cowpea, soybean and groundnut to synthesize and accumulate phytoalexins upon attack by fungi. Three genotypes each of cowpea, soybean and groundnut were used in the study. Using three fungi species (*Fusarium* sp., *Colletotrichum* sp. and *Aspergillus niger*) as biotic elicitors, phytoalexins were educed in the adaxial cotyledon surfaces of these genotypes, after which inoculated surfaces and their control treatments were analysed for phytoalexins accumulation. Lesions were produced in all fungus-inoculated cotyledons 36 h after incubation. Results obtained from radial growth bioassay using the fungus, *Curvularia lunata,* showed that, concentrated cotyledon extracts obtained from fungus-inoculated cotyledons had a significantly higher percent inhibition of the test fungus than extracts derived from sterile distilled water-inoculated cotyledons (control treatments). 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. These results prove that, the compounds were not preformed in the plant genotypes, but were produced *de novo* in response to the fungus challenge.

Key words: *Arachis hypogaea*, biotic elicitors, *Glycine max*, phytoalexins, *Vigna unguiculata*

INTRODUCTION

Leguminous crops serve as inimitable sources of income for resource-poor farmers, with a considerable manifold utilisation in many households in West and Central Africa (Tarawali *et al.*, 2002). They are utilised as an important substitute for animal protein for many people in Africa, where main delicacies are characterised by high starch fractions with little or no animal protein (Taylor *et al.*, 2005). Important grain legumes such as soybean (*Glycine max* (L.) Merr) and groundnut (*Arachis hypogaea* L.) supply more than 35% of the world's processed vegetable oil (Graham and Vance, 2003).

However, the overall average yields of notable grain legumes cultivated in sub-Saharan Africa including cowpea (*Vigna unguiculata* (L.) Walp), soybean and groundnut are less than 1 ton per ha (CGIAR, 2011). Both biotic and abiotic constraints account for these very low yields of grain legumes in Africa. Biotic constraints related to diseases, are key among the factors militating against profitable legume production.

Fungal diseases are very widespread in crop plants and their control has been sought through the use of fungicides. The use and the misuse of 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 occurrence and spread of microbial resistance due to the misuse of these fungicides, have consequentially led to the promotion of new and potent antimicrobials of natural and synthetic origin (Nascimento *et al.*, 2000).

One of the most extensively studied natural defense mechanisms against fungi pathogens in plants is inducible defenses, whereby plants produce toxic chemicals, pathogen-degrading enzymes and deliberate cell suicide in order to restrict, slow down or overcome an invading pathogen (Dangl and Jones, 2001). The understanding of defense mechanisms has led to the development of many highly resistant cultivars against fungi pathogens. Most research works on inducible resistance mechanisms have shown that, plants activate these defenses after infection to stop pathogen development (Dixon and Harrison, 1990).

The induced synthesis and accumulation of secondary metabolites such as phytoalexins is one of the most extensively studied chemical plant defense responses to pathogen infections (Hammerschmidt, 1999). Phytoalexins are a diverse group of natural 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). Phytoalexins accumulate at the site of infection of pathogen and are believed to restrict ingress of invading pathogen (s). This study provides evidence for the validity of this assertion by delineating the probable utility of detected phytoalexins as a chemical marker of resistance among some notable legume crops in Ghana. Specifically, the study sought to firstly ascertain the crop species' reaction to fungi challenge-inoculation, determine the antifungal activity of legume cotyledon extracts and finally detect the presence of UV absorbing compounds in the cotyledon extracts.

MATERIALS AND METHODS

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, Kwame Nkrumah University of Science and Technology (Knust), Kumasi, Ghana.

Source of experimental materials and their characteristics: Three genotypes of cowpea, soybean and groundnut were used in the study. All genotypes used in the present study were obtained from the Council for Scientific and Industrial Research-Crop Research Institute (CSIR-CRI), Fumesua, Kumasi, Ghana, except Jenguma (a soybean genotype), which was obtained from a farmer at Navrongo in the Upper East Region of Ghana.

Cowpea genotypes: Asontem, Bengpla and Nhyira were the cowpea genotypes used for the cowpea phytoalexin studies.

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.

Bengpla is an early maturing (65 days) variety. It is resistant to pod shattering and lodging. It has medium sized seeds with smooth white shiny testa and black hilum. Asontem and Bengpla are resistant to major diseases of cowpea caused by a wide array of pathogens. These include anthracnose, web blight, brown blotch, Cercospora leaf spots, Septoria leaf spot, scab, bacterial blight etc.

Nhyira is known to be early maturing (65-68 days), high yielding (2.3 ton ha^{-1}), 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.

Soybean genotypes: Varieties of soybean namely Anidaso (TGX 813-6D), Jenguma (TGX 1830) and CRI-Nangbaare (TGX 1830-20E) were used for phytoalexin response study in soybean. 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 (Jackson, 2006).

Groundnut genotypes: 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 (Jackson, 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 (Jackson, 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 (Jackson, 2006).

Culture media preparation: The growth medium used was Potato Dextrose Agar (PDA) and was prepared by resuspending 39 g of PDA in 1 L of sterile distilled water. A broad-spectrum antibiotic (chloramphenicol, 250 mg L^{-1}), was added to the suspension to suppress the growth of bacteria. The volumetric flask containing the mixture was corked with a non-absorbent cotton wool and sterilisation was done by autoclaving at 121°C at 15 psi for 20 min. The sterilised PDA nutrient medium was then dispensed into sterilised glass Petri dishes with a diameter of 8 cm at aliquots of 50 mL, under the laminar flow hood and allowed to solidify before used.

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.

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.

Preparation of the cowpea, groundnut and soybean 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 separate 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 and 10 for groundnut were placed in each Petri dish separately. This was replicated five times for each genotype.

Preparation of *Fusarium* sp., *Colletotrichum* 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 1×10^5 spores/mL as described by Hargreaves *et al.* (1977). The host-pathogen combinations used were cowpea-*Fusarium* sp., soybean-*Colletotrichum* sp. and groundnut-*Aspergillus niger*. One set of adaxial cotyledon surfaces of the legumes (cowpea, soybean and groundnut) were inoculated with 30 μL drops (1×10^5 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.

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°C to half the initial volume.

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, 50 µL 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 percent inhibition was calculated based on the radial growth on the sixth day because maximum growth was observed in the control.

Analysis of cotyledon extracts by thin layer chromatography: A pipetted volume of 20 µL of the concentrated cotyledon extracts of each legume genotype was spotted on a pre-coated 10 cm origin Thin Layer Chromatography (TLC) plate (Merck Kieselgel 60 F254 Silica gel). The concentrated cotyledon extracts were derived from both sterile-distilled-water and fungus-inoculated cotyledons. 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 (25:1 v/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.

Parameters determined: Radial growth (cm) of *Curvularia lunata*. Percent inhibition of *C. lunata* and Rate of flow (Rf) values of UV absorbing compounds.

Statistical analysis: The Genstat (2007) was used for the statistical analysis. The percent 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 of differences between treatments was determined, using the least significant difference (lsd) at 5% probability.

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

RESULTS

Crop species reactions to fungi: The three genotypes each of cowpea, soybean and groundnut reacted similarly to the fungus and sterile-distilled-water inoculation. Fungus-inoculated adaxial surfaces of cotyledons of cowpea, soybean and groundnut resulted in the formation of lesions and browning of tissues (Table 1). The colour reaction ranged from brown in cowpea and groundnut to dark brown in the soybean genotypes (Fig. 1). Lesions formed were confined to the inoculum

Asontem Bengpla Nhyira

Adnidaso Jenguma Nangbaare

Adepa Azivivi Nkosour

Fig. 1(a-c): Symptoms on sterile distilled water inoculated (Control) and fungus inoculated (Elicited). cotyledons of legumes. (a) Cowpea genotypes (b) Soybean genotypes and (c) Groundnut genotypes Left: Control, Right: Elicited

Table 1: Response of adaxial cotyledon surfaces to challenge inoculation

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

+: Present, -: Absent

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. The adaxial cotyledon surfaces of soybean. 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 (Fig. 1b) and some cowpea cotyledons showed signs of rot, especially, adaxial cotyledons of Bengpla inoculated with sterile-distilled water (Fig. 1a).

Antifungal activity of the selected legume cotyledon extracts

Cowpea: The percent inhibitions of *C. lunata* were significantly different ($p<0.05$) among the cowpea cotyledon extracts (Fig. 2). Control cotyledon extracts (obtained from sterile-distilled-water-inoculated adaxial cotyledon surfaces) had significantly ($p<0.05$) lower percent inhibitions than the elicited cotyledon extracts (obtained from *Fusarium* sp., inoculated adaxial cotyledon surfaces) except Nhyira control and Nhyira elicited (Fig. 2). Among the three genotypes of cowpea used in the study, Bengpla had the highest percent 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 percent inhibition for both the control (22.0%) and the elicited (28.2%). However, there was no significant difference between per cent inhibition of Asontem elicited (26.5%) and Nhyira Control (27.0%).

Soybean: There were significant differences ($p<0.05$) between the soybean cotyledon extracts in relation to the inhibition of *C. lunata* (Fig. 3). 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 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. 3).

Groundnut: The mean percent inhibition of *C. lunata* was significantly different ($p<0.05$) from each of the groundnut cotyledon extracts. The elicited cotyledon extracts (adaxial cotyledon surfaces inoculated with *Aspergillus niger*) had significantly ($p<0.05$) higher percent inhibitions than control cotyledon extracts (adaxial cotyledon surfaces inoculated with sterile distilled water) in

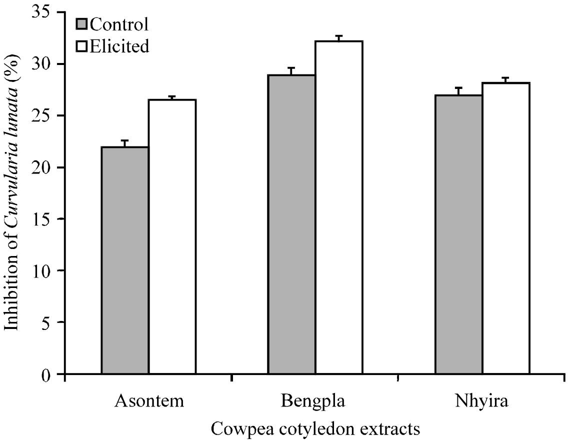

Fig. 2: Effect of cowpea cotyledon extracts on the growth of *Curvularia lunata*

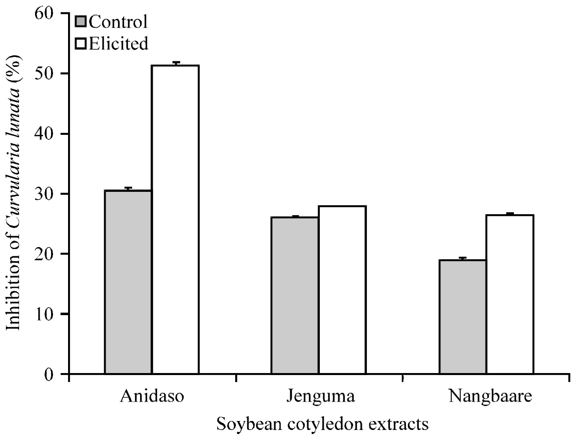

Fig. 3: Effect of soybean cotyledon extracts on the growth of *C. lunata*

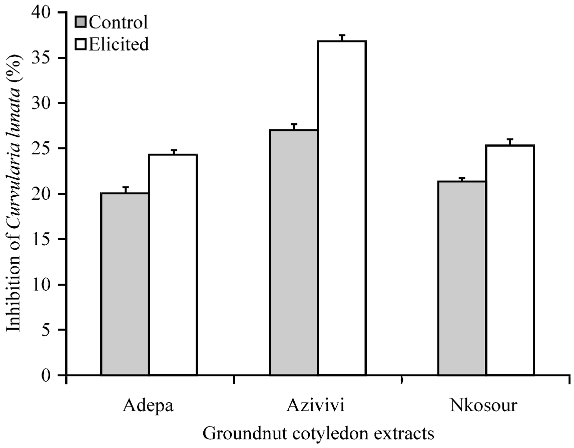

Fig. 4: Effect of groundnut cotyledon extracts on the growth of *C. lunata*

Fig. 4. Azivivi had the highest percent inhibition for both the control (27.0%) and the elicited (36.8%) among the three groundnut varieties. Although, Adepa control recorded the lowest percent

inhibition, there were no significant difference between Adepa control (20.0%) and Nkosuor control (21.3%). There was no significant difference between the per cent inhibition of Adepa elicited (24.3%) and Nkosuor elicited (25.4) (Fig. 4).

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

Cowpea: Five different compounds were recovered from the cowpea cotyledon extracts (Table 2). 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.

Soybean: 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 *Colletotrichum*-challenged cotyledons (elicited) of the three varieties of soybean used. Compound S-III (R_f 0.40) occurred in elicited Andaso and Nangbaare. 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).

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

	Rate of flow (R_f) of UV absorbing compounds				
	Compounds				
Cowpea cotyledon extracts	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

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

	Rate of flow (R_f) of UV absorbing compounds					
	Compounds					
Soybean cotyledon extracts	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

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

	Rate flow (R_f) of UV absorbing compounds				
	Compounds				
Groundnut cotyledon extracts	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

Table 5: Correlation between the number of UV absorbing compounds and the percent inhibition of *C. lunata*

Crop species	Correlation coefficient (r)
Cowpea	0.7
Soybean	0.7
Groundnut	0.9

Groundnut: Five different compounds were produced by groundnut cotyledons after challenge with *Aspergillus niger* (Table 4). 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_f0.14) also occurred in Nkosuor for control and elicited extracts (Table 4). Compound G-III (R_f 0.20) occurred 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).

Genotype response to accumulation of antimicrobial compounds: The number of UV absorbing compounds in the cotyledon extracts of each crop species was positively correlated (r = 0.7, 0.7 and 0.9) with the per cent inhibition of *C. lunata* of the same cotyledon extracts (Table 5) for cowpea, soybean and groundnut, respectively.

DISCUSSION

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 confirms the findings of 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 agrees with 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 the findings by Boue *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).

Lesions produced in the cowpea cotyledons inoculated with sterile distilled water may be attributed, partly, to the small nature of the seeds. Compared to the seeds of soybean and groundnut, the cowpea seeds had limited reserves due to their small size and were thus, predisposed to physical injury. Such mechanical injuries could cause the lesions observed in those cotyledons, as reported by Soylu *et al.* (2002).

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 percent 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). This 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 percent 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, 2002).

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 implies that, phytoalexins are produced after infection by a pathogen and are virtually absent in healthy host tissues (Mansfield, 2000). 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 exists 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.

Genotype response to accumulation of antimicrobial compounds: The strong positive correlation between number of UV absorbing compounds and percent 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 percent 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 and groundnut 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.

CONCLUSION

It can be concluded from the present study that, adaxial cotyledon surfaces reacted to fungal elicitation by producing lesions. Extracts obtained from fungus-inoculated cotyledons (elicited) had significantly higher percent 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. Based on the percent inhibition of *C. lunata* and the number of UV absorbing compound(s) produced by the cotyledon extracts, Bengpla (cowpea), Anidaso (soybean) and Azivivi (groundnut) showed the best response to induced accumulation of phytoalexins.

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