Exploring the Changes of Resistant Genes Expression in Groundnuts (*Arachis Hypogea*) In Response to *Aspergillus Flavus* Exposure at Seedling Stage

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Abstract: *Aspergillus flavus* **infect groundnut seeds and produce secondary metabolites, aflatoxins. The aflatoxins are associated with various diseases in domestic animals and humans globally. Mitigating the aflatoxin contamination in crops through the development of cultivars tolerant to fungus colonization and aflatoxin contamination has been considered the most cost-effective measure. This research was conducted to ascertain that the resistance genes identified in the previous transcriptome analysis were involved in groundnut defense mechanisms to *A. flavus* infection. Eight genes were selected for additional scrutiny through the real time PCR on a groundnut seedling at an interval of 2 days within a 7-day period. The results indicate a network of gene expression patterns in a sequential order in both resistance and susceptible lines at a seedling stage. The peak expression level per gene indicates the time gene action was crucial. We conclude that these genes are involved in groundnut resistance to *A. flavus* infection and provide important targets for the molecular marker screening.**

Key words: *Aspergillus flavus*, **groundnuts, Real Time PCR, gene expression, aflatoxin, seedling.**

I. INTRODUCTION

*A*spergillus flavus and *Aspergillus parasiticus* are known to synthesize large quantity of aflatoxin that compromise the quality of wide range of agricultural produce. This has been regarded as a major drawback in attaining food security and a major concern to human and animal health (Andrade and Caldas, 2015). Aflatoxin B1 on groundnut (*Arachis hypogaea* L.) has been documented as a causative agent of liver cancer (Nayak *et al*., 2017).

Maize (*Zea mays* L.) and groundnut are the most important source for human exposure to aflatoxin (Nayak *et al*., 2017). In groundnuts, the infection occurs at the farm, during harvesting, drying, storage and transportation (Waliyar *et al*., 2015). Globally, numerous initiatives have been undertaken to mitigate this problem. The development of resistant genotypes had been deemed a cost-effective and practical approach (Holbrook *et al*., 2010).

The progress in germ plasm improvement in groundnuts have progressed slowly in comparison to crops such as maize because of dearth of resistant checks, linkage drags, paucity of information on plant microbe interaction and grater genotype by environmental interaction (Burow *et al*., 2008). Globally, limited breeding programs in groundnuts have realized varieties capable of resisting *A. flavus* colonization and aflatoxin contamination (Holbrook *et al*., 2009; Nigam *et al*., 2009).

Mixon and Rogers, (1973) conducted the initial screening of groundnuts against *A. flavus* infection. They characterized various resistant proteins in two groundnut lines upon *A. flavus* infection. The initial investigations focused more on the synthesis of novel proteins in the tolerant genotypes. Proteomics study by Szerszen and Pettit, (1990) in a tolerant versus susceptible groundnuts genotype discovered four important pathogenesis-related proteins 24 hours upon fungus infection. Four unique proteins were also identified in proteomic analysis of runner type groundnuts against Spanish bunch groundnuts under artificial inoculation with the *A. flavus* by Liang *et al*. (2006).

Currently, next generation sequencing, gene chip and comparative proteomics are applied in investigating the resistance level in plants. With these technologies, novel genes markers and additional proteins essential in plant breeding have been discovered (Guo *et al*., 2008; Chen *et al*., 2009). Transcriptomics analysis have been done for late leaf spot (Luo *et al*., 2007), *A. flavus* and bacterial wilt in groundnuts (Nayak *et al*., 2017).

Previously, we conducted gene expression profiling using peanut microarray. We discovered genes with *A. flavus,* and aflatoxin resistant properties based on their homology compared to the annotated entries and database searches. In order to provide addition information necessary for cultivar development, the expression analysis of the 8 resistant genes

was conducted during fungal infection at a seedling stage. The main aim was to examine the changes and sequence of resistant gene activation in a seven-day period upon *A. flavus* infection.

II. MATERIALS AND METHODS

2.1 Plant Genotypes and Fungal Isolate

The resistant GBK005111 and susceptible GBK036397 genotypes of groundnuts were used as a contrasting genotype. Toxigenic *A. flavus* strain, *A. flavus_JOOUST5* isolated and characterized in our previous study was used to inoculate these genotypes. It was sub-cultured on Potato Dextrose Agar until sporulation. The spores were washed over the surface of growth media with distilled water and a suspension for inoculation was prepared.

2.2 Seedling preparations

From each genotype line, 100 blameless seeds were picked and sterilized through immersion in 0.75% sodium hypochlorite for three minutes and rinsed 3 times in distilled for 5 minutes each. The seeds were then hydrated up to 20% water content by soaking in distilled water for 30 minutes. The hydrated seeds were then plated in petri-dishes lined with 6 sheets of sterile tissue papers moistened with sterile water. The plated seeds were incubated at $27\ ^0$C for 3 days in high relative humidity chambers for germination. Fifty seedlings of each genotype were immersed in sterile distilled water and were considered controls. The second sets of 50 seedlings were immersed in a spore suspension and were considered infected samples. Both the sets were transferred back into their respective petri-dishes and incubated for additional 7 days.

2.3. RNA isolation

The RNA isolation was done according to the protocol developed by Yin *et al*., (2011). Disinfected scalpel was used to cut and collect the root and shoot tissues in Eppendorf tube on the 1, 3, 5- and 7-days post-inoculation. The samples were instantly frozen in liquid nitrogen and kept at -80^0C freezer. After collecting the samples from all the replicates, the sample per genotype, treatment and time points were pooled together and ground in precooled mortar and pestle. One hundred

milligram of the ground tissues was weighed and put in 10 ml tube and cells lysed with 5ml of the extraction buffer. The contents were vortexed fully to mix the contents and incubated at 65^0C for 40 minutes with gentle whirling in a water bath and allowed to cool on the bench. 2ml of chloroform was added, vortexed and incubated in ice for 5 minutes before centrifuging at 8,000 rpm for 30 minutes. The clear supernatant was gently moved to the new RNase free tube and 1.5ml of LiCl added mixed by inversion, incubated at -80 ^{0}C for 1.5 hours. The solution was then centrifuged at 8,000 rpm for 20 minutes in a refrigerated centrifuge to pallet the RNA. The pellets were washed with 0.75 ethanol solution and centrifuged at 8,000 rpm for 20 minutes. The RNA pellets were allowed to dry on the bench and dissolved in 100 µL RNase free water.

2.4. Reverse transcriptase PCR

The reverse transcription was done in 20 µl Nucleases free microcentrifuge tube containing I µl of primer pairs, 5 µl of isolated RNA, 1 µl of each dNTP mix at a neutral pH and 10 µl of distilled water. The mix was then incubated at in a water bath for 60 ^{0}C for 6 minutes and then transferred to ice for 5 minutes. Centrifugation was then done briefly to collect the mix at the bottom and 4ul of the strand buffer, 1 µl of dithiothreitol, 1ul of RNase inhibitor and superscript were added and pipetting up and down to mix them. The incubation was then done for 45 minutes at 55 ^{0}C and the reaction inactivated by warming the contents in a water bath at 70 ^{0}C for 15 minutes.

The RT-PCR was done according to the parameters of Rodrigues *et al*. (2009): 1 initial cycle for denaturation was set for 4 minutes at 94 ^{0}C. The subsequent 30 denaturation's cycles were done for 1 minutes at 94 ^{0}C, annealing was done for 1min at 55 – 60 ^{0}C for 30 cycles each, the extension on each cycle was done at 72 ^{0}C for 1 minutes and the final extension at 72 ^{0}C for 6 min.

Primers were designed using the Primer Express 3.0 plus tool and presented in the Table 1. The expression analysis of the 8 resistant genes was conducted. The actin gene from groundnut kernels was used to quantify the gene expression using the ▩▩▩ method (Claire *et al*. 2004).

Table 1: Outline of 10 genes and primer sets used in RT_PCR.

Accession No.	Gene Analysed	Primer set (5' 3')	Fragment length (bp)
P83595	Trypsin inhibitor	F: CGAGATACAGGGTTGGTTTGAG R: CCAAAAGCCTATTTCCCTCATC	128
AAF602701	Lipoxygenase (LOX)	F: TGGTGAAGAGTCACCAAAAGG R: TCCAATGTGGATTATCCCTCTC	141
gi|110810624	Late embryogenesis abundant protein B19.3 (LEA B19.3)	F: TAGTTCGGGTTGTAGTAGCAGGGT R: AAGGTTCCATCTTCTCGCCGATGT	99
gi|146771807	Glycinin	F: TATGATGATGACGATCGACGACCACG R: TGCATAGTGTTTCCTCCACTCCGT	82
NP_188086	Cytochrome P450	F: TGGGCTTACTCGAAATACCG R: GCATTATCACCCCAAAGTCC	123
P11670	Pathogenesis-related protein 1 (MSPR10-1)	F: AACACTCCATGGGGCCTTAC R: TGTAATGCAGGCACTCATCG	147

NC_037620.1	Endochitinase	F: TGCCAATACACTGCTGGAGG R: GTAAGGGCAGTTGCAGGGAT	146
NC_037622.1	12-oxophytodienoate reductase 2	F: CAGTCCCATGCTGCCTTGTA R: AGTTGTGGAAGGGTGTGTACC	112
NC_037620.1	Disease resistance response protein 206_PUTATIVE	F: GTAAAACCCTTGCGCTCGTC R: CGTCTTTGACGACCCCATCA	73
NC_037635.1	Defensin like proteins	F: CACGTTGCTGCCTCACTTTG R: ATTGCTGGCCCCTAGCATTC	96
DQ8273525.1	Peanut Actin gene	F: GTTCCACTATGTTCCCAGGCA R: CTTCCTCTCTGGTGGTGCTACA	85

III. RESULTS

3.1. Expression levels as detected by RT-PCR

The expression analysis as obtained by RT-PCR in this study were in harmony with the preceding transcriptomic study. All the 8 genes investigated were upregulated in at least two time points (Fig 1) and is consistence with their upregulation in the transcriptomic study (Table 2). The RT-PCR discovered varied trends in gene activation upon fungal infection in groundnuts seedling stage in a seven-day period.

Table 2: A comparison of the peak gene activation of defense related genes within 7 days at a seedling stage with microarray output conducted on mature groundnut crops previously upon fungal infection.

Gene Name	RT-PCR Peak expression level	$\log_2 \geq 1.5$ (Microarray) (From the microarray study)
Lipoxygenase	4.14	3.1
Cytochrome P540	2.32	3.1
Endochitinase	5.23	3.5
Trypsin inhibitors	2.44	2.3
Pathogenic related proteins	7.38	4.2
Defensin like proteins	5.98	3.6
Disease resistance response proteins	8.22	2.5
Glycinin	1.23	2.8

3.2. Gene expression patterns

The results reveal a varied pattern of gene expression at a seedling stage after *A. flavus* inoculation (Fig 2). The peak values shown in the table 2 identify the highest expression levels per gene. All genes had positive peaks that occurred at different time points, (Fig1). The expression levels of genes varied from the time of inoculation to day 7 with some registering down regulation at some points. The level of expression correlates with the requirement of the gene action with the highest peak indicating the time in which the gene is most critical in the defense against the *A. flavus* infection.
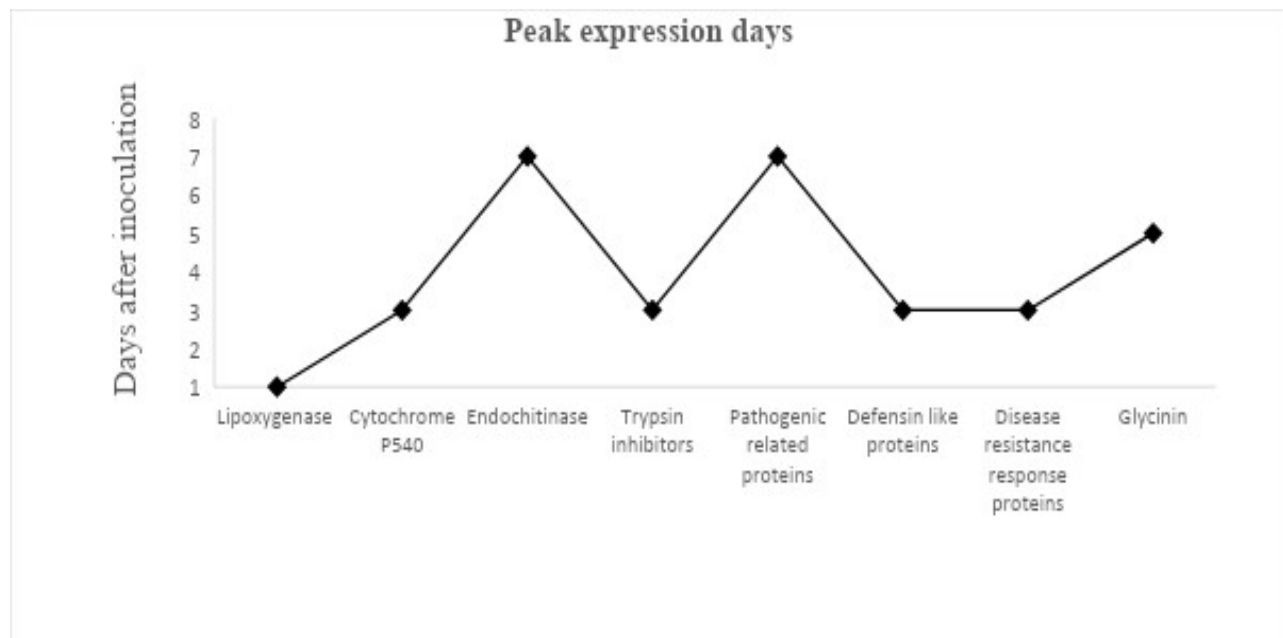

Figure 1: Gene expression peak times of the selected resistant genes in two contrasting groundnut genotypes (resistance and susceptible) challenged by *A, flavus* in a seven-day time period.
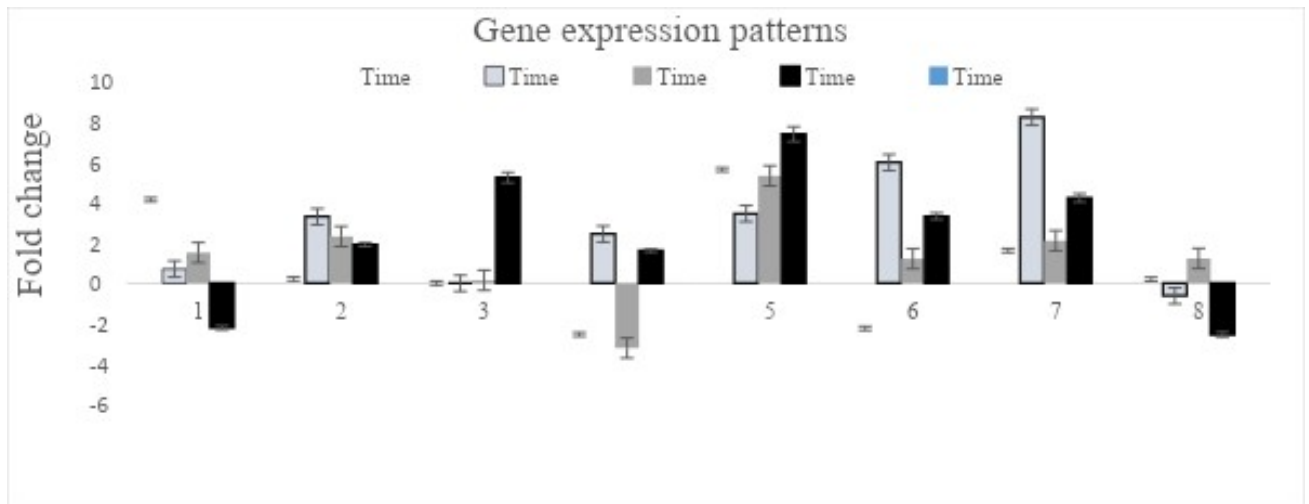
Figure 2: Gene expression patterns using RT-PCR in groundnuts at a seedling stage of resistant\susceptible genotype in response to *A, flavus* colonization in a seven-day time period. The pathogenic related proteins and disease response proteins had higher overall fold change while the glycinin had the lowest fold change.

Lipoxygenase (LOX) gene had its peak expression on day one after inoculation, expression levels plummeted at day three, then short up slightly at day five, and finally repressed at day seven. This suggests the involvement of LOX in the first line of defense during pathogenesis.

Cytochrome P540, Trypsin inhibitors, Defensin like proteins and Disease resistance response proteins had their peak expression levels on day three after inoculation. Even though they had their peak expression on day three, they never had a pattern of expression. A common feature of these four genes is that they had the lowest expression level on day one. However, in Cytochrome P540, the expression levels fell steadily from day three to day seven while Trypsin inhibitors, Defensing like proteins and Disease resistance response proteins had their expression falls to the lowest at day five but increases in day seven.

Unlike the other genes described earlier, Glycinin had its expression peak at day five after inoculation and had almost insignificant expression for the rest of the time points (Fig 2).

The slowest genes to show peak expression were Endochitinase and Pathogenic related proteins at day seven. The activity of the Endochitinase was silent during the early stages of pathogenesis up to day five. However, its expression short up to the highest up to fivefold at day seven after inoculation. Pathogenic related proteins had a slightly higher expression levels at day one, gradually fall to day three, and short up gradually up to the highest level at day seven (Fig 2).

IV. DISCUSSION

The study reveals that the resistance genes under investigation were up regulated in most of the time points. The maximum peak and deep expression levels of these genes occurred at different days. This is in agreement with

phenomenon that genes are activated at different times upon perceiving or experience an attack (Liang *et al*., 2009). Our findings indicate that resistant genes under investigation acted in programmed manner in their resistance activity. The biological response to *A. flavus* infection in groundnuts is intricate. The genetic composition and the biochemical synthesis of the groundnut accessions facilitate the counter attack measure to invading fungi. This give rise to sequentially gene activation in both genotypes leading to a network of gene expression.

The Lipoxygenase (LOX) genes had its peak expression at day one. This indicates that the gene is critical in the first line of defense as an activator of signaling network. This agrees with the studies conducted by Liang *et al*., (2009), in which they discovered a rapid accumulation of LOX and elevated levels of membrane lipid peroxidation within 1-2 days upon fungal inoculation. LOX is non-heme-iron comprising of fatty acid dioxygenases that take part in lipid metabolisms. They are critical genes in oxidative degradation of lipids within the cells to confer resistance upon fungal attack. The lipid metabolites are believed to be essential soluble signal in plant counter attack to the invading fungal attack (Christensen and Kolomiets, 2011). LOX enzymatically converts polyunsaturated fatty acids into hydroperoxides. The hydroperoxides are eventually turned to oxylipins. The oxylipins takes part in hypersensitive response through the oxidative damage to the cell membranes (Christensen and Kolomiets, 2011). Oxylipins can also be processed into the traumatin, jasmonic acid and methyl jasmonates which are involved in the regulation of various physiological functions of the plants (Hwang and Hwang, 2010) The oxylipins produced through the catalytic action of 9-LOX and 13-LOX isoforms of LOX could mimic or interfere with the fungal oxylipins leading to cross kingdom communication (Gao *et al*., 2009). The study of LOX gene in *Capsicum annuum* by Hwang and Hwang, (2010), strengthened cross talk hypothesis when they revealed that *Xanthomonas campestris* thrived more on paper plants in which the LOX gene was knocked down.

Four genes, Cytochrome P540, Trypsin inhibitors, Defensin like proteins and Disease resistance response proteins genes had their peak expression on the third day after inoculation. These genes synthesize resistance molecules through oxidization of pre-cursers in response to fungal invasion. Cytochrome P540 comprise of largest and metabolically diverse protein families that take part in phytohormone homeostasis (Mizutani and Ohta, 2010). Cytochrome P450 regulates the production of Jasmonic acid (JA) in the cells for the plant defense (Park et al., 2002). Jasmonic acid offer plant protection through the suppression of the reproduction, development and production of secondary metabolites of plant pathogens (Mosblech et al., 2009). Cytochrome P450s are also involved in the Jasmonoyl-L-isoleucine (JA-IIe) turnover which activates the plant immune system (Koo et al., 2011).

Trypsin inhibitors, Defensin like proteins and Disease resistance response proteins genes function in various amino acids synthesis (Van Loon et al., 2006). Trypsin inhibitors function as protein inhibitors and consist of amino acid with varying sequences and sizes. They have been found to attenuate the growth of A. flavus and inhibit the activity of alpha-amylase (Chen et al., 2007; Mellon et al., 2007). Plant defensins like proteins are basic antimicrobial peptides consisting of 45 to 54 amino acid with eight conserved cysteine residues that form three to four coupling of the thiol groups (Halbach, 2002; Lay and Anderson, 2005). It is documented that defensins have constitutive expression pattern with high upregulation upon pathogen invasion and wounding (de Beer and Vivier, 2011). They offer plant immunity by forming a protective barriers to pathogen invasion (de Beer and Vivier, 2011). Our results were in synchrony to these findings in which upregulation was observed in this gene after fungal inoculation.

Disease Resistance Response protein is an inducible proteins that provides a non-host resistance (Choi et al., 2004). This type of resistance confers plant immunity against all races of a pathogen and is typically broad-spectrum and more durable. This gene was first discovered in pea, Pisum sativum, as a defense gene in which it was highly upregulated upon bacteria and fungal pathogen and elicitors exposure (Choi et al., 2004). Though the precise function of the defense gene remains elusive, previous reports indicates that it shares a significant amino acid sequence homology (77%) with a proteins that regulates the production of lignin and lignan (Choi et al., 2004; Wang et al., 1999). Lignan has antimicrobial properties while lignin is a structural constituent of cell walls that confers mechanical protection from penetration by fungal appressoria and cell wall degrading enzymes. Its peak expression at the third day may indicate that it is required to reinforce the cell wall to hinder the penetration of the developing fungus.

Glycinin is an 11S globulin that accumulates as the major storage protein in embryos or cotyledons of most dicotyledonous plants (Shewry, 1995). The hexameric protein has a mass of approximately 350 kDa and is composed of five subunits $A_{1a}B_{1b}$, $A_{1b}B_2$, A_1B_{1a}, A_3B_4 and $A_{5A}4_{B3}$ (Nielsen et al., 1989). A single disulfide bond links the acid and basic polypeptide chain that forms the subunits (Nielsen et al., 1989). According to Dhatwalia et al., (2009) the basic peptides have been showed to reduce the mycelia growth and spore germination of A. niger and Penicillium species. It activation on the fifth day may be due to response to the developing mycelium.

The last group of genes to have their peak upregulation were pathogenic related (PR) proteins and its related protein genes, endochitinase. These genes are involved in the cell wall mediated resistant factors (Glazebrook, 2005; Park et al., 2004). The PR compounds coagulates on the cell wall of plant species during the pathogen invasion to hinder the penetration (Dixon and Harrison, 1990). These genes function in the cell wall strengthening through saccharides biosynthesis (Durrant and Dong, 2004).

This research gives essential information on the pattern of gene activation in groundnut upon fungal attack. The host provides mechanisms of resistance that involves several genes activation in a sequential manner. The order of gene expression determines the level of resistance and is critical in exclusion of fungal colonization. The results obtained in this study were congruent to the microarray study and it establishes that the screening at seedling stage could provide quicker and reliable results. The study also validates the involvement of these genes in resistance of groundnuts to A. flavus. These genes therefore provide important probes for molecular screening and laid groundwork for the functional genetics' investigations in groundnuts.

Data Availability

Most of the data used to support the findings of this study are included in the article. Additional data are available from the corresponding author upon request.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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