Employing Peanut Seed Coat Cell Wall Mediated Resistance Against *Aspergillus flavus* Infection and Aflatoxin Contamination

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**Abstract:** Aflatoxins, which have been classified as a group 1 carcinogen, are the most well-known mycotoxins produced by *Aspergillus flavus*. Aflatoxins have been linked to liver diseases, acute hepatic necrosis, resulting in cirrhosis or hepatocellular carcinomas and loss of value in international trade for peanuts contaminated with it. The four main aflatoxins are B₁, B₂, G₁, and G₂, of which B₁ is the predominant one. The plant cell wall is the primary barrier against pathogen invasion. The host cell’s ability to rapidly repair and reinforce its cell walls results in a reduction of the penetration efficiency of the pathogen, which secretes cell wall degrading enzymes and inhibits host cell wall biosynthetic proteins. Cell wall fortifications such as deposition of callose, cellulose, lignin, phenolic compounds and structural proteins help to prevent pathogen infection. The peanut seed coat acts as a physical and biochemical barrier against both pre and post-harvest pathogen infection. The structure of the seed coat and the presence of polyphenolic compounds has been reported to inhibit the growth of *A. flavus*. Comprehensive research on peanut seed coat development and biochemistry will provide information to design efficient strategies for seed coat-mediated resistance to *A. flavus* infection and aflatoxin contamination.

**Keywords:** *A. flavus*; aflatoxin; peanut; seed coat; cell wall; disease resistance.
1. Introduction

There are significant real-world health, economic, agricultural and political issues that stem from the production of and contamination by of one single class of fungal natural product, aflatoxin. Since the discovery of aflatoxin approximately 50 years ago [3], entire scientific and government institutes have risen with the purpose of researching and reducing aflatoxin levels in our crops. With an apparent 25% contamination of all crops, entire countries have lost crop export markets due to the inability to maintain an adequate low aflatoxin presence [4]. In the US alone, there is an estimated $270 million loss annually due to aflatoxin contamination in food and feed crops [5]. A higher incidence of hepatocellular carcinomas is also tied into aflatoxin exposure in some countries [3]. In addition, aflatoxin reduces immune response and increases susceptibility to disease, especially HIV [6]. There is no doubt the damage and the potential damage that aflatoxin has on nations across the globe is enormous. Aflatoxin is therefore one of the most highly studied mycotoxins and its highly complex biosynthetic story is still not well understood.

1.1 Mycotoxins:

Generally, mycotoxins represent a class of low-molecular weight filamentous fungal secondary metabolites that have adverse effects on humans and animals [7, 8]. Mycotoxins are quite complex and difficult to characterize correctly. Fungal secondary metabolites that kill off bacteria are more accurately named antibiotics while those that focus on plants are termed phytochemicals. There is even a distinction of chemicals between micro- and macroscopic fungi, such as toxins in certain mushrooms that are no doubt deadly to humans but not characterized as mycotoxins. Mycotoxins are more widespread in areas that favor mold growth with hot and humid climates and are especially prevalent in developing countries [7, 9, 10]. Fungal growth and invasion can cause mycoses, common to severe diseases that often arise through opportunistic fungi. However, mycotoxins are most specifically correlated with mycotoxicoses, diseases related to exposure via dietary, respiratory or dermal contact to mycotoxins. Mycotoxicoses can arise through acute or chronic contact with the fungal toxic metabolites and, depending on the mycotoxin, can affect
various organs and tissues. Because of their biological importance, research has mainly focused on the classes of mycotoxins that have severe negative impacts on human and livestock health[11]. Of the roughly 300 mycotoxins, research primarily focused on aflatoxins, citrinin, ergot alkaloids, fumonisins, patulin, trichothecens, zearalenone, ochratoxin, T-2, and others and their derivatives. These various chemicals, mainly produced from a select few fungal species, are usually contaminants of feed (pre-harvest contamination) and fodder (post-harvest contamination). Predominantly they are produced from the genera, *Aspergillus*, *Alternaria*, *Claviceps*, *Fusarium*, *Penicillium*, and *Stachybotrys*. There is variation of toxicity and mycotoxin production across species of the same genera, especially so in the case of aflatoxin and *Aspergillus*.

Mycotoxins have far-reaching effects across various aspects of our society. Aflatoxin is arguably the most well-known mycotoxin and this is not without cause. Aflatoxins are considered a Group 1 carcinogen according to the International Agency for Research on Cancer[7]. Some strains of *Aspergillus flavus* can produce up to $10^6$ µg/kg of aflatoxin; this is not only impressive but a very real danger considering the U.S. Food and Drug Administration requires minimum aflatoxin concentration levels to be 0.5 µg/kg for milk products, 20 µg/kg for all other human food products, and even 300 µg/kg for cattle meal[7, 10]. Developed countries such as the United States devote a large amount of resources into the screening, management and quarantine of crops contaminated by aflatoxins. Paired with proper cultivation/storage techniques and procedures from farmers, the potential of human and livestock health being negatively affected is minimal. The higher realistic priority is the potential loss of millions of dollars of crop goods due to contamination, and the cost of screening to keep contaminated materials out of the food chain [12-14]. However, the scenario changes in developing countries, especially those that meet the ideal climate conditions for fungal growth, such as many African and South East Asian countries. Without the proper resources to be able to stop contamination, with ideal growth conditions and often substandard storage conditions, aflatoxin contamination remains a large problem in the health community. A higher occurrence of liver diseases in developing countries is difficult to blame
solely on aflatoxin contamination, but various reports do link higher hepatic carcinomas to possible aflatoxin contamination in China and African and South East Asian countries[15-18]. The high incidence of contamination also leads to the inability for countries to secure high value international trade markets with crop exports. Entire countries and populations of people have been affected by this single natural product that does not even have a known ecological role for its host [5].

1.2 Brief history of peanut aflatoxin contamination

Aflatoxins represent a significant milestone in the discovery of not only the possible adverse side effects of fungal secondary metabolites, but of mycotoxins in general. There had been previous reports of livestock death and human illness from the consumption of molded fodder and food, but as no toxin was isolated, these reports were deemed insignificant. It wasn’t until the early 1960s in England that the term “mycotoxin” was coined. In the spring of 1960 in South Eastern England, not far from London, poultry farmers noticed a significant outbreak of an unknown disease in their turkeys. Mortality was observed to be 100 % in many cases, with a wide range of older and young healthy turkeys being affected. By the time August had come, approximately 100,000 turkeys and other domestic birds had died, ending the first observed outbreak of the newly named “Turkey X Disease” [9, 19]. It wasn’t until shortly after that they determined the link between Turkey X Disease and the outbreaks were from contaminated peanut meal shipped from South America that was used as feed [9, 19, 20]. Closer inspection revealed a large contamination from the mold *Aspergillus flavus* on the peanut meal. After extract isolation of the mold, they characterized aflatoxin (*Aspergillus flavus* toxin). A hot methanol extraction was used on the toxic meal. Next, partitioning of the suspension of the extract into chloroform was done. Finally, concentration of the chloroform residues was performed by distribution in methanol, water and petroleum ether[21]. The extract was further purified by paper chromatography and was found to omit a blue fluorescence under UV light, hence the naming of aflatoxin B (blue fluorescence) and aflatoxin G (green fluorescence). Not only was a first crude extraction protocol defined, but so too was a chemical assay to screen for aflatoxin [21]. Shortly after this time, the discovery
of other new and previously observed mycotoxins took place. Within 9 years of the discovery and naming of aflatoxin, the FDA had already set action levels for contamination in food, aflatoxin carcinogenesis was revealed in rats and the structure, toxicology and metabolism were elucidated[20]. The stage was set for the discovery, research, and characterization of these new mycotoxins, with aflatoxin being the world front runner.

### 1.3 Evolutionary background of Aspergillus

To gain a better understanding of the aflatoxin pathway, it is important to look back at the evolutionary timeline of *Aspergillus*, the aflatoxin producing genus of filamentous fungi. A commonly heard misconception and previous way of thinking by some is that fungi and plants are closely related. Though some may group fungi and plants together based on the presence of a cell wall, evolutionarily speaking, the *Fungi (Mycota)* kingdom most likely evolved from eukaryotic aquatic protists around 900 million years ago (*Figure 1*). Fungi also have cell walls derived from chitin, while plant cell walls are composed of cellulose, lignin, hemicellulose and other related polymers. It is estimated that there are up to 1.5 million different species of fungi in the world, most of which have not been discovered[22, 23]. This creates issues in determining the lineages of fungal species, as most fungi have not been cultured or collected. New molecular tools have been used to recently better clarify the early

![Figure 1: Depiction of the fungal phylum Ascomycota.](image-url) Ascomycota along with Basidiomycota make up the sub-kingdom Dikarya, one of many sub-kingdoms in Fungi. Circled in red is the subclass containing the *Aspergillus* genus.
lineages of fungi; there are still conflicting and changing reports on certain taxa of early protozoa being considered fungi and vice versa[24]. The genus *Aspergillus* falls into subkingdom of Dikarya, phylum Ascomycota, subphylum Pezizomycotina, class Eurotiomycetes, subclass Eurotiomycetidae, order Eurotiales and family Trichocomaceae[24].

Ascomycota make up the largest phylum of fungi (currently based on discovered species) [22]. They are typically called the “sac fungi” due in part to the shared characteristic of an ascus, a sac-like structure where spores are formed. Many fungi in this phylum, however, are asexual and do not reproduce via asci. The Ascomycota fungi are monophyletic, sharing a common ancestral species. *Aspergillus* is a filamentous fungus, as described earlier; however, other Ascomycetes can be single celled, such as yeasts; others can be both single celled and filamentous (dimorphic) [22]. The subphylum Pezizomycotina contains approximately 90% of the Ascomycota and is without surprise a main focus area of research. Fungi in this subphylum are the hyphal species which give the characteristic filamentous growth. *Aspergillus* is one of 39 genera in Trichocomaceae. These genera are usually common soil and plant saprobes with cosmopolitan distribution.

*Aspergillus* is a very intriguing genus by itself, with many aflatoxin producing species and species that produce mycotoxins other than just aflatoxin such as ochratoxin and patulin[25]. However, in terms of aflatoxin productions, *A. flavus, A. parasiticus, A. bombycis, A. ochraceoroseus, A. nominus, A. niger,* and *A. pseudotamari* are the most important species[7]. It should also be noted that *Penicillium puberulum* has been reported to produce aflatoxin on certain crops and on media [21]. This gives us possible insight into the evolutionary history of aflatoxin as both *Aspergillus* and *Penicillium* are genera in the Trichocomaceae family. This could mean that the gene cluster for aflatoxin production formed before the split into two genera. Alternatively, horizontal gene transfer could be one possibility of why multiple phylogenetically-unrelated species have aflatoxin production; or the loss and gain of aflatoxin production ability could have also happened repeatedly over
the evolution of the genus or even at the family level[25]. *Aspergillus* has had many different species sequenced and so far, the genome appears to be relatively stable with all genomes showing 8 chromosomes ranging in size between 28-40 Mb[26]. Studying certain strains of *Aspergillus* that do not produce aflatoxin but still produce sterigmatocystin, an important immediate precursor to aflatoxin with its own carcinogenic properties, gives us more insight into the evolutionary history of the aflatoxin producing genes. *Aspergillus nidulans* produces sterigmatocystin but not aflatoxins and, based on protein similarities between *A. nidulans* and other species, is estimated to have a gene cluster as old as 450 million years old[5]. Evidence suggests that the aflatoxin genes present and intergenic distances in *A. flavus* species have been conserved for approximately 25 million years. Other evidence suggests that gene duplications and deletions in gene clusters, such as aflatoxin, explain the phenomenon of gene similarity in section *A. flavus* just as well as horizontal gene transfer[1], especially when

![Figure 2: A phylogenetic tree of the class Eurotiomycetes containing the *Aspergillus* genera. The arrows depict examples of horizontal gene transfer of single and clusters of genes[1].](image-url)
considering that the prime location of the aflatoxin gene cluster near the telomeric regions is optimal for recombination, DNA inversions, translocations and deletions.

### 1.4 Types of aflatoxins:

There are over 20 reported aflatoxins, most of which are slight derivatives of the four main aflatoxins, Aflatoxin B₁, B₂, G₁, and G₂[7, 11]. However, aflatoxin B₁ is the predominantly studied and most recognized aflatoxin and is typically the aflatoxin understood to be referred to when it is not otherwise specified. This is due to aflatoxin B₁ being the most potent carcinogen of them all; it is ten times more toxic than potassium cyanide and is labeled a Group 1 Carcinogen by the International Agency for Research on Cancer[7, 27]. The distinction between B and G aflatoxins represents the color (blue or green) they fluoresce under UV light and their relative chromatogram with thin-layer chromatography[9]. In general, aflatoxins are difuranocoumarin derivatives produced via the polyketide pathway from hexanoate [7, 28]; more detail into their biosynthesis will be discussed in a later section. Aflatoxin B₁ has a cyclopentenone ring structure and a double bond in the terminal furan ring of a bisfuran moiety[29]. The toxicity and carcinogenic properties of aflatoxin B₁ (Figure 3) have their origins from the double bond of the terminal furan ring[7, 20, 28]. Cytochrome P450 enzymes convert aflatoxin B₁ into the 8,9-epoxide form, which then intercalates with DNA and proteins, preventing DNA replication or transcription and induces mutagenic activity[28]. Aflatoxin B₂ is similar to B₁; however, B₂ is missing the double bond in the terminal furan ring, being a dihydro derivative. It is significantly less toxic and is less mutagenic but still fluoresces blue under UV light.

**Figure 3** Structures of different Aflatoxins produced by *A. flavus.*
Aflatoxin G₁ is similar to B₁ but, as stated earlier, fluoresces green/yellow under UV. Aflatoxin G₁ still possesses the unsaturated double bond at the 8,9 position of the terminal furan ring, however, the G series contain a fused lactone ring instead of the fused cyclopentenone ring to the lactone ring of the coumarin moiety as seen in the B series. Aflatoxin G₂, like B₂, lacks the unsaturated double bond in the terminal furan ring. G₁ possesses similar although lesser toxicity and carcinogenic activity compared to B₁. *Aspergillus flavus* has only been recorded to produce aflatoxin B₁ and B₂ while *Aspergillus parasiticus* has been reported to produce aflatoxin B₁, B₂, G₁ and G₂[11]. There are many other aflatoxins besides the “main four”; two notable variants are aflatoxin M₁ and M₂, which are present in milk due to cows metabolically bio-transforming aflatoxin B₁ into a hydroxylated form[7]. The difference between M₁ and M₂ is again the presence of a double bond in the 8,9 position of the terminal furan ring. M₁ is said to be as acutely hepatotoxic as aflatoxin B₁ but not as carcinogenic[11].

1.5 Biological significance of aflatoxins

Aflatoxins are the most carcinogenic natural substances produced with severe acute and chronic symptoms[7]. The main organ targeted by aflatoxins is the liver, causing acute hepatic necrosis, resulting in cirrhosis or hepatocellular carcinomas[10]. Reportedly, as little as a microgram per day/Kg body weight of dose will induce a carcinogenic response in liver cells[20]. Although it is hard to correlate outbreaks of disease or associate negative symptoms to aflatoxin contamination, there have been a few documented cases where aflatoxin ingestion was deemed the most probable cause. An outbreak of hepatitis in India in 1974 was associated with aflatoxin-contaminated maize; there were 100 recorded deaths[7, 20]. Another case resulted in acute encephalopathy (brain dysfunction) in children in Malaysia from contaminated noodles[20]. Kenya saw an outbreak in both 2004 and 2005 after maize contamination lead to approximately 150 deaths[20]. It is estimated that anywhere from 4-28% (25,200-155,000) of worldwide new hepatocellular carcinoma cases each year are a result of aflatoxin contamination[20], although there is also evidence of effects on
extrahepatic tissue as well (encephalopathy, etc.). Epidemiological studies have also determined an association between aflatoxin contamination and incidence of HIV or viral load in Africa[30, 31]. Reports also show aflatoxins used by Iraqi scientists in the 1980s, incorporated into warheads as part of their bioweapons program along with anthrax and botulinum[20]. History suggests that both acute and chronic exposure to aflatoxin are both crippling, however, the dietary chronic exposure generally proves more lethal. These toxins prove to be just as problematic in animals as well, although susceptibility is dependent on a variety of factors (species, weight, age, etc) [11].

We have already discussed aflatoxin B$_1$’s mechanism of intercalating within DNA, causing mutagenesis and damage. Aflatoxin also inactivates the P53 tumor suppressor gene by mutation at codon 249, changing guanine to thymine[7]. This inactivation gives aflatoxin B1 its potential for inducing hepatocellular carcinomas. Evidence exists of the aflatoxin DNA adducts being responsible for non-hepatic cancers as well, particularly in lungs most likely from dust inhalation and aflatoxin exposure. Apart from binding to DNA, aflatoxin B1-epoxide also binds to endogenous glutathione, creating a soluble glutathione-toxin adduct that can be excreted[28]. This can be a reliable biomarker in screening for early aflatoxin contamination as can the screening for a mutation at codon 249 in P53.

1.6 Biosynthetic pathway of aflatoxins

The biosynthetic pathway of aflatoxin is a long and complicated, but heavily studied, pathway originating from acetate then into the polyketide pathway. The first step in the pathway is the production of norsolorinic acid by type II polyketide synthase (Figure 4). Norsolorinic acid is created from a poly-β-chain derived from a hexanoyl-CoA starter and seven malonyl-CoA extenders[28]. The six-carbon chain of norsolorinic acid is then oxygenated and cyclized in order to give the ketal, averufin. The steps after averufin are unclear but there is rearrangement and a Bayer-Villiger oxidation to give rise to versiconal acetate. Two carbons are lost through hydrolysis to produce versicolorin B, which through oxidation becomes versicolorin A. Versicolorin A eventually will give sterigmatocystin, an
important precursor and mycotoxin, through a series of chemical changes. From here, an aromatic ring of sterigmatocystin is cleaved through oxidative cleavage; an additional carbon is lost and then recycлизation occurs. An O-methylation step is required to produce O-methylsterigmatocystin, from which aflatoxin B₁ and G₁ are synthesized. To produce aflatoxins B₂ and G₂, a branching from versicolorin B occurs that follows the same original pathway but with dihydro-derivatives.

1.7 Genes Involved in AF Biosynthesis

The genes involved in the biosynthesis of aflatoxins were among the first fungal secondary metabolites genes shown to be exclusive to a gene cluster on a single chromosome, which we now know to be fairly common [5]. The entirety of aflatoxin genes are located in a 70-80kb gene cluster located on the telomeric region of chromosome 3 (Figure 4).

![Figure 4](image)

**Figure 4**: The 80kb aflatoxin gene cluster found in *A. flavus* with the direction of individual gene transcription [2].

The *aflR* gene has been found to be the transcriptional activator important for aflatoxin biosynthesis and important in the expression of *aflE, aflC, aflJ, aflM, aflK, aflQ, aflP, aflR* and *aflG*. *AflR* binds a sequence-specific DNA-binding binuclear zinc cluster protein[5]. Flaherty and Payne [32] were able to show that *aflR* mutants lack the ability to produce important aflatoxin biosynthetic genes and as a result can be termed “afla-safe”, and are used for research on *Aspergillus* infection, aflatoxin biosynthesis, or for maize or peanut are used for application of atoxigenic competitive strains of *A. flavus* that are applied to commercial fields, or in the case of *A. sojae*, used in food production such as the fermentation of soy sauce. Another important regulatory gene is *aflS* (previously *aflJ*). Located next to aflR, this gene possibly shares the same binding sites. Although the exact role of *aflS* is unclear, it has
been suggested that it maintains a regulatory role in aflatoxin biosynthesis by enhancing transcription of various important genes in the cluster[5]. Though aflatoxin biosynthetic genes have evolved over the time, an equal resistance has been put forth by plants by the process of cell wall fortification through deposition of inhibitory biomolecules in the seed coat layer or testa.

2. The evolved function of seed coat and its significance in land plants

Plants have evolved to form seed coats and an outer shell to protect the seeds during dispersal from pathogen infections, animal consumption, mechanical damages and survive harsh weather conditions. For example, coconut seeds are one among the largest seeds that are protected by a hard seed coat (endocarp) and shell (exocarp), which makes them able to survive for years and spread across salty ocean waters to colonize different continents. In case of cotton seeds, the outer seed coat layer produces fibers that help in seed dispersal through wind. Hence, the seed coat mediated protection and dispersal drove plant evolution to environmental adaptation and generation of new species. In addition, the seed coat plays important roles in seed germination, vigor, longevity and storage potential of seed[33]. The thickness, form, shape and color of seed coats vary based on the plant species which evolutionarily developed to perform various functions based on the environment in which they grow. Seeds which lack a thicker seed coat, as seen in biomass crops, are chemically treated to prevent seed infection by pathogens, but when it comes to seeds that are directly used for human consumption e.g. peanut seeds, chemical treatment is not favored. Deposition of seed coats with active biochemicals is a viable solution to protect the seed infection. In general, the seed coat mediated disease resistance, mechanical strength and successful dispersal is primarily determined by the accumulation of hydrophobic polymers such as lignin and tannins[34]. The lignification of the seed coat found in endocarps of dry fruits, such as drupes, occurs as a part of secondary cell wall formation and can comprise up to twice the percentage of lignin present in wood tissue [35]. Apart from polymers, endocarp tissue also accumulates flavanoids and by-products of the phenylpropanoid pathway that have antibacterial and antifungal properties[36]. Detailed information of seed coat
development in different land plant seeds and its genetic basis has been reviewed elsewhere[34]. *Arabidopsis* has been a choice for functional research in dicot seed coat development, and genes involved in the process have been characterized[37, 38]. In *Arabidopsis*, the size of the seed coat was increased by manipulating gene expression of *AUXIN RESPONSIVE FACTOR 2* in the integument region of developing ovules[39]. Therefore, in food crops such as peanuts, it is critical to understand the detailed seed coat developmental process and find the genes involved in seed coat development. This will allow manipulating the seed coat for altered composition or size to enhance the protection of seeds from mechanical, biotic and abiotic stress.

2.1 *Protective layers of peanut seed against pathogen invasion (outer shell and seed coat)*

Peanut is one of the widely grown crops in the world and its prone to seed borne diseases primarily caused by soil borne pathogens[40]. To restrict seed borne diseases, peanut seeds have a hard shell that harbors the cotyledons (Figure 5). The fully matured/dried cotyledons are surrounded by a thin seed coat, also known as peanut skin or testa.

![Figure 5](image)

*Figure 5*: Transverse and cross section of peanut pod showing outer shell, seed coat and cotyledon inside (left image). Developmental picture of peanut seed from early pod to mature pod stages (right image).

The peanut shell has multiple uses such as a biomass in thermal energy production, packing material, animal feed, and as nitrogen rich gardening material. Soil borne diseases can be prevented by either improving the peanut shell or seed coat. During peanut processing, the shells are removed by mechanical breaking of outer shell to release the seeds. Any
increase in peanut shell thickness or toughness to improve the disease resistance would affect the shelling ability of peanuts. Further, in commercial outlets and markets, peanuts are sold mainly after shelling as weighed bags which are prone to post-harvest seed infection by pathogenic fungi since *A. flavus* is one of the most commonly-occurring pathogens in the environment. Therefore, to improve the peanut post-harvest resistance to pathogens, the strength and composition of the peanut seed coat, which acts as primary layer of defense of shelled peanut seeds, can be improved. The peanut seed coat is composed of multiple cell wall layers, and peanut varieties differ in composition of flavonoids and tannins which ultimately give different colors to the peanut seed coat. Since cell wall is the primary layer of defense and a sub-focus of this review, the following sections focus on the overall role of cell wall in diseases resistance and seed coat development and its composition and its role in resistance to *A. flavus* infection in peanuts.

### 2.2 Role of plant cell walls in resistance against fungal infections

Plants are repeatedly challenged by multiple pathogens and to defend themselves, plants have developed various strategies, which aid in rapid recognition of pathogens and activation of biochemical and structural defenses[41, 42]. Aflatoxin contamination involves successful penetration and colonization of plant cell walls by *A. flavus*. Therefore, host pathogen warfare begins at the cell wall, which is a primary barrier against the infection of pathogens. Plant cell walls are mainly composed of cellulose, hemicellulose, pectin, lignin, phenolic compound polymers and structural proteins[35]. Successful infection by the fungus involves secretion of cell wall degrading enzymes and inhibition of host cell wall biosynthetic genes[43, 44]. In response to pathogen attack, if the host cell can rapidly repair and reinforce its cell walls, it will reduce the penetration efficiency of the pathogen. Studies have clearly demonstrated that to prevent successful infection, a genome-wide defense response is initiated in the host plant, including battery of cell wall biosynthetic genes[45-47]. The majority of defense responses may be genus- or species-specific, while cell wall defense responses are ubiquitous[41, 42]. Several cell wall fortifications such as deposition of callose, cellulose, lignin, phenolic compounds and structural proteins have been reported to
occur directly below the point of attempted penetration to prevent the pathogen infection. Callose (β, 1-3 glucose polymer) is a cell wall component that is quickly deposited in response to pathogen infection[48, 49]. Callose deposition is the first line of host defense response and is measured by the amount of callose deposition. Callose deposition was first described as papillae [50] which are composed of (1,3)-β-glucan polymer [51] and deposited at the site of fungal penetration. Several studies on a wide variety of host-pathogen interactions involving successful prevention of pathogen colonization suggest that callose acts as a physical barrier and slows down pathogen invasion, which gives the plant time to activate downstream defense responses[52, 53].

In addition to callose deposition, other compounds such as proteins, peroxidase enzymes, phenolic compounds and other unclassified compounds are deposited in the cell walls[54-56]. Hydroxyproline-rich glycoproteins are produced ahead of the hyphal invasion and reinforce the cell walls to halt pathogen infection[57, 58]. Apart from the cell wall proteins, phenolic compounds containing cell wall polymers such as cellulose[59], lignin[60-62], and suberin [63] are synthesized for cell wall fortification. Lignin is known to physically bind hyphal tips and bacteria to restrain them and restrict the diffusion of their enzymes and toxins into the host cell[46, 47]. Lignified callose reinforcements ensheath invading hyphal tips at the cell walls providing a direct physical resistance against invading pathogens[57, 58]. The phenylpropanoid the key metabolic pathway that supplies metabolic compounds to the lignin and anthocyanin biosynthesis [64] and plays important role in plant defense against invading pathogens. Hence, the reinforcement of the cell walls, which can improve host resistance, is initiated as primary defense response early in the process of host pathogen interaction. The cell wall fortification is important for improving the post-harvest disease resistance as there is not active response from the dormant seed against pathogen infections. Manipulation of cell wall is an excellent strategy to improve the post-harvest disease resistance which acts as physical barrier against pathogen infection.
2.3 Seed coat cell wall composition and its role in fungal resistance

Seeds coats are formed during the seed developmental process that hosts polymers such as tannins, phenols, cellulose, hemicellulose, pectin and other pigments to protect the embryo from biotic, abiotic and mechanical stresses. In particular the seed coat plays an important role in host-pathogen interaction and thereby disease resistance. The waxy coating on the micropyle (small opening adjacent to hilum) plays a role in pathogen susceptibility of the seed[33]. Along with phenolic compounds, hydrophobic proteins accumulate in seed coat that altogether acts as deterrent/toxin for the feeding organism thereby protecting embryo within the seed. The phenolic composition of seed coat provides color to the seeds and influence the seed coat structure and thereby seed anatomy and plays a major role in water imbibition of seed coat.

Several studies indicate that the color of seed coat is associated with disease resistance against pathogens. For example, dark colored (anthocyanin) seed coat in common bean and pea seeds are resistant to pathogens compared to light colored seeds[65-67]. Further, extracts of black seed coats of Phaseolus vulgaris contained phenolic compounds and inhibited growth of Rhizoctonia solani [65] indicating the very important role of seed coat in disease resistance. The mechanism of seed coat development and pigmentation is thoroughly investigated in Arabidopsis[37]; however, there are not many reports on lignin or other phenolic compound biosynthesis. Recent studies showed that seed coat cell walls are reinforced with secondary cell walls during seed development and maturation in Arabidopsis[35, 68]. The percentage of lignin is very low in seeds[69]; however, the seed coats are fortified with various flavonoids, a highly diverse group of secondary metabolites consists of flavanols, anthocyanins, and proanthocyanidins (condensed tannins) with known antioxidant as well as antimicrobial properties in addition to other structural functions. Of the flavonoids, the condensed tannins, chemically flavan-3-ols [70] are highly enriched in the seed coats. The tannins deposited in the seed coats protects the seed from invading pathogens and predators in addition to influencing the seed coat-imposed seed dormancy[71, 72]. External addition of extracted tannins (proanthocyanidins) to the media reduced A.
parasiticus[73] colonization indicating a possibility to improve the disease resistance by increasing the tannin content of the peanut seed coat. The early stage of seed coat development represents a live and growing tissue with active gene expression that ultimately determines the nature of seed coat that will be formed at maturity.

Seed coat transcriptome profiling from Medicago truncatula showed genes involved in abscisic acid biosynthesis and suberin production in early stages of seed coat establishment[74]. In another study, the gene expression analysis of developing Pisum sativum seed coat showed expression of genes involved in secondary metabolite production and plays potential role in quenching ROS production under stress conditions[75]. Therefore, the seed coat developmental process in peanuts needs to be studied at both molecular and biochemical level to understand the pathways and mechanism by which seed coat mediated resistance can be improved against A. flavus infection.

2.4 Peanut Seed coat, biochemical composition and A. flavus resistance

The peanut seed contains three parts: outer seed coat layer (testa), endosperm (cotyledons) and embryo (Figure 4). The outer seed coat protects the embryo and endosperm from biotic (bacteria, fungi, insects or virus) and abiotic (mechanical, dehydration or UV) stresses. Thus far in peanut, previous studies were mainly focused on identifying the antioxidants present in seed coat and the extraction of seed pigments[76-80]. These studies showed that peanut seed coat with different colors were composed of different pigments; however, the role of these anthocyanins/proanthocyanidins in A. flavus resistance is not thoroughly investigated and has not been used to develop A. flavus-resistant peanut varieties.

Previous research reported some resistant factors such as structure of seed coat [81, 82] and the presence of polyphenol compounds [83, 84] inhibits the growth of A. flavus. Further researchers have also reported the induced defense mechanisms such as increased lignification[85], accumulation of phytoalexins [86] and production of Pathogenesis Related (PR) proteins[87] in response to A. flavus infection. Despite the compelling evidence, efforts
were not made to breed peanut cultivars for improving cell wall traits in the seed coat tissue to resist *A. flavus* infection. Recent studies showed that seed coat developmental study in peanut showed expression of several genes related to flavonoid biosynthesis at early stage of development resulting in accumulation of polymeric phenolic compounds in epidermal layer of seed coat[88]. Also, an EMS generated peanut mutant line was reported to contain higher amounts of melanin in seed coat which arise due to higher gene expression involved in secondary metabolism pathway which gave rise to increase in seed coat weight and cracking of seed coat[89]. Therefore, seed coat composition is very critical for maintaining an active biochemical layer that involves in several functions of seed related developmental process. Therefore, a comprehensive research on the seed coat development, gene expression and biochemical study is necessary to develop efficient strategies for the seed coat mediated *A. flavus* resistance and aflatoxin contamination.

3. Summary

*A. flavus* is an opportunistic fungal pathogen that infects peanuts resulting in the accumulation of potent carcinogenic aflatoxins[90]. Aflatoxin contamination in peanuts remains a major economic and food safety issue across the globe and particularly in semi-arid tropical countries. However, the lack of knowledge on the factors responsible for peanut resistance to *A. flavus* infection underlines huge challenge to researchers worldwide. Peanut seed coat acts as a physical and biochemical barrier against both pre and post-harvest pathogen infection[91]. Since *A. flavus* infects the seed during the seed development stage (transition to dormancy) and/or during seed storage, it does not encounter an active genetic resistance from live cell(s). *A. flavus* belongs to the family of pathogenic fungal species that produces cell wall degrading enzymes to break into the plant cell walls[92]. Seed coat extracts from colored Pea seeds showed resistance to pathogenic fungal infection including member from *Aspergillus* genus indicating the potential of seed coat cell wall composition in generating resistance. Therefore, understanding the seed coat development in peanuts and thereby developing an intact peanut testa could play an important role in reducing aflatoxin contamination by *A. flavus* infection[91].
Production of the mycotoxin aflatoxin has attracted scientific and economic importance due to its severe adverse effects on agriculture, livestock and human health. Its presence proves detrimental to financial and biological factors within a country, as well as across the globe. However, there are still a variety of factors that remain a mystery when dealing with aflatoxin: details of various parts of its biosynthesis, variation of production within different fungi, evolutionary origins, the complete biological and environmental factors inducing production, and the biological/ecological role that aflatoxin has within *Aspergillus*. The mutagenic nature of aflatoxin paired with the fact that *Aspergillus* is typically viewed as a “weak pathogen” gives rise to the theory that aflatoxin specifically is used to inhibit microbial competition on a food substrate[93]. Aflatoxin will continue to be studied, as it still possesses possible contamination threats, even with proper screening and handling of harvested crops as well as pre-harvested crops. Functional genomic, molecular biology and biochemical research will further solutions to this problem, providing insight into the genetic regulation of aflatoxin in *Aspergillus* as well as creating possible transgenic crops that have increased resistance to fungal infection. Peanuts, being largely the main crop focused with aflatoxin along with corn, have potential to curb aflatoxin accumulation with research into increasing certain characteristics (lignin accumulation, polyphenolic accumulation etc.) of the peanut seed coat (testa), which would prevent *Aspergillus* from pre- or post-harvest infection.

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Abbreviations: Aflatoxin: *Aspergillus flavus* toxin.

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