Plant Gene 32 (2022) 100381

Contents lists available at ScienceDirect

Plant Gene

journal homepage: www.elsevier.com/locate/plantgene

# Seed coat mediated resistance against *Aspergillus flavus* infection in peanut

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ARTICLE INFO

Editor: A Rooney

*Keywords:*
Aflatoxin
*Aspergillus flavus* toxin
Peanut
Cell wall
Seed coat

ABSTRACT

Toxic metabolites known as aflatoxins are produced via certain species of the *Aspergillus* genus, specifically *A. flavus*, *A. parasiticus*, *A. nomius, and A. tamarie*. Although various pre- and post-harvest strategies have been employed, aflatoxin contamination remains a major problem within peanut crop, especially in subtropical environments. Aflatoxins are the most well-known and researched mycotoxins produced within the *Aspergillus* genus (namely *Aspergillus flavus*) and are classified as group 1 carcinogens. Their effects and etiology have been extensively researched and aflatoxins are commonly linked to growth defects and liver diseases in humans and livestock. Despite the known importance of seed coats in plant defense against pathogens, peanut seed coat mediated defenses against *Aspergillus flavus* resistance, have not received considerable attention. The peanut seed coat (testa) is primarily composed of a complex cell wall matrix consisting of cellulose, lignin, hemicellulose, phenolic compounds, and structural proteins. Due to cell wall desiccation during seed coat maturation, post-harvest *A. flavus* infection occurs without the pathogen encountering any active genetic resistance from the live cell(s) and the testa acts as a physical and biochemical barrier only against infection. The structure of peanut seed coat cell walls and the presence of polyphenolic compounds have been reported to inhibit the growth of *A. flavus* and aflatoxin contamination; however, there is no comprehensive information available on peanut seed coat mediated resistance. We have recently reviewed various plant breeding, genomic, and molecular mechanisms, and management practices for reducing *A. flavus* infection and aflatoxin contamination. Further, we have also proved that seed coat acts as a physical and biochemical barrier against *A. flavus* infection. The current review focuses specifically on the peanut seed coat cell wall-mediated disease resistance, which will enable researchers to understand the mechanism and design efficient strategies for seed coat cell wall-mediated resistance against *A. flavus* infection and aflatoxin contamination.

## 1. Background

Aflatoxins pose significant real-world health, economic, and agricultural problems, and in the U.S. alone, there is an estimated annual economic loss of $270 million due to aflatoxin contamination of food and feed crops (Georgianna and Payne, 2009). *Aspergillus* infection is a common issue within production agriculture, and many crop species are afflicted during field exposure, harvest, storage, or the processing and transport stages (Kumar et al., 2017). *Aspergillus* species such as *A. flavus, A. parasiticus, A. nomius* and *A. tamarie* are the major aflatoxin-producing fungi and are ubiquitous, although more prevalent in temperate and humid conditions (Kurtzman et al., 1987). Aflatoxin

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https://doi.org/10.1016/j.plgene.2022.100381
Received 11 May 2022; Received in revised form 23 August 2022; Accepted 2 September 2022
Available online 7 September 2022

contamination is more prevalent in peanut (*Arachis hypogaea*), wheat (*Triticum aestivum*), walnut (*Juglans regia*), corn (*Zea mays*), rice (*Oryza sativa*), cotton seed (*Gossypium hirsutum* L.) and tree nuts (Goto et al., 1996; Jelinek et al., 1989; Severns et al., 2003), though *A. parasiticus* is more limited to peanut crop, while *A. flavus* occurs in cotton seed, corn, tree nuts and peanuts (Kumar et al., 2017).

At least 20 aflatoxin derivatives are known to exist with four natural forms ($AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$), along with derived forms of $M_1$ (hydroxylated AFB1) and $M_2$ (hydroxylated AFB2) (Inan et al., 2007; Giray et al., 2007; Hussain and Anwar, 2008). The level of toxicity depends on the type of toxin associated and increases from $G_2$, $B_2$, $G_1$ to $B_1$ (Jaimez et al., 2000). Despite aflatoxins being the most studied mycotoxin, their complex biosynthetic mechanism has not been well understood. Further, it is unknown how and when aflatoxin biosynthesis is induced in *A. flavus* by internal or external factors. Several strategies have been used to reduce the *A. flavus* infection and subsequent aflatoxin biosynthesis in peanut crops, yet it remains a major issue in peanut production. Since *A. flavus* infects both pre- and post-harvest peanuts, it poses a serious challenge compared to other fungal pathogens that infect the pre-harvested crop. Biocontrol mechanisms were very effective in controlling and managing an *A. flavus* strain in maize by competitive exclusion using nontoxic strains such as non-aflatoxigenic *Aspergillus flavus* strain, *Bacillus subtilus*, *Lactobacillus spp*, *Pseudomonas* spp., *Ralstonia* spp., and *Burkholderia* spp. (Abbas et al., 2006; Palumbo et al., 2006; Accinelli et al., 2012). Studies on delivering such bioplastic formulations of competitive strains of non-aflatoxin producing *A. flavus* strain showed decrease in the population of Aflatoxin producing *A. flavus*. The presence of various strains of *B. subtilus* and *P. solanacearum* in the non-rhizosphere soil of maize was reported to reduce *A. flavus* (Nesci et al., 2005). Studies in parallel lines in peanuts also reported that *Trichoderma* spp. effectively controls aflatoxins from 20 to 90% under laboratory conditions (Anjaiah et al., 2006; Brown and Bhatnagar, 2015).

## 2. Aflatoxin contamination and aflatoxicosis

Mycotoxins represent a class of low-molecular weight chemical compounds produced by filamentous fungi as secondary metabolites that adversely affect humans and animals (Bennett and Klich, 2003; Zain, 2011). Mycotoxins are more widespread in areas that favor mold growth, particularly in hot and humid climates (Bennett and Klich, 2003; Zain, 2011; Richard, 2007). These chemical compounds are specifically correlated with mycotoxicosis, a disease related to exposure via dietary, respiratory, or dermal contact to mycotoxins. Because of their biological importance, research has been focused mainly on the classes of mycotoxins that showed severe negative effects on human and livestock health (Hussein and Brasel, 2001). Of approximately 300 mycotoxins, research is primarily focused on aflatoxins, citrinin, ergot alkaloids, fumonisins, patulin, zearalenone, ochratoxin, trichothecenes (T-2 mycotoxin), and others, including their derivatives. Often, a single species can produce multiple mycotoxins and contaminate feed and fodder, resulting in mycotoxicosis in animals and humans. Predominantly, mycotoxins are produced from the genera *Aspergillus*, *Alternaria*, *Claviceps*, *Fusarium*, *Penicillium*, and *Stachybotrys*. Nevertheless, the level and toxicity of mycotoxin production across species of the same genera changes significantly in case of *Aspergillus*.

Aflatoxins are arguably the most well-known mycotoxins and are considered as Group 1 carcinogens according to the International Agency for Research on Cancer (IARC) (Richard, 2007). Some strains of *A. flavus* can produce up to $10^6$ ppb/per kg of seed (of aflatoxins). This can be highly toxic, considering the U.S. Food and Drug Administration (FDA) limits total aflatoxin concentration levels to 20 μg/kg for all human food products and 300 μg/kg for cattle meal (Richard, 2007; Bennett and Klich, 2003). Developed countries such as the United States and European Union devote enormous resources to screening, managing, and quarantining crops contaminated by aflatoxins. A combination of proper land management practices, cultivation, and storage techniques practiced by producers can minimize the risk of exposure to both humans and livestock. The highest priority is to prevent initial infection, reduce aflatoxin contamination, prevent the potential loss of millions of dollars in crop production, and reduce the cost of screening contaminated materials (Khlangwiset and Wu, 2010; Mitchell et al., 2016; N'dede et al., 2012).

The scenario and management strategies drastically change in developing countries, especially those with congenial climate conditions for fungal growth, such as many African and Southeast Asian countries. Due to the lack of adequate infrastructure and resources to prevent favorable fungal growth conditions, aflatoxin contamination remains a huge problem for the global health industry and stakeholders. A higher occurrence of liver diseases in developing countries may not be solely attributed to aflatoxin contamination, as malnutrition and lack of access to suitable drinking water can also have negative effects. However, various reports associate higher hepatic carcinomas due to aflatoxin contamination in China, Africa, and Southeast Asian countries (Ross et al., 1992; Lauvergeat et al., 2001; Peers and Linsell, 1973; Van Rensburg et al., 1985). A high incidence of contamination also leads to the inability of countries to secure high-value crop exports in international trade markets. Hence, there is a need to understand the nature of the aflatoxins produced, their biosynthetic processes, and the measures to reduce aflatoxin contamination in food crops.

Hence, a comprehensive strategy involving plant breeding, genetic, genomic, and molecular mechanisms, and management practices is required to effectively control or reduce *A. flavus* infection and aflatoxin contamination. These strategies were reviewed in our recent publication (Pandey et al., 2019). The current review focuses explicitly on the seed coat cell wall mediated physical and biochemical resistance against *A. flavus* infection and aflatoxin reduction in peanuts. This is an underexplored research topic which holds promise in *A. flavus* resistance and is gaining significance with the recent discoveries. A research paper proving the physical and biochemical resistance against *A. flavus* was published by our group recently (Commey et al., 2021). *A. flavus* infects the peanut in the later stages of seed development, seed maturation, and post maturation. During these stages, the seed is either entering or has transitioned into dormancy stage where there is no active genetic resistance, hence, providing an opportunity for *A. flavus* to successfully colonize the peanut seeds. Peanut dried seed coat, primarily composed of cell wall tissue, protects the cotyledons and embryo, and offers an excellent physical and biochemical barrier against *A. flavus* infection and aflatoxin contamination in pre-and post-harvest peanuts. Here we review the current understanding and future potential of the seed coat mediated *A. flavus* infection and aflatoxin contamination in peanuts.

## 3. Strategies for *A. flavus* resistance and aflatoxin contamination reduction in peanuts

Researchers worldwide are actively working on designing various strategies for *A. flavus* resistance and aflatoxin contamination. While there is no perfect strategy which can effectively reduce *A. flavus* infection and aflatoxin contamination, combining multiple strategies is highly effective due to the complex nature of the menace across the globe. We have recently reviewed current strategies, other than seed coat cell wall mediated resistance involved in reducing *A. flavus* infection and aflatoxin contamination (Pandey et al., 2019). Genetic, genomic, and molecular strategies have been used to understand host-pathogen interactions and aflatoxin production in peanut crop. Integration of phenotypic data with genetic, molecular, and genomic data greatly improved the understanding of the nature of *A. flavus* and peanut interactions and aflatoxin production. Genetic approaches using Quantitative Trait Loci (QTL) analysis have been extensively employed for discovering the *A. flavus* resistance and aflatoxin contamination associated genetic knowledge in peanut plants (Khan et al., 2020; Jiang et al., 2021). Also, soil physiochemical properties and topography alter

the symbiotic fungi composition and associated gene expression and metabolite composition of peanut pods that influence *A. flavus* colonization (Yao et al., 2021). While these are excellent discoveries that helped to understand *A. flavus* resistance and reduce aflatoxin contamination, these approaches were not based on seed coat mediated physical and/or biochemical resistance.

## 4. Peanut shell and seed coat mediated *A. flavus* resistance

The peanut shell and seed coat reduce *A. flavus* infection and aflatoxin contamination, and researchers across the globe employed them in selecting for resistance. The intact pod shell provides an effective barrier against *A. parasiticus* (Kushalappa et al., 1979); nevertheless, strengthening the peanut shell is not a feasible option, as discussed in the previous section. After shelling, only peanut seed coat is intact to protect cotyledons from *A. flavus* infection (Figs. 1 and 2). Peanut seed coat cell wall acts as a physical and biochemical barrier against both pre- and post-harvest pathogen infection (Xue et al., 2005; Dieme et al., 2018; Commey et al., 2021). We have previously shown that removal of the peanut seed coat increased *A. flavus* infection, and biochemicals extracted from seed coats prevented *A. flavus* growth (Commey et al., 2021). Since *A. flavus* infects the seed during pre- and post-harvest stages, it does not encounter any active genetic resistance from live cell(s) as the seed coat during pre-harvest is entering the dormant stage. Presence or absence of the intact peanut testa plays an important role in the colonization by *Aspergilli* (Xue et al., 2005). Peanut kernel skin color strength is positively correlated with total polyphenol level (Nayak et al., 2020) that provides antioxidant property. Previous research reported that some resistant factors such as the structure of the seed coat (LaPrade et al., 1973; Zhou and Liang, 1999) and the presence of phenolic compounds (Daigle et al., 1984; Azaizeh et al., 1990) inhibit *A. flavus* growth. Seed coat structural differences were observed between resistant and susceptible peanut lines (Zhou and Liang, 1999; Dieme et al., 2018). Genotypes with thicker seed coats, smaller hilum, and compact seed coat structure showed higher resistance against *A. flavus* infection. Seed coat transcriptomic studies of three seed development stages by RNA seq analysis of EMS generated *pscb* (peanut seed coat crack and brown color) mutant line, and wild type revealed higher expression levels of phenyl propanoid and flavonoid genes. Specifically, polyphenol oxidases, peroxidases were highly present in late seed development stages in addition to three commonly differentially expressed genes CCoAOMT1, kinesin, and MYB3 genes in all three seed developmental stages. These three common genes were identified to be responsible for seed cracking and brown color seed coat phenotype (Wan et al., 2016).

Further, presence of wax and cutin layers have been shown to confer *A. flavus* resistance in certain genotypes (Liang and Pan, 2003). In addition to seed coat structure, presence of biochemical compounds has been reported to confer *A. flavus* resistance. Extracted phenolic compounds from peanut embryo inhibited the growth of *A. flavus* in vivo (Lindsey and Turner, 1975). Association of a specific chemical such as trypsin and 5,7-dimethoxyisoflavone with *A. flavus* resistance was reported (Lindsey and Turner, 1975; Zhou and Liang, 1999), indicating the fact that the presence of certain inhibitory compounds confers *A. flavus* resistance. It is possible that these could also be structural compounds; for example, the phenolic compounds can be cell wall bound or free form. It is not known how these biochemicals function in conferring resistance to *A. flavus*. Further, researchers have also reported induced defense mechanisms such as increased lignification (Liang et al., 2001), accumulation of phytoalexins (Liang, 2002), and production of pathogenesis related (PR) proteins (Szerszen, 1990) in response to *A. flavus* infection. Despite the compelling evidence, efforts were not made to breed peanut cultivars for improving cell wall traits against *A. flavus* infection. Comprehensive research on the seed coat development and biochemistry is necessary to develop efficient strategies for seed coat mediated *A. flavus* resistance and aflatoxin contamination.

Phenolic compounds such as cinnamic acid and benzoic acid derivatives possess antifungal activity against *A. flavus* (Kim et al., 2004). A high-throughput radial growth bioassay using various cinnamic and benzoic derivatives showed that vanillic acid, a type of chlorogenic acid showed the highest inhibition of *A. flavus* followed by other cinnamic acid derivatives. Total phenolics extracted from peanut seed coats inhibited *A. flavus* growth in radial growth assays (Commey et al., 2021). Analysis of methanol extracts from peanut skin, hull, raw kernel, and roasted kernel flour showed that epicatechin has the highest inhibitory effect on *A. flavus* followed by p-coumaric acid, ferulic acids,


**Fig. 1.** Peanut pod and seed coat developmental stages.
A. Longitudinal cross section of peanut pod at R5 stage (beginning seed).
1: Outer pod shell. 2.Parenchymatous tissue 3: Seed coat (stained). Scale bar: 2 mm.
B. Peanut reproductive developmental stages (R).
Images representing different developmental stages of peanut pod. R1: Bloom, R2: Peg formation, R3: Pod formation, R4: Full pod, R5: Seed formation, R6: Full seed, R7: Maturity, R8: Harvest maturity. Scale bar: 2 cm.
C. Longitudinal cross section of peanut pod (R2-R5 stages).
Cross section images shows developmental stages of shell, parenchymatous tissue, seed coat and cotyledons.
Scale bar: 2 mm.

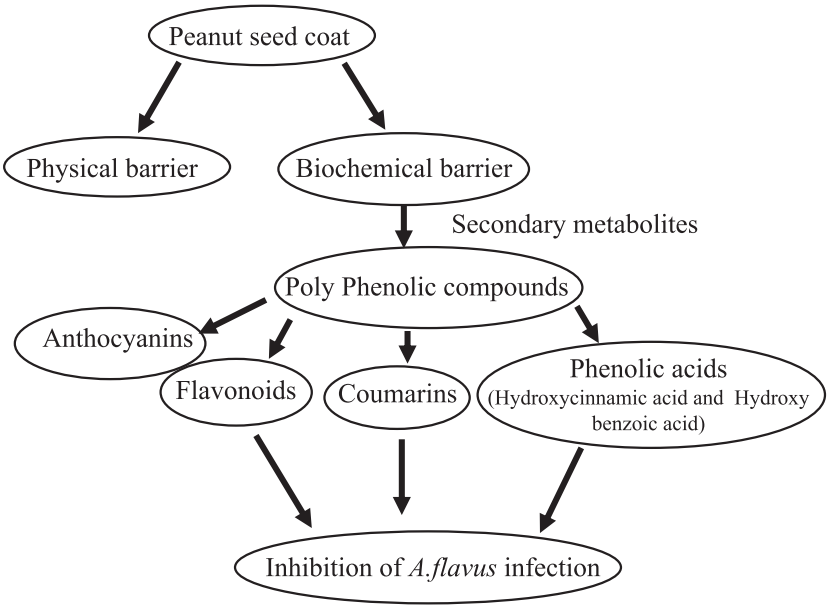


**Fig. 2. Peanut seed coat mediated physical and biochemical resistance against *A. flavus* infection.**

cholorogenic acid, and quercetin (Win et al., 2011). Further analyses of peanut seed coat phenolic compounds using high-performance liquid chromatography (HPLC) showed the presence of p-hydroxybenzoic acid, chlorogenic acid, p-coumaric acid, ferulic acid, resveratrol, epicatechin, and quercetin. Wound-induced stilbene phytoalexins from peanut seeds were shown to inhibit spore germination and hyphal extension of *A. flavus* (Wotton and Strange, 1985). Interestingly, the amount of these wound-induced phytoalexins were reduced in cultivars exposed to drought stress compared to non-stressed controls (Arora and Strange, 1991; Dorner et al., 1989). This phenomenon correlates with the fact that drought exposed peanuts are highly susceptible to *A. flavus* infection and aflatoxin contamination. This means multiple plant exposure to stress will eventually make the plant susceptible. Therefore, *A. flavus* infection of peanuts in the field followed by drought exposure will make peanuts more susceptible to *A. flavus* infection and aflatoxin accumulation. Also, the *A. flavus* pathogen thrives best under dry conditions induced by drought. The role of quercetin in inhibiting the proliferation of *A. flavus* and aflatoxin production has been investigated using *A. flavus* strains (Li et al., 2019). Transcriptome analysis using RNA-seq technology showed that quercetin induces cell death of *A. flavus* by inhibiting the proliferation and expression of developmentally related genes. Further analysis of the quercetin in the regulation of aflatoxin biosynthesis showed that quercetin represses the expression of aflatoxin production-related genes, resulting in lower aflatoxin production.

Polyphenols have been reported to play a critical inhibitory role in aflatoxin production (Holmes et al., 2008), and the role of specific phenolic compounds in inhibiting *A. flavus* growth was demonstrated (Kim et al., 2004). Various concentrations (5, 10, 15, 25 mM) of individual phenolics such as cinnamic acid, cinnamic and benzoic derivatives such as veratraldehyde, vanillin, vanillic acid, coumaric acid, and caffeic acid were added to Potato Dextrose Agar (PDA) media to study inhibitory effects on *A. flavus* strain NRRL 3357. Radial growth patterns observed after 7 days at 28 °C demonstrated that cinnamic acid, vanillic, and veratraldehyde, greatly inhibited hyphal growth at 5 mM. Vanillic acid and the three coumaric acids showed inhibition at 5 mM–25 mM concentrations, while caffeic acid showed limited inhibition at the highest concentration (Kim et al., 2004). Various phenolic compounds (p-coumaric acid, ferulic acid, hydroxybenzoic acid, chlorogenic acid), flavonoid compounds (epicatechin), and other chemical compounds such as quercetin and reservatrol have been demonstrated to be present in peanut seed coats using HPLC analysis (Win et al., 2011; Sobolev, 2008). It was reported that the relative abundance of the phenolic compounds was altered in response to fungal infection, indicating the fact that these compounds play a significant role in host-pathogen interactions and disease resistance. Another defense mechanism that could explain the role of the seed coat in deterring infection is phytoalexin production in peanut coats. Resveratrol is present in peanut seed coat and was the dominant phytoalexin produced after 24 h of incubation, but after 48 h arachidin-3 and SB-1 were the major phytoalexins (Sobolev, 2008). Wound-induced stilbene phytoalexins from peanut seeds were shown to inhibit spore germination and hyphal extension of *A. flavus* (Wotton and Strange, 1985). Overall, the seed coat cell wall structure and phenolic compounds present in peanut seed coat cell walls have been shown to confer *A. flavus* resistance and aflatoxin contamination reduction in peanuts. Metabolite profiling identified pipecolic acid (Pip) as an important component of peanut seed resistance against *Aspergillus flavus* infection (Sharma et al., 2021). Sharma et al. recently reported a strong association of pipecolic acid (Pip) accumulation with peanut resistance to *A. flavus* infection (Sharma et al., 2021). Pip triggered the production of the ALD1-like gene involved in its biosynthesis. The role of Pip was not only confirmed in many resistant cultivars but also reduced *A. flavus* infection in susceptible cultivars by coating with Pip (Sharma et al., 2021).

In peanuts, resistance is acquired at the seed coat, testa, and molecular defense levels. The seed coat thickness, testa composition of polyphenols, palisade cell layer thickness, and presence of a wax layer protects peanuts from aflatoxin production (Soni et al., 2020). Being the outermost layer of peanut kernels, the seed coat acts as the primary physical barrier against *A. flavus* infection. Seed coat thickness and permeability might contribute to resistance, as proposed by LaPrade's group (LaPrade et al., 1973). Studies have reported that resistant genotypes of peanut showed smaller hila made up of tightly packed palisade tissue with thick waxy coatings relative to susceptible peanut genotypes, indicating a role in fungal resistance (Kushalappa et al., 1979). Preliminary studies also showed that peanut testa with higher tannin content and polyphenol compounds like flavonoids act as *A. flavus* inhibitors (Turner et al., 1975; Sanders and Mixon, 1979). Later studies also indicated the role of wax and peanut seed coat cutin thickness in resistance against *A. flavus* infection. Additionally, tannins were reported to be higher in testa than in cotyledons which has shown significant reduction of *A. parasiticus* infection and aflatoxin reduction in peanut genotypes of PI337409 and TX-798736 (Liang and Pan, 2003;

Azaizeh et al., 1990). Tannins and 5–7 dimethoxyisoflavone were previously described as inhibitors of *A. flavus* infection. Electron microscopy and light microscopy studies by Zambettackis and Bockelee Morvan reported diversity in the peanut testa (Zambettakis and Bokelee, 1976). Further, membrane parameters also mediated resistance against aflatoxin producers. Research studies in these lines using gene expression identified upregulation of genes involved in lipid metabolism, oxidative signaling, and cell wall synthesis. Peanut lipid membrane genes such as desaturases, lipoxygenases, and encoded proteins act as defenses for *A. flavus* infection followed by oxalate oxidase, P450 monooxygenase, lipoxygenase, and amino cyclopropanecarboxylate oxidase genes. Cell wall defenses also include LOX, hydrogen peroxide, jasmonic acid, and salicylic acid as a second level of protection to the enzymes mentioned above (Zhang et al., 2015). It has been shown that levels of malondialdehyde and membrane peroxidation increased in response to inoculation by the aflatoxin producers.

A study of different peanut genotypes by protein profiling also revealed the presence of higher trypsin content in resistant lines relative to susceptible lines. Studies also reported that tryptophan has an inhibitory role on *A. flavus*-produced aflatoxin by changing aflatoxin biosynthetic gene expression while tyrosine promoted aflatoxin production (Wilkinson et al., 2007). There are various inducible molecular responses upregulated by pathogens such as lignin deposition, cell wall crosslinking, phytoalexins, hypersensitive response, reactive oxygen species (ROS), and pathogenesis related proteins. The precursor of lignin, phenylalanine ammonia lyase (PAL), was reported to be higher in resistant peanut cultivars than in susceptible cultivars.

## 5. Structure, composition, and functions of seed coats

Plants have evolved mechanisms such as the formation of seed coat cell walls and outer shells to protect the developing and mature seeds from mechanical stress, adverse environmental conditions, and biotic and abiotic stresses. Seed coat cell layers are compressed and desiccated during the seed coat development (Fig. 1), and the dried seed coats are primarily composed of cell wall material (Western et al., 2000; Sechet et al., 2018; Mendu et al., 2011a; Mendu et al., 2011b). Plant cell walls are mainly composed of cellulose, hemicellulose, pectin, lignin, phenolic compound polymers, and structural proteins. The composition of the cell wall plays an important role in protecting the seed from pre- and post-harvest pathogen infections. The phenolic composition of the seed coat imparts color to the seeds and influences the seed coat cell wall structure and functions. Several studies indicate that seed coat color is associated with disease resistance against pathogens. In addition to protection from biotic and abiotic stressors, the seed coat also plays an important role in seed germination, vigor, longevity, and the storage potential of the seed (Souza and Marcos-Filho, 2001). The thickness, form, composition, shape, and color of seed coats vary based on the plant species. For example, coconut seeds are one of unique seeds that are protected by a seed coat (exocarp) and a hard shell (endocarp) that help them survive under adverse environmental conditions.

The peanut seed coat plays an important role in the host-pathogen interaction and disease resistance, particularly against post-harvest pests and disease. The peanut seed contains three parts: outer seed coat cell wall layer (testa), endosperm (cotyledons), and embryo. The outer seed coat protects the embryo and endosperm from biotic (bacteria, fungi, insects, or virus) and abiotic (mechanical, dehydration or UV) stresses. In the later part of seed maturation and post maturation, seeds enter the stage of dormancy, hence, lack active genetic resistance, providing an opportunity for pathogens and/or saprophytes to successfully colonize the seeds. The later stage of developing seeds and post-harvest seeds depends on the physical resistance arising from the shell and/or seed coat cell walls. Dark colored (anthocyanin-containing) seed coats in common bean and pea seeds were resistant to pathogens compared to lighter colored seeds (Prasad and Weigle, 1975; Stasz and Harman, 1980; Islam et al., 2003). Further, extracts of black seed coats of *Phaseolus vulgaris* contained phenolic compounds and inhibited the growth of *Rhizoctonia solani* (Prasad and Weigle, 1975), indicating a very important role of the seed coat in disease resistance. Certain flavonoid compounds such as quercetin have also been reported to play a critical role in inhibiting *A. flavus* and aflatoxin production in tea (Zhou et al., 2015). Seed coat cell walls are reinforced with secondary cell walls in *Arabidopsis* (Mendu et al., 2011b; Mendu et al., 2011a; Stork et al., 2010). The mechanism of seed coat cell wall development and pigmentation has been thoroughly investigated in *Arabidopsis* (Haughn and Chaudhury, 2005); however, there are few reports on lignin or other phenolic compound biosynthesis. The percentage of lignin is very low in seeds (Liang et al., 2006); however, the seed coat cell walls are fortified with various flavonoids, a highly diverse group of secondary metabolites (Fig. 2) consisting 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, are highly enriched in the seed coats (Dixon et al., 2005). The tannins deposited in the seed coat protect the seed from invading pathogens and predators in addition to influencing the seed coat-imposed seed dormancy (Debeaujon et al., 2000; Shirley, 1998). Though seed development is comparable in many crop plants, it is important to study individual species to comprehensively understand the developmental program which helps in designing effective strategies for seed coat cell wall mediated disease resistance. Here, we have systematically described the peanut seed coat to understand its developmental program which will help in designing strategies for *A. flavus* resistance in peanut.

## 6. Developmental biology of peanut shell and seed coat

The Fabaceae family members have a common seed coat structure with interspecific variation based on the modes of cell differentiation, size, and orientation of cell wall layers (Lush and Evans, 1980). Legumes have two ovule integuments, the inner integument and the outer integuments which are composed of multiple cell layers, ultimately forming the protective seed coat (Smýkal et al., 2014). Despite seed coat variations in structure and composition among the Fabaceae family members, they follow similar embryo and endosperm developmental phases (Butler, 1996). The seed coat structure of most fabacean seeds is made up of an epidermal cell layer of the outer integument, scleroid cell layers, parenchymal cell layers, the micropyle and the strophiole. A combination of malpighian cells with either heavily or unevenly thickened cell walls and a cuticle covering the tangential cell walls encompasses the outer epidermis integuments. The sclereids serve as an intermediate between the outer epidermis and the endodermal layers. One of the critical roles of seed coat during development is the production and deposition of defense-related compounds including phytoalexins, anthocyanins, and cell wall- associated phenolics as structural components. The innermost cell layer endothelium is the active form of the seed coat, and it either contains proanthocyanidins (PAs) or compressed parenchyma cells.

Peanut seeds are formed inside a hard-shell structure known as a pod which is the first formed structure after fertilization. The lignified pods protect the developing embryo from mechanical, biotic, and abiotic stresses. The peanut pod is produced below ground; hence the pod is constantly challenged by soil-borne pathogens and needs to protect itself from various soil-borne diseases. To restrict soil-borne diseases, peanuts have a hard outer shell and a protective seed coat covering the cotyledons. Soil-borne diseases can be prevented by either improving the structure and composition of peanut pods and/or seed coat. During peanut processing, the shells are removed by the mechanical breaking of the outer shell to release the seeds; hence, altering the shell structure or composition will pose issues in the mechanical as well as hand shelling process. Therefore, the seed coat will serve as the only practical form of protection against *Aspergillus flavus* infection. The peanut seed is primarily composed of the seed coat, embryo, and cotyledons. During seed

development, the seminal integuments are made up of three dissimilar types of cells. The initial cell is in relation to vessels, the second cell type surrounds the vascular bundles which form the conductive tissue, and last the type of cell usually begins during seed formation, into parenchymal tissues which develop into a thick envelope around the seed (Zambettakis and Bokelee, 1976). These layers protect the seed during the development and after maturation. The outer epidermis of the seed coat is made up of a single layer of polygonal cells with thick cuticulated walls, while the inner walls are relatively thin after maturation. Investigation of the role of epidermal cells in pathogen infection suggested the significance of the wax layer, a junction between epidermal cells, the thickness of the cell, and intactness of seed coat in *A. flavus* invasion in peanuts (Zambettakis and Bokelee, 1976). Therefore, this suggest that the seed coat could be playing a significant role in mediating *A. flavus* resistance and aflatoxin contamination in peanuts.

## 7. Genetics and molecular biology of the seed coat cell wall development and disease resistance

It is well established that the seed coat cell wall structure and certain biochemicals play a significant role in disease resistance. It is important to understand the molecular and genetic mechanisms behind the seed coat cell wall development and biochemical production. Several studies have been reported on the identification of the QTLs associated with the *A. flavus* resistance and aflatoxin contamination in peanuts (Khan et al., 2020; Jiang et al., 2021); however, the molecular basis was not established due to the lack of genome sequence information. With the availability of the draft genomes of peanut diploid and tetraploid species, the molecular association of the QTL can be established to understand the molecular mechanism. Peanut seed coat cell wall developmental biology is not well understood. Anatomical studies using brightfield microscopy showed an interesting pattern in terms of the seed coat cell wall layer development at different stages of seed development and maturation. A systematic investigation using electron microscopy will reveal the developmental biology of the seed coat. Further, associating developmental biology with the transcriptomic analysis will help in understanding the molecular mechanism behind the seed coat cell wall development and biochemical production.

A comparative transcriptome analysis using RNA-seq was performed to understand the mechanism of peanut response to aflatoxin production by *A. flavus* (Wang et al., 2016a). The study was performed using post-harvest seeds which showed a higher level of induction of the phenylpropanoid-derived compound synthetic pathway genes. This is consistent with the biochemical studies which showed phenylpropanoid-derived compounds are involved in the *A. flavus* resistance and aflatoxin contamination reduction in peanuts. It will be interesting to see how these phenylpropanoid-derived compounds are synthesized during the peanut seed coat cell wall development. Identification of these genes will help in developing functional molecular markers for developing resistant lines. Comparative proteomic analysis of *A. flavus* susceptible and resistant varieties identified several secondary metabolism-related proteins that play a role in resistance response (Bhatnagar-Mathur et al., 2021). To understand the host-pathogen interactions in peanut seed coat in response to *A. flavus* infection (Zhao et al., 2019), transcriptome analysis using RNA sequencing was performed to identify genes expressed in the peanut seed coat of a resistant line (J-11) after infection with *A. flavus*. These data were coupled with proteome analysis to facilitate explaining transcriptomic data (Zhao et al., 2019). Though the correlation of the proteome and transcriptome data was poor, overall, the study indicated that the genes involved in pathogenesis- and/or defense-related proteins such as transcription factors, pathogenesis-related proteins, and chitinases were differentially expressed, indicating the fact that various mechanisms are involved in the peanut seed coat mediated resistance against *A. flavus* infection.

Wang et al. group used an RNA-seq approach on *A. flavus*-inoculated peanut resistant and susceptible cultivars and identified a higher number of differentially expressed genes (DEGs) involved in mycelial growth, penetration, conidial formation, development, biosynthesis, and accumulation of aflatoxins in the susceptible cultivar when compared with the resistant cultivar (Wang et al., 2016b). A report by Zhao et al. used RNA-sequencing and identified the gene expression changes specific to transcription factors, pathogenesis-related proteins, and chitinases involved in two basic mechanisms of oxidative stress and cell wall remodeling as peanut defenses against *A. flavus* infection (Zhao et al., 2019). Chitinases and β-1,3-glucanase were shown to increase in resistant cultivars compared to susceptible cultivars after *A. flavus* inoculation (Liang et al., 2005). Interestingly the resistant cultivars not only showed the increased activity of these enzymes but also had higher numbers of glucanase isoforms. Overall, a combination of resistant genotypes combining upregulation of enzymes involved in cell wall modification (chitinases, glucanases, lignin), testa composition changes to promote higher tannins and polyphenols, increase of wax and cutin layers, amino acids etc., might be more effective against *A. flavus* infection.

Several studies showed differential expression of several cell wall related and defense related genes in response to *A. flavus* infection. Transcriptome studies identified a total of 13,539 genes in which 663 showed differential response to *A. flavus* infection (Zhao et al., 2019). Transcriptomic studies in peanut preharvest seed coat reported transcription factors, pathogenesis-related proteins, and chitinases as key defense responsive factors (Nayak et al., 2017). Transcription factors like bZIP, WRKY proteins, ethylene response binding factors (ERF), MYB, MYC, and NAC involved in jasmonic acid (JA), salicylic acid (SA) and ethylene pathway were reported to play role in biotic and abiotic stress genes in peanut (Chen et al., 2014). It was reported that plant defense mechanism can be activated by higher expression of lipoxygenase gene that induces signal molecules such as jasmonic acid, methyl JA, lipid peroxides, fatty acids and secondary metabolites that target pathogens (Hammond-Kosack and Jones, 1996). ACC (1-aminocyclopropane-1-carboxylic acid) genes, forms key part of the ethylene biosynthetic pathway was also known to play role in *A. flavus* resistance (Arahy.78SDCB.1 and Arahy.G16PPK.1). PR proteins like bZIP transcriptional factors, were also upregulated by *A. flavus* infection which forms active component of systemic resistance mediated by salicylic acid (Mitsuhara et al., 2008). PR proteins were involved in peanut defenses against *A. flavus* infection. For example, PR-2 β-1,3-glucanase can digest fungal cell walls and produces higher levels of elicitors both at transcript and protein level during *A. flavus* infection. Pathogenesis Related proteins (PR) proteins, peroxidases, and chitinases, were reported as predominantly expressed in the resistant cultivars of peanut and their expression differences help in *A. flavus* resistance. Similarly, comparative transcriptomics and weighed gene co-expression network analysis (WGCNA) of peanut genotypes resistant (J-11, R) and susceptible (Zhongua-12, S) cultivars revealed 18 genes producing PR10, ACO1 (1-amino cyclopropane 1-carboxylate oxidase), MAPK kinase, STK (serine/threonine kinase), PRR's (pattern recognition receptors), cytochrome P450, SNARE protein, Pectinesterase, Phosphatidylinositol transfer protein and PPR (pentatricopeptide repeat) protein in *A. flavus* defenses (Cui et al., 2022). Two MAP kinases namely, *arahy. L410JY* and *arahy. BC5GM2* were identified to be highly expressed in peanut resistant cultivars along with Cytochrome P450 during *A. flavus* induced defense responses. Pattern recognition receptors such as *RPVOD7* were also identified in *A. flavus* defense response that aid in PTI (pattern triggered immunity) by the recognition of *A. flavus* pathogen associated molecular patterns. Plant defenses were also activated by R protein (resistant), STK (serine threonine kinase, *arahy. D2YYPY*) recognizes *A. flavus released* effectors and mediates Effector triggered Immunity (ETI). Six NBS-LRR genes (R proteins) were identified in cultivated peanut using High-through put sequencing. These genes were upregulated during *A. flavus* infection suggesting their role in ETI responses (Song et al., 2017).

## 8. Plant cell walls, disease resistance, and potential for cell wall engineering

Plants are repeatedly challenged by several pathogens, and to defend themselves, they have developed various strategies which aid in rapid recognition of pathogens and activation of biochemical and structural defenses (Ellinger et al., 2013; Thordal-Christensen, 2003). Host-pathogen warfare begins at the cell wall, which is a primary barrier against the infection of pathogens. Therefore, successful infection of fungi involves secretion of cell wall degrading enzymes and inhibition of host cell wall biosynthetic genes (Wu et al., 1997; Cook et al., 1999). In response to a pathogen attack, the plant cells rapidly repair and reinforce their cell walls to reduce the penetration efficiency of the pathogen and to prevent its entry into the cell. During host-pathogen interactions, a genome-wide defense response is initiated in the host plant, including a battery of cell wall biosynthetic genes (Rose et al., 2002; Veronese et al., 2003; Guest and Brown, 1997). Most defense responses may be genus or species specific, while cell wall defense responses are ubiquitous (Ellinger et al., 2013; Thordal-Christensen, 2003). Constitutive defense is one of the strategies adopted by plants, and involves activation of structural components such as cell wall, waxy epidermal cuticles, and bark which provides existing barriers and also provide support and rigidity to the plant (Freeman and Beattie, 2008). 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 the pathogen infection (Chen and Kim, 2009; Kortekamp et al., 1997). This is the first line of host defense response and is measured by the amount of callose deposition. Callose deposition was first described as papillae (deBary, 1863), composed of (1,3)-β-glucan polymers (Mangin, 1895) 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 pathogen invasion, which gives the plant time to activate downstream defense responses (Brown et al., 1998; Lamb and Dixon, 1997). Apart from callose, pathogen infection also triggers other cell wall components such as cellulose (Mach, 2008), lignin (Bi et al., 2011; Lauvergeat et al., 2001; Hano et al., 2006) and suberin (Thomas et al., 2007) for the cell wall fortification. Hydroxyproline-rich glycoproteins are also produced ahead of the hyphal invasion and reinforce the cell walls to prevent the pathogen infection (El-Gendy et al., 2001; Domingo et al., 1994). Lignified callose reinforcements sheath invading hyphal tips at the cell walls and provide a direct physical resistance against invading pathogens (El-Gendy et al., 2001; Domingo et al., 1994). Apart from the cell wall proteins, phenolic compounds containing cell wall polymers such as lignin and suberin are rapidly produced following infection to increase resistance to pathogens in many plants. 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 (Veronese et al., 2003; Guest and Brown, 1997). In addition, the phenylpropanoid metabolic pathway, which supplies metabolic compounds to lignin and anthocyanin biosynthesis, plays an important role in plant defense against invading pathogens (Vogt, 2010). Hence, reinforcement of cell walls, which can improve host resistance, is initiated as a primary defense response early in the process of host-pathogen interaction. We discuss the seed coat cell wall layer development, structure, composition, and its effect on disease resistance in the following sub-sections.

## 9. Application of seed coat cell wall mediated resistance in plant breeding and biotechnology

Screening of germplasm lines for the presence or relative abundance of *A. flavus* resistance compounds has not been performed. Wet chemistry methods such as gas chromatography–mass spectrometry (GC–MS) or HPLC could be used to examine the specific phenolic composition in peanut seed coat cell wall and possibly identify the specific phenolic compounds which account for resistance in peanut lines. Identification of the specific phenolics conferring *A. flavus* resistance and/or aflatoxin reduction will offer a novel strategy to improve the composition and quantity in peanuts using plant breeding and/or genetic manipulation. In addition, it paves a way to develop a dry chemistry method such as Fourier-transform infrared spectroscopy (FTIR) and/or Near Infra-Red (NIR) techniques which could be used as novel tools to identify *A. flavus* resistant lines. These lines can be further confirmed for the resistance using well established in vitro seed colonization assay (IVSC), preharvest aflatoxin contamination (PAC), and aflatoxin production (AP). The dry chemistry methods are particularly useful in countries where expensive equipment (GC–MS and HPLC) and wet lab skills are not extensively available. The dry chemistry does not need extensive processing or storage facilities as seed coat powder or perhaps even in fact seeds can be used for the analysis. With the availability of the whole genome sequence, QTL, eQTL, and transcriptome data, it is possible to develop gene-specific functional molecular markers for molecular breeding. Further, specific genes can be over-expressed or knocked out using genome editing for developing *A. flavus* resistance and aflatoxin reduction using biotechnological approaches.

## 10. Summary and future directions

*A. flavus* infection and aflatoxin contamination in major food crops attracted scientific and economic importance due to its severe adverse effects on agricultural products, livestock, and human health. With stringent regulations on the permissible levels of aflatoxins, peanut imports from Asia and Africa are adversely affected by *A. flavus* and aflatoxin contamination. Multiple strategies have been employed to reduce the pathogen infection and toxin contamination; however, the menace remains a major challenge for peanut producers across the globe. Several factors contributing to aflatoxin contamination remain a mystery: nature of biosynthetic genes, variation in toxin production within the fungal species, evolutionary origins, biological and environmental factors inducing toxin production, and the biological/ecological role of aflatoxin to the *Aspergillus* species. Aflatoxin contamination and *A. flavus* infections are to be further investigated, as they still pose contamination threats, even with proper screening and handling of harvested crops as well as pre-harvested crops. Genomic, molecular, and management practices have been employed to mitigate aflatoxin contamination and *A. flavus* infection, and the addition of biochemical approaches will offer an additional strategy to address this issue. Since *A. flavus* infects the seed during seed development (transition to dormancy) and/or during seed storage, it does not encounter any active genetic resistance from the live cell(s). The dried and desiccated peanut seed coat which is primarily composed of cell walls, acts as a physical and biochemical barrier against both pre- and post-harvest pathogen infections. Cell walls play a significant role in defending the plants against pathogen infections and the structure and composition of peanut seed coat has been shown to reduce *A. flavus* infection and aflatoxin contamination. Several cell wall fortifications such as deposition of callose, cellulose, lignin, phenolic compounds, and structural proteins help to prevent pathogen infection. Clearly, further studies on the combination of seed coat cell wall structure and composition of phenolic compounds are required to develop effective strategies against *A. flavus* infection and aflatoxin contamination. Recently, single cell transcriptomics technique was used to identify transcription factors in peanut leaf blade development, and such techniques can be expanded to understand spatial level changes in genes and proteins during peanut seed coat development and its potential in *A. flavus* resistance (Liu et al., 2021).

A comprehensive understanding of peanut seed coat development, genes involved, biochemistry, and their roles in disease resistance will

enable researchers to understand the mechanism and design efficient strategies for seed coat cell wall-mediated resistance against *A. flavus* infection and aflatoxin contamination. Prevention of post-harvest infection and contamination can be highly useful in developing countries where there is lack of proper and adequate storage facilities. Successful exploitation of seed coat cell wall mediated resistance in peanut can also be applied to crops belonging to the family Fabaceae. Once the compounds conferring resistance are identified through wet chemistry methods (HPLC and/or GC–MS), rapid dry chemistry techniques such as FTIR and/or NIR can be developed. The dry chemistry methods are less expensive and efficient methods that can be used to screen germplasm lines by peanut breeders to reduce *A. flavus* infection and aflatoxin contamination. This can provide an alternative and rapid method to screen germplasm lines compared to the three traditionally known measurer, viz. in vitro seed colonization, pre-harvest aflatoxin contamination, and aflatoxin production. Further, specific genes involved in seed coat cell wall structure and/or biochemical production can be overexpressed or knocked out using genome editing for developing *A. flavus* resistance and aflatoxin reduction.

## Acknowledgments

**Funding**: We thank the funding agencies USAID under CGIAR-US University linkage grant program (AID-BFS-G-11-00002-11)" and USAID-Peanut Lab (7200AA18CA00003) for supporting this study. We would like to thank Winifred Asbjornson Endowment and Montana State University for partial financial support for this study. We would like to thank Harman Kaur for proofreading the manuscript.

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