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RESEARCH ARTICLE

Enhancing resistance to aflatoxin in groundnut through seed coat-mediated lignification

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Abstract

Groundnut (*Arachis hypogaea* L.) is an important oilseed crop of the world. Aflatoxin contamination of seeds by *Aspergillus flavus* is limiting groundnut quality and trade in India. Thirty-six groundnut genotypes were evaluated for *A. flavus* incidence and aflatoxin accumulation using *in-vitro* seed colonization assay and under field conditions. The study identified one high oleic acid line ICGV 15090 with low *A. flavus* severity (1.0) and aflatoxin accumulation (1358.63 ppb) and infection percentage of 9.33 with aflatoxin contamination of 2.59 ppb under both the conditions. The genotype ICGV 15090 also recorded high cellulose (120 mg/g cell wall), anthocynanin (0.22) and lignin (3.55%) contents in the seed coat. The negative correlation between aflatoxin and lignin content (r = -0.67) indicated the role of seed coat cell wall lignin in conferring resistance to aflatoxin contamination in groundnut. Breeding for lignin fortification in seed coat can be explored to inhibit *A. flavus* infection and aflatoxin contamination in groundnut.

Keywords: Groundnut, *Aspergillus flavus*, aflatoxin, seed coat, lignin, resistance

Introduction

Groundnut (*Arachis hypogaea* L.) is an important legume and oilseed crop in tropical and sub-tropical regions of India and Sub-saharan Africa nations. India is the leading producer of groundnut with a production of 6.72 m t in an area of 4.73 m ha. However the productivity is low at 1422 kg/ha compared to USA (4426 kg/ha) and China (3900 kg/ha; FAOSTAT 2020). This is mainly attributed to cultivation in marginal soils under rainfed conditions and occurrence of biotic and abiotic stresses (Kadiyala et al. 2021). Among these, aflatoxin contamination of seeds due to *Aspergillus flavus* Link Ex Fries and *A. parasiticus* Spear is an important threat affecting groundnut quality and trade prospects (Ziyaee et al. 2021). Aflatoxins are considered as Group I carcinogenic compounds and can cause hepato-cellular carcinoma (Kortei et al. 2021). Exposure to aflatoxin reduces immunity, which can consequently increase susceptibility to other diseases such as HIV (Guan et al. 2021). The permissible limit for aflatoxin in food commodities in India is 30 µg/kg which is higher compared to the limits in European Union (4 µg/kg) and USA (20 µg/kg). The levels are even more stringent in dried nuts ranging from 2-12 µg/kg for B_1 aflatoxin and 4-15 µg/kg for total aflatoxins (Sharma and Parisi 2017; Habschied et al. 2021). Despite being the second largest producer of groundnut, India has low export of 800,00 tonnes per year due to aflatoxin contamination (Suneja 2019).

Groundnut resistance mechanisms to aflatoxins can be broadly categorized as resistance to seed colonization, pre- and post-harvest fungal contamination and aflatoxin production (Ncube and Maphosa, 2020). Though several genotypes with considerable resistance to *A. flavus* have been identified (Lai et al.2015; Yu et al. 2019), to date however, there is no genotype that combines all these resistance mechanisms to combat the pathogen and aflatoxins (Pandey et al. 2019). Groundnut breeding for aflatoxin resistance is challenging due to limited availability of improved

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germplasm, instability of pre-harvest resistance, lack of standardized screening protocols, significant genotype x environment interaction for aflatoxin contamination limited understanding of genetics of resistance and absence of correlation between *A. flavus* and *A. parasiticus* resistance under field and laboratory conditions (Nigam et al. 2009; Sharma et al. 2018; Yu et al. 2019).

In groundnut, cotyledons are the feeding site for *A. flavus* and this is where the aflatoxins are produced (Nigam et al. 2009). *A. flavus* has to overcome two physical barriers viz., pod shell and seed coat to reach the cotyledons. Though the pod shell confers resistance to pod infection by *Aspergillus*, groundnut is sold mainly after shelling and is hence prone to post-harvest seed infection and aflatoxin contamination in addition to pre-harvest infection during seed development stage (Nigam et al. 2009). Groundnut resistance to *A. flavus* is also attributed to seed coat characteristics such as thickness and density of palisade layers, presence of wax layers and absence of fissures and cavities (Liang et al. 2006; Nigam et al. 2009).

Aflatoxin control and prevention strategies mainly include blocking the infection process of *A. flavus*. In groundnut, the seed coat composition is very critical since the biochemicals are involved not only in developmental processes but also in defence responses. For instance, several genes related to flavonoid biosynthesis at early stage of seed coat development resulted in accumulation of phenolic compounds in epidermal layer of seed coat (Wan et al. 2018). Though the biochemistry of seed coat is well-studied in groundnut, there is a limited understanding of seed coat-mediated A. flavus resistance. A comprehensive understanding of the role of seed coat biochemical characteristics is necessary to develop efficient strategies for seed coat-mediated *A. flavus* resistance and to mitigate aflatoxin contamination. Keeping this in view, the study has been conducted to identify resistance source to *A. flavus* using in vitro seed colonization assay and under field conditions; and to estimate potential seed coat biochemical components for *A. flavus* resistance in groundnut.

Materials and methods

The experiment was carried out in the rainy and post-rainy seasons of 2019 and 2020 at Groundnut Research Unit, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, India. Thirty six groundnut genotypes were selected based on prior knowledge of their oil content and the level of resistance to *A. flavus* (Table 1). Seed material of the genotypes was obtained from Groundnut Breeding Unit, ICRISAT. The experiments were carried out for evaluating the *A. flavus* colonization severity and estimating aflatoxin production using in-vitro seed colonization assay; screening the genotypes for *A. flavus* infection and aflatoxin contamination under field conditions; and evaluating the genotypes for variation in seed coat cell wall components.

Evaluation of groundnut genotypes using in-vitro seed colonization assay

Groundnut seeds were evaluated for *A. flavus* colonization using *in vitro* seed colonization assay. Pure culture of *A. flavus* strain AF11-4 was obtained from Groundnut Pathology Laboratory, Research Program-Asia ICRISAT, Hyderabad, India. A spore suspension at a concentration of 1.0 x 10^6 spores/mL of sterile, distilled water containing 0.01% polysorbate surfactant Tween 20 was prepared for inoculation. For each genotype, 30 well-matured seeds with intact seed coat and devoid of any damage were selected and surface sterilized with 0.1% mercuric chloride for 3 minutes. Seeds were subsequently washed in sterile, distilled water for three times to remove any traces of mercuric chloride. Ten seeds dipped in *A. flavus* spore suspension for four minutes were placed on moist blotting paper in a petri dish. For each genotype, three Petri dishes were maintained as three replications. The plates were placed in semi-rigid plastic boxes lined with blotting paper and maintained at 27 ± 1°C and relative humidity of 95% in dark for 7 days. Individual seeds were scored for surface colonization by *A. flavus* and colonization severity was calculated for all the genotypes on a scale of 1-4 (Thakur et al. 2000) where 1: <5 per cent seed surface colonized with scanty mycelial growth and scanty sporulation; 2: 5–25% seed surface colonized with good mycelial growth and scanty sporulation; 3: 26–50% seed surface colonized with good mycelial growth and good sporulation and 4: >50 per cent seed surface colonized with heavy sporulation.

The aflatoxin content in the seeds was estimated by indirect competitive enzyme-linked immunosorbent assay (Reddy et al. 2001). For this, 20 g of finely powdered groundnut seeds were mixed in a solution containing 70% methanol and 0.5% KCl for 30 minutes at 300 rpm. The seed extract was filtered on a qualitative filter paper and the filtrate was stored in 15 mL centrifuge tube. Antibody production, sample preparation and ELISA procedure were conducted according to Reddy et al. (2001). Briefly,

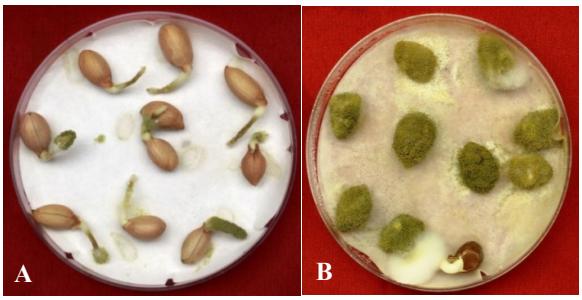

Fig. 1. Groundnut genotypes (A) ICGV 15090 and (B) ICG 10020 showing *Aspergillus flavus* strain AF11-4 colonization using *in vitro* seed colonization assay with AfB_1 concentration of 1358.63 ppb and 12663.50 ppb, respectively

Table 1. Details of the groundnut genotypes used in the study to evaluate the role of seed coat biochemical components for resistance to *Aspergillus flavus* and aflatoxin accumulation

S. no	Name of the genotype	BG	Pedigree	Remarks
1	ICGV 03043	SB	[ICGV 99160 × (ICGV 93124 × ICGS 44)]	High oil
2	ICG 1122	-	Landrace collection from Andhra Pradesh	>2% infection
3	ICG 1323	-	Landrace collection from Andhra Pradesh	>2% infection
4	ICG 3700	-	Landrace collection from Andhra Pradesh	>2% infection
5	ICG 1326	-	Landrace collection from Andhra Pradesh	>2% infection
6	ICGV 02206	SB	(ICGV 93280 × J 11) × ICGV 88145	Tolerant to A. flavus
7	ICG 10020	-	Landrace collection from Andhra Pradesh	>2% infection
8	ICGV 02207	SB	(ICGV 93280 × J 11) × ICGV 88145	Tolerant to A. flavus
9	ICGV 91278	SB	V4-7-5 × ICGS 11	0–7 ppb
10	ICG 4749	-	Landrace collection from Telangana	>2% infection
11	ICG 1994	-	Landrace collection from Telangana	>2% infection
12	ICG 3336	-	Landrace collection from Telangana	>2% infection
13	ICGV 181065	SB	ICGV 022066 × ICGV 15059	High oleic acid line
14	ICG 1859	-	Landrace collection from Maharashtra	>2 % infection
15	ICG 9407	-	Landrace collection from Maharashtra	>2% infection
16	ICG 10094	-	Landrace collection from Maharashtra	>2% infection
17	ICGV 07222	SB	[(ICGV 92069 × ICGV 93184) × (NCAC 343 × ICGS 44)]	High oil
18	ICGV 03042	SB	[ICGV 99160 × (ICGV 93124 × (L1 × ICGS 44)]	High oil
19	ICGV 181490	SB	DH 86 × Sunoleic 95	High oleic acid
20	ICGV 91284	SB	J 11 × ICGV 86184	0–7 ppb
21	ICG 1173	-	Landrace collection from Andhra Pradesh	>2% infection
22	ICGV 15083	VB	ICGV 06420 × Sunoleic 95R	High oleic acid
23	55-437	SB	Parent material	-
24	ICG 27	-	Landrace collection from Maharashtra	4% infection
25	ICGV 91283	SB	V4-7-5 × JL24	0–7 ppb
26	ICGV 181075	SB	ICGV 022066 × ICGV 15059	High oleic acid
27	ICGV 91279	SB	JL 24 × VRR 245	0–7 ppb
28	ICG 3267	-	Landrace collection from Maharashtra	>2% infection
29	ICGV 03331	SB	ICGV 91334 × ICGV 92028	Tolerant to A. flavus
30	ICGV 15090	VB	ICGV 06420 × Sunoleic 95R	High oleic
31	J 11(R)	SB	Ah 4218 × Ah 4354	Resistant check
32	TMV 2	SB	Mass selection from TN	>150 ppb
33	JL 24 (S)	SB	Selection from EC 94943 (1878)	Susceptible check
34	IVGV 171002	SB	ICGV 06110 × (ICGV 06110 × Sunoleic95 R)	High oil
35	ICGV 171010	SB	ICGV 07368 × (ICGV 07368 × Sunoleic 95 R)	High oil
36	ICGV 171024	SB	ICGV 06420 × (ICGV 06420 × Sunoleic 95 R)	High oil

BG= Botanical Group; SB= Spanish Bunch; VB= Virginia Bunch;

polyclonal antibodies to AFB_1 were produced to conduct an indirect competitive ELISA on Maxi-sorp (Nunc A/S, DK-4000 Roskilde, Denmark) ELISA plates and the Log_{10} values for aflatoxin concentration were determined in the sample extract (µg/kg).

Evaluation of groundnut genotypes for A. Flavus infection under field conditions

The field experiment was conducted in *A. flavus* sick plot maintained at ICRISAT, Hyderabad during the post-rainy seasons of 2019 and 2020. The field is characterized by tropical wet and dry climate of Telangana at 17.5' latitude and 78.27 E longitudes at an altitude of 542 m above the sea level. The seed bed was prepared as fine tilth after which sowing was taken up during second fortnight of November with 40 seeds sown in double-row plots of 4 m long at spacing of 30

x 10 cm. The field was provided with irrigation immediately after sowing. The field was inoculated with *A. flavus* strain AF 11-4 at 30, 45, 60 and 75 days after sowing (DAS). For each 4 m row, 100 g of inoculum was applied near the roots of the plants and covered with soil. Water-stress conditions were imposed at 75 DAS to facilitate *A. flavus* infection. However, one life-saving irrigation was given to save the crop from extreme water stress. The experiment was laid in alpha lattice design with three replications and each replication comprised of four homogenous blocks to reduce the heterogeneity among the experimental material and to reduce inter-block effect. To estimate *A. flavus* infection, 30 well-matured seeds with intact seed coat were selected for each genotype. The seeds were surface-sterilized using 0.1% mercuric chloride solution for one minute and washed in sterilized distilled water for three times. For each genotype, ten seeds were kept in a petri dish and incubated for 9 days after which *A. flavus* infection was estimated as percentage of seeds showing pathogen colonization as per Dieme et al. (2018).

Screening of selected groundnut genotypes for variation in seed coat cell wall components

Quantitative determination of crystalline cellulose in selected groundnut genotypes was performed according to Updegraff (1969). In this method, 300 mg of groundnut seed powder was added to 1 mL protein solubilisation buffer containing 50 mM Tris HCl buffer, pH 8.8, 0.5 mM EDTA, 10% sodium dodecyl sulphate was added to solubilise the proteins. The sample was centrifuged and the process was repeated twice. The supernatant was saved for further protein analysis. The pellet was washed with 1.0 mL distilled water two times followed by a series of washes in solutions of 70% ethanol, 100% methanol, chloroform/methanol (1/1, v/v), 100% ethanol at 13,000 rpm for 10 minutes. The pellet was dried at 37°C overnight and weighed for cell wall. To the dried cell wall of known weight, 1.5 mL of Updegraff reagent (acetic acid: nitric acid: water, 8:1:2 v/v) was added and heated for 100°C for 30 minutes. The sample was washed in 1 mL water, pelleted using 1 mL acetone and dried at 37°C. The dried pellet was solubilized in 1 mL concentric sulphuric acid for 1 hour at room temperature. After this, 190 µL of distilled water and 400 µL of 0.3% anthrone reagent prepared in concentrated sulphuric acid were added and incubated at 100°C for five minutes. The absorbance was measured at 620 nm and the glucose content was determined using a standard curve plotted for different quantities of glucose solution. Crystalline cellulose was estimated by dividing the values of glucose content by a factor of 1.11, taking into account the water loss during hydrolysis.

To estimate the anthocyanins, 10 mL of 0.1% HCl/ methanol was added to 1.0 g of finely ground fresh seed and kept overnight at 4°C on a shaker. Following centrifugation at 13,000 rpm for 10 minutes, the supernatant was filtered through 0.22 µm filter and the extract was then re-suspended in hexane or methanol for GC/MS (Pang et al. 2009). For lignin estimation, 200 mg of fresh seed coat of test lines were suspended in 0.8 mL of 80% methanol by vortexing and after centrifugation at 13,000 rpm for 10 minutes, the extracts were re-suspended in methanol. The process was repeated and the extract was filtered through 0.22 µm filter and vacuum-dried at 45°C. The dried extract was weighed and re-suspended in hexane for GC/MS analysis. One mL of acetone was added to the seed coat pellet and extracted after centrifugation at 13,000 rpm for 10 minutes. The process was repeated after which the pellet was dried overnight. The pellet was suspended in 1.0 mL thioglycolic acid and 3 M HCl solution and incubated at 80°C for 3 hours. The pellet was vacuum-dried at 30°C for 3 hours. The pellet was suspended in 1.0 mL NaOH solution and incubated overnight at 37°C and centrifuged at 13,000 rpm, for 10 minutes. The supernatant was suspended in 200 µL HCl and precipitated for 4 hours at 4°C. After centrifugation at 13,000 rpm for 10 minutes, the pellet was vacuum-dried at 30°C for 4 hours and resuspended in DMSO overnight at 37°C. After final centrifugation, the supernatant was collected and OD was estimated at 280 nm (Robinson and Mansfield 2009).

The analysis of variance (ANOVA) was calculated using SAS version 9.2, SAS Institute Inc., 2013.

Results and discussion

An attempt was made to identify potential groundnut genotypes with resistance to *A. flavus* colonization and aflatoxin production using *in-vitro* seed colonization assay and under field conditions. Further, these genotypes were evaluated for their seed coat cell wall biochemical components *viz.,* cellulose, anthocyanins and lignin for their role in *A. flavus* resistance and aflatoxin production.

Evaluation of groundnut genotypes using in-vitro seed colonization assay

Groundnut genotypes showed significant differences ($p < 0.01$) in *A. flavus* colonization severity and aflatoxin concentration levels (Table 2). None of the genotypes screened in this study were immune to the highly toxigenic *A. flavus* strain AF 11-4 reaffirming the virulence of the strain used in the present study. The *A. flavus* colonization severity among the genotypes ranged from 1 (ICGV 15090) to 4 (ICG 10020 and ICG 27) with a mean colonization severity of 2.37 (Fig. 1; Table 3). Genotypes, ICGV 181490 (1.10), ICGV 181065 (1.13), ICG 4749 (1.16) and JL 24 (1.16) recorded low colonization and genotypes, ICG 3336, ICG 10094 (3.80), ICG 1323 and ICGV 9407 (3.20) recorded high colonization severity. In addition to *A. flavus* colonization, the seeds were also tested for aflatoxin production. None of the evaluated genotypes were free from aflatoxin accumulation, which

ranged from 1358.63 ppb (ICGV 15090) to 16147.36 ppb (ICGV 03331) with a mean production of 11768.68 ppb. Following the genotype ICGV 15090, which recorded both low *A. flavus* severity and aflatoxin contamination, genotype ICGV 181075 (1767.70 ppb) had significantly less aflatoxin content. Genotypes ICG 3267 (15754.93 ppb) and ICG 1173 (15522.76 ppb) recorded high aflatoxin contents.

The variable performance of the genotypes in *A. flavus* colonization and aflatoxin accumulation are in agreement with previous findings (Mehan et al. 1986; Mehan et al. 1986b; Mehan et al. 1987). More recently, Commey et al. (2021) used the in vitro seed colonization assay, similar to the current study, and showed complete *A. flavus* colonization of groundnut without seed coat in both resistant and susceptible genotypes, thus demonstrating the role of intact seed coat in reducing *A. flavus* infection. They also reported stronger inhibition of *A. flavus* growth in resistant line compared to the susceptible line, with or without intact seed using both toxigenic and atoxigenic strains of *A. flavus*. Further, in their study, the genotype 55-437 showed 21.20 % *A. flavus* incidence which is similar to the colonization severity of 2.16 (equivalent to 5-25% surface colonization) obtained in the current study. On the other hand, they reported 52.29 % in susceptible line TMV 2 compared to the lower severity of 1.36 (equivalent to 26-50 % surface colonization) obtained in this study, which might be due to different toxigenic strains used in both the studies.

Within the evaluated genotypes, variations in *A. flavus* colonization and aflatoxin accumulation could be attributed to both physical (seed coat, wax and cutin deposition etc.) and chemical (tannins, isoflavones, chitinases, trypsins and lignin etc.) barriers (Soni et al. 2020). It is to be noted that the genotypes used in this study viz., ICGV 15090 and ICGV 181075, both of which had low *A. flavus* colonization and aflatoxin production, are high oleic acid lines (Shashidhar et al. 2020). This assumes significance since an earlier study by Shan et al. (2006) has reported that groundnut varieties with high oleic acid coupled with high protein, low linoleic and low fat contents were highly resistant to *A. flavus*. Furthermore, it has also been reported that the composition of fatty acids affects aflatoxin production and that resistance to *A. flavus* can be improved by altering the fatty acid composition (Zubair et al. 2011).

In groundnut, there are only few reports, with different findings, evaluating the effect of altered fatty acid composition of seeds on aflatoxin production. A laboratory study comparing the effects of different fatty acid-fortified media revealed that three individual fatty acids viz., myristic acid, palmitic acid and stearic acid stimulated aflatoxin B1 synthesis while two unsaturated fatty acids, oleic acid and linoleic acid inhibited the toxin synthesis (Priyadarshini and Tulpule 1980). A field study comparing groundnut genotypes with reduced versus normal linoleic acid composition revealed no measurable effect of these fatty acids on pre-harvest aflatoxin contamination (Holbrook et al. 2000). In contrast to both these studies, Xue et al. (2003) and Xue et al. (2005) reported that high-oleate (low-linoleic) Virginia-type of cultivars developed more aflatoxin than normal- to high-linoleate lines. This is in contrast to the study by Priyadarshini and Tulpule (1980) and our current finding on low aflatoxin in high oleic acid genotypes ICGV 15090 and ICGV 181075. Since the most abundant fatty acids in groundnut seeds are linoleic, oleic and plamitic acids (Fabbri et al. 1984; Passi et al. 1984), the fatty acid compositions of the high-oleic acid genotypes ICGV 15090 and ICGV 181075 can be explored further for aflatoxin management. The roles of key genes/transcription factors viz., linoleate 95-lipoxygenase, resveratrol synthase, chalcone synthase, defensins, chitinases involved in *A. flavus* resistance during in-vitro seed colonization (Nayak et al. 2017) in these genotypes can be further investigated for developing aflatoxin-free groundnut.

Table 2. Analysis of variance for *Aspergillus flavus* strain AF11-4 infection and aflatoxin accumulation in the groundnut genotypes evaluated using *in vitro* seed colonization assay and under field conditions during 2019 and 2020

***In vitro* seed colonization assay**			
Source of variation	Degrees of freedom	Colonization severity	AfB1 (ppb)
Treatment	32	2.45**	45277550**
Error	66	0.0096	316182
Total	98	2.46	45593732
** indicates significance at the 0.01 probability level; ppb: parts per billion			
Evaluation under field conditions			
Source of variation	Degrees of freedom	Infection percentage	AfB1 (ppb)
Treatment	35	509.1** (206.9**)	3.52**
Error	72	0.64 (0.26)	0.008
Total	107	509.7 (207.16)	3.52

** indicates significance at 0.01 and 0.05 probability levels; values in parenthesis are arcsine transformed values for A. flavus infection percentage; ppb = parts per billion.

Table 3. Mean performance of the groundnut genotypes for infection to *Aspergillus flavus* strain AF11-4 and aflatoxin accumulation using *in vitro* seed colonization assay and evaluation under field conditions during 2019 and 2020

S. no.	Name of genotype	*In-vitro* seed colonization assay		Evaluation under field conditions	
		Colonization severity	AfB_1 (ppb)	Infection percentage	AfB_1(ppb)
1	ICGV 03043	2.20	10894.56	15.66 (23.31)	1.56
2	ICG 1122	2.36	14631.96	20.66 (27.03)	1.53
3	ICG 1323	3.23	11932.56	30.66 (33.62)	1.05
4	ICG 3700	2.70	13114.43	15.66 (23.31)	1.88
5	ICG 1326	2.23	11729.20	20.66 (27.03)	1.102
6	ICGV 02206	3.20	9620.03	31.33 (34.03)	1.2
7	ICG 10020	4.00	12663.50	31.33 (34.03)	7.36
8	ICGV 02207	2.96	13614.60	33.66 (35.46)	2.35
9	ICGV 91278	2.8	12415.63	51.66 (45.95)	1.22
10	ICG 4749	1.16	11636.00	35.33 (36.47)	2.29
11	ICG 1994	2.70	11736.73	41.33 (40.00)	1.55
12	ICG 3336	3.83	12842.70	35.33 (36.47)	1.06
13	ICGV 181065	1.13	11929.20	36 (36.86)	2.13
14	ICG 1859	2.46	13455.36	21.33 (27.50)	2.12
15	ICG 9407	3.20	14941.73	21 (27.27)	1.28
16	ICG 10094	3.83	14821.16	21(27.27)	1.03
17	ICGV 07222	1.83	14429.66	35.33 (36.47)	1.00
18	ICGV 03042	2.86	16748.00	32.66 (34.85)	1.14
19	ICGV 181490	1.10	8461.43	15.33 (23.05)	2.82
20	ICGV 91284	1.33	7731.20	10.66 (19.06)	1.51
21	ICG 1173	2.03	15522.76	43.66 (41.36)	1.24
22	ICGV 15083	1.40	6065.43	49.66 (44.80)	1.34
23	55 - 437	2.16	11608.96	31.33 (34.03)	3.05
24	ICG 27	4.00	14328.80	55.66 (48.25)	1.83
25	ICGV 91283	2.53	13107.33	53 (46.71)	1.87
26	ICGV 181075	1.13	1767.70	35.33 (36.47)	2.26
27	ICGV 91279	2.30	14706.66	40.66 (39.62)	1.35
28	ICG 3267	2.76	15754.93	36.33 (37.06)	2.08
29	ICGV 03331	2.26	16147.36	24.33 (29.55)	2.13
30	ICGV 15090	1.00	1358.63	9.33 (17.78)	2.59
31	J 11 (R)	2.86	3821.50	15.66 (23.31)	2.14
32	TMV 2	1.36	10255.86	60.66 (51.15)	1.52
33	JL 24 (S)	1.16	14570.66	41.33 (40.00)	2.41
34	ICGV-171002	1.26	11768± 562.30	34.33 (35.86)	1.36
35	ICGV-171010	1.14	1358.63	42(40.39)	1.56
36	ICGV-171024	1.68	16748.00	43.66 (41.36)	2.08
	Mean ± S.E	2.36±0.098	4.78	32.6±0.80 (34.35±0.51)	1.89±0.092
	CV %	4.16	4.78	2.4 (1.5)	4.88
	CD at 5%	0.161	916.66	1.31 (0.84)	0.1505

Values in parenthesis are arcsine transformed values for *A. flavus* infection percentage; ppb = Parts per billion; R= Resistant check; S= Susceptible check

Table 4. Spearman's rank matrix correlation on *Aspergillus flavus* strain AF11-4 and aflatoxin accumulation in groundnut genotypes evaluated using *in vitro* seed colonization assay and under field conditions

***In-vitro* seed colonization assay**		
Traits	Colonization severity	AfB_1 (ppb)
Colonization severity	1.00000	0.44899**
AfB_1 (ppb)		1.00000
** indicates significance at 0.01 probability level; ppb: parts per billion		
Evaluation under field conditions		
Traits	Infection percentage	AfB_1 (ppb)
Infection percentage	1.00000	-0.099ns
AfB1 (ppb)		1.00000
Ns= non significant, p=0.56; ppb= parts per billion		

Table 5. Analysis of variance for seed coat biochemical components and aflatoxin accumulation in groundnut genotypes

Source of variation	Degrees of freedom	Cellulose (mg/g of cell wall)	Lignin (%)	Anthocyanin (average absorbance at 520 nm)	AfB_1 (ppb)
Treatment	32	337.62**	0.06**	0.006**	31232059.5**
Error	33	5.16	0.01	0.00003	4850.3
Total	65	342.78	0.07	0.00603	31236909.8

** indicates significance at 0.05 and 0.01 probability levels; ppb= parts per billion

Spearman's rank correlation test revealed a positive moderately weak association ($r = 0.44$, $p < 0.01$) between colonization severity and aflatoxin accumulation among the selected groundnut genotypes indicating *A. flavus* induced aflatoxin production in groundnut seeds (Table 4). For example, the colonization severity and aflatoxin accumulation were the lowest in genotype ICGV 15090 (1 and 1358.63 ppb, respectively) and ICGV 181075 (1.3 and 1767.70 ppb, respectively). On the other hand, in genotypes with similar *A. flavus* colonization severity like ICGV 181490 (1.10) and ICG 91284 (1.13), the aflatoxin accumulation was much higher with 8461.43 and 7731.20, respectively. This weak correlation between *in-vitro* seed colonization by *A. flavus* in resistant genotypes and aflatoxin accumulation, also reported by Dieme et al. (2018), suggested that these two mechanisms might be controlled by different genes and combining them on the same genetic background might provide resistance to both *A. flavus* colonization and aflatoxin accumulation.

Evaluation of groundnut genotypes for A. flavus resistance under field conditions

significant differences ($p < 0.01, 0.05$) were found among the genotypes for both *A. flavus* infection and aflatoxin accumulation under field conditions (Table 2). The infection of *A. flavus* ranged from 9.33 (ICGV 15090) to 60.66 % (TMV 2). High infection was also recorded in ICG 27 (55.66%) and ICGV 91278 (51.66%). Genotypes ICGV 03043 (15.66%), ICG 3700 (15.66%), J 11 (15.66%) and ICGV 181490 (15.33%) recorded low infection of *A. flavus* (Table 3). Aflatoxin accumulation in the genotypes ranged from 1 (ICGV 07222) to 7.36 ppb (ICG 10020). Genotypes ICG 10094 (1.03 ppb), ICG 3336 (1.06 ppb) ICG 1326 (1.01 ppb) and ICGV 03042 (1.14 ppb) also recorded low aflatoxin contents. The resistant check J 11 recorded 2.14 ppb and susceptible check JL 24 recorded 2.41 ppb aflatoxin. Spearman's rank correlation test between *A. flavus* infection and aflatoxin accumulation revealed a negative (non-significant) association ($r = -0.099$, $p > 0.01$) among the selected groundnut genotypes (Table 4) suggesting that *A. flavus* infection and aflatoxin accumulation are controlled by different genes and that the genetic mechanisms to these two traits are independent (Ozimati et al. 2014).

In both *in vitro* colonization assay and evaluation under field conditions, only one genotype *viz.,* the high oleic acid line ICGV 15090 consistently showed low *A. flavus* severity and less aflatoxin accumulation. Currently, there are no known reports of the presence of three resistance mechanisms *viz., in-vitro* seed colonization, pre-harvest aflatoxin contamination and aflatoxin production in a single genetic background (Pandey et al. 2019). The identification of ICGV 15090 in this study provides an excellent opportunity to achieve stable genetic resistance against *A. flavus* infection in the field.

In the present study, Spearman's rank correlation test revealed different associations between *A. flavus* infection and aflatoxin accumulation among the evaluated genotypes using *in-vitro* seed colonization assay and field evaluation. While the association was found to be positive and moderately weak with the *in-vitro* assay, it was non-significantly negative under field evaluation. These differential associations might be due to different factors influencing the resistance mechanisms such as thick layer of cutin, wax and high level of lignin affecting resistance to seed invasion and colonization of seed coat and factors such

Table 6. Mean performance of groundnut genotypes for seed coat biochemical components and aflatoxin accumulation

S. no	Genotypes	Cellulose (mg/g of cell wall)	Lignin (%)	Anthocyanin (Avg. absorbance at 520 nm)	AfB_1 (ppb)
1	ICGV 03043	73.50	3.15	0.06	10229.20
2	ICG 1122	74.00	3.00	0.06	14637.80
3	ICG 1323	86.00	3.05	0.05	11943.50
4	ICG 3700	86.00	3.10	0.11	14114.05
5	ICG 1326	80.50	3.05	0.07	11743.70
6	ICGV 02206	95.50	3.05	0.16	9619.90
7	ICG 10020	92.50	3.05	0.06	12670.10
8	ICGV 02207	78.00	3.35	0.14	9621.80
9	ICGV 91278	102.50	3.10	0.15	12423.30
10	ICG 4749	87.50	3.00	0.05	11652.40
11	ICG 1994	90.50	3.15	0.06	11730.00
12	ICG 3336	106.50	2.95	0.06	12863.90
13	ICGV 181065	77.50	3.10	0.15	11943.70
14	ICG 1859	103.00	3.10	0.12	13472.90
15	ICG 9407	89.50	3.00	0.07	14942.50
16	ICG 10094	96.00	3.35	0.15	8521.60
17	ICGV 07222	94.00	3.25	0.12	14444.50
18	ICGV 03042	80.50	2.90	0.06	16797.50
19	ICGV 181490	108.50	3.35	0.10	8467.10
20	ICGV 91284	110.50	3.20	0.19	7776.70
21	ICG 1173	97.00	2.95	0.05	15524.05
22	ICGV 15083	102.50	3.35	0.18	6073.05
23	55 - 437	116.00	3.45	0.21	6112.80
24	ICG 27	104.00	3.10	0.12	14328.10
25	ICGV 91283	96.50	3.45	0.21	9410.90
26	ICGV 181075	100.00	3.50	0.17	1771.40
27	ICGV 91279	109.00	3.25	0.23	14707.30
28	ICG 3267	88.00	3.00	0.17	15747.30
29	ICGV 03331	90.00	3.35	0.11	9164.00
30	ICGV 15090	120.00	3.85	0.22	1355.30
31	J 11(R)	117.50	3.30	0.20	3797.20
32	TMV 2	76.50	3.15	0.06	10273.60
33	JL 24(S)	81.00	3.25	0.06	14630.90
	Mean±S.E	94.25±2.27	3.17±0.10	0.11±0.005	10985.21±69.6
	CV %	2.41	3.28	4.71	0.58
	CD at 5%	4.62	0.21	0.01	141.60

Ppb= parts per billion; R= Resistant check; S= Susceptible check

Table 7. Pearson correlation matrix between seed coat biochemical components and aflatoxin accumulation in groundnut genotypes

Traits	Cellulose (mg/g of cell wall)	Lignin (%)	Anthocyanin (average absorbance at 520 nm)	AfB_1 (ppb)
Cellulose (mg/g of cell wall)	1.00000	0.41236*	0.63553**	-0.46557**
Lignin (%)		1.00000	0.60439**	-0.67288**
Anthocyanin (average absorbance at 520 nm)			1.00000	-0.39392*
AfB_1 (ppb)				1.00000

** indicates significance at 0.05 and 0.01 probability levels; ppb= parts per billion

as terminal drought, temperature of 25–30°C and moisture (>7 %) influencing resistance to pre-harvest aflatoxin contamination (Soni et al. 2020).

Estimation of seed coat biochemical components and aflatoxin concentration in groundnut genotypes

Significant differences in seed coat biochemical components viz., cellulose, lignin, anthocyanin and aflatoxin concentration were observed among the groundnut genotypes ($P < 0.01$) (Table 5). The cellulose content in the cell wall ranged from 73.5 mg/g (ICGV 03043) to 120 mg/g (ICGV 15090) (Table 6). Most of the genotypes had cellulose ranging between 80 to 100 mg/g of cell wall. Genotypes, J 11 (117.5 mg/g) and 55-437 (116 mg/g) have recorded high cellulose in the cell wall. The lignin content in the cell wall ranged from 2.9 (ICGV 03042) to 3.55 % (ICGV 15090). Except for three genotypes viz., ICGV 03042, ICG 3336 and ICG 1173, the rest of the genotypes recorded more than 3% lignin content in their seed coats. The anthocyanin content ranged from 0.05 (ICG 1173, ICG 4749 and ICG 1323) to 0.23 (ICGV 91279). While the genotypes TMV 2, ICGV 03043, ICG 10020, ICG 3336, JL 24, ICG 1122, ICG 1994 and ICGV 03042 recorded low anthocyanin content of 0.06, genotypes ICGV 15090 (0.22), 55-437 (0.21) and ICGV 91283 (0.21) recorded high anthocyanin content. The aflatoxin concentration ranged from 1355.30 (ICGV 15090) to 16797.50 ppb (ICGV 03042) among all the tested genotypes. Low aflatoxin concentrations were also recorded in the genotype ICGV 181075 (1771.45 ppb), J 11 (3797.20 ppb), and ICGV 15083 (6073.05 ppb).

Seed coat provides a protective layer to the developing zygote and is an important physical barrier for any pathogen entry. Successful penetration and colonization of cell wall by *A. flavus* are pre-requisites for aflatoxin contamination in groundnut (Soni et al. 2020). Host-mediated resistance involving the production of the natural phytoalexin resveratrol by the developing seed has been reported in groundnut (Pandey et al. 2019). Upon infection by *A. flavus,* a wide range of genes involved in reactive oxygen species (ROS) detoxification such as resveratrol synthase, phenylalanine ammonia lyase, chalcone synthase, catalses, superoxide dismutase, glutathione-S-transferease, senescence-associated protein etc. are expressed to block *Aspergillus* growth and aflatoxin production (Nayak et al. 2017). These resistance-conferring genes are involved in the production of compounds such as phenylpropanoids, coumarins, stilbenes, cinnamic acid, flavonoids, ascorbate etc. which are the primary constituents of groundnut seed coat (Wan et al. 2016; Wang et al. 2016).

In groundnut, flavonoid and phenylpropanoid biosynthesis pathways were reported to be related to aflatoxin resistance (Garcia et al. 2013; Wang et al. 2016). Lignins together with anthocyanins, flavonols and proanthocyanidins constitute the main group of plant phenylpropanoids (Fornalé et al. 2010). The low *A. flavus* incidence and aflatoxin accumulation in genotype ICGV 15090 might be due to high lignin and anthocyanin contents in the seed coat. Increase in lignin and insoluble proanthocyanin in the seed coat were also reported in groundnut resistant to *Aspergillus* (Cobos et al. 2018). In this context, knowledge in seed coat biochemical composition will not only help in understanding the genetic control of *Aspergillus* resistance and aflatoxin contamination, but also in improving groundnut seed quality (Wan et al. 2016).

Pearson correlation matrix test revealed a negative moderately weak association ($p < 0.01$) between cellulose ($r = -0.46$), anthocyanin ($r = -039$) with aflatoxin concentration among the selected groundnut genotypes. A negative association was found between aflatoxin concentration and lignin ($r = -0.67$) (Table 7). This has several implications for developing *A. flavus* resistance in groundnut through identification of candidate genes, pathways and regulatory networks associated with these biochemical components. For example, cell wall lignification, encoded by plantacyanins (blue copper proteins) has been reported as a major defence mechanism in groundnut against *A. flavus* infection (Zhao et al. 2019). Lignin also promotes the biosynthesis of precursors involved in the strengthening of cell wall (Bedin et al. 2020). The highest accumulation of lignin with 3.85% was recorded in genotype ICGV 15090 which also had lowest *A. flavus* incidence and aflatoxin contamination implying the probable role of lignin in aflatoxin resistance. The significant negative linear correlation between lignin content and AFB_1 concentration in the outer seed fraction in maize (Bartolić et al. 2022) corroborates that lignin fortification of cell wall might play a major role in conferring resistance to aflatoxin in groundnut.

In future studies, the relationship between lignin in the seed coat and aflatoxins can be explored using tools such as optical and electron paramagnetic resonance spectroscopy to understand the differential responses in groundnut seed fractions. Lignin content may be a used as a reliable indicator to screen for aflatoxin contamination and to identify *A. flavus* resistance in groundnut. Breeding efforts strengthening the lignin content in seed might inhibit *A. flavus* infection and aflatoxin contamination in groundnut.

Authors' contribution

Conceptualization of research (SSA, MVNK, VR); Designing of the experiments (SSA, MVNK, VR, HKS); Contribution of experimental materials (MVNK, HKS, PJ); Execution of field/ lab experiments and data collection (SSA, MVNK, HKS); Analysis of data and interpretation (SSA, MVNK, VR, VGS); Preparation of the manuscript (SSA, MVNK, VR, VGS)

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