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Assessment of Groundnut Varietal Tolerant to Aflatoxin Contamination in Indonesia

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Abstract

Aflatoxin is secondary metabolite produced by *Aspergillus flavus* and *A. paraciticus* that grow on the seed coat (*testa*) of groundnut. This toxin is a serious food safety issue throughout the world. The availability of resistant genotype to *A. flavus* infection and/or aflatoxin contamination urgently needed. The experiment found one genotype had aflatoxin contamination under the safe level (≤ 10 ppb), with <15% of seed number infected by *A. flavus*. Recently, the biggest peanut industry, where the main production is roasted-peanut (in shell) produced from fresh pods, grows and develops that variety.

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Keywords: aflatoxin, Aspergillus flavus, *groundnut, varietal tolerance*

Introduction

Aflatoxin contamination, especially the most potent toxic aflatoxin B_1 (mentioned as "aflatoxin" in the rest of this paper), in groundnut seeds is a serious food safety issue throughout the world. The toxin is carcinogenic, teratogenic, mutagenic, immune-suppressive [1] and therefore it hazards both human and poultry health. The aflatoxin-producing fungi, *A. flavus* and *A. parasiticus* [2], can invade groundnut seeds when groundnut pods are still standing in the field (pre-harvest infection), during curing and drying, and in storage and transportation (post-harvest infection) [3]. Pre-harvest infection occur when environmental conditions during crop maturation are unfavorable for crop growth because of elevated temperature (up to 35°C) and prolonged moisture deficit [4]. In semi-arid environment, dry condition during end of growing season when the crop experienced to drought is conducive to pre-harvest contamination [1, 5] whereas postharvest contamination is more prevalent in wet and humid areas. Adopting some cultural practices that create *geocarphosphere* and *rhizosphere* with abundant water supply and free from insect and fungal invasion during late generative growth phase, especially in the last 4-6 weeks of growing season, could minimize pre-harvest contamination. Whilst post harvest management is

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basically undertaken to shorten the length of pods processing by introducing machineries such as pod thresher, dryer, and Sheller/de-hulling [6] and improve the technology of handling (appropriate drying, pre-cleaning, and sorting), storage structure (improving storage practices and facilities), and transportation. In addition, the use of field-aflatoxin detection kit; regulations relating food, animal feed ingredients; education and extension activities on prevention of aflatoxin contamination are some other approaches where people in developing countries would successfully adopt [7]. However, those practices/approaches have not been widely and precisely adopted by small farmers in developing countries who contribute about 90% to the world groundnut production [FAO 2006 *in* 8], where most of them manage a limited capital for groundnut crop. One of cost-effective strategy for reducing aflatoxin contamination is the use of resistant cultivar [3]. The cultivar resistant to seed infection by *A. flavus* or *A. parasiticus* or to aflatoxin production would be of great value to farmers in both developed and developing countries. Therefore, breeding for groundnut resistance to aflatoxin-producing fungi and/or aflatoxin production can play a significant role in preventing aflatoxin contamination, especially for pre-harvest aflatoxin contamination under the condition when irrigation is unavailable [1]. The availability of improved breeding lines coupled with pre and post harvest aflatoxin management practices is the comprehensive tool for reducing aflatoxin contamination of groundnut kernels harvested from farmers [9].

Research on aflatoxin contamination have been starting since 1960s and have documented the importance of drought stress, high soil temperature, and pod damage as the risk factors for increasing aflatoxin production. Later efforts were the works focused on the development of screening techniques and the identification of sources of resistance to *.A flavus* and/or aflatoxin contamination as foundation of conventional resistance breeding program to develop groundnut breeding lines that have high pod yield and low aflatoxin contamination. The recent research efforts focused on the use of molecular genetics approaches to reduce aflatoxin contamination [10]. The purpose of the experiment was to examine the resistance of genetic resources from Indonesia to aflatoxin contamination under end of season drought stress during dry season in Indonesia.

Materials and Methods

The experiment took place at Muneng Research Station, East Java, Indonesia during the dry season. The experiment applied a Randomized Block design with 10 genotypes treatment and three (3) replicates, which resulted in 30 plots. Those ten Indonesia genotypes consisted of two local varieties (Local Pati, Local Blitar), three drought tolerant lines (GH 27, GH 51 and GH 57), one foliar disease tolerant line (GH 15) and four national-improved varieties (Sima, Turangga, Komodo and Tuban). Chemical fertilizers of 23 kg N + 45 kg P_2O_5+22.5 kg K_2O/ha were applied in the furrows at planting time. Dolomite at the rate of 500 kg/ha was broadcasted at flowering stage to ensure the success of pod filling. The plot size was 12.5 m x 5 m where we can obtain at least 5 kg of fresh pods. The trial was under dry condition but irrigation water was available to ensure the success of crop establishment, good vegetative and generative growth. Irrigation was applied at planting time, 19, 36, 52, and 66 days after sowing (DAS) with amount of 135, 147, 181 and 285 m^3 respectively. Irrigation stopped at 66 DAS and, thereafter, the crops were under dry condition (around 30 days) until harvesting time (95 DAS). Unfortunately, there was 37 mm rain (one rainy day) at two days before harvest. A number of measurements was undertaken *i.e.* pod-zone soil temperatures every 4 days; pod moisture content at 50, 60, 70 and 80 DAS and harvesting time (gravimetric method); seed moisture content (gravimetric method) just before ELISA; number of seeds infected by *A. flavus* using AFPA (*Aspergillus Flavus and Paraciticus Agar*) media (100 kernels obtained from each genotype x 3 replicates); weight of three kernel categories (**sound mature kernels-SMK**: intact, mature, clean/no fungal infection; **shriveled kernels**: intact, immature, >50% seed surface was shriveled, clean/no fungal infection; and **damaged kernels**: discolor because of fungal infection, cracked, rotten) done on 2.5 kg of sun-dried pods sample; and aflatoxin content using ELISA (Enzyme Linked-Immunosorbent Assay) method developed by Alice and Kennedy [11]. Aflatoxin contamination was then grouped based on its safety for human consumption based on SNI [12](9), which stated that the maximum level of aflatoxin in peanut and

their products is ≤ 15 ppb, while for total aflatoxin is 20 ppb, and therefore any amount that is higher than 15 ppb is unsafe and non-permissible for human.

Results and Discussion

Aflatoxin content

Aflatoxin content on groundnut kernels was various among Indonesian genotypes (Table 1). The highest level of aflatoxin content was in Tuban cultivar, while the lowest was in GH 51 with 21 and 5 ppb, respectively. Furthermore, aflatoxin content of GH 51, Local Pati, Local Blitar, Sima, Turangga, GH 27, and GH 57 were in the permissible level with toxin content <15 ppb. The rest three genotypes (Komodo, GH 15, and Tuban) were in the non-permissible level with toxin content higher than 15 ppb (Table 1). Those with permissible level (<15 ppb) of aflatoxin content were safe for human consumption as mentioned in the document of Codex Alimentarius Commison in [13].

Aflatoxin content of each kernel category in all genotypes was available (Table 2). In general, the aflatoxin contained by SMK was lower compare to those contained by shriveled and damaged seeds. The highest level of aflatoxin in SMK (13.50 ppb) was in Tuban cultivar with the range between 13.10-14.30 ppb. This narrow range with relatively high amount pointed out the consistently high of aflatoxin content in SMK of Tuban cultivar.

The drought tolerant line GH 51 had the lowest aflatoxin content (1.90 ppb in average) with narrow range and very low values (1.80-2.00 ppb). In other experiment under drought stress condition, genotype GH 51 performed low aflatoxin contamination *i.e*. 4.31 ppb in average and ranged from 1.65-6.35 ppb [14].

The SMK of Sima, Komodo, Turangga, and Tuban cultivars had aflatoxin content >15 ppb, despite the reading took soon after the pods were getting dry. This high content probably would easily increase with improper storage condition, ultimately under high relative humidity and high room temperature. Those cultivars together with other cultivars had aflatoxin content higher than 15 ppb in their shriveled and damaged seeds (Table 2).

As summary, GH 51, the drought tolerant line, performed best among other tested genotypes relates to aflatoxin contamination. It is necessary, therefore, to thoroughly discuss the condition or performance of three risk factors for aflatoxin contamination viz. soil temperature, soil moisture content and pod damage in supporting the incidence of aflatoxin contamination in all tested genotypes, especially to that potential genotype GH 51.

Soil Moisture Content and Soil Temperature

Observation at 50 DAS showed that soil moisture content was below permanent wilting point (PWP: 19 % g/g, Riyadi: pers. comm.) after the field was irrigated at 36 DAS. This dry condition supported increasing soil temperature at the pod zone at about the same time (Table 3). At 80 DAS, however, soil moisture content increased to the condition available to plant growth *i.e*. between PWP and field capacity, although the last irrigation was applied 14 days before (at 66 DAS) with high amount of irrigated water (353 m^3) (Table 3). High soil moisture content at harvesting time in all plots was mostly probable caused by 37 mm rainfall occurred two days before harvesting.

The progressive soil drying from 66 DAS through to harvesting time at 95 DAS might be generated drought stress to the seeds at certain period of time in that period. This dry condition could stimulate aflatoxin contamination for most of Indonesian genotypes. The fresh kernels (just harvesting) has already contaminated with aflatoxin even though still under the safe level. This is the potential for increase during post harvest if appropriate handling does not proceed.

In general, soil temperature during the growing season was mostly above 30°C, with the range from 26 to 33.2°C. The vegetative period was under high soil temperature, but started at around 35 days before harvest the temperature progressively went down to 29°C and further to around 26°C (Fig. 1). The previous works show that the range of temperature from 26°-29°C in the *geocarphosphere* during pod formation and pod filling had bigger risk for producing aflatoxin-contaminated groundnuts. Groundnut crops grown under drought stress mostly produced immature

pods, small pods and small seed size, which firstly invaded by *A. flavus* as well as aflatoxin contaminated [15].

Aflatoxin contamination exhibit when the crops suffered from water shortage for 30-50 days during pod maturation period and high (average 29-31^{o}C) soil temperature [16]. Despite the pods were under favorable soil temperature for aflatoxin production (around 28-30^{o}C, fig. 1) close to harvesting time, all genotypes had increasing pod moisture content (Table 3).

As summary, that soil temperature and soil moisture content during pod formation and pod filling periods were in the aflatoxin risk zone.

Physical quality of pods and kernels

Pod maturity illustrates its physical quality. The mature pods are the filled pods while the empty pods will dominate the immature pods. Shelling outturn tells the weight ratio of shells to the pods. The higher the value, the lower was the seeds weight as the pods got empty and shriveled kernels dominated. The data tells that shell weight contributed 29.5 – 39.5% to pods weight (Table 4). In other words, the seed weight of those Indonesia genotypes was between 60.5 to 70.5% to their pod weight. This highest percent of shelling outturn or the lowest ratio seed/pod weight belongs to Turangga together with Sima and Komodo variety as contributed the higher shell weight, higher damage and shriveled seeds and conversely the lower weight of SMK (Fig 2). Low values of shelling outturn of the rest genotypes (including GH 51) revealed the pod filling smoothly proceed during the generative growth phase. Pod yields of those genotypes correlated to the ratio of seed to pod weight following the equation of $Y=-0.004x^2+0.616x-19.86$, with $R^2=0.74$ means that 74% of pod yield was determined by the ratio of seed to pod weight (the level of pod filling).

The proportion of SMK, shriveled and damaged seeds weights indicates the quality of kernels. High SMK, low shriveled and damaged seeds perform high quality of kernels. Drought resistant genotype GH 51 had higher proportion of damaged seeds despite of their high proportion of SMK (Fig. 2). Among 10 genotypes, local cultivars and cv. Tuban in general had high proportion of SMK and low portion of either shriveled or damaged seeds. Whilst all released cultivars and promising lines, except GH 15, tended to have lower portion of SMK as there was higher amount either shriveled or damaged seeds (Fig. 2).

The important contributor for damaged seeds was the changing of seeds color (getting darker or spotted), moldy seeds and followed by split seeds developed in SMK kernels. Since the location of pathogenic fungi such as *Alternaria* spp, *Fusarium* spp, *Macrophomina phaseolina*, *Rizoctonia* spp, *Aspergillus* spp was in seed coat or *testa* [17], and therefore their infection were responsible for discoloring the seed coat. For example, *A. flavus* infection recognized by the presence of the yellow greenish mycelia, and it destroyed the seeds [18].

The invasion of *A. flavus* to the seeds was also possible because of physical damage of pods especially for late-harvesting pods [8]. The physical quality relates to aflatoxin contamination was damage seeds [18].

As summary, that GH 51 together with other six genotypes had high pod maturity and kernel quality excluding Turangga, Sima and Komodo variety. These three genotypes in fact had high shelling outturn, shriveled and damage seeds.

Aspergillus flavus *infection*

The fresh seeds of all genotypes obtained at harvesting time were free from *A. flavus* infection as pointed out by zero and low percent values (Table 5). Surprisingly, these genotypes with low *A. flavus* infection produced high aflatoxin. Based on the data, *A. flavus* infection correlated to aflatoxin contamination followed the equation of $Y=0.063x^2-1.263x+13.62$ with R^2: 0.438. This correlation explained that fungal growth and aflatoxin contamination were the consequence of an interaction among the fungus, the host and the environment, where the appropriate combination of these factors determine the infestation and colonization of the fungus, and the amount of aflatoxin produced. Therefore, the presence of *A. flavus* in groundnut seeds does not automatically mean the presence of aflatoxin, but rather, that a potential for contamination exists. Conversely, the absence

of *A. flavus* does not guarantee that groundnut seeds free from aflatoxin. Other reports [1, 7] also mentioned that the condition of *A. flavus* colonization and fungal growth on seeds poorly correlated to aflatoxin production.

It is important to be underlined that the weight of damage seeds did not linearly correlates to the number of seeds infected by *A. flavus*. For example in GH 51 genotype, the weight of damage seeds was high (Table 6), but the infection rate of *A. flavus* was low (Table 5), the damage was mainly because of split seeds. The invasion of *A. flavus* to the seeds was possible because of physical damage of pods especially for late-harvesting pods [8].

Data shows that Indonesia genotypes in general had higher aflatoxin contamination (ranged from 20-24 ppb) with very little (almost free) of *A. flavus* infection. Gridthai *et al* [1] have had compiled many reports, and concluded that aflatoxin production was dominantly affected by environment. In addition, genotypes that resistant to aflatoxin production showed inconsistency across various growing environments.

Seed moisture content

Dorner *et al*. (1989) *in* [19] reported that kernel moisture content is crucial in the incidence of aflatoxin contamination where the range of 18 to 28 % moisture content is critical level suitable for aflatoxin production. Other publication mentioned the number of between 15 to 30 % and soil temperature that higher than 28 ^{o}C during pod filling period in the pod zone [20]. Below 15% and above 30% of moisture content were the safe levels. The observation shows that GH 51 with low aflatoxin contamination contained 6-9% of moisture in their seeds immediately before ELISA suggesting that the pods and seeds got dry to safe moisture content (Table 6). This condition indicated that seeds were beyond the aflatoxin-risk zone, which was 15-30% of moisture content [20]. In case of Local Blitar, Sima, Turangga, Tuban, and GH 57, their seed moisture contents were in the aflatoxin-risk zone (Table 6) and most probably these moisture contents as one reason for aflatoxin production.

Supartini (1994) *in* [18] reports that seed moisture content of ≤8% was able to inhibit *A. fl*avus infection and aflatoxin contamination in groundnut seeds compared to those with seed moisture content >10%. Based on that figure, most Indonesian genotypes (Table 7) were susceptible to *A. flavus* infection and aflatoxin contamination. This phenomenon also revealed in genotype GH51 and Local Lamongan, and 12 ICRISAT genotypes grown in the same place under end of season drought stress [14]. Based on those two conditions, it strongly predicted that genotypes with low aflatoxin contamination or under the permissible level generally had low seed moisture content.

Pod Moisture Content

Pod moisture content went down with increasing the plants age from 50 DAS through to 90 DAS. It seems that 37 mm rainfall did not absolutely influence the moisture content of the pods. Local cultivars generally contained less moist than the improved cultivars tested especially cult. Sima and Turangga (Table 8).

Gridthai *et al* (1) summarized that pod moisture content under drought stress has been proposed as the main condition that can help to maintain the capacity of plants to produce *stilbene phytoalexin* to prevent pre-harvest aflatoxin contamination. Gridthai *et al* [21] proposed that drought resistance traits *i.e.* specific leaf area, relative water content in the leaves, chlorophyll density and drought stress ratings are promising as indirect selection tools for selecting genotypes for resistant pre-harvest aflatoxin contamination. These drought resistance traits were associated well with those related to pre-harvest aflatoxin contamination under drought condition. Arunyanark *et al* [22] emphasized drought tolerance traits: specific leaf area and root length density, and kernel colonization by *A. flavus* could be used as selection criteria for aflatoxin resistance.

Table 1. Aflatoxin content of Indonesia genotypes.

Genotype	Aflatoxin B_1 content (ppb)	
	Average	Range
Local Pati	13	6 – 20
Local Blitar	12	4 – 22
Sima	12	3 – 24
Komodo	19	9 – 23
Turangga	13	3 – 22
Tuban	21	20 – 22
GH 15	18	10 – 20
GH 27	10	5 – 20
GH 57	15	6 – 20
GH 51	5	3 – 6

Table 2. Aflatoxin content on three kernels' groups at 10 Indonesia genotypes.

Genotype	Aflatoxin content on					
	SMK (ppb)		Shriveled (ppb)		Damaged (ppb)	
	Avg	Range	Avg	Range	Avg	Range
Local Pati	6.8	1.7-12.0	12.5	12.1-13.1	8.2	5.3-12.7
Local Blitar	6.2	1.2-13.8	15.0	11.3-18.4	7.3	1.2-16.2
Sima	7.5	1.2-16.3	10.6	1.2-15.6	6.6	1.2-14.6
Komodo	11.4	4.1-15.2	9.7	1.2-16.0	15.3	14.6-15.9
Turangga	9.9	1.2-16.0	3.4	1.0-7.9	10.9	4.4-15.1
Tuban	13.5	13.1-14.3	5.3	1.2-13.0	10.5	6.3-13.7
GH 15	10.5	8.5-11.8	14.9	12.5-17.9	18.2	16.9-19.8
GH 27	5.3	1.2-9.4	14.7	11.9-18.2	10.2	5.5-12.9
GH 57	9.2	1.2-13.4	15.6	12.4-18.9	9.5	5.7-13.2
GH 51	1.9	1.8-2.0	10.3	3.8-15.7	9.1	4.0-7.2

Avg: Average

Table 3. Soil moisture content at plot where 10 Indonesia genotypes grown.

Genotypes	Soil moisture content at… (% g/g)		
	50 DAS	80 DAS	Harvesting time
Local Pati	14.6	27.7 b	27.6 ab
Local Blitar	14.3	29.9 ab	27.2 b
Sima	14.3	30.8 ab	24.9 b
Komodo	13.8	27.2 b	22.6 ab
Turangga	12.8	26.9 b	24.3 b
Tuban	15.8	29.7 ab	26.5 ab
GH 15	14.3	31.4 a	26.6 b
GH 27	13.3	28.4 b	25.3 b
GH 57	13.4	30.1 ab	27.2 b
GH 51	15.1	28.2 b	22.7 a

Numbers in the same column followed by different letters are significantly different at p=5% level
Permanent wilting point (PWP): 19 % g/g, Field Capacity (FC): 32% g/g (Riyadi: pers. comm.)

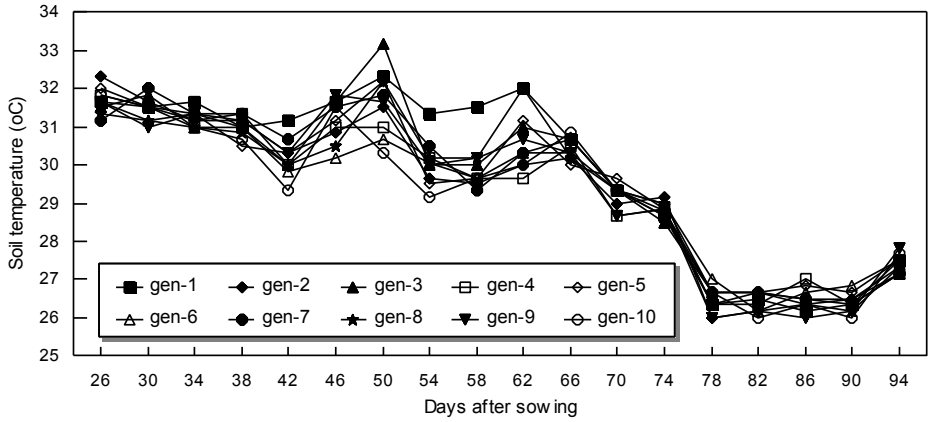

Figure 1. Soil temperature at pod zone of 10 peanut genotypes during the growing season.

Table 4. The shelling outturn of Indonesia genotypes.

Genotype	Shelling outturn (%)
Local Pati	30.9
Local Blitar	29.5
Sima	35.7
Komodo	35.6
Turangga	39.5
Tuban	33.3
GH 15	33.3
GH 27	31.3
GH 57	31.8
GH 51	31.1

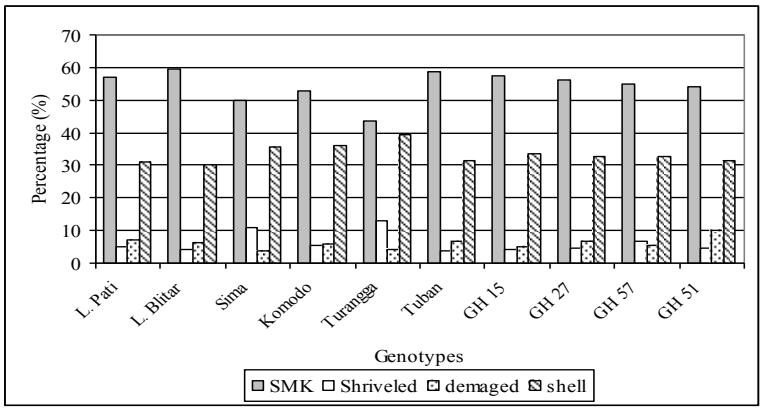

Figure 2. The distribution of sound mature kernel (SMK), shriveled, damaged seeds and shell of 10 genotypes.

Table 5. *Aspergillus flavus* infection of seeds of Indonesia genotypes.

Genotypes	*A. flavus* infection (%)	
	Average	Range
Local Pati	0	0
Local Blitar	0	0
Sima	0.3	0 - 0.3
Komodo	0	0
Turangga	0.6	0 - 0.6
Tuban	0.2	0 - 0.2
GH 15	0	0
GH 27	0.1	0-0.1
GH 57	0	0
GH 51	0	0

Table 6. The percentage of damage kernel of Indonesia genotypes.

Genotype	Damage seed (%)
Local Pati	6.9
Local Blitar	7.8
Sima	3.7
Komodo	5.9
Turangga	4.4.
Tuban	6.5
GH 15	5.0
GH 27	6.6.
GH 57	5.3
GH 51	10.0

Table 7. Seed moisture content of Indonesia genotypes just before ELISA test.

Genotype	Seed moisture content before Elisa test (%)
Local Pati	8.8
Local Blitar	12.4
Sima	12.8
Komodo	9.2
Turangga	19.7
Tuban	11.6
GH 15	8.7
GH 27	8.2
GH 57	11.3
GH 51	8.9

Table 8. Pod moisture contents at 10 Indonesia genotypes.

Genotypes	Pod moisture content at … (%) *)					
	50 DAS	60 DAS	70 DAS	80 DAS	90 DAS	95 DAS
Local Pati	85.1	80.4 cd	64.0 d	53.0 cd	45.1 c	37.6 d
Local Blitar	83.4	79.0 d	64.7 cd	59.9 abc	45.6 c	38.0 cd
Sima	87.3	84.0 abc	73.0 a	64.5 ab	58.7 a	47.3 a
Komodo	83.1	86.8 a	72.1 ab	66.4 a	51.3 abc	42.0 abcd
Turangga	86.5	84.6 ab	70.3 abc	67.3 a	57.4 ab	46.9 a
Tuban	85.1	81.6 bcd	64.0 d	51.7 d	48.7 c	36.8 d
GH 15	86.0	83.4 abc	66.4 bcd	61.4 ab	46.9 c	40.5 abcd
GH 27	85.8	84.2 abc	67.0 bcd	60.5 abc	47.2 c	45.3 abc
GH 57	86.2	83.5 abc	66.9 bcd	66.2 a	50.0 bc	46.0 ab
GH 51	85.3	83.5 abc	66.1 cd	58.5 bcd	53.2 abc	38.7 bcd

Numbers in the same column followed by different letters are significantly different at p=5% level *) obtained from 3 sampling plants

Conclusion

Assessment of Indonesia genotypes tolerance to aflatoxin contamination the drought resistant line GH 51 had the lowest aflatoxin content. This genotype also performed low *A. flavus* infection. This genotype was released by the Minister of Agriculture, The Republic of Indonesia in year 2013 as genotype with the main character of low *A. flavus* infection and aflatoxin contamination. Recently, the big peanut industry, where the main production is roasted-peanut (in shell) produced from fresh pods, grows and develops that variety.

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