Research Application Summary

In-vitro determination of kernel resistance to *Aspergillus flavus* and aflatoxin accumulation in groundnut

Ozimati, A.[1], Rubaihayo, P.R.[1] & Ntare, B.[2]
[1]Department of Agricultural Production, School of Agricultural Sciences, Makerere University, P. O. Box 7062, Kampala, Uganda.
[2] International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), P. O. Box 320, Mali, Bamako
Corresponding author: alfredozimati@yahoo.com

Abstract

The study determined the level of resistance to *Aspergillus flavus* and to accumulation of aflatoxins in potential new breeding sources from ICRISAT Mali and in locally adapted groundnut genotypes commonly grown by Ugandan farmers. Genotypes were significantly different in their responses to *A. flavus* and to aflatoxin accumulation at P≤0.001. Locally adapted genotypes that had both low *A.flavus* and accumulation of aflatoxins included Serenut 4, AGRA 99044 and erudu red, while AGRA 99033, Serenut 2, acholi white and red beauty had the highest levels of *A.flavus* and aflatoxin accumulations. High level of variations in the genotypes can be further used for genetic studies in order to establish effective and efficient breeding strategy for resistance to *A.flavus* and to the accumulation of aflatoxins in Ugandan lines.

Key words: Aflatoxins, *Aspergillus flavus*, groundnuts

Résumé

L'étude a déterminé le degré de résistance à *Aspergillus flavus* et à l'accumulation des aflatoxines dans les sources potentielles de nouvelle reproduction de l'ICRISAT au Mali et dans les génotypes d'arachides adaptés aux conditions locales couramment cultivés par les agriculteurs ougandais. Les génotypes étaient significativement différents dans leurs réponses à *A. flavus* et à l'accumulation des aflatoxines à P <0,001. Les génotypes adaptées aux conditions locales, qui avaient à la fois l'*A.flavus* faible et l'accumulation d'aflatoxines faible incluaient Serenut 4, AGRA 99044 et erudu rouge, tandis que l'AGRA 99033, Serenut 2, Acholi blanc et la beauté rouge avaient les plus hauts niveaux d'*A. flavus* et d'accumulations des aflatoxines. Le niveau élevé de variations dans les génotypes peut être en outre utilisé pour des études génétiques afin d'établir la stratégie de reproduction efficace et efficiente pour la résistance à *A. flavus* et à l'accumulation des aflatoxines dans les lignes ougandaises.

Mots clés: Aflatoxines, *Aspergillus flavus*, arachides

Background

Groundnut is prone to infestation by two closely related species of fungi, *A. flavus* and *A. parasiticus*. These fungal species produce mycotoxins known as aflatoxins. Consumption of products contaminated with aflatoxins results in suppressed immune systems in both humans and animals, leading to diseases such as cancer in humans (Brown *et al*., 1998). The most recent and largest outbreak occurred in Kenya in January to June of 2004, when 317 cases of acute hepatic failure and 125 deaths were attributed to aflatoxins consumed from contaminated maize (Eduardo *et al*., 2005). Contamination also markedly reduces the economic value of the grain (Kwemoi, 2011). Due to the dangers they pose, food and feed levels of aflatoxin are controlled by regulations in more than 50 countries (Agag, 2004). For example, the United States Food and Drug Administration (USFDA) have set limits of 20ppb for total aflatoxins for interstate trade in food and feeds (Agag, 2004). The Uganda National Bureau of Standards (UNBS) has set limits for aflatoxins in all Ugandan foods and feeds at 10 ppb to certify the quality of the produce intended for the export market (Kaaya & Warren, 2005). The European Union (EU) countries have established maximum levels for aflatoxins as low as 2ppb. Most samples of groundnuts sold in Ugandan markets contain aflatoxins levels of more than 20ppb, the minimum level recommended by UNBS for interstate food trade (Kaaya *et al*., 2005). Attempts to mitigate the problem of aflatoxins using good post-harvest handling practices has not proved to be very effective because aflatoxins contamination can occur in the field as well in storage, and the use of fungicides is not cost effective for a small scale farmer (Kwemoi, 2011). Thus it is thought that use of genotypes resistant to *A.flavus* and to accumulation of aflatoxins is the best option for the farmers. Therefore, the objective of this study was to identify sources of such resistance in locally adapted genotypes and to confirm whether genotypes resistant to aflatoxin accumulation elsewhere would confer the same resistance to biotypes in Uganda.

Literature Summary

In groundnut, resistance to aflatoxin-producing fungi may be of three types: a) resistance to pod infection (pod wall); b) resistance to seed invasion and colonization (seed coat); and c) inhibition of aflatoxin production (Utomo *et al.,* 1990; Upadhyaya *et al*., 1997). The fungi have to penetrate the pod wall and the seed coat to reach the cotyledons from which they derive their sustenance. Resistance to pod infection is attributed to pod-shell structure. Resistance to seed invasion and colonization is also mostly physical, and has been correlated with the thickness,

density of palisade cell layers, absence of fissures and cavities, and the presence of wax layers in the seed coat (Mehan *et al.*, 1987). Recent studies have focused on resistance to seed invasion and colonization, and on inhibition of aflatoxin production, because pod-wall resistance is associated with hard pods, making shelling difficult.

Study Description

Two aflatoxin-resistant varieties from ICRISAT, Mali, and 16 locally-adapted varieties, including one susceptible check (Acholi white), were evaluated at Makerere University's School of Food Technology, Nutrition and Bio-Engineering microbiology laboratory. The level of resistance to infection by *A.flavus* and to aflatoxins accumulation was tested in the 18 genotypes, in a randomized complete block design, with 3 replications, each replication containing 30 seeds per genotype in 3 petri-dishes. A.*flavus* was isolated from naturally-infected groundnut samples obtained from the markets of Kalerwe, Owino and Bwaise in Kampala, Uganda and cultured on potato-dextrose agar (Difco 1Becton Drive, Franklin Lakes, New Jersey (NJ), 07417, USA) for 14 days at a temperature of 28^0C with 12 hours of light. The pure culture was blended with distilled water and then filtered. Conidial concentrations were estimated using a hemacytometer, and adjusted with distilled water. Two drops of Tween 20 per 100 ml were added. The resulting suspension was adjusted to a concentration of about 10^6 spores per ml (Francis, 2011). In each petri-dish, a total of 1 ml of the conidial suspension was surface applied to the kernel without wounding the seed (Li, 2004).

Severity of the fungal growth (colonization) on the kernels was rated on a scale of 1-5 (Francis, 2011), with some modification. The severity was rated according to the percent of the kernel surface covered by visible mycelia growth, where; 1 = none, 2 = 1 to 20%, 3 = 21 to 50%, 4 = 51 to 70%, and 5 = 71 to 100%.

After scoring for severity of colonization, samples that had been artificially inoculated with *A. flavus* were oven dried at a temperature of 50-60^{o}C for 1 day, the seed coats removed and the samples ground. The samples were then passed through a 1–mm mesh and 25g of the sample was used to quantify the total aflatoxin concentration in the sample, measured in parts per billion (ppb), using VICAM Aflatest (VICAM,Watertown, MA,USA) immune-affinity flouro-metric method. The 25 g of the sample and 5 g of sodium chloride were mixed with 125

mls of diluted methanol in the ratio of 7:3 methanol: distilled water, respectively. The filtered mixture was passed through the immune-affinity column at a rate of 1-2 drops per second. The columns were then washed with 20 mls of distilled water and the aflatoxins recovered using 1 ml of methanol. The extract was read using a calibrated VICAM Series-4 fluorescent set at 360 nm excitation and 450 nm emissions, which gave a direct level of aflatoxin in each sample each.

Research Application

The research showed wide variation in genotypes' response to *A. flavus* and aflatoxin accumulation, and identified potential sources of resistance to both *A.flavus* and aflatoxins accumulation among the locally adapted genotypes in Uganda (Table 1). This information can be used for inheritance studies in crosses of these sources with other locally adapted genotypes that have farmer-preferred attributes. The genetic studies would establish a foundation for an effective breeding strategy. Further, the research observed that two newly released genotypes, from the same parentage (Serenut 1 x Serenut 2), showed maximum contrast for the two forms of resistance: with AGRA 99044 resistant to seed colonization and to aflatoxins accumulation, with AGRA 99031 susceptible to both *A.flavus* and aflatoxins accumulations. This information could be used to identify molecular markers associated with QTL for resistance. Subsequently, validation of those markers with populations arising from the different parents could allow markers to be used for cheaper screening of large numbers of genotypes for resistance to *A. flavus* and aflatoxin accumulation, since the phenotypic screening for aflatoxins is very expensive.

Table 1. Genotypes with the most extreme ratings.

Genotypes	*A flavus* (%) Kernel rating	Aflatoxins (ppb)	Response
Serenut 4	13	23	Resistant
AGRA 99044	18	16	Resistant
Erudurudu	23	18	Resistant
Serenut 2	46	38	Resistant
AGRA 99031	61	385	Susceptible
AGRA 99064	72	316	Susceptible
Red beauty	93	350	Susceptible
Acholi white	100	277	Susceptible
LSD		14.46	28.02
CV		2.9	1.1

AGRA 99044(R) Serenut 4(R) Acholi White (S)

***A. flavus* and aflatoxin accumulation were significant at p≤ 0.001 Picture 1; Kernel cover with *A.flavus.* (note the grey-green fungal growth on the susceptible check)**

References

Agag, B.I. 2004. Mycotoxin in Foods and Feeds. Assuit University. *Bulletin for Environmental Research* 7:1.

Brown, R.L and Cleveland, T.E. 1998. Advances in the development of host resistance in corn to aflatoxin contamination by *Aspergillus flavus*. *Phytopathology* 89:113-117.

Eduardo, A., Lindblade, K., Gieseker, K., Schurz, K.R., Kieszak, S., Njapau, H., Schleicher, R., Leslie, F., Misore,A., Rubin, C., Slutsker, L. and the aflatoxin investigating group. 2005. Case –control study of an acute aflatoxicosis out break, Kenya, 2004. *Enviromental Health Perspective* 113:1779-1783.

Francis, T. 2011. Characterization of locally adapted groundnut germplasm for resistance to *Aspergillus flavus*. MSc thesis, submitted to Makerere University, Kampala, Uganda.

Kaaya, N. A. and Warren, H. L. 2005. A review of past and present research on aflatoxins in Uganda. *African Journal of Food Agriculture and Nutritional Development* 5:1

Kwemoi, B.D. 2011. Characterisation of a diverse set of maize germplasm for resistance to infection by *Aspergillus flavus*. MSc thesis, Makerere University, Kampala, Uganda.

Li, R. 2004. A genetic study of resistance to kernel infection by *Aspergillus flavus* in maize. Secondary Traits. PhD Dissertation, Texas A & M University.

Mehan, V.K. McDonald, D. and Rajagopalan, K. 1987. Resistance of peanut genotypes to seed infection by *Aspergillus flavus* in field trials in India. *Peanut Science* 14:14–17.

Upadhyaya, H.D., Nigam, S.N., Mehan, V.K. and Lenne, J.M. 1997. Aflatoxin contamination of groundnut – prospects of a genetic solution through conventional breeding. pp. 81–85. In: Aflatoxins contamination problems in groundnut in

Asia: Proceedings of the First Working Group Meeting, 27–29 May 1996, Ministry of Agriculture and Rural Development, Hanoi, Vietnam.

Utomo, S.D., Anderson, W.F., Wynne, J.C., Beute, M.K., Hagler, W.M. Jr. and Payne, G.A. 1990. Estimates of heritability and correlation among three mechanisms of resistance to *Aspergillus parasiticus* in peanut. *Proceedings of the American Peanut Research and Education Society* 22:26. (Abstract.).