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Breeding for Aflatoxin Resistance in Maize – A Review

Vaibhav P. Chaudhary[1,*], Rohini Bansode[2] and Sandeep Kumar[3]

[1]Deptt. of Plant Breeding and Genetics, A.A.U., Jorhat 785013 (Assam), India
[2]Deptt. of Plant Biotechnology, K.A.U., Thrissur 680656 (Kerala), India
[3]Deptt. of Environmental Sciences, IARI, New Delhi 110012, India

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Abstract

Crowned with the rhetoric phrase “the queen of cereals” maize (*Zea mays* L.) is not only of worldwide valued as a food, feed and as a supply of diverse industrially vital products, but is also a model genetic organism with gigantic genetic diversity but the escalating problems resulting to death and diseases has been of a great concern aroused due to the contamination of maize grain by aflatoxin globally. Aflatoxin contamination in maize occurs above safe levels in many countries and even aflatoxin exposure at low levels can result in reasonable human health impacts to both humans and animals. Aflatoxin contamination in maize can affect the agricultural sector output, generally, along with each of the four pillars of food security (availability, access, utilization and stability), specifically. In recent past, a signiûcant research efforts are been put forth for generating resistant maize inbred lines along with identifying the resistance mechanisms. Here, we present before you the overall progress made for resistance towards Aflatoxin resistance in maize, both at conventional and non-conventional levels. We also propose few future directions, towards the healthy growth of maize. Joint venture research by the plant breeders, pathologists and biotechnologists will result in the reduction and may even discard the problem in a brief time period.

Keywords : Developing world, aflatoxin, maize, inbred lines, resistance

Introduction

The queen of cereals - corn, has become the most important agricultural crop not only in the temperate regions but also in the tropical and sub-tropical areas of the globe. Maize is an excellent supplier of carbohydrates, protein, iron, vitamin B and minerals. If not dried immediately after harvest, high moisture content (32-35%) in the cob can encourage the growth of the fungi *Aspergillus flavus,* and also result in a soaring quantity (up to 15%) of broken seed at shelling (Tastra et al., 1990). A broken seed, a good source for *A. flavus* growth produces aflatoxin in the seed. The ubiquitous survey has emphasized the huge problem offered by aflatoxin to human health and to the economics of crop production (CPC 2004). Food and Agriculture Organisation (FAO) has specified, the maximum permissible limit for aflatoxin in maize grain as 30ppb (parts per billion) and also according to Hamilton (1986) and Wilson (1978), food contaminated by aflatoxin (>30ppb) can cause fatal diseases. Daily intake of low doses of aflatoxins over time causes chronic aflatoxicosis (illness from aflatoxin poisoning), resulting in impaired food consumption, stunted growth, immune suppression, and possible liver cancer development (Cardwell and Henry, 2004; Gong et al., 2004 and Farombi, 2006). Contaminated maize had been the cause behind the numerous deaths in Kenya in recent years (Lewis et al., 2005 and Yu et al., 2008). Drying the grain is therefore imperative to prevent *A. flavus* infection.

Corresponding authors- e-mail : vpcmaize@gmail.com
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The genus *Aspergillus* was first described by P. A. Micheli (1729) and the species *A. flavus* was ûrst described by Link (1809) (Amaike and Keller, 2011). The aflatoxins were discovered in England (1962), where thousands of poultry died upon eating aflatoxin-contaminated peanuts, as a causative agents of ‘‘Turkey X’’ disease, (Blount, 1961; Forgacs and Carll, 1962 and Wogan, 1966). Investigations revealed that toxicity was associated with the presence of *A. flavus*, and further that extracts of cultures of the fungus isolated from the meal were capable of inducing the ‘‘turkey X’’ syndrome. The name “afl-atoxin” comes from the genus *Aspergillus*, which is where the letter “a” in afl-atoxinis derived and “fl-a” from the species name *-flavus* (Sargeant et al., 1963; Jacobsen et al., 1993; Rustom, 1997; Devero, 1999 and Abbas et al., 2011). There after screening of the feed initiated, the chemical structures of the major aûatoxins (B1, B2, G1, and G2) were clarified, and steps were taken to prevent post-harvest contamination of grain crops (Asao et al., 1965 and Trenkand Hartman, 1970). Surprisingly it was found that *A. ûavus* colonize and produce aûatoxin on developing maize kernels prior to harvest (Lillehoj et al., 1975 and Diener et al., 1983, 1987) on Texas field (1920) (Taubenhaus, 1920), in Southern Indiana (1971-72) and in Missouri (1972).

Gigantic losses since the 1970s due to contamination of the aflatoxin, has made the researchers to take pain staking efforts to spot the source of host plant resistance to prevent the attack by *A. flavus.* However, investigations have highlighted that resistance to aflatoxin contamination is a polygenic phenomena. Therefore, attempts to transfer resistance from inbred lines into commercial varieties with desirable agronomic characteristics are the need of hour with the accessibility of biomarkers has been highlighted in this review.

Toxicity and importance

Aflatoxins are both acutely and chronically toxic. Aflatoxin B1 is one of the most toxic metabolite and a potent hepato-carcinogen and their long-term exposure to extremely low levels in the diet is an important consideration for human health. Acute aflatoxin toxicity has been demonstrated in a wide range mammels. Aflatoxins have received greater attention than any other mycotoxins because they have a potent carcinogenic effect in laboratory rats and their acute poisonous effects in humans.

Aflatoxin

Aflatoxins, naturally occurring carcinogenic by-products of common fungi on grains and other crops, occurs seldomly in the tropics, particularly in lipid-rich seeds. It can be documented by a yellow-green or gray-green mold developing on kernels. It is produced by the fungal strains like *Aspergillus flavus* and *Aspergillus parasiticus* (Cotty, 1994 and Bhatnagar et al., 2003). The fungal strains are soil born and propagate on food finally producing aflatoxin (Horne et al., 1991). Aflatoxins are classified as a group 1 carcinogen (IARC 1993).

Originally, two toxic constituents (AFB and AFG) of aflatoxin were known on thin layer chromatography plates and were characterized due to the blue and green fluorescence, respectively (Sargeant et al., 1963). A total of 17 aflatoxins have been isolated (W HO, 1979), but only 4 are known popularly. The most familiar aflatoxins are M1 and M2 because of their presence in milk of animals previously exposed to B1 and B2 (Bennett and Klich, 2003) AFB2, AFG1 and AFG2 do not occur in the absence of AFB1. In most cases AFG1 is observed in escalating quantity than AFB2 and AFG2 (Weidenborner, 2001).

Asao et al., 1963; Van Dorp et al., 1963 and Van der Zijden et al., 1962, categorized the chemical and physical nature of the aflatoxins B1, B2, G1 and G2. Chemically, aflatoxins are difurocoumarolactones (difurocoumarin derivatives). Structure consists of a bifuran ring fused to a coumarin nucleus with a pentenone ring (in B and M aflatoxins), or a six-membered lactone ring, in G aflatoxins, (Patterson et al., 1978 and Devero, 1999). Structures based largely on interpretation of spectral data were proposed for aflatoxins B1 and G1 in 1963 (Asao et al., 1963 and Asao et al., 1965) and for B2 (Chang et al., 1963 and Van Dorp et al., 1963) and G2 subsequently.

The minimum temperatures for the invasion of aflatoxins by *A. flavus* and *A. parasiticus* are reported as 12 to 41°C, with optimum production occurring between 25 and 32°C (Boller and Schroeder, 1974 and Sorenson et al., 1967). Synthesis of aflatoxins in feeds are amplified at temperatures above 27°C (80 F), humidity levels greater than 62% and moisture levels in the feed above 14%. Moisture levels above 17.5% and at temperatures of 24^0C or warmer, aflatoxins were formed by *Aspergillus flavus* present in the epiphytic mycoflora by the study conducted by Trenk and Hartman (1970).

Aflatoxin B1, the most toxic compound among all and has been found to be one of the most potent carcinogens occurring naturally (Vasanthi, 1993; Vasanthi and Bhat, 1998; FAO, 1997). Health penalties of aflatoxin are much more serious problem in developing countries than in the developed (Brankov et al., 2013). Because of frequent contagion of aflatoxin B1 in agricultural produces such as peanuts, corn and animal feed stuffs, aflatoxin problem become a possible threat to human (Wogan et al., 1966 and Gong et al., 2002) and animal fitness (Busby and Wogan, 1979; Wogan, 1965 and Shashidhar et al., 2005).

Yield losses due to aûatoxin contamination have been reported in million-dollar in the United States (Rubens and Cardwell, 2003; Vardon et al., 2003 and Rubens and Cardwell, 2005).

Penetrance of fungi in kernel:

Seed adulteration by *A. flavus* depends on factors like the seed's intrinsic susceptibility, environmental factors, the fungal community and the capacity of the fungus (Horn, 2003). Study conducted by Semeniuk, (1954) reported that testa restricts the fungal penetration of hypha into the seed which becomes thicker as the seed matures, except over the embryo. Infections by *Aspergillus* were thought to occur at either the silk scar or hilar layer (Payne, 1987). Nevertheless, evidence suggests that the fungus penetrates into the kernel via the hilar layer (Fennell et al., 1973 and Diener et al., 1987).

Microscopic examination of ears of a non-resistant maize line that were wound-inoculated (Smart et al., 1990) indicated that *A. flavus* spread 14 days post-inoculation (dpi) and could be found throughout tall rachis tissues except the pith and lignified fibers at 28 dpi. Infection of kernels was always through the rachilla, and hyphae did not enter the endosperm through the outside of the pericarp. Spread of the fungus through the rachis was a vital infection instrument in wound-inoculated maize ears. Aflatoxin was not detected in non-infected kernels indicating that it was not translocated through the rachis (Smart et al., 1990). Marsh and Payne, (1984) showed that *A. flavus* infection through the silk, occurred after pollination, which initiates silk senescence. After progressing through the silk, hyphae penetrated the kernels through the pedicel, which appears to provide the primary route for fungal invasion (Lillehoj, 1983). A study carried out by the prevalence of colonization is higher on silk of mature maize ears than young ears (Zuber and Lillehoj, 1979).

Where does it occur ?

1. **In Raw Agricultural Products :** Aflatoxins are often found in crops before harvest where contamination can occur if crop drying is delayed or crop is stored in moist condition. They are also detected in milk, cheese, corn, peanuts, cottonseed, nuts, almonds, figs, spices, and a variety of other foods and feeds. Consumption of aflatoxin-contaminated feed by animal leads to the contamination of milk, eggs, and meat products. The highest risk of aflatoxin contamination is found in the commodities such as corn, peanuts, and cottonseed (Siwela et al., 2011)

2. **In Processed Foods :** Corn is the staple food of many countries and is grown in climates that are likely to have perennial contamination with aflatoxins. However, procedures used in the processing of corn such as alkaline conditions or oxidizing steps help to reduce contamination of the resulting food product. Milk and milk products, including non-fat dry milk, cheese, and yogurt contains aflatoxin M1 contamination in (Jayramchandran et al., 2013).

Concentration of aflatoxinis more in food crops grown and stored in the warmer areas of the world, where the international trading of these important commodities tells that aflatoxins are not only a problem for the producing nations but are also of concern for importing countries. Biological control, control of insect pest, development of resistant cultivar, etc. stratagies are available to manage aflatoxins in crops but these are having its own disadvantages. The most widely used strategy for controlling aflatoxin contamination in food and feed grains is by pre harvest prevention, especially through host resistance (Lillehoj, 1987). Good cultural and management practices can reduce pre harvest aflatoxin contamination to a certain extent but cannot eliminate it completely. Identification of resistant genotypes in corn through plant breeding is a time consuming approach for aflatoxin contamination. There is an urgent need for aflatoxin contamination by employing currently available modern technologies. The present paper discuss about application of biotechnology in developing corn varieties that are resistant to aflatoxin and can prevent contamination by *Aspergillus* species. Therefore, an attempt to transfer resistance from inbred lines into commercial varieties with desirable agronomic characteristics is the need of hour with the accessibility of biomarkers has been highlighted in this review.

KSA (kernel-screening assay) :

A rapid laboratory-based kernel-screening assay (KSA) developed by Brown et al. (1995) constructs higher and more identical levels of contamination and aflatoxin production and permits variation of resistant and susceptible maize genotype. Endorsed with several advantages, compared to traditional breeding techniques: 1) it can be performed and repeated several times throughout the year and outside of the growing season; 2) it requires few kernels; 3) it can detect/identify different kernel resistance mechanisms expressed; 4) it can dispute or confirm field evaluations (e.g. identify escapes); and, 5) relationships between laboratory findings and inoculations in, the field have been demonstrated (Brown et al., 1995). It has also been proven to be a valuable complement to standard breeding practices in the evaluation of germplasm for aflatoxin-resistance.

GT-MAS : gk was found to be the resistant corn population against aflatoxin production through KSA technique (Windstorm et al., 1987). A clear correlation was proved between fungal fluorescence and aflatoxin levels with the use of KSA technique (Rajasekaran et al., 2013). It was also concluded that once the fungus has entered through the pedicel, it spreads quickly through the open spaces between the pericarp and the aleurone layer, ultimately colonising the endosperm and scutellum and finally, the embryo. The KSA is designed to address the fact that aflatoxin build up occurs in the mature and not developing kernels.

Resistant Germplasms :

Identifying the germplasm with either genetic resistance to infection and growth of *A. flavus* the ability to suppress fungal production of aflatoxin after infection is the need of the hour. Genetic variation is known to exist for aflatoxin build-up and *A. flavus* resistance in maize, and firmly resistant breeding lines have been established. Screening by the pin bar inoculation method helped in identification of two resistant inbred lines (Mp420, SC54, Tex6 and Mp313E) at multiplications (Scott and Zummo, 1988 and Hamblin and White, 2000) and released as the source of resistant germplasm (Windham and Williams, 1998). Exception to the Mp313E, expression of resistance in the above sources of germplasm was fluctuating with the environmental conditions. However, early studies done (Widstrom et al., 1984; Betran et al., 2002; Naidoo et al., 2002; Williams et al., 2008 and Williams et al., 2008) by with these lines concluded that much of the resistance was highly quantitative tended to be inherited and led to high general combining ability (GCA) in the hybrids. Specially epistatic, dominant, and reciprocal effects were also seen in diallel experiments, which limited the value of resistance in hybrids.

Genotype × environment (G×E) interaction, quantitative nature and phenotyping particularly at the very low levels at which aflatoxins can be problematical has been acting as the barriers at various levels for the low heritability of the resistance into the hybrids. Multi locational screening conducted (McMillian et al., 1993; Guo et al., 2001; Williams and Windham, 2001; Williams and Windham, 2006) showed that the germplasms, Mp715, Mp717, GT-MAS: gk, CML176, CML269, CML322, and Tx114 were still posing the resistance mechanism in it. Recently the lines gMp718, Mp719, Tx736, Tx739, and Tx740 show a much better plant type (agronomical characters) and high resistance mechanism has been released (Mayfield et al., 2012 and Williams and Windham, 2012). Guo et al., (1998) suggested that kernel proteins were important in two resistant genotypes, (GT-MAS: gk and Mp420) to *A. flavus* infection and aflatoxin contamination. Mexican maize landrace, Tuxpeno, has been the pedigree of a majority of the lines identified as resistant. This landrace has been used extensively in the creation of many of the maize breeding pools and populations of the International Maize and Wheat Improvement Centre (CIMMYT) because it is a high yielding, agronomically superior dent population with a good GCA (Warburton et al., 2013).

Identification of resistance-associated proteins (RAPs) in Corn

Developing resistance to fungal infection in wounded as well as intact kernels would go a long way toward solving the aflatoxin problem (Payne, 1998). Examination of kernel proteins of several genotypes revealed differences between genotypes resistant and susceptible to aflatoxin contamination (Guo et al., 1998). Both Zeamatin and RIP have been shown to inhibit *A. flavus* growth *in vitro* (Guo et al., 1997). Also two kernel proteins were identified from a resistant corn inbred (Tex6) which may contribute to resistance to aflatoxin contamination (Huang et al., 1997). The identification of these proteins can be used as markers, and may facilitate the cloning and introduction of antifungal genes through genetic engineering into aflatoxin susceptible crops.

Genetic Engineering Strategies :

Large germplasm pool along with differential resistance in the crops is required for plant breeding strategy. However, genetic engineering for resistance may be essential for crops which seem to have little resistance to aflatoxin contamination. Genetic engineering strategies can be utilized to enhance host resistance to mycotoxin contamination (Brown et al., 2010). Genes encoding antifungal proteins effective against mycotoxigenic fungi have been identified. Genes encoding for fungal resistance such as bacterial chloroperoxidase (CPO), small lytic peptide, D4E1 have been identified.

Identification of RAPs through proteome analysis

Proteomics approaches have been used for identification of resistance associated proteins. This approach attained more importance due to increased protein resolution and detection sensitivity by 10 to 20 fold over conventional approaches and also, increased ability to identify more constitutively-expressed RAPs.2-D gel electrophoresis method has been used for comparison of kernel proteins from several resistant and susceptible genotypes. Various protein spots which are unique or up regulated in resistant lines were detected, isolated from 2-D gels and identified using ESI-MS/MS after in-gel digestion with trypsin (Chen et al., 2002, 2007). These proteins can be grouped into three categories based on their peptide sequence homology: (1) storage proteins like globulins and late embryogenesis abundant proteins (LEA3, LEA14); (2) stress-responsive proteins, such as aldose reductase (ALD), glyoxalase I (GLX I) and heat shock proteins, and

(3) antifungal proteins, including TI. In total, 21 proteins up regulated in resistant versus susceptible lines have been identified using comparative proteomics (Table 1).

RAPs that have been identified can be further investigated to understand their potential nature of resistance. These includes (1) aldose reductase (ALD), (2) glyoxalase I (GLX-I), (3) pathogenesis related protein 10 (PR-10), (4) peroxiredoxin antioxidant (PER1), (5) cold-regulated-like protein (ZmCOR), (6) trypsin inhibitor, ZmTI, and 14 kDa TI (Chen et al., 2002, 2007).

Table 1. List of RAPs in Maize

Antifungals	Stress-related	Storage
Zeamatin	Aldose reductase (ALD)	Globulin I
Trypsin Inhibitor 14 kD	Cold-regulated (ZmCORp)	Globulin II
Trypsin inhibitor 10 kD	Water stress inducible(WSI)	Cupin domain (Zmcup)
Ribosome inactivating (RIP)/ Mod-1	Anionic peroxidase	Late embryogenesis (LEAIII)
B-1,3,-glucanase	Small heat shock protein	LEA 14
PR 10 Glyoxalase (GLX I)	PR 10.1 Peroxiredoxin (PER1)	

Mapping genes

Mapping genes associated with aflatoxin resistance in Maize have been identified. “Resistant” lines such as (R001, LB31, and Tex6) used for breeding program, where mapping populations were developed using B73 and/or Mo17 elite inbreds as the “susceptible” parents. 2L, 3L, 4S, and 8S regions on chromosome arms may prove promising for improving resistance in commercial lines through marker assisted breeding.

Table 2. RAPs as breeding markers (Brown et al., 2009)

RAP Gene	Activity vs. *A. flavus*	Resistance- related enzyme rx	Mapping Bin	Other
Heat Shock a	nda	nda	1.03	
PR-10	+[1]	Rnase	1.03	Knockout=suscept
TI-14 kDa	+high	Inhib, trypsin	2.06	Inhib, amylase
WSI	nda	nda	3.07	
Zeamatin	+low	Inhib, trypsin	7.04	
Heat Shock b	nda	nda	8.01	
ZmCorp	+	Lectin	8.04	
GLXI	nda	Forms D- Lactate	10.3	knockout
RIP	+	Lytic	nda	
PER 1	nda	Peroxidase	nda	
Â-1,3 glucanase	+	glucanase	nda	

Candidate Genes

Many bio-macromolecules and low molecular weight compounds candidate genes have been identified as antifungal in kernel tissues at various stages of kernel development in grain crops (Brown et al., 2004, 2010). Candidate antifu ngal compounds *viz*., RIPs, lectins, polypeptides, cell-surface glycoproteins, hydrolases, and certain basic proteins have been identified by several workers (Brown et al., 2010; Chen et al., 2007).

Maize kernel proteins which are found to be inhibitory in action towards *A. flavus* and aflatoxin accumulation have been reported (Table. 1). These represents PR -10, a pathogenesis related protein with antifungal and RNase activity (Chen et al., 2010) and glyoxalase I. Stress -related proteins are involved in inhibition of aflatoxin accumulation. RIP-1 (ribosome inactivating protein) from maize functions as antifungal activity that has been shown to be useful against *A. nidulans in vitro* (Nielsen et al., 2001). Tissue

specific expressions of maize kernel PR proteins which mainly function during the normal process of seed germination are mainly accumulated in response to fungal infection (Cordero et al., 1992).

Future prospects of breeding for aflatoxin resistance

Unable to locate germplasm lines showing complete resistance level to fungi, but aiming further for pyramiding resistance genes from diverse sources. Unfortunately, the success stories made so far in conventional breeding has not been able to meet the expectations for complete resistance. The recourse to biotechnology, through modification of the aflatoxin biosynthesis pathway against infection by aflatoxin-producing fungi may help in gaining maize free from aflatoxin. Genetic resistance alone may not be enough to eliminate the problem of aflatoxin contamination in maize. It will have to be accomplished with excellent crop husbandry and post-harvest practices.

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