Obliging Tactics to Mitigate the Intricate Problem of Aflatoxin Contamination in Peanut: A Review

ABSTRACT
Peanut (Arachis hypogaea L.) grown throughout the globe for its protein and oil contents. Its kernels are consumed as raw, boiled or roasted, and also in the form of culinary oil. Being a rich source of human diet (antioxidants, minerals and vitamins), animal feed (oil pressings, green straw and pods), industrial raw material (oil cakes and fertilizer), and soil fertility (atmospheric nitrogen fixation), peanut is a brilliant cash crop for both domestic markets as well as international trade. Having crystal clear importance in food and feed security peanut products are severely contaminated by aflatoxins (AFs). AFs produced mainly by Aspergillus flavus (A. flavus) and Aspergillus parasiticus (A. parasiticus), are secondary metabolites that jeopardize both human and animal health. There is no magic bullet found yet to solve this problem. Several techniques have been tested to minimize and control AFs contamination including different physical, chemical, and biological preventions. Many biological control agents, including nontoxigenic fungal strains, yeasts, and bacteria have been applied and considerable achievements gained. However, for complete eradication, a surge of studies is required to deeply investigate this intricate problem at gene and nucleotide levels and discover a permanent solution through elucidating its mechanism. The current review is focused on knowledge about A. flavus, its optimal growth conditions, growth promoting factors, factors affecting the level of AFs production, AFs biosynthesis pathway, mechanisms involved in resistance against fungal infection, various techniques and some simple precautionary recommendations to minimize AFs production.

Keywords: Aflatoxins, Aspergillus flavus, Aspergillus parasiticus, Peanut, Secondary metabolites
1. INTRODUCTION

Peanut (*Arachis hypogaea*) is grown in most parts of the world and more than 100 countries share its cultivation. It is cultivated mostly in the arid and semi-arid regions of the globe, where the two major growing partners are China and India. The top ten producers of peanut globally are China, India, Nigeria, United States, Sudan, Myanmar, Tanzania, Argentina, Indonesia, and Senegal (Figure 1) [1, 2]. Peanut is a good source of protein and oil. The importance and value of peanut oil is extremely vibrant due to the presence of low level of saturated fat and rich in antioxidants, resveratrol, which are the main contributor in cardiovascular health. Peanut share more than $35 billion in world economy in terms of production. The integral part of global peanut cultivation (~ 95 %) belong to Asia and Africa, where farmers are doing cultivation with very negligible farming resources and the most part is under rain-fed conditions. This indicates that there is a great space for its yield and quality improvement in the future. Peanut kernels contain about 48 to 50 % oil and 25 to 28 % protein, providing a rich source of energy for a large group of the human community throughout the world. Beside a promising supplier of human food, peanut also provide significant share in animal feed in the form of haulms [3]. Peanut kernels are a good source of essential nutrients including various antioxidants, minerals, vitamins and more importantly a valuable source of mono-unsaturated fatty acids. It contains p-coumaric acid and resveratrol antioxidants, the important vitamin E and other B-complex groups of pantothenic acid, thiamine, vitamin B-6, niacin and folates, a decent source of poly phenols and flavonoids. Peanut being an important source of vital nutrients, recommend as a ray of hope to eradicate the micronutrient malnutrition in different part of the developing world. A best example is its role in Niger hunger, where peanut helped in saving thousands of lives.
1.2 Hot spot potential threat in peanut food products and feed consumption

No doubt peanut having a sheet anchor role in the world economy, but its production and quality are greatly threatened by aflatoxin (AFs) contamination. AFs, produced mainly by Aspergillus flavus (A. flavus) and Aspergillus parasiticus (A. parasiticus) as a secondary metabolite, are carcinogenic for both humans as well as animals [4, 5]. AFs contamination of crops is a worldwide food safety concern, refers to a group of four mycotoxins (B₁, B₂, G₁, and G₂). Chemical structures of these four types of AFs are some what related to each other (Figure 2). Strains of A. flavus show great variation in their ability to produce AFs. Toxigenic strains of A. flavus typically produce only two types of AFs, B₁ and B₂, but most strains of A. parasiticus can produce all the four toxins [6]. Since AFs are potential carcinogens, their quantity in food and feed is closely monitored and regulated in many countries. European Union has set a maximum level of 2μg kg⁻¹ for B₁ and 4μg kg⁻¹ for total AFs in crops [7].

![Chemical structures of B₁, B₂, G₁, and G₂ AFs](image_url)

**Fig. 2** Chemical structures of B₁, B₂, G₁, and G₂ AFs
Peanut production and quality are severely affected by *A. flavus* during both pre- and post-harvest conditions [8, 9]. Prior to harvesting *A. flavus* infection frequently occurs when the pods come in direct connection with its spores in the soil. The population density of the fungus, moisture level and temperature of soil at different levels of pods development are the various factors responsible for the intensity of *A. flavus* infection [10]. It is very difficult and almost impossible to remove the AFs once the food item is contaminated with it, even with different types of food processing and cooking practices. Roasting, however, is found helpful up to some extent to minimize the AFs contamination in peanut. But, it is crystal clear that the prevention of AFs contamination is better than the application of various costly strategies which are applied to remove the contamination. The most promising footstep to overcome this problem is growing resistant cultivars, but in case of peanut development of resistant cultivars to AFs contamination is a challenging job due to its very young genomic research. Currently improvement in peanut is mainly dependent on a few promising genetic cultivars, different cultural management strategies and various techniques to control and minimize the intensity of disease and pest infestation. Several cultivars of peanut were developed through conventional breeding systems but their resistance level is still not enough to completely prevent AFs contamination. Now a day the contamination of various agro-products by AFs is the hot spot threat in most of the developing countries to both human as well as animal, because of having worse effect on food and feed safety and security [11, 12].

1.2.1 Worse experiences from the past about AFs contamination

The problem of food items contamination with AFs is not a new one to be solved, its prevention strategies has been started since 1960s. The main focus for its prevention was due to the famous “turkey X disease” which caused more than 100,000 deaths of turkey poults near London, England. The main cause of these deaths was AFs contaminated feed [18]. The focus on prevention strategies was further intensified by the 1970s outbreak in maize occurred in US, followed by a more serious outbreak in Kenya involved 317 cases caused 200 human deaths in 2004. These deaths were confirmed due to direct aflatoxicosis caused by consumption of maize contaminated with AFs [16, 19, 20]. The consumption of contaminated food in low doses are the root cause of cancer and suppression of various immunological responses.
Mostly the main and primary target of carcinogenic and toxic AFs remained the liver, where the end story includes the deadly liver cancer (Figure 4) [21]. AFs also have the ability to suppress the activity of those cells which are responsible for boosting human and animal immunity [22]. The level that how much these AFs are carcinogenic depends upon the amount and exposure time of the victim to it. Mainly due to these two factors two types of carcinogenic effects are found in the affected personals. (A) Acute illness and death, which is the result of consuming contaminated food containing very high levels of AFs. People died as a result of jaundice and liver failure, example is that of 2004 in Kenya where more than 200 people died. No animal species found yet to have resistance to acute toxic effects of AFs [16]. (B) Chronic illnesses or Cancers, caused due to the exposure to AFs of low level for a long time. International Cancer Research Institute (ICRI) categorizes AFs as a Class 1 carcinogen. Beside, AFs are also responsible for the interference in normal functioning of different cells, being capable of binding to various proteins, RNA and DNA to restrict their normal expression and thus causing cancer, mutations and necrosis in both human and animals [16]. AFs contamination is directly responsible for the economic loss in different crops especially in peanut, causing great reduction in market value. Increase occurs in the cost of product due to increase in application of healthcare and high regulatory principles. There are different regulatory principles applied by U.S. Food and Drug Administration (FDA) on levels of AFs. These levels are 20 ppb in food and feed items while 0.5 ppb in milk. These regulatory guidelines have put a great economic load of over US$932 million on agriculture worldwide due to crop losses caused by mycotoxigenic fungi including A. flavus. In extreme cases the food and feed items contaminated with AFs are completely rejected from the market. For example Africa alone pay more than US$670 million per annum to fulfill the EU principles for all food exports [12]. So, learning from the past worse experiences of AFs contamination, it is the order of the day to find out an environmental friendly solution to this distressing problem as soon as possible. The current review is focused on knowledge about A. flavus, its optimal growth conditions, growth promoting factors, AFs biosynthesis pathway, various techniques and some simple precautionary recommendations to minimize AFs production in peanut.
2. Shape, ecology and geographical distribution of
A. flavus

Due to indistinct differences in morphological and biochemical characteristics of Aspergillus species, its exact identification is a tedious job. Anyhow, A. flavus is believed to have velvety, brown or yellow to green mold with conidiophores of various lengths and mostly are pitted, rough and spiny either uniseriate or biseriate (Figure 5), covering the entire vesicle with pointed out phialides in every direction. Conidia are subglobose to globose, clearly echinulate, of having diameter within the range of 3.5 to 4.5 mm. Based on the characteristics of the produced sclerotia, A. flavus isolates can be distributed into two phenotypic types S and L. The S strain yields numerous small sclerotia having an average diameter less than 400 µm, while the L strain produces fewer but larger sclerotia. Within the S strain, some isolates, termed SB, produce only B AFs, whilst others, named SBG, produce both B and G AFs [23]. The S strain isolates have been referred to as a typical [24], producing microsclerotium [25] and A. flavus var. parrvisclerotigen [26]. The microsclerotial strains differ from A. flavus and therefore it has been suggested that they represent a taxon separated from A. flavus [26, 27]. Molecular phylogenetic analysis suggests that SB isolates are closely related to the A. flavus type culture and other L strain isolates [28].

A. flavus is distributed throughout the globe just like other related species of the Aspergillus genus. Its distribution is encouraged by airborne conidia as well as by insect activities. Similarly, humidity and other atmospheric elements also provide good support for mold vigorous growth. Water activity (aw) range of 0.86 to 0.96 also offers optimal growth condition to A. flavus. Normally A. flavus grow well at 37°C but its growth can also be experienced with in the temperature range of 12 to 48°C. The main strength of A. flavus due to which it wins the competition for substrate in plant/soil over other pathogens, is its ability to withstand a broad range of harsh environmental conditions. A. flavus form structures like mycelium or sclerotia, making it capable of overwintering. Then under favorable conditions, sclerotia either form further hyphae or asexual spores, called conidia, helping in the dispersion of fungus in the soil and air [29, 30].

Fig. 4 A=human liver in normal shape, B=infected liver by AFs in initial stage, C=completely worn-out liver after AFs infections

Fig. 5 Filamentous body structure of A. flavus
2.1 Factors affecting the level of AFs production
Fungal growth and AFs contamination are the consequences of interactions among the fungus, host and environment (Figure 6). Although some of the molecular mechanisms remains unclear, many biotic, abiotic, nutritional and environmental factors can affect the production of AFs [14]. Nutritional elements such as carbon source, nitrogen source, amino acids, fats, and trace elements can affect the production of AFs. Monosaccharide like Glucose, sucrose and maltose can promote the formation of AFs, though peptone, sorbitol and lactose cannot. However, the mechanism of the carbon source involved in the regulation of AFs biosynthetic pathway gene expression is still poorly understood. Nitrogen source affects the synthesis of AFs in different ways, when A. flavus lives in the medium of nitrate and nitrite, the levels of toxin varies [31]. Certain amino acids can also be counterproductive to the production of AFs. It has been found that tryptophan can inhibit AFs production, while tyrosine can promote the production of toxins [32]. It is reported that metal ions can affect both the growth of A. flavus and production of AFs at the cellular and molecular level [33]. Lipids make a great impact on the formation of AFs, it is not only a source of nutrition, but also a metabolic substrate [34] and signaling molecule [35] of the Fatty acyl-CoA. Some environmental factors such as temperature, pH, drought and other stresses are also believed to affect the production of AFs [36, 37]. Studies have shown that G-protein signaling catenation mediated by protein kinase A, can lead to AFLR gene transcription. This signaling pathway may respond to impact of the environment, thus affecting AFs biosynthesis [38]. When the temperature is close to 30°C, AFs are most prone to be produced. The production of AFs is closely related to changes in pH, when the media is acidic, AFs can be formed, but in alkaline media the formation would be inhibited like the fungal growth, along with the secondary metabolites, such as sporulation and sclerotia formation [39]. Secondary metabolism and sporulation require similar environmental conditions. In addition, it is reported that the secondary metabolites are formed at the same time of sporulation [38, 40]. Mutant strains with no sporulation cannot produce AFs. Some compounds which can inhibit A. flavus producing spores are also been shown to inhibit the production of AFs. The oxidative stress can induce the production of AFs. After treated by tert-Butylhydroquinone, the production of AFs increase significantly [37]. There are also endogenous phytochemical constituents, capable of inhibiting AFs production of A. flavus, and the bioactivity resided in a complex of hydrolyzable tannins. These tannins can be hydrolyzed by a fungal tannase present in A. flavus, yielding gallic acid and ellagic acid, testing of which showed that gallic acid had potent inhibitory activity towards AFs biosynthesis [41].

The main contributors in increased level of AFs contamination are highly depended upon biotic (biological) and abiotic (environmental) factors providing optimal conditions for Aspergillus to produce AFs in high amount. The production of AFs is greatly encouraged by flood, heavy rain and poor storage conditions. The AFs production level is further increased by mechanical damage through various pest and different types of stress conditions. Variation in seasons, geographical conditions for example the penetration of fungal spores to different crop parts occurs due to extreme variation in weather conditions, the kind of fungal strain present in that area, disturbance from other pest and organisms, moisture level of the soil and temperature are the elements which are involved in boosting AFs contamination [12, 40, 42, 43].

Fig. 6 Environment, A. flavus and Host interactions triangle

2.2 Schematic representation of AFs synthesis pathway
From the initial days of AFs identification, efforts have been started to control this problem [44-48]. The frontline discovery of a color mutant that stores the brick-red pigment, norsolorinic acid (NOR) in A. parasiticus, clear a milestone to understand the chemistry of AFs biosynthesis [49-52]. Since NOR discovery, the first
one and stable AFs precursor in the AFs biosynthetic pathway [53-55], provided a vital role in identification of other intermediates in AFs synthesis pathway. It opened the opportunity for isolating the first AFs pathway gene which encoding a reductase for the conversion of NOR to its final product in the form of AFs [54, 56, 57]. After some important genes being cloned, the AFs pathway gene cluster was identified in *A. flavus* and *A. parasiticus* [58]. The discovery of the cluster stimulated renewed interest to understand AFs biosynthesis throughout the globe. Substantial progress has been gained in elucidating the biosynthetic pathway of AFs, the pathway intermediates, genes, their corresponding enzymes, and different regulatory mechanisms [59-72]. There are about 30 genes which putatively involved in AFs biosynthesis. Different studies have found that AFs synthesis pathway genes are clustered within a 75-kb region in *A. flavus* and *A. parasiticus* on chromosome III approximately 80 kb away from telomere [55, 72-78]. Throughout the globe, AFs synthesis pathway has been extensively studied by different scientists and they have got promising achievements, but the complete and sound basis of the AFs synthesis pathway are still ambiguous. A better way to deeply get into insights of the mechanism of AFs production, a comprehensive investigative approach must be applied, including classical gene cloning combined with modern whole genome sequencing approaches. AFs synthesis pathway is long and complex process governed by various other regulatory mechanism, but here it is expressed in a simple and short form, which can be easily understood (Figure 7). A chains of highly intricate oxidation-reduction reactions cause the formation of AFs. The given schematic diagram is currently the most putative scheme for AFs biosynthesis, which involves the formation of NOR from polyketide acting as a first and basic step towards AFs synthesis. This step is followed by the conversion of NOR to averatin (AVN) leading the pathway to its final products in the form of AFs production.

![AFs Synthetic Pathway Diagram](image)

*Fig. 7* The schematic AFs synthetic pathway. Where NOR=Norsolorinic acid, AVN = Averantin, HAVN=5'-hydroxyaveratin, OAVN=oxoaveratin, AVF=averufin, VHA=versicoloral hemiacetal acetate, VHOH(VAL)=versicoloral, VER-B=versicolorin-B, VER-A=Aversicolorin-A, DMDHST=demethyldihydrosterigmatocystin, DMST=demethylsterigmatocystin, DHST=dihydrosterigmatocystin, ST=sterigmatocystin, OMST=O-methylsterigmatocystin, DHOMST=dihydro-o-methylsterigmatocystin, AFB1=aflatoxin B1, AFB2=aflatoxin B2, AF-G1=aflatoxin G1, AFG2=aflatoxin G2
3. Genetics basis of resistance mechanisms
Resistance against *A. flavus* infection and its subsequent AFs production mechanisms are quantitative in nature [79]. In peanut, resistance to AFs may be attributed to three different levels: it may be due to resistance against fungal infection at pod wall, or resistance to seed invasion and colonization at seed coat, or it may be the result of resistance to AFs production in cotyledons of the seed. To infect peanut, *A. flavus* have to penetrate the pod wall and then pass through seed coat to get entry into the cotyledons from where it derives its food and cause AFs contamination. Pod-shell structure and seed coat thickness, density of palisade cell layers, and presence of wax layers are the key traits contributing in resistance to pod infection and seed invasion and colonization [80]. Resistance to fungal infection can be achieved at three different levels. 1): In case of peanut, mostly AFs contamination occurs at pre-harvest stage and management practices of the crop. In this case resistant cultivars to fungal infection can play vital role in elimination of AFs contamination [81]. Resistance cultivars will provide great assistance in screening for resistance germplasm through using genomics-assisted breeding (GAB). 2): Seed coat thickness and its permeability contribute significantly in resistance against *A. flavus* infection acting outermost layer of seed defense [82]. Similarly, more compact arrangement of palisade like layer of testa accompanied by thicker waxy surface subsidizes resistance to *A. flavus* infection. Higher wax and cutin deposition are key elements contributing significantly in resistance level against *A. flavus* infection and AFs contamination, because wax content was found in significantly higher amount in resistant genotypes as compared to susceptible ones [83]. 3): Plants are endowed with several inducible defense responses like lignification and cell wall cross-linking, hypersensitive response, phytoalexins, and production of active oxygen and numerous pathogenesis related proteins in response to pathogen attack [84]. Peanut seeds contain resveratrol, which is an antifungal secondary metabolite. It was noticed that the level of resveratrol was higher up to several folds even after three days of inoculation as compared with that of susceptible genotypes [85]. Keeping in view the above three factors during developing peanut commercial cultivars can greatly support the mission of AFs contamination elimination at both pre- and post-harvest stages of the crop.

4. Obliging techniques to reduce AFs contamination in peanut
Several techniques have been tested to minimize, prevent, eliminate or decontaminate different products from AFs contamination in peanut and other crops at various growth stages. Among these prevention measures the most affective one is to minimize and prevent AFs contamination at pre-harvest stage of the crop [86]. The main focus now is to minimize and prevent the contamination of AFs through fully exploring and understanding the interrelationship between the crop and the fungus. *Aspergillus* infection is divided into three parts i.e., in the first part it damage the cell wall of the host through different enzymes, secondly, fungal machinery is developed inside the host and at third stage the production of AFs occurs [87, 88]. Techniques which can minimize AFs contamination to a significant level includes using several biological agents, advanced cultural practices and more importantly the use of modern plant breeding techniques to develop resistant cultivars against *A. flavus* and other fungi responsible for AFs production.

4.1 Handy traits against AFs contamination
There are some traits the evaluation of which can control the AFs production to a significant level. In case of peanuts, *A. flavus* to get access to the cotyledons, from where it derive its nutritions, have to penetrate the pod wall and the seed coat [80]. Here pod-shell structure can play key role in resistance to pod infection, while resistance to kernel infection and colonization is generally physical, and mostly related with thickness, density level of palisade cell layers, absence of cavities and fissures, and due to wax layers. So the structure and characteristics of the pod can be used as a source of screening for AFs resistance traits. Throughout the world, drought stress before harvest is the main reason for AFs contamination in peanut. Drought resistance traits are promising as indirect selection tools for improving resistance to preharvest AFs contamination. Traits related to drought resistance were associated well with those related to preharvest AFs contamination under drought conditions. Besides, specific leaf area, relative water content, chlorophyll density and drought stress ratings are also the best traits can be used as indirect selection tools for lower preharvest AFs contamination. Breeding for drought tolerance using these traits as selection criteria may help to accelerate progress in developing re-
sistance to preharvest AFs contamination [89]. Because of different evaluation criteria, selecting the resistance source directly is more complexed, usually resistance to AFs producing fungi can be divided in to three types i.e., bringing resistance to pod infection (pod wall), to kernel infection (seed coat), and resistance to AFs production (cotyledons) [90]. There are contradictory reports on the relationship among invito seed colonization by A. flavus (IVSCAF)-resistance and under natural conditions in open field. Sources of all the three types of resistance have been reported, but mostly the results obtained under IVSCAF were not conformed when tested under natural environment [91]. This contrast in the resistance level evaluated under laboratory conditions compared with that of open field under natural environment make it a more complex, because the value of a resistant source mainly depends upon the stability of its resistance under various envirnmental conditions. The resistance level to pod infection has been found to be highly variable and the resistance found through IVSCAF-resistance is not the absolute one, because even the best sources show about 15 % seed colonizatoin. Though, a few lines shown stable resistance but the resistance levels found are not very high [91], due to highly significant interections of genotype by environment for this trait.

4.2 Control of AFs contamination in susceptible crops

No doubt most promising elimination strategy of AFs is to develop resistant cultivars against it, but in case of susceptible one, we can also control and minimize its contamination following some simple precautionary procedures. The use of various fungicides which minimizes the fungal attack during growth season of the crop, providing proper storage conditions, including the use of different anti-mold preservatives, following reliable transport and distribution practices. A strict observation should be kept on aeration, temperature and moisture level of the storage facility, because these are the root cause of fungal growth. The entry of various insect under storage condition should be stopped. Proper storage conditions play key role in minimizing the risk posed by AFs contamination, because most of the products reach to consumers through a specific kind of storage condition. Following these simple precautionary measures, we can control and minimize the level of AFs production up to a significant level.

4.3 Control of AFs production via biological agents

Among the different techniques used for the control of AFs contamination, one and the most environment friendly is biological control or bio-control, which in simple words means control of life by life. Different bacterial species have been used to control and minimize the invasion of A. flavus and other related species which are responsible for AFs production. Among these bacterial species the famous one are Bacillus subtilis, Pseudomonas spp., Lactobacillus spp., Burkholderia spp. and Ralstonia spp., which inhibit Aspergillus and consequently AFs production under laboratory conditions [92]. Similarly, many Bacillus subtilis and Pseudomonas solanacearum strains are helpful to minimize AFs contamination when these were isolated from the maize grown soil other than the rhizosphere [93]. However, at field level in minimization of AFs contamination these bacterial strains were comparatively less affective [6]. Besides these bacterial strains, there are also some yeast species i.e., Candida krusei and Pichia anomala which were tested and found to have biocontrol properties against A. flavus at laboratory level [94]. As these microorganisms having the potential to control AFs under field conditions, so, there is a great need for these trains to be tested under natural environment in the field. One big threat in applying the biological control is due to the presence of biological control agent, where A. flavus and other related AFs producing fungi may accelerate their reproduction and start rearrangement of their genes, which will enable them to beat the biological control strength. This threat alarm us that providing a biological control strategy, it is very crucial that it should be a complete and a robust one. We must engineer our biological control agent by keeping in mind that there may be re-invasion from the fungus with more threatening capability, due to the rearrangement of its genome overtime. So, the need is to develop more highly sophisticated and defensive strategy for the near future in terms of biological control [40]. Some promising achievements have also been made in term of biological control of AFs contamination by using the nontoxicgenic competitive strains of A. parasiticus and A. flavus. Due to this strategy 70 to 90 % reduction in AFs contamination reported in peanut and cotton fields [6, 95, 96]. Owing to these successes two products obtained from nontoxicgenic strains have got the approval from US Environmental Protection Agency (EPA) and are being under use as a bio-pesticides in
peanut and cotton fields in different states of the US [6].

4.4 Application of advanced agricultural practices
Advanced agricultural practices include timely planting, which can be very helpful to escape the invasion of the fungus, maintaining proper plant to plant distances, providing such a conditions in which there is no threat of drought, supply of all essential nutrients at proper time, controlling weed to such a level having no adverse affect on main crop. Similarly, providing strategies which will help to control different insect pest. More importantly, to have proper and on time harvesting. These strategies will greatly minimize the level of AFs contamination at both field and under storage conditions [97, 98]. Crop rotation practices with time to time, proper disposal and management system for the crop residues can also provide assistance in control of A. flavus for upcoming season crop. Among other nutrients calcium is so much vital for peanut, because it is responsible for the thickness of the peanut cell wall and speeding up the processes of pod filling. Similarly, in case of peanut 50 to 90 % AFs reduction can be obtain through applying lime to the soil, using residues of cereal crops and applying farm yard manure. The farm yard manure helps to provide optimal growth conditions for various beneficial microorganisms having important role in suppressing the soil infections [97, 99, 100]. These strategies are extremely important because they are beneficial and are not so much demanding in terms of cost as well as these are environmental friendly. So, these can be used more frequently as compared to others, to minimize the contamination of various peanut products by AFs.

4.5 The role of classical plant breeding in reducing AFs contamination
An important way to win the combat against A. flavus infection and AFs contamination, is to develop highly resistant cultivars. But unfortunately, resistant cultivar development is a bottleneck in case of peanut due to its narrow genetic diversity. There is also great need of robust, consistent and an efficient techniques for screening the available resources. Various efforts have been made to find indirect methods to select resistant genotypes against pre-harvest contamination by AFs. The aim was to cut down the price, spent on screening various product for removing the contaminated ones [13, 101]. The level of significance of any resistant genotype mainly depends upon that how much it is stable. Resistant genotypes against A. flavus and other related species are very important to control the production of these toxins, but the genetic mechanism underlying this resistance is still thirsty for elucidation. On theoretical scale, important interaction have been found between the resistant genotype and the environment, but at field level they are not so significant. The great obstacle in development of a good resistant backup source against AFs is that the allelic association among different sources for resistance traits, that can be helpful for breeders to pyramid the non-allelic genes for each resistant mechanism, is still unknown. Under laboratory conditions some promising results obtained but they were not satisfactory when tested under field conditions [102, 103]. There is a great need to find resistant source which will give stable results under both laboratory and field conditions.

4.6 The role of Biotechnology in combat against AFs contamination
Bringing resistance in genotypes against AFs through classical methods is not so much an efficient and fast to get rid of these toxins. As a ray of hope, biotechnology offer more fast and more efficient way to win the contest against AFs. Through biotechnology, we can get help through studying the three main aspects related to AFs contamination, a): To further strengthen our knowledge about the mechanism of AFs biosynthesis i.e., knowledge of the fungus, b): Knowledge about the environmental factors which are involved in AFs productions and c): How to bring host-plant resistance.

4.6.1 Knowledge about Aspergillus
There is a great need to fully explore and understand each and every aspect of the life cycle of different fungi, responsible for the production of AFs. Currently, the available literature and research has been extensively reviewed and various future possibilities has been predicted. Valuable progress has been made in exploring biosynthetic pathway of the AFs and several genes have been figure out to have role in AFs production pathway. Several enzymes which speed up this production system and other regulatory systems, have been figured out [71, 104, 105]. Genome editing and manipulation has been carried out to control and guide the AFs production regulation with in the fungus. At more advanced level different genes are identified and cloned having significant role in the biosynthesis pathway of the AFs production.
These genes could be used to inhibit the biosynthesis pathway of AFs production. Two important Aspergillus species i.e., \textit{A. flavus} and \textit{A. parasiticus} have been mapped and sequenced in pinning down a 75 kb gene family, which contain about thirty genes. These genes control the AFs biosynthesis pathway [14, 104, 106]. These information has greatly facilitated and opened the opportunity to find out resistant mechanisms which will prevent fungal progression as well as the production pathway of AFs.

4.6.2 Environmental factors responsible
Environmental factor are of so much importance, greatly affecting AFs production. Among the environmental factors, drought is the major one effects both AFs production as well as appropriate development of seeds [107]. Drought causes reduction in moisture level of the seed as a result of which the property of seed hormone to produce phytoalexins is greatly reduced. Due to the reduction of these phytoalexins, fungal infection occur which cause great economic losses [14]. So, understanding the interaction between the fungus and different environmental factors will be very fruitful to minimize the risk posed by AFs contamination.

4.6.3 Mechanism of host-plant resistance
From decades plant breeders and reasearchers are trying to minimize and get rid of AFs contamination through conventional techniques of plant breeding. Although they also have got some promising results but the progress is still very limited to win the combat against Aspergillus and AFs. So, the need to overcome this problem as soon as possible has shifted the research trust from classical breeding to modern plant biotechnology. Through genomic manipulation techniques accompanied by good agricultural practices, provides some golden spark to prevent AFs contamination. In the modern era in various techniques like microarray, sequencing of the whole genome and expressed sequence tags (EST), valuable achievements have gained through finding out different genes involved in host-plant interaction as well as AFs contamination. A few plant factors are also found to have some defensive properties against Aspergillus infection. This defensive nature of the plant is found in three form i.e., Seed proteins which are involved in defense against host cell wall degrading enzymes of the fungus, some natural products found in the seeds or kernels which have a vital role minimize the fungal growth (AFs production) and some other proteins which comes in activation when the plant is under stress conditions [91].

4.6.4 Candidate resistant genes, key towards permanent solution of AFs
Permanent and most effective way to control AFs production is to find out some inhibitory compound against them which may be inhibitory proteins, small molecular weight polypeptides, lectins, hydrolases and cell-surface glycoproteins. Through cDNA several genes are found to have resistance against AFs contamination when they were up-regulated. Similarly, some valuable achievement has been made through proteomic methods [108-110]. In a study carried out by Guo et al. [109] more than 21,777 expressed sequenced tags (ESTs) were generated in peanut to figure out resistant genes, having role in the host plant defense mechanism against Aspergillus and AFs contamination. These genes were then used to develop markers and genetic maps. These genes can be used to profile the transcript and find out candidate genes for various traits of interest. Studies have been carried out to identify the miRNA having role in the gene expression during post-transcriptional stage. Gene expression in resistant peanut cultivar as well as that of susceptible one was profiled and 62 genes were found to have resistance against AFs contamination when they were up-regulated. Along with these 62 genes other twenty-two putative resistant genes against Aspergillus were figured out. In resistant cultivars these genes were highly expressed as compared to that of susceptible one [111].

4.6.5 Targeting Induced local lesions in genomes (TILLING)/mutagenesis
Germplasm are the backup tool for any crop having a good source of resistance to different types of stress conditions and various infectious diseases, having variability among them for the same trait. Sometime the natural variation found among these germplasm is not enough to cope with the sudden invasion from different insect pest and diseases. So, to add to the natural capabilities of the germplasm induced mutation play a very vital role. Various sources have been developed of induced mutation for peanut. Knoll et al. [112] using TILLING techniques screened 3400 mutant lines which were developed through Ethylmethane Sulfonylate (EMS) application. This population developed through TILL-
ING will be helpful for functional studies of the genomic, also for recovering unwanted and unintentional mutations [112]. Similarly, using this technique we can also develop such mutated lines which will have the ability to resist against Aspergillus infection and AFs contamination.

4.7 Molecular breeding, a competent approach to get rid of AFs

Classical breeding no doubt has its own importance but molecular breeding has played and still playing an important role in coping with the growing food demand from the rapidly growing population of the world. Molecular markers are the keys of molecular breeding which have enabled the breeders to transfer a trait like resistance to a variety which was susceptible prior to this transformation. These tools can be used to limit the traits which are undesirable. These techniques are so much important to get rid of the Aspergillus and AFs contamination [113]. In case of peanut, very minute variability has been explored for AFs resistance at DNA level using molecular markers, even various agromorphological traits have also been found with very negligible variation among the various cultivars. At earlier attempts SDS-PAGE was used to find out some differences at protein level to AFs resistance but no promising success was gained. After that an Amplified Fragment Length Polymorphism (AFLP) marker, having resistance to Aspergillus infection, was converted to Sequence Characterized Amplified Region (SCAR) to get promising results in future breeding programs. Then many cultivars of peanut, having promising resistance to AFs, have been used to develop such SCAR markers and one of those having promising results i.e., SCAR “AFs-412” [114]. So, using such type of molecular markers in future breeding programs we can minimize the contamination level of AFs. These markers will be helpful in screening the germplasm prior to the development of commercial peanut cultivars.

The key tool of modern biotechnology and molecular breeding is the ability of altering the genome of an organism which in short terms known as genetic engineering. Through using this technique a molecular plant breeder can bring desirable change in the genetic makeup of a cultivar, which will enable it to survive various stress conditions and show resistance to different invading insect pests. Using molecular transformation tools, we can develop such peanut cultivars which will have resistance to AFs production [115, 116]. Currently scientists are working on various genes and genes constructs to develop resistant cultivars against those fungi which are responsible for AFs production. Some lytic peptides have been found which are capable of inhibiting A. flavus and are the ray of hope to develop resistant cultivars to AFs contamination. These lytic peptides include D4E1 and D5C, but incase of natural lytic peptide, used to develop stable resistant cultivars, researchers are facing the controversy of effecting non-targeted organism in case of transgene escape to the natural environment [108, 117].

4.7.1 QTL mapping, a golden gift of molecular breeding to mitigate AFs contamination

QTL mapping is one of the golden gifts provided by molecular breeding and is comprehensively under use in modern plant breeding to improve different quality and quantity traits. Most of the important morphological and physiological traits are controlled by several genes working in groups called quantitative traits. These traits are also called complex, multifactorial or polygenic traits. The genomic regions which control the expression of these complex traits are called QTLs. QTL mapping is one of the reasons which shifted the thinking from classical breeding to molecular breeding and one of the great breakthrough in that respect was the development of molecular or DNA markers. DNA markers are the building blocks of genetic linkage map, while these maps have a critical role to find out the specific genomic regions which control the expression of the quantitative traits [118]. Through QTL analysis tightly linked molecular markers to the trait of interest like A. flavus resistance can be developed and will be deployed in molecular breeding to develop resistant cultivars, and also to screen the susceptible ones. QTL mapping in peanut was slow compared to other crops, because of its complicated genome, but recently due to the advent of more advanced sequencing techniques like Specific Length Amplified Fragment sequencing (SLAF-seq), this process accelerated. SLAF-seq technology is a new, highly precise and robust as compare to other sequencing techniques. More importantly its cost is much lower than its counterparts. It is a combination of locus-specific amplification and high-throughput sequencing, been subjected to a series of critical trials to assure its high accuracy, efficiency, and density. Being in its
young age, SLAF-seq has been fruitfully applied to construct high-density genetic map and important QTLs have been identified harboring significant putative candidate genes for different traits of interest in various crops and animal [119-128]. Till now in peanut, very few QTL mapping studies have been reported against AFs contamination. The first study based on QTL mapping against A. flavus invasion reported six QTLs located on chromosome A01, A02, A03, A04, B05, and B08 which contributed 22.7 %, 11.2 %, 6.2 %, 6.6 %, 10.5 %, and 7.3 % in PVE, respectively [129]. Individual QTLs were identified for aflatoxin AFB₁, AFB₂, and PSII via a RIL population obtained from crossing Zhonghua 10 and ICG 12625. In this study, they identified two QTLs for PSII one located on chromosome A03 sharing 8.0 % in phenotypic variation (PVE) and second located on chromosome A10 with 13.0 % PVE. For AFB₁, 7 QTLs were mapped including two major QTLs located on chromosome A05 and B06 sharing 17.9 and 16.3 % in PVE, respectively. For AFB₂ they also mapped seven QTLs located on chromosome A07, B05, B06 and B07 with PVE contribution of 12.2 %, 11.1 %, 21.0 % and 14.5 %, respectively [130]. QTL mapping via a RIL population obtained from a cross of two highly contrasting nature to A. flavus resistance Yueyou 92 (YY92) and Xinhuixiaoli (XHXL) during in-vitro seed colonization (IVSC) mapped two major QTLs located on chromosome A03 and B04 shared 19.0 % and 5.1 % in PVE, respectively (unpublished data). Genome-wide association studies using ICRISAT reference set identified a marker associated with IVSC and with more than 24.7 % contribution in PVE [131]. Even these studies are of great importance and the QTLs/genomic regions identified (Table 1) can be used in future studies to bridge the gap of AFs contamination in peanut but still these findings are very few and further findings needed to find solid and consistent solution to this alarming problem.

Table 1: Mapped QTLs of AFs resistance in peanut

<table>
<thead>
<tr>
<th>Trait</th>
<th>LG</th>
<th>Position</th>
<th>Marker Interval</th>
<th>LOD</th>
<th>PVE %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistance to A. flavus invasion</td>
<td>A01</td>
<td>20.35</td>
<td>TC11H06–TC4H07</td>
<td>4.30</td>
<td>22.7</td>
<td>Liang et al. 2009</td>
</tr>
<tr>
<td>A02</td>
<td></td>
<td>9.31</td>
<td>gi-716–TC1E05</td>
<td>2.26</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td>A03</td>
<td></td>
<td>5.31</td>
<td>pFGSseq18E7–Seq4E08</td>
<td>2.60</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>A04</td>
<td></td>
<td>12.76</td>
<td>pFGPseq2H8–PM3</td>
<td>2.1</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>B05</td>
<td></td>
<td>25.01</td>
<td>pFGPseq7G2–TC5A06</td>
<td>2.91</td>
<td>10.5</td>
<td></td>
</tr>
<tr>
<td>B08</td>
<td></td>
<td>6.78</td>
<td>TC11A04–PM137</td>
<td>2.4</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>Percent seed infection</td>
<td>A03</td>
<td>28.5</td>
<td>AHGS2058–AGGS0052</td>
<td>3.1</td>
<td>8.0</td>
<td>Yu et al. 2019</td>
</tr>
<tr>
<td>A04</td>
<td></td>
<td>51.1</td>
<td>AHGS1245–AGGS0876</td>
<td>3.2</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>A05</td>
<td></td>
<td>80.3</td>
<td>ARS734–GM2156</td>
<td>6.0</td>
<td>17.9</td>
<td></td>
</tr>
<tr>
<td>B06</td>
<td></td>
<td>42.5</td>
<td>AGGS1515–AGGS1587</td>
<td>6.4</td>
<td>16.3</td>
<td></td>
</tr>
<tr>
<td>B06</td>
<td></td>
<td>69.5</td>
<td>AHGS1464–HAS0969</td>
<td>3.1</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>B07</td>
<td></td>
<td>39.2</td>
<td>AGGS1581–GM2067</td>
<td>3.6</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>B07</td>
<td></td>
<td>86.0</td>
<td>TC3B4–AHGS2233</td>
<td>3.1</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>B07</td>
<td></td>
<td>103.7</td>
<td>AHGS1081–AHE0615</td>
<td>3.2</td>
<td>7.5</td>
<td></td>
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<tr>
<td>Aflatoxin B₁</td>
<td>A10</td>
<td>43.5</td>
<td>AGGS1425–ARS710</td>
<td>5.0</td>
<td>13.0</td>
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</tr>
<tr>
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<td></td>
<td>51.1</td>
<td>AHGS1245–AGGS0876</td>
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<td>8.0</td>
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</tr>
<tr>
<td>A05</td>
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<td>ARS734–GM2156</td>
<td>6.0</td>
<td>17.9</td>
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<tr>
<td>B06</td>
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<td>42.5</td>
<td>AGGS1515–AGGS1587</td>
<td>6.4</td>
<td>16.3</td>
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<tr>
<td>B06</td>
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<td>69.5</td>
<td>AHGS1464–HAS0969</td>
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<td>7.8</td>
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</tr>
<tr>
<td>B07</td>
<td></td>
<td>39.2</td>
<td>AGGS1581–GM2067</td>
<td>3.6</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>B07</td>
<td></td>
<td>86.0</td>
<td>TC3B4–AHGS2233</td>
<td>3.1</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>In-Vitro seed colonization</td>
<td>A03</td>
<td>50.2</td>
<td>AGGS1139–AHGS2025</td>
<td>3.5</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>A07</td>
<td></td>
<td>74.3</td>
<td>AHGS1454–HAS1360</td>
<td>4.0</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td>A07</td>
<td></td>
<td>83.5</td>
<td>ARS734–GM2156</td>
<td>5.1</td>
<td>12.2</td>
<td></td>
</tr>
<tr>
<td>B05</td>
<td></td>
<td>45.4</td>
<td>AGGS0979–TC19E1</td>
<td>4.9</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td>B06</td>
<td></td>
<td>43.1</td>
<td>GM2444–AHGA335472</td>
<td>3.8</td>
<td>9.3</td>
<td></td>
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<tr>
<td>B06</td>
<td></td>
<td>43.2</td>
<td>GM2444–AGGS0983</td>
<td>8.8</td>
<td>21.0</td>
<td></td>
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<tr>
<td>In-Vitro seed colonization</td>
<td>B07</td>
<td>80.8</td>
<td>TC3B4–AHGS2233</td>
<td>5.3</td>
<td>14.5</td>
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<tr>
<td>A07</td>
<td></td>
<td>1.673</td>
<td>Marker8555604–8633509</td>
<td>10.54</td>
<td>19.03</td>
<td>(unpublished data)</td>
</tr>
<tr>
<td>B04</td>
<td></td>
<td>1.338</td>
<td>Marker4154940–4158241</td>
<td>2.85</td>
<td>5.15</td>
<td>Pandey et al. 2014</td>
</tr>
</tbody>
</table>

In-Vitro seed colonization
4.8 Phenotyping, a compulsory call for AFs resistance evaluation

The biosynthesis of AFs in almost all crops is a result of intricate fungus-environment interactions. High level of AFs contamination at field has been reported when the growing season accompanied by drought and hot weather conditions [132]. Studies have shown that maize crop grown under optimum irrigation resulted in reduced fungal infection and AFs contamination, especially when the irrigations were applied in drought stress conditions [133, 134]. Similarly, greater AFs contamination was shown in peanuts during drought-stressed conditions accompanied by high soil temperatures, moreover affecting pre-harvest infection [135]. Mostly it was found that the genotypes reported as resistant under in vitro conditions when tested in natural environment in the field were not so much promising. This threat further compels the need to develop such a high throughput phenotyping assays which will provide field-like environmental conditions for resistance evaluation against AFs [136, 137]. Keeping in mind that nothing is impossible in science, and more importantly due to our lanced sequenced genome for cultivated peanut (http://peanutgr.fafu.edu.cn) [138], we are hopeful that in near future a promising and consistant solution to the problem of AFs contamination may be found.

5. CONCLUSION AND RECOMMENDATIONS

AFs contamination is an extremely intricate problem which is strongly influenced by numerous external factors as well as genetic resistance. Resistant cultivar development is still being a challenging job in peanut. In addition, to prevent AFs contamination in peanut good management practices during pre-and post-harvest stages are extremely critical. In the past various techniques have been applied to minimize and eradicate the AFs contamination. Though some encouraging achievements have been made but non of them was found to have 100 % efficacy in elimination of AFs contamination, where the main hurdle found was the availability of very little knowledge of the molecular mechanism of AFs production. As alternative various conventional breeding techniques and strategies were found helpful up to some extent but no one was found adequate for complete solution of this problem. To further speed up the contest against AFs contamination a sound and deep investigation is required at molecular level to find out more resistant genes against AFs production. Significant control of AFs contamination needs a multipronged method comprise of biological control, more advance agronomic and cultural practices along with high genetic resistance by the host plant. Following are some simple recommendations which can reduce AFs contamination to significant level: Use of lime (0.5 t/ha), cereal crop residue (5 t/ha) and farm yard manure (10t/ha) at sowing time to reduce A. flavus infection and AFs contamination from 50 to 90 % [139]. Select comparatively highly resistant cultivar to A. flavus infection and AFs contamination and with better tolerance to drought [140]. Harvest the crop at optimum maturity. Harvesting before optimum maturity or making delay in harvesting causing poor quality seed which provide opportunity for A. flavus to produce AFs. Try to avoid any physical damage to pods at the time of harvest due to which A. flavus infection occurs more frequently. For drying the harvested pods, clean sheets should be used instead of direct drying on the ground. Better to remove the immature and infected pods before drying from the mature and healthy pods. Sustain proper storage facilities having proper ventilation. The pods should be dried and more importantly have low relative humidity.Having proper control on the entry exit of insect pest and rodents.

AUTHOR CONTRIBUTION STATEMENT

SAK wrote the manuscript and ZW modified the manuscript.

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