Chapter 5

Novel Methods for Preventing and Controlling Aflatoxins in Food: A Worldwide Daily Challenge

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1. Introduction

Talking about Aflatoxins is not a new issue. Aflatoxins are a big problem that day by day turns more important due to their implication in crop production, food quality and human and animal health. Aflatoxins are also everywhere because those toxic secondary metabolites are mycotoxins produced by a large number of Aspergillus species, being *A. flavus*, and *A. parasiticus* the main producers; nevertheless, species like *A. nomius, A. pseudotamarii, A. parvisclerotigenus, A.bombycis, A. ochraceoroseus, A. rambellii, Emericella astellata* and *E. venezuelensis* are aflatoxin generators too [1,2]. Since those toxins have been recognized as a significant worldwide problem in 1960 (because of being isolated and identified as the causative toxins in "Turkey-X-disease" after 100,000 turkeys died in England from liver acute necrosis and bile duct hyperplasia after consuming groundnuts infected with *Aspergillus flavus*) [3-5], researchers have studied lots of ways to fight against this threat; however, after more than a half century, aflatoxins are still a big problem that has not been easy to deal with, because humans are not able to manipulate essential factors that affect aflatoxin contamination like the region weather, the crop genotype, the soil type, the minimum and maximum daily temperatures and the daily net evaporation [5].

Aflatoxins (AF) affect almost everything we eat: cereals (maize, wheat and rice principally) and their derivates; oilseeds (cotton, peanut, rapeseed, coconut, sunflowers and others), cassava, nuts, dry fruits, delicatessen products, spices, wines, legumes, fruits, milk and milk de-

rivates [6,7], and even chocolates [8]. In order to find a solution for this problem, some organizations and institutions have purposed prevention strategies in order to reduce the risks given by this public problem especially in low-income countries, but those strategies are not enough to give a real solution to this worldwide daily problem.

2. The global problem of AF in crops and food

The prevalence of AF in crops and livestock is a serious problem in many parts of the world, undermining public health and development efforts. AF are highly toxic, cancer causing fungal metabolites known to cause immune-system suppression, growth retardation, liver disease, and death in both humans and domestic animals. According to the United Nations Food and Agriculture Organization (FAO), 25% of world food crops are affected, and countries that are situated between 40ºN and 40ºS are most at risk. Over 4.5 billion people in developing countries are at risk of chronic AF exposure [9]. Unless AF levels in crops and livestock are effectively managed, international development efforts to achieve greater agricultural development, food security and improve health will be undermined.

AF are very stable and persistent, so they are difficult to remove. Due to they are contained in many crops that are consumed by animals, AF have turned into a serious animal problem too. The most susceptible animals are rabbits, turkeys, chickens, pigs, cows and goats [10]. AF can be transmitted from animals to human food (by eggs, meat and dairy) with the consequent risk to human health.

Even non-mouldy foods or raw materials may contain AF. Spores can be transferred by insects (especially flies, wasps and bees) or by birds to foods where the spores germinate, produce mycelium, and AF are excreted. Seeds can contain AF by infection of the egg-cells of the flowering plants. The spores of *A. flavus* and *A. parasiticus* can germinate on the stigma surfaces of plants, then the germ tube penetrates to the developing embryo mimicking pollen germ tubes. The mycelium can establish an endotrophic relationship which is not harmful for the healthy plant. However, if the plant is under drought stress, then significant levels of AF may be produced in the plant tissue during growth in the field. Under these circumstances food commodities may already be contaminated at harvest and, although the concentrations are never as high as those formed in stored commodities, they can be economically significant [11, 12].

The danger of AF lies in their mode of action by inhibiting the incorporation of precursors for the synthesis of DNA, RNA and proteins; they also block the action of some enzymes that are responsible for the synthesis of nucleic acids, causing centrilobular necrosis in the liver, polymorphonuclear infiltration and fatty degeneration. AF toxicity depends on the dose, the exposure degree, the age, the nutritional status of the animal and the possible synergic effects of the chemical agents to which they are exposed [13]. Some secondary metabolites produced by Aspergillus species are harmful for animals too. That's the case of cyclopiazonic acid (CPA), which causes necrosis of liver or gastrointestinal tissue and necrotic changes in skeletal muscle and kidney [14, 15].

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The economic impacts of AF contamination can vary greatly among affected food and feed commodities. These differences include the severity of the contamination problem, the geographic range of AF problems, the types of AF control methods available, and which sectors bear the burden of the cost of AF contamination. All of these factors affect whether AF control methods are adopted [16]. AF and mycotoxins in general have not been widely prioritized from a public health perspective in low-income countries. This is because knowledge of mycotoxins and the full range and scale of their adverse health effects is incomplete and the known risks are poorly communicated to governments in regions where the contamination is greatest [17]. Matters that have to be considered by government to avoid diseases from aflatoxicosis are: an opportune and nonexpensive analytic detection, unifying worldwide government regulations, deviation of AF-contaminated commodities from the food supply, improving research on the biosynthesis and molecular biology of AF, and designing new control strategies for the abolition of AF contamination of food crops, inter alia [10].

3. How to prevent pre-harvest AF?

It was established in about 1970 that fungal contamination could start in the field before harvest [9]. Although the highest levels of AF are undoubtedly associated with post-harvest spoilage of food commodities stored under inappropriate conditions of water activity and temperature, the aflatoxigenic fungi have more complex ecologies [12]. Factors that influence the incidence of fungal infection and subsequent toxin development include invertebrate vectors, grain damage, oxygen and carbon dioxide levels, inoculum load, substrate composition, fungal infection levels, prevalence of toxigenic strains and microbiological interactions. Insect damage on crops allows fungi to access in them, increasing the chances of AF contamination, especially when loose-husked maize hybrids are used [18, 19].

Controlling or reducing infection by regulating the factors that increase the risk of AF contamination in the field contributes extensively in managing AF. Management practices that reduce the incidence of AF contamination in the field include timely planting, maintaining optimal plant densities, proper plant nutrition, avoiding drought stress, controlling other plant pathogens, weeds and insect pests and proper harvesting [20]. Pre-harvest measures that are efficient in reducing AF levels are the same as those that will enhance yields. Crop rotation and management of crop residues also are important in controlling *A. flavus* infection in the field. Tillage practices, fertilizer application, weed control, late season rainfall, irrigation, wind and pest vectors affect the source and level of fungal inoculum, maintaining a disease cycle in crops like maize [19, 21]. Lime application, use of farm yard manure and cereal crop residues as soil amendments have shown to be effective in reducing *A. flavus* contamination as well as AF levels by 50-90%. Calcium, which is part of lime, thickens the cell wall and accelerates pod filling, while manure facilitates growth of microorganisms that suppress soil infections [21].

In order to minimize the levels of AF and mycotoxins in general, the National Institute of Agricultural Technology of Argentina (INTA), recommends to make early plantings, to

plant resistant genotypes, to do good farming practices, to avoid stress conditions, to minimize insect damage, to harvest early in order to avoid delays, to avoid damaged kernels and to storage at less of 13% moisture in a clean, fresh and airy place with no insects [22]. As mentioned before, it is important to avoid product moisture, high temperatures (between 25 and 32°C) and high relative humidity in storage and seeds preservation. Weeds have to be removed and crop rotation should be done routinely. Prior to the preparation of the ground, dead organic matter has to be disabled or burned; product mechanical damage has to be avoided; crops have to be collected at full maturity; storage places should be dry and the entry of water has not to be allowed; storage health standards have to be fulfilled (pallets, proper humidity levels, adequate ventilation and lighting, etc.), and periodic inspection of the stored product should be done [23].

To avoid risks to human and animal health, INTA also suggests to avoid feeding animals with crops in poor condition (especially corn), not to use fractions of discarded corn fodder, and to make good manufacturing practices [22].

4. Traditional AF control methods

Since AF have been recognized as a significant worldwide problem, researches have proposed some ways of detoxification. AF detoxification refers to those post-harvest treatments directed to eliminate or diminish the toxic effects of toxins. Those strategies can be divided into three different groups: natural methods, physical methods and chemical methods, which are focused on destroying, modifying or adsorbing AF [24]. There is variety of tools such as post-harvest drying (which is economically accessible), adequate storage, shelling, dehulling, product sorting, early harvest, regionally adjusted planting dates, and insect control. However, even when storage conditions are generally good, AF frequently form prior to harvest while the crop is maturing and/or awaiting harvest, which can result in significant losses [5].

4.1. Natural methods

The natural methods used to avoid AF are principally: seed cleaning, sorting and seed division by screening and extrusion. Nevertheless, those techniques are neither practical nor efficient at all, and food micronutrients content get diminished [24]. Since 1989, the FAO has supported some decontamination processes like the UK-Thai Project (UTP) System, which showed to reliably produce low AF-content maize during the rainy season. With the UTP system, maize is first field dried on the stalk for one to two weeks before harvesting to reduce moisture content to 20%. It is next shelled within 24 to 48 hours of harvest, and loaded into a drier within 12 hours of shelling. Thus, within 48 hours, it is dried to 14% moisture content, with no part exceeding 15%. AF content is monitored rapidly by a special adaptation of the bright greenish-yellow fluorescence (BGYF) test. Maize dried to 14% moisture content by the UTP system can be safely stored for a minimum of two months with no increase in AF content [25].

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By the other hand, cleaning of stores before loading in the new harvests has been correlated with reduction in AF levels. Separating heavily damaged ears (those having greater than 10% ear damage) also reduces AF levels in crops like maize. Wild hosts, which constitute a major source of infestation for storage pests, should also be removed from the vicinity of stores. For some crops like peanuts, the standard practice is drying of pods in the sun. Often pods are left in the field after uprooting for up to four weeks to partially dry prior to home drying [19].

AF are unevenly distributed in a seed lot and may be concentrated in a very small percentage of the product. Sorting out of physically damaged and infected grains (known from colorations, odd shapes and size) from the intact commodity can result in 40-80% reduction in AF levels [19]. The advantage of this method is that it reduces toxin concentrations to safe levels without the production of toxin degradation products or any reduction in the nutritional value of the food. This could be done manually or by using electronic sorters. Some studies have also looked at the use of local plant products for the control of fungi mostly proving their efficacy in-vitro but these products have not been sufficiently tested for their efficiency in controlling AF in stored crops [19, 26].

4.2. Physical methods

Although natural methods are cost-effective, the fungal contamination in grains is often unavoidable, so there is the need to apply a suitable process to inactivate the toxin. Sorting can remove a major part of AF contaminated units, but levels in contaminated commodities may also be reduced through physical food processing procedures like dehulling (which reduces AF contamination by 92%), roasting, baking, frying, X-radiation, extrusion cooking and nixtamalization, being the last two the most studied because of their effectiveness [27-29].

Roasting, baking and frying are three common methods used in some low-income countries, and all of them involve heath. Nevertheless, the heat used as the only factor for the mycotoxins destruction is ineffective because the temperatures reached during the detoxification process affect vitamins and food proteins. In contrast, heath can be used for increase the reactive capacity of some food molecules such as acids, alkalis and other chemical agents [30].

Radiation has also been used against AF. X-rays are capable of producing a high issuance of energy, which causes the breakdown of stable molecular structures. It has been established that AFB1 and AFG1 are the most sensitive to X-rays [30, 31].

Extrusion cooking is a processing technology that involves pushing a granular food material down a heated barrel and through an orifice by a rotating, tight fitting Archimedean screw. The shear forces created by the rotating action of the screws, together with frictional, compressive and pressure forces provide the necessary environment for rapidly cooking and transforming the food into visco-elastic melt. Extrusion cooking is an efficient high temperature short time process, and it is used to produce a wide variety of foods and ingredients. To destroy or inactivate AF, the extrusion cooking conditions need to be severe (high shear, high temperature, and the right pH) in order to provide the necessary environment in the

barrel, but such treatments to destroy or inactivate AF in peanuts may affect essential nutrients and compromise the nutritional quality of the food product [32].

In 2011, Saalia & Philips reported that extrusion of artificially contaminated food degrade AF to varying degrees depending on the extrusion conditions without altering nutritional quality. They extruded naturally contaminated peanut meal by varying the moisture (20, 28, 35 g/ 100 g); pH (7.5, 9.5) and extruder die diameter (2.5, 3, 3.5, 4.0 mm). The highest AF reduction in naturally contaminated peanut meal was 59% at feed moisture content of 35 g/100 g. Higher (91%) reduction was achieved in the artificially contaminated peanut meal at moisture of 20 g/100 g. In-vitro protein digestibility and Fluorodinitrobenzene (FDNB)-available lysine of the extrudates were not significantly different from non-extruded peanut meal, and extrusion conditions for AF reduction did not adversely affect protein nutritional quality. Extrusion conditions that reduced throughput in the single screw extruder promoted greater AF reduction. Those conditions also marginally reduced the protein nutritional quality of the extrudates. High moisture conditions provided extrudates with the least in-vitro protein digestibility and lowest available lysine. Decontamination of naturally contaminated peanuts using extrusion cooking was less successful (59%) than artificially contaminated peanut meal (91%) [32].

Nixtamalization (TNP) is an alkaline cooking process original from ancient Mexico which is applied in corn tortillas. Alkalinity largely destroys AF in corn. TNP consists on the cooking of the grain in abundant water and lime (2–3 L of water/kg of maize processed, with 1–3% $CaOH_2$) at boiling temperatures for 35–70 min, with a steeping period of 8–16 h. After the steeping, the lime cooking solution (nejayote) is decanted, and the grain is thoroughly washed to leave the grain ready for milling to obtain the maize dough for making tortillas [33, 34]. It has been shown that traditional nixtamalization is capable of destroying 85% of the AF present in maize, and 15% of AF remaining in mass does not retain its fluorescence properties, but can be recognized by the monoclonal antibodies used for recent studies detection [35]. Mendez-Albores and collaborators reported that traditional nixtamalization can reduce AF concentrations in 94% even in highly contaminated maize, being more effective than extrusion cooking; nevertheless, this finding has been widely questioned because other authors suggest that AF lactone rings, which are opened during nixtamalization alkaline process, can be closed when tortillas are acidified in stomach [34, 35]. It is important to mention that some authors have reported nixtamalization as a chemical method [24].

4.3. Chemical methods

Chemical AF control methods are principally those which involve the use of chemical reagents for different purposes. Most investigators are looking for new sources of materials to control spoilage caused by fungi in food. However, the application of synthetic preservatives has led to a number of environmental and health problems because they are themselves carcinogenic, teratogenic, and highly toxic with long degradation periods [36, 37].

Insecticides and fumigants were the first chemicals to be used to deal with aflatoxigenic fungi. The DOA Division of Plant Pathology and Microbiology screened since several decades ago, seven reagents in the laboratory for effectiveness in preventing or reducing AF contam-

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ination of maize. Only three of the reagents were found to be effective: sodium bisulphite, ammonia, and propionic acid. Sodium bisulphite and ammonia treatments resulted in grain with a strong residual odor; the ammonia treatment also produced darker grain. The most promising regent was the propionic acid-based fungicide formulation, which effectively controlled both mould growth (*A. flavus*) and AF formation, while not adversely affecting the physical quality of the grain [25]. Nowadays, the use of insecticides for this purpose has been abandoned due to the toxic residues that they generate [19]. About fumigants, only two were in common use in the last decade: methyl bromide and phosphine. Methyl bromide has been identified as a major contributor to ozone depletion, which casts a doubt on its future use in pest control. There have been repeated indications that certain insects have developed resistance to phosphine, so its use is now doubtful [30, 38]. It has also been reported that propionic acid, sodium propionate, benzoic acid, ammonia, urea and citric acid are the best anti-fungal chemical compounds tested in feeds [39].

Organic solvents can be used to remove AF in food because mycotoxins have the physicochemical characteristic to be soluble in them. Combinations such as hexane-acetone-water or isopropanol-water, inter alia, have been reported to be effective mycotoxins draggers. Some acids such as hydrochloric acid, sulfuric acid and their derivatives have the capability to react with the lactone groups of AFB1, AFG1, and with non-aromatic double bonds present in AF. Toxicologically, the addition reaction of the acids with the double bonds structures appears to be most effective in terms of detoxification because the reaction products are polar substances that can be eliminated in the urine. Alkalis like monoethylmethylamine, hydroxide and calcium chloride, sodium hydroxide and ammonium carbonate, are reactive with the lactone group of AF. Oxidant agents such as ozone, peroxides and permanganates in alkaline solutions are reactive with non-conjugated double bonds of AF. The ozonolysis reaction leads to the creation of smaller molecules, but some of the obtained products could be toxic. The glycosylation reaction results in the creation of two hydroxyl groups that can subsequently form hydrogen bonds; nevertheless although this mechanism is effective for AF detoxification, it should be used in combination with polymers or silicates capable of adsorbing physically AF through hydrogen bonds [30].

Adsorption of mycotoxin molecules has been studied recently. It can be done by different inert chemicals, such as some complex indigestible carbohydrates (cellulose, polysaccharides in the cell walls of yeast and bacteria like glucomannans, peptidoglycans and others), synthetic polymers (such as cholestyramine and polyvinylpyrrolidone), humic acid and vegetable fibers, and clays or synthetic silicates, which can sequester mycotoxins. The pyrrolidone mechanism of action is due to both, physical adsorption effect and the bridges establishment of hydrogen and nitrogen in its structure [30, 40, 41]. The adsorptive capacity of the carbohydrate complexes in the yeast cell wall offers an interesting alternative to inorganic adsorbing agents. Modifications in manufacturing techniques have enabled the production of specifically modified yeast cell wall preparations with the ability to adsorb a range of mycotoxins. Several reports indicate the possibility of there being more than one target for mycotoxin binding in cell wall preparation. However, it is too early to interpret the mechanistic aspects and more basic studies are needed on the interaction of individual

mycotoxins with different components of *S. cerevisiae* cell wall. More studies are needed on the chemistry of binding and stability of the complex, especially under the harsh conditions of the gastrointestinal tract. Moreover, several studies suggest that yeasts or esterified glucomannan products may not be effective in reducing AFM1 concentrations. Further *in vivo* studies are needed to confirm the effectiveness of yeasts and derivative products in suppressing absorption of AF in ruminants. Results on the efficacy of synthetic polymers or vegetable fibers in sequestering mycotoxins are highly promising, although this field is still in its infancy and further research is needed [40].

The aluminum silicates belong to clays, highlighting bentonite, sepiolite and zeolite. These compounds possess a three-dimensional structure formed by the junction core of SiO_4 tetrahedra, wherein some ions such as aluminum ions are intercalated. Nowadays, between of all the chemical methods of detoxification, silicates are the most used because they don't create waste problems, they don't destroy food vitamins and proteins, they don't generate partial reactions, they don't create toxic metabolites, and their prices are not elevated. Not only natural aluminum silicates but also Hydrated Sodium Calcium Aluminosilicates (HSCAS) are used, because the last ones have a greater adsorption capability because of being refined products. In its structure, not only aluminum ions, but also calcium and sodium ions are intercalated, increasing the distance between silicon ions and improving adsorption capacity. Since 1988 there are numerous publications that demonstrate the use of HSCAS as adsorbents for mycotoxins, at *in vivo* and *in vitro* level [30, 41]. HSCAS clay can adsorb AFB1 with high affinity and high capacity in aqueous solutions (including milk) and in the meantime it can markedly reduce the bioavailability of AF in poultry; it can greatly diminish the effects of AF in young animals, i.e., rats, chicks, poults, ducklings, lambs, and pigs; and it can decrease the level of AFM1 in milk from lactating cows and goats [40].

5. Novel AF control methods

Although there are a lot of methods that have been practiced in order to fight against aflatoxigenic fungi and their toxins, they have been criticized because of their low effectiveness or due to their contaminant nature as mentioned before. That is why in recent years researchers have chosen new ways to deal with this threat involving microbiological and biotechnological methods that are promising because of the good results that have been obtained with them.

5.1. Microbiological methods

The use of microorganisms is a strategy that has been used recently. There have been reported some processes such as the action that ruminal flora has over mycotoxins. It was found that it is capable of esterifying ochratoxin A, turning it into ochratoxin C. The isolated action of bacteria and fungi such as *Corynebacterium rubrum*, *Aspergillus niger*, *Trichoderma viride* and *Mucor ambiguus* in the modification of the structure of AFB1 has been studied too [30].

The most studied microbiological decontamination is the fermentation process, which is used during the production of bread from wheat kernels contaminated with deoxynivalenol. After fermentation, a reduction in toxins levels is observed, and this is attributed to fermentation *per se* and to the thermal process to which the product is subjected. Decontamination occurs because yeast adsorb toxins [42]. Some reviews report that experiments of alcoholic fermentation by *Saccharomyces cerevisiae* with contaminated must with deoxynivalenol (DON) and zearalenone, showed results where after 7 to 9 days of fermentation the DON was stable to the process, the initial content of zearalenone was converted to β-zearalenol (β-ZEL), and α-zearalenol; most of the metabolization of zearalenone occurred in the first and second days of fermentation, showing the instability of the toxin to this process [42]. Not only *Saccharomyces cerevisiae* but also some lactic bacteria and yeasts are used widely in food fermentation because they have wall structures which are capable to adhere mycotoxins. Mycotoxins can be degraded by specific enzymes, as the case of ochratoxin A, which peptidic group is attacked by proteases [30]. Other researches have shown good inhibition results in AF production using microorganisms such as Bacillus spp (98%), *A. flavus* (90%), *A. parasiticus* (90%) and Trichoderma spp (75%) [42].

5.2. Biotechnological methods: Biological Control

Biotechnological methods are those in which biological systems or their derivates are used in order to obtain better products. From among them, talking about AF control, we can highlight the biological control, the use of natural extracts and essential oils and genetic engineering to mention a few.

5.2.1. Biocompetition

An option to supplement, but not to supplant the traditional methods of AF control is biological control. Most AF biological control programs can truly be defined as biocompetition since they do not utilize parasites or diseases of the pest, but instead use atoxigenic Aspergillus species to competitively exclude toxigenic fungi [43]. Augmentative biological control is as a pest management tactic that utilizes the deliberate introduction of living natural enemies to low the population level of invasive pests. Biological control has been utilized for more than 100 years in efforts to control a wide number of agricultural pests including fungi, insects and weeds [44]. Biocontrol strategies have been implemented to control AF contamination in several important agricultural crops, such as peanut, cotton and corn [43, 45, 46]. Some authors have reviewed some biological methods using bacteria, yeasts and fungi as competitors for containment of *A. flavus* growth and/or toxin production [46, 47]. Natural population of fungi like *A. flavus,* consists of toxigenic strains that produce copious amount of AF and atoxigenic strains that lack the capacity to produce AF. In the competitive exclusion mechanism, introduced atoxigenic strains out compete and exclude toxigenic strains from colonizing grains thereby reducing AF production in contaminated grains [48]. The use of *A. flavus* atoxigenic strains (afla–) reduce AF contamination in many crops; nevertheless, the mechanism by which a non-aflatoxigenic strain interferes with AF accumulation of toxigenic strains has not been definitively elucidated [49, 50].

Since the last decade of the past century, some yeasts and bacteria have shown to be effective on controlling fruits and vegetables postharvest diseases. In the early nineties, biological control of grain fungi was studied only to a limited extent. Most of the studies had dealt mainly with the interaction between mycotoxigenic strains (mostly aflatoxigenic ones) and other fungi, occurring naturally on grains, grown in competition. A limited number of fungi (especially *Aspergillus niger* van Tieghem), yeasts and bacteria were found to inhibit, detoxify or metabolize AF; however, it was determined that their antagonistic effect was highly dependent on cultural and environmental conditions [51]. There has been found that the yeast *Pichia guilliermondii* is effective in controlling major citrus fruit rots [52]. Based in those studies, in 1993, Paster and collaborators evaluated the efficacy of *Pichia guilliermondii* Wickerham for the control of the common *Aspergillus flavus* storage fungus and the natural microflora of soya beans, obtaining good results. The ability of *Pichia guilliermondii* to inhibit growth of grain microflora was studied using naturally contaminated soya beans and sterilized soya beans artificially inoculated with *Aspergillus flavus.* When *A. flavus* (at a spore concentration of 10^2 spores ml^{-1}) and *P. guilliermondii* (at concentrations of 10^7 or 10^9 spores ml^{-1}) were applied simultaneously to sterilized soya beans, fungal proliferation was inhibited during 16 days of storage. Application of yeast cells 3 days prior to fungal inoculation resulted in decreased inhibitory activity. The inhibitory effect of the yeast was compared with that of propionic acid using naturally infested soya beans at two levels of moisture content (11 and 16%). At both levels the yeast prevented fungal proliferation on the grain for a limited period, but propionic acid showed better fungistatic activity [51].

During 1994 and 1995, studies were conducted in the environmental control plot facility at the National Peanut Research Laboratory in Georgia to determine the effect of different inoculum rates of biological control agents on preharvest AF contamination of Florunner peanuts. Biocontrol agents were nontoxigenic color mutants of *Aspergillus flavus* and *Aspergillus parasiticus* that were grown on rice for use as soil inoculum. Those results were published three years later [53]. Findings like these were the basis of further studies focused on the use of aflatoxigenic Aspergillus species that researchers are still investigating with more detail.

In recent years, some antagonists have been applied in biocontrol of postharvest diseases of agricultural products. Naturally occurring populations of atoxigenic strains are considered reservoirs from which to select strongest biocompetitors. The atoxigenic strains colonizing the environment where crops are affected by repeated AF outbreaks should have adapted to, and hence acquired, a superior fitness, for the relevant environment. Selecting biocontrol strains is not straightforward, as it is difficult to assess fitness for the task without expensive field trials. Reconstruction experiments have been generally performed under laboratory conditions to investigate the biological mechanisms underlying the efficacy of atoxigenic strains in preventing AF production and/or to give a preliminary indication of strain performance when released in the field [54]. The mechanisms by which afla– strains interfere with AF accumulation has not yet been definitively established. The prevalent opinion is that it depends on the competitive exclusion of AF producer (afla+) strains from the substrate as a result of (a successful) physical displacement and competition for nutrients by afla– strains. However, different hypotheses may still be taken into consideration [55].

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Biological control is a promising approach for reducing both preharvest and postharvest AF contamination. There are some studies that report reductions in AF that are achieved by applying nontoxigenic strains of *A. flavus* and *A. parasiticus* to soil around developing plants, especially in peanuts. When late-season drought conditions make peanuts susceptible to invasion and growth by these fungi, the applied nontoxigenic strains competitively exclude toxigenic strains present in the soil and thereby reduce subsequent AF concentrations. Reductions in AF contamination with the use of nontoxigenic strains, has also been demonstrated in corn and cottonseed [56-59].

In 2003, Dorner and collaborators reported the results of a study that was conducted to evaluate the efficacy of three formulations of nontoxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* to reduce preharvest AF contamination of peanuts during two years. Formulations included a solid-state fermented rice, fungal conidia encapsulated in an extrusion product termed Pesta and conidia encapsulated in pregelatinized corn flour granules. Analysis of soils for *A. flavus* and *A. parasiticus* showed that a large soil population of the nontoxigenic strains resulted from all formulations. In the first year, the percentage of kernels infected by wild-type *A. flavus* and *A. parasiticus* was significantly reduced in plots treated with rice and corn flour granules, but it was reduced only in the rice-treated plots in year two. There were no significant differences in total infection of kernels by all strains of A. *flavus* and *A. parasiticus* in either year. AF concentrations in peanuts were significantly reduced in year two by all formulation treatments with an average reduction of 92%. Reductions were also noted for all formulation treatments in year one (average 86%), but they were not statistically significant because of wide variation in the AF concentrations in the untreated controls. Each of the formulations tested, therefore, was effective in delivering competitive levels of nontoxigenic strains of *A. flavus* and *A. parasiticus* to soil and in reducing subsequent AF contamination of peanuts [59]. The maize endophyte *Acremonium zeae* is antagonistic to kernel rotting and mycotoxin producing fungi *Aspergillus flavus* and *Fusarium verticillioides* in cultural tests for antagonism, and interferes with *A. flavus* infection and AF contamination of preharvest maize kernels. In 2005, Wicklow, reported results of chemical studies of an organic extract from maize kernel fermentations of *Acremonium zeae* (NRRL 13540), which displayed significant antifungal activity against *Aspergillus flavus* and *F. verticillioides,* and revealed that the metabolites accounting for this activity were two newly reported antibiotics pyrrocidines A and B. Pyrrocidines were detected in fermentation extracts for 12 NRRL cultures of *Acremonium zeae* isolated from maize kernels harvested in different places. Pyrrocidine B was detected in whole symptomatic maize kernels removed at harvest from ears of a commercial hybrid that were wound-inoculated in the milk stage with *A. zeae* (NRRL 13540) or (NRRL 13541). The pyrrocidines were first reported from the fermentation broth of an unidentified filamentous fungus LL-Cyan426, isolated from a mixed Douglas Fir hardwood forest on Crane Island Preserve, Washington, in 1993. Pyrrocidine A exhibited potent activity against most Gram-positive bacteria, including drug-resistant strains, and was also active against the yeast Candida albicans. In an evaluation of cultural antagonism between 13 isolates of *A. zeae* in pairings with *A. flavus* (NRRL 6541) and *F. verticillioides* (NRRL 25457), A. zeae (NRRL 6415) and (NRRL 34556) produced the strongest reaction, in-

hibiting both organisms at a distance while continuing to grow through the resulting clear zone at an unchanged rate. [60].

In 2005, Bandyopadhyay reported a test of twenty-four atoxigenic *A. flavus* isolates under field conditions in Nigeria to identify a few effective strains that could exclude toxigenic strains. These atoxigenic strains were evaluated for a set of selection criteria to further narrow down the numbers to a few for further use in biocontrol field experiments. Good criteria of selection will ensure that the candidate atoxigenic strains belong to unique vegetative compatibility groups (for which testers have been developed) that are unable to produce toxigenic progenies in the natural environment. Propensity to multiply, colonize and survive are other selection criteria to make sure that few reapplications will be required once the atoxigenic strains are introduced in the environment [48].

In 2006, Palumbo and collaborators isolated bacteria from California almond orchard samples to evaluate their potential antifungal activity against AF-producing *Aspergillus flavus.* Fungal populations from the same samples were examined to determine the incidence of aflatoxigenic *Aspergillus species*. Antagonistic activities of the isolated bacterial strains were screened against a neither nonaflatoxigenic nor mutant of *A. flavus*, which accumulates the pigmented AF precursor norsolorinic acid (NOR) under conditions conducive to AF production. 171 bacteria isolated from almond flowers, immature nut fruits, and mature nut fruits showed inhibition of A. *flavus* growth and/or inhibition of NOR accumulation. Bacterial isolates were further characterized for production of extracellular enzymes capable of hydrolyzing chitin or yeast cell walls. Molecular and physiological identification of the bacterial strains indicated that the predominant genera isolated were Bacillus, Pseudomonas, Ralstonia, and Burkholderia, as well as several plant-associated enteric and nonenteric bacteria [61].

Chang & Hua in 2007, from screening subgroups of nonaflatoxigenic *A. flavus,* identified an *A. flavus* isolate, TX9-8, which competed well with three *A. flavus* isolates producing low, intermediate, and high levels of AF, respectively. TX9-8 has a defective polyketide synthase gene (pksA), which is necessary for AF biosynthesis. Co-inoculating TX9-8 at the same time with large sclerotial (L strain) *A. flavus* isolates at a ratio of 1:1 or 1:10 (TX9-8:toxigenic) prevented AF accumulation. The intervention of TX9-8 on small sclerotial (S strain) *A. flavus* isolates varied and depended on isolate and ratio of co-inoculation. At a ratio of 1:1 TX9-8 prevented AF accumulation by *A. flavus* CA28 and reduced AF accumulation 10-fold by *A. flavus* CA43. No decrease in AF accumulation was apparent when TX9-8 was inoculated 24 h after toxigenic L- or S strain *A. flavus* isolates started growing so the competitive effect likely is due to TX9-8 outgrowing toxigenic *A. flavus* isolates [62].

In 2009, it was reported that *Serratia plymuthica* 5-6, isolatedfromthe rhizosphere of pea reduced dry rot of potato caused by *Fusarium sambucinum* [63]. In 2009, a new strain of *Bacillus pumilus* isolated from Korean soybean sauce showed strong antifungal activity against the AF-producing fungi *A. flavus* and *A.parasiticus* [64].

In 2010, a strain of marine *Bacillus megaterium* isolated from the Yellow Sea of East China was evaluated by Kong and collaborators for its activity in reducing postharvest decay of peanut kernels caused by *Aspergillus flavus* in *in vitro* and *in vivo* tests, this, because microor-

ganisms are capable of producing many unique bioactive substances, and therefore could be a rich resource for antagonists [65]. The results showed that the concentrations of antagonist had a significant effect on biocontrol effectiveness *in vivo*: when the concentration of the washed bacteria cell suspension was used at 1×109 CFU/ml, the percentage rate of rot of peanut kernels was 31.67%±2.89%, which was markedly lower than that treated with water (the control) after 7 days of incubation at 28 °C. The results also showed that unwashed cell culture of *B. megaterium* was as effective as the washed cell suspension, and better biocontrol was obtained when longer incubation time of *B. megaterium* was applied. When the incubation time of B. megaterium was 60 h, the rate of decay declined to 41.67%±2.89%. Furthermore, relative to the expression of 18S rRNA, the mRNA abundances of aflR gene and aflS gene in the experiment group were 0.28±0.03 and 0.024±0.005 respectively, indicating that this strain of *B. megaterium* could significantly reduce the biosynthesis of AF and expression of aflR gene and aflS gene [66].

In 2011, Degola and collaborators conducted a study in order to evaluate the potential of the different atoxigenic *A. flavus* strains, colonizing the corn fields of the Po Valley, in reducing AF accumulation when grown in mixed cultures together with atoxigenic strains; additionally, they developed a simple and inexpensive procedure that might be used to scale-up the screening process and to increase knowledge on the mechanisms interfering with mycotoxin production during co- infection [54].

Farzaneh and collaborators reported in this year, an investigation in which *Bacillus subtilis* strain UTBSP1 was isolated from pistachio nuts and studied for the degradation of AFB1. The results indicated *B. subtilis* UTBSP1 could considerably remediate AFB1 from nutrient broth culture and pistachio nut by 85.66% and 95%, respectively. Cell free supernatant fluid caused an apparent 78.39% decrease in AFB1 content. The optimal conditions for AFB1 degradation by cell free supernatant appeared at 35 and 40°C, during 24 h. Furthermore, the results indicated that AFB1 degradation is enzymatic and responsible enzymes are extracellular and constitutively produced. They found that destructive AFB1 differed from standard AFB1 chemically, and lost a fluorescence property [67].

It was found that *A. flavus* K49 produces neither AFs nor cyclopiazonic acid (CPA) and is currently being tested in corn-growing fields in Mississippi. Its lack of production of AF and CPA results from single nucleotide mutations in the polyketide synthase gene and hybrid polyketide nonribosomal peptide synthase gene, respectively. Furthermore, based on single nucleotide polymorphisms of the AF biosynthesis omtA gene and the CPA biosynthesis dmaT gene, it is known that K49, AF36 and TX9-8 form a biocontrol group, appear to be derived from recombinants of typical large and small sclerotial morphotype strains [50].

Not only Aspergillus, but also other pathogens have been faced to biocontrol. For example, it is known that the plant pathogen *Fusarium solani* causes a disease root rot of common bean (*Phaseolus vulgaris*) resulting in great losses of yield in irrigated areas. Species of the genus Trichoderma have been used in the biological control of this pathogen as an alternative to chemical control. To gain new insights into the biocontrol mechanism used by *Trichoderma harzianum* against the phytopathogenic fungus, *Fusarium solani,* it was performed a transcriptome analysis using expressed sequence tags (ESTs) and quantitative real-time PCR

(RT-qPCR) approaches. A cDNA library from *T. harzianum* mycelium (isolate ALL42) grown on cell walls of *F. solani* (CWFS) was constructed and analyzed. A total of 2927 high quality sequences were selected from 3845 and 37.7% were identified as unique genes. The Gene Ontology analysis revealed that the majority of the annotated genes are involved in metabolic processes (80.9%), followed by cellular process (73.7%). Genes that encode proteins with potential role in biological control have been tested. RT-qPCR analysis showed that none of these genes were expressed when *T. harzianum* was challenged with itself. These genes showed different patterns of expression during in vitro interaction between *T. harzianum* and *F. solani* [68].

It is a fact that several papers have been published about AFB1 reduction by some bacterial isolates. Lactic acid bacteria such as Lactobacillus, Bifidobacterium, Propionibacterium and Lactococcus were found to be active in removing AFB1 primarily by the adhesion method. In addition, some bacteria such as *Rhodococcus erythropolis*, Bacillus sp., *Stenotrophomonas maltophilia*, *Mycobacterium fluoranthenivorans* and *Nocardia corynebacterioides* were reported to degrade AFB1 [67].

5.2.2. Natural products and essential oils

Plants produce lots of secondary metabolites as part of their normal growth and development in order to fight against environmental stress, pathogen attack or other adversities. One of the most important secondary metabolites are essential oils (EOs), which are extracted from plants, commonly by a distillation process [69] and then used as natural additives in different foods to reduce the proliferation of microorganisms and their toxins production due to their antifungal, antiviral, antibacterial, antioxidant and anticarcinogenic properties [70-72]. They have received major consideration in regard to their relatively safe status and enrichment by a wide range of structurally different useful constituents [73]. Until 1989, more than 1340 plants were known to be potential sources of antimicrobial compounds, which are safe for the environment and consumers, and are useful to control postharvest diseases, being an excellent alternative to reduce the use of synthetic chemicals in agriculture. The majorities of the essential oils are classified as Generally Recognized As Safe (GRAS) and have low risk for developing resistance to pathogenic microorganisms [74, 75].

There is a large number of different groups of chemical compounds present in EOs, that is why antimicrobial activity is not attributable to one specific mechanism but to the existence of several targets in the cell [76, 77]. There is a relationship between the chemical structures of the most abundant compounds in the EOs and the anitimicrobial activity; minor components have a critical part to play in antimicrobial activity, possibly by producing a synergic effect between other components [78]. Not only EOs but also alkaloids, phenols, glycosides, steroids, coumarins and tannins have been found to have antimicrobial properties [79]. Generally, the extent of the inhibition of the oils could be attributed to the presence of an aromatic nucleus containing a polar functional group [80], being phenols the majority group. For example, in 2008, Bluma and Etcheverry, based in the principle that phenolics are secondary metabolites synthesized via phenylpropanoid biosynthetic pathway which build blocks for cell wall structures serving as defense against pathogens, found that phenolic

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compounds such as acetocyringone, syringaldehyde and sinapinic acid inhibit AFB1 biosynthesis by *A. flavus* in PDA and reduce norsolinic acid production, because the presence of phenolic OH groups are able to form hydrogen bonds with the active sites of target enzymes increasing antimicrobial activity [69].

There is a wide list of natural products from the entire world (summarized in Table 1) used in the last decade to diminish Aspergillus populations to counteract the effect of AFs in food or to test fumigant activity in feed at specific inhibitory concentrations [81]. It has been demonstrated that the antifungal capability of those EOs depend on the concentration in which they are applied and the conditions around them. In 2001, Varma and Dubey reported that EOs from plants like *Caesulia axillaris* and *Mentha arvensis* have fumigant activity in the management of biodeterioration of stored wheat samples by *A. flavus* showing the same efficacy as postharvest fungicides used for this purpose [38]. In 2002, Soliman and Badeaa tested inhibitory activity of essential oils from 12 medicinal plants against *A. flavus, A. parasiticus, A. ochraceus* and *Fusarium moniliforme*, finding that the oils of thyme and cinnamon (at a 4500 ppm concentration), marigold (42000 ppm), spearmint, basil and quyssum (3000 ppm) completely inhibit all the test fungi. Caraway was inhibitory at 2000 ppm against A. *flavus, A. parasiticus* and 3000 ppm against *A. ochraceaus and F. moniliforme*. *A. flavus, A. ochraceus, A. parasiticus* and *F. moniliforme* were completely inhibited by anise at 4500 ppm, being chamomile and hazanbul essential oils just partially effective against the test toxigenic fungi [71].

NATURAL PRODUCT	COMMON NAME	PRINCIPAL METABOLITE	PATHOGEN INHIBITED	INHIBITORY CONCENTRATION	REFERENCE
Achillea fragrantissima	Qyssum	Polyphenolic compounds	A. flavus, A. parasiticus, A. ochraceus	3,000 ppm	[71]
Agave asperrima	Maguey Cenizo	Polyphenolic compounds	A. flavus A. parasiticus	< 2 mg ml^{-1}	[15]
Agave striata	Maguey Espadín	Polyphenolic compounds	A. flavus A. parasiticus	< 2 mg ml^{-1}	[15]
Ageratum conyzoides	Goatweed	Precocene, Cumarine, trans-Caryophyllene	A.flavus	0.10 µg ml^{-1}	[91]
Azadirachta indica A. Juss	Neem	Aromatic compounds	*A. parasiticus*	"/ 10% (v/v)	[81]
Caesulia axillaris	Pink Node Flower	Aromatic compounds	*A. flavus*	nd	[38]

NATURAL PRODUCT	COMMON NAME	PRINCIPAL METABOLITE	PATHOGEN INHIBITED	INHIBITORY CONCENTRATION	REFERENCE
Calendula ofricinalis L.	Marigold	Carfone	A. flavus, A. parasiticus, A. ochraceus	< 2,000 ppm	[71]
Carum carvi L.	Caraway	Carfone	A. flavus, A. parasiticus, A. ochraceus	2,000 – 3,000 ppm	[71]
Cicuta virosa L. var. latisecta Celak	Umbelliferae	γ-Terpinene p-Cymene Cumin Aldehyde	A. flavus	5 µl ml^{-1}	[75]
Cinnamomum cassia	Cassia	Aromatic compounds	*A. parasiticus*	2.5 % (v/v)	[79]
Cinnamomum zeylanicum L.	Cinnamon	Cinnamic aldehyde O-methoxy-cinnamaldehyde Carfone	A. flavus, A. parasiticus, A. ochraceus	200 – 250 ppm, < 500 ppm	[71, 83, 85]
Citrus limon	Lemon	Limomene	A. flavus	2, 000 ppm	[13]
Cymbopogon citratus	Lemongrass	Citral, geraniol, eugenol, α-pinene, linalool	A.flavus	1 – 5%, 1,200 ppm	[81, 83]
Eucalyptus globulus	Blue Gum	1,8-cineole	A.flavus A. parasiticus	nd	[86]
Hedeoma multiflora Benth	Mountain Thyme	α-Terpinene ð-Terpinene ρ-Cimeno o-Cimeno Borneol Thymol Carvacrol	A. flavus, A. parasiticus	2,000 – 3,000 µg g^{-1}	[69]
Laurus nobilis	Bay Leaf	Aromatic compounds	*A. parasiticus*	1 – 5 % (v/v)	[79]

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NATURAL PRODUCT	COMMON NAME	PRINCIPAL METABOLITE	PATHOGEN INHIBITED	INHIBITORY CONCENTRATION	REFERENCE
Lippia turbinate var. integrifolia (griseb)	Poleo	β-Cariofilene α-Humulene Camfene Sabinene	A. flavus, A. parasiticus,	2,000 – 3,000 µg g^{-1}, 2500 µl l^{-1}	[69, 95]
Mentha arvensis	Wild Mint	Menthone Menthol	A. flavus	nd	[38]
Mentha viridis	Spearmint	Menthone Menthol β-pinene α-pinene	A. flavus, A. parasiticus, A. ochraceus	3,000 ppm	[71]
Ocimum basilicum	Sweet Basil	β-pinene α-pinene Ocimene Methyl Chavecol	A. parasiticus	5% (v/v)	[71, 79]
Ocimum basilicum L	Basil	β-pinene α-pinene Ocimene Methyl Chavecol	A. flavus, A. parasiticus, A. ochraceus	3,000 ppm	[71]
Ocimum gratissimum	Clove Basil	γ-terpinene Methyl cinnamate	F.moniliforme, A.flavus A. fumigatus	800 ppm	[83, 93]
Origanum vulgare	Oregano	γ-terpinene p-cimeme Linalool Cariophyllene	A. flavus	500 µg g^{-1}, 100 – 2,000 ppm	[81, 85]
Pëumus boldus	Boldo	α-Pinene β-Pinene α-Terperpine ρ-Cimene Terpinen-4-ol α-Terpinolene	A. flavus, A. parasiticus	2,000 – 3,000 µg g^{-1}, 2500 µl l^{-1}	[69, 95]
Pimpinella anisum L.	Anise	Metilchavicol Anethol	A. flavus, A. parasiticus, A. ochraceus	< 500 ppm	[71]
Satureja hortensis L.	Winter Savory	Carvacrol Thymol	*A. parasiticus*	~0.5 mM	[81, 87]

NATURAL PRODUCT	COMMON NAME	PRINCIPAL METABOLITE	PATHOGEN INHIBITED	INHIBITORY CONCENTRATION	REFERENCE
Syzygium aromaticum	Clove	Humulene Cariophyllene Eugenol	A. flavus, A. parasiticus	1500 µl l^{-1}	[95]
Thymus eriocalyx	Avishan	Thymol β-phellandrene cis-sabinene hydroxide 1,8-cineole β-pinene	A. parasiticus	250 ppm	[84]
Thymus vulgaris L.	Thyme	β-pinene α-pinene Thymol p- cymene	A. flavus, A. parasiticus, A. ochraceus	< 500 ppm, 1000 ppm	[71, 83]
Thymus X-porlock	Thyme	Thymol β-phellandrene cis-sabinene hydroxide 1,8-cineole β-pinene	A. parasiticus	250 ppm	[84]
Trachyspermum ammi (L.)	Ajowan	Aromatic compounds	A. flavus	1 g ml^{-1}	[92]
Zingiber officinale	Ginger	Polyphenolic compounds	A.flavus	800 – 2,500 ppm	[83]

Table 1. Metabolites obtained from some natural products which are used to diminish fungal populations and AF production (nd= no data).

EOs and other natural products have been tested not only against Aspergillus species but also Fusarium species, which most of the times are developed in parallel. In 2003, Vellutti and collaborators reported the effect of cinnamon, clove, oregano, palmarose and lemongrass oils on fumonisin B1 growth and production by three different isolates of *F. proliferatum* in irradiated maize grain at 0.995 and 0.950 aw and at 20 and 30°C. The five essential oils inhibited growth of *F. proliferatum* isolates at 0.995 aw at both temperatures, while at 0.950 aw only cinnamon, clove and oregano oils were effective in inhibiting growth of *F. proliferatum* at 20°C and none of them at 30°C. Cinnamon, oregano and palmarose oils had significant inhibitory effect on FB1 production by the three strains of *F. proliferatum* at 0.995 aw and both temperatures, while clove and lemongrass oils had only significant inhibitory effect at 30°C [81]. In 2004, Nguefack and his group of researchers tested the inhibitory effect of EOs extracted from *Cymbopogon citratus*, *Monodora myristica*, *Ocimum gratissimum*, *Thymus vulgaris* and *Zingiber officinale* against *F. moniliforme*, being *O. gratissimum*, *T. vulgaris* and *C. citratus* the most effective over conidial germination and fungal growth at 800, 1000 and

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1200 ppm, respectively. Moderate activity was observed for the EO from *Z. officinale* between 800 and 2500 ppm, while the EO from *M. myristica* was less inhibitory. These effects against food spoilage and mycotoxin producing fungi indicated the possible ability of each EO as a food preservative [83].

In 2005, Sánchez and collaborators prepared ethanolic, methanolic and aqueous extracts of flowers from mexican *Agave asperrima* and *Agave striata,* in order to diminish growth and production of AF from *A. flavus* and *A. parasiticus* at in vitro and in vivo level. All extracts, but specifically the methanolic one, showed an effective inhibition growth (99%) [15]. In the same year, Rasooli & Owlia extracted the EOS from *Thymus eriocalyx* and *Thymus X-porlock* in order to test antifungal activity against *A. parasiticus* growth and AF production. *T. eriocalyx* showed lethal effects at 250 ppm while *T. X-porlock* was lethal at 500 ppm [84].

EOs from common spices have been also investigated, that is the case of cinnamon (*Cinnamomum zeylanicum*) and oregano (*Origanum vulgare*) which shows antifungal activity against *A. flavus* at 2000 ppm and 1000 ppm respectively in a malt-agar medium and a fungistatic activity at 100 ppm. [85]. Eucalyptus (*Eucalyptus globules)* is effective against the storage fungi *A. flavus* and *A. parasiticus* [86]. Lemon EO (*Citrus limon*), applied in food AF-contaminated samples, results in a strong antiaflatoxigenic and antifungal substance, reducing AF concentrations in food samples for broilers up to 73.6% [13]. Sweet basil (*Ocimum basilicum*), cassia (*Cinnamomum cassia*), coriander (*Coriandrum sativum*) and bay leaf (*Laurus nobilis*) at 1–5% (v/v) concentration were studied in palm kernel over the aflatoxigenic fungus *A. parasiticus* CFR 223 and AF production. Sweet basil oil at optimal protective dosage of 5% (v/v) was fungistatic on *A. parasiticus*; in contrast, oils of cassia and bay leaf stimulated the mycelia growth of the fungus in vitro but reduced the AF concentration (AFB1+AFG1) of the fungus by 97.92% and 55.21% respectively, while coriander oil did not have any effect on both the mycelia growth and AF content of the fungus. The combination of cassia and sweet basil oils at half their optimal protective dosages (2.5% v/v) completely inhibited the growth of the fungus. It was found that the addition of whole and ground basil leaves markedly reduced AF contamination; however, 10% (w/w) of whole leaves was more effective as the reduction in AF was between 89.05% and 91% [79].

In 2008, Bluma and Etcheverry found that *Pimpinella anisum* L. (anise), *Pëumus boldus* Mol (boldus), *Hedeoma multiflora* Benth (mountain thyme), *Syzygium aromaticum* L. (clove), and *Lippia turbinate* var. *integrifolia* (griseb) (poleo) had an inhibitory effect on Aspergillus section Flavi growth rate, and their efficacy depended mainly on the water activity and EOs concentration. Boldus, poleo, and mountain thyme EOs completely inhibited AFB1 at 2000 and 3000 $\mu g\ g^{-1}$ [69]. *Satureja hortensis* L. has been also reported as a potent inhibitor of AFB1 and AFG1 produced by *A. parasiticus* at concentrations from 0.041 to 1.32 mM [87]. In 2009, Kumar and collaborators found that *Cymbopogon flexuosus* EO and its components were efficient in checking fungal growth and AF production, inhibiting absolutely inhibited the growth of *A. flavus* and AFB1 production at 1.3 μlml^{-1} and 1.0 μlml^{-1} respectively, due to the principal component: eugenol [88]. Razzaghi-Abyaneh and his investigation group found that *Thymus vulgari* and *Citrus aurantifolia* inhibit both *A. parasiticus* and AF production. The EOs from *Mentha spicata* L., *Foeniculum miller*, *Azadirachta indica* A. Juss, *Conium maculatum*

and *Artemisia dracunculus* only inhibited fungal growth, while *Carum carvi* L. effectively inhibited AF production without any obvious effect on fungal growth. *Ferula gummosa, Citrus sinensis, Mentha longifolia* and *Eucalyptus camaldulensis* had no effect on *A. parasiticus* growth and AF production at all concentrations used [89]. There are other investigations of the potential use of antifungal component eugenol for the reduction of AFB1. Komala and collaborators reported some findings in stored sorghum grain due to fungal infestation of sorghum results in deterioration of varied biochemical composition of the grain. In this study, three genotypes (M35-1; C-43; LPJ) were inoculated with two highly toxigenic strains of *Aspergillus flavus* with three different eugenol treatments in order to evaluate the AFB1 production. From this study it was found that at 8.025 mg/g concentration, eugenol completely inhibited the AFB1 production. The lowest amount of AFB1 was observed in genotype M35-1, whereas higher amount AFB1 was observed in LPJ followed by C-43. In all sorghum genotypes there was a significant positive correlation existing between protein content and AF produced, the r values being 0.789 and 0.653, respectively. Starch in three genotypes was found to have a significant negative correlation with AF produced. The starch content decreased whereas the protein content in all sorghum varieties increased during infection [90].

Ageratum conyzoides EO is other specie that has been studied recently. It acts directly on the mycelial growth and AFB1 production by *A. flavus*, inhibiting fungal growth to different extents depending on the concentration, and completely inhibiting AF production at concentrations above 0.10 μg/mL, because this EO acts affecting mainly the fungal mitochondria [91]. This EO acts similarly than Ajowan extract (*Trachyspermum ammi* L., which acts directly over AFB1, AFB2 and AFG2 [92]. In 2011, it was found that *Ocimum gratissimum* EO acts a nontoxic antimicrobial and antiaflatoxigenic agent against fungal and AF contamination of spices infected with *A. flavus* isolated from *Piper nigrum* and *Myristica fragrans* respectively at 0.6 μl/ml and 0.5 μl/ml, as well as a shelf life enhancer in view of its antioxidant activity, playing a prominent role in the development of an ideal plant based food additive [93]. It was found too that EOs extracted from the fruits of *Cicuta virosa* L. var. latisecta Celak acts against *A. flavus, A, oryzae, A. niger,* and *Alternaria alternata*, having a strong inhibitory effect on spore production and germination in all tested fungi proportional to concentration. The oil exhibited noticeable inhibition on dry mycelium weight and synthesis of AFB1 by *A. flavus*, completely inhibiting AFB1 production at 4 μL/mL [75].

Because of the great results obtained with this kind of AFs biocontrol, researchers are still investigating new natural products and their active compounds in order to deal with those toxins ad the fungi which produce them, and avoiding the use of fumigants that are toxic for plants and for plant consumers. In this year, EOs from plants like *Zanthoxylum alatum* Roxb have been studied, because it has been proved that its two major constituents (linalool and methyl cinnamate) inhibit the growth of a toxigenic strain of *A. flavus* (LHP-10) as well as AFB1 secretion at different concentrations. *Zanthoxylum alatum* Roxb EO has also showed strong antioxidant activity with an IC50 value at 5.6 μl/ml [94]. EOs from boldo, clove, anise and thyme are still studied against aflatoxigenic Aspergillus strains in specific cultures like peanut-based medium, finding that those EOs have influence on lag phase, growth rate, and AFB1 accumulation [95]. The EO extracted from the bark of *Cinnamomum jensenianum* Hand.

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Mazz has been tested for antifungal activity against *A. flavus*. Mycelial growth and spore germination was inhibited by the oil in a dose-dependent manner. The oil also exhibited a noticeable inhibition on the dry mycelium weight and the synthesis of AFB1 by *A. flavus*, completely restraining AFB1 production at 6 μl/ml. The possible mode of action of the oil against *A. flavus* is discussed based on changes in the mycelial ultrastructure [37]. Nevertheless, most research is needed in order to understand the mechanisms of action of the essential oils over aflatoxigenic fungi, turning them into potential sources for food preservation.

5.3. Genetic Engineering: Molecular biology and genetics proposals

The genome of plants has significant influence on fungal contamination and the subsequent biosynthesis of mycotoxins, hence, the importance of developing new varieties through genetic engineering, capable of withstanding the fungal attack or inhibiting toxin production. Several researchers have found some seed varieties with significant differences in regard to contamination by *Aspergillus flavus* and its subsequent AF production. These differences may be due to different factors, and the plant genome can influence the expression of the mycotoxin biosynthesis [95]. Various approaches have been suggested for genetic control of preharvest AF contamination including the development and use of crops with resistance to insects and resistance to plant stress (especially for tolerance to drought and high temperatures). Several sources of resistant germplasm have been identified and released for crop genetic improvement [95]. Using a combination of genetic, genomic and proteomic approaches to elucidate crop defense mechanisms and their genetic regulation will significantly improve the efficiency of genetic breeding for better crop cultivars [98].

One of the most important challenges in AFs genetic engineering has been the identification of the genes that are present in aflatoxigenic strains but not in the non-toxigenic ones, in order to design in the laboratory non-toxigenic strains by manipulating the genes of toxigenic strains. The AF pathway genes are found to be clustered in the genome of *A. flavus* and *A. parasiticus.* These genes are expressed concurrently except for the regulatory gene *aflR*. In this gene cluster, a positive-acting regulatory gene, *aflR*, is located in the middle of the gene cluster. Adjacent to *aflR* a divergently transcribed gene, *aflS* (*aflJ*), was also found to be involved in the regulation of transcription. Other physically unrelated genes, such as *laeA* and *veA*, also have been shown to exhibit a "global" regulatory role on AF biosynthesis [98]; nevertheless, although the basis of the toxigenic activity of AF are being well investigated, more research is still needed in order to get more information about how to manipulate genes in the different strains present in different crops and foods.

AF are synthesized by enzymes encoded within a large gene cluster. The initial step in the generation of the polyketide backbone of AF is proposed to involve polymerization of acetate and nine malonate units (with a loss of CO2) by a polyketide synthetase in a manner analogous to fatty acid biosynthesis. AF synthesis is controlled by different enzymes which are expressed through gene expression processes. Genetic studies on AF biosynthesis in *Aspergillus flavus* and *Aspergillus parasiticus* led to the cloning of 25 clustered genes within a 70 kb DNA region responsible for the enzymatic conversions in the AF biosynthetic pathway.

Regulatory elements such as *aflR* and *aflS (aflJ)* genes, nutritional and environmental factors, fungal developmental and sporulation were also found to affect AF formation [31].

Aflatoxigenic *Aspergillus flavus* isolates show four DNA fragments specific for *aflR, nor-1, ver-1, and omt-A* genes. Non-aflatoxigenic A. flavus strains give variable DNA banding pattern lacking one, two, three or four of these genes. Recently, it has been found that some AF non-producing *A. flavus* strains show a complete set of genes. Some studies suggest that 36.5% of non-aflatoxigenic *A. flavus* strains show DNA fragments that correspond to the complete set of genes (quadruplet pattern) as in aflatoxigenic *A. flavus*; 32% shows three DNA banding patterns grouped in four profiles where *nor-1, ver-1 and omt-A* are the most frequent profile; 18.7% of non-aflatoxigenic *A. flavus* strains yield two DNA banding pattern whereas 12% of the strains show one DNA banding pattern [99]. The *aflR* gene, encoding a 47 kDa sequence-specific zinc-finger DNA-binding protein is required for transcriptional activation of most, if not all, the structural genes of the AF gene cluster. Like other Gal4-type regulatory proteins that bind to palindromic sequences, functional AflR probably binds as a dimer. It binds to the palindromic sequence 5'-TCGN5CGR-3' in the promoter regions of the structural genes. The AflR-binding motifs are found to be located from 80 to 600 bp, with the majority at the 100 to 200 bp, relative to the translation start site. AflR binds, in some cases, to a deviated sequence rather than the typical motif such as in the case of *aflG* (*avnA*). When there is more than one binding motif, only one of them is the preferred binding site such as in the case of *aflC* (*pksA*). Deletion of *aflR* in *A. parasiticus* abolishes the expression of other AF pathway genes. Overexpression of *aflR* in *A. flavus* up-regulates AF pathway gene transcription and AF accumulation. AflR is specifically involved in the regulation of AF biosynthesis [98].

The *aflS* (*aflJ*) gene, although not demonstrating significant homology with any other encoded proteins found in databases, is necessary for AF formation. In the *A. parasiticus aflR* transformants, the production of AF pathway intermediates was significantly enhanced in transformants that contained an additional *aflR* plus *aflS.* Quantitative PCR showed that in the *aflS* knockout mutants, the lack of *aflS* transcript is associated with 5- to 20-fold reduction of expression of some AF pathway genes such as *aflC* (*pksA*), *aflD* (*nor-1*), *aflM* (*ver-1*), and *aflP* (*omtA*). The mutants lost the ability to synthesize AF intermediates and no AFs were produced. However, deletion of *aflS* (*aflJ*) did not have a discernible effect on *aflR* transcription, and vice versa. Overexpression of *A. flavus aflS* (*aflJ*) does not result in elevated transcription of *aflM* (*ver-1*), *aflP* (*omtA*), or *aflR*, but it appears to have some effect on *aflC* (*pksA*), *aflD* (*nor-1*), *aflA* (*fas-1*), and *aflB* (*fas-2*), which are required for the biosynthesis of the early AF pathway intermediate, averantin [98, 100, 101].

The global regulatory gene, *laeA* (for lack of *aflR* expression), is well conserved in fungi as shown by its presence in the genomes of all fungi so far sequenced. LaeA is a nuclear protein which contains an S-adenosylmethionine (SAM) binding motif and activates transcription of several other secondary metabolism gene clusters in addition to the AF cluster. It also regulates some genes not associated with secondary metabolite clusters, but this mechanism is not known yet. One proposed regulatory mechanism is that LaeA differentially methylates histone protein and it alters the chromatin structure for gene expression [98]. Recent analyses of nonaflatoxigenic *A. parasiticus sec-* (for secondary metabolism negative) variants

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generated through serial transfer of mycelia of the *sec+* parents show that *laeA* is expressed in both *sec+* and *sec-* strains, suggesting that LaeA only exerts its effect on AF biosynthesis at a certain level and is independent of other regulatory pathways that are involved in fungal development [102].

The *veA* gene is initially found to be crucial for light-dependent conidiation. The light dependence is abolished by a mutation (*veA1*) which allows conidiation of *A. nidulans* to occur in the dark. A comparison of the light effect on sterigmatocystin production by *A. nidulans veA+* and *veA1* strains showed that both strains produced sterigmatocystin but the highest amount was produced by the *veA+* strain grown in darkness. However, *veA*-deleted *A. flavus* and *A. parasiticus* strains completely lost the ability to produce AF regardless of the illumination conditions [103, 104]. Under normal growth conditions, some *A. flavus* and all *A. parasiticus* strains produce conidia in both dark and light conditions. VeA contains a bipartite nuclear localization signal (NLS) motif and its migration to the nucleus is light-dependent and requires the importing α carrier protein. In the dark VeA is located mainly in the nucleus; under light it is located both in cytoplasm and nucleus. VeA has no recognizable DNA-binding sequences and likely exerts its effect on sterigmatosyctin and AF production through proteinprotein interactions with other regulatory factors. Post- translational modifications such as phosphylation and dephosphorylation may modulate its activity. Lack of VeA production in the *veA*-deleted *A. flavus* and *A. parasiticus* strains consequently abolishes AF production because a threshold concentration of nuclear VeA might be necessary to initiate AF biosynthesis [98, 104]. One of the approaches in the field of AF research with regard to proteomics is to study the AF resistance proteins in host plants such as corn. The investigation on proteins associated with host resistance has been shown to be a possible strategy for controlling AF contamination of plants [105, 106].

An important factor affecting the agricultural commodities is the drought stress. Pre-harvest contamination of maize, peanuts and other products with AFs has been observed to be higher especially in the drought years, having devastating economical [106]. Guo and collaborators reviewed the potential of genetics, genomics and proteomics in understanding the relationship between drought stress and preharvest AF contamination in agricultural products. Different proteomic approaches revealed that resistant lines have elevated levels of stress-related proteins, antifungal and storage proteins in comparison to susceptible lines [95]. The use of proteomic tools has made possible to find different categories of resistance associated proteins which can be divided into 3 groups: stress-responsive proteins, storage proteins and antifungal proteins indicating that storage and stress-responsive proteins may play an important role in enhancing stress-tolerance of host plant [106, 107]. The use of proteomics is still a new tool to understand plant resistance against fungal contamination, so it promises to become an important field for understanding fungal genetic behavior.

5.4. Storage and packing technologies

As mentioned before, it is well known that AF contamination of foods increase with storage period. That is why proper selection of packaging materials is necessary to prevent absorption of moisture and AF formation which will influence the overall product quality and safe-

ty [19, 108]. Postharvest contamination of grain can also take place during transportation, so grains need to be well covered and/or aerated during transportation [19]. Storage prior and during marketing has to be done in appropriate bagging, preferably sisal bags, because this kind of material facilitates aeration in transit. The use of containers made from plant materials (wood, bamboo, thatch) or mud placed on raised platforms and covered with thatch or metal roofing sheet is another way to prevention. The stores should be constructed to prevent insect and rodent infestation and to prevent moisture from getting into the grains. While new storage technologies such as the use of metal or cement bins by small-scale farmers would serve better, their uptake has been slow due to their high cost. Many farmers nowadays store their grains in bags, especially polypropylene which are not airtight, but there is evidence that this method facilitates fungal contamination and AF development [19, 109, 110]. Presently there are efforts to market improved hermetic storage bags in Africa, based on triple bagging developed for cowpea which has been or is being tested for other commodities [19].

Not only optimal storage plastic bagging and container materials have been proposed. Shakerardekani and Karim reported in 2012 a short communication in which they studied the effect of five different types of flexible packaging films (low density polyethylene (LDPE) which served as the control, food-grade polyvinyl chloride (PVC), nylon (LDPE/PA), polyamide/polypropylene (PA/PP) and polyethylene terephthalate (PET)) on the moisture and AF contents of pistachio nuts during storage at room temperature (22–28 °C) and relative humidity of 85–100%. Samples were analyzed at 0, 2, 4, 6, 8 and 10 months during the storage period. Results showed that there was an increase in moisture content with the increase in storage time of pistachio nuts. The increase in moisture content was associated with the AF level of pistachio nuts during storage time. All the packaging materials except LDPE delayed the moisture absorption and AF formation of the product. The most suitable packaging materials for maintaining the quality and safety of pistachio nuts were PET films followed by nylon, PA/ PP and PVC. The shelf-life of pistachio showed to be extended from 2 months (Control) to 5 months when PET was used as the packaging material [108].

In the market, there are some products that have been proved recently on grain shelf-life extension. This is the case of Mater-Bi® (MB), a bioplastic product composed of starch, polycaprolactone (e-caprolactone) and a minor amount of a natural plasticizer, being a reliable and readily adaptable product currently used for making shopping bags, biofillers, agricultural films and a number of other commercial products [111]. Moreover, MB is completely biodegradable, having a rate of breakdown similar to that of cellulose, having a highly favorable low environmental impact profile [112]. Based in MB properties and reviewing previous research that demonstrated that AF contamination in corn is reduced by field application of wheat grains pre-inoculated with the non-aflatoxigenic *Aspergillus flavus* strain NRRL 30797, Accinelli and collaborators in 2009 conducted a series of laboratory studies on the reliability and efficiency of replacing wheat grains with the novel bioplastic formulation Mater-Bi® to serve as a carrier matrix to formulate this fungus. Mater-Bi® granules were inoculated with a conidial suspension of NRRL 30797 to achieve a final cell density of approximately log 7 conidia / granule. Incubation of 20-g soil samples receiving a

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single Mater-Bi® granule for 60-days resulted in log 4.2–5.3 propagules of *A. flavus* / g soil in microbiologically active and sterilized soil, respectively. Increasing the number of granules had no effect on the degree of soil colonization by the biocontrol fungus. In addition to the maintenance of rapid vegetative growth and colonization of soil samples, the bioplastic formulation was highly stable, indicating that Mater-Bi® is a suitable substitute for biocontrol applications of *A. flavus* NRRL 30797 [43].

Nowadays, the use of biopolymer covers on seeds has been a successful and economic biocontrol method. The most used is chitosan, a biopolymer which is found naturally in cell walls of certain fungi, but which primary production source is the hydrolysis of chitin in alkaline medium at high temperatures [113]. Chitosan is known for its antifungal and antimicrobial properties, and it can be used in solution, films, spheres, hydrogels, nanoparticles, fibers and coatings, which makes it useful for a variety of applications in different areas [114]. Since the nineties, chitosan has been used to coat fruits and vegetables because of its bactericidal and fungicidal properties, and its ability to form films favoring the preservation of products due to the modification of the internal atmosphere and reduced transpiration losses. In addition, the coating gives the fruit more firmness and promotes the reduction of microbial development [113, 115, 116]. Due to the success of the results obtained using chitosan as a biocide, a large number of researchers all over the world have applied chitosan in seeds under storage conditions, reporting a favorable decrease on storage fungi even under high humidity conditions and thereby decreasing the amount of mycotoxins developed in the grain [116, 117].

In 2011, Lizárraga-Paulín and collaborators reported their findings about the use of chitosan in maize against *Aspergillus flavus* and *Fusarium moniliforme.* The objective of this research was to determine the protective effect of chitosan in maize seedlings subjected to the fungi mentioned above. In order to achieve the aim, after some quality tests, three groups of seeds were separately subjected to attacks by *Aspergillus flavus* and *Fusarium moniliforme*. A first group was considered as a positive control, another was coated with chitosan solution and, a final group was mechanically damaged before application of the biopolymer. In the fifth week of growth, leaf structures of the seedlings were planted in agar PDA in order to determine the presence of stressful-fungi. It was found that leaves from the seeds treated with chitosan developed no fungal burden, suggesting that chitosan acts as an activator of defense mechanisms in maize seedlings, preventing infection by the pathogenic fungi and turning chitosan recovering into a good method to storage maize seeds under adverse conditions [118]. More research is needed in order to determine if not only *A. flavus* and *F. moniliforme* but also AF and fumonisins development can be prevented since seed level.

6. Conclusions

The use of biotechnological methods is a promising tool based on the use of biological systems, living organisms or their derivatives, and focused not only on increasing agricultural products quality, but also on the development of new approaches for fighting against AF

and avoiding diseases caused by this threat. The use of new materials like biopolymers and biodegradable plastics on crops seems to be more effective against toxins, and moreover, they have the capability to replace substances that are harmful for health, avoiding contamination and offering the consumer better and uncontaminated products.

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