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Introducing an Indigenous Non-toxigenic Aspergillus flavus Strain Isolated from Iraqi Corn Grains as a Bio-Control Agent to Reduce Aflatoxin Contamination in Corn Grains

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Introducing an Indigenous Non-toxigenic *Aspergillus flavus* Strain Isolated from Iraqi Corn Grains as a Bio-Control Agent to Reduce Aflatoxin Contamination in Corn Grains

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by

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ABSTRACT

Mycotoxin contamination of cereal crops, such as maize and sorghum, is a global concern because of the potential health effects on humans and animals. Although substantial research has been conducting regarding mycotoxin prevention and mitigation, little information is available about the association of mycotoxin-producing fungi with corn and sorghum grain in Iraq. Identifying and refining indigenous atoxigenic strains to reduce mycotoxin contamination of maize and sorghum has the potential to enhance the nutritional value of these grains while reducing economic losses. However, to our knowledge, this tactic has not yet been adopted by agricultural authorities and farmers in Iraq. To survey mycotoxigenic fungi associated with corn and sorghum grain in Iraq and identify potential biological control agents 'customized' for Iraqi production conditions, a collection of corn and sorghum grain samples were imported from Iraq. DNA-based diagnostic analyses were integrated with morphology-dependent methods to identify seedborne mycoflora in corn and sorghum samples collected from different regions in Iraq (north, central, and south). The most common fungal genera, i.e., *Fusarium spp.*, *Alternaria spp.*, *Chaetomium spp.*, *Penicillium spp.*, and *Aspergillus spp.* were found frequently in both kinds of grains. However, *Exserohilum*, *Anthracosystis, Bipolaris, Sporisorium, Curvularia, Sarocladium, Humicola, Byssochlayms, and Stenocarpella* were found associated with some samples, which is the first such reporting of these genera associated with corn in Iraq. Moreover, species borders of *Aspergillus spp.* and *Fusarium spp.* were delimited successfully through multi-locus sequence typing analysis of four conserved genes within each genera (*ITS*, *B-tub, CaM,* and *RPB-2* for *Aspergillus spp.* and *EF-1a, CaM*, and *H3* for *Fusarium spp.*).

Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of Iraqi corn and sorghum samples revealed that most samples were contaminated with relatively low levels of fumonisin-B_1, ranging from 0.01-8.6 ppm. However, neither corn nor sorghum samples were

contaminated with aflatoxin-B_1. Also, analyses of mycotoxigenic potential of *Aspergillus flavus* (27 isolates) identified three non-toxigenic *A. flavus* isolates (A-1-9, A-4-54, and A-4-75), whereas the rest varied in aflatoxin-B1 production. However, all *Fusarium* species (86 isolates) produced fumonisin-B_1 at different levels, ranging from high to low concentrations. This is potentially the first report of *F. incarnatum* and *F. acuminatum* (well-known as deoxynivalenol – DON- and T2 producers, respectively) producing fumonisin mycotoxins.

Co-inoculation experiments between Iraqi native nontoxigenic isolates (A-1-9, A-4-54, and A-4-75) and native Iraqi toxigenic isolate (A-15-49) or the reference *A. flavus* NRRL 3357 substantiated the competitive ability of the Iraqi native nontoxigenic isolates to suppress aflatoxin biosynthesis in toxigenic strains. Aflatoxin-B_1 was suppressed by approximately 99.2% compared to control treatments in which toxigenic isolates were inoculated individually. This is the first report of indigenous non-toxigenic *A. flavus* isolates from Iraq with documented biological control activity against toxigenic strains. Further investigations are required to evaluate the performance of these isolates in corn fields in Iraq.

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Global importance of corn and sorghum

Corn (*Zea mays* L.) and sorghum (*Sorghum bicolor* L.) are cereal crops planted worldwide because of their edible grains. They supply humans and animals with nutritious energy for life and are considered staple crops for global food security (Aseefa et al., 2014). The Food Agriculture Organization (FAO) notes that more than 50% human caloric intake is supplied by corn, wheat, and rice. Corn is cultivated worldwide for the quality and the quantity of yields, convenient planting, and adaptability to diverse agro-ecological regions (Fandohan et al., 2003). In recent decades, corn production has increased dramatically along with the expansion of the world's population and it's demanded more nutritional resources. Based on estimates from the United States Department of Agriculture (USDA 2019), the world corn production for 2019/2020 was 1,113.02 million metric tons (MMT), which was slightly higher than production in 2017/2018 (1,080.09 MMT). The USA accounted for approximately 31% of global production (347.78 MMT). In Iraq, corn is an important agricultural commodity, and is a primary income source for 11% of the Iraqi population (World Food Programme, 2019). Corn ranks with wheat, barley, dates, and rice as a top inland crop in Iraq, along with fruit and vegetable crops such as citrus, tomato, and potato (Lucani, 2012).

Global sorghum production is not as widely distributed as global corn production. Sorghum is commonly cultivated in arid and semiarid regions of the world due to its heat and drought tolerance. Global sorghum production in 2019/2020 (57.26 MMT) was substantially less than corn. According to the FAO, sorghum ranked 5th in world cereal production after wheat, barley, rice, and maize. However, in some regions of the world, such as Africa, Central America, and South Asia, people with low incomes may rely heavily on sorghum for their daily caloric intake (Pariona, 2019). Sorghum grain is widely used for human nutrition and livestock feed, and

recently has become more widely utilized for the production of alcohol (ethanol), oil, paper, and biofuels (Stevens, 2019). In Iraq, there is little information about sorghum in terms of disease pressure, as sorghum grain is primarily utilized for animal nourishment. Historically, Iraqi sorghum acreage is significantly less corn (for example, 20.ooo vs 60.ooo hectare, respectively, in 2019) according to Index Mundi (n.d).

Many food industries utilize corn and sorghum in their products because of their starch and oil content. A key concern is that these crops could potentially be invaded by plant pathogens before or after harvest, leading to reduced grain quality. In this context, a greater understanding of fungal disease pressure in Iraqi production conditions would provide valuable, baseline information to protect and optimize grain yield and quality.

Plant pathogenic fungi

Fungi are eukaryotic organisms that have been classified into an autonomous kingdom (Fungi) (Blackwell, 2011). The nutritional content of cereal crops concurs with the nutritional requirements of many fungi, which is a primary reason that some fungi have evolved to become plant pathogens (Bennett & Klich, 2003). Even though a large number of fungal species are associated with plants in various ways, less than 10% of known fungal species are able to colonize plants and establish disease (Carris et al., 2012). Based on the stage of crop production at which fungal pathogens cause the greatest impact, diseases of cereal grains can be grouped into two major categories: field or storage diseases. For many field diseases, relative humidity and ambient temperature are key environmental conditions that influence disease initiation and progression. Additionally, grain moisture and insect infestation are important contributing factors (USDA, 2006). For corn and sorghum, fungal genera that are frequently isolated from grain include *Aspergillus*, *Alternaria*, *Fusarium*, *Cladosporium*, and *Penicillium* (Magan, & Lacey,

1985). Field fungi can access stored grains through pre-harvest infection, dust, contaminated transportation containers or storage bins, or damaged/insufficiently contained storage facilities (Youssef *et al*., 2008). For storage diseases of corn and sorghum, some of the most frequently encountered pathogens belong to the genera of *Aspergillus*, *Fusarium*, and *Penicillium* (Orsi et al., 2000). The major factors that influence germination of quiescent fungal spores during grain storage are moisture content and storage temperature. Additionally, insect activity can promote fungal dissemination within stored grain (Suleiman & Omafe, 2015).

Many studies pertinent to pathogens of corn and sorghum grain require identification of fungi as an initial step. In maize samples collected from different storage conditions in Ethiopia, *Aspergillus spp.* were the most prevalent fungi, followed by *Fusarium spp.* (Tsedaley & Adugna, 2016). Similarly, grain molds in corn samples collected from seven provinces in Iraq predominantly contained *Aspergillus flavus* 18.57% followed by *Fusarium spp.* 12.8 %, *Asp. ochraceous* 9.96%, *Asp. terreus* 9.07%, *Asp. fumigatus* 8.46%, *Alternaria spp.* 6.40%, *Rhizopus spp.* 4.98%, *Asp. oryzae* 4.80%, *Penicillium spp.* 4.53%, *Asp. versicolor* and *Rhizoctonia spp.* 4.27%, and *Asp. tamari* and *Mucor spp.* 3.20% (Hassan et al., 2014). In the same context and earlier, Alrawi et al. (2010) found that *Fusarium spp.*, *Aspergillus spp.*, and *Penicillium spp.* were predominant stored corn grains from Iraq. In India, fungi most frequently associated with sorghum grains included *Curvularia lunata*, *Fusarium verticillioides, Drechslera longirostrata*, *Alternaria alternata* and *Aspergillus flavus* (Panchal & Dhale, 2011). Notably similar results were obtained by El Shafie and Webster (1981) who surveyed seed-borne fungi of sorghum in Sudan.

Impact of fungal pathogens on corn and sorghum grain

Fungi can live in a diverse range of natural habitats, with conditions ranging from normal to extreme (Hawksworth, 2006; Mueller & Schmit, 2006; Sancho et al., 2007). A majority of fungi are saprophytic, utilizing dead materials such as residues of plants and insects as sources of nutrients. However, a minority of fungi are classified as parasitic, i.e., require a living host to survive and/or complete their life cycle (Voegele & Mendgen, 2011). In this context, plant pathogenic fungi attack virtually every crop species and cause considerable economic losses annually. Deterioration of grain can happen upon fungal infection, and can include abnormal appearance (discolored grains), lack taste and odor, lower nutritional value, lost weight, reduced yields, reduced seed viability (ability to germinate), deformation or lost vigor of seedlings, and contamination with mycotoxins (Ogaraku, 2010). Today, tremendous improvements have been achieved in terms of plant disease management and grain storage technology. However, costs incurred by pre/postharvest diseases in cereal crops remain high worldwide as fungi continually threaten the world's five fundamental crops (wheat, rice, maize, potato, and soybean) (Fisher et al., 2012; Almeida et al., 2019).

Maize is third in terms of crop importance worldwide after wheat and rice. However, demand for maize has steadily increased as the world's population has increased exponentially. Recent projections suggest global demand for corn will double from 2010 to 2025 (Rosegrant et al., 2008; Kyenpia et al., 2009). To date, more than 65 diseases have been recorded on corn by different causal agents (bacteria, viruses, and fungi), and these diseases are estimated to reduce global corn production by approximately 11% annually (Carlos, 2004; Suleiman & Omafe, 2015). According to Tsedaley (2016) fungi are the most devastating pathogens of corn, as global yield reductions could reach 80% annually without disease mitigation/management (Orsi et al.,

2000). Many fungal pathogens attack different plant tissues of corn throughout all stages of plant development. Of extreme importance are diseases associated with mycotoxin production in grains, such as Aspergillus ear rot, caused by *Aspergillus flavus* and *A. parasiticus*, and Fusarium ear rot, caused by *Fusarium verticillioides* and related species. Both diseases are common wherever corn is cultivated, especially in humid and warm conditions (CAST, 2003; Bentley et al., 2006).

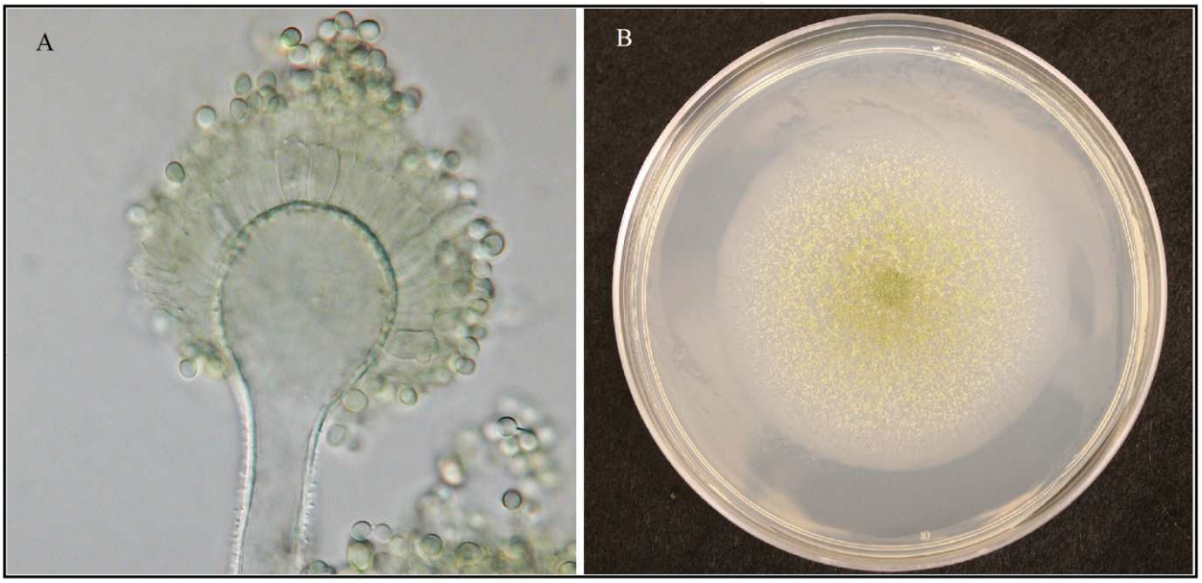
Contamination of corn and sorghum grain with aflatoxins and fumonisins has been reported in many regions of the world. In the Philippines, major genera of mycotoxigenic fungi (*Aspergillus spp.*, *Fusarium spp.*, and *Penicillium spp.*) were isolated from cereal crops including corn and sorghum, and mycotoxins such as aflatoxin, fumonisin, ochratoxin, nivalenol, and zearalenone were detected in unprocessed grain (Balendres et al., 2019). An investigation of 114 farmsteads in Zambia found farmers and consumers in these areas to be at high risk of exposure to aflatoxins and fumonisins; *A. flavus* and *F. verticillioides* were frequently isolated from grain, and the concentrations of aflatoxins and fumonisins were tenfold higher than allowable limits (Mukanga et al., 2010). The occurrence of multiple mycotoxins in sorghum has been reported in Ethiopia; when 70 samples of sorghum grains were evaluated, LC-MS/MS analysis confirmed a high incidence and concentration of aflatoxins and fumonisins (Chala et al., 2014). In Europe, aflatoxin and fumonisin contamination of maize has profoundly impacted human and animal health and caused considerable economic loses. Fumonisin contamination of corn is periodically common in regions of southern Europe, where environmental conditions are often favorable (Logrieco et al., 2002). Contamination of maize with *A. flavus* is increasingly common in Europe, most likely due to climatic change affecting the continent (Masiello et al., 2014). Aflatoxins and fumonisins also affect maize production in regions of South America. In

Argentina, *Fusarium spp.* are most commonly associated with corn, followed by *Penicillium spp*. and *Aspergillus spp*. (Camiletti et al., 2017). In northern Argentina, a study assessing yellow corn, white corn, and corn-based products revealed *F. verticillioides* to be present in all samples. However, only two samples of corn or corn-based products were contaminated with fumonisins, with concentrations ranging from 603 to 1888 ppb (Torres et al., 2001).

Aspergillus flavus Link

Aspergillus flavus mainly inhabits soil and is primarily saprophytic in terms of nutrient acquisition. However, *A. flavus* is also an opportunistic plant pathogen (Klich, 2007). Some seed crops such as maize, sorghum, peanut, cottonseed, and tree nuts are particularly vulnerable to *A. flavus* infection, which causes economic losses to due reduced yield and contamination with aflatoxins (Dorner, 2004). *A. flavus* represents the organism's anamorphic (asexual) phase of life, in which the fungus propagates exclusively through asexual structures such as conidia (Samson, 2016). However, the teleomorph (*Petromyces flavus*), the sexual phase of this species has been reported recently after observation of two individuals with opposite mating type genes formed sclerotia (fruiting bodies) containing fertile ascospores after hyphal fusion (Horn et al., 2009).

Phenotypically, *A. flavus* in culture media (such as potato dextrose agar; PDA) often display yellow-green powdery masses representing massive numbers of asexual spores (conidia), whereas the reverse sides of colonies display a reddish-gold color (Fakruddin et al., 2015). Microscopically, conidiophores proliferate from septated hyphae and end with spherical vesicles. Metulae and phialides are biseriated and cover three quarters of the head (vesicle) and give rise to chains of spherical to subspherical conidia with finely roughened or, rarely, smooth walls (Pitt & Hocking, 2009) (Figure 1).



(A) *A. flavus'* head (biseriated vesicle with masses of conidia. (Source: Wikimedia).
(B) *A. flavus* colony on 0.2X PDA at 7 day of age. (Photo by Ali Almatakeez).

Figure 1: Microscopic features of *Aspergillus flavus* (A) and colony appearance on PDA medium (B).

With respect to taxonomy, the most important aflatoxin-producing *Aspergillus* species aggregate in the sub-genus *Circumdati* (Frisvad & Samson, 2000). Among the seven sections within the subgenus *Circumdati*, section *Flavi* includes an assemblage of genetically related aflatoxigenic and non-aflatoxigenic species (e.g. *A. flavus* and *A. parasiticus*). Also, other species such as *A. oryzae* and *A. sojae* belong to this section are industrially important as they utilized in producing soy sauce (Peterson, 2008; Tsang et al., 2018).

Populations of *A. flavus* often have high levels of genetic diversity that account for phenotypic variation, including aflatoxin biosynthesis (Horn et al., 2009). Morphologically, *A. flavus* strains can be further classified according to the size of sclerotia; *A. flavus* L strains form large sclerotia (≥ 400µm in diameter), whereas *A. flavus* S strains form small sclerotia (< 400µm in diameter). Both morphotypes (*i.e.*, L and S) have the ability to produce aflatoxins, and it is

notable that S strains generally produce more sclerotia and aflatoxins, whereas the quantities of sclerotia and aflatoxins are lower among L strains. Additionally, non-aflatoxigenic strains more frequently L-type strains (Probst et al., 2009).

Aflatoxins

Aflatoxins are considered to be the most potent naturally produced toxins associated with agricultural products that pose hazards to human and animal health (Kumar et al., 2017). Aflatoxins are secondary metabolites produced by certain species of fungi grouped in *Aspergillus* section *Flavi*. *A. flavus* Link and *A. parasiticus* Spear are the main producers of aflatoxins. However, other species that belong to the two sections *Nidulantes* and *Ochraceorosei* are also reported to produce aflatoxins (Sarma et al., 2017). Chemically, aflatoxins are difuranocoumarin derivatives synthesized from polyketide precursors (Figure 2). Depending on their fluorescence properties under UV light, aflatoxins have been classified into four major types: aflatoxin-B_1 (AFB_1) and aflatoxin-B_2 (AFB_2), which exhibit blue fluorescence, and aflatoxin-G_1 (AFG_1) and afaltoxin-G2 (AFG_2), which exhibit green fluorescence (Geiser et al., 2000). Strains of *A. flavus* primarily produce $AFB_1$and AFB_2, while *A. parasiticus* produces all four types of aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2) (Amaike & Keller, 2011).

The aflatoxin biosynthetic gene cluster

The aflatoxin biosynthesis pathway in *A. flavus* is governed by at least 27 genes clustered in about 80 kb of genomic DNA near the telomeric region of chromosome III (Figure 3). The enzymatic reactions involved in the biosynthesis of aflatoxin are well described (Georgianna & Payne, 2009). Genes in aflatoxin biosynthetic cluster are regulated by transcriptional factors (i.e. *aflR* and *aflS*) co-localized within the gene cluster (Caceres et al., 2020).

The aflatoxin biosynthetic pathway is complex and involves many oxidative-based reactions (Minto & Townsend, 1997). Impaired expression of transcription within the aflatoxin gene cluster (*aflR* and *aflS*) reduces or abolishes production of aflatoxins. Flaherty and Payne (1997) reported that fusing the coding region of *aflR* to the strong, constitutive promoter of *adh-1* from *A. flavus* genetically complemented a Δ*aflR* mutant *A. flavus*. In general, partial and/or complete deletion of aflatoxin biosynthetic cluster genes is almost always associated with atoxigenicity among natural populations of *A. flavus*. In this context, Wei et al., (2014) found that 76 of 323 *A. flavus* isolates evaluated were unable to produce aflatoxin due to various deletions in aflatoxin cluster genes. Nearly 97% of the non-aflatoxigenic strains had at least one deletion in *aflT*, *nor-1*, *aflR*, or *hypB*, which suggested that these genes could be utilized as biomarkers of atoxigenicity in natural populations.

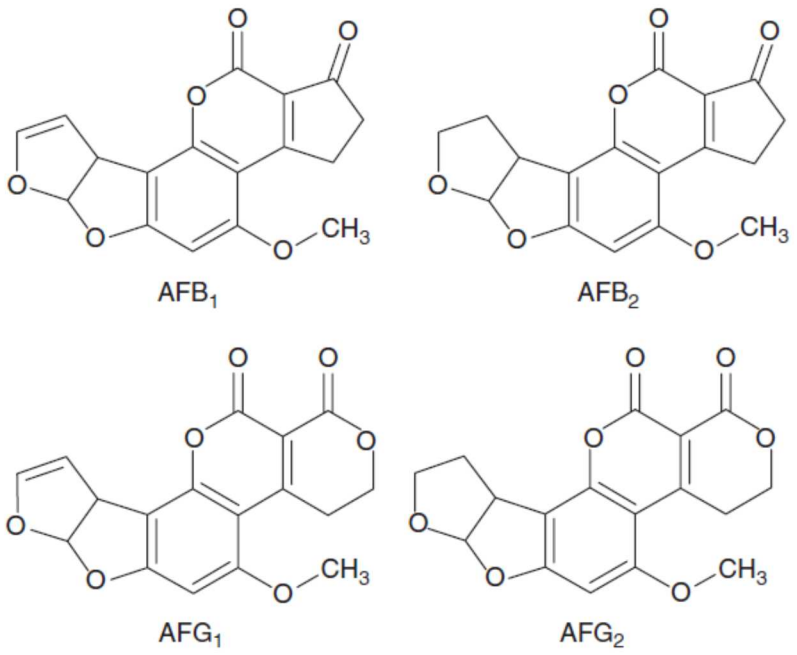


Figure 2: Structural formulas of the four major types of aflatoxins**.** Reference: Coppock & Christian, (2007).

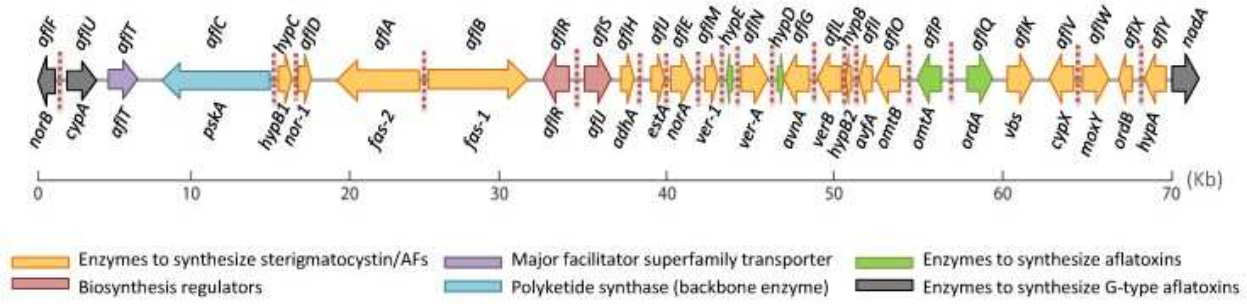


Figure 3: Aflatoxin gene cluster includes the new and old gene nomenclatures (above and below arrows representing genes, respectively). Red dotted lines represent the binding sites of *AflR* in the above pathway. Reference: Caceres et al., (2020).

Significance of aflatoxin contamination

Aflatoxin contamination can occur before and after grain harvest. Entry of aflatoxins into human and animal feed supplies is a major concern for growers, grain processors, and consumers of grain and grain-based food products. Outbreaks of aflatoxicosis have been reported in many countries where aflatoxin-contaminated crops have been documented. Aflatoxicosis describes a multi-symptom malady resulting from consumption of food products contaminated with aflatoxins. Chronic aflatoxicosis resulting from exposure to small amounts of aflatoxins over extended periods of time is associated with stunting during childhood, hepatocarcinoma, and immune system impairment. In contrast, acute aflatoxicosis results from consuming high doses of aflatoxins within a short amount of time (Gong et al., 2004; Amaike & Keller, 2011). Acute aflatoxicosis causes severe illness in humans such as acute hepatitis, abdominal pain, vomiting, and can be fatal (Azziz-Baumgartner et al., 2005). Outbreaks of aflatoxicosis on the African continent are more frequent than in other regions of the world because climatic factors (temperature and humidity) are conducive for aflatoxin biosynthesis, corn and sorghum are dietary staples, grain storage facilities are inadequate or lacking in many locations, and high quality grain products are allocated for export, which leaves lower quality products (i.e.

aflatoxin-contaminated grains) for domestic consumption (Darwish et al., 2014; Matumba et al., 2014; Misihairabgwi et al., 2017). In Kenya, widespread incidences of aflatoxicosis have been reported many times (1981-1982, 2001, 2004-2006, and 2008), and aflatoxin-contaminated maize was directly linked to at least 125 deaths in Kenya in 2004 (Probst et al., 2007; Probst et al., 2010). Similarly, aflatoxicosis cases in India resulting from consuming contaminated corn are reported sporadically yet consistently (Reddy & Raghavender, 2007). In the US, more safeguards are in place as the US Food and Drug Administration (FDA) has determined 100–300 ppb of AF_s as action levels in commodities for feeding mature nonlactating animals, 20 ppb for commodities destined for human consumption, dairy and immature animal consumption, pet food, and interstate commerce, and 0.5 ppb for milk, yet an outbreak of acute aflatoxicosis killed at least 100 dogs because of aflatoxin-contaminated dog food (Groopman et al., 2013; Lang, 2006).

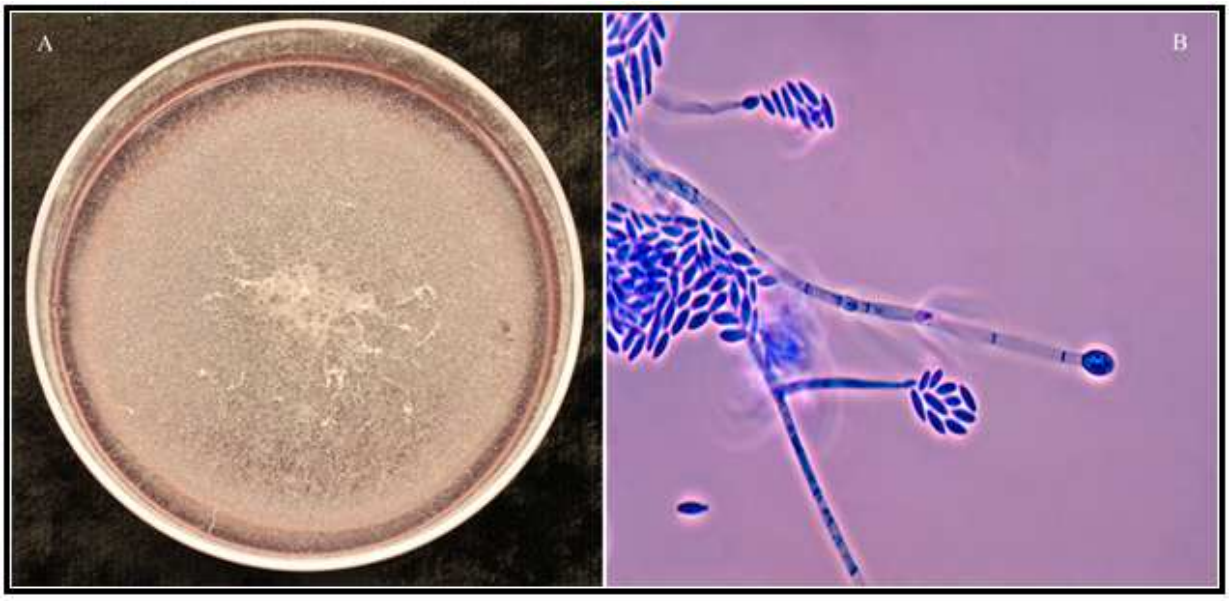
The outbreak of Turkey X disease in 1960 that killed 100,000 birds in England due to aflatoxin-contaminated peanuts in feed launched initial aflatoxin research efforts. Since then, aflatoxins have been studied extensively. Although four types of aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2) have been were documented as toxic contaminants of food and feed products, the hierarchy of aflatoxin toxicity starts with AFB_1, followed by AFG_1, AFB_2, and AFG_2. AFB_1 is the most carcinogenic and teratogenic aflatoxin analog isolated from food commodities and forages (IARC, 2002; Coppock & Christian, 2007). The toxicity of AFB_1 vary among mammals in general and even between individuals of the same species depending on factors such as sex, detoxification capacity, and nutritional factors (Williams et al., 2004). Generally, large farm animals (cattle, horses, sheep, and pigs) tend to be more sensitive to AFB_1 than smaller animals (turkeys, ducks, chickens, and chicks) (Haschek & Voss, 2013).

Along with hepatotoxicity and hepatic carcinogenicity, aflatoxins cause other deleterious effects. For instance, aflatoxins can induce mutations in DNA, incite teratogenic effects, and cause congenital malformations (Wangikar et al., 2005). Also, maladies of the central nervous system, gastrointestinal tract, respiratory system, cardiovascular system, urinary system, and endocrine system have been associated with exposure to aflatoxin (Bbosa et al., 2013). Beyond human health, aflatoxins incur substantial economic losses when consumed by farm animals, which suffer reduced egg, milk, and meat production, or even premature death (Grace et al., 2015).

Fusarium verticillioides **(Sacc) Nirenberg**

Fusarium verticillioides is the most frequent cause of stalk and ear rot diseases of maize worldwide (Danielsen et al., 1998; Gai et al., 2018). Taxonomically, *F. verticillioides* (as an anamorphic species) belongs to division *Ascomycota*, class *Ascomycetes*, order *Hypocreale*s, genus *Fusarium*, and the *Gibberella fujikuroi* species complex within section *Liseola* (Reyes-Velázquez et al., 2011). The nomenclature *F. verticillioides* (Sacc) Nirenberg, suggested by Gelach and Nirenberg, was adopted by the *Fusarium* research community as an updated name for *F. moniliform* (Moretti & Susca, 2010).

Morphologically, features of *F. verticillioides* colonies on PDA are white and cottony at early stages of growth, with funiculose mycelia. At mature growth stages, colonies develop pink and pale salmon coloration. Colony appearance is powdery because of microconidial production (Pitt & Hocking, 2009) (Figure 4). Microconidia are abundant, unicellular, oval to club shaped, with a flattened base, and carried as long chains on single phialides (Glenn, 2006). Macroconidia and chlamydospores are generally absent (Glenn et al., 2004) although some strains produce limited numbers of macroconidia (Pitt and Hocking, 2009).



(A) *F. verticillioides* colony on 0.2X PDA after seven days of growth. (Photo by Ali Almatakeez).
(B) Septated mycelium and singular phialides with gathered microconidia of *F. verticillioides*. (Reference: CDC Public Health Image Library).

Figure 4: *F. verticillioides* colony appearance on PDA medium (A). Microscopic features of *F. verticillioides* (B).

Pathogenic fungi have been classified into three main groups depending on lifestyle: biotrophs, hemibiotrophs, and necrotrophs (Oliver et al., 2004). *F. verticillioides* is hemibiotrophic, which means that the pathogen penetrates the host, establishes a close relationship, yet remains concealed (biotrophic phase) causing symptomless disease. Once physiological changes or senescence occurs in the host, a necrotrophic phase begins in which the pathogen aggressively kills and consumes host tissues (Koecka et al., 2011; Lanubile et al., 2014). Asymptomatic infections caused by *F. verticillioides* have been studied with a transgenic isolate expressing green fluorescent protein (GFP) (Oren et al., 2002).

Mycotoxin profiling revealed that diverse secondary metabolites are produced by members of the *Gibberella fujikuroi* species complex (Kim et al., 2012). Of these metabolites,

fumonisins have attracted the attention of the world due to health complications in humans and animals who consume contaminated crops (especially corn) and derived commodities (Heng et al., 2012; Oldenburg et al., 2017). At least 15 *Fusarium* species have the ability to synthesize fumonisins (Proctor et al., 1999). The three predominant species associated with fumonisin-contaminated grains worldwide are *F. fujikuroi* (teleomorph *Gibberella fujikuroi*), *F. proliferatum* (teleomorph *G. intermedia*), and *F. verticillioides* (teleomorph *G. moniliformis*). Of these, *F. verticillioides* has earned the most scrutiny, as this species produces large amounts of fumonisins, is widely distributed in the world, is most dominant in maize fields, and is most frequently associated with outbreaks of mycotoxicosis in domestic animals (Rheeder et al., 2002; Kim et al., 2012).

Fumonisins

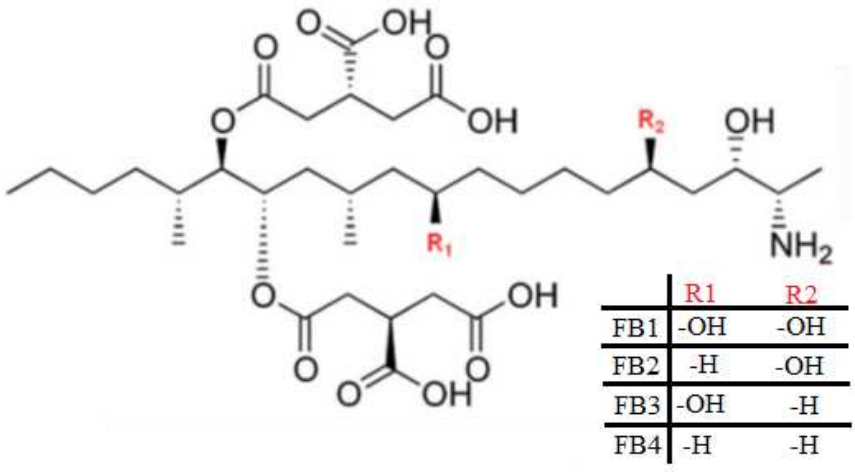
Fumonisins are a group of mycotoxins comprised of at least 28 structural analogs that be categorized into four series: A, B, C, and P. Fumonisins are well-documented as food-borne carcinogenic metabolites associated with corn and various other crops (Rheeder et al., 2002). Research endeavors spanning decades started in 1970 when an outbreak of equine leukoencephalomalacia (ELEM) occurred in South Africa due to horses consuming contaminated maize. By 1988, the causal mycotoxins were given the names fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) (Gelderblom et al., 1988). The B series of fumonisins (FB_1, FB_2, FB_3, and FB_4) are the most toxic analogs and also predominate in crops. Fumonisin B analogs vary in percentage of natural incidence; FB_1 is most common (typically 70% or more of total fumonisins present in a given sample) compared to other analogs (FB_2, FB_3, FB_4) that each typically comprise 05% to 20% of total fumonisin content (Nelson et al., 1993; Marasas, 1996).

Fumonisins are polyketide-derived metabolites that contain a 20-carbon backbone with an amino functional group at C-2` and methyl groups at C-12` and C16`. Other functional groups in the fumonisin core structure include 6-carbon tricarballylic esters that attach at C-14` and C-15` and a hydroxyl group at C-2`. However, the presence or absence of two more hydroxyl groups at C-5` and/or C-10` differentiates the four B-series fumonisin analogs (Munkvold et al., 2019) (Figure 5).

The fumonisin biosynthetic gene cluster

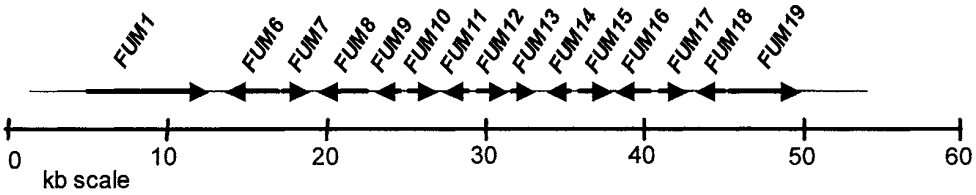
Numerous studies have been performed to elucidate the molecular basis of fumonisin biosynthesis. Similar to other mycotoxins such as aflatoxins, trichothecenes, ochratoxins and zearalenone, fumonisin biosynthesis results from the coordinated regulation of genes clustered together within a specific genomic region (Blacutt et al., 2018). The final delineation of the fumonisin biosynthesis cluster represented the culmination of many collaborative studies. The first report came from Desjardins et al. (1996) who found that defects in *FUM2* and *FUM3* caused fumonisin-B_3 and fumonisin-B_2 phenotypes in *Gibberella fujikuroi*, and that the two genes showed close genetic linkage with *FUM1*, an important gene in fumonisin production. Later, disruption analyses by Seo et al. (2001) identified four more genes designated *FUM6*, *FUM7*, *FUM8*, and *FUM9* that are required for fumonisin formation; these genes were adjacent to the *FUM5* gene previously identified by Proctor et al. (1999) to encode a polyketide synthase (PKS) essential for fumonisin biosynthesis. Shortly thereafter, Proctor et al. (2003) stated that the fumonisin biosynthetic gene cluster consisted of 15 genes in close physical proximity that encoded most the essential enzymes involved in fumonisin production (Figure 6). The genome of *F. verticillioides* has been sequenced (Ma et al., 2010), and the gene clusters underlying numerous *Fusarium* mycotoxins (including fumonisins) have been characterized extensively

(Desjardins & Proctor, 2007). However, the exact delineation of mycotoxin gene clusters is subject to change as more functional analyses are published. As of today, according to Woloshuk and Shim (2013), 23 genes are thought to participate in the biosynthesis of fumonisin in *F. verticillioides*, and these genes are clustered within an 80 kb region of chromosome 1.



	R1	R2
FB1	-OH	-OH
FB2	-H	-OH
FB3	-OH	-H
FB4	-H	-H

Figure 5: Core structure of fumonisins: B_1, B_2, B_3, and B_4.



Gene	Predicted function
FUM1	Polyketide Synthase
FUM6	Cytochrome P450 Monooxygenase/Reductase
FUM7	Dehydrogenas
FUM8	Aminotransferase
FUM9	Dioxygenase
FUM10	Fatty Acyl-CoA Synthetase
FUM11	Tricarboxylate Transporter
FUM12	Cytochrome P450 Monooxygenase
FUM13	Carbonyl Reductase
FUM14	Condensation Domain Peptide Synthetase
FUM15	Cytochrome P450 Monooxygenase
FUM16	Fatty Acyl-CoA Synthetasse
FUM17	Longevity Assurance Factore
FUM18	Longevity Assurance Factore
FUM19	ABC Transporter

Figure 6: Map of the fumonisin biosynthetic gene cluster. The predicted function of each gene is listed beside the gene designation. (Reference: Yu et al., 2004)

A crucial component of mycotoxin biosynthesis is the master regulator of biosynthetic cluster genes. This regulatory gene is usually located within the same gene cluster it regulates (Keller et al., 2005). Interestingly, none of the original 15 predicted genes of the fumonisin gene cluster pathway of *F. verticillioides* appeared to be a regulatory gene. BLASTX analyses of a novel open reading frame (ORF), designated ORF-21 or *FUM 21*, revealed putative zinc finger domains typically associated with transcription factors (Proctor et al., 2003). Shortly thereafter, expressed sequence tags (EST) data confirmed that *FUM21* encoded a full-length Zn(II)2Cys6 protein. Involvement of *FUM21* in the transcriptional regulation of most *FUM* genes was confirmed by functional analyses; *Δfum21* mutants were unable to produce fumonisin (Brown et al., 2007). In addition to *FUM21,* other genes in *F. verticillioides* have been implicated in the regulation of fumonisin biosynthesis. For instance, *FCK1* encodes a cyclin-dependent kinase in *F. verticillioides* that is activated by physical association with a C-type cyclin encoded by *Fcc1* (Shim and Woloshuk, 2001). Although *FCK1* is physically located outside of the *FUM* cluster, disruption of *FCK1* drastically reduced fumonisin-B_1 production and caused pleiotropic morphological defects such as enhanced colony pigmentation (Bluhm & Woloshuk, 2006; Brown et al., 2007).

Significance of fumonisin contamination

F. verticillioides is ubiquitous and predominantly isolated from corn, and consequently, high concentrations of fumonisins often occur in corn and corn-based food and feed products (Bullerman, 1996; Moss, 2009). Fumonisins endanger human and animal health when they contaminate food chains (Oldenburg et al., 2017). Many cases of mycotoxicoses associated with fumonisin-contaminated feed have been reported in domestic animals (Munkvold et al., 2019). The first report of fumotoxicosis accompanied an outbreak of equine leukoencephalomalacia

(ELEM) in South Africa in horses because of consumption of corn contaminated with *F. verticillioides*, and the pathological changes in the liver cells of horses such as bile duct proliferation, increased numbers of mitotic figures, multinucleated hepatocytes, and large, bizarre hyperchromatic nuclei strongly suggested the potential carcinogenicity of *F. verticillioides* metabolites (Kellerman et al., 1972). In 1981, rudimentary evidence was introduced that high rates of human esophageal cancer in the southwestern districts of Transkei were associated with consuming corn infected by *F. verticillioides* (Marasas et al., 198; Rheeder et al., 1992). Also, 68 cases of human esophageal cancer were reported in Italy from utilizing potentially contaminated corn for brewing beer (Franceschi et al., 1990). Similarly, corn samples collected from a region of Iran with a high rate of esophageal cancer (Mazandaran Province) were highly contaminated with FB_1, FB_2, and FB_3 (Shephard et al., 2000). However, establishing a conclusive link between fumonisin consumption and esophageal cancer has proven to be somewhat elusive.

After elucidating the molecular structure of fumonisins (Gelderblom et al., 1988), research was conducted to explore the basis of fumonisin toxicity. When six levels of FB_1 and FB_2 were administrated to pigs via contaminated corn, symptoms included depleted sphingolipids in hepatocytes, increased concentrations of free-sphinganine and free-sphingosine in tissues (liver, lung, and kidney) and serum, pulmonary interstitial odema, and hepatic injuries (Riley et al., 1993). Additionally, a study of pregnant women exposed to fumonisins by consuming contaminated corn-based products (tortillas) indicated increased risks of neural tube defects (NTDs) (Missmer et al., 2006). In countries such as Brazil, Guatemala and China where corn is a main staple, a high incidence of NTDs has been documented, and craniofacial anomalies were reported as possible side effects of the interferences between fumonisins and

folic acid (Marasas et al., 2004; Waśkiewicz et al., 2012). The ability of fumonisin to induce a broad range of morbidities (liver and renal toxicity and carcinogenesis, neurotoxicity, induction of pulmonary edema, and others) is attributed to disruption in sphingolipid metabolism resulting from the inhibition of ceramide synthase due to structural similarity between fumonisins and sphingoid bases (Desai et al., 2002). Relatedly, the level of sphingoid bases in serum and tissues is an effective biomarker to monitor fumonisin exposure (Schertz et al., 2018). The incidence of hepatocellular carcinoma increased significantly after administering FB_1 to rats, which pointed to the potential involvement of fumonisin exposure in hepatocellular carcinoma in human cases (Abdel-Wahhab et al., 2010). However, other studies in China postulated that the occurrence of liver cancer in humans was due to the synergistic effects of multiple toxins (aflatoxin-B_1, fumonisin-B_1, and trichothecenes) in maize samples collected from the affected areas (Ueno et al., 1997). Due to the absence of an unequivocal evidence linking fumonisins to human hepatocarcinoma, the International Agency for Research on Cancer (IARC) categorized FB_1 as a group-B_2 carcinogen that is potentially carcinogenic to humans (Ostry et al., 2016). Because of such mischievous consequences most countries have set the allowable levels of fumonisins in commodities as in the US, FDA has set 2 to 4 ppm to industry for total fumonisins in human food products, and from 5 to 100 ppm in animal feeds (Groopman et al., 2013).

Management of mycotoxins in maize grains

Cereal crops, including maize, are prone to mycotoxin contamination during three main stages of crop development: pre-harvest growth, harvest and drying, and storage (Alshannaq & Yu, 2017). Important factors influencing the magnitude of mycotoxin overlap to some degree between these three stages, including environmental conditions (temperature and relative humidity), insect infestation, and presence of bio-competitors/biological control organisms.

However, across all stages, environmental conditions appear to be the most influential factors (Mannaa & Kim, 2017; Munkvold et al., 2019). In this context, many practices have been employed to manage mycotoxin contamination in all three stages. Because fungal infestation starts in the field, and soil is an important reservoir of fungal inoculum, emphasizing mycotoxin control during pre-harvest conditions is a primary management goal. Moreover, unsatisfactory pre-harvest disease management often reduces yield and/or grain quality. Hence, post-harvest management is generally focused on mitigating further mycotoxin contamination of grains (Mahuku et al., 2019). In the field, mycotoxin management relies heavily on tactics that prevent or at least reduce infections of mycotoxigenic fungi. However, existing management tools often only mitigate, and cannot prevent, disease development and mycotoxin severity (Munkvold et al., 2019).

Cultural practices (planting date, tillage practices, crop rotation, plant population, and irrigation) are well-known to reduce many crop diseases. The central rationale is to change ambient conditions to favor plant health and prevent infection (Munkvold, 2003). Among cultural practices, fungicides and insecticides can serve as preventative measures and/or disease treatments (Rose et al., 2019). However, controlling plant disease with chemicals and/or agronomic approaches is not always effective, and thus genetic resistance is advantageous (Lanubile et al., 2017; Andersen et al., 2018). To generate resistant crops, genetic resistance must be identified and introgressed into commercially viable hybrids (Reid et al., 1994; Brown et al., 2013). However, effective genetic resistance to mycotoxin contamination has not yet been deployed successfully in corn or sorghum (Smith et al., 2019).

Considering the limitations of conventional mycotoxin management practices, alternative approaches have been explored. In this vein, novel biological control strategies have been

developed to manage pre-harvest aflatoxin contamination of maize (Dorner, 2009; Savić et al., 2020). In the most effective approach, non-toxigenic *A. flavus* strains are broadly applied to maize fields in order to exclude/inhibit toxigenic, naturally occurring *A. flavus* strains (Udomkun et al., 2017). The first atoxigenic *A. flavus* strain was introduced as a potential biocontrol agent by Cotty (1990) when seven atoxigenic isolates, originally isolated from cotton fields in Arizona, were co-inoculated with toxigenic strains on cotton. Six of these strains significantly reduced AFB_1 contamination. Later, the most effective strain (AF36) successfully decreased aflatoxin contamination of corn, pistachio, almond, and fig (Brown et al., 1991; Ortega-Beltran et al., 2019). Interestingly, pre-harvest application of atoxigenic *A. flavus* strains may provide protection in post-harvest conditions (Dorner and Cole, 2002). Pre-harvest treatment of peanuts in the U.S. with atoxigenic strains of *A. flavus* and *A. parasiticus* reduced aflatoxin contamination in storage and field conditions by 77-98% (Horn & Dorner, 2009). In sub-Saharan Africa, where *Aspergillus* species belonging to section *Flavi* are predominant in staple crops and aflatoxin contamination is consistently problematic, application of biological control in groundnut and maize enhanced yields and reduced aflatoxin contamination. Long-term (10 year) studies of the biological control product Aflasafe in maize fields showed consistent limitation of aflatoxin contamination, with > 95% of treated maize containing less than 20 ppb of aflatoxin, and of this, > 90% of samples had < 4.0 ppb aflatoxin (Bandyopadhyay et al., 2019).

Interestingly, aflatoxin contamination of maize by African strains was suppressed more by native African atoxigenic strains compared to the American atoxigenic strain AF36 (Cardwell & Henry, 2004). However, atoxigenic *A. flavus* strains display variation in their ability to prevent aflatoxin contamination in maize. Applying multiple genotypes of atoxigenic strains instead of a single genotype is newly adopted in Africa for biological control of aflatoxin (Atehnkeng et al.,

2016; Bandyopadhyay et al., 2016). With the stability and efficacy of biological control, this technology has been adopted worldwide, and many studies have documented the effectiveness of this approach to manage aflatoxin contamination in corn crop and other important crops.

Justification and objectives

Mycotoxin contamination of cereal crops, particularly maize, is a global concern because of the potential health effects on humans and animals. Although substantial research has been conducting regarding mycotoxin prevention and mitigation, there is a dearth of knowledge about the association of mycotoxin-producing fungi with corn and sorghum grain in Iraq. Aflatoxin contamination of maize and aflatoxicosis in broiler flocks have been reported and documented periodically in Iraq (Ali et al., 2017; Shareef, 2010). In the context of climate change, the incidence of aflatoxin contamination in cereal crops in Iraq may increase in the future. Moreover, effective pre-harvest strategies to manage mycotoxin contamination are most likely dependent on durable host resistance, which is currently not available. Identifying and refining indigenous atoxigenic strains to reduce mycotoxin contamination of maize and sorghum has the potential to enhance the nutritional value of these grains while reducing economic losses. However, to our knowledge, this tactic has not yet been adopted by agricultural authorities and farmers in Iraq. To survey mycotoxigenic fungi associated with corn and sorghum grain in Iraq, and identify potential biological control agents 'customized' for Iraqi production conditions, a collection of corn and sorghum grain samples were imported from different regions of Iraq and utilized to address the following research objectives:

Objective 1: Identify mycotoxigenic (and other) fungi associated with corn and sorghum grain samples collected from different regions of Iraq.

Objective 2: Differentiate toxigenic and atoxigenic isolates of *Aspergillus* and *Fusarium* by LC-MS/MS.

Objective 3: Assess the capability of native atoxigenic isolates to suppress mycotoxin production by toxigenic isolates on maize grain.

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Chapter 2: Survey of fungi associated with corn and sorghum in Iraq

Abstract

Sixteen samples of corn and sorghum grain, collected from different locations in Iraq, were surveyed for fungal microflora, with emphasis on identifying mycotoxigenic fungi. Fungi were identified via a combination of traditional methods (morphological parameters) and phylogeny analysis. A total of 468 fungal isolates belonging to twenty-two genera were isolated and identified. The most dominant groups of fungi, in descending order, were *Fusarium, Chaetomium, Alternaria, Penicillium, Aspergillus, Rhizopus, Trichothecium,* and *Nigrospora* (ranging from 5.4 to 0.8 % of samples). Additional isolates were identified as species of *Cladosporium, Exserohilum, Ulocladium, Neosartorya, Anthracosystis, Bipolaris, Sporisorium, Curvularia, Sarocladium, Trichoderma, Humicola, Byssochlayms,* and *Stenocarpella* (0.5 to 0.1% of samples). Interestingly, *Exserohilum*, *Anthracosystis, Bipolaris, Sporisorium, Curvularia, Sarocladium, Humicola, Byssochlayms, and Stenocarpella* have not been recorded previously as seed-borne fungi in Iraq. To accurately identify isolates of *Aspergillus spp.* (35) and *Fusarium spp*. (86) to the species level, a multi-locus sequence typing (MLST) analysis was conducted by sequencing three loci within *Aspergillus* genomes (*B-tub, CaM,* and *RPB-2*), and three other loci (*EF-1a, CaM*, and *H3*) within *Fusarium* genomes. The MLST analysis identified six *Aspergillus* species (*A. flavus, A. fumigatus, A. terreus, A. pseudoglaucus, A. wentii,* and *A. amstelodami*) and four *Fusarium* species (*F. verticillioides, F. thapsinum F. acuminatum,* and *F. incarnatum*) associated with corn and sorghum grains. This study provided one of the first surveys of fungi associated with corn and sorghum in Iraq and highlighted the potential for mycotoxin contamination in both commodities.

Introduction

Cereal crops and their products are considered humankind's most important source of food and energy. Given the increasing world population and concomitant demand for food, the susceptibility of cereal crops to pathogens is a serious threat to public health (Pal, 2017). The kingdom Fungi includes about 80,000 to 120,000 formally described species. However, projections suggest that the number of species in this kingdom could be as high as 1,500,000. To date, approximately 8000 species of fungi are known to be plant pathogens (Hawksworth, 2001).

According to the FAO (2009), maize and sorghum rank second and fifth, respectively, in total world production of cereal crops. Moreover, numerous industries rely on these grains to produce human staple foods such as corn flour, starch, and grits, as well as animal feed (Roige, *et al*., 2009). As rich sources of carbohydrates, proteins, and fats (Gao & Kolomiets, 2009), they are subject to attack by diverse pathogenic fungi in the field, during field conditions, storage, transport, processing, and potentially as food products (Kumar, *et al.,* 2008).

Mycotoxins are secondary metabolites produced by fungi, and because of the worldwide distribution of mycotoxigenic fungi, approximately 25% of food and agriculture products are contaminated by mycotoxins at some level (FAO, 2009). About 300-400 fungal species have been identified as mycotoxigenic, and threaten human and animal health when their toxins enter food chains (Cole & Cox, 1981). To date, over 350 compounds have been identified as harmful mycotoxins, and most of these toxins are produced by three fungal genera: *Aspergillus spp*. (aflatoxins), *Fusarium spp*. (fumonisin and deoxynivalenol), and *Penicillium spp*. (patulin) (Pal, 2017). Mycotoxin contamination of cereal crops, such as corn and sorghum, is considered one of the biggest challenges facing modern agricultural production (Gaag, et al., 2003). Many factors can facilitate fungal infection of cereal crops, such as ambient temperature, relative humidity

(RH), moisture content, CO_2 concentration, physical damage, and the presence of other microorganisms (Marín, et al., 1996; Richard et al., 2007). Given the widespread distribution of mycotoxigenic fungi, mycotoxin contamination of agricultural products is a chronic concern (Perrone et al., 2007).

Monitoring the quality of cereal grains consistently is a crucial step to ensure acceptable levels of mycotoxins and suitability. In 1991, an economic embargo was imposed on Iraq by the United Nations, and these sanctions isolated Iraq from most developed nations. Consequently, Iraqi universities and research centers lagged in the development and implementation of modern agricultural techniques until the first decade of 21st century. Unfortunately, as a result, for more than a decade the identification of food- and grain-associated fungi relied heavily on traditional, morphology-based techniques. To date, modern identification techniques have not been utilized to describe fungi associated with corn and sorghum in Iraq. Therefore, the goal of this study was to survey fungal microbiota associated with corn and sorghum grains from different regions of Iraq and apply DNA-based diagnostic methods to define species of *Aspergillus* and *Fusarium*.

Materials and methods

Sample collection:

A total of 18 samples (11 corn, 7 sorghum) were collected from seven different locations in Iraq. Erbil, Dohuk, and Akree represent northern Iraq; Baghdad and Kirkuk represent central Iraq, and Karbala and Najaf represent southern Iraq (Figure 1). Samples weighted 200 to 250 gm and were stored in the fridge at 4^0C until analysis. Each sample was assigned a number for convenient labeling (Table 1).

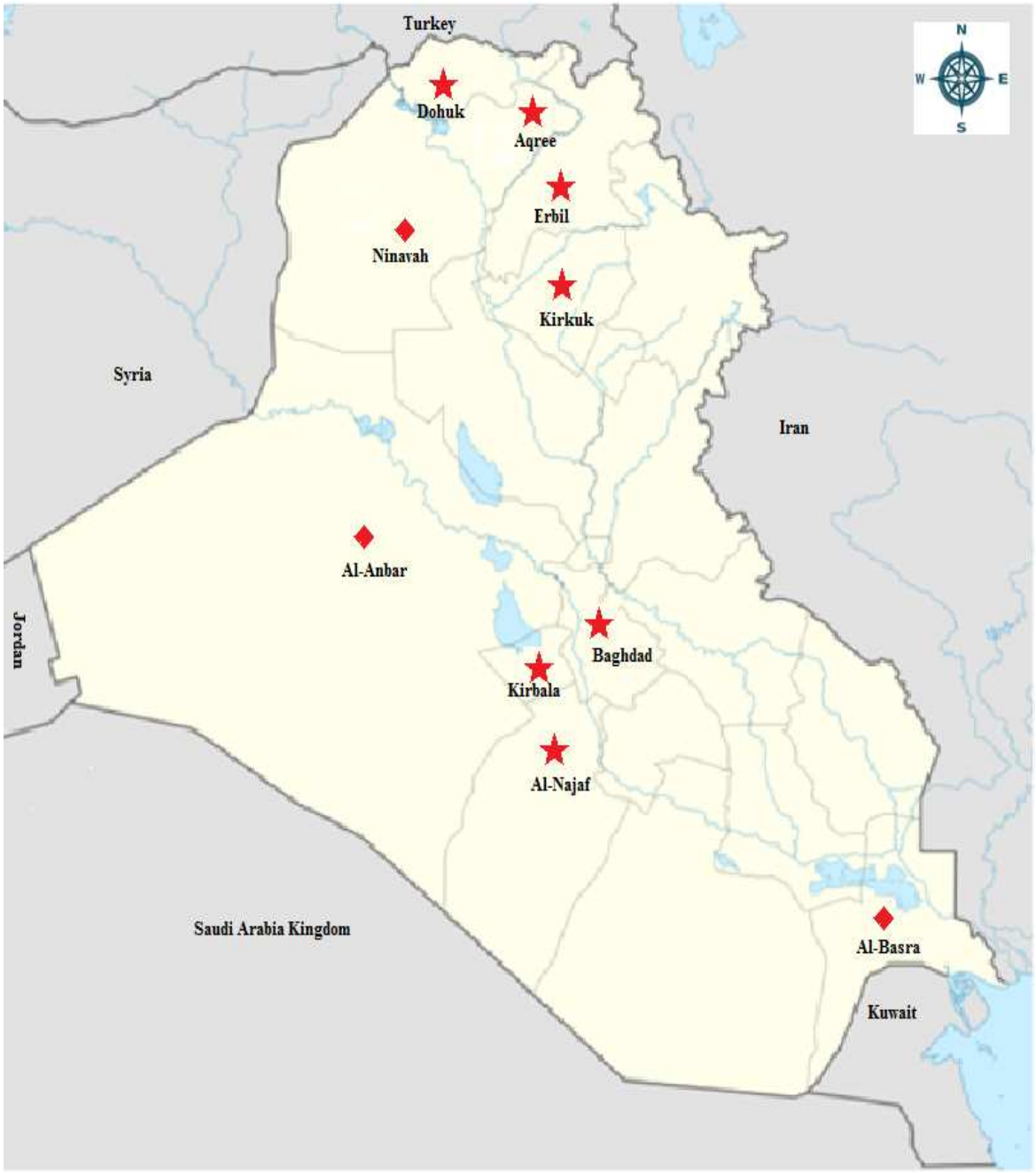

Figure 1: Geographic map of Iraq showing the locations at which corn and sorghum samples were collected for this study.

Table 1: Corn and sorghum samples and name of the city (source of sample).

Region in Iraq	Sample No.	Source of sample	Type of sample
North	8	Erbil	Yellow corn (YC)
	9	Erbil	Yellow corn (YC)
	10	Erbil	Yellow corn (YC)
	16	Dohuk	Sorghum (S)
	17	Akree	Sorghum (S)
	18	Akree	Yellow corn (YC)
Middle	7	Kirkuk	Yellow corn (YC)
	11	Kirkuk	Sorghum (S)
	14	Kirkuk	Yellow corn (YC)
	12	Baghdad	Yellow corn (YC)
	13	Baghdad	Yellow corn (YC)
South	1	Karbala	Yellow corn (YC)
	2	Karbala	Yellow corn (YC)
	3	Karbala	Yellow corn (YC)
	4	Karbala	Sorghum (S)
	5	Karbala	Sorghum (S)
	6	Kabala	Sorghum (S)
	15	Najaf	Sorghum (S)

Isolation and preliminary identification of seed-borne fungi

Fungi were isolated from grain samples following International Seed Testing Association protocols (ISTA, 1979). For each corn or sorghum sample, 100 seeds were selected arbitrarily and sterilizing externally by submerging in 2% sodium hypochlorite for 3 minutes. Then, seeds were rinsed three times in sterile ddH_2O and plated (three seeds/plate) on petri dishes (100mm x 15mm) containing 0.2X potato dextrose agar medium (PDA; Difco Laboratories, Sparks MD) and 70 ppm glutamycin. Cultures were incubated at room temperature for 14 days in 12:12 hr light:dark photoperiod. Every 24 hours, cultures were evaluated and fungi emerging from seeds were transferred to new PDA plates to avoid overgrowth by saprophytic fungi (e.g. *Zygomycetes*). Finally, all fungi were preliminarily identified based on colony morphology on PDA media, microscopic characteristics of mycelia and conidia, and other parameters outlined in standard classification keys (Pitt and Hocking, 2009).

DNA extraction and analysis

Preparation of fungal tissue:

For each isolate, three petri dishes with 0.2X strength PDA were overlaid with sterile cellophane membranes and inoculated with 500 µl spore suspension ($1x10^6$ spores/ml) and incubated at 25°C for 14 days.

Extraction of genomic DNA:

For DNA extraction, cellophane membranes covered with fungal mycelia were transferred to a pre-chilled mortar containing 1 mg of glass beads (150-212 µm; Sigma Chemical Co., St. Louis, MO). Then, fungal tissue was frozen by adding liquid nitrogen and ground thoroughly with a pestle. Ground tissues of mycelium were either kept in 15 ml conical screw-cap centrifuge tubes (USA Scientific Inc., Ocala, FL) and stored at -80 °C or subjected immediately to DNA extraction. For DNA extraction, a CTAB protocol described by Gonita-Mishra et al., (2014) was utilized with some modifications. Briefly, ~500 mg of freshly ground tissue was transferred to a 2 ml sterilized Eppendorf tube containing 500 µl of CTAB extraction buffer (100 mM Tris HCl at ph 8.0; 1.4M NaCl; 20 mM E.D.T.A; 2% CTAB), 4% polyvinylpyrrolidone (PVP), and 2.5 µl beta-mercaptoethanol (BME). Tubes were incubated in a water bath at 60 °C for 30-60 minutes and vortexed every 10 minutes to homogenize contents thoroughly. Then, 500 µl of chloroform:isoamyl alcohol (24:1; v/v) was added to each tube and homogenized with a vortex mixer for 20 minutes. Tubes were centrifuged at 12000 rcf for 5 minutes and supernatants transferred to new Eppendorf tubes. After a second wash with chloroform:isoamyl alcohol as described above, 25 µl of RNase (Sigma) was added to each supernatant and incubated at 37 °C for 60 minutes. To remove RNase and digested RNA, the volume of each supernatant was adjusted to 800 µl with ddH_2O, 800 µl of chloroform was added

and mixed by inverting the tube for 1 minute, and tubes were centrifuged at 12000 rcf for 5 minutes to separate organic and aqueous phases. The aqueous phase was carefully transferred into fresh tubes containing an equal volume of isopropanol and 0.33% volume of 5 M NaCl, gently mixed by inversion, incubated at room temperature for 5 minutes, and then centrifuged for 5 minutes at 12000 rcf. After discarding the aqueous phase, the DNA pellet was washed with 500 µl of ice-cold 70% ethanol, incubated at room temperature for 5 minutes, and centrifuged at 12000 rcf. This step was repeated by washing with 500 µl ice-cold 100% ethanol. The supernatant was discarded, and pellets were air dried for 5-10 minutes. Finally, DNA pellets were dissolved in 50-100 µl TE buffer or ddH_2O and stored at -20 ºC until PCR analysis.

PCR and DNA analysis:

The internal transcribed spacer (ITS) regions of rDNA were amplified from all fungal isolates. These regions are located between the small sub-unit rRNA (18S) and the large sub-unit rRNA (28S) of rDNA (El Aaraj et al., 2015). In fungi, the ITS amplicon is broadly utilized for genus-level taxonomic identification (Nilsson et al., 2009). Two widely-used primers were selected to amplify the ITS region in this study: *ITS1* (F-5′TCC GTA GGT GAA CCT GCG G-3′) and *ITS4* (R-5′TCC TCC GCT TAT TGA TAT GC-3′) (Gardes & Bruns 1993). For PCR, each reaction (50 µl) contained: 10 µl 5X Buffer (0.3 M Tris base; 0.1 M $(NH4)_2SO4$; 0.01 M MgSO4; 15% Glycerol; 2.5% Tween-20; 0.12% Orange G; pH 9 at 25 °C), 1.25 µl of 10 mM dNTPs, 1.25 µl each of 10 µM *ITS1* and *ITS4*, 0.65 µl Taq DNA polymerase, 34.6 µl ddH_2O, and 1.0 µl DNA template. PCR was performed with a GeneAmp PCR System 9700 thermocycler and the following conditions: 94 ºC for 3.0 min (one cycle); 94 ºC for 30 sec, 56 ºC for 30 sec, and 72 ºC for 1.0 min (35 cycles); 72 ºC for 5.0 min (one cycle). PCR amplicons were evaluated via

gel electrophoresis in 0.7-1% agarose gels with 1X TEB buffer and GelRed Nucleic acid stain (1 µl/20 ml). Amplicons were visualized and photographed under UV light.

Amplicons were sequenced (Sanger approach) by GENEWIZ (South Plainfield, NJ). Sequences were evaluated via BLAST analyses using the GenBank database hosted by the National Center for Biotechnology Information (NCBI). Query sequences (obtained in this study) were compared to sequences previously deposited in the database. Species-level identification was based on the highest identity score(s) between reference sequences. Final determination of taxonomic identity was achieved through the combination of morphological-based identification and ITS sequence analysis.

Multi-locus sequence typing (MLST) of *Aspergillus* and *Fusarium* isolates:

All *Aspergillus* and *Fusarium* isolates were subjected to MLST analysis, which utilized three additional loci for each genus. For MLST analysis, PCR primers are described in Table 2, and cycling conditions are summarized in Figure 2.

Table 2: Primers utilized for MLST analysis.

Species-specifity	Locus*	Primer sequence (5′-3′)	Primer Abbrv.	Annealing temp.(^{0}C)	Amplicon's size(bp)
Aspergillus	Calmodulin-CaM [1]	F-CCG AGT ACA AGG ARG CCT TC R-CCG ATR GAG GTC ATR ACG TGG	CMD5 CMD6	55	500-600
	RPB-2[2]	F-GAY GAY CGK GAY CAY TTC GG R-CCC ATR GCY TGY TTR CCC AT	5Feur 7CReur	55	700-800
	Tubulin(B-tub)[3]	F-GGT AAC CAA ATC GGT GCT GCT TTC R-ACC CTC AGT GTA GTG ACC CTT GGC	Bt-2a Bt-2b	58	400-500
Fusarium	Calmodulin [4]	F-GAG TTC AAG GAG GCC TTC TCC C R-TGC ATC ATG AGT TGG AC	CL1 CL2A	55	500-600
	EF-1α [5]	F-ATG GGT AAG GAA GAC AAG AC R-GGA AGT ACC AGT GAT CAT GTT	EF-1H EF-2T	55	600-700
	Histone (H3)[6]	F-ACT AAG CAG ACC GCC CGC AGG R-GCG GGC GAG CTG GAT GTC CTT	H3-1a H3-1b	68	500-600

*Reference: 1, 2, and 3 Samson *et al.,* (2014); 4 and 5 Wang *et al.,* (2011); 6 Glass & Donaldson (1995)

Construction of phylogenetic trees:

Phylogenetic analyses were conducted with DNA sequence data initially obtained as ab1 extension files. Sequences were imported into Geneious (version 9.1.8) for analysis (Kearse et al., 2012). Multi-locus sequence data of the four loci from each *Aspergillus* or *Fusarium* isolate were aligned separately with Geneious and then concatenated. A Tamura-Nei model was utilized to construct phylogenetic trees via neighbor-joining (NJ) with a bootstrap value of 1000. Phylogenetic trees were generated as topologies. The placement of each *Aspergillus* isolate within the phylogenetic tree was achieved by including ex-type strains reported by Samson et al. (2014): *A. wentii* NRRL 375, *A. terreus* NRRL 225, *A. fumigatus* NRRL 163, *A. flavus* NRRL 1957, and *A. pseudoglaucus* NRRL 40. For *Fusarium*, the ex-type strains employed for taxonomic placement were *F. acuminatum* BUF036, *F. icarnatum* F9, *F. verticillioides* H06A-2A-4, and *F. thapsinum* A73.

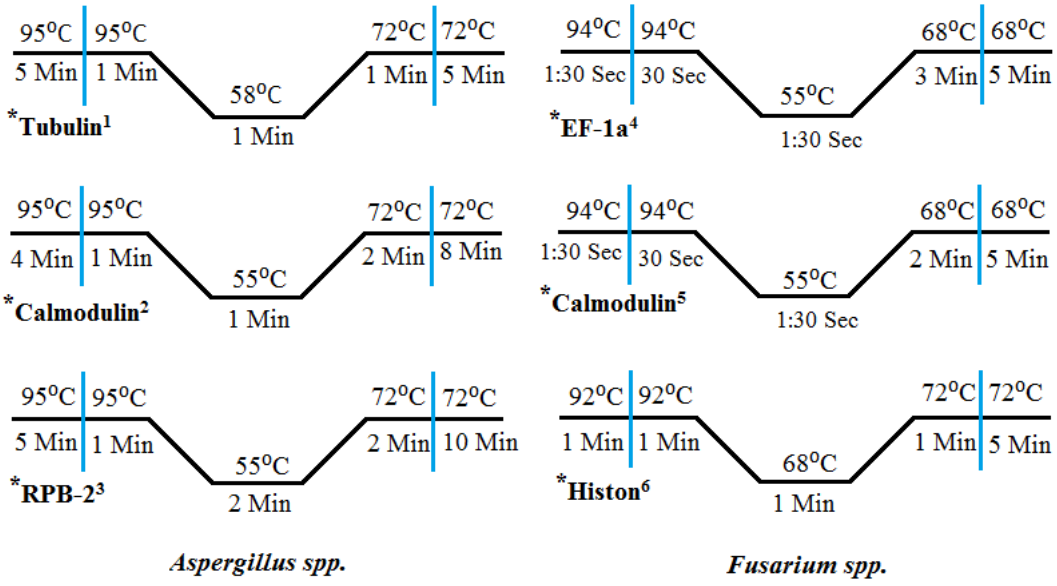

Figure 2: Shows PCR conditions of each primer's pair in MLST analysis.

Results and discussion

In this study, 22 genera of fungi were associated with seeds with varying percentages of isolation (Table 3; Figure 4). Based on the visual identification and DNA-based technique, the highest percentages of isolation were with *Fusarium spp.*, *Chaetomium spp.*, and *Alternaria spp.*, (84, 82, and 82 percent, respectively) followed by *Penicillium spp.*, *Zygomycetes spp.*, *Aspergillus spp., A. niger*, and *Trichothecium spp.* (46, 44, 34, 30, and 19 percent, respectively). Fungi from these genera commonly invade various plant hosts, especially rice, barely, wheat, and maize, and can be isolated from different conditions (*e.g.*, field or storage) (Abubakr, 2017). *Fusarium* is a well-known genus that includes many broad host-range pathogens that cause wide-spread infestation of cereal crops. Moreover, many species in this genus produce various mycotoxins (Geng et al., 2014). *Fusarium spp.* were present in 10 of 16 samples (Figure 3), a high frequency that is compatible with findings of other researchers. Al-Rawi (2010) noted in a survey of corn-associated pathogens in Iraq that *Fusarium* species were most frequently present, which could be explained by the fact that many *Fusarium* species are common in soil (Bentley et al., 2006) and can by disseminated by dust, infected plant residues, or mechanical transport (Youssef et al., 2008). Similarly, of the mycobiota associated with maize kernels in Guatemala, *Fusarium* and *Aspergillus* predominated, which implied that maize from this region is likely to be contaminated with mycotoxins (Mendoza et al., 2017). In the current study, most *Fusarium spp.* (81%) were isolated from corn seeds, whereas the rest (19%) were isolated from sorghum seeds. This observation may be due to sorghum being less susceptible than corn to pathogenic *Fusarium spp*. (Bhat et al., 1997). Interestingly, the next most common fungi were *Chaetomium spp.* and *Alternaria spp*., which were isolated at the same frequency from corn and sorghum seeds (35, and 65 % respectively) (Table 3). Both genera were mostly isolated from the same

samples (6, 9, 10, 11, 13, 14, and 15), as well as a few other samples individually (1, 2, and 7 for *Alternaria*, and 4, 5, and 16 for *Chaetomium*) (Figure 3).

Table 3: Fungal genera identified in this study and their percentage of isolation**.**

Genera	Total No.	In corn	In sorghum
		No. isolates (%)	No. isolates (%)
Fusarium spp.	86	64(74)	22(26)
Chaetomium spp.	82	29(35)	53(65)
Alternaria spp.	82	29(35)	53(65)
Penicillium spp.	46	43(93)	3(7)
Zygomycetes	36	16(44)	20(56)
Aspergillus spp.	35	13(37)	22(63)
Asp. niger	33	22(67)	11(34)
Trichothecium spp.	18	------	18(100)
Nigrospora spp.	13	13(100)	------
Cladosporium spp.	8	2(25)	6(75)
Exserohilum spp.	6	6(100)	------
Ulocladium spp.	5	1(20)	4(80)
Neosartorya spp.	4	4(100)	------
Anthracosystis	3	3(100)	------
Bipolaris spp.	3	2(66)	1(33)
Sporisorium spp.	2	2(100)	------
Curvularia spp.	1	1	------
Sarocladium spp.	1	1	------
Trichoderma spp.	1	1	------
Humicola spp.	1	1	------
Byssohlamys spp.	1	1	------
Stenocarpella spp.	1	1	------

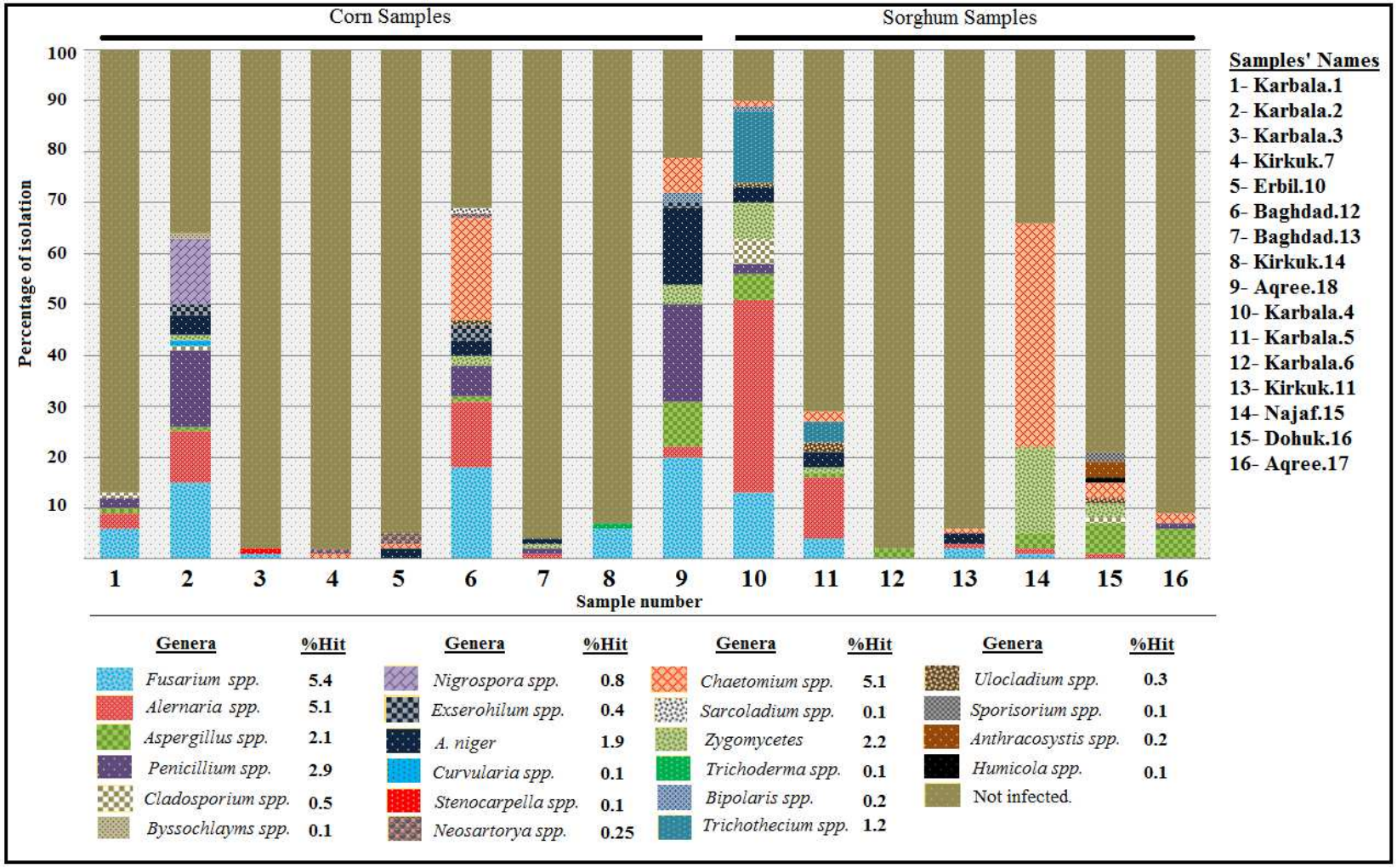

Figure 3: Fungal genera isolated from corn and sorghum seeds and their percentage of isolation.

Chaetomium species are mainly considered to be saprophytic occupants of soil (Domsch & Gams, 1972). Interestingly, a previous report indicated this genus is commonly associated with sunflower seeds in Iraq, but not corn or sorghum (Abdullah & Al-Mosawi, 2010); thus, the high percentage of *Chaetomium spp.* observed in sorghum and corn seeds is novel but not completely unexpected. Comparably, *Alternaria* species also have been found associated with corn seeds in Iraq (Al-Rawi, 2010) and with sorghum seeds at a relatively a high frequency in India along with *Chaetomium spp*. (Sreenivasa et al., 2010).

Penicillium spp. were identified in seven samples (1, 2, 6, 7, 9, 10, and 16), and 93% of isolates were obtained from corn seeds. This finding concurs with a previous study in Iraq, which documented this genus in stored maize grain (Hassan et al., 2014). *Penicillium* has been classified among the most toxigenic fungi that contaminate sorghum grain with mycotoxins (Kange et al., 2015). Zygomycetes (*Rhizopus* and *Mucor*) were also recovered from four corn samples (2, 6, 7, and 9) and four sorghum samples (10, 11, 14, and 15). In a study from Libya, zygomycetes were isolated with relatively high percentages from corn and sorghum grain (Attitalla et al., 2010). Thus, these fungi may be regionally important as contaminants in corn and sorghum in the Middle East.

Aspergillus species were also isolated from the two kinds of seeds at a relatively high percentage. However, 73% of *A. niger* isolates were recovered from corn, which is consistent with results of other studies conducted around the world (Palencia et al., 2010; Tsedaley and Adugna, 2016; Hussain et al., 2013).

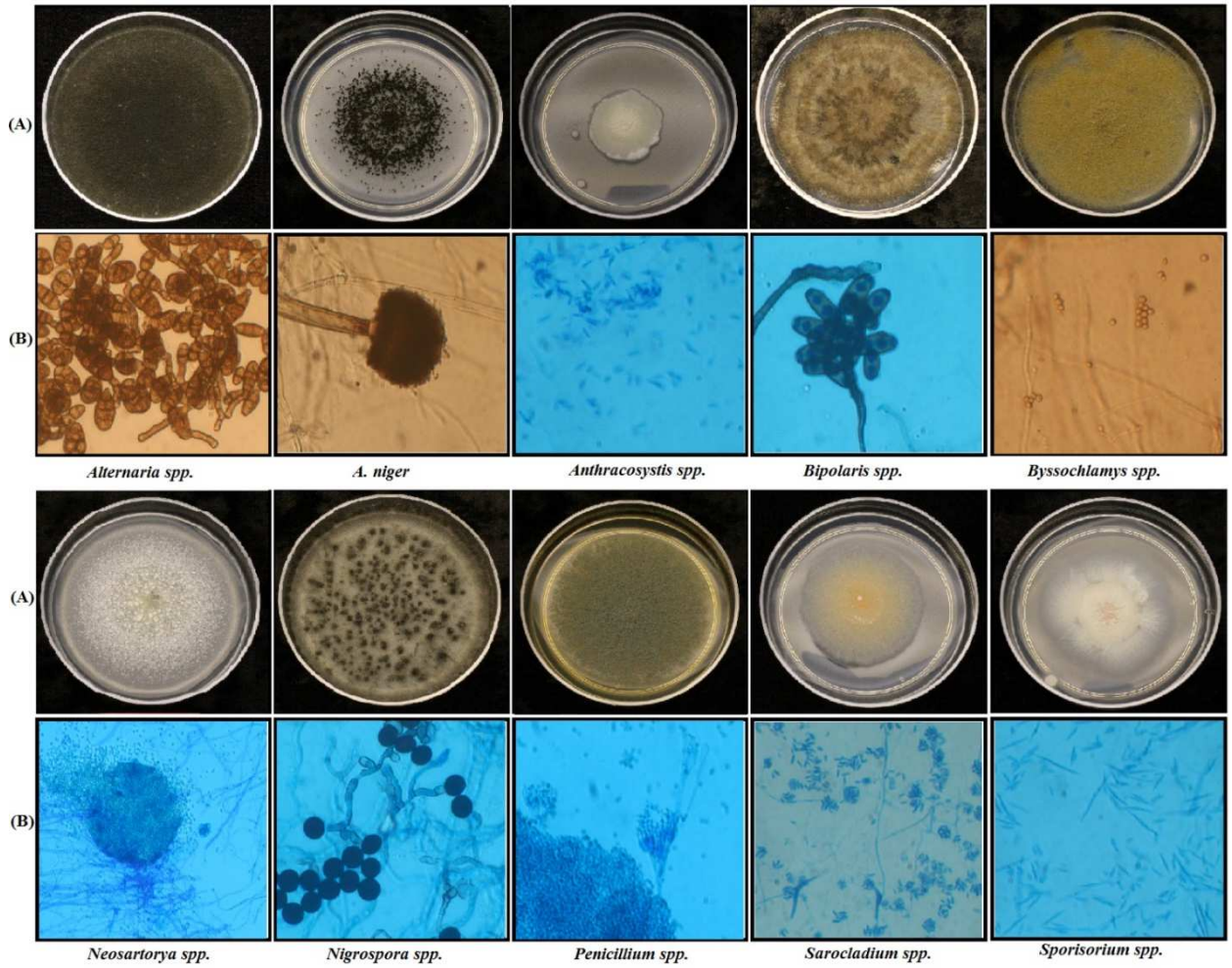

Figure 4: Fungal genera associated with Iraqi corn and sorghum grain. Colony appearance on PDA medium (A). Morphological features shown under 40X magnification (B).

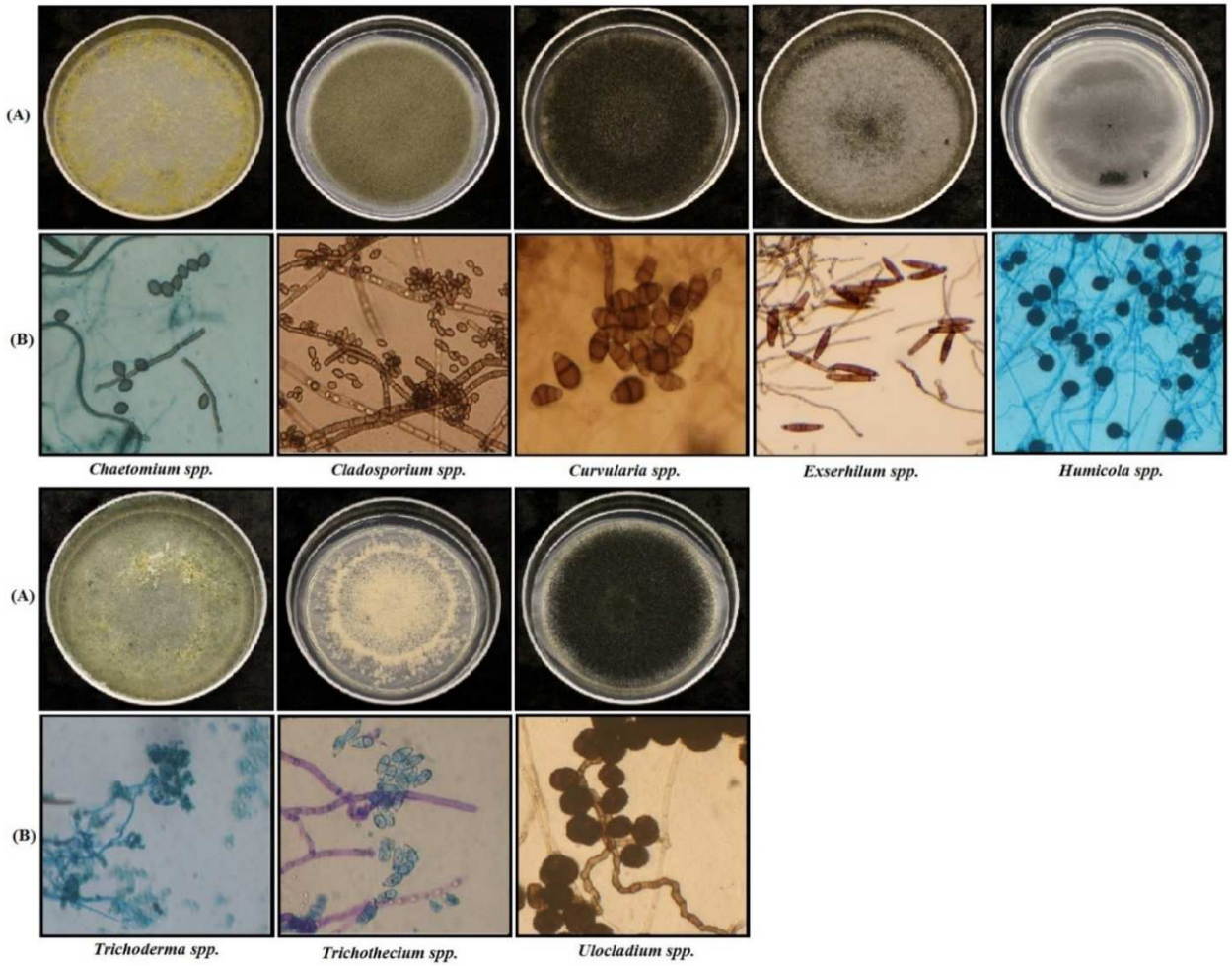

Figure 4 (Cont.): Fungal genera associated with Iraqi corn and sorghum grain. Colony appearance on PDA medium (A). Morphological features shown under 40X magnification (B)

Somewhat surprisingly, all of the *Trichothecium* and *Cladosporium* isolates were obtained from sorghum seeds (Table 3; Figure 2), whereas previous studies indicated that these fungi are commonly associated with corn seeds. However, these genera have been documented in sorghum (Al-Doory & Domson, 1984; Hashmi & Ghaffar, 2006). In contrast, all *Nigrospora* and *Exserohilum* isolates were isolated from corn seeds (Table 3; Figure 2), and *Nigrospora* was exclusively isolated from a single sample. Moreover, *Curvularia spp.*, *Trichoderma spp.*, *Ulocladium spp.*, *Neosartorya spp.*, *Anthracosystis spp.*, *Bipolaris spp.*, *Sporisorium spp.*, *Humicola spp.*, *Byssochlamys spp.*, and *Stenocarpella* spp. were isolated at very low frequencies as a part of the corn-associated mycoflora (Table 3). Although these genera have been linked with diseases on corn and sorghum in various regions of the world, it is pertinent to mention that most of these genera have not been recorded or even referenced as pathogens associated with cereal crops in Iraq.

Curvularia and *Bipolaris* have been reported on maize grains and cause reduced germination (Akonda et al., 2016). In Pakistan, a survey conducted on maize grain identified many genera of fungal pathogens, including *Nigrospora*, *Curvularia*, *Trichoderma*, and *Bipolaris* (Niaz & Dawar, 2009). Also, seed decay and seedling blight in corn caused by *Bipolaris spp*. and *Ulocladium spp*. has been documented in Iraq (Merjan, 2006). Head smut, caused by *Sporisorium spp*., has been reported in many countries and can cause substantial yield losses of corn (Ya et al., 2014). *Humicola spp.*, are well known to be soil-inhabiting fungi (Yu-Lan et al., 2016) and have been isolated, although perhaps not frequently, from corn seeds in preceding studies (Gonzalez et al., 1995). *Byssochlamys* species have been reported as causal organisms of silage deterioration and as potential producers of the mycotoxin patulin. Silage contaminated with *Byssochlamys* threatens animal health directly and human health indirectly

(Driehuis & Dude Elferink, 2000). Likewise, species of *Stenocarpella* have been studied extensively (i.e. *S. maydis* and *S. macrospora*) as they cause Diplodia ear rot of maize and have been documented wherever corn crops are grown (Lamprecht et al., 2011).

Additional to 33 isolates of *A. niger*, *Aspergillus* isolates obtained in this study (35 in total) grouped into six species: *A. flavus* (27 isolates), *A. fumigatus* (2 isolates), *A. amstelodami* (2 isolates), *A. pseudoglaucus* (2 isolates), *A. terreus* (1 isolate), and *A. wentii* (1 isolate) (Figure 5). All *A. flavus* isolates shared similar colony features which included a granular appearance, dense production of spherical conidia with finely to slightly roughened walls, and green to yellowish-green pigmentation (Rodrigues et al., 2007). Also, *A. fumigatus* isolates are well-known in forming velutinous blue-green or dark turquoise colonies and produced uniseriate phialides in a columnar pattern on conidial heads with parallel conidial chains (Zulkifli & Zakari, 2017). *A. amstelodami* isolates were unambiguously identified by their umbonate colonies with a combination of colors: yellowish cleistothecia, dull green conidial heads, and white mycelia at colony margins (Pitt & Hocking, 2007; Gautam & Bhadauria, 2012). Similarly, *A. pseudoglaucus* isolates formed floccose colonies, yellowish sulfur or orangish in color, with pallid to deep green or olivaceous conidia (Chen et al., 2017). *A. terreus* formed tan to brown, velvety colonies; conidial heads were pear-shape and biseriate with a columnar pattern upon vesicle heads (Zulkifli & Zakari, 2017). The colony attributes of *A. wentii* were as mentioned by Pitt and Hocking (2009); mycelia were white to light yellow, floccose in texture, with moderate to dense grayish yellow to orange yellow conidia.

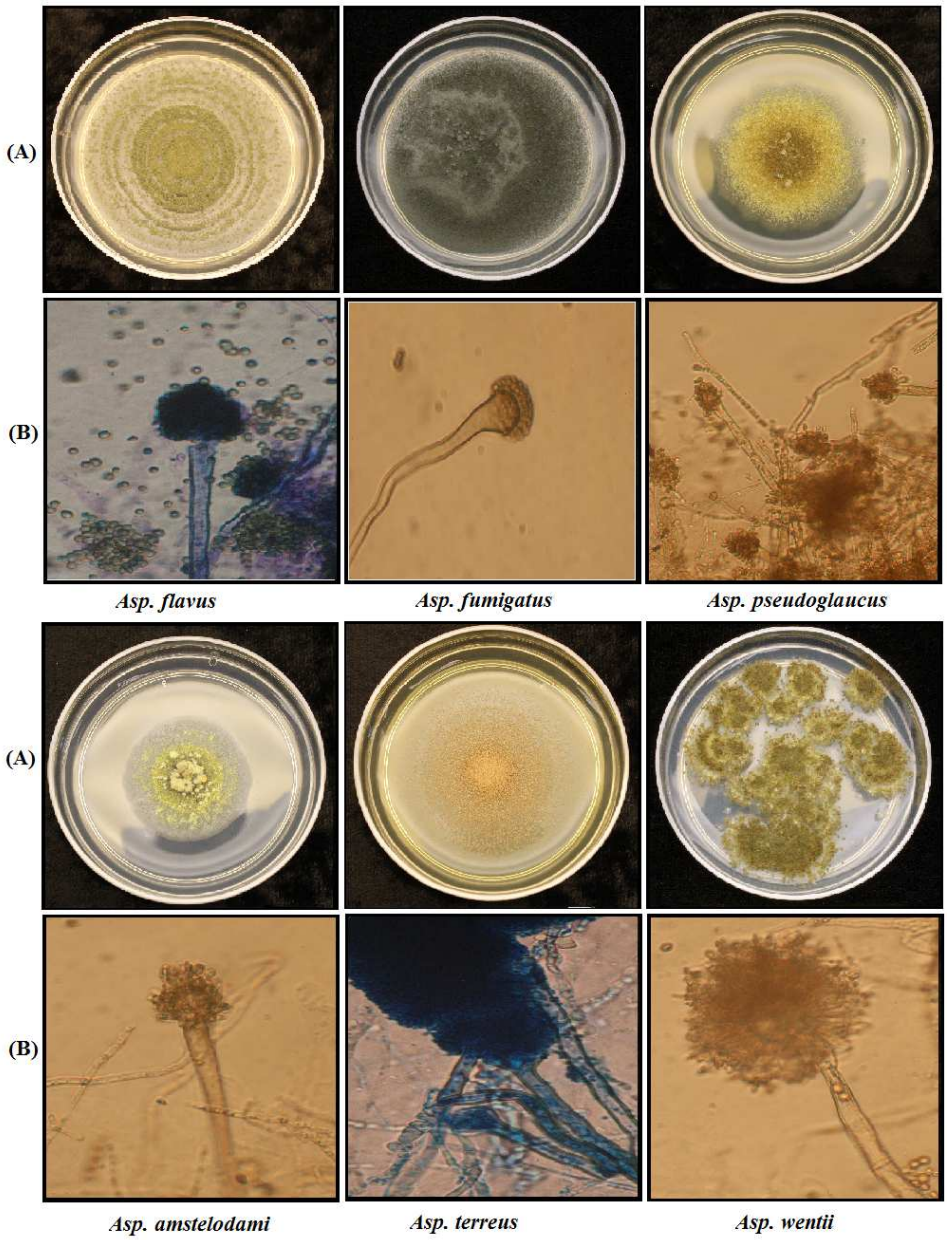

Figure 5: *Aspergillus* species isolated from corn and/or sorghum seeds. Colony appearance on PDA medium (A). Morphological features shown under 40X magnification (B).

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Similarly, 86 *Fusarium* isolates of species were initially identified based on morphological characteristics (Leslie and Summerell, 2006; Pitt and Hocking, 2007), which delineated four species associated with corn and sorghum seeds: 49 isolates of *F. verticillioides*, 21 isolates of *F. incarnatum*, 17 isolates of *F. thapsinum*, and 1 isolate of *F. acuminatum*. The

morphological features of *F. verticillioides* were consistent with the description of Leslie and Summerell (2006): cottony, white mycelia with orange- to violet-gray pigmentation with age; macroconidia not observed; microconidia were abundant, non-septate, oval or club shaped, and arranged in long chains on monophialides or diphialides. The morphological identification of *F. incarnatum* was confirmed by white floccose mycelia turning brown to orange with age, orange and granular sporodochia were formed within mycelia, macroconidia were numerous, elongate with two pointed ends, and septate with 3 to 5 cells. *F. thapsinum* was identified based on colony characteristics and microscopic features, including white, grainy mycelia with abundant microconidia, neither sporodochia nor macroconidia were formed, microconidia were club-shaped with flattened bases. The most distinctive attribute of this fungus was prolific yellow pigmentation in culture media. Finally, the one isolate of *F. acuminatum* was recognized morphologically by its floccose, white to greyish rose or greyish ruby red mycelia, and abundant macroconidia that were crescent-shaped with two acuminated ends and 3-5 septa (Figure 6).

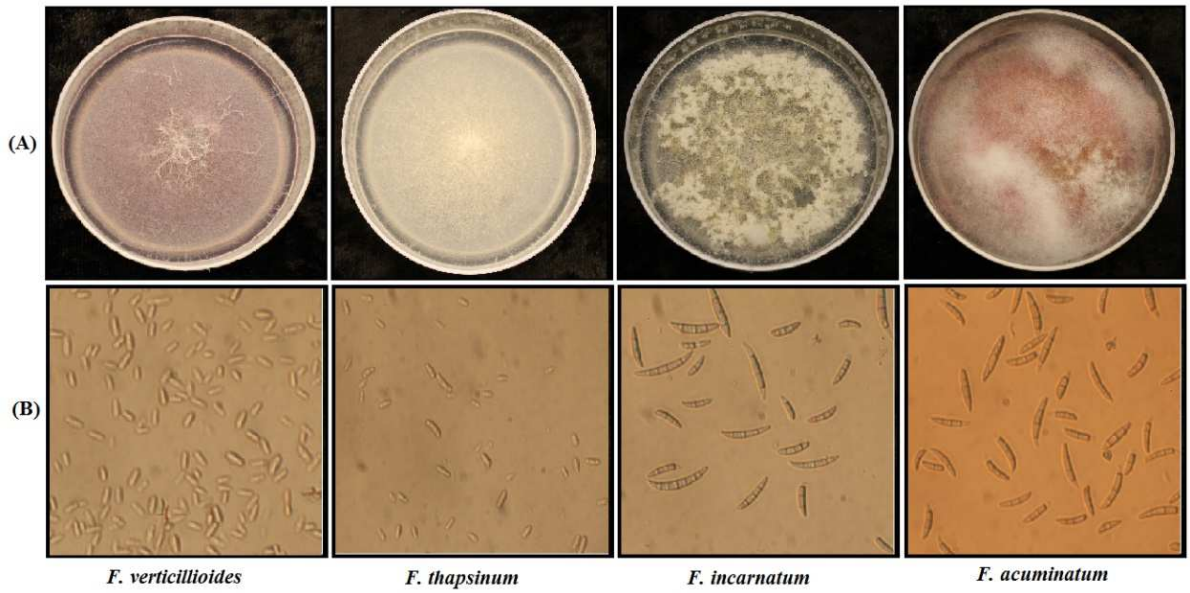

Figure 6: *Fusarium* species isolated from Iraqi corn and/or sorghum seeds. Colony appearance on PDA medium (A). Conidia shown under 40X magnification (B).

PCR is a reliable approach to amplify DNA markers for molecular barcoding and taxonomic identification. In this study, all isolates of *Aspergillus* and *Fusarium* were evaluated with ITS sequence data, which was insufficient for species-level taxonomic resolution even convinced in the terms of species identification and classification with such super speciose organisms i.e. *Aspergillus* and *Fusarium* (Samson et al., 2014; Raja et al., 2017). Sequencing amplicons in one direction with the forward PCR primer of each gene provided sequencing data ranging from 400-800 bp. Homology searches of GenBank via BLAST revealed 97-100% homology between the query and deposited sequences, and consistently corroborated morphology-dependent species-level identifications within each genus.

A neighbor-joining phylogenetic tree constructed from concatenation of *ITS*, *CaM*, *B-tub*, and *RPB-2* sequences grouped all of the *Aspergillus* isolates within five distinct clades (Figure 7). All *A. flavus* isolates (27) grouped with the ex-type strain of *A. flavus* (NRRL 1957) in base clade V. Clades I and II included the only isolates of *A. wentii* and *A. terreus* that grouped with high bootstrap values of 99.7 and 99.5 with reference isolates *A. wentii* NRRL 375 and *A. terreus* NRRL 225, respectively. Similarly, the two isolates of *A. fumigatus* grouped in clade III with bootstrap values of 97.5 and 99.2 with the reference isolate *A. fumigatus* NRRL 163. *A. pseudoglaucus* and *A. amstelodami* grouped together within the same clade (III) with bootstrap values with the reference isolate *A. pseudoglaucus* NRRL 40. This could be explained by the fact that *A. pseudoglaucus* and *A. amstelodami* are basionyms of *Eurotium repens* and *E. amstelodami*, respectively, which belong to the same Section (*Aspergillus*) within *Aspergillus* (Peterson et al., 2008). Findings of this study robustly agreed with previous studies and confirmed that *CaM, B-tub*, and *RPB-2* genes are useful DNA barcoding markers for species

identification within Aspergillus (Samson et al., 2014; Zulkifli & Zakari, 2017; Raja et al., 2017).

Similarly, a neighbor-joining phylogenetic tree constructed from aligning and concatenating ITS*, CMD, EF-1a,* and *H3* loci grouped the *Fusarium* isolates obtained in this study into three major species complexes (Figure 8). The single isolate of *F. acuminatum* grouped into the *Fusarium avenaceum/acuminatum/tricinuctum* species complex (clade I) with the reference strain of *F. acuminatum* (BUF036). All of the *F. icarnatum* isolates (19) grouped with the reference isolate *F. icarnatum* F9, repesenting the *Fusarium incarnatum/equiseti* species complex in sub-clade II*b*1. The third species complex represented in this study (*Gibberella fujikuroi* species complex) localized on sub-clade II*b*2, which was further divided into two branches. Branch *b*2i included all 49 isolates of *F. verticillioides* and the reference isolate *F. verticillioides* (H06A-2A-4). Branch *b*2ii included 17 isolates of *F. thapsinum* and the reference isolate of *F. thapsinum* (A73). and appeared at *b*2ii branch. The nesting of *F. verticillioides* and *F. thapsinum* within the same sub-clade II*b*2 indicates close taxonomic relatedness, as proposed in pervious studies (Leslie et al., 2004; Klittich et al., 1997). Overall, the results were consistent with preceding studies that indicated the four loci utilized in this study for phylogenetic identification are highly informative for intra-species differentiation among *Fusarium* spp. (Steenkamp et al., 1999; Wang et al., 2011).

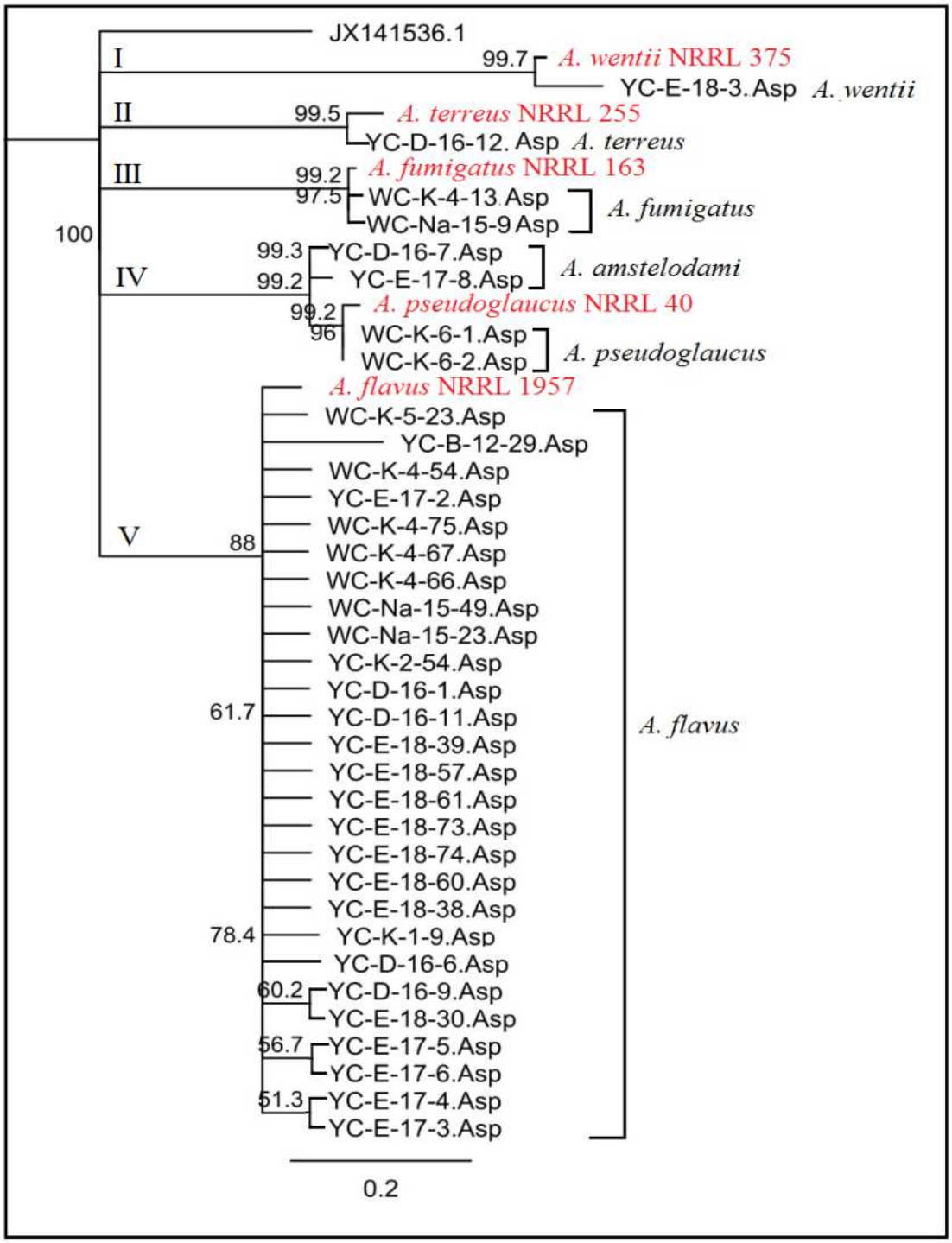

Figure 7: Neighbor-joining tree constructed from concatenated sequences of *ITS*, calmodulin (*CaM*), b- tubulin (*B-tub*), and *RPB-2* genes of *Aspergillus* species. Iraqi samples are in black font, and reference isolates are in red font. Bootstrap values are indicated as percentages based on 1000 replications.

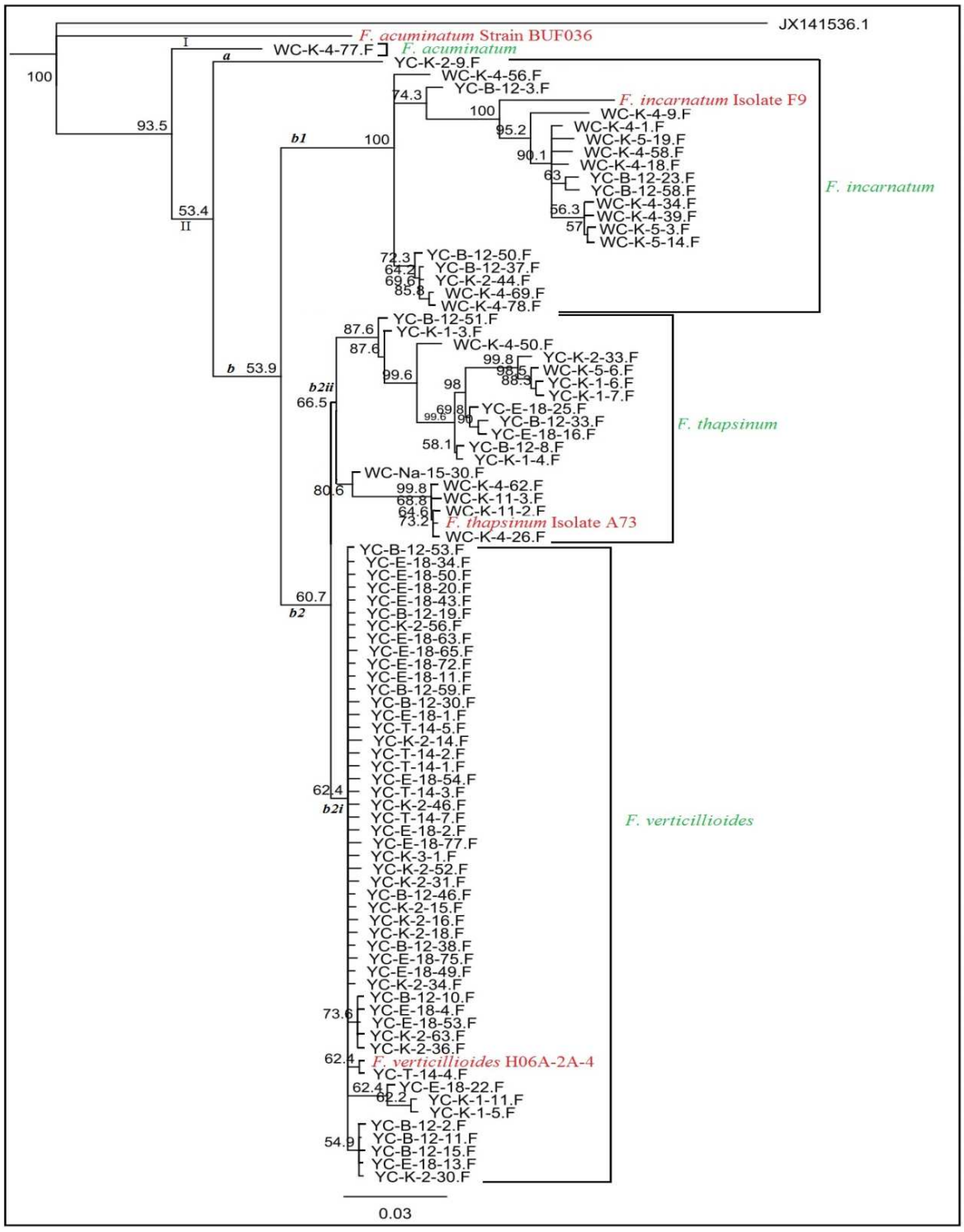

Figure 8: Neighbor-joining tree constructed from concatenated sequences of *ITS*, calmodulin (*CaM*), histone 3 (*H3*), and elongation factor-1α (*EF-1a*) genes of *Fusarium* species. Bootstrap values are indicated as percentages based on 1000 replications.

In conclusion, effort to produce pathogen-free crops through adopting various strategies such as using disease-resistance lines, applying fungicides and pesticides, recruiting a natural bio-control agent to combat pathogenes. however, checking the quality of stored grains i.e. corn and sorghum grains routinely as yet is considered as a precaution step precedes the food industries processes the identification of seed-borne fungi depending on the colony's morphology and microscopic characteristics is an essential procedure and intrinsically a daunting task since it needs high quality of expertise, long-term training, laborius, and time consuming; yet the combination between the morphology-dependent approaches and revolutionary DNA-based strategies have ushered into a new era of high resolution-identification of fungal pathogens and deeper vision in fungal taxonomy. Harboring such pathogens e.g. *Aspergillus spp*., *Fusarium spp*. and *Penicillium spp*. in grains alludes to more health complications in humans and animals as some species of these pathogens are well known as mycotoxigenic.

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Chapter 3: The mycotoxigenic potential of *Aspergillus flavus* and four *Fusarium* species isolated from corn and sorghum grain from Iraq

Abstract

Corn and sorghum consistently face the threat of infection by mycotoxigenic fungi, particularly aflatoxigenic and fumonigenic species. Biological control of mycotoxin contamination via application of atoxigenic strains has proven effective, and has been optimized regionally, in some areas of the world. However, biological control of mycotoxigenic fungi has not yet been attempted in Iraq. The goal of this study was to identify atoxigenic strains of mycotoxigenic species of *Aspergillus* and *Fusarium* from Iraqi corn and sorghum samples. To accomplish this goal, an LC-MS/MS assay was optimized to simultaneously quantify fumonisins, aflatoxin, and ergosterol (a fungal membrane sterol that allows quantification of fungal biomass). LC-MS/MS analysis revealed that 15 of 18 Iraqi grain samples evaluated were contaminated with various levels of FB_1 (max. 8.6 µg/gm), but none of the samples contained quantifiable levels of AFB_1. A total of 113 isolates of *Aspergillus flavus* and *Fusarium spp*. obtained from Iraqi grain samples were evaluated on cracked corn kernel medium to evaluate their mycotoxigenic potential. Three isolates of *A. flavus* were atoxigenic (A-1-9, A-4-54, and A-4-75), whereas the rest produced AFB_1 from low (0.2-1.0 µg/gm) to high levels (36-2174 µg/gm). In contrast, no atoxigenic *Fusarium* isolates were identified. *F. verticillioides* isolates averaged the highest levels of FB_1 production (1707 µg/gm), followed by *F. thapsinum* (789 µg/gm), *F. incarnatum* (223 µg/gm), and *F. acuminatum* (32 µg/gm). In addition to providing a novel LC-MS/MS technique to simultaneously quantify aflatoxins, fumonisins, and ergosterol, this study identified three atoxigenic isolates of *Aspergillus flavus* that could potentially be deployed as biological control agents to mitigate mycotoxin contamination of grain in Iraq.

Introduction

Worldwide, corn and sorghum face constant attack by fungal pathogens and consequently are at risk of being contaminated with mycotoxins. Mycotoxins are secondary metabolites produced by a wide range of toxigenic fungi and are associated with numerous health disorders in humans and animals (Galvano & Ritieni, 2005). Two of the most important categories of mycotoxins are aflatoxins and fumonisins, which are primarily produced by species of *Aspergillus* and *Fusarium*. The four major types of aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2) are mainly produced by two well-known species: *A. flavus* Link and *A. parasiticus* Speare. The toxicity of AFB_1 is well established (Peraica et al., 1999). Aflatoxins have been reported as contaminants of many cereal crops (e.g. corn, cotton, and barley) and other agricultural commodities as well (e.g., tomato, pumpkin, and peanuts) (Sahar et al., 2009). Similarly, fumonisins affect a wide variety of important crops and have caused economic losses in the U.S. ranging from $10-40 million USD in epidemic years (Wu, 2007). There are three main fumonisin analogs (FB_1, FB_2, and FB_3), and they are primarily produced by *F. verticillioides* (Sacc.) Nirenberg, and *F. proliferatum* (Matsushima) Nirenberg. Along with suppressing immune responses, fumonisins have been linked to numerous human and animal health concerns (Voss et al., 2009).

Many factors can influence mycotoxin contamination of crops. Influencing factors include environmental conditions in the field and/or storage, insect damage, and exposure of developing crops to heat and drought (Payne, 1992; Atanda et al., 2011). Many of these factors are difficult to control, and thus careful monitoring of mycotoxin levels in grain is required. Relatedly, many countries have strict limits for mycotoxins in cereal grains and derived products destined for human or animal consumption (FAO, 2004).

Corn and sorghum are susceptible to infection by fumonigenic and aflatoxgenic fungi, and many cases of such infections have been documented around the world (Leslie and Summerell, 2006). In 37 corn samples collected from two regions of Argentina, *Fusarium* and *Aspergillus* were the most frequently isolated pathogens, and relatively high levels of aflatoxin were detected (Camiletti et al., 2016). Comparably, *Aspergillus* and *Fusarium* species were frequently associated with maize kernels collected from the highlands of Guatemala (Mendoza et al., 2017). Although some studies have suggested that sorghum is less susceptible to mycotoxigenic fungi, reports exist of aflatoxin being detected in sorghum grain (Bandyopadhyay et al., 2006; Ayalew et al., 2006).

Globally, a considerable amount of research effort has focused on mycotoxins in corn because of the diversity and importance of corn-based industries (food, feed, by-products, and biofuel) (Edgerton, 2009). Comparatively less focus has been placed on sorghum, as it represents a substantially lesser, yet still significant, percentage of world cereal production (Van Rensburg, 2012). In this context, many analytical techniques have been developed to identify and quantify mycotoxins in a wide range of matrices (Shepard, 2008). However, there are no published reports of analytical protocols that simultaneously quantify multiple mycotoxins and biomarkers associated with fungal growth, such as ergosterol.

In the previous chapter, diverse species of *Aspergillus* and *Fusarium* were found to be associated with corn and sorghum in Iraq. In this chapter, isolates of *Aspergillus flavus* and *Fusarium spp.* described in Chapter 2 were evaluated for their mycotoxigenic potential. To accomplish this goal, a novel LC-MS/MS protocol was developed to simultaneously quantify aflatoxins, fumonisins, and ergosterol.

Materials and methods

Optimization of mycotoxin extraction and analysis

Overview of analytical approach:

Conventional methods employed for mycotoxin analyses (e.g. HPLC and LC-MS) are generally optimized for only one class of toxin. Because of the frequent co-occurrence of multiple mycotoxins in the same matrix, developing extraction and analysis protocols that facilitate the simultaneous quantification of multiple categories of mycotoxins is a high priority (Sadhasivam et al., 2017). For the protocol developed in this study, the goal was to develop a robust method to simultaneously quantify aflatoxins, fumonisins, and ergosterol in cereal grain samples.

Preparation of analytical standards:

An analytical standard mixture containing 100 ppm each of aflatoxin-B_1, fumonisin-B_1, and ergosterol (Sigma-Aldrich, St. Louis, MO) was prepared in absolute methanol and serially diluted to individual stocks of 10, 1, and 0.1 ppm. All analytical standards were stored at -20ºC until use.

Optimizing mycotoxin extraction parameters:

Mycotoxin-spiked corn samples were prepared by homogenizing corn kernels (0.5 gm; previously sterilized and toxin-free) with 50 µL of mixed toxin stock (100 ppm). Four extraction solvents were evaluated (2 mL per scintillation vial): methanol, methanol:water (80:20 v/v), chloroform, and acetonitrile:water (50:50 v/v). For each extraction solvent tested, four scintillation vials containing spiked corn were evaluated, with a fifth vial was treated with sterile diH_2O serving as the control. After adding extraction solvent, vials were shaken overnight at 180-200 rpm with an orbital shaker (Lab-Line instruments Inc., Melrose Park, IL). The next day,

vials were allowed to settle, and 1 mL from each vial was passed through a 0.22 µm nylon filter (Membrane Solutions Company, Aubum, WA). All extracts were stored in auto-sampler vials at -20ºC until analysis.

LC-MS/MS setting and analysis:

LC-MS/MS analysis was performed with a Shimadzu UPLC-20A/LC-30A and a Shimadzu 8060 triple quadruple mass spectrometer with a heated electrospray source in positive ion mode (Shimadzu, Kyoto, Japan). Chromatographic separation was performed with a C18 column (2.1 x 50 mm, 1.9 µm particle size, Shimadzu UHPL check out kit) with a linear gradient comprised of 0.1% formic acid (FA) in HPLC grade water/ 0.1% FA in methanol ramped at a rate of 8% methanol/min over 5 min and then 2% methanol/min over 5 min. the flow rate was 0.3 mL/min. Sample volume of 1 µL were injected. In the collision compartment or second quadruple (Q2), the collisionally induced dissociation (CID) was conducted by utilizing argon gas at a pressure of 270kPa. To detect and determine each analyte or precursor ion the most frequent product ions were selected and measured. Two product ions (reference) were chosen for identification and most abundant fragment ion was used for quantification. Unknown samples were required to be within 30% of the reference ion ratio of the standard (Diaz Perez et al., 2019).

Assessment the mycotoxin-content of corn and sorghum samples

Moisture content determination:

The moisture content of corn and sorghum samples was determined according to Alrawi (2011) by transferring 15 gm of grain from each sample to glass dishes (weights recorded empty and full). Dishes containing grain were incubated at 105ºC for 1h in a drying oven, and weights

were recorded. Sample heating and weighing was repeated every 15 minutes until weights stabilized. The percentage moisture content (MC) was calculated as follows:

$$MC\% = \frac{Loss\ in\ wieght\ (gm)}{Original\ wieght\ of\ sample\ (gm)} \text{ X } 100$$

Preparing samples for extraction:

An aliquot of 20 gm from each Iraqi grain sample was ground into fine powder with a Tekmar A-10 analytical mill grinder at 20000 RPM (Tekmar Company, Cincinnati, OH). Ground samples were stored in plastic bags at 4°C until analysis.

Mycotoxin extraction and LC-MS/MS analysis:

Based on optimization of extraction solvents and conditions, ground samples were extracted with 2 mL of 100% methanol per 0.5 gm ground corn. The remaining extraction steps and parameters for LC-MS/MS analysis were as described above.

Differentiation between toxigenic and atoxigenic isolates

Preparation of cracked corn kernel medium:

Scintillation vials containing cracked corn kernel medium were individually inoculated with 0.5 mL of spore suspension (1 x 10^6 spore/mL) from 27 isolates of *A. flavus* and 86 isolates of *Fusarium spp.*, and incubated at room temperature for 14 days. During the first two days of incubation, vials were periodically shaken to ensure thorough dissemination of fungal inoculum. After 14 days, inoculated kernels were ground in a pre-chilled mortar with liquid nitrogen. Ground samples were stored at -20 ^{0}C until time of extraction. Each isolate was assigned an ID code consisting of A for *Aspergillus* and F for *Fusarium*, followed by a number indicating the sample origin as stated in the previous chapter, and finally a number representing the isolate's order within each sample.

Results and discussion

Optimization of mycotoxin extraction and analysis:

As analytical instrumentation continually improves, more sensitive, accurate, and cost-effective protocols are evolving to measure mycotoxins in food and feed (De Santis et al., 2017). Effective extraction methods to purify target analytes are a crucial step, and oftentimes challenge, in mycotoxin analysis due to the complexity of matrices and interferences requiring elimination. In this context, developing a reliable extraction protocol to determine the co-occurrence of aflatoxins and fumonisins is an important aspect to consider. The quantifier ion and qualifier ions (ions defragmented from the target compound in the mass spectrometry analysis) derived from the precursor ions of AFB_1 and FB_1 during LC-MS/MS in MRM mode are shown below (Table 1; Figure 1).

Table 1: UPLC-MS/MS analysis parameters.

Analyte	Precursor ion (m/z)[1]	Quantifier(qualifier) ion (m/z)	Std. conc.(ppm)	LOD[2] (ppm)	R.T [3] (Min)
AFB_1	313.0707[M+H][4]	241.09 (285.08)	0.1,1.0,10,100	0.1,1.0,10,100	2.51
FB_1	722.3957[M+H]	704.4 (352.3)	0.1,1.0,10,100	0.1,1.0,10,100	4.32

(1) m/z: An expression represents mass divided by the charge number of the parent ion in the MS.
(2) LOD: Is the lowest concentration of the target analyte that can be detected in the MS.
(3) R.T: Retention time.
(4) Protonated ion.

Evaluating different organic solvents in spiked corn samples revealed that absolute methanol (100%) had the most efficient recovery of AFB_1 and FB_1 (84.2 and 77.4%, respectively) compared to other solvents (Table 2). These outcomes could be explained by the fact that aflatoxins (B_1, B_2, G_1, and G_2) and fumonisins (B_1, B_2, and B_3) are highly soluble in methanol, but not as soluble in chloroform and acetonitrile-based solvents (Zhang et al., 2018).

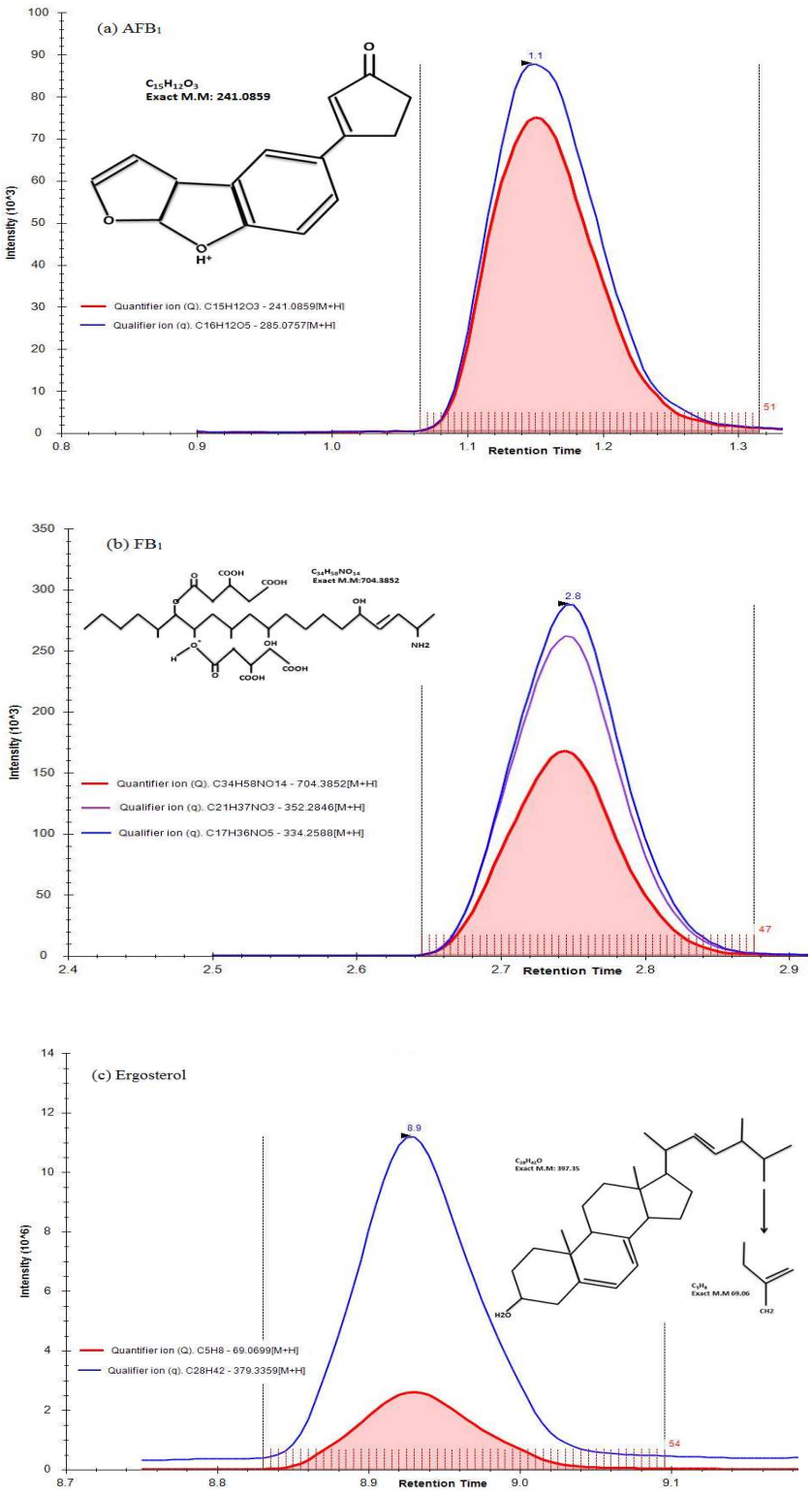

Figure 1: UPLC-MS/MS MRM chromatograms of parameters in the mycotoxins profile screen.

Table 2: Efficacy of different organic solvents for mycotoxin extraction.

Solvent	Recovered toxin out of 2.5 ppm	
	AFB_1 (ppm)	**FB_1 (ppm)**
Chloroform	1.56	0.013
Methanol	2.105	1.936
MeOH 80%	1.38	1.57
ACN:H_2O	0.141	1.568
Control	ND*	ND

*ND: Not Detected.

Assessment the mycotoxin-content of corn and sorghum samples:

Moisture content (MC) of grain has a crucial role in post-harvest grain quality. Many studies have confirmed that post-harvest mismanagement of moisture content can support growth and metabolism of mycotoxigenic fungi (Pardo et al., 2004) and insects (Mendoza et al., 2017). The moisture content of 18 Iraqi samples (11 corn and 7 sorghum) ranged from 1.5 (in Najaf-15^S) to 4.1 (in RC Erbil-10^{YC}) (Table 3). These low values are below the threshold required to support active fungal growth, and are somewhat remarkable considering that corn and sorghum farmers in Iraq mostly use sunlight to dry grain inasmuch as the weather is dry and hot during the whole planting season of these two crops (July through October) (W.F.P, 2019).

Regarding the mycotoxin profile of Iraqi corn and sorghum grain (18 samples), fumonisin-B1 was detected at various concentrations in 12 samples, with levels in corn samples generally higher than in sorghum samples. The highest levels of FB_1 (8.6 and 3.3 µg/gm) were found in Baghdad-12^{YC} and Aqree-18^{YC} samples respectively, which corresponds with the two highest number of *Fusarium* isolates (16 and 20 alternatively) among the 18 samples (Figure 3). Moreover, three other samples (Karbala-1^{YC}, Karbala-5^S, and Kirkuk-11^S) contained very low

concentrations of fumonisin-B1 (≤ 4 ng/gm), whereas the remaining samples (Karbala-4 ^{S}, Karbala-6 ^{S}, and RC Erbil-10^{YC}) did not contain detectable levels of fumonisin-B_1. These results are consistent with the findings of Ayalew et al. (2006) who stated that sorghum is less susceptible to mycotoxin contamination than other crops.

Interestingly, all the corn and sorghum samples were free of aflatoxin-B_1, which is unusual considering that 27 *A. flavus* isolates were obtained from these samples. This observation may result from the competition between *A. flavus* and other species during pre-harvest crop development as other organisms, such as *Aspergillus niger*, *Cladosporium spp*., and *Alternaria spp*. associated with the same samples. Some strains of these organisms have been shown to behave antagonistically against toxigenic *A. flavus* strains (Cvetni and Pepeljnjak, 2007). Hence, other fungi collected in this study might potentially restrict and/or inhibit the growth and/or toxin production by *A. flavus*. Moreover, the low percentages of moisture content (2.5-4%) of the samples evaluated in this study lowers the water activity (a_w), which directly suppresses growth of mycotoxigenic fungi.

Although 83% of samples (15 out of 18) were contaminated with fumonisin-B1, values of the toxin were generally within allowable limits for human consumption (2 - 4 ppm) (Azizi & Rouhi, 2013). This could possibly be explained by the relatively low occurrence of *F. verticillioides* in the samples (Figure 2). The low moisture content of the samples could explain why all samples were free of AFB_1 and contaminated with only low levels of FB_1 (except Baghdad-12^{YC} and Aqree-18^{YC}) due to the fact that storing starchy cereal crops at low moisture content (≤ 14) strongly suppresses fungal growth (Sweets , 2020; Tsurta, 1987).

Table 3: Mycotoxin content (AFB_1 & FB_1), ergosterol, and moisture content (MC) in Iraqi corn and sorghum samples.

Region	Samples	AFB_1 (µg/gm)	FB_1 (µg/gm)	Ergost (µg/gm)	% (MC)
North	**RC Erbil-8^{YC}**	**ND**	0.11	0.01	2.5
	RC Erbil-9 ^{YC}	**ND**	0.01	0.00*	2.8
	RC Erbil-10^{YC}	**ND**	**ND**	0.00*	4.1
	Dohuk-16 ^{S}	**ND**	0.01	0.2	2.3
	Aqree-17 ^{S}	**ND**	0.01	0.1	3.3
	Aqree-18 ^{YC}	**ND**	3.3	0.5	2.3
Middle	**Kirkuk-7^{YC}**	**ND**	0.01	**ND**	2.6
	Kirkuk-11 ^{S}	**ND**	0.00*	0.6	3.0
	Kirkuk-14 ^{YC}	**ND**	0.5	0.4	3.1
	Baghdad-12^{YC}	**ND**	8.6	0.3	4.0
	Baghdad-13^{YC}	**ND**	0.01	0.02	2.5
South	**Karbala-1^{YC}**	**ND**	0.00*	0.02	3.0
	Karbala-2^{YC}	**ND**	0.7	0.3	3.3
	Karbala-3^{YC}	**ND**	0.2	0.7	3.0
	Karbala-4 ^{S}	**ND**	**ND**	2.3	3.5
	Karbala-5 ^{S}	**ND**	0.00*	0.4	3.0
	Karbala-6 ^{S}	**ND**	**ND**	0.1	3.0
	Najaf-15 ^{S}	**ND**	0.04	2.5	1.5

*****The Products have been detected in very low concentrations (≤4ng).
YC stands for yellow corn. **S** stands for sorghum. **ND:** Not detected.

Differentiation between toxigenic and atoxigenic isolates

A. flavus isolates:

LC-MS/MS analysis of the 27 *A. flavus* isolates revealed that 88% (24 of 27) were aflatoxin-B1 producers, and only three isolates were atoxigenic (A-1-9, A-4-54, and A-4-75) (Table 4). Three mycotoxigenic phenotypes were observed: atoxigenicity, represented by three isolates (mentioned above), low levels of toxigenicity, represented by seven isolates (A-5-23, A-7-2, A-17-3, A-17-4, A-17-5, A-17-6, and A-18-61), and high levels of toxigenicity, represented by the remaining 17 isolates.

Table 4: Aflatoxin-B1 and ergosterol production by *A. flavus* isolates.

A flavus code ID	AFB1 productivity		Ergosterol productivity	
	Conc. (µg/gm)	RT**	Conc. (µg/gm)	RT
nA-1-9	**ND***	-----	2.9	8.516
tA-2-54	824	1.623	2.7	8.509
nA-4-54	**ND***	------	5.9	8.509
tA-4-66	402	1.628	1.9	8.511
tA-4-67	358	1.628	8.7	8.516
nA-4-75	**ND***	------	2.8	8.506
tA-5-23	0.3	1.621	2.8	8.507
tA-12-29	822	1.623	2.6	8.505
tA-15-23	318	1.626	2.8	8.509
tA-15-49	2174	1.628	1.5	8.508
tA-16-1	36	1.626	2.5	8.513
tA-16-6	176	1.628	1.3	8.509
tA-16-9	2142	1.627	2.3	8.511
tA-16-11	938	1.624	1.9	8.503
tA-17-2	0.4	1.623	3.7	8.509
tA-17-3	0.3	1.627	2.4	8.509
tA-17-4	0.2	1.625	9.6	8.516
tA-17-5	0.2	1.634	7.1	8.508
tA-17-6	0.2	1.623	1.7	8.510
tA-18-30	76	1.626	2.2	8.503
tA-18-38	113	1.631	3	8.516
tA-18-39	818	1.627	2.4	8.509
tA-18-57	340	1.632	1.7	8.516
tA-18-60	218	1.625	4.7	8.505
tA-18-61	1	1.626	2.8	8.507
tA-18-73	312	1.633	1.6	8.514
tA-18-74	296	1.627	1.8	8.508

ND: Not detected. **n:** atoxigenic. **t:** toxigenic. **RT:** Retention time.

Variations among *A. flavus* isolates in terms of aflatoxin-B1 production were striking (Figure 4), as low amounts of AFB_1 (0.2-1.0 µg/gm) were produced by isolates associated with Karbala-5, Aqree-17, and Aqree-18 samples (A-5-23, A-7-2, A-17-3, A-17-4, A-17-5, A-17-6, and A-18-61). Substantially higher production of AFB_1 (36-2174 µg/gm) was distributed within other toxigenic isolates, with the highest productivity recorded by the A-15-49 isolate (2174 µg/gm).

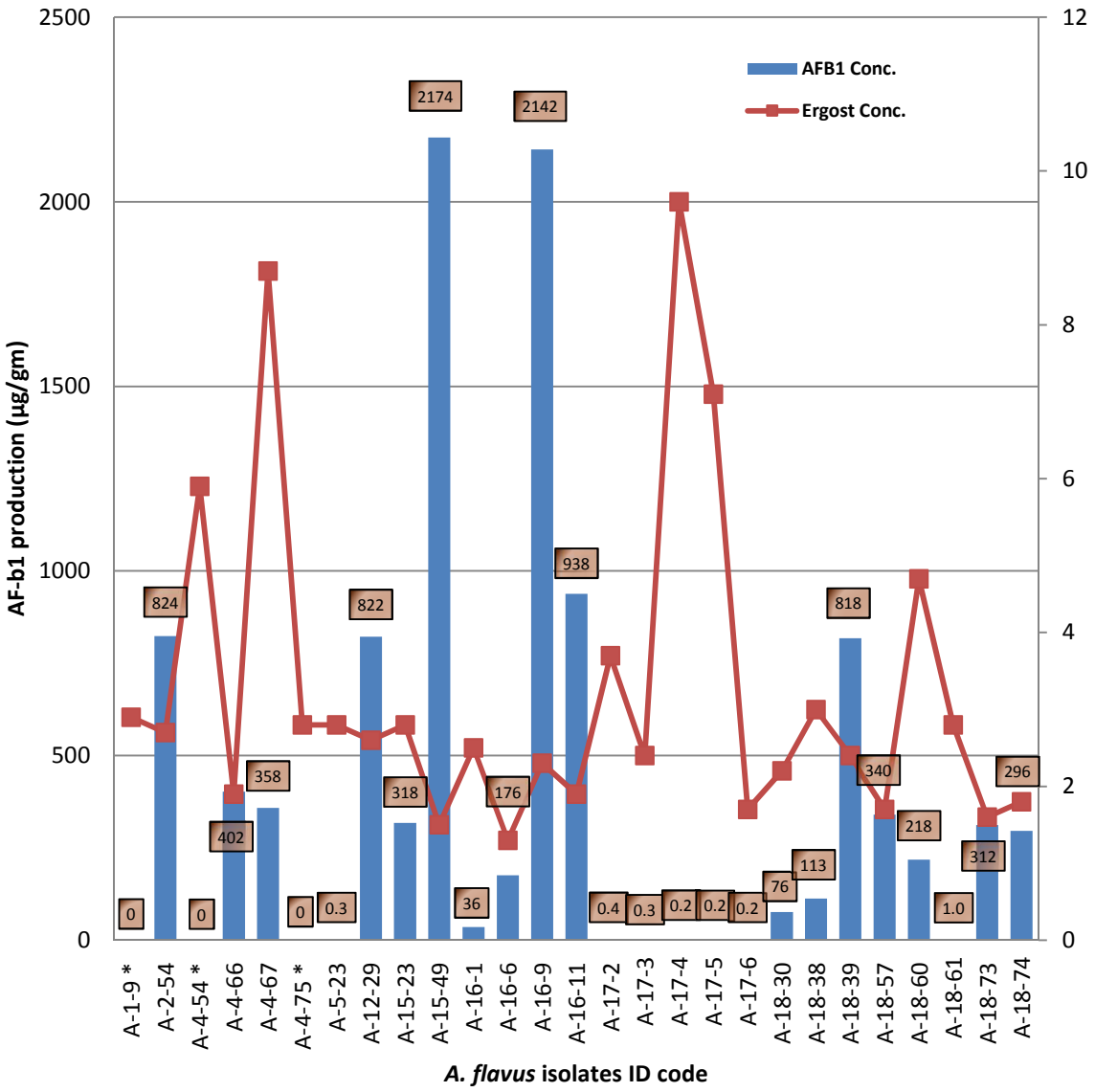

Figure 2: Aflatoxin-B_1 and ergosterol production by *A. flavus* isolates obtained from Iraqi corn and sorghum seed.

Remarkably, an *A. parasiticus* isolates was not recovered in this study. However, *A. flavus* was the *Aspergillus* species most frequently isolated. This observation is consistent with findings by Pitt and Hocking (2004) that *A. parasiticus* is less prevalent than *A. flavus*. Also, Horn et al. (2014) mentioned that *A. flavus* is more frequently isolated from cereal crops such as corn and cotton seeds, whereas *A. parasiticus* is more prevalent in ground crops, such as peanuts.

Variances in AFB_1 productivity within the toxigenic phenotype could be explained by morphotype, isolate-specific requirements for nutritional or environmental conditions, or other cryptic factors (Probst et al., 2010; Payne, 2016; Mannaa & Kim, 2017).

The capability of *A. flavus* to synthesize aflatoxins (B_1 and B_2) genetically maps to a 75kb region of chromosome III that encompasses at least 30 clustered genes directly involved in aflatoxin formation (Li & He, 2018). Having two categories of *A. flavus* in terms of aflatoxin production (i.e. producers and non-producers) implies that aflatoxin producing strains possess all the genes of the aflatoxin cluster. Many studies have dissected the molecular basis of aflatoxin biosynthesis (Yu et al., 1995; Yu et al., 2004). Conversely, the existence of atoxigenicity suggests that naturally occurring genotypic alterations block aflatoxin biosynthesis. The inability of *A. flavus* isolates to synthesize aflatoxin has been documented extensively and has been found to be caused by deletion of the entire gene cluster, deletion of crucial genes within the cluster, or single nucleotide polymorphisms within the aflatoxin cluster genes (Adhikari et al., 2016).

***Fusarium* isolates:**

Among plant pathogenic fungi, *Fusarium* has received extensive attention in terms of taxonomic delimitation and mycotoxigenesis, as they cause extensive economic losses during pre- and post-harvest conditions (Nelson et al., 1992). *Fusarium* pathogenicity is often compounded by the production of fumonisins (Aiyaz et al., 2016). The biosynthesis of fumonisins is regulated by an 80 kb region of chromosome I; this region contains 23 genes encoding essential enzymes for fumonisin biosynthesis (Alexander et al., 2009). The approach of inoculating cracked corn kernel medium to differentiate toxigenic and atoxigenic isolates of *Fusarium* in terms of fumonisin-B_1 production was effective and efficient. LC-MS/MC analysis indicated that all four *Fusarium* species (*F. verticillioides*, *F. acuminatum*, *F. thapsinum*, and *F.*

incarnatum) were able to produce FB_1. However, huge disparities in mean FB_1 production were recorded between the four species and even within each taxon. The descending order of mean production values were 1707, 789, 223, and 32 µg/gm of FB_1 produced by *F. verticillioides*, *F. thapsinum*, *F. incarnatum*, and *F. acuminatum*, respectively. Notably, *F. verticillioides* was not isolated from any sorghum samples (Figure 3), possibly due to higher levels of innate genetic resistance (Bhat et al., 1997; Bandyopadhyay et al., 2006). *F. verticillioides* predominated among *Fusarium* isolates (56.9%), and it is not surprising that FB_1 production by this species was considerably higher than other species (ranging from 17-7593 µg/gm) (Figure 4). Within the *Fusarium fujikuroi* species complex, *F. verticillioides* is one of the most important producers of fumonisins toxins, as this species is globally distributed, colonizes corn frequently, and often produces high level of fumonisins (Ross et al., 1992). Not only in Iraq, but also worldwide, previous researches have reported this species to be consistently associated with corn (Placinta et al., 1999; Jabbar et al., 2015; Fallahi et al., 2019).

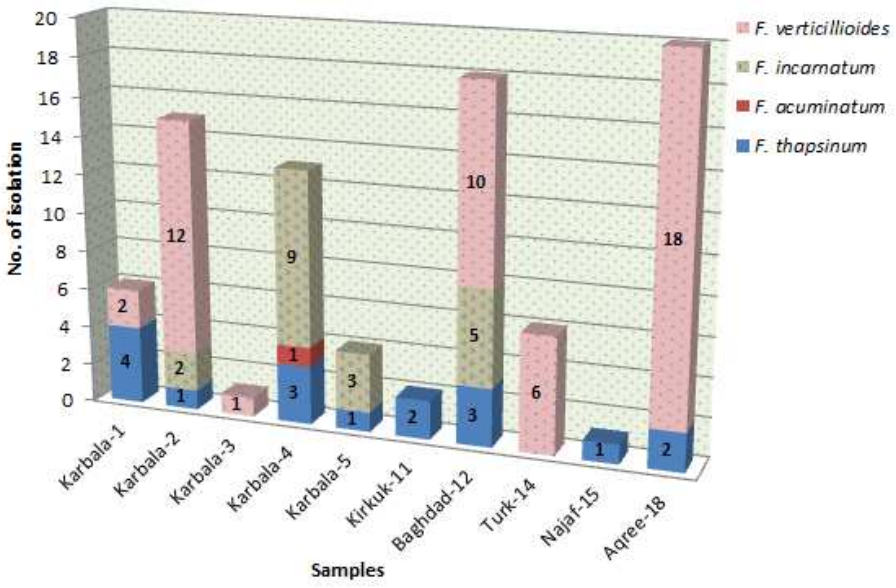

Figure 3: Distribution of *Fusarium* species isolated from corn and sorghum seeds

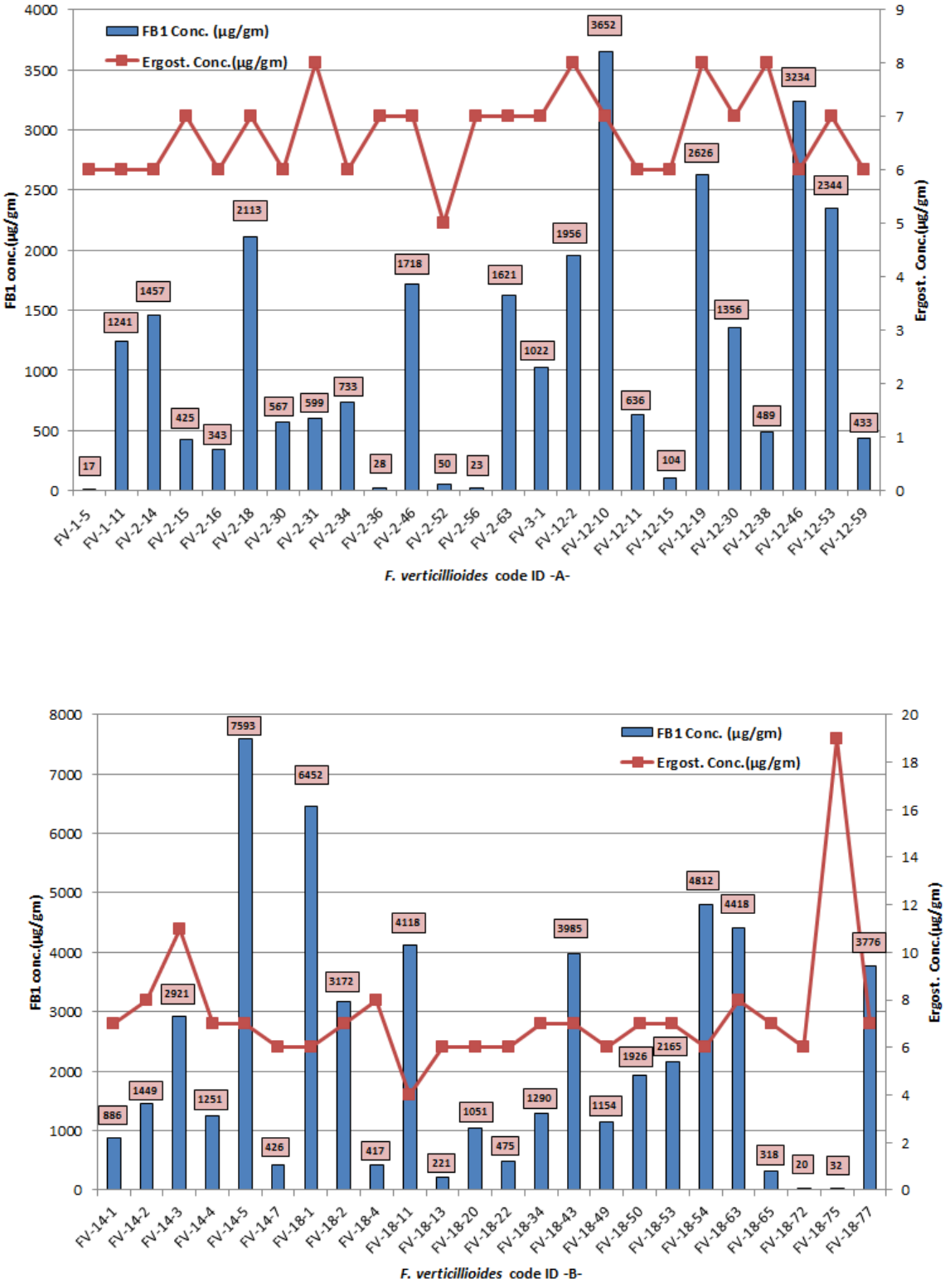

Figure 4: Fumonisin-B_1 and ergosterol production by *F. verticillioides* (batch A & B) isolated from Iraqi corn and sorghum seeds.

F. thapsinum isolates produced FB_1 at levels ranging from 23 to 4013 µg/gm, with a relatively wide variance in the toxin production among isolates (Figure 5). This species was isolated from four samples of corn and sorghum with similar frequencies. Even though this species is well known as a moniliformin (MON) producer on sorghum grain, this study indicated that *F. thapsinum* also produces FB_1. This finding was consistent with previous studies listing *F. thapsinum* among fifteen known *Fusarium* species that produce fumonisins (Rheeder et al., 2002; Glenn, 2007).

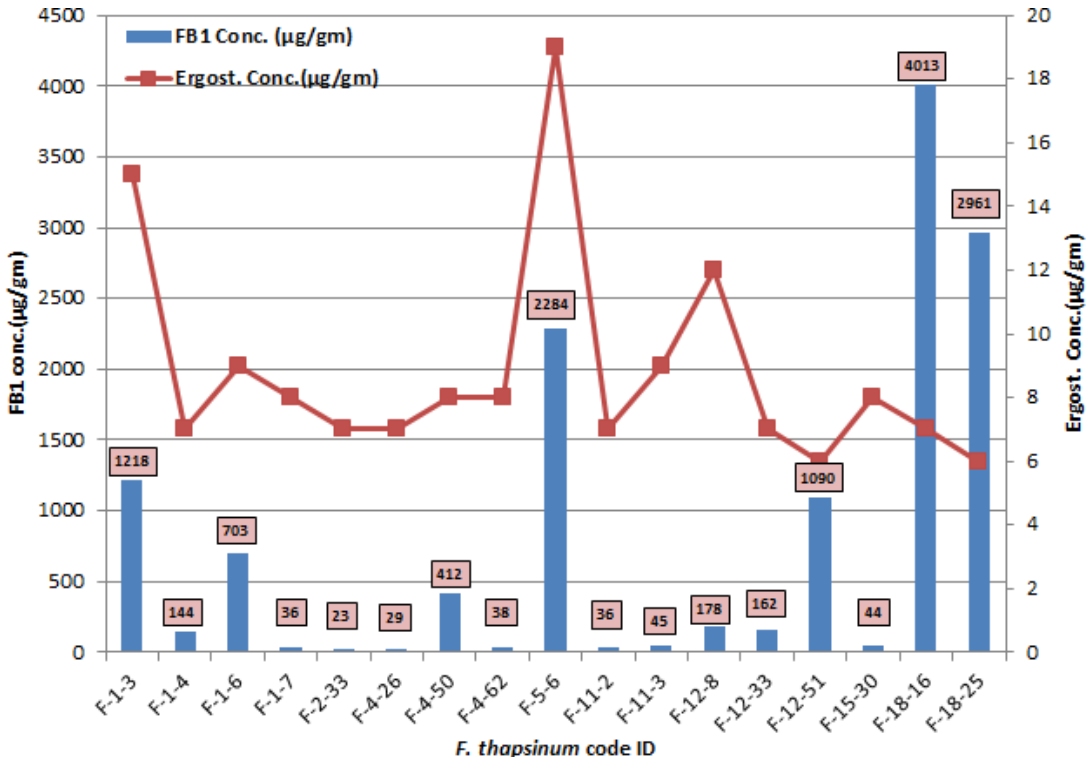

Figure 5: Fumonisin-B_1 and ergosterol produced by *F. thapsinum* isolates from Iraqi corn and sorghum seeds.

In this study, *F. incarnatum* (19 isolates) and *F. acuminatum* (1 isolate) were able to synthesize fumonisin-B1. However, relatively low levels of FB_1 were produced by both species, with the exception of isolate F-12-3, which produced high levels of FB_1 (Figure 6). These results

may conflict with previous molecular studies which found that some isolates of *F. incarnatum* failed to produce detectable levels of fumonisin because of deletions within the fumonisin gene cluster (Divakara et al., 2014; Aiyaz et al., 2016). Similarly, the inability of *F. acuminatum* to produce FB_1 has been documented (Thiel et al., 1991), which has also been identified as a deoxynivalenol (DON) and T2 producer (Wang et al., 2010; Marín et al., 2012). In future work, the genomes of these fungi should be sequenced and compared to the genomes of reference isolates (fumonisin producers and non-producers) to confirm taxonomic identification and confirm the presence/absence of an intact fumonisin gene cluster.

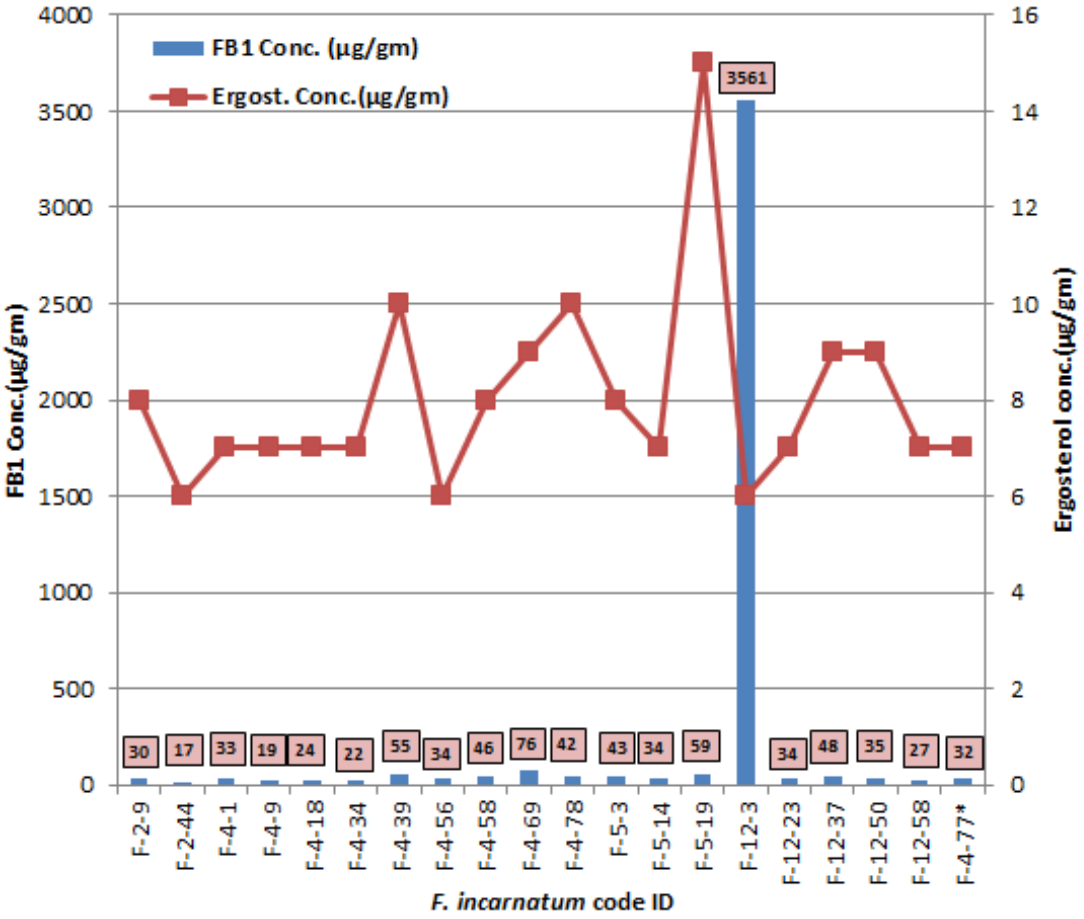

Figure 6: Fumonisin-B_1 and ergosterol production by *F. incarnatun and F. acuminatum* (*) isolates from Iraqi corn and sorghum seeds.

In conclusion, *Aspergillus spp.* and *Fusarium spp.* are very important pathogens because they include toxigenic strains that can produce dangerous mycotoxins (i.e. aflatoxin-B_1 and fumonisin-B_1). In this study, screening the mycotoxins profile in corn and sorghum samples revealed that fumonisin-B_1 predominated in both categories, whereas aflatoxin-B_1 was not detected in any sample even though aflatoxigenic isolates of *Aspergillus. flavus* were associated with corn and sorghum samples. Three *A. flavus* isolates were non-toxigenic and the rest were toxigenic, whereas all *Fusarium* isolates were able to produce FB_1. Interestingly, this might be the first report that *F. incarnatum* and *F. acuminatum* have the ability to produce FB_1 in Iraq.

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Chapter 4: Evaluation of Iraqi atoxigenic *Aspergillus flavus* isolates as potential bio-control strains to suppress aflatoxin contamination of grain

Abstract

Aspergillus flavus is a well-known aflatoxin producer that causes crop deterioration and public heath complications in humans and animals. However, some strains are naturally unable to produce aflatoxins, and concurrently have the ability to suppress aflatoxin biosynthesis by toxigenic strains. To demonstrate this phenomenon, four Iraqi strains of *Aspergillus flavus* were evaluated in this study; three non-toxigenic domestic isolates (A-1-9, A-4-54, and A-4-75) and one toxigenic domestic isolate (A-15-49). The reference *A. flavus* strain NRRL 3357 was also included as a positive control. Co-inoculation with each of the atoxigenic strains and a toxigenic isolate (wild type or A-15-49) on cracked corn kernel medium significantly ($p \leq 0.05$) suppressed aflatoxin-B_1 production. The average AFB_1 concentration in co-inoculation experiments with an Iraqi atoxigenic strain and toxigenic isolate A-15-49 was 12.0 μg/gm, and with the Iraqi atoxigenic strains and the reference strain was 42.0 μg/gm. In control samples (toxigenic strains only), strain A-15-49 produced 2252 μg/gm and the wild type produced 6196 μg/gm AFB_1. Even though significant differences appeared between the capabilities of the Iraqi atoxigenic strains to suppress aflatoxin-B_1 production, they all reduced aflatoxin levels substantially (99.2 to 99.8% reductions). Strain A-1-9 was the most effective at suppressing the two toxigenic strains. This study is the first identification of viable biocontrol strains of *A. flavus* identified from Iraq, which could have substantial utility mitigating aflatoxin contamination of corn, sorghum, and other crops in the region.

Introduction

Before the 1960 Turkey X disease outbreak in the United Kingdom, aflatoxins were unknown as contaminants of cereal crops. Turkey X disease was ultimately linked to fodder contaminated with high concentrations of a toxic compound now known as aflatoxin (Richard, 2008). Aflatoxins are a family of secondary metabolites produced primarily by *A. flavus* and *A. parasiticus* (Diener et al., 1987). These mycotoxins threaten human and animal health through contaminated food and the induction of aflatoxicosis (Fink-Gremmels & van der Merwe, 2019). Acute aflatoxicosis generally manifests in the liver and causes fatty liver, hepatic hemorrhage and necrosis, biliary disorders, and potentially death (Williams et al., 2004). However, chronic aflatoxicosis is more common, as it is initially symptomless yet culminates in symptoms such as kidney and liver cancer, birth defects, and immunosuppression (WHO, 2018). The International Agency for Research on Cancer (IARC) has ranked aflatoxin-B_1 among the top 5 human carcinogens based on evidence from many clinical studies (CAST, 2003; Wu & Santella, 2012; Adilah & Redzwan, 2017). In addition to health effects, aflatoxin contamination of nuts and cereal crops (especially in maize) is a global production concern and has been documented worldwide reduce crops yield, grain quality, and economic profitability (CAST, 2003; Atehnkeng et al., 2008; Ostadrahimi et al., 2014).

The ability of some microorganisms (bacteria, yeasts, and fungi) to reduce aflatoxin production has been exploited as a biocontrol strategy. Some of the most promising biocontrol agents are atoxigenic *A. flavus* isolates; as early as 1990, atoxigenic strains were found to reduce aflatoxin contamination in cotton and peanut fields (Yin et al., 2008). Subsequently, lab and field experiments worldwide confirmed the utility of non-toxigenic strains to reduce aflatoxin by 80 -

90% in cottonseed, peanuts, and corn crops (Atehnkeng et al., 2008; Richard, 2008; Abbas et al., 2011).

The goal of this study was to evaluate atoxigenic *A. flavus* isolates originating from Iraq as potential biocontrol organisms for aflatoxin management. To this end, three atoxigenic strains from Iraq (described in Chapters 2 and 3) were co-inoculated with toxigenic strains (from Iraq and the wild-type reference strain) of *A. flavus*.

Materials and methods

Fungal strains and culture maintenance:

The toxigenic profile of 27 isolates of *A. flavus* from Iraqi corn and grain samples were documented in Chapter 3. Relevant information about the non-toxigenic domestic isolates ($NTDI_s$; isolates A-1-9, A-4-54, and A-4-75) and the toxigenic domestic isolate (DTI; isolate A-15-49) utilized in this Chapter is presented in Table 1. The non-toxigenic isolates ($NTDI_s$) of *Aspergillus flavus* were isolated from corn seeds imported from Karbala province, Iraq (A-1-9, A-4-54, and A-4-75), whereas the toxigenic domestic isolate (TDI) of *A. flavus* was isolated from sorghum seeds obtained from Najaf province, Iraq (A-15-49). Also, *A. flavus* strain NRRL 3357 was utilized as the reference strain for this study. The stability of non-toxigenicity in isolates A-1-9, A-4-54, and A-4-75 was confirmed in the 5^{th} generation after 20 cycles of single spore culture starting with the original stock (Atehnkeng et al., 2008). For long-term preservation, cultures were stored in 25% glycerol at -80 ºC. *Aspergillus spp.* were maintained on V8 agar medium and/or 0.2X potato dextrose agar medium (PDA, BD Difco, USA) supplemented with carbenicillin (100 µg/mL) to suppress bacterial contamination (Bluhm, 2006).

Table 1: Fungal isolates utilized in co-inoculation experiments.

ID code	Fungal species	Crop category	Sample location	Toxigenicity
A-1-9	*A. flavus*	Corn	Karbala province	Non-toxigenic
A-4-54	*A. flavus*	Corn	Karbala province	Non-toxigenic
A-4-75	*A. flavus*	Corn	Karbala province	Non-toxigenic
A-15-49	*A. flavus*	Sorghum	Najaf province	Toxigenic
W.T NRRL 3357	*A. flavus*	N/A	N/A	Toxigenic

Preparation of fungal inoculum:

To prepare fungal inoculum for the co-inoculation experiment, each isolate was cultured on fresh 0.2X PDA and incubated for 5-7 days in darkness at room temperature. For each plate, conidia were harvested with an L-spreader (Thomas Scientific, NJ, USA) in 5 mL of sterile 0.154 M sodium chloride solution with 0.02% Tween 20. Conidial suspensions were adjusted to $1x10^6$ conidia/mL with a hemocytometer. All conidial stocks stored at 4 ºC until co-inoculation.

Co-inoculation of corn seeds:

The ability of $NTDI_s$ (A-1-9, A-4-54, and A-4-75) to suppress aflatoxin production by toxigenic isolates (A-15-49 and *A. flavus* NRRL 3357) was assessed by conducting co-inoculation experiments as proposed by Atehnkeng et al. (2008) with minor modifications. Scintillation vials containing cracked corn kernel medium were autoclaved at 120 ºC for 20 minutes. Vials were inoculated with 200 µL of spore suspension ($1x10^6$ conidia/mL) of each isolate individually as controls for each category in this experiment (toxigenic and non-toxigenic). Co-inoculation treatments consisted of mixing spore suspensions (100 µL each of toxigenic and non-toxigenic isolates) for the inoculum. Inoculated vials were vortexed for 1 minute to ensure even spread of inocula on kernels. The co-inoculation step was conducted simultaneously and non-simultaneously (after 1 hour) between each NTDI and TDI (A-15-49)

and the reference strain NRRL 3357. Two periods of incubation were set for co-inoculation treatments (7 and 14 days) at 28 ºC. There were three replications in this experiment.

Aflatoxin extraction and LC-MS/MS analysis:

For each sample, kernels were pooled and ground in pre-chilled mortars with liquid nitrogen. Three aliquots (about 0.5 gm for each represent three replications) of each pulverized sample (treatment) were extracted as mentioned in the previous chapter (2^{nd}). The final extracts were placed in 2 mL auto-sampler vials and at -20 ºC until analysis.

LC-MS/MS setting and analysis:

Aflatoxin-B_1 extracts of the co-inoculation experiment were analyzed with a Shimadzu 30AUPLC-20A/LC coupled to a Shimadzu 8060 triple quadrupole mass spectrometer with a heated electrospray source set on positive ion mode (Shimadzu, Kyoto, Japan). LC-MS/MS conditions were as follows: Separation was performed with a C18 column (2.1 x 50 mm, 1.9 µm particle size) with a linear gradient comprised of 0.1% formic acid (FA) in HPLC grade water/ 0.1% FA in methanol ramped at a rate of 8% methanol/min over 5 min and then 2% methanol/min over 5 min. the flow rate was 0.3 mL/min. A sample volume of 1 µL was injected. In the collision compartment or second quadrupole (Q2), collisionally induced dissociation (CID) was conducted by utilizing argon gas at a pressure of 270kPa. To detect and quantify the precursor ion of AFB1, the most frequent product ions were selected and measured. Two product ions (reference) were chosen for identification and most abundant fragment ion was used for quantification. Unknown samples were required to be within 30% of the reference ion ratio of the standard (Diaz Perez et al., 2019).

Statistical analysis:

Data were analyzed with SPSS statistical analysis software, IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, NY, USA) (Gouda, 2015). For four sets of data ($NDTI_s$ + DTI-Simul., $NDTI_s$ + DTI- After 1h., $NDTI_s$ + W.T-Simul., and $NDTI_s$ + W.T- After 1h.), differences of the means were analyzed via ANOVA: Two-Way with replication analysis. Fisher's Least Significant Difference (LSD) was employed to calculate mean separation and statistical significance. Mean values of AFB_1 concentrations were considered significantly different at $P \leq 0.05$. The descriptive statistical analysis included AFB_1 concentrations (Mean ± SD) and the percentage (%) of AFB_1 suppression in isolate A-15-49 and NRRL 3357 (Table 1).

Results and discussion

LC-MS/MS analysis of the 5th generation of each NTDI confirmed that they were atoxigenic, thus confirming their stability as non-toxigenic strains. The ANOVA: Two Way analysis conducted in the co-inoculation experiment carried out to evaluate the performance of $NDTI_s$ (A-1-9, A-4-54, and A-4-75) in excluding the toxigenic isolates (TDI-A-15-49 and/or *A. flavus* NRRL 3357) and blocking aflatoxin-B_1 production on (CCKM) at two major levels, inoculated simultaneously and after 1 hour. Further, within each level two periods of incubation (7d and 14d) were assessed comparatively (Table 2).

Raw data as shown in table (3) displayed that co-inoculation between $NDTI_s$ (A-1-9, A-4-54, and A-4-75) and TDI-A-15-49 had reduced AFB_1 production by TDI-A-15-49 vigorously from 2252 µg/gm, when TDI inoculated alone, into relatively very low total average (12.5µg/gm). Moreover, neither prolonging the incubation period to 14 days nor type of $NDTI_s$ had reduced the AFB_1 production significantly from corresponding values when the incubation time was 7 days through all the $NDTI_s$ (Figure 1).

Table2: Co-inoculation of $NTDI_s$ with toxigenic isolates (DTI A-15-49 and W.T *A. flavus* NRRL 3357 strain) in aftoxin-B_1 production on cracked corn kernels media (CCKM).

	Co-inoculation with DTI (A-15-49) isolate[1]		
	NTDI- A-1-9	**NTDI- A-4-54**	**NTDI- A-4-75**
Treatment Type	**AFB-1 conc. (µg/gm) Mean ± SD [4] (%)[3]**	**AFB-1 conc. (µg/gm) Mean ± SD (%)**	**AFB-1 conc. (µg/gm) Mean ± SD (%)**
Simul. (7D &14D)	8 ± 0.0 [a] (99.6) *	14.5 ± 0.5 [a] (99.4) *	15 ± 1.0 [a] (99.4) *
After 1h (7D & 14D)	7.5 ± 0.5 [c] (99.7) *	12 ± 1.0 [b] (99.5) *	15 ± 0.0 [a] (99.3) *
	Co-inoculation with *A. flavus* NRRL 3357 strain[2]		
Simul. (7D &14D)	16 ± 3.0 [b] (99.8) *	85 ± 29.0 [a] (99.2) *	28 ± 1.0 [b] (99.6) *
After 1h (7D & 14D)	18 ± 2.0 [b] (99.7) *	77 ± 24.0 [a] (99.3) *	28 ± 0.0 [b] (99.6) *

1: AFB_1 Conc. of A-15-49 alone in CCKM is 2252 µg/gm.
2: AFB_1 Conc. of *A. flavus* NRRL 3357alone in CCKM is 6196 µg/gm.
3: Percentage of reducing AFB-1 production.
4: Means followed by different lower-case letters indicate to significant differences between groups at the significant level (≤0.05).
*****There are no significant differences between simul. & after 1h co-inoculation through all types of treatments.

Similarly, the productivity of TDI-A-15-49 was dropped significantly from 2252 µg/gm into total average 11.5 µg/gm, and there were not significant differences between the two periods of incubation (7D and 14D) when non-simultaneous inoculation (1hour later the toxigenic inocula was added) was conducted (Figure 2). However, significant differences ($p \leq 0.05$) appeared between the ability of $NDTI_s$ in reducing the AFB_1 production as the A-1-9 was the most powerful in reducing AFB_1 production followed by A-4-54 and lastly A-4-75 (7.5, 12.0, and 15.0 µg/gm respectively) (Table 3).

Table 3. The effect of two periods of incubation (7D and 14D) and their interaction with simultaneous and non-simultaneous co-inoculation (After 1h) between NDTI$_s$ and DTI A-15-49 on the AFB$_1$ production.

NTDI Teatment	Co-inoculated with DTI A-15-49		
	AFB-1 conc. (µg/gm)		
	7 D Simul.	**14 D Simul.**	**Avg (µg/gm)**
A-1-9	8	8	**8***
A-4-54	15	14	**14.5***
A-4-75	16	14	**15***
Avg (µg/gm)	**12***	**11***	**Total Avg. 12.5**
NTDI Teatment	Co-inoculated with DTI A-15-49		
	AFB-1 conc. (µg/gm)		
	7 D after 1h	**14 D after 1h**	**Avg (µg/gm)**
A-1-9	8	7	**7.5****
A-4-54	13	11	**12****
A-4-75	15	15	**15****
Avg (µg/gm)	**12***	**11***	**Total Avg. 11.5**

*No significant differences between treatments' means at significant level (≤0.05)
** significant differences between treatments' means at significant level (≤0.05)

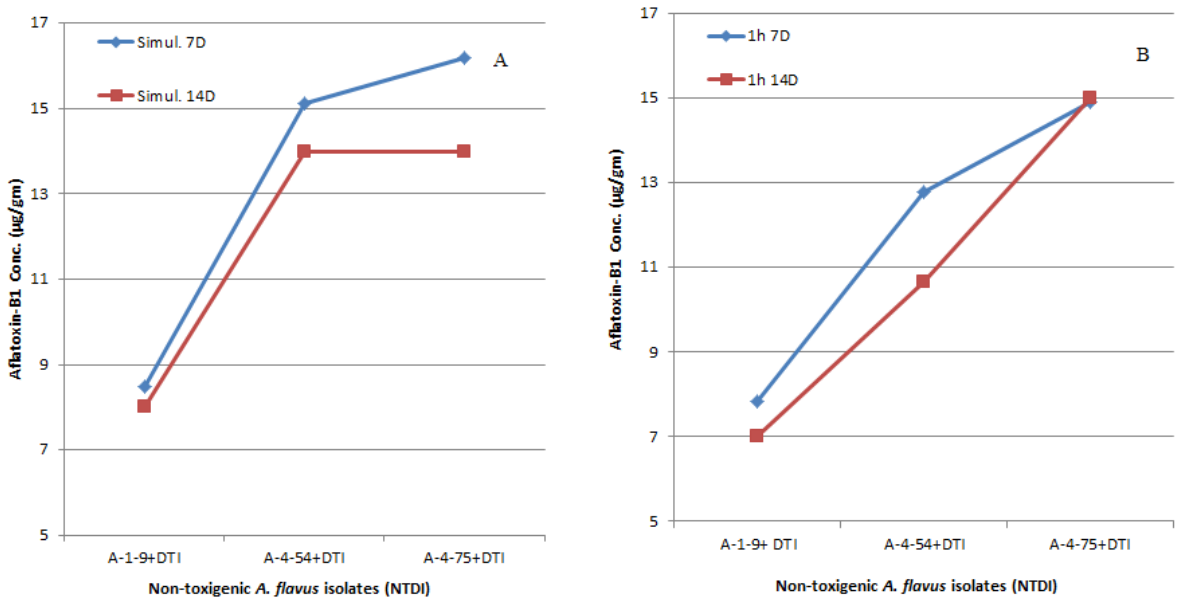

Figure 1. The efficacy of co-inoculation NTDI$_s$ with DTI A-15-49 simultaneously (A) and after 1h (B) in reducing AFB$_1$ production at two periods of incubation (7D and 14D).

The same experimental design was adopted in the co-inoculation between $NTDI_s$ and the reference *A. flavus* NRRL 3357 strain to evaluate the antagonistic behavior of $NTDI_s$ against a wild type and non-domestic isolate. Two-Way ANOVA analysis of the raw data of co-inoculation between $NTDI_s$ and W.T strain simultaneously had revealed decrease in the AFB_1 productivity of the *A. flavus* NRRL 3357 from 6196.0 µg/gm (inoculated alone treatment) into average total 43.0 µg/gm. Generally, time of incubation did not influence significantly on the aflatoxin-B1 exclusion as AFB_1 concentration at 7D was 54 µg/gm, whereas at 14D it was 32 µg/gm. However, the $NTDI_s$ exhibited blocking of AFB_1 production dramatically and significantly as the A-1-9 was the most potent followed by A-4-75 and A-4-54 (16.0, 28.0, and 85.0 µg/gm respectively) (Table 4). Likewise, the co-inoculation between $NDTI_s$ and *A. flavus* NRRL 3357 non-simultaneously (after 1h) had sharply reduced the productivity of the W.T strain into 41µg/gm, and interestingly protraction the incubation time to 14 days had lowered AFB_1 concentration significantly from 50 41µg/gm at 7D into 32 µg/gm at 14D. Similar scenario was observed with $NDTI_s$ treatments which presented significant differences ($p \leq 0.05$) between them as A-1-9 recoded as the strongest and A-4-75 came next and lastly A-4-54 (18.0, 28.0, 77.0 µg/gm respectively) (Figure 2 &Table 4).

Carrying out ANOVA: Two-Way analysis (without replication) on the overall average data of simultaneous and non-simultaneous co-inoculation within each toxigenic treatment (DTI A-15-49 and W.T A. flavus NRRL 3357) revealed that for both toxigenic treatment, posterior inoculation (after 1h) with the toxigenic strains had no significant impact in aflatoxin-B_1 contamination in corn grains comparing with co-inoculation simultaneously (11.0 and 13.0 µg/gm respectively). However, analyzing data according to $NTDI_s$ treatment type the outcomes

confirmed that the NTDI A-1-9 was significantly the most potent in reducing the AFB_1 contamination in corn grains followed by NTDI A-4-74 and NTDI A-4-54 (Table 5& Figure 3).

Table 4: The effect of two periods of incubation (7D and 14D) and their interaction with simultaneous and non-simultaneous co-inoculation (After 1h) between $NDTI_s$ and W.T *A. flavus* NRRL 3357 on the AFB_1 production.

NTDI Teatment	Co-inoculated with W.T *A. flavus* NRRL 3357 AFB-1 conc. (µg/gm)		
	7 D Simul.	**14 D Simul.**	**Avg (µg/gm)**
A-1-9	18	13	**16****
A-4-54	114	56	**85****
A-4-75	29	27	**28****
Avg (µg/gm)	**54***	**32***	**Total Avg. 43**
NTDI Teatment	Co-inoculated with W.T *A. flavus* NRRL 3357 AFB-1 conc. (µg/gm)		
	7 D after 1h	**14 D after 1h**	**Avg (µg/gm)**
A-1-9	20	16	**18****
A-4-54	101	54	**77****
A-4-75	28	27	**28****
Avg (µg/gm)	**50****	**32****	**Total Avg.41**

*No significant differences between treatments' means at significant level (≤0.05)
** significant differences between treatments' means at significant level (≤0.05)

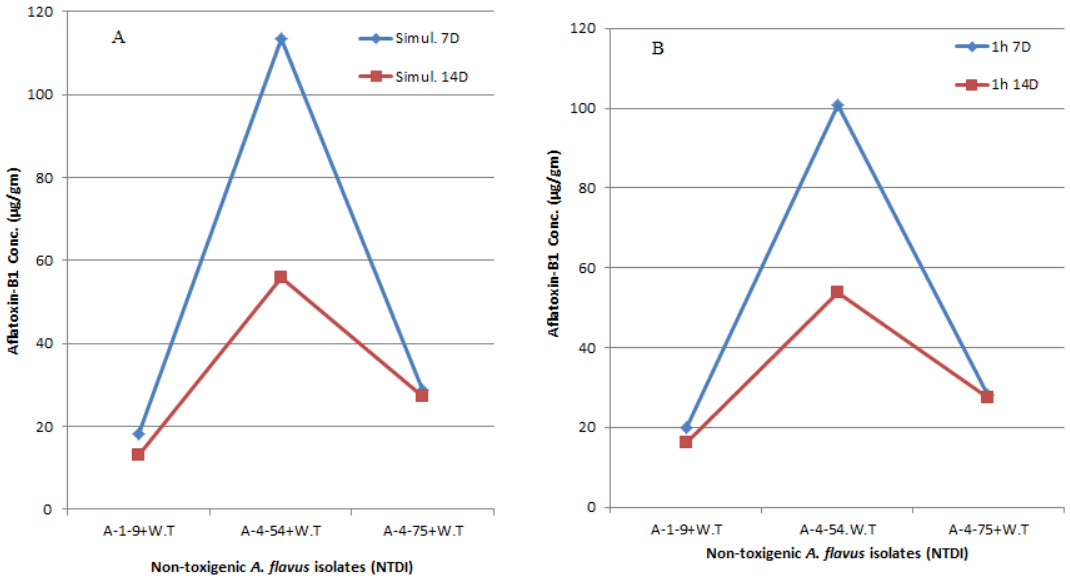

Figure 2. The efficacy of co-inoculation $NTDI_s$ with *A. flavus* NRRL 3357 simultaneously (A) and after 1h (B) in reducing AFB_1 production at two periods of incubation (7D and 14D).

The co-inoculation of $NDTI_s$ (A-1-9, A-4-54, and A-4-75) and with DTI (A-15-49) greatly reduced aflatoxin-B1 production compared to the control treatment (DTI inoculated alone) was ranged from 99.3% to 99.7%. Moreover and in the same context, delayed inoculation of the $NTDI_s$ (1hour) did not significantly reduce aflatoxin-B_1 contamination in corn grains from the simultaneous treatments. However, significant impact was noticed in the non-simultaneous co-inoculation treatment between the $NDTI_s$ treatments.

In general, the lowest AFB_1 contamination in corn grains was recorded with the co-inocualtion with NTDI (A-1-9) followed by A-4-54 and A-4-75 (7.5, 13.5, and 15.0 µg/gm respectively). The same scenario was observed when the $NDTI_s$ co-inoculated with the *A. flavus* NRRL 3357 simultaneously and non-simultaneously (after 1h) as the percentage of AFB_1 formation inhibition was high and ranged from 99.2% to 99.8%, and the NTDI A-1-9 also surpassed the rest of $NTDI_s$ significantly. However, in this scenario, the NTDI A-4-75 came secondly then A-4-54 (17, 28, and 81 µg/gm respectively) (Table 5 & Figure 3).

Cereal crops' contamination with aflatoxins in has becaome a global concern as these toxins are potent carcinogens and hepatotoxins, hence many countries have set regulations to minimize such toxins in the crops and insure the edibileness of their by-products (van Egmond & Jonker, 2004). Aflatoxin contamination management through adopting bio-control approaches have enhanced widely as the outcomes of such bio-safed organism-based methods promissing as an alternative way of chemicals-based methods which have increased population's concerns about the chemical residuals in food supplies (Cole & Cotty, 1990).

Table 5: The effect of simultaneous and non-simultaneous co-inoculation (After 1h) of NDTI$_s$ with DTI A-15-49 and *A. flavus* NRRL 3357 on the AFB$_1$ production.

NTDI Teatment	Co-inoculated with DTI A-15-49		
	AFB-1 conc. (µg/gm)		
	Simul.	**After 1h**	**Avg (µg/gm)**
A-1-9	8	7	**7.5****
A-4-54	15	12	**13.5****
A-4-75	15	15	**15****
Avg (µg/gm)	**13***	**11***	**Total Avg 12**
NTDI Teatment	Co-inoculated with W.T *A. flavus* NRRL 3357		
	AFB-1 conc. (µg/gm)		
	Simul.	**After 1h**	**Avg (µg/gm)**
A-1-9	16	18	**17****
A-4-54	85	77	**81****
A-4-75	28	28	**28****
Avg (µg/gm)	**43***	**41***	**Total Avg 42**

*No significant differences between treatments' means at significant level (≤0.05)
** significant differences between treatments' means at significant level (≤0.05)

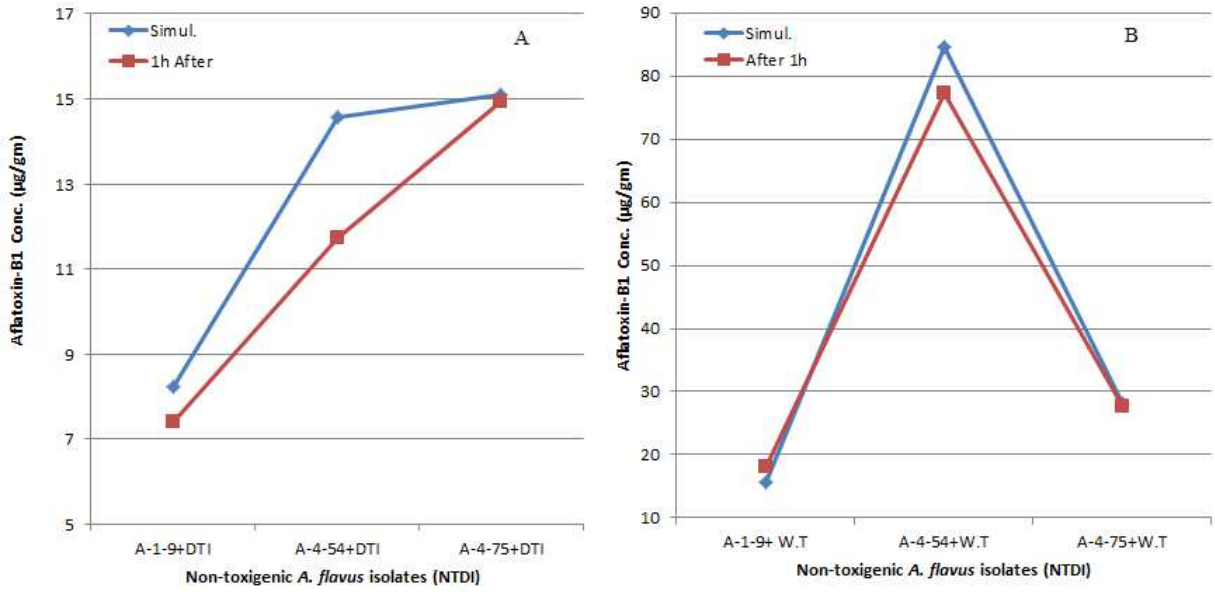

Figure 3: The efficacy of simultaneous and non-simultaneous (after 1h) co-inoculation NTDI$_s$ with DTI A-15-49 (A) and with *A. flavus* NRRL 3357 (B) in reducing AFB$_1$ production on corn grains.

Three machnisms were suggested for discriping the competions between plant pathogenic fungi and colonizing in the infection site of host plant; competitive exploitation, intereference competition, and parasitic fitness (Reid et al., 1999; Wicklow, 1981). However, on maize kernels the competitive exploitation seemed feasible and more likely exestence when the non-toxigenic *A. flavus* (AF36) co-inoculated with the wild-type toxigenic AF70 labeled with green fluorescent protein (GFP). The outcomes of this study indicated significant decreasing in AF70 population with vigorous expansion of the AF36 on maize kernels (Hruska et al., 2014).

Without consideration to simultaneous and non-simultanneous co-inoculation treatments, the presented outcomes in table (2) have a similar trend to the results in a study of Tehnkeng et al. (2008) when the ability of eleven atoxigenic isolates of *A. flavus* to inactivate aflatoxin (B_1 and B_2) production was evaluated on corn. Relatively high percentages of inhibition in aflatoxin-B_1 and B_2 contaminations were recorded in corn grains (96.4- 99.9%) when the atoxigenic isolates co-inoculated with the toxigenic one. Similarly, in a previous study by Cotty (1990) wounded cotton bolls co-inoculated simultaneously by atoxigenic and toxigenic *A. flavus* isolate showed significant decrease in the aflatoxin production comparing with the toxigenic strain alone. Moreover, the non-simultaneous co-inoculation (36 h after inoculating with atoxigenic isolate) revealed relatively significant decontaminated corn kernels comparing with simultaneous co-inoculation treatments. However, the aflatoxin production wasn't inhibited when the toxigenic inoculated was inoculated 24h after inoculation with the atoxigenic isolate. In the same context, there were comparable observations in the non-simultaneous co-inoculation between NTDIs and the two toxigenic isolates (i.e. *A. flavus* NRRL 3357 and DTI A-15-49) as the outcomes indicated that there are further decontamination status but not significant between the simultaneous and the non-simultaneous co-inoculations, and this may be explained and

suggests at the same time that the more time in the anterior inoculation with atoxigenic isolate, the more affects could be observed in terms of aflatoxin inhibition in corn kernels and vice versa. Back to table (2), it is evident that conducting co-inoculation protocol triggered by non-toxigenic inoculation step is preferable to gain desirable less aflatoxin-contaminated products. Contrarily, in the Atehnkeng et al., (2008) study which stated that even though an anterior inoculation (1h) with the toxigenic strain, significant levels in aflatoxin reduction was recorded in the co-inoculation experiments. The differences and similarities among studies of co-inoculation toxigenic and atoxigenic strains of *A. flavus* suggests that the biodiversity within *A. flavus*' population is such wide that improbable to introduce a cosmopolitan bio-control agent with a unique genotype to manage aflatoxin contamination concomitantly with diversities in host crops, climatic changes, and soil-inhabitants microbiomes (Ehrlich, 2014).

It seems that not only initiating the co-inoculation process with atoxigenic and/or toxigenic treatment will affect on the bio-control agent efficiency, but also additional factors should be considered as previous studies indicated such as direct delivering (sprayable formula or needle injection) the non-toxigenic *A. flavus* suspension spore to the target plant (Lyn et al., 2009; Williams et al., 2013) or indirect way by mixing the suspension spore with soil before planting (Cardwell & Henry, 2004). Further, the efficacy of the bio-control agent was found impacted by the inoculum rate and time of application (in the soil) as well. Studies indicated that more aflatoxin inhibition achieved in peanuts fields by increasing the inoculum concentrations of the atoxigenic *A. flavus* (Dorner et al., 1998) and not adding the inoculum to soil until the temperature reaches 20 ºC in the soil (Dorner, 2004).

Even though each of the $NTDI_s$ has showed high percentages of aflatoxin-B1 reduction when either co-inoculated with DTI A-15-49 or *A. flavus* NRRL 3357 (Table 2), significant

differences were observed between the $NTDI_s$ in terms of performance of aflaoxin-B_1 reduction (Table 5), which are owing to differences in the non-toxigenic fungi' ability to impede aflatoxin contamination in grains (Cotty, 1990), and this in turn suggests there might be different levels of specificity between $NTDI_s$ when they co-inoculated with each of toxigenic strain in this study. Moreover and interestingly, all $NTDI_s$ have displayed a similar propensity to competitively exclude two toxigenic isolates (DTI A-15-49 and *A. flavus* NRRL 3357) which are from two different agro-ecologies though. These outcomes could be at variance relatively with what mentioned in Cardwell and Cotty (2000) review that two native and nontoxigenic isolates of *A. flavus* in Benin (BN-22 and BN-30) along with the US AF-36 non-toxigenic isolate were assessed in terms of precluding native and exotic aflatoxin-producing fungi (BN-40, BN-48, and AF-13 from US respectively). The results showed that the African non-toxigenic isolate (BN-30) was the only that reduced the aflatoxin production by African toxigenic isolate (BN-40). However, all the African non-aflatoxigenic isolates effectively reduced the aflatoxin production by native African toxigenic isolate (BN-48). This situation may propose that these $NTDI_s$ (A-1-9, A-4-54, and A-4-75) may have (geneticaly) high dgrees of similarity with the local and exotic toxigenic *A. flavus* populations which in turns can improve their efficacy in mitigating aflatoxins contamination in corn kernels (Atehnkeng et al., 2016).

In concluding, in the scope of aflatoxins decontamination in corn crops efforts still exert in worldwide to come up with a geographical native non-toxigenic isolate and deploye this strain as bio-control agent given an intellect that native non-toxigenic *A. flavus* isolates are more efficient in blocking the propagation of toxigenic *A. flavus* and consequently reducing aflatoxin contamination in corn crops. Concurrent findings of this study sugget that at least one the $NTDI_s$

could potentially be deployed as a region-wide bio-control agent to competitively exclude aflatoxin-producing fungi in maize and other cops as well in Iraq.

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Conclusion

Among crops that highly support food security in the world are maize and sorghum crops which constitute occupy the 4th and 5th rank in the world productivity of cereal crops after wheat, barley, and rice as these two crops can be cultivated in wide range of environmental conditions, and more importantly that these crops fundamentally are driving daily calories of low-income people in many countries in Africa and Asia wherein food scarcities have been stated. Maize and sorghum grains predisposed to pathogenic fungi infections, especially the mycotoxigenic species of *Aspergillus* and *Fusarium*, during developing stage at fields and post-harvest stage (storage and marketing phases) if conditions at storages are improper and rotten. Consequently, mycotoxins contaminations (i.e. with aflatoxins and fumonisins) occur that pose health risks to consumers and cause economic turmoil. In Iraq, maize cultivation appears more common than sorghum as the late only intended for animal feed and the association of aflatoxigenic and fusariogenic fungi in maize grains is well documented. Interestingly, aflatoxicosis cases in human are seldom and/or non-existed. However, sporadic aflatoxicosis in farm animals have been reported in many cities in Iraq because of nourishments contaminated with aflatoxin(s).

Worldwide, attempts have been taken to enhance corn crop's quality and quantity and reach free-aflatoxin maize grains through appropriate farm practices, development pathogen-resistant cultivars, adding fungicides, and adopting bio-control agents which appear the most promising tactic in eliminating aflatoxigenic fungi and reducing aflatoxin contamination in grains. Yet, in Iraq deploying a non-toxigenic strain of *A. flavus* has not been adopted as serious solution for managing pre-harvest maize contamination with aflatoxin. Thereupon, this study came to identify an indigenous non-toxigenic *A. flavus* strain and evaluate its performance in

excluding toxigenic strains and mitigating aflatoxin contamination on maize grains. To achieve this goal, first objective was identifying seed-borne fungi that associated with corn and sorghum grains that imported from Iraq. Seed health testing boosted with sequence-based identification i.e. amplified Internal Transcript Spacer (*ITS*) sequences revealed that 468 pathogens distributed on twenty-two genera were associated with corn and sorghum grains. The most predominance genera were *Fusarium*, *Chaetomium*, and *Alternaria* followed by *Penicillium*, and *Aspergillus*. Furthermore, other genera such as *Bipolaris, Byssochlamys, Exserohilum*, *Humicola, Sarocladium, Sporisorium,* and *Stenocarpella* were recorded at the first time in Iraq as seed-associated fungi. Conducting multi-locus sequences typing (MLST) analysis was vigorous in delineating boundary between species of *Aspergillus* and *Fusarium*, as the phylogeny tree exhibited that all *Aspergillus* species (36 isolates) were distributed between five clades and *A. flavus* was the biggest clade which included 27 isolates. Similarly, 86 isolates of *Fusarium* were disseminated onto four species (*F. verticillioides, F. thapsinum F. acuminatum,* and *F. incarnatum*), and 49 isolates gathered in the *F. verticillioides* clade. Interestingly, this the first report of *F. thapsinum F. acuminatum,* and *F. incarnatum* as pathogens isolated in Iraq from maize and sorghum grains.

In the second object, the toxigenic profile of all *Fusarium* species revealed that the four species have the ability to produce fumonisin-B_1 at different levels, and not coincidentally that high levels of fumonisin-B_1 productions were observed with *F. verticillioides* isolates (the well-known fumonisins producers). However, this might be the first report of *F. acuminatum,* and *F. incarnatum* as FB_1 producers in Iraq. In addition, only three isolates of *A. flavus* had shown lack the ability to synthesize AFB_1, which means that these isolates can be potentially recruited as bio-control agents to control aflatoxin contamination in maize grains, whereas the rest isolates

evinced their ability to form AFB_1 by producing different levels of the toxins. Paradoxically enough, screening the mycotoxin profile of all maize and sorghum samples displayed that the 15 out of 18 samples were contaminated with acceptable levels of fumonisin-B_1 (≥4ppm), and noteworthy that neither maize nor sorghum samples were contaminated with aflatoxin-B_1, and this probably due to low moisture content of grains in all samples which prevented activation of such toxigenic fungi.

The last objective was investigation the ability of the three Iraqi non-toxigenic domestic *A. flavus* isolates ($NTDI_s$)(A-1-9, A-4-54, and A-4-75) in decontaminating AFB_1 on maize grains when each was co-inoculated with the reference strain *A. flavus* 3357 NRRL and with the most toxigenic domestic isolate (TDI)(A-15-49) in another time alternatively. The outcomes manifested that all NTD isolates have successfully excluded either toxigenic isolates, and sturdy decreasing in AFB_1 contamination (reached 99.8%) was noticed in maize grains comparing with the control groups (toxigenic only inoculated).

Even though that aflatoxicosis not pandemic neither in farm animals nor human in Iraq, inevitable maize contamination with AFB_1 and mortality in poultry and farm animals are sporadically reported in Iraq. Hence, pressing need to improve and provide maize yield in safe form for consumers still requisite, and therefore this study is introducing indigenous atoxigenic *A. flavus* isolates as potent bio-control agents to govern aflatoxin-contaminated maize. Additional works demanded to evaluate the efficiency of these isolates in handling pre-harvest aflatoxin contamination in maize crops in Iraq.