INFLUENCE OF TEMPERATURE, WATER ACTIVITY, AND OIL CONTENT ON GROWTH AND AFLATOXIN PRODUCTION ON OIL SEEDS BY *ASPERGILLUS FLAVUS* AND *A. PARASITICUS*

by

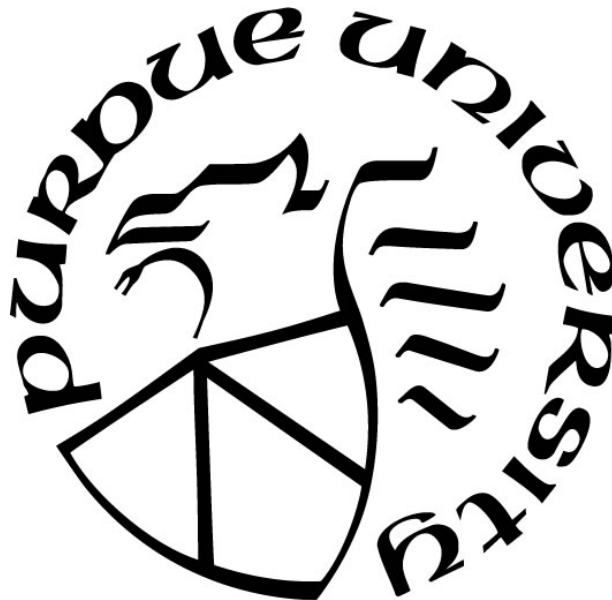**Chih-Hsuan Chang**

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ABSTRACT

Aflatoxins (AFs) are highly toxic second metabolites produced by *Aspergillus flavus* and *A. parasiticus*. They are widely detected in cereals, spices, and drinks worldwide. Aflatoxin contamination of foods and crops poses a high health risk for humans and livestock. It is well known that environmental conditions and substrates could influence fungal growth and aflatoxin production. This study tested the effect of water activity (0.82, 0.86, 0.90, 0.94, and 0.98 a_w) and incubation temperatures (20°, 27°, and 35°C) on the growth and aflatoxin production of *A. flavus* and *A. parasiticus* on ground flax seeds and ground niger seeds. The effect of oil contents of ground niger seeds on fungal growth and aflatoxin production was also investigated in this study.

These two fungal species could not grow on any of the tested substrates with 0.82 a_w at 20°, 27°, or 35°C. *Aspergillus flavus* grew most rapidly on flax seeds with 0.90 a_w at 27°C and also 0.94 a_w at 27° or 35°C. However, on niger seeds, *A. flavus* grew best at 0.90 or 0.94 a_w incubated at 35°C as well as at 0.94 or 0.98 a_w incubated at 27°C. *Aspergillus parasiticus* showed the optimum growth on flax seeds with 0.90 a_w at 35°C, whereas on niger seeds, the optimum occurred on seeds with 0.90 a_w at 35°C and also on seeds with 0.94 a_w at 27° or 35°C. The optimum conditions for *A. flavus* to produce high levels of aflatoxins (270-299 μg/kg) on flax seeds were 0.90 a_w at 35°C; whereas, the optimum conditions for *A. flavus* to produce aflatoxin (203-278 μg/kg) on niger seeds were 0.90 or 0.98 a_w at 27°C and also 0.90 a_w at 35°C. *Aspergillus parasiticus* produced high levels of aflatoxins (284-365 μg/kg) on flax seeds under the following three conditions, 0.86 or 0.98 a_w at 35°C and 0.94 a_w at 27°C; *A. parasiticus* produced 200-384 μg/kg of aflatoxins on niger seeds under nine out of 12 tested incubation conditions.

Reducing mean oil contents from 35.2 to 10.5% of ground niger seeds had very little effect on the growth of the two fungi but significantly decreased their aflatoxin production under certain incubation conditions. On de-oiled niger seeds inoculated with *A. flavus*, only 13μg/kg of AFB1 was found on seeds with 0.94 a_w at 27°C; whereas, on de-oiled niger seeds inoculated with *A. parasiticus*, high levels of aflatoxins (245-345 μg/kg) were only detected under the three following incubation conditions, 0.90 or 0.94 a_w at 27°C, and 0.86 a_w at 35°C.

This study showed that the optimum growth and aflatoxin production by *A. flavus* and *A. parasiticus* were not identical and influenced by incubation conditions, including temperature,

water activity, and growth substrates. The results of this study could help establish guidelines for post-harvest and storage conditions for oil seeds to prevent fungal growth and aflatoxin formation.

CHAPTER 1.   INTRODUCTION

1.1   Aflatoxins

Mycotoxin contaminated crops and foods post a high health risk for humans and livestock. Aflatoxins, a group of mycotoxins, are highly toxic second metabolites produced by *Aspergillus spp.* on various agricultural commodities. *Aspergillus flavus* and *A. parasiticus* are the two primary producers of aflatoxins (Klich, 2007; Cary et al., 2005; Li et al., 2009; Cotty and Jaime-Garcia, 2007; Frisvad et al., 2005; Nierman et al., 2015; Yu et al., 2011). Aflatoxins represent one of the most poisonous and widespread mycotoxins in agricultural commodities in the world (Li et al., 2009; Gizachew et al., 2016). Consumption of aflatoxins may lead to acute poisoning (aflatoxicosis) or other life-threatening diseases (WHO, 2018; Eaton and Gallagher, 1994; IARC, 2002). Humans are exposed to mycotoxins by consuming contaminated food (Marino et al., 2014; Ali et al., 2013). Aflatoxins have been widely detected in foods from plants and animal sources, such as grains, crops, spices, meats, and dairy products (Pardo et al., 2005; Ribeiro et al., 2006). Kensler et al. (2011) summarized the history timeline of aflatoxins discovery, as shown in Figure1.
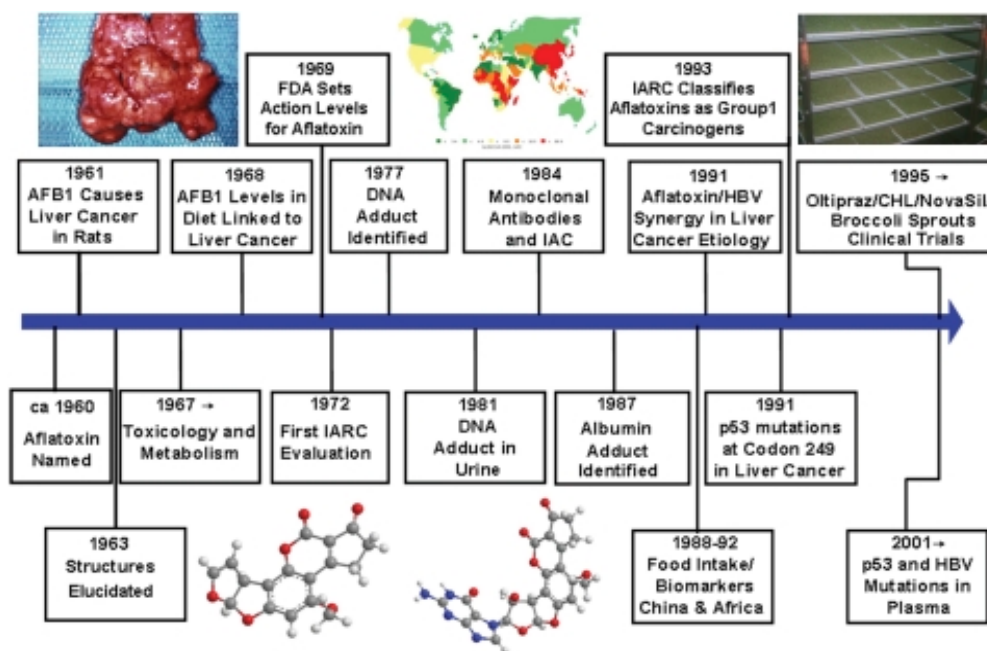
Figure 1. History timeline of aflatoxins discovery (Kensler et al., 2011)

1.1.1 Chemical Properties of Aflatoxins

More than 14 forms of aflatoxins are known; of these, AFB1, AFB2, AFG1, AFG2, AFM1, and AFM2 are the groups of aflatoxin mainly found on food (Kumar, 2018; Da Silva et al., 2018; WHO, 2018). Figure 2 shows the most dominant forms of aflatoxins in the world. The aflatoxin B and G groups were differentiated by their fluorescent colors when observed on the thin-layer chromatography (TLC) plates under ultraviolet light. Blue fluorescent color indicates AFB1 and AFB2; green fluorescent color indicates AFG1; and green-blue fluorescent color indicated AFG2 (Iqbal et al., 2013; Kumar, 2018; IARC, 2002). Aflatoxin M1 and M2 are the hydroxylated metabolite form of AFB1 and AFB2 respectively. The molecular formula (molecular weight) of AFB1, AFB2, AFG1, AFG2, AFM1, and AFM2 are $C_{17}H_{12}O_6$ (312 g/mol), $C_{17}H_{14}O_6$ (314 g/mol), $C_{17}H_{12}O_7$ (328 g/mol), $C_{17}H_{14}O_7$ (330 g/mol), $C_{17}H_{12}O_7$ (328 g/mol) and $C_{17}H_{14}O_7$ (330 g/mol) respectively (Kumar, 2018; NCBI, 2020; Han et al., 2010) (Figure 2). Aflatoxins are soluble in several polar organic solvents, such as chloroform, menthol, and methanol. It is slightly soluble in water and insoluble in non-polar solvents. (IARC, 2002; Kumar, 2018)
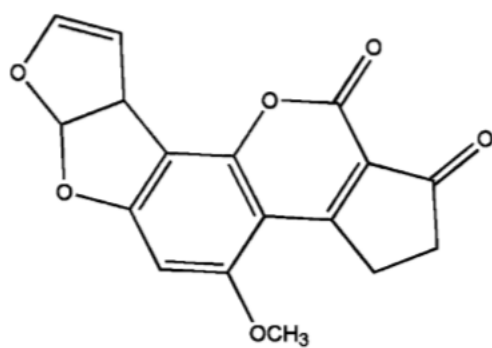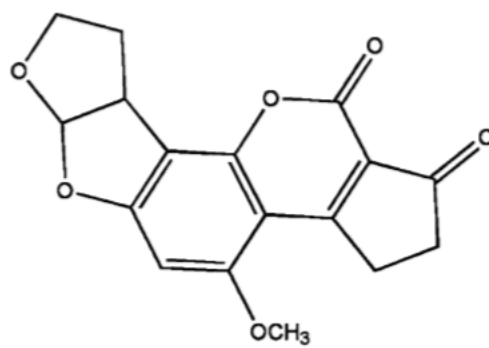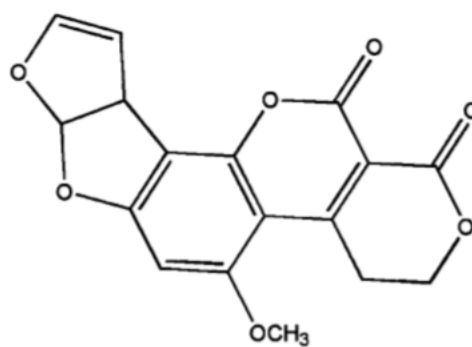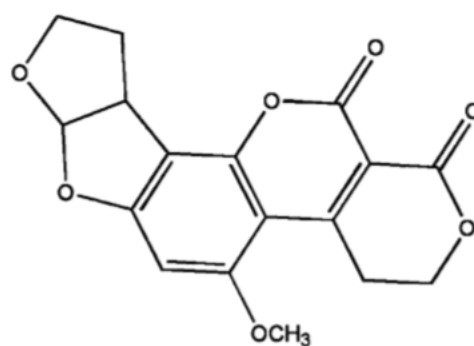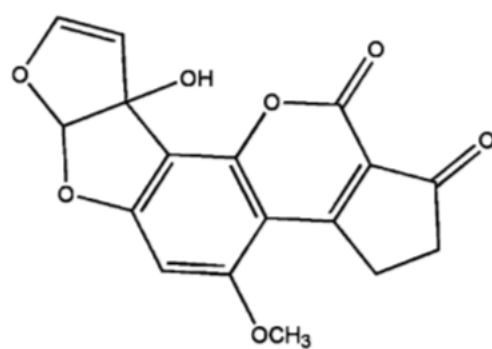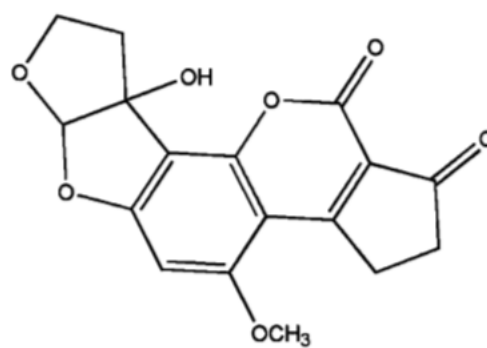
Figure 2. Chemical structure of the most dominant forms of aflatoxins (Han et al., 2010)

1.1.2 Toxicity

Aflatoxins are considered a human carcinogen with potent carcinogenicity, teratogenicity, mutagenicity, nephrotoxicity, genotoxicity and hepatotoxicity (Eaton and Gallagher, 1994; Dahal et al., 2016; Ribeiro et al., 2006; Hua al., 2019; Kumar, 2018; Li et al., 2009; Bircan, 2006). Moreover, the International Agency for Research on Cancer (IARC) classified AFB1, AFB2, AFG1, AFG2 and, AFM1 as a group I carcinogen (International Agency for Research on Cancer 2002; WHO, 2018). Jaimez et al. (2000) stated that the toxicity of aflatoxin varies among different forms of aflatoxin. Among these, AFB1 is the most toxic, followed by AFG1, AFB2, and AFG2 respectively.

Abdel-Fattah et al. (1982) reported that the aflatoxin toxicity in domestic animals was associated with the consumption of mold-contaminated feeds. Animals can hydroxylate AFB1 and AFB2 in their organs and transform into AFM1 and AFM2 respectively, and excrete into their milk or meat production. Kumar (2018) stated that the ingestion of aflatoxins leads to aflatoxicosis, the disease caused by aflatoxins intake. Acute aflatoxicosis is life-threatening, and the symptoms include bile duct proliferation, hemorrhagic necrosis of the liver, edema, and lethargy. Chronic aflatoxicosis has a major effect on immunotoxicity (Williams et al., 2004). The severity of aflatoxicosis is determined by the concentration of aflatoxins intake and animal maturity (Williams et al., 2004).

Eaton and Gallagher (1994) found that the consumption of aflatoxin-contaminated dietary products could attack human liver and kidney. They also stated that ingestion of aflatoxins could increase the human risk of hepatotoxicity, liver cancer and kwashiorkor, and Reye's syndrome. Food and Agriculture Organization of the United Nations (2004) stated that the consumption of aflatoxins might also damage DNA and result in genotoxic cancer. In recent years, the application of human aflatoxin biomarkers has been used to monitor the exposure of aflatoxins in human urine and blood (Kensler et al., 2011). Figure 3 summarized the possible pathways of AFB toxicity in affecting animal and human health (Williams et al., 2004; Seid and Mama, 2019; World Health Organization, 2018).
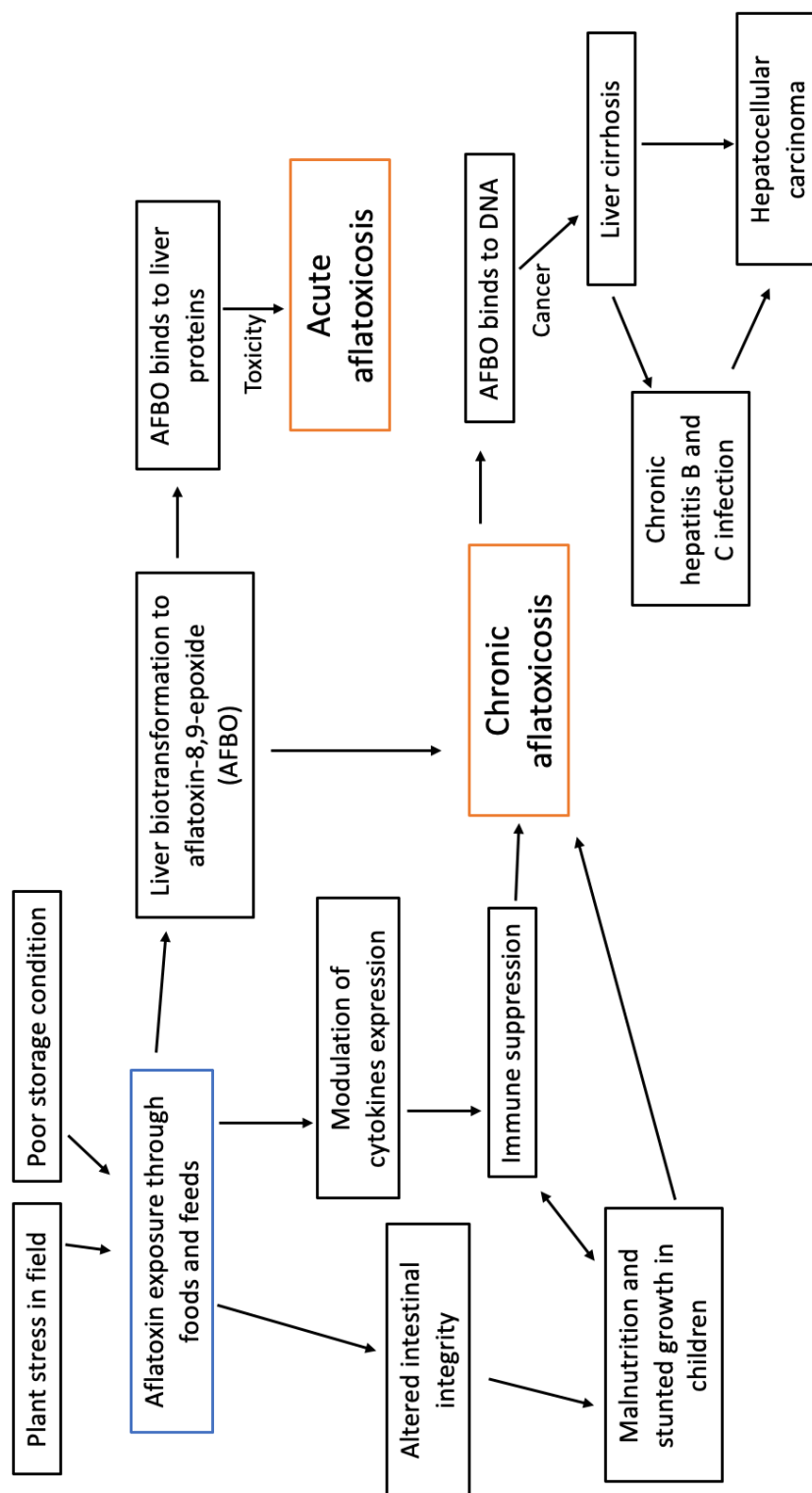
Figure 3. Possible pathways leading to AFB toxicity in animals and humans

1.2   Aflatoxins in Food and Feeds

Aflatoxin has been widely detected in cereals (corn, maize, barley, oats, rice, sorghum, and wheat), oilseeds (soybean, peanut, sunflower and cotton seeds), spices (chili peppers, black pepper, coriander, turmeric, and ginger), dried fruits, tree nuts (pistachio, almond, walnut, coconut and Brazil nut) and a variety drinks (coffee, milk, and wines) (Pardo et al., 2005; World Health Organization, 2018; Food and Agriculture Organization of the United Nations, 2004). Chulze et al. (1989) reported that 3.3% out of 150 corn samples were contaminated with AFB1 in Argentina. A previous study also found that 82% of peanut butter and 37% of sesame paste samples were contaminated by aflatoxins in China (Li et al., 2009). Previous studies found heavy aflatoxin contamination in milk when cows were fed with aflatoxins contaminated feed (van Egmond, 1983; Gizachew et al., 2016). When animals consume aflatoxins contaminated feed, aflatoxins can later be transferred to eggs, meat, and milk (Ribeiro et al., 2006; Food and Agriculture Organization of the United Nations, 2004). Gizachew et al. (2016) found that the average of 0.092µg/L of AFM1 was detected in milk samples after dairy cows were fed with aflatoxins contaminated feeds in Ethiopia. A previous study reported that aflatoxins were detected in raw milk (15.9%), eggs (45.2%), breast milk (4.8%), and serum from liver cancer patients (63.9%) (Tchana, 2010).

1.3   Current Regulation of Aflatoxin in Foods and Feeds

The regulations of aflatoxins in foods and feeds have been established since the 1960s. (World Health Organization, 2018; European Commission, 2010). In 2003, more than 99 countries set the regulation of mycotoxin for foods or feed. The Codex Alimentarius Commission (CAC), composed of 168 countries, is supported by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO). The purpose of CAC is to set the international standards for foods and feeds to maintain world trade and protect consumers' health at the same time (Food and Agriculture Organization of the United Nations, 2004). For example, the maximum levels for aflatoxins set by CAC in crops, dried fruits, nuts, and milk range between 0.5 to 15 µg/kg (Food and Agriculture Organization of the United Nations, 2004). The US Food and Drug Administration (USFDA) (1994) also set the aflatoxins regulation for food and feed to help minimize the risk in the U.S. Current regulation set by the different organizations for aflatoxins in food and crops are summarized in Table 1 (Eskola et al., 2020; Food and Agriculture Organization of the United

Nations, 2004; Food and Agriculture Organization of the United Nations, 2004; US Food and Drug Administration, 1994).

Table 1. Maximum levels of aflatoxins in foodstuffs, dairy products and animal feedstuffs set by different regulatory agencies.

Foods or feeds	Maximum levels (µg/kg)	
Codex Alimentarius standard	AFB1	**Total aflatoxins**
Almonds, Brazil nuts, hazelnuts, peanuts, pistachios for processing	—	15
Almonds, Brazil nuts, hazelnuts, pistachios for direct human consumption	—	10
Commission Regulation (EC) 1152/2009 maximum and guidance levels	AFB1	**Total aflatoxins**
Groundnuts peanuts, hazelnut, Brazil nuts and other oilseeds for processing	8	15
Almonds, pistachios, apricot kernels for processing	12	15
Tree nuts, other than the tree nuts above for processing or hazelnuts and Brazil nuts for direct human consumption	5	10
Groundnuts (peanuts), other oilseeds, other three nuts below for direct human consumption or use as an ingredient in foodstuffs	2	4
Almonds, pistachios, apricot kernels for direct human consumption	8	10
All cereals except maize and rice	2	4
Maize, rice and dried fruit for processing	5	10
Milk	—	0.05 (AFM1)
Infant formulae and dietary foods	0.1	0.025
Spices (chili powder, cayenne, paprika, white and black pepper, nutmeg, ginger, turmeric)	5	10
Food and Drug Administration (FDA) standard	AFB1	**Total aflatoxins**
All foods except milk	—	20
Milk	—	20 (AFM1)
Corn and peanut products intended for finishing beef cattle or cottonseed meal intended for beef cattle, swine, or poultry	—	300
Corn, peanut products, cottonseed meal and other animal feeds and feed ingredients, intended for immature animals or dairy animals or when the intended use is not known	—	20

1.4　Current Control of Aflatoxins

Foods and feed may be contaminated by molds during production, harvesting, processing, transportation and storage (Bordin et al., 2014; World Health Organization, 2018). The World Health Organization (2018) suggested that the best method to minimize the risk of aflatoxins is to inhibit the fungal growth on agricultural commodities. Winter and Pereg (2019) showed that the development of aflatoxins could be monitored and inhibited from "field to fork" in pre-harvest and post-harvest periods. During the pre-harvest period, bio-control, improved plant varieties and integrated pest management are all key elements of aflatoxin management strategies. On the other hand, foods and feed handling, improved storage environment, and quality assurance of the food chain are also critical during the post-harvest period (Winter and Pereg, 2019; World Health Organization, 2018; Food and Agriculture Organization of the United Nations, 2004). Previous studies showed that fungal growth could be controlled by lactic acid bacteria (LAB) (Perczak et al., 2018; Taroub et al., 2019). Moreover, LAB may also facilitate mycotoxin reduction or removal (Luz et al., 2018; Elsanhoty et al., 2014). Fung (1977) observed common antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) could influence the growth of *A. flavus* and aflatoxin production. Fanelli and Fabbri (1989) further suggested that BHA and BHT were able to inhibit aflatoxin production.

1.4.1　Reduce Fungal Contamination and Aflatoxin Production

Mannaa and Kim (2017) stated that *Aspergillus* spp. are classified as storage fungi and xerophilic fungi that mainly occurred when water activity of crops is below 0.95 during the pre-harvest and post-harvest stages. World Health Organization (2018) stated that the most efficient procedure to minimize fungal contamination is to keep the foods and feeds in their dry state. Storage environments with warm and humid conditions generally support fungal growth and increase the risk of mycotoxin contamination (World Health Organization, 2018; Food and Agriculture Organization of the United Nations, 2004; Cotty and Jaime-Garcia, 2007). Bordin et al. (2014) found that removing visible contaminated seeds or grains in a pre-clean process after harvesting could also reduce aflatoxin content by 40-80%. World Health Organization (2018) also stated that discarding damaged, discolored, or shriveled crop plants could decrease the exposure to aflatoxins because damaged crops are much more likely to support mold growth. Gerbaldo et

al. (2012) found that *Lactobacillus rhamnosus* L60 and *L. fermentum* L23 were able to inhibit 90% growth of *A. flavus* (RC 2053 and RC 2055) and *A. parasiticus* (RC 2062) in De Man Rogosa Sharpe (MRS) agar.

1.4.2   Remove Aflatoxin Production

Kumar (2018) stated that aflatoxins can be detoxified or removed by physical, chemical or biological methods. However, it is hard to preserve the nutritional value of the food during the detoxification process (World Health Organization, 2018; Food and Agriculture Organization of the United Nations, 2004). The physical method of washing the crops can remove 40% of AFB1 (Kumar, 2018). Goldblatt (1971) reported that aflatoxins in oil seeds could be removed by extraction with various solvents, such as hexane, methanol, ethanol and acetone.

Previous studies also showed that LAB could bind aflatoxins on the cell surface, inhibit aflatoxin production, and internalize aflatoxins (Biernasiak et al.,2006; Peltonen et al., 2001). Huang et al. (2018) found that 38.38% of AFB1 was degraded by *Bacillus subtilis* (a probiotic bacteria) in the LB medium. Biernasiak et al. (2006) observed that *L. casei* (LOCK 0920), *L. brevis* (LOCK 0944), *L. plantarum* (LOCK 0945), and *Saccharomyces cerevisiae* (LOCK 0142) could decrease 29-49% of aflatoxins in barley and wheat flour-based medium after 24 hours of incubation. Peltonen et al. (2001) observed that 56.6-73.2% AFB1 were adsorbed by *L. amylovorus* and *L. rhamnosus* after 72 hours of incubation.

1.5   Method of Aflatoxin Detection

There are various methods to detect aflatoxins in food and feeds, such as gas chromatography (GC), thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), liquid chromatography-mass spectroscopy (LC-MS), and enzyme-linked immune-sorbent assay (ELISA) (Wacoo et al., 2014). The most simple and rapid detection method is thin-layer chromatography (TLC), which was developed in the 1960s, uses ultraviolet (UV)–vis spectral analysis to identify and quantitate aflatoxins (Wacoo et al., 2014; De Iongh et al., 1964; Iqbal et al., 2013). Thin-layer chromatography is the most popular and cheapest method, which can screen large amounts of samples (Iqbal et al., 2013).

The developed extraction method, QuEChERS (quick, easy, cheap, effective, rugged and safe), was commonly used for liquid and solid sample extraction from agricultural commodities. This method enhances the qualification of samples during analysis. Another detection method, HPLC, was used to detect aflatoxin fluorescence appearance under UV radiation by the equipped fluorescent detector or UV detector. However, AFB1 and AFG1 need to get derivatization by bromination or irradiation to be seen on chromatograms (Wacoo et al., 2014). Preparing the analysis samples before HPLC using the extraction and immunoaffinity column (IAC) can enhance the sensitivity for aflatoxins detection (Wacoo et al., 2014; Iqbal et al., 2013). Although HPLC may not be the best detection method, it provides high identified results (Iqbal et al., 2013).

The LC-MS method, which uses confirmatory assays, provides more specific, quantitative, and qualitative detection than HPLC and can test multiple mycotoxin contamination in a single sample (Wacoo et al., 2014; Iqbal et al., 2013). Another popular detection method, ELISA, is an antibody testing method using a primary antibody that explicitly targets the aflatoxin molecules and presents the fluorescents.

1.6   Aflatoxin Producers

The majority of aflatoxin producers are found in the *Aspergillus* section *Flavi* (Mannaa and Kim, 2017; IARC, 2002). Among these, *A. flavus* and *A. parasiticus* represent the most dominant aflatoxin producing species in this section. Both fungi mainly contaminated maize, rice, grapes, sorghum, and nuts (mostly peanuts, pistachio, and almond nuts) (Ribeiro et al., 2006; Mannaa and Kim, 2017; Eaton and Gallagher, 1994; Cotty and Jaime-Garcia, 2007; Battilani et al., 2020). Other aflatoxin producing species in the *Flavi* section include *A. nomius*, *A. pseudotamarii*, *A. bombysis*, *A. tamarii*, and *A. parvisclerotigenus* (Kumar, 2018; Cotty, P. J., and Jaime-Garcia, 2007; Sanchis and Magan, 2004; IARC, 2002). However, not all *Aspergillus* spp. in section *Flavi* produce aflatoxin. For example, *A. oryzae* and *A. sojae* do not produce any aflatoxin (Klich, 2007). There are several *Aspergillus* species outside the *Aspergillus Flavi* section, also known to make aflatoxins. These include *A. ochraceoroseus*, *A. rambellii*, *A. fumigatus*, *A. australis*, *Emericella astellata*, and *E. venezuelensis* (Frisvad et al., 2005; Kumar, 2018; Cary et al., 2005; Cottyand Jaime-Garcia, 2007; IARC, 2002).

1.7 Environmental Conditions Influence Aflatoxin Production

It is well known that mold growth and aflatoxin production are influenced by environmental factors such as temperature, water activity, relative humidity, and rainfall (Pardo et al., 2005; Ribeiro et al.,2006; Bowen and Hagan, 2015; Cotty and Jaime-Garcia, 2007). Among these environmental conditions, temperature and water activity (a_w) are the most critical factors influencing fungal growth and aflatoxin production (Marino et al., 2014; Pardo et al., 2005; Ali et al., 2013; Ribeiro et al., 2006; Mannaa and Kim, 2017; Pose et al., 2010; Cuero et al., 1987; Bouras et al., 2009; OBrian et al., 2007; Nazari et al., 2014). Moreover, temperature and water activity influenced the gene expression of the aflatoxin biosynthesis gene (Mannaa and Kim, 2017; Geiser et al., 2000; Yu et al., 2011).

1.7.1 Water Activity

Ribeiro et al. (2006) reported that barley rootlets with 0.80 a_w supported the best AFB1 production by *A. flavus* (NRRL 5520), followed by 0.90 and 0.95 a_w at 25°C respectively under incubation conditions at 0.80, 0.90, and 0.95 a_w at 25° and 30°C. The optimum water activity on polished rice for the growth of *A. flavus* (NRRL 2999) was 0.95 a_w (Sorenson et al., 1967). Lahouar et al. (2016) found that the optimum growth condition for three *A. flavus* strains was observed on sorghum seeds with 0.99 a_w at 37°C under incubation conditions range at 0.85-0.99 a_w at 15°, 25° and 37°C. The minimum water activity for the growth of *A. flavus* was 0.78–0.84 a_w; the minimum water activity for *A. parasiticus* was 0.81–0.82 a_w (Mannaa and Kim, 2017). The optimum water activity supported the most aflatoxin production by *A. flavus* and *A. parasiticus* was 0.99 a_w (Mannaa and Kim, 2017).

1.7.2 Temperatures

Temperature can influence both fungal growth and aflatoxin biosynthesis. Mannaa and Kim (2017) found that the optimum temperature for the growth of *A. flavus* was 35°C, and the optimum temperature for the biosynthesis of aflatoxins by *A. flavus* was 28°C. Moreover, the optimum temperature for the growth of *A. parasiticus* was 35°C, and the optimum temperature for the biosynthesis of aflatoxins by *A. parasiticus* was 33°C. Sorenson et al. (1967) observed that the optimum temperatures for aflatoxin B (184,000-760,000 µg/kg) and aflatoxin G (64,000-758,000

μg/kg) production by *A. flavus* (NRRL 2999) on rice were at 28°-32°C and 28°C respectively. Furthermore, they also stated that fewer AFB1 was found on rice at 34°C (18,000-29,000 μg/kg) and 37°C (300-700 μg/kg).

1.8 Aflatoxin Production on Oil Seeds

A previous study reported AFB1 contamination in broken rice (14-20 μg/L), corn grains (6-11 μg/L), corn gluten feed (6-57 μg/L), and cottonseed cake (6-10 μg/L) in Pakistan (Afzal et al., 1979). Another research group also observed aflatoxin contamination on peanut butter (82%) and sesame seeds (37%) in China (Li et al., 2009). Aflatoxins were also detected in oil after extracted from aflatoxin contaminated oil seeds. It has been found that 10-20% of the total aflatoxins present in the original oil seeds could be transferred to crude oil during the extraction process (Bordin et al., 2014). *Aspergillus* spp. often cause discoloration, necrosis, and morphological change on oil seeds (Bordin et al., 2014). Moreover, damaged feedstuffs or agricultural commodities have a higher incidence to support mold growth and aflatoxin production (Afzal et al., 1979). Since oil seeds and oil seed products are widely consumed, aflatoxin contamination on oil seeds, such as flax and niger seeds, is a global concern.

1.8.1 Oil Contents

Some studies showed that fungal growth and aflatoxin production on the oil seeds could be enhanced by fatty acids such as oleic acid, linoleic acid, and palmitic acid. The whole grains of niger seeds naturally contain 35-46% of oil, mainly linoleic acid (Mekonnen et al., 2018; Bhatnagar and Krishna, 2013; Dagne and Jonsson, 1997). However, the oil extraction method can only extract 31-42% of oil from niger seeds (Bhatnagar and Krishna, 2013). Different types of oil also influenced aflatoxin production. Liu et al. (2016) observed that a 3-fold decrease in AFB1 production was detected on defatted corn and defatted peanut as compared to full-fat corn (120 μg/kg) and full-fat peanut (10,140 μg/kg). Moreover, lipid oxidation can enhance aflatoxins biosynthesis (Bircan et al., 2006; Passi et al., 1984).

1.8.2 Flax Seeds

Flax seeds, also called golden linseeds, are seeds of the flax plant, *Linum usitatissimum*, which originated from Asia. Flax plants usually grow in moist soil composed of sand, silt and clay (Daun et al., 2003). Flax seeds have been considered functional food ingredients in recent years because they are rich in omega-3, lignans, digestible fibers, and high-quality proteins (Daun et al., 2003; Bernacchia et al., 2014). Researchers also stated that flax seeds have the function of preventing cardiovascular disease (Bernacchia et al., 2014). Flax seeds have about 38-45% oil content, and their fatty acid composition is mainly polyunsaturated fatty acids (United States Department of Agriculture, 2019; Pellizzon et al., 2007; Mercier et al., 2014). Flax seeds are also commonly added to baked goods, such as bread, pasta, cakes, muffins, cookies, and cereal bars (Mercier et al., 2014).

1.8.3 Niger Seeds

Niger seeds, also called Nyjer or thistle seeds, are oil seeds of the herb *Guizotia abyssinica*, which closely related to sunflowers (Gizachew et al., 2019b; Ramadan, 2012). Niger seeds are mainly produced and used as animal feeds in Ethiopia and India (Getinet and Sharma 1996). Niger seed oil is also used for cooking and accounted for 50-60% of Ethiopia's oil consumption (Mekonnen et al., 2018). After oil extraction (de-oil process), the seed residues, called noug cake, are usually used as feed supplements for dairy cows to increase milk production. However, noug cake has been found to contain high concentrations of aflatoxins (290-397 μg/kg). After dairy cows ingested aflatoxin contaminated noug cake, they produced AFM1 milk. Moreover, a previous study found that 100% of milk samples collected contained AFM1 in Ethiopia (Gizachew et al., 2016).

1.9 Objective

In order to control aflatoxin contamination on oil seeds and the products derived from these seeds, it is necessary to know the range of storage temperatures and water activities supporting fungal growth and aflatoxin formation. However, the environmental conditions that favor fungal growth and aflatoxin production on different oil seeds have not yet been extensively studied.

This research project studied the effect of temperature and water activity on growth and aflatoxin production by *A. flavus* and *A. parasiticus* on flax seeds and niger seeds. Additionally, the influence of oil content on growth and aflatoxin production by the two fungi on niger seeds under various growth conditions was also investigated.

MATERIALS AND METHODS

1.10 Fungal Cultures

Aspergillus flavus (NRRL 3357) and *A. parasiticus* (NRRL 465) were obtained from the US Department of Agriculture (USDA, Peoria, IL). Both fungi were grown on potato dextrose agar (PDA, Sigma-Aldrich, St. Louis, MO) or Czapek yeast extract agar (CYA) (Himedia laboratories pvt. ltd. Mumbai India) slants at 27°C for five days. The conidia suspension was prepared in an aqueous solution of 0.05% Tween 80 and harvested by scraping with a sterile inoculation loop on the surface of the slant. The final concentration of spore suspension was adjusted to 0.25 $OD_{540\ nm}$, which contained approximately 4.9-7.8 x 10^6 conidia/mL. The spore count in each inoculum was verified using a hemocytometer.

1.11 Preparation of Seed Samples

Flax and niger seeds were purchased from local stores in Indiana, USA. Both seeds were ground by a coffee grinder for five seconds twice and sterilized using autoclave at 121°C/ 15 psi for 15 mins before use. The oil of the niger seeds was extracted using a pre-heated automatic oil press machine (Vevor, CXZZC00, Shanghai, China). The seed residues were grounded into powder by a blender (Waring Commercial, USA) for five seconds twice and sterilized as described previously. The water activity value was determined using a water activity meter (Aqualab, Pullman, WA, USA). Initial water activity was 0.75 a_w after sterilization. The sterilized seed samples were adjusted to 0.82, 0.86, 0.90, 0.94 or 0.98 a_w by adding approximately 1.2, 1.7, 2.0, 3.7, and 8.0 mL sterile dH_2O respectively.

1.12 Oil Content Evaluation of Niger Seeds

The amount of oil naturally presented in niger seeds and oil remaining in the seed residues were determined using the Bligh and Dyer Lipid Extraction Method (Bligh and Dyer, 1959; Dasari and Goud, 2013). Ten grams of seed samples were mixed in a 90 mL chloroform/methanol mixture with volumetric ratios 1:2 (v/v) for 10 mins using a medium-high speed on a stir plate. Then, 30 mL chloroform and 30 mL dH_2O were added to the mixture and mixed for additional 10 mins. Finally, the seed mixture was filtered through a grade 1 (pore size 11µm) Whatman® Qualitative

Filter Paper with a vacuum pump. The filtrate was allowed to separate into two phases inside a separation funnel. The lower phase, which contained lipids, was transferred into a pre-weighed glass bottle. The lipid weight was measured, and then lipid content was calculated using the formula below (Dasari and Goud, 2013). The mean of triplicate measurements was used for calculation.

$$\text{Oil content (\%) = Weight of lipid/ Weight of seed sample x 100\%}$$

1.13 Inoculation, Incubation and Growth Measurement

The sterilized seeds (2.0 g) were placed in a 60mm petri dish (VWR, Cat. number: 470199-974). The inner diameter of the petri dish was 52mm. Each plate was point inoculated with 10 µL of spore suspension and incubated at 20°, 27°, and 35°C. In order to maintain the water activity of the seeds throughout the 30 days of incubation, each culture petri-dish was sealed with Corning Sealing Tape (Sigma–Aldrich, St. Louis, MO, USA) and incubated in a closed glass jar with a thick absorbent pad (Millipore Sigma™, Cat. numberAP30034P0). The diameter of mycelial growth on each plate was measured after incubation for 5, 10, 15, 20, 25, and 30 days.

1.14 Aflatoxins Extraction and Purification

The triplicate seed samples were collected after 5, 10, 15, 20, and 30 days from each incubation condition. All the seeds in the petri-dish were transferred into a 50 mL centrifuge tube containing 5 mL of 80% methanol. After shaking the tube at 200 rounds/min for 90 mins at room temperature, the seed suspension was filtered with a 0.22 µm membrane filter (Fisher, Cat. number: 09-719C). Then, triplicate filtrate samples collected from each incubation condition were combined into one, which was used for aflatoxin analysis.

One milliliter of the pooled sample was mixed with 4 mL PBS buffer and purified with the AflaTest immune-affinity column (VICAM, Cat. number: G1010), followed by an additional washing with 5 mL of PBS and 5mL of dH_2O. The flow rate of the purification process was controlled at one drop per second. The column was then extracted by 1.5 mL HPLC grade methanol (Fisher Scientific, USA).

1.15 Determination of Aflatoxin Production

The purified aflatoxin samples were detected and quantified using HPLC (Thermo Scientific, UltiMate 3000) equipped with a fluorescent detector. The chromatogram was recorded with excitation at 365 nm and emission at 455nm wavelength. The aflatoxin concentration was identified and quantified by peak area related to a standard curve of aflatoxin, which was prepared using 0, 5, 10, 20, 50, 100, 250, 500, and 1,000 μg/kg of *A. flavus* aflatoxin standard (Sigma-Aldrich, Milwaukee, WI, USA). The limit detection of aflatoxins was <1 μg/kg, and the limit quantification of aflatoxins was <5 μg/kg using this HPLC system.

1.16 Statistical Analysis

Two-way analysis of variance (ANOVA) of each incubation condition was used to compare the growth by the two fungal species under various combinations of water activity and incubation temperature. A p-value ≤0.05 was considered statistically significant.

1. Fungal culture and spore suspension

A. flavus *A. parasiticus*
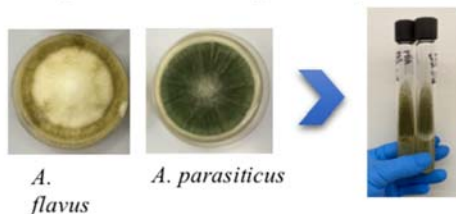
- *flavus* and *A. parasiticus* were grown on PDA or CYA slants.
- Spore suspensions were prepared with 0.05% Tween 80 and adjusted to 0.25 (OD540nm).

2. Oil Content Evaluation of Niger Seeds

- Niger seed oil was extracted from niger seeds using an automatic oil press machine.
- Oil content was determined using Bligh & Dyer method.

3. Preparation of seeds and inoculation

Sterilized seeds (flax, niger, and de-oiled niger seeds) were adjusted to different water activity values by adding sterile water.

Sterilized seeds (2g) were placed in petri-dish and point inoculated with 10 μL spore suspension.

All culture petri dishes were sealed with sealed tape and incubated in a closed jar with a thick absorbent pad .

4. Fungal growth measurement and AF analysis

Mycelial growth on each plate was recorded.

Aflatoxins were extracted by 80% methanol and filtered with a 0.22 μm filter.

Filtrates were purified using HPLC clean-up columns. Aflatoxins were quantified by HPLC.
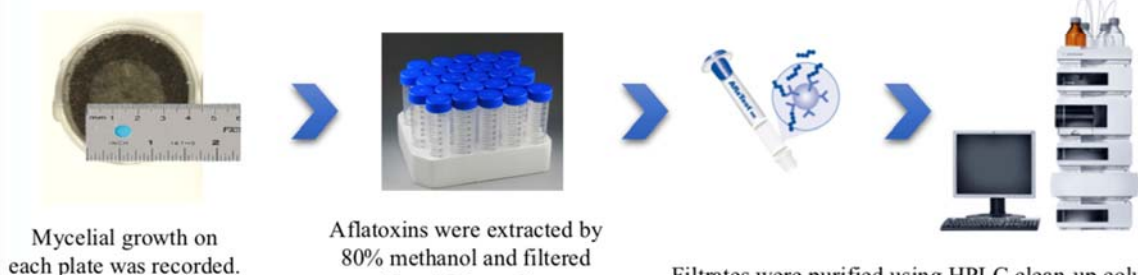
Figure 4. Flowchart of materials and method

CHAPTER 2.   RESULTS

2.1   Mycelial Growth on Flax Seeds

Figure 5 shows the mean colony diameters of *A. flavus* and *A. parasiticus* on ground flax seeds with 0.86, 0.90, 0.94, and 0.98 a_w at 20°, 27°, and 35°C. The optimum conditions for *A. flavus* to grow on flax seeds were 0.90 a_w at 27°C, 0.94 a_w at 27°C, and 0.94 a_w at 35°C (Figure 5b, c). The optimum growth condition for *A. parasiticus* to grow on flax seeds were 0.90 a_w at 35°C (Figure. 5b, c). No visible growth was detected on ground flax seeds with 0.82 a_w at all three temperatures throughout the 30 days of incubation (not shown in Figure 5).

On flax seeds with 0.86 a_w (Figure 5a), both fungi showed the most rapid growth at 35°C, followed by 27° and 20°C. At 35°C, confluent growth (52mm) of *A. flavus* and *A. parasiticus* were reached within 20 and 30 days respectively. At 27°C, *A. parasiticus* showed faster growth than did *A. flavus*; the mean colony diameters of the two fungi were 43 and 21mm respectively at the end of 30 days. Long lag phases for both fungi were observed at 20°C. No visible growth was detected at 20°C during the first 15 days. The mean colony diameters of *A. flavus* and *A. parasiticus* were 12 and 8mm respectively at the end of 30 days of incubation.

On flax seeds with 0.90 a_w (Figure 5b), *A. parasiticus* showed significantly faster growth at 35°C (P<0.028) as compared to 27° and 20°C. At 35°C, *A. parasiticus* showed slightly faster growth than *A. flavus,* and it reached confluent growth on day 15; whereas, *A. flavus* showed the confluent growth on day 20. At 27°C, *A. flavus* showed significantly rapid growth (P<0.02), on which confluent growth was detected on day 10, while the mean colony diameters of *A. parasiticus* were only 33mm at the end of 30-day incubation. At 20°C, neither fungi showed visible growth during the first 5 days, and then the mean colony diameters of *A. flavus* and *A. parasiticus* grew to 40 and 28mm respectively at the end of 30-day incubation.

On flax seeds with 0.94 a_w (Figure 5c), *A. flavus* grew much faster than did *A. parasiticus* at all three temperatures tested. *Aspergillus flavus* showed confluent growth on day 15 at 27° or 35°C, and on day 30 at 20°C; whereas, *A. parasiticus* reached confluent growth on day 30 at 27° or 35°C and did not grow at 20°C over the 30-day incubation period. It was also observed that *A. parasiticus* did not grow at 27°C during the first 10 days and then grew rapidly between 15 and 30

days of incubation. *Aspergillus parasiticus* showed a significant difference in colony diameters (p<0.0057) among three different temperatures.

On flax seeds with 0.98 a_w (Figure 5d), *A. flavus* grew more rapidly than did *A. parasiticus* at all three temperatures. *Aspergillus flavus* showed significantly faster growth than *A. parasiticus* at 20°C (P<0.03) and 27°C (P<0.02). The mean colony diameters of *A. flavus* reached 52, 47, and 22mm at 35°, 27°, and 20°C respectively at the end of a 30-day incubation. *Aspergillus parasiticus* showed a lag period with no visible growth during the first 10 and 20 days of incubation at 27° and 20°C respectively and the mean colony diameters were 41, 8, and 3mm at 35°, 27°, and 20°C respectively at the end of 30 days of incubation.
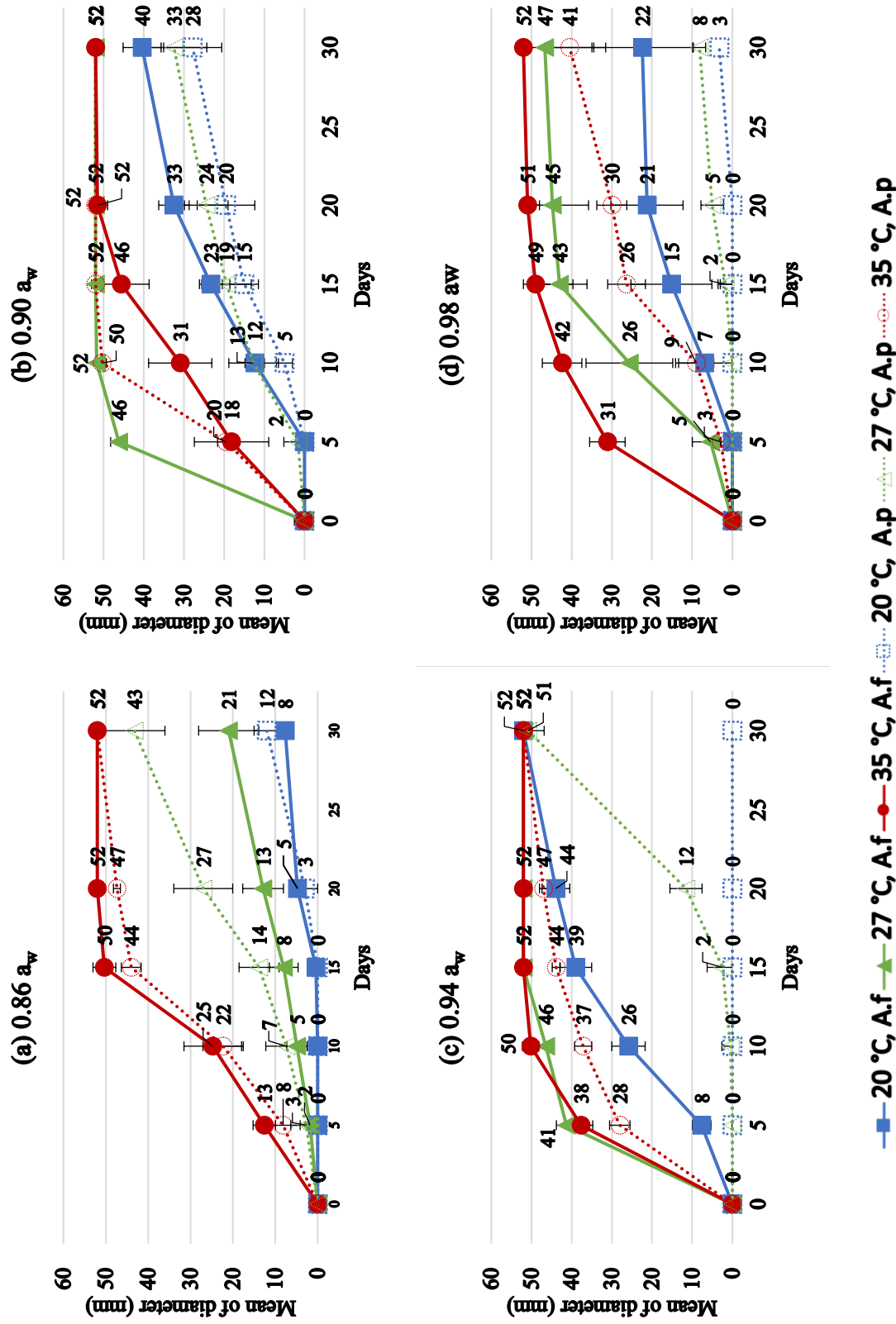
Figure 5. Mean colony diameters of *A. flavus* (A.f) and *A. parasiticus* (A.p) after incubation at 20°, 27°, and 35°C on ground flax seeds with (a) 0.86, (b) 0.90, (c) 0.94, and (d) 0.98 a_w for 30 days.

2.2 Aflatoxins Production on Flax Seeds

The study showed the effect of water activity and incubation temperature on AFB1, AFB2, AFG1, and AFG2 production by *A. flavus* and *A. parasiticus* on ground flax seeds. Aflatoxins were only found on ground flax seeds incubated under selected conditions in the study. Among the four types of aflatoxins, AFB1 and AFG1 were the two dominant aflatoxins produced by *A. flavus* and *A. parasiticus*. AFB2 concentrations were very low (<17 µg/kg) and AFG2 concentrations were all below quantifiable level (5 µg/kg) (Table 2). Therefore, only the data of AFB1, AFB2, and AFG1 are shown in Figure 6. In general, *A. flavus* showed better growth but produced lower levels of aflatoxins than did *A. parasiticus* on ground flax seeds.

A wide range of AFB1 (<5-247 µg/kg), AFG1 (<5-51 µg/kg), and AFB2 (<5–15 µg/kg) concentrations were found on flax seeds inoculated with *A. flavus* during the 30-day incubation at 27° and 35°C. Aflatoxins were not found on flax seeds with 0.86, 0.90, 0.94, or 0.98 a_w at 20°C, nor on flax seeds with 0.86, 0.90 or 0.98 a_w at 27°C. High levels of AFB1 (247, 232 µg/kg) produced by *A. flavus* were found on flax seeds with 0.90 a_w at 35°C on 20 and 30 days, respectively. The highest AFG1 (51 µg/kg) levels produced by *A. flavus* were also found on flax seeds with 0.90 a_w at 35°C incubated for 10-30 days. Less than 50 µg/kg of AFB1 was found on flax seeds with 0.90 and 0.94 a_w at 27°C (Table 2a, Figure 6a).

Various levels of AFB1 (<5-324 µg/kg), AFG1 (<5-165 µg/kg), and AFB2 (<5-18 µg/kg) were also detected from flax seeds inoculated with *A. parasiticus*. Aflatoxins were not found on flax seeds with 0.86 or 0.98 a_w incubated at 20°C nor on flax seeds with 0.90 or 0.94 a_w at 35°C. High concentrations of AFB1 (217-324 µg/kg) were detected from flax seeds with 0.86 and 0.98 a_w at 35°C and 0.94 a_w at 27°C. Additionally, 165 and 110 µg/kg of AFG1 were found on flax seeds with 0.90 a_w at 20°C and 0.86 a_w at 27°C respectively (Table 2b, Figure 6b).

Overall, *A. flavus* only produced a high level of total aflatoxins (>200 µg/kg) on ground flax seeds with 0.90 a_w at 35°C; whereas, *A. parasiticus* formed high levels of total aflatoxins (>200 µg/kg) on flax seeds with 0.86 and 0.98 a_w at 35°C, 0.90 a_w at 20°C, and 0.94 a_w at 27°C. It appears that high incubation temperature (35°C) was in favor of aflatoxin production by both fungi (Figure 6).

(a) *A. flavus*
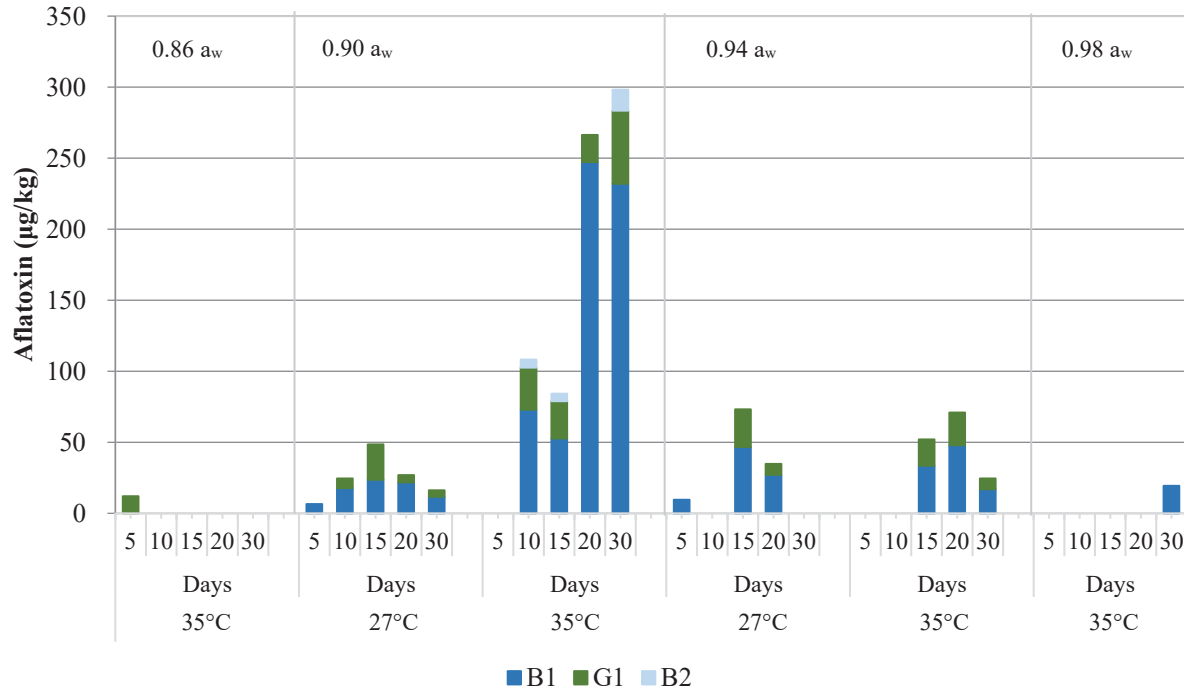
(b) *A. parasiticus*
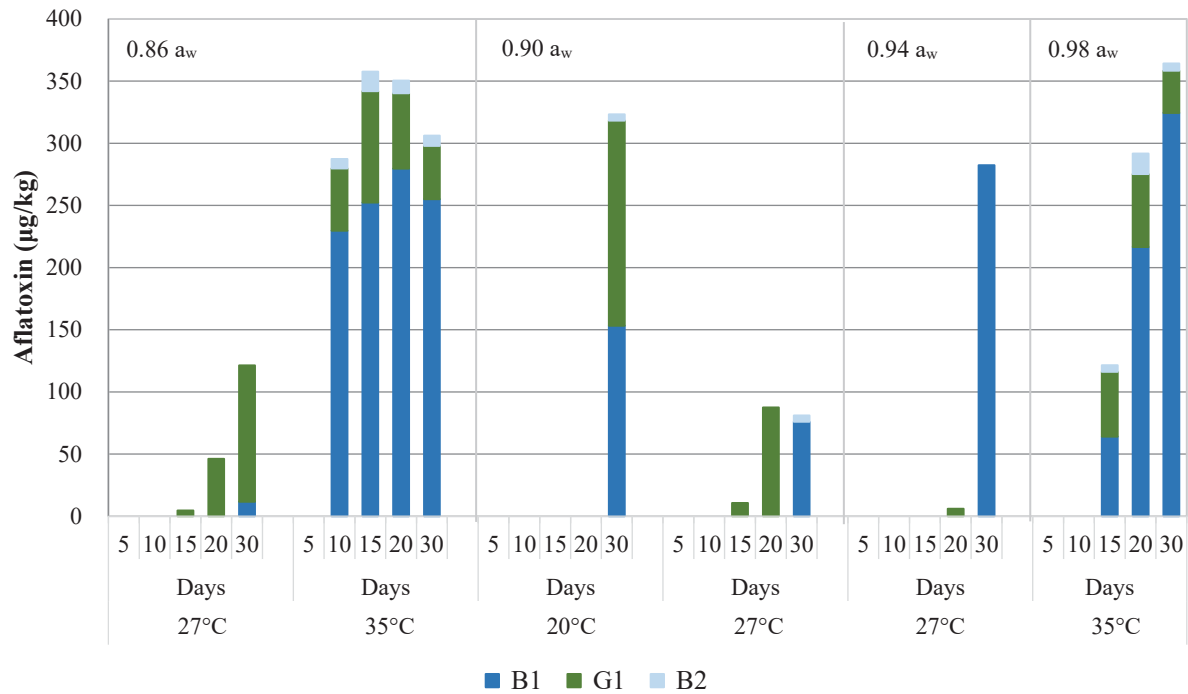
Figure 6. Aflatoxin production of the five sampling times (5, 10, 15, 20, and 30 days) by (a) *A. flavus* and (b) *A. parasiticus* at 20°, 27°, and 35°C on ground flax seeds with 0.86, 0.90, 0.94, and 0.98 a_w. Conditions that did not produce toxins were not included in Figure.

Table 2. Aflatoxins of the five sampling times produced by (a) *A. flavus* and (b) *A. parasiticus* at 20°, 27°, and 35°C on ground flax seeds with 0.86, 0.90, 0.94, and 0.98 a_w.

(a) *A. flavus*

Temp.	Day	0.86 a_w						0.90 a_w						0.94 a_w						0.98 a_w					
		Dia-meter	B1	G1	B2	G2	Total AFs	Dia-meter	B1	G1	B2	G2	Total AFs	Dia-meter	B1	G1	B2	G2	Total AFs	Dia-meter	B1	G1	B2	G2	Total AFs
20°C	5	0	0	0	0	0	0	0	0	0	0	0	0	15	0	0	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	11	0	0	0	0	0	38	0	0	0	0	0	4	0	0	0	0	0
	15	0	0	0	0	0	0	21	0	0	0	0	0	48	0	0	0	0	0	12	0	0	0	0	0
	20	0	0	0	0	0	0	28	0	0	0	0	0	49	0	0	0	0	0	17	0	0	0	0	0
	30	3	0	0	0	0	0	40	0	0	0	0	0	52	0	0	0	0	0	17	0	0	0	0	0
27°C	5	0	0	0	0	0	0	48	6	4	0	0	6	47	10	3	0	0	13	4	0	0	0	0	0
	10	2	0	0	0	0	0	52	18	7	1	0	18	52	3	0	0	0	3	28	0	0	0	0	0
	15	3	0	0	0	0	0	52	24	25	1	0	24	52	47	26	2	0	75	36	0	0	0	0	0
	20	8	0	0	0	0	0	52	22	5	1	0	22	52	27	8	1	0	36	38	0	0	0	0	0
	30	18	0	0	0	0	0	52	12	5	1	0	12	52	0	0	0	0	0	41	0	0	0	0	0
35°C	5	12	0	12	0	2	14	18	0	0	0	0	0	43	0	0	0	0	0	23	0	0	0	0	0
	10	25	0	0	0	0	0	30	73	30	6	2	111	50	0	0	0	0	0	36	0	0	0	0	0
	15	50	0	0	0	0	0	44	53	26	5	3	87	52	34	18	3	2	57	46	0	0	0	0	0
	20	52	0	0	0	0	0	51	247	19	4	0	270	52	48	23	4	0	75	50	0	0	0	0	0
	30	52	0	0	0	0	0	52	232	51	15	1	299	52	17	7	2	1	27	52	19	0	0	0	19

Not detectable level of aflatoxin (<1μg/kg). Not quantifiable level of aflatoxin (2-4 μg/kg).

Table 2. continued

(b) *A. parasiticus*

Temp.	Day	0.86 a_w						0.90 a_w						0.94 a_w						0.98 a_w					
		Dia-meter	B1	G1	B2	G2	Total AFs	Dia-meter	B1	G1	B2	G2	Total AFs	Dia-meter	B1	G1	B2	G2	Total AFs	Dia-meter	B1	G1	B2	G2	Total AFs
20°C	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	20	3	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	30	15	0	0	0	0	0	32	153	165	5	1	324	0	0	0	0	0	0	0	0	0	0	0	0
27°C	5	5	0	0	0	0	0	3	2.6	0	0	0	2.6	0	0	0	0	0	0	0	0	0	0	0	0
	10	10	0	0	0	0	0	11	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	15	18	0	5	0	0	5	19	0	11	0	0	11	5	0	0	0	0	0	1	0	0	0	0	0
	20	28	0	46	0	0	46	22	0	87	0	0	87	10	0	6	0	0	0	3	0	0	0	0	0
	30	44	12	110	0	0	122	28	76	4	5	0	85	50	282	0	2	0	284	6	0	0	0	0	0
35°C	5	16	0	0	0	0	0	39	3.5	0	0	0	3.5	39	0	0	0	0	0	0	0	0	0	0	0
	10	33	230	50	8	1	289	51	0	0	0	0	0	48	0	0	0	0	0	3	0	0	0	0	0
	15	51	252	90	16	1	359	52	0	0	0	0	0	51	0	0	0	0	0	16	64	52	5	3	124
	20	52	280	61	10	1	352	52	0	0	0	0	0	52	0	0	0	0	0	18	217	59	17	1	294
	30	52	255	43	8	0	306	52	0	0	0	0	0	52	0	0	0	0	0	29	324	34	6	1	365

Not detectable level of aflatoxin (<1μg/kg). Not quantifiable level of aflatoxin (2-4 μg/kg).

2.3   Mycelial Growth on Niger Seeds

Figure 7 shows the mean colony diameters of *A. flavus* and *A. parasiticus* on ground niger seeds (35% oil) with 0.86, 0.90, 0.94 and 0.98 a_w at 20°, 27°, and 35°C. The optimum conditions for *A. flavus* to grow on ground niger seeds were 0.90 and 0.94 a_w at 35°C as well as 0.94 and 0.98 a_w at 27°C (Figure 7b, c, d). The optimum conditions for *A. parasiticus* to grow on the ground niger seeds were 0.90 and 0.94 a_w at 35°C and also 0.94 a_w at 27°C (Figure 7b, c). Both fungi showed a 5 to 10 days lag phase at 0.86 a_w (Figure 7a) and did not grow at 0.82 a_w on ground niger seeds at all three temperatures throughout the 30-day incubation.

On niger seeds with 0.86 a_w (Figure 7a), both fungi grew most rapidly at 35°C, followed by 27° and 20°C. *Aspergillus parasiticus* only showed lag phase at 20°C, whereas *A. flavus* did not show any visible growth on day 5 at 35°C or day 10 at 27° or 20°C. At 35°C, although the two fungi did not grow at 0.86 a_w during the first 5 days, they still reached confluent growth on day 30. At 27°C, *A. parasiticus* showed a significantly faster growth (P<0.05) as compared to *A. flavus*. At 20°C, *A. parasiticus* showed a slightly faster growth on niger seeds than did *A. flavus,* and the mean colony diameters of *A. flavus* and *A. parasiticus* grew to 29 and 22mm respectively at the end of 30-day incubation.

On niger seeds with 0.90 a_w (Figure 7b), same as at 0.86 a_w, both fungi showed the most rapid growth at 35°C, followed by 27° and 20°C. At 35°C, *A. flavus* showed slightly faster growth than did *A. parasiticus*; the confluent growth was observed on 15-day and 30-day incubation respectively. At 27°C, confluent growth of *A. flavus* and *A. parasiticus* were reached within 15 and 20 days respectively. At 20°C, the mean colony diameters of *A. flavus* and *A. parasiticus* were 39 and 46mm respectively on 30-day.

On niger seeds with 0.94 a_w (Figure 7c), *A. flavus* and *A. parasiticus* showed similar growth at 27° and 35°C. At 35°C, the confluent growth of *A. flavus* and *A. parasiticus* were reached within 15 and 20 days respectively. At 27°C, confluent growth of *A. flavus* and *A. parasiticus* were reached within 10 and 15 days respectively. At 20°C, *A. flavus* showed a faster growth initially than did *A. parasiticus*, but *A. parasiticus* showed more rapid growth after 15-day incubation. The mean colony diameters of *A. flavus* and *A. parasiticus* were 48 and 50mm respectively at the end of 30 days of incubation.

On niger seeds with 0.98 a_w (Figure 7d), both fungi showed the most rapid growth on niger seeds at 27°C, followed by 20° and 35°C, respectively. At 35°C, both fungi grew rapidly initially,

and then the growth slowed down after 5 days of incubation. The mean colony diameters of *A. flavus* and *A. parasiticus* reached 31 and 36mm respectively at the end of incubation. The most rapid growth of *A. flavus* occurred on niger seeds at 27°C, on which confluent growth was detected on day 10. Only *A. flavus* on niger seeds at 27°C reached confluent growth at the end of 30-day incubation. At 27°C, the mean colony diameters of *A. parasiticus* was 48mm on day 30. At 20°C, the mean colony diameters of both fungi were 48mm after 30 days of incubation.

These results showed that at high temperature (35°C) with a low water activity (0.86 a_w) helped shorten the lag phase on niger seeds (Figure 7a). In contrast, it is possible that lower water activity needed more time to show visible growth.
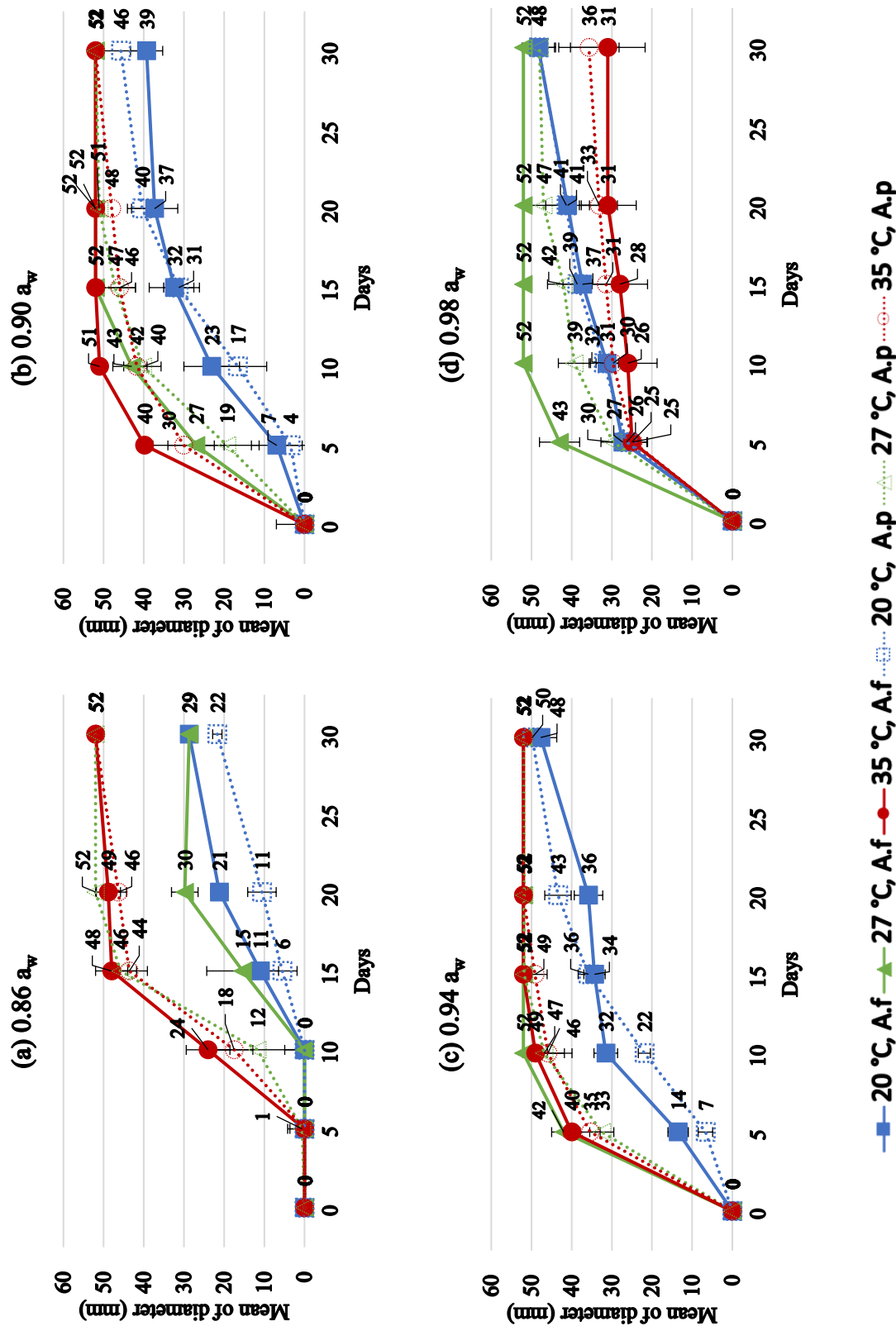
Figure 7. Mean colony diameters of *A. flavus* (A.f) and *A. parasiticus* (A.p) after incubation at 20°, 27°, and 35°C on ground niger seeds with (a) 0.86, (b) 0.90, (c) 0.94, and (d) 0.98 a_w for 30 days.

2.4   Aflatoxins Production on Niger Seeds

The study shows the effect of water activity and incubation temperature on AFB1, AFB2, AFG1, and AFG2 production by *A. flavus* and *A. parasiticus* on ground niger seeds. Aflatoxin B1 and G1 were the two major aflatoxins among the four types of aflatoxins produced by *A. flavus* and *A. parasiticus* detected on ground niger seeds. *Aspergillus flavus* and *A. parasiticus* showed similar growth patterns under most incubation conditions; however, the much higher aflatoxins produced by *A. parasiticus* were observed on niger seeds than did *A. flavus*.

As shown in Figure 8a (Table 3a), aflatoxins produced by *A. flavus* were only observed on ground niger seeds incubated under four conditions throughout the 30 days of incubation. Aflatoxins were only found on niger seeds with 0.90, 0.94, or 0.98 a_w at 27°C and 0.90 a_w at 35°C. An extensive variety concentration of AFB1(<5-270 µg/kg), AFG1 (<5-134 µg/kg), and AFB2 (<5–9 µg/kg) were found on niger seeds during 30 days of incubation. High levels of total aflatoxins (203-278 µg/kg) produced by *A. flavus* were detected on niger seeds with 0.90a_w at 27° and 35°C and also on seeds with 0.98 a_w at 35°C. The highest AFB1 (278 µg/kg) and AFG1 (134 µg/kg) produced by *A. flavus* were found on niger seeds with 0.90 a_w at 27°C. Low levels of AFB2 (<5-9 µg/kg) and AFG2 (< 5 µg/kg) were detected on niger seeds with 0.90 and 0.98 a_w at 27°C.

As shown in Figure 8b (Table 3b), a wide range of AFB1(<5-256 µg/kg), AFG1 (<5-150 µg/kg), AFB2 (<5–17 µg/kg), and AFG2 (<5-15 µg/kg) were found on niger seeds inoculated with *A. parasiticus* during 30 days of incubation. Aflatoxins were observed under all incubation conditions inoculated with *A. parasiticus* on niger seeds. High levels of total aflatoxins (≥200 µg/kg) produced by *A. parasiticus* were detected on niger seeds in 19 out of 60 samples (32%) extracted from each sampling incubation time. *Aspergillus parasiticus* did not produce any detectable aflatoxins on niger seeds in 16 out of 60 samples (27%) extracted from each sampling incubation time. Very high concentrations of AFB1 (200-256 µg/kg) were detected on niger seeds with 0.86, 0.90, 0.94, and 0.98 a_w at 27°C and also 0.98 a_w at 20°C as well as 0.86 and 0.90 a_w at 35°C. High levels of AFG1 (136, 121, and 129 µg/kg) produced by *A. parasiticus* were found on niger seeds with 0.86, 0.90, and 0.98 a_w at 27°C respectively.

Overall, the optimum condition for *A. flavus* to produce aflatoxins on ground niger seeds was 0.90a_w at 27°C. Whereas, *A. parasiticus* showed high levels of total aflatoxins production on niger seeds with 0.86 a_w at 27° and 35°C as well as 0.98 a_w at 20° and 27°C. Although *A. flavus*

and *A. parasiticus* showed similar growth patterns in most incubation conditions, the aflatoxins production had nothing in common.

(a) *A. flavus*
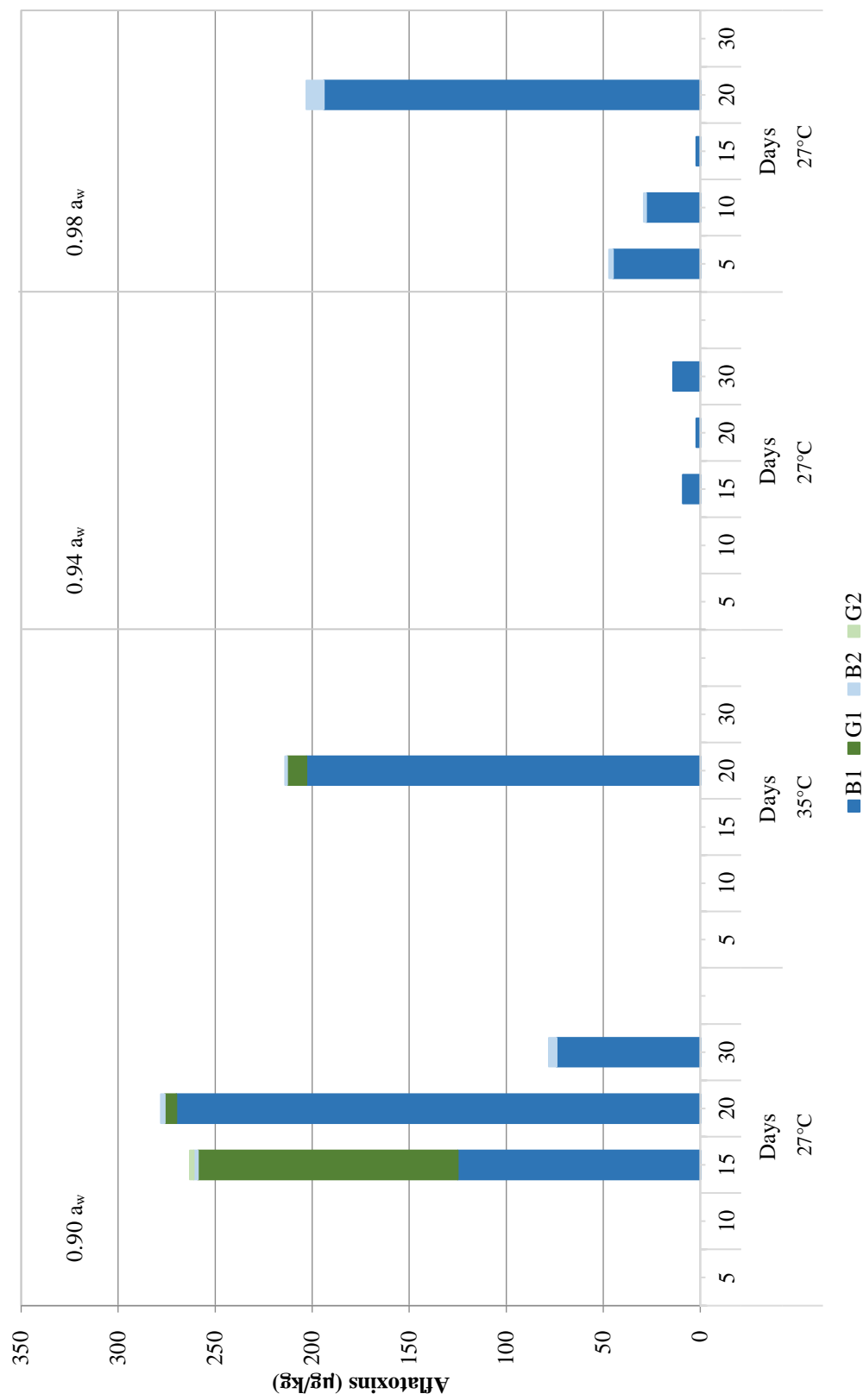
Figure 8. Aflatoxins produced by (a) *A. flavus* and (b) *A. parasiticus* on ground niger seeds with 0.86, 0.90, 0.94, and 0.98 a_w after incubation at 20°, 27°, and 35°C for 30 days.

Figure 8. continued
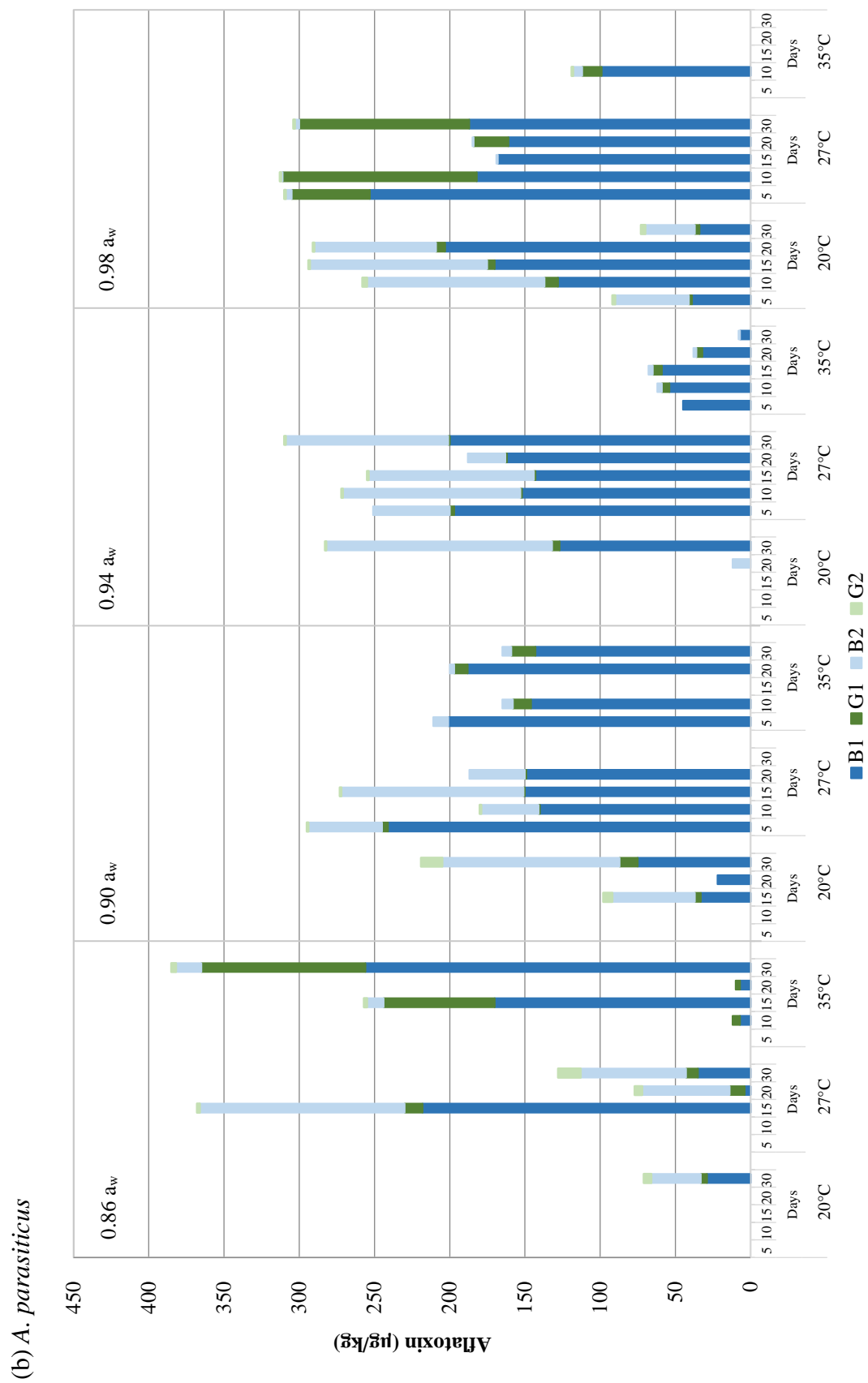
(b) *A. parasiticus*

Table 3. Aflatoxins of the five sampling times produced by (a) *A. flavus* and (b) *A. parasiticus* at 20°, 27°, and 35°C on ground niger seeds with 0.86, 0.90, 0.94, and 0.98 a$_w$.

(a) *A. flavus*

Temp.	Day	0.86 a$_w$						0.90 a$_w$						0.94 a$_w$						0.98 a$_w$					
		Dia-meter	B1	B2	G1	G2	Total AFs	Dia-meter	B1	B2	G1	G2	Total AFs	Dia-meter	B1	B2	G1	G2	Total AFs	Dia-meter	B1	B2	G1	G2	Total AFs
20°C	5	0	0	0	0	0	0	7	0	0	0	0	0	14	0	0	0	0	0	27	0	0	0	0	0
	10	0	0	0	0	0	0	23	0	0	0	0	0	32	0	0	0	0	0	31	0	0	0	0	0
	15	11	0	0	0	0	0	32	0	0	0	0	0	34	0	0	0	0	0	37	0	0	0	0	0
	20	21	0	0	0	0	0	37	0	0	0	0	0	36	0	0	0	0	0	41	0	0	0	0	0
	30	29	0	0	0	0	0	39	0	0	0	0	0	48	0	0	0	0	0	48	0	0	0	0	0
27°C	5	0	0	0	0	0	0	27	0	0	0	0	0	42	0	0	0	0	0	43	45	2	0	0	47
	10	0	0	0	0	0	0	43	0	0	0	0	0	52	0	0	0	0	0	52	28	1	0	0	29
	15	15	0	0	0	0	0	52	125	2	134	2	263	52	9	0	0	0	9	52	2	0	0	0	2
	20	30	0	0	0	0	0	52	270	2	6	0	278	52	2	0	0	0	2	52	194	9	0	0	203
	30	29	0	0	0	0	0	52	74	4	0	0	78	52	14	0	0	0	14	52	0	0	0	0	0
35°C	5	0	0	0	0	0	0	40	0	0	0	0	0	40	0	0	0	0	0	25	0	0	0	0	0
	10	24	0	0	0	0	0	51	0	0	0	0	0	49	0	0	0	0	0	26	0	0	0	0	0
	15	48	0	0	0	0	0	52	0	0	0	0	0	52	0	0	0	0	0	28	0	0	0	0	0
	20	49	0	0	0	0	0	52	203	1	10	0	214	52	0	0	0	0	0	31	0	0	0	0	0
	30	52	0	0	0	0	0	52	0	0	0	0	0	52	0	0	0	0	0	31	0	0	0	0	0

Not detectable level of aflatoxin (<1μg/kg). Not quantifiable level of aflatoxin (2-4 μg/kg).

Table3. continued

(b) *A. parasiticus*

Temp.	Day	0.86 aw						0.90 aw						0.94 aw						0.98 aw					
		Diameter	B1	B2	G1	G2	Total AFs	Diameter	B1	B2	G1	G2	Total AFs	Diameter	B1	B2	G1	G2	Total AFs	Diameter	B1	B2	G1	G2	Total AFs
20°C	5	0	0	0	0	0	0	4	0	0	0	0	0	7	0	0	0	0	0	26	39	2	49	2	93
	10	0	0	0	0	0	0	17	0	0	0	0	0	22	0	0	0	0	0	32	128	9	118	3	257
	15	6	0	0	0	0	0	31	33	4	55	6	99	36	0	0	0	0	0	39	170	5	118	1	294
	20	11	0	0	0	0	0	40	22	0	0	0	22	43	0	0	12	0	12	41	203	6	81	1	290
	30	22	29	4	33	5	72	46	75	12	118	14	220	50	127	5	150	1	283	48	34	3	33	3	73
27°C	5	0	0	0	0	0	0	19	241	4	49	1	295	33	197	3	51	0	251	30	253	4	52	1	310
	10	12	0	0	0	0	0	40	140	1	38	1	180	47	152	1	118	1	271	39	182	1	129	1	313
	15	46	218	12	136	2	367	47	150	1	121	1	273	52	143	1	110	1	255	42	168	1	0	0	169
	20	52	4	10	58	5	77	51	149	1	37	0	188	52	162	1	25	0	188	47	161	1	23	0	185
	30	52	35	8	70	15	127	52	0	0	0	0	0	52	200	1	108	1	311	48	187	3	113	1	303
35°C	5	0	0	0	0	0	0	30	201	10	0	0	212	35	45	0	0	0	46	25	0	0	0	0	0
	10	18	7	0	5	0	12	42	146	7	12	0	166	46	54	3	5	0	62	30	99	6	13	1	119
	15	44	170	11	74	2	258	46	0	0	0	0	0	49	59	3	6	0	69	31	0	0	0	0	0
	20	46	7	0	3	0	11	48	188	3	9	0	200	52	32	2	4	0	37	33	0	0	0	0	0
	30	54	256	17	109	3	384	52	143	6	16	0	165	52	7	1	0	0	8	36	0	0	0	0	0

Not detectable level of aflatoxin (<1µg/kg). Not quantifiable level of aflatoxin (2-4 µg/kg).

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2.5   Mycelial Growth on De-oiled Niger Seeds

The oil content in de-oiled niger seeds was determined as 9.3, 11.4, and 10.7% from the three trials. The mean oil (lipid) content of de-oiled niger seeds was 10.5% after the oil extraction process.

Figure 9 shows the mean colony diameters of *A. flavus* and *A. parasiticus* on de-oiled niger seeds with 0.86, 0.90, 0.94, and 0.98 a_w at 20°, 27°, and 35°C. The most rapid growth of *A. flavus* and *A. parasiticus* occurred on seeds with 0.90 and 0.94 a_w at 35°C (Figure 9c). No visible growth was detected on seeds with 0.82 a_w at all three temperatures throughout the 30 days of incubation (not shown in Figure 9).

On de-oiled niger seeds with 0.86 a_w (Figure 9a), the incubation temperatures significantly influenced the growth of *A. flavus* ($P< 0.023$) and *A. parasiticus* ($P<0.043$). *Aspergillus flavus* and *A. parasiticus* showed similar growth curves at each temperature. The most rapid growth for both fungi was found at 35°C, followed by 27° and 20°C respectively. At 35°C, confluent growth of *A. flavus* and *A. parasiticus* were reached within 20 and 30 days respectively. At 27°C, the mean colony diameters of *A. flavus* and *A. parasiticus* grew to 46 and 41mm respectively at the end of 30- day incubation. Both fungi showed a lag phase period with no visible growth during the first 10 days of incubation at 20°C.

On de-oiled niger seeds with 0.90 a_w (Figure 9b), both fungi showed the confluent growth at 27° and 35°C during 30 days of incubation. However, at the beginning of the incubation, *A. flavus* showed better growth at 20°C, followed by 35° and 27°C. *Aspergillus parasiticus* has a faster growth at 35°C initially, followed by 20° and 27°C. At 35°C, confluent growth of *A. flavus* and *A. parasiticus* were reached within 20 and 15 days respectively. At 27°C, confluent growth of *A. flavus* and *A. parasiticus* were reached within 30 and 20 days respectively. Interestingly, *A. flavus* showed a lag phase at 27°C during the first 5 days of incubation. *Aspergillus parasiticus* also showed slower growth at the beginning of incubation at 27°C, but no lag phase was observed. At 20°C, the mean colony diameters of *A. flavus* and *A. parasiticus* were 43 and 37mm respectively at the end of 30 days of incubation.

On de-oiled niger seeds with 0.94 a_w (Figure 9c), both fungi showed very similar growth among all three temperatures. At 35°C, the confluent growth of both fungi was reached within 20 days of incubation. At 27°C, *A. flavus* grew faster than did *A. parasiticus* at the beginning of 5-day incubation; however, *A. parasiticus* showed better growth than did *A. flavus* at 10-day

incubation. Both fungi reached confluent growth at the end of the 30-day incubation. At 20°C, *A. parasiticus* showed faster growth than did *A. flavus* at the beginning. Confluent growth of *A. flavus* and *A. parasiticus* were reached within 20 and 30 days respectively.

On de-oiled niger seeds with 0.98 a_w (Figure 9d), neither fungi reached confluent growth at the end of 30 days of incubation under all incubation temperatures. The mean colony diameters of *A. flavus* were 33, 47, and 36mm at 35°, 27°, and 20°C respectively at the end of 30-day incubation. On the other hand, the mean colony diameters of *A. parasiticus* were 34, 48, and 41mm at 35°, 27°, and 20°C respectively. Both fungi showed the most rapid growth at 27°C, followed by 20° and 35°C.

Overall, de-oiled niger seeds with 0.86, 0.90 and 0.94 a_w at high temperature (35°C) supported rapid growth, whereas on seeds with a high water activity (0.98 a_w) at 27°C also promote fast growth.
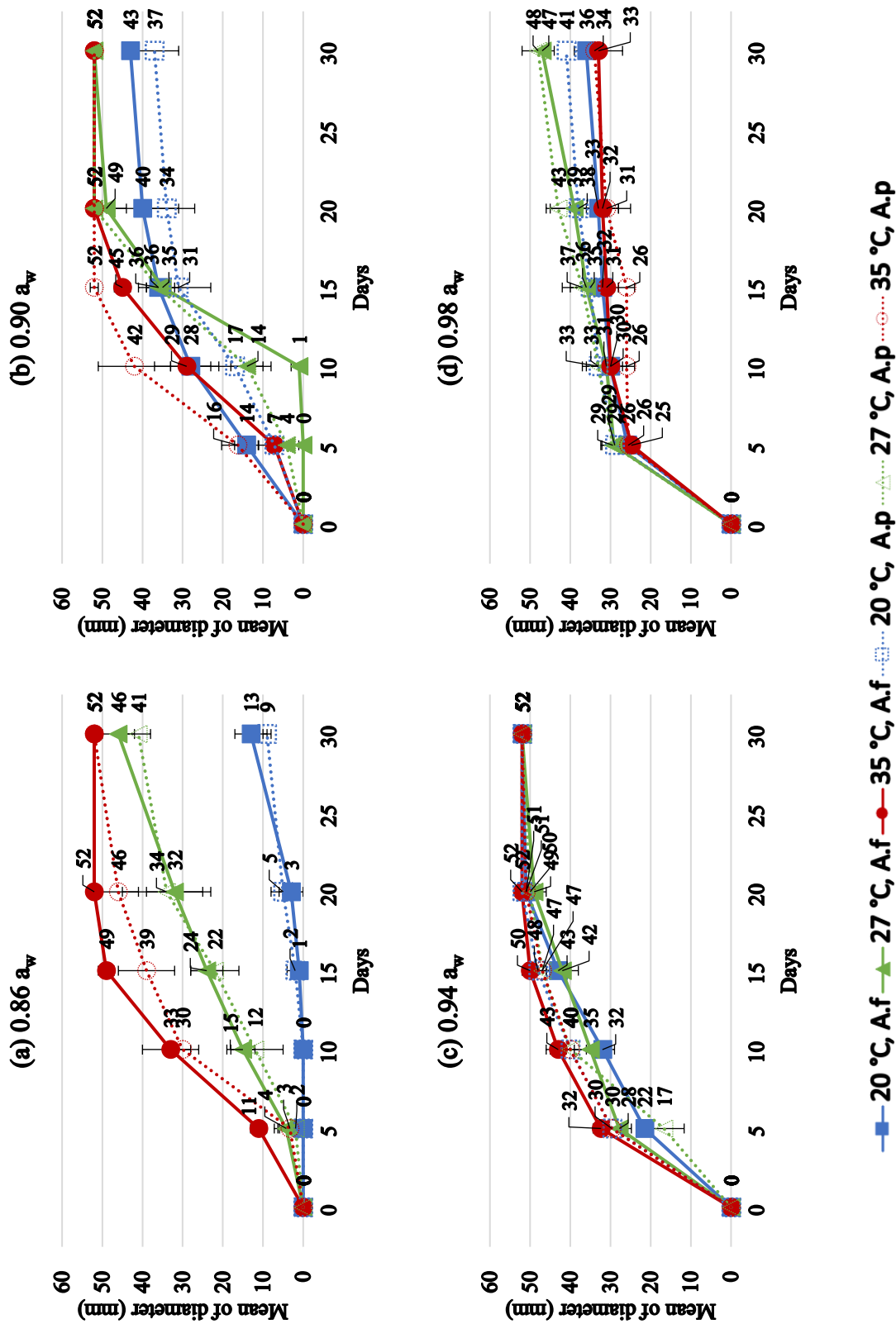
Figure 9. Mean colony diameters of *A. flavus* (A.f) and *A. parasiticus* (A.p) after incubation at 20°, 27°, and 35°C on de-oiled niger seeds with (a) 0.86, (b) 0.90, (c) 0.94, and (d) 0.98 a_w for 30 days.

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2.6 Aflatoxins Production on De-oiled Niger Seeds

The study showed the effect of water activity and incubation temperature on AFB1, AFB2, AFG1, and AFG2 production by *A. flavus* and *A. parasiticus* on de-oiled niger seeds. Aflatoxins were only found on de-oiled niger seeds incubated under selected conditions in this study. *Aspergillus flavus* only produced a low level of AFB1 (13 µg/kg) on de-oiled niger seeds with 0.94 a_w at 27°C after incubation for 30 days (Figure 10a, Table 4a). *Aspergillus parasiticus* produced a higher level of aflatoxins than did *A. flavus* on de-oiled niger seeds. As shown in (Figure 10b, Table 4b), a wide range of AFB1 (6-175 µg/kg), AFG1 (<5-190 µg/kg), AFB2 (<5–10 µg /kg), and a trace amount of AFG2 (≤5 µg/kg) were found on seeds inoculated with *A. parasiticus* during the 30-day incubation. There was no toxin production by *A. parasiticus* on seeds with 086, 0.90, 0.94, or 0.98 a_w at 20°C or 0.94 a_w at 35°C, nor on seeds with 0.98 a_w at all three temperatures. *Aspergillus parasiticus* produced high levels of total aflatoxins (332, 345 µg/kg) on seeds with 0.90 and 0.94 a_w at 27°C respectively. Moreover, *A. parasiticus* produced high levels of AFB1 (175 µg/kg) and AFG1 (190 µg/kg) with 0.90 a_w at 27°C. Furthermore, *A. parasiticus* also produced high levels of AFB1 (149 and 153 µg/kg) and AFG1 (170 and 190 µg/kg) with 0.94 a_w at 27°C on 20-day and 30-day.

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Figure 10. Aflatoxin production of the five sampling times (5, 10, 15, 20, and 30 days) by (a) *A. flavus* and (b) *A. parasiticus* at 20°, 27°, and 35°C on de-oiled niger seeds with 0.86, 0.90, 0.94, and 0.98 a_w. (The conditions did not produce toxin were not shown in Figure)

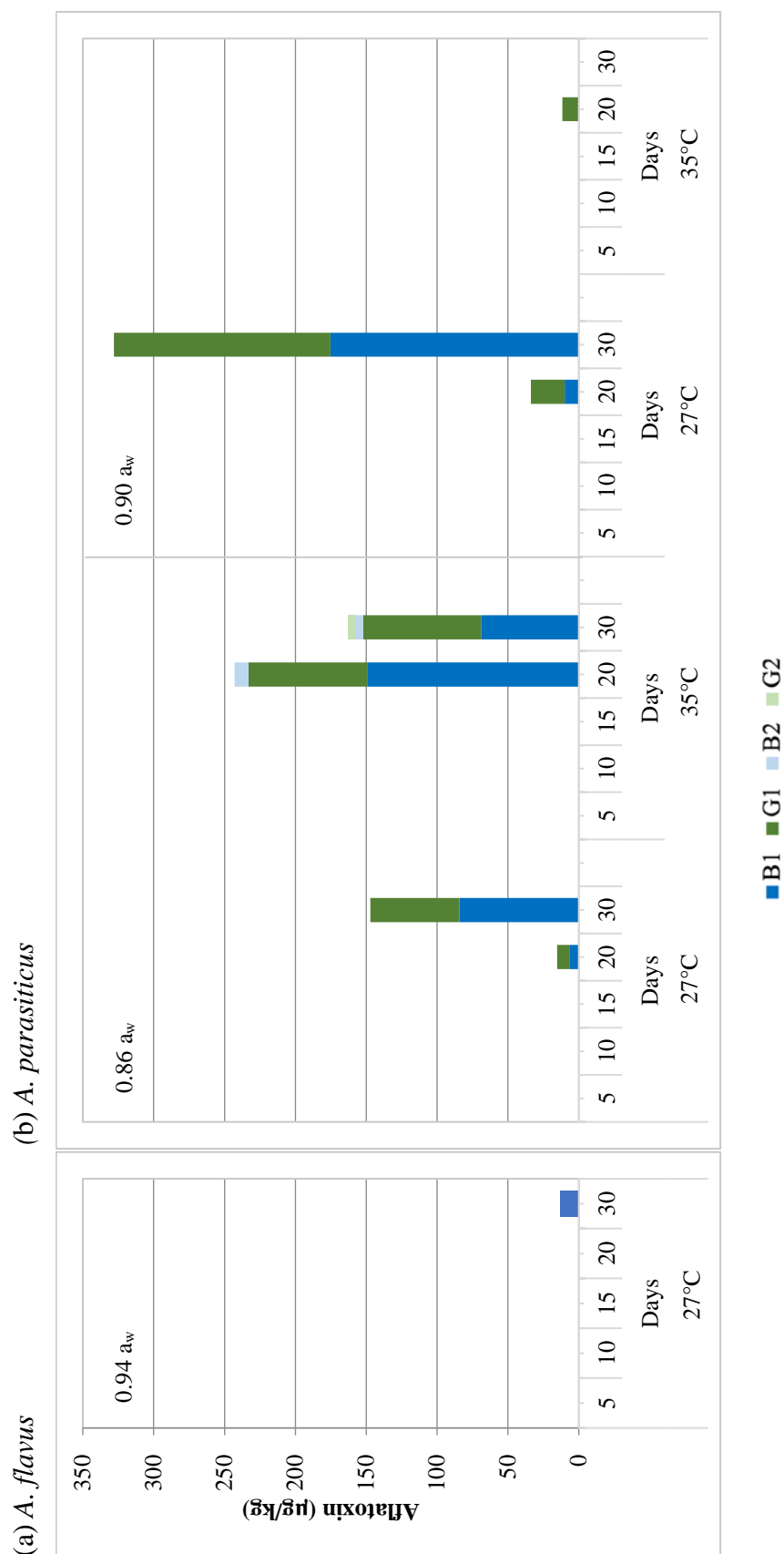Table 4. Aflatoxins of the five sampling times produced by (a) *A. flavus* and (b) *A. parasiticus* at 20°, 27°, and 35°C on de-oiled niger seeds with 0.86, 0.90, 0.94, and 0.98 a_w.

(a) *A. flavus*

Temp.	Day	0.86 a_w						0.90 a_w						0.94 a_w						0.98 a_w					
		Diameter	B1	B2	G1	G2	Total AFs	Diameter	B1	B2	G1	G2	Total AFs	Diameter	B1	B2	G1	G2	Total AFs	Diameter	B1	B2	G1	G2	Total AFs
20°C	5	0	0	0	0	0	0	14	0	0	0	0	0	22	0	0	0	0	0	26	0	0	0	0	0
	10	0	0	0	0	0	0	28	0	0	0	0	0	32	0	0	0	0	0	30	0	0	0	0	0
	15	1	0	0	0	0	0	36	0	0	0	0	0	43	0	0	0	0	0	32	0	0	0	0	0
	20	3	0	0	0	0	0	40	0	0	0	0	0	51	0	0	0	0	0	33	0	0	0	0	0
	30	13	0	0	0	0	0	43	0	0	0	0	0	52	0	0	0	0	0	36	0	0	0	0	0
27°C	5	4	0	0	0	0	0	0	0	0	0	0	0	28	0	0	0	0	0	29	0	0	0	0	0
	10	15	0	0	0	0	0	1	0	0	0	0	0	35	0	0	0	0	0	31	0	0	0	0	0
	15	24	0	0	0	0	0	35	0	0	0	0	0	42	0	0	0	0	0	36	0	0	0	0	0
	20	32	0	0	0	0	0	49	0	0	0	0	0	49	0	0	0	0	0	39	0	0	0	0	0
	30	46	0	0	0	0	0	52	0	0	0	0	0	52	0	0	0	13	13	47	0	0	0	0	0
35°C	5	11	0	0	0	0	0	7	0	0	0	0	0	32	0	0	0	0	0	25	0	0	0	0	0
	10	33	0	0	0	0	0	29	0	0	0	0	0	43	0	0	0	0	0	30	0	0	0	0	0
	15	49	0	0	0	0	0	45	0	0	0	0	0	50	0	0	0	0	0	31	0	0	0	0	0
	20	52	0	0	0	0	0	52	0	0	0	0	0	52	0	0	0	0	0	32	0	0	0	0	0
	30	52	0	0	0	0	0	52	0	0	0	0	0	52	0	0	0	0	0	33	0	0	0	0	0

Not detectable level of aflatoxin (<1μg/kg). Not quantifiable level of aflatoxin (2-4 μg/kg).

Table 4. continued

(b) *A. parasiticus*

Temp.	Day	0.86 aw						0.90 aw						0.94 aw						0.98 aw					
		Dia-meter	B1	B2	G1	G2	Total AFs	Dia-meter	B1	B2	G1	G2	Total AFs	Dia-meter	B1	B2	G1	G2	Total AFs	Dia-meter	B1	B2	G1	G2	Total AFs
20°C	5	0	0	0	0	0	0	7	0	0	0	0	0	30	0	0	0	0	0	29	0	0	0	0	0
	10	0	0	0	0	0	0	17	0	0	0	0	0	40	0	0	0	0	0	33	0	0	0	0	0
	15	2	0	0	0	0	0	31	0	0	0	0	0	48	0	0	0	0	0	35	0	0	0	0	0
	20	5	0	0	0	0	0	34	0	0	0	0	0	52	0	0	0	0	0	38	0	0	0	0	0
	30	9	0	0	0	0	0	37	0	0	0	0	0	52	0	0	0	0	0	41	0	0	0	0	0
27°C	5	2	0	0	0	0	0	4	0	0	0	0	0	17	0	0	0	0	0	29	0	0	0	0	0
	10	12	0	0	0	0	0	14	0	0	0	0	0	40	0	0	0	0	0	33	0	0	0	0	0
	15	22	0	0	1	1	2	36	0	3	0	0	0	47	0	7	0	1	8	37	0	0	0	0	0
	20	34	6	9	1	2	18	52	10	24	2	4	40	50	153	170	4	1	329	43	0	0	0	0	0
	30	41	84	63	4	0	152	52	175	153	3	1	332	52	149	190	4	2	345	48	0	0	0	0	0
35°C	5	3	0	0	0	0	0	16	0	0	0	0	0	30	0	0	0	0	0	26	0	0	0	0	0
	10	30	0	0	0	0	0	42	0	0	0	0	0	40	0	0	0	0	0	26	0	0	0	0	0
	15	39	0	0	0	0	0	52	0	0	0	0	0	47	0	0	0	0	0	26	0	0	0	0	0
	20	46	149	84	10	2	245	52	0	12	0	0	12	51	0	0	0	0	0	31	0	0	0	0	0
	30	52	69	83	6	5	163	52	0	4	0	0	4	52	0	0	0	0	0	34	0	0	0	0	0

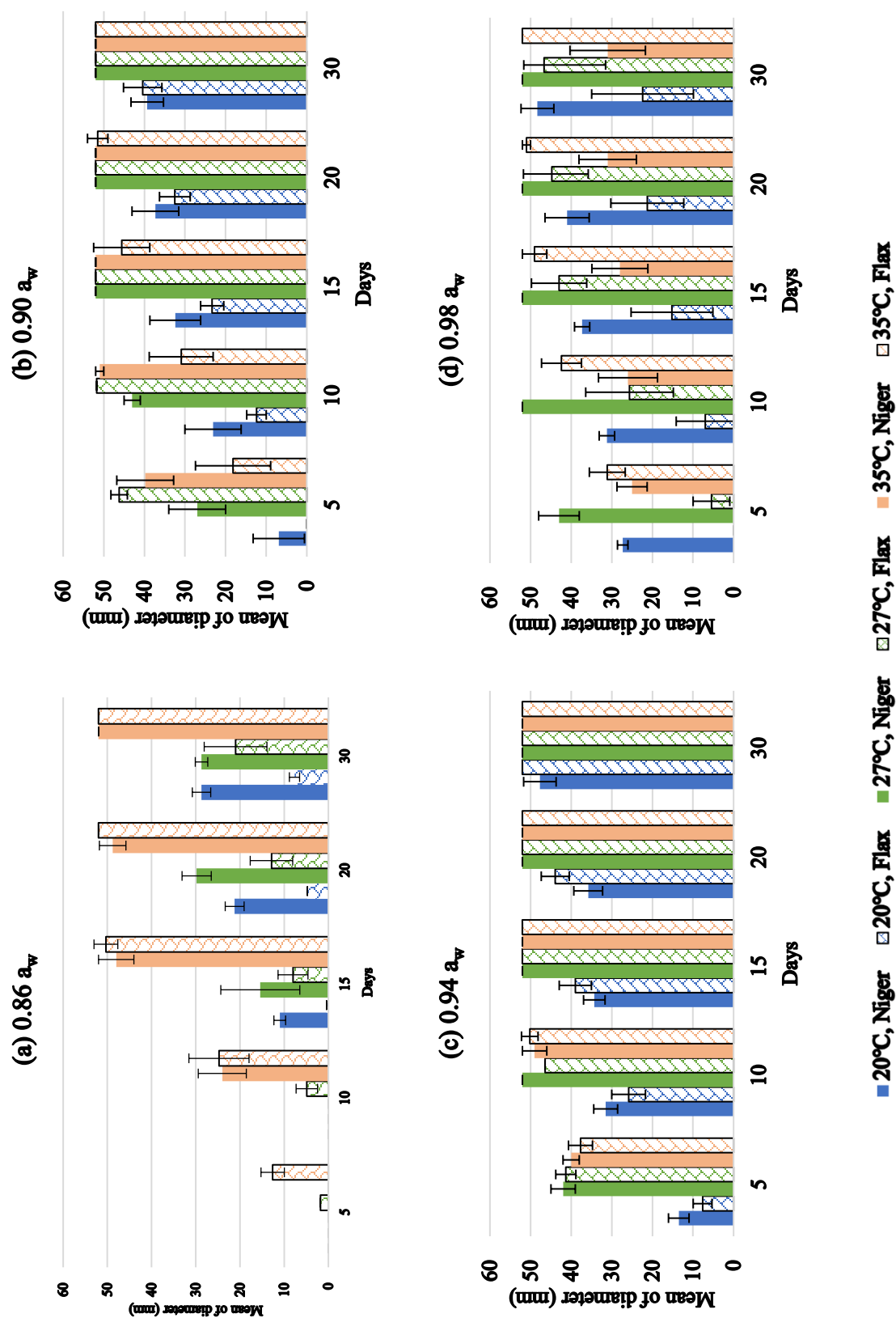Not detectable level of aflatoxin (<1μg/kg). Not quantifiable level of aflatoxin (2-4 μg/kg).

Figure 11. Mean colony diameters of *A. flavus* after incubation at 20°, 27°, and 35°C on ground flaxseeds and niger seeds with (a) 0.86, (b) 0.90, (c) 0.94, and (d) 0.98 a_w for 30 days.
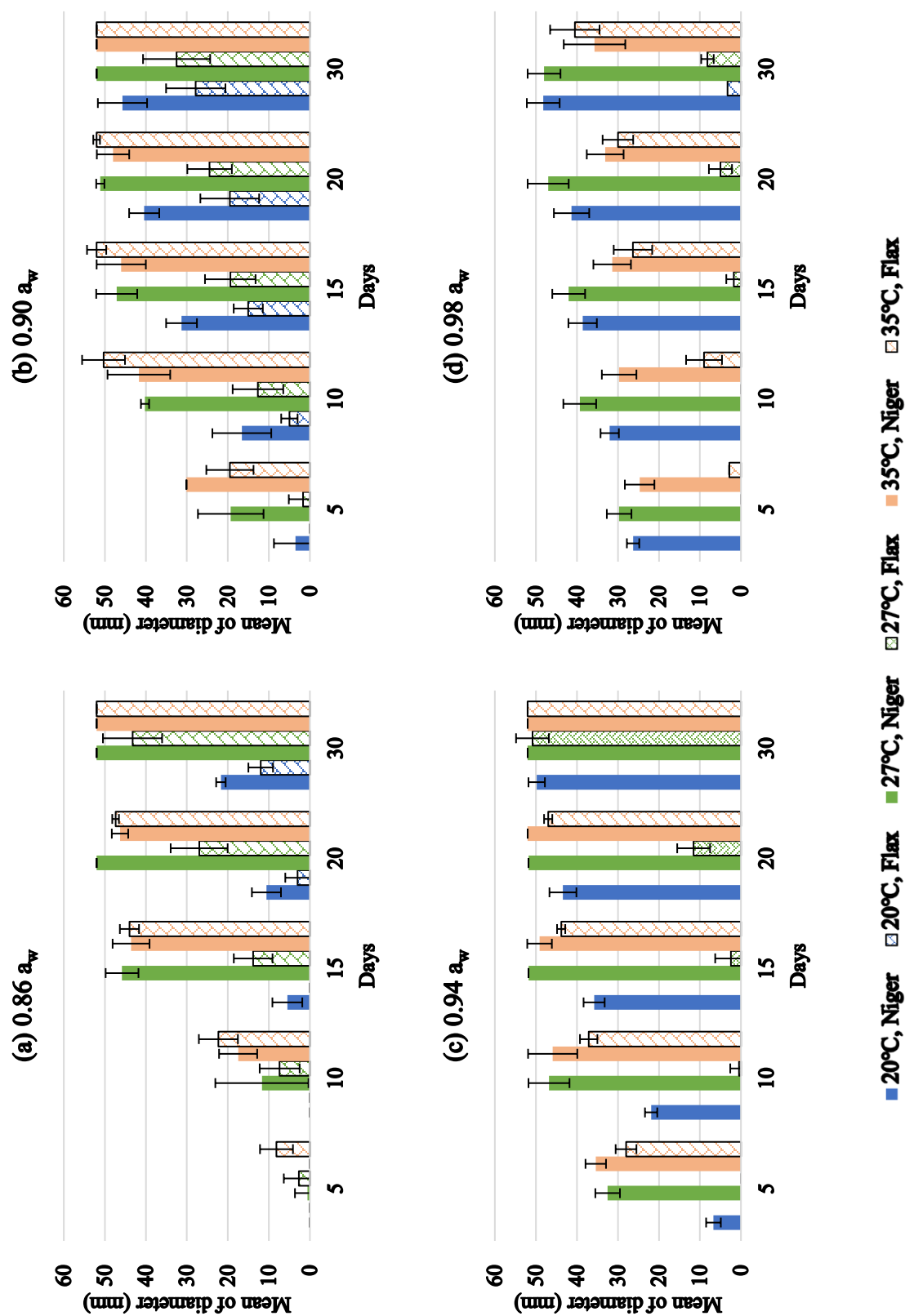
Figure 12. Mean colony diameters of *A. parasiticus* after incubation at 20°, 27°, and 35°C on ground flaxseeds and niger seeds with (a) 0.86, (b) 0.90, (c) 0.94, and (d) 0.98 a_w for 30 days.

Figure 13. Mean colony diameters of *A. flavus* after incubation at 20°, 27°, and 35°C on ground niger seeds and de-oiled niger seeds with (a) 0.86, (b) 0.90, (c) 0.94, and (d) 0.98 a_w for 30 days.

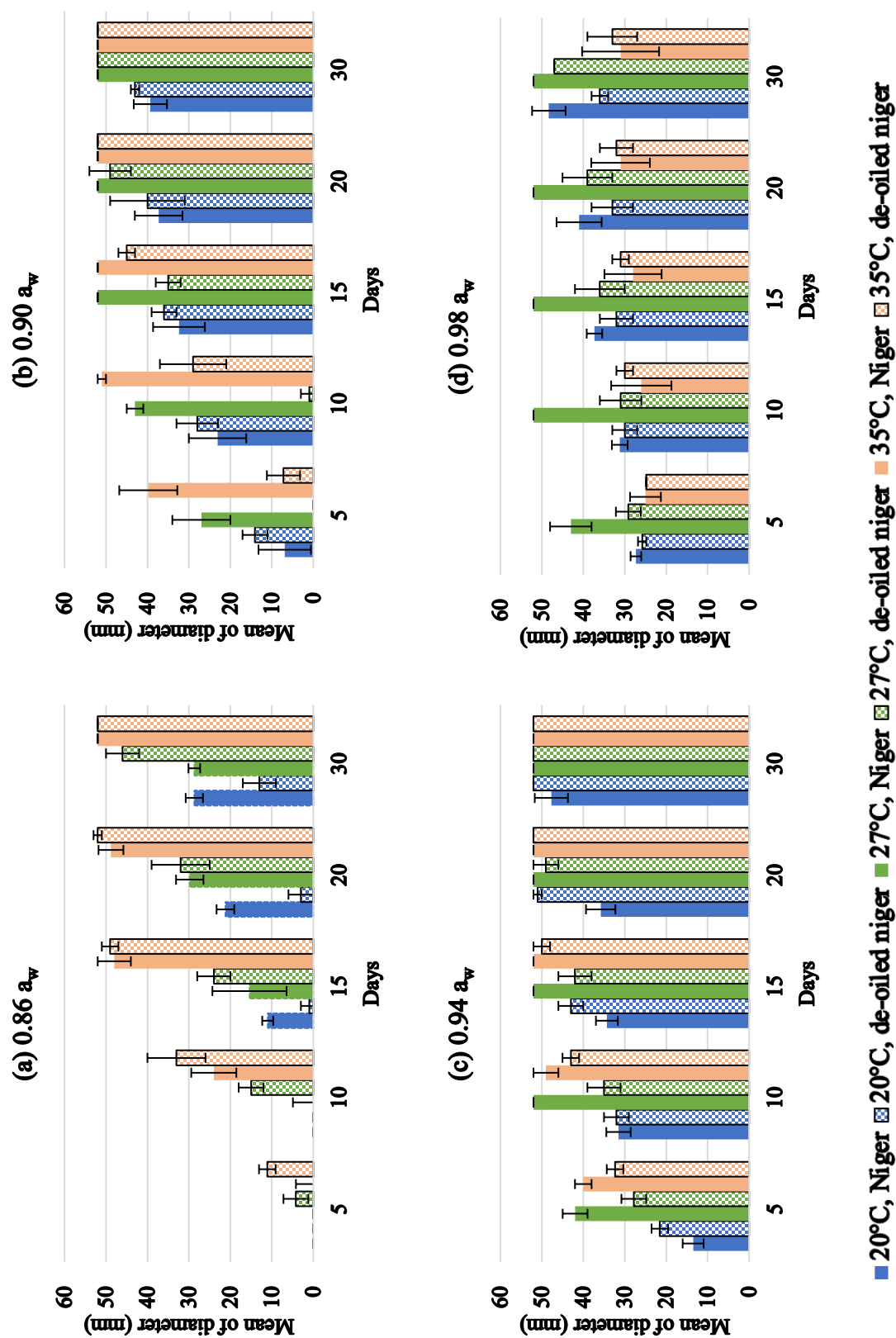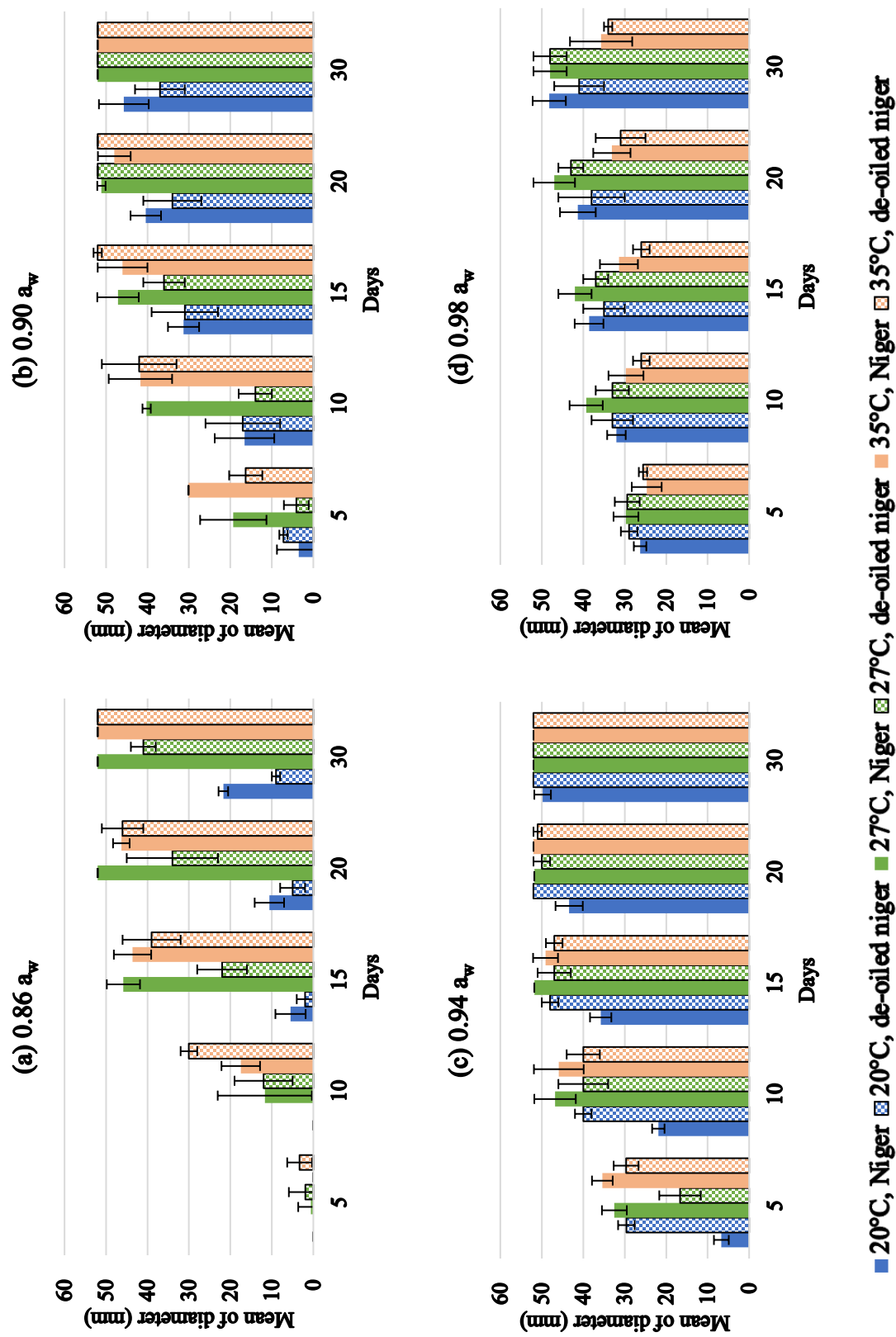Figure 14. Mean colony diameters of *A. parasiticus* after incubation at 20°, 27°, and 35°C on ground niger seeds and de-oiled niger seeds with (a) 0.86, (b) 0.90, (c) 0.94, and (d) 0.98 a_w for 30 days.
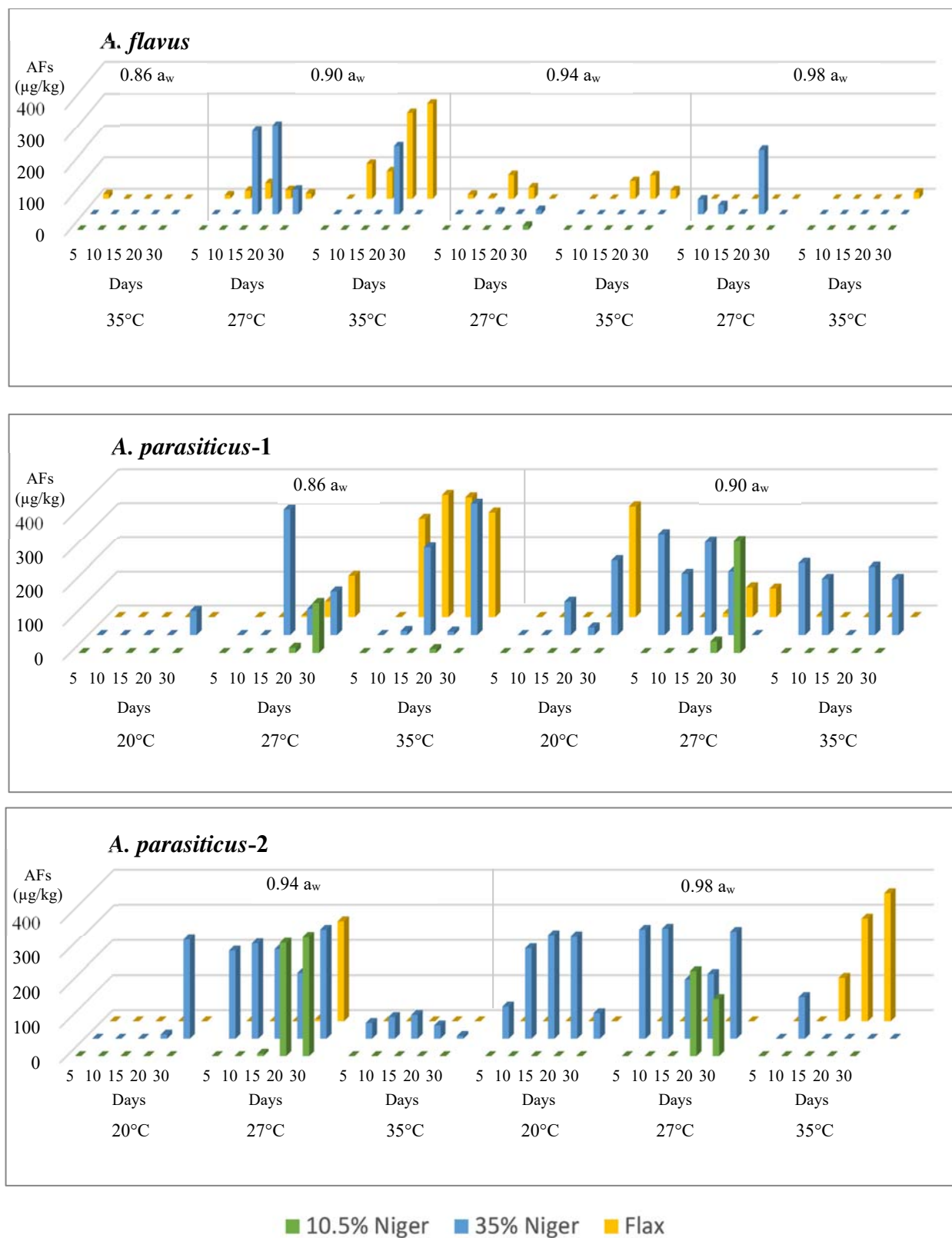
Figure 15. Total aflatoxins of the five sampling times produced by (a) *A. flavus* and (b) *A. parasiticus* at 20°, 27°, and 35°C on de-oiled niger seeds with 0.86, 0.90, 0.94, and 0.98 a$_w$.

Table 5. Comparison and discussion for growth and aflatoxin production

Growth	Flax	Niger	De-oiled niger
A. flavus OPT Growth	0.90 a_w/ 27°C 0.94 a_w/ 27°C 0.94 a_w/ 35°C	0.90 a_w/ 35°C 0.94 a_w/ 27°C, 35°C 0.98 a_w/ 27°C	0.90 a_w/ 35°C 0.94 a_w/ 35°C
A. parasiticus OPT Growth	0.90 a_w/35°C	0.90 a_w/ 35°C 0.94 a_w/ 27, 35°C	0.90 a_w/ 35°C 0.94 a_w/ 35°C
A. flavus in low and high a_w	0.86 a_w/ 35°C 0.98 a_w/35°>27°>>>20°C	0.86 a_w/ 35°C 0.98 a_w/ 27°>20°>35°C	0.86 a_w/ 35°C 0.98 a_w/ 27°C
A. parasiticus in low and high a_w	0.86 a_w/ 35°C (poor growth at 20°C) 0.98 a_w – poor growth (35-slow, little growth-at 20°, 27°C)	0.86 a_w/ 35°C =27°C 0.98 a_w/ 27°≥20°>35°C	0.86 a_w/ 35°C 0.98 a_w/ 27°C

Other studies	Incubation condition	Strain/ substrate	Growth result
Cuero et al. (1987)	0.90, 0.95, 0.98 a_w/ 16°C, 25°C	*A. flavus* (IMI 102566)/ irradiated cracked maize	0.98 a_w/ 16°C (OPT)
Lahouar et al. (2016)	0.85, 0.88, 0.91, 0.94, 0.97, 0.99 a_w/ 15°, 25°, 37°C	three *A. flavus* isolates/ sorghum seeds	0.99 a_w/ 37°C (OPT)
Yogendrarajah et al. (2016)	0.826–0.984 a_w/22°, 30°, 37 °C	*A. flavus* (UG AF60), *A. parasiticus* (UG AF542)/ whole black peppercorns	0.857 a_w/ 37°C (Poor growth) 0.984 a_w/ 22°C (Poor growth)

Aflatoxin production	Flax	Niger	De-oiled niger
A. flavus	0.90 a_w/35°C (OPT)	0.90 a_w/ 27, 35°C (OPT) 0.98 a_w/ 27°C (OPT)	0.94 a_w/ 27°C (13 µg/kg)
A. parasiticus	0.86, 0.98 a_w/ 35°C (OPT) 0.90 a_w/ 20°C (OPT) 0.94 a_w/ 27°C (OPT)	0.90-0.98 a_w/ 20°C (OPT) 0.86-0.98 a_w/ 27°C (OPT) 0.86-0.90 a_w/ 35°C (OPT)	0.86 a_w/ 35°C (OPT) 0.90 a_w/ 27°C (OPT) 0.94 a_w/ 27°C (OPT)

Other studies	Incubation condition	Strain/ substrate	AF production
Faraj et al. (1991)	0.90, 0.95, 0.98 a_w/ 25°, 30°, 35°C	*A. flavus* (CMI 102566), *A. parasiticus* (NRRL 2222) irradiated maize	*A. flavus* (408-5450 µg/kg) *A. parasiticus* (405-3717 µg/kg)
Yogendrarajah et al. (2016)	0.826–0.984 a_w/ 22°, 30°, 37 °C	*A. flavus* (UG AF60) *A. parasiticus* (UG AF542) whole black peppercorns	*A. flavus* (non-detected AF) *A. parasiticus* (3.1-6.4 µg/kg)
Sorenson et al. (1967)	8°-37 °C	*A. flavus* (NRRL 2999) rice	28°C (AFB1-OPT) (184000-760000 µg/kg) 32 °C (AFB1-OPT) (633000-760000 µg/kg) 28°C (AFG1-OPT) (64000-458000 µg/kg)
Ribeiro et al. (2006)	0.80, 0.90, 0.95 a_w/ 25°, 35°C	*A. flavus* isolates barley rootlets	AFB1 (200 µg/kg) 0.95 a_w /25°C
Liu et al. (2016)		*A. flavus* (NRRL 3357)/ soybean, peanut, corn, wheat, corn endosperm, and corn germ	defatted corn germ was 7-fold less compared to full-fat corn germ (48210 µg/kg)
Mellon et al. (2000)		*A. flavus*/ defatted cottonseeds	defatted cottonseeds 829-fold less as compared to ground cottonseeds

OPT: Optimum condition; OPT aflatoxin: >200 µg/kg of aflatoxin

CHAPTER 3. DISCUSSIONS

This study demonstrated that both mycelial growth and aflatoxin production by *A. flavus* (NRRL 3357) and *A. parasiticus* (NRRL 465) were affected by temperature and water activity. Additionally, temperature and water activity influenced the growth of these species, while their capacity to produce aflatoxin depended on the substrate (flax seeds, niger seeds versus de-oiled niger seeds) used, which varied for each of these fungal strains. This work builds on previous work of our research group investigating the influence of water activity, temperature, and incubation period on the growth of aflatoxin producing fungal strains (Gizachew et al., 2019a; Ting et al., 2019).

3.1 Effect of Temperature and Water Activity on Growth of *A. flavus* and *A. parasiticus*

It is well known that temperature and water activity influences the optimum conditions for fungal growth. In this study, it was found that at high water activity (0.98 a_w), both fungi showed rapid growth on flax seeds at 35°C and on niger seeds at 27°C (Figure 11 and 12, Table 5). In contrast, on flax or niger seeds with a low water activity (0.86 a_w), both fungi grew rapidly at 35°C. Similar results were reported in previous studies. Cuero et al. (1987) studied the effect of water activity (0.90, 0.95, and 0.98 a_w) on the growth of *A. flavus* (IMI 102566) on irradiated cracked maize and rice at 16° and 25°C. Their result also indicated that at the high water activity (0.98 a_w) more rapid growth of *A. flavus* was supported at the lower temperature (16°C). Yogendrarajah et al. (2016) observed that both *A. flavus* (UG AF60) and *A. parasiticus* (UG AF542) showed poor growth on whole black peppercorns with a low water activity (0.857 a_w) at a higher temperature (37°C); however, they also observed that *A. flavus* grew poorly on whole black peppercorns with a high water activity (0.984 a_w) at a low temperature (22°C), while *A. parasiticus* did not grow at all under these conditions. The findings of Yogendrarajah et al. (2016) differed from the results of this study. Lahouar et al. (2016) tested the effects of temperature (15°, 25° and 37°C) and water activity (0.85, 0.88, 0.91, 0.94, 0.97 and 0.99 a_w) on the growth of three *A. flavus* isolates on sorghum seeds. They reported that the optimum growth conditions for the three *A. flavus* isolates were on sorghum seeds with a high water activity (0.99 a_w) at a high temperature (37°C). Based

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on these results, the optimum temperature and water activity appear to be an important factor influencing fungal growth for the various substrates tested.

3.2 Effect of Temperature and Water Activity on Aflatoxin Production of *A. flavus* and *A. parasiticus*

On flax and niger seeds, the median water activity (0.90 and 0.94 a_w) at 27° or 35°C supported the highest levels of aflatoxin production (Figure 15). However, flax and niger seeds with a low water activity (0.86 a_w) did not generally support aflatoxin production when the fungal strains were incubated at low temperature (20°C). Other research groups also found that on rice, the optimum temperatures for *A. flavus* (NRRL 2999) to produce AFB1 (184,000-760,000 µg/kg) were at higher temperatures (28° and 32°C); the optimum temperature for AFG1(64,000-458,000 µg/kg) production being 28°C (Sorenson et al., 1967). Ribeiro et al. (2006) showed that on barley rootlets, the optimum conditions for *A. flavus* to produce AFB1 (200 µg/kg) was with 0.95 a_w at 25°C. Aflatoxin production is clearly influenced by temperature and water activity, according to the fungal strains being tested.

It is well known that the optimum condition for fungal growth do not coincide with the maximum levels of aflatoxin production for a given strain (Mannaa and Kim, 2017). This study also found that the optimum aflatoxin production conditions were not congruent with optimum growth conditions for the fungal strains we tested (Table 5). Lahouar et al. (2016), however, did find that the optimum AFB1 production was associated with the optimum growth for the three *A. flavus* isolates they tested. Our study also observed that aflatoxins were only detectable on seeds within a short window of time (Figure 15), which was especially true for some strains grown on particular substrates at certain temperatures for a given level of water activity (e.g., see day 20 for *A. flavus* at 0.90 a_w and 35°C). The four types of aflatoxins (AFB1, AFG1, AFB2, and AFG2) might increase and decrease during the 30-day incubation. Similarly, Ribeiro et al. (2006) also observed that AFB1 was only detected on barley rootlets within a short period time, while Giorni et al. (2011) stated that the decrease of aflatoxins might be due to re-absorption, utilization, or degradation by the fungi.

3.3 Comparison of Growth and Aflatoxin Production on Flax Seeds and Niger Seeds

Previous studies found that incubation substrates are critical factors influencing fungal growth (Bircan, 2006; Cotty and Jaime-Garcia, 2007; Magan et al.,1984; Ribeiro et al., 2006; Liu et al., 2016). Overall, *A. flavus* and *A. parasiticus* grew similarly or more rapidly on niger seeds than on flax seeds under most incubation conditions. However, *A. flavus* showed more rapid growth at 35°C on flax seeds than on niger seeds with 0.98 a_w (Figure 11 and 12). As shown in Figure 15, *A. parasiticus* produced higher levels of aflatoxins under a broader range of incubation conditions than did *A. flavus.* Up to 299 and 278 μg/kg aflatoxins were produced by *A. flavus* on flax and niger seeds*,* whereas up to 365 and 384 μg/kg aflatoxins were produced by *A. parasiticus* on flax and niger seeds respectively. Overall, niger seeds supported higher levels of aflatoxin production under wide range of incubation conditions than did flax seeds regardless of the fungal strain. Flax seeds contain 38-45% fat, which include 53% α-linolenic acid, 17% linoleic acid, and 19% oleic acid, and 29% carbohydrate (Bernacchia et al., 2014; Epaminondas et al., 2011; Pali and Mehta, 2014; Pellizzon et al., 2007; Mercier et al., 2014). Niger seeds contain 42-44% fat, which include 54.3-72.7% of linoleic acid and 5.4-26.8% of oleic acid, and 34-39% carbohydrate (Ramadan, 2012). It has been reported that sugar contents, trace elements, and amino acid contents in substrates are a factor in influencing mycelial growth and aflatoxin production (Liu et al., 2016). Niger seeds contain more carbohydrate, which offers an explanation for our results.

3.4 Effect of Oil Content on Growth and Aflatoxin Production on Niger Seeds

The two fungal strains showed similar growth on niger and de-oiled niger seeds under most incubation conditions. However, *A. flavus* grew more rapidly on niger seeds than did on de-oiled niger seeds under the following incubation conditions: 0.90, 0.94 or 0.98 a_w at 27°C. *Aspergillus parasiticus* also grew more rapidly on niger seeds than did on de-oiled niger seeds, but under different incubation conditions (0.86 or 0.90 a_w at 27°C). This result suggested that oil content is important for fungal growth overall.

The oil content of niger seeds was also found to influence aflatoxin production. As shown in Figure 15, *A. flavus* produced 14-278 μg/kg aflatoxins on niger seeds under four incubation condition (0.90, 0.94, or 0.98 a_w at 27°C as well as 0.90 a_w at 35°C), whereas it only produced 13 μg/kg aflatoxins on de-oiled niger seeds under one incubation condition (0.94 a_w at 27°C).

Aspergillus parasiticus produced 8-384 µg/kg aflatoxins on niger seeds under all incubation conditions tested; however, it produced 8-345 µg/kg aflatoxins on de-oiled niger seeds at 27° or 35°C in the following incubation conditions (0.86, 0.90, 0.94 a_w at 27°C as well as 0.86 and 0.90 a_w at 35°C) on (Figure 15). Liu et al. (2016) found that on defatted corn, *A. flavus* (NRRL 3357) produced 6,820 µg/kg AFB1, which was 7-fold less than 48,210 µg/kg produced in full-fat corn germ. Furthermore, adding lipid back to the defatted substrates could bring back the AFB1 production. On defatted corn germ supplemented with 10 % corn oil, *A. flavus* produced 33,030 µg/kg of AFB1. Mellon et al. (2000) also showed that AFB1 production by *A. flavus* (AF 13) on defatted cottonseeds was 829-fold less than on ground cottonseeds. A previous study stated that unsaturated fatty acids such as linoleic acid could enhance growth of *Aspergillus* spp. as well as their lipid peroxidation and aflatoxin production (Fanelli and Fabbri, 1989). Taken together, these findings suggest a key role for lipid content in the production of aflatoxin.

3.5   Comparison of Growth and Aflatoxin Production by *A. flavus* and *A. parasiticus*

Results of this study showed that *A. flavus* (NRRL 3357) grew slightly faster than did *A. parasiticus* (NRRL 465) under most incubation conditions on the three tested substrates. However, *A. parasiticus* produced high levels of aflatoxins under a wider range of incubation conditions than did *A. flavus*. Similar results were reported by several research groups. Yogendrarajah et al. (2016) found that *A. flavus* (UG AF60) showed a faster growth rate than did *A. parasiticus* (UG AF542) but produced less aflatoxin than did *A. parasiticus* (3.1-6.4 µg/kg) on whole black peppercorns under most incubation conditions. Faraj et al. (1991) found that *A. flavus* (CMI 102566) produced lower levels of aflatoxins (408-5,450 µg/kg) than did *A. parasiticus* (NRRL 2222) (405-3,717 µg/kg) under several incubation conditions (0.90, 0.95, 0.98 a_w at 25°, 30°, and 35°C) on irradiated maize. Similar to results seen for a single aflatoxin producing strain showing variance between growth optima versus peak aflatoxin production, differences between growth optima and peak aflatoxin production also appear to vary according to the particular fungal strain given the same temperature and water activity. These findings highlight the importance of understanding growth and aflatoxin production dynamics at the species level, as all aflatoxin-producing fungi cannot be expected to behave in the same manner under environmental condition seen in the field.

CONCLUSIONS

This research clearly illustrated that temperature, water activity, incubation substrates, and fungal strains affected growth and aflatoxin production. The optimum growth condition for *A. flavus* was observed on flax seeds with 0.90 or 0.94 a_w at 27°C as well as 0.94 a_w at 35°C; on niger seeds with 0.90 or 0.94 a_w at 35°C and also with 0.94 or 0.98 a_w at 27°C; on de-oiled niger seeds with 0.90 or 0.94 a_w at 35°C. On the other hand, the optimum growth condition for *A. parasiticus* was detected on flax seeds with 0.90 a_w at 35°C; on niger seeds with 0.94 a_w at 27°C and also 0.90 or 0.94 a_w at 35°C; on de-oiled niger seeds with 0.90 or 0.94 a_w at 35°C. Moreover, there was no visible mycelial growth and aflatoxin production on seeds with 0.82 a_w at 20°, 27°, and 35°C. The finding suggested that the best storage condition for flax seeds and niger seeds should be maintained below 0.82 a_w, eliminating the possible fungal growth and aflatoxin production.

This study also showed that the most favorable growth conditions did not support the maxima for aflatoxin production during the 30-day inoculation period. The optimum conditions for *A. flavus* to produce high levels of aflatoxins (>200 µg/kg) on flax seeds were 0.90 a_w at 35°C, on niger seeds with 0.90 or 0.98 a_w at 27°C as well as 0.90 a_w at 35°C. However, only 13 µg/kg aflatoxins were detected on de-oiled niger seeds with 0.94 a_w at 27°C. The optimum conditions for *A. parasiticus* to produce high levels of aflatoxins (>200 µg/kg) on flax seeds were 0.86 or 0.98 a_w incubated at 35°C and also 0.90 a_w incubated at 20°C as well as 0.94 a_w incubated at 27°C. Moreover, on de-oiled niger seeds, high aflatoxin concentration was detected on de-oiled seeds with 0.86 a_w incubated at 35°C and also 0.90 or 0.94 a_w incubated at 27°C. High levels of aflatoxins (>200 µg/kg) were observed on niger seeds under most of the incubation conditions. Furthermore, de-oiled niger seeds are often used as a supplement of animal feeds, and are good substrates for fungal growth and aflatoxin production.

The results of this study could help in establishing guidelines for storage conditions of the seeds and de-oiled seeds to minimize fungal growth and aflatoxin production, with our findings highlighting the need to consider individual factors, including individual fungal species, substrate qualities and the potential for ambient environmental influences, when selecting storage conditions that will minimize risks associated with aflatoxin production. However, this study does confirm the results of previous research showing that in considering all other factors, food and feed storage under the driest of conditions (i.e., at or below 0.80 a_w) is likely the best practice.

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