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EFFECTS OF TEMPERATURE, WATER ACTIVITY, AND CARBON DIOXIDE ON AFLATOXIGENIC *Aspergillus flavus* GROWTH AND AFLATOXIN PRODUCTION IN PEANUTS

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ABSTRACT

Unappropriate postharvest handling of peanuts can facilitate the occurrence of fungi and mycotoxin contamination, especially *Aspergillus flavus* which can produce aflatoxins. The main factors affecting the growth of *A. flavus* during storage are temperature, relative humidity, moisture content, water activity, and carbondioxide (CO_2). The objective of this research was to test and make a model describing the effect of temperature, water activity (a_w) and CO_2 levels on *A. flavus* growth and aflatoxin production *in vitro* and in stored peanuts. Methodology of this research were 4 stages, i.e. 1) culturing of strain toxigenic *A. flavus* BIO 3338, 2) in vitro testing on Potato Dextrose Agar (PDA) mediua, and 3) in vivo testing in peanuts at various temperature, a_w and CO_2 using 6 replications for each treatments, and 4) statistical analysis using Completely Randomized Factorial Design for water activity and temperature treatments, and Completely Randomized Design for carbondioxide treatment. Determination of aflatoxin was conducted using Thin Layer Chromatography (TLC) for aflatoxin of *A. flavus* culture and Liquid Chromatography Mass Spectro (LCMS). Result showed that temperature, a_w, and CO_2 could control toxigenic *A.flavus* BIO 3338 growth and aflatoxin production in PDA (in vitro) and in peanuts (in vivo). Increasing of *A. flavus* growth was influenced by increasing of water activity. The optimum *A. flavus* growth was found on PDA and peanuts with 0.97 a_w at 30°C and 35°C, respectively. Diameter of *A. flavus* and aflatoxin production decreased with increasing CO_2 concentrations. Formation of conidia and sclerotia of *A. flavus* also decreased with increasing of CO_2.

Key words **:** *aflatoxin, Aspergillus flavus, peanuts, temperature, water activity, carbondioxide*

1. INTRODUCTION

1.1. Background

Peanuts is one of agricultural commodities in Indonesia that has successfully entered the world market. Indonesian peanuts exports reached 1584 tonnes since January until May 2019. Some peanuts importer countries are Malaysia, Saudi Arabia, United States of America, Canada, England and Holland (Dirgantara 2019). Based on Ministry of Agriculture (2016) the highest productivity of peanuts in Indonesia was found in West Java (16.30 qu/ha), followed by Gorontalo (16.07 qu/ha), West Sumatra and West Nusa Tenggara (14.40 qu/ha). Hirschmann (2020) reported that the production of Indonesian peanuts was 963.4 thousand tonnes in the period 2015. North Sumatra's Central Bureau of Statistic (2020) also reported that in the period 2019, peanuts production from North Sumatra was 4888.5 tonnes.

According to Dharmaputra *et al.* (2007) most of peanut growing environments was dry land conditions and mixed cropping with maize or cassava. Farmers dried peanut pods by sun-drying on bamboo mat or paved floor, while the collectors also dried them by sund-drying on woven paved floor. As much as 47% retailers stored peanuts in jute bags, while 32% retailers stored peanuts in recylable plastic bags for less than one week. Not only peanuts, retailers sold other commodities such as milled rice, maize, soybean, mungbean, garlic, onion, sugar palm, *kerupuk, emping* and cashew. Their postharvest handling methods can affect *Aspergillus flavus* infection and aflatoxin B_1 (AFB1) contamination in peanuts.

Micromorphology of *Aspergillus* section *Flavi* is uni or biseriate conidial head, production of dark-colored sclerotia by certain species, and yellow green to brown shades conidia. *Aspergillus* section *Flavi* includes 33 species, and most of them are natural producers of aflatoxin (Frisvad *et al.*2019). Aflatoxins are toxins produced by *Aspergillus flavus* and *A. parasiticus*. Aflatoxins are considered dangerous due to their association with various diseases in humans and animals, such as aflatoxicoses and liver cancer. There are four naturally occurring aflatoxins in many commodities, i.e. aflatoxin B_1, B_2, G_1, and G_2. The most common and toxic of aflatoxins is aflatoxin B_1 (Basappa 2009). Aflatoxins are highly toxic and cause disease in livestock and humans (Lien *et al.*2019). Aflatoxins are found in many tropical and subtropical countries where temperature and humidity conditions are optimal for mold growth and for production of these toxins (Rustom 1997). Dharmaputra *et al.* (2013a) reported that the mean of *Aspergillus flavus* populations in peanuts collected from Pasar Anyar and Pasar Bogor in Bogor, West Java were 8194 and 983 cfu/g, respectively, while AFB1 contents were 2.0 and 91.4 ppb, respectively. Other research

conducted by Lien *et al.* (2019), a total of 1089 samples of peanut candies, peanut butter, and groundnuts were analyzed for aflatoxin since 2011 to 2017. The aflatoxin contents of AFB1, AFB2, AFG1, and AFG2 were 2.40, 0.41, 0.91, and 0.03 µg/kg, respectively. According to Ambarwati *et al.*(2011) estimated dietary for AFB1 found in children was 15.2 ng/kg/bw/day and 95th percentile exposure was 38.9 ng/kg/bw/day, while in adults 9.0 ng/kg/bw/day and 95th percentile exposure was 27.0 ng/kg/bw/day. The excess cancer risk of AFB1 exposure in children and adults in Bogor was 193 and 115 cancers/year per 100,000 population, respectively. European Commission (2013) determined the maximum tolerable limits (MTL) of total aflatoxin and AFB1 in peanuts are 15 and 8 ppb, respectively. Petersen (2018) also reported that based on Codex, MTL of total aflatoxin was 15 ppb in peanuts, while that of based on EU Regulation No.1881/2006, the MTL of total aflatoxins and AFB1 were 15 and 8 ppb, respectively. The MTL of total aflatoxin and AFB1 in processed peanut products for direct consumption were 4 and 2 ppb, respectively.

According to Medina *et al.* (2014) the expression of *A. flavus* pathway is influenced by some environmental factors. The interactions of water activity (a_w) and temperature are related to the ratio of two key regulatory genes (*aflSlafR*). The high ratio of *aflSlafR* correlated with the *A. flavus* production level. Magan and Aldred (2007) reported that stored food is usually alive and respiring actively after harvest. Not only temperature, relative humidity and water activity, but also CO_2 levels influence the fungal growth (Giorni *et al.* 2008). Pateraki *et al.* (2007) reported that up to 50% CO_2 had only a slight impact on ochratoxin A production by *Aspergillus carbonarius* over a range a_w conditions, with a_w being a more important factor than CO_2. Based on Medina *et al.* (2017) climate change scenarios are predicted to get significant effects on the security of commodities. FAO (2012) also explained that climate change may be due to natural internal processes or external forcings, or to persistent anthropogenic changes in the composition of the atmosphere or in land use.

Interactions between abiotic and biotic factors including pest immigration and emigration and changes in intergranular atmosphere are complex in stored food. Under modified and elevated temperature and humidity conditions their volatility may increase resulting in less effective coverage and thus less control of mycotoxigenic fungi. However, studies on *A. flavus* population and aflatoxin content in peanuts under various storage conditions are limited. The combinations of CO_2 levels, water activity and temperature which will be treated in this research can be a promising method to inhibit *A. flavus* growth, aflatoxin contamination, and preserve intact peanut kernels. The concept model will estimate

A. flavus growth rates and aflatoxin production in peanuts, however, it will be optimistic and will provide somewhat conservative predictions.

1.2. Objectives

- To test the effect of temperature, water activity (a_w) and CO_2 levels on *A. flavus* growth and aflatoxin production of artificial media and stored peanuts.
- To make a model describing the effect of temperature, water activity (a_w) and CO_2 levels on *A. flavus* growth and aflatoxin production of stored peanuts.

1.3.Expected output

It is expected, that the research results:

- could obtain the modelling predicted an appropriate storage conditions (temperature, water activity and CO2 level) for peanuts related to *Aspergillus flavus* growth and aflatoxin production.
- could be effectively implemented in minimizing the risk of aflatoxin contamination of stored peanuts.

2. BENEFIT AND IMPORTANCE OF RESEARCH

This research is important to be conducted, because the main causes of the decreasing of peanuts quality during storage and transportation are the infection of aflatoxin producing fungus (*Aspergillus flavus*) and aflatoxin contamination. This research could obtain the modelling predicted an appropriate storage conditions (temperature, water activity and CO2 level) for peanuts related to *A. flavus* growth and aflatoxin production, to minimize aflatoxin contamination risk in stored peanuts.

3. METHODOLOGY

3.1. Time and location of research

Preparation and research activity will be conducted from March up to November 2021 at SEAMEO BIOTROP, Bogor.

3.2. Strain (isolate) of *Aspergillus flavus*

Strain of *A. flavus* BIO 3338 isolated from peanuts was used in this research, because it produced total aflatoxins 137.55 µg/kg in 10% Coconut Extract Medium after 10 days of incubation under dark conditions at 28°C. The strain was obtained from Culture Collection Phytopathology Laboratory, SEAMEO BIOTROP, Bogor, Indonesia.

3.3. Effect of interacting climate change factors (a_w, temperature and CO_2) on *Aspergillus flavus* BIO 3338 growth and aflatoxin production *in vitro*

Petri dishes (9 cm diam.) containing Potato Dextrose Agar (PDA) adjusted with glycerol-water solutions at 0.90, 0.92, 0.95 and 0.97 a_w (Giorni *et al*. 2008; Belbahi *et al.* 2016) was centrally inoculated with 2.5µL of *A. flavus* suspension (10^6 conidia mL^{-1}). The plates were incubated at 30, 35 and 37°C (Belbahi *et al.* 2016). Six replications were used for each treatment. The diameter of fungal colonies was determined after 7 and 14 days of incubation. Aflatoxin production of *A. flavus* culture was analyzed after 14 days of incubation, using Thin Layer Chromatography (TLC) method (Bainton 1980).

The treatments that produce the highest *A. flavus* growth and aflatoxin production, was placed into a 3 L glass jar, then CO_2 at concentrations of 25, 50 and 75% were introduced into the glass jar (Giorni *et al*. 2008). Six replications were used for each treatment. The diameter of fungal colonies was determined after 7 and 14 days of incubation. Aflatoxin production of *A. flavus* culture was analyzed after 14 days of incubation.

3.4.Effect of interacting climate change factors (a_w, temperature and CO_2) on *Aspergillus flavus* BIO 3338 growth and aflatoxin production (*in vivo*)

3.4.1. Preparation of peanuts

Raw kernels (moisture content ± 8%) of peanut (local variety) was obtained from Balai Besar Penelitian Bioteknologi dan Sumber Daya Genetik Pertanian (BB Biogen) in Bogor. Before irradiation, peanut kernels were selected and calculated the quality based on the percentage of intact and damaged kernels. The calculation result, the intact and damaged kernels were 79.3 and 20.7%, respectively. About 15 kg peanut kernels were irradiated with a dose range of 13.3-15.5 kGy and mean of 14.3 kGy at Pair Layanan Iradiasi BATAN in Jakarta, for killing the microbes in the kernels. Before use, some species of fungi and aflatoxin content analyses were performed on peanut kernels to check the presence of fungi and aflatoxin.

3.4.2. Inoculation of *Aspergillus flavus* BIO 3338 and treatments of tempertature, water activity and carbon dioxide in peanuts

About 40 g irradiated peanuts with aw level of 0.90, 0.92, 0.95 and 0.97 were inoculated with 160 µL of *A. flavus* suspension (10^6 conidia mL^{-1}) (Pratiwi *et al.* 2015). Water activity of peanuts was adjusted using saturated salt solution. To adjust 0.90, 0.92, 0.95 and 0.97 a_w were used $BaCL_2$, $NH_4H_2PO_4$, K_2HPO_4 and K_2SO_4 (200g salt in 200 mL water), respectively, that placed into a 3 L glass jar. The peanuts were then incubated at 30, 35 and 37^{o}C. *Aspergillus flavus* population and aflatoxin content on peanuts were determined after 7 and 14 days of incubation. Six replications were used for each treatment.

The treatments that produce the highest *A. flavus* growth and aflatoxin content, were placed into a 3 L glass jar, then CO_2 at concentrations of 25, 50 and 75% were introduced into the glass jar. 2008). Six replications were used for each treatment. *Aspergillus flavus* population and aflatoxin content on peanuts were determined after 7 and 14 days of incubation using dilution method followed by pour plate method on PDA and Liquid

Chromatography Mass Spectro (LCMS) method, respectively. Aflatoxin analysis was conducted at PT. Saraswanti Indo Genetech (SIG) in Bogor.

3.5.Experimental design

The data of the growth of *Aspergillus flavus* BIO 3338 and aflatoxin production were analyzed using Completely Randomized Factorial Design for water activity and temperature treatments with 2 factors, and Completely Randomized Design for carbondioxide treatment (Mattjik and Sumertajaya 2002).

3.5. Empirical model of *Aspergillus flavus* BIO 3338 growth rate

Growth curves will be developed by plotting the colony diameters (mm) of each of fungal against incubation time (days) to determine the growth rate. The growth rate is calculated using the linear model of the growth curves (Yogendrarajah *et al*. 2016; Belbahi et al. 2016), as follow:

$$\mu = \frac{(d_t - d_0)}{(t - \lambda)} \text{ for } t > \lambda \quad (1)$$

Where, μ is *A. flavus* growth rate (mm/day), dt is diameter at time t (days), d_o is initial diameter and λ is the lag phase or the time to visible growth (λ, days).

In accordance with monofactorial design, the combined effects of environmental factors such as temperature, aw and CO_2 on *A. flavus* growth rate are estimated using multi-cardinal model (γ-concept) (Zwietering *et al*. 1996; Belbahi *et al*. 2016):

$$\mu = \mu_{opt}.\lambda_T.\lambda_{a_w}.\lambda_{CO_2} \quad (2)$$

Where,

$$\lambda_T = \left(\frac{T_{max} - T}{T_{opt} - T_{max}}\right)^2 \quad (3)$$

$$\lambda_{a_w} = \left(\frac{a_{w_{max}} - a_w}{a_{w_{opt}} - a_{w_{max}}}\right)^2 \quad (4)$$

$$\lambda_{CO_2} = \left(\frac{CO_{2\,max} - CO_2}{CO_{2\,opt} - CO_{2\,max}}\right)^2 \quad (5)$$

The terms T, aw, CO_2 correspond to the values of temperature, water activity and carbon dioxide respectively. Moreover, the T_{opt}, aw_{opt} and CO_{2opt} are the values of temperature water activity and carbon dioxide at which the μ is at its optimal value (μ_{opt}). While T_{max}, aw_{max} and CO_{2max} are the level of factors above which no-growth occurs.

Climate change is expected to impact more on transportation, drying and storage. To manage the risk of aflatoxins after harvest, conditions such as storage should be optimal. An optimal condition of storage will be achieved by minimizing of fungal growth rate, stated by the following formula:

$$\mu = \mu_{opt}.min(\lambda_T.\lambda_{a_w}.\lambda_{CO_2}) \quad (6)$$

Practically, this can be achieved by adopting technology to modify and maintain of temperature, water activity and CO_2 concentration in the storage.

The coefficient of determination (R^2) and root mean square error (RMSE) will be used to assess the fit of the models. RMSE measures the residual variability between predicted and the experimental values of growth rate. The equation used to estimate RMSE was based on the sample standard error of the differences between the square root of predicted and experimental values as shown below:

$$RMSE = \sqrt{\frac{\Sigma(\mu_{prediction} - \mu_{experiment})^2}{n}} \quad (7)$$

where $\mu_{prediction}$ is the colony growth rate predicted by cardinal model, $\mu_{experiment}$ is the colony growth rate obtained by the experiment and n is the number of samples.

4. RESULT AND DISCUSSIONS

4.1 Toxigenic *Aspergillus flavus* BIO 3338 growth and aflatoxin production at various water activities and temperatures *in vitro*

Water activity (a_w) and temperature could affect *A.flavus* growth and aflatoxin production in *Potato Dextrose Agar* (PDA) medium (Tables 1 and 4). The growth of *A.flavus* and aflatoxin production were inhibited with the decrease of a_w, either after 7 or 14 days of incubation. At 35^{o}C, *A. flavus* could not grow at 0.90 and 0.92 a_w. At 37^{o}C, *A. flavus* could not grow at 0.90 a_w, while at 30^{o}C, *A. flavus* could grow at all of a_w level. The optimum growth of *A. flavus* at the three of temperature was at 0.97 a_w. According to Adamaraja *et al.*(2018), water activity could affect growth of *A. flavus* mycelia. The optimum growth of *A. flavus* mycelia was at 0.98 a_w. Pratiwi *et al*. (2015) reported that the maximum growth of *A.flavus* BIO 2237 and aflatoxin production either in *Czapek Dox Agar* (CDA) or soybean were reached at 30^{o}C with relative humidity (RH) of 90%, and it neither could not grow, nor produced aflatoxin in soybean at high temperature (40^{o}C) and low RH (70%).

The growth of toxigenic *A. flavus* BIO 3338 on PDA with various a_w and temperature levels after 7 and 14 days of incubation is presented in Figure 1.

Table 1. Diameter of *Aspergillus flavus* BIO 3338 colony (mm) at various temperatures and water activities after 7 and 14 days of incubation

Temperature (^{o}C)	Water activity (a_w)							
	After 7 days of incubation				After 14 days of incubation			
	0.90	0.92	0.95	0.97	0.90	0.92	0.95	0.97

30	9.9 e	10.2 e	19.9 c	28.6 a	10.1 e	11.8 e	25.8 c	41.2 a
35	0 f	0 f	16.9 d	24.8 b	0 f	0 f	17.3 d	35.8 b
37	0 f	10.4 e	18.7 cd	26.8 ab	0 f	12.1 e	22.7 c	41.6 a

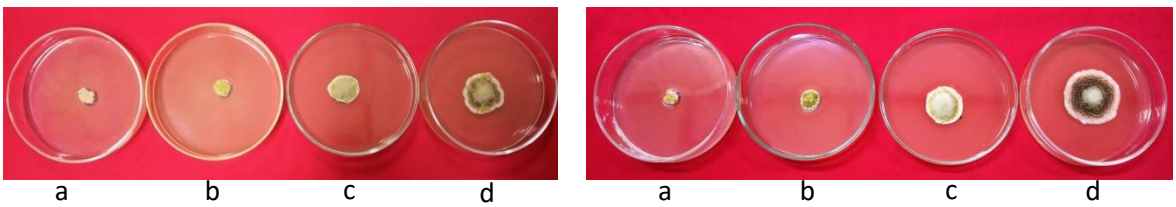
Numbers followed by the same letter (in each incubation time) do not differ significantly according to Duncan's Multiple Range Test at 95% confidence level

Temperature of storage influenced on the formation of conidia and sclerotia of *A. flavus* (based on score) after 7 and 14 days of incubation. Formation of conidia of *A. flavus* at 30 °C was lower than 35 and 37 °C, while its formation of sclerotia at 30 °C was higher than at 35 and 37 °C (Table 2). Water activity also influenced conidia and sclerotia formation of *A. flavus* (based on score) after 7 and 14 days of incubation. The highest formations of conidia and sclerotia were found in 0.95 and 0.97 a_w, respectively (Table 3).

Table 2. Conidia and sclerotia formation of *Aspergillus flavus* BIO 3338 at various temperatures after 7 and 14 days incubation

Incubation time (days)	Formation	Temperature (°C)		
		30	35	37
7	Conidia	+	++	++
	Sclerotia	++	+	+
14	Conidia	+	++	++
	Sclerotia	+++	++	+

Notes :
+ = low
++ = middle
+++ = high



A1 A2

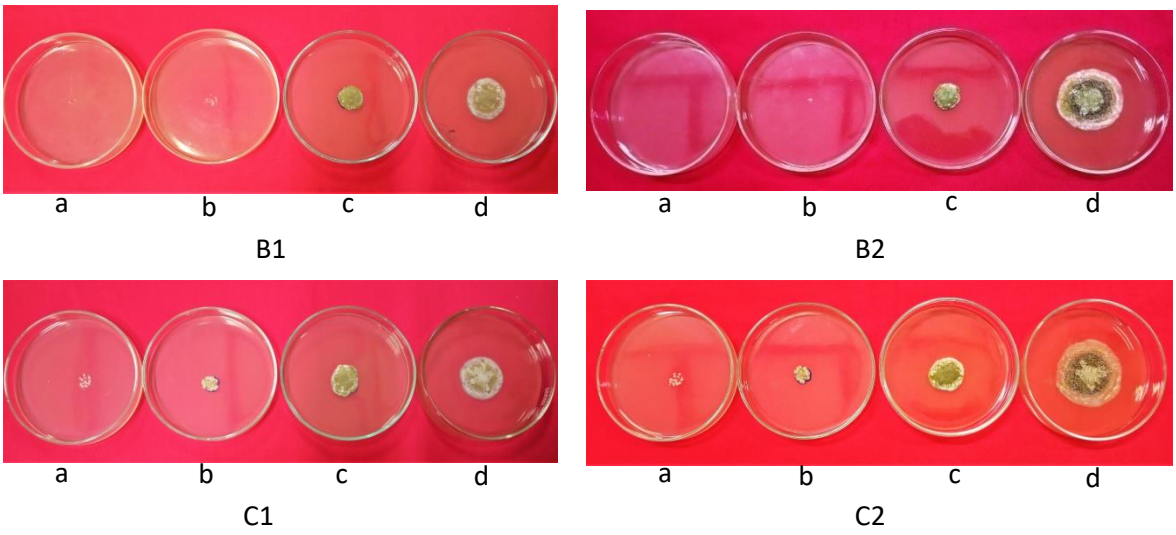


Figure 1. Toxigenic *Aspergillus flavus* BIO 3338 growth on Potato Dextrose Agar (PDA) at various water activities (a_w) and temperatures. A= 30°C, B= 35°C, C= 37°C; 1= 7 days of incubation 2= 14 days of incubation; a= 0.90 a_w; b= 0.92 a_w; c= 0.95 a_w; d= 0.97 a_w

Table 3. Conidia and sclerotia formation of *Aspergillus flavus* BIO 3338 at various water activities after 7 and 14 days of incubation

Incubation time (days)	Formation	Water activity			
		0.90	0.92	0.95	0.97
7	Conidia	-	+	++	+
	Sclerotia	-	-	-	+
14	Conidia	-	+	++	+
	Sclerotia	-	-	+	+++

Notes :

- = not formed
+ = low
++ = middle
+++ = high

Table 4. Aflatoxin production of *Aspergillus flavus* BIO 3338 at various temperatures and water activities after 14 days of incubation

Temperature (°C)	Aflatoxin production (µg/kg)
	Water activity (a_w)

	0.90	0.92	0.95	0.97
30	1969 ef	3642 e	8072 d	16260 c
35	*	*	2716 ef	29229 a
37	*	665 ef	808 ef	19418 b

Numbers followed by the same letter do not differ significantly according to Duncan's Multiple Range Test at 95% confidence level

* Analysis of aflatoxin was not conducted, because *A. flavus* was not grow (diameter of colony was zero)

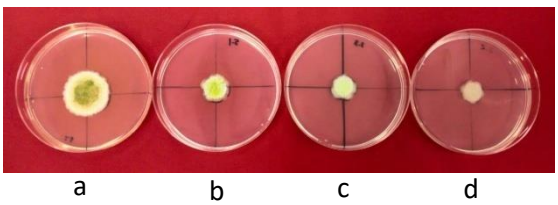
4.2 Toxigenic *Aspergillus flavus* BIO 3338 growth and aflatoxin production at various concentrations of carbon dioxide *in vitro*

Result of temperature and water activity treatments on *A. flavus* BIO 3338 growth and aflatoxin production showed that the highest of *A. flavus* growth and aflatoxin production were found at temperature of 30°C and 0.97 a_w. Concequently, temperature of 30°C and 0.97 a_w were used for carbon dioxide treatments. Diameter of *A. flavus* and aflatoxin production decreased with increasing CO_2 concentrations (Tables 5 and 7). Formation of conidia and sclerotia of *A. flavus* also decreased with increasing of CO_2 (Table 6).

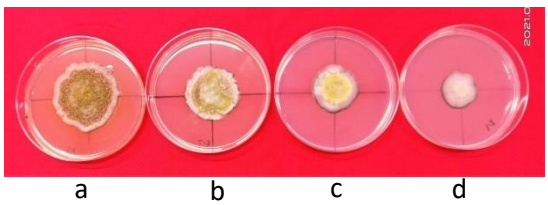
Table 5. Diameter of *Aspergillus flavus* BIO 3338 colony at various carbon dioxide concentrations after 7 and 14 days of incubation

CO_2 concentration (%)	Diameter of *A. flavus* colony (mm)	
	After 7 days of incubation	After 14 days of incubation
25	20.9 a	40.6 a
50	18.7 b	30.0 b
75	16.2 c	25.6 c

Numbers followed by the same letter do not differ significantly according to Duncan's Multiple Range Test at 95% confidence level



A



B

Figure 2. Toxigenic *Aspergillus flavus* BIO 3338 growth at various carbon dioxide concentrations on Potato Dextrose Agar (PDA) with 0.97 a_w at 30°C. A = after 7 days of incubation, dan B = after 14 days of incubation; a= control (air), b= 25% CO_2, c= 50% CO_2, d= 75% CO_2

Table 6. Conidia and sclerotia formation of *Aspergillus flavus* BIO 3338 at various carbon dioxide concentrations after 7 and 14 days of incubation

Incubation time (days)	Formation	CO_2 (%)		
		25	50	75
7	Conidia	+++	++	+
	Sclerotia	-	-	-
14	Conidia	+++	++	+
	Sclerotia	+	-	-

Keterangan:
- = not formed
+ = low
++ = middle
+++ = high

Table 7. Aflatoxin production of *Aspergillus flavus* BIO 3338 at various carbon dioxide concentrations after 14 days of incubation

CO_2 concentration (%)	Aflatoxin production (µg/kg)
25	18.4 a
50	11.0 ab
75	0.0 b

Numbers followed by the same letter do not differ significantly according to Duncan's Multiple Range Test at 95% confidence level l

4.3 Toxigenic *Aspergillus flavus* BIO 3338 growth and aflatoxin production in peanuts at various water activities and temperatures *in vivo*

Aspergillus flavus growth was determined based on its population in peanuts at various water activities and temperatures. Population of *A. flavus* increased with increasing water activity, and the highest population was at 0.97 a_w either after 7 or 14 days of incubation

(Table 8). The optimum growth of *A. flavus* and aflatoxin production in peanuts were found at 35°C. At 35°C, aflatoxin production in peanuts increased with increasing a_w (Table 9). The growth of *A. flavus* and aflatoxin production had a positive correlation with moisture content of peanuts. The highest moisture content of peanuts was at 35°C. At 35°C, moisture content increase with increasing a_w (Table 10).

Tabel 8. Population of *Aspergillus flavus* BIO 3338 (cfu/g) in peanuts at various temperatures and water activities after 7 and 14 days of incubation

Temperature (°C)	Water activity (a_w)							
	After 7 days of incubation				After 7 days of incubation			
	0.90	0.92	0.95	0.97	0.90	0.92	0.95	0.97
30	$1.03x10^2$	9.67x10	$1.08x10^2$	$6.44x10^3$	$8.89x10^2$	$9.44x10^2$	$2.28x10^3$	$2.33x10^3$
35	$2.87x10^2$	$2.06x10^3$	$2.10x10^4$	$1.46x10^5$	$7.39x10^4$	$3.64x10^5$	$8.89x10^6$	$9.22x10^6$
37	$5.91x10^2$	$1.43x10^3$	$1.22x10^4$	$5.61x10^4$	$6.33x10^4$	$2.71x10^5$	$5.33x10^6$	$6.94x10^6$

Table 9. Aflatoxin production (µg/kg) of peanuts at various temperatures and water activities after 14 days of incubation

Temperature (°C)	Water activity (a_w)			
	0.90	0.92	0.95	0.97
30	0.01	0.09	0.24	0
35	120,58	235,33	803,58	946,08
37	1,25	15,44	110,60	39,02

Table 10. Moisture content (% w/w) of peanuts at various temperatures and water activities after 7 and 14 days of incubation

Temperature (°C)	Water activity (a_w)							
	After 7 days of incubation				After 7 days of incubation			
	0.90	0.92	0.95	0.97	0.90	0.92	0.95	0.97
30	6.69	6.87	9.10	21.0	7.64	9.18	8.40	20.59

35	10.42	11.73	13.46	20.65	11.06	12.21	14.03	21.21
37	10.25	10.94	12.94	20.42	10.41	11.63	12.69	20.28

4.4 Toxigenic *Aspergillus flavus* BIO 3338 growth and aflatoxin production in peanuts at various concentracions of carbon dioxide *in vivo*

Result of temperature and water activity treatments on *A. flavus* growth and aflatoxin production showed that the highest of *A. flavus* growth and aflatoxin production were found at temperature of 35ºC and 0.97 a_w. Concequently, temperature of 35ºC and 0.97 a_w were used for carbon dioxide treatments. The effect of CO_2 concentracion on moisture content and population of *A. flavus* in peanuts tended not be significant (Tables 11 and 13), while aflatoxin production was inhibited at 50% CO_2 (Table 12). Pateraki *at al.* (2007) reported that as much as 50% CO_2 only had little impact on ochratoxin A production by *A. carbonarius* under various conditions. Water activity (a_w) is more important factor compared to CO_2. According to Giorni *et al*. (2008) up to 75% CO_2 resulted in an inhibition of the *A. flavus* growth in the media and maize grains.

Table 11. Population of *Aspergillus flavus* BIO 3338 (cfu/g) in peanuts at various concentrations of carbon dioxide after 7 and 14 days of incubation

CO_2 concentration (%)	After 7 days of incubation	After 7 days of incubation
25	$1.27x10^5$	$1.41x10^5$
50	$1.48x10^5$	$1.76x10^5$
75	$5.0x10^4$	$1.64x10^5$

Table 12. Aflatoxin production of peanuts at various concentrations of carbon dioxide after 14 days of incubation

CO_2 concentration (%)	Aflatoxin production (µg/kg)
25	15.04
50	0.41
75	0.36

Table 13. Moisture content (% w/w) of peanuts at various concentrations of carbon dioxide after 7 and 14 days of incubation

CO_2 concentration (%)	After 7 days of incubation	After 7 days of incubation
25	15.18	17.50
50	15.47	17.02
75	15.06	16.42

Giorni *et al*. (2008) reported that fungal growth on artificial media was highly influenced by both CO_2 and a_w level. The growth of *A.flavus* based on maize media *in vitro* at 0.92 and 0.95 a_w were 25 and 41 mm, respectively, while AFB1 production at those water activity conditions were 541 and 470 ng/g, respectively. The lowest population of *A. flavus* (6 cfu/g) and AFB1 production (40 ng/g) on maize grains (*in vivo*) was found at 0.92 a_w. Up to 75% CO_2 resulted in an inhibition of the *A. flavus* growth in the media and maize grains. The growth of *A. flavus* and AFB1 production *in vitro*, i.e. 7 mm and 9 ng/g, respectively. The population of *A. flavus* and AFB1 *in vivo* were 6 cfu/g and 128 ng/g, respectively. The efficacy of interactions between controlled atmospheres and a_w showed that treatment with 25% CO_2 could be sufficient to efficiently reduce *A. flavus* development but at least 50% CO_2 was required to obtain a significant reduction of aflatoxin synthesis. According to Gilbert *et al.* (2018) AFB1 production had positive correlation with water activity and carbon dioxide (CO_2) levels of maize kernels. The highest AFB1 production (7 000 ng/g) was found in maize at water activity condition 0.99, temperature 30 °C and elevated levels of CO_2 650 ppm.

Magan *et al.* (2011) explained that the range of *A. flavus* growth at 0.95 and 0.90 aw, i.e. 6 – 9 mm/day and 2 – 9 mm/day, respectively, while the range of AFB1 production at those conditions, i.e. 2 278 – 3 082 and 331.5 – 448.5 ng/g, respectively. According to Dharmaputra *et al.* (1992) both CO_2 concentrations and the three isolates of *A. flavus* (BIO-16, BIO-17 and BIO-18) affected mycelial growth, sporulation, spore germination and aflatoxin production significantly. The increase in colony size of isolates BIO-16, BIO-17 and BIO-18 before and after fumigation were 1362.9, 973.8 and 1166.0 mm^2, respectively. Colony size of *A. flavus* based on various CO_2 concentration, i.e. 20% (1137.2 mm^2), 40% (614.1 mm^2), 60% (478.3 mm^2), and 80% (199.1 mm^2). The lowest colony size of *A. flavus* before and after fumigation based on interaction between *A. flavus* isolates and CO_2 concentrations was found in *A. flavus* BIO-17 with CO_2 concentration 80% (31.9 mm^2).

Belbahi *et al.* (2016) reported that based on interaction between temperature, water activity (a_w) and CO_2 levels, the highest growth rate of *A. niger* (22.6 mm/day) was found at temperature 35 °C, a_w 0.952, and 9.4% CO_2. The lowest *A. niger* growth at various temperature was found at 10 °C (1.1 mm/day), while at various water activity was 0.818 a_w (0.4 mm/day) and at various CO_2 levels was 55.1% CO_2 (3.2 mm/day). Mousa *et al.* (2011) conducted a research using two isolates of *A. flavus* (DISF 10 and DISF 15) with range temperature (15 – 37 °C) and range a_w (0.89 – 0.99). *Aspergillus flavus* could grow conducively to particularly high aflatoxin at temperature 25 and 30 °C. The highest aflatoxin content (1344 ng/g) was produced by isolate DISF 10 at temperature 25 °C and 0.98 a_w. According to Adaramaja *et al.* (2018) water activity significantly affected lag phases and growth rates in all strains of *A. flavus*. The shortest mean lag phase (1.2 days) was at 0.98 and 0.995 a_w, while the longest (2.5 days) was found at 0.90 a_w. The growth of aflatoxigenic strains was similar over the 0.90 - 0.98 a_w, but the optimal condition was found at 0.98 a_w. Non-aflatoxigenic strain had maximum growth at 0.95 a_w. There were no growth response under severe water activity stress (0.85 a_w).

5. CONCLUSIONS

Temperature, water activity (a_w), and carbon dioxide karbondioksida (CO_2) could control growth of toxigenic *Aspergillus flavus* BIO 3338 and aflatoxin production in Potato Dekstose Agar (PDA) in vitro. The growth of *A. flavus* (based on diameter of colony) and aflatoxin production were inhibited with decrease a_w of medium. At 35°C, *A. flavus* could not grow at 0.90 and 0.92 a_w. At 37°C, *A. flavus* could not grow at 0.90 a_w, while at 30°C, *A. flavus* could grow at all of a_w level. The optimum growth of *A. flavus* at the three of temperature was at 0.97 a_w the optimum of *A. flavus* growth and aflatoxin production were

found at 30°C and 0.97 a_w. Diameter of *A. flavus* and aflatoxin production decreased with increasing CO_2 concentrations.

Temperature and water activity (a_w) could control toxigenic *A.flavus* BIO 3338 growth aflatoxin production in peanuts (in vivo), while the effect of CO_2 concentration on moisture content and population of *A. flavus* tended not be significant. Aflatoxin production was inhibited at 50% of CO2. Population of *A. flavus* increased with increasing water activity, and the highest population was at 0.97 a_w either after 7 or 14 days of incubation (Table 8). The optimum growth of *A. flavus* and aflatoxin production in peanuts were found at 35°C. At 35°C, aflatoxin production in peanuts increased with increasing a_w (Table 9). The growth of *A. flavus* and aflatoxin production had a positive correlation with moisture content of peanuts. The highest moisture content of peanuts was at 35°C. At 35°C, moisture content increase with increasing a_w.

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APPENDICES

Appendix 1. Analisys of variance of diameter of *Aspergillus flavus* BIO 3338 colony at various temperatures and water activities after 7 days of incubation

Source	DF	Sum of square	Mean square	F value	Pr>F
Temperature	2	541.241944	270.620972	69.89	<.0001**
a_w	3	6264.436667	2088.145556	539.26	<.0001**
Temperature x a_w	6	345.485833	57.580972	14.87	<.0001**
Error	60	232.333333	3.872222		
Total	71	7383.497778			

Appendix 2. Analisys of variance of diameter of *Aspergillus flavus* BIO 3338 colony at various temperatures and water activities after 14 days of incubation

Source	DF	Sum of square	Mean square	F value	Pr>F
Temperature	2	991.55583	495.77792	40.41	<.0001**
a_w	3	14258.85819	4752.95273	387.36	<.0001**
Temperature x a_w	6	336.41639	56.06940	4.57	<.0001**
Error	60	736.20833	12.27014		
Total	71	16323.03875			

Appendix 3. Analisys of variance of aflatoxin production of *Aspergillus flavus* BIO 3338 culture at various temperatures and water activities after 14 days of incubation

Source	DF	Sum of square	Mean square	F value	Pr>F
Temperature	2	104076168	52038084	7.18	0.0016**
a_w	3	5313410932	17711366977	244.48	<.0001**
Temperature x a_w	6	675616605	112602767	15.54	<.0001**
Error	60	434675894	7244598		
Total	71	6527779599			

Appendix 4. Analisys of variance of diameter of *Aspergillus flavus* BIO 3338 colony at various carbon dioxide after 7 days of incubation

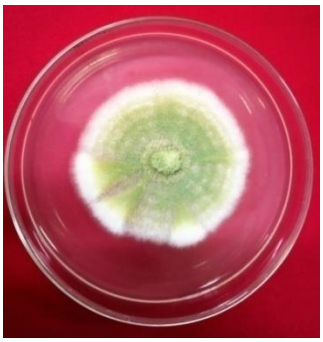
Source	DF	Sum of square	Mean square	F value	Pr>F
Treatment	2	66.34111111	33.17055556	21.72	<.0001**
Error	15	22.91000000	1.527333333		
Total	17	89.25111111			

Appendix 5. Analisys of variance of diameter of *Aspergillus flavus* BIO 3338 colony at various carbon dioxide after 14 days of incubation

Source	DF	Sum of square	Mean square	F value	Pr>F
Treatment	2	712.2100000	356.1050000	258.86	<.0001**
Error	15	20.6350000	1.3756667		
Total	17	732.8450000			

Appendix 6. Analisys of variance of aflatoxin production at various carbon dioxide after 14 days of incubation

Source	DF	Sum of square	Mean square	F value	Pr>F
Treatment	2	1031.582500	515.791250	6.12	0.0114*
Error	15	1265.056100	84.337073		
Total	17	2296.638600			



Appendix 7. Toxigenic *Aspergillus flavus* BIO 3338 on *Potato Dextrose Agar* (PDA) after 7 days of incubation

Appendix 8. Inoculation of *Aspergillus flavus* BIO 3338 on PDA at various water activity (a_w)

Appendix 9. Covering of plastic petridish containing *Aspergillus flavus* BIO 3338 culture using steril paper. A= covering of plastic petridish, B= plastic petridish covered by steril paper

Appendix 10. Incubation of *Aspergillus flavus* culture at various carbondioxide concentrations at 30^{o}C in incubator

Appendix 11. Measuring of carbondioxide using *Cosmotector* CO_2

A

B

Appendix 12. Peanuts in a plastic and glass jars containg salt solution for treatments of (A) temperature and water activity, and (B) carbondioxide

Appendix 13. Carbondioxide treatment on peanuts in a glass jar containing salt solution