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The effect of temperature and relative humidity for *Aspergillus flavus* BIO 2237 growth and aflatoxin production on soybeans

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Abstract

Aspergillus flavus (A. flavus) producing aflatoxin frequently contaminates crops such as soybeans. The growth of this mold on soybeans and other foodstuffs is affected by temperature and relative humidity (RH). The aim of this study was to measure the growth of *A. flavus* BIO 2237 and aflatoxin production at different temperatures and RH. *Aspergillus flavus* BIO 2237 was isolated from Indonesia origin foodstuffs. *Aspergillus flavus* BIO 2237 was inoculated in Czapek Dox Agar (CDA) and soybeans for 10 days at a temperature of 20, 30, and 40°C with RH of 70, 80, and 90%. Aflatoxin analysis was conducted using RP-HPLC equipped with fluorescence detector and post column photochemical reactor. The limit of detection (LoD) for aflatoxin of B_1, B_2, G_1, and G_2 was 0.45, 0.26, 0.05, and 0.13 ng/mL (ppb), while their limit of quantification (LoQ) was 1.50, 0.88, 0.18 and 0.43 ng/mL (ppb) respectively. The maximum growth for *A. flavus* BIO 2237 in CDA and soybeans was reached at a temperature of 30°C with RH of 90%, and this was based on the highest diameter of colony and amount of cell mass formed in that condition. The maximum level of aflatoxin in contaminated soybeans was found at 999 ng/g (ppb), and this was produced at the same condition as its fungi's growth. *Aspergillus flavus* BIO 2237 can not grow as well as produce aflatoxin in soybeans at high temperature (40ºC) with low RH (70%). There was a significant difference (sig<0.05) in aflatoxin content (AFB1, AFB2, AFG1, and AFG2) between temperature and RH, meanwhile the difference on the growth of *A. flavus* BIO 2237 in CDA and soybeans caused by RH.

Introduction

Aspergillus flavus is a fungus that is widely distributed in nature and is mostly found at cereal grains and legumes such as peanuts, corn, and rice. *Aspergillus flavus* can grow at agricultural crops before harvest or during storage (Saini and Kaur, 2012). Its growth is influenced by the environmental condition such as temperature and relative humidity (RH) until produce aflatoxin (Giorni *et al.*, 2012). The relative humidity that is higher than 85% is a very supportive environment for *A.flavus* growth (Al-Shikli *et al.*, 2010).

The presence of aflatoxin in food will significantly affect its quality and safety. Furthermore, it will result in economic decrease. Apart from that, aflatoxin outbreaks occurred in Kenya, India, and Thailand (CAST, 2003) and 317 poisoning cases were reported by CDC (2004) from which 125 of them led to death. The health problems affected by aflatoxin can happen due to the fact that aflatoxin is carcinogenic, teratogenic, toxigenic, immunotoxigenic, and mutagenic to human and animal (Wild and Gong, 2010). This is relevant to IARC (2002) provision that classifies aflatoxin B_1 as a member of group I carcinogens whose toxicity is the most dangerous to human's health.

Aflatoxin contamination is more common in the tropics and sub-tropics, such as Indonesia, and this conditions are relate well to temperature and rainfall that may strongly suitable for the growth of *A. flavus*. During the rainy season, *A. flavus* and *Rhizopus oryzae* that were more predominated as compared to other fungus (Hussaini *et al.*, 2009). Moreover, Indonesia is one of the countries affected by extreme climate conditions that have taken place around the world for the last decade. In November 2013, the temperature in Indonesia ranged from 22-37°C, and RH ranged from 43-98% (Indonesian Agency for Meteorological, Climatological, and

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Geophysical, 2013). Such conditions are expected to support the growth of *A. flavus* as the aflatoxin producer. The temperature increment on earth in the future is predicted to affect the growth of *A. flavus* and the formation of aflatoxin during food storage. Therefore, in this research the effects of storage under different conditions towards the growth of *A. flavus* and production of aflatoxin were investigated.

Materials and Methods

The main materials used in this research were toxigenic fungus as aflatoxin producers that is *A. flavus* BIO 2237 (Collection of Microbiology Laboratory of SEAMEO BIOTROP-Bogor, Indonesia) and soybeans (Wilis variety). To adjust the relative humidity, the saturated salts (ammonium chloride (20˚C with RH 70%), barium chloride (20˚C with RH 80%), ammonium sulfate (30˚C with RH 70%), potassium nitrate (30˚C with RH 80%; 30˚C with RH 90%; 30˚C with RH 70%), sodium nitrate (30˚C with RH 70%) and potassium sulfate (20˚C with RH 90%; 30˚C with RH 90%) were used (modification was made in Greenspan 1976; Wexler and Hasegawa 1954). The materials used to analyze aflatoxin were chemical standard of aflatoxin B_1, B_2, G_1, G_2 (Sigma, USA), 1 mL of immunoaffinity column Afla test (Vicam, USA), NaCl (Merck, Germany), aquabidest (Kalbe, Indonesia), methanol (Merck), and acetonitril (Merck). Aflatoxin analysis was conducted using RP-HPLC with fluorescence detector (Agilent Technologies, USA).

The effect of temperature and relative humidity towards the growth of A. flavus *BIO 2237 as the aflatoxin producer at Czapex Dox Agar medium and Soybeans*

The influence of temperature and RH for the growth of *A. flavus* BIO 2237 by following a modification of Kokkonen *et al*. (2010). Modification done was media, temperature, and RH that was adjusted. Before treatment, the soybeans were irradiated to avoid fungus contamination. As much as 2.5 µL of *A. flavus* BIO 2237 spore suspension (10^6 CFU/mL) was inoculated in the center of CDA medium, and 100 µL of *A. flavus* BIO 2237 spore suspension (10^6 CFU/mL) was inoculated into several points of soybeans (25 g) in petridish. The petridishes were placed in mini desiccator and the desiccators were placed in incubator and incubated at a temperature of 20, 30, 40°C and RH of 70, 80, 90% for 10 days. The observation of growth of *A. flavus* BIO 2237 in CDA medium was conducted by measuring the diameter colony (in mm) every 24 hours. Whereas in soybeans, it was conducted by measuring the mass of the cell that was formed within incubation period. All processes were conducted in two replicates. The aflatoxin content of all incubated soybeans was measured quantitatively.

Aflatoxin analysis by HPLC method

The concentration of aflatoxin in soybeans was analyzed by RP-HPLC method with immunoaffinity column cleanup (AOAC 991.31, 2012). The sample (± 25 g) and NaCl (5 g) were mixed with 125 mL of methanol – water (7:3 v/v) and then were crushed with dry blender with high speed for 2 minutes. The result was filtered using fluted filter paper. Fifteen milliliters of sample was taken out using a pipette and put into Erlenmeyer flask, and then 30 mL aquabidest was added and homogenized. The homogeneous filtrate was re-filtered using microfiber paper, and 15 mL of it was taken and put inside siring barrel correction which was already connected to IAC Afla test for purification. The column was washed with 20 mL of aquabidest, and aflatoxin was eluted with 1.0 mL of methanol at a rate of 1 drop/second. The eluent was then added with 0.5 mL of aquabidest and vortexed before injected into HPLC. Each experiment was conducted in duplicate, and the concentration of aflatoxin in the sample was determined by calculating its concentration using standard curves and bringing the result into the following formula:

$$\text{Aflatoxin concentration (ppb)} = \frac{\text{concentration in standard curve x latest solution volume (mL) x DF}}{\text{Sample's mass (g)}}$$

Note: DF (Dilution factor)

Statistical analysis

The descriptive statistic and analysis of variance (ANOVA) were employed using SPSS (version 16.0, Microsoft Corp, USA). A probability value of 0.05 was used to determine the statistical significance.

Results

The growth of A. flavus *BIO 2237 at Czapex Dox Agar (CDA)*

The effect of temperature and RH towards *A. flavus* BIO 2237 growth pattern was observed in 10 days of incubation period where the different condition was at a lower temperature (20°C) and at a higher temperature (40°C) than the room temperature (30°C). During incubation period, the growth of *A. flavus* BIO 2237 increased. This indicates that *A. flavus* BIO 2237 grew up at a certain condition as

Table 1. The cell mass and aflatoxin content in soybeans inoculated with *A. flavus* BIO 2237 incubated at various temperatures and relative humidities (RH)

T (°C), RH (%)	Cell mass (mg)	AFB_1 (ppb)	AFB_2 (ppb)	AFG_1 (ppb)	AFG_2 (ppb)	Total (ppb)
Control	1155±1.6^{bc}	47.9±2.4^{cd}	2.3±0.2^{a}	20.2±1.7^{c}	0.2±0.0^{a}	70.6±4.0^{c}
(20°C, 70%)	361±0.1^{a}	12.4±0.0^{ab}	2.7±0.5^{a}	0.5±0.0^{a}	0.5±0.0^{a}	16.1±0.4^{a}
(20°C, 80%)	439±0.0^{a}	73.2±0.1^{e}	19.3±0.4^{c}	4.6±0.3^{ab}	3.8±0.2^{c}	100.8±0.3^{d}
(20°C, 90%)	1116±1.3^{c}	381.4±11.1^{f}	64.7±1.4^{e}	10.4±0.2^{b}	0.3±0.0^{a}	456.8±9.6^{e}
(30°C, 70%)	335±0.1^{a}	0.6±0.1^{a}	0.1±0.0^{a}	0.6±0.4^{a}	0.1±0.0^{a}	1.4±0.5^{a}
(30°C, 80%)	2350±0.2^{e}	31.5±0.3^{bc}	32.5±1.0^{d}	1.1±0.1^{a}	0.1±0.0^{a}	65.3±0.6^{bc}
(30°C, 90%)	2635±0.3^{f}	935.5±3.8^{g}	6.1±2.0^{ab}	57.0±2.7^{d}	0.4±0.0^{a}	999±0.7^{d}
(40°C, 70%)	nd	nd	nd	nd	nd	nd
(40°C, 80%)	2255±0.4^{de}	3.6±0.8^{a}	0.4±0.0^{a}	1.4±0.0^{a}	nd	5.5±0.8^{a}
(40°C, 90%)	2471±0.0^{ef}	60.1±1.1^{de}	7.1±1.1^{b}	11.2±0.2^{b}	1.3±0.0^{b}	80.5±0.3^{cd}

Note: nd (not detected)
Control (30ºC, 75%)
Different letters define significant differences (sig<0.05) in column

presented in Figures 1-3. The figure shows that the growth of *A. flavus* BIO 2237 continued to rise sharply from the incubation period ranging from 1 to 7 days; however, after 7 days of the incubation period, the growth of *A. flavus* BIO 2237 began to slow down.

Aspergillus flavus BIO 2237 formed a colony with a diameter of 55, 69, and 71 mm at a temperature of 30°C and at RH of 70, 80, 90% (day 7th) respectively. Therefore, the maximum growth was 71 mm which occurred at temperature 30°C with RH of 90%, based on the highest diameter of colony that was formed. On the other hand, *A. flavus* BIO 2237 in room condition (as control) with the same temperature and with lower RH (75%) formed a smaller colony with a diameter of 63 mm. This result indicated that at the same temperature, the lower RH gave the lower diameter of *A. flavus* BIO 2237 colony. At a lower temperature (20°C) or higher temperature (40°C) with low RH (70%), the diameter tended to be smaller. The statistical analysis in day 7th indicated significant difference RH (Sig<0.05) on the growth of *A. flavus* BIO 2237 in CDA (Figure 1-3). However, the temperature and also the interaction between two factors were not statistically significant.

The growth of A. flavus *BIO 2237 in soybeans*

The cell mass of *A. flavus* BIO 2237 on soybeans ranged from 0 to 2635 mg. The highest cell mass of *A. flavus* BIO 2237 in soybeans occurred at the same condition as that of CDA medium, i.e. at a temperature of 30°C with RH of 90%. The moist condition during incubation will allow *A. flavus* BIO 2237 to grow in soybeans. The moisture content of soybeans which initially was at 13% increased during incubation period. In this research, the cell mass was only seen to be formed at RH of 80 and 90% (temperatures of 20, 30, and 40°C) and at RH of 70% (temperature of 20 and 30°C) (Table 1). At the temperature of 40°C with RH of 70%, *A. flavus* BIO 2237 cannot grow as shown by the undetected cell mass. Based on the results, it can be concluded that the RH takes an important role for the formation of *A. flavus* BIO 2237 cell mass in soybeans (sig<0.05), i.e. *A. flavus* BIO 2237 grows less at lower RH and vice versa.

The formation of aflatoxin in soybeans

Aflatoxin of B_1, B_2, G_1, G_2 (AFB1, AFB2, AFG1, AFG2) can be observed at retention times of 13, 12, 11, and 9 minutes respectively. The linearity of standard curve for each aflatoxin was determined by injecting standard solutions 7 times at concentrations of 0, 2, 5, 10, 20, 30, 40, and 50 ppb for AFB1 and at concentrations of 0, 3, 5, 10, 20, 30, and 40 ppb for AFB2, AFG1, AFG2 with correlation coefficient (R^2) of 0.999. Limit of detection (LoD) of AFB1, AFB2, AFG1, and AFG2 was 0.45, 0.26, 0.05, and 0.13 ppb respectively, whereas the limit of quantification (LoQ) was respectively 1.50, 0.88, 0.18 and 0.43 ppb. LoD was calculated with a signal to noise ratio of S/N=3, and LoQ used S/N=10.

Aflatoxin contamination can occur due to the potential growth of *A. flavus* BIO 2237 in soybeans. The results in Table 1 indicated that the highest cell mass of *A. flavus* BIO 2237 (2635 mg) gave the highest aflatoxin concentration in soybeans (999 ppb), given by the sample at the same temperature and RH (30°C and 90%). On soybeans, aflatoxin (B_1, B_2, G_1, and G_2) was significantly influenced by both

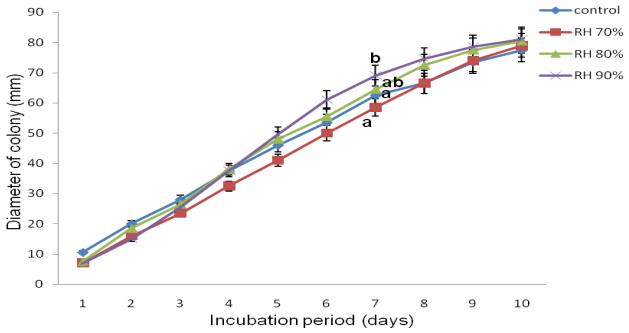

Figure 1. The growth of *A. flavus* BIO 2237 in CDA medium at the temperature of 20ºC with RH of 70, 80, and 90%. The error bars showed the standard deviations

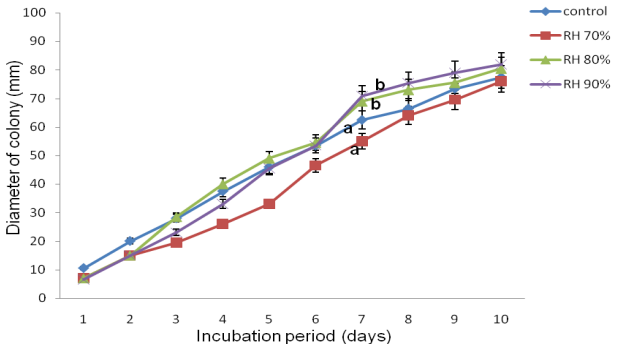

Figure 2. The growth of *A. flavus* BIO 2237 at the CDA medium at a temperature of 30°C with RH of 70, 80, and 90%. The error bars showed the standard deviations

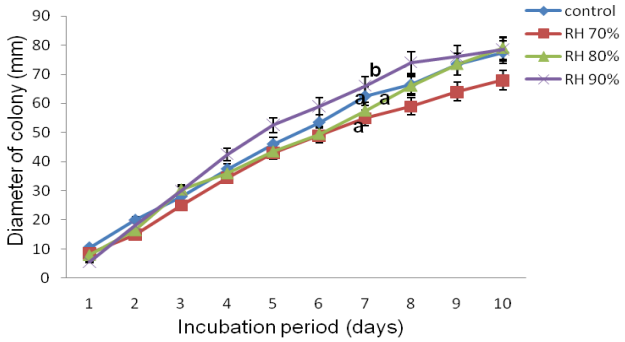

Figure 3. The growth of *A. flavus* BIO 2237 at CDA medium at a temperature of 40°C with RH of 70, 80, and 90%. The error bars mentioned the standard deviations

temperatures and RH (sig<0.05).

The growth of *A. flavus* BIO 2237 will affect the amount of aflatoxins produced by the fungus, and therefore, a high contamination of *A. flavus* BIO 2237 in soybeans will contribute to the high concentration of aflatoxin. In this research, high temperature and dry environment resulted in the low formation of aflatoxin. This is related to the growth of *A. flavus* BIO 2237 which is more dominant in high RH.

Discussion

The presence of *A. flavus* BIO 2237 in CDA and soybeans was affected by nutrition, aw, moisture content, and period of incubation. Moreover, environmental factor such as temperature and relative humidity also highly influences the growth of *A. flavus* BIO 2237 and aflatoxin production in medium. The growth of *A. flavus* BIO 2237 gave the orange colour in CDA. Similarly, Das *et al.* (2012) shown the maximum growth of *A. flavus* MTCC 2798 occurs in 7 days. After that, *A. flavus* MTCC 2798 will actively produce aflatoxin and the synthetic medium will appear orange. Aflatoxin will be formed after *A. flavus* passes the log phase of its growth period or enters its stationery phase (static). In the static phase, the nutrition is reduced because it has been used extensively in the log phase; therefore, in this phase, the amount of living *A. flavus* is directly proportional to the dead. At this condition, *A. flavus* can no longer breed, and it will try to survive by continuously producing aflatoxin (Milani, 2013).

The existence of *A. flavus* BIO 2237 in CDA and soybeans was highly detectable at temperature of 30°C and RH of 90%, and likewise for aflatoxin production in soybeans. Das *et al*. (2012) stating that *A. flavus* is a mesophilic fungus which grows well on a temperature of 30°C and thus the production of aflatoxin is expected to occur at the same temperature, and Al-Shikli *et al.* (2010) stating that RH in which *A. flavus* growth is optimally above 85%. A research result obtained by Kusumaningrum *et al.* (2010) exhibited that relative humidity can affect the growth of *A. flavus* in maize significantly. Based on the experiment data, the contamination of *A. flavus* BIO 2237 and aflatoxin production were more dominantly found in the sample that were stored in higher RH. The condition of storage environment such as high RH will migrate water in the air to the sample (Kabak *et al.*, 2006; Cotty and Garcia, 2007). Dharmaputra *et al*. (2007) reported that the highest aflatoxin B_1 content was found in peanut kernels stored at RH 94% after 8 weeks of storage at room temperature (± 28ºC). In the moist environment, the relative humidity of an environment ranges from 70 to 90% (Das *et al.*, 2012), while at the dry environment, the relative humidity ranges from 50 to 60% (Arzandeh *et al*., 2009). The height of moisture content can be effectively for *A. flavus* to growth and to produces aflatoxin in soybeans. A research from Hussaini *et al.* (2009) found that sorghum stored in the moist environment was more highly contaminated by *A. flavus* than that stored in the dry environment. Atehnkeng *et al*. (2008) and Kaaya *et al.* (2006) also found that corn and peanut from a moist area contain higher aflatoxin compared to those that come from a warm and dry area.

The formation of aflatoxin in food will surely differ across countries depending on their weather.

Cotty and Gracia (2007) state that the fluctuation of temperature and relative humidity in tropical countries may cause aflatoxin contamination. The given extreme condition i.e. temperature 40°C with RH 70% could stop *A. flavus* BIO 2237 to grow and to produce aflatoxin in soybeans. This condition can be used to storage the soybeans for long-term, in order to avoid from contamination of *A. flavus* and aflatoxin production. Soybeans is a very important commodity for Indonesia. According to the national data, 2.5 million tons of soybeans is consumed per year, mostly as food products such as tempeh, tofu, tauco, kecap (soybean sauce), and etc. (Indonesian Ministry of Trade, 2013). The role of soybeans towards national food is huge, therefore it must be protected from mold and mycotoxin contamination.

Conclusion

The best growth of *A. flavus* BIO 2237 either in the laboratory medium (CDA) or in foodstuff (soybeans) was found at a temperature of 30°C with RH 90%. At higher temperature i.e. 40°C with lower RH i.e. 70%, the *A. flavus* BIO 2237 cannot grow in soybeans. The highest concentration of aflatoxin was found in soybeans with the highest growth of *A. flavus* BIO 2237 respectively.

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