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Evaluation of the Effect of Water Activity and Temperature on Lag Phase and Growth Rate of Aflatoxigenic *Aspergillus* section *Flavi* Strains Isolated from Stored Rice Grain

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Abstract

This study examined the effect of environmental factors water activity (a_w) and temperature on lag phase prior to growth and growth rate of six aflatoxigenic fungi, *Aspergillus* section *Flavi,* strains were isolated from stored paddy rice grain in Thailand. Statistical analysis indicated that both studied stress factors significantly affected lag phases and radial growth rates at the a_w and temperature regimes studied ($P<0.05$). Overall, the growth of each strain was similar over the 20-40°C and 0.90-0.98 a_w ranges but optimal condition was found to be around 0.95 a_w and 30°C. Under severe a_w stress (0·90 a_w) with elevated temperature (40°C), differences were observed in growth responses, with some strains unable to grow. The lag phases were significantly increased at marginal temperature and a_w levels. The combined factors showed statistical interaction for growth rate ($P<0.05$), while there was no evidence of statistical interaction effect on the lag phases prior to growth ($P>0.05$). Growth rate under more freely available water conditions (>0.95 a_w), the disordinal interaction was observed when strains were cultured at higher temperatures (>30°C). In contrast, growth rate with cooler temperatures at <30°C showed ordinal interaction. Growth rates were fastest at 0.95 a_w and 30°C. However, this was not significantly different from that at 0.98 and 0.90 a_w ($P>0.05$).

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Keywords: Aspergillus section *Flavi*; aflatoxin; water activity; temperature; rice grain

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1. Introduction

Contamination with mycobiota in stored rice grains during pre- or post-harvest periods is less commonly reported than that for wheat or corn. However, these can have a significant impact on grains damage, milling quality, seed germination, dry matter and nutritional value as had been found for other commodities (Reddy et al., 2009; Siruguri et al., 2012; Kumar and Kweera, 2013; Fagbohun and Oluwaniyi, 2015) A potential serious risk to human health can occur when members of this mycobiota are mycotoxin producing species. It is known that species of *Aspergillus*, *Penicillium* and *Fusarium* are predominant mycobiota which were isolated from paddy rice, polished rice and husked rice. The focus on these species is linked to mycotoxin production in rice grains (Tonon et al., 1997; Park et al., 2005; Makun et al., 2007; Nguyen et al., 2007). There are also some reports in Thai rice that has been contaminated with mycotoxins (Tanaka et al., 2007; Anukula et al., 2013; Songsermsakul, 2015). *Aspergillus* section *Flavi* is well known as the main producer of aflatoxins (B1, B2, G1 and G2). Aflatoxin B1 has been categorized as a Group IA carcinogenic agent and also have been described as mutagenic and teratogenic agent (IARC, 2002). There are several factors affecting growth and mycotoxin production by mycotoxigenic fungi. These include temperature, water activity (a_w), fungal strains, microbial ecology and post-harvest handling conditions (Magan et al, 2004; Park et al., 2005; Makun et al., 2007; Nguyen et al., 2007). Under high temperature stress when combined with drought conditions, higher amounts of aflatoxins were formed by *A. flavus* (Jones et al., 1980; Payne et al., 1988; Craufurd et al 2006; Kebede et al, 2012). Mycelial growth is also affected by these critical factors; temperature and a_w (Cairns et al., 2005; Pardo et al., 2006; Akbar and Magan, 2014). Due to the combined effects of high temperature and water stress in some regions, the contamination of mycotoxigenic fungi in major commodities becomes a serious problem in food security and food nutritional quality in tropical developing countries and indeed also temperate countries (Magan et al., 2011). Thailand is one of the major rice producing tropical countries. However, poor post-harvest handling of the rice grains, sometimes serious problems have occurred. The key ecological factors of a_w and temperature influence and determine colonization of this commodity by both mycotoxigenic and non-mycotoxigenic spoilage fungi (Magan et al., 2010; Akbar and Magan, 2014). The objectives of this study was to evaluate the interacting environmental factors of a_w and temperature on lag phase and relative growth rate of aflatoxigenic *Aspergillus* section *Flavi* on a milled paddy rice-based medium.

2. Materials and Methods

2.1 Fungal strains

The aflatoxin producing fungal strains used in this study were *Aspergillus* section *Flavi* (SS4, SS6, SS7, SS17, SS20 and SS23) which were previously isolated from paddy rice collecting from Nakhonratchasima rice research center and Chanthaburi province in Thailand.

2.2 Preparation of mycelial inoculum

The inoculum was prepared from 7-days-old cultures of fungal strains grown on Malt Extract Agar (MEA) at 25°C. A mycelium plug (5 mm diameter) was cut from the margin of growing colonies using a sterilized cork borer which was placed in the center of Petri dishes (9 cm diameter) of each treatment.

2.3 Media, inoculation and growth measurement

An experiment was designed to determine the effect of a_w and temperature on lag phase prior to growth and growth rate by the selected strains. A full 3x3 factorial design was applied with MINITAB version 16 (Minitab, Inc., USA). The basic media 3% milled paddy rice agar was used in this study. The a_w of media was modified by adding increasing amounts of glycerol to obtain the following a_w treatment levels of 0.90, 0.95 and 0.98. These were checked with the a_w meter (Aqualab, Decagon devices, Inc., USA). After inoculation, the Petri plates were sealed with parafilm tape and kept in closed polyethylene bags at the tested incubation temperatures (20, 30 and 40°C). Each treatment was carried out in triplicate. The diameter of the colonies was measured daily in two directions at

right angles to each other (Marin et al., 1996). Lag phase and radial growth rate for each studied combination treatment were calculated from linear regression slopes of the growth curves.

2.4 Statistical analysis

The effect of the experimental factors on lag phase and growth rate were analysed statistically by using MINITAB version 16.0 (Minitab Inc., USA). ANOVA was adopted to test the significant differences among the studied treatments. Post-hoc inter factor differences were calculated with Tukey multiple comparison tests at statistical significant level of $P<0.05$ evaluating statistical interactions.

3. Results and Discussion

3.1. Effect of a_w and temperature on Aspergillus section Flavi strains lag phase and growth

The effect of different temperatures and a_w levels on the lag phases prior to growth of 6 strains was shown in Fig. 1. This showed that at marginal temperatures (20°C), regardless of a_w level, a longer initial lag phase was observed when compared to other treatments. The shortest average lag times (<1day) were at 30°C and 0.95-0.98 a_w. Fig. 2 showed the effect of a_w and temperature on the relative growth rate of each strain. Overall, the growth rates were generally high at 0.95-0.98 a_w and 30°C. The highest growth rate was 14.85±0.17 mm/day at 40°C and 0.98 a_w by strain SS4. This strain and strains SS6 and SS23 also exhibited more noticeable tolerance response to elevated temperatures than isolates SS7, SS17 and SS20 which were more sensitive to high temperature stress (40°C). With regard to the influence of temperature stress, the observed higher growth rates of fungal strains at optimum temperature (30°C) at nearly all a_w levels used is supported by the view that the greatest tolerance to lowered a_w occurs at optimum growth temperatures (Ayerst, 1969; Aldred et al., 1999; Magan, 2007). However, this finding can indicate the tolerance to lowered a_w was strain dependent as observed in strain SS4 which showed optimal growth at 30-40°C. The most studies suggest that *A. flavus* can only grow slowly at high temperature (40°C). The tolerance of some strains to 40°C in the present study could be related to tolerance developed over a long period to elevated temperatures. And these could become important under climate change conditions where extreme changes in temperature and drought stress and elevated CO_2 conditions can occur. Medina et al. (2014) showed a stimulation of aflatoxin production under climate change conditions although growth was relatively unaffected. Moreover, the optimum and marginal conditions of a_w and temperature on growth and aflatoxin production by *A. flavus* can be very different (Mousa et al., 2013; Medina et al., 2014; Choi et al., 2015).

3.2. Evaluation the effect of a_w and temperature on lag phase on Aspergillus section Flavi strains.

Based on the higher growth rate of selected fungi at different temperature levels, they were divided into two groups; high- temperature (40°C) tolerant strains (strain SS4, SS6, SS23) and moderate temperature-tolerant strains (20-30°C; strain SS7, SS17, SS20). Therefore, statistical analysis was extended to evaluate the effect of a_w and temperature on lag phases prior to growth and growth rate of the combined experimental data sets based on these two groups as mentioned above. For lag phase, normality of the distribution of the population was tested using the Kolmogorov–Smirnov test. The test revealed that the response variable was not normally distributed and needed to be normalized by transformation prior to analyses. The data sets were transformed by the Johnson transformations ($P>0.05$). The original data sets of lag phase were thus transformed according to the following equation:
For high- temperature (40°C) tolerant strains (strain SS4, SS6, SS23),

$$Y^* = -0.804455 + 0.730743 \times A\sinh\left(\frac{\left(x - 0.405189\right)}{0.2221359}\right) \quad (1)$$

For moderate temperature-tolerant strains (20-30°C; strain SS7, SS17, SS20),

$$Y^* = -1.89305 + 1.99922 \times A\sinh\left(\frac{(x + 0.249935)}{1.19561}\right) \quad (2)$$

In the equation, Y^* represents the transformed lag phase and x represents the original data.

An ANOVA analysis was carried out on the transformed experimental data for two groups. These showed that both of studied factors including a_w and temperature had a significant effect on lag phase within the range used in this study ($P<0.05$). However, the combined factors showed no statistical interaction effect on the lag phase of both groups ($P>0.05$) which can imply that the effect of one factor on the response does not depend on the level of the other factor.

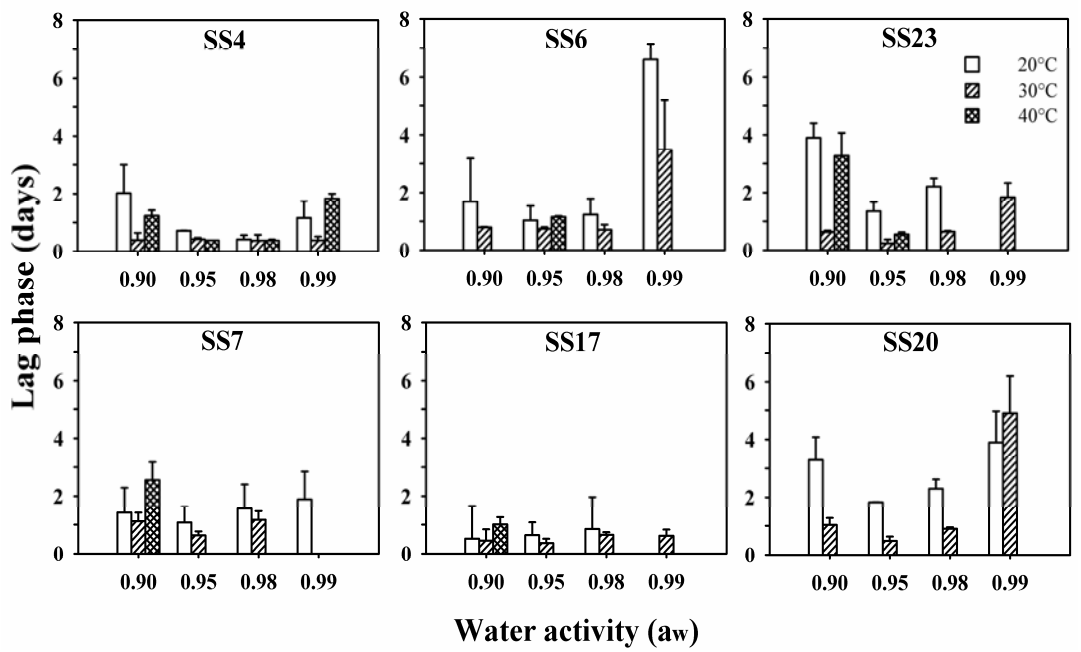

Fig. 1. Comparison of the lag phases (days) of six strains of *Aspergillus* section *Flavi* at different a_w and temperature levels on a 3% milled paddy rice agar. Bars indicates standard error of the mean.

The main effects plot was useful in the practical interpretation of the results. This could be used to examine the changes in the mean level providing the tendency of the response on the tested level of factors. Fig. 3 represented the main effects of a_w and temperature in term of back-transformed data. The results showed that as water stress (drier conditions) and low temperature (20°C) were imposed, longer initial lag phase duration became more pronounced. The shortest lag times prior to growth were obtained at 0.95 a_w and 30°C and the longest were at 0.90 a_w and 20°C for both groups. The strong inhibitory effect of high temperature (40°C) on growth was observed among temperature-sensitive strains, where extended lag phases prior to growth was also obtained.

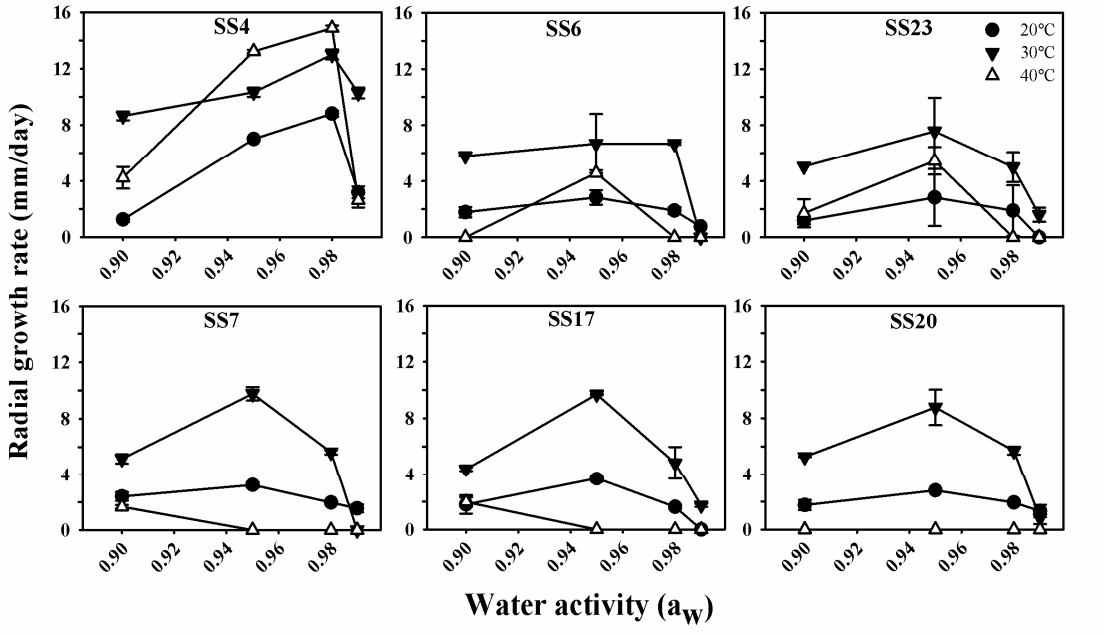

Fig. 2. Effect of a_w and temperature on radial growth rates of six strains of *Aspergillus* section *Flavi* on a milled paddy rice agar.

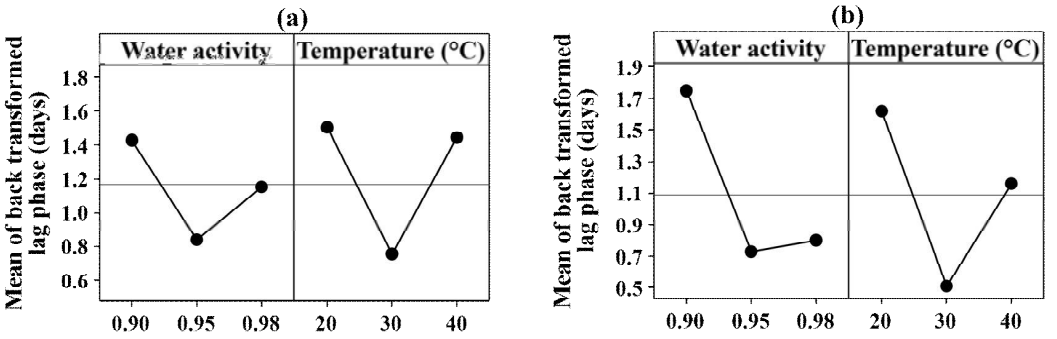

Fig. 3. Main effects plot of the mean of back-transformed lag phase of combined experimental data of (a) high-temperature (40°C) tolerant strains (strain SS4, SS6, SS23) and (b) moderate temperature-tolerant strains (20-30°C; strain SS7, SS17, SS20).

3.3. Evaluation the effect of a_w and temperature on growth rate on Aspergillus section Flavi strains.

Evaluation of the effect of a_w and temperature on growth rate of the combined sets of experimental data was also investigated. As the response variables were not normally distributed, transformation of the experimental data was performed by Johnson transformations (P>0.05) according to the following equation:
For high-temperature (40°C) tolerant strains (strain SS4, SS6, SS23),

$$Y^* = 0.854173 + 0.758968 * \ln\left(\frac{(x + 0.457416)}{18.1163 - x}\right) \quad (3)$$

For moderate temperature-tolerant strains (20-30°C; strain SS7, SS17, SS20),

$$Y^* = -3.40545 + 1.93853 * \ln(x + 2.68357) \quad (4)$$

In the equation, Y^* represents the transformed lag phase and x represents the original data

An ANOVA analysis was performed on the transformed data. Both quantitative variables including a_w and temperature were observed as significant changes in colony radial growth rate for both groups ($P<0.05$). However, a significant effect on the interaction did occur, but only in the group of strains which tolerated moderate temperatures ($P<0.05$). Main effects plot of a_w and temperature in term of the back-transformed data of high- temperature (40°C) tolerant strains was shown (Fig. 4a). The results showed that under the most severe water stress (0.90 a_w), strong inhibitory effect of radial growth occurred. A sharp decline in average growth rate was observed as a function of low temperature condition (20°C). Growth at 30°C was a more favourable condition but no statistically significant differences of growth rate occurred from 40°C ($P>0.05$). The interaction effect plot was then performed on the back transformed data of moderate temperature-tolerant strains (Fig. 4b). The ordinal interactions between a_w and temperature were found at lower levels in the experimental temperature (20-30°C). The smaller change in radial growth rate did occur under such conditions at 0.98 and 0.90 a_w. However, when the cultured temperature increased (>30°C), their interactions on responses became disordinal interaction especially under high water availability condition (>0.95a_w). The effect of each a_w levels was influenced by the presence of the high temperature used showing the different effect on the responses. Post hoc analysis was extended to evaluate the interaction effects. It also revealed that growth at 0.95 a_w with 30°C, the fastest growth rate was obtained but not significantly from that at 0.98 and 0.90 a_w ($P>0.05$). There have been detailed studies which have examined the optimum a_w and temperature conditions for growth of *A. flavus* isolating from Korean rice. Choi et al. (2015) had reported lower optimum temperature for growth (21°C, 0.97 a_w) and no growth was observed at 21°C and 85% RH (0.85 a_w). These optimal conditions are lower than that found by present study (30°C, 0.95 a_w), although those strains were isolated from rice samples. The different location of strains and adaptation to different types of stress conditions may be the additional explanation for this observation. In addition, the maximal growth rate of fungi influenced by of cultured medium was when water stress was imposed (Garcia et al., 2011; Astoreca et al., 2012).

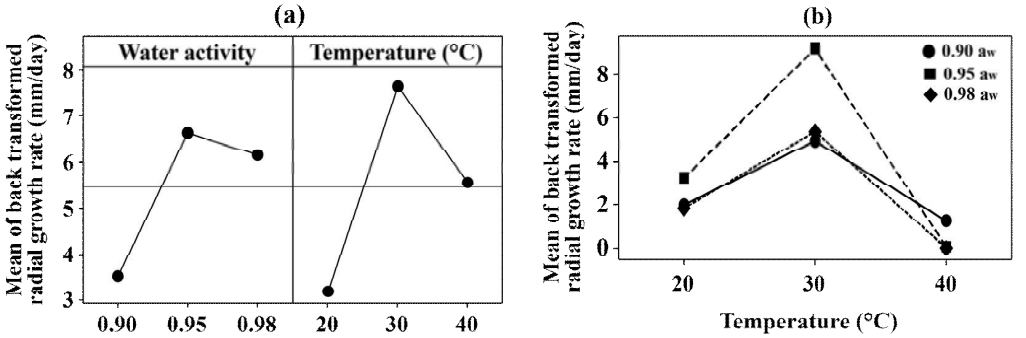

Fig. 4. (a) Main effects plot of the mean of back-transformed radial growth rate (mm/days) of combined experimental data of high-temperature (40°C) tolerant strains (strain SS4, SS6, SS23). (b) Interaction effects plot of the mean of back-transformed radial growth rate of combined experimental data of moderate temperature-tolerant strains (20-30°C; strain SS7, SS17, SS20).

As both groups of *Aspergillus* section *Flavi* were part of the mycobiota of rice grains, integrating the data from both groups was performed. The corresponding 2D contour plot of the relative lag phase profile was constructed (Fig. 5a). This provided the boundary conditions for lag times prior to growth based on the data range used. Minimum a_w and temperatures to delay the initial growth (>2 days) were 0.90-0.91 a_w and 20-22°C. Fig. 5b presents contour plot of radial growth rate profile. Examining this plot, it showed the optimal conditions for growth (>7.5 mm/day) depending on the temperature. Under moderate temperature (30°C), a wider optimum a_w range (0.92-0.97

a_w) was observed. This was wider than that found at high temperature (40°C) where more freely water available (0.96-0.98 a_w) condition was optimum. When the temperature was reduced to 20°C, the impact of a_w on responses became less significant**.** Under optimal temperature conditions, the response depended almost entirely on a_w. A number of studies have detailed the optimum and marginal conditions of a_w and temperature for growth of *A. flavus*)Niles et al., 1985; Mousa et al., 2013; Choi et al., 2015(. These studies have compared a wide range of strains of *A. flavus* which were isolated from different type of rice samples and different regions. Generally, *A. flavus* was able to grow over a wider range of a_w levels and appears to be more tolerant of drier conditions. Studies by Pitt and Miscamble (1995) reported that the minimum a_w for growth of *A. flavus* was 0.82 a_w (25°C(, 0.81 a_w (30°C(and 0.80 a_w (37°C). Mousa et al. (2013) found slight growth of *A. flavus* (0.1-0.2 mm./day(at 0.84 a_w)20°C(when optimum temperature was 30°C. They also compared the growth with different types of rice. Growth with brown rice, a lower minimum a_w level (0.82 a_w) was observed comparing with milled rice which was lower than in the present study of 0.85 a_w regardless temperature (data not shown). Niles et al. (1985) found a slight higher optimum temperature for growth of *A. flavus* at 35°C when cultured with wheat grains. They also reported no growth at 42.5°C and 20°C (0.85 a_w) however, under lower stress temperature (15°C and 20°C), growth was observed at more freely water conditions (>0.975 a_w).

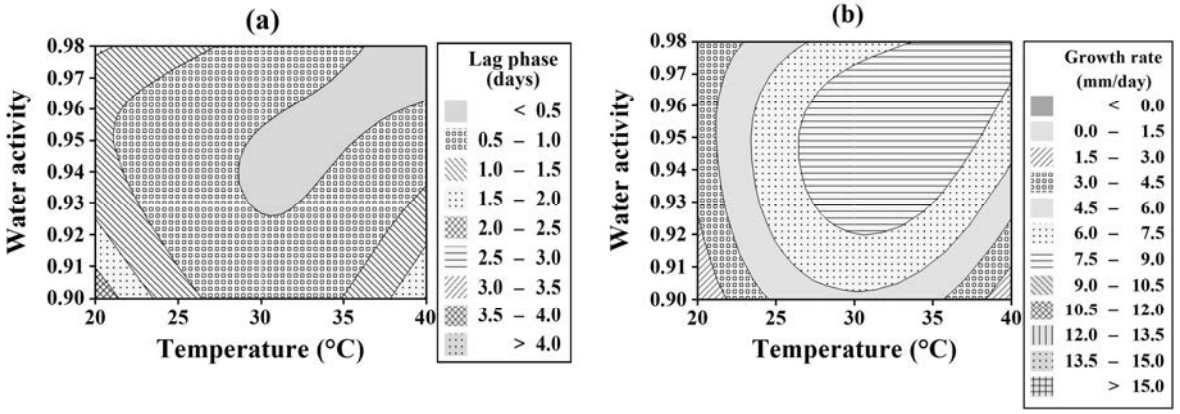

Fig.5. Mean of contour plot of (a) relative lag phase profile and (b) growth rate profile of the combined experimental data from both groups of *Aspergillus* section *Flavi* strains in relation to a_w x temperature on a milled paddy rice agar.

4. Conclusion

This study provided ecophysiological growth patterns of *Aspergillus* section *Flavi* in the response to the combined environmental stresses (a_w X temperature) on a milled paddy rice agar medium. The maximum, optimum and minimum conditions for growth have been detailed. This useful information may be the additional explanation in the interaction effects on growth of *Aspergillus* section *Flavi* which can occur during post-harvest handling of rice grains minimizing fungal contamination. Moreover, i*n vivo* studies need to be performed in order to have a better understanding of the growth response and aflatoxin production under environmental stress.

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