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EFFECT OF TEMPERATURE, WATER ACTIVITY AND CINNAMON ESSENTIAL OIL ON THE GROWTH OF *ASPERGILLUS FLAVUS* LA01 AND *ASPERGILLUS NIGER* LA04

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ABSTRACT

The aim of the study was to evaluate the influence of temperature, water activity and cinnamon essential oil on the growth of *Aspergillus flavus* LA01 and *Aspergillus niger* LA04 and these factors were further optimized to inhibit their growth. Both strains were isolated by Potato Dextrose Agar medium (PDA) and identified by sequencing method from rice collected in Long An province. Evaluating these factors' inhibition on the fungi growth was based on growth rate at the various points of water activity (aw) (v/v) such as 0.85, 0.90, 0.95, 0.99 and at 25 ^{o}C, 30 ^{o}C, 37 ^{o}C, 42 ^{o}C; at various concentrations of the oils 0 %, 1 %, 3 %, 5 %, 7.5 %, 10 % and 15 % at 30 ^{o}C incubated for 5 days - the disc diffusion method. The results showed that 30 ^{o}C and aw = 0.95 - 0.99 were optimum conditions for *Aspergillus flavus* LA01 and *Aspergillus niger* LA04 growth. However, at 42 ^{o}C, 37 ^{o}C and aw = 0.85 - 0.9 could control *Aspergillus flavus* LA01 and *Aspergillus niger* LA04 growth. At concentration of 10 - 15% cinnamon essential oils (EOs), 30 ^{o}C and aw = 0.9 - 0.95 could also inhibit them.

Keywords: *Aspergillus flavus* LA01, *Aspergillus niger* LA04, temperature, water activity, cinnamon essential oils (EOs).

1. INTRODUCTION

A. flavus and *A. niger* are the most widely studied fungal species. These species contaminate in many agricultural products such as rice, maize, etc. They infect in many steps from pre-harvest to storage and transformation. Hence, mycotoxins could be available in pre- and post-harvest agricultural products.

The growing of fungi and the accumulating of mycotoxins in food are impacted by many factors such as temperature, water activity, substrate, and so on [1]. Generally, water activity and temperature are considered as most critical factors during drying and storage [1]. A lot of chemical and physical methods have been applied to reduce mycotoxins, but only a

few have been accepted. Therefore, preventing growth of fungi and producing mycotoxin represent important steps in risk management. The influence of abiotic and biotic factors on fungal growth and mycotoxins production have been widely studied [1 - 3].

The antifungal properties of essential oils have been known for a long time, and studies on such effects on several postharvest phytopathogens have been reported [4-6]. Antifungal tests *in vitro* conditions of several EOs [5] showed that they can be active against some types of fungi. Furthermore, the potential use of EOs to control postharvest diseases requires.

Rice (*Oryza glaberrima*) is an important food resource in terms of nutritional and economic values. Rice grains can be colonized by several fungal species during growing stages [7] . Several genera such as *Aspergillus, Fusarium* and *Penicillium* are among the most prevalent mold in food [7].

The Vietnamese climate is characterized by high temperature and relative humidity that could stimulate toxigenic fungal growth and mycotoxin production. With high level of rain and humidity, the average relative humidity is high, up to 90 % [6]. Moreover, Vietnamese farmers and small retailers have badly stored, and even sold without packing; it is directly exposed to moist warm air. Fungi and mycotoxin contamination is highly risky. It can negatively influence consumer health. Therefore, it is important to develop prevention and control strategies to reduce mycotoxins contamination in Vietnamese rice. The main objective of the present study was to determine the effect of water activity, temperature and cinnamon essential oil on the *Aspergillus flavus* LA01 *and Aspergillus niger* LA04 isolated from rice in Long An (southern Vietnam) and selected for high mycotoxins production.

2. MATERIALS AND METHODS

2.1. Fungal isolates

Two isolates of *A. flavus*LA01 and *A. niger* LA04 were used in this study. The two isolates were obtained from rice samples collected from the farmers living in Long An province in Vietnam in 2017 and were kept in the laboratory of the Ho Chi Minh city university of Food Industry. The isolates were previously found to produce Aflatoxin B1 and Ochratoxin A when cultivated in potato dextrose agar (PDA).

A. flavus LA01 and *A. niger LA04* isolates were sub-cultured on potato dextrose agar (PDA) plates and incubated at 30 ^{o}C for 10 days to enable significant sporulation. After incubation, a sterile inoculation loop was used to remove the conidia from the PDA plates. The conidia were suspended in an aqueous solution of 0.05 % (w/v) tween 80. After homogenizing, the spore concentrations were determined using the Thomas cell counting chamber. The suspensions were diluted to adjust the final 10^{6} spores.ml^{-1}.

2.2. Effect of temperature and water activity on growth of fungi

Glycerol was used to modify the water activity of sterilizing medium PDA to 0.85; 0.9; 0.95; 0.99 [5], then poured this medium into petri-dish (20 ml/dish) and cultured 10 µl spore solution (the density of 1.10^{6} spore ml^{-1}) for each petri-dish. These dishes were surveyed at different temperatures 25 ^{o}C, 30 ^{o}C, 37 ^{o}C, and 42 ^{o}C in 10 days, measured diameter daily to evaluate the growth rate of fungi (cm.day^{-1}). All experiments repeated three times to have

average value. The growth rate of fungi was calculated via a formula: A = $\frac{\sum_{K=0}^{7}(d_{k+}}{7}$. In there, A is the growth rate of fungi (cm day^{-1}); d is diameter of growing colonies (cm).

2.3. Effect of temperature and cinnamon essential oil content on growth of fungi

Antimicrobial activity of essential oils was determined by disc diffusion method [8]. Potato dextrose agar (PDA) medium was cultured 10 µl spore solution (the density of 1.10^6 spore ml^{-1}) for each petri-dish. Then, 10 µl of each cinnamon essential oil (Song Natural Medicinal Oil Co., Ltd, P. R. China.), diluted in Tween 80 to get final dilution (1 %, 3 %, 5 %, 7.5 %, 10 % and 15 % (v/v) were added to 6 mm diameter sterile blank filter discs and placed in the center of the cover of the Petri. The dishes were then sealed using sterile laboratory parafilm to avoid eventual evaporation of the essential oils, followed by incubation at 30 ^oC for 5 days. Blanks were prepared by adding 10 µl of tween 80 solution to the filter discs. The effectiveness of the essential oils was calculated by measuring the diameter (cm) of the zone of fungal growth inhibition above the disc.

3. RESULTS AND DICUSSION

3.1. Effect of temperature and water activity on growth of *Aspergillus flavus* LA01 and *Aspergillus niger* LA04

The Fig.1A shows that the growth of *A. flavus* LA01 on PDA media (pH = 5.7) at different temperatures (25 ^oC, 30 ^oC, 37 ^oC and 42 ^oC) and water activities (a_w) (0.85, 0.90, 0.95, 0.99). At 0.85 a_w, *A. flavus* LA01 was completely inhibited at all temperatures, whereas at 0.90 a_w, its growth rate significantly increased (0.24 ± 0.003 cm day^{-1}) at 30 ^oC. Specifically, the maximum growth rate was observed at 0.95 a_w and 30 ^oC (1.72 ± 0.02 cm day^{-1}) while the figure for others such as *Aspergillus niger* was 0.99 a_w (Figure 1B). Moreover, this result demonstrated that the temperature affected the growth rate of *A. flavus* LA04. At three a_w of 0.90, 0.95 and 0.99, the maximum growth temperature was detected at 30 ^oC (Fig. 2). The results were similar compared to some previous studies. Das *et al*. (2012) [9] found the optimum growth a_w and temperature of *A. flavus* being 0.95 - 0.99 a_w and 30 ^oC respectively. Furthermore, Rosso *et al.* (1995) [10] reported that at the range of 0.834 - 0.845 a_w, the growth rate of *A. flavus* was inhibited by high temperature.

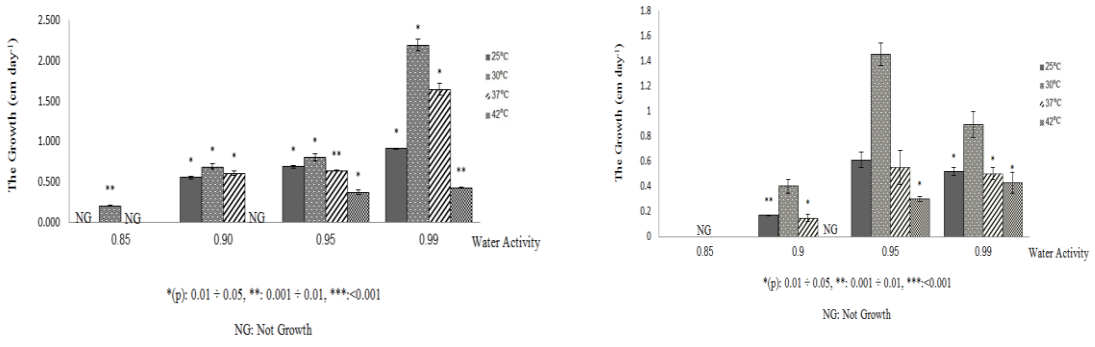

Figure 1. Effect of temperature and water activity on the growth of *Aspergillus flavus* LA01 (A) and *Aspergillus niger* LA04 (B) on PDA media.

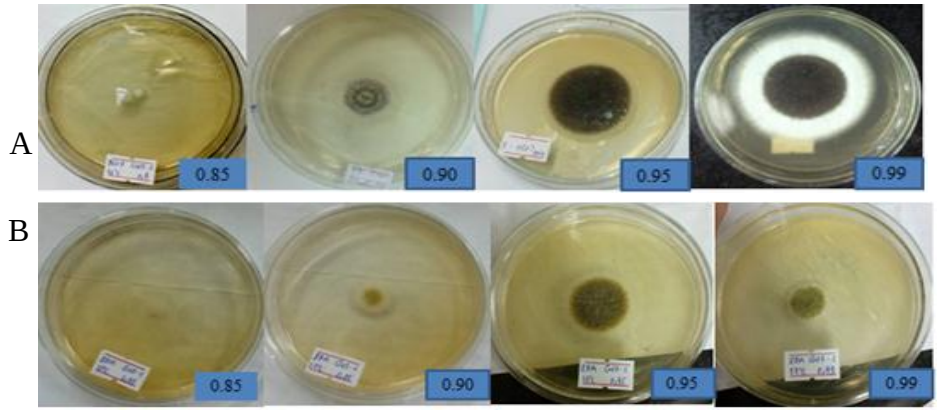

Figure 2. The growth of *Aspergillus flavus* LA01 (A) and *Aspergillus niger* LA04 (B) on PDA at 0.85 – 0.99 a_w, 30 ^oC after 5 days.

The study of Nesci *et al*. (2004) [11] also showed that the survival percentage of *A. flavus* considerably rose from 20 % to 80 % at 0.95 and 0.99 a_w. To sum up, the growth of *A. flavus* was affected by a_w and temperature. In this study, *A. niger* LA04 was also investigated at the same conditions as *A. flavus* LA01 (Fig. 1A). Generally, its growth rate decreased as increasing the incubation temperature. Although the optimum growth temperature of *A. niger* LA04 was also at 30 ^oC, it was faster than that of *A. flavus* LA01. At 42 ^oC, *A. niger* LA04 slowly was grown at 0.95 – 0.99 a_w and was not grown at 0.85 - 0.90 a_w. However, the optimum conditions was observed at 0.99 a_w and 30 ^oC (2.2 ± 0.043 cm day^{-1}). This result was similar to the researches of Marin *et al.* (2009) [12]. Moreover, Roberto Parra *et al.* (2004) [13] also illustrated that *A. niger* was grown well at 0.90-0.99 a_w and at temperature between 30 ^oC and 35 ^oC, especially at 30 ^oC.

Consequently, a_w was an integral factor to control the growth of mold on substances and there was a correlation between a_w and the growth rate of *Aspergillus sp* isolated from rice. Hence, we should manage the a_w of rice at less than 0.85 combined with high temperature (> 37 ^oC) during transport and storage to inhibit the growth of *Aspergillus* sp.

3.2. Effect of essential oil on the growth of *Aspergillus flavus*LA01 *and Aspergillus niger*LA04

According to the results, the optimum temperature and a_w for growth of *Aspergillus* sp. Strains isolated from rice were observed at 30 ^oC and 0.90 – 0.99 a_w, respectively. However, at 0.99 a_w both species were not inhibited at all different concentrations. Thus, in this study, antimicrobial activity of cinnamon essential oil was evaluated by agar well diffusion method at 30 ^oC and a_w of 0.90 -0.95.

A.flavus LA01 and *A. niger* LA04 were treated with cinnamon essential oil at various concentration of 0 %, 1 %, 3 %, 5 %, 7.5 %, 10 % and 15 % and different two a_w of 0.90 and 0.95 before incubating at 30 ^oC for a 5-day cultivation. As mentioned in the Table 1, at the concentrations between 5 % and 15 %, the cinnamon essential oil displayed the inhibition potential of *A. flavus* LA01.

Table 1. Inhibition zone diameter of *Aspergillus flavus* LA01 and *Aspergillus niger* LA04 on PDA media at 30 ^{o}C and a_w of 0.90 and 0.95 after 5-day cultivation.

Concentration of essential oil (%)	**Inhibition zone diameter (cm)**			
	***Aspergillis flavus* LA01**		***Aspergillus niger* LA04**	
	0.90 a_w	**0.95 a_w**	**0.90 a_w**	**0.95 a_w**
0	-	-	-	-
1	-	-	-	-
3	-	-	-	-
5	2.33 ± 0.11	0.5 ± 0.07	0.83 ± 0.14	0.54 ± 0.125
7.5	2.9 ± 0.07	1.56 ± 0.106	1.17 ± 0.07	0.64 ± 0.118
10	2.95 ± 0.106	2.01 ± 0.301	1.49 ± 0.05	0.87 ± 0. 129
15	3.78 ± 0.071	2.5 ± 0.212	1.50 ± 0.05	0.98 ± 0.03

(-) No inhibition zone diameter.

Noticeably, at concentration of 15 % essential oil, the largest inhibition zone diameters were observed for both a_w of 0.90 and 0.95, 3.78 ± 0.071 cm and 2.5 ± 0.212 cm, respectively. By contrast, the essential oil did not seem to inhibit the growth of *A. flavus* LA01 at a concentration less than 5 %.

In a similar way, antibacterial activity of *A. niger*LA04 was also examined (Table 1). The result showed that the growth of *A. niger* LA04 on two media of 0.90 and 0.95 a_w was inhibited by the cinnamon essential oil at a concentration greater than 5 %. As for 0.90 a_w, the inhibition zone at 5 % was the smallest with 0.83 ± 0.14 cm diameter. The figure for 15 % rose to 1.50 ± 0.05 cm, but it was not different significantly compared with 10 % ($p > 0.05$). Likewise, at 0.95 a_w, there was a correlation between the zone diameter and concentration of essential oil. When raising the concentration from 5 % to 15 %, the inhibition zone diameter also increased from 0.54 ± 0.125 cm to 0.98 ± 0.03 cm.

In addition, the result also demonstrated that the antimicrobial potential of cinnamon essential oil at 0.90 a_w was better than that of 0.95 a_w. Radwan *et al.* (2014) [14] reported that the cinnamon essential oil of 2 %, 3 % and 4 % did not considerably affect the growth of *A. niger*, and only inhibited at a concentration of 6 %. Another research of Lopez *et al.* (2005) [15] also indicated that *Aspergillus niger*LA04 was considerably inhibited by this essential oil. Scientists have revealed that some essential oils were likely to attack and break apart the cell membrane because of its hydrophobic property [16]. Beside they could also be able to affect enzymes that lead to respiratory depression and cell death [16]. This was so-called "interrupt mechanism" [16].

On the other hand, analyzing chemical composition of essential oils showed that theirs major bioactive content were phenolic compounds, terpenes, aldehydes and ketones, which primarily attacked cytoplasmic membrane of microorganisms [16]. Specifically, cinnamaldehyde was a major antimicrobial compound in cinnamon essential oil. It could deplete cytoplasm and destroy mitochondrial membrane as well as making protein folding and cell wall instability of microorganisms [16]. Moreover, it was able to inhibit some enzymes such as β-(1,3)-glucan synthase and chitin synthase [16].

In conclusion, the cinnamon essential oil could inhibit the growth of *A. flavus* LA01 and *A. niger* LA04 at a concentration greater than 5 %. Its antimicrobial potential of *A. flavus* LA01 at 0.90 a_w and 30 ^oC was better than that of *A. niger* LA04. Therefore, we can use it as an antimicrobial compound combined with a temperature (greater than 37 ^oC) and a_w (less than 0.85) condition to control the growth of mold in agricultural products and foods in Viet Nam.

4. CONCLUSIONS

Water activity, a_w was one of the most important factors to control the growth of mold on substances and there was a correlation between a_w and the growth rate of *Aspergillus sp* isolated from rice. At 30 ^oC and a_w = 0.95- 0.99 were optimum conditions for *Aspergillus flavus* LA01 and *Aspergillus niger* LA04 growth. However, at 42, 37 ^oC and a_w = 0.85 - 0.9 could control *Aspergillus flavus* LA01 and *Aspergillus niger* LA04 growth. The cinnamon essential oil could be used to inhibit the growth of *A. flavus* LA01 and *A. niger* LA04 at a concentration greater than 5 %. Its antimicrobial potential of *A. flavus* LA01 at 0.90 a_w and 30 ^oC was better than that of *A. niger* LA04. At concentration of 10 - 15 % cinnamon essential oil, 30 ^oC and a_w = 0.9 - 0.95 could strongly inhibit them.

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TÓM TẮT

ẢNH HƯỞNG CỦA NHIỆT ĐỘ, HOẠT ĐỘ NƯỚC VÀ TINH DẦU QUẾ LÊN SỰ SINH TRƯỞNG CỦA *ASPERGILLUS FLAVUS* LA01 VÀ *ASPERGILLUS NIGER* LA04

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Mục đích của nghiên cứu này là đánh giá ảnh hưởng của hoạt độ nước, nhiệt độ và nồng độ tinh dầu lên sự sinh trưởng của *Aspergillus flavus* LA01 và *Aspergillus niger* LA04. Cả hai chủng này được phân lập và định danh từ các mẫu gạo thu thập từ Long An bằng phương pháp đại thể, vi thể và giải trình tự gen. Sau đó tiến hành khảo sát ở các yếu tố như: hoạt độ nước 0,85; 0,90; 0,95; 0,99 với nhiệt độ 25 ^oC, 30 ^oC, 37 ^oC, 42 ^oC. Đồng thời cũng tiến hành thí nghiệm trên các nồng độ tinh dầu quế 0 %, 1 %, 3 %, 5 %, 7,5 %, 10 % và 15 % ở nhiệt độ 30 ^oC và hoạt độ nước a_w = 0.95- 0.99. Kết quả thu được như sau: Ở 30 ^oC và a_w = 0.95 - 0.99 là điều kiện tối ưu cho *Aspergillus flavus* LA01 và *Aspergillus niger* LA04 sinh trưởng mạnh. Tuy nhiên, ở nhiệt độ 42 ^oC, 37 ^oC và a_w = 0.85 - 0.9 có thể ức chế sự sinh trưởng của *Aspergillus flavus* LA01 và *Aspergillus niger* LA04. Ở nồng độ tinh dầu 10 - 15 %, 30 ^oC và a_w =0.9 - 0.95 có thể kìm hãm sự sinh trưởng của nấm mốc.

Từ khóa: Aspergillus flavus LA01, *Aspergillus niger* LA04, nhiệt độ, hoạt độ nước, tinh dầu quế.