The antagonist effect of Pichia kluyveri against the aflatoxigenic Aspergillus flavus

Wafa Masoud

Department of Agricultural Biotechnology, Faculty of Agricultural Sciences and Technology,

Palestine Technical University (Kadoorie), Tulkarem- Palestine

w.masoud@ptuk.edu.ps

Abstract

Aspergillus flavus is one of the main fungi that are known to produce Aflatoxin B1 (AFB1). AFB1 has been detected in many food products and reported to be a carcinogenic. Using a biocontrol agent against A. flavus can safely eliminate or reduce the occurrence of AFB1 in food. Therefore, the effects of four yeast strains of Pichia kluyveri on the growth and spore germination of A. flavus were studied. In addition, the effect of exposure of A. flavus to the yeast's headspace, as well as the effects of different headspace concentrations of 2-phenyl ethyl acetate and ethyl acetate, on the growth of A. flavus were investigated. Growth of A. flavus and spore germination were significantly reduced by P. kluyveri. The yeast's headspace significantly reduced the fungal growth. Furthermore, the volatile compound 2-phenyl ethyl acetate significantly reduced growth of A. flavus at a concentration of 5 µg/l with a complete growth inhibition at 60 µg/l. On the other hand, fungal growth was only significantly reduced at 40 µg/l concentration of ethyl acetate. It can be demonstrated that P. kluyveri is an effective biocontrol agent against growth of the aflatoxigenic A. flavus.

Keywords: Aspergillus flavus, Aflatoxin B1 (AFB1), Pichia kluyveri, Biocontrol, 2-Phenyl ethyl acetate, Ethyl acetate

Introduction

Aflatoxin B1 (AFB1) is one of the main fungal secondary metabolites, which is toxic, mutagenic, and carcinogenic (Ostry et al., 2017). AFB1 has been detected in large numbers of food and agricultural products (Sarma et al., 2017). There are many possible control methods that can be applied to prevent the occurrence of AFB1 in food, such as the usage of fumigants (Rajendran, and Sriranjini, 2007) fungicides (Sipos et al., 2021), or biocontrol agents (Fredlund et al., 2004). However, the fungicide residues in food and feedstuffs are of great concern to food safety (Steinberg, and Gurr, 2020). Therefore, application of a biological control against AFB1 producing fungi can be the method of choice, where no harm to human and environment will take place. Biological control of the aflatoxigenic fungi in food products will help to reduce the accumulation of AFB1 in those products.

The aim of the present study was to investigate the effects of four strains of *P. kluveri* on the growth of AFB1 producing strain of *A. flavus*. In addition, the effects of 2-phenyl ethyl acetate and ethyl acetate, the main volatile compounds produced by *P. kluyveri* on growth of *A. flavus* were investigated.

Literature review

Aflatoxins B1 and B2, G1, G2 and M1 are the most relevant for food safety (Afsah-Hejri et al., 2013). Aflatoxins can contaminate crops before harvest or post-harvest due to inadequate storage conditions where moisture and temperature are suitable for mold growth. Aflatoxins were detected in corn, cotton seed, cereals nut, rice, figs, wheat, almonds, spices and others (Sarma et al., 2017). Animal feed contaminated with aflatoxin may sometimes cause contamination of milk, egg and meat products (Kang'ethe, and Lang'a, 2009). The International Agency for Research on Cancer (IARC) classified aflatoxin as a group 1 of the causative agents of cancer (Ostry et al., 2017). Aflatoxin B1 may affect organs like the liver and kidneys (Alvarez et al., 2020). Furthermore, the consumption of high levels of aflatoxins within a short period was reported to cause the life threatening aflatoxicosis (Williams et al., 2004). *A. flavus* is one of the main fungi that was reported to produce AFB1 in many food products (Sarma et al., 2017). The control of *A. flavus* is very important to protect food and feedstuffs from aflatoxin contamination.

Yeasts have been reported as biocontrol agents against various plant pathogens. Yeasts have simple requirements to be cultivated and are considered safe, which make them ideal biocontrol agents. *Pichia kluyveri* is one of the yeast species that was reported to have antimicrobial activities against a number of microorganisms.

Mewa-ngongang et al. (2019) found that *P. kluyveri* inhibited the growth of several spoilage yeast species in raw fruits. Furthermore, the growth of the post-harvest fruit fungi *Botrytis cinerea* and *Monilinia laxa* was eliminated by *P. kluyveri* (Mewa-ngongang et al., 2021). In addition, *P. kluyveri* was among the wild yeast species that prevented growth of *Aspergillus carbonarius* and *Aspergillus ochraceus* and their ability to produce ochratoxin A (OTA) (Souza et al., 2017). The volatile compounds produced by *P. kluveri* and *Pichia anomala* were reported to inhibit growth of *A. ochraceus* and OTA production in fermented green coffee beans (Masoud et al., 2005).

The hypothesis of the study

In the present study, it is assumed that *P. kluyveri* has an antagonist effect against growth of the aflatoxigenic *A. flavus*. Furthermore, the volatile organic compounds, mainly 2-phenyl ethyl acetate and ethyl acetate are assumed to play a major role in the antifungal activity of *P. kluyveri*.

The Methodology

Cultures

Four strains of *P. kluyveri* (S4Y3, S7Y1, S8Y4, S13Y4), which were isolated from fermented coffee beans (Masoud et al., 2004) and an aflatoxin B1- producing strain of *A flavus* ATCC 367 (ATCC, Washington DC, NV, USA) were used in the present study.

Effect of *P. kluyveri* on growth of *A. flavus*

Each Strain of *P. kluyveri* was propagated in 25 ml of MYGP broth (Difco, Detroit, MI, USA) at 25 °C for 48 hours. Then the suspension was centrifuged at 3000 x g for 10 min, and the yeast cells were harvested and resuspended in saline (8 g NaCl/l distilled water). The number of *P. kluyveri* cells was measured under microscope using a haemocytometer (Neubauer). Then the yeast suspensions were diluted to final concentrations of 10^6 cells/ml. Twenty ml of melted MYGP agar were added to petri dishes and mixed with the diluted suspensions of *P. kluyveri* and left to solidify at room temperature for 2 h. Fungal spores were collected from previously inoculated plates of Potato dextrose agar (PDA) (Difco, Detroit, MI, USA) with *A. flavus* and suspended in saline and spores' concentration was adjusted to 10^6 spores/ml as described above for yeast cells. Spots of 10 µl of the fungal spore suspension (10^6 spores/ml) were placed on three sites of each MYGP plate that was inoculated with *P. kluyveri* as described above. A control was used by adding spots of *A. flavus* spore suspension on three sites of uncultivated MYGP plate.

The petri dishes were incubated at 25 °C for 7 days. Then the fungal colony diameter was measured (Masoud, and Kaltoft, 2006). For each strain of *P. kluyveri*, the experiment was conducted in triplicates.

Effect of *P. kluyveri* on spore germination of *A. flavus*

In this experiment, 10^6 spores/ml of *A. flavus* and 10^6 cells / ml of *P. kluyveri* were inoculated in 10 ml of MYGP broth and incubated at 25 °C. In addition, spores of *A. flavus* (10^6 spores/ml) were inoculated in *P. kluyveri* free supernatant and incubated at 25 °C in order to investigate the effect of yeast metabolites on fungal growth. The yeast free supernatant was prepared from a 24 h old culture of *P. kluyveri* in MYGP broth, which was centrifuged at 3000 x g for 10 min and the supernatant was filtered through a 0.22 µm nitro-cellulose filter. A control was prepared by inoculating spores of *A. flavus* (10^6 spores/ml) in MYGP broth and incubated at 25 °C. Germination of fungal spores was inspected by microscopy after 24, 48 and 72 h (Masoud, and Kaltoft, 2006).

Effect of exposure of *A. flavus* to *P. kluyveri* headspace

A volume of 100 µl from a suspension of 10^6 cells/ml of *P. kluyveri* was spread on the MYGP plates. On the other hand, a volume of 100 µl from 10^6 spores/ml suspension of *A. flavus* was spread on PDA plate. After that, the lids of the two plates were removed and the plates were sealed with tape and parafilm and incubated at 25 °C for 7 days. A negative control was prepared by taking a volume of 100 µl from 10^6 spores/ml suspension of *A. flavus* that was spread on PDA plate and was sealed with yeast free MYGP plates. This experiment was conducted in triplicates for each strain of *P. kluyveri*. The fungal biomass was estimated by cutting agar with fungal growth and mixing it with 200 ml of distilled water then the residual agar was melted using a microwave. The fungal biomass was obtained using a pre-weighted filter paper, which was then dried at 80 °C for 24 h. Afterwards, the dry weight of the fungal biomass was estimated (Fredlund et al., 2004).

Effects of ethyl acetate and 2-phenyl ethyl acetate on growth of *A. flavus*

A volume of 100 µl (10^6 spores/ml) of *A. flavus* was spread on PDA plates then the plate was placed facing down another plate containing either ethyl acetate or 2-phenyl ethyl acetate with concentrations of 5, 10, 15, 20, 40, 60, 80 and 100 µg/l. The two plates were sealed as just described in the previous section and incubated at 25 °C for 7 days. This experiment was performed in triplicates for each treatment. The dry weight of the fungal biomass was estimated as described in the previous section (Fredlund et al., 2004).

Data Analysis

Data was analyzed using Excel 2016 (Microsoft Corporation, USA). For the effects of *P. kluyveri* on growth of *A. flavus*, which were determined by agar-diffusion test and the fungal biomass weight, the mean values and standard deviations were calculated. For the effects of exposure of 2-phenyl ethyl acetate and ethyl acetate on growth of *A. flavus*, the T-test was performed.

Results and Discussion

The effect of four strains of *P. kluyveri* on growth of *A. flavus* is shown in Figure 1. It can be seen that the four strains of yeast strongly reduced the colony diameters of *A. flavus*. Differences were observed between strains of *P. kluyveri* in their antagonist effects against fungal growth. The strongest growth inhibition was observed with strain S13Y4 followed by S7Y1, S4 Y3 and S8Y4, respectively.
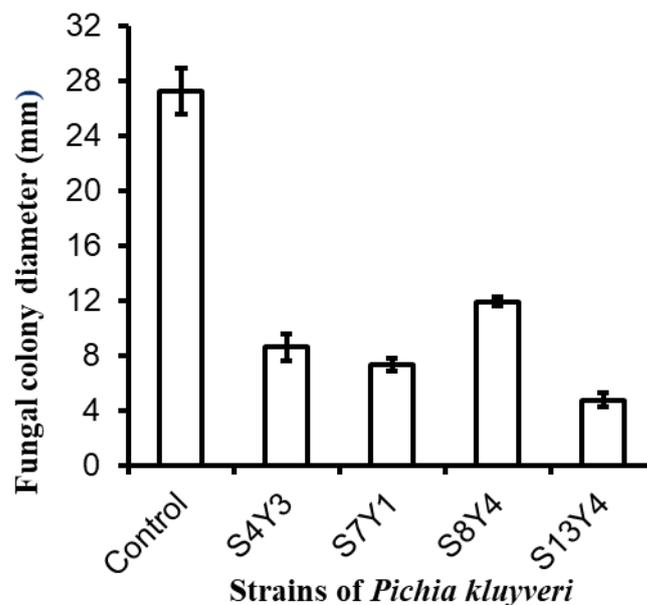

Figure 1: Growth inhibition of *A. flavus* on MYGP plates inoculated with four strains of *P. kluyveri* (S4Y3, S7Y1, S8Y4, S13Y4). Control represents growth of *A. flavus* on PDA plates without *P. kluyveri*. Inhibition is expressed as the fungal colony diameter. Bars represent standard deviations.

Pichia kluyveri has been detected in a number of fermented food and is considered safe. It was one of main yeast species that was isolated during coffee fermentation and participated in modification of coffee flavor (Wang et al., 2020; Pereira et al., 2021). *P. kluyveri* was also applied as a starter culture for cocoa fermentation and was demonstrated to play a role in chocolate flavor through its volatile organic compounds (Moreora et al., 2021).

The strains of *P. kluyveri* used in this study were isolated from all stages of coffee fermentation and were reported to inhibit growth of *A. ochraceus* and to prevent production of OTA (Masoud and Kaltoft, 2006). In addition, *P. kluyveri* was found to inhibit the spoilage yeasts and fungi in raw fruits (Mewa-ngongang et al., 2019).
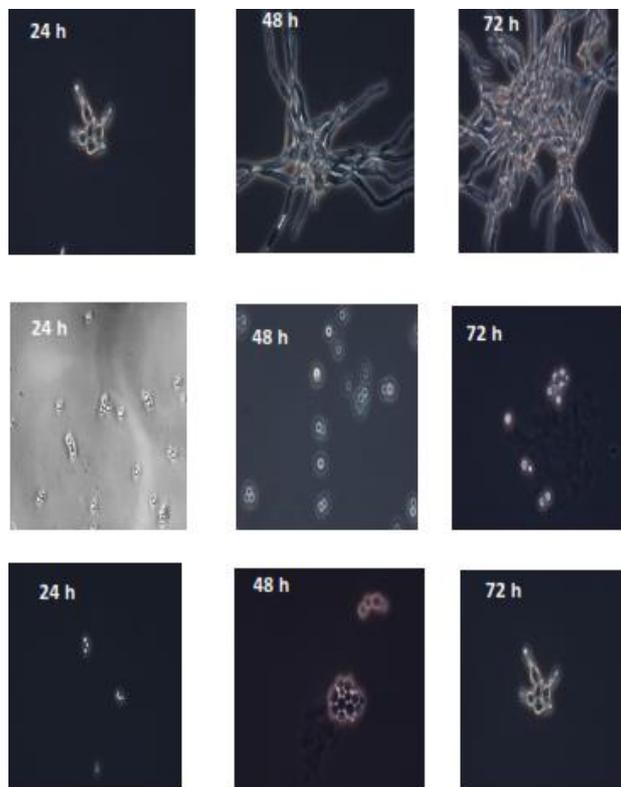

Figure 2: Germination of spores of *A. flavus* after 24, 48 and 72 h in MYGP broth (1ˢᵗ row); in MYGP broth inoculated with *P. kluyveri* S13 Y4 (2ⁿᵈ row); in MYGP *P. kluyveri* free supernatant (3ʳᵈ row).

Figure 2. Shows the effect of co-culturing *A. flavus* spores and *P. kluyveri* in MYGP and as well as culturing of *A. flavus* spores in yeast free supernatant. When co-cultured with *P. kluyveri*, the fungal spore germination was totally inhibited after 24, 48 and 72 h of incubation. Furthermore, spores of *A. flavus* did not germinate in yeast free supernatant, after 24 h (Figure 2). Spores of *A. flavus* were observed to be enlarged after an incubation period of 48 h, but no germ tube was seen. On the other hand, short germ tubes arise from spores of *A. flavus* after 72 h in yeast free supernatant, compared to the control with long normal germ tubes. Knowing the antagonist mechanism is very important for development of successful biocontrol methods. However, due to complex interactions between host, pathogen and biocontrol agent, it is not easy to identify the mechanism behind the antimicrobial activity. Different modes of action of the antagonist activity of yeasts against filamentous fungi has been suggested such as competition for nutrients and space, production of killer toxins,

production of volatile metabolites, production of enzymes parasitism and resistant induction in the host tissue (Freimoser et al., 2019). In the present work, the initial pH of MYGP was 5.6, which was then decreased to 4.3 in the yeast free supernatant. Germination of *A. flavus* spores in MYGP broth adjusted to pH 4.3 was investigated and found to be normal. Thus, it seems that inhibition of *A. flavu*s spore germination in *P. kluyveri* free supernatant was not due to changes in the pH of MYGP. Depletion of nutrients by *P. kluyveri* or production of extra cellular metabolites might be the causative agent behind inhibition of *A. flavus* spore's germination. Jayamurthy et al. (2014) reported that extracellular metabolites produced by *Streptomyces* sp. Inhibited growth of a number of fungi and yeast species.
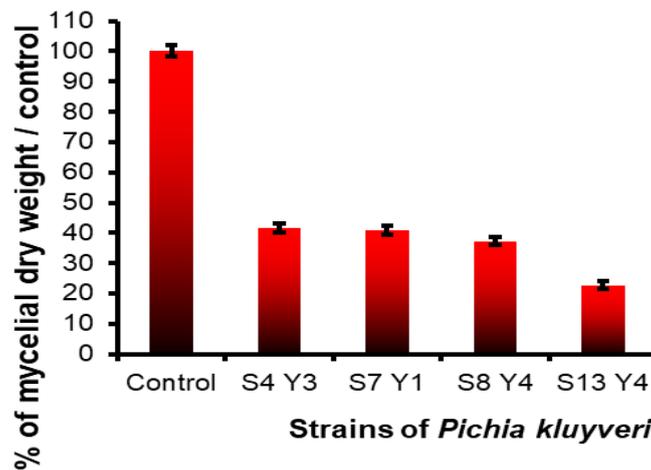

Figure 3: The effects of exposure of four strains of *P. kluyveri* (S4Y3, S7Y1, S8Y4, S13Y4) to *A. flavus*. Control represent growth of *A. flavus* on PDA plates exposed to MYGP plate not inoculated with *P. kluyveri*. Inhibition is expressed as the fungal biomass dry weight. Bars represent standard deviations.

Table 1: The effects of exposure of A. flavus cultivated on PDA plates to ethyl acetate and 2-phenyl ethyl acetate

Dry weight of fungal biomass (g) after exposure to ethyl acetate and 2-phenyl ethyl acetate		
Headspace concentration ($\mu g / l$)	Ethyl Acetate	2-Phenyl Ethyl Acetate
0	80	80
5	80	61 [s]
10	78	50 [s]
15	80	43 [s]
20	76	23 [s]
40	68 [s]	13 [s]
60	59 [s]	0 [s]
80	46 [s]	0 [s]
100	35 [s]	0 [s]

S: Significant at *p < 0.05* level.

Exposure of *A. flavus* to plates inoculated with *P. kluyveri* reduced the fungal biomass with strain S13Y4 showing the highest growth reduction (Figure 3). Mewa-Ngongang et al. (2019) demonstrated that the volatile organic compounds produced by *Candida pyrali*dae and *P. kluyveri* have antimicrobial activity against spoilage microorganisms of fruits. *Pichia kluyveri* was reported to produce different volatile compounds from which 2-phenyl ethyl acetate and ethyl acetate having the strongest antifungal activity against growth of *A. ochraceous* (Masoud et al., 2005). Hua et al. (2014) demonstrated that 2 phenyl ethanol was the major volatile compound behind the antagonist effect of P. *anomala* against growth of *A. flavus* and its ability to produce AFB1.The effect of applying different concentrations of ethyl acetate and 2-phenyl ethyl acetate on growth of *A. flavus* was investigated (Table 1). It can be seen that 2-phenyl ethyl acetate started to have a significant effect on growth of *A. flavus* at a concentration of 5 $\mu g / l$ with a complete growth inhibition at 60 $\mu g / l$. On the other hand, ethyl acetate significantly reduced fungal growth at 40 $\mu g / l$, but it did not completely inhibit *A. flavus* growth at the highest concentration investigated *i.e.* 100 $\mu g / l$ (Table 1).

Implications, conclusions and recommendations

From the results obtained, the antagonist activity of *P. kluyveri* against growth of *A. flavus* seems to be due to volatile organic compounds, mainly 2-phenyl ethyl acetate. The application of *P. kluyveri* as a biocontrol agent in fermented food and feed can be regarded as a promising tool to reduce their contamination with the aflatoxigenic *A flavus*. However, further studies are needed in real life food fermentations.

Acknowledgement

References

1. Afsah-Hejri, L., Jinap, S., Hajeb, P., Radu, S., and Shakibazadeh, S. (2013). A review on mycotoxins in food and feed: Malaysia case study. Comprehensive Reviews in Food Science and Food Safety. 12(6). PP 629–651.

2. Alvarez, C. S., Hernández, E., Escobar, K., Villagrán, C. I., Kroker-Lobos, M. F., Rivera-Andrade, A., Smith, J. W., Egner, P. A., Lazo, M., Freedman, N. D., Guallar, E., Dean, M., Graubard, B. I., Groopman, J. D., Ramírez-Zea, M., and McGlynn, K. A. (2020). Aflatoxin B 1 exposure and liver cirrhosis in Guatemala: A case-control study. BMJ Open Gastroenterology. 7(1). PP 1–7.

3. Fredlund, E., Druvefors, U. A., Olstorpe, M. N., Passoth, V., Schnurer, J. (2004). Influence of ethyl acetate production and ploidy on the anti-mould activity of *Pichia anomala*. FEMS Microbiology Letters. 238. PP 133–137.

4. Freimoser, F. M., Rueda-Mejia, M. P., Tilocca, B., and Migheli, Q. (2019. Biocontrol yeasts: mechanisms and applications. World Journal of Microbiology and Biotechnology. 35 (10). PP 154-172.

5. Hua, S. S., Beck, J. J., Sarreal, S. B., and Gee, W. (2014). The major volatile compound 2 phenylethanol from the biocontrol yeast, *P. anomala*, inhibits growth and expression of aflatoxin biosynthetic genes of *Aspergillus flavus*. Mycotoxin Research. 30. PP 71-78.

6. Jayamurthe, H., Sajna, K. V., Dastagar, S. G., and Pandey A. (2014). Anti-fungal potentials of extracellular metabolites of Western Ghats isolated Streptomyces sp. NII 1006 against moulds and yeasts. Indian Journal of Exoerimental Biology. 52 (11) PP 38-46.

7. Kang'ethe, E. K., and Lang'a, K. A. (2009). Aflatoxin B1 and M1 contamination of animal feeds and milk from urban centers in Kenya. African Health Sciences. 9 (4). PP 218-226.

8. Masoud, W., Cesar, L. B., Jespersen, L., and Jakobsen, M. (2004). Yeast involved in fermentation of Coffea arabica in East Africa determined by genotyping and by direct denaturating gradient gel electrophoresis. Yeast. 21. PP 549-556.

9. Masoud, W., and Kaltoft, C. H. (2006). The effects of yeasts involved in the fermentation of *Coffea arabica* in East Africa on growth and ochratoxin a (OTA) production by *Aspergillus ochraceus*. International Journal of Food Microbiology. 106. PP 229-234.

10. Masoud, W., Poll, L., and Jakobsen, M. (2005). Influence of volatile compounds produced by yeasts predominant during processing of *Coffea arabica* in East Africa on growth and ochratoxin A (OTA) production by *Aspergillus ochraceus*. Yeast. 22,. PP 1133–1142.

11. Mewa-ngongang, M., Plessis, H. W., Karabo, S., Ntwampe, O., Chidi, B. S., Hutchinson, U. F., Mekuto, L., and Jolly, N. P. (2019). The Use of *Candida pyralidae* and *Pichia kluyveri* to Control Spoilage Microorganisms of Raw Fruits Used for Beverage Production. Foods. 8. PP 454-467.

12. Mewa-ngongang, M., Wilbur, H., Chidi, B. S., Hutchinson, U. F., Seteno, K., Ntwampe, O., Okudoh, V. I., Jolly, N. P., Box, P. O., Town, C., and Africa, S. (2021). Physiological and Antagonistic Properties of *Pichia kluyveri* for Curative and Preventive Treatments Against Post-Harvest Fruit Fungi. Polish Journal of Food and Nutrition Sciences. 71 (3). PP 245–253.

13. Moreira, I. Costa, J., Vilela, L., Lima, N., Santos, C. and Schwan, R. (2021). Influence of *S.* cerevisia*e* and *P. kluyveri* on chocolate flavour. Journal of the Science of Food and Agriculture. 101 (10). PP 4409-4419.

14. Ostry, V., Malir, F., Toman, J., and Grosse, Y. (2017). Mycotoxins as human carcinogens—the IARC Monographs classification. Mycotoxin Research. 33 (1). PP 65–73).

15. Pereira, P. V., Bravim, D. G., Grillo, R. P., Betroli, L. D., Osorio, M. M., Oliveira, D. S., Miguel, M. G. P., Schwan, R. F., Silva, S. A., Coelho, J. M., and Bernardes, P. C. (2021). Microbial diversity and chemical characteristics of *Coffea canephora* grown in different environments and processed by dry method. World Journal of Microbiology & Biotechnology. 37 (3). PP 51-59.

16. Rajendran, S., and Sriranjini, V. (2007). Use of fumigation for managing grain quality. Stewart Postharvest Review. 6(9). PP 1-8.

17. Sarma, U. P., Bhetaria, P. J., Devi, P., and Varma, A. (2017). Aflatoxins: Implications on Health. Indian Journal of Clinical Biochemistry. 32(2). PP 124–133.

18. Sipos, P., Peles, F., Brasso, D. L., Beri, B., Pusztahelyi, T., Pocsi, I., and Gyori, Z. (2021). Physical and chemical methods for reduction in aflatoxin content of feed and food. Toxins. 13 (204). PP 1-17.

19. Souza, M. L. De, Reinis, F., Passamani, F., Luiza, C., Batista, L. R., Schwan, R. F., and Silva, C. F. (2017). Use of wild yeasts as a biocontrol agent against toxigenic fungi and OTA production. Microbiologia Agrícola. 39(3). PP 5-17.

20. Steinberg, G., Gurr, S. J. (2020). Fungi, fungicide discovery and global food security. Fungal Genetics and Biology.144. PP 1-4.

21. Wang, C., Sun, J., Lassabliere, B. Yu, B and Liu, S. (2020). Coffee flavour modification through controlled fermentation of green coffee beans by *Saccharomyces cerevisiae* and P*ichia kluyveri*: PartII. Mixed cultures with or without lactic acid bacteria. Foode Research International. 136. PP 1-11.

22. Williams, J. H., Phillips, T. D., Jolly, P. E., Stiles, J. K., Jolly, C. M., and Aggarwal, D. (2004). Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences, and interventions. American Journal of Clinical Nutrition. 80(5). PP 1106–1122.

التأثير المضاد لبشيا كلايفيري ضد أسبرجيلاس فلافاس المنتج للأفلاتوكسين

وفاء مسعود

قسم التكنولوجيا الحيوية الزراعية، كلية العلوم والتكنولوجيا الزراعية، جامعة فلسطين التقنية (خضوري)، طولكرم- فلسطين

w.masoud@ptuk.edu.ps

ملخص

أسبرجيلاس فلافس هو أحد الفطريات الرئيسية المعروفة بإنتاجها لمركب الأفلاتوكسين ب 1. وتم اكتشاف مركب الأفلاتوكسين ب 1 في عديد من المنتجات الغذائية. وتم التوثيق؛ كونه مادة مسرطنة. والمكافحة الحيوية لفطر الأسبرجيلاس فلافس، يمكن أن تؤدي إلى تقليل مركب الأفلاتوكسين ب 1 في الغذاء، أو القضاء عليه. وتمت دراسة أربع سلاسل من الخميرة بيشيا كلايفيري، وتأثيرها في نمو جراثيم الفطر أسبرجيلاس فلافس وإنباتها. كما تمت دراسة تأثير المواد المتطايرة، التي تنتجها خميرة بيشيا كلايفيري، في نمو الفطر. وتبين أن الخميرة قادرة على منع نمو جراثيم الأسبرجيلاس فلافس، ومنع إنباتها. كما تم منع نمو الفطر بواسطة تعريضه للمواد العضوية المتطايرة، التي تنتجها خميرة بيشيا كلايفيري. وتبين أنّ المادة المتطايرة 2– فينيل أثيل أسيتات، هي أكثر مادة فعالة ضد نمو الفطر، بالمقارنة مع مادة الأثيل أستات.

إنّ خميرة بيشيا كلايفيري هي عامل حيوي فعال ضد فطر الأسبرجيلاس فلافس، المنتج لمركب الأفلاتوكسين ب 1.

الكلمات الدالة: أسبرجيلاس فلافس، أفلانوكسين ب 1، بيشيا كلايفيري، المكافحة الحيوية، 2– فينيل أثيل أستات، أثيل أستات.