Sains Malaysiana 54(1)(2025): 151-164
http://doi.org/10.17576/jsm-2025-5401-12

Biological Control of *Aspergillus flavus* with *Pseudozyma hubeiensis* Yeast from Nutmeg (*Myristica fragrans* Houtt.)

(Kawalan Biologi *Aspergillus flavus* dengan Yis *Pseudozyma hubeiensis* daripada Buah Pala (*Myristica fragrans* Houtt.))

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Received: 11 March 2024/Accepted: 28 October 2024

ABSTRACT

Yeasts are potential biocontrol agents for *Aspergillus flavus,* an aflatoxin-producing fungus that is present in various agricultural products, including nutmeg. This study aimed to obtain yeast isolates from nutmeg (seeds, pulps, and leaves), characterise them, and identify their antagonistic effects on *A. flavus.* The antagonistic activities toward *A. flavus* were determined by a dual-culture method. Moreover, the possible mechanism responsible for these antagonistic effects was also analysed. The results showed that 51 yeast isolates were successfully isolated from nutmeg. The inhibition percentages of 47.25 ± 1.66% (isolate DP 1341a) and 55.98 ± 1.31% (isolate DP 1342) were statistically significant ($p < 0.05$). The antagonistic mechanisms of the DP 1341a isolate were associated with the production of volatile organic compounds (32.79 ± 1.01%), a chitinolytic index (2.51 ± 0.55), and hyperparasitism but not toxin activity. Moreover, the DP 1342 isolate produced volatile organic compounds (54.33 ± 3.13%), exhibited toxin activity (2.74 ± 0.22) and exhibited hyperparasitism but did not exhibit chitinase activity. Molecular identification showed that the two yeast isolates (DP 1341a and DP 1342) were identified as *Pseudozyma hubeiensis* with sequence similarity > 99%. Therefore, the selected yeast isolates, *P. hubeiensis* DP 1341a and DP 1342, could be further developed as biological control agents for *A. flavus.* This finding will also be useful for improving biological control agents as an eco-friendly and economically viable disease management strategy.

Keywords: Antagonist: *Aspergillus flavus*; *Myristica fragrans*; *Pseudozyma hubeiensis*; yeasts

ABSTRAK

Yis adalah agen kawalan biologi yang berpotensi untuk *Aspergillus flavus*, iaitu sejenis kulat penghasil aflatoksin, yang hadir dalam pelbagai produk pertanian termasuk buah pala. Kajian ini bertujuan untuk memperoleh pencilan yis daripada buah pala (biji, pulpa dan daun), dan kemudian mencirikan serta mengenal pasti aktiviti antagonis terhadap *A. flavus*. Aktiviti antagonis terhadap *A. flavus* ditentukan dengan kaedah dwi-kultur. Selain itu, mekanisme yang mungkin bertanggungjawab bagi kesan antagonis juga dianalisis. Hasil kajian menunjukkan 51 pencilan yis berjaya dipencilkan daripada buah pala. Peratusan perencatan sebanyak 47.25 ± 1.66% (pencilan DP 1341a) dan 55.98 ± 1.31% (pencilan DP 1342) adalah signifikan secara statistik ($p < 0.05$). Mekanisme antagonis pencilan DP 1341a dikaitkan dengan pengeluaran sebatian organik meruap (32.79 ± 1.01%), indeks kitinolitik (2.51 ± 0.55), hiperparasitisme, tetapi tidak menghasilkan aktiviti toksin. Manakala, pencilan DP 1342 menghasilkan sebatian organik meruap (54.33 ± 3.13%), menunjukkan aktiviti toksin (2.74 ± 0.22) dan hiperparasit, tetapi tidak menunjukkan aktiviti kitinase. Pencirian molekul menunjukkan bahawa kedua-dua pencilan yis tersebut (DP 1341a dan DP 1342) telah dikenal pasti sebagai *Pseudozyma hubeiensis* dengan persamaan jujukan > 99%. Oleh itu, pencilan yis *P. hubeiensis* DP 1341a dan DP 1342 yang terpilih boleh dibangunkan sebagai agen kawalan biologi bagi *A. flavus*. Penemuan ini juga berguna untuk penambahbaikan agen kawalan biologi sebagai strategi pengurusan penyakit yang mesra alam dan berdaya maju dari segi ekonomi.

Kata kunci: Antagonis; *Aspergillus flavus; Myristica fragrans; Pseudozyma hubeiensis*; yis

INTRODUCTION

Nutmeg (*Myristica fragrans* Houtt.) is native to Indonesia and has become a highly valuable spice plant. Nutmeg has been utilised as a household spice in Southeast Asian countries (Cao et al. 2020). The export value of Indonesian nutmeg in 2019 reached USD138 million, with an export volume of 20 thousand tons. The most exported part of nutmeg comes from whole nutmeg seeds produced by 88.93% of smallholder plantations (Directorate General of Plantations 2021).

Nutmeg seeds and fruits have multipurpose value because they have been widely used in various industrial fields, both food and nonfood, so their quality needs to be considered (Farag, Mohsen & Abd El Nasser 2018; Pesireron et al. 2019). Nutrients in nutmeg include vitamins, minerals, and organic substances related to essential oils (Gupta Azhar & Kalam 2020). In addition, nutmeg is reported to have insecticidal, antifungal, and antibacterial properties (Dhaslin, Issac & Prabha 2019; Ibrahim et al. 2020).

Aflatoxin contamination in agricultural products causes approximately 25% or more of global plants to be destroyed annually (World Health Organization 2018). Aflatoxin is known as a strong carcinogen, immunosuppressive substance, and mutagenic agent. Aflatoxin is a toxic secondary metabolite produced by several types of *Aspergillus* spp. including *A. flavus, A. parasiticus,* and *A. nominus* (Aiyama, Trivittayasil & Tsuta 2018). Four aflatoxins are known as B1, B2, G1, and G2 types (Kim et al. 2017). The aflatoxin producer, *A. flavus*, is reported to contaminate various plantation crops, including nutmeg. In the last few years, nutmeg export in Indonesia has decreased due to aflatoxin contamination. In 2014 - 2016, there were 38 cases of rejection of Indonesian nutmeg seeds by the European market due to aflatoxin contamination (Sembiring 2020). Aflatoxin contamination has been reported to exceed the European standard limit which is a maximum total aflatoxin concentration of 10 µg/kg and aflatoxin B1 concentration of 5 µg/kg (Supriadi 2017). At the farmer level, 56% of the nutmeg seed samples from North Sulawesi were contaminated with aflatoxin. The aflatoxin levels at the farmer level and exporters were 141 µg/kg and 50 µg/kg, respectively. Hence, high aflatoxin contamination in postharvest nutmeg seeds must be handled seriously (Dharmaputra et al. 2015).

Many studies have shown that *A. flavus* growth can be prevented by utilising biological control agents (BCA), including yeast (Freimstreakr et al. 2019). According to Citanirmala, Rahayu and Hariyadi (2016), aflatoxin contamination can be prevented from the preharvest period by implementing standard operation procedures in crop management. One of the efforts to control aflatoxin can be made by inhibiting the growth of *A. flavus*, which produces aflatoxin postharvest. The growth of aflatoxin-producing *A. flavus* can be controlled by utilising BCA, which is considered efficient and environmentally friendly (Moradi et al. 2020). Several BCAs derived from bacterial isolates, such as *Bacillus subtilis, Pseudomonas* spp., *Lactobacillus* spp., *Ralstonia* spp., and *Burkholderia* spp., can inhibit the growth and production of aflatoxin *in vitro* (Nesci, Bluma & Etcheverry 2005; Palumbo, Baker & Mahoney 2006). However, because Aspergillus strains outcompete bacteria, they are unable to reduce aflatoxin production under field conditions (Dorner 2004).

Yeast has high potential as a BCA because of its ability to produce extracellular polysaccharides such as complex glucan, mannoprotein, and chitin, which can bind mycotoxins of *A. flavus* (Campagnollo et al. 2020). Furthermore, it is easy to cultivate in simple nutrition, can spread rapidly, does not produce allergic conidia, can colonise dry surfaces for long periods, and can grow on various substrates and under various temperature conditions (Abdel-Kareem, Rasmey & Zohri 2019; Jaibangyang, Nasanit & Limtong 2021; Suvarna et al. 2018). Studies on the ability of yeast to inhibit the growth of aflatoxin-producing *A. flavus* have been reported. Tayel et al. (2013) reported that the yeast *Pichia anomala* ATCC 34080 has antifungal properties and produces exochitinase and β-1,3-glucanase enzymes that can inhibit the growth of aflatoxin-producing *A. flavus* contaminants on corn kernels for animal feed. The antagonistic yeast *Aureobasidium melanogenum* from *Moringa oleifera* leaves was also reported to inhibit *A. flavus* by 43% (Sukmawati et al. 2020).

Jaibangyang, Nasnit and Limtong (2020) reported that 46 out of 49 yeast strains isolated from the surfaces and tissues of plant leaves produced volatile organic compounds (VOCs) that inhibited the mycelial growth and conidial germination of *A. flavus* A39. The most effective yeasts were *Candida nivariensis* DMKU-CE18, *Naganishia liquefaciens* DMKU-CE84, *Kwoniella heveanensis* DMKU-CE82, *Hannaella sinensis* DMKU-CP430, and *Wickerhamomyces anomalus* DMKU-RP25. *Saccharomyces cerevisiae* produces an exochitinase enzyme that can also inhibit *A. flavus* growth (Abdel-Kareem, Rasmey & Zohri 2019), whereas *P. pastoris* produces β-1,3-glucanase, which inhibits hyphal growth in *A. flavus* (Zhang et al. 2019).

The studies mentioned showed that plant-inhibiting yeasts are prospective BCAs of *A. flavus*. Information on the effectiveness of yeast-mediated inhibition of *A. flavus* on nutmeg plants is still limited; therefore, this research aimed to isolate and characterise the antagonistic potential of yeast for controlling *A. flavus* on nutmeg. This study aimed to obtain antagonistic yeast isolates from nutmeg plants that have the potential to suppress *A. flavus*-producing aflatoxin and to determine the pathogenicity potential of this fungus so that it can be used as a bioactive material for the development of biofungicide formulations for further research.

MATERIALS AND METHODS

NUTMEG SAMPLING

Yeast was isolated from various parts of the nutmeg plant, such as the pulp, seeds, and leaves. The healthy nutmeg of two trees was sampled by the purposive method. Each tree was sampled for four nutmeg fruits and leaves in duplicate. The selected sample trees that showed no sign of rot on the fruit or the leaves were cleaned with tap water. Each nutmeg sample was split and excreted for its seeds. The nutmeg pulp was then diced, and the seeds were smashed in a sterilised mortar. Each nutmeg pulp and seed sample was weighed (10 g), while the nutmeg leaf samples were cut into small parts (1 cm × 1 cm) and weighed (1 g). Each sample was submerged for 1 min in a sterilised jar bottle half-filled with sterile distilled water. Each sample was drained using filter paper. Furthermore, the fruit and seed samples were put into 90 mL of yeast malt broth (YMB) media supplemented with 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% gluc-streak, and 0.01% chloramphenicol in a jar bottle. Moreover, the leaf samples were put into 9 mL of YMB in a threaded tube. YMB samples were cultured on a rotary shaker (FinePCR) at 150 rpm for 24 h at room temperature.

YEAST ISOLATION AND PURIFICATION

The plant parts of the nutmeg were dipped in sterilised physiological NaCl solution (0.85%). The solution was gradually diluted to 10^{-3} and spread (100 µL) following the spreader technique on yeast malt agar (YMA) consisting of 20 g (g/L) agar, 10 g dextrose, 3 g malt extract, 5 g peptic digest of animal tissue, and 3 g yeast extract. The inoculated YMA was incubated at 30 °C for 24 h. A single yeast colony was reisolated on fresh YMA to purify and obtain a single culture following morphological characteristics and microscopic examination as described by Wang, Jia and Bai (2006). A single purified yeast colony was stored in a slanted YMA as a stock and working culture. Standard yeast procedures were used to determine the morphological properties of the strains (Kurtzman, Fell & Boekhout 2011).

ANTAGONISTIC TEST

The research design used was a completely randomized design (CRD) in which the percentage inhibition of *A. flavus* by treatment with antagonistic yeast isolates was determined in triplicate. Aflatoxin-producing *A. flavus* BIO 33210 was obtained from the Bogor Research Center for Spices and Medicinal Plants Culture Collection. The fungus originated from contaminated nutmeg seeds. The *A. flavus* isolate was grown on PDA and then incubated at 27 °C for 7 days. One streak of 7-day-old *A. flavus* inoculant was put into sterile 1.5 mL of Tween 20, homogenised, mixed in a rotary shaker (150 rpm), and incubated at room temperature for 24 h.

The dual culture method used was described by Sukmawati et al. (2020). One loop full of yeast culture was streaked 6 cm long on the PDA medium on plates 3 cm from the right edge of the dish on the PDA. A total of 2 µL of *A. flavus* suspension was grown on PDA at a 1 cm distance from the left side of the yeast isolate and incubated for 48 h. A total of 2 µL of *A. flavus* suspension inoculated on PDA media without yeast isolates was added to the control media. Antagonistic test media and control media were incubated simultaneously at 27 °C for 7 days in duplicate. The percentage of yeast inhibited by *A. flavus* was calculated using the following formula:

$$\text{Inhibition percentage (\%)} = \frac{(R1-R2)x\ 100\%}{R1}$$

where R1 is the growth diameter of *A. flavus* in the control treatment; and R2 is the growth diameter of *A. flavus* with treatment.

CHITINOLYTIC TEST

The chitinolytic test was performed based on Susilowati et al. (2021) by inoculating yeast isolates on solid chitin media (g/100 mL: a mixture of 0.3% colloidal chitin, 0.1 g of K_2HPO_4, 0.01 g of $MgSO_4.7H_2O$, 0.05 g of yeast extract, 0.1 g of peptone, 0.5 g of NaCl, 0.1 g of $(NH_4)_2SO_4$, and 1 g of agar). The antagonist potential of the yeast was assessed at 48 h by diluting a loop full of the culture in a 1.5 mL microtube containing 100 µL of sterilised distilled water. The solution was vortexed to homogeneity, and then a sample solution (5 µL) was taken and spotted on the surface of the chitin media in a Petri dish. The sample was incubated at room temperature (± 28 °C) for 2 days. A negative control was performed using distilled water, and a positive control was performed using a chitinase standard solution. Subsequently, the chitin medium on the petri dish was flooded for 2-3 min with 0.3% Congo Red solution and then rinsed with 0.1% NaCl solution. The chitinolytic activity, as indicated by the clear zone surrounding the colony, was measured. The chitinolytic index value was determined using the following formula:

$$\text{Chitinolytic index} = \frac{\text{clear zone diameter}}{\text{yeast colony diameter}}$$

DETECTION OF VOLATILE ORGANIC COMPOUNDS (VOCS)

The double dish set (DDS) method was performed as described by Dennis and Webster (1971) and Parafati et al. (2015). The ability of volatile compounds to inhibit pathogens was observed using two uniform petri dishes. A total of 20 µL (OD_{660} = 0.8 containing approximately 10^7 cells/mL) of the 48-h-old yeast suspension was inoculated on the centre of 90 mm petri dishes containing 18 mL of

PDA, whereas 72-h-old *A. flavus* BIO 33210 colonies were inoculated on the second dish, inverted, and placed on top of the first dish. Both parts of the dishes were wrapped and sealed with two layers of Parafilm® (Merck) and incubated for 8 days at 26 °C. This technique creates a tightly closed chamber where VOCs concentrate and move freely to and from both strains. A nontreated pathogenic fungus was prepared as the control. The volatile compound activity test was performed based on the fungal growth inhibition percentage (IP), which was calculated using the following formula:

$$\text{The inhibition percentage (\%)} = \frac{(R1-R2)x\ 100\%}{R1}$$

where R1 is the diameter of *A. flavus* in the control treatment; and R2 is the diameter of *A. flavus* with treatment.

TOXIN ACTIVITY

The toxin activity test was conducted on selected yeast isolates following the procedure of Santos, Sanchez and Marquira (2004). In brief, 48-hour-old yeast cultures of YMA-MB media consisting of 30 mg of 1-1 methylene blue, 6% (w/v) NaCl, and 2% agar were suspended in sterilised water and spotted (5 µL) at the center of the agar media. *A. flavus* culture (72-h-old) was inoculated against yeast spot at a 3 cm distance. The petri dish was incubated for 7 days at 20 °C. The parameter measured was the inhibition zone diameter surrounding the yeast colony marked by a clear zone. The inhibition zone index of toxin activity was calculated using the following formula:

$$\text{Toxin activity} = \frac{\text{clear zone diameter}}{\text{yeast colony diameter}}$$

HYPERPARASITISM TEST

The hyperparasitism activity of the yeast against the pathogenic fungus *A. flavus* was assessed using the water agar blocking method (Allen, Burpee & Buck 2004). A 5-day-old yeast isolate was grown on one side of a water agar block (0. 6 cm long), and the other side was inoculated with a 10-day-old fungus. The agar block was placed on a sterilised glass object, covered with a sterilised cover glass, placed on a sterile petri dish, and incubated for 4-6 days. The hyperparatism of the yeast on *A. flavus* was observed under a light microscope. The pathogenic fungal mycelial characteristics with and without antagonistic treatment were compared to observe the interaction between the fungus and yeast. The observation of yeast types with high potential for hyperparasitism was followed by scanning electron microscopy (SEM) observation. The sample was prefixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature for 2 h and then fixed in 2% osmium tetraoxide for 1 h. The samples were dehydrated in serial concentrations of ethanol (20%, 50%, 70%, and 100%) for 5 min each. The dried samples were glued to a specimen stub and then coated with carbon and gold. Observations were carried out using SEM (type JSM-5310 LV, Japan) at 10 kV at magnifications ranging from 2,000 to 10,000×. The resulting images were captured digitally.

YEAST IDENTIFICATION

MACROSCOPIC AND MICROSCOPIC IDENTIFICATION

Macroscopic and microscopic identifications were performed on a selected antagonist yeast isolate. The yeast isolate was inoculated on YMA using the streak method and incubated for 48 h at 30 °C. Grown yeast was macroscopically identified by observing colony morphology which were colour, texture, form, edge, surface, and colony elevation. Microscopic observation was carried out using a light microscope (Olympus BX51) at 1,000× magnification. A 20-h-old culture of yeast (10 µL) was dripped on the surface of a sterilised glass and then covered with a sterilised cover glass. Yeast characteristics such as cell size, form, and asexual reproduction type (budding, fission, blasto-conidia, stigmata-conidia, or hypha formation) were observed (Kurtzman, Fell & Boekhout 2011).

MOLECULAR IDENTIFICATION

DNA extraction was performed using the Wizard Genomic DNA Purification Kit. DNA concentration and purity measurements were performed using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Yeast DNA amplification was conducted using the polymerase chain reaction (PCR) method with a thermal cycler. The PCR reaction had a mixture of 25 µL consisting of 0.5 µL (10 pmole/µL) of primer ITS 5 (forward) (5'-GGA AGT AAA AGT CGT AAC AAG G-3'), 0.5 µL (10 pmole/µL) of primer ITS 4 (reverse) (5'-TCC TCC GCT TAT TGA TAT GC-3') (White et al. 1990), 6 µL of DNA template sample, 5.5 µL of nuclease-free water, and 12.5 µL of Go Taq® Green Master Mix (Promega). The PCR conditions were including 5 min of initial denaturation at 94 °C, 30 s of 35 cycles of denaturation at 94 °C, 30 s of annealing at 52 °C, 30 s of extension at 72 °C, and 7 min of final extension at 72 °C. Visualisation of the ITS rDNA region amplification was conducted by electrophoresis with 1.7% agarose comprising 1.2 g of agarose, 100 mL of 1× TAE buffer, and 2 µL of red gel. The electrophoresis products were visualised under a UV transilluminator (White et al. 1990).

DATA ANALYSIS

The data obtained from the yeast antagonist test against *A. flavus* were analysed using one-way analysis of variance (ANOVA) at the 95% confidence level via the SPSS version 20 application program. Significantly different results were

further tested using Duncan's multiple range test (DMRT) at the 5% confidence level ($\alpha = 0.05$). Macroscopic and microscopic yeast morphology data are presented in the form of images and tables.

The sequencing analysis was carried out following the Sanger method, and sequence data in FASTA format were edited by the ChromasPro application version 2.6.2 followed by the nucleotide Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov) to determine species based on the highest percentage of sequence similarity (% identities) with a range of 98-100% between the tested yeast sequences and the known yeast ITS region sequence database in GenBank NCBI. The phylogenetic tree was constructed using the MEGA 7 application. The CLUSTAL X program was used to align the sequences. Using the neighbor-joining approach (Saitou & Nei 1987), phylogenetic trees were built using evolutionary distance data derived from Kimura's two-parameter model, and 1,000 bootstrap analyses were carried out (Felsenstein 1985).

RESULTS AND DISCUSSION

YEAST ISOLATION FROM NUTMEG PLANTS

A total of 51 yeast isolates were obtained from the nutmeg seeds (14 isolates), pulp (26 isolates), and leaves (11 isolates) (Table 1). This result showed that yeast isolates favour the nutmeg pulp compared to the seed and leaf pulp. In line with this study, Ling et al. (2020) also reported that the pulp may contain more nutritive materials that are suitable for supporting the growth of yeast in the pulp. Phytochemical analysis showed that nutmeg pulp contains essential oils, proteins, lipids, starch, and various residues (Rahardiyan, Poluakan & Moko 2020). In addition, nutmeg contains carbohydrates, minerals, proteins, vitamins (A, B, C), folic acid, riboflavin, niacin, thiamine, minerals (sodium, calcium, magnesium, phosphorus, zinc), β-carotene, and β-cryptoxanthin (Susilowati et al. 2021). Nutritive nutrients such as glucose are important carbon sources for yeast (Hyun et al. 2014).

Yeast growth and abundance may vary between plants which are influenced by the host plant. According to Ling et al. (2020), yeast growth and abundance can be strongly affected by various environmental factors including host, pH, nutritional content in the media, temperature, humidity, solar radiation, plant exudates, and rainfall.

YEAST ANTAGONISTIC TEST AGAINST *A. flavus*

An antagonistic test was performed on PDA media for all 51 yeast isolates against *A. flavus*. An antagonistic test showed that five yeast isolates, BJ 1222, DB 3341, DB 1331, DP 1341a, and DP1342 inhibited *A. flavus* growth by 23.7-55.98%. Table 2 summarises the inhibition percentages of yeast isolates, showing that DP 1342 exhibited the highest antagonistic activity (55.98 ± 1.31%). The five isolates had significantly different inhibition abilities based on the Duncan test ($p < 0.05$). Following the antagonistic assay, two yeast isolates with good average inhibition percentages namely isolates DP 1341a (47.25 ± 1.66%) and DP 1342 (55.98 ± 1.31%), were further selected.

The most potent isolates (DP 1341a and DP 1342) were obtained from the nutmeg leaf surface. The greatest ability of yeast to inhibit colony *A. flavus* growth was projected by the zone of inhibition between the two test microbes, as shown in Figure 1, where *A. flavus* colony growth was less than that of the control.

As shown in Figure 1, the growth of fungi was retarded compared with that in the control treatment (without yeast). This condition was probably due to competition in terms of space. Zhang et al. (2020) reported that yeast and *A. flavus* require nutrition and space to grow and develop. Hence, antagonistic activities occur in the form of competition between nutrients and space. The antagonistic activities are shown by the growth inhibition of one test microorganism. *A. flavus* growth inhibition in the antagonist test was observed by mycelial growth reduction and the existence of an inhibition zone. The ability of the yeasts, DP 1341a and DP 1342 to inhibit *A. flavus* production clearly indicated an antibiotic mechanism. Both yeasts were expected to produce metabolites that inhibited *A. flavus* mycelial growth.

DETECTION OF VOLATILE ORGANIC COMPOUNDS (VOCs)

The volatile compound activity test was performed on the yeast isolates, DP 1341a and DP 1342 against *A. flavus* with the two uniform petri dish methods. The antagonistic mechanism occurred without physical contact between the yeast and pathogenic fungi. The highest average inhibition percentage against *A. flavus* was shown by the yeast isolate DP 1342 (54.33 ± 3.13%). Meanwhile, the average inhibition percentage was shown by the yeast isolate DP 1341a was 32.79 ± 1.01%. The growth reduction may also be affected by secondary metabolites (certain substances) that suppress pathogen growth (Sofiana, Susilowati & Putra 2020).

The antagonistic effects of VOCs can be affected by various factors, including the medium type, antagonist microbe concentration, and incubation time. *A. flavus* mycelium and reduced spore growth were observed in the yeast isolate treatment groups. Moreover, the growth of *A. flavus* mycelia and spores in the control petri dish was full (Figure 2).

The growth of VOCs is a vital factor in the biocontrol of pathogenic moulds. VOCs are lipophilic substances with low molecular weights (<300 Da) (Farbo et al. 2018) and high vapour pressures and are produced by microbes during primary and secondary metabolism (Contarino et al. 2019). Volatile compounds are grouped into several chemical groups namely alcohols, esters, aldehydes, ketones, terpenes, aromatic hydrocarbons, and lactones (Choińska et al. 2020). Jaibangyang, Nasanit and Limtong (2021)

TABLE 1. The number of yeasts isolated from nutmeg seeds, pulp, and leaves

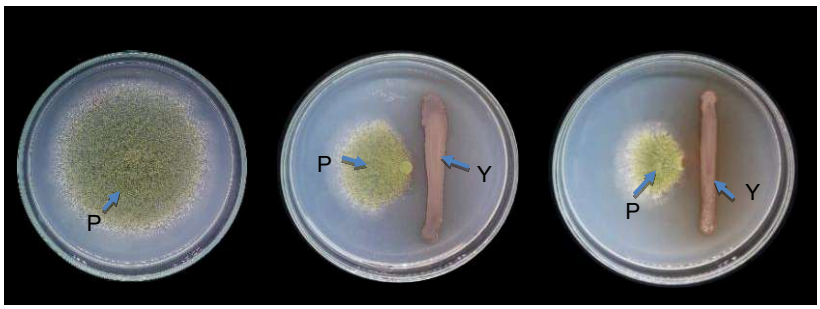
Origin of plant part	Isolates code	Number of isolates (percentage of total isolates)
Seed	Bj 1221, Bj 1222, Bj 1332, Bj 1342, Bj 2321, Bj 2331, Bj 2332, Bj 3321a, Bj 3322, Bj 3331, Bj 3332, Bj 3342, Bj 6321a, Bj 7331	14 (27.45)*
Pulp	Db 1232, Db 1232a, Db 1232b, Db 1232e, Db 1331, Db 1341, Db 1342b, Db 2231b, Db 2231c, Db 2321a, Db 2332a, Db 2332b, Db 2342, Db 3232a, Db 3321, Db 3331, Db 3332, Db 3341, Db 3342, Db 4132a, Db 132b, Db 4342, Db 5322a, Db 6332, Db 8321, Db2232b	26 (50.98)
Leaf	Dp 1142, Dp 1231, Dp 1232, Dp 1341, Dp 1341a, Dp 1342, Dp 2141, Dp 2142, Dp 2242, Dp 2341, Dp 2342	11 (21.57)
Total isolates		51 (100)

*The percentage represented in parentheses is calculated based on the proportion of the total number of isolates obtained from each plant part (n) divided by the total isolates (N) following the formula: $\text{Percentage} = \frac{n}{N} x\ 100\%$

TABLE 2. Antagonistic test of inhibition percentage of yeasts against *A. flavus*

Yeast isolates	Inhibition percentage ± SD (%)*
BJ 1222	23.75 ± 1.25 [d]
DB 3341	36.25 ± 1.25 [c]
DB 1331	17.50 ± 1.25 [e]
DP 1341a	47.23 ± 1.66 [b]
DP 1342	55.98 ± 1.32 [a]
Control treatment	0.00 ± 0.00 [f]

[a]Means followed by the same letter are not significantly different according to the Duncan Multiple Range Test (DMRT) $p < 0.05$ *The inhibition percentage (%) = $\frac{(R1-R2)x\ 100\%}{R1}$ where: R1 = growth diameter of *A. flavus* on the control treatment, R2 = growth diameter of *A. flavus* with treatment. SD = standard deviation



Arrows indicate P = pathogen *A. flavus*, and Y = yeast

FIGURE 1. Yeast antagonistic mechanism against a pathogen (*A. flavus*); A. *A. flavus* isolate alone (control treatment), B. *P. hubeiensis* DP 1341a against *A. flavus*, C. *P. hubeiensis* DP 1342 against *A. flavus*. All treatments were incubated at 27 °C for 7 days

reported that the yeast *Kwoniella heveanensis* produced VOCs that produce excellent antagonistic activities against the pathogen *A. flavus*, which produces aflatoxin B1 on corn kernels.

KILLER TOXIN SUBSTANCE ACTIVITY TEST

Figure 3 shows the results of the killer toxin activity test, where Killer toxin activity occurred only on yeast isolate DP 1342, with an average inhibition zone (index of the killer toxin) of 2.74 ± 0.22, while isolate DP 1341a did not produce an inhibition zone. Killer toxin activity was indicated by an inhibition zone surrounding the yeast colony and a decrease in the growth of *A. flavus* mycelia and conidia towards the yeast. The arrow shows the yeast isolate inhibition zone observed on YMA-MB media incubated at 27 °C for 7 days.

The antifungal activity of *Pseudozyma* species is determined by the secretion of cellobiose lipids with fungicidal activity against a very broad spectrum of fungi. Golubev, Pfeiffer and Golubeva (2006) reported that the yeast species *P. tsukubaensis* produces a killer toxin (mycocin) with a very narrow, taxonomically specific action spectrum and is active only against some representatives of the orders *Microstromatales* and *Ustilaginales.*

The *A. flavus* growth reduction and inhibition zone formation are likely affected by antagonist mechanisms such as the use of a toxic substance or hydrolytic enzyme diffusion. As stated by Belda et al. (2017), yeast excretes deadly toxins containing deadly proteins against fungi. Killer toxins are proteins that are glycosylated and bind to specific receptors on the surface of the target microorganism, which can then be killed (Mannazzu et al. 2019). In addition to *Pseudozyma*, several yeasts, such as *Saccharomyces cerevisiae*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Ustilago*, *Torulopsis*, *Williopsis*, *Zygosaccharomyces*, *Aureobasidium*, *Zygowilliopsis*, and *Mrakia*, reportedly produce deadly toxins (Liu et al. 2015).

CHITINOLYTIC TEST

A chitinolytic test was carried out on potential antagonist yeast isolates (DP 1341a and DP 1342) to determine their ability to hydrolyse chitin substances. A chitinolytic test demonstrated that the DP 1341a isolate could hydrolyse chitin substances with an indicator of clear zone formation on chitin media with a chitinolytic index of 2.51 ± 0.55, while the DP isolate 1342 did not show any chitinolytic activity. Interestingly, this isolate did not produce chitinase but produced a deadly toxin. According to Joubert and Doty (2018), yeast acquired from plants commonly produces extracellular enzymes due to their ability to degrade the cell wall. The absence of chitinolytic activity in the DP 1342 isolate may be due to other factors.

Extracellular enzymes are usually regulated under nutrient-poor conditions. Chitinolytic enzyme secretion is preferred as an antagonist mechanism for biocontrol yeast since this enzyme can be used to degrade fungal cell walls (Zajc et al. 2019). The cell wall is a crucial component of fungal cell homeostasis. Most pathogenic fungi have cell walls composed of chitin complex polymers (Khunnamwong et al. 2020). The *A. flavus* cell wall is mainly composed of β-D-glucan and chitin (Lima, Colombo & de Almeida Jr. 2019). The chitinase enzyme produced by microbes cleaves the 1,4-glycosylic bond between C1 and C4 from 2 N-acetylglucosamine and hydrolyses chitin into oligomers and monomers of N-acetyl-β-d-glucosamine (Akocak, Churey & Worobo 2015).

HYPERPARASITISM TEST

The antagonist yeast isolates, DP 1341a and DP 1342, exhibited a hyperparasitism mechanism. The mechanism was indicated by damaged *A. flavus* hyphae and yeast adhesion to *A. flavus* hyphae (Figures 4 & 5). Figure 5(C) shows that there was one spot indicating cell lysis, even though the majority of yeast cells accumulated along the hyphae. The adhesion process was marked by abundant yeast colonisation due to the antagonistic effects of the yeast. The activities include direct contact with both microorganisms in terms of space, lack of nutrition, and extracellular substances produced by the antagonist agent.

In line with this study, Safitri, Wiyono and Sokarno (2021) also reported a hyperparasitism mechanism from an isolate of *Rhodotorula mucilaginosa* yeast on fungal pathogenic *Phytophthora capsici* hyphae. The antagonist yeast tends to absorb nutrients from pathogen cells and causes pathogen cell death (Zhang et al. 2020). The hyperparasitism mechanism also involves the production of extracellular enzymes such as 1,3-β-glucanase, chitinase, and proteases, during pathogen cell wall degradation (Spadaro & Droby 2016). Montesinos and Bonaterra (2019) suggested that the hyperparasitism mechanism occurs due to direct interaction between yeast and the pathogen's cell wall degradation process.

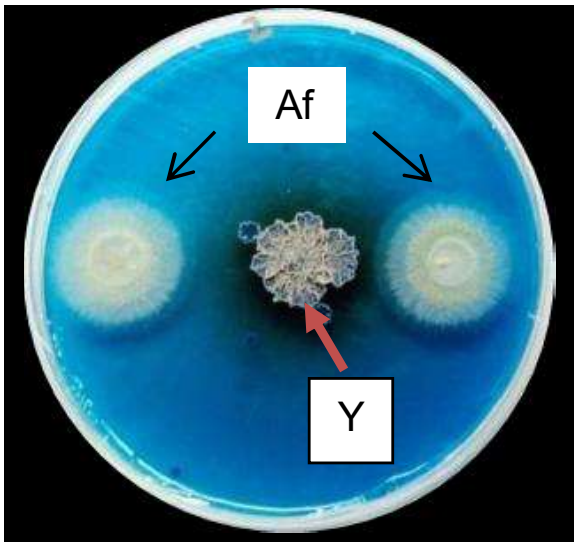
MACROSCOPIC AND MICROSCOPIC IDENTIFICATION OF YEAST ANTAGONIST

Macroscopic and microscopic identifications were conducted on yeast isolates with greater antagonistic ability (DP 1341a and DP 1342). The DP 1341a yeast isolate had macroscopic characteristics similar to those of the DP 1342 isolate which were pale white in colour, a circular shape, an umbonate elevation, no glossiness, a smooth surface texture, and flat edges (Figure 6). Based on their distinct colony and morphology, Kurtzmann, Fell and Boekhout (2011) described the genus *Pseudozyma* as they relate to them. On YM agar, after 1 month of incubation at 20 °C, the streak culture was whitish to cream, butyrous, dull, smooth, or somewhat wrinkled (Wang, Jia & Bai 2006).

Our results showed that the DP 1341a yeast isolate had microscopic characteristics similar to those of the

Arrows indicate Af = *A. flavus*, and Y = yeast

FIGURE 2. Performance of double dish set assay showing *A. flavus* colony growth inhibited by yeast isolates; A. *A. flavus* isolate (control treatment without yeast isolates), B. Treatment with yeast *P. hubeiensis* DP 1341a, C. Treatment with yeast *P. hubeiensis* DP1342

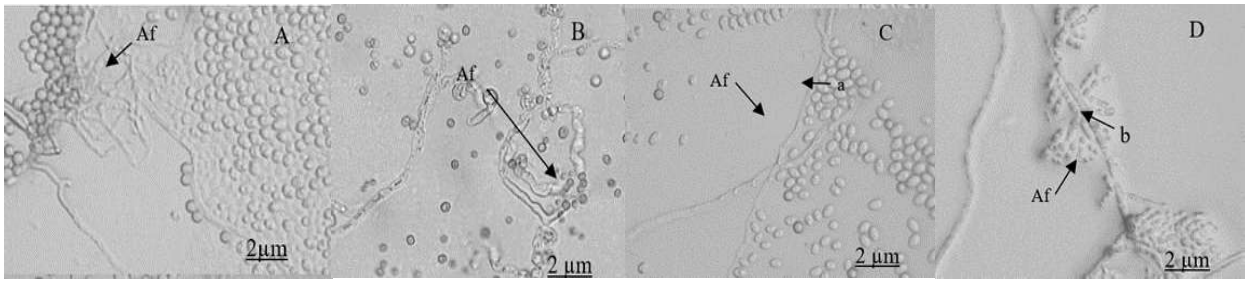


Af = *A. flavus* (indicated by the black arrow), and Y = yeast (indicated by the red arrow)

FIGURE 3. Killer toxin substance activity of yeast *P. hubeiensis* DP 1342 against *A. flavus*

TABLE 3. Molecular characterisation of selected yeast isolates based on ITS Region

Isolate code	Top BLAST match in NCBI database	Max score	Query coverage (%)	E-value	Accession	Sequence similarity (%)
DP 1341a	*Pseudozyma hubeiensis*	1208	100	0.0	KY828936.1	100
DP 1342	*Pseudozyma hubeiensis*	1177	100	0.0	MK508798.1	99.84



a: yeast *P. hubeiensis* DP 1341a, b: *P. hubeiensis* DP 1342, Af: *A. flavus*

FIGURE 4. (A&B) *A. flavus* hypha showing lysis due to yeast cell hyperparasitism, (C&D) yeast cell adhesion on *A. flavus*

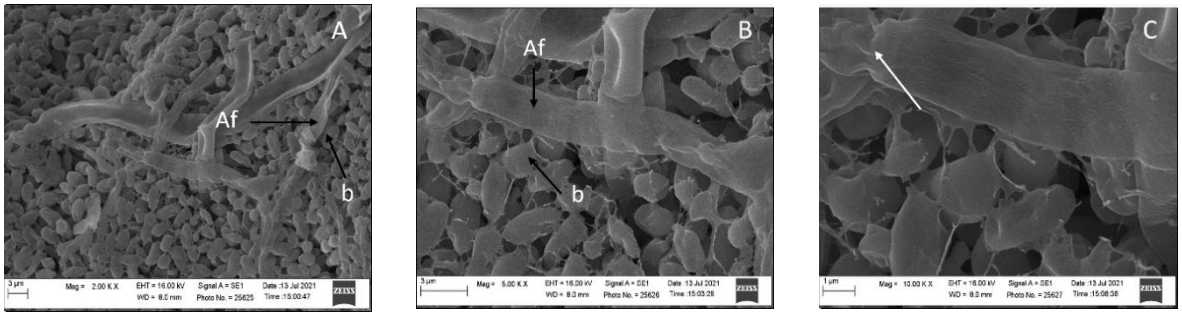


FIGURE 5. (A & B) yeast cell adhesion on *A. flavus* on 2,000× & 5,000× magnifications, (b: yeast *P. hubeiensis* DP 1342, Af: *A. flavus*), and (C). *A. flavus* hypha damage on a 10,000× magnification, (the white arrow shows parasitism symptoms emerged)

DP 1342 isolate, i.e., the cells were cylindrical, single, or in pairs. The budding shape is on the shorter side. Based on the macroscopic and microscopic morphological characteristics, both isolates (DP 1342 and DP 1341a) were categorised as *Pseudozyma* spp. *Pseudozyma* spp. yeast has a dimorphic form that belongs to the *Basidiomycota* phylum, *Ustilaginomycetes* class, and *Ustilaginales* order. The *Pseudozyma* genus can live well on the surface or in plant tissue (Hartati et al. 2023). *Pseudozyma* is also reported as a potential antagonist agent that produces fatty acids that inhibit pathogenic fungi (Avis & Bélanger 2002). Typically, *Pseudozyma* species are referred to as saprotrophic epiphytes. It is highly probable that the release of antifungal drugs enhances their competitiveness within the phylloplane microbiota and serves as a crucial natural defense mechanism for plants against harmful fungi (Golubev, Pfeiffer & Golubeva 2006). Yeasts that colonise leaves are frequently employed as BCAs to guard against a variety of foliar diseases, including *A. flavus, Ustilago maydis*, and powdery mildew fungi. The fungal yeast *P. flocculosa* is a basidiomycetous species that has been well studied for its ability to suppress powdery mildew, which is a common phyllosphere disease of a wide range of greenhouse and field crops. *P. flocculosa* was initially discovered to antagonise powdery mildew on cucumbers in various environmental settings (Avis & Bélanger 2002).

P. flocculosa is a powerful antagonist of powdery mildew fungi and appears to target only and specifically members of *Erysiphales.* Over the years, understanding the exact mode of action of BCA has been challenging. Antibiosis, through the secretion of the glycolipid flocculosin, initially gained much traction because of the strong antimicrobial activity of the molecule *in vitro* (Mimee, Labbe & Bélanger 2009).

MOLECULAR IDENTIFICATION

The visualisation results of the yeast DNA bands were obtained through electrophoresis (Figure 7). The DNA samples of both isolates were well amplified, as indicated by the presence of a DNA band with a size of ±700 bp.

Blast analysis as shown in Table 3 to identify the nucleotide bases of the DP 1341a and DP 1342 isolates showed that both isolates were *P. hubeiensis,* with similarity values greater than 99% and an E-value of 0.00 for the closest species, showing that the yeast sequence significantly matches the data in the NCBI GenBank.

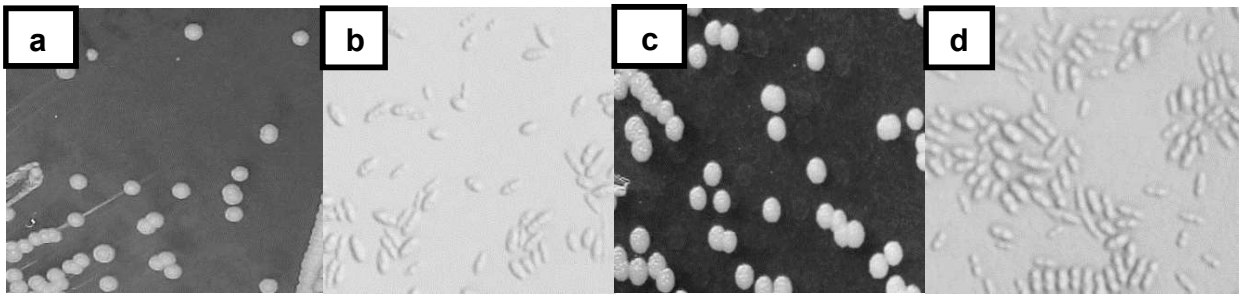


FIGURE 6. Characteristics of antagonistic yeast isolate; (a). Macroscopic and (b). phase contrast micrograph of *P. hubeiensis* DP 1341a; (c). Macroscopic and (d). Phase contrast micrograph of *P. hubeiensis* DP 1342 (1,000× magnification)

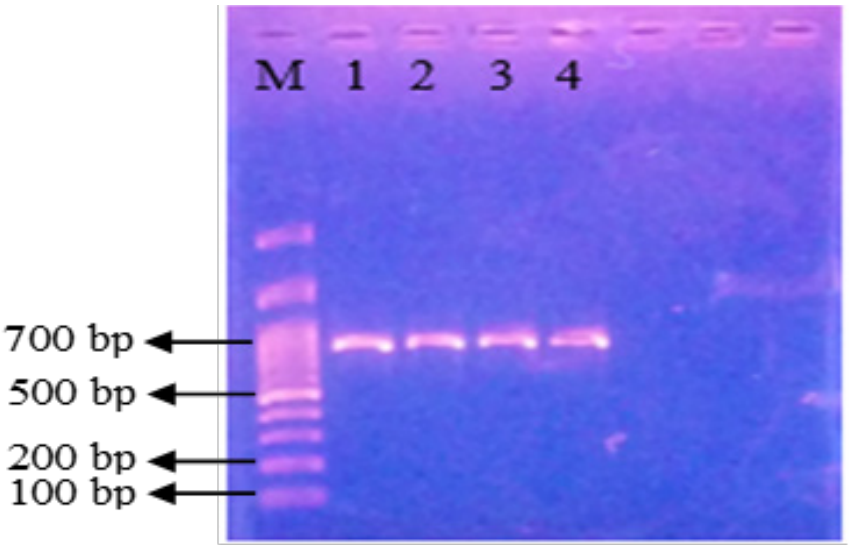


FIGURE 7. Electrophoresis of yeast amplification products. M= 100 bp marker; lane 1 and 2= Isolate DP 1342; lane 3 and 4= Isolate DP 1341a

This result agreed with that of Johnson et al. (2019), who reported that a sequence homology value greater than 97% could represent the same species. The sequence of the nucleotide bases from sequencing was as follows: DP 1341a (756 bp) and DP 1342 (758 bp). Maryati and Ferniah (2021) explained that the ITS rDNA region of yeast highly varies between species, with a range of 300-900 bp.

Sequence alignment of the muscle program in MEGA 7 software showed no gaps in the sequence of the nucleotide bases. Isolates, DP 1341a and DP 1342, were in the same clade as *P. hubeiensis* strain ABS1, *P. hubeiensis* strain ABS2, and *P. hubeiensis* isolate AA62 with a bootstrap value of 100% (Figure 8). The higher the bootstrap value is, the greater the confidence of the clade formed (Johnson et al. 2019). A bootstrap value above 90% is considered stable, while a bootstrap value below 70% indicates that phylogenetic instability can change (Syaifudin et al. 2019). According to Konsue, Dethoup and Limtong (2020), *P. hubeiensis* is known to have antagonistic effects on pathogenic fungi.

This study showed that *P. hubeiensis* yeast isolated from nutmeg, particularly the DP 1341a and DP 1342 isolates, can be applied as a biological control agent against *A. flavus*, which resulted in reduced growth of the fungal pathogen on postharvest storage seeds and thus improved quality of the nutmeg as well as improved sustainable crop production. This study also suggested that the yeast isolates obtained in this study have the potential to control *A. flavus*. These isolates can be applied to reduce postharvest disease development caused by *A. flavus* on nutmeg. However, it is necessary to optimise the effectiveness and mode of action as well as the appropriate application. Therefore, bioformulations can be developed to improve disease control under different environmental conditions.

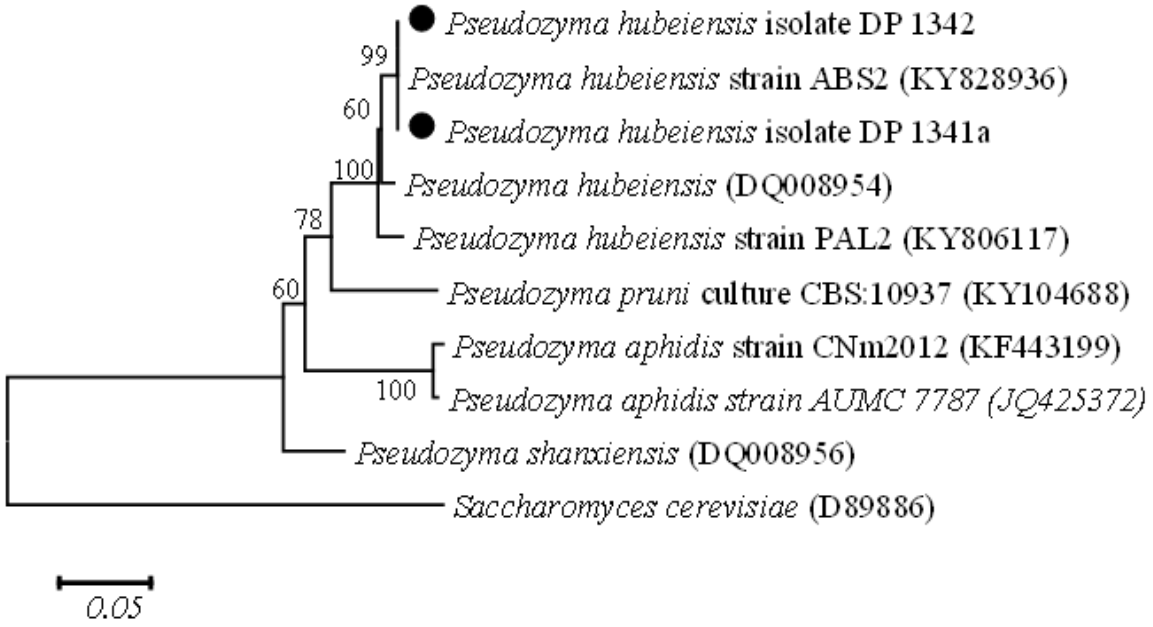


FIGURE 8. Phylogenetic tree of selected yeast isolates from nutmeg plant based on ITS region sequence analysis using the Neighbor-joining 1,000× Bootstrap method (MEGA 7). *Saccharomyces cerevisiae* is the outgroup

CONCLUSIONS

The present study was the first to report the activity of the yeast *P. hubeiensis* isolated from nutmeg against aflatoxin-producing *A. flavus*. The antagonistic mechanisms on the DP 1341a isolate included VOC (32.79 ± 1.01%), chitinolytic index (2.51 ± 0.55), and no killer toxin activity, but showing hyperparasitism. The antagonist mechanisms on the DP 1342 isolate included VOC (54.33 ± 3.13%), killer toxin activity (2.74 ± 0.22), and no chitinase activity, but showing hyperparasitism. Molecular identification showed that both the DP 1341a and DP 1342 isolates were identified as yeast *P. hubeiensis* with sequence similarity values ranging from 99.84-100%.

This research outlined the impact of yeast as a BCA with antagonistic effects. It showed that it was almost equally effective in managing *A. flavus,* the causal agent of aflatoxin, on nutmeg. These two BCAs of *P. hubeiensis* isolates were effective at suppressing the growth of fungi, which is particularly important for small-scale nutmeg farmers to reduce the use of pesticides in nutmeg and thus improve food safety.

ACKNOWLEDGEMENTS

The author would like to thank Mr. Jajang and Mrs. Siti Aminah, who helped carry out this activity at the ICABIOGRAD-Microbiology Laboratory, Bogor. The author expresses gratitude to the Dept. of Biology, Faculty of Science and Technology, Syarif Hidayatullah Islamic University Jakarta for providing the research grant.

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