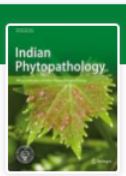

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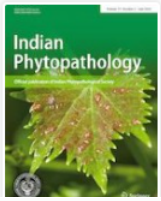

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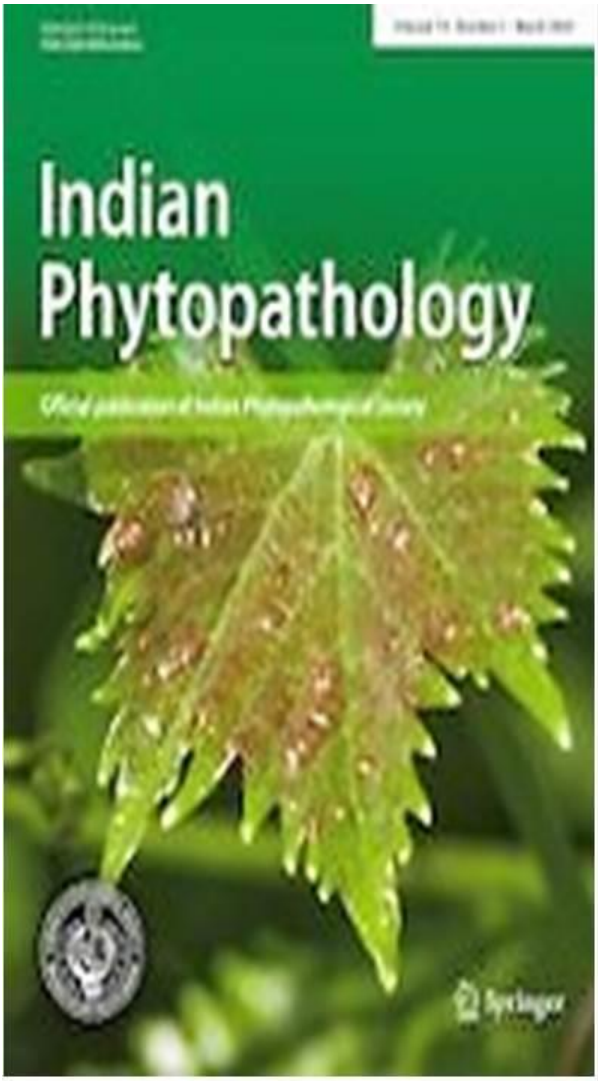

Research Article | Published: 07 February 2020

Antagonistic activity of phylloplane yeasts from *Moringa oleifera* Lam. leaves against *Aspergillus flavus* UNJCC F-30 from chicken feed

Dalia Sukmawati, Marsha Hanin Andrianto, Zico Arman, Nuniek Ina Ratnaningtyas, Shabrina Nida Al Husna, Hesham Ali El-Enshasy, Daniel Dailin & Ahmed Atta Kenawy

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Abstract

Aspergillus flavus is widely known as an aflatoxin-producing fungus that frequently contaminates feed and affects livestock, which leads to severe health problem for animal and human. Biological agents have been proven to prevent this contamination since they can produce metabolites which have antagonistic activity. In this study, phylloplane yeasts isolated from *Moringa oleifera* leaf have shown an ability to inhibit the growth of *Aspergillus flavus* UNJCC F-30 collected from chicken feed. This research was conducted in three stages: (1) yeast isolation (leaf washing and direct method), followed by (2) antagonistic test using dual culture method, and (3) molecular identification using D1/D2 region of 26S rDNA. In the first stage, 38 yeast isolates have been succesfully obtained. These isolates were of different colors: peach pigment (60.5%), the non-pigmented yeast (26.5%), cream (10%), and orange (3%). Antagonistic activity against *A. flavus* UNJCC F-30 was tested based on growth, sporulation, and the presence of clear zones. Screening result showed that 12 yeast isolates are capable of inhibiting *A. flavus* UNJCC F-30. Among them, 4 isolates with the code K4, K10, K15, and K26 showed the highest antagonist ability. Molecular identification resulted that the 4 isolates show a similar identity with *Aureobasidium pullulans* UWFP 993 (100%), *Aureobasidium melanogenum* QCC:M017/17 (99%), *Aureobasidium melanogenum* QCC:M017/17 (100%) and *Rhodotorula taiwanensis* CBS:11729 (99%), respectively. Isolate K10 exhibited the highest percentage of inhibition activity among all isolates which is potential for application as biocontrol agent against *A. flavus*. As *A. pullulans* is a common yeast found on leaf surfaces of many Indonesian flora, therefore it can be considered as safe and alternative to reduce fungal contamination from *A. flavus* in feed chicken.

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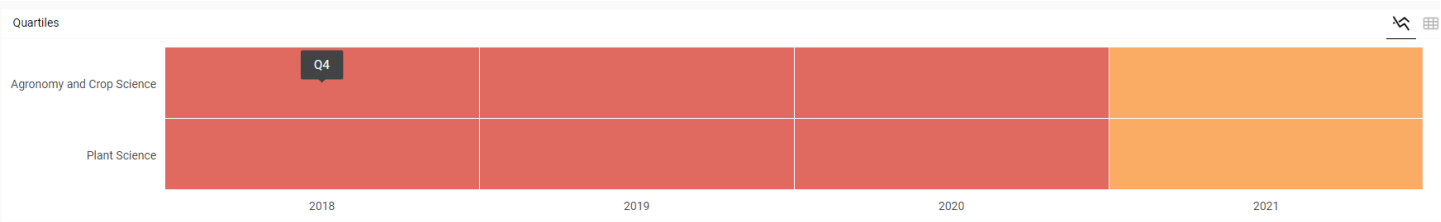

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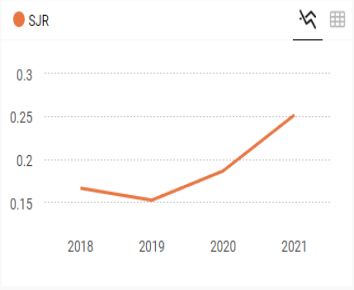

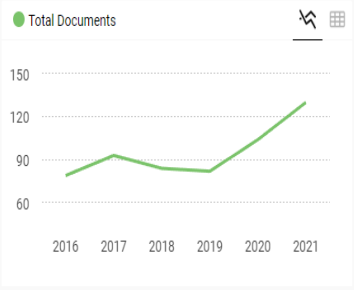

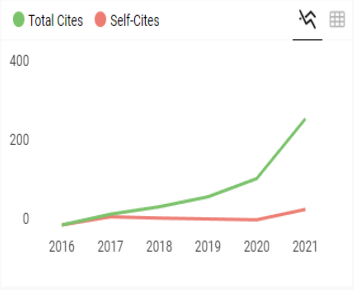

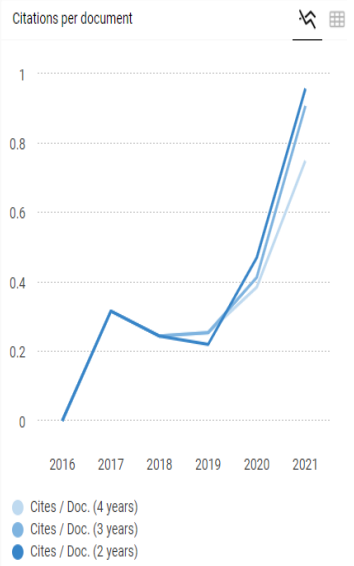

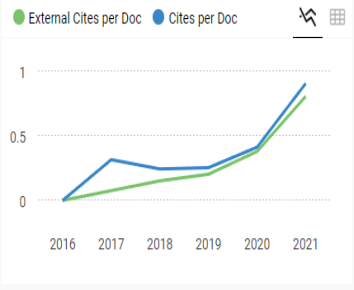

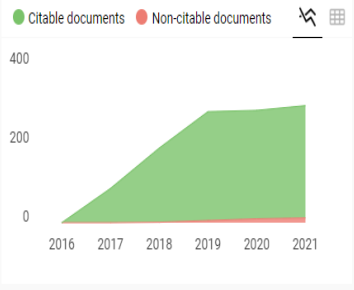

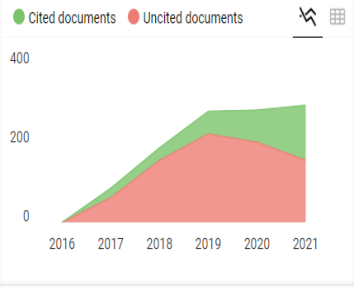

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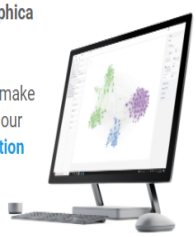

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RESEARCH ARTICLE

Antagonistic activity of phylloplane yeasts from *Moringa oleifera* Lam. leaves against *Aspergillus flavus* UNJCC F-30 from chicken feed

Dalia Sukmawati[1,2] · Marsha Hanin Andrianto[1] · Zico Arman[1] · Nuniek Ina Ratnaningtyas[3] · Shabrina Nida Al Husna[4] · Hesham Ali El-Enshasy[5,6] · Daniel Dailin[5] · Ahmed Atta Kenawy[6]

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Abstract

Aspergillus flavus is widely known as an aflatoxin-producing fungus that frequently contaminates feed and affects livestock, which leads to severe health problem for animal and human. Biological agents have been proven to prevent this contamination since they can produce metabolites which have antagonistic activity. In this study, phylloplane yeasts isolated from *Moringa oleifera* leaf have shown an ability to inhibit the growth of *Aspergillus flavus* UNJCC F-30 collected from chicken feed. This research was conducted in three stages: (1) yeast isolation (leaf washing and direct method), followed by (2) antagonistic test using dual culture method, and (3) molecular identification using D1/D2 region of 26S rDNA. In the first stage, 38 yeast isolates have been succesfully obtained. These isolates were of different colors: peach pigment (60.5%), the non-pigmented yeast (26.5%), cream (10%), and orange (3%). Antagonistic activity against *A. flavus* UNJCC F-30 was tested based on growth, sporulation, and the presence of clear zones. Screening result showed that 12 yeast isolates are capable of inhibiting *A. flavus* UNJCC F-30. Among them, 4 isolates with the code K4, K10, K15, and K26 showed the highest antagonist ability. Molecular identification resulted that the 4 isolates show a similar identity with *Aureobasidium pullulans* UWFP 993 (100%), *Aureobasidium melanogenum* QCC:M017/17 (99%), *Aureobasidium melanogenum* QCC:M017/17 (100%) and *Rhodotorula taiwanensis* CBS:11729 (99%), respectively. Isolate K10 exhibited the highest percentage of inhibition activity among all isolates which is potential for application as biocontrol agent against *A. flavus*. As *A. pullulans* is a common yeast found on leaf surfaces of many Indonesian flora, therefore it can be considered as safe and alternative to reduce fungal contamination from *A. flavus* in feed chicken.

Keywords Antangonistic · *Asperillus flavus* · Yeast · *Morinaga oleifera* · *Aureobasidium*

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Introduction

Extensive research over the years has made it obvious that mycotoxins are commonly prevalent in majority of feed and food contamination. This type of toxins are produced by a wide range of fungi, especially from the group of *Aspergillus*. Mycotoxins can be found in crops and foodstuffs containing carbohydrates and proteins such as cereal grains, legumes, spices, dried fruits, apples, coffee beans, and tree nuts, as well as chicken feed ingredients such as maize, often under warm and humid conditions (Pietsch and Burkhardt-Holm 2015; Sukmawati et al. 2018). Aflatoxins (AF), zearalenone (ZEN), ochratoxin A (OTA), fumonisins (FUM), trichothecenes such as deoxynivalenol (DON), and T-2 toxin are some of the mycotoxins that can significantly impact the health and productivity of poultry species (Ashiq et al. 2014; Pietsch and Burkhardt-Holm 2015; Wu et al. 2011). Aflatoxins, a class of mycotoxins produced by the species of *Aspergillus*, including *A. flavus, A. parasiticus, A. nomius* (Ehrlich and Cotty 2004), are often found in feed ingredients (corn and peanut products) used for poultry rations. One case happened when Turkey-X disease of 1960, which resulted in the loss of several thousand turkey poults in the United Kingdom (Chen et al. 2013). Most prevalent forms of AF include B1, B2, G1, and G2, with aflatoxin B1 (AFB1) is detected as the highest concentration in chicken feed contamination (Wu et al. 2011). Research conducted by Sukmawati et al. (2018) showed that fungi from species of *Aspergillus* and *Penicillium* are found in chicken feed contamination.

The presence of *A. flavus* and its metabolites in food and feed can cause severe economic losses for the farmers and are potentially harmful to poultry and human health. Contamination by *A. flavus* in maize production in Pakistan for example, has resulted considerable losses for corn farmers due to long-term crop failure (Ashiq et al. 2014). In addition, aflatoxin can be accumulated both in poultry and human after long-term consumption. Aflatoxins cause a variety of effects in poultry, including decreased weight gain, poor feed efficiency, reduced egg production and egg weight, increased liver fat, changes in organ weights, reduction in serum protein levels, carcass bruising, poor pigmentation, liver damage, decreased activities of several enzymes involved in the digestion of starch, protein, lipids, and nucleic acids, and immunosuppression (Varga et al. 2015; Chen et al. 2016; Niessen et al. 2018; Frisvad et al. 2019). There are also multiple effects to the human health, including the acute or chronic disease episodes, such as carcinogenic, mutagenic, teratogenic, estrogenic, hemorrhagic, nephrotoxic, hepatotoxic, neurotoxic (Pereira et al. 2014) which lead to aflatoxicosis and cause death (Pietsch and Burkhardt-Holm 2015). Morover aflatoxin may also lead to deformities resulted by the interference in bone metabolism (Bbosa et al. 2013).

Fungal growth and subsequent mycotoxin formation is dependent on a range of factors including seasons, location of grain cultivation, drought and time of harvest. Long term analysis of grain and feed samples worldwide has indicated that it is possible to have grains with extremely high concentrations of aflatoxin, although the overall mycotoxin concentration is low (Pereira et al. 2014). The prevention of *A. flavus* contamination has been done so far with several methods such as utilization of chemical substances, manipulating its growing environment (Bhatnagar-Mathur et al. 2015), and application of biological agents, including antagonistic activity of microorganisms. Besides its safety, the use of yeast as a biological agent to prevent aflatoxin contamination showed several consideration and effectiveness. However, this method of control and prevention has not been developed commercially (Ashiq et al. 2014). In other research, phylloplane yeasts, epiphytic microorganisms that are commonly found on plant surfaces (Schmit and Lodge 2004; Sukmawati et al. 2015; Kaewwichian and Limtong 2014), have been reported to present antagonistic properties against *Aspergillus* sp. (Sukmawati and Miarsyah 2017).

It is known that the surface of plant leaves represents a complex terrestrial habitat with the presence of natural compounds which can be used as nutrients for microorganisms. Sukmawati (2016) has reported that yeast isolated from *Cerbera manghas* leaves have ability to inhibit the growth of *Aspergillus* and *Penicillium.* Moringa leaf (*Moringa oleifera* Lam.) is known to have nutrient content that is suitable for yeast to grow. Moringa leaves are known to have high concentrations of nutrients such as α-tocopherol, riboflavin, nicotinic, folic acid, pyridoxine, β-carotene, and other nutrients (Fatima et al. 2014) which are considered as the secondary metabolites produced by phylloplane yeasts. Moringa leaf extract is also reported effective in inhibiting *A. flavus* with a concentration of 12.5 mg/ml (Jeff-Agboola and Awe 2016). Research on *M.oleifera* is limited to the exploration of plant extracts for human health. However, Moringa plants are reported containing 539 compounds known in traditional African and Indian medicine, and have been used in traditional medicine to prevent more than 300 diseases (Fuglie 2001).

In this study, yeasts isolated from Moringa leaf were investigated for their antagonistic activity against *A. flavus* UNJCC F-30 collected from chicken feed contamination. Macroscopic and microscopic observations were performed to identify the isolates. Molecular identification was conducted using D1/D2 region of 26S rDNA with 600 bp gene in length (Makene 2014). Studying biodiversity of mycobiota is very important for identifying and documenting changes and similarities between species. Moreover, various novel natural compounds can be isolated and identified from such mycobiota with promising potential biological, medical and industrial applications. Therefore, this study

was expected to support findings in the study of the potential of phylloplane yeast from Moringa leaves to overcome the contamination of *A. flavus* using biological agents.

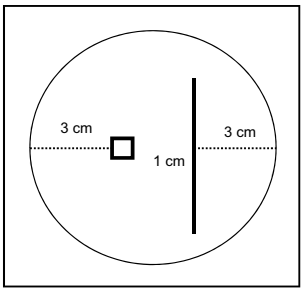
Materials and methods

Yeast isolation from *Moringa oleifera* leaf surfaces

A total of 16 *Moringa oleifera* leaves have been collected from four different trees. Isolation was done using washing method and direct method (Sukmawati et al. 2015). Each of leaf samples was cut by 1 × 1 cm in average and 1 g of it was then added to 9 ml of yeast-peptone-dextrose (YPD) broth, 1% of yeast extract, 2% of peptone, 2% of dextrose until reaching the total volume of 10 mL with pH 4.5. The solution was then put into the shaker at 100 rpm for 30 min to get the fully dissolved solution. 100 µL of liquid yeast suspensions were spotted onto the surface of YPD agar containing 10% dextrose and chloramphenicol (50 µg/mL) and incubated at 30 °C for 2 days for observation. For direct method, leaf samples from the same solution were taken and placed directly on the CA medium, followed by incubation under the same conditions. All colonies with yeast-like morphology were stored at the Universitas Negeri Jakarta Culture Collection (UNJCC) with preservation using L-drying method and 10% glycerol at −20 °C. All steps were done in aseptic techniques and under sterile condition.

Screening and antagonistic test of yeast isolates from *Moringa* leaves against *A. flavus* UNJCC F-30

Preparation of mold sample was done by cultivating *Aspergillus flavus* UNJCC F-30 on coconut agar (CA) medium for 4 days at 28 °C. The spore suspension (10^7 spore/ml) was made in 0.5 ml tween 20 solution. A total of 38 yeast isolates from Moringa leaves were picked and cultivated on Yeast Malt Agar medium in duplicates. After 4 days of incubation, antagonistic activity test was performed for all isolates using dual culture modified method (Sukmawati and Miarsyah 2017). 10 µl of yeast suspension was inoculated on the surface of CA medium following 6 cm line in length. 1 µl of *A. flavus* UNJCC F-30 spore suspension was added into the middle of the medium surface with 1 cm apart from each isolate (Fig. 1). The interaction between each yeast isolate and *A. flavus* UNJCC F-30 such as the presence of clear zone was observed for 10 days at 28 °C (Alemu 2016). Other antagonistic parameters were considered, such as the sporulation, the presence of mycelium and the growth rate of *A. flavus* UNJCC F-30 compared to untreated control. Percent growth of inhibition was calculated based on Korsten et al. (1995), as follows:



Notes: ▫ Mold pathogen (*Aspergillus* sp.)
| Yeast isolates (with 6 cm long)

Fig. 1 Yeast isolates were tested for their antagonistic activity against *Aspergillus* sp. using dual culture method

$$\mathrm{GI} = \frac{K_r - r_1}{k_r} \times 100\%$$

where K_r represents the distance (measured in mm) of fungal growth from the point of inoculation to the colony margin on control plates, r_1 is the distance of fungal growth from the point of inoculation to the colony margin in the direction of the antagonist, while GI is the percent growth of inhibition. After the data was collected, it was then subjected to analysis of variance (ANOVA). Differences between means were tested by Tukey's test.

Macroscopic and microscopic identification of potential yeast inhibitors of *A. flavus* UNJCC F-30 isolated from Moringa leaves

Four isolates out of 38 isolates showed the highest potential inhibiting activity against *A. flavus* UNJCC F-30. These isolates were collected for macroscopic and microscopic identification. Colony morphological features such as texture, color, surface, profile, and the edge of colony's. Microscopic observation of the colonies was done using a phase contrast microscope (Olypmpus) at 400× magnification to determine their budding type and cell shape.

Molecular identification of yeast isolates using D1/D2 region of 26S rDNA

Four isolates with the highest inhibition activity (isolates K4, K10, K15, and K26) were collected for molecular identification. Genomic DNA of yeast isolates were used for amplification of D1/D2 region of LS1 gene. Molecular identification was performed using forward primer NL1 (5'-GCATATCAATAAGCGGAGAAAG-3′) and reverse primer NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) based on Makene (2014). Colony of each yeast isolate was inoculated into YMA medium and incubated overnight at 37 °C. DNA was extracted as decribed previously by Sukmawati et al. (2015).

3 µL of DNA template was added with nuclease free water (8.5 µL), Go Taq Green Mastermix (12.5 µL), NL1 forward primer (0.5 µL), NL4 reverse primer (0.5 µL) for PCR reaction. PCR conditions were set for 35 cycles as follows; predenaturation at 95 °C for 2 min, post denaturation at 95 °C for 30 s, annealing at 58.0 °C for 30 s, elongation at 72 °C for 1 min, final elongation of 72 °C for 10 min, and extension at 4 °C for final condition. Visualization of PCR products was performed using electrophoresis with 1% agarose gel and 1 × TAE buffer (Tris Acetate EDTA) based on Sambrook and Russell (2001). PCR products were sent to First Base DNA sequencing services to obtain the sequence reads. Sequence data result was then edited using the ChromasPro version 2.6.2 application program followed by analysis using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov) to get the closest homologous species with the yeast isolates. Phylogenetic tree was constructed using MEGA 7 application program with 1000 times bootstrap with Neighbor Joining method (Tamura et al. 2013).

Results and discussion

Yeast isolates obtained from *M. oleifera* leaves

In this study, 38 phylloplane yeast isolates were obtained from Moringa leaves. Result showed that most of yeast isolates found in *Moringa* leaf were pigmented (73.5%), while non-pigmented yeasts were only a fourth (26.5%). Peach-pigmented yeast was accounted for 60.5% of all isolates, followed by cream and orange which were 10% and 3%, respectively. Sukmawati et al. (2015) reported that phylloplane yeast was predominantly peach (Astaxanthin) and cream pigments. Peach pigments are commonly found in *Candida* sp. and cream pigments in *Cryptococcus* sp. (Hospenthal et al. 2002), while the orange pigment (Torularhodin) is in *Rhodotorula sperm* sp (Ungureanu and Ferdes 2012). Pigments are used by fungi to protect against extreme conditions such as humidity, extreme temperatures, and high UV intensity.

Antagonistic activity test of yeast isolates from *M. oleifera* against *A. flavus* UNJCC F-30

Screening was performed to determine antagonistic activity of 38 isolates against *A. flavus* UNJCC F-30 using Coconut Agar medium which is known suitable for optimal aflatoxin growth. Coconut Agar medium has high glucose content, which is required for the growth of *A. flavus* (Nair et al. 2014). Glucose functions as energy source to express aflatoxin gene and plays a role in the aflatoxin biosynthetic pathway (Yu et al. 2002). Sugar in the cluster group of hexose transporters, glucosidase, NADH oxidase, and Cys_6Zn_2 (regulatory genes) are used as carbon sources at the end of aflatoxin biosynthesis (Ehrlich et al. 2005). The 38 yeast isolates were subjected to antagonist test, and 12 of all yeast isolates were found to have ability to inhibit the growth of *A. flavus* UNJCC F-30 (Table 1). The ability of yeast to inhibit the growth of *A. flavus* was shown by the presence of clear zone between the two microbes. Potential yeast also shows inhibition ability in mold sporulation.

Yeast isolates with the code K10 showed the most potential antagonistic activity based on diameter of clear zones, sporulation and mycelium formation (Table 1). The presence of clear zone by isolate K10 can be shown in Fig. 2. The ability of yeast to inhibit growth can be indicated by reduction of mycelium growth, reduction of sporulation process, and indicated by the presence of clear zone (Sibounnavong et al. 2009). The clear zone can also be caused by the presence of secondary metabolites produced by yeast and is known as antibiosis mechanism. The leaf surface is an interkingdom crossroads between plants and microorganisms, and secretion of antimicrobial biochemicals to aerial surfaces is thought to be one defensive strategy by which plants deter potential pathogens (Shepherd and Wagner 2007). This is also considered to play a role as antibiotic for the growth of other microorganisms.

The presence of clear zone is due to the antagonistic properties produced by yeast. This is explained by previous research showing that yeast has antagonistic properties towards *Aspergillus flavus* mold which has the ability of mycoparasites (Megeed 2013). The ability of these mycoparasites can occur due to the stimulation of chemical compounds released by *A. flavus*, and yeast has a chemotropic response of these stimuli. Previous research reported that *A. flavus* experienced growth inhibition due to the presence of secondary metabolites, such as harzianolide and butenolide. These compounds can be produced by *Trichoderma harzianum* that inhibit almost 90% of *A. flavus* growth (Megeed 2013). This mechanism has degradation effect on dioctyl phthalate, methyl jasmonate, butabarbitol, and cyclopentanyone found in *A. flavus* mycelium. This degradation causes releases of cyclopentane ring from aflatoxin (Mostafa et al. 2013). The inhibition zone can also be formed by the space and nutrition competition between mold and yeast (Rosa-Magri et al. 2011). The ability of yeast to inhibit pathogenic fungi shows the mechanism of antibiosis. Yeast produces organic metabolite compounds which can inhibit the growth process of mycelium.

Identification of molecular potential yeast isolates, macroscopic and microscopic morphological observation

Currently, yeasts deserve particular attention as biological control agents since they can apply an effective control of postharvest diseases. In this context, yeast can be considered

Table 1 Measurement of inhibition activity of 12 yeast isolates from *M. oleifera* leaves in duplicates (1 and 2) using dual culture method

Isolate codes	Diameter (mm)		Inhibition percentage (%)		(Sporulation; Mycelium; clear zone)	Antagonistic activity (mm) (mean ± SE)
	1	2	1	2		
K10	19.32	20.3	44.63	41.82	+; +; present	$43.22^{f} \pm 1.40$
K26	20.88	21.32	40.15	38.89	+; ++; present	$39.52^{ef} \pm 0.63$
K4	20.75	21.3	40.53	38.95	++; ++; few	$39.74^{ef} \pm 0.79$
K13	22.27	22.06	36.17	36.77	++; ++; almost clear	$36.47^{def} \pm 0.30$
K15	23.52	22.44	32.59	35.68	++; +++; almost clear	$34.13^{cdef} \pm 1.54$
K14	23.54	23.78	32.53	31.84	++; +++; almost clear	$32.18^{bcd} \pm 0.34$
K33	25.02	23.38	28.29	32.99	+++; +++; clear	$30.64^{cde} \pm 2.35$
K32	24.09	25.28	30.95	27.54	+++; ++++; passed	$29.24^{cd} \pm 1.70$
K6	24.06	27.73	31.04	20.52	+++; ++++; passed	$25.78^{b} \pm 5.26$
K25	25.85	26.11	25.91	25.16	++++; ++++; passed	$25.53^{b} \pm 0.37$
K37	25.31	27.05	27.46	22.47	++++; +++++; passed	$24.96^{a} \pm 2.49$
K30	27.15	32.48	22.18	6.91	+++++; +++++; passed	$14.54^{a} \pm 7.63$

The inhibition percentage was calculated based on Korsten et al. (1995). Sporulation, mycelium and the presence of clear zone were also observed. Means of antagonist activity followed by different letters are significantly ($P < 5\%$) different according to Tukey's test

+ little, ++ few, +++ many, ++++ so many, +++++ uncontrollable indicate the sporulation parameters which means that it is seen less or many. The presence of clear zone is observed and indicated by "present", "few", "almost clear", and "passed". Measurements were conducted in duplicates (1 and 2). Means of antagonist activity followed by different letters are significantly ($P < 5\%$) different according to Tukey's test

Fig. 2 Antagonistic activity test **a** untreated control; **b** isolate of K10; **c** Control of *A. flavus* in CA medium, incubation at 30 °C for 5 days. The inhibition zone corresponds to the clear area for which there is a possibility of presence of secondary metabolites

as a safe and environmentally friendly alternative to manage contamination by other microorganisms. Yeast identification in this study was performed based on molecular approach, macroscopic and microscopic observation. Identification was carried out for four yeast isolates; K4, K10, K15, and K26, that could potentially inhibit *A. flavus* F-30. Identification results showed that isolate K4 has percent homology very close to *Aureobasidium pullulans* UWFP993 with maximum identity value reaches 100%. Isolate K15 has percent homology very close to *Aureobasidium melanogenum* QCC:M017/17 with maximum identity value of 100%. Isolate K10 and K26 were identified as *Aureobasidium melanogenum* QCC:M017/17 and *Rhodotorula taiwanensis*, respectively, with maximum identity value of 99%. BLAST results on 4 isolates showed E-value of 0.00, which indicates that the data has significant match (Table 2).

The result of phylogenetic analysis showed that isolates K10 and K15 were identified as *Aureobasidium melanogenum* QCC:M017/17 since they are in one monophyletic clade, with 61% bootstrap value, as well as *Aureobasidium pullulans* UWFP 993 which has sequence proximity (Fig. 3). K26 isolate was identified as *R. taiwanensis* CBS:11729,

Table 2 BLAST results of yeast isolates based on D1/D2 regional sequencing analysis

Isolate codes	BLAST results	Max score	Query (%)	*E* value	Accession number	Identity (%)	Gaps
K4	*Aureobasidium pullulans* UWFP 993	1116	98	0.0	FJ515219.1	100	2/712 (0%)
	Aureobasidium melanogenum QCC:M017/17	1109	1109	0.0	KY781749.1	99	4/712 (0%)
K10	*Aureobasidium melanogenum* QCC:M017/17	992	98	0.0	KY781749.1	99	6/706 (0%)
	Aureobasidium pullulans UWFP 993	992	98	0.0	FJ515219.1	99	9/706 (0%)
K15	*Aureobasidium melanogenum* QCC:M017/17	1085	95	0.0	FJ744598.1	100	5/712 (0%)
	Aureobasidium pullulans UWFP 993	1105	99	0.0	AY213693.1	99	7/712 (0%)
K26	*Rhodotorula taiwanensis* CBS:11729	1114	100	0.0	KY109163.1	99	5/712 (0%)
	Rhodotorula mucilaginosa FK1	1042	98	0.0	JQ695909.1	99	8/712 (0%)

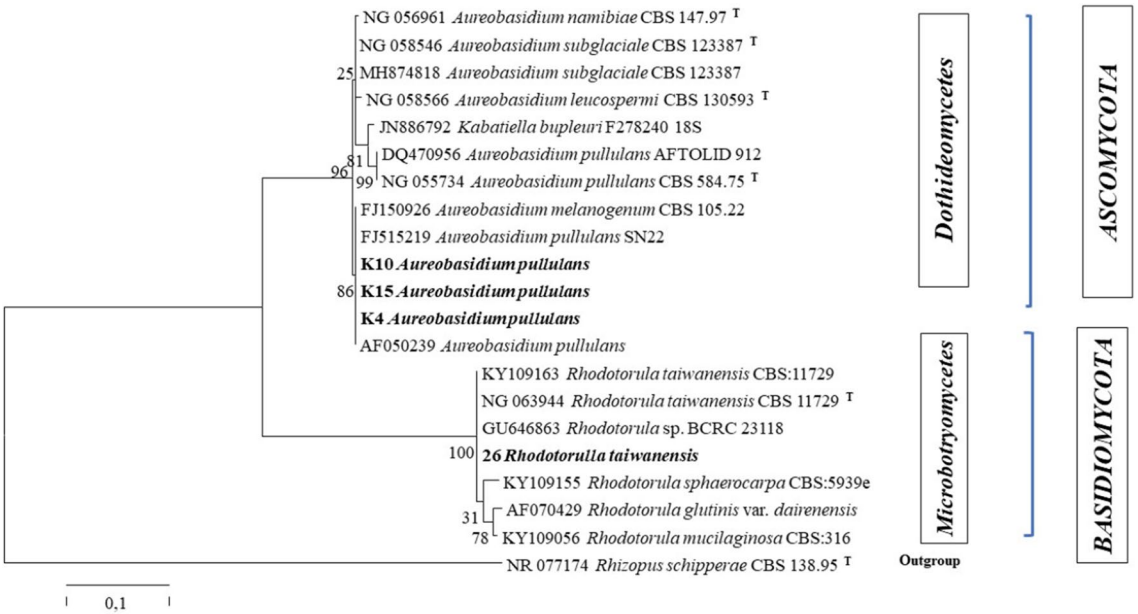


Fig. 3 Phylogenetic tree of yeast isolates from *Moringa oleifera* was reconstructed by maximum likelihood algorithm based on the distance calculated by Kimura's two-parameter model from sequences of D1/D2 regions of rDNA.Bootstrap values greater than 50% from 1000 replicate bootstrap resamplings. *Rhizopus schipperae* ATCC 96514T was used as an outgroup

has a bootstrap value of 99%. *Aureobasidium pullulans* has variants that have been analyzed based on regions D1/D2, β-tubulin, RNA Polymerase 2 (RNAP2), Translation Elongation Factor-1α (EFI-1α), and Internal Transcribed Spacer (ITS). The variant is *A. pullulans* var. pullulans, *A. pullulans* var subglaciale, *A. pullulans* var namibiae, and *A. pullulans* var melanogenum (Liu et al. 2011). Previous studies reported that yeast *R. taiwanensis* was analyzed based on DI/D2, ITS, and cob regions having proximity to *Rhodotorula mucilagosa* and *Rhodotorula dairenensis*, this is similar to this study seen in the phylogeny tree, all of which have proximity to high bootstrap values (Zhao et al. 2012).

Aureobasidium pullulans is a common yeast phylloplane. Previous research reported that *A. pullulans* was found on the leaf surface of *Broussonetia papyrifera* (Sukmawati et al. 2015). *Aureobasidium pullulans* is also found on leaf surfaces in several regions in Indonesia (Sjamsuridzal et al. 2010). Yeast is also reported to have antagonistic properties towards mold *Aspergillus carbonarius* which causes acid rot in grapes (Dimakopoulou et al. 2008). The study reported that *A. pullulans* can reduce the levels of ochratoxin contamination that occur in wineries (Dimakopoulou et al. 2008).

Aureobasidium pullulans (De Bary) Arnaud, a yeast-like fungus, is one of the most promising BCAs; it resides in different environments such as the surface of fruit from the early development stages to maturity, or in woody tissues and leaves (Gonzalez and Tello 2011). It can also survive under different conditions: dry and wet environments, controlled atmosphere and a wide range of temperatures (Mari et al. 2012). Previous works revealed that competition for nutrients (Bencheqroun et al. 2007), induction of host defence, antibiosis, parasitism and production of lytic

enzymes (exochitinase, endochitinase and β-1,3-glucanase) (Zhang et al. 2010) are the main mechanisms responsible for yeast efficacy. Promising results were also obtained with *A. pullulans* isolated from the surface of 'Redhaven' peaches, active against brown rot of stone fruit (Mari et al. 2012); however, besides the recently reported production of volatile organic compounds (Di Francesco et al. 2015), little is known about the mechanisms of action involved in the biocontrol potential of *A. pullulans*.

Research on *R. taiwanensis* has not shown antagonistic activity yet, but other species of the same genus, namely *Rhodotorula fragaria* and *Rhodotorula hinula* are reported to have antagonistic activity against *A. flavus* (Hejri et al. 2013). Both species were able to reduce levels of aflatoxin, each at 1.18 ng/ml and 1.17 ng/ml. Both species produce almost the same secondary metabolite compounds, so the decrease in aflatoxin levels was also not significant (Hejri et al. 2013). There are two groups of phylloplane fungi: residents and casuals. Residents multiply on the surface of healthy leaves without harming the host plant or affecting it. While, casuals though existing on the surface of the leaf, cannot grow on it (Elkhateeb 2018).

Yeast isolates obtained have different morphological characteristics (Table 3; Fig. 4). Pigmented yeasts are commonly found in yeast phylloplane (Sukmawati et al. 2015). The pigments in yeast are formed due to stress conditions caused by excessive UV light, so that the pigment serves as a protector of photooxidative damage (Moliné et al. 2010). The four yeast isolates identified in this study were all pigmented. Isolates K10 and K15 at 48 h were peach-pigmented, but after 10-15 days incubation, they turned black (Fig. 4). This pigment change is caused by melanin contained in *A. melanogenum*. This black pigment is not only found in *A. melanogenum*, but is also found in other species of *Aureobasidium* with different types of melanin. Differences in levels and types can be caused by different

Table 3 Macroscopic characteristic of potential yeast isolates

Isolate codes	Colony colour	Colony surfaces	Colony texture	Colony profile	Colony edge	Cell shape	Germination	Cell size (μm)
K4	Peach	Soft	Butyrous	Convexed	Filamented	Oval	Monopolar	(3–8) × (3–5)
K10	Peach	Soft, dull	Butyrous	Scored	Filamented	Round, oval	Monopolar	(4–6) × (3–5)
K15	Peach	Soft, dull	Butyrous	Scored	Filamented	Round, oval	Monopolar	(2–5) × (2–4)
K26	Orange	Sparkling, slippery	Mucoid	Convexed	Scored	Round, oval	Monopolar	(4–6) × (3–4)

Each isolate was observed with respect to its colony colour, colony surface, colony texture, colony profile, colony edge, cell shape, characteristic of germination and its cell size (μm)

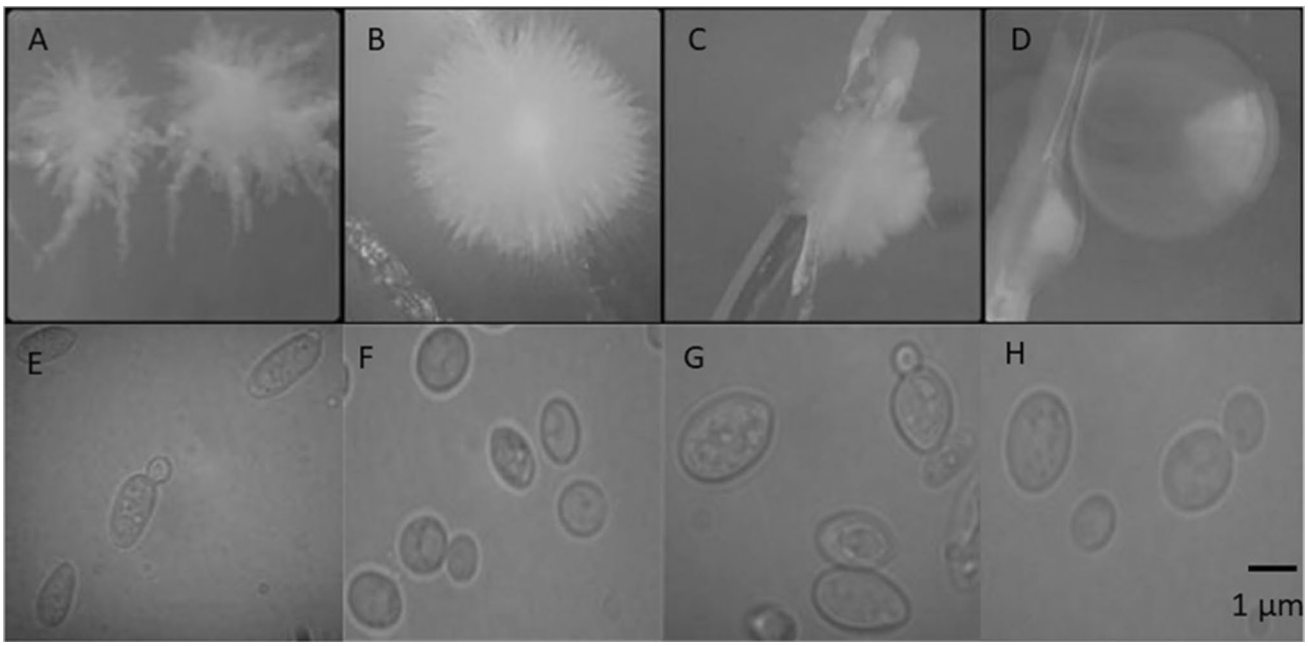


Fig. 4 Macroscopic and microscopic characterization of each potential yeast isolate. Isolate K4 (**a**, **e**); isolate K10 (**b**, **f**); isolate K15 (**c**, **g**); isolate K26 (**d**, **h**)

conditions, such as UV light and oxidizing agents (Nieuwenhuijzen et al. 2016).

The morphology of *A. pullulans* cells with oval or elliptical forms and monopolar sprouting (Fig. 4), is in line with previous reports (Punnapayak et al. 2003). Macroscopic characteristics of yeast also have similarities with previous studies, but A. *pullulans* isolated from air has different levels of melanin, so it can turn blackish red after long incubation (Punnapayak et al. 2003).

The size of phylloplane yeast cells in this study was smaller than that of apples and pears, but other characteristics were similar (Mirzwa-Mróz et al. 2014). This is due to the differences in nutrients found in fruits and leaves which shows that nutrients in the fruit are more than in the leaves part (Nachtigall and Dechen 2006). This difference allows the difference in cell growth and size. Morphological characteristics of yeast *R. taiwanensis* are known to have similarities to the morphology of *R. mucilaginosa*, which has an orange pigment, shiny, mucoid, with flat edge colonies. The cell size of both also has the same size. The difference between them lies in the colony profile, *R. taiwanensis* has a mountainous profile, while *R. mucilaginosa* is flat (Chang and Wang 2002).

Conclusion

In this study, phylloplane yeast isolates obtained from Moringa leaves showed 12 out of total 38 isolates have potential to inhibit *A. flavus*. Isolate K10 which was identified as *Aureobasidium melanogenum* QCC:M017/17 has the highest percentage inhibition of 43%. It is showed that yeast isolates from *M. oleifera* have an inhibition ability against *A. flavus*. They reside in different environments such as the surface of fruit from the early development stages to maturity and in woody tissues and leaves. They can also survive under different conditions: dry and wet environments, controlled atmosphere and a wide range of temperatures. Yeast isolates of high inhibition growth capacity against *A. flavus* were *A. pullulans, A. melonogenum*, and *R. taiwanensis*. For the last few years, the need to address aflatoxin presence by using bio-agents as inhibitors has become urgent. Further studies related to high biomass production and formulation of biological control agents based on strains are now carried out in our laboratories.

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