BioControl (2020) 65:377–386
https://doi.org/10.1007/s10526-020-09996-9

Biological control of aflatoxin-producing *Aspergillus flavus* by volatile organic compound-producing antagonistic yeasts

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Received: 9 September 2019 / Accepted: 20 January 2020 / Published online: 29 January 2020

Abstract *Aspergillus* spp. are the most common phytopathogenic fungi able to produce various types of aflatoxins. Yeasts can produce volatile organic compounds (VOCs) that may be used as biocontrol agents against mycotoxigenic fungi. In this study, we aimed to evaluate antagonistic yeasts that are potentially capable of producing active VOCs against the aflatoxin-producing fungus, *Aspergillus flavus* A39. In total, 366 epiphytic and endophytic yeast strains isolated from leaves of rice, sugarcane, and corn in Thailand were screened for their potential. Only 49 yeast strains were able to produce antifungal volatile organic compounds (VOCs). *Candida nivariensis* DMKU-CE18 was the most effective yeast strain to inhibit the mycelium growth (64.9 ± 7.0% inhibition) and conidial germination (49.3 ± 3.3% inhibition) of *A. flavus* A39, and to reduce aflatoxin production (74.8 ± 6.5% reduction) in corn grains. The analysis results of headspace gas chromatography/mass spectrometry (GC/MS) revealed that the major VOC produced by this yeast strain was closest to 1-pentanol.

Keywords Aflatoxin · *Aspergillus flavus* · *Candida nivariensis* · Volatile organic compounds · Yeasts

Handling Editor: Jane Debode

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10526-020-09996-9) contains supplementary material, which is available to authorized users.

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Introduction

Phytopathogenic fungi present one of the most significant problems in agriculture. They can cause damages and reduce the quality of agricultural products. Various fungal species can also produce mycotoxins (Bu'Lock 1980). *Aspergillus*, one of most important phytopathogenic fungi found in agricultural products, can produce various types of aflatoxins. *A. flavus*, *A. parasiticus*, *A. nomius*, and *A. tamarii* are commonly found in or on foods and feedstuff (Moss 1998). Contamination of agricultural products by aflatoxins leads to the annual destruction of an estimated 25% or more of the world's food crops and feed (World Health Organization 2018). In Thailand, aflatoxins have been detected in food and feed products. The highest rate of

contamination was found in peanuts (36% of all contaminated foods), followed by milk (20.7%), and poultry (17.5%) (Waenlor and Wiwanitkit 2003). Consumption of aflatoxin-contaminated food/feed products can pose a serious health threat to humans and livestock. It has been demonstrated that aflatoxins are carcinogenic agents in many animal species (Amaike and Keller 2011; Waliyar et al. 2015). The four main types of aflatoxins are B1, B2, G1, and G2 (Zain 2011). Aflatoxin B1 (AFB1) is most frequently found in agricultural crops, such as peanuts, corn, rice, soybeans, spices, etc. (Guchi 2015; Magan et al. 2003; Mannaa and Kim 2016; Waenlor and Wiwanitkit 2003). Moreover, the International Agency of Research on Cancer (IARC) categorized AFB1 into a group I carcinogen for humans (Min et al. 2011).

Yeasts are easily cultivated, fast growing, and readily found in a variety of substrates and conditions (Türker 2014). Several yeast strains have been identified as potential biocontrol agents against mycotoxigenic fungi. For example, *Kluyveromyces thermotolerans* strains were able to control *A. carbonarius*, *A. niger*, and ochratoxin A (OTA) in grapes (Ponsone et al. 2011). When used as a dip treatment during coffee processing, *Saccharomyces cerevisiae* reduced the incidence of ochratoxigenic mold, such as that of *A. niger*, *A. ochraceus*, and OTA, without affecting cup quality (Velmourougane et al. 2011). A supplement of 2% *S. cerevisiae* in the control diet containing AFB1 (200 ng g^{-1}) was found to partly counteract some of AFB1's toxic effects in growing chicks (Çelýk et al. 2003). The antagonistic characteristics of yeasts have been attributed to mechanisms such as competition for nutrients (Raspor et al. 2010); secretion of antifungal compounds, such as killer toxins (Petersson and Schnürer 1995); and production of hydrolytic enzymes (Comitini and Ciani 2010), siderophores (Ismail et al. 1985), and volatile organic compounds (VOCs) (Rezende et al. 2015). Therefore, yeast could be a promising antagonistic agent against mycotoxigenic fungi in post-harvest biocontrol.

In recent years, increased focus has been placed on VOCs produced by microorganisms as biological control agents. For instance, 3-methyl-1-butanol and 2-methyl-1-butanol produced by *S. cerevisiae* could inhibit the development of *Phyllosticta citricarpa* which causes citrus black spot (Toffano et al. 2017). The VOCs produced by *Lachancea thermotolerans* have revealed their potential to protect tomatoes inoculated with *Fusarium oxysporum* (Zeidan et al. 2018). In addition, Grzegorczyk et al. (2017) hypothesized that VOCs could be one of the main mechanisms of antagonistic *Debaryomyces hansenii* KI2a and *Wickerhamomyces anomalus* BS91 against *Monilinia fructigena* and *M. fructicola* which cause considerable economic losses in stone fruit crops.

Saprophytic yeasts are common on surfaces and tissues of plant leaves and fruits (Khunnamwong et al. 2018; Limtong and Nasanit 2017; Srisuk et al. 2019). As previously mentioned, some yeast strains effectively compete with post-harvest fungal pathogens. Saprophytic yeasts' potential capability has also been shown when used in several applications to reduce aflatoxin contamination in food and agricultural products (Hua et al. 1999; Masoud and Kaltoft 2006). However, to the best of our knowledge, only a few articles have reported the use of antifungal VOCs produced by yeasts (Chen et al. 2018; Oro et al. 2018; Payne et al. 2000; Zeidan et al. 2018). In this study, we therefore aimed to evaluate the potential capability of saprophytic yeasts isolated from plant leaves to produce active VOCs against aflatoxin-producing fungi.

Materials and methods

Microorganisms

Three hundred and sixty-six yeast strains (165 epiphytic and 201 endophytic) were previously isolated from the surfaces and tissues of leaves of economic crops including rice, sugarcane, and corn in Thailand (Khunnamwong et al. 2018; Srisuk et al. 2019; Into et al. pers. communication). An aflatoxin-producing fungus, *Aspergillus flavus* A39 isolated from bael, was obtained from Dr Amara Chinaphuti, Department of Agriculture (DOA), Thailand. Other strains of *A. flavus*: CH016, CH033, CH271, CH307, and CH464, were obtained from Kasetsart University Research and Development Institute (KURDI). The yeast strains and fungi were maintained at 4 °C on yeast malt (YM) extract agar (0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 1.0% dextrose, and 1.5% agar) and potato dextrose agar (PDA) (Titan Biotech LTD, India), respectively.

Screening of antagonistic yeast strains against aflatoxin-producing fungi

All yeast strains were tested for their potential against *A. flavus* A39 by performing the dual culture method (Rosa et al. 2010). Yeast culture (48 h old grown on PDA at 28 °C) was streaked on PDA, 2 cm from one dish edge. A 5 mm diameter disc of *A. flavus* A39 mycelium (seven days old grown on PDA at 28 °C) was placed 2 cm from the opposite edge of the dish. A control experiment was prepared by inoculation of the fungi without yeast. The dishes were sealed with parafilm and incubated at 28 °C in the dark for seven and 14 days. After incubation, the mycelium growth was measured. Each treatment was conducted in duplicate. Yeast strains that inhibited fungal mycelium growth were selected for further screening of antifungal VOC-producing yeasts.

Screening of VOC-producing yeasts against aflatoxin-producing fungi

To evaluate the production of antifungal VOCs by the selected yeasts, two-partition polystyrene Petri dishes were used (Rosa et al. 2010). Yeast culture (48 h old grown on PDA at 28 °C) was streaked onto PDA on one side of a Petri dish and incubated at 28 °C for 48 h. After incubation, a 5 mm diameter disc of *A. flavus* A39 mycelium (seven days old grown on PDA at 28 °C) was inoculated on the other side of the dish. The control experiment was performed without yeast inoculation. The dishes were sealed with parafilm and incubated at 28 °C in the dark for seven and 14 days. Two replications were conducted. The diameter of the fungal colony was measured and the fungal growth inhibition (%) was calculated compared to the control. The antifungal VOC-producing yeasts were then selected for further determination with other strains of *A. flavus*.

Efficacy of VOC-producing yeasts on fungal mycelium growth inhibition

The efficacy of VOC-producing yeasts against *A. flavus* A39 was determined in accordance with Farbo et al. (2018). Aliquots of 100 µl of yeast cell suspension [10^7 cells ml^{-1} prepared from yeast culture grown on yeast extract peptone dextrose (YPD) broth (1% yeast extract, 2% bacteriological peptone, 2% dextrose) at 28 °C, 150 rpm for 24 h] were evenly spread on PDA agar dishes and incubated at 28 °C for 48 h. The dish cover was then replaced by a PDA dish. Twenty microliters of fungal spore suspension (10^6 spores ml^{-1}) prepared in Ringer's solution [9% (w/v) NaCl, 0.2% (w/v) $CaCl_2$, 0.2% (w/v) KCl, and 0.1% (w/v) Tween 20] were spotted onto the center of the PDA dish. The dishes were sealed with parafilm and incubated at 28 °C for seven days in the dark. Each treatment was conducted in triplicate. The control experiment was performed without yeast inoculation. The radial growth of fungal mycelium was measured and the fungal growth inhibition (%) was calculated compared to the control.

Efficacy of VOC-producing yeasts on fungal conidial germination inhibition

Yeast strains were cultured in YPD broth at 28 °C, 150 rpm for 24 h. The slide culture technique was applied in this experiment. Briefly, a cotton pad was placed at the center of a Petri dish and a glass slide was placed on top of the cotton pad. Five ml of sterile distilled water were added to soak the cotton pad. A 5 mm square block of PDA was then placed in the center of the glass slide. Fungal spores (seven days old grown on PDA at 28 °C) were inoculated on four sides of the agar square with a teasing needle. A sterile cover slip was placed on the upper surface of the agar cube. The dish was then covered by a dish containing 48 h-old yeast culture and sealed with parafilm. The control experiment was performed without yeast inoculation. Three replications were conducted. The conidial germination was investigated after incubation at 28 °C for 21 h (Zhou et al. 2018). At least 100 conidia per treatment were observed at 100× magnification with a light microscope (Olympus CX21). The germinated spores were counted with the inhibition of conidial germination calculated and compared to the control, in accordance with Gong et al. (2015). In addition, the germ tube length was measured and the germination rating scale was recorded (germination rating scale: 1 = no germination; 2 = germ tube < 2× conidium size; 3 = germ tube 24× to 4× conidium size; 4 = germ tube > 4× conidium size) (Zhou et al. 2018).

Efficacy of VOC-producing yeasts on aflatoxin B1 reduction in corn grains

Ten g of corn grains were placed in one side of a two-partition Petri dish and inoculated with 1 ml of fungal conidial suspension (10^6 spores ml^{-1}). Then, 5 ml of sterile distilled water were added to a cotton pad on the other side of the dish. The dish was covered by a dish containing 48 h-old yeast culture and sealed with parafilm. The dish was incubated at 28 °C for 14 days in the dark. The control treatment was carried out the same way but without yeast inoculation on the PDA dish. All treatments were conducted with three replications. For aflatoxin extraction, 10 g of corn grain sample were ground and then mixed with 50 ml of 70% methanol. The samples were shaken for 30 min at 300 rpm. The extract was filtered through a Whatman No. 4 filter paper. The filtrate was analyzed by ScreenEZ® Aflatoxin ELISA Test Kit (Siam Inter Quality, Thailand) (Chinaphuti et al. 2002).

Analysis of volatile organic compounds (VOCs)

An efficient yeast strain was cultured in a 20 ml headspace vial containing 7 ml of PDA and incubated at 28 °C in the dark for two days. The VOC compositions were then analyzed by headspace-gas chromatography/mass spectrometry (GC/MS). The GC/MS analysis was conducted, in accordance with Suwannarach et al. (2017), with slight modifications. The volatiles in the air space above the yeast culture were trapped using headspace for 45 min at 30 °C. The headspace was inserted into the splitless injection port of a gas chromatograph [Agilent 7890A for gas chromatography (GC) and 5975C MDS for mass spectrometry (MS), Agilent Technologies, USA] equipped with a DB-WAX capillary column (30 m × 0.25 mm, film thickness 0.25 µm) (Supelco, USA). The column temperature was programmed at 40 °C for 2 min and then to increase to 200 °C at 5 °C min^{-1}. Helium was the carrier gas. Prior to trapping the volatiles, the headspace was cleaned at 250 °C for 57 min under the flow of helium gas. All mass spectra were compared with the data system library [National Institute of Standards and Technology (NIST08)]. Blank sample analysis (growth medium without yeast inoculation) was performed under the same conditions. Three replications were conducted.

Statistical analysis

Statistical analysis was performed using one-way ANOVA with IBM SPSS Statistics 20.0. Differences were considered significant when $p \leq 0.05$.

Results

Antagonistic yeasts against *A. flavus* A39

The results of primary screening using the dual culture assay revealed that, of the total 366 yeast strains, 127 (39 epiphytic and 88 endophytic) could inhibit the mycelium growth of *A. flavus* A39. Only 49 yeast strains of 13 species (34 strains) belonging to Ascomycota and 14 species (15 strains) in Basidiomycota were able to produce antifungal VOCs against *A. flavus* A39 (see Supplementary Table S1). In addition, only *Candida tropicalis* DMKU-RE01 had the capability to inhibit the other five tested strains of *A. flavus*. *Saitozyma flava* DMKU-RE67 and *W. anomalus* DMKU-RP25 were able to inhibit four strains, while the other 11, 15, and 11 yeast strains inhibited three, two, and one strains of *A. flavus*, respectively. Moreover, nine yeast strains were unable to inhibit these *A. flavus* strains (see Supplementary Table S1).

Efficacy of VOC-producing yeasts on fungal mycelium growth inhibition

Of the total 49 yeast strains, 46 were able to produce antifungal VOCs against the growth of fungal mycelium when using face-to-face plate assay. The fungal growth inhibition varied from 1.9 ± 2.6 to 64.9 ± 7.0%. The statistical results revealed that the efficacy of the VOCs produced by these yeasts was statistically clustered into ten groups ($F_{48,98}$) = 9.362, $p \leq 0.001$) (Table 1). The most effective strain against the growth of *A. flavus* A39 was *Candida nivariensis* DMKU-CE18 (64.9 ± 7.0% inhibition), followed by *Naganishia liquefaciens* DMKU-CE84 (38.9 ± 6.9% inhibition), *Kwoniella heveanensis* DMKU-CE82 (34.4 ± 3.0% inhibition), *Hannaella sinensis* DMKU-CP430 (34.4 ± 7.4% inhibition), and *W. anomalus* DMKU-RP25 (31.1 ± 21.0% inhibition) (Table 1).

Table 1 Inhibition of fungal growth, conidial germination, and percentage germination (after 21 h incubation at 28 °C) by VOC-producing yeasts

Species	Strain	Inhibition of fungal growth (%)	Germinated spores (%)*			
			Scale 1	Scale 2	Scale 3	Scale 4
Candida nivariensis[A]	DMKU-CE18	64.9 ± 7.0[a]	49.3 ± 8.7[a]	16.0 ± 14.5[a]	20.3 ± 11.1[abcde]	14.3 ± 20.6[c]
Naganishia liquefacien[B]	DMKU-CE84	38.9 ± 6.9[b]	20.7 ± 15.5[a]	1.7 ± 2.1 [cd]	12.0 ± 12.5[bcde]	65.7 ± 23.9[ab]
Kwoniella heveanensis[B]	DMKU-CE82	34.4 ± 3.0[bc]	43.0 ± 25.0[a]	1.7 ± 1.2 [cd]	16.0 ± 4.6[abcde]	39.3 ± 21.6[abc]
Hannaella sinensis[B]	DMKU-CP430	34.4 ± 7.4[bc]	39.7 ± 25.5[a]	2.0 ± 3.5 [cd]	20.3 ± 14.5[abcde]	38.0 ± 22.3[abc]
Wickerhamomyces anomalus[A]	DMKU-RP25	31.1 ± 21.0[bcd]	18.7 ± 12.7[a]	9.7 ± 4.9[abc]	38.3 ± 9.3[a]	33.3 ± 3.5[ab]
Rhodotorula sp.[B]	DMKU-SE21	26.6 ± 3.0[cde]	30.3 ± 24.8[a]	1.0 ± 1.0 [cd]	18.3 ± 10.3[abcde]	50.3 ± 14.4[abc]
Sporobolomyces carnicolor[B]	DMKU-CE25	23.3 ± 3.0[cdef]	22.3 ± 25.0[a]	4.3 ± 3.2[bcd]	19.3 ± 11.6[abcde]	54.0 ± 12.1[ab]
Rhodotorula mucilaginosa[B]	DMKU-CE130	22.7 ± 3.0[cdef]	37.7 ± 2.1[a]	2.7 ± 2.5 [cd]	23.0 ± 9.2[abcde]	39.7 ± 10.7[abc]
Candida intermedia[A]	DMKU-CP705	22.7 ± 1.1[cdef]	34.3 ± 20.8[a]	2.0 ± 2.0 [cd]	16.3 ± 3.5[abcde]	47.3 ± 20.5[abc]
Trichosporon asahii[B]	DMKU-SE62	22.7 ± 6.3[cdef]	31.7 ± 11.5[a]	1.0 ± 1.0 [cd]	8.7 ± 3.1[de]	58.7 ± 11.9[ab]
Meyerozyma caribbica[A]	DMKU-RP91	22.0 ± 2.0[defg]	32.3 ± 33.3[a]	1.7 ± 1.5 [cd]	16.7 ± 9.5[abcde]	49.3 ± 25.5[abc]
Meyerozyma caribbica[A]	DMKU-RP168	21.4 ± 3.0[defg]	33.0 ± 16.5[a]	1.0 ± 1.0 [cd]	27.0 ± 11.3[abcde]	39.0 ± 15.1[abc]
Candida citri[A]	DMKU-RE11	20.7 ± 6.0[defg]	28.3 ± 33.0[a]	1.3 ± 1.5 [cd]	20.0 ± 17.7[abcde]	50.3 ± 14.2[abc]
Pichia myanmarensis[A]	DMKU-CP657	20.7 ± 7.4[defg]	27.7 ± 16.8[a]	1.7 ± 2.9 [cd]	22.0 ± 27.8[abcde]	48.7 ± 20.0[abc]
Candida tropicalis[A]	DMKU-RE01	20.1 ± 2.0[defg]	43.0 ± 7.2[a]	4.0 ± 4.6[bcd]	11.3 ± 7.0[bcde]	41.7 ± 4.7[abc]
Meyerozyma caribbica[A]	DMKU-SE16	20.1 ± 3.4[defg]	29.0 ± 14.8[a]	2.0 ± 1.7 [cd]	21.3 ± 18.5[abcde]	47.7 ± 6.0[abc]
Candida glabrata[A]	DMKU-CE39	20.1 ± 3.4[defg]	9.3 ± 13.6[a]	2.3 ± 2.5 [cd]	16.7 ± 11.9[abcde]	71.7 ± 2.5[a]
Meyerozyma caribbica[A]	DMKU-SE97	17.5 ± 3.0[efgh]	38.0 ± 27.2[a]	1.0 ± 1.0 [cd]	11.0 ± 1.7[bcde]	50.0 ± 29.1[abc]
Candida intermedia[A]	DMKU-CP791	17.5 ± 4.9[efgh]	32.3 ± 4.5[a]	1.0 ± 1.0 [cd]	6.3 ± 2.1[de]	60.3 ± 7.1[ab]
Meyerozyma caribbica[A]	DMKU-RP47	16.8 ± 3.0[efgh]	44.0 ± 11.3[a]	0.7 ± 1.2 [cd]	16.7 ± 6.1[abcde]	38.7 ± 6.0[abc]
Rhodotorula sp.[B]	DMKU-CE58	16.8 ± 6.3[efgh]	29.3 ± 4.5[a]	0.3 ± 0.6[d]	16.0 ± 10.6[abcde]	54.33 ± 11.0[ab]
Meyerozyma caribbica [A]	DMKU-SP178	16.8 ± 4.1[efgh]	22.3 ± 15.3[a]	1.0 ± 1.0 [cd]	18.0 ± 9.9[abcde]	58.7 ± 4.5[ab]
Rhodotorula toruloides[B]	DMKU-CP699	16.2 ± 14.1[efgh]	21.0 ± 9.5[a]	3.3 ± 3.5[bcd]	26.0 ± 4.6[abcde]	49.7 ± 10.1[abc]
Kodamaea ohmeri[A]	DMKU-RE27	15.5 ± 15.5[efgh]	23.7 ± 8.1[a]	11.7 ± 18.5[ab]	23.0 ± 19.5[abcde]	41.7 ± 31.1[abc]
Candida intermedia[A]	DMKU-CP03	14.9 ± 11.4[efghi]	30.7 ± 39.6[a]	0.3 ± 0.6[d]	6.7 ± 5.7[de]	62.3 ± 35.0[ab]
Meyerozyma caribbica[A]	DMKU-CE77	14.9 ± 1.1[efghi]	17.7 ± 10.7[a]	3.3 ± 3.2[bcd]	35.0 ± 19.9[abc]	44.0 ± 20.0[abc]
Candida pseudointermedia[A]	DMKU-CE59	13.6 ± 7.9[fghi]	32.3 ± 20.8[a]	2.7 ± 1.5 [cd]	12.0 ± 7.0[bcde]	53.0 ± 14.0[ab]
Meyerozyma caribbica[A]	DMKU-SP461	13.6 ± 4.5[fghi]	32.0 ± 25.5[a]	0.3 ± 0.6[d]	9.3 ± 8.5[cde]	58.3 ± 16.6[ab]
Meyerozyma guilliermondii[A]	DMKU-RP26	13.6 ± 4.1[fghi]	19.0 ± 10.4[a]	5.3 ± 3.1[bcd]	36.7 ± 20.6[ab]	39.0 ± 15.4[abc]
Candida parapsilosis[A]	DMKU-SP64	12.9 ± 1.1[fghi]	33.3 ± 21.7[a]	1.0 ± 1.0 [cd]	19.7 ± 12.7[abcde]	46.0 ± 8.7[abc]
Papiliotrema sp.[B]	DMKU-CP117	12.9 ± 1.1[fghi]	32.7 ± 33.1[a]	0.3 ± 0.6[d]	19.0 ± 15.4[abcde]	48.0 ± 18.4[abc]
Meyerozyma caribbica[A]	DMKU-CP710	12.3 ± 5.2[fghij]	38.3 ± 9.6 [a]	3.3 ± 4.0[bcd]	21.0 ± 5.0[abcde]	37.3 ± 7.1[abc]
Candida parapsilosis[A]	DMKU-SP434	11.6 ± 3.0[fghij]	35.7 ± 15.2[a]	4.0 ± 3.6[bcd]	24.7 ± 4.2[abcde]	35.7 ± 18.2[abc]
Saitozyma sp.[B]	DMKU-SE54	11.0 ± 7.4[fghij]	45.0 ± 22.9[a]	1.3 ± 1.5 [cd]	21.3 ± 9.5[abcde]	32.3 ± 12.9[ab]
Meyerozyma caribbica[A]	DMKU-CP724	11.0 ± 6.0[fghij]	38.3 ± 11.1[a]	2.0 ± 1.7 [cd]	11.7 ± 10.0[bcde]	48.0 ± 13.8[abc]
Meyerozyma caribbica[A]	DMKU-SP44	11.0 ± 9.3[fghij]	28.7 ± 26.5[a]	2.7 ± 1.5 [cd]	7.3 ± 2.5[de]	61.3 ± 25.5[ab]
Candida floricola[A]	DMKU-CP149	11.0 ± 1.1[fghij]	20.0 ± 5.2[a]	1.3 ± 1.5 [cd]	10.7 ± 1.2[cde]	68.0 ± 6.2[ab]
Meyerozyma caribbica[A]	DMKU-RE86	9.0 ± 5.6[ghij]	12.0 ± 2.7[a]	4.0 ± 1.0[bcd]	23.0 ± 3.5[abcde]	61.0 ± 2.0[ab]
Occultifur plantarum[B]	DMKU-SE24	9.0 ± 4.1[ghij]	11.7 ± 9.3[a]	2.3 ± 1.5 [cd]	28. 7 ± 10.7[abcd]	57.3 ± 13.3[ab]
Meyerozyma caribbica[A]	DMKU-RE75	7.1 ± 4.5[hij]	35.3 ± 38.8[a]	6.7 ± 10.7[bcd]	23.0 ± 26.5[abcde]	35.0 ± 26.9[abc]
Saitozyma flava[B]	DMKU-RP128	7.1 ± 5.6[hij]	18.3 ± 6.7[a]	2.0 ± 2.0 [cd]	23.3 ± 9.6[abcde]	56.3 ± 5.8[ab]
Meyerozyma caribbica[A]	DMKU-CP629	5.8 ± 1.1[hij]	33.0 ± 13.9[a]	0.7 ± 0.6 [cd]	18.0 ± 15.6[abcde]	48.3 ± 15.0[abc]
Meyerozyma caribbica[A]	DMKU-SP248	4.5 ± 7.6[hij]	36.3 ± 45.7[a]	1.0 ± 1.7 [cd]	2.0 ± 1.7[de]	60.7 ± 43.3[ab]
Kodamaea ohmeri[A]	DMKU-CP51	4.5 ± 6.2[hij]	21.3 ± 23.3[a]	1.7 ± 2.9 [cd]	23.0 ± 22.3[abcde]	54.0 ± 22.3[ab]
Saitozyma flava[B]	DMKU-RE67	2.5 ± 4.7[ij]	38.3 ± 11.6[a]	2.7 ± 3.1 [cd]	21.7 ± 2.5[abcde]	37.3 ± 12.1[abc]
Candida tropicalis[A]	DMKU-RP15	1.9 ± 2.6[ij]	32.3 ± 17.9[a]	2.0 ± 2.7 [cd]	15.7 ± 6.4[abcde]	50.0 ± 15.5[abc]

Table 1 continued

Species	Strain	Inhibition of fungal growth (%)	Germinated spores (%)*			
			Scale 1	Scale 2	Scale 3	Scale 4
Meyerozyma caribbica[A]	DMKU-CE17	0 ± 0.33^{j}	23.3 ± 22.5^{a}	5.0 ± 4.6^{bcd}	35.0 ± 12.8^{abc}	36.7 ± 15.1^{abc}
Kodamaea ohmeri[A]	DMKU-RE114	0 ± 0.0^{j}	35.3 ± 20.7^{a}	5.0 ± 7.8^{bcd}	24.0 ± 25.2^{abcde}	35.7 ± 21.6^{abc}
Papiliotrema laurentii[B]	DMKU-SP67	0 ± 0.0^{j}	24.3 ± 8.1^{a}	1.3 ± 0.6^{cd}	14.7 ± 9.1^{abcde}	59.7 ± 7.1^{ab}

Yeast species are ordered according to their percentage of inhibition of fungal growth. Each value comprises the means of three replicates ± SE. In the same column, data followed by the same letters are not significantly different according to the least significant difference test ($p > 0.05$)

A phylum Ascomycota, *B* phylum Basidiomycota

*Germination rating scale: 1 = no germination; 2 = germ tube < 2× conidium size; 3 = germ tube 2× to 4× conidium size; 4 = germ tube > 4× conidium size: 100 conidia per treatment were counted

Efficacy of VOC-producing yeasts on fungal conidial germination inhibition

The inhibition of conidial germination varied from 9.3 ± 13.6 to 49.3 ± 8.7% among the 46 yeast strains that could inhibit mycelial growth (Table 1). The most effective strain for reducing conidial germination was *C. nivariensis* DMKU-CE18 (49.33 ± 8.7% inhibition), followed by *Saitozyma* sp. DMKU-SE54 (45.0 ± 22.9% inhibition) and *Meyerozyma caribbica* DMKU-RP47 (44.0 ± 11.3% inhibition). Although, no significant difference was found in the inhibition of conidial germination by the VOCs produced by the tested strains (see scale 1 in Table 1) ($F_{48,98} = 0.560$, $p > 0.05$), *C. nivariensis* DMKU-CE18 seemed to be the most effective in reducing spore germination as it resulted in a low percentage of germinated spores in each germination rating scale.

Reduction of aflatoxin in corn grains

Five potential yeast strains, based on the previous results, were selected for in vivo assay in corn grains (Fig. 1). The most effective strain in reducing AFB1 in corn grains was *C. nivariensis* DMKU-CE18 (74.8 ± 6.5% reduction), followed by *K. heveanensis* DMKU-CE82 (62.7 ± 10.5% reduction), *N. liquefaciens* DMKU-CE84 (60.2 ± 7.3% reduction), *H. sinensis* DMKU-CP430 (52.6 ± 22.4% reduction), and *W. anomalus* DMKU-RP25 (51.4 ± 10.3% reduction). In addition, all strains significantly reduced aflatoxin in corn grains when compared to the control experiment ($F_{5,12} = 9.182$, $p \leq 0.001$). However, the efficacies of VOCs in reducing aflatoxin were not significantly different among these yeasts ($p = 0.104$).

Volatile organic compound (VOC) compositions

The most effective antifungal VOC-producing yeast strain, *C. nivariensis* DMKU-CE18, was selected for HS-GC/MS analysis. Three volatile compounds were detected from the yeast strain. The compound that was closely matched to 1-pentanol was the major volatile compound produced by *C. nivariensis* DMKU-CE18 (Table 2).

Discussion

Phytopathogenic fungi cause serious damages to and low quality in agricultural products. Fungal growth destroys pre- and post-harvest fruit, vegetables, and grains (Aidoo 1993; Spadaro and Gullino 2004; Wilson and Pusey 1985). Chemical fungicides have been widely used to control pathogenic fungi in agriculture. However, this strategy affects consumer health and raises environmental concerns. Antagonistic microorganisms have been successfully applied against fungal growth in food and agricultural products. This strategy generates benefits, not only in biocontrol efficacy, but also in safety for human beings (Gotor-Vila et al. 2017; Huang et al. 2012; Leelasuphakul et al. 2008).

Numerous microorganisms may be used as biocontrol agents against mycotoxigenic fungi. Several yeast strains have been identified as potential biocontrol agents. For instance, Hua et al. (1999) reported

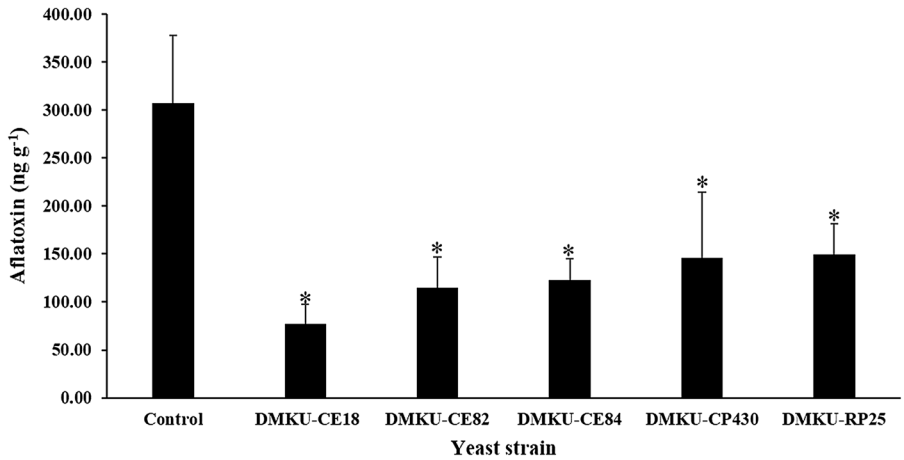

Fig. 1 Aflatoxin reduction in corn grains by VOC-producing yeasts. The experiment without yeast inoculation was prepared as the control. Significant differences between yeast treatments and control are indicated by * with $p \leq 0.001$

Table 2 VOC compositions of *C. nivariensis* DMKU-CE18 by HS-GC/MS

Retention time (min)	Peak area ± SE[a] (%)	Possible compound	Molecular weight (g mol^{-1})	Quality match (%)
6.36	3.17 ± 1.06	1-Propanol, 2-methyl-	74.12	50
6.74	4.68 ± 1.67	1-Butanol, 3-methyl-	88.15	90
9.27	92.16 ± 0.88	1-Pentanol	88.15	56

[a]Mean values of the percentage of the peak area over the total area of the peaks in the chromatogram from three replicates of yeast strain grown on potato dextrose agar (PDA)

saprophytic yeasts, *Pichia anomala* WRL-076 and *C. krusei* WRL-038, isolated from the fruits of almond, pistachio, and walnut trees, were inhibitors to aflatoxin biosynthesis by *A. flavus*. In addition, Afsah-Hejri (2013) reported that five saprophytic yeasts comprising *Pseudozyma fusiformata*, *Cryptococcus albidus*, *Rhodotorula fragaria*, *Cryptococcus hungaricus*, and *Rhodotorula hinula* showed the highest level of biocontrol activities against *A. flavus* PTCC 5006. Their inhibitory effects on sporulation, colony expansion, biomass production, and prevention of AFB1 production were evaluated. *P. fusiformata* was the most effective yeast strain in the reduction of fungal spores (84.6%) and the inhibition of AFB1 production (89.1%).

In the current study, several yeast strains in Ascomycota and Basidiomycota were able to produce antifungal VOCs against *A. flavus* A39. The production of VOCs seemed to be strain-dependent not species-dependent. The most effective strain to inhibit the mycelium growth and conidial germination of *A. flavus* A39 and to reduce aflatoxin B1 production by this fungus in corn grains was *C. nivariensis* DMKU-CE18. This yeast strain was isolated from corn leaf tissue (Khunnamwong et al., 2018). A few previous studies on the ability of *Candida* spp. to produce antifungal VOCs have been reported. For example, *C. intermedia* C410 produced volatile compounds that effectively suppressed the conidial germination and mycelial growth of *Botrytis cinerea.* This could control Botrytis fruit rot in strawberries (Huang et al. 2011). *Candida sake* 41E and F36A produced VOCs which inhibited the growth of five pathogens of apples, comprising *Penicillium expansum*, *B. cinerea*, *Alternaria alternata*, *A. tenuissima*, and *A. arborescens* (Arrarte et al. 2017). To the best of our knowledge, the current study is the first to reveal that *C. nivariensis* could produce VOCs against the growth and conidial germination of *A. flavus.* It also reduced the production of aflatoxin when co-inoculated into the fungal-contaminated corn grains. However, *C. nivariensis* has been found as a pathogenic strain in humans

(Alcoba-Flórez et al. 2005). This strain has been reported to have antifungal resistance including against azoles (Borman et al. 2008; Figueiredo-Carvalho et al. 2016; Fujita et al. 2007). Therefore, the utilization of VOCs from this strain by excluding yeast cells could be an alternative approach for safe treatment against the growth of *A. flavus* in agricultural products. In recent years, Parafati et al. (2017) reported the utilization of commercial hydrogel spheres as a support for VOC-producing yeasts including *W. anomalus*, *Metschnikowia pulcherrima*, *Aureobasidium pullulans,* and *S. cerevisiae*. These immobilized yeast cells could effectively produce VOCs against mold decay on strawberry and mandarin fruits caused by *B. cinerea*, *Penicillium digitatum*, and *P. italicum.*

The VOC profiles of *C. nivariensis* DMKU-CE18 comprised compounds closely matched to 1-propanol-2-methyl, 1-butanol-3-methyl-acetate, and 1-pentanol in the database. The one closest to 1-pentanol was the major volatile compound produced by this yeast strain. These compounds were also produced by an endophytic yeast, *Nodulisporium* sp., that was isolated from *Myroxylon balsamum* found in the upper Napo region of the Ecuadorian Amazon (Mends et al. 2012). This strain produced several VOCs, such as 1-butanol-3-methyl, 1-propanol-2-methyl, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, and 1-nonanol along with phenylethyl alcohol, all of which were active against the pathogenic fungi *Aspergillus fumigatus*, *Phytophthora cinnamomic*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*. Furthermore, *S. cerevisiae* produced 3-methyl-1-butanol and 2-methyl-1-butanol. A mixture of these VOCs showed antimicrobial activity against various phytopathogens in vitro (Rezende et al. 2015). Masoud et al. (2005) reported that volatile compounds produced by *W. anomalus*, *Pichia kluyveri,* and *Hanseniaspora uvarum* during coffee processing could inhibit the growth of *A. ochraceus*, thus leading to the prevention of OTA production. The most effective VOC compound on fungal growth inhibition was 2-phenyl ethyl acetate. Hua et al. (2014) revealed that 2-phenylethanol, produced by *W. anomalus* WRL-076 affected spore germination, growth, toxin production, and gene expression in *A. flavus*. To date, various new microbial products, including yeast-based products, have reached the commercial market as biocontrol agents (Janisiewicz and Korsten 2002). However, no yeast product has been registered on the commercial market as a biological control agent for mycotoxin-producing fungi.

In conclusion, our research results showed that various saprophytic yeast strains isolated from plant leaves produced antifungal VOCs against *A. flavus* A39. Our study is the first to report that VOCs produced by *C. nivariensis* could inhibit the mycelial growth and conidial germination of *A. flavus* and reduce aflatoxin production in contaminated corn grains. Therefore, the VOCs produced by this yeast have potential in agriculture as a biocontrol agent to manage aflatoxin contamination by *A. flavus* during grain storage. However, further research is needed on the appropriate methodology for using the VOCs produced by *C. nivariensis.*

Acknowledgements This work was supported by the Thailand Research Fund (TRF) through the TRF Research-Team Promotion Grant (RTA 6080004). The authors would like to thank Dr Amara Chinaphuti, Department of Agriculture (DOA), Thailand, for providing *Aspergillus flavus* A39.

Compliance with ethical standards

Conflicts of interest The authors declare that there are no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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