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Biofumigant potential and inhibition mechanism of *Trichoderma asperelloides* SKRU-01 volatile organic compounds for controlling aflatoxigenic *Aspergillus parasiticus* and *Aspergillus flavus* in stored peanuts

Sawai Boukaew [a,*], Wanida Petlamul [a], Siriporn Yossan [b], Sirasit Srinuanpan [c], Karistsapol Nooprom [d], Zhiwei Zhang [e]

[a] Center of Excellence BCG for Sustainable Development, College of Innovation and Management, Songkhla Rajabhat University, Songkhla, 90000, Thailand
[b] Division of Environmental Science, Faculty of Liberal Arts and Science, Sisaket Rajabhat University, Sisaket, 33000, Thailand
[c] Center of Excellence in Microbial Diversity and Sustainable Utilization, Chiang Mai University, Chiang Mai, 50200, Thailand
[d] Faculty of Agricultural Technology, Songkhla Rajabhat University, Songkhla, 90000, Thailand
[e] Institute of Materia Medica, Xinjiang University, Urumqi, 830017, People's Republic of China

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ABSTRACT

Microbial volatile organic compounds (VOCs) have emerged as promising alternatives to synthetic fungicides for managing stored grain. In this study, the inhibitory effects of *Trichoderma asperelloides* SKRU-01 VOCs on hyphal growth and spore germination of *Aspergillus parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 were investigated. The results demonstrated significant suppression of hyphal growth and spore germination in both strains by SKRU-01 VOCs. By using solid-phase microextraction (SPME) coupled with gas chromatography-mass spectrometry (GC-MS), the SKRU-01 VOCs were identified to contain 23 compounds, with acetophenone being predominant (14.41%). Antifungal experiments revealed that acetophenone, at a minimum inhibitory concentration (MIC) of 1.0 μL/mL (solid media assay) or 1.0 μL/L (vapor phase assay), completely inhibited the growth of both strains. Moreover, the application of SKRU-01 VOCs effectively reduced mold density and aflatoxin B_1 (AFB_1) production in peanuts and also exhibited superior efficacy compared to acetophenone at a 1/2 MIC concentration. Acetophenone exhibited inhibitory effects on both fungal strains through multiple mechanisms of action. This included its ability to inhibit ergosterol biosynthesis, reduce the aflatoxin inducer methylglyoxal, impair antioxidant defense enzymes, and disrupt non-enzymatic defense molecules. Overall, this research highlights the biofumigant potential of SKRU-01 VOCs in stored grain management and provides novel insights into their antifungal mechanism against *A. parasiticus* and *A. flavus*. Furthermore, these findings contribute to the development of sustainable strategies for grain preservation and underscore the broader applications of microbial VOCs in agricultural and food safety practices.

1. Introduction

Aflatoxin-producing fungi, namely *Aspergillus flavus* and *Aspergillus parasiticus*, are fungi that pose a substantial risk to stored grains and their derived products, including crops such as peanuts, rice, and maize (Yu et al., 2004). These fungi are well-known for their ability to produce harmful aflatoxins (AFs) in both crops and food, which can have serious consequences for human and animal health globally (Waliyar et al., 2015). Aflatoxin contamination jeopardizes the quality and safety of peanut products, which serve as essential sources of edible oil, protein, and play a pivotal role in global agricultural production and trade (Von Hartwig et al., 2020).

To prevent mycotoxin-induced food spoilage, the food industry routinely inspects stored cereals and grain products for contamination and employs methods to inhibit fungal growth. While the conventional approach involves the use of chemically synthesized antibiotics to prevent food deterioration, this method has limitations due to the potential emergence of antibiotic-resistant microorganisms (Farag et al., 2020). Furthermore, the utilization of chemical fungicides can result in significant adverse effects on humans, animals, other organisms, and the environment (Zhong et al., 2021). Therefore, it is important to explore alternative antimicrobial agents that are effective, non-toxic, and

* Corresponding author.
E-mail address: sawai.bo@skru.ac.th (S. Boukaew).

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biodegradable to ensure food safety and protect human health (Aparicio-García et al., 2021). Recently, there has been a growing emphasis on utilizing volatile organic compounds (VOCs) emitted by biocontrol agents (BCAs) as a sustainable and promising method for postharvest disease management (Contarino et al., 2019; Zheng et al., 2019). This method is being explored as a replacement for chemical interventions and for the implementation of integrated control programs to effectively control postharvest decay in fruits, vegetables, grains, and seeds (Oufensou et al., 2023; Zhang, Li, Zhang, et al., 2020).

VOCs are characterized by their low molecular weight (<300 Da), low polarity, and high vapor pressure (Korpi et al., 2009). They are commonly employed for postharvest disease control in fruits, vegetables, and grains due to their non-toxic nature, easy evaporation, and minimal risks involved (Farbo et al., 2018). Rather than relying on chemicals, utilizing effective antifungal agents can serve as an alternative approach to control fungal diseases. Several microorganisms have been investigated for their capacity to produce antimicrobial VOCs, potentially enabling the suppression of phytopathogenic fungi. For instance, *Bacillus* species (Yousefvand et al., 2023; Zhang et al., 2023), *Pseudomonas* species (Chandrasekaran et al., 2023; Parlapan et al., 2023), and *Streptomyces* species (Boukaew et al., 2013, 2021; Mirsonbo et al., 2023) have been found to produce antimicrobial VOCs. Additionally, fungi such as *Noduliosporium* species and *Muscodor* species (Kaddes et al., 2019), *Geotrichum candidum* (Mitra et al., 2023), and yeast such as *Pichia anomala* (Oufensou et al., 2023) have also demonstrated success in controlling food spoilage pathogens.

Trichoderma species are recognized for their ability to inhibit the growth of plant pathogenic fungi by producing potent fungitoxic antibiotics, extracellular cell wall-degrading enzymes, and VOCs (Ayyandurai et al., 2023; Pascale et al., 2017). These findings highlight the potential of *Trichoderma* species as biofungicides. Consequently, there is a rising interest in investigating the potential of utilizing microbial VOCs as biocontrol agents (Ayyandurai et al., 2023; Nawrocka et al., 2023). As an example, *T. koningiopsis* has been shown to effectively inhibit *Botrytis cinerea* through the emission of VOCs (You et al., 2022), while VOCs emitted by *T. afroharzianum* were found to significantly reduce *Fusarium* contamination in fresh chilies (Khruengsai et al., 2021). Additionally, *T. atroviride* IC-11 demonstrates promise as a potential approach for protecting blueberries against *B. cinerea* (Bello et al., 2022).

In a recent study, a significant finding emerged, indicating that *T. asperelloides* SKRU-01 effectively inhibited the spoilage of food and feed by aflatoxin-producing fungi in peanuts through the production of antimicrobial secondary metabolites (Boukaew et al., 2023). However, the potential of VOCs in preventing aflatoxin-producing fungi is still a subject of ongoing discussion. The aim of this study was to evaluate the efficacy of *T. asperelloides* SKRU-01 VOCs and, specifically focusing on the main active ingredient acetophenone, in controlling the mycotoxins produced by *A. flavus* and *A. parasiticus* in peanuts. Furthermore, the study aimed to investigate the mechanism of action of acetophenone, the primary active ingredient, against these two fungal strains responsible for mycotoxin production. These findings hold significant implications for the advancement of biological preservatives capable of effectively controlling both mycotoxins, thereby expanding the potential for biological protection in peanut storage.

2. Materials and methods

2.1. Culture media and microorganisms

Strain SKRU-01 was isolated from loam soil samples collected from cultivation plots at the Faculty of Natural Resources, Prince of Songkla University, Hatyai, Thailand and identified to be *Trichoderma asperelloides* (Boukaew et al., 2023). The strain was cultured on Difco™ potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) at 30 °C for 7 days. Spores were collected from the agar surface using a sterile spreader glass and suspended in a solution of sterile distilled water (5 mL) and 0.1% (v/v) Tween 80 (Sigma-Aldrich). The spore suspensions were filtered through sterile cheesecloth to remove residual mycelia, and the spore count was determined using a hemocytometer under a microscope (Nikon Eclipse E100 LED, Tokyo, Japan). The concentration was adjusted with sterilized water to achieve the desired final concentration ranging from 10^4 to 10^7 spores/mL, as per experimental requirements.

The two aflatoxigenic fungal strains, *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4, were previously identified as high aflatoxin producers (Boukaew et al., 2020a). For spore collection, the fungi were cultured on PDA plates at 30 °C for 7 days, and spores were collected using the aforementioned method.

2.2. Suppression of hyphal growth and spore germination in mycotoxin-producing fungi using SKRU-01 VOCs

2.2.1. Preparation of volatile organic compounds from T. asperelloides SKRU-01 (SKRU-01 VOCs)

The preparation of SKRU-01 VOCs involved transferring five μL of a spore suspension (10^7 spores/mL) of the *T. asperelloides* SKRU-01 onto PDA (Kluger et al., 2013). This mixture was then incubated for 7 day at 30 °C, after which the organic compounds activity of the *T. asperelloides* SKRU-01 culture was investigated.

2.2.2. Suppression of hyphal growth and spore germination

The antifungal activity of SKRU-01 VOCs against mycotoxin-producing fungi was assessed by evaluating their ability to inhibit hyphal growth. This was done using a modified version of the method described by Archana et al. (2021) in Petri dishes. Briefly, a Petri dish containing strain SKRU-01 (as described in section 2.2.1) was covered with another Petri dish containing PDA medium inoculated with a 5 μL suspension of each mycotoxin-producing spore at a concentration of 10^5 spores/mL. Both sets of plates were securely sealed to create a controlled environment conducive to fumigation facilitated by fungal growth. As a control, a PDA medium without inoculation of the SKRU-01 strain was used. Three replicates were conducted for each treatment. After a five-day incubation at 30 °C, the diameters of colonies produced by each fungus capable of producing mycotoxins were measured. To quantify the percentage inhibition of hyphal growth, the following formula was employed: Percentage inhibition (%) = [(Dc × Dt)/Dc] × 100, where Dc corresponds to the hyphal growth of the fungus on the control plate, and Dt refers to the mycelial growth of the fungus on the test plate.

The antifungal activity of SKRU-01 VOCs against mycotoxin-producing fungi was assessed by evaluating their ability to inhibit spore germination. Briefly, a Petri dish containing strain SKRU-01 (as described in section 2.2.1) was covered with another Petri dish containing PDA medium. Then, 10 μL of a suspension of each mycotoxin-producing spore (at a concentration of 10^5 spores/mL) was spread on separate PDA plates using a sterile glass spreader. Both sets of plates were then placed together and sealed to allow for fumigation, which was activated by fungal growth. As a control, a PDA medium without inoculation of the SKRU-01 strain was used. Three replicates were conducted for each treatment. After a 24-h incubation at 30 °C, the number of spores that had germinated in each plate producing mycotoxin was calculated. The percentage of spore germination inhibition was calculated using the previously mentioned method.

2.3. Effects of strain SKRU-01 spore concentrations on VOCs production against both mycotoxin-producing fungi, and identification of the resulting VOCs

To assess the impact of different spore concentrations of strain SKRU-01 on the growth of mycotoxin-producing fungi on PDA, an antifungal bioassay was performed following the procedure outlined below. The PDA plates were contented with 50 μL of a spore suspension containing various concentrations of 10^4, 10^5, 10^6 or 10^7 spores/mL of strain SKRU-01 and incubated at 30 °C for 7 days. Additionally, PDA plates were

inoculated in the center with a 5 μL suspension of each mycotoxin-producing spore (at a concentration of 10^5 spores/mL). Both sets of plates were then sealed together to create a fumigation effect, which was activated by fungal growth. A control plate without SKRU-01 strain inoculation was used for comparison. Each treatment was replicated three times. The incubation period and calculation of the percentage inhibition of hyphal growth followed the procedure described earlier.

In order to quantify the volatile organic compounds (VOCs) produced by the strain SKRU-01, the methodology outlined by Lee et al. (2016) was adopted with slight modifications. Briefly, 10 μL of a 10^7 spore/mL suspension of strain SKRU-01 was inoculated onto the headspace above a 3 mL PDA culture in a 5 mL glass flask. This culture was then incubated at 30 °C for a period of 7 days. Subsequently, VOCs produced by strain SKRU-01 in the PDA headspace samples were captured and quantified using a purge and trap method, a technique previously described by Lee et al. (2015). Negative controls were obtained from sterile PDA headspace samples. The collection of volatile compounds was performed using Solid-phase micro-extraction (SPME). Quantification of the VOCs was carried out through gas chromatography-mass spectrometry (GC-MS) analysis using a gas chromatograph mass spectrometer (Trace GC Ultra/ISQMS, Thermo Scientific Inc., USA). The TR-5MS column (30 m × 0.25 mm, film thickness 0.25 μm, Agilent, Santa Clara, CA, USA) was selected for chromatographic separation. Mass spectra were acquired in scan mode (total ion count, 20–350 m/z) for compound identification, and their concentrations were determined by comparing the peak areas to calibration curves generated from known standard compounds. To ensure the accuracy of our quantification, multiple calibration standards were used across a range of concentrations.

2.4. In vitro evaluation of the inhibitory effects of selected individually identified VOCs on the growth and spore germination of mycotoxin-producing fungi

To determine the optimal dosages of VOC components, their inhibitory effects on the mycelial growth of mycotoxin-producing fungi were assessed following the method outlined by Ling et al. (2022). A 5 μL spore suspension of mycotoxin-producing fungi was inoculated at the center of PDA plates. Five pure chemicals representing the major components of the SKRU-01 VOCs, namely linalool (Sigma–Aldrich, 95% purity), acetophenone (Fluka, 99.5% purity), phenylethyl alcohol (Sigma–Aldrich, 99% purity), heneicosane (Sigma–Aldrich, 98.0% purity), and geosmin (Sigma–Aldrich, 97.0% purity), were dropwise added at a concentration of 100 μL per liter of airspace volume onto 6 mm filter paper placed on the inner cover of a Petri dish. The dish was sealed, inverted, and incubated at 30 °C for 5 days. Three replicates were performed for each dose, including a control with 0.5% Tween-80. Hyphal growth inhibition was calculated as described in the previous procedure.

To determine the effective dosages of VOC components against mycotoxin-producing spores, separate PDA plates were inoculated with a suspension of each mycotoxin-producing spore (at a concentration of 10^5 spores/mL). Pure standards of the VOC components were then dropwise added (onto 6 mm filter paper) and attached to the inner cover of the Petri dish. The dish was sealed and incubated in an inverted position. After a 24-h incubation at 30 °C, the number of germinated spores on each mycotoxin-producing plate was counted. The percentage inhibition of spore germination was determined using the same method as described previously.

To determine whether the VOCs that were individually identified for their growth suppression activity or spore germination inhibition had killed or inhibited the two mycotoxin-producing fungal strains under investigation, the VOCs were removed from the bioassay plates where complete growth inhibition (100%) was observed. The PDA plates (showing complete growth inhibition) were then incubated at 30 °C for five days. If no growth appeared during this time, it can be inferred that the pathogen was killed by the VOCs.

2.5. Determining the minimum inhibitory concentration (MIC) of the selected VOCs component against mycotoxin-producing fungi in solid media and vapor phase

For the solid media assay, the selected VOCs were mixed with PDA at final concentrations ranging from 0 to 1.2 μL/mL, following the method of Li et al. (2021) with some modifications. A 5 μL suspension of mycotoxin-producing fungi (at a concentration of 10^5 spores/mL) was then applied to the center of PDA plates containing the VOCs. The plates were incubated at 30 °C for 48 h, and the diameter of each mycotoxin-producing fungi colony was measured every 24 h to determine the minimum inhibitory concentration (MIC). The mycelial growth inhibition was calculated as described previously. Each concentration was tested in triplicate.

For the vapor phase assay, sterile 50 mm diameter Petri dishes containing 10 mL of PDA medium were inoculated with 5 μL of a suspension of mycotoxin-producing fungi (at a concentration of 10^5 spores/mL) and inverted. Different concentrations of individually identified VOC solutions ranging from 0.2 to 1.2 μL/L (compound volume/airspace volume) were applied to the Petri dish lids. The Petri dishes were sealed with film and incubated at 30 °C for 48 h. A control was included using PDA medium without fumigation of the selected VOCs. The diameter of each mycotoxin-producing fungi colony was measured every 24 h to determine the MIC, and the mycelial growth inhibition was calculated as described previously. Each concentration was tested in triplicate.

2.6. Comparison of the biofungicidal ability of SKRU-01 VOCs and acetophenone for inhibiting the growth of mycotoxin-producing fungi on peanuts

2.6.1. Peanut preparation

Peanut kernels obtained from a local supermarket were stored at −20 °C. Before testing, the peanuts were surface sterilized by immersing them in a 3% (v/v) sodium hypochlorite solution for 3 min, followed by several rinses with sterile distilled water, following the procedure outlined by Abd-Alla (2005). Afterward, they were autoclaved at 121 °C for 15 min.

2.6.2. Effect of SKRU-01 VOCs and acetophenone on peanuts inoculated with each mycotoxin-producing fungal spores

An experiment was conducted to investigate the effect of VOCs on mycotoxin-producing fungi-induced mildew during peanut storage, following the methodology described by Ling et al. (2022). Two Petri dishes of different diameters (140 mm and 60 mm) were utilized. In the experiment, a small Petri dish with a 60 mm diameter was inoculated with PDA media, followed by the addition of 10 μL (at a concentration of 10^7 spores/mL) of strain SKRU-01 on the PDA medium, and incubated at 30 °C for 7 days. In a larger Petri dish (140 mm), two layers of sterile filter paper were placed on PDA media, and 2 mL of sterile water was added to maintain humidity. A plastic bag containing 30 peanuts was mixed with 1 mL of each mycotoxin-producing fungi spore suspension (10^5 spore/mL). The mixture was then transferred to the larger Petri dish (140 mm). Subsequently, the small Petri dish containing the inoculated strain SKRU-01 or acetophenone at concentrations of 1/2 MIC, MIC, and 2 MIC was placed in the center of the larger Petri dish (140 mm) on sterile filter paper, and the dish was sealed with Parafilm. The dish was incubated at 30 °C for 5 days, following the procedure outlined by Zhang, Li, Bi, et al. (2020). After a five-day incubation period, the peanuts inoculated with the two strains of aflatoxigenic fungi were divided into two equal batches: one for assessing mold density and the other for estimating aflatoxin production. The experiment was conducted in triplicate, with each replicate consisting of 30 peanuts.

To determine the mold density in each peanut sample, 5 g of the sample was mixed with 45 mL of 0.85% NaCl saline solution and agitated on a rotary shaker at 200 rpm for 2 h at 30 °C. The resulting suspension was serially diluted two-fold in 0.85% NaCl saline solution,

and 0.1 mL of the diluted suspension was plated on PDA plates. The plates were incubated at 30 °C for 48 h, and the mold density was calculated as the logarithm of spores per gram of peanuts. The percentage of spore reduction was calculated using a previously described method. Each treatment was performed in triplicate.

For the measurement of Aflatoxin AFB_1 concentration, a modified version of the method described by Sidhu et al. (2009) was employed to extract aflatoxin from peanuts. Ground peanut powder (10 g) was mixed with 25 mL of 70% aqueous methanol and homogenized for 3 min using a laboratory homogenizer. The resulting mixture was filtered through Whatman No. 1 filter paper to obtain a clean sample. The concentration of AFB_1 was quantified using High-performance liquid chromatography with fluorescence detection (HPLC-FLD; Agilent Technologies, Wilmington, DE, USA), following the procedure outlined by Choochuay et al. (2018). The entire experiment was conducted in triplicate for each treatment.

2.7. Mechanism action of acetophenone against two mycotoxin-producing fungal strains

The antifungal properties of acetophenone were assessed at different concentrations: 0.5 μL/mL (1/2 MIC), 1.0 μL/mL (MIC), and 2.0 μL/mL (2 MIC). To evaluate its effects, acetophenone was added to PDB, except for the control group that received no acetophenone. The fungal strain capable of producing mycotoxins was then inoculated with a 10 μL spore inoculum (at a concentration of 10^5 spores/mL). The flasks containing the inoculated cultures were incubated for 5 days at 30 °C on a rotary shaker (150 rpm). After the incubation period, the culture broth was divided into two equal portions. One portion was used to determine the mycelial mats of the two mycotoxin-producing strains, following the method described by Li et al. (2011). The other portion was used to investigate the antifungal and antiaflatoxigenic mechanisms of action.

2.7.1. Acetophenone-induced changes in plasma membrane ergosterol biosynthesis

The ergosterol content of two mycotoxin-producing strains was assessed using the procedure outlined by Das et al. (2020). This involved measuring the wet weight of the sterilized mycelial mat. Subsequently, a 5 mL solution of ethanolic KOH was mixed with the mycelial mat, agitated for 2 min, and then heated for 1 h at 80 °C. Following this, a mixture of n-heptane and distilled water (2:5 v/v) was added to each flask, with agitation for 2 min. The flasks were then left undisturbed at room temperature to allow for the extraction of membrane sterols. Sterol content was measured by scanning the upper n-heptane layer between 230 and 300 nm using a UV–Visible spectrophotometer (Tian et al., 2012). Each treatment was replicated three times.

2.7.2. Acetophenone-induced modulation of cellular methylglyoxal levels

For each mycotoxin-producing fungus strain, 10 μL of spore inoculum at a concentration of 10^5 spores/mL was added to 10 mL of PDB medium. Acetophenone was then added at concentrations of 1/2 MIC, MIC, and 2 MIC, while the control group remained untreated. After incubating the samples at 30 °C for 5 days on a rotary shaker (150 rpm), the biomass was collected and ground in 3 mL of 0.5 M $HClO_4$. Subsequently, the mixture was centrifuged at 8880×*g* for 20 min, and the resulting supernatant was neutralized by drop-wise addition of K_2CO_3 solution, followed by another round of centrifugation. The supernatant was neutralized by adding K_2CO_3 solution drop by drop, followed by centrifugation. The collected supernatant was used for the estimation of cellular methylglyoxal levels as described by Yadav et al. (2005). Methylglyoxal content was determined by sequentially adding 3,3-diaminobenzidine (DAB), $HClO_4$, and supernatant, followed by measuring the optical density at 341 nm. The methylglyoxal concentration was calculated using a standard calibration curve of methylglyoxal (Upadhyay et al., 2018). Three replicates were performed for each treatment.

2.7.3. Effects of acetophenone on the enzymatic and non-enzymatic defense systems

2.7.3.1. Preparation of fungal biomass and enzyme extracts. Following exposure to acetophenone at 1/2 MIC, MIC, and 2 MIC concentrations, the biomass of each mycotoxin-producing fungus was homogenized in 3 mL of phosphate-buffered saline (PBS, 100 mM, pH 7.4). Subsequently, centrifugation was performed at 8880×*g* for 20 min. The control group was not treated with acetophenone. Biochemical tests were then conducted using the supernatant obtained from both the treated and untreated samples.

2.7.3.2. Determination of cellular ROS, CAT, SOD, and glutathione (reduced and oxidized). Determination of cellular reactive oxygen species (ROS) (Keston & Brandt, 1965), catalase (CAT) (Beers & Sizer, 1952), superoxide dismutase (SOD) (Kostyuk & Potapovich, 1989; Lowry et al., 1951), and glutathione (reduced and oxidized) levels (Hissin & Hilf, 1976) was conducted following the established procedures described by Boukaew et al. (2023).

2.8. Statistical analysis

The experimental procedures were performed in triplicate, and the collected data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 26 (IBM Corp; IBM SPSS Statistics for Windows, ver. 26.0, Armonk, NY). The data were subjected to analysis of variance (ANOVA), and when necessary, mean comparisons were conducted using Tukey's HSD (Honestly Significant Difference) test, with a significance level set at $p < 0.05$.

3. Results

3.1. Suppression of hyphal growth and spore germination in mycotoxin-producing fungi using SKRU-01 VOCs

Fumigation with SKRU-01 VOCs had significant effect ($p < 0.05$) on the mycelial growth and spore germination of aflatoxin-producing fungi (*A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4) (Fig. 1). The stain TISTR 3276 showed stronger inhibition (75.67%) than strain PSRDC-4 (71.33%) (Fig. 1A). Fig. 1B illustrates spore germination inhibition percentages of 70.88% and 67.41% for strains TISTR 3276 and PSRDC-4, respectively. The colony morphology of both strains after treatment with SKRU-01 VOCs is illustrated in Fig. 1C.

3.2. Effects of strain SKRU-01 spore concentrations on VOCs production against both mycotoxin-producing fungi, and identification of the resulting VOCs

Fig. 2 displays the effect of varying concentrations of strain SKRU-01 spores on VOCs production against both TISTR 3276 and PSRDC-4 strains. The results indicate a significant impact ($p < 0.05$) of escalating concentrations of SKRU-01 spores on the mycelial growth of both pathogen strains. At a concentration of 10^6 spores/mL, SKRU-01 VOCs inhibited TISTR 3276 and PSRDC-4 by 71.00% and 64.67%, respectively. Although the inhibitory effect was statistically insignificant ($p > 0.05$) difference at concentrations between 10^6 and 10^7spores/mL (71.00% and 70.33%, respectively). The data suggested that SKRU-01 VOCs have potential as a biocontrol agent against these pathogens.

The VOCs produced by the SKRU-01 strain were analyzed using SPME GC-MS. Following a seven-day cultivation period (Table 1), a total of 23 VOCs were detected in the SKRU-01 culture. The identified VOCs showed a high degree of similarity (over 90%) to the compounds listed in the NIST library. These VOCs encompassed diverse categories such as alkenes, aromatic hydrocarbons, alcohols, ketones, alkanes, and acids. Among the detected VOCs, acetophenone constituted the major

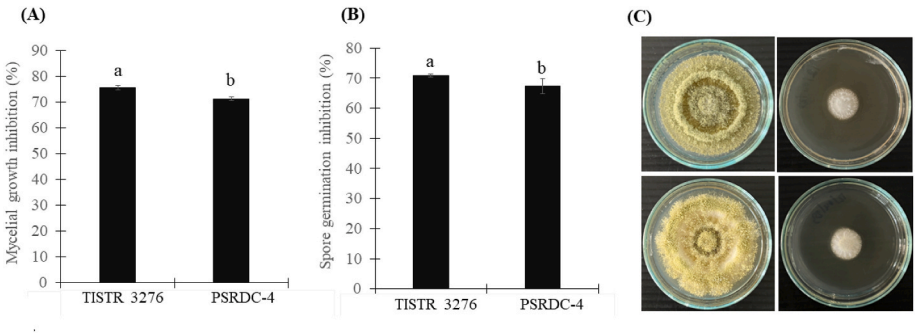


Fig. 1. Inhibition of **(A)** mycelial growth and **(B)** spore germination of *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 by volatile compounds produced by *T. asperelloides* SKRU-01 (SKRU-01 VOCs) after inoculation at 30 °C for five days for mycelial growth and 24 h for spore germination. **(C)** shows the colony morphology of both strains TISTR 3276 and PSRDC-4 after being treated with SKRU-01 VOCs. The presented values represent the means of three replicates (±SD). Different letters above the bars, indicating no statistically significant difference after Tukey's HSD test (ANOVA, $p > 0.05$), were observed for mycelial growth and spore germination of both strains TISTR 3276 and PSRDC-4 when exposed to SKRU-01 VOCs.

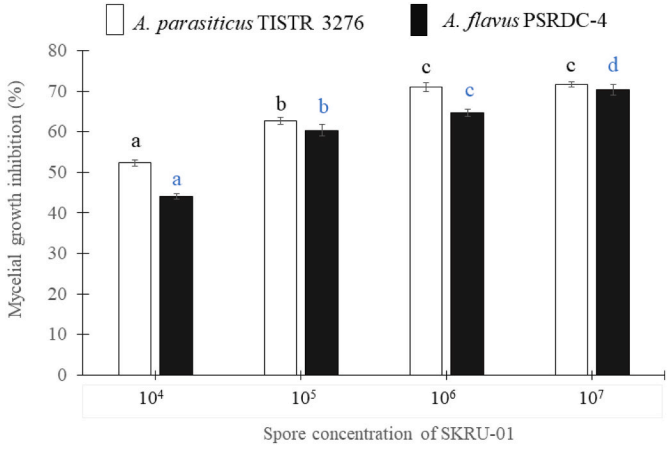


Fig. 2. Effects of *T. asperelloides* SKRU-01 spore concentrations (10^4 to 10^7 spores/mL) on volatile compound production and its impact on mycelial growth of *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 after inoculation at 30 °C for five days. The presented values represent the means of three replicates (±SD). Different letters above the bars, indicating no statistically significant difference after Tukey's HSD test (ANOVA, $p > 0.05$), were observed for mycelial growth of both strains TISTR 3276 and PSRDC-4 when exposed to different spore concentrations of SKRU-01 VOCs.

component, accounting for 14.41% of the total VOCs in the SKRU-01 culture (Fig. 3), followed by linalool (5.43%), phenylethyl alcohol (5.33%), heneicosane (3.97%), and geosmin (2.56%), respectively.

3.3. *In vitro evaluation of the inhibitory effects of selected individually identified VOCs on the growth and spore germination of mycotoxin-producing fungi*

The antifungal activity of four pure VOCs against the mycelial growth of both TISTR 3276 (Table 2A) and PSRDC-4 (Table 2B) strains is presented in Table 2. Table 2 demonstrates the significant inhibitory effect ($p < 0.05$) of all four pure VOCs on the mycelial growth of both pathogenic strains, with only linalool and acetophenone achieving complete inhibition (100%).

Table 2 presents the antifungal activity of four pure VOCs against the spore germination of both TISTR 3276 (Table 2A) and PSRDC-4 (Table 2B) strains. The four VOCs demonstrated significant inhibition ($p < 0.05$) of spore germination of both pathogenic strains on PDA plates. Linalool and acetophenone fumigation exhibited complete inhibition (100%) on the germination of both strains. Geosmin displayed lower activity, with similar inhibitions (57.79%–58.18%). These viability results aligned with the inhibitory effects observed on mycelial growth.

A verification trial substantiated the absence of mycelial growth or spore germination in both pathogenic strains when exposed to acetophenone or linalool treatments on agar plates, resulting in complete inhibition (100%). Removing pure VOCs from the bioassay indicates that acetophenone could kill both strains TISTR 3276 and PSRDC-4, while linalool could only inhibit their growth. Therefore, only acetophenone was selected for further studies, as it demonstrated efficacy against both strains TISTR 3276 and PSRDC-4.

Table 1
Tentative identification of volatile organic compounds produced by *T. asperelloides* SKRU-01 using SPME GC-MS analysis.

No.	R_T (min) [a]	Possible compound	Formula	Match Factor (%) [b]	Area (%)
1	1.66	Acetaldehyde	C_4H_8O	92.3	1.03
2	2.57	Heneicosane	$C_{21}H_{44}$	90.3	3.97
3	3.55	Isobutyric acid	$C_6H_{12}O_2$	91.2	0.93
4	4.08	3-Pentanone	$C_6H_{12}O$	95.6	0.58
5	5.80	Butanoic acid	$C_7H_{14}O_2$	90.1	0.55
6	7.67	Pentanoic acid	$C_8H_{16}O_2$	90.7	0.47
7	8.65	β-Myrcene	$C_{10}H_{16}$	90.8	0.56
8	9.75	d-Limonene	$C_{10}H_{16}$	92.2	0.10
9	10.11	3-Octanon	$C_9H_{18}O$	91.6	0.84
10	10.82	Isoamylalcohol	$C_5H_{12}O$	94.3	0.73
11	11.72	2-Heptanone	$C_8H_{16}O$	90.6	0.37
12	14.34	4D-Methylhexanoic acid ethyl ester	$C_9H_{18}O_2$	90.3	0.16
13	21.83	2-Furanmethanol	$C_{10}H_{18}O_2$	97.7	1.25
14	26.14	Acetophenone	C_8H_8O	94.5	14.41
15	28.22	Cyclohexane	$C_{15}H_{24}$	93.9	0.55
16	28.43	Geosmin	$C_{12}H_{22}O$	97.8	2.56
17	29.69	3-Cyclohexene	$C_{10}H_{18}O$	96.2	1.76
18	31.23	4β-5α-Eremophila	$C_{15}H_{24}$	94.1	0.64
19	32.19	Linalool	$C_{10}H_{18}O$	95.8	5.43
20	31.45	Acetic acid	$C_{10}H_{12}O_2$	95.7	2.01
21	33.18	Benzyl alcohol	C_7H_8O	94.5	0.63
22	33.80	Benzeneethanol	$C_8H_{10}O$	96.9	1.16
23	34.29	Phenylethyl alcohol	$C_8H_{10}O$	90.8	5.33

Note.
[a] R_T, retention time.
[b] Volatile organic compounds produced by the strain SKRU-01 were detected with a match factor of less than 90%. These compounds are not included in this table.

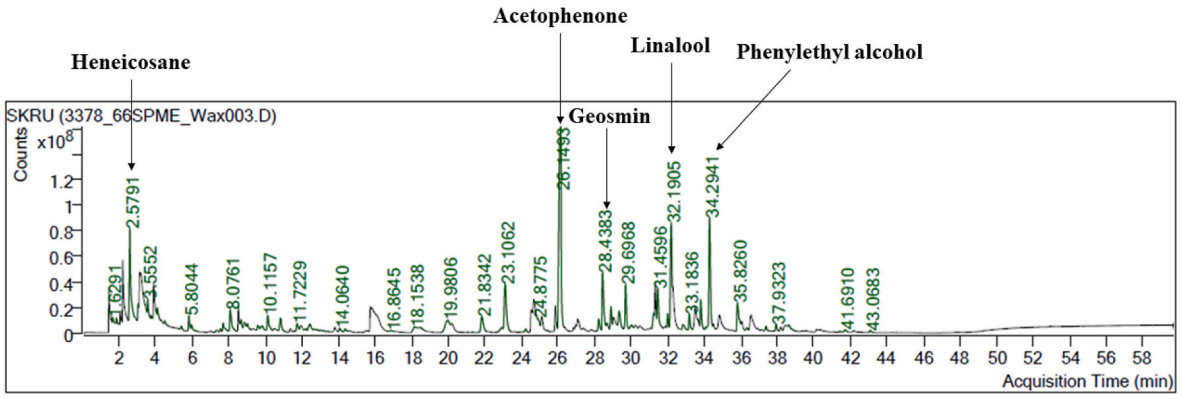
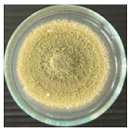
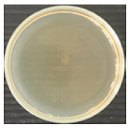
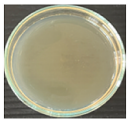
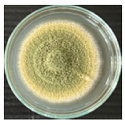
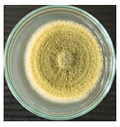


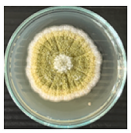
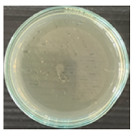
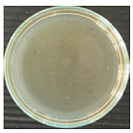
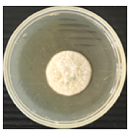
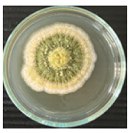
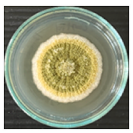
Fig. 3. Mass spectra illustrating the volatile organic compounds (VOCs) emitted by *T. asperelloides* SKRU-01, as detected through SPME GC-MS analysis of a 7-day-old culture. The spectra highlight the presence of five major VOCs.

Table 2
Effects of selected pure volatile organic compounds on the growth of **(A)** *A. parasiticus* TISTR 3276 and **(B)** *A. flavus* PSRDC-4 after an incubation at 30 °C for 5 days.

(A)

Parameters	Pure compounds					
	Control	Linalool	Acetophenone	Phenylethyl alcohol	Heneicosane	Geosmin
Radial growth (cm)	4.57^{a} ± 0.09	0.00^{d} ± 0.00	0.00^{d} ± 0.00	2.60^{c} ± 0.43	4.03^{b} ± 0.05	3.97^{b} ± 0.05
Inhibition of radial growth (%)	–	100 ± 0.00	100 ± 0.00	43.07 ± 4.32	11.68 ± 0.71	13.14 ± 0.47
Colony morphology						
Spore germination inhibition (%)	–	100^{a} ± 0.00	100^{a}± 0.00	67.50^{b} ± 7.79	6.82^{d} ± 0.57	58.18^{c} ± 3.21
[a] Role of pure compounds	–	Inhibited	Killed	Inhibited	Inhibited	Inhibited

(B)

Parameters	Pure compounds					
	Control	Linalool	Acetophenone	Phenylethyl alcohol	Heneicosane	Geosmin
Radial growth (cm)	3.58^{a} ± 0.05	0.00^{c} ± 0.00	0.00^{c} ± 0.00	2.58^{b} ± 0.62	3.28^{a} ± 0.05	3.20^{a} ± 0.08
Inhibition of radial growth (%)	–	100 ± 0.00	100 ± 0.00	27.97 ± 1.85	8.39 ± 0.50	10.49 ± 0.82
Colony morphology						
Spore germination inhibition (%)	–	100^{a} ± 0.00	100^{a}± 0.00	65.40^{b} ± 2.27	12.80^{d} ± 1.63	57.79^{c} ± 7.21
[a] Role of pure compounds	–	Inhibited	Killed	Inhibited	Inhibited	Inhibited

Note: Reported values represent means ± standard deviation from three replicates. Data followed by the same letter within each column show no statistically significant difference after Tukey's HSD test (ANOVA, $p > 0.05$).

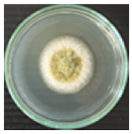
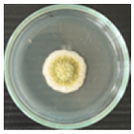
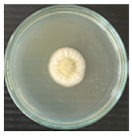
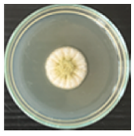
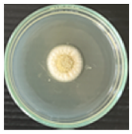
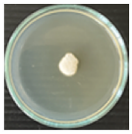
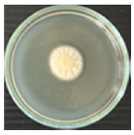
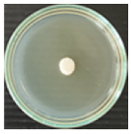
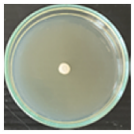
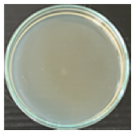
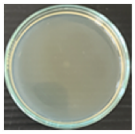
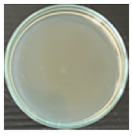
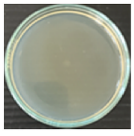
[a] Viability of both pathogenic fungi was determined five days after removing the acetophenone or phenylethyl alcohol.

3.4. Determining the minimum inhibitory concentration (MIC) of acetophenone against mycotoxin-producing fungi in solid media and vapor phase

The inhibitory concentration of acetophenone in the mycelial growth of both TISTR 3276 and PSRDC-4 strains was tested using a solid medium bioassay, and the results are presented in Table 3. Increasing the concentrations of acetophenone significantly ($p < 0.05$) inhibited the mycelial growth of both pathogen strains. Acetophenone strongly inhibited the mycelial growth of both pathogens at 24 h with concentrations of 0.80 μL/mL (inhibition percentages greater than 79%). At 48 h, both strains TISTR 3276 and PSRDC-4 exhibited continued growth, resulting in lower inhibition percentages (approximately 73.47% and 75.90%, respectively). However, mycelial growth was completely inhibited by acetophenone at concentrations of 1.0 and 1.2 μL/mL for the entire 48-h duration. Consequently, the minimum inhibitory concentration (MIC) of acetophenone on solid media for both pathogenic strains was determined to be 1.0 μL/mL.

The inhibitory concentration of acetophenone on the mycelial growth of both fungal strains was further validated through a vapor-phase bioassay, and the results are presented in Table 4. The vapor-phase bioassay demonstrated significant ($p < 0.05$) inhibition of mycelial growth in both pathogens as the concentration of acetophenone increased. At 0.80 μL/mL for 24 h, acetophenone exhibited higher inhibition on strain TISTR 3276 (84.85%) than strain PSRDC-4 (67.78%). However, at 48 h, growth of the two strains was continued, resulting in

Table 3
Antifungal activity of acetophenone concentrations in solid media assay against *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 following a 48-h incubation at 30 °C.

Acetophenone concentrations (μL/mL)	Inhibition of Radial growth (%)					
	A. parasiticus TISTR 3276			*A. flavus* PSRDC-4		
	24 h	48 h	Colony morphology (48 h)	24 h	48 h	Colony morphology (48 h)
0	–	–		–	–	
0.2	$47.92^{e} \pm 2.63$	$18.37^{e} \pm 0.82$		$10.87^{e} \pm 0.50$	$3.61^{e} \pm 0.50$	
0.4	$58.33^{d} \pm 1.65$	$37.76^{d} \pm 0.50$		$26.09^{d} \pm 1.00$	$20.48^{d} \pm 1.29$	
0.6	$65.33^{c} \pm 0.68$	$47.68^{c} \pm 0.50$		$56.52^{c} \pm 3.58$	$32.53^{c} \pm 1.41$	
0.8	$79.17^{b} \pm 1.00$	$73.47^{b} \pm 0.58$		$85.78^{b} \pm 0.50$	$75.90^{b} \pm 0.00$	
1.0	$100^{a} \pm 0.00$	$100^{a} \pm 0.00$		$100^{a} \pm 0.00$	$100^{a} \pm 0.00$	
1.2	$100^{a} \pm 0.00$	$100^{a} \pm 0.00$		$100^{a} \pm 0.00$	$100^{a} \pm 0.00$	

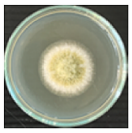
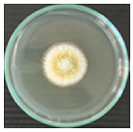
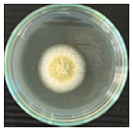
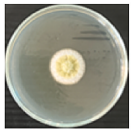
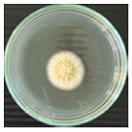
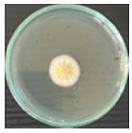
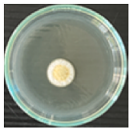
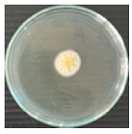
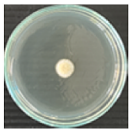
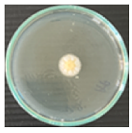
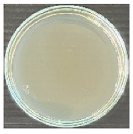
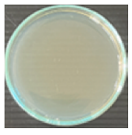
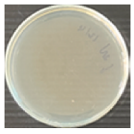
Note: Reported values represent means ± standard deviation from three replicates. Data followed by the same letter within each column show no statistically significant difference after Tukey's HSD test (ANOVA, $p > 0.05$).

lower inhibition values (70.34% and 57.50%, respectively). Complete inhibition of mycelial growth was achieved at concentrations of 1.0 and 1.2 μL/mL for a duration of 48 h. Therefore, the MIC of acetophenone was confirmed as 1.0 μL/mL (solid media assay) or μL/L (vapor phase assay) for both pathogenic strains.

3.5. Comparison of the biofungicidal ability of SKRU-01 VOCs and acetophenone for inhibiting the growth of mycotoxin-producing fungi on peanuts

Peanuts infected with both TISTR 3276 (Table 5A) and PSRDC-4 strains (Table 5B) were utilized to assess the impact of SKRU-01 VOCs and its primary component, acetophenone, on the prevention of aflatoxin contamination in peanuts (Fig. 4). The results in growth of both pathogen strains were significantly declined ($p < 0.05$) compared to the controls (Table 5). The inhibitory effect of SKRU-01 VOCs on growth was dose-dependent and showed greater efficacy than acetophenone (at 0.5 μL/L (1/2 MIC)) in inhibiting spore reduction and total AFB_1 production of both strains TISTR 3276 (83.66% and 19.68 μg/kg, respectively) and PSRDC-4 (90.59% and 15.34 μg/kg, respectively). Acetophenone also exhibited a dose-dependent inhibitory effect on the growth of both TISTR 3276 and PSRDC-4 strains, albeit with a lower

Table 4
Antifungal activity of acetophenone concentrations in vapor phase assay against *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 following a 48-h incubation at 30 °C.

Acetophenone concentrations (μL/L)	Inhibition of Radial growth (%)					
	A. parasiticus TISTR 3276			*A. flavus* PSRDC-4		
	24 h	48 h	Colony morphology (48 h)	24 h	48 h	Colony morphology (48 h)
0	–	–		–	–	
0.2	29.00^{e} ± 0.86	24.24^{e} ± 1.57		26.26^{e} ± 0.56	21.88^{e} ± 1.04	
0.4	49.49^{d} ± 0.29	40.00^{d} ± 3.77		40.00^{d} ± 1.98	34.38^{e} ± 0.50	
0.6	67.68^{c} ± 0.29	62.76^{c} ± 3.07		60.00^{c} ± 0.50	46.25^{d} ± 1.04	
0.8	84.85^{b} ± 0.50	70.34^{b} ± 1.89		67.78^{b} ± 0.29	57.50^{c}± 1.76	
1.0	100^{a} ± 0.00	100^{a} ± 0.00		100^{a} ± 0.00	100^{b} ± 0.00	
1.2	100^{a} ± 0.00	100^{a} ± 0.00		100^{a} ± 0.00	100^{a} ± 0.00	

Note: Reported values represent means ± standard deviation from three replicates. Data followed by the same letter within each column show no statistically significant difference after Tukey's HSD test (ANOVA, $p > 0.05$).

efficacy. After treatment with a dose of 1.0 μL/L (MIC) and storage for 5 days, the peanuts were completely protected against contamination from the strain PSRDC-4. However, a dose of 2.0 μL/L (2 MIC) was required to prevent contamination from the strain TISTR 3276 during storage.

3.6. Action mechanism of acetophenone on the antifungal and antiaflatoxic properties against two mycotoxin-producing fungal strains

The presence of acetophenone led to a significant ($p < 0.05$) reduction in the mycelial dry weights of both aflatoxin-producing fungal strains (Table 6). Remarkably, acetophenone exhibited stronger antifungal activity against the PSRDC-4 strain than the TISTR 3276 strain. At the MIC, acetophenone demonstrated more than 89% inhibition on the growth of both strains (89.33% and 93.04%, respectively) and achieved complete inhibition (100%) of both aflatoxin-producing fungal strains at 2 MIC (Table 6A). Consequently, fungal cells at control, 1/2 MIC, and MIC concentrations were utilized to investigate the mechanisms underlying the antifungal and antiaflatoxigenic properties.

3.6.1. Acetophenone-induced changes in plasma membrane ergosterol levels the two fungal strain

The effect of different acetophenone concentrations on membrane ergosterol biosynthesis in cells of both TISTR 3276 and PSRDC-4 strains

Table 5
Comparison the biocontrol efficacy of SKRU-01 VOCs with acetophenone (1/2 MIC, MIC, and 2 MIC) to inhibit spore and aflatoxin formation by **(A)** *A. parasiticus* TISTR 3276 and **(B)** *A. flavus* PSRDC-4 growing in peanuts after five days incubation at 30 °C.

(A)

Treatments	Viable spores (log spores/g)	Spore reduction (%)	AFB_1 production (μg/kg)
Control	$5.63^{a} \pm 1.02$	–	$158.61^{a} \pm 3.45$
SKRU-01 VOCs	$0.92^{c} \pm 0.51$	83.66	$19.68^{c} \pm 2.68$
1/2 MIC	$2.78^{b} \pm 0.92$	50.62	$78.59^{b} \pm 1.49$
MIC	$0.22^{d} \pm 0.12$	96.09	$12.16^{d} \pm 2.41$
2 MIC	ND^{e}	100	< LOD^{e}

(B)

Treatments	Viable spores (log spores/g)	Spore reduction (%)	AFB_1 production (μg/kg)
Control	$4.89^{a} \pm 1.05$	–	$163.47^{a} \pm 3.89$
SKRU-01 VOCs	$0.46^{c} \pm 0.13$	90.59	$15.34^{c} \pm 1.02$
1/2 MIC	$1.38^{b} \pm 0.21$	71.78	$59.57^{b} \pm 2.98$
MIC	ND^{d}	100	< LOD^{d}
2 MIC	ND^{d}	100	< LOD^{d}

Note: Reported values represent means ± standard deviation from three replicates. Data followed by the same letter within each column show no statistically significant difference after Tukey's HSD test (ANOVA, $p > 0.05$). ND = not detected. Limit of detection (LOD) = 0.9 μg/kg. Acetophenone was added to the filter paper in mouth-to-mouth plates to reach concentrations of 0.5 μL/L (1/2 MIC), 1.0 μL/L (MIC) and 2.0 μL/L (2 MIC), respectively, and the plates were sealed with a sealing film and incubated at 30 °C for five days.

was examined, and the results are summarized in Table 6. Acetophenone exhibited a significant ($p < 0.05$) dose-dependent inhibition of ergosterol biosynthesis in both strains. Ergosterol reduction in the TISTR 3276 strain treated with 1/2 MIC and MIC concentrations of acetophenone was 45.90% and 50.10%, respectively, while in the PSRDC-4 strain, it was 59.71% and 63.85%, respectively (Table 6B).

3.6.2. Acetophenone-induced modulation of cellular methylglyoxsal levels

In Fig. 5, it is evident that the levels of methylglyoxal (MG) in both the TISTR 3276 and PSRDC-4 strains cultured under 1/2 MIC and MIC acetophenone exposure were significantly lower than the control samples. In the control group, the MG level for both strains, TISTR 3276 and PSRDC-4, was measured at 2018.93 μM/g FW and 1899.89 μM/g FW, respectively. Notably, a significant ($p < 0.05$) reduction was observed at the MIC concentration of acetophenone, resulting in MG levels of 1514.20 μM/g FW and 1526.44 μM/g FW for the respective strains.

3.6.3. Effects of acetophenone on the enzymatic and non-enzymatic defense systems

The analysis of ROS level, SOD level, CAT level, reduced GSH activity, oxidized GSSG activity, and GSH/GSSG ratio in both the TISTR 3276 and PSRDC-4 strains, treated with 1/2 MIC and MIC concentrations of acetophenone, is presented in Fig. 6. Treatment with various concentrations of acetophenone led to a significant ($p < 0.05$) elevation in the production of ROS, SOD, CAT, GSH, and the GSH/GSSG ratio compared to the control. In Fig. 6A, the control ROS levels in the PSRDC-

Table 6
Influence of acetophenone at concentrations of 0.5 μL/mL (1/2 MIC), 1.0 μL/mL (MIC), and 2.0 μL/mL (2 MIC) on **(A)** percent growth inhibition and **(B)** percent inhibition of ergosterol production in *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 cultured in potato dextrose broth (PDB) at 30 °C for five days.

(A)

Treatments	*A. parasiticus* TISTR 3276		*A. flavus* PSRDC-4	
	Mycelial dry weights (mg)	Mycelial growth inhibition (%)	Mycelial dry weights (mg)	Mycelial growth inhibition (%)
Control	$227.95^{a} \pm 7.42$	–	$178.80^{a} \pm 14.99$	–
1/2 MIC	$121.25^{b} \pm 3.89$	46.80	$82.10^{b} \pm 7.35$	54.08
MIC	$24.30^{c} \pm 6.08$	89.33	$12.45^{c} \pm 1.06$	93.04
2 MIC	$0^{d} \pm 0.00$	100	$0^{d} \pm 0.00$	100

(B)

Treatments	Mean of percentage inhibition of ergosterol production ± SD	
	A. parasiticus TISTR 3276	*A. flavus* PSRDC-4
0 (Control)	$0.00^{c} \pm 0.00$	$0.00^{c} \pm 0.00$
1/2 MIC	$45.90^{b} \pm 0.07$	$59.71^{b} \pm 0.02$
MIC	$50.10^{a} \pm 0.06$	$63.85^{a} \pm 0.11$

Note: Reported values represent means ± standard deviation from three replicates. Data followed by the same letter within each column show no statistically significant difference after Tukey's HSD test (ANOVA, $p > 0.05$).
At 2 MIC, complete inhibition (100%) of fungal cells was observed in both pathogenic fungi, and it was not used to examine ergosterol production.

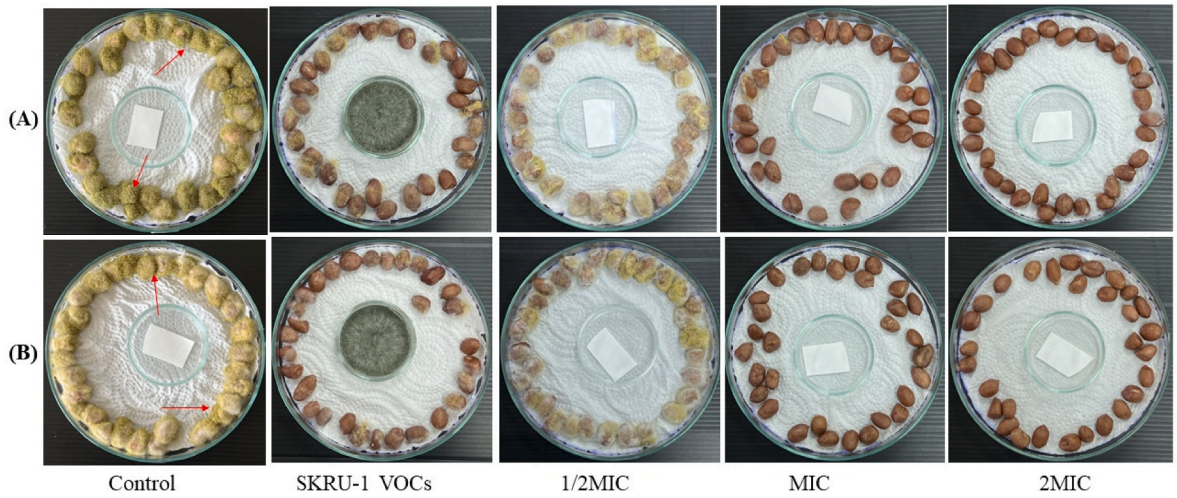


Fig. 4. Growth of **(A)** *A. parasiticus* TISTR 3276 and **(B)** *A. flavus* PSRDC-4 in peanuts fumigated with sterile distilled water (control), volatile compounds produced by *T. asperelloides* SKRU-01 (SKRU-01 VOCs), and acetophenone at concentrations of 0.5 μL/mL (1/2 MIC), 1.0 μL/mL (MIC), and 2.0 μL/mL (2 MIC) after storage at 30 °C for five days. The arrow indicates the presence of dark green spores.

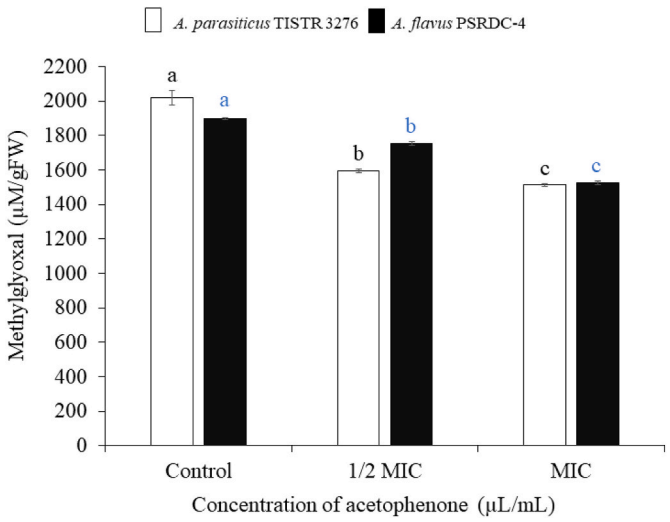


Fig. 5. The impact of acetophenone on cellular methylglyoxal (MG) levels in *Aspergillus parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 cells cultured in potato dextrose broth (PDB) at 30 °C for five days. Acetophenone concentrations of 0.5 µL/mL (1/2 MIC), and 1.0 µL/mL (MIC) were tested. The presented values represent the means of three replicates (±SD). Different letters above the bars, indicating no statistically significant difference after Tukey's HSD test (ANOVA, $p > 0.05$), were observed for MG levels of both strains TISTR 3276 and PSRDC-4 when exposed to different concentrations of acetophenone.

4 and TISTR 3276 strains were 2.49 and 1.44 unit/min/mg protein, respectively. Treatment with MIC acetophenone resulted in a substantial ($p < 0.05$) increase in the ROS level. Specifically, the ROS level reached 5.12 and 1.63 unit/min/mg protein for the PSRDC-4 and TISTR 3276 strains, respectively.

Fig. 6B and C illustrate that acetophenone significantly ($p < 0.05$) elevated the levels of cellular antioxidant enzymes, namely SOD and CAT. In the absence of acetophenone (control), the levels of SOD and CAT in the TISTR 3276 and PSRDC-4 strains were 4.82 and 0.90 unit/min/mg protein (for SOD) and 81.26 and 31.14 unit/min/mg protein (for CAT), respectively. However, upon treatment with acetophenone at MIC, the levels of SOD and CAT noticeably increased to 9.70 and 3.07 unit/min/mg protein (for SOD) and 109.27 and 34.78 unit/min/mg protein (for CAT) in the respective strains.

Following exposure to acetophenone, significant ($p < 0.05$) changes were observed in the levels of GSH, GSSG, and GSH/GSSG ratio in both PSRDC-4 and TISTR 3276 strains. Fig. 6D and F illustrate that the GSH and GSH/GSSG ratio in the treated groups were significantly higher compared to the control groups. For instance, in the control and MIC acetophenone treatment, the GSH levels of the TISTR 3276 strain increased from 4.34 to 9.07 µM/mg protein, and those of the PSRDC-4 strain increased from 6.19 to 7.98 µM/mg protein. The observed dose-dependent increase in GSH and GSH/GSSG levels suggests an enhanced mechanism in the fungal cells to counteract oxidative stress (Das et al., 2020). However, unlike other enzyme activities, the cellular GSSG activity decreased as the concentration of acetophenone increased (Fig. 6E). Our study revealed significant changes in the levels of GSH, GSSG, and the GSH/GSSG ratio in response to acetophenone exposure in both PSRDC-4 and TISTR 3276 strains. These findings suggest an enhanced mechanism within fungal cells to counteract oxidative stress. However, GSSG activity showed a contrasting response. This sheds light on the intricate cellular responses to acetophenone-induced stress.

4. Discussion

This paper discusses the need for safe and eco-friendly control agents against *Aspergillus* contamination in crops, highlighting the research on *T. asperelloides* SKRU-01 VOCs, specifically acetophenone, to mitigate peanut deterioration caused by aflatoxigenic *Aspergillus* species. Even without direct contact, the VOCs emitted by the SKRU-01 strain inhibit

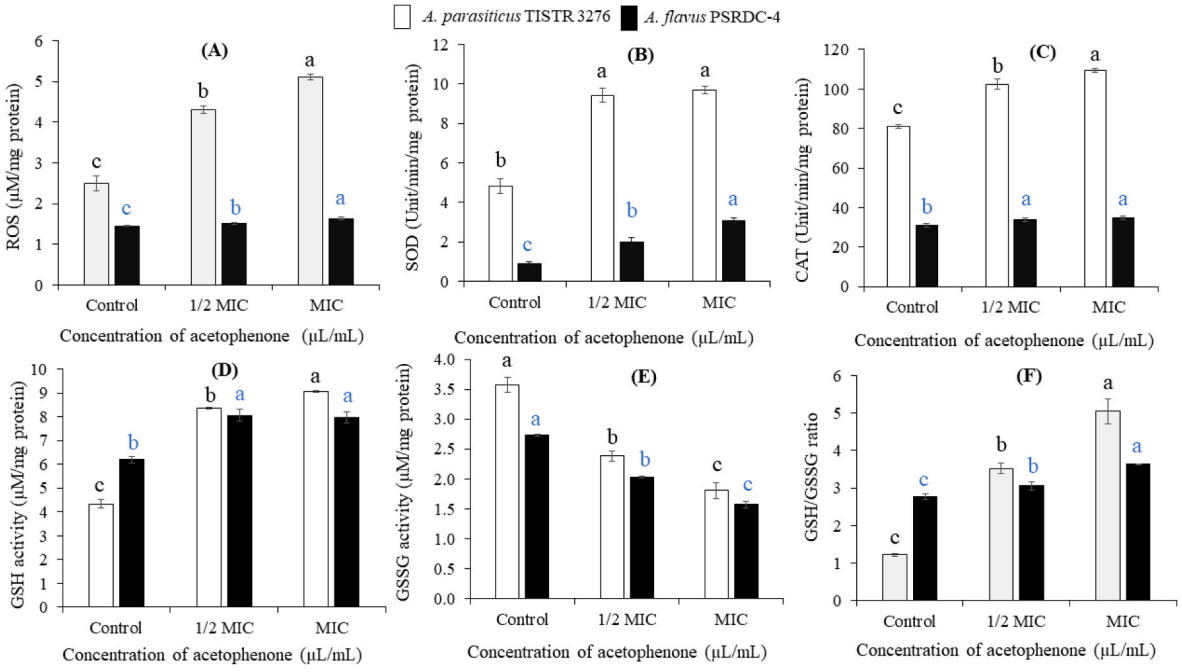


Fig. 6. The impact of acetophenone on cellular reactive oxygen species (ROS) level **(A)**, superoxide dismutase (SOD) level **(B)**, catalase (CAT) level **(C)**, reduced glutathione (GSH) activity **(D)**, oxidized glutathione (GSSG) activity **(E)**, and GSH/GSSG ratio **(F)** of *Aspergilllus parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 cells cultured in potato dextrose broth (PDB) at 30 °C for five days. Acetophenone concentrations of 0.5 µL/mL (1/2 MIC), and 1.0 µL/mL (MIC) were tested. The presented values represent the means of three replicates (±SD). Different letters above the bars, indicating no statistically significant difference after Tukey's HSD test (ANOVA, $p > 0.05$), were observed for ROS, SOD, CAT, GSH, GSSG, GSH/GSSG levels of both strains TISTR 3276 and PSRDC-4 when exposed to different concentrations of acetophenone.

the hyphal growth and spore germination of *A. flavus* and *A. parasiticus*. These findings are consistent with previous research, reinforcing Trichoderma's ability to produce VOCs that are effective against a variety of pathogens. *Trichoderma*-derived VOCs are a promising biofumigant for preventing fungal deterioration in food and feed, according to multiple studies (Barakat et al., 2014; Intana et al., 2021; Li et al., 2018; Rajani et al., 2020; Ruangwong et al., 2021; You et al., 2022).

In this study, the analysis of VOCs emitted by the SKRU-01 strain identified five compounds, including linalool, acetophenone, phenylethyl alcohol, heneicosane, and geosmin, with potent antifungal properties. Some of these compounds, including linalool, phenylethyl alcohol, and geosmin, have been previously identified as antifungal agents in other *Trichoderma* isolates. Utilizing the primary antifungal constituents, namely acetophenone, phenylethyl alcohol, geosmin, and henicosane, derived from the SKRU-01 strain could provide a more efficient strategy for the development of natural fungicides. Among the identified VOCs, acetophenone exhibited significantly increased antifungal activity, effectively eliminating both pathogenic strains. Solid media and vapor phase assays confirmed that it significantly inhibited hyphal growth in both strains. Even at modest concentrations of 1.0 μL/mL (solid media assay) or 1.0 μL/L (vapor phase assay), acetophenone inhibited the growth of both fungi completely. This method is especially promising for preventing the spread of *Aspergillus* species during the storage of agricultural products. In addition to *Cytospora* sp., *B. cinerea*, and *Magnaporthe grisea*, investigations have demonstrated acetophenone's efficacy as an antifungal agent against other pathogens, such as *B. cinerea* and *M. grisea* (Dan et al., 2018). Additionally, fumigation of chili fruit with 100 μL/L acetophenone for 12 h completely inhibited the growth of *Colletotrichum gloeosporioides* PSU-NY8 over a 12-day incubation period (Boukaew et al., 2018). These results highlight the significance of acetophenone as the primary antifungal component of the SKRU-01 strain, indicating its potential application in controlling various fungal infections (Boukaew et al., 2018; Brilli et al., 2019; Dan et al., 2018; La Guerche et al., 2005; Li et al., 2022; Prakash et al., 2015; Wang et al., 2013a).

Microbial volatiles have a number of applications in the storage and preservation of fruits, vegetables, and cereals. The volatile organic compounds of *Corrallococcus* sp. were effective at protecting citrus from *P. digitatum* deterioration (Ye et al., 2020). Similarly, a 6-h fumigation of postharvest chili fruit with *S. philanthi* RM-1-138 VOCs eradicated anthracnose disease during a 12-day incubation period (Boukaew et al., 2018). The VOCs produced by *S. philanthi* RL-1-178 served as a biofumigant against aflatoxin-producing fungi in soybean seeds that had been preserved (Boukaew & Prasertsan, 2020b). Additionally, *Enterobacter asburiae* Vt-7, in conjunction with 1-pentanol and phenylethyl alcohol, effectively inhibited the proliferation of aflatoxin-producing fungi and prevented aflatoxin formation in stored peanuts (Gong, Dong, et al., 2019). The study on stored peanuts revealed that acetophenone produced by the SKRU-01 strain exhibited intensive antagonistic activity by inhibiting fungal growth (mold density) and decreasing aflatoxin B_1 production. The SKRU-01 VOCs could provide a more effective biocontrol against mycotoxin-producing fungi, potentially reducing mycotoxin-induced food spoilage. However, it's important to note that the utilization of both VOCs and isolated pure compounds offers viable approaches in the field of food preservation. These two approaches can be complementary, and the choice between them depends on specific preservation goals, such as the type of food product, desired shelf life, and the spectrum of microorganisms to be controlled. These findings are in line with previous studies that have demonstrated the effectiveness of fumigation with various microbial volatiles, such as *B. subtilis* CL2 (Ling et al., 2022), *S. yanglinensis* 3-10 (Lyu et al., 2020), *E. asburiae* Vt-7 (Gong, Dong, et al., 2019), and *Alcaligenes faecalis* N1-4 (Gong, Wu, et al., 2019), in preventing food spoilage caused by *Aspergillus* spp. in grains. The volatile acetophenone from strain SKRU-01 could serve as novel biocontrol agent for managing food and crop pathogens and mitigating mycotoxins in storage.

AFB_1 is recognized as the most harmful toxin among all aflatoxins. Aflatoxigenic strains, specifically *A. parasiticus* and *A. flavus*, are responsible for the production of this mycotoxin, which poses substantial health hazards to both animals and humans (Awuchi et al., 2021). The results revealed that fumigants containing SKRU-01 and the main volatile constituent acetophenone significantly inhibited the production of AFB_1 on peanuts by both pathogenic strains. Notably, all regimens exhibited anti-aflatoxigenic properties, with the exception of the 1/2 MIC of acetophenone. The levels of AFB_1 production were determined to be below the public health concern threshold of 20 μg/kg. It is important to note that the maximum levels of total aflatoxin in food and feed can vary among different countries. For example, permissible levels of aflatoxins in foods vary by country and food type. In Thailand, food products are permitted to contain up to 20 μg/kg of total aflatoxins (Panrapee et al., 2016). In the United States, the Food and Drug Administration (FDA) has set stringent regulations limiting the AFB_1 content in maize and maize products to a maximum of 20 μg/kg (Wang et al., 2023b European Union has instituted stricter standards, establishing maximum levels of 4 μg/kg and 2 μg/kg for total AFs and AFB_1 in peanuts, respectively (European Commission, 2010). The SKRU-01 VOCs exhibit significant antifungal and antiaflatoxigenic properties, effectively preventing food deterioration in *in vitro* and in peanuts caused by aflatoxin-producing fungi. This demonstrates that SKRU-01 VOCs have the potential to be effective natural biofumigation fungicides.

To gain additional understanding, the impact of acetophenone on the enzymatic and non-enzymatic antioxidant defense systems of fungal cells was also investigated. The growth tests revealed significant differences in the dry cell weight of both pathogen strains when exposed to varying concentrations (1/2 MIC to 2 MIC) of acetophenone, demonstrating its essential function in inhibiting the growth of these pathogens. In order to comprehend the underlying mechanisms, this study examined the effect of acetophenone on membrane ergosterol in fungi. Inhibition of ergosterol biosynthesis ranged between 50.10% and 63.85%, indicating a direct interaction between acetophenone and key enzymes implicated in ergosterol biosynthesis. This finding suggests that acetophenone targets the plasma membrane of fungi, inhibiting ergosterol biosynthesis and resulting in membrane integrity changes. These results are consistent with previous research by Li et al. (2022), which demonstrated the reduction of ergosterol levels via the targeted action of linalool on a key enzyme in ergosterol biosynthesis. These results strongly support the hypothesis that the plasma membrane is the principal target of acetophenone's antifungal activity. This study provides valuable insights into the inhibitory effects and underlying mechanisms of acetophenone's antifungal activity against aflatoxins-producing fungi. These findings have implications for prospective applications in fungus control and mycotoxin contamination mitigation.

Acetophenone was more effective than the control at inhibiting the biosynthesis of MG at 1/2 MIC and MIC concentrations. This inhibition of MG biosynthesis provides a novel mechanism of action with antiaflatoxigenic effects, which may be associated with the impairment and downregulation of the *afl*-R and *nor*-1 genes. MG is a cytotoxic byproduct of glycolysis and a substrate that induces AFB_1 in *A. flavus* cells. According to Chaudhari et al. (2020), it stimulates the production of ROS by affecting glutathione levels. These findings are consistent with a study conducted by Upadhyay et al. (2018), which demonstrated that *Cistus ladanifer* essential oil inhibits MG biosynthesis.

The observed alterations in GSH and GSSG concentrations, as well as the decrease in GSSG activity, indicate that acetophenone has the potential to influence the protein and nucleic acid profiles of aflatoxin-producing fungal cells. The presence of MG as a cellular stress marker during mycotoxin biosynthesis results in the upregulation of important regulatory genes, such as *afl*-R and *nor*-1, which promote AFB_1 production (Chen et al., 2004). The decrease in MG biosynthesis observed after treatment with acetophenone may be attributable to a dose-dependent decrease in AFB_1 production via the down-regulation of

multiple aflatoxin regulatory genes, including *nor*-1 and *afl*-R. In addition, the inhibition of cellular MG by the bioactive components of acetophenone demonstrates its potential for developing aflatoxin-resistant food crops using environmentally benign transgenic technology.

ROS accumulation is linked to apoptosis and perturbation of cellular oxygen metabolism (Hwang et al., 2012). On the basis of this knowledge, it was hypothesized that the elevated ROS levels induced by acetophenone treatment could result in apoptosis in TISTR 3276 and PSRDC-4 cells. Results demonstrated that at 1/2 MIC and MIC concentrations, acetophenone significantly increased ROS levels in comparison to the control. In addition to nucleic acid fragmentation, cellular tissue enlargement, chromatin fibril condensation, ATP pool depletion, inhibition of mitochondrial ATPase activity, and degradation of the phosphatidylserine lipid layer, elevated levels of ROS can have detrimental effects. Yoo et al. (2005) report that these effects can ultimately result in cell death or apoptosis. In addition, antioxidant enzymes such as SOD and CAT were more active in cells treated with acetophenone. The increased antioxidant activity observed after treatment with acetophenone alleviated oxidative stress, resulting in a dose-dependent decrease in AFB_1 content. Treatment with acetophenone also increased cellular SOD levels, which may influence reactive hydroxyl radicals via its dehydrogenase activity, thereby disrupting the mitochondrial respiratory system and inhibiting AFB_1 biosynthesis (Furukawa & Sakuda, 2019). In addition, acetophenone treatment altered the levels of GSH, GSSG, and the ratio of GSH/GSSG. The distribution of thiol groups among cellular organelles influences the interconversion of GSH and GSSG, modulating the cell's redox state and overall oxidative defense capacity (Sies & Jones, 2020). According to previous research by Grintzalis et al. (2014), reducing oxidative stress can inhibit AFB_1 biosynthesis by modulating GSH/GSSG activity. These findings are consistent with this theory.

5. Conclusion

This study demonstrated the effective control of *A. flavus* and *A. parasiticus* growth in stored peanuts using VOCs from *T. asperelloides* SKRU-01. *In vitro*, studies identified acetophenone as the main constituent responsible for the antifungal activity of VOCs against both fungal strains. The mechanism of action of acetophenone includes the inhibition of cellular ergosterol biosynthesis on the plasma membrane, reduction in methylglyoxal (aflatoxin inducer), confirming a novel antiaflatoxigenic mechanism, and significant impairment of antioxidant defense enzymes (SOD and CAT) and nonenzymatic defense molecules (GSH and GSSG), revealing its biochemical mechanism of action. These findings highlight the potential of acetophenone as an effective antifungal agent against *A. flavus* and *A. parasiticus* and contribute to the understanding of its underlying mechanisms.

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Sawai Boukaew: Funding acquisition, Conceptualization, Data curation, Supervision, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Wanida Petlamul:** Data curation, Formal analysis, Writing – review & editing. **Siriporn Yossan:** Formal analysis, Writing – review & editing. **Sirasit Srinuanpan:** Writing – review & editing. **Karistsapol Nooprom:** Formal analysis, Writing – review & editing. **Zhiwei Zhang:** Formal analysis, Writing – review & editing.

Declaration of competing interest

The authors declare no competing interests regarding the publication of this study.

Data availability

No data was used for the research described in the article.

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