Biocontrol Activity of Volatile-Producing *Bacillus megaterium* and *Pseudomonas protegens* against *Aspergillus flavus* and Aflatoxin Production on Stored Rice Grains

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Abstract   In our previous study, three bacterial strains, *Bacillus megaterium* KU143, *Microbacterium testaceum* KU313, and *Pseudomonas protegens* AS15, were selected as effective biocontrol agents against *Aspergillus flavus* on stored rice grains. In this study, we evaluated the inhibitory effects of the volatiles produced by the strains on *A. flavus* growth and aflatoxin production on stored rice grains. The three strains significantly reduced mycelial growth of *A. flavus* in dual-culture assays compared with the negative control strain, *Sphingomonas aquatilis* KU408, and an untreated control. Of these tested strains, volatiles produced by *B. megaterium* KU143 and *P. protegens* AS15 markedly inhibited mycelial growth, sporulation, and conidial germination of *A. flavus* on agar medium and suppressed the fungal populations in rice grains. Moreover, volatiles produced by these two strains significantly reduced aflatoxin production in the rice grains by *A. flavus*. To our knowledge, this is the first report of the suppression of *A. flavus* aflatoxin production in rice grains using *B. megaterium* and *P. protegens* volatiles.

Keywords   Aflatoxin, *Aspergillus flavus*, *Bacillus megaterium*, Bacterial volatiles, *Pseudomonas protegens*

Rice (*Oryza sativa* L.) is one of the most important food crops worldwide. Stored rice grains are often subjected to contamination by a wide range of fungi, causing significant economic losses and poor grain quality [1]. Fungi belonging to the genera *Aspergillus* and *Penicillium* are the most detected in stored rice grains [2, 3]. Of these fungi, *Aspergillus flavus* is one of the most significant, since it is prevalent on stored food products and produces the harmful aflatoxins. It usually exists as a saprophyte in the soil, where it is associated with decaying organic matters [4]. The xerophilic nature of *A. flavus* allows it to tolerate the dry conditions

found in storage facilities and to dominate the fungal populations found on rice grains [5, 6].

Aflatoxins are a group of secondary metabolites, primarily produced in nature by a few species of the genus *Aspergillus*, and are considered one of the most dangerous mycotoxins, based on their high toxicity to humans and animals that have directly or indirectly consumed contaminated food [7]. Geographical areas with high level of dietary intake of aflatoxins have increased levels of liver disease and cancer [8]. Consumption of food highly contaminated with aflatoxins has caused several outbreaks of aflatoxicosis, including those in western India and Kenya where several hundred individuals were affected and many lost their lives [9, 10]. Hence, management of levels of aflatoxins in stored rice grains is critically important.

One of the most effective methods for controlling aflatoxin levels on stored rice grains is to avoid contamination by eliminating or reducing *A. flavus* populations [3]. Several chemical and physical methods have been applied for the control of *A. flavus* and aflatoxins on stored grains. A number of fungicides, including benomyl, iprodione, and thiabendazole, are used for controlling fungi on grains in storage [11, 12]; however, because of their adverse effects on grain quality and health concerns associated with the residues from chemical application, there is an urgent need to identify alternative, non-toxic approaches to control

fungal and aflatoxin contamination of grains [13]. Biological control is therefore emerging as a promising alternative to chemical control methods, as it offers an effective, environmentally sound approach to the management of fungal and aflatoxin contamination of stored grains. Several biocontrol agents have been tested against *A. flavus* and aflatoxin production on stored grains, including strains of *Bacillus subtilis*, *Rhodococcus erythropolis*, and *Pseudomonas fluorescens*; these microorganisms were reported to inhibit the growth and aflatoxin production of *A. flavus* on grains [14, 15]. Further, several volatile compounds from plants were reported to inhibit aflatoxin production by *Aspergillus* spp. [16, 17].

In our previous study, three potential biocontrol bacterial strains (*Bacillus megaterium* KU143, *Microbacterium testaceum* KU313 and *Pseudomonas protegens* AS15) were selected from a total of 460 bacterial strains [18, 19], isolated from stored rice grains collected from 11 different rice-processing complexes in Korea [2]. These three selected bacterial strains exhibited biocontrol activity against *A. flavus* and aflatoxin production on stored rice grains; in particular, *P. protegens* AS15 could degrade aflatoxin B_1 and utilize it in liquid nutrient-deficient media [19]. The objectives of this study was to evaluate the inhibitory effects of the volatiles produced by these antagonistic bacterial strains on *A. flavus* growth and aflatoxin production on stored rice grains.

To prepare bacterial suspensions, the three bacterial strains, *B. megaterium* KU143, *M. testaceum* KU313, and *P. protegens* AS15, and a negative control strain *Sphingomonas aquatilis* KU408 [19] were streaked on nutrient agar (NA) and incubated at 28°C for 2 days; then, single colonies from each strain culture were transferred to nutrient broth and incubated in a rotary shaker (160 rpm) in the dark at 28°C for 2 days. After incubation, bacterial cells were harvested by centrifugation at 5,000 ×g at for 15 min, suspended in a 10-mM $MgSO_4$ solution, and washed twice with the same solution by centrifugation. Bacterial cells were adjusted to 10^8 cells/mL ($OD_{600} = 0.5$) in 10-mM $MgSO_4$ solution using a spectrophotometer. In addition, *A. flavus* AF57 isolated from stored rice grains [2] and *A. flavus* KCCM 60330 (an aflatoxigenic strain), obtained from the Korean Culture Center of Microorganisms (KCCM; Seoul, Korea) were used in this study. The latter strain was used for assessment of aflatoxin production. Conidial suspensions were prepared by culturing fungal isolates on potato dextrose agar (PDA) for 5 days at 28°C. Conidia were harvested in sterile distilled water (SDW) containing 0.03% Tween 20 and adjusted to 10^7 conidia/mL using a hemocytometer. Stored unhulled rice grains (cv. Ilpum) from Korea University Farm (Namyangju, Korea) were used in this study and surface-sterilized using 1% sodium hypochlorite for 3 min, followed by 70% ethanol for 5 min [20]; then, washed three times in SDW and blotted dry on filter papers (Whatman No. 1) before fungal inoculation.

To test the antifungal activities of the bacterial strains against *A. flavus* AF57, a dual-culture assay was conducted on PDA as previously described [19]. Briefly, bacterial strains or SDW (control) were line-streaked in the center of PDA plates, which were inoculated on the opposite edges with 2 μL of the prepared conidial suspensions. When the mycelia of *A. flavus* AF57 in the control plates almost reached the streaked lines, mycelial inhibition (mm) was measured; at the same time, mycelial growth area (cm^2) was assessed using ImageJ software after photography [21].

To examine the antifungal effects of the volatiles produced by the three bacterial strains on mycelial growth, sporulation, and conidial germination of *A. flavus* AF57, *in vitro* tests were conducted using I-plates (Petri plates 90 mm in diameter, separated in the middle with a partition that allowed only volatiles to pass from one side to the other, without any direct contact between the cultured organisms on either side) (Fisher Scientific, Pittsburgh, PA, USA). For the *in vitro* tests, 100 μL of bacterial suspensions of the test and negative control strains and 10-mM $MgSO_4$ solution (untreated control) were smeared on the NA side of the I-plates and incubated at 28°C. After 24 hr of incubation, 2 μL of the conidial suspension (10^7 conidia/mL) of *A. flavus* AF57 were drop-inoculated onto the opposite side of the I-plates, which contained PDA. Plates were then sealed with Parafilm M (Sigma-Aldrich, St. Louis, MO, USA) and incubated in the dark at 28°C. When mycelia almost reached the center of the control plates, the mycelial growth area (cm^2) of *A. flavus* AF57 on the PDA side of the I-plates was measured, as described above. To assess fungal sporulation, fungal conidia in each I-plate were harvested in SDW containing 0.03% Tween 20 and vortexed for 1 min. Then, numbers of conidia were determined using a hemocytometer; the total numbers of the conidia harvested from each I-plate were divided by the mycelial growth area (cm^2) of the plate. Sporulation was expressed as the log number of conidia per mycelial growth area (cm^2) of each sample. For evaluation of *A. flavus* AF57 conidial germination, conidia from the PDA side of the I-plates were harvested in SDW containing 0.03% Tween 20. The harvested conidia were filtered through two layers of sterile cheesecloth and transferred to potato dextrose broth at a final concentration of 10^5 conidia/mL. After stationary incubation of each sample (three sub-replicates) at 28°C in the dark for 10 hr, the numbers of germinated conidia among a total of 100 conidia per sample were determined.

To examine the effect of the volatiles produced by the tested bacterial strains against the population of *A. flavus* AF57 on unhulled rice grains, *in vivo* tests were conducted using I-plates as follows. Aliquots (100 μL) of the prepared treatments described above were smeared on I-plates containing NA and incubated at 28°C. After 24 hr, surface-sterilized unhulled rice grains (2 g) were inoculated with the 200 μL of the *A. flavus* AF57 conidial suspension (equivalent to 10^6/g rice grains) and placed on the other side of the I-plates. The plates were then sealed with Parafilm and incubated for 7 days at 28°C. The populations of *A. flavus* AF57 on rice grains were assessed as previously described

[19]. Briefly, rice grains were finely ground using an analytical mill (IKA A11 basic, IKA Works, Wilmington, DE, USA), 1 g of grains were suspended in 10 mL of SDW, and were incubated in a rotary shaker (160 rpm) at 28°C for 30 min. After incubation, 10-fold serial dilutions of rice suspensions were prepared and 200 μL of the diluted suspensions were smeared on 18% glycerol agar (DG18; Fluka 40587, Sigma-Aldrich) [22]. These smeared DG18 plates were incubated for 4 days at 28°C for assessment of the fungal population. The fungal population was expressed as the log number of colony-forming units/g dry weight of rice grains.

To examine the effects of bacterial volatiles on aflatoxin production, the aflatoxigenic *A. flavus* strain KCCM 60330 was used in this test rather than *A. flavus* AF57, which produces low levels of aflatoxins. This experiment was conducted using I-plates as described above. Treatments and assessment of fungal population were conducted as shown above. Total aflatoxins, including aflatoxin B_1 and B_2 produced by *A. flavus* KCCM 60330 were analyzed using a competitive direct enzyme-linked immunosorbent assay with Veratox HS (Neogen Co., Lansing, MI, USA), following the procedure described by Mannaa *et al.* [19].

Statistical analysis of the data was conducted using Statistical Analysis Systems (SAS Institute, Cary, NC, USA). All experiments were performed twice with three replications

Table 1. Antifungal activities of the bacterial strains, *Microbacterium testaceum* KU313, *Bacillus megaterium* KU143, *Pseudomonas protegens* AS15, and *Sphingomonas aquatilis* KU408 (negative control), on mycelial growth of *Aspergillus flavus* AF57 using dual-culture assays on PDA

Treatment	Mycelial growth inhibition (mm)[a]	Mycelial growth area (cm²)
Control	-[b]	20.4 ± 0.1 a[c]
KU408	0.5 ± 0.4 d	20.1 ± 0.2 a
KU313	7.6 ± 0.8 c	14.3 ± 0.4 b
KU143	13.7 ± 0.5 b	8.4 ± 0.2 c
AS15	17.4 ± 1.2 a	6.8 ± 0.1 d

[a]Mycelial growth inhibition (mm) was determined using a dual-culture assay on potato dextrose agar (PDA) plates, which were streaked with the bacterial strains or sterile distilled water (control) in the center. The plates were then inoculated with conidial suspension of *A. flavus* AF57 on the opposite edges from the bacteria. Mycelial inhibition was evaluated when mycelia reached the centers of the control plates and mycelial growth area (cm²) was determined using ImageJ software.
[b]Not detected.
[c]Values are presented as the means ± standard errors of six replicates from repeated experiments. Different letters in each column indicate significant ($p < 0.05$) differences according to the least significant difference test.
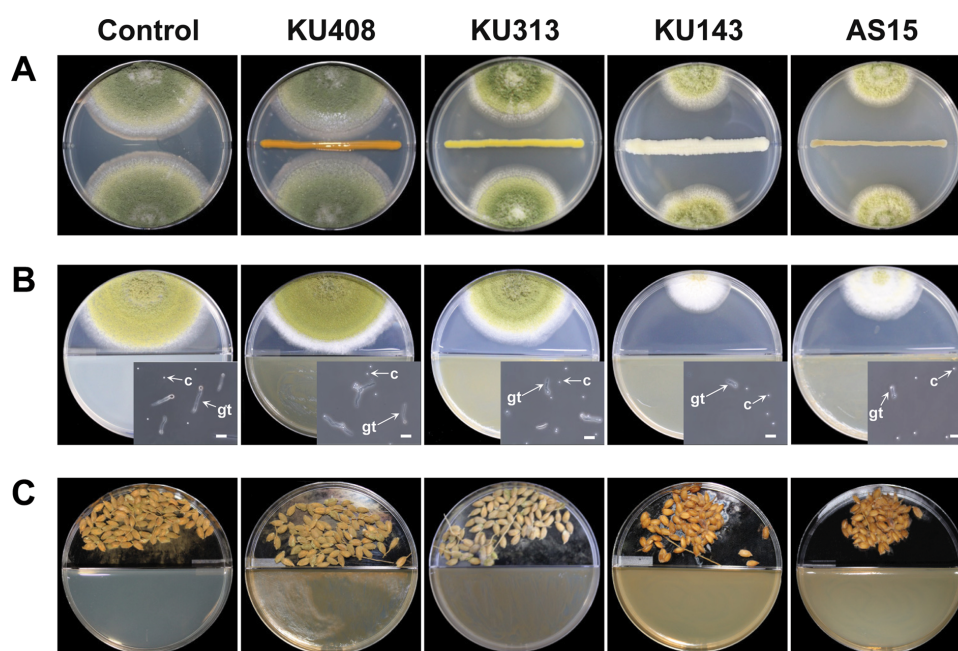

Fig. 1. Effects of the antifungal or volatiles produced by the bacterial strains, *Microbacterium testaceum* KU313, *Bacillus megaterium* KU143, *Pseudomonas protegens* AS15, and *Sphingomonas aquatilis* KU408 (negative control), on *Aspergillus flavus* AF57 mycelial growth determined using dual-culture assays on potato dextrose agar (PDA) (A) and PDA on I-plates (B), and unhulled rice grains on I-plates inoculated with *A. flavus* AF57 (C). In the dual-culture assays, bacterial strains or sterile distilled water (control) were line-streaked in the center of the plates, which were then inoculated with conidial suspension of *A. flavus* AF57 on the opposite edges from the bacteria. In I-plate tests, bacterial strains or 10-mM $MgSO_4$ solution (untreated control) were smeared on one side (nutrient agar) of the I-plates and the other side (PDA or rice grains) was inoculated with conidial suspension of *A. flavus* AF57 24 hr after the bacterial treatment. Insets of Fig. 1B are photographs of the conidial germination of *A. flavus* AF57 affected by the bacterial volatiles and untreated control. c, conidia; gt, germ tube (scale bars: B inset = 20 μm).

per treatment. Combined data from repeated experiments were analyzed after confirmation of homogeneity of variances, using Levene's test [23]. For analysis of fungal populations, data were analyzed after logarithmic transformation. Analysis of variance was determined using the general linear model procedure and means were compared using the least significant difference at $p < 0.05$.

The results of dual-culture assays exhibited that the three tested antagonistic strains, *B. megaterium* KU143, *M. testaceum* KU313, and *P. protegens* AS15, caused significant ($p < 0.05$) reduction in mycelial growth compared with the negative control strain, *S. aquatilis* KU408, and SDW control plates (Table 1, Fig. 1A). Among the tested bacterial strains, *P. protegens* AS15 caused the most marked reduction in mycelial growth (Table 1). However, the negative control bacterial strain did not inhibit mycelial growth, producing results similar to those of the SDW control (Table 1).

For the effects of bacterial volatiles against mycelial growth, sporulation, and conidial germination of *A. flavus* AF57 on I-plates (Fig. 1B), volatiles produced by *B. megaterium* KU143 and *P. protegens* AS15 significantly ($p < 0.05$) reduced the mycelial growth area compared with *S. aquatilis* KU408 (bacterial negative control) and MgSO$_4$ solution (untreated control) (Fig. 2A). Volatiles produced by *B. megaterium* KU143 and *P. protegens* AS15 significantly ($p < 0.05$) inhibited sporulation of *A. flavus* AF57 compared with negative and untreated controls (Fig. 2B). In addition, volatiles generated by *B. megaterium* KU143 and *P. protegens* AS15 significantly ($p < 0.05$) inhibited conidial germination (%) of *A. flavus* AF57 compared with negative and untreated controls (Fig. 2C). In contrast, volatiles produced by *M. testaceum* KU313 did not significantly ($p > 0.05$) inhibit mycelial growth, sporulation, or conidial germination of *A. flavus* AF57 compared with the bacterial negative control or untreated
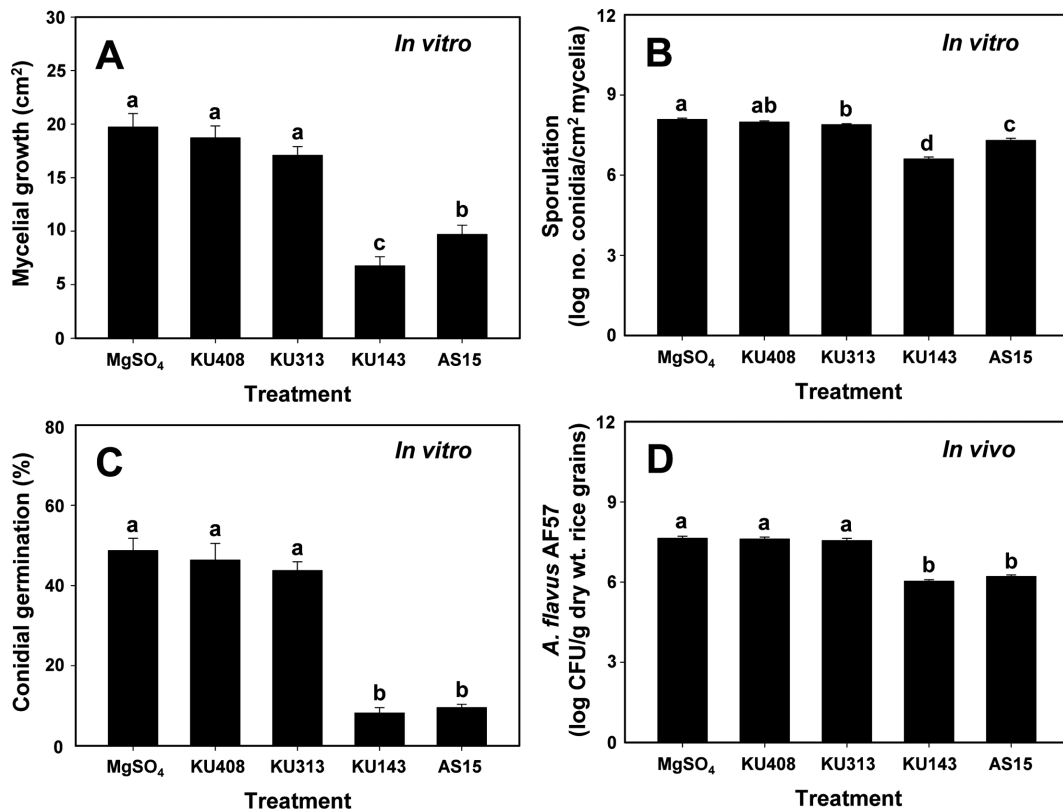

Fig. 2. Antifungal activities of the volatiles produced by the bacterial strains, *Microbacterium testaceum* KU313, *Bacillus megaterium* KU143, *Pseudomonas protegens* AS15, and *Sphingomonas aquatilis* KU408 (bacterial negative control), on mycelial growth (A), sporulation (B), and conidial germination on potato dextrose agar (C), and fungal population on unhulled rice grains inoculated with *Aspergillus flavus* AF57 (D). Bacterial strains were smeared on one side of the I-plates and the other side was inoculated with conidial suspension of *A. flavus* AF57 24 hr after bacterial treatment. Mycelial growth area (cm²) was measured using ImageJ software after the fungal mycelia reached the center of 10-mM MgSO$_4$ solution-treated plates (untreated control). Sporulation was determined as total harvested spores divided by the mycelial growth area. For conidial germination, the numbers of germinated conidia among a total of 100 conidia were determined 10 hr after incubation in potato dextrose broth at 28°C in the dark. At 7 days after inoculation, fungal populations on rice grains were determined by assessment of colony-forming units (CFU) on DG18 plates, which were smeared with the diluted rice suspensions and incubated for 4 days at 28°C in the dark. Different letters on vertical bars with error bars (standard errors, $n = 6$) indicate significant ($p < 0.05$) differences between treatments according to the least significant difference test.

control (Fig. 2A–C). Testing of the effects of bacterial volatiles on the population of *A. flavus* AF57 on unhulled rice grains (Fig. 1C) showed that those produced by *B. megaterium* KU143 and *P. protegens* AS15 significantly (*p* < 0.05) reduced fungal populations compared with bacterial negative and untreated controls (Fig. 2D). However, volatiles produced by *M. testaceum* KU313 failed to inhibit the fungal growth in rice grains (Fig. 2D).

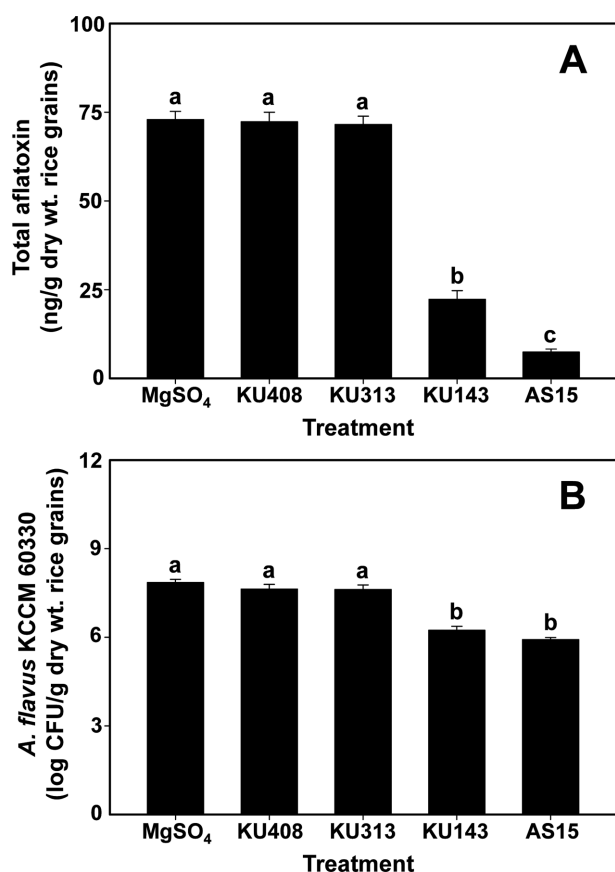When the effect of bacterial volatiles on aflatoxin

Fig. 3. Biocontrol activities of the volatiles produced from bacterial strains, *Microbacterium testaceum* KU313, *Bacillus megaterium* KU143, and *Pseudomonas protegens* AS15, and *Sphingomonas aquatilis* KU408 (bacterial negative control), on total aflatoxin production (A) and the population of the aflatoxigenic *Aspergillus flavus* KCCM 60330 in stored rice grains (B). In I-plate tests, bacterial strains or 10-mM MgSO$_4$ solution (untreated control) were smeared on one side (nutrient agar) of the I-plates and the other side (rice grains) was inoculated with conidial suspension of *A. flavus* KCCM 60330 24 hr after the bacterial treatment. Total aflatoxins in rice grains were quantified using a Veratox HS ELISA kit. Fungal populations were determined 7 days after inoculation by assessment of colony-forming units (CFU) on DG18 plates smeared with the diluted rice suspensions and incubated for 4 days at 28°C in the dark. Different letters on the vertical bars with error bars (standard errors, *n* = 6) indicate significant (*p* < 0.05) differences between treatments according to the least significant difference test.

production was examined on unhulled rice grains using the aflatoxigenic *A. flavus* KCCM 60330, volatiles produced by *B. megaterium* KU143 and *P. protegens* AS15 significantly (*p* < 0.05) reduced total aflatoxin production compared with the bacterial negative control strain, *S. aquatilis* KU408, and untreated MgSO$_4$ control (Fig. 3A). In particular, volatiles generated by *P. protegens* AS15 and *B. megaterium* KU143 markedly inhibited total aflatoxin production by *A. flavus* KCCM 60330 to 7.4 and 22.3 ng/g dry weight rice grains (percent total aflatoxin reduction relative to untreated control, 90.4% and 69.9%) compared with 72.9 ng/g dry weight rice grains for the untreated MgSO$_4$ control, respectively (Fig. 3A). In contrast, volatiles produced by *M. testaceum* KU313 did not inhibit total aflatoxin production in rice grains (Fig. 3A). In addition, the bacterial volatiles generated by *B. megaterium* KU143 and *P. protegens* AS15 significantly (*p* < 0.05) inhibited the fungal growth of *A. flavus* KCCM 60330 compared with bacterial negative and untreated controls (Fig. 3B), similar to the observations in the experiment using *A. flavus* AF57 (Fig. 2D).

Production of antimicrobial or systemic resistance-inducing volatile compounds by antagonistic bacterial strains is one of the biocontrol mechanisms against plant pathogens [24, 25]. Volatiles from antagonistic rhizobacteria were reported to stimulate pepper fruit ripening and to inhibit mycelial growth, sporulation, and spore germination of *Colletotrichum acutatum* and *Phytophthora capsici* [25, 26]. There were also reports demonstrating the influence of volatiles of plant or microbial origin on the growth of fungal species, including *A. flavus* [27, 28]. In our previous study [19], the antagonistic bacterial strains, *B. megaterium* KU143, *M. testaceum* KU313, and *P. protegens* AS15, were selected as effective biocontrol agents against *A. flavus*. The results of the current study demonstrate that, among these strains, *B. megaterium* KU143 and *P. protegens* AS15 produce antifungal volatile organic compounds (VOCs), which can inhibit the mycelial growth, sporulation, and conidial germination of *A. flavus* AF57 on artificial medium and fungal populations on unhulled rice grains inoculated with *A. flavus* AF57 (rice-originated isolate) or KCCM 60330 (aflatoxigenic isolate). In contrast, our results do not provide evidence that *M. testaceum* KU313 can produce antifungal volatiles, although it was found to be an effective antagonistic strain against *A. flavus* on rice grains [19]. The biocontrol activity of *M. testaceum* KU313 may be attributable to the production of non-volatile antifungal compounds, as indicated by the results of the dual-culture assay in this study and evaluation of effective colonization ability on rice grains in our previous study [19]. Further, in our previous study, the cell-free culture filtrates from these bacterial strains were proven to inhibit conidial germination, germ-tube lengths, and mycelial dry weight of *A. flavus*, indicating that these strains can produce extracellular antifungal compounds [19].

Since aflatoxins were discovered as early as 1960, various chemical compounds, natural products, and microbial extracts have been screened as potential inhibitors of aflatoxin

biosynthesis [29]. Extensive research efforts have been directed toward selection of microorganisms with aflatoxin inhibition ability in co-cultivation with aflatoxigenic *Aspergillus* spp. [30]. Previously, specific plant volatiles, including alcohols, aldehydes, and ketones have been reported to inhibit fungal growth and aflatoxin production by *A. flavus* [16, 17, 31, 32]. Roze *et al.* [32] demonstrated that volatiles, including formic acid pentyl ester, hexanoic acid ethyl ester, pentanoic acid methyl ester, 3,5-octadien-2-one, and 2-pentenal from willow bark inhibited aflatoxin production in *Aspergillus parasiticus* grown on a minimal medium; aflatoxin inhibition was correlated with a two-fold reduction in expression of the *ver-1* gene, which encodes an enzyme to the aflatoxin biosynthesis pathway. In another study, soybean volatile compounds, including aldehydes, (E)-2-hexenal, and (E)-2-heptenal effectively inhibited *A. flavus* growth and aflatoxin production [31]. The biocontrol yeast, *Pichia anomala*, was reported to produce 2-phenylethanol, which inhibited aflatoxin production by down regulation of expression of aflatoxin biosynthesis genes in *A. flavus* [33]. In this study, volatiles produced by *B. megaterium* KU143 and *P. protegens* AS15 significantly reduced aflatoxin production by *A. flavus* KCCM 60330 in rice grains. Considering the suppression of *A. flavus* growth and toxin production by these bacterial volatiles, *P. protegens* AS15 inhibited aflatoxin production more than fungal population, indicating the possible degradation of the aflatoxin or suppression of toxin production by *A. flavus*. However, reduction of the aflatoxins by the treatment with *B. megaterium* KU143 may result from the inhibitory effect on the fungal growth. Further study to identify the VOCs responsible for the suppression of aflatoxin production will be needed to facilitate understanding of the related processes and to develop an effective and applicable method to control aflatoxins in stored crop grains.

Taken together, the results of the present study suggest that the antagonistic bacterial strains, *B. megaterium* KU143 and *P. protegens* AS15, produce effective antifungal volatile compounds that inhibit *A. flavus* growth on artificial medium and unhulled rice grains. Furthermore, the volatiles from these two bacterial strains reduced aflatoxin production by *A. flavus* in stored rice grains. To our knowledge, this is the first report of the inhibition of *A. flavus* aflatoxin production in rice grains using the volatiles produced by *B. megaterium* and *P. protegens*.

ACKNOWLEDGEMENTS

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