Biocontrol Activity of *Bacillus megaterium* BM344-1 against Toxigenic Fungi

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**ABSTRACT:** Mycotoxins are secondary metabolites of some fungal species and represent important contaminants of food and feed. This study aimed to explore the biological control activity of *Bacillus megaterium* BM344-1 volatile organic compounds (VOCs) on the growth and mycotoxin production of single representatives of the toxigenic species *Aspergillus flavus*, *Aspergillus carbonarius*, *Penicillium verrucosum*, and *Fusarium verticillioides*. In vitro co-incubation experiments indicated the *P. verrucosum* isolate as the most sensitive one, with a growth inhibition ratio of 66.7%, followed by *A. flavus* (29.4%) and *F. verticillioides* (18.2%). Exposure of *A. flavus*, *P. verrucosum*, and *F. verticillioides* to BM344-1 VOCs resulted in complete inhibition of aflatoxins (AFB1, AFB2, and AFG1), ochratoxin A, and fumonisin B1 (FB1) synthesis on artificial media, respectively. *In vivo* experiments on maize kernels showed 51% inhibition of fungal growth on ears simultaneously infected with *A. flavus* spores and exposed to BM344-1 volatiles. Likewise, AF synthesis by *A. flavus* was significantly (*p < 0.05*) inhibited (25.34 ± 6.72 μg/kg) by bacterial volatiles as compared to that in control maize ears (91.81 ± 29.10 μg/kg). Gas chromatography–tandem mass spectrometry-based analysis of headspace volatiles revealed hexadecanoic acid methyl ester (palmitic acid) and tetracosane as bioactive compounds in the BM344-1 volatilome. Bacterial volatiles have promising potential to control the growth and mycotoxin synthesis of toxigenic fungi and may present valuable aid in the efforts to warrant food and feed safety.

1. INTRODUCTION

Mycotoxins are important contaminants of agriculture and food industries and are mainly produced by some species of *Aspergillus*, *Penicillium*, and *Fusarium*. After the first discovery of aflatoxin (AF) in 1960s, there has been a tremendous effort to dissect mycotoxin nature, toxicity, and mycotoxigenic species. At present, the list of known mycotoxins covers over 400 compounds, including toxins produced by *Aspergillus* and *Penicillium* (such as AFs, ochratoxins, patulin, etc.) and *Fusarium* (e.g., zearalenone, fumonisins, deoxynivalenol, and T-2/HT-2). AFB1, produced by *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* is widely known for its hepatotoxicity and has been classified as a group 1A human carcinogen. Ochratoxin A (OTA), a nephrotoxic metabolite, is found in many food commodities and is synthesized by some *Aspergillus* (*Aspergillus carbonarius*, *Aspergillus ochraceus*, *Aspergillus westerdijkiae*, *Aspergillus niger*, etc.) and *Penicillium* (such as *Penicillium verrucosum* and *Penicillium nordicum*) species. Fumonisins (FB1 and FB2) are among the most important mycotoxins produced by *Fusarium* species (*Fusarium verticillioides* and *Fusarium proliferatum*) and induce neurotoxic effects on the exposed animal and human.

Pre- and post-harvest contamination of food crops with toxigenic fungi and the accumulation of their toxins remain ever challenging for food and feed regulatory authorities. Agricultural husbandry practices such as crop rotation, proper sowing and harvesting timing, insect and pest control, grading and segregation of products, proper irrigation and the use of effective fungicides result in significant control of fungal infection and mycotoxin accumulation. However, persistence of fungicide residues in food and emerging fungicide-resistant fungal populations are major concerns associated with chemical fungicides. Likewise, some physical control methods in spite of having significant potential to degrade mycotoxins may affect the quality of cereal-derived food and feed. UV irradiation of toxin-contaminated food not only has limited applicability but also compromises the nutritional and organoleptic characteristic of food.

Over the recent past, several efforts have been devoted to define alternate and safer strategies to minimize the impact of mycotoxins and to control fungal infection in crops. Microbial...
control by living and inactivated yeasts and bacterial cells, their diffusible and volatile compounds, and enzymes are being explored for their antagonistic potential against fungi.\textsuperscript{13,14}

In our previous studies, we have reported yeast\textsuperscript{15–18} and bacterial\textsuperscript{19–21} cultures possessing strong antifungal potential against toxigenic fungi. This study was designed to investigate \textit{in vitro} as well as \textit{in vivo} effects of \textit{Bacillus megaterium} (BM344-1) against the growth and toxin production potential of toxigenic isolates of \textit{A. flavus}, \textit{P. verrucosum}, and \textit{F. verticillioides}. Additionally, the chemical nature of the BM344-1 volatilome was investigated to identify the bioactive molecule(s) in the bacterial volatilome.

### 2. RESULTS AND DISCUSSION

#### 2.1. Optimum Conditions for the Efficient Production of Bacterial Antifungal Volatiles

Lipophilicity, low polarity, high vapor pressure, and low molecular weight are the main characteristics of microbial volatile organic compounds (VOCs) that are gaining momentum for their potential application against fungal contamination of food and feed commodities.\textsuperscript{22,23} The precise mode of action of VOCs is not yet well understood and probably varies with the chemical nature of molecules and their microbial sources.\textsuperscript{24} However, interference with the fungal metabolic pathways by alteration in the expression of key genes is generally an accepted mechanism of their antifungal activity.\textsuperscript{15,16} Three media [tryptic soy agar (TSA), Luria-Bertani (LB), and nutrient agar (NA)] and four bacterial cell dilutions ($10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$) were preliminarily tested to explore the appropriate requirements for an efficient antagonistic activity of BM344-1 against \textit{Aspergillus carbonarius} AC82. The volatiles produced by BM 344-1 on TSA at $10^{-1}$ dilution showed the highest inhibitory effect on a colony size of \textit{A. carbonarius} as measured at day 7 of co-incubation (Figure 1). The composition of growth media, particularly protein- and sugar-contents, plays a key role in the bacterial volatilome. On protein-rich media, \textit{Lysobacter} sp. produced bioactive compounds such as pyrrole, decanal, and pyrazines as compared to inactive compounds on sugar-rich media.\textsuperscript{25} In the present study, the antagonistic activity of BM344-1 was linked to protein richness with the highest inhibitory efficacy measured on TSA (15 g of pancreatic casein and 5 g of soy peptone in 1 L), followed by that on LB (10 g of tryptone, 5 g of yeast extract), and the least on NA (5 g of peptone and 2 g of yeast extract). In the study of Bruce \textit{et al.},\textsuperscript{26} VOCs produced by bacterial cultures on TSA showed a complete inhibition of fungal growth, whereas inhibition was minimal when bacteria were grown on other media. In fact, amino acids acting as components of antagonistic volatiles are found in particular high-protein media compared to others.

#### 2.2. Antagonistic Spectrum of \textit{B. megaterium} BM344-1 Volatiles against \textit{A. flavus}, \textit{F. verticillioides}, and \textit{P. verrucosum}

Exposure of different mycotoxigenic fungi to BM344-1 volatiles resulted in a significant decrease in the colony diameter as compared to that of unexposed control fungi. \textit{P. verrucosum} showed the highest sensitivity to bacterial volatiles, followed by \textit{A. flavus} and \textit{F. verticillioides}. The growth inhibition ratios (%) calculated with comparison to control fungi were 66.7, 29.4, and 18.2% for \textit{P. verrucosum}, \textit{A. flavus}, and \textit{F. verticillioides}, respectively (Figure 2). The higher sensitivity of \textit{P. verrucosum} compared to that of \textit{A. flavus} to bacterial volatiles was previously observed by Ul Hassan \textit{et al.}.\textsuperscript{19}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Effect of the type of growth media and bacterial cell dilution on the antifungal activity of \textit{B. megaterium} BM344-1. Bacterial cells at dilutions $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$ were spread inoculated on three types of media (TSA, LB agar, and NA) and sealed with fungal inoculated plates.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Spectrum of antifungal activities of \textit{Bacillus megaterium} BM344-1 against toxigenic \textit{Aspergillus}, \textit{Fusarium}, and \textit{Penicillium} fungi. The fungi in the second row are the control (unexposed to bacterial volatiles), while those in the third row showing significant effects on colony size and sporulation are exposed to \textit{B. megaterium} BM344-1 volatiles for 3 days.}
\end{figure}
Exposure of *P. verrucosum* and *A. flavus* to *Bacillus licheniformis* volatiles (the major antagonist compound was 3-methyl-1-butanol) resulted in 53 and 49% reduction in the colony diameter when compared to that of the unexposed control, respectively. In line with this study, Zeidan et al. 17 found that the highest sensitivity is of *Penicillium*, followed by that of *Aspergillus*, and the least by *Fusarium* to yeast VOCs. The observed differences in fungal colony diameters among the three fungi (each from different genus) in response to bacterial volatiles may be associated with their cell wall structure. The cell wall composition of fungi varies according to their microenvironmental stressors and plays a significant role in the fungal resistance.27,28 Antagonistic *Bacillus* volatiles (such as those of *Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus cereus*, and *B. megaterium*) against phytopathogenic and toxigenic *Aspergillus* and *Penicillium* spp. have been reported by several authors.29–31

2.3. Reversibility in BM344-1-Induced Fungal Growth Inhibition. After removal from the bacterial volatile environment, all three fungi showed normal growth and sporulation, suggesting that microbial volatiles effects were transient and the presence of antagonistic bacteria or their VOCs is needed for consistent inhibition. In a study by Wheatley et al.,32 similar reversibility to physiological growth and sporulation was observed in fungi after removal from the bacterial environment. In a similar study, after removal from the VOC environment, Fiori et al.33 observed the reversibility of sporulation in *A. carbonarius* which was completely inhibited upon exposure to yeast volatiles.

*B. licheniformis* BL350-2 producing 3-methyl-1-butanol as a bioactive compound caused significant growth inhibition in *Aspergillus* westerdijkiae BA1 (62%), *A. carbonarius* MG7 (60%), *P. verrucosum* MC12 (53%), *Aspergillus* niger MC05 (50%), *A. flavus* CMS (49%), *A. parasiticus* SB01 (47%), and *Aspergillus ochraceus* MD1 (44%), which showed complete reversal upon removing the fungi from the bacterial VOC environment.19

2.4. Inhibitory Effect of *B. megaterium* BM344-1 on Mycotoxin Synthesis. Exposure to BM344-1 volatiles not only inhibited the vegetative growth but also affected the mycotoxin biosynthesis potential of toxigenic fungi (Table 1). At day 7 of co-incubation, *A. flavus* showed a significant reduction in AFB2 synthesis, while the production of other classes of AFs (AFB1, AFG1, and AFG2) was totally inhibited. Similarly, OTA synthesis by *P. verrucosum* and FB by *F. verticillioides* were also completely inhibited by bacterial volatiles. *F. verticillioides* exposed to BM344-1 was able to synthesize FB2, but the concentration of this mycotoxin in the medium was significantly lower than that of unexposed control fungi.

The inhibition/reduction in mycotoxin synthesis by toxigenic fungi in the bacterial9,30 or yeast5–18 VOCs-saturated environment could be associated with changes in the expression of biosynthetic-cluster genes,34 protein profiles,16,34 or altered enzymatic activities of the target fungi.32 The volatiles synthesized by *B. megaterium* KU143 resulted in the inhibition of AF accumulation on stored rice grains colonized by *A. flavus*.30

2.5. Biological Control Activity of *B. megaterium* BM344-1 against *A. flavus* Growth and AF Synthesis on Maize Kernels. *In vivo* exposure of *A. flavus*-infected maize ears to BM3441-1 volatiles showed a significant inhibition of fungal growth as well as AF synthesis (Table 2). In the control maize ears (without BM344-1 VOCs), the spread of *A. flavus*, measured as number of kernels with visible fungal growth, was significantly higher (22.5 ± 0.7 kernels) compared to that in the ears infected with fungi and exposed to bacterial VOCs (11 ± 1 ears), showing 51% inhibition in the fungal growth as a consequence to bacterial VOC exposure. In line with the present study, Mannaa et al. (2017)30 reported a significant decrease in the *A. flavus* population on un-hulled rice grains exposed to *B. megaterium* BM344-1 volatiles. The VOCs (3-methyl-1-butanol as compound) produced by *B. licheniformis* showed a similar inhibitory effect on the growth of *A. flavus* on infected maize ears.19

In line with the fungal growth, the levels of AFs in the VOCs-exposed *A. flavus*-contaminated maize ears were significantly (p ≤ 0.05) lower [25.34 ± 6.72 (± standard deviation)] than that in the unexposed maize ears (91.81 ± 29.10). TSA alone showed no effect on fungal growth and its mycotoxin production ability (Table 2). These *in vivo* results are in line with the *in vitro* antagonistic activity of BM344-1 against mycotoxin synthesis potential of *A. carbonarius, P. verrucosum*, and *F. verticillioides* (Section 4.4). Inhibition of AF synthesis by *A. flavus* on exposure to volatiles emitted by *B. megaterium* KU143 and *B. licheniformis* 350-2 on un-hulled rice and maize ears has been reported by Mannaa and Kim31 and Ul Hassan et al.,19 respectively. However, in the present study, it is unclear if the observed reduced mycotoxin synthesis is associated with fungal growth slowdown or with specific

### Table 1. Effect of *B. megaterium* BM344-1 Volatiles on the Mycotoxin Biosynthesis by Different Toxigenic Fungi

| fungi              | mycotoxin (µg/kg) | control | VOCs-exposed |
|--------------------|-------------------|---------|--------------|
| *A. flavus*        | AFB1 199.44 ± 16.40o | n.d*    |              |
|                    | AFB2 84.82 ± 11.00o  | 13.91 ± 2.45o |              |
|                    | AFG1 37.26 ± 4.50o   | n.d     |              |
|                    | AFG2 14.21 ± 2.12o   | n.d     |              |
| *P. verrucosum*    | OTA 84.80 ± 9.50o   | n.d     |              |
| *F. verticillioides*| FB1 1.04 ± 0.07o    | n.d     |              |
|                    | FB2 11.85 ± 2.36o    | 1.62 ± 0.01b |              |

*Effect of *B. megaterium* BM344-1 volatiles on mycotoxin production of *A. flavus, P. verrucosum*, and *F. verticillioides*. Mycotoxin production of the control (fungi not exposed to BM344-1 volatiles) and VOCs-exposed fungi are shown as mean ± SD obtained from three replicates. Different superscript letters on values in rows indicate the significant difference at p ≤ 0.05. *Not detected (below the limit of detection of the analytical system).
mechanisms such as effects on the expression of genes involved in mycotoxin biosynthesis or alteration in the enzymatic activities.

2.6. GC−MSMS Analysis of BM344-1 Volatiles. Bacterial volatiles analysis performed by gas chromatography−mass spectrometry (GC−MS) revealed the presence of hexadecanoic acid methyl ester (palmitic acid) and tetracosane. Both these compounds are well-known microbial volatiles holding strong antagonistic activities against toxigenic as well as phytopathogenic fungi (Figure 3).

The absence of these compounds in the control flasks [tryptic soy broth (TSB) without bacteria] suggests that both the fungal growth and mycotoxin synthesis inhibition were due to single or synergistic/additive interaction of the two compounds (Table 3). Hexadecanoic acid was the major compound in the microbial VOC mix of Bacillus atrophaeus HAB-5 inhibiting Colletotrichum gloeosporioides and sea-weeds suppressing Aspergillus, Penicillium, and Fusarium. Likewise, tetracosane was the major constituent of antifungal volatiles produced by Chaetomium globosum.

3. CONCLUSIONS The volatiles produced by B. megaterium BM344-1 have shown high potential against the growth and mycotoxin biosynthesis in three representative isolates of A. flavus, P. verrucosum, and F. verticillioides. The antifungal activity of BM344-1 was enhanced by increasing the protein content in the growth medium. During in vitro co-incubation experiments, P. verrucosum showed the highest sensitivity, with a growth inhibition ratio of 66.7%, followed by A. flavus (29.4%) and F. verticillioides (18.2%). Exposure of A. flavus, P. verrucosum, and F. verticillioides to BM344-1 VOCs resulted in complete inhibition of AFs (AFB 1, AFG 1, and AFG 2), OTA, and fumonisin B1 (FB1) synthesis on artificial media, respectively. Under in vivo testing on maize ears, BM344-1 showed significant inhibition of A. flavus growth and AF synthesis on infected kernels. The headspace analysis of bacterial volatiles indicated hexadecanoic acid methyl ester (palmitic acid) and tetracosane as bioactive compounds. These results suggest potential application of bacterial culture for the preservation of food commodities.

4. MATERIALS AND METHODS

4.1. Microbial Cultures and Growth Media. B. megaterium BM344-1 was isolated from strawberry jam (imported from Turkey) marketed in Qatar and identified by its protein spectrum using matrix-assisted laser desorption ionization time-of-flight. A. flavus CECT 2687 was obtained.
from the culture collection center, University de Valencia Spain; A. carbonarius AC82, F. verticillioides FV04, and P. verrucosum PV11 were isolated from animal feed.\textsuperscript{22} TSA was prepared by adding 15 g of pancreatic casein, 5 g of soy peptone, 5 g of sodium chloride, and 15 g of agar in 1 L of distilled water. LB agar for Bacillus sp. was prepared by mixing 15 g of agar, 10 g of tryptone, 5 g of yeast extract and 5 g of NaCl in 1 L of distilled water. NA was prepared by adding peptone (5 g), meat extract (1 g), yeast extract (2 g), sodium chloride (5 g), and agar (15 g) in 1 L of distilled water.

4.2. Optimization and Investigation of B. megaterium BM344-1 for Its Antifungal Activities. In order to find appropriate conditions for the optimal production of antifungal volatiles, different dilutions of B. megaterium BM344-1 were preliminarily inoculated on three types of bacterial growth media. In each case, 100 μL of bacterial cell suspension \(10^{-1}\) \((\sim2.5 \times 10^7 \text{ cfu/mL}), 10^{-2}\) \((\sim2.5 \times 10^6 \text{ cfu/mL}), 10^{-3}\) \((\sim2.5 \times 10^5 \text{ cfu/mL}), 10^{-4}\) \((\sim2.5 \times 10^4 \text{ cfu/mL}), \) and \(10^{-5}\) \((\sim2.5 \times 10^3 \text{ cfu/mL})\) was plated on TSA, LB, and NA. Inoculated plates were incubated at \(30^\circ\text{C}\) for 24 h. In an Eppendorf tube, fungal spores of A. carbonarius were prepared by transferring inocula from the freshly sporulating fungal colony to 1 mL of saline solution, amended with 0.05% Tween 80. A 10 μL aliquot of the spore suspension (adjusted at \(\times10^6\)) was inoculated at the center of PDA plates. The cover of the fungal inoculated plates was replaced with the base plate of bacterial inoculated plates. The two plates were sealed face-to-face with a double layer of Parafilm and then an additional layer of scotch tape. The sealed plates were incubated at \(26^\circ\text{C}\) for 72 h before measuring the diameter of the fungal colonies and the extent of sporulation. Fungal growth inhibition was calculated as

\[
\text{fungal growth inhibition (\%) } = \frac{C - T}{C} \times 100
\]

\(C\) = Number of infected kernels in A. flavus-inoculated maize ear. \(T\) = Number of infected kernels in A. flavus-inoculated and BM344-1 volatiles-exposed maize ear.

4.6. Effect of BM344-1 Volatiles on AF Contamination on Maize Kernels. Maize ears were removed at the site of fungal inoculation from the treated kernels (Section 2.5) and thoroughly mixed. The AF contents of representative (2 g) samples were extracted in 10 mL of 70% methanol.\textsuperscript{19} Enzyme-linked immunosorbent assay (ELISA) kits (RIDASCREEN Aflatoxin Total, Art no. R4701) obtained from R-Biopharm AG, Darmstadt, Germany, were used for AF analysis. An ELISA plate reader (Multiskan FC, Thermo Scientific, Waltham, MA, USA) installed with Skanlt software (Version 4.1. Thermo Scientific, MA, USA, 2015) was used to obtain the absorbance values of unknown standards solutions, and the absorbance values of unknown samples were added to the calibration curve to calculate the amount of toxins in our samples. For this purpose, the software Z9996 RIDA-SOFT Win (R-Biopharm, Darmstadt, Germany) was used.

4.7. Analysis of BM344-1 Volatile Bioactive Compounds. Bacterial volatiles were captured on activated charcoal (AC) and analyzed by GC–MS/MS as described by UI Hassan et al.,\textsuperscript{19} with little modification. Briefly, in 250 mL Erlenmeyer flasks, bacterial cell suspension was added to 100 mL of TSB media. Two valve rubber-corks were fitted to allow the passage of glass tubing. To the outer end of one tube, a volatile trap (glass Pasteur pipette filled with AC) was attached, while the other end was kept inside the flask at the neck level. The inner end of the second tube was placed \(\sim1\) cm above the TSB level, and the outer end was sealed with Parafilm. Flasks were incubated at \(30^\circ\text{C}\) in a shaking incubator for 72 h. A gentle stream of nitrogen gas was introduced into the flask through the open end of the second tube for the removal of headspace volatiles to be trapped on AC. Captured volatiles on AC were eluted in dichloromethane and analyzed by GC with the set parameters as described UI Hassan et al.\textsuperscript{19} The mass spectral libraries of Wiley and NIST were used to compare the obtained spectra of unknown compounds. The control flasks were maintained with TSB without adding bacterial cells.
4.8. Statistical Analysis. The effect of bacterial VOCs on fungal development (colony size) in vitro and on maize kernels was presented as the fungal growth inhibition (%) as compared to that of unexposed fungi calculated by the formula given in Section 2.2. The mean values of mycotoxin synthesis inhibition in VOCs-exposed fungi were compared with that of the control using Student’s “t-test”. The data for mycotoxin synthesis inhibition on maize kernels was subject to ANOVA, followed by post hoc multiple comparison by Duncan’s multiple range test at p ≤ 0.05. Statistical software IBM SPSS (IBM SPSS Version 25 for macOS; SPSS Inc., Chicago, IL, USA) was used for these analyses.

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Notes
The authors declare no competing financial interest.

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