Antifungal strains M1-8 and M6-4 as biocontrol agents against *Aspergillus flavus* on peanut kernels

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RESEARCH ARTICLE

Abstract

Aspergillus flavus is the main pathogenic fungi for peanut kernels, and it has highly negative economic and health impacts. To explore the biological control agent against *A. flavus,* two antagonistic strains were screened from 70 bacteria isolates from moldy stuff and identified as *Burkholderia plantarii* M1-8 and *Burkholderia glumae* M6-4. *In vitro* pathogen inhibition determination indicated culture filtrates of M1-8 and M6-4 had distinct inhibition zones and could decrease the mycelial growth of *A. flavus*. Furthermore, the biocontrol assay showed moldy rates of peanut seeds treated with culture filtrate were much lower than that of the control group. The biocontrol efficacy of M1-8 and M6-4 culture filtrate reached 88.6% and 84.2%, respectively, which were higher than that of the sorbic acid treatment group. Moreover, culture filtrate of M1-8 was tolerant to proteinase K, high light, pH and temperature (below 70°C), and had good stability of antifungal activity. The results indicate that these two antifungal strains could be used as biocontrol agents for controlling *A. flavus* during the peanut supply and storage period.

Keywords: Antifungal strain; *Aspergillus flavus*; Biocontrol; Identification; Peanut

Introduction

As an important industrial and oil crop worldwide, peanut is easily contaminated by *Aspergillus flavus* during storage (Hertwig *et al*., 2020). *A. flavus* is considered an opportunistic food spoilage fungal that can survive under harsh conditions and might invade a wide range of agricultural products during storage, handling and shipment (Mamo *et al*., 2020). In infected peanut kernels, *A. flavus* can cause moldy decay and also produce aflatoxin B1 (AFB1), which is the most common and toxic fungal toxin, posing a threat to food contamination (Lyu *et al*., 2020; Xu *et al*., 2023). Physical and chemical methods are widely used for controlling the contamination of *A. flavus* (Moon *et al*., 2018; Sun *et al*., 2021). However, physical control is not suitable on a large scale and chemical control often leads to environmental pollution (Mohd Azuar Hamizan *et al*., 2022).

With the increasing use of grains and grain-based products, it is very important to ensure their safety. The adverse effects of chemical preservatives and the restrictions imposed by the food industry on their application as food additives have reignited interest in finding effective and economical ways to control *A. flavus* in post-harvest storage and food processing (Kumar

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et al., 2021; Shabeer *et al*., 2022; Zadravec *et al*., 2022). Biological control offers a safer, more environmentally and economically sound alternative. Many antagonistic bacteria have shown the inhibition of the growth of moulds (Muhialdin *et al*., 2020; Cardoso Gimenes *et al*., 2023). Among the microorganisms, most *Burkholderia* species can be used as biological control agents against plant pathogenic fungi, including *Botrytis cinerea*, *A. flavus*, *Aspergillus niger*, *Penicillium expansum*, *Sclerotinia sclerotiorum* and *Phytophthora cactorum* (Elshafie *et al*., 2012; Perin *et al*., 2006). For instance, *B. gladioli* pv. agaricola strain ICMP 12322 was able to enhance disease protection and improve the consistency of biological control against tomato wilt caused by *Verticillium dahliae* (Elshafie *et al*., 2017). *Burkholderia cepacia* strain (QBC03) was examined and exhibited *in vitro* biocontrol activity against various fungi of *Aspergillus*, *Penicillium* and *Fusarium* (Zeidan *et al*., 2019). Andrade *et al*. (2021) reported that *Burkholderia cepacia* may also be beneficial as a biocontrol agent. Different species of *Burkholderia* spp. have important biological and metabolic characteristics that can be used as an antagonistic biological control agent for soil bioremediation and plant growth promotion (Elshafie *et al*., 2021).

Though *Burkholderia* spp. is scientifically important and potential practical application, very little is known about the effect of *Burkholderia plantarii* and *Burkholderia glumae* in the post-harvest control of *A. flavus* contamination in peanut kernels. Therefore, the main objective of this work was to assess the inhibition of *B. plantarii* M1-8 and *B. glumae* M6-4 on *A. flavus*, evaluate the biocontrol potential of two antagonistic strains against *A. flavus* on peanut kernels, and investigate the antifungal activity stability of culture filtrate under protease K, light, pH and temperature. To our knowledge, this is the first report that the antifungal action of *B. plantarii* and *B. glumae* was confirmed for controlling *A. flavus*, and our results will enrich the microbial strains resources against *A. flavus* and lay a foundation for exploiting efficient biocontrol agents to control *A. flavus* in peanut.

Materials and Methods

Materials

The seeds of peanut (Yuhua 65) were purchased from the seed market (Henan Qiule Seed Industry Science and Technology Company, Ltd.) in Zhengzhou city, Henan province. *A. flavus* was preserved at Henan University of Technology (Zhengzhou, China) and maintained at 4°C on potato dextrose agar (PDA) medium (200-g potato, 20-g dextrose, 17-g agar, 1000-mL distilled water, pH 7).

Isolation of antagonistic bacteria

The method of Shi *et al*. (2017) was referenced to isolate and screen the bacteria. Briefly, the moldy stuff was placed in 1000-mL sterile saline and shaken at 160 rpm at 37°C for 60 min. Then, it was serially diluted up to 10^{-6}, coated on Luria-Bertani (LB) medium and incubated at 37°C for 48 h. Strains with better antagonistic effects on the plates were screened out.

Identification of antagonistic bacteria

Antagonistic bacteria M1-8 and M6-4 were cultured on LB medium (5-g yeast extract, 10-g NaCl, 10-g peptone, 15–20-g agar, 1000-mL distilled water, pH 7) and placed at 37°C for 24 h to observe colony characteristics, such as morphology, color and growth properties. Physiological and biochemical tests were performed using universal methods (Guerrero, 2001), including the methyl red (M-R) test, the Voges-Proskauer (V-P) test, nitrate reduction and glucose decomposition. Protease and chitinase produced by antagonistic bacteria were detected according to the protocols of Jamali *et al*. (2020).

Genomic DNA of antagonistic strains was extracted with the phenol-chloroform method (Zakry *et al*., 2012). The 16 Svedberg ribosomal ribonucleic acid (*16S rRNA*) gene sequence was amplified using the specific primer pairs 16SF (5′-AGA GTT TGA TCA TGG CTC AG-3′) and 16SR (5′-ACG GTT ACC TTG TTA CGA CTT-3′) (Yi *et al*., 2021). The polymerase chain reaction (PCR) reaction followed the condition of 94°C for 4 min, followed by 30 cycles of 30 s at 94°C, 40 s at 54°C and 90 s at 72°C, with a final extension step of 10 min at 72°C. Then the PCR product was sent to Sangon Biotech Co., Ltd. (Shanghai, China) for sequencing. The *16S rRNA* gene sequences of M1-8 and M6-4 were submitted to GenBank (NCBI, USA; http://www.ncbi.nlm.nih.gov) under the accession numbers MZ664043 and MZ664044, respectively. Through the analysis of homology on GenBank, similar gene sequences were used to construct the phylogenetic tree by the maximum likelihood method using MEGA 7.0 software (Kumar *et al*., 2016).

In vitro inhibition tests of antagonistic strains and culture filtrate on A. flavus

In vitro inhibition tests of antagonistic strains M1-8 and M6-4 were performed on the PDA plates through plate confrontation method (Li *et al*., 2019). To prepare the culture filtrate, antifungal strains were inoculated into liquid LB medium, cultured with 180 r/min at 37°C for 2 d, and filtered with 0.22-μm filter. Then, *A. flavus* disks

(9 mm) were added into three flasks with liquid PDB medium, including 10 mL of sterile water (control), 10 mL of M1-8 culture filtrate and M6-4 culture filtrate, respectively. After incubation at 28°C for 3 d, a small number of pathogenic mycelia was picked for morphological observation under the light microscope. The experiment was repeated three times.

Biocontrol assay of culture filtrate on moldy peanut kernels

The biocontrol assays of M1-8 and M6-4 culture filtrate were performed according to the method of Sun *et al.* (2021). Intact peanut kernels without mold contamination were selected and disinfected with 0.1% sodium hypochlorite for 2 min, then rinsed with sterile water and dried under aseptic conditions. About 80 peanut kernels were divided into four groups and transferred into petri dishes. *A. flavor* spores suspension (10^6 spore/mL) was inoculated on peanut kernels. Then 1 mL of liquid LB medium (control), sorbic acid (SA), M1-8 and M6-4 culture filtrate were evenly sprinkled on the surface of the peanut kernels, respectively. The peanut kernels were incubated at 28°C for 5 d and their moldy incidents were recorded. Every treatment had four replicates and the process was conducted three times.

The number of moldy peanut kernels contaminated by *A. flavus* was recorded every 24 h. Visible mycelia growing on the surface of the peanut kernels was taken as the moldy standard. The moldy rate and biocontrol efficacy were calculated in accordance with the following formula:

$$\text{Moldy rate (\%)} = (Nm/Nt) * 100 \quad (1)$$

$$\text{Biocontrol efficacy (\%)} = [(Mc - Mt)/Mc] * 100 \quad (2)$$

Where Nm represents the number of moldy peanut kernels Nt represents the total number of peanut kernels, Mc represents the moldy rate in the control group and Mt represents the moldy rate in the culture filtrate or SA treatment group.

Stability evaluation of the culture filtrate of strain M1-8

The stability of *B. plantarii* M1-8 culture filtrate was determined according to the method of mycelial growth rate (Yi *et al.*, 2022). Inhibition rates of culture filtrate were detected after incubating with 10-mg/mL protease K (Merck, Darmstadt, Germany) for 0.5, 2, 4, 8 and 16 h, exposure to illumination (4500 ± 500 lx) for 0.5, 2, 4, 8 and 16 h, adjusting the pH to pH 7 (control, CK), 2, 4, 6, 10 and 12 with 1-mol/L HCl and 1-mol/L NaOH, and treating under different temperatures (20°C (control, CK), 60°C, 70°C, 80°C and 90°C) for 2 h, respectively. M1-8 culture filtrate without any treatment was set as the control. Each experiment was conducted three times. Inhibition rates of culture filtrate were calculated according to the following formula:

$$\text{Inhibition rate (\%)} = (Dc - Dt)/Dc \times 100 \quad (3)$$

Where Dc represents the mycelial radial growth diameter of *A. flavus* in the control group, Dt represents the mycelial radial growth diameter of *A. flavus* in the culture filtrate or SA treatment group.

Statistical analysis

All data were expressed as the means ± SD error and analyzed by one-way analysis of variance at ANOVA using SPSS 20.0 (SPSS Co. Ltd., Chicago, IL, USA). Significant differences between means at $p<0.05$ were determined by multiple comparisons with Duncan's multiple range test.

Results

Isolation and identification of antagonistic strains M1-8 and M6-4

Among the 70 bacteria isolated from the moldy stuff, two strains, M1-8 and M6-4, showed significant inhibition activity against *A. flavus*. After 48-h incubation, colony characteristics of two strains exhibited Gram-negative, slimy shape, yellowish appearance with a smooth surface and overall roundness. In the physiological and biochemical tests, the V-P test, nitrate reduction and glucose decomposition are all positive, but the M.R. test is negative. In addition, both strains produce protease but not chitinase (Figure 1).

16S rRNA gene sequences of strains M1-8 and M6-4 were 1,499 bp and 1,500 bp, and were deposited in the GenBank nucleotide database on the NCBI website with the accession numbers MZ664043 and MZ664044. Twelve and eleven of the *16S rRNA* gene sequences of known bacteria were used to construct phylogenetic trees, respectively. Phylogenetic relationships were inferred through the *16S rRNA* gene sequence alignment and cladistic analysis of homologous nucleotide sequences (Figure 2). Based on the phylogenetic tree, strain M1-8 was similar to *Burkholderia plantarii* PG1 (CP002581) with a homology of 99.33% and was identified as *Burkholderia plantarii* M1-8, and strain M6-4 was similar to *Burkholderia glumae* GX1 (MN400210) with a homology of 98.93% and was identified as *Burkholderia glumae* M6-4.

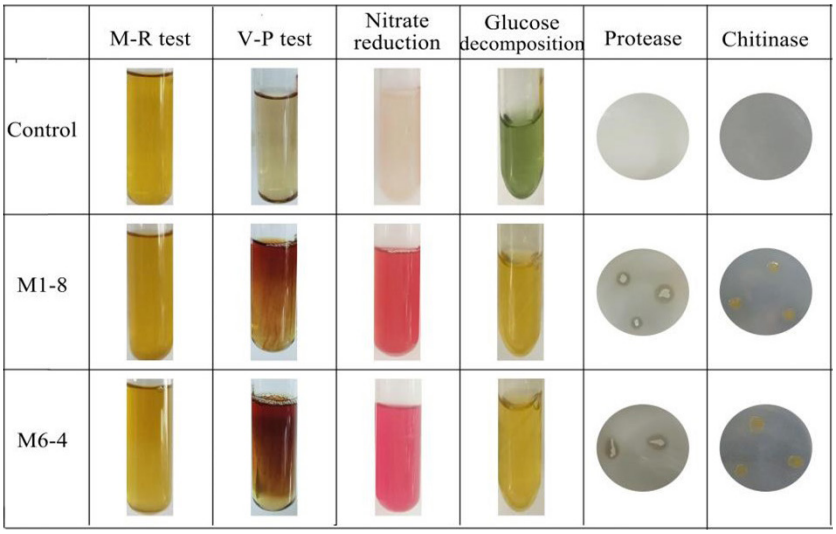

Figure 1. Physiological, biochemical and hydrolases tests of strains M1-8 and M6-4.

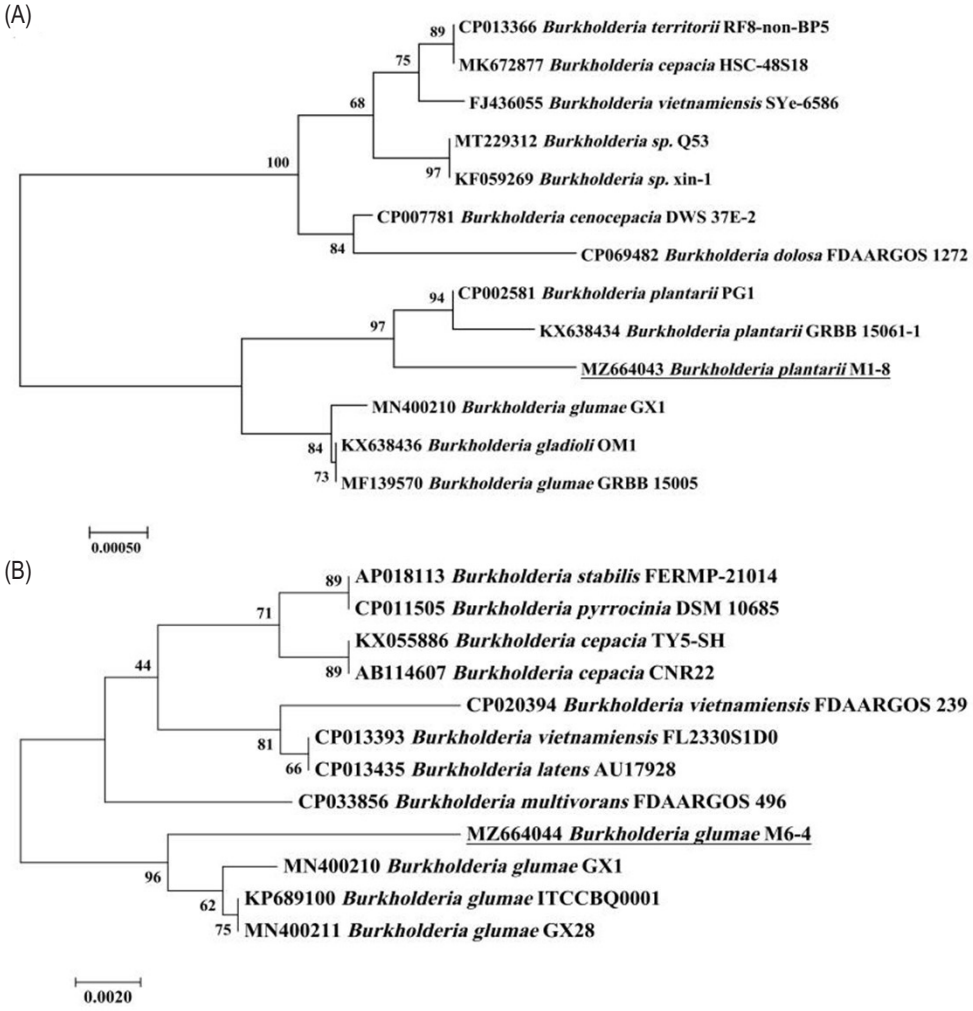

Figure 2. Phylogenetic trees constructed based on *16S rRNA* gene sequences. (A) The phylogenetic tree of M1-8. (B) The phylogenetic tree of M6-4.

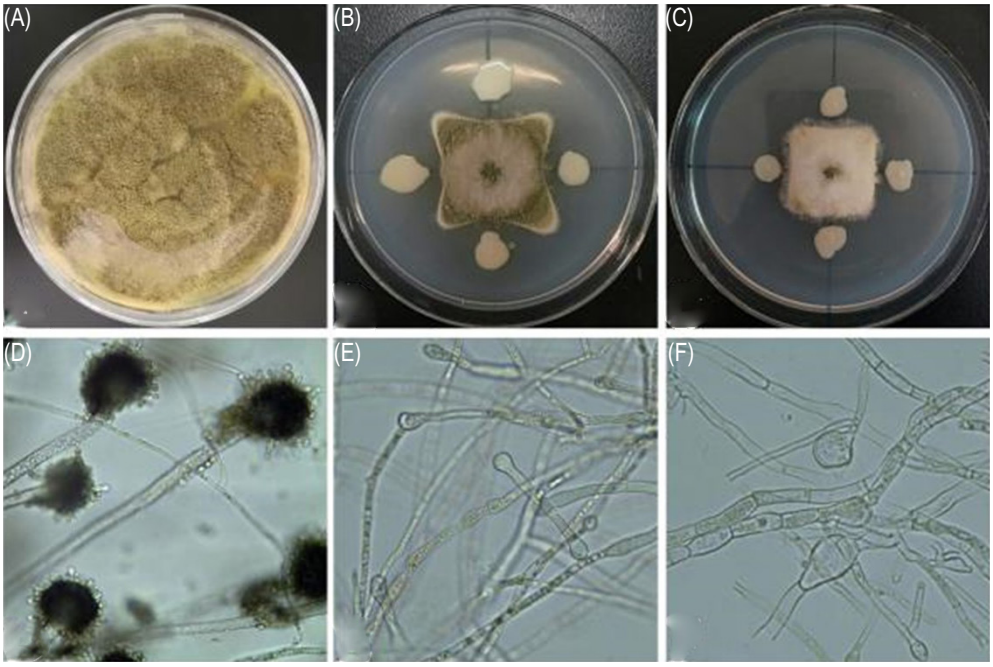

Figure 3. Inhibitory effects of *B. plantarii* M1-8 and *B. glumae* M6-4 and their culture filtrate on *A. flavus*. (A) Normal growth of *A. flavus* (Control). (B) Inhibition of M1-8 on *A. flavus*. (C) Inhibition of M6-4 on *A. flavus*. (D) Mycelial morphology of normal *A. flavus*. (E) Mycelial morphology of *A. flavus* treated by M1-8 culture filtrate. (F) Mycelial morphology of *A. flavus* treated by M6-4 culture filtrate. Images were taken at 400× under a light microscope.

In vitro inhibition analysis of strains and their culture filtrate on *A. flavus*

In vitro inhibition effects of M1-8, M6-4 and their culture filtrate on *A. flavus* are shown in Figure 3. In PDA plates, *B. plantarii* M1-8 and *B. glumae* M6-4 both had inhibition zones and showed strong inhibitory effects on *A. flavus* (Figures 3B, C). Compared to the normal mycelia and propagules in Figure 3D, no significant propagules were found in the culture filtrate treatment group (Figures 3E, F). The treated mycelia did not grow well and showed roughness on the surface and swelling at the tip.

Biocontrol effects of culture filtrate on moldy peanut kernels

In the biocontrol test, peanut kernels treated by SA, M1-8 and M6-4 culture filtrate exhibited a slight moldy infection after 5 d of incubation. Only a small amount of *A. flavus* was found on the peanut kernels in the treatment group, while the peanut kernels in the control group were significantly infected with *A. flavus*. (Figure 4A). As shown in Figure 4B, the moldy rates of peanut kernels treated with culture filtrate gradually increased with increasing days and much lower as compared to of the control group. Moreover, biocontrol of the culture filtrate of M1-8 and M6-4 reached 88.6% and 84.2%, respectively, which were higher than that of the SA treatment group

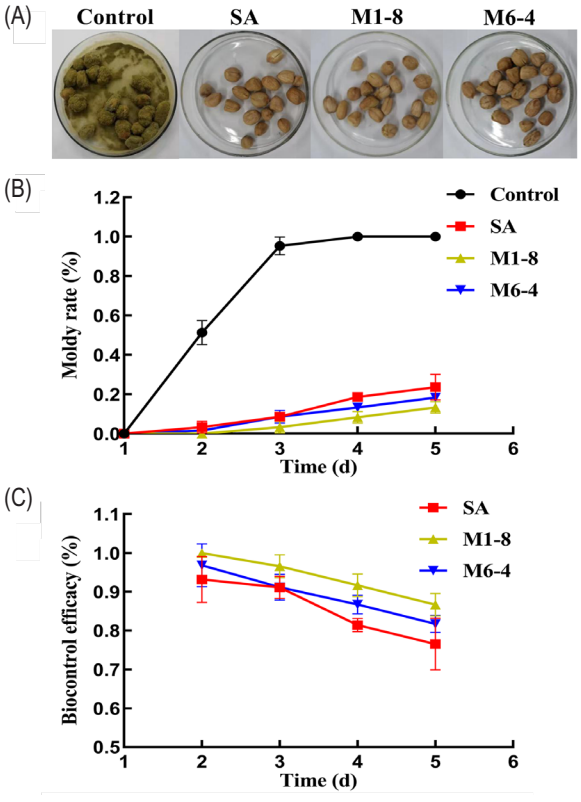

Figure 4. Biocontrol effects of M1-8 and M6-4 culture filtrate and SA on *A. flavus*. (A) Moldy appearance on peanut kernels. (B) Moldy rate of peanut kernels. (C) Biocontrol efficacy of SA, M1-8 and M6-4 culture filtrate.

(Figure 4C). These results showed that strains M1-8 and M6-4 had good control effects on *A. flavus* in moldy peanut kernels.

Antifungal activity stability of M1-8 culture filtrate

B. plantarii M1-8 culture filtrate treated with proteinase K and high light showed no significant change in inhibitory activities and exhibited stable antifungal activity against *A. flavus* (Figures 5A, B). The antifungal activity of the culture filtrate treated with different pH was relatively stable at pH 2~10. But a strong alkaline level resulted in a significant decrease in the antifungal activity (Figure 5C). When the culture filtrate was treated at different temperatures, there was no significant difference in antifungal activities between 60°C and 70°C and the antifungal activity remained at 59.63% at 70°C (Figure 5D). However, as the temperature increased above 70°C, the inhibition rate decreased significantly. These results indicated that the culture filtrate was tolerant to proteinase K, high light, normal pH and temperature (below 70°C), and had good stability of antifungal activity.

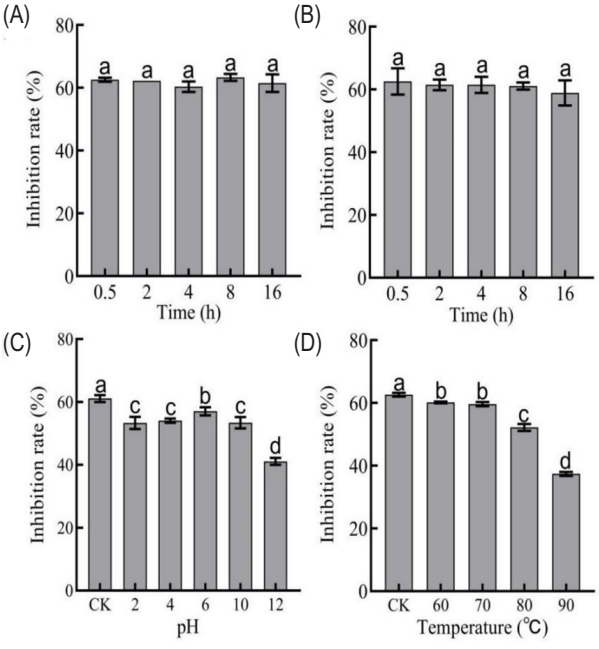

Figure 5. Stability of antifungal activity of *B. plantarii* M1-8 culture filtrate treated with protease K, light, pH and temperature. (A) Protease K treatment. (B) 4500 ± 500 lx illuminated light treatment. (C) pH treatment. (D) Temperature treatment. CK represents the control. Different lowercase letters on the bars indicate the significant difference at $p<0.05$. The same letter represents no significant differences between treatments.

Discussion

With the high-quality development of the food industry, exploring green and effective ways to control *A. flavus* contamination in post-harvest peanuts became a new orientation in this field (Ma *et al*., 2021; Sun *et al*., 2021). Biocontrol methods appear to be promising in reducing mycotoxin contamination of cereals to improve the sustainability of food and feed production. In order to isolate the antagonistic bacteria against *A. flavus* for controlling aflatoxin, the bacteria in the moldy samples were isolated and screened. The method is based on the concept that biocontrol agents isolated from the object to be applied are more suitable than microorganisms isolated from other sources. This approach has been used in many studies, including the selection of an endophytic *Streptomyces* spp. DEF09 from wheat roots as an antagonist against *Fusarium graminearum* (Colombo et al., 2019), 13 yeast strains isolated from pistachio samples inhibiting the growth of *A. flavus* (Moradi *et al*., 2020), 16 bacteria isolates against *Aspergillus* spp. screened from idli batter and other fermented foods (Santosh *et al*., 2021), and yeasts isolated from plants being tested as potential biocontrol agents to reduce AFB1 production on hazelnuts (Dikmetas *et al*., 2023). In this study, antagonistic bacteria against *A. flavus* were isolated from the moldy stuff and identified as *B. plantarii* M1-8 and *B. glumae* M6-4.

The plate confrontation method can assess the growth inhibition on the pathogen of the potential antagonist strain (Mohd Azuar Hamizan *et al*., 2022). As shown in Figure 3, *B. plantarii* M1-8 and *B. glumae* M6-4 showed inhibition effects on the growth of *A. flavus*. The results are in agreement with those reports of the inhibition on the fungal growth of *Burkholderia* spp. (Zeidan *et al*., 2019; Andrade *et al*., 2021). The pathogenic mycelia treated with culture filtrate exhibited abnormalities and no significant propagules. Based on Figure 1, protease was observed to be produced by two strains. It is speculated that protease may play a role in inhibiting the growth of pathogens. This is consistent with the report that active substances produced from culture filtrate have great practical value (WoldemariamYohannes *et al*., 2020).

To further investigate the biocontrol potential of *B. plantarii* M1-8 and *B. glumae* M6-4, in the present study, biocontrol test showed that the culture filtrates of M1-8 and M6-4 both had strong control efficacy on *A. flavus* and were more effective than SA, indicating that some bioactive metabolites in the culture filtrate could play an inhibitory role on moldy peanut kernels. Strains M1-8 and M6-4 should be suggested as potential growth inhibitors against *A. flavus*.

Effective application of a biocontrol agent depends upon being familiar with the biological environment where the agent is to be used (Wang *et al*., 2020). Previous reports indicated that the inhibitory activity of antagonistic bacteria culture filtrate may be affected after treatment with different factors, including protease, natural light, ultraviolet light, pH and temperature (Hasani *et al*., 2018; Jiang *et al*., 2019). The inhibition rate of the M1-8 culture filtrate had no obvious decrease after protease K treatment, which indicated that proteases K did not affect the inhibition activity of the culture filtrate, which agrees with the previous report of Zhang *et al*. (2020). When the culture filtrate was exposed to light, the inhibition rate showed no significant changes. It indicated that M1-8 culture filtrate was tolerant to light, which was consistent with the results of Jiang *et al*. (2019). The inhibition rate of *Streptomyces deccanensis* QY-3 culture filtrate remained more than 80% after treatment at 100°C for 1 h (Gu *et al*., 2020). Ahsan *et al*. (2017) reported that the antifungal activity of the cultural filtrate of the *Streptomyces* strain KX852460 remained the same at 60–90°C. However, the inhibition rate of M1-8 culture filtrate gradually decreased when the temperature was above 70°C, indicating the culture filtrate cannot endure high temperatures. In addition, M1-8 culture filtrate did not have tolerance to strong alkali, which was in agreement with the results of intolerance to strong acids and bases in the culture filtrate (Jiang *et al*., 2019). The results are important for developing the new biocontrol agents for controlling *A. spergillus* during peanut storage.

Conclusions

This study aimed to screen out antagonistic strains for effective control of *A. flavus*. Two antagonistic strains were identified as *Burkholderia plantarii* M1-8 and *Burkholderia glumae* M6-4. Culture filtrate of both strains could decrease the mycelial growth of *A. flavus*, and their biocontrol efficacies were higher than of the SA treatment group. Furthermore, M1-8 culture filtrate is relatively stable to proteinase K, high light, pH 2~10 and temperature (below 70°C) and has good stability of antifungal activity. Further research is needed to extract and identify the active substances for developing effective fungicides to control *A. flavus* contamination.

Authors' contributions

Yanjie Yi and Youtian Shan wrote the manuscript and designed the research; Youtian Shan, Yu Lou, Zhiwen Ning, Qingyao Zhang, Yingao Yang and Yuqian Liang performed the experiments; Youtian Shan, Zhipeng Hou and Jinsheng Shi made the data analysis; Yanjie Yi revised the manuscript. All authors read and agreed to the published version of the manuscript.

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Conflicts of interest

The authors declare no conflict of interest.

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