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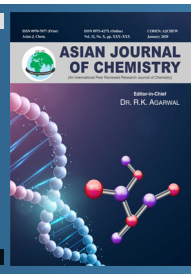

Screening, Identification and Controlling Effect of Antifungal Compound from *Bacillus megaterium* LB01-16

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In order to screen, identify high active compound and investigate its controlling effect on anthracnose disease in postharvest mangos, the bioassay-guided fractionation was used to screen and isolate antifungal components from fermented broth of *Bacillus megaterium* LB01-16 and the inhibition zone diameter of active components isolated against *Colletotrichum gloeosporioides* were measured with a disk diffusion test. The results revealed that four monomer compounds were separated from the fermentation broth, and only one monomer with strong antifungal activity, numbered 007, was obtained and identified as glucopyranosyl 12-hydroxyjasmonic acid by ^{1}H & ^{13}C NMR and by comparing with literature. This study confirmed that the glucopyranosyl 12-hydroxyjasmonic acid produced from *Bacillus megaterium* LB01-16 can inhibit the anthracnose disease of postharvest mangos by directly reducing mycelium growth and inhibiting the spore germination of *Colletotrichum gloeosporioides*, thus providing a promising strategy for controlling of anthracnose disease in postharvest mangos.

Keywords: *Bacillus megaterium*, Anthracnose disease, Postharvest mangos, *Colletotrichum gloeosporioides*, Controlling effect.

INTRODUCTION

Bacillus megaterium is an important microorganism in the food industry, has been widely used in the control of rice sheath blight, tomato gray mold and *Aspergillus flavus* in the storage period of peanut [1-3]. Most important of all, the glutamic acid decarboxylase produced by *Bacillus megaterium* CICC 10055 can catalyze the production of γ-aminobutyric acid (GABA) from glutamic acid [4]. GABA, as an important neurotransmitter in the mammalian nervous system, possesses the functions of reducing blood pressure, sedation, and diuresis [5,6]. Moreover, the fatty acid and its derivatives produced by *Bacillus megaterium* L2 had strong antimicrobial activity against EC-1 in *Amorphophallus konjac* K. Koch during storage [7]. Recently, the fermentation filtrate from *Bacillus megaterium* LB01 that have been reported better antifungal effect against *Colletotrichum gloeosporioides* in postharvest mango [8].

In recent years, applications of jasmonic acid and its derivatives (*e.g.* methyl jasmonate, jasmone, propyl dihydrojasmonate, 12-hydroxyjasmonic acid and its 12-O-β-D-glucoside, *etc.*) have attracted much more attention in the food industry [9]. Many researchers have found that jasmonic acid and its derivatives can effectively decrease the ethylene release of blueberry as well as reduce the decay rate of blueberry [10], prolong the anti-aging activity of fresh-cut pine apples [11] and its derivatives have strong inhibition effect on *Colletotrichum gloeosporioides* in grapes [12], soft rot fungus in kiwi fruits [13] and green plum pathogen in apples [14]. In addition, jasmonate derivatives can interact with mitochondria of human cancer cells, resulting in swelling of mitochondria [15]. Among them, 12-hydroxyjasmonic acid has a good inhibitory effect on *Pseudomonas aeruginosa*, which is easily infected by cancer patients in clinic [16]. At the same time, 12-hydroxyjasmonic acid is often used as flavouring agent in tropical flavour food [17]. Various studies have shown that the novel jasmonate derivatives could be applied in medicines and pharmacology [18,19]. Therefore, jasmonic acid and its derivatives with antimicrobial effect can be used safely in the prevention and treatment of food microbial diseases.

In continuation of our ongoing research on the development of novel eco-friendly fungicide that can replace traditional chemicals, *Bacillus megaterium* LB01-16 was chosen as research

interest. Bioassay-guided isolation and purification of active components 005-008 in the fermentation filtrate of the strain LB01-16 were performed. Finally, compound 007 is screened by Kirby-Bauer diffusion method and its inhibitory effect on *Colletotrichum gloeosporioides* in postharvest mango *in vitro* and *in vivo* is further tested. This study provides the resource of active component for the control of anthracnose disease in postharvest mango fruits.

EXPERIMENTAL

The chemical reagents utilized were of reagent grade in the process of isolation and purification of active compounds produced by *Bacillus megaterium* LB01-16. The purity of active components was monitored by TLC and HPLC. NMR spectra were recorded at 600 (^{1}H) on a Bruker-Avance III Plus 600 spectrometer in DMSO-d_6 using TMS as internal reference. *Colletotrichum gloeosporioides* used as pathogen and the strain LB01-16 was obtained from the Key Laboratory of Regional Ecological Environment Analysis and Pollution Control in Western Guangxi, P.R. China.

Typical procedure of bioassay guided isolation and purification: Strain LB01-16 fermentation broth (48 L) was filtered to collect fermentation filtrate, which was concentrated under reduced pressure to obtain the extractum [8]. The extractum was extracted three times with AcOEt to obtain 29 g extract. The extract was separated by silica gel column chromatography, CH_2Cl_2-CH_3OH (60:1, 15:1, 1:15, 6:1, v:v) was used for gradient elution. Inactive fraction was rejected. The active fractions were combined into fraction 1 and 2 according to TLC, HPLC and Kirby-Bauer analysis [20,21]. In order to isolate the monomers from active fraction 1 or 2 again, silica gel, Sephadex-LH20 column chromatography and TLC were applied alternatively. The activity of each fraction or monomer was screened according to the diameter of inhibition zone measured by Kirby-Bauer diffusion method [21]. Finally, four antifungal compounds (005~008) were obtained, however compound 007 possessed stronger antifungal activity against *C. gloeosporioides*.

Structural identification of antifungal compound 007: Compound 007 isolated from the fermentation broth was known. Therefore, ^{1}H & ^{13}C NMR techniques were used to identify its structure compared with the data reported in the literature.

Effect of compound 007 on conidial germination rate of pathogen: *in vitro* bioassay of effect of compound 007 on conidial germination rate was performed on the basis of previous work [8]. Compound 007 of different concentrations, PDB liquid medium and conidial suspension were added together to a 1.5 mL sterile centrifuge tube to make the final concentrations 5, 6 and 7 mg/L, respectively. Mixed solution (100 µL) was applied on the slide, and then put it into a culture dish with wet filter paper, and cultured at 25-26 ºC. When the length of conidial germ tube exceeds the width, is regarded as germination. The germination rate and elongation of conidial germ tube after treated with compound 007 at different time were counted and found that the total number of conidia in random observation field was not less than 100. The same amount of sterile water was added as the control. Each process is set up two parallel groups and the experiments were performed twice.

$$\text{Conidial germination rate (\%)} = \frac{\text{Conidial germination number}}{\text{Total number of investigated conidia}} \times 100$$

Effect of compound 007 on mycelium growth: *in vitro* antifungal effect of compound 007 was tested according to mycelium growth rate method [22]. Compound 007 (10 mL) of different concentrations were added to 90 mL PDA medium under aseptic conditions and the final concentration was 5, 6 and 7 mg/L, respectively. The edge of a 7-day old colony was pressed with sterile puncher whose diameter is 5 mm. The fungus cake was transferred to the center of the drug containing medium, which was sealed and cultured at 24-26 ºC. Colony diameters were measured with cross method on 1, 3 and 5 d, respectively. The same amount of sterile water was added as control. Each process is set up three parallel groups and the experiment was performed three times.

Effect of compound 007 on lesion extension on mango fruits: Mango fruits with uniform size and colour and no mechanical damage were selected and soaked in 2% sodium hypochlorite solution (bleach solution) for 2 min, then washed with sterile water and dried in the air. Mango was wounded with a sterile injector in the waist of each fruit. Conidia suspension (10 µL) of pathogen [9] was injected into the wound of mango, and 1 h later, the injected solution was dried, followed by 20 µL compound 007 (75 , 150 mg/L, respectively). After drying, mango was placed in a sterilized fresh box and stored at 24-26 ºC. On 2, 4, 6 and 8 days, the incidence of fruit disease was recorded and the lesion diameter was measured by cross method. The same amount of sterile water was injected into the wound as control and the experiment was replicated three for each 20 fruits.

Treatment of data: The data were processed by SPSS 19.0 software. Duncan multiple comparison method was adopted to analyze significant difference ($p < 0.05$) and extremely significant difference ($p < 0.01$).

RESULTS AND DISCUSSION

Isolation of compound 007 with strong fungistasis: It can be observed by the method of Kirby-Bauer that filtered paper containing 0.5 mg/L of compounds 005-008 cannot produce the inhibition zone against the pathogen, while that containing 5.0 mg/L of compound 005-008 can produce the inhibition zone with a certain diameter.

On the basis of activity results, highly effective fractions 1 & 2 were screened, whose diameter of inhibition zone were 22.18 ± 0.02 and 18.16 ± 0.09 mm, respectively. The fractions 1 & 2 were further isolated and compounds 005-008 were obtained, where compound 007 possessed strong fungistasis as compared to compounds 005, 006 and 008 (Table-1).

Structural identification of compound 007: The ^{1}H & ^{13}C NMR data of the isolated compound 007 were compared with the literature [23], which confirmed as 3*R*,7*R*-(-)-β-D-glucopyranosyl 12-hydroxyjasmonic acid. White crystal (recrystallized from ethanol). ^{1}H NMR (600 MHz, DMSO-d_6): 1.53 (1H, m, H-4b), 2.24 (1H, d, J = 8, 11 Hz, H-7), 2.29 (1H, d, J = 10, 15 Hz, H-2b), 2.33 (1H, d, J = 10, 19 Hz, H-4a), 2.50 (1H, m, H-3), 2.54 (2H, q, J = 8 Hz, H-11), 2.57 (2H, q, J = 8

TABLE-1
INHIBITION ZONE DIAMETERS PRODUCED BY SM 005-008 AGAINST *Colletotrichum gloeosporioides* BY KIRBY-BAUER DIFFUSION METHOD

Compound (mg/L)	005 (5.0)	006 (5.0)	007 (5.0)	004 (5.0)	Control (0.0)
Inhibition zone diameter (mm)	9.52 ± 0.12^{b}	8.72 ± 0.10^{b}	21.00 ± 1.64^{a}	10.28 ± 0.14^{b}	–

There are significant differences between different letters (a, b) in the same line ($p < 0.05$).

Hz, H-8), 2.71 (1H, d, J = 6, 15 Hz, H-2a), 3.28 (1H, d, J = 9, 10 Hz, H-14), 3.40 (1H, t, J = 10 Hz, H-16), 3.46 (1H, d, J = 3, 7 Hz, H-17), 3.48 (1H, t, J = 10 Hz, H-15), 3.73 (1H, d, J = 7, 13 Hz, H-18b), 3.83 (1H, d, J = 8, 11 Hz, H-12b), 3.91 (1H, d, J = 3, 13 Hz, H-18a), 4.03 (1H, d, J = 11 Hz, H-12a), 4.47 (1H, d, J = 9 Hz, H-13), 5.60 (1H, d, J = 8, 12 Hz, H-9), 5.65 (1H, d, J = 8, 12 Hz, H-10); ^{13}C NMR (200 MHz, DMSO-d_6): 24.6 (C-11), 26.8 (C-4), 27.8 (C-8), 39.2 (C-2), 40.2 (C-5), 41.2 (C-3), 53.8 (C-7), 64.1 (C-18), 68.7 (C-12), 71.7 (C-16), 75.1 (C-14), 77.7 (C-17), 77.8 (C-15), 105.2 (C-13), 127.5 (C-9), 130.42 (C-10), 185.0 (C-1), 229.6 (C-6).

Inhibitory effect of compound 007 against conidial germination rate of pathogen: Compound 007 can effectively inhibit conidial germination and tube elongation (Fig. 1). Control conidial was germinated after 6 h and length of the germ tube was grown to 345.44 µm after 12 h. However, 6.17% of conidia germinated and the length of the germ tube was 5.96 µm when treated with 6 mg/L of compound 007 for 12 h, however, the conidia did not germinate when treated with 7 mg/L of compound 007 for 12 h.

Inhibitory effect of compound 007 against mycelial growth: Compound 007 has a significant inhibitory effect on the mycelial growth of pathogen and the difference between different concentration is also significant (Fig. 2). The antifungal effect increased with the increase of concentration. The colony diameter of the control was 65.29 mm on 5th day however, the colony diameter when treated with 5, 6 and 7 mg/L compound 007 was 51.6, 37.24 and 26.72 mm, respectively.

Inhibitory effect of compound 007 against Lesion extension on mango fruits: The lesion diameter of mango fruit in each group increased gradually with storage time (Fig. 3). However, the lesion diameter when treated with 75 and 150 mg/L of compound 007, was significantly smaller than that in the control (Fig. 3a) and the difference of each concentration was significant ($p < 0.05$). The antifungal effect increased with the increase of concentration. On 8th day, the lesion diameter

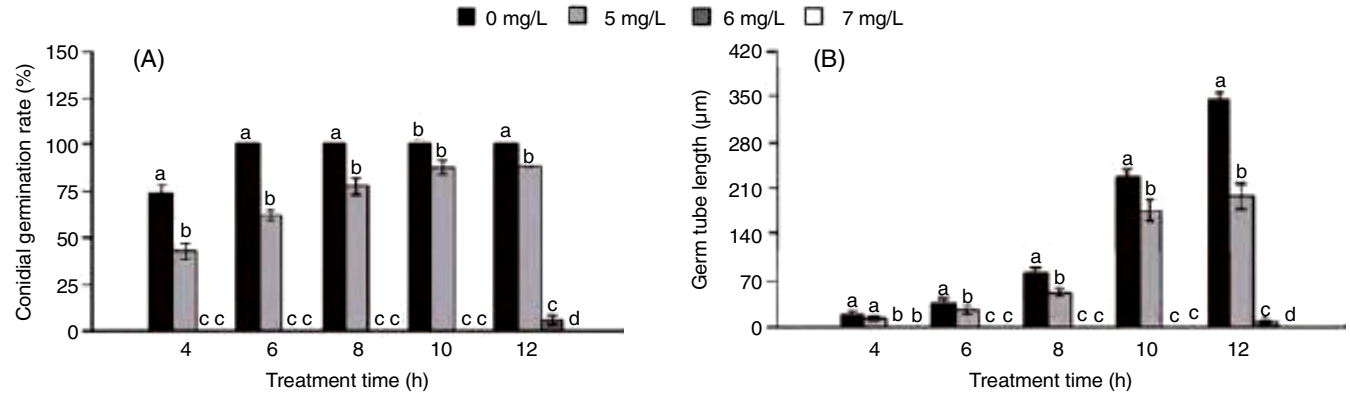

Fig. 1. Effect of compound 007 on conidial germination and germ tube elongation of *Colletotrichum gloeosporioides*. Process followed by different letters (a, b, c, d) are significantly different by the Duncan's multiple range test ($p < 0.05$)

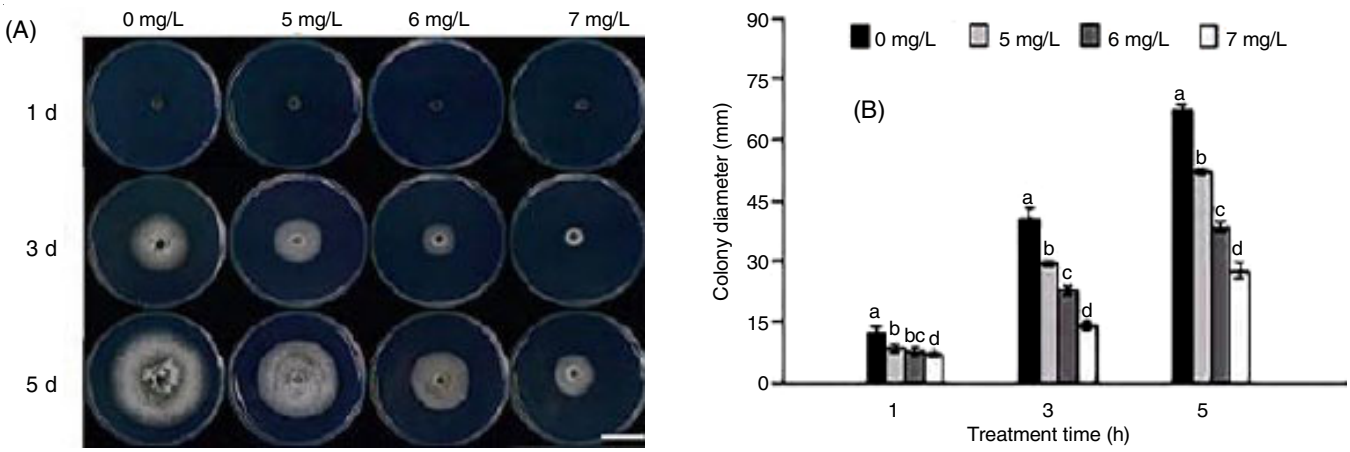

Fig. 2. Effect of compound 007 on colony growth of *Colletotrichum gloeosporioides*. Process followed by different letters (a, b, c, d) are significantly different by the Duncan's multiple range test ($p < 0.05$)

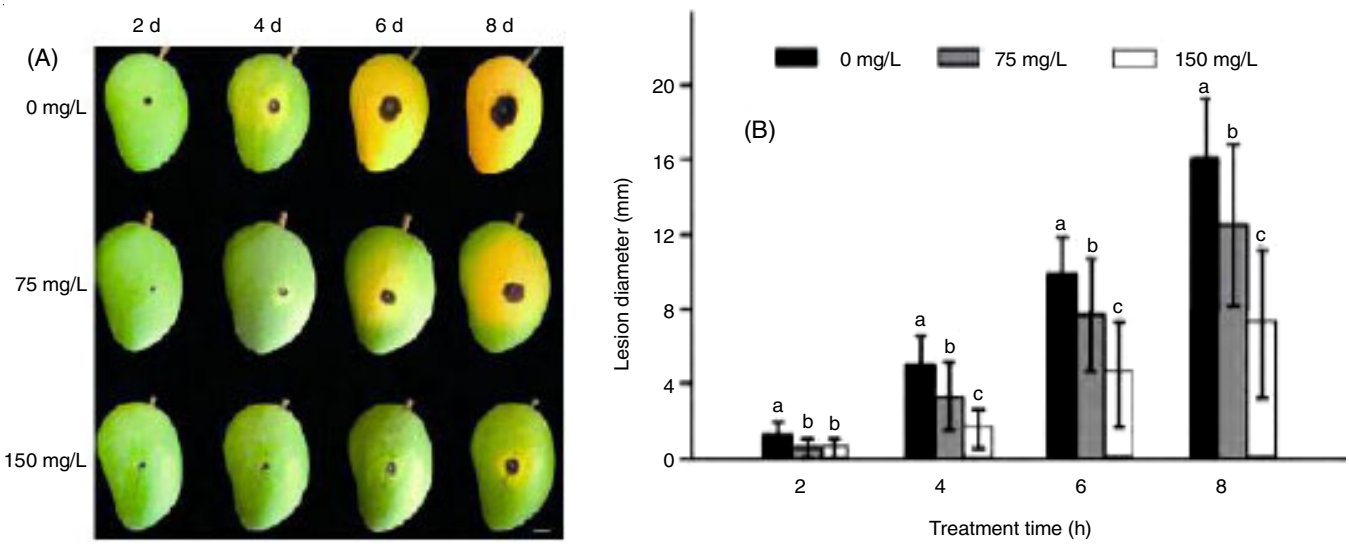

Fig. 3. Effect of compound 007 on the lesion extension of anthracnose in mango fruits. Process followed by different letters (a, b, c) are statistically different by the Duncan's multiple range test ($p < 0.05$)

of the control was 15.80 mm, which when treated with 75 and 150 mg/L compound 007 was only 11.7 and 7.04 mm, respectively, thus a decrease of 23.18 and 53.26%, respectively was observed.

Conclusion

In this work, four compounds 005-008 were isolated by chromatography from the fermentation broth of *Bacillus megaterium* LB01-16 in the special environment of Guangxi karst area of P.R. China. Compound 007 possessed stronger antifungal action agaist *Colletotrichum gloeosporioides* and its structure were identified as 3*R*,7*R*-(-)-β-D-glucopyranosyl 12-hydroxyjasmonic acid by ^{1}H & ^{13}C NMR. Further, glucopyranosyl 12-hydroxyjasmonic acid (compound 007) was used for the control of postharvest anthracnose in mango fruits. The results showed that it had dose-dependence and exhibit significant inhibitory effect. It could effectively inhibit the conidial germination, germ tube elongation and mycelium growth, indicating that compound 007 had the potential to be applied to the control of postharvest anthracnose in mango fruits.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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