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Study on the antifungal effect and mycolytic activity of the biocontrol agent *Chaetomium subaffine* LB-1

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Abstract: The antifungal effect and mucolytic activity of a newly screened biocontrol strain *Chaetomium subaffine* LB-1 were researched in this study. The results found that LB-1 has good antifungal effects on the test plant pathogenic fungi *Botrytis cinerea* Pers. ex Fr., *Fusarium oxysporum* f. sp. *cucumerinum* Owen and *Alternaria solani* (Ellis & Martin) Sorauer in dual culture assay, with the inhibition rates of 61.39, 50.34 and 51.22%, respectively. Flocculated and dissolved hyphae of the phytopathogenic fungi were observed at the interaction zone on a dual-cultured PDA plate, but the hyphae of LB-1 were normal. The LB-1 cell-free filtrate has significant inhibitory effects on the three tested fungi in the poison plate assay; dissolved colonies, vesiculated and flocculated hyphae of the test pathogenic fungi were also found on the PDA plates supplemented with the LB-1 cell-free filtrate. Clear halo zones around the LB-1 colonies were found on the protease test plate, pectinase test plate and cellulose test plate, indicating that LB-1 could produce mucolytic enzymes of protease, pectinase and cellulase. However, the activities of chitinase and β-l,3-glucase were not detected on their test plates from LB-1. An obvious oil-displaced circle was formed in the oil spreading test, indicating that a surface-active substance might be contained in the LB-1 cell-free filtrate. These results proved that the biocontrol agent of *C. subaffine* LB-1 could exert its antifungal effects via living competition and mycolysis, and the latter may be obtained by production of mycolytic enzymes and surface-active substances.

Keywords: biocontrol strain LB-1; cell-free filtrate; hyphae dissolution; living competition; mycolytic enzyme; surface--active substance

Biocontrol agents have proven to be an eco-friendly strategy to control plant diseases without negative effects on plants (Cook 1993). Now, many biocontrol agents of plant disease, such as *Trichoderma* spp. (Monte 2001; Prasad et al. 2016), *Pseudomonas* spp. (Stephan et al. 2016), *Bacillus* spp. (Narasimhan & Shivakumar 2016), *Streptomyces* spp. (Lu et al. 2016) etc., have been extensively reported upon. It is generally proven that biocontrol agents could obtain their biocontrol effects via living competition, mycoparasitism, resistance induction and mycolysis (Gao et al. 2005; Abdallah et al. 2016).

Mycolysis is an important mode of action for biocontrol agents and could lead to the hyphae dissolution of the pathogen (Mitchell & Alexander 1963; Alfonso et al. 1994). There are usually three ways for biocontrol agents to achieve their mycolytic activity (Aktuganov et al. 2008): The first one is the production of antibiotics, which could inhibit the synthesis of the fungal cell wall (Debono

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& Gordee 1994); the second one is the secretion of mycolytic enzymes, which could lead to the degradation of the fungal cell wall (Sivan & Chet 1989; Parafati et al. 2015); the third one is the production of surface-active substances, which could destroy the permeability of the fungal cell membrane (Maget-Dana & Peypoux 1994). Mycolytic enzymes of proteases, pectinase, cellulose, chitinase and β-1,3-glucanase play important roles in the mycolysis of biocontrol agents and have been analysed extensively (Viterbo et al. 2002; Hernandez-Montiel et al. 2018). Protease and β-1,3-glucanase could degrade the laminarin and destroy the integrity of the fungal cell wall (Flores et al. 1997; Vazquez-Garciduenas et al. 2018), pectinase could degrade α-1,4 glycosidic bonds of the pectin polymers and induce the plants' resistance (Kikot et al. 2009; Abdallah et al. 2016), cellulase and β-1,3-glucanase can degrade the cell wall of pathogenic fungi and are of important in mycoparasitism (Ramot et al. 2000; Markovich & Kononova 2003). Chitin is a structural polymer of many fungi and can be degraded by chitinase, which could open the C_1 and C_4 bonds in N-acetyl-glucosamine (GlcNAc) and take effect in the interaction between biocontrol agents and pathogens (Sahai & Manocha 1993). At present, biocontrol agents, such as *Chaetomium spirale* ND35 (Guo et al. 2005), *Bacillus subtilis* JN032305 (Ashwini & Srividya 2014), *Trichoderma viride* NBAII Tv 23 (Parmar et al. 2015), *Streptomyces albospinus* CT205 (Wang et al. 2016) and *S. griseorubens* E44G (Al-Askar et al. 2015), etc., have been reported to produce mycolytic enzymes in controlling plant disease.

Chaetomium spp. are considered to be promising in plant disease control (Soytong et al. 2001). In recent years, the biocontrol effects of *Chaetomium* spp. have been constantly reported on (Aggarwal et al. 2004; Shanthiyaa et al. 2013; Phong et al. 2016), and the antifungal effects of *Chaetomium subaffine*, one species of *Chaetomium* spp., have also been found (Xu et al. 2014; Abro et al. 2019). LB-1 is a newly isolated biocontrol strain identified as *Chaetomium subaffine*, which exhibited prominent antagonistic effects on plant pathogenic fungi of *Exserohilum turcicum*, *Fusarium oxysporum* f. sp. *niveum*, *Bipolaris maydis*, *Botrytis cinerea* and *Phytophthora infestans* in our previous research (Liu et al. 2015, 2018). The aim of this study was to further clarify the antifungal effect and mycolytic activity of the LB-1 strain on plant pathogenic fungi.

MATERIAL AND METHODS

Fungal isolates and culture medium. *Chaetomium subaffine* Sergeeva LB-1 is a novel biocontrol strain screened by our research group, which has been preserved both in our laboratory and in the China General Microbiological Culture Collection Center (CGMCC). *Botrytis cinerea* Pers. ex Fr., *Fusarium oxysporum* f. sp. *cucumerinum* Owen and *Alternaria solani* (Ellis & Martin) Sorauer were used as the test pathogenic fungi in this research. *B. cinerea*, *F. oxysporum* f. sp. *cucumerinum* and *A. solani* are common plant pathogenic fungi and have been preserved and used for experimental research in our laboratory for many years, they can cause tomato grey mould, cucumber wilt and potato early blight, respectively. The biocontrol strain LB-1 and the three species of test pathogenic fungi were precultured on potato dextrose agar (PDA; 20% filtered potato juice, 2% dextrose, 2% agar) for three days before being used in this research.

Analysis of the antifungal effect and mycolytic property of LB-1. The antifungal effect of LB-1 was determined using a dual culture assay with *B. cinerea*, *F. oxysporum* f. sp. *cucumerinum* and *A. solani* as the test phytopathogenic fungi. Briefly, mycelial discs (6 mm in diameter) of LB-1 and the three test phytopathogenic fungi were obtained from their three-day-old colonies. Two mycelial discs (one each for LB-1 and the test fungi) were placed upside-down and opposite to each other on a PDA plate (90 mm in diameter) and cultured (25 °C, 10 h dark and 14 h light per day for 5 days. A mycelial disc of each test pathogenic fungi was inoculated on a PDA plate (90 mm in diameter) and served as the corresponding control. The radii of the test pathogenic fungi on the control PDA plates (A) and dual-cultured PDA plates prolonged toward LB-1 (B) were measured, the inhibition rates were calculated per the following Equation (1) and the antifungal effect of LB-1 was evaluated (Siameto et al. 2010). Five replicates were set for each treatment.

$$Inhibition\ rate(\%) = \frac{(A-B)\times 100}{A} \quad (1)$$

The mycelia of LB-1 and the test fungi at the colony contact surface on the dual-cultured PDA plate were obtained, wet-mount slides were made and observed under a phase-contrast inverted microscope (TS100, Nikon, Japan).

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Analysis of the inhibitory effect and mycolytic property of LB-1 cell-free filtrate. The cell-free filtrate of LB-1 was obtained as follows: LB-1 was cultured by shaking (25 °C, 130 rpm) for 10 days in a potato dextrose broth (PDB; 20% filtered potato juice, 2% dextrose). The cultured broth was filtrated with three layers of sterile gauze, the filtrate was centrifuged (4 °C, 12 000 rpm) for 15 min and the supernatant was used as the cell-free filtrate of LB-1.

A poison plate assay was used to detect the inhibitory effect of the LB-1 cell-free filtrate as described by Zhang et al. (2010): The LB-1 cell-free filtrate was sterilised with a 0.22 μm Millipore and added in the PDA culture medium to get the final concentration of 25% (v/v). A 6-mm mycelial disc of each test plant pathogenic fungi was placed individually in the centre of the PDA plate (90 mm in diameter) modified with the LB-1 cell-free filtrate and incubated for 5 days (25 °C, 10 h dark and 14 h light per day). A PDA plate (90 mm in diameter) without any supplement was used as the control. Five replicates were set for each treatment. The colony diameters of the test pathogenic fungi were measured, the inhibitory effects of the LB-1 cell-free filtrate were analysed according to the inhibition rates calculated as follows (Equation 2).

The mycelia of the test plant pathogenic fungi on the LB-1 cell-free filtrate modified PDA plate were obtained, wet-mount slides were made, the characteristics of the mycelia were observed and the mycolytic property of the LB-1 cell-free filtrate was evaluated.

Detection of the mycolytic enzymes produced by LB-1. A test plate assay was employed to detect the mycolytic enzymes produced by LB-1 in this study. The protease activity of LB-1 was detected per the method described by Folman et al. (2003): A culture medium with a yeast extract (1.5 g/L), agar (18 g/L) was prepared. 20 mL of a 5% skim milk solution was mixed with the sterilised culture medium and a protease test plate was made. A 6-mm mycelial disc of LB-1 was inoculated in the centre of the test plate (90 mm in diameter) and incubated for 5 days (25 °C, 10 h dark and 14 h light per day), the appearance of a clear halo zone around the LB-1 colony indicates a positive result. Five test plates were set for the protease detection.

The pectinase activity of LB-1 was assayed per the method described by Ramzan et al. (2016): a culture medium with K_2HPO_4 (0.1 g/L), $MgSO_4$ (0.5 g/L), $NaNO_3$ (3 g/L), $FeSO_4$ (0.01 g/L), pectin (2 g/L), agar (18 g/L) was autoclaved and a 6-mm mycelial disc of LB-1 was inoculated in the centre of the test plate (90 mm in diameter) and incubated for 5 days (25 °C, 10 h dark and 14 h light per day). The plate was stained with a 0.2% Congo red solution and rinsed with a 1 mol/L NaCl solution, a clear halo zone around the LB-1 colony indicates a positive result. Five test plates were set for the pectinase detection.

The cellulase activity of LB-1 was assayed per the method described by Ramzan et al. (2016): A culture medium was made with carboxymethyl cellulose sodium (10 g/L), Na_2HPO_4 (2 g/L) and agar (18 g/L). The culture medium was autoclaved and a 6-mm mycelial disc of LB-1 was inoculated in the centre of the test plate (90 mm in diameter) and incubated for 5 days (25 °C, 10 h dark and 14 h light per day). The plate was stained with a 0.2% Congo red solution and rinsed with a 1 mo/L NaCl solution, a clear halo zone around the LB-1 colony indicates a positive result. Five test plates were set for the cellulase detection.

β-l,3-glucase activity of LB-1 was assayed with an agar medium supplemented with K_2HPO_4 (1 g/L), Na_2HPO_4 (3 g/L), $FeSO_4{\cdot}7\ H_2O$ (0.5 g/L), glucose (1 g/L), tuckahoe (0.01 g/L). The culture medium was autoclaved and 0.06 g/L aniline blue was added (Wang et al. 2014). A 6-mm mycelial disc of LB-1 was inoculated in the centre of the test plate (90 mm in diameter) and incubated for 5 days (25 °C, 10 h dark and 14 h light per day), a clear halo zone around the LB-1 colony indicates a positive result. Five test plates were set for the β-l,3-glucase detection.

The chitinase activity of LB-1 was assayed per the method described by Roberts et al. (1988): A 6-mm mycelial disc of LB-1 was inoculated in the centre of the culture plate (90 mm in diameter) with colloidal chitin (10 g/L) as the only carbon source, supplemented with $NH_4H_2PO_4$ (1 g/L), KCl (0.2 g/L), $MgSO_4{\cdot}7H_2O$ (0.2 g/L) and agar (18 g/L), pH 7.0, and incubated for 5 days (25 °C, 10 h dark and 14 h light per day), a clear halo zone around the LB-1 colony indicates a positive result. Five test plates were set for the chitinase detection.

Detection of the amphiphilic property of LB-1 cell-free filtrate. An oil spreading test was used to detect the amphiphilic property of the LB-1 cell-free

$$Inhibition\ rate(\%) = \frac{(diameter\ of\ control\ colony - diameter\ of\ treatment\ colony) \times 100}{diameter\ of\ control\ colony} \quad (2)$$

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filtrate as described by Chen et al. (2017): 1 mL liquid paraffin was added to the surface of distilled water in a petri dish to form a thin oil layer. Then, a 20 µL LB-1 cell-free filtrate was dripped in the centre of the oil layer. If an oil drainage ring is formed, it indicates that the LB-1 cell-free filtrate contains surface-active substances and has an amphiphilic property, and vice versa.

Statistical analysis. The least significant difference test (LSD) was conducted using the SAS system (version 8.1) with the statistical significance threshold set at $P = 0.05$. The data distribution were tested with the Shapiro-Wilk test of the SAS system.

RESULTS

Antifungal effect of LB-1 and its mycolytic property. The dual-cultured PDA plate assay showed that the colony extension of the test fungi *B. cinerea*, *F. oxysporum* f. sp. *cucumerinum* and *A. solani* were inhibited by the rapid growth of LB-1, with inhibition rates of 61.39, 50.34 and 51.22%, respectively (Table 1). Dissolved and flocculated hyphae of the plant pathogenic fungi were observed at the interaction zone between the two dual-cultured strains on the PDA plates under a microscope, while the hyphae of LB-1 were normal (Figure 1).

Inhibitory effect of the LB-1 cell-free filtrate and its mycolytic property. The statistical analysis indicated that, at a test level of $P = 0.05$, there were significant differences between the colony diameters of the same pathogenic fungi cultured on the PDA plates supplemented with the 25% (v/v) LB-1 cell-free filtrate and the corresponding controls. The inhibition rates of the LB-1 cell-free filtrate on the pathogenic fungi of *B. cinerea*, *F. oxysporum* f. sp. *cucumerinum* and *A. solani* were 24.92, 10.81, 20.70%, respectively (Table 2). The Shapiro-Wilk test showed that data of each treatment group and control group was consistent with the normal distribution. Obvious colony dissolution, hyphae swelling and flocculation of the plant pathogenic fungi on the LB-1 cell-free filtrate modified PDA plates could be observed (Figure 2).

Table 1. Antifungal effects of *Chaetomium subaffine* LB-1 on the three species of test plant pathogenic fungi

Plant pathogenic fungi	Inhibition rate (%)
B. cinerea	61.39
F. oxysporum f. sp. *cucumerinum*	50.34
A. solani	51.22

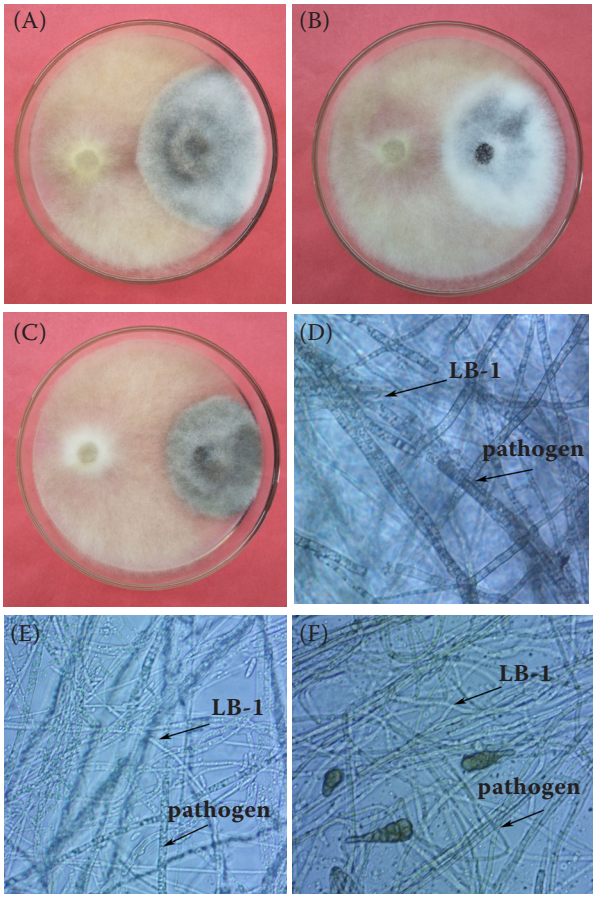

Figure 1. Colony extension and hyphae characteristics of the three species of test pathogenic fungi and *Chaetomium subaffine* LB-1 on a dual-cultured PDA plate

On each plate, LB-1 is on the left side, the pathogenic fungus is on the right; (A) dual-cultured *B. cinerea* and LB-1; (B) dual-cultured *F. oxysporum* f. sp. *cucumerinum* and LB-1; (C) dual-cultured *A. solani* and LB-1; (D) hyphae of *B. cinerea* and LB-1; (E) hyphae of *F. oxysporum* f. sp. *cucumerinum* and LB-1; (F) hyphae of *A. Solani* and LB-1

These results proved the antifungal effects and mycolytic property of the LB-1 cell-free filtrate.

Mycolytic enzymes produced by LB-1. The results of test plate assay showed that there were clear halo zones around the LB-1 colonies both on

Table 2. Inhibitory effects of *Chaetomium subaffine* LB-1 cell-free filtrate on the three species of test plant pathogenic fungi

Plant pathogenic fungi	Inhibition rate (%)
B. cinerea	24.92
F. oxysporum f. sp. *cucumerinum*	10.81
A. solani	20.70

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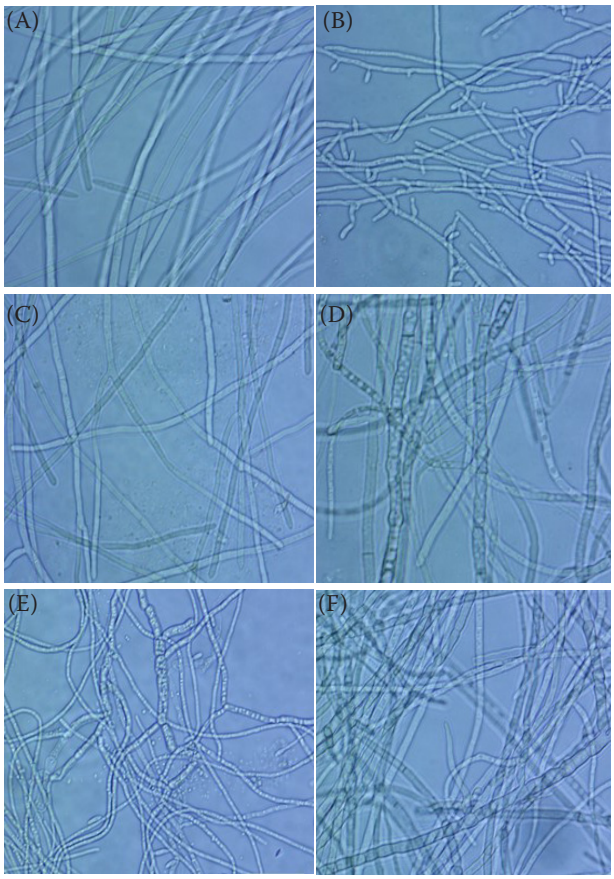

Figure 2. Characteristics of hyphae of the three species of test plant pathogenic fungi on PDA plates amended with/without LB-1 cell-free filtrate

(A) mycelia of *B. cinerea* on PDA plate without LB-1 cell-free filtrate; (B) mycelia of *F. oxysporum* f. sp. *cucumerinum* on PDA plate without LB-1 cell-free filtrate; (C) mycelia of *A. solani* on PDA plate without LB-1 cell-free filtrate; (D) mycelia of *B. cinerea* on PDA plate amended with LB-1 cell-free filtrate; (E) mycelia of *F. oxysporum* f. sp. *cucumerinum* on PDA plate amended with LB-1 cell-free filtrate; (F) mycelia of *A. solani* on PDA plate amended with LB-1 cell-free filtrate

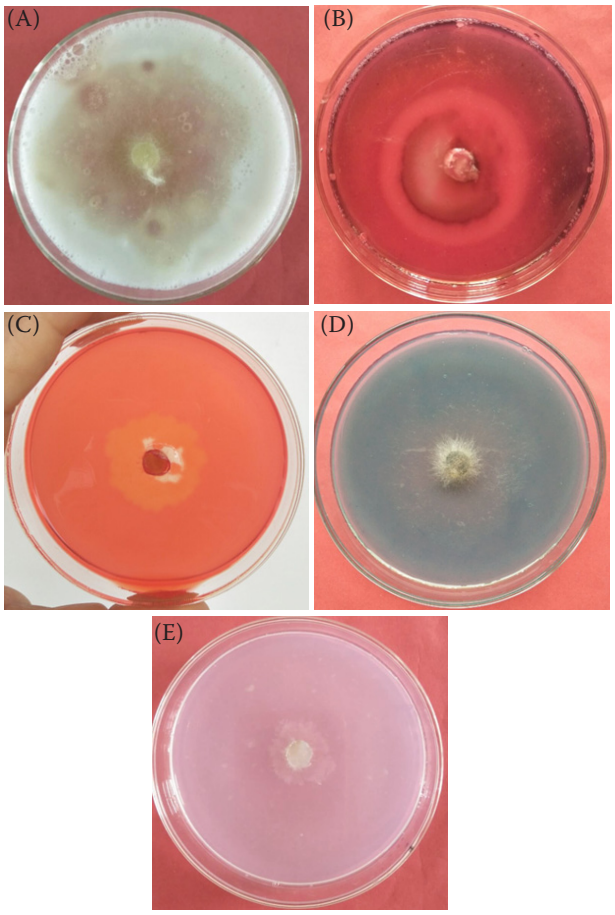

Figure 3. Formation of the halo zones around LB-1 colonies on test plates for mycolytic enzymes detection: (A) protease, (B) pectinase, (C) cellulase, (D) β-l,3-glucase and (E) chitinase

protease test plate and pectinase test plate. A halo zone surrounding the LB-1 colony also appeared on the cellulase test plate, but it was fuzzy. There was no distinguishable halo zone on the β-l,3-glucase test plate and chitinase test plate (Figure 3). This indicates that LB-1 has a strong ability to produce mycolytic enzymes of protease and pectinase, but not in producing β-l,3-glucase and chitinase.

Amphiphilic property of LB-1 cell-free filtrate. The oil spreading test in this research found that the liquid paraffin film on the surface of the distilled water in the petri dish was squeezed around to form an oil-displaced circle when the LB-1 cell-free filtrate was dripped into it (Figure 4). This indicated that the LB-1 cell-free filtrate contains a surface-active substance, which gives the amphiphilic property of the LB-1 cell-free filtrate.

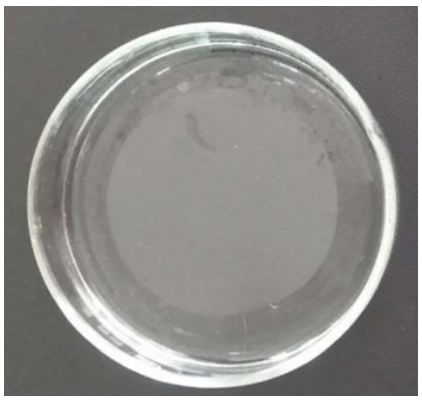

Figure 4. Drainage ring formed on the surface of the distilled water covered with a liquid paraffin film

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DISCUSSION

In this study, the antifungal effects and mycolytic property of the biocontrol agent *C. subaffine* LB-1 as well as its cell-free filtrate on three kinds of common plant pathogenic fungi: *B. cinerea*, *F. oxysporum* f. sp. *cucumerinum* and *A. solani* were clarified with a dual culture assay and poison plate assay. The results of the suppressed colony extension, mycelia degradation and flocculation of the plant pathogenic fungi (Tables 1 and 2, Figures 1 and 2) demonstrated that compete for nutrition and living space, secret mycolytic substance are the ways for LB-1 to obtain its antifungal effects.

Besides the mycolytic substance, the prominent antifungal effects of the LB-1 cell-free filtrate in our research (Table 2) indicated that some antibiotic substance might be produced by LB-1. At present, antibiotic substances, such as chaetoviridin (Park et al. 2005), chaetomugilin (Yamada et al. 2011), chaetoglobosin (Mcmullin et al. 2013), etc. have been detected from *Chaetomium globosum*. Thus, the antibiotic substance contained in the cell-free filtrate of *C. subaffine* LB-1 needs to be further studied.

The production of mycolytic enzymes of the biocontrol agents could be detected via the test plate assay rapidly and accurately. In this method, development of a clear halo zone surrounding the colony of the biocontrol strain indicates the production of mycolytic enzymes (Folman et al. 2003; Ashwini & Srividya 2014). The test plate assay in this study showed that there were clear halo zones around the LB-1 colonies on the individual protease test plate and pectinase test plate, this indicated that LB-1 could produce mycolytic enzymes of protease and pectinase.

Oil spreading, drop collapse, and blood agar lysis are common methods to detect surface-active substance produced by microorganisms, but the oil spreading technique is more sensitive and reliable than that of drop collapse and blood agar lysis (Youssef et al. 2004). Thus, the oil spreading test was used in our research and the results proved that LB-1 could produce surface-active substance and give the amphiphilic property of the LB-1 cell-free filtrate. However, surface-active substances are structurally diverse biomolecules, which include glycolipids, lipopeptides, polysaccharide, phospholipids, fatty acids, neutral lipids, etc. These substances are amphiphilic molecules that could decrease the interfacial tension, and effect the permeability of the fungal cell membrane, which is often applied in plant disease control and the bioremediation of polluted environment (Georgiou et al. 1992; Maget-Dana & Peypoux 1994; Song et al. 2013). Zhu et al. (2011) found that the biocontrol agent *B. amyloliquefaciens* XZ-173 could produce lipopeptide, which reduced the surface tension of water and showed a stable emulsification capability and strong inhibition effects against *Rhizoctonia solani* and *Ralstonia solanacearum*. Chen et al. (2017) screened the biocontrol agent *Bacillus* sp. FJAT-14262 and identified that its fermentation supernatant contains a surface-active substance of surfactin, which exhibited strong inhibitory effects on *F. oxysporum*. In this research, the surface-active substance contained in the cell-free filtrate of the biocontrol agent LB-1 was studied, but the chemical composition of the surface-active substance needs to be clarified in the future.

In conclusion, research on the antifungal effect and mycolytic activity of the biocontrol agent *C. subaffine* LB-1 in this study showed that LB-1 and its cell-free filtrate have significant antifungal effects on three species of common plant pathogenic fungi of *B. cinerea*, *F. oxysporum* f. sp. *cucumerinum* and *A. solani* and could lead to the hyphae dissolution of the phytopathogenic fungi through mycolysis, which might be obtained via the production of mycolytic enzymes (protease and pectinase) and a surface-active substance.

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