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ARTICLE

Antifungal activity of *Streptomyces albidoflavus* L131 against the leaf mold pathogen *Passalora fulva* involves membrane leakage and oxidative damage

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Abstract *Passalora fulva* (or *Fulvia fulva*) is the causal microorganism of tomato leaf mold, the outbreak of which occurs worldwide in greenhouse especially when humidity is high. However, studies on antifungal agents of *P. fulva* are still very limited. In this study, a marine-derived *Streptomyces albidoflavus* strain L131 showing potent inhibitory activities against *P. fulva* was identified and characterized. The active antifungal components were obtained, and studies on the antifungal mechanisms of the crude extract showed that the antifungal metabolites of L131 caused damage of hyphae and spore development, as well as plasma membrane of *P. fulva*. In addition, accumulation of endogenous reactive oxygen species of the leaf pathogen was also observed after treatment by culture extracts of L131. To our knowledge, this is the first report on the studies of the antifungal mechanisms against *P. fulva*, which benefit further development of biocontrol agent against tomato leaf mold disease.

Keywords Marine-derived *Streptomyces albidoflavus* · Tomato leaf pathogen · *Passalorafulva* (syn. *Fulvia fulva*, *Cladosporium fulvum*) · Antifungal activity · Reactive oxygen species (ROS)

Introduction

Plant diseases caused by fungal pathogens have large negative impact on agricultural productivity, and investigation of fungicide, either chemically or naturally synthesized, has aroused increasing worldwide attention (El-Tarabily et al. 2009; Zhao et al. 2012; Castano et al. 2013; Boukaew and Prasertsan 2014). *Passalora fulva* (Cooke) U. Braun & Crous, previously named as *Cladosporium fulvum* Cooke, or *Fulvia fulva* (Cooke) Ciferri, is the causal microorganism of tomato leaf mold (Cooke 1883; Thomma et al. 2005), the outbreak of which occurs worldwide in greenhouse especially when humidity is high (Latorre and Besoain 2002; Iida et al. 2010). Infection of this fungus inhibits the photosynthesis and respiration of the plant, thus affects the fruit quality and results in severe economic losses (Smith et al. 1969). Although the introduction of *Cf* resistance genes into tomato cultivars provided effective control on *P. fulva* (Rivas and Thomas 2005), outbreaks were still reported in countries where tomato cultivars without *Cf*-resistant genes are grown, and new races of *P. fulva* are also emerging in tomato varieties (Enya et al. 2009; Iida et al. 2010; De Wit et al. 2012).

Chemical fungicides can be used to control mold, however, they usually induce notable emergence of drug-resistant microorganisms (Ruddock et al. 2005) and cause environmental problems. In addition, the toxic residues in

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food are also an increasing concern for safty problem (Baysal et al. 2008). As a promising alternative, biocontrol strategy is effective and environmentally friendly to combat plant pathogens (Soylu et al. 2010; Castano et al. 2013), and crude extracts from various plants and microorganisms were investigated for their potential as biological control agents (Mahlo et al. 2010; Zaker and Mosallanejad 2010; Zacky and Ting 2013). However, antifungal activities of microbial strains against *P. fulva* have not been well studied.

Actinomycetes are one of the most widely distributed groups of microorganisms in nature, which are important producers of bioactive compounds against plant pathogens (El-Tarabily et al. 2009; Xue et al. 2013; Zeng et al. 2013). *Streptomyces* species are well known for their potential to produce natural products with diverse structures and activities, and marine-derived streptomycetes have been reported to exhibit antagonistic activities against various fungal strains (Liu et al. 2009; Kim et al. 2012; Thenmozhi et al. 2013). However, studies on marine-derived streptomycetes against plant fungal pathogens including *P. fulva*are are very limited. The only example on the activity against *P. fulva* is the work on *S. rimosus* MY02 isolated from soil sample (Liu et al. 2009), and studies on marine-derived streptomycetes to combat tomato leaf mold pathogen have not been reported so far.

With the emergence of resistant fungal pathogens, novel biological control agents are urgently required. Studies on the antifungal mechanisms are important for developing more efficient biocontrol agents to combat plant pathogen infections. Until now, mechanisms of antifungal action of *Streptomyces* strains involve changing of the membrane permeability and cytoskeleton structure, interacting with the organelles (Xiong et al. 2013), coiling of the fungal mycelia, and penetration into the fungal mycelium and induction of cell lysis (Palaniyandi et al. 2013), as well as production of antifungal enzymes such as chitinases and glucanases (Trejo-Estrada et al. 1998; Yang et al. 2005). However, studies on the antifungal action against *P. fulva* are still not reported so far.

We are interested in exploration of marine-derived streptomycetes with antifungal activities against *P. fulva* and have screened active strains that suppress the growth of *P. fulva*. In this study, we report strains with superior activities against *P. fulva*, and also characterized a streptomycete strain L131. The antifungal mechanism of the active metabolites from L131 against *P. fulva* was also studied.

Materials and methods

Bacterial strains and media

S. albidoflavus strain L131 used in this study was deposited in China General Microbiological Culture Collection Center with the accession number of CGMCC No. 5291, which was cultured on A1 agar (yeast extract 4 g, soluble starch 10 g, peptone 2 g, agar 20 g in 0.75 L of seawater, and 0.25 L of distilled water) at 28 °C for 7 days. *P. fulva* was purchased from China Forestry Culture Collection Center (CFCC) 80924, and was cultured on PDA (potato 200 g, glucose 20 g, and agar 15 g in 1 L of distilled water) at 28 °C for 2 days. For seed culture, TSB medium (tryptone 17 g, soya peptone 3 g, glucose 2.5 g, NaCl 5 g, and K_2HPO_4 2.5 g in 1 L of distilled water) was used. The optimized fermentation medium was corn flour 15 g, soya peptone 15 g, yeast extract 1.5 g, $CaCO_3$ 0.5 g, and glycerin 2 ml in 1 L of distilled water.

Antagonism assay against *P. fulva*

To perform the antagonism assay against *P. fulva* on agar plate, marine-derived streptomycetes were inoculated on the center of A1 agar plate. After cultivation at 30 °C for 48 h, *P. fulva* was inoculated 1.5 cm away from strain L131 on the A1 agar plate. The growth status of the strains was photographed after cultivating at 30 °C for 120 h.

Tomato leaves were freshly collected from the same height of a healthy tomato plant, and were then disinfected with 70 % alcohol for 1 min and rinsed with sterile water. Subsequently, the disinfected tomato leaves were completely soaked by L131 fermentation broth for 30 min. The tomato leaves which soaked in the same volume of sterile water were served as control. After air dried at room temperature, an agar block (10 mm in diameter) cut from *P. fulva*-growing agar plate was inoculated on each leaf. The leaves were incubated in sterile plates at 28 °C for 72 h. The control leaves were treated with ddH_2O and stored under the same condition. Three leaves were used for each group of experiment, and similar results were obtained. After the incubation, the leaves were photographed, and the effect of active components in the fermentation broth of L131 against *P. fulva* was evaluated.

Determination of stability and polarity of the fermented broths

For pH stability test, pH values of the fermentation broths were adjusted at the range of 1–14, respectively, with 1 N HCl or 1 N NaOH. After 10 h incubation at room temperature, the fermentation broth was adjusted to original pH value (pH 6.4). The antifungal activity against *P. fulva* of the treated fermentation broth was tested using the agar plate diffusion method with 100 µl broth (Huh et al. 2004). The non-treated fermentation broth of L131 was used as control.

For polarity test, the fermentation broth was treated with the same volume of ethyl acetate, chloroform, and hexane, respectively. The organic phases were separated, evaporated to dryness, and re-dissolved in the same volume of

ddH_2O. The antifungal activity against *P. fulva* of the treated fermentation broth was tested using the method mentioned before (Huh et al. 2004). The non-treated fermentation broth of L131 was served as control.

For protease stability test, the fermentation broth was treated with protease K (1 mg/mL) or chymotrypsin (1 mg/mL) at 37 °C for 1 h, and then heated at 100 °C for 2 min to inactivate the enzymes. The antifungal activity against *P. fulva* of the treated fermentation broth was tested using agar diffusion assay and the non-treated fermentation broth of L131 was served as control.

Purification of the active fractions from L131

Erlenmeyer flasks (500 mL) containing 200 mL of fermentation medium were incubated with the L131 seed culture (2 %, v/v), and cultured on a rotary shaker (150 rpm) at 30 °C for 168 h. Then the culture broths were centrifuged at 12,000 rpm for 3 min and the supernatants were collected. Preliminary purification using resins was performed according the method described previously (Jiao et al. 2014). Four macroporous resins D001, AB-8, X-5 (Sanxing Resin Tech, Anhui, P. R. China), and HP-20 (Mitsubishi, Tokyo, Japan) were added to the supernatants at a final concentration of 0.3 mg/mL, respectively. The mixture was then shaken at 150 rpm for 24 h. After washing by ddH_2O, the resins were desorbed by 50 and 100 % methanol, respectively, shaking at 150 rpm for 24 h. The eluent was concentrated by rotary evaporator to dryness and re-dissolved in the same volume of ddH_2O, which was designated as active fractions in the following text. At the same time, the Minimum inhibitory concentration (MIC) of the crude extract of the active fractions against *P. fulva* was determined using the method described before (Liu et al. 2009) with minor modifications. MIC was determined as follows. Spores of *P. fulva* (10^8 spores/ml) were inoculated in potato dextrose agar (PDA) plates, and the agar plates were punched using 1 ml blue tips to hold 100 µl serial dilution of the active fraction, which was added to the agar plates before incubation at 28 °C for 96 h. The culture in PDA without the addition of active fraction was used as control. The MIC was defined as the lowest concentration of the active fraction that could inhibit the growth of any visible fungal of *P. fulva*. All experiments were repeated twice. The diameter of the agar block to hold the active fraction is 6 mm, therefore, when the inhibition zone is 6 mm, there is no antifungal activity.

Effect of antifungal compounds from L131 on morphology of *P. fulva*

The antifungal activity of the crude extract of L131 against *P. fulva* was performed using agar diffusion method. After 48 h, spores and mycelia of *P. fulva* on the edge of the inhibition zone were collected and used for scanning electron microscopy (SEM) analysis following the method described previously (Xiong et al. 2013). Samples treated with ddH_2O were used as control, and the samples were visualized by Quanta 450 SEM.

Determination of lesion of plasma membrane

The spores of *P. fulva* were harvested by adding 2 mL sterile ddH_2O to each agar plate with 7-day-old cultures and scraping the mycelia surface to free the spores. The spore suspension of *P. fulva* was diluted in the MA-medium (Mandels and Reese 1957) to a final concentration of 2×10^4 spores/mL. Then the active fractions from L131 culture broth were added into the suspension with a final concentration of 100 µg/mL. The sample without the active fractions was considered as control. After incubation at 28 °C for 48 h, the cells were washed and re-suspended in 500 µL PBS containing 1 µg/mL propidium iodide (PI) solution for 30 min at room temperature. All the experiments were carried out in the dark. The fluorescence of the cells was detected by fluorescence microscope using excitation and emission wavelengths of 535 and 615 nm, respectively.

Determination of endogenous ROS production

The spore suspension of *P. fulva* was diluted in MA-medium to a final concentration of 2×10^4 spores/mL. Then the active fractions from L131 culture broth were added into the suspension with a final concentration of 100 µg/mL. The sample without adding the active fractions was considered as control. After incubation at 28 °C for 48 h, 10 µL fluorescent dye dichlorodihydrofluorescein diacetate (DCFH-DA) (2.5 mg/mL in ethanol) was added into 1 mL of the mixture, mixed, and incubated for 4 h at 28 °C. Then the cells were washed and re-suspended in 500 µL PBS. All the experiments were carried out in the dark. The fluorescence of the cells was detected by fluorescence microscope using excitation and emission wavelengths of 485 and 535 nm, respectively.

Results

Significant inhibitory effect of L131 against *P. fulva* on agar plate was observed (Fig. 1). The edge of the *P. fulva* colonies proximal to strain L131 was visibly suppressed; in contrast, the region of *P. fulva* colonies that were distant from L131 grew normally. Strong suppression of cell growth of *P. fulva* indicates the potential of L131 as biocontrol agent against this tomato leave mold.

Fig. 1 Antifungal activity of L131 against *P. fulva*. Strain L131 was inoculated on the center of A1 agar plate. After cultivation at 30 °C for 48 h, *P. fulva* was inoculated 1.5 cm away from strain L131 on the same agar plate. The growth status of the strains was photographed after cultivating at 30 °C for 120 h

Experiment using tomato leaves also demonstrated that the fermentation broth of L131 inhibited the growth of *P. fulva* (Fig. 2). The control leaves were healthy, while the leaves treated with *P. fulva* exhibited the typical symptoms of fungal infection. The infected area on the leaves developed intensive and obvious disease spots with deep color, and browning region were found on the back of the area; however, there were no obvious spots and browning observed on the leaves treated with L131 fermentation broth (Fig. 2).

The stability of the antifungal compounds of strain L131 is shown in Fig. 3. The average diameter of the inhibition zones was about 13 mm when the pH value of culture broth was between 4 and 8. The inhibition diameter became smaller under strong acidic or alkali conditions but the activity still remained.

The antifungal compounds cannot be extracted by ethyl acetate, chloroform, and normal hexane, and the antifungal activity was only observed in water phase. At the same time, no decrease of the antifungal activities was observed after protease K and chymotrypsin treatments (data not shown).

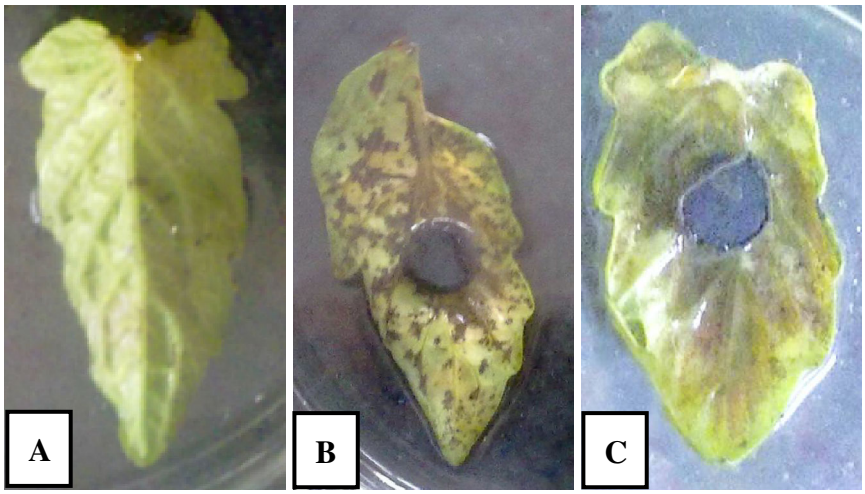

Fig. 2 Disease suppression of L131 on tomato leaves infected with *P. fulva*. **a** Leaves treated with ddH_2O; **b** leaves treated with fungus only; **c** leaves treated with fungus and L131 fermentation broth

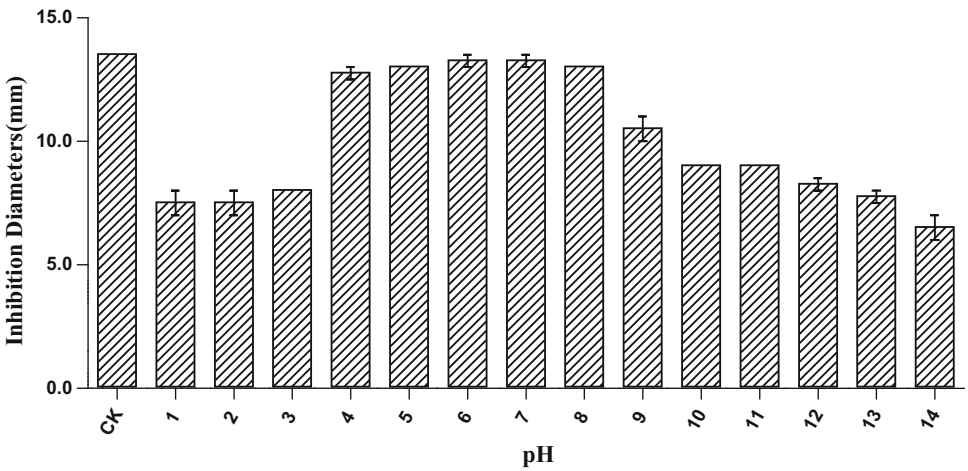

Fig. 3 pH stability of the L131 fermentation broth. L131 fermentation broths were adjusted to different pH and the activity against *P. fulva* was tested using the agar plate diffusion method with 100 µl broth. The diameter of the inhibition zone was measured after incubating the plate at 30 °C for 120 h, and was used to compare the stability of the broth under different pH

Table 1 The inhibitory activity of fractions absorbed by different resins against *P. fulva*

Resin	Solutions (Inhibition zone diameter in mm)			
	Fermentation medium	Residue	Eluent	
			100 % methanol	50 % methanol
HP-20	16.0	13.0	14.0	6.0
D101	16.0	15.0	6.0	6.0
AB-8	16.0	14.0	15.0	6.0
X-5	16.0	15.0	6.0	14.0

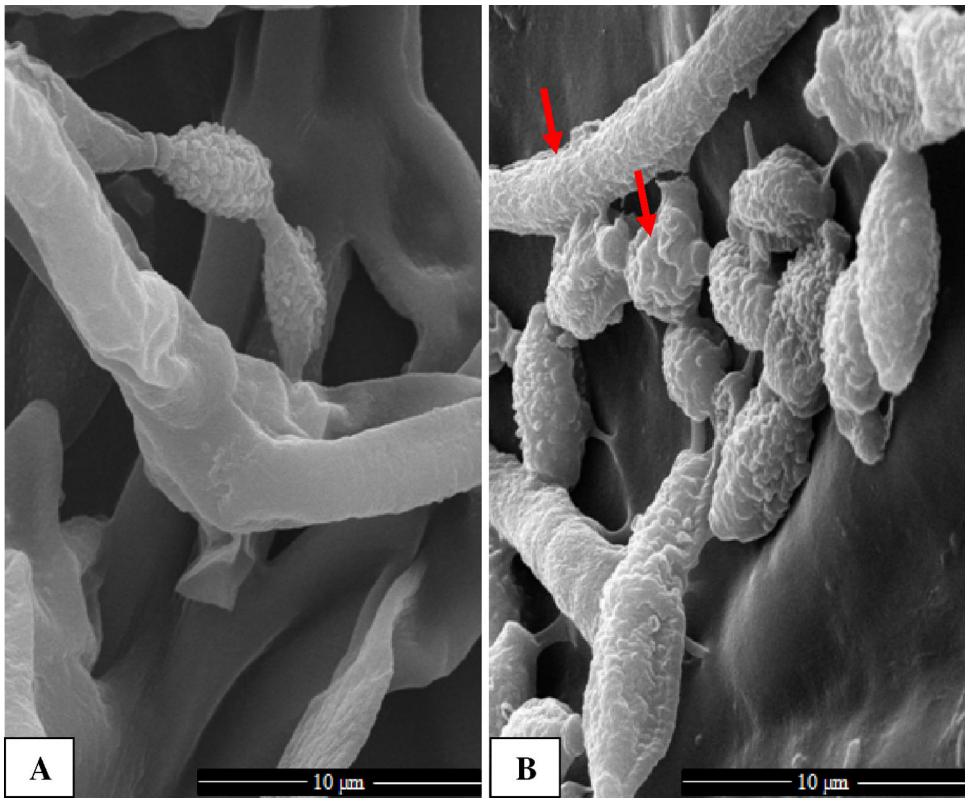

Fig. 4 Effect of the active fractions from L131 on the growth of mycelia and spores. Morphological changes of *P. fulva* on mycelia and spores were observed using scanning electron microscopy (×12,000). **a** *P. fulva* treated with ddH_2O; **b** *P. fulva* treated with L131 active fractions. Arrows indicate the increased roughness on mycelia and inhibition on spore development by the active fractions from L131

According to the stability and polarity of the active fractions and the better solvent tolerance of the macroporous resins, four macroporous resins D001, AB-8, X-5, and HP-20 were used to purify the active fractions from the culture broth and to further study the inhibitory activity against *P. fulva*. The antifungal activity of the active substances adsorbed by AB-8 and HP-20 was higher than the other two resins. The AB-8-adsorbed substances, which were desorbed by 100 % methanol, showed the highest inhibitory activity. At the same time, it was shown that when the concentration of methanol was 50 %, the active substances cannot be eluted by AB-8, therefore, crude extract containing antifungal components can be washed by 50 % methanol, after which it was desorbed by 100 % methanol using AB-8 macroporous resin (Table 1). The MIC of the crude extract of the active fractions against *P. fulva* was 32 μg/mL.

Due to the polarity and complexity of the active fractions of L131, we were not able to obtain pure compounds despite extensive attempts to purify the active substance(s). However, biocontrol can be achieved using both the whole cells and the crude culture extracts. The purification method established here provide a basis for preparation of biocontrol agents for further evaluation.

The margins of the inhibition zone were visualized by SEM. The hyphal morphology of *P. fulva* treated with ddH_2O as the control was smooth and the spore morphology was regular and smooth (Fig. 4a). On the contrary, increased roughness of the hyphae of *P. fulva* treated with the crude extract of the active fractions from L131 was observed, and the treatment also caused severe inhibition of spore development (Fig. 4b).

PI is a nucleic acid binding fluorescent probe which can be adsorbed by injured membranes or dead cells.

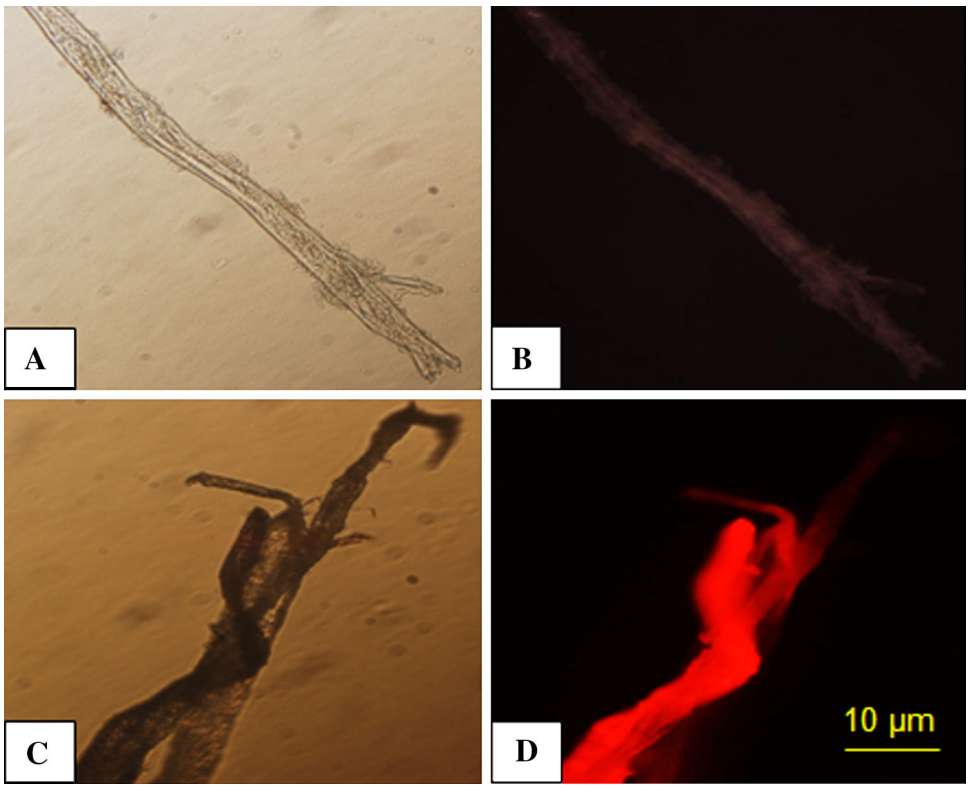

Fig. 5 Effect of the L131 active fractions on the lesion of plasma membrane caused by *P. fulva*. The spores of *P. fulva* were harvested by adding 2 mL sterile ddH_2O to each agar plate with 7-day-old cultures and scraping the mycelia surface to free the spores, and the spore suspension was diluted to a final concentration of 2×10^4 spores/mL. Then the active fractions from L131 culture broth were added into the suspension with a final concentration of 100 μg/mL. The sample without the active fractions was considered as control. After incubation at 28 °C for 48 h, the cells were washed and re-suspended in 500 μL PBS containing 1 μg/mL propidium iodide (PI) solution for 30 min at room temperature. The fluorescence of the cells was detected by fluorescence microscope using excitation and emission wavelengths of 535 and 615 nm, respectively. **a** and **b** Control culture under bright field and fluorescence microscope, respectively; **c** and **d** samples treated with active fractions loading at 100 μg/mL under bright field and fluorescence microscope, respectively

Compared with untreated cells, the cells treated with the active fractions exhibited strong fluorescent intensity. The morphological changes of the mycelium can also be observed (Fig. 5). Results in this figure indicate that the active fractions of strain L131 caused damage of the cell membrane of *P. fulva*.

DCFH-DA is a fluorescent dye which can be used to assay ROS production. After treatment of the active fractions, the fluorescence intensity the *P. fulva* cells was markedly increased, while untreated cells exhibited almost invisible fluorescence (Fig. 6). Based on these results, it can be concluded that the active fractions induced the accumulation of ROS in *P. fulva*cells.

Discussion

In recent years, studies on biocontrol agents have received much more attentions. There is broad consensus that biocontrol is environmentally friendly and effective to control plant pathogens. In spite of the urgent need, few antifungal microorganisms against *P. fulva* have been developed. We found strong inhibitory activity of strain L131 against *P. fulva*, suggesting that marine-derived streptomycetes are promising source for biocontrol of plant fungal pathogens. This is also the first time that *S. albidoflavus*-related strain was found to have the inhibitory effect on *P. fulva*.

In the previous report, culturable bacteria were isolated from tomato leaves, and it was found that bacterial strains that were inferred to be *Bacillus subtilis* and some bacterial strains belonging to *Pseudomonas* and *Pantoea* have strong antifungal activity against *P. fulva* (Lee et al. 2005). However, the antifungal mechanisms of the active strains against *P. fulva* have not been explored in the previous studies (Enya et al. 2007; Liu et al. 2009). We observed clear damage on mycelia and spores of *P. fulva* treated with the crude extract of the active fractions from L131, and accumulation of endogenous reactive oxygen species

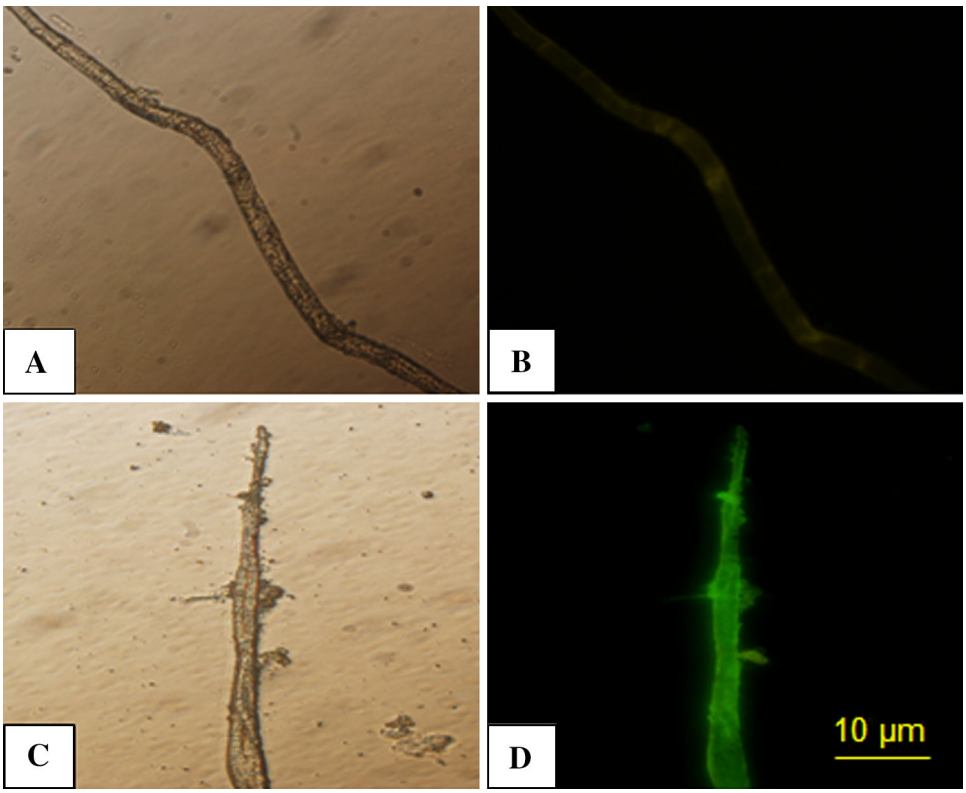

Fig. 6 Effect of the L131active fractions on the endogenous ROS of *P. fulva*. The spore suspension of *P. fulva* was prepared with a final concentration of 2×10^4 spores/mL. Then the active fractions from L131 culture broth were added into the suspension with a final concentration of 100 μg/mL. The sample without adding the active fractions was considered as control. After incubation at 28 °C for 48 h, 10 μL fluorescent dye dichlorodihydrofluorescein diacetate (DCFH-DA, 2.5 mg/mL in ethanol) was added into 1 mL of the mixture, mixed, and incubated for 4 h at 28 °C. Then the cells were washed and re-suspended in 500 μL PBS. All the experiments were carried out in the dark. The fluorescence of the cells was detected by fluorescence microscope using excitation and emission wavelengths of 485 and 535 nm, respectively. **a** and **b** Control culture under bright field and fluorescence microscope, respectively; **c** and **d** samples treated with active fractions loading at 100 μg/mL under bright field and fluorescence microscope, respectively

(ROS) of the leaf pathogen was also observed after treatment by culture extracts of L131. To our knowledge, this is the first report that *S. albidoflavus*-related strain exhibits activity against leaf mold pathogen *P. fulva*.

Plasma membrane is an important component of cell which plays an essential role in material exchange and energy transfer. The morphological changes and the strong fluorescent intensity assessed by PI exhibited by the cells treated with the purified fractions indicated that the plasma membrane is an important target of active compounds produced by strain L131. ROS include a number of reactive molecules and free radicals derived from molecular oxygen; they were produced as byproducts and have the potential to cause many deleterious cellular events. In the recent research, the induction of ROS formation by antifungal agents in fungi has been observed (Qin et al. 2010; Mello et al. 2011). The markedly increasing of the fluorescence intensity in treated cells proved that the active fractions leaded to the accumulation of ROS in *P. fulva* cells which might cause cell death. Combined with the changes of the morphology and the inhibitory effect on cell growth and spore development, it was deduced that ROS accumulation was an important symbol of the antifungal effects of active fractions produced by strain L131.

Although the pure active compound was not obtained, a large proportion of impurities was removed by purification steps mentioned above and biological control could be applied using crude extracts (Ilic et al. 2007; Liu et al. 2009; Soylu et al. 2010; Mello et al. 2011). Spore suspension of L131 and its culture crude extracts have the potential to be further developed as biological agents. The current work is the first time that *S. albidoflavus*-related strain was observed to produce active compounds with strong inhibitory activity against leaf mold pathogen *P. fulva*. Our results showed that the active fractions induced death of *P. fulva* cells by damaging the plasma membrane and inducing the accumulation of endogenous ROS. The results in this study provide basis for further development and application of agricultural antibiotics as biocontrol agents against tomato leaf mold pathogen.

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