In this paper, rhamnolipids are investigated, for the first time, for their feasibility for inhibiting dimorphic fungi. Rhamnolipids were found to effectively inhibit a dimorphic fungus isolated from tomato plants which was identified as *Mucor circinelloides* according to characterizations by morphologies as well as 28S rDNA sequences. Rhamnolipids markedly reduced growth of this fungus in both the yeast-like form and the filamentous form. Such an inhibitive effect was similarly obtained with *Verticillium dahliae*, a representative member of dimorphic fungi, confirming the effectiveness of rhamnolipids in the two growth forms of dimorphic fungi. Interestingly, rhamnolipids showed a greater inhibitive function in the case of the pathogenic growth mode of dimorphic fungi, such as the mycelium growth for *M. circinelloides* and the yeast-like growth for *V. dahliae*, than their non-pathogenic modes. The use of rhamnolipids might greatly reduce the frequently-reported drugresistance to the common anti-fungal agents by deterring the possible switch between the two modes of dimorphic fungi. Overall, rhamnolipids as environment-friendly biocontrol agents have a potential use in protecting plants from dimorphic fungi infections, and could also offer guidance toward future research into controlling dimorphic disease infection in humans.

Key Words: antifungal; dimorphism; *Mucor circinelloides*; rhamnolipids; *Verticillium dahliae*
Lopez et al., 2003; Khan et al., 2009). *M. circinelloides*, like *V. dahliae*, is a dimorphic microorganism which can simply switch between filamentous branching hyphae and multipolar budding yeasts by adapting to changing environmental cues (Nadal et al., 2008). The pathogen mode of dimorphic fungi occurs either in mycelia form (Fingeroth et al., 1994; Mahlert et al., 2006), yeast-like growth (Neumann and Dobinson, 2003), or switching between the two forms (Nemecek et al., 2006). Usually, chemically synthesized fungicide has exhibited differential inhibitions against the two growth modes, as well as a differential resistance, of the dimorphic fungi (Zwiers and De Waard, 2000).

Besides the known antifungal activities, however, rhamnolipids have never been investigated for their antifungal activities against the dimorphic growth of fungi. There is an obvious need to search for alternative biocontrol agents that are nontoxic to animals and nonpolluting environmentally to control both the yeast-like and filamentous growth forms of dimorphic fungi for agricultural applications. The present study, presents, for the first time, an examination of the possibilities of using rhamnolipids to control dimorphic plant pathogens by considering an isolated dimorphic fungus from a severely infected tomato field, and another devastating fungus *V. dahliae* ATCC 7611.

**Materials and Methods**

**Fungal strain isolate and cultivation.** Samples of pathogen-infected tomato plants from a severely contaminated tomato field (Wenzhou, China) were collected in a sterilized polythene bag and delivered to the laboratory. The tomato plants were swabbed in 75% ethanol for 2 min, rinsed several times with distilled water and then blotted dry with sterile filter papers. Lesions were aseptically cut and plated on sterile potato dextrose agar (PDA) in 9 cm Petri Plates for incubation at 28°C. Two days later, the mycelium was transferred onto fresh agar plates (PDA, Czapek yeast extract agar and Czapek’s agar) to determine the most appropriate agar plates at 28°C. According to morphological observations, this fungus was found to grow fast and well on PDA and Czapek yeast extract agar, but not on Czapek’s agar. Hence, the convenient and effective PDA was used for subculture every 7 days. During the investigation of spore generation using a light microscope (XSS-2A, Nanjing, China), it was found that spore generation was of quite different morphology in shaking tubes than that in statically cultured tubes. Spores generated into germ tubes in a shaking (aerobic) culture following the germination was of quite different morphology in shaking tubes. Spores generated into germ tubes in a shaking (aerobic) culture following the multiplication and filamentous growth forms of dimorphic fungi. Light microscopy was routinely used to examine the spore generation and mycelium. For an observation with high resolution, samples were fixed by immersion in 5% (v/v) glutaraldehyde for 24 h at 4°C and post-fixed with 1% (w/v) osmium tetroxide for 2 h at 4°C before assay with SEM. The fixed samples were dehydrated in a graded acetone series and covered with gold prior to examination in a Scanning Electron Microscope (TM-1000, Hitachi, Tokyo).

**Molecular testing and phylogenetic analysis.** DNA extraction and PCR amplification of the isolated strain were conducted in Guangdong Detection Center of Microbiology (Guangzhou, China) following previous protocols (Sambrook et al., 1989), and the forward primer NL1 (5’-GCATATCAAAGCGGA GGAAG-3’) was used. The amplified fragment was sequenced by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). Its 28S rDNA sequence having a length of 660 bp was then submitted for a BLAST search at the GenBank database (National Center for Biotechnology Information, Washington, DC, http://www.ncbi.nlm.nih.gov). This sequence was subsequently assigned the accession number JF823517 by GenBank.

The above sequence was aligned using the CLUSTALX program (version 2) (Yoon et al., 2011) and adjusted using the BioEdit program (version 7.0.9), while ambiguous bases and alignment gaps were not taken into consideration. The obtained sequence was further analyzed by the MEGA4 software. The evolutionary distances were determined according to the Kimura two-parameter model using bootstrap values based on 1000 replications. The similarity values were calculated and the neighbor-joining tree was generated using the same software.

**Preparation of rhamnolipids.** *Pseudomonas aeruginosa* strain ZJU211 (CCTCC M209237) was isolated from heavily oil-contaminated soil as described previously (Tang et al., 2007) and applied to produce rhamnolipids using colza oil (6%, v/v) as the sole carbon source. 60 mL of medium (Tang et al., 2007) contained in a 250 mL Erlenmeyer flask was inoculated with 3% (v/v) of bacteria, then cultured in a reciprocating shaker (ZHWY-1102C, ZHICHENG, Shanghai, China) at 180 rpm at 37°C. After incubation for 2 days, the complete culture volume was used as inoculums for a 10-L bioreactor (GUIJS-10C, Eastbiotech, Zhenjiang, China) with a working volume of 5 L which was aered by a sparger at 5 L air min⁻¹ and stirred at a speed of 300 rpm at 37°C. At the end of fermentation (96 h), the culture broth was heated to 121°C for 20 min, centrifuged at 5000 rpm for 20 min to obtain a cell-free culture broth, then acidified to pH 2.0 by sulfuric acid (1 mol/L). The rhamnolipids precipitation was then collected for subsequent extraction by chloroform:methanol (2:1, v/v). After evaporation under vacuum, rhamnolipid extract (with a purity of 98.9%) was used for the following experiments. The rhamnolipid content was determined by an anthrone-sulphate assay and expressed as μg/mL (Sha et al., 2012).

**Inhibition of colony growth of mycelium on PDA plates.** Rhamnolipids were added to the molten PDA and poured into the 9-cm Petri plates. Un-amended PDA plates served
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as controls. The center of the agar plates was inoculated with mycelial plugs of 5 mm in diameter obtained from the 7-day-old culture plate. The plates were sealed with Parafilm “M” (Pechiney, Chicago, IL, USA) and incubated at 28°C. In each test, three replicate plates were run. At the end of the culture (around 7 days), the diameter of the mycelial growth zone was measured with a vernier until the control plates were completely full of mycelia and the inhibition rate relative to the control was calculated.

Inhibition on spore germination and biomass formation in dimorphic growth. The inoculum of sporangiospores was prepared as follows. The hyphae collected from 7-day-old culture plates of either *M. circinelloides* or *V. dahliae* ATCC 7611 were firstly washed by 5 mL of sterile water and the solution was then sieved through a double layer of lens paper for removal of hyphae. The concentration of spores in the filtrate was measured by counting with a haemocytometer. The final spore solution was obtained by dilution to a concentration of $5 \times 10^8$ spores/mL.

The inhibitory effects of rhamnolipids on spore germination and biomass formation of dimorphic fungi were conducted with either hyphal form or yeast form. Fungus isolate could develop mycelium growth in a shaking (aerobic) culture or yeast growth in a static (microaerophilic) culture. Contrary to the fungus isolate, *V. dahliae* ATCC 7611 presented mycelium growth under static and yeast bud growth modes under shaking cultures, corresponding well with that previously reported (Nadal et al., 2008). By inoculation, 1 mL of spore solution of fungus isolate or *V. dahliae* ATCC 7611 was added into 10-mL glass test tubes with 4 mL of Potato Dextrose Broth (PDB) with or without rhamnolipids. For shaking culture, the inoculated glass tubes were immediately put into, and cultured in, a reciprocating shaker in dark at 28–30°C at a speed of 200 rpm. In a static culture, the inoculated glass tubes were sealed with Parafilm “M” membrane and maintained at 28–30°C. Three repetitions were run simultaneously for each rhamnolipid dose tested.

After 8 h in shaking cultures, or 12 h in static cultures, the germination was observed under a light microscope and the numbers of germinated or un-germinated spores were individually counted with a haemocytometer for calculating the spore germination rates. A spore was considered to be germinated when the germ tube length was 1.5 times the spore diameter. In each measurement, four areas of the haemocytometer were recorded. After 5 d in either shaking, or static, culture, the fungal mycelia were collected by filtration and dried at 60°C for 24 h before the determination of biomass accumulation. The growth inhibition rates were expressed relative to the control without rhamnolipid exposure.
Statistical analysis. Data were subjected to an analysis variance using SPSS 16.0 for a Windows program. In order to investigate the differences between rhamnolipid treatments, the percentages with respect to the control were analyzed to normalize the data. One-way ANOVA analyses and Duncan’s multiple range test were used to detect significant differences between treatments and differences were considered significant when $p < 0.05$.

Results

Identification of fungus isolate

The unknown dimorphic fungus isolated from tomato plants was subcultured on PDA plates for further morphological identification and molecular testing.

The formed colonies on PDA plates initially appeared white and became a light gray as the mycelium developed at 2 days, which later turned into dark gray at 7 days. The center of the colony was raised and the circumference of the colony was flat and white. The grayish white aerobic mycelia grew fast and covered the whole plates after 2 days of culture. According to observation with a light microscope, the aerial hyphae were nonseptate, broad and branched followed by the formation of sporangiophore, columellae and sporangia. Sporangiophores were generated directly from the mycelium at a form of sympodial branch. Columellae were either subglobose or obovoid at a length of 10–30 μm and width of 12–40 μm. By assay with SEM, it could be seen that the globose sporangia had patterned walls with a diameter ranging 36 to 65 μm (Figs. 1a and 1b) and conidia were in a morphology varying from ellipsoidal to subglobose in a diameter range of 4–7 μm (Figs. 1c and 1d). According to the microscopic morphology above, as well as the physiological observations described in Section “Materials and Methods”, the isolate was preliminarily identified as a Mucor species.

In molecular testing, the 28S rDNA sequence was assayed. This isolated strain showed a high similarity of 99.8% with Mucor circinelloides (GeneBank accession number FM246460). And all other species of the family Mucor revealed a 28S rRNA gene sequence similarity of more than 99% with the fungus isolate. Therefore, this fungus isolate could be recognized as Mucor circinelloides. Molecular phylogenetic data were obtained from 28S rDNA sequence analysis and are presented in Fig. 2. According to the distinct phylogenetic position, the isolate was thus confirmed to be M. circinelloides, and the novel isolated strain was deposited in China Center for Type Culture Collection (CCTCC), named as M. circinelloides CCTCC M210113.

Inhibition of the isolated M. circinelloides CCTCC M210113 by rhamnolipids

The inhibition of mycelial growth by rhamnolipids was first investigated on PDA plates. As shown in Fig. 3, rhamnolipids inhibited the colony growth of mycelia of M. circinelloides CCTCC M210113 in a distinct concentration-dependent manner. Exposure to rhamnolipids at 10 μg/mL and 200 μg/mL markedly reduced the colony...
growth of *M. circinelloides* CCTCC M210113 by 19% and 58%, respectively.

The inhibition of spore germination and biomass accumulation of mycelial growth by rhamnolipids was then examined with a shaking culture. As shown in Fig. 4a, in a concentration-dependent manner, rhamnolipids significantly reduced spore germination and biomass accumulation by 95% and 91%, respectively, in the presence of 200 μg/mL of rhamnolipids.

The inhibition on yeast growth of *M. circinelloides* CCTCC M210113 was investigated with a static culture. As shown in Fig. 4b, rhamnolipids treatment obviously inhibited spore germination as well as biomass accumulation in a concentration-dependent manner. Severe inhibitions by 65% and 85% were observed for spore germination and mycelium growth, respectively, at 200 μg/mL of rhamnolipids.

A comparison of Figs. 4a and 4b, show that rhamnolipids resulted in a greater inhibition of the mycelium growth than the yeast-like growth of *M. circinelloides* CCTCC M210113.

**Inhibition of V. dahliae ATCC 7611 by rhamnolipids**

As shown in Fig. 5a, rhamnolipids demonstrated an obvious inhibition of colony growth of *V. dahliae* ATCC 7611. As an example, the inhibition rate of colony growth reached 73% at 60 μg/mL of rhamnolipids. Similarly, rhamnolipids significantly reduced the biomass production of *V. dahliae* ATCC 7611 under either mycelium growth in a static culture or yeast growth modes in a shaking culture (Fig. 5b). As an example, rhamnolipid treatment at 120 μg/mL reduced biomass accumulation by about 60% and 70% for mycelium and yeast-growth, respectively (Fig. 5b), while inhibiting about 50% of spore germination in each growth mode (data not shown). Contrary to *M. circinelloides* CCTCC M210113, it can be seen from Fig. 5b that the yeast-like growth was more sensitive to rhamnolipids than mycelium growth.

**Discussion**

Unlike common fungi, dimorphic fungi are quite distinct for their properties of dimorphism. So pathogenicities caused by dimorphic fungi are peculiar due to their dimorphic switches in adapting to changes in temperature, nutrients, or other environmental factors (Mahlert et al., 2006; Nadal et al., 2008). In this paper, a fungus isolated from severely-infected tomato plants, and *V. dahliae* ATCC 7611, were used as two representative dimorphic plant pathogens to examine the feasibility of rhamnolipids as biocontrol agents.

First of all, the isolated strain from the infected tomato field was identified. The observation of its morphology under both light microscopy and scanning electron microscopy suggested it being a *Mucor* species. The determined similarity of 99.8% by a 28S rDNA sequence, and its distinct phylogenetic position, further confirmed this strain to be *M. circinelloides* (accession number JF823517). The observed dimorphic properties of the isolated fungus (Fig. 1) further confirmed *M. circinelloides*, which is characterized by dimorphic growth (Lubbehusen et al., 2004), similar to other *Mucor* species such as *M. racemosus, M. rouxii* and *M. genevensis* (Orlowski, 1991).

In this study, rhamnolipids, for the first time, has been investigated for their antifungal activities against both yeast-like growth and filamentous growth of dimorphic plant pathogens. Rhamnolipids effectively reduced the growth of *M. circinelloides* CCTCC M210113 in both the mycelium mode or yeast mode (Fig. 4), showing its potential in the treatment of plant pathogens. *M. circinelloides*, like most zygomyces, frequently causes mycotic diseases and, in particular, functions as a postharvest pathogen on numerous fruits and vegetables (Moline and Kuti, 1984). In our previous research, *M. circinelloides* was also shown to be sensitive to crude rhamnolipids in the form of a cell-free culture broth on petri plates containing PDA medium, whilst the yeast-like growth of *M. circinelloides* was not mentioned there (Sha et al., 2012). The feasibility of using rhamnolipids to inhibit dimorphic fungi was confirmed by its effective performance on a typical dimorphic plant pathogen of *V. dahliae* ATCC 7611. Like performance on *M. circinelloides*
CCTCC M210113, rhamnolipids showed an effective antifungal activity in the case of *V. dahliae* ATCC 7611, in both yeast-like forms (under shaking culture) or filamentous forms (under static culture). As *V. dahliae* has a wide host range, including over 300 woody and herbaceous plant species, rhamnolipids could be of great potential use in biocontrol due to their capability of inhibiting such devastating fungi. Besides, it was shown that rhamnolipids can also effectively reduce the growth of *Ustilago maydis*, another dimorphic pathogen fungi, in either mycelium mode or yeast mode.

Interestingly, rhamnolipids showed a greater inhibitive function against the pathogenic growth mode of dimorphic fungi. As shown in Fig. 5b, rhamnolipids showed more inhibition of the yeast-like growth, the pathogenic mode (Neumann and Dobinson, 2003) of *V. dahliae*, than its non-pathogenic mode in mycelium growth. The trend occurred similarly to *M. circinelloides* whereas rhamnolipids exhibited a better inhibitive performance on mycelium growth (Figs. 5a and 5b), the pathogen mode of this strain (Fingeroth et al., 1994; Gauthier, 2015; Lee et al., 2013). As dimorphic fungi can switch morphology between pathogenic and non-pathogenic forms (Nemecek et al., 2006), the inhibition of rhamnolipids on both the mycelium and yeast-like modes could greatly reduce the spreading of the pathogenic infection. Moreover, such inhibitions on two growth modes might reduce the frequently-reported resistance of dimorphic fungi to fungicides. It has been well reported that dimorphic fungi, like *M. circinelloides* CCTCC M210113, could show resistance to some conventional antifungal agents (Gomez-Lopez et al., 2003; Khan et al., 2009) or are refractory to the common antifungal drugs (Galgoczy et al., 2005) partially by switching between a hyphal mode and a yeast-like mode for better adjustment to a harsh environment (Cooper, 1987). It seems that rhamnolipids not only produce a direct inhibition of the pathogenic mode of dimorphic fungi, but also reduce the possible switch between the two growth modes, thereby enhancing their sensitivity.

Rhamnolipids with potent antifungal activities against plant pathogenic fungi might have a potential in the biocontrol of human pathogenic diseases. Dimorphism is common among pathogens because the different growth forms represent different advantageous strategies for the pathogen: the yeast form is ideal for distribution in fluid circulation around the host (water-conducting in plants, blood or lymphatic circulation in animals), whilst the hyphal growth form can benefit its penetration across solid tissues (Nadal et al., 2008; Yangui et al., 2010). It is well accepted that many human opportunistic pathogenic fungi are dimorphic and could be similarly controlled by the azole fungicides, typical agents in the treatment of plant pathogenic fungi. However, most zygomycota species show a resistance to typical azole family agents (Gomez-Lopez et al., 2003; Khan et al., 2009). Even the combinational use of two drugs, such as itraconazole and terbinafine, exhibited no significant synergistic effect against *M. circinelloides*, one of the most resistant fungi in the zygomycota species (Gomez-Lopez et al., 2003). The susceptibility of *M. circinelloides* to rhamnolipids suggests their potential use in the treatment of resistant pathogenic fungi in both humans and plants.

Lastly, we would like to mention that di-rhamnolipid purified from rhamnolipid mixtures showed a similar inhibition efficacy against the two growth modes of both *M. circinelloides* CCTCC M210113 and *V. dahliae* ATCC 7611, while mono-rhamnolipid showed much less function, indicative of the dominant antifungal activity of di-rhamnolipid. We found that the IC$_{50}$ (an inhibition rate of 50%) values of di-rhamnolipid and mono-rhamnolipid against the *M. circinelloides* CCTCC M210113 mycelium mode were 25 $\mu$g/mL and 316 $\mu$g/mL, respectively, while IC$_{50}$ values for the yeast mode were 120 $\mu$g/mL (di-rhamnolipid) and 1800 $\mu$g/mL (mono-rhamnolipid). The IC$_{50}$ values of di-rhamnolipid and mono-rhamnolipid against the *V. dahliae* ATCC 7611 mycelium mode were 102 $\mu$g/mL and 1250 $\mu$g/mL, respectively, while IC$_{50}$ values for the yeast mode were 41 $\mu$g/mL (di-rhamnolipid) and 522 $\mu$g/mL (mono-rhamnolipid).

The antifungal effect of rhamnolipids on inhibiting the two growth modes of dimorphic fungi might be largely associated with a strong surface activity of di-rhamnolipid, which could disrupt the cell membrane of spores. However, a nonionic surfactant of Tween-80 with a low CMC of 13 $\mu$g/mL (Nayak et al., 2009) showed no inhibition of *M. circinelloides* CCTCC M210113 in agreement with our observations (data not shown), but, in turn, stimulated growth at a high concentration of 840 $\mu$g/mL (Lubbehusen et al., 2003). Hence, the surface activity of fungicides should not be the sole factor for illustrating antifungal activity, and the specific interactions between surfactants and the targeted membranes of fungi might also play a role (Abalos et al., 2001; Costa et al., 2010).

**Conclusion**

In summary, rhamnolipids exhibited remarkable antifungal activity against *M. circinelloides* CCTCC M210113 and *V. dahliae* ATCC 7611 in both yeast-like and filamentous forms, suggesting a high potential as agrochemicals in protecting crops and, possibly, against dimorphic disease infections.

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