African Journal of Microbiology Research Vol. 6(50), pp. 7572-7578, 28 December, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.2290
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

A new endophytic *Paraconiothyrium brasiliens* LT161 shows potential in producing antifungal metabolites against phytopathogens

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Accepted 29 November, 2012

A new endophytic strain of *Paraconiothyrium brasiliens* LT161 was isolated from the healthy stems of *Cinamonum camphora* collected from Nanjing, China, and was identified based on morphological characteristics and internal transcribed spacer (ITS) sequence analysis. The culture filtrate and its ethyl acetate extract of strain LT161 showed strong growth inhibition activity *in vitro* against fungal phytopathogens such as *Rizoctonia solani, Alternaria alternate, Glomerella glycines, Phytophthora capsici, Fusarium oxysporum, Fusarium graminearum* and *Cryphonectria parasitica.* The bioactive metabolites in the filtrate were relatively thermally stable, but sensitive to strong alkaline conditions. Simulation test within the dish indicated that the bioactive composition of strain LT161 pose considerable ability in controlling rice sheath blight disease, *Fusarium* root rot and wheat *Alternaria* leaf blight. The antifungal compounds in the filtrate of strain LT161 might be novel based on liquid chromatography/mass spectrometry (LC/MS) analysis and comparison to the Syngenta natural product dereplication database. These results suggest that the strain LT161 could be a promising candidate for producing leading compound of pesticide development or used directly as biological control agent in the sustainable agriculture system.

Key words: *Paraconiothyrium brasiliens*, antifungal activity, biological control, endophytic fungi.

INTRODUCTION

Phytopathogens cause huge agriculture losses all over the world every year. Synthetic fungicides are one of the cheapest and most effective approaches for the control of plant diseases. Indeed, the tremendous increase in crop yields associated with the 'green' revolution would not have been achieved without the contribution of these synthetic compounds (Damm et al., 2008). However, due to the pathogen resistance mutations and the emergence of new pathogens, many of the traditional chemical pesticides have gradually failed; development of new drugs for plant diseases are urgently needed. In view of the negative effects on the environment and human health of traditional chemical pesticides, scientists have to find new approaches. Biocontrol agents along with their multiple antimicrobial active metabolites have become a very promising alternative (Strobel, 2003). As potential resources, endophytes may play a special role in preventing soil-borne diseases and vascular tissue diseases considering their advantages in ecological niche (Verma et al., 2009; Aly et al., 2011; Pimentel et al., 2011).

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Endophytes are microorganisms that live in the intercellular spaces of stems, petioles, roots and leaves of plants causing no discernible manifestation of their presence and have typically gone unnoticed (Strobel et al., 1998). Some endophytes are able to produce certain active metabolites which could protect their hosts from the attack of pathogens, insects or animal, or they have direct or indirect effects on plant growth promotion or crop yields improvement (Strobel and Daisy, 2003; Barrow et al., 2008; Zhang et al., 2012). Many endophytes could produce some biologically active substances which are similar or the same as the active substances that are produced by their hosts. Even more, some endophytes are the real producer of the biologically active substances that are found in the host plant. For example, it has been evidenced that toxins which discourage insects and other grazing animals found in tall fescue were produced by endophytes (Guo et al., 2008). Therefore, isolating endophytes from some medicinal plant which are able to produce certain bioactive substance has become an efficient method to screen broad-spectrum, and stable and low plant toxicity biocontrol agents (Strobel et al., 2003). *Cinamonum camphor* is a kind of traditional medicinal plant in China. Several secondary metabolites of it have been found with a variety of biological effects, such as antibacterial, anti-inflammatory effects, as pest control and heart stimulant (Yeh et al., 2009; Wang et al., 2012).

In this study, we report an endophytic strain of *Paraconiothyrium brasiliens* LT161 that was isolated from the medicinal plant *C. camphor*. The filtrate of strain LT161 exhibited strong antifungal activity against many phytopathogens. Moreover, the active component in the filtrate of strain LT161 was isolated and preliminary identified, which we speculated might be a new compound.

MATERIALS AND METHODS

Isolation and screening of endophytic fungi

Fresh stems of *C. camphor* were collected from Zijing mountain of Nanjing, as described by Liu et al. (2001). Several segments were randomly cut from each stem, washed in running water and sterilized successively with 75% ethanol and 40% formaldehyde for 3 min each. The segments were washed again using sterilized water and cut into 1 cm long sections and placed on potato sucrose agar (PSA: potato, 200 g; sucrose, 20 g; agar, 15 g; sterilized water, 1 L; streptomycin, 50 mg/ml). The same medium without agar (PS) was used as a liquid medium. After incubation for 2 to 7 days at 28℃, colonies appeared on the plates and were isolated individually as single colonies on PSA, and stored in 4℃. In order to prove fungi isolated in this way, endophytes instead of epiphytes, 2 to 3 stems without cutting were placed in the same PSA (contained streptomycin 50 mg/ml) as negative control.

Species identification

Strain LT161 was identified by morphological observation on hyphae, spores, colony formation and pigment production as well as partial ribosomal DNA sequence analysis (Liu et al., 2007). The specific primer pair ITS1/ITS2 was used to perform polymerase chain reaction (PCR) amplification and the PCR product was sequenced and compared with similar sequences retrieved from the DNA databases by using the BLAST search program in the National Center for Biotechnology Information (NCBI) (Altschul et al., 1997).

Antifungal metabolites extracted by various organic solvents

Strain LT161 was transferred to 400 ml of PS liquid and cultured at 28℃, and 100 rpm for 72 h. The obtained culture broth was filtered and divided into four equal parts. Three of them were extracted exhaustively with petroleum ether, ethyl acetate and n-butanol respectively (filtrate: organic solvents = 1:3 vol/vol) at room temperature. Each part was extracted for three times. Another part was extracted exhaustively with petroleum ether, ethyl acetate and n-butanol successively. The solvent in each extract was removed using a rotary vacuum evaporator R-124 under reduced pressure to yield a dark-brown tarry residue, which was dissolved in methanol to obtain the concentration of 1 mg/ml.

To test the antifungal activity of the extract, 200 μL of *A. niger* spore suspension was spread evenly on the solid PSA medium plate and dried naturally for 30 min. 10 μL of each extract (1 mg/ml) or 75% Daktarin WP (1 mg/ml) was added to the sterile paper disks (diameter, 5 mm), placed evenly onto the plate surface and incubated at 28℃ for 48 h to measure the growth inhibition zone of *A. niger*. This experiment was repeated for thrice.

Effective dose (ED50) values of ethyl acetate extract

Antifungal effects of strain LT161 extracts against seven phytopathogens were tested in Petri dishes containing the PSA medium, following the published procedure (Gong et al., 2006). The ethyl acetate extract was mixed with 10 ml of melting PDA medium and poured into Petri dishes (9 cm in diameter) with the final concentrations of 0.01, 0.07, 0.14, 0.28, 0.35, 0.50, 0.75, 1.00, 1.50 and 2.00 mg/ml, respectively. Each of the seven test fungal discs (7 mm in diameter), which were taken from the fresh margin of the mycelia, were transferred equally onto the Petri dishes. After cultivating at 28℃ for 48 h, the colony size was measured and the growth inhibition percentage relative to the negative control was calculated at increasing fungicide concentrations and a regression analysis was performed. ED50 values are the concentration of extract at which relative growth is reduced by 50%. Each inhibition experiment was replicated thrice. The seven target phytopathogenic fungi were *Rhizoctonia solani* (RS), *Fusarium oxysporum* (FO), *Fusarium. graminearum* (FG), *Alternaria alternate* (AA), *Phytophthora capsici* (PC), *Glomerella glycines* (GG) and *Cryphonectria parasitica* (CP), which were maintained in our laboratory.

Simulated *in vivo* disease control

The simulated *in vitro* disease control was performed using the method described by Zhao et al. (2010). Briefly, 50 seeds of wheat and 100 seeds of rice uniform in size and breed were sorted, then sterilized with 70% ethanol for 7 min, rinsed thrice with sterilized water and germinated aseptically in an incubator at 28℃ until the root length of each seed was longer than 10 mm. Batches of 10 germinated seeds were placed evenly on water-soaked filter papers in Petri dishes. A fresh mycelial disc (6 mm in diameter) of the phytopathogens was placed directly on the seminal roots of wheat and rice (50 seeds for each kind of phytopathogens) and 2

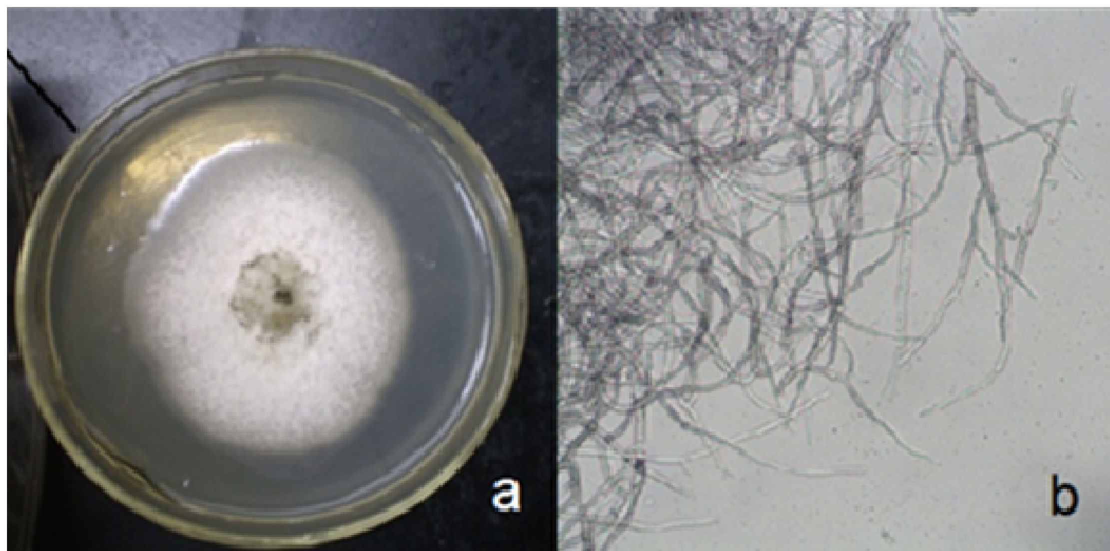


Figure 1. Colony (A) and mycelia (B, 40×10) of strain LT161 cultivated at 28 °C for 12 days.

ml of the ethyl acetate extract solution (1 and 2 mg/ml), sterile water (negative control) and 75% Daktarin WP (1mg/ml, positive control) were spread on the germinated seed roots respectively. The mycelial discs of the phytopathogens were removed after 24 h. The disease indexes (DI) and disease control efficiency (DCE) was assessed in terms of the length of cortical browning on the seminal roots at 48 h after incubation at 28°C.

The stability of the antifungal ethyl acetate extract

The effects of pH and temperature on the stability of antifungal filtrate were conducted by the method described by Zhao et al. (2010). Briefly, the ethyl acetate extract of strain LT161 was adjusted to different pH values in the range of 1.0 to 14.0 using 2 M HCl or 2 M NaOH, then incubated at 4°C for 24 h. Antifungal activity was assayed after the samples were readjusted to pH 7.0. Each experiment was replicated thrice. Similarly, the ethyl acetate extracts was held at temperatures of 25, 80, 100 and 121°C respectively for 30 min, and their antifungal activity was tested after cooling to room temperature. The relative remaining activity was measured by comparing with the samples held at pH 7.0 and room temperature. To assess the effects of metal ions on antifungal activity, each ion (Ca^{2+}, Mg^{2+}, Mn^{2+}) was added to the ethyl acetate extracts to get the final concentrations of 0.003, 0.010, 0.030 and 0.100 mol L^{-1} respectively and maintained at room temperature for 24 h to determine their antifungal activities thereafter.

Isolation of active compounds

With the help of bioautographic technique (Valgas et al., 2007), the bioactive spots containing the antifungal compounds against *A. niger* were collected from the TLC plates, dissolved in methanol, purified by silica gel column chromatography and Sephadex-LH-20, and identified by reverse phase HPLC (Waters Alliance 2695) analysis coupled to UV (UV Detector: Waters 2996 Photo Diode Array) and MS (Mass Spectrometer: Thermo Finnegan LCQ Deca XP Plus. Electrospray ionization) detection and the results were compared to the data of standard compounds in the Syngenta natural product dereplication database.

Data analysis

The results of the experiments were analyzed with GraphPad Prism (version 5.01) software to estimate the significance of the differences ($p < 0.05$ or $p<0.01$) using one-way ANOVA and the Tukey test.

RESULTS

Characterization of strain LT161

Four endophytic strains were isolated from the health stem of *C. camphora* and strain LT161 showed a broad spectrum of antifungal activities against all the seven target phytopathogens; *R. solani*, *F. oxysporum*, *F. graminearum*, *A. alternate*, *P. capsici*, *G. glycines* and *C. parasitica* (data not shown). Strain LT161 grows slowly on PSA medium with a flat, round and felt-like colony at 28°C; the colony diameter is 1.8-2.1 cm 4 days after inoculation and 9.0 cm at 14 days. The colony color initially was white and then changed to brown with the cultivation extension. The fresh aerial mycelia are white and become olive when they grow older. The colony color on the back of Petri dish was black in the center and brown in the margin. Many uneven discrete black conidiomata appeared in the colonies at 28°C for 5-12 days (Figure 1). Partial sequence analysis of the ribosomal DNA amplified by primer pair of ITS1 and ITS2 indicated that strain LT161 had 99% homology to that of *P. brasiliense* (Figure 2). According to the morphological observation and ITS sequence analysis, strain LT161 was identified to be *P. brasiliens* LT161.

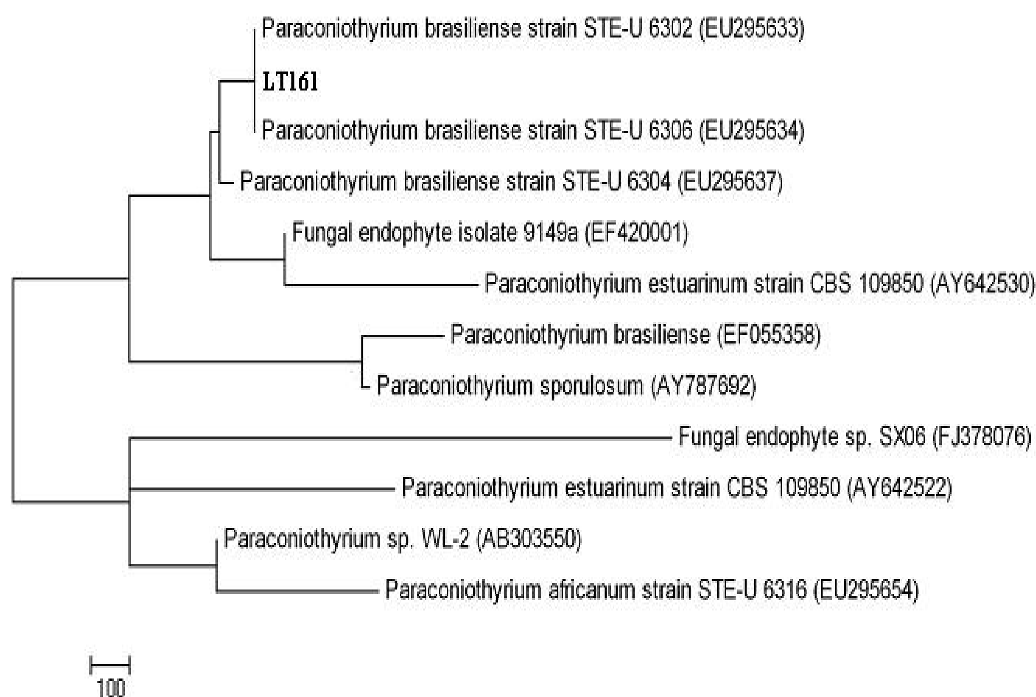


Figure 2. Phylogenetic tree based on partial ribosomal DNA sequences. Boot values (1 000 replicates) are reported on or adjacent to each branch. Scale is nucleotide substitutions per base.

Organic solvent extraction of antifungal fractions

Various organic solvents were used to extract the bioactive components in the culture filtrates of strain LT161. The results show that the ethyl acetate extract and the n-butanol extract exhibited antifungal activity with growth inhibition zone of 16 and 14 mm respectively, while the petroleum ether extract did not. The antifungal activity of ethyl acetate extract was higher than the positive control of Daktarin (growth inhibition zone, 12 mm). Moreover, successful extraction by different organic solvents showed that the active components could be completely extracted by ethyl acetate and did not need to do the following extraction by n-butanol solvent. In other word, ethyl acetate is the appropriate organic solvent for bioactive extraction from strains LT161 filtrate.

ED_{50} of ethyl acetate extract

Table 1 shows the dose-dependent growth inhibition regression equations and ED_{50} of the ethyl acetate extract of strain LT161 against seven test phytopathogens. The strain LT161 extract exhibited strong antifungal inhibition against *C. parasitica* with ED_{50} 0.058 as well as other phytopathogens (ED_{50} 0.212-0.439).

Simulated *in vivo* disease control

The active components in the ethyl acetate extract of strain LT161 showed strong DCE against the rice sheath blight and *Fusarium* root rot as well as wheat *Alternaria* leaf blight diseases at the concentration of 2 mg/ml; about 80.0, 64.3and 80.0% respectively, which were significantly higher than that of Daktarin ($p<0.05$). However, at concentration of 1 mg/ml, the DCE against the three test diseases was decreased significantly; about 60.0, 47.0 and 28.0%, correspondingly. It seemed that the disease control capacity of the ethyl acetate extract of strain LT161 varied among the test diseases. For example, the DCE of rice sheath blight reduced to 25.9% at 1 mg/ml compared with it at 2 mg/ml, however, for wheat *Alternaria* leaf blight, it was about 63.4%. Similarly, the DI of each disease was reduced significantly at the high concentration compared with them at low concentration (Table 2; Figure 3).

Factors affecting the antifungal activity of strain LT161 extract

Figure 4 demonstrates that the active compounds in the culture filtrate of strain LT161 were quite pH resistance. The antifungal activity of the extract against *A. niger* remained almost unchanged when the culture was

Table 1. ED_{50} and the dose-dependent antifungal activity of strain LT161 extract.

Target phytopathogen	Regression equation	ED_{50}
Alternaria alternate	$logX^a=0.01921y^b-1.318$	0.439
Fusarium graminearum	logX=0.01897y-1.317	0.428
Rizoctonia solani	logX=0.01898y-1.515	0.272
Phytophthora capsici	logX=0.01842y-1.387	0.342
Fusarium oxysporum	logX=0.01974y-1.66	0.212
Glomerella glycines	logX=0.0134y-1.142	0.337
Cryphonectria parasitica	logX=0.02806y-2.636	0.058

[a] X represents the concentration of ethyl acetate extract of strain LT161 filtrate; [b] Y represents the growth inhibition percentage relative to the negative control

Figure 3. Photograph of wheat *Alternaria* leaf blight disease controlled by ethyl acetate extract of strain LT161. **a**, blank control; **b**, negative control; **c**, positive control (75% Daktarin WP); **d** and **e**, treated by ethyl acetate extract at 1mg/m and 2mg/mL respectively.

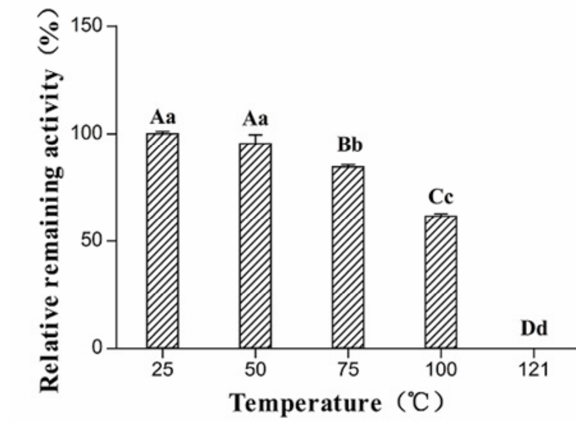
exposed to conditions in pH<12, but significantly reduced when pH>12, and the antifungal activity was totally lost when pH=14. Besides, the active metabolites of LT161 had considerable temperature stability. When handled in 100℃ for 30 min, its anti-*A. niger* activity declined by less than 40%. In addition, the active metabolites were not sensitive to Ca^{2+}, Mg^{2+} and Mn^{2+} (the antifungal activity did not significantly change at the given concentrations).

Isolation of the active fractions and compounds

24.5 g of ethyl acetate extract were achieved from 25 L of strain LT161 filtrate, of which 0.37 g of antifungal fractions were obtained based on bioautographic TLC guided isolation. LC/MS analysis showed that there were several main peaks which appeared at about 13.30, 17.43, 21.75 and 23.27 s respectively. The MS data at these retention times (RT) were compared to the Syngenta natural product dereplication database, but no similar compounds were found based on molecular weight, RT and UV spectrum, which indicated that the structure of the active compounds in the extract might be new.

DISCUSSION

C. camphora has been widely used as fine timber, oil crops, and medicinal species for a long time in south China. It has been reported that the extracts or essential oils from *C. camphora* leaves have antibacterial and antifungal activities *in vitro*, but the potential effect of associated endophyte fungi has not been discussed



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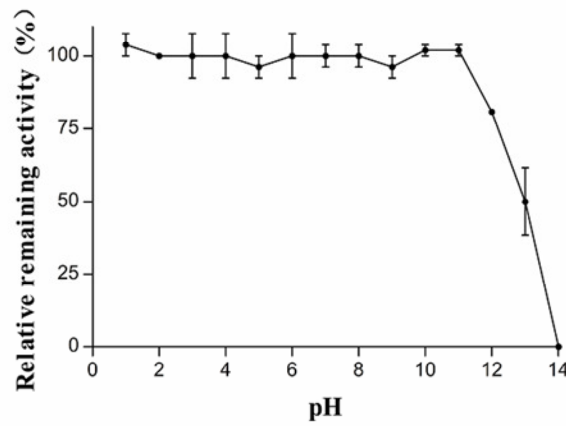

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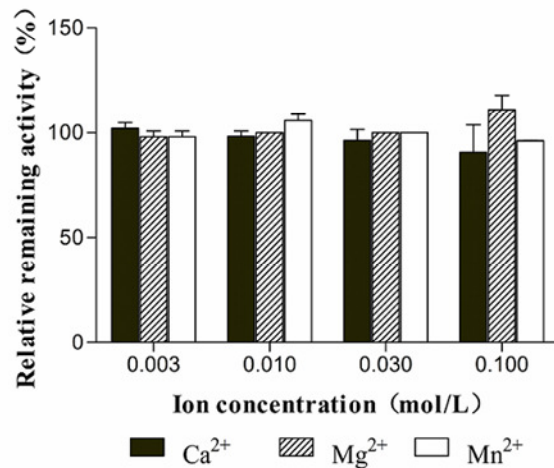

C

Figure 4. Effects of temperature (a), pH (b) and metal ions (c) on the stability of antifungal metabolites. Data were analyzed using one-way ANOVA and Tukey multiple test. The same letters indicate no significant differences (a, b, c, d; $P<0.05$) or highly significant difference (A, B, C, D; $P<0.01$) among the data in the same group of treatment.

(Mishra et al., 2008; Costa et al., 2010). However, there are only a few reports regarding the endophyte diversity (36 ascomycetes and 3 basidiomycetes) in *C. camphor* (He et al., 2012), without antimicrobial activity detection. In this study, we first report an endophytic strain LT161 that showed strong and broad spectrum of antifungal activity against seven phytopathogens *in vitro*. The ethyl acetate extract of strain LT161 exhibited a strong potency nearly equal to that of commercial fungicide Daktarin. Besides, the antifungal metabolites of strain LT161 are quite stable under different temperatures, pH conditions or Ca^{2+}, Mg^{2+} and Mn^{2+} concentrations. With these abilities, strain LT161 joins the growing list of endophytes that have potential for biological control of plant diseases.

Based on the morphological characteristics and rDNA-ITS sequence analysis, strain LT161 was identified as *P. brasiliens*. *Paraconiothyrium* spp. have been proved to produce extracellular laccase, ascotoxin, sesquiterpenoids and other bioactive compounds (Liu et al., 2010; Forootanfar et al., 2011; Khan et al., 2012) which inhibiting influenza virus replication (Fukami et al., 2000) and anticancer activities (Turbyville et al., 2006). Moreover, the distribution of *P. brasiliense* is very wide, which has been detected in USA, China, Canada, Italy, Japan and South Africa in a wide range of host plants (Damm et al., 2008). This suggests that *P. brasiliense* as a promising biocontrol agent and may possess necessary symbiosis ability with a variety of agriculture plants and great environmental adaptations.

Based on the bioautographic and TLC guided isolation strategy, the active fraction showed similar antifungal activities against the test fungi at 15 µg/ml to that of the ethyl acetate extract at 1 mg/ml. LC/MS analysis indicated that the active compounds in the fraction might be new based on comparison of the molecular weight, RT and UV spectrum of the compounds to the chemicals in the Syngenta natural product dereplication database. Thus, the structure of the antifungal compounds in the extract of strain LT161 needs to be elucidated in the future and the DCE in the field requires testing as well.

ACKNOWLEDGEMENT

This work was supported by the Jiangsu Science and Technology Support Program (BE2011355; BE2012372), the Special Fund for the Public Service Sector of the National Environmental Protection Ministry (201009023), the Natural Science Foundation of Jiangsu Province (BK2009234), the Fundamental Research Funds for the Central Universities (1082020803, 1092020804), and the National Training Program for Fundamental Scientists (J1103512).

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