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Author(s): Liqing Sun, Chung-Yun Hse, Todd Shupe, Mingjing Sun, Xiaohua Wang, and Kai Zhao
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Isolation and Characterization of an Endophytic Fungal Strain with Potent Antimicrobial and Termiticidal Activities From Port-Orford-Cedar

LIQING SUN,[1,2] CHUNG-YUN HSE,[2] TODD SHUPE,[3] MINGJING SUN,[1] XIAOHUA WANG,[1] and KAI ZHAO[1,4]

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ABSTRACT Termites are responsible for an estimated US$1 billion annually in property damage, repairs, pest control, and prevention. There is an urgent need of finding a better alternative way to control and prevent termites. Port-Orford-Cedar (POC) has been known to have significant levels of natural durability and termiticidal activities due to its extractive contents. In this study, 25 endophytes including 22 fungal and 3 bacterial strains were isolated from the POC. Four strains, namely, HDZK-BYF21, HDZK-BYF1, HDZK-BYF2, and HDZK-BYB11, were chosen to test their termiticidal activities. The fermentation broth of strain HDZK-BYF21 displayed the potent antimicrobial and termiticidal activities. Morphological examination and 18 S rDNA sequence analysis demonstrated that strain HDZK-BYF21 belonged to the genus *Aspergillus*. This finding indicates the existence of an interesting chemical symbiosis between an endophytic fungus and its host. This is also the first report on endophytes isolated from the POC that may have potential termiticidal activities. Endophytes with termiticidal activities can be grown in bioreactor to provide an inexhaustible supply of bioactive compounds and thus can be exploited commercially.

KEY WORDS termite, endophyte, fermentation metabolite, biocontrol, antitermiticidal activity

Termites are a class of pests in the tropics and can cause considerable problems in fabrics to even noncellulosic materials such as asbestos, asphalt bitumen, lead, and metal foils (Bultman et al. 1979, Yoshimura and Takahashi 2000, Verma et al. 2009, Anurag et al. 2012). Termites have been discovered in at least 11 states in the United States and are responsible for an estimated US$1 billion annually in property damage, repairs, pest control, and prevention, and US$250–300 million cost in China (Zhang et al. 2008, Zhao et al. 2011). Furthermore, termites are continuing to spread worldwide, as witnessed by the diffusion of several *Reticulitermes* species across both Europe and North America (Chris 2010, Morina et al. 2010). Although a few physical and chemical methods have been tried to eliminate termite populations, they all have limitations such as low effectiveness, highly toxic residues, environmental harmfulness, and high cost.

Some species of heartwood have the inherent ability to resist biological degradation (Eaton and Hale 1993). For example, Port-Orford-Cedar (POC), a heartwood species widely recognized for its horticultural uses and the quality of its wood, has been known to have significantly high levels of natural durability and termiticidal activities due to the extractive contents (McDaniel 1989, Du et al. 2011). The antifungal properties and termiticidal activities of POC extracts have been evaluated and reported (Liu 2004, Gao et al. 2008). While POC extracts can be used as termiticidal phytochemicals, large-scale harvest of POC will result in rapid depletion of the species in its natural habitat, as POC is native to only a small area of southwest Oregon and northwest California. To find another alternative way to produce the same termiticidal phytochemicals in a nondestructive manner will be of significance for conserving this valuable tree taxon.

Several studies have identified endophytes as a promising source of the natural secondary metabolite products with different biological activities (Rosa et al. 2011). Moreover, endophytes are more metabolically active than their free counterparts are due to their specific functions in the nature and activation of various metabolic pathways to survive within their host tissues (Strobel and Daisy 2003, Strobel 2006). Additional studies have shown that endophytic fungi isolated from the inner bark (phloem-cambium) of *Taxus cuspidate*, can produce taxol (Zhou et al. 2001; Sun et al. 2003; Ge et al. 2004; Zhao et al. 2008, 2009). Therefore, according to the theory of horizontal transfer from the host plant to its microbial symbiotic, we assumed that endophytes could exchange their genes with host and produce the same metabolites as their hosts do (Strobel 2006) Based on the previous reports, we hypothesized

[1] School of Life Science, Heilongjiang University, Harbin 150080, China.
[2] Southern Research Station, Adhesive and Composite Laboratory, Pineville, LA 71360.
[3] Louisiana Forest Products Development Center, School of Renewable Natural Resources, Louisiana State University, Baton Rouge, LA 70803.
[4] Corresponding author, e-mail: zybin395@126.com.

that endophytes isolated from the POC could also produce antitermitic compounds as their host could. In this study, we isolated 25 endophyte strains from the POC, and found that some of them had antimicrobial activities against index strains isolated from the gut of termites which can digest cellulose and support a termite's life, suggesting the existence of an intrinsic relationship between the endophytes and their host (Rosa et al. 2013). Strains HDZK-BYF21, HDZK-BYF1, HDZK-BYF2, and HDZK-BYB11 were chosen to test their termiticidal activities. A fungus HDZK-BYF21 with potent antimicrobial and termiticidal activity was identified by both morphological observation and 18 S rDNA sequences analysis. We demonstrated that the strain HDZK-BYF21 belonged to the genus *Aspergillus*. This is the first report on endophytes isolated from the POC that have potential termiticidal activities. Our results have provided a novel alternate way to produce environmentally friendly and low-cost termiticidals for controlling termites.

Materials and Methods

Insects and Strains for Bioassays. POC was collected in Oregon, USA.

The tested termite species, *Odontotermes formosanus* (Shiraki) (*O. formosanus*), was collected from Logia Hill of Wuhan, Hubei Province in China. Termites were maintained in an incubator at 26.5°C and 80% relative humidity (RH) and supplied with water and newspaper.

Index strains with degradation activities for lignin and cellulose, including HDZK-BYTB3, HDB-BYTB5, HDZK-BYTF1, and HDZK-BYTF2, were isolated from the gut of *O. formosanus* and used for the antimicrobial assay. These strains were stored at Key Laboratory of Microbiology, College of Life Science, Heilongjiang University, Harbin, China.

Isolation and Culture of Endophytes from the POC. The phloem of POC bark was selected and cut into 1.5- by 1.0- by 0.5-cm sections. These sections were treated with 75% ethanol for 3–5 min, and their surfaces were then washed with sterile distilled water. The disinfected wood sections were planted into potato dextrose agar (PDA) medium plate containing potato (200 g/liter), glucose (10 g /liter), and agar (15 g/liter) at 37°C for 10–24 h (Shen and Chen 2007) and cultured at 28°C for 3–5 d. At the same time, the disinfected wood sections were also planted into beef extract and peptone medium plate containing nystatin and cultured at 37°C for 2–3 d. Other pieces of wood treated by the same procedures and rolled on the surface of the PDA medium or beef extract and peptone medium plate were used as the blank culture control.

The fungal colonies from PDA medium plate were transferred to PDA medium at 28°C for 3–5 d, and the bacterial colonies from beef extract and peptone medium plate were transferred to beef extract and peptone medium containing beef extract (3 g/liter), peptone (10 g/liter), and NaCl 5 (g/liter) at 37°C for 10–24 h (Shen and Chen 2007). Some purified single colonies were obtained by standard plate-streaking technique.

Antimicrobial Activity. The agar well diffusion method, as described previously (Okeke et al. 2001), was followed for estimating the antimicrobial activity of the isolated strains fermentation broth. After incubation, the diameters of the inhibition zones were measured for each microorganism tested. Strong antimicrobial activities were considered when the diameters of the inhibition zones were >20 mm. All the assays were performed in triplicates.

Termiticidal Activity. The no-choice bioassay method (Kang et al. 1990) was used to evaluate the termiticidal activities of fermentation broth of the isolated strains. Samples of the fermentation broth were applied to 1 g filter paper samples (Whatman No. 3, 8.5 cm in diameter). A piece of filter paper treated with distilled water only was used as a control. Three hundred active termites were put on each piece of filter paper in a petri plate (9 cm in diameter by 1.5 cm in height). The dishes with covers were then placed in an incubator at 26.5°C and 80% RH. A few drops of water were periodically dripped onto the bottom edge of each petri dish. Termiticidal activities of fermentation broth of the strains tested were analyzed daily by recording the mortality of the termites for 14 d. Five biological replications were made for each sample.

Identification of Strain with the Strongest Antimicrobial and Termiticidal Activities. *Morphological Examination.* The obtained endophytic fungal strains were activated at 28°C on a plate of PDA medium. The mycelia were inoculated at three different spots in a 9-cm plate with PDA medium and incubated at 28°C for 12 d (Shen and Chen 2007). Termiticidal strains were detected and identified to the genus and species levels based on their morphological characteristics, as described previously (Gordon 1973, Coppuccino and Natalie 1983, John et al. 1994, Dong and Cai 2001, Shen and Chen 2007). The morphology of endophytic fungi was examined with both light microscopy and transmission electron microscopy (TEM). Digital micrographs of the colonies were taken with a Coolpix 995 camera (Nikon, Tokyo, Japan).

Molecular Analysis. The collection and culture of mycelia were carried out as previously described (Liu et al. 2008). For the sequence analysis, genomic DNA was extracted from the strain with the strongest antimicrobial activities and termiticidal function and identified with the previously described methods (Sambrook and Maniatis 2002).

The 18 S rDNA sequences of the strains tested were amplified by polymerase chain reaction (PCR) with primer pairs 5'- GGA TCA GAA TTC TAT TCT GGT TGA TCC TGC CAG -3' (forward) and 5'- CTC AGT AAG CTT GAT CCT TCC GCA GGT TCA CC -3' (reverse). The PCR reactions were carried out with one cycle of heat treatment at 94°C for 5 min, followed by denaturation at 94°C for 35 s, annealing at 55°C for 33 s, and extension at 72°C for 1 min for a total of 35 cycles, and by a final extension at 72°C for 7 min. The PCR products were stored at 4°C, later separated by 0.8% agarose gel electrophoresis, and then sequenced.

Sequencing of the PCR products was performed by Bo-ya Company Ltd. (Shanghai, China). The fragment of 18 S rDNA was sequenced in both directions with an ABI PRISM 377-18 DNA Sequencer (Applied Biosysterns, Foster City, CA) according to the manufacturer's instructions. The obtained sequences were submitted to GenBank for homology search with BLAST (http://rdp.cme.msu.edu and http//ncbi.nim.nih.gov). The sequences of 18S rDNA were aligned with those of related strains retrieved from GenBank with CLUSTAL X. A phylogenetic tree was constructed from the evolutionary distances by PHYLIP (version 3.57 c, distributed by Felsenstein, J., Department of Genetics, University of Washington, Seattle, WA).

Antitermiticidal Function of the Strain with the Strongest Antimicrobial and Termiticidal Activities. The method of Zhao et al. (2011) was employed to distinguish the termiticidal and repellent functions of fermentation broth of the cultured strain. In the test A, filter paper (Whatman No. 3, 8.5 cm in diameter) was impregnated with the fermentation broth and distilled water. In the test B, one half of the filter paper was impregnated with the fermentation broth and distilled water while the other half was impregnated with distilled water only. In the test C, a piece of filter paper was treated with beef extract and peptone medium only and used as a control. The procedures used were the same as those described in section anti-termitical activity. Triplicates were made for each test.

Characterization and Identification of Antitermiticidal Compounds Produced by the Strain with the Strongest Antimicrobial Activities and Termiticidal Function. Characterization and identification of antitermitical compounds produced by strain with the strongest antimicrobial activities and termiticidal function were performed as described previously (Zhao et al. 2011). Fermentation broth was treated with macroporous resin column chromatography according to Soemmering's extraction methods to obtain crude product, which was then used for extracting the antitermitic compounds with ethyl acetate in our study. Twenty grams of the crude product were extracted by using 500 ml of ethyl acetate with continuous shaking on a rotary shaker at 150–180 rpm for 48 h. The filtrates were concentrated using a rotary evaporator at 40°C and then stored at 4°C in airtight containers for further use.

The extracted product was further analyzed by gas chromatography-mass spectrometry (GC-MS) to identify the structure of the newly extracted antitermitic compounds. For the analysis, the samples were injected into a DB-17MS capillary column (30 cm in length, by 0.25 mm in diameter, and by 0.25 μm in film thickness; Agilent Technologies, Santa Clara, CA). A GC-MS model consisting of 6890 N gas chromatograph coupled with 5973 insert mass selective detector was used. The temperature for injector was set at 250°C and that for the detector was set at 280°C. The stepped temperature program was set as the follows: the temperature was set firstly at 50°C for 2 min, and then increased from 50 to 280°C at a rate of 10°C/min and finally held at 280°C for 5 min. The total running time was 30 min. The GC-MS interface temperature was 280°C. The injection volume was 1 μl. The solvent delay was 2 min and injected with a split ratio of 1:10. The MS scan range was from 15 to 500 amu. Compounds were identified by comparing their retention times with those of authentic compounds and the spectral data obtained from library data of corresponding compounds.

Statistical Analysis. The Scheffe multiple comparison procedure from the SAS statistical program was employed to evaluate differences in percentage of mortality for the antitermitic tests. The differences in mean values between groups with $P < 0.05$ were considered statistically significant. All the results were expressed as mean ± SD.

Results

Antimicrobial Activities of Fermentation Broths of the Isolated Endophytes. We isolated a total of 25 endophytes including 22 fungal strains and 3 bacterial strains from POC bark. Antimicrobial assay showed that all the isolated endophytes exhibited different levels of antimicrobial activities against index strains isolated from the gut of termites which can digest cellulose and support a termite's life (Table 1), suggesting the existence of an intrinsic relationship between the endophytes and their host. As seen from Table 1, strains HDZK-BYF21, HDZK-BYF1, HDZK-BYF2, and HDZK-BYB11 displayed the higher levels of antimicrobial activity as indicated by their diameters of the inhibition zones of >20 mm and were, thus, chosen to test their termiticidal activities.

Termiticidal Activities of Fermentation Broth of Isolated Endophytes. As seen from Table 2, the fermentation broths of strains HDZK-BYF21, HDZK-BYF1, HDZK-BYF2, and HDZK-BYB11 were capable of killing termites as reflected by significantly higher rates of the termite mortality caused by the fermentation broths of these four strains than that of the control group at 14 days ($P < 0.05$). The fermentation broth of strain HDZK-BYF21 displayed the highest rate of termite mortality compared with those of the strains HDZK-BYF1, HDZK-BYF2, and HDZK-BYB11 ($P < 0.05$). Moreover, the termiticidal activity was maintained for the shortest time. It becomes evident that the endophytic fungus HDZK-BYF21 from the POC displayed the strongest antimicrobial and termiticidal activities, suggesting that the fermentation broth of this strain can be further exploited as a potential natural biotermiticide against termites.

Identification of Strain HDZK-BYF21. Strain HDZK-BYF21 grew faster on the PDA medium, and a large number of spores were formed during 8–12 d. Colonies were smooth and yellow in color, but after 5 d of culturing, the color was changed to green, and red pigment was deposited. The spores were globular and smooth in the initial stage and kermesinus in the later stage with the diameters of conidia in the range of 2.5–7.5 μm. The photographic pictures of mycelium, conidia, vesicles, and foot cells were shown in Figure 1a–d, respectively.

Table 1 Antimicrobial assay of isolated strains fermentation broth against index strains isolated from the gut of *O. formosanus*

Index strains	Inhibition zone diameter (mm)[a]			
Isolated strains	HDZK-BYTB3	HDB-BYTB 5	HDZK-BYTF1	**HDZK-BYTF2**
HDZK-BYB11	19.6 ± 2.4	—	20.6 ± 2.5	**21.3 ± 3.7**
HDZK-BYB52	18.5 ± 4.2	—	17.5 ± 0.4	**16.5 ± 4.4**
HDZK-BYB152	13.5 ± 1.7	15.8 ± 1.9	19.4 ± 3.1	**18.5 ± 0.4**
HDZK-BYF1	21.4 ± 3.06	—	19.0 ± 1.20	**22.2 ± 3.40**
HDZK-BYF 2	20.1 ± 3.12	—	20.5 ± 1.02	**22.3 ± 3.16**
HDZK-BYF 9	16.5 ± 3.18	—	—	**—**
HDZK-BYF11	11.8 ± 1.94	11.8 ± 1.90	—	**—**
HDZK-BYF12	18.3 ± 3.24	18.3 ± 2.86	—	**—**
HDZK-BYF13	12.2 ± 2.88	—	13.4 ± 4.16	**11.2 ± 2.00**
HDZK-BYF14	15.5 ± 3.08	—	—	**14.2 ± 3.10**
HDZK-BYF15	19.5 ± 3.26	13.5 ± 2.10	14.9 ± 2.32	**—**
HDZK-BYF17	17.9 ± 3.04	—	—	**19.0 ± 3.78**
HDZK-BYF18	18.6 ± 3.90	15.6 ± 2.06	18.1 ± 2.64	**18.7 ± 2.82**
HDZK-BYF21	25.3 ± 3.24	23.2 ± 2.82	22.0 ± 4.12	**22.1 ± 4.02**
HDZK-BYF30	17.3 ± 3.22	—	17.5 ± 2.84	**—**
HDZK-BYF45	12.5 ± 1.80	—	11.0 ± 1.92	**12.6 ± 2.04**
HDZK-BYF133	16.2 ± 2.34	15.2 ± 1.78	—	**16.2 ± 1.98**
HDZK-BYF136	11.2 ± 1.26	12.3 ± 2.00	11.2 ± 2.04	**—**
HDZK-BYF221	19.5 ± 2.46	11.2 ± 1.86	—	**18.6 ± 1.68**
HDZK-BYF224	12.6 ± 2.28	11.0 ± 1.90	12.6 ± 1.42	**14.6 ± 2.44**
HDZK-BYF937	16.3 ± 1.80	16.3 ± 1.68	—	**—**
HDZK-BYF1161	15.0 ± 1.00	14.3 ± 1.02	14.8 ± 2.24	**14.6 ± 0.92**
HDZK-BYF11451	—	—	—	**12.3 ± 1.66**
HDZK-BYF11517	—	—	—	**15.4 ± 2.08**
HDZK-BYF12751	—	—	15.8 ± 3.02	**12.2 ± 1.90**

[a] Inhibition zone diameter was presented as mean ± standard deviation of three experiments. "—" indicates negative reaction.

Table 2 Antitermitic activities of isolated strains fermentation broth for 14 d

Strains	The number of dead termites after feeding fermentation broth					Termite mortality (%)[a]
	1	2	3	4	5	
HDZK-BYF21	277	258	270	289	284	91.87 ± 4.06[a]
HDZK-BYF1	148	126	138	131	122	44.33 ± 3.43[b]
HDZK-BYF2	122	136	123	110	118	40.60 ± 3.15[b]
HDZK-BYB11	102	70	67	80	83	26.80 ± 4.60[c]
Control	2	1	4	0	1	0.53 ± 0.51[d]

Data with lower case alphabets show significant difference ($P < 0.05$).
[a] Termite mortality was presented as mean ± standard deviation of five experiments.

The 18 S rRNA of strain HDZK-BYF21 was successfully amplified by PCR with an expected size of 1,645 base pairs. Homology searching against GenBank revealed that the sequences of strain HDZK-BYF21 shared 99% similarity with those of *Aspergillus flavus* KC907367. A phylogenetic relationship was established through the alignment and analysis of homologous nucleotide sequences among these species (Fig. 2), which indicated that the strain HDZK-BYF21 was closest to the genus *Aspergillus*. According to the phylogenetic analysis, strain HDZK-BYF21 was classified to the genus *Aspergillus*.

Antitermitic Function of Strain HDZK-BYF21 Fermentation Broth. The fermentation broth of the strain HDZK-BYF21 displayed termiticidal activities in both termite tests A and B. The termite mortality rates were 95.3 ± 3.4, 60.4 ± 3.7, and 4.8 ± 2.9 % in termite tests A, B, and C after 10 d, respectively. In addition, most of these dead termites in test B lied on the side of the filter paper impregnated with the fermentation broth, suggesting that the antitermitic function of the fermentation broth of strain HDZK-BYF21 is attributed to its toxic effects on termites. Therefore, our results showed that fermentation broth of the strain HDZK-BYF21 was capable of killing termites and had significant antitermitic functions ($P < 0.05$), while it did not have significant repellent function for *O. formosanus* ($P > 0.05$).

Antitermiticidal Compound Extracted from Strain HDZK-BYF21. The fermentation broth from strain HDZK-BYF21 was extracted with ethyl acetate, and the extracts displayed a number of peaks in the GC-MS spectrum. The known antitermite compound, α-terpineol, was found at the retention time of 19.074 min with 99% similarity (Fig. 3).

Discussion

The antimicrobial activities of plant endophytic fungi have been reported previously (Liu et al. 2010). As the

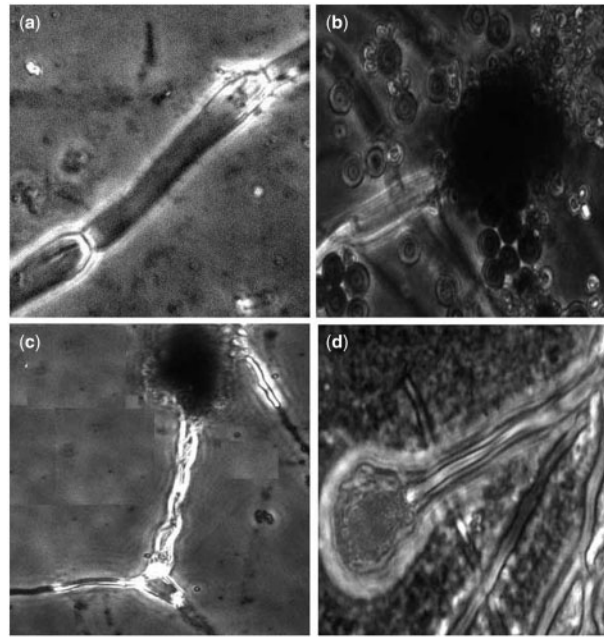

Fig. 1. Morphology of strain HDZK-BYF21 under a transmission electron microscopy at the magnitude of 20, 500. (a) mycelium; (b) conidium and spherical; (c) spherical vesicle and foot cell; (d) spherical vesicle.

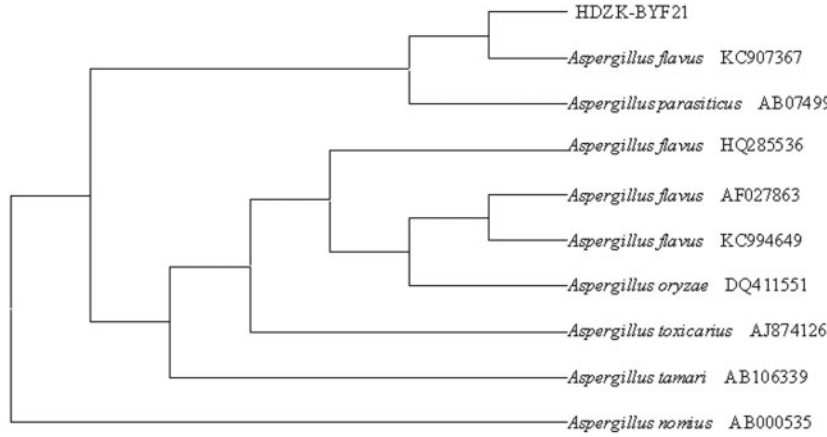

Fig. 2. Phylogenetic tree about the relationship of strain HDZK-BYF21 with other related microbiological species from GenBank based on their homologous sequences of 18S rDNA.

previous researches on endophytes were mainly focused on searching for the metabolites of the host-plant in the endophytic partner (Puri et al. 2006, Kusari et al. 2009, Qadri et al. 2013), the theory of horizontal transfer from the host plant to its microbial symbiotic received much impetus (Strobel 2006). Therefore, endophytes are synergistic to their host. At times, they are known to prevent their host from successfully attacking fungi and pests by producing special substances such as secondary metabolites which in turn demand nutrition (Strobel and Daisy 2003). Unlike their host plant, many endophytes are capable of surviving under quite extreme and inhospitable conditions (Bacon and James 2000). It has been known that the numbers of secondary metabolites produced by the fungal endophytes are larger than those of any other endophytic microorganism classes.

In this study, a total of 25 endophytes were isolated from the POC trees and shown to have natural termite resistance as being displayed in an antimicrobial assay. According to the diameters of their inhibition zones, the strains HDZK-BYF21, HDZK-BYF1, HDZK-BYF2, and HDZK-BYB11 were chosen to further test their termiticidal function. The result showed that the fermentation broth of the strain HDZK-BYF21 caused the highest rate of termite mortality. Thus, this strain

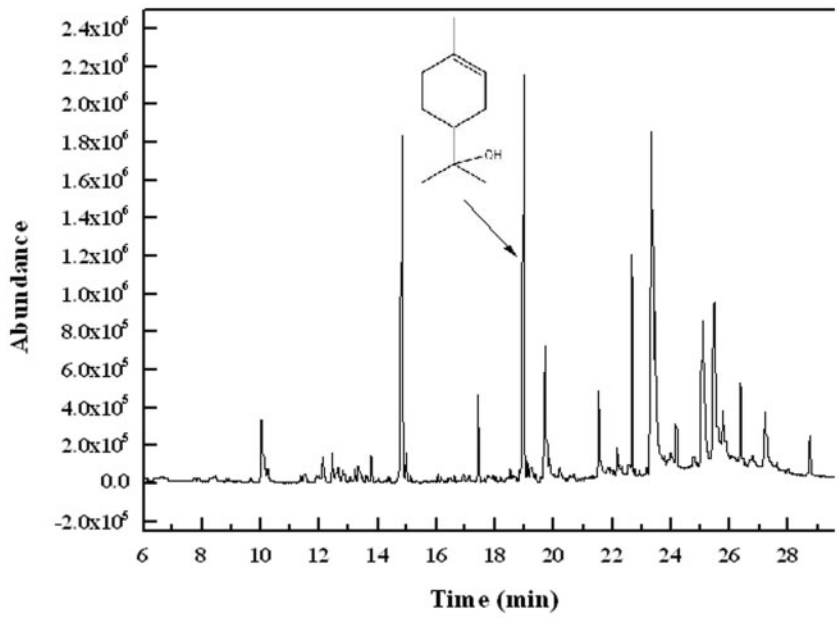

Fig. 3. GC-MS spectrum of the extracts from strain HDZK-BYF21. The arrow indicates the molecular ion of α-terpineol at the retention times of 19.074 min.

was identified as *Aspergillus* by both morphological characterization and 18 S DNA sequencing analysis.

Previous researches showed that α-terpineol, torreyol, τ-muurolol, τ-cadinol, and α-cadinol isolated from the POC displayed termiticidial activities (McDaniel 1973). In a previous study, we isolated endophytes from Eastern Red-Cedar, *Juniperus virginiana*, and found that it had the natural termite resistance and produced α-terpineol to kill termites (Zhao et al. 2011). In the present study, we also extracted α-terpineol from the antitermitic compound-producing endophytic HDZK-BYF21 isolated from the POC. Together, these results indicate that α-terpineol is one of the potent phytochemicals responsible for the termiticidal activities of endophytes present in host plants.

It is worthy of pointing out that a wide variety of natural products produced by endophytic fungi present in their host plants possess unique structures and bioactivities, thus representing a huge reservoir which offers an enormous potential for exploitation of their use and applications in agricultural and industrial areas (Tan and Zou 2001). Fermentation of endophytic fungi with the potential for production of bioactive compounds confers several advantages including reproducible and dependable productivity. These endophytic fungi can be grown in the bioreactor to provide an inexhaustible supply of bioactive compound and thus can be potentially exploited in large-scale commercial application.

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