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Nematicidal endophytic bacteria obtained from plants

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Abstract - Two hundred and six bacterial isolates were obtained from leaf, flower and stem of three healthy plants, *Euphorbia pulcherrima* Willd, *Pyrethrum cinerariifolium* Trev. and *Heracleum candicans* Wall. The nematicidal activity experiment showed that a total of 92 isolates displayed activity against *Caenorhabditis elegans* (Maupas) Dougherty, and 70 isolates resulted active against *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle. Strain LCB-3 exhibited the strongest nematicidal activity against both two nematodes. According to the 16S rDNA, the strain LCB-3 was identified as *Brevundimonas diminuta*. Based on bioassay-guided fractionation, a nematicidal metabolite (*R*)-(**-**)-2-ethylhexan-1-ol was obtained from LCB-3. The median lethal concentrations (LC_{50}) of the compound were 542.0 mg l^{-1} against *C. elegans* and 168.1 mg l^{-1} against *B. xylophilus* 48 h after treatment.

Key words: *Brevundimonas diminuta*, endophytic bacteria, nematicidal, (*R*)-(**-**)-2-ethylhexan-1-ol.

INTRODUCTION

Parasitic nematodes represent the largest source of essentially uncontrollable biotic stress. Plant parasitic nematodes have caused serious damages to agriculture and forest. For example, it has been reported that plant parasitic nematodes caused as much as $100 billion in annual losses of crops worldwide (Koenning *et al.,* 1999), and the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle causes disastrous diseases in the pinewood. Therefore, it is necessary to search for more resources to control plant parasitic nematodes.

Several strategies are used to control plant parasitic nematodes, including cultural practices such as crop rotations, developing and planting resistant varieties, and applying chemical nematicides or biological nematicidal agents. In the past few years, the efficacy of synthetic pesticides is constantly weakened by development of resistance in parasitic nematodes. At the same time, commercially successful nematicides have caused both environmental problems and human health concerns due to their toxic residues, so some of them have been restricted (Yagi *et al.*, 1993). These problems are addressing the search for new antiparasitic agents with more environmental friendly and less toxic natural compounds (Kraouti *et al.*, 1995).

A way of searching for such environmentally friendly biological agents is to screen naturally occurring compounds in microorganisms. Among all known producers of natural products, microorganisms represent a rich source of biologically active metabolites that find wide-ranging applications as agrochemicals, antibiotics, antiparasitics and anticancer agents (Gunatilaka, 2006). Likewise, microorganisms have attracted the attention as potential nematode bio-control agents due to their nematicidal activities and environmental friendliness. Microorganisms produce a wide range of secondary metabolites, many of them have been identified and intensively investigated for their nematicidal toxins (Li *et al.*, 2007a).

In the past few decades, it has been realised that plants may serve as a reservoir of untold numbers of microorganisms known as endophytes. Partial endophytes are being intensively studied since some of them can produce unique or novel bioactive substances. Endophyte is a large unexplored source, especially as nematode bio-control agents. Information in the literature about nematicidal activity of endophytes is scarce. Up to the present, only two nematicidal metabolites produced by endophytes have been reported. Pregaliellalactone, a hexaketide metabolite which had been isolated from several fungi, including an endophytic fungus E99297 (**AF485074**), showed nematicidal activity (Köpcke *et al.*, 2002a; 2002b). 3-Hydroxypropionic acid, obtained from several endophytic fungi, showed selective nematicidal activity against the plant-parasitic nematode *Meloidogyne incognita* (Schwarz *et al.*, 2004). There is no report about nematicidal activity of endophytic bacteria. This represents a novel field to be explored to detect nematicidal resources from endophytic bacteria.

MATERIALS AND METHODS

Plant materials. Thirty samples of three plants (ten samples of per plant), *Euphorbia pulcherrima* Willd, *Pyrethrum cinerariifolium* Trev. and *Heracleum candicans* Wall., were collected from Kunming Institute of Botany P. R. China in March 2007. The plant samples were processed within 24 h after collection.

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Isolation of endophytic bacteria. Plant samples were washed with mild detergent and thoroughly washed in running tap water, and air dried before sampling. Before surface sterilisation, plant materials were cut into small segments using sterile knife blade (Phongpaichit *et al.*, 2006). In our experiment, sample segments were successively surface sterilised by immersion in 75% ethanol for 30 s, and 0.1% hydrargyric dichloridum (containing 0.1% Tween 20) for 2-10 min according to different materials with vigorous shaking. In order to remove the disinfectant, sections were rinsed 3 times in sterile water for 10 min. The sections were air dried under a sterile flow. The sterilised segments of leaf, flower and stem were placed on plates of LB, NB and YMG media (LB: 10 g peptone, 5 g yeast extract, 10 g NaCl and 18 g agar in 1 l H_2O, pH 7.0 before autoclaving; NB: 10 g peptone, 3 g beef extract, 5 g NaCl and 18 g agar in 1 l H_2O, pH 7.0-7.2 before autoclaving; YMG: 4 g yeast, 10 g glucose, 10 g malt extract and 18 g agar in 1 l H_2O, pH 7.0-7.2 before autoclaving). Then the plates were incubated at 28 °C until the outgrowth of microbe. After isolation of bacteria from plant materials, each isolate was checked for purity and transferred to a new medium. All isolated strains were deposited in the Key Laboratory for Conservation and Utilization of Bio-resources of Yunnan Province, China.

Nematicidal activity. The culture methods of the saprophytic nematode *Caenorhabditis elegans* (Maupas) Dougherty and the pine wood nematode *B. xylophilus* (Steiner et Buhrer) Nickle were based on the references (Li *et al.*, 2005).

The isolated strains were grown in shake culture (100 ml per 250 ml triangular flask) on LB medium. After fermentation for 5 d at 37 °C at 180 rpm, the fermentation broth was centrifuged at 8000 rpm for 15 min to discard bacterial cells and to obtain the culture supernatant. Each fermentation broth sample (2 ml) was added in a Petri dish (6 cm diameter) containing 150-200 nematodes. Nematicidal activity (NA) was calculated using the formula:

$$NA = IN/SN \times 100\%$$

where IN is the number of immobile nematodes, SN is the sum of all nematodes counted (SN > 100). Each treatment was replicated 3 times and calculated the average. The NA ≥ 80%, 50% ≤ NA < 80%, 20% ≤ NA < 50% or NA < 20% at 48 h were respectively considered to have strong, moderate, slight or no NA.

16S rDNA analysis. Genomic DNA was extracted and amplified from bacterium according to the procedure described by Xu *et al*. (2003). 16S rDNA was amplified by PCR using TaKaRa Ex Taq (TaKaRa Biotechnology) with the following primers: A 20f (5′-AGAGTTTGATCCTGGCTCAG-3′) and B 1500r (5′-GGTTACCTTGTTACGACTT-3′). The PCR reaction was performed by 5.5 min pre-denaturation at 95 °C, followed by 35 cycles of 1 min at 94 °C, 40 s at 55 °C, 2 min at 72 °C and a final extension of 10 min at 72 °C and then cooled to 4 °C. The amplified 16S rDNA fragment was purified with a PCR fragment recovery kit (TaKaRa) and directly sequenced using an ABI PRISMTM 377 DNA sequencer (Applied Biosystems Inc.) with the PCR primers (20f and 1500r) as the sequencing primers. The 16S rDNA sequence of the test strain was aligned manually against corresponding sequences of most related strains obtained from the GenBank database.

Spectroscopic and chromatographic analysis. Column chromatography was performed on silica gel G (200-300 mesh; Qingdao Marine Chemical Factory, Qingdao, P.R. China) and Sephadex LH-20 (Pharmacia). Thin-layer chromatography (TLC) was carried out on silica gel (Si gel G; Qingdao Marine Chemical Factory). Spots were made visible by spraying with 5% vitriol ethanol solution followed by heating. Nuclear magnetic resonance (NMR) experiment was carried out on a Bruker DRX-500 spectrometer. Mass spectrum was recorded on a VG Auto-Spec-3000 mass spectrometer. Optical rotation was measured on a Jasco DIP-370 digital polarimeter.

Extraction and isolation of nematicidal compound from strain LCB-3. LCB-3, obtained from the stem of *Euphorbia pulcherrima*, was cultured as described previously. After centrifugation at 8000 rpm for 15 min, the culture supernatant was concentrated to 1 l, and then extracted with n-butanol. After filtration and removal of the solvents under rotary evaporation at 50 °C, a dark brown crude extract was obtained and dissolved in methanol. The isolation and characterisation of bioactive substances from the extract were done using bioassay guided fractionation.

The residue (3.12 g) was chromatographed over a silica gel G column (90 g, 200-300 mesh) eluting with petroleum ether/acetone (20:1 to 4:1, v/v) to afford eight subfractions (Fr. A1-A8). Fr. A2 (0.4 g) was repeatedly purified on a Sephadex LH-20 column (30 g) eluting with acetone to obtain 25 mg of the active substance defined compound **1**.

Nematicidal activity of compound 1. The *in vitro* activity of compound **1** was assayed against two nematodes *C. elegans* and *B. xylophilus*. Compound **1** was dissolved in acetone and diluted with distilled water to 800, 400, 200 and 100 mg l^{-1} for nematicidal activity assay based on the method described by Li and coworkers (2005). Each treatment was replicated 3 times, then the mean percentage of mortality at 24 and 48 h was calculated. Distilled water containing 5% acetone and distilled water were used as control.

The median lethal concentration (LC_{50}) of compound **1** against *C. elegans* and *B. xylophilus* was calculated according to Microsoft Excel (version 2003 software, USA). Regression analyses were also conducted by Excel for linear model. Data on proof mortality of nematodes were transformed into probit value, and concentrations (C) of compound were also changed to $\log_{10}$ (C) before analysis.

RESULTS AND DISCUSSION

Isolation of endophytic bacteria

The three plants, *E. pulcherrima*, *P. cinerariifolium* and *H. candicans*, used for the isolation of the endophytic bacteria in our experiment, are characterised by distinct biological activities. *Euphorbia pulcherrima* was found to possess antibacterial activity (Li *et al.*, 2007b) and *H. candicans* has been commonly used for medical purpose in China (Klaus, 2002). Both of them showed nematicidal activity in our screening experiment. *Pyrethrum cinerariifolium* is a famous pesticidal plant which has been proven to possess nematicidal effects as well (Mbaria *et al.*, 1998; Mochizuki, 2003). From the three plants, a total of 206 bacterial isolates were obtained (Table 1). Among them, 37 were isolated from *E. pulcherrima*, 117 from *P. cinerariifolium*, and 52 from *H. candicans*. From LB, NB and YMG media, 78, 74 and 54 isolates were obtained respectively, which suggested that LB and NB media are compatible with bacteria isolation.

Nematicidal activity of endophytic bacteria

Between the 206 bacterial isolates obtained in the present work, 92 (accounting to 44.7%) showed NA against *C. elegans* at 48 h,

TABLE 1 - Number of endophytic bacterial isolates from different media and plants

Host plant	LB medium			NB medium			YMG medium			Total
	Flower	Leaf	Stem	Flower	Leaf	Stem	Flower	Leaf	Stem	
Euphorbia pulcherrima	5	5	7	4	3	6	2	1	4	37
Pyrethrum cinerariifolium	11	12	18	25	9	11	14	7	10	117
Heracleum candicans	9	4	7	9	3	4	8	4	4	52
Total		78			74			54		206

TABLE 2 - Number of active bacterial isolates (NA* > 20%)

Host plant	Total number of isolates	Number of NA positive isolates							
		Caenorhabditis elegans				*Bursaphelenchus xylophilus*			
		Strong	Moderate	Slight	Total	Strong	Moderate	Slight	Total
Euphorbia pulcherrima	37	9	10	3	22	6	10	5	21
Pyrethrum cinerariifolium	117	15	31	17	63	18	15	10	43
Heracleum candicans	52	2	3	2	7	1	4	1	6
Total	206	26	44	22	92	25	29	16	70
Percentage		12.6	21.4	10.7	44.7	12.1	14.1	7.8	34.0

* Nematicidal activity: NA ≥ 80%, 50% ≤ NA < 80%, 20% ≤ NA < 50% at 48 h were respectively considered to have strong, moderate or slight NA.

and 70 (accounting to 34.0%) displayed NA against *B. xylophilus* at 48 h (Table 2). Most of them were isolated from *P. cinerariifolium*, the traditional pesticidal plant. Of the 206 isolates 12.6% and 12.1% showed strong NA (≥ 80%) against *C. elegans* and *B. xylophilus,* respectively at 48 h. These isolates may be potential candidates for developing novel nematicidal agents.

Strain LCB-3 was isolated from the stem of *E. pulcherrima*. Among the bacterial isolates with nematicidal activity, strain LCB-3 displayed 100% NA both against *C. elegans* and *B. xylophilus* at 48 h, thus it was selected for further studies.

Identification of strain LCB-3

16S rDNA of the strain LCB-3 was amplified and sequenced (**EU368739**). It is identical with many *Brevundimonas diminuta* 16S ribosomal RNA genes (**DQ857897.1, X87274.1, DQ979376.1, FM180525.1, AJ227778.1, AB021415.1** and **EU430084.1**)**.** The matching rate is 99%. According to this, LCB-3 was identified as *B. diminuta*.

Brevundimonas diminuta was found to colonise aortic aneurysms and might play a role in their development (Silva *et al.*, 2006). This is the first report of *B. diminuta* with nematicidal activity.

Chemical identification of compound 1

Compound **1** was obtained as colourless oil: $[\alpha]^{16,D}$ = - 36 (*c* = 30, $CHCl_3$). EIMS *m/z*: M^+ not observed, 112 (M^+ - H_2O, 4%), 98 (M^+ - CH_3OH, 6), 83 (60), 57 (100). ^{1}H-NMR (500 MHz, CDCl3) : δ 3.53 (2H, d, J = 7.2 Hz, H-1), 1.66 (1H, m, H-2), 1.26-1.37 (8H, m, H-3,4,5,7), 0.89 (6H, t, J = 7.0 Hz, H-6,8); ^{13}C NMR (125 MHz, CDCl3) : δ 65.7 (C-1), 42.3(C-2), 30.5 (C-3), 29.5(C-4), 23.7 (C-5), 14.5 (C-6), 23.5(C-7), 11.5 (C-8). Compound **1** was identified as (*R*)-(**-**)-2-ethylhexan-1-ol (Fig. 1) by comparing its spectroscopic data with those reported in the literature (Baczko and Larpent, 2000).

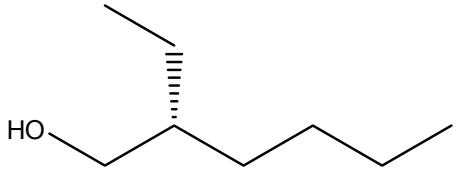

FIG. 1 - Structure of (*R*)-(**-**)-2-ethylhexan-1-ol.

Nematicidal activity of compound 1

The compound (*R*)-(**-**)-2-ethylhexan-1-ol was tested for nematicidal activity. Bioassay results showed that compound **1** caused significant mortality on *C. elegans* and *B. xylophilus* at the concentrations of 800 and 400 mg l^{-1} (Table 3). The nematicidal effect of the compound **1** was increased by augmented concentration and exposure time. LC_{50} of compound **1** were 542.0 mg l^{-1} against *C. elegans* and 168.1 mg l^{-1} against *B. xylophilus* 48 h after treatment. NA of distilled water containing 5% acetone and distilled water was under 5% at 48 h.

It has been reported that compound (*R*)-(**-**)-2-ethylhexan-1-ol has been obtained by chemosynthesis. However, that the molecule has not yet been isolated from natural material before. The similar compound, 2-ethyl 1-hexanol, had been showed to have antifungal activity (Fernando *et al.*, 2005). However, nematicidal activity of (*R*)-(**-**)-2-ethylhexan-1-ol has not been reported previously.

Bioactive natural products still retain an immense impact on modern medicine and agriculture though synthetic products have been improving. During the evolutionary process, endophytic microorganisms have developed the biochemical ability to produce bioactive natural products similar or identical to their host plants. Nematodes are the most abundant animals on the earth, and bacteria also possess a biologically and phylogenetically wide diversity (Bongers and Ferris, 1999). As one of the enormous and relatively untapped source of new medicinal and agricultural products, endophytic microbes represent a huge potentiality for the research of new bioactive natural products. The results of the present work clearly confirmed that endophytic bacteria can be an abundant resource for searching novel nematicidal agents.

TABLE 3 - Nematicidal activity (%) of (*R*)-(**-**)-2-ethylhexan-1-ol

Concentrations (mg l^{-1})	*Caenorhabditis elegans* (%)		*Bursaphelenchus xylophilus* (%)	
	24 h	48 h	24 h	48 h
800	47.6	58.5	90.1	96
400	38.5	45.2	66	73
200	21.3	26.6	42	53.8
100	10.1	21.7	25	35.1
LC_{50}	636.8	542.0	231.2	168.1

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