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Nematicidal Activity of *Paecilomyces* spp. and Isolation of a Novel Active Compound

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Many species of *Paecilomyces* are entomogenous fungi and several are efficacious toward nematodes. To study the potential of *Paecilomyces* species in controlling nematodes, fungal extracts of 40 *Paecilomyces* spp. were evaluated for their nematicidal activity against *Bursaphelenchus xylophilus* and *Panagrellus redivivus*. The extracts of six *Paecilomyces* spp. exhibited the nematicidal activity against *P. redivivus*, and 11 species exhibited the nematicidal activity against *B. xylophilus*. The methanol extract of strain 1.01761 incubating on Czapek solid medium killed more than 95% *P. redivivus* in 24 h at 5 mg/ml, and the filtrate of strain 1.01788 cultured in Sabouraud's broth medium resulted in 90% mortality of *B. xylophilus* in 24 h at 5 mg/ml. A novel nematicidal compound, 4-(4'-carboxy-2'-ethyl-hydroxypentyl)-5,6-dihydro-6-methyl-cyclobuta[b]pyridine-3,6-dicarboxylic acid, was isolated from *Paecilomyces* sp. YMF1.01761. The LD_{50} value of the compound within 24 h against *P. redivivus* was 50.86 mg/L, against *Meloidogyne incognita* was 47.1 mg/L, and against *B. xylophilus* was 167.7 mg/L.

***Keywords*:** *Paecilomyces* spp., nematicidal activity, novel compound

Many species of *Paecilomyces* are entomogenous fungi and some play important roles in pest management (Jalata *et al*., 1979; Liang *et al*., 2003). Metabolites from these fungi are not only detrimental towards other pests, but can also stimulate plant growth.

Plant-parasite nematodes inflict serious damage on agricultural crops and plants. *P. lilacinus* suppresses nematode reproduction and a commercial product (containing fungal spores) is now available for grower use (Jalata, 1986; Shahzad and Ghaffar, 1987; Reddy and Khan, 1989). Dijian found that *P. lilacinus* produces acetic acid, which suppresses growth of nematode larvae and eggs (Djian *et al*., 1991). Nematicidal leucinostatin was isolated from an Australian strain of *P. lilacinus* (Park *et al*., 2004). Additionally, purified Paeciloxazine from *Paecilomyces* sp. BAUA3058 exhibits nematicidal activity against *Rhabditis pseudoelongata* in 2004 (Kanai *et al*., 2004). We evaluated 40 different *Paecilomyces* strains isolated from China for nematicidal activity against *Bursaphelenchus xylophilus* and *Panagrellus redivivus*. A nematicidal compound was isolated from one of the active strains of *Paecilomyces*.

Materials and Methods

Culture and extraction of fungi

Forty strains of *Paecilomyces* deposited in the culture collection of Key Laboratory for Conservation and Utilization of Bio-resource, Yunnan University (from No. 1.01761 to No. 1.01800) were evaluated for nematicidal activity against two nematodes. All of them were isolated from soil in Yunnan Province, China, except for eight strains (No. 1.01771 to No. 1.01778) which were isolated from insects in Yunnan. All fungal strains were initially cultured in PDA medium at 25℃. Each strain was then sub-cultured on eight different kinds of media: liquid and solid media of modified Sabouraud's (SDBY, SDAY), Czapek (CB, CA), Potato Dextrose (PDB, PDA), and Corn meal (CMB, CMA). The liquid cultures were incubated in 500 ml flasks at 180 rpm and 25℃ for 8 days. The solid cultures were incubated on 90 cm plates at 25℃ for 15 days.

The solid cultures (5 plates each) were extracted 3 times with 80% methanol (100 ml) at room temperature (48 h each time). The methanol extracts were filtered and concentrated under vacuum, and the residues were then dissolved in 5% methanol at 5 mg/ml concentration. The liquid cultures (500 ml) was filtered, the supernatant concentrated under vacuum and the residues dissolved in 5% methanol at 5 mg/ml concentration. All samples were stored at 4℃ prior to use.

Isolation and structural characterization of active compound from *Paecilomyces* YMF1.01761

Column chromatography was carried out on silica gel (200~300 mesh; Qingdao Marine Chemical Factory, China), Sephadex LH-20 (Pharmacia, USA) and C_{18} (25~40 μm, Merck, Germany). Thinlayer chromatography (TLC) was performed on silica gel (Si gel G; Qingdao Marine Chemical Factory), and the spots were visualized under 5% vitriol ethanol solution. NMR experiments were carried out on a Bruker DRX-500 spectrometer. Mass spectra were recorded on a VG Auto- Spec-3000 mass spectrometer. Infrared (IR)

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spectra were measured on a Paragon 1000pc spectrometer.

The Czapek solid cultures (18 L) of *Paecilomyces* YMF 1.01761 were extracted using the method described above to produce a crude residue (5.8 g). The residue was applied to a silica gel chromatography column (200 g, 200~300 mesh) gradient eluted with petroleum ether/acetone to yield Ly1 to Ly14. A bioassay guide was used to determine which fraction had nematicidal activity. Active fraction Ly8 was then purified on a silica gel column (10 g, 200~300 mesh) gradient eluted with chloroform/acetone to give fractions Ly81 to Ly83. Active fraction Ly82 was further separated on a Sephadex LH-20 column with acetone to obtain active fraction Ly822 which was purified on a C18 column eluted with methanol/water (1/4, v/v) to yield 86 mg of active compound (Ly-1). The compound was a colourless crystal, EI-MS (70 eV) m/z: 365$[M]^+$; IR (KBr): 3578, 2980, 2936, 2892, 1852, 1772, 1668, 1462, 1387, 1270, 1226, 1050, 954, 732 cm^{-1}; NMR spectral data are given in Table 3.

Assay of nematicidal activity

Panagrellus redivivus: The saprophytic nematode was cultured on oatmeal medium (oatmeal: 20 g, water 80 ml) at 25°C for 7 days, and then refrigerated at 4°C prior to use.

Bursaphelenchus xylophilus: *Botrytis cinerea* was cultured on a PDA plate at 25°C when the fungus was fully grown, the plate was inoculated with the pine nematode, and then cultured until the fungal mycelia had been completely consumed.

Meloidogyne arenaria: The root-knot nematode was cultured on tomatoes in greenhouse conditions. Second stage juveniles were extracted and stored, according to the methods of Kerry and Bourne (2002).

All cultured nematodes were separated from the culture medium using the Baerman funnel technique (Gray, 1984), and an aqueous suspension of nematodes was prepared to use as a working stock.

Each extract was diluted in 5% methanol to a final concentration of 5 mg/ml. An aliquot (2 ml) was pipetted onto a 6 cm diameter Petri dish containing 150~200 nematodes. The Petri dishes were incubated at 25°C. Each treatment was replicated 3 times. The control treatment consisted of methanol without fungal extract. Dead and alive nematodes were separately counted at 24 h. The nematodes were considered to be dead when they did not move on physical stimuli with a fine needle (Barron and Thorn, 1987; Kwok *et al.*, 1992).

Five percent methanol water solutions of the purified compound at 800, 600, 400, 200, 100, 50, 20, and 10 mg/L were assayed for nematicidal activity by the method described above. Dead and alive nematodes were separately counted at 12 h, 24 h, and 36 h.

The final mortality was calculated as: mortality % = [$\sum$ (mortality of extract–mortality of control) /n]×100%.

Statistical analysis software (SPSS/version13.0 software, USA) was used to evaluate nematicidal activity of compound Ly-1. Regression analyses were conducted by SPSS to produce a linear model. Data on proof mortality of nematodes were transformed into probit values and LC_{50} was calculated according to probit analysis.

Results and Discussion

Nematicidal activity of fungal extracts

Nematicidal activity varied among the *Paecilomyces* strains tested (Table 1 and 2). Percentage of proof mortality that was less than 40% for a given fungal strain was recorded as inactive and was not listed in Table 1 and 2. Six strains (15.1% of all tested strains) exhibited nematicidal activities against *P. redivivus*. Eleven strains (27.5% of total strains tested) showed nematicidal activity against *B. xylophilus*. The extract from *Paecilomyces* sp. 1.01780 showed nematicidal activities against both *P. redivivus* and *B. xylophilus* after 24 h exposure. The methanol extract of mycelia of 1.01761 cultured on Czapek solid medium resulted in more than 95% mortality against *P. redivivus* at 5 mg/ml within 24 h. Strain 1.01788 displayed the strongest nematicidal activity against *B. xylophilus*. The filtrate of SDBY broth medium causes over 90% mortality against *B. xylophilus* within 24 h.

Mortality results differed among the eight selected media tested. The extracts against *P. redivivus* were cultured on both solid and liquid of Czapek and corn meal, whereas the extracts against *B. xylophilus* were cultured on both solid and liquid of Czapek and Sabourauds'. Czapek medium is usually used to observe taxonomic characterizations of *Paecilomyces* spp., and Sabouraud's medium contains more N source than corn meal. Further investigation to determine the effect of N source activity against different nematodes is warranted.

This is the first report that metabolites of *Paecilomyces* spp. having lethal activity against these two nematode species.

Table 1. Percentage of proof mortality of *Paecilomyces* fungal extracts[a] from eight different media against *Panagrellus redivivus* (24 h, n=3)

Strain number	Medium[b]							
	PDA	CMA	CA	SDAY	PDB	CMB	CB	SDBY
	Mortality (%±SD)							
1.01761	-	81.02±0.32	95.69±0.58	-	-	91.05±0.45	-	-
1.01771	-	-	-	-	-	-	-	58.37±0.28
1.01780	-	-	57.28±0.02	-	-	-	-	-
1.01784	-	95.13±0.24	-	-	-	-	69.84±0.61	-
1.01798	71.56±0.65	-	-	-	-	-	-	-
1.01799	-	-	-	-	87.36±0.37	-	-	-

[a] The concentration of each extract is 5 mg/ml in 5% methanol; -, mortality <40%

[b] PDA=potato dextrose agar; CMA=corn meal agar; CA=czapek agar; SDAY=sabouraud's agar; PDB=potato dextrose broth; CMB=corn meal broth; CB=czapek broth; SDBY=sabouraud's broth

Table 2. Percentage of proof mortality of *Paecilomyces* fungal extracts[a] from eight different media against *B. xylophilus* (24 h, n=3)

Strain number	Medium[b]							
	PDA	CMA	CA	SDAY	PDB	CMB	CB	SDBY
	Mortality (%±SD)							
1.01762	-	-	-	72.22±0.23	-	-	72.35±0.65	-
1.01763	-	-	-	57.15±0.54	-	-	-	-
1.01773	-	-	-	82.64±0.25	-	-	-	-
1.01780	76.64±0.22	-	-	-	-	-	-	-
1.01782	-	-	-	-	-	-	64.66±0.24	-
1.01786	-	-	-	58.26±0.06	-	-	-	-
1.01788	-	-	-	-	-	-	78.85±0.51	94.65±0.11
1.01791	-	-	-	-	-	72.98±0.51	-	-
1.01792	-	-	68.23±0.31	-	-	-	-	-
1.01793	-	-	65.20±0.13	-	-	-	-	-
1.01794	-	-	70.03±0.44	-	-	-	-	-

[a] The concentration of each extract is 5 mg/ml in 5% methanol; -, mortality <40%
[b] PDA=potato dextrose agar; CMA=corn meal agar; CA=czapek agar; SDAY=sabouraud's agar; PDB=potato dextrose broth; CMB=corn meal broth; CB=czapek broth; SDBY=sabouraud's broth

We suggest that more species belonging to this family should be screened to search for new sources of nematicidal substances. Results also indicate that the nematicidal effect varies among the different culture medium tested, which may suggest that nutrient availability affects the production of various chemical metabolites.

Isolation and structure determination of active compound from *Paecilomyces* YMF1.01761

A nematicidal compound was isolated from *Paecilomyces* strain 1.01761 and designated as "Ly-1". This compound was purified as colourless crystal. ^{13}C NMR and DEPT spectra (Table 3) indicated that the compound contained a total of 18 carbon signals, including three CH_3, three CH_2, four CH and eight quaternary carbons. The IR (KBr) spectrum showed strong absorption at 852 cm^{-1}, 1772 cm^{-1}, and 1668 cm^{-1}, indicated the presence of carboxyl groups. The EI-MS (*m/z*: 365$[M]^+$) revealed the presence of odd number of N. The molecular formula was determined to be $C_{18}H_{23}O_7N$. Seven unsaturated bonds can be attributed to aromatic or heterocyclic ring. According to the correlation from HMBC, there should be a pyridine ring. Based on the HMBC experiment, protons of methylene at δ 3.40 (Hα-6 CH_2) and 3.91 (Hβ-6 CH_2) correlated to neighbouring quaternary carbons and also correlated to a methyl and a carboxyl. It suggested that the neighbouring quaternary carbons on pyridine ring combined

Table 3. NMR data of Ly-1 (in CD_3COCD_3)

	$\delta^{13}C$[a]	$\delta^{1}H$[a]	HMBC ($C_{NO.}$)[a]
1	-	-	-
2	150.84(d)		
3	164.73(s)		
4	148.33(s)		
5	144.38(s)		
6	130.77(s)		
3-COOH	166.42(s)		
6-COOH	175.56(s)		
6-CH_3	27.58(q)	1.78(m)	6-CH_2, 6-C
6-CH_2	32.94(t)	3.91(d, J=12.95) 3.40(d, J=13.10)	5, 6, 6-C, 6-COOH
6-C	48.64(s)		
1'	66.26(d)	5.11(d, J=25.45)	
2'	54.50(d)	2.20(m)	4, 1', 4', 3', 2'-CH_2, 2'-CH_3
3'	28.82(t)	1.63(m)	2', 4', 5'
4'	38.80(d)	3.22(m)	
5'	13.10(q)	0.95(m)	4', 3'
2'-CH_2	20.28(t)	1.75(m)	2', 2'-CH_3
2'-CH_3	13.85(q)	1.16(m)	2'-CH_2
4'-COOH	166.75(s)		

[a] Recorded at 500 MHz

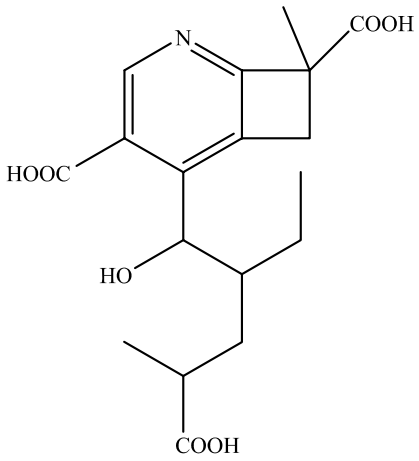

Fig. 1. Structure of the compound Ly-1.

with a cyclic methyl substituted butane. The presence of dissociated hydroxyl was proofed by sharp absorption peak at 3578 cm^{-1}. Methine (δ66.26) is influenced by the hydroxyl, downfield shift. The proton of methine at δ2.20 (H-2') displayed correlation with C4 (δ148.33), C1' (δ66.26), C4' (δ38.80), C3' (δ28.82), C2'-CH_2 (δ20.28), and C2'-CH_3 (δ13.85). It supported the substitution of three branches. Methine (δ148.33) on one of branch downfield shift by influence of carboxyl substituted.

Finally, the proposed structure of Ly-1 was approved by detailed HMQC and HMBC experiments (Table 3). The metabolite was identified as 4-(4'-carboxy-2'-ethyl-hydroxy-pentyl)-5,6-dihydro-6-methylcyclobuta[*b*]-pyridine-3,6-dicarboxylic acid, and the chemical structure of the compound had not been previously reported (Fig. 1).

Nematicidal activity of 4-(4'-carboxy-2'-ethyl-hydroxy-pentyl)-5,6-dihydro-6-methylcyclobuta-[*b*]pyridine-3,6-dicarboxylic acid

Percentages of proof mortality caused by Ly-1 against *P. redivivus*, *B. xylophilus,* and *M. incognita* are listed in Table 4. According SPSS analysis, the LD_{50} values of the compound within 24 h were 50.86 mg/L against *P. redivivus*, 47.1 mg/L against *M. incognita*, and 167.7 mg/L against *B. xylophilus*. The isolating process yielded 86 mg Ly-1 from 5.8 g extract. Calculated with a 20% loss of one time silica chromatography, a 20% loss of recrystal and another 5% loss, the yield of Ly-1 should be 3.6%, which is a very high yield in natural production.

Twenty six metabolites were isolated from 13 *Paecilomyces* strains from 1997 to 2006. The metabolites included cyclopeptides, alkaloids, pigments, polysaccharides, trichothecanes, polykitides, and other classes, and include metabolites with antitumor, neurotrophic, enzyme inhibitor, and cytotoxic activity. Beauvericin and cyclodepsipeptides were isolated from *P. tenuipes* (Nilanonta *et al*., 2000) and pesticidal dipicolinic acid was obtained from *P. fumosoroseus* (Asaff *et al*., 2005). Lang reported cytotoxic paecilosetin from *P. farinosus* (Lang *et al*., 2005) and Cheng isolated neurotrophic alkaloid (+)-N-Deoxymilitarinone A from *Paecilomyces farinosus* (Cheng *et al*., 2006). As for nematicidal metabolites, leucinostatins were isolated from an Australian strain of *P. lilacinus* (Park *et al*., 2004). Yoshinori purified a Paeciloxazine with nematicidal activity from *Paecilomyces* sp. BAUA3058 (Yoshinori *et al*., 2004). Gupta *et al*. (2006) investigated nematiciadal activity of secondary metabolites from *P. lilacinus*. All research indicates that *Paecilomyces* spp. produces numerous metabolites, but that only a few of them have been shown to exhibit nematicidal activity. Additional *Paecilomyces* spp. and their associated metabolites need to be further investigated for their potential use in managing plant-pathogenic nematodes.

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Table 4. Effect of Ly-1 on the proof mortality of three nematode species *in vitro*

Concentration (mg/L)	*Panagrellus redivivus*			*Meloidogyne incognita*			*Bursaphelenchus xylophilus*		
	12 h	24 h	36 h	12 h	24 h	36 h	12 h	24 h	36 h
10	3.3	5.8	5.8	2.1	4.4	4.3	0	0	0
20	5.0	24.0	27.2	20.2	28.9	29.7	2.6	4.3	6.7
50	41.3	48.6	50.2	55.6	57.8	58.9	19.7	21.2	22.1
100	41.0	58.4	58.1	59.1	60.2	61.4	22.4	30.6	37.2
200	75.7	92.4	91.8	79.8	93.4	96.6	40.6	47.8	47.9
400	97.1	100	100	100	100	100	67.7	72.9	77.8
600	-	-	-	-	-	-	79.9	90.1	92.3
800	-	-	-	-	-	-	81.1	92.7	93.3
LD_{50}	88.2	52.4	51.0	59.1	47.1	45.1	226.0	167.7	148.9

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