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SHORT COMMUNICATION

Nematicidal activity of *Trichoderma* spp. and isolation of an active compound

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Abstract *Trichoderma* spp. play an important role in biotic control, and several are efficacious against nematodes. To study the potential of *Trichoderma* species in controlling nematodes, fungal filtrates of 329 *Trichoderma* strains were evaluated for their nematicidal activity against *Panagrellus redivivus* and *Caenorhabditis elegans.* Fifteen strains exhibited nematicidal activity against *P. redivivus*, and 14 strains showed activity against *C. elegans.* The strain YMF1.02647 showed strong nematicidal activity against both nematodes, and the culture broth could cause more than 90% mortality to the tested nematodes within 48 h. A nematicidal compound was isolated from ethyl acetate extract of *Trichoderma* YMF1.02647 based on bioassay-guided fractionation. The compound was identified as trichodermin according to the spectroscopic data, which could kill more than 95% both *P. redivivus* and *C. elegans* in 72 h at 0.4 g l^{-1}.

Keywords *Trichoderma* spp. · Nematicidal activity · Trichodermin · Biocontrol

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Introduction

Trichoderma is a genus of fast-growing fungi widely existing in the soil, which play an important role in biotic-control. Numerous strains of this genus are rhizosphere competitors and are able to degrade hydrocarbons, chlorophenolic compounds, polysaccharides and the xenobiotic pesticides used in agriculture (Harman and Kubicek 1998; Harman et al. 2004). *Trichoderma* spp. are opportunistic, avirulent plant symbionts, and can produce metabolite to inhibit soil pests, plant-parasite and phytopathogenic fungi (Schirmböck et al. 1994; Stefanova et al. 1999; Harman et al. 2004). These secondary metabolites are bacteriostatic and nematicidal agents. *Trichoderma* spp. have been described as biocontrol agents against nematodes which are able to suppress *Meloidogyne* spp. populations (Suarez and Llobell 2004). Some nematicidal compounds have been obtained from *Trichoderma* spp. Acetic acid was identified as the nematicidal principle in the culture filtrate of *Trichoderma longibrachiatum* (Djian et al. 1991). Gliotoxin has been isolated from a large number of fungi including a strain of *Trichoderma virens* which showed nematicidal activity (Anitha and Murugesan 2005). A peptide cyclosporin A possessing nematicidal activity against *M. incognita* was obtained from *Trichoderma polysporum* (Li et al. 2007). Viridin was obtained from *Trichoderma* spp. which was found to possess weak activity against *Anguillula aceti* (Watanabe et al. 2004; Anitha and Murugesan 2005).

In the present work, we evaluated the toxicity of culture filtrates of 329 *Trichoderma* strains for nematicidal activity against *Panagrellus redivivus* and *Caenorhabditis elegans*. From a strain YMF1.02647, a nematicidal compound was isolated by bioassay-guided fractionation and identified as trichodermin based on the spectroscopic data.

Table 1 The strains of *Trichoderma* for testing nematicidal activity

	Strain number
T. viride	YMF1.00137,1.00139,1.00143,1.00144,1.00146,1.00148,1.00150–1.00152,1.00157,1.00159,1.00161–1.00163, 1.00179,1.00185–1.00188,1.00192,1.00194,1.00197,1.00198,1.00201,1.00203,1.00205,1.00206,1.00208,1.00213, 1.00222–1.00225,1.00232,1.00234,1.00236–1.00238,1.00242,1.00244,1.00252,1.00254,1.00258,1.00260,1.00273, 1.00274,1.00276,1.00280,1.00282,1.00283,1.00286,1.00289–1.00292,1.00294,1.00298–1.00302,1.00306,1.00307, 1.00312–1.00315,1.00317,1.00318,1.00321–1.00323,1.00330–1.00332,1.00334–1.00339,1.00342,1.00344,1.00345, 1.00347,1.00355,1.00356,1.00359,1.00361,1.00362,1.00363,1.00365,1.00368–1.00371,1.00377,1.00380,1.00382, 1.00387,1.00390,1.00392,1.00394–1.00397,1.00401,1.00402,1.00405,1.00407,1.00411,1.00412,1.00414,1.00416, 1.00418,1.00420
T. harzinaum	YMF1.00138,1.00142,1.00149,1.00154,1.00165,1.00176–1.00178,1.00183,1.00184,1.00191,1.00193,1.00195, 1.00196,1.00199,1.00202,1.00204,1.00210–1.00212,1.00214,1.00216,1.00217,1.00219,1.00220,1.00226,1.00227, 1.00229,1.00230,1.00241,1.00245,1.00248,1.00250,1.00251,1.00261,1.00268,1.00271,1.00304,1.00305,1.00308, 1.00309,1.00319,1.00324,1.00326–1.00329,1.00349,1.00352,1.00353,1.00358,1.00366,1.00373,1.00374,1.00381, 1.00383–1.00386,1.00389,1.00393,1.00403,1.00404,1.00406,1.00408,1.00413,1.00415,1.00419
T. hamatum	YMF1.00141,1.00147,1.00153,1.00156,1.00158,1.00189,1.00190,1.00207,1.00209,1.00215,1.00218,1.00221, 1.00228,1.00235,1.00239,1.00243,1.00246,1.00247,1.00249,1.00253,1.00255–1.00257,1.00259,1.00269,1.00270, 1.00272,1.00275,1.00279,1.00281,1.00284,1.00287,1.00293,1.00296,1.00316,1.00140,1.00325,1.00333,1.00340, 1.00348,1.00351,1.00354,1.00357,1.00360,1.00364,1.00367,1.00372,1.00376,1.00378,1.00388,1.00400
T. longbrachiatum	YMF1.00233,1.00240,1.00288,1.00295,1.00297,1.00346,1.00379
T. piluliferum	YMF1.00303,1.00310,1.00311,1.00375,1.00398,1.00410
T. virens	YMF1.00160,1.00200,1.00231,1.00277,1.00320,1.00341,1.00417
T. yunnanensis	YMF1.01695,1.01696
T. polysporum	YMF1.00278
T. koningii	YMF1.00343
T. atroviride	YMF1.00350
T. compactus	YMF1.01693
T. appressus	YMF1.01694
Trichoderma sp.	YMF1.00428,1.00432,1.00449,1.00468,1.00473,1.00502,1.00517,1.00717,1.00719,1.00726,1.00729,1.00818, 1.00860,1.00868,1.00869,1.00902–1.00904,1.00906,1.00907,1.00917,1.00924,1.00929,1.00944,1.00945,1.01358, 1.01367,1.01383,1.02609–1.02647

Materials and methods

General experimental procedures

Column chromatography (CC) was performed on silica gel G (200–300 mesh, Qingdao Marine Chemical Factory, China), and on Sephadex LH-20 (Amersham Pharmacia, Sweden). TLC was performed on silica gel GF_{254} (10–40 μm, Qingdao). All solvents were distilled before use. NMR spectra: Bruker AM-400 spectrometer, chemical shifts δ in ppm rel. to Me_4Si, coupling constants J in Hz. ESI–MS: Finnigan LCQ-Advantage, in *m/z*.

Fungi, media and culture conditions

329 strains of *Trichoderma* (Table 1) were deposited in the culture collection of the Key Laboratory for Conservation and Utilization of Bio-resource, Yunnan University and were evaluated for nematicidal activity. All fungal strains were initially maintained on potato dextrose agar (PDA, 20% potato, 2.0% dextrose, 2.0% agar, pH 7.0) slants at 4°C and transferred twice for rejuvenation. All of them were cultured on PDA plates at 28°C for 7 days, and then these strains were inoculated into 250-ml triangular flasks each containing 100 ml of the PDB (potato dextrose broth) medium. These flasks were cultured on a rotary shaker (180 rev min^{-1}) at 28°C. After 7 days later, culture broths were filtered, and the filtrates served as stock solutions for screening nematicidal activity (Dong et al. 2006).

Nematodes

Panagrellus redivivus and *Caenorhabditis elegans*: Since the free-living nematodes *P. redivevus* and *C. elegans* were easy to cultivate, they were chosen as tested nematodes in our screening test. The two nematodes were 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: Fungus *Botrytis cinerea* was cultured on 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.

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.

Isolation and purification of active compound (**1**)

Strain *Trichoderma* sp. YMF1.02647 was grown in shake culture (200 ml per 500-ml triangular flask) on PDB medium. After fermentation for 7 days at 26°C at 180 rev/min, 18 l fermentation broth of YMF1.02647 was filtered. The fermentation broth was condensed and extracted by ethyl acetate. The ethyl acetate extract was concentrated to produce a crude extract (3.28 g). The extract was applied to a silica gel G chromatography column (300 g) eluted with petroleum ether/acetone (20:1 to 1:1, v/v) to yield sixteen fractions Fr1-16. Nematicidal bioassay showed Fr2 had nematicidal activity. Fr2 was further separated on a Sephadex LH-20 column eluting with acetone to obtain eight subfractions $Fr2_{1-8}$. Active $Fr2_3$ was then purified on a silica gel column (20 g) eluted with petroleum ether/ethyl acetate (20:1, v/v) to yield 1.045 g of active compound (**1**).

Compound **1:** Colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 5.48 (1H, dd, $J = 7.8$, 3.6 Hz, H-4), 5.31 (1H, d, $J = 5.4$ Hz, H-10), 3.71 (1H, d, $J = 5.2$ Hz, H-2), 3.50 (1H, d, $J = 5.4$ Hz, H-11), 3.02 (1H, d, $J = 4.0$ Hz, H-13a), 2.73 (2H, m, H-13b, H-3a), 2.46 (1H, dd, $J = 15.4$, 7.8 Hz, H-3b), 1.81–1.97 (3H, m, H-7a, H-8a, H-8b), 1.62 (3H, s, H-16), 1.33 (1H, brd, $J = 12.1$ Hz, H-7b), 0.83 (3H, s, H-15), 0.61 (3H, s, H-14). ^{13}C-NMR ($CDCl_3$,100 MHz) δ: 170.6 (s, C-1′), 139.8 (s, C-9), 118.3 (d, C-10), 78.8 (d, C-2), 74.7 (d, C-4), 70.1 (d, C-11), 65.2 (s, C-12), 48.6 (s, C-5), 47.5 (t, C-13), 40.1 (s, C-6), 36.3 (t, C-3), 27.7 (t, C-8), 24.1 (t, C-7), 23.0 (q, C-16), 20.9 (q, C-2’), 15.7 (q, C-15), 5.5 (q, C-14). ESIMS: 315 $[M + Na]^+$, 607 $[2 M + Na]^+$.

Bioassay of nematicidal activity

Fermentation broth samples (2 ml) of the tested strains were added to a petri dish (6 cm diameter) containing 150–200 nematodes, respectively. Each treatment was replicated three times. Sterilized water was used as control. All dishes were incubated at 25°C. The nematodes were considered to be dead when they did not move on physical stimuli with a fine needle. The numbers of live and inactive nematodes were counted after different incubation times (12, 24, 48, 72 h). Toxicity was estimated according to the mean percentage of dead nematodes and proof mortality (Total of mortality in test group minus the total of mortality in control) was calculated. Nematodes *P. redivevus* and *C. elegans* were used in the screening test of strains.

The in vitro activity of the compound was assayed against three nematodes *P. redivevus*, *C. elegans* and *B. xylophilus*. The compound was dissolved in acetone and diluted with distilled water to 400, 200, 100 and 50 mg l^{-1} for nematicidal assay. Each treatment was replicated three times, then the mean percentage of mortality and proof mortality were calculated. Distilled water containing 5% acetone was used as control. The data at two time-steps (48 and 72 h) was subjected to Independent-Sample *T*-Test using ANALYZE (SPSS Statistics Data Editor17.0 software, USA). Data on proof mortality (M) were changed to $\sin^{1/2}(M)$ before analysis.

Results and discussion

A total of 329 strains of *Trichoderma* were assayed for their nematicidal activity against *P. redivevus* and *C. elegans*. Nematicidal activity varied among the *Trichoderma* strains tested (Table 2). Percentage of proof mortality that was less than 50% for a given fungal strain was recorded as inactive and was not listed in Table 2. 15 strains exhibited nematicidal activities against *P. redivivus* and 14 strains showed activity against *C. elegans.* Among them, eight strains exhibited nematicidal activity both against *P. redivivus* and *C. elegans*. These active strains belonged to *T. viride*, *T. harzianum*, *T. piluliferum*, *T. compactus* and unidentified species. In the genus of *Trichoderma*, *T. longibrachiatum* (Djian et al. 1991), *T. viride* (Zhang and Zhang 2009), *T. harzianum* (Siddiqui and Shaukat 2004), *T. hamatum* (Girlanda et al. 2001), *T. virens* (Meyer et al. 2001) and *T. koningii* (Sankaranarayanan et al. 1997) have been reported to possess nematicidal activity. It is the first report about the species of *T. compactus* with nematicidal activity. Among these active strains, eight strains showed strong nematicidal activity, causing more than 80% mortality against the tested nematodes. YMF1.02647 was the most potential strain which could kill more than 95% *P. redivivus* and *C. elegans* within 48 h, respectively, and so was selected for study of its nematicidally active component.

According to bioassay-guided fractionation, an active compound (**1**) was obtained from the ethyl acetate extract of strain YMF1.02647. The compound (**1**) was identified to be trichodermin (Fig. 1) based on spectroscopic data. Three nematodes *P. redivevus*, *C. elegans* and *B. xylophilus* were used to estimate the nematicidal activity of the compound. Bioassay experiments indicated that the effect of trichodermin on nematode mobility varied with length of exposure time. The result showed that trichodermin could kill more than 95% both *P. redivivus* and *C. elegans* in 72 h at 400 mg l^{-1}, but only showed 54.2% mortality against *B. xylophilus* in 72 h at the same concentration (Table 3). This implied that the nematicidal activity of the compound

Table 2 Proof mortality of fungal filtrates of *Trichoderma* spp. against *P. redivivus* and *C. elegans* [mortality (% ± SD)]

Strain number (species)	*P. redivivus*				*C. elegans*			
	12 h	24 h	48 h	72 h	12 h	24 h	48 h	72 h
1.00157 (*T. viride*)	–	–	–	–	36.44 ± 0.03	50.32 ± 0.02	61.89 ± 0.02	70.85 ± 0.01
1.00187 (*T. viride*)	2.00 ± 0.01	5.30 ± 0.01	32.00 ± 0.01	52.33 ± 0.02	–	–	–	–
1.00197 (*T. viride*)	6.63 ± 0.30	17.13 ± 0.19	23.46 ± 0.06	65.54 ± 0.05	14.55 ± 0.01	18.67 ± 0.02	21.57 ± 0.08	70.43 ± 0.11
1.00198 (*T. viride*)	15.01 ± 0.02	18.71 ± 0.01	26.46 ± 0.02	62.86 ± 0.02	29.29 ± 0.10	30.71 ± 0.01	42.14 ± 0.01	78.58 ± 0.01
1.00244 (*T. viride*)	19.04 ± 0.02	44.29 ± 0.01	56.04 ± 0.06	64.24 ± 0.03	–	–	–	–
1.00251 (*T. harzianum*)	–	–	–	–	2.00 ± 0.01	3.75 ± 0.01	22.00 ± 0.02	50.60 ± 0.10
1.00261 (*T. harzianum*)	1.00 ± 0.01	10.48 ± 0.02	45.71 ± 0.03	54.29 ± 0.09	3.60 ± 0.02	16.19 ± 0.20	41.57 ± 0.02	75.23 ± 0.02
1.00294 (*T. viride*)	29.33 ± 0.15	38.70 ± 0.21	54.67 ± 0.05	66.00 ± 0.56	30.00 ± 0.10	44.00 ± 0.12	61.70 ± 0.05	67.00 ± 0.04
1.00302 (*T. viride*)	–	–	–	–	40.78 ± 0.01	78.01 ± 0.02	83.35 ± 0.01	89.47 ± 0.01
1.00311 (*T. piluliferum*)	–	–	–	–	4.85 ± 0.01	30.21 ± 0.02	51.42 ± 0.02	83.10 ± 0.01
1.00337 (*T. viride*)	8.45 ± 0.05	31.55 ± 0.01	84.26 ± 0.04	90.25 ± 0.05	–	–	–	–
1.00347 (*T. viride*)	3.12 ± 0.02	25.47 ± 0.04	61.00 ± 0.02	89.58 ± 0.04	–	–	–	–
1.00361 (*T. viride*)	–	–	–	–	9.18 ± 0.01	23.45 ± 0.19	65.87 ± 0.03	71.24 ± 0.06
1.00371 (*T. viride*)	–	–	–	–	3.62 ± 0.02	24.39 ± 0.03	54.28 ± 0.01	84.14 ± 0.01
1.00409 (*Trichodrama* sp.)	3.45 ± 0.01	4.12 ± 0.05	41.32 ± 0.02	53.29 ± 0.04	3.15 ± 0.01	11.81 ± 0.02	63.47 ± 0.01	83.15 ± 0.02
1.00416 (*T. viride*)	4.89 ± 0.01	14.80 ± 0.03	25.16 ± 0.01	62.35 ± 0.01	10.14 ± 0.02	65.75 ± 0.02	80.16 ± 0.01	87.23 ± 0.02
1.00432 (*Trichodrama* sp.)	2.48 ± 0.01	13.19 ± 0.01	16.24 ± 0.02	50.48 ± 0.03	3.52 ± 0.02	10.55 ± 0.01	17.25 ± 0.02	54.18 ± 0.02
1.01693 (*T. compactus*)	7.24 ± 0.01	19.70 ± 0.01	37.00 ± 0.03	55.03 ± 0.15	–	–	–	–
1.02614 (*Trichodrama* sp.)	2.00 ± 0.01	17.62 ± 0.02	59.52 ± 0.02	69.05 ± 0.02	–	–	–	–
1.02616 (*Trichodrama* sp.)	3.57 ± 0.02	16.43 ± 0.02	31.43 ± 0.01	70.71 ± 0.02	–	–	–	–
1.02647 (*Trichodrama* sp.)	63.84 ± 0.03	97.19 ± 0.01	*	*	17.44 ± 0.04	67.14 ± 0.06	96.39 ± 0.01	*
Control	0	2.81 ± 0.01	4.01 ± 0.015	6.52 ± 0.01	0	2.53 ± 0.02	3.61 ± 0.01	5.72 ± 0.02

– mortality < 50%

* After the mortality attained maximum 100%, proof mortality stayed the same

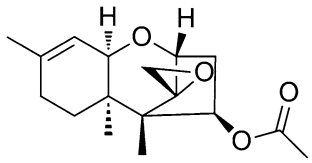

Fig. 1 Chemical structure of trichodermin (Nielsen et al. 1998)

Table 3 Effect of compound (**1**) on the proof mortality of three nematodes in vitro (%)

	Concentration (mg/l)			
	50	100	200	400
P. redivivus				
12 h	1.0	2.6	10.4	36.4
24 h	2.1	5.3	22.3	63.0
48 h	2.4	10.2	53.0	94.3
72 h	5.7	24.5	70.2	98.1
C. elegans				
12 h	1.0	2.3	8.3	30.3
24 h	2.0	5.1	19.8	49.1
48 h	3.5	9.0	33.2	72.6
72 h	4.0	15.1	50.4	95.3
B. xylophilus				
12 h	0.0	0.1	2.9	11.4
24 h	0.1	1.2	10.6	29.5
48 h	0.8	3.1	18.3	40.4
72 h	2.0	4.3	27.2	54.2

The control (5% acetone) did not show obvious activity against the tested nematodes (mortality <10%)

Table 4 Influence of exposure time (48 and 72 h) on the mortality of nematodes at each concentration of trichodermin

	50 mg l^{-1}	100 mg l^{-1}	200 mg l^{-1}	400 mg l^{-1}
P. redivivus	-9.087^{***a}	-15.091^{***}	-21.259^{***}	-4.914^{+}
C. elegans	-3.949^{*}	-14.285^{***}	-21.119^{***}	-1.02^{+}
B. xylophilus	-4.547^{**}	-4.648^{***}	-12.945^{***}	-10.305^{*}

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, $^{+}$ $P > 0.05$

[a] Values were t values from independent-sample T-Test

trichodermin was selective. Statistical analysis showed that exposure time was a key factor for affecting nematicidal activity of the compound against *P. redivivus*, *C. elegans* and *B. xylophilus* at low concentration (from 50 to 200 mg l^{-1}), but it had no significant difference at high concentration (400 mg l^{-1}) (Table 4). Trichodermin had been isolated from several species of *Trichoderma* including *T. viride*, *T. harzianum*, *T. longibrachiatum* and *T. reesei*, and other fungi such as *Stachybotrys cylindrospora*, *Memnoniella echinata* etc. (Godtfredsen and Vangedal 1964; Watts et al. 1988; Nielsen et al. 1998; Reino et al. 2008). The trichothecenes are one of the most important groups of mycotoxins, and trichodermin as a member of the trichothecene group showed diversiform bioactivities, such as being a potent antifungal antibiotic and an inhibitor of protein synthesis in mammalian cells (Ueno 1983; Jaradat et al. 2006; Valero et al. 2007). In the nematicidal treatment, the nematodes wriggled slowly after 12 h treatment with trichodermin (400 mg l^{-1}), some of them were inactive, and no give response to physical stimuli at 24 h, most of them were inactive at 48 h. After 72 h, the inactive nematodes had not been disassembled. In our experiment, 1.045 g pure trichodermin was isolated from 3.28 g crude extract of YMF1.02647, which indicated that trichodermin was responsible for the nematicidal activity of strain YMF1.02647. It is suggested that utilization of this nematicidal screening model in searching for active resource from *Trichoderma* spp. should find more nematicidal strains. These isolates may be potential candidates for developing novel nematicidal agents.

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