Annals of Microbiology, **56 (2)** 163-166 (2006)

Preliminary results on nematicidal activity from culture filtrates of Basidiomycetes against the pine wood nematode, *Bursaphelenchus xylophilus* (Aphelenchoididae)

Jin Yan DONG[1], Xing Ping LI[2], Lei LI[1], Guo Hong LI[1], Ya Jun LIU[1], Ke Qin ZHANG[1]*

[1]Key Laboratory for Conservation and Utilization of Bioresources, Yunnan University, Kunming, Yunnan, 650091;
[2]Department of Mathematics, Yunnan Normal University, Kunming, Yunnan, 650092, P.R. China

Received 23 December 2006 / Accepted 13 April 2006

Abstract - One hundred and eighty one fungal species that were isolated from the fresh fruiting bodies collected in the Mountains of Pu Er County of Yunnan Province, China were tested on the pine wood nematode, *Bursaphelenchus xylophilus in vitro*. Each fungal species was grown in Czapek broth and potato dextrose broth (PDB). Fifteen filtrates from *Amauroderma austrosinense, Amauroderma macer, Filoboletus* sp., *Laccaria tortilis, Lactarius gerardii, Lentinula edodes, Oudemansiella longipes, Oudemansiella mucida, Peziza* sp., *Pleurotus* sp., *Sinotermitomyces carnosus* (two strains), *Strobilomyces floccopus, Termitomyces albuminosus, Tylopilus striatulus* grown on PDB were found to be pathogenic to the tested nematodes. Eleven filtrates from *Amanita junguillea, Amanita* sp., *Daedalea sepiaria, Fistulina hepatica, Omphalotus olearius, Oudemansiella mucida, Peziza* sp., *Pleurotus pulmatus, Ramaria* sp., *Tricholoma conglobatum, Tylopilus striatulus* grown on Czapek broth were also pathogenic to the nematodes. When screening for nematicidal potential of fungi, it is important to study the growth medium conditions necessary to obtain the optimal nematicidal effect as fungal filtrates growing on different liquid media showed a very inconsistent toxicity towards nematodes.

Key words: nematicidal activity, mortality, culture filtrate, fungi.

INTRODUCTION

Since pine wilt disease caused by the pine wood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner *et* Buhrer 1934) was discovered in Nanjing in 1982 (Cheng *et al*., 1983), it has spread rapidly in China and become a serious problem for pine forests in China. There has been no effective means to control the disease. Although there are several registered nemacides in Japan, they are used only to protect valuable trees in parks because of their high cost (Jiang, 1994). There is increasing need to find more acceptable alternatives. Fungi appear to be a source of effective pesticidal compounds and may come to be regarded as an inexhaustible source of harmless pesticides having low plant and human toxicity and being easily biodegradable (Siddiqui and Mahmood, 1996). Consequently several groups of investigators have surveyed toxicity of culture filtrates of fungi against *B. xylophilus, Heterodera glycines* (Ichinohe), *Meloidogyne incognita* (Kofoid et White), *Meloidogyne javanica* (Treub), *Caenorhabditis elegans, Panagrellus redivivus* (Goodey), *Rotylenchulus reniformis* (Linford et Oliveira) by means of immersion test and isolated some nematicidal metabolites such as alkaloids, peptides, terpenes, fatty acids etc. (Desai *et al*., 1972; Alam *et al*., 1973; Sing *et al*., 1983; Khan and Hussain, 1989; Khan and Kgan, 1992; Chattopadhyay and De, 1995; Pathak and Kumar, 1995; Anke and Sterner 1997; Sankaranarayanan *et al.*, 1997; Li *et al*., 2004; Dong *et al*., 2004, 2005a, 2005b). One of the more interesting, selective nematicides reported lately is omphalotin A, a cyclic dodecapeptice isolated from *Omphalotus olearius*. Omphalotin A is very potent against the plant parasite *Meloidogyne incognita* (LD_{50}: 0.75 ug/mL). It is considerably more active than the commercially available nematicide ivermectin and it is 50 times less active against the saprophytic *Caenorhabditis elegans* (Mayer *et al*., 1997; Sterner *et al*., 1997). There may be many more fungi, not yet tested, which could prove to be effective for the control of plant parasitic nematodes. Therefore, the objectives in the present investigation were to determine the pathogenic effect of fungi isolated from the fresh fruiting bodies collected in Mountains of Pu Er County of Yunnan Province on the pine wood nematode, *B. xylophilus*.

MATERIALS AND METHODS

Fungi, media, and culture conditions. One hundred and eighty one fungal strains mainly belonging to the genera *Agaricus, Agrocybe, Amanita, Amauroderma, Boletus, Bulgaria, Calvatia, Cladosporium, Clitocybe, Collybia, Coltricia, Cordyceps, Craterellus, Cystoderma, Daedalea, Filoboletus, Gastroboletus, Gyrodon, Helvella, Hemitrichia, Hydnum, Inocybe, Isaria, Laccaria, Lactarius, Leatiporus, Lentinus,*

* Corresponding author. Phone: +86-871-5034878.
Fax: +86-871-5034838. E-mail: zhangkq126@sina.com.

Lycoperdon, Mycena, Nidularia, Omphalotus, Oudemansiella, Paxillus, Peziza, Phylloporus, Pleurotus, Pluteus, Polyporus, Pulveroboletus, Ramaria, Russula, Scleroderma, Sinoboletus, Sinotermitomyces, Stereum, Strobilomyces, Termitomyces, Thelephora, Tremella, Tricholoma, Tylopilus, Xerocomus and *Zygorhynchus* were isolated. Mycelial cultures from basidiomycetes were derived from the fresh fruiting bodies or their basidiospores collected in Mountains of Pu Er County of Yunnan Province in August 2000 and were identified by Prof. Zang Mu, Senior Taxonomist, Kunming Institute of Botany, the Chinese Academy of Sciences, Kunming, Yunnan, People's Republic of China. All cultures were maintained on potato dextrose agar (PDA, 20% potato, 2.0% glucose, 2.0% agar, pH 7.0) slants at 4 °C and transferred every 6 months.

Two liquid media, Czapek medium (3% sucrose, 0.1% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $FeSO_4 \cdot 7H_2O$, 0.2% $NaNO_3$) and potato dextrose broth (PDB, 20% potato, 2.0% glucose, pH 6.0) were used to culture these fungi. Slant tube cultures were inoculated into four 250 mL Erlenmeyer flasks each containing 70 mL of the above media. These flasks were cultured on a rotary shaker (200 rpm) at 28 °C. Maximum fungal growth was observed after different periods (10, 24, 48, 72 h) of incubation and one flask culture on PDB or Czapek medium was used to obtain filtrate. The culture filtrates served as stock solutions for screening nematicidal activity.

Nematodes. The fungus, *Botrytis cinerea* Pers., was cultured on PDA in Petri dishes (Ø 90 mm) at 26 °C. Petri dishes with fully grown fungus were inoculated with *B. xylophilus* and left until fungal mycelia were completely consumed. The cultured nematodes (mixed stage) were separated from the culture medium by the Baerman funnel technique and enumerated on a grid under a microscope (x 20). An aqueous suspension of the nematode (*ca*. 15000 nematodes mL^{-1}) with all life stages was prepared by appropriate dilution for use as a working stock.

Bioassay of nematicidal activity. The nematotoxin bioassay was made in 6 cm Petri dishes. Three hundred nematodes in 20 µL of aqueous suspension were transferred to Petri dishes containing 2 mL of fungal extracts and gently mixed. All dishes were incubated at 25 ºC. The numbers of live and inactive nematodes were counted under a binocular microscope after different incubation times. Toxicity was estimated according to the mean percentage of dead nematodes. Nematodes were considered dead if they gave no response to physical stimuli such as mechanical stirring and pricking with the point of a needle. Tow controls were used for comparison: one in pure distilled water, the other in the uninoculated sterilized culture medium. Each treatment was replicated four times and the data obtained were analysed statistically (ANOVA, LSD Procedure) at 5 and 1% levels of probability.

RESULTS

Out of 181 fungal strains fifteen cultural filtrates of fungi grown on PDB medium were found to immobilize over 50% of nematodes following 72 h exposure *in vitro* screening (Table 1). After 72 hours, over 80% of nematodes were immobilized in five filtrates of *Amauroderma austrosinense*

TABLE 1 – Effect of cultural filtrates from fungi on PDB medium on the mortality of *Bursaphelenchus xylophilus in vitro* (mean of 4 replicates)

Control/Name of fungi	Per cent mortality after different exposure periods (h)					
	10	24	48	72	L.S.D. at 5% level	L.S.D. at 1% level
Amauroderma austrosinense	19.4	52.4	73.8	85.6	7.77	10.89
*Amauroderma macer**	13.5	48.7	65.8	71.4	3.74	5.25
*Amauroderma macer***	35.8	81.2	95.6	100	4.81	6.74
Filoboletus sp.	4.7	37.9	50.7	58.4	8.60	12.06
Laccaria tortilis	63.5	95.6	83.2	63.4	4.67	6.55
Lactarius gerardii	6.8	29.4	48.1	66.9	8.11	11.37
Lentinula edodes	11.4	28.6	47.2	57.6	5.26	7.37
Oudemansiella longipes	42.5	59.4	66.8	76.8	12.08	16.93
Oudemansiella mucida	22.8	41.8	52.4	58.9	5.06	7.10
Peziza sp.*	7.8	29.8	38.5	65.4	5.67	7.95
Peziza sp.**	32.7	85.4	94.6	100	4.28	6.00
Pleurotus sp.	11.4	24.3	48.9	64.1	6.01	8.42
Sinotermitomyces carnosus	2.9	25.4	44.7	56.7	6.85	9.60
Sinotermitomyces carnosus	2.1	24.5	48.6	52.4	7.72	10.82
Strobilomyces floccopus	42.3	56.7	78.9	82.7	8.11	11.38
Termitomyces albuminosus	49.2	52.4	54.4	53.4	8.89	12.46
Tylopilus striatulus	3.1	35.1	67.5	82.1	5.01	7.03
Control (broth alone)	0.0	1.3	4.7	7.5	–	–
Control (water alone)	0.0	2.7	6.6	9.1	–	–
	0.0	1.1	4.8	7.6	–	–
L.S.D. at 5% level	4.82	6.46	7.85	7.01	–	–
L.S.D. at 1% level	6.76	9.06	11.01	9.83	–	–

* Adults (≥0.5 mm) (The length of the mature pine wood nematode is 1 mm by average. Thus, the adults were appointed to the nematodes which body was longer than 0.5 mm).
** Larvae (< 0.5 mm) (The length of the mature pine wood nematode is 1 mm by average. Thus, the larvae were appointed to the nematodes which body was shorter than 0.5 mm).

TABLE 2 – Effect of cultural filtrates from fungi on Czapek broth medium on the mortality of *Bursaphelenchus xylophilus in vitro* (mean of 4 replicates)

Name of fungi	Per cent mortality after different exposure periods (h)					
	10	24	48	72	L.S.D. at 5% level	L.S.D. at 1% level
Amanita junguillea	4.1	12.1	34.2	50.2	4.96	6.96
Amanita sp.	2.1	14.3	24.6	65.8	6.96	9.76
Daedalea sepiaria	2.4	21.4	43.8	60.1	6.14	8.61
Fistulina hepatica	6.8	20.7	68.5	51.3	7.87	11.03
Omphalotus olearius	5.1	12.4	46.1	53.3	6.93	9.71
Oudemansiella mucida	8.7	53.4	71.2	86.7	7.32	10.26
Peziza sp.	5.4	52.1	66.5	75.0	8.78	12.31
Pleurotus pulmatus	8.7	33.8	58.9	83.1	6.24	8.74
Ramaria sp.	3.2	11.5	20.5	65.8	5.20	7.29
Tricholoma conglobatum	1.2	32.1	70.3	50.1	5.52	7.74
Tylopilus striatulus	25.3	49.1	59.6	85.6	5.95	8.34
Control (broth alone)	0.0	1.1	4.8	7.6	–	–
Control (water alone)	0.0	2.7	6.6	9.1	–	–
L.S.D. at 5% level	10.89	7.56	7.61	6.51	–	–
L.S.D. at 1% level	15.27	10.60	10.67	9.13	–	–

(85.6%), *Amauroderma macer* (100%), *Peziza* sp. (100%), *Strobilomyces floccopus* (82.7%), *Tylopilus striatulus* (82.1%). The pathogenic effect of both *Amauroderma macer* and *Peziza* sp. isolates on the pine wood nematodes were interesting as they had different biological activity on the adults and larvae of *B. xylophilus*. When the tested nematodes were larvae, 100% pathogenicities within 72 h of exposure were observed with both. On adult nematodes, nematicidal activities of the cultural filtrates of *Amauroderma macer* and *Peziza* sp were only 71.4%, and 65.4% respectively.

Out of 181 fungal strains eleven cultural filtrates of fungi grown on Czapek broth medium were found to immobilize over 50% of nematodes following 72 h exposure *in vitro* screening (Table 2). Culture filtrates of other fungal strains grown on Czapek broth medium showed no or little (£º50%) nematode pathogenicity during the tested period. After 72 h, a maximum mortality of 86.7% of individuals was recorded using isolates of *Oudemansiella mucida*, followed by *Tylopilus striatulus* (85.6%) and *Pleurotus pulmatus* (83.1%).

It was observed that effects of cultural filtrates on nematodes mobility varied with length of exposure time. In the first case, as illustrated in the treatment with most fungi that were inactive, the nematodes were unaffected and moved at each observation over the duration of the test. In the second case, the nematicidal activity of the culture extracts of *Amauroderma austrosinense*, *Amauroderma macer*, *Oudemansiella longipes*, *Amanita junguillea, Oudemansiella mucida,* etc. increased with the time of exposure. In the third case the nematodes were afflicted initially and continually became inactive after 24 h of exposure, but recovered during the remainder of the exposure period as the cultural filtrates of *Laccaria tortilis* and *Tricholoma conglobatum* showed. In the fourth case, e.g. cultural filtrate of *Termitomyces albuminosus*, the number of immobilized nematodes was relatively stable from the start to the end of counting.

It was also observed that the growth and nematicidal activity of fungi were dependent from the nutrient component of the culture medium tested. On the basis of the growth conditions and nematicidal effects of fungi grown on PDB or Czapek broth, the fungal species tested could be grouped into four distinct categories. In the first category, most fungi grew well in both PDB and Czapek broth but their cultural filtrates were inactive. In the second category the growth and the nematicidal activities were observed in cultural filtrates from fungi grown on PDB and Czapek broth as illustrated in the cultural filtrates of *Tylopilus striatulus*. In the third category, the fungi grew well in PDB medium and had been found to be pathogenic to nematodes, whereas they were all inactive because no growth was observed in the Czapek broth medium. The fungi were *Amauroderma austrosinense, Laccaria tortilis, Lentinula edodes, Oudemansiella longipes, Oudemansiella mucida, Sinotermitomyces carnosus, Strobilomyces floccopus* and *Termitomyces albuminosus.* In the fourth category, the nematicidal effect of the fungi was not proportional to their growth condition. For example, the growth of *Amanita junguillea* and *Amanita* sp. on PDB was better than Czapek broth, but their nematicidal activities were significantly less toxic than those on Czapek broth.

DISCUSSION

The influence of the culture medium on toxin production was very important as the filtrates from different liquid media showed varying toxicities towards nematodes. Different effects of culture filtrates of the fungi tested on nematode pathogenicity suggest that the different active nematicidal principles and/or various amounts of toxins were produced in media. That is in agreement with some other papers according to which there were obvious differences in the growth and toxicity of the fungi depending on the nutritional conditions of the culture media tested (Smith and Moss, 1985; Cayrol *et al*., 1989; Anke *et al*., 1995; Chen *et al*., 2000). Hence it appears that the conditions necessary for mycotoxin production (media, culture conditions, age, etc.) vary from one fungus to the other. Consequently when the ability of fungi to produce any toxins is studied, it is absolutely necessary to test several parameters. Also to establish the identity of the toxic metabolite in the near future, it is

absolutely necessary to define a well-known synthetic media, which gives optimal nematicidal results. Likewise, the toxic properties of the fungus were greatly influenced by the culture conditions. Modification of fermentation parameters has been proved to be a valuable tool to increase the number of new nematicidal metabolites (Anke *et al*., 1995). The knowledge of the biosynthetic pathways, the understanding of their regulation, and the ecological functions of fungal secondary metabolism may greatly help make use of this rich natural resource.

The results of the present study regarding the nematicidal effects of fungal filtrates o*n B. xylophilus* are not directly proportional to the length of exposure which are in agreement with our previous paper (Dong *et al*., 2004) on the pine wood nematode, but in contradiction with some others papers (Alam *et al*., 1973; Khan and Hussain, 1989; Khan and Kgan, 1992; Chattopadhyay and De, 1995; Pathak and Kumar, 1995; Sankaranarayanan *et al*., 1997) according to which there was distinct increase in the toxicity of the cultural filtrates on *Meloidogyne incognita*, *Meloidogyne javanica,* etc. with increase in time of exposure. This could be attributed to the selection of *B. xylophilus* as test nematode instead of species such as *M. incognita*, *M. javanica,* etc. Differences in susceptibility of the different species of nematodes could be responsible for this.

What is interesting in these preliminary findings is the specificity of the toxic metabolite against adults and larvae of nematodes tested. The reasons for such specificity and the biochemical mechanisms of recognition involved on the nematode are also of interest. This specificity appears as another problem in the nematicidal mycotoxins research.

Acknowledgements

We are grateful to Pu-Qiu Song for providing cultures of the tested fungi and Prof. Mu Zang for taxonomic advice. This study was financially supported by the National Natural Science Foundation of China (NSFC30570059 and NSFC20562015) and Yunnan Provincial Natural Science Foundation (2005C0005Q).

REFERENCES

Alam M.M., Khan M.W., Saxena S.K. (1973). Inhibitory effect of culture filtrates of some rhizosphere fungi of okra on the mortality and larval hatch of certain plant parasitic nematodes. Indian J. Nematol., 3: 94-98.

Anke H., Stadler M., Mayer A., Sterner O. (1995). Secondary metabolites with nematicidal activity from nematophagous fungi and ascomycetes. Can. J. Botany, 72: 932-939.

Anke H., Sterner O. (1997). Nematicidal metabolites from higher fungi. Curr. Org. Chem., 1: 427-440.

Chattopadhyay P.R., De A.B. (1995). Inhibitory effects of certain fungi on the larval hatch of *Meloidogyne incognita*. Indian J. Nematol., 25: 204-205.

Cayrol J.C., Djian C., Pijarowski L. (1989). Study of the nematicidal properties of the culture filtrate of the nematophagous fungus *Paecilomyces lilacinus.* Rev. Nematol., 12 (4): 331-336.

Chen S.Y., Dickson D.W., Mitchell D.J. (2000). Viability of *Heterodera glycines* exposed to fungal filtrates. J. Nematol., 32: 190-197.

Cheng H.R., Lin M.S., Li W.Q., Fang Z.D. (1983). The occurrence of pine wilt disease by a nematode found in Nanjing. For. Pest Dis., 4: 1-5.

Dong J.Y., Zhao Z.X., Cai L., Liu S.Q., Zhang H.R., Duan M., Zhang K.Q. (2004). Nematicidal effect of freshwater fungal cultures against the pine-wood nematode, *Bursaphelenchus xylophilus*. Fungal Divers., 15: 123-133.

Dong J.Y., Li R., He H.P., Zhang K.Q. (2005a). Nematicidal sphingolipids from the fresh water fungus *Paraniesslia* sp. YMF1.01400. Eur. J. Lipid Sci. Technol., 107: 779-785.

Dong J.Y., He H.P., Shen Y.M., Zhang K.Q. (2005b). Nematicidal epipolysulfanyldioxopiperazines from *Gliocladium roseum*. J. Nat. Prod., 68: 1510-1513.

Desai M.V., Shah H.M., Pillai S.N. (1972). Effect of *Aspergillus niger* on root-knot nematode, *Meloidogyne incognita.* Indian J. Nematol., 2: 210-214.

Jiang L.Y. (1994). The history and control strategy of pine wilt disease in Japan. For. Pest Dis., 3: 45-47.

Khan T.A., Hussain S.I. (1989). Effect of culture filtrates of soil fungi on the mortality and hatching of reniform nematode (*Rotylenchulus reniformis*). Indian J. Nematol., 19 (1): 35-40.

Khan S.T., Kgan T.A. (1992). Effect of culture filtrates of soil fungi on the hatching and mortality of root knot nematode (*Meloidogyne incognita*). Curr. Nematol., 3 (1): 53-60.

Li G.H., Shen Y.M., Zhang K.Q. (2004). Nematicidal activity and chemical component of Poria cocos. J. Microbiol., 43 (1): 17-20.

Mayer A., Anke H., Stemer O. (1997). Omphalotin, a new cycle peptide with potent nematicidal activity from *Omphalotus olearius.* I. Fermentation and Biological activity. Nat. Prod. Lett., 10: 25-32.

Pathak K.N., Kumar B. (1995). Nematotoxic effects of *Trichoderma harzianum* culture filtrate on second stage juveniles of rice root knot nematode. Indian J. Nematol., 25: 223-224.

Sankaranarayanan C., Hussaini S., Sreeramakumar S., Prasad R.D. (1997). Nematicidal effect of fungal filtrates against root-knot nematodes. Biol. Control, 11: 37-41.

Siddiqui Z.A., Mahmood I. (1996). Biological control of plant-parasitic nematodes by fungi: a review. Bioresource Technol., 58: 229-239.

Sing S.P., Pant V., Khan A.M., Saxena S.K. (1983). Inhibitory effect of culture filtrate of some rhizosphere fungi of tomato as influenced by oil cakes on mortality and larval hatch of *Meloidogyne incognita*. Nematologia Mediterranea, 11: 119-123.

Smith J.E., Moss M.O., Eds (1985). Mycotoxins, Formation Analysis and Significance, Wiley and Sons, Chichester.

Sterner O., Etzel W., Mayer A. (1997). Omphalotin, a new cycle peptide with potent nematicidal activity from *Omphalotus olearius.* II. structure elucidation. Nat. Prod. Lett., 10: 33-38.