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Activity of fungal culture filtrates against soybean cyst nematode and root-knot nematode egg hatch and juvenile motility

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Summary – Fungi were isolated from soybean cyst nematode (SCN, *Heterodera glycines*) eggs collected in China, and 253 fungal isolates were assayed for production of compounds active against SCN and root-knot nematode (RKN, *Meloidogyne incognita*). Fungal isolates were grown for 3 and 7 days in potato dextrose broth (PDB), the culture broths were sterile-filtered to remove fungal biomass, and the filtrates were placed into 24-well plates to test for effects on egg hatch and juvenile motility. *Meloidogyne incognita* egg hatch ranged from 2 to 121% of hatch in PDB controls and *H. glycines* hatch from 15 to 224%. Activities of filtrates harvested after 3 and 7 days were significantly correlated. Only four isolates produced filtrates that significantly inhibited juvenile motility of SCN, RKN or both nematodes. This study identified fungal isolates capable of producing compounds active against these nematodes, and demonstrated that there was a low correlation in activity against SCN and RKN. The active fungal isolates are candidates for studies on identification of potential nematicides.

Keywords – China, fungi, *Heterodera glycines*, *Meloidogyne incognita*, natural products.

Many fungi are known to produce nematicidal or nematistatic compounds (Anke *et al.*, 1995; Hallmann & Sikora, 1996; Anke & Sterner, 1997; Chen *et al.*, 2000; Meyer *et al.*, 2000; Köpcke *et al.*, 2001). For example, the fungus *Omphalotus olearius* produced omphalotin A, a nematicidal compound that demonstrated greatest activity against the root-knot nematode (RKN) *Meloidogyne incognita* (Kofoid & White) Chitwood (Buchel *et al.*, 1998; Mayer *et al.*, 1999). Fungal endophytes of tall fescue produced (or induced production of) compounds including loline alkaloids, pyrrolopyrazine, and organic acids that may account for activity against some phytoparasitic nematodes (Rowan & Gaynor, 1986; Porter, 1994; Bush *et al.*, 1997). Secondary metabolites from other endophytic fungi, such as *Fusarium oxysporum*, were toxic to *M. incognita* (Hallmann & Sikora, 1996), and toxins from various *Fusarium* spp. reduced nematode viability (Ciancio, 1995; Anke & Sterner, 1997; Nitao *et al.*, 2001). The wood-rotting basidiomycete *Pleurotus ostreatus* produced the nematotoxin *trans*-2-decenedioic acid (Kwok *et al.*, 1992); acetic acid was an active component from culture filtrates of *Paecilomyces lilacinus* and *Trichoderma longibrachiatum* (Djian *et al.*, 1991); and linoleic acid was identified as a nematotoxic compound from the nematophagous fungi *Arthrobotrys conoides* and *Arthrobotrys oligospora* (Anke *et al.*, 1995). *Pochonia chlamydosporia* secreted proteases, one of which hydrolysed proteins from *M. incognita* egg shell (Segers *et al.*,

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1994, 1999) and nematicidal activity in a *P. chlamydosporia* culture was primarily from phomalactone, which acted against *M. incognita* (Khambay *et al.*, 2000).

Active compounds from fungal cultures that are deleterious to plant-parasitic nematodes have potential for application as novel nematicides. This approach has already been employed with the fungus *Myrothecium*, which was originally isolated from *Heterodera glycines* Ichinohe (soybean cyst nematode, SCN). Activity of culture products from the fungus includes nematicidal effects on adult nematodes, alteration of rhizosphere ecology and inhibition of egg hatch, juvenile development and root-finding (Warrior *et al.*, 1999; Perry *et al.*, 2000; Twomey *et al.*, 2000, 2002; Fernández *et al.*, 2001). The fungus is now cultured to generate the novel nematicides that are utilised in the product DiTera® (Valent BioSciences Corporation, Libertyville, IL, USA).

Soybean and SCN are believed to be indigenous to the People's Republic of China (Liu *et al.*, 1995, 1997). Consequently, fungus strains long associated with SCN should be located in the region as well. It is possible that over a long association, fungal strains more adapted to SCN may have developed. Such strains might provide material for new control measures. To obtain a sample of some of these native fungal strains, fungi were isolated from SCN eggs collected in Chinese agricultural fields. Filtrates of culture broths from these fungal isolates were then screened in the laboratory for effects on SCN and on RKN, *M. incognita*. The assay was designed to test whether, under the experimental conditions, the fungal isolates produced compounds that were inhibitory or stimulatory to egg hatch, that affected second-stage juvenile (J2) motility, and that were active against two different plant-parasitic nematodes.

Materials and methods

Collection and isolation of fungi

Cysts of SCN were collected during late summer from fields known to be infested with SCN and located in major soybean-growing regions of China near Anda City and Beijing. Site 1 (Anda) was about 1 km south of Anda City; Site 2 (Institute) was located at the Alkaline Soil and Crop Breeding Institute, Heilongjiang Academy of Agricultural Sciences, Anda Institute, about 1 km from Site 1; Site 3 (Chang Ping) was in Chang Ping County, near the northern part of Beijing; and Site 4 (Tong) in Tong County, near the eastern part of Beijing. Soybean plants and soil surrounding the roots were dug from each field and placed in plastic bags for transport to laboratory facilities, where females and cysts were washed from the roots and soil and collected on a 250 μm aperture sieve. The females and cysts were separated from the debris by flotation in a 1 M sucrose solution, returned to the sieve, washed in water, and surface-sterilised in 70% ethanol. Eggs were removed from females and cysts and placed onto one of three agar media in Petri dishes: 1.5% water agar; corn meal agar (Difco Laboratories) + 0.05 g/l streptomycin sulphate, and potato dextrose agar (PDA; Difco Laboratories) + 0.05 g/l streptomycin sulphate. All three media were used for eggs from each collection site. The females and cysts were discarded. The number of females and cysts sampled per site were: 96 (Anda), 63 (Institute), 95 (Chang Ping), and 36 (Tong).

Fungi growing from the nematode eggs were isolated and are being maintained at the Nematology Laboratory, Beltsville, MD, USA, and at the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF), US Plant, Soil and Nutrition Laboratory, Ithaca, NY, USA. Some *Fusarium* isolates are also maintained at the Fusarium Research Center, Pennsylvania State University, University Park, PA, USA.

Microwell assay procedures

Microwell assays were conducted to determine activity of fungal exudates against eggs of *H. glycines* and *M. incognita* using procedures similar to those described by Nitao *et al.* (1999). Fungal cultures in potato dextrose broth (PDB; Difco Laboratories) were grown for 3 and 7 days in Erlenmeyer flasks at 25°C on rotary shakers (240 rpm), the fungal biomass was removed by centrifugation (37 000 *g* for 30 min) and sequential filtration through GD/X series syringe filters: 1.0 μm GF/B filter (only used when obvious fungal matter was still present in the supernatant); 0.45 μm GMF filter; and a sterile 0.2 μm PES filter (Whatman, Clifton, NJ, USA). This ensured that there was no fungus growth in the microwell assays. The pH of each culture filtrate was determined. SCN was cultured on soybean plants (*Glycine max* cv. Essex) and RKN on tomato plants (*Lycopersicon esculentum* cv. Orange Pixie) grown in a glasshouse. For these cultures, 2-week-old soybean plants were inoculated with SCN eggs, and cysts were collected 3 months later. Three-week-old tomato seedlings were inoculated with RKN eggs and egg masses were collected after 2 months.

After harvest, SCN cysts were placed in 0.5% sodium hypochlorite in an autoclaved tissue grinder and gently crushed. The eggs were surface-disinfested by agitation in a sterile vial for 2.5 min, then pipetted onto an autoclaved 25 μm aperture sieve and rinsed with sterile water. Egg masses of RKN were similarly rinsed with sterile water, placed into sodium hypochlorite, agitated and rinsed on a sieve. The nematode eggs were then placed into culture filtrates, sterile water or control PDB (not inoculated with fungus) in 24-well tissue culture plates. The water controls were used to monitor egg and J2 viability. As the fungi had been cultured in PDB, egg hatch in the PDB controls was used for comparison with egg hatch in the filtrates. Hatch of SCN is negatively affected by PDB (Nitao *et al*., 1999), so the culture broth filtrates were tested at a lower concentration against SCN than against RKN, to correct for the interference of PDB while maximising the test concentration. Culture broth concentrations of 75 and 90% were found to give satisfactory egg hatch for SCN and RKN, respectively (Nitao *et al*., 1999, 2001). Each bioassay trial consisted of five replicate wells per treatment, *ca* 200 eggs per well. Motility was determined by counting spontaneously moving, hatched J2 in each well 3-4 days after eggs were placed in the filtrates. To determine effects of filtrates on egg hatch, numbers of motile and non-motile J2 were counted after 14 days in the treatments. Assays were repeated with filtrates from 40 of the isolates whose effects on hatch ranged from inhibitory to stimulatory, and with all filtrates that inhibited J2 motility. Repeated trials were combined for analysis. In addition, each treatment that inhibited J2 motility was tested again in three more trials (with 100, 200 and 200 eggs per well, respectively) in which motile J2 in filtrates, water and PDB were counted at 3 days, the treatments and controls were replaced with water the following day, and motile J2 counted again the next day. During these toxicity trials, immotile J2 were prodded with a needle to check for a response.

Statistical analyses

Percentage of hatched eggs in a well was calculated as: number of hatched J2/number of eggs originally placed in the well $\times$ 100. Egg hatch after 14 days in fungal broth filtrate relative to PDB controls was then calculated for each filtrate as: (average % hatch in test broth filtrate/average % hatch in control PDB) $\times$ 100. Averages were calculated using each well as a replicate. The percent motile J2 was calculated as: (average % motile J2 in test broth filtrate/average % motile J2 in control PDB) $\times$ 100. Percent hatch and J2 motility relative to controls were both calculated for broth filtrates harvested from 3- and 7-day-old cultures. Results of microwell assays were analysed by t-test, comparing hatch in each filtrate with that in the PDB control for that filtrate. To highlight those filtrates most active against the nematodes, results are only reported for treatments that had 'strong' effects on hatch and/or motility, 'strong' effects being defined as inhibition, stimulation or toxicity that were both 60% and significantly ($P \leqslant 0.05$) different from the PDB control.

Correlations between SCN and RKN egg hatch in 3- and 7-day-old fungal culture broth filtrates were analysed using Spearman Rank Order Correlation (SigmaStat software, SPSS, Chicago, IL, USA). Since RKN egg hatch data had unequal variances that could not be corrected by standard transformations, analyses were applied to ranked data. The Spearman correlation coefficient (r_s) can range between +1 and −1, a high positive coefficient indicating that fungal isolates inhibitory to one species or at one culture age are also inhibitory to the second species or at the other culture age. A negative value indicates that isolates inhibitory to one species or at one culture age are stimulatory towards the second species or at another culture age.

Results

A total of 253 fungal isolates, representing at least 17 genera and 23 species, were obtained from *H. glycines* eggs and assayed for production of natural compounds that affected egg hatch and J2 motility of *H. glycines* and *M. incognita*. Effects on egg hatch ranged from stimulatory to inhibitory. One or more isolates of each of the following fungi produced culture broths that strongly inhibited egg hatch of SCN and/or RKN (Table 1): *Acremonium* sp. (one of one isolate tested), *Aspergillus ochraceus* K. Wilh. (1/4), *Aspergillus* sp. (3/4), *Chaetomium globosum* Kunze:Fr. (1/1), *Cladosporium cladosporioides* (Fresen.) G.A. De Vries (3/8), *Cylindrocarpon* sp. (2/2), *Epicoccum nigrum* Link (1/1), *Fusarium acuminatum* Ellis & Everh. (1/3), *Fusarium compactum* (Wollenw.) Gordon (2/3), *Fusarium equiseti* (Corda) Sacc. (11/20), *Fusarium oxysporum* Schlechtend. Fr. (1/34), *Fusarium solani* (Mart.) Sacc. (4/21), *Mortierella* sp. (1/21), *Paecilomyces farinosus* (Holmsk.) A.H.S. Brown & G. Sm. (1/1), *Penicillium* sp. (7/13), *Ramicandelaberlongisporus* Y. Ogawa, S. Hayashi, Degawa & Yaguchi (1/2), *Trichothecium ro-*

Table 1. *Isolates of fungi (from* Heterodera glycines *cysts collected from four fields in China) producing culture filtrates that strongly affected hatch (average hatch ± 60% different at* P ⩽ *0.05 from that in non-inoculated potato dextrose broth) of* H. glycines *and* Meloidogyne incognita *eggs after 14 days immersion in filtrates from 3- and 7-day-old cultures, and pH of filtrates.*

Fungus	NL isolate[1)]	Field[2)]	Hatch (%)					
			H. glycines		*M. incognita*		Filtrate pH	
			3-day	7-day	3-day	7-day	3-day	7-day
Acremonium sp.	L209	Anda	–[3)]	–	30	–	6.5	5.1
Aspergillus ochraceus	L296	Anda	36	–	–	18	4.5	6.3
Aspergillus sp.	L192	Anda	–	–	4	8	6.0	5.4
	L354	Chang Ping	28	–	–	–	4.1	7.1
	L378	Anda	–	–	30	35	4.8	6.4
Chaetomium globosum	L250	Institute	–	15	–	2	5.3	5.2
Cladosporium cladosporioides	L189	Anda	–	–	–	34	6.8	6.7
	L338	Anda	–	–	–	34	6.6	6.4
	L377	Institute	25	–	3	5	6.2	6.5
C. sphaerospermum	L208	Anda	161	166	–	–	7.0	6.9
	L218	Anda	170	207	–	–	7.4	7.5
Cylindrocarpon sp.	L125	Institute	–	–	34	–	4.9	6.3
	L341	Anda	–	–	36	34	5.1	4.9
Epicoccum nigrum	L217	Anda	208	–	–	16	6.3	6.2
Fusarium acuminatum	L193	Anda	–	–	35	24	6.5	6.3
F. compactum	L175	Anda	–	–	15	12	6.7	7.1
	L251	Anda	31	40	26	18	5.8	6.0
F. equiseti	L128	Anda	39	31	19	36	7.1	7.5
	L130	Anda	–	–	29	26	6.6	6.9
	L134	Anda	–	–	26	–	6.4	6.8
	L135	Anda	–	–	9	32	6.3	6.6
	L136	Anda	–	–	23	37	6.4	6.9
	L140	Anda	–	–	19	34	6.3	6.8
	L142	Anda	–	–	32	–	6.6	6.7
	L152	Anda	–	–	28	40	6.2	6.6
	L159	Anda	–	35	34	25	6.9	7.2
	L215	Anda	–	–	16	33	6.5	6.7
	L317	Anda	–	–	28	31	6.7	6.7
F. oxysporum	L126	Anda	164	–	–	–	5.9	6.2
	L131	Chang Ping	–	–	34	36	5.5	5.8
	L176	Anda	177	–	–	–	5.2	5.4
	L195	Anda	162	183	–	–	4.7	4.8
	L212	Anda	–	161	–	–	5.5	5.7
F. solani	L141	Tong	–	–	–	40	5.9	6.4
	L144	Anda	–	168	–	–	4.3	4.2
	L151	Anda	29	–	33	23	5.2	5.2
	L319	Anda	–	30	–	–	4.1	3.9
	L328	Anda	36	32	–	–	4.2	4.5
Mortierella sp.	L369	Anda	–	16	–	39	6.3	3.8
Paecilomyces farinosus	L160	Anda	–	–	–	19	4.0	3.8
Penicillium sp.	L120	Chang Ping	25	27	28	24	4.9	4.9
	L121	Institute	–	–	–	40	3.7	3.6
	L294	Chang Ping	35	32	–	35	2.9	2.8

Table 1. *(Continued).*

Fungus	NL isolate[1)]	Field[2)]	Hatch (%)					
			H. glycines		*M. incognita*		Filtrate pH	
			3-day	7-day	3-day	7-day	3-day	7-day
	L305	Chang Ping	–	–	28	16	3.2	3.4
	L343	Institute	181	40	–	–	5.1	4.6
	L350	Chang Ping	–	39	–	–	4.8	4.1
	L352	Chang Ping	19	15	–	22	5.0	4.4
Pochonia chlamydosporia	L247	Anda	–	162	–	–	7.2	7.4
Ramicandelaber longisporus	L355	Tong	–	166	29	31	5.8	5.6
Trichothecium roseum	L248	Anda	37	–	–	–	6.9	7.0
Unidentified	L137	Anda	–	–	33	37	4.1	5.2
	L138	Anda	–	–	–	38	6.4	6.4
	L198	Anda	–	161	–	–	6.3	6.2
	L203	Anda	–	28	36	22	4.3	5.9
	L213	Institute	172	–	35	–	4.2	5.6
	L216	Chang Ping	–	–	30	14	5.1	6.3
	L219	Anda	–	–	37	29	4.9	4.7
	L223	Anda	–	170	–	–	6.4	6.9
	L225	Institute	224	172	39	29	5.1	4.7
	L242	Anda	–	–	20	–	4.3	4.8
	L281	Anda	37	–	–	–	5.8	6.2
	L306	Anda	–	32	–	–	5.5	3.6
	L330	Tong	36	20	–	39	4.0	3.5
	L362	Anda	34	–	–	–	6.3	6.2
	L368	Anda	38	39	–	–	5.9	5.6
	L370	Anda	–	–	–	37	5.9	6.1
	L374	Anda	–	–	32	31	4.8	4.1

[1)] NL isolate: USDA ARS Beltsville Nematology Laboratory Isolate Number.
[2)] Field locations: Anda, near Anda City; Institute, Alkaline Soil and Crop Breeding Institute, Heilongjiang Academy of Agricultural Sciences, near the Anda site; Chang Ping: Chang Ping County; Tong: Tong County.
[3)] not ±60% different from hatch in PDB control.

seum (Pers. Fr.) Link (1/1), and 15 of 101 unidentified isolates.

Filtrates that strongly stimulated SCN egg hatch were produced by isolates of *Cladosporium sphaerospermum* Penz. (2 of 3 isolates), *E. nigrum* (1/1), *F. oxysporum* (4/34), *F. solani* (1/21), *Penicillium* sp. (1/13), *Pochonia chlamydosporia* (Goddard) Zare & W. Gams (1/2), *R. longisporus* (1/2), and by four of 101 unidentified isolates (Table 1). Compounds from one isolate each of *Aspergillus* sp., *F. compactum*, *F. equiseti*, and *Penicillium* sp. inhibited J2 motility. Taxa that produced filtrates with no strong effects on hatch or motility were: *Chrysosporium* sp. (two isolates), a *Fusarium* that was not identified to species (one), *Idriella* sp. (one), *Paecilomyces lilacinus* (Thom) R.A. Samson (one), *Ramichloridium schulzeri* (Saccardo) de Hoog (one), *Trichoderma viride* Pers.:Fr. (one), and 84 unidentified isolates.

In the microwell assays, mean SCN egg hatch (± standard deviation) in controls was 29 ± 11.6% for eggs in water, and 24 ± 10.1% for eggs in PDB. Filtrate affect on SCN egg hatch varied from 85% reduction to more than doubling hatch, compared to PDB controls. In filtrates from 3-day-old fungal cultures, SCN hatch ranged from 19 to 224% (mean = 90 ± 35.5%) that in PDB controls (Fig. 1A). Hatch in filtrates from 7-day-old cultures presented a similar overall response, with hatch ranging from 15 to 207% (mean = 88 ± 35.6%) that in PDB controls (Table 1). Filtrates from 168 isolates (66.4% of

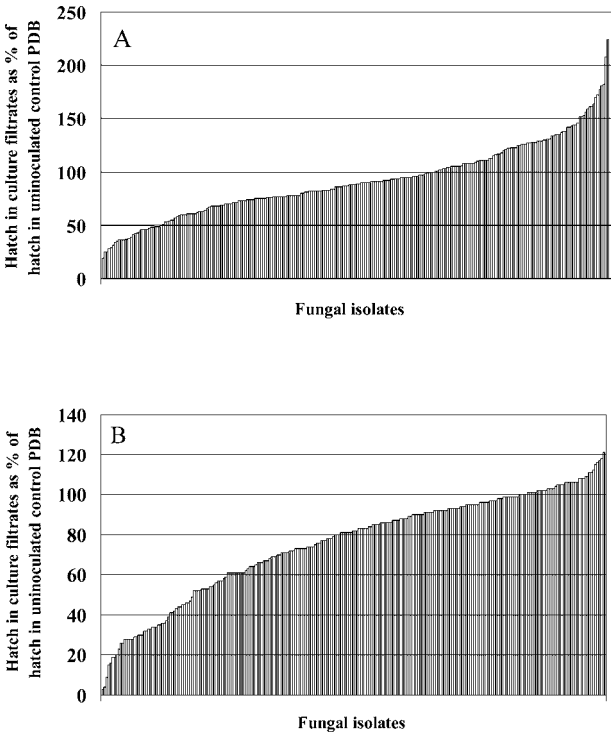

Fig. 1. *Percent hatch of nematode eggs in culture filtrates from 253 isolates of fungi grown for 3 days in potato dextrose broth (PDB), compared to hatch in non-inoculated PDB controls. A:* Heterodera glycines*; B:* Meloidogyne incognita*. Each vertical bar represents one fungal isolate.*

those tested) reduced or increased ($P < 0.05$) SCN hatch compared to that in the PDB controls. So many filtrates significantly affected hatch that only those filtrates that induced a 'strong' inhibition or stimulation are listed in Table 1. A total of 23 isolates (9.1% of those tested) produced filtrates that strongly inhibited SCN egg hatch under the assay conditions (Table 1). Some of these were strongly inhibitory to SCN when harvested at either 3 days or at 7 days, but not at both days. For such isolates, the mean SCN egg hatch in the filtrates not meeting the strong inhibition criteria was 68.9 (range 41-181)%. The least hatches were recorded in 7-day-old *C. globosum* (L250) filtrate, with 85 and 98% hatch reductions for SCN and RKN, respectively.

Fifteen isolates (5.9%) produced compounds that strongly stimulated SCN egg hatch compared to PDB controls (Table 1). Filtrates from most of these increased SCN hatch to some extent from both 3- and 7-day harvests. Filtrates of the 15 isolates that were not 'strongly' stimulatory at one of the broth harvest times nevertheless induced a mean egg hatch of 124 (40-159)% compared to PDB controls. When results from filtrates produced by all of the 253 tested fungal isolates were combined, SCN egg hatch in 3-day culture filtrates was correlated with SCN egg hatch in 7-day culture filtrates ($r_s = 0.74$, $P \leqslant 0.001$). However, there were fungi that produced highly active compounds (or effective amounts of active compounds) at only one time period, so that not all filtrates were equally effective at both harvest times (Table 1). For example, SCN egg hatch in filtrates from *Epicoccum nigrum* (L217) and *Penicillium* sp. (L343) was, respectively, 208 and 181% in 3-day broths but 44 and 40% in the 7-day broths.

Mean RKN egg hatch ($\pm$ standard deviation) in controls was 43 ± 14.4% for eggs in water, and 67 ± 17.1% for eggs in PDB. Hatch of RKN in 3-day-old culture filtrates ranged from 3 to 121 (mean 75 ± 26.5)% of that in PDB controls (Fig. 1B) and from 2 to 118 (73 ± 25.9)% in 7-day-old filtrates. Filtrates from 180 isolates (71.1% of those tested) significantly affected RKN egg hatch and 47 of those isolates (18.6% of those tested) produced filtrates that strongly decreased RKN egg hatch (Table 1). Of the 23 isolates that produced filtrates strongly inhibitory to SCN egg hatch, 13 also strongly inhibited RKN egg hatch. Overall, there was a low correlation of activities against SCN and RKN, as indicated by low r_s coefficients, at both fungal culture ages (3-day $r_s = 0.24$, $P \leqslant 0.001$; 7-day $r_s = 0.22$, $P \leqslant 0.001$). As observed with SCN, some of the isolates in Table 1 produced filtrates that were strongly inhibitory to RKN when harvested at either 3 or 7 days, but not at both. For those isolates, the mean RKN egg hatch in the filtrates that did not meet the strong inhibition criteria was 61 (41-106)%. However, despite some exceptions, RKN egg hatch in culture filtrates harvested after 3 days of fungal growth was significantly correlated with RKN egg hatch in culture filtrates collected after 7 days ($r_s = 0.80$, $P \leqslant 0.001$).

Although filtrates from six isolates stimulated ($P < 0.05$) RKN egg hatch compared with PDB controls, the effect was not as pronounced as with SCN and none of these filtrates strongly stimulated RKN egg hatch. The largest increase in RKN egg hatch (121% hatch compared to PDB controls) occurred in 3-day-old filtrate of *Mortierella* sp. (L283), which had no other significant influence on egg hatch of either nematode. Of the 15 fungal isolates that produced filtrates strongly stimulating egg hatch of SCN, four produced filtrates that strongly inhibited RKN egg hatch (Table 1).

Filtrates from only four fungal isolates (1.6% of those tested) were strongly toxic to J2. Removal of filtrate did

not result in enhanced motility in any of these treatments. Motilities (compared to PDB controls) in strongly toxic filtrates were: *Aspergillus* sp. (L192, Anda): 13 and 19% for SCN (3- and 7-day filtrate, respectively); *F. compactum* (L175, Anda): 9 and 14% for SCN and RKN, respectively, in 3-day filtrate (this was the only isolate with filtrate strongly toxic to J2 of both SCN and RKN); *F. equiseti* (L317, Anda): 34 and 15% for RKN in 3- and 7-day filtrates, respectively; *Penicillium* sp. (L353, Chang Ping): 16 and 17% for RKN (3- and 7-day filtrates). All of these isolates except *Penicillium* sp. (L353) also strongly inhibited RKN egg hatch (Table 1).

The pH of filtrates that strongly inhibited SCN and RKN egg hatch ranged from 2.8 to 7.5, while that of filtrates that strongly stimulated SCN hatch was 4.2 to 7.5 (Table 1). The pH of filtrates that inhibited J2 motility varied from 4.8 to 7.1.

Results with multiple isolates of a single fungus species were often variable. For example, of eight isolates of *C. cladosporioides* only one produced filtrates strongly inhibitory to both SCN and RKN (Table 1); two isolates strongly inhibited RKN hatch only, while filtrates from the other five isolates did not strongly influence hatch of either nematode. Of the 20 tested isolates of *F. equiseti* (all from Anda), two strongly inhibited hatch of both nematodes and nine inhibited that of RKN (Table 1). Filtrate from only one of these isolates was strongly toxic to RKN J2. Filtrate from one of 21 *F. solani* isolates strongly inhibited egg hatch of both nematodes (Table 1), two filtrates strongly inhibited SCN only, one inhibited RKN only, and one strongly stimulated SCN egg hatch (Table 1).

Discussion

A wide range of activities was recorded from the filtrates of the tested fungal isolates. More than half the 253 isolates produced culture broths that significantly affected egg hatch of SCN or RKN, and 27% strongly inhibited or stimulated hatch and/or exhibited strong toxicity to J2. Activity was not fungal species-dependent, but varied among isolates within a species, even when these had been collected from the same field.

The strong stimulatory effect of some filtrates on SCN hatch may have been caused by compounds that directly stimulated egg hatch, but it is also possible that hatch was enhanced because fungi utilised substances in PDB inhibitory to SCN egg hatch (Chen *et al.*, 2000). None of the filtrates had a correspondingly strong stimulatory effect on RKN in our study; this would be expected if PDB nutrient breakdown was a factor since RKN hatch was not inhibited by PDB. Alternatively, SCN might be more sensitive to fungus-produced stimulatory compounds or the compounds might have specific activity against SCN. For example, filtrates from isolates of *E. nigrum* (L217) and *Penicillium* sp. (L343) strongly stimulated SCN egg hatch when 3-day-old filtrates were used, but not when 7-day-old filtrates were used, indicating that the early hatch stimulation was caused directly by active products and not PDB breakdown.

For hatch of each nematode, there was a strong correlation in activity between filtrates from 3- and 7-day-old cultures. There was, however, a low correlation in filtrate activity between *H. glycines* and *M. incognita.* Twice as many isolates produced culture filtrates that strongly inhibited RKN hatch than produced filtrates that strongly inhibited SCN hatch (47 *vs* 23 isolates). Of these, only 13 isolates strongly inhibited egg hatch of both nematodes, ten inhibited SCN only, and 34 inhibited RKN only. Some of these differences may have been due to the culture broths being tested at a somewhat lower concentration against SCN than RKN, to compensate for the negative effect of PDB on SCN egg hatch. This complicates direct comparison of fungal broth activities against the two nematode species. However, variable activity of compounds applied against *Heterodera* and *Meloidogyne* has been reported in previous studies (Gourd *et al.*, 1994; Mayer *et al.*, 1999), and in our study, the possibility of a nematode species-specific effect of some filtrates on the two nematodes is reinforced by the observation that several isolates produced filtrates that strongly stimulated SCN hatch but strongly inhibited RKN hatch. Compounds selected for future study will be tested at a range of concentrations to more fully examine nematode-specific activity.

The recorded pH varied among filtrates. Pike *et al.* (2002) demonstrated that pH was not a hatching signal for SCN, so pH differences might not have directly altered SCN egg hatch. While it has been reported that fungal culture filtrates toxic to SCN J2 had lower pH than other culture filtrates (Chen *et al.*, 2000), a study with RKN demonstrated that activity of acetic acid on J2 paralysis increased at lower pH, although pH was not the direct cause of the antagonistic effect (Djian *et al.*, 1991). Culture filtrates selected for studies on identification of active compounds could be tested after neutralisation to determine whether pH was a factor affecting either the nematodes or the activity of potentially useful compounds.

Most of the fungal species isolated in this study have been reported from SCN (Morgan-Jones *et al.*, 1981; Gintis *et al.*, 1982, 1983; Carris & Glawe, 1989; Carris *et al.*, 1989; Meyer *et al.*, 1990; Liu, 1991; Liu *et al.*, 1992; Chen *et al.*, 1994; Mizobutsi *et al.*, 1999). *Ramicandelaber longisporus* was recently described by Ogawa *et al.* (2001), and is first reported from nematode eggs in our study. Isolates of some species tested in our study are known to produce nematicidal or nematotoxic compounds against *H. glycines* and/or *M. incognita*, with results dependent upon such parameters as fungal isolate, culture medium and method, pH and concentration of compound(s). For example, culture filtrates from two isolates of *P. lilacinus* inhibited egg hatch and juveniles of *H. glycines*, with one filtrate more active than the other (Sun *et al.*, 2002). *Fusarium solani*, *F. oxysporum*, *P. lilacinus* and *P. chlamydosporia* produced toxic filtrates, activity varying with culture medium and with species of fungus (Chen *et al.*, 2000). Filtrates from cultures of *A. ochraceus*, *F. solani*, *F. oxysporum*, *P. lilacinus*, *T. viride* and *P. chlamydosporia* were toxic to *M. incognita* juveniles, inhibited hatching, and/or suppressed egg or J2 populations on plants (Ameen, 1991; Hallman & Sikora, 1996; Khan, 1999; Sharma, 1999; Wang *et al.*, 1999; Costa *et al.*, 2000, 2001; Randhawa *et al.*, 2001). Extensive biocontrol work has been conducted with two of the tested species: *P. lilacinus* is marketed as a biocontrol product for nematodes on various crops, and *P. chlamydosporia* is being intensively studied as a biocontrol agent for root-knot nematodes on vegetable crops in southern Europe (Kerry & Bourne, 2002; Neethling, 2002). The isolates of *P. lilacinus* and *P. chlamydosporia* tested in our study did not exhibit strong nematicidal characteristics; this could be due to the assay conditions or to the isolates themselves.

The assays of SCN-associated fungi reported in this study have identified fungal isolates that produce compounds active against SCN and against RKN. Collection of fungi from one nematode species did not necessarily lead to isolation of fungal strains that produced compounds with greatest activity against the host nematode. A number of fungi may attack multiple hosts and be more aggressive against some hosts than others. Some isolates produce compounds with broad-spectrum activity, as was demonstrated when two of the fungi that strongly suppressed egg hatch of both SCN and RKN were selected for bioassay-directed isolation and identification of antagonistic products. Trichothecenes and flavipin, which are toxic to various organisms, were identified as the active components from *Fusarium equiseti* (L128) and *Chaetomium globosum* (L250) filtrates, respectively (Nitao *et al.*, 2001, 2002). Compounds active against more than one plant-parasitic nematode might be utilised against multiple nematode targets, increasing the scope of usefulness as pesticides. For a different focus, filtrates highly active against only one nematode species may yield natural products that have narrower biological activity, with fewer undesirable effects on non-target organisms.

Fungi from any soybean field can be used as a possible source for nematode-antagonistic products. As soybean has been transported worldwide, fungi in each new region have adapted to SCN as a host and many produce compounds active against SCN. Additionally, some fungi that originally evolved in association with SCN have most likely been carried with soybean, SCN and soil around the world (this would be less common with seed transport, and more frequent with plants and soil). However, it is probable that only a subset of the population that evolved in association with SCN would have been transported elsewhere, and these strains would then have adapted to the new environmental conditions. Collection of fungi from the source of origin of SCN and soybean provides potential for isolation of strains that are not available in other locations. These isolates can then be utilised in studies for identification of possible biobased nematicides, or as candidates for use as live microbial pest control agents, introducing genetic material for application in locales where the fungal strains are not naturally available.

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References

AMEEN, H.H. (1991). [Nematicidal effect of *Aspergillus ochraceus* filtrate.] *Bulletin of Faculty of Agriculture, University of Cairo* 42, 963-970.

ANKE, H. & STERNER, O. (1997). Nematicidal metabolites from higher fungi. *Current Organic Chemistry* 1, 361-374.

ANKE, H., STADLER, M., MAYER, A. & STERNER, O. (1995). Secondary metabolites with nematicidal and antimicrobial activity from nematophagous fungi and Ascomycetes. *Canadian Journal of Botany* 73, S932-S939.

BUCHEL, E., MAYER, A., MARTINI, U., ANKE, H. & STERNER, O. (1998). Structure elucidation of omphalotin, a cyclic dodecapeptide with potent nematicidal activity isolated from *Omphalotus olearius*. *Pesticide Science* 54, 309-311.

BUSH, L.P., WILKINSON, H.H. & SCHARDL, C.L. (1997). Bioprotective alkaloids of grass-fungal endophyte symbioses. *Plant Physiology* 114, 1-7.

CARRIS, L.M. & GLAWE, D.A. (1989). *Fungi colonizing cysts of* Heterodera glycines. Bulletin 786, College of Agriculture, Agricultural Experiment Station, University of Illinois at Urbana-Champaign and US Department of Agriculture, 93 pp.

CARRIS, L.M., GLAWE, D.A., SMYTH, C.A. & EDWARDS, D.I. (1989). Fungi associated with populations of *Heterodera glycines* in two Illinois soybean fields. *Mycologia* 81, 66-75.

CHEN, S.Y., DICKSON, D.W., KIMBROUGH, J.W., MCSORLEY, R. & MITCHELL, D.J. (1994). Fungi associated with females and cysts of *Heterodera glycines* in a Florida soybean field. *Journal of Nematology* 26, 296-303.

CHEN, S.Y., DICKSON, D.W. & MITCHELL, D.J. (2000). Viability of *Heterodera glycines* exposed to fungal filtrates. *Journal of Nematology* 32, 190-197.

CIANCIO, A. (1995). Observations on the nematicidal properties of some mycotoxins. *Fundamental and Applied Nematology* 18, 451-454.

COSTA, M.J.N., CAMPOS, V.P., PFENNING, L.H. & OLIVEIRA, D.F. (2000). Pathogenicity and reproduction of *Meloidogyne incognita* in tomato plants (*Lycopersicon esculentum*) with application of fungal filtrates or plant and animal manure extracts. *Nematologia Brasileira* 24, 219-226.

COSTA, M.J.N., CAMPOS, V.P., PFENNING, L.H. & OLIVEIRA, D.F. (2001). Fungus filtrates toxicity to *Meloidogyne incognita*. *Fitopatologia Brasileira* 26, 749-755.

DJIAN, C., PIJAROWSKI, L., PONCHET, M., ARPIN, N. & FAVRE-BONVIN, J. (1991). Acetic acid: a selective nematicidal metabolite from culture filtrates of *Paecilomyces lilacinus* (Thom) Samson and *Trichoderma longibrachiatum* Rifai. *Nematologica* 37, 101-112.

FERNÁNDEZ, C., RODRÍGUEZ-KÁBANA, R., WARRIOR, P. & KLOEPPER, J.W. (2001). Induced soil suppressiveness to a root-knot nematode species by a nematicide. *Biological Control* 22, 103-114.

GINTIS, B.O., MORGAN-JONES, G. & RODRÍGUEZ-KÁBANA, R. (1982). Mycoflora of young cysts of *Heterodera glycines* in North Carolina soils. *Nematropica* 12, 295-303.

GINTIS, B.O., MORGAN-JONES, G. & RODRÍGUEZ-KÁBANA, R. (1983). Fungi associated with several developmental stages of *Heterodera glycines* from an Alabama soybean field soil. *Nematropica* 13, 181-200.

GOURD, T.R., SCHMITT, D.P. & BARKER, K.R. (1994). Differential sensitivity of *Meloidogyne* spp. and *Heterodera glycines* to selected nematicides. *Journal of Nematology* 25 (Supplement), 746-751.

HALLMANN, J. & SIKORA, R.A. (1996). Toxicity of fungal endophyte secondary metabolites to plant-parasitic nematodes and soil-borne plant-pathogenic fungi. *European Journal of Plant Pathology* 102, 155-162.

KERRY, B.R. & BOURNE, J.M., EDS (2002). *A manual for research on* Verticillium chlamydosporium*, a potential biological control agent for root-knot nematodes*. Gent, Belgium, IOBC OILB, 84 pp.

KHAMBAY, B.P.S., BOURNE, J.M., CAMERON, S., KERRY, B.R. & ZAKI, M.J. (2000). A nematicidal metabolite from *Verticillium chlamydosporium*. *Pest Management Science* 56, 1098-1099.

KHAN, T.A. (1999). Studies on the toxic effect of culture filtrate of some fungi on root-knot nematode. *Bionotes* 1, 38-39.

KÖPCKE, B., WOLF, D., ANKE, H. & STERNER, O. (2001). New natural products with nematicidal activity from fungi. *British Mycological Society International Symposium, Bioactive Fungal Metabolites – Impact and Exploitation, UW Swansea, UK, 22-27 April 2001*, p. 72. [Abstr.]

KWOK, O.C.H., PLATTNER, R., WEISLEDER, D. & WICKLOW, D.T. (1992). A nematicidal toxin from *Pleurotus ostreatus* NRRL-3526. *Journal of Chemical Ecology* 18, 127-136.

LIU, W.Z., LIU, Y., DUAN, Y.X., HONG, Q.C. & WANG, K.N. (1995). Studies on the soybean cyst nematode (*Heterodera glycines*) in China. *Korean Journal of Applied Entomology* 34, 83-88.

LIU, X.Z. (1991). *Taxonomic and ecological studies on nematophagous fungi in China*. Ph.D. dissertation, Beijing, China, Department of Plant Protection, Beijing Agricultural University, 86 pp. + 162 figs.

LIU, X.Z., DING, L., WU, X.Y. & SHEN, C.Y. (1992). Nematophagous fungi in China. *Mycosystema* 5, 117-126.

LIU, X.Z., LI, J.Q. & ZHANG, D.S. (1997). History and status of soybean cyst nematode in China. *International Journal of Nematology* 7, 18-25.

MAYER, A., KILIAN, M., HOSTER, B., STERNER, O. & ANKE, H. (1999). *In-vitro* and *in-vivo* nematicidal activities of the cyclic dodecapeptide omphalotin A. *Pesticide Science* 55, 27-30.

MEYER, S.L.F., HUETTEL, R.N. & SAYRE, R.M. (1990). Isolation of fungi from *Heterodera glycines* and *in vitro* bioassays for their antagonism to eggs. *Journal of Nematology* 22, 532-537.

MEYER, S.L.F., MASSOUD, S.I., CHITWOOD, D.J. & ROBERTS, D.P. (2000). Evaluation of *Trichoderma virens* and *Burkholderia cepacia* for antagonistic activity against root-knot nematode, *Meloidogyne incognita*. *Nematology* 2, 871-879.

MIZOBUTSI, E.H., FERRAZ, S., RIBEIRO, R.C.F. & MENEZES, M. (1999). Isolation of fungi from *Heterodera glycines* eggs collected in different soyabean-producing areas of Brazil. *Nematologia Brasileira* 23, 69-75.

MORGAN-JONES, G., GINTIS, B.O. & RODRÍGUEZ-KÁBANA, R. (1981). Fungal colonization of *Heterodera glycines* cysts in Arkansas, Florida, Mississippi and Missouri soils. *Nematropica* 11, 155-164.

NEETHLING, D. (2002). The commercialisation of *Paecilomyces lilacinus* as an agent for the control of plant-parasitic nematodes. *Nematology* 4, 152. [Abstr.]

NITAO, J.K., MEYER, S.L.F. & CHITWOOD, D.J. (1999). *In-vitro* assays of *Meloidogyne incognita* and *Heterodera glycines* for detection of nematode-antagonistic fungal compounds. *Journal of Nematology* 31, 172-183.

NITAO, J.K., MEYER, S.L.F., SCHMIDT, W.F., FETTINGER, J.C. & CHITWOOD, D.J. (2001). Nematode-antagonistic trichothecenes from *Fusarium equiseti*. *Journal of Chemical Ecology* 27, 859-869.

NITAO, J.K., MEYER, S.L.F., OLIVER, J.E., SCHMIDT, W.F. & CHITWOOD, D.J. (2002). Isolation of flavipin, a fungus compound antagonistic to plant-parasitic nematodes. *Nematology* 4, 55-63.

OGAWA, Y., HAYASHI, S., DEGAWA, Y. & YUKIO, Y. (2001). *Ramicandelaber*, a new genus of the Kickxellales, Zygomycetes. *Mycoscience* 42, 193-199.

PERRY, R.N., TWOMEY, U., ROLFE, R.N. & WARRIOR, P. (2000). Effects of DiTera® on aspects of the life cycle of *Globodera rostochiensis*. *Aspects of Applied Biology* 59, 53-58.

PIKE, S.M., HEINZ, R., WALK, T., JONES, C., KRAUS, G.A., GASSMANN, W., NOVACKY, A.J. & NIBLACK, T.L. (2002). Is change in electrical potential or pH a hatching signal for *Heterodera glycines*? *Phytopathology* 92, 456-463.

PORTER, J.K. (1994). Chemical constituents of grass endophytes. In: Bacon, C.W. & White, Jr, J.F. (Eds). *Biotechnology of endophytic fungi of grasses*. Boca Raton, FL, USA, CRC Press, pp. 103-123.

RANDHAWA, N., SINGH, P., SANDHU, K.S. & BHATIA, A. (2001). Effect of culture filtrates of soil fungi on hatching of *Meloidogyne incognita*. *Plant Disease Research* 16, 280-282.

ROWAN, D.D. & GAYNOR, D.L. (1986). Isolation of feeding deterrents against Argentine stem weevil from ryegrass infected with the endophyte. *Journal of Chemical Ecology* 12, 647-658.

SEGERS, R., BUTT, T.M., KERRY, B.R. & PEBERDY, J.F. (1994). The nematophagous fungus *Verticillium chlamydosporium* produces a chymoelastase-like protease which hydrolyses host nematode proteins *in situ*. *Microbiology* 140, 2715-2723.

SEGERS, R., BUTT, T.M., CARDER, J.H., KEEN, J.N., KERRY, B.R. & PEBERDY, J.F. (1999). The subtilisins of fungal pathogens of insects, nematodes and plants: distribution and variation. *Mycological Research* 103, 395-402.

SHARMA, D.D. (1999). Effect of culture filtrates of biocontrol agents on larval mortality of *Meloidogyne incognita*, in comparison with Rugby 10G. *Indian Journal of Sericulture* 38, 152-154.

SUN, M.H., LIU, X.Z. & JIN, Z.B. (2002). Effects of *Paecilomyces lilacinus* on egg hatching and juvenile mortality of *Heterodera glycines*. *Acta Phytophylactica Sinica* 29, 57-61.

TWOMEY, U., WARRIOR, P., KERRY, B.R. & PERRY, R.N. (2000). Effects of the biological nematicide, DiTera®, on hatching of *Globodera rostochiensis* and *G. pallida*. *Nematology* 2, 355-362.

TWOMEY, U., ROLFE, R.N., WARRIOR, P. & PERRY, R.N. (2002). Effects of the biological nematicide, DiTera®, on movement and sensory responses of second stage juveniles of *Globodera rostochiensis*, and stylet activity of *G. rostochiensis* and fourth stage juveniles of *Ditylenchus dipsaci*. *Nematology* 4, 909-915.

WANG, L.F., YANG, B.J. & LI, C.D. (1999). Evaluation of pathogenicity of parasitic fungi to root-knot nematodes. *Scientia Silvae Sinicae* 35, 41-47.

WARRIOR, P., REHBERGER, L.A., BEACH, M., GRAU, P.A., KIRFMAN, G.W. & CONLEY, J.M. (1999). Commercial development and introduction of DiTera®, a new nematicide. *Pesticide Science* 55, 376-379.