Annals of Microbiology, **58 (3)** 369-373 (2008)

The efficacy of nematicidal strain *Syncephalastrum racemosum*

Jianhua SUN[1*], Haikuan WANG[2], Fuping LU[2], Lianxiang DU[2], Gaofeng WANG[1]

[1]College of Chemistry and Life Science, Tianjin Normal University, Tianjin 300387; [2]College of Biotechnology, Tianjin Key Laboratory of Industrial Microbiology, Tianjin University of Science and Technology, Tianjin 300222, P.R. China

Received 11 January 2008 / Accepted 8 May 2008

Abstract - Nematicidal activity using a pure culture of *Syncephalastrum racemosum* was studied. The results showed that the metabolites of *S. racemosum* have pretty high nematicidal activity. The nematicidal principles were soluble in water and had high thermal stability. The major acid metabolites extracted from cultures of *S. racemosum* identified from HPLC were oxalic acid dihydrate and tartaric acid. Soil application with culture filtrate of *S. racemosum* significantly ($P < 0.05$) reduced nematode population densities and subsequent root-knot development in tomato compared with the controls.

Key words: biosynthesis, environmental preservation, fermentation; filamentous fungi, nematicides, *Syncephalastrum racemosum.*

INTRODUCTION

Plant-parasitic nematodes are invertebrate worm-like animals that require feed from the susceptible host plants in order to complete their life cycles. So far, more than 1200 species of nematodes have been found to attack plants with virtually every crop being susceptible to at least one particular assortment (Mcbride *et al*., 2000), which causes great damage to the production of crops and vegetables with 100 billion US dollars each year in the world nowadays (Akhtar and Malik, 2000). Although the most effective method to control the plant-parasitic nematodes is the use of chemical nematicides, such as ongoing bromomethane or organo-phosphorous insecticide (Anke and Sterner, 1997), application of commercial nematicides, however, has been severely restricted because of problems associated with ground water contamination, food safety and worker protection. The pesticides of the current century will be routinely required not only more active but also environmentally safe and cost-effective. Increasing awareness of environmental and human health concerning with chemical nematicides and, as a consequence, the removal of several efficacious products from the world market recently provide great impetus for the search of new environmentally compatible products for nematode management (Jagdale *et al*., 2002).

Concurrently, biological agents such as fungi and bacteria, could also be anticipated to apply as part of integrated pest management program to control plant-parasitic nematodes, although only a few commercial formulations have been developed, and the use is limited so far (Stadler *et al*., 1994; Tsao *et al*., 2000; Momin and Nair, 2002; Wei *et al*., 2003; Chomeheon *et al.,* 2005; Quang *et al.,* 2006; Oliveira *et al.,* 2007; Stadler *et al.,* 2007). The most significant progress in this area is the discovery of the natural product omphalotin series from the basidiomycete *Omphalotus olearius*, which belong to a family of cycle dodecapeptides (Mayer *et al*., 1997). Subsequently, isolation, X-ray structural characterisation and total synthesis of these compounds have been further performed by chemists (Sterner *et al*., 1997; Buchel *et al*., 1998; Thern *et al*., 2002). This made people pay more attention to investigate the production of new biologically nematicidal active metabolites from microorganism and the interest in the metabolites of fungi is increasing (Butt *et al*., 2001). The metabolites from filamentous fungus *Syncephalastrum racemosum* showed very strong and wide range nematicidal activity against some kinds of important plant-parasitic nematodes, such as cyst nematodes (*Heterodera glycines*), root-knot nematodes and pine wood nematode (*Bursaphelenchus xylophilus*) during our research (Sun *et al*., 1999).

In this contribution, we firstly report nematicidal activity and the nematicidal principles characteristics of strain *Syncephalastrum racemosum.*

MATERIALS AND METHODS

Screening of fungal cultures for nematicidal activity. One gram of soil sample infected by root-knot nematodes was suspended in 10 ml sterile water. After shaking, 5 ml suspension was added in a 500 ml shaking flask containing 100 ml PSM medium (300 g l^{-1} potato, 20 g l^{-1} sucrose, malt extract 2 g l^{-1}). The mixture was incubated at 27 °C with 170 revolutions per minute (rpm) for 48 h. The grown microorganisms were

* Corresponding author. Phone: +86-22-23766539;
Fax: +86-22-23766556; E-mail: jianhuasun55@yahoo.com.cn

isolated on potato sucrose malt-extract agar plates (PSMA: 300 g l^{-1} potato, 20 g l^{-1} sucrose, malt extract 2 g l^{-1}, agar 15 g l^{-1}) at 27 °C for 96 h. Eighty-eight strains of fungi were obtained from the plates and were maintained on PSMA medium at 4 °C. Culture broth was prepared by inoculating 10 ml PSM medium in a cotton plugged test tube (25 x 200 mm) with growth from a stock slant, and incubated with shaking at 27 °C for 4 days. The culture broth was separated by centrifuged at 3500 g for 15 min to supernatant and cells. One tenth of the supernatant was freeze-dried, dissolved in 0.1 ml of water, and injected into a cotton ball. The cotton ball after drying in *vacuo* in a desiccator was placed into the centre of the Petri dish (Ø 4.5 cm) where *Botrytis cinerea* fungus was growing. The nematode suspension (0.1 ml, 1500 heads) was injected by a microsyringe into the cotton ball and the dish was kept at 26 °C for 116 h, then the nematodes were collected for counting and the reproduction rate of nematodes (G) was calculated from the equation [the number of living nematodes in the sample / the number of living nematodes in the control] (Kawazu *et al*. 1980).

All the cells were soaked in acetone-methanol (1:1) at room temperature for 1-3 days, and the extract was concentrated to 0.1 ml and subjected to the nematicidal activity assay as above stated.

Another culture broth of the same strain was kept standing in a test at 27 °C for 2 weeks after the shaking mentioned above. This culture broth was also worked up in a similar way, and subjected to the nematicidal activity assay. These are samples indicated by "shaken and subsequent stationary incubation" in Table 1.

Inoculum preparation. *Syncephalastrum racemosum* was subcultured on PSMA slants, incubated at 27 °C for 5-6 days and the spore suspension was used to inoculate 100 ml of PS liquid medium (300 g l^{-1} potato, 20 g l^{-1} sucrose) to a final concentration of 10^7 spore l^{-1} in a 500 ml shaking flask. The flask was rotated in a rotary shaker at 27 °C with 170 rpm for 96 h.

Nematicidal substance production and characterisation. Fermentations were carried out in a 30 l fermenter containing PS liquid medium with aeration (1:0.5 vvm) and stirring (100-400 rpm) at 27 °C for 42 h. The production medium was inoculated with 4% (v/v) inoculum. After the complete consumption of the carbon source and pH 2.0, the fermentation was stopped up to 42 h, and culture filtrate and mycelium were separated by filtration.

The nematicidal activity test of culture filtrate was repeated three times and the active nematicidal principles of *Syncephalastrum racemosum* were characterised.

Heat resistance of *Syncephalastrum racemosum* metabolites were tested by sterilising the culture filtrate for 30 min and subsequently tested for nematicidal activity. Culture filtrate (pH 2.0) was adjusted to pH 7.0 (NaOH) and was tested for nematicidal activity.

To determine the solubility of active principles, the culture filtrate lyophilised was extracted with the solvents including water, methanol, ethanol, acetonitrile, acetone, ethyl acetate, n-butyl alcohol. The extract was centrifuged at 10000 *x g* for 15 min. The supernatant evaporated by heating and solid substance dissolved in water was tested for nematicidal activity.

The culture filtrate of strain *S. racemosum* (5 ml) was extracted with 12 ml ethanol, the extract was centrifuged at 3500 *x g* for 15 min and the supernatant was subjected to HPLC analyses with a ion exchange SCR-101 H column (4.6 x 250 mm), applying a water at a flow rate of 0.6 ml/min.

Nematicidal activity assays. An in vitro test for nematicidal activity was carried out using 96-well Nunclon plates. In each well, 0.2 ml samples were poured, and then 20 µl of the nematode suspension (containing 300 heads of pine wood nematodes, *Bursaphelenchus xylophilus*) was added. The number of living and dead (paralyzed) nematodes was counted under a binocular microscope after incubating at 26 °C for 5 days. Each treatment was replicated at three times. Nematicidal activity was estimated according to the mean percentage of dead nematodes after the due time.

Glasshouse experiment. The upper 15 cm soil surface was removed. The soil was poured with 2.5 ml/m^2 culture filtrate of *S. racemosum*. Soil applied with 2.5 ml/m^2 sterile distilled water served as controls. After 1 week soil treatment, 3-week old tomato seedlings raised in sterilised soil were planted. Three replicates used for each treatment were randomized on a glasshouse bench. The temperature of the glasshouse throughout the experiments ranged between 24-30 °C. The root-knot nematodes in *S. racemosum* culture filtrate-treated and untreated soil were extracted using a modified Baermann funnel technique

TABLE 1 - Nematicidal activity of cultures of 12 strains of fungi

Fungal strain	Shaken incubation Activity[a], Growth rate[b] (%)		Shaken and subsequent stationary incubation Activity[a], Growth rate[b] (%)	
	Cells	Supernatant	Cells	Supernatant
1	+, 10.1	+, 8.0	+, 11.0	+, 8.5
2	+, 0	+, 0	+, 0	+, 0
3	—	—	+, 5.1	—
4	+, 6.1	—	—	—
5	+, 7.7	—	+, 1.7	—
6	—	—	+, 2.7	—
7	+, 9.3	+, 8.9	—	—
8	+, 12.1	+, 8.6	+, 24.3	+, 18.1
9	+, 16.3	+, 17.5	+, 15.1	+, 16.5
10	+, 10.8	+, 12.2	+, 11.1	+, 13.2
11	+, 21.1	+, 18.1	+, 24.4	+, 19.1
12	+, 9.6	+, 8.6	+, 10.1	+, 8.1

[a] Activity observed from superficial appearances of fungal mat: +: active, —: inactive.
[b] Growth rates calculated from nematode numbers.

TABLE 2 - Index standard of infection degree of tomato by root-knot nematodes

Level	Infection degree of tomato by root-knot nematodes	Evaluation standard
0	No	The phenotypes of roots were normal, no gall was found
1	Slight	A little galls were found in some roots that accounted for 1~25% of all the roots
2	Moderate	Some galls were found in some roots that accounted for 26~50% of all the roots
3	Serious	Many galls were found in some roots that accounted for 51~75% of all the roots
4	Worst	Large quantitative of galls were found in some roots that accounted for 76~100% of all the roots

Disease index % = $\sum (A \times B)/(C \times D)$
where: A is the number of tomatoes of each "Level", B is the number of index standard of each "Level", C is the number of the index standard of the highest "Level", D is the number of all the tomatoes.

(Rodriguez and Pope, 1981) and were counted with the aid of low power stereomicroscope 1 week after treatment. Plant growth parameters including plant height and fresh weights of shoot and root were determined 5 weeks after treatment. The experiment was terminated 4 months after treatment, the root system was washed and number of galls by *Meloidogyne incognita* on entire root system was recorded. The index standard of infection degree of tomato by root-knot nematodes is indicated in Table 2.

Statistics. All experiments were repeated three times. The data were the mean values of the experiments. The Tukey test was used for mean comparison. Statistical differences referred to in the text are significant at P = 0.05.

RESULTS

Screening of cultures of 88 strains of fungi

A total of 88 fungal isolates from soil were screened for nematicidal activity, among which 12 were obtained based on nematicidal activity, while the others were discarded based on their comparatively poor nematicidal activity. The result of screening 12 fungi is shown in Table 2. Of the 12 isolates, the entry 2 with the maximum nematicidal activity was chosen to use for following experiments.

Identification of the strain was done using morphology method and rDNA ITS sequences analysis. Identification studies on the strain revealed clearly the presence of *Syncephalastrum racemosum.* Scanning electron microscopic features of *Syncephalastrum racemosum* is indicated in Fig. 1.

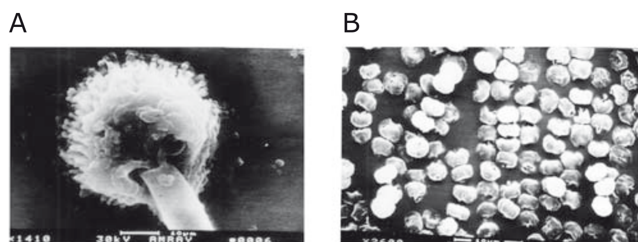

FIG. 1 - Scanning electron microscoic features Fungal strain of *Syncephalastrum racemosum*. A: spore apex (x1410), B: spores (x2600). Bars: 10 μm.

Characteristics of the active nematicidal principles and the acid compounds from cultures in the fermenter

The culture broth pH was 2.0 after 42 h fermentation. Second-stage pine wood nematode juveniles were employed for the preliminary biological test of culture filtrates of *Syncephalastrum racemosum*. Bio-assay results show that unsterilised and sterilised culture filtrates respectively have 98.38 and 96.25% nematicidal activity after 120 h incubation with nematode. Culture filtrate (pH 2.0) and culture filtrate adjusted to pH 7.0 respectively exhibited 100 and 91.6% nematicidal activity after 120 h incubation with nematode (Fig. 2). The five solvent fractions including water, methanol, ethanol, acetone, acetonitrile has above 90% nematicidal activity. The major acid metabolites extracted from cultures of *S. racemosum* were identified from HPLC to be oxalic acid dihydrate, tartaric Acid by comparison with spectra of standard samples.

Glasshouse experiment

Soil application with culture filtrate of *S. racemosum* caused significant (P < 0.05; 94.68% compared to controls) reduction in nematode population. Similarly, the disease index was reduced (P < 0.05; 53.52% compared to controls). Plant height and fresh weight was significantly (P < 0.05; 7.71 and 26.62% compared to controls) increased. Interestingly, yield was significantly (P < 0.05; 28.71% compared to controls) elevated (Table 3 and Fig. 3).

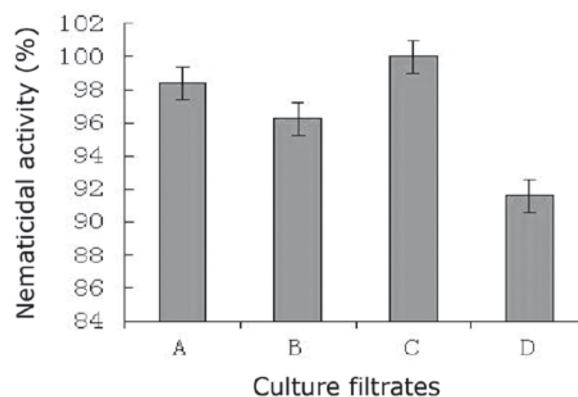

FIG. 2 - The nematicidal activity of different culture filtrates after 120 h incubation with second-stage pine wood nematode juveniles. A: unsterlised culture filtrate, B: sterelised culture filtrate, C: culture filtrate (pH 2.0), D: culture filtrate (pH 7.0).

TABLE 3 - Effects of soil application with culture filtrate of *Syncephalastrum racemosum* on tomato

Group	NP (heads/kg)	DI	PH (cm)	FW (g)	YD (kg/667m^2)
Experiment (n = 50)	60a	27.50a	128.26a	167.36a	4931.42a
Control (n = 50)	995b	59.17b	119.08b	132.18b	3831.42b

NP: nematode population (numbers of *M. javanica* J2 per 1000 g soil); DI: Disease Index; PH: average of plant height; F: fresh weight of each plant; Y: average yields of the tomatoes.
n: number of samples. Values (means) followed by different letters within each column are significantly different based on the Tukey test (P = 0.05, n = 50).

DISCUSSION

The fungi as a source of biologically active metabolites represent an enormous source for natural products with diverse chemical structures and activities. The culture broths of the some fungi screened for nematode-antagonistic activity have been reported. One hundred and eighty one fungal species isolated from the fresh fruiting bodies were tested on the pine wood nematode, *Bursaphelenchus xylophilus in vitro* (Dong *et al*., 2006). *Pleurotus ferulae* Lenzi, a species of edible fungus, was found to have nematicidal activity in experiments searching for nematicidal fungi. Three nematicidal metabolites were isolated based on bioassay-guided fractionation from the extracts of the fungus *P. ferulae* (Li *et al*., 2007). The 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*). 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 (Meyer *et al*., 2004). An isolate of the fungus *Chaetomium globosum* produced culture broths that inhibited *in vitro* egg hatch and juvenile mobility of root-knot nematode (*Meloidogyne incognita*) and hatch of soybean cyst nematode (*Heterodera glycines*). Extraction and bioassay-directed fractionation of the culture broth filtrate determined that flavipin, a low molecular weight compound, was the fungus metabolite responsible for most of the nematode-antagonistic activity (Nitao *et al*., 2002). A strain of the fungus *Fusarium equiseti* isolated from soybean cyst nematode secretes nematode-antagonistic compounds. Bioassay-guided fractionation of an extract of the culture broth was undertaken to identify the compounds. Fractions were assayed for activity against a root-knot nematode (*Meloidogyne incognita*), a plant pathogen that attacks the roots of numerous plant species. Two trichothecene compounds were isolated that inhibited egg hatch and immobilized second-stage juveniles of this nematode: 4,15-diacetoxy-12,13-epoxy-3,7-dihydroxytrichothec-9-en-8-one and 4,15-diacetoxy-12,13-epoxy-trichothec-9-en-3-ol. (Nitao *et al*., 2001). There is no report on the efficacy of nematicidal strain *Syncephalastrum racemosum* as we know so far. This study showed that the metabolites of *Syncephalastrum racemosum* have pretty high nematicidal activity. In any case, this is a really exciting result due to the many unprecedented advantages, comparing with the applied nematicides in the market, such as environmentally safe, cost-effective, soluble in water and high thermal stability, making it has the great potential for practical application for nematode control

Some of the active compounds being synthesised by chemical industries are also fungi metabolites such as some organic acids which have a direct effect on nematode mortality. The result about characteristics of the active nematicidal principles indicate that acid compounds are not all nematicidal components that fungus *Syncephalastrum racemosum* produced, and it means that more research is needed to identify the other kinds of nematicidal compounds and the nematicidal activity of these compounds for other types of nematodes.

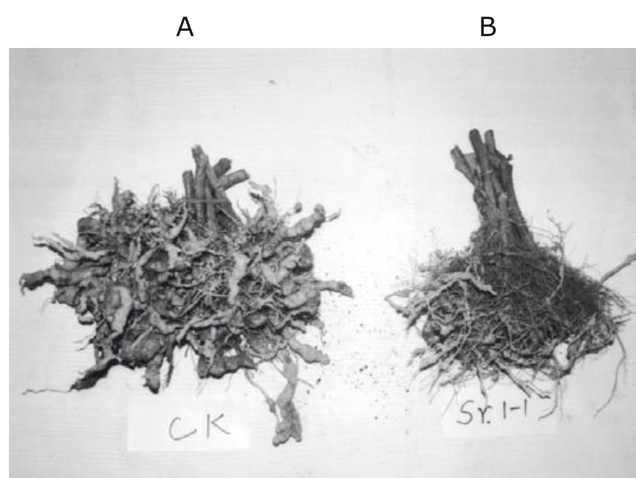

FIG. 3 - Photos of tomato roots untreated (A) and treated with Syncephalastrum racemosum metabolites (B) in glass-house experiments.

Acknowledgements
The authors wish to acknowledge the financial support provided by the National Natural Science Foundation of China (No. 30270877), and the Project of Key Technology R&D Program (No. 05YFGZGX04400) supported by the Tianjin Municipal Science and Technology Commission of China.

REFERENCES

Akhtar M., Malik A. (2000). Roles of organic soil amendments and soil organisms in the biological control of plant-parasitic nematodes: A review. Bioresource Technol., 74: 35-47.

Anke H., Sterner O. (1997). Nematicidal compounds from higher fungi. Curr. Org. Chem., 1: 361-374.

Buchel E., Martini U., Mayer A., Anke H., Sterner O. (1998). Omphalotins B, C, and D nematicidal cyclopeptides from *Omphalotus olearius*: Absolute configuration of omphalotin A, Tetrahedron, 54: 5345-5352.

Butt T.M., Jackson C.W., Magan N. (2001). Fungi as biocontrol agents progress, problems and potential. CABI Publishing, UK, 1-9: 311-334.

Chomeheon P., Wiyakrutta S., Sriubolmas N., Ngamrojanavanich N. Isarangkul D., Kittakoop P. (2005). 3-nitropropionic acid (3-NPA), a potent antimycobacterial agent from endophytic fungi: Is 3-NPA in some plants produced by endophytes. J. Nat. Prod., 68: 1103-1105.

Dong J.Y., Li X.P., Li L., Li G.H., Liu Y.J., Zhang K.Q. (2006). Preliminary results on nematicidal activity from culture filtrates of Basidiomycetes against the pine wood nematode, *Bursaphelenchus xylophilus* (Aphelenchoididae). Ann. Microbiol., 56: 163-166.

Jagdale G.B., Somasekhar N., Grewal P.S., Klein M.G. (2002). Suppression of plant-parasitic nematodes by application of live and dead infective juveniles of an entomopathogenic nematode, *Steinernema carpocapsae*, on Boxwood (*Buxus* spp.). Biol. Control, 24: 42-49.

Nitao J.K., Meyer S.L.F., Schmidt WF., Fettinger J.C., Chitwood D.J. (2001). Nematode-antagonistic trichothecenes from *Fusarium equiseti*. J. Chem. Ecol., 27: 859-869.

Nitao J.K., Meyer S.L.F., Oliver J.E., Schmidt WF., Chitwood D.J. (2002). Isolation of flavipin, a fungus compound antagonistic to plant-parasitic nematodes. Nematology, 4: 55-63.

Kawazu K., Nishii Y., Ishii K. (1980). A convenient screening method for nematicidal activity. Agric. Biol. Chem., 44: 631-635.

Li G.H., Wang X.B., Zheng L.J., Li L., Huang R., Zhang K.Q. (2007). Nematicidal metabolites from the fungus *Pleurotus ferulae* Lenzi. Ann. Microbiol., 57: 527-529.

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

Mcbride R.G., Mikkelsen R.L., Barker K.R. (2000). The role of low molecular weight organic acids from decomposing rye in inhibiting nematode populations in soil. Appl. Soil Ecol., 15: 243-251.

Momin R.A., Nair M.G. (2002). Pest managing efficacy of trans-asarone isolated from *Daucus carota* L. seeds. Agr. Food. Chem., 50: 4475-4478.

Oliveira D.F., Campos V.P., Amaral D.R., Nunes A.S., Pantaleao J.A., Costa D.A. (2007). Selection of rhizobacteria able to produce metabolites active against *Meloidogyne exigua*. Eur. J. Plant Pathol., 119: 477-479.

Quang D.N., Hashimoto T., Asakawa Y. (2006). Inedible mushrooms: A good source of biologically active substances. Chem. Rec., 6: 79-99.

Rodriguez K.R., Pope M.H. (1981). A simple incubation method for the extraction of nematodes from soil. Nematropica, 11: 175-186.

Stadler M., Anke H., Sterner O. (1994). Six new antimicrobial and nematicidal bisabolanes from the Basidiomycete *Cheimonophyllum candidissimum*. Tetrahedron, 50: 12649-12654.

Stadler M., Fournier J., Quang D.N., Akulov A.Y. (2007). Metabolomic studies on the chemical ecology of the *Xylariaceae* (Ascomycota). Nat. Prod. Comm., 2: 287-304.

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

Sun J.H., Yu K.L., Bi P., Peng D.L. (1999). Study on biological control of plant parasitic nematodes by using fungal metabolites. J. Yunnan Agric. Univ., 14: 122-123.

Meyer S.L.F., Huettel R.N., Liu X.Z., Humber R.A., Juba J., Nitao J.K. (2004). Activity of fungal culture filtrates against soybean cyst nematode and root-knot nematode egg hatch and juvenile motility. Nematology, 6: 23-32.

Thern B., Rudolph J., Jung G. (2002). Total synthesis of the nematicidal cyclododecapeptide omphalotin A by using racemization-free triphosgene-mediated couplings in the solid phase. Angew. Chem. Int. Edit., 41: 2307-2309.

Tsao R., Yu R.Q., Friesen I., Potter J., Chiba M. (2000). Factors affecting the dissolution and degradation of oriental mustard-derived sinigrin and allyl isothiocyanate in aqueous media. Agric. Food Chem., 48: 1898-1902.

Wei J.Z., Hale K., Carta L., Platzer E. (2003). *Bacillus thuringiensis* crystal proteins that target nematodes. Proc. Natl. Acad. Sci., USA, 100: 2760-2765.