A Root-penetration Bioassay for the Screening of Nematode-Control Principles

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ABSTRACT

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In vitro test allows the direct observation of paralyzation and death of nematodes, which is good for studying the mode of action of the natural nematicides. However, it is not as ideal a screening method as the root-penetration bioassay because it is more strenuous than the latter and has the opportunity to miss some natural nematode- control principles which require bioactivation or have a different mode of action other than paralyzation and direct killing of nematodes. The bioassay results showed that natural nematode-control principles exist widely among spices and vegetables, and revealed a wealth of sources of phytochemicals for the future synthesis of relatively safe nematicides. Among the materials tested, wasabi, garlic, shallot, chili pepper, Chinese chive, radish, and green tomato fruit all resulted in 100% reduction of root penetration of *Meloidogyne javanica*. Cabbage, onion, green onion, ripe tomato fruit were also highly effective in preventing nematode infection. Bitter gourd was the least effective one, with only 55.5% reduction of root penetration. The nematicidal effect of garlic extract was reduced by 54% after boiling for 5 min, but that of shallot extract was not affected.

Key words: bioassay, garlic, natural nematode-control principles, spice plants, *Meloidogyne javanica*

INTRODUCTION

Plant-parasitic nematodes cause severe crop losses every year. The control of these serious pests still depends mainly on nematicides, in spite of the concern for environmental pollution and the pesticide residues in fruits and vegetables. The search for alternatives for nematode control has become an urgent matter. Soil amendments with green manures, crop residues, industrial by-products have been studied extensively [2,15]. The phytochemicals in green manures and crop residues opened a new area for synthesis of plant-based nematicides or so called botanical pesticides [3,9,15]. The existence of natural nematicides has been documented since more than four decades ago. Rohde and Jenkins [10] found that asparagus roots contained a certain glycoside which was toxic to *Trichodorus christie*. It was not only effective on direct contact with the nematodes but was also effective systemically in plants. Uhlenbroek and Bijloo [12,13] reported that marigold contained two nematicidal principles, terthienyl and bithienyl. This plant significantly reduced galling on tomato by *Meloidogyne incognita* in pot tests [15]. Calabar bean was found to contain an alkaloid which killed *Ditylenchus dipsaci* through systemic action [4]. The search for natural nematicides from locally available plants and other natural sources is one of the major efforts in my laboratory. The comparison of screening methods and the effect of some herbs, spices, and vegetables on *Meloidogyne javanica* are reported here.

MATERIALS AND METHODS

Preparation of nematodes

The root-knot nematode *M. javanica* was originally isolated from a taro patch in the experimental station of the National Taiwan University and cultured on mung bean (*Vigna radiata* (L.) Wikzek) seedlings in Seed-Pack Growth Pouch (Mega International of Minneapolis). Egg masses were dissected from roots and hatched on two layers of facial tissue on a small screen in a small Petri dish (5.4 cm in diam.) with a thin layer of distilled water. Fresh second-stage juveniles were collected every day and kept at 15℃. They were used within three days. The number of nematodes in 1 ml of the collection was counted under a dissecting microscope (SZH, Olympus) and the concentration of nematodes was then adjusted to 400 nematodes/ml by concentrating the suspension or adding tap water to the suspension. The actual volumn of inoculum was within the range of 1-1.5 ml with 400 nematodes for each root- penetration bioassay. The nematodes for *in vitro* test were hand-picked into each Petri dish to avoid dilution of the test liquid.

Plant materials for screening of nematode-control principles

Chinese parsley, basil, onion, and garlic were used in the initial *in vitro* test. They were chosen because of their pungent aroma. For the same reason other spices and herbs were included in the later tests.

All the materials were obtained from a local supermarket with the exception of pogostemon which was purchased from a Chinese drug store. Some vegetables were also tested to see if nematode-control principles existed widely among edible plants. Plant materials used for root-penetration bioassay included onion, green onion, shallot, Chinese chive, garlic, cabbage, chili pepper, carrot, wasabi (commercial paste), green and red tomato fruits, mint, bitter gourd, pogostemon, and petals of radish (see Table 2 for scientific names).

For *in vitro* tests, each plant material was weighed (approximately 120 g for each test) and cut into small pieces (approximately 1 cm long). Equal amount of distilled water was added. The mixture was then homogenized in a regular food blender (Philips) at high speed for 30 sec. The liquid was then filtered through four layers of cheesecloth and then filter paper (Whatman No. 1). The aliquat of 5 ml of the filtrate was pipeptted into a small Petri dish for the test.

For root-penetration bioassay, the plant materials were homogenized as described above but used directly in the test without filtration and the dilution factor varied among different materials. Most of the dilution factors were 1:2 (plant: distilled water) but some were 1:1 when the material was less effective and 1:12 or 1:15 when the material was more effective (see Table 2 for dilution factors). The aliquat of 30 ml of the diluted homogenate was used in the tests.

In vitro tests

Fifty juveniles of *M. javanica* were hand-picked and placed in each small Petri dish containing 5 ml of the filtrate prepared as described above. The number of paralyzed nematodes was counted under a dissecting microscope 24 h after the treatment. Nematodes which were lying motionless in the Petri dish were considered paralyzed. The paralyzed nematodes were then transferred to distilled water for reactivation. After 24 h, those which did not respond to the touch of a nematode pick under a dissecting microscope were considered dead. Nematodes similarly treated with distilled water were used as the control. There were four replicates in each experiment. The incubation temperature was 28℃.

Bioassay with adzuki bean seedlings

Five-day old adzuki bean (*Phaseolus angularis* Wright) seedlings were each transplanted into 200 g sterile sands in a plastic cup (7 cm diameter). Each seedling was inoculated with 400 second-stage juveniles of *M. javanica*. Thirty mililiter of the diluted plant homogenate was then poured into each cup. Distilled water was used in place of the plant homogenate in the control. Seedlings were kept at 28℃ in a growth chamber. Three days after treatment, seedlings were examined for phytotoxicity. Their roots were stained with acid-fuchsin [6] and the number of nematodes penetrated the roots were counted under a dissecting microscope (Wild, M3Z). The phytotoxicity was classified into three categories: (1) severe – root system rotted or severely stunted, shoot stunted; (2) light – fewer lateral roots or shorter root system; (3) none – root system and shoot looked normal comparing to the control.

Physical properties of the nematicidal principles in garlic and shallot extracts

The filtered garlic extract, 1:10 (w/v) dilution, was boiled for 5 min and cooled to room temperature. The nematicidal effect was tested in Petri dish as described above.

One batch of shallot extract, 1:4 (w/v) dilution, was boiled for 5 min. Another batch of the same dilution was kept frozen at -20℃ for 10 days. Their effect on the penetration of *M. javanica* juveniles was bioassayed with adzuki bean seedlings as described above. There were four replicates in each experiment.

RESULTS

Among the four spicy vegetables tested, garlic extract was most effective in paralyzing *M. javanica*, followed by extracts of Chinese parsley and onion (Table 1). Basil was least effective. Garlic extract also was most effective in causing the death of *M. javanica*, followed by Chinese parsley extract (Table 1). Extract from basil and onion were not very toxic to the nematodes tested.

Most of herbs, spices and vegetables tested, were very effective in reducing root infection of adzuki bean by *M. javanica*. Wasabi, garlic, shallot, Chinese chive, radish, chili pepper and green tomato all had 100% reduction of the infection of *M. javanica* juveniles on the adzuki bean roots (Table2). The infection was reduced by more than 95% with pogostemon, cabbage, onion , green onion, and ripe tomato. Carrot, mint and bitter gourd were less effective with 83.6%, 78.4%, and 55.5% reduction of infection, respectively. When diluted to 1: 12 (w/v), wasabi still maintained high level of effectiveness (99.9% reduction of infection) while chili pepper became relatively ineffective (3.5%reduction of infection).

Boiled garlic extract caused only 38% paralization and 45%death of nematodes (Table3). Boiling reduced the nematicidal activity of garlic extract by 54% and the

Table 1. Paralytic and lethal effects of extracts from some spicy vegetables on the second-stage juveniles of *Meloidogyne javanica*

Plant extract [1]	Nematodes Paralyzed (%) [2]	Nematodes Killed (%) [2]
Chinese parsley (*Coriandrum sativum*)	61.4	27.8
Basil (*Ocimum basilicum*)	9.7	10.3
Onion (*Allium cepa*)	28.9	6.2
Garlic (*Allium sativum*)	100.0	100.0
Water Control	0	0

1. Dilution factor – plant material : distilled water =1:1 (w/v)
2. Mean of four replicates. Nematodes lying motionless in the Petri dish were considered paralyzed, while those failing to respond to the touch of a nematode pick were considered dead.

paralization effect by 60%. On the contrary, shallot extract was heat stable (Table 4). Freezing had no effect on its activity either.

DISCUSSION

The *in vitro* test is good for studying paralyzation and lethal effect of the test materials. However, the *in vitro* test is more time consuming than the bioassay described because of the requirement of filtration of the plant extract and the touching of each nematode to determine its death. Some plant extracts remained cloudy after filtration and was difficult to observe the nematodes in the *in vitro* test. Moreover, the test may also miss some natural nematode-control principles which require bioactivation or have different mode of actions other than paralyzation and direct killing of nematodes. For example, the *in vitro* test of onion extract (Table 1) would rule it out as a good source for natural nematicide, yet the bioassay (Table 2) showed that it was very effective for nematode control by reducing 97% of root penetration. Birch *et al.* [(5)] also found that some plant compounds, such as DMDP, a polyhydroxy alkaloid isolated from tropical legume seeds and leaves, have low activity *in vitro* but high activity *in vivo* for nematode control. The bioassay method has an additional advantage of being able to determine the phytotoxicity effect of the test materials. Phytotoxicity is an important reference in the practicality of field application.

Since the plant materials showing nematode-control effect do not necessarily "kill" nematodes directly, it may be more appropriate to say that they contain natural nematode-control principles than natural nematicides. This study showed that natural nematode-control principles exist widely

Table 2. Bioassay on the effectiveness of herbs, spices, and vegetables on the penetration of *Meloidogyne javanica* [1]

Plant	Dilution factor (Plant: distilled water)	No. nematode penetrated [2]		Reduction in infection (%)	Phytotoxicity
		Treatment	Control		
Allium cepa (onion)	1:2	1.3	45	97.1	Light [3]
A. fistulosum (green onion)	1:2	3	93	96.8	Severe [4]
A. fistulosum var. *caespitosum* (shallot)	1:2	0	117	100	Severe
A. odorum (Chinese chive)	1:2	0	60	100	Light
A. sativum (garlic)	1:10	0	126	100	Severe
Brassica oleracea var. *capitata* (cabbage)	1:1	1.9	134	98.6	Light
Capsicum annuum (chili pepper)	1:3	0	71	100	Severe
	1:12	106	163	35.0	Light
Daucus carota var. *sativa* (carrot)	1:2	23	140	83.6	None
Wasabia japonica (wasabi)	1:3	0	198	100	Severe
	1:12	0.3	265	99.9	Severe
Lycopersicum esculentum (tomato) Large green fruit	1:1	0	187	100	Severe
Red mature fruit	1:1	4	91	95.6	Light
Mentha arvensis var. *vulgaris* (mint)	1:15	46	213	78.4	None
Momordica charantia (bitter gourd)	1:2	61	137	55.5	None
Pogostemon cablin (pogostemon)	1:2	0.3	120	99.8	None
Raphanus acanthiformis (radish) petal	1:1	0	152	100	Severe

1. Azuki bean seedlings grown in plastic cups with sterile sand were used as the bioassay hosts. Each plant was inoculated with about 400 second-stage juveniles of *M. javanica* and kept at 28℃ in a growth chamber for three days.
2. Mean of four replicates.
3. Fewer lateral roots or shorter root system.
4. Root system rotted or severely stunted, shoot stunted.

Table 3. Effect of heat treatment on the nematicidal property of garlic extract on *Meloidogyne javanica* second-stage juveniles [1]

Treatment	Nematode paralyzed (%) [2]	Nematode killed (%) [2]
Boiling for 5 min	38	45
Untreated extract	96	98
Reduction of effectiveness (%) [3]	**60**	**54**

1. 1:10 (w/v) dilution.
2. Mean of four replicates. Nematodes lying motionless in the Petri dish were considered paralyzed, while those which did not respond to the touch of a nematode pick were considered dead.
3. 100-(Boiling/Untreated)×100 (%)

Table 4. Effect of heat and freezing treatment on the control efficiency of shallot extract [1] on *Meloidogyne javanica* with adzuki bean root-penetration bioassay.

Treatment	No. nematode penetrated [2]	% reduction in penetration [2]
Boiling for 5 min	0	100
Frozen for 10 days	0.7	99.3
Untreated extract	0.9	99.1
Control (distilled water)	95	

1. 1: 4 (w/v) dilution.
2. Mean of four replicates.

among herbs, spices, and vegetables. This is a useful discovery in the search for safe nematicides. Phytochemicals have become a popular trend in the recent development of pesticides in view of their being safer than the conventional chemicals. However, not all plant products are safe. Poisonous plants do exist in the nature, such as oleander, derris, and poisonvetches, to name a few. The natural nematicides in the herbs, spices, and vegetables offer a safe alternative as they are all edible. Akhtar [(1)] reported that vegetable and fruit processing waste reduced root galling of tomato by *M. incognita* in pots with field soil. Contrary to the beneficial effect, Walker [(14)] discovered that low rates of Indian mustard residues increased the population of *Tylenchulus semipenetrans*. The requirement of high concentration of wasabi, shallot, Chinese chive, etc. may hinder their direct application in the field. However, their nematode-control principles are worth purifying and eventually synthesized in large mass for field use, since they were all 100% effective in controlling nematode (Table 2). Cabbage, onion, green onion, tomato and pogostemon are also good candidates for purification and synthesizing phytochemicals as they exerted 96-99% nematode control. Since the bioassay was conducted in sterile sands in the laboratory, the reduction of nematode penetration was most likely due to the effect of phytochemicals in the extracts and not the result of increasing natural nematode enemies as they did not exist in the sterile sands. Phytochemicals in those plant products may control nematodes by direct killing, preventing penetration by causing paralyzation, causing the lose of host finding ability, repulsion, or by some other unknown mechanisms. The low lethality and paralyzation effect yet high reduction of penetration of *M. javanica* by the onion extract (Table 1&2) indicated that the mechanism of nematode control involved more than just killing or paralization. Rishitin, a phytoalexin from potato tubers with anti- fungal and anti-bacterial activities, was found to have repellent activity on *Xiphinema diversicaudatum* [(5)]. Garlic extract had 100% paralyzation and lethal effect on *M. javanica* second-stage juveniles (Table 1) and 100% reduction of root penetration (Table 2) even at 1: 10 (w/v) dilution. This suggested that garlic cloves may contain very powerful nematicidal principles. These results collaborated with the reports of Sukul *et al.* [(11)] and Nath *et al.* [(8)]

Boiling garlic extract for 5 min reduced its nematicidal effect by 54%(Table 3), indicating that the nematicidal principle may be volatile or somehow heat unstable. Chatterjee *et al.* [(7)] reported that crude extract of *Ocimum sanctum* leaves killed 100%of *M. incognita* second-stage juveniles while the boiled extract only killed 10%of them. They concluded that the volatile oil eugenol was the prime candidate for the nematicidal principle in *O. sanctum*. Steam distilled oil fraction has also been reported to be responsible for the nematicidal activity of garlic. [(8)] This may explain the reduction of nematicidal activity of garlic extract by boiling.

Boiling did not change the effect of shallot extract on the reduction of root penetration by nematodes (Table 4). Obviously the nematicidal principle in shallot is different from that in garlic. It is possible that the herbs, spices, and vegetables which exhibited high effectiveness in these tests possess different type of phytochemicals for nematode control and hereby present a wealth of sources for the future synthesis of safe nematicides, although the direct application of the plant materials in the field is not practical due to the requirement of great bulk to reach effective concentration. Those with severe phytotoxicity can be used as pre-planting treatment and those with little or no phytotoxicity can be used as post-planting treatment.

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LITERATURE CITED

1. Akhtar, M. 1993. Utilization of plant-origin waste materials for the control of plant-parasitic nematodes. Bioresour. Technol. 46: 255-257.
2. Akhtar, M., and Alam, M.M. 1993. Utilization of waste

materials in nematode control: A review. Bioresour. Technol. 45: 1-7.

3. Akhtar, M., and Mahmood, I. 1994. Potentiality of phytochemicals in nematode control: A review. Bioresour. Technol. 48: 189-201.
4. Bijloo, J. D. 1965. The "pisum test": A simple method for the screening of substances on their therapeutic nematicidal activity. Nematologica 11: 643-644.
5. Birch, A. N. E., Robertson, W. M., and Fellows, L. E. 1993. Plant products to control plant parasitic nematodes. Pestic. Sci. 39: 141-145.
6. Byrd, D.W., J., Kirkpatrick, T., and Barker, K. R. 1983. An improved technique for clearing and staining plant tissue for detection of nematodes. J. Nematol. 15: 142-143.
7. Chatterjee, A., Sukul, N. C., Laskar, S., and Ghoshmajumdar, S. 1982. Nematicidal principles from two species of Lamiaceae. J. Nematol. 14: 118-120.
8. Nath, A., Sharma, N.K., Bhardwaj, S., and Thapa, C.D. 1982. Nematicidal properties of garlic. Nematologica 28: 253-255.
9. Prakash, A., and Rao, J. 1997. Section B: Botanical pesticides against nematodes. Pages 299-344 *in*: Botanical Pesticides in Agriculture. A. Prakash & J. Rao eds. CRC Press, Florida, 461 pp.
10. Rohde, R.A. and Jenkins, W.R. 1958. The chemical basis of resistance of asparagus to the nematode *Trichodorus christiei*. Phytopathology 48: 463.
11. Sukul, N. C., Das, P. K., and De, G. C. 1974. Nematicidal action of some edible crops. Nematologica 20: 187-191.
12. Uhlenbroek, J. H., and Bijloo, J. D. 1958. Investigation on nematicides. I. Isolation and structure of a nematicidal principle occurring in *Tagetes* roots. Rec. Trav. Chim. Pays-Bas Belg. 77: 1004-1008.
13. Uhlenbroek, J. H., and Bijloo, J. D. 1959. Investigation on nematicides. II. Structure of a second nematicidal principle isolated from *Tagetes* roots. Rec. Trav. Chim. Pays-Bas Belg. 78: 382-390.
14. Walker, G. E. 1997. Effects of *Brassica* residues and other organic amendments on abundance and sex ratio of *Tylenchulus semipenetrans* in soil. Aust. J. Exp. Agric. 37: 693-700.
15. Yen, J. H., Lin, C. Y., Chen, D.Y., Lee, M. D., and Tsay, T. T. 1998. The study of antagonistic plants in the control of south root-knot nematode, *Meloidogyne incognita*. Plant Pathol. Bull. 7: 94-104. (in Chinese)

摘 要

蔡碧雲. 2000. 應用根部侵入生物檢定法篩選天然線蟲防治劑. 植病會刊 9:131-136. (台北市 國立台灣大學 植物病理學系，電子郵件 bieyntm@ccms.ntu.edu.tw；傳真 02-23636490)

在培養皿中可直接觀察天然殺線蟲劑對線蟲之麻痺致死效果，有助於了解其作用機制，但此法易錯過一些需透過寄主才能發揮作用，或經由其他機制來防治線蟲之天然線蟲防治劑。根部侵入之生物檢定(bioassay)為較理想之篩選方法。利用此法測試之結果顯示，天然線蟲防治劑普遍存在於藥草、辛香料及蔬菜之中。芥末、蒜頭、紅蔥頭、辣椒、韭菜、蘿蔔葉柄及未熟番茄果實之抽出液皆對根瘤線蟲 *Meloidogyne javanica* 侵入寄主根系具完全之抑制效果。廣藿香、甘藍菜、洋蔥、青蔥、紅熟番茄果實之效果次之。效果最差者為苦瓜，抑制率僅 55.5%。煮沸五分鐘可將蒜頭抽出液之殺線蟲效果降低 54%，但對紅蔥頭抽出液則無影響。

關鍵詞：生物檢定、蒜頭、天然線蟲防治劑、辛香料植物、根瘤線蟲