Pakistan Journal of Biological Sciences 10 (17): 2910-2914, 2007
ISSN 1028-8880

Study of Sugar Beet Cyst Nematode Life Cycle Using Plant Tissue Culture Method

[1,2]Atena Shadmehr, [3]Payman Nouroozi, [1,2]Ghasemali Garousi and [4]Ali-Reza Ahmadi
[1]Department of Agricultural Biotechnology, Faculty of Engineering,
Imam Khomeini International University, Qazvin, Iran
[2]Department of Nematology,
Plant Protection National Research Institute, Tehran Iran
[3]Sugar Beet Seed Improvement Institute, Karaj, Iran
[4]Department of Biomedical Sciences, Alzahra University, Tehran, Iran

Abstract: After optimization of sterilizing of cyst and larva second stage of *Heterodera schachtii*, possibility of using nematode on seedlings of sugar beet (*Beta vulgaris* L.) in *in vitro* conditions were studied using sterilized larvae of beet cyst nematode. For this purpose, non sterile cysts were extracted from infected soil and hatched into zinc chloride solution with concentration of 0.5 g L^{-1}. Then, for preparation of sterile second stage larvae, several sterilizing treatments were used. Mean comparisons were performed between sterilized live larvae number by Duncan's method. Results showed that 70% ethanol for 1 min followed by 2.5% hypochlorite sodium for 5 minutes and 0.1% hypochlorite sodium for 20 min were best treatments for disinfecting cysts and larvae, respectively. In parallel, two nematode susceptible sugar beet varieties were applied to produce seedlings in *in vitro* culture. PG_{oB} medium containing different hormonal compositions was used for producing of hairy roots and inoculation of seedling with sterilized larvae. After nematode inoculation tests, daily observations were done by counting cysts and stained roots and larvae under stereomicroscope. Between 5-12 cysts formed on the roots of each seedling from two varieties 40 days after inoculation. As a result, it seems that this technique can be used for sugar beet germplasm evaluation to screen nematode resistant genotypes in *in vitro* controlled condition.

Key words: Cyst nematode, inoculation, sugar beet, *in vitro*, hairy roots

INTRODUCTION

The sugar beet cyst nematode, *Heterodera schachtii* schmith., is widespread in all main sugar beet growing areas worldwide and causes yield loss when population is large.

The cyst nematode is also widespread in most of the sugar beet growing areas of Iran specially in Khorasan, Western Azarbayjan, Esfahan, Kermanshah and Fars previanses (Behdad, 1998), where it can cause losses, over 10% of sugar beet yield (Whitney and Duffus, 1991).

This plant parasite nematode live inside the root which is called endoparasite. They invade the host roots as juvenile and then induce the formation of highly specialized feeding structures within the vascular cylinder that serve to satisfy the nutritional demands of the developing animals. The sugar beet cyst nematode feed from a syncytium, which is composed of numerous cells after fusion of protoplasts. The life cycle of the beet cyst nematode includes five stages, The first stage is completed inside the egg the first molt occurs soon after the embryo becomes elongated and wormlike. The second-stage larva is completely formed before it hatches from the egg. After penetrating the root of the host these larvae undergo three molts before reaching the adult stage. The development of the adult males requires about 20 days after which time they leave the roots. The female larvae develops after the third molts as a flask-shaped body with a rounded posterior end in which a gelatinous matrix forms into which egg are deposited. The brown cysts form in about 36 days and hatching begins a few days later. Two to three generation per year may result (Paul, 1990).

The culture of plant parasitic nematode on whole plants or excised roots grown on agar in sterile conditions has been used the variety of purposes (Asbach *et al*., 2004). This include studies on the feeding behavior of nematode, their effects on host metabolism

Corresponding Author: Atena Shadmehr, Department of Agricultural Biotechnology, Faculty of Engineering,
Imam Khomeini International University, Qazvin, Iran

(Jones, 1980) life cycles and population dynamics, nematode reproduction on transformed roots, nematode development (Lauritis *et al*., 1983), instructions among nematodes, plant and physical environment (Hashmi, *et al*., 1994; Sudirman and Webster, 1995), investigation with electromacroscopy (Orion *et al*., 1995) and observation of fungal parasitism of nematode egg in situ, additionally, nematodes from monoxenic culture have been used for screening nematode resistance in plant (Ferris and Williamson, 2004) and for looking at effect on resistance when compounds are added to the media (Huettel and Hammerschlag, 1986), for assays of nematode biocontrol agents (Meyer *et al*., 1990; Meyer and Heuttel, 1996; Meyer and Meyer, 1995; Verdego and Jaffee, 1988) and for cyst nematode production in root explants culture.

Media happen use for monoxinic culture of variety of cyst nematode by White (1943) and Tiner (1960) media low salt lauritis medium, (Lauritis *et al*., 1983; Hooper, 1986), Gamborg's B5 media (Gamborg *et al*., 1976) without cytokinins or auxins (Huettel, 1990) and Skoog, Tsui and White's media (Orion *et al*., 1995).

A major difficulty in monoxenic culture is the development of a sterilization method to kill contaminant bacteria and fungai while being harmless to cyst nematode. Various disinfectants an antibiotics were tested for the ability to eliminate contaminant organism by Verdejo (1988), Sijmons *et al*. (1991), Paul *et al*. (1990), Narayanan *et al*. (1999), Lauritis *et al*. (1983), Goverse *et al*. (2000), Cho *et al*. (2000), Nour *et al*. (2003) and Sobszak *et al*. (2005).

Objectives of this study were to optimize a new method for sterilization of cyst nematode larvae and to study life cycle of sugar beet cyst nematode by using plant tissue culture method.

MATERIALS AND METHODS

This study was carried out at the international University of Imam Khomeiny and Sugar Beet Seed Institute.

Cyst nematode extraction and Sterilization: Soil sampling was done in autumn of 2004. Soil and sugar beet roots from ten infested farms were sampled in west Azerbaijan (Oromiyeh), Iran. Soil samples (500 to 1000 g) and root material from each farm were fractionated with flowing water throw 850 and 200 μm-pore-size stacked sieves. Then 2 g kaoline powder was added to 100 mL of soil solution witch collected from extraction stage. Cyst were collected by into fallowing two stages. at first the sample was centrifuged for 10 min in 3700 rpm. Then the pellet was resuspended in 50 mL 63% sucrose and centrifuge for 1 min in 3500 rpm. The supernatant was transferred to 200 μL pore size stacked sieves. Using a low magnification stereomicroscope 10000 cyst were manually collected from all samples. Cyst were surface sterilized by immersion, for 5 min in 2.5% sodium hypocolorite, followed by seven rinse in sterile water. for determination of the best time of hatching of cyst they were incubated in 0.5 g L^{-1} of $ZnCl_2$ (Southey, 1970) at 25°C for 23 days (Grunlder *et al*., 1997). Cyst were incubated under dark condition after 5-7 days, second stage juveniles nematodes were collected dally for sterilization.

Surface sterilization method for *H. schachtii* juveniles: Juveniles which hatched from cyst were separated from debris as the migrated through the filter in to the water nematode suspension were collected and stored at 15°C until required, no longer than 2 days. Sterilization presidior for nematodes were conducted in a lamina flow cabinet and all apparatus, equipments and sterilands were autoclaved at 121°C and 10^3 kpa for 15 min. Healthy cyst were surface sterilized by submersion in 70% ethanol for 1 min, rinsed in autoclaved distill water, submerged in 0.1% sodium hypocolorite, with 0.05% of triton X-100 for 5 min and rinsed 3 times in sterile double distill water. Cyst were incubated at 25°C in dark condition on sterilized solution of $ZnCl_2$ (0.5 g L^{-1}) for 5-7 days, second stage juvenile nematode were collected. larvae (10^3 larvae per sample) were treated with 0.1, 0.2, 0.3, 0.4, 0.6 and 0.8% sodium hypocolorite, with 0.05% of triton X-100 for 20 min, at 25°C. Determination of larvae viability and cyst disinfection after treatment with various sodium hypocolorite was done using the sample of larvae and cyst for demonstration of bacterial and fungal carrying capacities. A sample from each hypoclorite sodium treatment containing 1000 larvae was added in to falcon tube 50 mL which contains potato dextrose broth media and also another sample was added into loria broth media and incubated on shaker incubator with 125 rpm, at 25°C for 3 day.

Hairy root induction (preparation): Seeds from sugar beet (*Beta vulgaris* L.) cultivars 191 (monogerm and sensitive to cyst nematode) and 7233 (multigerm and sensitive to cyst nematode), kindly provided by (sugar beet seed institute) were washed in soapy water, surface sterilized by submersion in 70% ethanol for 20 sec and then rinsed with trap water and incubated in table shaker for 3 h, after that seeds were submerged and shacked in 2% sodium

hypocolorite for 20 min. After rinsing 3 times with sterile double distill water, seeds were incubated in sterilized plates with filter paper at 25°C in growth chamber for 2 days. Germinated seeds were transferred to agar plates, containing PG_{oB} (De Greef and Jacobs, 1979) culture medium, supplemented with 3% sucrose, 9 g L^{-1} agar, 0.1 mg L^{-1} IBA and 0.1 mg L^{-1} NAA. Plant were grown at 25°C with 16 h light.

Hairy root inoculation: *In vitro* culture of 14 days old sugar beet hairy root plants were inoculated with sterile preparasitic j_2 of the sugar beet cyst nematode were use per Petri plates for inoculation hairy root culture. Plates were incubated at 25°C with 16 h of low lights, (500, lux).

Analysis of cyst nematode development: For analysis nematode development on beet roots they were excised and then were stained with fushin acid (0.035 g which is diluted in 7.5 mL /dw plus, 2.5 mL acetic acid (100%). Nematode development on the beet (both cultivars) was monitored *in vitro* by stereomicroscope (Zeiss, stemi s.v 11) normally, root infection by preparasitic j_2 result in the formation of a feeding cell. The parasitic j_2 subsequently develops into two other juvenile stages, j_3 and j_4. Four to six weeks after inoculation, adult males and females appear.

RESULTS AND DISCUSSION

Cyst nematode were successfully were extracted from soil and infected plant. Results of treatment solutions shows that treatment with 70% of ethanol for 1 min plus hypocolorite sodium 5% with 0.05% of triton x-100 for 5 min was the best treatment for surface sterilization of cyst nematode. There was significant different between the number of alive larvae and also producing of larvae free of contaminating fungi and bacteria (Table 1).

Results of incubation of cyst in 0.5 g L^{-1} $ZnCl_2$ solution were showed that the highest number hatching of cyst to form larvae was between 7-10 days after incubation (Fig. 1).

Treatment with high concentration (5%) of hypocolorite sodium were more effective than other treatments. Treatment with law concentration of hypocolorite sodium (2.5%) with out ethanol were unable to complete decontamination, of cyst.

Treatment of larvae with 0.1% hypocolorite sodium for 20 min were more effective foe surface sterilization of larvae. Results of treatment with law concentration of hypocolorite sodium and also treatment times of law than 20 min were more less effective. The viability of the second stage larvae of *H. schachtii* in different percentages of hypocolorite sodium are shown in Table 2.

H. schachtii larvae were shown to tolerate and survive in the 80% survive range in solution containing of to 0.1% hypocolorite sodium was 90% lethal for *H. schachtii* larvae at a 0.4% concentration (Table 2). Results of this study shown that all concentration of hypocolorite sodium was were useful for bacterial sterilization of the larvae.

The first sugar beet plants to be tested in this study for infection with *H. schachtii* were germinated in *in vitro* PG_{oB} plates which were incubated semi vertically, were produce more hairy roots. Attraction of the juvenile to the root tips could be observed but only a few invaded the root and established a feeding site. We set out to optimize the culture condition so that high infection rates could be established reproducibly.

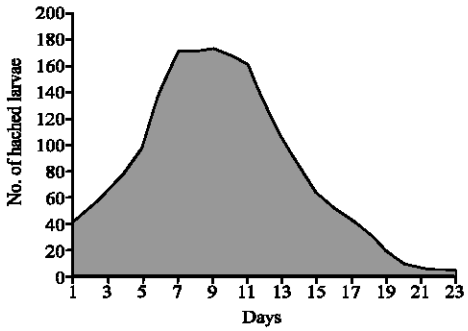

Fig. 1: Hatching period of cyst nematode on $ZnCl_2$

Table 1: Effect of sterilant solution on the number of cyst free of contaminant and the percentage of cyst forming larvae

	Five day after hatching				Six day after hatching				Eleven day after hatching			
Treatments	Live	Dead	Total	Live (%)	Live	Dead	Total	Live (%)	Live	Dead	Total	Live (%)
A	62	104	166	37	55	88	143	38	32	135	167	19
B	120	333	453	26	130	341	471	38	76	431	507	14
C	2	7	9	22	6	18	24	25	1	7	8	12
D	77	263	340	22	80	241	321	24	132	233	365	36
E	325	26	351	92	368	18	386	95	421	32	453	92

A: Sodium hypocolorite 5% plus triton 0.05% for 5 min; B: Sodium hypocolorite 5% plus ethanol 70%(1 min) plus triton 0.05% for 5 min; C: $HgCl_2$ 0.001% plus triton 0.05% for 5 min; D: Eanol 70% (1 min) plus sodium hypocolorite 5% plus triton 0.05% for 5 min; E: Non steril cysts

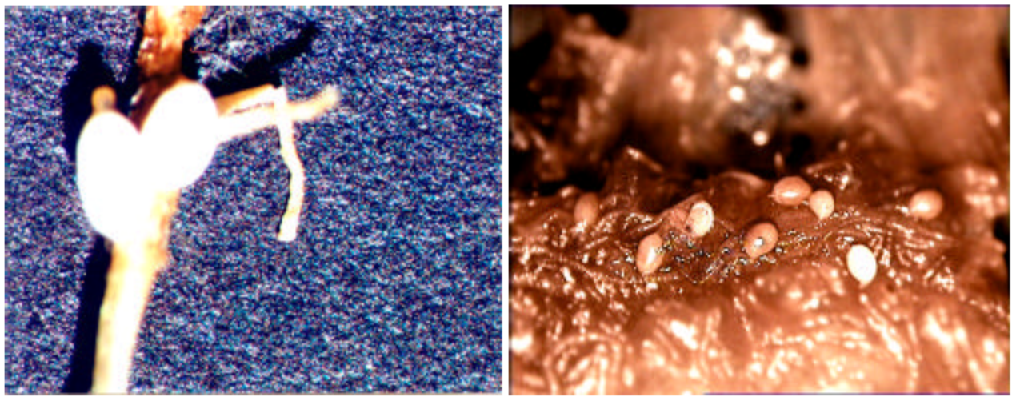

Fig. 2: Cyst formation of *Heterodera schachtii* on sugar beet roots on sterile culture

Table 2: Viability of the second stage larvae of *H. schachtii* in different percentages of hypocolorite sodium

Treatments	Live	Dead	Total	Live (%)
A1	200	54	254	79.0
A2	146	112	258	56.5
B1	94	182	276	34.0
B2	21	233	254	8.0
C1	32	210	242	13.0
C2	25	304	329	75.0
D1	10	341	351	3.0
D2	19	290	309	6.0

A: (0.1% hypocloroid sodium); B: (0.2% hypocloroid sodium); C: (0.3% hypocloroid sodium); D: (.4% hypocloroid sodium)

Different concentration of PG_{oB} media was tested for attraction of larvae to hairy roots. The medium composition was further optimized for pH, sucrose concentration and matrix strength. Quarter concentration of the PG_{oB} medium with 1% of sucrose and 0.9% g L^{-1} agar with media acidity of media adjusted to pH = 6.

The time of inoculation was routinely 7-10 days after germination. Earlear infection 2-4 days let to two much root damage and plant growth was reduced to such an extent that cyst development could not be maintained to maturation. Root infections of sugar beet were developed normally in tissue culture condition. Mature cyst filled with eggs developed on sugar beet in 4-6 weeks and could be used for subsequence. Infection indicating that the life cycle had been completed (Fig. 2).

In conclusion the simple sterilization technique for second stages larvae of cyst nematode as well developed in this study. Compression the results of larvae and cyst sterilization show that it is better to sterilized only second stage larvae because the disinfectant has effect on reduction rate of hatching cyst. Second stage of larvae of *H. schachtii* has been successfully sterilized by this simple method. In addition nematode developed on hairy root of sugar beet was successfully obtained. There were no differences in cyst between two sensitive sugar beet cultivars. The best conditions for development and reproduction of cyst nematode on sugar beet hairy root on agar containing (PG_{oB}) media were obtained following sterilization by simple method. Reproduction of the beet cyst nematode *H. schachtii* and of the root knot nematode *Meloidogyne javanica* on hairy roots have been reported by Paul *et al*. (1987) and Verdego *et al*. (1988), respectively.

ACKNOWLEDGMENTS

This research was performed at the sugar beet seeds Institute (Karaj) and I am incredibly thankful to Laboratory members for their help. I thank Dr. R. Amiri for assistance in the analysis of the data and Ms. P. Yavari gave valuable advice on the root culture technique. The author is grateful for Dr. B. Mahmoody, assistance.

REFERENCES

Asbach, K., D. Cai, C.H. Jung and I. Pauselius, 2004. Investigation on the interaction between beet cyst nematode and *Arabidopsis thaliana* carrying resistance from sugar beet. Plant Nematol., 2: 256-270.

Behdad, E., 1998. Pathogenesis Agent and Iranian Plant Important Disease. Yadbood Press. Esfahan, pp: 456.

Cho, H.J., S.K. Farrand, G.R. Noel and J.M. Widholm, 2000. High efficiency induction of soybean hairy roots and propagation of the soybean cyst nematode. Dep. Crop Sci., 210: 195-204.

De Greef, W. and M. Jacobs, 1979. *In vitro* culture of sugar beet: Description of a cell line with high regeneration capacity. Plant Sci. Lett., 17: 55-61.

Ferris, P.A.R. and V.M. Williamson, 2004. Host Plant Resistance (HPR) against nematodes. Biol. Approaches to Manage. California, 14: 21-30.

Gamborg, O.L., R.A. Miller and K. Ojima, 1976. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res., 50: 151-158.

Goverse, A., H. Over mars, J. Engelbertink, A. Scots, J. Baker and J. Helder, 2000. Both Induction and morphogenesis of cyst nematode feeding cells are mediated by Auxin. Molecular Plant-Microbe Interactions, 13: 1121-1129.

Grunlder, F.M.W., M. Sobczak and S. Iange, 1997. Defense responses of *Arabidopsis thaliana* during invasion and feeding site induction by the plant-parasitic nematode *Heterodera glycines*. Physiol. Mol. Plant Pathol., 50: 419-429.

Hashmi, G., R.N. Huettel, F.A. Hammerschlag and L.R. Krusberg, 1994. Optimal levels of *Meloidogine incognita* inoculums for infection of tomato and peach *in vitro*. J. Nematol., 26: 531-534.

Hooper, D.J., 1986. Extraction of Free-living Stages from Soil. In: Laboratory Methods for Work with Plant and Soil Nematodes. Southey, J.F. (Ed.), London: Ministry of Agriculture, Fisheries and Food, pp: 5-30.

Huettel, R.N. and FA. Hammerschlag, 1986. Influence of cytokinin on *in vitro* screening of peaches for resistance to nematodes. Plant Dis., 70: 141-144.

Huettel, R.N., 1990. Monoxenic Culturing of Plant Parasitic Nematodes, Using Carrot Disc, Callus Tissue and Root Explants. In: Plant Nematology Laboratory Manual. Zuckerman, B.M., W.I. Mai and M.B. Harrison (Eds.), London, pp: 273-276.

Jones, M.G.K., 1980. The Interaction of Plant Parasitic Nematodes with Excised Root and Tissue Cultures. In: Tissue Culture Methods for Plant Pathologists. Ingram, D.S. and J.P. Helgeson (Eds.), Blackwell Scientific Publications, Oxford, pp: 161-166.

Lauritis, J.A., R.V. Rebois and L.S. Graney, 1983. Development of *Heterodera glycines* ichinohe on soybean, *Glycine max* L. Merr., under genotobiotic conditions. J. Nematol., 15: 272-281.

Meyer, S.L.F., R.N. Huettel and R.M. Sayre, 1990. Isolation of fungi from *Heterodera glycines* and *in vitro* bioassays for their antagonism to eggs. J. Nematol., 22: 532-537.

Meyer, S.L.F. and R.J. Meyer, 1995. Effects of a mutant strain and a wild type strain of *Verticillium lecanii* on *Heterodera glycines* populations in the greenhouse. J. Nematol., 27: 409-417.

Meyer, S.L.F. and H. Robin, 1996. Application of a sex pheromone, pheromone analogs and *Verticillium lecanii* for management of *Heterodera glycines*. J. Nematol., 28: 36-42.

Narayanan, R.A., R. Atz, R. Denny, N.D. Young and D.A. Somers, 1999. Expression of soybean cyst nematode resistance in transgenic hairy roots of soybean. Cell Biol. Mol. Genet., 2: 54-62.

Nour, S.M., J.R. Lawrence, H. Zhu, G.D.W. Swerhone, M. Welsh, T.W. Welacky and E. Topp, 2003. Bacteria associated with cyst of the soybean cyst nematode (*Heterodera glycines*). Southern Crop Protection and Foot Research Center. Applied Environ. Microbiol. 69: 607-615.

Orion, D., W.P. Wergin, D.J. Chitwood and E.F. Erbe, 1995. Low temperature scaning microscope observation of the *Meloidogyne incognita* egg mass: The gelatinous matrix and embryo development. J. Nematol., 26: 402-411.

Paul, H., J.E.M. Deelen, B. Henken, S.M. Bock, W. Lang and F.A. Krens, 1990. Expression *in vitro* of resistance to *Heterodera schachtii* in hairy roots of an alien monotelosomic addition plant of *Beta vulgaris*, transformed by *Agrobacterium rhizogenes*. Euphytica, 48: 153-157.

Sijmons, P.C., F.M.W. Grundler, N. Mende, P.R. Burrous and U. Wyss, 1991. *Arabidopsis thaliana* as a new model host for plant parasitic nematodes. Plant J., 1: 245-254.

Sobczak, M., A. Avrova, J. Jupowicz and M. Phillips, 2005. Characterization of susceptibility and resistance responses to potato cyst nematode (*Globodera* spp.) Infection of tomato lines in the absense and presence of the broad spectrum nematode resistance hero gene. Mol. Plant Microbe Interac., 18: 301-308.

Southey, J.F., 1970. Laboratory methods for work with plant and soil nematodes. Ministry of Agriculture, Fisheries Food. London. pp: 72.

Sudirman and J.M. Webster, 1995. Effect of ammonium ions on egg hatching and second-stage juveniles of *Meloidogyne incognita* in axenic tomato root culture. J. Nematol., 27: 346-352.

Tiner, J.D., 1960. Cultures of plant parasitic nematode genuse *Pratylenchus* on sterile excised roots. I. Their establishment and maintenance. Exp. Parasitol., 9: 121-126.

Verdejo, S., B.A. Jaffee and R. Mankau, 1988. Reproduction of *Meloidogyne javanica* on plant roots genetically transformed by *Agrobacterium rhizogenes*. J. Nematol., 20: 599-604.

White, P.R., 1943. A Handbook of Plant Tissue Culture. Journal Cattel, Lancaster, Pa.

Whitney, E.D. and E. Duffus, 1991. Compendium of Beet Diseases and Insects. The American Phytopathological Society Press, USA.