EXCISED ROOT CULTURE FOR MASS PRODUCTION OF *HOPLOLAIMUS COLUMBUS* SHER (NEMATA: TYLENCHIDA)

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ABSTRACT

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Experiments with a monoxenic culture of *Hoplolaimus columbus* on excised roots were conducted to evaluate effects of temperature and initial population density (Pi) on final population numbers (Pf), evaluate host range, and compare virulence and host specificity to that in field populations. The nematodes fed and reproduced on excised root cultures, with average reproductive factors (Pf/Pi) of 254 on alfalfa and 121 on soybean after 90 days. Incubation at 30°C and an initial population of 10 nematodes per 9-cm petri dish were optimal for reproduction. Nematodes maintained in excised root culture for one year retained their virulence and host specificity in the greenhouse when compared to extracted field populations.

Key words: alfalfa, culture, excised root, *Hoplolaimus columbus*, host, lance nematode, *Medicago sativa*, reproductive factor, soybean, virulence.

RESUMEN

Supramana, S., S. A. Lewis, J. D. Mueller, B. A. Fortnum y R. E. Ballard. 2002. Cultivo de secciones de raíces para la producción masiva de *Hoplolaimus columbus* Sher (Nemata: Tylenchida). Nematrópica 32:5-11.

Experimentos de cultivos monoxenico de *Hoplolaimus columbus* utilizando secciones de raíces se establecieron para evaluar el rango de hospedera, efecto de la temperatura y densidad inicial de población sobre la densidad final, y comparar virulencia y especificidad de hospedera de los nematodos criados con poblaciones de campo. Los nematodos se alimentaron y reprodujeron en cultivos de secciones de raíces, presentando un factor reproductivo de 254 y 121 en alfalfa y soya, respectivamente, después de 90 días. Incubación a 30°C y poblaciones iniciales de 10 nematodos por placa Petri de 9 cm fueron óptimas para la reproducción del nematodo. Nematodos criados en cultivos asépticos de secciones de raíces por un año conservaron su virulencia y especifícidad de hopederas en el invernadero al compararlos con nematodos extraídos del campo.

Palabras claves: alfalfa, cultivo, nematodo lanceta, secciones de raíces, *Hoplolaimus columbus*, hospedera, *Medicago sativa*, factor reproductivo, soya, virulencia.

INTRODUCTION

The Columbia lance nematode, *Hoplolaimus columbus* Sher, is a serious pathogen of soybean (*Glycine max* (L.) Merr.) and cotton (*Gossypium hirsutum* L.) in the Coastal Plains of South Carolina, North Carolina, and Georgia (Fassuliotis *et al.*, 1968; Motsinger *et al.*, 1974; Lewis and Smith, 1976). In addition, more than fifteen agronomic crops and weeds are known hosts of the species (Fassuliotis, 1974; Högger and Bird, 1974; Lewis and Smith, 1976). In previous studies the nematodes grew poorly in monoxenic and greenhouse cultures, making the inability to produce large quantities of inoculum a major impediment to conducting research.

Monoxenic culture is an excellent means for mass production of certain plant-parasitic nematodes (Griffin, 1968; Faulkner *et al.*, 1974; Bingefors and Bingefors, 1976; Jones, 1985). Our objective was to investigate the potential of excised root cultures for producing large quantities of *H. columbus* for inoculum. Success in culturing will lead to more precise and effective experiments to assess host status of crop species, cultivars, and genotypes to *H. columbus*. Moreover, nematodes from sterile culture are less likely to be contaminated by other nematode species and microorganisms that may result in problems determining causal agents in plant disease.

MATERIALS AND METHODS

Excised root culture: Alfalfa (*Medicago sativa* L. 'Wrangler') seeds were sterilized in concentrated sulfuric acid for 20 min and washed three times in sterilized distilled water. The seeds then were germinated in Petri plates containing a solidified 1% water agar. Three root tips (30 mm) were excised and transferred onto solidified Gamborg's B5 medium, pH 5.7-6.0 (Gamborg *et al.*, 1976) in 1.3% agar on a 9-cm plastic Petri dish. Fresh nematodes extracted by centrifugal flotation were sterilized by soaking them in 0.5% streptomycin sulfate and 0.5% chlorhexidine diacetate (Hibitane), each for 5 min, followed by washing three times with sterilized distilled water. The disinfested nematodes (25/petri dish) then were transferred onto 7-day-old excised root cultures. Petri dishes were double sealed with Parafilm strips and kept inverted in a 30°C incubator (Riedel *et al.*, 1988). This served as the nematode stock culture, which was used as the inoculum source for all experiments in the laboratory and greenhouse. For the experiments on the effect of initial population on final population, initial populations of 10, 20 and 30 eggs, juveniles and adults of *H. columbus* were inoculated on 7-day-old excised roots. After double sealing with Parafilm strips, the cultures were kept inverted in a 30°C incubator in a completely randomized design with six replications. Final populations were recovered, using a mist chamber, 90 days after inoculation. The second series of experiments were conducted using 'Hutcheson' soybean excised root cultures. Initial populations of 10, 20, 30, 40, and 50 eggs, juveniles, and adults of *H. columbus* were inoculated onto petri dishes. The experiments were replicated five times and terminated 90 days after inoculation. Nematodes were recovered from root cultures by incubation in a mist chamber.

Host suitability: 'Wrangler' alfalfa, 'Jackson' lima bean (*Phaseolus lunatus* L.), 'Hutcheson' soybean, and 'Rutgers' tomato (*Lycopersicon esculentum* L.) were selected for the host suitability experiment. Alfalfa excised root cultures were prepared as described previously. Other seeds were sterilized in 95% ethanol followed by 25% commercial Clorox (1.3% sodium hypochlorite) each for 10 min followed by two rinses in sterile distilled water. Seeds then were germinated in petri dishes containing solidified 1% water agar. One root tip was transferred to Gamborg's B5 medium to established soybean and lima bean cultures and three root tips were transferred for tomato. A 2-mm cube of agar with approximately 25 nematodes from an *H. columbus* stock culture containing eggs, juveniles, and adults on excised 'Hutcheson' soybean roots was transferred aseptically onto 7-day-old cultures. Petri dishes were double-sealed with Parafilm strips and placed inverted in a 30°C incubator. The experiment was arranged in a completely randomized design with 10 replications per plant species. Ninety days after inoculation, all of the agar and plant tissue from the cultures were removed from the petri dishes and placed in a mist chamber for 7 days to extract the nematodes and determine the reproductive factor (Rf: final population/initial population, or Pf/Pi) for each plant species.

Virulence: Reproduction of *H. columbus* generated from 1-year-old alfalfa excised root culture was compared with that of *H. columbus* extracted directly from soil by centrifugal flotation (Jenkins, 1962). 'Hutcheson' soybean, a host of *H. columbus*, served as the host and was grown in 1-liter plastic pots filled with pasteurized gravel and river sand media (1:3, v/v). Pots were maintained in a Wisconsin water tank at 30°C. The pots were infested with nematodes at three initial population levels: 100, 200, and 300 juveniles and adults, 7 days after seeding. Controls consisted of soybean planted in pots and treated identically, but without nematodes. The experiment was a 3 by 2 factorial design (three inoculum levels and two nematode sources) with three replications and was terminated 90 days after inoculation. Centrifugal flotation and a mist chamber were used to extract *H. columbus* from sand and roots, respectively. The numbers of nematodes extracted by these procedures were combined to get a total number of nematodes per pot.

Host specificity: Two experiments were conducted using *H. columbus* extracted directly from soil samples and from 1-year-old alfalfa excised root cultures. Pots containing selected crop cultivars were infested with 100 or 300 nematodes per one-liter pot 7 days after seeding. The nematodes were added to pots containing 'Wrangler' alfalfa, 'Jackson' lima bean, common bermudagrass (*Cynodon dactylon* (L.) Pers.), 'Iowa Chief' and 'Pioneer 3163' corn (*Zea mays* L.), 'Delta Pine 90' cotton, 'Hutcheson' soybean, 'Pioneer × S 530' sorghum (*Sorghum vulgare* L.), sudangrass (*Sorghum drummondii* (Nees ex Steudel) Millsp. & Chase), 'Rutgers' tomato, and 'Coker 9835' wheat (*Triticum aestivum* L.). Crops were grown in 1-liter plastic pots containing pasteurized gravel and river sand media (1:3, v/v) and maintained in Wisconsin water tanks at 30°C. The experiments were arranged in a completely randomized design with six replications and were terminated after 90 days. Nematodes were recovered as described previously. Data were analyzed with analysis of variance (ANOVA), and differences in means ($P = 0.05$) were separated by Least Significant Difference (LSD).

RESULTS

Excised Root Culture: H. columbus fed and reproduced well on alfalfa excised root culture. Females laid eggs readily without the presence of males, and males were not observed at any time. An initial population of 10 nematodes/petri dish resulted in a significantly greater Rf (data not shown) than an initial population of 20 or 30 nematodes (LSD = 0.05). In succeeding experiments with a broader range of Pi, the Rf for 10 nematodes/dish was greater than for other initial populations, though not significantly. The nematodes generally fed ectoparasitically on the cortical cells and occasionally endoparasitically, initiating necrosis of the affected cells (Fig. 1A).

Host suitability: The nematodes fed and reproduced in all selected hosts. Necrosis of the cortical cells occurred on soybean (Fig. 1B) and other hosts. The reproductive factor was significantly greater on

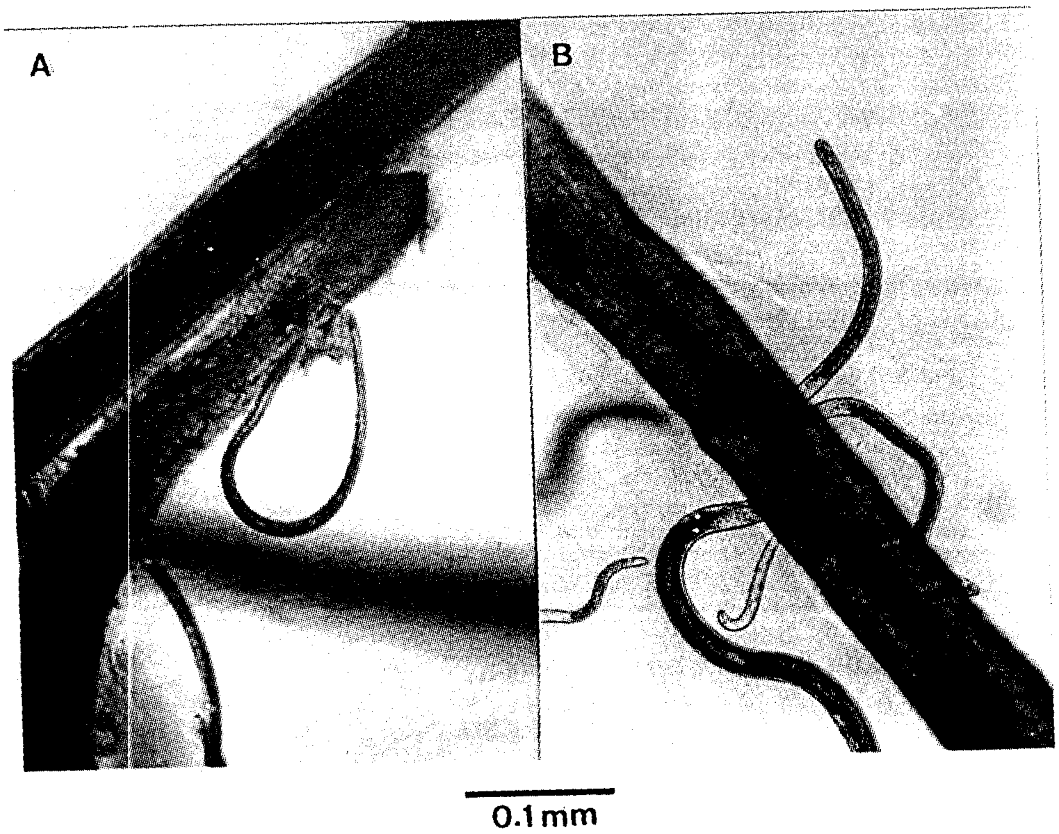

Fig. 1. *Hoplolaimus columbus* fed on the subepidermal cells causing necrosis of the cortical cells of alfalfa (A) and soybean (B).

alfalfa, followed in order by soybean and lima bean where the Rfs were approximately one-half that on alfalfa, and tomato where the Rf was approximately one-tenth that on alfalfa. The average reproductive factor of 254 on alfalfa was obtained 90 days after inoculation (Table 1).

Virulence: Hoplolaimus columbus maintained on sterile alfalfa culture for one year (four subculturings) retained its virulence on greenhouse grown soybean. Average nematode numbers were higher in pots infested with nematodes generated from monoxenic culture than with those extracted directly from soil samples when Pi = 300 (Table 2). However, no difference occurred at Pi = 100 or 200. Comparison of three Pi's within the same source of nematode inoculum indicated little difference among the three population levels with soil derived inoculum. With nematodes derived from sterile culture (MI), final populations increased with increasing Pi.

Host specificity: Hoplolaimus columbus maintained in alfalfa culture for one year (four sub-culturings) generally retained host specificity on the nine crop cultivars tested in the greenhouse. Comparable results were obtained in experiments in 1997 and 1998, indicating that monoxenically cultured *H. columbus* maintained its host preferences (Table 3).

Table 1. Reproductive factors (Rf) of *Hoplolaimus columbus* Sher on alfalfa, lima bean, soybean, and tomato excised root cultures 90 days after inoculation with a mixture of 25 eggs, juveniles and adults.

Common name	Scientific name	Cultivar	Rf
Alfalfa	*Medicago sativa*	Wrangler	254.13 a
Lima bean	*Phaseolus lunatus*	Jackson	112.65 b
Soybean	*Glycine max*	Hutcheson	121.35 b
Tomato	*Lycopersicon esculentum*	Rutgers	27.20 c

Data are means of ten replications. Means within a column followed by a common letter are not different (LSD = 0.05).

DISCUSSION

Hoplolaimus columbus reproduced on excised root culture of all hosts evaluated at 30°C at different initial population densities. The average reproductive factors of 254 and 121 obtained on alfalfa and soybean, respectively, were more than 100-fold higher than in a previous report of *H. columbus* culture (Riedel *et al.*, 1988).

Inoculation of *H. columbus* onto host plants after maintenance in excised root culture for one year revealed that the nematodes maintained their ability to reproduce on those plants. Although significantly greater nematode recovery was obtained by using nematode inoculum at higher levels from excised roots than directly from soil, further investigations are needed to determine the duration of the nematode's reproductive potential under monoxenic culture. In the interim, the nematode should be maintained on soybean roots, for example, if the nematodes will serve as inoculum for experiments on soybean.

Hoplolaimus columbus from 1-year old culture also retained its host specificity to nine selected crops in the greenhouse. Comparable reproductive rates were dem-

Table 2. Final population (Pf) of *Hoplolaimus columbus* Sher from 'Hutcheson' soybean using inoculum from alfalfa excised root culture (CI) and from soil (SI) at initial populations of 100, 200, and 300 nematodes/liter 90 days after inoculation.

	H. columbus 90 days after inoculation (Pf)		
Initial population (Pi)	CI	SI	Mean
100	2,666.67 a, x	5,360.00 a, x	4,013.33 a
200	6,586.67 a, x	5,466.67 a, x	6,026.67 ab
300	10,480.00 b, x	6,453.33 a, y	8,466.67 b
Mean	6,577.78 a	5,760.00 a	

Data are means of three replications. Means within columns (a, b) and rows (x, y) followed by the same letter are not different (LSD = 0.05).

Table 3. Reproductive factors (Pf/Pi) of *Hoplolaimus columbus* Sher from selected crop cultivars with inoculum from soil (SI) and alfalfa excised root culture (CI) in the greenhouse 90 days after inoculation.

Common name	Scientific name	Cultivar	*H. columbus*[y] Rf	
			SI	CI
Alfalfa	*Medicago sativa*	Wrangler	—	4.7 cz
Bermuda-grass	*Cynodon dactylon*	—	5.4 b	4.8 c
Corn	*Zea mays*	Iowa Chief	13.3 ab	—
		Pioneer 3163	—	3.8 cd
Cotton	*Gossypium hirsutum*	Delta Pine 90	5.6 b	3.3 cd
Lima bean	*Phaseolus lunatus*	Jackson	5.0 ab	10.6 b
Sorghum	*Sorghum bicolor*	Pioneer XS 530	19.4 a	12.2 ab
Soybean	*Glycine max*	Hutcheson	14.6 ab	15.5 a
Tomato	*Lycopersicon esculentum*	Rutgers	0.04 c	0.2 d
Wheat	*Triticum aestivum*	Coker 9835	4.8 ab	4.3 c

[z]Data are means of six replications. Means within a column followed by a common letter are not different (LSD = 0.05).
[y]Pi = 300 *H. columbus*/liter from naturally-infested field soil (SI) and 100 *H. columbus*/liter soil from one-year old alfalfa culture (CI). Broken line (—) indicates plant-nematode not tested.

onstrated in pots infested with cultured nematodes and from soil samples from the original collection site. The highest and the lowest nematode recoveries on soybean and tomato, respectively, were consistent with previous studies on the host range of *H. columbus* (Fassuliotis, 1974).

Monoxenic culture has been the major source of inoculum of *Ditylenchus dipsaci* for alfalfa resistance screening programs. The nematode retained its host specificity and virulence after it had been maintained in monoxenic culture for over 10 years (Faulkner *et al.*, 1974; Bingefors and Bingefors, 1976). With the techniques that we have developed for successful mass culturing of *H. columbus*, screening of cotton and soybean germplasm and breeder lines can be accomplished. This approach is possible because of the numbers of nematodes that can be reared, and because the nematodes retain their ability to reproduce on their field hosts. Monoxenic culture also has the distinct advantage of providing a known nematode isolate with no contaminating nematode, fungal or bacterial species. Resistance or tolerance screening programs for soybean are in place for other nematodes and insects, and the addition of *H. columbus* in these programs should be beneficial as new cultivars are developed. However, for tolerance assays additional research is needed to determine applicability of excised root response to whole plant response in a field situation.

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