A Technique for Quantitative Use of Nematodes from Monoxenic Tissue Culture as Soil Inoculum

M. J. MARTIN, Research Associate, Department of Plant Pathology, Oklahoma State University, Stillwater 74078; R. M. RIEDEL, Professor, Department of Plant Pathology, Ohio State University, Columbus 43210; and R. C. ROWE, Associate Professor, Department of Plant Pathology, Ohio Agricultural Research and Development Center, Wooster 44691

ABSTRACT

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A technique for using monoxenic cultures of *Pratylenchus penetrans, P. scribneri*, and *P. crenatus* directly as soil inoculum is discussed. The technique has been used successfully to infest both organic and mineral soils. The technique can be used to infest large quantities of soil for use in field microplots. Practical applications, advantages, and disadvantages of the technique are discussed.

Plant-parasitic nematodes can easily be reared in sufficient quantities in monoxenic tissue culture to permit their use in field studies. The chief problems surrounding their use in the field stem from difficulties in 1) extracting nematodes from culture and adding them to soil in a viable condition, 2) mixing them evenly throughout large volumes of soil, and 3) reproducing similar population densities at different field sites and in different test years. These problems can be minimized, however, by not extracting nematodes from cultures but instead by adding cultures directly to the soil and standardizing culture procedures.

For 5 yr, we have been using *Pratylenchus* spp. from tissue cultures in potato microplot studies (3). In these studies, we have successfully incorporated *Pratylenchus penetrans* (Cobb) Filipjev & Schuur. Stekh., *P. crenatus* Loof, and *P. scribneri* Steiner in Rifle peat, Kibbie fine sandy loam, and Wooster silt loam. With modifications, the techniques described here should be adaptable to any crop/soil combination by using any of the species of plant-parasitic nematodes that have been reared in monoxenic culture.

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MATERIALS AND METHODS

Pratylenchus species were cultured in (25 × 150 mm) culture tubes at 20 C for 3 mo on Ranger alfalfa callus (5). Nematode inoculum was prepared by emptying the agar and callus tissue contents of 16 tubes into a one-speed Waring Blendor. Each tube was then rinsed with 1 ml of tap water to ensure complete collection of nematode aggregations from tube walls. To blend the tube contents, the blender switch was flipped once on and off as rapidly as possible. The contents were stirred by hand and blended once more by flipping the blender switch. This resulted in a semihomogenous mixture of callus and media and 0.5- to 1-cm segments of uncallused alfalfa seedlings. Complete homogenization would destroy too many of the nematodes.

Blended contents of 64 culture tubes were added directly to 10 L of 57% methyl bromide- and 43% chloropicrin-fumigated

soil and mixed well by hand, resulting in 10 L of highly concentrated nematode-infested soil. Soil moisture should be slightly less than potting consistency (about 50% of field capacity) before adding the nematodes. Soil that is too wet will become sticky and difficult to use, whereas soil that is too dry will result in decreased nematode survival. Concentrated nematode-infested soil was held in plastic bags for 12–24 hr at about 25 C to permit redistribution of nematodes from callus bits into soil.

After incubation, measured volumes of concentrated nematode-infested soil were added to 20-L batches of fumigated soil in metal bushel baskets to generate low, medium, and high treatments. Each 20-L batch was first mixed by hand, then mixed further for 10 revolutions in a 28-L-capacity twin-shell blender (without beater bars) to ensure uniform dispersal of inoculum. After mixing, two 100-cm^3 samples were taken, from which actual viable populations in the mixed soil were determined by 24-hr incubation in water in modified Baermann funnels. The mixed soil was covered with plastic, stored overnight, and placed in microplots the following day.

Table 1. Initial populations of *Pratylenchus penetrans* in three soil types infested with callus culture inoculum

Treatments[x]	Number of nematodes per 100 cm^3 final mixed soil		
	Rifle peat	Kibbie fine sandy loam	Wooster silt loam
1979			
High	260 a[y,z]	223 a	185 a
Medium high	118 b	120 b	45 b
Medium	75 bc	61 bc	30 b
Low	42 c	12 c	10 b
1980			
High	146 a	140 a	106 a
Medium	51 b	39 b	30 b
Low	16 c	14 c	9 b
1981			
High	361 a	257 a	150 a
Medium	137 b	93 b	24 b
Low	47 c	17 c	8 c
1982			
High	229 a	209 a	245 a
Medium	92 b	78 b	84 b
Low	25 c	30 c	38 c

[x] In 1979, high, medium high, medium, and low treatments were generated by adding 700, 350, 165, and 80 cm^3, respectively, of concentrated nematode-infested soil to 20-L batches of uninfested soil. In 1980, 1981, and 1982, high, medium, and low treatments were generated by adding 700, 300, and 100 cm^3, respectively, of concentrated nematode-infested soil to 20-L batches of uninfested soil.

[y] Values in 1979 are the means of eight replicate 20-L soil batches; values in 1980 are the means of 45 replicates; values in 1981 and 1982 are the means of 24 replicates.

[z] Within a test year, values in one soil type followed by the same letters are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

Table 2. Initial populations of *Pratylenchus scribneri* and *P. crenatus* in Rifle peat and Kibbie fine sandy loam infested with callus culture inoculum

Treatments[w]	Number of nematodes per 100 cm^3 mixed soil			
	P. crenatus		*P. scribneri*	
	RP[x]	KFSL	RP	KFSL
1980				
High	···	195 a[y,z]	···	···
Medium	···	79 b	···	···
Low	···	28 c	···	···
1981				
High	119 a	118 a	282 a	124 a
Medium	66 b	56 b	141 b	54 b
Low	24 b	11 c	54 c	13 c
1982				
High	65 a	···	124 a	···
Medium	27 b	···	42 b	···
Low	9 c	···	22 b	···

[w] High, medium, and low treatments were generated by adding 700, 300, and 100 cm^3, respectively, of concentrated nematode-infested soil to 20-L batches of uninfested soil.

[x] RP = Rifle peat, and KFSL = Kibbie fine sandy loam.

[y] Values in 1980 are the means of 15 replicate soil batches; values in 1981 are the means of eight replicates, and values in 1982 are the means of 24 replicates.

[z] Within the same test year, species, and soil type, values followed by the same letters are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

In summary, the steps in this technique are: 1) shake agar and alfalfa callus contents of 16 3-mo-old monoxenic culture tubes of *Pratylenchus* into a Waring Blendor; 2) wash each tube with 1 ml of tap water to collect all nematodes; 3) to mix, flip blender switch on and off quickly; 4) mix briefly by hand and repeat step 3; 5) empty contents into beaker; 6) repeat steps 1–5 three times; 7) mix blended contents of 64 culture tubes with 10 L of fumigated organic soil to make 10 L of concentrated nematode-infested soil; 8) incubate 12–24 hr at about 25 C in a plastic bag; 9) add volumes of concentrated nematode-infested soil to 20-L batches of fumigated soil in metal bushel baskets (we added 100, 300, and 700 cm^3 to establish field populations ranging from about 15 to 350 *Pratylenchus* per 100 cm^3 of soil); 10) mix well by hand, followed by 10 turns in a twin-shell soil blender; 11) two 100-cm^3 samples are taken immediately after mixing each 20-L batch; initial nematode populations in mixed soil are determined by a 24-hr extraction in modified Baermann funnels in water; and 12) infested soil may be used immediately or be covered and stored 12–36 hr in a cool place.

Initial populations in the mixed soil may be varied by altering the volume of concentrated nematode-infested soil added to the 20-L soil batches, by varying the number of alfalfa seedlings per culture tube, or by lowering the number of cultures added to the 10 L of soil for making the concentrated nematode-infested soil. Sixty-four cultures are the maximum that can be added without the soil getting sticky.

RESULTS AND DISCUSSION

This technique of soil infestation with *P. penetrans* has been used successfully in field microplot studies in three soil types during four seasons (Table 1). The results of using *P. scribneri* and *P. crenatus* to infest Rifle peat and Kibbie fine sandy loam during three seasons are given in Table 2.

Using callus inoculum of *P. penetrans* directly generally gave similar population densities between soil types within a test year (1979–1981) (Table 1); however, populations always tended to be lower in the silt loam. We originally attributed lower populations to greater nematode attrition in the heavier silt loam soil during mixing in the twin-shell blender, but we found recently that lower populations resulted from preparing concentrated nematode-infested soil from the same type of soil as it would be used to inoculate. For example, concentrated nematode-infested organic soil (Rifle peat) was used to infest the 20-L batches of Rifle peat and concentrated nematode-infested silt loam soil was used to infest the 20-L batches of Wooster silt loam. In the case of the silt loam, this resulted in a very sticky concentrated

nematode-infested soil, poor nematode survival, and low population densities. Further experimentation demonstrated that using concentrated nematode-infested soil prepared from organic soil resulted in high population densities in silt loam. In this experiment, which was repeated once with similar results, the population density in silt loam infested with concentrated nematode-infested organic soil was 245 *P. penetrans* per 100 cm^3 soil, which was not significantly different from the population density (249/100 cm^3) in Rifle peat infested with the same inoculum soil. The population density in silt loam infested with concentrated nematode-infested silt loam soil was significantly lower at 80 *P. penetrans* per 100 cm^3 soil.

Concentrated nematode-infested organic soil was used as inoculum for all three soil types in 1982. Results of this modification are in Table 1. The population densities in the three soil types are very similar. In the future, we will use only Rifle peat organic soil (90% organic matter) to make concentrated nematode-infested soil.

Successful use of callus cultures of nematodes for field use requires standardization at each step in the culture process. For example, population densities of *P. penetrans* in these tests (Table 1) varied from year to year with the number of alfalfa seedlings used per culture tube. Culturing in 1979 was not standardized; anywhere from six to 12 seedlings per tube were used. In 1980, however, eight to 10 seedlings were used, and in 1981 and 1982, an average of 12 and 10 seedlings, respectively, were used. Populations varied accordingly.

There are advantages and disadvantages to the use of nematodes from tissue culture in field studies. Proper maintenance and culture of nematodes in sterile plant tissue require considerable time spent in repetitive technical work with careful attention to detail. For example, the midwinter subculture that generates 1,500 culture tubes for the season's microplots requires one full-time person for 5 wk. On the other hand, the alternative method of generating sufficient greenhouse pot cultures requires extensive greenhouse space, labor support for greenhouse maintenance, and a large energy input in heat and lighting. Using our technique, a single 0.38-m^3 incubator containing 1,500 culture tubes (25 × 150 mm) can provide enough inoculum to infest 1,500 cylindrical tile microplots (30 × 30 cm) with field levels of *Pratylenchus* spp.

Nematodes cultured under standardized conditions can be relied on to provide the same quantities of nematodes every year, whereas nematode reproduction in pot cultures may be erratic (1). Greenhouse pot cultures are also difficult to keep free of general fungal and bacterial contamination as well as nematode cross-contamination—problems that are readily avoided with tissue culture.

Perhaps the most serious hindrance to the widespread use of nematodes from tissue culture is that to date only 46 plant-parasitic species have been monoxenically cultured (5). For the migratory parasites, a representative species of many genera has been successfully cultured, and it is likely that other species of the same genera can be easily cultured with a few modifications of host and culture conditions. The sedentary endoparasites, however, represent the biggest problem. Although some can be cultured successfully in excised root culture (1,2,4), such cultures can require 2-wk subcultures for maintenance. It is quite possible, however, given recent advances in tissue culture, that further research will result in a successful easy to maintain tissue culture for species of *Meloidogyne, Heterodera*, and similar sedentary endoparasitic nematodes.

The greatest advantage of using nematodes from tissue culture in field studies is increased experimental control. Use of nematodes from standardized sterile cultures generates statistically well-defined initial population densities of a single nematode species. Effects on growth and yield of the host plant can then be directly attributed to a well-defined population density of one particular species. Nematode reproduction can be measured during the growing season and/or at the end. Once the effects and reproduction of single species on a host are known, it is easy to make the test more realistic by adding known numbers of more than one tissue-cultured nematode species, as well as other components. Use of tissue-cultured nematodes can increase precision in studies involving nematode reproduction, interspecific competition, rotation effects on nematode populations, and crop loss assessment.

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