Advanced Journal of Microbiology Research Vol. 2012
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

An improved method for the extraction of nematodes using iodixanol (OptiPrep ™)

Dewang Deng[1], Allan Zipf[1], Y. Tilahun[1], G.C.Sharma[1], J. Jenkins[2] and K. Lawrence[3]

[1]Department of Natural Resources and Environmental Science, Alabama A&M University, Normal, AL.
[2]USDA-ARS-CSRL, Mississippi State, MS
[3]Entomology and Plant Pathology, Auburn University, Auburn, AL

Accepted 16 April, 2012

A new nematode extraction technique was established, which is based on an iso-osmotic density- gradient medium (OptiPrep™). This technique resulted in significantly higher numbers of clean eggs and vermiform nematodes that retain higher viability (48.6%) than samples processed with the sucrose method (28.7%). Nematodes survived exposure to OptiPrep™ for 22 hours without significant mortality whereas all nematodes died in the sucrose medium. OptiPrepTM provided a suitable, non-toxic alternative to the traditional density gradient material for the isolation of nematodes. This technique is convenient and relatively simple, with the added benefit of yielding cleaner samples compared to traditional isolation techniques.

Key words: nematode extraction, density gradient, nematology.

INTRODUCTION

A rapid, reliable and highly efficient method for the extraction of viable nematodes from soil is of particular importance not only for field diagnostics but also laboratory research. Nematodes are most frequently extracted using modifications of the Baermann funnel (Walker and Wilson, 1960; Thorne, 1961; Hooper, 1990), live bait (Fan and Hominick, 1991) or centrifugal flotation techniques (Byrd et al., 1966; Saunders and All, 1982). The mechanical separation of nematodes from soil by the Baermann funnel is on nematode motility, while the live bait method relies on infectivity and the flotation relies on the physical properties of the juvenile. The Baermann funnel method could only recover live nematodes; however, it takes longer. Sucrose solutions as density-gradient media for centrifugal flotation technique have proven to be valuable tools to isolate nematodes quickly, making the centrifugal flotation method one of the most preffered methods for the extraction of nematodes. Density centrifugation (Jenkins, 1964) extracts living and dead nematodes, so that a higher extraction rate may theoretically be expected (Curran and Heng, 1992).

However, the recovery rate could still be less than 50% (Viglierchio and Schmitt, 1983).

Research on sperm and cell/macromolecular separation has revealed that density gradients generally suffer from a lack of resolving power due to their high osmolarity, and the viscosity of the sucrose gradients often leads to long centrifugation times for equilibrium density banding (Smith et al., 1997). Iodixanol offers some important advantages over sucrose gradients, notably that gradients can be produced that are iso-osmotic over their entire density ranges (Ford et al., 1994). This research dealt with the extraction of reniform nematodes using an iodixanol solution, OptiPrep™, including the determination of its toxicity during the preparation of nematode samples and evaluation, of its efficiency when used in the centrifugal flotation extraction technique. The reniform nematode, *Rotylenchulus reniformis,* Linford and Oliveira (1940) , an important phytonematode species that is widely distributed throughout the tropical and subtropical regions, was used in the study.

MATERIALS AND METHODS

Nematode populations

Three germinated cotton seeds of Deltapine 5415 a variety from Delta and Pine Land Company (Scott, MS), were planted in one 12 oz Styrofoam cup filled with a silt clay loam soil, previously oven-

*Corresponding author. E-mail: yontil@gmail.com.

treated at 135ºC for 12 h and planted in a12 oz styrofoam cup. Eggs were isolated as per below from the laboratory standard reniform nematode population of Auburn University (Alabama, US) and approximately 5,000/cup were used.

Primary nematode collection

After removal of aboveground stems, the infested soil and roots were removed from the cup and placed in a 5 liter bucket and gently rinsed in 3 volumes of tap water. After soaking the roots for 1 - 2 min, most of the remaining soil was removed by gentle mas-saging, and the roots were transferred into another container full of water. After gently rinsing 2 - 3 times, roots were suspended in the water for over 1 hour to remove the last few remnants of soil from the roots. The contents of both containers were combined and mixed vigorously until the soil was sufficiently dispersed. The super-natant in the bucket, after settling for three minutes, was poured through stacked 106 microns and 45 micron (opening size) sieves. The settled sediment was washed and sieved 3 times in this fashion. The collection on the 45 micron (opening size) sieve was transferred into a 250/500 ml beaker where it was allowed to settle for at least 5 min.

Concentrating nematode samples

Vermiform nematodes

The supernatant in the 250/500 ml beaker was poured through a small 25 micron sieve. The collection was equally divided into three 50 ml centrifuge tubes for later processing.

Eggs

The roots were continually stirred in 10% bleach for 4 min, and then poured through 100 meshes, 45 and 25 micron sieves, respectively. The collection on the 25 micron sieve was washed thoroughly with water and then was washed out of the sieve into a 50 ml centrifuge tube.

Centrifugation of both egg and vermiform tubes was carried out at 3000 rpm for 3 min in a Marathon 21 k benchtop centrifuge (Fisher Scientific, Suwanee, GA). The supernatant in the tube was poured out, and the pellet was used in the following treatments.

Sample cleaning

Three different cleaning methods were used as treatments being repeated 4 times. The pellet was resuspended in 10 ml distilled water and, from now on, it was referred to as the Control sample. A similar sample, prepared as the control sample, was then layered onto the surface of undiluted OptiPrep™ (60% iodixanol, Axis-Shield Company Oslo, Norway) in a 15ml conical centrifuge tube, of which the volume of OptiPrep™ was about 2 - 3 ml, depending on how dirty the tested sample was. After centrifugation at 3000rpm for 1 min, ½ the volume of supernatant was discarded. The remainder of the supernatant, above the interface, was the OptiPrep™-treated sample. The last treatment was the standard sucrose treatment. Briefly, freshly prepared 40% sucrose solution was added to the centrifuge tube with pellet, shaken, and centrifuged at 3000rpm for 1min. The supernatant was immediately poured through the 106 micron and 25 micron sieve set. The sieve set was thoroughly washed with distilled water until the sucrose solution was sufficiently rinsed out. The collection was the Sucrose – treated sample The whole process associated with sucrose cleaning was completed within 5 min.

Nematode number and viability

For each sample, 8 to ten 25 µl aliquots of vermiform nematodes were randomly selected and observed under a dissecting microscope. The total number of nematodes counted were converted to number/ml for data analysis. For viability studies, a nematode was counted as "moving" = viable, if its body shifted within 1 min.

Data analysis

The observed data were calculated by following formulas, which were derived and modified according to previous research (Campbell and Gaugler, 1992; Jenkins, 1964):

$$\text{Motility Rate}(\%) = \frac{\text{Number of Moving Nematodes}}{\text{Total Nematodes}} \times 100$$

$$\text{Recovery Rate}(\%) = \frac{\text{Total Treatment Nematodes/ml}}{\text{Total Control Nematodes/ml}} \times 100$$

The extraction efficiency, which combined the motility and recovery rates, was here defined as:

$$\text{Relative Extraction Efficiency}(\%) = \frac{\text{Moving Rate X Recovery Rate for the Treatment}}{\text{Moving Rate X Recovery Rate for the Control}} \text{ X } 100$$

All of the data were analyzed as the GLM Procedure with both actual and log-transformed data using SAS 8.0 (SAS, Cary, NC). If there was a significant difference among treatments based on F Test, Duncan's Multiple Range Test was applied.

To further determine the toxicity of OptiPrep™ during the preparation of nematode samples, a unique study on long-term adaptability was carried out. Samples from the same OptiPrep™-treated collection were placed in distilled water as a control, OptiPrep™ and 40% Sucrose. Four repeats of nematode samples were observed for 22 h to determine the viability of the nematodes in the different solutions.

RESULTS

The motility rates of OptiPrep™-treated samples were similar to those of the unprocessed Control and both differed significantly of the Sucrose treatment (Table 1). Values determined for the sucrose treatment (28.7%) actually averaged almost half of the Control values (51.3%). However, the values for the sucrose- treated samples averaged only half of the Control values (Table 1). Using OptiPrepTM as a gradient medium, the extraction efficiency reached 85%, while that of the sucrose method was only 24%. Of course, the highest extraction efficiency was that of the Control, but these samples were dirty and possibly useless for any research pur-poses. OptiPrep™-treated samples not only provided a high efficiency of recovery, but the samples were also cleaner than the sucrose-treated ones for vermiform specimens (Figure 1 A vs. B) and for eggs (Figure 1c).

Table 1. Motility rate, recovery rate, and relative extraction efficiency for the different nematode preparation cleaning treatments.

Cleaning Treatment	Motility Rate (%)	Recovery Rate (%)	Relative Extraction Efficiency (%)
Control (Not Cleaned)	51.3±2.7 a	100.0±0.0	100
OptiPrep™	48.6±3.0 a	89.9±0.7	85
Sucrose	28.7±0.3 b	43.7±2.0	24

Mean ± Standard Deviation.

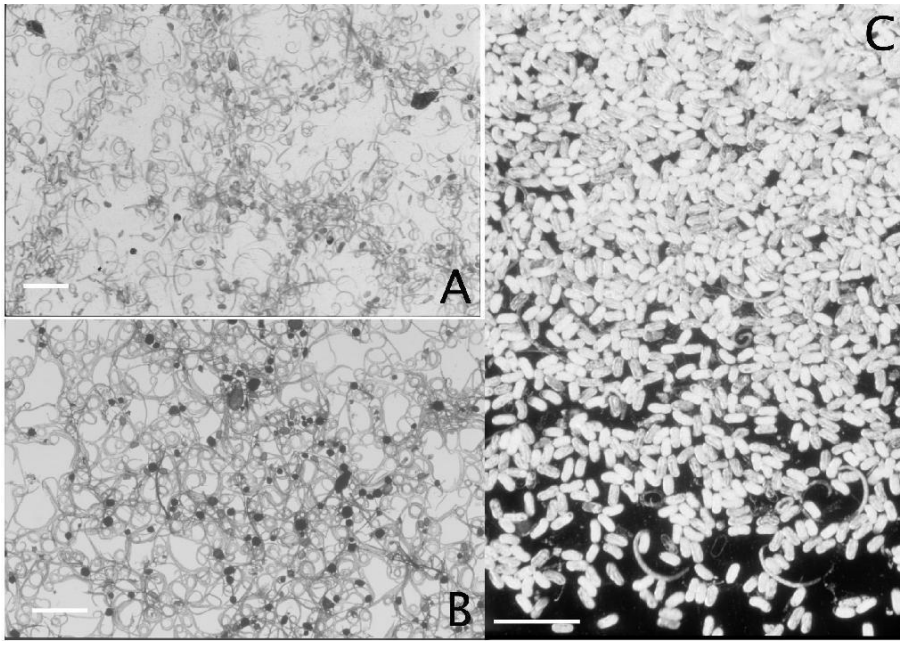

Figure 1. Representative samples of cleaned nematode preps. Scale bar = 400 m. A = Sucrose, B = OptiPrep™, C = Eggs after extraction with the OptiPrep™ method.

With regard to OptiPrep™ toxicity to nematodes during the preparation of samples, the differences in nematode motility rate calculated were highly significant according to ANOVA analysis (Table 1) with no significant differ-rence between the OptiPrep™- treated and Control while all the nematodes were dead after 22 h of exposure to 40% Sucrose.

DISCUSSION

The method is very reliable and reproducible after optimizing for centrifuges and types of collection tubes. It must be kept in mind that one does not collect nematodes/eggs from the interface. The combination of centrifugal time and speed used allows for the nematodes/eggs to clear the upper half of the water column. The absolute depth of this nematode-clear area needs to be examined initially, but once determined, can be reliably used as the standard during nematode extraction. The present study revealed that the nematodes/eggs were within half the volume above the interface after contrifugation using our optimized parameters.

Compared with the Sucrose method, it is not necessary to prepare fresh media each time nor there is time pressure for subsequent quick rinsing with water because the nematodes can tolerate exposure to OptiPrep™. Recovery rates reached here were comparable to, or eventually higher than those provided with other nematode extraction techniques (Viglierchio and Schmitt, 1983, Curran and Heng, 1992).

Although this method was not extensively tested for egg isolation, this method can probably be applied to collect eggs, at least for those species whose females lay a large majority of the eggs outside their bodies. The OptiPrepTM method, allowed high recovery of clean and viable specimens of reniform nematode (*R. reniformis*) to be achieved and we believe that it can easily be adapted to other nematodes that live or at least spend part of their

lives in the soil as well as to other soils with different textural composition.

This research is supported by a grant from the Applied Genomics for Cotton Improvement (Alabama State Grant ALAX-2280143164-0188544). The authors would like to express sincere appreciation to Dr. Altaf Khan, Ms. Jan Wang, and Mr. Tom Smith for their assistance in this endeavor. This manuscript was edited by Dr. Lynn Boyd and Dr. Robert Robbins.

REFERENCE

Campbell LR, Gaugler R (1992). Effect of exsheathment on motility and pathogenicity of two entomopathogenic nematode species. J. Nematol. 24: 365-70.

Curran J, Heng J (1992). Comparison of three methods of estimating the number of entomopathogenic

nematodes present in soil samples. J. Nematol. 24: 170-176.

Fan X, Hominick WM (1991). Efficiency of the Galleria (wax moth) baiting technique for recovering infective stages of entomopathogenic rhabditids (Steinernematidae and Heterorhabditidae) from sand and soil. Revue de Nematologie 14: 381-387.

Ford T, Graham J, Rickwood D (1994). Iodixanol: a nonionic iso-osmotic centrifugation medium for the formation of self-generated gradients. Anal Biochem. 220:360-6.

Hooper DJ (1990). Extraction and processing of plant and soil nematodes. In:Luc M, Sikora, RA, Bridge J (eds.) Plant parasitic nematodes in subtropical and tropical agriculture. CAB International, Wallingford 45-68.

Jenkins WR (1964). A rapid centrifugal-flotation technique for separating nematodes from soil. Plant Disease Reporter. 48:692.

Saunders MC, All JN (1982). Laboratory extraction methods and field detection of entomophilic rhabditid nematodes from soil. Environ. Entomol. 11: 1164-5.

Smith TT, Byers M, Kaftani D, Whitford W (1997).The use of iodixanol as a density gradient material for separating human sperm from semen. Arch Androl. 38: 223 -230.

.