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EXTRACTING VIABLE RENIFORM NEMATODES: AN IMPROVED METHOD

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Abstract

A reniform nematode isolation technique was established, which is based on an iso-osmotic density-gradient medium (OptiPrep™). This technique results 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 survive exposure to OptiPrep™ for 22 hours without significant mortality whereas all nematodes died in the sucrose medium. OptiPrep™ 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. This is the first literature report of the use of the OptiPrep™ density-gradient medium for the isolation of nematodes. It could be expected to apply for extraction of other soil-borne nematodes.

Introduction

Rotylenchulus reniformis Linford & Oliveira, commonly known as the reniform nematode, is found in 11 states in the Cotton Belt of the US (Overstreet, 1999). The incidence of reniform nematode has greatly increased within the last decade and an estimate was made that reniform nematode has suppressed statewide yields by 9% in Alabama in 2001 (Gazaway and McLean, 2003).

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; Yhorne, 1961), 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 contingent on nematode motility, while the live bait method relies on infectivity, whereas 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 more common methods for the extraction of nematodes. Density centrifugation (Jenkins, 1964) extracts living and dead nematodes, so it gives a higher extraction rate theoretically (Curran and Heng, 1992). However, the recovery rate still could 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).

The purpose of this poster is to report present studies on the extraction of reniform nematodes using an iodixanol solution, OptiPrep™, determination for the toxicity of OptiPrep™ during the preparation of nematode samples, and evaluation for the use of OptiPrep™ in the centrifugal flotation extraction technique.

Materials and Methods

Nematode Populations

Three germinated cotton seeds of Deltapine 5415 from Delta and Pine Land Company (Scott, MS), were planted in a silty clay loam soil, which was oven-treated at 135ºC for 12 hours and planted in one 12 oz styrofoam cup. Eggs were isolated as per below from the laboratory standard reniform nematode population of Auburn University and approximately 5,000/cup were used.

Primary Nematode Collection

After removal of aboveground stems, the infested soil ball was removed from the cup and placed in a 5 liter bucket and gently rinsed in 3 volumes of tap water. After soaking for 1-2 minutes, most of the remaining soil was removed by gentle massaging, 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 supernatant in the bucket, after settling for three minutes, was poured through stacked 100 mesh/325 mesh (opening size) sieves. The settled sediment was washed and sieved 3 times in this fashion. The collection on the 325 mesh 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 500 mesh 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 mesh, 325 mesh and 500 mesh sieves, respectively. The collection on the 500 mesh 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 21K 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 as treatments were developed and were repeated 4 times. The pellet was resuspended in 10 ml distilled water and was called 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 (Sulston and Hodgkin, 1988). 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 100 mesh and 500 mesh 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 minutes.

Nematode Number and Viability

For each sample, 8-10 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 was defined in the research, which combined the moving and recovery rate:

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

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 Tests were processed.

Results

The motility rates among the treatments were highly significantly different according to ANOVA analysis. The motility rates of OptiPrep™-treated samples were similar to those of the unprocessed control. However, the values for the sucrose-treated samples averaged only half of the Control values (Table 1). According to Duncan's Multiple Range Test, the motility rates were not significantly different between the cleaning by OptiPrep™ and the Control, but were significant when sucrose was used. Extraction efficiency as the combination of motility and recovery rates was defined in this research. Using OptiPrep™ as a gradient medium, the extraction efficiency reached 85%, while that of the sucrose method was only 24%. Of course, the highest extraction efficiency is that of the Control, but it is absolutely dirty and almost not useable for any research purposes. OptiPrep™-treated samples not only provided a high efficiency of recovery, but the samples were also cleaner (Figure 1 A vs. B) than the sucrose-treated ones for both vermiform nematode and its eggs (Figure 1C).

To further determine the toxicity of OptiPrep™ during the preparation of nematode samples, a 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 for 22 hours with four repeats. The differences in motility rate were highly significant according to ANOVA analysis (data not shown). Similarly, there was no significant difference between the OptiPrep™-treated and Control while all the nematodes were dead after overnight exposure to 40% sucrose (Figure 2).

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 initially examined, 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 is there time pressure for quick rinses because the nematodes can tolerate exposure to OptiPrep™. Recovery rates reach levels which are comparable or much higher than other nematode extraction techniques (Viglierchio and Schmitt, 1983; Curran and Heng, 1992) for the recovery.

Although we did not extensively test this method for egg isolation, we feel this method can also be applied to collect eggs, at least from those species with externally presented eggs. In fact, the OptiPrep™ method is now our standard method for reniform nematode egg collection in our lab.

This method offers the advantage of high recovery of clean and viable nematodes, a combination essential for population surveys and molecular biology techniques, as well as for such mundane tasks as population building and identification. We believe this method can easily be adapted to other nematodes in other soil types and prove advantageous for future studies.

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Table 1. 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	89.9±0.7	85
Sucrose	28.7±0.3	43.7±2.0	24

[a] Mean ± Standard Deviation

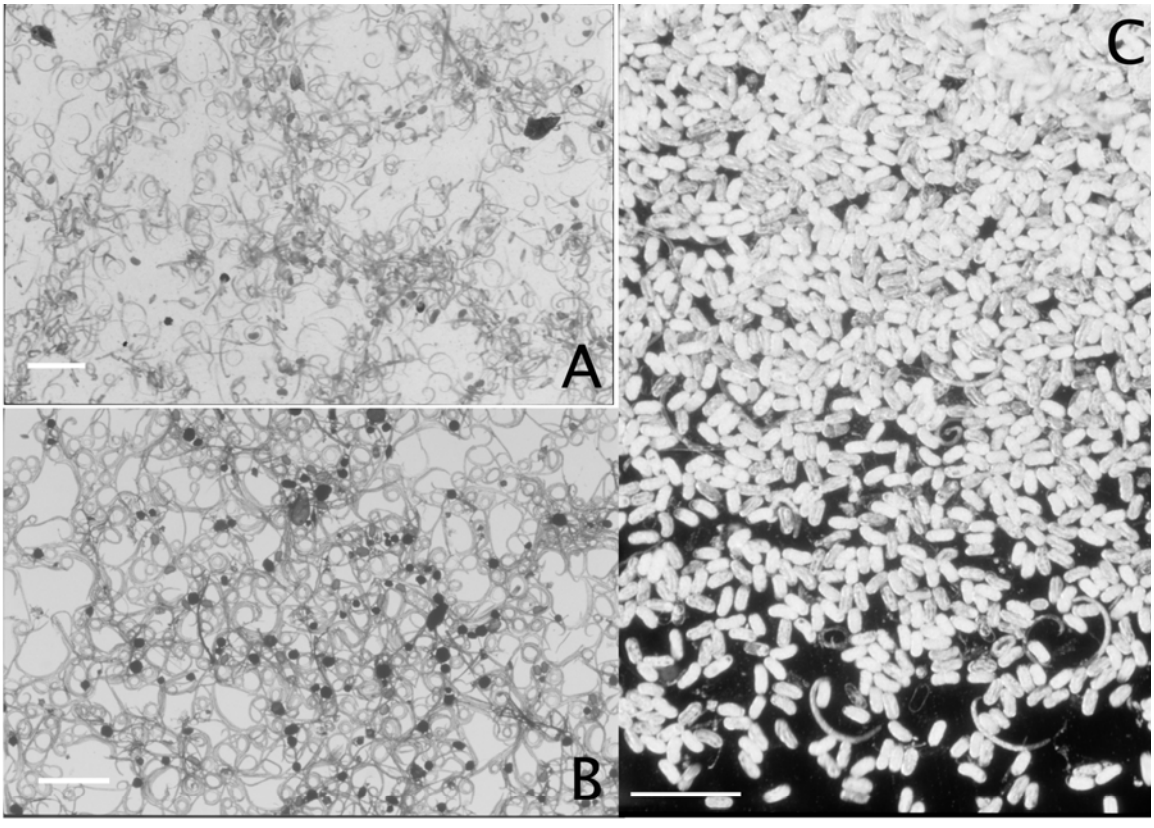

Figure 1. Representative samples of cleaned nematode preps. Scale bar = 400µm. A=Sucrose, B=OptiPrep™, C=Eggs after extraction with the OptiPrep™ method.

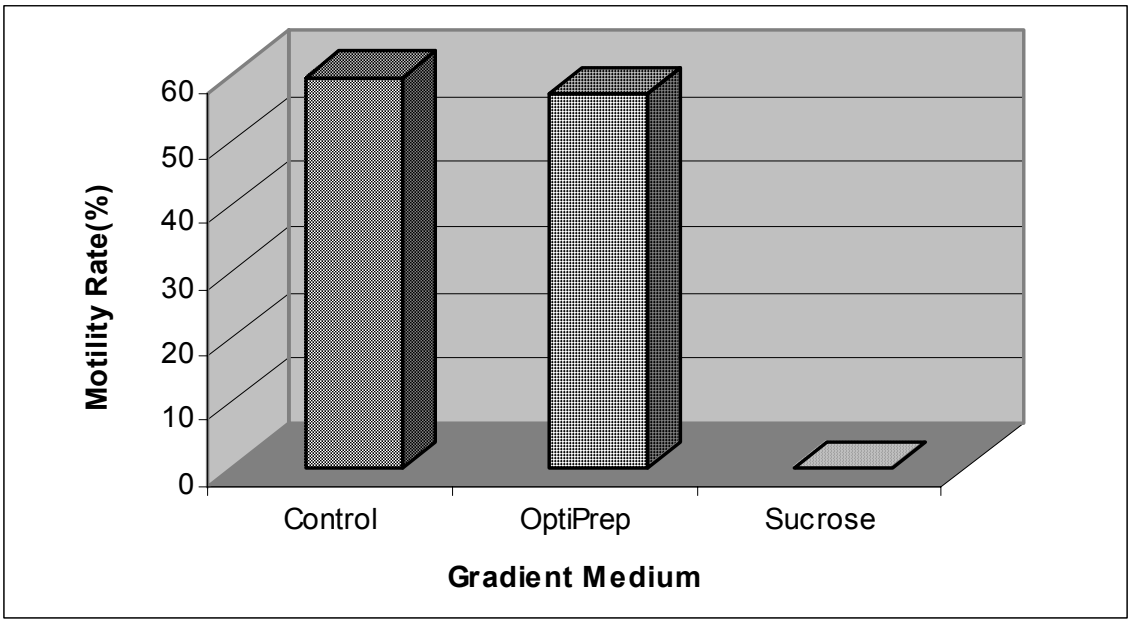

Figure 2. Comparison of motility rates after 22 hour treatment exposure in water, Optiprep and 40% Sucrose, respectively. Bars with the same letter are similar to each other based on Duncan's Multiple Range Test ($P<0.05$).