

## Efficient Procedure for Extracting *Tylenchulus semipenetrans* from Citrus Roots

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**Abstract:** Investigations were undertaken to determine the suitability of sucrose and magnesium sulphate solutions and a silica colloidal suspension with centrifugation for extracting *Tylenchulus semipenetrans* from citrus roots. The efficiency of incubation, sodium hypochlorite, centrifugation, and maceration methods was also compared. Numbers of females recovered by centrifugation with colloidal silica were greater than those from sucrose or magnesium sulphate. Incubation, sodium hypochlorite, and centrifugation methods were satisfactory for extracting eggs, second-stage juveniles, and males, whereas the maceration-sieving method was less efficient. Combining the sodium hypochlorite method with a 15-second maceration followed by centrifugation in colloidal silica reduced the recovery of *T. semipenetrans* females from citrus roots.

**Key words:** *Citrus aurantium*, citrus nematode, extraction method, sour orange, *Tylenchulus semipenetrans*.

Population densities of semi-endoparasitic nematodes are determined by counting the number of nematode life stages in soil and roots. The nematode population densities obtained are expressed as numbers per cubic centimeter soil and gram fresh or dry root weight. *Tylenchulus semipenetrans* Cobb eggs, males, and second-stage juveniles (J2) are extracted from soil; these stages and females can also be extracted from citrus roots (11). The determination of *T. semipenetrans* population density from roots and soil provides a more complete evaluation of nematode infection than that obtained from soil alone. Root maceration alone (1,4,6) and with centrifugal flotation (7) are used to extract all life stages of *T. semipenetrans*. Other methods allow the extraction of selective *T. semipenetrans* life stages, such as males and J2 with an incubation technique (11), or males, J2, and eggs with a sodium hypochlorite method (5). The efficiency of the maceration plus centrifugal-flotation method is influenced by maceration time and separation agent and its specific gravity during the centrifugation (10). Magnesium sulphate solution (sp gr 1.16) is suitable for extracting most of the nematode stages (2), but it is not suggested for species of *Longidorus* and *Xiphinema*, which can be extracted using a colloidal silica suspension having the

same specific gravity but no osmotic pressure effect. Therefore, a study was conducted to compare the effect of different techniques on the extraction efficiency of all *T. semipenetrans* life stages from citrus roots.

### MATERIALS AND METHODS

Sour orange (*Citrus aurantium* L.) roots infested with *T. semipenetrans* (averaging 271 eggs and juveniles and 85 females per gram roots) were collected in November 1988, gently washed in water, cut in segments 0.5 cm long, thoroughly mixed, and divided into 56 samples (10 g each).

In experiment 1, each root sample was placed in a 250-ml glass container with 150 ml water. Six replicate samples were macerated for 15 seconds and six for 30 seconds at 8,000 rpm in an electric miniblender. The water suspension was sieved through 250- $\mu$ m-pore and 10- $\mu$ m-pore sieves. Root debris and nematodes on the 10- $\mu$ m-pore sieve were collected in 650-ml centrifuge tubes, with colloidal silica (Du Pont De Nemours International S. A.) at 1.16 sp gr. Volumes of the root suspension were adjusted to 500 ml each. To facilitate the formation of the pellets during centrifugation, 8 g kaolin was added and suspended with a vibromixer. The suspensions were centrifuged one time at 2,200 rpm (1,600 g) for 5 minutes. The supernatants were poured on a 10- $\mu$ m-pore sieve and gently rinsed with water; the remain-

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ing debris was collected in beakers. Three 1-ml aliquots of the nematode suspension were used to count males, eggs, and J2, and two 5-ml portions were used to count young and adult female nematodes.

In experiment 2, 18 root samples were divided into three groups of six samples each and macerated as described for 15 seconds. Each group was centrifuged once separately with colloidal silica, sucrose, or magnesium sulphate at 1.16 sp gr. Nematodes were recovered by the same procedure as in experiment 1.

In experiment 3, eight root samples were macerated for 15 seconds and four replicates were extracted in colloidal silica by single centrifugation as in experiment 1. The remaining four replicates each received 8 g kaolin, and the suspension was centrifuged in water at 2,200 rpm for 5 minutes. The supernatants were then discarded, and the pellets were resuspended in 500 ml colloidal silica and centrifuged again for 5 minutes. Nematodes in the supernatants were collected and counted.

In experiment 4, 18 root samples were divided in three groups of six replicates. Samples of one group were macerated for 15 seconds and nematodes were extracted by single centrifugation in colloidal silica as described before. Samples of the second group were placed in 0.5-liter glass containers with 25 ml water. The containers were kept 4 days at 19 C. Eggs, J2, and males were collected every 48 hours; those in three 1-ml suspensions were counted. Samples of the third group were placed in 500-ml plastic bottles containing 150 ml of a 1% sodium hypochlorite solution and shaken for 3 minutes at 550 shakes/minute with an electrical shaker. The suspension was sieved through 250- $\mu$ m-pore and 10- $\mu$ m-pore sieves and washed free of sodium hypochlorite; nematodes on the 10- $\mu$ m-pore sieve were collected and counted.

In experiment 5, the efficiency of double centrifugation in colloidal silica was compared with the three most common extraction methods and a combination of two methods. Infested roots (averaging 165 eggs and J2 and 73 females per gram) were

collected from the same orchard in September 1989 and 30 samples (10 g each) were prepared. Six root samples each were processed by the double centrifugation method described in experiment 3 and incubated at 25 C for 4 days. Nematodes were collected daily and processed by the sodium hypochlorite method (5). Another group of six root samples was first processed by the sodium hypochlorite method and thereafter by double centrifugation in colloidal silica. The remaining six root samples were processed by the maceration-sieving technique (1), except that 250- $\mu$ m-pore and 10- $\mu$ m-pore sieves were used to eliminate root debris and collect nematodes.

Data were analyzed by Duncan's multiple-range test or Student's *t*-test.

#### RESULTS AND DISCUSSION

Extraction efficiency of *T. semipenetrans* was not affected ( $P \leq 0.05$ ) by maceration time or number of centrifugations.

Total numbers of *T. semipenetrans* J2, males, eggs, and females collected by single centrifugation with colloidal silica were greater ( $P \leq 0.05$ ) than those extracted with sucrose or magnesium sulphate, but they were not different between the sucrose and magnesium sulphate solution (Table 1). The small numbers of *T. semipenetrans* females obtained in sucrose and magnesium sulphate probably were due to the detrimental effect of these chemicals on the nematode and especially on female tonicity. Females can be greatly reduced in size, wrinkled (9), and plasmolized (3) by sucrose and magnesium sulphate solutions, which are hypertonic with respect to nematode body content. This may result in their loss during the retrieval process.

More ( $P \leq 0.05$ ) J2, males, and eggs were recovered from the sodium hypochlorite method than from single centrifugation with colloidal silica (Table 2). Total numbers of J2, males, and eggs obtained from incubation were intermediate and not different from the other methods.

When data from extracting methods were compared, fewer ( $P \leq 0.05$ ) J2 and

TABLE 1. Efficiency of three solutions for extracting *Tylenchulus semipenetrans* from citrus roots (10 g) by single centrifugation.

Solution	Juveniles, males, and eggs	Females
Colloidal silica	3,575 a	946 a
Sucrose	1,865 b	7 b
Magnesium sulphate	1,213 b	10 b

Means followed by the same letter are not different ( $P \leq 0.05$ ) according to Duncan's multiple-range test.

males were recovered by the maceration-sieving and sodium-hypochlorite methods than by double centrifugation with colloidal silica or incubation (Table 3). The combination of sodium hypochlorite and double centrifugation methods gave intermediate recovery of J2 and males. The greatest number of females was obtained by double centrifugation in colloidal silica followed by a combination of the sodium hypochlorite and the double centrifugation in colloidal silica methods. Fewer ( $P \leq 0.05$ ) females were recovered by the incubation, sodium-hypochlorite, and maceration-sieving methods.

Data from these experiments demonstrate that double centrifugation in colloidal silica was the most efficient method of extracting *T. semipenetrans* females from citrus roots. Most of the females were not damaged. This method could be useful when extraction of females is required for investigating the biology of *T. semipenetrans*, especially reproduction potential and control.

Based on data from experiment 4, we expected that combining the sodium hypochlorite method with double centrifugation

TABLE 3. Effect of methods on extraction of *Tylenchulus semipenetrans* from citrus roots (10 g).

Method	Eggs	Juveniles and males	Females
Double centrifugation (Dc)†	1,330 a	322 a	638 a
Incubation	1,414 a	388 a	63 c
Sodium hypochlorite (Sh)	1,271 a	124 b	61 c
Sh + Dc†	1,384 a	261 ab	208 b
Maceration-sieving	905 b	123 b	77 c

Means followed by the same letter are not different ( $P \leq 0.05$ ) according to Duncan's multiple-range test.

† In colloidal silica.

gation in colloidal silica would have increased the retrieval of *T. semipenetrans* females. Data from experiment 5 demonstrated that the methods combined did not increase numbers of extracted females. Most probably, many females treated with sodium hypochlorite are damaged during maceration and lost during centrifugation.

Macerating root pieces for 15 seconds followed by double centrifugation in colloidal silica provided more females than, and numbers of eggs and J2 similar to, those obtained by the incubation method; this confirms other results (8). This procedure is rapid and unaffected by environmental conditions, egg hatch, and egg laying, and it provides a reliable extraction method for all stages of *T. semipenetrans* infesting citrus roots.

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TABLE 2. Efficiency of different methods for extracting *Tylenchulus semipenetrans* from citrus roots (10 g).

Method	Juveniles, males, and eggs
Single centrifugation in colloidal silica	1,029 b
Incubation	1,429 ab
Sodium hypochlorite	1,634 a

Means followed by the same letter are not different ( $P \leq 0.05$ ) according to Duncan's multiple-range test.

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