Collection and Selection of Cysts of the Sugar Beet Nematode, Heterodera Schachtii[1]

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Introduction

The large scale collection of nematode cysts has remained an onerous procedure since the discovery of the cyst-forming nematodes about the middle of the 19th century. In the interest of expediency, separation methods have frequently been manifests of needless compromises in cyst quantity, quality, and/or purity. A resolution of the difficulty is becoming of increasing importance in view of the growing interest in a number of the cyst-forming plant parasitic nematodes.

The cyst, a distinguishing, characteristic stage in the life cycle of these nematodes, is an immotile particle which can harbor viable, abeyant larvae amidst a soil matrix. The cultivated zone of a heavily infested sugar beet field in California can contain on the order of two cysts per gram of soil. If 50,000 cysts were needed for a moderate hatching assay, for example, it would require every cyst from 25 Kg. of soil. Since frequently on the order of 80 to 90 percent of such cysts are empty or otherwise non-viable, the amount of soil processed, neglecting recovery losses, must be increased to from 125 to 250 Kg. Few farmers would be disposed to permit their fields to become so heavily infested; consequently the nematode population is likely to be lower. If one also considers the probable losses from the separation procedures, it is evident that it may be necessary to process a ton or more of field soil to obtain the desired number of cysts. An increase in the amount of soil processed for a given number of cysts correspondingly increases the amount of general debris collected. Moreover, the increase in general debris increases as well the amount of debris similar to cysts in several physical properties, thereby necessitating more elaborate separatory procedures.

Since the purpose of the operation is to obtain viable cysts, one is restricted to gentle separation procedures usually dependent upon physical properties. A purification problem can be approached either by the sufficient refinement of a separation procedure to increase the sensitivity to a given property, or by

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the utilization of a series of less sensitive procedures each based upon a different property. Theoretically the first approach (i.e. by extensive refinement) would be capable of separating cysts from debris as long as any difference, however small, existed in their properties. The natural variability of cysts precludes the requisite *unique* value of any property (i.e. there is usually a range of values for a property); the approach becomes impractical, and one is compelled to use the alternative. Since the over-all enrichment is the product of the enrichments achieved in each procedure of the series, it is possible to be as selective as one wishes, with a sufficient number of steps.

Procedures currently used for cyst separation have been recently reviewed (6)[3]. The present report is concerned with the modification, extension, and/or combination of these procedures together with the suggestion of new ones as they may apply to large scale cyst collection.

Methods of Separation

Sieving: Sieving normally involves the presentation of particles of varying size and shape for passage through a lattice, usually square, with openings of known size. Since the lattice can be considered essentially two-dimensional, sieving is theoretically capable of rejecting entirely only those particles whose minimum projected area is larger than the lattice opening. In practice long particles, e.g. fibers, capable of lengthwise passage, are often caught. Frequently sieves are operated in an overloaded condition so that the efficiency is seldom optimum.

In view of these observations sieving for the separation of cysts from soil is impractical, but the screening method can often be used to advantage in conjunction with other operations. The decant-sieve method (1) whereby, the water (after infested soil, thoroughly mixed with water, is allowed to settle momentarily) is decanted through the appropriate screens for the collection of cysts, illustrates the principle. The decant-wet screen technique is virtually the only method (other than elutriation for small samples) whereby cyst concentrate may be obtained from wet soil.

Flotation: Flotation depends upon the specific gravity of a particle (the cyst, being less than that of a liquid, water) as is the case when the cyst is dry or nearly dry. This method is preferred for the extraction of large quantities of soil since it is very efficient and easily adaptable to large scale operations. Dry or nearly dry soil is well mixed with several volumes of water

[3] Numbers in parentheses refer to literature cited.

and then the whole allowed to settle for a short time. The float containing the cysts is then collected and the water and sediment discarded. The operation requires only minutes so that with the proper equipment large quantities of soil may be processed easily. Since this method separates most of the organic matter from the inorganic, the enrichment decreases as the organic content of the soil increases; consequently it is very effective in mineral soils but almost useless in peaty soils.

The common Erlenmeyer flask is a satisfactory separating tank for occasional small samples, but for large scale operations, workers usually construct extraction tanks most suitable to the individual laboratory routine. The nematology laboratory of the Rothamsted Experimental Station, Harpenden, Herts, England, uses the Fenwick can (5) or the modified Fenwick can (6) which is simply a large metallic Erlenmeyer flask with added pouring collar and spout, sloping bottom and drain plug. The soil is washed through the screen insert of a funnel extending into the neck of the full can of water whereupon the float rises and flows down the spout.

The Golden Nematode Laboratory on Long Island, New York, has a 60 gallon tank with three water inlet nozzles and drain plug at the bottom and a pouring spout at the top (10). Water supplied by a 2-inch main at about 40 p.s.i. violently mixes with the 75 lb. charge of soil; when the tank is nearly full the water is shut off and the heavy material allowed to settle momentarily. The float is then washed out the spout with a gentle current of water, after which the remaining water and sediment is drained into a sump.

At the nematode laboratory at the University of California at Davis, a smaller 30-gallon version of the Long Island tank is used. Water from the mains is supplied through a 2½-inch fire hose and the drain is at the side near the bottom of the drum. The unit is portable and can be operated conveniently in a field within range of a fire hydrant as has been done at several of the Spreckels sugar factories.

Since most sugar beet nematode cysts will be passed by a U. S. Series No. 20 sieve but retained by a No. 60 sieve, the float which is separated in any of these devices is so sieved to remove the larger and the smaller particles (Figure 1). The material collected in these large scale operations may contain considerable amounts of material more dense than water, and it is advisable to repeat the procedure in suitable apparatus. This second operation can be combined conveniently with the following selection procedure.

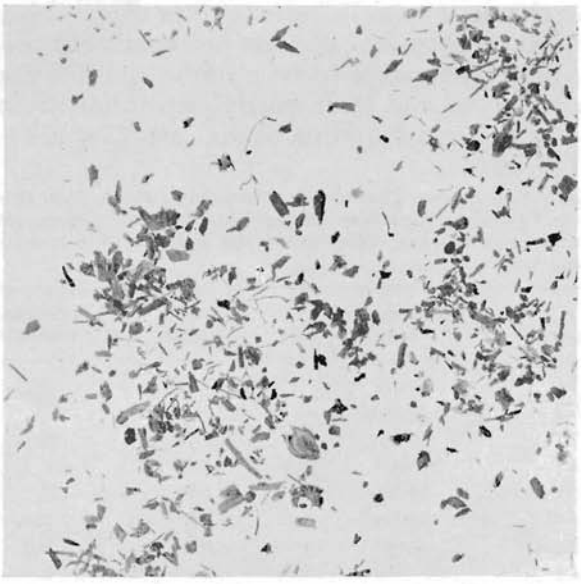

Figure 1.—Cysts and debris (Float) separated in the mixing of dry or nearly dry field soil with water.

Hydration: The dry or nearly dry cyst, according to Archimedes principle, floats on water because its weight, as a particle, is less than that of the corresponding volume of water. The low specific gravity of the cysts can be explained in part by the cyst's internal structure. If the cyst is considered as being a relatively large casing permeable to water and gases (3) within which there are a variable number of smaller casings, also permeable to water and gases, which in turn contain the second stage larvae, then the drying of a "wet" cyst could be visualized as a gas for liquid exchange within the cyst casing from the colloidal gel (8) enveloping the larval casings. Extensive drying could likely result in the dehydration of the larvae with corresponding gaseous vacuole formation and/or shrinkage of the worm. The over-all result, replacement of water within the cyst by gases, would effect a decrease in the specific gravity of the particle. It would be reasonable to expect the specific gravity of cysts containing more larvae (or organic material little permeable through the cyst wall) and therefore less gas, to be relatively greater than the empty ones. Since it is known that the specific gravity of saturated cysts is greater than that of water, in the reversal of the drying process, hydration, the liquid for gas exchange would permit the specific gravity of the "fuller" cysts to exceed that of water and sink before the less full ones, as has long been believed to be the case. The organic debris obtained along with the cysts in the float possesses a different

structure so that the change in specific gravity due to gas-water exchange and/or water absorption should be small. Consequently a hydration procedure ought to be a useful tool for the selection of the "fuller" cysts and their partial separation from associated debris. This has been found to be the case (Table 1).

Table 1.—Typical Sedimentation Analysis Obtained by Hydration of Crude Field Cyst Float Concentrate. The Float Concentrate Has Been Shaken with Water in a Stoppered Flask, then Poured Into a Large Funnel Partially Filled with Water and Allowed to Hydrate and, after Intermittent Stirring, Sink.

Material	Wt.(g)	Sedimentation Time	No. Cyst/gm.	Percent Viability[2]
Float cyst conc.	27.4			
A	16.7	10 min.	0	
B	3.7	6 hrs.	2,025	36
C	0.7	10 hrs.	14,700	35
D	0.7	30 hrs.	24,300	31
E	0.3	72 hrs.	33,900	3.4
F	2.1	Float	2,230	0.9
Loss[1]	2.9		0	

[1] This loss represents organic and inorganic particles passing through 60 mesh screen used to retain cysts and associated larger particles of the desired fractions.

[2] A cyst is considered viable when dissection reveals a larvae manifesting the characteristics presumed to indicate viability. This is a crude estimation at best (4). The effect upon the contents of different cysts, of varying periods of hydration and dehydration is as yet poorly understood but is a factor to be considered.

It has been mentioned in the preceding section that the float obtained in the large scale procedure contains a considerable amount of dense matter; this can be seen in Fraction A. This material is carried over in part by strong water currents and in part as bound to light organic matter. The predominant portion of Fraction B consists of aggregates of cysts, sand and other debris not disintegrated by the stirring and shaking. The discard of Fraction B would manifest a significant loss of viable cysts, but retention would add a considerable amount of undesirable debris. If, however, the float remaining after the removal of A is exposed to the action of a Waring blendor for 20 to 30 seconds, the aggregates are broken up; the particles then will become more favorably redistributed. The new Fraction B contains a negligible number of cysts so that if the material sedimenting after 6 but before 30 hours is collected, one selects the "fuller" cysts with their correspondingly greater larval potential while discarding up to 90 percent of the undesirable debris. An additional washing benefit is derived from the blendor treatment; the cyst surface is cleaned of clay and silt particles and other

matter which tend to interfere with its rolling properties, the basis of a subsequent procedure.

Elutriation: As a method for the separation of particles, elutriation involves complex hydrodynamic relations of particles, fluid and apparatus, not well understood or analyzed. The basis for separation is the settling velocity of a particle which represents the combined effect of the size, shape, and density of the material and of the density and viscosity of the fluid. It is not easily obtained by calculation except for special cases. The settling velocity, V, of spherical cysts could be estimated by the application of Stoke's law (11)

$$V = \frac{2}{9} \frac{g\ r^2\ (\rho_2 - \rho_1)}{\mu_1}$$

(where g = gravitational constant, ρ_2 = cyst density, ρ_1 = water density, μ_1 = water viscosity and r = spherical cyst radius) if the cysts were spherical, of constant density, and of sufficiently small, constant radius. However, neither the cysts nor the debris meets these restrictions well enough to make velocity calculations profitable.

As a result, the development of the elutriation technique has been empirical. Settling velocity carries the implication of the motion of a body through a fluid at rest, however it is the reverse situation, a moving fluid around a body at rest, which is more amenable to practical application. If the fluid is moving *perfectly uniformly* there can by the laws of mechanics be no difference between the two cases, as the superposition of a common uniform motion (equal and opposite to the velocity of the body, so that the latter is brought to rest) makes no difference to mechanical phenomenon. Therefore, to imitate the behavior of a body moving in a fluid at rest by means of a body at rest in a moving fluid, it is necessary to make the flow of fluid as uniform as possible by suitable means (12).

In nematology, Seinhorst, the leading proponent for the use of elutriation, has designed several models of apparatus for the separation of nematode worms from soil with an efficiency of the order of 90 percent (13). Hesling (8) has constructed a simple elutriating device which he recommends for the extraction of wet cysts of *Heterodera major*. In this unit the desired fluid velocity is determined by visual observation of the cyst behavior in the rising current of water. The same apparatus could be used to collect the cyst forms of other nematodes. In the Davis laboratory a simple glass cylinder with a diffusing device at the inlet has been found useful in separating cysts from debris (Figure 2). The water flow rate is determined by visual

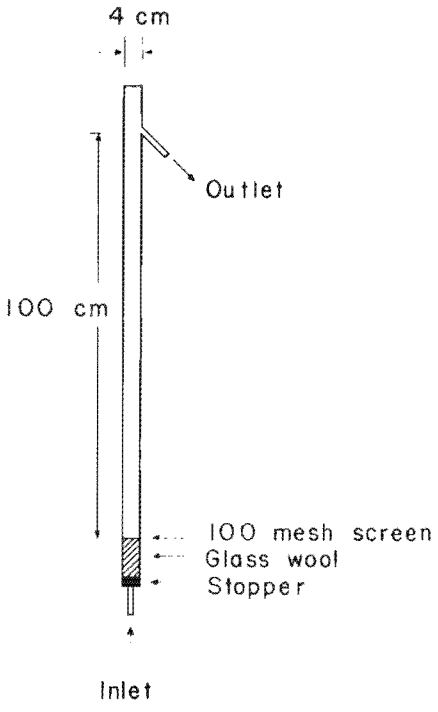

Figure 2. — Glass tube elutriator used for purification of cysts of *Heterodera schachtii*.

observation after the method of Hesling. This device is limited in that it can remove only debris particles whose settling velocity is less than that of the slowest cyst (Figure 3) [1]. It was designed for use with the wet cyst material selected in the hydration procedure. Since the cyst material from hydration contains cysts and debris of comparable density, the separation achieved by elutriation depends upon other properties. This can perhaps be indicated by illustration.

It is known that a flat object of uniform density tends to fall with its greatest projected area perpendicular to the direction of relative motion, sometimes with spiraling or tipping motion. Accordingly, if one compares particles of equal volume and specific gravity, the fall velocity will be greatest for spherical objects. If one compares flat objects, the fall velocity will decrease as the projected area departs from the circular form.

Rolling: Separation by rolling depends upon resistance to the motion of particles down a relatively smooth inclined plane.

[1] At the higher flow rates the nature of the tube and the settling material lead to less idealistic flow behavior; however the method is still useful. [2] To avoid interference from gases coming out of solution, water from high pressure mains ought to be deaerated.

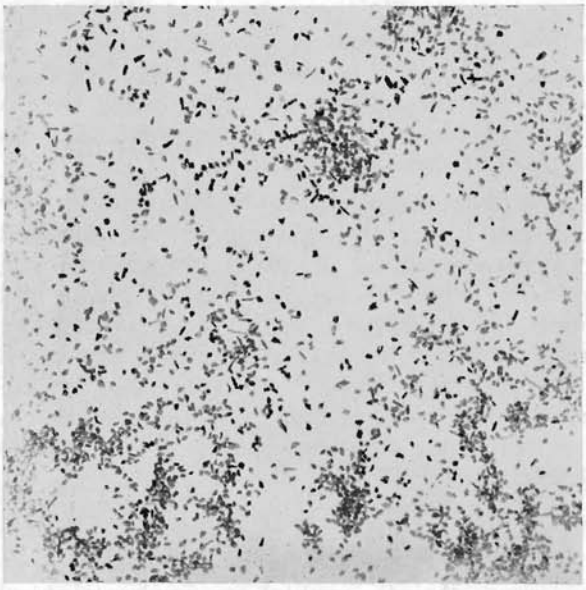

Figure 3.—Sugar beet nematode cyst concentrate obtained from field soil by flotation, hydration and elutriation.

The critical forces involved are frictional. It is well known that frictional resistance to the motion of static or sliding bodies is usually much larger than that of rolling bodies; consequently the technique effects a separation of rolling bodies from sliding ones. As one might expect the spherical particle would separate the most satisfactorily in this procedure. In practice, however, only in the most favorable species are the cysts more or less spherical so usually some gentle type of vibration is provided to dislodge them from other particles and to encourage rolling, but not sliding, motion. A number of devices have been made for this purpose depending upon local needs. They range from a sheet of cardboard, tapped with a pencil (6) to an elaborate gravity table arrangement (2). Hesling (6) has devised a simple mechanical unit employing an inclined rubber belt and an electric bell vibrator. The cysts roll downward and off the lower edge while the debris is carried upward and off the upper edge. The apparatus suffers somewhat from the electrostatic properties of the rubber belt and the cysts. It may be possible to minimize some of these troubles with commercially available anti-static solutions. The efficiency of the technique depends upon the proportion of viable, poorly rolling cysts in the population as well as the amount of rolling debris. Lemon or oval shaped cysts are therefore less efficiently separated by the rolling method.

Electrostatic: This approach to separation utilizes the susceptibility of the cyst to electrostatic charging, a property here-

tofore considered a nuisance. The generation of electrostatic charge on small particles is an extremely complex problem. It appears to be frictional in nature, but the mechanism by which it is produced is poorly understood and the experimentation is difficult. The literature on the subject is voluminous with reports frequently in apparent contradiction (7).

In any event cysts of *Heterodera schachtii* migrate preferentially in an electrostatic field (14). Dry cyst concentrate obtained by previously described procedures, flotation, hydration, and elutriation, is introduced between charged parallel plates and allowed to fall through the electrostatic field. While descending the cysts migrate preferentially towards one plate and the debris towards the other. In practice a band near either plate is entirely cysts or debris. Since a single pass requires less than a minute, a cyst-debris mixture may be passed repeatedly to achieve the desired separation.

Cyst Supply Source

Inasmuch as the most efficient method or combination of methods can separate only the cysts present in a given sample, it is necessary either to increase the quantity of source material processed or to improve the quality to obtain large numbers. Since cysts differ from field to field in quantity, quality, and even biotype (9), the only recourse is to the rearing of nematodes. Rather than to consider a simple population build-up in field soil, it would be desirable to exploit the rearing method more fully with the prospect of simplifying and shortening the separation procedures as well as improving the cyst quality.

An increase in the number or the ratio of viable to nonviable cysts and/or the elimination of debris would be steps in this direction. The viable cyst increase can be handled easily by effecting a large population build-up in a short time (one generation if necessary) with an initial infestation of larvae. The debris could be reduced by growing plants hydroponically; however a soil matrix is of advantage for plant support, nematode infection and plant nutrition. An examination of the cyst separation procedures previously discussed will reveal that relatively large inorganic particles can be completely separated from cysts quite easily and that it is the organic matter which is troublesome. If, therefore, cyst-forming nematodes are reared upon plants grown in coarse washed sand with the aid of a mineral nutrient solution, one ought to achieve maximum benefits with minimum effort (Figure 4). The improved growing conditions enable the plants to support a greater parasite population than

Figure 4.—Concrete block tanks filled with sand in which the sugar beet nematode is reared upon sugar beets.

can plants in soil. It has been possible to build up to a population of the order of 750,000 cysts from an initial infestation of 750 larvae within 6 months in a tank containing 3.5 cu. ft. of sand. The cyst separation can be accomplished easily by the appropriate conventional procedures. A small amount of organic debris originating from the root system of the growing plants is also found in the sand (Figure 5). The cysts so reared are firm, full, clean, and in general larger than those obtained from a field source (Table 2) (Figure 6)[5].

Table 2.—Comparison of Field Cysts and Sand Reared Cysts of *H. schachtii*.

Source	Percent Retained by Sieve Size						Total Wt.
	U.S. 40		U.S. 50		U.S. 60		
	by No.	by Wt.	by No.	by Wt.	by No.	by Wt.	
Field[1]	24.1	39.0	63.2	54.3	12.7	6.7	1.06 g
Sand	57.1	71.0	40.3	28.0	2.6	1.0	1.58 g

[1] Field cysts were those selected by flotation, hydration, elutriation and rolling.

[5] For practical considerations the cysts of Fig. 6 were dried quickly, an expediency highly detrimental to the viability of the encysted larvae.

Figure 5.—*Heterodera schachtii* cysts obtained by decant-sieving of moist sand from the rearing tanks.

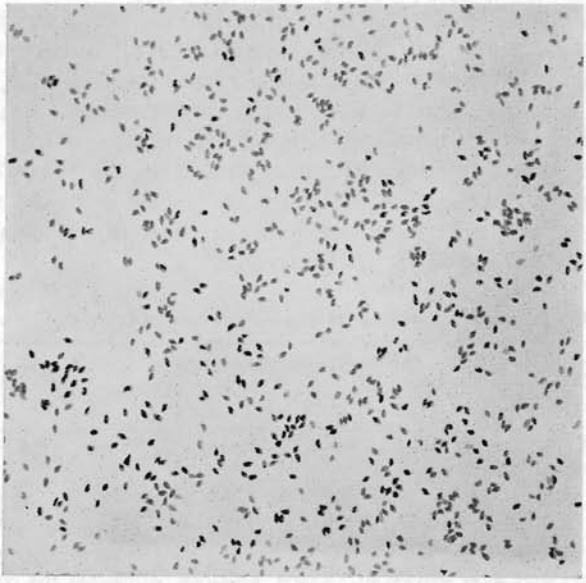

Figure 6.—Clean cysts of *Heterodera schachtii* from rearing tanks sized by U. S. Series No. 40/No. 50 sieves. (Aperture 0.417-0.295 mm)

Discussion

To enable a research worker to secure cysts of the highest quality with a minimum of difficulty, he must have at his disposal the best source and the most efficient separatory methods available. For small numbers he can simply choose the quality and quantity by picking individual cysts directly from root or soil samples with forceps, but for large numbers the task would be formidable. The selection of the separatory method or combination of methods depends upon the choice of source material and upon the physical and physiological properties of the desired cyst nematodes. It is apparent, therefore, that the highly variable nature of cysts and soil matrix may not permit any one refining procedure to be applicable to all situations.

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