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A QUANTITATIVE METHOD FOR EXTRACTING RESTING SPORES OF TWO NEMATODE PARASITIC FUNGI, *NEMATOPHTHORA GYNOPHILA* AND *VERTICILLIUM CHLAMYDOSPORIUM*, FROM SOIL

BY

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A method is described for quantitative extraction of *Nematophthora gynophila* and *Verticillium chlamydosporium* spores from soil. These two species of fungi can prevent populations of the cereal cyst-nematode, *Heterodera avenae*, increasing when susceptible cereal hosts are grown. The soil sample is wet sieved and the spores separated by centrifugation on a high specific gravity solution of magnesium sulphate. The spore suspension is counted directly under a compound microscope (125 ×). About 80% of spores added to three soils of different texture were recovered. In a survey of 23 soils, spore numbers of both fungi were large where *H. avenae* failed to multiply on susceptible hosts, and small where the nematode was increasing or causing damage.

INTRODUCTION

The parasitic fungus, *Nematophthora gynophila* Kerry & Crump, attacks females of several species of cyst-nematode and prevents cyst formation (Kerry & Crump, 1980). The fungus is widespread in soils infested with the cereal cyst-nematode, *Heterodera avenae* Woll., and can prevent nematode multiplication on susceptible cereal crops (Kerry & Crump, 1977; Kerry *et al.*, 1980). The cuticle of parasitized females is destroyed within about 7 days at 13° leaving a mass of resting spores which are readily dispersed in the soil (Crump & Kerry, 1977). Estimates of *N. gynophila* in soil based on the recovery of diseased females necessitates frequent sampling. Also, recovery of the parasitised nematodes whose cuticle has been destroyed is difficult using wet sieving techniques, and rates of parasitism are underestimated (Kerry & Crump, 1977; Kerry, 1980). There is a need for a quantitative method to estimate *N. gynophila* which does not rely on the recovery of diseased females from soil. The method described below extracts the fungal resting spores from soil and determines their numbers.

Most techniques for the estimation of fungal densities in soil rely upon germination of spores and growth of a mycelium on artificial media (Blair, 1944) neither of which has been achieved with *N. gynophila*. Spores of *Plasmodiophora brassicae* Wor., which also cannot be cultured were extracted from soil using centrifugation and sedimentation on sucrose (Buczacki & Ockenden, 1978). Although the spores are small (2.4-3.9 μm diam.) they occur in large numbers

($> 10^5$ spores/g air-dried soil) allowing spore suspensions to be greatly diluted to facilitate counting without reducing accuracy. Initial tests of the recovery of *N. gynophila* indicated that spore densities in soil were much less than those of *P. brassicae* and a more complete separation of spores from soil is necessary.

While the method for extracting resting spores of *N. gynophila* was being developed chlamydospores of *Verticillium chlamydosporium* Goddard were also recovered. This fungus is the most important egg pathogen of the cereal cyst-nematode (Kerry & Crump, 1977) and of the beet cyst-nematode (Tribe, 1979) and the chlamydospores were counted in all the tests done.

MATERIALS AND METHODS

Spore suspensions of *N. gynophila* and *V. chlamydosporium* were found to float in a concentrated solution of magnesium sulphate when centrifuged at low speeds, but when soil particles were present most spores were carried with the soil mineral fractions to the bottom of the tube. This effect was minimised by wet sieving the sample to remove most of the soil before centrifugation. The optimum specific gravity of magnesium sulphate and centrifuge speeds and times were determined and the following method adopted.

The extraction method. The soil is passed through a 4 mm aperture sieve to remove large particles. After thorough mixing of the soil, a 25 g and 100 g sample are taken for spore extraction and for moisture assessment respectively. The 25 g sample is washed with a fine spray through three sieves of decreasing aperture size (1000 μm, 150 μm and 53 μm) and is collected on a 10 μm aperture nylon sieve (Henry Simon Ltd., P.O. Box 31, Stockport). The flow of water through the 10 μm aperture sieve is enhanced by using a vibromixer (A.G. für, Chemie-Apparatebau, Zürich) or by placing a sponge against the underside of the mesh. The sediment remaining is washed onto 250 ml magnesium sulphate (S.G. 1.30, concentration 650g/litre anhydrous $MgSO_4$) in a centrifuge bottle, so that it forms a separate upper layer, and is centrifuged at 39 relative centrifugal force (R.C.F.) for 4 minutes. The supernatant, containing particles between 10 and 53 μm diameter and of specific gravity < 1.30, is immediately poured onto a 10 μm aperture sieve and washed with water. The diluted magnesium sulphate passing through the sieve is collected and reused after adjusting its specific gravity. The suspension containing the spores is washed into a 10 ml tube and centrifuged at 850 R.C.F. for 5 minutes after which the supernatant is discarded leaving the sediment containing the spores in 0.5 ml water. The sediment is mixed by stirring with a pipette while blowing air through it, and a 0.01 ml sub-sample is placed in a small drop of 50% glycerol in water, on a graduated perspex slide, and covered with a coverslip (d. 13 mm); the glycerol reduces evaporation. Spores of *N. gynophila* and *V. chlamydosporium* are counted at 125-200 × magnification. Two or three sub-samples are counted and the mean number of spores per gram air-dried soil calculated.

Resting spores of *N. gynophila* are identified by their size (d. 20 μm), pitted thick wall (4 μm) and spherical (d. 9 μm) central to sub-central reserve body (Fig. 1), see Kerry & Crump (1980).

Efficiency of the method in recovering spores from different soils

Large numbers of *N. gynophila* and *V. chlamydosporium* spores were obtained by washing a bulk of soil from Butt Close, Woburn through the sieves but not centrifuging on magnesium sulphate as above. The spore/soil suspension was mixed with a vibromixer and 20 ml aliquots pipetted onto three replicates each of a 100 g sample of a sandy loam, clay and peat soils (9 samples in total). Three 20 ml replicates of the spore/soil suspension were used for determining the number of spores being added, and three 100 g samples of each soil were included for determining the residual numbers of spores present.

In this test the samples were processed as described above except that 100 g, not 25, g soil samples were wet sieved and the sediment on the bottom sieve (10 μm aperture) was washed into a beaker in 100 ml of water, mixed with the vibromixer, and a 20 ml sub-sample pipetted onto the magnesium sulphate. After centrifuging the sediment was washed on a 10 μm aperture sieve, re-suspended on magnesium sulphate and centrifuged again, the supernatant being kept separate for each spore count. This was repeated until no more spores were recovered; in practice this was achieved after resuspending the sediment from the initial extraction four times (five suspensions in all).

Comparison of recovery of spores from 25 g and 100 g soil samples. Because samples of 100 g of soil were difficult and time consuming to wash through the sieves but smaller samples might result in greater variation between counts, the variability between samples was measured using a sandy loam and clay soil both known to contain *N. gynophila* and *V. chlamydosporium.*

Each soil was screened through a 4 mm aperture sieve and thoroughly mixed. Five replicates of 25 g and 100 g samples were taken for each soil (20 samples in total). The 100 g samples were washed through the sieve and sub-sampled as in the previous test whereas the 25 g sub-samples were extracted in total. For all samples the sediments from the 10 μm aperture sieve were suspended on magnesium sulphate twice, both supernatants being combined for counting.

Survey of N: gynophila *and* V. chlamydosporium *in fields infested with* H. avenae *and* H. schachtii

25 g soil samples from 23 fields known to be infested with the nematodes were extracted as above. Nematode numbers were determined by extracting cysts from moist soil in a fluidising column (Trudgill, *et al.*, 1972) as described by Kerry (1975). The spore and nematode densities, and cropping histories are in Table IV.

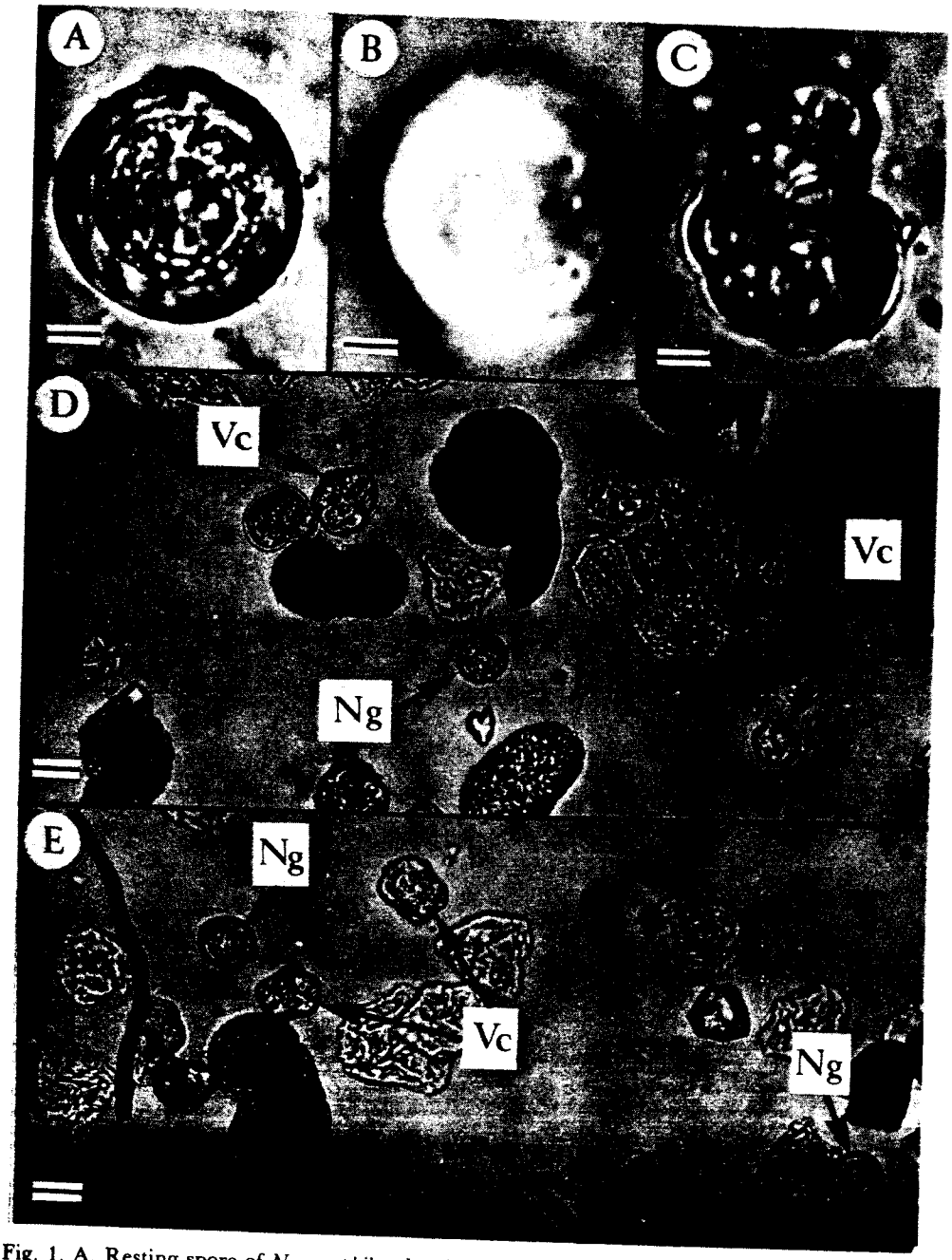

Fig. 1. A, Resting spore of *N. gynophila*, showing spherical central to sub-central reserve body; B, Resting spore of *N. gynophila*, showing pits on the surface; C, Chlamydospore of *V. chlamydosporium*; D, E, Spores of *N. gynophila* (Ng) and *V. chlamydosporium* (Vc) amongst debris on counting slide. A-C, scale bar = 5 μm; D, E, scale bar = 20 μm.

RESULTS

Efficiency of the method in recovering spores from different soils

All the spores of *N. gynophila* (Table I) and *V. chlamydosporium* (Table II) added to soil were recovered and soil texture did not affect the number of spores extracted. Most spores ($p < 0.001$) of both fungal species were recovered in the initial extraction, and few were found after the second re-suspension on magnesium sulphate. After one re-suspension 89% (82-94) of *N. gynophila* and 78% (71-86) of *V. chlamydosporium* spores were recovered from all soils.

TABLE I

The number of* N. gynophila *spores/g soil recovered after being added to three soils of different texture, (means of three replicates)*

	Spore suspension	Sandy loam soil	Peat soil	Clay soil	Mean	± SE_D
Initial extraction	101	75	67	82	81	
Re-suspension 1	32	52	55	50	47	
Re-suspension 2	6	12	18	9	11	6.5
Re-suspension 3	3	5	5	3	4	***
Re-suspension 4	1	1	3	2	2	
Soil mean	28	29	30	29		
± SE_D		5.8 N.S.				

*** = indicates a significant difference at $p < 0.001$. * = number of spores recovered is determined by subtracting the total recovered from the number of spores already present in each soil.

TABLE II

The number of* V. chlamydosporium *spores/g soil recovered after being added to three soils of different texture, (means of three replicates)*

	Spore suspension	Sandy loam soil	Peat soil	Clay soil	Mean	± SE_D
Initial extraction	52	47	54	29	45	
Re-suspension 1	20	32	31	25	27	
Re-suspension 2	8	11	22	16	14	4.3
Re-suspension 3	3	5	11	3	6	***
Re-suspension 4	1	1	2	2	2	
Soil mean	17	19	24	15		
± SE_D		5.8 N.S.				

*** = indicates a significant difference at $p < 0.001$. * = number of spores recovered is determined by subtracting the number of spores already present in each soil from the total recovered.

Comparison of recovery of spores from 25 g and 100 g soil samples

When 25 g and 100 g samples of sandy loam soil were extracted, the numbers of *N. gynophila* and *V. chlamydosporium* spores recovered was similar, although the 25 g samples gave greater variation between replicates (Table III). A greater number ($p < 0.001$) of *N. gynophila* spores was extracted from the 100 g sample of the clay soil, but sample size did not affect the recovery of *V. chlamydosporium*.

Survey of N. gynophila *and* V. chlamydosporium *in fields infested with* H. avenae *and* H. schachtii

In the survey of 23 soils (Table IV) those where *H. avenae* was decreasing had small nematode populations and large spore counts whereas in soils where the nematode was increasing, populations were greater and spore counts smaller. No previous data were available on nematode population changes for the eight soils from Woburn or from the two soils with sugar beet in rotation from Broom's Barn. In most soils, but not those at Woburn, the density of *V. chlamydosporium* spores was greater than that of *N. gynophila*. Four soils infested with *H. avenae* in microplots at Rothamsted, treated with formalin (38% formaldehyde) at a rate of 3,000 l/ha for five years, contained few spores and many nematode eggs. In untreated soils, the nematode populations failed to multiply when cereals were grown continuously and many spores were extracted from soil.

TABLE III

Comparison of the number of spores/g soil of N. gynophila *and* V. chlamydosporium *extracted from 25 g and 100 g soil samples, (means of five replicates ± S.E.)*

	Clay loam soil		Clay soil	
	25 g	100 g	25 g	100 g
N. gynophila	161 ± 19.4	163 ± 15.9	12 ± 1.2	18 ± 2.8
V. chlamydosporium	78 ± 9.0	74 ± 4.4	16 ± 1.8	15 ± 3.3

DISCUSSION

All the spores not recovered in the first extraction were recovered after four re-suspensions, but this was time consuming and re-suspending the sediment once was sufficiently accurate, and adding the supernatant to that from the initial extraction, left only one spore count per sample. This shortened method recovered approximately 80% of the spores of *N. gynophila* and *V. chlamydosporium* added to soil. Whether such an efficient recovery is possible from field soils, where the spores may be bound to soil particles, although most soil aggregates are dispersed during wet sieving, is uncertain. 25 g soil samples were processed more rapidly than 100 g samples with little increase in varia-

TABLE IV

The spore density of N. gynophila (N.g.) *and* V. chlamydosporium (V.c.) *in soils containing* H. avenae *and* H. schachtii

Site	No. of samples	Cropping history	Nematode status*	Mean eggs/g soil	Mean *N.g.* spores/g soil	Mean *V.c.* spores/g soil
Woburn	8	Intensive cereals	*H. avenae*	4(0-24)	53(0-143)	21(4-46)
Overton	1	Intensive cereals	*H. avenae* in decline	<1	35	365
Overton	2	Intensive cereals	*H. avenae* increasing	18	5	40
Herriard	1	Intensive cereals	*H. avenae* damaged patch	—	0	29
Microplots	4	Intensive cereals untreated	*H. avenae* in decline	13(1-26)	180(11-407)	312(23-463)
Microplots	4	Intensive cereals, + formalin	*H. avenae* increasing	76(28-113)	9(0-31)	39(3-74)
Broom's Barn	1	Continuous sugar beet	*H. schachtii* increasing	18	9	33
Broom's Barn	1	Sugar beet in rotation	*H. schachtii*	30	0	96
Broom's Barn	1	Sugar beet in rotation	*H. schachtii* + *H. avenae*	6	63	22

* Based on population changes in previous years.

bi... etween replicates and so these were used in the standardized method (...).

...the survey, differences in spore numbers were observed between soil ...ples; large spore densities being associated with decreasing nematode ...opulations. In pot experiments, poor nematode multiplication has been associated with heavy female parasitism (Kerry & Crump 1977; Kerry *et al.*, 1980). Formalin increased multiplication of *H. avenae* and Williams (1969) suggested that it was removing a competitor or parasite that was limiting reproduction. Later, Kerry *et al.* (1980) showed that formalin killed fungi parasitic on females and eggs of *H. avenae*. In the survey, fewer spores were

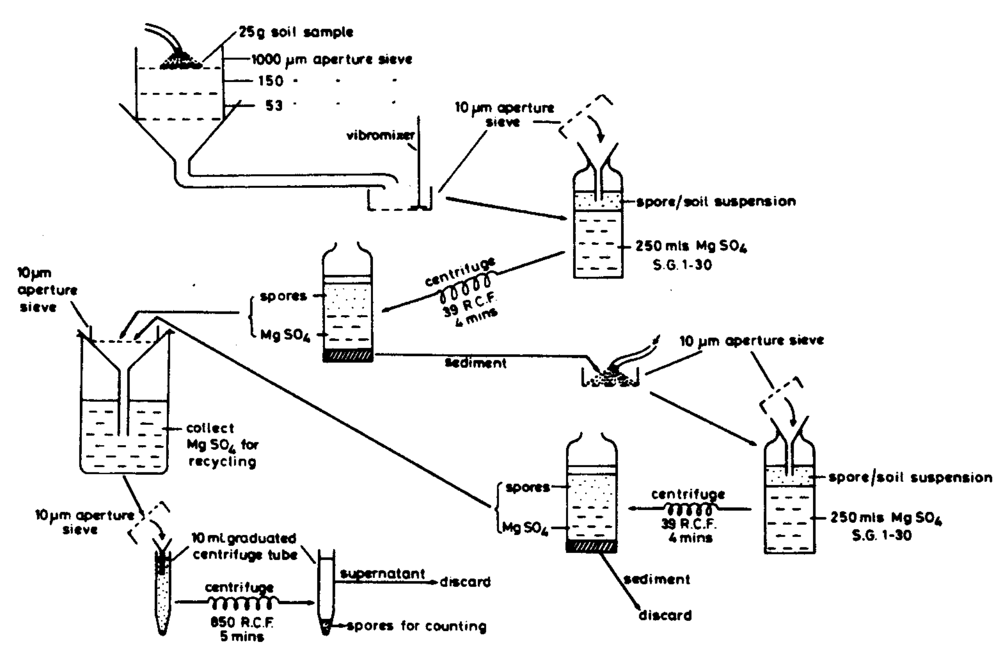

Fig. 2. Summary of the method for extracting spores of *N. gynophila* and *V. chlamydosporium* from soil.

extracted from microplots which had been treated with formalin, and these had denser nematode populations than untreated microplots. Spores of both fungi were found at Broom's Barn where sugar beet had been grown continuously, and infected females of *H. schachtii* Schmidt were observed in pots containing soil from the same site (Crump, unpublished data). This is the first recording of *N. gynophila* in soil not infested with *H. avenae*.

Previously *N. gynophila* could only be detected by growing a susceptible host plant in field soil and examining females on roots for parasitism; a method that was not quantitative and took several weeks. The method described provides a rapid, quantitative technique for assessing the spore densities of *N. gynophila*

and *V. chlamydosporium* in soil and with a little experience one person can process about four samples per day.

Using this method spores of *N. gynophila* have been extracted from soil infected with *H. avenae* in Denmark, *H. schachtii* in Holland and Poland, and *H. glycines* Ichinohe in U.S.A. Spores of *V. chlamydosporium* were found in the samples from Denmark and Holland.

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ZUSAMMENFASSUNG

Eine quantitative Methode zur Gewinning von Dauersporen zweier nematodenparasitärer Pilze, Nematophthora gynophila *und* Verticillium chlamydosporium, *aus dem Boden*

Es wird eine Methode zur quantitativen Extraktion der Sporen von *Nematophthora gynophila* und *Verticillium chlamydosporium* aus dem Boden beschrieben. Diese beiden Pilze können einen Populationsanstieg des Getreidezystenächens, *Heterodera avenae*, beim Anbau von Wirtspflanzen verhindern. Die Bodenprobe wird in feuchtem Zustand gesiebt und die Sporen durch Zentrifugieren in einer konzentrierten Magnesiumsulfatlösung abgetrennt. Die Sporensuspension wird unter einem Mikroskop bei 125-facher Vergrößerung direkt ausgezählt. Rund 80% der drei Böden unterschiedlicher Textur zugesetzen Sporen wurden mit diesem Verfahren wiedergefunden.

Bei einer Untersuchung von 23 verschiedenen Böden war die Anzahl der Sporen beider Pilze dort hoch, wo sich *H. avenae* an Wirten nicht vermehrte und dort gering, wo die Nematoden zunahmen oder Schäden anrichteten.

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