

AN AGAR DISC METHOD FOR ISOLATION OF FUNGI COLONIZING NEMATODE EGGS

A. K. Culbreath, R. Rodríguez-Kábana, and G. Morgan-Jones

Department of Botany, Plant Pathology and Microbiology, Auburn University, Agricultural Experiment Station, Auburn, Alabama 36849, U.S.A.

Accepted:

17.X.1984

Aceptado:

ABSTRACT

Culbreath, A. K., R. Rodríguez-Kábana, and G. Morgan-Jones. 1984. An agar disc method for isolation of fungi colonizing nematode eggs. *Nematropica* 14:145-154.

A method was developed for estimating the level of fungal colonization of nematode eggs in soil and for isolation of involved fungi. A 1.4-mm mesh fiberglass screen was glued between two 5.6-cm diam rings cut from polyvinyl chloride (PVC) pipe. Together, the rings formed a 1-cm long cylinder bearing a screen across its middle. The cylinder was filled with water agar bearing *Meloidogyne arenaria* eggs in suspension. Cylinders were buried singly in pots of soil treated with two levels of chitin (0 or 2% w/w) and 6 levels of sucrose (0 to 2%). After 10 days the rings were retrieved, and eggs in the agar were examined for fungal colonization. Eggs in discs buried in soil with chitin had higher rates of fungal invasion than eggs buried in soil with no chitin. Representative colonized eggs were removed and placed on chitin agar to isolate and identify associated fungi. Fungi encountered were mostly those previously known to be consistently associated with eggs of heteroderid nematodes. A field study with the method demonstrated a significant level of egg colonization by fungi in a soil with 5 different fertilizer treatments; soils deficient in N had higher levels of egg colonization than those with adequate N fertilization.

Additional key words: biological control, nematode pathology, methodology, population dynamics, fungal and nematode ecology.

RESUMEN

Culbreath, A. K., R. Rodríguez-Kábana, y G. Morgan-Jones. 1984. Un método con discos de agar para aislar hongos colonizadores de huevos de nematodos. *Nematropica* 14:145-154.

Se desarrollo un método para aislar hongos de huevos de nematodos y poder determinar el grado de colonización de los huevos por los hongos en el suelo. Con estos fines se pegó un pedazo de malla de fibra de vidrio [perforaciones cuadradas de 1.4 mm de yado] entre dos anillos de tubería de cloruro de polivinilo (CPV) de 5.6-cm de diámetro para formar un cilindro de 1-cm de altura con la malla en el medio. Después de tapar herméticamente la base del cilindro con una tapa de plástico se virtió dentro del mismo una suspensión de huevos de *Meloidogyne arenaria* en agar-agua (1.0%, p/p) tibia (30 C). Los cilindros con el agar solidificada se enterraron en macetas con suelo que había sido tratado con 2 niveles de quitina (0 y 2% p/p) y 6

concentraciones de sacarosa (0-2% p/p). Después de 10 días se recuperaron los anillos y después de lavarlos se sacaron dos discos de agar de cada cilindro descartándose uno. Se examinó cada disco para determinar el número de huevos colonizados. Los huevos de discos provenientes de suelo con quitina mostraron un grado de colonización más alto que los provenientes de discos enterrados en suelos sin la enmienda de quitina. También se tomó una muestra representativa de los huevos colonizados para aislar los hongos presentes usándose para ello un agar con quitina. La mayoría de las especies fungosas aisladas resultaron ser conocidas como asociadas a los huevos de nematodos en la familia Heteroderidae. Un experimento de campo en un suelo con 5 niveles de fertilización demostro que existía un nivel significativo de colonización de los huevos por los hongos y que el nivel fué más alto en parcelas deficientes en N que en las que tenían un nivel adecuado de fertilización con N.

Palabras claves adicionales: combate biológico, patología de nematodos, metodología, dinámica poblacional, ecología de nematodos.

INTRODUCTION

Eggs of plant-parasitic nematodes belonging to the genera *Heterodera* Schmidt and *Meloidogyne* Goeldi, when exposed to the soil, may sometimes be colonized by opportunistic, soil-borne fungal species, particularly those belonging to the form-class Deuteromycetes (1, 2, 4, 11). Isolation and recognition of these fungi usually has been performed by incubation of colonized eggs on some suitable agar medium (1, 2, 3, 4) following surface sterilization. *Heterodera* eggs were extracted from cysts, and those of *Meloidogyne* from disintegrating root gall tissue bearing egg masses. Systematic exploration of soils for fungi capable of colonizing eggs and quantification of fungal colonizing activity have not been possible due to lack of appropriate methodology. Ideally a procedure should be able to measure the ability of fungi to colonize eggs *in vivo* in a given soil. Methods developed for studying fungal activity range from the use of buried glass slides coated with selective agar media to the use of capillary tubes and nylon gauze impregnated with substrate (7). These procedures aim to provide information on hyphal growth in particular, rather than document the level of fungal inoculum, some of which may be in the form of ungerminated fungal spores. Information on the latter aspect is more easily obtained by dilution plate techniques. Johnson and Shamiyeh (6) proposed the use of an agar-slide technique to study the destruction of *M. incognita* (Kofoid and White) Chitwood eggs by microbial species in soil. Published methods to study the activities of specific soil microorganisms are frequently complicated, require specialized equipment, or do not provide for adequate gas (O₂) exchange and three dimensional contact between the substrate (e.g. nematode eggs) and the organism (7). In this paper we describe a simple but effective method for sampling soils

for fungi capable of colonizing nematode eggs and for estimating their level of activity.

MATERIALS AND METHODS

Cylinders were made from 5.6-cm-diam PVC pipe, each bearing a median 1.4-mm mesh fiberglass screen. The pipe was cut with a band saw to form rings 0.5 cm high, two rings being used to make each cylinder. A piece of the fiberglass screen was glued between the 2 rings to form a cylinder 1 cm long with the screen through the middle (Fig. 1). After the glue dried, excess screen on the outside of each cylinder was trimmed off and the cylinders were submersed for 30 min in 10% (v/v) Chlorox® [alkaline 5.25% (w/w) NaOCl] followed by washing in running tap water overnight to remove all traces of glue solvent and Chlorox®. The cylinders were then air-dried. Each cylinder was closed at one end with a tight-fitting plastic lid (Solo No. 603 P, Solo Cup Company, Chicago, Illinois, USA). Molten (approx. 30 C) 1% (w/v) water agar containing 150 µg/ml streptomycin sulfate and *Meloidogyne arenaria* (Neal) Chitwood eggs was poured into the closed cylinders. The agar was allowed to solidify after which the lid was removed from each cylinder and excess agar scraped off. *M. arenaria* eggs were obtained from tomato roots as described by Godoy et al. (3) and the aqueous egg suspension was mixed with 2% (w/w) water agar containing streptomycin sulfate added just prior to pouring the agar; this provided 100-200 eggs/cylinder.

Prepared cylinders were buried for 10 days, one per pot, in a series of 10-cm-diam 1-L capacity pots containing 1 kg of a soil from a peanut field near Headland, Alabama. The soil was a sandy loam (pH 6.2, less than 1% organic matter) and was amended with several levels of chitin, sucrose, or combinations of both materials. The soil was treated with two levels (w/w) of chitin [0 and 2%] and 6 levels of sucrose: 0, 0.25, 0.5, 1.0, 1.5, and 2.0% (w/w). There were 8 replications per treatment in a factorial design. After 10 days, the cylinders were removed from the soil, washed, and two agar discs were removed from each cylinder. The bottom agar disc was scraped away with a curved spatula to allow the top disc to be lifted from the cylinder. Nematode eggs in the top disc were examined with a microscope for colonization and the percentage of eggs bearing fungi was recorded. Ten invaded eggs from each disc were removed and placed, two per dish, on acidified 0.2% chitin agar containing 150 µg/ml of streptomycin sulfate. The dishes were incubated for 72 hr at 25 C, then examined, and the fungi associated with the eggs were identified.

The effectiveness of the method for determining the level of colonization of *M. arenaria* eggs by fungi under field conditions was tested in a

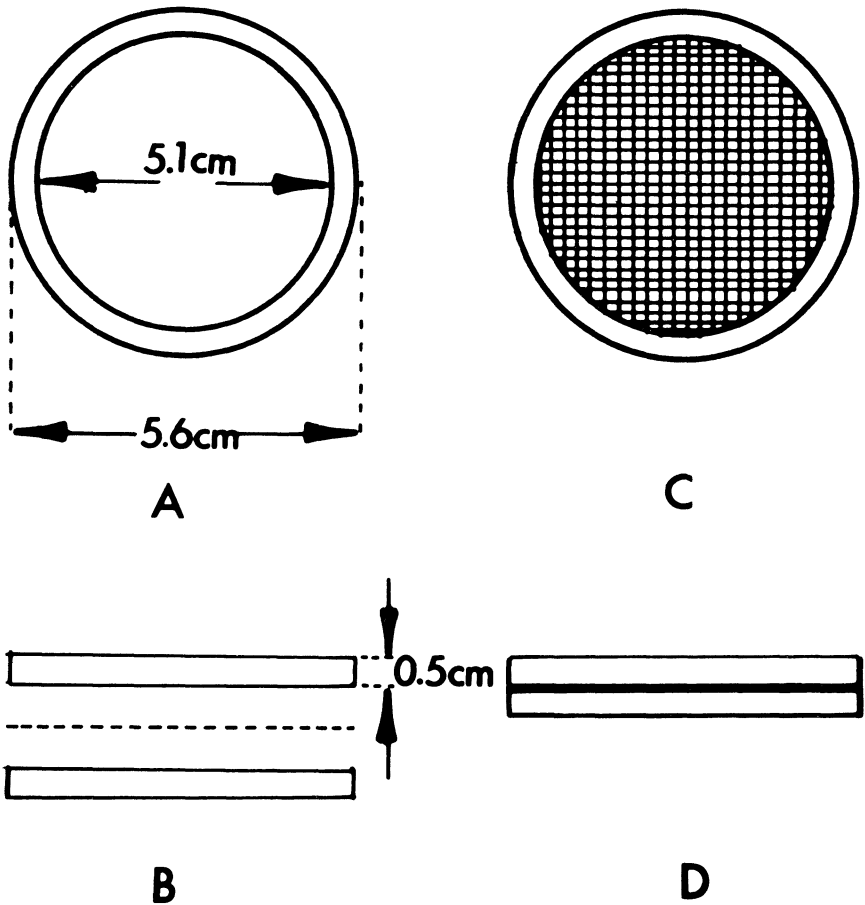


Fig. 1. Diagram showing dimensions and parts used to make cylinders for the agar disc method. A. PVC ring; B. Side view of PVC rings and fiberglass screen in the middle; C, D. Top and side views of finished cylinder.

field experiment at the Agronomy farm near the Auburn University campus. The experiment was a fertility study known as the Cullars' rotation, established in 1905 to compare the effect of several fertilization regimes on the yields of cotton, corn, soybean, and wheat. Fertilization treatments included the additions of mineral fertilizers for nitrogen, phosphorus, potassium, and minor elements. Some treatments in the study included green manuring by turning under a mixture of 'Autauga' crimson clover and common vetch in the spring every third year. A detailed description of the experiment has been published (9). Plots in the

experiment were 6 x 29 m. The discs were buried in June 1984 in plots with 'Auburn' 56 cotton. Five fertilization treatments were chosen for the test (Table 1). In each plot a total of 10 cylinders prepared as described were buried to a depth of 18 cm following a completely random arrangement. Following burial, the soil around each cylinder was moistened (approx. 60% field capacity). After 10 days the cylinders were removed and the agar discs with the eggs were examined as described for the chitin amendments study.

All data were analyzed following standard procedures for analysis of variance (10); Fisher's least significant differences were calculated also following standard procedures.

RESULTS

Agar discs with *M. arenaria* eggs retained their shape and clarity after 10 days in the soil. Colonization of the eggs by fungi was readily observed and it was possible to estimate the degree of invasion. Data on percent of *M. arenaria* eggs colonized by fungi are presented in Table 2. Factorial analysis of the data revealed no significant interaction between the effects of sucrose levels and chitin concentrations on the degree of colonization of the eggs. Sucrose amendments had no effect on colonization of the eggs, but colonization was significantly ($P = 0.01$) increased by the addition of chitin to the soil.

The number of fungal species colonizing eggs in soil without chitin was higher than the number for soil with chitin. However, with some

Table 1. Fertilization treatments in a field experiment chosen to assess the effectiveness of the disc method for determining differences in the level of colonization of *M. arenaria* eggs by fungi in response to soil fertility.

Mineral Fertilization ^z	Green Manure ^y	Factor Studied
N P K	+	Complete fertilization
N K	+	Phosphorus deficiency
P K	+	Organic nitrogen
P K	-	Nitrogen deficiency
None	-	No fertilization

^zN(kg/ha) as $\text{NH}_4\text{NO}_3 = 135$; P at 75 kg P_2O_5 /ha; K at 100 kg K_2O /ha.

^y+ = green manure (common vetch + crimson clover); - = no green manure.

Table 2. Effect of sucrose and chitin amendments to soil on colonization by fungi of *Meloidogyne arenaria* eggs embedded in water-agar discs buried in the amended soil.

Percent sucrose added	Percent of eggs colonized	
	0% chitin	2.0% chitin
0.00	21.95	25.80
0.25	15.49	23.06
0.50	16.77	23.85
1.00	22.36	22.90
1.50	18.78	17.85
2.00	18.04	26.50
LSD (P = 0.05)		6.24
LSD (P = 0.01)		8.28

exceptions the species found in soil without chitin were the same as those in soil with chitin amendment. The most common fungal species colonizing the eggs, irrespective of type of amendment, were: *Chaetomium* spp., *Fusarium oxysporum* Schlecht., *F. solani* Mart., *Humicola fuscoatra* Traaen, *Paecilomyces lilacinus* (Thom) Samson, *Penicillium* spp., *Phoma eupyrena* Sacc., *Sporothrix schenckii* Hektoen and Perkins, and *Trichoderma harzianum* Rifai. *Arthrobotrys oligospora* Fres., *Fusarium moniliforme* Sheldon and *Malbranchea aurantiaca* Sigler and Carmichael were isolated only from eggs in discs buried in chitin-amended soil, whereas *Cladosporium sphaerospermum* Penz., *Gliocladium roseum* Bain., and *Xenokylindria obovata* Morgan-Jones were encountered only in discs buried in soils without chitin. The amount of sucrose added exerted no effect on the frequency of occurrence of fungal species in soil with or without chitin.

Results from the field study (Fig. 2) indicated that soils that received no fertilization or nitrogen had higher percentages of egg colonization than the other soils of the study; there were no other significant differences in egg colonization in response to soil fertility. The species of fungi observed colonizing the eggs in this study were essentially the same as those observed in the chitin amendment experiment.

DISCUSSION

The method described permits isolation of fungi associated with colonized eggs and an estimation of egg colonization in different soils. To

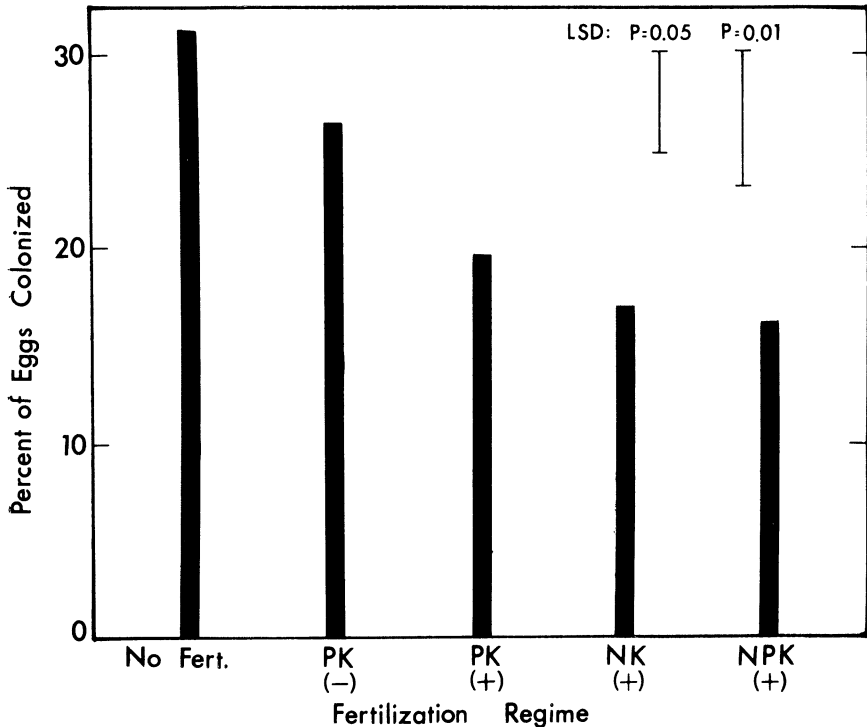


Fig. 2. Relation between fertilization regime and the level of colonization of eggs of *M. arenaria* by soil fungi in a field experiment at Auburn, Ala. (+ = green manure; - = no green manure).

contact the agar discs, and subsequently colonize the embedded eggs, hyphal growth into the agar must be taking place. Water agar was used so that the primary source of nutrient would be the eggs. Other agar media can be used when the goal is to provide selectivity. In other studies (unpublished) we have, however, noticed that when chitin agar (3), or sugar-based media were used, the discs disintegrated and could not be satisfactorily recovered after 1 week. The method can be used with eggs of other nematode species and could be extended for use in the study of colonization of cysts of *Heterodera*. Following recovery and study, the discs can be dried and retained as a permanent record.

Most species of fungi isolated in the sucrose/chitin study have been observed colonizing eggs of *Meloidogyne* spp., and eggs and cysts of *Heterodera glycines* Ichinohe in previous studies (1, 2, 4). The method described here is useful as a means of determining which nematode colonizing fungi are present in a particular soil. Our results also indicate

that the disc method can be used to determine the effects of various amendments on the spectrum of fungal species participating in nematode egg colonization. We were able to detect a decrease in the number of species implicated in colonization in response to chitin amendments to the soil. This was expected since chitin amendments are known to selectively stimulate development of a specific chitinolytic soil mycoflora (5, 8).

Results from the field study indicated that a significant level of colonization of eggs of *M. arenaria* existed in all soils tested. The results also indicated that the method can be used to detect differences between treatments in the level of egg colonization by fungi. Although the fertility study was very limited in scope, the data obtained do suggest that N deficiency in a soil may be stimulatory of the fungi capable of colonizing eggs of *M. arenaria*. A possible explanation may be that in N-deficient

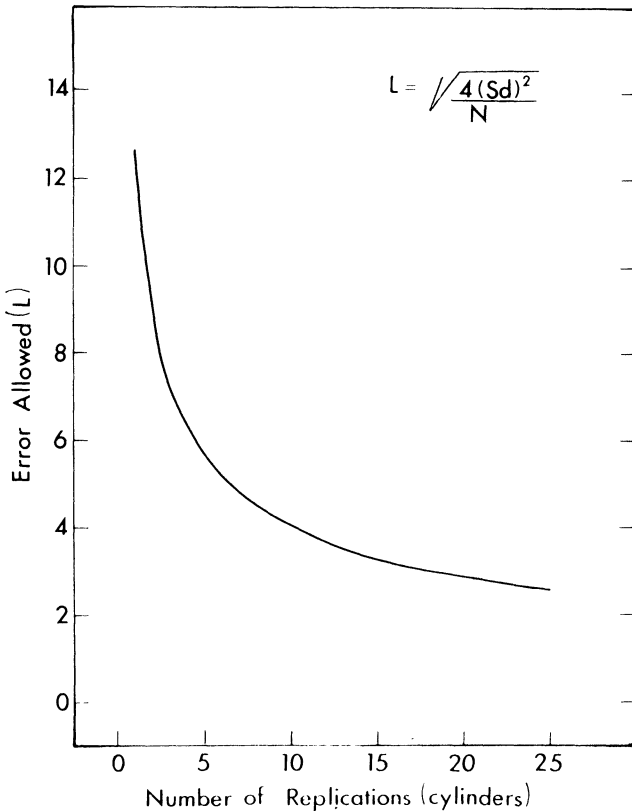


Fig. 3. Relation between allowable error (L) and number of replications (N) at 5% level of probability (Sd = standard deviation).

soils, organisms with enzyme systems capable of decomposing complex organic matter (e.g. chitin) to release bound N may be more numerous than in soils with more complete fertility. This hypothesis will need further testing.

Data from the field experiment were used to calculate the relation between the number of replications (N) and allowable error (L) using the formula (10):

$$L = 4(Sd)^2/N$$

where Sd = the standard deviation. Our calculations indicated that 8-10 cylinders per soil could be sufficient to detect significant ($P=0.05$) differences of 4-5% between soils (Fig. 3). It is likely that these figures will be different for other soils and we recommend that estimation of the number of replications needed for individual soils be made.

We make no claim that our water agar disc method accurately reflects a natural situation where colonization of eggs in masses or singly is concerned. It documents the presence of fungi capable of colonizing eggs and gives some estimate of what might be occurring in a given soil where natural egg masses are exposed to fungal antagonists.

LITERATURE CITED

1. GINTIS, B. O., G. MORGAN-JONES, and R. RODRIGUEZ-KABANA. 1982. Mycoflora of young cysts of *Heterodera glycines* in North Carolina soils. *Nematropica* 12:295-303.
2. GINTIS, B. O., G. MORGAN-JONES, and R. RODRIGUEZ-KABANA. 1983. Fungi associated with several developmental stages of *Heterodera glycines* from an Alabama soybean field soil. *Nematropica* 13:181-200.
3. GODOY, G., R. RODRIGUEZ-KABANA, and G. MORGAN-JONES. 1982. Parasitism of eggs of *Heterodera glycines* and *Meloidogyne arenaria* by fungi isolated from cysts of *H. glycines*. *Nematropica* 12:111-119.
4. GODOY, G., R. RODRIGUEZ-KABANA, and G. MORGAN-JONES. 1983. Fungal parasites of *Meloidogyne arenaria* eggs in an Alabama soil. A mycological survey and greenhouse studies. *Nematropica* 13:201-213.
5. GODOY, G., R. RODRIGUEZ-KABANA, R. A. SHELBY, and G. MORGAN-JONES. 1983. Chitin amendments for control of *Meloidogyne arenaria* in infested soil. II. Effects on microbial population. *Nematropica* 13:63-74.
6. JOHNSON, L. F., and N. B. SHAMIYEH. 1968. Evaluation of an agar-slide technique for studying egg hatching of *Meloidogyne incog-*

- nita* in soil. *Phytopathology* 58:1665-1668.
7. JOHNSON, L. F., and E. A. CURL. 1972. *Methods for research on the ecology of soil-borne plant pathogens*. Burgess Publishing Co., Minneapolis, MN. 247 pp.
 8. MIAN, I. H., G. GODOY, R. A. SHELBY, R. RODRIGUEZ-KABANA, and G. MORGAN-JONES. 1982. Chitin amendments for control of *Meloidogyne arenaria* in infested soil. *Nematropica* 12: 71-89.
 9. RODRIGUEZ-KABANA, R., and R. J. COLLINS. 1979. Relation of fertilizer treatments and cropping sequence to populations of two plant parasitic nematodes. *Nematropica* 9:151-166.
 10. STEEL, R. G. D., and J. D. TORRIE. 1960. *Principles of Statistics*. McGraw-Hill Book Co., New York. 481 pp.
 11. TRIBE, H. T. 1979. Pathology of cyst-nematodes. *Biol. Rev.* 52:477-507.

Received for publication:

27.VIII.1984

Recibido para publicar: