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Optimization of Mitochondrial DNA-based Hybridization Assays to Diagnostics in Soil[1]

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Abstract: Nucleic acid hybridization among root-knot nematode mitochondrial DNAs can be used to identify several *Meloidogyne* species. Research was initiated to optimize mitochondrial DNA-based molecular diagnostics for the demanding environments likely to be encountered in field isolates. DNA hybridization using reconstituted DNA-soil mixtures revealed a loss of assay sensitivity ranging from 34% to 92% with four agronomic soils tested. This problem was alleviated by the addition of exogenously added DNA. Variation in nematode egg lysis procedures also affected hybridization efficiency, with NaOCl treatment most effective at disrupting *Meloidogyne* eggs. These optimized conditions permit detection of mtDNA released from one to five *Meloidogyne* eggs using standard nucleic acid hybridization procedures.

Key words: DNA hybridization, *Meloidogyne* spp., mitochondrial DNA, root-knot nematode.

Reliable detection and identification of *Meloidogyne* spp. populations are essential prerequisites for effective management. Accurate diagnosis of root-knot nematodes has generally relied on discrimination among anatomical features such as perineal pattern and stylet structure (5,7) or on host-range testing (7). However, variable morphology and the occurrence of intraspecific races that propagate only on defined plant hosts have confounded identification procedures (9). Recently, biochemical approaches including serology (13), protein gel electrophoresis (6,14), and monoclonal antibodies (20) have been employed that complement information assembled from available taxonomic characters.

Physical analysis of nematode DNA also has proven useful in distinguishing among several *Meloidogyne* spp. (3,4). Because of its rapid evolution and high cellular copy number, mitochondrial DNA (mtDNA) provides a most convenient marker for nematode populations (10). Nematode mtDNA analysis has been employed to estimate genetic divergence among sibling species of soybean cyst nematodes (17) and four root-knot nematode species (16).

We have evaluated previously the utility of mtDNA as a reagent in the development of molecular diagnostics for *Meloidogyne* populations (15). The successful application of molecular approaches to problems of nematode detection and identification will require adapting these fastidious procedures to analysis of crude field samples. We describe here the optimization of nucleic hybridization assays in an environment likely to be encountered in field samples. Specifically, we address the effect of various soil types on our ability to detect the presence of a limited number of nematode eggs.

Materials and Methods

Nematode cultivation: Meloidogyne incognita and *M. chitwoodi* were maintained on tomato (*Lycopersicon esculentum* L. cv. Tropic), pepper (*Capsicum frutescens* L. cv. California Wonder) and wheat (*Triticum aestivum* L. cv. NuGaines). *Meloidogyne incognita* eggs were isolated as described by Hussey (8).

DNA labeling and hybridization: Meloidogyne spp. mtDNA was isolated by isopycnic centrifugation as described previously (15). Restriction enzymes were purchased from a variety of commercial sources and used according to manufacturers' recommendations. Purified plasmid or mtDNA was linearized by restriction enzyme cleavage,

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TABLE 1. Soil varieties tested in hybridization assays.

Soil variety	Description
Field 15A	Coarse sandy loam
UC mix	50% blow sand, 50% peat and fir bark
UCLA krilium #2	Pelleted clay soil
Peat and sand	50% organic material, 50% sand
Blow sand	11% 1–2 mm, 87% 0.1–1 mm, 2% < 0.1 mm
Silica sand	Fine sand, 0.25–0.1 mm

denatured by adding one-third volume of 1.5 M NaOH and 4.5 M NaCl (12), and immobilized onto nitrocellulose filters with a Bio-Rad dot blot manifold. Plasmid or mtDNA was ^{32}P-labeled in vitro by nick-translation (18). Hybridization and autoradiography were performed as described previously (15). Hybridization signal intensities from the resultant autoradiographs were quantified with a LKB UltroScan XL laser densitometer interfaced with integrative software. Several independent exposures were analyzed to ensure measurements were obtained within the linear response range of the X-ray film.

Detection of egg mtDNA in soil: Eggs were counted and added to a mixture of 100 mg soil, 50 µl of water, and 100 µl of 5% NaOCl. Because of the uncompromising specificity of DNA hybridization, use of autoclaved soil was not required. After gentle mixing, samples were incubated at room temperature for 1 hour. Fifteen microliters of 6.7 M $Na_2S_2O_3$ was added to neutralize the NaOCl. Alternatively, eggs were broken by freezing egg–soil suspensions at −80 C for 2 hours followed by thawing at room temperature (one freeze–thaw cycle). Seventy microliters of denaturation solution (4.5 M NaCl, 1.5 M NaOH) was added and the preparations were boiled for 10 minutes. After brief centrifugation to remove particulate matter that might otherwise clog filter pores, DNA in the supernatant was immobilized on nitrocellulose paper and hybridized with ^{32}P-labeled mtDNA as described.

Effects of soil on mtDNA hybridization assays: To quantify how contaminating soil might compromise the sensitivity of mtDNA-based diagnostic assays, reconstituted preparations containing known quantities of purified DNA and several soil types were tested. Our soil types varied with respect to granularity, degree of desiccation, and clay content (Table 1). The bacterial plasmid pBR322 (21) was used in these initial mixing experiments because it is easily obtained in preparative yields.

Increasing amounts of linearized pBR322 plasmid DNA were mixed with various soils and the resultant preparations were immobilized on nitrocellulose paper. Filter-bound DNA was hybridized with ^{32}P-labeled pBR322 DNA, and the degree of hybridization was monitored by the intensity of the autoradiographic signal.

DNA-soil binding assay: To investigate the reduction in assay sensitivity by added soil, highly purified *M. chitwoodi* mtDNA was radiolabeled and mixed with type 15A soil (Arlington sandy loam). Type 15A is of a coarse, sandy loam consistency representative of agronomic soils in western Riverside County, California. The recovery of exogenously added DNA was then followed through each step of our assay procedure. *Meloidogyne chitwoodi* mtDNA labeled with ^{32}P was added to 100 mg soil and mixed with either 250 µl water alone or 150 µl water plus 100 µl 5% NaOCl. The suspension was then incubated for 1 hour at 25 C. Fifty microliters of 6.7 M $Na_2S_2O_3$ was subsequently added. After boiling the sample for 10 minutes, the soluble and particulate fractions of the sample were separated by centrifugation for 30 seconds in an Eppendorf microcentrifuge. Radioactivity retained in the resultant supernatant and pellet was determined by liquid scintillation counting with aqueous scintillant.

Egg lysis: An additional parameter influencing the sensitivity of mtDNA-based molecular diagnostics is the efficient lysis of target organisms within field isolates for optimal exposure of cellular DNA. *Meloidogyne incognita* eggs were systematically

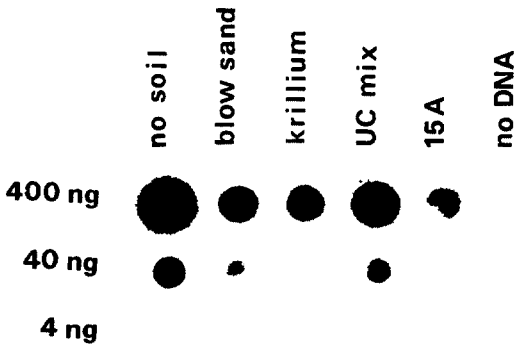

FIG. 1. Effects of soil samples on hybridization efficiency. Vertical columns designate soil samples; horizontal rows indicate amount of pBR322 target DNA added to reconstituted mixtures. Faint signals detectable by laser densitometry were observed with 40 ng of DNA in the krilium and 15A soil samples, as well as in the 4 ng no soil control preparation but do not appear in this reproduction.

subjected to enzymatic treatment with lysozyme (E.C. 3.2.1.17, 500,000 U/mg), papain (E.C. 3.4.22.2, 60 U/mg), chitinase (E.C. 3.2.1.14, 150 U/mg), chymotrypsin (E.C. 3.4.21.1, 90 U/mg), and pronase (164 U/mg) either individually or in combination. All enzymes were purchased from Boehringer Mannheim with the exception of chitinase (United States Biochemicals). Physical insult of eggs (freeze–thaw cycles or nitrogen pressure cell explosive decompression) or exposure to 2.65% (v/v) NaOCl for 2 hours were also evaluated as lysis techniques. Efficacy of egg breakage and concomitant dissolution of juveniles encased within eggs was monitored by microscopic inspection.

The efficacy of egg lysis in the presence of soil was monitored using filter hybridization assays. *M. incognita* eggs were hand counted, mixed with soil type 15A, and lysed by either bleach or freeze–thaw treatment. The samples were annealed with ^{32}P-labeled *M. incognita* mtDNA, and hybridization signals were visualized by autoradiography.

RESULTS

Reduction in hybridization assay sensitivity by soil: When 400 ng of pBR322 target DNA was mixed with various soils, DNA was most easily detected in the presence of either the UC mix, blow sand, or UCLA krilium, whereas soil sample 15A extensively inhibited hybridization (Fig. 1).

Densitometric measurements revealed that 40 ng of pBR322 DNA can be detected in UC mix at 66% of the "no soil" control level. Corresponding values were 49% with blow sand, 32% with UCLA krilium, and only 8% with 15A. Similar relative results were obtained with 4 ng of pBR322 added to these soils.

Partitioning of DNA between aqueous and soil phases: Results of ^{32}P-mtDNA partitioning into particulate and soluble fractions during sample preparation are itemized in Table 2. In four separate trials, approximately 77% of the exogenously added mtDNA was recovered in the supernatant and would be available for hybridization assays. Assay sensitivity would therefore be diminished about 23% as a direct consequence of this specific clearing step in our sample preparation procedure. We tested the possibility that target DNA was nonspecifically interacting with soil components by adding excess nonradioactive calf thymus (ctDNA) directly to soil samples along with ^{32}P-labeled *M. chitwoodi* mtDNA. If nematode DNA was adventitiously binding to soil particles, we anticipated that ctDNA would effectively compete for these nonspecific binding sites and release additional nematode DNA into supernatant, thereby increasing the availability of target DNA for hybridization assays. Recovery of ^{32}P-labeled *M. chitwoodi*

TABLE 2. Recovery of ^{32}P-labeled *Meloidogyne chitwoodi* mtDNA in cleared supernatants.

Components	DNA recovered (%)†	
mtDNA alone	100	(4)
mtDNA + NaOCl	100	(4)
mtDNA + soil	76.7 ± 3.2	(4)
mtDNA + soil + NaOCl	73.1 ± 2.4	(4)
mtDNA + soil + ctDNA	88.7 ± 0.2	(2)

^{32}P-labeled *M. chitwoodi* mtDNA was mixed with reconstituted components as described in text, including calf thymus (ct) DNA.

† Values represent mean ± one standard deviation. Number of trials is indicated in parentheses.

TABLE 3. Lysis of *M. incognita* (race 1) eggs.

Treatment	Hours	Lysis
Lysozyme, 0.5 μg/μl	16	None
5.0 μg/μl	16	None
Pronase, 50 μg/μl	48	None
Papain, 100 μg/μl	48	None
Chitinase + chymotrypsin, 20 mg/ml of each	0.5 min	None
Pretreatment with 0.75% NaOCl (15 min) then chitinase + chymotrypsin	33 min	100%
2.65% NaOCl	2	100%
Freeze–thaw, one cycle		30%
NO_2 explosive decompression		None

Results were judged by microscopic observation.

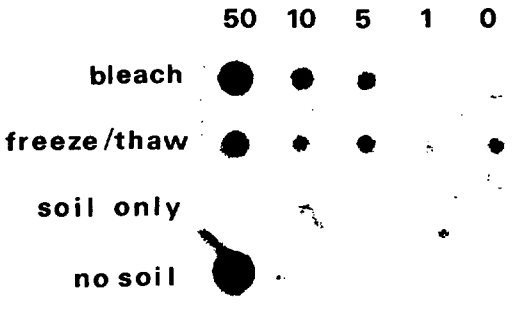

FIG. 2. Detection of *Meloidogyne incognita* egg mtDNA by filter hybridization. Vertical columns designate precise number of eggs employed in each sample. Horizontal rows indicate lysis treatment. The spot in the 0 freeze–thaw preparation (lacking eggs) is an artifact, but it serves to demonstrate that caution must be exercised when interpreting hybridization experiments.

mtDNA in the supernatant fraction significantly improved to 88% when calf thymus DNA was added to the sample (Table 2).

Egg lysis: Efficacy of egg breakage, including lysis of cells from juveniles developing within individual eggs, was monitored by microscopic inspection. Among the experimental lysis conditions tested, 30% of the eggs were lysed by freeze–thaw (one cycle) while total egg breakage occurred after a 30-minute incubation at room temperature in 2.5% NaOCl (Table 3).

When efficacy of the lysis procedures was evaluated with hybridization assays on nitrocellulose filters, the strongest hybridization signal was obtained from egg DNA immobilized to filters in the absence of soil (Fig. 2, bottom row, first sample). Notably, this prominent signal was observed only in a control sample preparation of high egg density that was not subjected to lysis treatment, indicating that annealing must have occurred with target mtDNA released from a few damaged eggs present within this preparation. This result further exemplifies the inhibitory behavior of soil in these hybridization assays. (Compare this result with row 1, in which eggs were efficiently lysed but a reduced level of mtDNA was detected in these soil mixtures.) Unambiguous detection of mtDNA was also observed in the presence of soil after the entire mixture had been subjected to bleach treatment (Fig. 2, row 1) or exposed to a single freeze–thaw cycle (Fig. 2, row 2). In each of these experiments, signals were reliably obtained with five or more eggs. Hybridization to DNA derived from one egg was observed on occasion but was not reproducibly detected.

Densitometric quantification of mtDNA hybridization varied among multiple trials of these experiments, although reproducible trends were recognized (Fig. 2). If the signal generated by 50 eggs in the absence of soil is arbitrarily assigned 100%, then the amount of hybridization from the same number of eggs with soil was 37% when samples were incubated in NaOCl, 9.3% when eggs were treated by a single freeze–thaw cycle, and 2.7% when egg breakage was not attempted. These results were consistent with findings made by direct microscopic observation of egg lysis (Table 3).

DISCUSSION

We have evaluated mtDNA-based hybridization technology for nematode egg identification within agronomic soils. This work extends the results of several investigations that established the feasibility of distinguishing among *Meloidogyne* spp. by nematode mtDNA analysis (15,16). These studies, however, involved hybridization experiments with highly purified mtDNA.

Therefore, it became necessary to refine hybridization technology and to adapt these diagnostic reagents to crude field samples.

Diagnostic analysis of field isolates probably would encounter substantial contamination with soil or plant debris. It has been demonstrated repeatedly that root material does not interfere with DNA hybridization when several nematodes are embedded within galls or cysts (2,15). Therefore, we focused on how soil might affect the hybridization reaction. Different types of soil altered the sensitivity of this assay to various degrees. Arlington Sandy Loam 15A inhibited hybridization to the largest extent and was subsequently employed to present the most difficult of conditions with which to refine these assays. The loss of hybridization sensitivity in the presence of soil is due in part to nonspecific binding of target DNA with soil components. This problem can be reduced by adding a large excess of nonhomologous DNA which cannot be exclusively provided by the soil microflora. By competition with nematode DNA in these samples, the exogenously added nucleic acid can increase the amount of target mtDNA available on filters for hybridization.

Several procedures are currently available for extraction of nematodes and eggs from soil (1). Enrichment of target organisms prior to hybridization analysis might provide an important alternative toward circumventing the inhibitory effects of soil in mtDNA-based hybridization assays. However, adaption of nematode extraction methodologies should not compromise the rapid, simultaneous processing of numerous samples in easily manipulated volumes which are advantages characteristic of molecular diagnostic assays. Given that we cannot reliably detect one egg reconstituted with 100 mg soil, a level that would otherwise represent a considerable population density in the field, nematode enrichment would appear to be a useful step preparatory to hybridization analysis. Centrifugal flotation (11) may be most readily interfaced with the mtDNA-based hybridization protocols described in this report, as a single step employing the microcentrifuge would be added to our processing procedure. It is recognized, however, that at low population densities, inefficient enrichment may result in a negative response that might otherwise generate a positive hybridization signal if the sample remained unfractionated.

New diagnostic tests for plant-pathogenic nematodes must be able to detect all life stages of the parasite. Eggs are among the most difficult of nematode life stages to lyse efficiently. We reasoned that if assay conditions were refined to effectively detect egg mtDNA in the presence of contaminating soil, more easily manipulated nematode forms such as infective second-stage juveniles could also be identified. Several chemical and mechanical treatments were tested to lyse eggs and improve detection of nucleic acids. A simple treatment with NaOCl (bleach) was the most effective. Given the high lysis efficiency observed with NaOCl, a combination of bleach and ctDNA as additives to sample preparations currently presents the most sensitive of hybridization assay conditions.

We have demonstrated that five nematode eggs could be detected with soil contamination. Given successful hybridization with a limited number of *Meloidogyne* adults (15) and the elevated concentration of mtDNA in nematode oocytes (19), we posit that one egg should be reliably detected. Our inability to detect fewer than five eggs in a reproducible fashion probably reflects incomplete lysis of eggs in these reconstituted samples.

We recognize that the most useful diagnostic assay should reliably detect a single nematode within randomly selected field samples. Sensitivity at this level may be particularly relevant to nematode populations of limited density, as when overwintering in agronomic soils. Employment of the procedures described in this report coupled with established enrichment techniques (1) should ultimately permit unambiguous detection of infectious root-knot nematodes within crude field preparations.

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