A Quantitative Method for Estimation of Teliospores of *Tilletia indica* in Soil

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ABSTRACT

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A bubbling-flotation-sieving method utilizing 50% glycerol was developed for the extraction of teliospores of *Tilletia indica* from artificially and naturally infested soil. A standard curve was prepared to estimate the number of teliospores recovered from soil artificially infested with about 50–5,000 teliospores per 10 g of soil. A $\log_{10}$ transformation stabilized the variances of the number of teliospores recovered relative to the number actually present in soil. A prediction equation then was developed to estimate the number of teliospores in a naturally infested Mexican soil (X) based on the number of teliospores recovered (Y): $\log_{10} X = (\log_{10} Y + 2)/1.47$. The mean number of teliospores in Mexican soil was estimated to be between 476 and 1,617 per 10 g of soil. The sample sizes required for margins of error within 5, 10, 15, and 20% of the mean number of teliospores recovered also were determined. This methodology for the extraction and enumeration of teliospores of *T. indica* from Mexican clay soil will be useful in future studies on the ecology and epidemiology of the fungus.

Karnal bunt or partial bunt of wheat (*Triticum aestivum* L.) caused by *Tilletia indica* Mitra (= *Neovossia indica* (Mitra) Mundkur) was first reported and described on wheat in India in 1931

(10,18). The pathogen since then has spread to Iraq, Mexico, Nepal, and Pakistan (18).

Teliospores of *T. indica* are introduced naturally into the soil by infected or contaminated seed and debris at harvest (5,10). These persistent propagules are reported to remain viable on seed in soil up to 45 mo (11,18). Teliospores on the soil surface will germinate, producing a promycelium bearing a whorl of about 65–185 primary sporidia (10). These sporidia or mycelia from primary sporidia will produce secondary sporidia and infect the wheat floret, causing Karnal bunt under conducive environmental conditions (10,18).

Little information is available on the survival of Karnal bunt teliospores at different soil depths and with different crop rotations (11). No information exists on the distribution of teliospores of *T. indica* in soil or on how many teliospores are needed to initiate infection. This paucity of information is due partly to the lack of a method to quantify the inoculum density of this pathogen. The objective of this study was to develop an inexpensive and reliable method for quantitatively extracting teliospores from the soil so that populations could be estimated within Mexican wheat fields. This method then can be used to determine the relationship between teliospore density and disease incidence and to study the ecology of this pathogen. Preliminary reports of this work have been published (5,6).

MATERIALS AND METHODS

Soil collection. A commercial wheat field near Cuidad Obregon, Sonora, Mexico, was selected on the basis of a previous report of high incidence of Karnal bunt. A portion of the field was divided into 90 contiguous 3 × 3 m quadrats from which 45 were sampled systematically before harvest (mid-April 1986) based on the methods of Punja et al (13). Twenty samples of clay soil (pH 8.0; organic matter 1.5%; 22% sand, 30% silt, 48% clay) were taken randomly throughout each quadrat with a soil sampling auger (Forestry Supplies Inc., Jackson, MS 39204) 2.03 cm in diameter inserted to a depth of 7.5 cm. Samples from each quadrat were placed into a polyethylene bag, labeled with the quadrat coordinates, and carried to Frederick, MD, under permits issued by PPQ-APHIS and the Maryland Department of Agriculture. The samples were assayed in the containment laboratory and greenhouse facilities of the USDA-ARS, Foreign Disease-

Weed Science Research Unit (12).

Since uninfested soil was not collected in Mexico, a Houston black clay (pH 7.9; organic matter 2.7%; 19% sand, 31% silt, 50% clay) similar in texture to the Mexican soil was obtained from C. L. Girdner, USDA-SCS, Temple, TX. Soils from both locations were sieved through a 2-mm (No. 10 mesh) sieve and allowed to air-dry on a greenhouse bench for 5–7 days.

Inoculum and standard curve preparation. One-year-old teliospores were removed, using the method of Royer and Rytter (14), from infected seeds of the

Table 1. Recovery of teliospores (TS) of *Tilletia indica* from artificially infested, nonsterile Houston black clay soil by the bubbling-flotation-sieving method

Actual no. of TS/10 g soil[a]	TS recovery		
	Mean no./ 10 g soil[b]	Range/ 10 g soil	%
41.5	10.9	0–72	26.4
180.5	40.8	0–114	22.5
443.0	100.7	0–277	23.3
907.5	328.1	82–762	38.1
4,727.5	2,426.8	1,254–4,513	52.0

[a] Number of TS added to the soil by atomization; residual TS found in atomizing tube and adhering to the polyethylene bag were subtracted from estimated TS. Values represent means from two experiments. TS per 10 g of soil was based on oven-dry (gravimetric) soil weight determined by drying soil at 105 C for 24 hr.

[b] Mean recovery for each value was determined from four TS counts for each of three 10-g soil samples for each of four replications.

wheat cultivar Sonalika that had been infected with *T. indica* obtained from Mexico. Teliospores were enumerated using a Hawksley eelworm cell (Hawksley-Sons, Ltd., Lancing, West Sussex Bn15 8TN, England) with a holding capacity of 1 ml. Predetermined quantities of teliospores then were applied consistently by atomization into 100 g of sieved Houston black clay soil samples contained within polyethylene bags. These artificially infested (AI) soil samples subsequently were mixed by hand. Estimated teliospore populations in AI soil samples were about 0, 50, 200, 500, 1,100, and 5,000 teliospores per 10 gm of soil. However, the actual number of teliospores determined to have been added to the soil by atomization was calculated by subtracting the residual teliospores found in the atomizing tube and adhering to the polyethylene bag from each estimated teliospore concentration (Table 1). The number of teliospores per 10 g of soil was based on oven-dry (gravimetric) soil weight determined by drying soil at 105 C for 24 hr. Teliospore recovery at each inoculum level from AI soil was determined from four teliospore counts for each of three 10-g soil samples for each of four replications. Teliospore recovery from naturally infested Mexican soil was determined from four teliospore counts for each of three 10-g soil samples per quadrat. Teliospore counts represent the number of times a 1-ml sample from infested soil is examined using a Hawksley eelworm cell.

Extraction procedure. The procedure described herein was a modification of the methods of Daniels and Skipper (4)

and Furlan and Fortin (7). A 350-ml fritted-glass filter funnel (Thomas Scientific Co., Swedesboro, NJ 08085-0099) was extended from 7.75 to 15.5 cm by a glassblower. The fritted-glass funnel had an internal diameter of 8.8 cm, a medium porosity, and a pore size ranging from 10 to 15μm. The glass funnel was mounted onto a Flexaframe support set at a height of 610 mm (Thomas Scientific Co.). The glass funnel narrowed down to a small opening for compressed air to enter. This opening was attached by vacuum tubing to a Bourdon pressure gauge connected to a compressed air outlet. The compressed air was regulated to 15 psi. Between 60 and 70 ml of a 50% glycerol solution (v/v) (J. T. Baker Chemical Co., Phillipsburg, NJ 08865) was poured into the funnel, and, subsequently, the equivalent of 10 g oven-dry weight of infested soil was placed into the bubbling solution (Fig. 1). Because soil particles adhered to the sides of the glass funnel during the bubbling, the insides of the funnel were gently washed with 50% glycerol discontinuously during the process. After the soil suspension had been agitated for 5 min, the air pressure was turned off. While the liquid was settling, the sides of the funnel were again gently rinsed with 50% glycerol. The suspension was allowed to settle for 10 min. The supernatant then was removed by suction with a 36-mm-diameter Pasteur capillary pipette attached to a series of two-flask systems connected to a Gast rotary portable air pump (Thomas Scientific Co.). The supernatant was removed to a level approximately 5 mm above the settled soil. The bubbling, settling, and supernatant-removal procedure was repeated two more times with clean glycerol solution for each sample to maximize teliospore recovery. Finally, the internal surface of the Pasteur pipette and tubing were rinsed thoroughly by plunging the pipette tip into two washes each of 100 ml of distilled water.

Teliospores were recovered for counting by pouring the combined supernatant through a 20-μm nylon sieve that was backwashed with distilled water. The concentrated teliospores in the sieve were collected in ≈ 20 ml of water in a 50-ml beaker. The teliospores were pipetted onto a Hawksley eelworm cell for enumeration at 100×. Funnels were cleaned between extractions with 2.625% NaOCl and rinsed with 1–3% sodium thiosulfate (Sigma Chemicals, St. Louis, MO 63178) and two washes of distilled water for 5 min each, respectively.

Teliospore germination. Teliospores were germinated to determine viability and to confirm identification. Initially, the concentrated teliospore suspensions from AI Houston black clay and naturally infested Mexican soil were placed into 15-ml centrifuge tubes, and 0.1 ml of a 0.5% NaOCl solution was
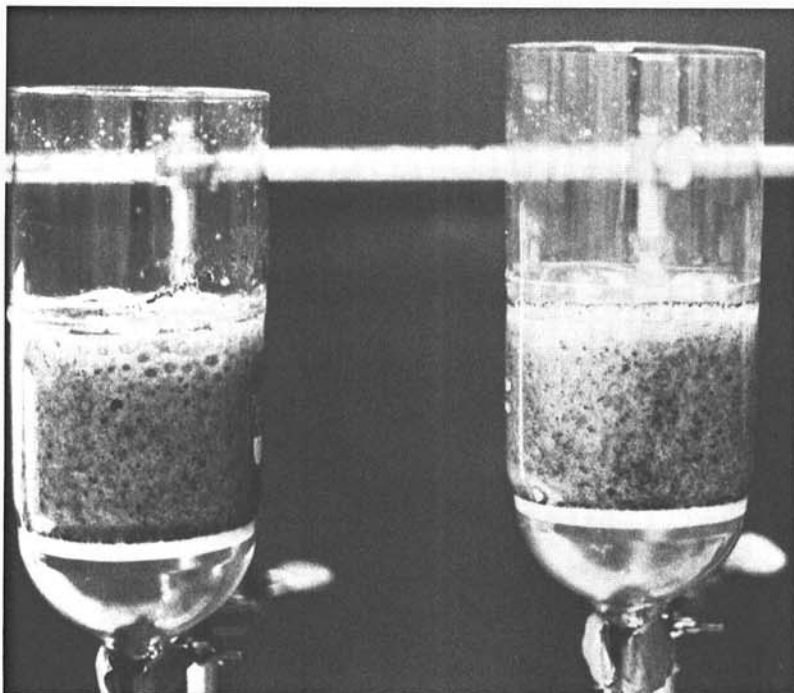

Fig. 1. Fritted glass filter funnels containing 10 g each of infested soil in 60–70 ml of a bubbling 50% glycerol solution.

added to prevent the spores from adhering to the wall of the tube during centrifugation. The suspension was centrifuged for 2 min at 3,500 g to pellet the teliospores. This pellet was resuspended in 0.5% NaOCl for 2 min and rinsed twice by centrifugation with distilled water. A 0.1-ml aliquot of the teliospore suspension was plated onto 2% water agar amended with 100 mg/L of streptomycin sulfate. These plates were incubated under alternating 12 hr light/12 hr dark at 15 C (16). Teliospores were examined for germination over a 10-day period 4–14 days after initiation of incubation.

Each standard curve and teliospore germination experiment was repeated at least once.

RESULTS

Successful recovery of teliospores for quantitative studies was possible using the bubbling-flotation-sieving procedure described. The number of teliospores recovered relative to the number added increased with increasing inoculum densities (Table 1). The percentage of teliospores recovered was not equal across estimated inoculum densities of 50–5,000 teliospores per 10 gm of AI soil. In addition, variances were not constant over inoculum densities as determined by heterogeneity of the plot of residuals vs. predicted values when estimated inoculum densities were regressed on actual inoculum densities (15). To stabilize the variances, a log transformation [$\log_{10}$ (teliospores + 1)] was used, since one of the assumptions for linear regression is to have a common (constant) variance. After transformation, regression coefficients for the two experiments were compared by Student's t test and were not significantly different at $P = 0.05$. Data from the two experiments were combined, and the linear regression of teliospore recovery per 10 gm of AI soil (Y) on teliospore populations per 10 gm of AI soil (X) was computed. The regression solution was: $\log_{10} X = (\log_{10} Y + 2)/1.47$ (Fig. 2). This prediction equation was used to estimate the number of teliospores per 10 gm in naturally infested Mexican soil (X_1) based on the number of teliospores per 10 gm recovered (Y_2). From this equation the estimate of X_1 was determined by (17) (Table 2): $X_1 = \bar{X} + q$, where $\bar{X} = [\log_{10}$ (mean teliospore population)/10 gm soil, 2.63], $q = \bar{Y} - Y/b$, where $Y = \log_{10}$ teliospore recovery, $\bar{Y} = \log_{10}$ (mean teliospore recovery, 1.86), and b = slope, 1.47. The 95% confidence limits for X_1 was determined by (17) (Table 2): $x = X_1 \pm (ts/b)(1 + 1/n + q^2/\Sigma x^2)^{\frac{1}{2}}$, where t = 5% level for $(n - 2)$ df, 2.03; s = mean square error, 0.05; b = slope, 1.47; n = sample size, 40; $q^2 = (Y - \bar{Y}/b)^2$; and $\Sigma x^2 = [\Sigma X^2 - (\Sigma X^2/n)]$, 18.93.

The MS error term of soil sample (ss) and MS error term of teliospore counts

(tc) were tested for significance at $P <0.50$. The F test was not significant and the SS_{ss} and SS_{tc} were combined to calculate a new MS_{tc}. Afterward, analysis of variance was performed to examine the variation within inoculum levels (replications) and between samples (teliospore counts) at inoculum levels (Table 3). Thus, the required degree of precision in terms of the margin of error of mean teliospore recovery per 10 g of soil sampled could be determined. In such a case, sample size is computed by (8): $N = (Z)^2(V_s)/[r(D)^2(\bar{X})^2 - (Z)^2(V_p)]$, where N = sample size for number of teliospore counts per 10 g of soil; $(Z)^2$ = normal distribution statistic corresponding to the 5% level of significance, 1.96; V_s = experimental error (SS_{error}/df$_{error}$); r = number of replications (number of 10-g soil samples assayed); $(D)^2$ = % margin of error about the treatment mean; $(\bar{X})^2$ = treatment mean; and $V_p = MS_2 - MS_1/n$, where MS_2 = mean square of replicate, MS_1 = mean square of error, and n = number of teliospore counts per replication.

For example, a researcher wishes to determine the sample size for the number of teliospore counts per 10 g of soil for an
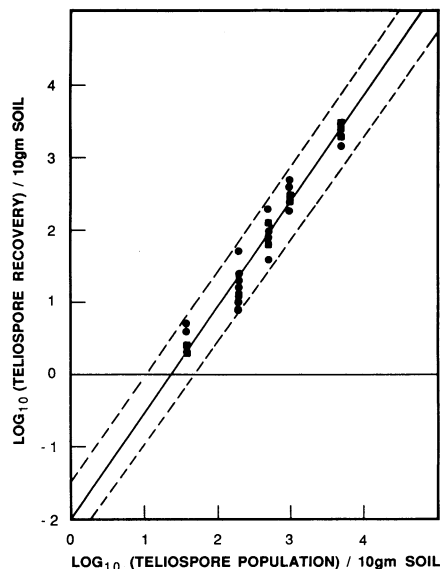
Fig. 2. The linear regression of $\log_{10}$ (teliospore recovery)/10 gm of soil on the $\log_{10}$ (teliospore population)/10 gm of artificially infested Houston black clay soil. The prediction equation, $\log_{10} X = (\log_{10} Y + 2)/1.47$, $R^2 = 0.96$, was used to estimate the mean number of teliospores per 10 gm of naturally infested Mexican clay soil.

Table 2. Estimated mean teliospore population per 10 g of naturally infested Mexican soil from two 9-m^2 nonadjacent quadrats in a wheat field

Teliospore population	Confidence limits
475.8	410.6–551.4
1,617.4	1,393.9–1,876.7

experiment with four replications that can achieve an estimate of the treatment mean within 10% of the true value. Using the variances from Table 3, $V_p = 0.03 - 0.31/12 = -0.02$ and $V_s = 0.31$; and $\bar{X} = 1.86$, $D = 0.1$, and $Z = 1.96$, the sample size for satisfying this requirement at the 10% level of significance is computed as: $N = (1.96)^2 (0.31)/[4 (0.1)^2 (1.86)^2 - (1.96)^2 (-0.02)] = 5.2 \approx$ five teliospore counts per 10-g soil sample. Thus, five teliospore counts per replication should be measured to satisfy the requirement that the estimate of the treatment mean would be within 10% of the true value 95% of the time. Estimated sample sizes for teliospore counts per replication were determined within 5, 10, 15, and 20% of the true value (Fig. 3). These results, however, do not include field sampling error or potential differences resulting from nonuniform samplings.

From naturally and artificially infested soil, all teliospores that germinated produced typical promycelia bearing a

Table 3. Analysis of variance for the $\log_{10}$ number of teliospores of *Tilletia indica* recovered per 10 g of artificially infested soil with four replications and 12 spore counts per replication with the bubbling-flotation-sieving method

Source[a]	df	Mean squares	Expectation of mean square[b]
Replication	3	0.03	$\sigma^2 + 12 \sigma^2_\epsilon$
Error	472	0.31	σ^2
Inoculum levels	4	124.16	...
Total	479	1.35	...

[a] Replication = replication within teliospore concentration, error = pooled error of teliospore counts and soil sample.
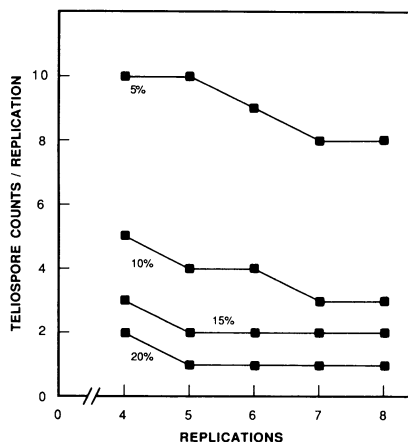[b] σ^2_ϵ = Among-replication variance; σ^2 = variance due to error.

Fig. 3. The relationship between teliospore counts per replication and the number of replications needed to satisfy the requirement that the estimate of the treatment mean would be within 5, 10, 15, and 20% of the true value 95% of the time.

whorl of primary sporidia. Plates were observed to contain ungerminated teliospores, however.

DISCUSSION

These results indicate that the bubbling-flotation-sieving method is effective for extracting and enumerating teliospores in naturally infested soil. The air passing through the fritted disk of the funnel breaks into tiny, well-distributed bubbles that disrupt and mix the infested soil within the 50% glycerol solution. Teliospores are dislodged from the soil and remain in suspension after the air supply is disconnected because the specific gravity of 50% glycerol is higher than that of the teliospores. Glycerol has been successfully used for separating and extracting other soilborne pathogens from soil and has not caused any deleterious effects (1,3).

Datnoff et al (5) reported a higher mean percent recovery of teliospores from AI soil in an earlier study. The soil in the preliminary study had a lower clay content than the soils from Mexico and Texas (25% vs. 48 and 50%, respectively). Teliospores probably bind more to the clay particles than sand, and as teliospore populations decreased in soil, available binding sites increased, making it more difficult to extract the teliospores from the Mexican and Texan soils. Soil texture also has been demonstrated to influence the assay precision for the recovery of vesicular-arbuscular mycorrhizal fungal spores (9). This same general relationship was observed between known inoculum densities and propagules recovered of *Plasmodiophora brassicae* Woron. (2). For this reason, a standard curve will be necessary for soil of each texture where wheat is grown in order to accurately estimate the true mean population of teliospores of *T. indica*.

There was some difficulty in enumeration, because organic particles were the same color and size range as the teliospores. However, teliospore morphology, especially the characteristic episporium adorned with thick, truncated projections, helped to distinguish teliospores from soil particles. In the event of confusion, teliospore identification was confirmed by germination.

The development of this assay was intended for use in countries where Karnal bunt is found. A relatively inexpensive and reliable method was needed. The equipment necessary for establishment of this technique can be purchased for approximately $1,500. Although glycerol was not recycled in our experiments, it could be (7), which would reduce the cost of each soil assay.

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