A Method for Extraction and Enumeration of Teliospores of *Tilletia indica*, *T. controversa*, and *T. barclayana* in Soil

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ABSTRACT

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A sucrose-centrifugation method was developed to extract teliospores of *Tilletia indica*, *T. controversa*, and *T. barclayana* from soil. Six soil types were artificially infested with teliospores of each of the three fungi separately to produce 10^2 , 10^3 , 10^4 , or 10^5 teliospores per 10 g. Each 10 g of infested soil was suspended in 200 ml of water with one drop of Tween 20 and shaken for 30 s. The soil suspension was first passed through a 117- μ m sieve and then through a 53- μ m mesh filter, and the filtrate was collected. The filtrate was then passed through a 20- μ m mesh filter, and materials caught on the mesh were washed into two 50-ml centrifuge tubes and spun for 3 min ($1,200 \times g$). The pellet was suspended in 1.6 M sucrose solution and centrifuged for 40 s ($200 \times g$). The supernatant was passed through a 20- μ m mesh filter. The materials caught on the 20- μ m mesh were collected, and the number of teliospores was determined. This procedure was initially used to extract teliospores of *T. indica* in soil. For extraction of teliospores of *T. controversa* and *T. barclayana*, 1.0 M and 1.3 M sucrose solutions, respectively, were used, and the 20- μ m mesh was replaced with a 13- μ m mesh filter. Teliospores of *T. indica*, *T. controversa*, *T. barclayana*, and *T. indica*-like fungus on rye grass were successfully extracted from naturally infested soils. The relationships between number of teliospores recovered from the soil and number of teliospores incorporated into the soil were $\hat{Y} = -0.60 + 1.28(X) - 0.04(X^2)$, $\hat{Y} = -1.25 + 1.56(X) - 0.07(X^2)$, and $\hat{Y} = -0.71 + 1.33(X) - 0.04(X^2)$ for *T. indica*, *T. controversa*, and *T. barclayana*, respectively, where $\hat{Y} = \log_{10}$ of the number of teliospores recovered from soil and $X = \log_{10}$ of the actual number of teliospores in soil.

Additional keywords: dwarf bunt, Karnal bunt, kernel smut, *Lolium*, *Neovossia horrida*, *Neovossia indica*, *Oryza sativum*, rice, *Triticum aestivum*, wheat

Tilletia indica Mitra (= *Neovossia indica* (Mitra) Mundkur) is the causal agent of Karnal bunt or partial bunt of wheat (*Triticum aestivum* L.) (11). The disease was reported initially from India in 1931 (11) and subsequently has been recorded in Afghanistan, Iraq, Mexico, Nepal, and Pakistan (15). In 1996, Karnal bunt was found in the United States (17) and Iran (5). Teliospores of *T. indica* introduced into the soil at harvest persist up to 45 months in soil (10,15) and are an important source of primary inoculum. Teliospores on the soil surface germinate and produce primary and secondary sporidia, which under conducive environmental conditions cause infection in the plants during the 2- to 3-week period at flowering (14).

In 1996, a *Tilletia* species morphologically similar to *T. indica* was found to cause kernel bunt in rye grass (*Lolium* sp.) in the United States (13). It is yet to be determined whether the causal fungus of

the rye grass bunt is *T. indica* or a new species in the genus *Tilletia*.

Tilletia controversa Kühn is the causal organism of dwarf bunt of winter wheat (4). Dwarf bunt of wheat was first discovered in Montana in 1935 (18) and since then has been reported in Canada, South America, Europe, and Asia. Occurrence of the disease is limited to areas where winter wheat is subject to prolonged snow cover (7). Teliospores of *T. controversa* are deposited on soil at the time of harvesting. The teliospores remain viable in soil up to 10 years. Infection of the plants originates from teliospores germinating under snow at or near the soil surface during seedling emergence (7).

Tilletia barclayana (Bref.) Sacc. & Syd. (= *Neovossia horrida* (Takah.) Padwick & A. Khan) causes kernel smut of rice (*Oryza sativum* L.). The disease was initially described in Japan in 1989 and since has been reported from major rice growing areas of the world (16). Teliospores of *T. barclayana* are released from smutted kernels before or during harvest and contaminate soil. After planting, teliospores float to the surface of paddy water, germinate, and produce primary and secondary sporidia, which are forcibly discharged into the air and carried onto rice panicles. These sporidia infect ovaries

through open florets and cause smutted kernels (16).

Since soilborne teliospores serve as the primary source of inoculum for the initiation of all three diseases mentioned, reliable methods are needed to evaluate survival of the teliospores at different soil depths, to quantify the inoculum density in soil, and to determine density of teliospores needed to initiate infection. There is little information on extraction and enumeration of teliospores of *Tilletia* species in soil. Datnoff et al. (3) developed a bubbling-flotation-sieving method utilizing 50% glycerol to extract and quantify teliospores of *T. indica* in soil from Sonora, Mexico. In our preliminary studies, we found this method to be inefficient in recovering teliospores of *T. indica* from soil.

The objective of this study was to develop a simple and reliable method for evaluating the presence of teliospores of *T. indica* in various agricultural soil types. Teliospores must survive extraction with no significant decrease in germinability. Since working with live teliospores of *T. indica* in Montana was not allowed, we initially used heat-killed teliospores of *T. indica* supplied by our collaborators in the United States Department of Agriculture, Agriculture Research Service, Foreign Disease-Weed Science Research Unit (USDA-ARS-FDWSRU) in Fort Detrick, Maryland. Subsequently, the procedure was evaluated using live teliospores of *T. indica* in the containment laboratory of the USDA-ARS-FDWSRU and by Animal and Plant Health Inspection Service (APHIS) personnel in Arizona. Live teliospores of closely related fungi, including *T. controversa* and *T. barclayana*, were also used to infest soil.

MATERIALS AND METHODS

Teliospores. Teliospores of *T. indica*, *T. controversa*, and *T. barclayana* used for artificial infestation of soil in this study were collected from India, Montana, and Arkansas, respectively. The average diameters (range) of teliospores of *T. indica*, *T. controversa*, and *T. barclayana*, averaged from 20 measurements for each fungus, were 35 μ m (26 to 43 μ m), 20 μ m (17 to 24 μ m), and 23 μ m (19 to 26 μ m), respectively. The average diameter (range) of teliospores of the *T. indica*-like fungus on rye grass, collected from Arizona and Oregon, was 33 μ m (24 to 38 μ m).

Extraction procedure. Five agricultural soils, including clay, clay loam, loam,

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sandy loam, and silty loam, and a sand, collected from various locations in Montana (Table 1), were used initially to develop the procedure for extraction of teliospores of *T. indica* in soil. There was no significant difference between the air-dried weight and the oven-dried weight of six soil types (10:9.7, wt/wt, air-dried/oven-dried) used in this study (Table 1). Thus, to avoid any negative effect of heat on soil structure during oven drying, we used air-dried soil samples.

Teliospores of *T. indica* removed from infected wheat grain produced in Hoshur, India, in 1996 were used in this study. Teliospores were enumerated using the Howard Mold Counting Chamber (Hauser Scientific, Blue Bell, PA). Samples of the six soil types were air-dried at room temperature on a laboratory bench for 14 days and passed through a 2-mm sieve. Predetermined quantities of teliospores were added to soil samples and thoroughly mixed. The artificially infested soil samples were estimated to have 10^2 , 10^3 , 10^4 , and 10^5 teliospores per 10 g of air-dried soil.

Each 10-g infested soil sample was suspended in 200 ml of tap water with one drop of Tween 20 and shaken by hand for 30 s. This soil suspension was passed first through a 117- μ m metal sieve and then through a 53- μ m Spectra/Mesh nylon filter (Spectrum, Houston, TX), and the filtrate was collected. The remaining debris on both sieves was washed using a fine sprinkler, and the filtrate was collected. This suspension (approximately 2 liters) was then passed through a 20- μ m Spectra/Mesh nylon filter. The materials caught on the 20- μ m mesh were washed into two 50-ml centrifuge tubes and spun for 3 min ($1,200 \times g$) using a bench-top Centra-CL2 centrifuge (International Equipment Company, Needham Height, MA). The supernatant was discarded, and the pellet was suspended in 35 ml of 1.6 M sucrose solution. This suspension was centrifuged for 40 s ($200 \times g$), during which it took 17 s for the centrifuge to reach full speed. The supernatant was passed through the 20- μ m mesh, and the pellet was saved. The procedure was repeated three times to maximize the teliospore recovery. The materials caught on the 20- μ m mesh were washed into a 50-ml centrifuge tube and spun for 5 min ($1,200 \times g$). The supernatant was removed using a Pasteur pipette, and the pellet was suspended in 1 to 3 ml of dis-

tilled water, depending on the original number of teliospores added to the soil. The suspension was pipetted onto the mold counting chamber, and the number of spores was enumerated at $\times 100$. Teliospore recovery at each inoculum level from artificially infested soils was determined from three teliospore counts for each of the 10-g soil samples for each of three replications (a total of nine counts for each inoculum level).

Samples of the six soil types were artificially infested with 10^3 live teliospores of *T. indica* per 10 g of air-dried soil. The teliospores had been separated from infected wheat grain harvested in Sonora, Mexico, in 1996. These artificially infested soil samples were tested to extract the teliospores using the sucrose-centrifugation procedure described above. The artificially infested soil samples were also processed for extraction of *T. indica* teliospores using the bubbling-flotation-sieving method developed by Datnoff et al. (3). However, we used a glass beaker and a stirring bar instead of a fritted-glass funnel to extract teliospores in 50% glycerol.

The sucrose-centrifugation procedure described for extraction of *T. indica* teliospores from soil was also used to extract teliospores of *T. controversa* and *T. barclayana* from artificially and naturally infested soils. However, because of the smaller size of teliospores of these two fungi, the 20- μ m mesh used to collect teliospores of *T. indica* was replaced with a 13- μ m Spectra/Mesh nylon filter. Also, because of the lower teliospore density, 1.0 M and 1.3 M sucrose solutions were used to separate teliospores of *T. controversa* and *T. barclayana*, respectively. The six soil types used to develop the procedure for extraction of *T. indica* teliospores in soil were also used to evaluate efficacy of the procedure for extraction of teliospores of *T. controversa* and *T. barclayana* from soil.

Teliospore germination. Teliospores of *T. indica* recovered from artificially infested soil samples at the USDA-ARS-FDWSRU were tested for germination. Teliospores of the original teliospore lot were germinated for comparison. After incubation of the teliospores in 15 ml of distilled water in 50-ml conical centrifuge tubes for 15 h at 20°C, the samples were centrifuged for 3 min ($200 \times g$). The pellets were suspended in 25 ml of 0.525%

sodium hypochlorite solution and immediately centrifuged for 1 min ($1,000 \times g$). The pellets were suspended in 25 ml of sterile distilled water and centrifuged for 3 min ($200 \times g$) to wash the spores. The teliospores were washed two more times. The pellets were then suspended in 1 ml of sterile distilled water, and 200 μ l of each suspension was transferred onto a 2% water agar (Difco Laboratories, Detroit, MI) plate containing 100 mg each of ampicillin and streptomycin sulfate per liter. The plates were incubated at 20°C under fluorescent light (12 h light/12 h darkness) for 12 days, and then teliospore germination was evaluated. Also, teliospores of *T. indica* extracted by using the bubbling-flotation-sieving procedure (3) were plated for germination. Teliospores of *T. controversa*, *T. barclayana*, and *T. indica*-like fungus from rye grass, recovered from soil, were tested for germination on water agar.

Field soil tests. Thirty-five soil samples were collected from 35 plots, each 1,400 m², in one wheat field in Arizona with an incidence of Karnal bunt in 1996, and were tested for the presence of *T. indica* teliospores. Soil samples were collected from seven wheat fields in different locations in Montana with a history of dwarf bunt and were assayed for the presence of *T. controversa* teliospores. Two soil samples collected from two separate rice fields in Arkansas with an incidence of rice kernel smut in 1996 were tested for the presence of *T. barclayana* teliospores. Also, four soil samples collected from four rye grass fields in Oregon and two soil samples collected from rye grass fields in Georgia suspected of having rye grass smut incidence in 1997 were assayed for the presence of teliospores of the *T. indica*-like fungus, using the procedure for extracting *T. indica* teliospores from soil.

RESULTS

Teliospores of all three fungi were successfully recovered from all of the six artificially infested soils. Overall, 70.3, 49.8, and 67.5% of teliospores of *T. indica*, *T. controversa*, and *T. barclayana*, respectively, incorporated into the soil samples, were recovered. Percentage of teliospores of *T. controversa* recovered from the soil was significantly lower than percentage of teliospores of *T. indica* or *T. barclayana*. There was no significant difference between recovery rates of teliospores of *T.*

Table 1. Soil types, collected in Montana, that were used to develop the procedure for extracting teliospores of *Tilletia indica*, *T. controversa*, and *T. barclayana* from soil

Soil texture	Location	Soil pH	Organic matter (%)	Sand (%)	Silt (%)	Clay (%)	Oven-dried wt of 10-g air-dried wt
Clay	Fort Ellis	5.5	4.26	14	39	47	9.6
Clay loam	Gallatin Valley	5.9	5.41	30	38	32	9.6
Loam	Ronan	8.6	1.12	43	48	9	9.9
Sand	Belgrade	9.2	<0.05	95	4	1	9.9
Sandy loam	Creston	7.6	4.10	51	39	10	9.8
Silty loam	Three Forks	7.0	24.37	33	61	6	9.2

indica and *T. barclayana* from the soil. Recovery rates of teliospores of *T. indica*, *T. controversa*, or *T. barclayana* from different soil types were not significantly different.

The average recovery rates of *T. indica* teliospores from six soil types were 0, 47.9, 68.8, 78.6, and 85.9% from soil samples containing 0, 10^2 , 10^3 , 10^4 , and 10^5 teliospores per 10 g, respectively (Fig. 1). Recovery of teliospores of *T. controversa* was 0, 19.3, 41.2, 65.1, and 73.8% from soil samples with 0, 10^2 , 10^3 , 10^4 , and 10^5 teliospores per 10 g, respectively. For *T. barclayana*, percent teliospore recovery was 0, 38.4, 67.0, 76.9, and 87.7 from soil samples containing 0, 10^2 , 10^3 , 10^4 , and 10^5 teliospores per 10 g, respectively. For all three fungi, percent recovery of teliospores from soil samples with 10^2 teliospores per 10 g was significantly lower than recovery from soil samples with 10^3 , 10^4 , or 10^5 teliospores per 10 g. Likewise, significantly lower percent teliospores was recovered from soil samples with 10^3 teliospores than from soil samples with 10^5 teliospores per 10 g. In *T. controversa*, recovery rates of teliospores from the soil samples with 10^3 teliospores were significantly lower than recovery rates from soil samples with 10^4 teliospores per 10 g. There was no significant difference between rates of teliospores recovered from soil samples with 10^4 and 10^5 teliospores per 10 g.

The relationships between number of teliospores recovered from soil and number of teliospores incorporated into the soil were $\hat{Y} = -0.60 + 1.28(X) - 0.04(X^2)$ ($R^2 =$

0.99, $P = 0.01$), $\hat{Y} = -1.25 + 1.56(X) - 0.07(X^2)$ ($R^2 = 0.98$, $P = 0.01$), and $\hat{Y} = -0.71 + 1.33(X) - 0.04(X^2)$ ($R^2 = 0.99$, $P = 0.01$) for *T. indica*, *T. controversa*, and *T. barclayana*, respectively, where $\hat{Y} = \log_{10}$ of number of teliospores recovered from soil and $X = \log_{10}$ of number of teliospores incorporated into soil.

Percent recovery of teliospores of *T. indica* from the artificially infested soils using the bubbling-flotation-sieving method (3) was significantly lower than that using the sucrose-centrifugation procedure (Table 2). The average germination rates (range) of teliospores were 43.2% (34.3 to 55.0%), 29.3% (21.3 to 49.3%), and 48.7% (46.0 to 51.0%) when teliospores were recovered using the sucrose-centrifugation procedure, the bubbling-flotation-sieving method, and from the original teliospore lot, respectively. Teliospores recovered from the soil using the bubbling-flotation-sieving method had significantly lower rates of germination than the rates of either the teliospores recovered from the soil using the sucrose-centrifugation method or the original teliospores.

Teliospores of *T. indica*, *T. controversa*, *T. barclayana*, and *T. indica*-like fungus from rye grass were successfully extracted from naturally infested soils using the sucrose-centrifugation procedure (Table 3). Teliospores of *T. indica* were found in 10 out of 35 soil samples from the wheat field in Arizona, although the level was low. Teliospores of *T. controversa* were extracted from all seven soil samples collected from seven wheat fields in Montana. The number of *T. controversa* teliospores

recovered from the naturally infested soil samples ranged from 16 to 15,250 teliospores per 10 g of soil. Both of the soil samples from the two rice fields in Arkansas were found to be infested with *T. barclayana*. The number of teliospores extracted averaged 11,250 per 10 g of soil. Three out of four soils from rye grass fields in Oregon had teliospores of *T. indica*-like fungus, and up to 15 teliospores per 10 g of soil were extracted. Teliospores of *T. indica*-like fungus were extracted from both of the soil samples from Georgia, and the number of teliospores recovered averaged 21 per 10 g of soil.

DISCUSSION

Density separation in sucrose solution and sieving has long been used to extract spores of mycorrhizal fungi from soil (1,2,8,12). Also, a centrifugal flotation technique using sucrose solution to separate nematodes from soil has been extensively used (9). The sucrose-centrifugation method described here is basically a density-dependent separating method similar to those used for extraction of spores of mycorrhizal fungi and nematodes from soil. Using this method, we are able to recover teliospores of *T. indica*, *T. controversa*, or *T. barclayana* from soil with a spore density as low as 10 teliospores per gram soil. We think that this procedure will be useful in accurately assaying soil for the presence of teliospores in areas with Karnal bunt and dwarf bunt of wheat, rice kernel smut, and rye grass smut, or in any area suspected of having any of these pathogens.

Since there was no significant difference in recovery rates of teliospores of either *T. indica*, *T. controversa*, or *T. barclayana* from all six different soil types, the formulas $\hat{Y} = -0.60 + 1.28(X) - 0.04(X^2)$, $\hat{Y} = -1.25 + 1.56(X) - 0.07(X^2)$, and $\hat{Y} = -0.71 + 1.33(X) - 0.04(X^2)$ can be used to estimate the actual numbers of teliospores of

Fig. 1. Relationship between number of teliospores in 10 g of soil and percent teliospores recovered using sucrose-centrifugation procedure. Data are from tests involving six soil types. For each fungus, columns with the same letter are not significantly different according to Duncan's multiple range test at $P = 0.05$.

Table 2. Efficacy of sucrose-centrifugation method (sucrose) and bubbling-flotation-sieving method (glycerol) in recovering *Tilletia indica* teliospores from artificially infested nonsterile soils^w

Extraction method ^x	Recovery (%)	Germination (%)
Sucrose	74.3 ^y	43.2 ^z
Glycerol	28.2	29.3
Original teliospores	...	48.7
LSD ($P = 0.05$)	6.5	10.4

^wNumber of teliospores incorporated into the soils was estimated to be 1,000 teliospores per 10 g of air-dried soil.

^xThe modified bubbling-flotation-sieving method utilizing 50% glycerol was developed by Datnoff et al. (3).

^yEach number represents the mean of nine spore counts.

^zEach number represents the mean of percent teliospores germinated in three agar plates.

T. indica, *T. controversa*, and *T. barclayana*, respectively, in naturally infested soils. In these formulas, $\bar{Y} = \log_{10}$ of number of teliospores extracted from soil and $X = \log_{10}$ of number of teliospores actually in the soil.

Datnoff et al. (3) reported a negative correlation between percent clay content of soil and percent recovery of *T. indica* teliospores from soil. They mentioned that the teliospores probably bind more to the clay particles than to sand. The results from our studies showed that there was no significant correlation between soil texture and percent recovery of teliospores of either *T. indica*, *T. controversa*, or *T. barclayana*. This may be due to the use of sucrose solution and Tween 20 in our procedure, as Ianson and Allen (8) found that using sucrose and dispersants dissociate and disperse clay particles attached to mycorrhizal fungal spores.

Furlan et al. (6) reported that sucrose was not a good separation medium for spores of endomycorrhizal fungi because spores become dehydrated and collapse. This may affect identification of the fungus and viability of spores. But Ianson and Allen (8) used 2 M sucrose solution to extract spores of vesicular-arbuscular mycorrhizal fungi from soil and found that exposing the spores to sucrose for 24 h did not cause significant damage. In the sucrose-centrifugation procedure, the spores are exposed to a maximum concentration of 1.6 M sucrose for less than 1 min, and we found neither collapse of cell walls nor reduction in germination of teliospores.

The bubbling-flotation-sieving method utilizing 50% glycerol (3) has been the only available procedure for extracting *T. indica* teliospores from soil. We found this procedure less efficient than the sucrose-centrifugation method in recovering teliospores of *Tilletia* species from Montana soils. The sucrose-centrifugation method described here is a simple and inexpensive method and appears to be reliable for extracting teliospores of *T. indica* and other *Tilletia* species from all types of soil. In addition to the six soil types used for artificial infestation with teliospores, live teliospores of *T. indica* were also incorporated into four other different soil types (10^4 teliospores per 10 g), collected from wheat

fields in Montana and Washington, and the spores were successfully recovered (79 to 97%) from the soils (data not presented). Also, researchers employed by the USDA-ARS-FDWSRU in Maryland and USDA-APHIS in Arizona have tested the sucrose-centrifugation method and extracted teliospores of *T. indica* from artificially and naturally infested soils with high efficiency (M. R. Bonde and T. Boratynski, *personal communications*). A comparison of the sucrose-centrifugation procedure with the bubbling-flotation-sieving procedure (3) showed that by using the sucrose-centrifugation method, not only a significantly higher number of *T. indica* teliospores was recovered from soil, but also less debris was associated with teliospores, which makes observation, identification, and enumeration of the teliospores much easier. Furthermore, higher rates of germination of teliospores recovered from soils using the sucrose-centrifugation method is another advantage of this method over the bubbling-flotation-sieving method.

In addition to using the sucrose-centrifugation procedure for extraction of teliospores of *T. indica* and *T. indica*-like fungus on rye grass from soil, we found that by changing the concentration of the sucrose solution and the mesh size, this procedure can be easily adapted to extract teliospores of other bunt and smut fungi from soil. We successfully extracted teliospores of *T. controversa* and *T. barclayana* from artificially and naturally infested soils by using lower concentrations of sucrose and the 13- μ m mesh filter. Also, using a 1.0 M sucrose solution and the 13- μ m mesh filter, we successfully recovered teliospores of *T. foetida*, the causal agent of common bunt of wheat, from artificially and naturally infested soils (data not presented).

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Table 3. Extraction of teliospores (TS) of *Tilletia* species from naturally infested soils

Field type, location	Fungus	TS extraction		Samples ^y tested (no.)	Samples with TS (no.)	Ave. TS/10 g of soil (no.) ^z
		Sucrose solution (molarity)	Mesh size (μ m)			
Wheat, Arizona	<i>T. indica</i>	1.6	20	35	10	0.4 (0-2)
Rye grass, Georgia	<i>Tilletia</i> sp.	1.6	20	2	2	21 (16-26)
Rye grass, Oregon	<i>Tilletia</i> sp.	1.6	20	4	3	8.5 (0-15)
Wheat, Montana	<i>T. controversa</i>	1.0	13	7	7	2,846 (16-15,250)
Rice, Arkansas	<i>T. barclayana</i>	1.3	13	2	2	11,250 (93-22,408)

^y All 35 soil samples from Arizona were from a single wheat field; each of the other samples represents one field.

^z The numbers inside the parentheses are the minimum and maximum numbers of teliospores extracted from 10 g of air-dried soil samples.

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