New Tests to Distinguish Teliospores of Tilletia controversa, the Dwarf Bunt Fungus, from Spores of Other Tilletia species

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ABSTRACT

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The transport of spores of *Tilletia controversa* on wheat shipments from one country to another is of international concern. By either light- or scanning electron microscopy, the morphological features of teliospores of various bunt and smut fungi are similar; thus, new distinctive identification tests were needed. Dry spores of *T. controversa* were shown to be spherical and normal in appearance in anhydrous propanol, whereas dry spores responsible for common bunt and grass smut were aspherical and deformed. A negative staining reaction (spores stained with methylene blue then mixed with India Ink) clearly showed the capsule on spores of smut fungi and aided identification. If the spores germinate in 1 wk on 3% water agar at 17 C, they are not spores of *T. controversa*. Spores of smut fungi first partially hydrolyzed with cellulase and then with protease showed a differential agglutination reaction with the lectin phytohemagglutinin-M, as well as with 4 M NaCl and 0.06 M $MgCl_2$; spores of *T. controversa* agglutinated strongly, whereas those of the common bunt fungus gave a weak agglutination reaction. A periodate oxidation reaction (0.16 M H_5IO_6, 0.1 M sodium acetate at pH 4.5, 57 C for 3.25 hr) removed the reticulum more readily from the outer wall of spores of common and grass smut fungi than from spores of the dwarf bunt fungus. The anhydrous propanol and the negative staining tests are recommended for routine use in identifying spores of smut fungi.

Additional key words: *Tilletia caries*, *T. fusca*, *T. elymi*, *T. holci*, and *T. scrobiculata*.

In the fall of 1973, wheat bunt diseases received international attention in connection with a large sale of wheat to the People's Republic of China. Some of the shipments of wheat to mainland China were contaminated with teliospores of the bunt fungi. Common bunt of wheat, caused by *Tilletia caries* (DC.) Tul., is present in mainland China, but dwarf bunt of wheat, caused by *Tilletia controversa* Kühn, has not been reported in that country. The People's Republic of China set a zero tolerance on spores of *T. controversa* and rejected several shipments of wheat containing spores that looked like those of the dwarf bunt fungus, when viewed with the light microscope. In the winter of 1974, all shipments of wheat from the Pacific coastal states to mainland China were stopped, because of the high probability that most would contain spores of the dwarf bunt fungus.

There is considerable similarity in the external appearance of spores of fungi responsible for dwarf, common, and some grass bunts (4, 5). The morphological overlap between these species is so great that when only a few spores are available for observation they cannot always be positively identified by light- or scanning electron microscopy. Thus, new tests are needed that are sensitive, precise, and rapid and which will differentiate clearly spores of *T. controversa* from those of fungi responsible for common and grass smuts, when only 5-100 spores are available.

Most tests described herein were developed using spores of three pathogenic races of the common bunt fungus, three races of the dwarf bunt fungus, and spores of one species of the grass bunt fungus, *Tilletia fusca* Ell. and Ev.. Since there are about 30 pathogenic races of *T. caries*, 17 races of *T. controversa*, and several reticulate-spored species of grass bunt fungi known in the Pacific Northwest, representative spore samples of these biotypes were gathered subsequently and tested by the methods described.

MATERIALS AND METHODS

Teliospores of the common bunt fungus, T-races, were obtained from infected wheat plants in the experimental field plots of R. J. Metzger, Agricultural Research Service, U.S. Department of Agriculture (ARS-USDA),

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Corvallis, Oregon. Teliospores of the dwarf bunt fungus, D-races, and of the grass smut fungi were obtained from J. A. Hoffmann, ARS-USDA, Logan, Utah. Spores were shaken from the broken sori, air-dried, and passed through a series of standard sieves to remove host-plant residues.

The following enzymes were used in hydrolysis of the spore wall: cellulase (type 1), amyloglucosidase, protease (type 6), hemicellulase, and lysozyme from Sigma Chemical Company, St. Louis, MO 63178; cellulase, laminaranase, chitinase, lysozyme, and pronase from Calbiochem Company, LaJolla, CA 92037. The enzyme reaction mixtures contained 50 mg spores and 25 mg enzyme in 50 ml of buffer. Protease and lysozyme reactions were buffered at pH 7.0 with 0.1 M potassium phosphate, and all other enzyme reactions were buffered at pH 5.0 with 0.1 M sodium acetate. All enzyme reactions that lasted longer than 4 hr contained sodium ethylmercurithiosalicylate (Thimersol) at 0.25 mM concentration to prevent microbial contamination.

All lectins used in the agglutination study were obtained from Calbiochem Company. The agglutination reaction mixture contained 1 mg teliospores and 0.1, 1.0 or 10 mg lectin per milliliter of buffer (0.15 M NaCl plus 0.2 M K_2HPO_4 at pH 7.0). The agglutination reactions were rapid, were carried out on microflocculation ring slides at room temperature, and were observed at ×25 - ×75 magnification.

In the periodate removal of teliospore walls, concentration, temperature, pH, and time all influenced the efficiency of the reaction. These four parameters were varied until a set of reaction conditions was found that caused spores of *T. controversa* to appear quite different from the other spore types. The concentrations of periodic acid tested were 0.12, 0.14, 0.15, 0.16, and 0.17 M; the temperatures tested were 55, 57.5, 60, and 70 C; the pH was buffered at 4.5 with 0.1 M sodium acetate; the reaction times tested were 1.5, 2.75, 3.0, 3.25, 3.5, and 4.0 hr. The spore concentration in these reaction mixtures did not exceed 2 mg/ml. The effects of the periodate reactions were observed with the light microscope and the scanning electron microscope.

The experiments that led to the development of the tests described herein were repeated at least three times.

RESULTS

Hydrolysis of the spore wall.—Our first experimental approach was based on the hypothesis that teliospore walls were chemically similar within species and different between species. If such differences exist they may be detected by treating the various spore types with enzymes, known to be capable of hydrolyzing portions of fungal cell walls, and then viewing the spores with the scanning electron microscope.

Based on the chemical constitution of fungal spore walls, seven different enzymes were chosen, each of which was potentially capable of hydrolyzing some of the teliospore wall material. The seven enzymes studied were cellulase, laminaranase, amyloglucosidase, chitinase, protease, hemicellulase, and lysozyme. The substrates hydrolyzed by these enzymes are, respectively: β-1,4 glucans, β-1,3 glucans, α-1,4 and α-1,6 glucans, chitins, proteins, polysaccharides (non-cellulose), and muramic acid polymers.

In addition to treating the spores with the individual enzymes various combinations of enzymes were tested for their combined effects. After 2 to 4 hr in the reaction mixture the spores were filtered from the suspension, dried in a vacuum oven, and viewed with a scanning electron microscope, usually at ×1,000 to ×10,000 magnification. No quantitative measurements were taken, but visual comparisons were made on the degree or extent of spore wall hydrolysis, and the relative effects of these enzyme treatments on the different spores were noted. Many treatments appeared to degrade the spore wall partially, but no enzyme treatment or series of treatments enabled a clear distinction of spores of *T. controversa* from those of *T. caries.* Spores of the grass smut fungi were not studied in this experiment. We tried to obtain an extracellular enzyme preparation that was capable of hydrolyzing spore walls of the bunt fungi by culturing *Trichoderma viride* Pers. ex Fr. on spores of *T. caries* and *T. controversa.* This omnivorous fungus grew poorly on these spores, and a suitable enzyme preparation was not obtained.

High concentrations of sulfuric acid (18 N) at 22 C removed about one-half of the reticulated spore wall of *T. controversa* in 2 hr, whereas 24 N H_2SO_4 at 22 C removed about three-fourths of this layer in the same time. A concentration of 10 N NaOH had an effect similar to 18 N H_2SO_4. Sodium hypochlorite, NaOCl, at 0.3 M and 22 C

TABLE 1. Agglutination reactions when teliospores of *Tilletia controversa* and *T. caries* were treated with cellulase for 2 hr, with protease for 2 hr, washed thoroughly, and then treated with phytohemagglutinin-M (Pha-M)

Pha-M concentration	Agglutination reactions	
	T. controversa spores	*T. caries* spores
10 mg/ml	++++[a]	++
1.0	++	+
0.1	+	+

[a]Symbols: ++++ = massive agglutination, and + = trace of agglutination.

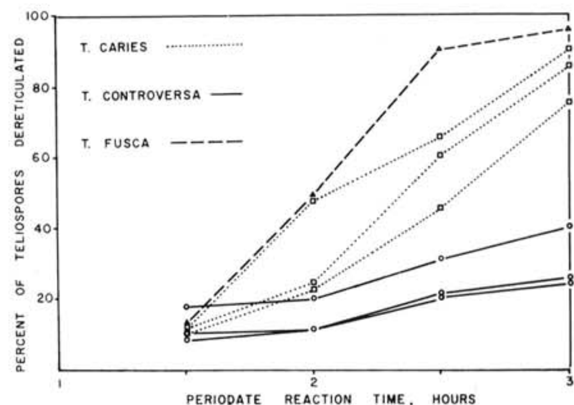

Fig. 1. Relative rates of removal of the reticulum from the walls of teliospores of *Tilletia controversa*, *T. caries* and *T. fusca* by the periodate reaction.

for 1 hr caused great swelling of the spores and partial wall fragmentation; if this reaction were allowed to continue for 3 hr all wall and wall fragments were dissolved and only the spherical central portions of the spores remained. Many reaction conditions were tested for these inorganic, hydrolytic reagents but no specific treatment was found that distinguished *T. controversa* from the other spore types.

Agglutination reactions.—Specific glycoproteins from plants including lectins, phytoagglutinins, or phytohemagglutinins may cause certain cells to agglutinate (6). These reactions are analogous to the antigen-antibody reactions in mammals. Lectins have multiple binding sites on each molecule and are highly specific for binding certain carbohydrate moieties. Thus, if a spore wall has a carbohydrate moiety exposed that can attach to a lectin molecule, then the lectin will bind several cells together in a three-dimensional matrix and cause agglutination. If a spore wall does not have the proper carbohydrate exposed for attachment to the lectin, no agglutination will occur. However, the spore wall may be partially hydrolyzed with enzymes, as indicated above, to expose new potential sites for lectin attachments. Five specific lectins were available commercially, and each was unique for carbohydrate specificity. These were: wheat germ agglutinin, phytohemagglutinin-M (Pha-M), concanavalin-A, anti-A-lectin, and anti-H-lectin.

No agglutinations were observed when untreated spores of the three fungi were reacted with any of the five lectins. Agglutination occurred, however, when walls of the spores were first altered by treatment with hydrolytic enzymes. The only lectin tested that had significant activity was Pha-M (Table 1). Hydrolytic enzyme treatments, prior to the agglutination reactions, were extended up to 48 and 72 hr, but no set of conditions was found superior to those described in Table 1.

In other experiments, spores were reacted with the lectins after treatment with the strong inorganic reagents, but again, only Pha-M, at 1 or 10 mg/ml had significant activity. After 2 hr at 37 C in either 18 N or 24 N H_2SO_4, the spores were washed thoroughly and reacted with Pha-M to give a +++ agglutination reaction. After 2 hr at 25 C in 10 N NaOH the spores gave a ++ agglutination reaction. However, the agglutination reactions of spores of *T. caries* and *T. controversa* were too similar to be a definitive test. No agglutination occurred if the spores were initially treated with any concentration of NaOCl tested.

Teliospores of all available races of *T. caries* and *T. controversa* were tested by the agglutination reaction described in Table 1, using a new commercial preparation of Pha-M. The following results were obtained in four replicated experiments: of 14 races of *T. controversa*, one gave a +++ reaction, 10 gave ++ reactions, and three gave + reactions; of 24 *T. caries* races, two gave ++ reactions, and 15 + reactions and seven gave no reaction. These results indicate that teliospores of *T. controversa* generally agglutinate more strongly than spores of *T. caries*, but the differences in these agglutination ratings are less than indicated in the initial tests. Results of this second series of tests also suggest some variation in the activity of the Pha-M lectin in different commercial preparations; e.g., several races of *T. controversa* that gave a ++++ reaction with the first preparation only gave a ++ reaction with the second Pha-M preparation.

During the agglutination experiments it was observed that certain inorganic salts also were capable of causing teliospores to agglutinate. Spores treated first with

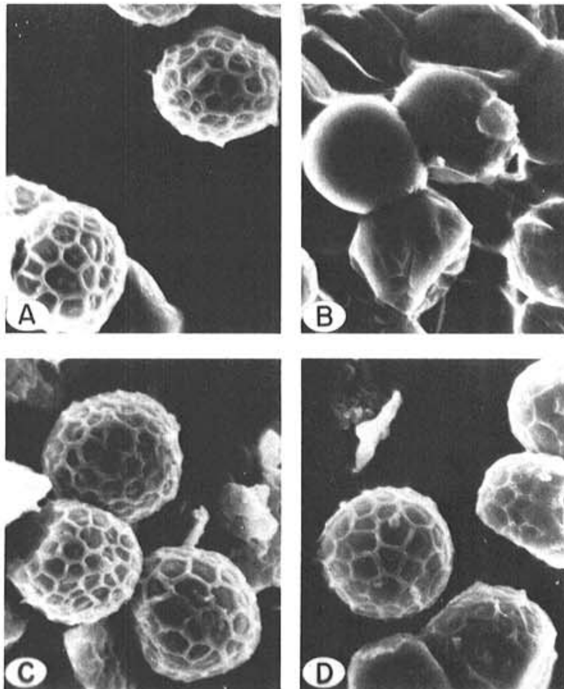

Fig. 2-(A to D). Scanning electron micrographs illustrating the effects of the periodate reaction on teliospores. **A and B)** *Tilletia caries* spores after 1.5 and 3 hr, respectively. **C and D)** *T. controversa* spores after 1.5 and 3 hr, respectively, in the periodate reaction mixture.

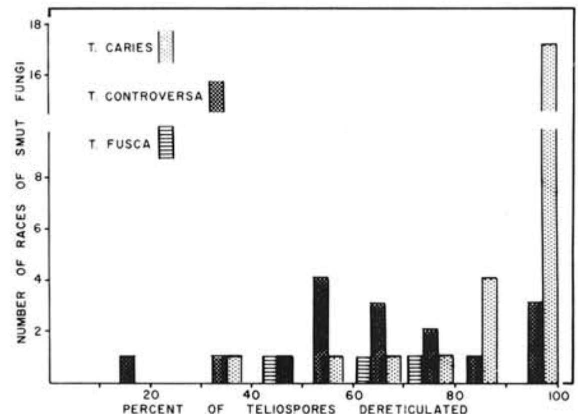

Fig. 3. Distribution plot of teliospores of 25 races of *Tilletia caries*, 16 races of *T. controversa*, and three species of grass smut fungi (*Tilletia fusca*, *T. bromi-tectorum*, and *T. guyotiana*) into relative percentage of teliospores in each race that had the reticulum removed by the periodate reaction. Data are plotted in increments of 10%.

hydrolytic enzymes, as indicated in Table 1, were observed to agglutinate in the presence of 4 M NaCl as follows: the 14 *T. controversa* races gave one ++++, 10 +++, and 3 ++ reactions; *T. caries* races gave 1 +++, 1 ++, 3 + reactions; and 18 races gave no agglutination reaction. The enzyme-treated spores also agglutinated in the presence of 0.06 M $MgCl_2$ as follows: *T. controversa* races gave 3 +++, 7 ++, 4 + reactions; *T. caries* races gave 11 + reactions and 13 races gave no agglutination reaction. These results were somewhat variable in different experiments, but always indicated that spores of *T. controversa* races as a group had a stronger agglutination reaction than spores of *T. caries* races. Agglutination reactions on spores of grass smut fungi were not performed due to insufficient spore samples. The salt concentrations of 4 M NaCl and 0.06 M $MgCl_2$ were chosen as optimum after testing a series of concentrations of each salt.

Periodate removal of spore wall.—Although experiments described above with NaOCl did not lead to any useful tests, they suggested that more refined experiments with selective oxidizing agents might be fruitful. Since most of the spore wall is composed of polysaccharides, oxidizing reagents were needed that would selectively attack these compounds. At concentrations less than 0.2 M, sodium periodate is known to oxidize simple sugars and polysaccharides specifically (3). At higher concentrations of periodate, nonspecific oxidations of other compounds may occur. The initial results indicated that the periodate oxidation reaction was a sensitive and selective way of slowly removing the cell wall of the spores of smut fungi. Also the spore walls of common and grass smut fungi were removed more quickly than were those of the dwarf smut fungus, suggesting that the spore walls of the dwarf smut fungus were thicker.

The following conditions were determined as near optimum for distinguishing between spores of dwarf, common, and grass smut fungi by the periodate reaction: 0.16 M periodic acid, 0.1 M acetate buffer at pH 4.5, and 3.0 to 3.25 hr at 57 C. Under these reaction conditions about 95% of the *T. fusca* spores and 75 to 90% of the *T.*

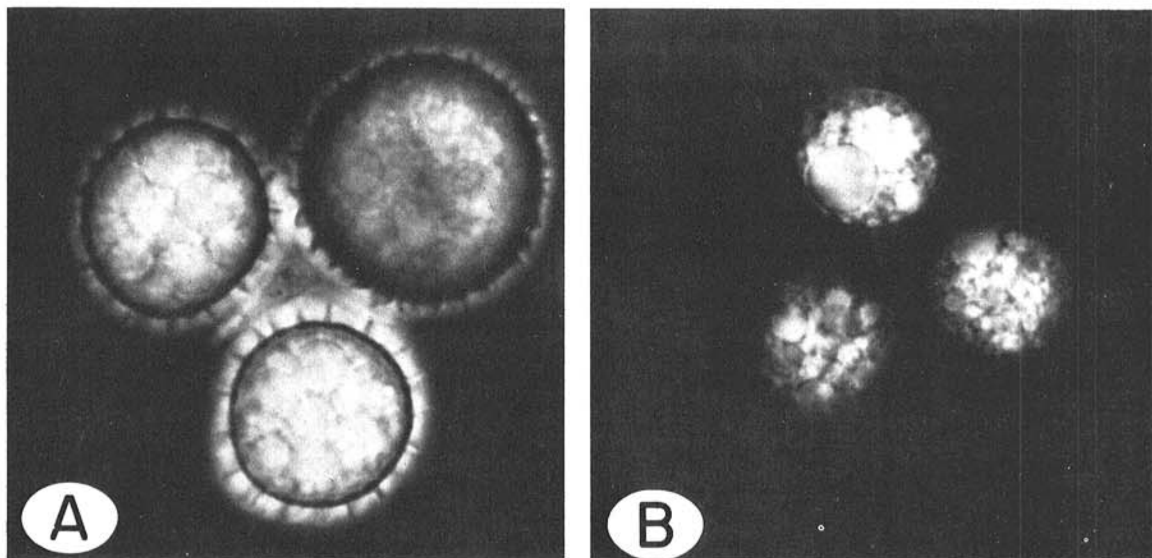

Fig. 4-(A, B). Photomicrograph of teliospores treated by the negative staining techniques, illustrating **A)** the capsule and the sharp spine-like ridges of the reticulum of *Tilletia controversa*, and **B)** *T. caries* spores without a capsule.

TABLE 2. Average spore diameters (including the capsules), thicknesses of the capsules, and thicknesses of the reticulations of hydrated mature teliospores of *Tilletia controversa*, *T. caries* and three species of grass smut fungi

Teliospores of:	Diameter (μm)	Capsule (μm)	Reticulations (μm)
T. controversa, dwarf bunt	23.05	3.14	2.26
T. caries, common bunt	20.29	1.53	1.00
T. fusca[a]	23.73	1.74	1.74
T. bromi-tectorum[a]	22.02	1.54	1.54
T. guyotiana[a]	24.42	2.52	2.52
LSD ($P = 0.05$)	0.33	0.14	0.12
LSD ($P = 0.01$)	0.43	0.18	0.16

[a]Grass smut fungi.

caries spores lost their surface reticulations and appeared to be smooth, whereas, only 20-25% of the *T. controversa* spores appeared to be smooth (Fig. 1 and 2). There also was a clear microscopic difference between the reticulations that remained on the *T. caries* and *T. controversa* spores. On *T. caries* the reticulations were low and rounded, whereas those that remained on spores of *T. controversa* were long, spike-like ridges. This variation in the reticulations that remained after the oxidation treatment was further accentuated by the use of any of the following stains in water: methylene blue (0.1 mg/ml), rose bengal (10 mg/ml), malachite green (0.1 mg/ml), safranine O (10 mg/ml), or nile blue A (1 mg/ml).

Teliospores of 16 races of *T. controversa*, 25 races of *T. caries*, three species of grass smut fungi (*T. fusca*, *T. bromi-tectorum* J. Urries and *T. guyotiana* Hariot) were tested by this method (Fig. 3). Twenty-one of 25 *T. caries* races and four of 16 *T. controversa* races had teliospores that were more than 80% dereticulated. Considerable variation in the percentage of spores that were dereticulated is evident within each species; nevertheless, the microscopic appearance of the spore samples that are partially dereticulated aids in the identification of the species.

Lead tetraacetate is reported (1) to be just as specific for oxidizing carbohydrates, but more rapid than the periodate oxidation. We attempted to use the best of these two oxidation reactions or to use them in series, and thus shorten the time required for this test, but we were never successful in removing the cell wall with the lead tetraacetate reaction.

Negative staining of spore capsule.—There was some variation in the existence and thickness of the capsule on the outside of the smut spores (Fig. 4). Our observations indicated that the capsule was more likely to be present and to be much thicker on spores of *T. controversa* than on spores of the common or grass smut fungi. This capsule, however, usually was difficult to observe. The capsular material was so fine that it was transparent to the electron beam (at 15 KV) of the scanning electron microscope. To use the capsule as a distinguishing character, a method was needed to make it more easily observed.

Teliospores in a drop of methylene blue dye (0.1 mg/ml) were mixed with a drop of India Ink (colloidal suspension of carbon particles) on a glass slide and observed with a microscope at ×400 to ×1,000 magnification. The capsules appeared as halos around the spores because the carbon particles blocked the light path, but the carbon particles could not penetrate the capsules and the capsules were transparent to light. This was an easy and efficient method of detecting the presence and thickness of the capsule and also permitted observation of the characteristic netlike surface of the spores (Fig. 4). The raised netlike surface of *T.*

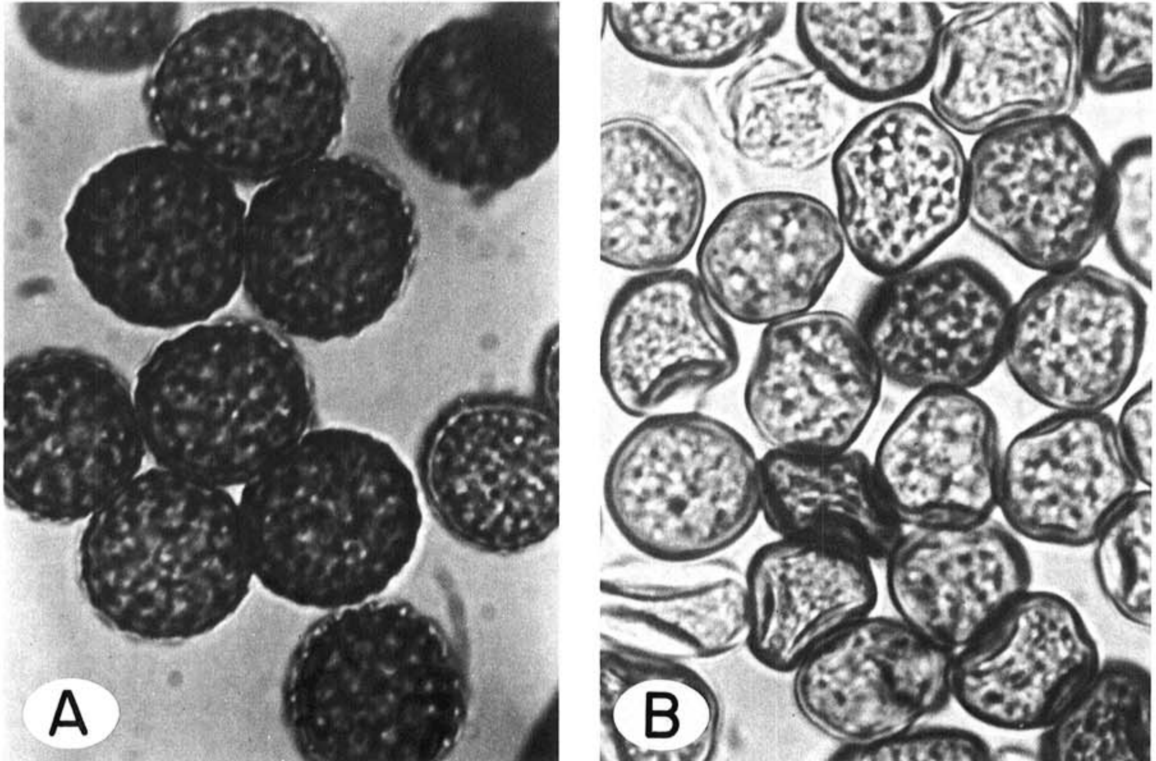

Fig. 5-(A, B). Photomicrograph of dry, **A)** spherical teliospores of *Tilletia controversa* and **B)** aspherical teliospores of *T. caries* in anhydrous l-propanol.

controversa spores appeared as long, sharp spines in a median view, in contrast to the shorter and blunter ridges of *T. caries* spores.

Observing 100 teliospores of each smut fungus, measurements were made on the diameter of the spores (including the capsule), the thickness of the capsule, and the thickness of the reticulations (Table 2). Random observations of 16 races of *T. controversa* and 25 races of *T. caries* teliospores were included in the values presented in Table 2. The average spore diameter and thickness of the reticulum of *T. controversa* was clearly different than *T. caries*, but the *T. controversa* data overlapped that of grass smut fungi. The average data on the thickness of the capsule, however, indicated a significant difference ($P = 0.01$) between the thick capsules of *T. controversa* spores and the much thinner capsules of teliospores of common and grass smut fungi.

Aspherical, distorted spores.—We have observed that dry teliospores of the common or grass smut fungi placed directly into some anhydrous organic solvents have an aspherical, deformed, distorted or shrunken appearance (Fig. 5). This first was observed with spores in dimethylsulfoxide and dimethylformamide, and we hypothesized that these potent solvents caused the cell walls to become soft and permeable. In subsequent tests with other solvents (e.g. methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, 1-octanol, glycerol, and glacial acetic acid) 30-90% of the teliospores of common and grass smut fungi were normally aspherical and distorted in the dry condition, whereas less than 5% of similarly treated *T. controversa* teliospores appeared to be aspherical. In a study of 25 races of *T. caries*, 16 races of *T. controversa* and six species of grass smut fungi [*T. fusca*, *T. bromi-tectorum*, *T. guyotiana*, *T. holci* (West.), de Toni, *T. elymi* Diet. and Holw., *T. scrobiculata* G. W. Fisch.] it was found that if more than 90% of the teliospores in a sample were spherical in 1-propanol the spores were *T. controversa*, whereas if less than 80% were spherical they were either from common bunt or grass smut fungi, (Fig. 6). When teliospores of dwarf bunt, common bunt, or grass smut fungi were suspended in either water or formic acid, they immediately rounded up and only 0-3% of the spores appeared to be aspherical.

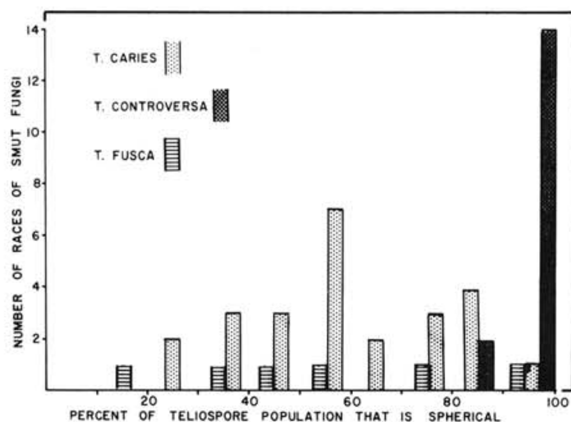

Fig. 6. Distribution plot of 25 races of *Tilletia caries*, 16 races of *T. controversa* and six species of grass smut fungi (*Tilletia fusca*, *T. bromi-tectorum*, *T. guyotiana*, *T. elymi*, *T. holci*, *T. scrobiculata*) into relative percentage of dry teliospores in each race that were spherical in 1-propanol. Data are plotted in increments of 10%.

If this method of distinguishing *T. controversa* from these other two types of smut fungi is used, only mature teliospores should be counted, and the spores must be dry. To ensure dry spores, they may be washed from the wheat sample with an anhydrous solvent such as 1-propanol or 1-butanol, or after spores are removed from the wheat sample by the usual water-wash method they may be dried in a vacuum oven for 24 hr at 70 C prior to observation in an organic solvent.

DISCUSSION

Although our objective is similar, our approach must be quite different from that taken by plant pathologists desiring to identify a sample of teliospores of smut fungi. A plant pathologist usually knows the host that the spores came from, often can observe the diseased plant, and has available relatively large numbers of spores. In contrast, the grain inspector concerned with monitoring spores in a shipment of wheat has no information on their origin and often has only a few spores available. The methods described in this report may be of value, not only to seed inspectors and scientists studying teliospores of the smut fungi, but also to others studying various thick-walled fungal spores.

In a sample containing only five to 100 spores it is important to note that 100 teliospores of smut fungi weigh about 0.2 μg. Obviously no conventional chemical analyses of amino acids (9), lipids (10), carbohydrates, proteins, etc., would be sensitive enough for the submicrogram samples. Furthermore, the walls of these teliospores are thick and difficult to break, hence attempts to rupture spores and monitor endogenous enzymes would be difficult if only a small sample of spores were available.

The method of spore germination commonly is used to distinguish *Tilletia* spp. (2, 8). *Tilletia* teliospores, upon germination, produce nonseptate promycelia which bear terminal filiform sporidia that commonly fuse in situ forming "H-shaped" fused sporidia. If several hundred or more teliospores are available for testing, and if the results are not needed immediately, it is advisable to check the germination characteristics of the spores. If the spores germinate in 1 wk on 3% water agar at 17 C they are not *T. controversa* spores (7, 8). We found under such conditions that spores of 23 of 25 races of *T. caries* germinated in 1 wk, whereas none of the 15 races of *T. controversa* or six species of grass smut fungi germinated. The spores of the grass smut fungi, however, were about 20 yr old and perhaps were not viable. A few races of *T. caries* and species of greaa smut fungi require a cold temperature and longer than 1 wk to germinate, but dwarf bunt teliospores will not germinate at 17 C.

Durán and Fischer (2) used comparative morphology of the teliospores and host symptomatology as the principal basis for species delimitation. They described the teliospores of *Tilletia* species as follows: *T. controversa*, spherical, usually 19-24 μm in diameter, reticulations 1.5 to 3 μm deep, covered by a hyaline sheath

1.5 to 5.5 μm thick; *T. caries*, spherical, less frequently aspherical, usually 14 to 23.5 μm in diameter, reticulations 0.5 to 1.5 μm deep; *T. fusca*, mostly spherical to nearly spherical, occasionally ovate to angular, 18 to 32 μm in diameter, reticulations 1.5 μm deep. These authors considered *T. guyotiana* and *T. bromi-tectorum* to be synonomus with *T. fusca*. These measurements are in agreement with those shown in Table 2, except that we found capsules (sheaths) on teliospores of *T. caries* and *T. fusca* whereas Durán and Fischer (2) described the sheath only on *T. controversa* teliospores. In all three species of grass smut fungi that were studied (Table 2) the depth of the reticulations exactly equalled the thickness of the capsules. This was in contrast to teliospores of *T. caries* and *T. controversa* in which the thickness of the capsules was greater than the depth of the reticulations. Durán and Fischer (2) noted that the thickness of the sheath often varied with the maturity of the spore, and they recommended that only mature spores be used for identification purposes. The grain inspector, however, has to deal with a mixture of teliospores of various ages.

The alcohol tests for distorted spores indicated that a high percentage of the nonhydrated teliospores of *T. caries* and *T. fusca* were aspherical and contorted, whereas more than 90% of the nonhydrated teliospores of *T. controversa* were spherical (Fig. 5). All of these spores quickly become spherical or nearly spherical when hydrated. We found that the geometrical designs of the reticulations on the three types of teliospores were too variable to aid in characterizing the species.

In a typical procedure used to monitor wheat shipments for teliospores, a 50 g sample of seed is added to 100 ml water, shaken, and the fine particulate matter collected either by centrifugation or filtration. In a washed preparation of this type, teliospores of the smut fungi often represent only a small fraction of the particulate matter found in the concentrated sample. Since some of the recommended tests (e.g., agglutination and periodate oxidation) require a clean spore suspension, the contaminating debris must be removed. This was done by passing the spore suspension (before the first centrifugation) successively through filters of 53 μm and 20 μm pore sizes. Teliospores passed through the 53 μm filter and were retained on the 20 μm filter. Then the material retained on the 20 μm filter was centrifuged in a sucrose gradient (20 to 50% sucrose) for 15 min at 40,500 *g*. The teliospores sedimented to the 30-35% sucrose zone, whereas any contaminating debris was found either above or below this zone. The spores were recovered from the centrifuge tube with a long needle attached to a syringe. This clean-up procedure recovered an average of 55% of the spores when the crude suspension contained 200 spores.

The agglutination data, obtained from cleaned teliospore samples, suggest that an unknown spore sample exhibiting a ++++ or +++ agglutination reaction with either 4 M NaCl or 0.06 M $MgCl_2$ is *T. controversa*, whereas spores that do not agglutinate are *T. caries*. The intermediate + or ++ reactions would not be as helpful in distinguishing teliospores of the smut fungi.

Results of the periodate removal of teliospore walls were encouraging in the initial tests (Fig. 1 and 2) but when spores of a large number of races were tested there was considerable variation (Fig. 3). The periodate reaction removed the reticulum from *T. caries* spores more effectively than from spores of *T. controversa* or the grass smut fungi. This was probably due to the thinner capsule and reticulum found on *T. caries* spores (Table 2). On spores partially dereticulated by the periodate reaction, the long spike-like ridges (median-view) on *T. controversa* are distinctly different than the low rounded ridges on *T. caries*; however, spores of grass smut fungi often appear similar to those of *T. controversa* in this test. Considering the time required for the periodate test and the lack of definitive results, we do not recommend this test as a routine procedure.

It is possible to gather information from the four recommended methods if 500 or more spores are available for study. At the other extreme, if only five to 10 spores are available for study, the inspector is limited to two tests, namely the anhydrous alcohol test in which the percentage of spherical spores in the sample is estimated (Fig. 6), and the negative staining test in which the appearance of the capsule, the reticulum, and the size of the spores are compared to average values (Table 2). For routine monitoring of a large number of samples containing teliospores of smut fungi, we recommend that the anhydrous alcohol test and the negative staining test be applied to all samples. If the results of these two tests are ambiguous for a particular sample, those spores should then be exposed to the germination test and/or the agglutination test.

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