Distinguishing Teliospores of *Tilletia controversa* from Those of *T. caries* by Fluorescence Microscopy

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ABSTRACT

Stockwell, V. O., and Trione, E. J. 1986. Distinguishing teliospores of *Tilletia controversa* from those of *T. caries* by fluorescence microscopy. Plant Disease 70:924-926.

Teliospores of the common bunt pathogen of wheat (*Tilletia caries*) and the dwarf bunt fungus (*T. controversa*) are difficult to distinguish by light and electron microscopy. An epifluorescence microscopy method was developed to identify teliospores of these fungal species. Teliospore samples were mounted in immersion oil and viewed with blue light. The reticulated wall layer of *T. controversa* teliospores fluoresced yellow-orange and the spores appeared spherical. Teliospores of *T. caries* had a nonfluorescing reticulated wall layer, yellow-fluorescing globules were present in the cytoplasm, and the teliospores were deformed. This rapid, sensitive method for teliospore identification should be useful to monitor international wheat shipments for teliospores of *T. controversa*.

The need to find observable morphological differences between teliospores of *Tilletia controversa* Kühn, dwarf bunt pathogen of wheat, and *T. caries* (DC.) Tul., common bunt pathogen, has intensified since 1974, when The People's Republic of China imposed a zero tolerance on teliospores of *T. controversa* in imported wheat shipments (4). Teliospores of these fungi have three easily discernible wall layers (2). The outermost wall layer is the translucent sheath, which can be seen by negative staining techniques (5). The reticulated layer appears as a network of ridges covering the teliospore. The innermost wall layer, adjacent to the cytoplasm, is called the endospore layer (2).

Numerous attempts have been made to devise a method applicable to routine monitoring of wheat shipments to distinguish between teliospores of *T. controversa* and *T. caries*. A germination test is an accurate method to distinguish viable teliospores of the two species; however, it requires 1 wk of incubation at 17 C to identify *T. caries* and 3–6 wk at 5 C to identify *T. controversa* (3). Teliospores of these wheat bunt fungi appear similar with light microscopy techniques. Trione and Krygier (5) found in morphological studies that the sheath and reticulated teliospore wall layers were, on the average, thinner on teliospores of *T. caries* than on those of *T. controversa*. Additionally, they found that 80% of teliospores of *T. caries* became aspherical in anhydrous 1-propanol, whereas only 5% of teliospores of *T. controversa* were deformed in organic solvents. Although these criteria for differentiating teliospores provided a usable test, a considerable amount of overlap in characteristics still existed between the two species, thus making an accurate identification of small samples difficult. At the ultrastructural level, differences in thickness and fine structure of the sheath and reticulated layer were apparent and could be used for identifying teliospores; however, the required techniques were too involved and time consuming for routine examination of teliospores in international wheat shipments (W. M. Hess, *personal communication*). Banowetz et al (1) produced monoclonal antibodies against teliospores of *T. controversa* and *T. caries*. Although they found quantitative differences in the binding ratios of the antibodies that developed against the teliospores, they did not detect any unique antibodies to distinguish dwarf bunt from common bunt teliospores.

Although all of the described techniques provided a method to identify teliospores of a single *Tilletia* species, the procedures were either too time consuming or the results too variable to identify teliospores from a small mixed sample obtained from a wheat shipment. The purpose of this investigation was to develop a rapid, sensitive method to distinguish teliospores of *T. controversa* from those of *T. caries* by fluorescence microscopy.

MATERIALS AND METHODS

Teliospore samples. Teliospores of the dwarf bunt fungus (*T. controversa* races D1 through D17) and the common bunt fungus (*T. caries* races T1 through T30) were obtained and stored as described previously (5).

Teliospore fluorescence. A drop of an aqueous suspension of teliospores was placed on a glass microscope slide and allowed to air-dry completely at room temperature. Samples were mounted in low-viscosity, nonfluorescing immersion oil and observed with a Zeiss Universal microscope with an RS-III epifluorescence attachment. Illumination was provided with a 50W mercury lamp. Zeiss filter set 487709 (485-nm excitation, 520-nm barrier filter) was used for routine examination of teliospore samples. About 500 teliospores were observed in each mounted sample, and the pattern of autofluorescence of teliospores of each race of the fungal pathogens was examined at least three times.

To determine the variability of autofluorescence patterns of teliospores of a single species of fungus, five samples of common or dwarf-bunt-infected wheat collected at different locations in the northwestern United States were chosen. A single bunt sorus was removed, rinsed three times with distilled water, and ruptured in distilled water. The teliospores were mounted as described. Five randomly chosen fields were examined on each slide with a 40× objective lens. The number of teliospores with a fluorescent reticulated wall layer, yellow globules in the cytoplasm, or an aspherical appearance was recorded. About 45 teliospores were examined per microscope field.

Teliospore germination. To determine the effect of immersion oil and microscopic observations on teliospore viability, teliospores of *T. caries* were treated by one of the following methods. Teliospores were immersed in distilled water or immersion oil for 30 min. A subsample of immersion-oil-treated teliospores was rinsed five times with

Contribution of the ARS, USDA, in cooperation with the Oregon Agricultural Experiment Station; Technical Paper 7654 of the latter.

Accepted for publication 11 April 1986 (submitted for electronic processing).

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distilled water. Other teliospores were mounted in immersion oil on glass slides and viewed by fluorescence microscopy. After observation, teliospores were scraped off the slides and rinsed five times with distilled water. After each treatment, teliospores were placed on 3% water agar with a sterile cotton swab and incubated at 17 C for 5 days. Average percent germination was determined by counting germinated and nongerminated teliospores in five random fields on each petri dish. About 200 teliospores were counted in each microscope field. This experiment was repeated three times.

RESULTS

Fluorescence microscopy. Teliospores of the two species were accurately distinguished when mounted in immersion oil and viewed with blue light. The sheath of teliospores of both species was nonfluorescent. The reticulated teliospore wall layer of *T. controversa* fluoresced orange-yellow and appeared as spikelike protusions when viewed at the median plane (Fig. 1A) or netlike when focused on the upper surface of the teliospores (Fig. 1B). The reticulated wall layer of teliospores of *T. caries* was nonfluorescent and appeared as a brown band around the teliospore when viewed in cross section (Fig. 2A) and as a dark net when focused on the upper teliospore surface (Fig. 2B). The endospore wall layer of teliospores of both fungal species fluoresced yellow to yellow-green. The teliospore cytoplasm of *T. controversa* fluoresced faintly with a dull green-yellow color. In contrast, the cytoplasm of teliospores of *T. caries* often contained bright, discrete, yellow to yellow-green fluorescing globules.

A single bunt sorus contains teliospores at different stages of development and maturity. In mature bunt sori of *T. caries*-infected wheat, immature teliospores constituted less than 1% of the sample (Table 1). The degree of maturity affected the appearance of teliospores with both transmitted light and epifluorescence microscopy. Mature teliospores appeared dark brown by light microscopy, whereas immature teliospores appeared hyaline. At all stages of immaturity, the reticulated wall layer of teliospores of *T. caries* appeared yellow when observed with fluorescence microscopy, similar to *T. controversa*, but the teliospores were collapsed and aspherical. As teliospores of *T. caries* matured, the fluorescence associated with the reticulated wall layer decreased.

The difference in fluorescence of the reticulated wall layer was the most consistent differential character observed in mature teliospores. An average of 98 ± 1% of teliospores of *T. controversa* from sori had a fluorescent reticulated wall layer, whereas none of the mature brown teliospores of *T. caries* from sori had a fluorescent reticulated wall (Table 1).

When mounted in aqueous solutions, mature teliospores of *T. controversa* and *T. caries* appeared similar by transmitted light microscopy. When mounted in immersion oil, 88 ± 11% of teliospores of *T. caries* appeared deformed, whereas only 8 ± 3% of teliospores of *T. controversa* appeared aspherical (Figs. 3 and 4, Table 1).

Germination of teliospores. Germination of distilled-water-treated teliospores of *T. caries* averaged 80 ± 5%. Teliospores of *T. caries* appeared collapsed and did not germinate while coated with immersion oil. Teliospores observed with epifluorescence microscopy or exposed to immersion oil, then rinsed with distilled water, appeared spherical and had germination values of 81 ± 2% and 79 ± 2%, respectively. Exposure to immersion oil or epifluorescence microscopy did not affect the ability of teliospores to germinate after the oil had been displaced.

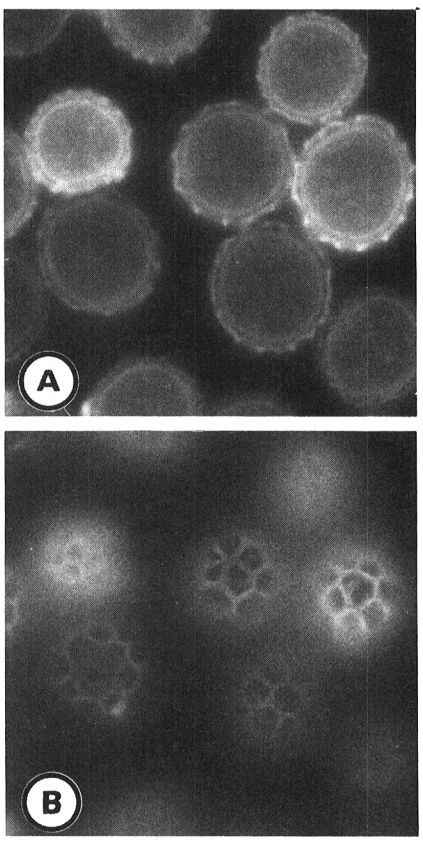


Fig. 1. Epifluorescence micrograph of teliospores of *Tilletia controversa* race D1. **(A)** Median plane through teliospores. Fluorescent reticulated wall layer appears spikelike (×785). **(B)** Upper surface of teliospores. Fluorescent reticulated wall layer appears netlike (×785).

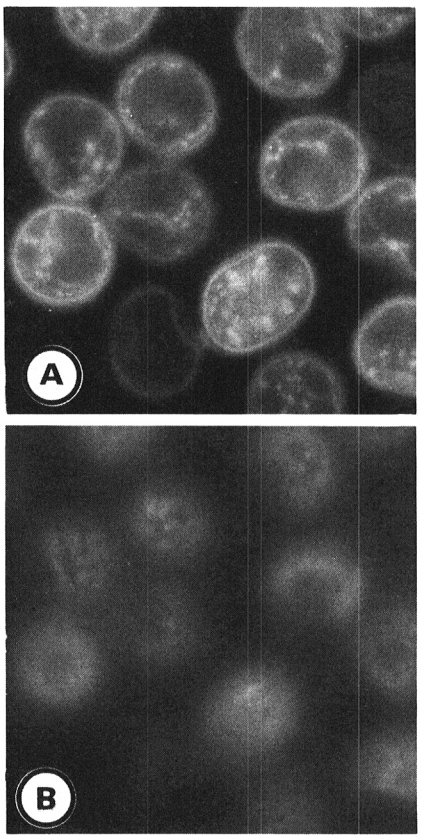


Fig. 2. Epifluorescence micrograph of teliospores of *Tilletia caries* race T7. **(A)** Median plane through teliospores. Reticulated wall layer is nonfluorescent, and cytoplasm contains fluorescing bodies (×785). **(B)** Upper surface of teliospores. Netlike reticulated wall layer appears dark (×785).

DISCUSSION

Aside from germination tests that require viable teliospores, previous methods to distinguish teliospores of *T. controversa* and *T. caries* were based on differences in the thickness of the sheath and reticulated teliospore wall layers (5). Because of variation within a sample and species, measurements of these characteristics could not be used as definitive criteria to determine the identity of a single teliospore. The described procedure for observing teliospores with epifluorescence microscopy provides a rapid and more accurate technique to identify mature teliospores of *T. controversa* and *T. caries*.

We suggest the following criteria to distinguish teliospores of *T. controversa* and *T. caries* by epifluorescence microscopy. Teliospores of *T. controversa* appear spherical when mounted in immersion oil and the reticulated wall layer fluoresces yellow-orange. Mature teliospores of *T. caries* are deformed in immersion oil, the reticulated wall layer is nonfluorescent, and the cytoplasm often contains yellow-fluorescing globules. Immature teliospores of *T. caries* may have a yellow-fluorescing reticulated wall layer; however, the teliospores will appear deformed. The described pattern of teliospore autofluorescence was consistent over all races of *T. controversa* and *T. caries* examined and was used to identify correctly 36 of 36 teliospore samples in a single blind test.

Wu and Warren (7,8) found that cytoplasmic autofluorescence observed within water-mounted spores of many

Table 1. Comparison of characteristics of teliospores of *Tilletia caries* and *T. controversa*

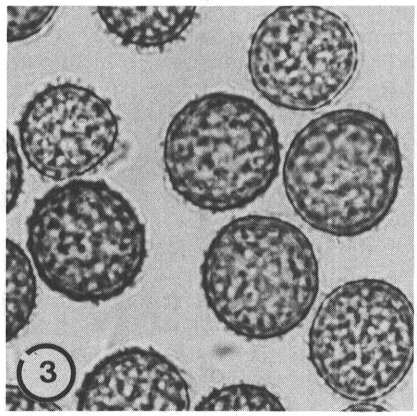
Sample	Number of teliospores examined[a]	Aspherical teliospores (%)	Teliospores with autofluorescent reticulated wall (%)	Teliospores containing yellow globules (%)
T. caries				
1	211	72	3[b]	58
2	222	83	2[b]	87
3	194	96	0	91
4	227	88	0	81
5	222	99	0	86
Average		88 ± 11	0.4 ± 0.5	81 ± 13
T. controversa				
1	214	12	97	1
2	248	9	99	12
3	287	3	97	3
4	210	8	99	0
5	224	6	99	9
Average		8 ± 3	98 ± 1	5 ± 5

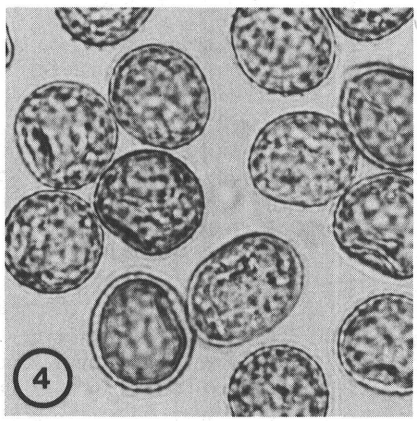
[a] Only teliospores with a reticulated wall were examined.
[b] Teliospores appeared hyaline under transmitted light microscope, i.e., immature teliospores.

species of fungi positively correlated with spore death. The cytoplasmic autofluorescence observed within teliospores of *T. caries* mounted in immersion oil should not be confused with the autofluorescence described by Wu and Warren. The yellow-fluorescing globules within teliospores of *T. caries* stained positively for lipids (*unpublished*). There was a positive correlation between the presence of visible lipid bodies in the teliospore cytoplasm and percent germination of these samples. A sample of teliospores that had lost its ability to germinate after prolonged storage at room temperature did not contain the fluorescing lipid bodies or general cytoplasmic autofluorescence described by Wu and Warren (7,8). Observing the presence of yellow globules or lipid bodies in the cytoplasm of teliospores of *T. caries* could be useful as a rapid general method to determine teliospore viability. Teliospores of *T. controversa* also contain lipid bodies within the cytoplasm; however, they are not clearly visible in mature spores under fluorescence microscopy, probably because of the presence of thick brightly fluorescent wall layers that mask the cytoplasmic autofluorescence.

There is a zero tolerance for teliospores of *T. controversa* in wheat shipments to The People's Republic of China; thus, detection of a single mature teliospore of this pathogen would result in rejection of the wheat shipment (4). None of the teliospores of *T. caries* had a fluorescent reticulated wall, so based on that character alone, it is unlikely that these teliospores would be misidentified as *T. controversa*. About 2% of the teliospores of *T. controversa* did not have a fluorescent reticulated wall and could possibly be misidentified as *T. caries*. The probability that all teliospores of *T. controversa* within a sample would be abnormal and not have a fluorescent reticulated wall can be calculated with a binomial expansion and expressed as $(0.02)^n$, where 0.02 is the observed frequency of teliospores of *T. controversa* with a nonfluorescent wall and n is the number of teliospores of *T. controversa* in the sample (6). If only one teliospore of *T. controversa* were present in a sample, then the probability that it would be misidentified as *T. caries* based solely on lack of fluorescence of the reticulated wall layer would be 0.02 or 2%. If three teliospores of *T. controversa* were present, then the probability that all would be abnormal would be decreased to 8×10^{-6} or 0.0008%. As the number of teliospores of *T. controversa* increases, the probability of all teliospores appearing abnormal and misidentified as *T. caries* decreases. Thus, considering only the appearance of the reticulated wall layer with fluorescence microscopy, this is a sensitive method to distinguish teliospores of *T. controversa* and *T. caries*. If other characteristics such as deformation of teliospores in immersion oil and presence of fluorescent cytoplasmic globules are also considered, then the probability of accurate identification would be increased.

The procedure for distinguishing teliospores of *T. controversa* from those of *T. caries* by epifluorescence microscopy is a rapid, accurate, and nondestructive technique. Teliospore samples free of debris are not required because the material is observed directly. The procedure does not affect teliospore viability, so germination tests can be used in conjunction with this procedure. This technique using epifluorescence microscopy should be useful in routine monitoring of international wheat shipments for the presence of *T. controversa* teliospores.





Figs. 3 and 4. Immersion oil-mounted teliospores of *Tilletia controversa* **(3)** race D1 (shown in Fig. 1) (×785) and *Tilletia caries* **(4)** race T7 (shown in Fig. 2) (×785).

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