A Biochemical Method for Estimating Viability of Teliospores of *Tilletia controversa*

T. G. Chastain and B. King

Department of Agronomy and Soils, Washington State University, Pullman 99164. Present address of first author: Columbia Basin Agricultural Research Center, Oregon State University, Pendleton 97801.
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ABSTRACT

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A rapid biochemical test was developed to assess indirectly the viability of teliospores of *Tilletia controversa*, the cause of dwarf bunt of wheat, that contaminate wheat grain. Lipase activity was detected consistently in extracts from viable teliospores by a fluorescein diacetate (FDA) assay. No fluorescence activity was observed in extracts from autoclaved spores. By comparison, lipase detection was inconsistent when 4-methylumbelliferyl-palmitate was used as substrate. Extracts of teliospores that did not germinate on soil extract agar exhibited no lipase or glucosidase activity. Viability of individual teliospores could not be assessed by lipase assay because spore coats were impermeable to FDA. The FDA assay reduced the time needed to assess the average viability of a teliospore population from 2 mo to 1 hr.

Dwarf bunt is a major constraint to expanding international markets for winter wheat (*Triticum aestivum* L.) grown in the Pacific Northwest. For example, the People's Republic of China will not accept wheat grain contaminated by teliospores of the dwarf bunt fungus *Tilletia controversa* Kühn (8). Even grain from cereals not susceptible to dwarf bunt often is infested by teliospores that are present in the local grain transport system. Developing a method for killing seedborne teliospores could expand export markets for wheat grain. Teliospores of *T. controversa* are dormant (6); consequently, the efficacy of potential agents for killing the spores is difficult to evaluate because it is unclear whether treated spores are dead or dormant. Germination is an unreliable measure of teliospore viability because most investigators (1,6,13) have reported low or variable germination percentages. Furthermore, the germination process requires at least 2 mo to complete (4). Thus, rapid methods for assessing teliospore viability are needed to screen treatments for eradicating teliospores from wheat grain and seed.

Most previous work on determining spore viability has focused on understanding and improving (1,7) teliospore germination. Few studies have been conducted to estimate the viability of teliospores by indirect methods. Yu and Trione (12) used 4-methylumbelliferyl-β-D-glucoside (4MBDG) in a fluorescent assay for glucosidase activity in extracts of teliospores of *T. controversa*. The relationship between 4MBDG assay results and teliospore germination still needs to be ascertained. Yu et al (13) reported that adenosine 5′-triphosphate content was considerably lower in autoclaved teliospores than in untreated spores. Autoclaved teliospores did not germinate, whereas 52% of untreated spores germinated.

Because lipids are major storage metabolites in teliospores of *T. controversa* (9) and are considered to be the major source of energy for teliospore germination, assays of enzymes that are essential in the metabolism of lipids would be likely candidates for rapid viability tests. We hypothesized that only viable teliospores have functional lipases (lipid esterases) that are capable of hydrolyzing triglycerides to fatty acids and glycerol. Fluorescein diacetate (FDA) is a good indicator of lipase activity (2) and has been used to determine the viability of pollen (3), plant cells (11), and plant protoplasts (5). Thus, the purpose of this study was to develop a rapid biochemical test for indirectly determining the viability of teliospores of *T. controversa*.

MATERIALS AND METHODS

Teliospores of *T. controversa* were extracted from bunt balls present in wheat grain harvested in 1988 from the Washington State University winter wheat nursery near Reardan, WA, and from stored grain provided by H. S. Fenwick, University of Idaho, Moscow. Teliospores also were obtained by placing contaminated grain samples (25–50 g) in a 100-ml buret containing 50 ml of distilled water and 1 drop of Tween 20. Air was bubbled through the buret to dislodge teliospores from the grain. Teliospores were recovered from the rinsate on a 0.5-μm nylon filter. A few teliospores of *T. caries* (DC.) Tul. & C. Tul. may have been present in some samples used for viability tests, but we determined that small, incidental quantities of spores of *T. caries* did not affect assay results.

Teliospores were presoaked in sterile distilled water for 0, 2, 4, 6, 8, or 24 hr at 22 C to activate enzymes. The control consisted of teliospore suspensions autoclaved for 30 min. Spores were recovered from suspensions on 0.5-μm nylon or 0.2-μm cellulose nitrate membrane filters by vacuum filtration.

Spores (0.5–20.0 mg) were homogenized for 1–2 min in a glass tissue grinder in 3 drops of one of the following: 1) cold sterile distilled water, 2) 5% sucrose, 3) 1% bovine serum albumin, 4) 0.1 M citrate phosphate buffer (pH 7), or 5) 0.06 M phosphate (KH_2PO_4 and Na_2HPO_4) buffer (pH 6.8). The homogenates were placed in 1.5-ml microcentrifuge tubes containing 0.5 ml of one of the following cold sterile buffers: 1) 0.05 M Tris HCl (pH 8), 2) 0.06 M phosphate (pH 6.8), 3) 0.1 M citrate phosphate (pH 7), or distilled water. Microcentrifuge tubes containing teliospore extracts were placed in a refrigerated centrifuge for 2 min at 11,800 *g*.

Aliquots of 100 μl of supernatant from untreated or autoclaved teliospore extracts were added to wells of 96-well microtiter plates. Buffer controls contained no spore extract. For determination of lipase activity, 10 μl of FDA was added to each microwell. FDA concentrations were 0.01, 0.1, 0.2, 0.3, or 0.5% (w/v) in acetone or in 2-methoxyethanol. An alternative method of measuring lipase activity involved adding 10 μl of 0.1 or 4.8 mM 4-methylumbelliferyl-palmitate (4MUP) substrate in 2-methoxyethanol or in acetone to microwells. Glucosidase activity in teliospore extracts was determined by the technique of Yu and Trione (12). Microwell plates were incubated at 20 and 30 C for lipase and glucosidase assays, respectively. All experimental procedures were repeated at least three times.

Lipase and glucosidase activity was evaluated visually under ultraviolet illumination after incubation. FDA fluorescence activity was measured with an Aminco-Bowman spectrophotofluorometer. Reference measurements were made by measuring the fluorescence of nonesterified fluorescein (Aldrich Chemical Co., Inc., Milwaukee, WI). The excitation wavelength was 470 nm. The concentration of FDA hydrolyzed to fluorescein by lipase present in teliospore extracts was calculated by the following formula:

$$C_e = \frac{RI_e}{RI_f} C_f$$

where C_e = concentration of fluorescein in extract, C_f = concentration of fluorescein in standard, RI_e = relative intensity of extract, and RI_f = relative intensity of standard.

Viability test results were compared with teliospore germination on soil extract agar (SEA). Aqueous suspensions of autoclaved and untreated teliospores were plated on SEA containing 5 mg of streptomycin sulfate/100 ml. Teliospores were incubated for 2 mo at 5 C and illuminated by fluorescent light (5). Six replicate plates each of autoclaved and untreated teliospores were examined at ×100. The surface of each plate was videotaped, and spores were examined in four representative 0.35-mm^2 areas to determine percent germination.

RESULTS AND DISCUSSION

No autoclaved teliospores germinated on SEA, whereas untreated spores averaged 47.4 ± 9.2% germination. Because the viability status of ungerminated teliospores of *T. controversa* is unknown, a control was needed that possessed a known viability level. Therefore, autoclaved teliospores were used as the control for enzyme assays in this study and by Yu and Trione (12). These autoclaved teliospores will be referred to as nonviable spores.

Preliminary experiments demonstrated that the viability of individual whole teliospores could not be determined with vital stains because the spores have highly impermeable spore coats (10). Consequently, we also encountered considerable difficulty in penetrating teliospores with FDA substrate. An attempt was made to increase infiltration by etching the spore coat with 2% NaOCl. Teliospores exposed to NaOCl for 20–25 min often were physically damaged, suggesting that NaOCl may have potential for killing teliospores on wheat grain.

Because teliospores were impermeable to FDA, spores needed to be homogenized to extract lipase for FDA substrate assay.

TABLE 1. Fluorescence response of extracts of teliospores of *Tilletia controversa* to indicator substrates for lipase activity

Lipase assay[a]	Solvent	Incubation time[b] (hr)	Relative intensity	
			Autoclaved	Nonautoclaved
FDA	acetone	7	0	14.9 ± 0.4[c]
FDA	2-MEtOH[d]	1	0	15.1 ± 0.6
4MUP	acetone	15	0	0.9 ± 0.4

[a] FDA = fluorescein diacetate; 4MUP = 4-methylumbelliferyl-palmitate. FDA and 4MUP concentration in incubation media was 0.027 and 0.018%, respectively.
[b] Incubation time required to observe visible fluorescence caused by enzymatic hydrolysis of nonfluorescent substrate. Fluorescence activity was measured spectrophotometrically.
[c] Values are means and standard errors of relative intensity.
[d] 2-MEtOH = 2-methoxyethanol.

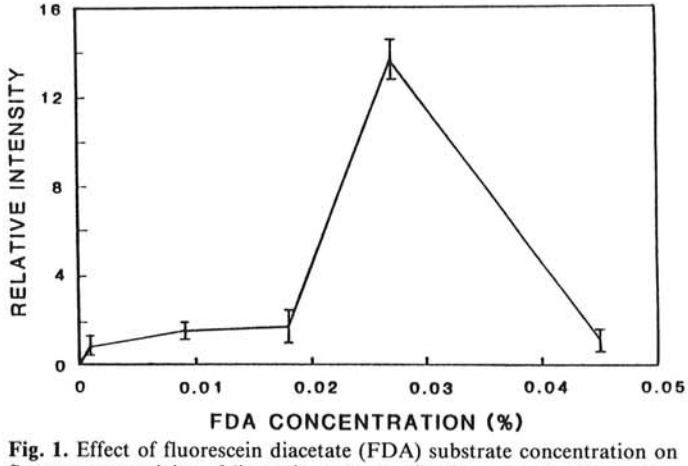

Fig. 1. Effect of fluorescein diacetate (FDA) substrate concentration on fluorescence activity of lipase in extracts of teliospores of *Tilletia controversa*. Teliospore sample weight was 1 mg. Relative intensity of nonesterified fluorescein = 90%. Each point represents the average of three observations. Bars represent standard errors.

TABLE 2. Influence of teliospore sample weight on hydrolysis of fluorescein diacetate (FDA) substrate by lipase in extracts of teliospores of *Tilletia controversa*

Teliospore sample weight (mg)	Fluorescence activity after incubation[a]	
	Relative intensity	Fluorescein detected (μg)
0.0	0 ± 0[b]	0
1.0	1.3 ± 0.3	0.4
5.0	2.0 ± 0.5	0.8
20.0	12.1 ± 0.5	4.6

[a]FDA concentration in incubation media was 0.007%. The fluorescein content present in spore extracts after incubation was determined spectrophotometrically.
[b]Values are means and standard errors of relative intensity. Relative intensity of nonesterified fluorescein = 88%.

Lipase activity was measured consistently in extracts from homogenized viable spores (Table 1). Extracts from nonautoclaved teliospores reacted with FDA substrate to release fluorescein, a fluorescent compound, whereas extracts from nonviable spores were not fluorescent. Hydrolysis of FDA by viable spore extracts indicated that considerable lipase activity was present and presumably indicated that no activity was present in nonviable spores.

Best viability estimates were obtained after presoaking teliospores for at least 4 hr. Viability test results were similar whether spores were ground in distilled water, bovine serum albumin, or sucrose. False positives for lipase activity were caused by spontaneous hydrolysis of FDA (2) and appeared to be pH dependent because they were detected at pH 8 but were eliminated at pH 6.8.

The acetone used as FDA solvent reacted with polystyrene microwell plates, prompting a search for a more suitable substitute. No similar reaction with microwell plates was evident for 2-methoxyethanol. Use of 2-methoxyethanol as FDA solvent also resulted in greatly increased accuracy and sensitivity, while reducing incubation time (Table 1). The longer incubation time required for FDA prepared in acetone presumably was caused by fluorescence quenching because adding acetone to porcine liver esterase reduced FDA fluorescence activity. By comparison, much less quenching was observed for 2-methoxyethanol. Stock solutions of FDA in 2-methoxyethanol must be stored at −15 C and used within 1 wk after preparation. The test also may be conducted directly in polypropylene microcentrifuge tubes (1.5 ml), thereby eliminating one step.

Because considerable palmitic acid is found esterified in teliospores of *T. controversa* (9), a lipase assay employing 4MUP could be a valuable viability test. The hydrolysis of 4MUP to fluorescent 4-methylumbelliferone revealed lipase activity in untreated spore extracts (Table 1). However, this method did not provide consistent detection of lipase activity in spore extracts. More work is needed to determine the efficacy of this assay.

Extracts from viable teliospores fluoresced in response to 4MBDG as previously published (12), indicating glucosidase activity in viable teliospores but not in nonviable spores.

We found that adding 10 μl of 0.3% FDA was best for assaying lipase present in teliospores of *T. controversa* (Fig. 1). The incubation media contained 0.027% FDA. It was evident that FDA fluorescence activity increased proportionally with the weight of teliospores examined. Extracts prepared from samples containing few teliospores may require a longer incubation period.

Fluorescein released from hydrolysis of FDA by lipase increased as teliospore sample weight (lipase) increased in a linear manner at low FDA concentration (0.007%) (Table 2). Nevertheless, detection of teliospore lipase activity by FDA was most limited by substrate availability because results using low sample weight (1 mg) were improved markedly by increasing substrate concentration (Fig. 1).

Teliospore extracts that exhibited no lipase or glucosidase activity were indicative of teliospores that did not germinate on SEA. Therefore, lipase and glucosidase assays serve as good indicators of the viability of a teliospore population of *T. controversa*. The minimum number of spores needed to conduct the test was about 700,000. The number of dormant or dead spores present in each sample is unknown because the viability of individual teliospores cannot be determined directly by the FDA assay. Indirectly, the number of viable teliospores present possibly could be determined by measuring the FDA activity of proportional mixtures of living and heat-killed spores and constructing a calibration curve for the assay. Work is under way to determine the feasibility of this approach.

Yu et al (13) suggested that other assays be used in addition to their glucosidase assay to obtain estimates of teliospore viability. Our FDA assay of the essential lipase enzyme could provide the needed verification of teliospore viability. Unfortunately, the inability to detect the viability of individual teliospores is a limitation of the present lipase and glucosidase assays.

The lipase assay reported here demonstrates excellent potential for rapid and reliable determination of the viability of teliospores of *T. controversa*. Moreover, this lipase assay could be used to screen treatments for disinfesting wheat grain and seed of teliospores, thereby providing a tool for solving quarantine restrictions imposed by dwarf bunt. The FDA lipase assay also could be adapted for determining the viability of teliospores of other *Tilletia* spp.

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