Enzyme Activities in Dormant Spores of Two *Tilletia* Species

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ABSTRACT

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A simple, sensitive microtest, based on fluorescence associated with enzyme activities in the presence of specific substrates, is described that may provide an accurate measure of dormant spore viability in many fungi. Esterase, sulfatase, and glycosidase activities were measured in extracts from teliospores of *Tilletia controversa* (TCK), and *T. caries* (TCT) in the presence of 15 different substrates. Except for four substrates, the measured activities were higher in TCK than in TCT. Activities of α-L-arabinopyranosidase and β-D-glucuronidase were found only in TCK. Temperature and pH optima were determined for the individual enzymes. The optimal temperatures for the enzyme activities were higher in all cases than the optimal temperatures for germination of teliospores of TCK or TCT. The temperature relationships are discussed.

Additional key words: *Triticum aestivum*, viability tests.

Germination is commonly used to monitor spore viability, but many fungal spores do not germinate readily, and rapid viability tests are needed. Enzyme activity is essential for the survival, growth, and development of fungi. Caltrider and Gottlieb (1) showed that the Embden-Meyerhof-Parnas (EMP) and hexose monophosphate shunt enzymes were active in some ungerminated fungal spores; but in spores of *Ustilago maydis* (DC) Corda, some of EMP system enzymes were active only during particular stages of germination. Knoche and Horner (7) reported some characteristics of a lipase in uredospores of a wheat rust fungus. The characteristics of enzymes from wheat bunt teliospores have not been reported, due perhaps to the low levels of enzyme activities. Guilbault et al (3–5) reported sensitive methods for examining enzyme activities in the presence of substrates that yield

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fluorescent products after the enzymatic reaction.

The aim of the present research was to measure enzyme activities in dormant spores and dead spores of the wheat bunt fungi, *Tilletia controversa* Kuhn and *T. caries* (DC) Tul. to aid in developing a test to monitor the viability of dormant teliospores.

MATERIALS AND METHODS

Spores of wheat bunt fungi, *T. controversa* (TCK), and *T. caries* (TCT), were provided by J. A. Hoffmann, USDA ARS, Logan, UT. The ears of the bunted wheat plants were harvested and stored for 1–3 yr at room temperature (17–35 C seasonal extremes). The spores were dry harvested by removing the bunt balls from the wheat head, breaking the bunt balls with gentle grinding in a mortar and pestle, and screening the spores through a 60-μm-mesh nylon cloth.

Enzyme preparation. TCK or TCT, 210 mg of teliospores per extraction, were placed in a glass-to-glass tissue grinder with 100 mg of glass powder and $(NH_4)_2SO_4$ (saturated at 4 C). The saturated $(NH_4)_2SO_4$ was added in three increments (1 ml, 1 ml, and 0.5 ml). This mixture was ground for 2–3 min after each increment of $(NH_4)_2SO_4$ using a rheostat-controlled motor-driven pestle. After the last $(NH_4)_2SO_4$ aliquot was added, the grinding was continued until essentially all of the spores were broken. The grinder and homogenate were maintained in an ice bath throughout the grinding operation. The homogenate was transferred to a centrifuge tube with additional saturated $(NH_4)_2SO_4$ solution to a final volume of 5 ml. Ethyl ether (10 ml) was added to the homogenate to partition the lipids; the mixture was shaken thoroughly and then centrifuged at 14,500 *g*, for 10 min at 4 C. The precipitate was recovered, and the enzymes were extracted by suspension of precipitate in 10 ml of deionized distilled water. The suspension was centrifuged at 7,700 *g* for 5 min (4 C); the supernatant was recovered, and the pellet was extracted one additional time. The second supernatant was combined with the first and then diluted with an additional 10 ml of deionized distilled water. The final volume of enzyme preparation was 30 ml. The enzyme concentrations were equivalent to those derived from a teliospore concentration of 7 mg/ml. Protein determinations were not made for each preparation, but the same equivalent weight of dry dormant spores was used in each assay. The enzyme preparations were held at 4 C prior to use, however only freshly prepared solutions were used to determine β-galactosidase activity. Separate enzyme preparations were made and used to monitor each enzyme throughout the pH and/or temperature ranges studied. Triplicate analyses were performed and each experiment was repeated three times. Results are presented as numerical averages.

Enzyme assay. A modification of the methods of Guilbault et al (3–5) employing fluorescent substrates was used for enzyme assays. The activities of enzymes derived from TCK and TCT teliospores were measured under various conditions of pH and reaction temperatures using 4-methylumbelliferyl derivatives as substrates. The concentration of substrates, the buffer solutions, and the times of incubation that were used in these studies are shown in Table 1. The buffer solution (2.5 ml) and the enzyme preparation (0.5 ml) were incubated together for 3 min at the selected temperature before the substrate solution (0.5 ml, at the reaction temperature) was added and stirred. Enzyme preparations heated in a boiling water bath for 10 min were used as controls. The change in fluorescence intensity was measured with a model 203 Perkin-Elmer fluorescence spectrofluorometer. For all enzyme assays, the sensitivity control was set at 4, the selector was set at $\times 1$ or $\times 10$, excitation wavelength was 334 nm, emission wavelength was 446 nm. A standard solution of 0.1 μg/ml of 4-methylumbelliferone was used to adjust to full-scale deflection, and distilled water was used for zero deflection. 4-Methylumbelliferone fluorescences strongly, whereas its derivatives fluorescence weakly. The change in fluorescence intensity was recorded automatically or was read manually. The enzyme activity was expressed as the change of fluorescence intensity per minute (Δ F/min), corrected for control activity.

The 4-methylumbelliferyl-heptanoate, and 4-methylumbelliferyl-β-D-cellobiopyranoside were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England; all other substrates were obtained from Sigma Chemical Company, St. Louis, MO 63178.

Fluorescence microtest for glucosidase. A drop of deionized distilled water and 2 mg of TCK teliospores were placed into a glass-to-glass tissue grinder and homogenzied for 1–2 min. An additional 1 ml of deionized distilled water was added, the suspension was mixed thoroughly, centrifuged at 2,000 *g* for 5 min (4 C), and the supernatant was recovered. Aliquots were prepared to contain enzyme preparations equivalent to either 1,000, 500, 200, 150, 100, 50, or 20 μg of spores per ml. Microtest II plates (Falcon 3042) were used in the following manner: microwells received 50 μl of 0.1 M citrate-phosphate buffer (pH 5.5) plus 50 μl of the appropriate aliquot of the spore extract. After 50 μl of the 0.1 mM 4-methylumbelliferyl-β-D-glucoside substrate was added to each well, the plate was incubated at 37 C for 2 hr. Wells which contained either a preparation from boiled teliospores or buffer solution with no spore extract were included as controls. The plates were examined visually under an ultraviolet lamp (maximum emission at 254 nm) and the relative fluorescence intensities were recorded.

Since our fluorescence assays did not use axenically cultured spores, there was a possibility that some of the enzyme activities measured resulted from contaminating microorganisms. We found, however, that in this assay no glucosidase activity was associated with viable whole or ruptured bacterial cells (142,000 to 1,420 cells per well) of *Erwinia herbicola* and *Bacillus phaseolicola*. We concluded that the small contamination of fungal spores by bacteria did not contribute significantly to the results of this fluorescence enzyme assay.

RESULTS

pH optima. The substrates, buffers and times of reactions used for enzyme assays are shown in Table 1. The effects of pH on the enzyme activities measured are illustrated in Figs. 1–14. Figs. 1–5 show that the esterase activities were optimum in the neutral or slightly alkaline range, and the optima depended more on the chain length of the moiety attached to 4-methylumbelliferone than on the source of the enzyme preparation. Short-chain moieties (acetate, butyrate, and heptanoate) were hydrolyzed optimally at pH 8.0–8.5, whereas the longer chain-length moieties (palmitate and stearate) were hydrolyzed best at pH 6.5–7.0. In contrast, the enzyme activities involving a glucosyl bond all had pH optima in the acidic range (4.5–6.0) with most of them in the pH range of 5.0–5.5 (Figs. 7–14). The sulfatase had a pH optimum of 8.0 (Fig. 6).

Temperature optima. The esterase-catalyzed hydrolysis of 4-methylumbelliferyl-heptanoate was measured over the range of 5 to 50 C. The results indicate an optimum temperature of 20 C for the enzyme derived from TCK spores and 30 C for the enzyme from TCT spores (Fig. 15). Both acid phosphatases (pH 5) and alkaline phosphatases (pH 8) from both TCK and TCT spores had temperature optima of 40 C. The precipitous decline in activity of the phosphatases above 40 C suggests enzyme denaturation (Figs. 16–17). β-D-glucosidase, β-D-galactosidase, and β-D-xylosidase activities were higher at 37 C than at 25 C. Compared with the β-D-galactosidase activity, β-D-glucosidase activity was more stable. Upon standing overnight at 4 C, about 75% of the β-D-galactosidase activity from the TCT spore preparation was lost, whereas the β-D-glucosidase activity did not change significantly.

Comparison of enzyme activity in TCK and TCT spore preparations. When the esterase and glycosidase activities were measured at their pH optima and temperature optima, there were wide variations in activity dependent both on the substrates and enzyme sources (Table 2). When 4-methylumbelliferyl-heptanoate was used as a substrate, the highest measured activity was esterase from TCK spores (112 Δ F/min). Most of the enzyme activities were higher in the preparations from TCK than in those from TCT, but sulfatase, *N*-acetyl-β-D-glucosaminidase, *N*-acetyl-β-D-galactosaminidase and acid and alkaline phosphatase exhibited greater activities in the TCT preparations. Two enzymes, α-L-arabinopyranosidase and β-D-glucuronidase, were detected in

homogenates from TCK spores but not in TCT spore preparations.

Comparison of enzyme activities in dormant and dead spores of *Tilletia* species. Enzymes prepared from the heated teliospores were compared with enzymes prepared from living dormant spores. Five different substrates, containing either ester or glucosyl bonds, were used in the assays. The results indicate that the enzyme preparations from heat-killed spores had little or no activity compared with enzyme preparations from living dormant spores.

Fluorescence microtest for glucosidase. After 2 hr of incubation, marked fluorescence was observed in wells of microtest plates that contained preparations from viable spores, but no fluorescence was observed in control wells containing material from boiled spores or citrate-phosphate buffer (Fig. 18). The fluorescence intensity diminished as spore preparations were diluted. The lowest visible fluorescence, after 2 hr, occurred in wells that contained material from 25 μg of spores. If incubation were continued for 15–20 hr rather than the standard 2-hr period, the fluorescence intensity increased and was visible in wells which contained material from 7 μg of spores. After 20 hr, there was still no fluorescence in the control wells.

DISCUSSION

There are many biochemical and physiological differences in the spores of *T. controversa* and *T. caries* even though their morphologies are quite similar. Teliospores of the two species require different conditions and time for germination and infection of wheat plants (10). In the work reported here, several different enzyme activities have been measured in spores of both TCK and TCT; the difference in activity, for the same enzyme, between the species was remarkable. Most of the activities of enzymes derived from TCK spores were higher than those prepared from TCT spores; but some of them, sulfatase, *N*-acetyl-β-D-glucosaminidase, *N*-acetyl-β-D-galactosaminidase, and acid and alkaline phosphatase, were higher in TCT spores. Two enzymes, α-L-arabinopyranosidase and β-D-glucuronidase were found in spores of TCK but were not detected in spores of TCT, even though the experiment was repeated many times using different enzyme preparations.

Two different pH ranges for optimum activity were found to depend on the type of bonds hydrolyzed. In general, esterases were most active in the pH range of 6.5–8.0, whereas the glycosidases were optimally active at pH 4.5–6.0. When butyrate and palmitate derivatives of 4-methylumbelliferone were used as substrates, the esterase from TCK spores required a one-half unit lower pH than the esterase from TCT spores for maximum activity.

The temperature optima for the different enzymes were also in two separate ranges. Most of the esterases showed maximal activity at 25 C, whereas the glycosidases required 37 C (Table 2). When 4-methylumbelliferyl-heptanoate was used as a substrate, the esterase prepared from TCK spores had maximal activity at 20 C, whereas the esterase from TCT spores showed a maximum at 30 C. Although morphologically similar, the many differences in enzyme properties show that the spores of TCK and TCT are biochemically quite different even when they are dormant.

Some relationships between the temperature optima of esterase (heptanoate) in TCK and TCT spores and the optimal temperatures of germination were evident. The optimal temperature for germination of TCK spores is 5 C, but TCT spores germinate best at 17 C (10,17). Although the optimal temperature of esterase activity (heptanoate) in TCK spores (20 C) was lower than that in TCT spores (30 C), in both cases these temperatures were higher than the optimal temperatures for germination. Both esterases may be relatively active (Fig. 15) during the germination of teliospores even though they are not at their respective temperature for maximal activity. Lipid esterases appear to be active during the early stages of germination of TCT spores (15). In uredospores of *Puccinia graminis* var. *tritici* Eriks. & E. Henn., the optimal temperature (15 C) for lipase activity was identical to the optimal temperature for germination (7).

The esterases from TCK and TCT spores when measured with 4-methylumbelliferyl-butyrate or heptanoate have the highest activities of all the enzymes assayed in this work. The esterase activity with 4-methylumbelliferyl derivatives varied with carbon chain length in the lipid-like fragment in the following order: $C_7 > C_4 > C_2 > C_{16} > C_{18}$. Palmitic acid ($C_{16:0}$) and linoleic acid ($C_{18:2}$) are the main components of the free fatty acids in dormant teliospores of TCK (13) and TCT, and they were found to decrease rapidly during germination of TCT teliospores (15).

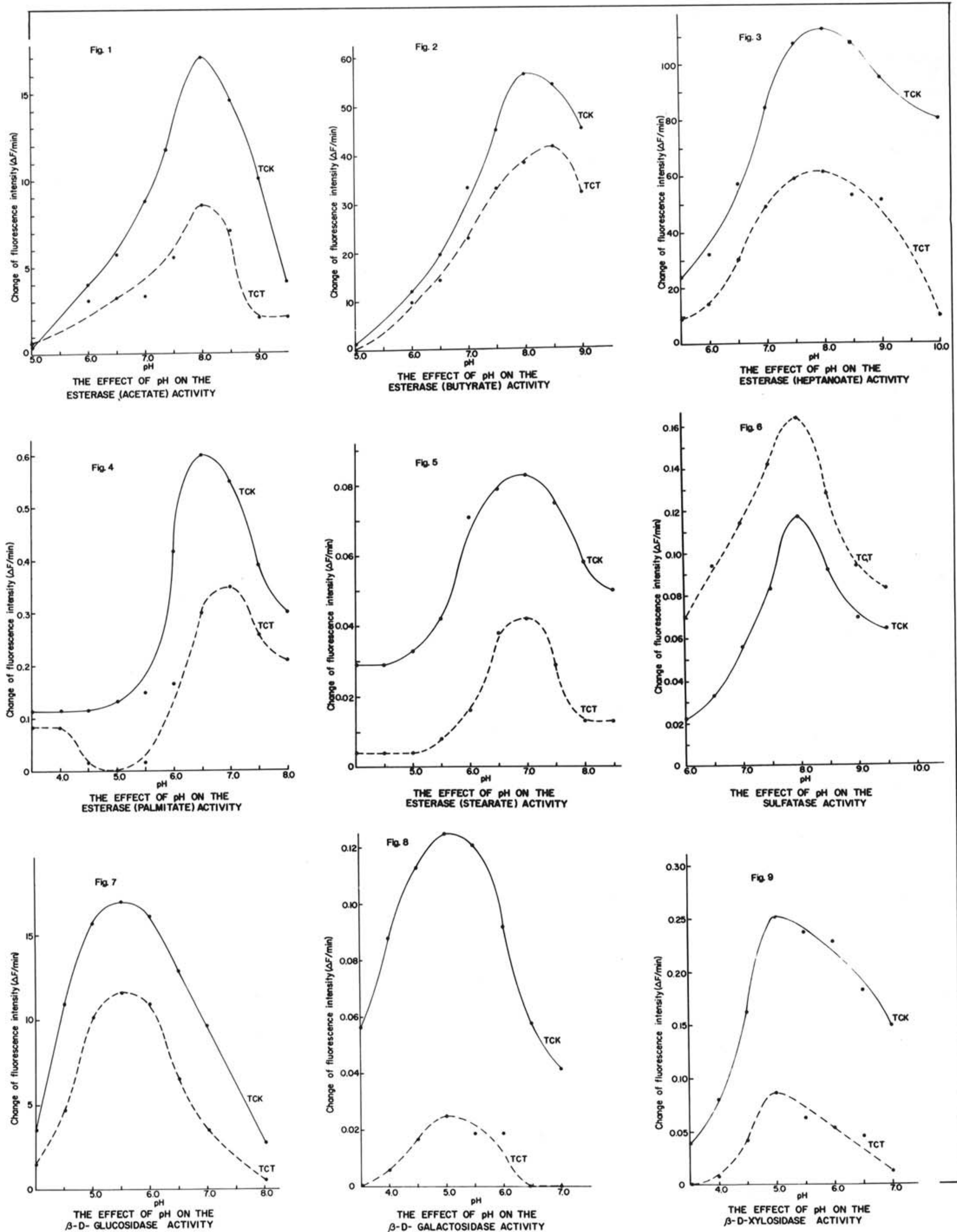
The moist spores heated to 100 C for 10 min were used as controls and were found to have either no enzyme activities or only about 5% of the level detected in unheated spores. This absence or low level of enzyme activities in heated spores was correlated with nonviability, since those spores also failed to germinate. In dormant fungal spores, some of the enzymes were either not present, inactive, or had very low activity (8). It has been shown (1), however, that the enzymes can be activated or synthesized at appropriate stages of germination.

The method of enzyme assay with a fluorescent substrate is rapid and sensitive. This technique has been used to examine the enzyme

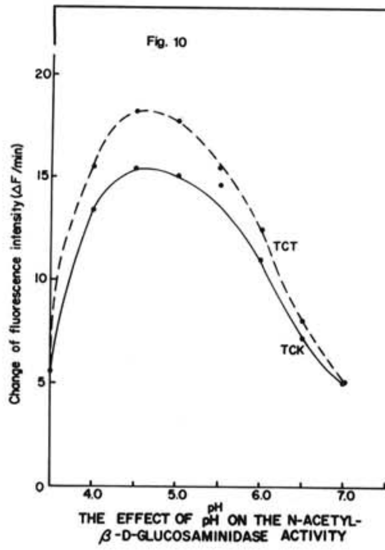
TABLE 1. The substrates, buffers, and reaction times used for enzyme assays

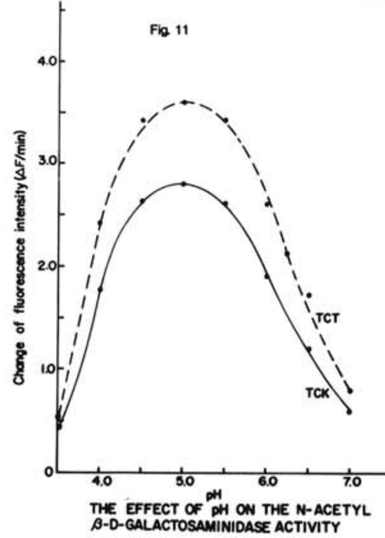
Substrate[a]	Substrate concentration[b] (mM)	Buffer Solution Concentration (M)	Buffer Solution Type	Time of reaction (min)	Reference
4-Mu-acetate	0.1[c]	0.1	Cit.PO_4[e] + Tris HCl	1	4
4-Mu-butyrate	0.1[c]	0.1	Cit.PO_4 + Tris HCl	1	4
4-Mu-heptanoate	0.1[c]	0.02	Glycine + PO_4	1	4
4-Mu-palmitate	0.1[c]	0.1	Cit.PO_4	30	4
4-Mu-stearate	0.1[c]	0.1	Cit.PO_4	120	4
4-Mu-sulfate	0.2[d]	0.1	Tris HCl	180	3
4-Mu-β-D-glucoside	0.2[d]	0.1	Cit.PO_4	30	11
4-Mu-β-D-galactoside	0.1[d]	0.1	Cit.PO_4	120	12
4-Mu-β-D-xyloside	0.2[d]	0.1	Cit.PO_4	60	2
4-Mu-*N*-acetyl-β-D-glucosaminide	0.1[d]	0.1	Cit.PO_4	1	14
4-Mu-*N*-acetyl-β-D-galactosaminide	0.1[d]	0.1	Cit.PO_4	30	16
4-Mu-β-D-cellobiopyranoside	0.1[d]	0.1	Cit.PO_4	30	
4-Mu-α-L-arabinopyranoside	0.1[d]	0.1	Cit.PO_4	120	
4-Mu-β-D-glucuronide	0.1[d]	0.1	Cit.PO_4	180	9
4-Mu-phosphate, acid	5.0[d]	0.1	Citrate, pH 5	60	6
4-Mu-phosphate, alkaline	5.0[d]	0.1	Tris HCl, pH 8	60	6

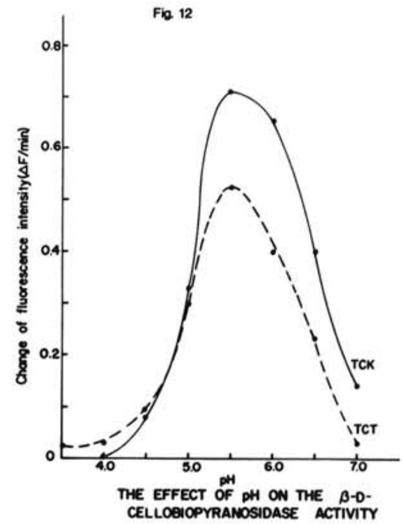
[a] 4-Mu is the abbreviation for 4-methylumbelliferyl.
[b] Final concentration in the reaction mixture.
[c] In 95% ethanol.
[d] In water.
[e] Cit.PO_4 buffer (0.1 M citric acid and 0.2 M K_2HPO_4).

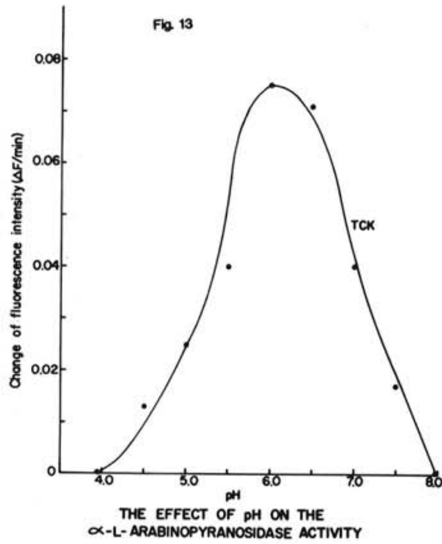


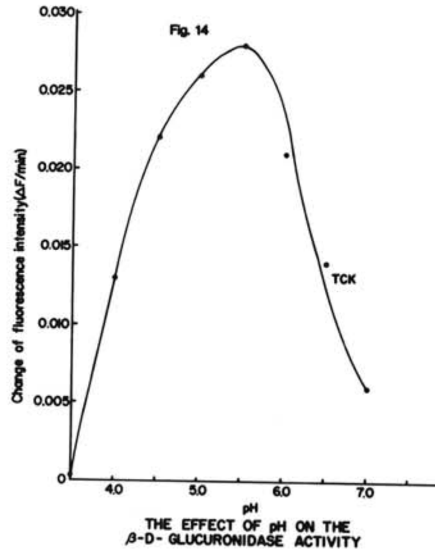
Figs. 1-14. The effect of pH on activities of certain enzymes from *Tilletia caries* (TCT) and *Tilletia controversa* (TCK).

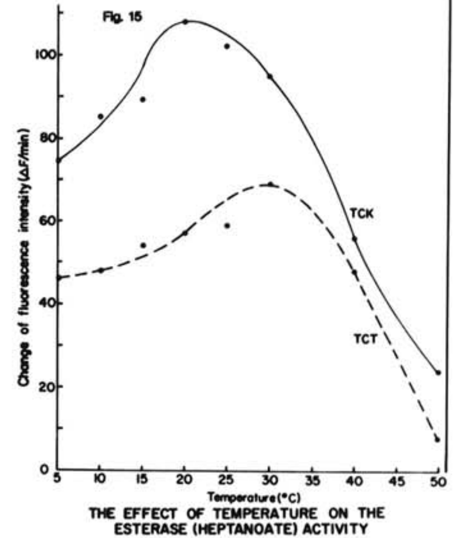


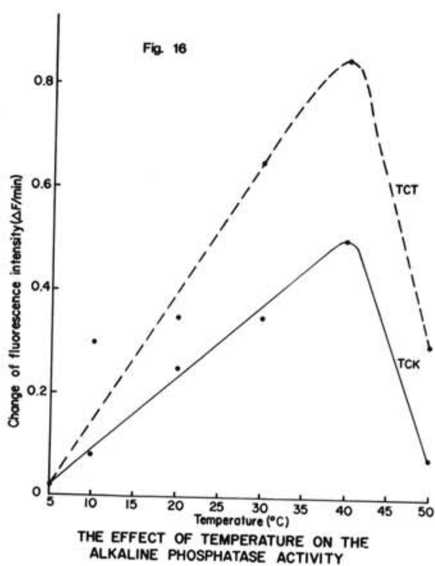


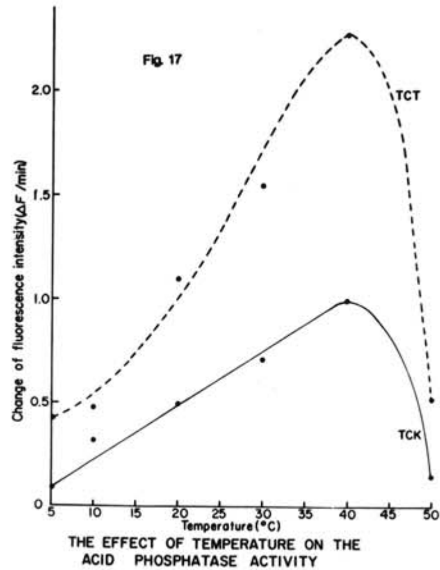












Figs. 15-17. The effect of temperature on activities of certain enzymes from *Tilletia caries* (TCT) and *Tilletia controversa* (TCK).

TABLE 2. The enzyme activities in teliospores of *Tilletia controversa* (TCK) and *T. caries* (TCT) at optimum conditions of pH and temperature

Enzyme	Optimum reaction conditions		Enzyme activity (ΔF/min.)	
	pH	Temp (C)	TCK	TCT
Esterase				
Acetate	8	25	17.00	8.50
Butyrate	8 (TCK) 8.5 (TCT)	25	56.50	41.50
Heptanoate	8	20 (TCK) 30 (TCT)	112.00	61.00
Palmitate	6.5 (TCK) 7.0 (TCT)	25	0.60	0.35
Stearate	7.0	25	0.08	0.04
Sulfatase	8.0	37	0.12	0.16
β-D-glucosidase	5.5	37	16.90	11.60
β-D-galactosidase	5.0	37	0.13	0.03
β-D-xylosidase	5.0	37	0.25	0.09
N-acetyl-β-D-glucosaminidase	4.5	37	15.40	18.25
N-acetyl-β-D-galactosaminidase	5.0	37	2.80	3.60
β-D-cellobiopyranosidase	5.5	37	0.72	0.53
α-L-arabinopyranosidase	6.0	37	0.08	0.00
β-D-glucuronidase	5.5	37	0.03	0.00
Phosphatase, acid	5.0[a]	40	1.00	2.28
Phosphatase, alkaline	8.0[a]	40	0.50	0.85

[a] According to Guilbault et al (5).

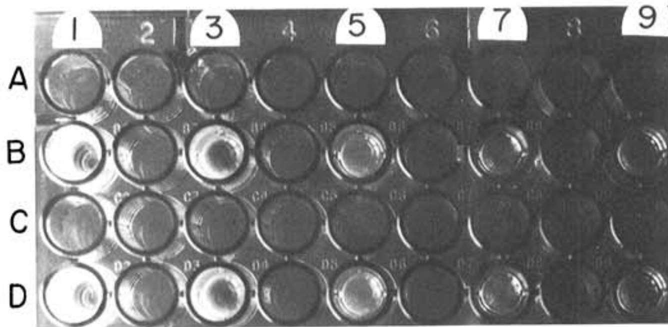


Fig. 18. The fluorescence microtest plate for monitoring glucosidase activity. Rows A and C were preparations from boiled spores of *Tilletia controversa*. Rows B and D were preparations from viable dormant spores. Columns 1, 3, 5, 7, and 9 contained extracts from different weights of spores in each well; 1 = 100 μg, 3 = 50 μg, 7 = 10 μg, and 9 = 5 μg. Columns 2, 4, 6, and 8 were buffer alone. The photo was taken under near ultraviolet light, using Kodak Tri-X Panchromatic film. White to grey on the photo are equivalent to a bright blue fluorescence. Black is equivalent to no fluorescence.

activities of individual molecules of β-D-galactosidase (12). The fluorescence microtest plate method for glucosidase activity used in our study required only 25 μg of teliospores per sample; this was simpler than the conventional assay since it did not require the use of a spectrofluorometer. The fluorescence microtest plate method may provide an accurate measure of spore viability and would be useful in monitoring the effects of fumigation or fungicidal treatments on fungi that have dormant spores.

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