# The Use of Isozymes to Identify Teliospores of *Tilletia indica*

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## ABSTRACT

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Single-teliospore cultures of *Tilletia indica*, causal agent of Karnal bunt of wheat, were examined by horizontal starch gel electrophoresis and staining for 50 enzymes. Thirty-two were present in high concentrations and could be resolved. Of 36 presumed isozyme loci, 15 (42%) were polymorphic (having allelic variation) among cultures. The high number of alleles in common (Rogers' coefficient of similarity = 0.83) among isolates of *T. indica* facilitated their differentiation from those of *Tilletia barclayana*, the causal agent of kernel smut of rice. Teliospores of the latter are frequently confused with those of *T. indica*. The average coefficient of similarity comparing *T. indica* with *T. barclayana* was extremely low (0.04), emphasizing the large difference in their isozymes. Isozymes are being used at the Foreign Disease-Weed Science Research Unit to distinguish between teliospores of *T. indica* and *T. barclayana* in stored grain, storage facilities, and transportation vehicles.

*Additional keywords*: chemotaxonomy, isozyme analysis, *Neovossia barclayana*, *Neovossia indica*, smut identification.

Karnal bunt of wheat was first discovered in 1930 by Mitra (11) near the town of Karnal, in the present state of Haryana, Northwest India. The pathogen, *Tilletia indica* Mitra (Neovossia indica (Mitra) Mundkur), has also been reported in Pakistan (12), Iraq (5), and Mexico (6). Although it has not been found in the United States, *T. indica* became of concern to the U.S. Department of Agriculture after several interceptions of teliospores in wheat entering the United States from Mexico (1,9).

Wheat movement into Karnal bunt-free countries is regulated on an international scale and subject to quarantine (15). A major problem in preventing movement of *T. indica* is the difficulty in distinguishing teliospores of this pathogen from the commonly found and morphologically similar teliospores of *T. barclayana* (Bref.) Sacc. and Syd., the causal agent of kernel smut of rice. Although the average diameter of teliospores of *T. indica* is greater than that of *T. barclayana*, after long periods in storage, *T. indica* teliospores apparently tend to shrink and cannot be readily differentiated from fresh spores of *T. barclayana* (Bonde and Peterson, personal observation). Teliospores of the rice pathogen sometimes become incorporated as contaminants in wheat or other grains during harvest, transport, or storage and can cause rejection of the shipment and even initiation of costly quarantine sanctions if mistakenly identified as *T. indica*. A method based on criteria other than morphology is required to distinguish teliospores of *T. indica* from those of *T. barclayana*.

We have briefly reported the identification of 29 enzymes and 31 presumed genetic loci coding for isozymes in *T. indica* that may be useful in comparing isolates of the pathogen from different areas of the world (2). A subsequent report described genetic studies confirming the identity of four representative and specific isozyme loci in *T. indica* (3).

This paper provides detailed information on enzymes present in cultures of *T. indica* and the best gel buffers for their resolution on starch gels. We compared 21 presumed isozyme loci in teliospore populations of *T. indica* from Mexico, India, Pakistan, and interceptions by the Animal and Plant Health Inspection Service (APHIS) to determine the degree of genetic diversity of the pathogen. (Teliospores from Iraq were not available.) These isozymes are compared with those of *T. barclayana* from the United States to determine the feasibility of distinguishing teliospores of the two pathogens.

## MATERIALS AND METHODS

**Cultures of fungi.** All cultures used in this study were derived from single teliospores. Cultures of *T. indica* were derived from five field collections from separate fields of infected wheat obtained in 1981, 1982, 1983, 1984, or 1985 in the state of Sonora, Mexico; from three field collections made in 1983 in separate areas in the state of Harayana, Northwest India; or from a field collection made in Pakistan in 1985. Also tested were cultures from interceptions of the pathogen in wheat at Mexicali, Mexico, and at the U. S. customs station at Calexico, CA, in addition to teliospores found in chicken feed at Calexico having entered the United States from Mexico. In addition to the 66 cultures used in the main comparative study reported here, at least 50 more monoteliospore cultures were used in earlier studies that gave similar results as those reported here. Twelve cultures of *T. barclayana* obtained from teliospores in stored rice or field collections made in Texas or California were included for comparison to determine whether they could readily be differentiated from *T. indica*.

**Preparing cultures for electrophoresis.** Individual infected seeds were agitated by hand in 20 ml of sterile distilled water in test tubes. The spore suspension then was filtered through a 60-$\mu$m screen to remove large debris and centrifuged at 260 *g* to form a pellet of teliospores. The pellet was resuspended in 12 ml of 0.525% NaOCl for surface sterilization and immediately centrifuged for 1 min at 2,800 *g*. The pellet was resuspended in 12 ml of sterile distilled water and centrifuged to wash the spores. This step was repeated, and an appropriate volume of sterile distilled water was then added to the pellet to give approximately $5.0 \times 10^3$ spores per milliliter. Two drops of the spore suspension were placed onto 2% water agar in a 35- × 10-mm petri plate and incubated at 19 C. Individual germinating teliospores were transferred to separate drops of sterile distilled water in separate 35- × 10-mm petri plates. Each

teliospore with attached basidiospores was teased to detach the basidiospores, and the basidiospores were spread over the surface of the water agar and incubated at 19 C. After 3–7 days, a block of water agar (about 3 $cm^2$) with mycelial growth was cut from the agar and placed inverted on the inside surface of the cover of a 100- × 15-mm petri plate. Sterile potato broth was placed in the bottom of the petri plate. As secondary sporidia were produced, they fell into the broth, where they floated and germinated. After 3–5 days' incubation at 19 C, mycelia and spores floating on the broth were skimmed from the surface, placed in Nunc cryotubes (Thomas Scientific, Swedesboro, NJ), and frozen at −196 C in a liquid nitrogen refrigerator. This technique minimizes possible competition among different genotypes in culture and loss of alleles (3).

**Extracting teliospore cultures for electrophoresis.** Each frozen sample was crushed with the aid of a glass rod, the end of which had been melted to form a bulb. Before crushing a sample, the tip of the rod was submerged in liquid nitrogen. This helped maintain the sample in a frozen state for the initial phase of crushing. Crushing continued until the sample completely thawed. The crushed samples were transferred to 12- × 77-mm disposable test tubes, brought up to 0.5 ml with 0.05 M Tris-HCl, pH 7.5, buffer, and centrifuged to pellet solid debris. The supernatant for each sample was drawn up into paper wicks cut from no. 470 filter paper (Schleicher and Schuell Inc., Keene, NH) and placed at the origin of starch gels.

**Gel electrophoresis of crushed sporidia and mycelia.** Horizontal starch gel electrophoresis and staining for specific enzymes followed procedures described by Micales et al (10).

**Genic nomenclature.** Genic nomenclature follows that described by Micales et al (10). Abbreviations with all capital letters refer to enzymes, whereas abbreviations with only the first letter capitalized refer to specific loci coding for the enzyme. Alleles at a particular locus were designated by the relative anodal mobility from the origin of the respective homomeric (all polypeptide components the same) protein products. The designation for each allele was relative to the protein product of one allele (usually the most common) designated 100. For example, Gpi-100 is the most common allele coding for glucosephosphate isomerase at the Gpi locus. Allele Gpi-180 is another allele at the Gpi locus coding for an enzyme molecule that migrates 80% further on the gel than the enzyme coded by allele Gpi-100.

**Analysis of data.** Analysis of isozyme data was performed with the use of the computer program Allozyme (compliments of R. Struss). Among other tests, the program determined the degree of similarity (coefficient of similarity) according to Rogers (14) and percent heterozygosity for each isozyme locus.

TABLE 1. List of enzymes for which high activity was found, their abbreviations, buffer systems for best resolution of bands, and whether polymorphism was detected for any of the loci coding for the enzyme

| Enzyme (E.C. no.) | Abbreviation | Buffer system | M or P[a] |
|---|---|---|---|
| Acid phosphatase (3.1.3.2) | ACP | M[b] | M |
| Aconitase (4.2.1.3) | AC | 4[c], C[d] | M |
| Adenylate kinase (2.7.4.3) | AK | C, R[e] | M |
| Aldolase (4.1.2.13) | ALD | R | M |
| Diaphorase (1.6.2.2) | DIA | R | P |
| Esterase-fluorescent (3.1.1.1) | EST-F | 4, R | P |
| Galactosaminidase (3.2.1.53) | GAM | C, 4 | M |
| Glucokinase (2.7.1.2) | GK | R, M | M |
| Glucose-6-phosphate dehydrogenase (1.1.1.49) | G6PDH | C | P |
| Glucose phosphate isomerase (5.3.1.9) | GPI | C, 4 | P |
| Glutamate dehydrogenase (1.4.1.2) | GDH | R, M | P |
| Glutathione reductase (1.6.4.2) | GR | R, M | P |
| Glycerate-2-dehydrogenase (1.1.1.29) | GLYD | R | ...[f] |
| Guanine deaminase (3.5.4.3) | GDA | R | M |
| Isocitrate dehydrogenase (1.1.1.42) | IDH | C, 4 | P |
| Lactate dehydrogenase (1.1.1.27) | LDH | C, 4 | P |
| Leucine aminopepidase (3.4.11.1) | LAP | R | M |
| Malate dehydrogenase (1.1.1.37) | MDH | M, 4 | M |
| Malic enzyme (1.1.1.40) | ME | C | M |
| Mannitol dehydrogenase (1.1.1.67) | MADH | R, C, 4 | P |
| Mannose phosphate isomerase (5.3.1.8) | MPI | R | P |
| Nucleoside phosphorylase (2.4.2.1) | NP | C | P |
| Peptidase with glycyl-leucine (3.4.11 or 3.4.13) | PEP-GL | R | M |
| Peptidase with leucyl-alanine (3.4.11 or 3.4.13) | PEP-LA | R | M |
| Peptidase with leucyl-leucyl-leucine | PEP-LLL | M | P |
| Peptidase with phenyl-alanyl-proline (3.4.11 or 3.4.13) | PEP-PAP | M | P |
| Phosphoglucomutase (2.7.5.1) | PGM | 4, R | P |
| Phosphogluconate dehydrogenase (1.1.1.44) | PGD | C | M |
| Phosphoglycerate kinase (2.7.2.3) | PGK | 4, C | P |
| Superoxide dismutase (1.15.1.1) | SOD | R | ...[f] |
| Triose phosphate isomerase (5.3.1.1) | TPI | R, M | M |
| Xanthine dehydrogenase (1.2.1.37) | XDH | R | M |

[a] M = locus or loci monomorphic; P = at least one locus coding for the enzyme was polymorphic.
[b] Electrode buffer according to Markert and Faulhaber (8); ≤275 V, ≤75 mA; electrode buffer diluted 1:4 for gel.
[c] Buffer according to R. K. Selander et al (16); 170 V, 3 hr.
[d] Electrode buffer according to Clayton and Tretiak (4); electrode buffer diluted 1:10 for gel buffer; ≤90 mA, 200 V.
[e] Buffer according to Ridgway et al (13); ≤250 V, ≤75 mA.
[f] Not enough isolates tested to determine if monomorphic or polymorphic.

## RESULTS

**Identification of presumed isozyme loci.** The presence of 50 enzymes was tested by using four separate gel buffer systems for resolution. Thirty-two enzymes were detected in high concentrations and resolved for interpretation. These enzymes and best buffer for resolution of each are presented in Table 1.

Of the 32 enzymes analyzed, subsequent analytical comparisons

TABLE 2. Identification of presumed alleles at 21 isozyme loci in teliospores of *Tilletia indica* and *T. barclayana*

| Isozyme locus | Total no. of alleles detected in *T. indica* | Isolates heterozygous for locus (%) | Alleles detected among isolates[a] | |
|---|---|---|---|---|
| | | | *T. indica* | *T. barclayana* |
| Acp | 1 | 0.0 | 100 | ... |
| Gdh | 3 | 1.7 | 100,110,130 | 100 |
| G6pdh | 2[b] | 5.2 | 100,126 | 100 |
| Gpi | 3 | 53.4 | 100,80,120 | 125 |
| Gr | 1 | 0.0 | 100 | 111 |
| Idh | 4 | 3.4 | 100,121,243,343 | 100,121 |
| Lap | 1 | 0.0 | 100 | ... |
| Ldh-1[c] | 3 | 9.9 | 100,133,67 | 33 |
| Ldh-2[c] | 1 | 0.0 | 100 | ... |
| Madh | 3 | 10.3 | 100,164,191 | 182,208 |
| Mdh | 1 | 0.0 | 100 | 121 |
| Mpi | 2 | 0.0 | 100,106 | 92,85 |
| Np | 2 | 12.7 | 100,73 | 73 |
| Pep-LA | 1 | 0.0 | 100 | 111 |
| Pep-LLL-1 | 2 | ...[d] | 100,110 | 86 |
| Pep-LLL-2 | 1 | 0.0 | 100 | 112 |
| Pep-PAP | 3 | ... | 100,108,133 | 100,108,133 |
| Pgd | 1 | 0.0 | 100 | 136,143 |
| Pgm-1 | 1 | 0.0 | 100 | ... |
| Pgm-2 | 4 | 14.8 | 100,94,87,106 | 94 |
| SOD | 1 | 0.0 | 100 | 153 |

[a] Sixty-six isolates of *T. indica* and 12 of *T. barclayana* were tested. Alleles for *T. barclayana* were given designations as if the isolates were of the species *T. indica* and the alleles were situated at the same loci. Blanks (···) for allele designations indicate probable null alleles. However, we treated blanks as missing data for comparisons with *T. indica*.
[b] A third (rare) allele was detected in a previous study but not in the comparison discussed in this paper.
[c] Where multiple loci, bands in lower zone of gel represent locus-1 and bands in upper zone represent locus-2.
[d] Scoring for heterozygosity sometimes was inaccurate because of insufficient resolution; therefore, results have been omitted.

identified 36 presumed loci. The teliospore population of *T. indica* tested was monomorphic (having no allelic variation) at 19 loci and polymorphic (having allelic variation) at 17 loci. Four enzymes (glutathione reductase [GR], lactate dehydrogenase [LDH], peptidase with leucyl-leucyl-leucine as substrate [PEP-LLL], and phosphoglucomutase [PGM]) were shown each to be coded by two loci, one monomorphic and one polymorphic.

The following enzymes each were found to be coded by a single monomorphic locus: acid phosphatase (ACP), aconitase (AC), adenylate kinase (AK), aldolase (ALD), galactosaminidase (GAM), glucokinase (GK), guanine deaminase (GDA), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), malic enzyme (ME), peptidase with glycyl-leucine as substrate (PEP-GL), peptidase with leucyl-alanine as substrate (PEP-LA), phosphogluconate dehydrogenase (PGD), triose phosphate isomerase (TPI), and xanthine dehydrogenase (XDH).

Alleles detected at 21 loci for 66 isolates of *T. indica* or 12 of *T. barclayana* are listed in Table 2. Three enzymes shown to be coded by polymorphic loci in *T. indica* in earlier studies were not tested because of limited amounts of fungal extracts. These were esterase (EST), diaphorase (DIA), and phosphoglycerate kinase (PGK).

The average intraspecific CS for all teliospores of *T. indica* was 0.83 (std. = 0.09) and for *T. barclayana* was 0.85 (std. = 0.10). Comparisons between individual teliospores of *T. indica* ranged from 0.72 to 1.00. When comparing individuals of *T. indica* with those of *T. barclayana*, the average CS was 0.04 (std. = 0.04), with a range of 0.00–0.29.

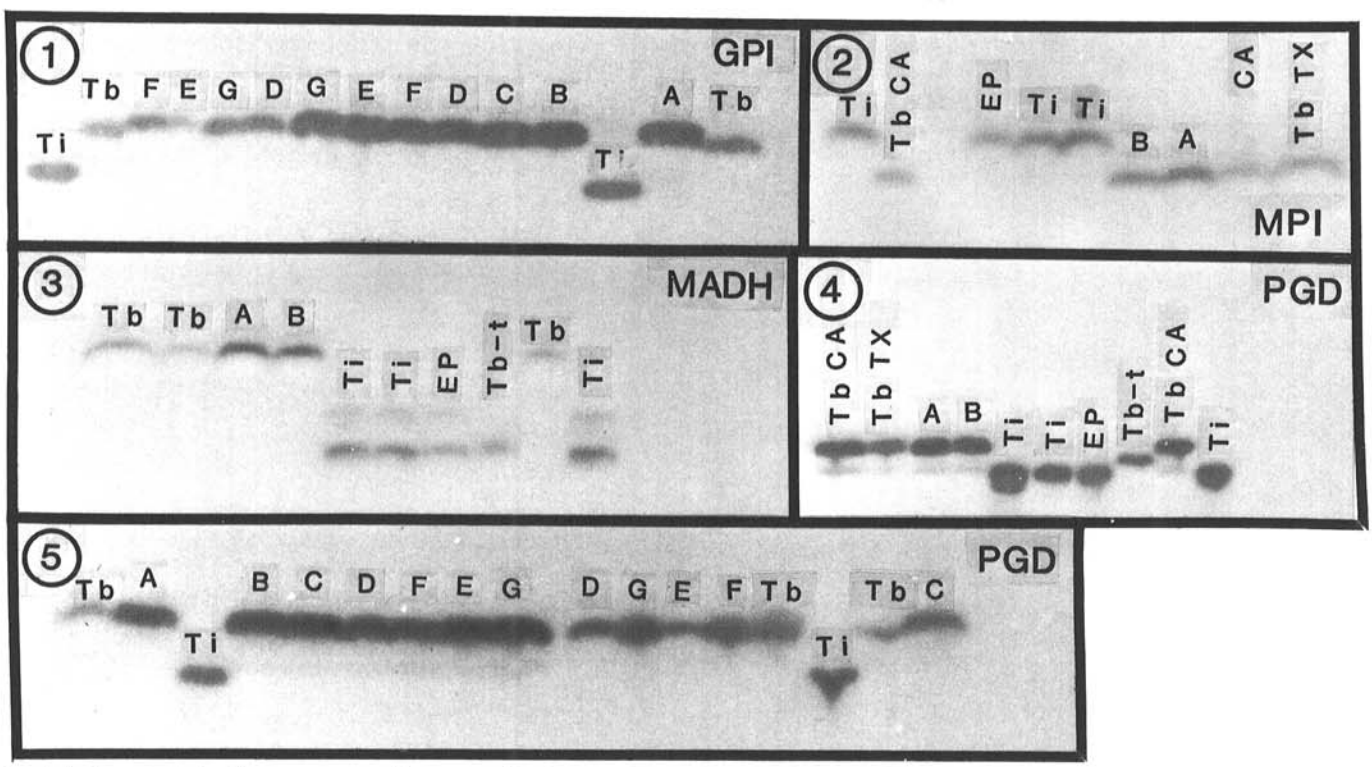
## DISCUSSION

With germination, the diploid teliospores of *T. indica* each produce a basidium upon which haploid basidiospores (primary sporidia) are formed (3). Basidiospores in turn germinate and grow eventually to produce large amounts of monokaryotic mycelia and secondary sporidia (3). During isozyme analyses, growth from single basidiospores usually gives rise to a single band of enzyme activity per locus. If secondary enzymes are present, they can be identified by simultaneous examination of the banding patterns of several isolates and the presence of series of characteristic bands produced per allele (10).

If a germinating teliospore with basidiospore progeny is isolated en masse and grown in culture, the phenotypic banding patterns of the represented genotypes necessarily are superimposed. Heteromeric (hybrid) enzyme bands are absent because only a single allele per locus is present in any individual fungal cell (3). The lack of heteromeric bands allows easy scoring of alleles; the detection of two bands for a locus indicates that the teliospore giving rise to the culture had been heterozygous, and detection of a single band indicates that the teliospore had been homozygous at the locus being examined (3). A previous study involving four polymorphic isozyme loci and 15 genetic crosses indeed showed that banding patterns for the respective loci were either one or two banded and genetically controlled (3).

In initial tests we recognized that isozyme alleles at heterozygous loci in teliospores often were not inherited with equal frequency by their progeny basidiospores (3). This caused the two isozyme bands representing a heterozygous locus of a teliospore to stain with unequal intensity when the culture was tested. In some instances, one of the bands could not even be detected.

A second problem was that during growth of teliospore cultures, sometimes one of the two bands was lost, because of apparent differences in growth rates of the genotypes arising from the teliospore (3). To minimize loss of bands, we developed a technique whereby competition during early growth of a culture presumably




**Figs. 1–5.** Phenotypic banding patterns used in a test to identify unknown teliospores in a plant pathogen regulatory situation. A–G are enzyme bands produced by intercepted teliospores. Some cultures were repeated on the gel to ascertain gel variability. EP represents a teliospore from an interception at Eagle Point, TX, previously shown to be *T. indica*. Ti are known teliospores of *T. indica*, and Tb known teliospores of *T. barclayana* from California (CA) or Texas (TX). Tb-t is *Tilletia bromus technicum* added for an additional comparison on some gels. **1**, Glucose phosphate isomerase test shows interceptions to be the same as standards of *T. barclayana*. Note that the edges of the Tb standards join with the edges of the bands for the unknowns. **2**, Mannose phosphate isomerase test shows interceptions A and B to be the same as standards of *T. barclayana* from California and Texas. Eagle Point interception produced the same isozyme as the two isolates of *T. indica*. **3**, Mannitol dehydrogenase test shows interceptions A and B to be the same as two standards of *T. barclayana* and different from standards of *T. indica*, Eagle Point, and isolates of *T. bromus technicum*. **4**, Phosphogluconate dehydrogenase test shows interceptions A and B to be the same as isolates of *T. barclayana* from California and Texas and different from *T. indica*, Eagle Point (*T. indica*), and isolates of *T. bromus technicum*. **5**, Same as Figure 4, with inclusion of all intercepted isolates.

was minimized (3). The cultures were grown for a maximum 12 days without subculturing, further minimizing shifts in allele frequencies and loss of bands. We used this technique throughout the study with confidence that most alleles were detected, but recognize that in a few instances a band may have been lost and the level of heterozygosity underestimated.

Of 36 presumed isozyme loci, 15 (42%) had detectable allelic variation (polymorphisms) among monoteliospore cultures. We considered any locus where at least one teliospore had a different phenotype to have been polymorphic. This relatively high level of polymorphism within *T. indica* is in contrast to the near absence for *Tilletia foetida* (Wallr.) Liro and *T. caries* (DC.) Tul. (Bonde and Peterson, *unpublished data*). The significance of the higher level of polymorphism in *T. indica* is unknown; however, it may be related to a different breeding system that promotes variation. Basidiospore fusion occurs primarily between basidiospores on the same basidium in *T. foetida* and *T. caries* (10), whereas fusion on the same basidium is rare or absent with *T. indica* (Bonde and Peterson, *unpublished data*). The greater frequency of outcrossing with *T. indica* presumably facilitates sexual recombination and greater genetic variability.

Only 12 isolates of *T. barclayana* were available for this study, and therefore we can say little about the magnitude of allelic variation. However, the organism is morphologically and biologically very similar to *T. indica* and therefore might also be expected to have a high level of isozyme variation.

Although there is considerable allelic variation in *T. indica*, 58% of the 36 putative isozyme loci had no detectable variation. Further studies may show some of these to be variable; however, most should be useful for identifying the pathogen. In our comparison of 66 isolates of *T. indica* from India, Pakistan, Mexico, and interceptions at Calexico, CA, the pathogen was monomorphic at nine of 19 loci. However, even with this imbalance toward polymorphic loci, it was easy to distinguish *T. indica* from the morphologically and biologically similar smut pathogen, *T. barclayana*. Furthermore, we found few isozyme bands in common between *T. indica* and either *T. foetida* or *T. caries* (Bonde and Peterson, *unpublished data*), emphasizing the potential of isozymes for smut identification.

A commonly used measure of genetic variation is the average level of heterozygosity for the population. The average for 19 loci (including monomorphic and polymorphic) for *T. indica* was 0.074 (= 7.4% heterozygosity), with a range of 0.000–0.534 among loci. This average heterozygosity is comparable to 0.063 over 104 loci for humans (7). Presumably, the latter is more representative of the entire genome because of a greater number of loci tested.

Isozyme variation in fungi can be great; however, even with the large variation in *T. indica*, it was easy to differentiate *T. indica* from the morphologically very similar *T. barclayana*. Individual monoteliospore cultures of *T. indica* expressed the most common allele for the majority of loci. This is emphasized by the average intraspecific CS value of 0.83 (range = 0.72–1.00) for 66 teliospores. Indeed, the pathogens could easily be differentiated by examination of only a few enzymes, e.g. GPI, MADH, MPI, and PGD (Figs. 1–5), where any one enzyme alone will differentiate and the other three are included for confirmation.

The technique of isozyme analysis is currently being used in our laboratory to assist the USDA Animal and Plant Health Inspection Service to distinguish between teliospores of *T. indica* and those of *T. barclayana* in plant pathogen regulatory situations. In every situation to date (approximately 15), the results were clear. Isozymes from intercepted teliospores were identical with those of standard known cultures of either *T. indica* or *T. barclayana*. Isozyme analysis provides a means to identify teliospores without conducting long-term pathogenicity tests. The time required is 3–14 days for teliospore germination, 3–7 days for growth, and one additional day for electrophoresis. Photographs of bands on actual gels used in a regulatory situation are shown in Figures 1–5. Of 11 enzymes used to identify the spores, all showed the teliospores could not have been *T. indica*, but were *T. barclayana*.

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