Inheritance of Isozymes in the Smut Pathogen *Tilletia indica*

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ABSTRACT

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Nineteen basidiospore lines of *Tilletia indica*, the causal agent of Karnal bunt of wheat, were tested by means of starch gel electrophoresis for specific isozyme alleles present at each of four putative polymorphic loci. These loci coded for the enzymes glucosephosphate isomerase, glucose-6-phosphate dehydrogenase, mannitol dehydrogenase, and nucleoside phosphorylase. The glucosephosphate isomerase and glucose-6-phosphate dehydrogenase loci each had three alleles, whereas the other loci each had two alleles in the isolates used in this study. The expressed genotypes of the parent basidiospores were compared with those of five single-teliospore progeny for each of 15 specific crosses. Electrophoretic results demonstrated inheritance of isozyme alleles by teliospores from the monobasidiospore lines. Basidiospores arising from germinating teliospores did not inherit alleles at heterozygous loci in the teliospores with equal frequency. Furthermore, some basidiospores inherited both alleles, indicating that many of the basidiospores may receive two haploid nuclei from the promycelium, or that some basidiospores may be aneuploids. All four loci can be used for genetic markers of this important pathogen and 12 other polymorphic loci, not examined in this study, also may prove valuable.

Additional keywords: fungal genetics, isozyme electrophoresis, *Neovossia indica*.

Tilletia indica Mitra is the causal agent of Karnal bunt of wheat, an important disease found in several tropical and subtropical areas of the world including India and Mexico; however, the pathogen is not known to be present in the United States (14). Because of several recent interceptions of teliospores in wheat entering the United States from Mexico, concern about *T. indica* has increased and centers on the possibility that the disease might someday become established in this country, with the primary effect being on U.S. wheat exports (14).

In an attempt to compare the pathogen from India and Mexico, and to develop techniques that can be used to distinguish teliospores of the pathogen from other morphologically similar organisms sometimes present in wheat shipments, we have been testing isozyme analysis (2). Although isozyme comparisons can be made without having made genetic crosses, more precise information can be obtained following evaluation of genetic crosses because it is then known for certain which isozyme bands are the results of which genetic loci. Identification of alleles and respective loci can be valuable as genetic markers for studying the genetics and life cycle of the organism (13).

The purpose of this study was to examine the inheritance of four specific enzymes coded by four presumed genetic loci in the smut pathogen *T. indica* and to use the information to gain information about its genetics. Twelve other polymorphic loci that we discovered in preliminary studies were not used. Because this pathogen is unusual for the genus in that it produces an extremely high number of basidiospores (up to approximately 180 per germinating teliospore), its genetics may be strikingly different than other smut pathogens studied (4).

MATERIALS AND METHODS

Cultures of fungi. All cultures of *T. indica* were derived from four collections of infected wheat seed from separate fields in 1981 and 1983 in the state of Sonora, northwest Mexico; or from three field collections made in 1983 in Amritsar, Patiala, and Sangrur, respectively, northwest India (Table 1).

Establishment of pure monobasidiospore lines. Individual infected seeds with sori were agitated by hand in 20 ml of sterile distilled water in test tubes. The spore suspension then was centrifuged at 700 *g* relative centrifugal force (rcf) to form a pellet, resuspended in 12 ml of 0.525% NaOCl to surface sterilize the teliospores and immediately centrifuged for 1 min at 700 *g* rcf. The pellet was resuspended in an appropriate volume of sterile distilled water to give approximately one spore per 3.4-mm^2 field in a light microscope following placement of two drops of the spore

TABLE 1. Isolate designations, geographic source, and presumed genotypes for four genetic loci for monobasidiosporelines used as parents in successful crosses

Isolate number	Geographic source	Genotypes for genetic loci			
		Gpi	Np	Madh	G6pdh
13	Mexico; 1981	75	100	100	100
18	Mexico; 1981	100	100	100	100
19	Mexico; 1981	100	100	100	100
20	Mexico; 1981	100	100	100	100
25	Mexico; 1981	75	100	100	100
27	Sangrur, India; 1983	75	100	100	100
29	Sangrur, India; 1983	100	75	100	75
39	Amritsar, India; 1983	125	100	100	100
41	Amritsar, India; 1983	125	100	100	100
44	Amritsar, India; 1983	100	100	100	100
47	Amritsar, India; 1983	100	100	100	100
57	Patiala, India; 1983	100	100	100	150
59	Mexico; 1983	75	100	180	100
67	Mexico; 1983	100	100	100	100
68	Mexico; 1983	100	100	100	100
70	Mexico; 1983	100	100	100	100
71	Mexico; 1983	100	100	100	100
72	Mexico; 1983	100	100	180	100
74	Mexico; 1983	100	100	180	100

suspension onto 2% water agar in a petri plate and spreading the spore suspension over the surface. When the first teliospores were observed to be germinating, but before formation of secondary sporida, single teliospores with attached basidiospores (primary sporidia) were transferred to drops of sterile water on the surface of separate agar plates and teased over the surface to release and spread the basidiospores. Then, approximately 12 basidiospores (from a total of about 150 basidiospores per germinated teliospore) were isolated per each of at least 20 teliospores and placed on water agar in individual plates and allowed to germinate. Each basidiospore was observed microscopically to ensure that only single basidiospores were isolated. These monobasidiospore cultures were transferred to potato-dextrose agar (PDA) slants (6).

The monobasidiospore cultures were seeded into potato broth in 250-ml flasks and grown as shake cultures at room temperature (approximately 18 C) for 3–7 days. Sporidia and mycelia from individual flasks were collected by centrifugation at 700 *g* rcf and frozen in Nunc cryotubes (Thomas Scientific Co., Swedesboro, NJ) submerged in liquid nitrogen. The frozen samples later were used directly for electrophoresis and for preserving the lines.

Genetic crosses of monobasidiospore lines. Genetic crosses were obtained as described by Royer and Rytter (14) by inoculating wheat (*Triticum aestivum* L. 'Olaf') with sporidial mixtures of pairs of monobasidispore lines previously shown to be compatible by their initiation of infection and formation of teliospores.

Determination of genotypes of teliospore progeny from monobasidiospore crosses. Teliospores resulting from the crosses were placed on 2% water agar at 18 C in 35-× 10-mm petri plates and later germinating teliospores individually transferred to separate 35- × 10-mm petri plates containing 2% water agar as described above. After 3–7 days, a block of water agar (surface area 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 containing sterile potato broth. After 3–5 days of incubation at 18 C, mycelia and spores floating on the broth were skimmed from the surface of the broth and frozen in a liquid nitrogen refrigerator, as described above, for later electrophoretic comparison with their respective parent basidiospore lines.

Determination of inheritance of isozyme alleles by basidiospores. Teliospores of known genotype with respect to each of the four isozyme loci were germinated and individual germinating teliospores transferred to separate 35-× 10-mm petri plates containing 2% water agar. After completion of teliospore germination, approximately 20 basidiospores for each of 10 germinated teliospores were transferred separately to the centers of individual 35-× 10-mm petri plates containing water agar. These cultures were grown for electrophoresis as described above. Monobasidiospore cultures derived from each specific teliospore were scored for their phenotypes at each of the four isozyme loci and compared side-by-side with the appropriate parents.

Electrophoresis. Each frozen sample was prepared for electrophoresis as previously described for other fungi (1), and horizontal starch gel electrophoresis and staining followed procedures described by Micales et al utilizing the continuous amino-citrate buffer system described by Clayton and Tretiak (3).

Genic nomenclature. Genic nomenclature followed that of May et al (12). Capital-lettered abbreviations 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 or cathodal mobility from the origin of their 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-125 refers to an allele that codes for an enzyme molecule that migrates 25% further than the enzyme molecule coded by the most common allele (Gpi-100) at the Gpi-locus. Gpi-75 refers to an allele that codes for an enzyme molecule that migrates 75% as far as that coded by Gpi-100.

RESULTS

Genetic crosses of monobasidiospore lines. The teliospore cultures derived from each of the 15 crosses produced the expected banding patterns for each locus (Fig. 1) except the cross 19 × 59 for locus-Gpi and the cross 27 × 57 for locus-G6pdh. In these two instances, one of the bands was missing for the expected heterozygous locus on each of the five progeny tested. In a few other instances one of the two expected bands for a given locus was missing for one of the five teliospores for that cross. More commonly, one band stained more intensely than the second band (Figs. 2 and 3). Crossing two monobasidiospore lines with the same allele always produced teliospores with the expected single band (Fig. 4).

Inheritance of isozymes by basidiospores. Data for the detection of isozyme alleles in monobasidiospore cultures derived from teliospores of known genotype are presented in Table 2. A 1:1 segregation ratio (not including basidiospores with two alleles per locus) was tested by means of the chi-square test for each pair of alleles. At locus-Glopdh and locus-Madh, the homogeneity of the data for all teliospores allowed the data to be pooled for the respective loci. In each instance, the segregation ratio was not 1:1 at $p < 0.05$. The homogeneity for teliospores 3, 4, and 5 for segregation of alleles Gpi-100 and Gpi-75 at the Gpi-locus allowed the data to be pooled. Again, the segregation ratio was not 1:1 at $p < 0.05$.

For locus-Np, the data could not be pooled. Teliospores 1, 2, and 7 segregated at a 1:1 ratio. Teliospore 6 had an expected frequency of less than 5 and therefore the data were not interpreted.

At the Gpi-locus, data for teliospores 1, 2, and 7 with genotype Gpi-125/100 could not be pooled because of lack of homogeneity. Teliospores 1 and 7 segregated at a 1:1 ratio; data for teliospore 2 were not interpreted because the expected frequency was less than 5. It was apparent that for at least some allele pairs the alleles were not inherited with equal frequency.

It was also apparent that a significant number of the basidiospores originating from some teliospores received both alleles for a locus heterozygous in the parent teliospore. For

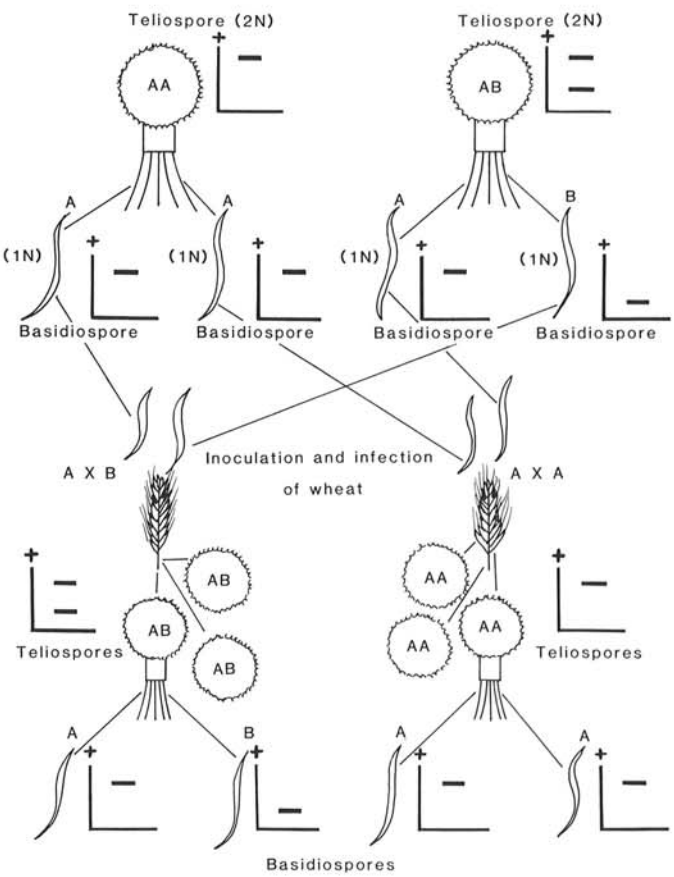


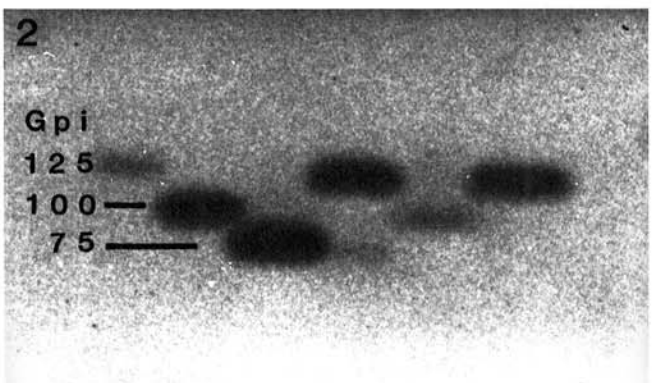
Fig. 1. Diagram depicting expected inheritance of specific isozyme alleles A and B by basidiospores from teliospores homozygous or heterozygous at a given isozyme locus, and genotypes for basidiospore crosses. Expected isozyme banding patterns for the various genotypes are depicted.

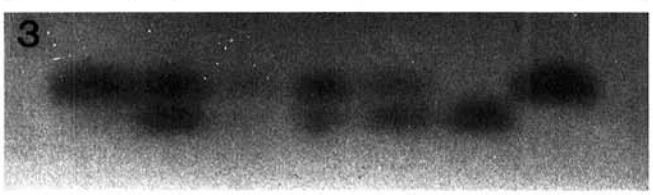
example, with teliospore No. 1 (Table 2), 7/17 basidiospores received Gpi-125, 7/17 Gpi-100, and 3/17 received both alleles. With teliospore No. 2, 3/14 inherited Np-100, 8/14 inherited Np-75, and 3/14 received both alleles. Surprisingly, at the Gpi locus five of the basidiospores inherited allele Gpi-125, and the remainder (9/14) both Gpi-125 and Gpi-100.

DISCUSSION

When basidiospore lines of known isozyme genotype were crossed, in general we obtained the results expected. For example, crossing a pure line containing allele Gpi-100 with a line carrying allele Gpi-125 produced a population of teliospores with a two-banded (Gpi-100/Gpi-125) phenotype. Of more than 75 monoteliospore cultures tested, arising from 15 specific crosses, none produced extra bands that could be interpreted as being coded by alleles not known to be present in the respective parent basidiospore lines. This in addition to the fact that banding patterns of monoteliospore cultures were comprised either of one or two bands (not three or more) indicates that the enzymes are coded by nuclear genes.

In early studies we observed that frequently one band stained more intensely than the second (Figs. 2 and 3) for monoteliospore cultures presumed heterozygous at the isozyme locus being examined. This suggested that perhaps one allele originating from the teliospore might have been inherited more frequently by its basidiospore progeny. A second explanation was that there might have been a population shift of the two different alleles (originating from the teliospore) during growth of the culture. We now feel confident that the unequal staining (and in some cases complete lack of a band) frequently is the combined result of one allele being inherited more frequently and a shift in the population of the two alleles during growth of the culture.





Figs. 2–4. 2, Alleles at the glucose-phosphate isomerase locus detected in monoteliospore cultures of *T. indica* as evidenced by isozyme bands. Center lane depicts both allele Gpi-125 and allele Gpi-75. The unequal staining of the bands is most likely at least partly a result of unequal inheritance of alleles by basidiospores arising from the parent teliospore. **3,** Isozyme gel after staining for the enzyme glucose-phosphate isomerase (GPI). Extract from monobasidiospore line #18 (with allele Gpi-100) was placed at origin at right, followed next to it by an extract from line #25 (allele Gpi-75). The next five lanes are extracts from five separate monoteliospore cultures derived from the cross between the two monobasidiospore lines. Each allele was detected in each of the five teliospore cultures although in some instances one or both produced a faint isozyme band. Where both bands are faint is frequently associated with a slow growing culture. **4,** Isozyme gel after staining for the enzyme glucose-phosphate isomerase. Extract from monobasidiospore line #67 (with allele Gpi-100) was placed at origin at left, followed next to it by an extract from line #74 (allele Gpi-100). The next five lanes are extracts from five separate monoteliospore cultures derived from the cross between the two monobasidiospore lines. Only a single allele was detected in each of the five teliospore cultures.

The unequal inheritance of alleles by basidospores of *T. indica* could result from post-meiotic segregation (PMS). This well-known phenomenon occurs with other fungi such as *Saccharomyces* and *Ascobolus*, where up to 15% of the tetrads can be affected (11). One effect of PMS is that a single haploid nucleus can have two different alleles (as a heteroduplex) following meiosis. The heteroduplex breaks down at the first mitotic division to give a mosaic of two types. A consequence of PMS is an aberrant segregation ratio (11) similar to what we see with basidiospores of *T. indica*.

We observed that when monoteliospore cultures were maintained on PDA for up to several weeks, usually one of the two bands of a two-banded phenotype disappeared. Subsequent studies showed that this loss occurred in as little as 1 wk when a culture was young and rapidly growing (Bonde and Peterson, *personal observation*) and presented a problem when testing teliospores for isozyme genotype. By adopting the technique used in this manuscript, we were able to minimize the frequency of missing bands, thus supporting our hypothesis that shifts in allele frequencies had been occurring. We believe this technique minimized competition of genotypically different individuals during early growth. Presumably each basidiospore was given the greatest chance of survival and the greatest chance to produce mycelium and secondary sporidia. Growth was allowed for only a short duration (3–7 days) with no subculturing, thus increasing the likelihood for minimal shift in allele frequency and therefore the correct determination of teliospore genotype.

The fact that in no instance over many experiments did we ever observe heteromeric (hybrid) isozyme bands with these four

TABLE 2. Alleles detected at four isozyme loci in teliospores and in their respective basidiospore progeny

	Alleles detected at loci			
	G6pdh	Madh	Np	Gpi
Teliospore 1	100/75	100/100	100/75	125/100
Progeny basidiospores	100(14)[a]	100(17)	100(11)	125(7)
	75(3)		75(6)	100(7)
	100/75(0)		100/75(0)	125/100(3)
Teliospore 2	100/75	100/100	100/75	125/100
Progeny basidiospores	100(11)	100(14)	100(3)	125(5)
	75(3)		75(8)	100(0)
	100/75(0)		100/75(3)	125/100(9)
Teliospore 3	100/100	180/100	100/100	75/100
Progeny basidiospores	100(13)	180(0)	100(13)	75(1)
		100(7)		100(8)
		180/100(6)		75/100(4)
Teliospore 4	100/100	180/100	100/100	75/100
Progeny basidiospores	100(11)	180(0)	100(11)	75(0)
		100(4)		100(9)
		180/100(7)		75/100(2)
Teliospore 5	100/100	180/100	100/100	75/100
Progeny basidiospores	100(12)	180(2)	100(12)	75(1)
		100(6)		100(5)
		180/100(4)		75/100(6)
Teliospore 6	100/75	100/100	100/75	100/100
Progeny basidiospores	100(8)	100(8)	100(8)	100(8)
	75(0)		75(0)	
	100/75(0)		100/75(0)	
Teliospore 7	100/75	100/100	100/75	125/100
Progeny basidiospores	100(1)	100(12)	100(6)	100(8)
	75(3)		75(6)	125(2)
	100/75(8)		100/75(0)	125/100(2)

[a] Number in parentheses equals number of basidiospores with that genotype.

enzymes in addition to 27 other enzymes used in other studies (not reported here) suggests that the mycelium and sporidia in culture were primarily, if not totally, haploid. Kirby and Mulley (10), studying inheritance of isozymes in *Ustilago bullata* Berk., frequently observed heteromeric bands in progeny arising from germinating teliospores, suggesting that basidiospores and resulting hyphae were dikaryotic (or diploid) and that the cultures remained in that condition. In all likelihood in our study, some of the enzyme molecules were dimers and would be expected to produce heteromic bands if two different alleles at a locus were present in the same fungal structures (9).

Frequently, both alleles in a teliospore of *T. indica* heterozygous at an isozyme locus apparently were inherited by some of the basidiospores. Further studies in which secondary sporidia from monobasidiospore cultures displaying a double-banded Gpi phenotype were tested for isozymes demonstrated that secondary sporidia each contained only a single allele for the Gpi-locus. (Morphological studies have showed only single nuclei in secondary sporidia (4).) Reduction at the locus must occur in these instances after production of the basidiospore but before or during formation of secondary sporidia.

There are several possible explanations as to why some basidiospores receive both alleles. One is that these basidiospores receive a diploid nucleus from the teliospore. This apparently is ruled out with *T. indica* by cytofluorometric studies of Davila (4) and Royer and Therrien (15) who showed that primary sporidia (basidiospores) are almost always, if not always, haploid.

A second explanation is that two haploid nuclei migrate into some basidiospores from the promycelium. Presumably, studies by Davila (4) showing single nuclei entering basidiospores from promycelia, rule this out. However, more recent cytological studies by Goates and Hoffmann (8) might support the hypothesis that some basidiospores indeed do receive multiple nuclei.

Goates and Hoffmann showed that following teliospore germination with *T. caries*, *T. foetida*, and *T. controversa*, haploid nuclei migrated into separate basidiospores; however, there usually were considerably fewer nuclei than basidiospores. The nuclei divided in the basidiospores, one of the daughter nuclei in each basidiospore exited the basidiospore to enter the promycelium, and these migrated into basidiospores lacking a nucleus. Extra nuclei lysed. Goates and Hoffmann (8) stated in the same manuscript that preliminary studies on *T. indica* showed the same general nuclear events.

Assuming this sequence to be correct, then the nuclear events in *T. indica* are not as simple as we once thought. Furthermore, with cytological techniques alone it would be exceedingly difficult to distinguish this sequence, as described by Goates and Hoffmann, from one where in some instances two genotypically different nuclei happen to enter a basidiospore, both surviving to produce a genotypically mixed culture. The isozyme data we obtained is consistent with this scenario. If two haploid nuclei did become incorporated in a basidiospore, segregation of alleles would be expected to occur at the time of formation of the first crop of secondary sporidia.

A major objection to this hypothesis might be that one would expect a few basidiospores to have both mating types (7) and therefore be solopathogenic. However, the observed rapid shift in allele frequencies in culture could readily prevent both mating types being represented in appreciable amounts and, therefore, prevent infection.

A third explanation for inheritance of two alleles by a basidiospore is that some basidiospores are aneuploids with one or more chromosomes unreduced. Aneuploidy could be caused in a number of ways including nondisjunction during meiosis in the teliospore (or promycelium) or to somatic segregation in fungal mycelium in the host (5). If aneuploidy was responsible for the inheritance of two alleles, segregation probably occurs early in the growth of the culture because of the lack of detectable heteromeric bands.

It is premature to make a statement as to the actual causes of the aberrant segregation ratios and inheritance of two alleles by some basidiospores. However, further studies should resolve these questions.

Isozyme analysis is a very valuable technique to study the genetics and life cycle of an organism, especially where a sexual cycle exists. With *T. indica*, at least 16 isozyme loci with genetic variability have been identified and these, in conjunction with cytological studies may someday give a more complete understanding of the life cycle of this important pathogen. Comparison of the isozyme banding patterns in this and other fungi also could be very useful in identification of these organisms.

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