Growth of Haploid *Tilletia* Strains in Planta and Genetic Analysis of a Cross of *Tilletia caries* × *T. controversa*

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ABSTRACT

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The growth of wild-type and genetically marked haploid strains of *Tilletia caries* and *T. controversa* was examined in susceptible and resistant wheat cultivars. Plants in the flag-leaf stage were inoculated by hypodermic injection of haploid strains of each fungus into the region of the developing head. Mutant and wild-type strains subsequently were recovered from wheat heads 3–6 wk after inoculation. Strains of compatible mating types of both fungi were crossed in planta and in wheat head organ culture, and the interspecific hybrid progeny were analyzed for segregation of mating type alleles and cycloheximide resistance. Drug resistance assorted independently of mating type among 101 F_1 progeny, which is consistent with Mendelian assortment of unlinked genes. In the interspecific hybrid, the optimum temperature for teliospore germination was controlled by one or more dominant genes from the strain of *T. controversa*. The onset of germination was intermediate relative to *T. caries* and *T. controversa* and may be controlled by genes that are expressing incomplete dominance or that interact additively. These findings indicate that further genetic analyses will be very informative for ascertaining the genetic relatedness and species designation for these and other related bunt fungi.

Tilletia species are the etiologic agents of bunt diseases of many species of gramineae. Common bunt of spring wheat and dwarf bunt of winter wheat are caused by *T. caries* (DC.) Tul. & C. Tul. and *T. controversa* Kühn in Rabenh., respectively. Spring wheat, which may contain no genes for resistance to dwarf bunt, escapes infection because the teliospores of *T. controversa* require a period of prolonged snow cover or low temperatures for germination. The teliospores of *T. controversa* usually are distinguished from those of *T. caries* by their broader and deeper reticulations and a gelatinous sheath (18). The disease symptoms incited by both fungi are similar, except that plants infected with *T. controversa* are stunted and have an increased number of tillers. Infection hyphae are produced in both species after fusion of two sexually compatible basidiospores and are thought to penetrate the coleoptile of germinating seeds and become established in the shoot meristem. At anthesis, the fungal mycelium proliferates and produces teliospores enclosed in sori that replace the kernels of the mature head.

Early investigations (8,9,13) demonstrated that field-grown wheat seedlings inoculated with germinating teliospores developed bunted heads upon maturity. Subsequently, Fernandez and Duran (5) demonstrated that bunted heads could be obtained within a period of only 4–5 wk after injection of germinating teliospores of *T. controversa* into the boot enclosing the developing wheat head. More recent research on infection and colonization of these *Tilletia* species has focused on genetic studies in axenic culture (1,12). Procedures have been developed for obtaining drug-resistant and auxotrophic mutants of *T. caries* and *T. controversa* (1,12). Heterokaryotic mycelia were recovered from a susceptible wheat cultivar after boot injection of genetically marked, sexually compatible haploid strains, but teliospores were not produced (2). Of particular interest, however, was the observation that both haploid strains were recovered occasionally from the wheat head, indicating that the haploid strain could survive in the host tissue.

This paper describes the growth of wild-type and genetically marked haploid strains of *T. caries* and *T. controversa* in susceptible and resistant wheat cultivars and the segregation of an antibiotic resistance marker and mating-type alleles among progeny of a hybrid cross of sexually compatible haploid strains of these two fungi. A wheat organ culture system that was used to obtain infected wheat heads is described, and its use is expected to expedite future genetic studies of this host-pathogen interaction.

MATERIALS AND METHODS

Strains and media. Wild-type haploid strains of *T. caries* were grown from single basidiospores isolated from germinating teliospores of race T9, whereas haploid strains of *T. controversa* were isolated from germinating teliospores of an unknown race obtained near Bozeman, MT. The isolation and characterization of cycloheximide-resistant strains 24c-cyc^r and 26b-cyc^r have been described elsewhere (1). The cycloheximide-resistant mutant of *T. controversa*, 66D-cyc^r, was obtained for this study using the same procedure. Wild-type cultures were grown in liquid T-19 minimal medium (T-19MM) (16) and maintained by transfer to fresh medium at 1-wk intervals. Drug-resistant strains, which grow slowly in liquid medium, were cultured on T-19MM containing cycloheximide at concentrations of 18 μg/ml for 66D-cyc^r and 50 μg/ml for 26b-cyc^r and 24c-cyc^r. Mycelia were recovered from sections of inoculated wheat heads that were placed on T-19 complete medium (T-19CM) (2) containing streptomycin (100 μg/ml) to retard bacterial growth.

Wheat inoculation and bioassay. Spring wheat (*Triticum aestivum* L.) used in these studies included the susceptible cultivar Chinese Spring and a derivative of the cultivar Red Bobs, which carries the *Bt*4 resistance gene to race T9 of *T. caries*. Seeds were surface sterilized in 0.5% sodium hypochlorite for 4–6 min, rinsed in sterile distilled water, and planted in a pasteurized soil

mix of equal parts of sand, soil, and peat. Plants were maintained in the greenhouse under daytime and nighttime temperatures of 20 C and 17 ± 5 C, respectively, with 18-hr high-intensity fluorescent lighting and weekly fertilization with Hoagland's solution (10).

Plants at or approaching the flag-leaf stage were inoculated above the uppermost node by hypodermic injection into the region of the developing head (5). Inocula (0.5 ml, 10^7–10^8 cells/ml) consisted of sporidia and hyphal fragments from a single haploid strain, or mixtures of equivalent inocula from two compatible haploid strains. Plants injected with mixtures of compatible strains were examined for bunted heads 4–6 wk after inoculation, whereas plants that were injected with a single haploid strain were examined 3–6 wk later for the presence of viable fungal mycelia. To examine for the presence of viable mycelia, heads were excised by cutting below the uppermost node and surface sterilized for 5 min in a solution of 0.5% sodium hypochlorite containing Tween 20 (100 μl/L) as described by Singh and Trione (14). Sections 1 cm in length then were removed from the uppermost node, the stem just below the head, the lower third of the rachis, the upper third of the rachis, and the embryo of a seed in the lower third of the head, transferred to T-19CM with and without cycloheximide, depending upon the strain, and incubated at 20 C with a 12-hr light/dark cycle. Plants that had a section that became contaminated were not included in these analyses, and heads that either died or failed to reach maturity after inoculation were not examined. Bunted heads were noted by visual inspection.

Organ culture. Wheat plants in the early boot stage were cut 2.5 cm below the uppermost node with a sterile scalpel, and the heads were surface disinfected with 70% ethanol. The cut ends were placed in 15-ml polyurethane test tubes filled with sterile organ culture medium (3,4) and incubated in a growth chamber at 20 C with high-intensity lighting and a 12-hr light/dark cycle. Heads were inoculated as described above with compatible haploid strains before being placed in the organ culture medium. At 7- to 10-day intervals, the lower 0.5 cm of the stem of each organ culture plant was removed with a sterile scalpel to facilitate nutrient uptake, and the stems were returned to fresh organ culture medium. Heads were examined daily for disease symptoms.

Analysis of hybrid progeny from a cross of *T. caries* and *T. controversa*. The kinetics of germination of teliospores of *T. caries* and *T. controversa* and the teliospores produced by crossing a haploid wild-type strain of *T. caries* and strain 66D-cyc^r of *T. controversa* were determined in duplicate experiments. The teliospores of each type were suspended in sterile water and distributed across the surface of four water agar plates to a density of 40–120 spores/mm^2. Two plates of each were incubated at 4 and 20 C, the optimal germination temperatures for teliospores of *T. controversa* and *T. caries*, respectively. Four hundred teliospores on each plate were identified, and their position was recorded by marking the bottom of the plate. The teliospores were monitored daily until approximately 50% had germinated, at which time the ungerminated teliospores could not be distinguished through the newly formed hyphae.

Segregation of mating type and drug resistance in the F_1 progeny obtained from the cross of *T. caries* and *T. controversa*. The hybrid teliospores obtained by crossing strain 66D-cyc^r of *T. controversa* with a wild-type strain of *T. caries* were used to analyze the segregation of mating-type alleles and cycloheximide resistance. To obtain haploid progeny, a petri dish containing germinating teliospores was inverted over another containing T-19 agar medium, and the basidiospores that collected on the lower plate were spread with a sterile glass rod and allowed to germinate. When germ tubes were visible, individual spores were cut out of the agar and transferred to fresh medium. Subsequently, sporidia from the monosporidial cultures were tested for mating type according to the procedure of Kollmorgen and Trione (11) and for resistance to cycloheximide. To ensure that the tester strains for these studies were mating competent, two monosporidial cultures from the F_1 progeny that were determined to be of opposite mating type were selected and arbitrarily designated tester strain 1 and tester strain 2. Ninty-nine other monosporidial isolates then were paired with these tester strains to determine their mating competence.

RESULTS

Growth of haploid strains of *T. caries* and *T. controversa* in planta. Although the plants were surface sterilized to inhibit fungal contaminants, a large percentage (approximately 25–70%) of plants in some experiments had at least one section that was contaminated, usually with *Aspergillus* spp., whereas in other experiments less than 10% were contaminated. To eliminate the possibility of overlooking the presence of *Tilletia* spp. among the hyphae of a contaminant, any plant from which a single section contained a fungal contaminant was not considered in this analysis. Furthermore, no attempt was made to recover the fungus from heads that failed to reach maturity or that died shortly after inoculation, and from areas wounded during inoculation. Of the heads analyzed 3–6 wk after inoculation, haploid mycelia of wild-type and cycloheximide-resistant strains were isolated from 6 to 25% of the heads of the susceptible wheat cultivar Chinese Spring (Table 1). Hyphae grew only from cut surfaces of the rachis, stem, and node and were never recovered from embryonic tissue, and frequently the fungus was isolated from more than one section per plant. Moreover, the wild-type strain of *T. caries* occasionally was observed growing out of the kernels and eventually formed a mycelial mat over the surface of the kernels. Drug-resistant mutant strains 24c-cyc^r and 26b-cyc^r of *T. caries* and 66D-cyc^r of *T. controversa* were positively identified by growth of the mycelium on medium containing cycloheximide. Although the recovery of both mutant strains of *T. caries* was less frequent than recovery of the corresponding wild-type strains, this was not true for 66D-cyc^r of *T. controversa* and its parental wild-type strain. The wild-type strain of *T. caries* also was recovered from 27% of the inoculated heads of the resistant cultivar Red Bobs, whereas 24C-cyc^r was not recovered from any of 26 inoculated plants.

Fungal crosses made in planta and in organ culture. The results of crossing sexually compatible, wild-type haploid strains of *T. controversa* and *T. caries* in all combinations in planta and of the heterologous cross in organ culture are presented in Table 2. Only the cross of wild-type strains of *T. caries* failed to produce bunted heads among the mature plants. The cross of strains of *T. controversa* and the heterologous cross made in planta produced 27 and 33% bunted heads, respectively, among the mature plants. To facilitate making crosses of haploid strains, an organ culture system of wheat heads was adopted. This method was used successfully to obtain teliospores from a heterologous cross

TABLE 1. Recovery of haploid strains of *Tilletia caries* and *T. controversa* from inoculated susceptible and resistant wheat cultivars

Haploid strain	No. heads analyzed	No. (%) successful isolations from analyzed heads
Chinese Spring (susceptible)		
T. caries		
wild-type	72	21 (25)
24c-cyc^r	24	2 (6)
26b-cyc^r	13	2 (15)
T. controversa		
Wild-type	26	5 (15)
66D-cyc^r	16	4 (25)
Controls[a]		
T-19 medium	24	0 (0)
Uninoculated	24	0 (0)
Red Bobs (resistant)		
T. caries		
Wild-type	45	13 (27)
24c-cyc^r	26	0 (0)
Controls[a]		
T-19 medium	10	0 (0)
Uninoculated	5	0 (0)

[a]Inoculated with T-19 growth medium and uninoculated plants.

of strain 66D-cyc^r of *T. controversa* and a wild-type strain of *T. caries*.

Kinetics of teliospore germination. Previous studies (17) have shown that teliospores of *T. caries* germinate at 18 C in less than 2 wk, whereas teliospores of *T. controversa* germinate within 4–6 wk at 4 C but not at temperatures above 15 C. Teliospores from the heterologous cross between a wild-type strain of *T. caries* and 66D-cyc^r (*T. controversa*) failed to germinate over a period of 10 wk at 18 C (data not shown) but germinated to maximum detectable levels (approximately 50–60%) within 3–4 wk at 4 C (Fig. 1). The latent period for the onset of germination was unique for the three types of teliospore, and for each type of teliospore, it varied only by 2–4 days in the two experiments. The onset of germination of teliospores of *T. controversa* occurred within 22–25 days, which was approximately 1 wk later than the onset of germination of the hybrid teliospores, whereas for teliospores of *T. caries*, it occurred within 11–12 days. Once initiated, the germination of teliospores of *T. caries* and hybrid teliospores reached 50–55% in approximately 5–7 days, whereas germination of teliospores of *T. controversa* required 9–10 days.

Segregation of cycloheximide resistance and mating-type alleles in a heterologous cross. To initiate studies of the genetic relatedness of these bunt fungi, the hybrid teliospores obtained in organ culture by mating strain 66D-cyc^r of *T. controversa* with a wild-type strain of *T. caries* were germinated, and random basidiospores were collected and analyzed for the segregation of the mating-type alleles and cycloheximide resistance (Table 3). Our results (15) indicated that these fungi lose mating competence when stored in culture. Therefore, to ensure that the tester strains used in the analysis of our F_1 progeny were mating competent, several single-spore F_1 progeny were crossed to identify two compatible strains, and these two strains were used in the analysis of all other F_1 progeny (Table 3). All progeny that failed to mate with one tester strain were shown to be competent to mate with the other. The results show that only two alleles were segregating in a 1:1 ratio (54:47) as expected for a bipolar mating system. The segregation of resistance and sensitivity to cycloheximide also showed 1:1 segregation although more sensitive progeny (59) were recovered than resistant progeny (42). Taken together, the F_1 progeny could be placed into four classes which occurred at approximately equal frequency ($P = 0.7$) as expected for Mendelian segregation and assortment of two heterozygous, unlinked genes.

DISCUSSION

Results presented in Table 1 clearly demonstrate that, after hypodermic injection, haploid strains of either fungus remain viable and grow in both susceptible and resistant wheat cultivars for a period of at least 6 wk. Furthermore, the recovery of the cycloheximide-resistant strains precludes the possibility that a contaminant was the source of the inoculum. Fernandez and Duran (5) first showed that the injection of germinating teliospores of *T. controversa* into the boots of susceptible wheat plants could result in the development of bunted heads. Using that technique, Churchill and Mills (2) attempted to cross bunt strains by injecting boot-stage wheat plants with secondary sporidia from two genetically marked haploid strains of *T. caries* that previously were shown to be sexually compatible (11). However, bunted heads did not form, but the parental strains were recovered from the heads, suggesting that haploid strains could grow for a period of several weeks. Although there has been long-standing acceptance that the heterokaryon of *T. caries* and *T. controversa* is the infectious stage that colonizes a susceptible host (7,16), it is now apparent that haploid strains of either fungus remain viable and grow in wheat once entry is gained.

The resistant cultivar Red Bobs was inoculated with haploid strains of *T. caries* to determine whether the haploid strains can grow in resistant cultivars. Although the wild-type strain of either fungus was recovered at approximately equal frequency from the resistant and the susceptible cultivar (Table 1), strain 24c-cyc^r was not recovered from the resistant cultivar. However, the cycloheximide-resistant strains grow more slowly in culture than wild-type strains and were consistently recovered at a lower frequency from the susceptible plant. Therefore, the slower growth rate of strain 24c-cyc^r could account for our failure to detect its growth in the resistant plant. The recovery of wild-type haploid strains from the resistant cultivar suggests that either the teliospores were heterozygous for at least one avirulence gene, or that resistance conferred by the *Bt4* gene does not restrict growth of the pathogen once entry is gained.

To circumvent potential problems of contamination frequently encountered in greenhouse studies, we adopted a wheat head organ culture system to facilitate mating of these fungi. Organ culture provides a means of working with obligate parasites in a controlled environment under sterile or near-sterile conditions when the use

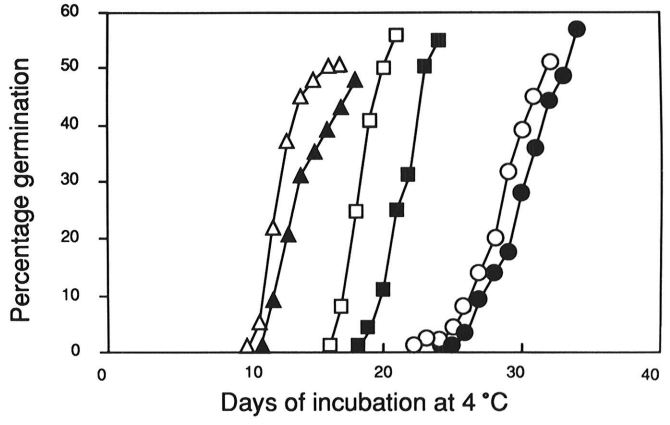

Fig. 1. Kinetics of germination of teliospores. Triangles = teliospores of *Tilletia caries*; squares = teliospores produced by crossing haploid strain 66D-cyc^r of *T. controversa* with a haploid wild-type strain of *T. caries*; circles = teliospores of *T. controversa*. Open and solid symbols represent results of two independent experiments.

TABLE 2. Production of bunted heads on a susceptible wheat cultivar grown in planta and in organ culture after inoculation with sexually compatible haploid strains of *Tilletia caries* (TCT) and *T. controversa* (TCK)

Crosses	No. heads inoculated	No. (%) heads reaching maturity	No. (%) bunted mature heads
In planta			
TCK × TCK	22	21 (95)	6 (27)
TCT × TCK	14	6 (42)	2 (33)
TCT × TCT	7	3 (42)	0 (0)
Controls			
T-19 medium	7	2 (29)	0 (0)
Uninoculated	18	18 (100)	0 (0)
In organ culture			
TCT × TCK (66D-cyc^r)	11	4 (36)	1 (25)
Controls			
T-19 medium	6	6 (100)	0 (0)
Uninoculated	4	4 (100)	0 (0)

TABLE 3. The segregation of resistance to cycloheximide and mating incompatibility among F_1 progeny derived from crossing *Tilletia caries* (TCT) with *T. controversa* (TCK)[a]

	F_1 progeny classes[b]			
	Mat(1), cyc^s	Mat(1), cyc^r	Mat(2), cyc^r	Mat(2), cyc^s
No. F_1 progeny[c]	35	19	23	24

[a] Parental strains TCT cyc^s × TCK cyc^r. Parental strains were determined to be compatible or incompatible according to the procedure of Kollmorgan and Trione (11).

[b] Progeny designated Mat(1) and Mat(2) are incompatible with tester strains 1 and 2, respectively. See Materials and Methods section for details.

[c] Chi-square value for four classes (3 df), $P = 0.7$.

of plant tissue culture is not feasible. It also should prove to be an efficient system for producing a supply of teliospores from which haploid, genetically marked strains may be obtained easily for genetic studies of these obligate pathogens.

Evidence presented here demonstrates that mating, heterokaryon formation, and the production of teliospores will occur after hypodermic injection of a mixture of compatible haploid strains of *T. caries* and *T. controversa* into the boot of the susceptible wheat cultivar Chinese Spring (Table 2). It was suggested previously (2) that loss of mating competence could have been a primary reason for not obtaining smutted wheat heads after the injection of mixtures of sexually compatible strains of *T. caries*. All of the strains used in this previous study (2) were maintained in storage for at least 1 yr before being used in crosses. Mating tests recently performed with each of these strains have shown that none is mating competent on agar medium (15). We have observed that strains of *T. caries* lose their competence to mate within a period of 3–4 mo of isolation if they are not subcultured continually. Although mating competence has been extended for some of our strains to more than 1 yr through continual subculturing, we have not found a method of storage that ensures long-term mating stability among haploid strains. Until it is developed, the genetic material to be used in subsequent crosses may be maintained as teliospores, which remain viable for years at room temperature.

Viable teliospores were obtained from a heterologous cross of *T. caries* × *T. controversa* in susceptible Chinese Spring wheat head organ culture. The onset of germination of these teliospores was intermediate when compared with teliospores of parental strains of *T. caries* and *T. controversa*, and they produced four classes of basidiospores with respect to mating type and sensitivity to cycloheximide (Table 3) as predicted by Mendel's law of random assortment of unlinked genes. Historically, these bunt fungi have been classified both as one species (19) and as two different species (6). Presently, they are considered to be different species, and, therefore, no attempt was made here for purpose of nomenclature to assign mating-type alleles to either fungus. Presently these fungi are distinguished by the kinetics and temperature optima for teliospore germination, symptomatology of the host, and the morphological characteristics of the sori. However, as shown in these studies (Fig. 1) and discussed elsewhere (see 12), these characteristics may be highly variable and greatly affected by the extent to which genes are recombined in their natural environment. The results presented in Table 3 demonstrate the potential for understanding the genetic relationship between these and other closely related fungi and suggest that genetic criteria will substantiate more definitively their species designations.

LITERATURE CITED

1. Churchill, A. C. L., and Mills, D. 1984. Selection and culture of auxotrophic and drug-resistant mutants of *Tilletia caries*. Phytopathology 74:354-357.
2. Churchill, A. C. L., and Mills, D. 1985. Heterokaryon formation in planta by genetically marked strains of *Tilletia caries*. Can. J. Bot. 63:1924-1925.
3. Donovan, G. R., and Lee, J. W. 1977. The growth of detached wheat heads in liquid culture. Plant Sci. Lett. 9:107-113.
4. Donovan, G. R., and Lee, J. W. 1978. Effect of the nitrogen source on grain development in detached wheat heads in liquid culture. Aust. J. Plant Physiol. 5:81-87.
5. Fernandez, J. A., and Duran, R. 1978. Hypodermic inoculation, a rapid technique for producing dwarf bunt in wheat. Plant Dis. Rep. 62:336-337.
6. Fischer, G. W. 1952. *Tilletia brevifaciens* sp. nov., causing dwarf bunt of wheat and certain grasses. Res. Stud. Wash. State Univ. 20:11-14.
7. Fischer, G. W., and Holton, C. S. 1957. Biology and Control of the Smut Fungi. Ronald Press Co., New York. 622 pp.
8. Flor, H. H. 1931. Indications of heterothallism in *Tilletia tritici*. (Abstr.) Phytopathology 21:107.
9. Flor, H. H. 1932. Heterothallism and hybridization in *Tilletia tritici* and *T. levis*. J. Agric. Res. (Washington, DC) 44:49-58.
10. Hoagland, D. R., and Arnon, D. I. 1950. The water culture method of growing plants without soil. Circ. Calif. Univ. Agric. Exp. Stn. 32 pp.
11. Kollmorgen, J. F., and Trione, E. J. 1980. Mating-type interactions between sporidia of a wheat-bunt fungus, *Tilletia caries*. Can. J. Bot. 58:1994-2000.
12. Mills, D., and Churchill, A. C. L. 1988. *Tilletia* spp., bunt fungi of the gramineae. Pages 401-414 in: Genetics of Plant Pathogenic Fungi. Advances in Plant Pathology. Vol. 6. G. S. Sidhue, ed. Academic Press, London.
13. Silbernagel, M. J. 1963. A method of using mycelial, monosporidial isolates of *Tilletia controversa* in pedigreed hybrid crosses. Phytopathology 53:1235-1236.
14. Singh, J., and Trione, E. J. 1969. In vitro culture of the dikaryon of the wheat bunt pathogens, *Tilletia caries* and *T. controversa*. Phytopathology 59:648-652.
15. Trail, F. 1985. The growth of wild-type and genetically-marked strains of *Tilletia caries* and *T. controversa* in susceptible and resistant wheat cultivars. M.S. thesis. Oregon State University, Corvallis. 59 pp.
16. Trione, E. J. 1964. Isolation and in vitro culture of the wheat bunt fungi, *Tilletia caries* and *Tilletia controversa*. Phytopathology 54:592-596.
17. Trione, E. J. 1982. Dwarf bunt of wheat and its importance in international wheat trade. Plant Dis. 66:1083-1088.
18. Wiese, M. V., ed. 1977. Compendium of Wheat Diseases. American Phytopathological Society, St. Paul, MN.
19. Young, P. A. 1935. A new variety of *Tilletia tritici* in Montana. Phytopathology 25:40.