The Ultrastructure and Nuclear DNA Content of *Tilletia indica*

C. D. Therrien, M. H. Royer, and J. A. Strauss

Associate professor, Departments of Biology and Plant Pathology, The Pennsylvania State University, University Park 16802; research plant pathologist, Foreign Disease-Weed Science Research Unit, USDA-ARS, Ft. Detrick, Building 1301, Frederick, MD 21701; and graduate assistant, Department of Biology, The Pennsylvania State University.

Part of this work was completed under Specific Cooperative Agreement 58-3615-4-012 with the Department of Biology, The Pennsylvania State University.

Accepted for publication 10 December 1987 (submitted for electronic processing).

ABSTRACT

Therrien, C. D., Royer, M. H., and Strauss, J. A. 1988. The ultrastructure and nuclear DNA content of *Tilletia indica*. Phytopathology 78:728-732.

The cytology of *Tilletia indica*, the incitant of Karnal or partial bunt of wheat, was investigated by transmission electron microscopy (TEM) and Feulgen-DNA (F-DNA) cytophotometry. Numerous mitoses followed meiosis in the teliospores, which were multinucleate when they germinated. The mean F-DNA content of the filiform primary sporidia was 0.115 arbitrary units (a.u.), that of the allantoid secondary sporidia 0.175 a.u. The mean F-DNA content of nuclei of binucleate teliospore initials was 0.217 a.u., that of postfusion nuclei 0.473 a.u. Apparently DNA replication in the teliospore initials occurred before nuclear fusion. Ultrastructural investigations of the teliospore initials showed that, whereas fusion nuclei were initially binucleolate, as teliospores developed they became uninucleolate.

Tilletia indica Mitra, a member of the Ustilaginales, causes Karnal or partial bunt of wheat. Originally described by Mitra (15), this species was subsequently transferred to *Neovossia* by Mundkur (16). However, recent evidence regarding the morphology, taxonomy, and cytology of the genus *Tilletia* (4–9) suggests that this organism belongs in *Tilletia*, where Mitra (15) originally placed it. The general morphology, taxonomy, and mode of inoculation of this organism has been discussed in a number of reports (7,11,15,17,20). Krishna and Singh (12) and Fuentes-Dávila and Durán (9) described the cytology of teliospore germination, the nuclear cycle, and development; however, neither report has established without equivocation the precise times of karyogamy and meiosis. In this report, we describe the use of transmission electron microscopy (TEM) and Feulgen-DNA (F-DNA) cytophotometry to further elucidate the cytology of *T. indica* and clarify some of the conflicting reports (9,12) regarding the nuclear cycle. All work with live material was performed in the containment laboratory and greenhouse facilities of the USDA Foreign Disease-Weed Science Research Unit at Ft. Detrick, Frederick, MD.

MATERIALS AND METHODS

Promycelia and sporidia. Teliospores of a Mexican collection of *T. indica* were obtained from J. M. Prescott, International Maize and Wheat Improvement Center (CIMMYT). Teliospores were increased by inoculating Olaf spring wheat and designated MEX81-374. Teliospores were recovered by agitating dried bunted kernels in sterile distilled water and filtering the suspension through a 60-μm nylon mesh screen. After pelleting by centrifugation, the teliospores were suspended for 2 min in a 0.5% aqueous solution of sodium hypochlorite, washed twice in sterile distilled water, and germinated on 2% water agar. Germination products used for F-DNA cytophotometry were fixed by flooding the culture dishes with a solution of 4% formaldehyde in 0.25 M sucrose, pH 7.0. The specimens were fixed for a minimum of 18 hr at 5 C, then postfixed in 70% ethanol for at least 24 hr at 5 C. The postfixative was removed by aspiration and the specimens washed twice in sterile distilled water. Teliospores with germ tubes (promycelia) were removed from the dishes with a dissecting needle and transferred to drops of distilled water on slides coated with albumin. The specimens were air dried at 42 C for 24 hr.

To obtain filiform primary sporidia, teliospores were recovered as above, but allowed to germinate for 7–10 days under a 10-hr photoperiod at 60 $\mu E \cdot m^{-2} \cdot sec^{-1}$. Sterile distilled water was poured over germinating teliospores and the primary sporidia dislodged with a rubber policeman. Drops of sterile distilled water containing primary sporidia were then smeared on slides coated with albumin. Secondary allantoid sporidia were obtained by inverting germinating teliospores over slides for 24 hr at 20 C, with a 10-hr photoperiod of light at 60 $\mu E \cdot m^{-2} \cdot sec^{-1}$. After air drying, the sporidia were fixed and postfixed as described above.

Infective hyphae and teliospores. Wheat plants were inoculated as described elsewhere (20) with paired monosporidial lines previously shown to be pathogenic. Approximately 10 days after inoculation, young caryopses were removed from the spikes, surface sterilized with 0.5% sodium hypochlorite for 2 min, rinsed twice in sterile distilled water for 1 min each, and dissected. Hyphae derived from the caryopses were then cultured on potato-dextrose agar. Cultures of the mycelium were fixed in sucrose-Formalin and postfixed in 70% ethanol. After a minimum period of 24 hr at 5 C in the postfixative, the specimens were rehydrated in sterile distilled water, and added in drops of sterile distilled water to albuminized slides. The specimens were air dried at 42 C.

To study development of teliospores from sporogenous hyphae in the host, kernels were removed at different times from the spikes and sectioned transversely. Sections 1–2 mm thick were fixed in sucrose-Formalin for at least 24 hr, washed several times with 70% ethanol, and postfixed in 70% ethanol for a minimum of 24 hr. After postfixation the sections were dehydrated in a graded ethanol series, cleared in xylene, and embedded in low melting point paraffin. The embedded material was oriented so that transverse sections 5 μm thick could be made. Before Feulgen-staining, the sections were deparaffinized in xylene, rehydrated in a graded ethanol series, and transferred to sterile distilled water.

Feulgen-staining and cytophotometry. The Feulgen reaction, as modified by Bryant and Howard (2), was used for the quantification of nuclear DNA. All specimens were hydrolyzed simultaneously in 5 N HCl at 23 C for 60 min and stained with Schiff's reagent (13) for 90 min. After three bisulfite rinses, the specimens were immersed in sterile distilled water, dehydrated in a graded ethanol series, cleared in xylene, and mounted in Cargille index of refraction oil with a refractive index of 1.532.

The two-wavelength method of Patau (18) was used for F-DNA

determinations. To minimize errors that may result from the hydrolysis and staining procedures, chicken erythrocyte nuclei, which have a nuclear genomic DNA content of 2.41 pg (24), were used as an internal standard. The need for an internal standard has been suggested by the investigations of Dhillon et al (3). To prepare the DNA standards, chicken erythrocytes were drawn in a heparinized syringe and smeared on clean microscope slides. The protocol for fixation and Feulgen staining was that described for *T. indica*; however, the refractive index of the oil used to mount the erythrocytes was 1.544.

Cytophotometric determinations were made with a Zeiss Universal microscope equipped with a Zeiss Type 01 microscope photometer. Instrument alignment and phototube linearity response were checked before recording measurements. The extinction coefficient for the F-DNA complex was omitted from the calculations, hence the DNA values are reported as arbitrary units (a.u.).

Although DNA values were recorded in arbitrary units, we determined the absolute DNA content of *T. indica* by F-DNA cytophotometry. To do this, chicken erythrocyte nuclei were used to calculate the extinction coefficient for the F-DNA complex. This value was determined to be 0.28 pg/μm^2 (23), and was substituted for k in the Patau (18) equation (where $DNA = kAL_1C$) to calculate absolute DNA values. This method was used to determine the unreplicated (1C) nuclear DNA content of *T. indica* was 0.1 pg.

Transmission electron microscopy. Sporogenous hyphae and developing teliospores for TEM studies were collected as described for cytophotometry. Specimens were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer for a minimum of 1 hr at room temperature, rinsed three times in cacodylate buffer, postfixed in 1% OsO_4 in 0.1 M cacodylate buffer for 1 hr, rinsed three times in cacodylate buffer, and immersed overnight in 1.5% aqueous uranyl acetate. The specimens were dehydrated in a graded ethanol series, passed through several changes of absolute ethanol/propylene oxide, propylene oxide/resin, and finally embedded in Spurr's low viscosity resin (21). Initially, sections 1 μm thick were cut with glass knives and stained with aqueous toluidine blue O to ensure that infected regions of the kernels were included in the material to be thin sectioned. After we oriented the blocks, gold sections (80–100 nm) were cut with a diamond knife and an LKB Ultrotome III (LKB-Produkter AB, Stockholm-Bromma 1, Sweden). The sections were picked up on Pelco 300 mesh Cu/palladium grids, stained for 20 min with 0.5% aqueous uranyl acetate (25), for 10 min with lead citrate (19), and examined with a Philips 300 electron microscope (N.V. Philips Gloeilampenfabrieken, Eindhoven, The Netherlands).

RESULTS

Data from F-DNA analyses of promycelia, sporidia, infection hyphae in culture, and prefusion and postfusion teliospore initials are summarized in Table 1 and Figure 1A–E. When the nuclear F-DNA content of promycelial nuclei was analyzed by absorption cytophotometry, the DNA values ranged from 0.083 to 0.245 a.u., except for one nucleus that had a value of 0.402 a.u. (Fig. 1A). Approximately 90% of the nuclei fell in the range of 0.11–0.24 a.u.

TABLE 1. Nuclear DNA content of *Tilletia indica* in arbitrary units

Developmental stage	N[a]	Mean DNA	S.E.[b]
Promycelium	50	0.154	0.010
Primary sporidia	50	0.115	0.007
Secondary sporidia	50	0.175	0.008
Infective hyphae	50	0.149	0.006
Binucleate teliospore initials	32	0.217	0.015
Post-fusion uninucleate teliospore initials	34	0.473	0.014

[a]Number of nuclei sampled.
[b]Standard error.

The range of F-DNA values in promycelia suggested that the nuclei were in 1-2C classes. Those with lower values, such as 0.1 a.u., were presumably arrested (presynthetic) (G_1) haploid nuclei, whereas nuclei with higher values, such as 0.2 a.u., were most likely postsynthetic (G_2) haploid nuclei. We determined that postfusion nuclei in teliospore initials had a mean F-DNA content of 0.473 a.u. (Table 1), which indicated that promycelial nuclei with a DNA content of approximately 0.1 a.u. were haploid G_1. According to Swift (22), such nuclei would have the 1C or minimum DNA content. Some nuclei, presumably in S phase of mitotic interphase, were intermediate in DNA content. There are two possibilities to explain the single promycelial nucleus with a F-DNA content of 0.402 a.u. It may have been an unreduced diploid nucleus, or a polyploid nucleus. Our observation that some sporidial nuclei appear to be polyploid (Fig. 1B) lends support to the latter hypothesis.

Analyses of the nuclear F-DNA content of sporidial and infective hyphal nuclei suggest a disparity in DNA values (Table 1, Fig. 1B and C). For example, filiform primary and falcate secondary sporidia had mean F-DNA values of 0.115 and 0.175 a.u. respectively, whereas infective hyphal nuclei had a mean F-DNA content of 0.149 a.u.

One way to explain the difference in DNA content between the primary and secondary sporidia is that the primary sporidial nuclei, being products of a finite number of mitoses, were predominantly haploid G_1, whereas secondary sporidia, which typically divide rapidly and indefinitely, were predominantly haploid G_2. Circumstantial evidence in support of this explanation was the result of measuring some binucleate secondary sporidia. In one such sporidium for instance, the two nuclei had individual

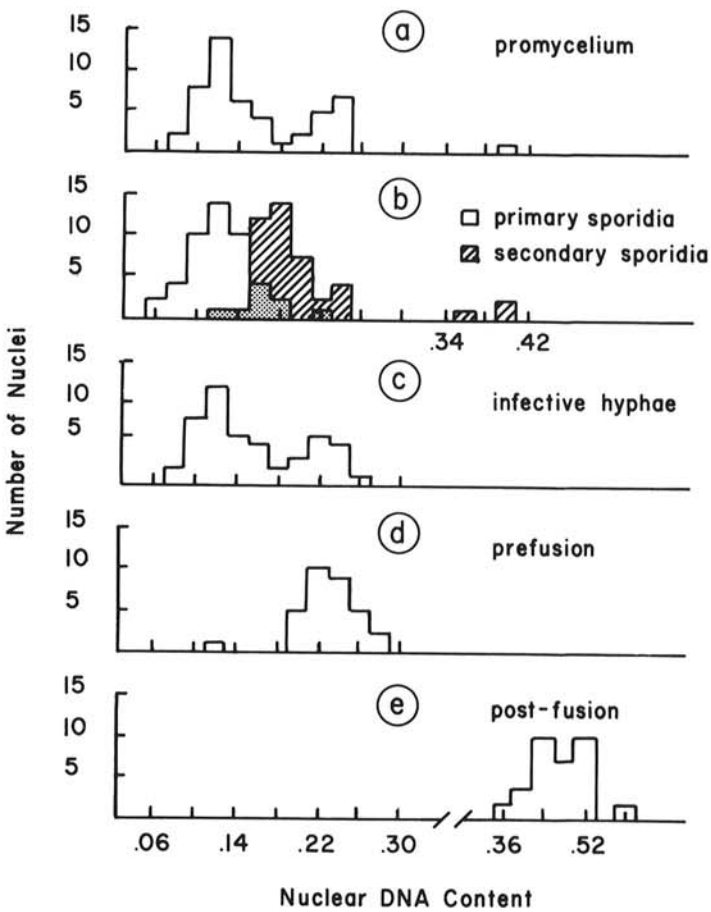


Fig. 1. Histograms representing nuclear Feulgen-DNA content of *Tilletia indica*. **A,** Feulgen-DNA content of promycelial nuclei. **B,** Feulgen-DNA content of sporidial nuclei. Shaded areas represent overlapping populations. **C,** Feulgen-DNA content of cultured infective hyphal nuclei. **D,** Feulgen-DNA content of prefusion nuclei in binucleate teliospore initials. **E,** Feulgen-DNA content of postfusion nuclei in uninucleate teliospore initials.

DNA values of 0.125 and 0.105 a.u. When averaged, these values equal the mean value of primary sporidia (Table 1). In another secondary sporidium, the two nuclei were so close together that they had to be measured as if they were one nucleus. When so measured, they had a combined F-DNA content of 0.229 a.u., the same as the previously described sporidium. Our observation that the primary and secondary sporidia differed in nuclear DNA content confirms the results of a previous investigation by Fuentes-Dávila (8). The value of 0.149 a.u. for infective hyphae was very likely due to mitotic activity. That is, some nuclei were presynthetic (G_1), some postsynthetic (G_2), and some intermediate in the S phase. The bimodal distribution seen in Fig. 1C is consistent with this hypothesis.

A light micrograph of a section stained with toluidine blue O showed sporogenous hyphae within the pericarp (Fig. 2). Sporogenous hyphae in kernels were analyzed by F-DNA cytophotometry (Fig. 1D and E) and TEM (Figs. 3–5). At 14,820× magnification, binucleate teliospore initials were observed (Fig. 3). In our studies, prefusion teliospore initials always either lacked a nucleolus or contained a single one, depending on the plane of sectioning. Prefusion cells had a mean F-DNA value of 0.217 a.u. and a range of 0.122–0.270 a.u., but only one nucleus had less than 0.201 a.u. (Table 1, Fig. 1D).

When we compared the F-DNA content of primary sporidia and prefusion nuclei, it appeared that most prefusion nuclei were in haploid G_2, containing the 2C or replicated DNA content. The single nucleus in which the F-DNA value was 0.122 a.u. apparently had not completed DNA replication. Teliospore initials in which both nuclei had apparently fused (Fig. 4) had two nucleoli, which suggests they were postfusion nuclei. F-DNA values of such postfusion nuclei ranged from 0.369 to 0.586 a.u., with a mean F-DNA value of 0.473 (Table 1, Fig. 1E). The mean value for such cells was approximately equivalent to the predicted 4C or diploid G_2 (replicated) value for *T. indica*. On the basis of the data from both prefusion and postfusion cells it appears that nuclei in the teliospore initials had undergone a single round of DNA replication before nuclear fusion.

In maturing teliospores the nucleoli fused; in so doing, they formed typically uninucleate teliospores with single nucleoli (Fig.

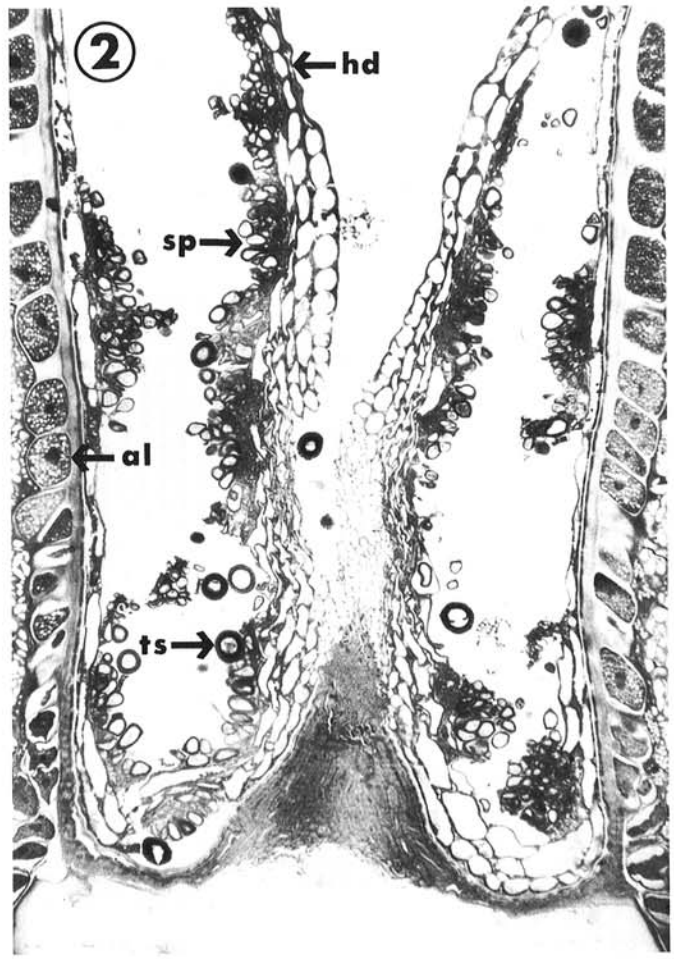
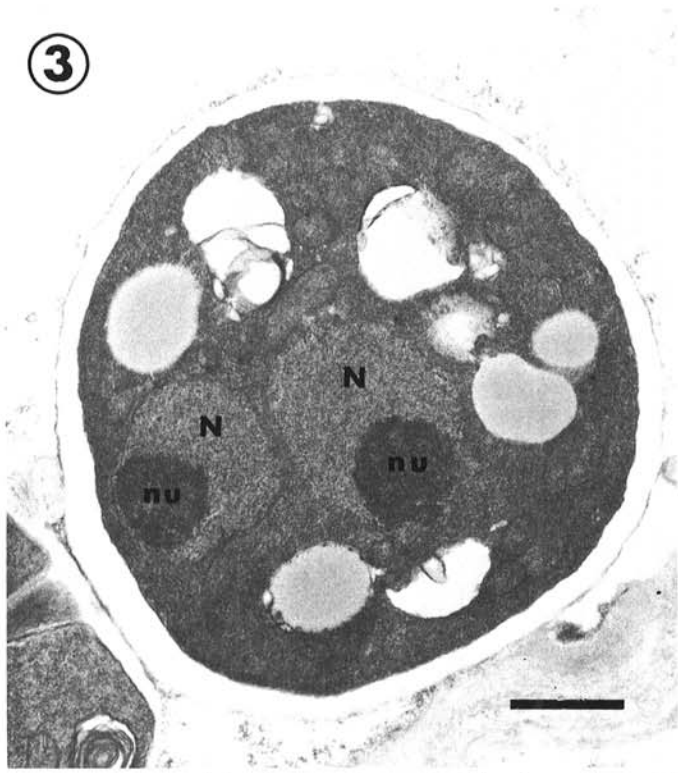
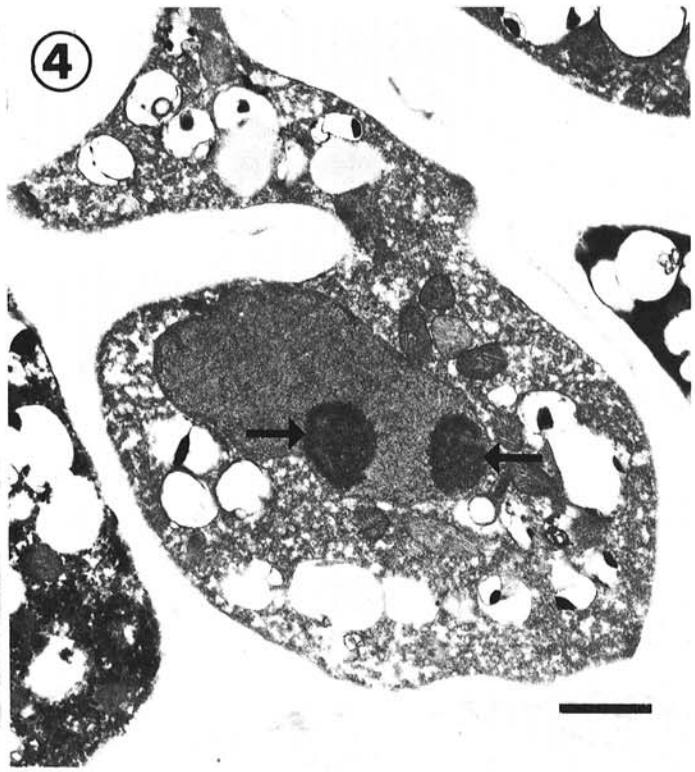


Fig. 2. Light micrograph of a sectioned caryopsis showing development of teliospores within the pericarp. al = aleurone layer; hd = hypodermis; sp = sporogenous mycelium; ts = teliospore.





Figs. 3 and 4. 3, Transmission electron micrograph of binucleate teliospore initial. Scale bar = 1 μm. N = nucleus; nu = nucleolus. **4,** Transmission electron micrograph of uninucleate teliospore initial. Scale bar = 1 μm. Nucleoli designated by arrows.

5). However, at the time of germination the teliospores were always multinucleate (Fig. 6).

DISCUSSION

This study demonstrates that germinating teliospores of *T. indica* are multinucleate, as has been observed in some other *Tilletia* species (10). Analyses of nuclei in postfusion teliospore initials and promycelia suggest that meiosis was followed by a number of mitoses in the teliospores. Fuentes-Dávila and Durán (9) placed the usual number of postmeiotic divisions at four, resulting in promycelia that typically had 64 nuclei. Our TEM observations and bright-field studies of Fuentes-Dávila and Durán (9) are at variance with the earlier studies of Krishna and Singh (12), who reported that a single meiosis occurred either in the teliospore or the promycelium and that all mitoses occurred in the promycelium.

In promycelia, nuclei fell principally into two categories, presynthetic (G_1) and postsynthetic (G_2). The data suggested that promycelial nuclei had replicated their DNA just before prophase. Previous observations (9,10) that promycelial nuclei migrate into primary sporidia, and that the nuclei of primary sporidia divide mitotically, are consistent with this suggestion.

We observed that teliospore initials were at first binucleate, then contained a single fusion nucleus with two nucleoli, which confirms a previous report (9) that karyogamy occurs before the development of the exospore. Similarly, the diameters of postfusion nuclei ranged from 3 to 4 μm, which is consistent with measurements reported by Fuentes-Dávila and Durán (9). In addition, F-DNA measurements of prefusion and postfusion nuclei (Table 1, Fig. 1D and E) suggest that DNA replication occurs before karyogamy, as has been reported in *Coprinus lagopus* (14) and *Sordaria fimicola* (1). As the exospore begins to develop the nucleoli then fuse, so that the final product of karyogamy is a diploid spore with a single fusion nucleolus. We often noted that the fusion nucleolus appeared to be a ringlike structure with a dense outer component surrounding a less electron-dense core of chromatinlike material. We have observed teliospores in later stages of development in which the nucleoli are more uniformly electron dense and, therefore, suggest that the

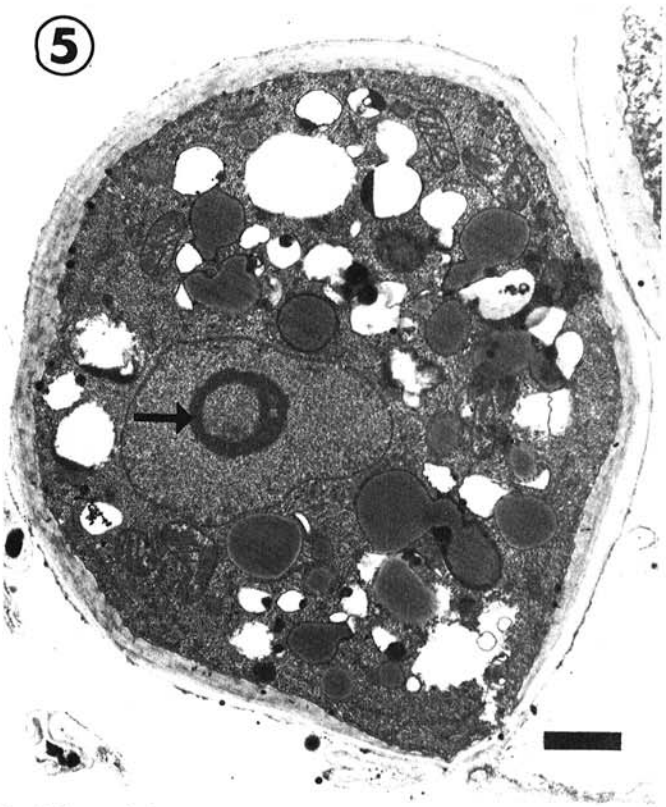


Fig. 5. Transmission electron micrograph of immature teliospore. Scale bar = 1 μm. Single ring-nucleolus designated by arrow.

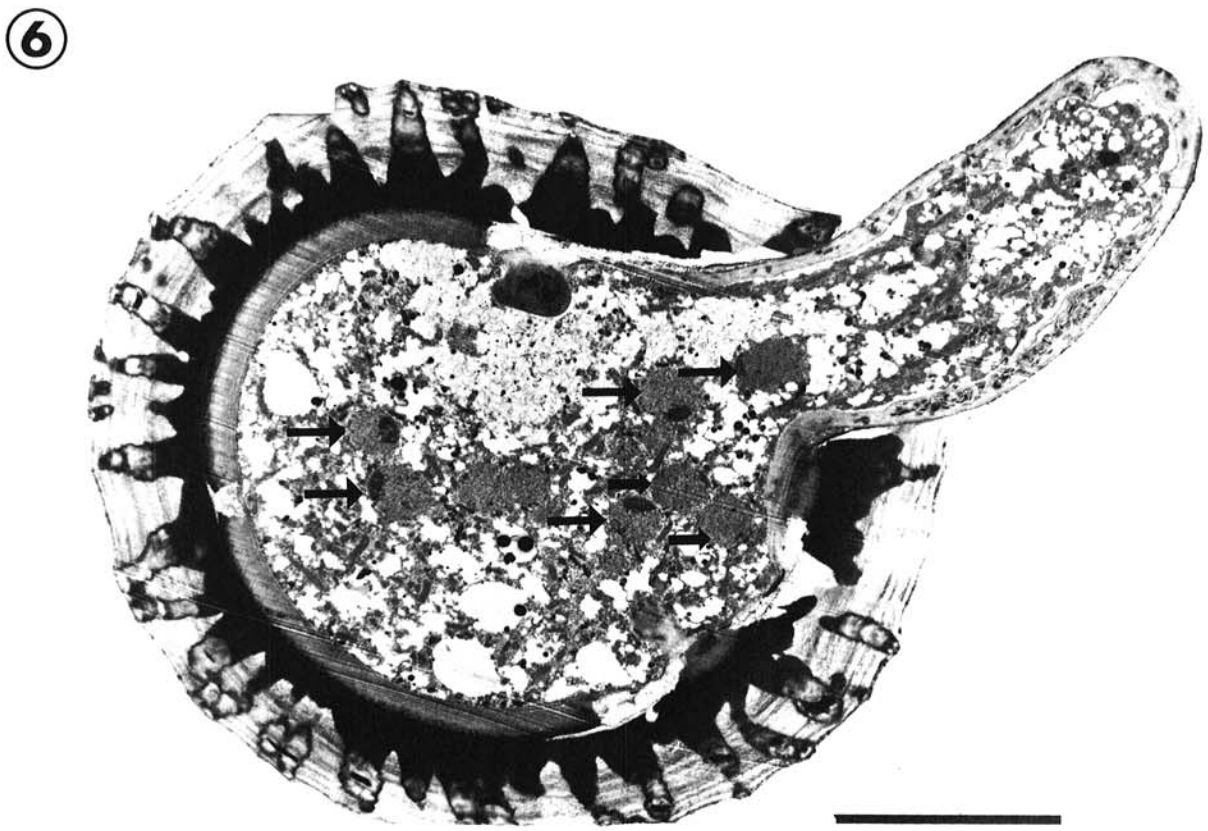


Fig. 6. Transmission electron micrograph of germinating teliospore. Scale bar = 10 μm. Nuclei designated by arrows.

ringlike structure may be a developmental stage of teliospores.

During the course of these investigations we have failed to demonstrate well-defined central and lateral elements of synaptonemal complexes, which would indicate the time of prophase I of meiosis in the developmental cycle of this species. The extreme difficulty we have encountered in infiltrating and thin sectioning the teliospores has contributed to this failure. Attempts to measure F-DNA content of mature teliospores also failed, largely because of the pigmented exospore. An analysis of the DNA content throughout the life cycle could also be used to determine the precise time of meiosis. Despite the absence of these important data, our observations that the nuclei of uninucleate teliospore initials are diploid G_2 (4C content) and the promycelial nuclei haploid 1C, are consistent with the hypothesis that both meiosis I and II occur at some stage between teliospore maturation and germination. It is our goal to develop techniques whereby we shall be able to elucidate this final stage in the life cycle of *T. indica*.

LITERATURE CITED

1. Bell, W. R., and Therrien, C. D. 1977. A cytophotometric investigation of DNA and RNA synthesis to ascus development in *Sordaria fimicola*. Can. J. Gen. Cytol. 19:359-370.
2. Bryant, T. R., and Howard, K. L. 1969. Meiosis in the Oomycetes. A microspectrophotometric analysis of nuclear deoxyribonucleic acid in *Saprolegnia terrestris*. Am. J. Bot. 59:1075-1083.
3. Dhillon, S. S., Berlyn, G. P., and Miksche, J. P. 1977. Requirement of an internal standard for microspectrophotometric measurement of DNA. Am. J. Bot. 64:117-121.
4. Durán, R. 1973. Ustilaginales. Pages 281-300 in: The Fungi. An Advanced Treatise. Vol. IVB. G. C. Ainsworth, F. K. Sparrow, and A. S. Sussman, eds. Academic Press, Inc., New York. 999 pp.
5. Durán, R. 1979. *Tilletia lycuroides*: Biological implications of nuclear behavior in the basidium. Mycologia 71:449-455.
6. Durán, R. 1980. *Tilletia aegopogonis*, a homo-heterothallic bunt fungus. Phytopathology 70:528-533.
7. Durán, R., and Cromarty, R. 1977. *Tilletia indica*: A heterothallic wheat bunt fungus with multiple alleles controlling incompatibility. Phytopathology 67:812-815.
8. Fuentes-Dávila, G. 1984. Biology of *Tilletia indica* Mitra. M.S. thesis. Department of Plant Pathology, Washington State University, Pullman. 65 pp.
9. Fuentes-Dávila, G. and Duran, R. 1986. *Tilletia indica*: Cytology and teliospore formation in vitro and in immature kernels. Can. J. Bot. 64:1712-1719.
10. Goates, B. J., and Hoffmann, J. A. 1987. Nuclear behavior during teliospore germination and sporidial development in *Tilletia caries*, *T. foetida* and *T. controversa*. Can. J. Bot. 65:512-517.
11. Holton, C. S. 1949. Observations on *Neovossia indica*. Indian Phytopathol. 2:1-6.
12. Krishna, A., and Singh, R. A. 1983. Cytology of teliospore germination in *Neovossia indica* the incitant of Karnal bunt of wheat. Indian Phytopathol. 36:115-123.
13. Lillie, R. D. 1951. Simplification of the manufacture of Schiff reagent for use in histochemical procedures. Stain Technol. 26:163.
14. Lu, B. C., and Jeng, D. Y. 1975. Meiosis in Coprinus. VII. The prekaryogamy S-phase and the postkaryogamy DNA replication in *C. lagopus*. J. Cell Sci. 17:461-470.
15. Mitra, M. 1931. A new bunt of wheat in India. Ann. Appl. Biol. 18:178-179.
16. Mundkur, B. B. 1940. A second contribution towards a knowledge of Indian Ustilaginales. Fragments XXVI-L. Trans. Br. Mycol. Soc. 24:312-336.
17. Munjal, R. L., and Chatrath, M. S. 1976. Studies on mode of infection of *Neovossia indica* incitant of Karnal bunt of wheat. J. Nuclear Ag. Biol., Biol. Abstr. 63:40-41.
18. Patau, K. 1952. Absorption microphotometry of irregular-shaped objects. Chromosoma 5:341-362.
19. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17:208-212.
20. Royer, M. H., and Rytter, J. 1985. Artifical inoculation of wheat with *Tilletia indica* from Mexico and India. Plant Dis. 69:317-319.
21. Spurr, A. R. 1969. A low-viscosity resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-43.
22. Swift, H. 1966. Desoxyribose nucleic acid content of animal nuclei. Physiol. Zool. 23:168-198.
23. Tooley, P. W., and Therrien, C. D. 1987. Cytophotometric determination of the nuclear DNA content of 23 Mexican and 18 non-Mexican isolates of *Phytophthora infestans*. J. Exp. Mycol. 11:19-26.
24. Vaughn, J. C., and Locy, R. D. 1969. Changing nuclear histone patterns during development. III. The deoxyribonucleic acid content of spermatogenic cells in the crab *Emerita analoga*. J. Histochem. Cytochem. 17:591-600.
25. Watson, M. L. 1958. Staining of tissue sections for electron microscopy with heavy metals. J. Biophys. Biochem. Cytol. 4:475-478.