

AN ABSTRACT OF THE THESIS OF

James Frederick Kollmorgen for the degree of Doctor of Philosophy
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Title: MORPHOLOGY, ULTRASTRUCTURE AND MATING OF SPORIDIA OF A
WHEAT-BUNT FUNGUS, TILLETIA CARIES (DC.) TUL.

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The teliospore of Tilletia caries germinates to produce a promycelium with a whorl of primary sporidia at its tip. The time between emergence of the promycelium and the development of primary sporidia averaged 2.3 h. Fusion between compatible sporidia occurred within an additional 3.8 h. Primary sporidia appeared to be blastic and at maturity were 37-57 μm long and c. 0.7, 1.8 and 1.2 μm wide at the tip, middle and base respectively. While still attached to the promycelium sporidia usually fused in pairs with a short tube near their midpoints. Sporidia in individual whorls were usually packed tightly together, and fused sporidia that were dislodged also remained close together along their entire length.

Promycelia contained many small vacuoles and mitochondria, and numerous lipid bodies. As the primary sporidia developed, the promycelial cytoplasm passed into the nascent cells. Septa developed between the bases of mature sporidia and the tips of the denticles. Sporidia that abscised from the denticles commonly had prominent birth scars at their bases. The sporidia had very thin walls, few vacuoles, attenuated mitochondria, and numerous lipid bodies. Conjugation pegs were generally produced by both members of a conjugating pair of

primary sporidia and there were bud scars where they emerged. The sporidial walls were apparently hydrolyzed during emergence of the pegs. Vesicles were sometimes present at the tips of the conjugation pegs and electron-dense accumulations were sometimes observed between the tips of advancing pegs just before fusion. The approaching conjugation pegs were precisely aligned, suggesting intercellular communication. The walls of the conjugation pegs fused and then were hydrolyzed. Fused sporidia were relatively homogeneous in content. The nucleus in a sporidium was often close to the conjugation tube and occasionally was partly within the tube.

When lunate secondary sporidia of opposite mating types were paired on agar, conjugation pegs developed as hyphal protuberances from sporidia or their germination tubes. Sometimes, germination tubes developed into conjugation pegs. A conjugation peg from one sporidium sometimes appeared to induce a peg in its partner, after contact. Conjugation pegs met precisely tip-to-tip before fusion. This often required, or caused, curvature of the pegs. Secondary sporidia of opposite mating types initially adjacent to each other were pushed apart during conjugation. Conjugation tubes were formed by the fusion of conjugation pegs from sporidia of opposite mating types or by fusion of a conjugation peg from a sporidium of one mating type with the body or germination tube of another sporidium of opposite mating type. The times required for development of conjugation pegs and formation of conjugation tubes depended on the relative orientations of the sporidia in a mating pair.

Conjugation was most rapid with sporidia positioned end-to-end. Conjugation was favored by a pH of 4.5-6 and at pH 6, conjugation occurred as readily on water agar as on a nutrient medium. The time required for conjugation increased as the initial distance between mating sporidia was increased.

On agar, there were most intersporidial contacts in pairs of sporidia of opposite (+ -) mating types than in pairs of like (+ + or - -) mating types. Pre-conjugation pegs were produced by sporidia after displacement of stimulating (opposite mating type) sporidia and the pegs continued to elongate. Sporidia of opposite mating types conjugated when subjected to a treatment that would disrupt fimbrial connections. Multiple hyphal tips were produced by differentiating sporidia separated from monokaryons (mycelia and sporidia) of opposite mating type by distances of 70-200 μm . The response was sex-specific. In some experiments the factors causing these responses were retained in the agar substrate. These factors may initiate the formation of conjugation pegs.

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Morphology, Ultrastructure and Mating of Sporidia
of a Wheat-Bunt Fungus, Tilletia caries (DC.) Tul.

by

James Frederick Kollmorgen

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MORPHOLOGY, ULTRASTRUCTURE AND MATING OF SPORIDIA
OF A WHEAT-BUNT FUNGUS, TILLETIA CARIES (DC.) TUL.

GENERAL INTRODUCTION

Tilletia spp. occur almost exclusively on the Gramineae and, from an economic viewpoint, those species which parasitize wheat are the most important. Diseases of wheat incited by Tilletia spp. are known as bunts. Tilletia caries (DC.) Tul. and Tilletia foetida Wallr. Liro cause common bunt of wheat; Tilletia controversa Kühn causes dwarf bunt. Common bunt is almost worldwide in distribution whereas dwarf bunt is restricted to certain areas in USA, Canada, Continental Europe, Argentina, USSR and Turkey.

Wheat-bunt poses as a constant threat to wheat production throughout the world because, in the absence of effective control measures, it causes marked yield reductions and renders grain unpalatable. There are also difficulties in marketing grain contaminated with spores of Tilletia spp. In the northwestern United States, bunt diseases are considered to be the most destructive of all diseases of wheat (Trione, 1977). Wheat bunt is controlled by sowing resistant cultivars and treating seed with fungicides. There is concern, however, that new virulent races of the bunt fungi may be selected in response to the selective action of wheats having new bunt resistance genes or gene combinations (Hoffmann and Metzger, 1976). It is also possible that fungicide-tolerant strains of the bunt fungi may develop. Consequently, a search for additional and perhaps more effective control measures is warranted. It is

possible that such control measures may be identified if the basic biology of Tilletia spp. pathogenic on wheat is more completely understood.

The three species of Tilletia that cause wheat bunt have essentially the same life cycle. There are many reports on various aspects of this life cycle (e.g. Buller and Vanterpool, 1933; Holton and Heald, 1941; Fischer and Holton, 1957; Trione, 1964, 1974; Kollmorgen, et al., 1978, 1979; Goates and Hoffman, 1979). The teliospore of T. caries germinates to form a promycelium with a whorl of primary sporidia at its tip. There are two mating types of primary sporidia, arbitrarily designated as + and -. Compatible (+ and -) sporidia usually fuse in pairs with a conjugation tube, while still attached to the promycelium. Fused primary sporidia germinate to produce binucleate hyphae or secondary sporidia. The binucleate stage of the bunt fungi is parasitic and teliosporogenic. Sporidia that do not mate, may germinate to form mononucleate hyphae or mononucleate secondary sporidia. The haploid, mononucleate stage is non-parasitic and can be readily cultured.

Teliospore germination, promycelial outgrowth, formation of primary sporidia, fusion of primary sporidia and germination of primary sporidia are possible focal points for the control of the bunt fungi (Trione, 1973). It is possible that the germination and post-germination developmental stages may be weak links in the whole life-cycle and thus most susceptible to control measures (Trione, 1973). The ultrastructure and physiology of both dormant and germinating teliospores have been investigated in considerable

detail (Lowther, 1950; Dewey and Tyler, 1958; Ettel and Halbsguth, 1964; Laseter, et al., 1968; Allen, et al., 1971; Trione and TeMay Ching, 1971; Hess, 1973; Trione, 1973; Hess and Weber, 1976; Trione, 1976; Gardner and Hess, 1977; Trione, 1977). However, there is no published information on the morphology of primary sporidial development in Tilletia spp. as revealed by electron microscopy, or on the ultrastructure of primary sporidia during their development and mating. The mating interaction between primary sporidia at the tip of a promycelium is very difficult to study, for it is almost impossible to observe the sequential interactions of sporidial fusion in situ. It is probable, however, that new information from studies of mating interactions between secondary sporidia on agar surfaces may be applicable to primary sporidia.

The purpose of the research described in this thesis was to obtain information on the morphology, ultrastructure and mating of primary sporidia of T. caries. Information is also presented on the morphology, timing and physiology of mating interactions between secondary sporidia.

CHAPTER I

MORPHOLOGY OF PRIMARY SPORIDIAL DEVELOPMENT^{*}

SUMMARY

The morphology and timing of sporidial development of Tilletia caries (DC.) Tul. were studied. The time between emergence of the promycelium from the teliospore and the formation of primary sporidia at the tip of the promycelium averaged 2.3 h, and fusion between compatible sporidia occurred within an additional 3.8 h. Primary sporidia appeared to be blastic and at maturity were 37-57 μm long and c. 0.7, 1.8 and 1.2 μm wide at the tip, middle and base respectively. While still attached to the promycelium sporidia usually fused in pairs with a short tube near their mid-points. Sporidia in individual whorls were usually packed tightly together, and fused sporidia that were dislodged also remained close together along their entire length.

INTRODUCTION

Tilletia caries (DC.) Tul. causes common bunt of wheat. Many aspects of the biology of the fungus have been studied in detail (Holton and Heald, 1941; Fischer and Holton, 1957; Duran and Fischer, 1961; Trione, 1973, 1974), but there is little recent information on the formation and fusion of primary sporidia.

^{*}Originally published in Transactions of the British Mycological Society 71: 223-229 (1978).

These events are prerequisites for infection of the wheat plant and are also possible focal points for control of the pathogen (Holton and Heald, 1941; Fischer and Holton, 1957; Trione, 1973).

The teliospore of T. caries germinates to form a promycelium with a whorl of primary sporidia at its tip and compatible sporidia usually fuse in pairs (McAlpine, 1910; Sartoris, 1924; Flor, 1932, Buller and Vanterpool, 1933; Hanna, 1934). A detailed account of these events as observed with a light microscope has been published by Buller and Vanterpool (1933).

The purpose of this investigation was to elucidate the morphology of sporidial development in T. caries using a scanning electron microscope and to obtain information on the timing of sporidial development.

MATERIALS AND METHODS

Teliospores of T. caries (race T-1) were obtained from R. J. Metzger, USDA-ARS, Corvallis, Oregon. The spores were surface sterilized in 0.3% sodium hypochlorite for 2-3 min, rinsed in three changes of sterile distilled water, seeded on agar (2.5 g/100 ml water) plates, and incubated at 18° for 89 h.

Germinating teliospores with attached promycelia and sporidia, were fixed and harvested for scanning electron microscopy by flooding the agar surface with glutaraldehyde-acrolein buffered with sodium cacodylate (Hess, 1966). A drop of Tween 20 in 12 ml of fixative facilitated wetting of the fungus. The resulting suspension was centrifuged to reduce the concentration of teliospores in the

supernatant. After fixation for 2 h at 22° the fungal material was collected by gentle vacuum aspiration on a silver membrane filter (Flotronics no. FM 13 0.45 μm), dehydrated through graded series of ethanol and acetone and dried by the carbon dioxide critical-point procedure (Anderson, 1951). Dried specimens were coated with c. 20 nm of gold.

Commencing 70.5 h after seeding, a plate was taken from the incubator every 0.5-1 h for 15.5 h and 100 spores on it examined for germination and production of sporidia. Material on the plate was then fixed as described above. Sporidia in various stages of development were stained with Fabil stain (Noel, 1964) and 100 whorls of sporidia were examined for fusions. The data were analyzed using linear regression and covariance analyses.

RESULTS

Figure 1 shows that teliospore germination was initiated after 71 h on 2.5% water agar at 18°. By 77.1 h, 50% of the spores had germinated. At 79.4 h primary sporidia at various stages of maturity were present on 50% of the promycelia and at 83.2 h, 50% of the whorls of sporidia at the tips of the promycelia contained fused sporidia. The rates of teliospore germination, primary sporidial formation and production of whorls with fused sporidia were the same ($p = 0.01$).

The promycelia emerged as tubes 3-4 μm in width with rounded tips and they often caused the teliospores to split (Fig. 2). Primary sporidia were produced when the promycelia were 14-57 μm in length and appeared to be blastic in origin. The primary sporidia from a

promycelium developed synchronously, at the same rate, and nearly all attained the same final length (Figs. 3-7, 9, 14, 15).

Mature sporidia were 37-57 μm long and c. 0.7, 1.8 and 1.2 μm wide at the tip, middle and base respectively, and were distinctly tapered at the tips (Figs. 7-15). When primary sporidia reached maturity the promycelium generally collapsed and annular thickenings were sometimes observed (Fig. 7). Conjugation tubes generally formed near the midpoints of the sporidia but sometimes the tubes were near the bases (Fig. 11). In most instances the sporidia fused in pairs forming a single conjugation tube but occasionally fusion of three cells with two conjugation tubes was observed (Fig. 12). Conjugation tubes were 1.4-2.1 μm wide and 0.9-1.4 μm long and perpendicular to the sporidia. Although fused pairs of sporidia were generally only connected at one point, the cells were commonly close together along their entire length (Fig. 10). Sporidia were rarely as widely spaced as those in Fig. 11, but even in this instance the cells were close together below the conjugation tube. Sporidia in individual whorls were generally packed tightly together and sometimes individual spores were difficult to discern (Fig. 14). Whorls of sporidia which had become detached from the promycelia generally retained their original configurations (Fig. 13).

Sterile, smooth spherical spores, 9-13 μm diam (Fig. 15), were often observed which contrast with the reticulate teliospores, 14-18 μm diam.

DISCUSSION

These results clarify the morphology of primary sporidial development in T. caries and provide new information concerning the timing of sporidial production. Few, if any, samples of teliospores of T. caries will germinate in less than 71 h and many will germinate at a slower rate (Lowther, 1950). The time required for teliospore germination is much greater than that required for germinated teliospores to produce fused sporidia.

Germinated teliospores were often split at the point of emergence of the promycelium (Fig. 2); this confirms a similar observation by Buller and Vanterpool (1933). It appears that, in addition to hydrolysis of the spore wall during emergence of the germ tube (Hess and Weber, 1976) a large internal mechanical pressure is exerted.

Buller and Vanterpool reported that the average width of the promycelium of T. caries was 8 μm and that the length varied from 20-500 μm . Trione (1973) observed that the promycelia of T. caries (race T-5) were 20-25 μm long prior to primary sporidial formation. Promycelia in the present study were generally smaller (3-4 μm wide and 14-57 μm long) than those observed by Buller and Vanterpool, but of similar length to those examined by Trione.

According to Buller and Vanterpool, the apex of the promycelium enlarges and produces peripherally a number of very short 'protuberances' some of which fork once. Primary sporidia grow from the ends of these structures. Other workers (Sartoris, 1924; Flor, 1932) referred to the sporidia as borne or produced at the tip of the

promycelium. The results presented in Figs. 2-6 are in agreement with the observations of Buller and Vanterpool and the 'protuberances' are often forked (Figs. 3, 5). As a result the primary sporidia are often borne in pairs. This may facilitate fusion. Holton (1953) demonstrated that the number of fusions between secondary sporidia of T. caries increased as their distance apart decreased.

The structures referred to as 'protuberances' may be more precisely called denticles (Barron, 1968; Kendrick, 1971). There were no morphological differences, however, between the tips of the denticles and the bases of the sporidia and it was impossible to tell whether promycelia were about to produce sporidia or had already done so. Sporidia seemed to be blastic in origin, without the appearance of pre-differentiated sterigmata.

Buller and Vanterpool recorded primary sporidia as 70-80 μm long, c. 4 μm wide in the middle and c. 2-3 μm wide at the base. The sporidia measured in the present study were much smaller, 37-57 μm long and c. 1.8 and 1.2 μm wide at the middle and base, respectively. This discrepancy may be due to shrinkage during dehydration and to variation in the race(s) of T. caries studied. It may also be due to natural variation.

Developing conjugation tubes (Fig. 8) were often observed but it was not possible to determine whether they were produced by one or both members of fused pairs. Buller and Vanterpool suggested that sporidia fuse via hyphal pegs produced by both members of a pair, which would indicate coordinated communication between the pair. Conjugation tubes normally occurred near the middle of sporidia,

but were also formed at the base or any distance upwards to within c. 15 μm of their apices (Buller and Vanterpool, 1933). In our study, however, fusion was never observed above the middle of sporidia. Hanna (1934) observed conjugation by two and three tubes and between three sporidia when the cells were paired on agar. Fusion of three sporidia by two conjugation tubes was observed in the present study, but this type of mating was not common. Fusion tubes were never more than 1.4 μm long and this is further evidence that sporidia are close together before fusion.

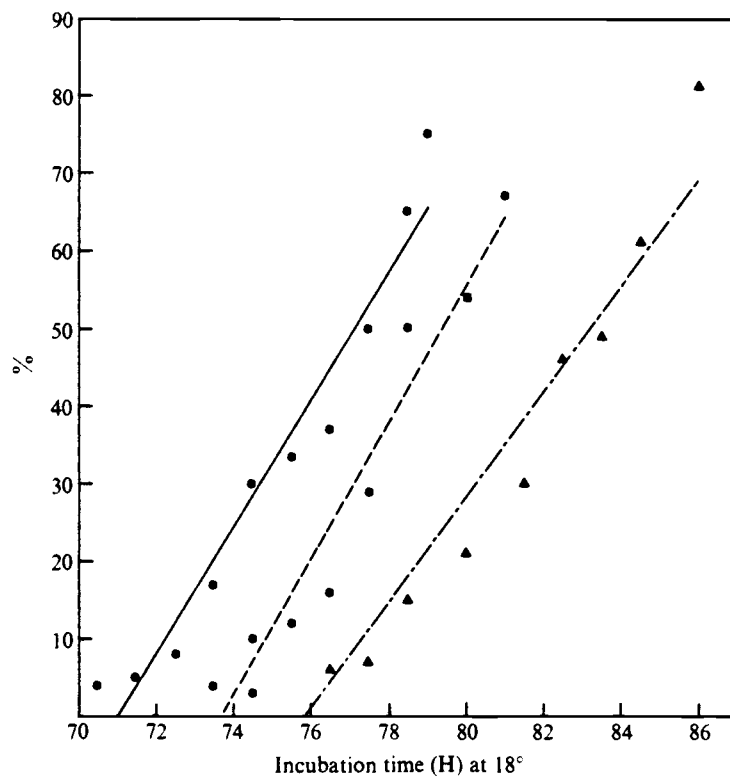
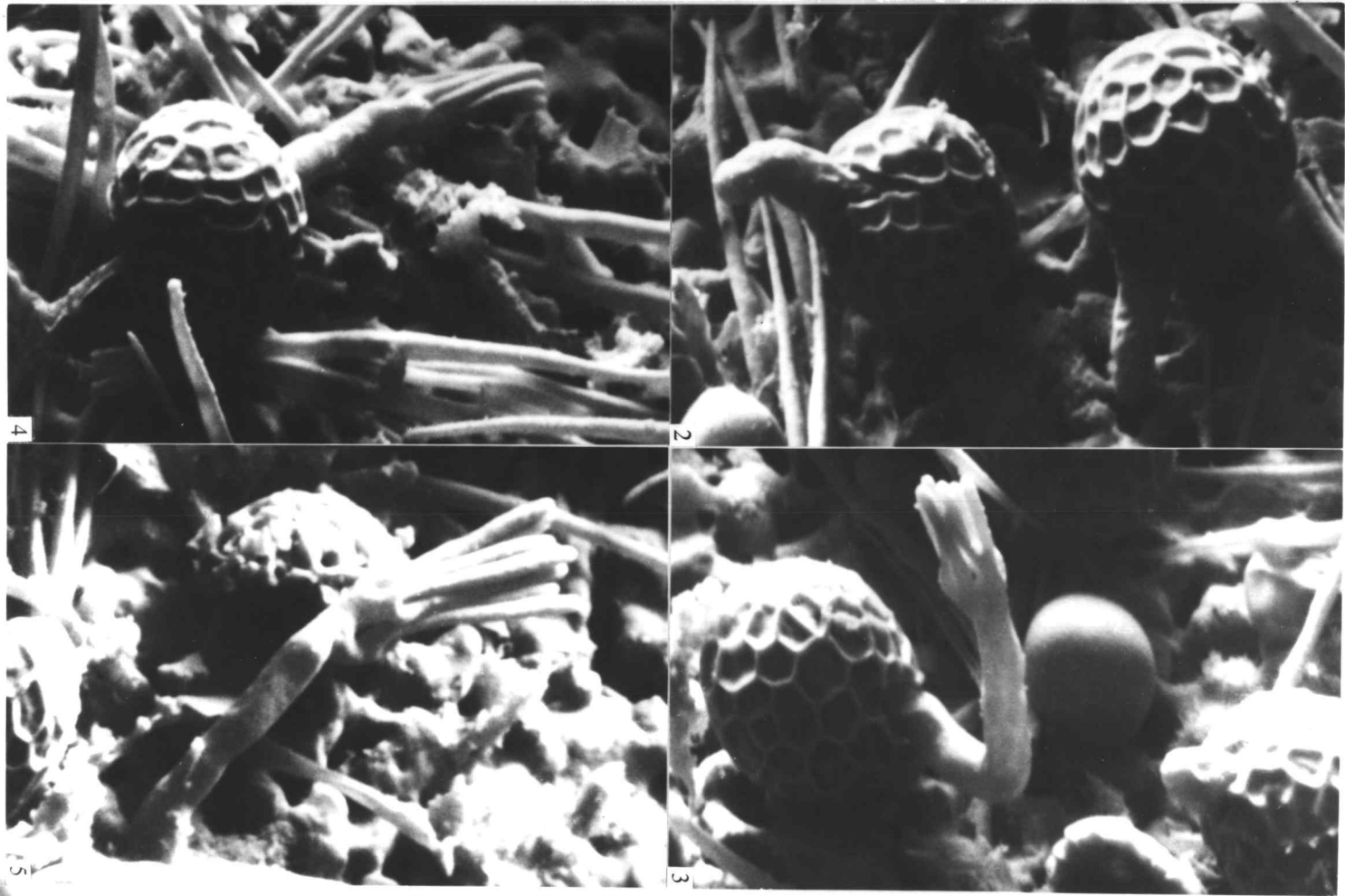


Fig. 1. Timing of primary sporidial development.

Teliospore germination (●—), promycelia with sporidia (■---), and whorls with fused sporidia (▲-·-·-) at indicated times on an agar surface at 18°. The correlation coefficients for the regression lines at 0.97, 0.97 and 0.96, respectively.

Figure 2. Germinating teliospores. (X 2,100).

Figures 3-5. Development of sporidia. (X 2,100).



Figures 6-7. Development of sporidia. (X 1,500).

Figure 8. Emergence of conjugation tube (centre of photograph).
(X 2,600).

Figure 9. Mature sporidia in juxtaposition ready for conjugation.
(X 800).



Figures 10-13. Fused primary sporidia.

Figure 10. Sporidia which have fused with a single conjugation tube near the midpoint of each sporidium. (X 1,900).

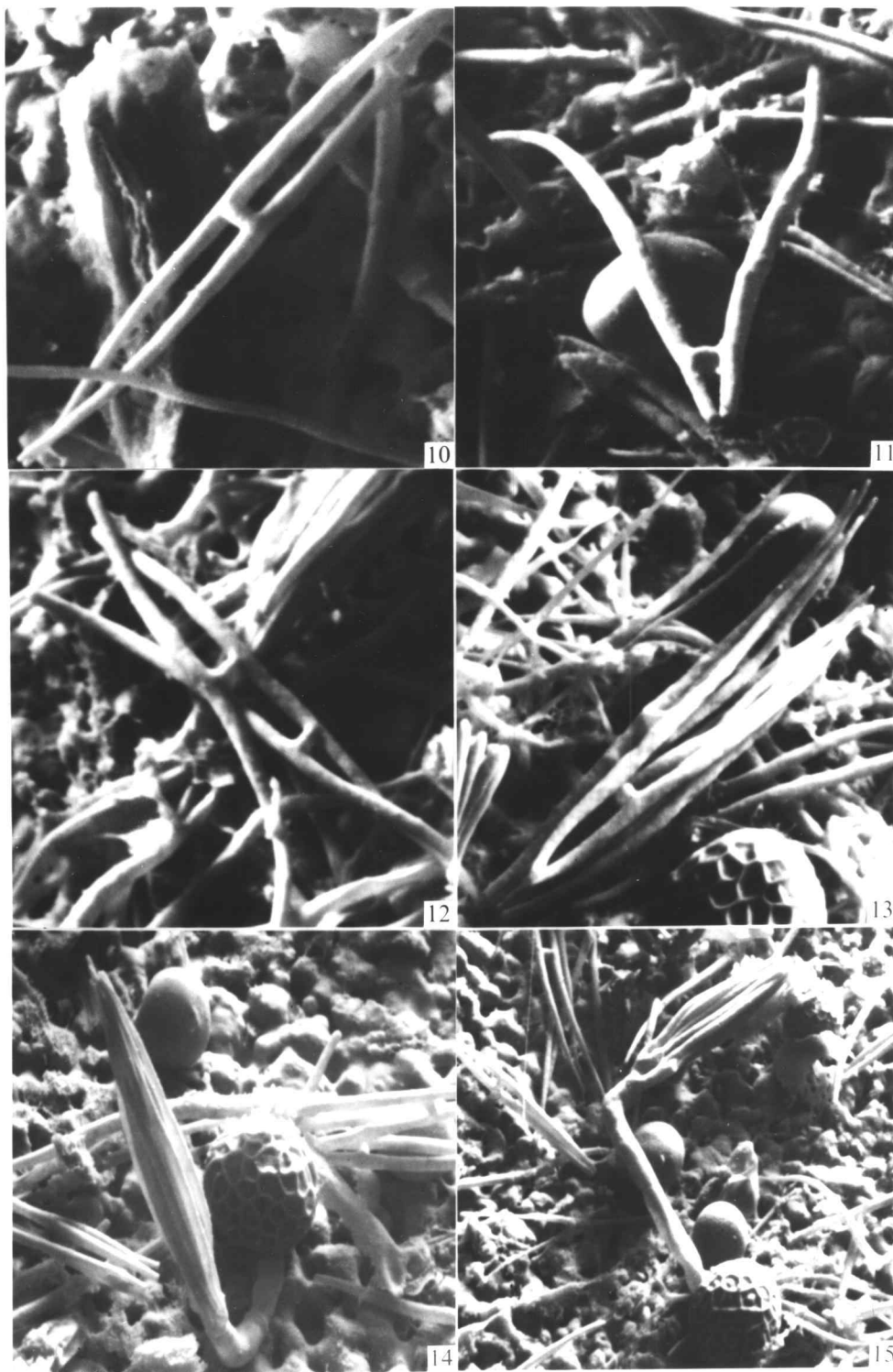
Figure 11. Sporidia which have fused with a single conjugation tube near the base of each sporidium. (X 1,900).

Figure 12. Fusion of three sporidia with two conjugation tubes. (X 1,900).

Figure 13. A whorl of conjugated sporidia detached from the promycelium. (X 1,800).

Figure 14. A whorl of primary sporidia which appear to be aggregated. (X 1,200).

Figure 15. Size relationships between a teliospore, sterile cells, promycelium and sporidia. (X 900).



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CHAPTER II

ULTRASTRUCTURE OF PRIMARY SPORIDIA
DURING ONTOGENY AND MATING*

SUMMARY

Primary sporidia of Tilletia caries (DC.) Tul. are borne on denticles at the tips of promycelia. The promycelia contain many small vacuoles and mitochondria and numerous lipid bodies. As the primary sporidia develop, the promycelial cytoplasm passes into the nascent cells. Septa develop between the bases of mature sporidia and the tips of the denticles. Sporidia that abscise from the denticles commonly have prominent birth scars at their bases. The sporidia have very thin walls, few vacuoles, attenuated mitochondria, and numerous lipid bodies. Conjugation pegs are generally produced by both members of a conjugating pair of sporidia and there are bud scars where they emerge from the sporidia. The sporidial walls are apparently hydrolyzed during emergence of the pegs. Vesicles are sometimes present at the tips of the conjugation pegs and, before fusion, electron-dense accumulations are sometimes observed between the tips of adjacent pegs. The approaching conjugation pegs are precisely aligned prior to fusion, suggesting polar communication. The walls of the conjugation pegs fuse and then are hydrolyzed. Fused sporidia are relatively homogeneous in content. The nucleus in a sporidium is often close to the conjugation tube and occasionally is partly within the tube.

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INTRODUCTION

Tilletia caries (DC.) Tul. causes common bunt of wheat. It is a pathogen of considerable economic importance in many wheat-producing areas of the world. There are many reports on the life cycle of T. caries (e.g. Holton and Heald, 1941; Fischer and Holton, 1957; Trione, 1964; Kollmorgen, et al., 1978). The teliospore of T. caries germinates to form a promycelium with a whorl of primary sporidia at its tip. There are two mating types of primary sporidia, arbitrarily designed as + and -. Compatible (+ and -) sporidia usually fuse in pairs with a conjugation tube, while still attached to the promycelium. Fused primary sporidia germinate to produce binucleate hyphae or secondary sporidia. Sporidia that do not mate, may germinate to form mononucleate hyphae or secondary sporidia.

Germination of teliospores, outgrowth of promycelia, and formation, fusion and germination of primary sporidia are possible focal points for the control of T. caries (Trione, 1973). New information gained from ultrastructural studies of these stages may therefore be of considerable value in investigations aimed at control of the pathogens.

The ultrastructure of dormant and germinating teliospores of T. caries has been investigated in considerable detail in a series of classic studies at the Electron Optics Laboratory, Brigham Young University (Laseter, et al., 1968; Allen, et al., 1971; Hess, 1973; Gardner, et al., 1975; Hess and Weber, 1976; Gardner and Hess, 1977; Allen, et al., 1978). There is no published information, however,

on the ultrastructure of T. caries during the various stages in the ontogeny and mating of primary sporidia. These stages are particularly important because they are prerequisites for the establishment of the pathogenic dikaryophase of T. caries (Trione, 1964).

This paper describes the ultrastructure of primary sporidia of T. caries during the various stages in their ontogeny and mating as revealed by transmission electron microscopy of thin-sectioned and freeze-etched preparations.

MATERIALS AND METHODS

Tilletia caries (race T-1) teliospores were kindly supplied by R. J. Metzger, geneticist, USDA-SEA-AR, Corvallis, Oregon. Primary sporidia were cultured as described by Kollmorgen et al. (1978).

Sporidia were harvested and fixed for thin-sectioning by flooding the surfaces of agar plates with glutaraldehyde-acrolein buffered with sodium cacodylate (Hess, 1966). The fixative contained either Tween 20 (one drop per 12 ml) or occasionally Teepol detergent (2 drops per 15 ml) to wet sporidial surfaces. The resulting suspension of basidial components was centrifuged at ca. 110 g for 3/4 minute to reduce the concentration of teliospores in the supernatant. The supernatant was then centrifuged at ca. 940 g for 15 minutes to pellet the sporidia. Sporidial pellets often contained germinating teliospores with attached promycelia. The material in the pellets was fixed for at least 2 hours and then processed for thin-sectioning using the procedures of Hess (1966) except that some specimens were embedded in Spurr (1969) resin.

For freeze-etching experiments the sporidia were harvested in distilled water containing Teepol detergent (2 drops per 15 ml) and concentrated as described above. The sporidia were then processed according to the methods of Hess, et al. (1968).

RESULTS

Promycelia have relatively thin, single-layered walls and numerous mitochondria and lipid bodies. Small vacuoles are also common (Figs. 1 and 2). The mitochondria vary in shape from spherical to branching and filamentous. The promycelia branch at their apices and produce primary sporidia (Fig. 3). At this stage there are attenuated mitochondria, large vacuoles, and lipid bodies in the promycelial tips (Fig. 4).

Primary sporidia are borne on short protuberances called denticles (Kollmorgen, et al., 1978) which are often arranged so that the sporidia are paired (Fig. 5). Sporidia that have attained their final lengths are readily dislodged from the promycelia. The abscission is aided by the formation of septa between the bases of the sporidia and the denticles (Figs. 5 and 6). After septum formation there are often large vacuoles in the denticles (Fig. 5) and occasionally cytoplasm is absent (Fig. 6).

Primary sporidia of T. caries have thin walls that appear to be single-layered and often fibrous (Figs. 5-7 and 13). The sporidia contain mitochondria that are often attenuated, with few vacuoles and numerous lipid bodies (Figs. 5-13). The lipid bodies are the major cytoplasmic components and often appear to be fused (Figs. 6,

8 and 11). Irregular electron-dense and electron-transparent patterns (Gardner and Hess, 1977) are occasionally observed in the lipid masses of primary sporidia. Sporidia that have budded from the denticles have prominent birth scars at their bases (Fig. 7). The sporidial walls appear to be hydrolyzed where the conjugation pegs emerge producing bud scars similar to the birth scars at the bases of the sporidia (Fig. 8). The nascent conjugation pegs are enveloped by new cell wall material and sometimes have several small vesicles in their tips (Fig. 8).

Conjugation pegs are generally produced by both members (+ and -) of a pair of mating sporidia (Fig. 10) and, just before fusion, electron-dense accumulations are sometimes observed between the tips of adjacent pegs (Fig. 9). The conjugation pegs are perpendicular to the long axes of the sporidia and meet precisely end to end (Fig. 10). The distal walls of the conjugation pegs apparently are hydrolyzed to produce conjugation tubes shown in Figs. 11 and 12. The nucleus in a sporidium is often close to the conjugation tube and occasionally it is partly within the tube (Fig. 11).

Fused sporidia have relatively homogeneous contents (Fig. 12). They germinate to form binucleate hyphae or secondary sporidia (Figs. 14 and 15). Like primary sporidia, secondary sporidia have abundant lipid bodies, few vacuoles and thin walls.

DISCUSSION

The present study provides new information on the ultrastructure of several important stages during the ontogeny and mating of primary

sporidia of T. caries. The results are consistent with previous observations on the morphology of primary sporidial development (Kollmorgen, et al., 1978) and results of studies on dormant and germinating teliospores of T. caries (Hess and Weber, 1976). There are also similarities between our observations and those of previous investigators with the yeast Saccharomyces cerevisiae Hansen and the basidiomycetes Schizophyllum commune Fries, Tremellea mesenterica Retzius ex Fr., and Ustilago violacea (Pers.) Roussel (Voelz and Niederpruem, 1954; Wells, 1965; Bandoni and Bisalputra, 1971; Osumi, et al., 1974; Poon and Day, 1976; Lipke, et al., 1976).

Promycelia

In number, type, and general distribution, organelles in promycelia of T. caries closely resemble those observed in germinating teliospores (Hess and Weber, 1976). This indicates that the cytoplasmic contents undergo little change as they pass from teliospores to promycelia. The fact that teliospores do not require exogenous nutrients to produce promycelia and primary sporidia supports this hypothesis.

One basic difference between teliospores and their promycelial outgrowth is the cell wall. Teliospores have thick multilayered walls, whereas promycelia have thin, single-layered walls. The promycelial wall begins to form within the germinating teliospore (Hess and Weber, 1976) and, at the magnifications used in the present study it is difficult to distinguish the wall from the plasma membrane.

Wells (1965) reported that after the initiation of sterigmata in S. commune, small vacuoles appeared in the proximal regions of the

basidia. He suggested that enlargement and coalescence of these vacuoles forced the cytoplasm and nuclei through the sterigmata and into the enlarging basidiospore initials. In the present study, the promycelia generally contained numerous small vacuoles although large vacuoles were sometimes present at their tips. It is probable that the small vacuoles are characteristic of immature promycelia and that they subsequently coalesce. This may force the cytoplasm and nuclei into the developing sporidia. However, the observation that promycelia generally collapse when the primary sporidia reach their final lengths (Kollmorgen, et al., 1978) suggests that as the sporidia enlarge, their cell walls are synthesized at a faster rate than the sporidial cytoplasm. This may create a partial vacuum which draws the promycelial cytoplasm into the sporidia and, in addition, promotes the coalescence of small vacuoles. A similar phenomenon occurs in elongating hyphae of T. caries, that is, cell walls are formed at a much faster rate than cytoplasm. This results in an elongating tip cell filled with cytoplasm, but followed by a series of evacuated cells sealed off by septa.

Sporidia

Kollmorgen, et al. (1978) studied the morphology of primary sporidial development in T. caries and proposed that the sporidia were borne on denticles. The observations in the present study are in agreement. The term "denticle" implies a short protuberance from which spores are released passively, whereas the more common term, "sterigma", implies a longer, tapered, pre-existing structure from

which spores are forcibly discharged (W. C. Denison, personal communication). Primary sporidia are continuous with the denticles and at maturity abscise at their basal ends. In contrast, secondary sporidia are borne on sterigmata and are forcibly discharged. It is possible that the denticles on which primary sporidia of T. caries develop, as vestigial sterigmata. Primary sporidia are frequently borne in pairs and, as previously suggested (Kollmorgen, et al., 1978), this arrangement may facilitate mating.

The thin apparently single-layered and often fibrous appearing walls of T. caries are similar in appearance to the sporidial walls of many rust fungi (Akai, et al., 1976) and to the basidiospore walls of S. commune (Voelz and Niederpruem, 1964).

Septa between the denticles and primary sporidia of T. caries are similar to those between mother and daughter cells in T. mesenterica (Bandoni and Bisalputra, 1971) and U. violacea (Poon and Day, 1976) but differ from septa between sterigmata and basidiospores in S. commune (Wells, 1965). This is probably due to the fact that primary sporidia of T. caries, and daughter cells of both T. mesenterica and U. violacea are released passively, whereas basidiospores of S. commune are forcibly discharged.

Primary sporidia of T. caries have distinctive birth scars which are similar to, but more conspicuous than, birth scars on daughter cells of U. violacea (Poon and Day, 1976). Distinctive birth scars are not visible on daughter cells of T. mesenterica (Bandoni and Bisalputra, 1971).

Sporidial Fusion

Conjugation pegs are not produced randomly on the surfaces of primary sporidia of T. caries but, as observed by Kollmorgen, et al. (1978) they usually occur at the midpoints of the cells. The observation that conjugation pegs from sporidia in mating pairs meet precisely end to end suggests polar communication between the sporidia preceding the actual fusion. The mechanism by which the specific sites for emergence of the conjugation pegs are determined is unknown and warrants further investigation.

Fungal fimbriae have been observed in U. violacea and it has been proposed that they are associated with mating in that fungus (Day and Poon, 1975; Poon and Day, 1974, 1975; Day, 1976). We hypothesized that fimbriae may be associated with mating between primary sporidia of T. caries. However, when shadow casting techniques based on those described by Poon and Day (1974, 1975) for visualization of fimbriae in U. violacea were used with sporidia of T. caries no fimbriae were observed. We did, however, observe fimbriae on an isolate of U. violacea kindly supplied by Dr. A. W. Day.

A second hypothesis is that hormones are associated with sporidial mating in T. caries. In S. cerevisiae, haploid cells of opposite mating type fuse via conjugation tubes produced by both cells in a mating pair (Osumi, et al., 1974). This sexual conjugation process is mediated by the action of diffusible mating hormones, two of which have been designated as a-factor and α -factor (Betz, et al.,

1977). Mating hormones also induce the formation of mating tubes in the yeast Rhodosporidium toruloides Banno (Abe, et al., 1975). Similarly, copulation between haploid cells of T. mesenterica is initiated by hormones that induce growth of conjugation tubes (Bandoni, 1965). Cox (1976) also reported that (+) and (-) strains of the heterothallic basidiomycete, Chionosphaera apobasidialis Cox each produced hormones which initiate conjugation pegs in the opposite strain. Reid and Bartnicki-Garcia (1976) suggested that the main action of the conjugation hormones of T. mesenterica may be to control the distribution of wall-synthesizing enzymes over the cell surface. An analogous mechanism might operate with T. caries sporidia. If primary sporidia of T. caries do communicate via hormones, these substances may determine the specific sites on the sporidia for emergence of the conjugation pegs. The hormones may also direct the subsequent growth of the conjugation pegs so that the pegs are precisely aligned before fusion.

Vesicles are present in the tips of conjugation pegs of primary sporidia of T. caries. According to Cabib (1975) numerous vesicles also occur very early in the development of yeast buds. He suggested that their function was connected with the formation of a new cell envelope at the bud site. According to Cortat, et al. (1972) local modifications of Saccharomyces cell walls are caused by the secretion of vesicles that contain β -glucanases into the wall at the site of new buds. Vesicles in T. caries may have a similar function, but may also carry enzymes to hydrolyze the walls of conjugation pegs during fusion.

Electron-dense material like that observed between conjugation pegs of T. caries before fusion, has been observed between conjugating haploid cells of S. cerevisiae (Osumi, et al., 1974) and at the tips of cellular extensions evoked by the α -factor in this yeast (Lipke, et al., 1976). Osumi, et al. (1974) suggested that the electron-dense material between conjugating S. cerevisiae cells may be a cementing substance produced in response to cell to cell contact. Lipke, et al. (1976) attributed the material to the cell walls becoming diffuse.

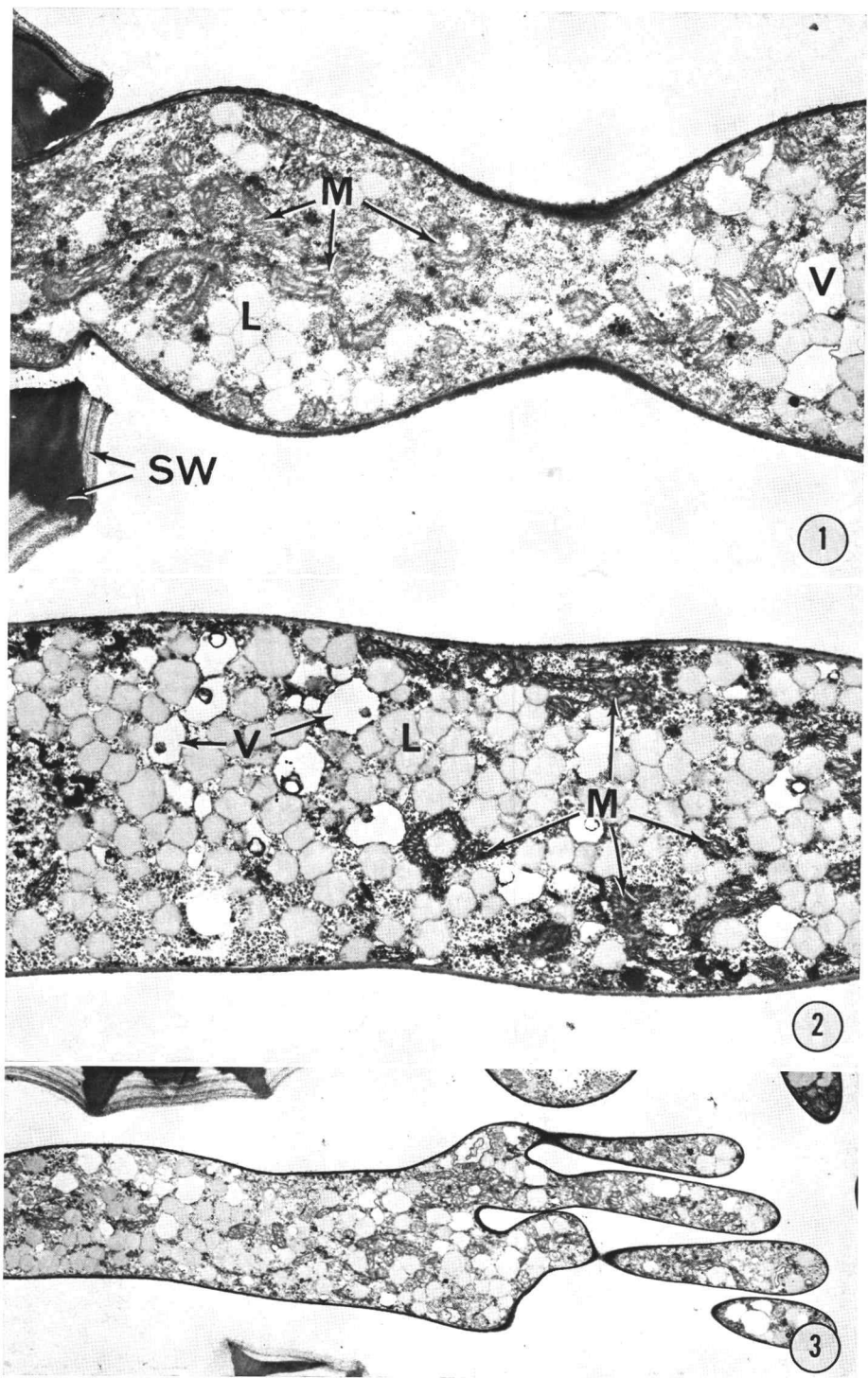
Nuclei in primary sporidia of T. caries are often close to the developing conjugation tubes and probably migrate through the tubes almost as soon as they form. Lipke, et al. (1976) suggested that nuclear migration was concomitant with elongation of S. cerevisiae cells exposed to the α -factor. In addition, Osumi, et al. (1974) reported that nuclei in S. cerevisiae seemed to migrate to the region of contact of haploid yeast cells before complete dissolution of the separating walls. Primary sporidia of T. caries are usually mononucleate and, after formation of a conjugation tube, the nucleus from one sporidium passes into the other to initiate the pathogenic dikaryophase (Trione, 1964). The nucleus in part of the conjugation tube pictured in Fig. 11 was probably migrating from one sporidium to the other. Further studies will be conducted to elucidate the relationship between the position of the nucleus in sporidia of T. caries and the site at which conjugation pegs emerge.

Figures 1-3. Promycelia of Tilletia caries.

Figure 1. Promycelium at the point of emergence from teliospore. A part of the teliospore wall (SW) may be seen. Note the presence of abundant mitochondria (M), lipid bodies (L) and vacuoles (V). X 8,600.

Figure 2. Typical section of a promycelium, showing abundant lipid bodies (L), mitochondria (M), and vacuoles (V). X 8,600.

Figure 3. Tip of a promycelium showing the branching that results in the development of primary sporidia. X 5,000.

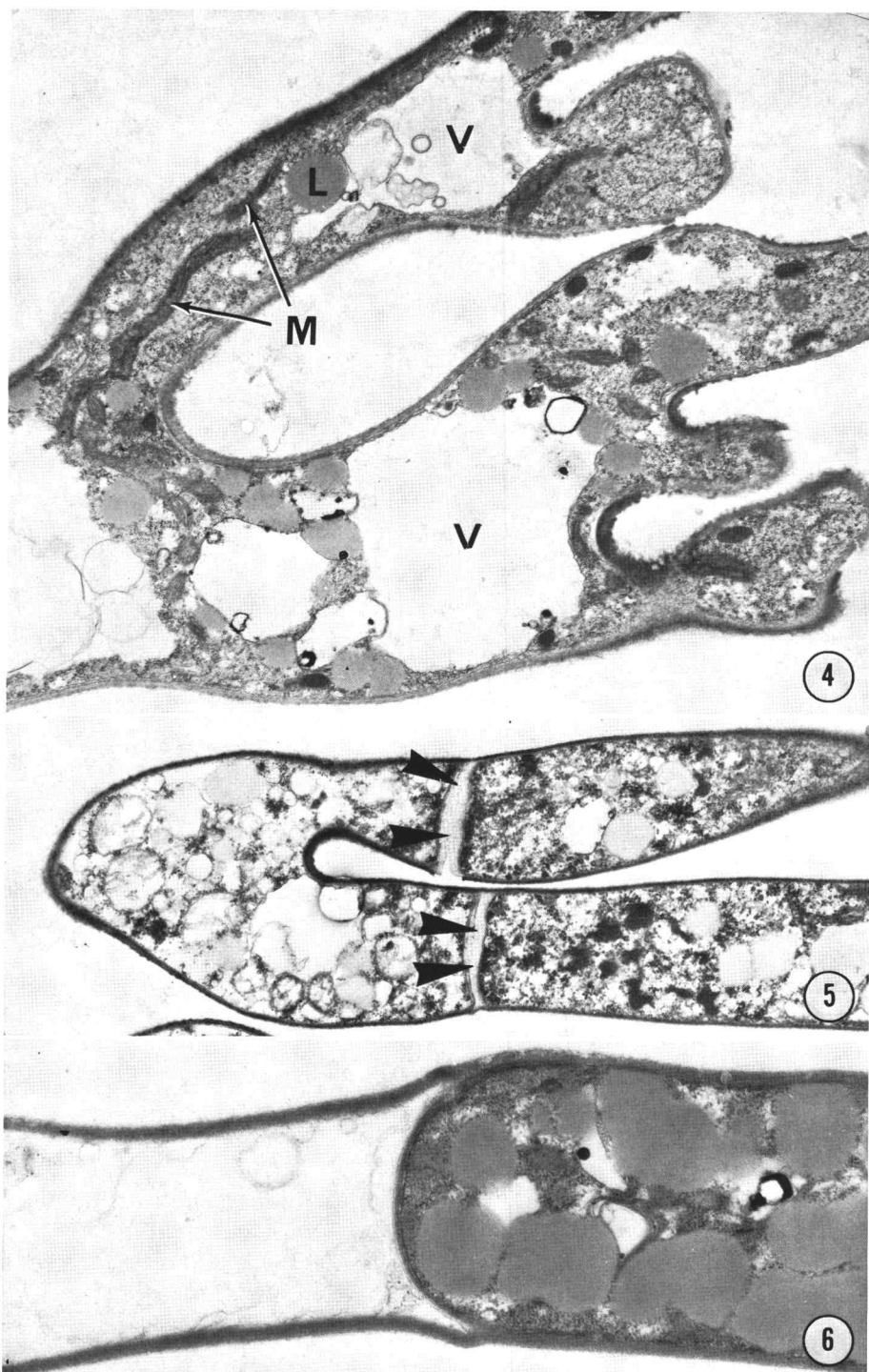


Figures 4-6. Promycelia and primary sporidia of Tilletia caries.

Figure 4. Oblique section of a tip of a promycelium, showing the vacuoles (V) that form as primary sporidia develop. Attenuated mitochondria (M) and lipids (L) are also evident. X 10,800.

Figure 5. Section of portions of two primary sporidia during formation of septa (arrows). X 13,500.

Figure 6. Portion of a primary sporidium (right) after septum formation. Note the absence of cytoplasm in the adjacent denticle. X 20,400.



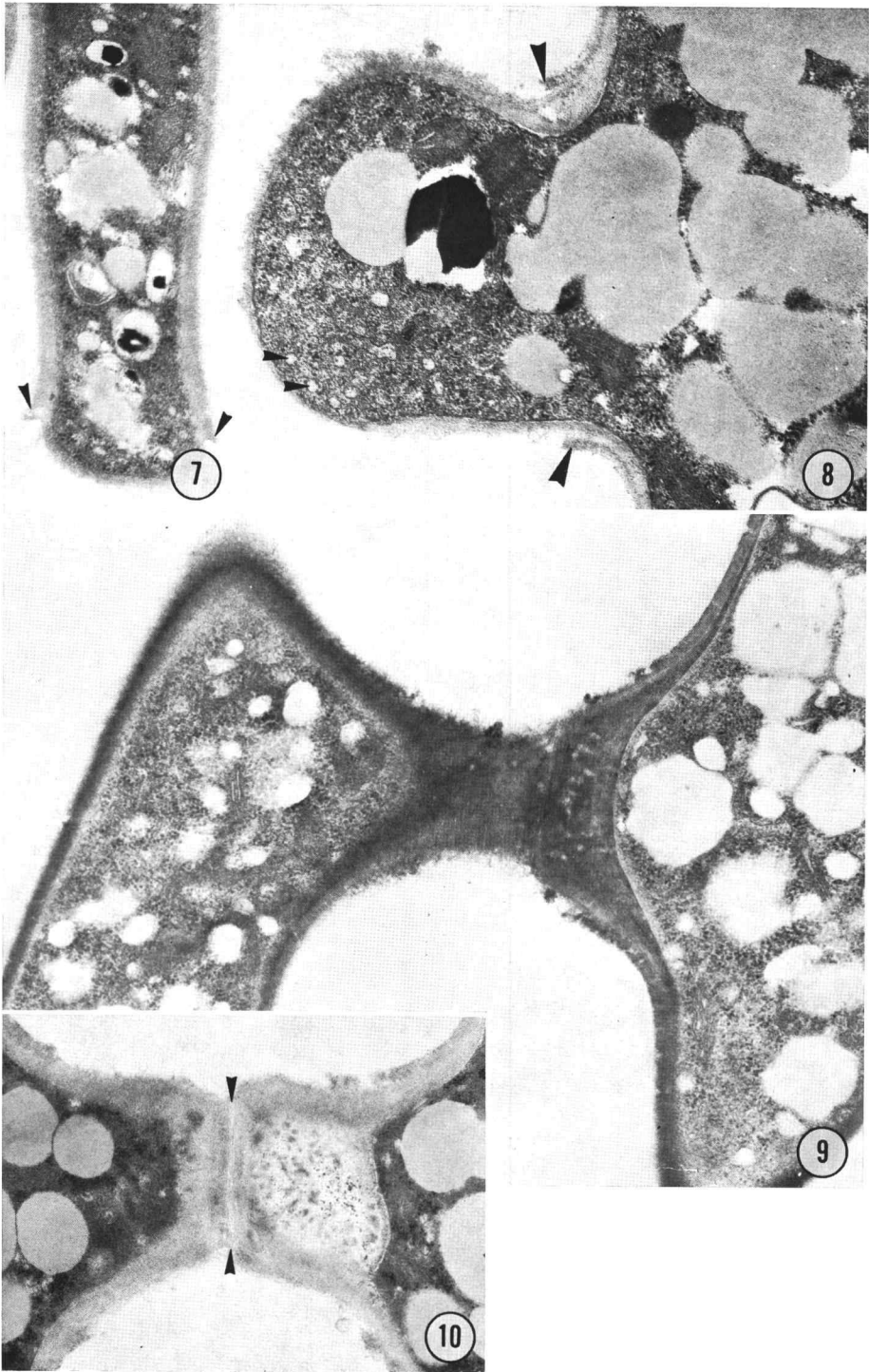
Figures 7-10. Primary sporidia of Tilletia caries.

Figure 7. Base of a primary sporidium after separation, showing birth scars (arrows). X 15,600.

Figure 8. Section of a sporidium with a conjugation peg before fusion, showing bud scars (large arrows) and vesicles in the expanding peg (small arrows). X 25,500.

Figure 9. Non-median section, showing electron-dense accumulations sometimes seen at the point of fusion between two primary sporidia. X 23,400.

Figure 10. Section showing the point of wall fusion (arrows) between two primary sporidia. X 23,400.



Figures 11 and 12. Fused primary sporidia of Tilletia caries.

Figure 11. Fused sporidia, showing a nucleus (N) in part of the fusion tube. X 20,500.

Figure 12. Fused sporidia, showing relatively homogeneous contents of the two sporidia and the fusion tube. X 13,500.

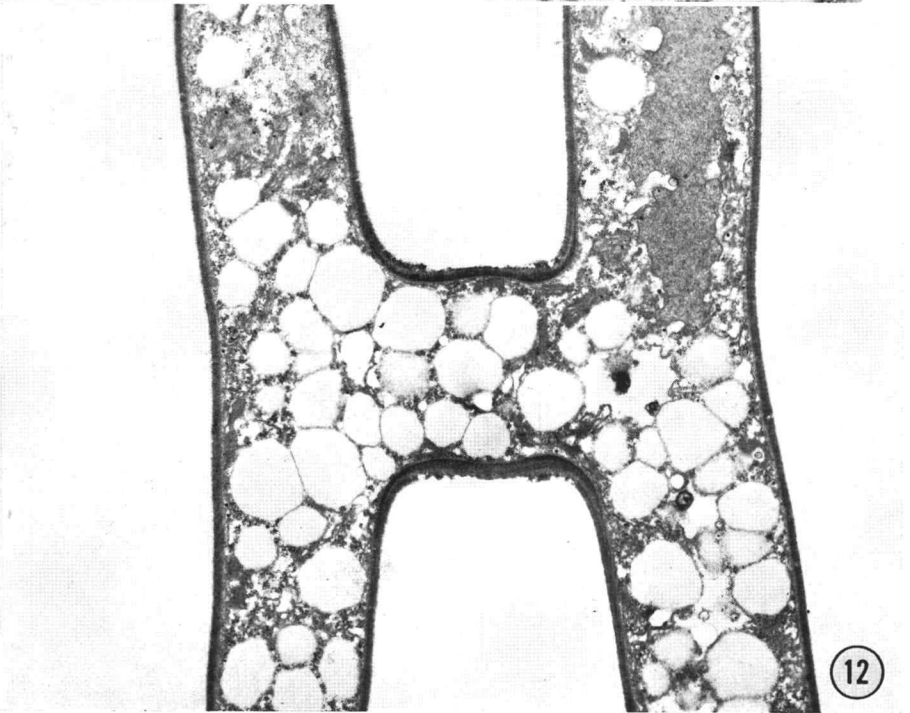
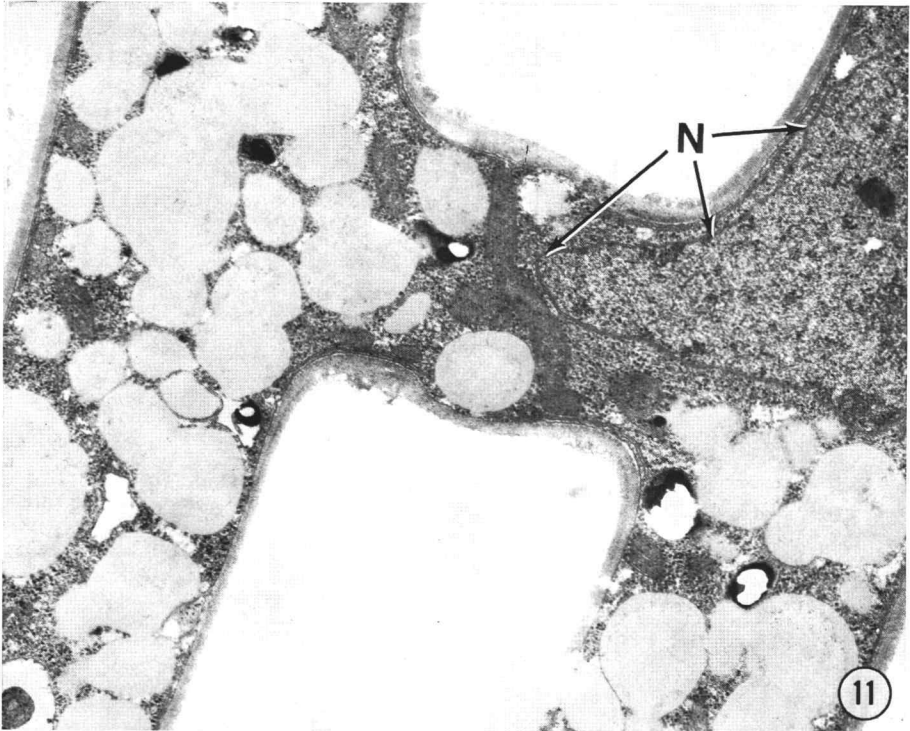
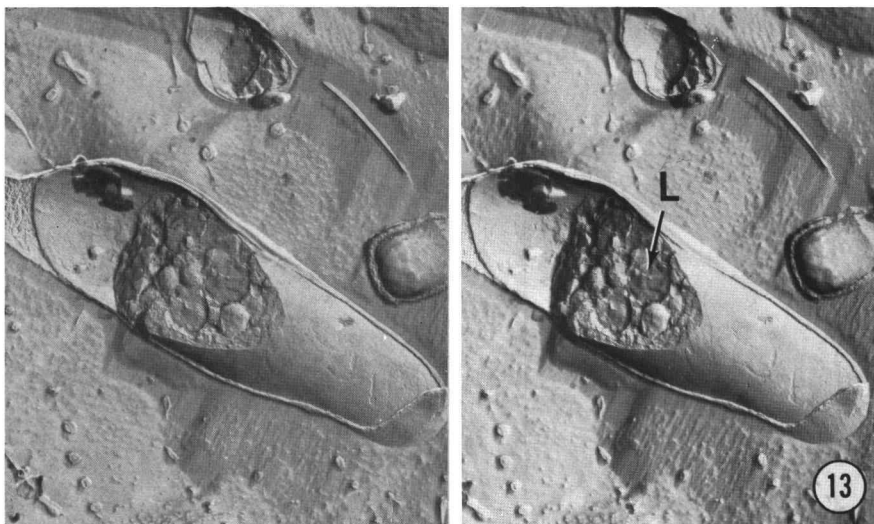


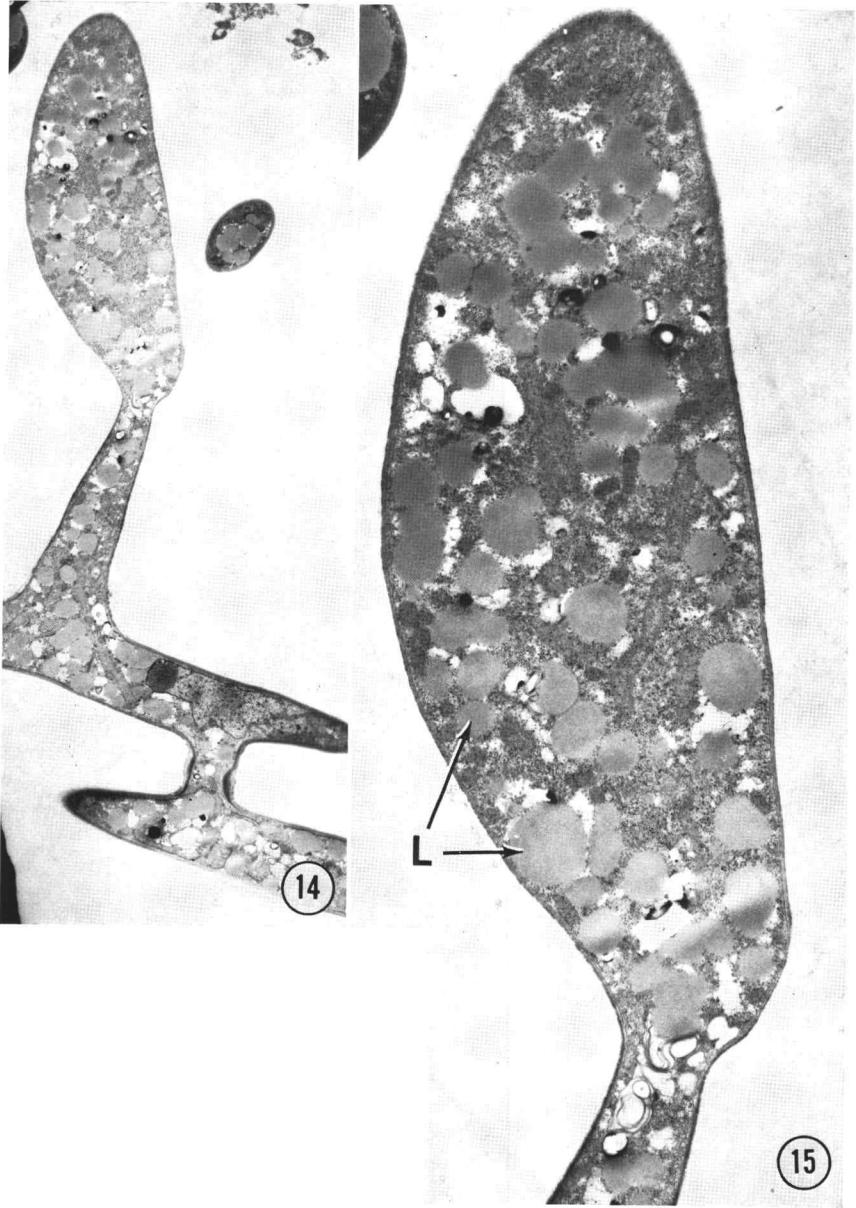
Figure 13. Stereo pair of a freeze-etch replica of an obliquely fractured primary sporidium of Tilletia caries, showing cross-fractured lipids (L) and characteristics of the membrane and the wall. X 9,000.



Figures 14 and 15. Tilletia caries secondary sporidium.

Figure 14. Section showing the development of a secondary sporidium from a fused pair of primary sporidia. Note the sterigma on which the secondary sporidium is borne. X 4,200.

Figure 15. Enlargement of secondary sporidium shown in Figure 14, showing the relatively thin spore wall and abundant lipid bodies (L). X 11,000.



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CHAPTER III

MORPHOLOGY AND TIMING OF SPORIDIAL MATING

SUMMARY

The morphology and timing of sporidial conjugation in Tilletia caries (DC.) Tul. are described. When lunate secondary sporidia of opposite mating types are paired close together on agar, conjugation pegs are formed by one or both mating types. Conjugation pegs may develop as hyphal protuberances from sporidia or their germination tubes. Sometimes, germination tubes develop into conjugation pegs. In studies at high magnification (X 870-990) a conjugation peg from one sporidium (+ or -) appeared to induce conjugation peg development in its partner after contact. Conjugation pegs meet precisely tip-to-tip before fusion. This often requires, or causes, a pronounced curvature of the pegs. When sporidia (lunate or filiform) of opposite mating types are placed adjacent to each other on agar the sporidial cells are pushed apart by the developing conjugation pegs. Conjugation tubes are formed by the fusion of conjugation pegs from sporidia of opposite mating types or by the fusion of a conjugation peg from a sporidium of one mating type with the body or germination tube of another sporidium of opposite mating type. The times required for the appearance of conjugation pegs and the formation of conjugation tubes depend on the relative orientations of the sporidia in a mating pair. Conjugation is most rapid with sporidia positioned end-to-end.

In our experiments, sporidial conjugation was favored by a pH

of 4.5 - 6. At pH 6, conjugation occurred as readily on water agar as on nutrient medium. The time required for sporidial conjugation increased as the initial distance between the sporidia in a mating pair was increased.

INTRODUCTION

Teliospores of the wheat-bunt fungus, Tilletia caries (DC.) Tul. germinate to produce a promycelium with a whorl of haploid primary sporidia at its distal end. Primary sporidia are either + or - with respect to mating type; compatible (+ and -) sporidia fuse with a conjugation tube near their midpoints. We have recently described the ultrastructure and morphology of primary sporidia of T. caries during mating (Kollmorgen, et al., 1978, 1979). The details of the mating interaction between primary sporidia at the tip of a promycelium are, however, very difficult to study. It is almost impossible to observe the sequential interactions of sporidial fusion in situ. Consequently, we only have limited information on the mating interaction per se.

To gain further information on sporidial mating in T. caries we studied mating interactions between sporidia on agar surfaces. The sporidia were derived from monokaryotic (haploid) cultures of T. caries established by isolating single primary sporidia from germinating teliospores. The cultures consisted of varying numbers of filiform secondary sporidia, lunate secondary sporidia and hyphal cells.

When compatible (+ and -) secondary sporidia are placed close

together (0-20 μ m) on an agar surface they generally form conjugation pegs and fuse to form a short-lived dikaryon. This mating event appears very similar to that described for primary sporidia at the tip of a promycelium (Buller and Vanterpool, 1937; Kollmorgen, et al., 1978, 1979). Indeed, if primary sporidia are removed from a promycelium before fusion and are placed adjacent to each other on an agar surface they will conjugate (Hanna, 1934; Kollmorgen, unpublished results).

There is little published information on the mating interaction between sporidia of T. caries on agar surfaces. Hanna (1934) paired primary sporidia on agar and noted that a conjugation tube united the two sporidia either at their midpoints (producing an H-shaped structure) or at their bases. Occasionally a pair of sporidia was united by 2 or even 3 conjugation tubes. According to Hanna, the pH of the agar medium had a decided influence on conjugation with lower and upper limits of 5.1 and 6.3 respectively. Temperature had little effect on conjugation however, because on agar at pH 6 the percentage of pairs of sporidia that conjugated was approximately the same at 10, 18 and 20° (Hanna, 1934).

Holton (1953) reported that the percentage of fusions between compatible pairs of secondary sporidia was influenced by proximity and the kind of medium. He showed that the sporidia fused more readily when lying next to each other than when further apart and that fusion occurred more readily on nutrient medium than on water agar.

In the present publication we describe the morphology and timing of events occurring during mating of sporidia of T. caries. We

confirm some of the earlier observations made by Hanna (1934) and Holton (1953) but our data is in disagreement with other aspects of their findings.

MATERIALS AND METHODS

(a) Isolates.

Monosporidial lines of T. caries (race T-1) were obtained by isolating single primary sporidia from germinating teliospores using the methods of Holton (1951). Stock cultures were maintained on potato-sucrose-agar. Filiform and lunate secondary sporidia were produced at 17° on T-19 medium (Trione, 1964) solidified with agar (2.5%).

(b) Sporidial Mating.

Sporidia (lunate and filiform) were mated on standard mating medium (SMM) consisting of agar (2.5%) buffered with 5 mM potassium phosphate at pH 6, modified mating medium (MMM) consisting of agar (2.5%) buffered with 10 mM citric acid and 10 mM potassium phosphate at pH 5, or T-19 solidified with agar (2.5%) at pH 6. The sporidia were positioned on a small section of agar medium on a cover slip using the procedure described by Holton (1951). This cover slip was usually inverted on a VanTieghem cell in a Petri dish with moist filter paper at its base. Unless stated otherwise, sporidia were incubated at room temperature. Pairs of lunate sporidia were positioned ca. 0-30 μ m apart and orientated according to three

different patterns (Fig. 1). In pattern a, the convex surface of one sporidium faced the concave surface of the other (Fig. 1a); in pattern b, the convex surfaces of both sporidia faced each other (Fig. 1b); in pattern c, the sporidia were positioned pole-to-pole (Fig. 1c).

(c) Observations and Microscopy.

For routine observations and time-lapse 35 mm photomicrography, the cover slip with the agar medium attached was generally inverted on a Van Tieghem cell mounted on the microscope stage. During observations over extended periods of time the cell was lined with moistened filter paper. For observations and photography at high magnifications the cover slip was placed directly on a microscope slide. To reduce evaporation and permit the use of oil immersion objectives a second cover slip was sometimes placed on the upper surface of the agar block.

(d) Photocinematicography.

Sporidia were prepared as described for 35 mm time-lapse photography and incubated at room temperature or at a constant temperature of 20°. Photographs (16 mm) were taken at 15-second frame intervals.

RESULTS

1. Definitions of conjugation pegs and conjugation tubes.

If lunate secondary sporidia (Fig. 2) are incubated on SMM they

normally germinate at one or both of their poles (Fig. 3). Branching of germination tubes in the initial stages of their growth on SMM is uncommon (Fig. 3). However, if lunate secondary sporidia of opposite mating types (+ and -) are incubated in close proximity (0-25 μ m) to each other on SMM the growth patterns described above are often modified. A major morphological change is due to the formation of conjugation pegs and conjugation tubes. In contrast, when filiform secondary sporidia (Fig. 4) are incubated on SMM they germinate as frequently at their sides as at their poles (Fig. 5). Like the lunate cells, filiform cells also produce conjugation pegs in the presence of the opposite mating type but the morphological changes preceding the actual fusion are far less marked. Thus, lunate sporidia are far better indicators of the presence of the opposite mating type than are filiform sporidia.

A conjugation peg is defined as a short hyphal protuberance produced by a sporidium of one mating type that fuses with a sporidium of the opposite mating type. Thus, any germination tube that contacts a cell of opposite mating type, even if by mere chance, and fuses with it is considered to be a conjugation peg. However, this classification can only be made after fusion has been observed. Conjugation pegs differ from germination tubes in two basic respects; they exhibit directed growth toward opposite mating type sporidia, and are capable of fusing with those sporidia. Conjugation pegs are always initiated on the sides of sporidia or germination tubes that are closest to the cells of opposite mating type.

The tube resulting from the fusion of two conjugation pegs or the fusion of a conjugation peg with the body of a sporidium or its germination tube is a conjugation tube.

2. Development of conjugation pegs and formation of conjugation tubes.

(a) Low magnification 35 mm time-lapse photomicrography. There were two similar experiments using lunate secondary sporidia arranged similar to those in pattern a. In the first experiment, the convex surface of the + sporidium faced the concave surface of the - sporidium while in the second experiment the positions of the + and - sporidia were reversed. Seventeen pairs of sporidia were studied in each experiment from photographs taken at 15-minute intervals after all the sporidia were paired.

Conjugation pegs were produced by + sporidia only (41% of the matings), - sporidia only (21% of the matings), or by both + and - sporidia (38% of the matings). Almost all the conjugation pegs exhibited directional growth toward a sporidium of opposite mating type. The mean times required for appearance of conjugation pegs in the first and second experiments were 1.0 and 0.9 h respectively, while the mean times required for conjugation were 1.5 and 1.7 h respectively.

(b) 16 mm Photocinemicrography. Pairs of lunate sporidia were positioned in each of the three different patterns shown in Fig. 1. The mean times for the appearance of conjugation pegs and for conjugation are shown in Table 1. The rates of conjugation peg formation and sporidial conjugation were faster ($p = 0.05$) with sporidia positioned pole-to-pole (pattern c). It was often very difficult to determine

whether conjugation pegs were produced by one or by both sporidia in a mating pair due to the poor resolution in this system. In many instances, however, a conjugation peg appeared to be produced initially by only one partner (+ or -) in a mating pair. This peg then grew toward the other sporidium and when it was very close to (or touching) it, that sporidium responded by initiating a peg of shorter length than the stimulating peg. Details of this interaction were obtained in 35 mm high magnification studies (section c). During conjugation displacement of one or both sporidia in a mating pair was sometimes observed. In some matings, the sporidia were pushed apart during growth of the conjugation pegs.

(c) High magnification 35 mm photomicrography. Lunate sporidia of opposite mating types were paired with their convex surfaces facing each other (Fig. 1b). Photographs were taken at several stages during the development and fusion of conjugation pegs. Detailed records were obtained for 10 matings and these records clarified some of the earlier observations.

The mean times for appearance of conjugation pegs in the + and - sporidia were estimated to be 1.1 and 1.3 h respectively. Conjugation pegs had met end-to-end after ca. 1.6 h and fusion was evident after ca. 1.9 h. In eight pairs, the dikaryon formed by fusion of the two sporidia, had germinated ca. 3.1 h after commencement of the experiment.

In all but one of the pairings, one of the sporidia germinated before either sporidium produced a conjugation peg (Fig. 6). In six matings, one of the sporidia (+ or -) did not germinate at its poles but as shown in Figs. 7-9 it produced a conjugation peg on its convex

surface that had a final length greater than the peg produced by its partner (Fig. 9). In one mating pair, neither sporidium germinated at a pole but each produced conjugation pegs. These observations suggest that in the presence of a sporidium of opposite mating type, formation of conjugation pegs is an alternative to polar germination. The opposite mating type may determine the mode of differentiation of its partner.

Conjugation pegs met precisely tip-to-tip before fusion (Figs. 10, 16, 19). This often required, or caused, a pronounced curvature of one or both pegs (Figs. 15, 16, 19). Sometimes the developing conjugation pegs pushed the lunate sporidia apart before fusion and also changed their relative orientations (Figs. 10-12, 18, 19). The locus for dikaryon germination (Figs. 12-14) was always at, or near to, the point of fusion of the two conjugation pegs. In the two pairings where a dikaryon was not formed, the nascent conjugation tube ruptured during fusion of the conjugation pegs and cytoplasm was exuded (Fig. 17).

In all 10 pairs of sporidia, the conjugation peg produced by one of the cells was much longer than the peg produced by the other. This was not related to mating type because the + sporidium had the longest conjugation peg in six of the matings and the - sporidium had the longest peg in the remainder. In seven pairs the conjugation peg from one of the sporidia appeared to contact its partner before that sporidium produced a conjugation peg (Figs. 18, 19). Thus, the conjugation peg from one sporidium may have induced peg formation in its partner, after contact. There was, however, a small bump on the

convex surface of the lower sporidium in Fig. 18 before contact. A conjugation peg subsequently formed at that site (Fig. 19).

3. Morphology of conjugation pegs and conjugation tubes.

Time-lapse 35 mm photomicrography and 16 mm photocinmicrography of interactions between sporidia of opposite mating types revealed considerable diversity in the morphology of mating.

If lunate sporidia of opposite mating types are orientated according to pattern a, conjugation pegs develop as follows. A germination tube from the sporidium with its concave surface facing the convex surface of its partner frequently curves toward its partner and fuses with its body, its germination tube or a conjugation peg from its germination tube (Figs. 20, 23). The fusion shown in Fig. 20 resulted in the formation and growth of a dikaryon (Figs. 21, 22). The original pair of mating sporidia now consist of a series of evacuated cells sealed off by septa. Sometimes, however, directed growth of the germination tube is not evident and contact, (resulting in fusion) with the opposite mating type, appears to occur by chance. Occasionally the germination tube from the sporidium with its convex surface facing the concave surface of its partner curves and fuses with its partner (Fig. 24). Conjugation pegs frequently develop as side-branches from germination tubes of sporidia orientated according to pattern a (Figs. 24, 25). Occasionally, a conjugation peg emerges from the body of one of the sporidia and it grows toward the other member in the mating pair (Fig. 26) but that is a very rare occurrence for sporidia in that orientation. Only one conjugation tube is usually

formed between sporidia initially 10 μm apart but occasionally two (Fig. 27) and very rarely, three tubes develop.

If lunate sporidia are orientated according to pattern b, are no more than ca. 10 μm apart and are positioned so that their poles are separated by the maximum distances, conjugation pegs frequently emerge from their convex surfaces (Figs. 7, 15, 18, 19). Sometimes, however, with sporidia orientated in this manner, conjugation pegs are produced as lateral branches from the germination tubes of one or both mating types. If the distance between the poles of the sporidia is reduced the frequency of this occurrence increases.

If lunate sporidia are positioned pole-to-pole (pattern c) conjugation pegs are almost always initiated at the adjacent poles of one or both of the sporidia. It is possible that the conjugation pegs develop from hyphal protuberances that were first initiated as germination tubes.

When filiform or lunate secondary sporidia are positioned on SMM so that they are touching at one discrete point a conjugation peg (or pegs) will generally only form at that point. However, if the sporidia are in contact along their surfaces conjugation pegs may form at one, two and sometimes three points. With filiform sporidia in this configuration (Fig. 28) the pegs are formed at random along the long axes of the sporidial cells (Fig. 29). In Fig. 29 at least two of the three conjugation tubes formed by fusion of the conjugation pegs have produced germination tubes. If lunate sporidia are paired according to pattern a, but are touching (Fig. 30) conjugation pegs almost always form at the poles of the sporidial cells (Fig. 31). If the sporidia

are arranged according to pattern b, however, and are touching (Fig. 32) conjugation pegs form on the convex surfaces of the sporidia. It is very uncommon for more than one conjugation tube to form under these conditions. In each of the three situations described above the sporidial cells are pushed apart during conjugation (Figs. 29, 31, 33).

Conjugation tubes may be formed in three different ways. A conjugation peg from a sporidium of one mating type may fuse with a similar peg from a sporidium of opposite mating type (Figs. 24, 25). In other matings, a conjugation peg is produced by only one sporidium in a mating pair and appears to fuse with the body of the sporidium of opposite mating type or with the germination tube from the sporidium of opposite mating type (Figs. 20, 26). In the experiment to determine the origin of conjugation pegs, 35.3% of the conjugation tubes were formed by the fusion of conjugation pegs, 35.3% from the fusion of conjugation pegs with the bodies of sporidia and 29.4% from the fusion of conjugation pegs with germination tubes. It is possible, however, that at the magnification (X 134) used to make these observations very small conjugation pegs (Figs. 9, 15, 19) were not identified. If so, the percentage of peg-peg fusions should be higher. In some of the matings the sporidia were not aligned parallel to each other. This enhances the possibility of a germination tube becoming a conjugation peg and fusing with a sporidium of opposite mating type.

4. Effects of nutrition, pH and distance on sporidial mating.

The effect of nutrition on sporidial mating was studied by pairing lunate sporidia with filiform sporidia ca. 10 μ m apart on SMM and T-19 agar media. After 5-12 h at 17^o the percentages of conjugated sporidia based on a total of ninety matings were 63.3% and 73.3% on SMM and T-19 respectively. However, this difference was not significant ($p = 0.05$).

The effect of pH on the mating of lunate secondary sporidia ca. 10 μ m apart on 2.5% water agar buffered with 10 mM potassium phosphate and 10 mM citric acid was studied. After 4 h incubation the number of contacts, the number of conjugations, and the number of pairs of cells in which both members had germinated or in which conjugation had occurred, was determined. The results in Table 2 show that sporidial conjugations and contacts were more frequent ($p = 0.01$) at pH values of 4.5, 5 and 6 than at pH 7 or 8. The percentage of matings in which both cells had germinated and/or conjugated was not affected ($p = 0.05$) by pH. However, at pH 8 the post-germination development of sporidia appeared somewhat reduced compared with sporidia on media of lower pH. Thus, at pH 8, the reduction in the percentage of contacts and conjugations could have been due to reduced growth.

To determine the relationship between sporidial conjugation and initial distance between the sporidia, lunate cells were paired at ca. 0, 10, 30, 50, 70 and 100 μ m on MMM and examined periodically for fusions. The times required for sporidial conjugation increased with increasing initial distances between the sporidia

(Table 3). For the incubation times of 7.5 and 8.5 h the percentages of fused sporidia were markedly less ($p = 0.05$) at distances greater than 30 μm . However, for times up to 24 h there was considerable conjugation even for sporidia initially 100 μm apart. It is probable, however, that the fusions occurring up to 24 h were due to random hyphal growth rather than the directed growth of conjugation pegs observed up to 8.5 h. In those matings with sporidia initially 30-100 μm apart multiple branching of the differentiating sporidia before conjugation was observed. The significance of this morphological effect will be discussed in another publication.

DISCUSSION

The present investigation has provided significant new information on sporidial mating in the economically important wheat-bunt pathogen, Tilletia caries.

Lunate secondary sporidia may produce conjugation pegs at one of several different sites although the pegs are most commonly associated with developing germination tubes. It is possible, however, to initiate the production of a conjugation peg from the body of a sporidium by carefully positioning the opposite mating type sporidium close to the test cell but at a maximum distance from its poles. It appears, therefore, that the poles of a lunate secondary sporidium are more sensitive to the presence of the opposite mating type cell than any other part of the sporidium. Studies on the formation of conjugation pegs in other fungi such as Tremella mesenterica Fries, Ustilago violacea (Pers.) Roussel and Rhodosporidium toruloides

Banno (Bandoni, 1965; Poon et al., 1974; Abe et al., 1975) have been concerned with yeast-like cells that reproduce by budding. With these fungi there is much less diversity in the origin of conjugation pegs than with the sporidia of T. caries. In addition, conjugation pegs can be identified by their morphologies before they fuse with the opposite mating type cell.

Conjugation tubes in T. caries may form between the bodies of two sporidia, between a germination tube from one sporidium and the body of another, or between two germination tubes. A similar series of fusion types was observed by Bowman (1946) in studies on sporidial fusion in Ustilago maydis (DC.) Cda.

The observation that opposite mating type lunate and filiform sporidia that are initially touching are pushed apart by nascent conjugation pegs has implications for the mating of primary sporidia at the tip of a promycelium. It is very probable that primary sporidia at the tip of a promycelium are initially touching so as to appear aggregated (Kollmorgen, et al., 1978) and are pushed apart by conjugation pegs thereby producing the classic H-shaped configuration of fused primary sporidia.

The present study shows that conjugation pegs may be produced by + sporidia only, - sporidia only, or by both + and - sporidia. It is possible however, that the microscope resolution in some of the experiments was insufficient to detect very small conjugation pegs. Conjugation pegs were always observed on both members of a mating pair in studies at high magnification with oil immersion objectives. In studies on the ultrastructure of primary sporidial mating in T. caries

we observed conjugation pegs from both + and - sporidia (Kollmorgen et al., 1979). Conjugation pegs may be produced by both mating types of T. mesenterica, U. violacea and R. toruloides (Bandoni, 1965; Poon et al., 1974; Abe et al., 1975). Furthermore, the conjugation pegs of T. mesenterica and R. toruloides meet precisely tip-to-tip (Bandoni, 1965; Abe et al., 1975) although in the case of R. toruloides a conjugation peg may fuse with the wall of the opposite mating type cell rather than with its conjugation peg. Bandoni (1965) also noted that the conjugation pegs of T. mesenterica curved near the point of contact allowing precise tip-to-tip fusion. These observations are consistent with our observations with T. caries although the curvature of the conjugation pegs may be due to a mechanical pressure as the pegs push against each other. It is considered that conjugation in T. mesenterica and R. toruloides is controlled by pheromones (Bandoni, 1965; Abe et al., 1975). In a later publication we will present evidence for pheromonal control of sporidial mating in T. caries.

Lunate sporidia conjugated over the pH range 4.5-8 although there was a sharp decline in the percentage of fusions above pH 6. This agrees with Hanna (1934) except for his report that the lower limit for conjugation was 5.1. This discrepancy could be due to the fact that Hanna worked with primary sporidia, whereas we used lunate secondary sporidia. In the course of the present study conjugation was observed to occur as often at 17° as at 25° thus supporting Hanna's temperature studies.

We have shown that the time for sporidial conjugation increases as the distance between sporidia increases. However, given sufficient

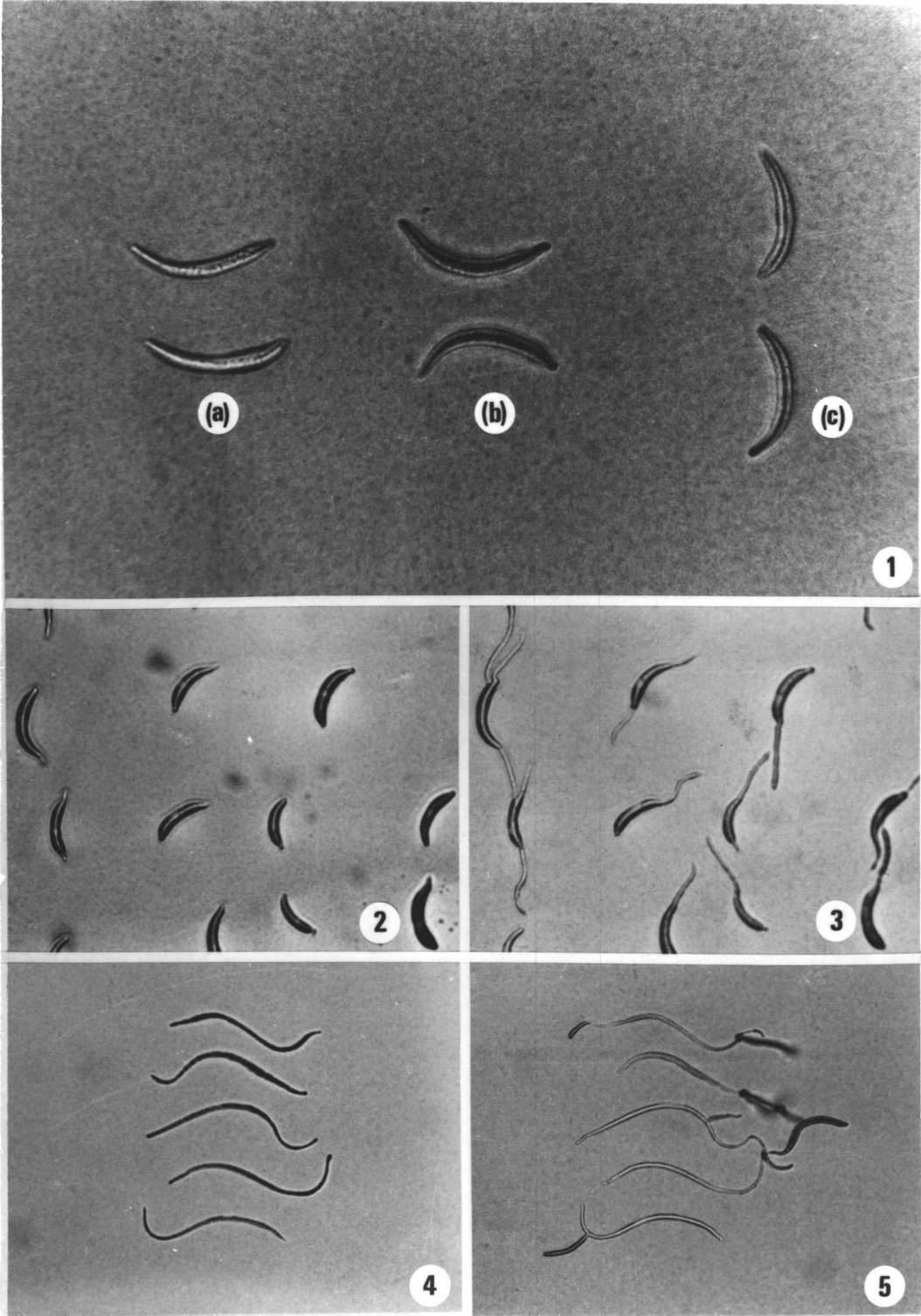
time conjugation will occur between sporidia initially 100 μ m apart. These results are in agreement with those of Holton (1953) for observations up to 8.5 h. However, in our studies, a substantial number of fusions occurred between 8.5 and 24 h in matings with sporidia initially 50-100 μ m apart. Holton (1953) does not report the time period over which he made his observations. Sporidial conjugation occurred as readily on the nutrient medium T-19 as on SMM whereas Holton (1953) reported that conjugation occurred more readily on nutrient medium than on water agar. However, Holton (1953) recorded only 10% fusions on water agar whereas we observed 63.3% fusions on water agar buffered with 5 mM potassium phosphate. In studies with U. maydis (Bowman, 1946) sporidial conjugation was favored by a non-nutrient medium. Holton (1941) also observed that sporidia of Ustilago avenae (Pers.) Jens. and U. levis (Kell. and Sw.) Magn. grown on plain agar responded more rapidly to the fusion stimulus than sporidia grown on potato-dextrose-agar. The sporidia from plain agar were termed "active" and those from potato-dextrose agar were termed "passive". According to Holton (1941) the increased tendency for sporidia of the smut fungi to fuse when placed under conditions of low nutrients is a generally recognized fact. This disagrees with his observations with T. caries (Holton, 1953) and with the results in the present study. Clearly, further studies on the effects of nutrition on sporidial conjugation are required. Results from these studies may have implications for infection of the wheat plant by T. caries in vivo.

Figure 1. Orientations of lunate sporidia during mating studies.
(a) convex-to-concave (pattern a); (b) convex-to-convex
(pattern b); (c) pole-to-pole (pattern c). (X 550)

Figures 2-5. Germination characteristics of secondary sporidia on SMM.

Figures 2,3. Lunate sporidia. (X 340)

Figures 4,5. Filiform sporidia. (X 280)



Figures 6-19. Conjugation of lunate sporidia. (X 870 - X 990)

Figures 6-9. Development of conjugation pegs.

Figure 10. Precise tip-to-tip meeting of conjugation pegs.

Figure 11. Fusion of conjugation pegs to form a conjugation tube.
Note that the sporidia have been pushed apart and their relative orientations have been changed.

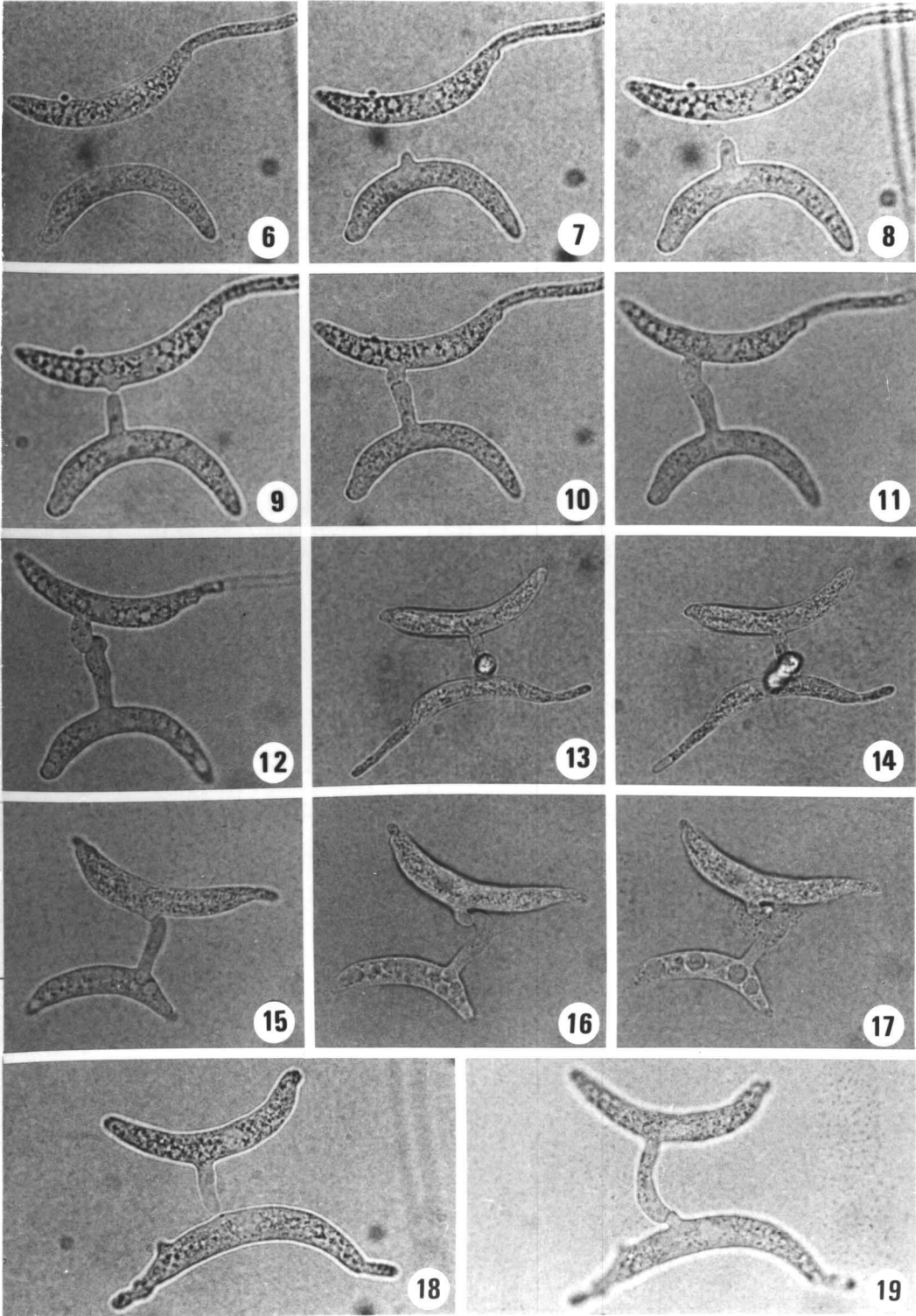
Figures 12-14. Germination of the dikaryon formed by fusion of two monokaryotic sporidia. The locus for germination is at, or near the point of fusion of the conjugation pegs.

Figures 15,16. Curvature of conjugation pegs before fusion.

Figure 17. Rupture of a conjugation tube.

Figure 18. Conjugation peg - sporidium contact preceeding formation of a conjugation peg on the lower cell. Note the slight swelling on the surface of the lower sporidium at the point of conjugation peg - sporidium contact.

Figure 19. Formation of a conjugation peg on the lower sporidium after contact with the conjugation peg from the upper sporidium. Note that the sporidia have been pushed apart and their relative orientations have changed.



Figures 20-27. Conjugation of lunate sporidia.

Figure 20. Conjugation by fusion of germination tubes. Note the pronounced curvature of the lower germination tube. (X 570)

Figures 21,22. Development of a dikaryon. Note the evacuated cells sealed off by septa in the differentiating sporidia. (Fig. 21, X 570; Fig. 22, X 350)

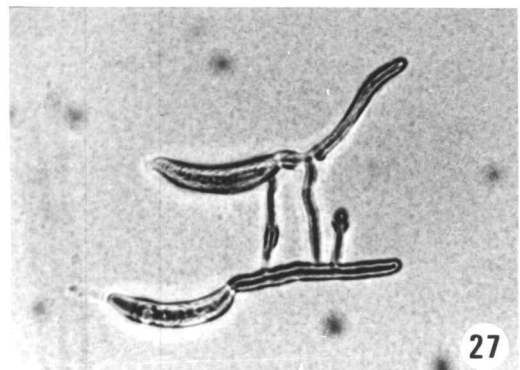
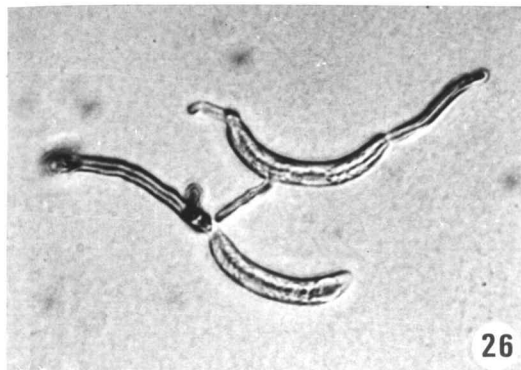
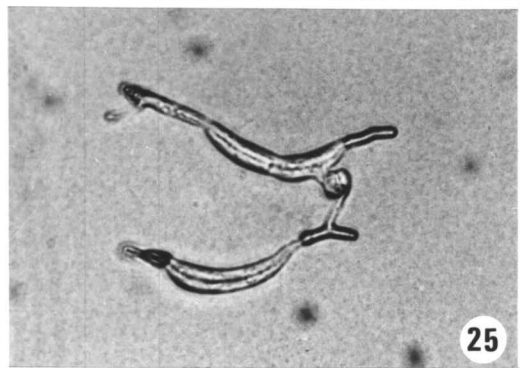
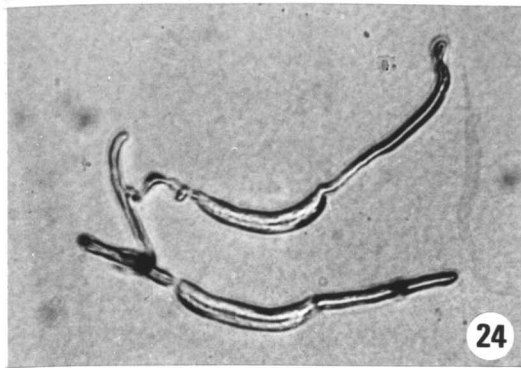
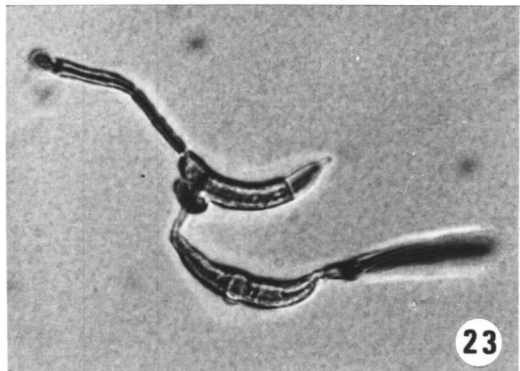
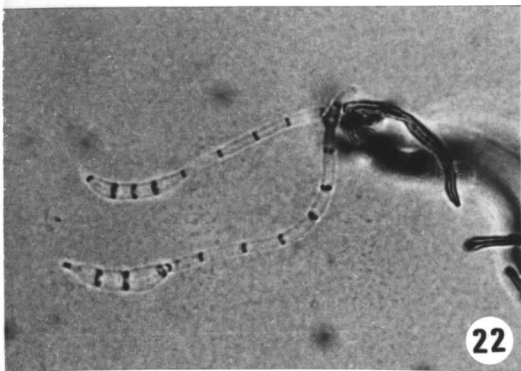
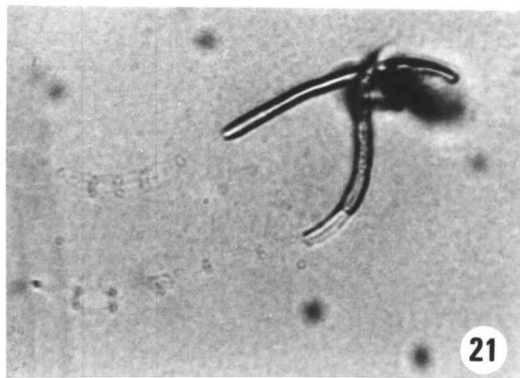
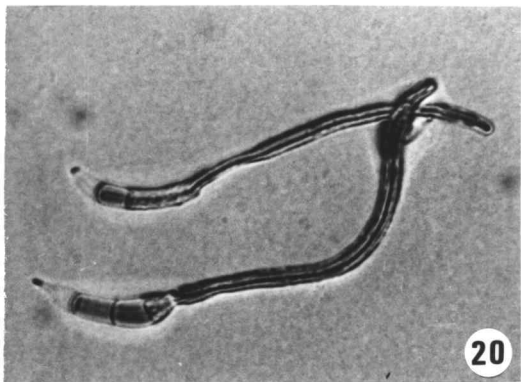
Figure 23. Fusion of a germination tube with the body of a sporidium or a conjugation peg produced by it. Note the sharp curvature of the germination tube. (X 570)

Figure 24. Downward curvature of a germination tube and fusion of it with a conjugation peg from a sporidium of opposite mating type. (X 570)

Figure 25. Fusion of a conjugation peg formed as a lateral branch from a germination tube (lower sporidium) with a conjugation peg from the body of the upper sporidium. (X 570)

Figure 26. Conjugation peg from the convex surface of the upper sporidium that has fused with the lower sporidium near its pole. (X 570)

Figure 27. Sporidial fusion by two conjugation tubes. The lateral hyphal protuberance from the germination tube of the lower sporidium may have been a conjugation peg that would have formed a third conjugation tube. (X 570)



Figures 28-33. Conjugation of sporidia that were initially touching.

Figures 28,29. Conjugation of filiform sporidia at three different loci. Note the germination pattern of the dikaryon. (Fig. 28, X 420; Fig. 29, X 440)

Figures 31,32. Conjugation of lunate sporidia arranged according to pattern 1. (X 560)

Figures 32,33. Conjugation of lunate sporidia arranged according to pattern 2. (X 560)

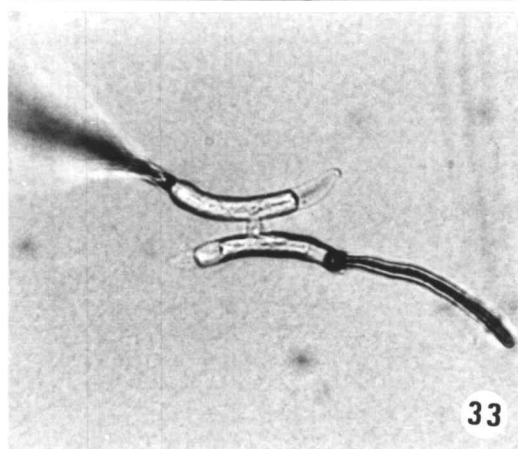
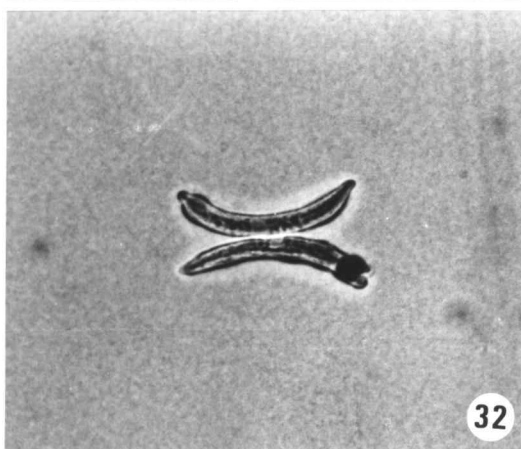
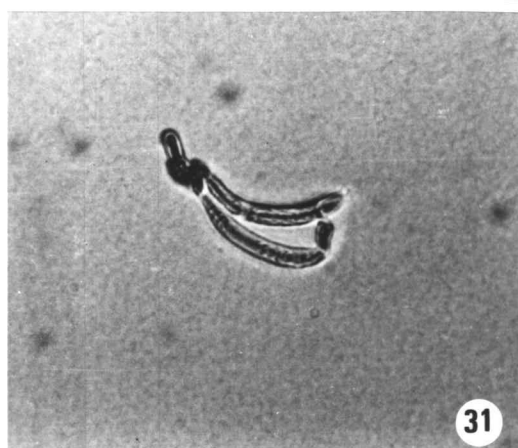
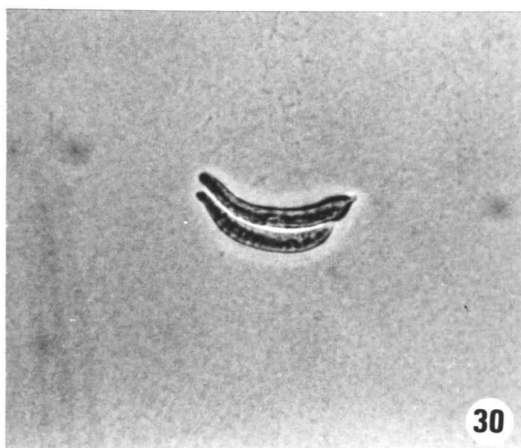
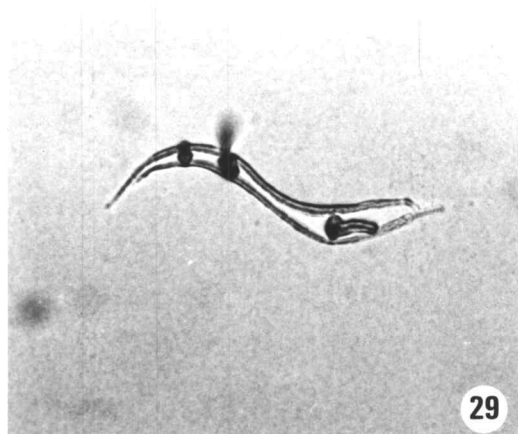
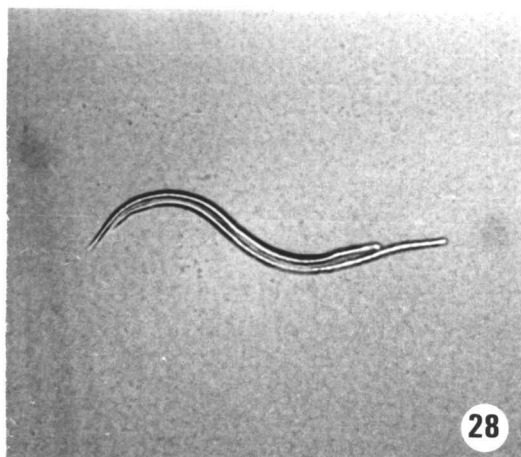


Table 1. Effect of sporidial orientation on the time for conjugation peg formation and conjugation.*

Orientation Pattern	Mean time (h) to :	
	Appearance of Conjugation Pegs	Conjugation
a (convex-to-concave)	2.5 a ⁺	2.7 a
b (convex-to-convex)	2.2 a	2.5 a
c (pole-to-pole)	0.5 b	0.8 b

*Values are means of 7-28 observations.

⁺Means in each column followed by the same letter are not significantly different by comparison of pairs ($p = 0.05$).

Table 2. Effect of pH on interactions between lunate secondary sporidia of opposite mating type.*

pH	% Conjugations	% Contacts	% Matings in which both sporidia germinated and/ or conjugated
4.5	89.5 a ⁺	89.6 a	96.4 a
5	100.0 a	100.0 a	100.0 a
6	85.7 a	85.7 a	92.9 a
7	6.9 b	14.1 b	93.3 a
8	6.9 b	20.2 b	92.9 a

*Values are means of 29 observations.

⁺Means in each column followed by a different letter are significantly different ($p = 0.01$) according to Duncan's multiple range test.

Table 3. Effect of initial distance between sporidia on timing of conjugation.*

Initial distance μm	% Conjugations after (h)			
	5.5	7.5	8.5	21-24
0	100.0 a ⁺	100.0 a	100.0 a	100.0 a
10	71.7 a	91.7 a	91.7 ab	91.7 a
30	50.0 b	70.8 a	75.0 b	100.0 a
50	12.5 bc	27.2 b	33.3 c	87.5 a
70	0.0 c	16.7 b	20.8 c	83.3 a
100	0.0 c	0.0 b	0.0 d	66.7 a

* Values are means of 24 observations.

⁺ Means in each column followed by the same letter are not significantly different ($p = 0.05$) according to Duncan's multiple range test.

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CHAPTER IV

MATING-TYPE INTERACTIONS

SUMMARY

Mating type interactions between monokaryons (secondary sporidia and mycelia) of Tilletia caries were studied. There were more inter-sporidial contacts in pairs of sporidia consisting of opposite (+ -) mating types than in pairs consisting of like (+ + or - -) mating types. Pre-conjugation pegs were produced by sporidia after removal of stimulating (opposite mating type) cells and the pegs continued to elongate. Sporidia of opposite mating types conjugated when subjected to a treatment that would disrupt fimbrial connections. Multiple hyphal tips were produced by sporidia separated from monokaryons of opposite mating type by distances of 70-200 μm . The response was sex-specific. In some instances the factors causing these responses were apparently retained in the agar substrate.

INTRODUCTION

The morphogenetic events during mating of sporidia of Tilletia caries (DC.) Tul. have recently been described (Kollmorgen, et al., manuscript in preparation). There are at least three stages: (1) initiation of conjugation pegs at specific sites on the sporidia; (2) directed growth of the conjugation pegs toward sporidia of opposite mating type, and; (3) fusion of the conjugation pegs with sporidia of opposite mating type to form conjugation tubes.

It is not known, however, how specific sites on sporidial surfaces are determined for the emergence of conjugation pegs nor how directed growth toward the opposite mating type is effected. Similar processes in other fungi are mediated by fimbriae or pheromones. Fimbriae occur in Ustilago violacea (Pers.) Roussel and are associated with cell pairing and the directed growth of conjugation tubes (Day and Poon, 1975; Day, 1976). Sex-specific induction of conjugation tubes by pheromones has been reported in: Rhodosporidium toruloides Banno (Abe, et al., 1975), Saccharomyces cerevisiae Hansen (Levi, 1956; Duntze, et al., 1970), and Tremella mesenterica (Retzius ex. Fr.) (Bandoni, 1965; Reid, 1974). There are many morphogenetic similarities between mating in T. caries and mating in R. toruloides, T. mesenterica and U. violacea. In each of these fungi conjugation pegs are formed by both mating types, mating occurs between cells separated on agar and conjugation pegs show directed growth.

The present investigation was conducted to obtain information on interactions between T. caries sporidia during mating. Attempts were also made to determine if fimbriae and/or pheromones are involved in sporidial interactions that precedes mating.

MATERIALS AND METHODS

(a) Isolates

Isolates used were #18 and #19 (+ mating type) and #24 and #26 (- mating type). They were obtained by isolating single primary

sporidia from germinating teliospores (race T-1) using the methods of Holton (1951). Stock cultures were maintained on potato-sucrose-agar. Filiform and lunate secondary sporidia were produced at 17°C on T-19 medium (Trione, 1964) solidified with agar (2.5%). Mycelium was grown in T-19 medium in flasks on a shaker at 20°C and was washed 2-3 times in distilled water before incubation on agar.

(b) Sporidial interactions

Sporidia were paired on standard mating medium (SMM) consisting of agar (2.5%) buffered with 5 mM potassium phosphate at pH 6 or modified mating medium (MMM) consisting of agar (2.5%) buffered with 10 mM citric acid and 10 mM potassium phosphate at pH 5. The sporidia were positioned on a small section of agar medium on a coverslip using the procedures described by Holton (1951). The coverslip was inverted on a Van Tieghem cell in a Petri dish with moist filter paper at its base. Blocks of MMM (1 cm X 1 cm unless stated otherwise) were used in studies on the branching of differentiating lunate sporidia and were treated as described above. The extent of branching was estimated by counting the numbers of hyphal tips on differentiating lunate sporidia after 3-6 h.

Experiments on sporidial interactions were conducted at room temperature (ca. 22-25°C) or constant temperature (23°C). Other specific methods used in studies on branching of differentiating sporidia are detailed in the results section.

RESULTS

(a) The frequency of intersporidal contacts.

When sporidia of opposite (+ and -) mating types are paired on SMM the developing conjugation pegs exhibit directed growth toward sporidia of opposite mating type (Kollmorgen, et al., manuscript in preparation). This directed growth suggests communication between the sporidia before conjugation. If there is directed growth of conjugation pegs there should be more inter-sporidial hyphal contacts in pairs of differentiating sporidia when the sporidia are of opposite rather than the same mating type. To test this hypothesis sporidia were paired 10 μ m apart on SMM in the combinations ++, --, and +-. The number of pairs of sporidia in which one differentiating cell made contact with its partner (with or without conjugating with it) was counted as well as the number of pairs that had conjugated. The results in Tables 1 and 2 show that intersporidial contacts and conjugations were much more frequent ($p = 0.01$) in (+ -) pairings than in (+ +) or (- -) pairings. These effects occurred when both sporidia in a pair were lunate as well as when one sporidium was lunate and its partner filiform. Sporidia of opposite mating types are obviously attracted to each other as they differentiate thereby enhancing the probability of fusion.

(b) Formation and growth of pre-conjugation pegs after displacement of stimulating sporidia.

Hyphal protuberances are produced on the convex surfaces of

lunate secondary sporidia in response to sporidia of the opposite mating type (Kollmorgen, et al., manuscript in preparation). Provided the stimulating sporidia are not displaced, the protuberances fuse with the opposite mating type cells and by definition are conjugation pegs. When a sporidium stimulates development of a conjugation peg in its partner but is displaced before the peg can fuse with its partner, the conjugation peg is referred to as a pre-conjugation peg. This peg has the morphological characteristics of a conjugation peg but because it does not fuse with a sporidium of opposite mating type it may continue to elongate.

We hypothesized that pheromones produced by differentiating sporidia of one mating type cause the formation of conjugation pegs in sporidia of opposite mating type. If this were true, it should be possible to displace one of the sporidia in a mating pair after it has secreted its pheromone but before its partner has shown any morphogenetic response. The sporidium that was displaced and/or its partner should subsequently produce a pre-conjugation peg.

To test this hypothesis, sporidia of opposite mating types were paired 5-10 μm apart on SMM with their convex surfaces facing each other. After 35-60 min one of the sporidia in each mating pair was displaced ca. 75 μm from its partner. In some of the pairs, one or both sporidia had already formed pre-conjugation pegs on their convex surfaces. Other pairs had already conjugated and displacement was not possible. Pre-conjugation pegs were sometimes formed on the convex surfaces of the displaced sporidia and/or their partners. Pegs already visible at the time of displacement continued to elongate.

Thus, the continued, proximity of a stimulating sporidium was not required for initiation and elongation of pre-conjugation pegs.

In a subsequent experiment, the sporidia in mating pairs were moved further and further apart after pre-conjugation pegs were initiated so that the pegs were never in contact. The most common response by the pre-conjugation pegs was to initiate branches that grew toward the opposite mating type. Curvature of the pegs was rarely observed.

It was reasoned that if pheromones travel between sporidia of opposite mating types through the agar substrate and have electrical charges, it may be possible to apply an electrical potential to the agar, displace the pheromones, and block mating. Experiments were conducted using an LKB horizontal slab-gel apparatus with agar at pH values of 5, 6 and 7.8. Bromophenol blue was used as an indicator of electrophoretic movement. The formation of conjugation pegs was not blocked suggesting that if pheromones were involved, they were not electrically charged under these experimental conditions.

(c) Attempts to block mating by breaking fimbrial connections

It was hypothesized that conjugation pegs may grow along fimbrial connections between opposite mating type cells as postulated for U. violacea (Day, 1976). To test this hypothesis, sporidia of opposite mating types (30 pairs) were positioned 25 μm apart on SMM. A glass needle 10 μm in diameter was drawn along the agar surface between the cells at ca. 15 min intervals until conjugation was imminent. This treatment would have disrupted fimbrial connections. In the control treatment, the 30 pairs of sporidia were undisturbed. The percentages

of conjugations in the control and test treatments were 50 and 33 respectively. Thus, conjugation was apparently not reliant on fimbrial connections.

(d) Sex-specific branching of differentiating sporidia

In an earlier study, (Kollmorgen, et al., manuscript in preparation) multiple hyphal tips were produced by differentiating lunate sporidia mated 30-100 μm apart on MMM. The hyphal tips were formed by the branching of hyphal protuberances (probably germination tubes) initiated at the poles of the sporidia (Figs. 1, 2, 5, 6). Experiments were conducted to determine if this response was sex-specific.

Sporidia of isolates #18 and #19 (+ mating type) and #24 and #26 (- mating type) were paired ca. 70 μm apart in the combinations + -, + + and - -. There were also control treatments with + sporidia and - sporidia not paired. Production of hyphal tips by both + and - sporidia, was stimulated by sporidia of the opposite mating type and not suppressed by sporidia of the same mating type (Tables 3, 4). Sporidia paired with cells of opposite mating type produced several hyphal tips during differentiation (Figs. 1, 2), whereas sporidia paired with cells of the same mating type usually produced only one or two hyphal tips (Figs. 3, 4). Sporidia that were not paired with other cells were similar in appearance to those in Figs. 3, 4. The + sporidium in Fig. 1 has produced one long branch that has grown toward the - sporidium and thus, is probably a pre-conjugation peg. Other branches (Figs. 1, 2) do not show directed growth.

A further experiment was conducted to determine if the factors

responsible for sex-specific branching would travel over distances of 100 or 200 μm . Sporidia were paired 100 and 200 μm apart in the combinations described above and similar control treatments were used. The + sporidia were stimulated to branch when 100 or 200 μm from the - sporidia but the - sporidia were not affected at either of these distances (Table 5).

It was hypothesized that the factors causing branching might exert a more pronounced effect and be effective over larger distances if they were present in higher concentrations at their origins. It was also anticipated that the concentrations of these factors would be proportional to the number of stimulating cells. To test these ideas, 20 sporidia (stimulating cells) of + mating type were positioned parallel to each other and in contact along the entire lengths of their long axes. Single test sporidia of like (+) or opposite (-) mating types were placed at 50 μm intervals for a distance of 750 μm away from the group of stimulating sporidia. This was repeated using sporidia of - mating type as the stimulating cells. Sporidia of opposite mating type to the stimulating cells and no more than 200 μm from them, produced several hyphal tips. This effect was not observed in the control treatments. Prolific branching was also observed within the group of 20 stimulating sporidia in those treatments where the test and stimulating sporidia were of opposite mating types.

The formation of multiple hyphal tips by differentiating lunate sporidia was first observed on MMM at pH 5 and had not been observed in previous investigations with sporidia paired on SMM at pH 6. It was of interest therefore, to determine if the formation of hyphal

tips was affected by pH. Sporidia (+ and -) were paired 70 μ m apart on MMM adjusted to pH 5 or pH 6 and the number of hyphal tips produced by each sporidium was counted. The results in Table 6 show that for both mating types there was more branching at pH 5 than at pH 6.

Lunate sporidia will conjugate with hyphae of opposite mating type (Kollmorgen and Trione, unpublished data). Because mycelium of T. caries can be more readily produced in large quantities and is easier to manipulate than single sporidia, experiments were conducted to determine if the sex-specific response described above occurred with mycelium as the source of stimulation. A very small clump (ca. 1 mm diameter) of mycelium was placed toward the end of a block of MMM, and sporidia were positioned at 50 μ m intervals away from the edge of the mycelium.

The results in Tables 7, 8 show that the number of hyphal tips was highest ($p = 0.05$) in those treatments involving opposite mating types. Thus the sex-specific response described for sporidial interactions also occurred with sporidial-mycelial interactions. The results from treatments including mycelium and sporidia of the same mating types as well as sporidia alone (Table 8), support the earlier finding that hyphal-tip formation is stimulated in + - pairings and not suppressed in + + or - - pairings.

(e) Sex-specific responses after removal of the stimulating sporidia or mycelium.

The factor produced by sporidia or mycelium of + mating type that stimulates production of hyphal tips by differentiating - sporidia

was called (+) factor. The factor produced by sporidia or mycelium of - mating type that stimulates production of hyphal tips by differentiating + sporidia was called (-) factor.

Experiments were conducted to determine if the (+) and (-) factors were present in an active form in MMM after growth of sporidia or mycelium. Sporidia (20 or 30) were micromanipulated to be adjacent to each other on blocks of MMM (ca. $\frac{1}{2}$ cm X 1 cm) and incubated for 3-4 h. These sporidia were then removed, 12 test sporidia were positioned where the first set had been and the size of the block of MMM was reduced to ca. $\frac{1}{2}$ cm X $\frac{1}{2}$ cm. There were four treatments involving four different sequences of sporidia viz. (i) + followed by +; (ii) + followed by -; (iii) - followed by -; (iv) - followed by +.

The results in Table 9 show that the number of hyphal tips from differentiating + sporidia was higher ($p = 0.05$) when the cells were preceded by - sporidia than when preceded by + sporidia. However, the extent of branching was not nearly as pronounced as in earlier experiments with sporidia or hyphae not removed. The number of hyphal tips on - sporidia was not significantly affected by the mating type of the preceding sporidia.

For studies on the production of (+) and (-) factors by mycelium, a block of MMM (1 cm X 1 cm) was cut in half and a small piece of mycelium positioned on one side of the cut. This was repeated for a total of 9 blocks. At 30 min intervals thereafter the two halves of a designated block were separated. The half with the mycelium was discarded and the other half was bioassayed for

(+) factor or (-) factor activity. For the bioassay, sporidia of opposite mating type to the mycelium were placed at ca. 50 μ m intervals for 500 μ m commencing 20-50 μ m from the edge previously adjacent to the block with the mycelium. There were two control treatments. In the positive control, the half-block with the mycelium was left in place for the duration of the experiment and the sporidia positioned as described above; in the negative control, sporidia were positioned at 50 μ m intervals from the cut edge of a block ($\frac{1}{2}$ cm X 1 cm) that had not been exposed to the mycelium.

The results in Table 10 indicate that (+) factor and (-) factor activities were transferred from one agar block to the other. Experiments were conducted to confirm this. The experimental methods were the same as those described above, except that the period of contact between the blocks (3-4 h) in the test treatments was not varied.

The results in Tables 11, 12 show that (+) factor and (-) factor activities were transferred to the test blocks. However, (+) factor activity in the test blocks was less than in the positive control (sporidia on block always in contact with + mycelium). The bioassay sporidia on agar blocks in contact with agar blocks with mycelium of opposite mating type generally produced several hyphal tips (Fig. 5). Some of the bioassay sporidia on blocks previously in contact with blocks with mycelium of opposite mating type also produced several hyphal tips (Fig. 6). The sporidia in Figs. 5, 6 have similar morphologies to those in Figs. 1, 2. These effects were not observed with sporidia on blocks not previously exposed to the mycelium (negative control).

Experiments were also conducted to determine if sex-specific factors were in culture filtrates from T. caries monokaryons. Filtrates were assayed before and after concentrating by lyophilization. Pieces of filter paper (2 mm X 8 mm) saturated with the filtrates were placed toward the ends of blocks of MM and sporidia positioned at 50 μ m intervals from them. No indications of pheromonal responses were observed.

DISCUSSION

This series of investigations provides strong evidence that opposite mating type sporidia of T. caries interact before conjugation and has identified a sex-specific response other than conjugation.

The observation that there are many more hyphal contacts between differentiating sporidia of opposite mating types than between sporidia of like mating types indicates sexual interactions preceding conjugation. These interactions apparently cause the formation and directed growth of conjugation pegs as well as the directed growth of germination tubes. Fusion between sporidia of the same mating types (+ + or - -) was observed. This has not previously been recorded. The finding that pre-conjugation pegs are sometimes formed on sporidia and may continue to elongate, after displacement of a stimulating (donor) sporidium strongly suggests that pheromones are involved in communication. This also provides evidence against initiation of pre-conjugation peg outgrowth by fimbrial connections. Mating was not blocked by passing a glass

needle between mating sporidia. This treatment would have disrupted fimbrial connections. Thus, unless fimbrial connections between conjugation pegs of T. caries are re-formed extremely rapidly (within ca. 15 min.) this result would suggest that fimbriae are not essential for mating in T. caries. Fimbriae have not been detected in ultra-structural studies on primary sporidia (Kollmorgen, et al., 1979) or secondary sporidia (R. Gardiner and A. W. Day, personal communication) of T. caries.

The discovery that lunate sporidia may produce multiple hyphal tips when separated from sporidia or mycelium of opposite mating type by 70 μ m or more has provided a valuable bioassay for sex-specific factors produced by T. caries monokaryons. Although it is not known if these factors are the same as those that induce conjugation pegs, the directed growth of some of the branches (Fig. 1) supports this idea. The lack of directed growth in other instances (Figs. 2, 5, 6) may be due to excesses of the factors. Haploid cells of T. mesenterica produced multiple conjugation tubes in response to a partially purified pheromone from that fungus (I. D. Reid, personal communication). We tested a sample of Tremella hormone kindly supplied by I. D. Reid for activity against T. caries sporidia. The hormone stimulated sporidial branching but the effect was not sex-specific (Kollmorgen and Trione, unpublished results).

Experiments to test whether the T. caries factors responsible for sex-specific branching were retained in agar or could be transferred from one agar block to another were somewhat inconclusive. Investigations with lunate sporidia as the source of these factors indicated

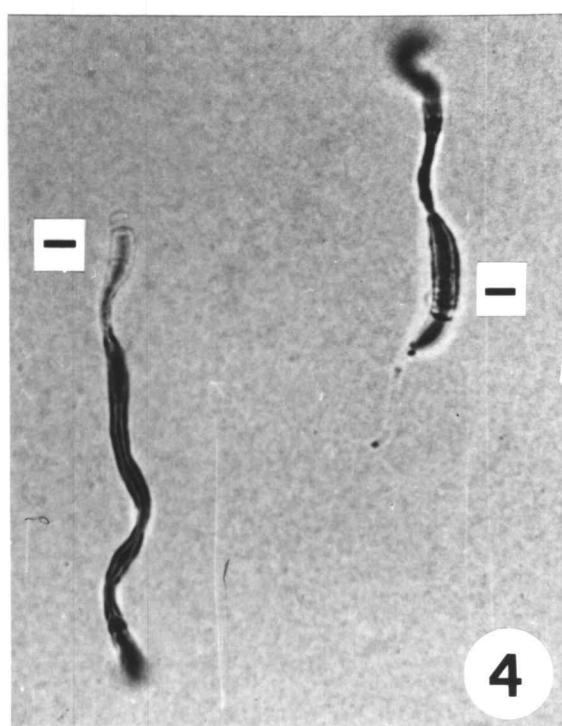
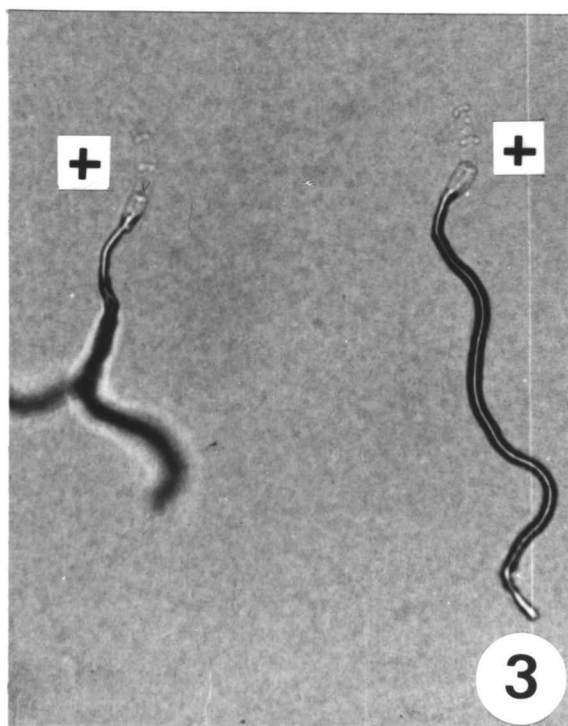
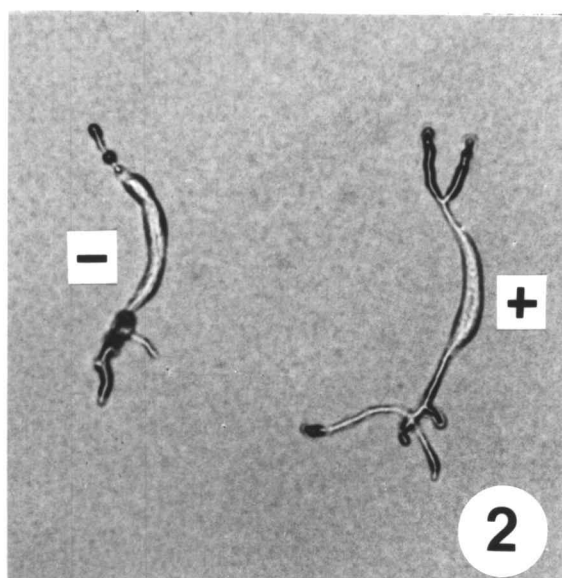
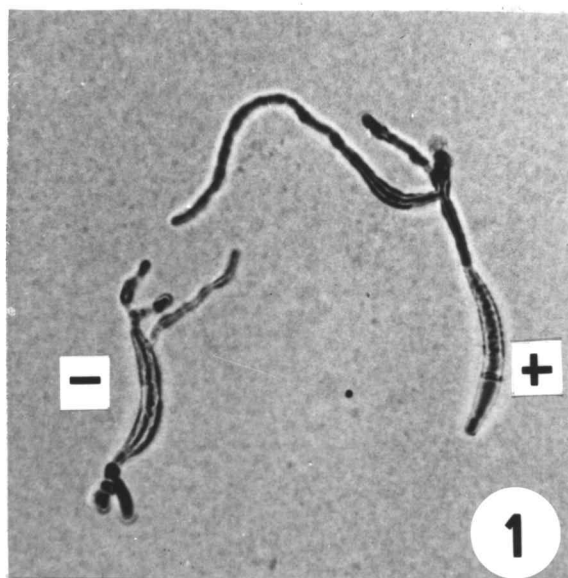
that only the (-) factor was retained in agar. A possible explanation is that the (-) factor is constitutive whereas the (+) factor is inducible. However, the extent of branching (Table 10) was too low to draw strong conclusions. In similar studies with mycelium as the source of the factors, there did appear to be transfer of the factors between agar blocks. Sporidia in the bioassay were sometimes markedly affected (Figs. 5, 6). It is possible that the factors are volatile, unstable, or are broken down by extracellular enzymes. Volatile pheromones have been reported in the Mucorales (Mesland, et al., 1974; Sutter, 1977) and the breakdown of acrasin (a pheromone produced by Dictyostelium discoideum Raper) by an extracellular enzyme (acrasinase) has been recorded (Bonner, 1969). Our inability to detect pheromonal activities in culture filtrates of T. caries monokaryons suggests that the compounds may be unstable, volatile or are inactivated. Another possibility is that the culture filtrates were not tested at concentrations required to evoke responses. It is noteworthy however, that pheromonal activities have been detected in crude culture filtrates of S. cerevisiae, T. mesenterica and R. toruloides (Duntze, et al., 1970; Reid, 1974; Abe, et al., 1975).

Figures 1-4. Production of multiple hyphal tips by lunate sporidia.

Figures 1,2. + and - lunate sporidia paired on MMM. Note the multiple hyphal tips. X 530

Figure 3. + sporidia paired on MMM. X 530

Figure 4. - sporidia paired on MMM. X 530



Figures 5,6. Production of multiple hyphal tips by lunate sporidia in response to (-) factor.

Figure 5. + sporidia on an agar block in contact with an agar block with - mycelium. Note the line of contact of the two blocks (arrows). The sporidia were stimulated by (-) factor to produce several hyphal tips. X 690

Figure 6. + sporidia on an agar block that had previously been in contact for 3 h with an agar block with - mycelium. The (-) factor apparently diffused into the agar block shown in the photograph and the sporidia were stimulated to produce several hyphal tips. X 690

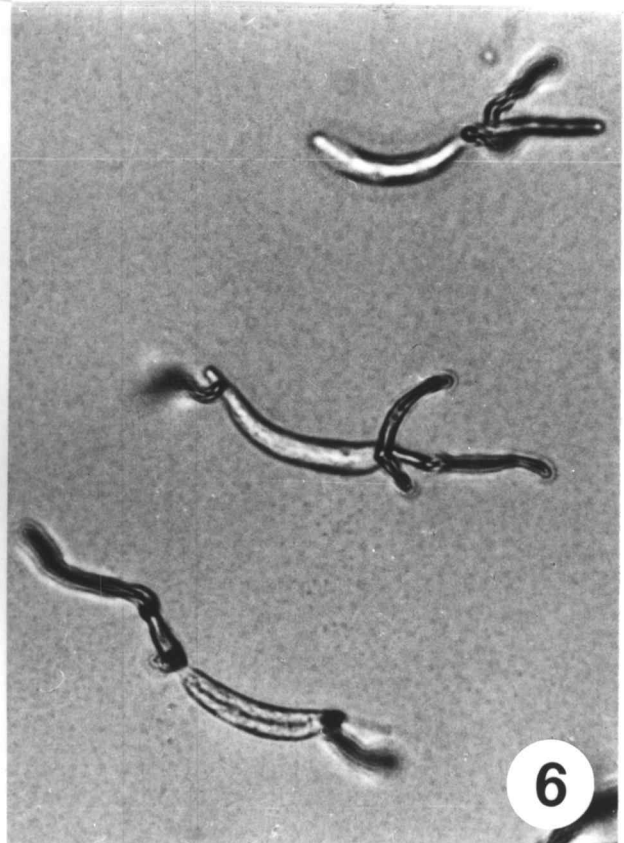
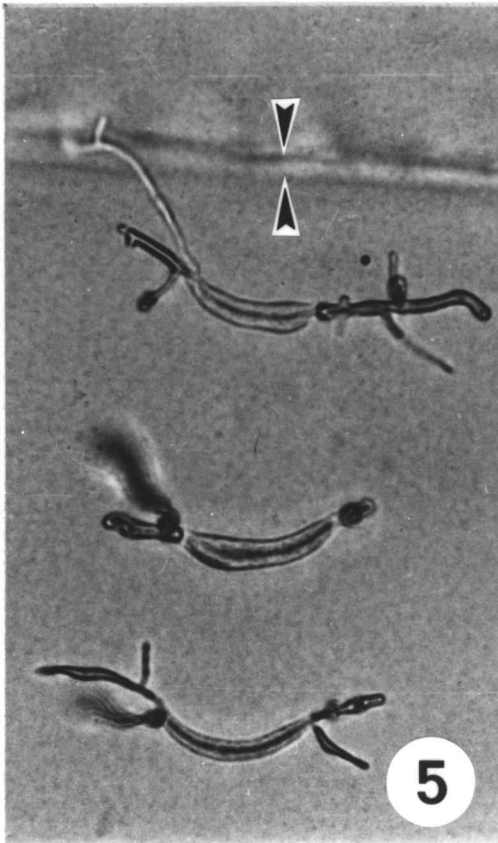


Table 1. Frequencies of hyphal contacts and conjugations in pairs of lunate secondary sporidia of like and opposite mating types.¹

Pair	% Contacts	% Conjugations
+ +	14.2 b ²	3.3 b
- -	8.3 b	5.8 b
+ -	87.1 a	87.1 a

¹Values are the means of 45 observations.

²Means in each column followed by a different letter are significantly different ($p = 0.01$) according to Duncan's multiple range test.

Table 2. Frequencies of hyphal contacts and conjugations in pairs of secondary sporidia (lunate and filiform cells) of like and opposite mating types.¹

Pair	% Contacts	% Conjugations
+ (lunate), + (filiform)	22.6 b ²	4.2 b
- (lunate), - (filiform)	13.1 b	0.0 b
+ (filiform), - (lunate)	88.1 a	73.8 a

¹Values are the means of 33 observations.

²Means in each column followed by a different letter are significantly different ($p = 0.01$) according to Duncan's multiple range test.

Table 3. The effect of mating type on production of hyphal tips by differentiating lunata sporidia (isolates #18 and #24) paired 70 μ m apart.

Stimulating sporidium	Number of hyphal tips on test sporidium of mating type ¹ :	
	+	-
None (control)	1.4 b	1.2 b
+	1.2 b	4.9 a
-	4.5 a	1.2 b

¹Values are the means of 22 observations.

²Means followed by the same letter are not significantly different ($p = 0.05$) according to Duncan's multiple range test.

Table 4. The effect of mating type on production of hyphal tips by differentiating lunate sporidia (isolates #19 and #26) paired 70 μm apart.

Stimulating sporidium	Number of hyphal tips on test sporidium of mating type ¹ :	
	+	-
None (control)	1.9 b	1.5 b
+	1.9 b	3.0 a
-	3.6 a	1.5 b

¹Values are the means of 24 observations.

²Means followed by the same letter are not significantly different ($p = 0.05$) according to Duncan's multiple range test.

Table 5. The effect of mating type on production of hyphal tips by differentiating lunata sporidia paired 100 and 200 μm apart.

Treatment	Hyphal tips per sporidium of mating type ¹ :	
	+	-
Control (no stimulating sporidium)	1.7 a	1.5 a
Stimulating sporidium 100 μm away	3.2 b	1.9 a
Stimulating sporidium 200 μm away	2.6 b	2.1 a

¹Values are the means of 13 observations.

²Means followed by the same letter are not significantly different ($p = 0.05$) according to Duncan's multiple range test.

Table 6. Effect of pH on the formation of hyphal tips by differentiating lunate secondary sporidia.

pH	Number of hyphal tips per sporidium of mating type ^{1,2} :	
	+	-
5	3.9	4.1
6	2.1	2.8

¹Values are the means of 12 observations.

²Means in each column are significantly different ($p = 0.05$).

Table 7. Production of hyphal tips by differentiating lunate sporidia in the presence of mycelium of opposite or like mating type.

Treatment	Hyphal tips per sporidium ¹
+ mycelium, + sporidia	2.1 b ²
- mycelium, + sporidia	3.6 a
- mycelium, - sporidia	1.2 c
+ mycelium, - sporidia	3.4 a

¹Values are the means of 60 observations.

²Means followed by a different letter are significantly different ($p = 0.05$) according to Duncan's multiple range test.

Table 8. Production of hyphal tips by differentiating lunate sporidia in the presence of mycelium of the same or opposite mating type.

Treatment	Number of hyphal tips per sporidium ¹
+ sporidia	2.6 c ²
+ mycelium, + sporidia	2.6 c
- mycelium, + sporidia	4.5 a
- sporidia	1.3 d
- mycelium, - sporidia	1.0 d
+ mycelium, - sporidia	3.8 b

¹Values are the means of 24 observations.

²Means followed by a different letter are significantly different (p = 0.05) according to Duncan's multiple range test.

Table 9. Effect of sporidial secretions on the formation of hyphal tips by differentiating lunata secondary sporidia.

Treatment	Number of hyphal tips ¹
+ followed by +	1.1 b
- followed by +	1.8 a
- followed by -	1.1 b
+ followed by -	1.3 ab

¹Values are the means of 48 observations.

²Means followed by the same letter are not significantly different ($p = 0.05$) according to Duncan's multiple range test.

Table 10. Migration of (+) factor and (-) factor activities between agar blocks in contact for different periods of time.

Treatment	Hyphal tips per sporidium in assay for: ¹	
	(+) factor activity	(-) factor activity
Sporidia on agar block never in contact with mycelium	1.7 b ²	1.6 e
Sporidia on agar block always in contact with mycelium	3.0 a	4.0 a
0.5 h contact between agar blocks	1.8 b	2.9 bcd
1.0 h " " " "	1.4 b	2.3 cde
1.5 h " " " "	2.0 b	3.3 abc
2.0 h " " " "	1.8 b	2.1 de
2.5 h " " " "	2.9 a	3.3 abc
3.0 h " " " "	3.2 a	3.5 ab
3.5 h " " " "	2.7 a	3.0 abcd
4.0 h " " " "	3.0 a	3.0 abcd

¹Values are the means of 10 observations.

²Means in each column followed by the same letter are not significantly different (p = 0.05) according to Duncan's multiple range test.

Table 11. Evidence for migration of (+) factor and (-) factor activities from agar blocks with mycelium to test blocks without mycelium, based on hyphal tips per sporidium.

Treatment	Hyphal tips per sporidium in assay for ¹ :	
	(+) factor activity	(-) factor activity
Sporidia on agar block never in contact with agar block with mycelium (negative control)	1.4 a ²	2.0 b
Sporidia on agar block always in contact with agar block with mycelium (positive control)	3.1 b	3.1 a
3-4 h contact between test agar block and agar block with mycelium	2.0 c	3.1 a

¹Values are the means of 40 observations.

²Means in each column followed by the same letter are not significantly different ($p = 0.05$) according to Duncan's multiple range test.

Table 12. Evidence for migration of (+) factor and (-) factor activities from agar blocks with mycelium to test blocks without mycelium, based on numbers of sporidia with five or more hyphal tips.

Treatment	Sporidia with five or more hyphal tips in assay for ¹ :	
	(+) factor activity	(-) factor activity
Sporidia on agar block never in contact with agar block with mycelium (negative control)	0	0
Sporidia on agar block always in contact with agar block with mycelium (positive control)	10	8
3-4 h contact between test agar block and agar block with mycelium	2	7

¹From a total of 40 sporidia.

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GENERAL CONCLUSIONS

The investigations described in this thesis have provided significant new information on the morphology, ultrastructure and mating of primary sporidia of T. caries. In addition, considerable information has been obtained on the morphology and timing of mating interactions involving secondary sporidia. It has also been shown that T. caries monokaryons (secondary sporidia and mycelia) produce sex-specific factors that cause multiple branching of lunate sporidia.

Promycelial outgrowth, formation of primary sporidia and the conjugation of these cells occur very rapidly and require less time than teliospore germination. Sporidial fusion is facilitated because primary sporidia at the tip of a promycelium are often borne in pairs and are packed tightly together so as to appear aggregated. Pairs of mating sporidia are probably in contact initially but are subsequently pushed apart by the developing conjugation pegs to form the classic H-shaped configuration of fused primary sporidia. The observation that secondary sporidia (lunate and filiform) of opposite mating types initially adjacent to each other on an agar surface are pushed apart during mating supports this idea.

Conjugation pegs are produced by both primary sporidia (i.e. + and -) in a mating pair. The pegs are nearly always at the midpoints of the sporidia and during conjugation they meet tip-to-tip. This suggests coordinated communication preceding the actual

fusion. It was hypothesized that fimbriae and/or pheromones might be involved in this communication event. However, fimbriae were not detected in an ultrastructural study.

The mating interaction at the tip of a promycelium is very difficult to study because it is almost impossible to visualize conjugation in situ. Further studies were therefore conducted with secondary sporidia and mycelia on agar surfaces. Mating patterns with secondary sporidia are much more diverse than with primary sporidia at the tip of a promycelium. Conjugation pegs are formed at several different sites on the sporidial cells, depending on the orientation of the sporidia relative to their mating partners. The conjugation pegs may be produced by + sporidia only, - sporidia only, or by both + and - sporidia. Conjugation pegs from secondary sporidia meet precisely tip-to-tip. This is consistent with the findings for primary sporidia and supports the concept of coordinated communication.

The studies on mating interactions between secondary sporidia strongly suggest that fimbriae are not involved in mating. This is further supported by the fact that fimbriae have not been visualized on secondary sporidia. It was hypothesized that pheromones may initiate the formation of conjugation pegs. The observation that sporidia produce pre-conjugation pegs after displacement of stimulating cells (i.e. sporidia of opposite mating type) and the finding that T. caries monokaryons produce diffusible, sex-specific factors that cause outgrowth of hyphal protuberances,

both support this hypothesis. Excesses of the stimulating factors may have been responsible for the lack of directed growth of many of the hyphal branches produced by differentiating sporidia. Studies aimed at isolating the sex-specific factors are warranted. Monokaryotic mycelium of both mating types (+ and -) has been shown to produce the sex-specific factors. Because this mycelium can be grown readily in large quantities it may be an excellent source of the factors.

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