Genetic and Cytological Evidence for a Diploid Life Cycle in Pythium aphanidermatum

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ABSTRACT

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A cytological and genetic examination was undertaken to determine the location of meiosis in the life cycle of *Pythium aphanidermatum*. The hyphal nuclei were examined in order to compare them with meiotic figures in the gametangia. A new model is suggested to explain the nonclassical fungal mitosis using the assumption of chromatin-nuclear-membrane attachment. Diplotene-diakinesis figures were photographed in both the antheridia and oogonia. A chloramphenicol-tolerant mutant was induced with ethyl methansulfonate and the oospore progeny segregated one drug-sensitive to 5.83 drug-tolerant. Zoospores from the same mutant failed to segregate, indicating that heterokaryosis was not responsible for oospore segregation. These results are most simply the product of heterozygous diploid somatic nuclei and gametangial meiosis. Both the cytological and genetic data suggest a life cycle with diploid nuclei in the mycelium.

The somatic nuclei in most fungi are haploid. Certain members of the oomycetes, however, are believed to possess diploid somatic nuclei which presumably undergo meiosis in the gametangia. Plasmogamy and karyogamy then immediately restore the diploid somatic nuclear condition. This scheme of gametangial meiosis first was suggested for the oomycetes in the late 19th century (43) and reported by Sansome (34, 35, 36, 37, 38) in more recent years. However, evidence in support of a haploid life cycle has been reported (9, 27, 42).

Cytological evidence of the location of meiosis in the majority of the oomycetes studied has relied on the following evidence: (i) multiple association of meiotic chromosomes; (ii) reduction in nuclear diameter as meiotic divisions proceed; (iii) bridges and fragments at anaphase, and (iv) a metaphase plate unique to meiosis (35, 36).

The observation of bivalents in gametangia was cited as evidence for meiosis in *Pythium* by Sansome (35, 36, 37, 38). However, the presence of bivalents should not be considered as proof of meiosis since mitotic track figures (double filaments) (7, 12, 31) can resemble pachytene bivalents. Further, the "ring of four chromosomes" seen by Sansome (35) could resemble the hyphal rings observed by Robinow (30) in *Basidiobolus*.

Additionally, nuclear size alone is not good evidence of meiosis (37). Meiosis reduces the concentration of DNA in each pre-replication nucleus by one-half. A sphere whose volume is halved will show a radius decrease of only 21%; such a decrease would be difficult to measure in view of the small size of oomycete nuclei [approximately 2 μm in diameter (38)].

Bridges and fragments have also been used as evidence of meiosis in the oomycetes (36). This may be a valid criterion for higher plants, but not for fungi, since similar figures occur in somatic cells of *Phytophthora* (42) and *Fusarium* (1).

Evidence of the diplotene-diakinesis stages of meiosis has recently been reported in the oomycetes (3, 37, 38). These stages do not resemble any mitotic figures and provide unmistakable evidence of meiosis.

Additionally, other approaches have been attempted to provide quantitative evidence for the site of meiosis. Microspectrophotometric evidence supports gametangial meiosis in *Apodachyla* and *Saprolegnia* (4, 19). Genetic evidence tends to support gametangial meiosis (14, 18, 21). However, the obtaining of genetic evidence has been hampered by the difficulty in obtaining mutants and the low percentage germination of oospores (18). Recently, Stanghellini et al. reported that snail ingestion of culturally grown oospores of *Pythium aphanidermatum* Edson (Fitzp.) increased the percentage germination from 20 to 94% (41). Thus, *P. aphanidermatum* was chosen to test the ploidy of this homothallic fungus by searching for segregation in the sexual progeny of a drug-resistant mutant. The cytology of somatic and gametangial cells also was studied.

MATERIAL AND METHODS

Cytology.—*Pythium aphanidermatum* was grown in centrifuged 5% V-8 juice for 2-5 days and fixed in ethyl alcohol-acetic acid (3:1) for 1 hr. Orcein (2 g in 100 ml 45% acetic acid, heated and filtered) and 5N HCl were mixed (1:1) in a Coor's spot dish. One cm^3 of the growing tips of the mycelial mat then was stained for 30 min followed by a 5-min rinse in 45% acetic acid and cleared for 10 min in a clearing agent (one part each of chloral hydrate, clove oil, lactic acid, xylene, and two parts phenol by volume).

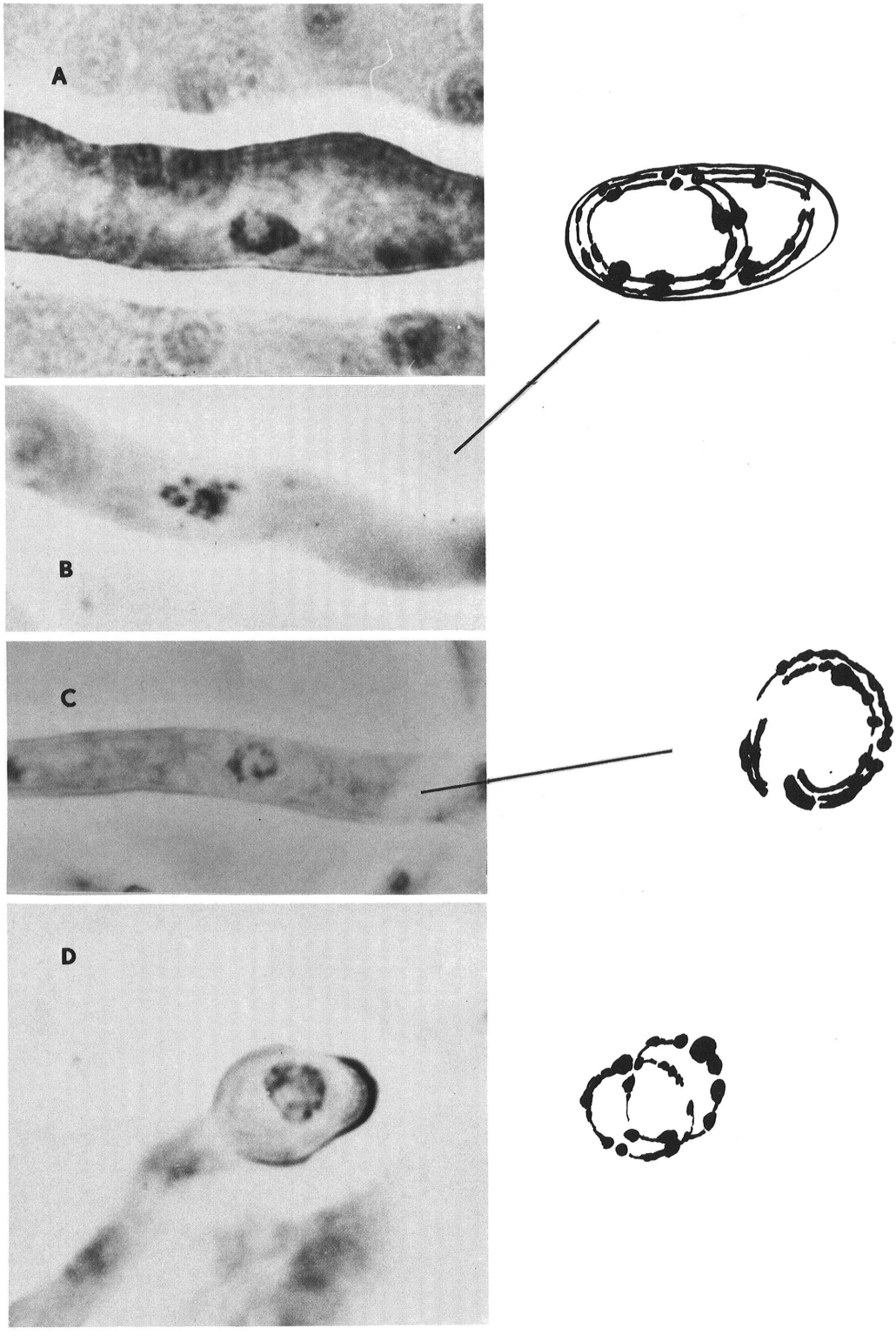

Fig. 1(A to D). Diplotene-diakinesis figures in gametangia of *Pythium aphanidermatum*. **A and B)** Antheridia ×1,600; **C)** oogonium ×2,000; and **D)** squashed oogonium ×4,800.

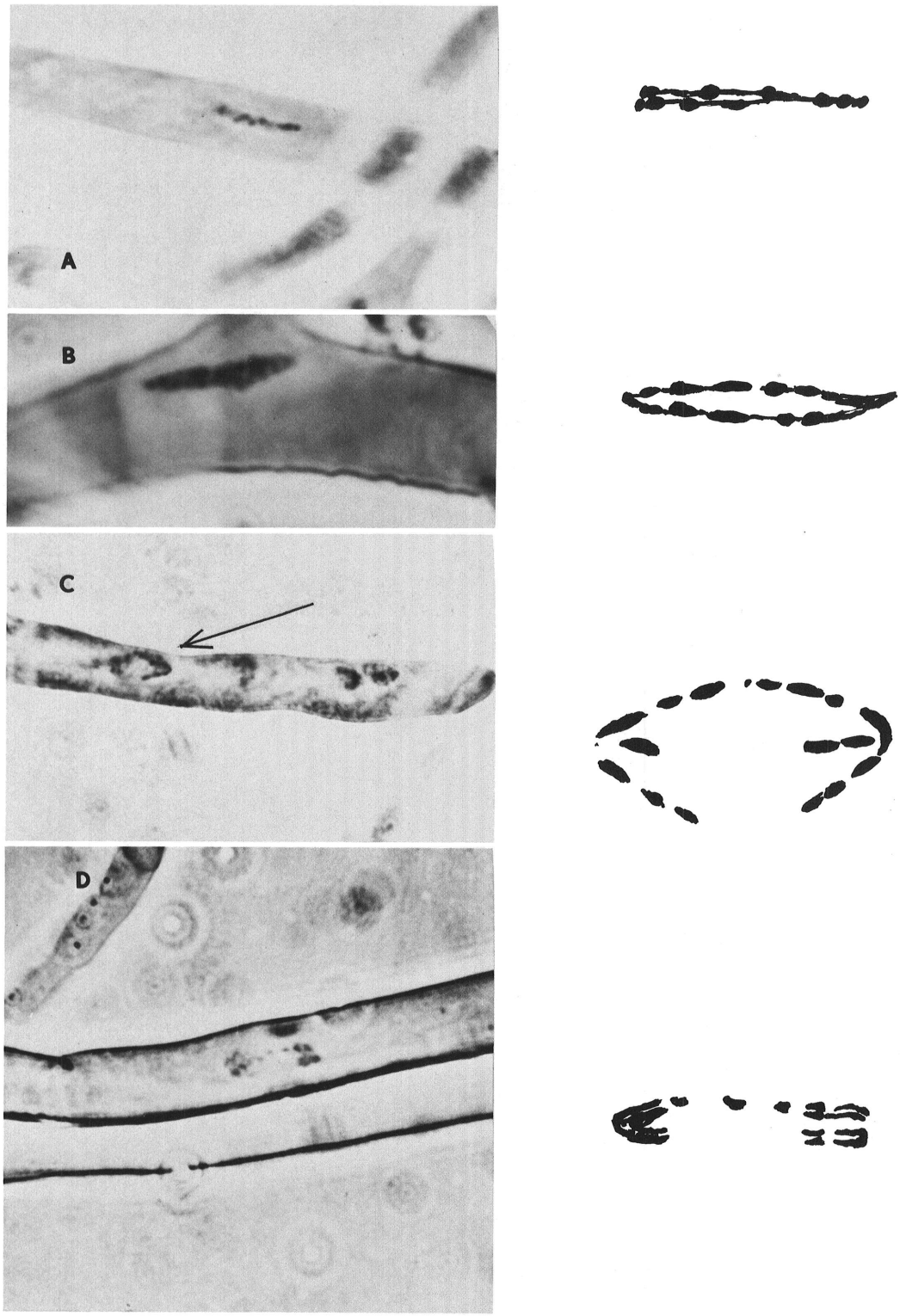

Fig. 2(A to D). Early hyphal nuclear division figures in *Pythium aphanidermatum*. **A)** Interphase nucleus with nucleolus ×2,800; **B)** track stage-double beaded filament ×1,800; **C)** filament oriented in ring ×1,500; and **D)** rings separating as division proceeds ×1,800.

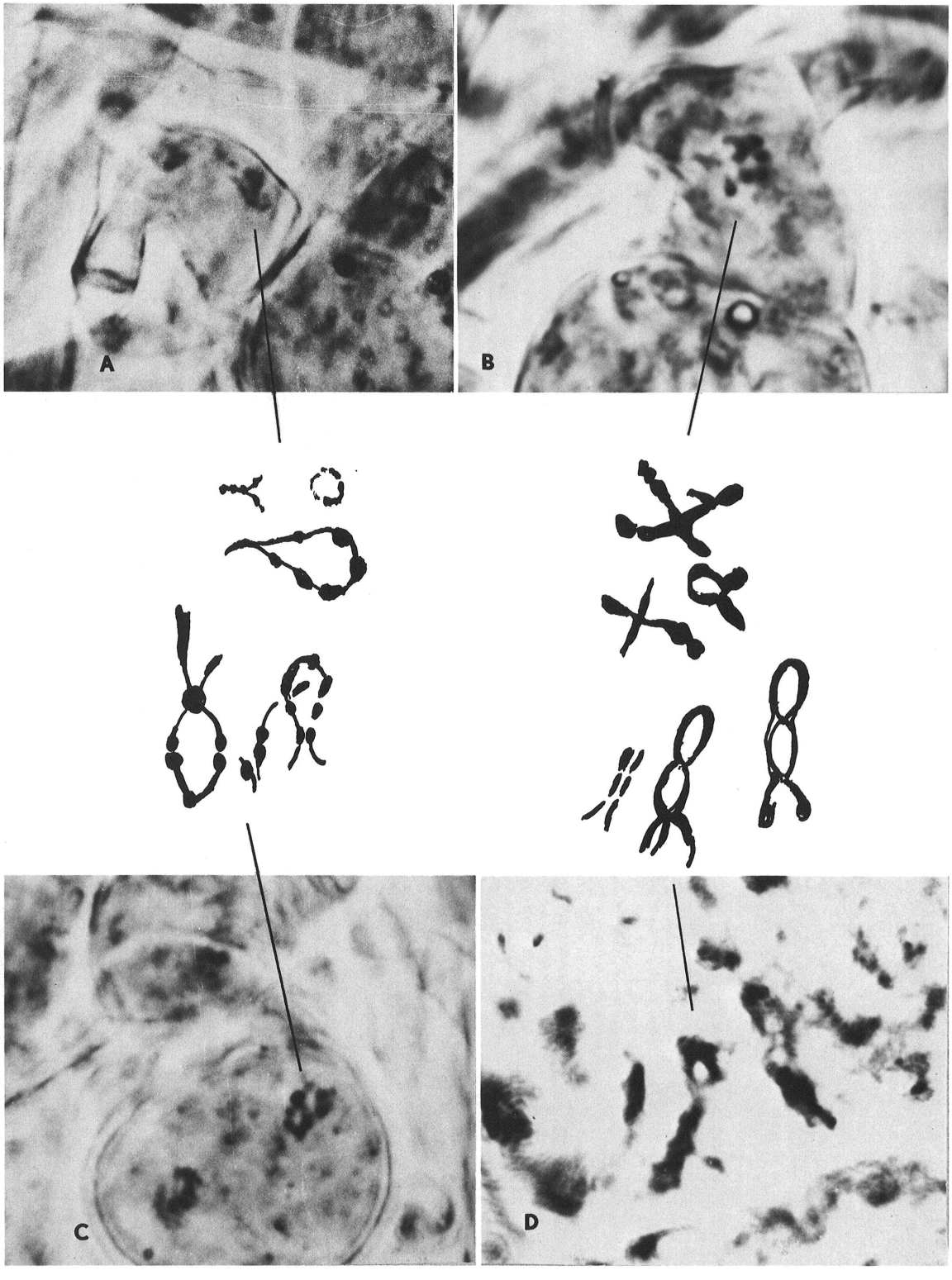

Fig. 3(A to D). Late hyphal nuclear division figures in *Pythium aphanidermatum*. **A)** Filament metaphase ×1,800; **B)** early anaphase ×3,400; **C)** late anaphase with "bridge-like" connections ×1,300; and **D)** early telophase with lagging chromatin bead ×1,300.

After clearing, the mycelium was rinsed in 45% acetic acid and mounted on a glass slide in the rinse solution or in water to improve contrast.

The Feulgen technique (4) and the carmine stain (42) gave equivalent results to the orcein stain.

Genetics.—A mutant of *P. aphanidermatum*, resistant to chloramphenicol (chloromycetin, Parke-Davis, Los Angeles, CA 90026) was obtained by treating 1 ml of ingested oospores (approximately 10^7 oospores) with 8 ml of 0.12 M ethyl methansulfonate (EMS) (Sigma Chemical Co., St. Louis, MO 63103) in sterile tap water for 2 hr at 22 C. The oospores were then washed by centrifugation. The supernatant liquid was discarded and the oospores transferred to a flask containing 200 ml of 10% V-8 juice and 600 μg chloramphenicol/ml and incubated at 22 C on a shaker. After 5 days of incubation, individual colonies (10-20 survived from 10^7 oospores) were transferred and tested for growth on corn meal agar (CMA) containing 600 μg chloramphenicol/ml. One of these mutants was selected and used in this study.

The chloramphenicol-resistant isolate was grown in liquid culture (deep petri plate with 30 ml water and four rolled oat flakes) for oospore production. Two 3-wk-old mycelial mats were removed, rinsed, and fed to water snails (41). Excreted oospores were washed in sterile distilled water, spread onto CMA plates and incubated at 35 C for 4-12 hr. Forty-one single germinating oospores, randomly selected from the population which showed a 94% oospore germination rate, were transferred to V-8 juice agar slants (10% Campbell's V-8 juice and 2% agar). Subcultures from such slants, as well as the parent isolate, were transferred to the testing medium. The testing medium contained 1,800 μg chloramphenicol/ml of CMA in order to clearly distinguish between chloramphenicol-tolerant and -sensitive isolates. The tolerant isolates grew 40 mm, whereas the sensitive (parent) isolate grew 0-5 mm in 100 hr, respectively.

Zoospores also were sampled randomly from the chloramphenicol-tolerant and -sensitive isolates. Zoospores were produced by transferring 5-mm diameter mycelial plugs, cut from the advancing margin of colonies growing on 10% V-8 juice agar, to sterile water contained in petri dishes. After 4-8 hr incubation at 22 C, zoopores were liberated and spread on water agar and isolated. Subcultures were made and transferred to the testing medium where growth was compared.

RESULTS

Gametangial cytology.—The general pattern of nuclear behavior in the gametangia of *P. aphanidermatum* agreed with that reported for related organisms (8). Many nuclei entered the developing oogonium until a septum was formed. Attachment of the antheridium occurred when there were approximately six nuclei in the oogonium and one to four nuclei in the antheridium. One-to-several meiotic events in each gametangium produced the gamete nuclei. A fertilization tube allowed a gamete to pass into the oogonium. Karyogamy was not observed because of stain clearing difficulties in the developing oospore. Several nuclei did remain within the antheridia and from six-to-eight nuclei were seen degenerating external to the developing oospore wall.

Diplotene-diakinesis figures were consistently observed in both developing antheridia (Fig. 1-A, B) and oogonia (Fig. 1-C, D). The synapsed chromosomes were in groups of three within the gametangia. This observation supports a diploid life cycle with a chromosome number of six.

Somatic cytology.—The interphase somatic nucleus (Fig. 2-A) was roughly spherical in shape and contained faintly visible threads of chromatin and a large Feulgen-negative nucleolus.

Nuclear division began with a thickening of the chromosomes until the nucleus contained tangled, double, parallel strands–the *track* stage (Fig. 2-B) (7, 12, 31).

Prophase contraction continued until metaphase was reached. The chromosomes then appeared as tiny (0.1 μm) beads attached to the membrane along two thirds of the equator. The appearance of the chromatin was probably determined by the space limitations as well as deformations caused by forces of cytoplasmic streaming or by independent nuclear motility forces (46). The latter forces are believed to originate in the microtubules that pass from the cytoplasm to the nucleus. In hyphae of wide diameter (~ 6 μm), antheridia, or oogonia, a ring (Fig. 2-C, D) [the "Saturn ring" of Robinow (30)] was observed from a polar view of the metaphase nucleus.

In the narrow confines of the hyphae, the metaphase nucleus resembled an oblong filament (Fig. 3-A). This "stretching" was caused, perhaps externally, by tension forces from the microtubules (16). The filament was seen here as a projection of the equatorial belt of chromatin. The stretched nucleus is approximately 7 μm long and resembled the beaded filament of Dowding and Weijer (11). Additionally, the metaphase nucleus sometimes appeared spherical and packed with heavily-staining chromatin beads.

Anaphase separation (Fig. 3-B, C, D) was probably of very short duration since it was rarely observed in stained material. This is in agreement with in vivo observations of *Fusarium* mitotic divisions (1). An anaphase duration of 13 sec out of a total mitotic duration of 5.5 min was reported. The separation began in a direction perpendicular to the hyphal axis (Fig. 3-B). Rotation of the nucleus (2) resulted in late anaphase separation directed parallel to the hyphal axis. The chromatin did not always separate unilaterally; occasionally "bridge like" connections occurred between daughter groups of chromatin (Fig. 3-Ç, D). These connections were evident in the division of filaments and correspond to the broken filament ends (Fig. 3-D), which were seen prior to separation (Fig. 3-C). The rings divided as complete double rings or crescents, without "lagging" chromatin material (Fig. 2-D) (45).

Genetics.—Oospore progeny of the chloramphenicol-tolerant isolate showed a segregation of six sensitive:35 tolerant in two trials (3:16 and 3:19). Zoospores were isolated to determine if this segregation resulted from segregation of a heterokaryon. No segregation was obtained in 51 single-zoospore isolates obtained from the chloramphenicol-tolerant isolate.

In order to expand these data, a second oospore generation was tested for segregation. This failed because of the almost complete oospore abortion found in 41

single oospore colonies. This loss of second generation fertility also was reported, although to a lesser extent, by Khaki and Shaw (21) and Castro and Zentmyer (5).

DISCUSSION

Genetic and cytological evidence both suggest diploid nuclei in the mycelium of *P. aphanidermatum*.

The diplotene-diakinesis stages observed in the gametangia pinpoint the location of meiosis just prior to plasmogamy. The association of these paired chromosomes in groups of three within the gametangia was evidence of six diploid chromosomes. The evidence for the meiotic character of the gametangial divisions was their larger size, the recognition of the distinctive diplotene-diakinesis figures, and the absence of similar figures in the hyphae.

The meiotic chromosomes were large; being about as long as the diameter of the entire somatic nucleus. In *Neurospora*, the tiny bead-like somatic chromosomes were about 0.1 - 0.3 μm whereas each of the seven bivalents at diakinesis was approximately 3.3 μm long (40). Although the somatic nuclei could be found with looping filaments and some with loops apparently crossing each other, the bivalents at diakinesis had two arms which looped and intersected at chiasma points and usually had two unpaired arms extending beyond this chiasma. Similar meiotic configurations in other oomycetes have been observed (3, 38).

Stained fungal chromatin generally exhibits the following mitotic figures which differ from mitosis of higher plants and animals (7, 11, 28, 29, 31): (i) apparent lack of a metaphase plate; (ii) nuclear membrane continuity throughout division; (iii) apparent absence of a well developed spindle; (iv) apparent beaded chains of chromatin in the form of filaments; rings and crescents; and (v) the track or double-filament stage.

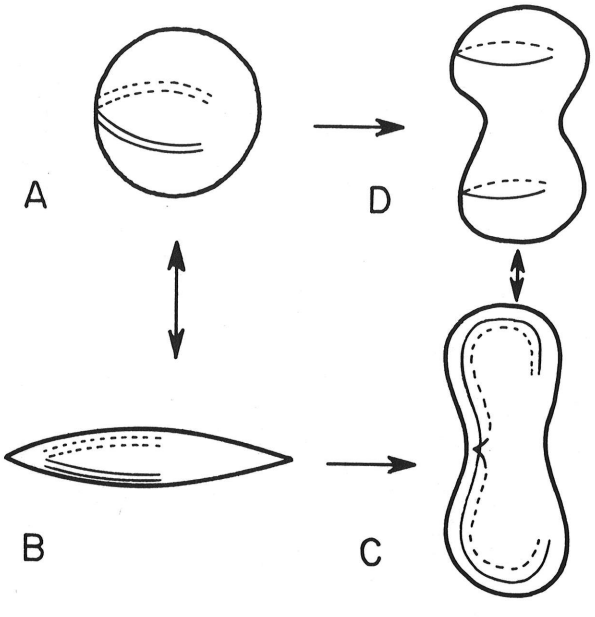

Fig. 4(A to D). Schematic diagram of the membrane-chromatin attachment model of fungal mitosis. **A)** Ring nuclear division of metaphase; **B)** filamentous nuclear division of metaphase; **C)** filamentous anaphase with apparent chromatin bridge; and **D)** late anaphase, common to both modes of division, with two separating daughter groups of chromatin.

Currently, there are two contrasting models of somatic nuclear divisions in fungi. One involves chromosomes linked in chains (10, 11, 12, 23, 26); the other is the classical scheme of independent chromosome segregation driven by a simple spindle apparatus (1, 15). The latter has been demonstrated in certain fungi by electron microscopy. These spindles seem to range from a single parallel band in *Saccharomyces* (32) and *Shizosaccharomyces* (25) to increasingly complex arrangements in *Saprolegnia* (16), *Fusarium* (1), *Phytophthora* (17), and *Catenaria* (20). Until more data are available by electron microscopy, the presence of a mitotic spindle cannot be assumed for all genera of fungi.

A new model, termed the membrane-chromatin attachment model, is proposed here which takes into account both of these models (Fig. 4). The new model relies on the membrane remaining intact during division and can explain chromosome segregation in the absence of a spindle apparatus. Other fungi with somatic chromatin figures similar to those observed in *P. aphanidermatum* also may conform to this model (2, 46). The mechanism of genome separation in the fungi without spindles could be due to differential membrane growth between the attachment points as suggested for bacteria and yeast (25), or a floating of attachment points on the membrane, as envisioned in the fluid mosaic membrane model (39).

Alternatively, the mechanism of genome separation could be similar to that of *Saccharomyces* (25). In this case, the formation and elongation of a single central band of spindles causes separation of the nuclear poles. This could also produce the anaphase figures seen in *P. aphanidermatum* (Fig. 3) as well as the separating filaments.

In higher organisms there are many reports of chromatin-membrane attachment and the concept is becoming increasingly popular in the literature (6). Permanent chromosome attachment to the nuclear membrane is well established in bacteria. The points of attachment function as DNA replication sites and probably in genome separation (6). The attachment seems permanent in *Gossypium* and it may have structural function as well as an involvement in DNA synthesis (6). Electron micrographs clearly show the attachment to the membrane of the permanently-condensed chromosomes of *Gynodinium cohnii* (22).

Nonrandom segregation of labeled DNA in bacteria (24), and in *Aspergillus* (33), provide further evidence of permanent association of parental DNA. An organelle which segregates along with the DNA – a kinetochore or possibly the nuclear membrane – could carry the parental DNA to one pole while the replicated (nonlabeled) DNA goes to the other.

Oospore segregation from the chloramphenicol-tolerant isolate can be explained with a diploid life cycle and a dominant mutation to chloramphenicol-tolerance. The wild-type recessive genes were carried in the diploid hyphal nuclei and produced the chloramphenicol-sensitive oospore segregants when two recessive gametes united. In *Aspergillus* (44) and *Phytophthora* (21), drug

resistance is frequently dominant. Zoospores obtained from the chloramphenicol-tolerant isolate produced colonies that failed to segregate, indicating that each hyphal nucleus was at least heterozygous, if not homozygous, for chloramphenicol-tolerance.

The expected segregation ratio of the oospores for chloramphenicol tolerance was 1:3, not the observed 1:5.83. The observed ratio could be produced if some nuclei were homozygous for the mutation and some heterozygous. The mutant chloramphenicol-resistant parents of the heterothallic *Phytophthora* used by Khaki and Shaw (21) were completely homozygous. They suggested a semi-dominant resistance allele which allows selection for the homozygous nuclei (of spontaneous or parasexual origin) because of their increased drug resistance over that of the heterozygous nuclei. The ratio observed here (1:5.83) could be produced by a parent with approximately one homozygous nucleus to three heterozygous ones.

Alternatively, a 1:5 segregation ratio could occur by fusion of the heterozygous gametangial nuclei thereby producing a tetraploid oospore (AAaa). Meiosis then occurs in the oospore and all but one of the nuclei degenerate. If this occurs randomly, a duplex segregation ratio of 1:5 will result. This explanation would change the location of meiosis, but would not change the diploid nature of the hyphae.

If chloramphenicol-tolerance is controlled by a single nuclear gene, then the recovery of the wild type from the mutant is incompatible with a haploid life cycle. Previously, genetic studies of *Phytophthora* (14, 18) have tended to support a haploid life cycle. The major criticism of previous work has been centered on the very low germination of the oospores, usually from 0.1% - 10%. The use of snail-ingested oospores of *Pythium aphanidermatum* increased germination to 94% and also eliminated propagules such as oogonia, antheridia, and hyphae. Two recent studies that do support a diploid life cycle in *Phytophthora* both had a high percentage oospore germination (13, 21).

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