# MORPHOGENESIS, NUCLEAR HISTORY, AND ULTRASTRUCTURE OF UROCYSTIS COLCHICI

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# ABSTRACT

# MORPHOGENESIS, NUCLEAR HISTORY, AND ULTRASTRUCTURE OF UROCYSTIS COLCHICI

By

Randolph Leroy Grayson

Development and ultrastructure of Urocystis colchici (Schlecht.) Rabenh. (=U. cepulae Frost) was studied in infected onion seedlings. The objectives of this study of U. colchici were: (1) to study the histology of the spore from sporogenesis through germination; (2) to study the nuclear history of the spore; and (3) to establish the genesis of the appendage cells and to discover, if possible, their function. The results of light and electron microscope techniques were compared using stained fresh, paraffin-embedded, or resin-embedded material from 12 to 16-day-old infected onion seedlings grown in artificially infested soil in the greenhouse.

Teliospores developed from the terminal cells of sporogenous hyphal branches which curved back upon themselves. Thus, the terminal cell in the center of the hyphal coil formed from the curving process became the central spore and surrounding hyphal cells became appendage cells of the teliospore. Both appendage cells and central

spores of young teliospores were dikaryotic initially, but central spores underwent karyogamy and became diploid before maturity. Appendage cells usually became mononucleate by apparent disintegration of one of the nuclei. Nucleoli were evident on both haploid and diploid nuclei.

Young central spores contained a ribosome-rich rough endoplasmic reticulum, numerous mitochondria, vacuoles, and few lipid bodies. As the central spores approached maturity, the endoplasmic reticulum became more granular and lipid bodies rapidly increased in number and size. In mature teliospores, the diploid nucleus was forced into a non-spherical lobed shape by the large lipid bodies. Central spore walls became differentiated into an electron-transparent inner layer, an electron-dense middle layer, and an electron-transparent outer layer.

Appendage cells had all normal cell constituents, but few lipid bodies. Appendage cell walls had only two distinguishable layers, and were separated from the central spore wall by an amorphous matrix.

Teliospores were aseptically removed from sori-containing surface-sterilized leaves of eight-week-old onions, suspended in sterile distilled water, spore clumps broken up by mechanical agitation, and the spore placed on malt extract phytone agar. Only appendage cells germinated under these conditions, usually one per teliospore. The exact roles of the central spore and appendage cells in pathogenesis remain to be elucidated.

# MORPHOGENESIS, NUCLEAR HISTORY,
# AND ULTRASTRUCTURE OF *UROCYSTIS COLCHICI*

By

Randolph Leroy Grayson

A THESIS

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# DEDICATION

This thesis is dedicated to Ben and Lavinia Grayson (my parents), Davetta and Wanda (my children), to Joan (my wife and inspiration) and to God who makes all things possible.

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# INTRODUCTION

The smut fungi cause diseases of economic importance in numerous angiospern hosts. In the family Gramineae, for example, millions of dollars worth of losses occur in cereal crops each year due to smuts. The term smut arose from the masses of dark powdery teliospores formed in sori (lesions) on the stems, leaves, or in the flowers of the host plants.

Throughout man's history there are references to famine and food shortages due to crop failures. Tillet (45) in 1755 succeeded in establishing that the disease called bunt of wheat resulted from contamination of seed with bunt fungus spores. However, Tillet erroneously concluded that the "dust" (spores) acted as a transmitting agent for a "poisonous entity" which infected the plant.

Prévost (35) in 1807 demonstrated a host-parasite relationship between the smut fungus and the wheat plant. Prévost was the first man to conceive the idea that the wheat bunt disease was a result of an interaction between a fungus pathogen and a host. This great achievement was not recognized until 28 years after his death in 1918. Both Tillet and Prévost clearly established that chemical treatment of wheat seed prior to planting inhibited development of the disease.

In 1853 de Bary published a classic comprehensive paper on the fungi, including the rusts and smuts. De Bary became a giant in the field of mycology and plant pathology, and students from all over the world came to his laboratory to study. Many of these students returned to their own countries and became important leaders in the field of modern plant pathology. One of these was Brefeld (1839-1925) who investigated the smut fungi and is credited for doing more than any other investigator in working out the life cycles of the cereal smuts (46).

The onion smut fungus, _Urocystis colchici_ (Schlecht.) Rabenh. (=U. cepulae Frost) belongs to the class Basidiomycetes, order Ustilaginales, and family Ustilaginaceae. This soil-borne plant pathogen was first reported in the Connecticut River Valley in 1869 (46). Infection of the onion seedling occurs in the cotyledon before it emerges above ground. The mycelium grows intercellulary, parallel to the vascular system of the host, without the formation of haustoria. During sporogenesis a sorus develops that pushes the host cells apart. As the sorus develops and the teliospores mature, the host cells disintegrate (46).

The teliospore is usually composed of a single, dark-pigmented, thick-walled central spore to which is attached an indefinite number of thinner walled appendage cells which have also been called nurse cells, sterile cells, or pseudospores (2,6,15,47). This type of spore morphology is characteristic of the genus *Urocystis*.

The pathogen cannot be easily eradicated from soil because of the longevity of the fungal spores. The disease is not important in the fall when the soil temperature is above 25° C. which inhibits pathogenesis (12,46,47). Since the common onion is not resistant to smut, it has been necessary to use chemical control in the onion growing areas of the northern states. The fungicide used must inhibit *U. colchici* until the onion plant develops beyond the three week susceptible period.

The few previous cytological investigations of *U. colchici* have all been accomplished with the light microscope. The limitations of light microscopy, the size, morphology, and pigmentation of the spore, and the lack of certain staining techniques led to some confusion among earlier investigators regarding the cytology of this fungus (2,6,43,49). These differences of opinion among the investigators concerned sporogenesis, nuclear size, nuclear condition of the spore, and its germination in culture. The only internal structure of the spore that these investigators identified was the nucleus.

Little attention has been given to the correlation of light and electron microscopy upon tissues fixed in the same or similar wasy. In this study an attempt was made to correlate the results obtained with light microscopy to those obtained in electron microscopy.

The objectives of this investigation of *Urocystis colchici* were: (1) to study the histology of the spores from sporogenesis through germination; (2) to study the nuclear history of the spore; and (3) to establish the genesis of the appendage cells and to discover, if possible, their function.

# LITERATURE REVIEW

The earliest paper dealing with sporogenesis and spore germination of Urocystis colchici was that of Thaxter (43). He examined thin sections of infected onions under the microscope and concluded that the spore ball originated from two or more lateral branches arising from the main hypha. He proposed that one branch gave rise to the main spore, whereas the pseudospores (also called nurse cells, acessory cells, sterile cells, or appendage cells) originated from the other branch, or branches, which surrounded it.

Thaxter (43) studied germinating spores utilizing several methods which were not clearly described. In one procedure, six month old smutted onions were mixed with moist soil and frozen for a week or more. This was followed by removal of the onions from the soil to a warm room where Thaxter said that teliospores germinated on the moist onion material. Thaxter reported that spores usually produced a single germ tube that often branched, terminating in production of sporidia (secondary spores). Spores treated similarly were also placed in Van Tieghem cells containing onion decoction, where germ tubes branched and were reported to produce sporidia larger in size than those in the frozen and thawed onion material. These sporidia were reported to germinate in water or nutrient solutions; however, further observations were impossible because of bacterial contamination.

Thaxter (43) further stated that sporidia were not produced by fresh teliospores from leaf sori placed in onion decoctions. These spores, in all cases, yielded only abundant mycelia. Also, hyphae in the early stages of sporogenesis taken from the living onion ceased spore development and continued as sterile mycelia.

In 1921 Anderson (2) described the development and pathogensis of Urocystis colchici, duplicating Thaxter's work and extending the investigation into new areas. Infected onion seedlings were surfaced-sterlized with 0.1% mercuric chloride followed by a sterile water wash before the lesions were opened, since all efforts to secure germination by Thaxter's method resulted in contamination of cultures. Spore germination was studied using fresh spores in a variety of solutions such as water, soil water with germinating onion seeds, dung decoction, onion decoction, and sugar solutions. In all of the above experiments, spores from mature lesions were put into a drop of water or solution on a slide kept in a petri dish moist chamber. Spore germination occurred in onion decoction, sugar solutions, and in one test next to a young cotyledon (2). Sterile conditions had to be maintained when using onion decoction, as it was easily contaminated by fungi and bacteria, that soon overran the slowly germinating smut spores.

Anderson (2) reported that an unstated percentage of fresh spores in 0.5-10% sugar solutions germinated at all sugar concentrations. The highest percent of germination was obtained in the 2% solution where 50% of the spores on the surface of the drops germinated. From these results Anderson concluded that oxygen as well as the presence of sugar was an important factor in germination.

Similar germination experiments were carried out using various types of agar media. The pattern was the same except that spore germination was highest in onion decoction agar. Anderson (2) noted that germination was non-synchronous, beginning on the third day and extending for as long as three weeks. He also found that exposing spores to freezing temperatures was not a prerequisite for germination.

Anderson's (2) detailed observation of spore germination using whole mounts varied from that of Thaxter's (43). Germination was initiated by the appearance of a hyaline hemispherical vesicle similar in size to one of the sterile cells. Anderson's opinion was that the vesicle originated from the central fertile spore through a pore or rupture of the spore wall. This vesicle, called a promycelium produced hyphal branches. Septation occurred and individual branches were soon lost in a dense mycelial mass. The older hyphal cells near the original spore lost their cytoplasm as growth progressed, became constricted at the septa, giving a bead-like appearance, while the terminal cells maintained their cytoplasm. Anderson (2) did not observe sporidial production from germinating spores contrary to Thaxter's (43) report.

Teliospores arose from dense mycelial masses growing between mesophyll cells parallel to the vascular bundles in infected onion seedlings according to Anderson (2). The spores began as lateral or terminal branches which curved back on themselves in a spiraling manner. The terminal cells probably became the central cell of the teliospore. This cell enlarged rapidly while the surrounding cells united with it and became appendage cells.

Anderson (2) reported that the central cell of each mature teliospore contained a single nucleus 3-4um in diameter with a nucleolus 0.6um in diameter. A single nucleus was reported in each nurse cell. The central cell of the teliospore was assumed to be viable and the attached appendage sterile.

Whitehead (49) in 1921 investigated germination of Urocystis colchici teliospores. All of Whitehead's attempts to induce germination of freshly collected teliospores failed. Spores from month-old dried onion leaves, subjected to freezing conditions at $-25^0$ C. for one day germinated to an unstated percent, as did sixteen-month-old air dried teliospore without the preliminary freezing step. Whitehead stated that teliospores were obtained in an uncontaminated state from sixteen-month-old dried plants by first immersing infected excised onion leaves into a solution of 0.1% mercuric chloride for five minutes and rinsing in sterilized distilled water. One, or occasionally two germ tubes arose from some spores incubated in water. The germ tubes ceased to grow after reaching a length of 40-50um. Whitehead stated that minute ovate sporidia developed laterally upon the germ tubes (49). He also reported that rapid spore germination occurred in onion decoction and colonies were formed within forty-eight hours (49).

Sporogenesis was studied by fixing infected seedlings in Carnoy's solution (49). Thick sections of 12-18um were stained with lactophenol blue and observed. Whitehead throught that the central spore arose from hyphae with swollen terminal ends. In his view the spore contained a single large nucleus, which increased in size until the surrounding hyphae became attached to the central spore. The walls of the appendage cells then thickened slightly and apparently lost their cytoplasmic content.

An investigation of the nuclear history of <u>Urocystis colchici</u> was carried out by Blizzard in 1926 (6). Onion seedlings in various stages of development were fixed in Fleming's weak chrom-osmic solution, embedded in paraffin, sectioned 3 to 5 u thick, and stained with Flemming's triple stain or iron-alum haemtoxylin. The former was the most effective stain. The dikaryotic condition of the parasitic mycelia and spore initials were observed by Blizzard (6) but he did not detect karyogamy due to the small size of the nuclei. After karyogamy presumably occurred, the central cell enlarged rapidly and came into contact with binucleate cells which attached and became the appendage cells. These cells were presumed to be sterile and to provide food for the main spore. Which binucleate cell would become the central spore was thought to be determined by the position of that cell in relationship to food supply during sporogenesis. Hyphae were mostly mononucleate at some distance from the sorus primordium, but became progressively more binucleate as they approached the sorus primordium. No haustoria were observed. Cell fusions between mononucleate hyphae which commonly lead to the dikaryotic condition were not observed.

For the cytological study of germinating spores, Blizzard used a method proposed by Harper (6). Blizzard's observations generally confirmed those of Anderson (2). Attempts to study the nuclear condition within germinating spores were unsuccessful due to spore wall pigmentation and interference of surrounding accessory spores. No nucleus could be recognized even in bleached, haemotoxylin-stained germinating spores. Blizzard assumed that the main spore nucleus went through meiosis prior to or during germination.

He thought nuclei migrated through the promycelium with one nucleus going into each promycelial branch, forming uninucleate hyphal cells in culture.

Evans (15) made a cytological study of the host-parasite relationship between *U. colchici* and young onion seedlings approaching immunity to infection. Neither appressoria nor haustoria were observed. Immunity was indicated by the inability of the fungus to penetrate deeper than the third sub-epidermal layer of cells. No new information on fungal cytology came from this study.

Tachibana and Duran (42) studied sexuality in *U. colchici*. Teliospores from smutted onions were placed on potato-sucrose agar. Germinating teliospores were moved individually by micromanipulation to individual agar blocks. Surface-sterilized seeds were inoculated with mycleia of single spore lines alone or in pairs. Of 100 single spore clones tested individually, only four gave infection of onion seedlings. When 10 clones were tried in all possible paired combinations, only certain pairs of clones resulted in infected seedlings. The authors concluded that teliospores gave rise to two mating types of mycelia which were not pathogenic until fusion occurred between mycelia of opposite type. This fusion was assumed to occur prior to, or immediately after, penetration into the host. Why teliospores of *U. colchici* functioned as unisexual entities, in contrast to other smut fungi whose sporidia contain nuclei of both mating types resulting from meiosis of a diploid nucleus could not be explained. Attempts to obtain dikaryotic mycelia by mating compatible single spore lines on agar media were unsuccessful.

Spore formation in the Ustilaginales in general and *U. colchici* in particular is a subject on which very little detailed information is available. Observations were made difficult by the tangled mass of mycelium, the small size of the teliospores, limited stain technology, and the resolution limits of light microscopy (2,6,43,49). No ultrastructural study of *U. colchici* has been reported. Only in recent years have attempts been made to study the ultrastructure of various stages of spore formation in other members of the Ustilaginales (18,29). These have been largely unsuccessful due to the nature of the material, limitations of histological techniques and inherent limitations of the electron microscope.

In the few studies done on different types of dormant thick-walled spores, investigators have used various methods in attempting to overcome the difficulties of fixation and infiltration of the embedding medium. They have included the use of devices to break spores open during fixation (14,40) or cracking them before fixation (18,31). In the present study, which employed thin sectioning techniques, no physical devices were used to facilitate the entry of fixatives or resins in an effort to avoid artifacts. Other researchers have studied spore surfaces using replica techniques (5,51) or scanning electron microscopy (19,25). These techniques did not yield information about internal morphology.

Freeze-etching has been used to study spore surfaces (23,24) as well as the internal structures of germinated and dormant spores (36,37). The advantage of freeze-etching is that no chemical treatment of the material occurs until the replica is formed on an "etched" fractured surface of the frozen specimen. Replicas consist of thin formvar or carbon films and a metal which corresponds exactly to the surface topgraphy of the specimen. The larger cell organelles are represented; freeze-etching will not give information on other structures such as the presence of ribosomes and differential staining of wall layers. Thin sections do provide this type of information (22).

Fine structure studies have their limitations. The amount of tissue from a population that one may examine in a working lifetime is infinitesimally small; thus the sampling problem is acute. In addition, under ideal conditions of tissue preparation, only the macromolecules are left in the specimen. Thus the architecture one observes represents only those structures which remain after fixation. The relationship, therefore, between the static image and the living dynamic cell is uncertain. This problem is basic to all histology.

Fungal tissues, including plant tissues, are more refractory than most vertebrate tissues. The hydrostatic pressure of the vacuoles, the thick hard walls, the low protein content of the cytoplasm in some cells, and the rapid change in the chemical composition and density of the material pose problems in all stages of fungal specimen preparation (9,28,32).

The more refactory nature of plant material demands variations in the methods of preparation. For example, material fixed in permanganate is exceptionally diagrammatic for membranes of the various organelles. Almost no detail can be seen in the cell wall, nucleus, or nucleolus. The stoma material, the ribosomes, and the bulk of the ground substances are not preserved (20). If it is difficult to attempt to relate structure to function when all of the major macromolecular structures are preserved within the cell, surely it is much more so when the bulk of these materials have been extracted or rendered unrecognizable. Similar considerations must be taken into account where other fixatives are used alone. Thus, one should compare various methods of fixation and staining before arriving at conclusions (16).

# METHODS AND MATERIALS

## Source of Smutted Onions

Onion seeds were planted in the greenhouse in flats containing steamed soil infested with field-collected teliospores. Infested seedlings at different stages of development were available at all times. Twelve to 16 day-old infected seedlings were collected for a portion of this study. Teliospores for germination studies were collected according to the method of Lacy (30).

Four-day old spores that appeared to be ready to germinate, or were beginning to germinate were removed, fixed in glutaraldehyde-acrolein and post-fixed in $OsO_4$ as described under the electron microscopy section.

## Light Microscopy

### Fixation

Sections of infected onion seedling containing sori were cut in ¼ inch lengths on top of a cold glass petri dish in a drop of 0.2M sodium cacodylate buffer (pH.7.2). These were immediately transferred to shell vials with caps containing modified CRAF (Navashins) solution (26), 3% glutaraldehyde-acrolein (21), FAA (26,27), or Carnoy's solution (26). Modified CRAF was prepared according to

Jensen (26) except for formalin being replaced by an equivalent amount of glutaraldehyde. Fresh material to be examined by the squash method was fixed in Carnoy's solution only. The rate of penetration of the fixatives were increased by placing the vials containing the tissues in a desiccator under reduced pressure. The tissues remained in the fixing preparations from 4 hours to overnight except for the fresh material which remained for 10 minutes in Carnoy's solution.

### Dehydration

Following a 2 hour rinse in water, dehydration was carried out in the same vials using an ethyl; tertiary-butyl alcohol series described by Johansen (27).

### Infiltration and Embedding

Methods of embedding plant material have been described (27). Briefly, specimens were placed in equal amounts of paraffin oil and tertiary-butyl alcohol (TBA) for 2 hours. The specimens in the TBA-oil mixture were placed in another vial 3/4 full of Bioloid semi-solid wax. This was put onto an oven (58-60° C) until the wax melted and specimens settled to the bottom. The mixture was poured off and pure paraffin added. The specimens were left for 16 hours in pure paraffin with several changes made. The entire contents of the vial were poured into aluminum dishes on a warming table (60°C). Then the specimens were floated on ice water until the paraffin congealed, then were immersed and left for 20 minutes. The dishes were moved, put into dust-free boxes and stored in the refrigerator until needed.

## Mounting and Sectioning

Paraffin-embedded were mounted on wooden (pre-paraffin soaked) blocks and sections (4-6 um thick) were cut on a rotary microtome. The ribbons were placed on slides coated with Haupts adjesive and partially flooded with 4% formalin solution. The slides were placed on a warming table (40°C) to expand the sections, dried and stored in dust-free boxes until examined.

## Stains and Tests

All stains used in this study have an affinity for nuclear materials. Two of the stains react with aldehydes produced by hydrolysis prior to the staining step. The stains used were Mayer's hematoxylin (27), acetocarmine (26), Pianexe 3b (38). Feulgen stain (10), and periodic acid-Schiff's reaction (PAS) (26). The staining schedules can be found in the references cited; however the best results were obtained when the materials were left in the stain for the maximum suggested time.

Removal of DNA and RNA by perchloric acid and the enzyme methods (26) were utilized to confirm the positive reactions of the stains with the nucleus of the spores.

A method for excellent nuclear staining of paraffin-infiltrated tissues, using acetocarmine, was developed in the following manner. Sections were deparaffinized with xylol and brought down to water through a graded ethanol series. The slide was flooded with acetocarmine and heated over an alcohol lamp, a rusty needle was placed along the margin of the hot stain until it turned dark red, fresh stain was added and the slide warmed again. The slide was removed, more stain added if necessary, and allowed to stand for ½ hour. The slide was washed in cold water, a cover slip added and sections examined. Care was taken at all steps not to allow the stain to boil or evaporate from the sections.

Tissues that have been fixed in glutaraldehyde, acrolein, or both, were not left in periodic acid longer than three seconds when using the PAS method. Longer exposure results in the over-staining of the sections.

Thick sections of EM material, fixed in glutaraldehyde-acrolein, and post-fixed in $O_sO_4$, were examined in the light microscope for the purpose of orientation, comparison, and identification of structures. These sections were cut on an ultramicrotome, transferred to glass slides, and attached by heating the slide to 70° C until dry.

Test for lipid localization on embedded sectioned material was done using Sudan III (26).

## Preparation of Fresh Material

Fresh materials were prepared by modifying the procedure of McIntosh (33). Small pieces of onion tissue (2mm by 6mm) were run rapidly through a series of decreasing concentrations of ethanol to water. After hydrolyzing in 1N HCL for 5 minutes at room temperature and 5 minutes at 60° C in the oven, tissues were washed and treated with Feulgen stain for 1 hour or acetocarmine for ½ hour. The Feulgen stained material was rinsed in distilled water, placed in 2% sodium bisulfite for 2 minutes, rinsed for 5 minutes, and placed on a slide in a drop of water. A cover slip was added and pressed firmly. The tissues were examined immediately. The acetocarmine-stained tissues were mounted in a drop of stain on a slide, teased with an iron needle while the slide was being warmed, and squashed under a cover slip. The tissues were examined within ½ hour.

## Photography

Photomicrographs were made using Kodak high speed Ektachrome film (ASA 125) in a Nikon Microflex microscope fitted with a AFM photomicrographic attachment or a Zeiss microscope equipped with phase-contrast and Nomarski interference-contrast optics.

## Electron Microscopy

### Fixation

Electron microscope observations indicated the following procedure was best for fungal tissues. Fixation was carried out at 4° C beneath a desiccator under reduced air pressure. Sections 2mm or less in diameter were placed for 1 hour in 3% glutaraldehyde-acrolein in 0.2M sodium cacodylate buffer (pH 7.2) in capped vials. Tissues were transferred through several changes of the same buffer, diluted 1:1 with distilled water for 1 hour. Post-fixation was in 1% $OsO_4$ prepared by diluting aqueous 2% $OsO_4$ 1:1 in the same buffer for ½ hour.

### Dehydration

Tissues were given two rinses in cold buffer diluted 1:1 with distilled water at room temperature. They were then moved through a graded ethanol series (30-100%); followed by a graded ethanol-propylene oxide series (30-100%) with a minimum of ½ hour per change. Tissues were left in each solution for 2 hours with 2 changes per hour in the 100% level of the graded ethanol series and the ethanol-propylene oxide series.

### Infiltration and Embedding

Tissues were left for 24 hours in each of the infiltration steps and then were transferred through a graded propylene oxide-ERL epoxy resin series (30-100%). The vials were uncapped at the final step of the series to allow the evaporation of propylene oxide. The tissues were transferred to flat embedding molds containing fresh ERL, placed in a desiccator under reduced pressure for 24 hours and polymerized at 70° C for 24 hours. All work was done under reduced air pressure except for the final embedding.

Sectioning and Staining

Ultrathin sections were cut with a diamond knife on a Porter-Blum ultramicrotome, flattened with xylene vapor, picked up on formvar coated grids, and stained in an alcoholic solution of uranyl acetate for 30 minutes followed by aqueous lead citrate for 7 minutes. Sections were examined and photographed in a Philips 300 transmission electron microscope using Kodak cut film (3¼x4).

$KM_nO_4$-Treated Tissues

Tissues were fixed in buffered 3% $KM_nO_4$ for 15 minutes at room temperature. These were washed, dehydrated, and embedded similar to the aforementioned materials.

# RESULTS

## Light Microscopy

Young developing sori were observed in longitudinal and transverse sections of infected onion cotyledons (Fig. 1,2). The parasitic mycelia grew intercellularly parallel to the vascular tissues of the host. Numerous lateral branches terminated in masses of sporogenous hyphae from which the spores arose (Fig. 1). Various stages of sorus development, with mature spores clearly evident in sori in the more advanced stages, were observed (Fig. 1). Within individual sori, spores were found in different stages of development (Fig. 2). The host mesophyll cells were pushed apart as the sorus developed. *U. colchici* did not produce haustoria or invade the vascular bundles of the onion cotyledon.

Sporogenesis was studied in hand-sectioned fresh material and prepared material by observing hyphae on the edge of sori primodia. Typically, a lateral branch arose from the mycelium which grew parallel to the vascular tissues of the host (Fig. 3). The branch was 1-1.5um in diameter with abundant lipid bodies present. As the lateral branch continued its growth (Fig. 4) the terminal cell enlarged into a bulbous shape 2um in diameter (Fig. 5).

Spore initials measured 3.4um ± 0.5um in diameter and contained two nuclei, each about 1.0um in diameter (Fig. 6).

The sporogenous branch curved inwardly upon itself so that the subterminal cells were wrapped around the enlarged spore initial (Fig. 7-11). The terminal cell greatly increased in size as it continued to spiral inwardly. This hooking over ofthe terminal branches was called a crozier by Anderson (2). Monitor sections (Fig. 8,9) post-fixed in osmium tetroxide illustrated the rapid increase in size of the terminal cells and lipid bodies. This spiraling growth of sporogenous branches continued (Fig. 8-10) until a terminal cell lay in the center of the coil composed of sporogenous subterminal branch cells (Fig. 11). The terminal cells became the central spore measuring 10um or more in diameter at maturity. The appendage cells arose from sporogenous hyphae that surrounded the central spore (Fig. 8,9). These hyphae adhered to the central spore as it expanded (Fig. 10). The appendage cells became appressed to the central spore, and became rounded at the end, flat on the side attached to the central spore, and torulose (Fig. 11). The result was oval-shaped appendage cells measuring 3-4um in length and 1.5-3um in width containing one or more small lipid bodies (Fig. 12). The appendage cells were almost hyaline and thin walled.

Mature compound spores averaged 17um in diameter, based on 50 measurements, and were usually well covered by appendage cells, due to the branching of the sporogenous hyphae after adhering to the central spore. Teliospores were divided into four arbitrary groups based upon degree of maturity and wall pigmentation for purposes of discussion. In fresh unstained materials mature spores were darkly pigmented, relatively large, with well-defined lightly colored nurse cells. The juvenile spores were the same size or slightly smaller than mature spores and were tan-colored with well-defined hyaline nurse cells. Young spores were translucent, usually smaller than juvenile spores, with no apparent appendage cells or only a few ill-defined ones. Spore initials appeared as enlargements at the tips of hyaline coils of mycelia.

In prepared sectioned materials (6um thick), it was found that Feulgen stain would clearly differentiate the four groups of spores by its reaction with spore walls.

Mature spore walls were dark brown with at least three wall layers clearly distinguishable. Appendage cells were light brown to nearly hyaline.

Walls of juvenile spores were reddish-brown with one or more wall layers present. Appendage cells were slightly red to hyaline and well-defined.

Young spores were pink colored, irregular in shape, with a spore wall that was homogeneous to partly differentiated (Fig. 14). They had a wide variety of shapes and sizes within the sorus. In fact, the diameter of some of these young spores was 8um which equals or exceeds the diameter of many juvenile spores. Appendage cell primordia were pink-colored and wrapped around the central spore.

Spore initials appeared as coiled mycelia with a swollen terminal end in the center. These were light pink in color.

The two nuclei in young spores were initially separated and surrounded by cytoplasm (Fig. 14). Lipids were located around the margin of the spores in small amounts. Lipid formation increased as the nuclei approached each other (Fig. 15).

In the early juvenile stage, nuclei were lying close to each other, surrounded by lipid bodies (Fig. 16). The nuclei gradually moved closer together until they contacted each other in the center of the spores. The nucleoli in prekaryotic nuclei were clearly evident in some sections stained with hematoxylin (Fig. 17, 18). The nuclei became appressed to each other (Fig. 19, 20) and soon fused together (Fig. 21) as the lipid bodies continued to encroach upon the remaining cytoplasmic area. As the spore lipid content continued to increase, the lipid-free cytoplasmic area around the nucleus decreased (Fig. 22, 23) until the free cytoplasm was displaced by lipids and disappeared (Fig. 24). When this process was finished the spore was considered to be mature (Fig. 24, 25).

Appendage cells underwent rapid changes from the young spore stage until maturity (Fig. 16-22). They became oval in shape with one side flattened against the central cells. In those sections where the stain reaction indicated the presence of nuclei in appendage cells, it was difficult to discern if one or two nuclei were present because they were small and lay close together. However, at a magnification of 5000 the appearance of appendage cells strongly suggested that some were binucleate and others mononucleate (Fig. 22).

All nuclear stains used in this study gave similar results in size and position of nuclei though the staining intensity varied among them. Perchloric acid and DNA-RNA tests were positive for DNA.

Wide variations existed in nuclear size depending upon the angle of cut through the section. Measurements were based on the mean of the longitudinal and transverse axis of each nucleus. Based on numerous observations using an optical micrometer, it appeared that haploid nuclei were approximately 1-1.25um in diameter and diploid nuclei 2um ± .25um in diameter in central spore cells.

A number of changes were noted in external morphology of spores prior to germ tube emergence with the light microscope. The first observable change was that spores originally 17um size increased to as much as 22.5um in diameter. Some appendage cells became twice as long as normal, and swelled until they had the appearance of "glass bubbles" on the spores. It was impossible to determine whether the germ tube arose from the central spore or from an appendage cell using the light microscope. A swollen hyaline appendage cell was always proximate to the germ tube. Appendage cells detached from the central spore were observed to germinate (Fig. 26). The same event was also observed by Tachibana (41). Sporidia were not produced by *U. colchici* in culture.

## Electron Microscopy

Terminal cells of sporogenous lateral branches were binucleate with the normal complement of cell organelles (mitochondria, ribasomes, endoplasmic reticulum, vacuoles, lipid bodies) when viewed with the electron microscope (Fig. 27-29). The nuclei were slightly larger than lum (1.14-1.19um) and were surrounded by mitochondria which exhibited polymorphism (Fig. 27,28). The cytoplasmic vacuolar system was highly developed, consisting of granular endoplasmic reticulum packed with ribosomes and vacuoles (Fig. 17-29). The plasmalemma was located very close to the electron transparent hyphal walls (Fig. 27-29). A relatively small amount of lipid was present at this stage of spore development.

As previously mentioned, the sporogenous hyphae turned inward forming a crook, and the terminal cell assumed a bulbous shape (Fig. 29), and became a binucleate central spore. The central spore wall appeared to be covered with a gelatinous matrix (Fig. 30). Curving of the sporogenous branch resulted in a coil with the central spore in the center of the coil surrounded by the other hyphal cells of the sporogenous branch. The outer hyphae cells became appendage cell primordia (Fig. 31, 32). The spore endoplasmic reticulum became more granular and more densely packed with lipid bodies.

The nuclei of the central spore then approached each other (Fig. 33) in preparation for fusion (karyogomy) in the central area encircled by lipid bodies (Fig. 34). After karyogamy in the juvenile spore, the nucleus remained centrally located as the lipid bodies increased in size and number (Fig. 35-38). The diploid nucleus was larger in diameter than the pre-fusion nuclei and the nucleolus was prominent (Fig. 35). The nuclear membrane was a characteristic two unit membrane (Fig. 36, 37) and had nuclear pores (Fig. 39). Mitochondria were conspicuous at all stages of sporogenesis.

Lipid bodies appeared in thin sections after $KMNO_4$ fixation as oval and circular electron transparent structures and appeared to be bounded by a single membrane (Fig. 39). This view was supported by the fact that in places where lipid bodies were in contact they did not coalesce. Instead, bulging of one lipid body caused indention of another lipid body at the point of contact, indicating the presence of a membrane. Lipid bodies varied in size from less than 1um to greater than 5um in diameter.

Without benefit of serial sections of nuclei in spores of different ages, it was difficult to absolutely determine the size of the nuclei or nucleolus in central spores. By viewing a large number of sections, one may estimate nuclear size. The haploid nuclei were slightly larger than 1um each (Fig. 27, 30, 34) with a variation from .81um to 1.7um. The median measurement was 1.25um ± 0.05um. The median measurement for diploid nuclei was approximately 2.0um ± 0.3um in diameter (Fig. 35-39). The nucleolus was approximately 0.9um ± 0.1um in diameter (Fig. 35).

Spore wall layers started to become evident during the juvenile stage (Fig. 40) when they were 0.3um and thicker. When the spores reached maturity the walls were composed of three distinct layers about 0.8um thick, covered with a matrix about 0.17um thick beneath the nurse cells and about 0.3um thick between the nurse cells (Fig. 41). The matrix originated from the gelatinous layer surrounding the central spore during its formation (Fig. 30).

It was this matrix that attached the nurse cells to the central spore. The outer layer was almost electron-transparent and was about 0.17um thick, as was the central layer which was electron dense. The inner layer was about 0.5um thick and electron-transparent layers plus the matrix were lum thick. The layers became distinct in some late juvenile spores (Fig. 46-48).

Mature appendage cell walls were composed of two layers approximately 0.46um thick (Fig. 41).

Study of the nuclear condition of appendage cells confirmed the observation by light microscopy that some cells contained one or two nuclei, and others none. The most consistent pattern revealed by E. M. was for younger spores to have appendage cells with two nuclei (Fig. 42,43) or one (Fig. 44-46), and older spores to have appendage cells with one (Fig. 48) or none (Fig. 47).

Because $OsO_4$-fixed spore material was so densely packed with osmophilic cell constituents, $KMnO_4$-fixed material was examined for some indication of the reason for the nuclear pattern in mature appendage (nurse) cells. Areas were found that possibly represented disintegrating nuclei (Fig. 49,50). Measurements of these areas were the same size as appendage cell nuclei. It appeared that disintegration might account for the variation in nuclear condition.

Appendage cells, like the central spores, contained the other usual cell constituents in all stages of development. The basic differences between appendage cells and central spores, other than size, were the smaller amount of sotred lipids in nurse cells (Fig. 47), the two layered spore wall (Fig. 41, 45) and perhaps a change in the nuclear number through disintegration. Measurements of nuclear size varied according to the cut of the sections (Fig. 35, 43-46, 48). The largest diameters were lum ± 0.lum. A nucleolus was present on a number of nuclei (Fig. 35, 43, 45). The complete cytological development of appendage cells to maturity is evidence against the concept that they serve as "food" for the central spore (6) or represent "sterile, probably dead, appendages" (38).

Non-synchronous spore germination occurred on agar media, starting on the third and continuing through the seventh day. Therefore, at any one time only a relatively small percent of the spore population was germinating. In addition, an estimated 25% of the spores failed to germinate on malt extract-phytone agar (MEPA) and a much larger percent on potato sucrose agar (PSA).

Numerous thin sections of spores revealed that central spores failed to germinate but appendage cells located near the agar surface germinated. Other appendage cells in the same area appeared to be capable of germination (Fig. 51), as indicated by a new wall layer formed within the existing wall, well preserved cytoplasm, and presence of small bodies which may represent lipid. These appendage cells (Fig. 51) can be compared to other appendage cells with their disorganized cytotplasm which suggest cell death (11). Chemical studies (1,8,17) indicated that lipids and carbohydrates were the energy reserve consumed during germination and germ tube growth. It appeared reasonable to consider the small electron-transparent bodies as storage lipid in the form in which it enters catabolic metabolism (Fig. 51,52,53).

Some germinated appendage cells contained very granular endoplasmic reticulum packed with ribosomes. A prominent nucleus (2.4um in diam.), nucleolus (1um in diam.), and a star-shaped dictyosome were evident in Fig. 54. Lomasomes were clearly evident in some sections and were considered to be involved in spore wall synthesis (Fig. 55).

Repeated attempts to locate a nucleus within the central spore of germinated teliospores failed. Other than the lipid bodies which almost filled the central spore, there appeared to be a complete absence of organelles in the remaining cytoplasmic area. High magnification revealed parallel membranes running at random throughout the cytoplasmic area (Fig. 56,57,58). Previous investigators (2,6,41,43,49) failed to find a nucleus or evidence of a meiotic process in the central spore of _U. colchici_. In addition, a nucleus was not discernible in the dormant spores of *Urocystis agropyri* (18,48).

# DISCUSSION

Some observations and results of this study agreed, but others were in direct conflict, with those obtained by earlier investigators (2,6,41,43,49). This comparative study, using light and electron microscopy, established that central spores arose from terminal cells of lateral sporogenous branches which curved back upon themselves, and that terminal cells became located in the center of a coil of hyphal cells. These hyphal cells became the appendage cells. This confirms some of the speculations of Anderson (2). There was no evidence that the origin of the appendage cells and central spores were from two different sporogenous branches (43,49), or that a sporogenous hyphal cell in a "favorable" position became the central spore, while the contents of the appendage cells served as "food" (6). All observations indicated that at maturity appendage cells and central spores alike were well-developed, viable cells.

Up to the present time, efforts to detect karyogamy in *U. colchici* have been unsuccessful, although previous investigators assumed that it occurred (2,6,41). The single nucleus observed in the central spore was considered to be diploid. Anderson (2) assumed that fusion occurred in a binucleate vegetative cell and Blizzard (6) assumed that it occurred in young spores. Karyogamy was shown in this investigation to occur in the central spore during the juvenile stage of teliospore development. After karyogamy occurred, the centrally located diploid nucleus, about 2um in diameter, was surrounded by a small amount of cytoplasm. The remaining volume of the central spore was filled with lipid bodies at maturity. Thus the nuclear condition of the sporogenous hyphae and young spores was dikaryotic whereas older juvenile spores and mature spores were diploid.

Prior investigators (2,6,43,46,49) had applied many names and some functions to the appendage cells that are attached to the central spore. They had been considered to be dead, sterile, appendage cells (46), sterile cells with one nucleus (2), sterile cells with 2 nuclei (6), which disappeared as the cell contents served as "food" for the developing central spore, pseudospores (6,43,49), and accessory cells (2). Names and functions were assumptions based on the investigations of pioneer mycologist, most of whom worked with other species of <u>Urocystis</u> or entirely different genera (2,6,49). The views of these pioneer investigators were extrapolated to apply to <u>U. colchici</u> by more recent investigators.

The nuclei of appendage cells were so small that they were difficult to distinguish with the light microscope. Light microscopy did infer that appendage cells were binucleate up to the juvenile teliospore stage and usually had one nucleus at spore maturity.

Observations of electron micrographs showed that the nuclear pattern of appendage cells was changing during the juvenile teliospore stage from appendage cells having one or two nuclei in the early juvenile stage to one or none in the cell at spore maturity. Permanganate-fixed material suggested that one of the nuclei decomposed because of what appeared to be nuclear debris in the cells. Appendage cells that lacked a nucleus showed no sign of nuclear or cytoplasmic disintegration and one could assume that the nucleus was missed in the longitudinal sections. The probability of missing both nuclei, where two were present which lay close together horizontally in dikaryotic cells, was considered to be small since the nucleus measured about 1um in diameter and appendage cells averaged 3um in diameter x 4um in length.

The length of exposure to $OsO_4$ was an important factor in differentiating layers of mature spore walls. Under-exposure to $OsO_4$ yielded entirely electron-transparent walls and over-exposure gave dark homogeneous walls. Optimum exposure showed that the central spore wall was composed of three distinct zones, similar to other thick-walled spores (13,44,50). It has been suggested (50) that the continuous dark layer represents the site for hydrophobic properties of the wall.

The matrix layer appeared to be a gelatinous material excreted by the wall to serve as a bond between wall and appendage cells. Teliospores prepared for light microscopy sometimes fractured along this line when hit by the microtome blade, releasing the appendage cells. This seemed to indicate that the matrix layer was weaker than the spore wall.

Recent research has established that fungal walls were composed of interwoven microfibrils embedded in an amorphous matrix substance (3,4). Chitin and cellulose form the cell walls for the majority of fungi with various polysaccharides acting as the cementing substance (3,4). Chemical studies provided ample evidence that fungal cells and spore walls were permeable to substances of low molecular weight (7). Therefore, it is highly probable that appendage cells and central spore cells were in chemical contact with each other through their walls.

Sporidia were not produced by germinated teliospores on agar media in this study. Other than the observations of Thaxter (43) and Whitehead (49), sporidia production has not been observed by other investigators. Both of these researchers worked with contaminated cultures, which may explain the presence of what they thought were sporidia. Sporidia are not formed in other species of _Urocystis_ except for _U_. _violae_ (46).

Previous investigators (2,6,41,42,43,49) have assumed that the germ tube from U. colchici teliospores arose from the central spore between appendage cells. This study has provided evidence that appendage cells germinated on agar media, whereas central spores did not germinate. Of numerous appendage cells attached to the central spore, usually one germinated and a few others appeared as though they were capable of germination as indicated by the appearance of cytoplasm and the synthesis of a new cell wall. These cells were always located near the agar surface. Germ tubes from appendage cells contained one nucleus and quickly branched upon contact with the agar surface. The germ tube wall was continuous with the new layer formed within the existing appendage cell wall prior to germ tube emergence. The majority of appendage cells on a given spore appeared to be dead as indicated by their disorganized cytoplasm. At no time was there any cytological evidence that karyogamy or meiosis occurred in appendage cells or germ tubes.

Nuclei were not found in the central spore of teliospores that germinated on agar, nor was there strong evidence for the meiotic process having occurred. The central spore contained some unusual membranes not observed under other conditions, however, the disorganization and disintegration associated with cellular breakdown was not present.

If the central spore went through meiosis, it would be unusual for all four nuclei to disintegrate. Since no nuclei were found in central cells of germinated teliospores, perhaps the membrane of the diploid nucleus degenerated, which is commonly observed in early meiosis in fungi (34), but the meiotic process was never completed on agar. The germination process may prove to be different under natural conditions in the presence of a susceptible host. Therefore meiosis, if it occurs in culture, remains undiscovered.

Of 100 monosporus teliospore lines of _U_. _colchici_ used individually by Tachibana (41), four caused smut in onions. He suggested that two teliospores of opposite compatibility had been obtained by accident during isolation procedures with the micromanipulator.

Tachibana (41) isolated 29 "sterile" cells (appendage cells) of which 18 grew on PSA and produced cultures. These were tested for pathogenicity as monosporic and polysporic lines. One "sterile" cell monosporic culture was pathogenic. When combined as polysporic lines, a higher percent (mean 98%) of smutted onions was observed than when polysporic lines from teliospores were used (mean 80.7%). Tachibana stated that "pathogenicity studies with these (appendage) cells showed them to be nonpathogenic as single cell lines" (41). This was true not only with appendage cell lines but also with the individual teliospore lines.

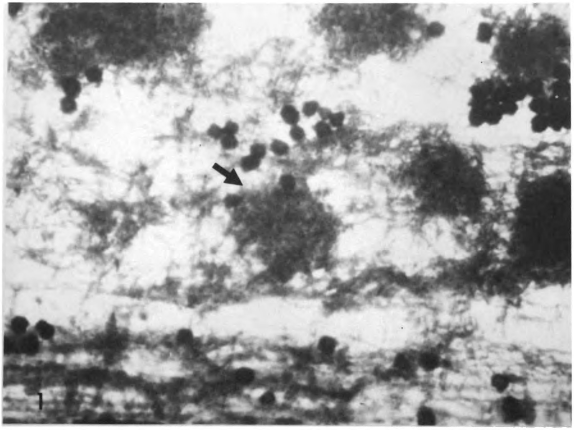
Tachibana concluded that *U. colchici* was heterothallic and the teliospores were of opposite mating types (+ and -). He assumed that meiosis occurred in the central spore with the disintegration of three of the resulting nuclei. The remaining haploid nucleus was thus assumed to be plus or minus. He ignored the possibility that appendage cells could germinate and provide mycelia of both mating types.

The results of this investigation indicated that meiosis in the central spore was not completed in culture, and that Tachibana (41) may have been dealing with haploid appendage cells. The pathogenic monosporic culture probably arose through the germination of a dikaryotic appendage cell or two appendage cells of opposite compatibility. It was interesting to note that 62% of appendage cells separated from teliospores produced cultures. A single teliospore has 20 to 40 appendage cells attached and only one, and rarely two, attached appendage cells germinated. The reason such a large number of the appendage cells' cytoplasms decomposed is also unknown.

It seems clear that appendage cells could play a role in pathogenesis in nature, but whether they do remains to be discovered. Experiments on the cytology of teliospores germinating on onion seedlings may reveal the roles that appendage cells and central spores play in infection.

Fig. 1. Lognitudinal section of young developing sori. X5100. Note the developing teliospores (arrow) in the masses of sporogenous hyphae. Stained using the periodic acid-Schiff's reaction (PAS).

Fig. 2. Transverse section of a sorus. X5100. Stained with Pianeze 3b. Within a sorus, teliospores were found in different stages of development. These stages consisted of spore initials (upper left arrow), young spores (lower left arrow), juvenile spores (middle arrow), and mature spores (tight arrow).



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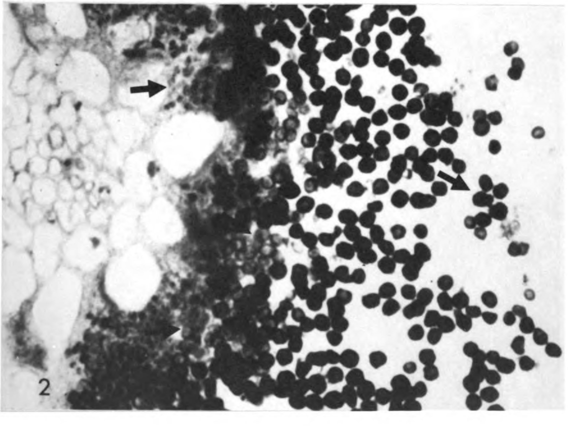

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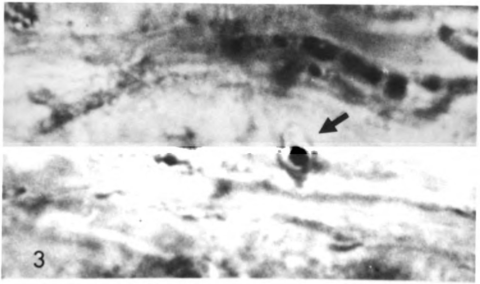
Fig. 3. Origin of the lateral branch. The lateral branch (arrow) arose from the parasitic mycelium and contained a prominent lipid body in the terminal cell. Fixed in glutaraldehyde-acrolein and post-fixed in $OsO_4$. X5100.

Fig. 4. Further development of the lateral branch. The lateral branch continued its growth as the terminal cell began to enlarge. Fixed in the same manner as Fig. 3. X5100.

Fig. 5. Terminal cells enlarged into bulbous shapes. These measured 2.0 um in diameter and contained prominent lipid bodies 0.8 to 1.0 um in diameter. X5100. The presence of lipid in the mycelia in Fig. 3-5 was verified by the positive reaction of $OsO_4$ and the Sudan test III.



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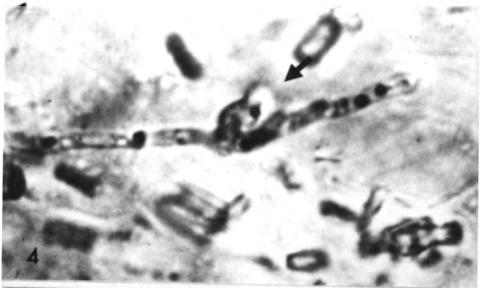

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Fig. 6. Binucleate terminal cells which represented the spore initials measured 3.4 um ± 0.5 um in diameter. X5100. Stained with PAS. The nuclei (N) measured about 1.0 um in diameter. A small lipid body (arrow) lay between them.

Fig. 7. Terminal cells of sporogenous branches starting to "hook-over." Terminal cells (arrow) started to "hook-over" as the sporogenous branch curved inwardly. X5300. Feulgen stained.

Fig. 8. Terminal cells of sporogenous branches in the "hook-over" or "U-shaped" configuration. In some sections terminal cells (spore initials) were observed "head-on" (a) and others in transverse positions (b). X5100. These terminal cells with small lipid bodies measured approximately 1.6 um in diameter. A direct comparison in development was the terminal cell at the bottom (c) which measured 3 um in diameter, contained a prominent lipid body; and had grown almost to the point where it came in contact with the remaining cells of its branch. Post-fixed in $OsO_4$.

Fig. 9. Continuation of the spiraling process and increased growth of the lipid containing terminal cells. The continued spiraling process, growth of the terminal cells, and lipid accumulation resulted in spores 4-5 um in diameter containing lipid bodies 1.0 um in diameter or larger. This process can be followed by starting with the top left arrow and moving counter clock-wise, a, b, c, etc. X5100. Post-fixed in $OsO_4$.

Fig. 10. A terminal cell coming in contact with the hyphal cells of the sporogenous branch. As the terminal cell (arrow) became larger, lipid bodies could not be observed due to cell wall thickness and pigment formation. X5100. Hematoxylin.

Fig. 11. The central spore lying in the center of a coil composed of sporogenous branch cells which became the appendage cells. The terminal cell became the central spore in the center of a coiled sporogenous branch (arrow). X5100. Post-fixed in $OsO_4$.

Fig. 12. Developing appendage cells (NC) on the central spore surface. X5300. Post-Fixed in $OsO_4$.

Fig. 13. A mature teliospore with appendage cells (NC). X7300. Unstained.


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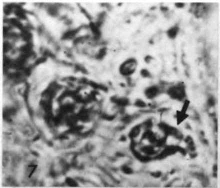
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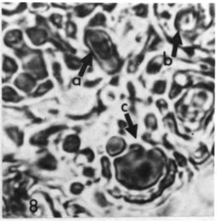
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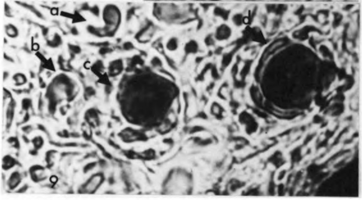
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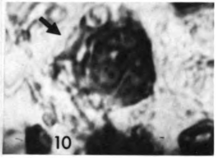
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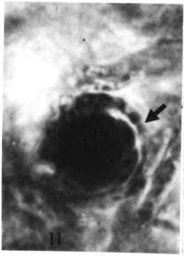
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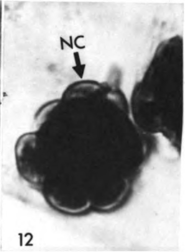
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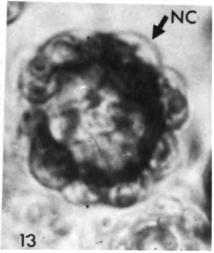
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Fig. 14. Binucleate young spore. X5100. Each of the nucleus (N) was surrounded by cytoplasm. The nuclei measured about 0.8 um each in diameter. Note the plastic state of the spore walls and the lipid in the background. Feulgen stained.

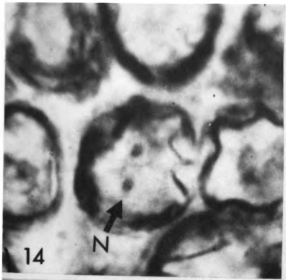
Fig. 15. Young spores with the nuclei approaching each other. The nuclei (N) approached each other as the teliospore developed. X5100. Hematoxylin stained.

Fig. 16. Binucleate early juvenile stage. The nuclei (N) were lying very close to each other in the early juvenile stage. X5100. Note the increased amount of lipid, the formation of the spore wall, and developing appendage cells. Stained with PAS.

Fig. 17. Early pre-karyogamy in juvenile spores. X5100. The nuclei (N) touched along their margins. The lipid bodies became globular and spore wall showed distinct layers in the juvenile stage of telispore development. Hematoxylin stained.

Fig. 18. Higher magnification of the teliospore in Fig. 17. X7000. Note the nucleoli (Nu) present on each nucleus (N). Hematoxylin stained.

Fig. 19. The nuclei (N) were a pressed along their margins prior to fusion (karyogamy). X5100. Hematoxylin stained.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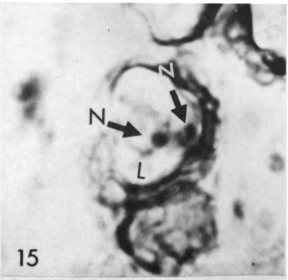
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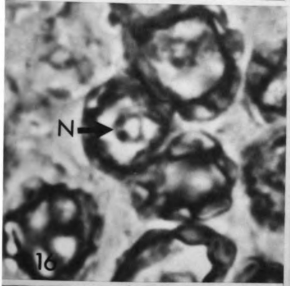
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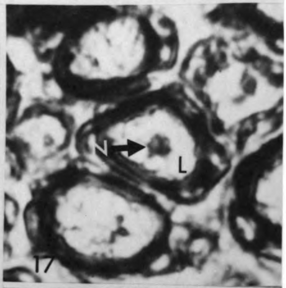
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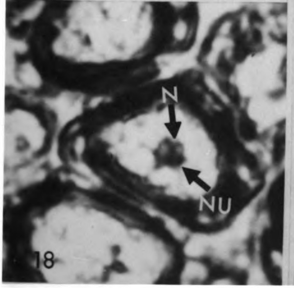
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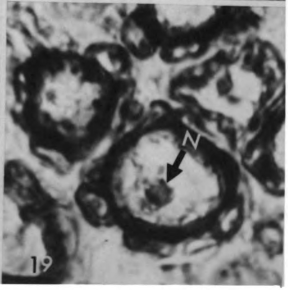
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Fig. 20. Higher magnification of Fig. 19. The prominent nucleoli (Nu) of each nucleus (N) are evident. X7600. Hematoxylin.

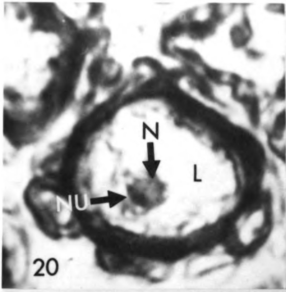
Fig. 21. Post-karyogamy in juvenile spores. Karyogamy has occurred resulting in a diploid nucleus (N). X5100. Acetocarmine.

Fig. 22. Bi- and uninculeate condition of the appendage cells during the juvenile stage. Some appendage cells (NCN) appeared to be binucleate whereas others were mononucleate. The cytoplasmic area (CA) around the diploid nucleus decreased as the lipids increased. X5100. Heamtoxylin.

Fig. 23. Lipid bodies encroaching upon the cytoplasmic area around the nucleus in teliospores. The cytoplasmic area (CA) around the nucleus (N) continues to be encroached upon by the lipid bodies as the teliospore matures. X6500. PAS.

Fig. 24. Nucleus surrounded by lipid bodies in a mature teliospore. X5100. Hematoxylin.

Fig. 25. Thick-walled mature teliospore with a nucleus. Notice that the entire cytoplasmic area was filled with lipid bodies as in Fig. 24. Note the thick spore wall (SW) was evident in the micrograph. X5100. Acetocarmine.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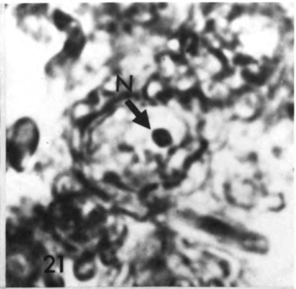
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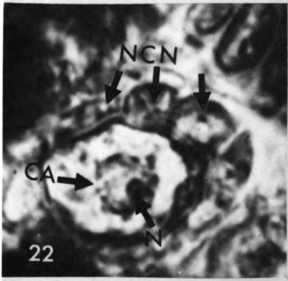
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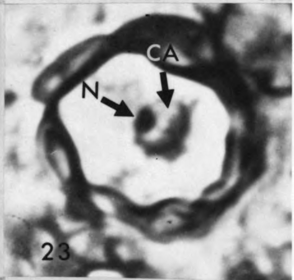
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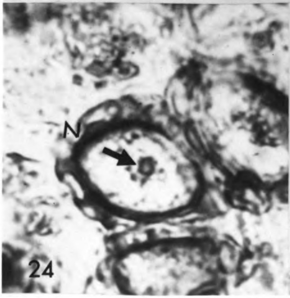
N
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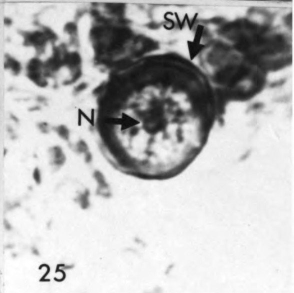
SW
N
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Fig. 26. Germentation of a detached appendage cell. Hyphae from a detached appendage cell that germinated. X5100.

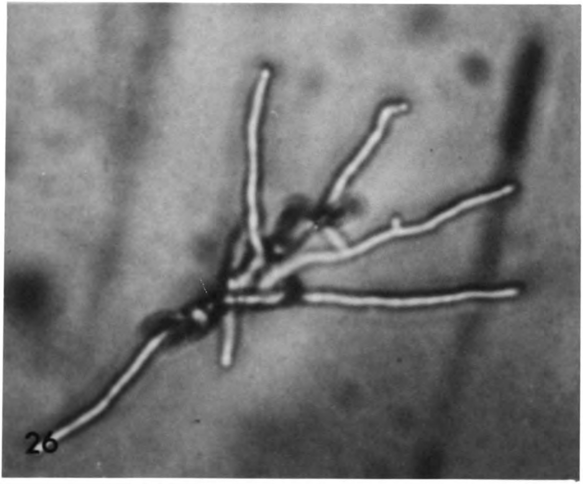

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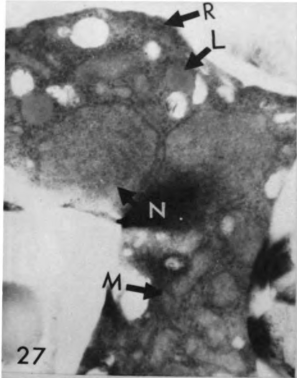
Fig. 27. Binucleate terminal cell of a sporogenous branch. Nuclei (N) were about 1.14 um each in diameter. Lipid bodies (L), mitochondria (M), and a ribosome (R)-packed endoplasmic reticulum were characteristic of terminal cells. X26,200.

Fig. 28. Terminal cell expanding. X26,200. The plasmalemma (P), vacuoles (va), and a nucleus (N) were evident, as well as lipid bodies (L) and ribosomes (R).

Fig. 29. Bulbous-shaped terminal cell. The cell is seen to be curving inwardly toward the sporogenous branch. X12,500. The endoplasmic reticulum was granular and rich in ribosomes with a prominent vacuole (Va) near the tip.

Fig. 30. The binucleate (N) central spore. This developed from a binucleate (N) terminal cell. X26,200. The spore wall (SW) was covered with a gelatinous layer which became the matrix.



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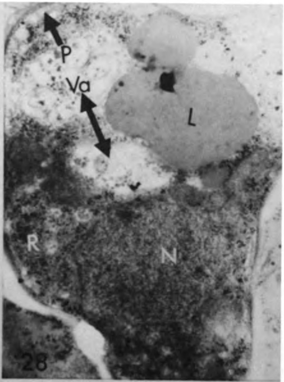

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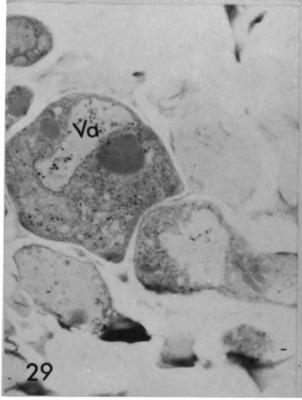

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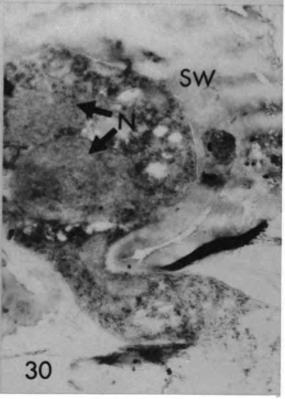

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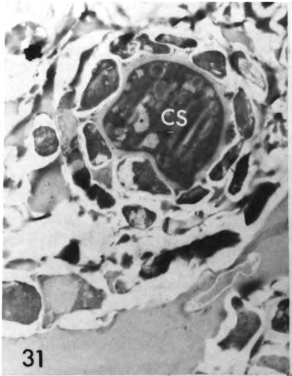
Fig. 31. Appendage cell premordia surrounding the central spore. This micrograph at low magnification (X5700) illustrated the position of the central spore (CS) after the inward curving of the sporogenous branch was finished.

Fig. 32. Appendage cell walls adhered to the central spore walls. The appendage cell primordia (NCP), composed of the surrounding sporogenous branch cells, adhered to the central spore (CS) walls through the matrix layer which was undifferentiated at this stage of development. X10,100.

Fig. 33. Binucleate early juvenile spore. Binucleate (N) juvenile spore with numerous lipid bodies (L), mitochrondia (M), and a very granular endoplasmic reticulum. X35,000.

Fig. 34. Pre-karyogamy in the juvenile spore. This was indicated by the position of the nuclci (N) which were making contact along their margins (arrows). The remaining cytoplasm between the nuclei will be squeezed out in the fusion process. X29,500.



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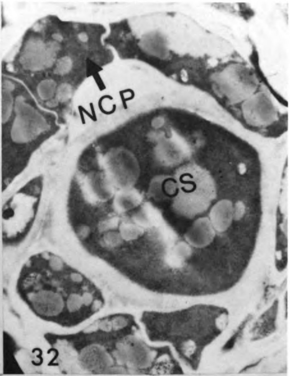

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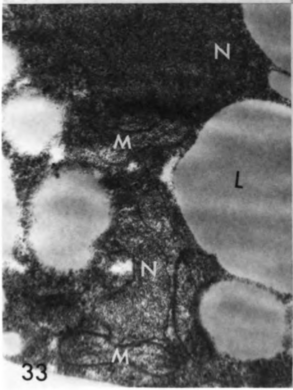

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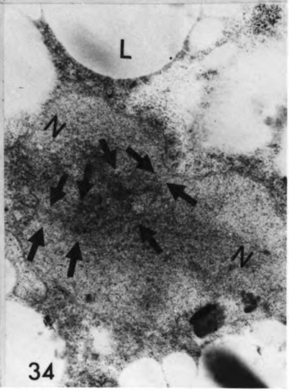

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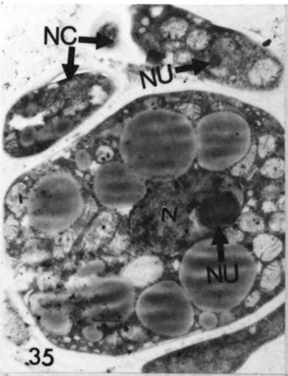
Fig. 35. Deploid central spore. After karyogamy, the diploid nucleus (N) remained centrally located in the juvenile spore. X10,100. A nucleolus (Nu) was present on both the diploid nucleus and the appendage cell (NC) nucleus. Note the many mitochondria scattered throughout the central spore.

Figs. 36 & 37. Nuclear membranes. The nuclear membranes were the usual double membranes (two unit) found in most forms of life. Fig. 36. X31,250. Fig. 37, X40,000.

Fig. 38. Nucleus surrounded by lipid bodies. Lipid bodies surround the centrally located nucleus as the teliospore matures. Note the differentiation of the spore wall. X9,600.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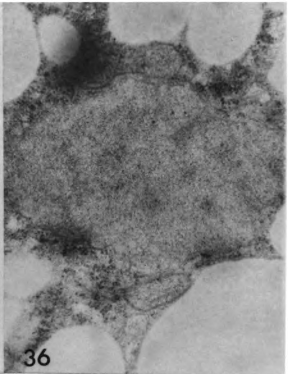
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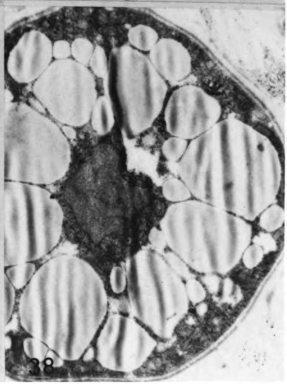
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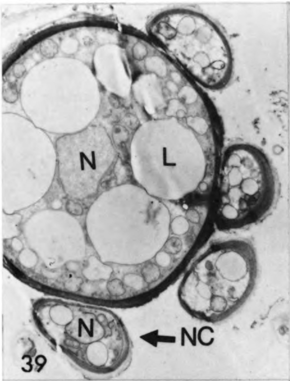
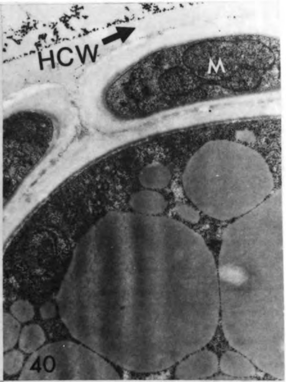
Fig. 39. Lipid bodies in thin sections after $KMNO_4$-fixation. $KMnO_4$-fixation indicated that the lipid bodies (L) were membrane bound. Nuclear pores were evident in both central spore and appendage cell (NC) nuclei (N). X9,600.

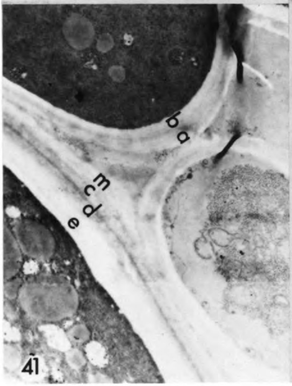
Fig. 40. Development of the teliospore walls. Juvenile spore wall layers are shown as two layers. X22,500. Differentiation of the spore wall layers began at this stage of development. Appendage cell walls pressed against the host cell wall (HCW) as the teliospore grows. M = mitochondria.

Fig. 41. Mature teliospore walls. X17,500. The central spore wall is represented by the inner layer (e), middle layer (d), and outer layer (c). The appendage spore walls were composed of an outer layer (a), and an inner layer (b). Appendate cells were cemented to the central spore by the matrix (m).

Fig. 42. Dikaryotic appendage cell $KMnO_4$ fixation. X29,500. Note pores in membranes of nuclei (a,b).


N
L
N
NC
39


HCW
M
40


b
a
m
c
d
e
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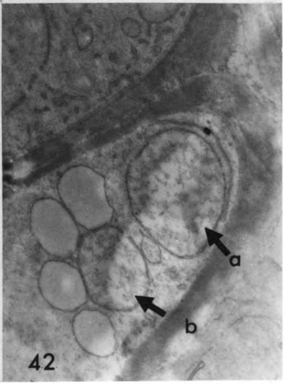
a
b
42

Fig. 43. Dikaryotic appendage cell post-fixed in $0_s0_4$. X23,000. N = nuclei.

Fig. 44-46. Uninucleate (haploid) appendage cells. N = Nucleus. Fig. 44 = X23,333
Fig. 45 = X21,000
Fig. 46 = X9000
Note three-layered spore wall (a) in Fig. 66.

Fig. 47. Appendage cell without a nucleus. X9000. L = lipid body.

Fig. 48. Uninucleate appendate cell. N = nucleus. X9000.

Fig. 49. Disintegrating nuclei of appendage cells. Perhaps region (X) represented a disintegrating nucleus of an appendage cell. In appearance and diameter, the x-area matched that of appendage cell nuclei fixed in a similar manner. X22,400.


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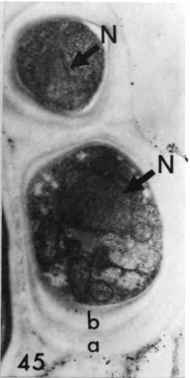
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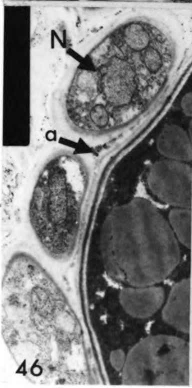
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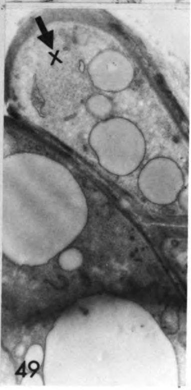
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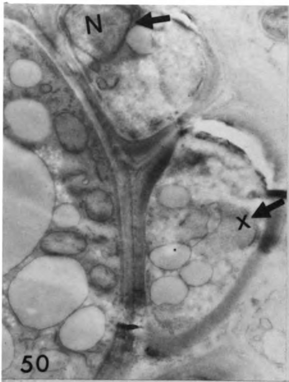
Fig. 50. Represents the same structure as Fig. 49 in a different appendage cell of another teliospore X11,000.

Fig. 51. Germinated appendage cell. Germinating appendage cell near the agar surface (left arrow). The appendage cell on the right (right arrow) and near the bottom (arrow) of the micrograph have synthesized new wall layers. Appendage cells a, b, c, cytoplasms appeared highly disorganized suggesting cell death. X7,500.

Fig. 52. Nucleus of a germinated appendage cell. N = nucleus. Nu = nucleolus. X10,340.

Fig. 53. The germ tube wall (GTW). This wall was 0.14-0.27 um thick and continuous with the newly synthesized wall. X29,500.



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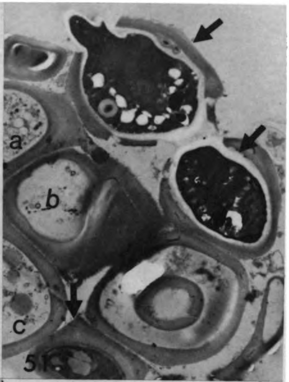

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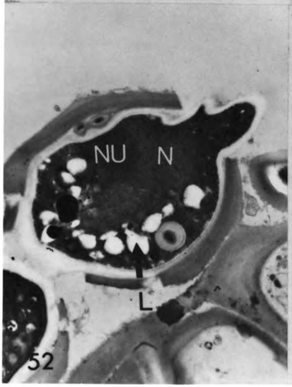

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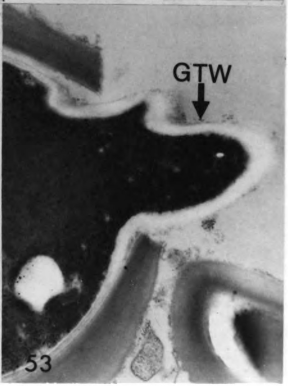

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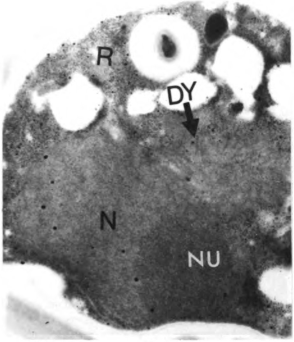
Fig. 54. Cell organelles of a germinated appendage cell. X29,400. N = nucleus. Nu = nucleolus. Dy = dictyosome. Perhaps the remains of lomasomes were left in the cell wall (arrow).

Fig. 55. Lomasomes (Lo) and plasmalemma (P) of a germinating appendage cell. The cell has probably aborted in germination. X14,000.

Figs. 56 & 57. Unusual membranes found in the central spore of germinated teliospores. X58,000. No cell organelles were evident in the non-lipid areas of the spore.



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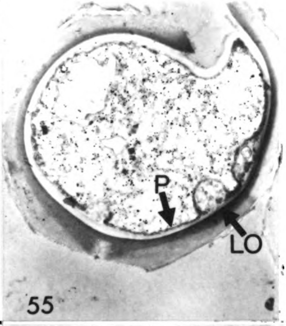

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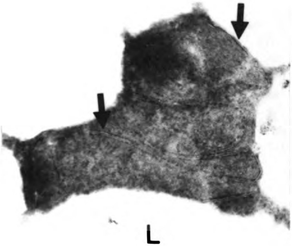

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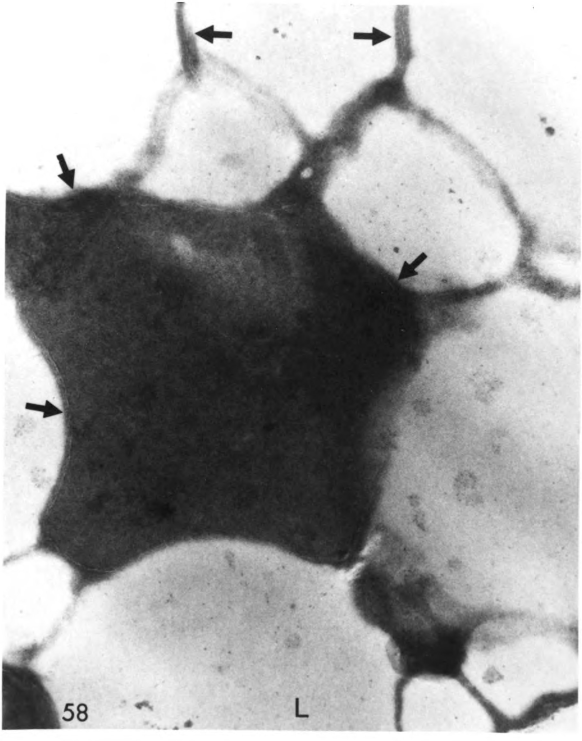
Fig. 58. Unusual membranes were present in the nuclear area of the central spore. X121,333.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