# MELANIZATION OF *VERTICILLIUM DAHLIAE* AT THE CELLULAR LEVEL

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ABSTRACT

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Melanization in Verticillium dahliae at the Cellular Level

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Microsclerotia are thick-walled, large globose cells which serve as survival structures in Verticillium dahliae. The globose cells are characteristic of older hyphal regions. The wild-type strain produces melanin which can be observed within and on the wall of the microsclerotia and extracellular fibers. Fungal melanin is considered necessary for the survival of microsclerotia during long periods of unfavorable conditions.

In order to examine the formation of microsclerotia as related to melanin production in wild-type (t-9), the albino-1 microsclerotia (alm-1) and the brown-1 microsclerotia (brm-1) mutants were grown on potato dextrose agar (PDA) over-laid with cellophane or polygalacturonic acid medium (PGAM) which induces synchronous development of microsclerotia. Albino-1 was induced to produce melanin by a brown substance (scytalone) in the culture filtrate of brm-1 mutant. Scytalone-induced albino (s-alm-1) was

compared cytologically with the development of melanin in t-9. Observations were made by phase contrast and transmission electron microscopy.

The cellular changes that accompanied the change of alm-1 to s-alm-1 were frequently observed mitochondria along with single and double membrane vesicles. There were electron-dense melanin granules similar to t-9 observed within extracellular fibers and on the wall of microsclerotia. The precipitate from the 3,3'-diaminobenzidine reaction was demonstrated on the inner mitochondrial membrane of alm-1, while s-alm-1 showed activity on the inner and outer mitochondrial membranes. The above mentioned changes in organellar structures and arrangement suggest a correlation with the production of melanin as seen in the wild type.

DEDICATION

To

My Wife

Ruth A. Hayward

My Parents

Mack and Lessie Hayward

My Parents-in-Law

Edward and Naomi Aiken

My Oldest Brother

Deacon James M. Hayward

My Oldest Sister

Mrs. Mary L. Miles

For

Their sincere encouragement, love, patience, and understanding during the period of my graduate study at Atlanta University.

A.G.H.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES

LIST OF FIGURES

LIST OF FIGURES

LIST OF FIGURES

LIST OF TABLES

# CHAPTER I

## INTRODUCTION

Verticillium dahliae, producing asexual spores as the only known means of reproduction, belongs to the form-class Fungi Imperfecti. This fungus, a soil-borne plant pathogen, affects over 300 plants including vegetables, flowers, fruit trees, field crops and forest crops by causing vascular wilt. It can be grown on potato-dextrose agar plates in a mycelial form, extending the colony diameter by a process of elongation at the hyphal tip (Green, 1969; Schreiber and Green, 1962; Nadakavukaren, 1962).

As one progresses from the hyphal tip into older regions, there is an occurrence of dark structures called microsclerotia (ms) which serve as survival structures. Microsclerotia are thick-walled melanized cells (Smith, 1965) that contain large lipid globules and other cytoplasmic organelles (Brown and Wyllie, 1970; Tolmsoff et al., 1973).

Fungal melanins appear to be necessary for the survival of propagative cells during prolonged periods that are unfavorable for growth. Fungal melanins also protect fungal cells from injury caused by irradiation or desiccation (Bell et al., 1976a). Protection of microsclerotia by fungal melanins from enzymes secreted by associated

soil microorganisms (Kuo and Alexander, 1967) is at least partially due to inactivation of glucanase in the microsclerotia cell wall.

The mechanisms whereby environmental factors influence microsclerotia production are still unknown. However, near-ultraviolet irradiation (Brandt, 1964), aluminum sulfate (Roth and Brandt, 1964), certain carbon sources, low oxygen levels, high carbon dioxide level (Ioannou et al., 1977a), high incubation temperatures and low water potentials (Ioannou et al., 1977b) all suppress microsclerotia formation.

Bell and his colleagues (1976a) observed that the number of microsclerotia in V. dahliae was directly related to the frequency of hyphal fusions. Fusions arise when small protuberances on one hypha coincide with similar outgrowths on an adjacent hypha. The protuberances grow toward each other, meet and then the walls break down so that bridges are formed between hyphae. Following the combining of the hyphae, microsclerotia initials are rapidly formed. These microsclerotia initials give rise relatively quickly to melanized microsclerotia, often within 3 to 8 days (Mayfield and Taylor, 1978; Gaskins and Mayfield, 1980).

Both genetic and survival variations play important roles in the pathogenicity of V. dahliae. It lives in the soil and is able to survive adverse conditions of the

soil's environment because of its survival variation. Genetic variability is another means of assuring the survival of V. dahliae. The microsclerotial stage of the life cycle seems to be an excellent place to focus one's attention, if verticillium wilt is to be controlled.

Before 1968, studies on the structure of microsclerotia had been confined to techniques using light microscopy (Gordee and Porter, 1961; Schnathorst, 1965; Isaac, 1967), except for Nadakavukaren's (1963) observation with an electron microscope (EM). Since 1968, several EM studies have involved microsclerotia from cultures (Brown and Wyllie, 1970; Mayfield and Taylor, 1978; Gaskins and Mayfield, 1980) or host tissue (Wright and Abrahamson, 1970; Smith and Berry, 1974). Wheeler et al., (1976) used a medium, polyglacturonic acid, which promotes the synchronous development of microsclerotia. Synchronous growth conditions have been used to study the structural changes in nuclei, mitochondria, and other cytoplasmic structures during microsclerotial development of V. dahliae.

To date, only two reported ultrastructural studies on cellular changes associated with microsclerotia production in synchronous growth were conducted in this laboratory (Mayfield and Taylor, 1978; Gaskins and Mayfield, 1980). Wheeler et al., (1976) did an ultrastructural study of various melanin granules deposited in the cell wall of V. dahliae. However, there is a need for a more critical

study on the cellular changes that accompany melanin biosynthesis. This critical study should be done on wild-types and microsclerotial mutants.

Mutants which vary in their ability to produce normal melanin- containing microsclerotia have been isolated by Bell et al. (1976a). These mutants are ideal for studying the process of melanin production and microsclerotia formation in V. dahliae. To date, there has been only one reported ultrastructural study of cytoplasmic organization of the mutants (Gaskins, 1979).

The present study was undertaken to gain information which might indicate which cellular organelles, inclusion, or regions are involved in carrying out specific biosynthetic processes along the melanin pathway. Phase contrast and transmission electron microscopy with cytochemical techniques were employed to analyze microsclerotia development at the cellular level of V. dahliae's wild-type, albino-1, and precursor treated albino-1.

# CHAPTER II

## REVIEW OF LITERATURE

Verticillium dahliae is a soil-borne plant pathogen which affects a range of host plants (Johnson et al., 1979; Jordon and Tarr, 1978; Jordon, 1972; Hall and Ly, 1972). It can be grown on agar plates in a mycelium form, extending the colony diameter by a process of elongation at the hyphal tip (Nadakavukaren, 1962; Schreiber and Green, 1962; Green, 1969). As one progresses from the hyphal tip into older regions, there is an occurrence of dark brown to black structures called microsclerotia. Microsclerotia are thick-walled melanized cells (Smith, 1965) that contain large lipid globules and other cytoplasmic organelles (Typass and Heale, 1976; Tolmsoff et al., 1973; Puhalla, 1973; Brown and Wyllie, 1970).

Although V. dahliae's pathogenicity is non-specific (Isaac, 1967), it can be placed in at least two groups based on the severity and the type of disease. One group causes mild symptoms, resulting in most of its host's leaves remaining somewhat normal with turgid pressure until death. A second group causes severe defoliation of leaves and death (Schnathrost, 1969).

Genetic recombination (Hastie, 1968), diploidization and heritable gene repression-depression (Tolmsoff et al., 1973), heterokaryosis (Puhalla and Mayfield, 1974) and

mutations and parasexuality (Puhalla, 1973), are all responsible for morphological variation in V. dahliae. According to Typass and Heale (1976), acriflavine has been found to induce hyaline variants of Verticillium microsclerotia. Other variants such as brown mutants (brm-1 to -4) produce brown coloration rather than a black pigmented microsclerotium. In variants that produce microsclerotia, each type was again capable of producing many new types of variants such as haploid and diploid.

V. dahliae has a haploid dominant pathogenic stage and a diploid heritable variability stage, the latter being of notable concern, because it is responsible for the wilt in many economically important crops. Tolmsoff et al. (1973) suggested that the diploid stage is involved in the formation of microsclerotia development. An invaded plant will keep Verticillium in its pathogenic haploid state until the plant is killed. At this time Verticillium might rush into a preferred diploid state for dormancy within the microsclerotia.

Until 1974, little was known concerning the biochemical events associated with the development and formation of microsclerotia in V. dahliae and other closely related organisms. Smith and Berry (1974) proposed that in Sclerotina gladiolo and V. dahliae, intercalary segments of one or more hyphae develop by the production of numerous bud-like ourgrowths or side branches and formation of

additional septae. The formation of microsclerotia during development in V. albo-atrum was described by Brandt and his associates (Brandt, 1962; 1964; MacMillan and Brandt, 1966). These bud-like outgrowths are called microsclerotia initials. After the microsclerotia initiation, there is an increase in size by further interweaving of hyphae, extensive outgrowth of branching, and coiling of hyphae.

As microsclerotia continue to develop, the cells become swollen and globose, while their walls become thickened, cells clumped and become pigmented. During this time, reserved food material accumulates in the form of large lipid bodies.

By using metabolic inhibitors, sulphus-containing-compounds, and metal chelates, Chet et al. (1966) and Chet and Henis (1968) studied the developmental stages of sclerotia formation in Sclerotium rolfsii. They suggested that sclerotia formation, which is similar to microsclerotia was repressed by a protein containing copper and a sulphydryl group. Marukawa et al. (1975) concluded that a secondary phenolic metabolite produced by Sclerotinia sclerotiorum and S. libertiana plays a role in the induction of sclerotium initials and melanogenesis of sclerotia. Sclerin, a metabolite which is produced at the mycelium stage has been isolated and characterized to be a monohydricphenol (Kubota et al., 1966). During the formation of sclerotia initials, sclerin concentrations are increased,

reaching a maximum concentration soon after sclerotia are fully mature. When concentrated sclerin was introduced to cultures of S. liberiana that did not normally produce sclerotia, sclerotia initials developed, while the sclerin concentration decreased. Kubota et al. (1966) concluded that this suggests a sequence of events controlled by different factors. The activities of polyphenoloxidase and peroxidase were stimulated by addition of sclerin to culture media, while amino-benzoic acid, which inactivates polyphenoloxidase, inhibited the production of both sclerin and sclerotina (Smith and Berry, 1974).

A possible role of a peroxidative system in melanin synthesis in Verticillium was studied by MacMillan and Brandt (1966). They suggested that peroxidase initiates most melanin synthesis in Verticillium. They also looked at microsclerotia production and melanin synthesis at the biochemical level. From that experiment, they suggested that peroxidase activity was in Verticillium, while phenolase activity was absent or very weak in Verticillium. While Gafoor and Heale (1971) examined hyaline variants of V. dahliae and V. albo-atrum for peroxidase levels, they showed a similar level of enzyme activity. They, therefore, suggested that peroxidase activity plays a general role in metabolism in fungal development as well as melanin production.

Melanin production in animals generally involves the action of tyrosinase (Swan, 1973). One of the intermediate compounds, indole-5, 6-quinone, condenses to form a melanin polymer in animals according to Swan (1973). Some fungal melanins have been classified as "indole melanin" (Ellis and Griffiths, 1974; Bull, 1970), while other such melanins appear to be synthesized from catechol (Piattelli et al., 1965; Gafoor and Heale, 1971). Ellis and Griffiths (1974) suggested that melanin of V. dahliae, like several other fungi, was indolic in nature. They obtained a positive reaction for alkali fusion product with Ehrlich's reagent for indoles and based their suggestions primarily on this evidence. Indole melanin granules are synthesized from tyrosine or 3, 4-dihydroxyphenylalanine (DOPA) by tyrosinase, and usually contain 8 to 10.5% nitrogen (Thomas, 1958). Heale and Isaac (1964) found that tyrosinase was not involved in the synthesis of melanin in V. albo-atrum. Gafoor and Heale (1971) further found that purified melanin of V. albo-atrum contained 1.9% or less nitrogen. Thus, neither catechol nor DOPA appears to be involved directly in melanin synthesis by V. dahliae or V. albo-atrum, which refuted their earlier suggestion. Jepson (1960) warned that Ehrlich's reagent is not absolutely specific for indole the nucleus and certain phenols.

Recent genetic and chemical studies with V. dahliae suggest that some fungal melanins are formed by a penta-

ketide pathway (Bell et al., 1976a; Stipanovic and Bell, 1976, 1977; Tokousbalides and Sisler, 1979; Wheeler et al., 1976). 1,3,7-trihydroxynaphthalene (Bell et al., 1976a) (+)-scytalone), 1,3,8-trihydroxynaphthalene ( (-)-vermelone) and 1,8-dihydroxynaphthalene are intermediates in this melanin pathway (Wheeler and Stipanovic, 1979). Wheeler et al., (1978) also refuted earlier suggestions that catechol (Gafoor and Heale, 1971) and DOPA (Ellis and Griffiths, 1974) were intermediate in fungal melanin biosynthesis in V. dahliae.

Fungal melanin appears to be necessary for the survival or propagative cells during prolonged periods unfavorable for growth. Melanin granules occur as dense screens in both the cell walls and among the extracellular fibers that encapsulate the cell walls (Brown and Wyllie, 1970; Durrell, 1964; Griffith, 1970; Griffiths and Campbell, 1971; Nadakavukaren, 1963; Wheeler et al., 1976). Melanized spores and hyphae, but not hyaline or albino spores and hyphae are able to survive for prolonged periods in natural soil (Lockwood, 1960). Protection by melanin is at least partially due to the inactivation of $\beta$ - 1,3 glucanase and chitinase enzymes secreted by soil microorganisms (Bloomfield and Alexander, 1967). Melanin also protects fungal cells from injury caused by irradiation or desiccation (Durrell, 1964; Sussman, 1968; Zhdanova and Pokhodenko, 1973). Melanin acts as a physical barrier to

the cytoplasm in V. dahliae, according to Bull (1970), like lignin acting as a physical barrier for cellulose.

The electron microscope reveals that microsclerotia of V. albo-atrum consist of aggregations of spherical cells interconnected by septal pores. Individual microsclerotia become heavily pigmented and contain organelles that are necessary for germination, growth and survival. These aggregations of microsclerotia are enclosed in a mucilaginous matrix in which melanin granules may also be found. Brown and Wyllie (1970) suggested that the mucilaginous matrix acts as a cement which hold the cellular mass together.

Transmission and scanning electron microscopic studies have been directed at understanding how melanin granules are deposited in and on the cell wall (Wheeler et al., 1976). Wheeler et al. (1978) studied the morphology of various granules. There have been few reports on the cellular changes that accompany melanin synthesis (Mayfield and Taylor, 1978; Gaskins and Mayfield, 1980). Mayfield and Taylor (1978) reported ultrastructural observations of microsclerotia cells produced in synchrony at 2 to 20 days after inoculation. They were not able to find any changes in ultrastructure after 5 days, except for thickening of the cell wall. Most of their results came from the third day of growth. Gaskins and Mayfield (1980) also examined V. dahliae wild-type and two of its mutants,

brown-1 and albino-4, from 2 to 5 days (with emphasis on day-3) using transmission electron microscopy.

From their study, they reported that small lipid globules increased in size, while the mitochondria exhibited alterations of their cristae. By using 3,3-diaminobenzidine (DAB), localized activity was demonstrable only in the mitochondrial regions demonstrating intact cristae. The disappearance of such cristae seemed to coincide with the appearance of single membrane-bound granular vesicles which develop in plant materials (Frederick and Newcomb, 1969), V. dahliae, (Gaskins, 1979) and other fungi (Todd and Vigil, 1972).

Mayfield and Taylor (1978) studied the changes in the nucleus and mitochondria of wild-type. There is a need for a more critical study on albino-1 mitochondria, especially after albino-1 has been induced to produce melanin. This study should be focused at the functional state of the mitochondria, since they are apparently converted to membraned-bound granular structures.

The plan of this study entails a critical ultrastructural study of changes in cellular organelles that may occur when scytalone treated albino are induced to produce melanin. These changes in the induced albino will be compared with the cellular changes that occur in the melanin producing wild-type strains.

# CHAPTER III

## MATERIALS AND METHODS

The strain of Verticillium dahliae used in this study was the wild-type (t-9), and two of its mutants, brown-1 (brm-1) and albino-1 (alm-1). Stock cultures were maintained on potato dextrose agar (PDA). Potato dextrose agar was used as a solid medium, prepared by the rehydration of 39 g of dehydrated PDA in a liter of distilled water. The medium was autoclaved for 20 min at 15 psi and poured into disposable polystyrene petri dishes, while still hot, to reduce contamination. Polygalacturonic acid medium (PGAM) was used for microsclerotia production (Wheeler et a., 1976). Polygalacturonic acid medium contained steam-warmed polygalacturonate solution, 40 ml; Difco agar, 2 g; $KH_2PO_4$, 52 mg; $K_2HPO_4$, 65 mg; $MgSO_4 \cdot 7H_2O$, 20 mg; and distilled $H_2O$ to make up 1 liter. Polygalacturonic acid medium was dispensed in 50 ml quantities into 250 ml Erlenmeyer flasks and autoclaved at 15 psi for 30 min. The polygalacturonate solution was prepared as follows: 25 g of polygalacturonic acid was suspended in 300 ml of distilled $H_2O$; 1 N NaOH (about 112 ml) was added with stirring to bring the pH to 7.0, and distilled $H_2O$ was added to make 500 ml. The solution was refrigerated at 1 C until needed.

Cellophane over-laid on PDA was inoculated with small pieces of mycelia (approx. 2 mm sq) from the edge of the stock colonies on PDA. Inoculated PDA over-laid with cellophane petri plates were placed in an incubator at 26 C.

Polygalacturonic acid medium was inoculated with a hyphal tip region taken from the edge of colonies on PDA. Flasks of the inoculated medium were placed on a rotary shaker (180 rpm) and incubated at room temperature (24-26 C).

## Induction of Melanin Production in Albino Microsclerotia Mutant

Albino-1 microsclerotia and brm-1 microsclerotia mutants were grown on different PDA over-laid with cellophane petri dishes at room temperature or in the incubator at 26 C. The cultures were allowed to grow for 3 to 12 days to form a mycelial mat. Brown-1 mycelial mat on cellophane was removed from the PDA dishes under sterile conditions. Albino-1 mycelial mat and cllophane were removed from the PDA dish and placed on the PDA dish where brown-1 had grown. The albino-1 mycelial mat and cellophane were allowed to remain on the brown-1's PDA for 12 hr. The results were observed with phase contrast and transmission electron microscopy.

## Phase Contrast Microscopy

Wet mounts of V. dahliae cultures were prepared by placing a drop of PGAM culture suspension on a clean microscope slide with a sterile Pasteur pipette. Cultures grown on solid medium (PDA) over-laid with cellophane were removed and placed on a microscope slide in a drop of sterile distilled $H_2O$. In order to study the changes in mycelial and microsclerotial development after inducing melanin in alm-1, the microscopic samples were initially observed 24 hr after inoculation and 18 hr thereafter. All wet mount preparations were observed through a Wild phase contrast microscope equipped for photography.

## Transmission Electron Microscopy

Hyphae and microsclerotia of V. dahliae to be observed with the electron microscope were harvested at different intervals, ranging from 3 to 9 days, and prepared by fixation in 3% glutaraldehyde in 0.05 M sodium cacodylate buffer at pH 7.4 for 15 min at room temperature, then fixed for an additional 15 min on ice. The specimens were postfixed using cold 2% $OsO_4$ in 0.05 M cacodylate buffer (pH 7.4) for 2 hr; washed for 1 hr in cold distilled $H_2O$ prior to dehydration. They were dehydrated in a graded series of ethanol, and then to absolute acetone. The specimens were then infiltrated for 30 min to 24 hr in a mixture of acetone-Spurr's low viscosity resin (Spurr, 1969) at a ratio of 1:1.

The resin was polymerized in BEEM capsules at 65 C for 24 hr. Sections were cut on a LKB Bromma-Ultramicrotome III with a glass knife, and collected on uncoated copper grids. They were counter stained with 0.5% uranyl acetate and Reynold's (1963) lead citrate and viewed with a RCA EMU-4 electron microscope operating with an accelerating voltage of 50 or 100 kv.

### 3,3'-Diaminobenzidine Localization

The localization of 3,3'-diaminobenzidine (DAB) was based on the procedures of Morrison (1977) and Taylor (1978). Microsclerotia specimens t-9, alm-1, brm-1, and induced albino-1 (s-alm-1) 8 to 9 days old, were fixed for 15 min in 3% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4. The specimens were transferred to cold (1 C) glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4 for 15 min. After five washed over a period of 30 min in the same buffer solution, the specimens were placed in vials containing 1 ml of reaction medium. The reaction medium was composed of 3,3'-diaminobenzidine tetrahydrochloride (DAB), 2.5 mg/ml in 0.05 M cacodylate buffer at pH 7.4.

In order to minimize photo-oxidation (Hirai, 1971), DAB was added to the buffer in a darkroom illuminated with red light just prior to its use. The vials were completely covered with aluminum foil and incubated at

27 C for 1 hr. For controls, microsclerotial specimens were (1) incubated in the non-DAB medium (3% cacodylate buffer, pH 7.4), or (2) preincubated in 1mM of potassium cyanide (KCN) in the buffer solution for 30 min at 27 C before being placed in the reaction medium. Both KCN preincubated and the non-DAB buffer cultures were incubated for 1 hr in the reaction medium. All reactions were terminated by washing 6 times with cold 3% cacodylate buffered solution during a 1 hr period. The specimens were postfixed and dehydrated as previously stated in the Methods section for transmission electron microscopy.

# CHAPTER IV

# RESULTS

## General Features of Growth

Potato dextrose agar plates over-laid with cellophane were inoculated with hyphae from the apical region of stock cultures grown on PDA. After 9 days in culture, t-9 and alm-1 mutants were similar in their linear growth, 4.5 centimeters (cm) in diameter (Figs. 1a and 1c), while the brm-1 mutant culture had spread 3.6 cm (Fig. 1b). After 6 days of growth, t-9 had begun the conversion from hyaline to black and had abrittle texture from the center to approximately 1.5 cm from the periphery. The outer 0.9 cm of t-9 remained hyaline and had a soft texture. After 6 days, alm-1 had produced a mat of mycelium with hyaline hyphae from the outer perimeter to approximately 0.5 cm from the center. Beyond 0.5 cm inward the mat had changed to a brittle texture, but remained hyaline in color. After 6 days, the cultured brm-1 resembled t-9 with the exceptions of color and size of mycelial mat. From the center outward 1 to 1.5 cm, the brm-1 mat was brownish-red, with a tough and brittle texture. The mycelium mat was hyaline in color from approximately 1 cm outward to the periphery.

When grown on agar plates (Table 1), the medium containing t-9 and alm-1 underwent no color changes while that

of brm-1 was brownish-pink due to a metabolite produced by the brm-1 mutant (Fig. 1b). When the 8th day brm-1 mycelial mat, which had been grown on PDA, was removed and replaced by the 8-day old alm-1 mycelial mat, there was a conversion of the mat from hyaline (Fig. 2a) to dark brown (Fig. 2b) and finally to black (Fig. 2d). Replacing brm-1 with t-9 did not show any morphological or color change in t-9.

In order to determine if the brm-1 mutant would induce the alm-1 mutant to produce a compound which would facilitate melanin production in brm-1, alm-1 was used to replace brm-1 that had been growing for 8 days. The alm-1 was allowed to grow for 12 hr prior to adding back brm-1. The results showed no production of melanin in the brm-1 mutant.

The inner portion of the alm-1 that had been induced (Fig. 3a) turned black, thus resembling t-9 (Fig. 3b). The texture of the induced alm-1 (Fig. 3a) was identical to t-9 (Fig. 3b).

## Phase Contrast Microscopy

Fifty ml of PGAM in flasks were inoculated with a $1 \times 10^5$ conidia/ml suspension. After 24 hr the conidia had produced hyphae. These hyphae produced conidia by "pinching off" at their tips (Fig. 4a). After 24 hr, the amount of conidia had increased two-fold, with some conidia

Fig. 1. Hyphal cultures growing on PDA for 9 days.

a. alm-1

b. brm-1

c. t-9

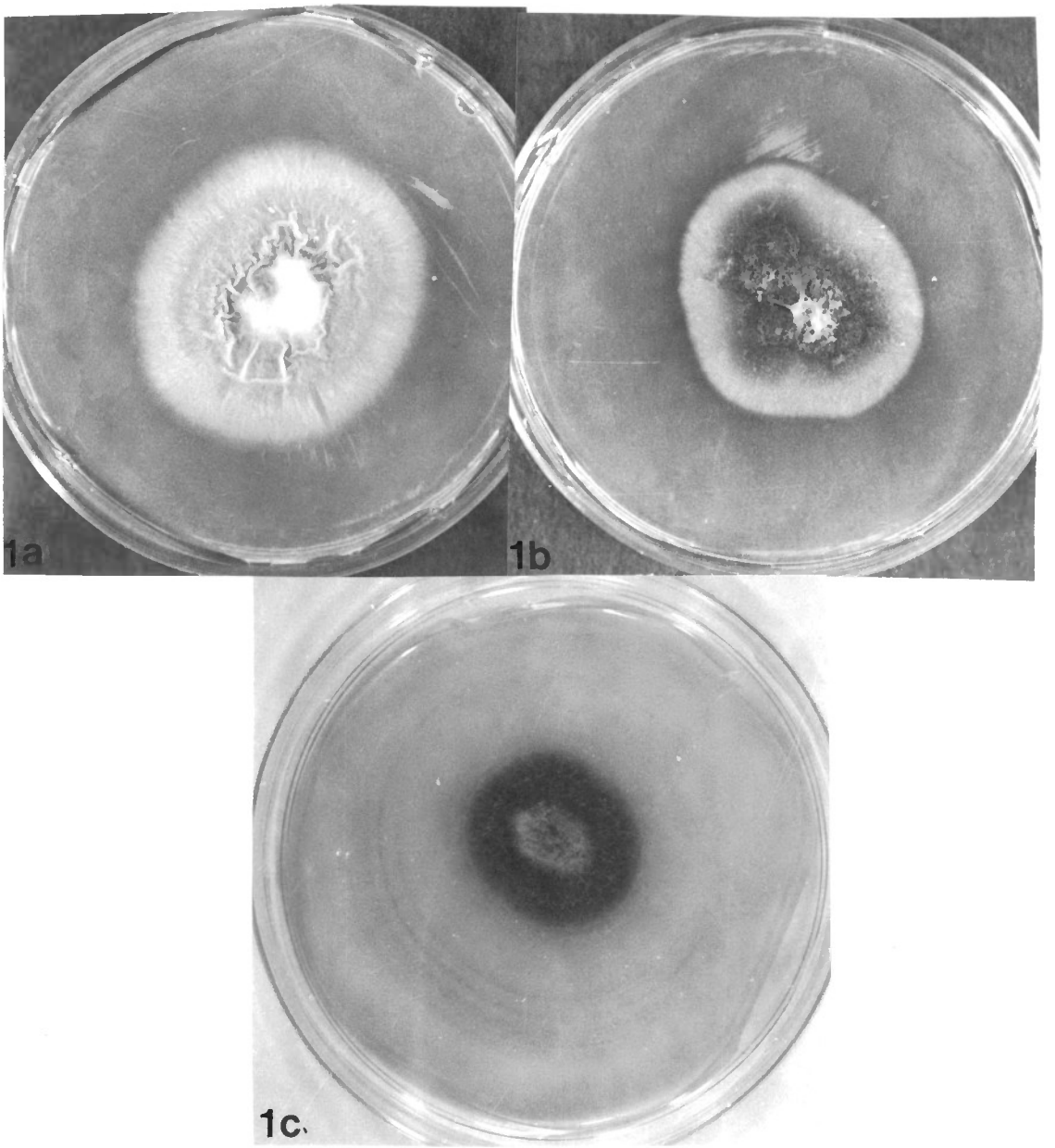

1a
1b
1c

Table 1. Characteristics of wild-type and melanin-deficient mutants of Verticillium dahliae

| Mutants | Microsclerotia formed | Microsclerotia color | | Medium color | (+)-Scytalone | |
|---|---|---|---|---|---|---|
| | | Agar | Liquid | | Accumulated in medium | Converted to melanin |
| Brm-1 | + | Brown-Red | Pale Pink | Pink | + | - |
| Alm-1 | + | Hyaline | Hyaline | Clear | - | - |
| Wild-type | + | Black | Black | Clear | - | + |

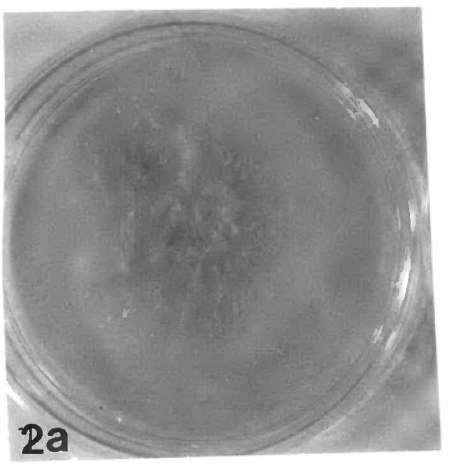
Fig. 2. Appearance of alm-1 on cellophane at various time intervals following their placement on the surface of PDA previously occupied by brm-1. The brm-1 mycelium was removed after 8 days of growth.

a. 1 min

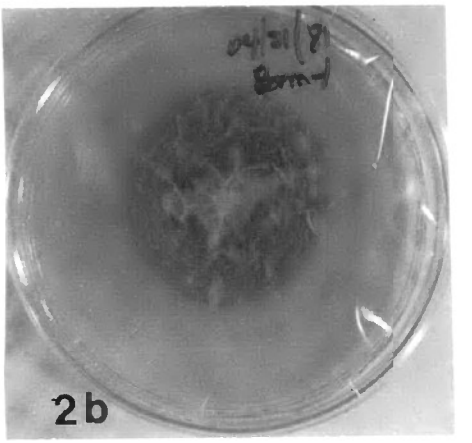
b. 15 min

c. 1 hr

d. 12 hr



2a



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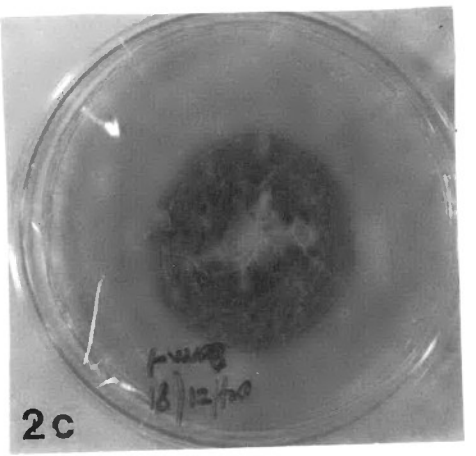
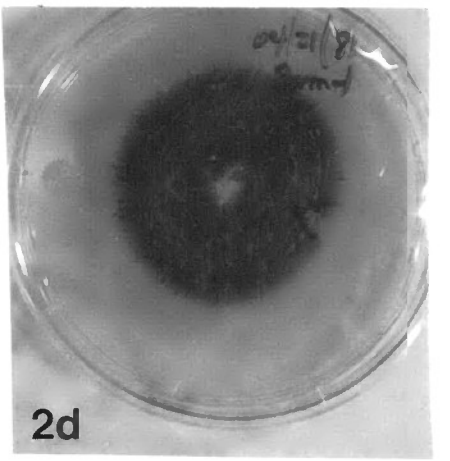


2c

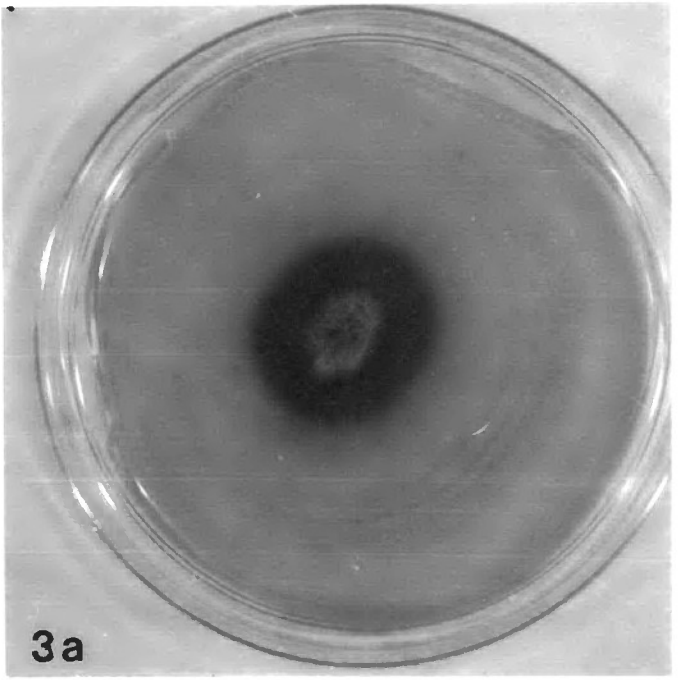


2d

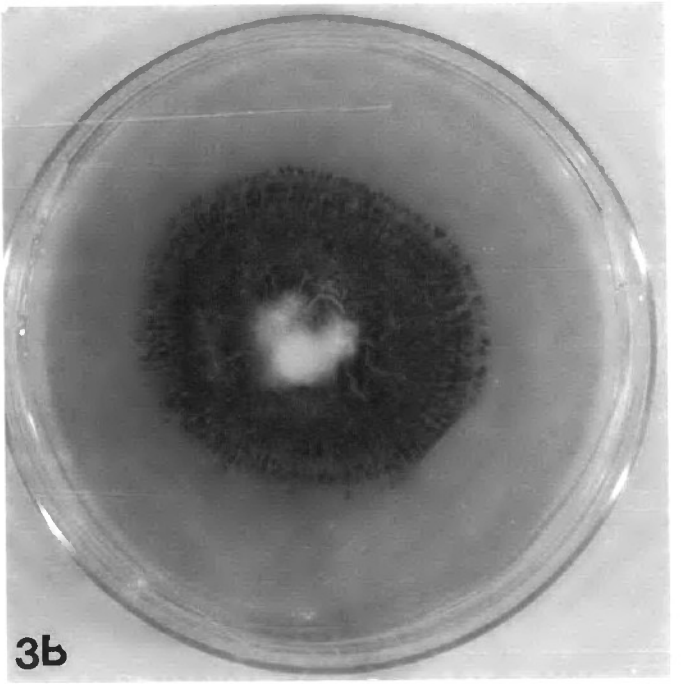
Fig. 3. *Verticillium dahliae* grown for 9 days on PDA.

a. induced alm-1

b. t-9



3a



3b

forming germ tubes (germination). Initially, microsclerotial development began when the germ tubes started to elongate. The number of germ tubes may vary from conidia to conidia, forming bipolar (Fig. 4c) or unipolar (Fig. 4d) germ tubes. As the germ tubes extend, they take on a chain-like appearance (Figs. 5a and 5b). In the chain-like structure, some cells begin to change their appearance by becoming globose (Figs. 5c and 5d). Further development takes place by cell enlargement, thickening of the cell wall and an increase in the size of lipid globules (Fig. 6).

Wild-type, alm-1 and brm-1 developed similar with the exception of the production of melanin in t-9 and a colored metabolite in the medium of brm-1.

## Transmission Electron Microscopy

With the aid of electron microscopy, t-9 strain and the alm-1, microsclerotia mutants of *V. dahliae* were compared as they developed in PGAM and PDA.

In alm-1 hyphae, there were few single membrane vesicles (Fig. 7), while in t-9 (Fig. 8) there were more single membrane vesicles which appeared to be attached to the cell membrane. There were also double membrane vesicles in t-9. At 3.5 days, fibrous structures appear on the wall of young microsclerotia of t-9 (Fig. 9) and also on 3.5 days old alm-1 (Fig. 7). As t-9 grew older, 4.5

Fig. 4. Production and germination of a conidium.

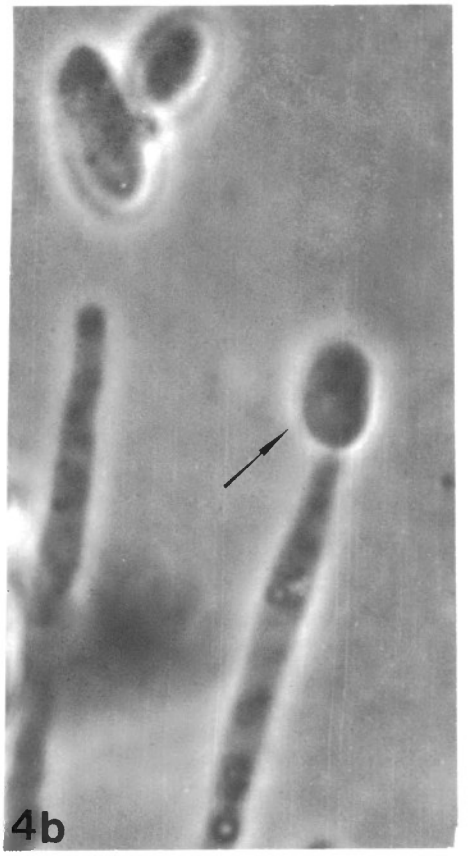
a. "pinching off" process (arrow)

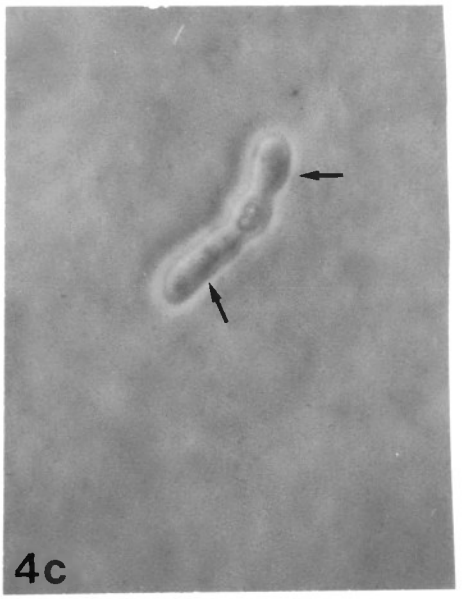
b. enlargement (arrow)

c. bipolar germination (arrow)

d. unipolar germination (arrow)


4a


4b


4c

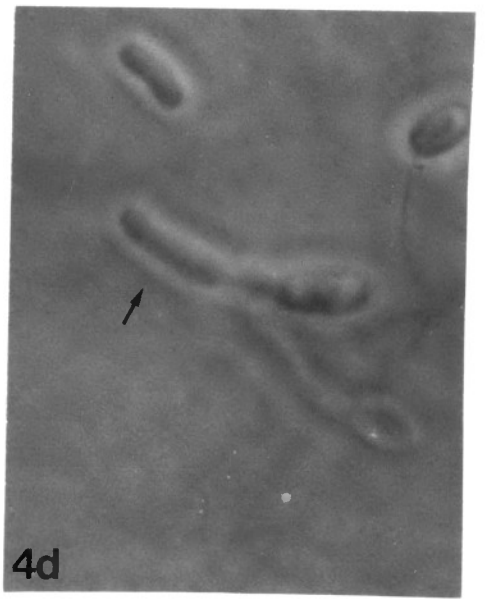

4d

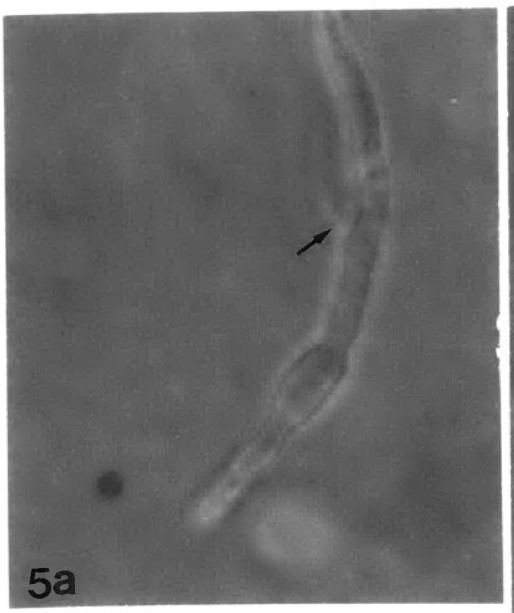
Fig. 5. Development of microsclerotium from a conidium

a. elongation of germ tube (arrow)

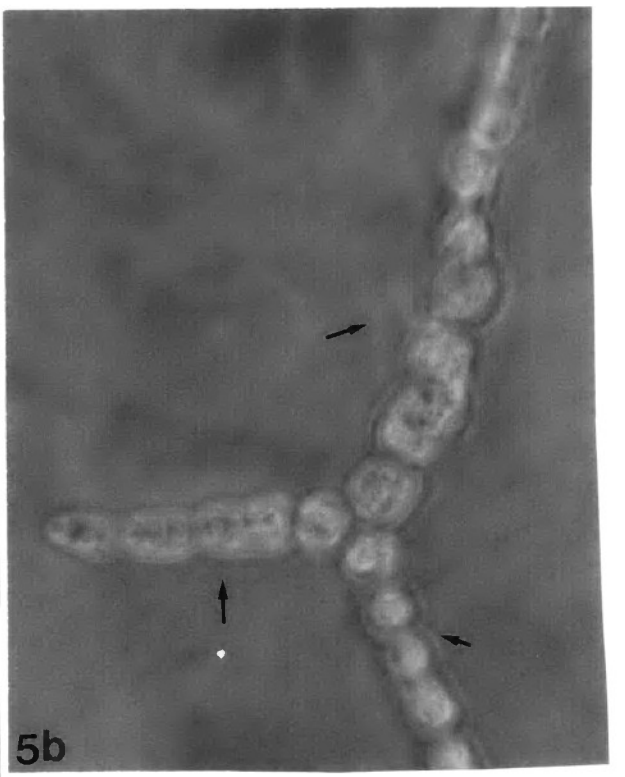
b. "chain-like" appearance (arrow)

c. young microsclerotia (arrow)

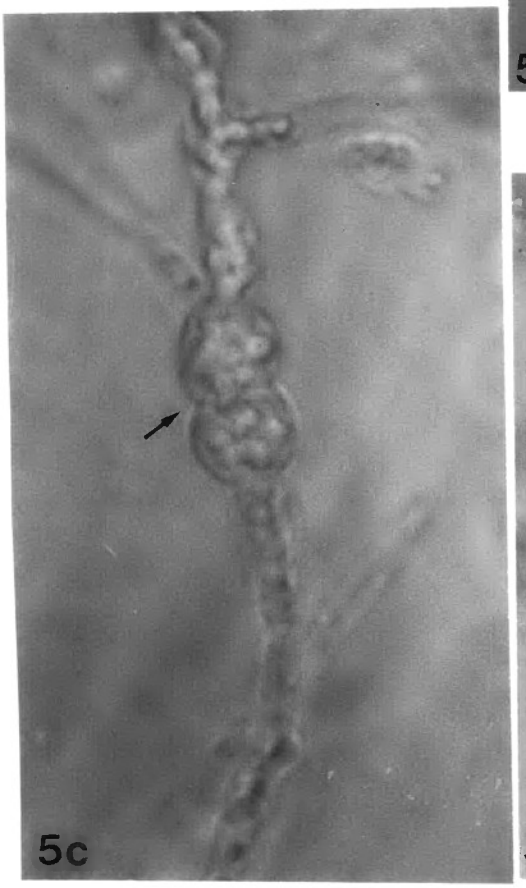
d. high magnification of young microsclerotia



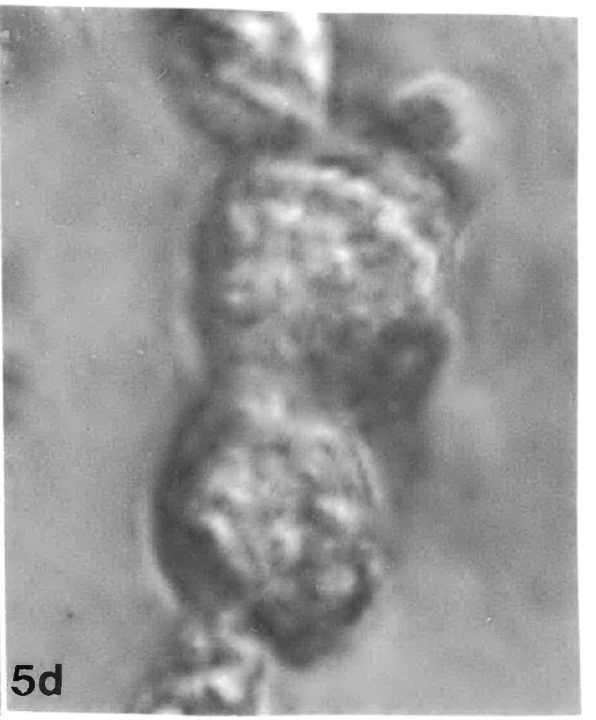
5a



5b



5c



5d

Fig. 6. Young microsclerotium at 72 hr. Note budding of microsclerotia (arrow).

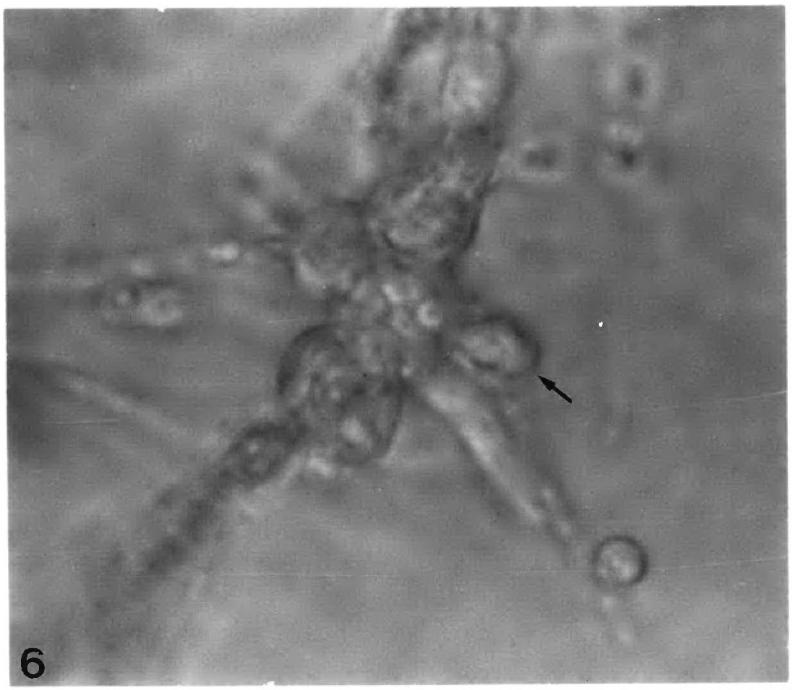
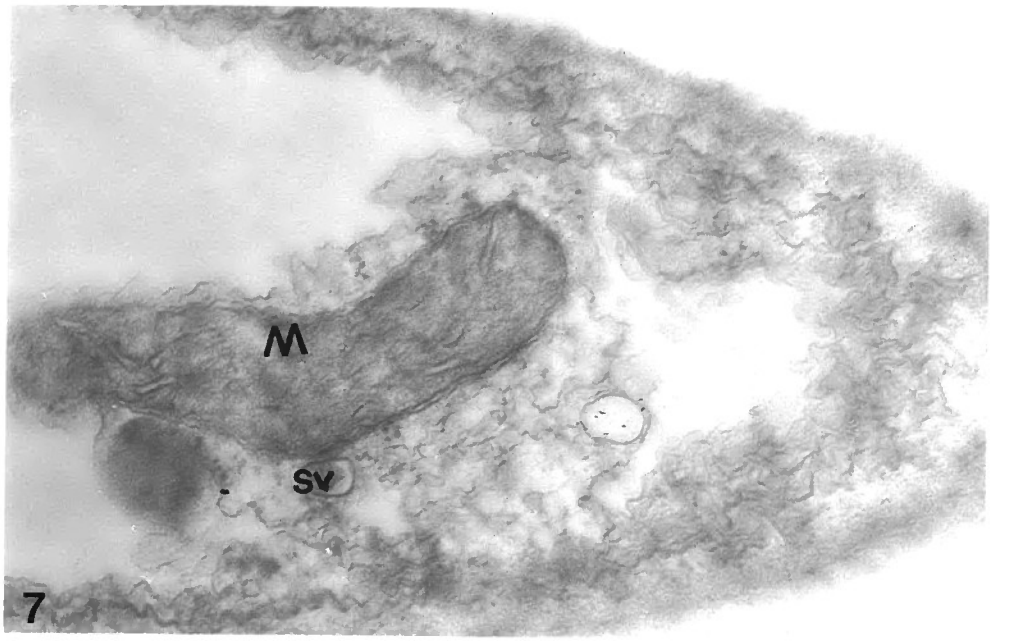

6

Fig. 7. Albino-1 hypha exhibiting altered mitochondrion (M) with nearby single membrane vesicles (sv). X105,000

Fig. 8. Wild-type hypha exhibiting double membrane vesicles (dv). Note altered mitochondrion (M). X105,000


M
sv
7

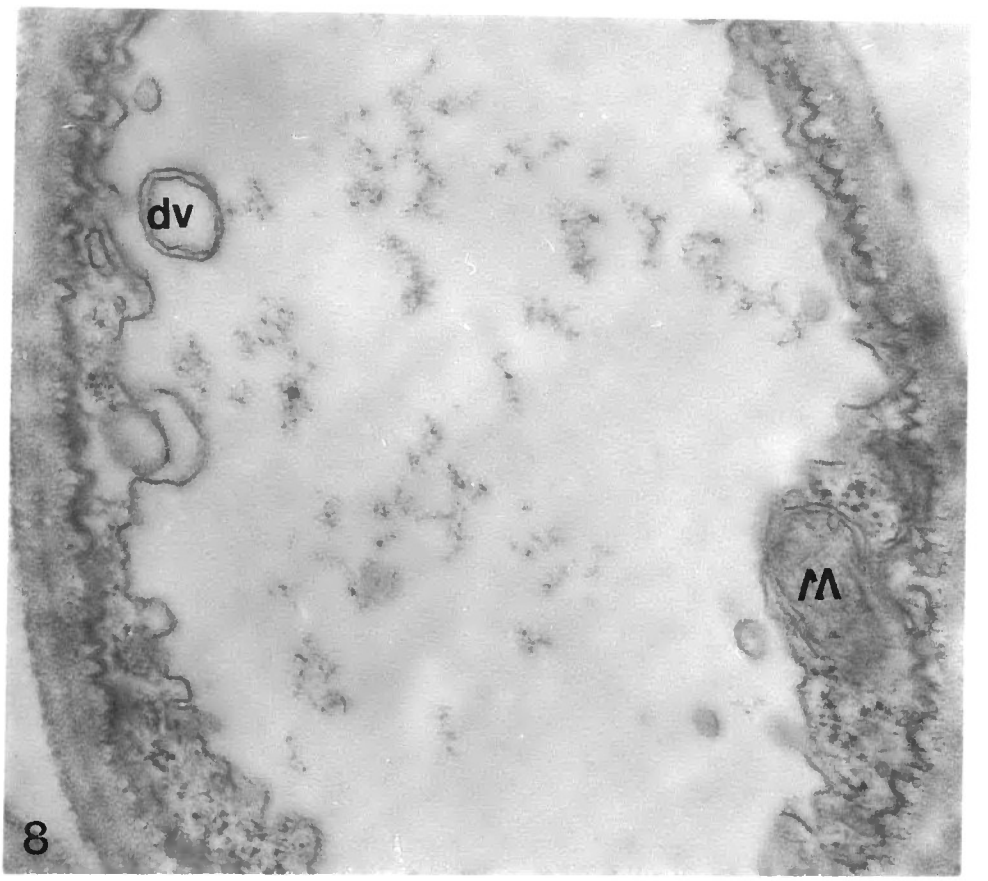
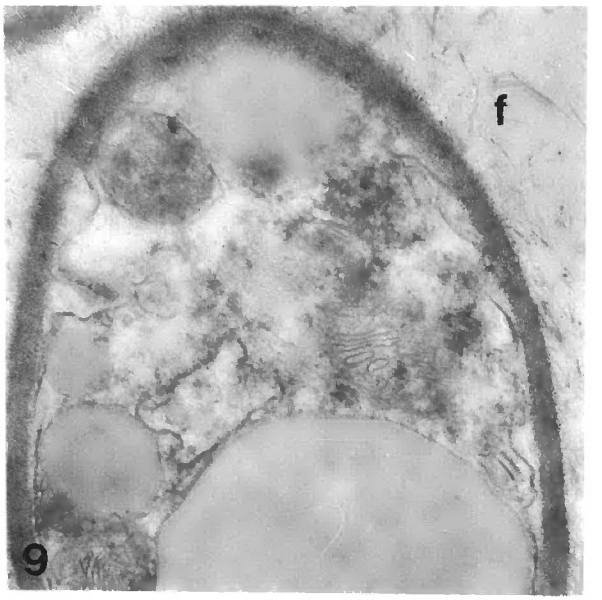
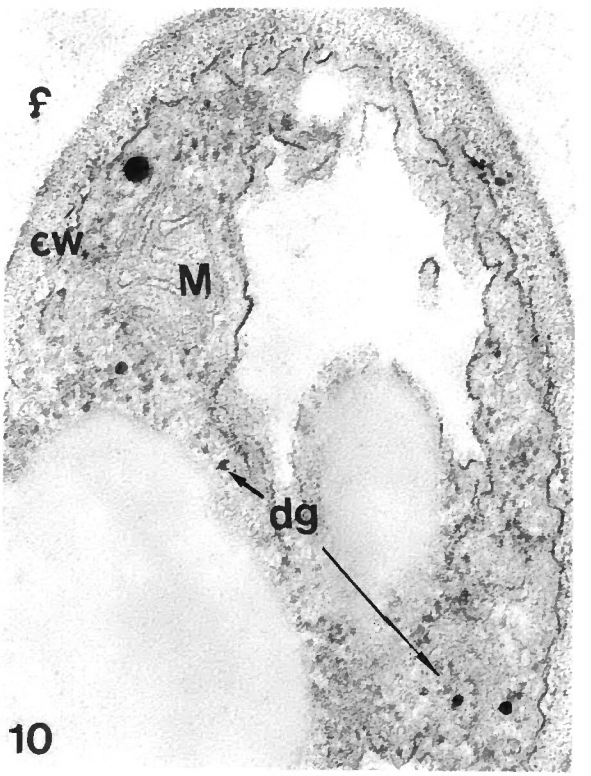

dv
M
8

Fig. 9. Wild-type cell exhibiting extracellular fibers (f). X75,000

Fig. 10. Young wild-type containing mitochondrion (M), microtubules (mt) and cytoplasmic dense granules (dg). Note fibers (f) outside of cell wall (cw). X105,000


f
9


f
cw
M
dg
10

days, a noticeable decrease in double membrane vesicles was observed (Fig. 9).

During the early stages of development, there were several different sized single membrane vesicles formed in alm-1 (Fig. 11c). Some of these observed vesicles were seen attached to the cell membrane (Fig. 12) of alm-1. Morphologically, the single membrane cytoplasmic vesicles are round to oblong in shape. No double membrane attached cytoplasmic vesicles were observed in t-9.

Lipid globules were observed varying in size from young microsclerotia of t-9 (Fig. 10) and alm-1 (Fig. 12) to older microsclerotia of t-9 (Fig. 21) and alm-1 (Fig. 22). In early stages, lipid globules tend to develop rapidly in t-9 (Fig. 9) and alm-1 (Fig. 13). As lipid globules develop, their increasing size pushes the cytoplasmic organelles and nucleus toward the cell membrane (Figs. 13 and 17).

As microsclerotia develop, mitochondria may vary in shape from spherical to oblong or elongated (Fig. 14). These different shapes of mitochondria may be in one young microsclerotium at the same time. The spherical or oblong mitochondria in alm-1 (Fig. 12 and t-9 (Fig. 14) have numerous cristae throughout their inner membrane, while the elongated single membrane or tubular-like structures that existed within the cytoplasm (Fig. 12). Endoplasmic reticulum of t-9 (Fig. 14) and alm-1 (Fig. 16) has been

Fig. 11. Albino-1 showing membrane vesicle in different part of the cell. X75,000

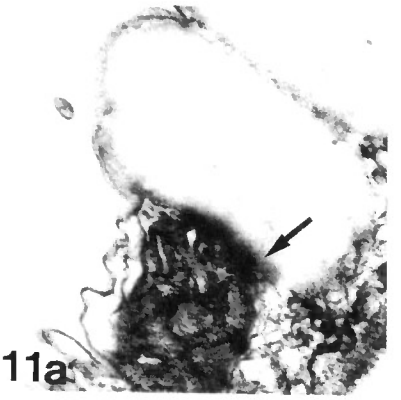
a. Altered mitochondrium with vesicle-like structure (arrow).

b. Single membrane vesicle with granules.

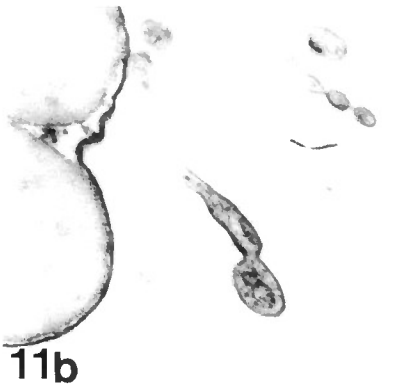
c. Vesicle near membrane (arrow).

d. Vesicles incorporated into cell membrane (arrow) with extracellular fibers (f).

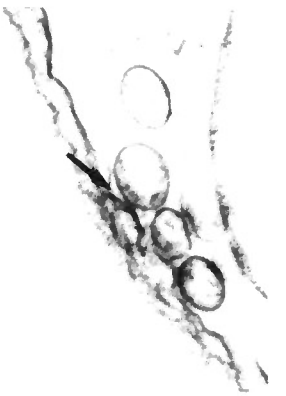
Fig. 12. Albino-1 exhibiting elongated mitochondrion (M) lipid globule (L) and single membrane vesicles (sv). Note the attachment of single membrane vesicle to cell membrane (cm). X105,000



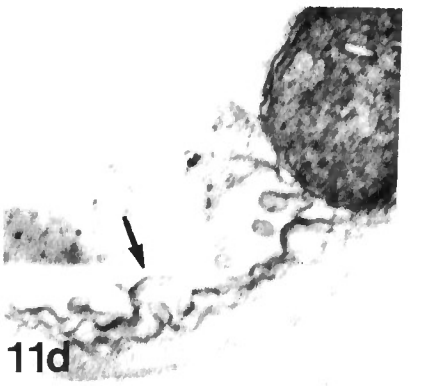
11a



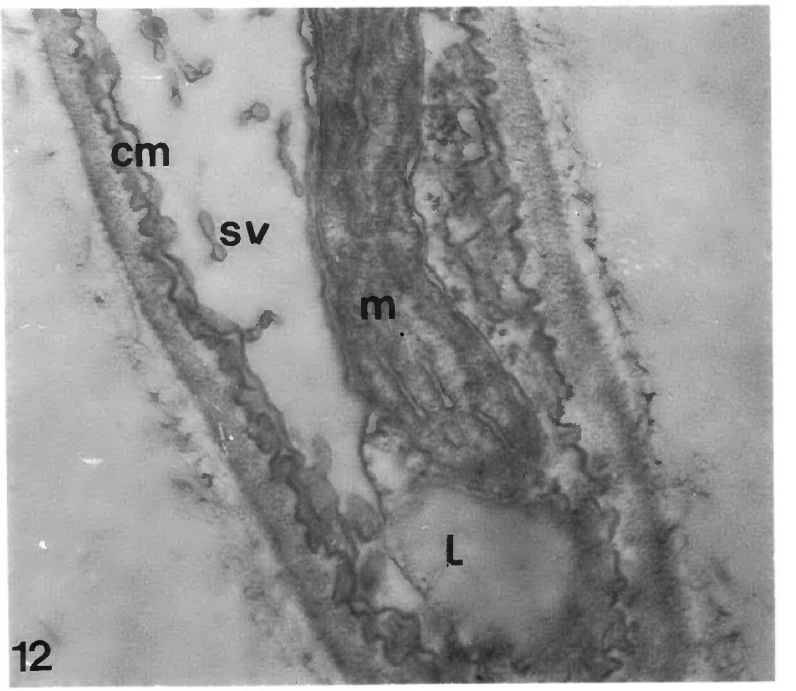
11b



11c



11d




12

Fig. 13. Albino-microsclerotium exhibiting a mitochondrion (M) and the nucleus (N) between lipid globules (L). Note discontinuity of cell membrane (arrow). X75,000

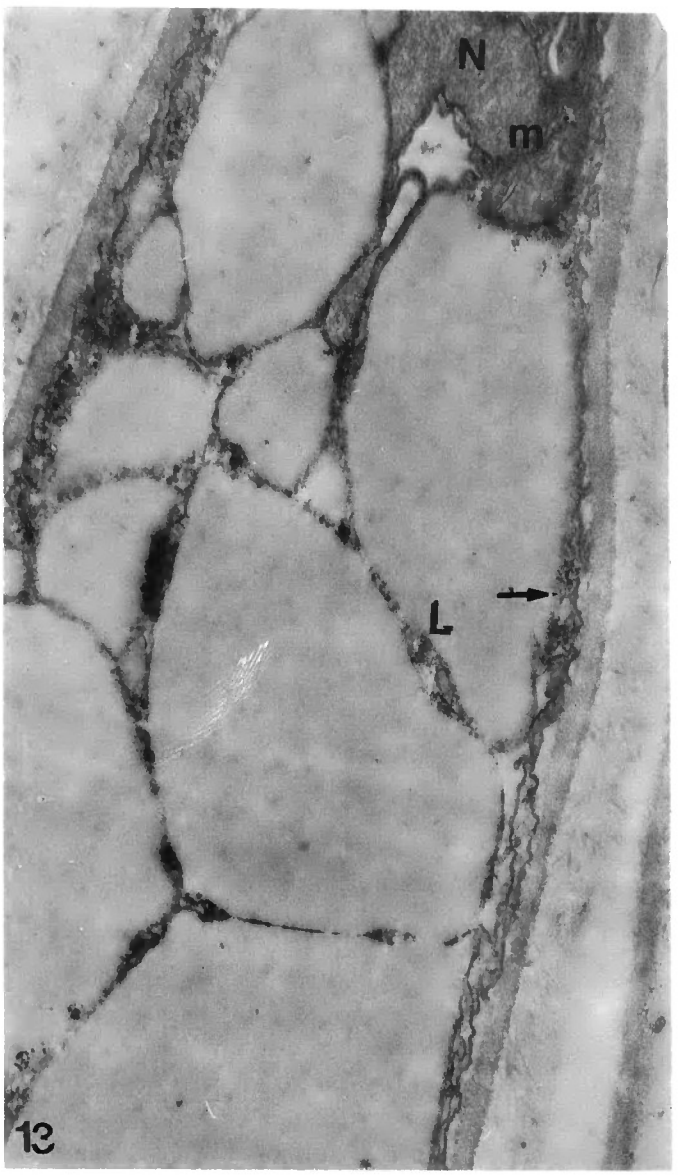

N
m
L
13

Fig. 14. Wild-type microsclerotium exhibiting mitochondrion (M), endoplasmic reiticulum (ER). X105,000

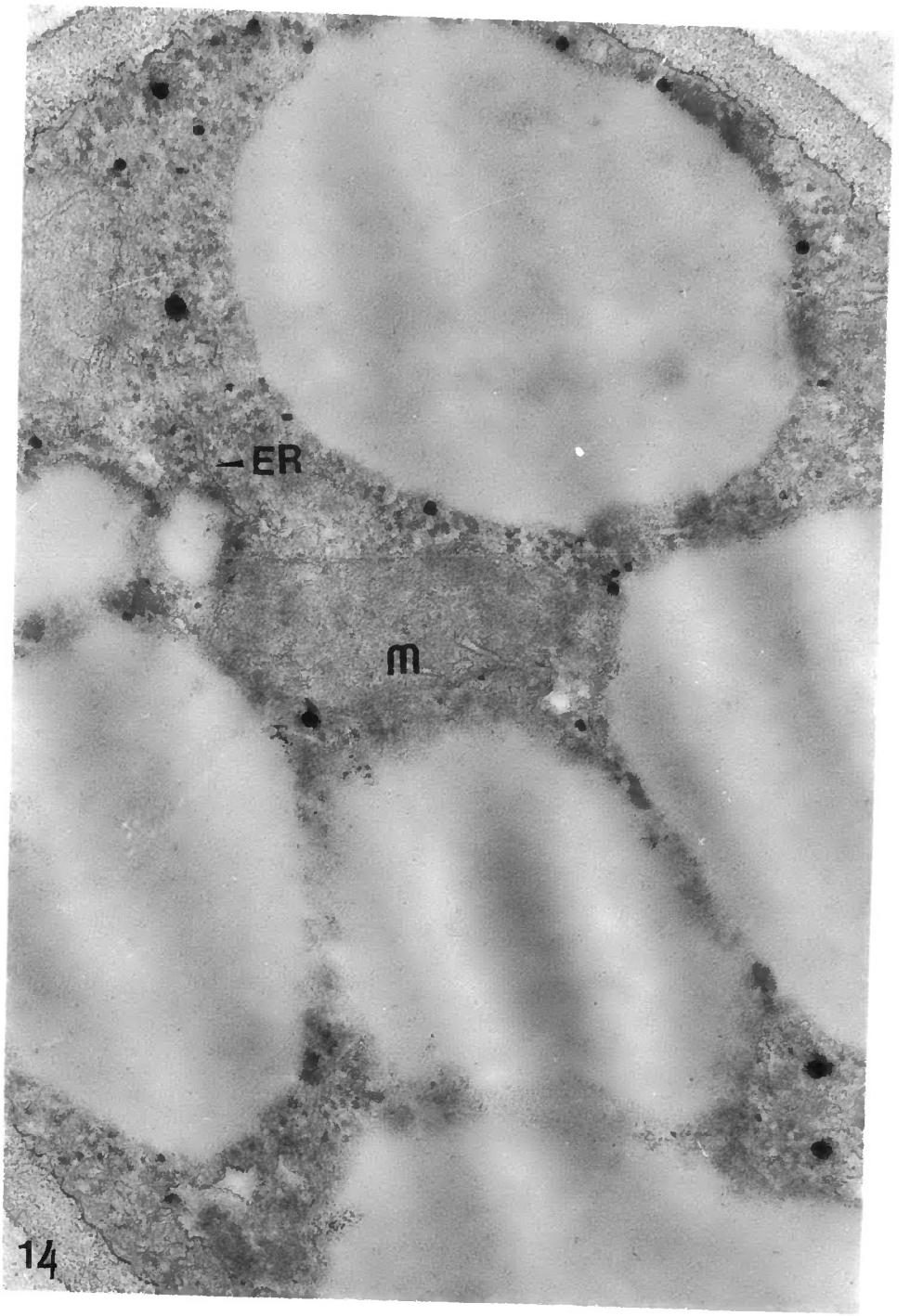
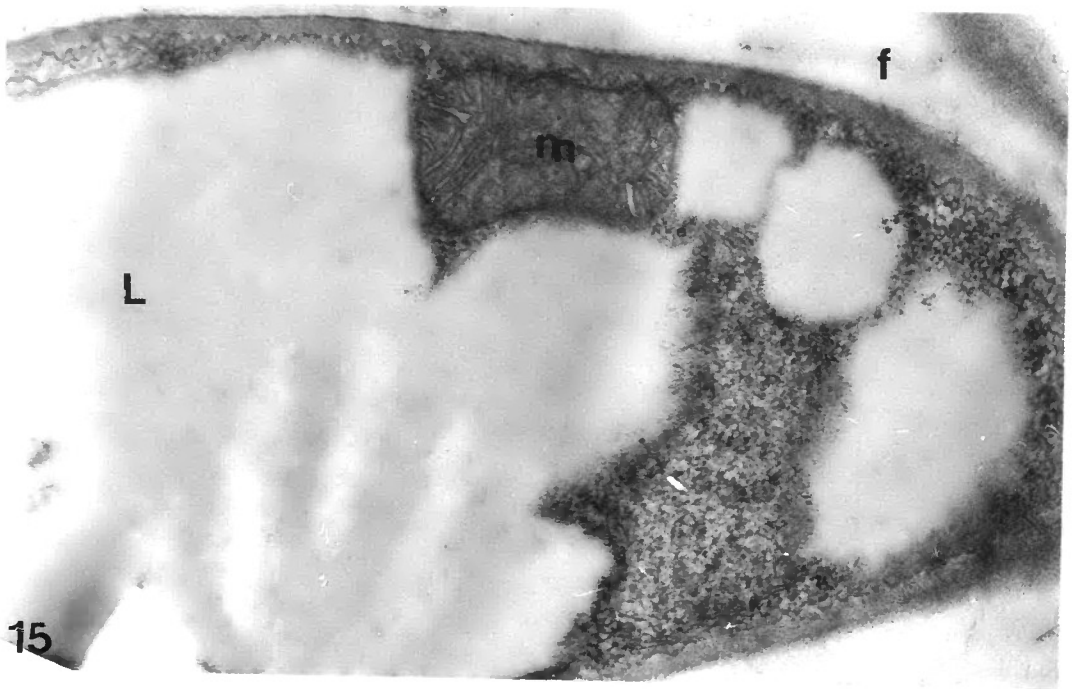
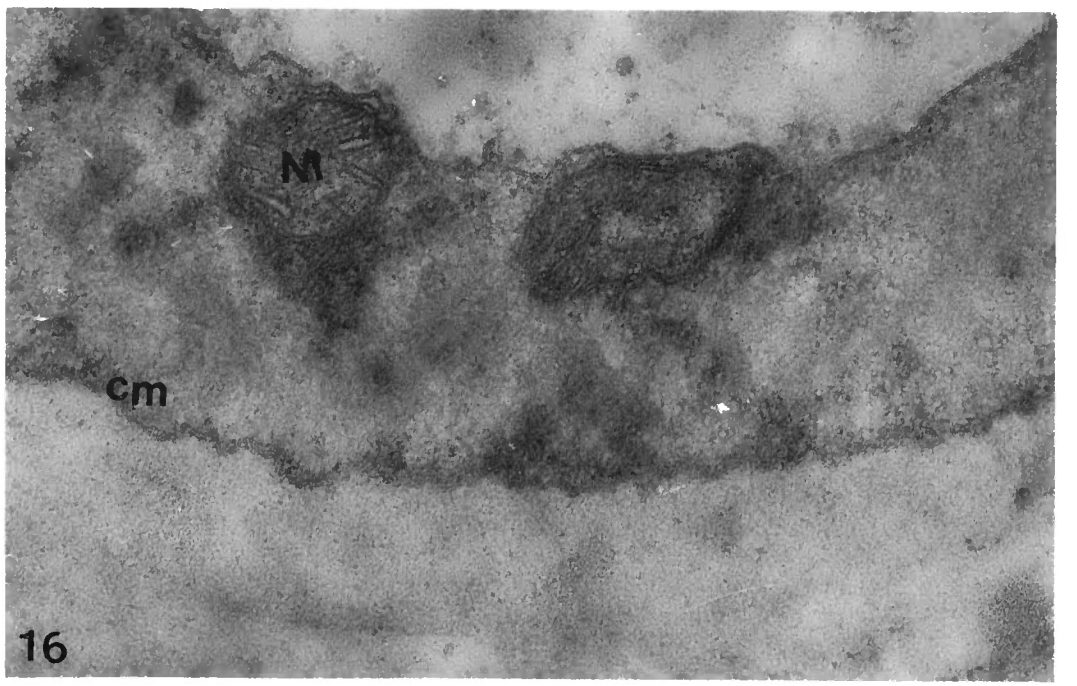

ER
m
14

Fig. 15. Albino-1 microsclerotium exhibiting altered mitochondrion (M) with increasing lipid globules (L). Note extracellular fibers (f). X75,000

Fig. 16. Albino-1 microsclerotium showing altered mitochondria (M) and discontinuity in cell membrane (cm). X130,000




15




16

observed stacked against each other, giving the appearance of tubules. This stacked-membrane like endoplasmic reticulum has been observed in 4 day to 6 day old microsclerotia.

In t-9 cells, electron-dense granules of varying densities were observed within the cytoplasm. Most of these electron-dense granules seem to be localized near large lipid globules (Figs. 10 and 14). Figure 17 shows electron-dense granules which were observed in localized areas near the mitochondria. These electron-dense granules are small and exist in groups. Further, these electron-dense granules have only been observed in developing t-9 microsclerotia.

During microsclerotial development, the nucleus may vary in position. The nucleus may be pushed toward the cell membrane (Figs. 18 and 20) or remain between the lipid globules (Fig. 19). In some instances, discontinuity in the nuclear membrane may be observed (Fig. 17). The nucleus may also be in close proximity with nearby mitochondria (Fig. 20).

The cell membranes of t-9 microsclerotia (Fig. 21) are double membranes and have few folds than alm-1 (Fig. 22). Between the cell membrane and the cell wall, unstained particles were observed in alm-1 (Fig. 22), while electron-dense particles were seen in t-9 (Fig. 23). The cell membrane of alm-1 (Fig. 11d) showed discontinuities

Fig. 17. Wild-type microsclerotium exhibiting circular (CM) and elongated mitochondria (EM), nucleus (N), cell membrane (cm) and lipid globules (L). Note vesicular bodies in vacuole (v). X51,000

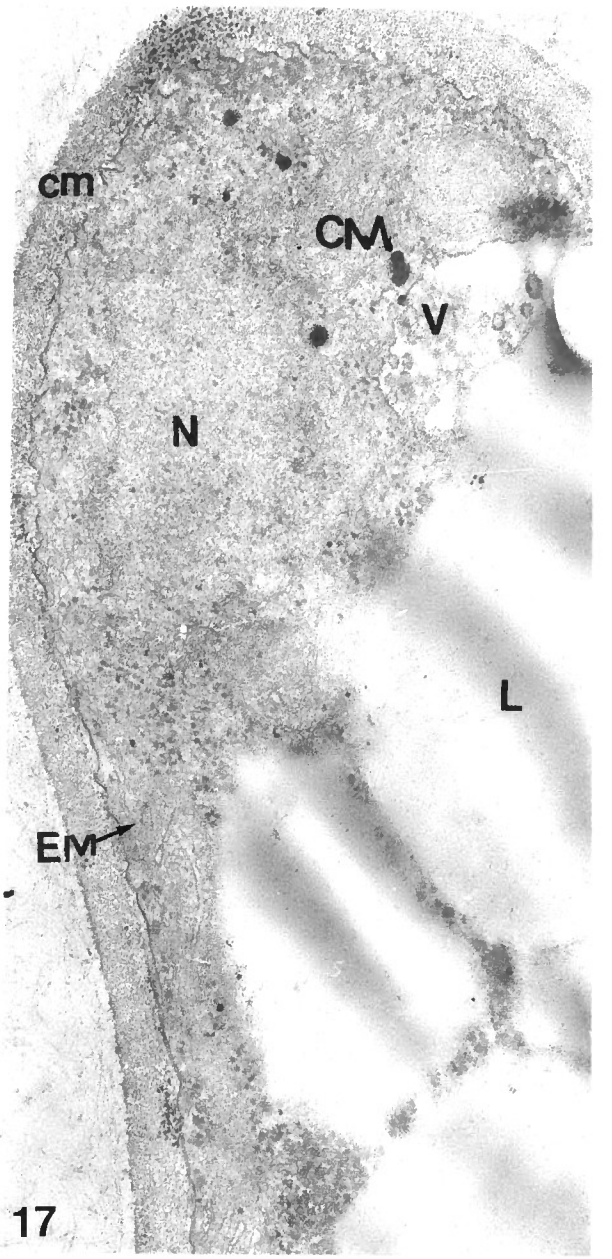
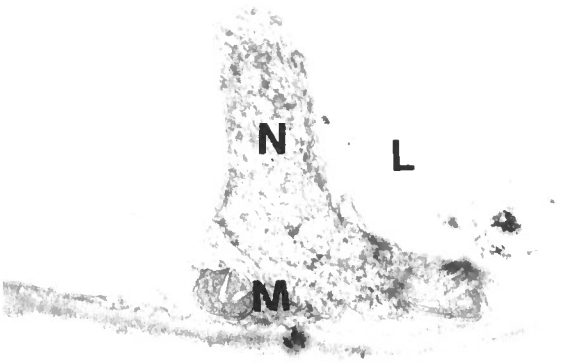
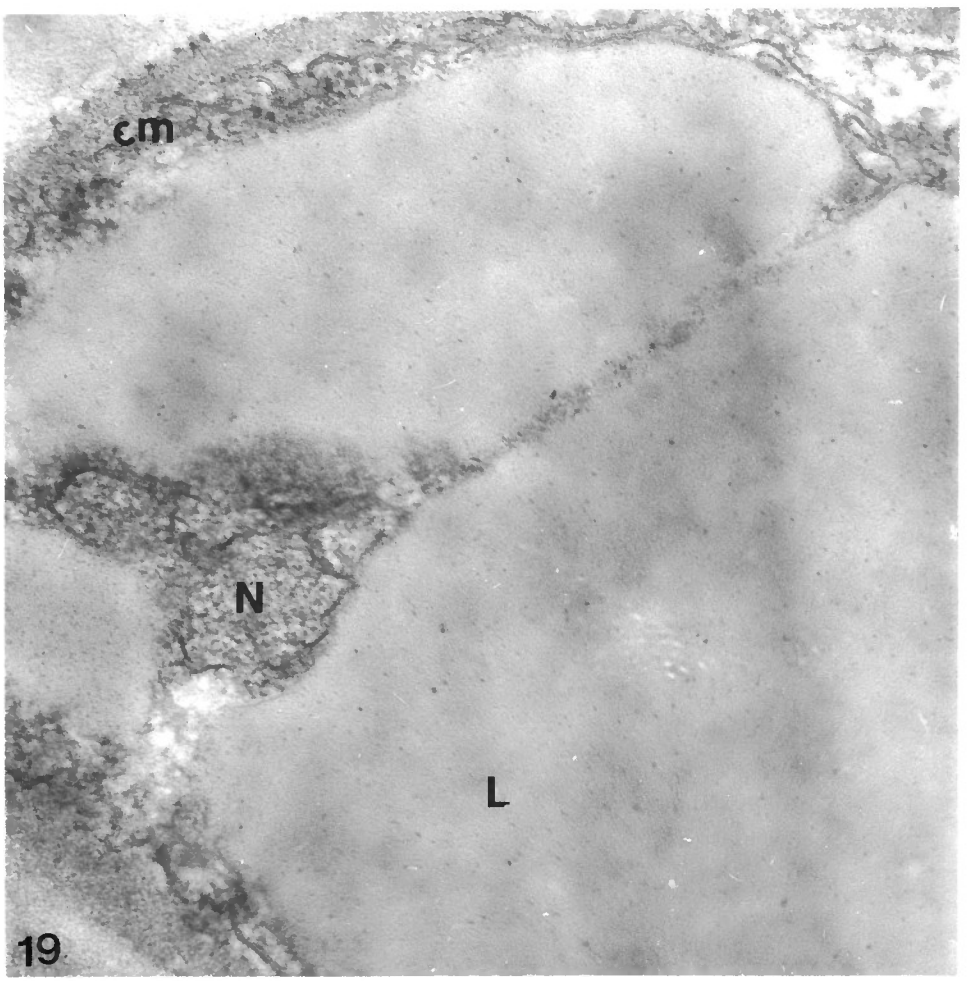

cm
CM
V
N
L
EM
17

Fig. 18. Albino-1 nucleus (N) and mitochondrion (M) are pushed toward the cell membrane (cm) by lipid globules (L). X31,000

Fig. 19. Albino-1 exhibiting nucleus (N) being squeezed by lipid globules (L). Note discontinuity in cell membrane (cm). X130,000

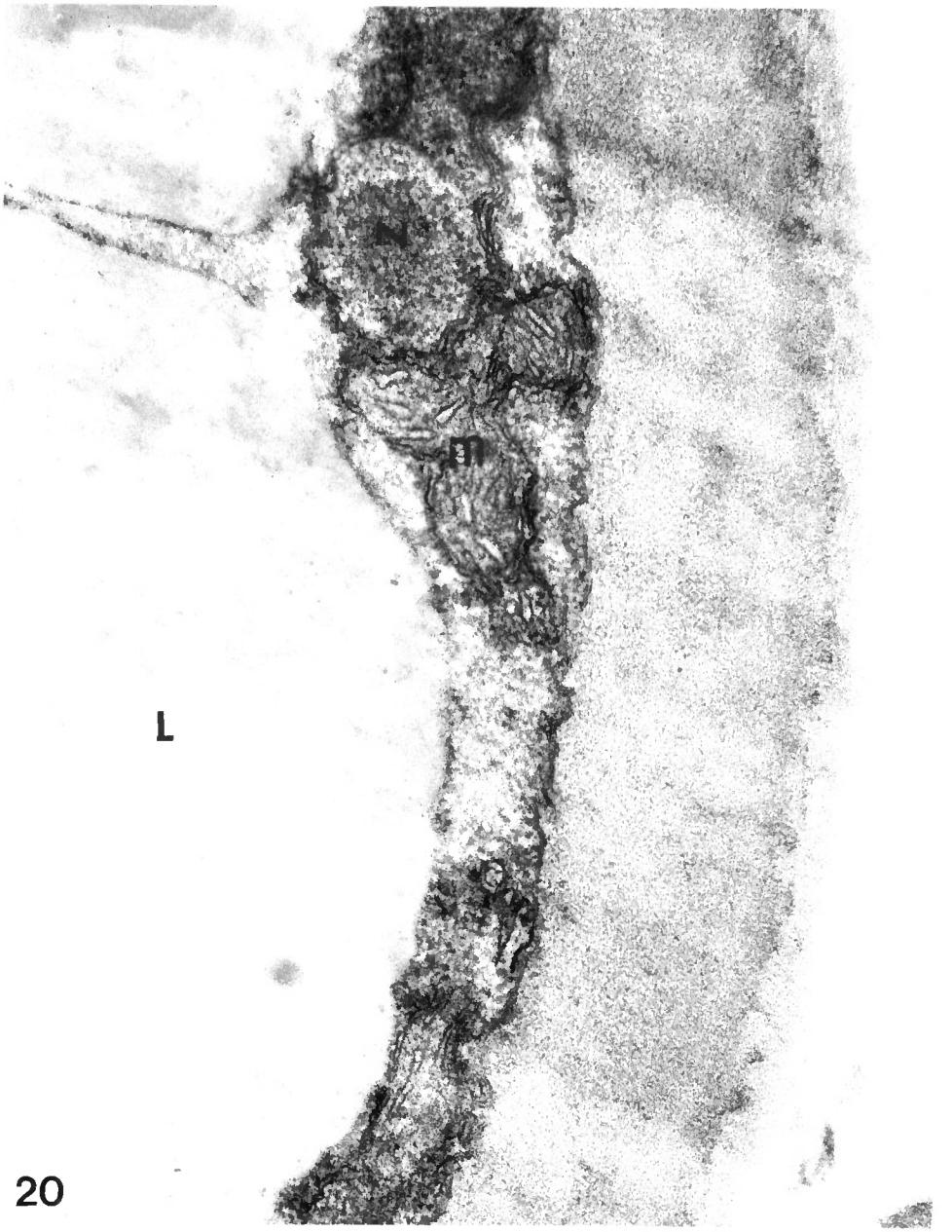



18




19

Fig. 20. Wild-type microsclerotium exhibiting nucleus (N) with nuclear membrane and dense chromatin materials. Note the apparently fused mitochondria (M) profile. X260,000




20

Fig. 21. Wild-type microsclerotia exhibiting melanin granules (mg) on the cell wall (cw) and cell membrane (cm). Note the size of the lipid globules (L), while the cell appears to be devoid of other organelles. X75,000

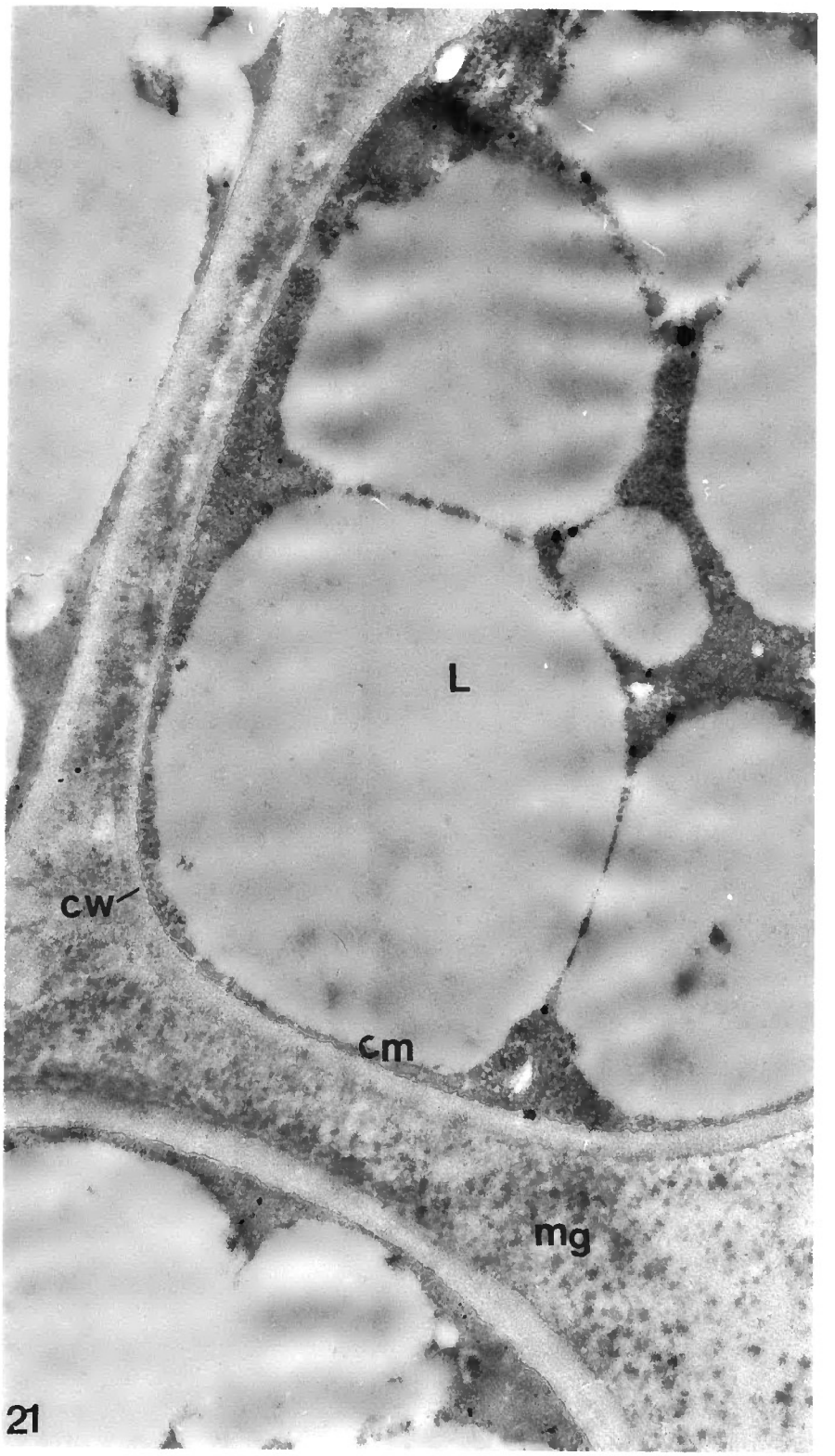

L
cw
cm
mg
21

Fig. 22. Albino-1 exhibiting small mitochondrial profiles (M), while particles are between cell wall (cw) and cell membrane (cm) with fold (arrow). X75,000

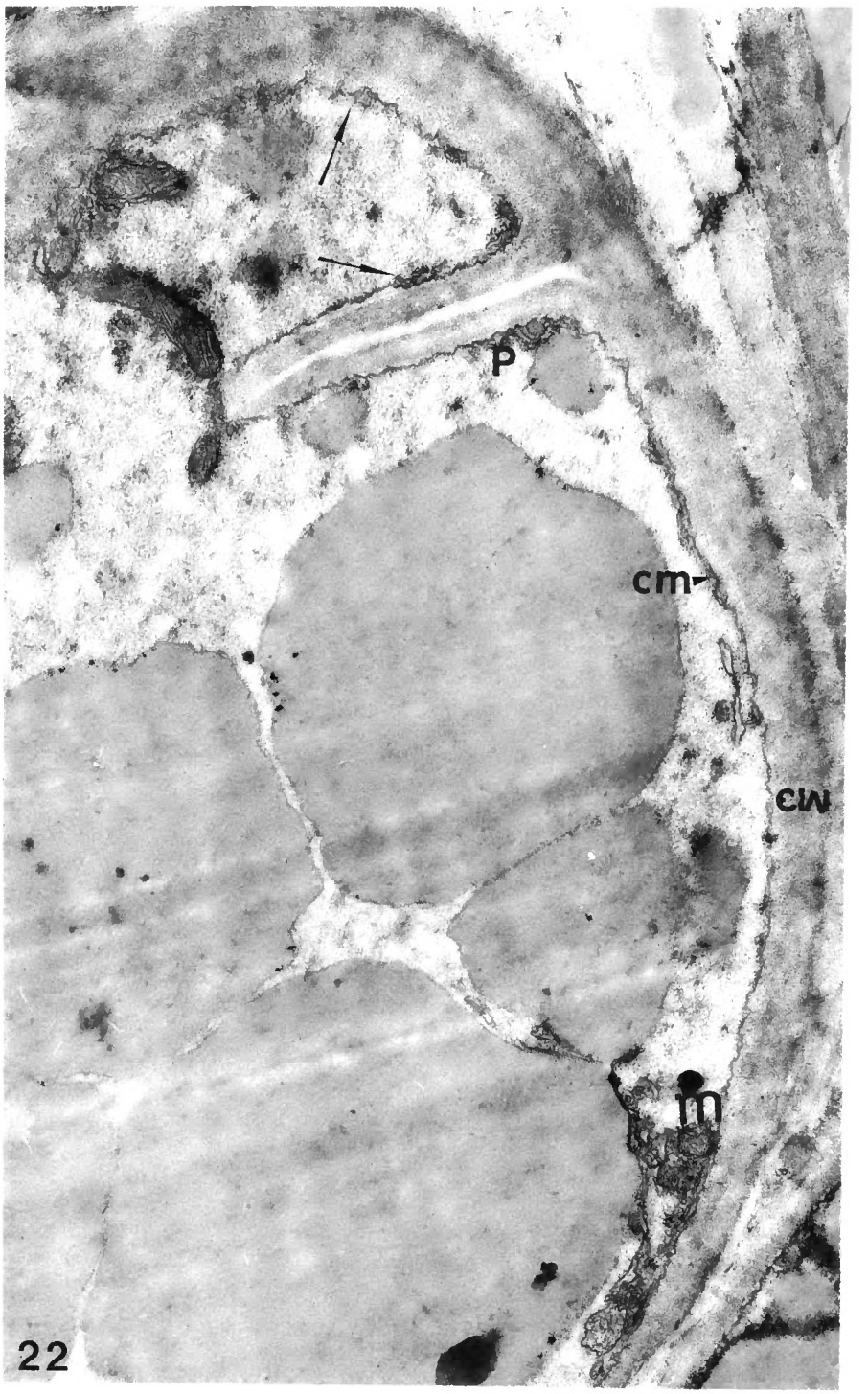
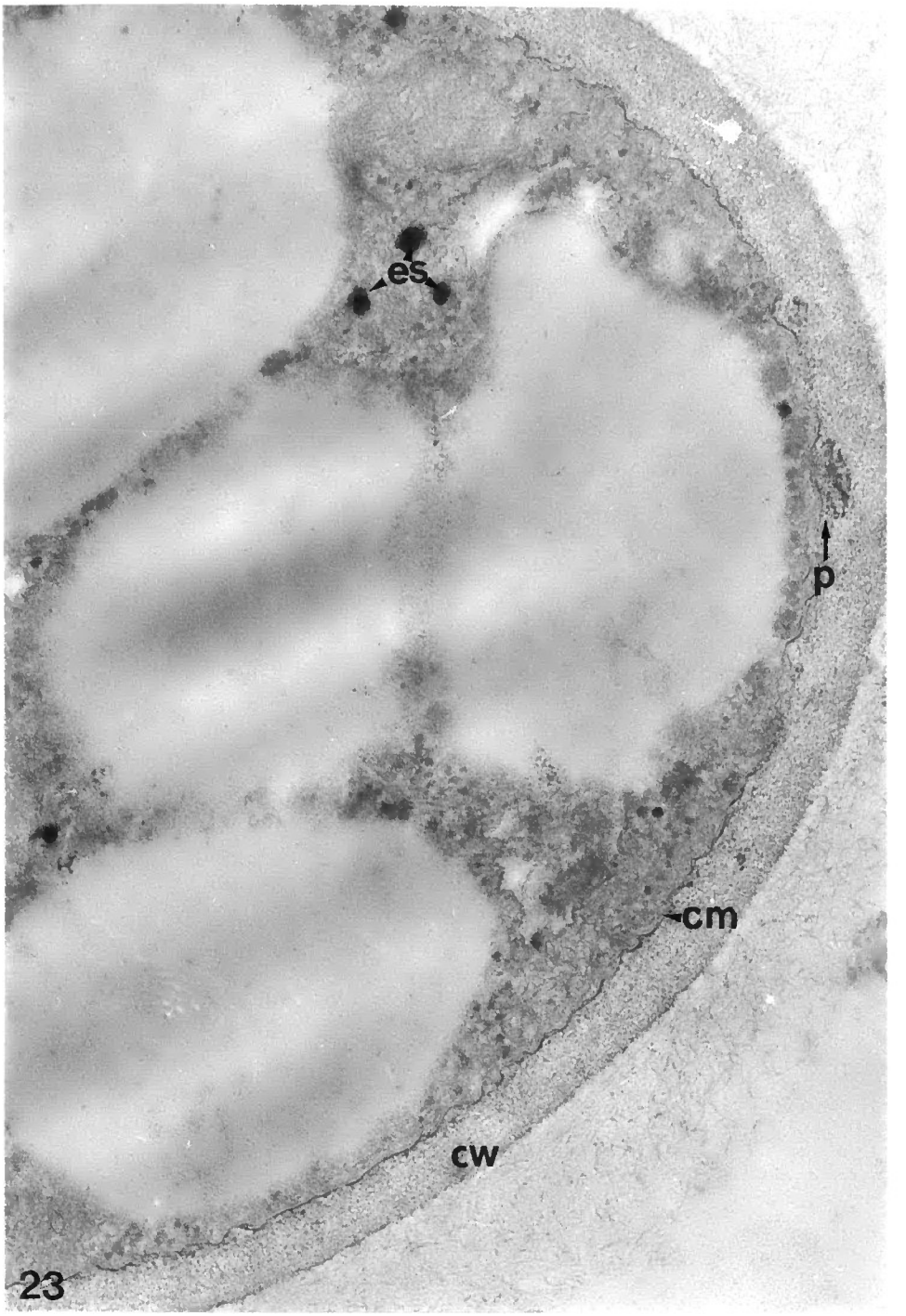

P
cm
cw
m
22

Fig. 23. Wild-type microsclerotium exhibiting particles (p) between the cell wall (cw) and cell membrane (cm). Note the electron-dense structure (es) in the cytoplasm. X105,000


es
p
cm
cw
23

at points where vesicles were seen attached.

The cell walls of both alm-1 and t-9 were similar in appearance, except the latter showed melanin granules within the outer cell wall (Fig. 10) and extracellular fibers at 4.5 days. Albino-1 also exhibited extracellular fibers at 4.5 days (Fig. 11d). The melanin granules were more abundant on the outer portion of the cell wall (Fig. 25) and in the extracellular fibers of t-9 (Figs. 21 and 24). Extracellular fibers of both, t-9 (Fig. 23) and alm-1 (Fig. 19) were similar in their appearances.

## Induction of Melanin Production in Albino-1 Microsclerotia Mutant

The scytalone-treated albino (s-alm-1) cells were observed in order to determine if the treatment had altered the cytoplasmic organelles or regions. Scytalone-treated albino-1 (Fig. 32) showed several different shapes of mitochondria like those observed in alm-1 (Figs. 12, 16) and t-9 (Fig. 14). Mitochondria were observed less frequently in alm-1 and t-9 than in s-alm-1. Wild-type mitochondrial membranes were observed in proximity with the nuclear membrane (Fig. 20), while s-alm-1 may show apparent nuclear-mitochondria contact (Fig. 30). Double and single membrane vesicles of s-alm-1 were also observed in close proximity to the nuclear membrane (Fig. 30). Elongated mitochondria of t-9 (Fig. 14) and alm-1 (Fig. 12) show a uniform arrangement of cristae, but s-alm-1 shows an

Fig. 24. Wild-type microsclerotium exhibiting melanin (mg) in the extracellular fiber region (f). Note electron-dense granules (eg) near mitochondrion (M). X105,000

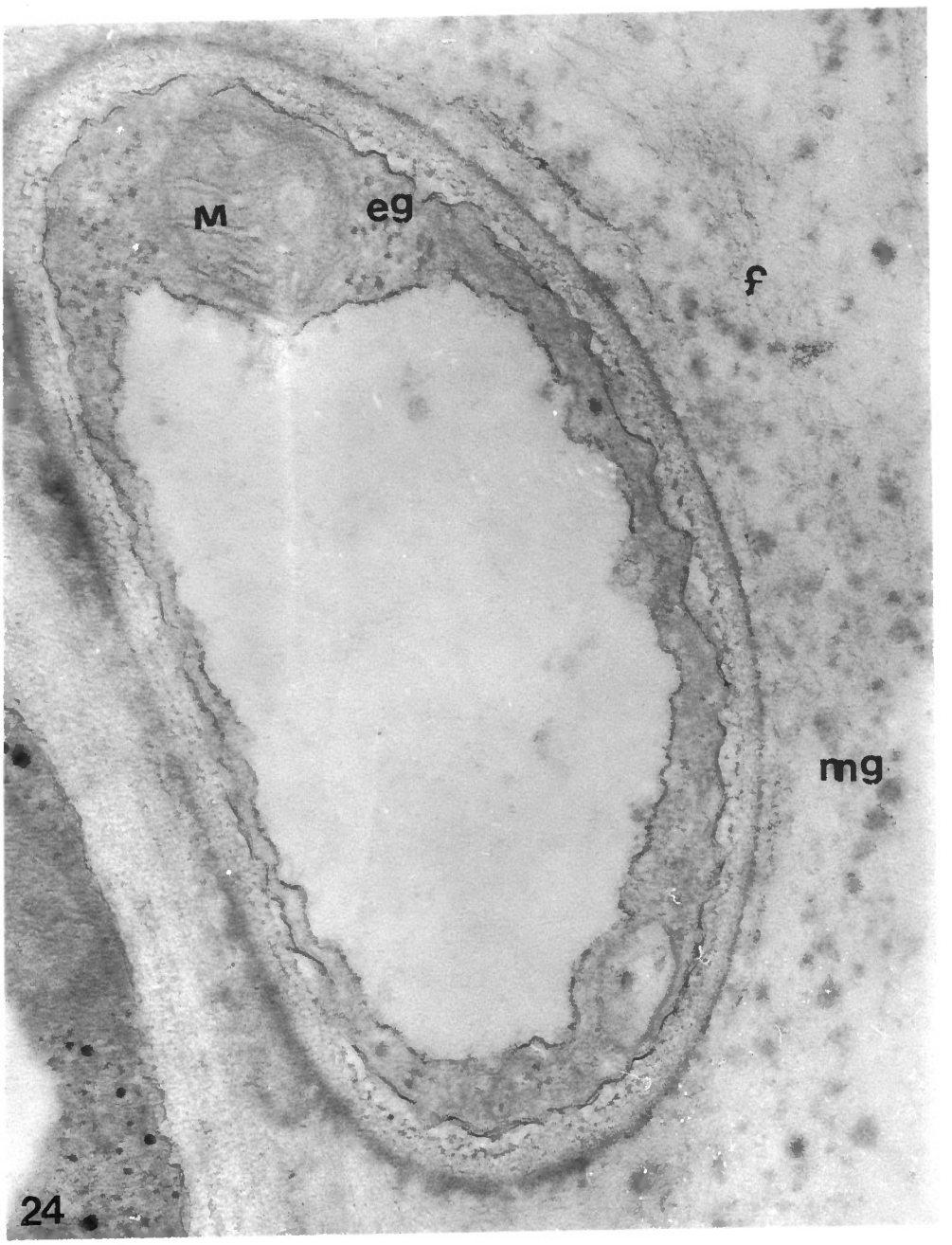

M
eg
f
mg
24

Fig. 25. Wild-type microsclerotia (upper half of micrograph) exhibiting melanin granules (mg) on and in the cell wall (cw), while young cell (lower half of micrograph) shows vesicles (v) with the cell wall (cw) devoid of melanin granules. X150,000

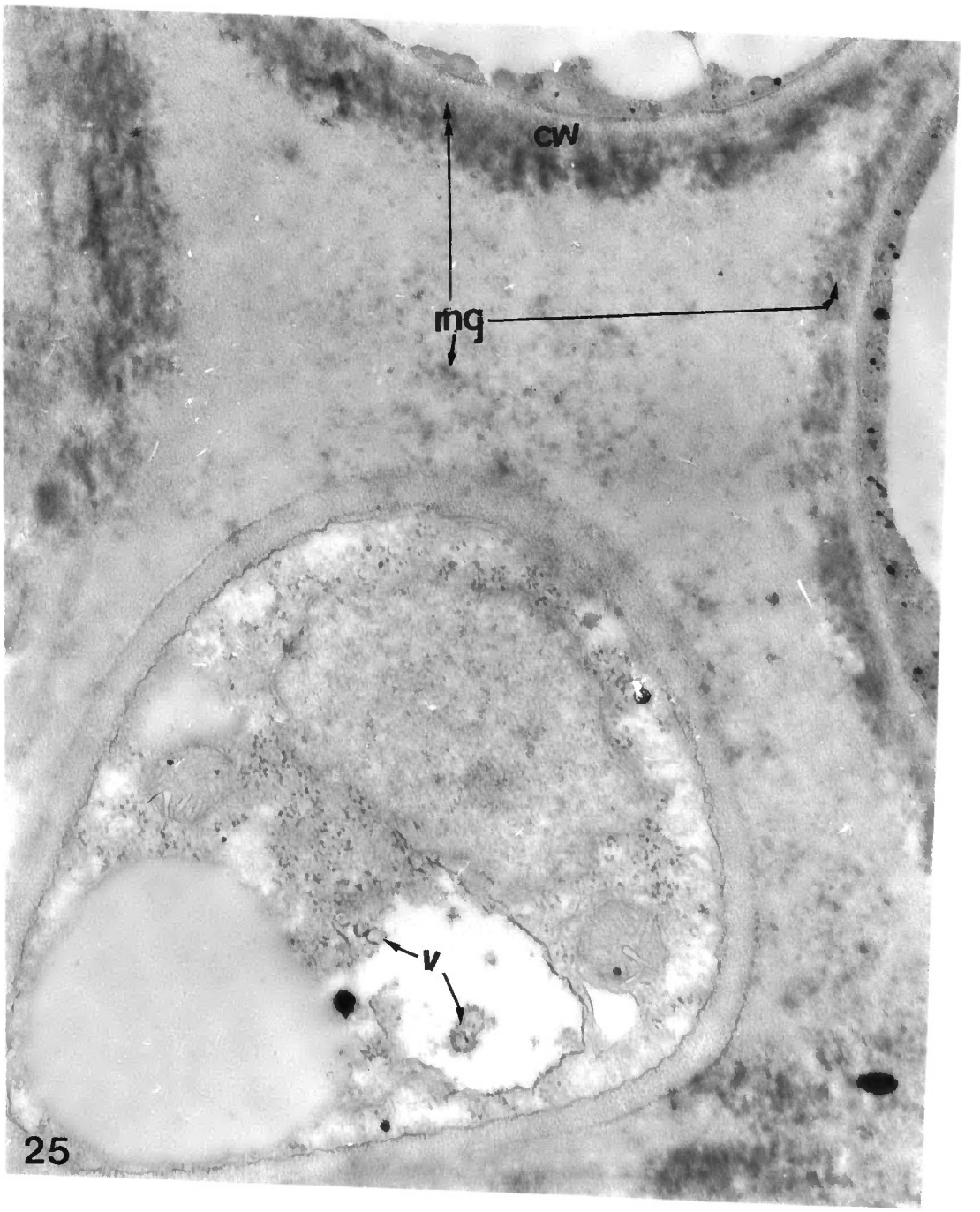
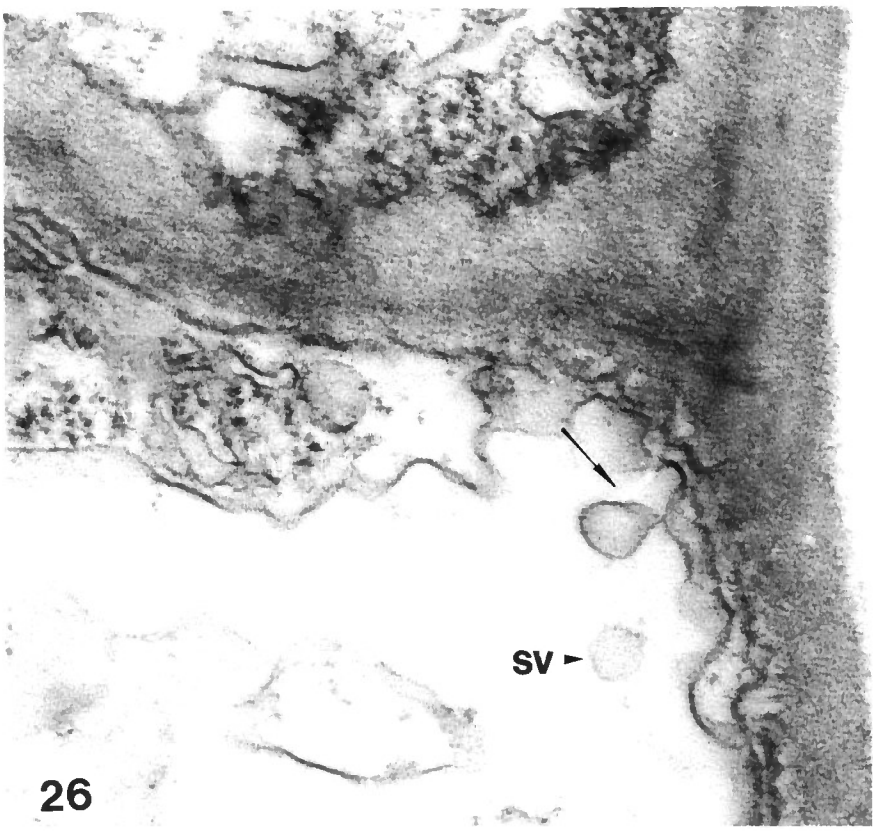
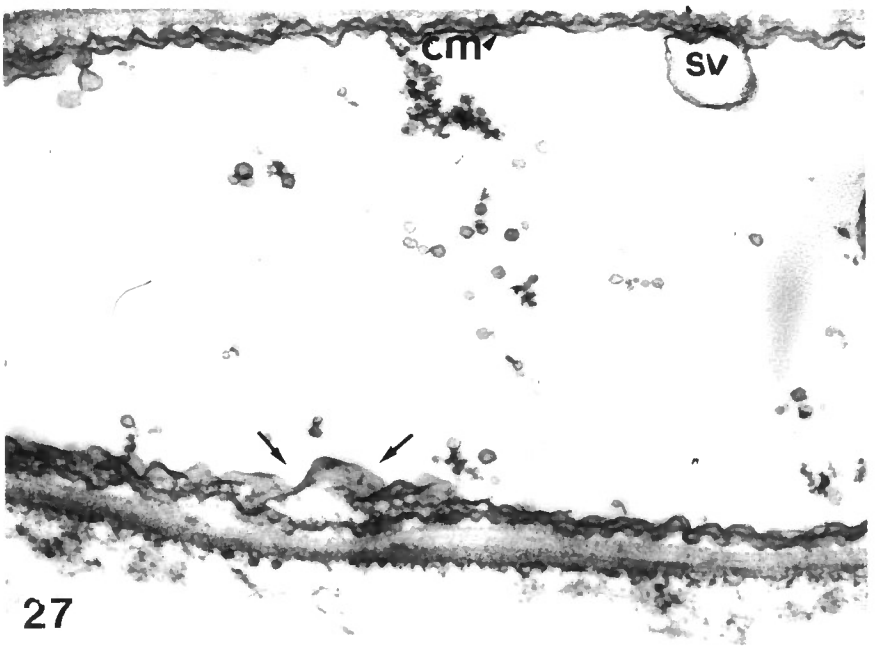

cw
mg
v
25

Fig. 26. Scytalone induced albino-1 young microsclerotium exhibiting single membrane vesicle (sv). Note some vesicles appear to be attached to each other and the cell wall (cw) (arrow). X75,000

Fig. 27. Scytalone induced albino-1 young microsclerotia exhibiting small single membrane vesicle (sv) throughout the cytoplasm. Note the discontinuity of the cell membrane (cm) (arrow). X42,000



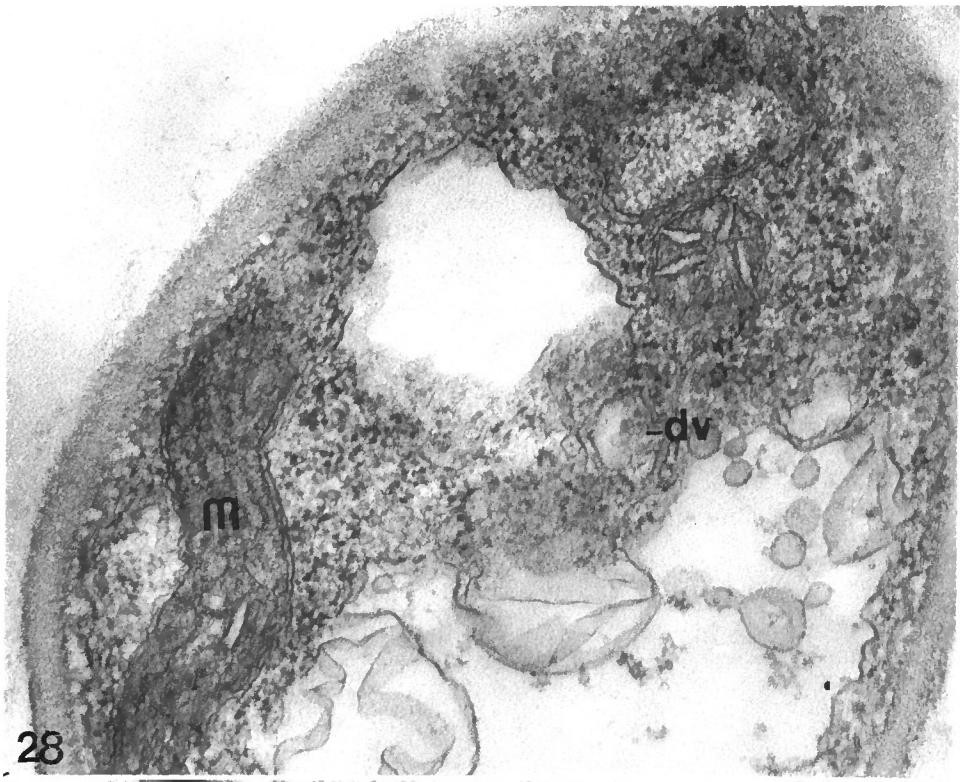

26



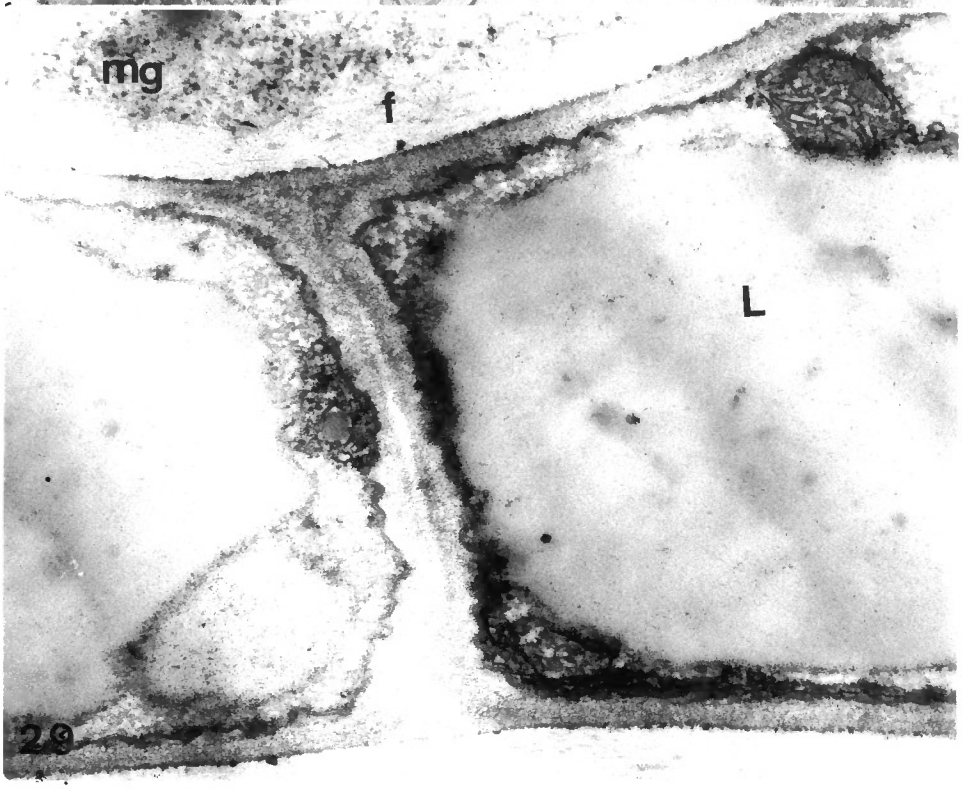

27

Fig. 28. Scytalone induced albino-1 microsclerotium exhibiting mitochondrion (M). Note double membrane vesicle (dv). X105,000

Fig. 29. Scytalone induced albino-1 yound microsclerotia exhibiting extracellular fibers (f) and melanin granules (mg). Note lipid globules and mitochondrion (M). X42,000



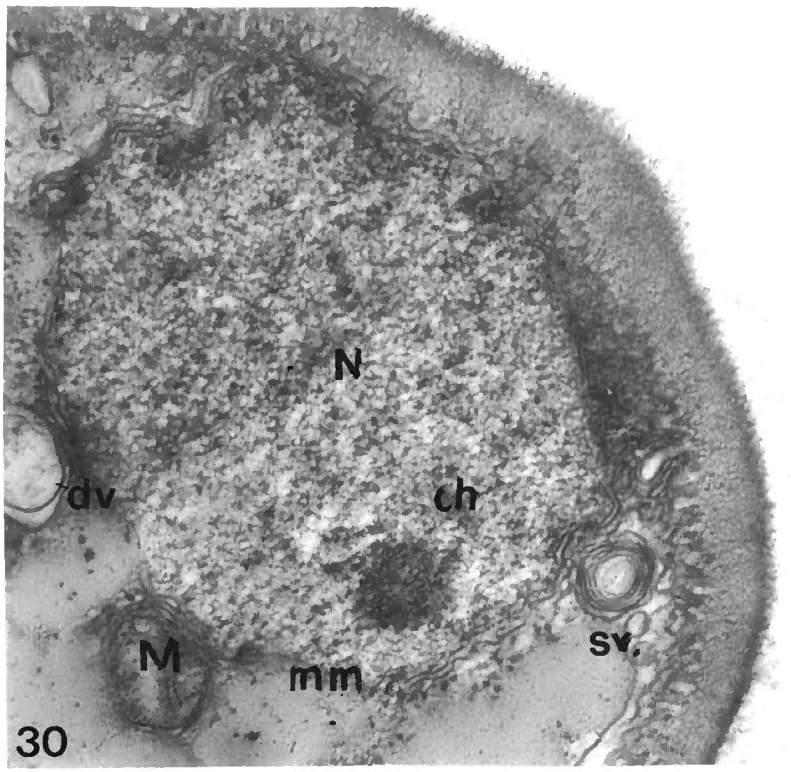

28




29

Fig. 30. Scytalone induced albino-1 exhibiting nucleus (N) with dense chromatin (ch). Note single membrane vesicle (sv) double membrane vesicles (dv) nuclear membrane (nm) and mitochondria (M). X105,000

Fig. 31. Scytalone induced albino-1 exhibiting altered mitochondria (M), and single membrane vesicles (sv) close to the cell membrane (cm). X45,000


N
dv
ch
M
mm
sv
30

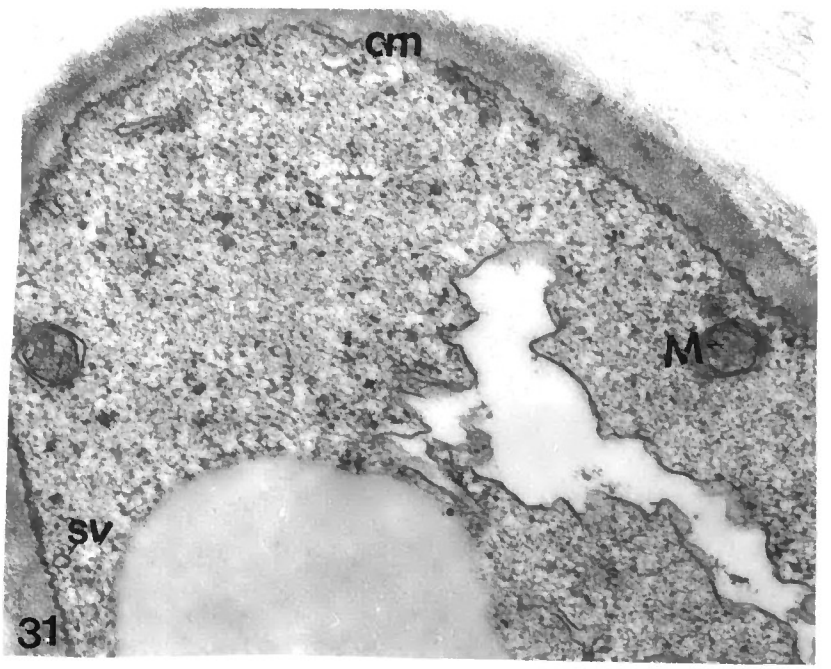
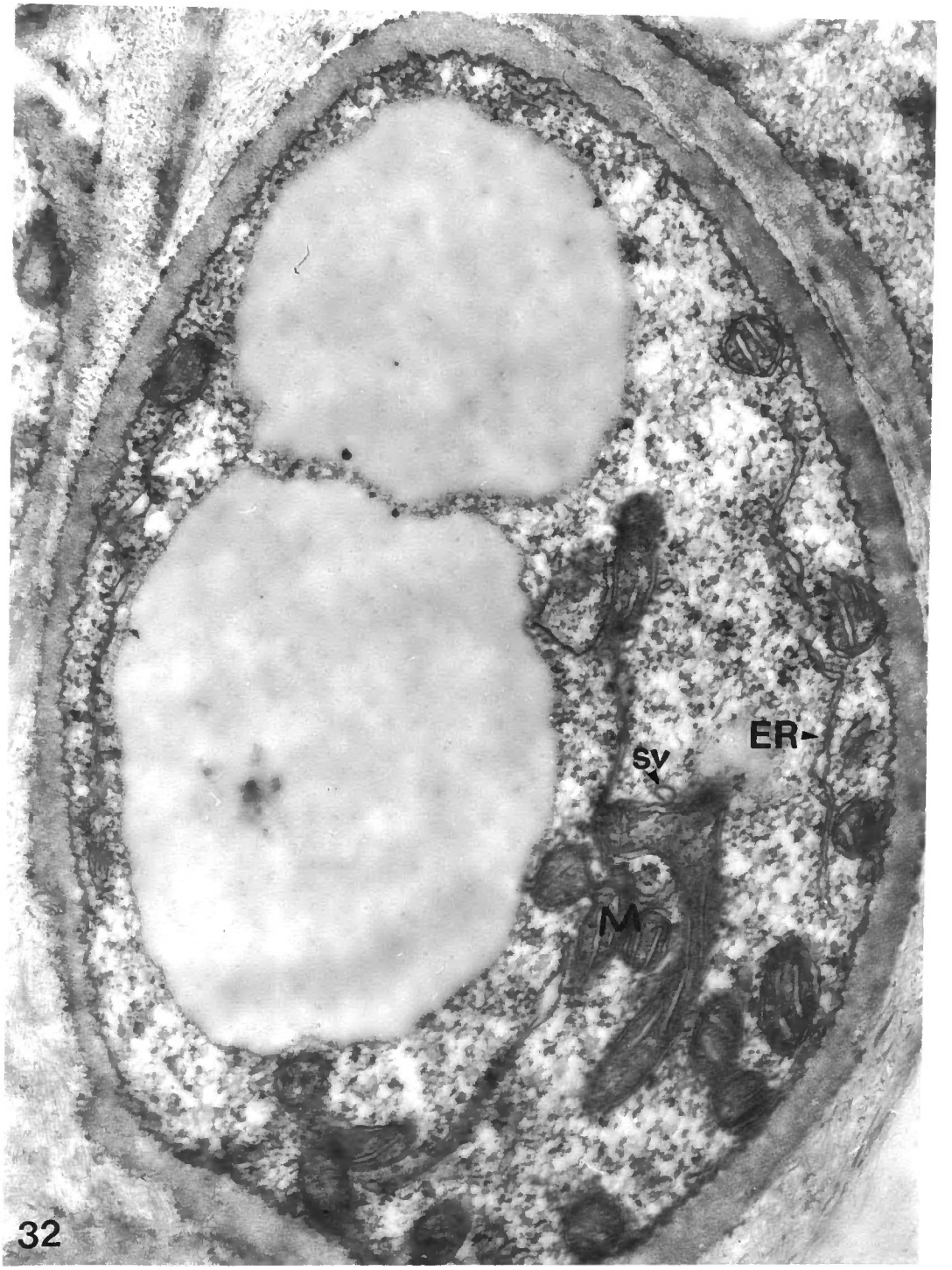

cm
M
sv
31

Fig. 32. Scytalone induced albino-1 microsclerotium showing endoplasmic reticulum (ER) and other cellular organelles. Note single membrane vesicles (sv) close to altered mitochondrion (M). X45,000


ER
sv
M
32

unevenness of its cristae in some of its elongated mitochondria (Fig. 32). In the vicinity of the altered mitochondria in s-alm-1 are single and double membrane vesicles (Fig. 30). In some developing microsclerotia (Fig. 33), multivesicular bodies were observed in s-alm-1, but were not observed in alm-1 and t-9. In spherical and oblong mitochondria, the cristae were observed to be intact and smooth in most cells.

As in untreated alm-1 (Fig. 11c), s-alm-1 shows single membrane vesicles. Some of s-alm-1 vesicles were attached to each other, while other single membrane vesicles were attached to the cell membrane (Figs. 26, 27). In earlier stages of microsclerotial development, s-alm-1 exhibits more single membrane vesicles (Fig. 27) than alm-1 (Fig. 7). However, in s-alm-1, double membrane vesicles have been observed. These double membrane vesicles (Fig. 28) are similar to those double membrane vesicles seen in t-9 (Fig. 9) and may be seen throughout the s-alm-1 cytoplasm.

In developing microsclerotia of s-alm-1, tubular-like structures (Fig. 32) resulted from the pushing together of vacuoles by developing lipid globules (Fig. 31). However, endoplasmic reticulum was throughout the cytoplasm of young microsclerotia.

As the microsclerotia develop, lipid globules were also seen developing (Fig. 33). These developing lipid

Fig. 33. Scytalone induced albino-1 microsclerotia exhibiting small mitochondria profiles (M) and multivesicular bodies (mv). X45,000

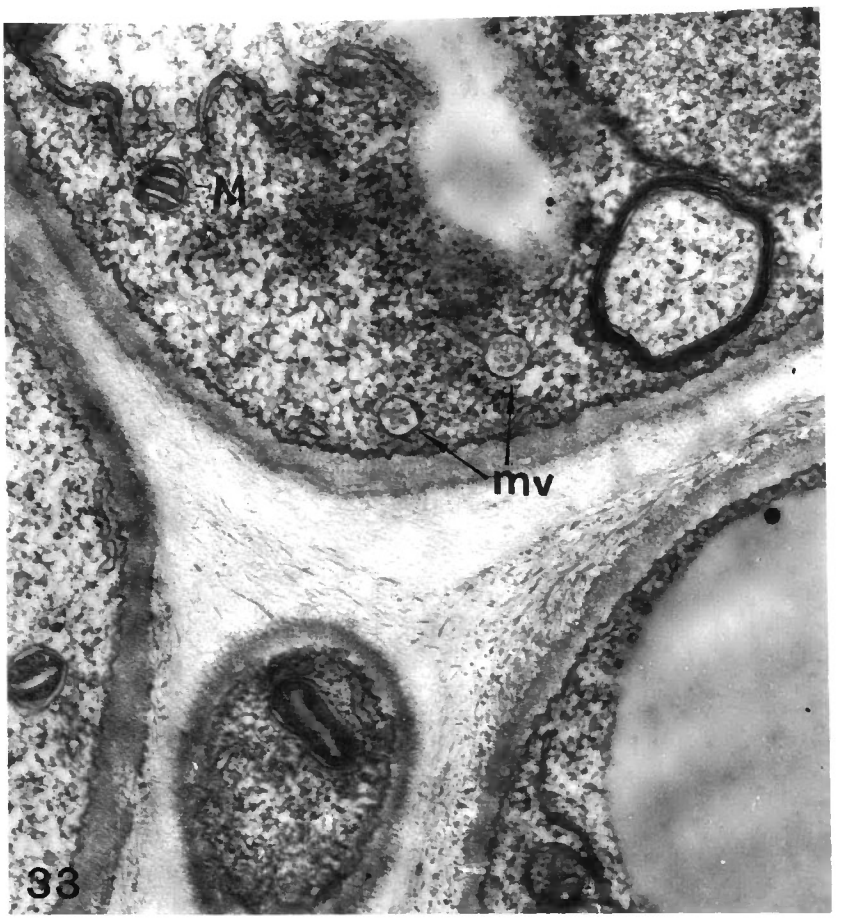

M
mv
33

Fig. 34. Scytalone induced albino-1 microsclerotium exhibiting nucleus with dense chromatin (ch) and discontinuity in the membrane. Note the similarity of the cytoplasmic granules and nuclear materials (arrow). X130,000

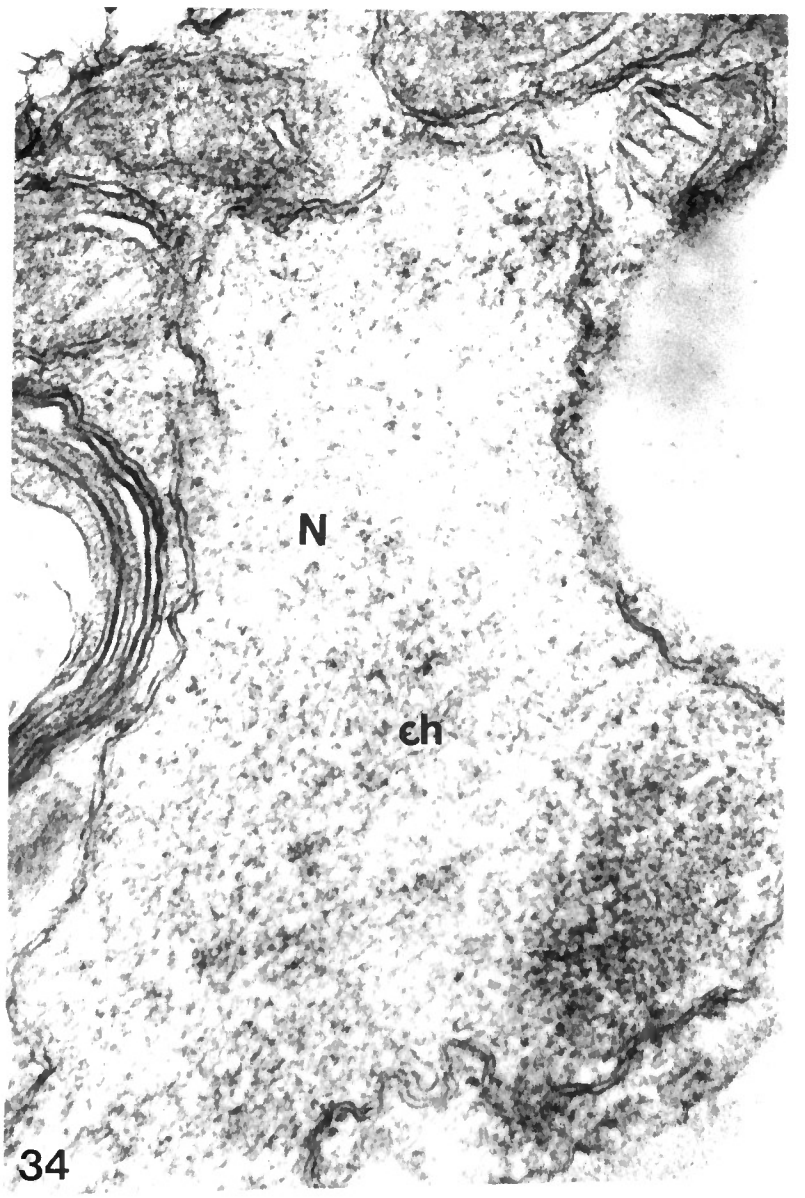
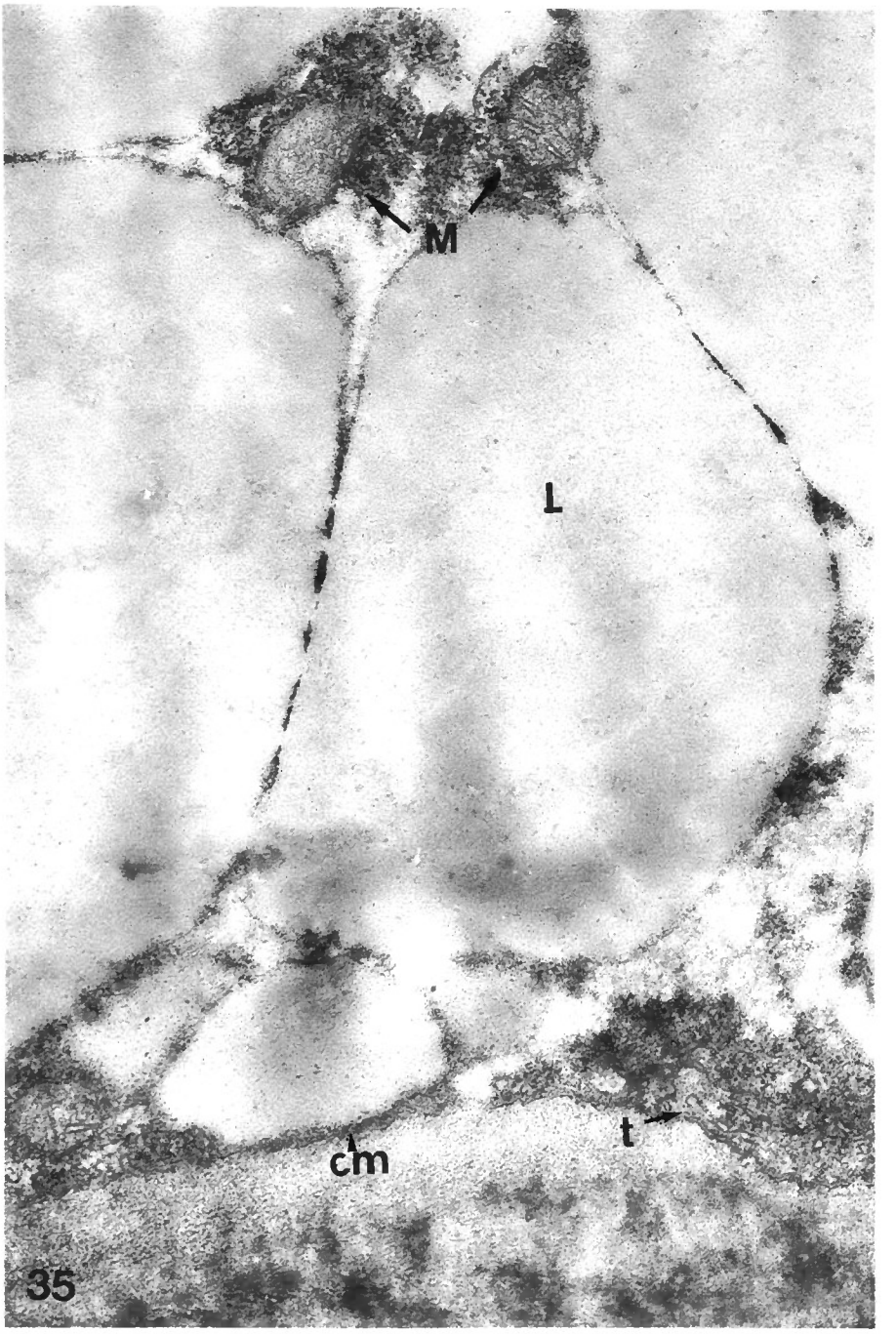

N
ch
34

Fig. 35. Scytalone induced albino-1 microsclerotium exhibiting organelles compressed by lipid globules (L). Note tubules (t) near cell membrane (cm). X44,200


M
L
cm
t
35

globules appear to compress the cytoplasmic organelles to the cell membrane (Fig. 35). There were no observed morphological differences in the size of the lipid globule in 9-day-old t-9 (Fig. 21), alm-1 (Fig. 22), or s-alm-1 (Fig. 39).

Electron-dense granules of s-alm-1 (Fig. 31) that are located near the lipid globules edge are similar in appearance to those seen in t-9 (Fig. 14). In s-alm-1, the electron-dense granules were only observed in developing microsclerotia and not in older microsclerotia. The observed nuclei contained dense chromatin materials and exhibited a double membrane (Fig. 30). The nuclear membrane of s-alm-1 had folds and showed discontinuity. Figure 34 shows interruptions in s-alm-1 nuclear membrane, with inner nuclear electron-dense granules being similar to those of the cytoplasm. Observed variations in intramembrane spaces and differences in the degree of staining of the nuclear membranes were seen in s-alm-1 (Fig. 34), while t-9 and alm-1 appeared unstained.

As for the cell membrane of the s-alm-1 cell (Fig. 30) of the microsclerotia (Fig. 33), it is highly folded like alm-1 (Fig. 22). There were discontinuities in parts of the cell membrane of t-9 (Fig. 21). No particles or granules were observed between the cell membrane and cell wall as was the case of t-9 (Fig. 23) and alm-1 (Fig. 22).

In developing microsclerotia, extracellular fibers were commonly observed in t-9 (Fig. 10), alm-1 (Fig. 11) and s-alm-1 (Fig. 29). In some cases, collectively, extracellular fibers may appear more dense in some areas than in others, due to direction of growth of extracellular fibers as well as their thickness (Fig. 31). Forty-five minutes after alm-1 had been treated with the scytalone, melanin granules were seen in the extracellular fiber region (Fig. 29). After 1 hr, melanin granules were seen throughout the extracellular region and on the cell walls of young developing microsclerotia (Figs. 27 and 29). And finally, the melanin could be seen in the cell wall (Fig. 36). These melanin granules of s-alm-1 were similar to those melanin granules produced by t-9 (Figs. 24 and 25).

## 3,3'-Diaminobenzidine Localization

By using 3,3'-diaminobenzidine (DAB), cytochrome c localization was demonstrated in the mitochondria and other membrane vesicles in t-9, alm-1, and s-alm-1. In t-9, the outer membrane of the mitochondria showed total staining (Fig. 39), partial staining (Fig. 40) or non-staining (Fig. 32), while the inner membranes did not stain. When alm-1 was observed, there were no instances of outer mitochondrial membrane staining (Fig. 41). However, the staining of the inner mitochondrial membranes varied from

exhibiting only partial staining (Fig. 43) to some of the cristae of the inner mitochondrial membranes showing staining (Fig. 42). In a single microsclerotium of alm-1, staining of mitochondria varied from part of one inner membrane being densely stained, while another mitochondrion showed total staining of its membrane (Fig. 46). Developing microsclerotia of s-alm-1 showed mitochondria densely stained on both the inner and outer membrane (Fig. 46). Also observed in s-alm-1 were stained inner membrane-like structures, without an outer mitochondrial membrane (Fig. 48). When DAB was used on developing t-9 microsclerotia, electron-dense stained cell membranes were observed (Fig. 37). Albino-1 exhibited parts of its cell membrane as being densely stained (Fig. 43). Scytalone-treated albino-1 exhibited a uniformly stained cell membrane (Fig. 44). Young developing microsclerotia of t-9 showed separation of the cell membrane from the cell wall (Fig. 40). In alm-1, the cell membrane was separated from the cell wall at different regions (Figs. 42 and 43). At those points where separation occurred, there always seemed to be an existing vesicle or particle (Fig. 42), whereas in s-alm-1, the cell membrane tended to stay in close proximity to the cell wall and was continuous (Fig. 40).

In alm-1, single membrane vesicles were observed in the cytoplasm (Fig. 49), with inner regions exhibiting compact granular material. This granular material appears to

Fig. 36. Scytalone induced albino-1 microsclerotia exhibiting melanin granules (mg) on and in the cell wall (cw), and within the extracellular fiber region (f). X130,000

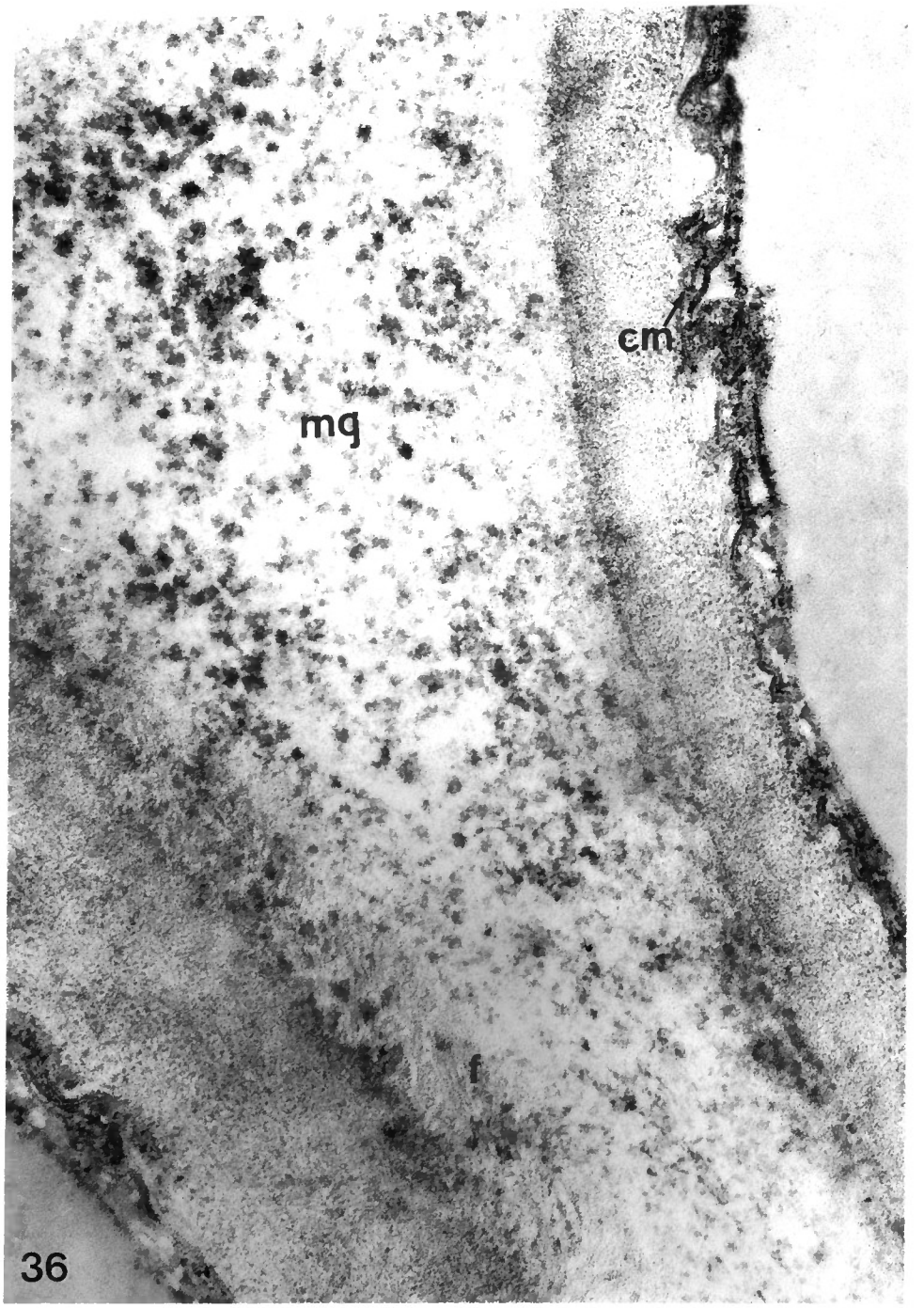

em
mg
36

Fig. 37. DAB treated wild-type microsclerotium. Note densely stained cell membrane (cm) and parts of double membrane vesicle (dv). X105,000

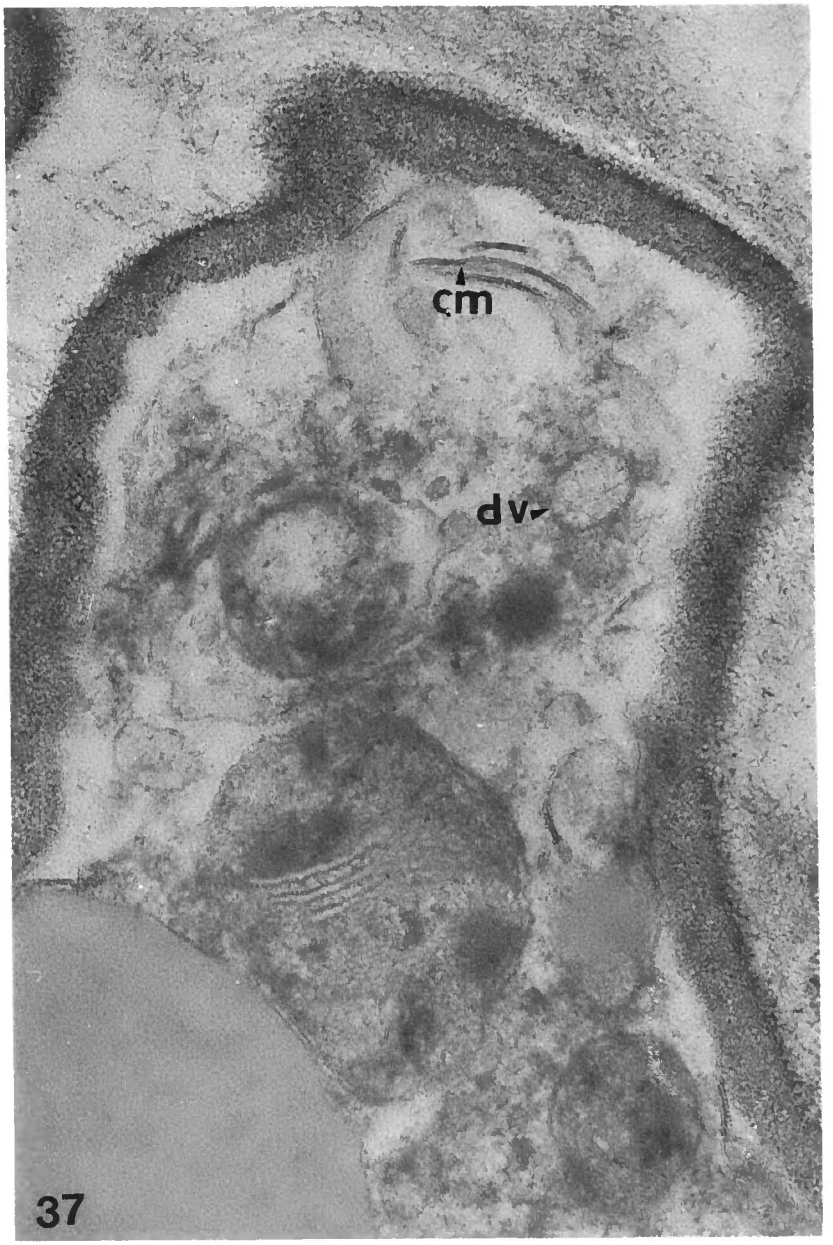

cm
dv
37

Fig. 38. DAB treated wild-type microsclerotium. Note staining of cell membrane (cm) and double membrane vesicles (dv). X150,000

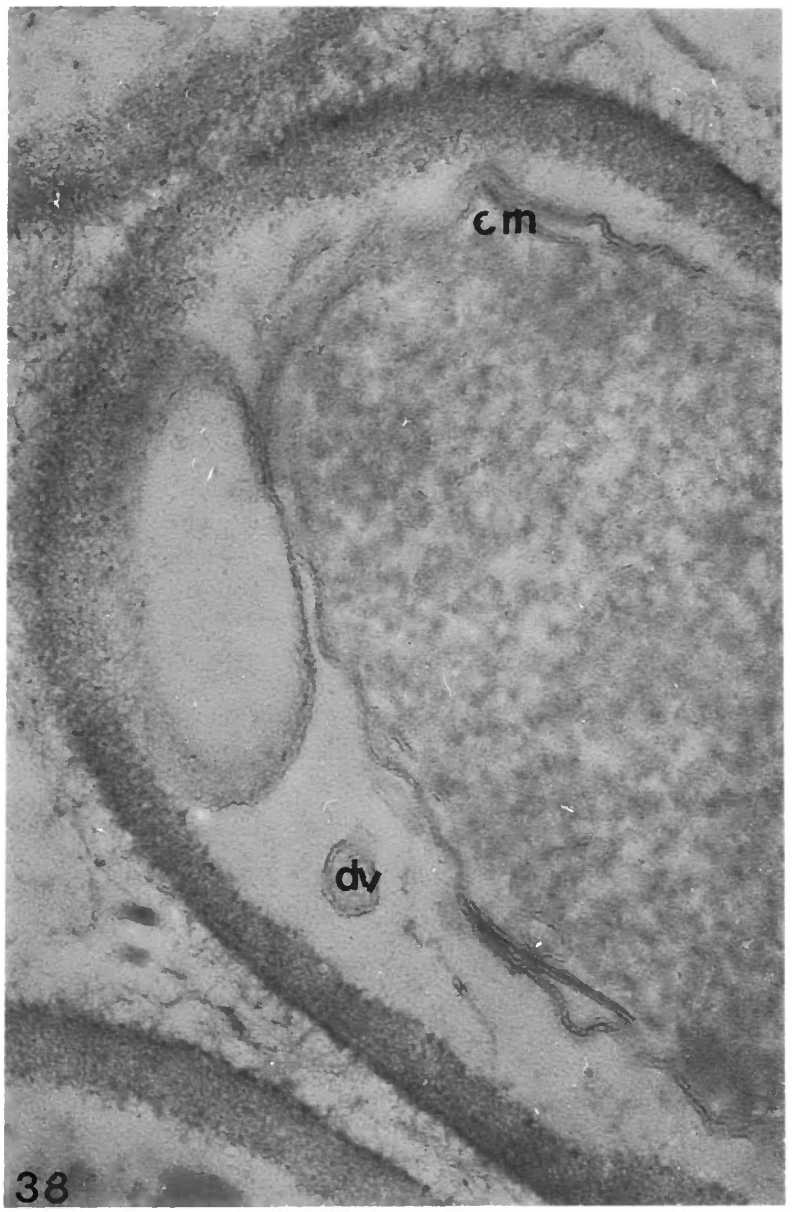
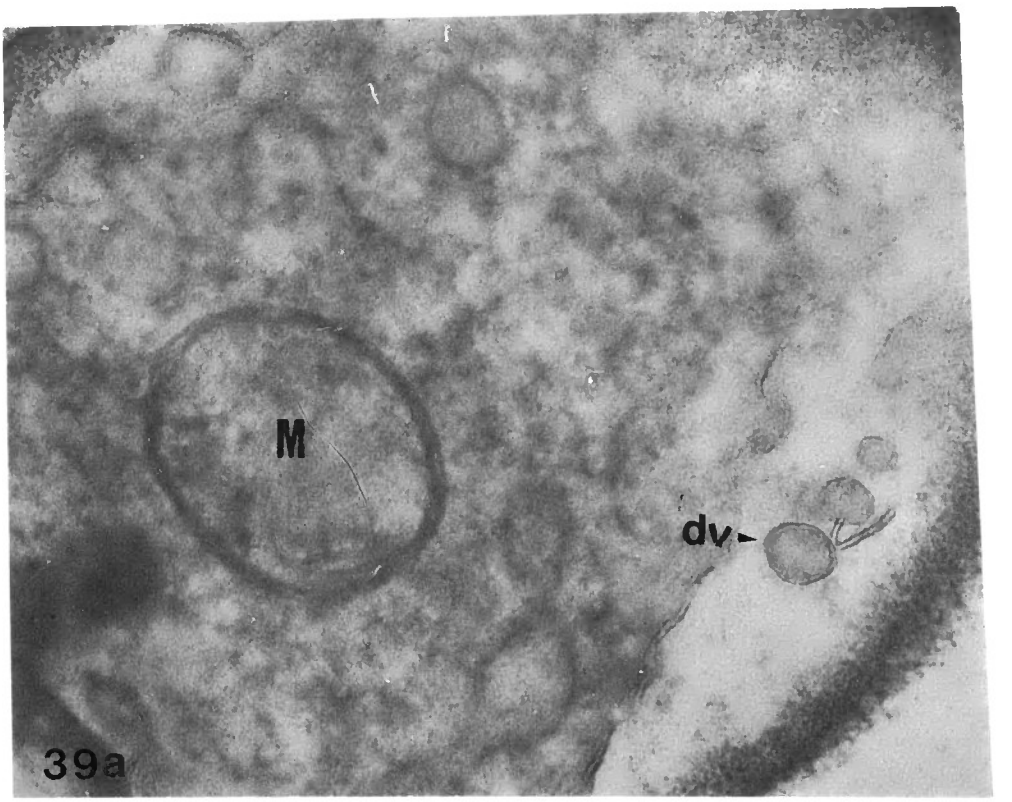

cm
dv
38

Fig. 39. DAB-treated (A) and non-treated (B) wild-type microsclerotia. Note the staining of the outer membrane of the mitochondrion (M) and the double membrane vesicles (dv) in A. The non-treated, figure B, shows unstained mitochondrion (M) and double membrane vesicles (dv) as compared with Fig. A above. X105,000


M
dv
39a

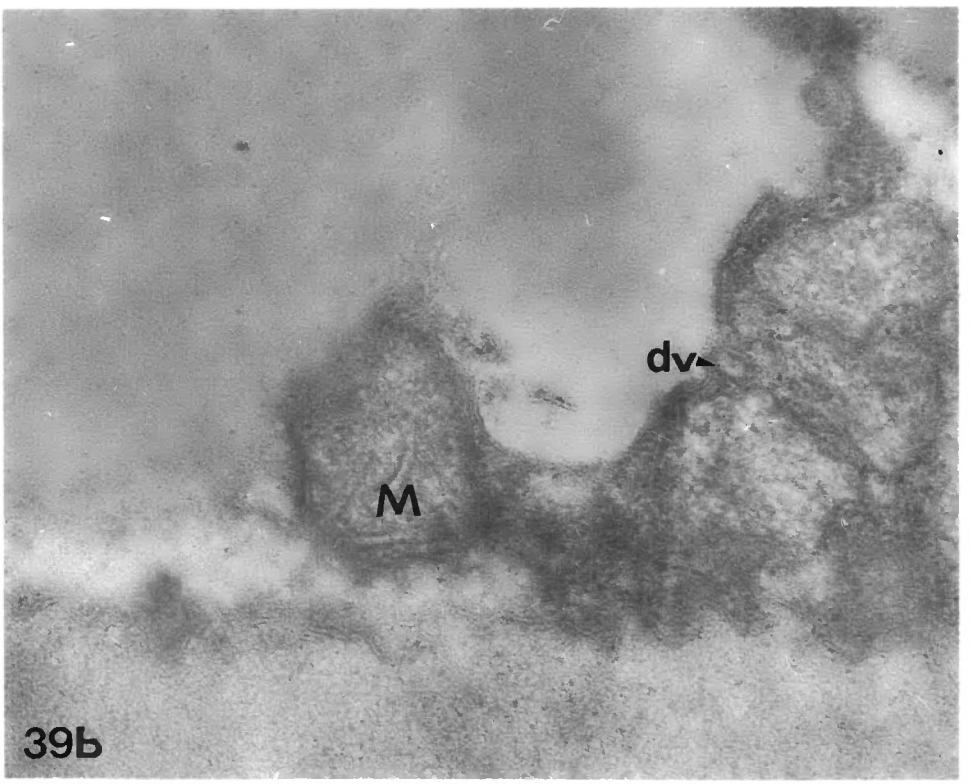
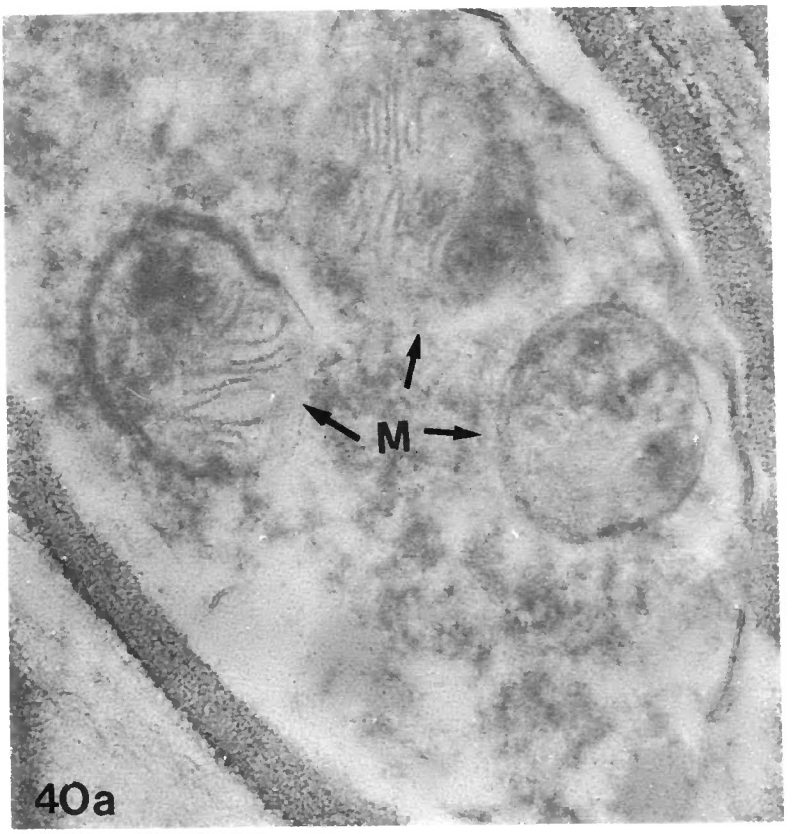
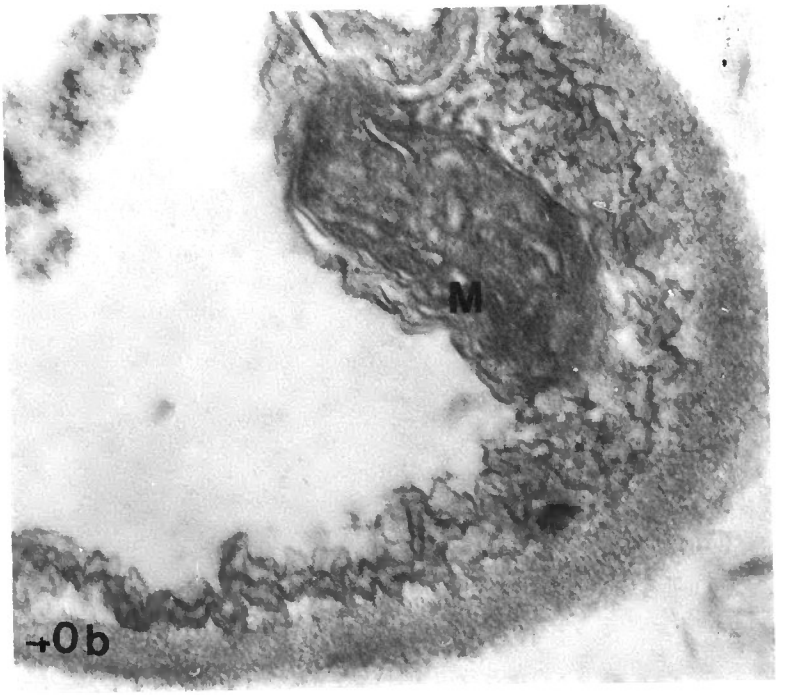

dv
M
39b

Fig. 40. DAB-treated (A) and non-treated (B) albino-1 microsclerotia. Note the part of the mitochondrion (M) that has dense-stained membrane (arrow) in A. The non-treated, Figure B, shows unstained mitochondrion (M) as compared with Fig. A above. X105,000



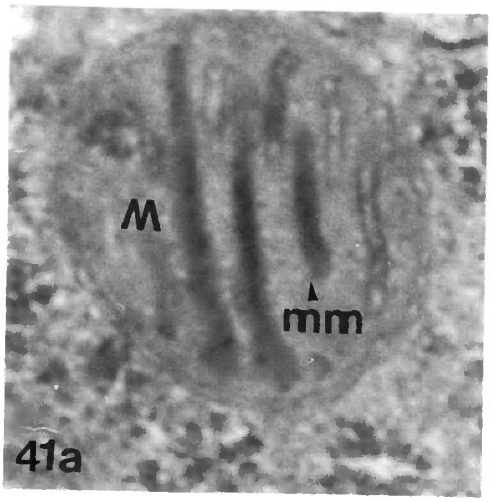

40a



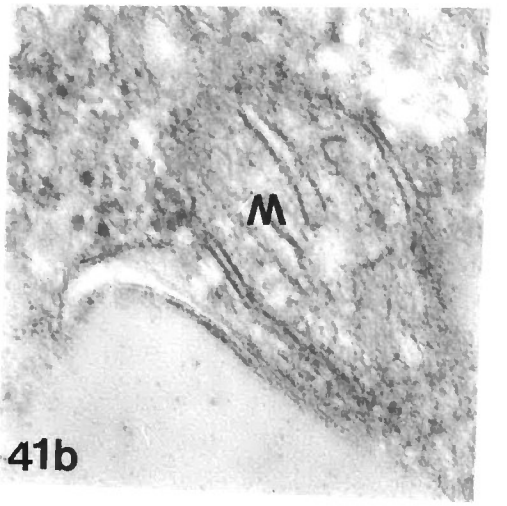

40b

Fig. 41. DAB-treated (A) and non-treated (B) albino-1 microsclerotia. Note the partially stained inner mitochondrial membrane (mm) in A and dense-stained areas (DA) of the cytoplasm. The non-treated, Figure B, shows unstained mitochondria (M) as well as the absence of densely stained cytoplasm as compared with Fig. A above. X105,000

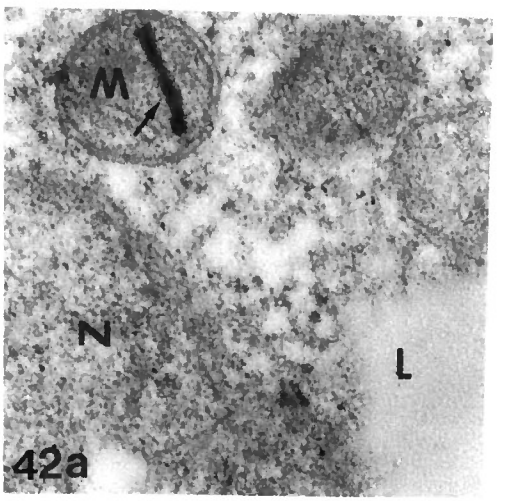
Fig. 42. DAB-treated (A) and non-treated (B) albino-1 microsclerotia. Note the nucleus (N), staining of the inner membrane of the mitochondrion (arrow) in A and the developing lipid globules (L). The non-treated, Figure B, shows mitochondrion (M) with densely stained inner membrane as compared with Fig. A above. X51,000



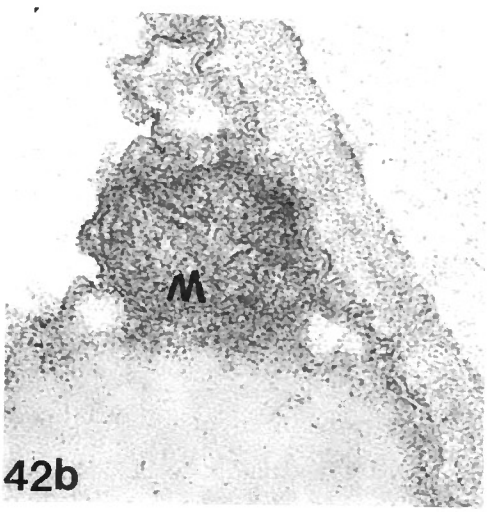

41a




41b

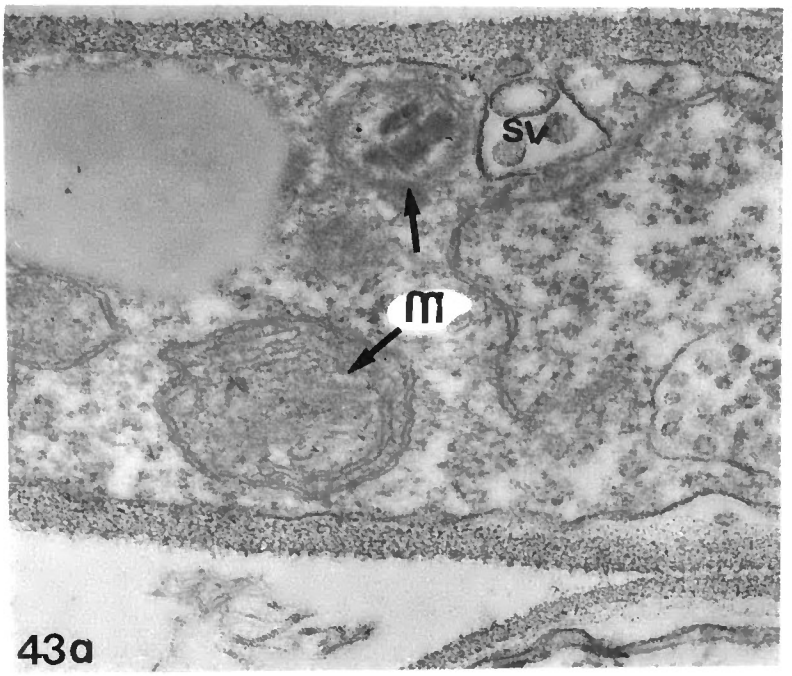



42a

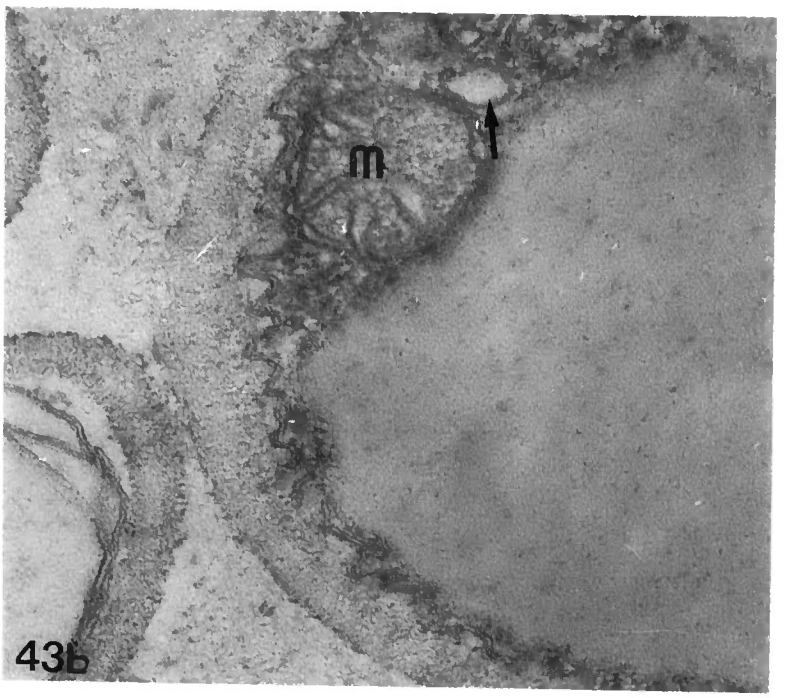



42b

Fig. 43. DAB-treated (A) and non-treated (B) albino-1 microsclerotia. Note the altered mitochondrion (lower right arrow), staining of the single membrane vesicles (sv) in A and partially stained inner membrane (arrow) of the mitochondrion (m). The non-treated, Figure B, exhibits altered unstained mitochondrion (m) as compared with Fig. A above. X75,000




43a

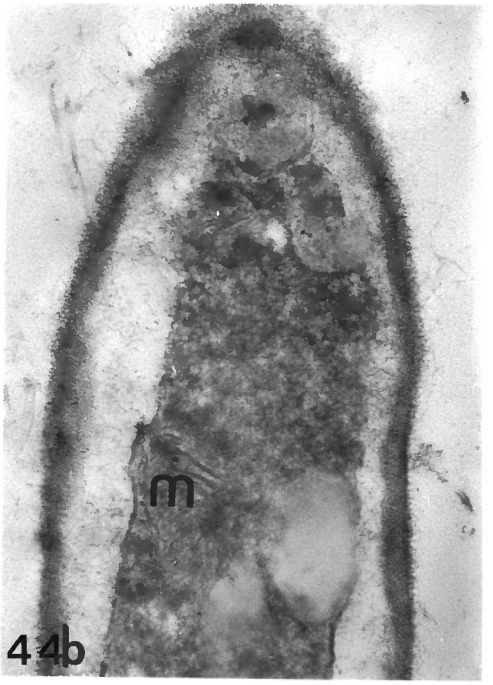


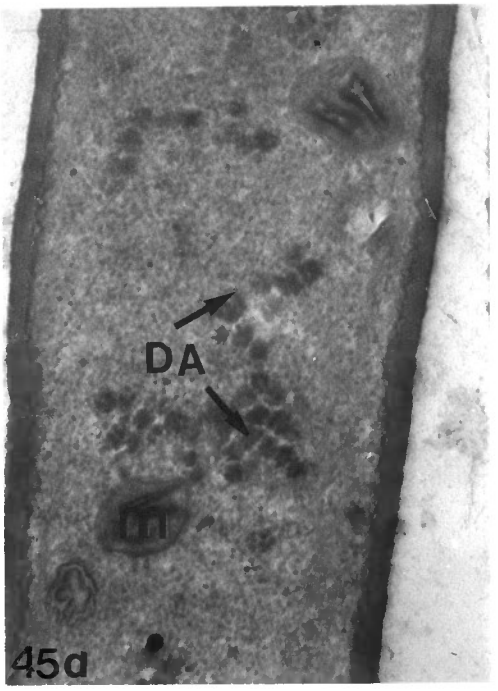

43b

Fig. 44. Scytalone-induced, DAB-treated (A) and non-induced, non-treated (B) albino-1 young microsclerotia. Note the stained mitochondria (M) in A. The non-induced, non-treated Figure B shows unstained mitochondrial membrane (M) as compared with Fig. A above. X31,000

Fig. 45. Scytalone-induced, DAB-treated (A) and non-induced, non-treated (B) albino-1 cells. Note the stained inner membrane (mm) of the mitochondria (M) in A and the densely stained areas (DA) of the cytoplasm. The non-induced, non-treated Figure B shows unstained mitochondrion (M) as well as the absence of densely stained areas in the cytoplasm as compared with Fig. A above. X51,500


m
44a


m
44b


DA
m
45a

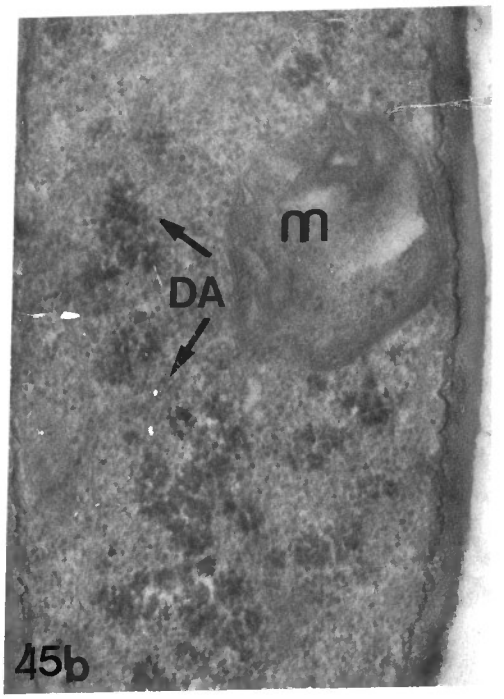
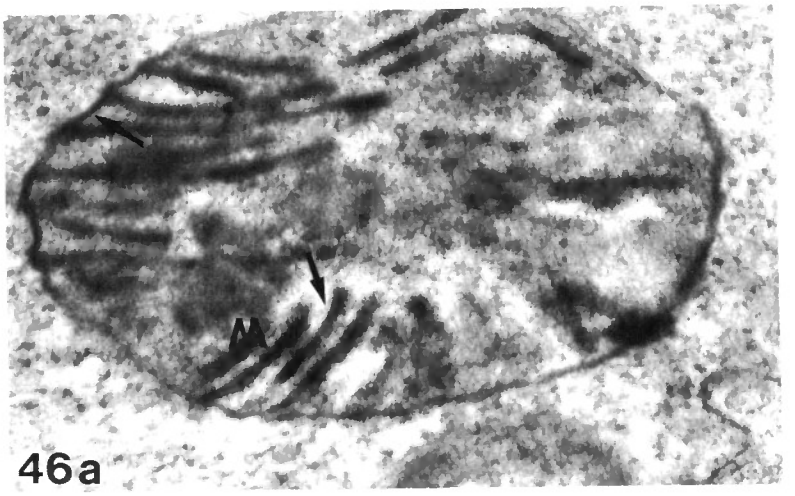
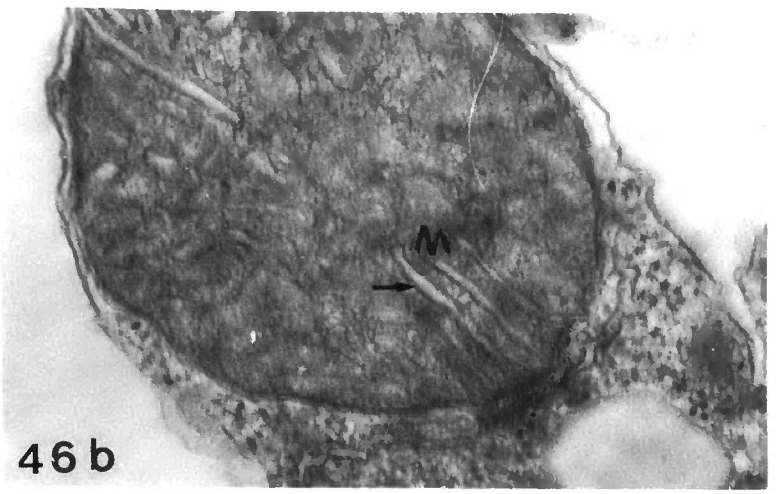

m
DA
45b

Fig. 46. Scytalone-induced, DAB-treated (A) and non-induced, non-treated (B) albino-1 mitochondria. Note the altered mitochondrion (M) with densely stained inner membrane ( M ) in A. The non-induced, non-treated Figure B shows unstained altered mitochondrion (M) as compared with Figure A above. X75,000



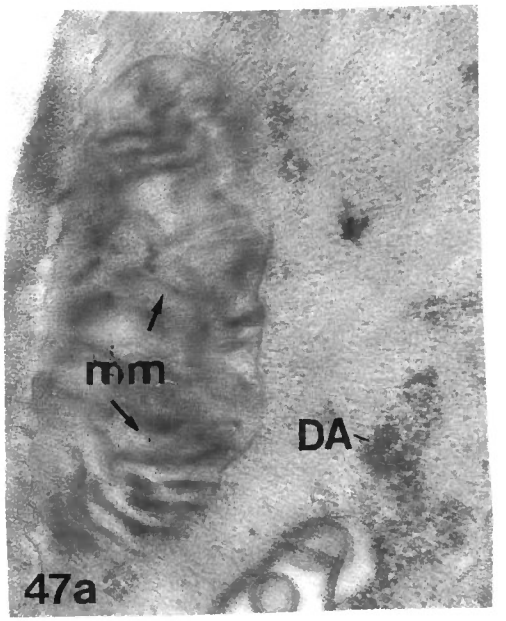

46a



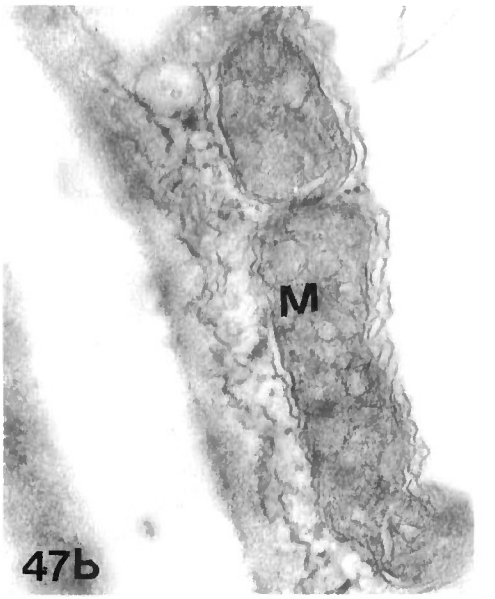

46b

Fig. 47. Scytalone-induced, DAB-treated (A) and non-induced, non-treated (B) albino-1 microsclerotia. Note the dense stained areas (DA) of the cytoplasm in A and stained inner mitochondrial membrane (mm). The non-induced, non-treated Figure B shows an altered mitochondrion (M) as compared to Fig. A above. X105,000

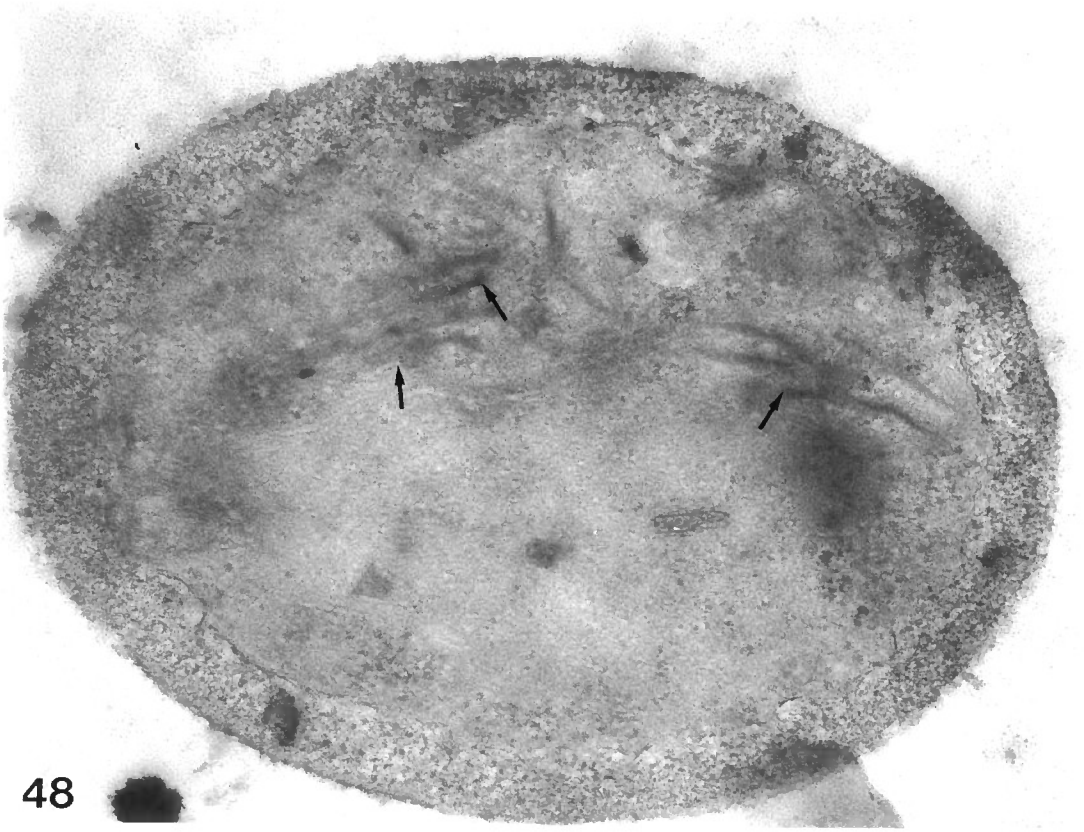
Fig. 48. Scytalone-induced, DAB-treated albino-1 young microsclerotium. Note dense-stained structures which resemble the inner mitochondrial membrane (arrow). X105,000




47a

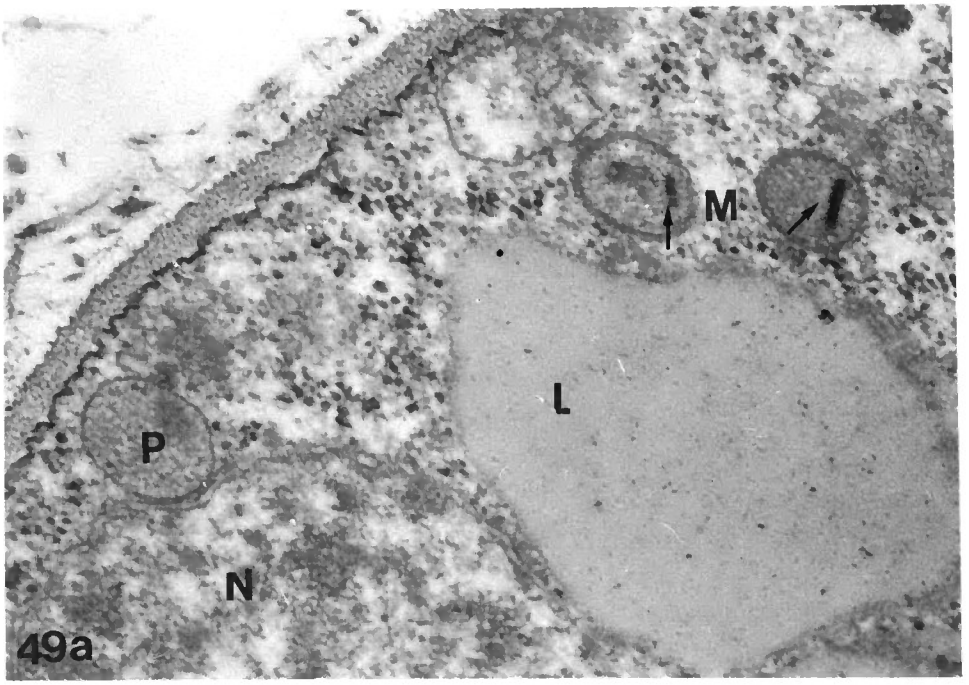



47b

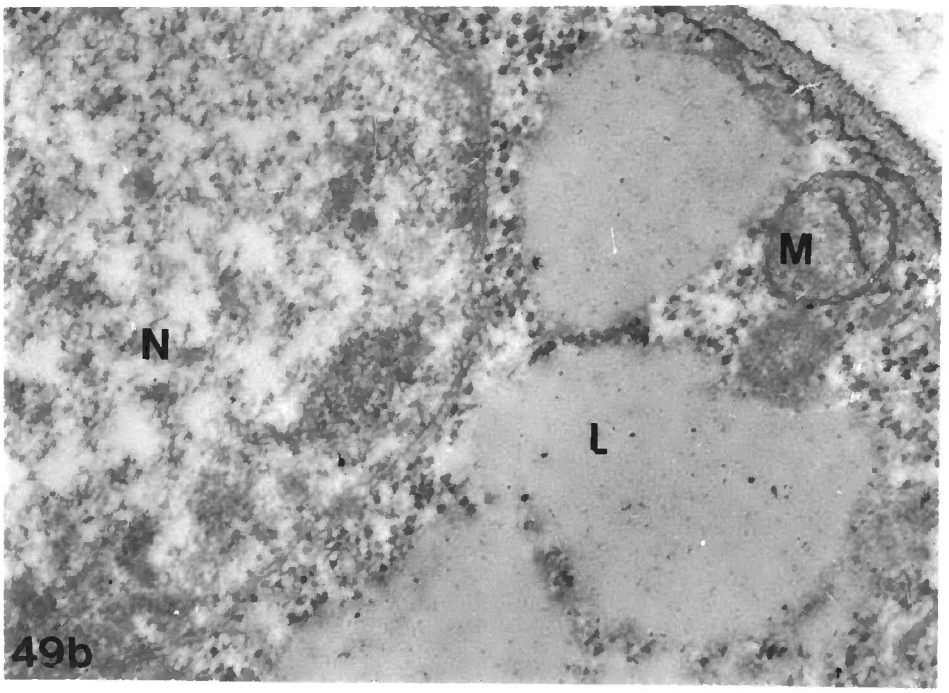


48

Fig. 49. DAB-treated (A) and non-treated (B) albino-1 microsclerotia. Note lipid globules (L), nucleus (N), mitochondrion (M) and large peroxisome-like (P) structure in A. The non-treated Figure B exhibits an unstained mitochondrion (m) as well as a nucleus (N) with a nucleolus (Nu) as compared with Figure A above. X75,000




49a

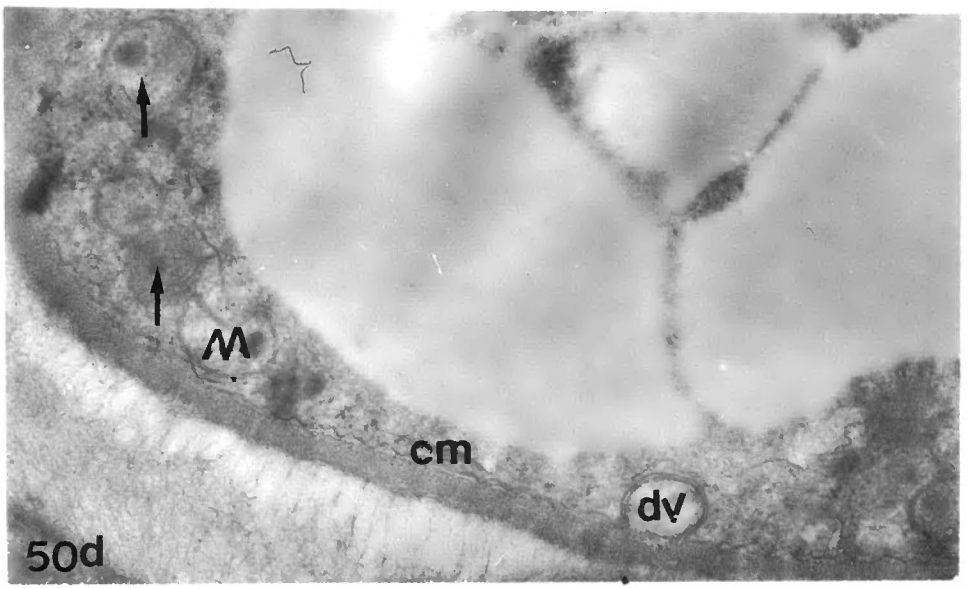


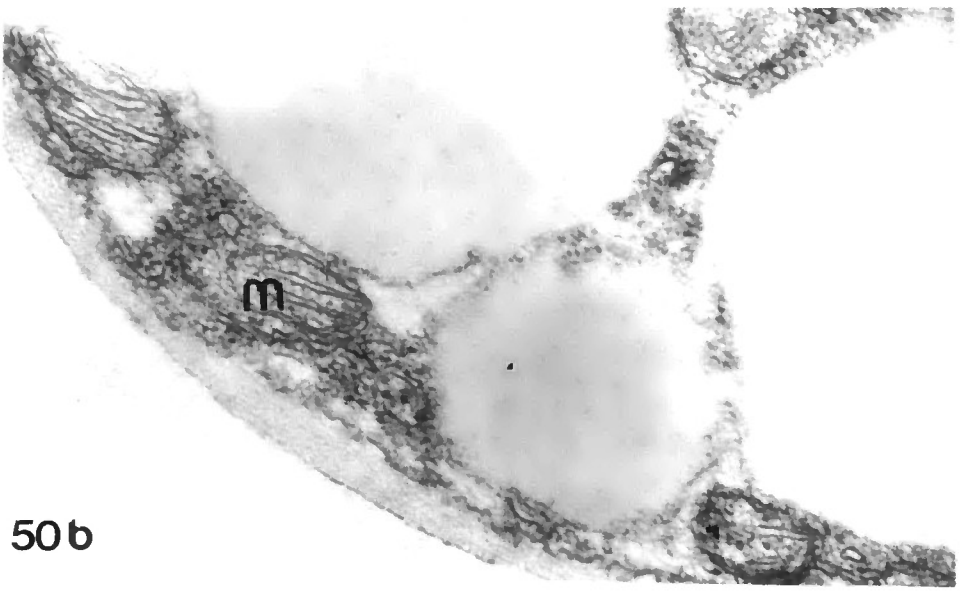

49b

be similar in content to the mitochondrial inner matrix materials. The size of the single-membrane vesicles is similar to the observed size of mitochondrial profiles (Fig. 49). Scytalone treated albino-1 also showed larger single membrane vesicles near the wall (Fig. 46). Figure 50 shows a double-membrane vesicle attached to the cell membrane in t-9. These double-membrane vesicles are densely stained lik the surrounding mitochondria.

During early stages of microsclerotial development, very noticeable electron-dense granules were observed in the cytoplasm of s-alm-1 (Figs. 46 and 47). In t-9 or alm-1, these large areas of electron-dense granules were not observed (Figs. 40 and 43). The development of lipid globules was similar in t-9 (Fig. 50), alm-1 (Fig. 42) and s-alm-1 (Fig. 40). Melanin granules in the extracellular fiber area and within the cell walls of t-9 (Fig. 50) and s-alm-1 (Fig. 46) were similar in their appearances. Albino-1 did not produce any melanin granules within the cell wall or in the extracellular fiber region (Fig. 43).

Fig. 50. DAB-treated (A) and non-treated (B) wild-type microsclerotia. Note the double membrane vesicle (dv) attached to the cell membrane (cm) in A and the altered mitochondrion (m) with small dense staining in the matrix (arrows). The non-treated Figure B exhibits unstained mitochondria (M) as compared with Fig. A above. X75,000


M
cm
dv
50d


m
50b

# CHAPTER V

## DISCUSSION

The results obtained in this investigation suggest that many of the alterations in the cellular structure of the melanin producing strain (wild-type) of V. dahliae are similar to the changes that occurred in the non-melanin producing microsclerotial mutant when treated with a melanin precursor, scytalone. In fact, some of the ultrastructural features of scytalone-treated albino-1 (s-alm-1) were more like the wild-type than the non-treated, normal albino-1 (alm-1). The ability of alm-1 to produce melanin when treated with scytalone is in agreement with the findings described by Bell et al. (1976b), in that s-alm-1 produced melanin was indistinguishable from the melanin produced by the wild-type. The fact that 8-day-old cultures of alm-1 were optimum for the induction of melanin, suggests that a threshold amount of scytalone must be produced by the brown-1 (brm-1) mutant. Since the induction of melanin is expressed within minutes after the introduction of scytalone to alm-1, these mutants represent an ideal system for looking at melanin development in V. dahliae. This type of system can be easily compared to the use of the morphogenetic system of Blastocladiella emersonii described by Cantino and Lovett (1964), in which they manipulated the environment to switch metabolic activities of the fungus

into different pathways.

Genetic studies with V. dahliae suggest that some fungal melanin is formed via the pentaketide pathway (Bell et al., 1976a; Stipanovic and Bell, 1976, 1977; Wheeler et al., 1976). The genetic lesion in the brm-1 microsclerotial mutant affects an enzyme that converts scytalone to 1,3,8-trihydroxynaphthalene (1,3,8 THN), the next compound in the pentaketide pathway of melanin synthesis. The wild-type isolates did not show any accumulation of scytalone in their culture medium. However, Bell et al. (1976b) recovered trace amounts of scytalone from cultures undergoing active melanin synthesis. The failure of melanin production in brm-1, when it replaced alm-1 that had been previously exposed to brm-1's metabolite (extract), suggests that the genetic lesion in alm-1 is before scytalone production in the pentaketide pathway. This suggestion correlates with the study of Bell et al. (1976b). The lack of pigmentation in the brm-1 mutant that was replaced by alm-1 is apparently due to alm-1's inability to produce metabolites or the retention of products inside of its cells.

With the above assumptions in mind, the alm-1 strain was selected to investigate changes in the cellular structures associated with melanin formation. Since it is assumed that the mutant with an early block in melanin synthesis is more likely to be altered in its cellular structures, a system is provided in which melanin production can

be "turned on" and compared to the same system when melanin is not being produced. Albino-1 utilizes scytalone by readily converting it into 1,3,8-THN which cannot be reconverted to scytalone. The compound, 1,3,8-THN is then converted to vermelone which in turn is converted to 1,3-dihydroxynaphthalene (1,3-DHN), one of the last compounds in the conversion to melanin (Bell et al., 1976b; Wheeler et al., 1978).

The observation of melanin granules in s-alm-1, which is similar to the wild-type, is in agreement with the results reported by Wheeler and his associates (1978). The location of the melanin granules in s-alm-1, like those of the wild-type strain, occurred only in the outer walls and the surrounding matrix of the microsclerotial cells. This observation was also in agreement with Bell et al. (1976b). Since there were no real distinguishing features between the melanin granules deposited in the scytalone-treated and the wild-type microsclerotial cells, they may be expected to follow a similar pattern of development. While the mechanisms of extracellular melanin granule deposition in fungi are not known, highly magnified electron micrographs do, however, suggest that networks of extracellular fibers keep the melanin granules together by forming a dense screen. The idea of melanin binding by extracellular fibers is supported by the observation of fibers appearing before melanin granules in the

wild-type. Similar results were reported and described by Wheeler et al. (1976).

The results from phase-contrast microscopy indicated that with increasing age, up to 3 days, there is an increased number of germinating conidia which develop into differentiating hyphae. These results agree with the findings of Taylor (1978) and Gaskins (1979). The conidia will germinate to produce hyphae (conidophores), which may be "pinched off" in rapid succession, forming more conidia which will later germinate to develop into hyphae. Hyphae may take on the shape of swollen chain-like, globose structures which are microsclerotial initials. Gaskins (1979) indicated that the conidiation pattern of albino-4, brown-1, and the wild-type was similar when observed by phase-contrast microscopy.

The hyphae of V. dahliae wild-type and its alm-1 microsclerotial mutant contain membrane vesicles, which may vary greatly in size and shape due to age and/or type of mutant. It is important to note that single membrane vesicles were first reported to occur in V. dahliae only prior to the accumulation of melanin granules (Taylor, 1978). In young alm-1, the single membrane vesicles were fewer than in the wild-type. Some single membrane vesicles were attached to the cell membrane of the wild-type and alm-1 mutant, however, during later stages of wild-type development, double membrane vesicles became obvious in the

cytoplasm. Bell and Muhletaler (1964) described evaginations of double membrane structures in fern archegonia. These evaginations were said to have arisen from large vesicles limited by two membranes of which the internal one developed evidence of folding, which Bell and Muhletaler considered to be mitochondrial cristae. However, the external mitochondrial membrane was distended to a great degree. In several places it formed vesicles which exhibited the ability to become detached. Data from this report showed a large number of vesicles throughout the cytoplasm in the active melanin producing strain (wild-type), while the s-alm-1 and normal alm-1 had fewer membrane bound vesicles. It is difficult to say whether the vesicles are derived from the endoplasmic reticulum (ER), since no vesicles were observed in continuity with ER. It has recently been pointed out that there is still some controversy as to how these vesicles are derived (Novikoff and Goldfischer, 1969).

Double membrane vesicles are seen in alm-1 only after it has been treated with scytalone, whereas the wild-type always exhibited double membrane vesicles throughout its cytoplasm. The proximity of these double membrane vesicles to the altered mitochondria in the s-alm-1 suggests a developmental relationship between the two organelles. Mayfield and Taylor (1978) described stages of the mitochondrial alteration leading to the development of single

membrane vesicles prior to melanin granule production in the wild-type strain of V. dahliae. This is also in keeping with the views of Kilarski and Jasinski (1970), who postulated that the membrane vesicles formed in association with the cellular changes in fern cells may move away from the parent organelles, while retaining the original internal membrane characteristics. Since the newly formed vesicles contain membranous material identical to the parent organelles, they (Kilarski and his associates) considered the organelles as the origin of the vesicles. Membrane vesicles like those described by Taylor (1978) and Kilarski and Jasinski (1970) were observed in greater frequency after the scytalone treatment. It is suggested that the vesicles observed in s-alm-1 strain of V. dahliae originated from the mitochondria by a similar process as that described by Kilarski and Jasinski (1970).

During melanin granule production in s-alm-1, single membrane vesicles increased along with an increase in the double membrane vesicles, which indicated a similarity to the pattern of the wild-type vesicle development. A similar type of phenomenon has been postulated by Lange and Olson (1976) in zoospores of Olipidium eassicae. They described microbodies (single membrane vesicles) connected and/or showing close associations with the mitochondria. Lange and Olson (1976) noticed that some of the mitochondria may become altered, followed by a swelling at one

end. They suggested that the swollen part of the altered mitochondria detaches itself, thereby leading to a single membrane vesicle which becomes part of the cytoplasm. Gaskins (1979) has already suggested that membrane vesicles derived from altered mitochondria were produced in young wild-type microsclerotial cells. The increased number of single membrane bound vesicles and the occurrence of altered mitochondria in s-alm-1 leads one to conclude that the mode of vesicle production in the induced strain is similar to that in the wild-type. The lack of the above observations in alm-1 (non-scytalone-induced) provides further evidence linking the presence of single membrane vesicles to melanin production, thus supporting the earlier suggestions of Taylor (1978) and Gaskins (1979).

The increased frequency in the occurrence of mitochondria in s-alm-1, with alterations in the inner (cristae) and outer membranes, suggests that the mitochondria may be responsible for the increase in number of single membrane vesicles and the production of double membrane vesicles. The occurrence of double membrane vesicles of s-alm-1 are similar to that of the wild-type. It is possible that the increased number of mitochondria in s-alm-1 may be in response to the production of two types of vesicles. A dual transport system is suggested since scytalone accumulates in the medium and is transported into the alm-1 cell, where melanin synthesis is believed to occur. On

the other hand, the preformed melanin accumulates primarily in the cell wall of the microsclerotia and extracellular fiber regions. Therefore, the two types of vesicles seen in s-alm-1 could be associated with the melanin transport and/or compartmentalization of different components in the overall synthesis and secretion of melanin.

Grove and Bracker (1970) postulated that the vesicles may act as transport elements for the cell wall formation of Gilbertella persicaria. Bracker (1966) proposed that cisternal rings were Golgi equivalents in some higher fungi which lacked the stacked cisternae typical of the Golgi apparatus in plants. Due to alteration of the mitochondria in s-alm-1, the produced vesicles are thought to act as transport structures. Since V. dahliae lack Golgi, it is possible that the presumed mitochondria-derived vesicles act similar to the Golgi in higher plants. Since there are few observed altered mitochondria in normal alm-1, it is suggested that mitochondria undergo a functional change in response to scytalone treatment. Such a change could be a decline in their respiratory rate or other metabolic changes.

Since Gaskins (1979) suggested that peroxisomes were single membrane vesicles resulting from altered mitochondria, it was of interest to determine if the mitochondria underwent functional changes associated with scytalone induction. Therefore, an osmiophilic polymer, 3,3-diamino-

benzidine (DAB), which gives a dense stain in the presence of cytochrome c oxidase activity, was used. The rationale for DAB localization is that if mitochondrial function is changed during scytalone induction, then the localization pattern for DAB should be different. If there is a decrease in normal mitochondrial functions, then a decrease in DAB product is expected. The localization of DAB reaction product was along the inner mitochondrial membrane of alm-1 (non-scytalone-treated) while DAB product was found concentrated along the outer mitochondrial membrane of the wild-type (melanin-producing) strain. This difference in localization of DAB suggests that there are functional differences between the mutant (alm-1) and the wild-type (melanin-producing) strain. However, s-alm-1 DAB localization pattern seems to follow the observation of Taylor (1978), who showed DAB localization reaction pattern on the inner and outer membranes of actively melanin producing wild type. The localization studies clearly demonstrate that mitochondrial functional changes occur in association with melanin production. The localization pattern of DAB along with extra-mitochondrial membrane elements of s-alm-1 suggest that there is a relationship between altered mitochondria and other membrane elements. Since there are few altered mitochondria in alm-1 and many in s-alm-1, this might suggest that the inner membrane system is associated with cytoplasmic vesicles.

This suggests that DAB-stained vesicles may have originated from altered mitochondria and these vesicles could contain a similar type of oxidative enzymes as the mitochondria or other enzymes essential for melanin production.

The increase in mitochondria within the s-alm-1 can be explained in a number of ways. Morrison (1977) postulated three ways by which this may occur: (1) mitochondria may be moved into or out of areas by enlarging secondary lysosomes; (2) mitochondria may actively move to new areas; and (3) movement by cytoplasmic streaming, which could be responsible for the reapportionment of the mitochondria to one area. However, since there is an increase in the number of mitochondrial profiles during melanin production in s-alm-1, the apparent increased mitochondrial profiles could play an important role by replacing the existing altered and deteriorating mitochondria.

Another indication of similarity in the pattern of melanin development in s-alm-1 and the wild-type strain, is cell membrane discontinuity. Taylor (1978) reported highly discontinuous cell membranes in the early stages of wild-type microsclerotial development of V. dahliae. Data from this report indicated that there were frequent discontinuities in older microsclerotial cell membranes of s-alm-1, while normal alm-1 showed none to a few discontinuous regions in their cell membranes.

Once microsclerotia have reached their maximum size, they become melanized within a short time, often only a few days. During this time an increase in deposition of structural and storage material, mainly lipid globules, became obvious. The close structural association between the accumulating lipid globules and the endoplasmic reticulum is involved in the formation of lipid globules. This is similar to the role of the endoplasmic reticulum in the formation of triglycerides described in other fungi (Weet, 1974; Power et al., 1981). Gordee and Porter (1961) indicated that the lipid globules serve as a source of energy during dormant and germinative stages of the microsclerotia.

Before day-5, electron-dense granules resembling glycogen were observed in the s-alm-1 and the wild type, while only a few were observed in normal alm-1, suggesting a change in the cytoplasmic content of alm-1. This change indicates another similarity between the wild-type strain and the s-alm-1. Since microsclerotia are survival structures in which lipid globules are used during their dormant and germinative stages, then it could be assumed that glycogen may act like ready storage structures in the development of the microsclerotia. Due to the accumulation of glycogen prior to lipid globule formation, it is assumed that glycogen utilization precedes lipid globule metabolism. Griffin (1981) stated that while the function of glycogen is unknown, it might serve as ready storage reserves.

All of the previously discussed information suggests that the changes in cytoplasmic organelles, that occur during the production of melanin granules in s-alm-1, follow a similar pattern to the cytoplasmic organelle changes that occurred in the production of melanin granules in the wild-type. The observed changes in the cytoplasmic organization as the alm-1 mutant (non-scytalone-treated) is converted to a melanin producing organism (s-alm-1) indicated that specific changes in organelles can be associated with melanin synthesis. The demonstrated functional and morphological changes in mitochondria due to scytalone treatment was of special interest in that they seem to reflect alterations in metabolic activities that accompany melanin production. Since scytalone is used in melanin synthesis and mitochondria alteration appears to accompany this process in alm-1, one may conclude that mitochondrial alterations are essential during melanin synthesis in V. dahliae. The mitochondria changes could also be linked to an increase in the population of vesicles which are correlated with melanin synthesis. This research has established the utility of the albino microsclerotial mutant in the investigation of melanin synthesis of V. dahliae.

Since the biochemical function and characteristics of the vesicles and altered mitochondria are unknown, further studies should be designed to develop the

biochemical activity associated with their presence. It would be of interest to determine if the induced double membrane vesicles in the s-alm-1 might play a role in cellular transport like the Golgi apparatus in other lower fungi as suggested by Bracker et al. (1976). For example, to answer such questions, one might utilize the localization of the marker enzyme such as thiamine pyrophosphatase (Steward, 1960). Through the combined techniques of biochemistry and electron microscopy, the alm-1 mutant can provide a better understanding of melanin synthesis in the filamentous fungi.

# CHAPTER VI

## SUMMARY

The development of microsclerotia in V. dahliae growing on potato dextrose agar (PDA) and polygalacturonic acid medium (PGAM) was studied by a combination of phase contrast and transmission electron microscopic techniques. Samples were taken from PGAM 24 hr following inoculation, then every 18 hr thereafter up to 96 hr. Conidiation, germination of conidia, hyphal elongation and microsclerotia production had all occurred in less than 78 hr.

Albino-1 on PDA over-laid with cellophane for 8 days was induced by brown-1 culture filtrate (scytalone) to produce melanin granules similar to the melanin granules in the wild-type. At the electron microscopic level, the most noticeable cytoplasmic organellar changes in albino-1 following induced synthesis of melanin were alterations in the increasing number of mitochondria and an increase in the single membrane vesicles with the production of double membrane vesicles. These changes resembled those seen in the wild-type. Electron-dense granules were observed in the induced albino-1 and the wild-type, which differ from albino-1. Cell membranes of the induced albino-1 and the wild-type were highly folded, differing from the albino-1 cell membrane by its non-folded pattern.

The pattern of 3,3'-diaminobenzidine (DAB) reaction product differed for the s-alm-1, alm-1 and the wild-type strains of V. dahliae utilized in this study. Localization of the DAB reaction was in the inner membrane of the mitochondria, while in the wild-type DAB localization was in the outer membrane of the mitochondria. Scytalone-induced albino-1 differed from alm-1 and the wild-type in that s-alm-1 had DAB localization on the inner and outer membranes of the mitochondria. This dual DAB localization reaction in alm-1 correlates with the findings of Taylor (1978).

All of the above evidence suggests that the cytoplasmic organellar changes that occur during the production of melanin, in induced albino-1, follow a pattern similar to the cytoplasmic organellar changes that occur in the production of melanin granules in the wild-type.

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