CYTOLOGY AND ULTRASTRUCTURE OF EUSTIGMATOPHYCEAE

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ABSTRACT

Twelve species of the algal class Eustigmatophyceae were studied by means of light and electron microscopy, with particular reference to structural aspects of the vegetative cells and the flagellar apparatus of the zoospores.

The vegetative cells are shown to have microfibrils (probably cellulose) in the cell wall (*Vischeria stellata*), lamellate vesicles in the cytoplasm of all the species observed and a clear connection between the chloroplast endoplasmic reticulum and the nuclear envelope only in representatives of the Monodopsidaceae. Microfibrils (probably cellulose) were also found in the cell wall of the tribophycean species *Ophiocytium maius*.

The most significant results on uni- and biflagellate zoospores include the observation of a Golgi body for the first time in a eustigmatophycean zoospore (*Vischeria helvetica*) and the first reconstruction of the system of flagellar roots in the Eustigmatophyceae (*V. stellata*). This consists of a rhizoplast and four microtubular roots: roots R1 (3 MTs) and R2 (2 MTs) originate at basal body B1 and run anteriorly around the flagellar swelling; root R3 (5 MTs) arises between the basal bodies and runs to the posterior end of the cell; root R4 (2 MTs) originates at basal body B2 and curves around the eyespot.

For comparison, zoospores of the tribophycean species *Heterococcus marietanii* and *H. protonematoides* were also studied. A system of flagellar roots consisting of a small rhizoplast and three microtubular roots, two directed anteriorly and one posteriorly was confirmed. A double helix was shown to be typical of the transition region of the flagella in this genus.

The few observations on settling cells showed the withdrawal of the complete flagellar apparatus including the swelling and the possibility of reformation of the pyrenoid in *Vischeria* from material stored in the spiral vesicles during the motile stage.

In preliminary observations on mitosis and cytokinesis it was found that, at early stages, basal bodies appear near the nuclear surface and the chloroplast and the pyrenoid divide. Cytokinesis seems to occur by a cleavage furrow.

My reconstruction of the flagellar root system in Eustigmatophyceae shows sufficient similarities with the flagellar roots of other heterokont algal and fungal classes to justify its inclusion with them in a single division, the Heterokontophyta. On the basis of this observation and the main ultrastructural features known for these classes, a phylogeny is constructed for the whole group and the probable characteristics of the common ancestor are proposed.

CONTENTS

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O livro GORDO da Diana

CHAPTER I. INTRODUCTION

A. GENERAL

The Eustigmatophyceae is a very small class of yellow-green algae, separated from the coccoid Tribophyceae (Xanthophyceae) in 1971 (Hibberd & Leedale, 1971a) on the basis of cytological and ultrastructural evidence (Hibberd & Leedale, 1970, 1972). Several unique features of zoospore organization, particularly the large size and special construction of the eyespot and the location of the flagellar swelling were found, together with a characteristic form of pyrenoid in the vegetative cells. This segregation was subsequently supported by differences in chloroplast pigment composition (e.g. Whittle, 1976; Whittle & Casselton, 1975a,b), the class containing the unique combination of chlorophyll a, β-carotene and violaxanthin and vaucheriaxanthin ester as major xanthophylls.

All known forms are coccoid in organization, obligate photoautotrophs, either freshwater or marine. The vegetative cells are spherical, polyhedral, stellate or ovoid in shape and may vary in size between 2µm and 32µm; they contain one to several nuclei, a parietal lobed chloroplast, usually with a conspicuous pyrenoid, often a red- pigmented body and a vacuole with vibrating granular contents. The zoospores, produced in some species, are especially diagnostic; they

are elongate, with a single anterior flagellum in most species, a unique type of eyespot, very large and independent of the chloroplast, and a unique type of flagellar swelling.

The group has been reviewed by Loeblich & Loeblich (1978) and Hibberd (1980a, 1982a,b), the former concentrating on biochemical and physiological aspects, the latter on morphology.

B. SYSTEMATICS

The new class has been generally accepted by phycologists, with a few exceptions (e.g. Fott, 1974; Dogadina, 1986). Its separation, however, gave rise to a series of taxonomic and nomenclatural problems, as pointed out by Silva (1979), and some authors, despite recognizing the new class, kept the eustigmatophycean species within the Xanthophyceae (e.g. Ettl, 1978) because of the absence of a proper systematic treatment of the group.

In 1981, a classification at every taxonomic level was proposed by Hibberd. Following this classification, and including the species recently described or others studied here, the class comprises 15 species, placed within 7 genera and 4 families (Table I). The different families were separated on the basis of cell shape and size, formation of zoospores or not and type of zoospore produced (uni- or biflagellate).

Depending on the authors, the new class has been placed either in a common division with some heterokont algae and fungi (e.g. Cavalier-Smith, 1986) or in a separate division,

the Eustigmatophyta (Leedale, 1974; Loeblich & Loeblich, 1978; Hibberd, 1981).

TABLE I. List of species included in the class Eustigmatophyceae, *sensu* Hibberd (1981).

FAMILIES	GENERA	SPECIES
EUSTIGMATACEAE	*Eustigmatos*	*E. magnus* *E. polyphem* *E. vischeri*
	Vischeria	*V. helvetica* *V. stellata* *V. punctata*
PSEUDOCHARACIOPSIDACEAE	*PSEUDOCHARACIOPSIS*	*P. minuta* *P. ovalis*
	BOTRYOCHLOROPSIS	*B. similis*
CHLOROBOTRYACEAE	*CHLOROBOTRYS*	*C. regularis*
MONODOPSIDACEAE	*MONODOPSIS*	*M. subterranea* *M. unipapilla*
	NANNOCHLOROPSIS	*N. gaditana* *N. oculata* *N. salina*

C. HABITAT

Most of the recent work on this group has been done on strains established in culture a long time ago by classic phycologists such as Vischer (see Hibberd, 1981). Species

need to be re-isolated and studied before the relative importance of the Eustigmatophyceae in nature can be assessed.

The few species described so far occur in either freshwater or marine habitats. Those placed in the Eustigmataceae were all isolated from soil (Petersen, 1932; Vischer, 1945), one species having been found very abundant in swimming pools (Adamson & Sommerfeld, 1978). The species with stipitate cells included in the Pseudocharaciopsidaceae were isolated from running water (Hibberd, 1982b, Lee & Bold, 1973), while the colonial species recently described by Preisig & Wilhelm (1989) was collected from a tank. *Chlorobotrys regularis*, only representative of the Chlorobotryaceae, was found in acid waters (Hibberd, 1974). The members of the genus *Monodopsis* were isolated both from soil and from a stream (Hibberd, 1982b); the other small members of this family, included in the genus *Nannochloropsis*, have been found in marine habitats (Droop, 1955; Bourrelly, 1958; Lubian, 1982a; Maruyama et al., 1986).

D. REPRODUCTION AND LIFE HISTORIES

The Eustigmatophyceae reproduce primarily by the formation of 2-4 autospores. This appears to be the only means of reproduction in species of the two families Chlorobotryaceae (Hibberd, 1974) and Monodopsidaceae (Hibberd, 1969; Antia et al., 1975; Maruyama et al., 1986). Members of the two other families can also reproduce by formation of uni- or biflagellate zoospores (Hibberd, 1969;

Lee & Bold, 1973; Preisig & Wilhelm, 1989). Sexual reproduction has not yet been observed in any species.

E. ULTRASTRUCTURE

Most of the information on the structure of the zoospores or vegetative cells was reported in the early seventies (Hibberd & Leedale, 1970, 1972) and was the basis for the separation of this new class of organisms, as previously mentioned. Six species were investigated, five producing zoospores with a single emergent flagellum and one producing biflagellate zoospores. Subsequently, other eustigmatophycean species were described, two biflagellate (Lee & Bold, 1973; Preisig & Wilhelm, 1989) and five azoosporic (Hibberd, 1974; Antia et al., 1975; Lubian, 1982a; Maruyama et al., 1986). Further details on flagellar structure and on vegetative cells were given by Hibberd (1980a).

In vegetative cells the main diagnostic features are the chloroplast organization, the presence and type of pyrenoid and the presence of lamellate vesicles in the cytoplasm or around the pyrenoid. The chloroplasts are bounded by a double-membraned envelope and by a layer of endoplasmic reticulum (CER) reported to be continuous with the nuclear envelope in the small azoosporic species (Antia et al., 1975; Lubian, 1982a; Maruyama et al., 1986). The chloroplast lamellae are composed of three thylakoids and no girdle lamella is present.

The large zoosporic species and *Chlorobotrys regularis* have stalked, polyhedral pyrenoids (Hibberd & Leedale, 1972; Hibberd, 1974) or spherical pyrenoids (Lee & Bold, 1973) in the vegetative cells but never in the zoospores; in these species, the pyrenoid matrix is devoid of thylakoids and lamellate vesicles can be seen against the pyrenoid faces. In the small azoosporic species, a terminal pyrenoid is present in *Monodopsis subterranea*, its matrix also not traversed by thylakoids (Hibberd, 1969, 1980a); in representatives of the genus *Nannochloropsis*, the presence of pyrenoids has been reported by Antia *et al.* (1975) but not observed by other authors (Lubian, 1982a; Maruyama *et al.*, 1986).

Lamellate vesicles are shown in the cytoplasm of most of the species studied but were not observed in *Nannochloropsis gaditana* (Lubian, 1982a,b) and their presence in the cytoplasm, around the pyrenoid or within the chloroplast matrix of both *N.oculata* and *N. salina* (Antia *et al.*, 1975), has not been clearly illustrated. In a later study of *N. oculata* (Maruyama *et al.*, 1975), lamellate vesicles are reported and shown only in the cytoplasm.

The zoospores have unique organization and were the main reason for establishing the class Eustigmatophyceae. The most important distinctive features are the eyespot and the flagella, in addition to the chloroplast organization and the presence of lamellate and spiral vesicles in the cytoplasm . The eyespot is very large, is not associated with the chloroplast and is located at the extreme anterior end of the cell; it consists of several droplets of different

size, with no membrane surrounding the whole complex; and it is associated with a flagellar swelling, always located at the base of the long, anterior and mastigoneme-bearing flagellum, either in uniflagellate zoospores (Hibberd & Leedale, 1972) or in biflagellate (Hibberd & Leedale, 1972; Lee & Bold, 1973; Preisig & Wilhelm, 1989). In biflagellate species the second flagellum is short, posteriorly directed and does not bear mastigonemes. A transitional helix has been clearly illustrated only in the long flagellum of *Vischeria helvetica* and in both flagella of *Pseudocharaciopsis ovalis* (Hibberd, 1979).

The lamellate vesicles in the cytoplasm of the zoospores are similar to those in the vegetative cells (see above) but the spiral vesicles, with fibrous, spirally wound contents can be found only in the zoospores (Hibberd, 1969, 1980a; Preisig & Wilhelm, 1989).

F. PIGMENT COMPOSITION

Relatively numerous studies on chloroplast pigment composition have been done, showing a unique distribution of pigments in the Eustigmatophyceae. The members of this class are most easily distinguished from those included in the Tribophyceae by the presence of violaxanthin as the major carotenoid and chlorophyll *a* as the only chlorophyll (Whittle & Casselton, 1969; 1975a,b; Stransky & Hager, 1970; Guillard & Lorenzen, 1972; Norgard et al., 1974; Whittle, 1976; Antia & Cheng, 1982; Lubian & Establier, 1982); in addition, both groups contain free and ester forms of

vaucheriaxanthin as well as β-carotene and other minor carotenoids. The high content of violaxanthin (up to 60% of the total carotenoid) in the Eustigmatophyceae is explained by the major role of this pigment in light harvesting for photosynthesis, possibly together with vaucheriaxanthin-ester (Brown, 1987; Owens *et al.*, 1987), in a way similar to the function of fucoxanthin in the Bacillariophyceae and Phaeophyceae or peridinin in the Dinophyceae (Larkum & Barrett, 1983; Anderson & Barrett, 1986).

The Eustigmatophyceae are normally green in culture but a variation in colour occurs, from green to brown and finally bright orange, when the cultures become older (Belcher & Miller, 1960; Stransky & Hager, 1970; Lubian & Establier, 1983). These changes in culture colour were seen to be related to changes of relative amounts of pigments during growth, the total carotenoids/chlorophyll a ratio rising and the proportion of keto-carotenoides such as cantaxanthin increasing in relation to total carotenoids, despite violaxanthin and vaucheriaxanthin remaining as the major ones (Antia & Cheng, 1982; Lubian & Establier, 1983).

Since establishment of the class, pigment composition and ultrastructure have therefore been the two major aspects used to classify an organism as a new eustigmatophycean species (Antia *et al.*, 1975; Lubian, 1982a,b; Maruyama *et al.*, 1986; Preisig & Wilhelm, 1989).

G. STORAGE PRODUCTS

Despite the importance of the nature of storage metabolites in algal taxonomy, nothing is yet known of the chemical composition of the storage metabolite in the Eustigmatophyceae. In transmission electron microscopy it appears as a lamellate structure in vesicles surrounding the pyrenoid or scattered in the cytoplasm of vegetative cells and zoospores, the lamellate vesicles. This photosynthate is not starch, as it gives a negative reaction with dilute iodine in potassium iodide (Hibberd, 1969; 1980a; Maruyama et al., 1986). A negative reaction to Thiery' s method for cytochemical demonstration of polysaccharides was also reported, together with insensibility to enzymatic extractions performed with pronase, lipase and phospholipase (Mesquita et. al, 1986).

Most of the species also accumulate a large amount of lipidic material, to judge from the osmiophilic nature of droplets and globules seen in electron micrographs (e.g. Hibberd, 1969) or from treatment with Sudan Black (Lubian, 1982a).

H. GROWTH, PHYSIOLOGY AND NUTRITION

Most of the fragmentary and early information on species now placed in the Eustigmatophyceae has been reviewed by Loeblich & Loeblich (1978). Studies with a few

representatives of the Eustigmataceae show growth rates and maximum cell densities in some species (Belcher & Miller, 1960) or that several tested organic substrates do not support growth of these organisms in the dark (Belcher & Miller, 1960; Casselton, 1966). All the other data concern species now placed in the Monodopsidaceae (see below).

In *Monodopsis subterranea*, mineral requirements and the effect of several organic substances were investigated (Miller & Fogg, 1957; 1958); it was found that requirement for potassium and tolerance to phosphate were low and that growth was improved by addition of sodium nitrate but not by exogenous supplies of organic substrates.

Most studies have been done on the small marine forms of the genus *Nannochloropsis*, either in the past or more recently. Earlier reports on *Nannochloropsis oculata* indicate that growth is not enhanced by the tested organic substances (Droop & Mc Gill, 1966; Bentley-Mowat & Reid, 1969) but is reduced by some organic acids or bases and inhibited by acetic and propionic acids (Droop, 1966); this species was found to be extremely tolerant to different salinities (Droop, 1955) and Ca/Mg ratios (Droop, 1958), to use several compounds as nitrogen sources (Droop, 1955) and to excrete vitamin B12 (Droop, 1968). A more recent study shows that light, temperature, salinity and nitrogen source in factorial combination influence growth of this organism (Terlizzi & Karlander, 1980).

In *Nannochloropsis salina* the excretion of high amounts of citrulline during the exponential phase has been reported (Berland *et al.*, 1970).

In other two species of *Nannochloropsis*, nitrogen deficiency was seen to promote lipid synthesis (Suen et al., 1987) and cells grown in saturating light were characterized by a high content of lipid, fatty acids and carbohydrate compared with those grown in light-limiting conditions (Sukenik & Carmeli, 1989).

In *Nannochloropsis gaditana*, growth was estimated under different culture and nutritional conditions, such as salinity, pH, temperature and nitrogen sources and optimum conditions were established (Lubian, 1979); another study shows that, in the range of values tested, an increase of light intensity can increase the exponential growth rate, whereas the maximum cell density reached by the cultures is dependent on the nitrate concentration in the medium (Lubian et al., 1986).

Another organism possibly belonging to this genus, known in the literature as *Stichococcus* sp., clone GSB Sticho (Ryther, 1954; Yentsch & Guillard, 1969; Norgard et al., 1974), also tolerates a wide range of salinity and temperature and grows well on several nitrogen sources (Ryther, 1954).

From these studies, it seems possible to generalize that the Eustigmatophyceae are obligate phototrophs, able to grow on a wide variety of nitrogen sources and without the requirement of external organic compounds, the marine species tolerating a wide range of salinities.

I. PHYLOGENY

Phylogeny of the Eustigmatophyceae has been considered from a biochemical standpoint (Ragan & Chapman, 1978) and on the basis of structural features (e.g. Hibberd, 1979; Moestrup, 1982; Cavalier-Smith, 1986). General agreement is that the line leading to the Eustigmatophyceae probably diverged before separation of all the heterokont groups and Hibberd (1979) considers this to have occurred before evolution of a girdle lamella in the chloroplast and a chrysophycean type of photoreceptor apparatus, involving a swelling at the proximal end of the short flagellum and an intraplastidial eyespot.

The definition of which classes of organisms to consider as heterokonts in order to include them in a common division Heterokontophyta is, however, a controversial matter. Thus, Hibberd (1969) placed in this new division the Xanthophyceae, Chrysophyceae, Phaeophyceae and Bacillariophyceae, previously found related by other authors (Pascher, 1914; Bourrelly, 1957), removing the Prymnesiophyceae and the Eustigmatophyceae. More recently, Leedale (1974) and Van den Hoek (1978) add the oomycete fungi and the Raphidophyceae (=Chloromonadophyceae). In 1986, Cavalier-Smith places all the mentioned groups in the new division Heterokonta (including Eustigmatophyceae, Prymnesiophyceae, oomycetes and other heterokont fungi).

A general phylogenetic relationship between the Eustigmatophyceae and the heterokont algae and fungi seems therefore well established but more detailed information on

these groups is necessary to clarify their relative positions during evolution.

J. AIMS OF THE STUDY

At the beginning of this research programme, zoospore structure in the Eustigmatophyceae was relatively well-known (Hibberd, 1980a; Hibberd & Leedale, 1972; Lee & Bold, 1973) but observations on vegetative cells were less detailed though representatives of each of the families comprising the class had been examined.

Information on vegetative cell structure was therefore scarce, though this was likely to be valuable for the systematics of the group, particularly the azoosporic species as pointed out by Hibberd in 1980 (Hibberd, 1980a). Processes of zoospore formation and settling remained to be studied, as did details of flagella structure with possible phylogenetic importance, in particular details of the flagellar roots. Nothing was also known about mitosis, meiosis, cell wall formation and structure.

This research was therefore developed to investigate some of these aspects in existing isolates of Eustigmatophyceae and related Tribophyceae, grown under various conditions. The results obtained are presented in éight main sections concerning the structure of vegetative cells, zoospore formation, structure and settling, mitosis and cytokinesis (preliminary observations) and cell wall structure and chemistry. These results are expected to be a valuable contribution in algal cytology, taxonomy and

phylogeny, amplifying knowledge on this particular group of organisms.

CHAPTER II. MATERIALS AND METHODS

A. SPECIES STUDIED

Thirty strains of Eustigmatophyceae and Tribophyceae used in this research programme were obtained from the Culture Collections of Algae at Göttingen (Schlösser, 1982) and at the University of Texas (Starr and Zeikus, 1987) (Tables II and III).

TABLE II. List of strains obtained from Göttingen and media used for growth.

CLASS	SPECIES	STRAIN	MEDIUM
EUSTIGMATOPHYCEAE	*Eustigmatos vischeri*	860.1	1b
	Eustigmatos polyphem	38.84	1
	Monodus subterraneus	848.1	1b
	Monodus unipapilla	8.83	1
	Nannocloropsis oculata	38.85	6
	Nannocloropsis salina	40.85	5
	Polyedriella helvetica	876.1	1b
	Vischeria punctata	887.1	1b
	Vischeria stellata	887.2	1b
	Vischeria stellata	33.83	1
TRIBOPHYCEAE (coccoid)	*Botrydiopsis callosa*	30.83	1
	Botrydiopsis intercedens	806.3	1b
	Botrydiopsis pyrenoidosa	31.83	1
	Bumilleriopsis filiformis	809.2	1b
	Bumilleriopsis peterseniana	809.3,	1b
	Chloridella neglecta	813.1b	1b
	Chloridella neglecta	48.84	1
	Ophiocytium capitatum	7.83	1
	Ophiocytium maius	855.1	1
	Ophiocytium parvulum	37.84	1
	Pleurochloris meiringensis	860.3	1b
(filamentous)	*Bumilleria sicula*	808.1	1
	Heterothrix montana	836.3	1
	Heterococcus marietanii	837.7	1
	Heterococcus protonematoides	835.9	1

TABLE III. List of strains obtained from the University of Texas and media used for growth.

CLASS	SPECIES	STRAIN	MEDIUM
EUSTIGMATOPHYCEAE	*Eustigmatos magna*	B2351	1/1b
	Nannochloropsis oculata	LB2164	6
	Pseudocharaciopsis texensis	2113	1/1b
	Vischeria punctata	86	1/1b
TRIBOPHYCEAE	*Chlorocloster enganidensis*	307	1/1b

The strain *Nannochloropsis gaditana* was obtained from Cadiz, Spain.

B. CULTURE METHODS

1. General

The species were maintained on media 1, 1b, 5 and 6 (Tables II, III), with the chemical composition described by Schlösser (1982) and reproduced in Table IV, either on agar slopes (test tubes) or liquid media (100 ml in 250 ml Erlenmeyer flasks), at 12°C and in a regime of constant light. During experimental work, the species were usually subcultured every two weeks but could be kept up to three months on the agar slopes without subculturing. Most of the

strains were also cultivated in soil water medium, supplied with cheese, wheat, NH_4MgPO_4 or $CaCO_3$.

TABLE IV. Chemical composition of the media used for growing the different eustigmatophycean and tribophycean species (after Schlosser, 1982).

	STOCK SOLUTION (ml)	NUTRIENT SOLUTION (ml)*			
		1	1b	5	6
KNO3	1.0	20	20	20	20
KH2PO4	0.1	20	20	20	20
MgSO4.7H2O	0.1	20	20	20	20
Micronutrient solution **		5	5	5	5
Soil Extract freshwater		30	30	-	30
-seawater		-	-	30	-
Distilled water		905	905	-	450
Filtered seawater		-	-	905	455
Proteose-peptone (g)		-	1	-	-
Vitamin B12 ($5x10^{-6}$g)		-	-	1	1

* Except for proteose-peptone.
** Details of preparation are given in Appendix 1.

2. Manipulation of cultures and growth estimation

Most of the listed species were tested several times to induce the production of zoospores by altering the conditions of culture growth, either by controlling the light regime,

the quality of the medium, the temperature or combinations of these factors at the same time.

The method which, after several attempts, proved successful in producing zoospores in sufficient quantity for electron microscopical studies in some species was to add fresh liquid medium to cultures on solidified medium (more than 1 month old) and to subject the culture to a long period of darkness (about 60 hours). Following this procedure, zoospores of *Vischeria helvetica* (=*Polyedriella helvetica*), *Vischeria punctata*, *Vischeria stellata*, *Eustigmatos magnus* (=*Eustigmatos magna*), *Pseudocharaciopsis minuta* (=*Pseudocharaciopsis texensis*), *Heterococcus marietanii* and *Heterococcus protonematoides* were obtained and studied.

In an attempt to synchronize cultures for studying mitosis, duplicate cultures of *Vischeria stellata* (887.2) were maintained in an 8:16h dark-light period at 18-23^{o}C for fifteen days and culture growth estimated by means of cell counts with a haemocytometer. A sample of 5 ml was fixed each day, after shaking the cultures thoroughly to minimize sampling error, and the cells on four entire haemocytometer grids were counted, a growth curve being obtained by plotting numbers of cells against time.

C. LIGHT AND ELECTRON MICROSCOPY

For light microscopy, zoospores or vegetative cells were normally examined alive. However, during preparation of the material for electron microscopy, a small drop of the cell suspension in Spurr's resin prepared for embedding was always

mounted on a slide, covered with a coverslip and polymerised at 70°C for 24h. This provided permanent preparations of the material that could be examined with the light microscope at any time.

Direct preparations of zoospores and cell wall material were prepared by mounting a drop of zoospore or cell wall suspension on a formvar/carbon-coated grid, fixing the material by exposure to 2-3% osmium tetroxide vapour, allowing to dry and shadowcasting with gold/palladium before observation with the electron microscope.

For sectioning, vegetative cells of some tribophycean species (genera *Ophiocytium* and *Botrydiopsis*) and all the eustigmatophycean species were fixed in 2.5%-3% glutaraldehyde (GA) in 0.2M cacodylate buffer, pH 7.2, at room temperature, for 2h and post-fixed in 2% osmium tetroxide in the same buffer for 1h30m-2h. Zoospores of both eustigmatophycean and tribophycean species were fixed in different ways (Table V), mostly in a mixture of 2.5% GA and 2% OsO_4 in 0.2M cacodylate buffer, pH 7.2, at 4°C, for 2h. Material was dehydrated in a graded ethanol series, embedded in Spurr's resin and sectioned on a Reichert OMU4 ultramicrotome, using glass or diamond knives. Sections were stained with 0.5% alcoholic uranyl acetate and Reynold's lead citrate and examined on a JEOL 1200 EX electron microscope.

For SEM, the material was fixed in 2% OsO_4 in 0.2M cacodylate buffer, pH 7.2, at room temperature, overnight and was dehydrated in a graded series of acetone. Samples were dried in a Polaron Critical Point dryer, fixed onto stubs with adhesive made from sellotape, then coated with gold in

a Polaron Sputter Coater Unit and viewed with a CamScan 3/30 VM scanning electron microscope.

TABLE V. Different fixation schedules used to fix zoospores.

BUFFER	FIXATIVES
Cacodylate 0.2M, pH 7.2	2%OsO4, 4°C, 1h 1.25%Ga + 1%OsO4, 4°C, 2h 2.5%Ga + 2%OsO4, 4°C, 2h 2.5-3%Ga, Room temp., 2h+2%OsO4, Room temp., 1h30min
Phosphate 0.1M, pH 7	2%OsO4, 4°C, 1h 1.25%Ga+ 1%OsO4, 4°C, 2h

D. CELL WALL STUDIES

1. Extraction of cell wall material

The general method used for cell wall extraction, is diagrammatically illustrated in Fig.1. About 700 ml of liquid cultures of *Vischeria stellata* and *Ophiocytium maius* were reduced to a pellet, after centrifugation at 4000 r min^{-1}. This pellet was placed in the cylinder of a French press and submitted to a pressure of 1000 Pascal. This operation was repeated 4-6 times to break the maximum number of cells. After centrifugation, the supernatant was discarded and the white top layer of the pellet removed with a Pasteur pipette

and placed in a round-bottom flask to be freeze-dried.

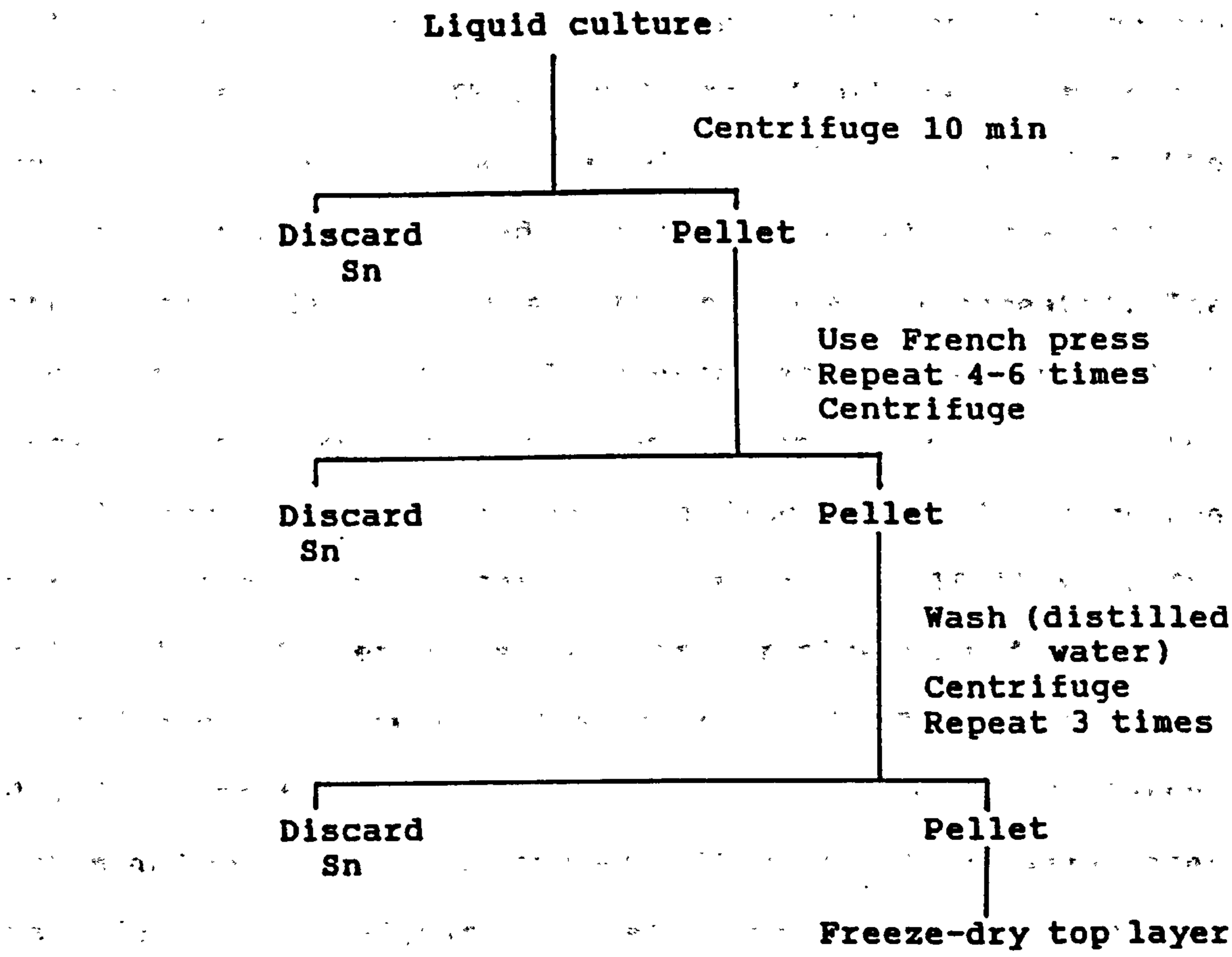


Fig. 1. Protocol used for preparing cell wall material from *Vischeria stellata* and *Ophiocytium maius*. Sn, supernatant.

Some of this material was diluted in distilled water and prepared for direct observation with the TEM (as previously indicated) and the remaining cell wall material was used to identify the insoluble fraction of carbohydrates present.

2. Hydrolysis of polysaccharides

The freeze-dried material of *Vischeria stellata* and *Ophiocytium maius* was hydrolysed by addition of aqueous trifluoracetic acid (TFA, 1 ml), the flask being stoppered securely and heated in an oven at 90-95°C for about 20h. The hydrolysate was evaporated to dryness on a rotary evaporator, then water added (1 ml) and the evaporation repeated. The addition of water followed by evaporation was repeated at least twice more. The final residue was dissolved in water (2 ml) and cations removed by adding cation ion exchange resin (micro spatula tip) and shaking for 10-15 min. The material was filtered using a Pasteur pipette and the resin washed once with water (less than 1 ml). The solution was divided into two equal volumes, placed into two different tubes and evaporated to dryness. These solutions were later used for sugar analysis, either by paper chromatography or gas liquid chromatography.

3. Analysis of sugar mixtures

a. Paper chromatography

Paper chromatograms were prepared using several capillary drops of a known standard mixture of sugars and of the hydrolysed cell wall material, dissolved in distilled water. The papers were irrigated in a mixed solvent of ethyl acetate (8 parts), pyridine (2 parts) and

water (1 part) for about 20 h. After drying, the sugars were located by the the following procedures:

a) Silver nitrate: the dry paper was dipped in a silver nitrate solution prepared by adding a saturated aqueous solution of $AgNO_3$ (1 ml) to acetone (500 ml) followed by sufficient water to redissolve the precipitate. After being dried in air, the paper was dipped in a freshly prepared solution of sodium hydroxide (40%, 1 ml) in methanol (40 ml) and allowed to become just dry in air. Finally, the paper was dipped in a solution of aqueous sodium thiosulphate (25% w/v), washed thoroughly with tap water and allowed to dry.

b) p-Anisidine hydrochloride: the dry paper was dipped in a solution of p-anisidine (3% w/v) in n-butanol (containing a little water) and heated in an oven (3-5 min) at 110°C.

b. Gas-liquid chromatography

After adding ice-cold distilled water (1-2 ml), potassium borohydride (10 mg) and checking that the pH was more than 8, the hydrolysate was left overnight at 2°C. Some drops of glacial acetic acid were added to remove excess of borohydride, then methanol (1 ml) and the solution was evaporated to dryness. The addition of methanol followed by evaporation was repeated six times. The solid was dried in a vacuum oven at 60°C for 30 min, followed by addition of acetic anhydride (1 ml), anhydrous sodium acetate (5 mg) and heated in a stoppered tube for 2 h at 90°C. The solution was concentrated to dryness on the rotary evaporator, followed

by addition of chloroform (2 ml) and its extraction three times with water (0,5 ml). The top layer was discarded after each washing and, finally, the chloroform solution was evaporated to dryness and kept for analysis by GLC. This analysis was performed in a PYE series 104 chromatograph, at 220^{o}C.

CHAPTER III. RESULTS

A. STRUCTURE OF THE VEGETATIVE CELLS

1. Eustigmataceae

Most representatives of this family have been studied either with the light microscope or with the transmission electron microscope (Hibberd, 1969; Hibberd & Leedale, 1972) and the results presented here mostly confirm those studies.

The vegetative cells of the species included in the genus *Eustigmatos* (*E. magnus*, *E. polyphem* and *E. vischeri*) and those placed in the genus *Vischeria* (*V. helvetica*, *V. punctata* and *V. stellata*) can be distinguished only by the morphology of the cell wall (Hibberd, 1981); the cell wall is smooth and featureless in the first genus (Figs 2, 3) but is raised into projections or ridges in the second (Figs 4, 5), though in culture these are sometimes infrequent in some species. Except for this feature, all the species are very similar in organization; they are spherical or polyhedral, 7-15 µm in diameter, uninucleate, containing a single parietal chloroplast (Figs 2, 4); a large pyrenoid can usually be seen against the internal face of the chloroplast, together with a vacuole with granular contents and a red-pigmented globule (Fig. 2).

General aspects of cell structure (Figs 6, 7) are also very similar in all the mentioned species. The cell wall is usually dense, composed of several layers (see Fig. 7); the nucleus is more or less spherical, with a nucleolus, and lies near the parietal chloroplast; a large, angular pyrenoid is attached to the inner face of the chloroplast by a narrow stalk; a large vesicle with osmiophilic globular contents (see also Fig. 8), probably corresponding to the red-pigmented body seen with the light microscope, is usually present in the cytoplasm of these cells; the remaining cytoplasm is filled with vacuoles, lipid droplets, mitochondria and other normal cell organelles, in addition to the typical lamellate vesicles (illustrated in Fig. 9).

A single Golgi body, consisting of three cisternae can occasionally be seen close to or parallel with the surface of the nucleus (Fig. 10).

The chloroplast lamellae are evenly spaced and run through the whole length of the chloroplast, a girdle lamella being absent (Fig. 11). The layer of endoplasmic reticulum that surrounds the chloroplast (CER) is usually more evident at the base of the pyrenoid together with a well-developed periplastidial reticulum (Fig. 12). The pyrenoid matrix appears finely granulated, not penetrated by chloroplast lamellae or individual thylakoids, and lamellate vesicles lie against its faces (Figs 12, 13).

Figs 2-7. Vegetative cells of *Eustigmatos vischeri*, *Vischeria stellata* and *V. punctata*.

Fig. 2. Spherical cells of *E. vischeri* showing the parietal chloroplast, the angular pyrenoid, the vacuole with some refractile granules and the red body. Anoptral contrast. x 1800.

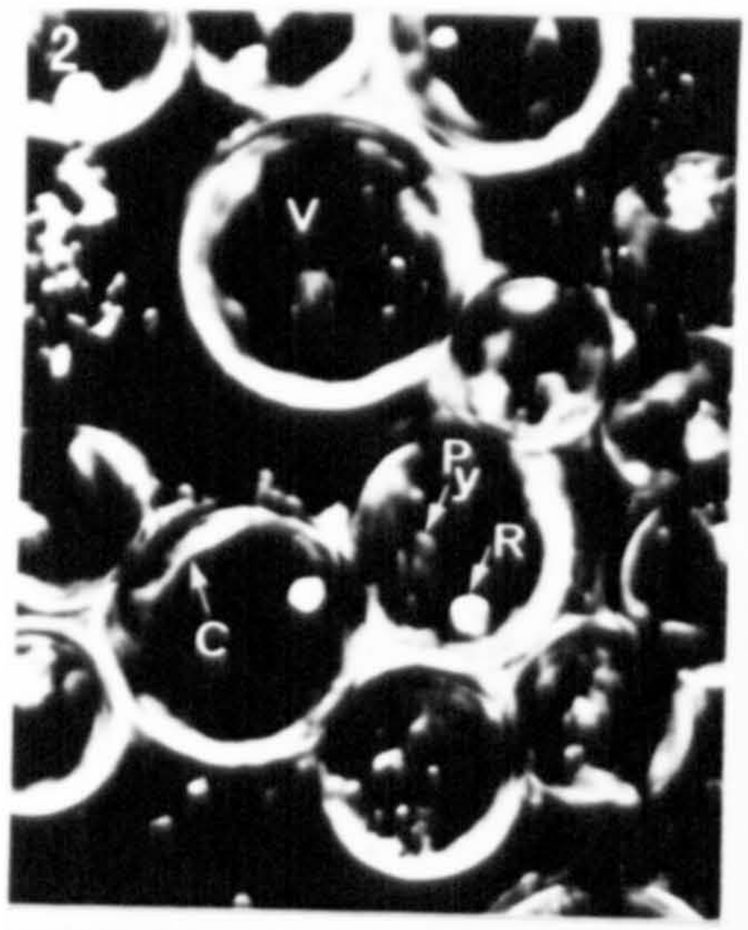
Fig. 3. Smooth cell walls of *E. vischeri*, shown by SEM. x 2500.

Fig. 4. Cells of *V. stellata* with a spiky shape. Bright field. x 1800.

Fig. 5. Nodules on the cell wall of *V. stellata*, as seen by SEM. x 3500.

Fig. 6,7. Sections of *Eustigmatos visheri* (Fig. 6) and *Vischeria punctata* (Fig. 7) showing the general cell structure. Fig. 6 x 15000; Fig. 7 x 20000.

Abbreviations used in figures: C, chloroplast; LV, lamellate vesicle; N, nucleus; Nu, nucleolus; OG, osmiophilic globule; PR, periplastidial reticulum; Py, pyrenoid; R, red body; V, vacuole; W, cell wall.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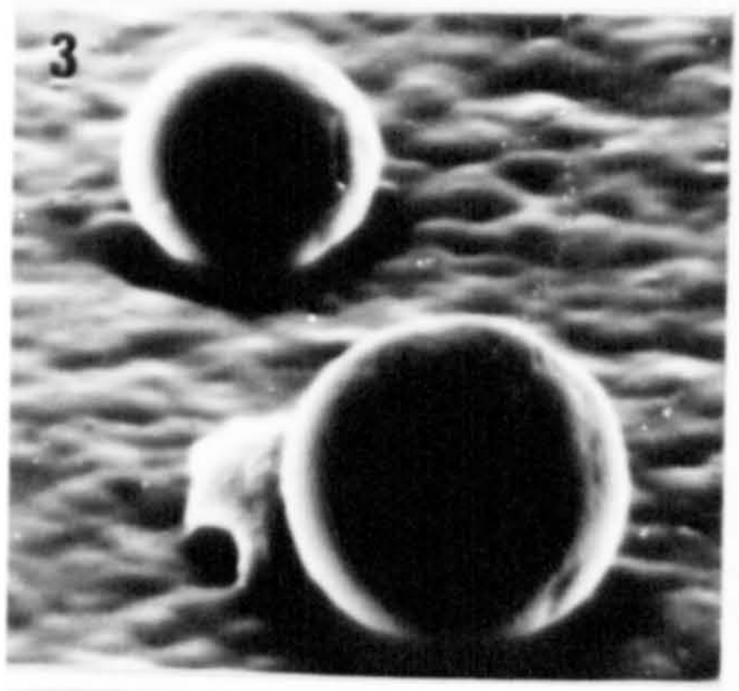
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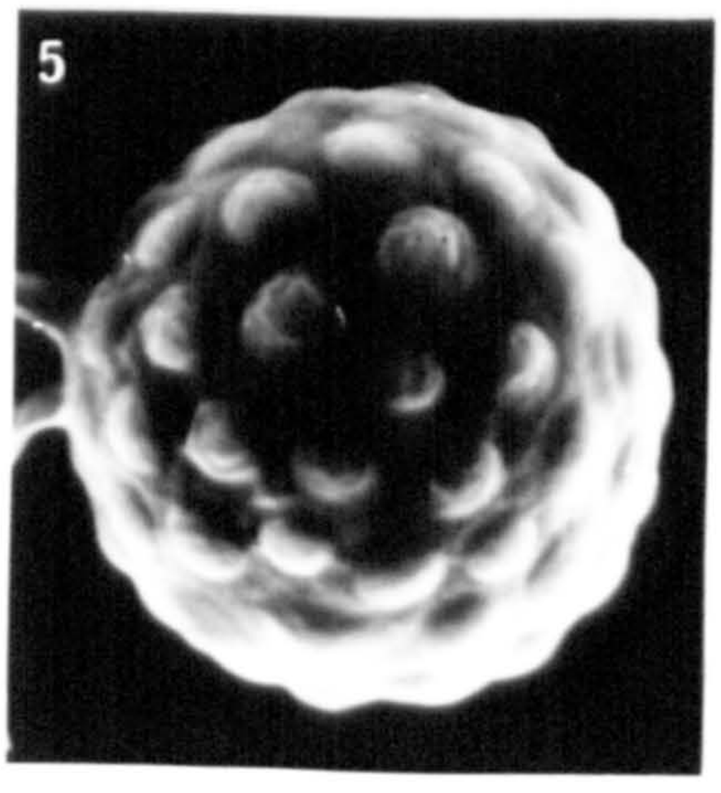
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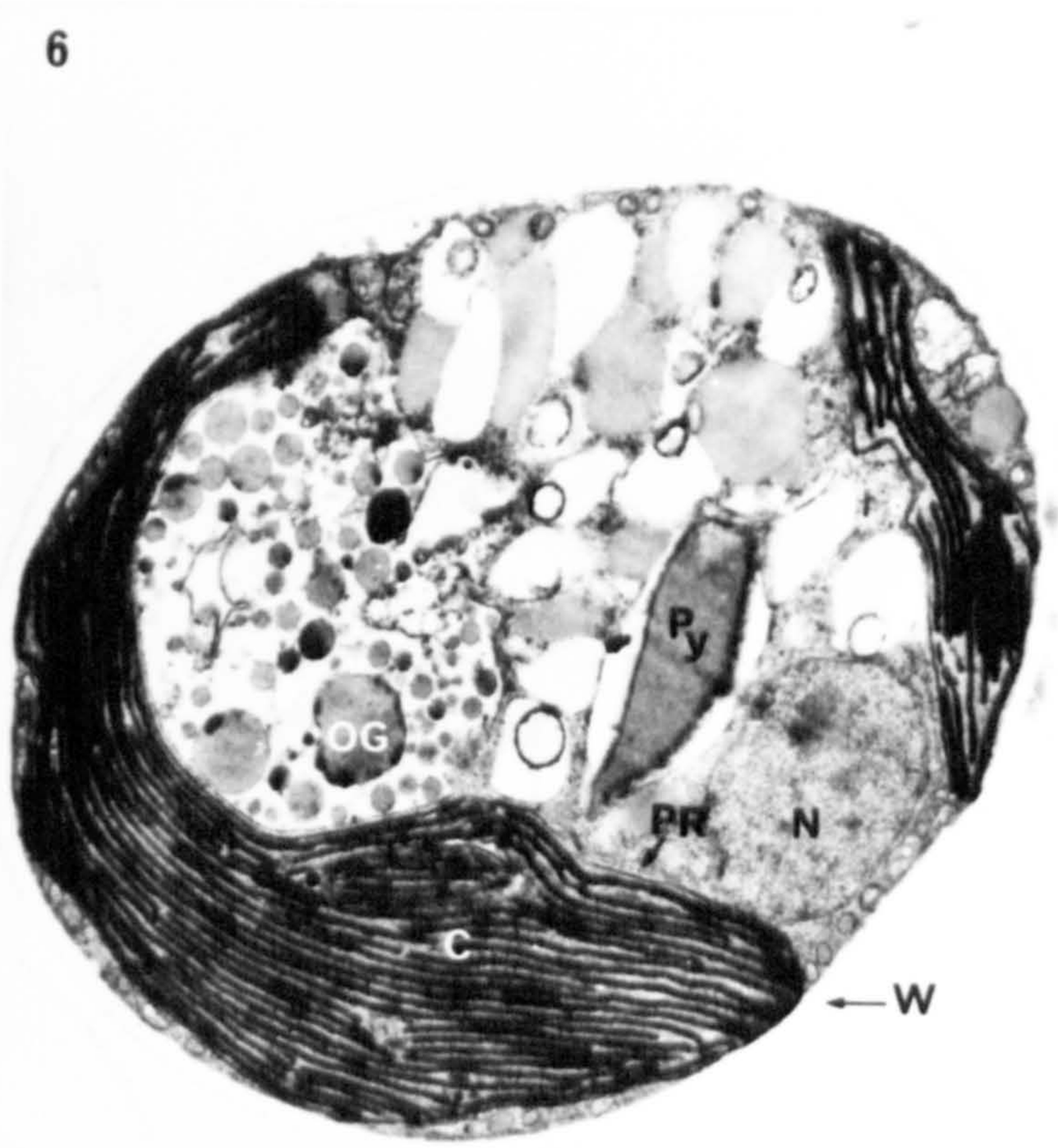
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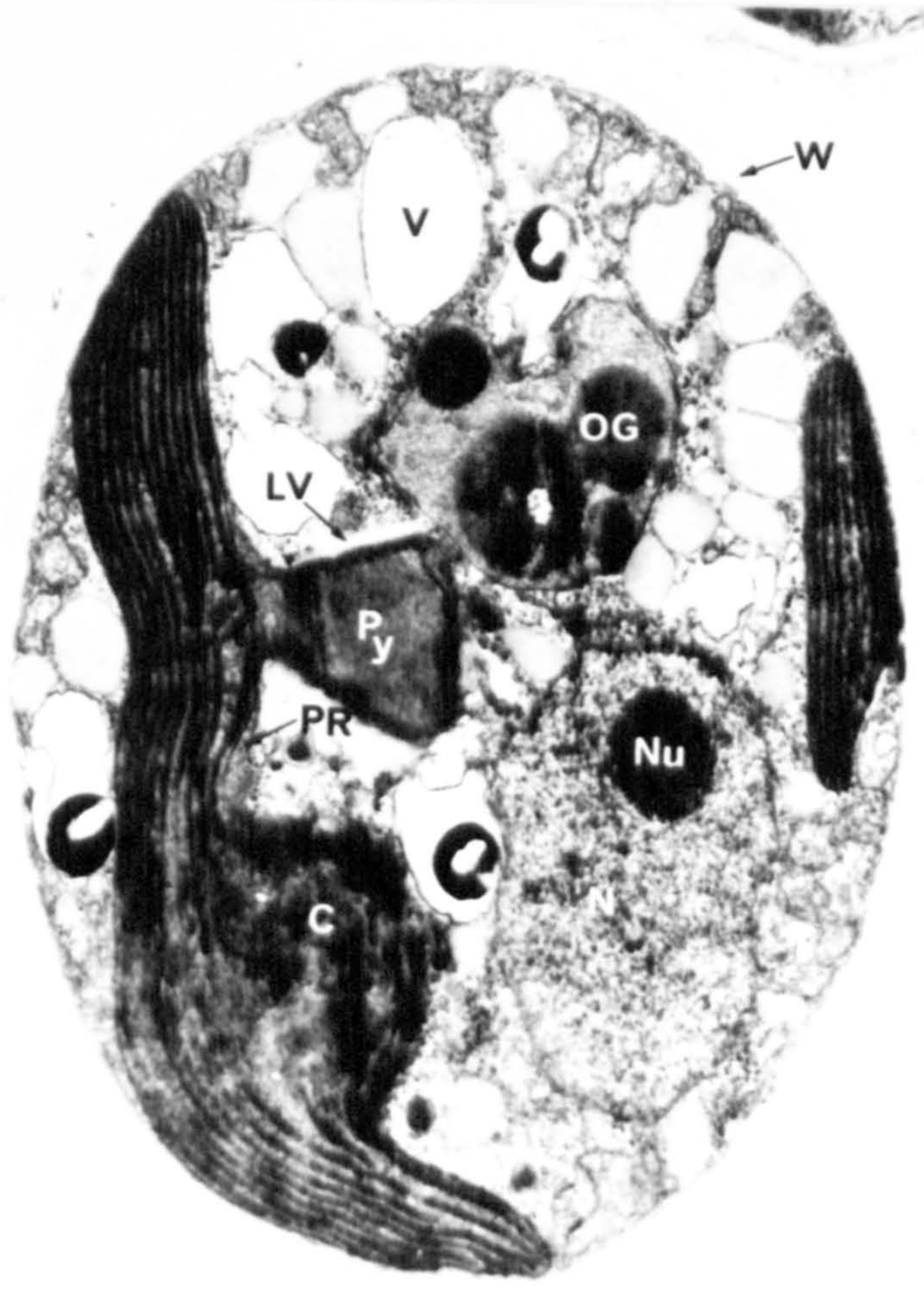
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Figs 8-13. Sections of some representatives of the genera *Eustigmatos* and *Vischeria*, showing some structural aspects in detail.

Fig. 8. Vesicle with osmiophillic globules (red body) in *E. polyphem*. x 27000.

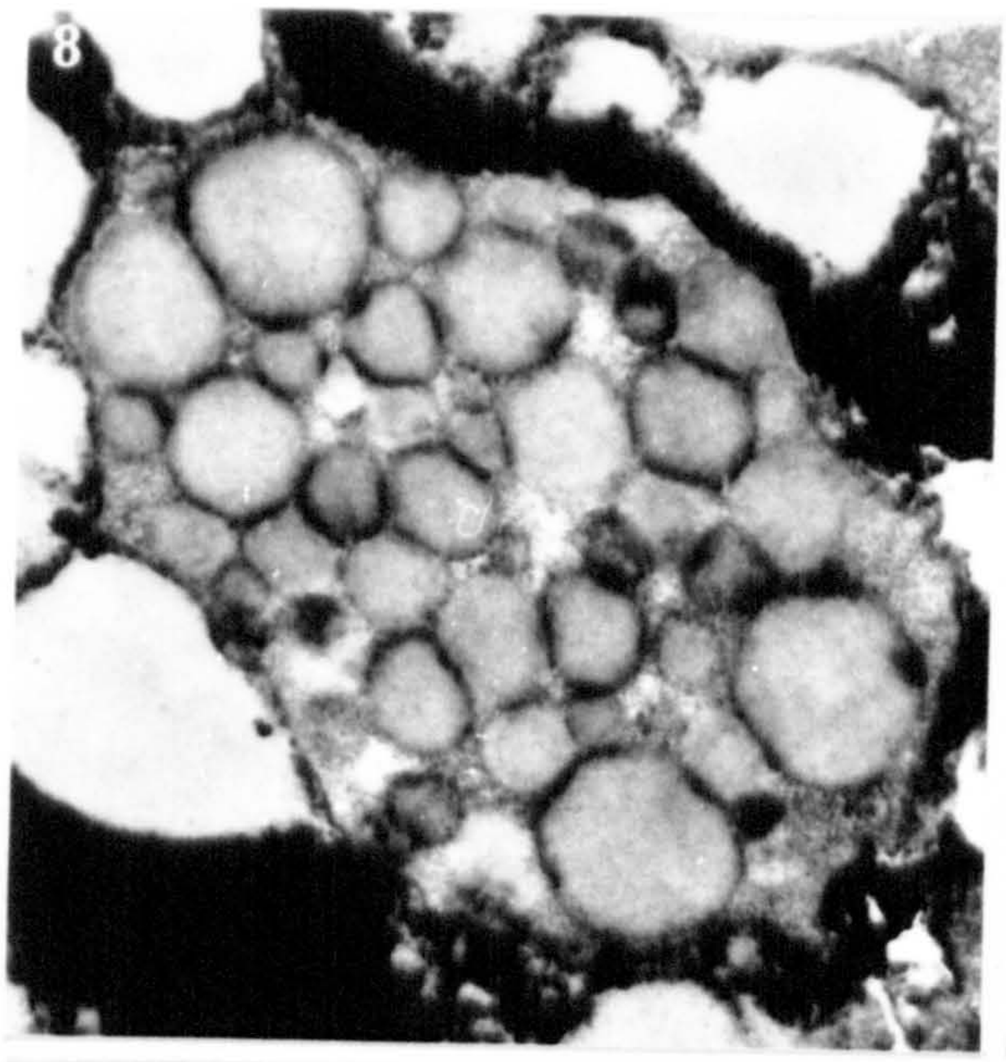
Fig. 9. Lamellate vesicles in *V. stellata*. x 54000.

Fig. 10. Small Golgi body (arrows) near the nucleus (N) in *E. vischeri*. x 45000.

Fig. 11. Chloroplast in *E. vischeri*. x 30000.

Fig. 12. Pyrenoid in *V. punctata*. Note the CER, together with the periplastidial reticulum at the basis of the pyrenoid (small arrows) and a lamellate vesicle against its face (large arrow). x 45000.

Fig. 13. Pyrenoid in *E. polyphem*. Note the lamellate vesicles (arrows). x 27000.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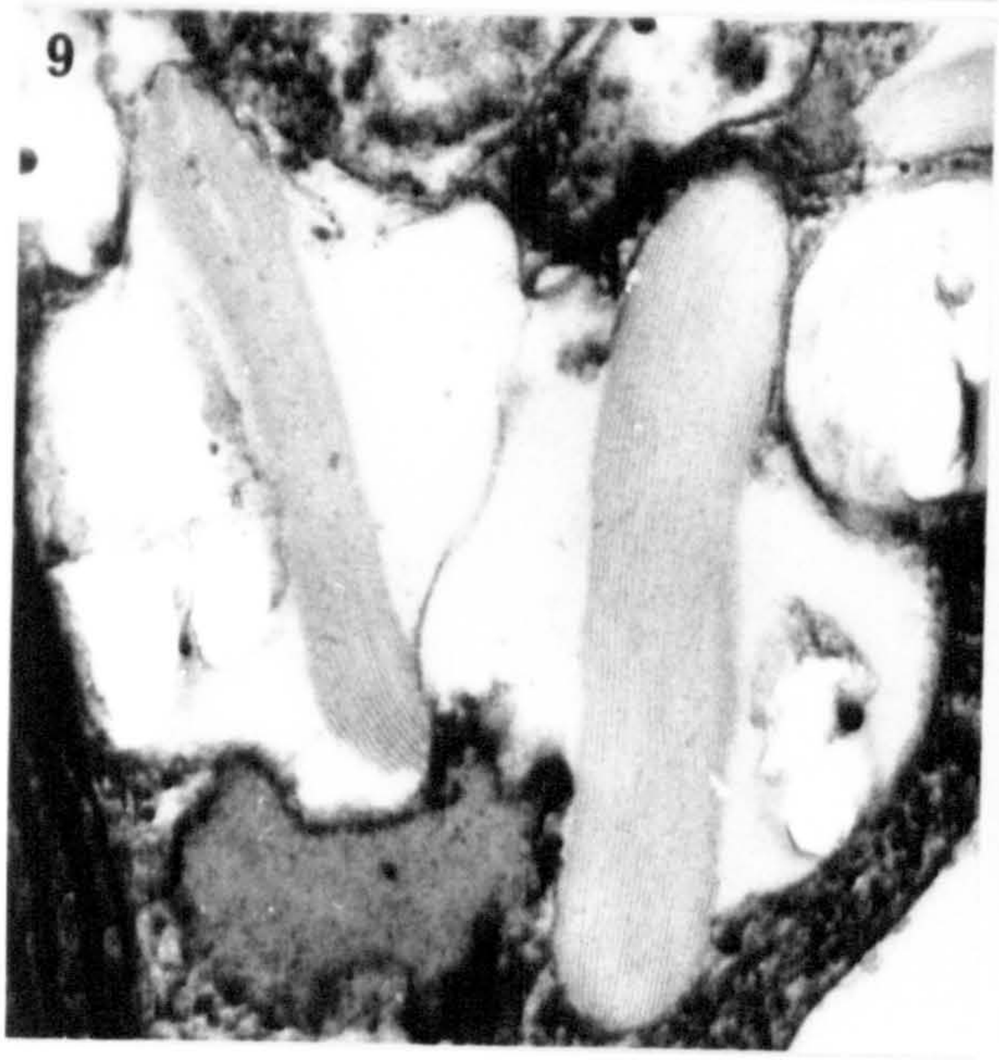
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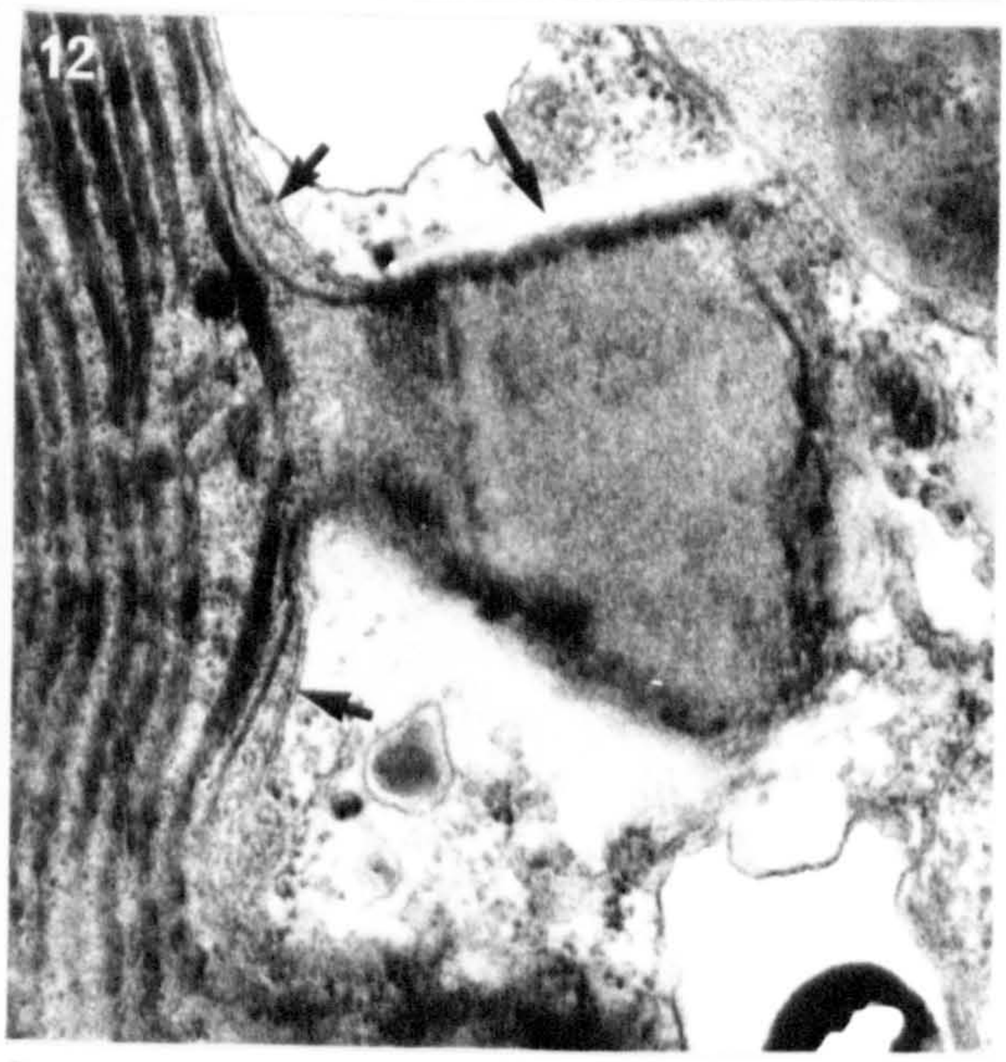
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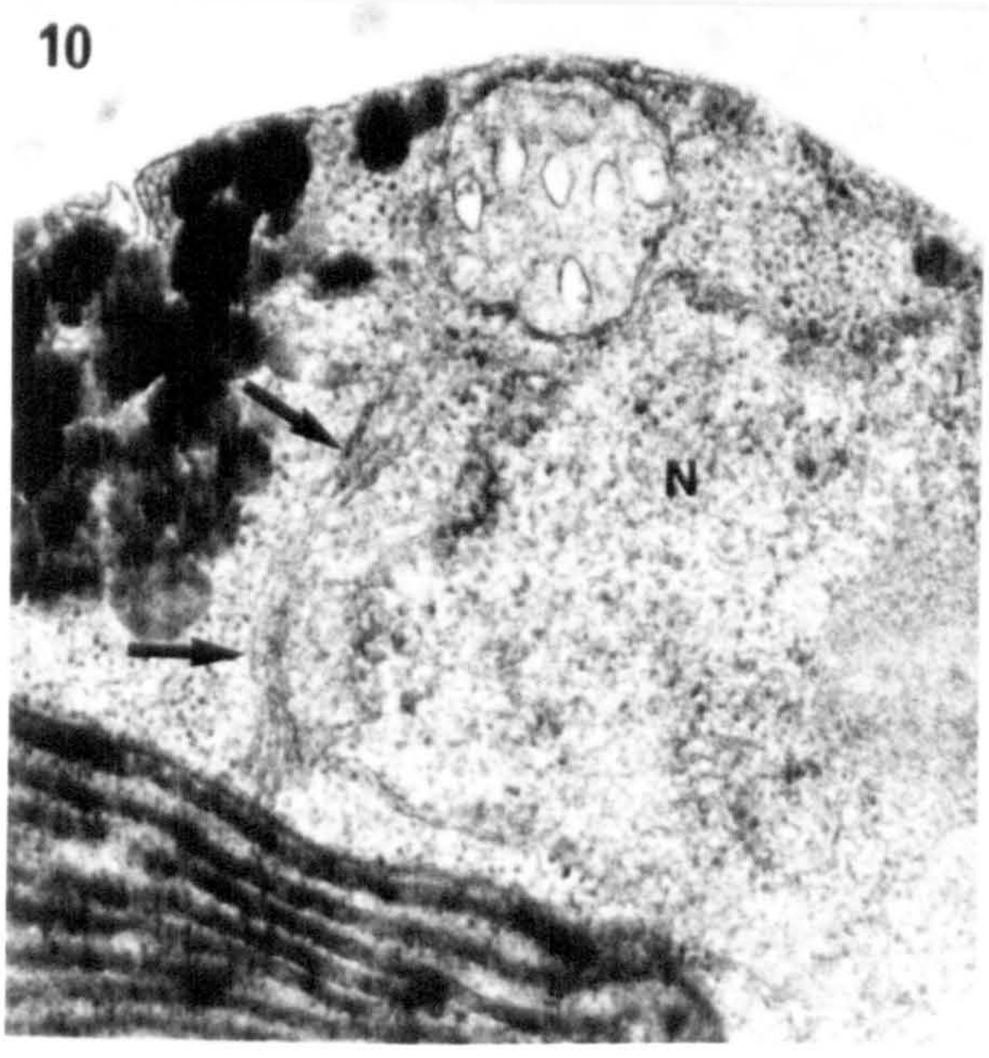
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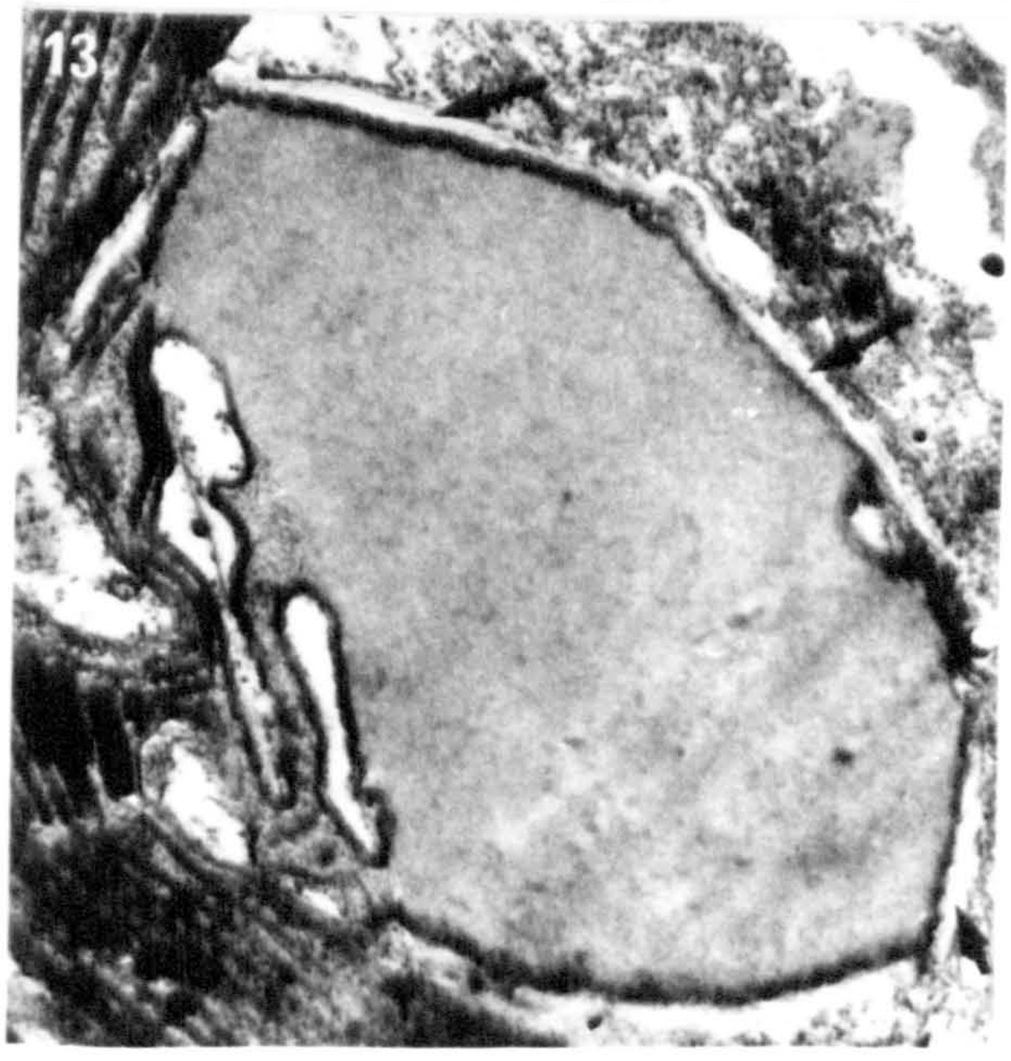
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2. Pseudocharaciopsidaceae

The species *Pseudocharaciopsis minuta* was first described as a member of the Eustigmatophyceae by Lee & Bold (1973), with the designation of *Pseudocharaciopsis texensis*. The general structure was described but details of the main organelles were not shown.

The vegetative cells of this species are elongated, cylindrical, up to 32µm long and 12µm wide, with an attenuate tip at the anterior end and a short stalk with an attaching disc (holdfast) at the posterior end (Figs 14, 15). A single parietal chloroplast, with one or more spherical or angular pyrenoids, can easily be seen in light microscopy (Fig. 15).

Thin sections (Figs 16-18) show the dense cell wall with an irregular outline (arrows), several nuclei, with no obvious connection with the parietal chloroplast, and more than one pyrenoid projecting from the internal face of the chloroplast, either spherical or angular in shape; the cytoplasm is filled with lamellate vesicles, large numbers of osmiophillic globules, different in size and density, and other normal cell organelles. One or more small Golgi bodies, with 3-5 cisternae, are usually present close to the nucleus (see Fig. 19).

Ultrastructural details of the chloroplast and the pyrenoid (Fig. 20) are as described for the previous eustigmatophycean species, lamellate vesicles also surrounding the pyrenoid; in this species, the CER is clearly visible not only at the base of the pyrenoid,

Figs 14-18. Vegetative cells of *Pseudocharaciopsis minuta*.

Fig. 14. SEM. Note the cell shape and the holdfast (arrow). x 2000.

Fig. 15. Light microscopy. Note the parietal chloroplast and the pyrenoid. Bright field. x 2000.

Figs 16-18. Longitudinal (Figs 16, 18) and transverse (Fig. 17) sections, showing the general structure. Note the outline of the cell wall (arrows) and the abundance of lamellate vesicles. Fig. 16 x 8000; Fig. 17 x 9000; Fig. 18 x 65000.

Abbreviations used in figures: C, chloroplast; H, holdfast; LV, lamellate vesicle; N, nucleus; OG, osmiophillic globule; Py, pyrenoid.

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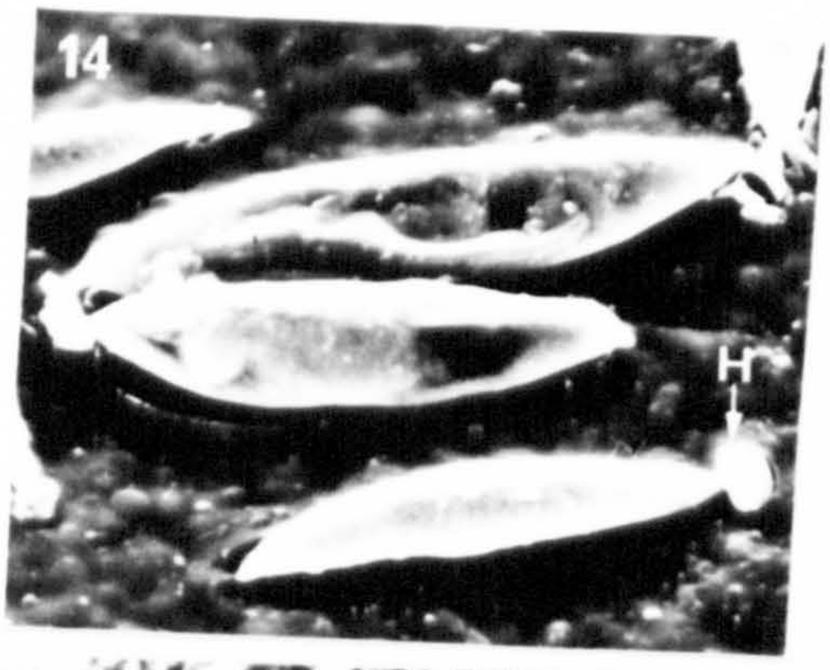

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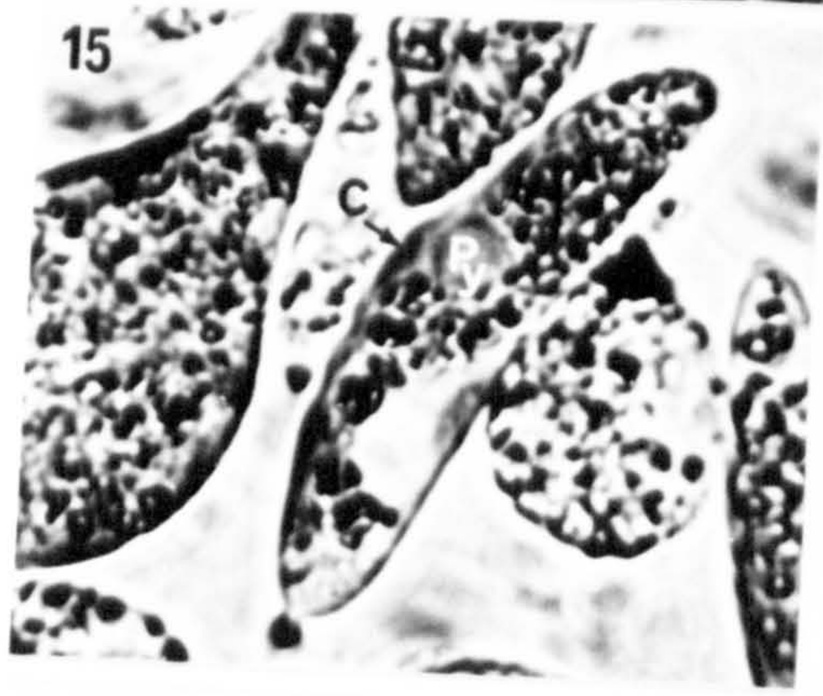

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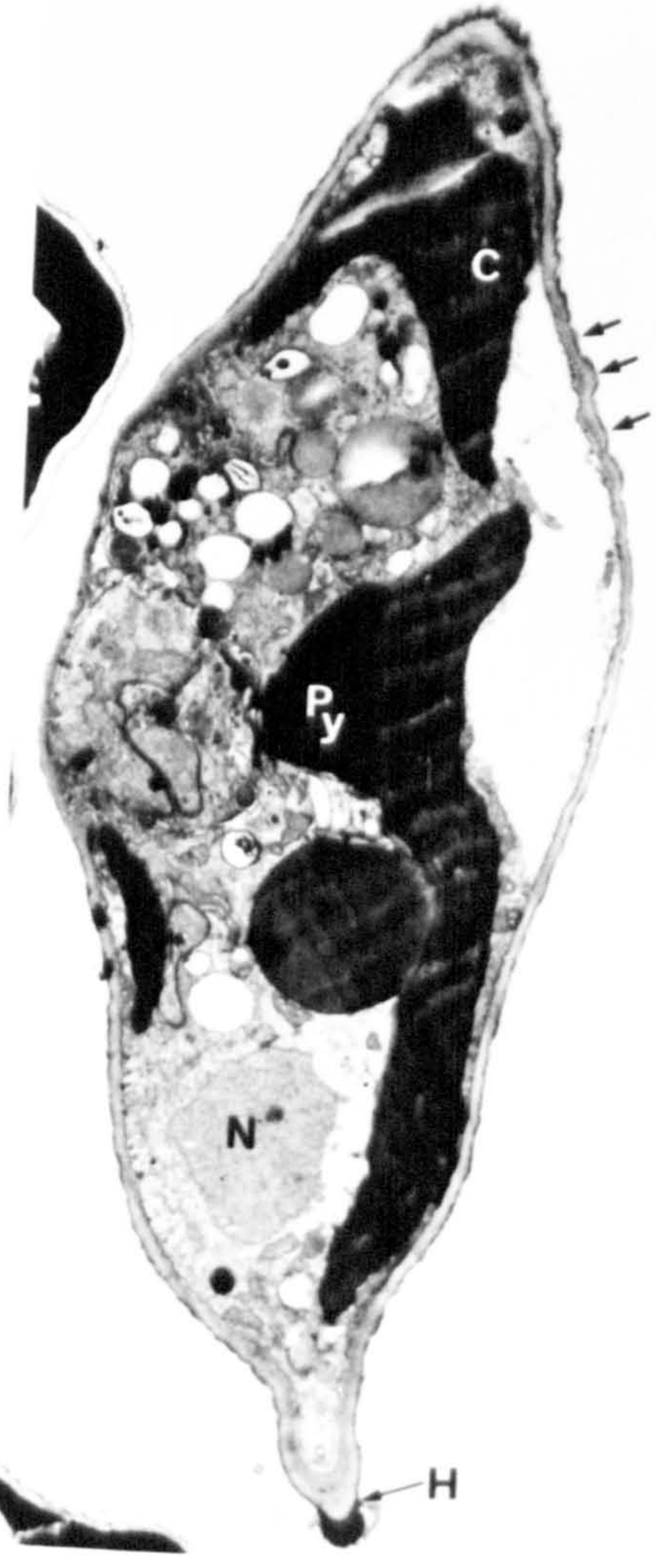

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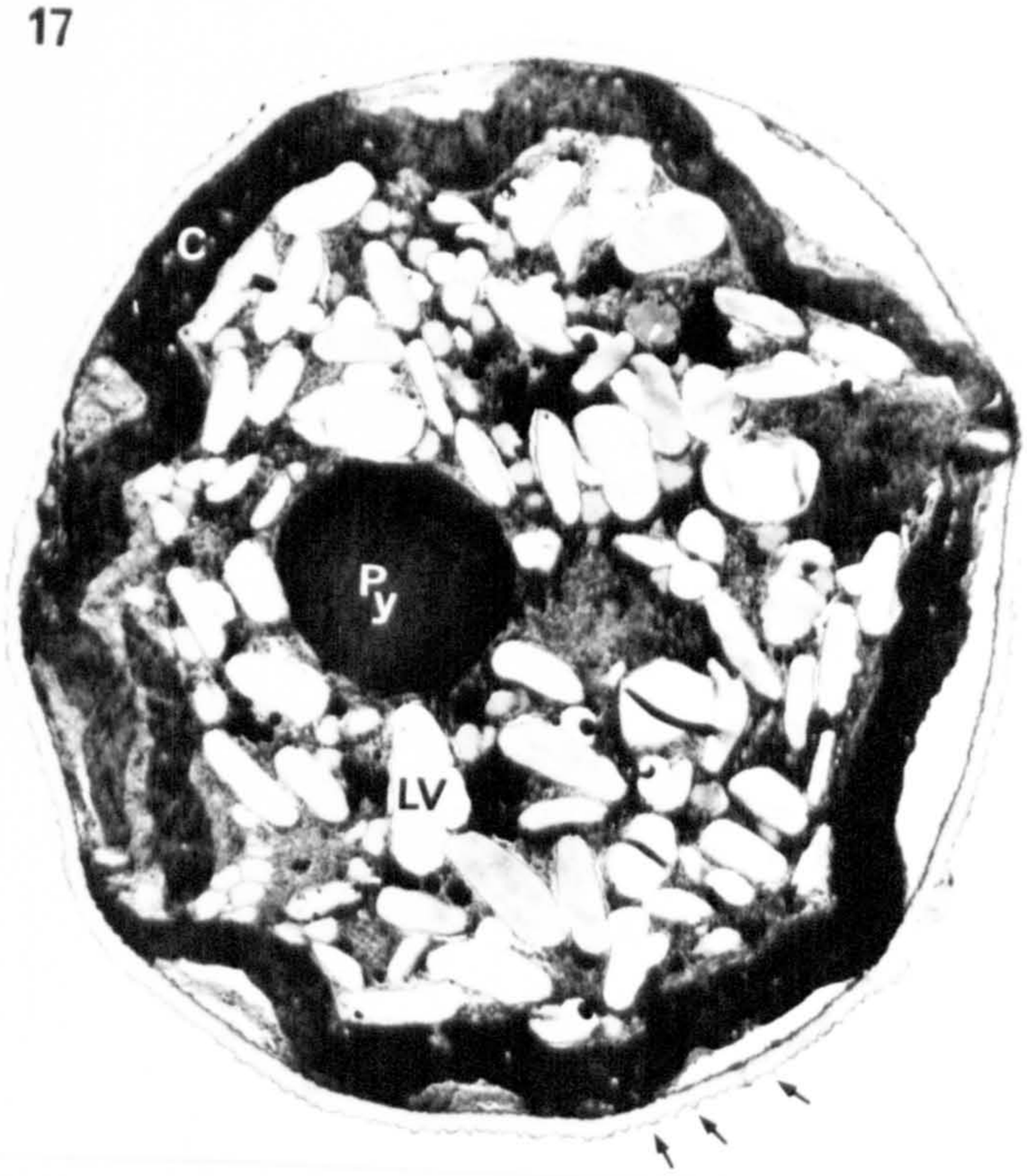

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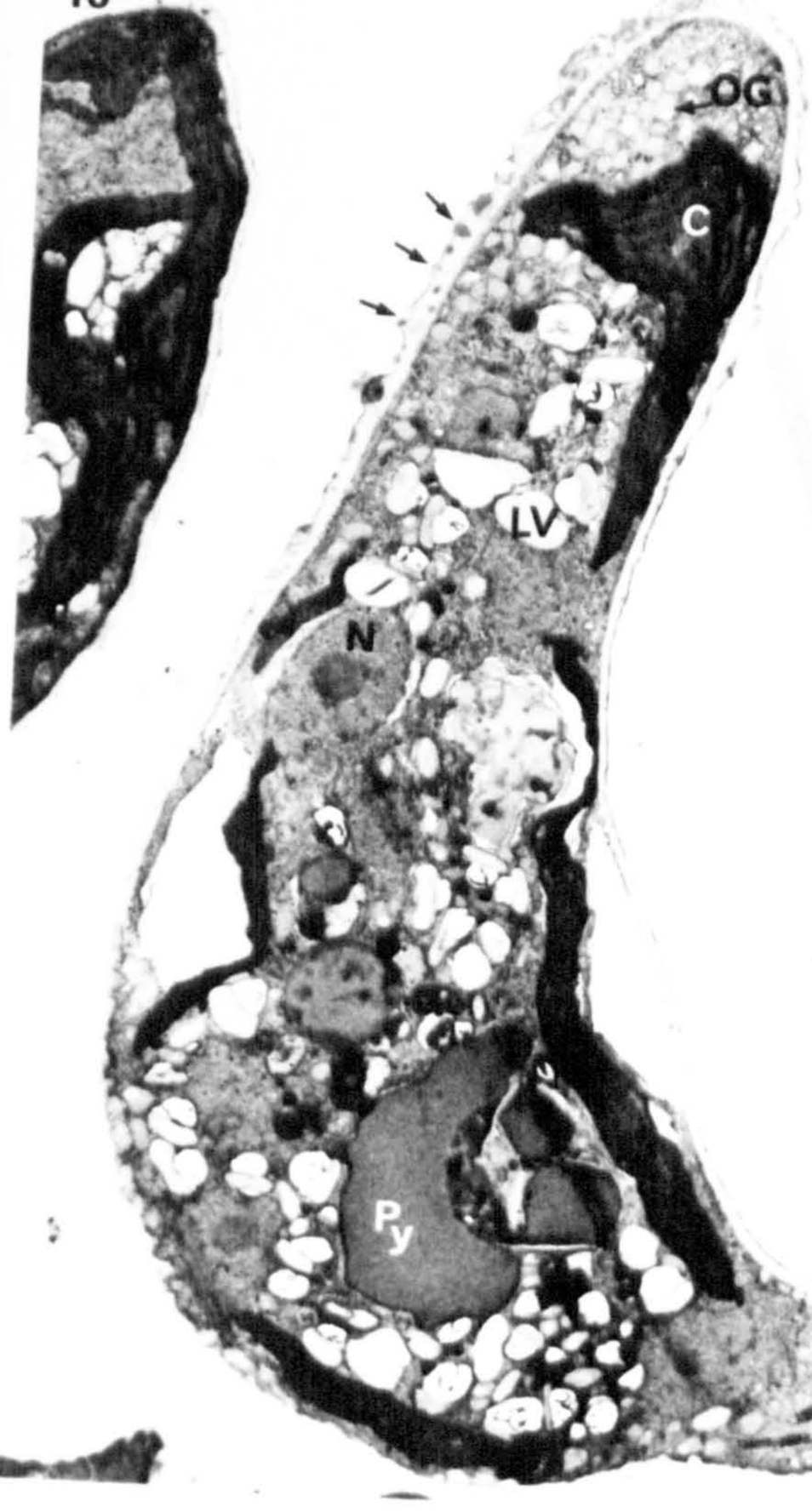

Figs 19-24. Details of fine structure in Pseudocharaciopsis minuta.

Fig. 19. Golgi body (arrow) and nucleus. x 38000.

Fig. 20. Chloroplast and pyrenoid. Note the developed periplastidial reticulum, the CER around the chloroplast and the pyrenoid (arrow) and a lamellate vesicle close to the pyrenoid. x 37500.

Fig. 21. Body in the cytoplasm surrounded by mitochondria and small vesicles (arrows). x 26000.

Fig. 22. Lamellate vesicles. x 45000.

Fig. 23. Osmiophillic globules in the cytoplasm. x 45000.

Fig. 24. Holdfast. Fig. 24a x 18500; b x 30000.

Abbreviations used in figures: C, chloroplast; LV, lamellate vesicle; M, mitochondrion; N, nucleus; PR, periplastidial reticulum; Py, pyrenoid.

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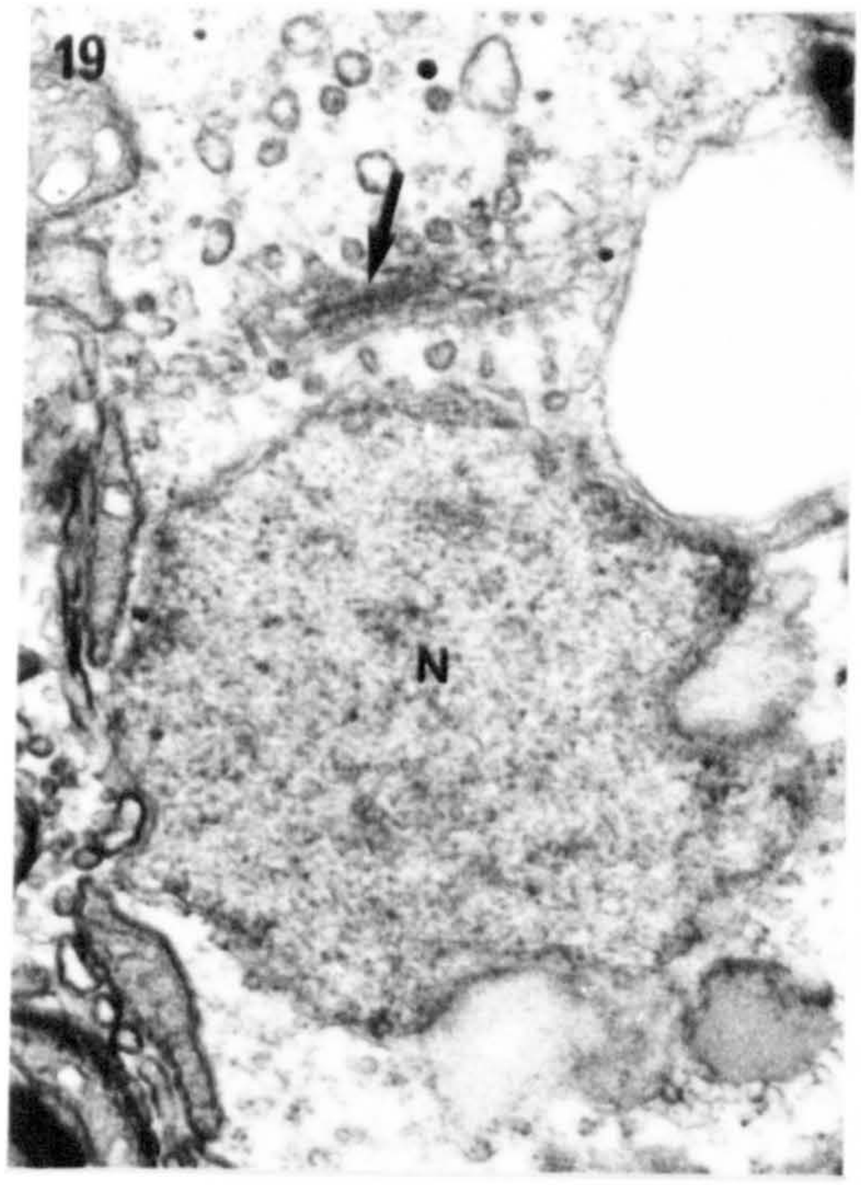

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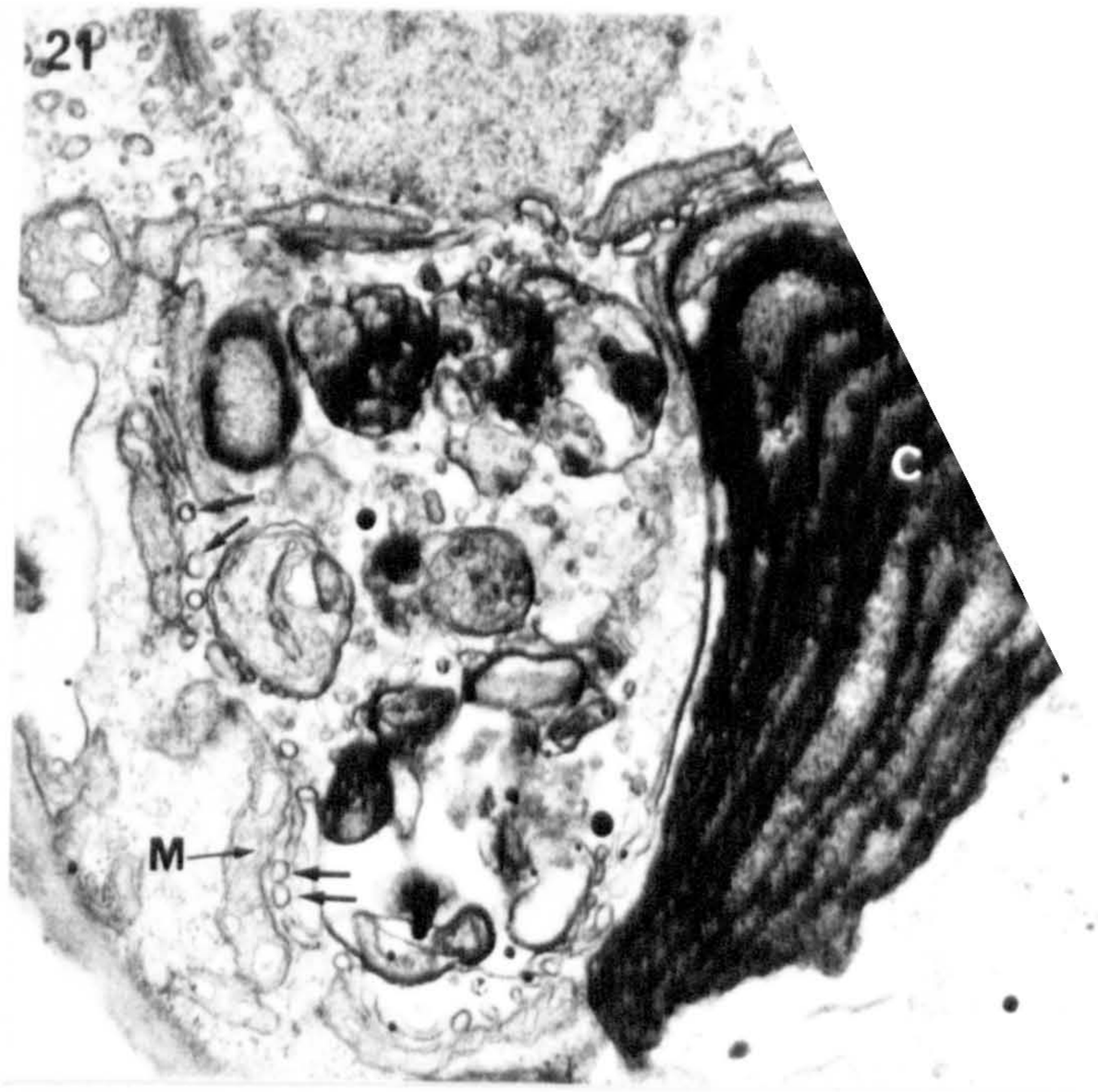

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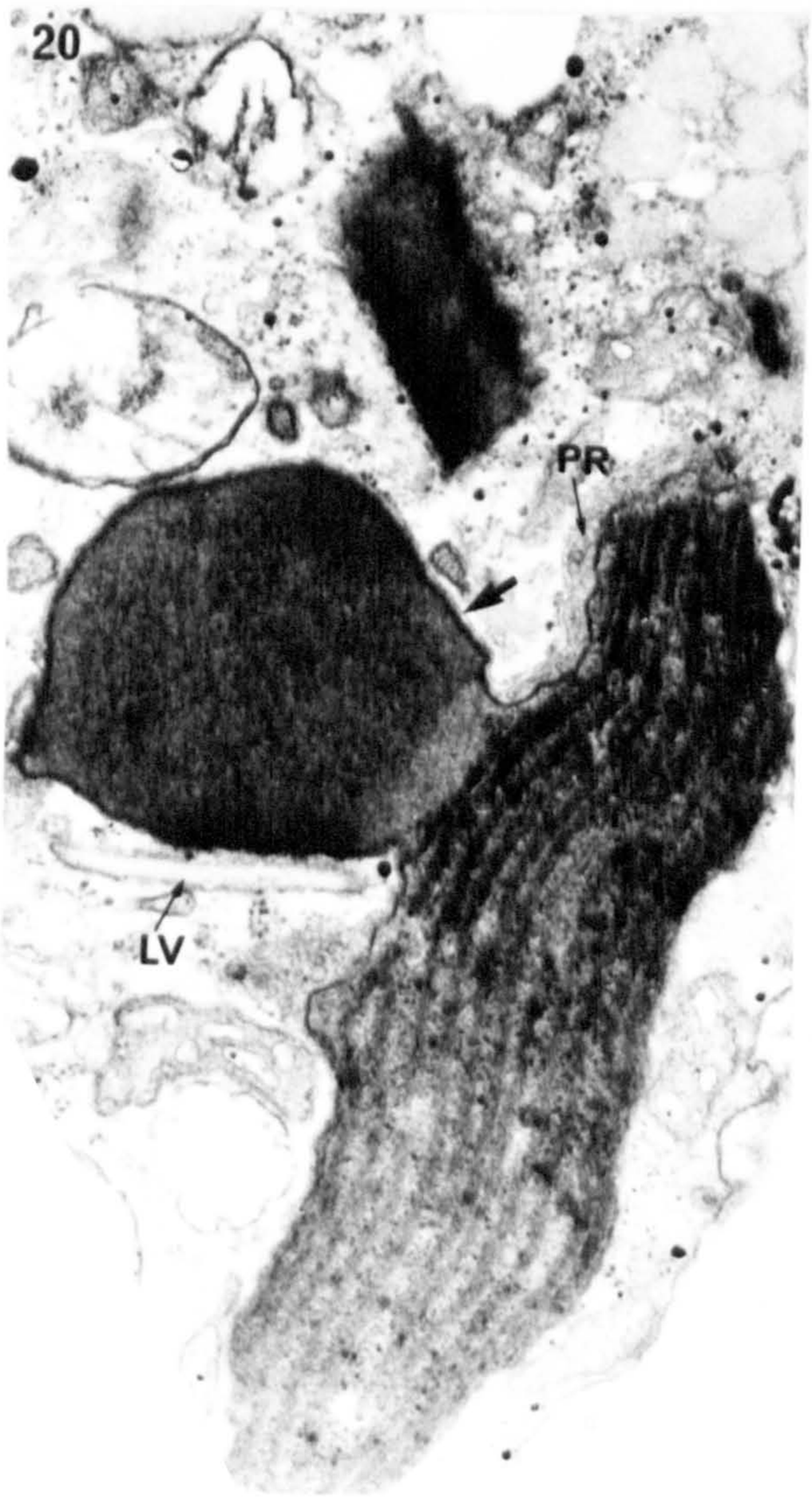

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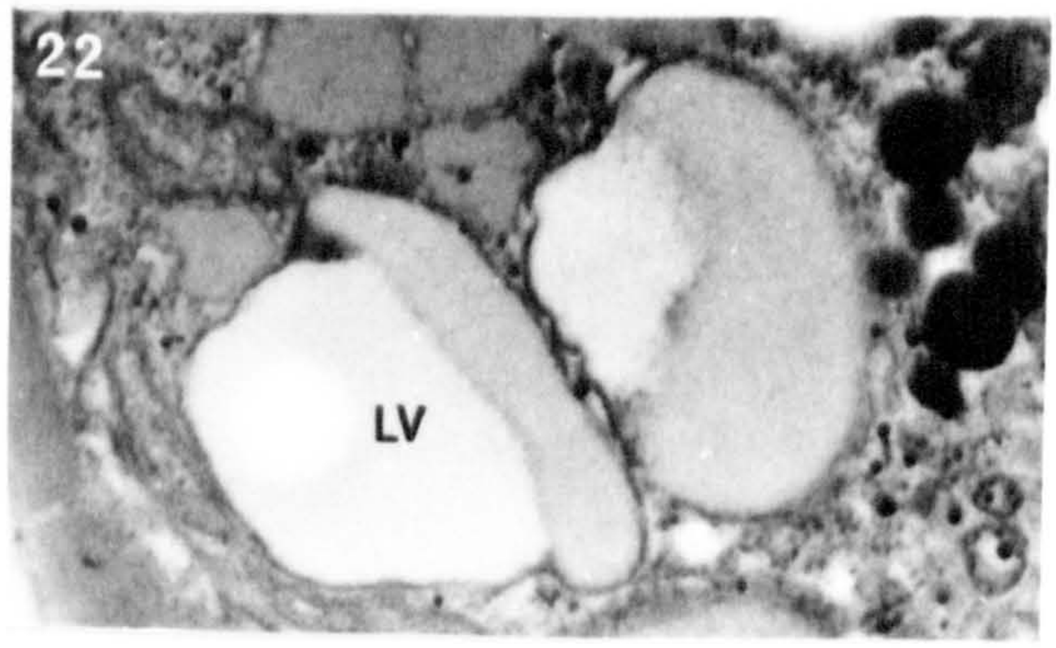

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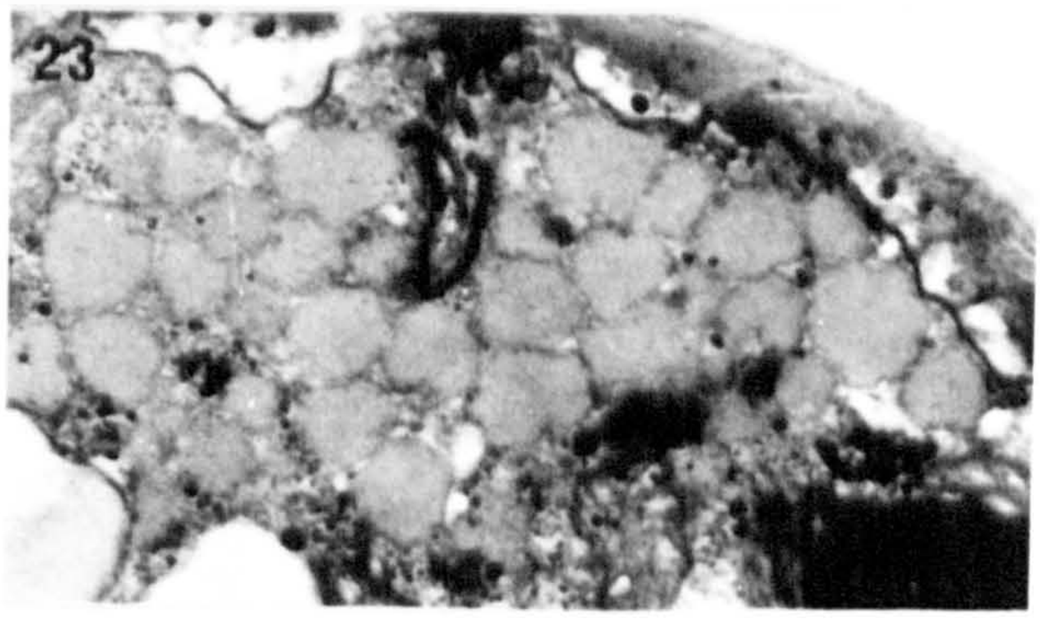

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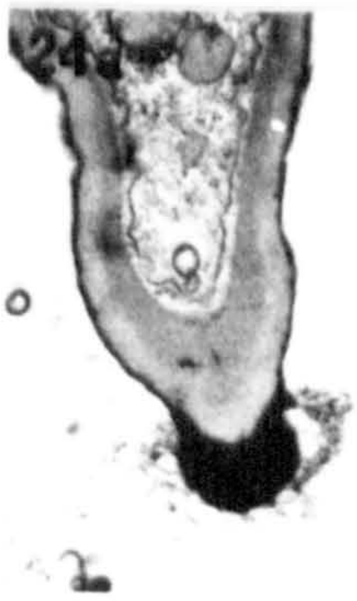

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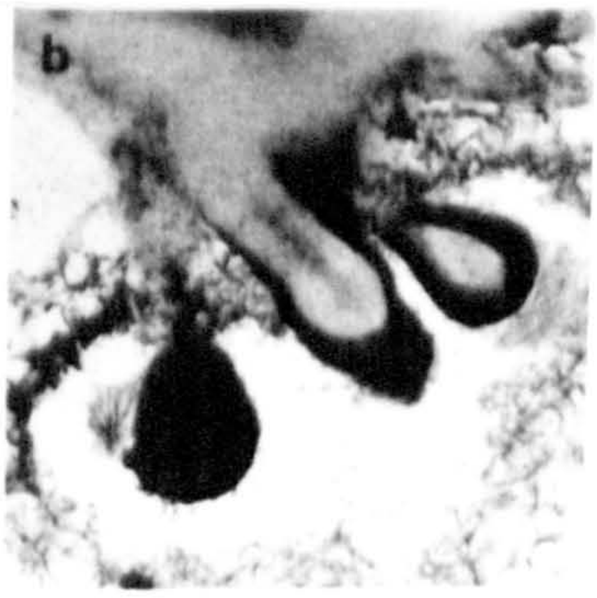

together with a well-developed periplastidial reticulum, but also around it (arrow in Fig. 20).

A particular aspect of this species is the presence of a body in the cytoplasm, surrounded by vesicles, membranes and mitochondria, full of material possibly in degradation (Fig. 21).

The lamellate vesicles (Fig. 22) have the same structure as in the Eustigmataceae but are particularly abundant free in the cytoplasm of this species (see Fig. 17).

Large globules of osmiophillic material (Fig. 23) accumulate in the cytoplasm, in particular at the anterior end of the cell.

In thin sections, the attaching disc of this organism appears to be composed of several branches capped with a very dense material and surrounded by possible mucilage (Fig. 24).

3. Monodopsidaceae

a. *Monodopsis*

The cells of *Monodopsis subterranea* (Fig. 25) and *M. unipapilla* (Fig. 26) are spherical, ovoid or cylindrical, normally measuring about 3.5-5µm x 7-9µm, and contain a single chloroplast and small refractile granules.

The general cell structure (Figs 27, 28) is similar in the two species. The cell wall appears irregular, with dense contents and small projections; a central nucleus, with a

Figs 25-33. Vegetative cells of *Monodopsis subterranea* and *M. unipapilla*.

Fig. 25. Cells of *M. subterranea*. Bright field. x 1800.

Fig. 26. Cells of *M. unipapilla*. Bright field. x 1800.

Figs 27, 28. General cell structure in *M. subterranea* (Fig. 27) and *M. unipapilla* (Fig. 28). Note the terminal pyrenoid in both species. Fig. 27 x 27000; Fig. 28 x 34000.

Fig. 29. Connection between the CER and the nuclear envelope (arrow) in *M. subterranea*. x 40000.

Fig. 30. Same connection (arrow) in *M. unipapilla*. x 47500.

Fig. 31. Vesicle with osmiophillic globules in *M. subterranea*. x 30000.

Fig. 32. Same in *M. unipapilla*. x 30000.

Fig. 33. Lamellate vesicle in *M. subterranea*. x 30000.

Abbreviations used in figures: C, chloroplast; LV, lamellate vesicle; N, nucleus; Nu, nucleolus; OG, osmiophilic globule; Py, pyrenoid; V, vacuole; W, cell wall.

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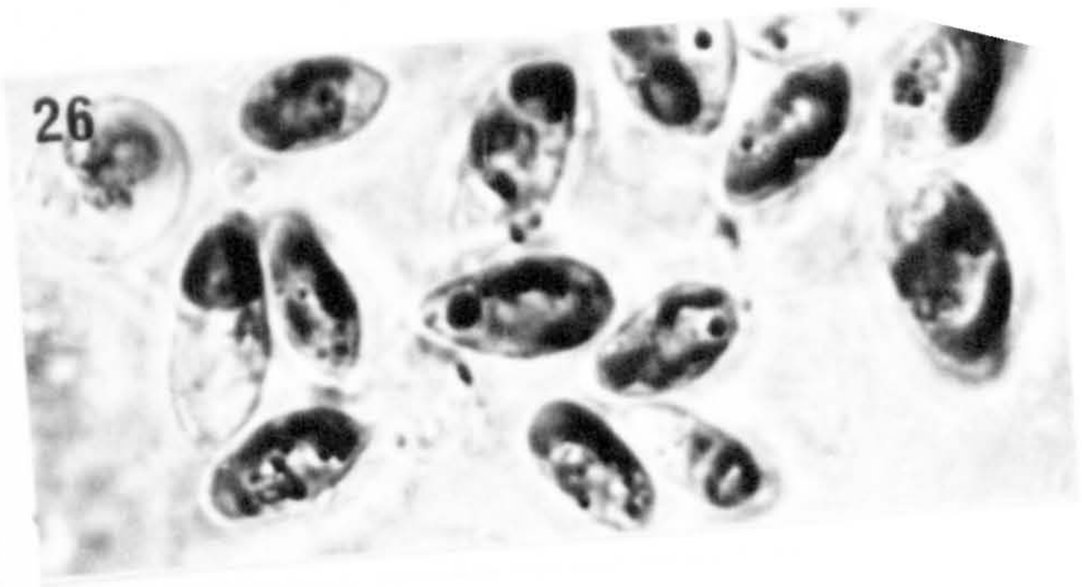

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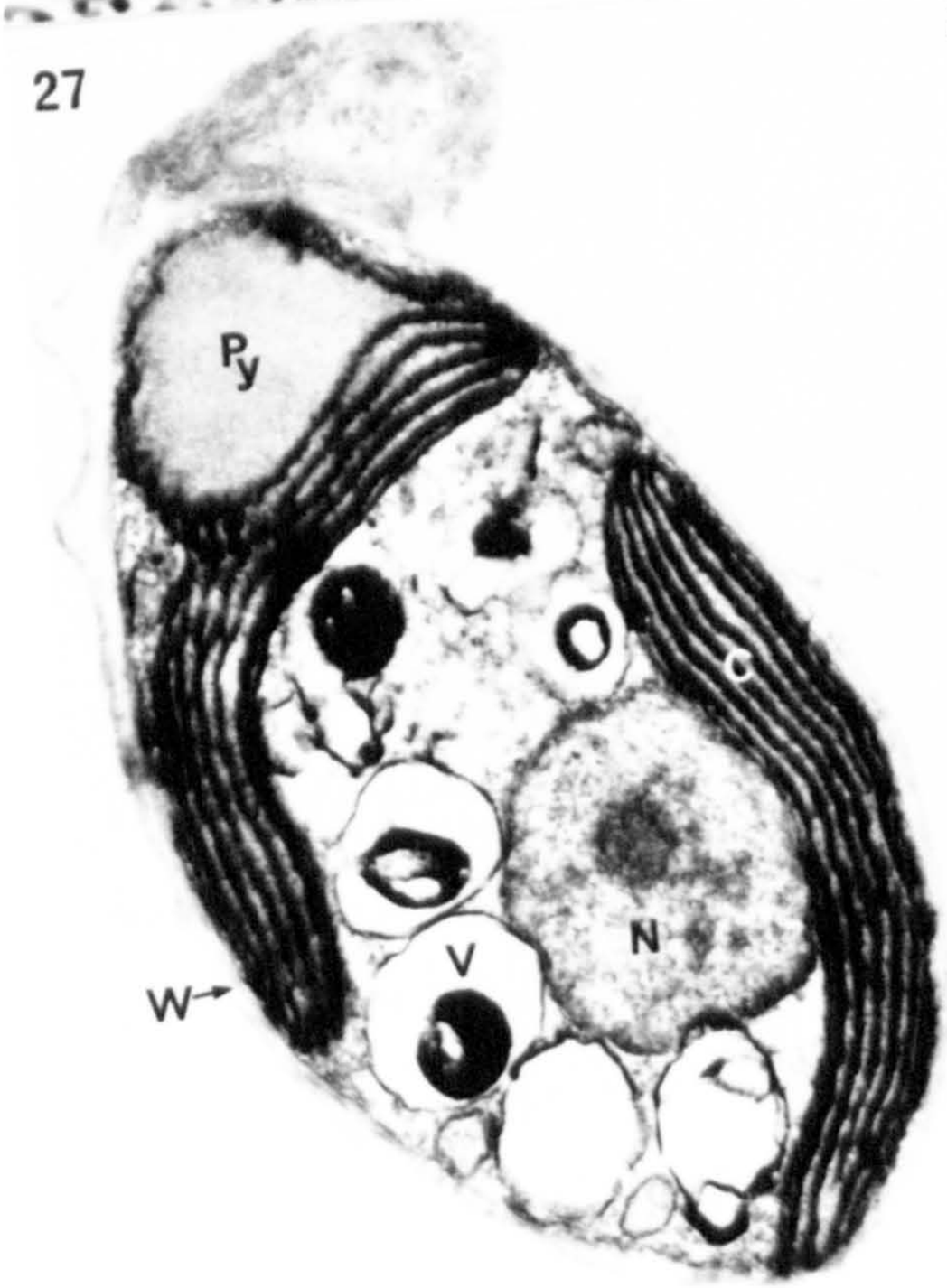

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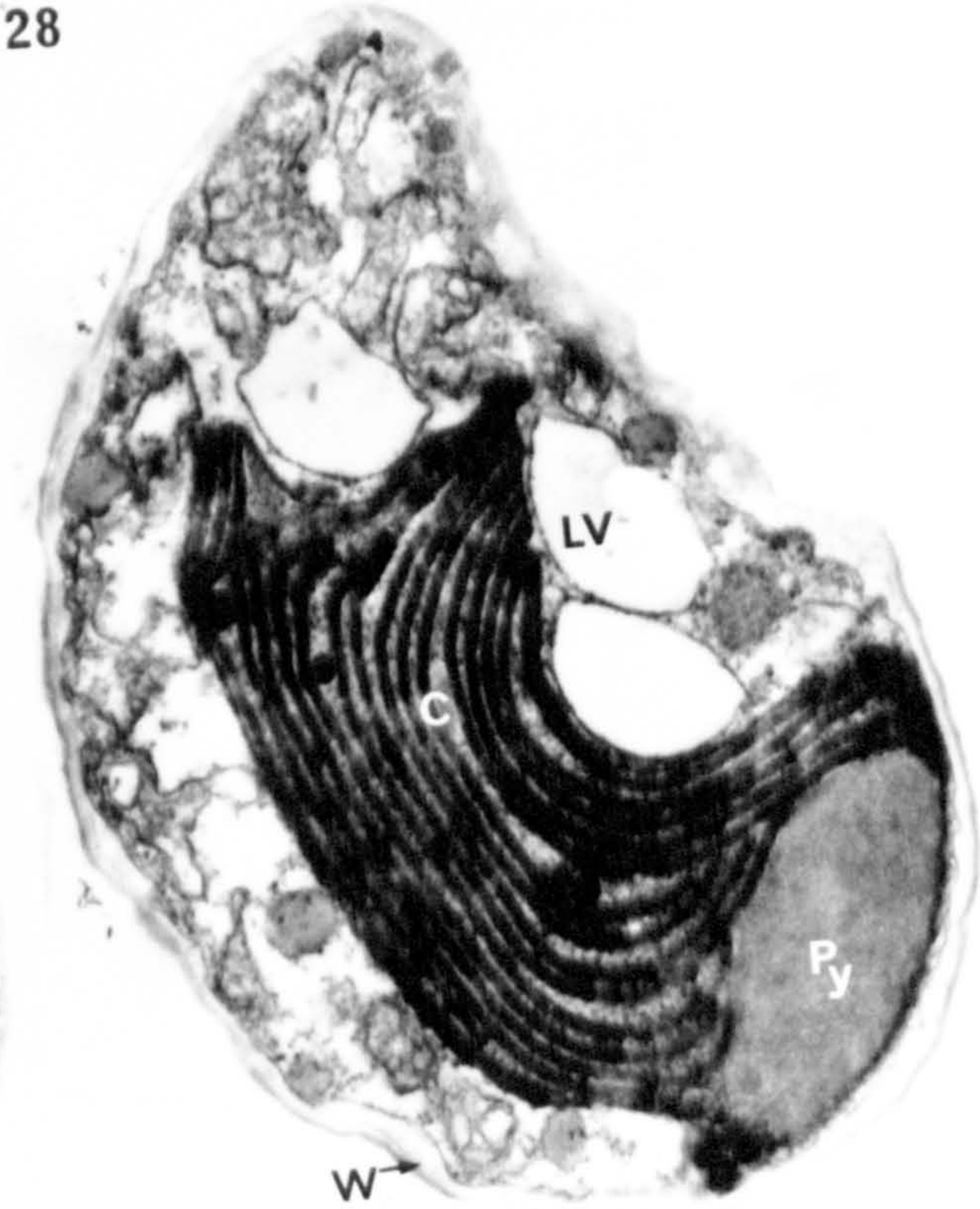

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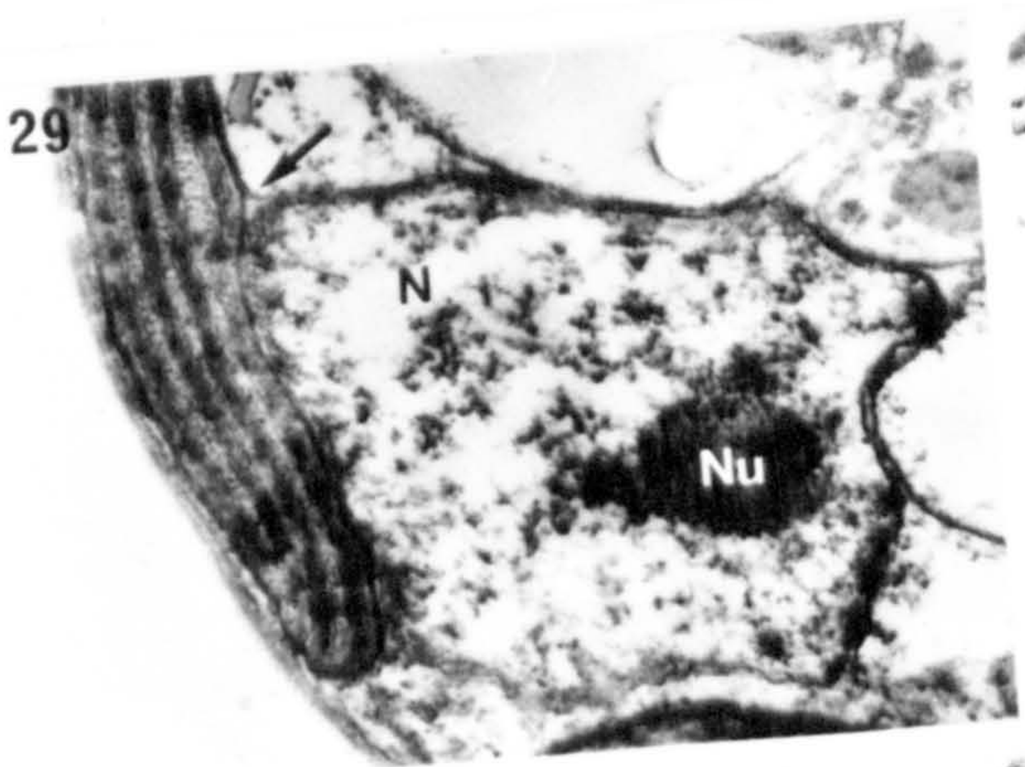

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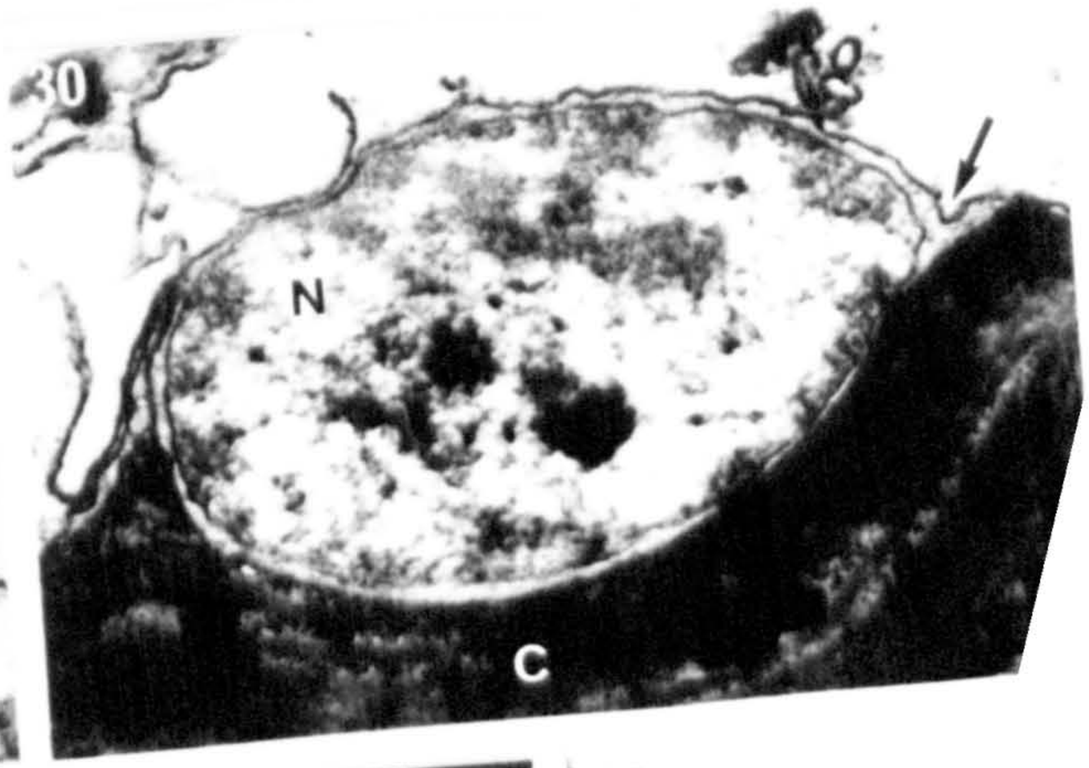

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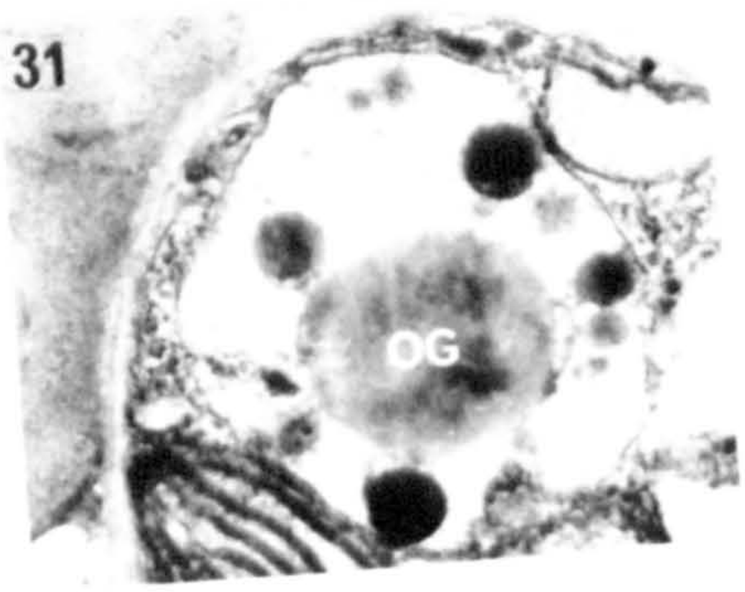

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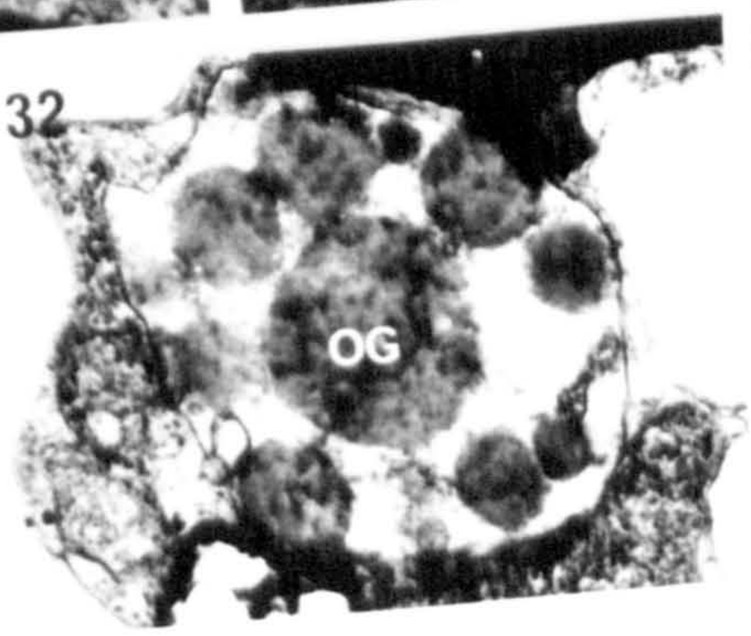

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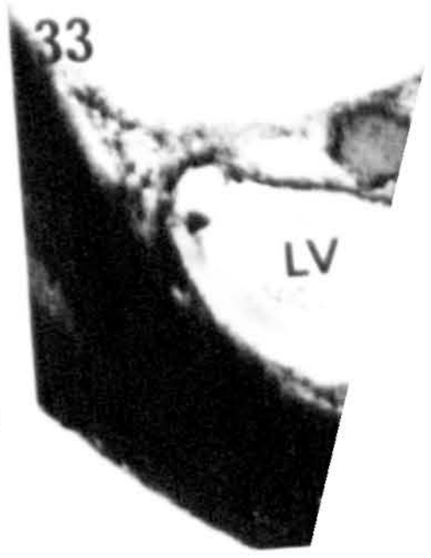

nucleolus, lies close to the chloroplast (see Fig. 27); the chloroplast lamellae are regularly spaced, a girdle lamella is absent and there is a terminal pyrenoid region not traversed by thylakoids (Figs 27, 28); lamellate vesicles (Fig. 28; see also Fig. 33), others with electron-dense contents and vacuoles (Fig. 27) can be seen in the cytoplasm.

A relevant ultrastructural feature of both species is the connection existing between the chloroplast endoplasmic reticulum and the nuclear envelope (Figs 29, 30), not reported by Hibberd in his preliminary study of *Monodopsis subterranea* (Hibberd, 1969, 1980a). The presence of a large vesicle containing dense globules (Figs 31, 32) and similar to the red-pigmented body of the Eustigmataceae (compare with Fig. 8) is also usual in the cytoplasm of both species and has not been shown before.

b. *Nannochloropsis*

Apart from size differences, the three strains studied are morphologically similar. The cells of *N. oculata* are subspherical, 2-4µm in diameter (Fig. 34) and those in *N. salina* (Fig. 35) and *N. gaditana* (Fig. 36) are cylindrical, 3-4 x 1.5-3µm, all with a single, parietal chloroplast.

Cell structure is illustrated in Figs 37-39. In general, my observations agree with previous studies on species of this genus (Antia *et al.*, 1975; Lubian, 1982a; Maruyama *et al.*, 1986) but some aspects are clarified. The nucleus, with a single nucleolus, lies near the chloroplast and a

connection between the CER and the nuclear envelope is present in all the species (arrows in Figs 37-38; not illustrated in *N. gaditana*); the chloroplast structure is typically eustigmatophycean but no pyrenoid was ever seen in these cells; profiles of mitochondria and lipid globules occur throughout the cytoplasm, in addition to the lamellate vesicles.

These vesicles were observed in the cytoplasm of all the species and are particularly well developed (Figs 40-42); they were never seen inside the chloroplast, as previously reported by Antia *et al.* (1975).

A small Golgi body can occasionally be seen close to the surface of the nucleus (Fig. 43).

Spherical organelles, penetrated by channels of cytoplasm or convoluted cysternae, similar to those described in *Ophiocytium maius* as stellate vesicles (Hibberd, 1969), are also common in the cytoplasm of these organisms (Fig. 44).

In thin sections, the cell wall in all three species appears dense, sometimes layered and often with plug-like structures (Figs 45, 46).

Figs 34-39. Vegetative cells of *Nannochloropsis oculata*, *N. salina* and *N. gaditana*.

Figs 34-36. Light microscopy. Fig. 34. *N. oculata*. Bright field. x 2250. Fig. 35. *N. salina*. Anoptral contrast. x 2250. Fig. 36. *N. gaditana*. Anoptral contrast. x 2500.

Figs 37-39. General cell structure in *N. oculata* (Fig. 37), *N. salina* (Fig. 38) and *N. gaditana* (Fig. 39). Note the connection between the CER and the nuclear envelope (arrows). Fig. 37 x 45000; Fig. 38 x 34000; Fig. 39 x 44000.

Abbreviations used in figures: C, chloroplast; LV, lamellate vesicle; M, mitochondrion; N, nucleus; Nu, nucleolus; OG, osmiophilic globule; V, vacuole; W, cell wall.

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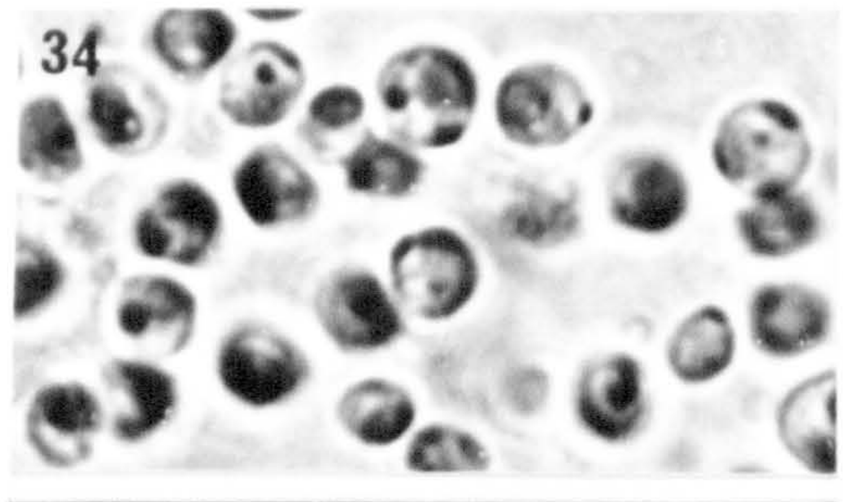

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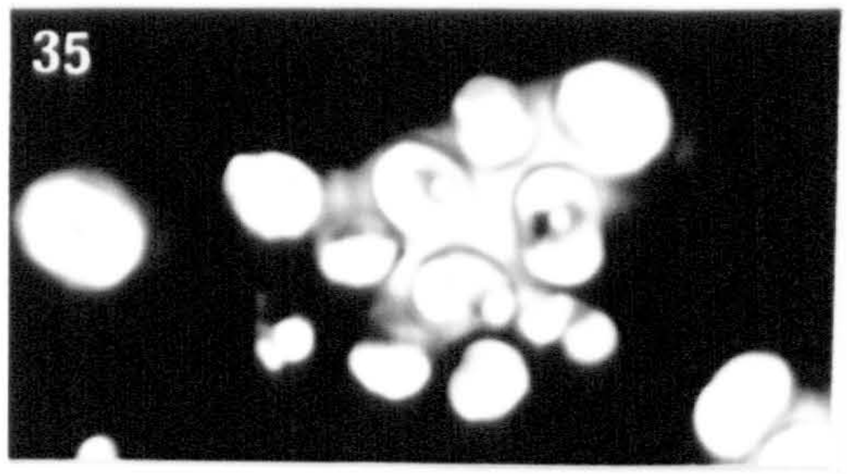

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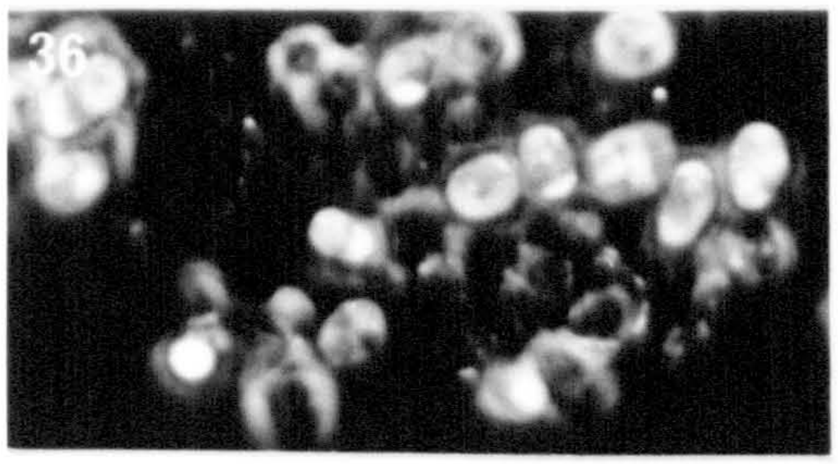

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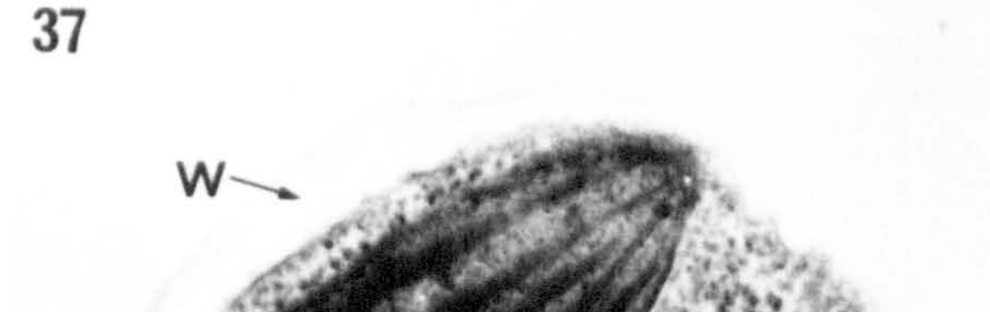

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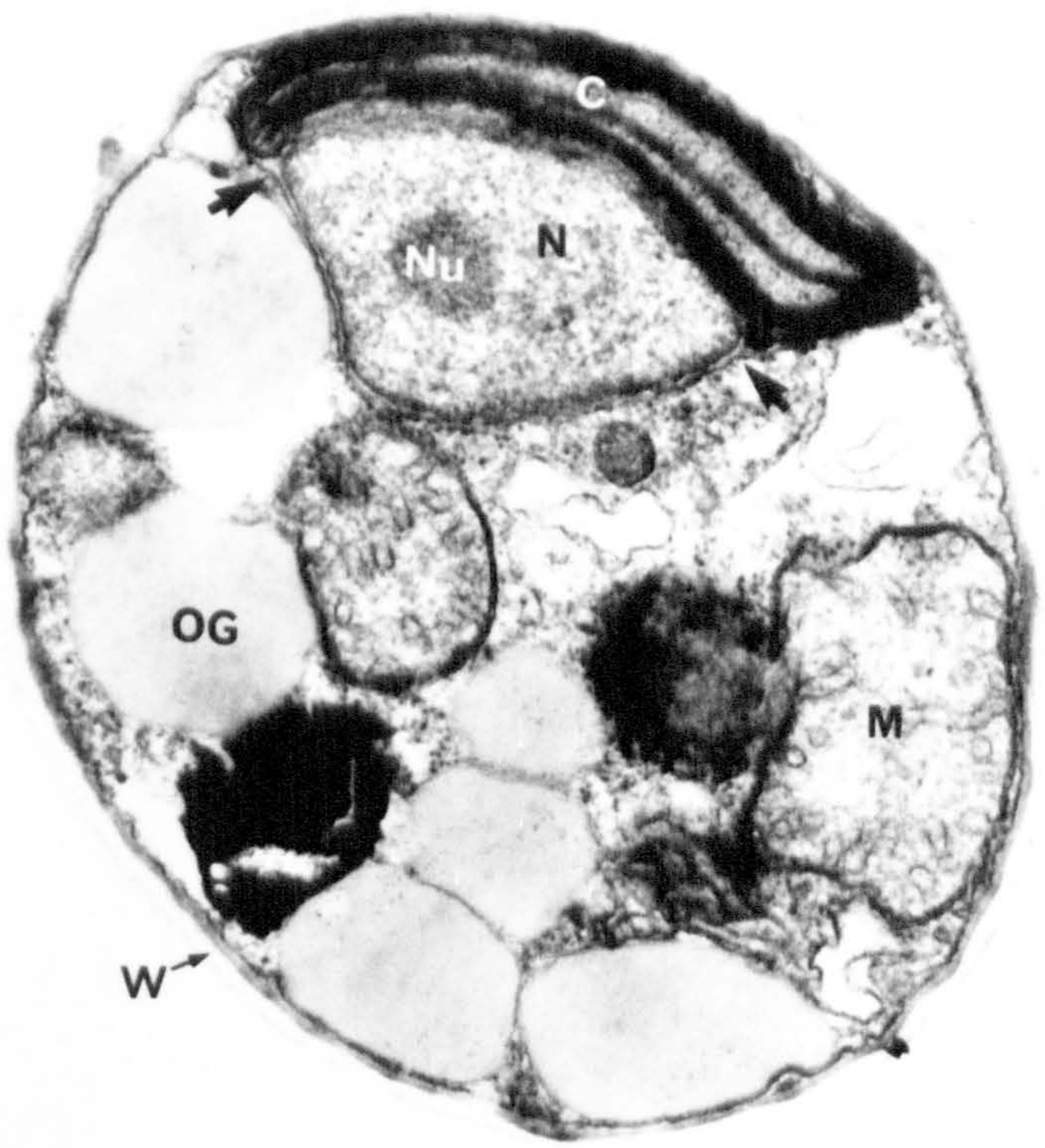

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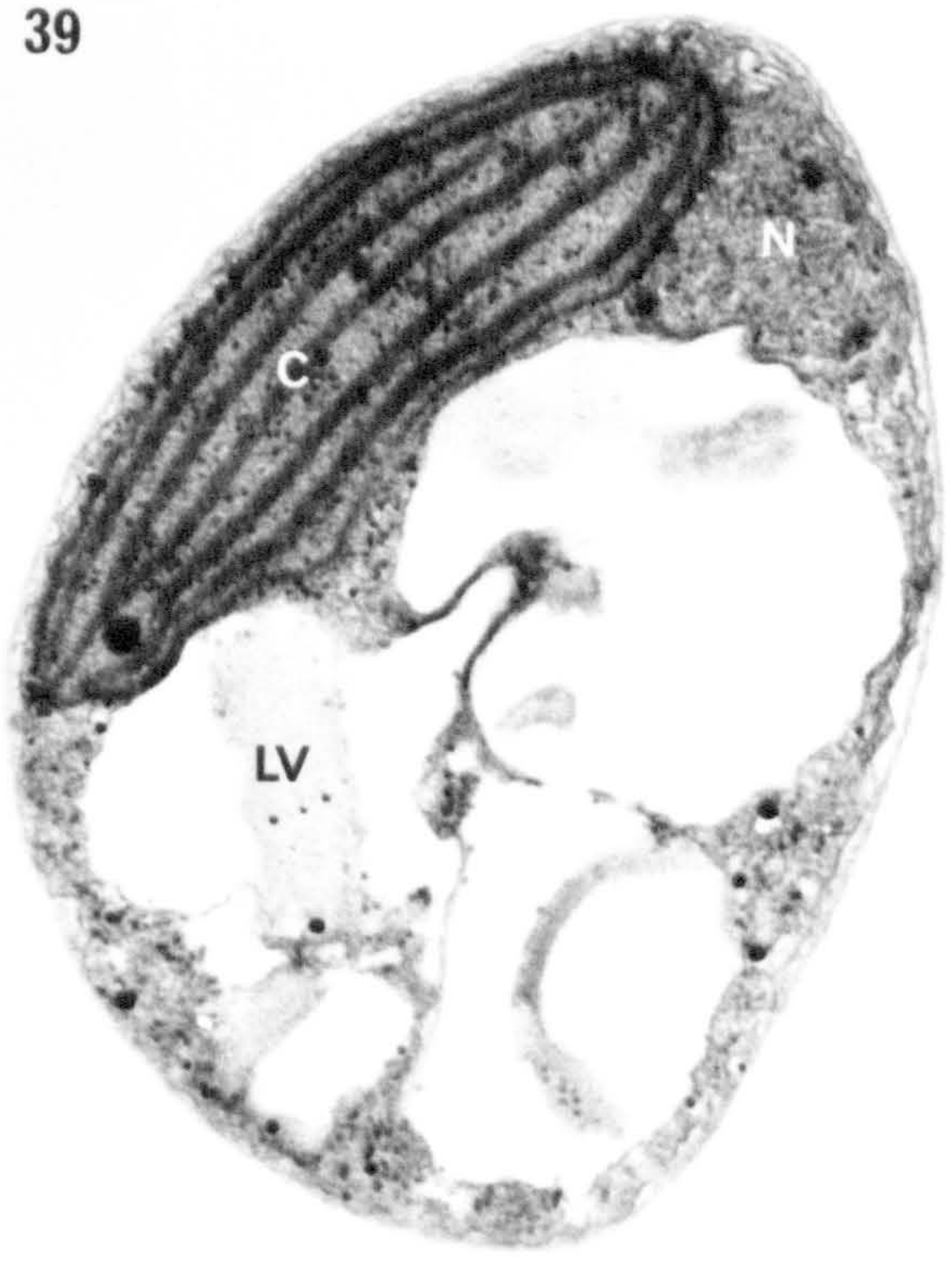

Figs 40-45. Some ultrastrutural details in cells of *Nannochloropsis oculata*, *N. salina* and *N. gaditana*.

Fig. 40-42. Lamellate vesicles in *N. oculata* (Fig. 40), *N. salina* (Fig. 41) and *N. gaditana* (Fig. 42). Fig. 40 x 63000; Fig. 41 x 50000; Fig. 42 x 66000.

Fig. 43. Golgi body (G) close to the nucleus (N) in *N. salina*. x 50000.

Fig. 44. Stellate bodies in the cytoplasm of *N. salina*. x 42000.

Fig. 45, 46. Plug-like structures (arrow) in the cell wall (W) of *N. salina*. Fig. 45 x 62500; Fig. 46 x 28500.

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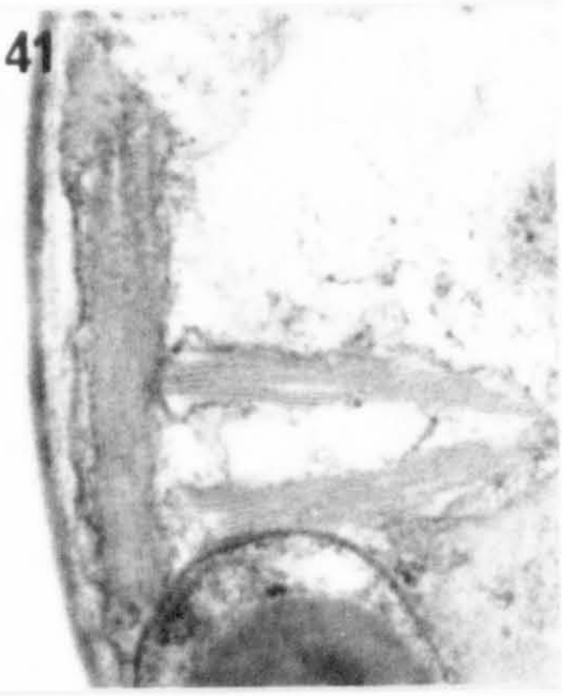

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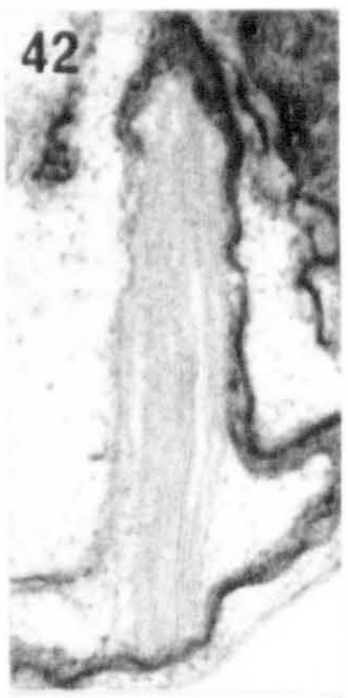

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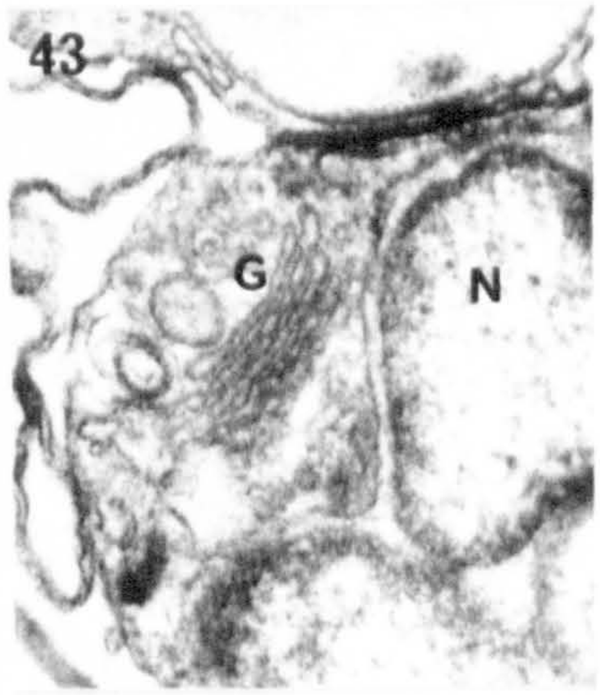

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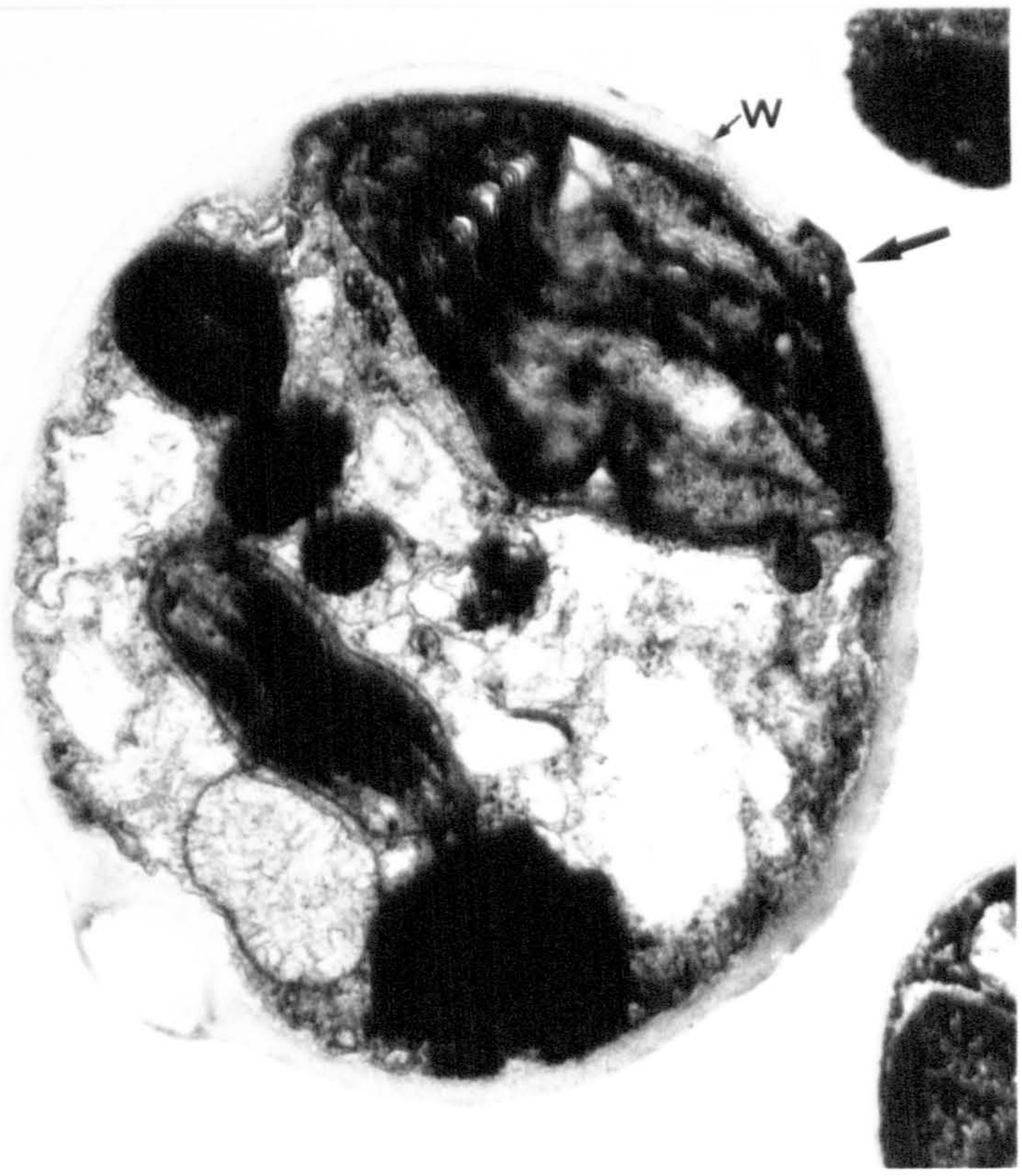

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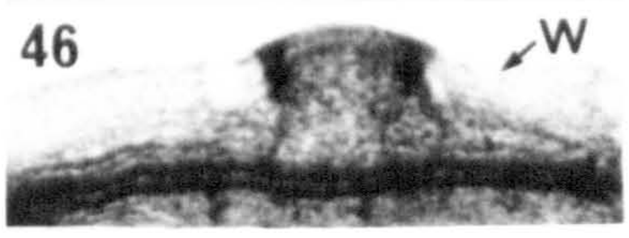

B. ZOOSPORE FORMATION IN *VISCHERIA STELLATA*

Some stages of zoospore formation were observed in *Vischeria punctata* and *V. helvetica* but poor fixation of the material prevented a better understanding of the process. Only cultures less than ten days old give a reasonable fixation but to produce zoospores the cultures have to be more than one month old.

Sections of mature zoosporangia (Figs 46-48) show several profiles of zoospores, with a nucleus, a chloroplast and the developing flagella between the cell membrane and the wall. Profiles of Golgi bodies are larger and more frequently seen than in the vegetative cells (Fig. 49). The lamellate vesicles are also more abundant and seem associated with dense material (Fig. 48; see also Fig. 51). No pyrenoid or pyrenoid remains have been seen in the cells at this stage.

The arrangement and ultrastructural details of some organelles in the forming zoospores are already similar to those subsequently observed in the free-living zoospores, such as the presence of spiral and lamellate vesicles (Fig. 48) and the existence of a flagellar swelling appressed against an extraplastidial eyespot (Fig. 50).

The vesicle with osmiophilic globules seen in the vegetative cells appears very large in some sections (Fig. 46) and can be seen as part of already well delimited zoospores (Fig. 47); it was not possible to elucidate, however, whether or not it breaks during the process and divides its material among the zoospores.

Delimitation of individual zoospores probably begins with profiles of endoplasmic reticulum (ER) being positioned along what may later become cleavage lines (Fig. 51) and zoospore release must occur by a general disintegration of the cell wall, but this aspect was not observed.

Figs 46-51. Mature zoosporangia of *Vischeria punctata* and *V. helvetica*.

Figs 46-48. Sections of *V. punctata* with 2 or more profiles of zoospores. Note the large vesicle with osmiophillic globules in Fig. 46, the nucleus and remains of that vesicle in Fig. 47. Fig. 46 x 9000; Fig. 47 x 12500; Fig. 48 x 17000.

Fig. 49. Section of *V. helvetica* showing two Golgi bodies (arrows) close to the nucleus and the chloroplast. x 30000.

Fig. 50. Anterior end of a zoospore of *V. punctata* showing the structure of the flagellum. x 45000.

Fig. 51. Cleavage lines (arrows) in *V. punctata*. Note also the dense material associated with the lamellate vesicles. x 25000.

Abbreviations used in figures: C, chloroplast; E, eyespot; F, flagellum profile; FS, flagellar swelling; LV, lamellate vesicle; N, nucleus; OG, osmiophilic globule; SV, spiral vesicle; W, wall of zoosporangium.

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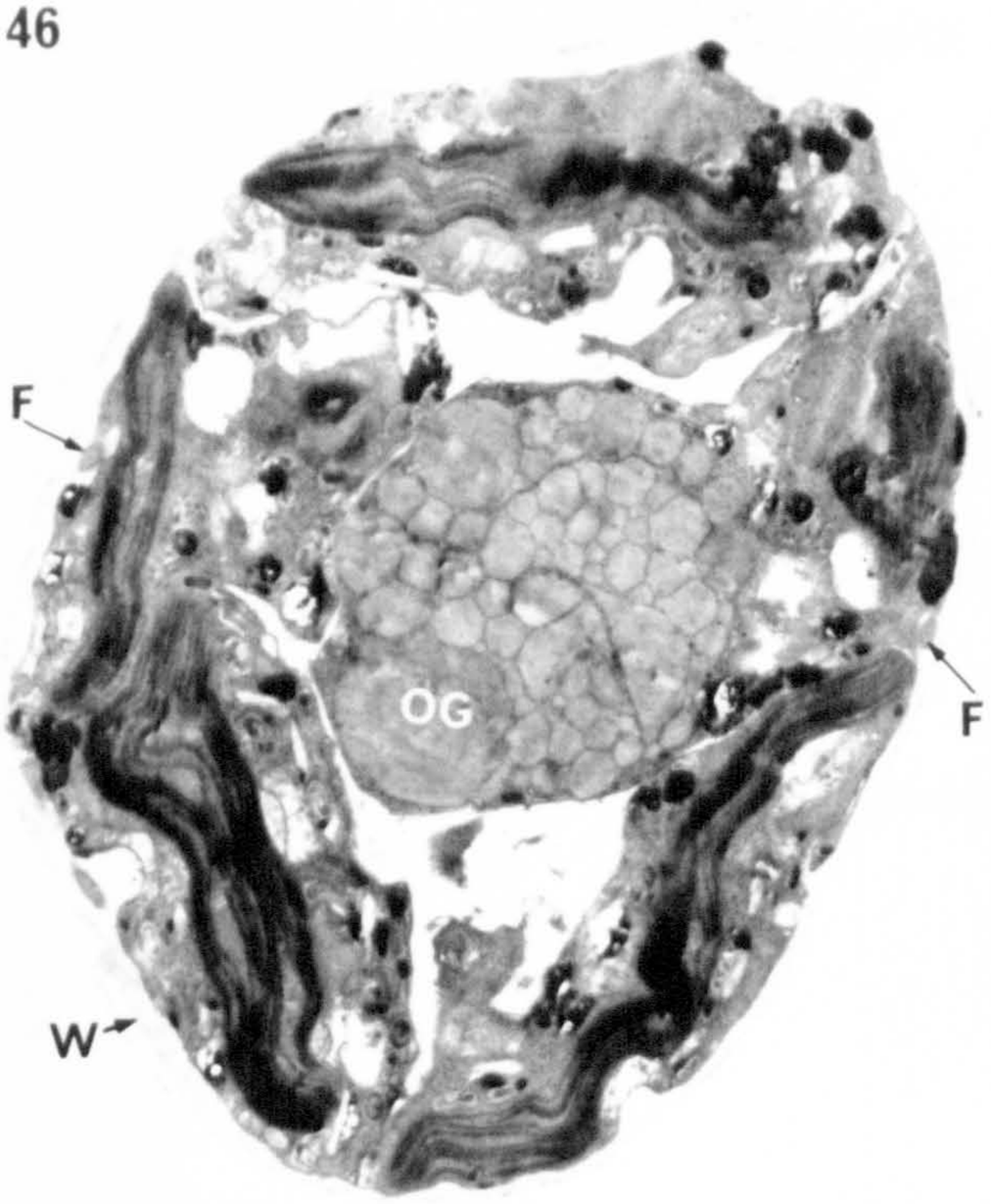

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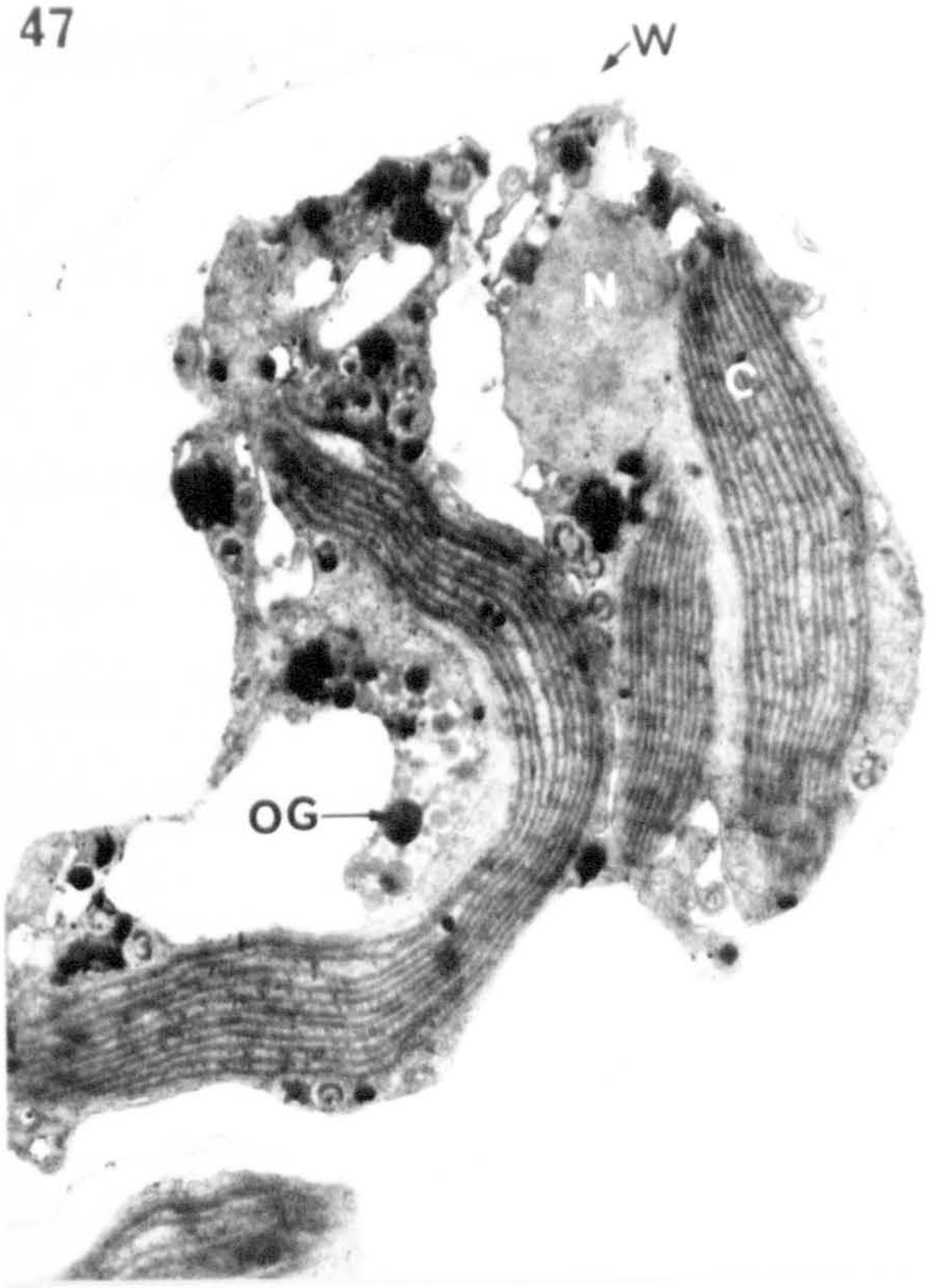

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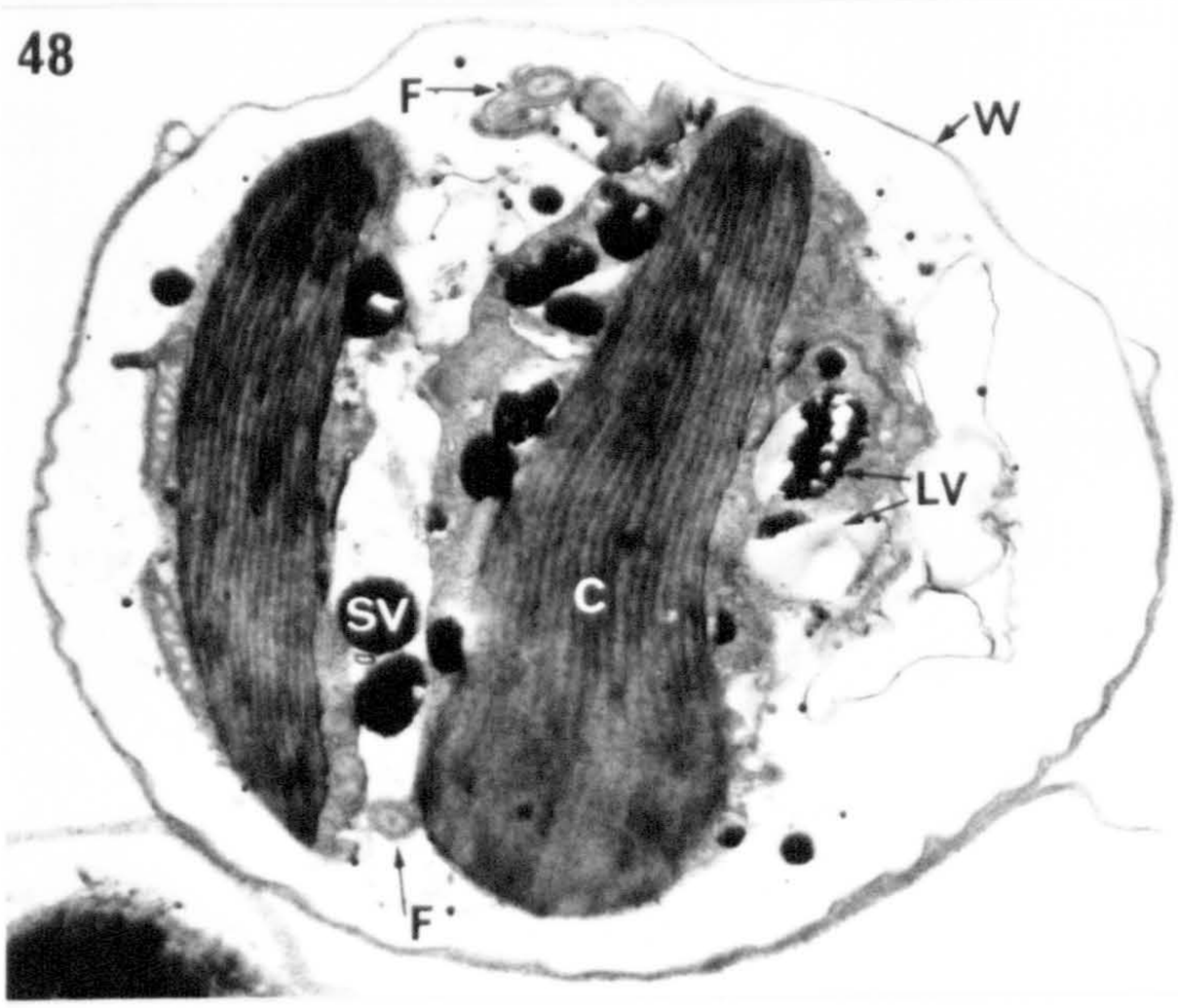

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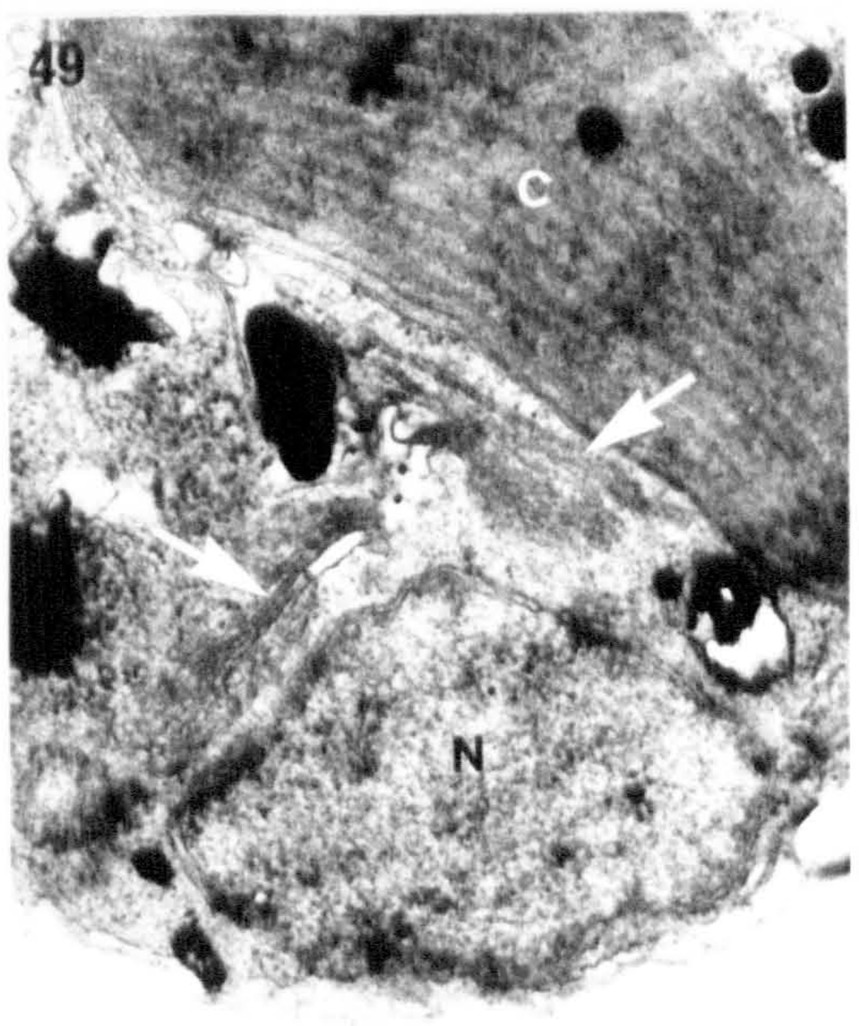

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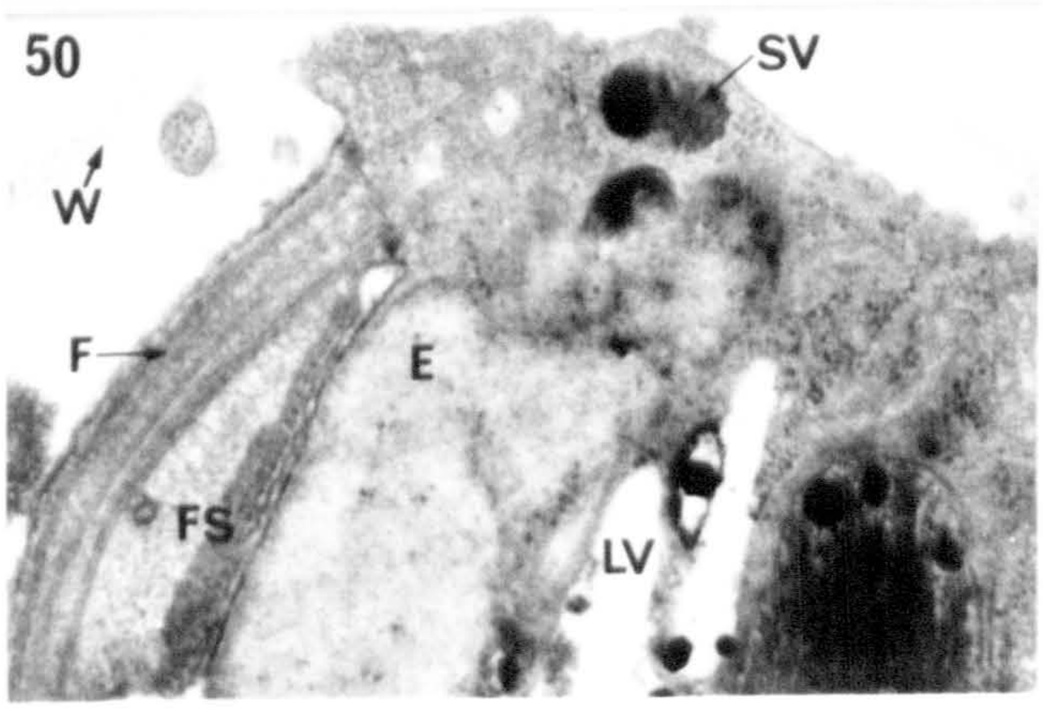

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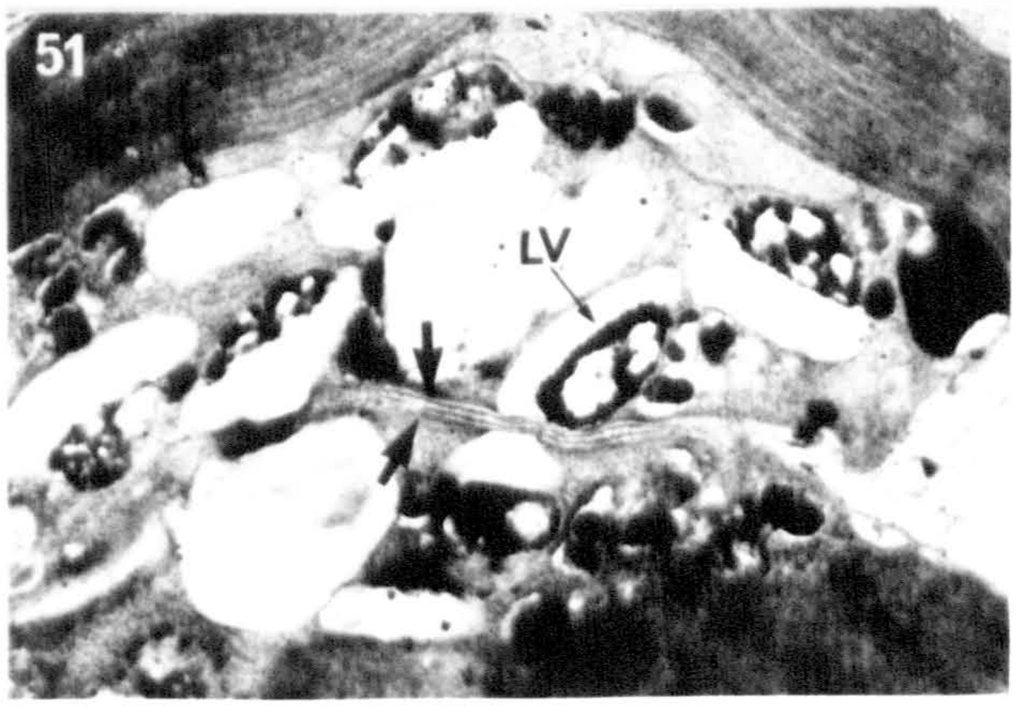


C. STRUCTURE OF UNIFLAGELLATE ZOOSPORES

Zoospores of *Eustigmatos magnus*, *Vischeria stellata*, *V. helvetica* and *V. punctata* have been investigated. More or less detailed observations on these species have been made before (Hibberd, 1969; Hibberd & Leedale, 1972) and the results presented here confirm those studies and give new evidence on the structure of the flagellar apparatus.

1. General

All the zoospores are very similar in organization, either when observed with the light or with the electron microscope. In light microscopy (Figs 52-54; see also Fig. 59), the zoospores appear as flask-shaped naked cells, 10-15µm long and about 5µm at maximum width, with one emergent flagellum almost as long as the cell body, a single chloroplast and a large eyespot, red-orange in colour, independent of the chloroplast.

The general cell stucture of these zoospores is illustrated in Figs 55-57 and Fig. 60. The single flagellum is inserted subapically; the large extraplastidial eyespot, consisting of osmiophilic globules of different size and not membrane-bounded, occupies the anterior part of the cell, together with a pyriform nucleus; the chloroplast is situated at the posterior end, its structure similar to that of the chloroplasts in the vegetative cells but without the pyrenoid; the rest of the cell is filled with normal organelles, such as mitochondria, and with different sorts

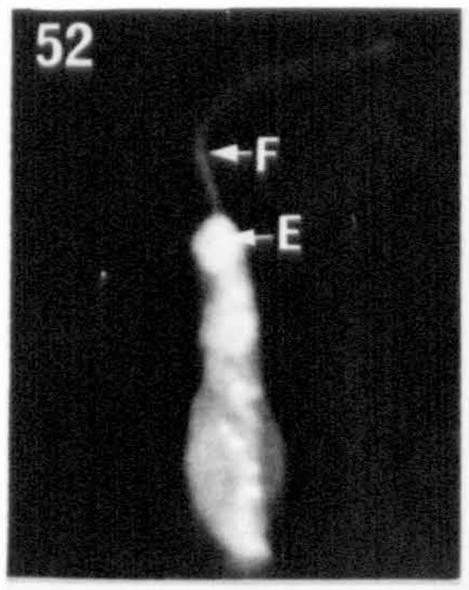
Figs 52-58. Zoospores of *Eustigmatos magnus*, *Vischeria helvetica* and *V. punctata*.

Figs 52-54. Light micrographs of *E. magnus* (Fig. 52), *V. helvetica* (Fig. 53) and *V. punctata* (Fig. 54) showing the characteristic shape, the single flagellum, the large, anterior eyespot and the elongate chloroplast. Anoptral contrast. Fig. 52 x 2500; Fig. 53 x 2200; Fig. 54 x 2200.

Figs 55-57. Longitudinal sections *E. magnus* (Fig. 55), *V. helvetica* (Fig. 56) and *V. punctata* (Fig. 57) showing the general cell structure. Fig. 55 x 30000; Fig. 56 x 21000; Fig. 57 x 8000.

Fig. 58. Section of *V. helvetica* showing a small Golgi body close to the nucleus. x 38000.

Abbreviations used in figures: C, chloroplast; E, eyespot; F, emergent flagellum; FS, flagellar swelling; G, golgi body; LV, lamellate vesicle; M, mitochondrion; N, nucleus; SV, spiral vesicle.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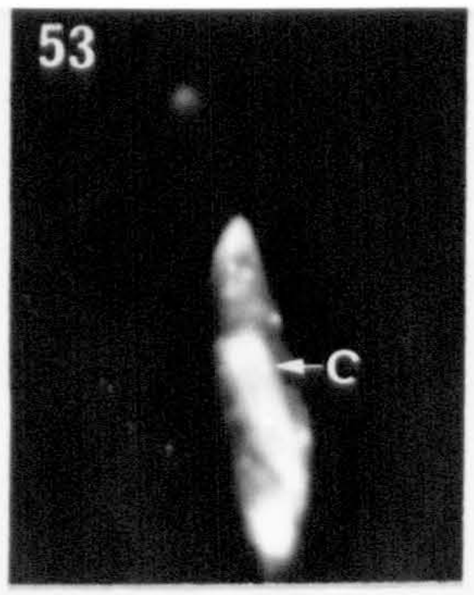
F
E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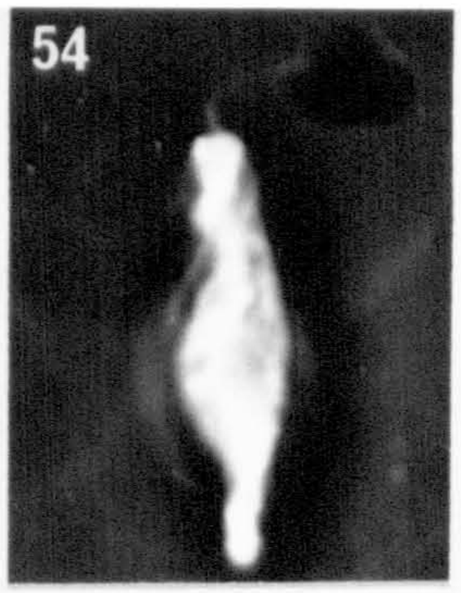
C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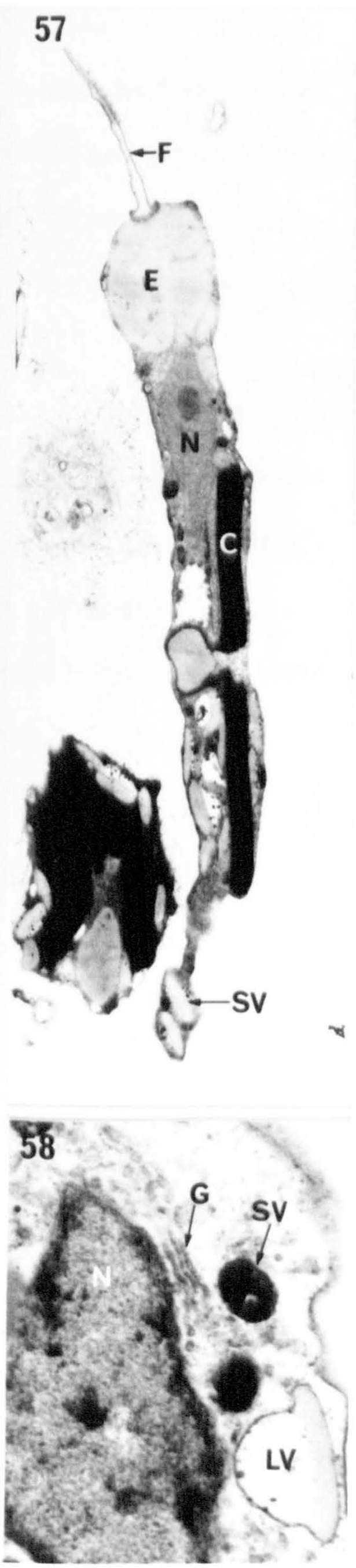
57
F
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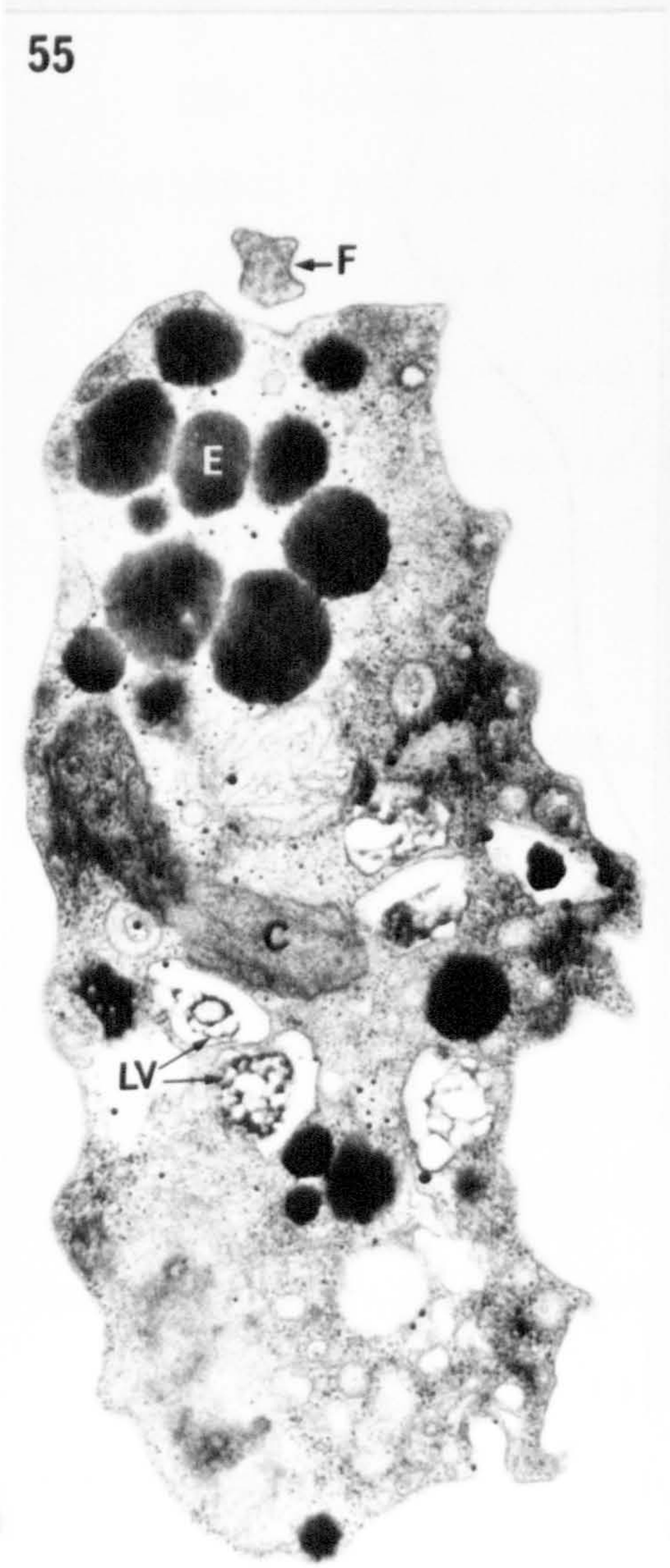
55
F
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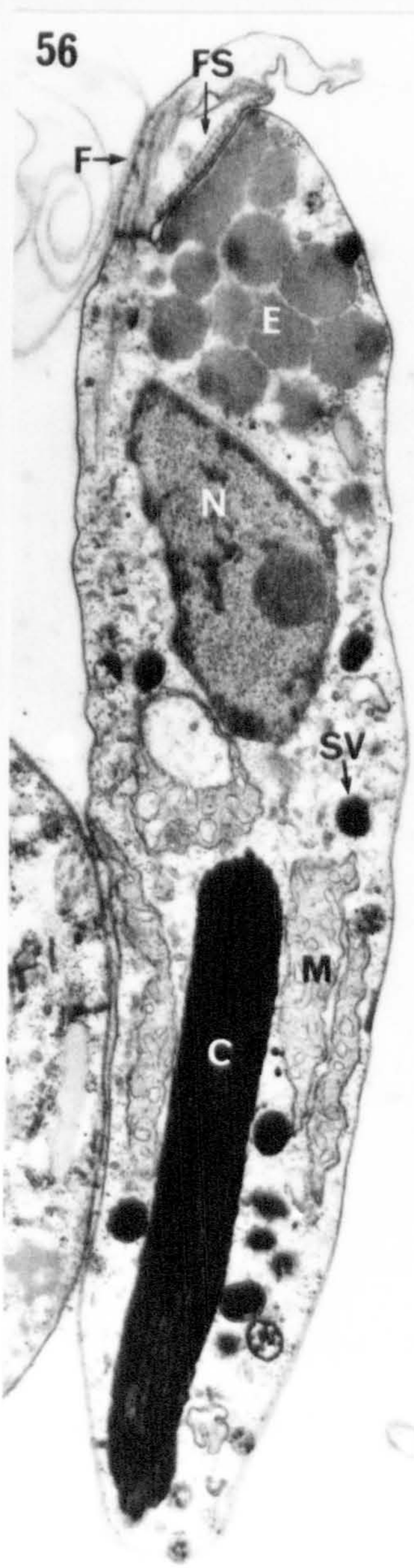
56
FS
F
E
N
SV
C
M

58
G
SV
N
LV

of vesicles, in particular the lamellate and spiral vesicles (see also Fig. 58) typical of eustigmatophycean zoospores.

Possible Golgi vesicles are usually seen between the nucleus and the eyespot but a Golgi body, previously reported to be absent in uniflagellate zoospores (*e.g.* Hibberd, 1980a, 1981), has been clearly observed only in zoospores of *Vischeria helvetica* (Fig. 58).

The ultrastructure of the flagellar apparatus is identical in all the studied species. For this reason it will be described and illustrated in detail in *Vischeria stellata* (see below and Figs 61- 85) while description of the same structures in the other species will be confined to the legends of the corresponding figures (Figs 86-110).

2. External flagellum, transition region and basal bodies

The external part of the flagellum (Fl) has tubular mastigonemes on its surface (Fig. 61), a swelling with electron-dense material close to the cell surface and eyespot (Fig. 62; see also Fig. 73) and consists of the usual 9+2 pattern of microtubules (MTs) in the axoneme (Fig. 63).

The transition region, between the external flagellum and the basal body (Figs 62-64), consists of a transverse partition slightly above the cell surface and a transitional helix with 4-5 gyres (Fig. 62) that lies above the transverse partition, inside the peripheral duplets (Figs 62, 63). Faint fibrous structures (transitional fibres) connect the axonemal duplets with the plasmalemma in this region (Fig. 64).

Figs 59-69. Zoospores of *Vischeria stellata*.

Fig. 59. Light microscopy. Anoptral contrast. x 2200.

Fig. 60. Longitudinal section of the zoospore, showing its general structure. x 23000.

Fig. 61. Mastigonemes on the surface of the emergent flagellum. x 8500.

Fig. 62. Longitudinal section of the base of the emergent flagellum, showing the transitional helix. Note the number of gyres in section (arrows), 4 on one side and 5 on the other. x 42000.

Figs 63-69. Seven selected transverse sections of two different series (Figs 63-66 and Figs 67-69) through the anterior end of the cell, to show the transition region of the flagellum, the two basal bodies and their associated roots. x 72000.

Fig. 63. Axonemal duplets, transitional helix, roots R1 and R2.

Fig. 64. Transitional fibres connecting duplets with the plasmalemma (arrows), roots R1 and R2.

Fig. 65. Triplet MTs in basal body B1 and root R1.

Fig. 66. Basal bodies. Note the possible 2 MTs of root R2 (arrow) and the striated fibrous band, connecting both basal bodies .

Fig. 67. Microtubular roots R1, R4 and striated band. x 72500.

Fig. 68.. 4 MTs of root R3 can be seen between both basal bodies. Roots R1 and R4 are still visible.

Fig. 69. Cartwheel pattern in basal body B1 and root R3.

Abbreviations used in figures: C, chloroplast; E, eyespot; F, emergent flagellum; FS, flagellar swelling; LV, lamellate vesicle; M, mitochondrion; R1, R2, R3 and R4, microtubular roots; SB, striated band; SV, spiral vesicle.

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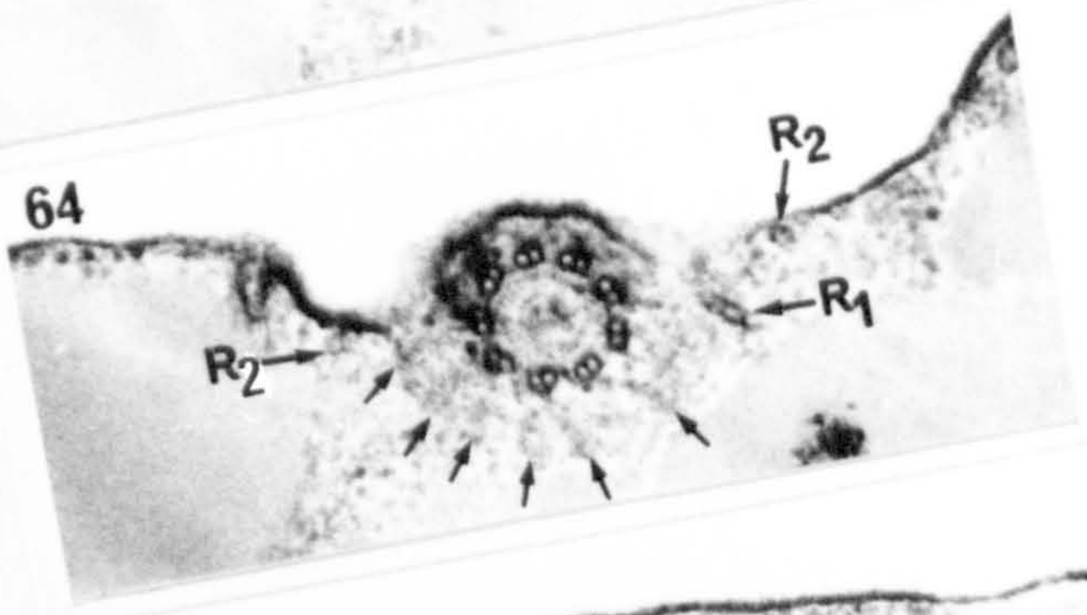

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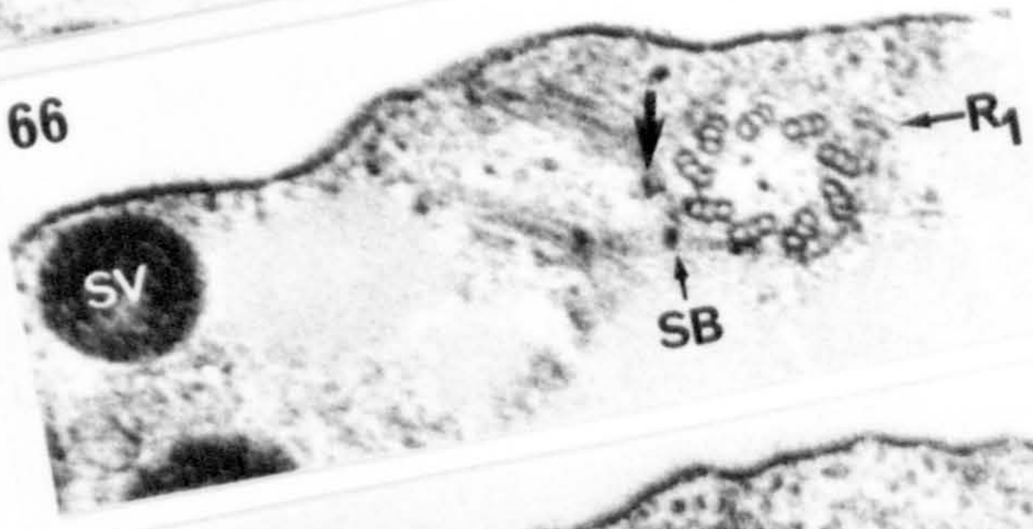

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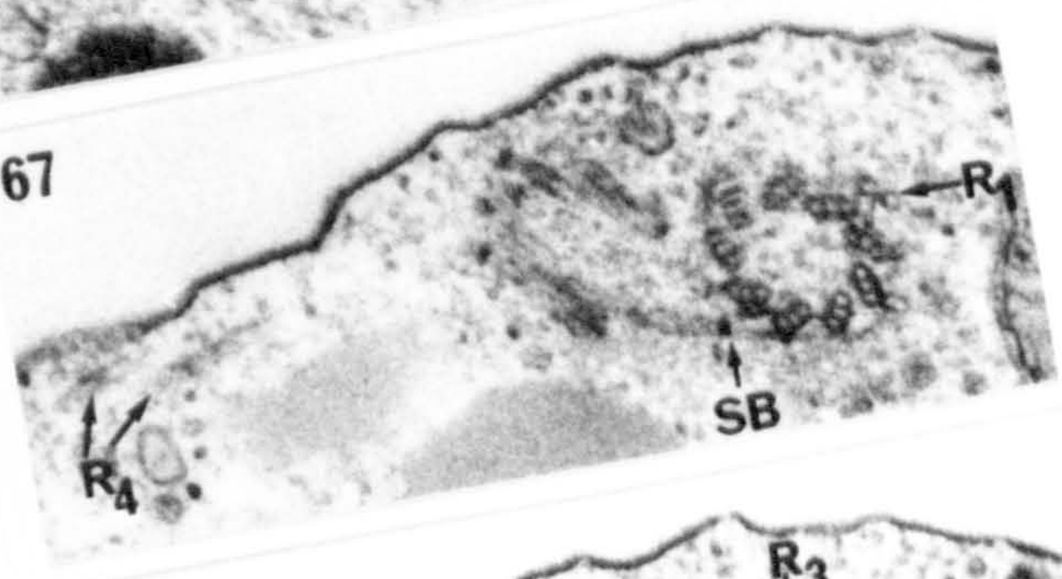

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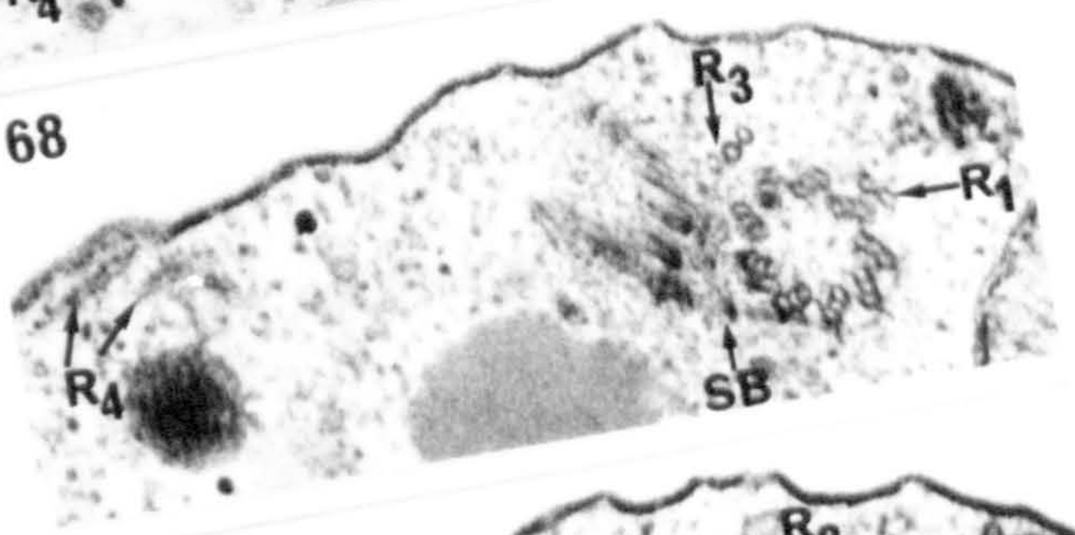

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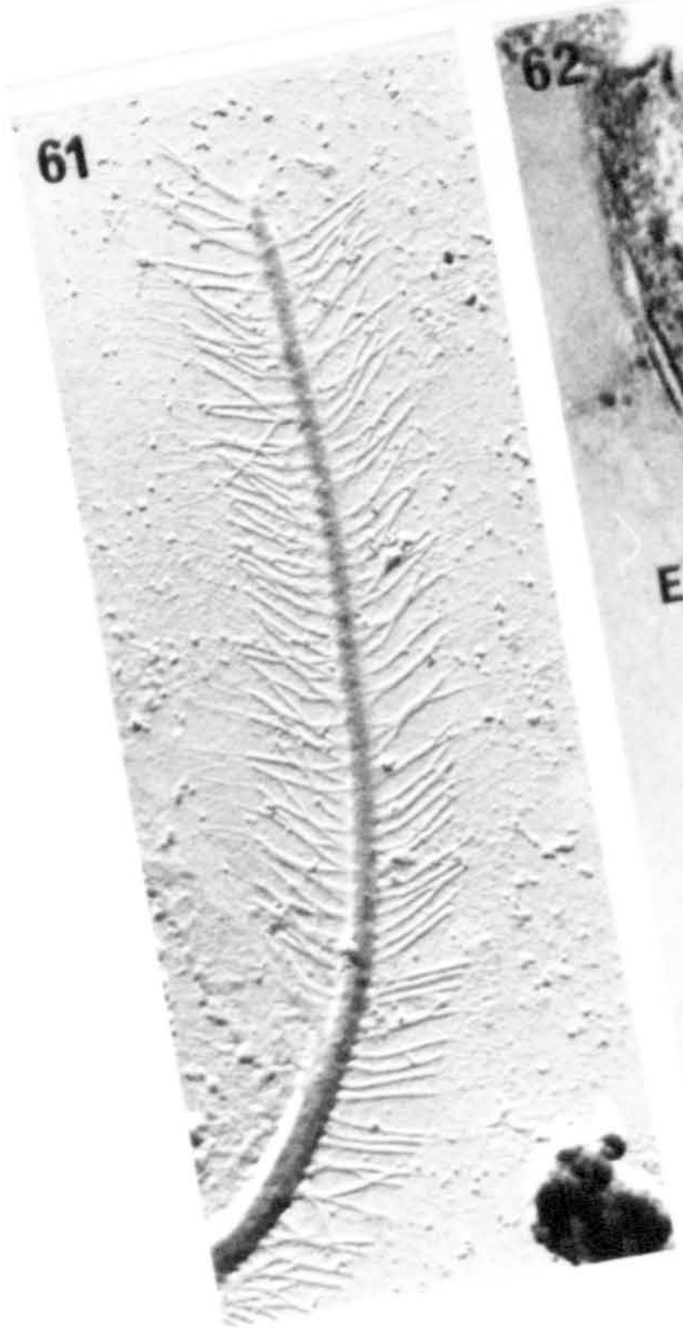

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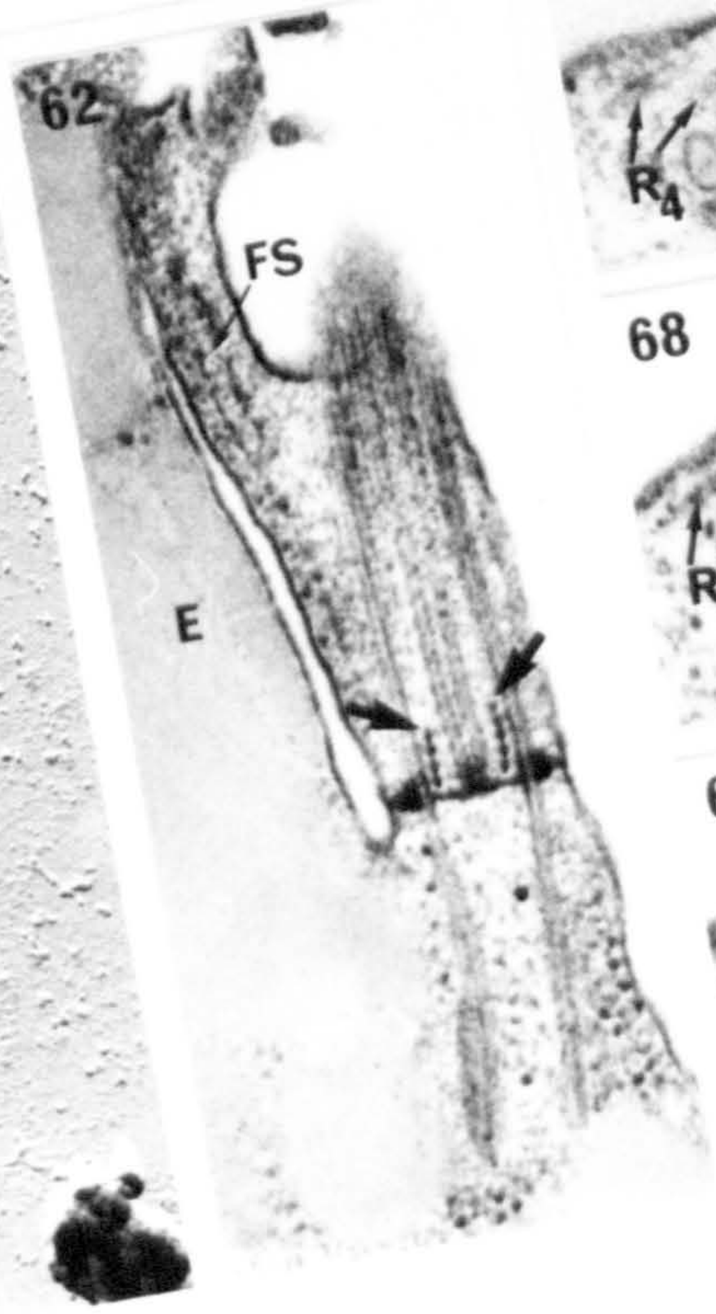


The basal body (B1), inside the cell, consists of nine triplet MTs (Fig. 65) also connected to the plasmalemma by transitional fibres. A second basal body (B2) lies more or less perpendicular to the first and is connected to it by a fibrous striated band (Figs 66-69). A cartwheel structure can be seen at the proximal end of basal body B1 (Fig. 69).

3. Flagellar roots

The flagellar roots present in the zoospores of all the mentioned species were studied in detail. A model showing their distribution has been reconstructed from serial sections of these zoospores, in particular those of *Vischeria stellata*, and is diagrammatically illustrated in Fig. 70; four microtubular roots (R1-R4) and one fibrous root, the rhizoplast (Rh), are present.

Root R1 originates at basal body B1 (Figs 71, 72) and runs anteriorly, close to the plasmalemma and around the flagellar swelling for a considerable extent, if not quite completing a loop around it (Fig. 73). Transverse sections of the zoospores always show this root to consist of 2 MTs (Figs 63-68); in longitudinal sections, however, the number of MTs observed is 2 in some sections (Fig. 71) and 3 in others (Figs 72, 73). One section appears to indicate that root R1 generates cytoskeletal MTs (see Appendix 3).

Root R2 extends from the opposite side of basal body B1 and also runs close to the plasmalemma and possibly around the swelling (Fig. 74). It appears to consist of 2 MTs (Figs 63-66).

Root R3 is associated with dense material between the basal bodies and runs close to the plasmalemma to the posterior end of the cell (Figs 71, 74, 75). It is composed of a band of 5 MTs which appear in cross section as a semicircular arc (Figs 76, 77; see also Figs 68, 69) and, in some sections (Figs 71, 75), it seems to be connected to basal body B1 by dense fibrous material.

Root R4 (Figs 78, 79; see also Figs 65, 67-69) consists of 2 MTs and extends from basal body B2 to run close to the plasmalemma and around the eyespot, first anteriorly and then turning posteriorly.

The only fibrous flagellar root seen in these zoospores is the rhizoplast (Figs 80-85). It is a cross-banded root which originates near the basal bodies and passes deeply into the cell, forming a characteristic association with the nucleus and possibly the Golgi body (Figs 80, 81). Near the nucleus, the rhizoplast splits into several branches spreading over the nuclear surface (Figs 82, 83; see also 84, 85). Near the basal bodies it appears also branched and divided into two parts that connect with the basal bodies (Figs 81, 85).

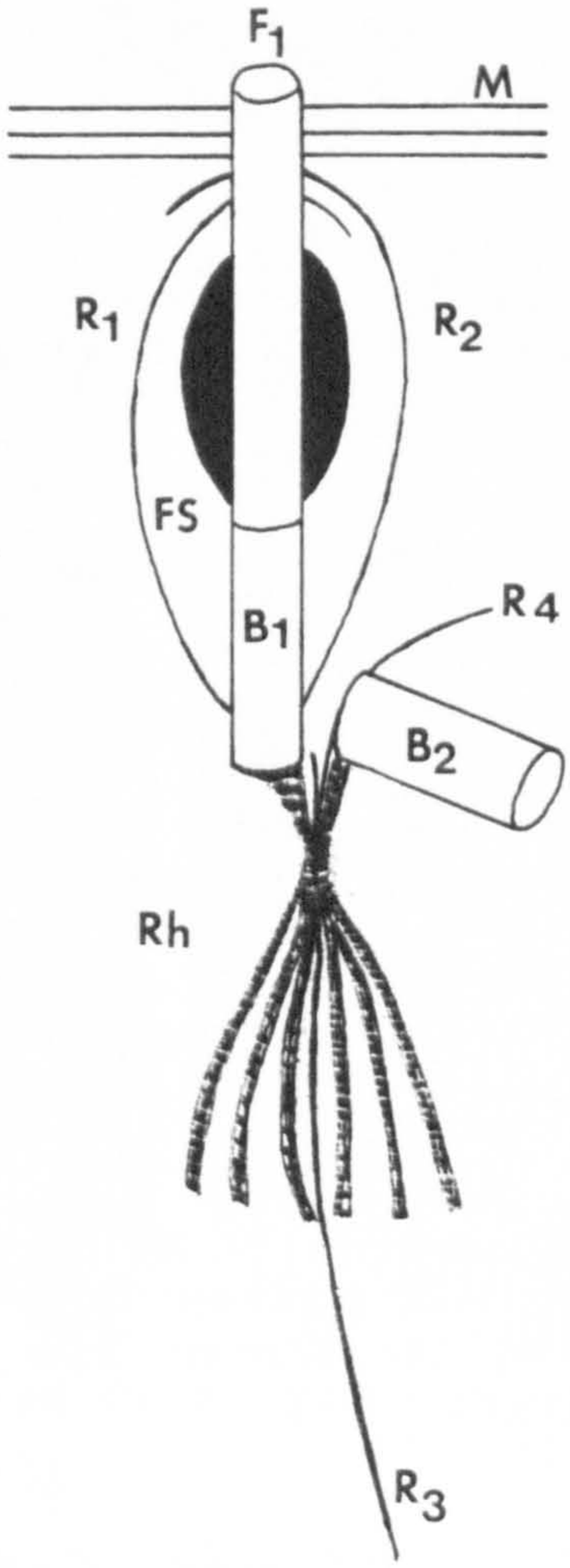


Fig. 70. Schematic drawing showing the relative positions of basal bodies, flagellar roots and swelling in zoospores of the eustigmatophycean species *Vischeria stellata*. View from the ventral to the dorsal side of the zoospore. B1, basal body of the emergent flagellum; B2, second basal body; F1, emergent flagellum; FS, flagellar swelling; M, mastigonemes; R1, R2, R3 and R4, microtubular roots; Rh, rhizoplast.

Figs 71-79. *Vischeria stellata*. Selected longitudinal and transverse sections of the zoospores, showing the different microtubular roots.

Fig. 71. Roots R1, R3 and R4 shown in relation to the basal bodies. x 42000.

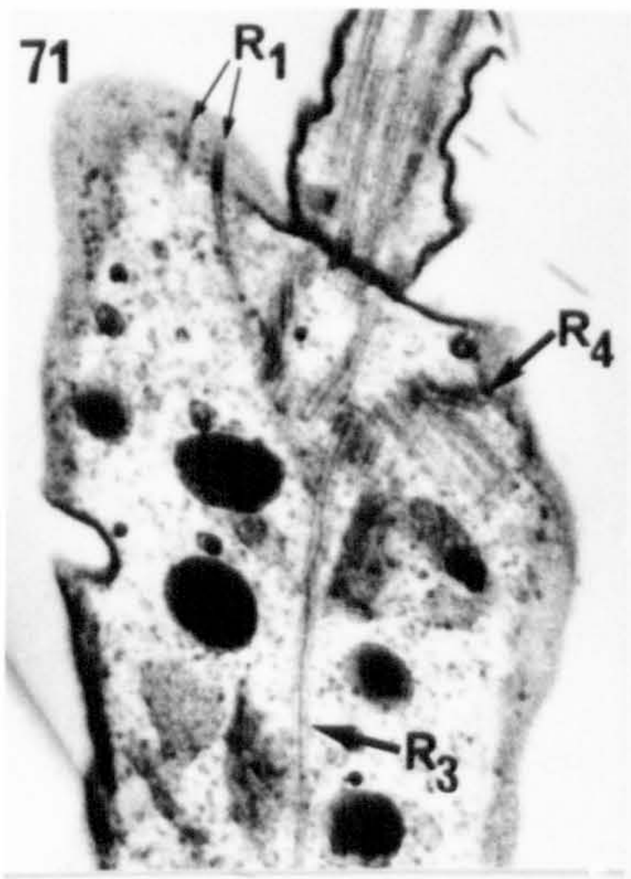
Fig. 72. Roots R1 and R4, ditto. x 70000.

Fig. 73. Flagellar swelling in oblique section. Note the 3 MTs of root R1, passing around it (arrows). x 48000.

Figs 74, 75. Two consecutive sections of a series, showing roots R2, R3 and R4. Note the dense material connecting both basal bodies and root R3 with basal body B1 (arrows, Fig. 75). Fig. 74 x 54000; Fig. 75 x 60000.

Figs 76, 77. Sections showing the 5 MTs of root R3 in a semicircular arc near the basal bodies (Fig. 76) and lower in the cell when the basal bodies are no longer seen and the tip of the nucleus (N) appears (Fig. 77). Fig. 76 x 100000; Fig. 77 x 90000.

Figs 78, 79. Two consecutive sections, showing root R4. x 37500.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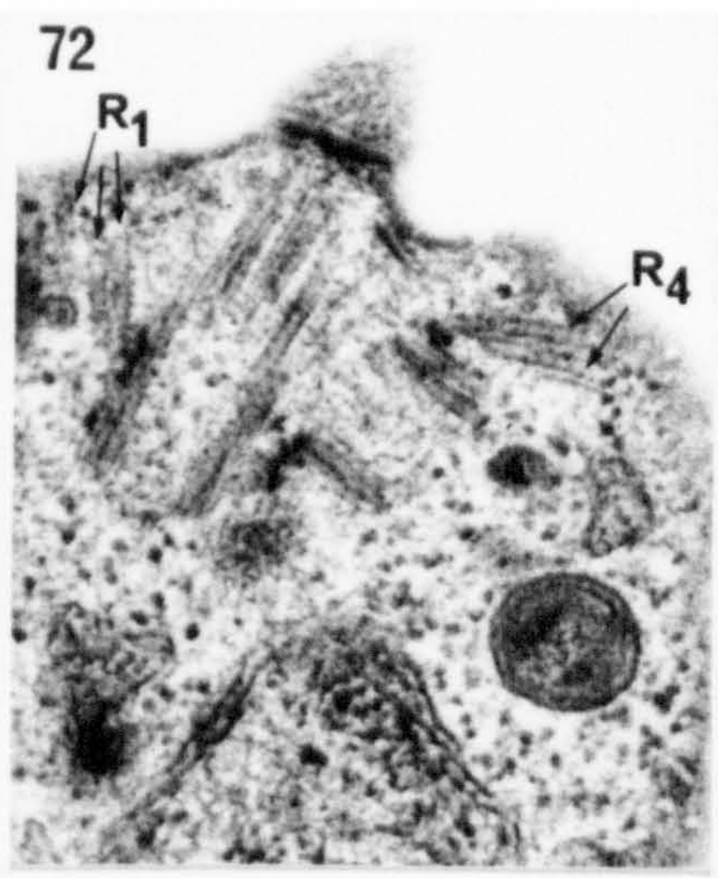
R1
R4
R3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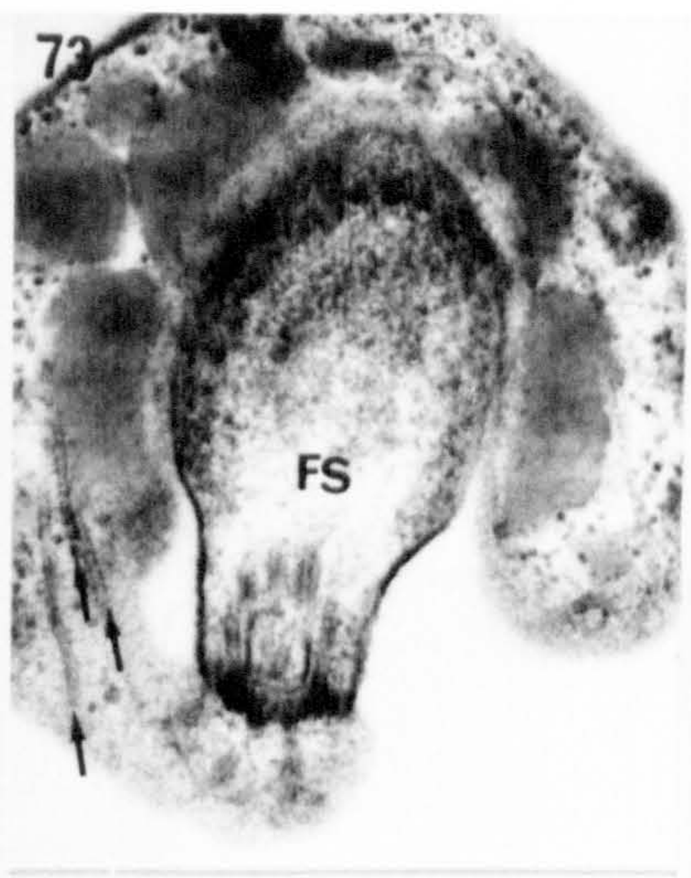
R1
R4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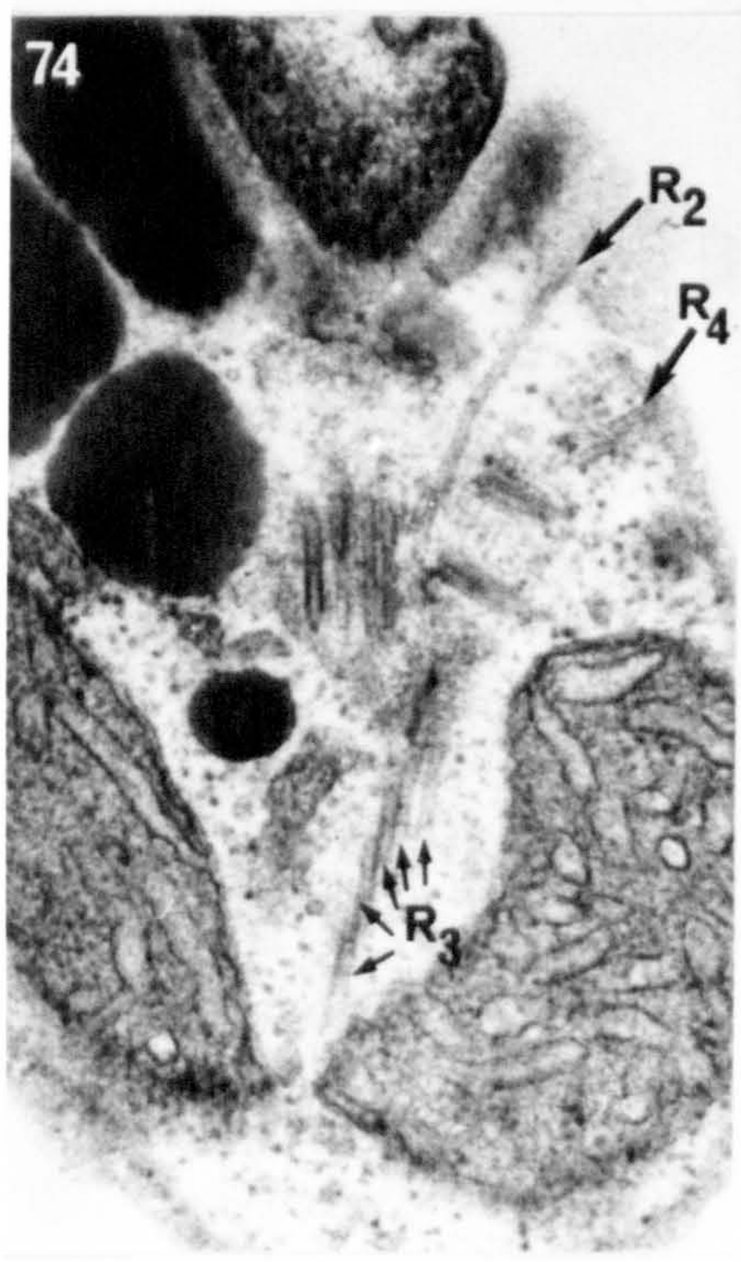
FS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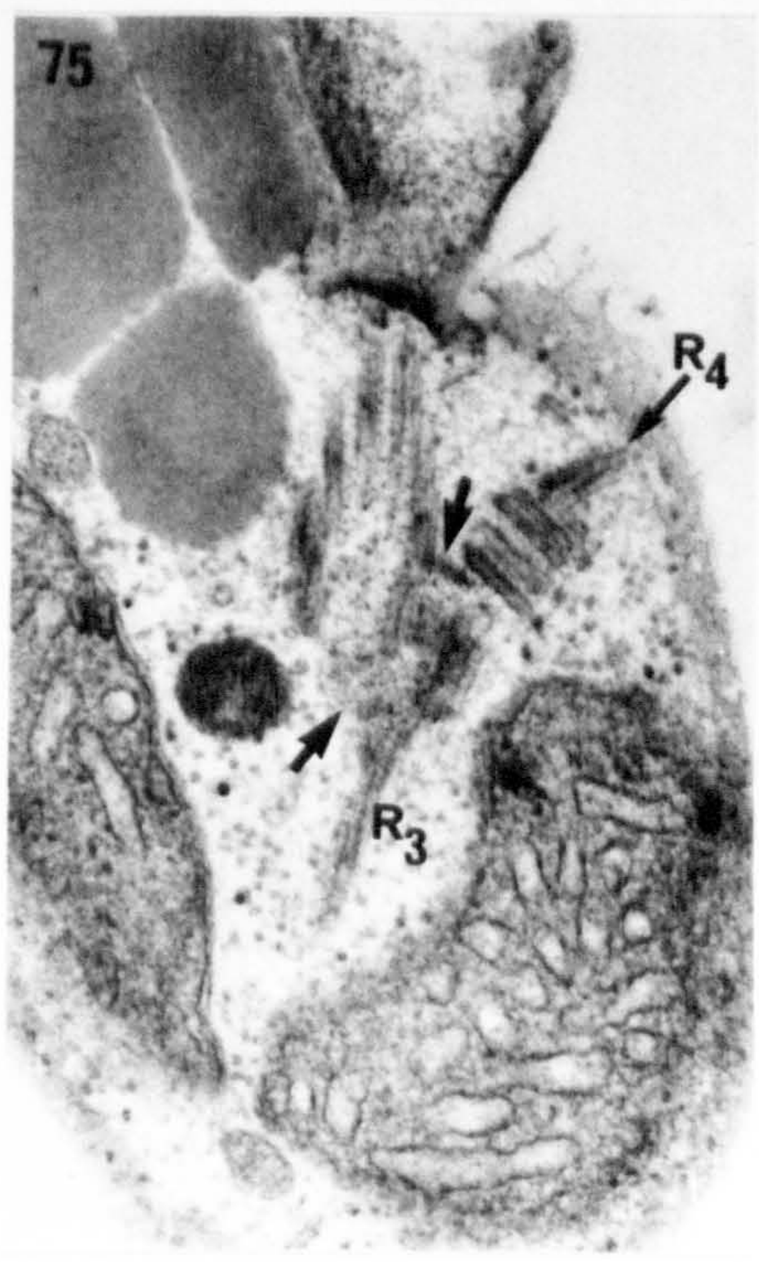
R2
R4
R3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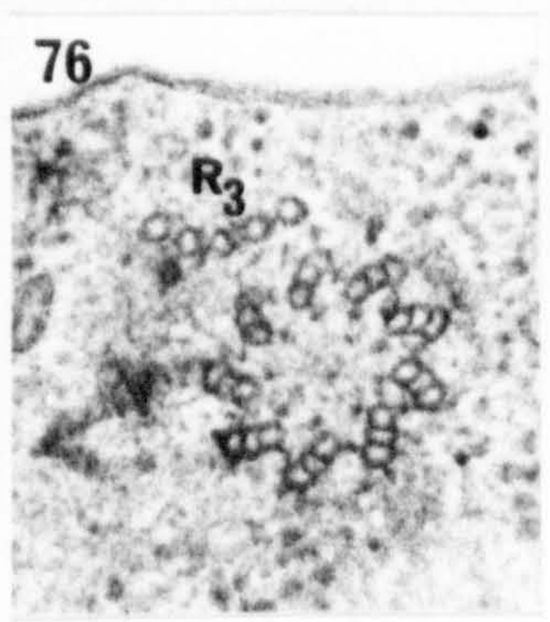
R4
R3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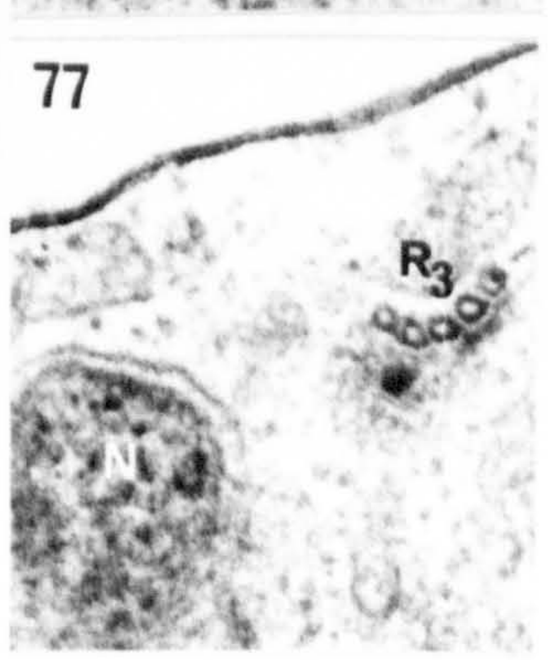
R3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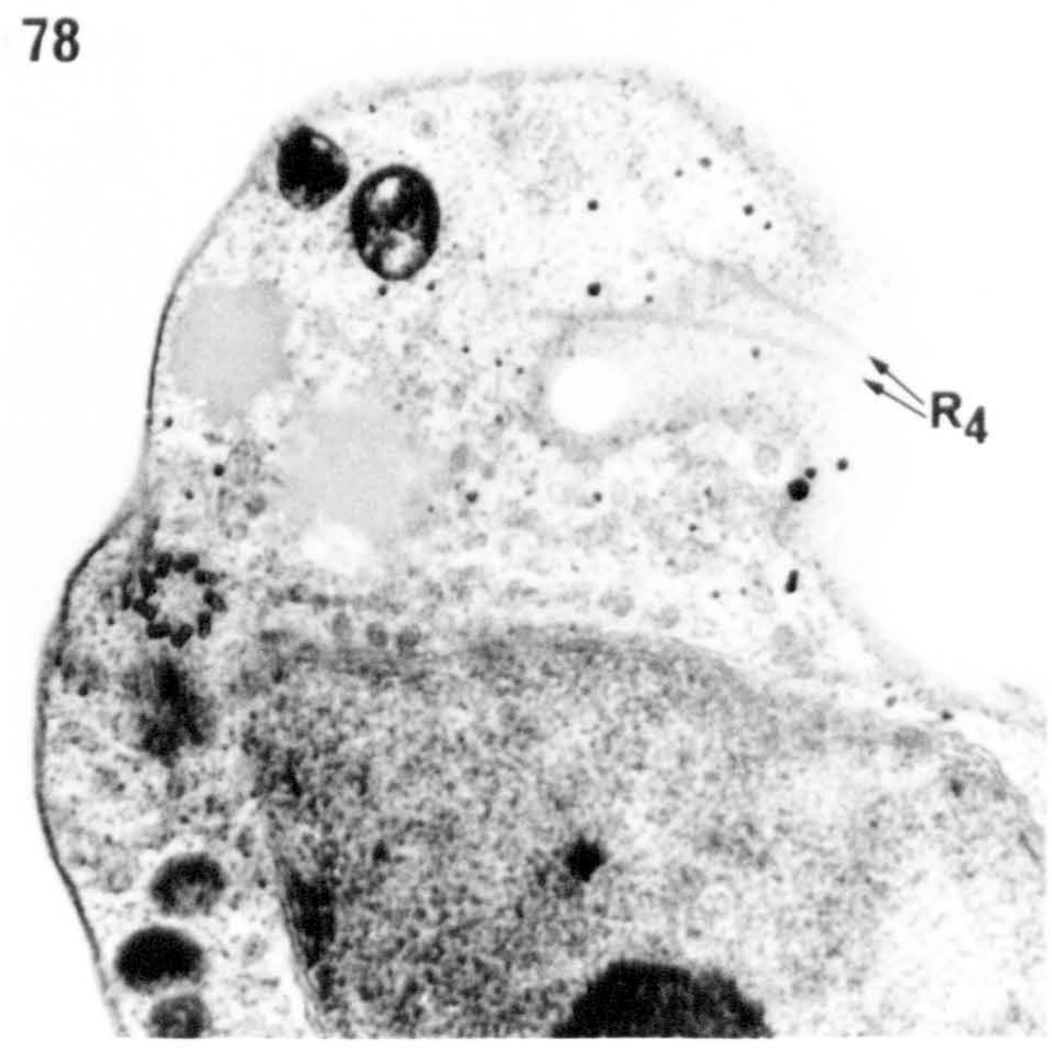
R3
N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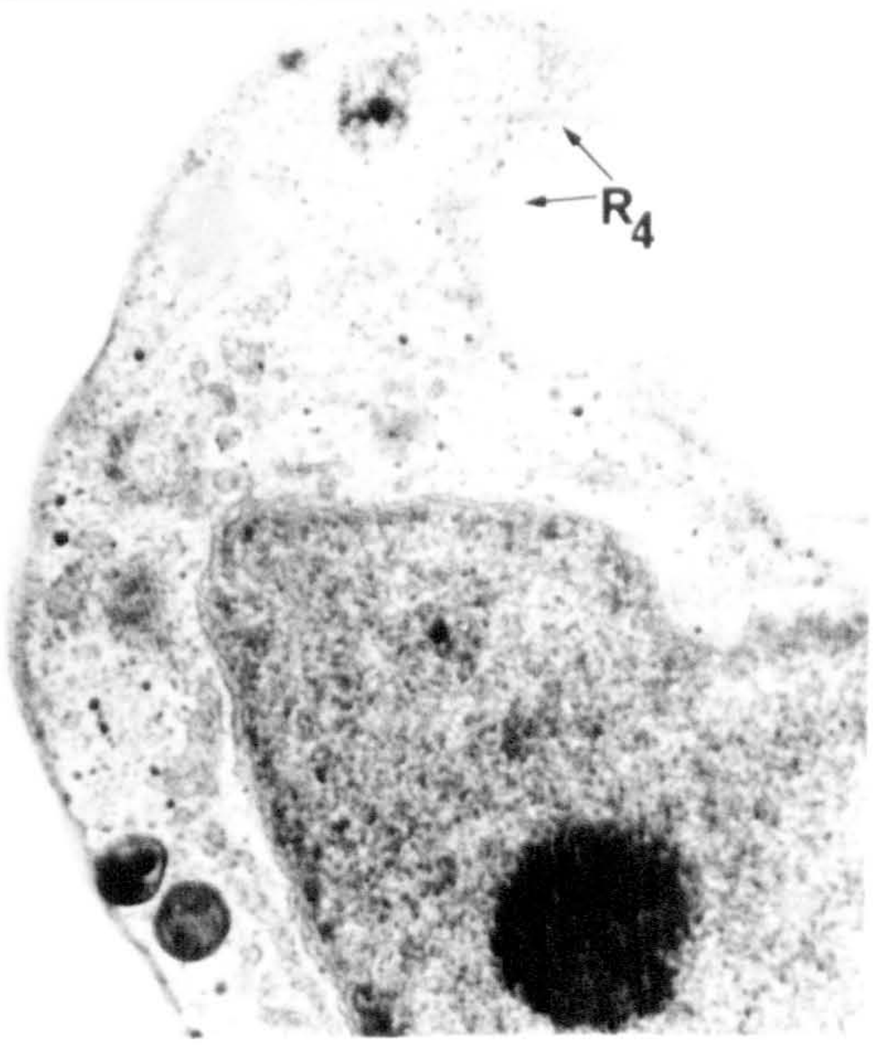
R4


79
R4

Figs 80-85. *Vischeria stellata*. Selected longitudinal sections of two series (Figs 80-83 and Figs 84, 85), showing the rhizoplast in the zoospores.

Fig. 80. Note the position of the cross-banded structure (large arrow) near the basal bodies and a fibre (small arrows) between the nuclear envelope and several Golgi vesicles. x 50000.

Fig. 81. Note the two branches that connect with the basal bodies (arrow). x 50000.

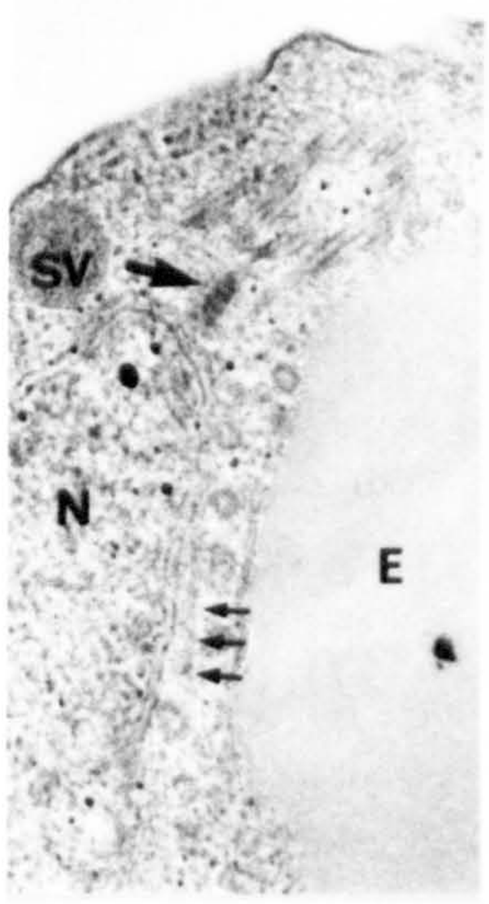
Fig. 82. The rhizoplast divides (large arrow) near the nucleus and several fibres (small arrows) can be seen spreading over the nuclear surface. x 47500.

Fig. 83. Some fibres (arrows) seen immediately after the nuclear surface leaves the plane of sectioning and surrounded by Golgi vesicles. x 60000.

Figs 84, 85. Note a fibre (small arrows) near the nucleus and the branch connecting with the second basal body (large arrow). x 52500.

Abbreviations used in figures: E, eyespot; N, nucleus; SV, spiral vesicle.

80



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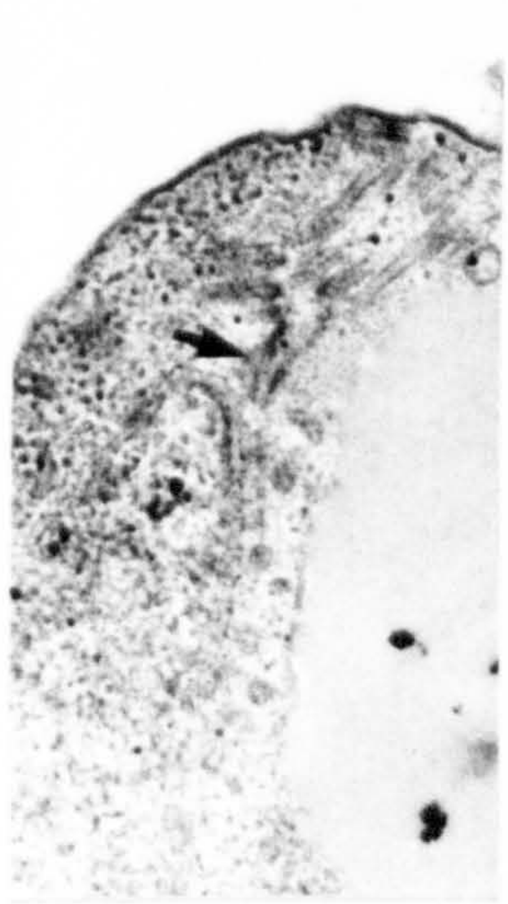

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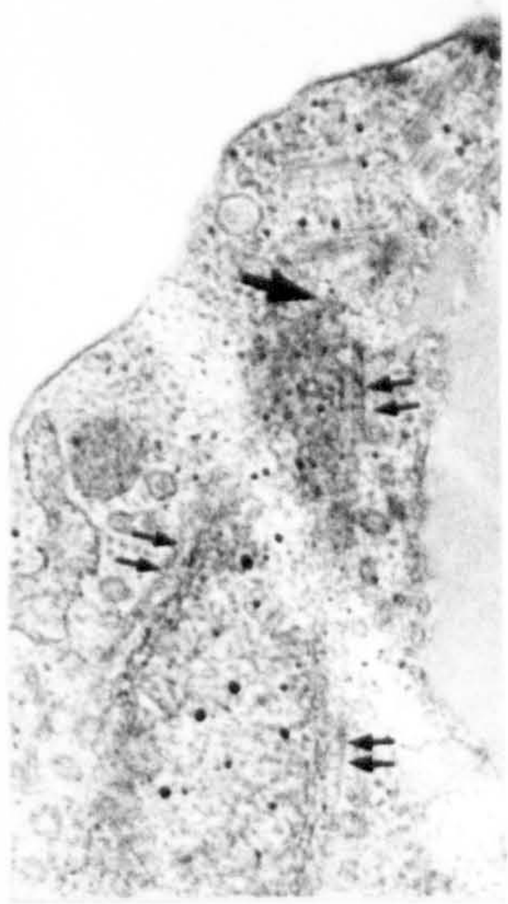

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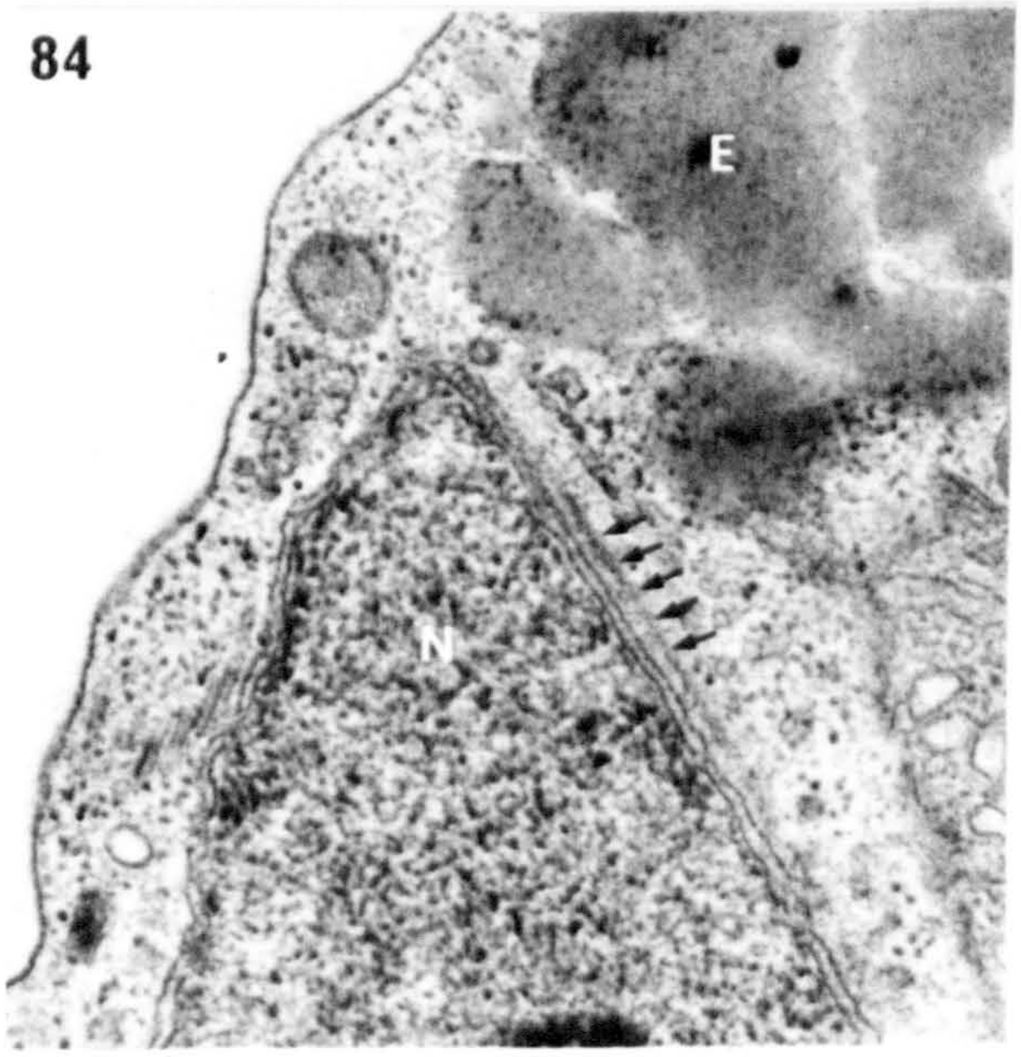

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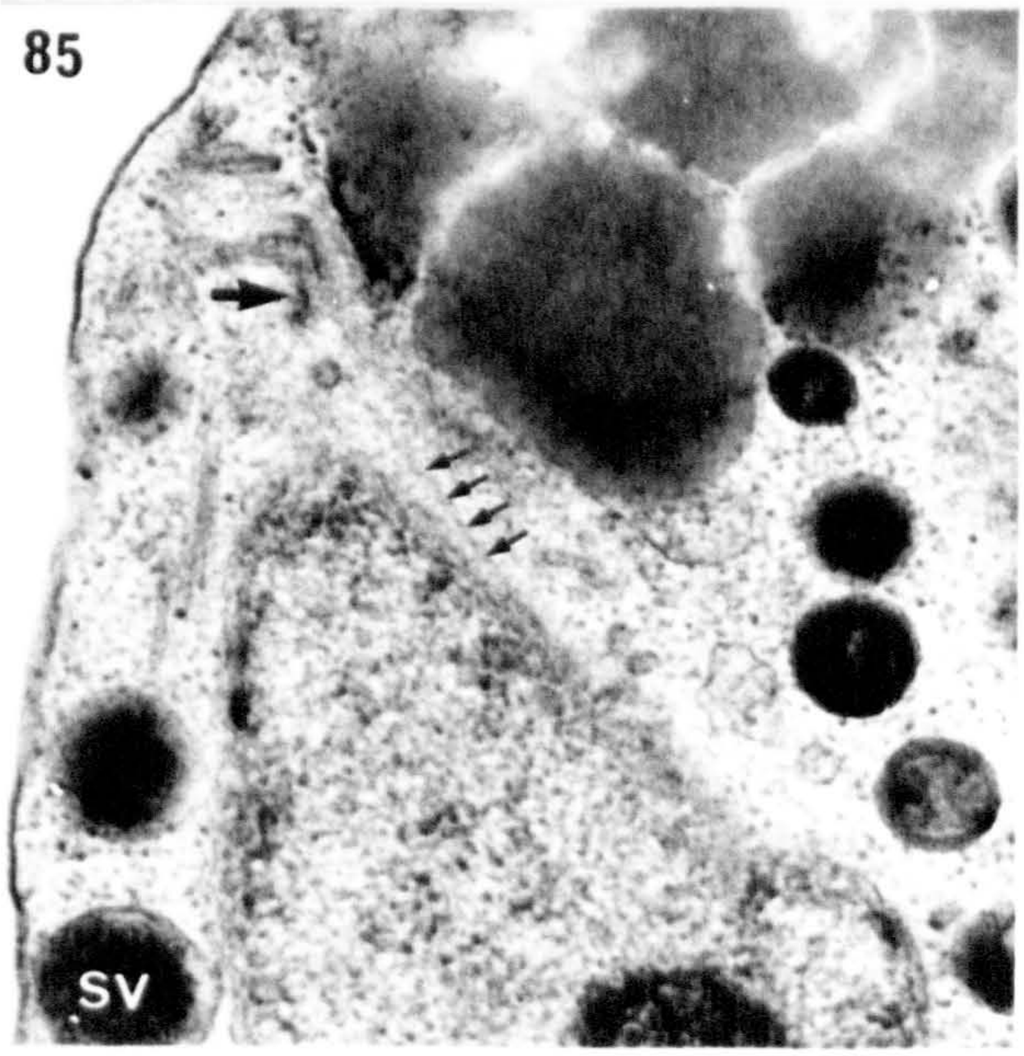


Figs 86-99. Details of the flagellar apparatus in zoospores of *Vischeria helvetica*.

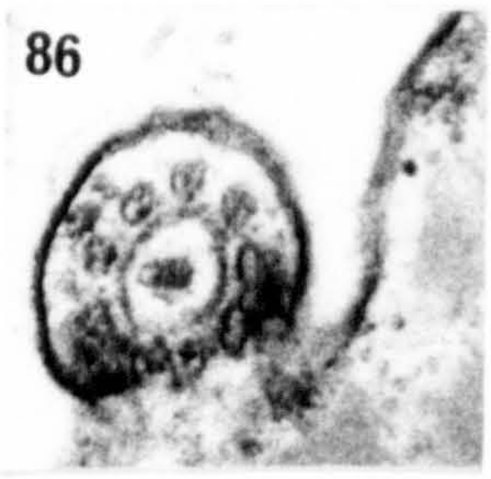
Figs 86-89. Selected transverse sections of a series through the anterior end of the cell, to show the transition region of the flagellum with a helix (Fig. 86), the two basal bodies and root R3 (Figs 87-89). Note the striated band (SB) in Fig. 87. Fig. 86 x 64000; Figs 87-89 x 62500.

Figs 90-95. Selected oblique sections of two series (Figs 90-92 and Figs 93-95), showing the 4 microtubular roots and their relation to the basal bodies and/or to the flagellar swelling. x 32500.

Figs 96- 99. Longitudinal sections showing root R3 and fibres of the rhizoplast (arrows) (Figs 96,97) and root R4 (Figs 98,99). Fig. 96 x 16000; Fig. 97 x 50000; Fig. 98 x 47500; Fig. 99 x 33000.

Abbreviations used in figures: E, eyespot; F, emergent flagellum; FS, flagellar swelling; N, nucleus; R1, R2, R3 and R4, microtubular roots; SB, striated band.

86



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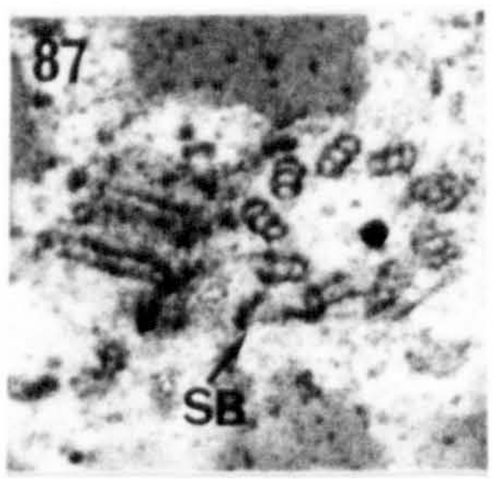

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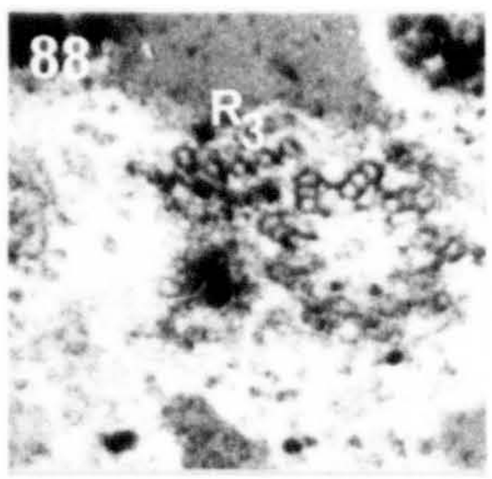

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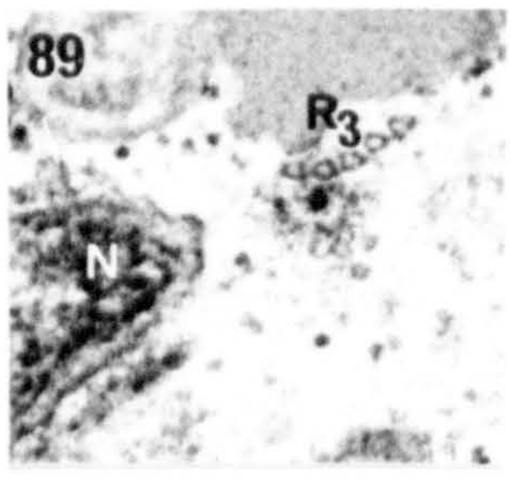

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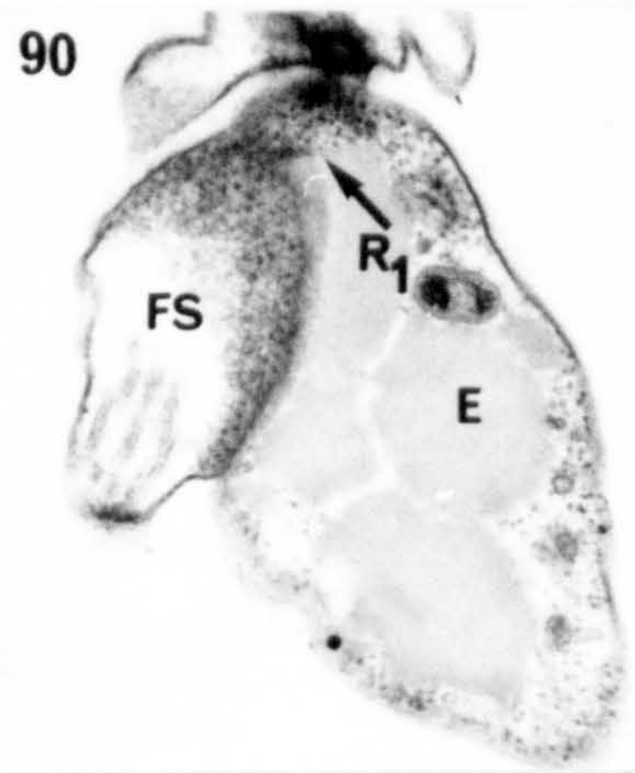

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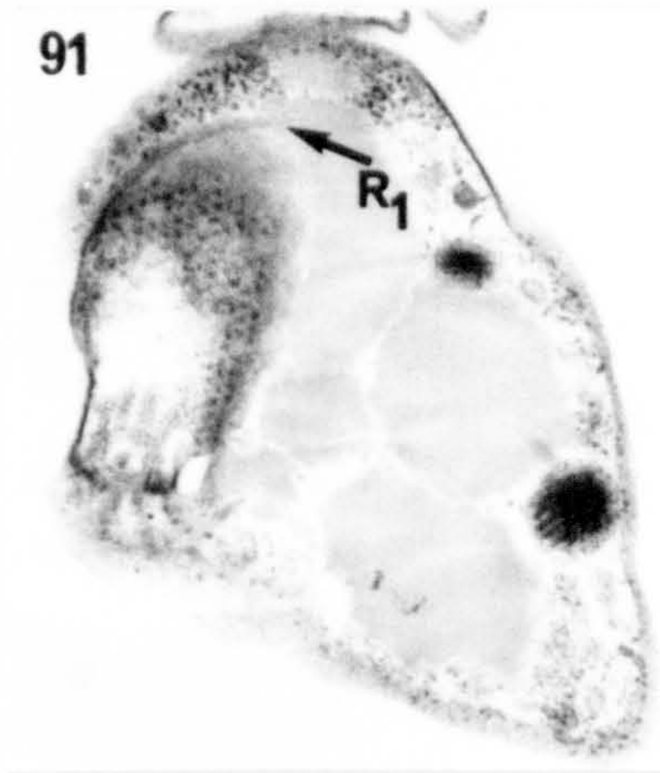

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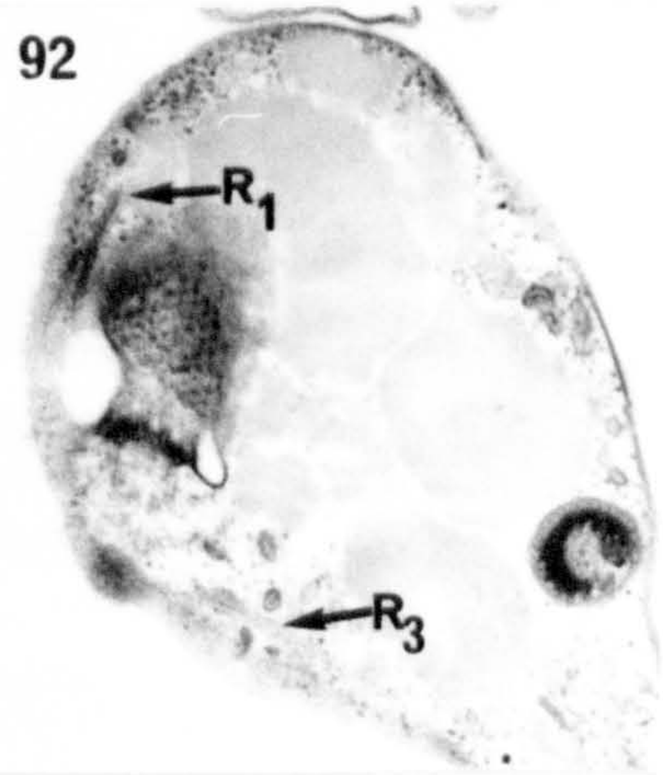

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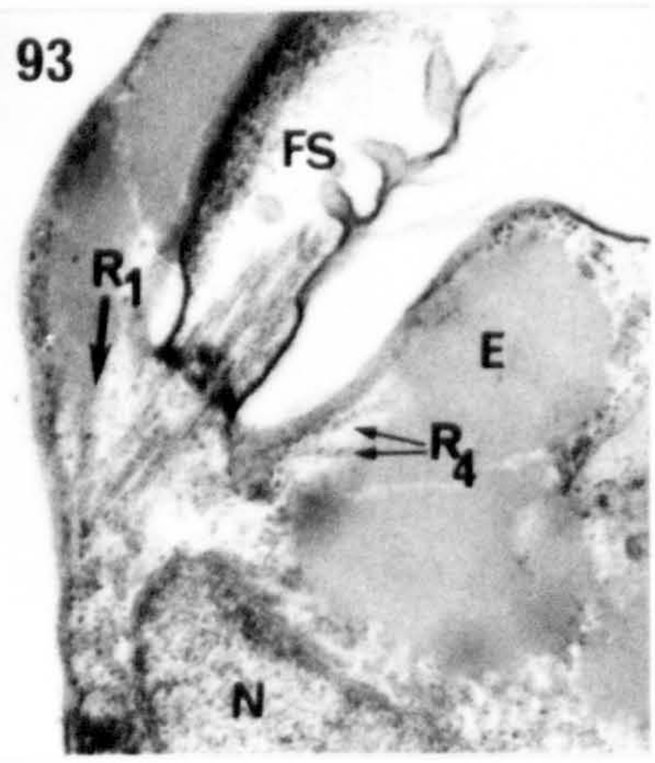

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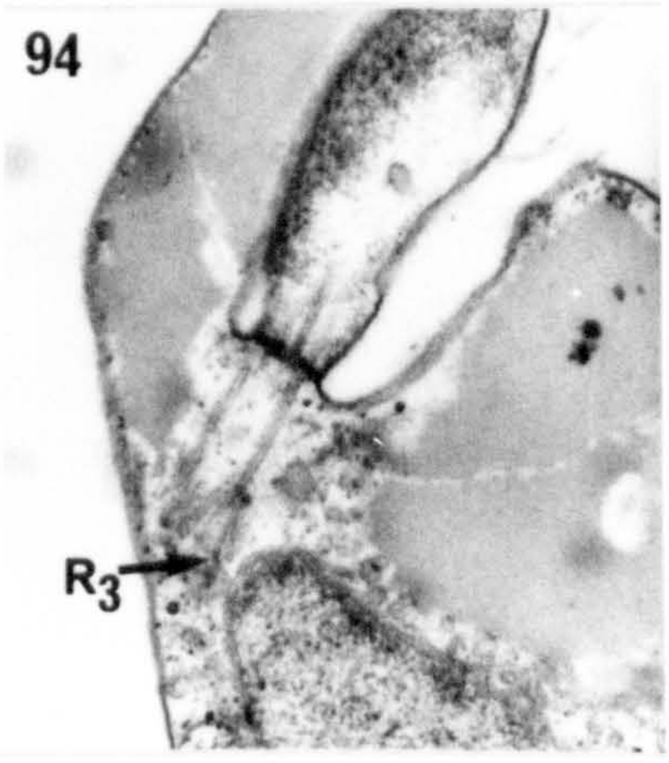

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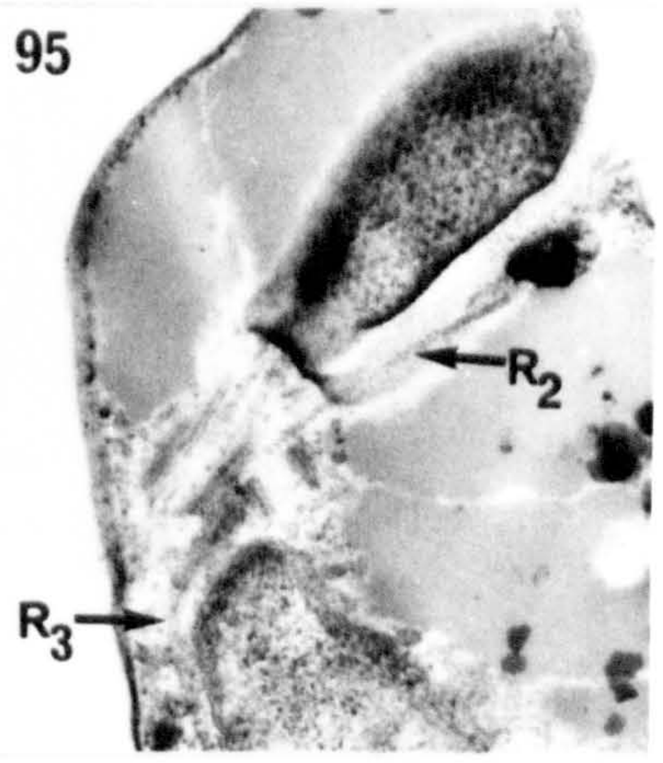

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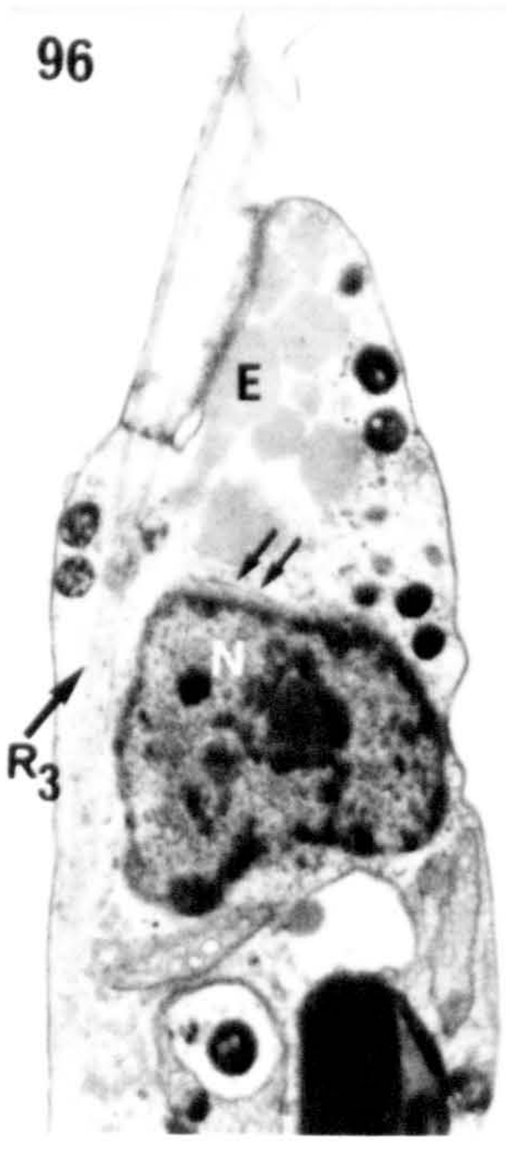

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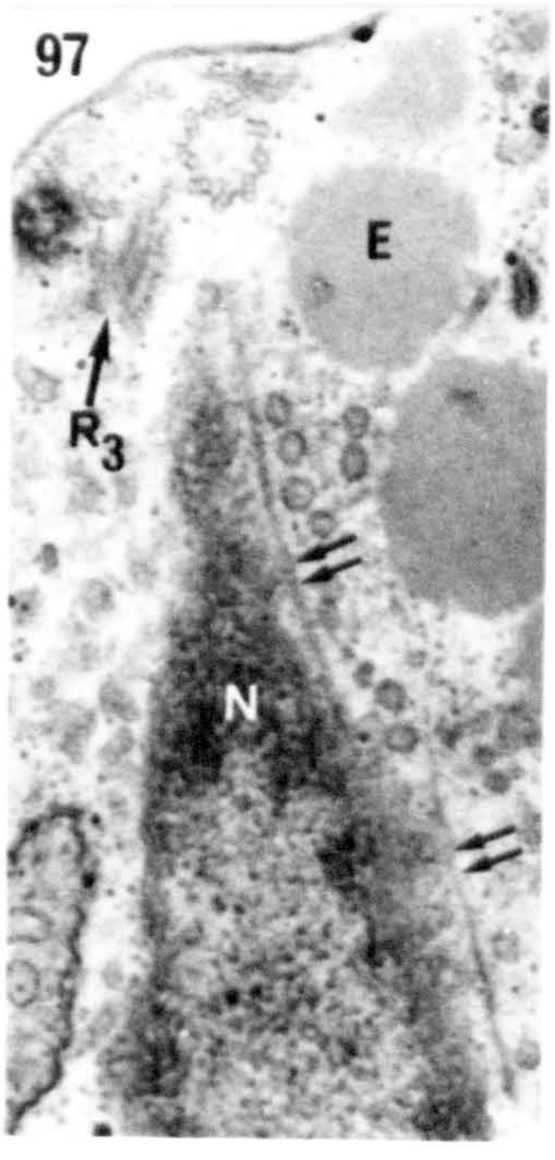

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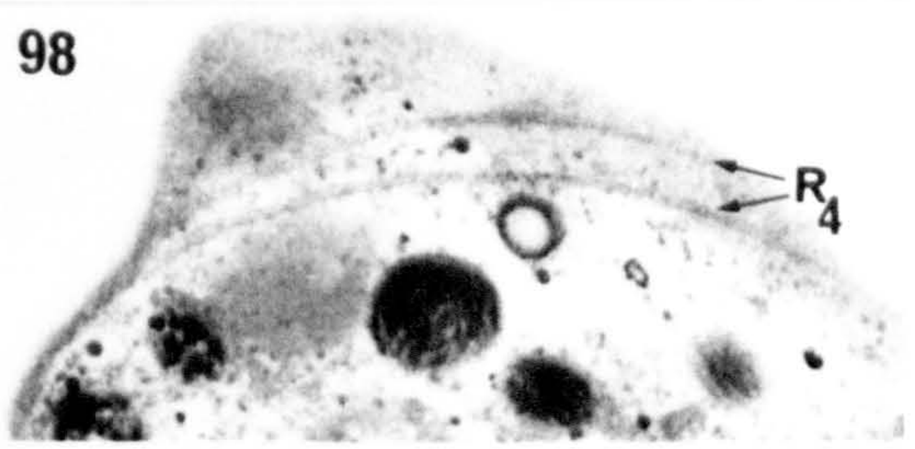

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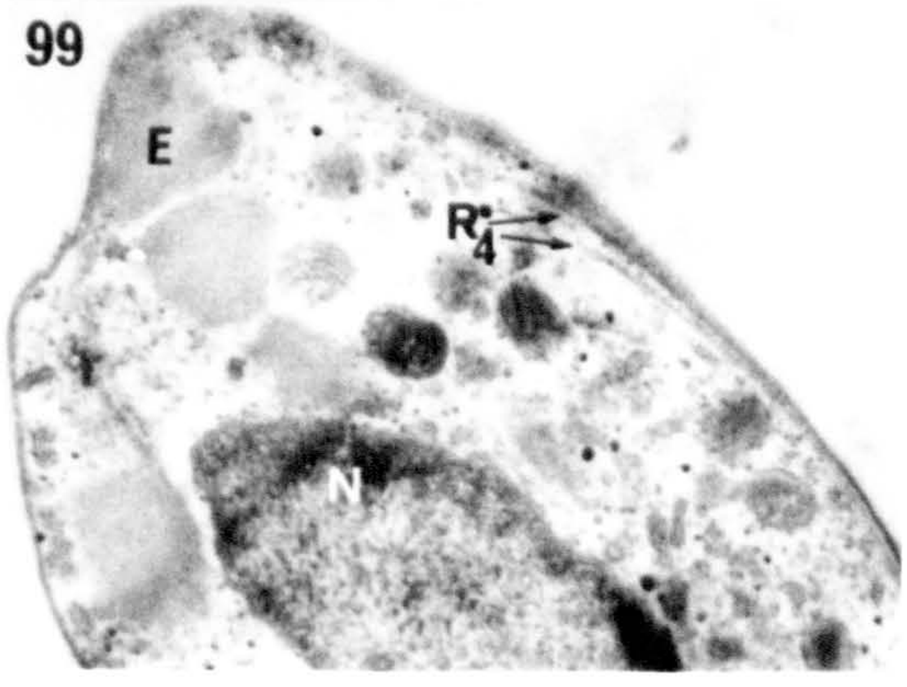


Figs 100-110. Details of the flagellar apparatus in zoospores of *Eustigmatos magnus*.

Figs 100-103. Consecutive oblique sections, showing the 4 microtubular roots and their relation to the basal bodies. Note also the transitional helix in Fig. 101 (arrow) and a fibre of the rhizoplast in Fig. 102 (arrow). Figs 100-102 x 45000; Fig. 103 x 42500.

Figs 104-110. Selected transverse sections of a series through the anterior end of the cell, to show the transition region of the flagellum, the two basal bodies and their associated roots R1 and R3. x 72000.

Fig. 104. Axonemal duplets and transitional helix.

Fig. 105. Transitional fibres connecting duplets with the plasmalemma (arrows).

Fig. 106. Transitional fibres connecting triplet MTs with the plasmalemma (arrows).

Fig. 107. Basal bodies and root R1.

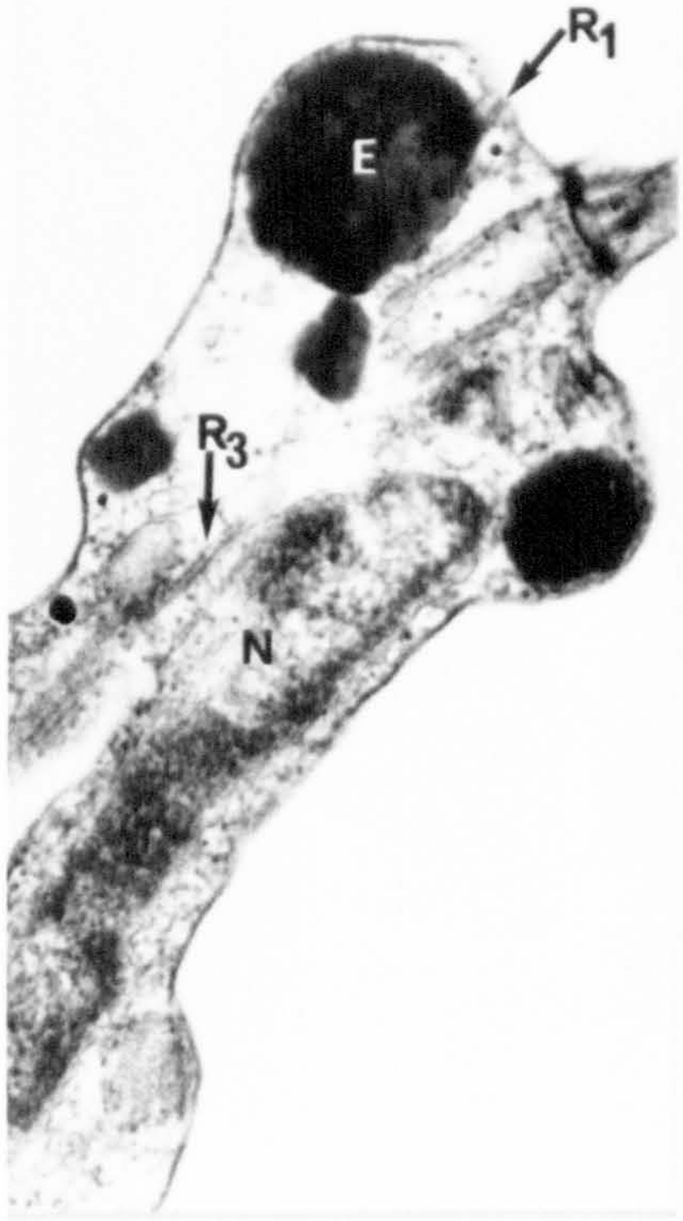
Fig. 108. Basal bodies connected by a striated fibrous band and the 5 MTs of root R3.

Fig. 109. Cartwheel pattern in basal body B1 and root R3.

Fig. 110. Root R3 lower in the cell when the basal bodies are no longer seen and the profile of the nucleus appears.

Abbreviations used in figures: E, eyespot; F, emergent flagellum; FS, flagellar swelling; N, nucleus; R1, R2, R3 and R4, microtubular roots; SB, striated band.

100



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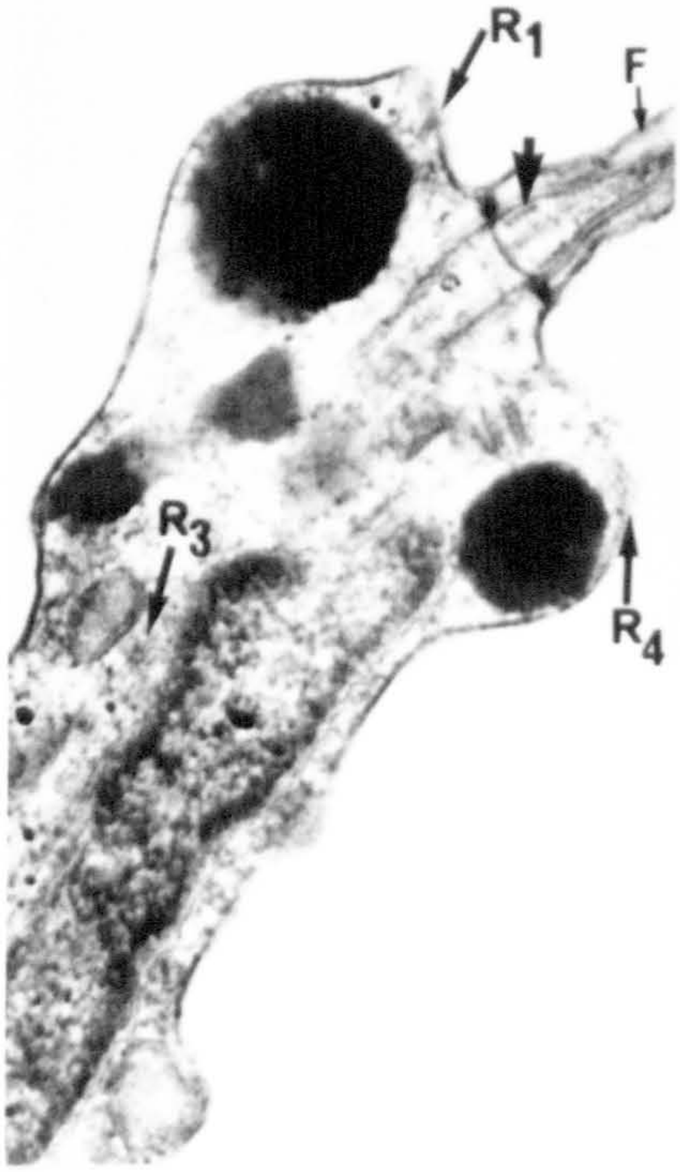

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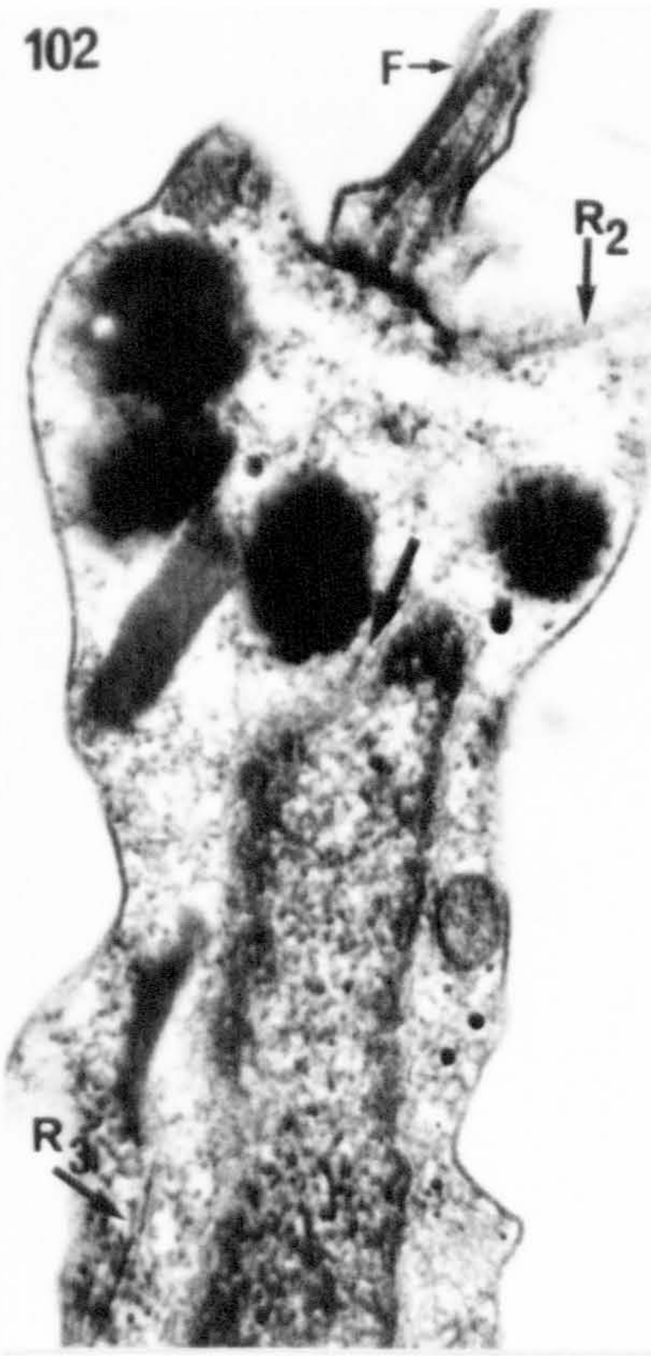

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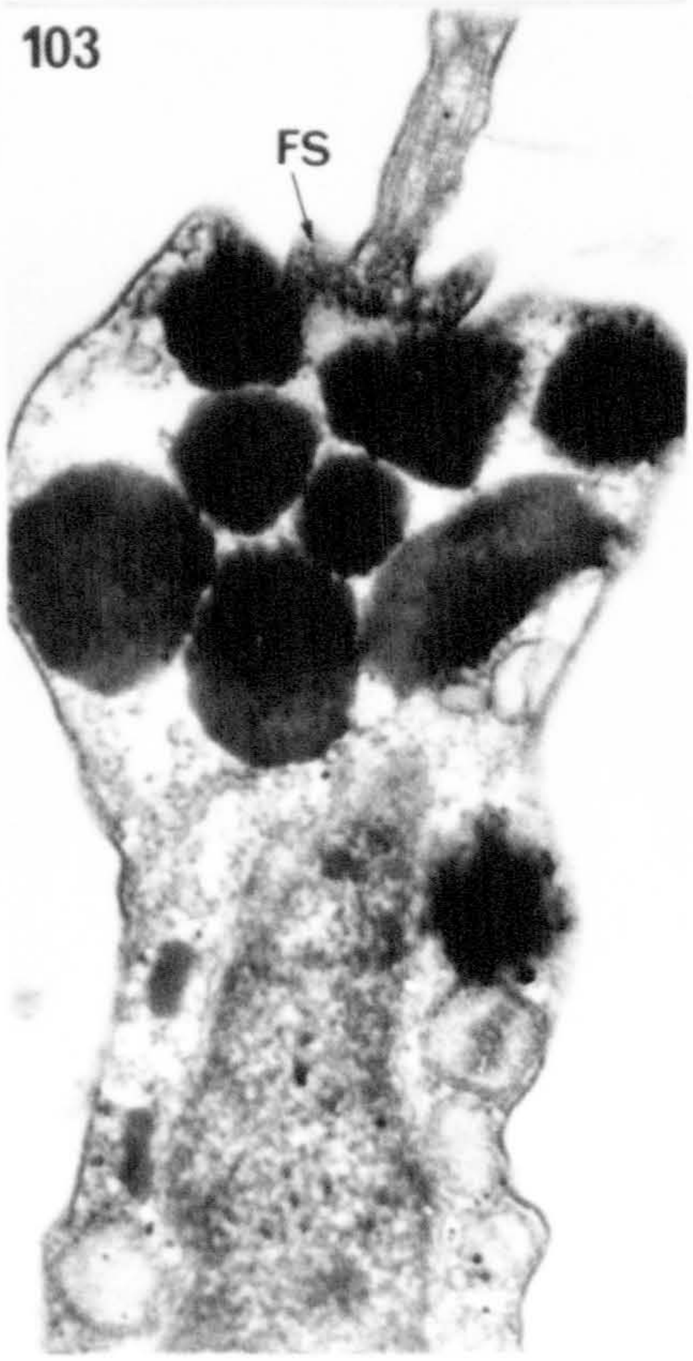

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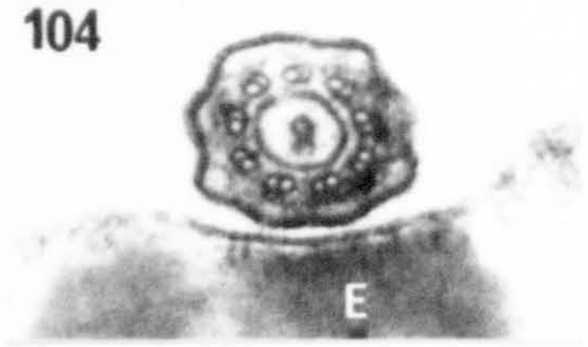

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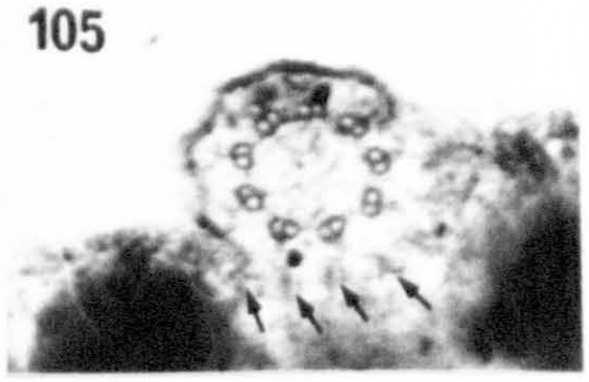

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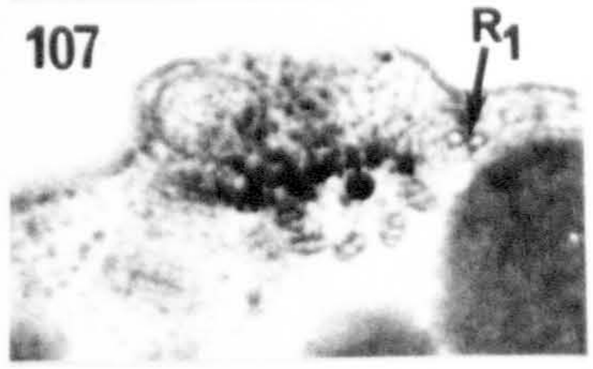

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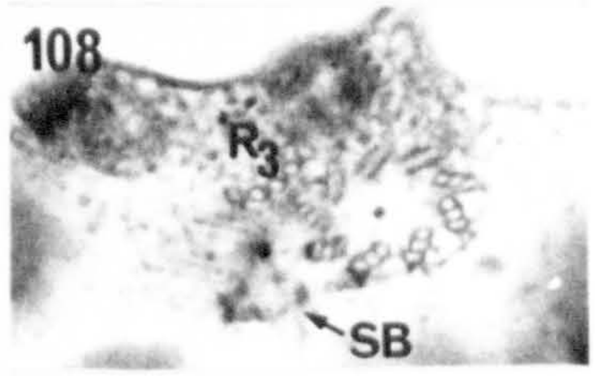

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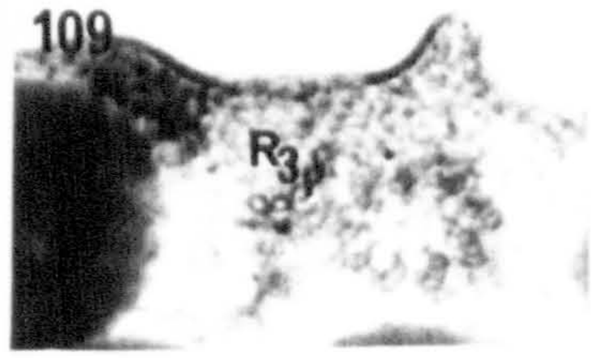

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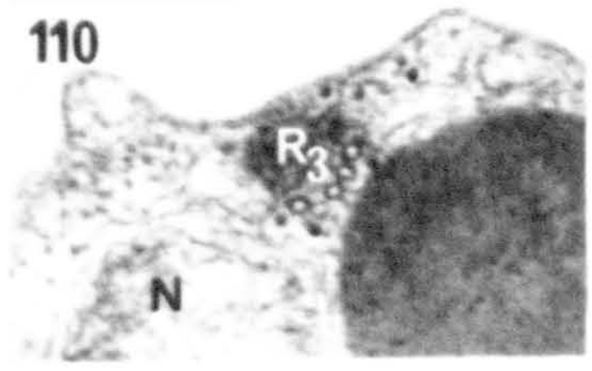

D. STRUCTURE OF BIFLAGELLATE ZOOSPORES

Observations on the structure of the biflagellate zoospores of *Pseudocharaciopsis minuta* mostly confirm a previous study on this organism (Lee & Bold, 1973) and add new information on the structure of the flagellar apparatus.

1. General

In light microscopy (Fig. 111), the zoospores appear elongated, 8-11µm long and 2-5µm wide, with one flagellum visible, directed anteriorly and a large eyespot at the anterior end.

The general cell structure is illustrated in Figs 112-114. The two flagella are inserted at more than ninety degrees to each other (Fig. 112); a pyriform nucleus with a nucleolus is placed between the eyespot and the chloroplast (Figs 112, 113) and a small Golgi body is usually present between the eyespot and the nucleus; the chloroplast is situated at the posterior end of the cell (Figs 112, 113), is typically eustigmatophycean in structure and never has a pyrenoid; the eyespot occupies the extreme anterior end of the cell, is composed of several lens-shaped droplets and is not membrane-bounded (Figs 113-118); the cytoplasm (Figs 112, 113) is filled with mitochondria, lamellate and spiral vesicles and two other large and osmiophilic vesicles (Fig. 114), usually seen between the chloroplast and the eyespot.

2. Flagellar apparatus

The structure of the flagellar apparatus is similar to that described for *Vischeria stellata*, with the exception that this species has two emergent flagella.

The external part of the anterior flagellum also bears mastigonemes on its surface (Figs 113, 115) and has a flagellar swelling at its base, containing dense material and in close association with the eyespot (Figs 116-118; see also Figs 127 and 131). In some sections (Figs 116-118), fibrous material seems to be present.

The external part of the second flagellum is smooth and bears no swelling (Fig. 119; see also Fig. 122); its axoneme, near the cell surface, consists of 9+2 duplet MTs (Fig. 115) but it was not possible to confirm if the flagellum width was reduced away from its base, as has been reported in all the biflagellate species (Hibberd & Leedale, 1972; Lee & Bold, 1973; Preisig & Wilhelm, 1989).

The transition region in both flagella (Figs 119-123; see also Figs 117, 125 and 129) consists of a transverse partition slightly above the cell surface and a transitional helix with 4-5 gyres surrounding the central pair of MTs.

It was not possible to reconstruct clearly the system of flagellar roots present, as the number of zoospores produced was insufficient for this type of study. However, in a few sections (Figs 116-118 and 124-132), MTs can be seen associated with the basal bodies in a way that is consistent with all the roots present in *Vischeria stellata*; in Figs 116-118, several MTs originate between both basal

bodies and run posteriorly, in a similar way to root R3; also some MTs originate at both sides of basal body B1 and run anteriorly around the flagellar swelling (Figs 124, 125-130; see also Figs 131, 132), possibly equivalent to roots R1 and R2. Another microtubular root originates at basal body B2 (see one MT in Figs 126- 128) and is possibly root R4, as described before.

Cross-banded fibrous material was also observed close to the basal bodies (Figs 117, 118) and the nuclear surface (Fig. 124).

Figs 111-118. Zoospores of *Pseudocharaciopsis minuta*.

Fig. 111. Light microscopy. Anoptral contrast. x 2500.

Figs 112, 113. Longitudinal sections, showing the general cell structure. Fig. 112 x 25000; Fig. 113 x 23000.

Fig. 114. Section showing the two dense vesicles between the eyespot and the chloroplast. x 31500.

Figs 115-118. Four selected longitudinal sections of a series through the anterior end of the cell, to show the transition region of the long flagellum and the flagellar swelling.

Fig. 115. Note the posterior flagellum in transverse section and the mastigonemes on the surface of the anterior flagellum. x 35000.

Figs 116-118. Note the fibrous material in the flagellar swelling and root R3; note also the transitional helix in Fig. 117 (large arrow) and a possible fibre of the rhizoplast in Figs 117 and 118 (small arrow). Figs 116 & 118 x 42000; Fig. 117 x 36000.

Abbreviations used in figures: C, chloroplast; DV, dense vesicle; E, eyespot; F1, anterior flagellum; F2, posterior flagellum; FS, flagellar swelling; LV, lamellate vesicle; M, mastigoneme; N, nucleus; R3, microtubular root; SV, spiral vesicle.

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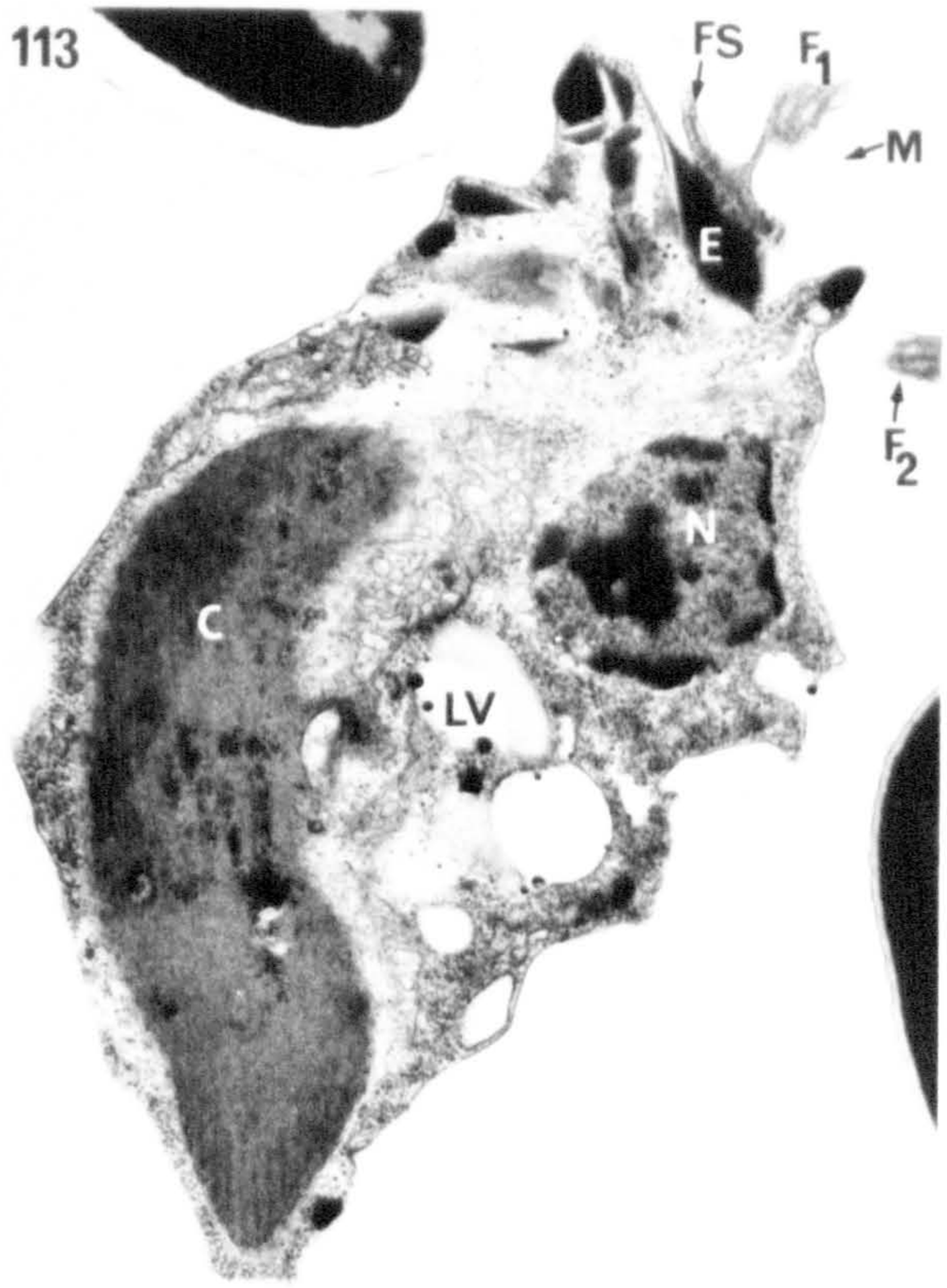
113



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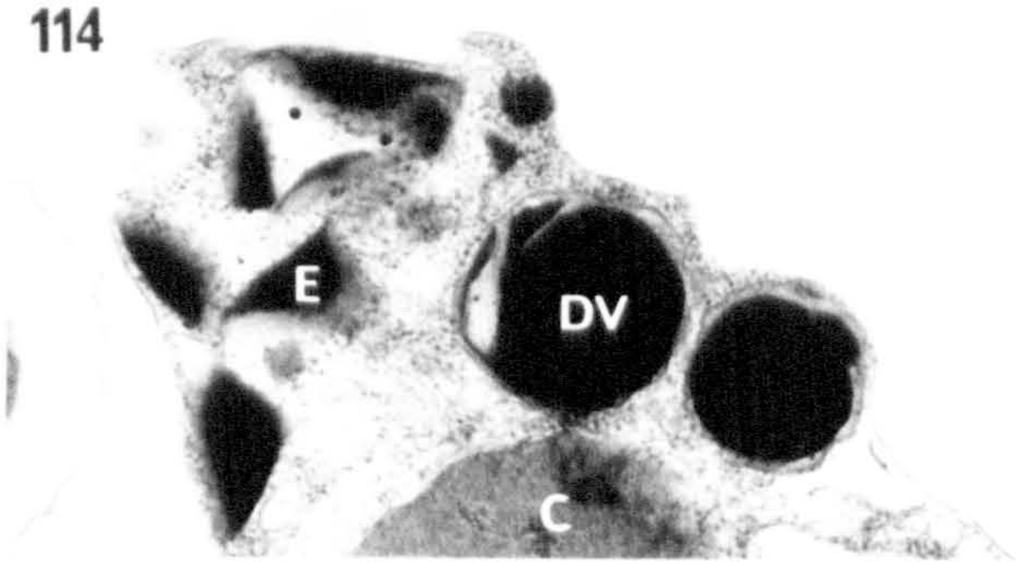

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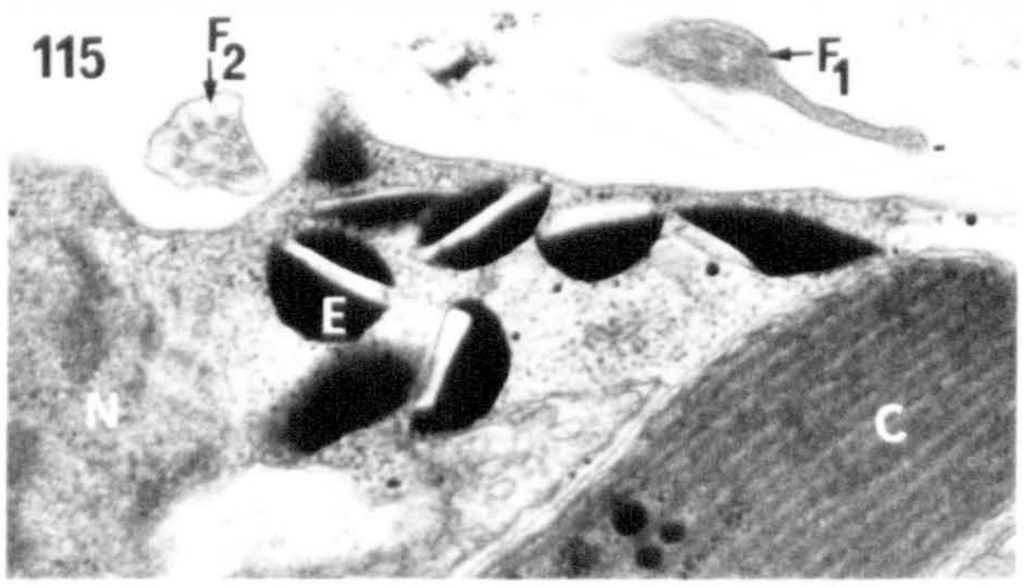

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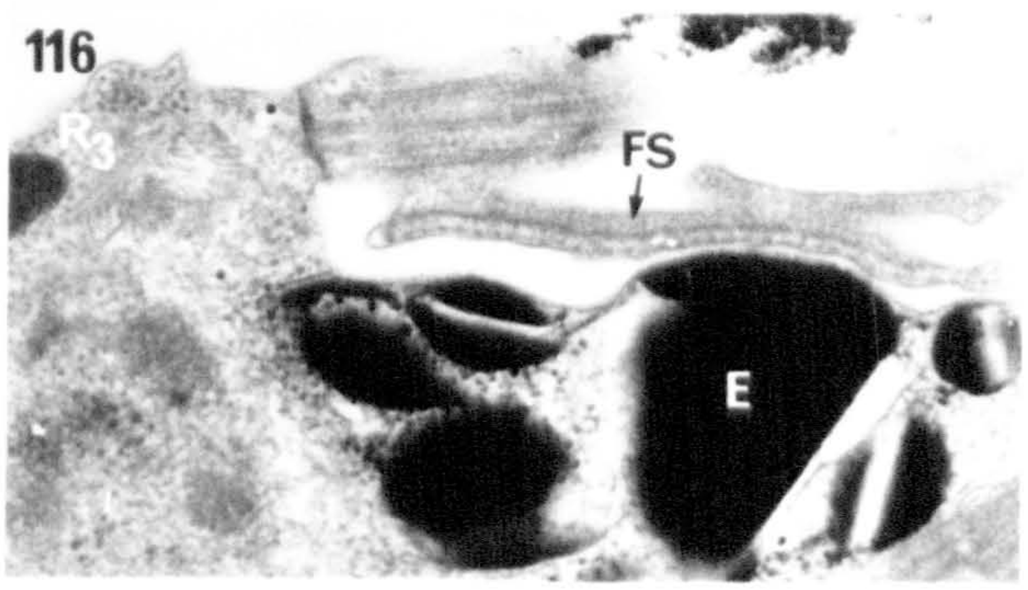

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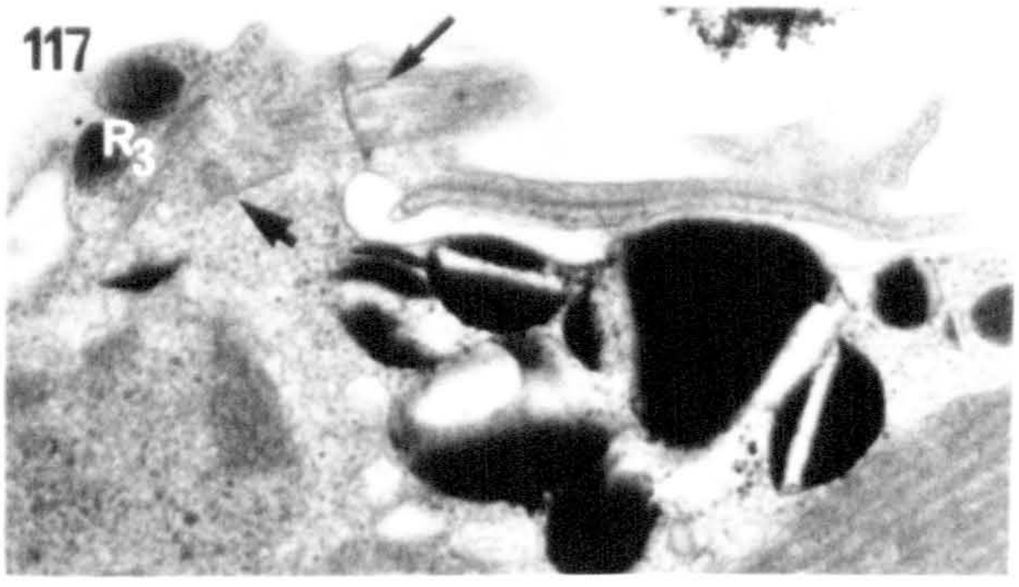

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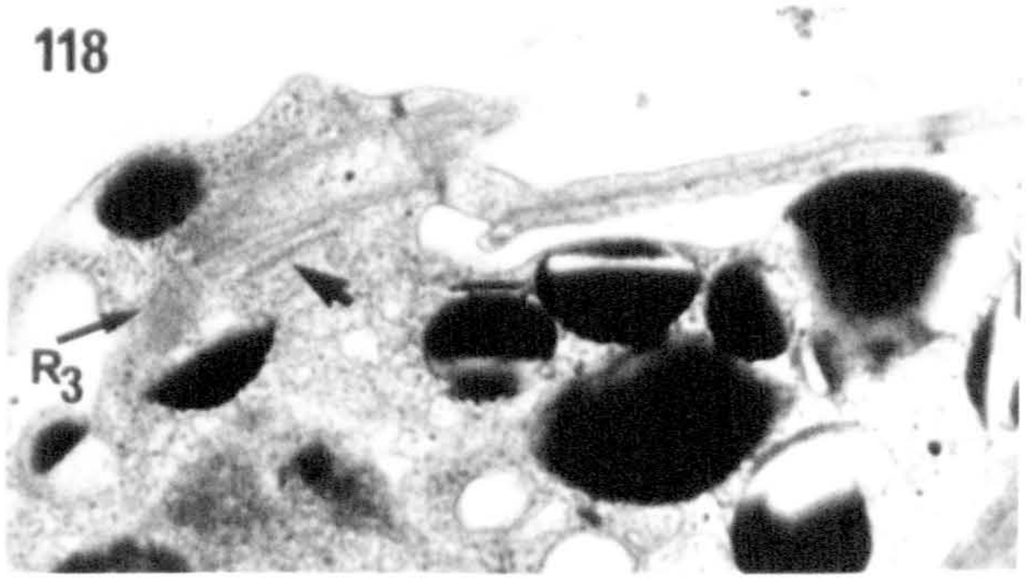


Figs 119-132. Zoospores of *Pseudocharaciopsis minuta*. Selected sections of different series, to show details of the flagellar apparatus.

Figs 119-121. Selected sections of a series showing the transition region of both flagella and the two basal bodies. Note the presence of a transitional helix in both flagella (arrows). Fig. 119 x 36000; Fig. 120 x 42000; Fig. 121 x 45000.

Figs 122, 123. Transition region in the posterior flagellum, in longitudinal and transverse section. Note the number of gyres in the helix (arrow). Fig. 122 x 42000; Fig. 123 x 55000.

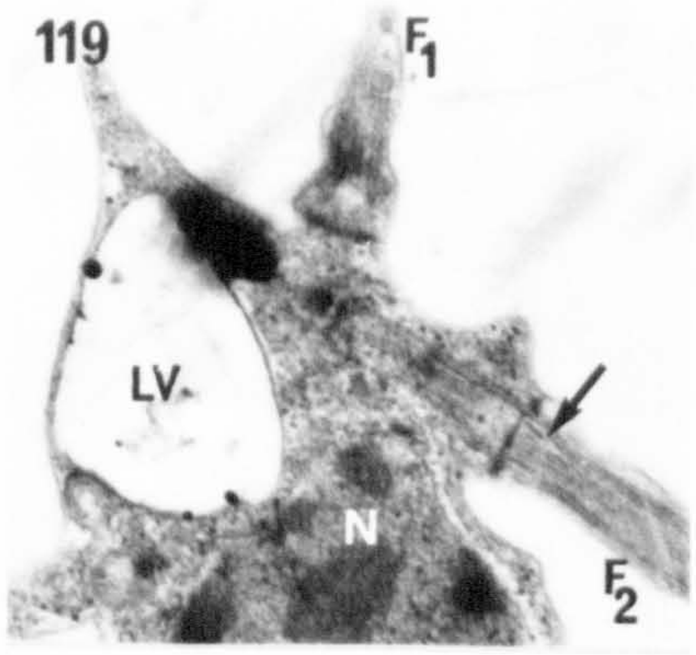
Fig. 124. Longitudinal section showing basal body B1 with associated root R1 and a fibre (arrow) close to the nucleus. x 50000.

Figs 125-130. Selected oblique sections of a series, to show the transition region of both flagella, the two basal bodies, the microtubular roots and their relation to the basal bodies and/or to the flagellar swelling. Note the number of gyres of the transitional helix in the posterior flagellum (arrow, Fig. 125). Figs 125 & 127 x 39000; Figs 126 & 128 x 30000; Fig. 129 x 45000; Fig. 130 x 50000.

Figs 131, 132. Flagellar swelling in two consecutive oblique sections. Note the MTs of root R1, passing around it (arrows), its position in relation to the eyespot and other cell components. x 30000.

Abbreviations used in figures: DV, dense vesicle; E, eyespot; F1, anterior flagellum; F2, posterior flagellum; FS, flagellar swelling; LV, lamellate vesicle; N, nucleus; R1, R2, R3, R4, microtubular roots.

119



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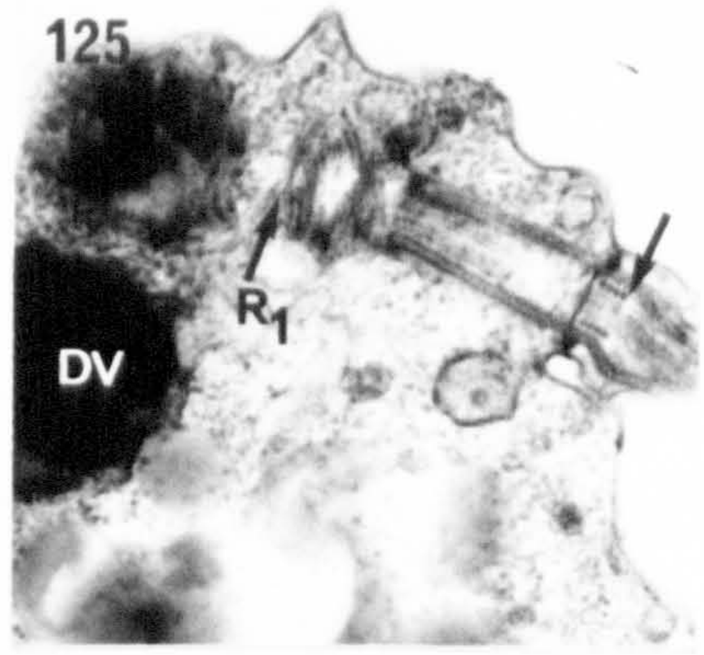

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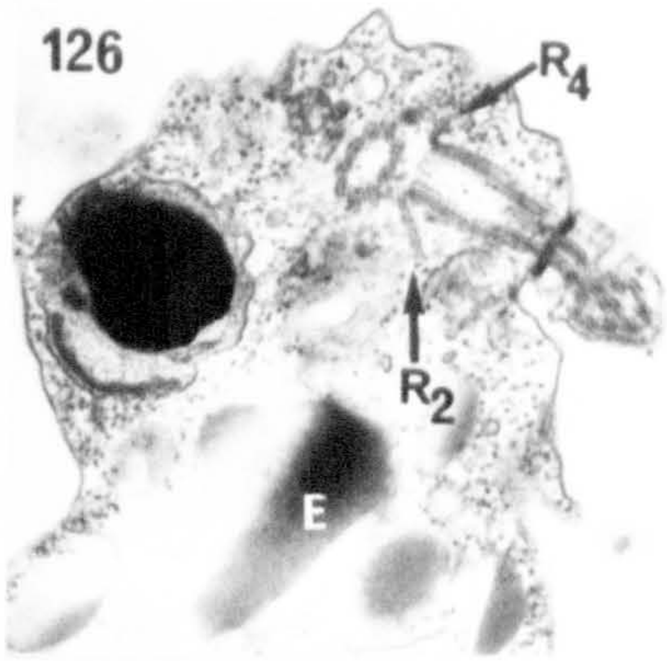

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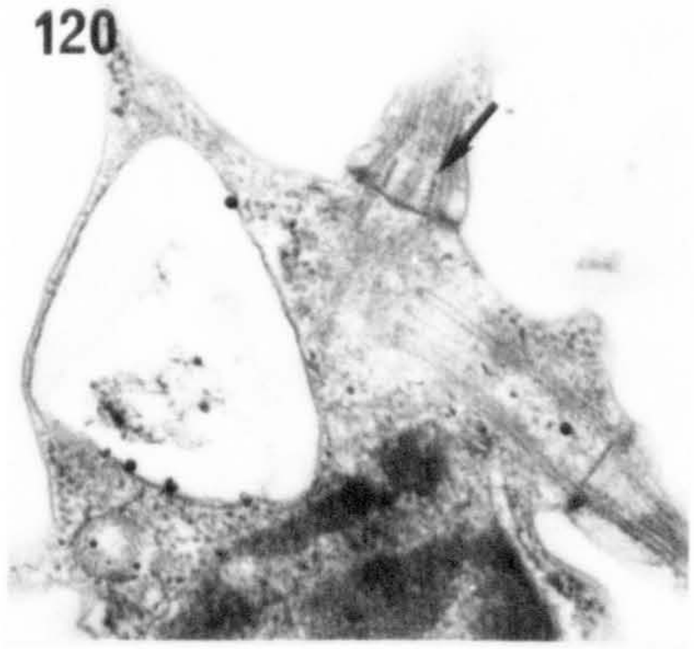

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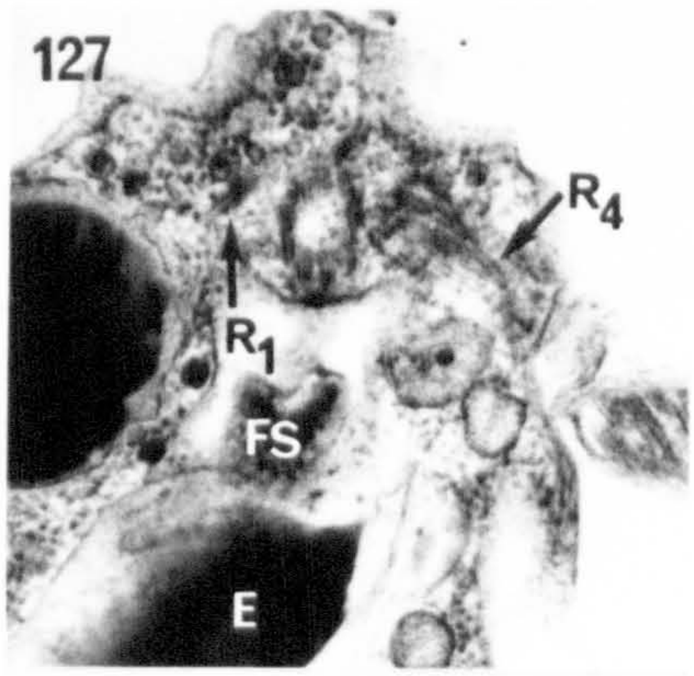

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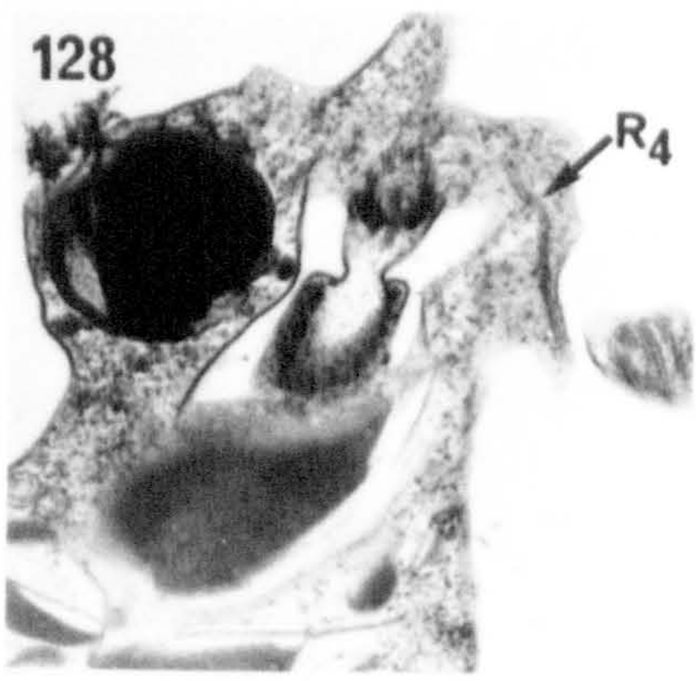

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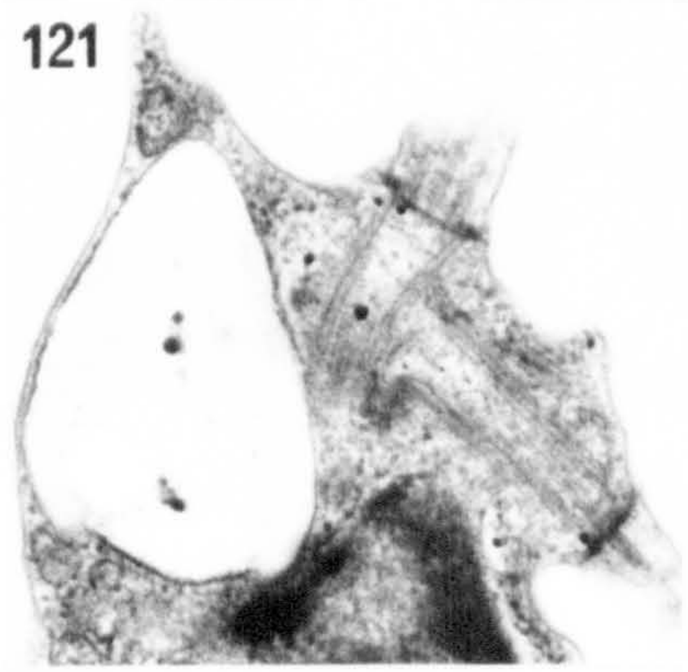

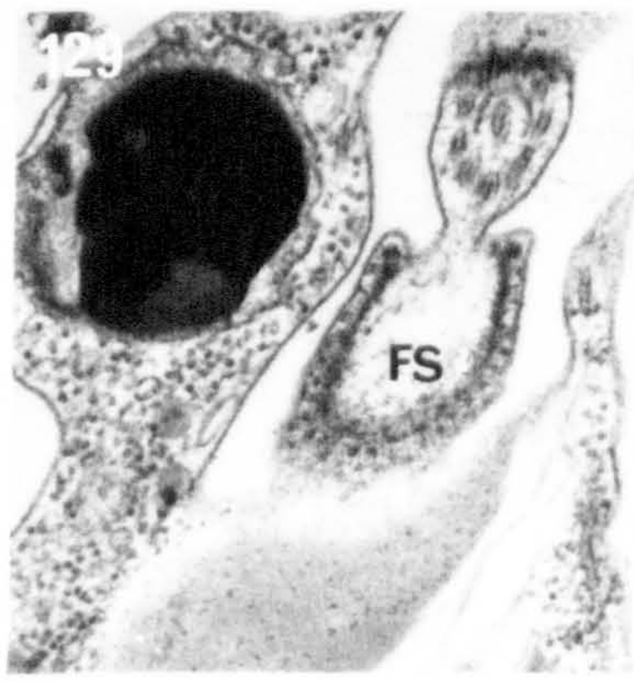

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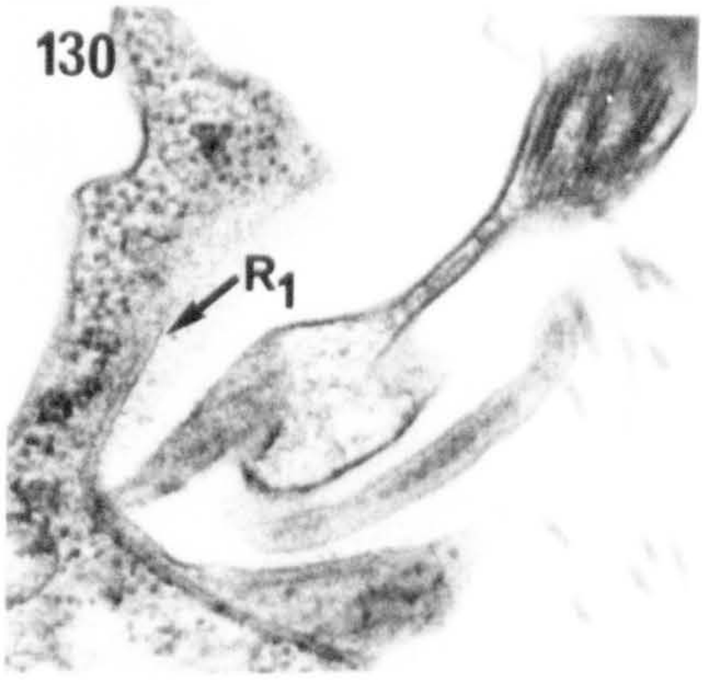

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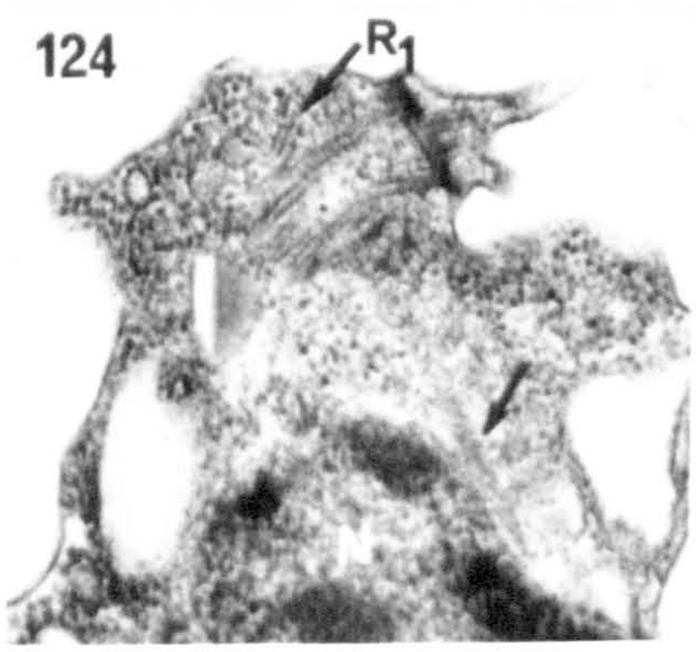

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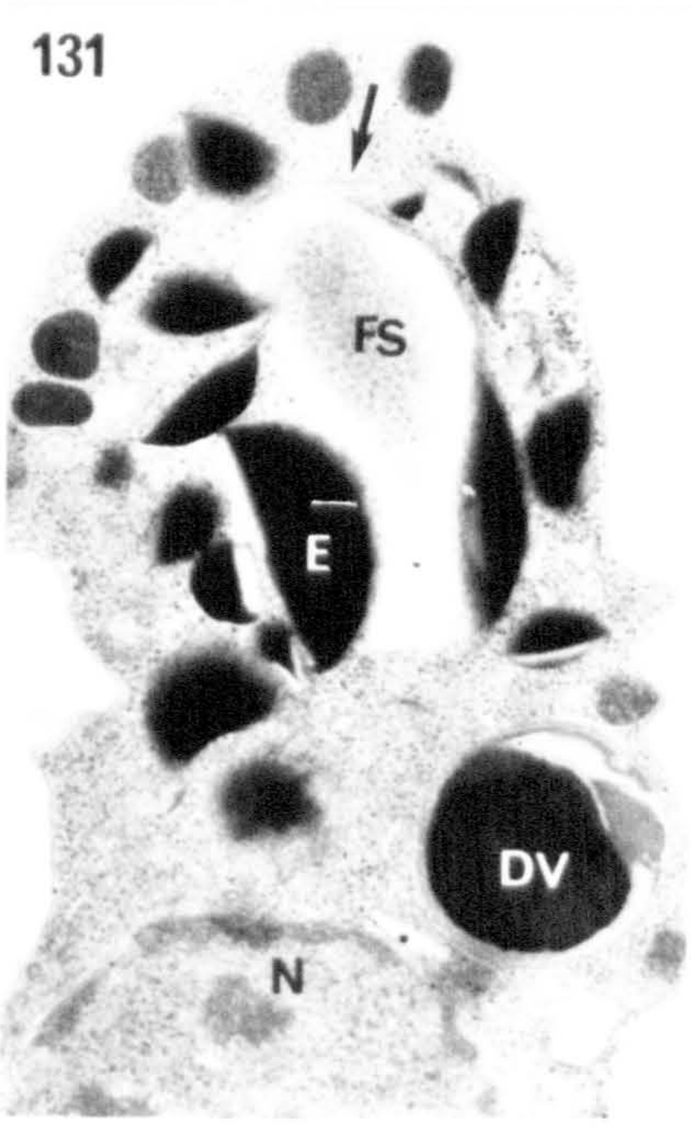


132

E. STRUCTURE OF THE ZOOSPORES IN THE TRIBOPHYCEAN SPECIES *HETEROCOCCUS MARIETANII* AND *H. PROTONEMATOIDES*

The results presented here confirm previous observations on these two species (Massalski, 1969) and add new information on the flagellar apparatus. Both species are very similar and detailed descriptions and illustrations are given for *H. marietanii* (Figs 133-164); the same ultrastructural aspects in *H. protonematoides* are confined to the legends of the corresponding figures (Figs 165-171).

1. General

The general structure of the zoospores is illustrated in Figs 133-135. They are elongated cells with two flagella inserted at an obtuse angle to each other, one directed anteriorly and the other posteriorly; the nucleus is situated at the anterior end of the cell, lateral to the chloroplast and a narrow projection is directed towards the basal bodies; a single Golgi body can be seen close to this projection (Fig. 134); a single chloroplast, with a girdle lamella and surrounded by CER is always connected to the nuclear envelope (see Fig. 134); an eyespot lies at the anterior end of the chloroplast, opposite to the flagellar swelling on the short flagellum (see Figs 133b, 134) and consists of a layer of osmiophilic globules immediatly below the chloroplast envelope (Fig. 135); in the cytoplasm, together with mitochondrial profiles and large osmiophilic globules, a contractile vacuole is always present near the

basal bodies (Fig. 134; see also Figs 136-138); mastigoneme-containing cisternae, other vacuoles and microbody-like structures (see Figs 155, 165) are also frequent. A pyrenoid was not clearly observed; however, some thylakoid-free regions are present in the chloroplasts (arrow in Fig. 135) that could be interpreted as a pyrenoid.

2. External flagellum, transition region and basal bodies

The anterior flagellum (Fig. 133) bears mastigonemes and no swelling on its surface while the free part of the second flagellum (Figs 133, 134, 136) is devoid of mastigonemes, has a pronounced swelling with dense contents closely pressed to the cell surface in the region of the eyespot (see also Fig. 140) and terminates in a hair-point (arrow in Fig. 133b). The standard configuration of 9+2 duplet MTs is present in the axoneme (*e.g.* Fig. 140).

The transition region of each flagellum (Figs 137-142) consists of a transverse partition at the cell surface level (Figs 137-139) and a double helix of 4-6 gyres and different apertures that lies above the transverse partition and inside the peripheral duplets (Figs 138, 139, 141). Transitional fibres connect the axonemal duplets with the plasmalemma at the point of entry into the cell (Fig. 142).

The basal bodies diverge from each other at an obtuse angle (see Figs 137-139), consist of the usual nine triplet MTs and are also connected to the plasmalemma by faint fibrous material (Fig. 143). Cartwheel structures were not observed but dense spheres of fibrous material are always

Figs 133-145. Zoospores of *Heterococcus marietanii*.

Fig. 133. Longitudinal section of the zoospore, showing its general structure (a) and the posterior flagellum (b). Note the flagellar swelling and the hair point of the posterior flagellum (arrow). a,b x 20000.

Fig. 134. Section showing the position of the Golgi body and the eyespot in relation to the nucleus and the flagellar swelling. Note the connection between the CER and the nuclear envelope (arrow). x 32000.

Fig. 135. Note the "thylakoid-free outpocketing" in the chloroplast (arrow). x 40000.

Figs 136-139. Longitudinal sections of the bases of both flagella showing the transition region and the basal bodies. Note the dense material between both basal bodies and inside them (small arrows), the number of gyres in the transitional helices (long arrows) and roots R1 and R3. Figs 136-138 x 32000; Fig. 139 x 40000.

Fig. 140. Transverse section of the axonemal duplets and the flagellar swelling. x 64000.

Figs 141-145. Five selected transverse sections of two different series (Figs 141-144 and Fig. 145) through the anterior end of the cell, to show the transition region of the flagellum and the two basal bodies. x 64000.

Fig. 141. Axonemal duplets and transitional helices.

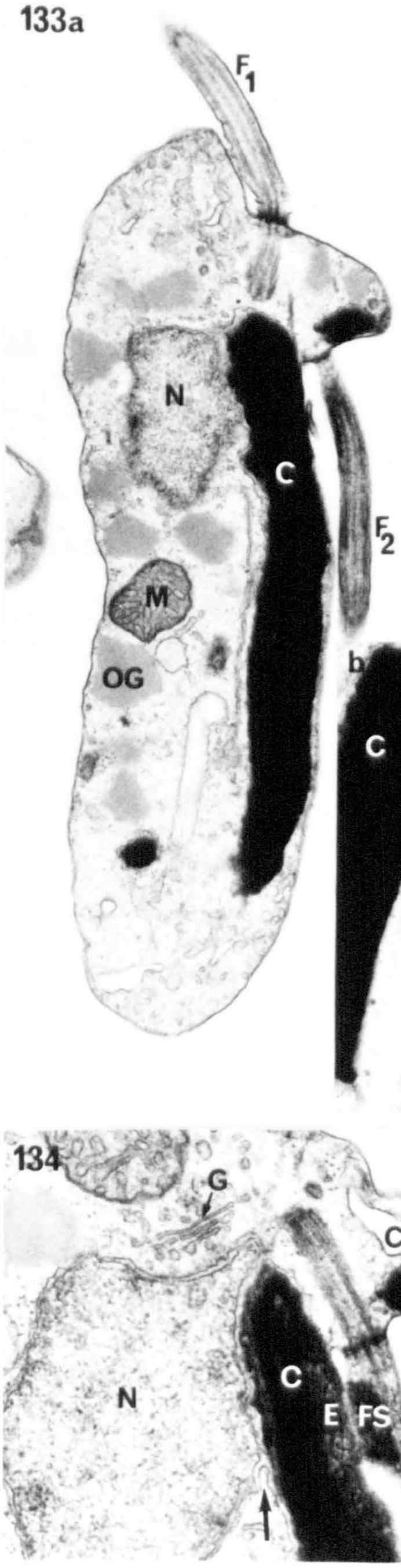
Fig. 142. Transitional fibres connecting duplets with the plasmalemma (arrows).

Fig. 143, 144. Triplet MTs in basal body B1. Note transitional fibres (arrows in Fig. 143) and sphere of dense material inside the basal body (Fig. 144).

Fig. 145. Basal bodies. Note spheres of dense material inside them.

Abbreviations used in figures: C, chloroplast; CV, contractile vacuole; E, eyespot; F1, anterior flagellum; F2, posterior flagellum; FS, flagellar swelling; G, golgi body; M, mitochondrion; N, nucleus; OG, osmiophilic globule; R1 and R3, microtubular roots.

133a



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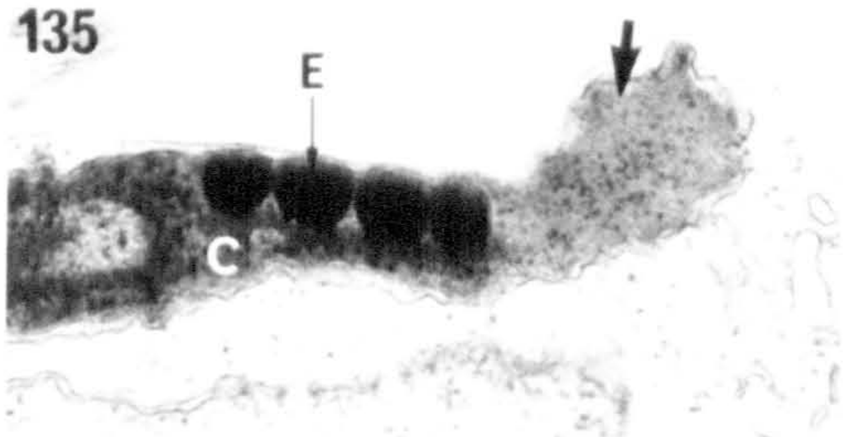

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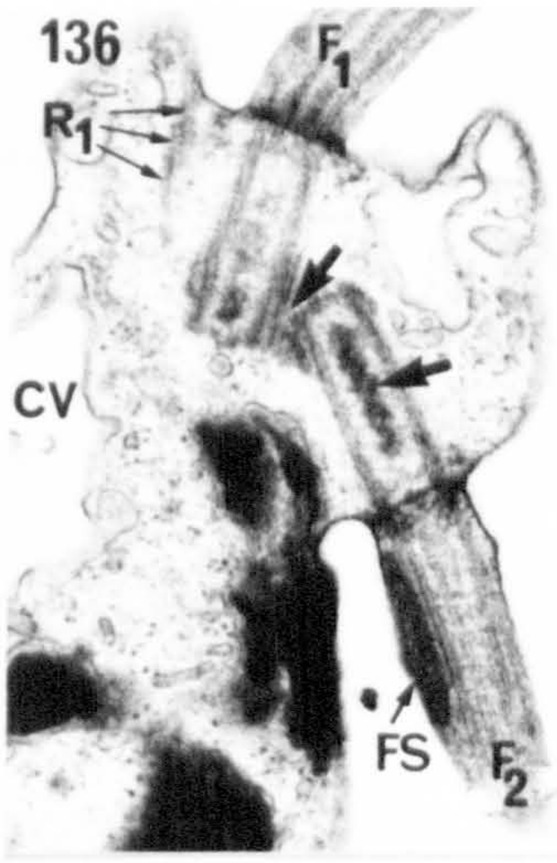

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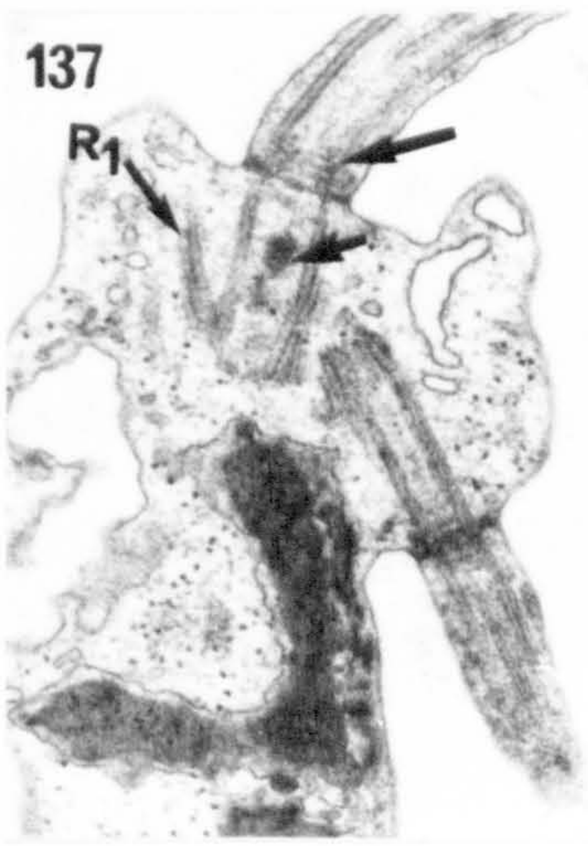

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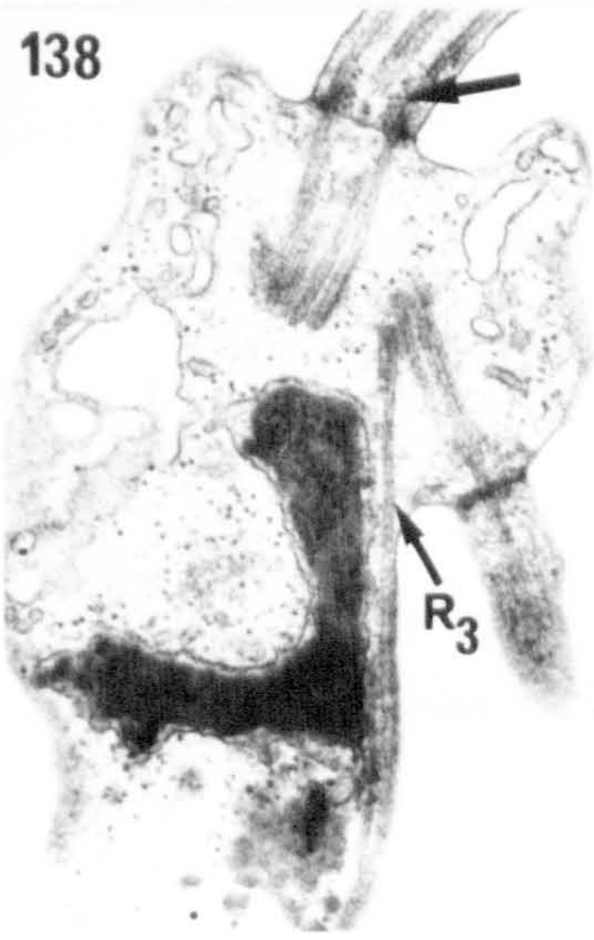

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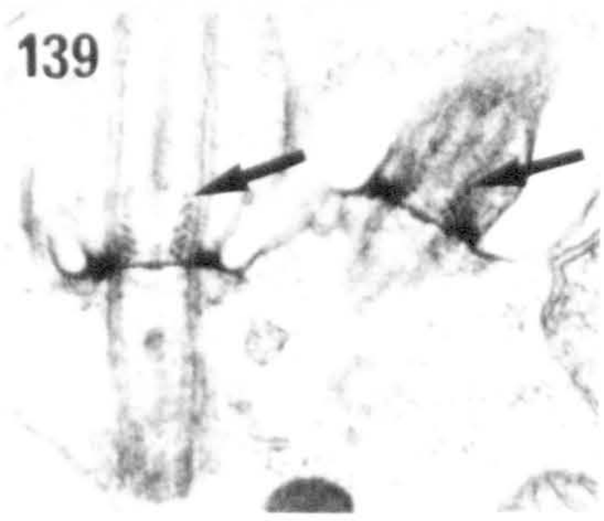

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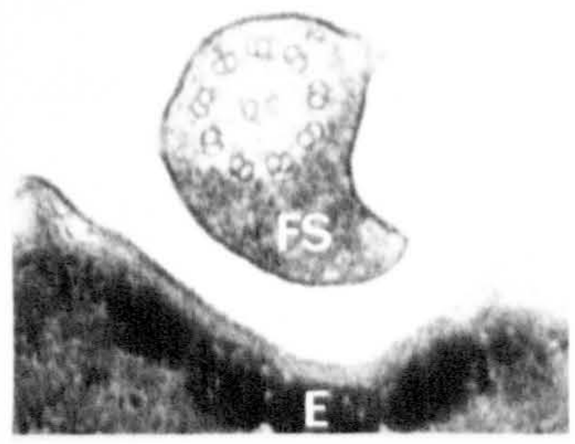

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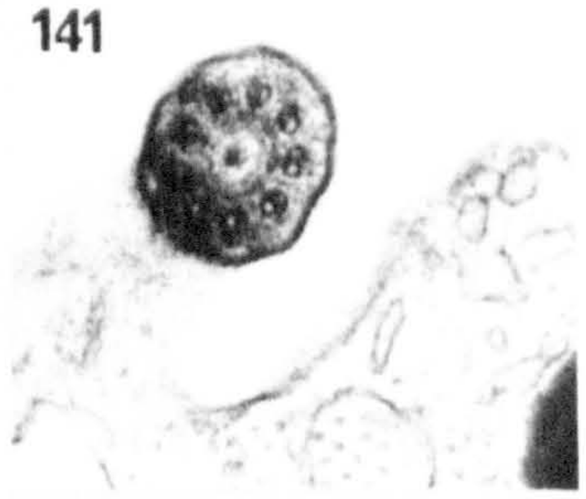

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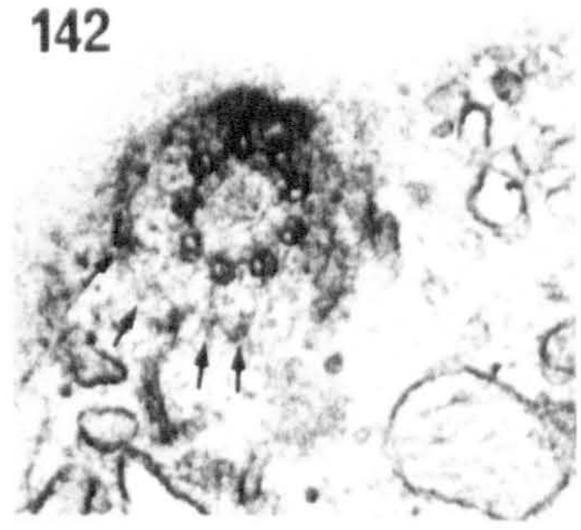

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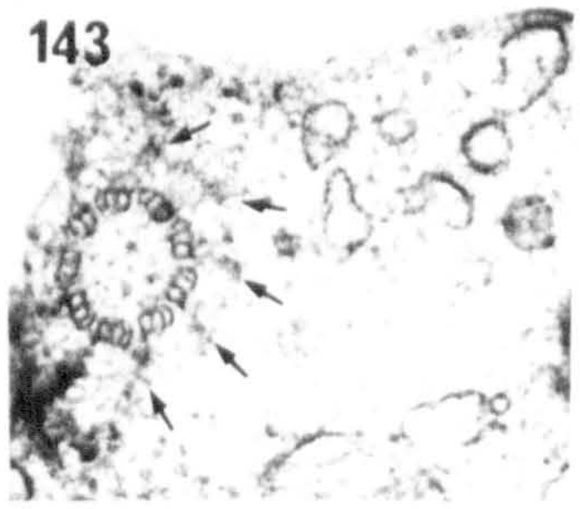

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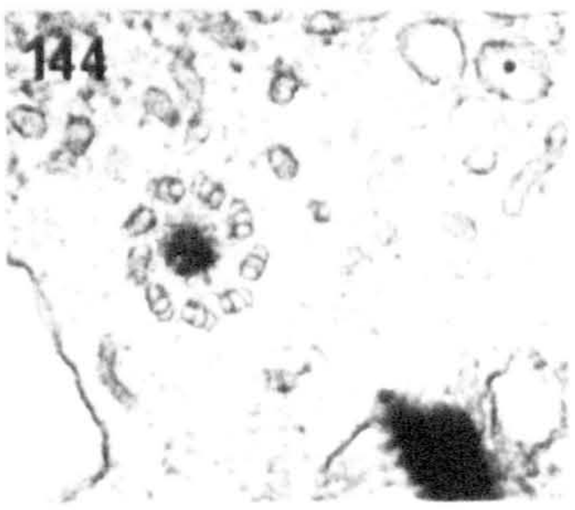

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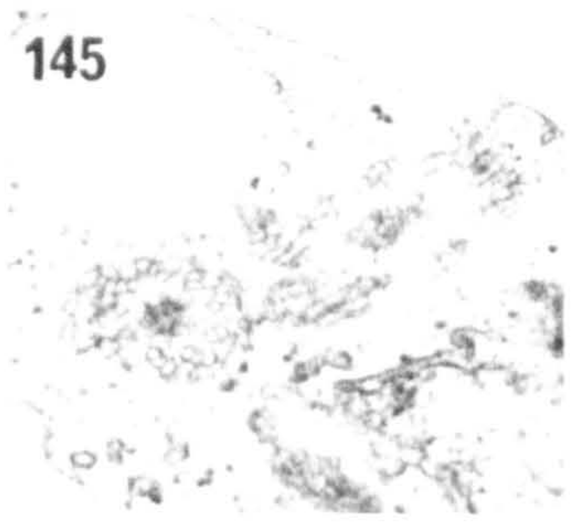


present in the centre of the basal bodies (Figs 144, 145), in some cases partly fused into a beaded rod or zigzag structure (Fig. 136). Dense structures appear to connect both basal bodies in some sections (see Figs 163, 164).

3. Flagellar roots

A system of three microtubular roots (R1-R3) and a small rhizoplast is present in these zoospores and diagrammatically illustrated in Fig. 146.

Root R1 (Figs 147-151; see also Figs 136, 137) originates at the basal body of the anterior flagellum (B1) and runs anteriorly in a clockwise direction, close to the plasmalemma and around the flagellar groove; it apparently consists of 3 MTs (Fig. 136) and originates cytoskeletal MTs (arrows in Figs 147-151).

Root R2 (Figs 147, 150) originates from the opposite side of basal body B1 and also runs anteriorly, close to the plasmalemma but in an anticlockwise direction to meet root R1 at some point. The number of MTs was not determined.

Finally, root R3 (Figs 152-154; see also Figs 138, 147, 149 and 151) originates at basal body B2 and runs posteriorly, between the chloroplast and the plasmalemma. It consists initially of 2 MTs but one terminates at a shorter distance than the other (see Figs 152-154).

A small rhizoplast is present in these zoospores. In some sections, dense striated material connects the basal bodies with the nuclear surface (Figs 155-156; see also Fig. 162); other fibrous material is present at the anterior

end of the nucleus, originating at the basal bodies (Figs 157, 158-161); dense material also appears to connect both basal bodies in some sections (Figs 163-164), as previously mentioned. It was not possible to determine if all these structures are part of a rhizoplast, either very short and terminating on the anterior projection of the nucleus or running deeper into the cell between the nucleus and the Golgi body (see also Figs 168, 169).

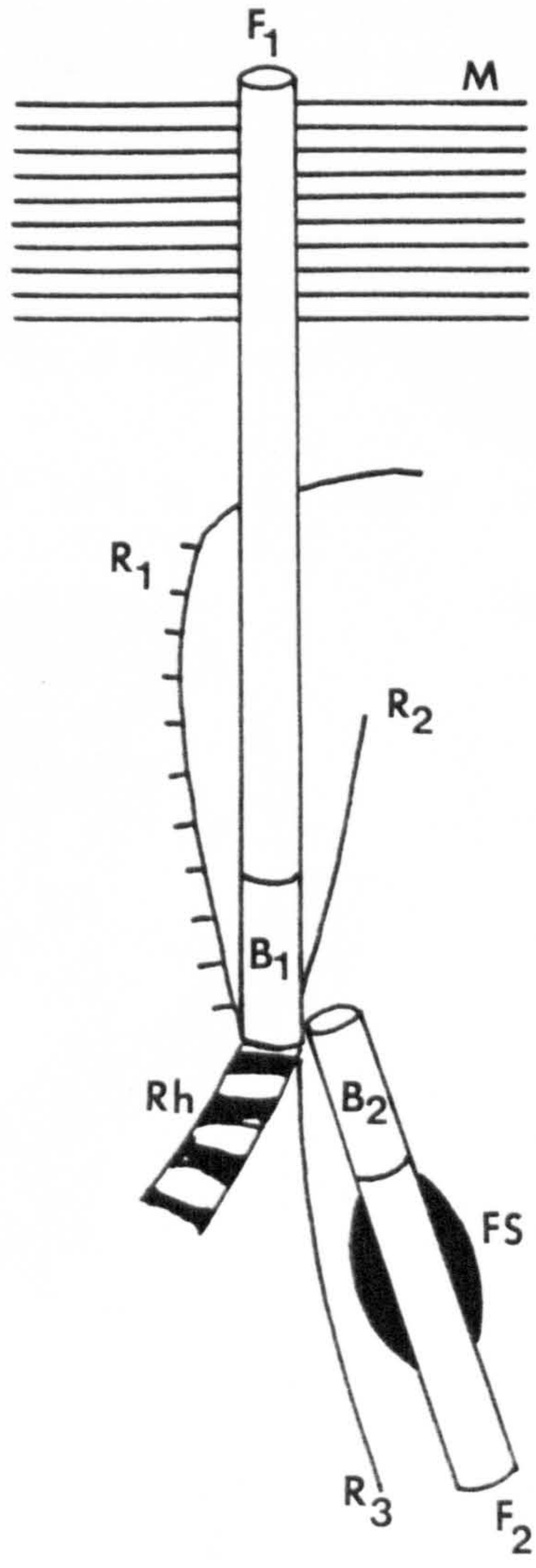


Fig. 146. Schematic drawing showing the relative positions of basal bodies, flagellar roots and swelling in zoospores of the tribophycean species *Heterococcus marietanii*. View from the ventral to the dorsal side of the zoospores. (Adapted from Andersen's drawing, 1989a). B1, basal body of the anterior flagellum; B2, basal body of the posterior flagellum; F1, anterior flagellum; F2, posterior flagellum; FS, flagellar swelling; M, mastigonemes; R1, R2, and R3, microtubular roots; Rh, rhizoplast.

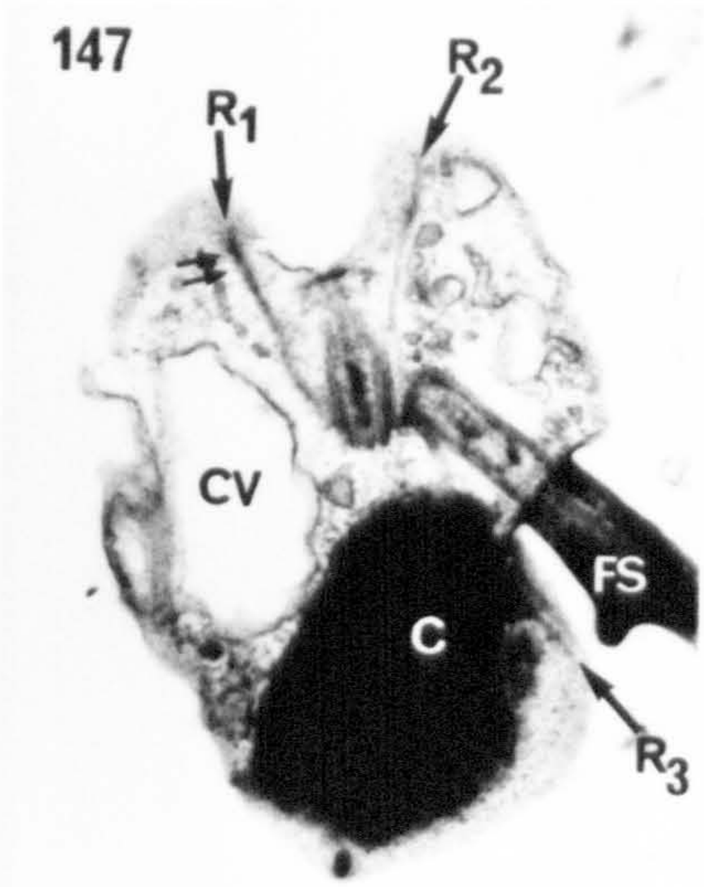
Figs 147-154. *Heterococcus marietanii*. Selected longitudinal sections of the zoospores, showing the different microtubular roots.

Fig. 147-151. Roots R1, R2 and R3 shown in relation to the basal bodies. Note cytoplasmic MTs originating from root R1 (arrows). Figs 147 & 148 x 24000; Fig. 149 x 48000; Figs 150 & 151 x 32000.

Fig. 152-154. Root R3, ditto. Figs 152 & 153 x 46000; Fig. 154 x 20000.

Abbreviations used in figures: C, chloroplast; CV, contractile vacuole; E, eyespot; FS, flagellar swelling; N, nucleus.

147



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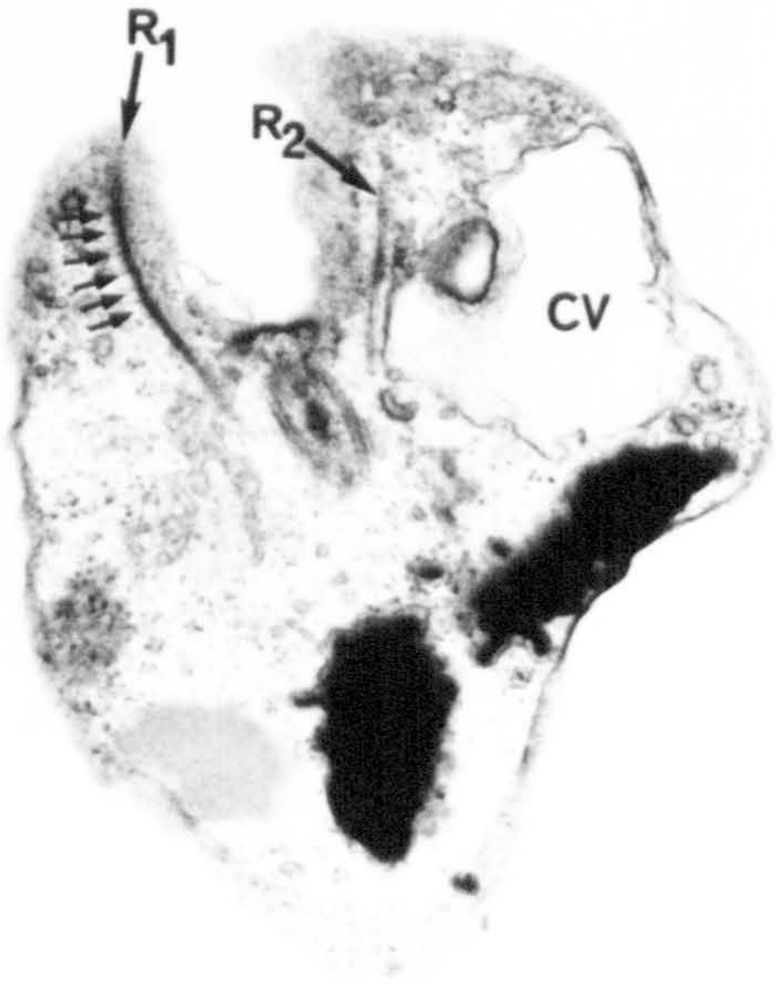

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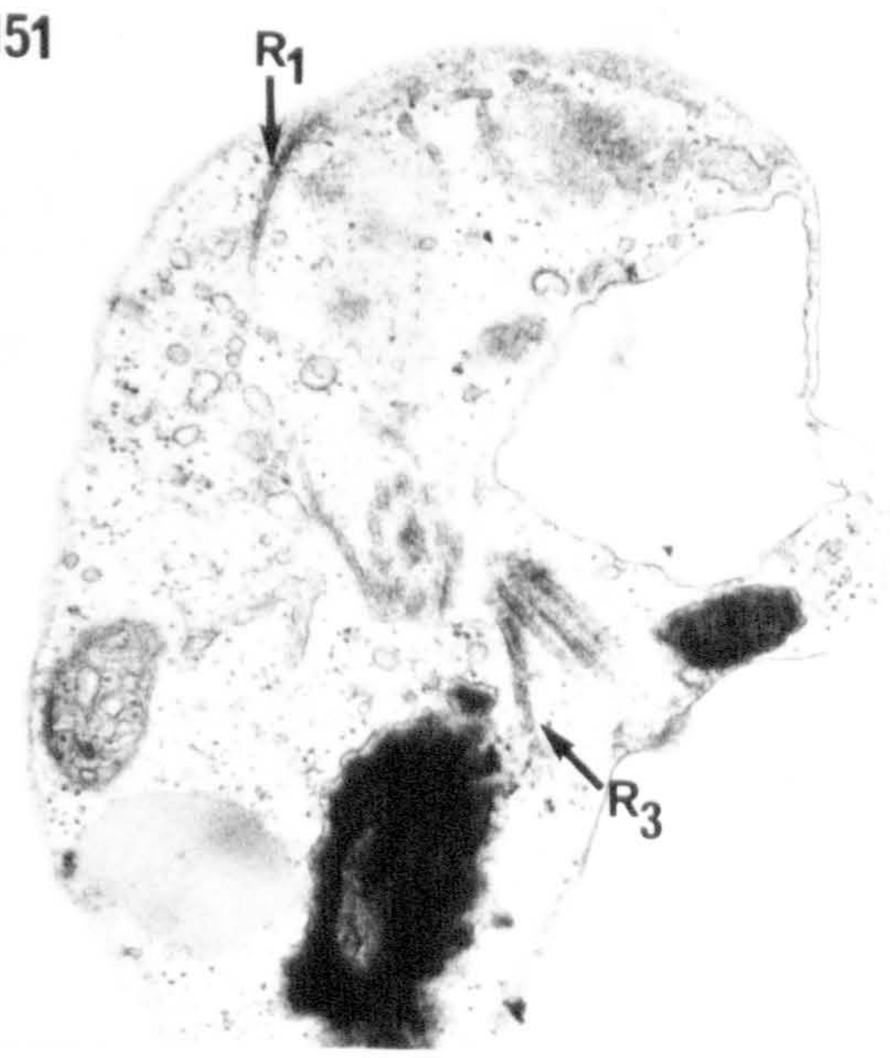

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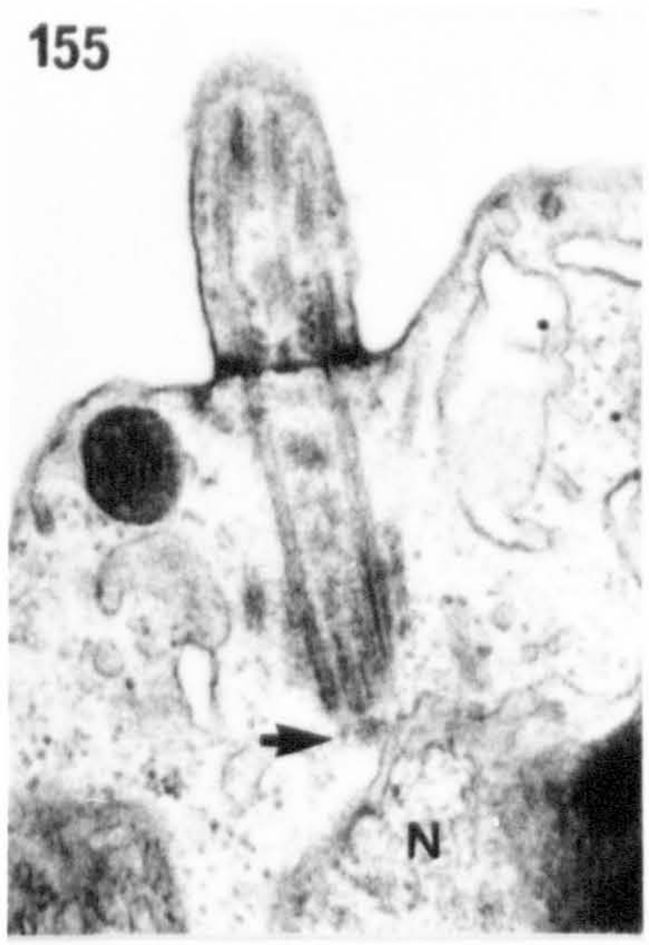
Figs 155-164. *Heterococcus marietanii*. Selected longitudinal sections showing a small rhizoplast.

Fig. 155. Striated fibrous material (arrows) connects basal body B1 with the nucleus (N). x 50000.

Fig. 156-164. Sections of different series showing striated fibrous material connecting basal body B2 with the nucleus (N) or close to the nucleus surface (small arrows) and dense, arc-shaped material between both basal bodies (large arrows). Figs 156,157,159 & 160 x 47500; Fig. 158 x 38000; Fig. 161 x 50000; Figs 162 & 163 x 42500; Fig. 164 x 68000.

155



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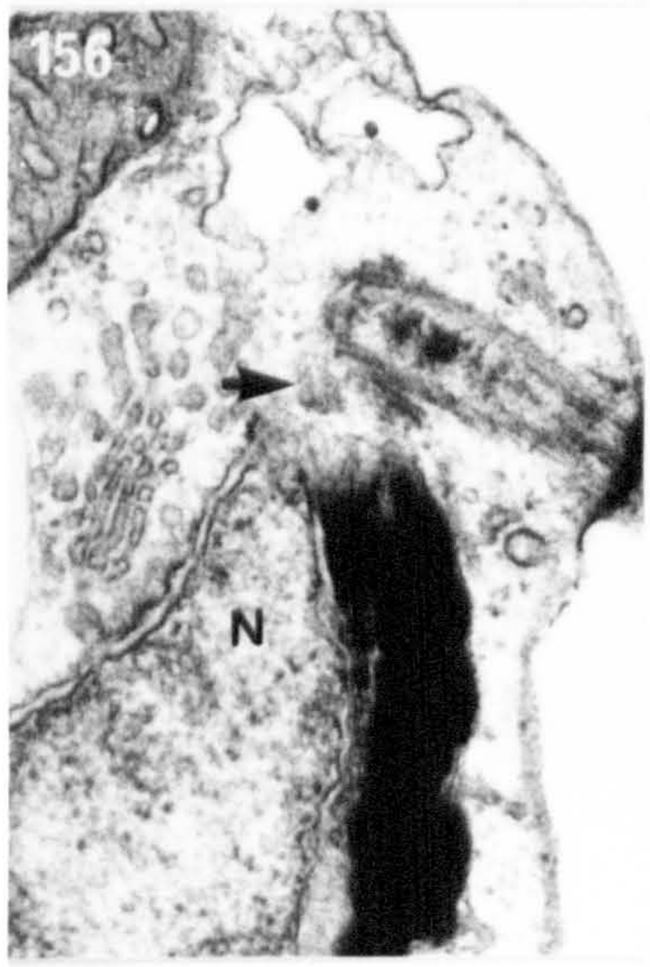

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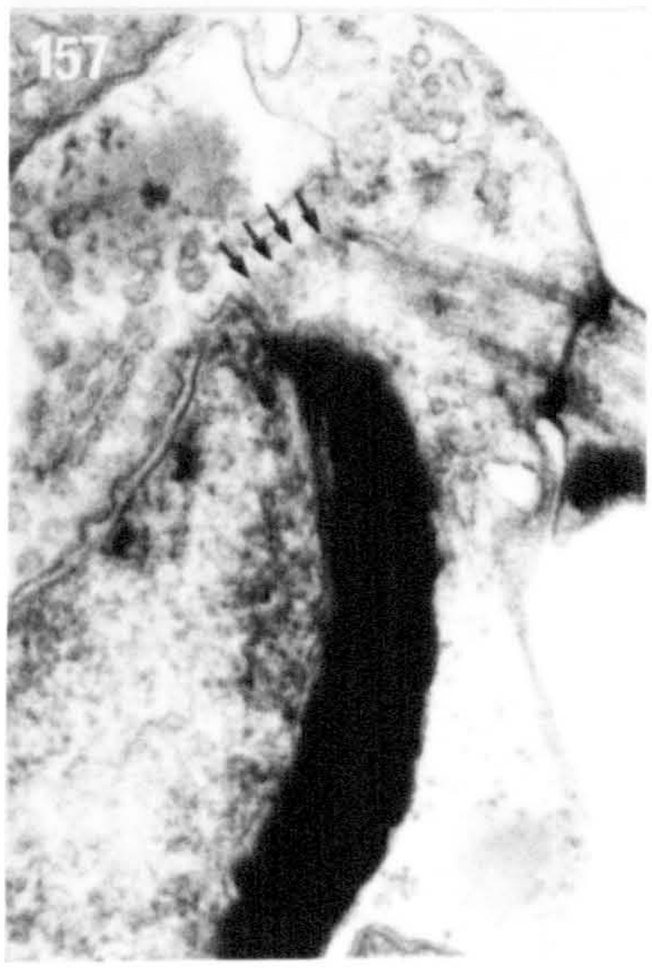

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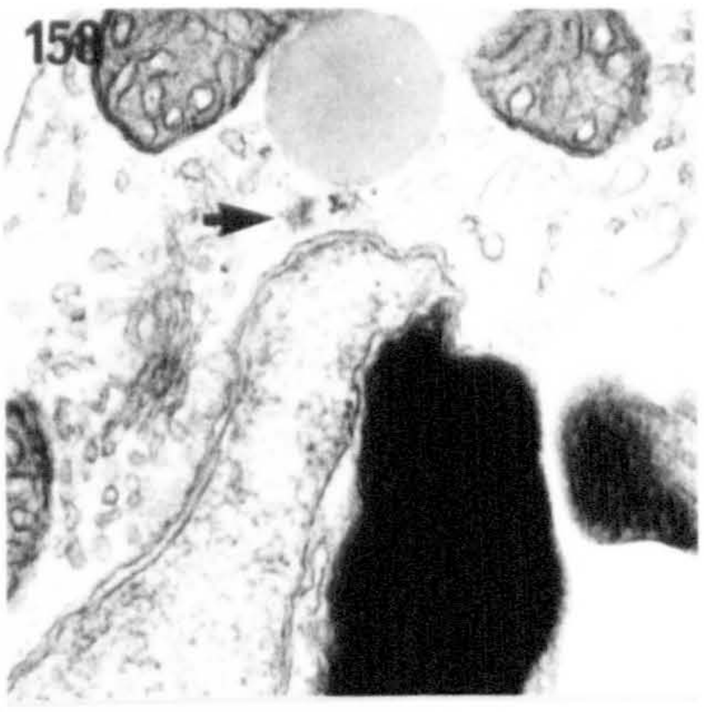

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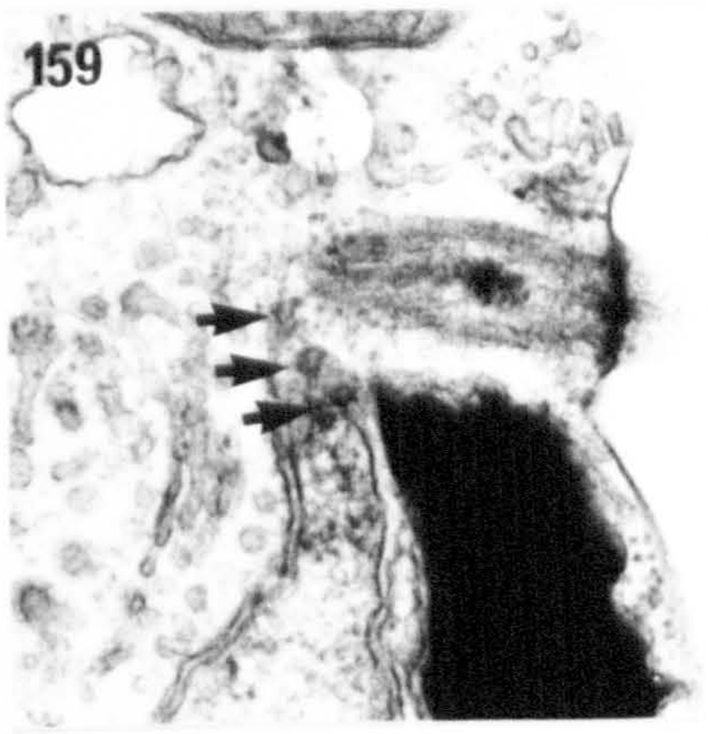

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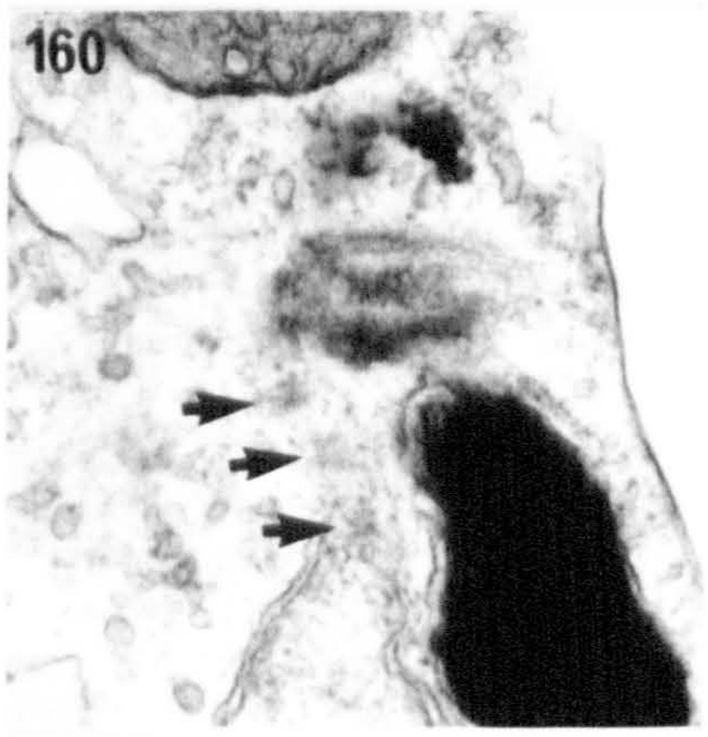

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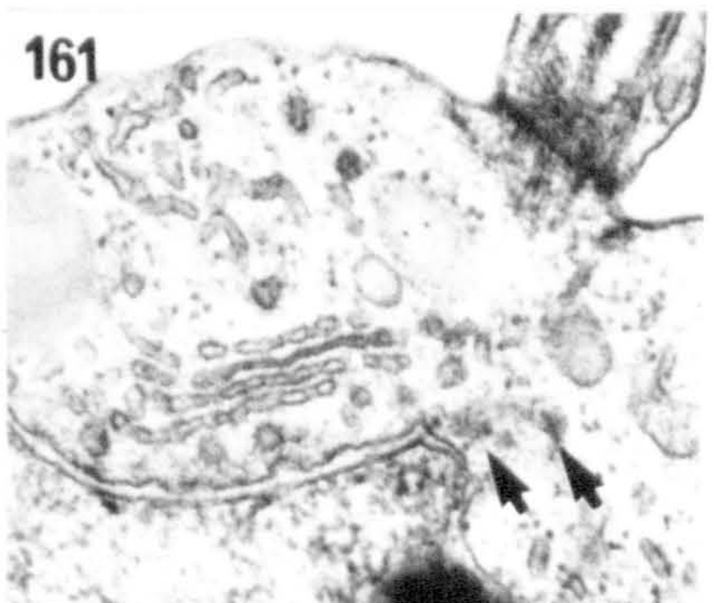

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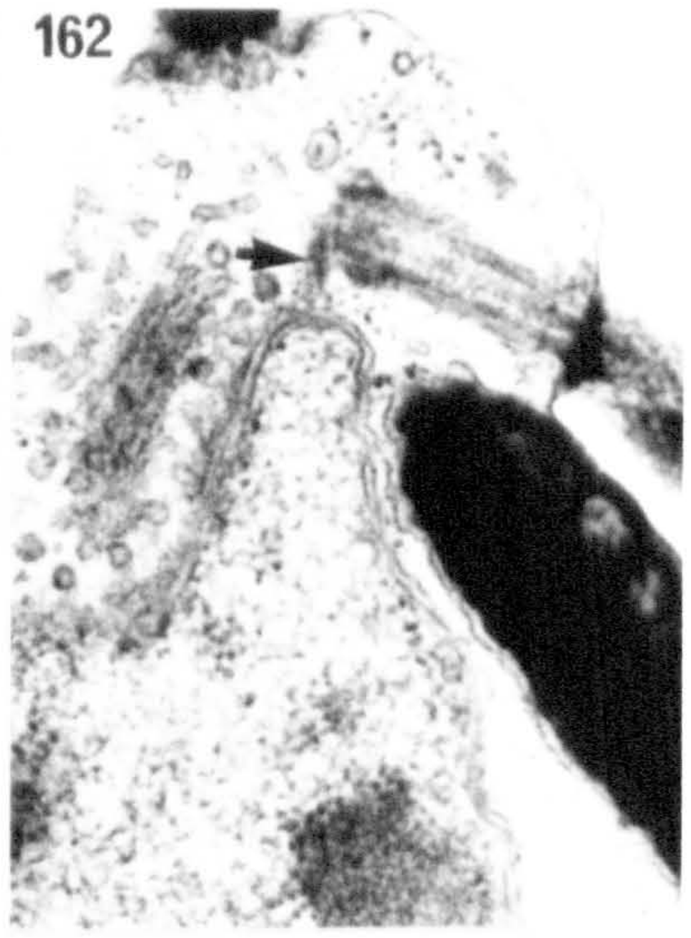

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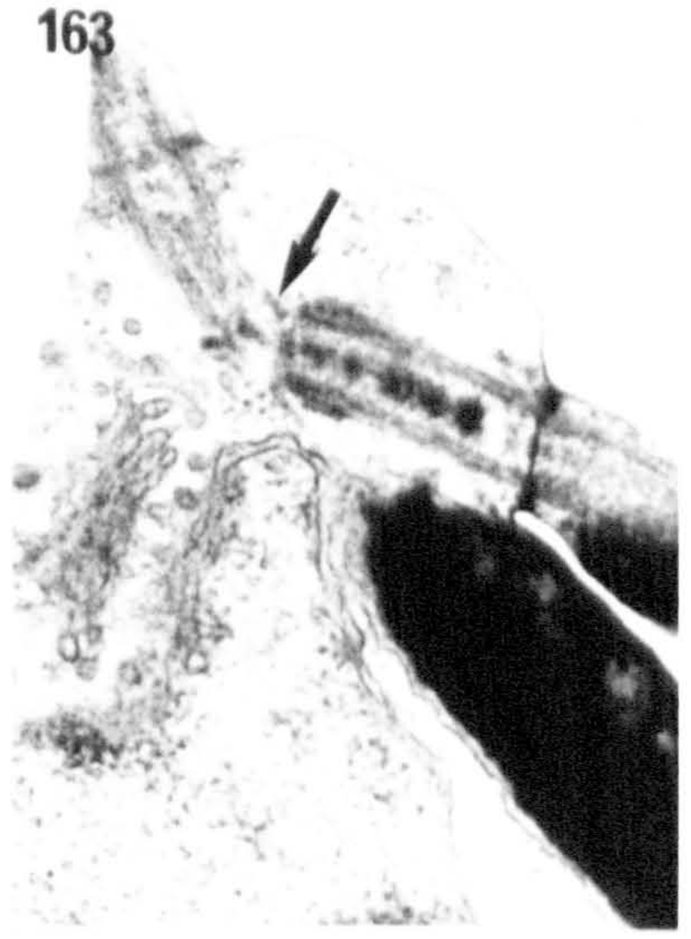

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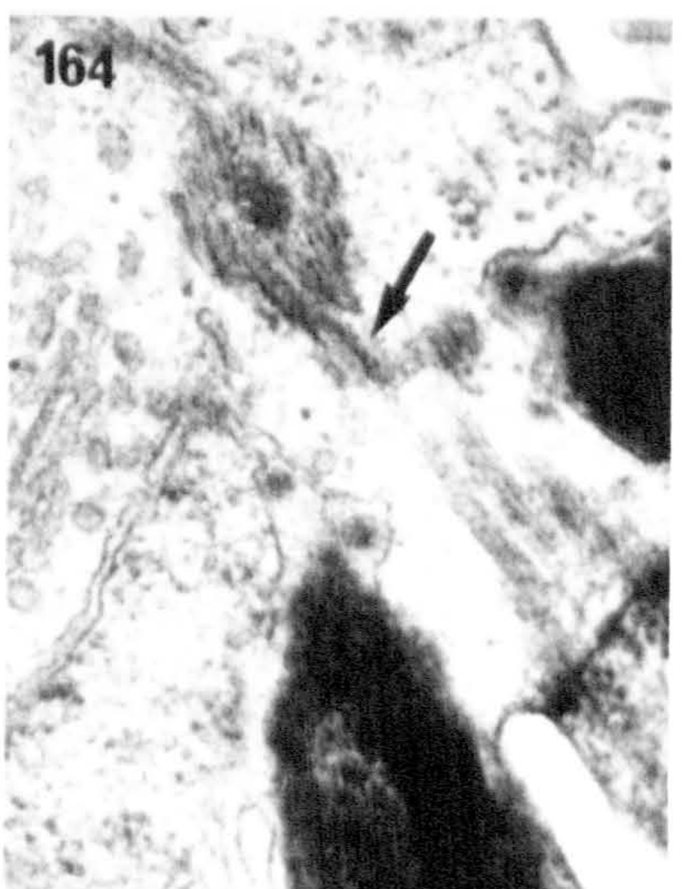


Figs 165-171. Zoospores of *Heterococcus protonematoides*.

Fig. 165. Longitudinal section of the zoospore, showing its general structure. x 20000.

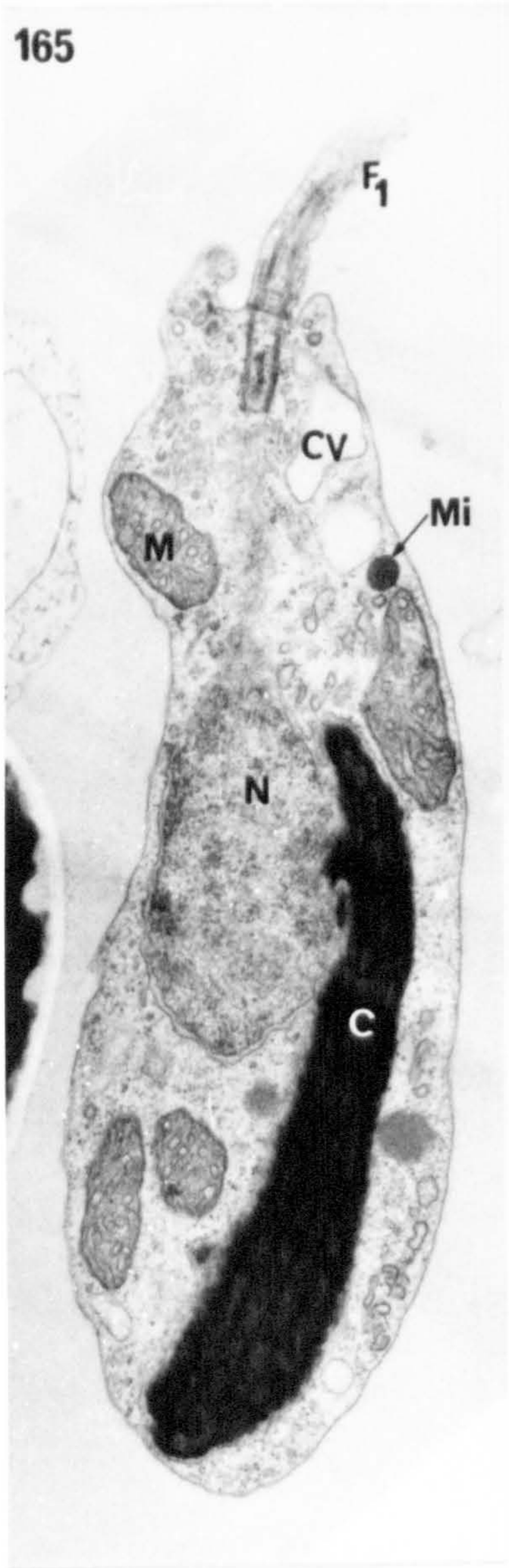
Fig. 166-169. Selected longitudinal sections of two series (Figs 166, 167 and Figs 168, 169) showing microtubular roots R1 and R3, and striated material connecting the basal bodies with the nucleus (arrows). Figs 166 & 167 x 42000; Figs 168 & 169 x 52500.

Fig. 170. Transverse section, showing the posterior flagellum in relation to the chloroplast and the nucleus. x 54000.

Fig. 171. Longitudinal section of the bases of both flagella showing the transition region and the basal bodies. Note dense material inside the basal bodies and number of gyres in the transitional helices (arrows). x 54000.

Abbreviations used in figures: C, chloroplast; CV, contractile vacuole; E, eyespot; F1, anterior flagellum; F2, posterior flagellum; FS, flagellar swelling; G, golgi body; M, mitochondrion; Mi, microbody-like structure; N, nucleus; R1 and R3,; microtubular roots.

165



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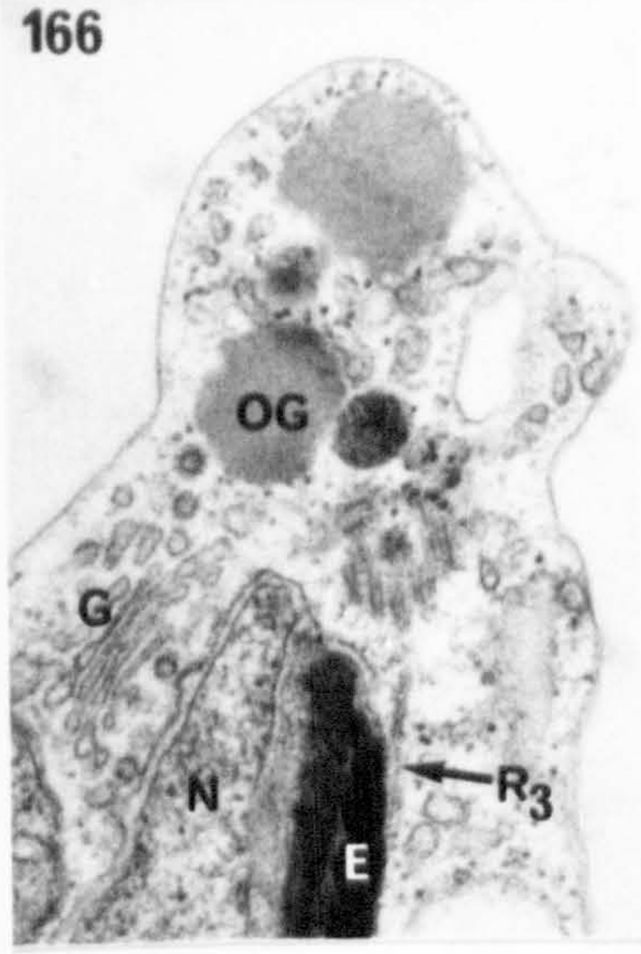

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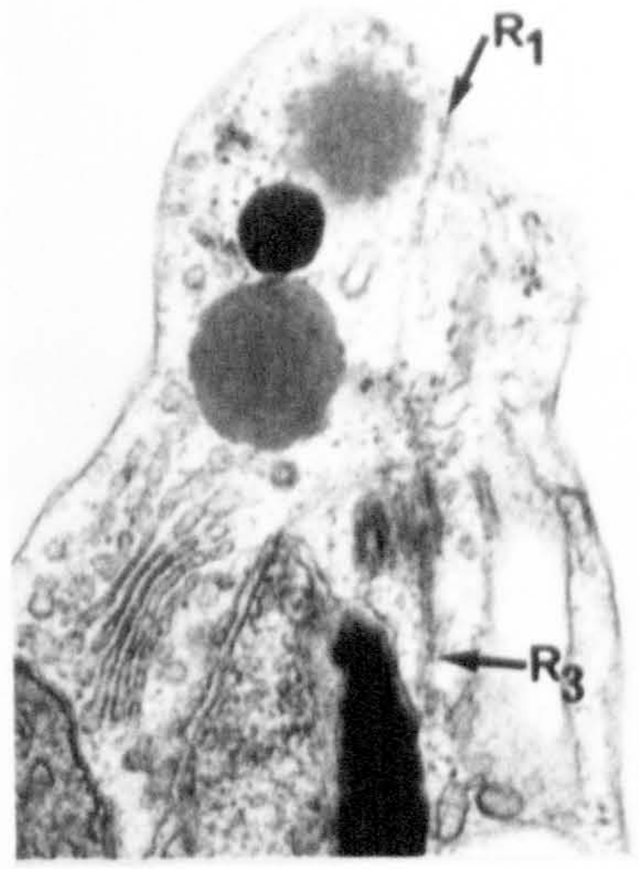

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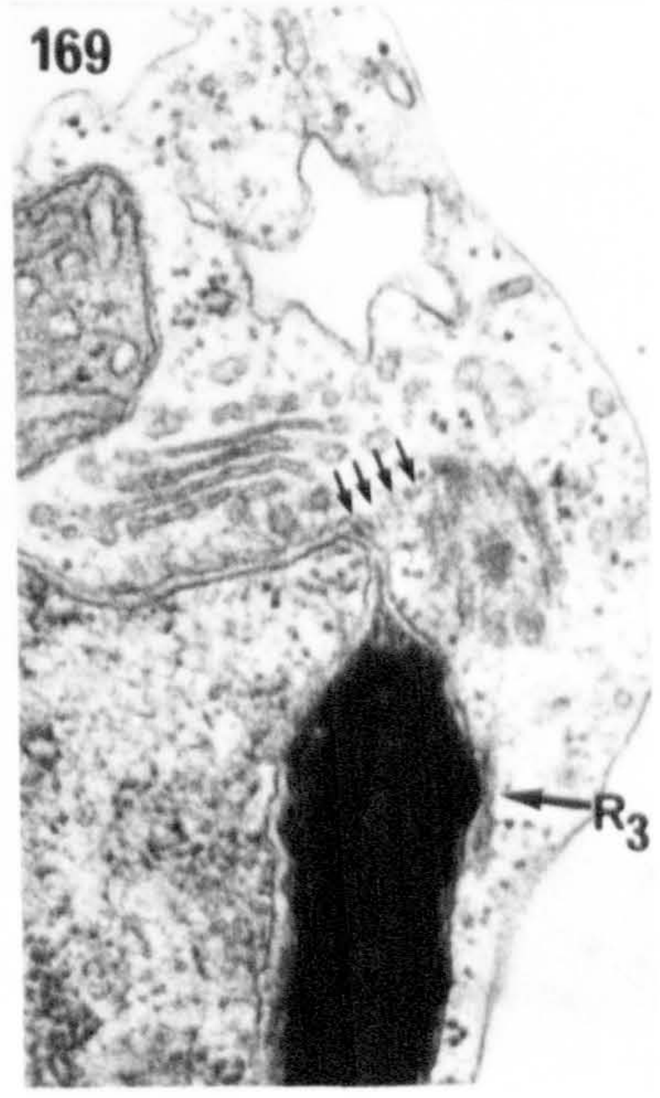

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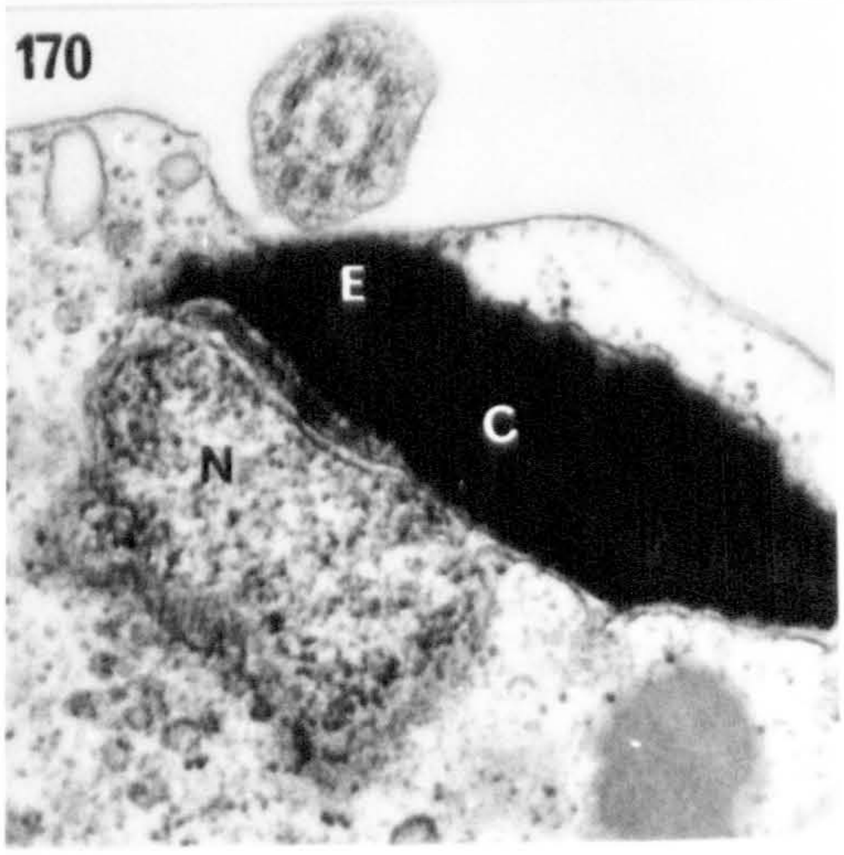

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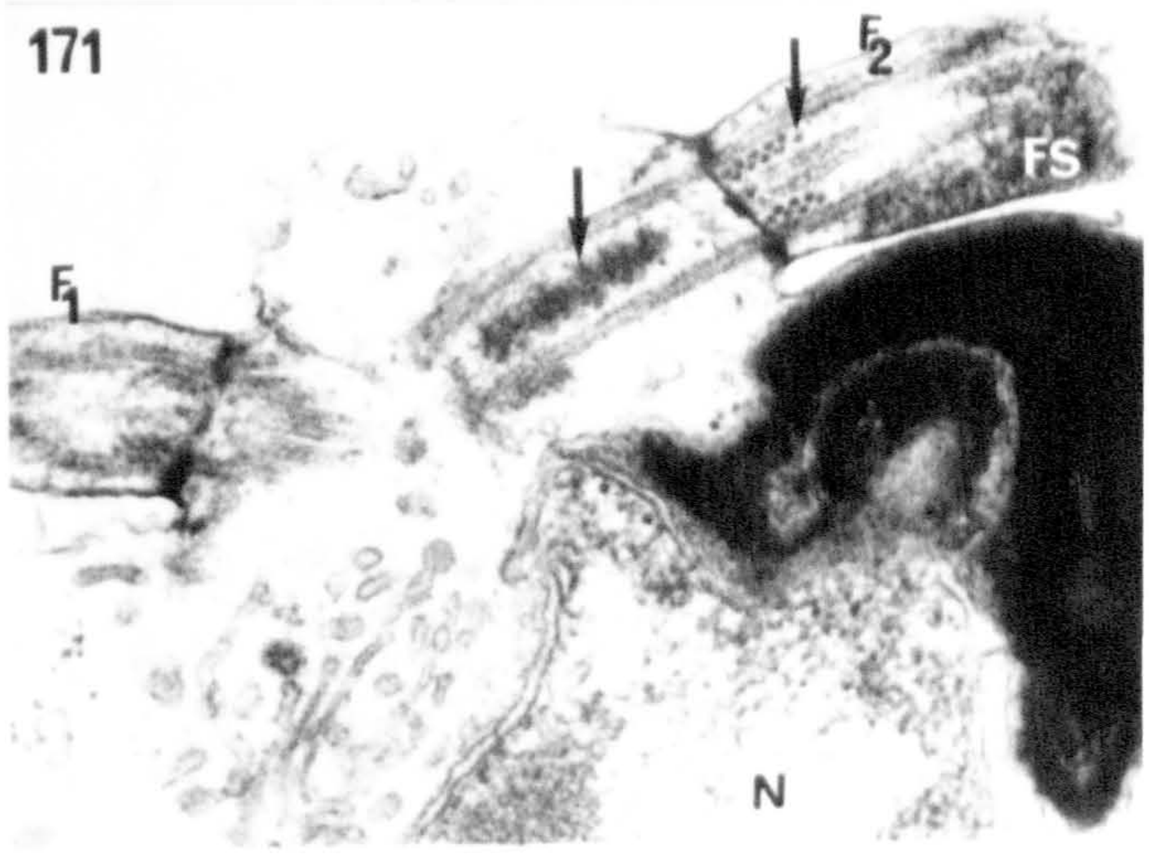


F. ZOOSPORE SETTLING

1. *Vischeria*

Recently settled zoospores of *Vischeria* (Figs 172-178) appear as rounded cells, containing the usual organelles together with profiles of withdrawn flagella and surrounded by an adhesive substance, mucilage-like, by means of which settling zoospores stick to any available surface (arrows in Fig. 172).

The withdrawal of the entire flagellum, including the flagellar swelling, is probably the onset of the process, since profiles of the free flagellar axis are evident in many sections together with mastigoneme-containing cisternae (Fig. 173; see also Fig. 176) and a dense layered structure similar to the swelling (Figs 174, 175). Clear 9+2 profiles persist until a late stage of wall formation (Fig. 176).

The Golgi body, seen only once in free zoospores, becomes very prominent in the settling cells, with numerous vesicles being produced (Fig. 172); several small vacuoles containing tubular and/or vesiculate material are also quite common close to and in the area later occupied by the cell wall (Figs 176, 177), both aspects suggesting a connection of these structures with the process of wall formation. Another related aspect is the presence of large number of mitochondria in the cytoplasm (Fig. 178).

A pyrenoid begins to be formed soon after settling (see below) and a red body appears in settled cells at a later

Figs 172-178. Settling cells of *Vischeria punctata* (Figs 172, 175, 178) and *V. stellata* (Figs 173, 174, 176, 177).

Fig. 172. Section showing a large and vesiculate Golgi body and profiles of the flagellum. Note the fibrillar material around the cell (arrows). x 30000.

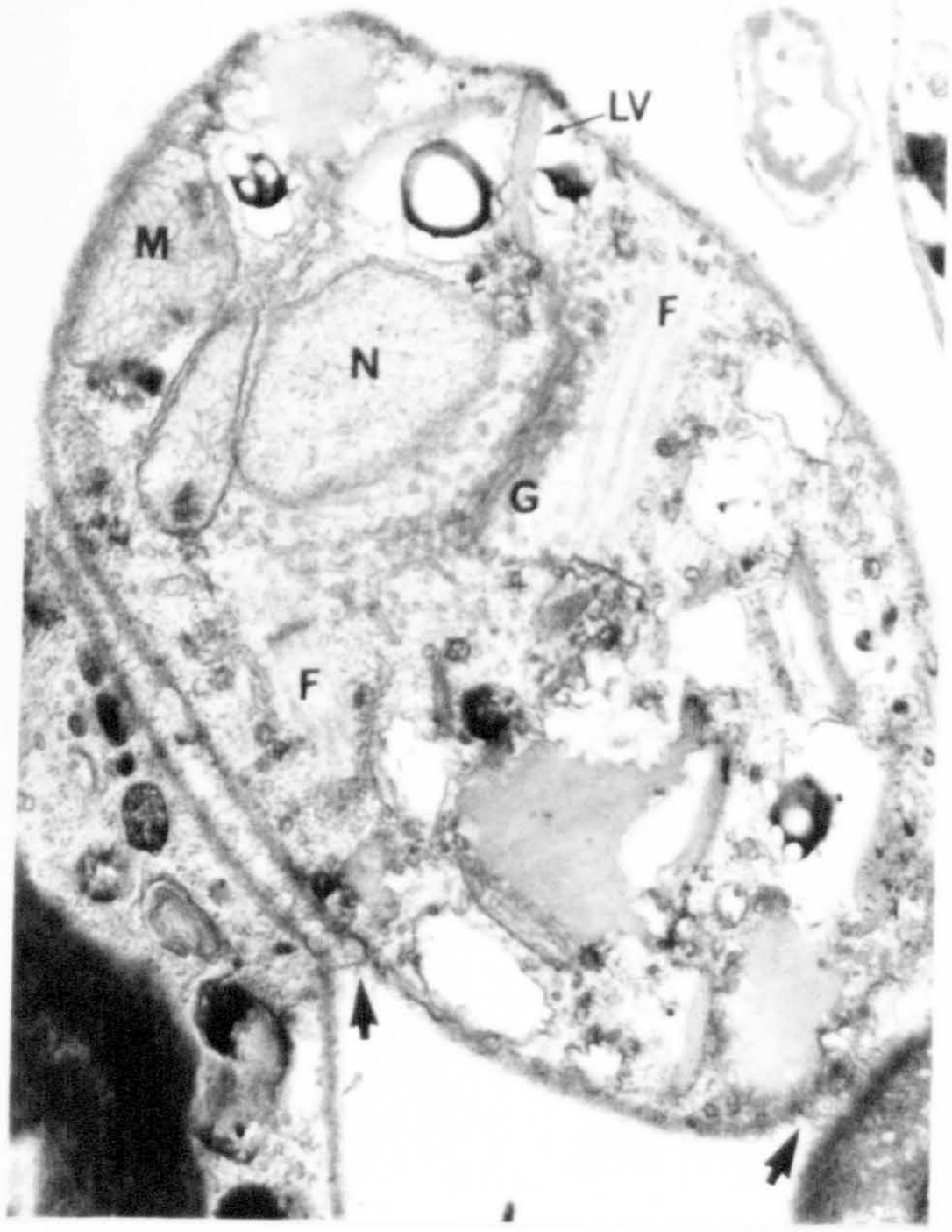
Figs 173-175. Sections showing the withdrawal of the entire flagellum, its mastigonemes and swelling. Fig. 173 x 24000; Fig. 174 x 50000; Fig. 175 x 40000.

Figs 176, 177. Note the presence of vesiculate and/or tubular material (arrows), possibly involved in the formation of the cell wall. x 45000.

Fig. 178. Note the close association of mitochondria with the chloroplast. x 21500.

Abbreviations used in figures: C, chloroplast; F, withdrawn flagellum; FS, flagellar swelling; LV, lamellate vesicle; M, mitochondrion; Ms, mastigonemes; N, nucleus; SV, spiral vesicle; W, cell wall.

172



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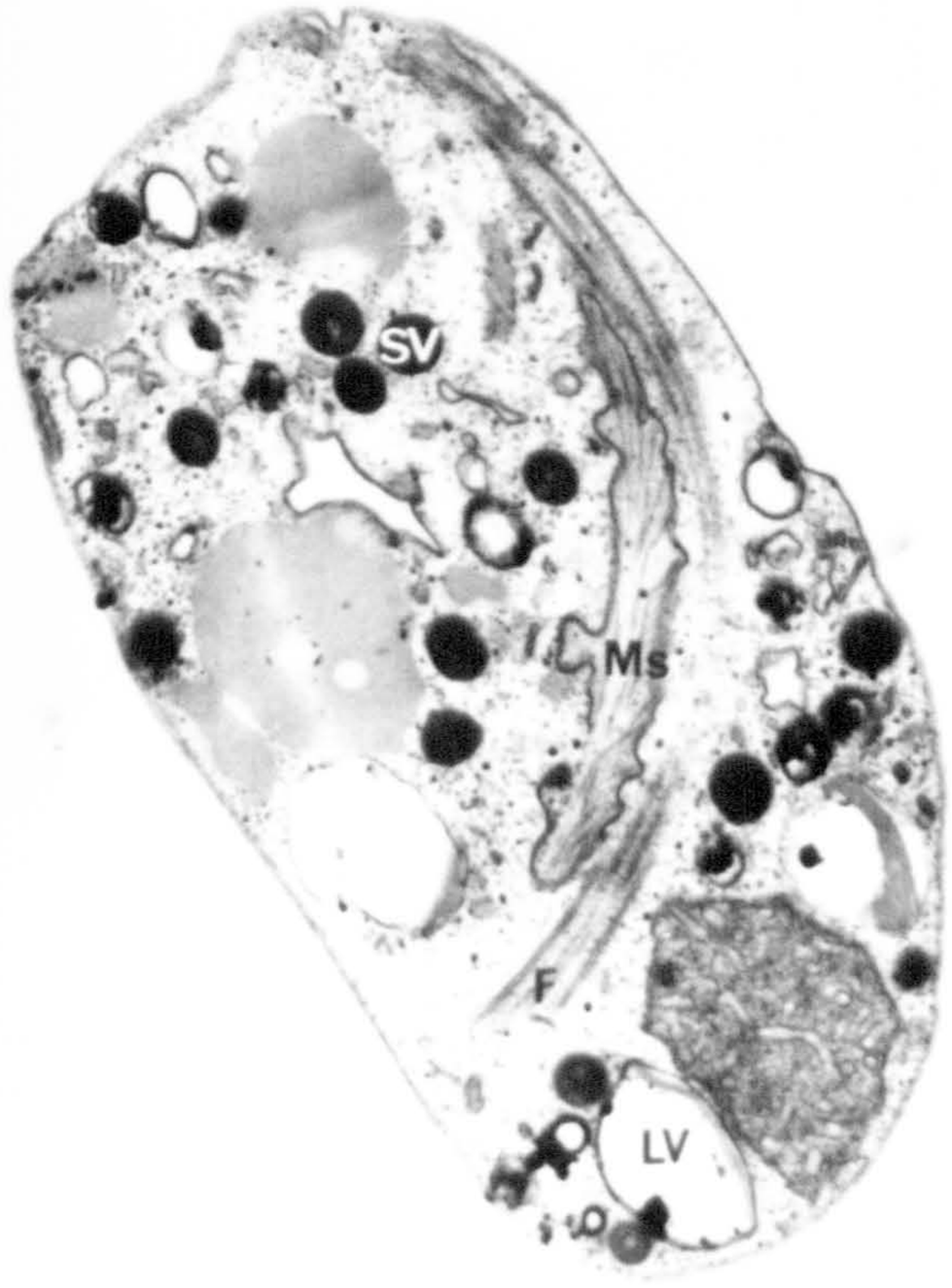

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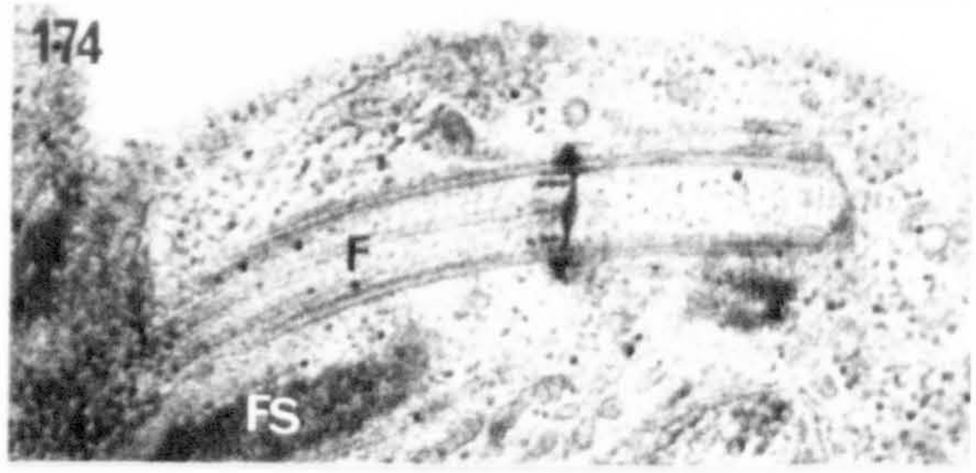

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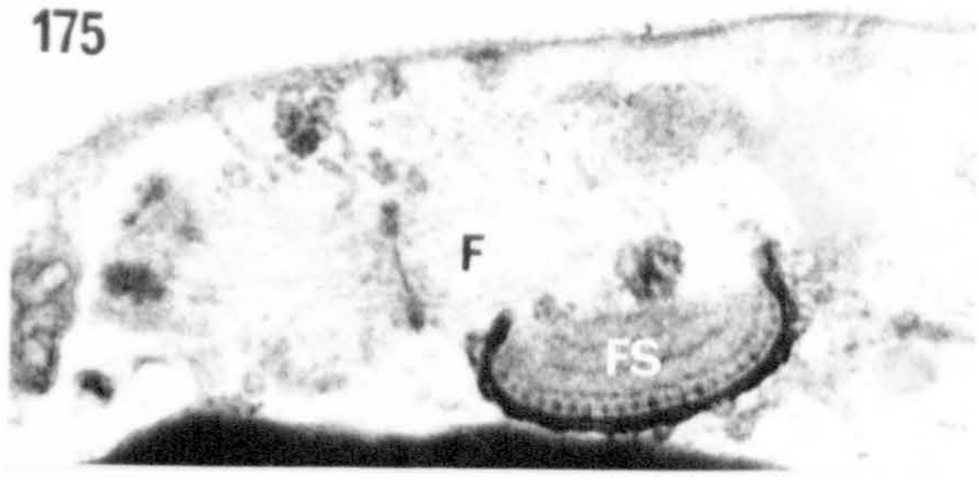

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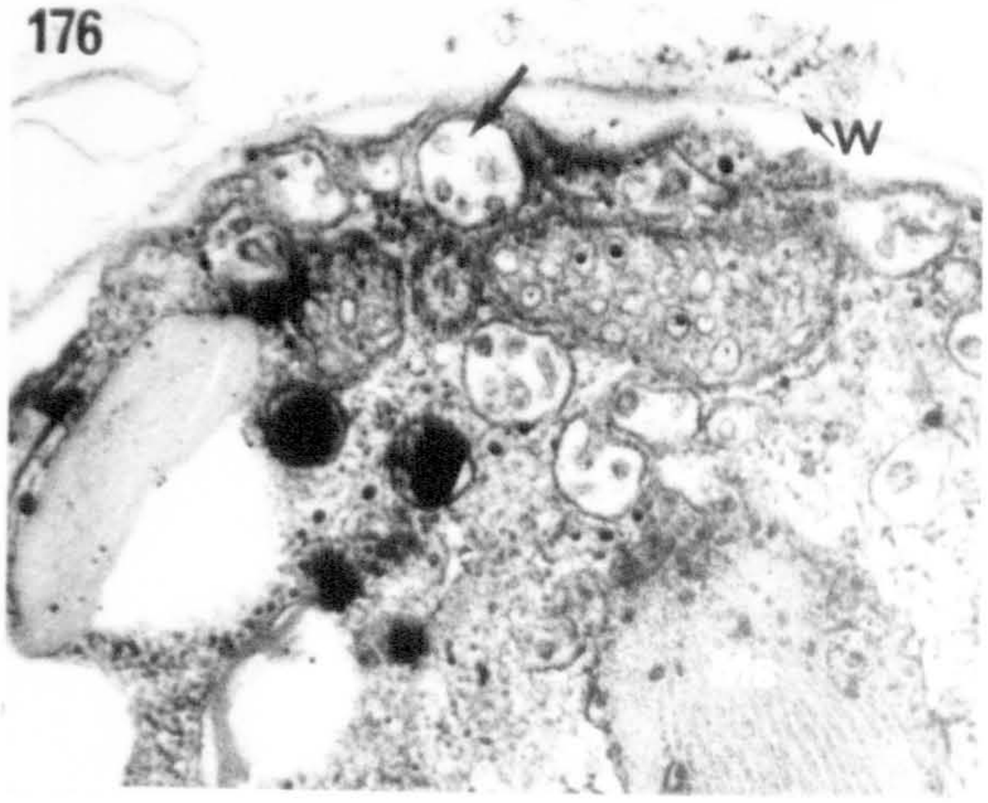

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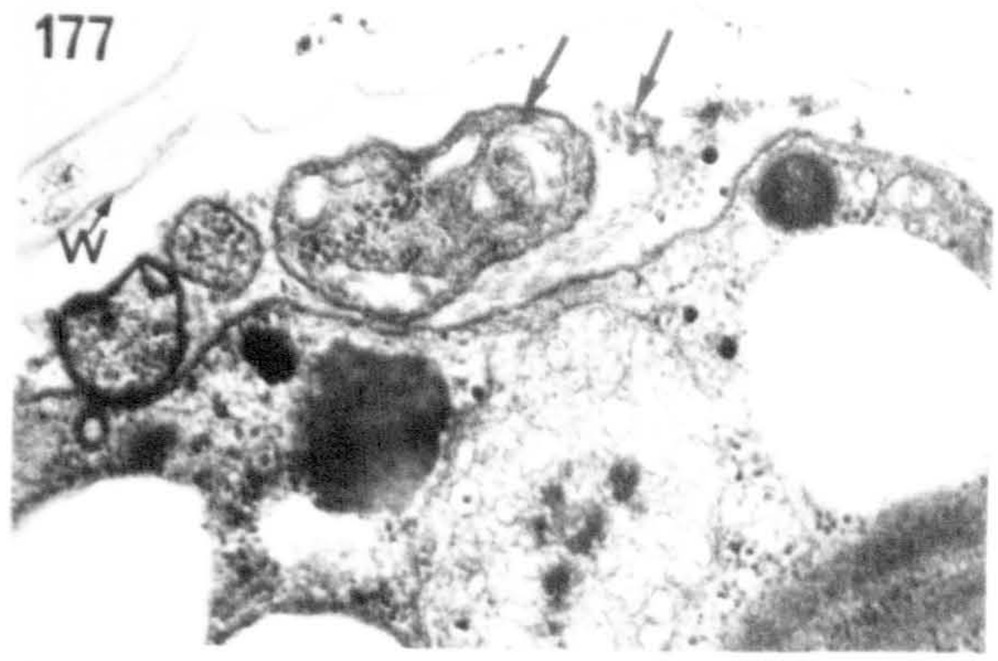

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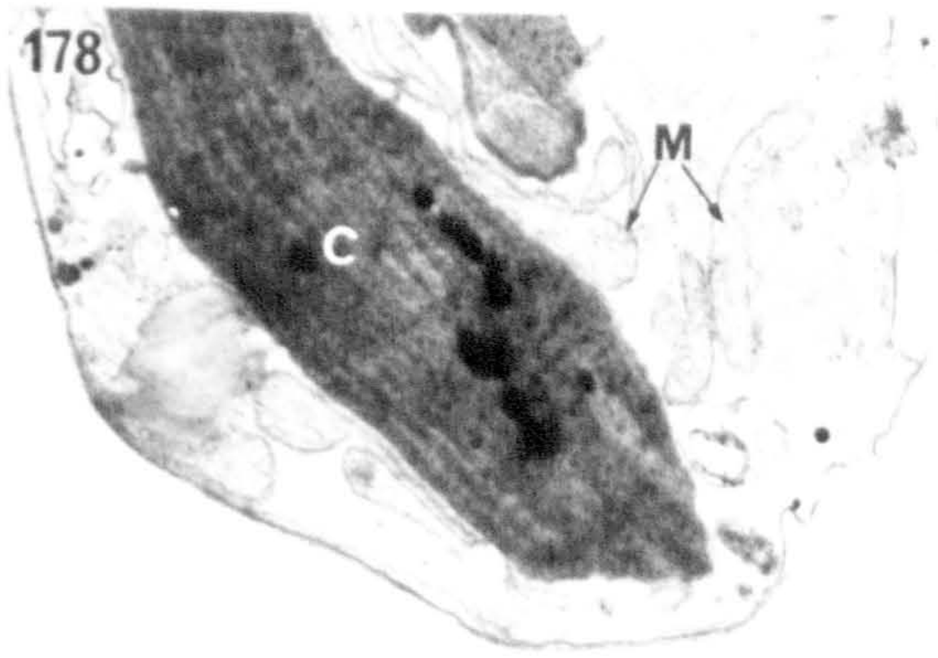
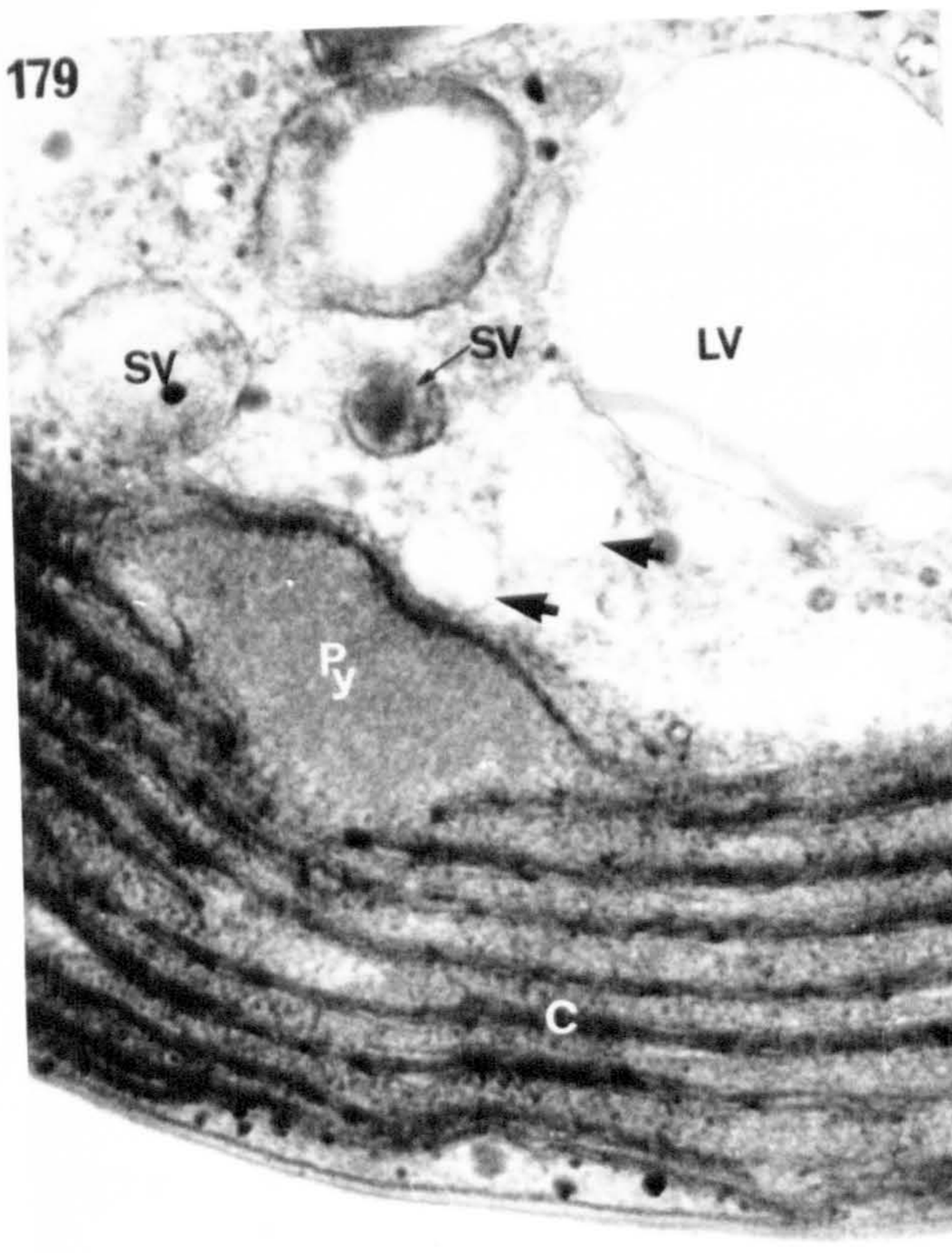


Figs 179-182. Pyrenoid formation in settling cells of *Vischeria stellata*.

Figs 179-181. Selected sections of a series. Note the vesicles fusing in the region of pyrenoid formation (arrows). Figs 179 & 180 x 57000; Fig. 181 x 47500.

Fig. 182. Note the proximity of spiral vesicles to the mentioned region. x 68000.

Abbreviations used in figures: C, chloroplast; F, withdrawn flagellum; LV, lamellate vesicle; Py, pyrenoid; SV, spiral vesicle.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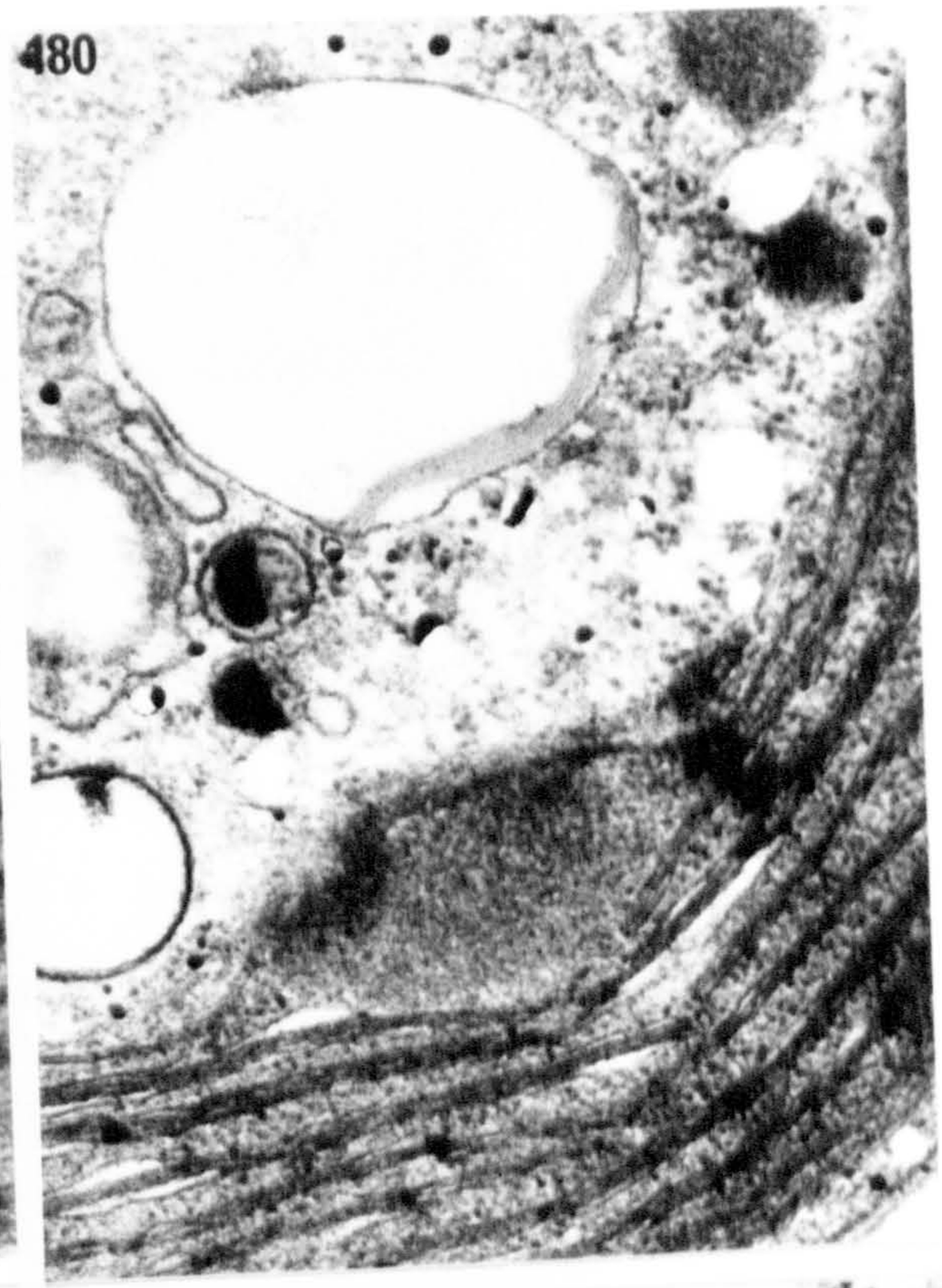
SV
SV
LV
Py
C


180

181
F

182
SV

stage of wall formation (not illustrated). Lamellate and spiral vesicles are frequent in the cytoplasm (Figs 173, 176) throughout the whole process.

The vegetative cells of species included in the family Eustigmataceae have a large angular pyrenoid attached to the internal face of the chloroplast and surrounded by lamellate vesicles, as previously indicated (see Section A.1, Chapter III). During pre-mitotic stages this pyrenoid appears to divide as a consequence of chloroplast division (see Section G, Chapter III). A pyrenoid is absent, however, in the uniflagellate zoospores produced by these species but begins to grow from the internal surface of the chloroplast soon after settling.

At this stage, in *Vischeria stellata* (Figs 179-182), vesicles similar to the spiral vesicles found in the zoospores fuse with the chloroplast in the region where the pyrenoid is being formed (Figs 179, 181); several spiral vesicles can also be found close to this region which suggests their possible role in storing the pyrenoidal contents in the zoospores. Cytochemical experiments with pepsin and protease did not show, however, digestion of the material either in the forming pyrenoid or in the spiral vesicles.

2. *Pseudocharaciopsis minuta*

The process of settling is similar to that just described for *Vischeria*, apart from the initial stages where the two dense bodies described in the vegetative cells

appear to be involved; in some sections (Fig. 183) these structures are seen very close to the cell surface or protruding from it; later in the process, their dense contents seem to have been released to originate the attaching disc present in the vegetative cells (Fig. 184); the settling cell elongates and the cell wall begins to form, vesiculate material similar to that seen in *Vischeria* also appearing close to the future wall (Figs 184, 185). In a similar way, the flagella are entirely withdrawn, including the swelling (Figs 186, 187). Stages of pyrenoid formation were not observed.

Figs 183-187. Settling cells of *Pseudocharaciopsis minuta*.

Fig. 183. Note the position of both dense bodies (arrows), close to the plasmalemma (a) or protruding from it and forming a short stalk (b). a x 38000; b x 28000.

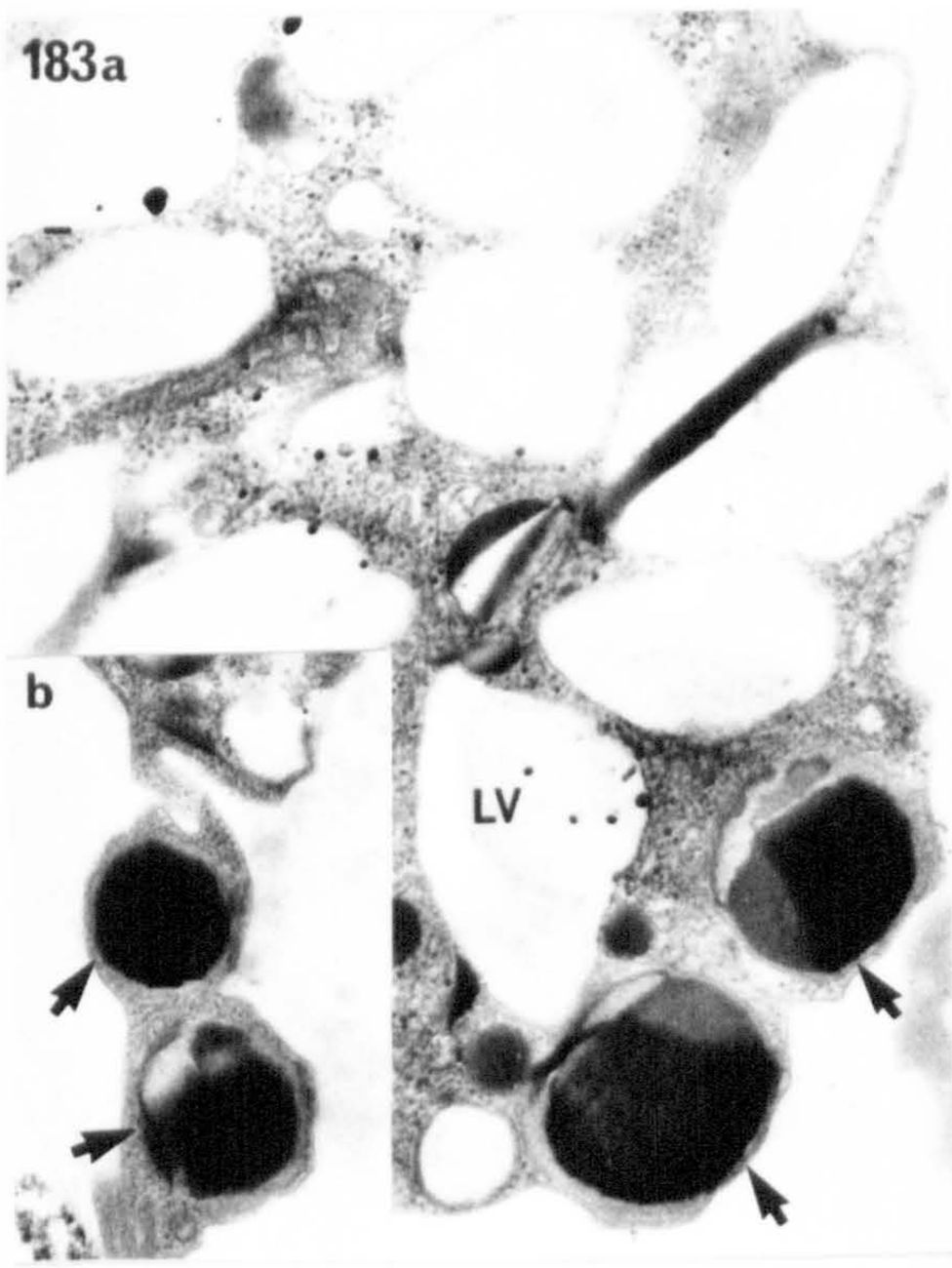
Fig. 184. Section showing the presence of a holdfast similar to that present in vegetative cells, several mitochondrial profiles near the chloroplast and a thin cell wall already formed. Note the vesicles with posible cell wall material (arrows). x 21500.

Fig. 185. Detail of the vesicles with tubular and/or membraneous material possibly involved in the formation of the cell wall. x 45000.

Figs 186,187. Sections showing the withdrawal of the flagellum. Fig. 186 x 54000; Fig. 187 x 36000.

Abbreviations used in figures: C, chloroplast; F, withdrawn flagellum; FS, flagellar swelling; H, holdfast; LV, lamellate vesicle; M, mitochondrion; W, cell wall.

183a



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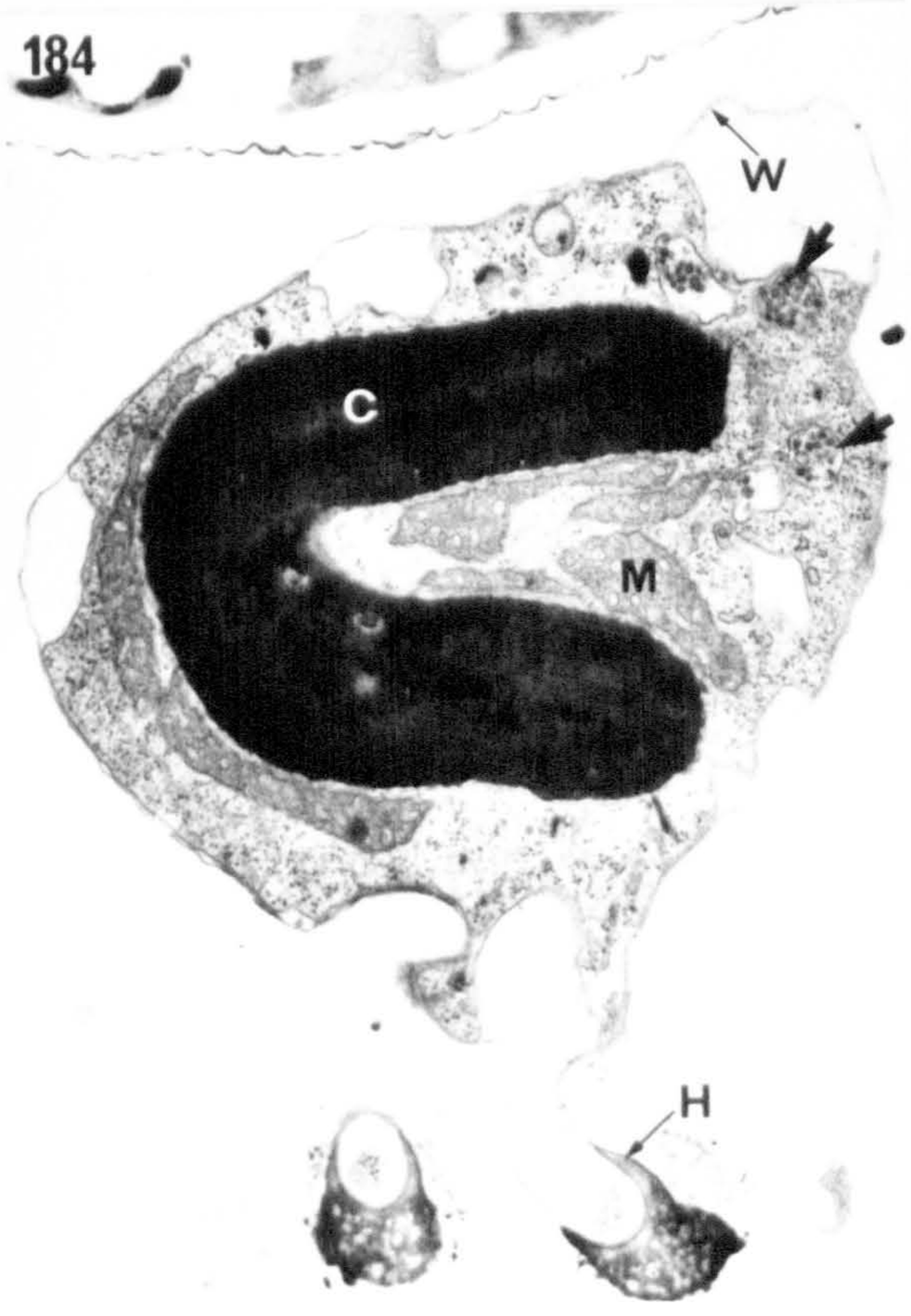

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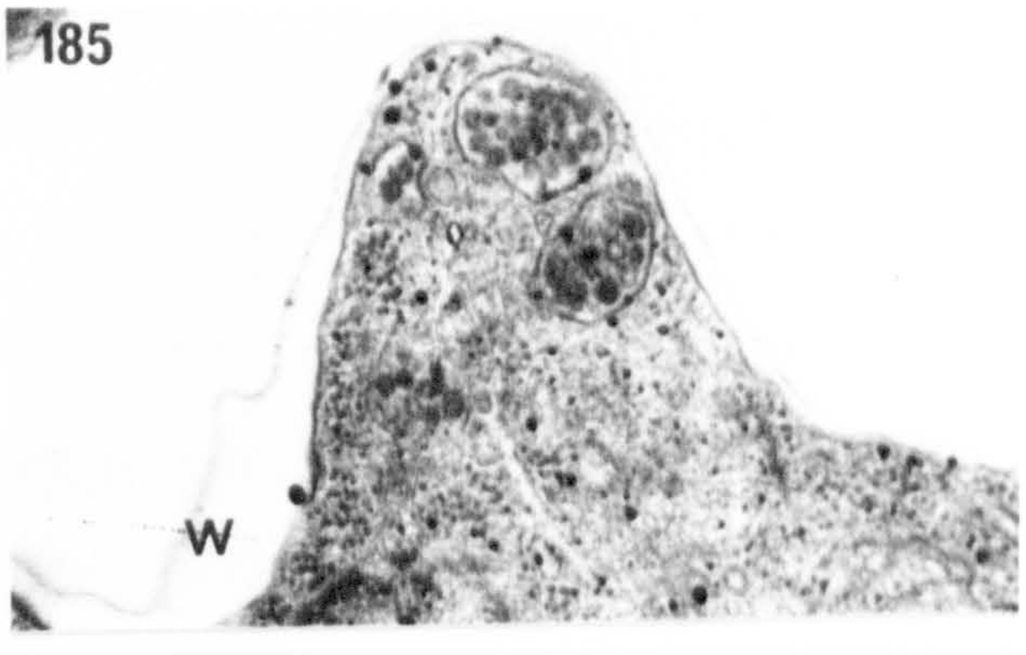

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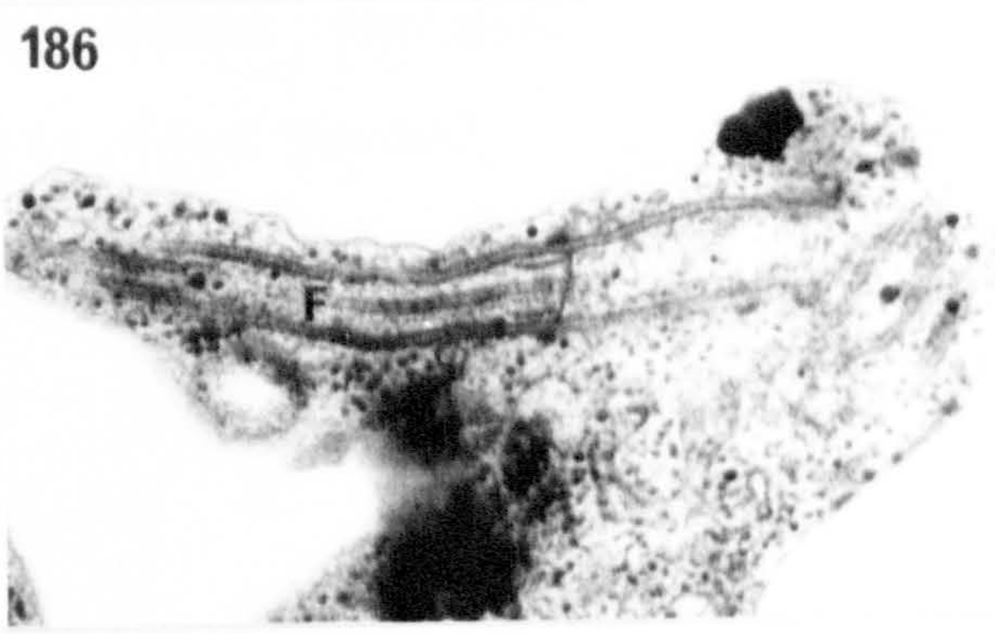

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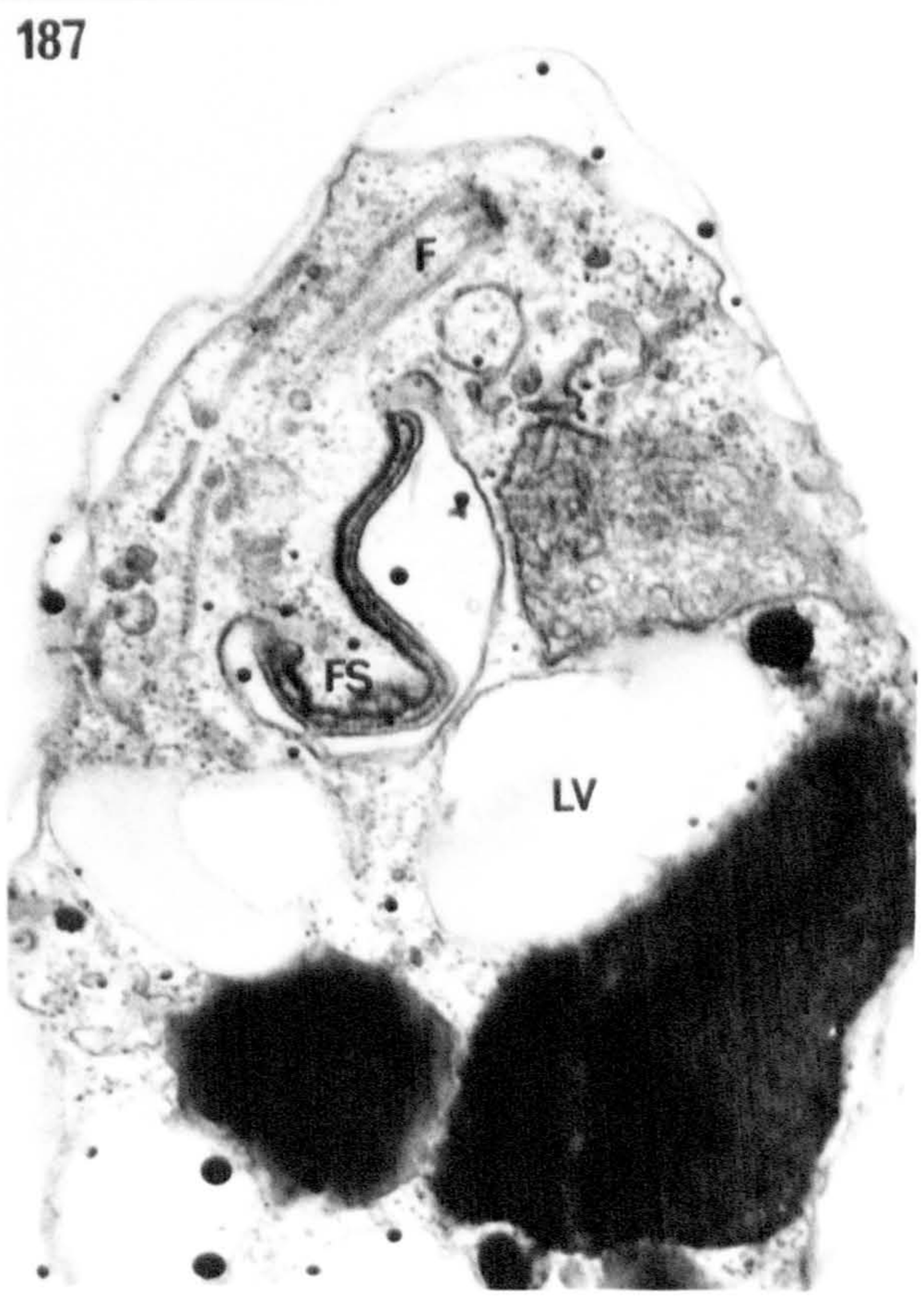


G. PRELIMINARY OBSERVATIONS ON MITOSIS AND CYTOKINESIS

Experiments to synchronize cultures have failed as an almost linear growth of the cultures tested was observed (Fig. 188). However a larger number of cells appeared to divide on the 3rd and 11th days after inoculation; for this reason, attempts to study mitosis were made in non-synchronized cultures, grown under the same conditions, and fixation of the material was done during the 11th dark period of growth.

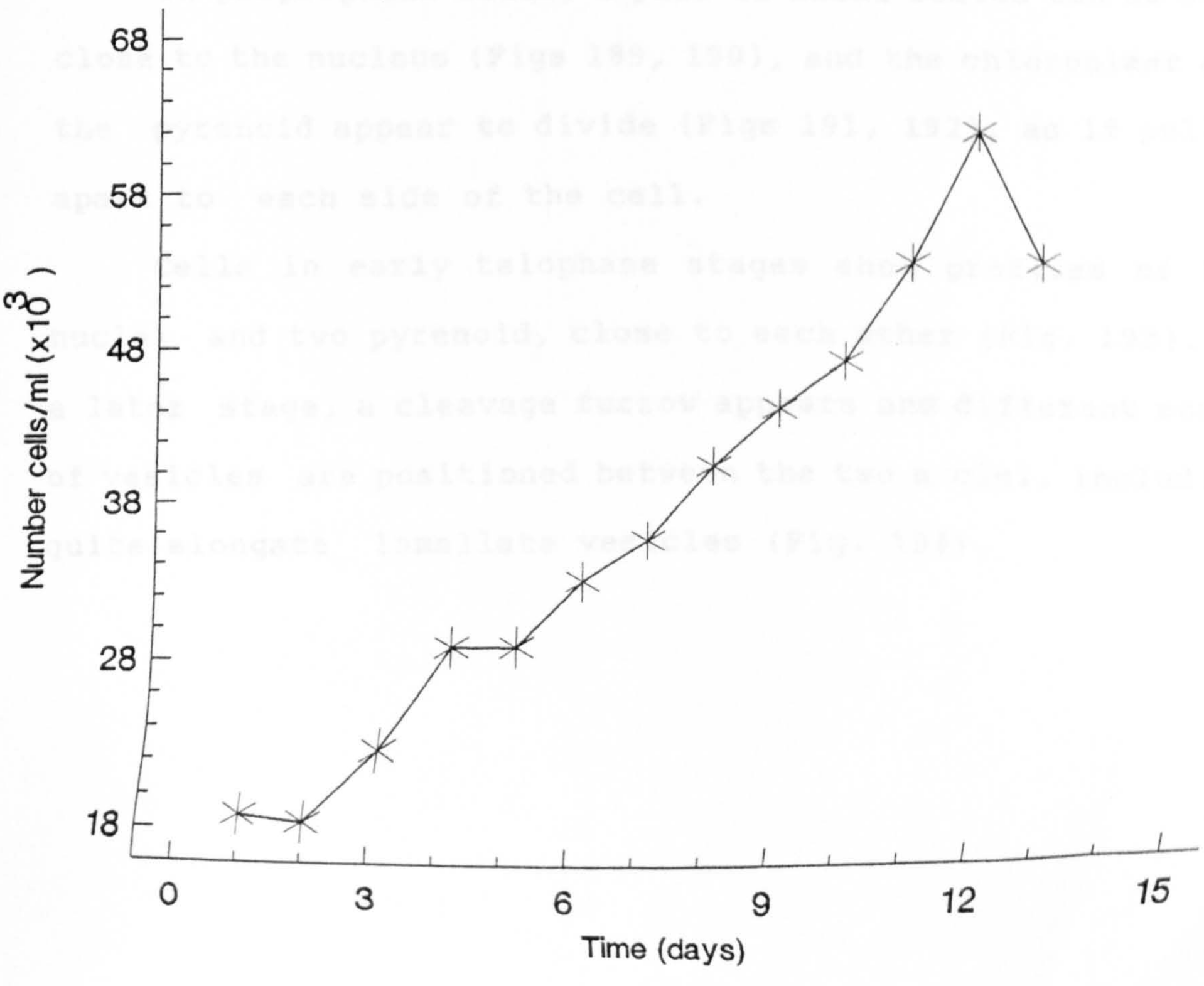


Fig. 188. Mean growth of duplicate cultures of Vischeria stellata in medium 1b (see Appendix 2 for values), at 12-18°C, during a fortnight.

Preliminary experiments with *Vischeria punctata* indicated that mitosis would take place 1h 30min - 2h after the onset of the dark period, since at a later time of fixation the sections showed cells already divided.

In later experiments, cells of *V. punctata*, *Eustigmatos vischeri* and *E. polyphem*, fixed at 10min intervals during that 1h 30min-2h period, showed a few preprophase (Figs 189-192) and telophase (Figs 193, 194) stages but no mitotic apparatus and/or other relevant mitotic phases were observed.

In preprophase cells, a pair of basal bodies can be seen close to the nucleus (Figs 189, 190), and the chloroplast and the pyrenoid appear to divide (Figs 191, 192), as if pulled apart to each side of the cell.

Cells in early telophase stages show profiles of two nuclei and two pyrenoid, close to each other (Fig. 193). At a later stage, a cleavage furrow appears and different sorts of vesicles are positioned between the two nuclei, including quite elongate lamellate vesicles (Fig. 194).

Figs 189-194. Some aspects of mitosis in *Vischeria* and *Eustigmatos*.

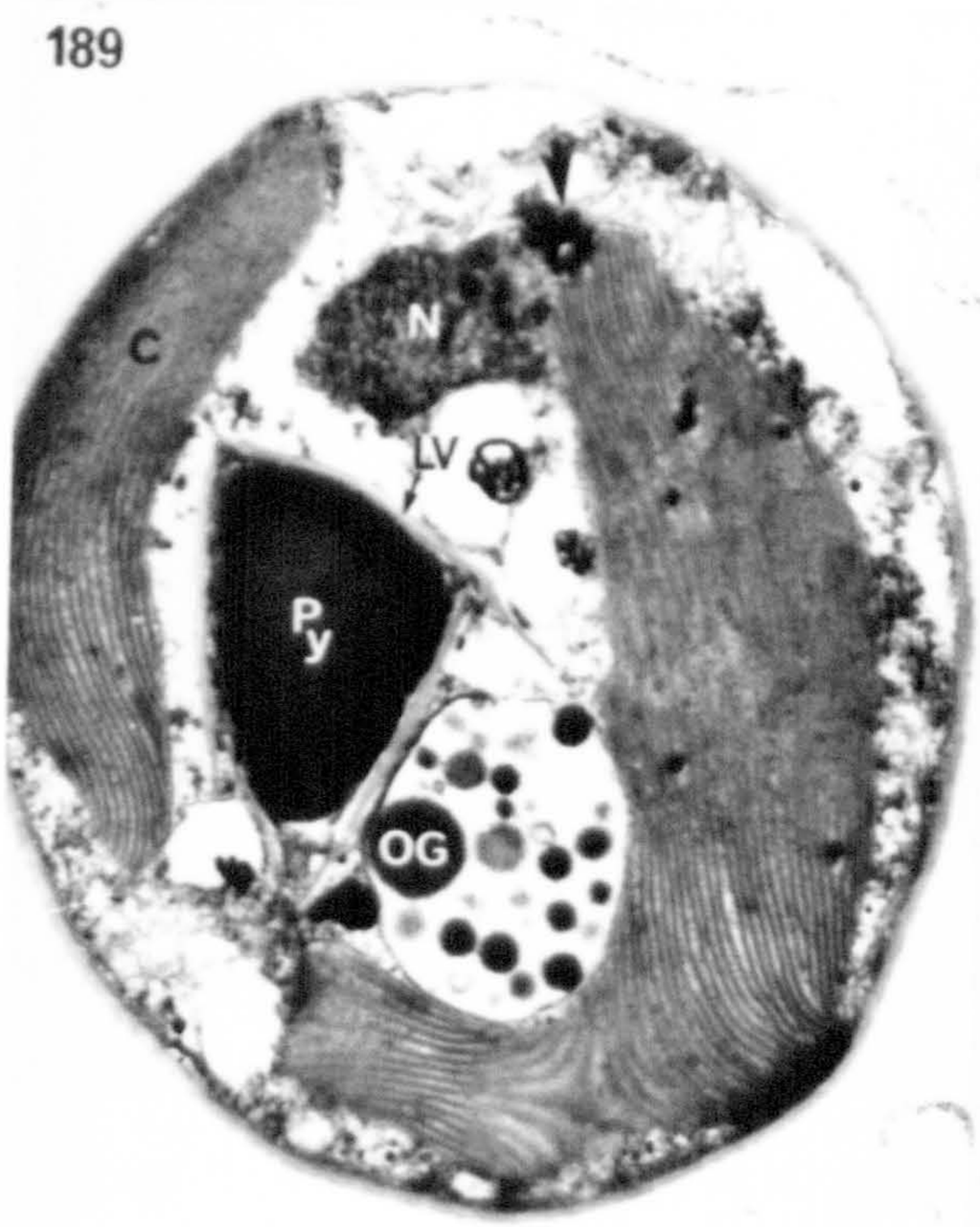
Figs 189-192. Cells of *V. punctata* (Figs 189-191) and *E. vischeri* (Fig. 192) in preprophase. Note the presence of two basal bodies near the nucleus surface (arrow) and the division of the pyrenoid. Fig. 189 x 16000; Fig. 190 x 31500; Fig. 191 x 15500; Fig. 192 x 16000.

Fig. 193. Cell of *E. polyphem* in telophase. x 14000.

Fig. 194. Cell of *V. punctata*. Note the beginning of the cleavage furrow (arrow) and the positioning of elongate lamellate vesicles between both nuclei. x 11000.

Abbreviations used in figures: C, chloroplast; LV, lamellate vesicle; N, nucleus; OG, osmiophilic globule; Py, pyrenoid.

189



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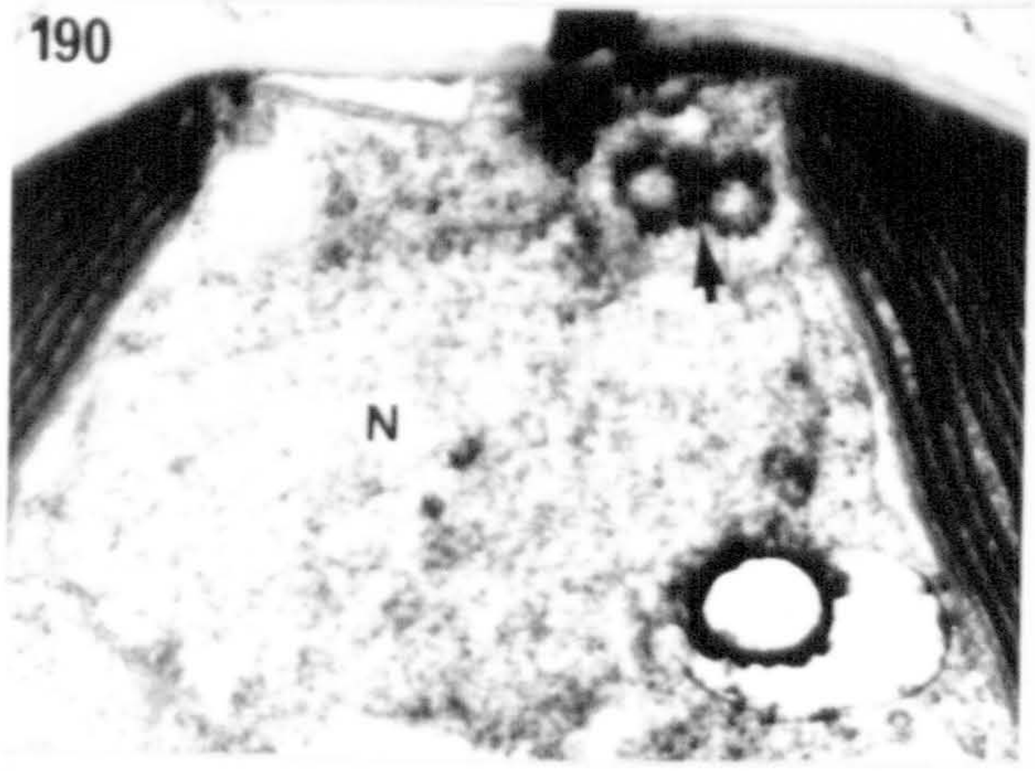

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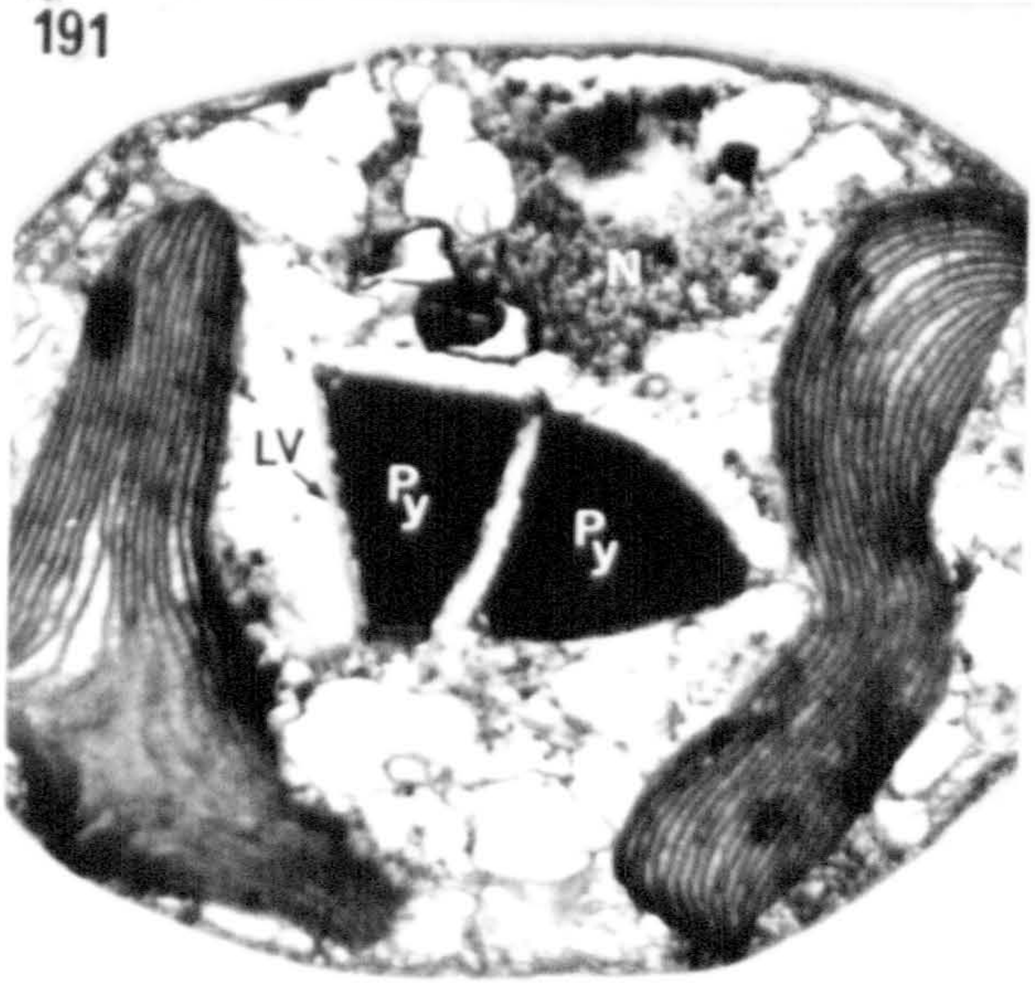

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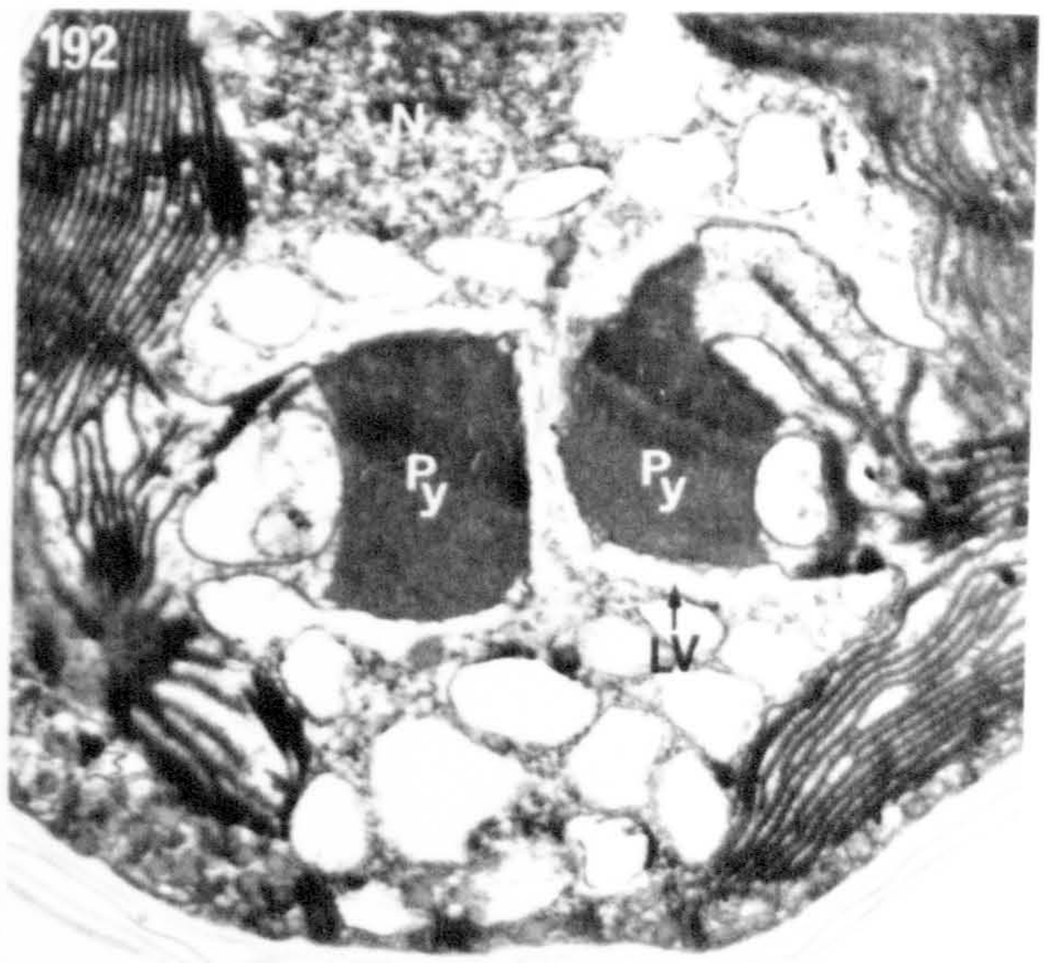

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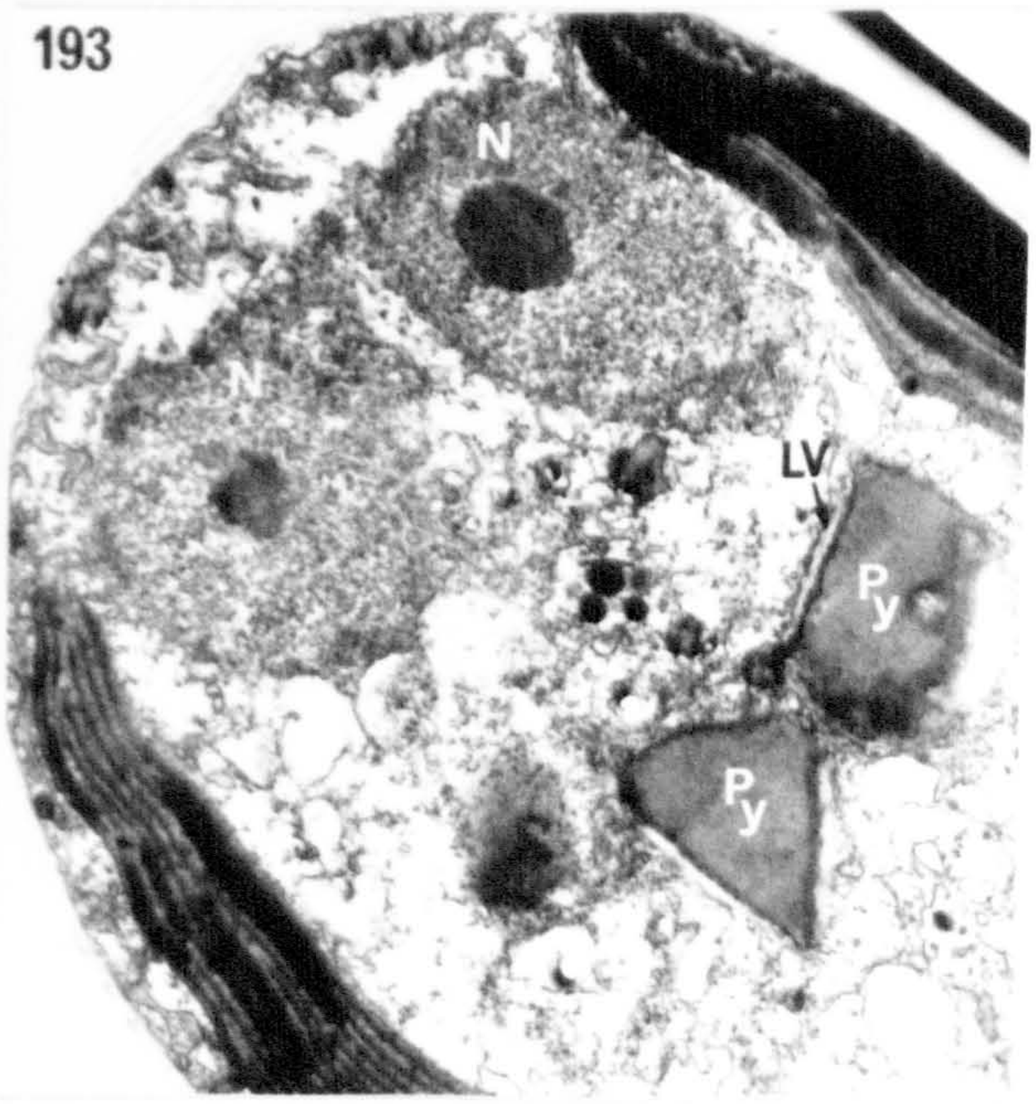

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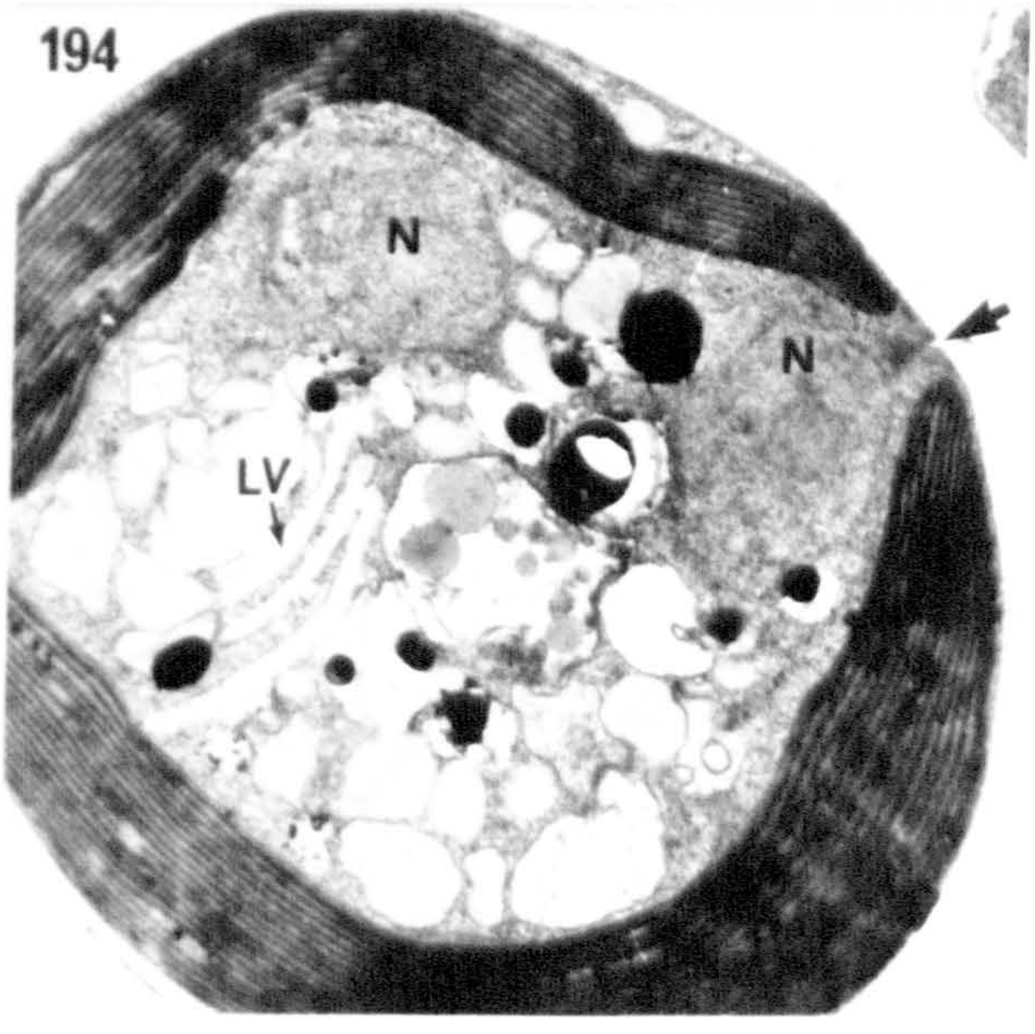

H. CELL WALL STUDIES IN *VISCHERIA STELLATA* AND *OPHIOCYTIUM MAIUS*

1. Structure

The cells of both species were difficult to break and only after being French-pressed 5-6 times and gently centrifuged could a small white layer of the pellet, containing mostly cell walls, be pipetted from the remaining green pellet, consisting of unbroken cells. This cell wall material of both species appeared as a white powder after being freeze-dried, insoluble in water.

In both species, direct preparations of this material showed the presence of numerous microfibrils (Figs 195, 196) while X-ray diffraction analysis revealed virtually nothing (Figs 197-199).

2. Analysis of sugars

Analysis of the sugars present in the cell wall material, made by paper and gas-liquid chromatography after complete hydrolysis with TFA, revealed glucose as the almost exclusive monosaccharide present in both species (Figs 200, 201); traces of rhamnose and arabinose were also present in the cell wall of *Vischeria* (Fig. 201A) and traces of arabinose in the cell wall of *Ophiocytium* (Fig. 201B).

Figs 195-199. Direct preparations and X-ray analysis of cell wall material of *Vischeria stellata* (Figs 195, 198) and *Ophiocytium maius* (Figs 196, 199).

Fig. 195. Microfibrils in the cell wall of *V. stellata*. x 80000.

Fig. 196. Microfibrils in the cell wall of *O. maius*. x 38500.

Fig. 197. X-ray diagram of cellulose I from paper tissue.

Fig. 198. X-ray diagram of the cell wall material in *V. stellata*.

Fig. 199. X-ray diagram of the cell wall material in *O. maius*.

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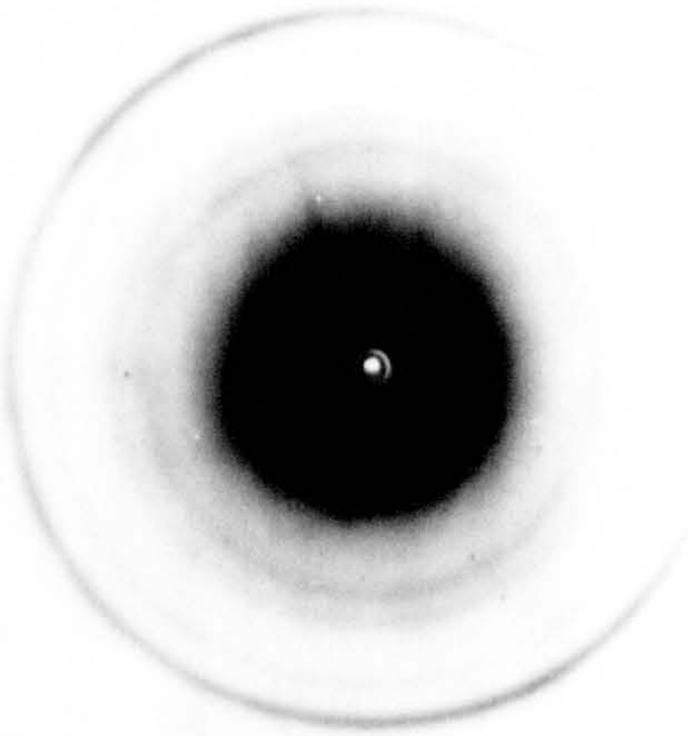

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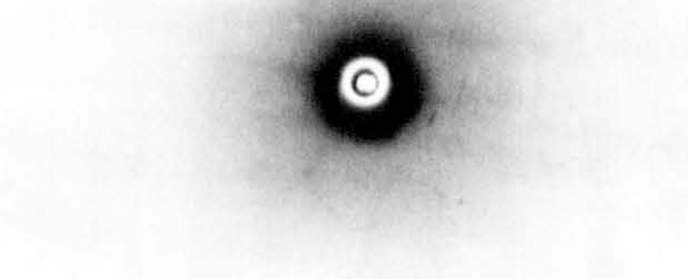

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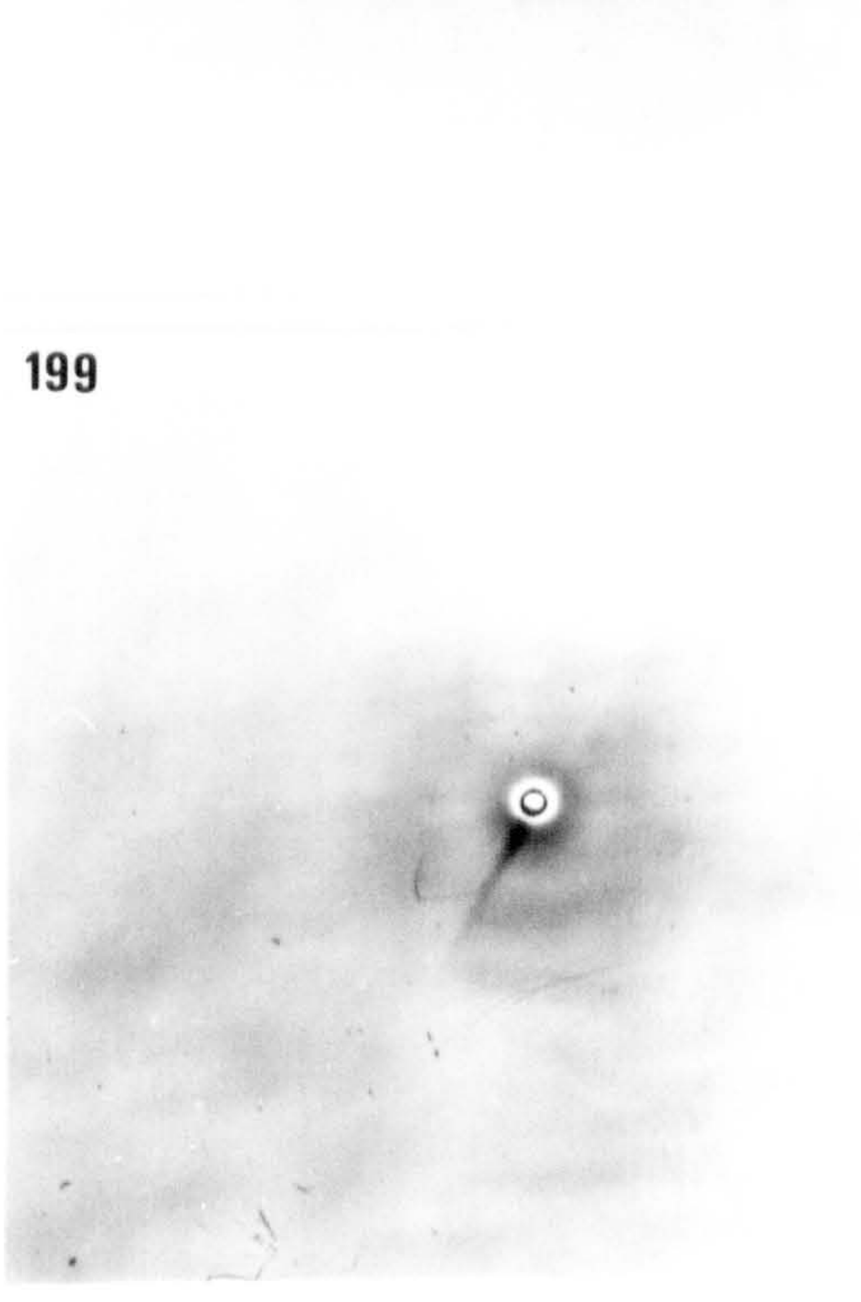

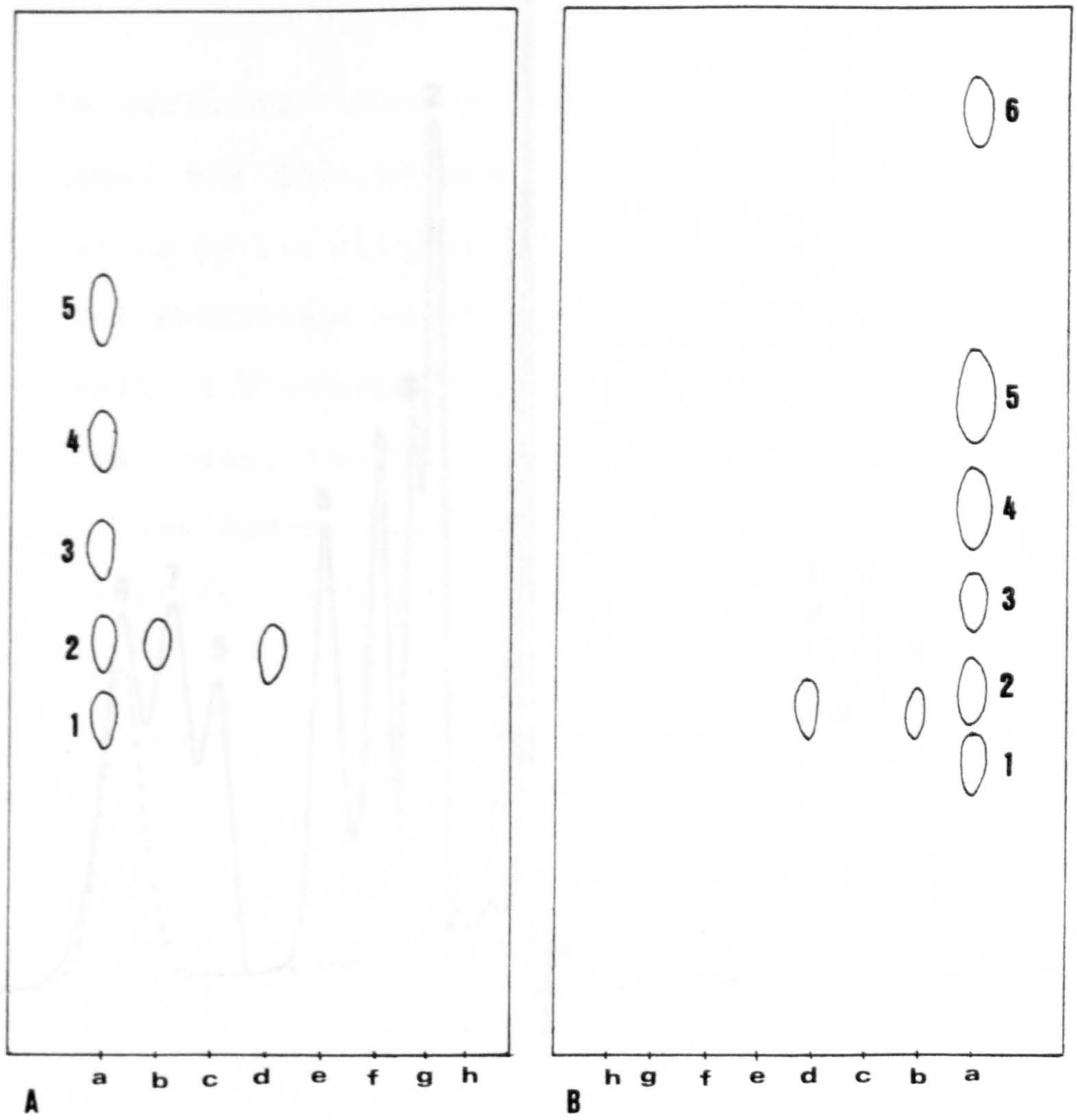

Fig. 200. Analysis of sugars by paper chromatography. A, p-anisidine treatment; B, Ag NO3 treatment. a, standard mixture of sugars; b, hydrolised cell wall material of *Vischeria stellata*; c, f, h, non-hydrolised cell wall material of *V. stellata*; d, hydrolised cell wall material of *O. maius*; e, g, non-hydrolised cell wall material of *O. maius*; 1, galactose; 2, glucose; 3, mannose; 4, arabinose; 5, fucose and xylose; 6, rhamnose.

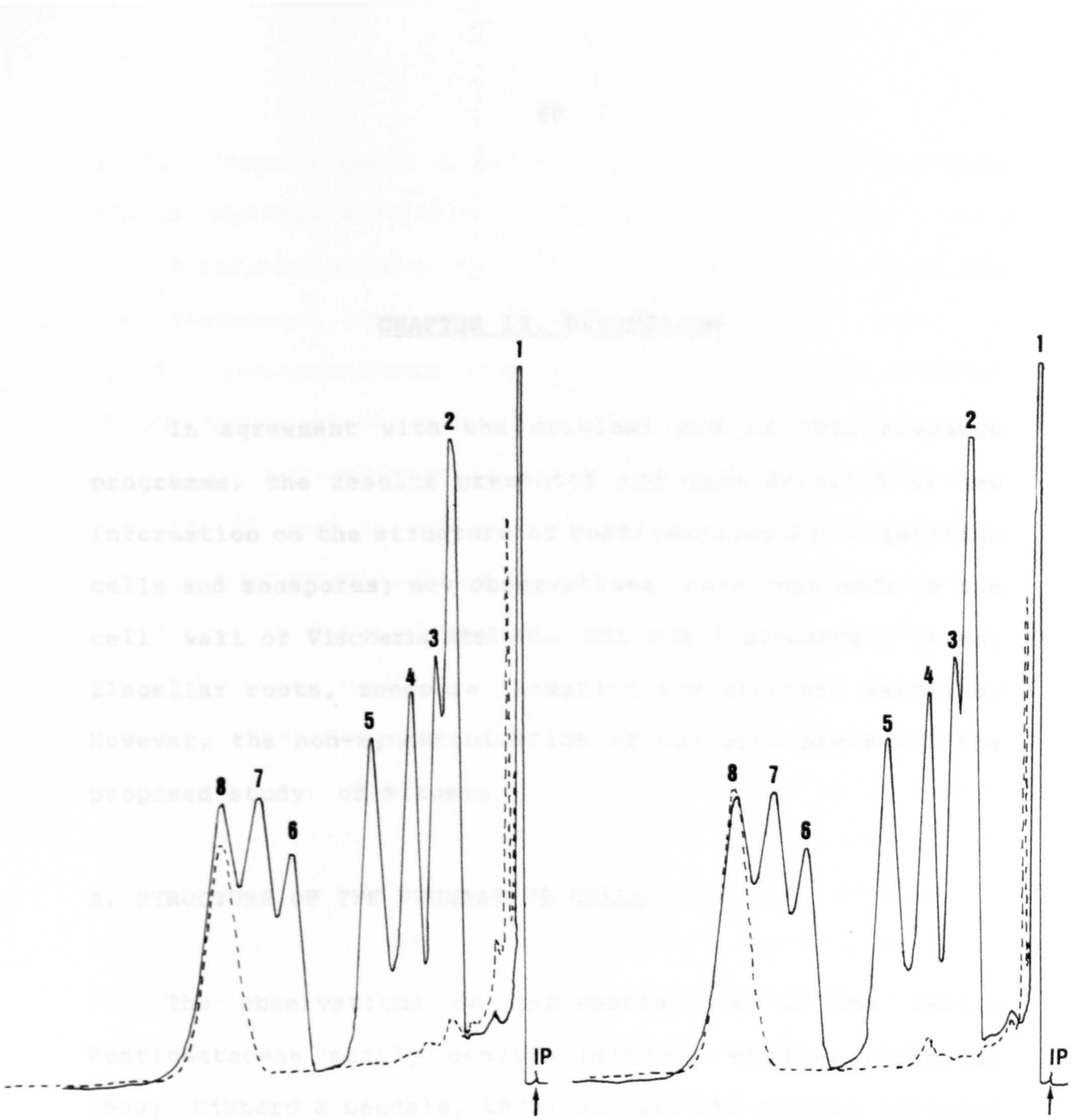

Fig. 201. Analysis of sugars by gas-liquid chromatography. A, hydrolised cell wall material of *V. stellata* (interrupted lines) and standard sugar mixture (continuous line); B, hydrolised cell wall material of *O. maius* (interrupted line) and standard mixture of sugars (continuous line); IP, injection point; 1, solvent peak; 2, rhamnose alditol acetate; 3, fucose alditol acetate; 4, arabinose alditol acetate; 5, xylose alditol acetate; 6, mannose alditol acetate; 7, galactose alditol acetate; 8, glucose alditol acetate.

CHAPTER IV. DISCUSSION

In agreement with the original aim of this research programme, the results presented add more detailed or new information on the structure of eustigmatophycean vegetative cells and zoospores; new observations have been made on the cell wall of *Vischeria stellata*, the small azoosporic forms, flagellar roots, zoospore formation and zoospore settling. However, the non-synchronization of cultures prevented the proposed study of mitosis.

A. STRUCTURE OF THE VEGETATIVE CELLS

The observations on representatives of the family Eustigmataceae mostly confirm previous studies (Hibberd, 1969; Hibberd & Leedale, 1972) and all six species included in this family (Hibberd, 1981) and studied here are basically similar in structure and only distinguishable by the ornamentation of the cell wall, shape and size of the cells.

The results on *Pseudocharaciopsis minuta*, the single species of the Pseudocharaciopsidaceae examined, also confirm previous observations on this species (Lee & Bold, 1973). However, the pyrenoids are either spherical or angular in shape and very similar to those of the Eustigmataceae. In the related species *P. ovalis* (Hibberd,

1969, 1980a; Hibberd & Leedale, 1972) and *Botryochloris similis* (Preisig & Wilhelm, 1989) pyrenoids were not shown.

A red body similar to that found in the cytoplasm of the Eustigmataceae and in *Monodopsis* was not seen in *Pseudocharaciopsis minuta*; the osmiophilic globules abundant in the cytoplasm and possibly equivalent to those in the red body are not enclosed in a vesicle (compare Fig. 23 with Figs 18, 31 and 32). In *P. ovalis* a red body was not shown; in *Botryochloropsis similis* the presence of a red body was indicated by the authors and shown in a settling cell; its structure is different both from the red body in the Eustigmataceae and the clusters of osmiophilic globules found in the cytoplasm of *Pseudocharaciopsis minuta*, consisting of homogeneous osmiophilic material as was reported also in the large azoosporic species *Chlorobotrys regularis* (Hibberd, 1974); unfortunately, details of the structure of the vegetative cells in *Botryocloropsis similis* were not given to allow further comparisons.

Despite the poor fixation achieved with representatives of the family Monodopsidaceae, some ultrastructural features previously reported were confirmed in all species, such as absence of a chloroplast girdle lamella and connection between the CER and the nuclear envelope in *Nannochloropsis* (Antia et al., 1975; Lubian, 1982a,b; Maruyama *et al.*, 1986); the same connection was observed for the first time in both species of *Monodopsis*.

The presence of lamellate vesicles in the cytoplasm, only clearly illustrated in *N. oculata* (Maruyama *et al.*, 1986), was established for the three strains of

Nannochloropsis and for both species of *Monodopsis*. However, the presence of lamellate material described within the chloroplast matrix in *Nannochloropsis* (Antia *et al.*, 1975) was never observed in any of the species studied.

A clear terminal pyrenoid whose matrix is devoid of chloroplast lamellae and a vesicle with osmiophilic globules, similar to the red body of the Eustigmataceae, are constant features in the cells of both species of *Monodopsis*, but were not found in any of the three studied representatives of *Nannochloropsis* or those reported previously; however, in the two species studied by Antia *et al.* (1975), a pyrenoid was occasionaly seen (though not convincingly illustrated, in my opinion) and suggested to be a transient structure.

The plug-like structures in the cell wall of all the representatives of the genus *Nannochloropsis* have no parallel in the other Eustigmatophyceae so far and have not been reported before. Their function is unknown; they may be related to the habitat of these species (marine or brackish organisms), being part of some osmoregulatory system present in the cell or they may simply appear at some stage during the cell cycle when the cell is encysting, if cysts are formed.

Concerning the Monodopsidaceae, Hibberd (1981) stated "The separation of this family into two genera largely on the basis of size is somewhat artificial but is necessitated by the lack of comparative ultrastructural studies". The results presented here not only confirm the position of this family within the Eustigmatophyceae but also agree with the placement of the known species in two different genera; in

fact, beyond the well established aspects of eustigmatophycean cell structure such as the presence of lamellate vesicles and absence of a girdle lamella, these are the only representatives studied so far where the connection between the CER and the NE is clearly present; on the other hand, together with differences of size and habitat, typical terminal pyrenoids and a red body are constant only in *Monodopsis*, not in *Nannocloropsis*, and plug-like structures have been observed only in *Nannochloropsis*.

B. STRUCTURE OF THE ZOOSPORES

1. General

Results on general zoospore structure in uni- and biflagellate zoospores mostly confirm previous observations (Hibberd, 1969; Hibberd & Leedale, 1972; Lee & Bold, 1973). However, a very small Golgi body was observed for the first time in the zoospores of *Vischeria helvetica*, close to the anterior end of the nucleus; considering that in the vegetative cells the Golgi body is also very small and difficult to observe and considering the high number of vesicles always present in the zoospores near the tip of the nucleus, it is my conviction that a small Golgi body is always present in the uniflagellate zoospores but extremely difficult to observe and possibly exists in a very vesiculate condition rather than being absent as reported previously (*e.g.* Hibberd, 1980a, 1981).

In the biflagellate zoospores of *Pseudocharaciopsis minuta*, the eyespot droplets occupy the same anterior position close to the long flagellum and independent from the chloroplast, but they are different from those in the Eustigmataceae and other Pseudocharaciopsidaceae studied (Hibberd, 1969, 1980a; Hibberd & Leedale, 1972; Preisig & Wilhelm, 1989) in possessing a central or peripheral transparent layer in the otherwise osmiophilic material. This particular structure may allow the eyespot to be strongly reflective in a way similar to that of multilayered eyespots found in other algae (for review see Foster & Smyth, 1980).

The two large dense vesicles found in zoospores of *P. minuta*, with no parallel in other eustigmatophycean species, possibly have a role in the settling stages (see Section F.2, Chapter III).

The general structure of the tribophycean zoospores agrees with previous studies on these same species (Massalski, 1969) or other related ones (Hibberd, 1980b; Massalski & Leedale, 1969; Hibberd & Leedale, 1971b; O'Kelly, 1989). The only report of a single-stalked pyrenoid in *Heterococcus* is given by O'Kelly (1989), resembling that of *Vaucheria woroniniana* (Marchant, 1972); a similar structure was observed in the chloroplast of *Heterococcus marietanii* and *H. protonematoides* but its occurrence in different parts of the chloroplast, together with its convoluted outline (see Figs 135, 150, 168), appears more like thylakoid-free outpocketings of the chloroplast.

In many sections of the zoospores studied here profiles of microbody-like organelles were observed (e.g. Figs 102, 131, 155, 165), never previously reported in the Eustigmatophyceae and observed only in *H. tectiformis* of the Tribophyceae (O'Kelly, 1989).

2. External flagellum, transition region and basal bodies

a. Eustigmatophycean zoospores

Most previous observations have been confirmed but, in all the species studied, some sections show the flagellar swelling as clearly crystalline in structure (e.g. Figs 90-95, 129, 131). Considering that the photoreceptor apparatus in the Eustigmatophyceae is morphologically similar only to that of the Euglenophyceae (for reviews, see Dodge, 1973; Foster & Smith, 1980; Greuet, 1982), the crystal in the flagellar swelling of the Eustigmatophyceae may be equivalent in function to the crystalline paraflagellar body of *Euglena* (Kivic & Vesk, 1972; Piccinni & Mammi, 1978; Wolken, 1977), acting as a photoreceptor in the process of phototactic orientation (for reviews, see Foster & Smyth, 1980; Smyth et al., 1988) and, possibly, also contains flavoprotein.

Green fluorescence has recently been observed in one of the isokont flagella of some prymnesiophytes and only in the short flagellum of chrysophytes, synurophytes and phaeophytes (Coleman, 1988; Kawai, 1988; Kawai & Inouye, 1989), indicating that a flavoprotein is likely to be the

receptor pigment for phototaxis in these organisms. In tribophycean organisms, autofluorescence was observed in both flagella of *Botrydiopsis*, but not in *Vaucheria* and not in the eustigmatophytes (*Pseudocharaciopsis*), cryptophytes or raphidophytes (Kawai & Inouye, 1989). Green fluorescence was also observed in the paraflagellar body of *Euglena* (Coleman, 1988); if both photoreceptors work in a similar fashion, it seems plausible to predict the localization of the photoreceptor pigment (likely to be a flavoprotein) in the flagellar swelling of the Eustigmatophyceae and, as a consequence, autofluorescence in this part of the long flagellum. Therefore, other species must be studied to support or reject this reasoning, and to compare with Kawai and Inouye's negative results on *Pseudocharaciopsis*.

The presence of MTs in the flagellar swelling of *Pseudocharaciopsis minuta*, previously reported by Lee and Bold (1973), was not confirmed; instead, fibrillar material seems to be present.

Concerning the transition region, a transitional helix with 4-5 gyres, reported before in *Vischeria helvetica* and both flagella of *Pseudocharaciopsis ovalis* (Hibberd, 1979, 1980a), was observed in the long flagellum of all the uniflagellate species studied and in both flagella of *P. minuta*, appearing therefore to be a constant feature in the eustigmatophycean zoospores.

Some ultrastructural aspects of the basal bodies common in other organisms (for reviews see Moestrup, 1982; Preisig, 1989), were observed for the first time in the Eustigmatophyceae, such as transitional fibres connecting

them to the plasmalemma, dense material and a striated band connecting them to each other, and the presence of a cartwheel structure in their proximal region.

b. Tribophycean zoospores

A double helix resembling the concertina-structure (or double helix) of heterokont fungi (Barr, 1981; Barr & Allan, 1985; Cooney et al., 1985; Barr & Desaulniers, 1987) was previously observed in the zoospores of *Botrydiopsis alpina* (Hibberd, 1979) and *Heterococcus tectiformis* (O'Kelly, 1989); one helix was reported by Massalski in zoospores of *Heterococcus*, but two are clearly seen in *H. moniliformis* (Massalski, 1969, Fig. 77). In both species of *Heterococcus* studied here, a double helix was present in the flagella, suggesting this feature to be constant in this tribophycean genus and possibly common to the group, rather than atypical.

Another aspect constant in this genus is the presence of several dense spheres of material inside the basal bodies, mentioned before only as two in number for *H. protonematoides* (Massalski, 1969) and also shown in micrographs of *H. tectiformis* (O'Kelly, 1989).

3. The flagellar roots

a. Eustigmatophycean zoospores

Four basic types of microtubular roots are now recognized in heterokont algae and fungi, referred to as roots R1, R2, R3 and R4 (for reviews see Andersen, 1987, 1989a,b; Preisig, 1989). These roots are present in the zoospores of *Vischeria stellata* and the other uni- and biflagellate species observed here in a system that, so far, seems typical for the class and quite similar to that displayed by the oomycete fungi (compare Fig. 70 with Fig. 1d in Andersen, 1987 and Fig. 35f in Andersen, 1989b).

Root R1 has the same origin and similar number of MTs and also seems to nucleate cytoskeletal MTs in the zoospores of *Vischeria stellata* as it does in the fungi and most of the other heterokont algae (Andersen, 1987; Preisig, 1989). This root R1, together with root R2, seems to loop around the flagellum (F1), as do roots R3 and R4 around the smooth flagellum (F2) in the Chrysophyceae; they presumably have a similar function of maintaining a close association between the flagellar swelling and the eyespot in both groups and, if this is so, it may be a feature common to other organisms with related types of photoreceptors. Root R1 in the Synurophyceae also loops but the photoreceptor consists only of paired flagellar swellings, not associated with the cell or chloroplast membranes, and an eyespot is absent (Andersen, 1985, 1987); the loop, therefore, may help to maintain the parallel position of the flagella and

swellings, possibly important in the photoreception of these organisms.

Root R3 is also associated with dense material between the two basal bodies and does not extend anteriorly beyond them, as is also the case in most of the other heterokonts. Transverse sections of the root show that the 5 MTs are arranged in a semicircular manner as in the thraustochytrids (Barr & Allen, 1985) but it was not possible to determine with certainty if it originates from basal body B2, basal body B1 or both; in some sections (e.g. Fig. 75) it seems to be connected to basal body B1 by one or two bands of dense fibrillar material. A function in maintaining the long flask-shaped form typical of the eustigmatophycean zoospores seems likely since some of the MTs were seen running to the very posterior end of the cell.

Finally, root R4 also originates on basal body B2 but runs in a unique way, anteriorly first and then posteriorly, around the bulging eyespot, presumably maintaining the characteristic shape of the latter and also of the anterior end of the cell, together with roots R1 and R2. The position of these roots in relation to the eyespot and the flagellar bases also suggests the involvement of these structures in the phototactic control of cell movements, as proposed for *Woloszynskia coronata*, a dinoflagellate with a similar type of eyespot (Dodge, 1984).

The rhizoplast in *Vischeria* appears basically similar to that described for other heterokont algae, namely Chrysophyceae, Synurophyceae and Raphidophyceae (for reviews see Moestrup, 1982; Preisig, 1989). Previous published

micrographs show a fibre of the rhizoplast and MTs of roots R4 and R3 in zoospores of *Vischeria helvetica* (Hibberd, 1980a; Hibberd & Leedale, 1972), root R4 in *V. stellata* (Hibberd & Leedale, 1972), roots R1 and R3 in *V. punctata* (Hibberd, 1980a; Hibberd & Leedale, 1972) and one MT of root R3 in *Pseudocharaciopsis ovalis* (Hibberd & Leedale, 1972).

A fundamentally different interpretation of my results has been suggested by Andersen (pers. comm.). Based on recent work on flagellar transformation (Beech *et al.*, 1988; Wetherbee *et al.*, 1988) and the fact that the long flagellum of the Eustigmatophyceae possesses the swelling and is associated with the eyespot, Andersen considers this flagellum could be equivalent to the short flagellum of other heterokont algae and one could therefore number the roots according to this assumption. However, why is re-interpretation of F1 and F2 necessary? Why should a short flagellum exist (in the biflagellate species) and not bear mastigonemes? Why should the short flagellum acquire mastigonemes and the cell reorientate so that F2 is long and directed anteriorly if it corresponds to the short flagellum of other heterokonts? Why are there heterokonts with two flagellar swellings, such as the synurophytes, and yet the long flagellum with mastigonemes is not also considered equivalent to the short flagellum of other groups? What is the initial justification for considering this more complicated explanation? In my opinion, this interpretation is much less likely than the one I have given.

b. Tribophycean zoospores

The results presented here for the two species of *Heterococcus* studied are in agreement with the reconstruction of the flagellar apparatus in *H. tectiformis* (O'Kelly, 1989) and previous studies on tribophytes, as concerns the microtubular roots (compare Fig. 146 with Figs 3 and 4 in O'Kelly, 1989 and Figs 2c and 35e in Andersen, 1989a,b, respectively); in fact, the number of microtubular roots, their origin and their orientation are all similar, though it was difficult to determine the exact number of MTs in each root of *H. marietanii* and *H. protonematoides*. Previous studies on these species (Massalski, 1969) do not show flagellar roots, but consider them to be similar to those in *Bumilleria sicula*.

It is possible that the rhizoplast in *H. marietanii* and *H. protonematoides* corresponds indeed to the cross-banded root described for *Bumilleria* as "composed of apparently rectangular blocks of dense material" and "always associated with the surface of the nucleus" (Massalski, 1969) and to the rhizoplast of *Ophiocytium maius* (Hibberd, 1980b), evident in Andersen's drawing of the tribophycean flagellar apparatus (Andersen, 1989a, Fig. 2c). It is also possible that the dense material that appears between both basal bodies in *H. marietanii* (Figs 163, 164) and the short fibrous band mentioned in *Heterococcus tectiformis* by O'Kelly (1989) as extending "from a central density at the proximal end of each basal body to the nuclear envelope" may also be parts of the same rhizoplast.

The other fibrous root in *Bumilleria* is perpendicular to the possible rhizoplast and described by Massalski as "slightly curved electron-dense bands"; a similar fibrous root does not seem to be present in *Heterococcus*.

C. STRUCTURE OF THE SETTLING CELLS

No previous studies exist on the settling stages of eustigmatophycean zoospores but the process appears similar to that of other algae (e.g. *Bumilleria*, Massalski, 1969), with attachment to a substrate, rounding up of the cell, withdrawal of the flagella and formation of the new cell wall; the presumptive cell wall material seen inside vesicles in the area of the future wall in *Vischeria* and *Pseudocharaciopsis minuta* closely resembles vacuoles shown in settling cells of *Bumilleria*.

The pyrenoid disappears at a certain stage during the process of zoospore formation and regenerates from the internal face of the chloroplast soon after settling. Considering the presence of numerous typical spiral vesicles in all the eustigmatophycean zoospores, and only in the zoospores, together with the observation that in settling cells similar vesicles seem to fuse with the chloroplast in the region of pyrenoid formation, it seems reasonable to suggest that pyrenoidal material might be stored in the zoospores inside the spiral vesicles. Unfortunately, experiments with enzymes designed to test this possibility, produced negative results. Since the pyrenoid matrix has been reported to consist of proteinaceous material in other

algae (e.g. Salisbury & Floyd, 1978; Kerby & Evans, 1978, 1981; Kuchitsu et al., 1988), sections of *Vischeria stellata* were exposed to proteolitic enzymes, but no digestion occurred either in the forming pyrenoid or in the spiral vesicles. A different role for the spiral vesicles was suggested by Preisig and Wilhem (1989): the provision of material for substrate attachment on settling.

In *Pseudocharaciopsis minuta*, the two large vesicles present in the zoospores may be important in the settling stages, perhaps giving rise to the stalk and attaching disc of the vegetative cells in this species; however, such vesicles were not reported in *P. ovalis* (Hibberd, 1969; Hibberd & Leedale, 1972), a species also with a short stipe and an attaching foot.

D. MITOSIS AND CYTOKINESIS

The pattern of mitosis displayed by the Eustigmatophyceae remains a major subject for future research. The few micrographs obtained show only that the chloroplast and pyrenoid seem to divide prior to mitosis and the presence of basal bodies near the nucleus. This indicates the possible role of basal bodies as microtubule-organizing centres during the process, giving origin to the MTs of the mitotic spindle, as is the case in many other algae (for reviews see Stewart & Mattox, 1980; Santos, 1986). Other aspects with phylogenetic importance, such as the type of spindle and behaviour of nuclear envelope and nucleolus, were not observed.

The cytokinetic process, also possibly important in algal phylogeny (Pickett-Heaps, 1976), appears to be accomplished in this group by a cleavage furrow.

E. CELL WALL STUDIES

Aspects of cell wall morphology, structure and chemistry are also used in classifying the algae. Unfortunately, in comparision with the Rhodophyceae, the Chlorophyceae and the Phaeophyceae the information available on the cell wall structure and chemistry for other groups, with few exceptions, remains extremely sparse (for reviews see Mackie & Preston, 1974; Percival & McDowell, 1981). This is particularly true in the case of the Eustigmatophyceae and the Tribophyceae.

So far, the only record on the chemical composition of the cell wall in the Eustigmatophyceae is given by Ford & Percival (1965), for *Monodus subterraneus*, previously considered a member of the Tribophyceae. The principal cell wall polysaccharide found was a B-D glucan containing both 1,3 and 1,4-linked units. Another reference to the cell wall of the Eustigmatophyceae in comparison with the Tribophyceae states that "eustigmatophycean cell walls differ from those in many Xanthophyceae in showing no swelling in dilute alkalis, no intense staining with basic dyes and in never giving a positive reaction with Schiff's reagent without prior acid hydrolysis" (Hibberd, 1980a). Negative reactions in chemical tests for cellulose were observed in *Chlorobotrys regularis* (Hibberd, 1974) and, on the basis of these

reactions, the refractile appearance of the wall, its flexibility and transparency to electrons, the author considers the cell wall probably not silicified but composed primarily of pectic materials with little cellulose.

In both species investigated here, the results indicate that the cell wall may be primarily cellulosic. The major monosaccharide found, after complete acid hydrolysis, was glucose and the nebulous X-ray diffraction diagrams could be consistent with cellulose in a fairly non-crystalline, amorphous state (Dr J. Lydon and Ms Julie Cox, pers. comm.). The chromatographic results also appear to indicate that microfibrils of cellulose are possibly linked together by polysaccharides consisting of arabinose and rhamnose, as is common in other cellulosic walls (Albersheim, 1975).

F. PHYLOGENY OF THE EUSTIGMATOPHYCEAE

The Eustigmatophyceae as a class has been generally accepted by phycologists but its elevation to divisional level as the Eustigmatophyta has been controversial. Several authors place the class in a separate division (e.g. Leedale, 1974; Loeblich & Loeblich, 1978; Hibberd, 1981) while others include it in the same division as other heterokont algal classes and aquatic fungi (e.g. Cavalier-Smith, 1986).

Flagellar differences have long been used for taxonomic and phylogenetic purposes and, recently, the ultrastructure of the motile cell, in particular the flagellar apparatus, has become the most useful indication of relationships within the protists in general and the algae in particular

(Hibberd, 1979; Moestrup, 1982; Melkonian, 1984; O'Kelly & Floyd, 1984; Preisig, 1989). Based on this information, Hibberd considers the most consistent classification to be that of Van den Hoek (1978), where the group is raised to the level of division; on the same information, Preisig (1989) maintains the Eustigmatophyceae within the Heterokonta *sensu* Cavalier-Smith (1986).

The main ultrastructural features shared by the Eustigmatophyceae and most of the classes included in this division are listed below. Information on other classes is primarily based upon several reviews, concerned with general algal ultrastructure (*e.g.* Dodge, 1973, 1974; Santos, 1986) or with the flagellar apparatus (Hibberd, 1979; Moestrup, 1982; Andersen, 1987, 1989a,b; Preisig, 1989), the photoreceptor system (Foster & Smyth, 1980; Greuet, 1982; Smyth *et al.*, 1988) or the chloroplast (Whatley & Whatley, 1981; Gibbs, 1981; Billard, 1985).

1) Presence of heterokont flagellation, with tubular mastigonemes on the long flagellum.
2) Similar transition region, with a transitional helix in both flagella, if the species is biflagellate.
3) Position of the two basal bodies at more or less a right angle to one another.
4) Similar basic system of flagellar roots.
5) Existence of a photoreceptor involving a flagellar swelling.

6) Chloroplast surrounded by endoplasmic reticulum (CER) sometimes connected with the nuclear envelope (NE).

7) Chloroplast lamellae consisting of 3 thylakoids.

The main differences of cell ultrastructure that separate the Eustigmatophyceae from those same groups are:

1) Unique type of photoreceptor, consisting of a flagellar swelling located at the base of the long flagellum (even if the species is biflagellate) in association with a very large, extraplastidial and non-membrane-bounded eyespot. In other heterokont algae where a photoreceptor is present, it is of the chrysophycean or synurophycean types (Andersen, 1987); the former includes a swelling on the short flagellum associated with an intraplastidial eyespot and the latter consists of paired flagellar swellings, with no eyespot.

2) Absence of a girdle lamella in the Eustigmatophyceae; however, a girdle lamella is also absent in some representatives of other classes (Raphidophyceae, Synurophyceae, Chrysophyceae and Tribophyceae).

In my opinion, the new information presented here on the flagellar apparatus, especially aspects such as the

system of flagellar roots and the constant presence of a transitional helix, supports a relationship of the Eustigmatophyceae not only with the heterokont algal classes Raphidophyceae, Tribophyceae (=Xanthophyceae), Chrysophyceae, Synurophyceae, Phaeophyceae and Bacillariophyceae, but also with the oomycete fungi; the existence of a clear connection of the CER with the nuclear envelope in the Monodopsidaceae is another feature shared with most heterokont algae. The Eustigmatophyceae should therefore be placed with these classes in a common division, the Heterokontophyta *sensu* Leedale (1980), with a heterokont cell as common ancestor.

Since the main difference between the groups is the type of photoreceptor, the derivation of the different classes from this ancestor can be interpreted as a result of evolution of different types of photoreceptors. If we consider that:

1) The biflagellate ancestor would be close to the eochromophyte of Cavalier-Smith (1986), a phagotrophic organism in which evolution of a photosensitive mechanism would favour a switch to phototrophy from phagotrophy.

2) A substance was present in <u>both</u> flagella which became organized for photoreception, perhaps in paired swellings (as in modern Synurophyceae);

3) Eyespots would be more recently acquired accessory structures of the photoreceptor apparatus (see Kivic & Walne, 1983).

4) The eustigmatophycean type of photoreceptor would evolve by retention of the swelling on the long mastigoneme-bearing flagellum and acquisition of an extraplastidial eyespot.

5) The tribophycean, chrysophycean and phaeophycean type of photoreceptor would evolve by retention of the swelling on the short smooth flagellum and development of an intraplastidial eyespot.

We may therefore assume that a synurophyte-like organism could be at the base of the heterokontophyta and divergence of the different classes occurred when more specialized types of photoreceptor were established (see Fig. 202). The proposed line of evolution for the heterokont classes is close to others proposed before (e.g. Dodge, 1979; Hibberd, 1979; Moestrup, 1982; Cavalier-Smith, 1986)

Accumulation of the photoreceptor pigment in two parallel swellings on the surface of the flagella without accessory structures such as eyespot, but probably with a transduction mechanism (TH?, roots?), would represent the first, simpler, more primitive photoreceptor apparatus to evolve and, therefore, the Synurophyceae must have branched off at this early stage.

With specialization, in the Tribophyceae, Chrysophyceae and Phaeophyceae, the swelling was kept on the short flagellum and an intraplastidial eyespot evolved, this flagellum being mainly concerned with photoreception and the long flagellum with locomotion. In the Eustigmatophyceae the

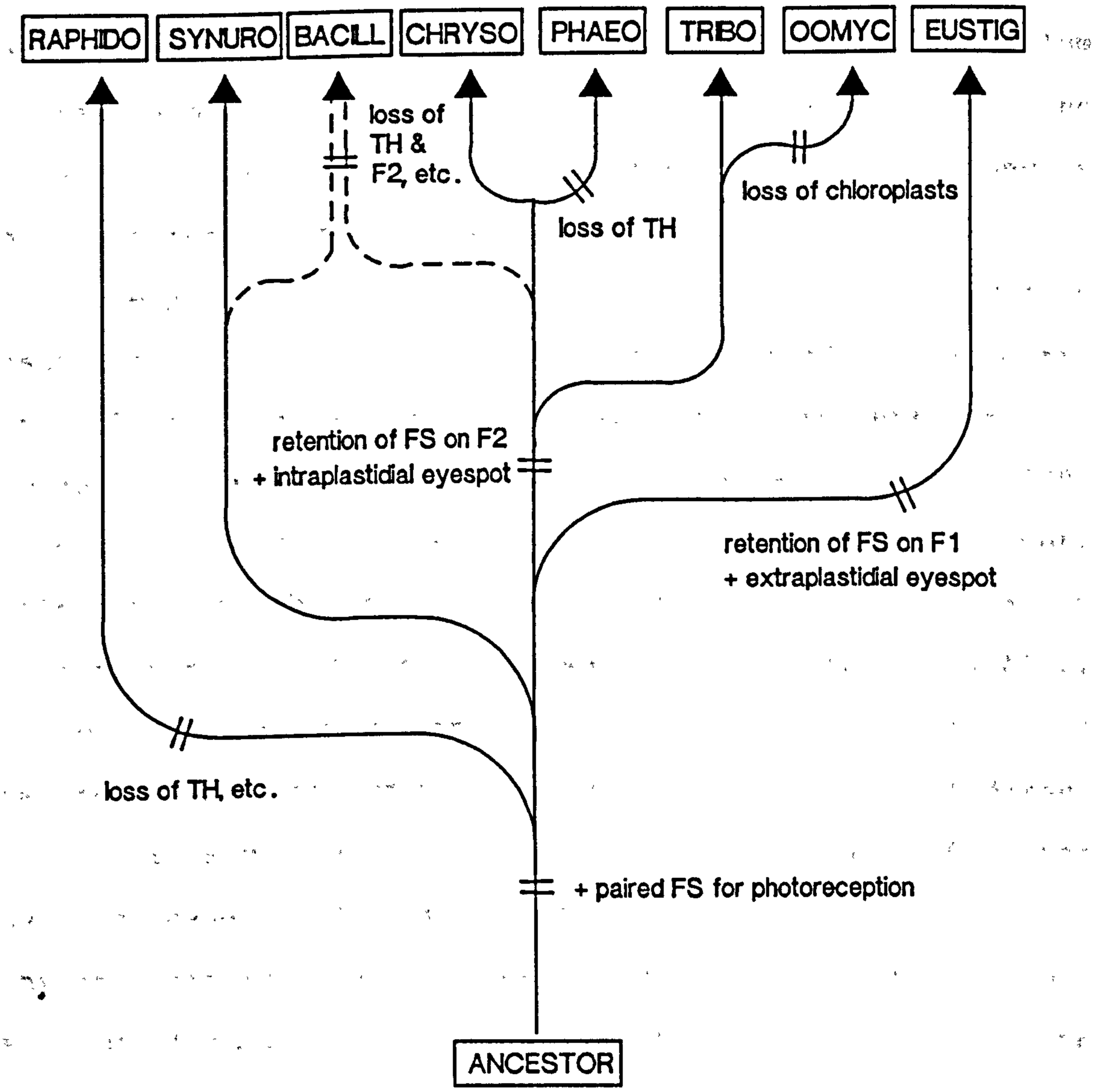

Fig. 202. Proposed phylogenetic tree for the Heterokontophyta. F1, flagellum with mastigonemes; F2, smooth flagellum; FS, flagellar swelling; MT, microtubular; TH, transitional helix; TMs, tubular mastigonemes;

swelling was kept on the long flagellum and an extraplastidial eyespot was acquired, the same flagellum therefore being both photoreceptive and locomotory, with no "need" for the second flagellum; the uniflagellate species may therefore be more advanced than the biflagellate.

The position of the Raphidophyceae is more difficult to establish. The presence of possibly primitive characters (periplast, extrusomes, some phagotrophic members) and the absence of cell wall and eyespot (see Heywood, 1980, 1989) appear to support the hypothesis that they are more primitive than the Tribophyceae, a group considered close by some authors (e.g. Ragan & Chapman, 1978; Dodge, 1979). Also, if swellings exist on both flagella, as pointed out with reference to *Vacuolaria* (see Heywood, 1983) by Andersen (1987), this may indicate that the group diverged from the Synurophyceae (with loss of the transitional helix) or is phylogeneticaly prior to them (and may have separated before a transitional helix became established). Since the Raphidophyceae also have a well developed fibrous root connecting the basal bodies to the nucleus that resembles more the conspicuous rhizoplast of the Synurophyceae than the small unbranched rhizoplast of the Tribophyceae, I consider this group as the most primitive within the heterokontophytes.

On the basis of the characters considered here (flagella, photoreceptor apparatus and chloroplast structure), the diatoms are another difficult group to place since the chloroplast features are shared by most of the algal groups under discussion, but the flagellar apparatus

is probably highly specialized (Manton & von Stosch, 1965). Until further information becomes available, and considering the similarity on other structural aspects between the Bacillariophyceae, Synurophyceae and Chrysophyceae (see Andersen, 1989a; Crawford & Round, 1989; Mann & Marchant, 1989), these three classes should be placed close together.

Finally, in relation to the heterokont fungi, the main results of this investigation show a very similar pattern of flagellar roots between the eustigmatophycean zoospores and the zoospores of these fungi, and that a double transitional helix is more widespread in tribophycean species than previously thought. Other aspects of cell structure show a striking morphological similarity between these three groups, in particular some vesicles that resemble the eustigmatophycean lamellate vesicles and the anterior dense bodies (see Beakes, 1989). Therefore the heterokont fungi probably evolved close to the divergence of the Eustigmatophyceae, Chrysophyceae and Tribophyceae, possibly from a tribophycean line with loss of chloroplasts.

In 1979, Hibberd considered that the possession of a transitional helix and tubular mastigonemes by the Eustigmatophyceae was more easily explained by assuming that these structures evolved prior to both the eustigmatophycean and the chrysophycean types of photoreceptor apparatus and, probably, the girdle lamella. Though this agrees with my previous discussion as far as the photoreceptor apparatus is concerned, my opinion in relation to the girdle lamella is different. Since several raphidophycean, synurophycean, chrysophycean and tribophycean species do not possess a

girdle lamella, this character seems to have been lost on several occasions. This implies that it could have been lost in the Eustigmatophyceae and that this class is not necessarily more primitive than those groups with a chrysophycean type of photoreceptor and girdle lamella.

In conclusion, the possible common ancestor may have been a flagellate cell, with two subequal or unequal flagella attached at the anterior end of the cell, with recently acquired mastigonemes on the longer, and, with recent symbiotic (phagotrophic) acquisition of chloroplasts, selective pressure to develop an efficient photoreceptor. The ancestor would also have or be in process of evolving a transitional helix, a descending fibrous flagellar root (rhizoplast), and a basic system of microtubular flagellar roots which could develop into the different patterns discussed above and summarized by Andersen (1987) and Preisig (1989). It would acquire a chloroplast organization characterized by four surrounding membranes (CER and chloroplast envelope), three thylakoids per lamella and possibly a girdle lamella. Selective pressure would result in evolution of a rudimentary type of photoreceptor, consisting of paired swellings (one on each flagellum) but no eyespot.

From this basic ancestor, the different groups within the Heterokontophyta would arise as a consequence of the further development of different types of photoreception, changes in the positions and the morphology of the flagellar apparatus (including the roots), and different cell shapes.

Interestingly, if one considers the prymnesiophytes,

their biochemistry (Ragan & Chapman, 1978), some aspects of the cell structure (e.g. Hibberd, 1976; Green et al., 1982), in particular displayed by the Pavlovales (see Green & Hibberd, 1977; Van der Veer, 1979; Green, 1980), green fluorescence (Kawai & Inouye, 1989), and the possible vestigial presence of mastigonemes in the ER of *Prymnesium* (Cavalier-Smith, 1986), the case could be argued for inclusion of this class in the same division with the heterokont algae and aquatic fungi and with the same possible ancestor. This would give a division that includes the organisms grouped by Cavalier-Smith (1986) in the subkingdom Chromophyta and with the heterokont ancestor positioned between the Cryptophyta and the Chromophyta *sensu* that author, after divergence of mitochondrial crista morphology has occurred (the eochromist of Cavalier-Smith).

They could thus represent an early off-shoot from the line leading to the Heterokontophyta which separated at a time when mastigonemes were establishing and neither the transitional helix nor girdle lamella had yet developed; they would have evolved a different photoreceptor mechanism, and a different flagellar apparatus (losing mastigonemes in both flagella, or never developing them, acquiring a different transition region and system of flagellar roots). In my opinion, these aspects are important enough to isolate them in a separate division and are reinforced by the presence of the haptonema, "peculiar" Golgi and unique scales.

G. CONCLUSIONS

The cytoplasm of all the eustigmatophycean cells, either vegetative or zoospores, always contain lamellate vesicles. This is so far the most typical character, displayed by all known species and the most reliable for identification at the ultrastructural level. The structure of the chloroplast is also constant but, contrary to the lamellate vesicles, chloroplasts surrounded by endoplasmic reticulum, with lamellae consisting of three thylakoids and no girdle lamella are found in members of other groups. Therefore, the lamellate vesicles are the only exclusive feature for diagnosis of the azoosporic forms with the electron microscope.

Another constant ultrastructural feature in the small azoosporic forms of the family Monodopsidaceae revealed by this research, is the connection of the CER with the nuclear envelope, not seen in other studied members of the Eustigmatophyceae. Pyrenoids are also highly diagnostic features for identification of the vegetative cells in the Eustigmataceae but these and other zoosporic members are more easily identified by the unique type of zoospores produced. Together with other ultrastrutural features previously known, such as the eyespot, the lamellate and spiral vesicles, the system of flagellar roots is similar in all the species studied so far and also appears to be diagnostic of eustigmatophycean zoospores.

Present knowledge of the Eustigmatophyceae indicates that this class of organisms is not, however, so different

from other groups of heterokont algae and fungi as to justify their being raised to divisional level. On the contrary, the most significant results of this investigation support the inclusion of the Eustigmatophyceae in a common division with all the other heterokont algae and fungi and a sharing of the same ancestor.

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APPENDIX 1

Preparation of the micronutrient solution

	Stock solution (%)	Applied solution
$ZnSO_4.7H_2O$	0.1	1.0 ml
$MnSo_4.4H_2O$	0.1	2.0 ml
H_3BO_2	0.2	5.0 ml
$Co(NO_3)_2.6H_2O$	0.02	5.0 ml
$Na_2MoO_4.2H_2O$	0.02	5.0 ml
$CuSO_4.5H_2O$	0.0005	1.0 ml
Distilled water		981 ml
$FeSO_4.7H_2O$		0.7 g
EDTA (Titriplex III, Merck)		0.8 g

Autoclave the components separately in two solutions which are united after cooling.

Solution I: 881 ml distilled water + stock solutions of salts without $FeSO_4$ + 0.4 g EDTA

Solution II: 100 ml distilled water + 0.7 g $FeSO_4.7H_2O$ + 0.4 g EDTA

APPENDIX 2

TABLE II. Growth of *Vischeria stellata* (duplicate cultures) in medium 1b, at 18-23°C, during a fortnight.

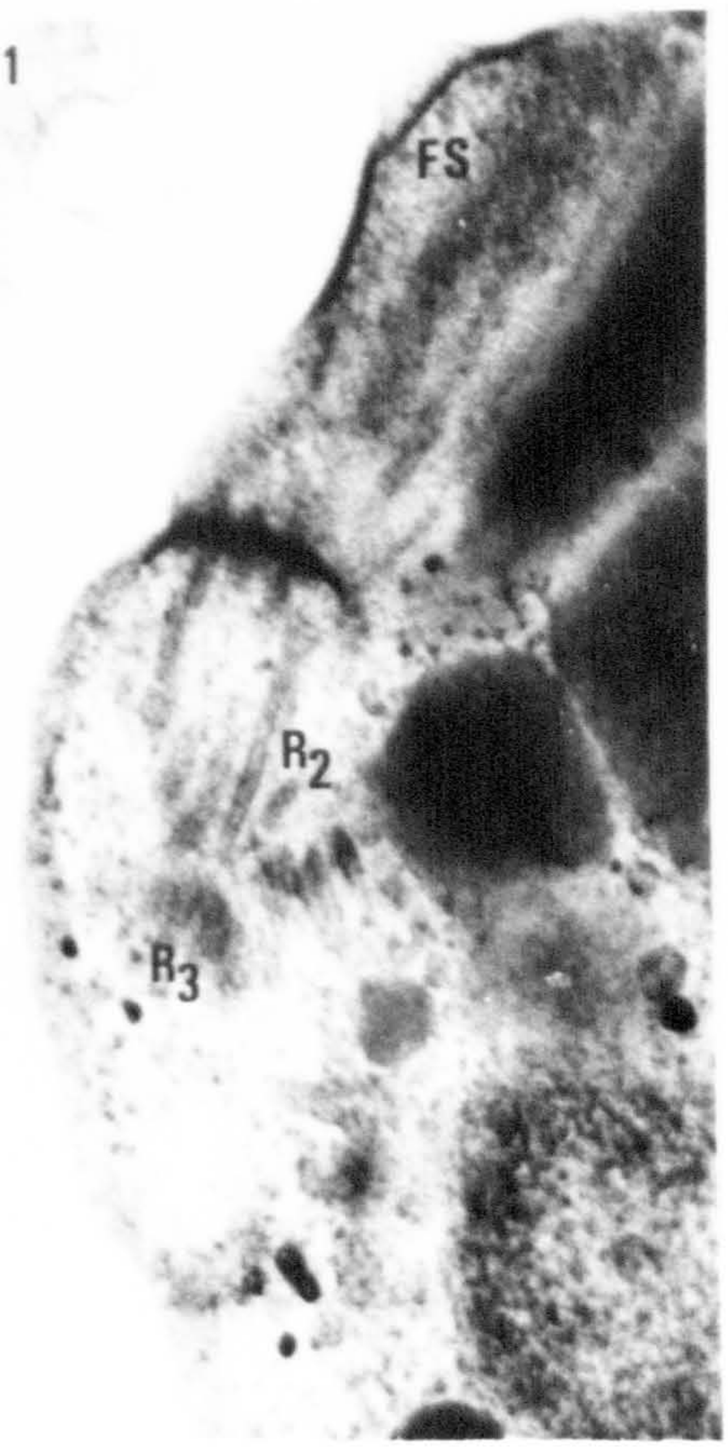
DAYS	CELLS / ml ($x10^3$)		
	Culture 1 (C1)	Culture 2 (C2)	C1+C2/2
1	20.9	16.7	18.8
2	21.8	14.9	18.4
3	24.6	20.4	22.5
4	31.8	24.9	28.4
5	32.3	24.3	28.3
6	36.4	28.2	32.3
7	37.0	32.8	34.9
8	44.1	35.5	39.8
9	47.7	39.4	43.6
10	49.6	43.7	46.7
11	59.4	47.0	53.2
12	67.0	56.4	61.7
13	74.1	32.5*	53.3
14	73.6	---	---

*Culture infected with fungi

APPENDIX 3

Two selected sections of the zoospores of Vischeria stellata showing the possible origin of cytoskeletal MTs (arrows) from root R1. 1, 2 x 62000.

1



2