THE STRUCTURAL BASIS OF

ZOOSPORE FORMATION

IN *PHYTOPHTHORA*

BY

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A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

OF THE AUSTRALIAN NATIONAL UNIVERSITY

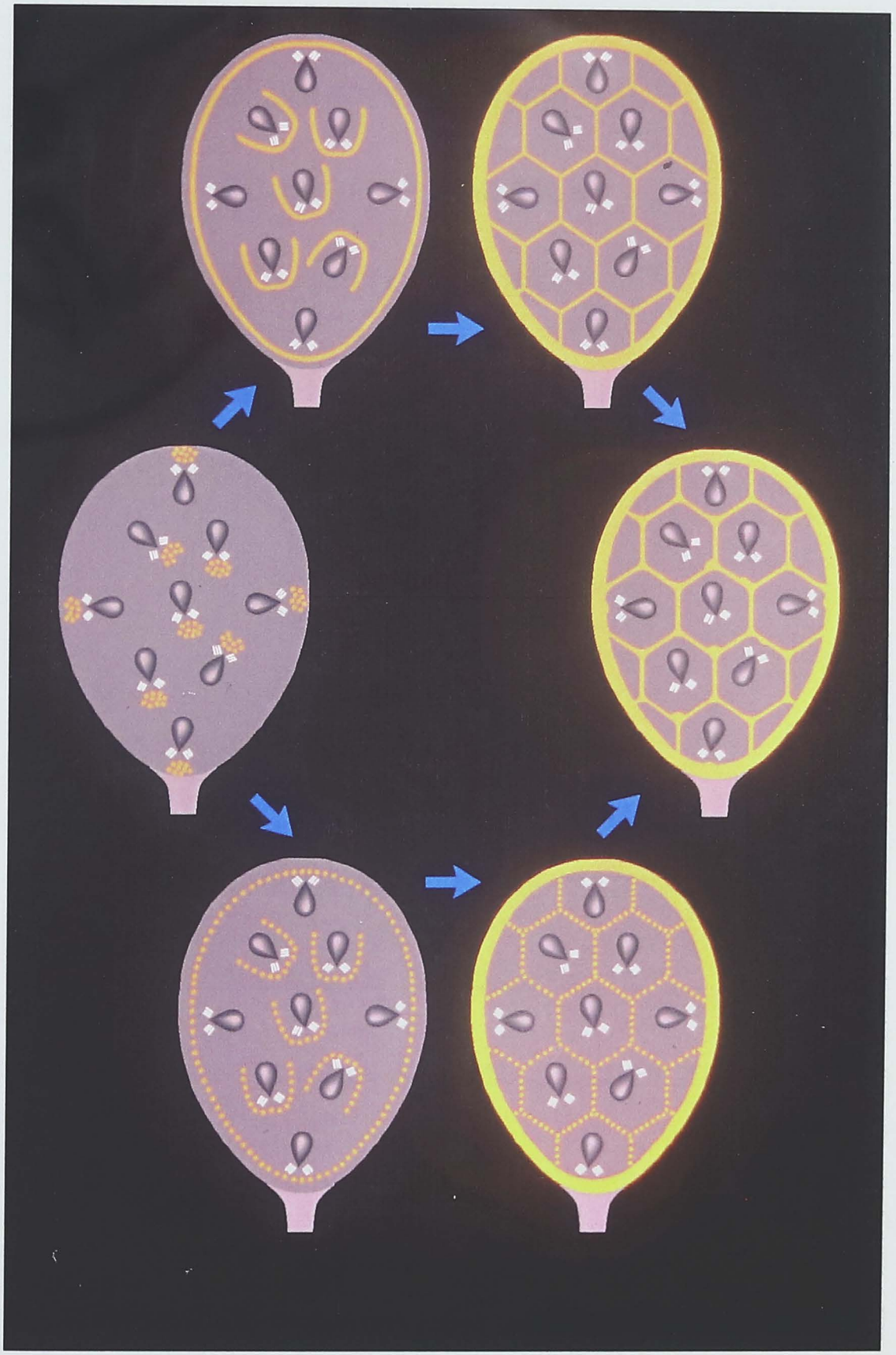
DECEMBER 1991

Dedication

This thesis is dedicated to the memory
of a man I loved most dearly,
my father, Ken Hyde,
and
to all my friends
who along the way have
sought the truth in whatever they did.

"Beauty is truth, truth beauty"

Frontispiece. This diagram summarises the most significant finding of this study. The bottom sequence shows the model of cleavage that was suggested by examination of sporangia of *Phytophthora cinnamomi* that had been prepared for electron microscopy by chemical fixation. This model was in agreement with previous studies of cleavage in this genus. The model of cleavage suggested by freeze-substituted sporangia (top sequence), however, was very different. It is believed that chemical fixation leads to artefactual vesiculation of the continuous cleavage planes seen in freeze-substituted sporangia. This finding has wide-ranging implications for eukaryotic cytokinesis. Also, see Fig 3.18.



STATEMENT

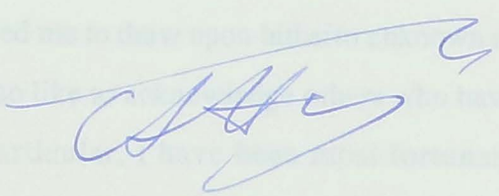
All research reported in this thesis

is original and my own

except where acknowledgement is given,

and has not been submitted

for any other degree.



Geoffrey J. Hyde

ACKNOWLEDGEMENTS

It is a great pleasure to have an opportunity to thank the many people who have contributed in so many ways towards the research and production of this thesis. Firstly I must thank Adrienne Hardham for her exemplary supervision and inspirational intellectual companionship during the course of my studies in the Plant Cell Biology Group at RSBS. Adrienne's unbounded passion for research generates a wave of energy around her. Like so many others at RSBS I have had the great fortune to be carried along in the wake of her enthusiasm. To my advisors, Frank Gubler and Brian Gunning, I am also most grateful. Frank is the happiest person ever to set foot in a lab, and was always available for some gentle guidance during the first half of my studies. Brian Gunning blesses our Group with his presence and is a constant reminder of the true potential of the human intellect. The last member of my supervisory team, Peter Hepler, joined it unofficially when I spent 9 weeks in his lab at Amherst, Massachusetts. Those were the hardest worked weeks of my thesis, and produced the most important results. It was the staggering generosity of Peter, the wondrous Peggy, and all the other happy Heplers that allowed me to draw upon hitherto unknown reserves!

I would also like to acknowledge others who have contributed intellectually to this thesis. In particular, I have been most fortunate to have had an Oomycete mycologist sitting at the desk beside me: Larry Lehnen has always been ready to share his encyclopaedic knowledge of fungi. Other resident and visiting academics have also been most helpful: Tobias Baskin, Les Watson, Andrew Staehelin and Hans Hohl. For getting me started along the scientific road, and introducing me to Adrienne, I thank Robyn Overall.

For technical assistance thanks to Jadwiga Duniec and Bruce Philip for keeping our cultures alive and ready for action. Also, thanks to Frank Sek for teaching me the secrets of running a gel. At UMASS I was given incredible support by Sue Lancelle and Dale Callaham, technicians extraordinaire. Other members of the Hepler lab, especially Zhang Dahong, were also most generous with their time and friendship. At RSBS, our electron microscope unit is one of our most valuable resources, not least because of the unfailingly knowledgeable and helpful staff. Thanks to Sally Stowe, Dave Llewellyn, David Vowles, Alan Lee and all the other good guys of the Unit.

On the production side of this thesis two people have been of immeasurable assistance. Firstly, a million thanks to Margaret Wigney who transformed my handwriting into this elegantly laid-out volume. Margaret, with her total professionalism and complete unflappability, was my rock of Gibraltar during the writing of this thesis. Secondly, James Whitehead. The exquisite illustrations that James has done for this thesis are testament enough to his creativity and skill; I would just like to add my appreciation of James' friendliness, approachability and patience.

I would also like to thank Ann Cleary and Jian Wang for helping me with some of the most tedious tasks of a thesis. Their assistance with letrasetting and compiling the bibliography was invaluable. To a little army of proofreaders, I am also in debt : John Dearnaley, Geoff Wasteneys, Michele Cope, Emer O'Gara, Dave Cahill, Brad Gabor and some of the people whom I have already thanked for other services. Thanks also to Dave Collings, Kerrong Zhang, Dave de Pury, Swati Baindur, Art Davis, Mary Webb, Don Horan, Cathy Busby, Ann Docherty and many others in RSBS who have helped along the way. Finally, the photography department, and especially Jeff Wilson, have done a fantastic job of reproducing the plates.

I would also like to thank some of my personal friends and family for their support. A PhD is quite an intense apprenticeship, and for keeping me sane over the years I thank Cameron Britten, for his most loyal and valued friendship. Woogie boogie, Cam! I thank my mother for her, as always, unconditional support; like her, I know how happy my father would have been to be part of all this. My nieces and nephews gave me unwitting encouragement by their excitement at the prospect of having a scientist in the family occasionally! To my friends, Jim and David Matthews, Luke Phuoc and An van den Borre, I now look forward to seeing more of you! As I do my other friends and family. But, in truth, the lack of hours spent outside RSBS has been amply compensated for by the pleasure of working in the atmosphere of cooperation and camaraderie of the Plant Cell Biology Group. Not to mention the volleyball!

ABSTRACT

The process of zoospore formation has been studied in the Oomycetes *Phytophthora cinnamomi* and *Phytophthora palmivora*. The aims of this study were to elucidate the structural mechanisms that bring about, firstly, the orderly cleavage of the multinucleate sporangia of these organisms and, secondly, the polarized distribution of organelles in the uninucleate zoospores formed during the cleavage process. This study was undertaken because of the limited nature of our general understanding of the formation of fungal zoospores and because zoosporogenesis is an ideal process for the study of cell differentiation.

The first part of this study was an ultrastructural examination of chemically-fixed sporangia of *P. cinnamomi*. The spatial distributions of three different vesicle types were monitored during cleavage. Identification of the vesicles was assisted by immunogold labelling, using monoclonal antibodies specific for each vesicle type. It was shown that the vesicles acquired their characteristically polarized distributions at different stages of cleavage. The process of cleavage itself appeared to involve a two-stage process. Specialized cleavage vesicles were firstly positioned at the boundaries of each future subdivision, then once alignment was complete, vesicle fusion brought about compartmentalization.

In the second part of this study sporangia of *P. cinnamomi* and *P. palmivora* were examined ultrastructurally after rapid-freezing freeze-substitution. The data from this approach suggested an entirely different mode of cleavage to that seen with chemical fixation. Cleavage elements were evident as vesicles only in preliminary stages, and therefore appeared as flattened membrane-bound structures that extended along the future subdivision boundaries. Their interconnection completed cleavage. It is proposed that freeze-fixation provides superior preservation of the cleavage systems and that the vesicles seen in late-stage chemically-fixed sporangia result from artefactual vesiculation of the extended cleavage elements. The fusion of pre-aligned vesicles has been proposed to explain the process of cytokinesis not only in other species of *Phytophthora* but in a wide variety of other eukaryotes. All these studies have, however, relied upon the examination of chemically-fixed material and must now be regarded with some suspicion.

The study of the cleavage process in sporangia prepared by both of the above methods was assisted by immunogold labelling, using a monoclonal antibody that bound to the contents of the cleavage elements. This antibody also bound to the sporangial dictyosomes, indicating that these were the source of the cleavage membranes. The antibody did not bind, however, to certain large vacuoles in the sporangium, a result which argues against previous suggestions of these vacuoles as sources of zoosporic

plasma membrane in *Phytophthora*. Immunoblotting indicated that the antibody bound to a number of high molecular proteins, possibly glycosylated. These proteins form part of a dense matrix material that is found inside the developing cleavage system, and which fills the extracellular spaces at the completion of cleavage. It is proposed that this matrix material may be a gel whose swelling produces the force to burst the sporangium and allow zoospore release.

The third part of this study used immunofluorescence microscopy to examine the distribution of microtubules in the sporangium of *P. cinnamomi*. Prior to the beginning of cleavage, extensive astral arrays of microtubules were seen, each array emanating from a point adjacent to the pear-shaped nucleus. These arrays defined equal-sized cytoplasmic domains around the nuclei. During the course of cleavage these arrays gradually changed into the characteristic arrays that have been previously described in the zoospore.

The fourth part of the study examined the possibility that these microtubules, and also actin microfilaments, might be involved in: (1) the regular spacing of sporangial nuclei; (2) structural regulation of the cleavage process and (3) polarization of the vesicles that had been examined in the first part of this study. Immunofluorescence microscopy of sporangia of *P. cinnamomi* that had been treated with the anti-microfilament drug, cytochalasin D, demonstrated that this drug caused cleavage to proceed in an abnormal manner, but did not effect nuclear positioning and the general disposition of the microtubular arrays. The three vesicle types examined in the first part of this study were concentrated along distinct subsections of the abnormal cleavage planes. These subsections could be identified by their spatial relationships to the nuclei, and in this regard, the vesicles exhibited analogous distributions to those seen in normally cleaved sporangia. In sporangia treated with oryzalin, an antimicrotubule drug, there was complete depolymerization of microtubules, randomization of nuclear positioning and abnormal cleavage. The three vesicle types were localized to the abnormal cleavage planes, but were not concentrated in any particular subsections. These results indicate that cytoskeletal elements and especially microtubules play a major role in many aspects of zoosporogenesis in *Phytophthora*. A number of new models are proposed to explain how microtubules might regulate cleavage and organelle positioning.

The findings of this study represent a major advance in our understanding of fungal zoosporogenesis. It is likely that when the techniques used in the present study are applied to other zoosporic systems there will be a similar clarification of the processes of cleavage to that which has occurred in *Phytophthora*.

LIST OF ABBREVIATIONS

CD	cytochalasin D
DAPI	4'-6-diaminophenylindole
DMSO	dimethylsulfoxide
EGTA	ethylene glycol bis (ß-aminoethylether) N',N',N',N' -tetraacetic acid
ESB	enzyme substrate buffer
FITC	fluorescein isothiocyanate
HP	high pressure
HPF	high pressure freezing
KD	kilodalton
LPV	large peripheral vesicle
M	molar
min	minute
PBS	phosphate buffered saline
PIPES	piperazine- N',N'-bis (2- ethane-sulphonicacid)
RF-FS	rapid freezing freeze substitution
SAM	sheep anti-mouse
SDS-PAGE	sodium dodecylsulphate - polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween 20
mAb	monoclonal antibody

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CHAPTER 7. ZOOSPOROGENESIS IN *PHYTOPHTHORA*: A DISCUSSION OF ITS IMPLICATIONS FOR THE LOWER FUNGI

1

STRUCTURAL ASPECTS OF FUNGAL ZOOSPORE FORMATION

1.1 INTRODUCTION

The most characteristic feature of those organisms commonly classified as the lower fungi is the production of motile zoospores: flagellated, uninucleate cells bounded only by a plasma membrane. Zoospores are one of many types of spores produced by these fungi, which most commonly exhibit a hyphal mode of growth. While other spore types are usually produced sparingly and are designed for long-term survival in adverse conditions, zoospores develop in large numbers and are suited to short-range dispersal in favourable conditions (Heath, 1976). Their production usually occurs within relatively large specialized structures called sporangia.

One of the hallmarks of fungal zoosporogenesis is that, with limited exceptions, the processes of cytokinesis and karyokinesis have been uncoupled. As a result the sporangium is typically multinucleate, and can remain in this state for some time, waiting until there is sufficient water in the immediate environment to allow dispersal of the zoospores (Lange & Olson, 1983). Once these conditions arise, the multinucleate

cytoplasm is rapidly cleaved in a simultaneous fashion to produce a number of uninucleate zoospores. The major significance of the zoospore in the life cycle of the lower fungi is that, once liberated from the sporangium, the zoospore can actively seek out a suitable environment for growth of the hyphal phase, which the zoospore is able to reform (Carlile, 1983). Such suitable environments often include other organisms, especially plants, whose viability may be weakened or destroyed by the invasion of fungal pathogens.

Of the lower fungi, it is the Oomycetes which cause more plant diseases than any other group (Alexopoulos & Mims, 1979), being responsible for serious losses in native and agricultural plant communities. Members of the Oomycete genus, *Phytophthora*, have, for example, been responsible for the catastrophic devastation of the Irish potato crops late last century (*P. infestans*) and the depletion of large areas of native bushland in Western Australia (*P. cinnamomi*). In diseases caused by *Phytophthora*, and most zoosporic pathogens, the zoospores are the primary units of dissemination. Understanding the mechanisms that underlie their development might therefore provide clues as to how to prevent their production and hence control the diseases they cause. As a result there is now a considerable body of work which pertains to structural aspects of fungal zoospore formation (reviewed by Heath, 1976; Olson *et al.* 1981).

Studies of zoospore formation have also been motivated by the recognition that zoosporogenesis provides a model process for the study of cell differentiation and developmental controls (Lange & Olson, 1983). The zoospore contains a variety of structures that facilitate motility, and a diverse array of other structures and organelles most of which exhibit a high degree of spatial ordering (reviewed by Heath, 1976; Lange & Olson, 1983). As such, the organization of the zoospore represents a major increase in complexity over that seen in the sporangium. The elucidation of how these changes occur in sporangia may shed light on similar processes in other systems which are not so amenable to experimental manipulations.

Despite the wealth of studies that have examined structural aspects of fungal zoosporogenesis, many key questions of this topic remain unanswered. For example, there is considerable confusion as to what is the true source of the membranes that partition the sporangium during cleavage (Heath, 1976). Also, little is known about how these membranes come to develop in the correct positions, or how each zoospore acquires a requisite complement of organelles (Olson *et al.* 1981). The lack of understanding of these and other issues of fungal zoosporogenesis probably results from the limitations of the techniques that have been used to address them. Studies to date have involved conventional electron microscopy of chemically fixed material. Thin section analysis is, however, not well suited to the assessment of spatial interrelationships in a structure as large as a sporangium. There have only been limited serial section analyses of sporangia (Olson *et al.* 1981; Armbruster, 1982). Also, it is difficult to evaluate the possible functions of cellular structures on purely morphological evidence. Lastly, membranes are sometimes poorly preserved by chemical fixation (Hausmann, 1977; Mersey & McCully, 1978). Some of these difficulties have been surmounted, at least partly, in other systems by the use of recently developed structural techniques, such as those involving monoclonal antibodies, freeze-substitution and fluorescence microscopy. The research of this thesis is an attempt to further our understanding of the structural basis of fungal zoosporogenesis by application of these new techniques in a study of sporangial cleavage in *Phytophthora*.

1.2 AN OVERVIEW OF SELECTED ASPECTS OF FUNGAL ZOOSPOROGENESIS

This section addresses various structural aspects of fungal zoosporogenesis which specifically pertain to the research of this thesis, but which also involve most of the key issues of this topic. The survey is based on the literature that has examined those fungi grouped within the Division Mastigomycota (*sensu* Alexopoulos & Mims, 1979),

the taxon in which almost all zoosporic fungi have traditionally been grouped. In some recent classifications (e.g., Margulis *et al.* 1989) the zoosporic fungi have been split into a number of different protoctistan phyla in consideration of their diverse evolutionary ancestry.

1.2.1 Sources of additional membrane required during cleavage

In any cell division process the membranous surface area of the resulting cells will be greater than that of the parent cell. Therefore, either the parent cell membrane must stretch or new membrane must be synthesized. While membrane stretching has at times been proposed to account for the relatively small increase in surface area that occurs during animal cytokinesis (Rappaport, 1986), in most cases of fungal zoosporogenesis there is such a dramatic increase that, with limited exception (Hoch & Mitchell, 1972, 1975), some other source of additional membrane has been suggested. The sources most commonly proposed are dictyosomes or dictyosome-analogoues (Hohl & Hamamoto, 1967; Lessie & Lovett, 1968; Elsner et al. 1970; Heintz, 1970; Williams & Webster, 1970; Travland & Whisler, 1971; Chong & Barr, 1973; McNitt, 1974; Schnepf *et al.* 1978; Taylor & Fuller, 1981; Lange *et al.* 1984, 1989; Lin & Leu, 1984; Martin & Miller, 1986; Sewall *et al.* 1986). Such suggestions have largely been based purely on morphological evidence but are in agreement with the proposed role of dictyosomal membranes in cytokinesis in other systems (Pickett-Heaps, 1975; Gunning, 1982).

In addition to dictyosomes, a great variety of other structures have been proposed as sources of the additional membrane required during fungal zoosporogenesis: endoplasmic reticulum (Lucarotti & Federici, 1984); vesicles pinched off either the plasma membrane (Renaud & Swift, 1964; Barron & Hill, 1974) or large central vacuoles (Williams & Webster, 1970); dense body vesicles (Gay & Greenwood, 1966; Gay *et al.* 1971; Olson *et al.* 1981; Armbruster, 1982); multivesicular bodies (Temmink & Campbell, 1968) and many other vesicles or vacuoles of unknown origin (Bland &

Amerson, 1973; Gotelli, 1974; Armbruster, 1982). As far back as 1976, Heath proposed that such a variety of sources for the zoosporic membranes might represent a confused state of affairs. This view is supported by the multiplicity of sources that have been proposed even in the same organism. For example, Armbruster (1982) proposed that three different vesicles supplied zoosporic membranes in all three Saprolegnialian species that she studied. In *Phytophthora capsici*, cleavage membranes have been proposed to be derived both from the membrane of large central vacuoles that are often seen in the sporangium of this species and also from dictyosomes (Williams & Webster, 1970). Heath (1976) suggested that identification of the true source of cleavage membranes would not be possible until specific morphological or histochemical markers were available. Some progress has recently been made in this regard, especially in the case of the Chytridiomycete, *Allomyces*. As discussed by Sewall *et al.* (1986), *Allomyces* lacks typical Golgi complexes composed of stacked cisternae, but studies using trans-Golgi markers (Feeney & Triemer, 1979) and Golgi-disrupting drugs (Sewall *et al.* 1986) indicate that in this genus dictyosomal analogues, consisting of single endomembrane elements, provide the additional membrane required during zoosporogenesis.

The difficulties of discerning these elements at the ultrastructural level are believed to be responsible for the proposals of numerous other sporangial structures as sources of zoosporic membrane in *Allomyces* (Sewall *et al.* 1986). It may well be that the use of membrane markers in further studies of zoosporogenesis in other fungi could also lead to a similar clarification to that which has occurred in *Allomyces*.

1.2.2 The mode of sporangial partitioning

The process of sporangial cytokinesis in the Mastigomycota is a more complex affair than normal cell division because, with the exception of the Orders Labyrinthulales and Thraustochytriales, (*sensu* Alexopoulos & Mims, 1979), the multinucleate sporangium is subdivided by a number of simultaneous subdivisions. In Section 1.2.3,

consideration will be given as to how cleavage might be regulated, but first the manner in which the membranous partitions actually form must be considered.

Data exist which support a variety of partitioning processes occurring during zoosporogenesis. The processes can be basically divided into three categories:

(a) *Fusion of pre-aligned vesicles.* In this model specialized vesicles line up along the future planes of cleavage, and when alignment is complete, fusion of these vesicles results in the formation of zones of separation, bounded on either side by membrane derived from that of the vesicles. The plane of vesicles may lie between the cytoplasmic domains of two developing zoospores, or, in the case of a zoospore forming in the sporangial cortex, between the cytoplasm of that zoospore and the sporangial plasma membrane. Such processes account for all, or most, of sporangial cleavage in each of the species of *Phytophthora* in which cleavage has been studied in detail (see review by Hemmes, 1983). Similar processes have been also reported in other Oomycetes and Chytridiomycetes (Blondel & Turian, 1960; Barron & Hill, 1974; Lunney & Bland, 1976). A somewhat less developed version of this process has been described in many other zoosporic fungi, in which vesicles or vacuoles are aligned along portions of the future cleavage planes. Completion of cleavage involves fusion and expansion of these structures (Gay & Greenwood, 1966; Heintz, 1970; Gay *et al.* 1971; Chong & Barr, 1973; Lange *et al.* 1984).

(b) *Progressive extension of cleavage furrows.* The most commonly reported mode of partitioning involves some sort of furrowing process. Such furrowing may be initiated (i) at the plasma membrane, proceeding centripetally (Heintz, 1970; Chong & Barr, 1973; McNitt, 1974); (ii) at the tonoplast, proceeding centrifugally (Gay & Greenwood, 1966; Gay *et al.* 1971; Hoch & Mitchell, 1972, 1975) or (iii) from any number of indeterminate, and often multiple, locations within the general system of developing cleavage planes (Gay & Greenwood, 1966; Temmink & Campbell, 1968; Lessie & Lovett, 1968; Heintz, 1970; Chong & Barr, 1973; McNitt, 1974; Olson *et al.* 1981; Armbruster, 1982; Lange *et al.* 1984). In furrowing processes, extension is

commonly reported to follow from the addition of small vesicles or cisternae either behind or at the leading edge of the furrow (Heintz, 1970; Travland & Whisler, 1971; Chong & Barr, 1973; Olson *et al.* 1981; Lange *et al.* 1984).

(c) Direct incorporation of existing membrane surfaces. As far as can be ascertained from what are often incomplete descriptions, it would appear that with few exceptions the plasma membranes of at least some zoospores in any particular fungal sporangium derive at least in part from direct incorporation of pre-existing membrane surfaces. The most extreme example is *Aphamomyces euteiches* in which it has been proposed that no new membrane synthesis occurs during cleavage; the zoosporic membranes are said to be derived from incorporation of the sporangial plasmalemma and an evaginating tonoplast (Hoch & Mitchell, 1972, 1975). In most cases, however, some contributions are reported to be made from both the existing surfaces and membranes formed *de novo*. In large sporangia, however, where some of the developing zoospores are surrounded solely by other zoospores, the central zoospores may lack any contribution from a pre-existing membrane. The opposite extreme to the case of *Aphanomyces* is found in several species of *Phytophthora*. In *P. infestans* and *P. capsici* it is thought that none of the zoosporic plasma membranes are formed by direct incorporation of existing membrane surfaces. In these species, the membranes are thought to originate from cleavage vesicles that themselves derive from dictyosomes and/or recycling of the membrane of the large central vacuole (Elsner *et al.* 1970; Williams & Webster, 1970). In *Phytophthora palmivora*, however, it would appear that the central vacuolar membrane is directly incorporated (Hohl & Hamamoto, 1967).

The literature on membranous partitioning during zoosporogenesis is confusing, for a number of reasons. Firstly, a great variety of processes have been proposed by which the partitioning membranes are thought to form. These include, as documented above, the fusion of pre-aligned vesicles, enlargement of vesicles, expansion of large central vacuoles, furrowing, and incorporation of existing membrane surfaces. In any one species up to four of these processes have been proposed (e.g., Gay & Greenwood,

1966). Secondly, different combinations of these processes have been proposed for closely related or even the same species. For example, in *Saprolegnia ferax* it has been described that the large central vacuole may sometimes be directly incorporated into the developing cleavage planes and at other times not (Gay & Greenwood, 1966). The same variation also exists between species of *Phytophthora* : in *P. palmivora (*formerly *P. parasitica*) the large central vacuole has been proposed to be directly incorporated (Hohl & Hamamoto, 1967), while this has not been described in any other species of this genus.

Another feature of possible concern in many descriptions of sporangial cleavage is the apparent lack of order in the process of partitioning. This is somewhat perplexing considering that one would expect that the subdivision of a such a complex system as a multinucleate sporangium would require an orderly mechanism.

1.2.3 Structural regulation of the cleavage process

Successful cell division requires that the planes of cleavage develop in an appropriate position. From a structural point of view, this achievement has several aspects: firstly, there must be some marking out of the appropriate positions, and subsequently the development of the partitioning membranes must be spatially restricted to these positions. Secondly, if any additional membrane is required for cleavage, then it must be brought to the region where cleavage is to occur. From studies of cytokinesis in plant and animal cells a considerable body of research has documented the roles of microtubules and microfilaments in these aspects of cleavage regulation (Gunning, 1982; Rappaport, 1986). There is strong evidence to suggest that these cytoskeletal structures are also involved in the regulation of sporangial cleavage. The strongest support comes from studies which have shown that antimicrotubule and antimicrofilament drugs disrupt the normal course of cleavage, causing the formation of abnormally-shaped, multinucleate masses of cytoplasm that are eventually released from the sporangium

(Slifkin, 1967; Schnepf *et al*. 1978; Schnepf & Heinzmann, 1980; Olson & Lange, 1983; Oertel & Jelke, 1986).

Ultrastructural evidence has also provided some support for the role of microtubules in the regulation of the cleavage process, with numerous reports of sporangial microtubules, most of which describe microtubular arrays radiating away from a point near each sporangial nucleus (Lessie & Lovett, 1968; Heath & Greenwood, 1971; McNitt, 1974; Hoch & Mitchell, 1975; Schnepf *et al*. 1978). The disposition of these arrays has led to several models for their role in cleavage, most notably that of Heath & Greenwood (1971). In this model, based on studies of Saprolegnia, the arrays of nucleus-based microtubules stabilize the cytoplasm around each nucleus, leaving less stable regions between the nuclei through which cleavage vacuoles preferentially expand. As discussed by Heath (1976) the ultrastructural evidence is not, however, entirely satisfactory because in some zoosporic fungi microtubular arrays are not seen in the sporangium. In addition, as noted by Heath *et al*.(1982) there is a lack of adequate data to assess how, or even if, the microtubules from adjacent nuclei might interact with each other. These problems probably derive in part from the notoriously inconsistent preservation of fungal microtubules and microfilaments in material prepared for electron microscopy (Lange & Olson, 1983; Cho & Fuller, 1989; Heath, 1990). In the case of actin, for example, there is a complete absence of reports of microfilaments in any fungal sporangium examined ultrastructurally. Preliminary studies using fluorescence techniques indicate, however, that actin is indeed present in sporangia of Saprolegnia (Heath, 1991). Even if microtubules and microfilaments were well preserved, they are extremely small, and this exacerbates the difficulties associated with serial sectioning enough of a sporangium to acquire the information necessary to deduce the spatial interrelationships of cytoskeletal elements and the developing cleavage planes.

1.2.4 Apportioning of organelles

The major objective of zoosporogenesis is to package the minimal ingredients for producing a new organism into an efficiently mobile unit (Heath, 1976). What might these "minimal ingredients" be and how does each zoospore obtain its requisite complement of them? The primary purpose of the zoospore is to carry the hyphal DNA to another location, so the nucleus is the most important structure in any zoospore. The presence of one, and only one, nucleus in each zoospore would be guaranteed if cleavage were controlled by the nucleus-based microtubule model proposed by Heath & Greenwood (1971) and discussed above. These arrays might also be involved in spacing the nuclei apart (Heath, 1976), thus ensuring that each nucleus acquires an adequate and equal portion of cytoplasm. Regular spacing of sporangial nuclei prior to the completion of cleavage has also been reported in zoosporic fungi (Hohl & Hamamoto, 1967; Schnepf *et al.* 1978; Armbruster, 1982). Unfortunately the paucity of information about the distribution of microtubules in these systems does not allow us to understand how the nucleus-based arrays may bring about the spacing of the nuclei.

A second critical set of structures are the flagella. Their presence in zoospores follows naturally from the intimate association, seen in the sporangium, between the nucleus and the centrioles or basal bodies from which the flagella develop (Hohl & Hamamoto, 1967; Elsner *et al.* 1979; Williams & Webster, 1970; Chong & Barr, 1973; Gotelli, 1974; Heath, 1976; Lunney & Bland, 1976; Schnepf *et al.* 1978; Armbruster, 1982; Lange *et al.* 1984). Mitochondria are always present in zoospores (Lange & Olson, 1983) and their presence may be assured by being randomly distributed in large numbers in the sporangium.

Regarding many other zoosporic structures, we can only speculate as to whether they represent some of the "minimal ingredients" required for functioning of the zoospore and/or reestablishment of the hyphal phase. It is difficult to be certain what may or may not be necessary. For example, while Oomycete zoospores regularly contain prominent water expulsion vacuoles which are thought to regulate internal osmolarity,

these structures are not found in all uniflagellate zoospores (Lange & Olson, 1983). Also, many structures within zoospores are proposed to be food stores, and while it is commonly assumed that zoospores rely totally upon stored reserves (Carlile, 1983) this is extremely difficult to prove. For zoospores to reform the hyphal phase, they rely upon their ability to encyst upon a suitable substrate. While Gubler & Hardham (1988) have demonstrated that specific vesicles within the zoospores of *P. cinnamomi* are involved in this process, there is as yet no evidence to prove that these vesicles are necessarily required for encystment to occur. While we are now acquiring valuable information about the function of these and many other zoosporic organelles, nothing is known about how they might be apportioned.

1.2.5 Spatial organization of organelles

It has been amply documented that the nuclei and organelles of zoospores are commonly positioned or distributed in a highly defined manner (see reviews by Bracker, 1967; Heath, 1976; Lange & Olson, 1983). Fig 1.1 illustrates diagramatically some of the previously described aspects of zoospore organization in *P. cinnamomi* (Hardham, 1987a), the species upon which most of the research of this thesis is focussed. In many fungi, some features of zoospore organization are already evident prior to the commencement of the cleavage process. In the previous section it was noted that basal bodies are commonly associated with sporangial nuclei and this association is retained throughout cleavage. Dictyosomes, also, are commonly found adjacent to the nucleus in zoospores (reviewed in Heath, 1976) and sporangia (Chapman & Vujicic, 1965; King *et al.* 1968; Williams & Webster, 1970; Schnepf *et al.* 1978; Miller *et al.* 1985) of many species. Indeed, the dictyosomes are usually found close to the nucleus-associated centrioles or basal bodies in all cells that contain these structures (Raven *et al.* 1981). While basal bodies are physically linked to the nuclei via microtubules, it is unclear how dictyosomal positioning is maintained.

Most other sporangial components, however, are usually described as having random spatial distributions prior to cleavage. There must therefore be mechanisms by which some of these structures acquire their highly polarized distributions in the mature zoospore. Unfortunately, nothing is known about what these mechanisms might be.

1.3 OBJECTIVES AND EXPERIMENTAL APPROACHES

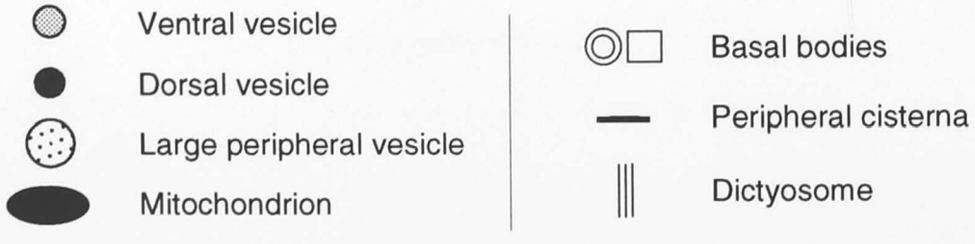
The objective of this thesis was to gather data that might provide answers to some of the unresolved questions of fungal zoosporogenesis. In particular, the research would address those issues that have been outlined in Sections 1.2.1-1.2.5. The Oomycete genus *Phytophthora* offered itself as the ideal system for this study for several reasons. Firstly, the availability of a range of monoclonal antibodies to many components of the released zoospore (Hardham *et al.* 1990) promised to facilitate the tracking of these components in the sporangium during the cleavage process. In particular it was hoped that some of these monoclonal antibodies might act as markers for the cleavage membranes, and thus help to identify their true source. Secondly, the zoospores of several species of this genus, and especially *P. cinnamomi* and *P. palmivora,* were well characterized and had proved to be amenable to some of the experimental procedures to be used, namely immunofluorescence microscopy and freeze- substitution (Hemmes, 1983; Hardham, 1987a, b: Cho & Fuller, 1987, 1989). Thirdly, most of the unresolved issues of fungal zoosporogenesis, such as confusion over the origins and mode of development of the cleavage membranes, also pertain to members of the genus *Phytophthora* (Sections 1.2.1-1.2.2). Resolution of these problems in *Phytophthora* therefore promised to shed some light upon those that exist across the spectrum of zoosporic fungi. Finally, although the main motivation for choosing the topic of zoosporogenesis was to use it as a model system for a study of cellular morphogenesis, *Phytophthora* contains some of the most economically and environmentally important

plant pathogens, and basic information about zoospore development may assist programs to control the diseases caused by these fungi.

The experimental approaches taken to achieve these aims were as follows. In the experiments of Chapters 2 and 3, electron microscopy was used to follow the ultrastructural changes that occur during the normal process of cleavage. Immunogold labelling, using the monoclonal antibodies referred to above, was used to assist these studies. The electron microscopy of Chapter 2 involved examination of sporangia preserved by chemical fixation. Some of the results of this approach, namely those involving the appearance of the cleavage membranes were, however, inconsistent. The experiments of Chapter 3 were undertaken partly to elucidate the source of the inconsistencies observed in Chapter 2. Rapid freezing and freeze-substitution were used as the methods of fixation for the experiments of Chapter 3 because these techniques are known to provide optimal preservation of membranes (McCully & Canny, 1985). Rapid freezing and freeze-substitution provide excellent preservation of most cellular components, so it was hoped that valuable information might be gained about many sporangial structures. The study of the normal course of cleavage was completed by the experiments of Chapter 5 in which immunofluorescence microscopy was used to study the distribution of sporangial microtubules during the process of cleavage. Some of the hypotheses suggested by the experiments of Chapters 2, 3 and 5 involved the possible involvement of microtubules and microfilaments in a range of cleavage processes, so these models were tested by examining the effects of anti-cytoskeletal drugs upon the cleaving sporangium (Chapter 6). Chapter 4 is a methodological study of a structural artefact that was noted in the experiments of Chapter 3. The findings of Chapters 2, 3 and 4 have been published as separate papers (Hyde *et al.* 1991 a, b and c respectively).

CHAPTER 1 FIGURE LEGEND

Fig 1.1 A diagrammatic representation of some structural features of the released zoospore of *P. cinnamomi*. The top diagram shows a surface view of the reniform zoospore, looking into the longitudinal groove that distinguishes the ventral surface. Two flagella originate in the groove. The anterior flagellum is coated with short projections (mastigonemes) which are not present on the posterior whiplash flagellum. The bottom diagram illustrates the distribution of certain organelles that might be seen if the zoospore was sectioned transversely, at right angles to the axis of the groove. The nucleus (N) is pear shaped and its narrow pole points towards the groove. Two basal bodies (for code, see below), from which the flagella extend, are associated with the narrow pole. Near the groove, a water expulsion vacuole (WEV) is present, and dictyosomes are concentrated around it. Other organelles are concentrated either at the ventral (ventral vesicles) or dorsal (dorsal vesicles, large peripheral vesicles and peripheral cisternae) surfaces. Mitochondrial profiles are also shown : these generally lie further away from the plasma membrane than profiles of other organelles, and are, in fact interconnected.



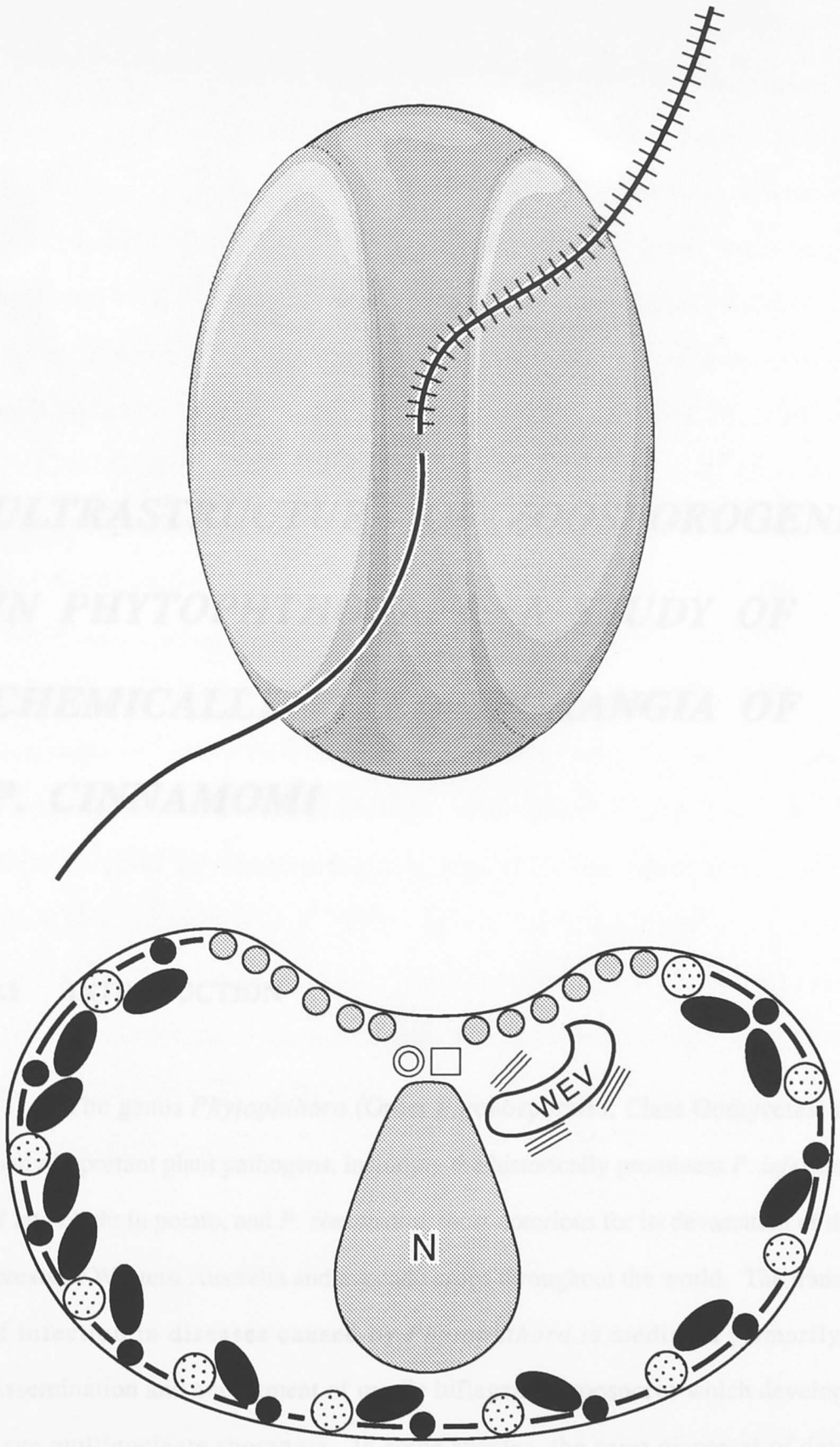


Fig. 1.1

2

ULTRASTRUCTURE OF ZOOSPOROGENESIS IN PHYTOPHTHORA. I. A STUDY OF CHEMICALLY FIXED SPORANGIA OF P. CINNAMOMI

2.1 INTRODUCTION

The genus *Phytophthora* (Order Peronosporales, Class Oomycetes) contains many important plant pathogens, including the historically prominent *P. infestans*, cause of late blight in potato, and *P. cinnamomi*, most notorious for its devastation of the jarrah forests of Western Australia and avocado crops throughout the world. The transmission of infection in diseases caused by *Phytophthora* is mediated primarily by the dissemination and encystment of motile biflagellate zoospores which develop within large multinucleate sporangia. In some species, the prior dispersal of detachable sporangia significantly widens the potential infective radius. One valuable approach in examining the roles of sporangia and zoospores in the infection process has been the study of their ultrastructure.

Zoosporogenesis, the formation of zoospores within the sporangium, has been examined in several species of *Phytophthora* (Chapman & Vujicic, 1965; Hohl & Hamamoto, 1967; King *et al.* 1968; Elsner *et al.* 1970; Williams & Webster, 1970; Hemmes, 1983). These studies indicate some features consistent within the genus but there are several important variations, most notably regarding the relationship between vesicles involved in the cleavage process and certain large cytoplasmic vacuoles. These vacuoles have been suggested as both a source (Williams & Webster, 1970) and a sink (Hohl & Hamamoto, 1967) for cleavage vesicles, while some studies report no relationship. Understanding these differences is one of many problems that remain unresolved, not only within *Phytophthora* but across the spectrum of zoosporic fungi. Central issues include identifying how cytoplasmic domains are established within the sporangium, and how each zoospore acquires the requisite complement of cellular structures. *P. cinnamomi* is an ideal system in which to address questions of zoosporogenesis since the ultrastructure of the zoospore itself is well characterised (Hardham, 1987 a,b) and there is a wide range of immunological markers available for this species (Hardham *et al.* 1990). In this, the first ultrastructural study of the sporangium of *P. cinnamomi*, I have employed conventional electron microscopy and immunogold labelling to describe some of the key events of zoosporogenesis, and have, for the first time, identified the source and fate of cleavage vesicles using immunospecific markers.

2.2 MATERIALS AND METHODS

2.2.1 Organism

The culture of *P. cinnamomi* (DAR 52646) used in this study was induced to produce sporangia and zoospores by the method of Hardham & Suzaki (1986). Briefly, a hyphal culture was grown on miracloth disks placed on nutrient agar. After 4-5d the disks were transferred to 250ml erlenmyer flasks containing nutrient broth. The

formation of sporangia was induced by replacing the broth with a mineral salts solution for 19h after which zoosporogenesis was induced by rinsing the culture in cold distilled water. Sporangia were collected in tufts of mycelium.

2.2.2 Light microscopy

Observations were made on zoosporogenesis in living sporangia using a Zeiss Photoscope III fitted with Nomarski interference contrast optics. Measurements of sporangial length were made using an eye-piece ocular fitted to the microscope.

DNA in sporangia fixed in a mixture of 0.2% glutaraldehyde and 4% paraformaldehyde in 50 mM Pipes buffer (pH 7) was stained with 20 μg/ml Hoechst 33662 (Sigma Chem. Co.) for 1h and observed using UG1 and UG5 exciter filters, an FT395 beam splitter and LP420 and KP560 barrier filters.

2.2.3 Electron microscopy

Observations were made on sporangia prepared by five methods:

A.Sporangia were fixed in 2% glutaraldehyde in 50 mM Pipes buffer for 2h at room temperature, then washed twice in 100 mM Pipes buffer, once in 25 mM sodium phosphate buffer, then postfixed in 1% OsO_4 in 25 mM sodium phosphate buffer and rinsed in buffer. All solutions were at pH 6.8-7.2. Cells were then dehydrated through a graded series of acetone solutions and embedded in Spurr's (Spurr, 1969) resin in flat-bottomed moulds.

B.Sporangia were processed as in A but were dehydrated through a graded ethanol series and embedded in LR White resin in beam capsules.

C.Sporangia were fixed in 2% glutaraldehyde in 100 mM cacodylate buffer containing 5 mM $CaCl_2$ (pH 6.8) for 2h at room temperature. After washing in 100 mM cacodylate buffer, cells were postfixed for 1h in a solution of 1% OsO_4, 0.8% KFeCN, 100 mM cacodylate buffer and 5 mM $CaCl_2$ (Hepler, 1981). Cells were then processed as in procedure above.

D.Sporangia were processed as in C but 10mM, instead of 100mM, cacodylate buffer was used at all steps.

E.Sporangia were fixed in 1% glutaraldehyde in 100 mM Pipes buffer (pH 6.8-7.2) for 2h at room temperature. After washing in buffer, sporangia were dehydrated in a graded ethanol series. Washing and dehydration steps up to 50% ethanol were at 4°C, while further dehydration was at - 20°C. The cells were then infiltrated with Lowicryl K4M resin for 24h at -20°C, then transferred to flat-bottomed aluminium weighing trays and polymerised under ultraviolet light for 48h at -20°C, followed by 24h at room temperature.

Embedded material was cut using a Reichert Ultracut E ultramicrotome and Diatome diamond knife. Sections were stained in 2% aqueous uranyl acetate for 10 min or 5 min and Reynolds' lead citrate (Reynolds,1963) for 5 min or 1 min, for Spurr's (Spurr, 1969) and Lowicryl K4M embedded material respectively. Immunogold labelling of sections from Lowicryl K4M embedded material followed the method of Gubler & Hardham (1988) and used monoclonal antibodies (mAbs) Cpa-2, Lpv-1, Cpw-1 and Vsv-1 (Table 2.1). These bind to the four different types of peripheral vesicles of the zoospore (Fig 1.1). The use of a number of different primary antibodies serves as a control for non-specific antibody binding, a problem that was not, in any case, suggested by the results. Photomicrographs of material studied by light microscopy were taken on Kodak Panatomic X film (differential interference optics) or Kodak T-Max 400 (fluorescence optics).

2.2.4 Sampling and examination of embedded material

The process of zoosporogenesis was examined by sampling sporangia at various times during the 70-75 min between induction and zoospore release. Sections of over 800 sporangia from 13 sequences of zoosporogenesis were examined. All preparation regimes provided good preservation of most cellular features, but as the mechanisms of zoosporogenesis were gradually deduced, it became evident that vesicles involved in the cleavage process were poorly preserved in up to 50% of sporangia prepared by methods A,B,C and E. Vesicle preservation was improved by method D. While general observations are based on consideration of sections from all 13 sequences, any numerical or otherwise definitive statements, unless otherwise indicated, relate to the inspection of

sections of 231 sporangia prepared by Method D and sampled at the following times: Pre-induction [number of sporangia (n) = 20]; Post-induction: 15 min (n=14); 20 min (n=12); 25 min (n=16); 30 min (n=20), 35 min (n=15); 40 min (n=13); 45 min (n=20); 50 min (n=25); 55 min (n=19); 60 min (n=17); 65 min (n=20) and 70 min (n=20). Sporangial cross-sections were chosen in a random manner and were used in a series of qualitative and semi-quantitative analyses. The synchrony of the cleavage process was high within any one run, and, generally, different sequences were comparable.

Table 2.1 Specificities of monoclonal antibodies, originally raised against components of zoospores of *P. cinnamomi*, to vesicles in the sporangium.

Monoclonal Antibody	Vesicle Labelled	Micrograph Label
Cpw-1	Cleavage vesicle (strong)	c
	Peripheral cisterna (weak)	arrows
	Dictyosomes	D
Cpa-2	Dorsal vesicle	d
Lpv-1	Large peripheral vesicle	L
Vsv-1	Ventral vesicle	v

2.3 RESULTS

2.3.1 The mature sporangium (pre-induction)

Living sporangia are a pale cream colour with a uniformly granular appearance. A basal septum separates the sporangial cytoplasm from that of the hypha and a thick translucent wall tapers sharply at the margin of a broad discoidal plug which caps the

apex (Fig 2.1). The sporangia are typically ovoid and 52.7 $\pm$ 13.5 μm (n=100) in length. Nuclei within the mature sporangia are regularly spaced (Figs 2.2-2.3). In small sporangia they occur in the cortical cytoplasm only, but in large sporangia some nuclei are internal (Fig 2.3). As is typical of biflagellate zoospores (Lange & Olson, 1983) the nuclei are pyriform in shape. The narrow poles of nuclei in the cortex are directed towards the sporangial wall (Fig 2.4), while those of the internal nuclei appear unoriented.

There is a marked polarisation in the distribution of certain components around the nuclei. In the cortex, basal bodies were always associated with the narrow nuclear pole (Fig 2.4). Ninety three percent of cortical nuclear profiles (n=44) had more dictyosomes in the region between the nucleus and the wall than in an equivalent area around the opposite pole of the nucleus (Fig 2.3). Clusters of small bodies, which were mostly vesicular (especially in material prepared by method C, as verified by serial sectioning) were also more common near the narrow pole than at the other pole of the nucleus in 75% (n=45) of cases (Fig 2.3). These vesicles had flocculent contents. Immunolabelling of these vesicles with mAb Cpw-1 (Fig 2.5) made it possible to track their fate throughout zoosporogenesis and subsequently they will be called cleavage vesicles. Small structures, morphologically similar to those referred to as microbody-like organelles in zoospores of *P. cinnamomi* (Hardham, 1987a), were also common in the regions of cytoplasm between the nuclei and the wall (Fig 2.4). These regions often appeared as clear zones, because apart from the four above- mentioned elements other large structures were less commonly seen in them (Fig 2.3). Clear zones containing cleavage vesicles, dictyosomes, basal bodies and microbody-like structures were also associated with the narrow poles of internal nuclei (Fig 2.3).

Of the four types of peripheral vesicles in the mature zoospore, three are already present in the pre-induction sporangium, being identifiable by morphological similarity and immunogold labelling. These vesicles are the large peripheral vesicles (Figs 2.6-2.7) and two types of small peripheral vesicle, the ventral (Fig 2.6) and dorsal (Fig 2.7) vesicles. They are labelled by mAbs Lpv-1, Vsv-1 and Cpa-2 respectively (Table 2.1).

The names of these vesicles refer to their relative size and location in the zoospore. No distributional patterns were evident in the sporangium at this stage, apart from the rarity of these vesicles in the clear zones. The fourth vesicle type, the peripheral cisterna, was not seen.

Other structures present in pre-induction sporangia include lipid bodies and mycolaminaran bodies, endoplasmic reticulum and mitochondria with short, tubular cristae (Figs 2.4,2.8). Presumptive mastigoneme packets are often seen in close association with mitochondria (Fig 2.8), an observation common in the Oomycetes (Gotelli, 1974; Martin & Miller, 1986). In 75% of sections, one to three vacuoles similar in size to the nuclei were seen in the sporangial interior (Fig 2.9).

The sporangial wall, in Spurrs' embedded material, appears to have up to five distinct layers, the outermost being an electron-dense fringe which continues over the apical plug (Figs 2.3,2.6,2.9). The inner sporangial wall is continuous with the inner layer of the basal septum (Fig 2.10). A thinner layer on the hyphal side of the septum is continuous with the inner layer of the hyphal wall. Sandwiched between these layers is a disk of material similar to that described as "slime substance" by Williams & Webster (1970), some of which is also entrapped within the inner layer of the septum. The papillar material at the apex appears homogeneous (Fig 2.3) except for the outer dense fringe.

2.3.2 Expansion of the cleavage vesicle clusters (first 20 min after induction)

During the first 20 min after zoosporogenesis is initiated by a cold shock, large clusters of cleavage vesicles appear throughout the sporangium (Figs 2.11-2.12). One large cluster is commonly seen between each cortical nucleus and the sporangial wall, filling up the area that appeared as a clear zone before induction. At the sporangial apex an especially large cluster appears to result from the merger of clusters associated with several nuclei whose narrow poles are preferentially angled towards the plug. A large

apical cluster was seen in 50% of sporangial profiles at 15 and 20 min. Each nucleus within the sporangial interior also appears to have an accompanying large cluster near the narrow pole. Some small clusters were seen near dictyosomes at other sites, most commonly at the broad poles of cortical and internal nuclei (Fig 2.13).

Immunolabelling with mAb Cpw-1 suggests that the cleavage vesicles may be derived from the dictyosomes. Material labelled by Cpw-1 occurred in both the cleavage vesicles and the cisternae of the dictyosomes (Fig 2.14). The level of binding to dictyosomes was low but consistent, with every membrane stack showing some staining. Gold particles bound to the flocculent contents of the cleavage vesicles and were closely associated with their membrane. Occasionally, profiles of vesicles morphologically similar to cleavage vesicles were seen that had no associated gold particles.

During this period flagellar growth began with extension of the microtubules of the basal bodies past a terminal plate into large cleavage vesicles, commonly called axonemal vacuoles (Fig 2.15). Cytoplasmic microtubules were associated with the basal bodies. The microbody-like organelles seen earlier were also common in the expanded clusters (Fig 2.12), especially at the apex of the sporangium. In 97% of cases at 15 and 20 min these organelles were also seen lined up along substantial portions of the sporangial perimeter (Fig 2.12). By the end of this period, the proportion of sporangia with one or more large central vacuoles had dropped to 58%.

2.3.3 Dispersal of the cleavage vesicle clusters (20-50 min post induction)

2.3.3.1 Organization of the cortical cleavage plane

Between 20 and 35 min after induction, the vesicle clusters associated with the narrow poles of cortical nuclei disappear, except for the large aggregation at the apex (Figs 2.16,2.19). Concurrently, a plane of cleavage vesicles gradually forms at the sporangial periphery, parallel to the the wall (Figs 2.16-2.17). These two developments are complete by 35 min (Fig 2.21). Microtubules fan out from the basal bodies of the

cortical nuclei, running close to the internal surface of the vesicles in the cortical plane (Fig 2.18). Flagellar growth continues throughout the sporangium at least until 35 min when the proportion of sections with one or more flagellar profiles first reaches 100%. In the cortex the axonemal vacuoles run obliquely towards the periphery of the sporangium (Fig 2.19). Axonemal vacuoles bind Cpw-1 as does the plasma membrane ensheathing the flagella (Fig 2.17).

Between 25 and 50 min after induction, the cleavage vesicles in sporangia prepared by procedures A, B,C and E often appeared fused. At about 30-35 min it was common to see a sizable shell of cytoplasm cut off by fusion of the cortical cleavage vesicles (Fig 2.20) and large spirally arranged vacuoles, apparently formed by fusion of the vesicles of the internal clusters (Fig 2.16). The thickness of the cortical shell of cytoplasm was less in material fixed late in this period of development than in material fixed earlier. Sporangia containing these features were also often strongly plasmolysed. When the buffer concentration was lowered (procedure D) fusion of cleavage vesicles at this stage of development was greatly reduced. The proportion of sporangia with a shell of cortical cytoplasm at 30 min dropped from 42% (n=17) in a sequence fixed in a high osmolarity buffer to 17% (n=20) using the diluted buffer.

2.3.3.2 Organization of the internal cleavage planes

Between 25 and 50 min after induction, cleavage vesicles of the internal clusters gradually disperse (Fig 2.22). At 25 min, 25% of sporangia have at least one internal cluster showing some sign of dissociation, a proportion that rises to 100% by 35 min. At the latter time, the most common pattern of dispersal involves two chains of vesicles which arch away from the narrow nuclear pole and then loop back on either side of the nucleus (Fig 2.22). Microtubules were sometimes seen radiating from the basal bodies of the internal nuclei (Fig 2.23) but their infrequent occurrence made it difficult to determine any relationship to the cleavage vesicle patterns. Flagella extend at approximately right angles to the long axis of the nucleus (Fig 2.24).

About the time internal chains of cleavage vesicles are first seen, there is a rapid onset of a close association between cleavage vesicles and the large peripheral vesicles (Fig 2.22). This is evident in 10% of sporangia at 30 min, rising to 85-90% at 35 min and later times up to 50 min. Co-localisation is more obvious in the sporangial interior than in the cortex (Fig 2.22).

As dispersal approaches completion, wavy chains of cleavage vesicles are seen surrounding areas of cytoplasm which often contain a centrally-placed nuclear profile (Fig 2.25). By 45 min, the cytoplasm of 50% of sporangia was partitioned by flexuous lines of vesicles. By 50 min, 84% of sporangia exhibited this or a more advanced pattern. Axonemal vacuoles lie in the system of vesicles (Fig 2.26). In the final stage of organization, chains of vesicles run in approximately straight lines, often meeting in groups of three separated by angles of about 120°. They form a network of polygons. Mitochondrial profiles occur predominantly near the boundaries of the polygonal domains. In most sporangial profiles at this stage, fusion of the vesicles of the cortical cleavage plane will have begun, or may even be almost completed (Fig 2.26).

While the last non-apical cortical clusters are seen at 30 min, clusters at the apex persist much longer, albeit in decreasing frequency, and only finally disappear at 45 min. From about this time onwards, the apical papillar material is thicker than previously. Between 20-50 min, the associated congregations of microbody-like organelles dissociate and near the end of this period, are only seen near the wall (Fig 2.27). They are no longer evident when cortical fusion is complete. The large central vacuoles were present in only 2 of 15 sporangia at 35 min and have completely disappeared by 45 min.

2.3.4 Cleavage and release (50-75 min post-induction)

During this period the sporangia become fully cellularised and the zoospores achieve their mature form prior to release. The external surface of the cleaved cytoplasm is furrowed by a mesh of grooves that divide the surface into a network of regular hexagons (Fig 2.28) within which nuclei are centrally located (Fig 2.29). Within each

hexagon a water expulsion vacuole can be seen expanding and contracting. Their cycles are not synchronised within the sporangium. Towards the end of this period flagellar beating can be detected at the periphery of the cytoplasm.

The cytoplasmic cleavage noted above results from fusion within and between the cortical and internal vesicle planes and the axonemal vacuolar system. Within the sporangial population fusion is first seen to commence at about 50 min in both the cortex and the interior (Figs 2.26,2.30). In any one sporangium however, fusion is always more advanced within the cortical plane and is sometimes complete, except for a section at the apex, before internal fusion has begun. Fusion of the axonemal vacuoles with other vesicles of the cleavage planes brings about externalisation of the flagella within the intercellular spaces (Figs 2.26,2.30). Fusion is typically retarded directly below the papillum (Fig 2.26) and may still be incomplete at 70 min when cleavage is finished in the rest of the sporangium.

In the sporangial interior, the membrane of the cleavage vesicles is partitioned between adjacent zoospore initials in the formation of their plasma membranes (Figs 2.30-2.33). In the cortex, the cleavage vesicles become appressed against the sporangial plasma membrane before fusing. Thus only some of the membrane of the cortical vesicles may contribute to that of the zoospores and the rest may remain with the sporangial plasma membrane (Fig 2.27). When cleavage is complete, the Cpw-1 positive antigen is closely associated with the plasma membrane of the zoospore initials and with the flocculent material which, having been released by vesicle fusion, now lies within the intercellular spaces (Fig 2.33). Gold particles are mainly associated with the outer surface of the membrane.

During the 50-70 min period many developments occur within each domain to bring about maturation of the zoospores. The ventral and dorsal vesicles move to their final locations, the latter type moving later than the former (Figs 2.31-2.33). At 60 min, in 82% of zoospore initials where the ventral surface is identifiable, the ventral vesicles are seen near it, while the dorsal vesicles are concentrated at the dorsal surface in only

47% of cases. The location of the large peripheral vesicles at the zoospore surface (Figs 2.31-2.32) follows automatically from their continued close association with the cleavage vesicles. Mitochondrial profiles show a similar distribution, but are not as close to the plasma membrane as the large peripheral vesicles (Fig 2.32). Their cristae appear to be longer than previously and membranous annuli are often seen within the matrix (Fig 2.32), features that become progressively more common during zoosporogenesis. Presumptive mastigoneme packets are still seen at the end of this period (Fig 2.32) but no mastigonemes were seen on any flagellum.

The fourth surface-associated vesicle of the zoospore, the peripheral cisterna, appears at about 50 min (Figs 2.32-2.33). It is seen directly below the newly-formed plasma membrane, even when other regions of the sporangium may not yet be cleaved. After immunolabelling with Cpw-1, small numbers of gold particles are seen on the peripheral cisternae (Fig 2.33). What is now an increasingly distinct inner layer of the thickened papillar material (Fig 2.34) also immunolabels with Cpw-1 (Fig 2.35). There was no staining of the papillar material at the pre-induction stage.

Initially after fusion, profiles of zoospore initials retain the polygonal geometry of the pre-fusion domains. Between 60-70 min the contours become more rounded and a groove develops in the zoospore surface adjacent to the narrow nuclear pole (Fig 2.36). This grooved surface is defined as ventral. When identifiable in the cortical zoospore initials it always (n=62) faces towards the sporangial wall. The large water expulsion vacuole lies adjacent to the groove (Fig 2.37). The area around the water expulsion vacuole contains numerous small vacuoles, vesicles and dictyosomes but lacks other large organelles. Ventral vesicles are common close to the groove (Fig 2.37) but the three other peripheral vesicles do not usually associate with the membrane opposite the narrow nuclear pole either before or after the groove develops. From the onset of fusion nuclear profiles become progressively more irregular and elongated than before (Figs 2.30,2.36). Of other large structures, the lipid bodies and mycolaminaran bodies appear

gradually closer to the nucleus from about 50 min on (Fig 2.36) and are uncommon in the cortex of the mature zoospore.

The release of zoospores in *P. cinnamomi* has been described at the light microscope level by Gisi *et al.* (1979). Ultrastructurally, the wall of the discharge vesicle (Figs 2.38-2.39) is fibrous and continuous with the tapered margin of the sporangial wall.

2.4 DISCUSSION

From a structural viewpoint there are seven major developments during zoosporogenesis in *P. cinnamomi*. Domains are established and then separated. After separation, the contours of the zoospore initials become more rounded, and the ventral surfaces of cortical initials face outwards. By the end of the process organelles are apportioned and correctly positioned within each zoospore initial. Finally, the papillar material at the sporangial apex undergoes changes before the zoospores are released.

The deployment of cleavage vesicles in the future planes of cleavage is the first definite indication that domain boundaries have been set. However, the regular spacing of the nuclei before induction suggests that domain boundaries may be set, but not identifiable using the present techniques, long before 50 min. Most, if not all, of the nuclei are probably transported into the sporangium from the hypha (Hemmes, 1983) and spacing of the nuclei must occur as the sporangium develops. Limited arrays of microtubules associated with each narrow pole have been noted in ultrastructural studies of *Saprolegnia ferax* (Heath & Greenwood, 1971) and may be involved in positioning the nuclei within the sporangium, as is the case for the extensive arrays noted in other syncytial systems (Menzel, 1986; Karr & Alberts, 1986; van Lammeren, 1988). Heath & Greenwood (1971) proposed that the cleavage elements might travel between regions of cytoplasm stabilised by microtubules around each nucleus. In *P. cinnamomi* no

microtubules were observed before induction although the pyriform shape of the nucleus may be indicative of their presence (Lange & Olson, 1983).

After induction, nuclear-associated microtubules run along, and possibly delimit, the inner boundary of the cortical plane of cleavage vesicles involved. Instead of restricting cleavage vesicle movement, microtubules could be involved in vesicle transport, as noted in other systems (Schnapp *et al.* 1985; Scholey *et al.* 1985). The striking patterns of cleavage vesicle dispersal in the sporangial interior also point to a dependence on some cytoskeletal infrastructure. Microtubules probably control the rounding up of the zoospore initials (Lange & Olson, 1983) and a possible tension in the microtubules that run between the nuclear- associated basal bodies and the zoospore surface could bring about the change in nuclear shape seen towards the end of cleavage.

The production of cleavage vesicles in *Phytophthora* species, as in other zoosporic fungi has most commonly been ascribed to the activity of dictyosomes (e.g. Hohl & Hamamoto, 1967; Elsner *et al.* 1970; Chong & Barr, 1973; Taylor & Fuller, 1981). Williams & Webster (1970) however considered the large central vacuole to be the main source of cleavage vesicles in *P. capsici*. Hohl & Hamamoto (1967) noted that cleavage vesicles coalesced after induction to form or enlarge the central vacuole of *P. palmivora* (formerly *P. parasitica).* Both cleavage vesicles and large vacuoles are present in *P. cinnamomi*. The immunogold labelling of sporangia with mAb Cpw-1 has revealed that the cleavage vesicles and dictyosomes share a common antigenic component which is not associated with the large vacuoles. The observations strengthen the case for the dictyosomal origin for the cleavage vesicles. Some vesicle profiles, morphologically similar to cleavage vesicles, however, had no associated gold particles and these may represent vesicles derived from the large vacuoles. The binding of mAb Cpw-1 to axonemal vacuoles supports the common opinion that they form by cleavage vesicle fusion (e.g. King *et al.* 1968; Williams & Webster, 1970).

The geometric arrays of the fully organized cleavage vesicle system have been noted in other fungi, including *Phytophthora* species (e.g. Hohl & Hamamoto, 1967;

Lunney & Bland, 1976). The polygonal patterning of fully cleaved sporangia can be attributed to the physics of packing (Thompson, 1942) and it provides the minimal surface area in relation to volume of the zoospore initials. Such geometry at the stage just before fusion, however, is unexpected, since the aligned planes of vesicles do not separate physically discrete units.

A number of studies of zoosporogenesis in *Phytophthora* (Hohl & Hamamoto, 1967; Elsner *et al* 1970; Williams & Webster, 1970) describe a cytoplasmic remnant which is cut off outside the plane of cortical vesicles and which is presumably resorbed before the end of zoosporogenesis. Such a remnant was also seen in *P. cinnamomi* when high osmolarity buffers were used during fixation. Lowering the fixative osmolarity decreased the frequency of occurrence of the remnant. Apparent vesicle fusion can be induced during fixation (Hausmann, 1977) and it is proposed that this remnant in chemically fixed sporangia of *P. cinnamomi* is an artefact. In vivo, vesicles of the cortical cleavage plane may gradually become positioned closer, and finally become appressed, to the sporangial plasma membrane before fusing. In this process no cytoplasmic remnant is formed and only the sporangial plasma membrane and some of the membrane of the cortical vesicles remain behind. This interpretation is supported by the decreasing thickness of the remnant in sporangia fixed at later times of the developmental sequence.

Early fusion in the sporangial interior was also judged to be artefactual primarily because unfused vesicles still predominated in many sporangia at later stages, and because lowering the fixative osmolarity reduced the occurrence of vesicle fusion in the sporangial interior. The examination of material fixed by non-chemical means, such as rapid freezing, is needed to evaluate these interpretations.

In the present study I have used mAb Cpw-1 as a marker for the cleavage vesicles within the sporangia. After cleavage, the Cpw-1 antigen becomes mostly located on the surface or between the zoospore initials. Peripheral cisternae were weakly labelled. In released zoospores, mAb Cpw-1 binds to the peripheral cisternae and the dictyosomes

but not to the plasma membrane (Hardham *et al.* 1990). Thus during or after release, the Cpw-1 antigen on the zoospore surface is apparently lost. Zoospores of *Chytridium confervae* possess a carbohydrate coat within the discharge vesicle (Taylor & Fuller, 1981) but this had not been noted in free-swimming zoospores (Barr & Hartmann, 1976). The material contained in the cleavage vesicles could be an osmoticum involved in the uptake of water into the sporangium before zoospore release, as suggested for *Pythium proliferum* (Lunney & Bland, 1976).

Apart from the peripheral cisternae, water expulsion vacuole and plasma membrane, the other structures of the zoospore are already evident in the pre-induction sporangium. In particular the large peripheral, ventral and dorsal vesicles are present and must either flow in from the hypha or be produced during sporangial maturation. Adequate allocation of many sporangial organelles amongst the zoospores during cleavage probably follows as a consequence of them being almost randomly scattered throughout the cytoplasm at the start of the sequence. The close association of the large peripheral vesicles and cleavage vesicles, also noted in *P. megasperma* (Hemmes, 1983), suggests a more sophisticated mechanism for regulating distribution whereby the large peripheral vesicles are tagged to elements that must be evenly aliquotted during compartmentalisation. Similarly, a continued association with the narrow nuclear poles throughout zoosporogenesis ensures the presence and correct positioning of the dictyosomes and basal bodies in each zoospore. Other elements, namely the dorsal and ventral vesicles, mitochondria, mycolaminaran and lipid bodies only take up their final positions after the zoospore initial domains have been clearly established. The processes involved in their movements are as yet unidentified.

The change in thickness and structure of the apical papillar material probably allows the eventual pressure-driven distension of this material in the formation of the discharge vesicle. The apical cytoplasm shows several specialized features which may relate to these changes. The especially large and persistent cleavage vesicle cluster seen at the apex appears to derive from dictyosomes of several nuclei preferentially angled

towards the plug. Such aggregations have been noted in other species (Williams & Webster, 1970; Lunney & Bland, 1976). The disappearance of these clusters coincides with changes in the structure of the papillar material, suggesting some involvement of the cleavage vesicles (Williams & Webster, 1970; Taylor & Fuller, 1981), a view supported by the heavy staining of the inner layer of the plug by mAb Cpw-1. If cleavage vesicles contain an osmoticum then the thickening of the papillar material may be swelling due to an induced uptake of water. The apical concentration of microbody-like organelles and the retarded fusion of the apical section of the cortical cleavage plane may be important for changes to the papillar material to allow release.

While the preferential angling of apical nuclei has not been described before, the more general feature of the orientation of the narrow nuclear pole of cortical nuclei towards the sporangial wall has been noted in several *Phytophthora* species (Williams & Webster, 1970; Elsner *et al.* 1970) and other Oomycetes (Armbruster, 1982). In *P. cinnamomi* the orientation of the nuclei, as well as their spacing, is established during maturation of the sporangia. The cortical nuclei maintain their orientation throughout zoosporogenesis, indicating that, even before induction, the future dorso-ventral axis of the zoospore is already established in a domain of cytoplasm surrounding each nucleus. The orientation of the cortical nuclei underpins the establishment of the cortical cleavage plane since cleavage vesicle production is concentrated at the narrow nuclear poles. The microtubule model proposed by Heath & Greenwood (1971) to explain the delimitation of cleavage vesicle dispersal might also probably require precise orientation of domains with respect to each other. The arrangement seen in P. cinnamomi might be one workable solution.

This study has shown that the mature sporangium of *P. cinnamomi* is structurally ordered at two levels of organization, the first involving arrangement of elements within the domains and the second involving orientation of the domains themselves. This hierarchical system is central to co-ordinating the highly co-operative processes by which the network of cleavage vesicle planes is established. Immunogold labelling has also

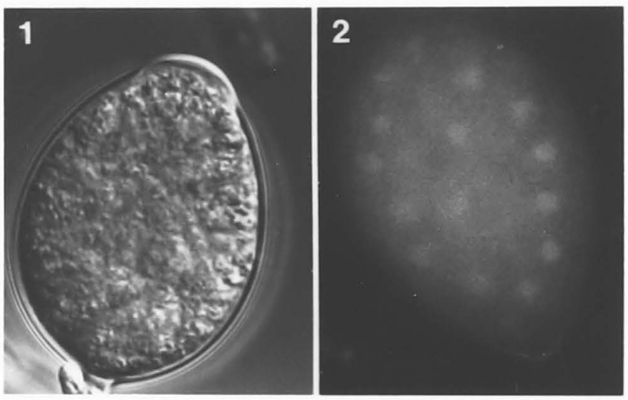
indicated that, in *P. cinnamomi*, dictyosomes and not large vacuoles are the source of cleavage vesicles. I believe that, unless optimal fixation methods are used, artefactual fusion of cleavage vesicles may occur and make it difficult to interpret possible relationships between these vesicles and other organelles. These findings suggest that the role of large vacuoles during cleavage in other *Phytophthora* species may need to be reassessed. Zoosporogenesis in *Phytophthora* may be more consistent than previously envisaged.

CHAPTER 2 FIGURE LEGENDS

Figs 2.1-2.2 Mature sporangia of *P. cinnamomi* before induction of zoosporogenesis.

Fig 2.1 The ovoid sporangium has a granular cytoplasm, separated from the hypha by a basal septum. At the apex is a plug of papillar material. Differential interference contrast optics. X1050.

Fig 2.2 Hoechst 33662 staining of sporangial nuclei. X700.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Figs 2.3-2.10 Structure of mature sporangia of *P. cinnamomi* before induction of zoosporogenesis. Figs 2.5-2.7 cells embedded in Lowicryl K4M resin; other figures, cells embedded in Spurr's resin.

Fig 2.3 Longitudinal section of sporangium with dense cytoplasm, basal septum (s) and apical papillar material (p). Near each nucleus is a clear zone (z), in which clusters of cleavage vesicles (c) and dictyosomes (D) are seen. Clear zones near cortical nuclei are close to the wall. X3500.

Fig 2.4 Nuclei are pyriform in shape and their narrow poles lie in clear zones with cleavage vesicles (c) and dictyosomes (D). Microbody-like organelles are also common here (m). They are typically smaller than ventral and dorsal vesicles (Figs 2.6-2.7), have a more irregular outline and a homogeneous matrix. One of a pair of basal bodies (arrow) is at the narrow pole. Narrow poles of nuclei in the sporangial cortex point towards the wall (w). Other structures include lipid bodies (Li), mycolaminaran bodies (my) and endoplasmic reticulum (E). X19,500.

Fig 2.5 Labelling of cleavage vesicles (c) with mAb Cpw-1 followed by sheep anti-mouse IgG-Au10 (Cpw-1-Au10). X27,000.

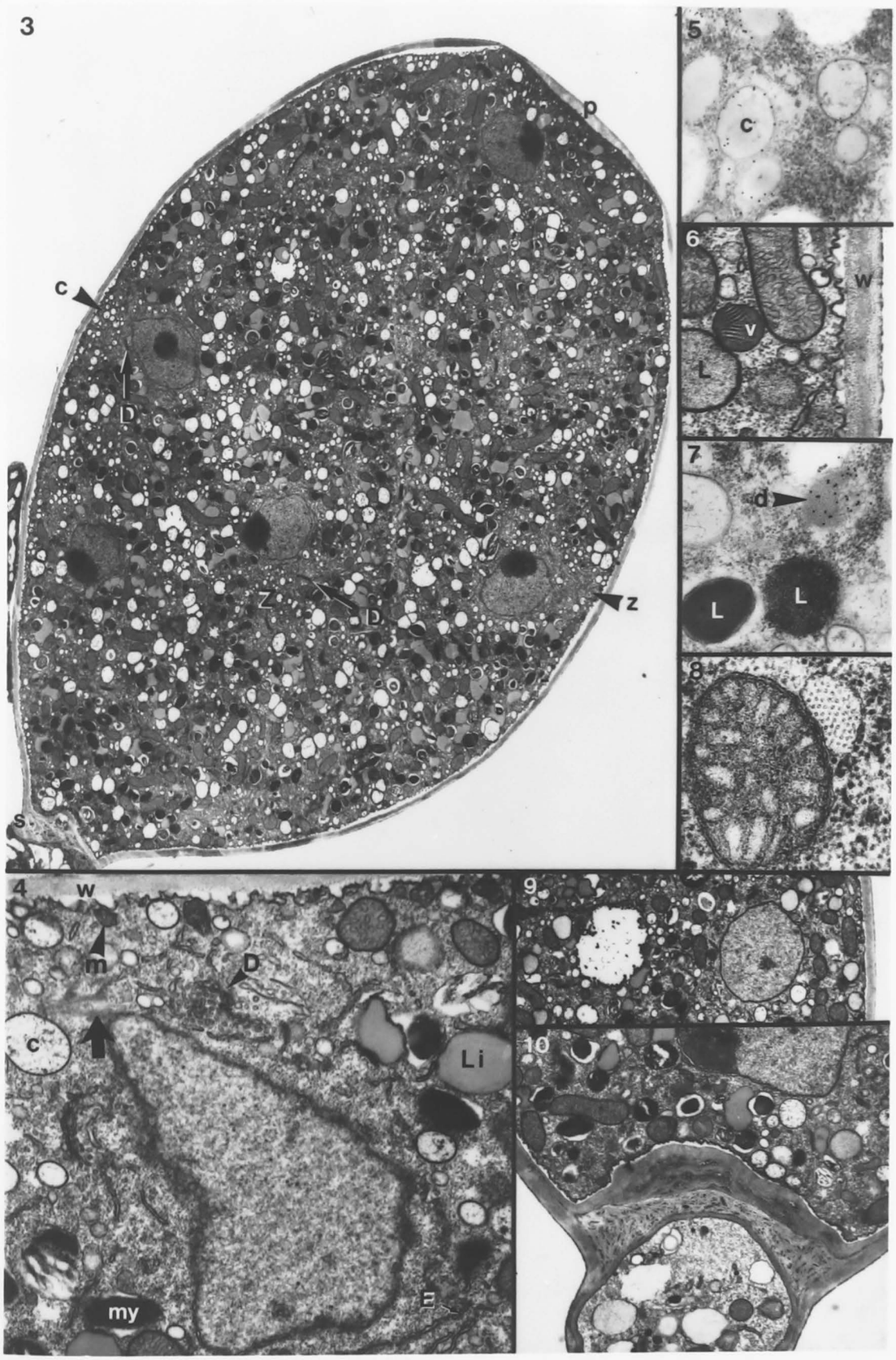
Fig 2.6 Large peripheral vesicles (L) and ventral vesicles (v) near wall (w). Ventral vesicles typically have a partly striated matrix in Spurr's material. X26,500.

Fig **2.7** Large peripheral vesicles (L). Dorsal vesicle (d) labelled with mAb Cpa-2 followed by sheep anti-mouse IgG-Au10. Part of the dorsal vesicles typically has a marbled appearance in all types of resin. X27,500.

Fig **2.8** Packet of tubular structures, probably mastigonemes, near mitochondrion. X63,000.

Fig **2.9** Large vacuole in sporangial interior. X4500.

Fig **2.10** Basal septum. A central layer of "slime substance" is surrounded by inner and outer layers continuous with the sporangial and hyphal walls respectively. X9,200.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V
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4
W
m
D
c
Li
my
E
9
10

Figs 2.11-2.15 Features of sporangia of *P. cinnamomi* during the first 20 min after induction of zoosporogenesis. Fig 2.14, cell embedded in Lowicryl K4M; other figures, cells embedded in Spurr's.

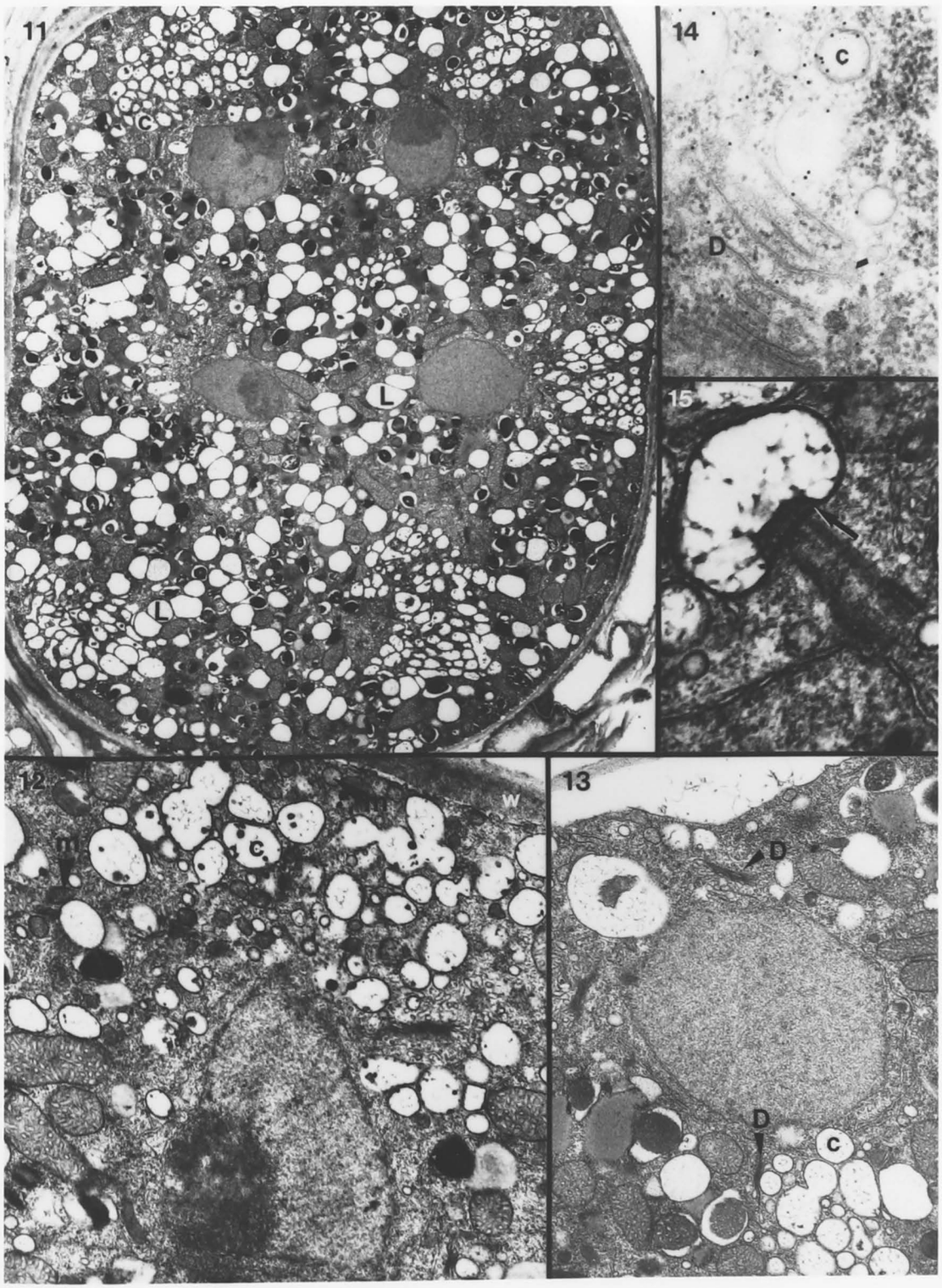
Fig 2.11 After induction large clusters of cleavage vesicles (c) are seen near each nucleus. Large peripheral vesicles (L) are evident. X5,000.

Fig 2.12 Microbody-like organelles (m) are common in the large clusters of cleavage vesicles (c) and also line up around the sporangial periphery. X15,000.

Fig 2.13 Small cluster of cleavage vesicles (c) at the broad pole of cortical nucleus. Dictyosomes (D) are seen nearby and close to the wall. X11,500.

Fig 2.14 Labelling of cleavage vesicles (c) and dictyosomal cisternae (D) with Cpw-1-Au_{10}. X44,000.

Fig 2.15 Flagellar growth begins by extension of the microtubules of the basal body past a terminal plate (arrow) into a large cleavage vesicle, the axonemal vacuole. X43,000.


11
C
L
L
14
C
D
15
12
W
M
C
13
D
D
C

Figs 2.16-2.20 Organisation of the cortical cleavage plane in *P. cinnamomi* (20-35 min post-induction). Figs 2.16,2.19,2.20, embedded in Spurr's. Figs 2.17-2.18, embedded in Lowicryl K4M.

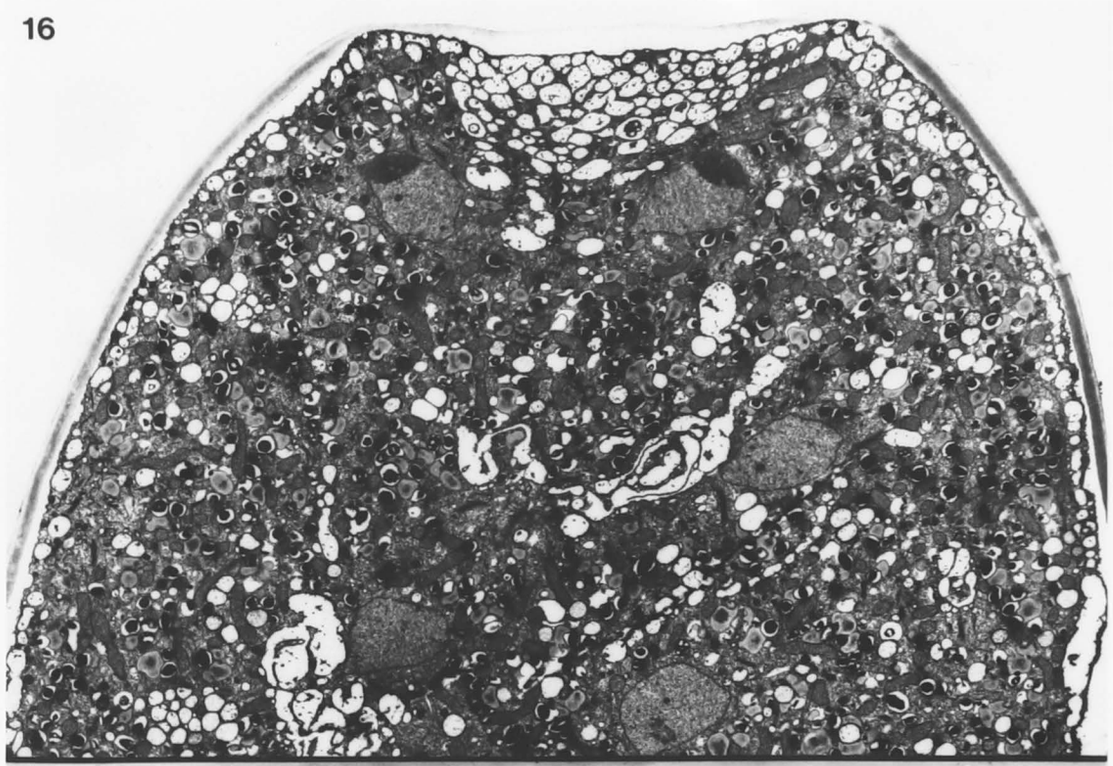
Fig 2.16 By 35 min cortical clusters of cleavage vesicles have dispersed to form a plane of vesicles parallel to the wall. One large cortical cluster remains at the apex. Internal clusters are still seen, and in this section appear fused, probably artefactually. X4500.

Fig 2.17 Cpw-1-Au_{10} labelling of vesicles of the cortical cleavage plane (c), axonemal vacuole and plasma membrane ensheathing the flagella (arrowhead). X20,000.

Fig 2.18 Microtubules (arrows) fan out from basal bodies (F) at the narrow pole of a cortical nucleus. Some microtubules run parallel to the cortical cleavage vesicles (c), labelled by mAb Cpw-1-Au_{10}. X27,500.

Fig 2.19 Flagellum (F) in an axonemal vacuole which runs obliquely towards the sporangial periphery, in this case the apex (p) with cleavage vesicle (c) cluster below. X18,000.

Fig 2.20 Shell of cytoplasm cut off by early fusion of the cortical cleavage plane in material fixed with high osmolarity buffer. X4300.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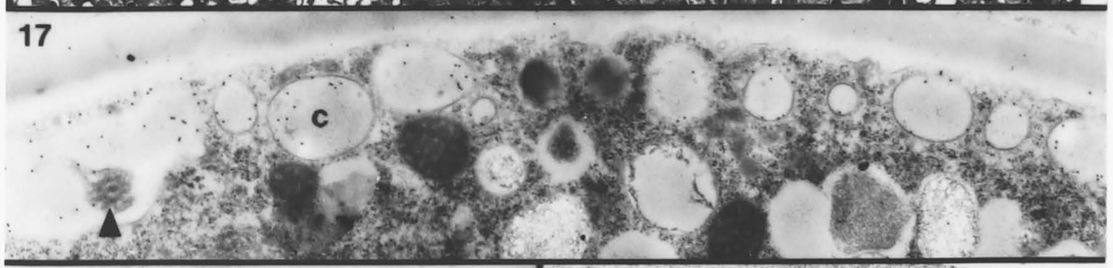
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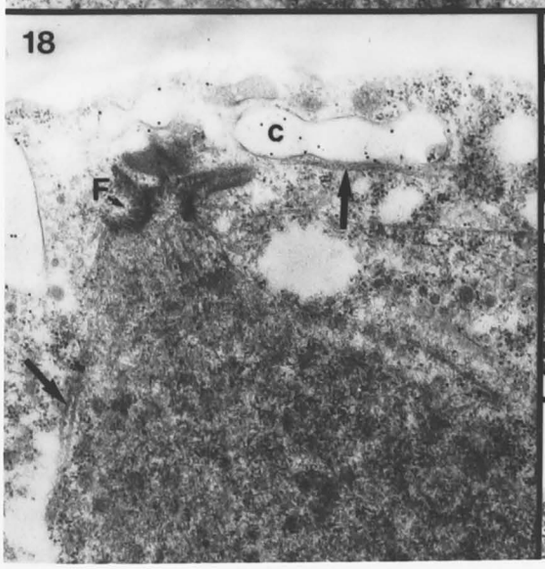
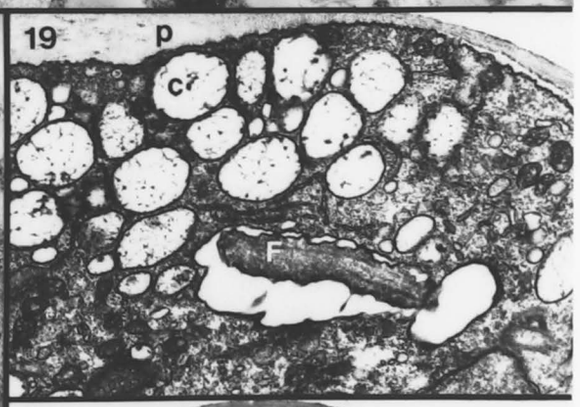
c


18
c
F


19
p
c
F

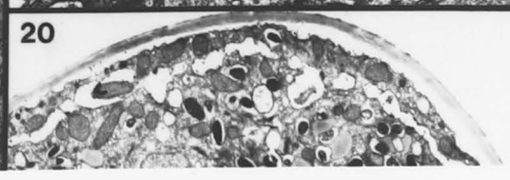

20

Fig 2.21 Organisation of the cortical cleavage plane. Between 20-35 min after induction the percentage of sporangial sections with a fully organised array of cleavage vesicles parallel to the wall rises sharply (▵). Concurrently, the large cleavage vesicle clusters (▫) that have developed earlier in the cortex, disappear. (For numbers of sporangia sampled at each time point see Materials and Methods).

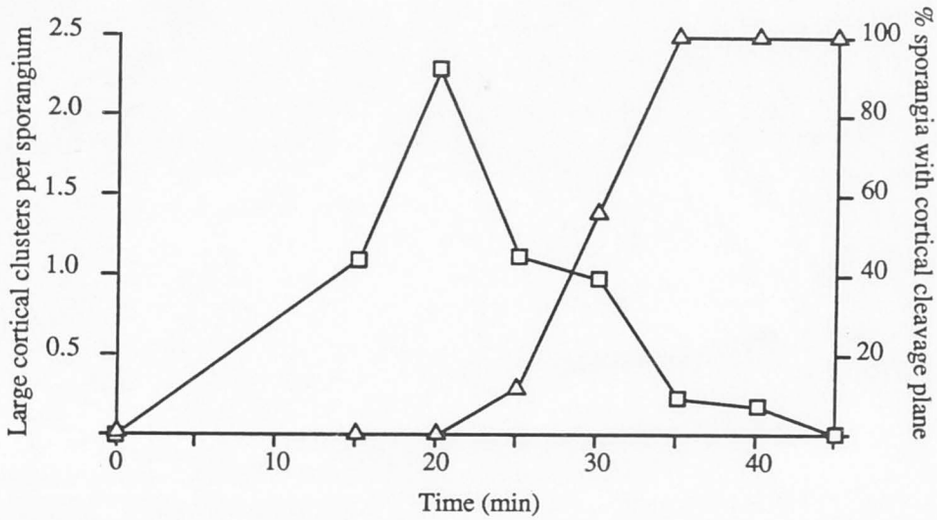

Large cortical clusters per sporangium
2.5
2.0
1.5
1.0
0.5
% sporangia with cortical cleavage plane
100
80
60
40
20
0
10
20
30
40
Time (min)

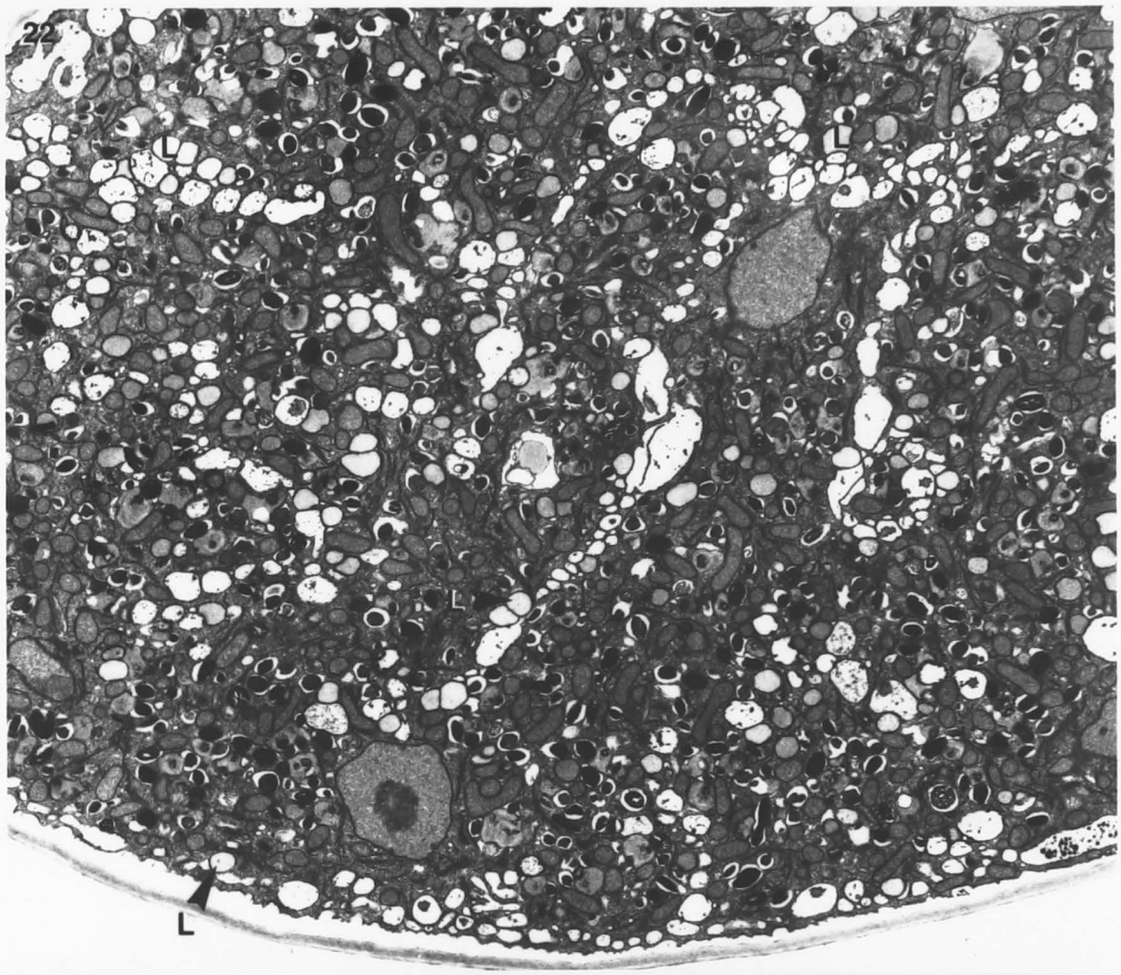
Fig. 2.21

Figs 2.22-2.24 Organisation of the internal cleavage planes in sporangia of *P. cinnamomi*. I. Early features (20-40 min after induction).

Fig 2.22 Cleavage vesicles which disperse from narrow poles of internal clusters often arch out and then loop back around the nucleus. Large peripheral vesicles (L) closely associate with the cleavage vesicles from this time on, especially in the sporangial interior. Spurr's resin. X5,300.

Fig 2.23 Microtubules (arrows) fanning out from the basal bodies (asterisk) of internal nucleus. LR White resin. X50,000.

Fig 2.24 Flagellar growth in the sporangial interior is approximately at right angles to the long axis of the nucleus. Spurr's resin. X15,000.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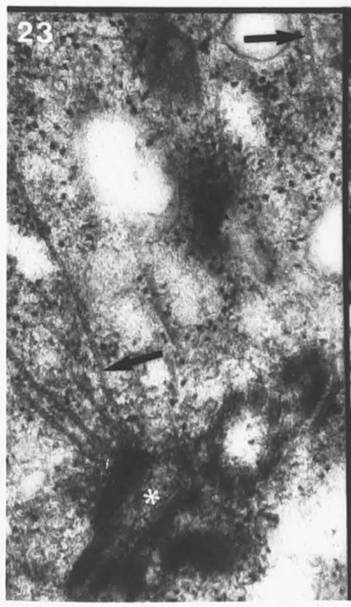
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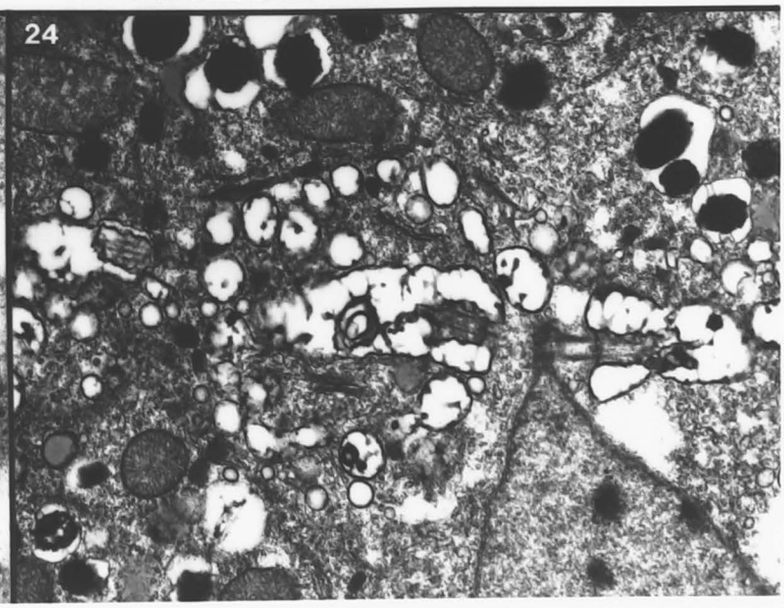
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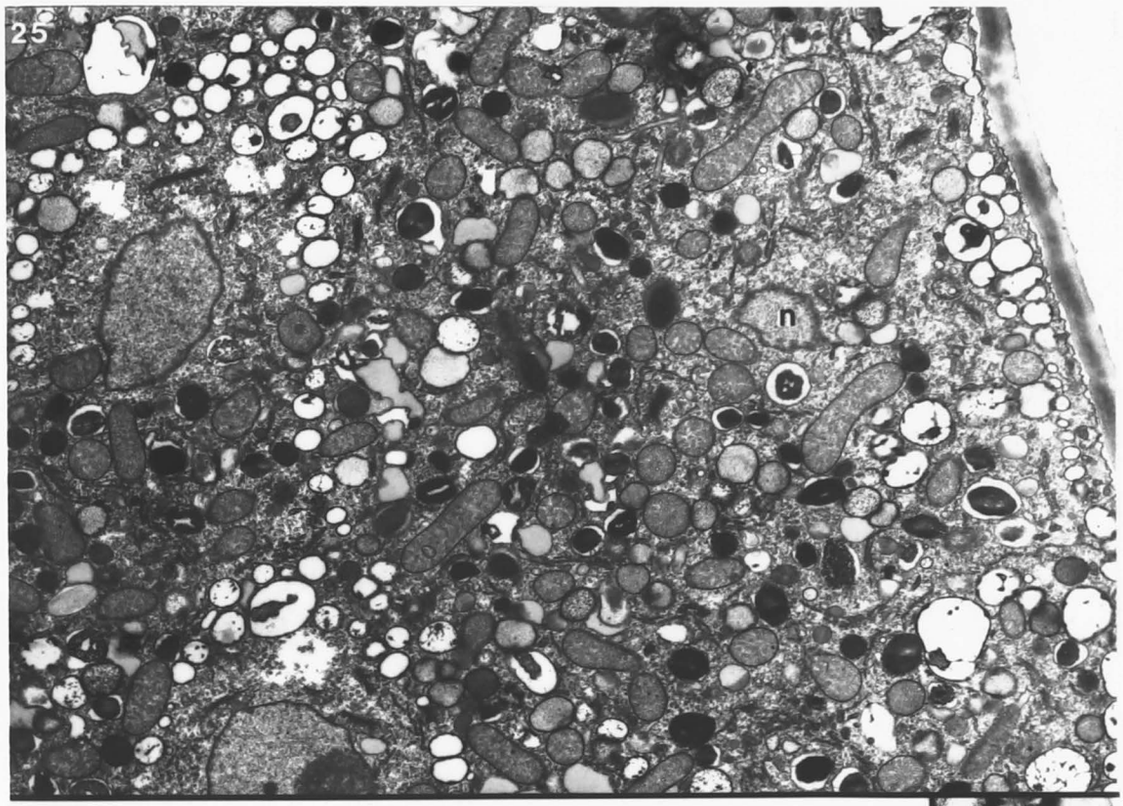
Figs 2.25-2.27 Organisation of the internal cleavage planes in sporangia of *P. cinnamomi*. II. Later features (40-50 min after induction). Spurr's resin.

Fig 2.25 At 40min, wavy lines of cleavage vesicles surround two internal nuclei. The cytoplasm around the small profile of a cortical nucleus (n) is bordered only by the cortical cleavage plane. X9300.

Fig 2.26 The internal planes are fully organised and border uninucleate domains. Cortical fusion is complete except at the apex (a). Some flagellar profiles are extracellular (large arrow), while others remain in axonemal vacuoles (small arrow). Mitochondria (arrowheads) are common near domain boundaries. X4000.

Fig 2.27 Before fusing, cleavage vesicles of the cortical plane apparently become appressed against the sporangial plasma membrane. Microbody-like organelles (arrow) and ventral vesicles (v) lie near the wall. X18,000.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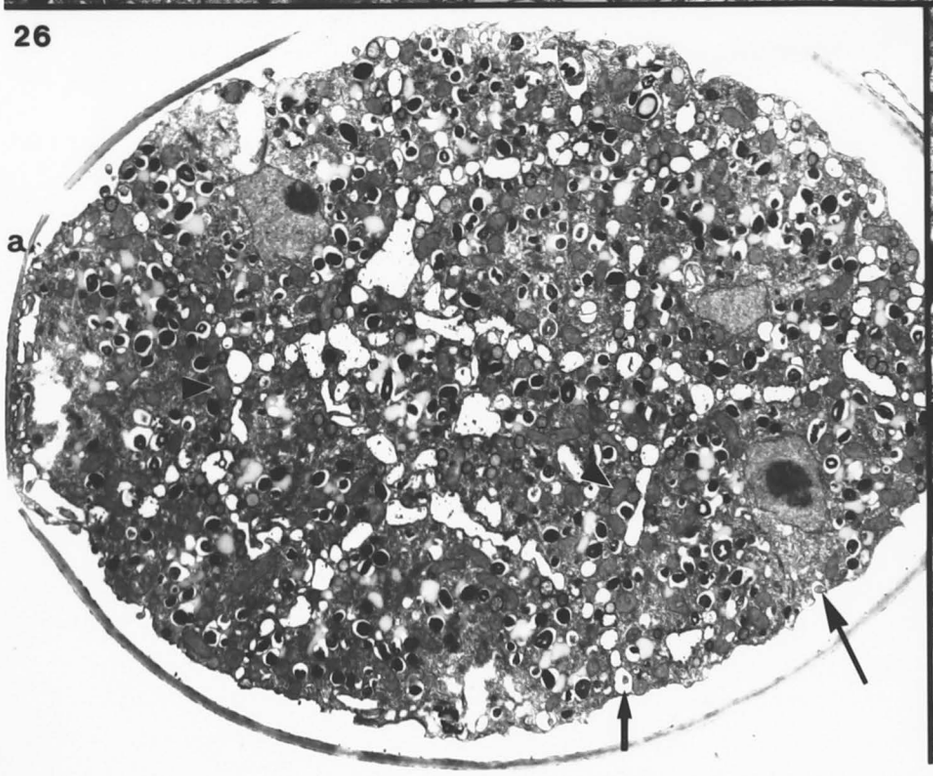
n

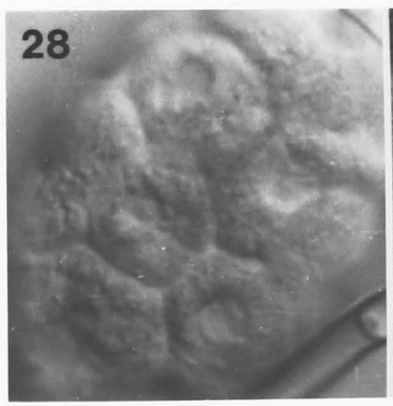

26
a


27
v

Figs 2.28-2.29 Fully cleaved sporangia of *P. cinnamomi.*

Fig 2.28 Hexagonal outlines border individual zoospore initials of the sporangial cortex. The prominent water expulsion vacuoles of cortical zoospore initials face outward. Differential interference contrast optics of live material. X1700.

Fig 2.29 Hoechst 33662 staining of nuclei centrally placed within the hexagonal outlines of zoospore initials. X2200.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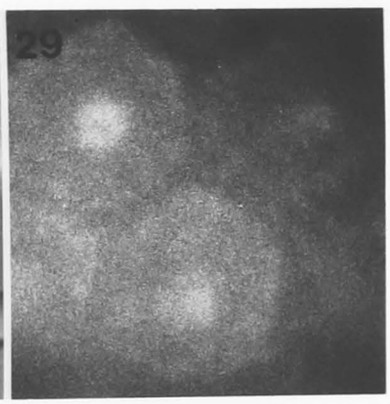
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Figs 2.30-2.35 Compartmentalisation of sporangia of *P. cinnamomi* (50-65 min after induction). Spurr's resin except Figs 2.33,2.35, Lowicryl K4M resin.

Fig 2.30 Fusion of the vesicles of the internal planes completes compartmentalisation. Profiles of flagella (arrow) lie in the intercellular space. X3500.

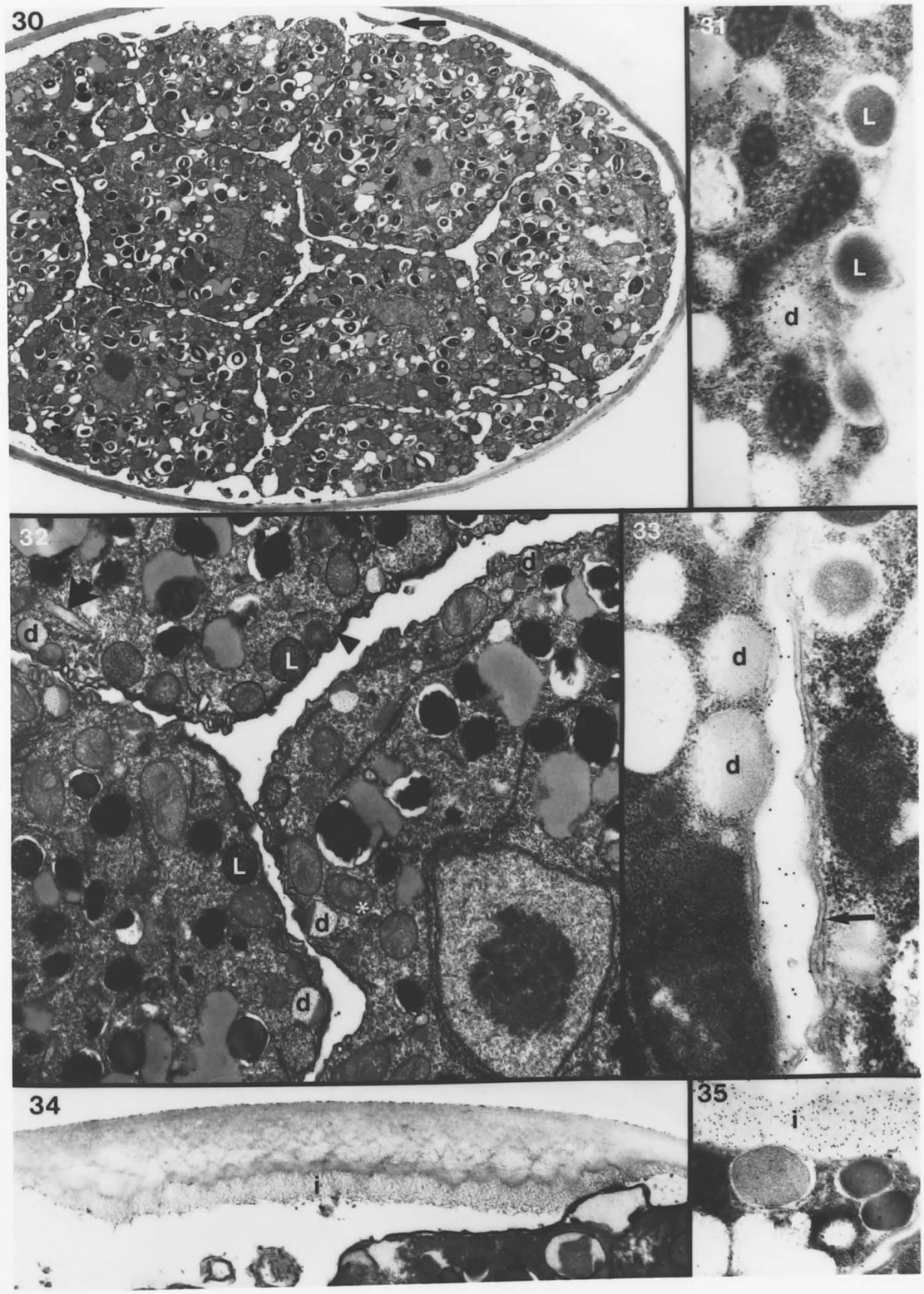
Fig 2.31 Large peripheral vesicles (L) at the surface of a newly formed zoospore initial. Dorsal vesicles (d), labelled with mAb Cpa-2-Au_{10}, are seen further from the surface. X24,500

Fig 2.32 At a later stage than Fig 2.31, dorsal vesicles (d) are seen near the plasma membrane as are the large peripheral vesicles (L). Peripheral cisternae (arrowhead) lie below the plasma membrane. Membranous annuli are evident in mitochondrial profiles (asterisks) which are common in the cortex of the zoospore initials. Mastigoneme packets are present (arrow). X16,000.

Fig 2.33 In a section labelled with Cpw-1-Au_{10}, gold particles are closely associated with the plasma membrane of the zoospore initials and the flocculent material in the intercellular space. Peripheral cisternae (arrow) bind Cpw-1-Au_{10} at low levels. Dorsal vesicles (d) lie in the zoospore cortex. X37,000.

Fig 2.34 Papillar material at maximum thickness with a distinct inner layer (i). X13,000.

Fig 2.35 Cpw-1-Au_{10} heavily labels the inner layer (i) of the papillar material. X16,000.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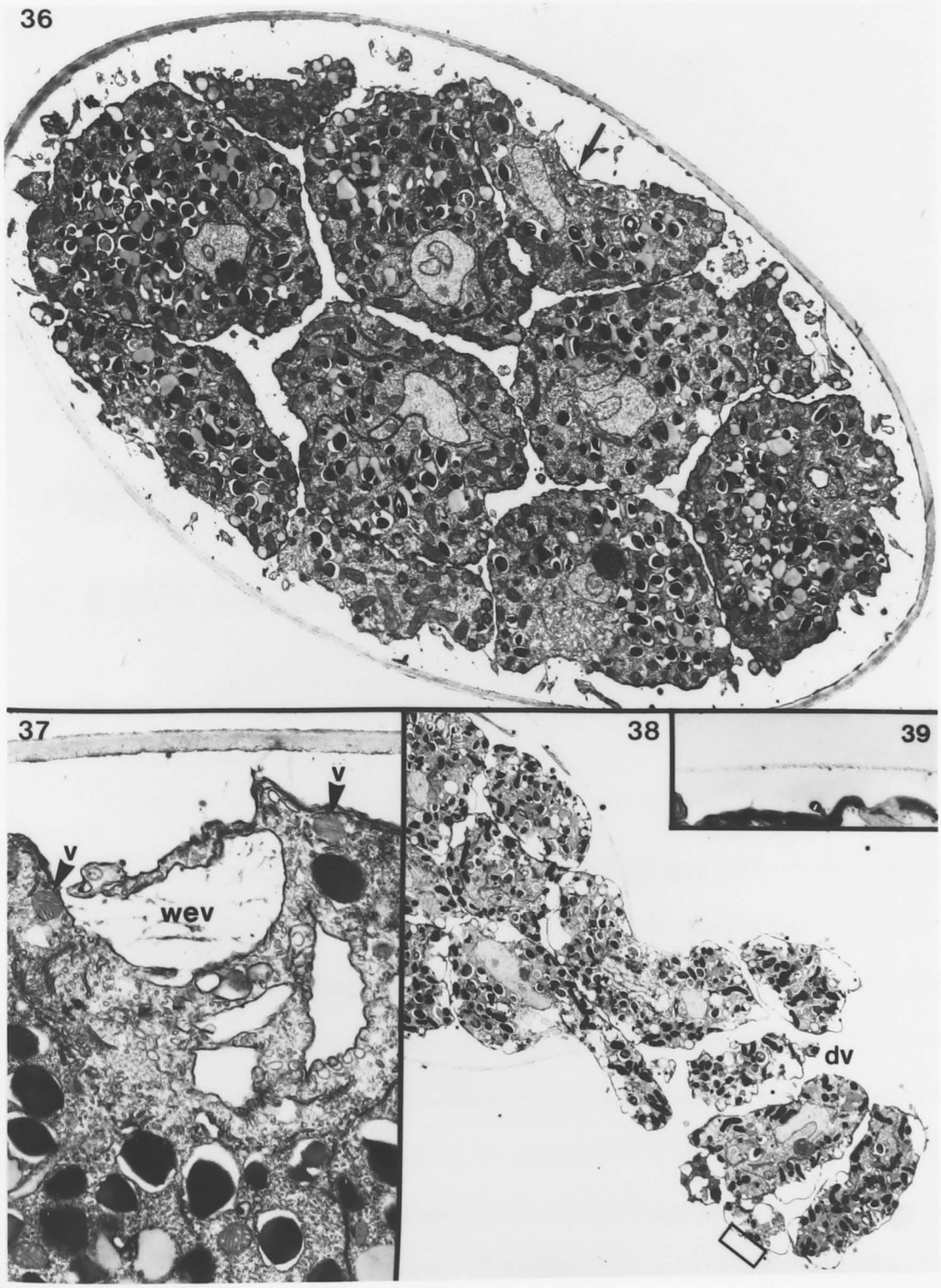
i

Fig 2.36 From about 65 min the zoospore initials round up and a groove develops (arrow) in the surface near the narrow pole of the nucleus. Nuclear profiles become irregular. Spurr's resin. X3,800.

Fig 2.37 Outward facing water expulsion vacuole (WEV) in the clear zone region of a cortical zoospore initial. Numerous vacuoles and small vesicles are seen nearby. Ventral vesicles (v) are seen at the surface in this region. Spurr's resin. X16,000.

Fig 2.38 Zoospores exit the sporangium into a fragile discharge vesicle (dv). Boxed area shown at higher magnification in Fig 2.39. Spurr's resin. X2,300.

Fig 2.39 The wall of the discharge vesicle. Spurr's resin. X16,000.


36
37
v
v
wev
38
dv
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3

ULTRASTRUCTURE OF ZOOSPOROGENESIS IN PHYTOPHTHORA. II. A STUDY OF FREEZE-SUBSTITUTED SPORANGIA OF P. CINNAMOMI AND P. PALMIVORA

3.1 INTRODUCTION

From the great number of ideas that have been put forward to explain the genesis of the additional plasma membranes required by cells formed during cytokinesis (Pickett-Heaps, 1972a; Rappaport, 1971; 1986), two principal models have emerged. The first involves a two stage process in which vesicles are aligned along some part or all of the future plane of cleavage, this part then being established by fusion of the vesicles. The second model involves the progressive extension or stretching of the partitioning membranes along some part or all of the developing cleavage plane, without any observed prealignment of vesicles. Examples supportive of both mechanisms have been reported in animal (Thomas, 1968; Rappaport, 1986), plant (Gunning, 1982;

Volker, 1972), fungal (Hawker & Gooday, 1967; Cole, 1986) and protoctistan (*sensu* Margulis *et al.* 1989: Rawlence, 1973; Lokhorst & Segaar, 1989) systems.

In a previous study of chemically fixed sporangia of the notorious plant pathogen, *Phytophthora cinnamomi*, I employed electron microscopy to examine the process by which the infectious, motile zoospores of this organism are formed. I concluded that formation of the zoosporic plasma membranes during sporangial cleavage followed the first of the models described above, that is, fusion of prealigned vesicles (Chapter 2). This process has also been described for *P. palmivora* (formerly *P. parasitica*, Hohl & Hamamoto, 1967) and all other species of *Phytophthora* for which the mechanism of zoosporic plasma membrane genesis has been described (review by Hemmes, 1983). In my previous study, however, I noted that the appearance of elements of the cleavage system of *P. cinnamomi* showed some marked variations which did not conform to the vesicle alignment model and which were related to the osmolarity of the fixative (Chapter 2). These variations led me to suggest that my conclusions be tested by examining sporangia prepared by rapid freezing, a procedure which is considered to provide better preservation of membrane morphology than chemical fixation.

In this study I re-investigate zoospore formation in *P. cinnamomi* and *P. palmivora* using rapid freezing, both at ambient and high pressure, followed by freeze substitution (RF-FS). My studies have been assisted by the use of a monoclonal antibody that labels cleavage elements in *P. cinnamomi* and *P. palmivora.* This is the first time, to my knowledge, that rapid freezing has been used to study cytokinesis in any eukaryotic organism for which vesicle alignment has been proposed to play a role in partitioning membrane formation. The results indicate that cleavage of the sporangium follows not from the fusion of prealigned vesicles as suggested by chemical fixation but rather from the progressive extension of partitioning membranes. I believe that the apparent misinterpretation of the cytokinetic process that has occurred in *Phytophthora* may reflect a much wider problem, involving studies of a great variety of eukaryotic

organisms. I also report other novel observations of sporangial structure and partially characterize an extensive extracellular matrix which derives from the contents of the cleavage system and which may play a role in zoospore release.

3.2 MATERIALS AND METHODS

3.2.1 Organisms and sampling

The cultures of *P. cinnamomi* (DAR 52646) and *P. palmivora* used in this study were induced to produce sporangia and zoospores by the methods of Hardham & Suzaki (1986). Briefly, sporangial formation was induced by transfer of mycelium to a nutrient poor medium; sporangial cleavage was induced by treatment with cold distilled water. Zoospores were released at about 50 min (*P. cinnamomi*) and 20 min (*P. palmivora*) after a cold shock. Samples were taken before induction of cleavage and at intervals between induction and release.

3.2.2 Freeze fixation

3.2.2.1 High pressure freezing

Wet tufts of mycelium with attached sporangia were placed in gold specimen holders (Balzers BB113142-1) with mineral salts solution (Hardham & Suzaki, 1986, for pre-induction) or distilled water (post-induction) filling the remaining space. Pairs of holders were clamped together and frozen in a Balzers HPM 010 hyperbaric freezer. After freezing, the holders were snapped apart under liquid nitrogen and transferred to the substitution medium.

3.2.2.2 Plunge freezing

Tissue was frozen on formvar covered loops following the procedures of Lancelle *et al.* (1986). To place tissue on the loop, a novel approach was used. The loop was held in place on the platform of a dissecting microscope by plasticine. About 8-

10µl of distilled water or mineral salts solution was placed on the upper face of the loop. A tiny tuft of lightly blotted mycelium was then placed in this drop and spread out using fine forceps. The water was then sucked up by a paper wick and the loop rapidly transferred to the plunging device.

3.2.3 Freeze substitution and preparation for electron microscopy

General procedure: Tissue frozen by the above methods was freeze substituted using the procedures of Lancelle *et al.* (1986). The method was modified by the inclusion of 0.05% uranyl acetate in the substitution medium, and after 36h at -80°C, the vials were first warmed to -30°C for 10h before being brought to room temperature. Tissue was then rinsed in acetone several times, and stained en bloc in 5% uranyl acetate in methanol for 2h. After rinsing with acetone, the tissue was infiltrated with Epon resin and polymerized. Sections were stained for 3-5 min in Reynolds' lead citrate (Reynolds, 1963)

Immunolabelling procedure: For material destined for immunogold labelling, the freeze substitution, infiltration and polymerization procedures of Lancelle and Hepler (1989) were followed with the inclusion of a 10h stage at -30°C prior to bringing the samples to room temperature during freeze substitution. The material was then embedded in LR White resin. Infiltration and polymerization with UV light were carried out at room temperature. Immunolabelling of sections on gold grids followed the methods of Gubler & Hardham (1988). Monoclonal antibody (mAb) Cpw-1 was used. This mAb has been previously shown to have a strong affinity for elements of the cleavage system and weaker binding to dictyosomes and peripheral cisternae in sporangia of *P. cinnamomi* (Chapter 2). After immunolabelling, sections were stained with 2% aqueous uranyl acetate for 20-30 min, followed by 2 min in lead citrate.

3.2.4 Immunoblot analysis

Proteins from freeze dried samples were solubilized in SDS sample buffer (63mM-Tris HCl buffer, pH 6.8, containing 2% SDS, 50mM dithiothreitol, 0.001%

bromphenol blue and 10% glycerol) for 5 min at 100°C. After heating, samples were centrifuged and the supernatant kept on ice until use. Solubilized proteins were separated by homogeneous (7% or 12% acrylamide) or gradient (5% to 20% acrylamide) SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose (Towbin *et al.* 1979). After transfer, the nitrocellulose sheets were stained with 0.2% Ponceau S in 3% trichloroacetic acid, cut into strips and blocked with 5% skim milk powder in Tris-buffered saline (TBS: 10mM Tris-HCl, pH 7.5 containing 150mM NaCl). After 1-2h, the strips were washed with TBS containing 0.5% Tween 20 (TBST). They were then incubated with antibodies in ascites fluid (diluted 1/800 in TBST containing 3% bovine serum albumin) for 1h, washed five times with TBST for 5 min each, and then incubated for 45 min with sheep anti-mouse IgG antibodies conjugated to alkaline phosphatase. After washing twice in TBST, twice in TBS and once in enzyme substrate buffer (ESB: 100mM Tris-HCl, pH 9.5 containing 100mM NaCl and 50mM MgCl), bound phosphatase was detected by immersing the strips in enzyme substrate containing 0.44% Nitro Blue Tetrazolium (Sigma, St. Louis, U.S.A.) and 0.33% 5-brom-4-chloro-3-indolyl-phosphate (Boehringer Mannheim, Germany) in ESB. Prior to antibody incubation, some strips were treated for 1h with either Pronase E (Sigma, 1 mg/ml in 50mM Tris-HCl, pH 7.5) or sodium periodate (20mM in sodium acetate buffer, pH 4.5).

3.3 RESULTS

3.3.1 Sporangia before and during early cleavage

The most notable feature of sporangia rapidly frozen before the induction of cleavage and during early post-induction stages was that these were the only sporangia in which large discrete cleavage vesicles were observed. These vesicles were most evident in regions of the cytoplasm that were close to the basal body associated pole of a nucleus and relatively free of other major organelles (Figs 3.1,3.3). In *P. cinnamomi*, cleavage vesicles were especially numerous in these regions (Fig 3.1). In both species the basal

body associated poles of nuclei in the sporangial cortex pointed towards the wall (Figs 3.1,3.3). During early post-induction, irregularly shaped cleavage elements were often seen around the nuclear pole (Fig 3.2).

A significant difference between the two species was the presence of a single large vacuole in the centre of the sporangium of *P. palmivora* (Fig 3.3). The vacuole was evident before induction, and was often ringed by a multilayered array of rough endoplasmic reticulum (Fig 3.3). Such arrays have not previously been described in *Phytophthora* and nothing comparable was seen in *P. cinnamomi*, which has a series of smaller central vacuoles (Chapter 2). In these and later stages microtubules were seen radiating from the basal bodies of both species (Fig 3.1). In *P. palmivora*, limited flagellar (Fig 3.3) and cleavage plane development was sometimes evident prior to induction.

The conformation of elements of the developing cleavage system, at these and later times, was directly comparable in high pressure and plunge frozen sporangia. With the exception of the large peripheral vesicles, other sporangial components were also equally well preserved by both techniques. The large peripheral vesicles were well preserved in plunge frozen sporangia but not in high pressure frozen material (Fig 3.3; Chapter 4).

3.3.2 Formation of cortical and internal cleavage planes

Subsequent stages of cleavage involved the formation of a single, cortical cleavage plane parallel to the sporangial wall and a series of internal cleavage planes separating uninucleate domains. In both species organization of the cortical plane proceeded more rapidly than that of the internal planes.

The first indications of the development of the cortical plane were extended cleavage elements which lay mainly parallel to the sporangial wall (Fig 3.4). These cleavage elements were frequently irregularly arranged (Fig 3.4), especially at the apex of the sporangium, and were apparently of greater surface area than would ultimately be

required. Several sporangial structures appeared to be associated with the development of the cleavage elements. The contents of elongated cisternae at the trans face of the Golgi apparatus often appeared similar to those of cleavage elements (Figs 3.2,3.5). A trans Golgi network sometimes appeared to interconnect these cisternae with cleavage elements (Fig 3.5). Small coated and uncoated vesicles were often numerous adjacent to cleavage elements and dictyosomes, and blebs were frequently seen on the surface of the elements (Figs 3.5,3.6). Discrete cleavage vesicles of the type seen earlier (Fig 3.1) were not evident at this or any later stages. Serial section analysis revealed that occasional circular profiles suggestive of such vesicles (Fig 3.4) were continuous with the extended cleavage elements.

By the time the cortical cleavage plane was fully developed (Fig 3.7), most of the irregular and superfluous elements of the cortical plane had disappeared. This reduction occurred more rapidly in *P. cinnamomi* than in *P. palmivora*. Flagella were evident within the developing (Fig 3.4) and completed (Fig 3.7) cortical cleavage plane which is part of the future extracellular space of the cleaved sporangium. The fully developed cortical cleavage plane cut off a shell of cytoplasm between itself and the sporangial plasma membrane (Fig 3.7). This shell occasionally had connections to the main cytoplasm. Apparent cytoplasmic islands (Figs 3.7-3.8) which were evident in the cortical cleavage plane often proved to be projections of the shell or of the main cytoplasm when checked by serial sectioning.

The cleavage planes which will surround the internal nuclei of *P. cinnamomi* appeared to develop initially as membranous sheets extending back behind the nucleus from the narrow pole region (Fig 3.9). The distal edges of these sheets were commonly dilated (Fig 3.9). As in the case of the developing cortical cleavage plane, there were often very irregular arrangements of cleavage elements around the narrow poles of internal nuclei. These irregularities disappeared as cleavage progressed. In *P. palmivora,* there was no direct evidence of a polar basis to cleavage element extension in the sporangial interior. This may, however, reflect the difficulty of capturing

intermediate stages of development in this species due to the rapidity of its cleavage process (Hohl & Hamamoto, 1967). In both species, cleavage progressed as the partitioning membranes continued to extend throughout the cytoplasm, interconnecting with each other to delineate the future zoospores (Figs 3.10-3.12). Serial sectioning indicated that the membranes extended as sheets, in this latter period at least. Just prior to release, the zoospores rounded up and the intercellular spaces became more obvious (Fig 3.13). The central vacuoles of both species disappeared during cleavage; there was no indication of their fate. The cortical shell of cytoplasm described earlier (Fig 3.7) had also disappeared in both species by the completion of cleavage. This occurred at a later stage of internal cleavage in *P. palmivora* than in *P. cinnamomi* (compare Figs 3.10 and 3.11). Prior to its disappearance the shell became thinner and often fragmented (Fig 3.8). My evidence suggests that this fragmentation involved localized fusion of the outer membrane of the cortical cleavage plane with the plasma membrane of the sporangium (Fig 3.8). Serial sectioning showed that the fragments were often interconnected and were occasionally continuous with the main cytoplasm. The inner membrane of the cortical plane was retained and formed part of the plasma membrane of the zoospores.

Apart from the cleavage system and an extracelllular matrix described below, two other novel features of sporangial structure were evident in this study. First, sinuous projections of cytoplasm were observed within the water expulsion vacuoles of both species and the membrane surrounding these vacuoles had numerous vesicles and blebs associated with it (Figs 3.11-3.12). Second, peripheral cisternae (flattened organelles previously described in sporangia of *P. cinnamomi* at late stages of cleavage only; Chapter 2) were evident in both species from very early cleavage stages (Fig 3.5) onwards and they were much more extensive in the zoospore initials (Figs 3.11,3.12) than previously observed.

3.3.3 The extracellular matrix

A notable and previously undescribed feature of sporangia was the dark, grainy appearance of material within the cleavage system (Figs 3.1-3.2,3.4-3.12). When

cleavage was complete, this material was external to the zoospore initials, forming an extracellular matrix (Figs 3.11-3.13). Variability in the apparent density of this material was marked in high pressure frozen material (e.g. Figs 3.3,3.8). In plunge frozen material, a consistent decrease in density was evident in sporangia sampled just prior to zoospore release (Fig 3.13).

The extracellular matrix of both species was labelled by mAb Cpw-1 (Figs 3.14-3.16). A thick layer of extracellular matrix was typically seen at the sporangial apex and showed particularly strong binding of mAb Cpw-1 (Fig 3.15). The sporangial wall also showed some binding of mAb Cpw-1, especially near the sporangial apex (Fig 3.15), suggesting that the antigen is leaking outwards. As in *P. cinnamomi* (Chapter 2), the central vacuole of *P. palmivora* was not labelled by mAb Cpw-1 (Fig 3.16). The antigen(s) recognized by Cpw-1 were identified on immunoblots of sporangial extracts. In *P. cinnamomi*, Cpw-1 bound to several broad bands with apparent molecular weights between 60 and 330kD (Fig 3.17). Similar results were obtained for *P. palmivora* (data not shown). The cleavage state of sporangia had no bearing on the number of bands detected. Pronase treatment of the transferred proteins of *P. cinnamomi* abolished antibody binding; treatment with periodate did not (Fig 3.17).

3.4 DISCUSSION

3.4.1 A new model for sporangial cleavage in *Phytophthora*

The results of this study describe a significantly different process of partitioning membrane formation to that previously reported during zoosporogenesis in *P. palmivora* and *P. cinnamomi* (Hohl & Hamamoto, 1967; Chapter 2). There was no evidence that cleavage follows from the fusion of prealigned vesicles, but rather the data indicate that subdivision of the sporangium results from the progressive extension and eventual interconnection of membranous sheets. These new findings are summarized and compared with the previous model in Fig 3.18. I consider that the discrepancy between

this and previous reports arises from the different techniques used to preserve sporangia, namely chemical fixation and RF-FS.

RF-FS is considered superior to chemical fixation for the preservation of cell structure generally and membranous components particularly (Lancelle *et al.* 1985; 1986; Gilkey & Staehelin, 1986; Cresti *et al.* 1987; Howard & O'Donnell, 1987). Chemical fixation may cause artefactual alterations in the morphology of membranous components. In a comparative study of wall-destined vesicles in freeze substituted and chemically fixed hyphae of the Oomycete *Saprolegnia*, for example, Heath *et al.* (1985) observed in frozen material densely staining tubular elements that became partially vesiculated and lost their contents following chemical fixation. Also, studies by McCully and co-workers have shown that in petiolar hairs of certain plants the membranous canalicular system, which is visible as elongated strands in living cells, becomes highly vesiculated during chemical fixation (O'Brien *et al.* 1973; Mersey & McCully, 1978) but retains its *in vivo* morphology when rapidly frozen (McCully & Canny, 1985). I propose that the data from sporangia preserved by RF-FS also more faithfully represent the structure of the living cell, and that the apparent alignment of vesicles seen in previous studies of cleavage in *Phytophthora* probably resulted from artefactual vesiculation during chemical fixation of membranous sheets similar to those seen in this study. Since the cleavage membranes of chemically fixed sporangia do not vesiculate during the final stages of zoosporogenesis, the network of aligned vesicles seen earlier (Fig 3.18) can readily be misinterpreted as an intermediate stage in the cleavage process. In addition, rather than occurring within specialized "axonemal vacuoles" (Hohl & Hamamoto, 1967; Hemmes, 1983; Chapter 2), flagellar development is shown by RF-FS to occur within the general system of cleavage planes.

3.4.2 Genesis of the cleavage membranes

The present study provides evidence that development of the cleavage planes, at least in *P. cinnamomi*, begins in regions near the basal body associated nuclear poles

where dictyosomes are concentrated and cleavage vesicles are initially clustered. The disappearance of the cleavage vesicles coincides with the formation of the first expanses of cleavage membranes, indicating that cleavage vesicles may be incorporated into the developing cleavage planes. Irregular cleavage elements that are seen in early post-induction stages may be transitional stages in the transformation of cleavage vesicles into more extended forms. Further expansion of the partitioning membranes appears to involve the contribution of membrane from dictyosomes, some of whose cisternae appear interconnected with developing cleavage elements through a trans Golgi network. This is the first report of a trans Golgi network in *Phytophthora*; the network may be disrupted during chemical fixation. Coated and uncoated vesicles, often seen near dictyosomes and cleavage elements, may also be involved in membrane augmentation during cleavage element extension. These observations are in agreement with previous proposals of a dictyosomal origin for cleavage elements in *Phytophthora* (Hohl & Hamamoto, 1967; Elsner *et al.* 1970; Chapter 2). Vesicles may be transported to the edge of the expanding system, which is characteristically dilated, or could be incorporated close to the nuclear pole. The vesicles and blebs could also play a role in membrane retrieval during development of the cleavage system.

The absence of any structural interconnection between the large vacuoles and the cleavage system and the lack of labelling of the vacuolar contents with mAb Cpw-1 indicate that the large vacuoles do not play a role in the cleavage process.

3.4.3 Implications of this study for cytokinesis in other eukaryotes

To my knowledge, this is the first published study to employ rapid freezing instead of chemical fixation to study cytokinesis in any system in which partitioning membrane formation would be expected to involve the fusion of prealigned vesicles. As such it represents the fairest test yet of this model, given the unpredictable preservation of membrane form by chemical fixation. The apparent failure of the alignment/fusion model in this study of zoosporogenesis in *P. cinnamomi* and *P. palmivora* has wide-

ranging implications for my understanding of cytokinesis in other eukaryotes. It has already been proposed (Schroeder, 1970; Rappaport, 1971; 1986) that the small body of studies which report vesicle alignment during cytokinesis in animal cells suffers from the use of a chemical fixation protocol that leads to vesiculation of the furrowing membranes. To my knowledge, there has been no consideration of similar vesiculation during cytokinesis in non-animal systems, although many studies have expressed concern over the difficulty of discerning whether apparent vesicles, seen in single cross-sections, might actually be part of a continuous system (Burr & West, 1970; Marchant & Pickett-Heaps, 1971; Zaar & Kleinig, 1975). Wilson *et al.* (1990) have recently outlined a theoretical basis for the general case of vesiculation of membranes by chemical fixatives. For reasons given below I believe that artefactual vesiculation of partitioning membranes may have occurred in many studies of a wide variety of eukaryotes, especially protoctists.

3.4.4 Absence of loosely arranged vesicles

Many of the studies that report vesicle alignment in protoctistan systems (e.g. Goodman & Rusch, 1970; Porter, 1972; Mims, 1973) have one, possibly critical, feature in common with the suspect animal reports and the chemical fixation studies of zoosporogenesis in *Phytophthora*. In all cases, a stage is described where vesicles are arranged in a row corresponding to the future plane of cleavage, but there is no prior stage described where the vesicles are loosely arranged in this region. A loose arrangement of vesicles is, however, typically described during early cell plate formation in plants (Hepler & Jackson, 1968; Gunning, 1982). It is commonly believed that this loose arrangement in plant cells arises because the vesicles are in the process of moving towards the future zone of cleavage from either side of it (Hepler & Jackson, 1968; Gunning, 1982). The absence of such a stage in some protoctistan systems might be explained by the possibility that cleavage vesicles move *along* the future plane of cleavage before coming to rest in that plane and fusing. In other cases, insufficient

sampling of cells from different stages of cleavage may have resulted in the loose arrangement of vesicles having gone undetected. Alternatively, if the neatly arranged stage is itself an artefact of preparation then a prior loose stage may not be required for whatever is the true process of membrane genesis. The present study, when viewed in the light of previous studies of zoosporogenesis in *Phytophthora*, suggests that the absence of a loosely arranged stage may well be a good indicator that the neatly arranged stage is an artefact.

3.4.5 Taxonomic heterogeneity

The taxonomic distribution of organisms in which vesicle alignment/fusion has been described is perplexing. Since this process is commonly considered an evolutionarily advanced feature of cytokinesis (Pickett-Heaps, 1972a; Rawlence, 1973), one might expect to trace clear lines of ancestry for this character back through the taxa in which it appears towards some primitive organism in which it first evolved. The impossibility of doing this is best illustrated by considering the protoctista. The vesicle alignment model has been proposed for a large number of phylogenetically distant protoctistan taxa, in many of which there have also been reports of the more primitive mechanism, namely progressive extension of the partitioning membranes (Table 3.1). In a number of cases the two mechanisms have been proposed in reports describing cytokinesis at different (e.g. *Labyrinthula* sp., *Plasmodiophora brassicae, Oedogonium cardiacum,* Table 3.1) or even the same (*Physarum polycephalum, Spirogyra* sp., Table 3.1) stage of the life cycle in the one genus or species.

While taxonomic heterogeneity such as this may have a variety of natural sources (Fowke & Pickett-Heaps, 1969; Coss & Pickett-Heaps, 1973; Watson *et al.* 1985) another possibility is that some variability has arisen from methodological complications such as those seen in this study. It is interesting that, with limited exception (Lucarotti & Federici, 1984), apparently the only eukaryotes that exhibit, during cytokinesis, a loose arrangement of vesicles similar to that seen in higher plant cell plate formation are

certain green algae (Phyla Conjugaphyta and Chlorophyta, *sensu* Margulis *et al.* 1989: e.g. Fowke & Pickett-Heaps, 1969; Pickett-Heaps & Fowke, 1970; Floyd *et al.* 1972; Pickett-Heaps, 1973; Marchant & Pickett-Heaps, 1973). The green algae and higher plants are commonly considered to be phylogenetically related (Pickett-Heaps, 1972a) and these two groups may represent the true evolutionary lineage of cytokinesis involving vesicle alignment/fusion. Cell plate formation has been proposed to occur in some brown algae (Rawlence, 1973; Markey & Wilce, 1975) but these descriptions do not include the loosely arranged stage and may be cases of artefactual vesiculation.

Table 3.1 Some protoctistan phyla in which both of the main modes of cell cleavage occur.

Phylum	Vesicular alignment/fusion	Progressive extension
Oomycota	***Phytophthora capsici*** Williams & Webster, 1970	***Saprolegnia ferax*** Gay & Greenwood, 1966
Labyrinthulomycota	***Labyrinthula sp.*** Porter, 1972	***Labyrinthula* sp.** Perkins & Amon, 1969
Plasmodiophoromycota	***Plasmodiophora brassicae*** Williams & McNabola, 1967	***Plasmodiophora brassicae*** Garber & Aist, 1979
Plasmodial Slime Moulds	***Physarum polycephalum*** Goodman & Rusch, 1970	***Physarum polycephalum*** Zaar & Kleinig, 1975
Chlorophyta	***Oedogonium cardiacum*** Coss & Pickett-Heaps, 1973	***Oedogonium cardiacum*** Coss & Pickett-Heaps, 1973
Xanthophyta	***Pseudobumilleriopsis pyrenoidosa*** Deason, 1971	***Botrydiopsis alpina*** Lokhorst & Segaar, 1989
Conjugaphyta	***Spirogyra* sp.** Fowke & Pickett-Heaps, 1969	***Spirogyra* sp.** Fowke & Pickett-Heaps, 1969
Phaeophyta	***Ascophyllum nodosum*** Rawlence, 1973	***Chorda tomentosa*** Toth, 1974

I believe that descriptions of cell plate formation in plants and certain green algae have features that make them distinct from, and more credible than reports of vesicle alignment/fusion in other eukaryotes. Nevertheless, the possiblity remains that cell plate membranes which have formed *in vivo* from the fusion of aligned vesicles may exhibit a temporary labile phase, similar to that shown by cleavage membranes in *Phytophthora*, during which they are susceptible to vesiculation by chemical fixation.

3.4.6 The extracellular matrix and its possible role in zoospore release

The results describe, for the first time in *Phytophthora*, the presence of a dense, grainy material filling elements of the developing cleavage system and forming an extracellular matrix which surrounds the zoospores in the fully cleaved sporangium (Fig 3.18). The passage of this material during cleavage was traced, in both species, by immunogold labelling using mAb Cpw-1. In chemically fixed material of *P. cinnamomi*, mAb Cpw-1 binds to flocculent material inside the cleavage system (Chapter 2). This material probably represents all that remains after chemical fixation of the dense matrix seen in freeze fixed sporangia. Polyacrylamide gel electrophoresis indicates that Cpw-1 binds to one, or more, proteins with apparent molecular weights between 60 and 330kD. Although antibody binding was not abolished by periodate treatment, the smearing of the bands is suggestive of extensive glycosylation of the antigen (Goldkorn *et al.* 1989).

Extracellular material, often referred to as mucilage, has been described surrounding the spores, sperms and similar structures of many protoctists (Moore, 1965; Pickett-Heaps, 1972b; Scott & Dixon, 1973; Toth, 1974; Lunney & Bland, 1976; Franke *et al.* 1977; Duckett & Peel, 1978) and fungi (Ingold, 1968) with several reports noting its origin from cleavage elements (Moore, 1965; Scott & Dixon, 1973; Lunney & Bland, 1976; Franke *et al.* 1977). The major significance of this material is its proposed capacity to act as a swelling gel (de Bary, 1887; Ingold, 1968; Pickett-Heaps, 1972b; Toth, 1974; Lunney & Bland, 1976; Duckett & Peel, 1978) or as an osmoticum (Ingold, 1971; Scott & Dixon, 1973) which may be involved in events leading to rupture of the

storage organ and release of its contents. Although the swelling gel model has been considered theoretically plausible to explain sporangial discharge in *Phytophthora* (MacDonald & Duniway, 1978; Gisi & Zentmyer, 1980) there has never, until now, been evidence of any extracellular material extensive enough to be seriously considered. There is indirect evidence that the extracellular matrix described herein might swell, since the volume occupied by it apparently increases just prior to zoospore release. If swelling does occur this may account for some or all of the force needed for bursting the sporangium.

Alternatively, the extracellular matrix material may operate as an osmoticum. Numerous studies of *Phytophthora* have proposed that sporangial discharge results from an osmotically driven increase in hydrostatic pressure (reviewed in Gisi, 1983). One deficiency of the hydrostatic model is that it relies on the existence of a semipermeable barrier across which the hypothetical osmoticum exerts its influence (Gisi & Zentmyer, 1980). The present study indicates that the sporangial membrane disappears too early (at least in *P. cinnamomi*) for it to have any role in this process. A similar dilemma in other systems has been addressed by examining the possibility that the sporangial wall itself may act as a semipermeable barrier (Money & Webster, 1988; 1989; Money, 1990). While further work is needed to assess the applicability of this proposal in *Phytophthora*, if swelling of an extracellular matrix is responsible for sporangial discharge, then no semipermeable barrier is required.

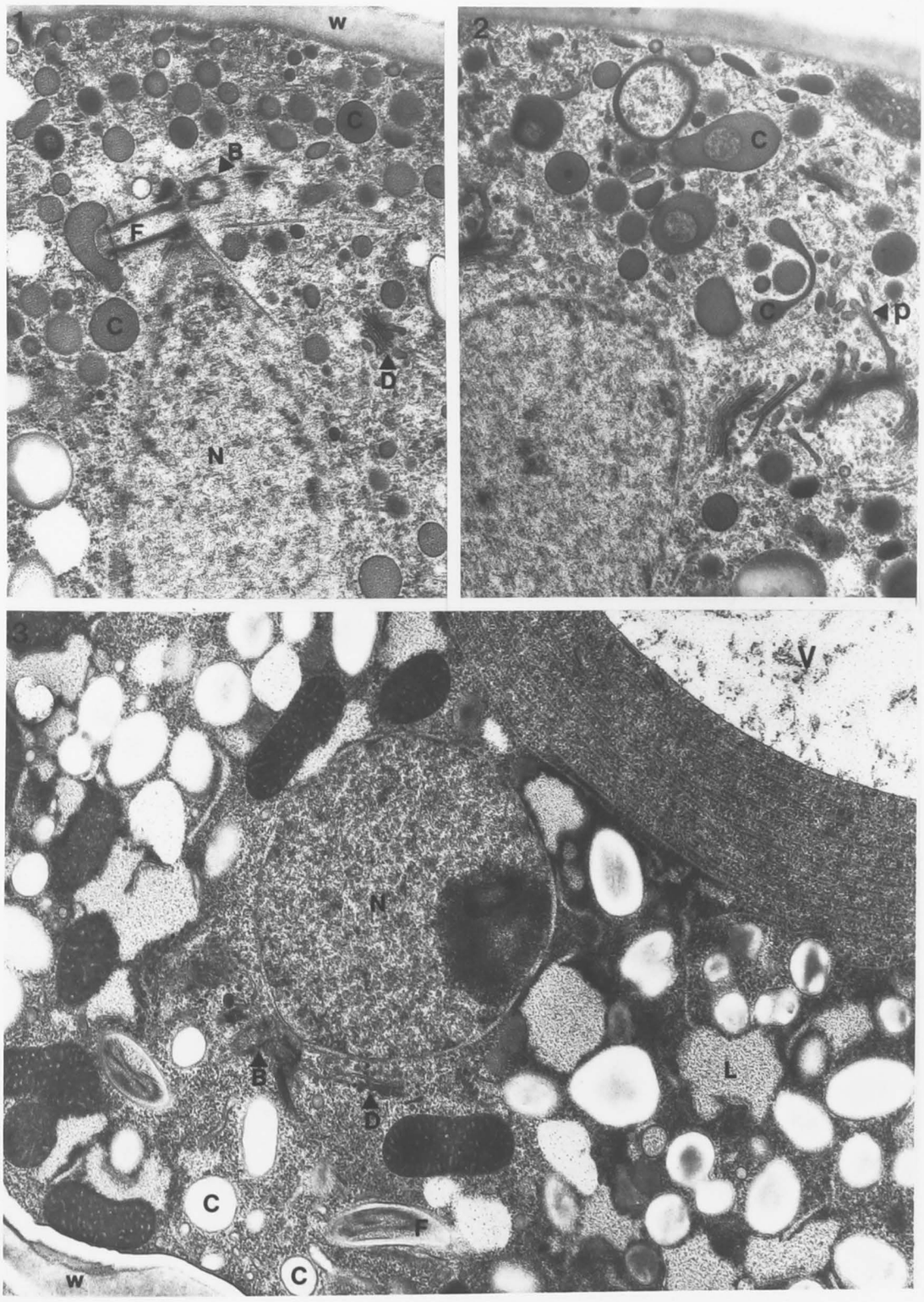
In conclusion I would like to point out that this study is another dramatic example of the superiority of rapid freezing procedures over chemical fixation for the preservation of cell structure. In particular, the maintenance of the apparent true form of the developing cleavage planes and the trans Golgi network in *Phytophthora* demonstrates again the effectiveness of rapid freezing in bringing to light extensive membrane systems which have gone undetected in chemically fixed material.

CHAPTER 3 FIGURE LEGENDS

Figs 3.1-3.3 Early stages of zoosporogenesis in *Phytophthora*, seen in freeze substituted sporangia from plunge frozen (Figs 3.1, 3.2) and high pressure frozen material (Fig 3.3).

Figs 3.1, 3.3 In *P. cinnamomi* (Fig 3.1) and *P. palmivora* (Fig 3.3), cleavage elements (C) evident as vesicles, and dictyosomes (D) lie near the basal body (B) associated pole of the nucleus (N) which, for these cortical nuclei, points to the sporangial wall (W). Development of the flagella (F) has begun. In the high pressure frozen cell (Fig 3.3) cleavage elements do not contain the dense material seen in the plunge frozen cell and large peripheral vesicles (L) are not well preserved. The sporangium of *P. palmivora* contains one large central vacuole (V), ringed by layers of rough endoplasmic reticulum (Fig 3.1: X28,000; Fig 3.3: X21,000).

Fig 3.2 Irregularly shaped cleavage elements (C) in *P. cinnamomi*. An elongated cisterna (P) at the trans face of a dictyosome has a similar appearance to the cleavage elements (X26,000).


1
W
C
B
F
C
D
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2
C
C
p
3
V
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C
F
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C

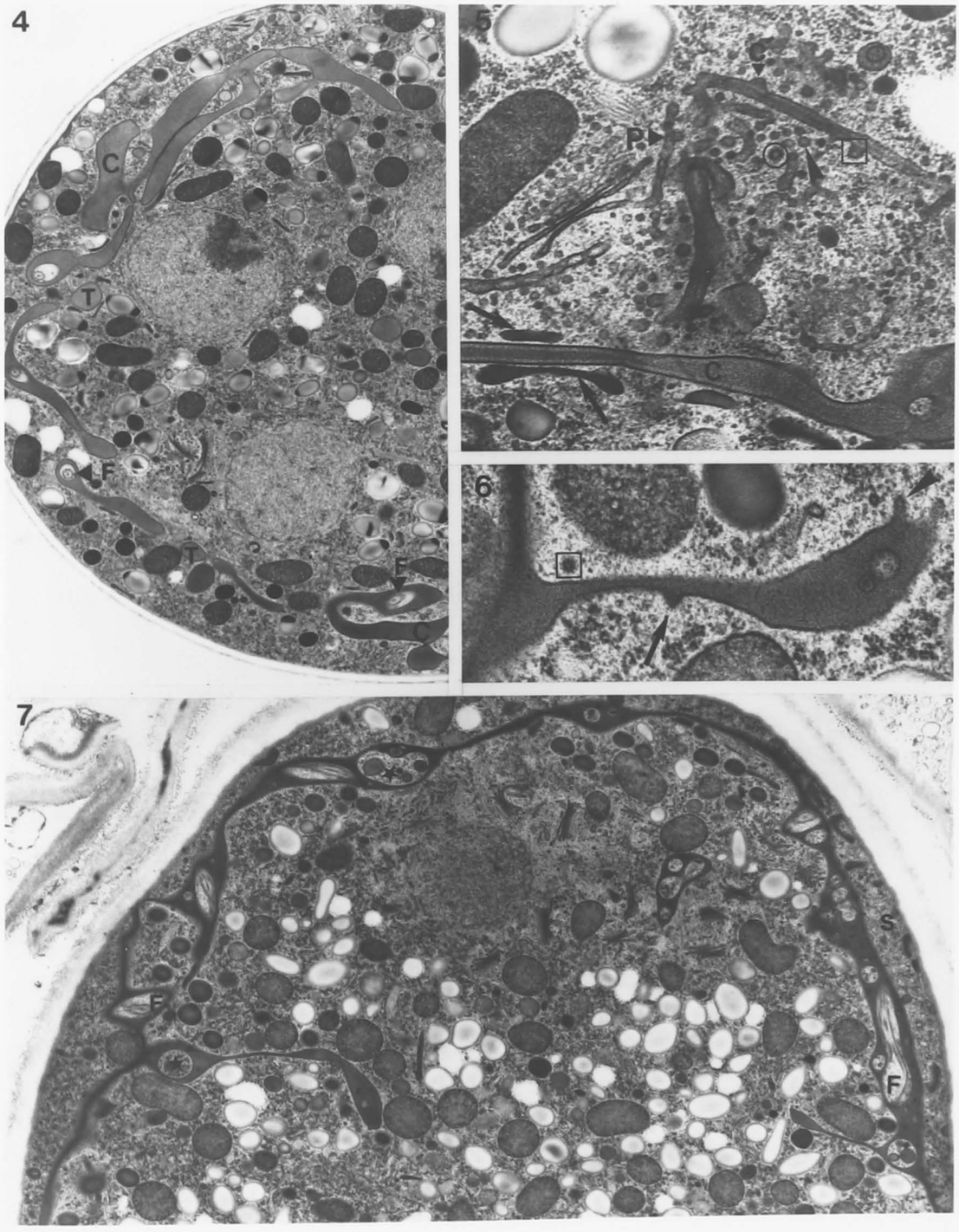
Figs 3.4-3.7 Development of the cleavage plane parallel to the sporangial wall in *Phytophthora* sporangia. Figs 3.4-3.5: *P. palmivora*; others: *P. cinnamomi*. All cells plunge frozen.

Fig 3.4 Extended cleavage elements (C), mainly parallel to the sporangial wall, seen in the sporangial cortex. Cross-sections of tubular portions (T) of the elements sometimes give a misleading impression (as confirmed by serial section analysis) of vesicle alignment. Flagella (F) are seen within the cleavage elements (X9,500).

Fig 3.5 An elongated cisterna (P) at the trans face of a dictyosome has a similar appearance to cleavage elements (C, C'). A trans golgi network appears to interconnect this cisterna with the uppermost cleavage element (C'). Blebs (boxed area) are seen on the surface of this cleavage element, and small coated (circled area) and uncoated (arrowhead) vesicles are seen nearby. Peripheral cisternae (arrows) run parallel to the lower cleavage element (X33,000).

Fig 3.6 Membrane blebs, coated (arrow) and possibly uncoated (arrowhead), seen on the surface of a cleavage element. A coated vesicle lies nearby (boxed area, X43,000).

Fig 3.7 Flagella (F) and apparent cytoplasmic fragments (stars) are seen within the fully developed cortical cleavage plane which cuts off a shell of cytoplasm (s) between itself and the wall (X10,000).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C
T
LF
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C
6
7
F
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F

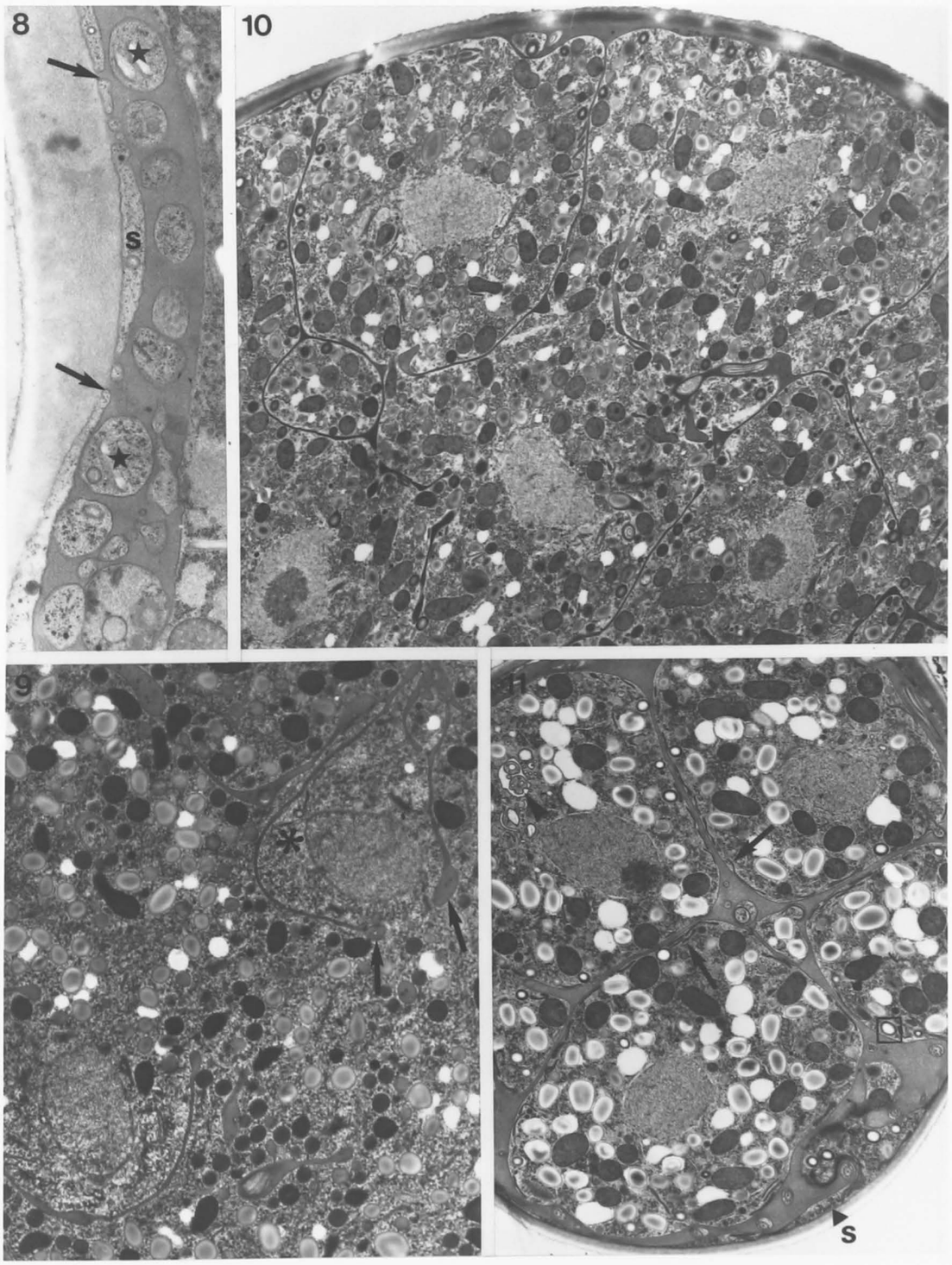
Figs 3.8-3.11 Development of the internal cleavage planes of *Phytophthora* sporangia. Fig 3.8: high pressure frozen cell; others: plunge frozen.

Fig 3.8 A fragmented cortical shell of cytoplasm (s) at the sporangial apex in *P. cinnamomi*. Fragmentation appears to involve localized fusion with the sporangial plasma membrane (arrows). Large cytoplasmic fragments (stars) are evident within the cortical cleavage plane (X17,000).

Fig 3.9 Extended cleavage elements run back behind the basal body associated pole of an internal nucleus (asterisk). The distal edges (arrows) are dilated (*P. cinnamomi*; X8,500).

Fig 3.10 At a later stage than Fig 3.9, cleavage elements almost delineate the future zoospore domains. The cortical cytoplasmic shell has disappeared (*P. cinnamomi*; X6,500).

Fig 3.11 In a sporangium of *P. palmivora*, in which internal cleavage is more advanced than that shown in *P. cinnamomi* in Fig 3.10, some of the cortical shell of cytoplasm is still evident (s). Peripheral cisternae (arrows) lie near the future plasma membranes of the zoospores. A water expulsion vacuole (arrowhead) is evident in one zoospore initial (X3,000).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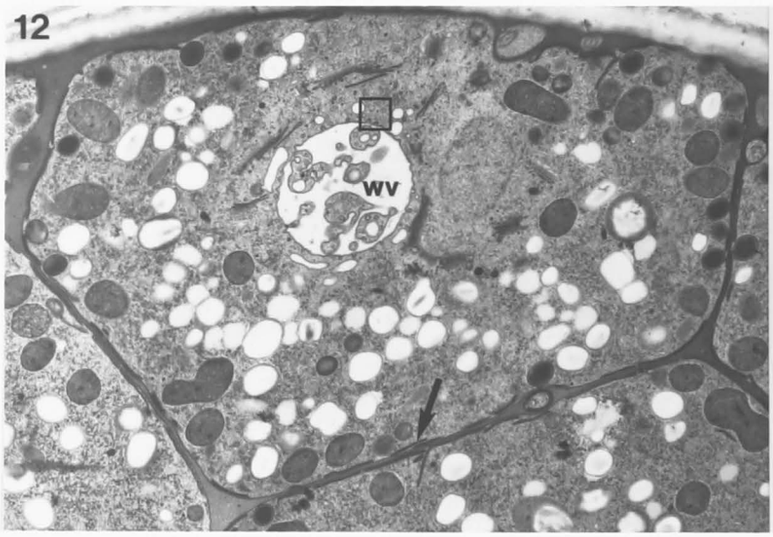
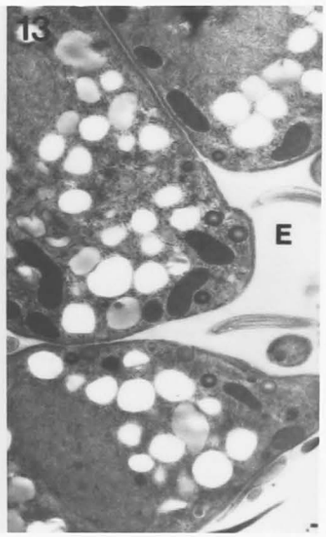
Figs 3.12-3.13 Final stages of cleavage in *Phytophthora*. Plunge frozen cells.

Fig 3.12 Fully cleaved zoospore initial of *P. cinnamomi*. Peripheral cisternae (arrows) lie parallel to the zoospore plasma membrane. Sinuous cytoplasmic projections are seen inside the water expulsion vacuole (WV). Blebs are evident on the vacuolar membranes and small vesicles (boxed area) are seen nearby (X9,500).

Fig 3.13 Rounded zoospores seen in the final stages of cleavage in *P. cinnamomi*. Extracellular matrix material (E) fills the spaces between the zoospores (X8,000).


12
WV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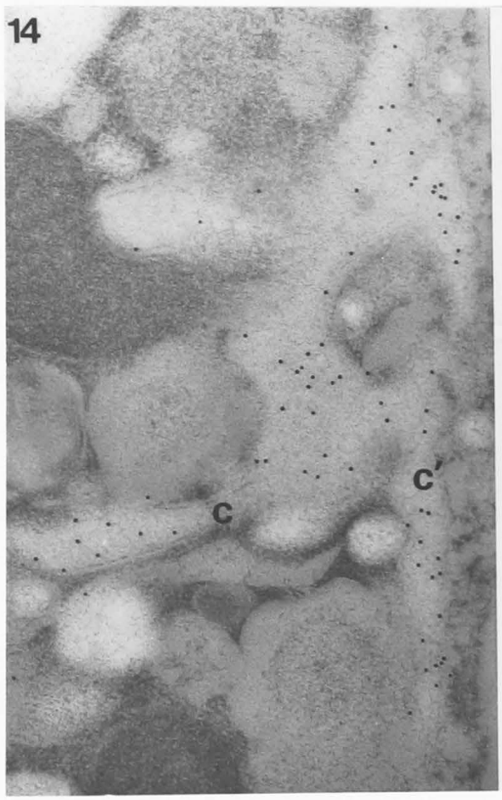
E

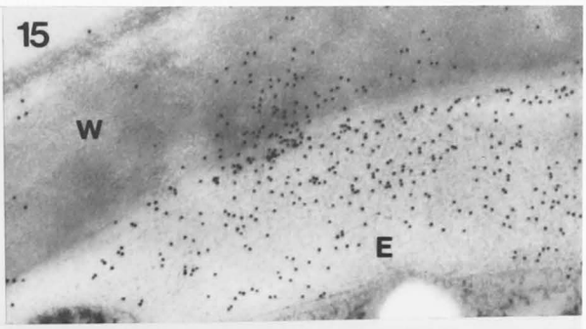
Figs 3.14-3.16 Labelling of contents of cleavage elements and extracellular matrix material with mAb Cpw-1, visualized using a second antibody, sheep anti-mouse IgG-Au_{10}. All cells high pressure frozen.

Fig 3.14 Labelling of cleavage elements of the internal (C) and cortical (C') cleavage planes in *P. cinnamomi* (X51,000).

Fig 3.15 Dense labelling of the thick plug of extracellular matrix material at the apex of a sporangium of *P. palmivora*. The sporangial wall (w) is also labelled (X41,000).

Fig 3.16 Labelling of part of the internal cleavage planes (C) of a sporangium of *P. palmivora*. The large central vacuole (V) is not labelled above background (X40,000).


14
C
C'


15
W
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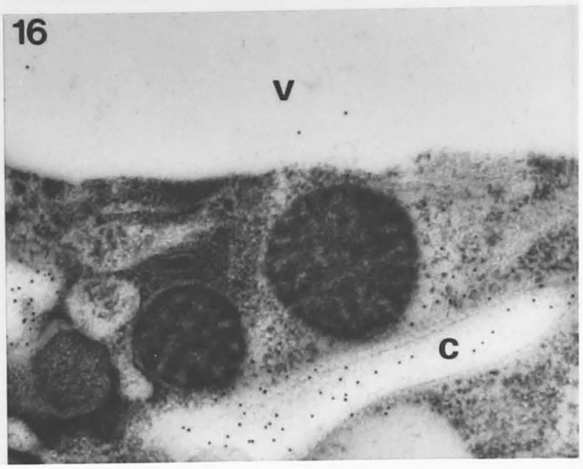

16
V
C

Fig 3.17 Immunoblot characterization of antigen(s) reacting with mAb Cpw-1. Sporangial proteins of *P. cinnamomi* were separated on a SDS-polyacrylamide gradient gel, electrophoretically blotted, stained with Ponceau S (lane a) and probed with Cpw-1, followed by peroxidase-conjugated secondary antibody (lane b). Lanes c and d show strips pretreated with Pronase and periodate, respectively, before antibody incubations. Positions of molecular weight markers (Mr x 10^{-3}) are shown on the right.

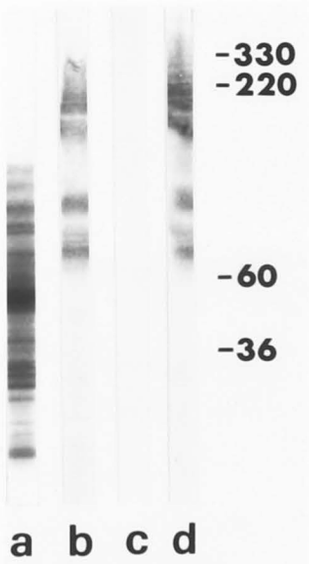


Fig.17

Fig 3.18 A diagrammatic comparison of the process of sporangial cleavage in *P. cinnamomi* suggested by rapid freezing-freeze substitution (A-B-C-D) with the previous model (A-B'-C'-D) based on observations of chemically fixed material. Many features of the first sequence are also true for *P. palmivora*; and many aspects of the second sequence (especially stage C') are shared by other *Phytophthora* species studied by chemical fixation. In these diagrams, shading represents extracellular matrix material. In actual sections, the matrix material appeared much denser in sporangia prepared by rapid freezing, as opposed to chemical fixation.

A: Before induction of cleavage and shortly afterwards, specialized cleavage vesicles (•) and dictyosomes (ᵐ) are found concentrated at definable poles of nuclei. The poles of cortical nuclei point towards the sporangial wall. Nuclei are evenly spaced within the coenocytic sporangium.

B, B': In the next stage, in rapidly frozen sporangia (B), paired sheets of membrane loop back from the poles of nuclei in the sporangial interior. In the cortex, one pair of membrane sheets runs parallel to the sporangial wall, and cuts off a shell of cytoplasm between the outer of the two membranes and the sporangial wall. In chemically fixed material (B') similar patterns are seen but these involve planes of discrete vesicles.

C, C': Next, in frozen sporangia (C), the membranous sheets in the sporangial interior extend and interconnect with each other and the inner of the two cortical membranes, thus subdividing the sporangium into uninucleate, membrane-bound domains. The outer cortical membrane and the cortical shell of cytoplasm disappear. In chemically fixed material (C'), the cortical plane of vesicles and the cytoplasmic shell have disappeared but planes of vesicles still demarcate domains around the nuclei.

D: In the final stage both rapidly frozen and chemically fixed sporangia contain fully formed zoospores with a rounded shape and a groove opposite the nuclear pole. Matrix material fills the extracellular space.

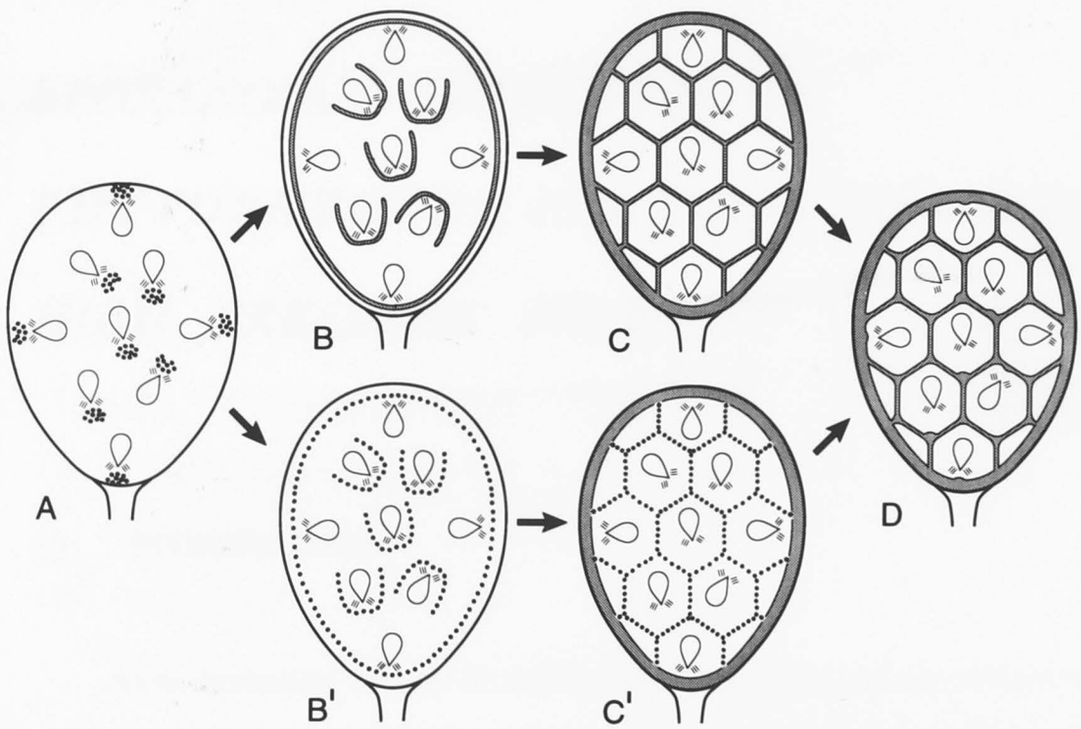


Fig.18

4

SPORANGIAL STRUCTURE IN PHYTOPHTHORA IS DISRUPTED AFTER HIGH PRESSURE FREEZING

4.1 INTRODUCTION

As an increasing number of studies indicates the superiority of rapid freeze fixation over chemical fixation for the preservation of cell structure (McCully & Canny, 1985; Lancelle *et al.* 1986; Cresti *et al.* 1987; Kiss *et al.* 1990), interest has grown in developing techniques for applying high hydrostatic pressure during the process of freezing. This has been motivated by knowledge of the capacity of high pressure (HP) to facilitate the freeze fixation of much larger samples of biological material than can be preserved, without ice-crystal damage, by freezing at ambient pressure (Moor & Riehle, 1968; Dahl & Staehelin, 1989). Most recent studies of high pressure freezing (HPF) have utilized the Balzers HPM 010 hyperbaric freezer and have obtained excellent results (Dahl & Staehelin, 1989; Lichtscheidl *et al.* 1990); any undesired effects of the high pressure (2100atm) applied in this apparatus have not been considered of significant

concern (reviewed in Kiss *et al.* 1990). Since these reports are limited, however, in number and in the range of organisms they examine, the general applicability of the technique will only be known as more studies are completed.

The sporangia of *Phytophthora cinnamomi* and *P. palmivora* are ideal systems in which to study the effects of HPF. Their ultrastructure has previously been studied in detail using chemical fixation (Hohl & Hamamoto, 1967; Chapter 2), and a range of monoclonal antibodies has been generated which show specificity for various cellular components of these species (Hardham *et al.* 1990). Most significantly, the small size of the sporangia makes them suitable for freeze fixation at ambient pressure, thus enabling a comparison and evaluation of the HP frozen material. In this study I compare the ultrastructure of sporangia of *P. cinnamomi* and *P. palmivora* that have been pressurized prior to fixation in a Balzers hyperbaric freezer or a French pressure cell to that of sporangia plunge frozen at ambient pressure.

4.2 MATERIALS AND METHODS

4.2.1 Organisms and sampling

The cultures of *P. cinnamomi* (DAR 52646) and *P. palmivora* (1732) used in this study were induced to produce sporangia and zoospores by the methods of Hardham & Suzaki (1986). Briefly, sporangial formation was induced by transfer of mycelium to a nutrient poor medium; sporangial cleavage was induced by treatment with cold distilled water. Samples were taken before induction of cleavage and at intervals between induction and release of zoospores.

4.2.2 Freeze fixation

4.2.2.1 High pressure freezing

Wet tufts of mycelium with attached sporangia were placed in gold specimen holders (Balzers BB113142-1) with mineral salts solution (Hardham & Suzaki, 1986,

for pre-induction) or distilled water (post-induction) filling the remaining space. Pairs of holders were clamped together and frozen in a Balzers HPM 010 hyperbaric freezer. After freezing, the holders were snapped apart under liquid nitrogen and transferred to the substitution medium.

4.2.2.2 Plunge freezing

Tissue was frozen on formvar covered loops following the procedures of Lancelle *et al.* (1986).

4.2.3 Freeze substitution and preparation for electron microscopy of freeze fixed material

General procedure: Tissue frozen by the methods above was freeze substituted using the procedures of Lancelle *et al.* (1986). The method was modified by the inclusion of 0.05% uranyl acetate in the substitution medium, and after 36h at -80°C, the vials were first warmed to -30°C for 10h, before being brought to room temperature. Tissue was then rinsed in acetone several times, and stained *en bloc* in 5% uranyl acetate in methanol for 2h. After rinsing with acetone, the tissue was infiltrated with Epon resin and polymerized. Sections were stained for 3-5 min in Reynold's lead citrate.

Immunolabelling procedure: HP frozen material destined for immunogold labelling was prepared using the freeze substitution, infiltration and polymerization procedures of Lancelle & Hepler (1989) with the inclusion of a 10h stage at -30°C, prior to bringing the samples back to room temperature during freeze substitution. Infiltration and polymerization with UV light were carried out at room temperature. Immunolabelling of sections on gold grids followed the methods of Gubler & Hardham (1988). Monoclonal antibodies (mAbs) Lpv-1, Cpa-2 and Vsv-1 were used. These mAbs have been previously shown to have affinities for three different vesicles found at the periphery of developing zoospores in sporangia of *P. cinnamomi* (Chapter 2). After

immunolabelling, sections were stained with 2% aqueous uranyl acetate for 20-30 min, followed by 2 min in lead citrate.

4.2.4 Pressurization of sporangia in a French pressure cell, their chemical fixation and preparation for electron microscopy

Between 40-50 min after the induction of cleavage, mycelia of *P. cinnamomi* were placed with distilled water in a French pressure cell (American Instrument Co., Maryland, USA) within which the pressure was raised to approx. 1360atm for about 1 s, then released. After retrieval from the French pressure cell, sporangia were fixed and infiltrated using techniques detailed in Method E of Chapter 2. Immunolabelling of sections was as described above except that sections were stained with 2% uranyl acetate and Reynold's lead citrate for 5 min and 1 min respectively.

4.3 RESULTS

4.3.1 Freeze fixed sporangia of *P. cinnamomi* and *P. palmivora*

Both HPF and plunge freezing provided excellent and comparable fixation of most sporangial structures in *P. cinnamomi* and *P. palmivora*. There were, however, some variations between sporangia prepared by the different procedures, the most significant of which are described below.

The first difference concerned structures described in previous studies of the two species (Hardham *et al.* 1990; Chapter 2) as large peripheral vesicles (LPVs). LPVs were present in plunge frozen sporangia (Fig 4.1; Table 4.1) at all stages of cleavage but were not seen in HP frozen sporangia. During early stages of cleavage in plunge frozen sporangia, LPVs showed no distinct distributional arrangement but in advanced stages they were close to the plasma membranes of future zoospores (Fig 4.1).

Table 4.1 Presence (+) or absence (-) of certain vesicles and irregularly shaped structures in normal and pressurized sporangia of *P. cinnamomi* and *P. palmivora*

	Monoclonal antibody bound by vesicle/structure	Plunge-freezing	High-pressure freezing (Balzers HPM 010)	High pressure-treated (French pressure cell)
P. cinnamomi				
Large peripheral vesicle	Lpv-1	+	-	+
Large irregular structure	Lpv-1	-	+	+
Dorsal surface vesicle	Cpa-2	+	+	+
Ventral surface vesicle	Vsv-1	+	+	+
P. palmivora				
Large peripheral vesicle		+	-	
Large irregular structure		-	+	

The second difference involved large irregularly shaped structures with granular contents that were observed at all stages of cleavage in HP frozen sporangia (Figs 4.2-4.5) but never in plunge frozen sporangia (Fig 4.1). A small portion of the perimeter of these structures was often bounded by membrane (Fig 4.2). During early stages of cleavage, the distribution of the irregular structures showed no distinct pattern (Fig 4.2). When cleavage was advanced, however, they had a distribution (Fig 4.3) similar to that described above for LPVs in cleaved plunge frozen sporangia. Immunolabelling of LR White embedded sections of HP frozen sporangia of *P. cinnamomi* showed that the irregular structures were the only sporangial elements labelled by mAb Lpv-1 (Fig 4.4), and that these structures were not labelled by mAbs Cpa-2 (Fig 4.5) or Vsv-1. Dorsal and ventral surface vesicles of *P. cinnamomi* (Chapter 2) were observed in both HP and plunge frozen sporangia of this species. They were identified by their characteristic morphologies and mAb binding (Fig 4.5; Table 4.1).

The third difference concerned the percentage of sporangia affected by ice-crystal damage. This was higher in material prepared by plunge freezing, especially that of *P. cinnamomi* which has, on average, larger sporangia than *P. palmivora*.

4.3.2 Sporangia of *P. cinnamomi* pressurized in a French pressure cell

In cleaved sporangia of *P. cinnamomi* that had been subjected to HP in a French pressure cell, very large mottled structures (Fig 4.6) were observed at the periphery of the developing zoospores. These structures were labelled by mAb Lpv-1 (Fig 4.6). Intact LPVs were also observed in these sporangia and were labelled by mAb Lpv-1 (Fig 4.6). Dorsal and ventral surface vesicles were evident, being identified by their characteristic morphologies and antibody binding (Table 4.1).

4.4 DISCUSSION

4.4.1 Disruption of sporangial structure in *Phytophthora* by high pressure freezing

The results suggest that the disruption of sporangial structure caused by the HPF procedure used in this study was brought about by the breakage of LPVs. LPVs, which were observed in plunge frozen sporangia and have previously been described in chemically fixed material of *Phytophthora* (Hardham *et al.* 1990; Chapter 2), consequently appear in HP frozen sporangia as large irregular structures. These structures retain the patterns of spatial distribution and, for *P. cinnamomi*, the mAb Lpv-1 binding characteristic of LPVs in this species (Hardham *et al.* 1990; Chapter 2). There was no evidence that any other cell structures were affected in the same fashion as the LPVs. In particular, two other vesicle types that have previously been noted to occur at the periphery of developing zoospores of *P. cinnamomi*, the dorsal and ventral surface vesicles (Chapter 2), were observed in HP frozen sporangia of this species. Nevertheless, since the LPVs represent a significant fraction of sporangial contents, their

apparent expansion following HPF causes considerable passive disruption of sporangial structure.

I believe that the feature of the HPF procedures most likely to have led to the disruption of LPVs is the pressure itself. The strongest evidence for this is the normal appearance of LPVs in sporangia which, apart from being plunge frozen, were prepared for electron microscopy by procedures identical to those used for HP frozen sporangia. Evidence which indicates that HP can directly cause expansion of LPVs is found in the observation in this study of very large mAb Lpv-1 binding structures in sporangia of *P. cinnamomi* that had been pressurized in a French pressure cell. The French pressure cell is not, however, an ideal method for assessing the effects of high pressure because, before being chemically fixed, the material is also exposed to the potentially deleterious effects of decompression. Below, I consider possible ways by which the application of high pressure may lead, directly or indirectly, to general structural damage in a sample.

Possible direct causes include shearing forces or shockwaves generated within the sample in the period between the commencement of pressurization and the completion of freezing. This period includes 20-30 msec at 2100atm before freezing commences (Gilkey & Staehelin, 1986). Another possible direct cause, proposed by Dahl & Staehelin (1989), is that air bubbles trapped in the specimen holders may cause deformation of samples during pressurization or thawing. Nevertheless, I feel it is unlikely that there was any systematic or incidental introduction of bubbles in this study because (1) the procedure I used for sample loading was the same that has previously been employed successfully with other tissues and (2) over thirty separate samples were frozen at HP and the artefact was evident in all sporangia examined in each sample. Additionally, preliminary evidence suggests that LPVs in germinating cysts of *P. cinnamomi* are disrupted by HPF (Hardham & Mendgen, unpublished data), even though the material was prepared by procedures that have previously been used to avoid the occurrence of air bubbles (Welter *et al.* 1988).

Dahl & Staehelin (1989) also suggest an indirect way inwhich high pressure may bring about structural damage. At 2100atm water solidifies as ice II and III, both of which are denser than water. These ices may undergo a phase transition to the more voluminous ice I during the long periods involved in freeze substitution.

If any of the possible causes outlined above are responsible for the expansion of LPVs seen in HPF frozen material, it is not clear how expansion of such an extensive nature may have been brought about. Previous chemical fixation studies have, however, indicated that LPVs of *P. cinnamomi* are especially sensitive organelles and will rupture if fixative osmolarity is too low (Hardham, 1985). It is possible that the same characteristic of LPVs that causes this sensitivity also makes them more liable to react to whatever is responsible for the damage seen in HP frozen material. This characteristic may concern the membrane of the LPV or its contents, which in *P. cinnamomi*, are rich in high molecular weight glycoproteins (Gubler & Hardham, 1990).

To ascertain whether the disruption of LPVs occurs during HPF or subsequent freeze substitution, examination of sporangia prepared by freeze fracture and freeze etching techniques should be helpful.

4.4.2 Implications for the future use of high pressure freezing

In the design of the Balzers hyperbaric freezer, 2100atm was selected as the operating pressure because, by its effects on the freezing properties of water, it minimizes the restrictions imposed by the poor heat conductance of water upon the size of samples that can be frozen without ice damage (Dahl & Staehelin, 1989). While long exposures to pressures of this magnitude are known to damage many biological tissues (Zimmerman, 1971), it has been thought that the brevity of exposure involved in the use of the Balzers freezer might avoid tissue damage of any major concern (Gilkey & Staehelin, 1986). While the excellent results obtained by previous use of the instrument indicate that this is true for a number of animal, plant and fungal tissues (Welter *et al.* 1988; Dahl & Staehelin, 1989; Lichtscheidl *et al.* 1990), the present study suggests that

the assumption is not universally valid. It is unlikely that this study will remain an isolated case because LPVs are found throughout the Oomycetes (Beakes, 1986); preliminary work already indicates that the LPVs of *Olpidiopsis* also do not survive the process of HPF-freeze substitution (Martha Powell, personal communication). Kiss *et al.* (1990) have reported minor bursting of mucilage vesicles in HP frozen *Arabidopsis* roots. This may be a less extreme example of the phenomenom I have described in this study. For any tissue that is found to be sensitive to freezing in the Balzers instrument, and which is too large to be frozen at ambient pressure without ice damage, then it may be profitable to freeze at a pressure lower than 2100atm.

In conclusion I would like to put the results of this study into perspective by pointing out that while HPF does not appear as good as plunge freezing for the fixation of *Phytophthora* sporangia, in several respects it provides better preservation of this material than I have previously obtained with chemical fixation. For example, I have never been able to achieve successful immunolabelling of LR White embedded sporangia that have been chemically fixed (personal observation) but I obtained excellent results with this resin in HP frozen material. More significantly, the conformation of the developing cleavage system was undisturbed even in those sporangia most affected by HPF whereas in chemically fixed material this system is severely disrupted (Chapter 3).

CHAPTER 4 FIGURE LEGENDS

Fig 4.1 Large peripheral vesicles (arrows) lined up along a cleavage plane in a plunge frozen sporangium of *P. cinnamomi* (X11,500).

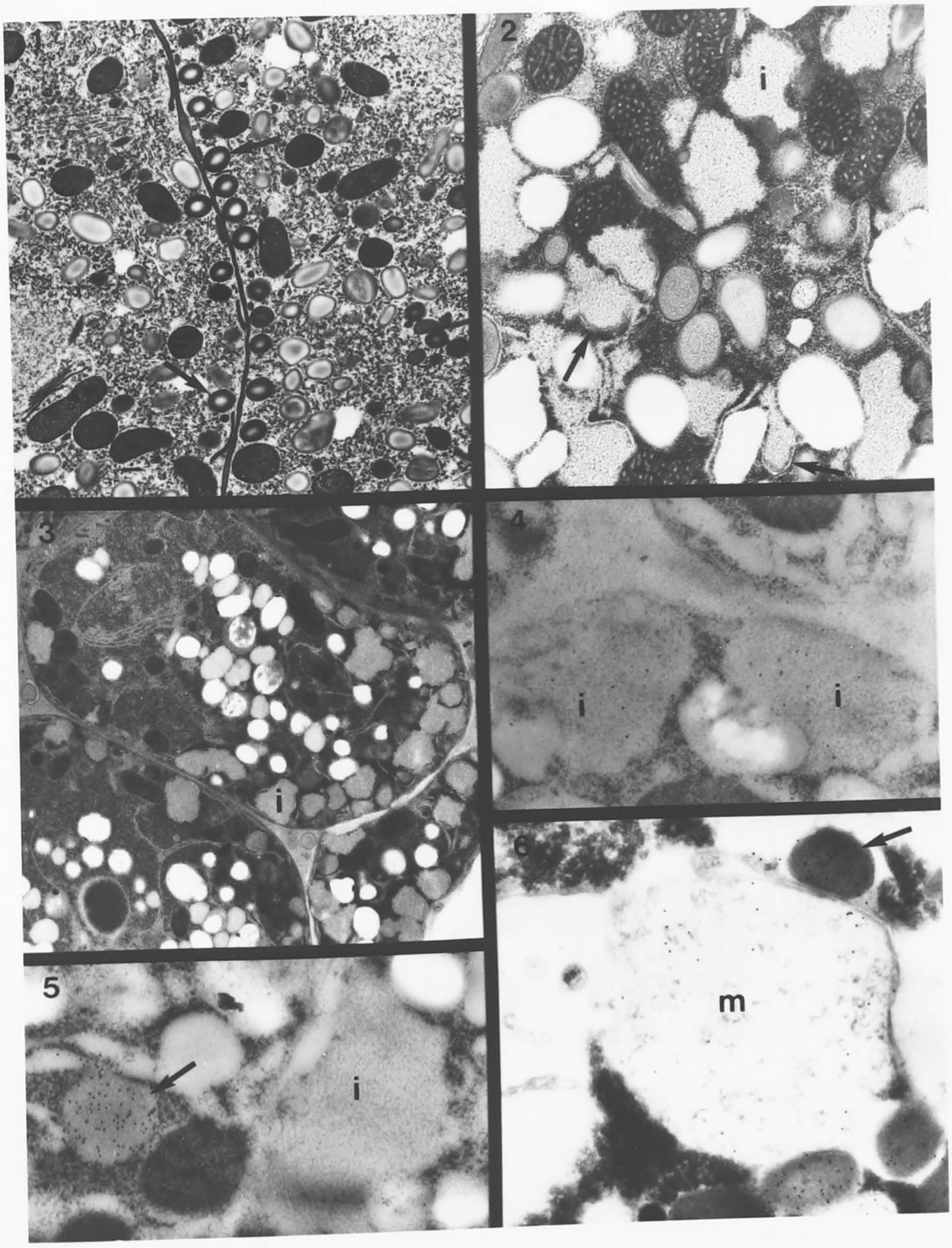
Fig 4.2 Large irregularly shaped structures (i) in a high pressure frozen, uncleaved sporangium of *P. palmivora*. Membrane (arrows) surrounds some areas of these structures (X23,000).

Fig 4.3 Large irregularly shaped structures (i) at the periphery of future zoospores in a fully cleaved, high pressure frozen sporangium of *P. cinnamomi* (X7,800).

Fig 4.4 Large irregularly shaped structures (i) immunolabelled by monoclonal antibody Lpv-1 and visualized using a second antibody, sheep antimouse IgG-Au_{10}. Cleaved, high pressure frozen sporangium of *P. cinnamomi* (X31,500).

Fig 4.5 Dorsal surface vesicle (arrow) immunolabelled by monoclonal antibody Cpa-2. A large irregularly shaped structure (i) nearby is not labelled with this antibody. Cleaved, high pressure frozen sporangium of *P. cinnamomi* (X33,500).

Fig 4.6 Very large mottled structures (m) and large peripheral vesicles (arrows), both labelled by monoclonal antibody Lpv-1, in a cleaved sporangium of *P. cinnamomi* that had been pressurized in a French pressure cell and then chemically fixed (X 34,000).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5

CONFOCAL MICROSCOPY OF DOMAIN-LIMITING MICROTUBULE ARRAYS IN CRYOSECTIONED SPORANGIA OF PHYTOPHTHORA CINNAMOMI

5.1 INTRODUCTION

The aim of this study is to increase our understanding of the structural mechanisms that regulate the orderly process of cleavage within the multinucleate sporangia of zoosporogenic fungi. There are several key questions that remain unresolved in this area. For example, what restricts the development of the cleavage membranes to within the zones of cleavage? Are the positions of the future cleavage planes predicted or established in some way prior to the onset of cleavage? Finally, what brings about and maintains the regular spacing of nuclei typically reported before and during cleavage (Heath & Greenwood, 1971; Hohl & Hamamoto, 1967; Williams & Webster, 1970; Chapters 2, 3).

From previous studies of the zoosporogenic fungi a commonly accepted model has emerged which addresses some of these questions (Heath & Greenwood, 1971; Olson *et al.* 1981; Lange & Olson, 1983). The fundamental proposal of this model is that, before and during cleavage, arrays of microtubules emanate from near each nucleus, marking out the sporangial cytoplasm into a number of equal-sized uninucleate domains, the boundaries of which exist at the junctions, or between the margins, of adjacent arrays. The ultrastructural evidence for this proposal has not, however, been altogether satisfying because, although nucleus-based arrays have been reported in the sporangia of many fungi (Heath & Greenwood, 1971; Gotelli, 1974; Hoch & Mitchell, 1975; Olson *et al.*, 1981; Armbruster, 1982; Lange & Olson, 1983) they are not always present at critical stages (Heath, 1976). Moreover, while microtubules have occasionally been described as extending to the domain boundaries (Olson *et al.*, 1981; Hoch and Mitchell, 1975), it has never been clearly demonstrated that these microtubules are present in the quantity one would expect if they were domain-limiting structures as it has, for instance, with other non-fungal systems (Karr & Alberts, 1986; Menzel, 1986).

Despite these shortcomings, the model still remains attractive, partly because there are no strong alternatives and also because a number of studies have demonstrated that treatment of sporangia with antimicrotubule drugs leads to a disruption of the orderly nature of cleavage and nuclear positioning (Slifkin, 1967; Schnepf & Heinzmann, 1980; Olson *et al.* 1981; Heath *et al.* 1982; Olson & Lange, 1983). It is possible that techninal problems may be responsible for the scarcity of observations of microtubules in those cytoplasmic regions where the domain boundaries would be expected to exist. Such problems include the difficulty of detecting, with thin-section analysis, cellular structures as small as microtubules and incomplete preservation of the cell's microtubule population. But even if we do accept the model, its predictive value suffers greatly from the lack of information regarding microtubular arrangements at the domain boundaries: it is impossible to determine what interactions the arrays may have at the boundaries either with each other or with the developing cleavage planes.

In this study I have further investigated the spatial distribution of microtubules and flagella within the pre-cleavage and cleaving sporangium of *P. cinnamomi*. In previous studies of the sporangium of this species (Chapters 2,3), electron microscopy has provided detailed information about the cleavage process and general cytoplasmic organization within the sporangium, but has failed to provide an overall picture of sporangial microtubule organization any more satisfactory than those from studies of other zoosporogenic fungi. Prompted by a recent study which used immunofluorescence microscopy to examine the flagellar rootlet system of the released zoospore of *P. cinnamomi* (Hardham, 1987b), I have applied this technique to the sporangium. The technique for the zoospore was modified by freezing and cryosectioning the chemically-fixed sporangia. This approach has provided new information regarding the extent of sporangial microtubular arrays, and should draw the attention of mycologists to the potential of cryosectioning as an adjunct to immunofluorescence studies of fungal structures.

5.2 MATERIALS AND METHODS

5.2.1 Organisms and sampling

The culture of *P. cinnamomi* (DAR 52646) used in this study was induced to produce sporangia by the methods of Hardham & Suzaki (1986). Briefly, sporangial formation was induced by transfer of mycelium to a nutrient poor medium; sporangial cleavage was induced by treatment with cold water. For fluorescence microscopy, samples were taken before induction of cleavage and at intervals between induction and release of zoospores.

5.2.2 Preparation of samples for fluorescence microscopy

5.2.2.1 Enzyme treated sporangia

Sporangia destined to be treated with wall-digesting enzymes were fixed in 4% paraformaldehyde in 50 mM Pipes buffer with 2 mM $MgSO_4$ and 5 mM EGTA for 1 hour at room temperature, then rinsed once in 100 mM Pipes buffer and twice in phosphate buffered saline (PBS : 150 mM NaCl, 20 mM sodium phosphate buffer). They were then treated with 1% Triton X100 in PBS for 1 h, rinsed three times in PBS and treated with various mixtures of commercially available wall-digesting enzymes such as Macerase, Cellulysin (Calbiochem, La Jolla, Ca.), Cellulase Onozuka R10 (Yakult, Honsha, Tokyo) and Driselase (Fluka, Buchs, Switzerland). All solutions were at pH 6.8, except for the enzyme mixtures which were at pH 4.5. None of these mixtures were effective in removing the sporangial wall.

After enzyme treatment, sporangia were incubated in Amersham anti-β-tubulin diluted 1:300 in PBS containing 1% bovine serum albumin and 0.02% NaN_3 at 37°C for 45-60 min, rinsed three times in PBS then incubated at 37°C for 45-60 min with a fluorescein isothiocyanate-(FITC) conjugated sheep antimouse antibody (SAM : Silenus Lab Pty Ltd, Dandenong, Australia) diluted 1:30 in PBS, with 1% bovine serum albumin and 0.02% NaN_3. After rinsing two times in PBS, the mycelial tuft containing the sporangia was lightly blotted and spread out on a slide in a drop of mowiol (Wick & Duniec, 1986) containing the nonfade additive paraphenylenediamine (0.1%). A coverslip was placed over the preparation, and the slide was left overnight before observation to allow penetration of the paraphenylenediamine and setting of the mowiol.

5.2.2.2 Cryosectioned sporangia

In the development of an immunofluorescence protocol for cryosectioned sporangia, various aspects of the procedure, such as fixative composition, section thickness, extracting detergent concentration and duration of antibody incubation were modified numerous times. The protocol which provided the most consistently effective

visualization of microtubules is detailed below. A tuft of mycelium was fixed for 2 h at room temperature in 4% paraformaldehyde, 2 mM $MgSO_4$, 5 mM EGTA, 10% dimethyl sulfoxide and Triton X100 in 50 mM Pipes buffer, pH 6.8, then rinsed as for post-fixed enzyme-treated cells. The tuft was then lightly blotted to compact the mycelium, placed in liquid Tissue-Tek (Miles Inc., Elkhart, IN, USA) inside a plastic mould (Cryomold, Miles Inc.) and plunged into liquid nitrogen. The frozen block was then stored at -20°C before sectioning the same, or following, day. Sectioning was done on a Reichert Cryotome (2800 Frigocut E) set to cut 10 µm sections. The sections readily transferred onto a breath-moistened gelatin-coated glass slide placed just above the ribbon. Gelatin coating of slides was done at least the night before and followed Jensen's (1962) method. Sections were left to settle onto the slide for at least 15 mins, then rinsed once in PBS, immersed in 5% Triton X100 in PBS for 1 h, rinsed three times in PBS and then incubated for 2 - 4 h in the primary antibody as described for enzyme-treated cells. Rinsings, secondary antibody incubation and mounting in moviol were also as described for enzyme-treated cells except that the incubation duration was either 4 h (at 37°C) or overnight (at 4°C). Some preparations were also stained, after rinsing off the secondary antibody, with 0.2 µg/ml DAPI in PBS for 25 mins at room temperature, in order to label DNA.

5.2.3 Microscopy

Microtubule-associated fluorescence was detected either by use of Zeiss Axioplan and Axiovert microscopes equipped with epifluorescence optics or by use of an Axiovert microscope adapted for confocal optics (Biorad MRC600). These approaches provided equivalent direct visualization of microtubule-associated fluorescence but less detail was lost in the process of photographically recording the confocal-generated images. DAPI staining was observed by fluorescence microscopy, using the Zeiss microscopes.

5.2.4 Observations and 3-D reconstruction of cleaved sporangia

To assess the spatial disposition of zoospore initials within cleaved sporangia, sporangia were sampled at a time when cleavage was complete but before rounding up had occurred (Chapters 2,3). Sporangia were fixed, dehydrated and embedded using the procedures detailed in Method D of Chapter 2. The sporangia were then serial sectioned (1.5 μm thickness), placed on glass slides, stained with Toludine Blue, and photographed. One sporangium was reconstructed to 85% of its full size (17 out of 20 sections) by tracing zoospore outlines from photographs into a 3-D reconstruction program (PC3D, Vers. 5.0, Jandel Scientific, Corte Madera, CA).

5.3 RESULTS

5.3.1 Microtubule arrays in sporangia prior to cleavage

In cryosectioned sporangia sampled prior to the induction of cleavage, tubulin-associated fluorescence indicated the presence of extensive astral arrays of microtubules (Figs 5.1-5.3). One array emanated from a distinct pole of each approximately pear-shaped nucleus (Figs 5.3 and 5.4). For those nuclei in the sporangial cortex, the poles from which the arrays emanated always pointed towards the sporangial walls (Fig. 5.4). The pattern of microtubule distribution appeared radial when sectioned in planes perpendicular to the polar axes of the nuclei (Figs 5.1-5.2). Other planes of section revealed different patterns (Fig 5.3). The various patterns evident within sporangia suggested a common form for all arrays, which is illustrated diagrammatically in Fig 5.5.

The relationship of arrays to each other, and to the domains they appear to demarcate, is best seen in Fig 5.2. Microtubules from each array extend to, and past, the hypothetical domain borders, and at times even appear to run as continuous structures between the arrays. The extent of microtubule distribution shown in Fig 5.2 and depicted in Fig 5.5 was apparent, when viewed directly through the microscope, in many sporangia sampled at this and some later times. The finer details of the arrays were,

however, usually extremely difficult to record photographically owing to high levels of background fluorescence and to swamping of the weak fluorescence at the array margins by the strong labelling of the flagella and more central regions of the arrays.

5.3.2 Basal bodies and early flagellar development

In whole, enzyme-treated sporangia and some sporangial cross-sections from cryosectioned material, there was no detectable labelling of cytoplasmic microtubules. In these preparations, flagella and flagellar-associated tubulin structures were, however, labelled, with details of their structure being all the more evident because of the lack of other fluorescence (Figs 5.6 - 5.8). In enzyme-treated sporangia sampled prior to cleavage, numerous spots of fluorescence were seen (Fig 5.6) and these had a similar distribution to the foci of the arrays described in the previous section. More detailed images of these spots, from confocal microscopy of cryosectioned sporangia, showed that each consisted of two small spots of intense fluorescence associated with a third more diffusely labelled region (Fig 5.7). The two intense spots are likely to correspond to the basal bodies seen at this stage with electron microscopy (Chapters 2,3). After the induction of cleavage, the basal body fluorescence presumably becomes continuous with that of the developing flagella (Fig 5.8).

Evidence suggests that the apparent absence of cytoplasmic microtubules in whole sporangia and some cryosectioned sporangia (such as those in Figs 5.6-5.8) is due to problems of antigen accessibility. When a block containing sporangia sampled at 20 min post-induction was sectioned at 10 μm thickness, 31% of cross-sections had the same pattern as that shown in Fig 5.8, while the remainder had staining similar to that shown in Fig 5.9. When the same block was sectioned at 25 μm, the percentage of sporangia only showing flagellar labelling (as in Fig 5.8) rose to 70%. It is possible that these sporangia had their cut surface against the glass slide, and that their upper surface was uncut and thus antibody access was restricted. The percentage of sporangia lacking a cut, exposed surface within the section would increase with greater section thickness.

Antibody penetration is likely to be limited in such cross-sections, since the sporangial wall appears to inhibit the entry of antibodies.

5.3.3 Microtubule arrays and flagella in cleaving and cleaved sporangia

In sporangia sampled at 10, 20 and 30 min after the induction of cleavage, microtubule arrays appeared similar to those seen at the precleavage stage. Aspects of these arrays can be seen in Figs 5.9-5.11. In sporangia that had prominent cytoplasmic microtubule labelling, flagellar development was first clearly evident in the 30 min samples (Figs 5.10 and 5.11); but it is likely that short stubs of fluorescence seen within arrays at 20 min (Fig 5.9) correspond to the flagellar initials seen in sporangia from this time that had no cytoplasmic microtubule labelling (e.g., Fig 5.8).

In sporangia sampled at 40 min (Figs 5.12-5.13), microtubule arrays were not so obviously radial as those seen earlier . Flagella from this time on had a more sinuous form (Figs 5.12-5.13) than that at 30 min. At 50 min, the array form was similar to that seen at 40 min, and some indications of sporangial cleavage were given by the disrupted appearance of cytoplasmic background fluorescence (not shown). By 60 min, cleavage was completed and the individual zoospores still possessed microtubular arrays (Figs 5.14-5.15), which in some cases clearly possessed some of the characteristic features of microtubule rootlets in released zoospores (Fig 5.15; Hardham, 1987b).

5.3.4 Hyphal microtubules

In cryosectioned hyphae, microtubules were predominantly linear and often appeared to emanate from, and run between, the evenly spaced nuclei (Figs 5.16-5.17).

5.3.5 Arrangement of zoospores in cleaved sporangia

The sporangium reconstructed using the 3D computer program was 62 μm long and contained 23 zoospore initials (Figs 5.18-5.19). Of these, only one had no contact

with the sporangial wall and it lay in the centre of the sporangium (Fig 5.19). Larger sporangia contained one or more internal zoospores, while smaller sporangia had none.

5.4 DISCUSSION

The results from this study indicate that the nucleus-associated microtubule arrays of precleavage and cleaving sporangia of *P. cinnamomi* are sufficiently extensive to be seriously considered as structural regulators of the cleavage process. Before discussing the details and implications of these findings, a general point needs to be made regarding how closely the microtubular distributions described in this study might reflect those that exist in the living sporangium.

Lancelle *et al.* (1987) have suggested that immunofluorescence procedures may cause artefactual bundling of microtubules that are spaced apart *in vivo*. In a previous immunofluorescence study of microtubule arrangements in the zoospore of *P. cinnamomi* (Hardham, 1987b), however, very consistent images were obtained of the complex flagellar rootlet system and these images correlated well with those from electron microscopic serial sectioning; these results argue against any gross distortion of microtubular arrangements in this system due to the preparatory procedures for immunofluorescence microscopy. Nevertheless, we cannot rule out the possibility that some of the fluorescent strands seen in the present study represent a condensation of adjacent microtubules within the astral arrays of the living cell; and that the astral arrays are therefore perhaps even more complex and extensive than described herein.

5.4.1 The possible roles of sporangial microtubule arrays in:

(a) Nuclear spacing and orientation

In Chapter 2 I stressed the significance of the regular spacing and orientation of nuclei in the precleavage sporangium for the later orderly progress of cleavage plane development. The precleavage relationships of the astral microtubular arrays, to the

nuclei and to each other, suggest possible ways in which nuclear spacing and orientation may themselves be established.

Several previous studies have implicated nucleus-based arrays in the maintenance of nuclear positioning (Slifkin, 1967; Olson *et al.* 1981; Heath *et al.* 1982) but the limited extent of the arrays has prompted the conclusion that microtubular anchoring of the nucleus is effected by interactions between the nucleus-associated microtubules and the cytoplasm (Heath *et al.* 1982). It is now clear from this study that the microtubules from adjacent arrays could also be interacting in some way, perhaps by static cross-linking (Tilney, 1971) or dynamic antiparallel interactions (Hogan & Cande, 1990) as has been proposed in models of mitosis. Both these models require some degree of overlapping and this is consistent with the arrays seen in *P. cinnamomi* (e.g., Fig 5.2). In combination with interactions between microtubules and the sporangial plasma membrane, interactions between microtubules of adjacent arrays would seem to provide an adequate model for the maintenance of the position of nuclei in the sporangial cortex. The radial aspects of the arrays of these nuclei are in direct opposition to each other (Fig 5.2, and diagrammatic representations, Figs 5.20-5.21).

The presence of extensive astral arrays in the precleavage sporangium also invites speculation as to their possible role in *establishing* nuclear spacing during sporangial development. Mechanisms have been proposed, in models of mitosis, by which microtubules of opposing arrays could generate pushing forces against each other, either by direct pushing as the microtubules extend (Salmon, 1989) or by dynamic antiparallel interactions (Hogan & Cande, 1990). In radial arrays capable of generating such forces, the amount of force per unit area that can be generated at the margin of the array decreases with increasing array diameter, since microtubule density at the margin also accordingly decreases. Such considerations indicate that, in a closed system with a number of spatially disordered array-generating nuclei, an equilibrium would be reached when all the available space was filled and when the lengths of the array radii were equal, or in other words when the nuclei were equidistantly spaced.

Such a model would seem particularly workable for establishing the orderly spacing of cortical nuclei during sporangial development in *P. cinnamomi* for the same reasons that made a microtubule-microtubule interaction model so attractive for maintaining the spacing of these nuclei. The model does not however explain the polarity of the cortical nuclei. This feature may be a consequence of the development of the sporangium as a hyphal swelling, since, at least in some other Oomycetes (Heath & Kaminskyj, 1989), the hyphal nuclei are similarly oriented with their basal-body poles towards the wall. It is unlikely that karyokinesis plays any role in nuclear spacing during sporangiogenesis in *P. cinnamomi*. Although there have been rare reports of mitosis within sporangia of *Phytophthora* (Laviola, 1974 [cited in Hemmes, 1983]; Jelke *et al.* 1987), it is believed that sporangial nuclei are, in this genus, most commonly derived solely from those already existing in the hypha (Hemmes, 1983).

The mechanisms I have described above could also be involved in the maintenance and/or establishment of nuclear spacing in hyphae of *Phytophthora*. The long, apparently continuous strands of fluorescence seen in this study, running between the evenly-spaced nuclei, might for example actually consist of two opposing sets of microtubules which originate at the nuclei and overlap somewhere in between. Some support for this model is provided by the descriptions of long bundles of hyphal microtubules in electron microscopic images of *Phytophthora infestans* (Temperli *et al.*, 1990) and *P. cinnamomi* (Dearnaley, unpublished observations). The reported shortness of nucleus-associated microtubules in the hyphae of some fungi (Heath & Kaminskyj, 1989) argues against the universality of this model.

The observations in this study of zoospore arrangement in cleaved sporangia also help to explain a commonly reported feature of nuclear arrangement in fungal sporangia, namely the cortical location of most, or all, nuclei (Williams & Webster, 1970; Heath & Greenwood, 1971; Chapter 2). The three-dimensional reconstruction demonstrates that in an average-sized sporangium of *P. cinnamomi* nearly all zoospores lie within the cortex. One can predict that such a sporangium would have, prior to cleavage, the same

disposition of undivided uninucleate domains (Figs 5.21-5.22). Hence the predominant location of nuclei in the cortex of sporangia of this, and no doubt other sizes, is a natural geometrical consequence of the relative dimensions of sporangia and the domains they contain.

(b) Marking out the positions of the future cleavage zones, and spatial restriction of cleavage membrane extension to the zones of cleavage

The arrangements of the microtubules of the astral arrays at various stages before and after the induction of cleavage indicate that the arrays may be involved in marking out the future zones of cleavage, and also, in causing, either directly or indirectly, the development of the cleavage system to be restricted to within these zones.

Figs 5.21 and 5.22 illustrate that the precleavage arrays described in this study are ideally suited for the marking out of the positions of the future cleavage planes.

Regarding microtubular regulation of sporangial cleavage, previous studies have suggested two models, and a third is proposed here. Schrader (1938) suggested that sporangial "asters" might act directly as physical barriers to restrict cleavage plane development to the appropriate zones. In the second model (Heath & Greenwood, 1971) microtubules are proposed to act indirectly. The cytoplasmic zones through which the cleavage membranes will migrate are proposed to contain few microtubules, and thus to lack an hypothesised stabilization existing in the remaining microtubule-rich cytoplasm as a consequence of many microtubular-cytoplasmic interactions. The third model draws upon the phragmoplast model suggested for cytokinesis in higher plants and some green algae (Gunning, 1982). In this model, the future plane of cleavage exists at the junction of two opposing sets of microtubules. Vesicles, from which the cleavage membranes are derived, migrate along the microtubules, stop in the junctional region and fuse to form the zone of separation.

One or more of these models could be involved in cleavage plane formation in *P. cinnamomi*. In the development of the cortical cleavage plane, the phragmoplast model

is obviously unworkable (see Fig 5.22), but either of the remaining models appears feasible, considering the extensive network of microtubules that lies in the cytoplasm beneath where the cortical plane will form. Evidence from electron microscopy indicates that microtubules are closely associated with the membranes of the developing cortical plane (Fig 2.18).

In the sporangial interior, the physical barrier model is generally the least attractive. It is hard to imagine, for example, that, during the development of cleavage planes between the cortical domains (Fig 5.22), the microtubules from directly opposing arrays retract somehow to open up a channel for cleavage planes to pass through. It is possible, however, that those microtubules arising from the internal nuclei and running parallel to the cleavage planes which develop near the narrow poles (Fig 5.22), could function as physical barriers in a similar fashion to that proposed for the analogous microtubules of the cortical nuclei.

Both of the remaining models appear feasible for some aspects of internal cleavage. The phragmoplast model seems ideally suited to formation of the planes that form between the cortical domains (Fig 5.22). Electron microscopic studies of higher plant phragmoplasts (Gunning, 1982) indicate that microtubules of the opposing arrays overlap in the junctional region: in *P. cinnamomi* overlapping was also evident in this region, albeit not in such a concentrated fashion as that seen in higher plants. Also, in *P. cinnamomi*, as in higher plants (Gunning, 1982), small Golgi-derived vesicles appear to be a source of cleavage plane membrane, and although no associations were seen between these vesicles and microtubules with electron microscopy (Chapters 2,3), this could be a consequence of poor preservation of microtubules and/or vesicles. An especially appealing feature of the phragmoplast model is that the dictyosomes are concentrated in the cytoplasm near the focus of each microtubule array (Chapters 2, 3). If some of the products of the dictyosomes are destined for incorporation into the cleavage planes, as was proposed in Chapters 2 and 3, then they must reach the sites at which they will be utilized. This movement could involve directed transport along the

astral microtubules towards the regions where cleavage planes are developing. Directed microtubular transport of cellular organelles has been proposed to occur in many systems (Williamson, 1986; McKerracher & Heath, 1987) and in addition to transporting cleavage material in *P. cinnamomi*, astral microtubules could also play a role in establishing the polarized distributions of the four peripheral vesicles of the zoospore (Fig 1.1).

Regarding the role of the cytoplasmic stabilization model in the sporangial interior, the data indicate that microtubules are *not* absent in all the regions where the cleavage planes will form : for example, between the cortical domains. Nevertheless, microtubular density would still be lowest in these regions, given the radial nature of the arrays, and this is consistent with Heath and Greenwood's model. It is possible, also, that the stabilization model might work in concert with the phragmoplast mechanism, with vesicles being fed into the advancing cleavage plane behind its leading edge. Olson *et al.*, (1981), in a study of zoosporogenesis in *Allomyces catenoides*, noted that the incorporation of cleavage vesicles followed this pattern. It is tempting to speculate that in *P. cinnamomi*, the motive force for the extension of the cleavage planes might be derived from swelling of the matrix material inside the cleavage elements (Chapter 3).

In the present study it was not possible to verify whether changes in array structure precede or follow any specific development of the cleavage system: while we could identify cleavage planes in fully subdivided sporangia, the developing planes were not readily apparent. Additionally, we had no success in using a monoclonal antibody (Cpw-1) to identify the developing cleavage planes in cryosectioned sporangia (Chapter 6) because although this antibody exhibits strong binding to the contents of cleavage elements in freeze-substituted sporangia, in chemically-fixed material not only are most of the contents apparently lost but the cleavage elements themselves become artefactually vesiculated.

Finally, it is also possible that astral microtubules exert their influence upon the spatial aspects of cleavage via their regulation of some other cytoskeletal network, for

instance an actin microfilament system. Studies using anti-microfilament drugs suggest that actin is involved in the regulation of cleavage (Schnepf *et al.* 1978; Oertel & Jelke, 1986; Heath, 1991) and preliminary results from an immunofluoresence study indicate that actin is distributed on either side of the developing cleavage planes in *Saprolegnia* (Heath, 1991). Further drug and immunofluorecence studies are needed to clarify what, if any, interactions may be occurring between networks of these two cytoskeletal elements.

5.4.2 Concluding remarks

As well as providing new and important details of microtubular arrangements in sporangia of *Phytophthora*, the present study demonstrates the potential value of cryosectioning as an adjunct to immunofluorescence studies of large fungal structures. With regard to further investigation of the ideas raised in this chapter it is to be hoped that rapid-freezing freeze-substitution used in combination with cryosectioning and immunofluorescence microscopy may allow a correlated study of cleavage plane development and changes in microtubular array structure. Such a study would be valuable not only for providing this specific information but as a means to investigate a much more general question: why did electron microscopic examination of rapidly-frozen freeze-substituted sporangia (Chapter 3) not indicate the extent of the microtubular arrays seen in this study?

Part of the answer to this question may lie in the difficulties of assessing the extent of microtubular distribution by thin-section analysis, but it is also possible that the total microtubular population was not preserved by the procedures used prior to examination. Cho & Fuller (1989) have doubted whether rapid-freezing freeze-substitution of zoospores of *P. palmivora* provides better preservation of microtubules than chemical fixation. In my electron microscopic studies of sporangia, this was not so : for example, with rapid-freezing at least some microtubules were evident prior to the induction of cleavage (Chapter 3), which was not the case with chemical fixation

(Chapter 2). In an immunofluorescence study of microtubules in hyphae of *Uromyces*, freeze-substituted material provided better results than chemically fixed (Hoch & Staples, 1985).

Taken together, these various results indicate that as yet we have no simple explanation as to why incomplete preservation of microtubules apparently occurs in electron microscopic studies of fungi, even, at least in some cases, when rapid-freezing freeze-substitution has been employed as the means of fixation.

CHAPTER 5 FIGURE LEGENDS

Figs 5.1-5.4 Microtubules and nuclei in the sporangium of *P. cinnamomi*, sampled before the induction of cleavage. Cryosectioned material treated with antitubulin-sheep antimouse-FITC (Figs 5.1-5.3) and DAPI (Fig 5.4).

Fig 5.1 Extensive arrays of microtubules, regularly spaced throughout the sporangial cytoplasm. X850.

Fig 5.2 Microtubules emanate from a focal point and extend beyond the boundaries of adjacent arrays. X2300.

Figs 5.3-5.4 In these photographs of the same double-labelled section, it is evident that the focal points of the arrays shown in Fig 5.3 are adjacent to distinct poles of the nuclei and that for nuclei in the sporangial cortex, these poles are adjacent to the sporangial periphery (Fig 5.4). X2100.

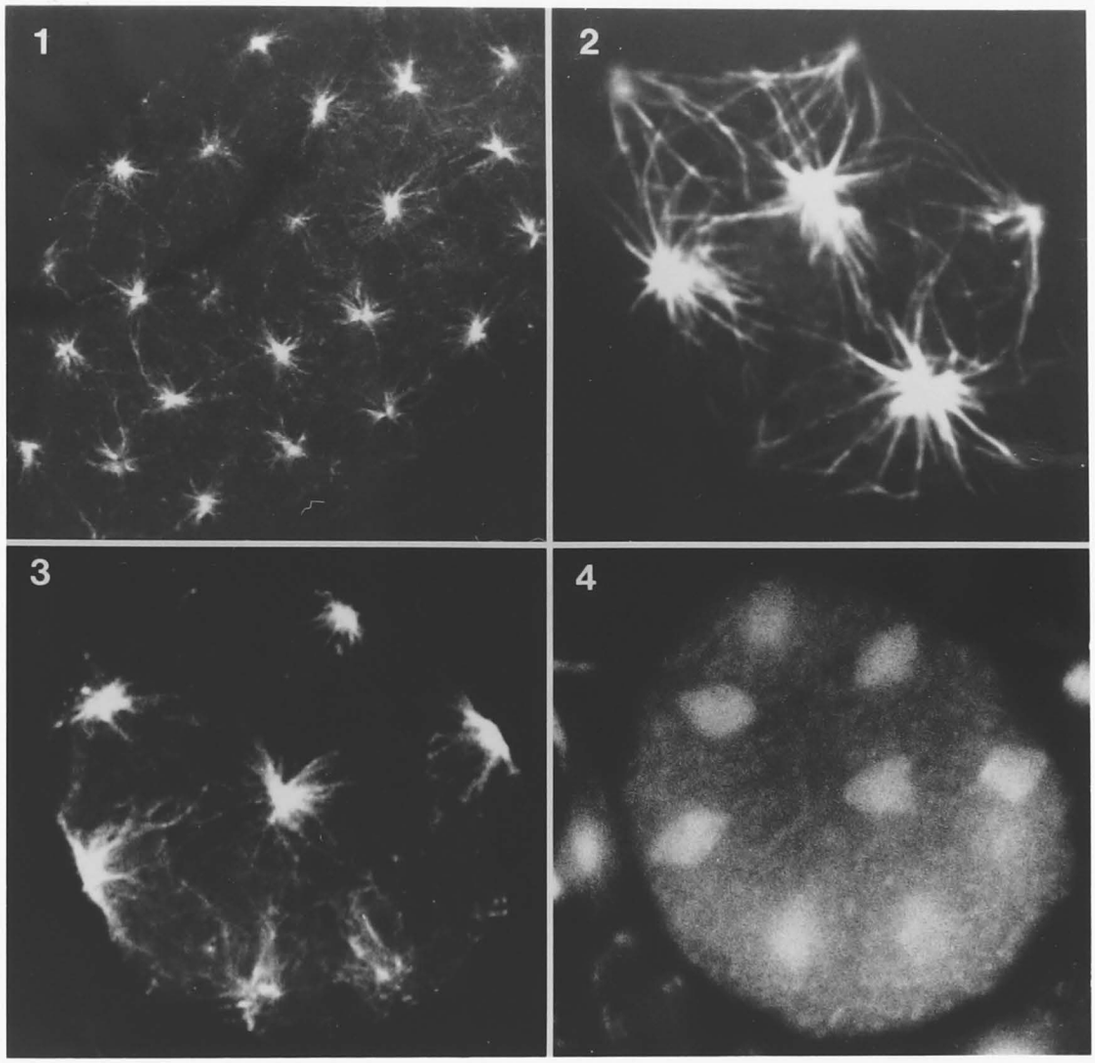
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Fig 5.5 A diagrammatic representation of the full extent of the nucleus-based microtubule arrays seen prior to, and up to 30 min after, the induction of cleavage.

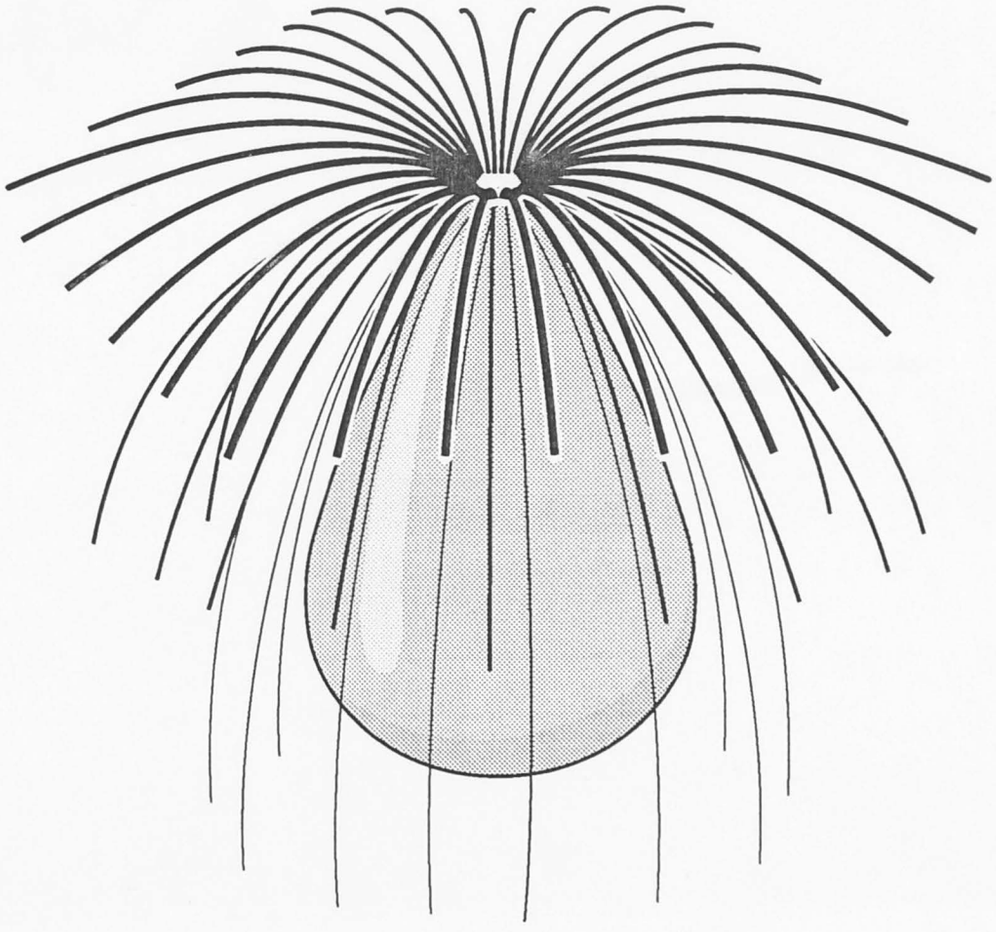


Fig.5.5

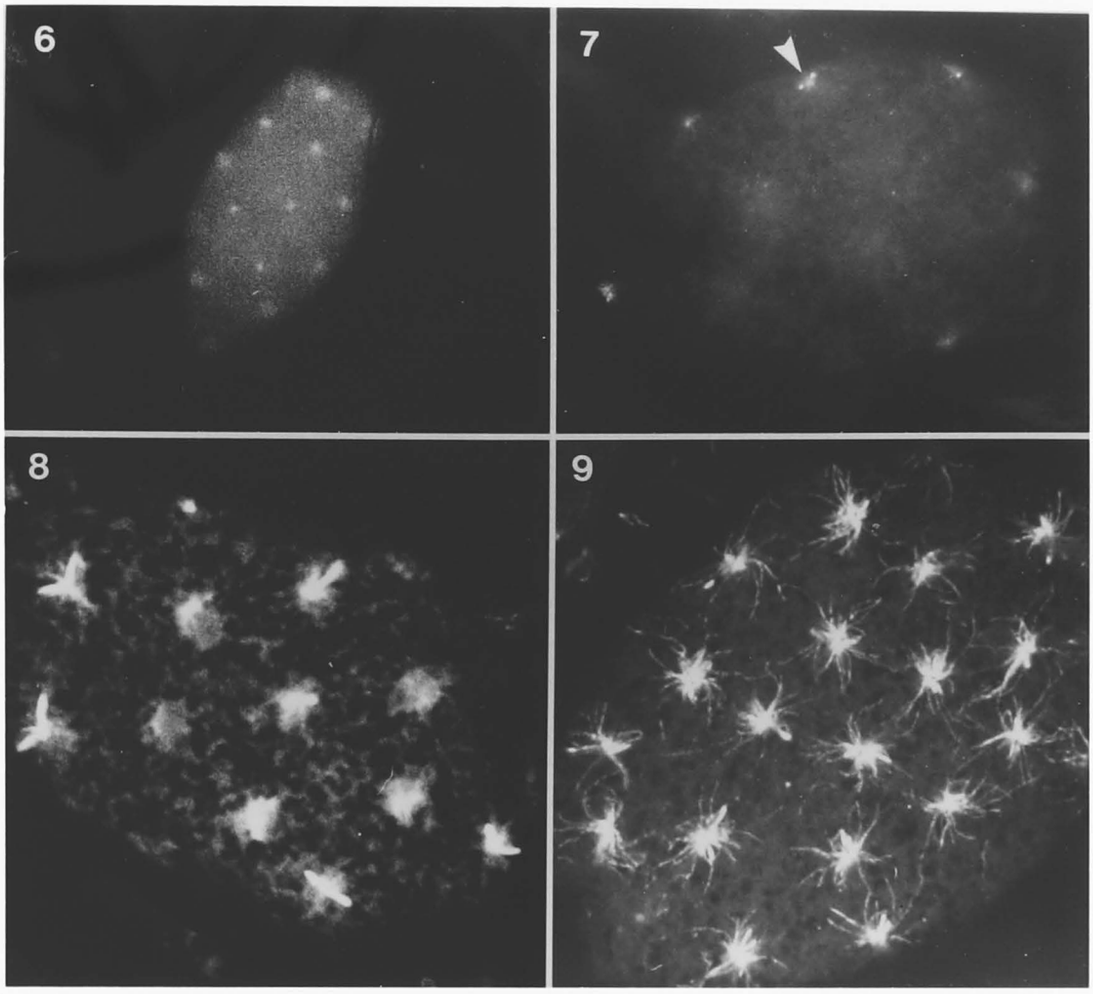
Figs 5.6-5.9 Variant patterns of tubulin-associated fluorescence in sporangia sampled prior to, and 20 min after, the induction of cleavage.

Fig 5.6 In an enzyme-treated whole sporangium sampled before induction no microtubule arrays are seen; instead regularly-spaced spots are evident. X760.

Fig 5.7 In a cryosectioned sporangium sampled before induction regularly-spaced structures are evident, consisting of two bright spots and a fuzzy area between the two spots. X1250.

Fig 5.8 In a cryosectioned sporangium sampled 20 min after induction, two short flagellar initials emanate from regularly-spaced foci. X1200.

Fig 5.9 In a cryosectioned sporangium sampled at 20 min, extensive microtubule arrays are evident. Short stubs of fluorescence seen in some arrays may be flagellar initials. X1200.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Figs 5.10-5.15 Tubulin-associated fluorescence in cryosectioned sporangia sampled between 30 and 60 min after the induction of cleavage.

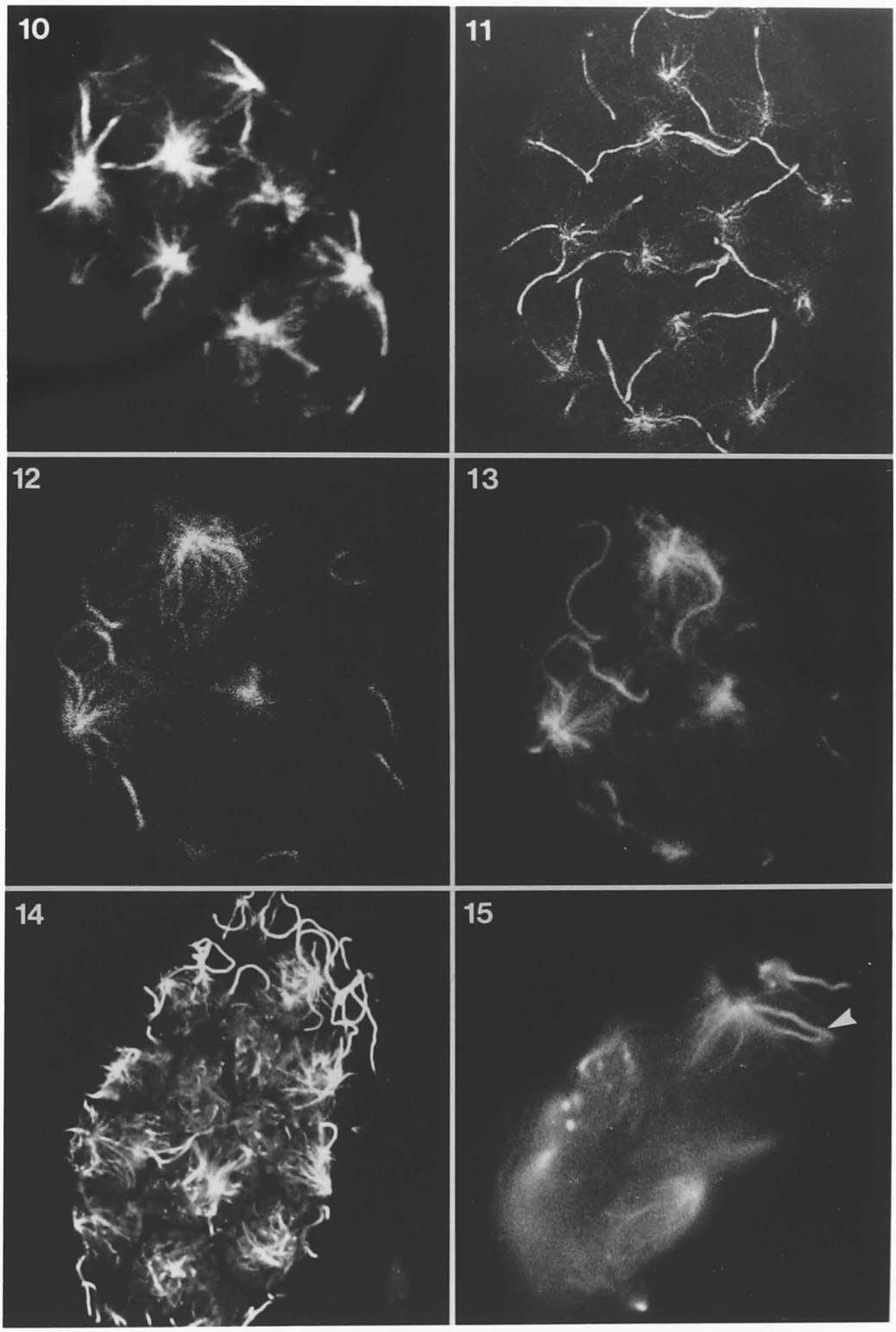
Fig 5.10 At 30 min flagellar initials extend beyond the boundaries of the radial arrays. X1600.

Fig 5.11 More developed flagellar growth, seen in another sporangium from a 30 min sample. X700.

Figs 5.12-5.13 Two optical sections of the same sporangium, from a 40 min sample. Microtubule arrays are evident but are not as obviously radial as at earlier times. Flagella now appear longer and more sinuous. X2000.

Fig 5.14 Completed cleavage in a 60 min sporangium. Microtubules are evident within the individual zoospores. X1100.

Fig 5.15 In some sporangia, zoospores showed evidence of the characteristic microtubular flagellar rootlet system. The posterior rootlets of one zoospore initial are seen here in a 60 min sporangium (arrowhead). X1800.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Figs 5.16-5.17 Two photographs of the same double-labelled section, showing, on the left, longitudinal arrays of microtubules which run between regularly spaced nuclei (on the right) in a hypha of *P. cinnamomi*. One nucleus appears to be in a short branch of the hypha. X1000.

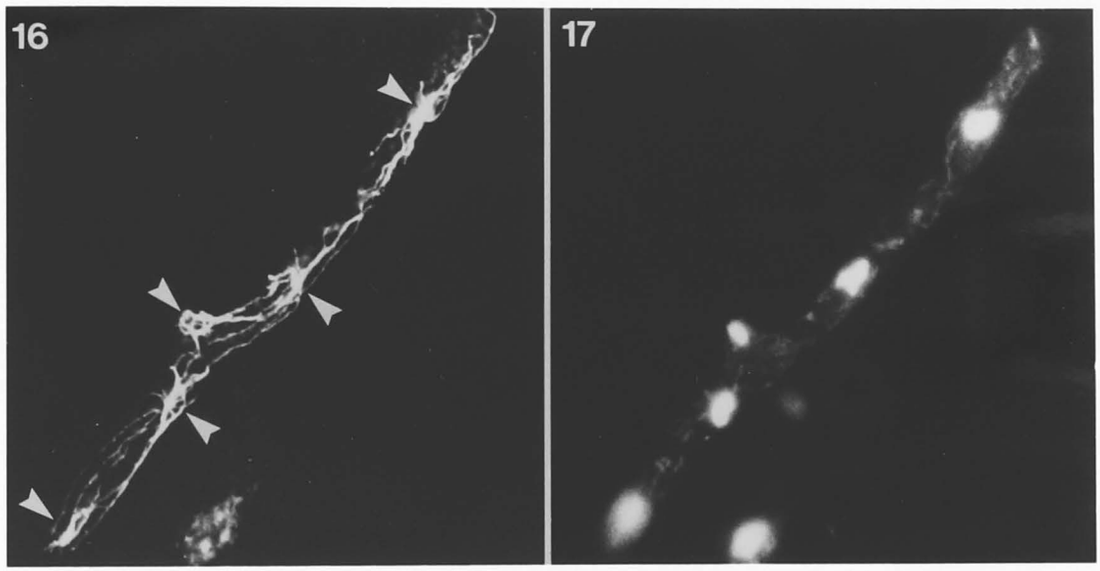
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Fig 5.18 Three dimensional reconstruction of a cleaved sporangium of *P. cinnamomi*, using 17 of 20 sections comprising the whole sporangium. Individual zoospores are shown in different colours; this sporangium contained 23 zoospores.

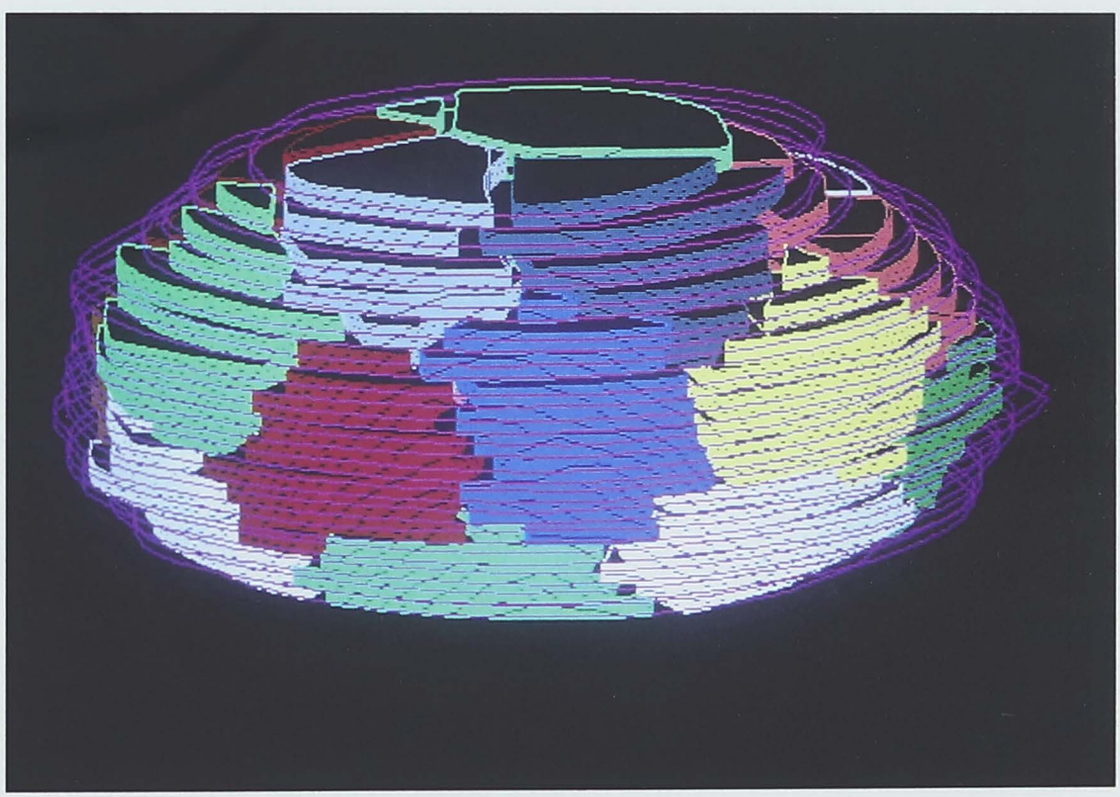
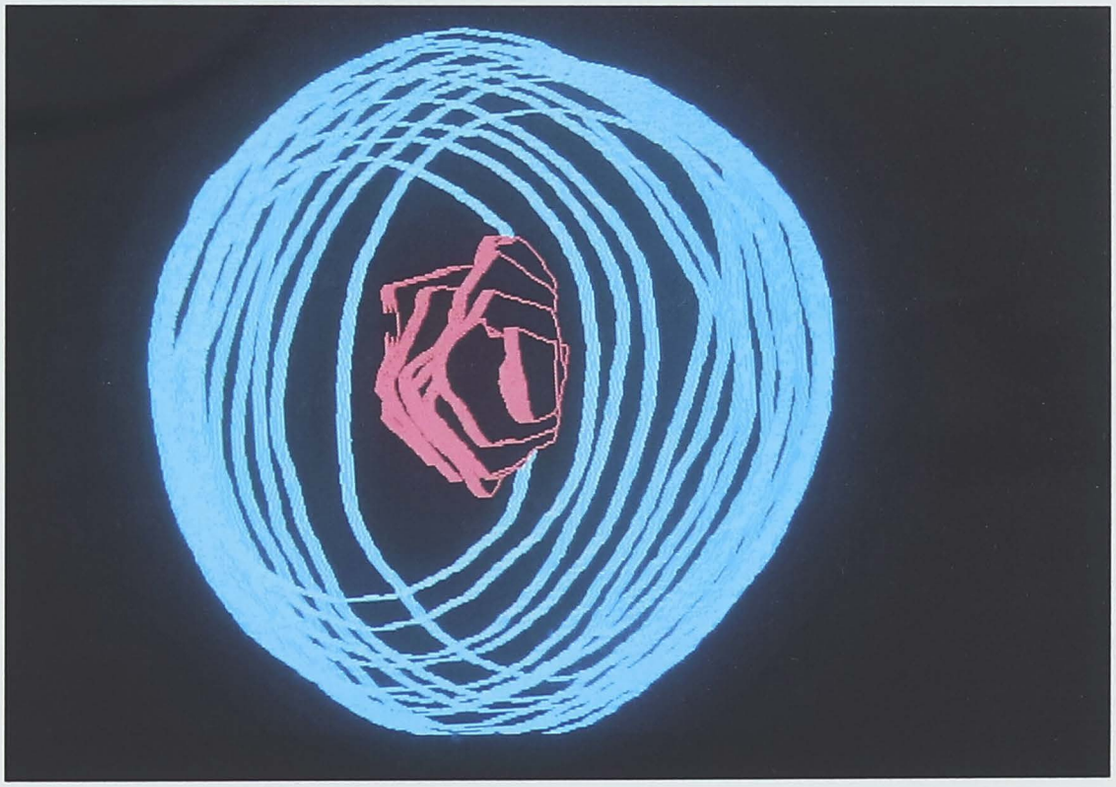


Fig 5.19 The same sporangium as shown in Fig 5.18, but rotated through 90°. Of the 23 zoospores in this sporangium, only one had no contact with the sporangial wall, shown here in blue.



Figs 5.20-5.22 Diagrammatic representations of the proposed relationships between microtubule arrays, nuclei, domain boundaries and cleavage planes at different stages of zoosporogenesis in a sporangium of *P. cinnamomi* with similar dimensions to that shown in Figs 5.18-5.19.

Figs 5.20-5.21 Pre-induction sporangium, shown in surface view and longitudinal section. The cortical nuclei have their astral arrays oriented towards the wall. The regions where adjacent arrays meet or overlap mark out the sporangium into a number of uninucleate, equal-sized domains.

Fig 5.22 This figure shows possible relationships between the microtubule arrays and the developing cleavage planes described in Chapter 3. Note that the cortical cleavage plane lies parallel to the outermost microtubules of the cortical arrays, while most of the internal planes are likely to develop between opposing sets of microtubules.

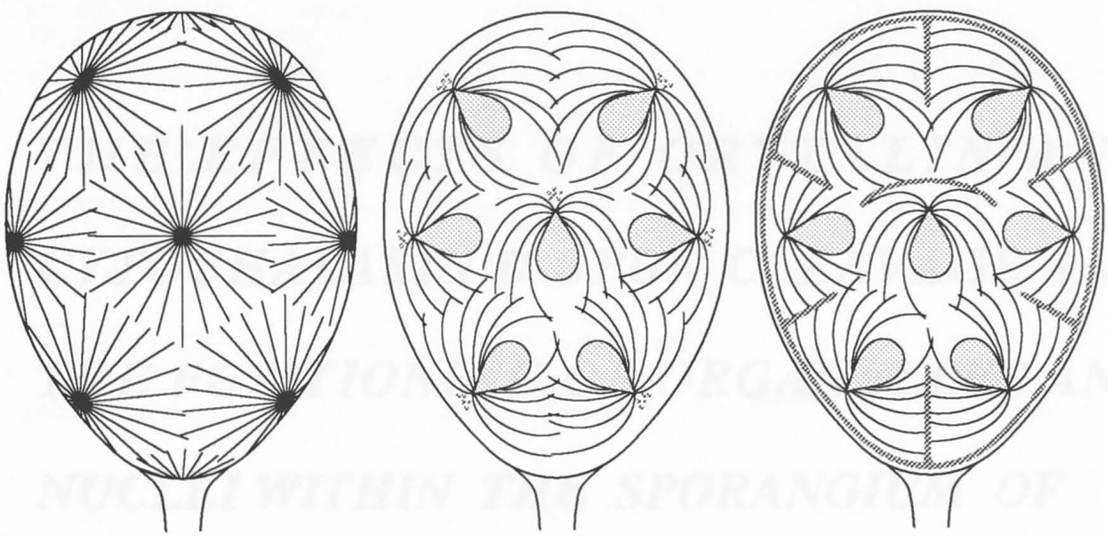


Fig. 5.20, 5.21, 5.22

6

THE EFFECTS OF ORYZALIN AND CYTOCHALASIN D UPON CLEAVAGE AND THE POSITIONING OF ORGANELLES AND NUCLEI WITHIN THE SPORANGIUM OF P. CINNAMOMI

6.1 INTRODUCTION

In Chapter 5 I have described and discussed the presence of extensive nucleus-based microtubule arrays within the uncleaved and cleaving sporangium of *Phytophthora cinnamomi*. It was suggested that these arrays may be involved in the structural regulation of some of the events of zoosporogenesis described in Chapters 2 and 3. In particular it was proposed that the arrays could play a role in the maintenance of nuclear spacing and orientation, cleavage plane development and the directed movement of various organelles within the sporangium. While previous studies (discussed in Chapter 5) have already indicated that nucleus-based microtubule arrays

may be involved in nuclear positioning and cleavage, nothing is known about the role of microtubules, or any other cytoskeletal element, in sporangial organelle movements. Since microtubules and microfilaments are thought to be involved in organelle movements in other fungal (McKerracher & Heath, 1987) and non-fungal (Schnapp *et al.* 1985; Scholey *et al.* 1985; Williamson, 1986) systems, further investigation of the role of the cytoskeleton in sporangial organelle positioning is attractive. The sporangium of *P. cinnamomi* is an ideal system in which to study this matter. It has been shown (Chapters 2, 3) that at least five types of structures (mitochondria, large peripheral vesicles, ventral vesicles, dorsal vesicles and peripheral cisternae) acquire, during the process of cleavage, highly concentrated distributions at, or near, the future zoospore periphery. The peripheral distributions of these structures also exhibit a second order of polarization : they are not distributed equally around the entire periphery but are localized at or near either the future ventral or dorsal surfaces of the developing zoospore (Chapter 2).

In this study I have investigated the roles of microtubules and microfilaments in the development of these polarities, and other aspects of zoosporogenesis, by examining sporangia of *P. cinnamomi* treated either with oryzalin or cytochalasin D, drugs known, respectively, for their anti-microtubule (Morejohn *et al.* 1987; Cleary & Hardham, 1988) and anti-microfilament (Yahara *et al.* 1982; Cooper,1987) properties. The effects of these drugs upon mitochondrial and vesicular distributions have been monitored by standard and confocal fluorescence microscopy of cryosectioned sporangia labelled with chemical dyes and monoclonal antibodies specific to three of the four vesicles detailed above.

6.2 MATERIALS AND METHODS

6.2.1 Organism

The culture of *P. cinnamomi* (DAR 52646) used in this study was induced to produce sporangia and zoospores as described previously (Chapter 2). In these experiments, zoospore release regularly occurred at about 65 min after exposure to cold shock.

6.2.2. Microscopy of living untreated and drug-treated sporangia

The process of cleavage and zoospore release was monitored in living sporangia that had been incubated either with various concentrations of cytochalasin D (CD:Sigma, St Louis, Mo.) or oryzalin (3-5-dinitro-N^4,N^4- dipropylsulfanilamide; Lilly Research Laboratories, Greenfield, IN), or (for controls) with appropriate concentrations of the chemicals that were used as solvents for the two drug treatments (dimethyl sulfoxide [DMSO] and acetone respectively). CD concentrations ranged between 10-100μM (0.025-0.25% DMSO) and oryzalin between 0.1-1.0μM (0.1-1.0% acetone). The drugs and solvents were added to the growth medium (mineral salts solution, Chapter 2) 20 min prior to the induction of cleavage and were included in the distilled water in which sporangia were incubated during the process of cleavage. Samples were taken at various times after the induction of cleavage and observed using differential interference contrast optics on a Zeiss Axioplan microscope.

6.2.3 Microscopy of fixed, cryosectioned sporangia

Sporangia destined for cryosectioning were sampled either before the induction of cleavage or between 60-65 min after induction. CD, oryzalin, DMSO and acetone were added to the incubation media as described above for living sporangia. The fixation and cryosectioning techniques have been described previously (Chapter 5). Sections were treated with five monoclonal antibodies (mAbs) whose specificities have been described previously (Vsv-1, Cpa-2, Lpv-1, Cpw-1: see Table 2.1; anti-ß-tubulin: see

Chapter 5). The incubations, rinsing and mounting procedures for sections treated with anti-β-tubulin were as described previously (Chapter 5). The general procedures for treatments with the other mAbs were similar except that shorter incubations were used (45 min for primary and secondary antibodies) and primary antibodies were used either as undiluted supernatants, or diluted to 1 μg/ml in the case of purified Cpa-2 antibody. Some preparations were also stained for DNA as described in Chapter 5. Fluorescence in sections was viewed using either a standard fluorescence microscope (Axioplan or Axiovert) or a microscope equipped with confocal optics (Biorad MRC600). When using the confocal microscope either single or multiple, accumulated optical sections were used to produce images which best illustrated characteristic patterns. Sections were also viewed using differential interference contrast optics either on standard or confocal microscopes. Most experiments were repeated at least twice; all the experiments involving sampling of drug-treated sporangia at 60-65 min were repeated three times, using concentrations that had been found to affect cleavage in a consistent fashion (1μM oryzalin; 100μM CD). To check that sporangial cleavage was not induced by the addition of drugs or solvents, some sporangia from each of the drug treatments and the controls were not cold-shocked, but were left in the incubating media for 60 min, after which 100 sporangia from each treatment were observed. There were no signs of cleavage in any of these sporangia, nor were there any released zoospores. Thus it would seem likely that all cleaved sporangia that were examined in the experiments of this study did not begin to cleave until after the application of the cold shock.

6.3 RESULTS

6.3.1 Effects of cytochalasin D and oryzalin on living sporangia

Observations of living sporangia that had been incubated in media containing either CD or oryzalin indicated that both of these drugs caused gross perturbations of the normal cleavage process. This was in contrast to the control treatments in which fully

cleaved sporangia appeared identical to those described in Chapter 2 (Fig. 6.1). In CD-treated sporangia viewed just before release, the cleavage planes were clearly evident but they lacked the polygonal relationships typical of those in control cells (Fig 6.2). A common feature of these sporangia was the presence of extensive uncleaved regions running parallel to the sporangial wall (Fig 6.2). CD-treated sporangia released their contents as irregularly-shaped cytoplasmic masses, usually possessing many active flagella. Smaller fragments were highly motile. Many active water expulsion vacuoles were evident within the released cytoplasm, two or three often being present even in some of the smaller fragments.

Abnormal cleavage was most consistently observed in sporangia incubated in 100μM CD. Lower concentrations of CD (20μM and 60μM) also caused abnormalities but not as effectively. In control treatments for the 100μM treatment, DMSO (0.25%) appeared to cause slight irregularities in the shapes of small numbers of the released zoospores.

When viewed just before release, oryzalin-treated sporangia appeared similar to those treated with CD. Upon release the sporangial contents consisted of highly irregular cytoplasmic masses (Fig 6.3) but, unlike those from CD-treated cells, these masses possessed no flagella and were non-motile. Active water expulsion vacuoles were, however, present (Figs 6.4 and 6.5). After release, the larger fragments sometimes proceeded to subdivide, pinching off small, spherical regions (Figs 6.4 and 6.5). After extended periods (15-20 min) this process was, however, reversed as small adjacent fragments fused together (Fig 6.6). This process appeared to relate to a loss of vitality, since water expulsion activity ceased and the fused masses eventually took up so much water that they became spherical and burst.

The shapes of the released zoospores from sporangia used as controls for the oryzalin treament (incubated in media with 1% acetone) were consistently normal.

6.3.2 Cryosectioned sporangia

6.3.2.1 Controls, pre-induction

There were no differences in the predominant patterns of DAPI and mAb associated fluorescence of acetone and DMSO treated control sporangia sampled prior to induction, so these results will be considered together. Tubulin-associated fluorescence was distributed in the characteristic astral arrays described in uncleaved sporangia in Chapter 5 (Fig 6.7). DAPI staining showed that, as previously described (Chapters 2, 3 and 5), the nuclei were regularly spaced, with the narrow poles of cortical nuclei pointing towards the wall (Fig 6.8). Small bright spots of fluorescence in DAPI-stained sporangia are likely to correspond to mitochondrial DNA, and will henceforth be referred to as mitochondria. Mitochondria were for the most part randomly distributed, but were usually less abundant near the narrow nuclear poles (Fig 6.8). The fluorescence associated with mAbs Vsv-1, Cpa-2 and Lpv-1 showed similar distributions to that of the mitochondria (Figs 6.9-6.11). The large gaps in fluorescence evident in many sporangia treated with the vesicle-specific mAbs (e.g. Figs 6.9 and 6.11) were shown, by combined DAPI-mAb staining, to correspond to regions occupied by nuclei and the clear zones, that, as previously described (Chapters 2 and 3) are associated with the narrow nuclear poles (Figs 6.8 and 6.9: same section, double-labelled).

6.3.2.2 Oryzalin treatment, pre-induction

Sporangial cryosections from sporangia incubated in 1μM oryzalin and labelled with anti-β-tubulin-SAM-FITC exhibited no microtubule-associated fluorescence (Fig 6.12). Roughly circular gaps in the background fluorescence of cryosections of these sporangia probably correspond to nuclei, and these had a dull ring of fluorescence around them (Fig 6.12). DAPI staining showed that the regular spacing of nuclei was lost; few nuclei were seen in the sporangial cortex, and when observed in this region they exhibited no polarity towards the sporangial wall (Fig 6.13).

Mitochondrial distribution in oryzalin-treated sporangia appeared similar to that of controls, but clear zones (Fig 6.13) did not regularly correspond to the presence of nuclei. Fluorescence associated with mAbs Vsv-1, Cpa-2 and Lpv-1 showed similar distributions to that of mitochondria (Figs 6.14-6.16).

6.3.2.3 CD treatment, pre-induction

The patterns of DAPI and mAb associated fluorescence in uninduced sporangia incubated in 100μM CD showed no significant differences from those of controls (Figs 6.17-6.20).

6.3.2.4 Controls, 60-65 min post-induction

There were no differences in the predominant patterns of DAPI and mAb associated fluorescence of acetone and DMSO treated control sporangia sampled just prior to release. Tubulin-associated fluorescence indicated the presence of flagella and microtubule arrays, as previously described in Chapter 5 (Fig 6.22). DAPI staining illustrated that the cortical nuclei retained their polarity during cleavage, and that the distribution of mitochondria became highly polarized towards the zoospore periphery (Fig 6.23). Mitochondria were not, however, evenly distributed along the entire periphery, being absent from much of those surfaces of the zoospores towards which the narrow nuclear pole pointed. These regions are the future ventral surfaces of the zoospores. As was seen in material prepared for electron microscopy (Chapter 2), the future ventral surfaces of cortical zoospores are always adjacent to the sporangial wall (Fig 6.23).

Fluorescence associated with mAb Vsv-1 showed exactly the reverse distribution to that shown by the mitochondria: it was localized to those regions identifiable (by combined DAPI-mAb staining) as the future ventral surfaces of the zoospores (Fig 6.24). The patterns associated with mAbs Cpa-2 and Lpv-1 were basically the same as that

described for the mitochondria (Figs 6.25 and 6.26), being concentrated on the future dorsal surfaces.

6.2.3.5 Oryzalin treatment, 60-65 min post-induction

Anti-β-tubulin- SAM-FITC labelling of oryzalin-treated sporangia sampled just prior to release indicated a complete absence of cytoplasmic microtubules and flagella (Fig 6.27). Background fluorescence indicated that the cytoplasm was cleaved, but in a highly irregular fashion (Fig 6.27). The patterns of abnormal cleavage were similar to those seen in living, oryzalin-treated sporangia. Optical serial sectioning with the confocal microscope showed that regions of cytoplasm that appeared as isolated fragments in single sections (e.g. Fig 6.27) were often interconnected. DAPI staining indicated that the nuclei lacked the spacing and polarity of those in control treatments and that the mitochondria were not localized along the abnormal cleavage planes (Fig 6.28).

In contrast to the mitochondria, the fluorescence associated with mAbs, Vsv-1, Cpa-2 and Lpv-1 did, in many sporangia, show strong localization to the abnormal cleavage planes (Figs 6.29-6.31). This fluorescence was, for the most part, distributed along the entire length of the abnormal planes (e.g. Figs 6.29 and 6.31). Combined DAPI-mAb staining indicated that gaps did not regularly correspond to the presence of nuclei.

6.2.3.6 CD treatment, 60-65 min post-induction

In CD-treated sporangia sampled just before release, flagella and cytoplasmic microtubules were evident (Fig 6.32). These structures emanated from a series of focal points, most of which were adjacent to the sporangial wall (Fig 6.32). DAPI staining indicated that cortical nuclei retained the spacing and polarity seen at the pre-induction stage (Fig 6.33). The patterns of abnormal cleavage, evident in DAPI-stained material, were similar to those seen in living, CD-treated sporangia. Mitochondrial distribution

was highly polarised, being localized to all portions of the abnormal cleavage planes except those opposite the narrow nuclear poles (Figs 6.33 and 6.37).

As was the case for control treatments from this time, Vsv-1 fluorescence showed the reverse distribution to that just described for mitochondria, being highly localized opposite the narrow nuclear poles, at least in the case of cortical nuclei (Figs 6.33 and 6.34 : same section, double-labelled). Cpa-2 and Lpv-1 fluorescence showed similar distributions to that of mitochondria, as was also the case for control treatments (Figs 6.35 and 6.36).

6.3.2.7 Cpw-1 labelling

Since mAb Cpw-1 labels the contents of the developing cleavage system in material prepared for electron microscopy (Chapters 2 and 3), an attempt was made to use it to track cleavage plane development by immunofluorescence microscopy. However, the fixative used, paraformaldehyde, appeared to cause vesiculation of the developing planes in the same manner as that described for glutaraldehyde (Chapter 3). In addition, at the completion of cleavage there was little labelling in the intercellular spaces (Fig 6.38), where one would expect it to be strongest. This is probably a consequence of the extraction of the antigen by the procedures involved with chemical fixation and preparation for electron microscopy, as proposed in Chapters 2 and 3.

Cpw-1 labelling in cleaved, cryosectioned sporangia material was mainly restricted to the zoospore periphery,reflecting, most likely, the adherence of some extracellular matrix material to the zoospore plasma membranes, and labelling of the peripheral cisternae (Fig. 6.38). This pattern was also seen in abnormally cleaved sporangia that had been incubated in oryzalin (Fig. 6.39). As was also seen in normally-cleaved sporangia prepared for electron microscopy (Chapters 2 and 3), a plug of extracellular matrix material at the sporangial apex was labelled by mAb Cpw-1 in, in this study, both normal and oryzalin-treated sporangia (Figs 6.38-6.39).

6.4 DISCUSSION

6.4.1 Establishment of the polarized distribution of the peripheral vesicles

The results of this study indicate that, in the establishment of the polarized distributions of the three peripheral vesicles examined herein, there are differing cytoskeletal requirements for, firstly, the general peripheral concentration of these vesicles and, secondly, the localization of these vesicles along either the ventral or dorsal sub-domains of the periphery. The reader is referred to Fig 6.40 for a diagrammatic summary of the results pertinent to this discussion.

6.4.2 Establishment of the general peripheral localization of peripheral vesicles

With regard to generating the first, general aspect of peripheral polarization the results indicate that neither microtubules nor microfilaments are necessarily required. In cleaved sporangia that had been incubated in media containing oryzalin or CD, all three vesicle types were concentrated along some part or all of the abnormal cleavage planes. There is strong evidence to suggest that this polarization occurred in the absence of any active microtubules or microfilaments. Firstly, while microtubule-associated fluorescence could be visualized in control sporangia, this was not the case in sporangia treated with oryzalin. With respect to the CD experiments, while the experiments do not demonstrate what effects this drug may have had on microfilaments, it is unlikely that any such structures that might exist in the sporangium before the drug treatment would still be functional after extended exposure to 100 μM concentrations of CD. CD is the most potent and specific of the anti-actin group of cytochalasins and affects actin-related processes in some systems at concentrations as low as 0.2 μM (Yahara *et al.* 1982; Cooper, 1987). Previous studies of Oomycete zoosporogenesis have shown that CD, or the less potent cytochalasin B, affects sporangial actin and/or cleavage at concentrations of between one fifth and one tenth of that used in this study (Oertel & Jelke, 1986; Heath 1991). These findings have led to proposals that actin is involved in regulation of the

cleavage process. Given the profound effects of CD upon cleavage in this study, it may well be that this effect also reflects a disturbance of some as-yet-undescribed actin network in the sporangium of *P. cinnamomi*.

The results of this study do not preclude the possibility that microtubules and/or microfilaments are directly involved in bringing about the general peripheral disposition of these vesicles under natural conditions. Nevertheless it now seems worthwhile to consider the possibility of alternative mechanisms: in any case we need to understand how this localization may have occurred under the experimental conditions. The model that seems most promising involves two processes: (1) the peripheral vesicles move randomly throughout the sporangial cytoplasm until (2) they come into contact with receptors on the zoospore plasma membrane which bind the vesicles and prevent their further movement. Ultrastructural support for this model is provided by the close contacts between all three vesicle types and the plasma membrane of the developing and fully-formed zoospores (see Figs 2.31-2.33, 2.37, 2.41). While not all vesicle profiles contact the plasma membrane, such contacts may occur outside the particular plane sampled by thin-sectioning. Interestingly, in my electron microscopic images of rapidly-frozen cleaved sporangia there are nearly always gaps in the extensive network of peripheral cisternae where peripheral vesicles are adjacent to the plasma membrane (e.g. Figs 3.12, 4.1). This pattern can also be seen in images of rapidly-frozen zoospores of *P. palmivora* (Cho & Fuller, 1989). Binding of vesicles by plasma membrane receptors has been suggested in other systems (Pearse, 1988). If this model is operating within the normally cleaving sporangium, then the sequential nature of localization shown by the three vesicles (Chapter 2) indicates perhaps that either three different receptors become activated sequentially or that the vesicles acquire their binding capacity at different stages.

6.4.3 Establishment of the ventral or dorsal surface localizaion of peripheral vesicles

While the results provide no evidence for cytoskeletal involvement in general aspects of peripheral localization, they do suggest that microtubules, but not microfilaments, are necessary for the distributional restriction of the ventral vesicles to the ventral surface domains, and of the dorsal and large peripheral vesicles to the dorsal surface domains. In oryzalin-treated sporangia that had undergone abnormal cleavage none of the three vesicle types were concentrated at any particular part of the aberrant planes. In CD-treated sporangia, however, although there was an equivalent disturbance of the cleavage process, each of the three vesicle types was restricted to a specific portion of the abnormal cleavage planes. The region occupied by each of the vesicle types was spatially oriented, with respect to the narrow nuclear pole, in a similar fashion to the ventral and dorsal surface domains occupied by the vesicles in normally cleaved sporangia.

Given that microtubules appear to be necessary for the localization of the three vesicle types to their respective peripheral domains, there are various possibilities as to what roles they may be playing in this process.

Firstly, if different membrane receptors are involved in binding each of the three vesicle types, as was proposed above, then microtubules may be involved in spatial restriction of the various receptors to either the ventral or dorsal surface domains. It is well known that membrane proteins of many different cell types can be restricted to localized domains within the plasma membrane, and cytoskeletal elements have often been proposed as effectors of such restrictions (Stya & Axelrod, 1983; McClosky & Poo, 1984).

In *P. cinnamomi*, the microtubule arrays of the cleaving sporangium are oriented in such a way that any contacts they might have with the developing dorsal and ventral surfaces will be of different sorts. Since the most radial portions of the arrays run parallel to the developing ventral surface (e.g. see Fig 5.22), the possibility arises of

many lateral contacts between microtubules and the plasma membrane in this region. Contacts of this type were seen in electron microscopic images (Fig 2.18). For the dorsal surface, however, there is only the possibility of much less frequent end-on type contacts with the distal ends of the microtubules. These differences may have some significance in any cytoskeletal mechanisms that establish the proposed restriction of membrane receptors to either the dorsal or ventral surface domains.

The different orientations of the array microtubules towards the dorsal and ventral surfaces suggest an additional way in which sorting of the vesicles to the different surfaces might occur. For example, if the dorsal and large peripheral vesicles could be selectively transported along the array microtubules to their distal ends, then these vesicles would naturally end up near some part of the dorsal surface. Once near this surface their position could be stabilized by interactions with some membrane receptor or the transporting microtubules.

6.4.4 Establishment of the polarized distribution of mitochondria

The results indicate that mitochondrial localization during cleavage requires the presence of an intact microtubule cytoskeleton. In oryzalin-treated sporangia sampled at 60-65 min, mitochondria were randomly distributed, whereas in control and CD-treated sporangia from this time mitochondria were concentrated along the cleavage planes, except for those portions opposite the narrow nuclear poles. Heath *et al.*(1982) have also reported that mitochondrial movements, in hyphae, are inhibited by anti-microtubule drugs but not by cytochalasin D. These findings suggest perhaps that the polarization of mitochondrial distribution during normal cleavage may involve transport along the microtubules, and/or microtubular stabilization of mitochondria in the zoospore cortex. While my electron microscopic studies have not indicated frequent contacts between mitochondria and microtubules, this could well be a consequence of poor microtubule preservation. In fungal hyphae microtubules have more associations with mitochondria than have any other organelles (Heath & Heath, 1978). The membrane

receptor model proposed above for peripheral vesicle polarization would not seem appropriate for mitochondria because profiles of these structures, as seen in electron micrographs, were rarely adjacent to the plasma membrane of developing zoospores (Chapter 2). Peripheral cisternae were usually interposed between the mitochondria and the plasma membrane.

6.4.5 Effects of oryzalin and CD upon cleavage and nuclear positioning

The effects of CD and oryzalin upon nuclear positioning and/or cleavage were generally similar to those that have previously been described in studies of Oomycete sporangia treated with cytochalasins and anti-microtubule drugs (Slifkin 1967; Schnepf *et al.* 1978; Olson *et al.* 1981; Heath *et al.* 1982; Olson & Lange, 1983; Oertel & Jelke, 1986; Heath, 1991). The results demonstrate for the first time, however, that the effects of cytochalasins upon sporangial cleavage are independent of any effect upon the microtubular cytoskeleton. As yet there are no clues as to the role played by actin in sporangial cleavage. The findings of this study support the proposals made in Chapter 5 regarding the roles of microtubules in cleavage and nuclear positioning in *P. cinnamomi*. The labelling, by mAb Cpw-1, of the abnormal cleavage planes in oryzalin-treated sporangia indicates that these abnormal planes still originate from the same source as normal cleavage membranes.

The continued cleavage of sporangial contents after release, seen in the oryzalin-treated material, has not been previously described. The mode of cleavage, involving a constriction process similar to that described for animal cells (Rappaport, 1986) was very different from that suggested by electron microscopy for regular sporangial cleavage, and warrants further investigation.

Finally, the efficacy of oryzalin in causing total depolymerization of cytoplasmic and flagellar microtubules should draw the attention of other mycologists to the potential of this drug for experimental purposes. There has been considerable inconsistency in the efficacy of other antimicrotubule agents in bringing about total depolymerisation of

microtubules (Heath, 1978; McKerracher & Heath, 1978; Temperli *et al.* 1991). Oryzalin should now be tested upon other fungal organsims, this being the first report of its use in such systems.

CHAPTER 6 FIGURE LEGENDS

Figs 6.1-6.6 Aspects of normal and drug-perturbed cleavage in living sporangia of *P. cinnamomi*. All sporangia (in figures of this chapter) that are specified as CD-treated have been incubated in 100μM CD and 0.25% DMSO; all oryzalin-treated sporangia have been incubated in 1μ M oryzalin and 1% acetone. Differential interference contrast optics.

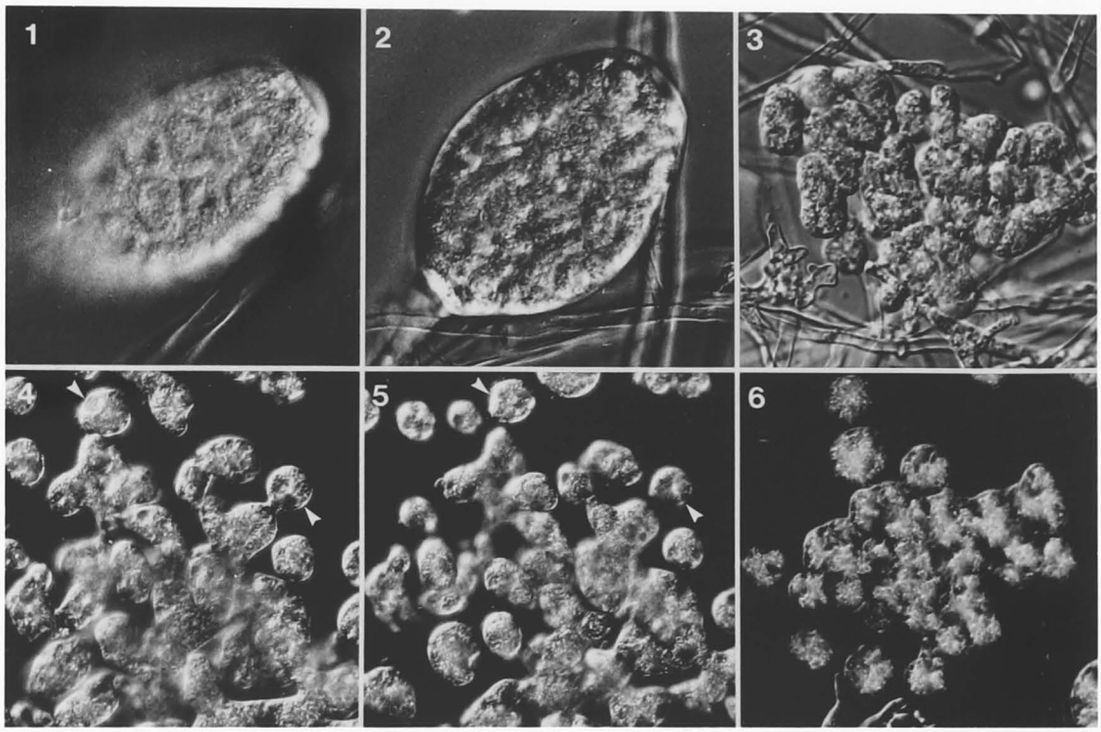
Fig 6.1 Cleaved sporangium from control treatment (0.25% DMSO) for CD experiments. X390.

Fig 6.2 Abnormally cleaved sporangium incubated in 100 μMCD. X550.

Fig 6.3 Released contents of an abnormally cleaved sporangium incubated in 1 μM oryzalin. X420.

Fig 6.4-6.5 Continued cleavage of released contents from a sporangium incubated in 1μM oryzalin (arrowheads). The fragments indicated by arrowheads in Fig 6.4 have prominent water expulsion vacuoles. X620.

Fig 6.6 Fusion of some of the fragments seen in Figs 6.4 and 6.5, seen 15 min after Fig 6.5 was photographed. X410.


1
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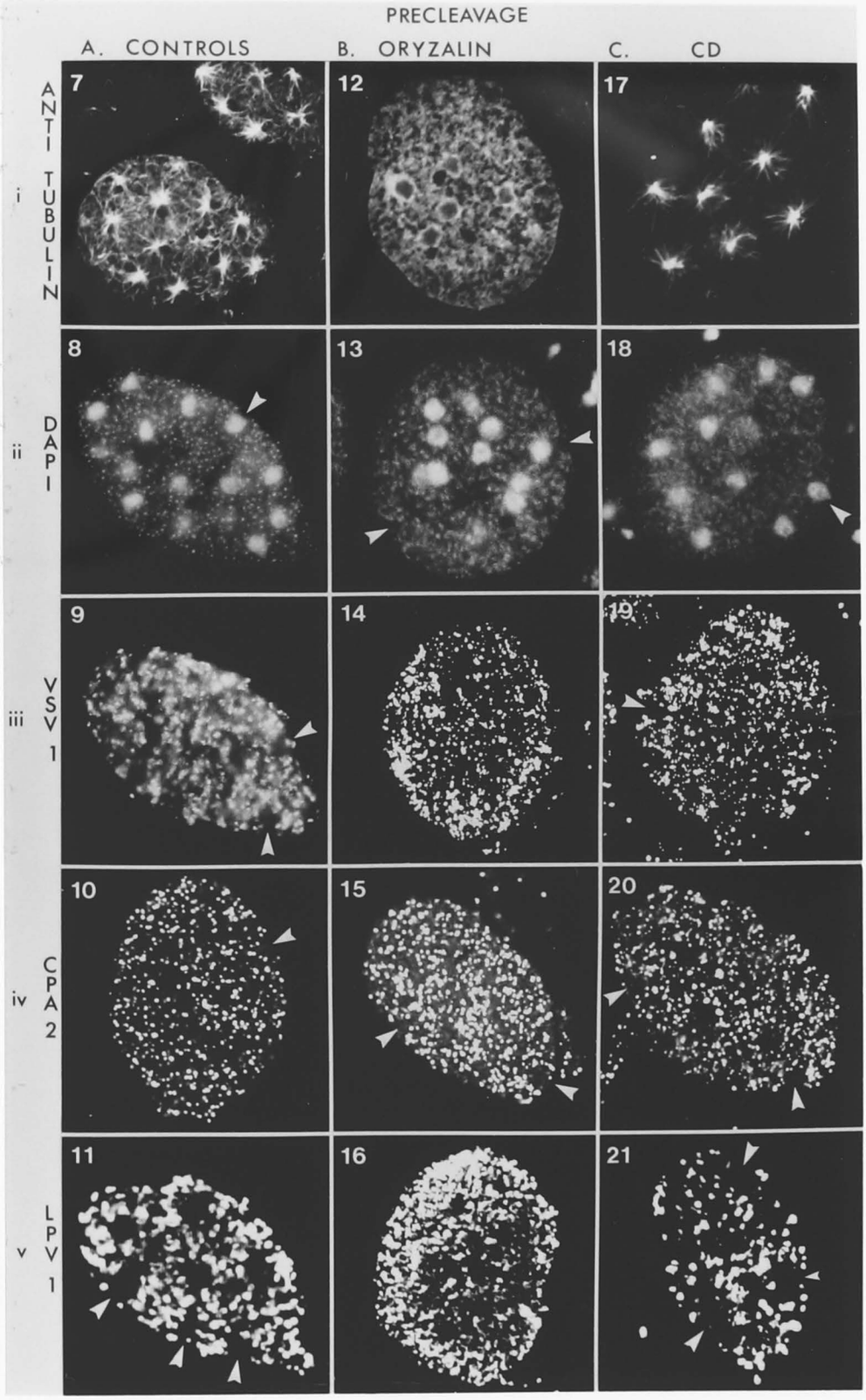
Figs 6.7-6.36 Patterns of fluorescence, either prior to the induction (Columns A-C) or at the completion (Cols D-F) of cleavage, in sporangia of *P. cinnamomi* that had been incubated either in 0.25% DMSO or 1% acetone control treatments (Cols A,D), 1 μM oryzalin (Cols B, E) or 100 μM cytochalasin D (Cols C,F) and then cryosectioned and labelled with sheep antimouse-FITC, following incubations with mAbs anti-β-tubulin (Row i), Vsv-1 (Row iii), Cpa-2 (Row iv) or Lpv-1 (Row v). Row ii shows sporangial cryosections labelled with DAPI. In Cols A-C, arrowheads point to gaps in either mitochondrial (Row ii) or vesicle-associated (Rows iii-v) fluorescence in the sporangial cortex. As shown in Figs 6. 8 and 6.9 (which are from one and the same section), the positions of gaps in vesicle-associated fluorescence regularly correspond to the positions of nuclei; but vesicle-free zones near the poles of nuclei also probably contribute in part to these gaps. In Cols D-F, arrowheads point to large gaps in cortical cleavage plane-associated fluorescence of either mitochondria (Row ii) or peripheral vesicles (Rows iii-v). These gaps are regularly found opposite the narrow nuclear poles (e.g. Figs 6.23 and 6.33). Figs 6.33 and 6.34 show the relationship between Vsv-1 associated fluorescence and narrow nuclear poles in the same section. The arrow in Fig 6.33 indicates a region of the sporangium in which mitochondrial polarization is most evident. All images are derived from confocal microscopy except DAPI stained sections and Fig 6.9. Magnifications Fig 6.7, X570; Fig 6.8, X760; Fig 6.9, X760; Fig 6.10, X690; Fig 6.11, X960; Fig 6.12, X1000; Fig 6.13, X960; Fig 6.14, X720; Fig 6.15, X550; Fig 6.16, X690; Fig 6.17, X880; Fig 6.18, X800; Fig 6.19, X700; Fig 6.20, X760; Fig 6.21, X700; Fig 6.22, X940; Fig 6.23, X710; Fig 6.24, X870; Fig 6.25, X620; Fig 6.26, X720; Fig 6.27, X840; Fig 6.28, X640; Fig 6.29, X860; Fig 6.30, X650; Fig 6.31, X860; Fig 6.32, X900; Fig 6.33, X630; Fig 6.34, X580; Fig 6.35, X880; Fig 6.36, X890.


PRECLEAVAGE
A. CONTROLS
B. ORYZALIN
C. CD
i ANTI TUBULIN
ii DAPI
iii VSV 1
iv CPA 2
v LPV 1
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Figs 6.22-6.36 For explanation, see previous legend.

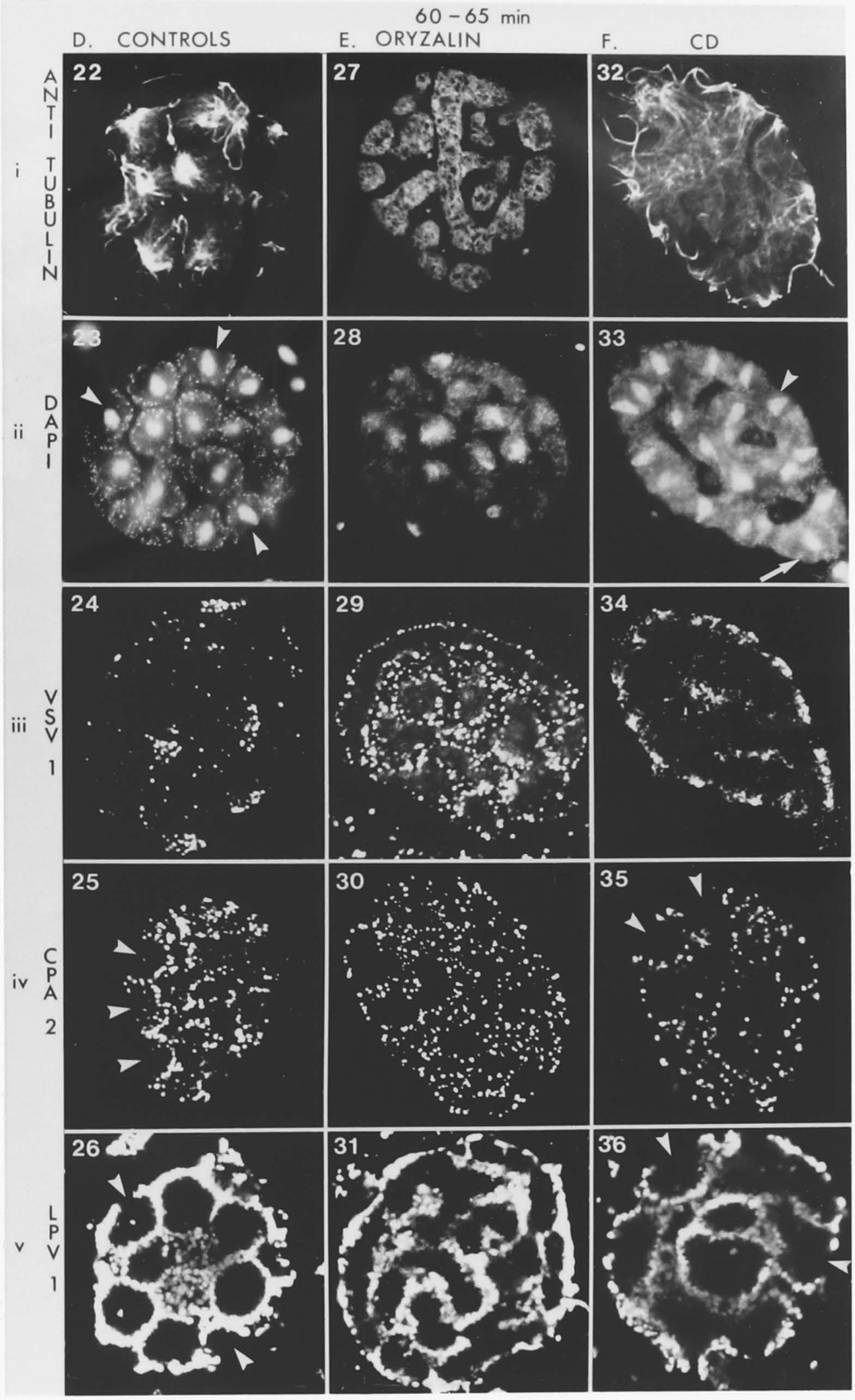
60 – 65 min
D. CONTROLS
E. ORYZALIN
F. CD
i ANTI TUBULIN
ii DAPI
iii VSV 1
iv CPA 2
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Fig 6.37 Cryosection of released contents of a cleaved sporangium incubated in 100 μM CD. DAPI staining shows peripheral polarization of mitochondria, and gaps in mitochondrial fluorescence near nuclear poles (arrowheads) X790.

Figs 6.38-6.39 Cryosections of cleaved sporangia incubated in 1% acetone (Fig 6.38) or 1μM oryzalin (Fig 6.39) and labelled with mAb Cpw-1. Fluorescence is mostly associated with the cleavage planes, and is also evident in association with an apical plug (arrowheads) Fig 6.38 X800; Fig 6.39 X670.

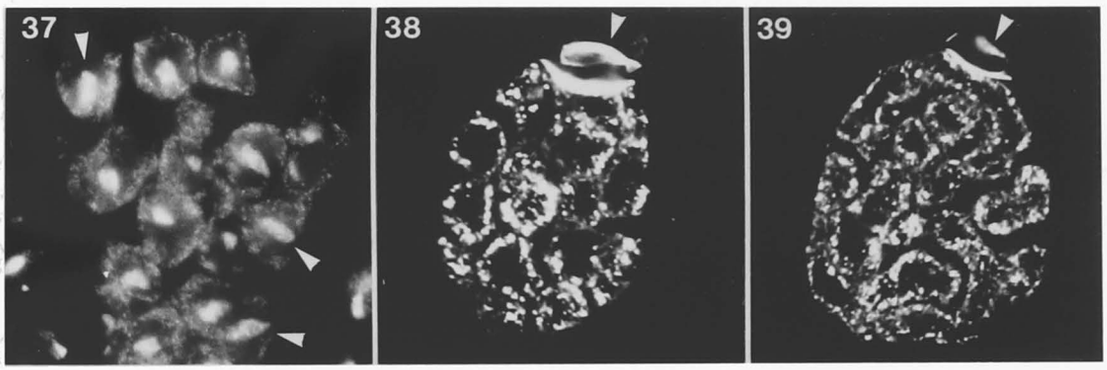
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Fig 6.40 The effects of oryzalin (ii) and cytochalasin D (iii) upon the normal (i) distributions of (a) microtubules; (b) nuclei; (c) mitochondria; (d) ventral vesicles; (e) dorsal vesicles and (f) large peripheral vesicles in cleaved sporangia of *P. cinnamomi*. These diagrams draw upon the results of this study the electron microscopic observations of vesicle and mitochondrial distributions in Chapters 2 and 3. In regions a,c,d-f the typical positions of nuclei are shown in light shading.

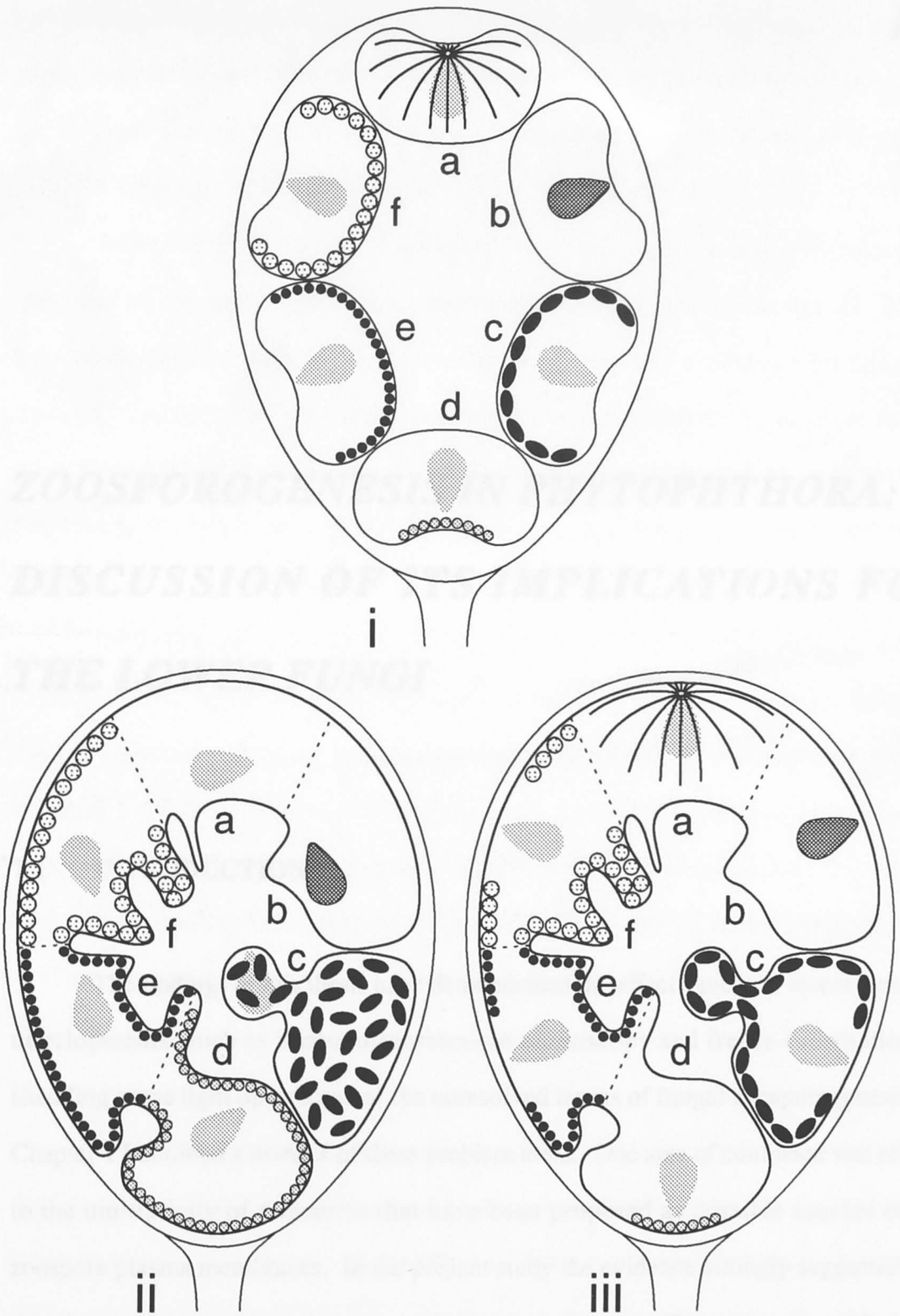

Fig. 6.40

7

ZOOSPOROGENESIS IN PHYTOPHTHORA: A DISCUSSION OF ITS IMPLICATIONS FOR THE LOWER FUNGI

7.1 INTRODUCTION

The findings of this thesis have demonstrated the effectiveness of recent technical developments, such as immunofluorescence microscopy and freeze substitution, in shedding some light upon some of the unresolved issues of fungal zoosporogenesis. In Chapter 1 I outlined a number of these problem areas. One area of confusion was related to the multiplicity of structures that have been proposed as possible sources of the zoospore plasma membranes. In the present study the evidence strongly suggested that the dictyosomes are the sole source of these membranes. There was no evidence to suggest that the membranes of large central vacuoles play any role in cleavage, either by being directly incorporated or recycled, as suggested in previous studies of *Phytophthora* (Hohl & Hamamoto, 1967; Williams & Webster, 1970). These findings parallel, to a large extent, those of Sewall *et al*, (1986) who have questioned the previously proposed

role of non-dictyosomal structures as sources of additional membrane in *Allomyces* zoosporogenesis. It is likely that any further studies of fungal zoosporogenesis which use cleavage element markers and/or freeze substitution will also be able to identify the source of cleavage membranes in a more convincing manner.

A second area of concern involved the variety of processes that have been suggested as being responsible for the formation of the partitioning membranes. It is all too evident that the results of the freeze substitution studies of this thesis must cast doubt over any description of sporangial cleavage that invokes either complete, or partial, alignment of "cleavage vesicles". I have already detailed the important implications of this finding for our understanding of cytokinesis in a wide variety of other eukaryotes (Chapter 3).

Regarding the other two partitioning processes that were outlined in Chapter 1, namely furrowing and direct incorporation of existing membranes, the results argue against the occurrence of the latter mechanism in *Phytophthora*, in contrast to previous evidence (Hohl & Hamamoto, 1967). Thus, in all species of *Phytophthora* studied so far it appears that all the zoospore membranes are formed completely *de novo*. Considering how freeze substitution has clarified this matter in *Phytophthora*, further investigation of this issue in other zoosporic fungi is now called for, especially since direct incorporation of existing membrane surfaces has been described or implied in every other study of fungal zoosporogenesis.

The research of this thesis has also broadened our understanding of several other aspects of zoosporogenesis and provided many results that confirmed the findings of previous studies. To conclude this thesis I would like to address these issues by trying to tie together the abundance of information that was revealed by the various experimental approaches. There is now, in the case of *P. cinnamomi*, arguably more information relating to the structural basis of zoosporogenesis than for any other zoosporic fungus. I believe it is worthwhile to now attempt to integrate this information, and try to evaluate its significance in understanding the process of fungal zoosporogenesis.

7.2 A NEW WAY OF LOOKING AT FUNGAL ZOOSPOROGENESIS

In presenting this synthesis I will be proposing a new way of conceiving the "problem" of zoosporogenesis. Reviews of this topic (e.g. Heath, 1976; Olson *et al*, 1981) have considered that the central issue of zoosporogenesis to be: "how does but one nucleus and the associated spore organelles come to be packaged together?" (Olson *et al*, 1981). Inherent, it would seem, in this question is the assumption that, at some stage, the sporangium is a jumbled collection of nuclei and organelles whose reorganization during the process of zoosporogenesis must be coordinated with the development of the cleavage planes. If one begins with such an assumption then zoosporogenesis appears to be quite a remarkable process, requiring considerable adaptation on the part of the organism. The results of the present study, however, lead one to question this assumption. The sporangium of *P. cinnamomi* is highly organized even before the process of cleavage begins. The future zoospore domains are already demarcated and we can even predict the location of their future ventral and dorsal surfaces. Microtubular arrays with several basic features in common with those of the zoospore are already present. Some organelles, namely the basal bodies and dictyosomes, occupy a similar location within the domains to that which they will have in the zoospores.

These organizational features of the uncleaved sporangium, and especially the existence of microtubule-defined uninucleate domains, could of course be considered as preparatory steps by which the sporangium is gearing up for cleavage. I believe, however, that a more accurate assessment of this organization is that it derives from an interaction between two "influences" unconnected with the requirements of orderly cleavage. These influences are first, the apparent requirement, in non-streaming coenocytic systems, for a constant volume of cytoplasm to be associated with each nucleus (see references and discussion in McNaughton & Goff, 1990); and second, the

likely evolution of many, if not all, lower fungi from a flagellate ancestor (Barr, 1983) and the continued utilization of a zoosporic life-cycle stage by these fungi.

Regarding the constant volume relationship, this has shown to be maintained, in a wide variety of non-fungal systems, by radial arrays of microtubules that emanate, in a symmetrical manner, from around the nucleus (Menzel, 1986; Brown & Lemmon, 1988, 1989; McNaughton & Goff, 1990). It is likely that these microtubules derive from the nuclear envelope (Bakhuisen *et al*, 1985). The volume of cytoplasm that appears to be defined by these arrays is in proportion to the DNA content of the nucleus (Goff, 1987; McNaughton & Goff, 1990) or even, in the case of abnormal mitosis, to the number of "lagging" chromosomes (Brown & Lemmon, 1989). Although there is no real understanding of why this phenomenon occurs it has been proposed that the nuclei of any given system interact effectively only with a certain volume of cytoplasm (Hartmann, 1928: cited in McNaughton & Goff, 1990).

Irregardless of what causes their development, the prevalence of microtubule-based, constant-volume nuclear domains in a wide variety of organisms would lead one to expect that they would also exist in a non-streaming coenocytic system such as a fungal sporangium. This has, of course, been borne out by the present study, and we can extrapolate, given the common reporting of nucleus-based arrays, that this phenomenon is widespread in the zoosporic fungi.

The arrays seen in the lower fungi, however, differ in two respects from those seen in other non-fungal coenocytic systems. They are asymmetrically located with respect to the nucleus, and, rather than deriving from the nuclear envelope, they emanate from material associated with the nucleus-associated basal bodies or centrioles (Heath & Greenwood, 1971). These features are also almost universally present in the microtubule rootlet systems of lower fungal zoospores (Heath, 1976; Olson & Lange, 1983). Since the lower fungi are believed to have evolved from flagellate ancestors (Barr, 1983) it seems likely that the microtubular arrays seen in the sporangium are in fact probably adaptations of "zoosporic" arrays, and not the other way around. The main motive

influence for the adaptation is likely to be the constant volume requirement, and not the events of cleavage. The constant volume requirement is also probably responsible for the observations, in this study, of regularly spaced hyphal nuclei. This phenomenon also appeared to involve nucleus-based arrays of microtubules.

The main influence of zoosporogenesis itself upon the organization of sporangial structures probably derives from the fact that it represents the persistence of the flagellate stage in the life cycle of the lower fungi. As such there is a pressure for the retention of the nucleus-associated centrioles, a constant feature of lower fungal hyphal nuclei (Heath, 1976). In eukaryotic tissues which do not produce motile cells, centrioles are not present (Raven *et al*, 1981).

From the above discussion it is evident that far from being a remarkable achievement, the production of equal-sized uninucleate flagellate zoospores is in fact almost inevitable. Indeed it would involve considerable effort on the part of the fungus to produce anything else. Of course, zoospores are much more than just flagellated, equal-sized, nucleated cells, and so the *true* problem of zoosporogenesis now emerges: given that zoospores are going to be formed by "cutting out" those portions of cytoplasm defined by the constant-volume microtubule arrays, how can these arrays be most efficiently utilized during zoosporogenesis?

The present study indicates that considerable use *is* made of the microtubular arrays in the process of cleavage and organelle positioning. The most elegant feature of the cleavage process in *Phytophthora* is the degree of "co-operation" between sporangial arrays. Adjacent domains do not cleave independently but utilise a common furrow. As proposed in Chapter 5, this may involve a phragmoplast-like system in which Golgi-derived membrane precursors are transported along the microtubules of opposing arrays. Alternatively, cleavage furrows might preferentially extend along the junctions of opposing arrays because microtubular density is lowest here. It is also possible that microtubules regulate a microfilament system which is itself the direct regulator of cleavage. Such a model is consistent with the observed effects upon cleavage of the

anti-microfilament drug, cytochalasin D (Chapter 6, and references therein). A particularly interesting aspect of cleavage was that any contacts between microtubules and the developing cleavage planes would be of two different types due the asymmetry of the microtubule arrays (Chapter 5). At the zoospore surface that develops opposite the focus of the array, any contacts would be lateral, whereas along the future dorsal surface only end-on interactions could occur. As discussed in Chapter 5, this may result in different mechanisms of cleavage regulation for these two surfaces.

While the asymmetrical nature of the microtubule arrays is perhaps a complicating factor for the process of cleavage, it appears to have been used to advantage in the regulation of organelle positioning. Recent studies of the zoospores of *Saprolegnia* and *P. cinnamomi* (Lehnen & Powell, 1989; Hardham & Gubler, 1990) have demonstrated that the polarized distribution of peripheral vesicles along either the ventral or dorsal surface of the zoospore has important consequences for the process of encystment. The present study indicates that sporangial microtubular arrays are involved in these polarizations. Moreover, both of the models proposed (Chapter 6) to effect these polarizations, namely localization of membrane receptors and directed transport, could take advantage of the different orientations or types of contact that the microtubules have with the ventral and dorsal surfaces.

P. cinnamomi, and no doubt other zoosporic fungi, use nucleus-associated microtubular arrays for a variety of purposes. In the zoospore they have been proposed to anchor the flagella and to determine cell shape (Heath *et al*, 1982). In the coenocytic stages they not only space the nuclei apart but assist in many ways in the transition back to the flagellate phase. The specific shape of the array shows considerable flexibility in adapting to the form most suited to the life-cycle stage in which it is present. This adaptability and the multiplicility of uses made of this structure are remarkable examples of cellular economy.

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