A cytological and ultrastructural study on the maturation and germination of oospores of *Plasmopara viticola* from overwintering vine leaves

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Observations on cytological and ultrastructural changes in *Plasmopara viticola* oospores were carried out during the overwintering period. Three types of oospores were observed. Type I, characterized by a thin inner oospore wall (IOW), large lipid globules and two nuclei, was recovered only in samples collected in October. These oospores were considered to be immature. Maturation occurred during November and involved a noticeable increase in thickness of the IOW, fusion of nuclei, formation of an ooplast and break up of large lipid globules into smaller ones (type II oospores). A few oospores (type III) showed abnormal organization with very large lipid globules and less frequently discernible nuclei. IOW solubilization, dissolution of the ooplast and lipid globules and nuclear division were the first detectable events during oospore germination. Germinating oospores produce a germ tube which was terminated by a sporangium. In its young stage, the sporangium had a thick wall and an unusual multi-layered membrane. During this phase, nuclear divisions took place in the sporangium. While sporangium development progressed, the ribosome density in the cytoplasm decreased and mitochondria, initially roundish with evident cristae, became their usual tubular profile. The plasma membrane had a typical structure and storage organelles, such as finger print vacuoles and lipid globules, became more numerous in the cytoplasm. Larger vacuoles contained the flagella of differentiating zoospores.

Plasmopara viticola (Berk. & M. A. Curtis) Berl. & De Toni, the causal agent of grapevine downy mildew, forms oospores as a result of sexual reproduction. In temperate climates, oospores, differentiated in infected leaf tissues, are the only surviving structures of the pathogen during the winter. The inoculum for new infections during the spring is, therefore, provided exclusively by oospore germination.

Due to the important epidemiological role of *P. viticola* oospores, their germination dynamics have been investigated throughout the overwintering period in different grapevine growing areas (Arens, 1929; Zachos, 1959; Burruano & Ciofalo, 1990; Vavassori, 1994). These studies showed that no germination occurs until November. Germination rates then increase and attain their highest value from February to April. Differences in germination dynamics observed from year to year and from various viticultural areas have been attributed to the climatic conditions occurring during the overwintering period (Ronzon, 1987; Serra & Borgo, 1995). In particular it seems that abundant rainfall and, to a lesser extent, low temperatures, have a positive effect on oospores germination. Since germination assays do not discriminate between immature and mature non-germinating oospores, it is still unknown if these climatic factors primarily influence oospore maturation or the germination of already mature oospores.

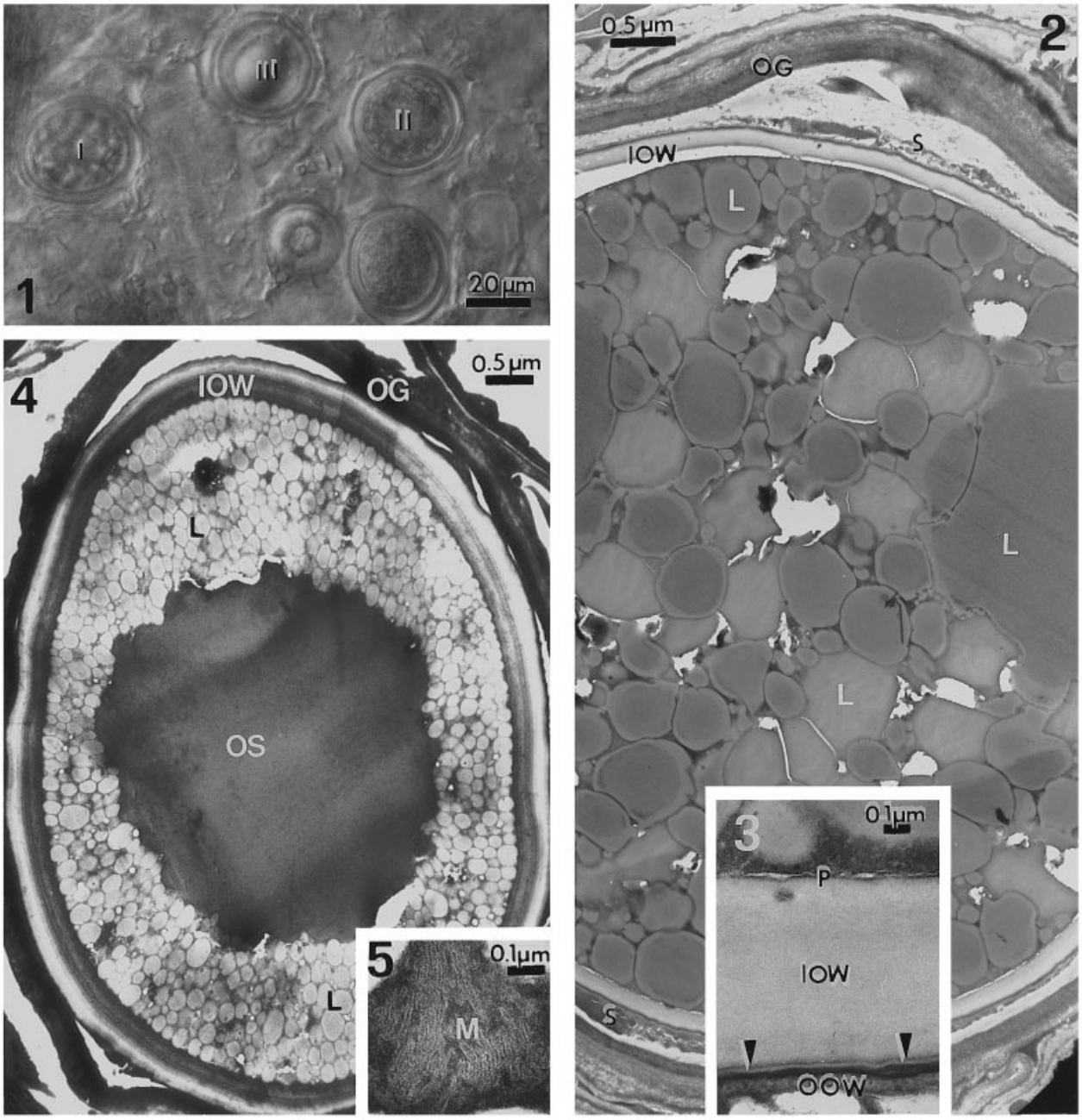
Cytological and ultrastructural studies may provide useful information on oospore development during overwintering. Unfortunately the well known difficulties encountered in processing oospores for cytological and ultrastructural observations, arising from the poor penetration of fixatives and resin through the oospore walls (Hemmes & Bartnicki-Garcia, 1975; Beakes, El-Hamalawi & Erwin, 1986), have hampered such investigations. Furthermore, since *P. viticola* does not grow on synthetic media, observations must be carried out on oospores naturally differentiated in grapevine leaves and overwintered in vineyards.

Preliminary investigations demonstrated acceptable oospore preservation when using recently introduced low viscosity resins (Vercesi, Tornaghi & Faoro, 1991). This provided the basis for an extensive cytological and ultrastructural examination of naturally occurring oospores of *P. viticola*, described here, which was carried out during the overwintering period, from their differentiation until the sporangium formation, with the aim of identifying the main steps in oospore development.

MATERIALS AND METHODS

Source of overwintering and germinating P. viticola *oospores*

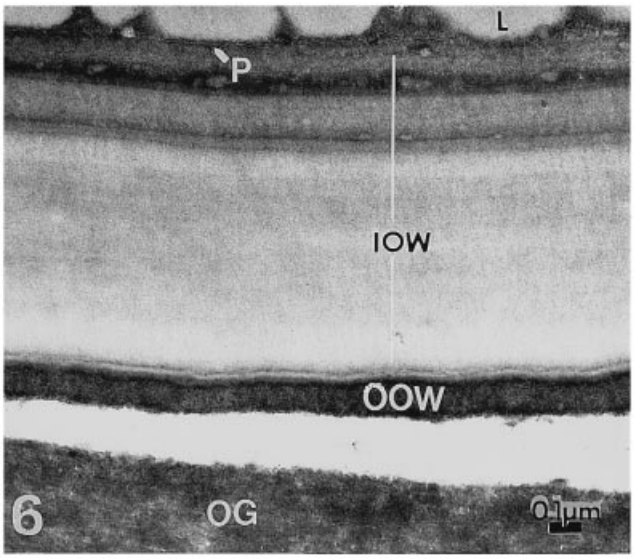
From October 1992 to May 1995, grapevine leaves showing mosaic symptoms of downy mildew infection were collected from a naturally infected vineyard of cv. Corvina located near



Figs 1–7. *P. viticola* oospores sampled in October. **Fig. 1.** Differential interference contrast microscopy of a cleared leaf fragment showing three oospore types (I, II, III). **Figs 2–7.** Median ultra-thin sections of the above oospore types. **Fig. 2.** Type I: lipid globules (L) of different sizes and electron-densities fill the whole cell lumen; the periplasmic space (S) between the oogonium wall (OG) and the outer oospore wall (OOW) contains electron-dense material; the detail of the oospore wall is visible in Fig. 3. **Fig. 3.** Type I oospore wall is formed by a thick inner layer (IOW) separated from a thin outer layer (OOW) by a third layer (arrowheads) which is probably the oosphere membrane (P = plasma membrane). **Fig. 4.** Type II: small and uniform lipid globules (L) surround a very electron-dense ooplast (OS). IOW is very thick and formed by a series of intergrading layers with different electron-density. **Fig. 5.** A quiescent mitochondrion (M) is visible among lipid globules of the oospore in Fig. 4.

Verona, Italy. Infected leaves were examined by light microscopy in order to excise the zones containing significant numbers of oospores. Twenty-four nylon (pore size = 100 µm^2) bags per year were each filled with ten oospore rich leaf fragments. Nylon was chosen since it would not interfere with environmental influences on the oospores and allowed rapid and easy recovery of leaf fragments from the field site. Oospore overwintering was carried out by laying the nylon bags side by side on the soil surface of the vineyard where the leaf samples were collected. Each month, from October to May, a nylon bag was randomly removed; some leaf fragments were processed directly for microscopy and some others utilized for oospore isolation. Oospore isolation was carried out by finely grinding the leaf fragments as described by Ronzon (1987).

In order to follow the different germination stages, from production of the germination tube onwards, 400 isolated oospores were incubated in Petri dishes on water agar (Noble



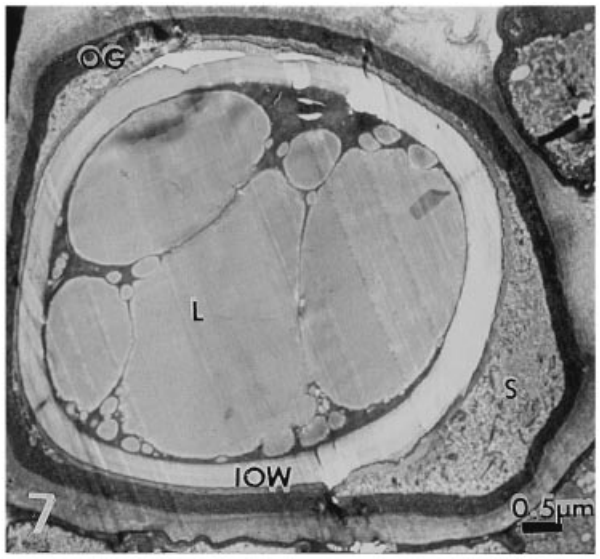


Fig. 6. Detail of the oospore walls in Fig. 4 (P = plasma membrane). **Fig. 7.** Type III: a few, very large lipid globules (L) almost completely occupy the cell lumen. The IOW is very thick but rather homogeneous.

Agar, Difco, 1% w/v) at 20 °C for 14 d and examined daily by light microscopy.

Light microscopy

Some leaf fragments were cleared with commercial sodium hypochloride (5% active sodium) and observed by differential interference contrast microscopy, according to Beakes *et al.* (1986), to assess the type of protoplasm organization in the oospores. Nuclear staining was carried out on isolated oospores, either immediately after isolation or at different incubation times (1–14 d) on water agar. The oospores were fixed with 30% ethanol for 5 min, washed repeatedly with distilled H_2O, treated for 15 min with 3 μg ml^{-1} DAPI fluorochrome in 0·05 M Tris HCl buffer, pH 7 (Brunk, Jones & James, 1979) and examined under epifluorescence. Semi-thin sections (1–2 μm) processed for TEM as described in the next section, but not treated with OsO_4, were stained with 1% toluidine blue in 0·5% Na_2CO_3 at 60° for a few seconds or 0·3% Sudan black B in 70% ethanol for 30 min (Bronner, 1975).

Electron microscopy

Overwintering oospores were processed for TEM directly within the leaf fragments, while germinating oospores were fixed after removal from the germination medium up to 48 h after the onset of germination (first detection of germination tube). Leaf fragments were fixed following different procedures; (i) 3% glutaraldehyde in 0·1 M phosphate buffer (pH 7) at room temperature for at least 4 h; (ii) 3% glutaraldehyde in 0·1 M phosphate buffer (pH 7), at 60° for 2 h followed by 2 h at room temperature, with or without vacuum using a water pump (Fineran, 1994) (iii) a mixture of 2·5% glutaraldehyde and 2·5% formaldehyde in 0·1 M cacodylate buffer (pH 7) under vacuum, at room temperature for 4–48 h; (iv) 1% $KMnO_4$ in 0·1 M cacodylate buffer (pH 7) for at least 48 h under vacuum.

Germinating oospores were covered with 2% Bacto-agar (Difco). After agar solidification, 1 mm^3 blocks containing a single oospore were cut out of the agar and fixed using procedure (iii), but without vacuum, as these structures are much more easily penetrated by fixatives.

In most cases, samples were post-fixed in 1% OsO_4 for 2–4 h. Samples were dehydrated in ethanol and embedded in either Epon-Araldite, L. R. White (London Resin Company, U.K.) and Bio-Acryl resin (BioCell, U.K.), infiltrated for 12–48 h under vacuum and polymerized for 24 h at 60°. Ultra-thin sections were cut, stained with 0·5% aqueous uranyl acetate and lead citrate and examined under a Jeol 100-SX electron microscope.

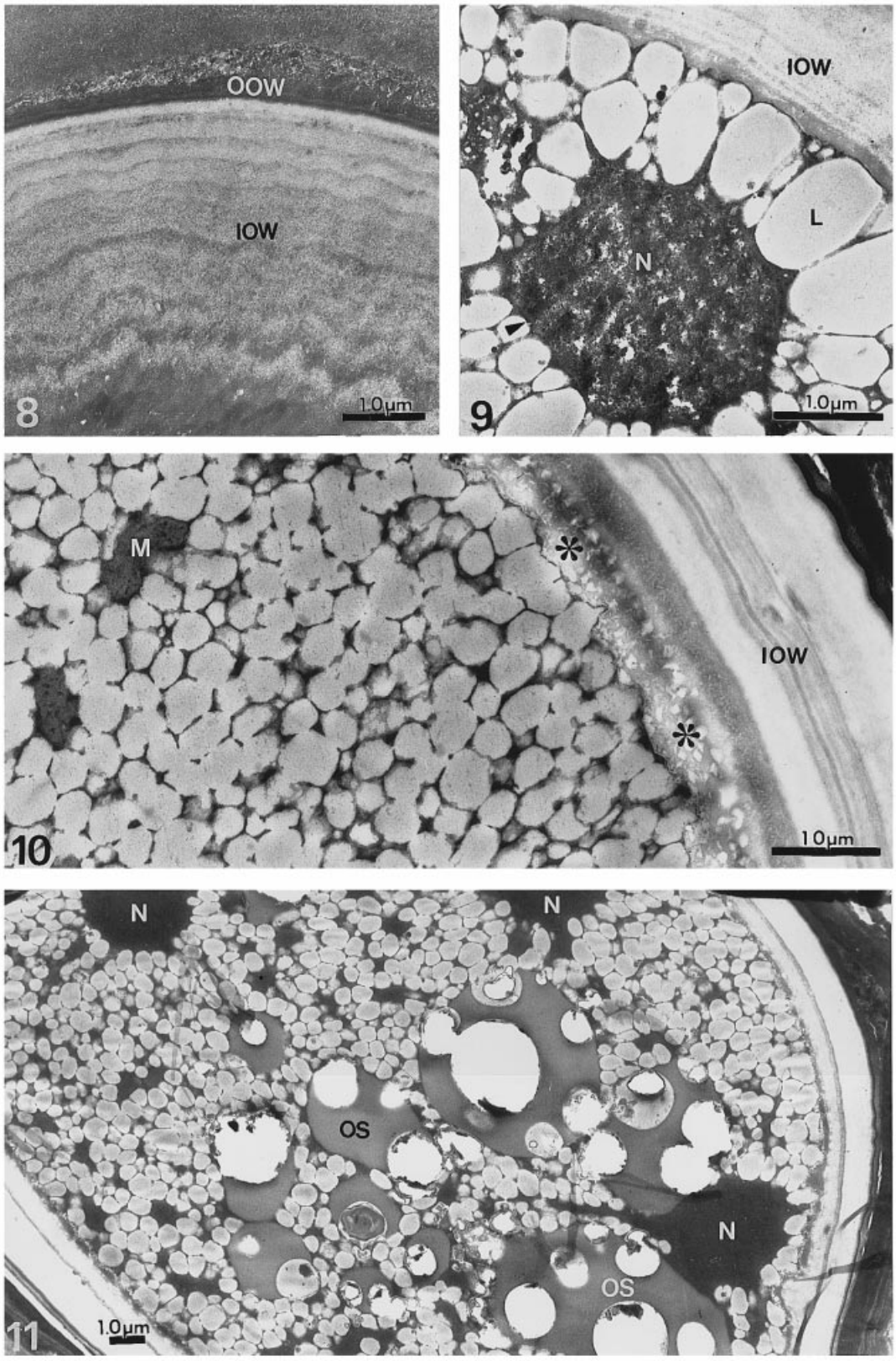
RESULTS

The best results were achieved when samples were fixed in a mixture of 2·5% paraformaldehyde and 2·5% glutaraldehyde in 0·1 M cacodylate buffer for at least 24 h under a slight vacuum at room temperature and post-fixed in 1% OsO_4 in the same buffer for 4 h. Thus all the figures, unless otherwise stated, are from samples processed in this way. Fixation carried out at 60° resulted in easier recognition of dehydrated organelles, particularly mitochondria. This method is not, however, generally recommended as it induced significant artefacts such as swelling of the mitochondria.

$KMnO_4$ fixation did not result in acceptable preservation of oospore protoplasm. As cell wall layers were more easily distinguishable following this treatment, however, this was routinely utilized in addition to aldehyde fixation. L. R. White resin proved to be the most suitable resin for embedding overwintering oospores, infiltrated over a period of 48 h under vacuum at 4° and cured for 24 h at 60°. No significant problems were encountered in fixing and embedding germination oospores.

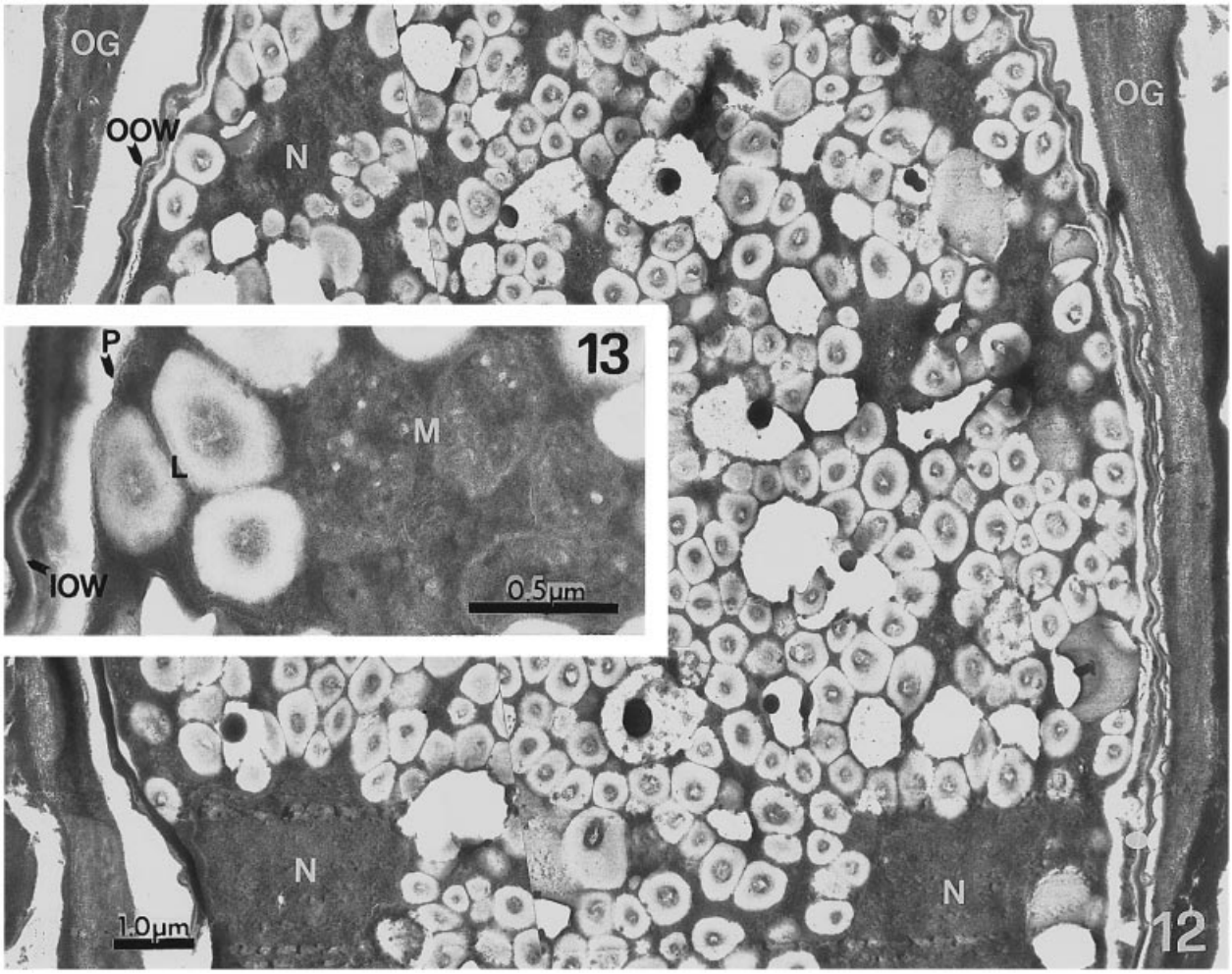
Overwintering oospores

In the samples processed in October, three different types of oospore organization were recognizable by light microscopy



Figs 8–9. Ultra-thin sections of *P. viticola* oospores collected in November. **Fig. 8.** Detail of the inner (IOW) and outer (OOW) oospore walls in a sample fixed with $KMnO_4$. The IOW is formed by intergrading numerous layers, some of which are discretely corrugated, probably because of inadequate fixation. **Fig. 9.** A nucleus (N), recognizable by the negatively-stained nuclear cisternae (arrowhead), among lipid globules (L) at cell periphery.

Figs 10–11. Ultrathin sections of *P. viticola* oospores collected in May. **Fig. 10.** An oospore showing incipient germination. The IOW inner layers (asterisks) are being digested, while mitochondria (M) are now easily recognizable. **Fig. 11.** Another oospore in incipient germination: numerous nuclei (N) appear at the cell periphery and the fragmented ooplast (OS) shows many already digested areas.



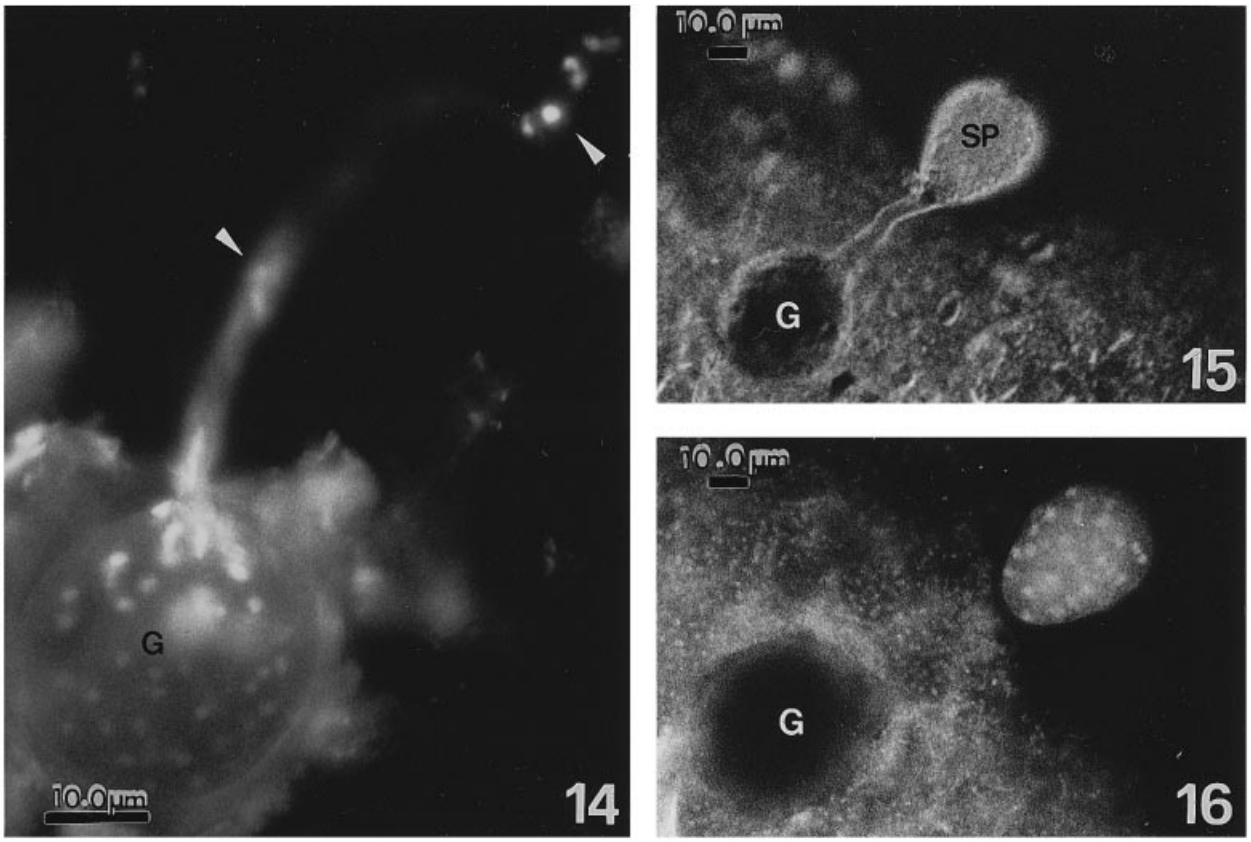
Figs 12, 13. Ultra-thin section of a germinated oospore when the germ tube had just emerged. **Fig. 12.** The outer wall (OOW) and the oogonium wall (OG) are unchanged. Numerous nuclei (N) and mitochondria (M) are visible among lipid globules. **Fig. 13.** Enlarged area of Fig. 12 showing that the inner oospore wall (IOW) is reduced to a very thin layer and that lipid globules (L) are partially digested; (P = plasmalemma).

(Fig. 1). Type I oospores (Fig. 2), which were most frequent, were characterised by their relatively thin inner oospore walls and large lipid globules distributed throughout the cytoplasm. A thick (0·5 µm) fibrillar oogonium wall (OG) was still present and was separated from the oospore by a periplasmic space, containing electron dense material. The oospore wall was divided into two layers, the outer oospore wall (OOW) and the inner oospore wall (IOW) (Beakes & Bartnicki-Garcia, 1989). The OOW was 70–100 nm thick and electron-dense. The IOW was electron-transparent and thicker (0·6–1 µm). Between the IOW and OOW, there is a layer, 30–40 nm thick, which was very electron-dense and often slightly undulating (Fig. 3). The protoplasm was surrounded by the plasma membrane, usually negatively stained, and it was almost entirely occupied by vacuoles of different size and of two different electron-densities. Sudan black B staining (not shown) demonstrated that they were mainly filled with lipids. The dense cytoplasm was confined to the reduced spaces among the vacuoles and did not contain clearly defined organelles.

In the oospores of type II (Fig. 4), whilst no changes are seen in the OG and in OOW, a series of intergrading layers appeared in the IOW adjacent the oospore cytoplasm, bringing its final thickness up to 3–3·5 µm (Fig. 6). The protoplasm of such thick-walled oospores was characterised by many small lipid droplets, usually localized at the periphery of the cell, surrounding an homogeneous and electron-dense central zone, similar to the already described ooplast (Hemmes, 1983). Multilayered vesicles were often observed in the electron-dense cytoplasmic spaces amongst the lipid globules; some of which probably represent quiescent mitochondria (Fig. 5).

A third small group of oospores, type III (Fig. 7), was characterized by the presence of 1–5 very large lipid globules, almost filling the cell lumen and probably deriving from the coalescence of the smaller ones. They closely resembled the abnormal oospores of *Phytophthora megasperma* proved to be non- or scarcely viable by Beakes *et al.* (1986).

In samples collected from November to April, only oospores belonging to types II and III were observed, in the ratio of 7–10 to 1 depending on the year of sampling. No further development was observed in either oospore type. In particular, the thickness of their walls remained about the same. In the type II oospores, however, the numerous layers forming the IOW became more visible. In $KMnO_4$ fixed oospores, up to 12–15 lamellae wee detected (Fig. 8).



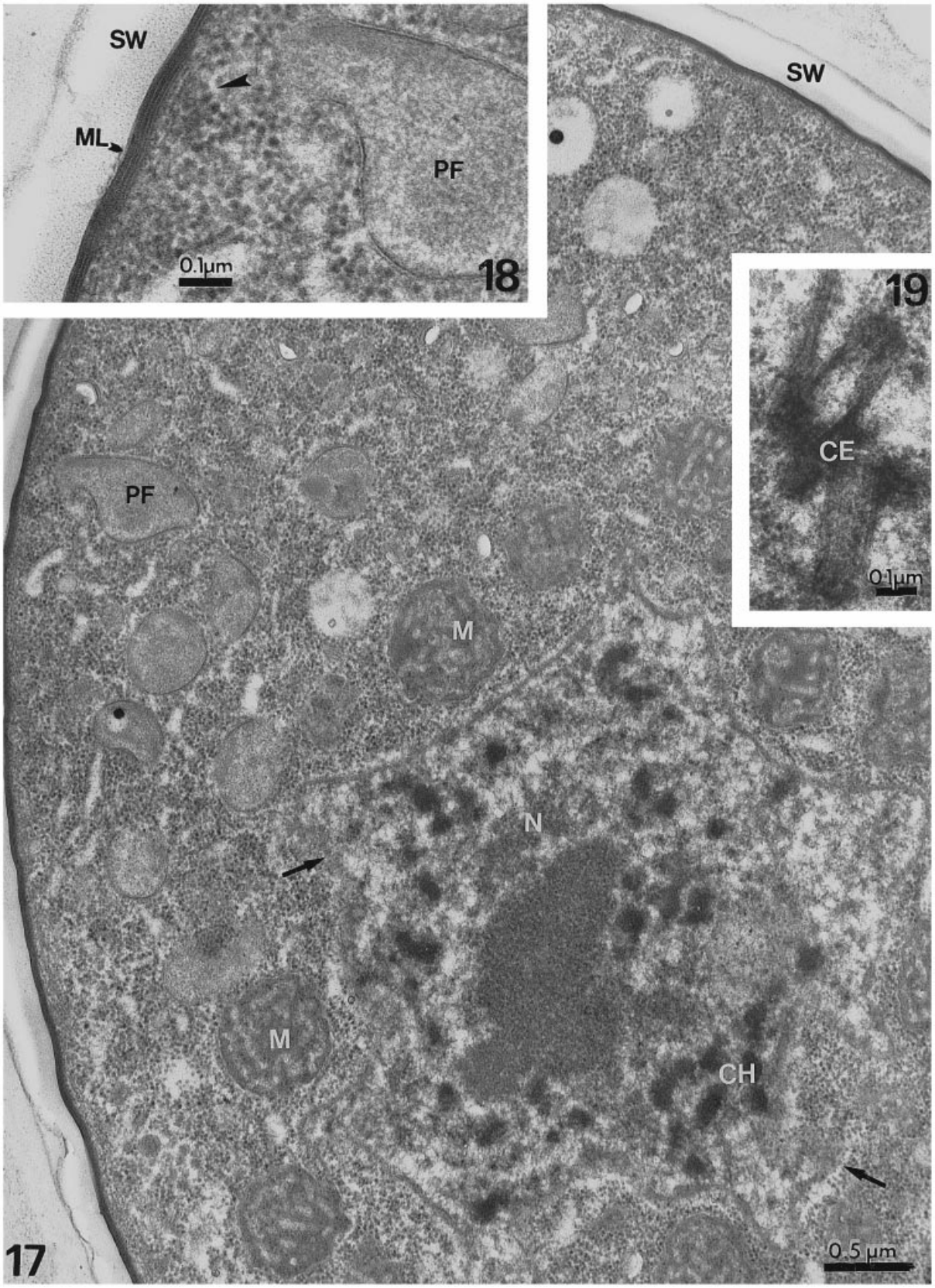
Figs 14–16. Light microscopy of germinated oospores (G). **Fig. 14.** Fluorescence microscopy of an oospore (G) stained with DAPI soon after the appearance of the germ tube: the bright spots are nuclei, often coupled possibly as the result of a recent division; some nuclei are possibly migrating through the germ tube (arrowheads). **Fig. 15.** Interference contrast microscopy of a germinated oospore showing a mature sporangium (SP). **Fig. 16.** The same sporangium as in Fig. 15 stained with DAPI and observed with UV light and at the same stage as Fig. 20.

Under TEM nuclei, usually localized at the cell periphery, were rarely visible in overwintering oospores (Fig. 9). Observations of a large number of oospores stained with the nuclear fluorochrome DAPI revealed the presence of two nuclei (probably prefusion, haploid nuclei) in type I oospores, while only a single nucleus was found in type II. In type III oospores, the nucleus was frequently not detected, although sometimes a diffuse fluorescence was observed in the cytoplasm, possibly due to the breakdown of the nuclear membrane.

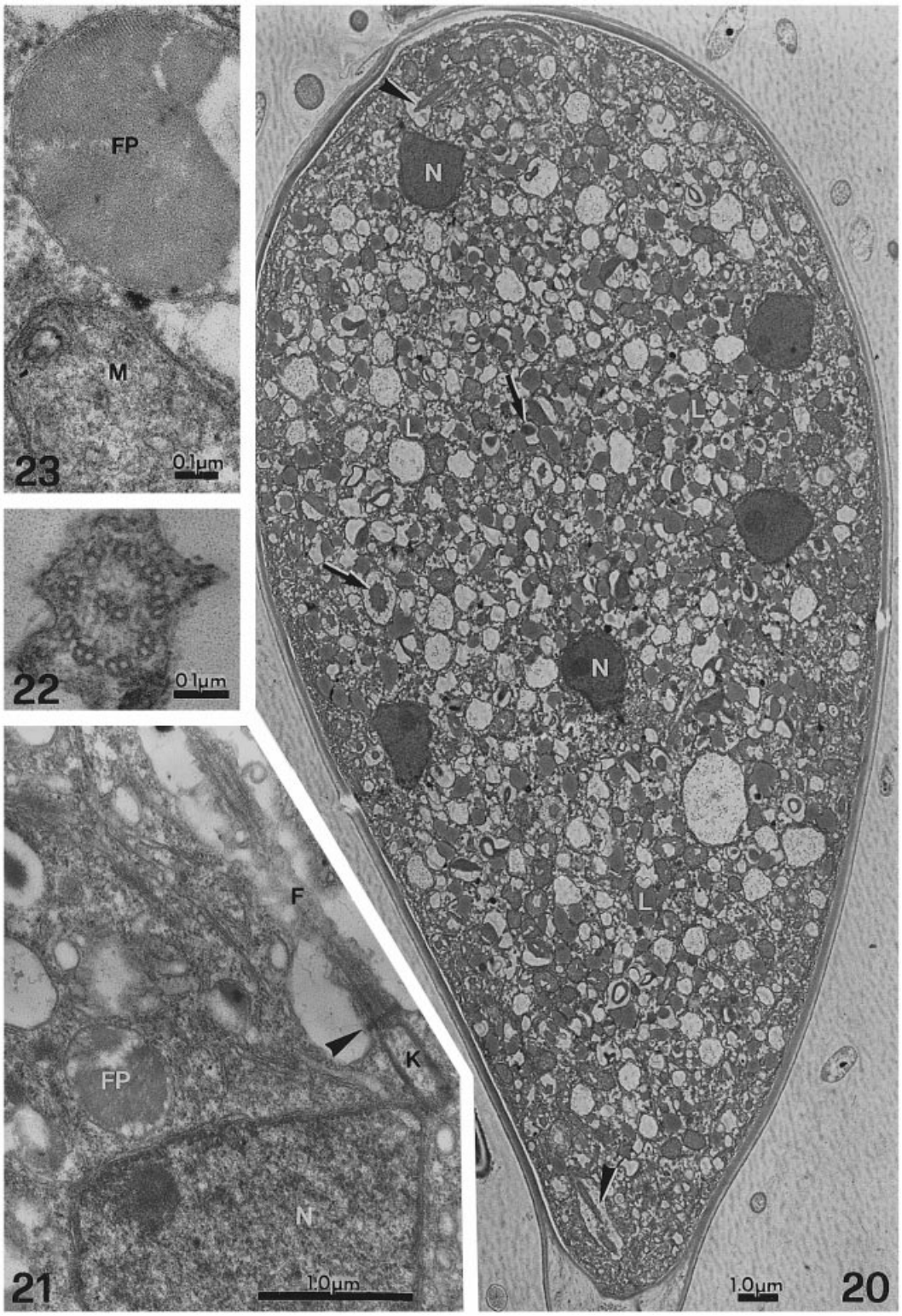
Germinating oospores

In May, a small number of oospores type II ultrastructure showed signs of incipient lysis of the IOW zone near the protoplasm (Fig. 10) indicative of the onset of germination. Moreover, the whole IOW appeared more electron-transparent and the number of visible layers apparently decreased. At the same time, two or more nuclei could be seen in the spore indicating nuclear divisions (Fig. 11). Numerous mitochondria were present in the interstices among the lipid globules (Fig. 10). The ooplast vacuole contents were less electron-dense and contained prominent electron-transparent spaces. As soon as the germ tube emerged, the overall ultrastructure of the oospore changed markedly. The thickness of IOW rapidly became greatly reduced and in some cases this wall layer had almost completely disappeared. The OG did not show appreciable change in comparison with resting oospores while the OOW appeared very crinkled (Fig. 12). In the oospore cytoplasm the first functional organelles, notably mitochondria, became evident (Fig. 13). The lipid globules became more electron-transparent at their periphery, with a more electron-dense central core (Fig. 12). This, together with loss of IOW was indicative of utilization of storage material. Numerous nuclei were observed in germinating oospores and they were readily detected with DAPI staining while probably migrating through the germ tube (Fig. 14). Germinating oospores always produced a germ tube (29–56 µm long) at the end of which a pyriform sporangium (27 × 45 µm) soon appeared (Figs 15 and 16).

Ten to twelve hours after germ tube emergence, the sporangium wall was almost electron-transparent and rather thick (Fig. 17). The protoplasm was surrounded by a multilayered layer underlying the sporangial cell wall. The cytoplasm contained many ribosomes, very often organized in polysome structures, suggesting enhanced synthetizing activity (Fig. 18). Vesicles, surrounded by a well-defined membrane, were distributed in the cytoplasm and showed a granular content (Fig. 18). At this stage, nuclear divisions were readily observed in the sporangium (Fig. 16). Pairs of centrioles, aligned end to end, were detected in the cytoplasm (Fig. 19). A very large number of mitochondria with a rather



Figs 17–19. Ultra-thin section of a very young sporangium. **Fig. 17.** At this stage the sporangium shows a thick wall (SW), a dense cytoplasm with many ribosomes, vesicles with a granular content (PF), and a multi-layered membrane. Many mitochondria (M) with tubular cristae surround a large nucleus with a partially dissolved membrane (arrows) and chromosome-like structures (CH). **Fig. 18.** Enlarged area of Fig. 17 showing the multi-layered membrane (ML), ribosomes aggregated in polysomes (arrowhead) and PF. **Fig. 19.** An enlarged area of Fig. 17 showing a pair of centrioles (CE) close to the nucleus.



Figs 20–23. Ultra-thin sections of an almost mature sporangium in which zoospore differentiation has begun. **Fig. 20.** A median longitudinal section through the whole sporangium which appears vacuolated; some of these vacuoles have a granular content (arrows), many other are filled with lipid globules (L) or with a lamellar material (finger print vacuoles, FP, see a detail in Fig. 23). Other vacuoles contain zoospore flagella (arrowheads) which are connected to the numerous pyriform nuclei (N). **Fig. 21**. Enlargement of a pyriform nucleus (N) with a kinetosome (K) attached to its apex; the kinetosome is connected to the flagellum (F) through the transitional plates (arrowhead) and is contained in a vacuole. Another vacuole (FP) contains an aggregate of lamellar material. **Fig. 22.** Cross section of a flagellum showing the 9 + 2 arrangement of the internal filaments. **Fig. 23.** Enlargement of a mitochondrion portion (M) and of a finger print vacuole (FP) containing lamellar material which is probably storage polysaccharides.

unusual round shape were localized very close to the nuclei and were characterized by prominent cristae (Fig. 17).

In a later stage of sporangium development, its wall became thinner and the multi-layered membrane was replaced by a typical plasma membrane. The nuclei showed an interphase status (Fig. 20). Staining with DAPI revealed that some of them appeared coupled. Close to the apex of the pyriform nuclei, kinetosomes, originating from the centrioles, were connected to the zoospore flagella (Fig. 21). The flagella in cross section appeared to consist of a 9 + 2 arrangement of internal filaments (Fig. 22) and were contained in a vacuole (Fig. 20). Mitochondria assumed their usual elongate tubular profile and the cristae were less evident. Also in these developing sporangia the ribosome density decreased and the cytoplasm appeared much more vacuolated. Other typical structures could be observed; lipid globules, sometimes with a dark core (Fig. 20) and finger print vacuoles (Fig. 23), which are thought to contain storage polysaccharides (Hemmes, 1983).

A septum (not shown) separated the mature sporangium from the germination tube. At this stage of the sporangium differentiation, the oospore appeared completely empty and recognizable only by the OG and OOW. The IOW and the cytoplasm were completely utilised for the formation of the sporangium and only a few residues of the previous structure remained in the oospore.

Unfortunately it was not possible to detect sporangium cleavage, probably because of the rapidity of its occurrence.

DISCUSSION

Difficulties encountered in investigating oospore ultrastructure have been pointed out by many authors and concern all the steps involved in sample preparation, particularly embedding (Hemmes & Bartnicki-Garcia, 1975; Beakes, 1980*a*, *b*; Beakes, 1981; Hemmes & Stasz, 1984). Hydrophilic low viscosity methacrylate resins such as L. R. White seem to be the best embedding media for the oospores of *P. viticola*. Epon-Araldite and Bio-Acryl, which is a mixture of epoxy and methacrylate resins, do not easily penetrate the wall layers even when infiltration is conducted under a moderate vacuum. Furthermore, use of L. R. White, instead of other uv-polymerizing methacrylate resins, allowed post fixation with OsO_4, which is essential for good preservation of the oospores.

Ultrastructural studies on the sexual structures of Oomycota have been carried out almost exclusively on species able to grow on synthetic media. The only exceptions being the studies on *Albugo candida*, an obligate parasite of *Brassica campestris* (Tewari & Skoropad, 1977) and *Bremia lactucae*, the causal agent of lettuce downy mildew (Sargent, Ingram & Tommerup, 1977). Other obligate biotrophs belonging to the Oomycota have been surprisingly neglected, in spite of the important role played by oospores in assuring survival of the pathogen (Populer, 1981).

Recently it has been pointed out that systematics of downy mildews needs to be defined on the basis of more accurate and precise criteria (Hall, 1996) but unfortunately numerous important species are still inadequately described (Dick, 1995). The ultrastructural approach has been recommended and seems to be particularly interesting both for phylogenetic and systematic purposes. The present study is the first report on the oospore ultrastructure of the grapevine downy mildew fungus, *P. viticola*, belonging to a genus with very poor information as to its morphological characteristics.

Wall layers of *P. viticola* oospores are more similar to those of Pythiaceae (Hemmes, 1983; Hemmes & Stasz, 1984; Beakes & Bartnicki-Garcia, 1989) than to the oospore wall complex of the other already described obligate parasites *A. candida* and *B. lactucae* (Tewari & Skoropad, 1977; Sargent *et al.*, 1977). Furthermore, the IOW of *P. viticola* oospores appears sometimes thicker and less homogeneous, being formed by numerous layers, characterized by different electron densities. Such wall organization has not been observed in other oomycetes which show a rather uniform appearance of the IOW, with the exception of *S. ferax* and *S. furcata* (Beakes & Gay, 1978; Beakes, 1980*b*). In these two species, however, the inner part of IOW is characterized by the presence of patches of electron-dense material.

The multi-layered structure of IOW in *P. viticola* might be related to the *in situ* production of the oospores. In fact, almost all investigations on the oospore ultrastructure have been carried out on samples differentiated on culture media or inoculated hosts under controlled conditions (Sargent *et al.*, 1977; Beakes, 1980*b*). By contrast, *P. viticola* oospores were formed under fluctuating conditions of temperature and wetness and it is possible that sudden environmental changes during differentiation induce a less homogenous development of the IOW. Also the genetic variation in the wild *P. viticola* population may have been greater than in the population maintained under controlled conditions.

Dense cytoplasmic appearance, the presence of membrane delimited components among the lipid globules and the ooplast in the centre of the cell are common characteristics of quiescent oospores (Hemmes, 1983). The inactive status of the oospore cytoplasm is confirmed by the erratic observation of constitutive organelles such as quiescent mitochondria (Beakes *et al.*, 1986).

Nuclear staining with DAPI allowed detection of two nuclei in type I oospores that were unable to germinate (Vercesi, Cortesi & Zerbetto, 1993). Fusion of nuclei and achievement of a definitive structure, together with germination capability, are particular characteristics of type II oospores. Sporangium formation by type II oospores supports the hypothesis of Jiang *et al.* (1989) that the presence of a single nucleus in oospores of *Phytophthora* spp. is an indispensable prerequisite for germination and that the fusion of gametangial nuclei is a useful criterion to distinguish mature oospores.

Diffuse fluorescence observed in oospores characterized by large lipid globules (type III) can be interpreted as a nuclear degeneration. Abnormal oospore structure and no discernible nuclei have already been associated, respectively, with scarce viability and absence of germination (Beakes *et al.*, 1986; Jiang *et al.*, 1989). It can, therefore, be assumed that type III oospores represent dead or degenerating *P. viticola* overwintering structures.

Oospores germinated at variable times after collection, depending on the year and the sampling month. Generally,

oospores isolated from samples collected in January or February germinated in 6–7 d, whilst those collected in April germinated in 3–4 d. Early germination events are represented by the rehydration of mitochondria, nuclear divisions and the progressive enzymic breakdown of the IOW which appears uniformly eroded in contrast with the large erosion cavities observed in *P. megasperma* (Beakes & Bartnicki-Garcia, 1989). The following unusual features were observed during the formation of *P. viticola* sporangia. At the beginning of germination, the sporangium wall appeared rather thick and divided into layers characterized by different electron transparency. In the same developmental stage a multi-layered structure was evident between the wall and the protoplasm and could be interpreted as a multi-stratification of plasma membrane. Though it is difficult to understand the significance of this stratification it could be the result of an intense synthetic activity of membrane and wall materials to allow the rapid enlargement of the sporangium. The same explanation could be put forward for the unusual thickness of the sporangium wall.

As already pointed out, *P. viticola* oospores play a very important role in grapevine downy mildew epidemiology. Germination of oospores provides the vital inoculum required for primary infections of susceptible hosts during the spring. In this season, the absence of primary foci in vineyards not treated against downy mildew, even after the occurrence of climatic conditions suitable for oospore germination, has been attributed to the still immature state of oospores.

The unchanged ultrastructure of *P. viticola* oospores from November onwards demonstrates that they are mature by the middle of November. Oospore maturation is not, therefore, the limiting factor for their germination and cannot account for the fluctuations in the germination rates reported by many authors and attributed to the effect of climatic factors (Zachos, 1959; Ronzon, 1987; Burruano & Ciofalo, 1990; Serra & Borgo, 1995). Following from these observations and the new results presented in this paper, it seems likely that the influence of rainfall and temperature occurring during overwintering are exerted on the physiological and/or biochemical processes leading to germination in already morphologically mature oospores.

REFERENCES

Arens, K. (1929). Untersuchungen über Keimung and Zitologie der Oosporen von *Plasmopara viticola* (Berl. *et* de Toni). *Jahrbucher für Wissenschaftliche Botanik* **70**, 57–92.

Beakes, G. W. (1980*a*). Electron microscopy study of oospore maturation and germination in an emasculate isolate of *Saprolegnia ferax*. 1. Gross changes. *Canadian Journal of Botany* **58**, 182–194.

Beakes, G. W. (1980*b*). Electron microscopy study of oospore maturation and germination of an emasculate isolate of *Saprolegnia ferax*. 2. Wall differentiation. *Canadian Journal of Botany* **58**, 195–208.

Beakes, G. W. (1981). Ultrastructural aspects of oospore differentiation. In *The Fungal Spore: Morphogenetic Controls* (ed. G. Turian & H. R. Hohl), pp. 71–90. Academic Press: London, New York, Toronto, Sydney.

Beakes, G. W. & Gay, J. L. (1978). Light and electron microscopy of oospore maturation in *Saprolegnia furcata*: wall development. *Transactions of the British Mycological Society* **71**, 25–35.

Beakes, G. W. & Bartnicki-Garcia, S. (1989). Ultrastructure of mature oogonium-oospore wall complexes in *Phytophthora megasperma*: a comparison of *in vivo* and *in vitro* dissolution of the oospore wall. *Mycological Research* **93**, 321–334.

Beakes, G. W., El-Hamalawi, Z. A. & Erwin, D. C. (1986). Ultrastructure of mature oospores of *Phytophthora megasperma* f.sp. *medicaginis*: preparation protocols and effects of MTT vital staining and permanganate pretreatment. *Transactions of the British Mycological Society* **86**, 195–206.

Bronner, R. (1975). Simultaneous demonstration of lipids and starch in plant tissues. *Stain Technology* **50**, 1–4.

Brunk, C. F., Jones, K. C. & James, T. W. (1979). Assays for nanograms quantities of DNA in cellular homogenates. *Analytical Biochemistry* **92**, 497–500.

Burruano, S. & Ciofalo, G. (1990). Studio sulla dinamica di germinazione delle oospore di *Plasmopara viticola* (Berk. *et* Curt.) Berl. *et* De Toni. *Notiziario Malattie Piante* **111**, 284–286.

Dick, M. W. (1995). Sexual reproduction in the *Peronosporomycetes* (chromistan fungi). *Canadian Journal of Botany* **73** (Suppl. 1), S712–S724.

Fineran, B. A. (1994). Hot fixation of fungal spores for transmission electron microscopy: application to thick-walled spores of the smut fungus *Entorrhiza*. *Mycological Research* **98**, 799–899.

Hall, G. S. (1996). Modern approach to species concepts in downy mildews. *Plant Pathology* **45**, 1009–1026.

Hemmes, D. E. (1983). Cytology of *Phytophthora*. In *Phytophthora, Its Biology, Taxonomy, Ecology and Pathology* (ed. D. C. Erwin, S. Bartnicki-Garcia & P.H. Tsao), pp. 9–40. The American Phytopathological Society: St Paul.

Hemmes, D. E. & Bartnicki-Garcia, S. (1975). Electron microscopy of gametangial interaction and oospore development in *Phytophthora capsici*. *Archives of Microbiology* **103**, 91–112.

Hemmes, D. E. & Stasz, T. E. (1984). Ultrastructure of dormant, converted and germinating oospores of *Pythium ultimum*. *Mycologia* **76**, 924–935.

Jiang, Jiping, Stephenson, L. W., Erwin, D. C. & Leary, J. V. (1989). Nuclear changes in *Phytophthora* during oospore maturation and germination. *Mycological Research* **92**, 463–469.

Populer, C. (1981). Epidemiology of downy mildews. In *The Downy Mildews* (ed. D. M. Spencer), pp. 57–105. Academic Press: London, New York, San Francisco.

Ronzon, C. (1987). Modélisation du comportement épidémique du mildiou de la vigne; étude du rôle de la phase sexuée de *Plasmopara viticola*. Thèse de doctorat, Université de Bordeaux II.

Sargent, J. A., Ingram, D. S. & Tommerup, I. C. (1977). Oospore development in *Bremia lactucae* Regel: an ultrastructural study. *Proceedings of the Royal Society London B* **198**, 129–138.

Serra, S. & Borgo, M. (1995). Indagini sulla maturazione e germinazione delle oospore di *Plasmopara viticola* svernate in condizioni naturali. *Petria* **5**, 91–103.

Tewari, J. P. & Skoropad, W. P. (1977). Ultrastructure of oospore development of *Albugo candida* on rapeseed. *Canadian Journal of Botany* **55**, 2354–2357.

Vavassori, A. (1994). Indagini sulla dinamica di germinazione delle oospore di *Plasmopara viticola* (Berk et Curt.) Berl. and De Toni. PhD Thesis, University of Milan.

Vercesi, A., Cortesi, P. & Zerbetto, F. (1993). La previsione del rischio epidemico da *Plasmopara viticola*: risultati e prospettive. *Atti del Convegno Nazionale 'Protezione delle colture: osservazioni, previsioni, decisioni'*, pp. 301–324.

Vercesi, A., Tornaghi, R. & Faoro, F. (1991). Preliminary investigations on the ultrastructure of *Plasmopara viticola oospores*. *Giornale Botanico Italiano* **125**, 1012–1013.

Zachos, D. G. (1959). Recherches sur la biologie et l'épidémiologie du mildiou de la vigne en Grèce. *Annales de l'Institut Phytophatologique Benaki, nouvelle série* **2**, 193–355.

(*Accepted 22 April 1998*)