Development of the Zygosporangium Wall in *Rhizopus sexualis* (Smith) Callen

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SUMMARY

As the zygosporangium of *Rhizopus sexualis* enlarges it develops a thick wall. Studies with the transmission and stereoscan electron microscopes show that this wall is formed in the following manner. (1) The gametangial septa increase in thickness by the deposition of new material, which is laid down more rapidly on the inner or zygosporangium side than on the suspensor side. These septa form the 'end walls' of the zygosporangium. Fine plasmodesmatal tubules remain at intervals in the wall and are in direct connexion with the tubules of a dense zone of endoplasmic reticulum situated on either side of the septa. It is considered likely that the plasmodesmata allow the passage of nutrients from the parent hyphae into the developing zygosporangium. (2) The 'side wall' of the zygosporangium is formed by the development of new patches within the original wall of the fused gametangia. At first these 'patches' are shaped like inverted saucers and are separate from each other, except that there is a more continuous zone adjacent to the end walls of the spore. Then, the 'patches' become larger, by the deposition of new material at the rim, finally becoming shaped like inverted flower pots and coalescing at the rims to give a continuous warted layer. Until this stage the zygosporangium wall is permeable to water and dissolved substances. Meanwhile the original wall first becomes gelatinous, then membranous, and finally tears and is sloughed off exposing the newly-formed sculptured wall. (3) Finally a new inner wall, impermeable to water and dissolved substances, is laid down within the ornamented wall.

INTRODUCTION

The delimitation of the gametangia of *Rhizopus sexualis* (Smith) Callen by the formation of septa (Hawker & Gooday, 1967) is quickly followed by the autodigestion of the fusion wall at the original area of contact between the progametangia. As the zygosporangium develops from the two fused gametangia it enlarges considerably and alters in shape. The septa between the gametangia and the suspensors are at first convex to the former (Fig. 1 A). When the fusion wall breaks down, the septa which now form the 'end walls' of the young zygosporangium bulge into the spore (Fig. 1 B) possibly as a result of the pressure of cytoplasm which can be seen to stream into the suspensors from the zygosporangia. At this stage the 'side wall' of the spore (i.e. the original walls of the progametangia) still shows a groove corresponding to the zone of contact of the progametangia, although the fusion wall has almost completely disappeared. This groove soon smooths out to give a more or less cylindrical spore with slightly concave end walls (Fig. 1 C). As the spore enlarges these end walls become stretched and flattened and the spore becomes almost spherical (Fig. 1 D). During this last process

the original side wall becomes stretched and gelatinized and is finally replaced by a new sculptured wall which develops *de novo* within it. Meanwhile, the end walls have become thickened by the deposition of new material on both sides of the original septa, but to a far greater extent on the inner or zygosporic side.

The present paper describes the processes of secondary wall formation in the developing zygosporic as determined by electron microscopy.

METHODS

The methods of growing and preparing material for examination under the transmission electron microscope were as described by Hawker & Gooday, (1967). In addition, observations of surface structure were made with a stereoscan electron microscope. The methods of preparation of the material were as described by Hawker (1968).

Fig. 1. Diagrams to indicate stages of development of zygosporic of *R. sexualis*, corresponding to the electron micrographs of Pls I-IV. (A) Fused progametangia, gametangia beginning to be delimited by primary septa (PS). (B) Gametangia fully delimited, septa now convex to suspensors, fusion wall (FW) still largely intact. (C) Fusion wall nearly dissolved, zygosporic enlarged, warts beginning to form. (D) Full-sized spherical, ornamented zygosporic (not necessarily mature since presence or absence of inner wall or endospore cannot be detected by light microscopy). Boxes 1 and 2 indicate positions of Pl. 1, fig. 1, 2 respectively.

RESULTS

Secondary thickening of the septa between gametangia and suspensors

It was reported in a previous paper (Hawker & Gooday, 1967) that secondary wall material was rapidly laid down on the gametangial side of the newly formed septa separating gametangia from suspensor cells and forming the end walls of the zygosporic. Plasmodesmata-like tubules were shown to extend through these septa (Hawker, Gooday & Bracker, 1966).

The present study shows that these plasmodesmata extend through the thickening walls at least until the spore becomes mature and are continuous with tubules of an extensive zone of complex endoplasmic reticulum extending along both sides of the walls (Pl. 1, fig. 1, 2). It is likely that the plasmodesmata are the channels by which food

material passes through the septa into the developing spore. It is otherwise difficult to account for the great increase in size of the zygospore and the accumulation within it of large quantities of food material, particularly lipids and glycogen (Hawker, Abbot & Gooday, 1968) as it matures. The dense zone of endoplasmic tubules bordering the end walls of the spore is probably associated with this translocation. We suggest that complex food materials, such as glycogen and lipid substances, are hydrolysed by these tubules on the suspensor sides of the septa and resynthesized by those on the zygospore side.

It has already been pointed out (Hawker & Gooday, 1967) that after the initial layers of secondary wall material are laid down on both sides of the septa, further deposition is more rapid on the inner or gametangial sides. As this unequal thickening progresses there is a tendency for slight bulges to develop between the plasmodesmata (Pl. 1, fig. 2), on the inner or zygospore side.

Development of the characteristic sculpturing of the lateral walls of the zygospore

The lateral walls of the mature zygospore can be seen by the light microscope to be covered with truncated conical wart-like projections. The rapid hardening of the walls and the development of black pigment make detailed observations of the formation of these warts difficult by light microscopy. Nevertheless, light microscopy affords some evidence that the 'warts' are formed *de novo* underneath the original gametangial wall and that the latter is eventually sloughed off. Similar observations were made by Vuillemin (1904), Dangeard (1906) and Ling-Young (1930) for other species of Mucorales, but their work has been overlooked by most mycologists. Thin sections observed by conventional electron microscopy and observations of whole and of broken zygospores of various ages by the stereoscan electron microscope confirm and amplify this evidence.

Stage 1. The warts are initiated soon after the completion of the septa forming the end walls of the young spore, before the fusion wall between the gametangia has completely dissolved and while a shallow equatorial groove still marks the zone of fusion of the original progametangia (Pl. 3, fig. 6 to 9). At this stage the primary outer wall of the zygospore initial becomes slightly thickened. Electron micrographs, such as Pl. 2, fig. 3, 5, suggest that this thickening is due to gelatinization and not to the deposition of new material. Beneath this original wall the warts are initiated as widely separated patches shaped like shallow inverted saucers, except that there is a more or less continuous band of similar material adjacent to the end walls of the spore and continuous with the secondary material laid down within them. This band is visible clearly in stereoscan electron micrographs of whole spores at this stage (Pl. 3, fig. 10). The cytoplasm fills the shallow domes of these 'saucers' and balloons out between them, enveloped by the plasmalemma. In stereoscan pictures at this stage the original wall can be seen stretched over the circular wart initials and extending between them in wrinkles, which are presumably the result of shrinkage of the cytoplasm during preparation for stereoscan viewing which involves desiccation (Pl. 3, fig. 7). Freeze drying of the material did not obviously improve the final result.

Stage 2. Soon after wart initiation the equatorial groove is filled out and the spore enlarges and becomes first barrel-shaped and finally more or less spherical, but with flattened or slightly concave end walls next to the suspensors.

During this period of zygosporic enlargement the saucer-like patches of new wall become at first cup-shaped (Pl. 3, fig. 9) and finally shaped like inverted flower pots, or irregular truncated cones (Pl. 2, fig. 3, 4; Pl. 3, fig. 11; Pl. 4, fig. 12, 13). It is suggested that growth of these projections takes place by the addition of new material at the base and that each new layer is of increasing diameter so that the bases of warts enlarge until they are finally touching one another. Stereoscan pictures of the insides of broken spores at this stage show the rims of the warts in contact with one another (Pl. 4, fig. 14, 15).

Cytoplasmic material containing organelles similar to those in the main body of the spore and enclosed within the plasmalemma fills the interior of the hollow warts of even full-sized spores (Pl. 2, fig. 4). As the rims of the warts extend, the portions of cytoplasm between adjacent warts become nipped in at the base (Pl. 2, fig. 3). It is not clear whether these are severed from the main body of the cytoplasm when the rims of the warts eventually touch each other or whether they are withdrawn into the interior, as the gaps close. In nearly mature spores the portions of cytoplasm projecting between the developing warts are covered with a mass of amorphous material (Pl. 2, fig. 3; Pl. 3, fig. 9), probably derived from the original wall by gelatinization. In slightly older specimens, in which the rims of the warts are in contact, thus completely sealing the spore, the inter-wart material outside the sculptured wall is not homogeneous, but there is no sign in any of our electron micrographs that any cytoplasm has been cut off and left to disintegrate.

The original gametangial wall is torn into scraps some time before the ornamented new wall is completed. Plate 4, fig. 12, 13 show a nearly mature zygosporic wall with scraps of the original wall adhering to the flattened tops of the flower-pot shaped warts.

Formation of a complete new inner wall

Up to and including the time when the bases of the warts are so closely adpressed to one another that they form a complete layer (Pl. 4, fig. 15) and for a varying period afterwards, the zygosporic wall is permeable to the fixatives used, and fixation of organelles with permanganate is satisfactory. Later, however, an inner wall layer (or layers) develops and this is impermeable to permanganate, with the result that the cytoplasm is not fixed satisfactorily, and, moreover, tends to fall out during sectioning. Examination of mechanically broken zygosporic walls by the light microscope and the stereoscan electron microscope (Pl. 4, fig. 17 and 18), shows that the new inner wall has projections fitting exactly into the interior of the warts. It has not so far been possible to determine the structure or the number of layers of this new inner wall. More satisfactory electron micrographs have, however, been obtained with *Mucor mucedo* in which several wall layers can be seen within the warted previously formed outer layer.

DISCUSSION

Vuillemin (1904) and Dangeard (1906) in optical microscope studies of the formation of the zygosporic wall in the Mucoraceae deduced much of the sequence of events which electron microscopy is now able to establish. They showed for several species that the ornamentations were formed beneath the original gametangial walls which were then fragmented and largely sloughed off. They also demonstrated the subsequent formation of yet another wall layer or layers beneath the ornamented wall. Their

findings were confirmed by Ling-Young (1930), but despite their very accurate drawings no modern text-book of mycology in general use gives a correct detailed description of the formation of the zygospore wall, and some authors have erroneously assumed that the warts develop by thickening of the original gametangial walls. Species of *Rhizopus* were not examined by Vuillemin (1904) and Dangeard (1906) but they studied wart formation in species of *Mucor*, *Zygorhynchus*, *Sporodinia* (syn. *Syzygites*) and *Spinellus*. Their illustrations and descriptions show that the process in these genera was essentially similar to that described in the present paper for *R. sexualis*. The warts of different species showed slight differences in final form but were all formed in a similar manner. The present writers have made a preliminary examination of species of *Mucor* and *Zygorhynchus* with the stereoscan microscope and have thereby confirmed the accuracy of the drawings of Vuillemin and Dangeard.

The structure and composition of the inner wall could not be decided by light microscopy alone or with the histochemical methods available at the beginning of the present century. Accordingly much confusion still exists. Vuillemin (1904) distinguished no less than five wall layers: (1) an innermost thin layer considered to be intermediate between 'active protoplasm' and an 'inert protective layer' and termed by him the 'mother membrane', which may correspond to the plasmalemma or cell membrane of the cytoplasm and therefore would not be part of the wall; (2) a thicker refringent elastic layer which he termed the 'cartilaginous layer', almost certainly the impermeable layer discussed above (p. 16); (3) a colourless pellicle which he termed the 'cuticle mediane' and which could have been cytoplasmic debris trapped between the 'cartilaginous' layer and the warted one; (4) the warty layer, pigmented, hard, inelastic and brittle and termed the 'carbonaceous layer' and finally (5) the 'cuticelle externe' described as a thin layer resting on the warts, becoming fenestrated and torn, and obviously corresponding to the original gametangial wall. Vuillemin discussed the confusion caused by the application of the terms epispore, perispore, mesospore and endospore to different wall layers by different authors and suggested that these terms had thereby become useless. Dangeard (1906), while agreeing in the main with Vuillemin's conclusions, used the term endospore for the wall, possibly multilayered, which develops at some time after the completion of the warted layer. This would seem to be the logical use of this term and should be retained. The 'carbonaceous' warty layer should be termed the mesospore, leaving the term epispore for the evanescent outer layer derived from the original walls of the gametangia. The chemical composition of the various layers of the zygospore wall has never been satisfactorily determined. Early claims (Mangin, 1899) that some layers contained cellulose have not as yet been checked by modern methods.

One of the features, indistinguishable by optical microscopy but brought out by the electron microscope, is the striking bands of endoplasmic reticulum or tubules bordering the end walls of the young zygospores on both the suspensor and spore sides and interconnected by plasmodesmata-like tubules. This complex system could be concerned either with wall deposition or with the hydrolysis, translocation and resynthesis of complex nutrients flowing into the spore from the parent zygothecia via the suspensors. The latter function seems to be the more likely (p. 15), since the bands of tubules are of approximately equal thickness on either side of the septa despite the very much greater amount of secondary wall material laid down on the zygospore side of the primary cross walls. Moreover, no such aggregation of tubules is seen at the

sites of wart formation or prior to the deposition of the final endospore layers. Mitochondria, however, are numerous at all sites of wall deposition, including that of secondary thickening of the septa where they are embedded in and invaginate the layer of tubules. It is likely that the mitochondria, rather than the complex tubule zone, are concerned with active deposition of secondary wall material.

The ornamentations on the walls of fungus spores are of importance in the determination of species in many groups. The use of spore-wall characters as a taxonomic criterion has some disadvantages, since the form of the sculpturing often changes radically during maturation of the spores. Moreover, in at least one example, the genus *Elaphomyces*, species have been based on differences in spore ornamentation (Lange, 1956; Eckblad, 1962) which were later shown to be within the range influenced by age and by rate of drying of the spores (Hawker, Fraymouth & de la Torre, 1967; Hawker, 1968). Thus a study of the development of ornamentation is of practical importance in the taxonomy of fungi. Light microscope studies of the mode of development of surface ornamentation of spores encountered difficulties in the small size of the ornamentations and/or the early development of pigmentation in many species. Electron micrographs of whole spores also yielded little information, although differences in the form of the conidial spines of different species of *Cunninghamella* (Kawakami, 1956), and the form of the wart-like projections on spores of some species of *Gasteromycetes* (Gregory & Nixon, 1950) were demonstrated. By studying immature spores, Gregory and Nixon concluded that during disintegration of the gleba the spores became coated with a gelatinous film which shrank and cracked while drying.

With the development of the technique of preparing ultrathin sections, electron microscopy provided a means of following the development of ornamentation from initiation to maturity. The work of Kawakami (1956) with yeast ascospores; Moore (1963) with ascospores of *Ascodesmis sphaerosporus* Obrist; Kukkonen & Vaissalo (1964) with smut spores of *Anthracoidea aspera* (Liro) Kukkonen; Thomas & Isaac (1967) with uredospores of wheat stem rust and Hawker *et al.* (1968) with ascospores of *Elaphomyces granulatus* Fr. shows that similar spore ornamentations may be formed in several different ways. Special methods, such as the carbon replica method, used by Swinburne & Matthews (1963) in a study of smut spores, give valuable information of the final form of spore sculpturing, but are less useful in studying its development. The stereoscan microscope, used in conjunction with the conventional transmission electron microscope, as in the present paper and in a study of *Elaphomyces* (Hawker, 1968), offers a satisfactory method of studying both the development and the mature form of spore ornamentation.

Studies of the walls of pollen grains (Harris, 1955; Heslop-Harrison, 1963; Pettit, 1966) suggest some similarities in the structure and development of the ornamented walls of certain pollen grains and fungus spores. Further work with a range of species and comparison with the spores of lower green plants is desirable.

It is too early to decide whether there is a limited number of clearly defined methods by which a particular form of spore ornamentation is achieved irrespective of the taxonomic position of the organisms concerned, or a general similarity in the pattern of development of spore ornamentation within particular families or genera. The present study suggests, however, that the development of warts on zygospores of the *Mucoraceae* is essentially similar in a number of genera.

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EXPLANATION OF PLATES

Plates 1 and 2 are of transmission electron micrographs. Plates 3 and 4 are stereoscan electron micrographs with the exception of fig. 18 (a phase contrast photomicrograph).

PLATE 1. Lines = 1 μ

Fig. 1. Part of end wall of nearly mature zygospore next to outer surface (as indicated in Fig. 1 D). *PS* = original primary septum; *SS* = secondary layers laid down on suspensor side of primary septum; *SZ* = secondary material laid down on zygospore side of primary septum; *P* = plasmodesmata; *ZT* = zones of tubules and/or membranes situated along both sides of wall; *L* = lipid bodies.

Fig. 2. Section of central part of end wall of slightly older zygospore. Lettering as in fig. 1. *SI* = inner layer of tertiary (?) wall material on zygospore side of septum; *X* = plasmodesma passing through this inner layer, apparently sheathed with similar material; *M* = mitochondrion.

PLATE 2. Lines = 1 μ

Fig. 3. Section through two developing 'warts' on lateral wall of nearly mature zygospore. *G* = Gelatinized original wall of gametangium; *W* = electron dense, pigmented wart; *C* = cytoplasm pinched between two flower-pot-shaped warts; *N* = nucleus. Other lettering as in fig. 1 and 2. Note: apparent stratification of wart material and comparative absence of endoplasmic tubules or membranes.

Fig. 4. Part of a wart enlarged. Note plasmalemma (*PL*) extending around cytoplasm filling cavity of wart. Other lettering as Pl. 1. Note apparent stratification of wart material.

Fig. 5. Tangential section through base of wart. Note gelatinized original wall (*G*).

PLATE 3

Fig. 6. Zygospore at stage shown in Text-fig. 1 C, showing fused gametangia; wall of suspensor wrinkled, owing to desiccation; note saucer-shaped wart initials covered by original wall of gametangia which is wrinkled between warts owing to contraction during preparation process. $\times 240$.

Fig. 7. Part of fig. 6 at larger magnification showing wart initials more clearly and original gametangial wall. $\times 850$.

Fig. 8 and 9. Slightly older zygospore showing continuous zone of warty wall adjacent to suspensor, projecting young warts now cup-shaped, and torn original wall. $\times 240$ and $\times 1000$.

Fig. 10. End view of slightly older zygospore showing nearly mature warts and continuous zone of warty projections next to collapsed suspensor. $\times 480$.

Fig. 11. Nearly mature zygospore (at stage shown, Text-fig. 1 D) showing warts shaped like inverted flower pots. $\times 240$.

PLATE 4

Fig. 12. Surface view of zygospore at same age as fig. 11. Note stratified, nearly fully formed warts, shreds of original outer wall (*G*) adhering to flat tops of warts, and amorphous remains of this wall in crevices between warts. $\times 1200$.

Fig. 13. Side view of warts at about same age as in fig. 11 and 12, showing apparent stratification. $\times 1200$.

Fig. 14. Part of zygospore wall of full-sized spore broken by mechanical means and contents removed by washing. $\times 200$.

Fig. 15. Part of same piece of wall at higher magnification. Warts, seen from inside, are hollow and joined at basal rims. $\times 2000$.

Fig. 16. Zygospore with suspensor (*S*) removed by mechanical means, showing smooth end wall (*EW*), i.e. the 'tympan' membrane of the 'drum' (Vuillemin, 1904) (cf Pl. 1, figs. 1, 2), and warted side wall. $\times 200$.

Fig. 17. Fully mature zygospore treated as in fig. 15 and 16, showing inner relatively smooth wall lining the warty layer (cf fig. 14 and 15). $\times 1200$.

Fig. 18. Phase-contrast photomicrograph of thick section of mature zygospore embedded in resin Note (i) inner wall (*IW*), which has torn away from warty layer but follows its shape, and (ii) absence of cytoplasm which has fallen out during sectioning, presumably owing to poor fixation. $\times 1200$.

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