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Post cleavage transformation of columella in *Rhizopus stolonifer* (Mucoraceae)

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Abstract. The post cleavage transformation of columella in *Rhizopus stolonifer* (Ehrenb. :Fr.) Vuill. was observed by scanning electron microscopy and transmission electron microscopy. Developmental biology of secondary wall formation, vacuolization, turnover of columella cytoplasm, and formation of the inverted pudding-bowl-shaped structure were critically studied at the ultrastructural level. The formation of the inverted pudding-bowl-shaped structure after collapse of the columella was attributed to the uneven thickness of the columella wall and the columella base strengthened by remnants of ruptured sporangial wall.

Keywords: Columella; *Rhizopus stolonifer*; SEM; TEM; Ultrastructure.

Introduction

Sporogenesis with multispored, columellate sporangium is a typical asexual reproduction of some members of mucoralean fungi. By cleavage of the cytoplasm, the sporangium is divided into a sporogenous region and a central, sterile, normally dome-shaped columella. Several studies have used electron microscopy techniques to study the spore formation process in sporangia (Bracker, 1968; Hammill, 1981; Fletcher, 1973; Beakes and Campos-Takaki, 1984). In these studies, however, the post-cleavage changes in columellae have received little attention. The genus *Rhizopus*, a member of the Mucoraceous fungi, is characterized by its black, large sporangium, apophysate with a very large columella. The columella normally collapses when exposed to air, so that it looks like an inverted pudding bowl attached to the end of a stiff sporangiophore (Ingold and Zoberi, 1963). Sexual reproduction of *Rhizopus* has been extensively studied in *R. sexualis* Callen (Callen, 1940; Hawker and Gooday, 1967, 1968, 1969; Hawker and Beckett, 1971), but less attention has been paid to electron microscopic studies of their asexual reproduction (Hawker and Abbott, 1963b; Buckley et al., 1968; Ho, 1988), particularly columella transformation. We used scanning electron microscopy and transmission electron microscopy to study the changes in the columella of *Rhizopus stolonifer* after it is completely separated from the sporogenous portion.

Materials and Methods

Fungal materials

The *Rhizopus stolonifer* used in this study was isolated from the atmosphere of Taipei and has been deposited in the Culture Collection and Research Center of FIRDI as CCRC 32449(+). Cultures of *R. stolonifer* were grown on Difco potato dextrose agar media in 5 cm petri dishes at 22°C in the dark.

Transmission electron microscopy

Different stages of columella development were selected under a dissecting microscope and fixed with 2.5% glutaraldehyde followed by 1% OsO_4, or with 1% $KMnO_4$. After fixation, the materials were washed, then dehydrated in a graded acetone series. The specimens were embedded in low viscosity epoxy resin (Spurr, 1969). A thin layer of resin (about 1 mm thick) was polymerized in a mold in a 70°C oven for 10–12 h. Selected stages were cut out, individually mounted, and then sectioned with a glass or diamond knife on a Reichert Ultracut E microtome. Sections about 80–90 nm thick were stained with 15% uranyl acetate and 0.4% lead citrate, then examined with a Hitachi H-600 or a Jeol 1200 EX-2 transmission electron microscope at 75 kV.

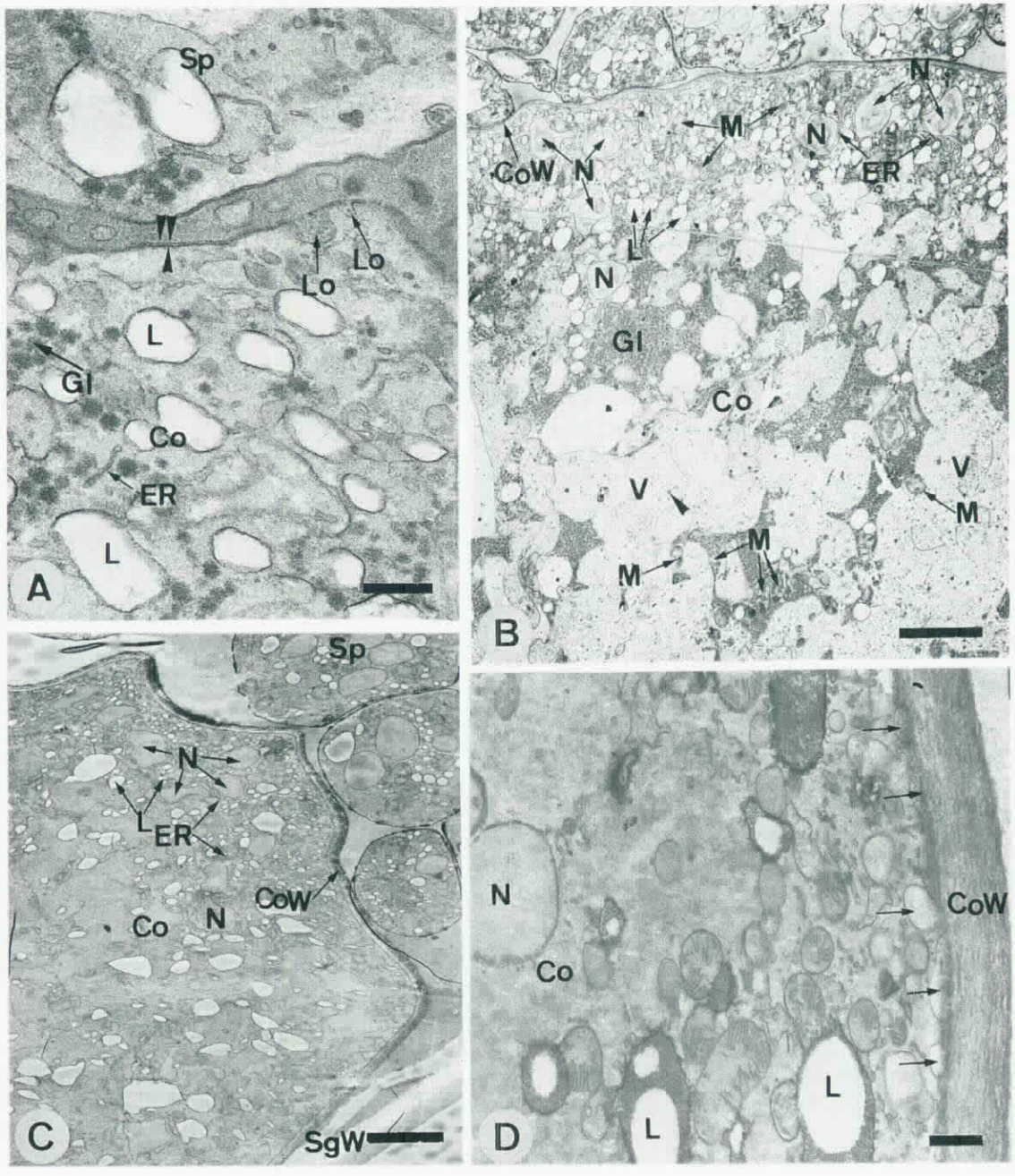

Figure 1. A–D. Longitudinal sections through sporangia during various stages of development after the columella has been delimited (TEM, $KMnO_4$ fixed). **A.** Portion of a nearly completely cleaved sporangium, showing the young spore above and the columella below. The newly formed columella is lined by a discontinuous layer (double arrow head) outside the plasmalemma (arrow head). Note the presence of lomasomes, glycogen, ER, and lipid globules. (bar = 400 nm). **B.** Longitudinal section through the columella in the early-stage of cytoplasmic turnover. Nuclei, mitochondria, lipid droplets, glycogen, and ER are scattered abundantly throughout the peripheral cytoplasm. Vacuoles occur at the center of the columella. In the central region, glycogen still occurrs, either in the cytoplasm or in the vacuole (arrrow head). Mitochondria are found within vacuoles. (bar = 4 μm). **C.** Portion of a columella in the early stage of turnover, wall getting thicker, showing columella with undulating outer surface. Nuclei, lipid globules, and ER are abundant in the peripheral cytoplasm. (bar = 3 μm). **D.** Periphery of columella at the same stage as in C. Note lomasomes (arrows) appearing along the inner side of fibrillar primary columella wall. (bar = 500 nm).

Abbreviations: AV, Autophagic vacuole; **Co**, Columella; **Col**, Collar; **CoW**, Columella wall; **DM**, Degenerated mitochondrion; **ER**, Endoplasmic reticulum; **Gl**, Glycogen; **L**, Lipid droplet; **Lo**, Lomasome; **M**, Mitochondrion; **Mb**, Microbody; **N**, Nucleus; **Nu**, Nucleolus; **RER**, Rough endoplasmic reticulum; **SgW**, sporangial wall; **Sp**, Spore; **SW**, Secondary wall; **V**, Vacuole.

Acid phosphatase localization

We used the method described by Gomori (1950).

Scanning electron microscopy

Young colonies with sporangia were examined under a dissecting microscope, and small mycelial agar squares (about 0.5 cm or less) were cut with a double-edged razor blade. These agar blocks were fixed with 2.5% glutaraldehyde and post fixed with 1% OsO_4. The materials were washed and dehydrated in a graded alcohol series. Specimens were then dried in a critical point dryer, coated with gold, and observed and photographed with a Hitachi S-520 scanning electron microscope at 20 kV.

Results

Early development of columellae of *R. stolonifer* was synchronized with the development of sporangiospores. At the late cleavage stage, the sporogenous tissue of the sporangium cleaved into individual spores. Meanwhile, the central portion of the sporangium was also delimited from the sporogenous region into a dome-shaped columella. The outer surface of newly formed columellae was irregular (Figures 1A–C). A discontinuous layer was observed to be situated outside the columella plasmalemma (Figure 1A). Lomasomes usually occurred along the inside of the columella plasmalemma (Figure 1A).

After completion of cleavage, the thickness of the columella wall began to increase. Throughout the wall thickening period, lomasomes were occasionally seen along the inner surface of the columella plasmalemma (Figures 1A, D). Later, this wall appeared fibrillar (Figures 1C, D). The outer surface of the columella wall initially undulated, because of the closely compressed young spores (Figures 1B, C), but later became smooth and oval-shaped when the columella wall thickened and hardened (Figures 3A, D).

The cytoplasm of newly delimited columellae was highly vacuolate in the central region, but there was a cytoplasmic dense area containing typical organelles near the columella wall (Figure 1B). A large amount of storage materials (mainly glycogen granules and lipid droplets), nuclei, mitochondria, and ER were ubiquitous in the peripheral region (Figure 1B). Nuclei, frequently appearing side by side, were usually accompanied by parallel ER. Continuities between ER and the nuclear membranes were occasionally observed (Figures 1B, C). Mitochondria were variously shaped, with profuse inner membranes (Figure 1B). With potassium permanganate preparations, the lipid droplets appeared as empty vesicles. These vesicles had characteristically distinct electron-dense membrane boundaries (Werner et al., 1964). These electron dense particles clumped in the cytoplasm, and had the general appearance described by Werner et al. (1964). Thus, they were considered to be glycogen (Figure 1B). The interior of the cytoplasmic

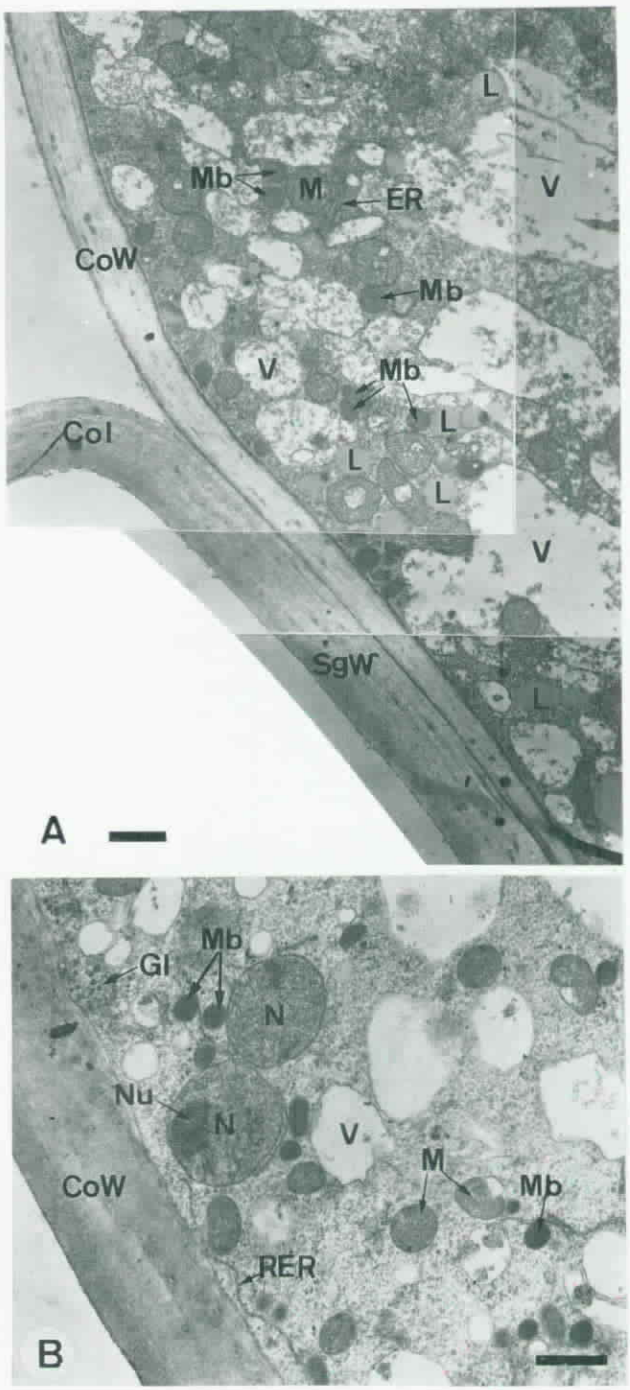

Figure 2. A–B. Longitudinal sections of columella in the early-stage of turnover (later than in Figure 1B.) (TEM, GA/OsO_4 fixed). (bar = 1 μm). **A.** Portion of columella near base. Peripheral cytoplasm begins to vacuolize; also contains typical mitochondria, lipid globules, and ER. Microbodies start to appear. Collar is also seen. Columella wall is laid down inside the sporangial wall and tapers. **B.** Portion of columella near columella wall at the same stage as in A. Note nuclei with nucleoli, mitochondria, microbodies, and RER in the cytoplasm. (see page 250 for key to abbreviations)

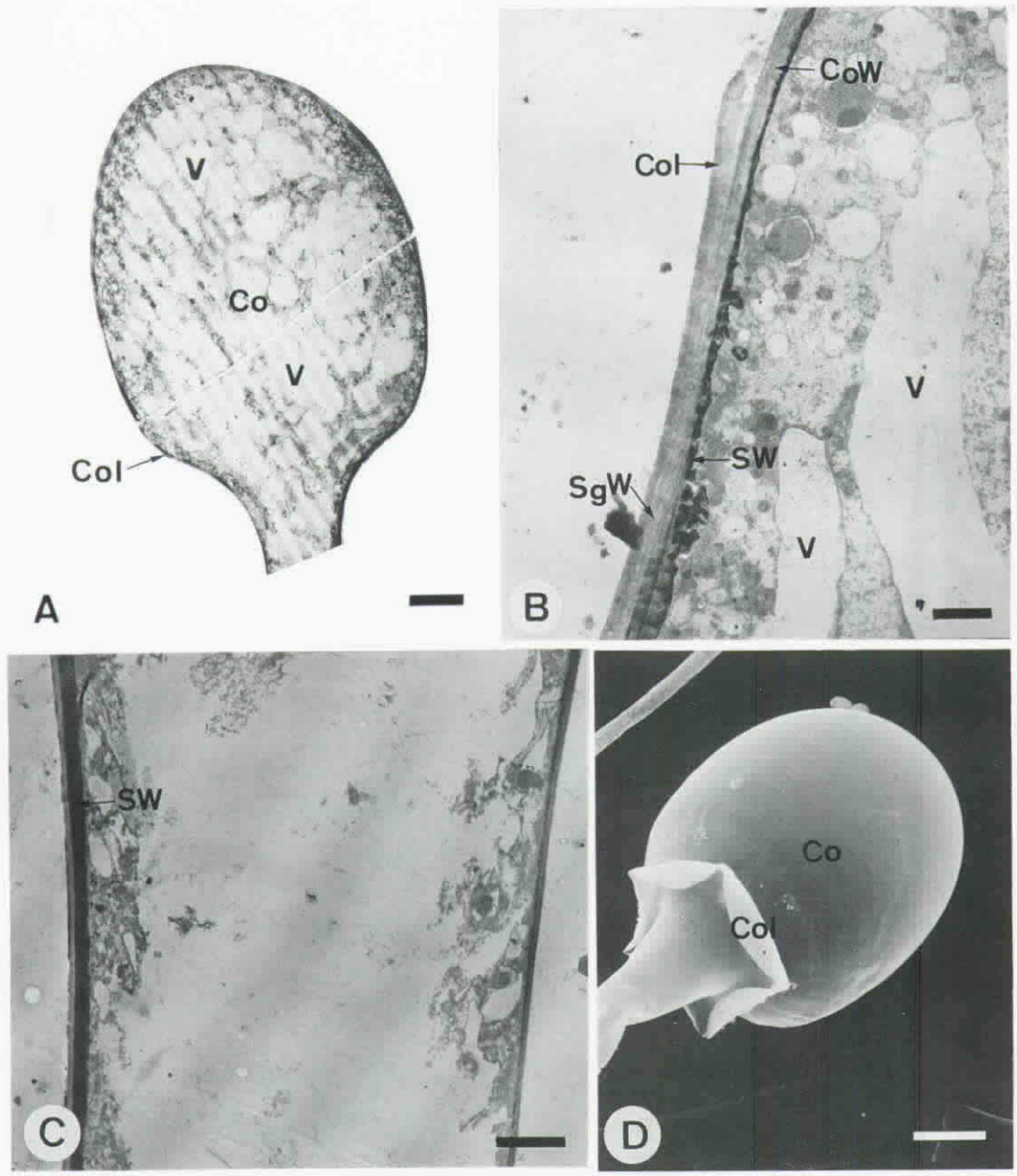

Figure 3. A–D. Longitudinal sections through columella in the early- to mid-stage of turnover (TEM, GA/OsO_4 fixed). **A.** The columella and part of the sporangiophore. Central portion of columella is occupied by vacuoles, with cytoplasmic dense area restricted at periphery. Note the presence of collar at the columella base. (bar = 20 μm). **B.** Magnification of portion of A, showing columella wall formed inside of the sporangial wall. Electron opaque secondary wall formed along the inside of columella and sporangial wall. (bar = 2 μm). **C.** Magnification of part of A, showing secondary wall along the inner side of sporangiophore wall. (bar = 5 μm). **D.** (SEM) An oblique side view of an oval-shaped columella, surface smooth, with a collar attached near the base. (bar = 24 μm). (see page 250 for key to abbreviations)

dense area was a vacuolate region where large vacuoles occupied the central part and the small ones were located near the outer cytoplasmic dense area. Numerous glycogen granules not only occurred between vacuoles but also could be found within vacuoles (Figure 1B). Mitochondria and lipid droplets were occasionally observed between vacuoles, but nuclei were rarely seen. This vacuolate area extended in a downward direction into the sporangiophore (Figures 3A, C).

The primary columella wall was observed to be thicker on the upper portion than near the base (Figures 3A, 7A). The thickness of the columella wall at this stage was around 0.4–2.2 μm. The lower portion of the columella wall came into close contact with the inside of the base of the sporangial wall (Figures 1C, 2A, 3B). After the formation of the primary fibrillar wall, the secondary

thickening of columella wall took place. This secondary wall was electron opaque, grew along the inner side of the fibrillar primary columella wall (Figure 3B), and extended downward into the sporangiophore (Figure 3C). This secondary wall was typically even, but occasionally it protruded unevenly into the internal cytoplasm (Figures 3B, C).

The turnover of columella cytoplasm could be divided into three stages. The early-stage began with the occurrence of autophagic vacuoles in the central region (Figure 1B). Mitochondria were found in these vacuoles soon after the delimitation of the columella was completed. Later, vacuoles smaller than those in the central region started to appear in the cytoplasm dense area (Figures 2A, B; 3B). Typical ER, nuclei, mitochondria, and storage material (such as lipid droplets and glycogen) were present at this stage. Microbodies began to appear, sometimes closely associated with ER (Figures 2A, B). The mid-stage of the turnover was distinguishable by the presence of many autophagic vacuoles in the

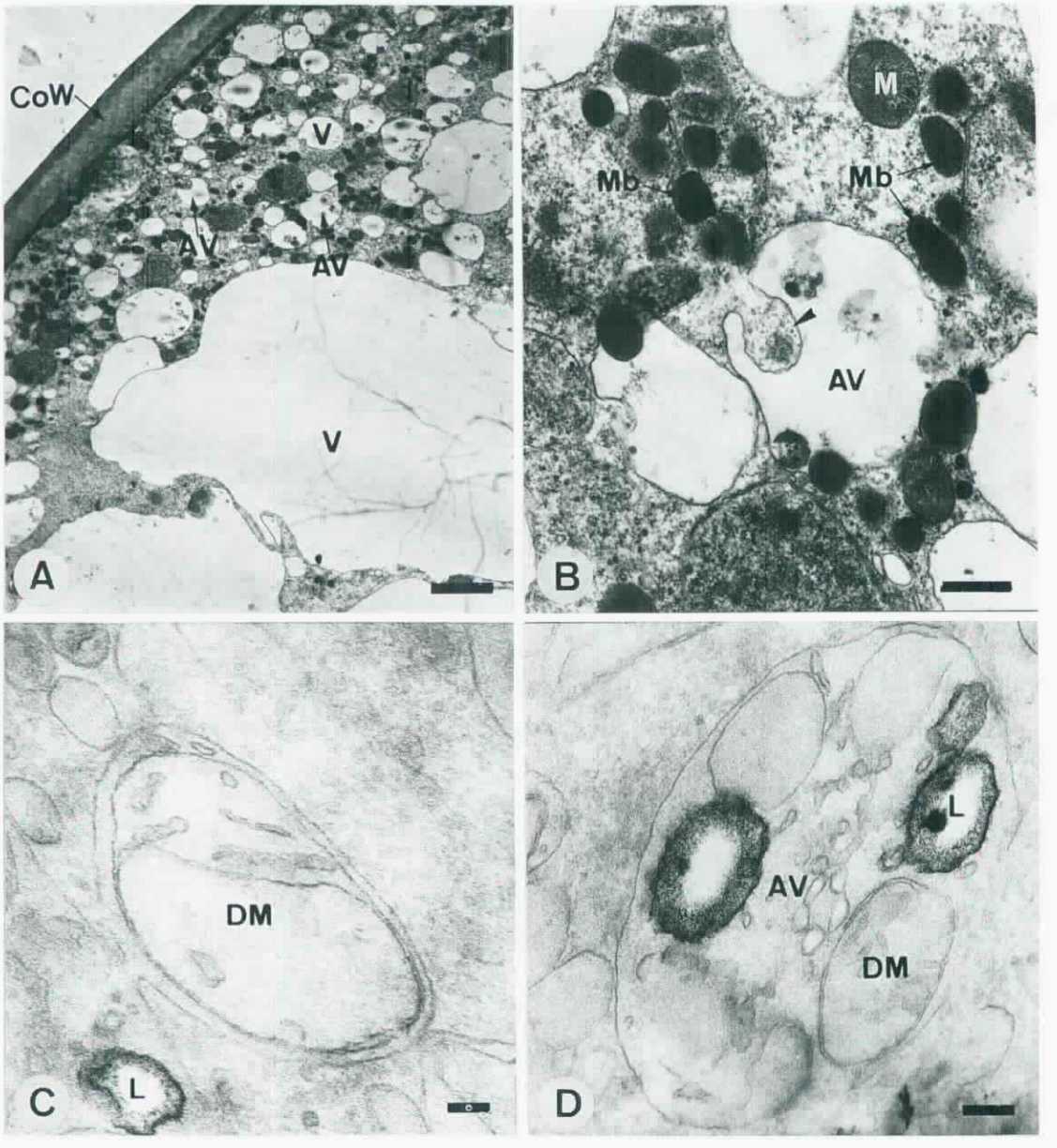

Figure 4. A–D. Longitudinal sections of columellae in early- to mid-stage of turnover. (TEM, A–B. GA/OsO_4 fixed, C–D. $KMnO_4$ fixed). **A.** Vacuolized columella periphery. Note the autophagic vacuoles and microbodies. (bar = 2. 5 μm). **B.** Autophagic vacuoles engulfing cytoplasmic materials (arrow head). Note the presence of microbodies. (bar = 500 nm). **C.** An autophagic vacuole contains a mitochondrion. (bar = 100 nm). **D.** Autophagic vacuole contains lipid droplets, a degenerated mitochondrion, and some membrane systems. (bar = 200 nm). (see page 250 for key to abbreviations)

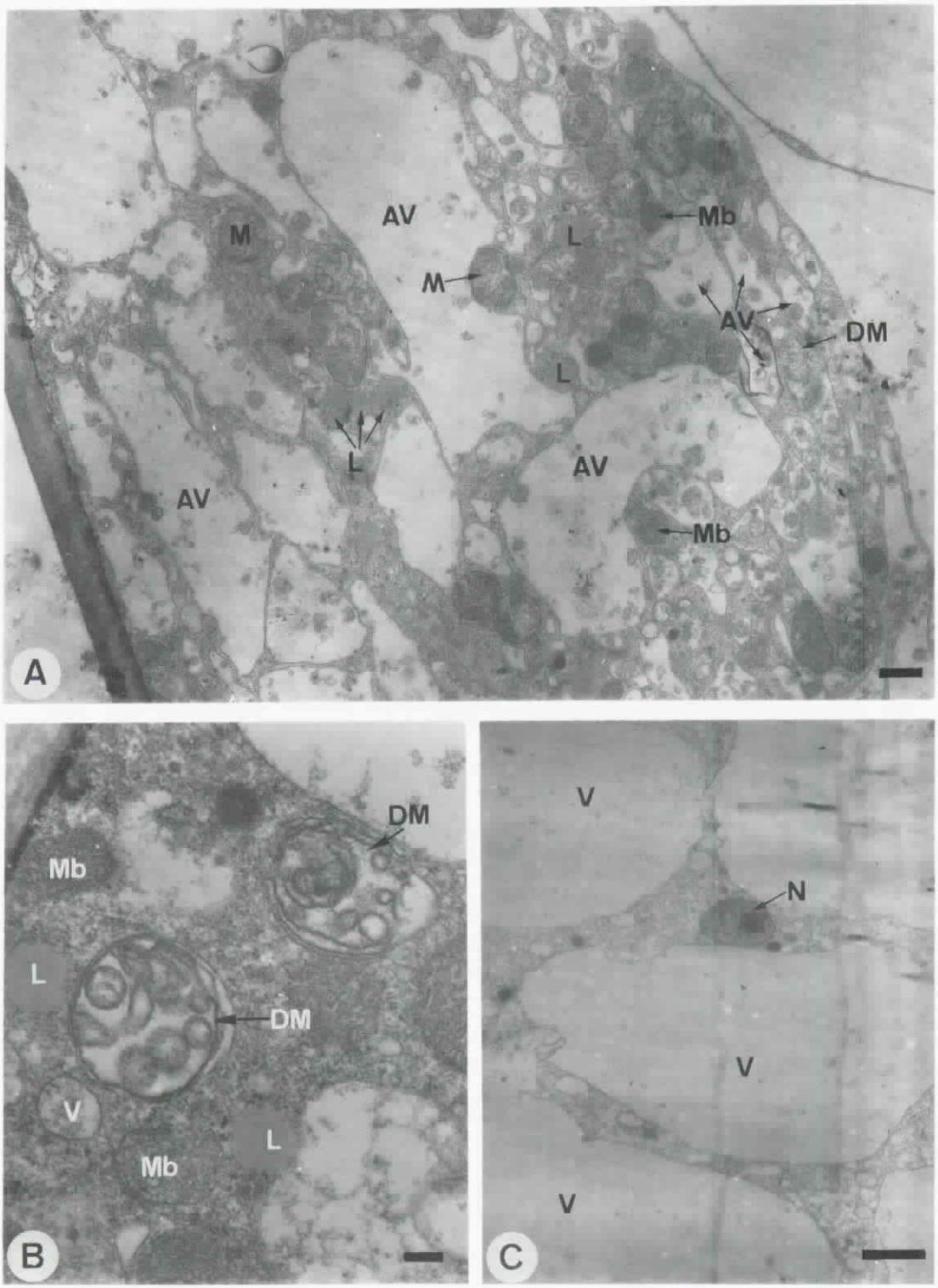

Figure 5. A–C. Longitudinal sections of columellae in the mid-stage of turnover. (TEM, GA/OsO_4 fixed). **A.** Highly vacuolate peripheral cytoplasm. Note typical mitochondria, lipid globules, microbodies, and autophagic vacuoles containing discernible membrane systems or just engulfing mitochondria and vacuoles. (bar = 800 nm). **B.** Portion of columella periphery showing degenerated mitochondria with distorted inner membrane, lipid droplets, and microbodies. (bar = 200 nm). **C.** Portion of columella in the central region. Note nuclei still found between vacuoles in the central region of columella. (bar = 2 μm). (see page 250 for key to abbreviations)

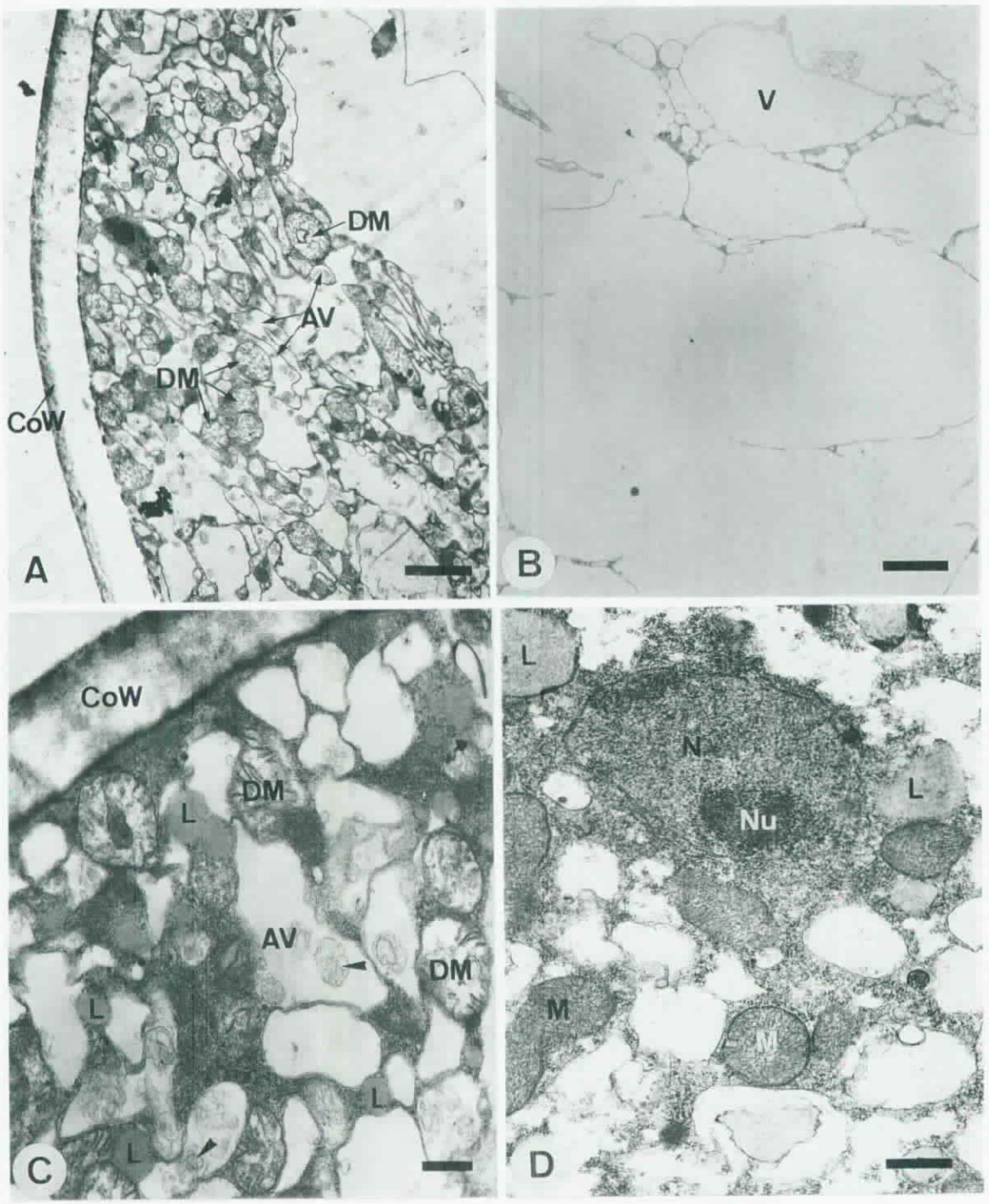

Figure 6. A–D. Longitudinal sections through columella in the late-stage of cytoplasmic turnover. (TEM, GA/OsO_4 fixed.) **A.** Peripheral cytoplasm contains mainly degenerated mitochondria and autophagic vacuoles. (bar = 2 μm). **B.** Central portion of columella, nearly empty; only trace of cytoplasm and vacuole membranes remain. (bar = 4 μm). **C.** Enlarged view of portion of A, showing degenerated mitochondria, lipid globules, and autophagic vacuoles containing remnants of mitochondria (arrow head) and discernible membranes. (bar = 500 nm). **D.** Portion of columella at the same stage as A, B, and C. Note typical nuclei, mitochondria, and lipid globules. (bar = 500 nm). (see page 250 for key to abbreviations)

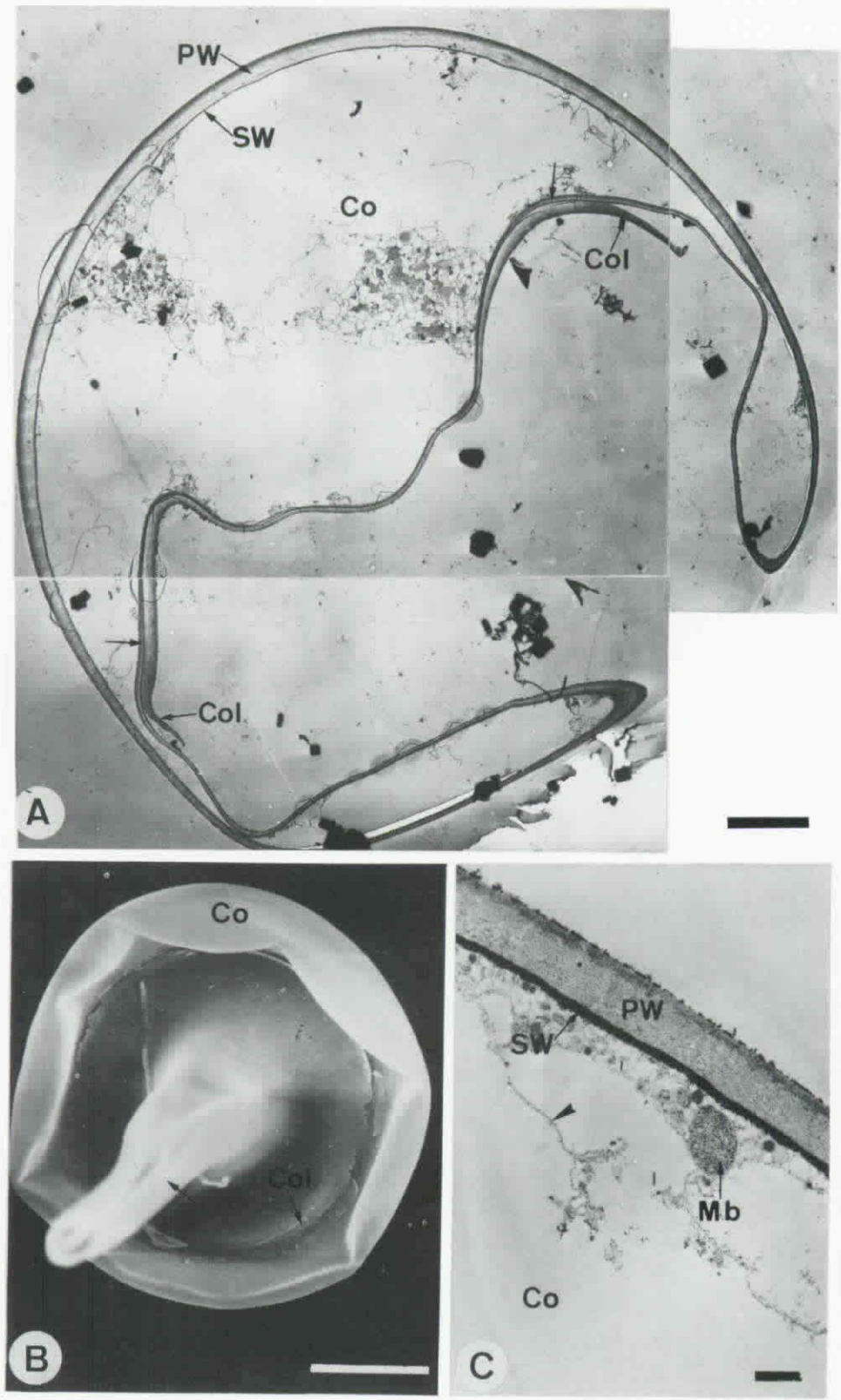

Figure 7. A, C. (TEM, GA/OsO_4 fixed, acid phosphatase localized). Oblique longitudianl section of a collapsed columella. **A.** Showing columella wall thickness decreasing from top down to the base and tapers, then merging into the sporangial wall (arrow). Columella wall distorted in an inward direction from the thinnest portion around collar attaching site. (bar = 10 μm). **B.** (SEM). Bottom view of a collapsed columella. Arrow indicates sporangiophore. (bar = 43 μm). **C.** Enlarged view of portion of A, showing dark lead phosphate precipitation, indicating the presence of acid phosphatase on microbodies, primary and secondary wall of columella, and vacuole membrane (arrow head). (bar = 1 μm). (see page 250 for key to abbreviations)

peripheral region (Figures 4A, 5A). Autophagic vacuoles were observed to form pseudopodia, which engulfed cytoplasmic material (Figures 4A, B). Degenerated mitochondria, lipid droplets, and discernible membrane systems were usually observed in autophagic vacuoles (Figures 4C, D). The number of microbodies increased, especially in early mid-stage. Degenerated mitochondria with distorted inner membrane also occurred in the cytoplasm (Figures 5A, B), but typical mitochondria and nuclei were ubiquitous (Figures 4A, C). When the late-stage of turnover was reached, the peripheral cytoplasm was filled mainly with degenerated mitochondria and with autophagic vacuoles containing remnants of lysed mitochondria and discernible membrane systems (Figures 6A, C). Lipid droplets, microbodies, some typical nuclei, and mitochondria were occasionally seen (Figure 6D), but there were fewer than in the previous stage. The central portion of columella became more and more empty, with only traces of cytoplasm and vacuole membrane remaining (Figure 6B). The turnover of columella cytoplasm was not synchronized, i.e. it proceeded faster in the center than at the periphery. Nevertheless, the entire columella would ultimately become almost completely empty (Figure 7A). A preliminary study of acid phosphatase localization in columellae indicated enzymatic activities in microbodies, along the columella wall (especially the secondary wall) and the tonoplast even after the columella collapsed (Figures 7A, C).

When the sporangial wall ruptured and liberated spores, the oval-shaped columella was then exposed to the outer air (Figures 3A, D). A collar and the basal part of the remnants of the ruptured sporangial wall could be seen attached to the base of columella (Figures 3A, B, D, 7A, B). This nearly empty, oval-shaped columella had a smooth surface (Figures 3A, D, 7A). Thin sections showed that the thickness of the columella wall decreased from top to the bottom, and was thinnest around the collar attaching site. The wall then tapered and merged with the original sporangial wall (Figure 7A). The base of the columella was strengthened by the remnants of ruptured sporangial wall. On exposure to dry air, columellae collapsed in a striking and definite manner. The wall was distorted in an inward direction at the weakest site around the collar and became like an inverted pudding bowl (Figures 7A, B). This structure persisted and was connected to the terminal end of the stiff sporangiophore (Figure 7B).

Discussion and Conclusion

In *R. stolonifer*, the delimitation of columellae is synchronized with spore formation. At the post cleavage stage of spore formation the delimitation of columellae is nearly completed. This differs from *Mucor* #218, in which the delimitation is completed before the post cleavage stage (Hammill, 1981), and *Gilbertella persicaria*, in which the delimitation is completed when the spore plasm is at the mid-cleavage stage (Bracker, 1968).

At the early delimitation stage, cytoplasmic remnants are seen in the space between spores and columella, but the mature columella surface is clean. This is in contrast with *Thamnidium elegans*, in which cytoplasmic debris attaches to the columella surface (Fletcher, 1973). This observation indicates that digestion or absorption of this cytoplasmic debris may occur in *R. stolonifer*.

After complete delimitation, the columella wall thickness increases faster than that in the spore wall, and quickly attains maximal thickness. Lomasomes (border bodies, named by Moore and McAlear in 1961) have been observed to occur just inside the plasmalemma during collumellal thickening. They were also observed in the columella of *T. elegans* (Fletcher, 1973). This structure is related to the cell wall formation, i.e. for transportation of wall material to cell wall (Marchant and Robards, 1968). The mature columella wall appears fibrillar, which is similar to the structure of the sporangiophore and mycelial wall of *R. stolonifer* (Hawker and Abbott, 1963a) and *R. rhizopodiformis* (Werner et al., 1964). Additionally, the same fibrillar appearance of the columella wall was also observed in the columella walls of *G. persicaria* (Bracker, 1968), *Mucor* #218 (Hammill, 1981) and *T. elegans* (Fletcher, 1973).

After formation of the columella primary wall, secondary wall formation takes place along the inner surface of the columella wall. This secondary wall is electron opaque, and does not increase evenly along the surface. It extends downward to the sporangiophore, and is joined to the sporangiophore secondary wall. The presence of a secondary wall in columellae was not reported in *G. persicaria* (Bracker, 1968), *Mucor* #218 (Hammill, 1981), or *T. elegans* (Fletcher, 1973). This is the first report of a secondary columella wall in Mucoralean fungi.

Autophagy of central vacuoles was found to occur early during columella transformation. Mitochondria were observed within vacuoles soon after the columella was delimited. This phenomenon has been reported likely to occur in *Mucor* #218 (Hammill, 1981) and *G. persicaria* (Bracker, 1968). In *G. persicaria*, degenerated mitochondria and crystals were present in vacuoles, but their presence might occur earlier during the mid-cleavage stage of spore formation (Bracker, 1968).

During the later transformation stage, autophagic vacuoles and microbodies are present in columellal cytoplasm. The autophagic vacuoles are especially pronounced. Perhaps both of these structures are involved in the turnover of columella contents. This is also found in *Mucor* #218 (Hammill, 1981), but in *G. persicaria*, only large sporangia are highly vacuolated, while the small sporangia are densely cytoplasmic and contain many microbody-like vesicles (Bracker, 1968).

Acid phosphatase activity along the columella wall, especially the electron opaque secondary wall and degenerated vacuole membrane, are observed even after the columella collapses. Acid phosphatase has been related to the secondary wall formation and autolysis of

plant cells in *Phaseolus vulgaris* xylem (Charvat and Esau, 1975). Therefore, transformation of post cleavage columellae is thought to be achieved either by autophagic vacuoles or by enzymatic activities.

Mucoraceous fungi normally have multi-spored sporangia with typical dome or oval shaped columellae. The columella wall delimits the sporogenous region along with the remaining sterile region. After the sporangial wall ruptures and spores are dispersed, this columella is exposed to the outside air. The inverted pudding-bowl-shape of the collapsed columella is a distinguishing feature of *Rhizopus* species (Ingold, 1940). The present study has revealed that the difference in columella wall thickness between the top and the base is one of the reasons for the formation of the bowl-shaped structure. The thinnest portion of the columella wall is near the base, around the collar attaching site. Consequently, it is relatively easy to distort inward from this thinnest portion when the columella becomes empty and dries. Another reason is that there is a sporangial wall remnant on the underside of the columella base, which strengthens the basal portion. Therefore, the columella does not collapse entirely but instead becomes a bowl-shaped structure.

Beakes and Campos-Takaki (1984) observed that the columella thickness of *Ellisomyces anomalus* is even, and that of *Mucor* #218 (Hammill, 1981) and *G. persicaria* (Braker, 1968) is reported to taper at the base. None of them, however, forms bowl-shaped columella after spores are liberated.

The columella is formed in the sterile part of the sporangium of *R. stolonifer*. Its cytoplasm and cell wall are connected to the sporangiophore. At the late degenerating stage of columellal cytoplasm, the empty core space of the columella expands and leaves a scattered cytoplasm containing few nuclei and ER. The enzymatic activities (acid phosphatase), however, are still present at this stage, or even after the collapse of the columella. We conclude that the active biological process continues throughout the development and transformation of columellae in *R. stolonifer*.

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匍枝根黴中軸形成後之變化

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本文以穿透式及掃描式電子顯微鏡觀察匍枝根黴（*Rhizopus stolonifer* Vuill.）中軸形成後之內部微細構造變化，有次生壁形成，液胞化及自我分解現象，最後幾全變空。文中並討論中軸塌陷後成傘狀，與中軸壁厚度之相關性。

關鍵詞：中軸；匍枝根黴；掃描式電子顯微鏡；穿透式電子顯微鏡；微細構造。