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Cell Wall Ultrastructure in Resting-Spores *of Polymyxa betae* KESKIN *(Plasmodiophoraceae)*

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With 12 Figures

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Summary

CIAFARDINI G., ARENA L.M.R., MARES D. & BRUNI A. 1995. Cell wall ultrastructure in resting-spores of *Polymyxa betae* KESKIN (*Plasmodiophoraceae*). – Phyton (Horn, Austria) 35 (1): 153–163, with 12 figures. – English with German summary.

The structure of the resting spore wall of *Polymyxa betae* KESKIN was investigated by cytochemical reactions at TEM and enzymatic treatments. Of the five layers making up the mature cell wall, the first two are intimately interconnected, the third is rather electron-transparent and fibrous, while PATAg reveals the fourth to be thin and rich in polysaccharidic materials. The fifth, innermost layer varies in appearance according to the various stages of maturation and to the area examined. During the maturation of the resting spore it forms in the upper area a discoidal area which disappears before germination. The wall, on the whole, is resistant to enzymatic treatment with pronase, lipase and phospholipase, and is destroyed by lysozyme. Our results provide evidence for a progressive modification of the upper area of the cell wall from a simpler to a more complex structure during the maturation of the spore and in relation to germination. Cytochemical tests and enzymatic studies yield no reliable evidence on the chemical composition of the wall, with the exception of the fourth layer, where the polysaccharidic components are localised.

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Zusammenfassung

CIAFARDINI G., ARENA L.M.R., MARES D. & BRUNI A. 1995. Die Ultrastruktur der Zellwand in Dauersporen von *Polymyxa betae* KESKIN (*Plasmodiophoraceae*). – Phyton (Horn, Austria) 35 (1): 153–163, mit 12 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die Wandstruktur der Dauerspore von *Polymyxa betae* KESKIN wurde mit cytochemischen Reaktionen im TEM sowie enzymatischen Behandlungen untersucht. Von den fünf Schichten, welche die ausdifferenzierte Zellwand aufbauen, sind die ersten zwei eng miteinander verbunden, die dritte ist eher elektronendurchlässig und faserig, während die PATAg-Reaktion darauf schließen läßt, daß die vierte Schicht dünn und reich an Polysacchariden ist. Die innerste fünfte Schicht variiert ihr Aussehen, entsprechend den verschiedenen Stadien der Differenzierung sowie jeweils an welcher Stelle sie untersucht wurde. Während der Differenzierung bildet die Dauerspore im oberen Bereich eine scheibenförmige Zone aus, welche vor der Keimung verschwindet. Die Wand als ganzes widersteht einer Enzymbehandlung mit Pronase, Lipase und Phospholipase, wird aber durch Lysozym zerstört. Unsere Ergebnisse liefern den Beweis für eine fortschreitende Modifizierung des oberen Bereichs der Zellwand von einer einfacheren zu einer komplexeren Natur während der Differenzierung und in Abhängigkeit von der Keimung. Cytochemische Testverfahren und Enzymbehandlungen liefern keine zuverlässingen Schlüsse auf die chemische Zusammensetzung der Zellwand mit Ausnahme der vierten Schicht, in welcher Polysaccharide lokalisiert sind.

Introduction

Polymyxa betae (Plasmodiophoraceae) is an obligate parasite of the roots of many *Chenopodiaceae* plants. In the sugar beet, it acts as a vector of beet necrotic yellow vein virus (BNYVV), the agent of a serious disease known as rhizomania (KESKIN 1964, FUJISAWA & SUGIMOTO 1977, RYSANEK & al. 1992). *P. betae* infection occurs via zoospores that penetrate into the root hairs and generate plasmodia in host cells. Later, plasmodia differentiate zoosporangi or sporosori that are constituted by aggregates of the resting spores, commonly called cysts, by which the fungus can survive in the soil for a very long period.

Several studies have been carried out on *Polymyxa betae* sporosori to ascertain their distribution in the soil with indirect methods (CIAFARDINI & MAROTTA 1989b, CIAFARDINI 1991) and the presence of BNYVV inside or outside the resting spore (see RYSANEK & al. 1992). However, only a few papers have been devoted to the structure and the chemical composition of cystosori cell wall. D'AMBRA & MUTTO 1977 described the presence of two layers in resting spore wall of *P. betae:* an outer electron-dense and an inner electron-transparent layer. Subsequently LANGENBERG & GIUNCHEDI 1982 confirmed this cell wall architecture. By scanning electron microscopy (SEM) CIAFARDINI & MAROTTA 1988, 1989a showed that morphological modifications occur in the cell wall during the maturation process of

sporosori and that the germination of the resting spores of *P. betae* takes place in particular fragile areas of the cell wall by cleavage.

In the present work we report about further studies exploring the architecture of the resting-spore wall by cytochemical and enzymatic procedures with transmission electron microscopy (TEM). The identification of the ultrastructural characteristics of cyst walls is one of the criteria for the differentiation of the species among the *Plasmodiophoromycetes,* even if they are primarily separated on the basis of the different host that they infect.

Moreover we have explored possible alterations that occur in the cell wall during the maturation of the resting spores. In fact, cell wall modifications are important events that determine the germination of resting spore, and therefore the capacity of the fungus to infect the host cells.

Materials and Methods

Fungus culture was performed following an improved procedure of the method described in CIAFARDINI & MAROTTA (1988, 1989a, b). *Polymyxa betae* KESKIN was cultured on the "Monomassa" hybrid (Society European Seed [SES], Italy) of sugar beet (*Beta vulgaris* L.). The seeds were released from their coating by soaking in distilled water for 2 h by gentle magnetic stirring, placed on a 2-mm mesh metal sieve, rinsed with new distilled water and disinfected with 70 % (v/v) ethyl alcohol for 5 min. After rinsing again with sterile distilled water, a treatment with 2 % sodium hypochlorite solution for 4 min ended the sterilisation procedure. Finally, the seeds were rinsed six times with sterile water, then dried on sterile blotting paper and placed on a sterilised inert substrate. For the sterilisation of river sand, vermiculite, glass cylinders and pans, as well as for germination we followed the procedure reported by CIAFARDINI 1991. After germination, the plantlets were transplanted to other pans containing 8 g inert substrate mixed with 4 g air-dried soil containing 60 infecting units (I.U.) per gram of *P. betae* evaluated with the most probable number (MPN) technique (CIAFARDINI & MAROTTA 1989b). The pans were moistened with 10 ml mineral nutrient solution and kept in the growing chamber for another 20 days. At the end of the incubation period, the presence of sporosori in the sugar-beet roots was determined and the sporosori were extracted as previously reported (CIAFARDINI & MAROTTA 1989a). The debris-free sporosori isolated from sugar beet used in this study were 98 % pure.

Resting spores were subjected to enzymatic digestion with pronase (from *Streptomyces griseus,* Boehringer), lipase (from wheat germ, Sigma), phospholipase A (from *Vipera russelli,* Sigma) and lysozyme (from chick egg white, Boehringer). For the enzymatic treatment we followed the procedure described by BUCZACKI & MOXHAM 1983 for the resting spores of *Plasmodiophora brassicae.*

Optical microscopic examinations were performed on debris-free sporosori employing a Zeiss Axiophot microscope equipped with conventional attachments.

During the preparation of samples for TEM, particular attention was devoted to procedures employed, because the resting spores of *P. betae* proved to be a hard to fix and embed material. After some attempts, we followed essentially the methods reported by MARES & al. 1990, extending the time of infiltration with the epoxy resin.

This procedure variation proved to be necessary for the thick-walled resting spores of *P. betae*. The sporosori were fixed with 5 % (v/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 4 h at 4° C and post-fixed with 1 % (w/v) OsO_4 in the same buffer for 6 h. After a brief wash in the buffer, the samples were harvested by gentle centrifugation at 440 x g, immobilised with 1 % (w/v) agar, dehydrated in a graded ethanol series and, finally, embedded in Epon-Araldite resin. Sections were cut on an LKB Ultratome III, stained with uranyl acetate and lead citrate and observed with a Zeiss EM 109 transmission electron microscope at 80 kV.

One portion of the sporosori sample was used for polysaccharide detection. The PATAg [periodic acid (PA), thiocarbohydrazide (TCH), silver proteinate (Ag)] method was used according to THIÉRY 1967 (for details see MARES 1982). The sections, collected on a gold grid, were exposed to TCH for 15 h. For reaction controls, ultrathin sections were processed omitting both PA oxidation, TCH and Ag treatment or replacing the PA with hydrogen peroxide (COURTOY & SIMAR 1974).

Results

The debris-free sporosori isolated from the sugar beet roots used in the various experiments were about 98 % pure. The cystosori of *P. betae* observed in the light microscope were irregular in shape, ranging from cuboidal or spherical to rectangular shape, and in size, being 10-90 µm width and 30-80 µm length (Fig. 1). Cysts were spherical, subspherical or ellipsoidal, with a depression in a pole visualised as upper area (hollow sphere or hollow ellipsoid) (Fig. 2), and had an average diameter of about 4 µm. The depression was characteristically observed in immature cystosori (Fig. 2); after the maturation period the resting spores appeared spherical and unhollowed (Fig. 3).

The cyst wall was finely verrucose,very thick (200-300 nm) and clearly multilayered (Fig. 4); the outer layers of the walls of contiguous cysts were fused only partially (Fig. 3). Special attention was given to the upper area

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Fig. 1. Debris-free sporosori of *Polymyxa betae* observed with the light microscope. Isolated sporosori are 98 % pure. Scale bar is 50 µm.

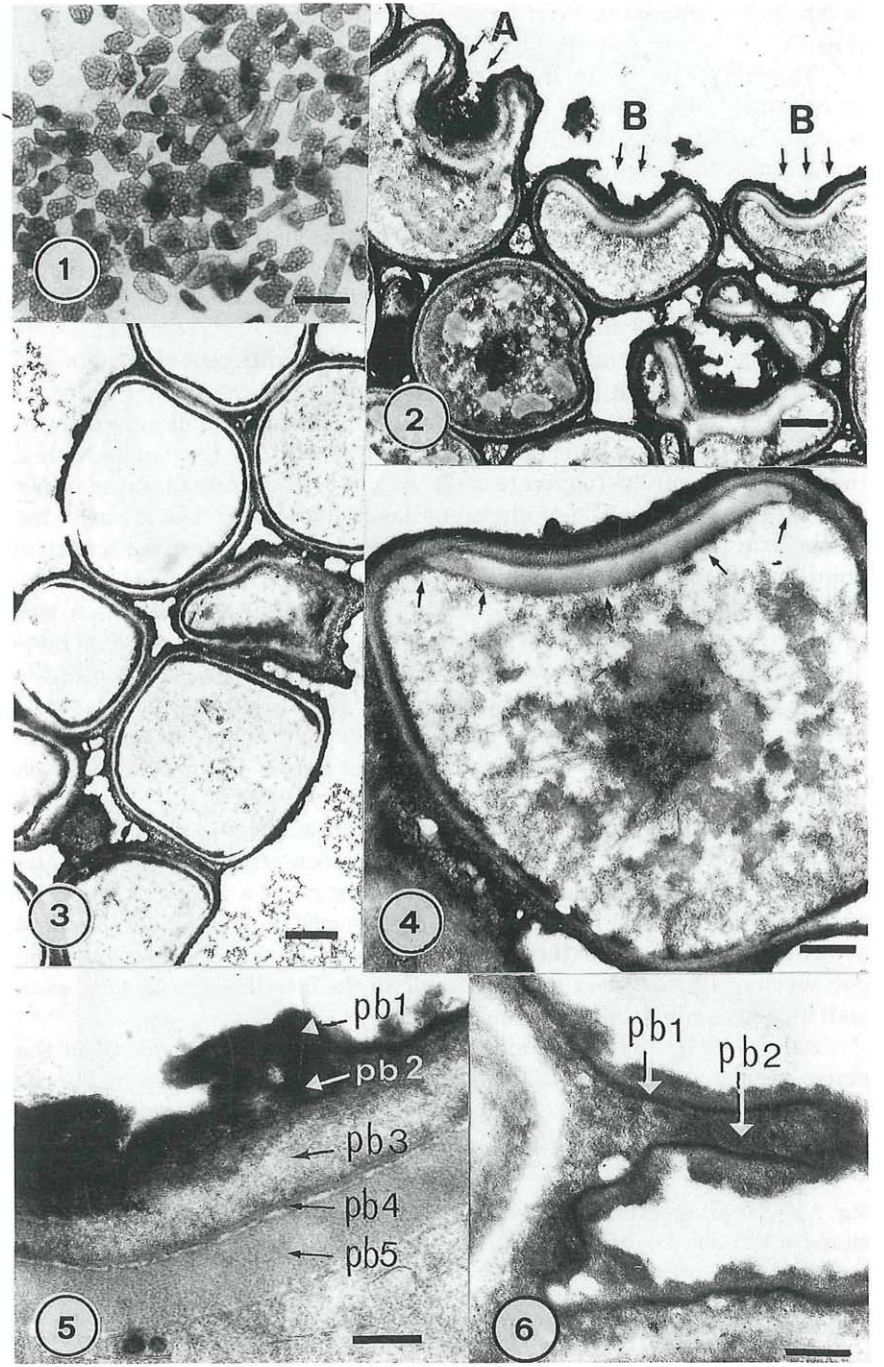
Fig. 2. Cistosorus of *P. betae* with resting spores in different stages of maturation. An evident depression is present in the upper area of the immature cyst (A), less evident in the more mature cysts (B). Bar is 1 µm.

Fig. 3. After germination, the resting spores appear spherical and unhollowed and the invaginations of the upper area are no more present. Bar is 1 µm.

Fig. 4. Mature resting spore in which a change in the cell wall architecture is in progress. In the upper area the innermost wall layer forms a electron-transparent zone, that protrudes towards the protoplasm (arrows). Bar is 500 nm.

Fig. 5. Magnification of the complex architecture of the cell wall of resting spore. Five layers are evident: Pb1 to Pb5. Pb1, Pb2 and Pb4 are thin and electron dense, while Pb3 and Pb5 are thick and more or less electron transparent. Bar is 200 nm.

Fig. 6. Portion of verrucose cyst wall with an irregular ornamentation formed by outer layers Pb1 and Pb2. Bar is 200 nm.



in which five successive layers were detected and indicated as Pb1 to Pb5 (Fig. 5).

The first layer (Pb1), the outermost part of the cell wall, was about 30 nm thick and consisted of very electron-opaque material. Pb1 showed several slight protrusions giving the wall an irregular external surface. The immediately underlying layer (Pb2) was electron-opaque and varied from 30 to 70 nm in thickness and often it extended into irregular spines (Figs. 5 and 6). The sublayer Pb3 appeared less electron dense, rather uniform in thickness (about 100 nm) and tightly pressed against layer Pb2 (Figs. 4 and 5). Layer Pb4 was thick -30 nm, highly electron-dense and with a clear margin between Pb3 and Pb5 (Fig. 5).

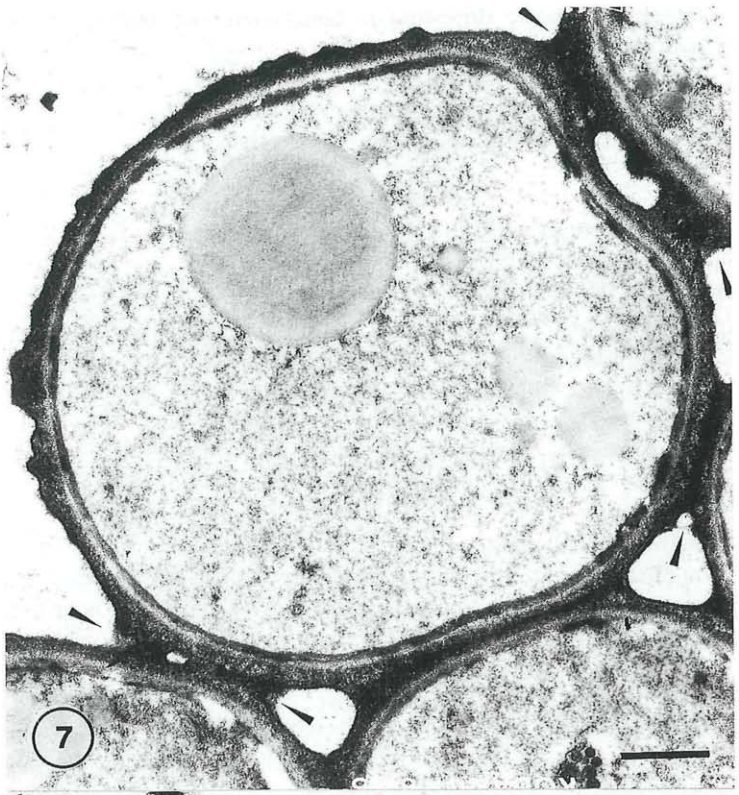
Finally, the innermost wall sheet, Pb5, had a different thickness and appearance according to the stage of maturation of the resting spore. In fact, in the immature cyst, Pb 5 formed a continuous osmiophilic sheet, uniformly electron-opaque along the entire girth of the spore with a thickness of about 80-100 nm (Fig. 7). At maturity it formed in the upper area an internal electron-transparent layer that protruded towards the protoplasm (Fig 4). This structure from a Pb5 layer began from a central point and then proceeded circularly towards the periphery.When the maturation process was complete a papilliform internal discoid region was present (Figs. 4, 5 and 8), with thickness reaching up to 240 nm in its middle portion. This upper area of the Pb5 layer was destroyed before the resting spore germination. On the left of Fig. 9 a still intact portion is visible, whereas on the right side demolition has begun. After the germination of the resting spore, this electron-translucent region of the Pb5 sublayer, was no longer visible (Fig. 10).

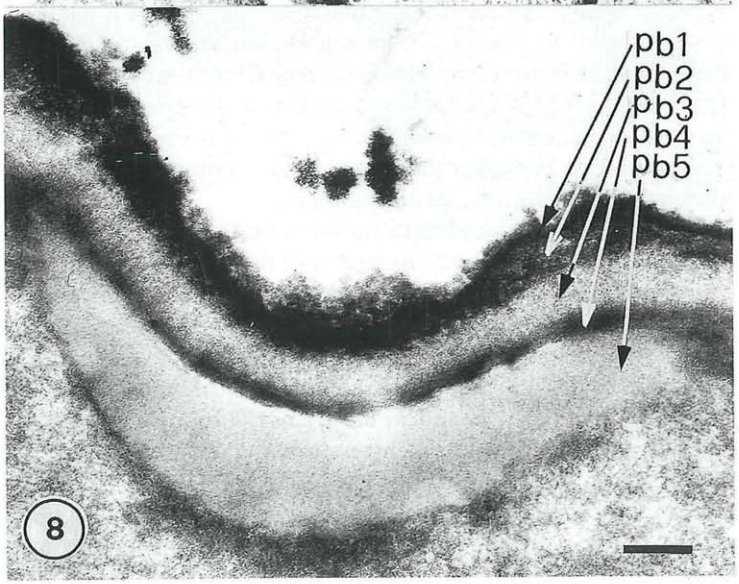
The analysis on the chemical composition of the five wall sublayers was attempted by means of the PATAg reaction on grids and by enzymatic digestion "in toto". Silver proteinate staining gives a strong reaction on Pb4 layer of the wall (Figs. 11 and 12). The figures show cysts that are kept at different stages of maturation; nevertheless silver granules, showing polysaccharides, are clearly recognisable in the intermediate layer of each wall of two partially fused resting spores.

After PATAg an intense electron scattering was also evident on the many storage vesicles that are located mainly at the periphery of the cytoplasm, indicating that, even if partially, they are polysaccharidic in nature (Figs. 9 and 12).

Fig. 7. Spherical immature resting spore: the walls of contiguous cysts are fused by means of Pb1 and Pb2 sublayers (arrowheads); Pb5 forms a uniformly osmiophilic sheet along the entire girth of the spore. Bar is 500 nm.

Fig. 8. Particular of the upper area of a mature cyst: the complex multilayered cell wall shows, only here, the Pb5 as a electron transparent zone, particularly conspicuous, gaining about 420 nm of thickness in the middle portion. Bar is 200 nm.





As far as enzymatic digestion is concerned, the resting spore wall showed a surprising resistance. Pronase, lipase and phospholipase used as individual treatment proved to be ineffective. Only with lysozyme some reaction was observed: the whole cell wall became spongious but no specific digestion of an individual layer was observed.

Discussion

The high concentration (98 %) of debris-free sporosori isolated from the sugar beet roots rendered easier the optical and electron microscope observations of the samples.

The TEM study permitted to acquire further details on the wall morphology of the resting spore in comparison with previous studies *in situ*, in which the sporosori were not separated from the roots of the host plants (TAMADA & BABA 1973, FUJISAWA & SUGIMOTO 1977).

Furthermore, this study permitted to follow some changes occurring in the cyst wall architecture during the maturation of the spore and in relation to germination.

The cell wall of the resting spore of *P. betae* is very complex. Of the five layers, the outermost two layers are interconnected so tightly that they can hardly be discerned at low magnification. The third one is fibrous in structure, while the fourth is thin and mainly consists of polysaccharides. The innermost layer is rather thick and osmiophilic.

The architecture of the wall differs from that of resting spores of other *Plasmodiophoromycetes* (MILLER & al. 1985), in which the ultrastructural characteristics of the cyst wall are one of the criteria employed to recognise the species. In particular the resting spore wall of *P. betae* is morphologically similar to that of *Ligniera verucosa* (MILLER & al. 1985) and most unlike that of *Plasmodiophora brassicae* (BUCZACKI & MOXHAM 1983, MOXHAM & BUCZACKI 1983). The cyst wall of *P. brassicae* consists of an outer layer of loose proteinaceous fibres, a fibrillar layer in which lipid-containing granules are enmeshed, and a predominantly chitinous wall with an inner, phospholipid-containing region.

Although we have been able to individuate the five layered structure of the *P. betae* wall, we have not succeeded in defining the chemical nature of each individual layer. We demonstrated the presence of polysaccharides with vyc-glycol groups oxidisable in the fourth layer, significantly reactive to PATAg test. However, the enzymatic digestion of the whole resting spore with pronase, lipase, phospholipase and the cytochemical tests did not furnish information on the chemical nature of the four other sheets.

It is known that *P. betae* has a chitinous wall (WATERHOUSE 1973). Since the Pb3 sublayer is fibrillar and poorly reactive to PATAg, it is possible that it is made of chitin (not reactive to PATAg), possibly interposed with other polysaccharide components. The enzymatic digestion by lysozyme can be interpreted as a non specific attack of peptidoglycans of the cell wall.

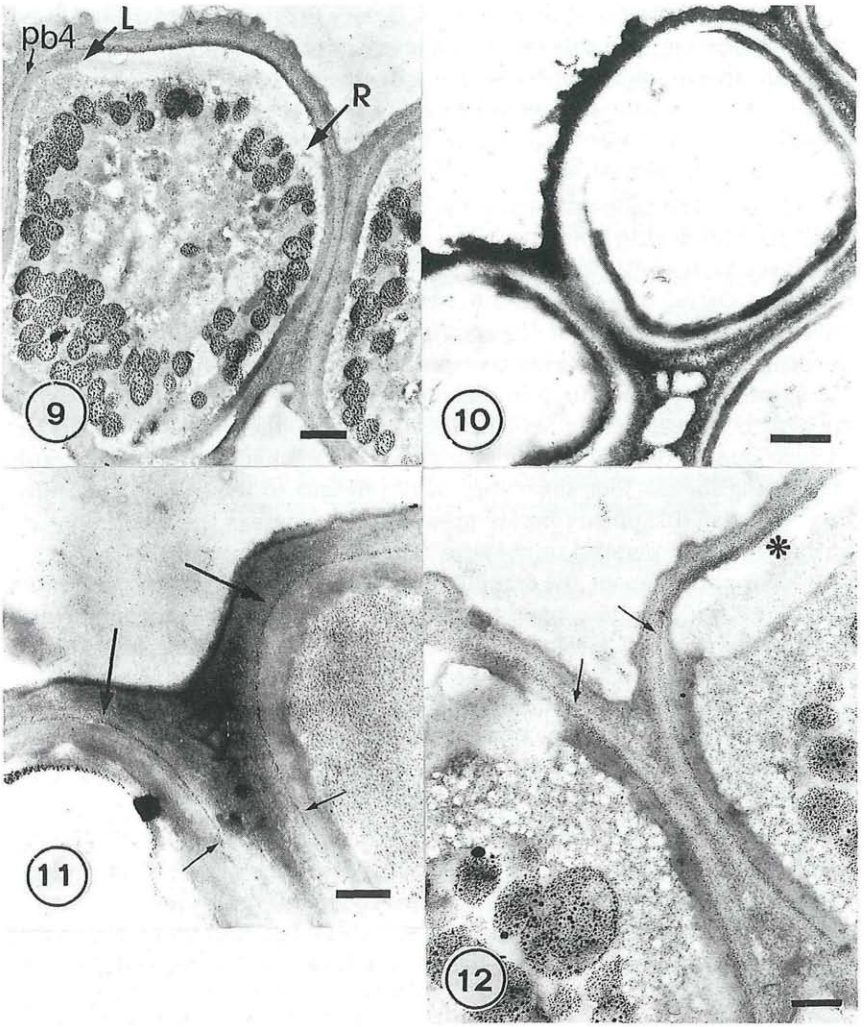


Fig. 9. Mature resting spore of *P. betae* stained with the PATAg procedure for polysaccharide detection. Particularly reactive are the many cytoplasmic vesicles of storage. L = Left, R = Right. Bar is 500 nm.

Fig. 10. Empty resting spore after the germination process. In the upper area of the cyst cell wall the discoidal area of the Pb5 layer is disappeared. Bar is 500 nm.

Fig. 11. Portion of cell wall of mature cysts after PATAg reaction. A deposition of electron-dense granules is evident in Pb4 (arrows), whereas the other layers are not or poorly reactive. Bar is 200 nm.

Fig. 12. A small increase of reactivity to PATAg test is showed by the Pb3 layer (arrows) just before germination, whereas Pb5 remains unreactive during all stages of maturation of the spore. Note, at this stage, the separation between Pb5 and the other layers in the upper area (asterisks). Bar is 200 nm.

With regard to the Pb1 and Pb2 it is very difficult to provide with our observations any indications on their chemical nature. On the basis of electron density and enzymatic and cytochemical reactivity we can only suppose that proteins and, perhaps, lipids are present in these outer layers, an interpretation in accordance with their protecting role against microbial attack and degradation.

An aspect to be mentioned is the change in morphology of the cell wall sublayer Pb5 during the maturation of the spore, since the morphology of this layer varies with the stage of maturation and area examined. The appearance during the maturation of a discoid electron-transparent area only in the upper area of the resting spore is of particular interest, indicating that this region may be related to the subsequent germination of the spore. Moreover, this papilliform area is very thick and very tightly packed, a characteristic which, together with the reduced upper-area surface due to the invagination, might limit exchange with the outside, accounting for the long dormancy of the fungus in the soil. Furthermore, this "papilla" disappears before germination, whereas the remaining part of the Pb5 layer persists unchanged. The disappearance of this area gives new information about the complex event which precedes the perforation of the cell wall in the upper area. The present results confirm previous SEM observations (Ciafardini & Marotta 1988, 1989a). The formation of an internal papillar layer in the upper area and its subsequent disappearance seems to be related to the increased fragility of this area inherent to a process of cell wall softening.

In conclusion, the exit-tube emission of the resting spore of *P. betae* is preceded by alterations of the cell wall architecture of the upper area. Our observations show the formation of a innermost cell layer at a certain stage of maturation of the spore, the disappearance of which seems to be related to the subsequent germination process. Our results also demonstrated a polysaccharide-rich cell wall sheet in the whole cell wall. Further research is in progress to investigate the chemical nature of the other wall layers of the resting spore.

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