

ULTRASTRUCTURE OF THE APICAL APPARATUS OF ASCI IN
OMBROPHILA VIOLACEA, NEOBULGARIA PURA AND
BULGARIA INQUINANS (LEOTIALES)

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The ultrastructure of the apical apparatus in asci of three species of the Ombrophiloideae (Leotiaceae) is compared. *Ombrophila violacea* and *Neobulgaria pura* show similarities in morphology and maturation pattern of the apical apparatus, suggesting a close relation between these species. In these respects and in development of the ascospore wall *Bulgaria inquinans* differs considerably from *O. violacea* and *N. pura*.

INTRODUCTION

The order Leotiales (Carpenter, 1988; Helotiales sensu auct.) is basically defined by the structure of the ascus. Its current classification is still largely based upon the system that Nannfeldt (1932) proposed for the inoperculate discomycetes. Students of Leotiales have disagreed on the arrangement of families for a long time now (Dennis, 1956, 1978; Korf, 1973; Barr, 1976; Hawksworth et al., 1983; Eriksson, 1983). Apparently it is difficult to create a natural system by applying the more conventional taxonomic criteria based on ascocarp anatomy, morphology of hymenial elements and ecology.

Light microscopic studies performed by Chadefaud (1964, 1973) and more recently by Baral (1987) provided valuable diagnostic data on the ascus morphology of various groups of inoperculate discomycetes. For studies on the structural variation of the ascus apex in taxa with small asci like the Leotiales the use of electron microscopy is necessary.

Bellemère (1977) made a comparative ultrastructural study of a selection of representatives of the Leotiales and proposed six types of apical apparatus. Occasionally, others studied some species as well (e.g. Corlett & Elliott, 1974; Schoknecht, 1975; Benny et al., 1978). Unfortunately these authors used different techniques and terminologies and had different ways of interpreting their electron micrographs. This has made the data less accessible to other mycologists. In order to reveal more about the variation in ultrastructure of the apical apparatus of asci within the Leotiales and its possible significance for the taxonomy of the group, a comparative study on selected species was initiated. In this first report data on three species of the Leotiaceae (Corda, 1842) are presented: *Ombrophila violacea* Fr., *Neobulgaria pura* (Fr.) Petr., and *Bulgaria inquinans* (Pers.) Fr.

Korf (1973) and Dennis (1978) treated *Ombrophila* Fr. in a restricted sense, arranging only a few species around *O. violacea*. Korf (l.c.) followed Gamundi & Dennis (1969)

and treated *Neobulgaria* Petrak, typified by *N. pura*, also in a restricted sense, separated from *Ascotremella* Seaver. More recently, Baral & Krieglsteiner (1985) proposed to place *Neobulgaria* in the genus *Ombrophila*. *Bulgaria inquinans* is the type species of a small genus showing a combination of characters that is quite unusual in Leotiaceae: pigmentation and size-differentiation of the ascospores within a single ascus and large ascocarp size.

The arrangement of *Ombrophila*, *Neobulgaria*, and *Bulgaria* together within a single tribe, the Ombrophiloideae sensu Dennis, suggests a close relationship of these genera. The classification of this tribe is largely based on anatomical features of the ascocarp, especially those concerning gelatinized tissues. Differences in other characters of the species assembled in this group are apparently considered less important. The Ombrophiloideae therefore seems to form a heterogeneous taxon and an interesting subject for a comparative study on the ultrastructure of the apical structures in the asci. Little is known about the ultrastructure of ascospore walls in the Leotiales. For this reason data concerning ascospore wall development are also included in the present paper.

MATERIALS AND METHODS

Fresh material was collected in the field. Parts of fruit-bodies were fixed for 3 hours using 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C, washed in buffer, and postfixed for 1 hour using 1% osmium tetroxide in cacodylate buffer at room temperature. Material was then dehydrated in a graded series of ethanol and embedded in Epon. During dehydration material was stained with 1% uranyl acetate for 10 minutes. Ultrathin sections were cut using a diamond knife on a Reichert Jung Ultracut E ultratome.

For PA-TCH-SP (periodic acid-thiocarbohydrazide-silver-proteininate), sections were picked up on uncoated 200 mesh golden grids and treated at room temperature as follows (modified from Thiéry, 1967): 1) 1% aqueous periodic acid (PA), 30 min.; 2) three rinses of water, 10 minutes each; 3) 0.2% thiocarbohydrazide (TCH) in 20% acetic acid, 60 min.; 4) rinses of 10%, 5%, and 2.5% acetic acid, 5 min. each; 5) three rinses of water, 10 min. each; 6) 1% aqueous silver proteininate (SP) (Prolabo, Paris), 25 min. in the dark; 7) three rinses of water, 10 min. each.

In other cases sections were contrasted with Reynolds' lead citrate and uranyl acetate, or, occasionally, with barium permanganate. Preparations were examined shortly after treatment, using a Philips EM 300 at 60 kV.

In the following list details are given about the origin of the collections.

Ombrophila violacea Fr. Bunderbos, Limburg, the Netherlands, on soil and plant debris, April 1990, H. Huyser.

Neobulgaria pura (Fr.) Petr. Fôret de St. Prix, Bois de la Canche, Morvan, France, on decaying wood, Oct. 1990, J. van Brummelen.

Bulgaria inquinans (Pers.) Fr. Mooshof, Dungou near Roith, Ober Pfaltz, Germany, on decaying wood, Sept. 1990, J. van Brummelen 7942.

The identification of specimens was performed according to Dennis (1978).

TERMINOLOGY

Clarification of the terminology employed

Corresponding terms of Bellemère (1977) and Bellemère et al. (1987) are given in brackets, as [1] and [2] respectively.

Apical apparatus: apical region of ascus wall that forms the functional spore-shooting apparatus. It includes the apical thickening and the central cylinder ('dôme apical' or 'appareil apical' [1], [2]).

Central cylinder (CC): central region in the apical apparatus. It operates as a pore by tearing or breaking during ascospore discharge (it includes 'coussinet apical', 'pseudomanubrium', 'cylindre axial' [1] and 'coussinet apical', 'pseudomanubrium', 'corps ombiliqué' [2]).

Annulus (A): annular (cylindrical) structure surrounding the central cylinder, from which it can be distinguished by differences in ultrastructure. Its outer limits are usually less distinct because of a more gradual change in structure, and therefore the annulus is considered to be a part of the apical thickening ('anneau inférieur', 'anneau supérieur' [1], [2]).

Annular protrusion (AP): part of the wall material in the apical thickening that is associated with the annulus and protrudes downwards into the ascus lumen. It may enclose the apical chamber to a variable extent ('pendentif' [1], [2]).

Apical chamber (AC): amount of epiplasm enclosed to a variable extent by the annular protrusion ('oculus' [1]; 'évacuation médiane du épiplasmé' [2]).

Apical thickening (AT): total region of increased thickness of the ascus wall, excluding the central cylinder, in which an annulus may be embedded.

Outer layer (OL): outer part of the ascus wall that does not increase in thickness in the apical apparatus.

Inner layer (IL): inner part of the ascus wall that increases in thickness in the apical apparatus.

Apex development: proces of apex formation and maturation.

Apex formation: addition of wall material to the apical region of the ascus wall, thus forming the apical apparatus.

Apex maturation: all changes in the ultrastructure of the apical apparatus that occur after apex formation is completed.

Stages in ascus development

Young ascus – The ascus initial is formed and elongates, meiosis and mitosis take place, resulting in (usually) eight ascospores delimited by two unit membranes. In the species under study the apex formation is completed within this stage.

Immature ascus – Ascospore maturation (Beckett, 1981b) occurs. The ascosporoplasm is enriched with organelles and lipid bodies, and the primary and secondary ascospore wall are formed. Towards the end of this stage, the various organelles decrease in number, while the number of vacuoles in the epiplasm increases. In the species under study most of the changes that characterize the apex maturation take place during this stage.

Mature ascus – Maximum vacuolization of the epiplasm is reached and no organelles remain in the epiplasm, ascospores are fully mature; the internal pressure increases rapidly, eventually leading to dehiscence.

Dehiscenced ascus – After the discharge of ascospores the ascus wall disintegrates rapidly.

RESULTS

Observations on the lateral ascus wall, the apical apparatus and ascospore wall are based on PA-TCH-SP material. Conventional staining procedures with uranyl and lead salts proved to be less suitable, because they did not provide the amount of contrast needed for a study on wall substructure. The applied procedure modified from Thiéry (1967) gave satisfactory results.

The electron micrographs presented here show the result of the uranyl staining during the ethanol dehydration series plus the specific binding of silver to the reactive (i.e. 'PAS-positive') polysaccharides in PA-TCH-SP procedure. When the periodic acid reaction step is omitted from this procedure, the binding of silver to the fungal wall is practically inhibited, indicating that levels of endogenous aldehydes or aldehydes introduced during fixation are very low. Thus, in the walls – and this can be stated for the walls only – nearly all contrast is in fact reactivity and this term will therefore be used rather than electron density.

Longitudinal median sections of young, immature, mature, and dehiscent asci were studied. The lateral ascus wall and the apical apparatus are described. Some observations on the development of the ascospore wall are included.

Ombrophila violacea*The ascus wall*

The shape of the ascus apex varies from truncate-rounded to rounded. In the lateral ascus wall two layers are observed. The outer layer 65–90 nm thick consists of two strata: a very reactive outer stratum which is associated with the reactive material of an extra-ascan periascus and a moderately reactive inner stratum with a rather rough granular appearance (Fig. 1C, 2C). The inner layer 180–250 nm thick of the lateral wall has an inner part that is somewhat more reactive and varies considerably in thickness (arrow, Fig. 2D). The thickness of the inner layer increases abruptly in the apical apparatus (Fig. 1A). The outer layer does not increase in thickness here. There seems to be a decreased reactivity in it at the tip of the apparatus and, especially in the mature ascus, the outer stratum may even be partly absent (arrows, Fig. 1C).

Young ascus – An apical apparatus with some distinct characters is already present shortly after ascospore delimitation. The apical thickening, which is mainly formed by an abrupt increase of the inner layer shows a random network of very fine reactive material (Fig. 1A). The central cylinder shows a similar substructure. The narrow, very reactive but discontinuous annulus is associated with an annular protrusion surrounding an apical chamber (Fig. 1A). The greatest annular reactivity is found in the annular protrusion. The

Abbreviations used in Figures 1–9: **A**, annulus; **AC**, apical chamber; **AP**, annular protrusion; **AS**, ascospore; **AT**, apical thickening; **AW**, ascus wall; **CC**, central cylinder; **E**, epiplasm; **ER**, endoplasmic reticulum; **G**, glycogen; **IL**, inner layer of ascus wall; **im**, investing membrane; **is**, inner stratum of **OL**; **iz**, inner zone of **pw**; **L**, lipid body; **m**, mitochondrion; **N**, nucleus; **OL**, outer layer of ascus wall; **os**, outer stratum of **OL**; **oz**, outer zone of **pw**; **P**, periascus; **Pa**, paraphysis; **pw**, primary wall of **SW**; **SP**, sporoplasm; **SW**, ascospore wall; **sw**, secondary wall of **SW**; **V**, vacuole; **Ve**, vesicle.

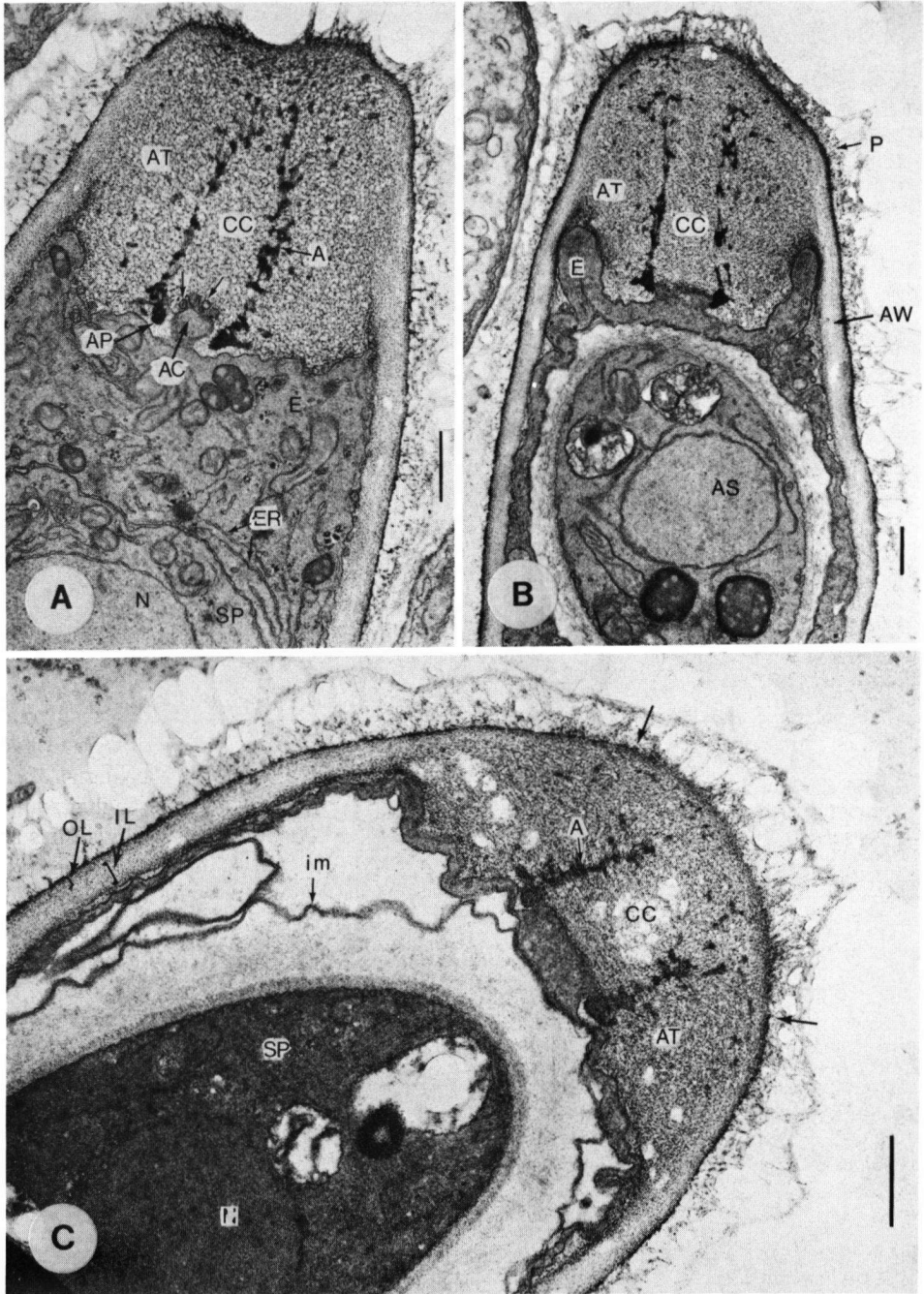


Fig. 1. *Ombrophila violacea*. Longitudinal median sections of apices in asci at different stages of development (bar equals 1 μ m). A. Young ascus, shortly after ascospore delimitation; B. immature ascus, ascospore wall development approximately halfway (compare Fig. 2C); C. mature ascus.

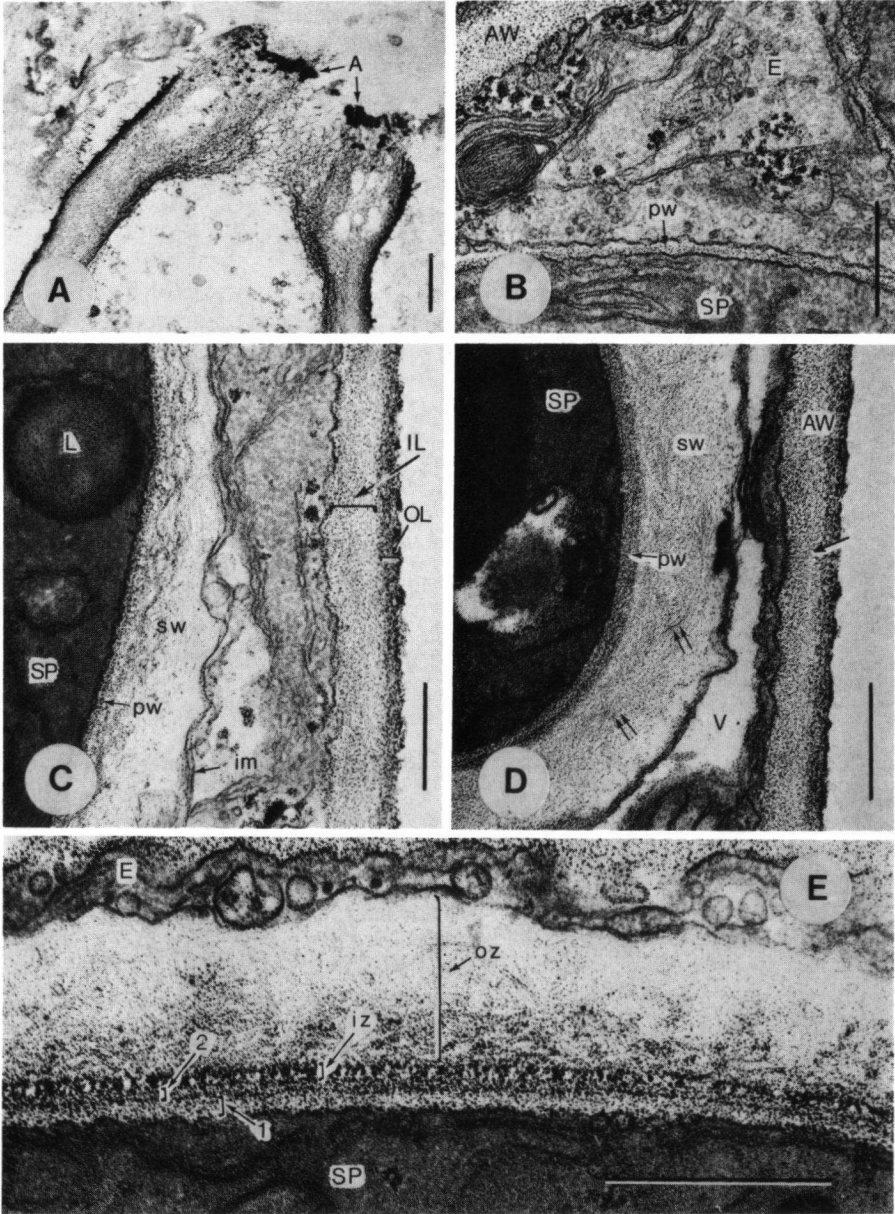


Fig. 2. *Ombrophila violacea*. A. Dehisced ascus (bar equals 1 μ m); B–E. development of ascospore wall (bar equals 0.5 μ m); B. development of primary wall; C, D. development of secondary wall; E. differentiation of primary and secondary wall.

patches of reactive material seem to spread out in the upper part of the annulus and are not found in the outer layer. Frequently some large vesicles are observed in the upper part of the apical chamber at this stage (arrows, Fig. 1A).

Immature ascus – The overall reactivity in the central cylinder slightly decreases. The boundary of the central cylinder with the epiplasm flattens (Fig. 1B). The irregularity of the boundary of the apical thickening with the epiplasm is probably caused by swelling.

Mature ascus – The apical apparatus is considerably compressed by the time the ascospore maturation is completed and the epiplasm is largely filled with vacuoles. The microfibrils in the central cylinder align in a 90° angle to the axis of the annulus. The annulus is a narrow, compact ring that seems to end abruptly on its upper side at a lower level than at the young and immature stage (Fig. 1C).

Dehiscid ascus – During discharge of the ascospores the annulus is everted over an angle of approximately 90°, hence giving the ascus apex a flattened appearance. Very little remnants of the central cylinder can be found. The remaining wall material swells and disintegrates very rapidly after dehiscence (Fig. 2A).

Ascospore wall development

At first the investing membrane and spore plasma membrane lie closely together and the first wall material appears in between these membranes shortly afterwards (Fig. 2B). This wall material is designated the primary wall. On further development the investing membrane is lifted from the primary wall by the deposition of a matrix substance. In this matrix 400–500 nm thick numerous reactive fibrils are found, at first mainly in the direct vicinity of the primary wall (Fig. 2C), later also in the outer parts of this matrix (double arrows, Fig. 2D). Finally, the primary wall (about 100 nm thick) becomes differentiated into two layers (1, 2 in Fig. 2E), while in the matrix of the secondary wall an inner zone (about 35 nm thick) of highly reactive bands is formed directly on the outer surface of the primary wall. It appears that this zone is formed from the deposition of the reactive material in the matrix (Fig. 2E). Shortly after discharge of the spores the secondary wall is still present, no longer surrounded by the investing membrane.

Neobulgaria pura

The ascus wall

Young asci could not be observed in the material available for this study. The shape of the ascus apex shows a distinct circular depression in the apical surface just over the annulus at the immature stage (Fig. 3A). In mature asci this depression is absent and the shape is rounded with a small flattened zone just over the central cylinder (Fig. 3B). The lateral ascus wall consists of two layers. In the outer layer approximately 60 nm thick two strata are observed. The outer stratum about 15 nm thick is very reactive and gives the surface of the ascus wall a rough appearance. The inner stratum about 45 nm thick is less reactive with a granular appearance (Fig. 4B). The outer layer is already considerably disintegrated towards the apex in the immature ascus (arrow, Fig. 3B). The inner layer 150–180 nm thick shows a fine granular reactivity. Its inner two-third to half is somewhat more reactive (Fig. 4B). The inner layer gradually thickens towards the apex to form the apical apparatus (Fig. 3A, B).

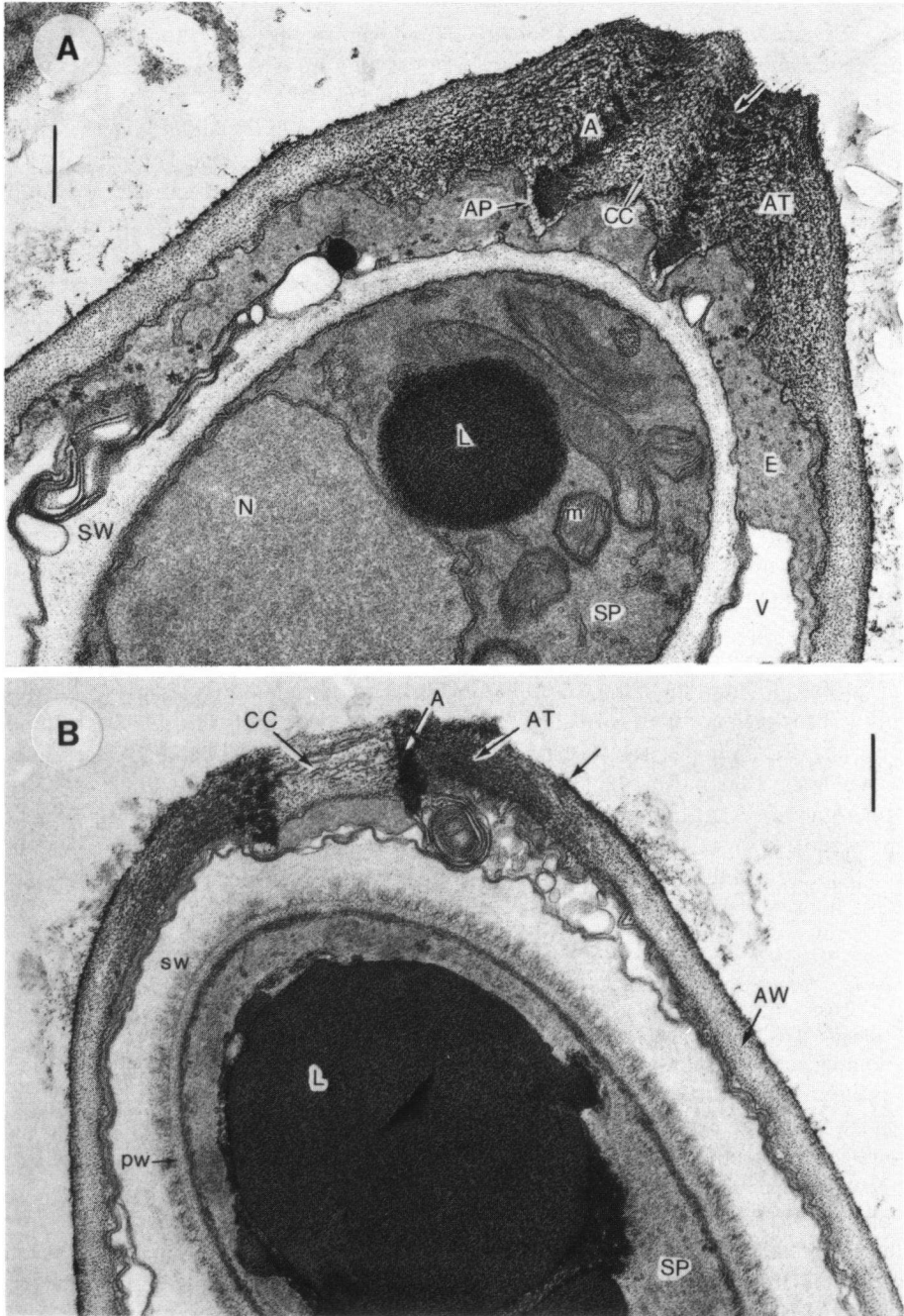


Fig. 3. *Neobulgaria pura*. Longitudinal median sections of apices of asci at different stages of development (bar equals 1 μ m). A. Immature ascus; B. mature ascus.

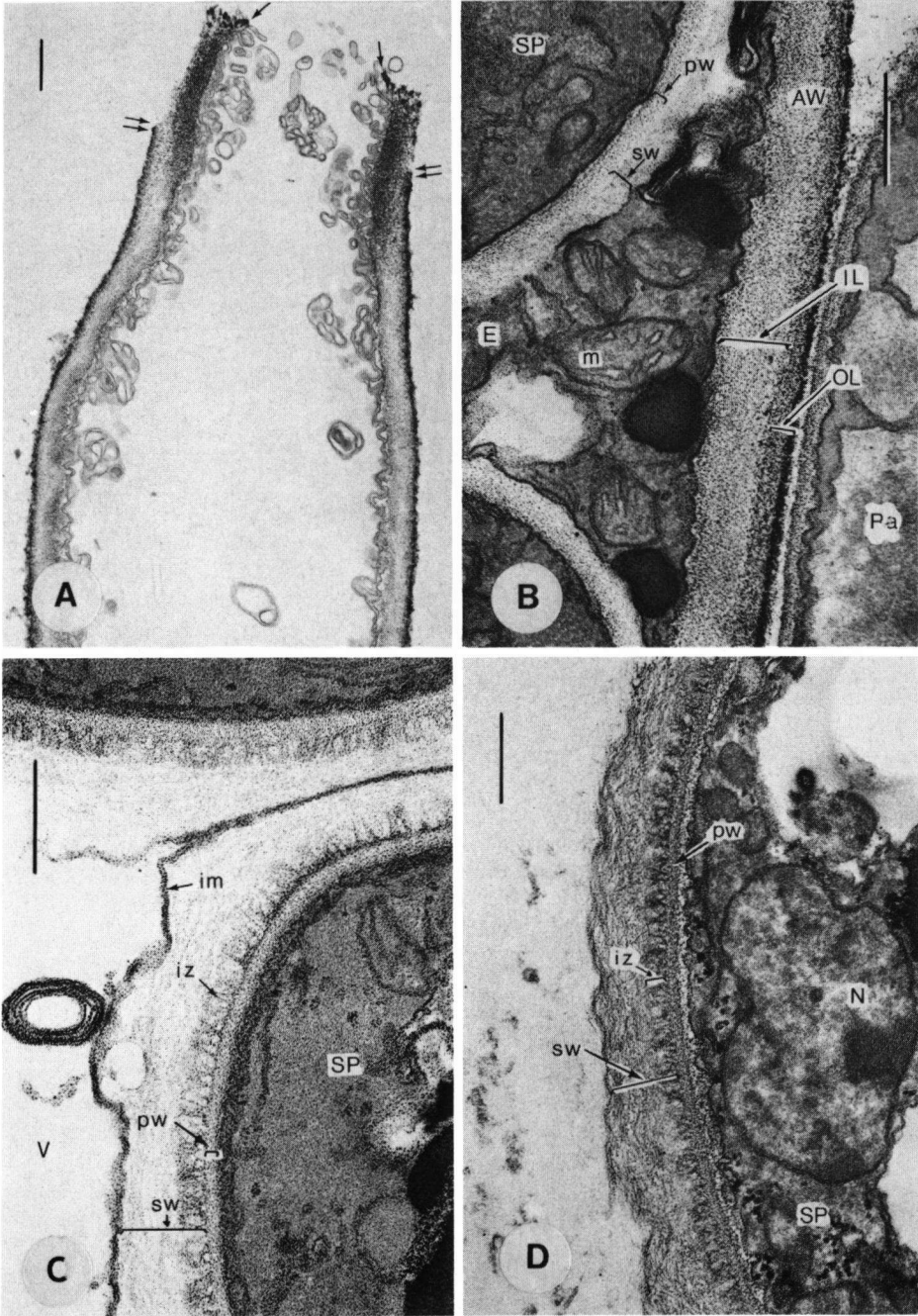


Fig. 4. *Neobulgaria pura*. A. Dehisced ascus (bar equals 1 μ m); B–D. development of ascospore wall (bar equals 0.5 μ m); B. development of primary and secondary wall; C. differentiation of secondary wall; D. ascospore after discharge.

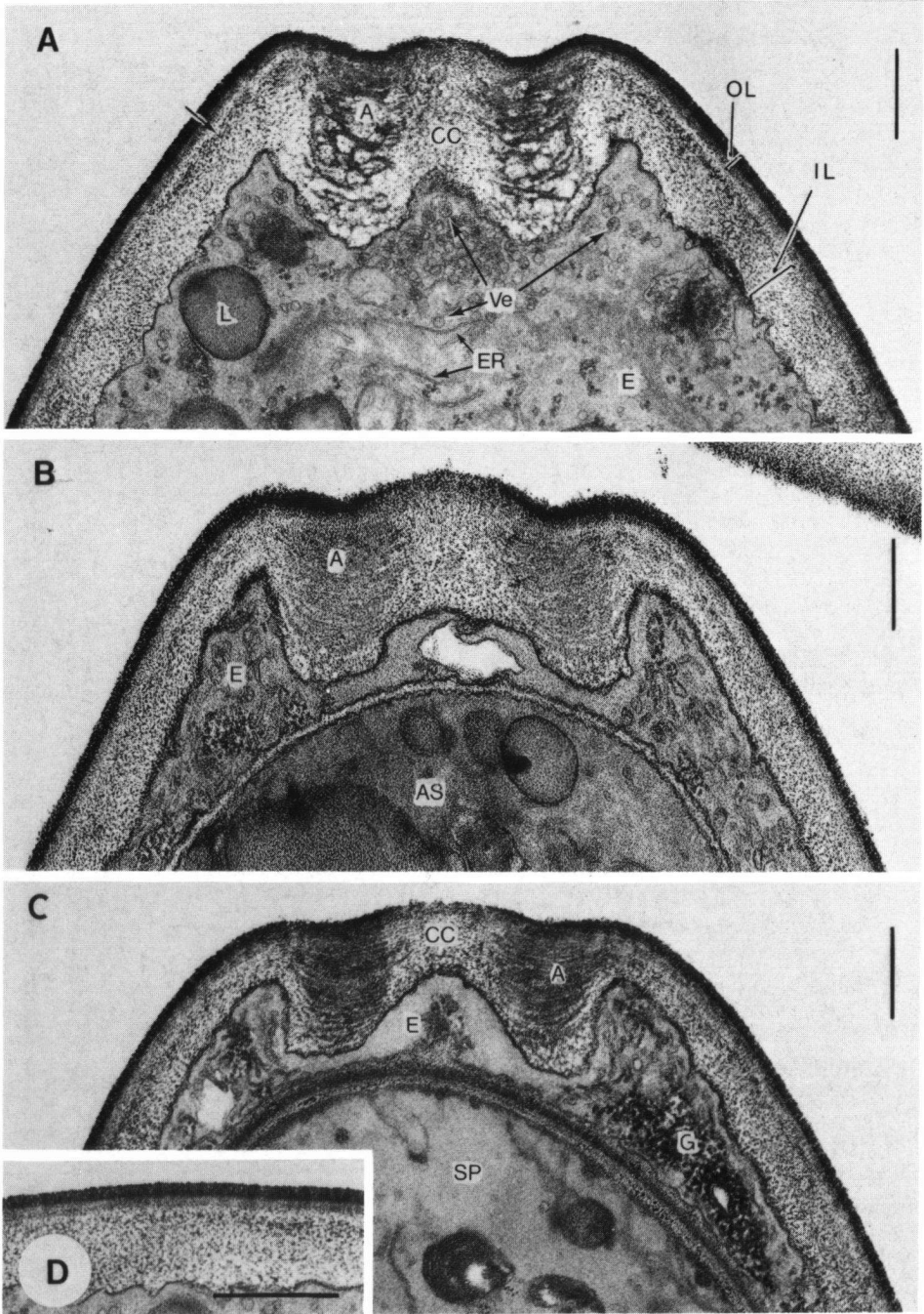


Fig. 5. *Bulgaria inquinans*. Longitudinal median sections of apices in asci at different stages of development (bar equals 1 μ m). A. Young ascus, before ascospore delimitation; B. immature ascus; C. as B, but more advanced state; D. detail of lateral ascus wall showing transverse microfibrils.

Immature ascus – The apical thickening which is mainly formed by a gradual increase of the thickness of the inner layer towards the apex contains several layers of very reactive material parallel to the ascus surface. This material increases in density to a variable degree in the annulus, giving it a rather irregular appearance (Fig. 3A). Also towards the inner side of the annulus the boundary with the central cylinder is less clear, because of the gradual decrease in reactivity here. The reactivity in the central cylinder is somewhat lower than in the apical thickening. Especially in the upper part of the annulus, where it increases in width the layered character is distinct (arrow, Fig. 3A), contrasting with the more compact mass of reactive material in the annular protrusion. The outer layer in the apical apparatus seems rather eroded. It does not fully cover the annulus and the central cylinder. The difference in reactivity that can be observed between an outer and inner zone of the inner layer in the subapical wall disappears in the apical thickening.

Mature ascus – When the ascospore walls are fully differentiated and the epiplasm is almost completely replaced by vacuoles the apical apparatus is strongly compressed (Fig. 3B). Furthermore changes in ultrastructure are observed: the reactive fibrils in the apical thickening lie closely together, parallel to the ascus surface. The annulus is more compact and the annular protrusion points straight downwards. The central cylinder has lost most of its reactivity and contains only a few distinctly reactive fibrils. The boundary of the central cylinder with the ascus lumen has flattened.

Dehisced ascus – In longitudinal section the inner layer points upwards surrounding a relatively large opening. The annulus is everted over an angle of approximately 90° during ascospore discharge. Its remnants are still clearly visible (arrows, Fig. 4A). The outer layer covers only the lower one-third part of the apical thickening (double arrows, Fig. 4A).

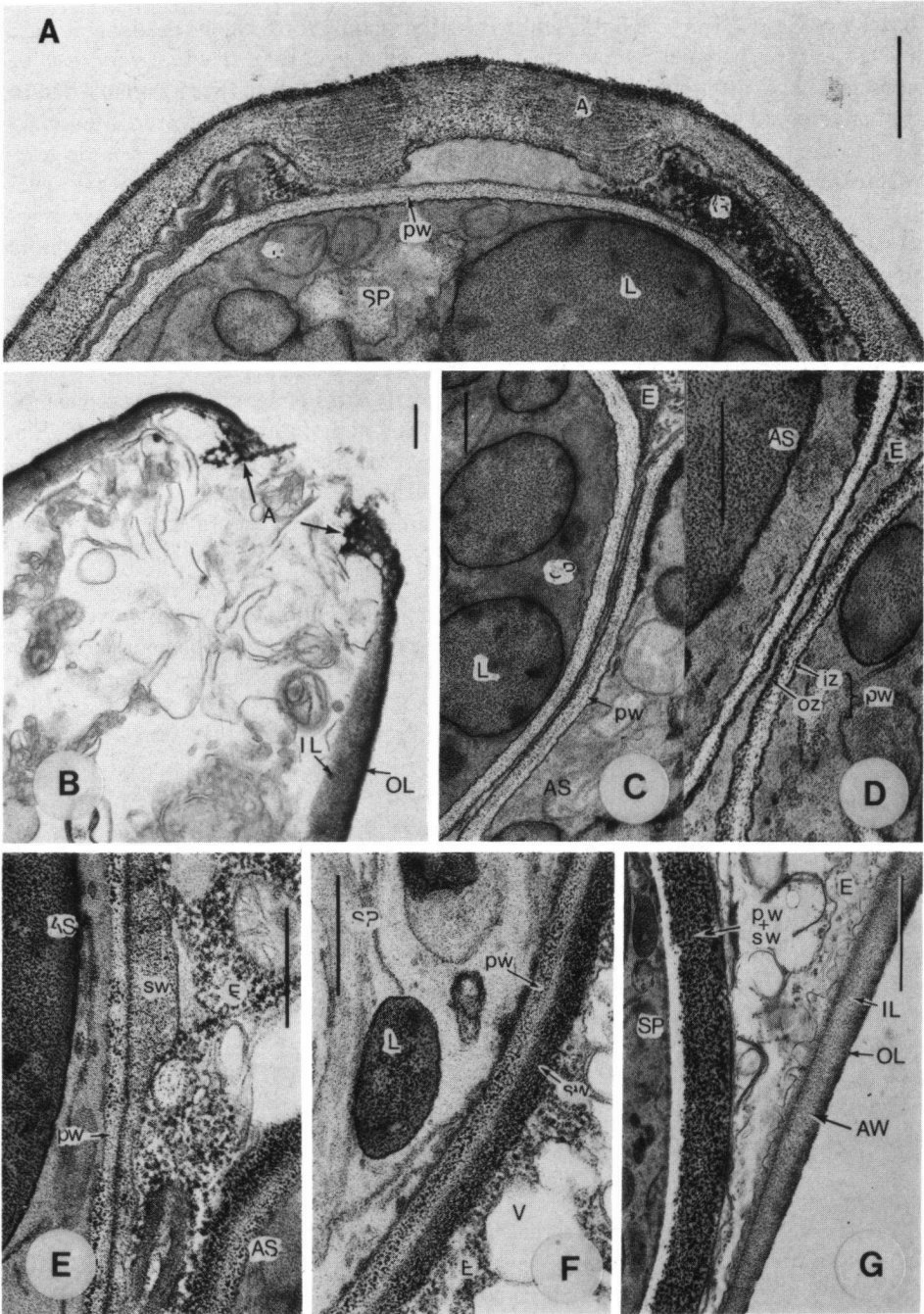
Ascospore wall development

In the youngest asci that could be studied there is already a considerable distance between the investing membrane and spore plasma membrane. A weakly reactive granular primary wall about 90 nm thick borders the spore plasma membrane and it is surrounded by an early secondary wall consisting of a matrix with a narrow non-reactive inner zone approximately 50 nm thick and an outer zone 100–200 nm thick containing moderately reactive fibrils (Fig. 4B). Later, in the inner zone of the secondary wall bristle-like reactive fibrils are deposited perpendicularly to the outer surface of the primary wall (Fig. 4C). The reactivity in the outer zone increases. Shortly after discharge of the ascospores the secondary wall is still present, no longer surrounded by the investing membrane (Fig. 4D).

Bulgaria inquinans

The ascus wall

Throughout the ascus development the circular depression in the apical surface over the annulus characterizes the shape of the ascus apex. In the lateral wall two layers can be observed. The outer layer about 120 nm thick is characterized by fibrils orientated perpendicularly to the surface of the ascus wall (particularly distinct in young and immature asci) (Fig. 5D). It seems to decrease markedly in thickness over the central cylinder, but it is difficult to find a clear boundary line with the inner layer there. The outer layer consists of a very reactive outer stratum and a less reactive inner stratum, both approximately 60 nm



thick (Fig. 5A). The inner layer 330–370 nm thick of the lateral wall shows a differentiation in reactivity in the part adjacent to the apical apparatus. There, a thin, lowly reactive outer zone and a considerably higher reactive inner zone ('strate annello-gène' of Chade-faud; arrow, Fig. 5A) can be distinguished. The reactivity of the inner zone decreases again inwards.

Young ascus – The apical thickening is formed by a rather abrupt increase of thickness of the inner layer. It is almost fully occupied by a broad annulus. Strictly, there is no annular protrusion (see definitions). In the upper part of the annulus the reactive material lies in parallel layers, while towards the base it is found in patches distributed at random (Fig. 5A). In the epiplasm near the apical apparatus high concentrations of vesicles containing reactive material are found in some asci (Fig. 5A). The central cylinder shows a fine granular reactivity, much like the subapical part of the inner layer (Fig. 5A).

Immature ascus – After the ascospore delimitation has been completed more of the reactive material in the annulus becomes oriented in fine fibrillar layers (Fig. 5B, C).

Mature ascus – The annulus now consists of very fine fibrils, oriented parallel to the ascus surface. The apical apparatus is compressed (Fig. 6A). Asci at corresponding stages of ascospore maturation sometimes show different stages of apex maturation (compare Figs. 5B and C).

Dehisced ascus – After dehiscence little remnants of the central cylinder can be found near the annular material. In longitudinal section the apical thickening and annulus show no eversion or any other marked change in position (Fig. 6B).

Ascospore wall development

In the earliest stage of ascospore wall development that could be observed wall material is deposited between the spore plasma membrane and investing membrane. This primary wall approximately 160 nm thick is of low reactivity and shows a fine granular line exactly in the middle separating two zones of about equal thickness (Fig. 6C). The outer zone soon differentiates into a layer of moderate to high reactivity (Fig. 6D). After this the investing membrane is irregularly lifted from the primary wall. In the resulting space secondary wall material 90–180 nm thick with a fine granular reactivity is deposited. In the beginning the reactivity in the secondary wall is lower than that of the outer zone of the primary wall (Fig. 6E). Some particles of reactive material are now present in the inner zone of the primary wall and soon in the secondary wall too. For some time the outer zone of the primary wall is practically devoid of these particles. The secondary wall now has a constant thickness of about 120 nm (Fig. 6F).

Finally, just before ascospore discharge the whole wall is incrustated with these large reactive particles (Fig. 6G).

Fig. 6. *Bulgaria inquinans*. A, B. Longitudinal median sections of ascus apices (bar equals 1 μ m); A. mature ascus; B. dehisced ascus; C–G. development of ascospore wall (bar equals 0.5 μ m, except G, 1 μ m); C. early development of primary wall; D. advanced development of primary wall; E. development of secondary wall and differentiation of primary wall; F. advanced differentiation of primary wall and advanced development of secondary wall; G. mature ascospore, differentiation of primary and secondary wall completed.

DISCUSSION

Although developed as a specific reaction for PAS-positive polysaccharides by Thiéry (1967), the PA-TCH-SP procedure has been used mainly for improvement of contrast in a number of studies on ascus wall ultrastructure of various taxonomic groups (Bellemère, 1977; Beckett, 1981a). Technical problems concerning the specificity of this reaction were not directly relevant to these studies.

In light microscopy, the ascus apices of *Ombrophila violacea*, *Neobulgaria pura*, and *Bulgaria inquinans* show regions blueing in Lugol's iodine or other iodine solutions (Dennis, 1978; Baral, 1987; and own observations). These regions correspond to the highly reactive annular structures observed in electron microscopy. In studies of the ascus apex one must be careful comparing details of light microscopic images with those of electron microscopic images.

The terminology used here for the description of the lateral ascus wall is in accordance with the one used in several other studies on operculate and inoperculate discomycetes (Schrantz, 1970; Griffiths, 1971; van Brummelen, 1981). Basically, the lateral ascus wall of the species under study contains an inner and outer layer in which strata can be designated. If the differentiation within the layers is not continuous in larger parts of the walls it is preferred to speak of zones. In his ultrastructural study of Helotiales Bellemère (1977) used a concept in which four layers in the ascus wall are distinguished, assuming the outer two, a and b, to correspond to the 'exoascus' and the inner two, c and d, to correspond to the 'endoascus' as described for light microscopy by Chadefaud (1973). The present results do not allow such a strict division to be utilised objectively.

A partly new terminology for the apical apparatus is introduced in the present study. It is a preliminary one and it may be adapted or extended in future studies if necessary. The terminology applied for the ascospore wall agrees with the work by Carroll (1966), Merkus (1976), Beckett (1981b), and van Brummelen (1986, 1989).

In the three species under study the apex formation, i.e. addition of wall material to the apical wall, is completed before ascospore delimitation. In a detailed study of this process in *Xylaria longipes* Beckett & Crawford (1973) described an apical body and a surrounding vesicle system. In *Bulgaria inquinans* no apical bodies were observed, but the high concentration of vesicles (Fig. 5A) resembles the vesicle system of *Xylaria longipes*.

Once apex formation has been completed, the wall ultrastructure in the apical apparatus continually changes on further ripening of the ascus. As yet little attention has been paid to the process of apex maturation. It is found that details of this process of maturation are essential for a comparative study of the Ombrophiloideae.

The relative reactivity and the interpretation of wall substructure in the apical apparatus of the asci are depicted in diagrammatic schemes (Fig. 7–9). Two patterns of apex maturation can be distinguished. In *Ombrophila violacea* and *Neobulgaria pura* the annulus condenses to a homogeneous, very reactive narrow ring occupying only a small part of the apical thickening. In *Bulgaria inquinans* the pattern is characterized by a reduced reactivity and an ordered arrangement of the microfibrils in the annulus occupying most of the apical thickening. In *O. violacea* and *N. pura* the structure of the outer layer of the ascus

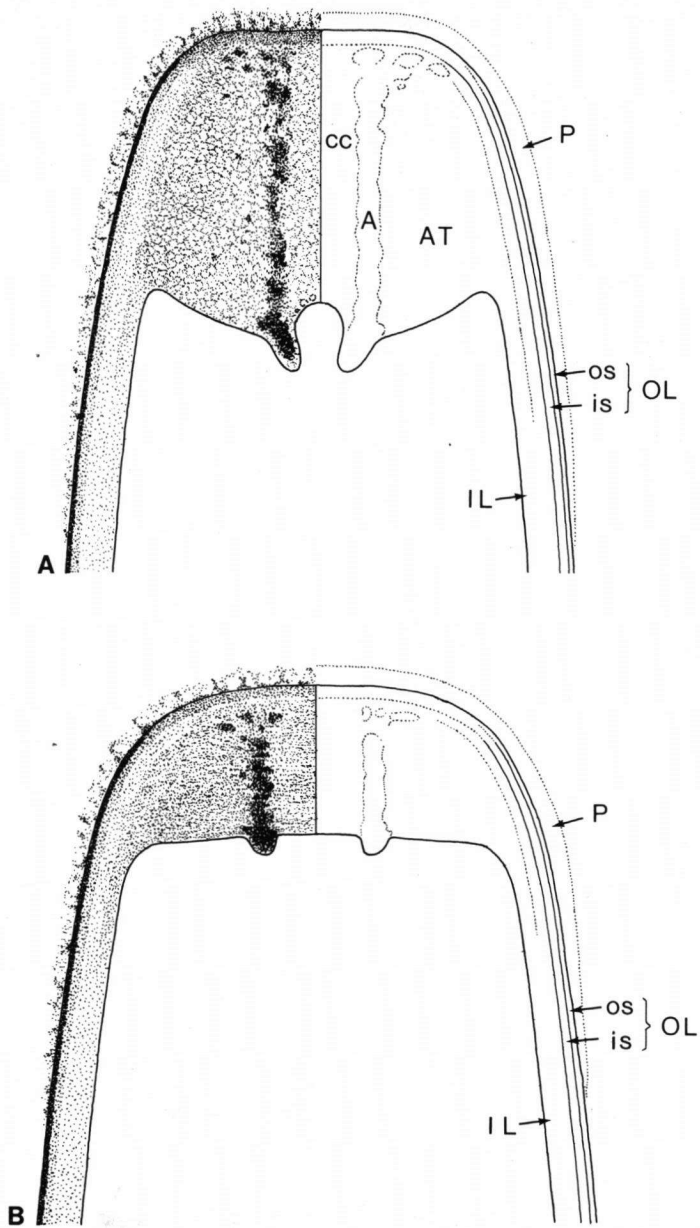


Fig. 7. Diagrammatic schemes of young (A) and mature (B) asci of *Ombrophila violacea*, demonstrating relative PA-TCH-SP reactivity on the left half and corresponding interpretation of layers on the right half of each scheme. See also the next two pages.

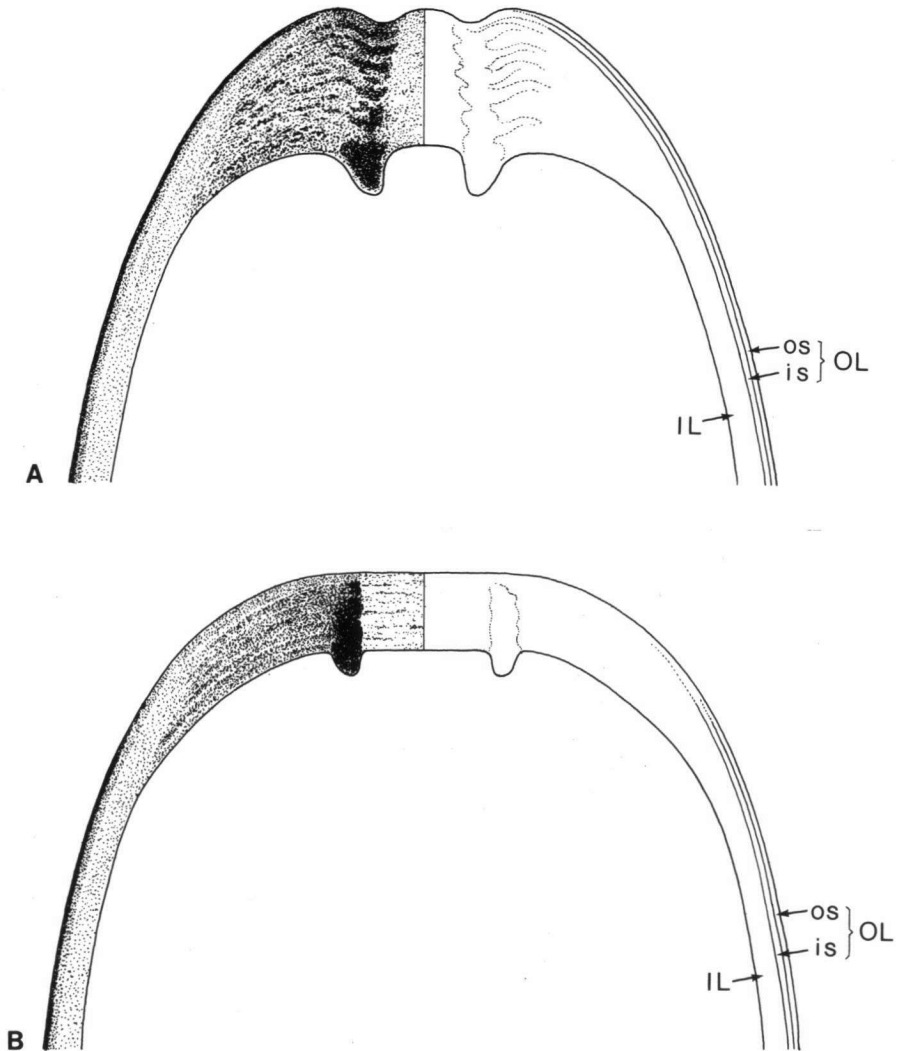


Fig. 8. Diagrammatic schemes of immature (A) and mature (B) asci of *Neobulgaria pura*, demonstrating relative PA-TCH-SP reactivity on the left half and corresponding interpretation of layers on the right half of each scheme.

wall is relatively simple, but in *B. inquinans* a rather remarkable outer layer with a transverse orientation of its constituents occurs. In *O. violacea* and *N. pura* the dehiscenced ascus is characterized by a 90° eversion of the annulus, while in *B. inquinans* no eversion is found. Furthermore, *O. violacea* and *N. pura* show similarities in development of the ascospore wall, while they both differ in this respect from *B. inquinans*. So, the ultrastructural data justify the conclusion that *O. violacea* and *N. pura* are closely related, while *B. inquinans* has no affinities that close to either of these two species.

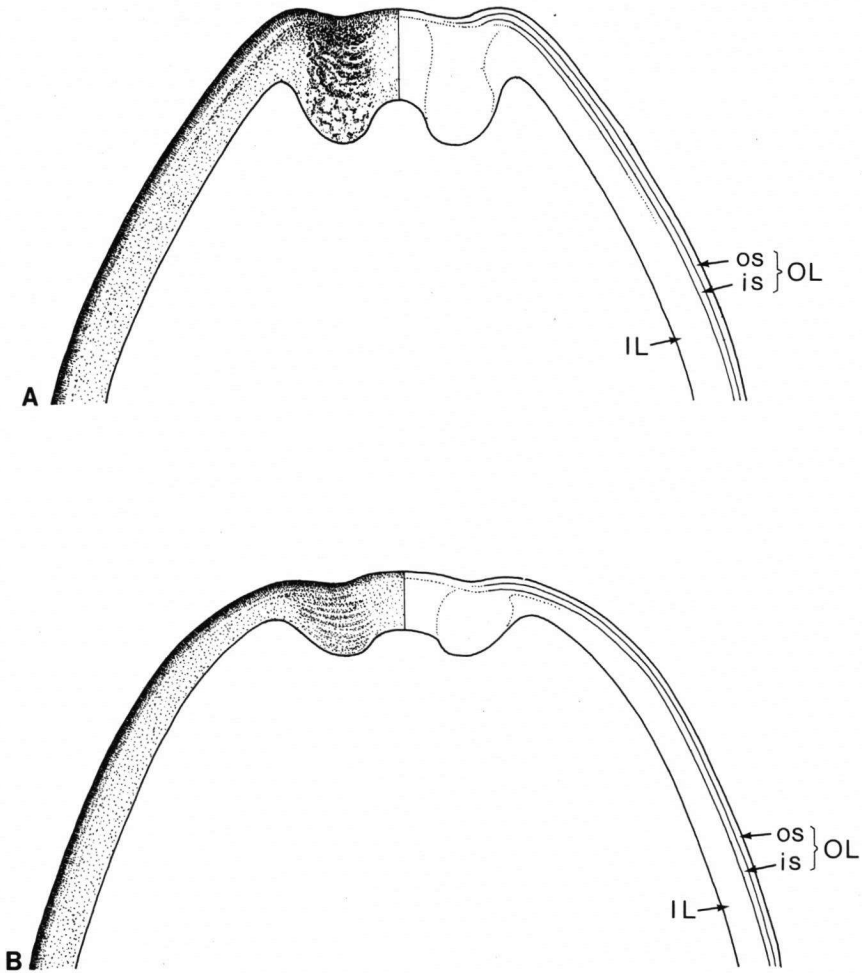


Fig. 9. Diagrammatic schemes of young (A) and mature (B) asci of *Bulgaria inquinans*, demonstrating relative PA-TCH-SP reactivity on the left half and corresponding interpretation of layers on the right half of each scheme.

The only data available on the apical ultrastructure in *N. pura* were published by Bellemère (1977). He shortly mentioned the species, stating it to be of the *Sclerotinia*-type. It is difficult to form an opinion about the substructure in the apical apparatus from the only electron micrograph shown. In the present study of *N. pura* it was not possible to clearly indicate in the apical apparatus a line of demarcation between two possible strata in the inner layer that could correspond to Bellemère's layers c and d. The eversion of the annulus during ascus dehiscence in *N. pura* found in the present study was also reported by

Bellemère (1977). This eversion is considered to be one of the distinctive characters of Bellemère's *Sclerotinia*-type. In *O. violacea*, not previously studied ultrastructurally, the inner stratum of the inner layer seems to be the most important one in the apical thickening. If it is assumed that this inner stratum corresponds to Bellemère's d layer, the apical apparatus would belong to the *Sclerotinia*-type sensu Bellemère.

Students of the ultrastructure of asci in species of *Sclerotinia* (*S. sclerotiorum*: Codron, 1974; Bellemère, 1977, and *S. tuberosa*: Schoknecht, 1975) did not report on changes related to apex maturation and it is not clear which stages are shown in the micrographs they published in their papers. Therefore it is difficult to make a good comparison.

The outer and inner strata of the outer layer in the ascus wall of *B. inquinans* are likely to correspond to the layers a and b as designated by Bellemère (1977). He clearly showed a "brosse apicale" over the apical apparatus that could not be observed in the specimens used in the present study. Bellemère did not mention the peculiar substructure of the outer layer in *B. inquinans*, although he stated earlier that as a general feature of the b-layer of the ascus wall "ses constituants sont orientés perpendiculairement à la surface de l'asque" (Bellemère, 1975). His interpretation of the layers c and d (inner layer?) cannot be confirmed here. There is a distinct differentiation in the inner layer in the subapical part of the ascus wall. The reactive zone (arrow, Fig. 5A) was called "strate annello-gène" by Chade-faud (1973) on the basis of Bellemère's micrographs. It is not clear why Bellemère (1977) excluded this zone from the scheme of his *Bulgaria*-type.

At this stage it can be concluded that *B. inquinans* certainly has a type of apical apparatus of its own. This was also recognized by Bellemère (1977), but additional features especially those concerning apex maturation need to be included in a schematic characterization of the type of apical apparatus in asci of *B. inquinans*.

Dennis (1978) mentioned the strong resemblance between *Ombrophila* and *Neobulgaria*, expecting the genera to be reunited in the future. Yet he did treat them separately, emphasizing differences in ascocarp anatomy. The proposal of Baral & Krieglsteiner (1985) to fuse *Neobulgaria* and *Ombrophila* is interesting in this respect. The present ultrastructural data confirm a close relation between *O. violacea* and *N. pura*, but it is too early to draw final conclusions concerning the taxonomic status of the two genera. The variation in ultrastructure of the apical apparatus and the development of the ascospore wall at the generic and family level within the Leotiales is still insufficiently known. For this reason a reconsideration on the position of *Bulgaria* within the Leotiaceae (Helotiaceae sensu auct.) will have to wait as well. Ultrastructural features of the ascus and features pertaining to ascocarp ontogeny and anatomy should be integrated into a modern system for the Ascomycotina. The data on ascus ultrastructure presented here do not confirm the relatedness suggested by the present arrangement of *Bulgaria* together with *Ombrophila* and *Neobulgaria* within the tribe Ombrophiloideae.

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