

Morphological and histochemical differentiation of the pollen wall of *Betula pendula* Roth. during dormancy up to anthesis

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Summary. Anthers of *Betula pendula* were collected at regular intervals during the dormancy period until anthesis. Ultrathin sections of maturing pollen grains were especially stained for polysaccharides and proteins and examined with TEM to determine whether structural or/and chemical changes in the pollen wall occur during the dormancy period of the plant life cycle. At the beginning of the dormancy period, the microspore wall consists of a well developed tectum, columellae and a foot layer. Spinules and supratectal elements are prominent. Microchannels are present in the tectum but not obvious in the foot layer. Some of the columellae are not clearly connected with the foot layer, but some connections are evident. Pores are filled with a thick fibrillar network "flocculent material". The cytoplasm is packed with starch grains and lipid globules. The stainability for acidic and neutral polysaccharides and protein was tested, and variations in the pollen wall are illustrated. As temperature increased towards the end of dormancy and before anthesis there is obvious differentiation in the morphology of the pollen wall. The granular fibrillar layer beneath the pore and the Zwischenkörper are the most variable part of the wall. Different histochemical reactions observed in different layers at the aperture sites indicate different functions of these layers.

Keywords: *Betula pendula*; Histochemistry; Pollen wall; Dormancy.

Introduction

The genus *Betula* includes c. 50 species, distributed mainly in cold parts of the northern hemisphere (Heywood 1978). *Betula pendula* Roth. = *B. verrucosa* Ehrh. (white birch), a common tree in the eastern part of Sweden, is among the most widespread species causing allergy. The male flowers are arranged in 3–6 cm drooping catkins and the female flowers in erect cylindrical catkins up to 4 cm long. *Betula* trees show

an active vegetative growth from spring through autumn and they have a dormancy phase in winter. The development of *Betula* pollen before and after dormancy was examined in detail by Dunbar and Rowley (1984). Other studies mainly concern the morphology and ultrastructure of mature *Betula* pollen grains (e.g., Erdtman et al. 1962, Doyle et al. 1975, Rowley 1981, Claugher and Rowley 1987).

Our main objective of this study was to demonstrate changes in the morphology and histochemistry of the wall of *Betula pendula* microspores and pollen grains during dormancy and until anthesis. It was also our aim to find out time and location of protein formation in the developing wall of *Betula* pollen.

Material and methods

The material for this study was collected from a *Betula* tree growing close to the Palynological Laboratory, Stockholm, during the winter period 1989–1990. The winter in this year was exceptionally mild. The dormancy period was comparatively short and the anthesis occurred at the beginning of April, about one month earlier than usual. Catkins were collected on: 20th Nov. 1989, 14th Dec. 1989, 16th Jan. 1990, 9th Feb. 1990, 9th March 1990, and 30th March 1990.

The anthers were fixed in 2% GA in 0.05 M Na-cacodylate buffer, pH 7.4, at room temperature for 24 h; some anthers were postfixed with OsO₄, while others were not. All anthers were dehydrated in a series of acetone, embedded in Spurr's medium (Spurr 1969) and sectioned. Ultrathin sections were stained in the following manner:

(1) General pollen wall stain: sections treated with 1% aqueous uranyl acetate for 5 min, washed with distilled water, and floated on lead citrate stain for 5 min.

(2) For protein: Sections on gold grids were stained with 5% phosphotungstic acid (PTA) in 10% acetone for 15 min (Benedetti and Bertolini 1963, Marinozzi 1968).

(3) For acidic polysaccharides: Sections on gold grids were stained

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with 0.1% PTA in 10% chromic acid for 5 min (Pease 1968, Marinuzzi 1967).

(4) For neutral polysaccharides and other carbohydrates: Sections on gold grids were treated for 30 min in 0.2% periodic acid (PA), 20 min in 0.2% thiocarbonylhydrazide (TCH), and 20 min in 1% silver proteinase (Thiery 1967, Rowley and Dahl 1977). As a control, sections were stained with TCH-silver proteinase without prior oxidation with PA and other sections were stained with only silver proteinase. TCH-silver proteinase stain following fixation with osmium was used to estimate the location of unsaturated lipids (Rowley and Dahl 1977).

Results

The results are arranged following the sequence of the dates catkins were collected. The temperatures are daily minimum and maximum.

20th November 1989, 0 °C / 3 °C

This stage probably represents a mid-microspore period with central nucleus (Fig. 1). The cytoplasm was packed mainly with lipid globules. The microspore wall was well developed, consisting of tectum ca. 0.5 µm thick, columellae and foot layer ca. 0.3 µm thick. The columellae were numerous, radially oriented (Figs. 2 and 3) and the intercolumnellar void contained fibrillar material (Fig. 3). The microchannels of the exine were generally masked and not easy to observe if OsO₄, UA-Pb stain was applied (Figs. 1 and 2), but if TCH-silver proteinase or PTA-acetone stain was applied, the microchannels were clearly visible (Figs. 6 and 7). A thick fibrillar network was observed at the pores (Figs. 2, 4, and 5). It is similar to the fibrillar-flocculent material described by Dunbar and Rowley (1984) surrounding the cytoplasm of *Betula pendula* during dormancy. The exine is stained moderately with PA-TCH-silver proteinase for neutral polysaccharides (Figs. 4 and 5). If periodic acid (PA) reaction was not used, the stainability of the exine increased (Fig. 6). The exine stained moderately for protein (Fig. 7) and densely for acidic polysaccharides (Fig. 8). A fine granular-fibrillar layer was visible at the proximal surface of the exine, thick under the aperture and thin at the interapertural parts (Figs. 2, 5, 7, and 8).

14th December 1989, -20 °C / -13 °C

The microchannels of the exine were clearly visible in this sample (Fig. 9), and even more obvious in material fixed without OsO₄ (Figs. 10 and 11). Microchan-

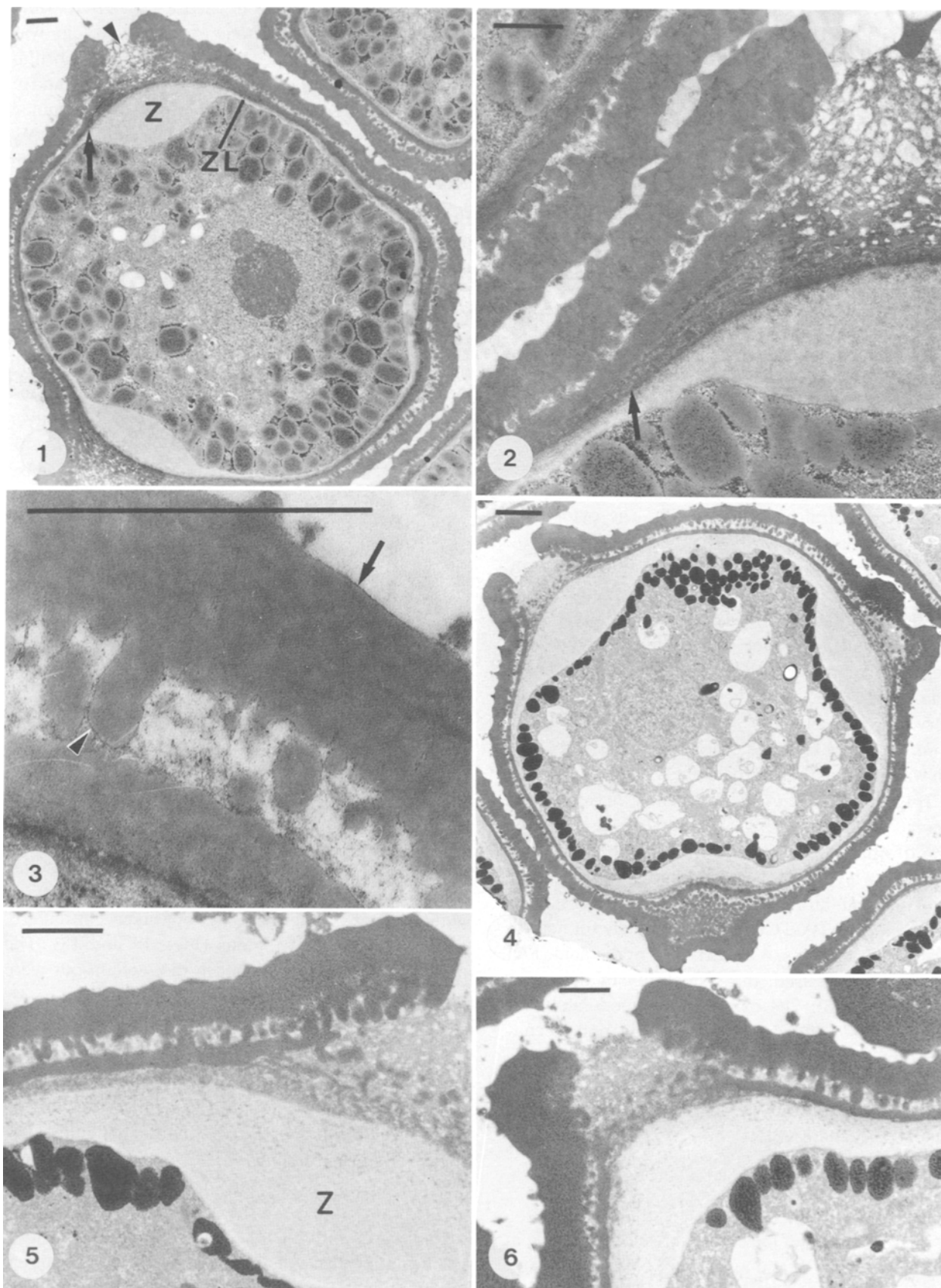
nels containing electron dense material were observed when PTA-acetone stain for protein was applied (Figs. 14 and 15). The fine granular-fibrillar layer between Zwischenkörper and the annulus and at the proximal surface of interapertural exine stained dark with OsO₄-UA-Pb (Fig. 9), and stained faintly with OsO₄-PTA-acetone (Fig. 14), but very dark with PTA-acetone without OsO₄ (Fig. 15). In section stained with TCH-silver proteinase to emphasize osmium and thus the possible sites of unsaturated lipids, the annulus, and the granular-fibrillar layer beneath the aperture were darkly stained (Fig. 12). The silver proteinase control moderately stains the exine, and the aperture units, which is considered to indicate the presence of phenols in the pollen wall (Fig. 13). The Zwischenkörper was not contrasted with OsO₄, UA-Pb (Fig. 9) and OsO₄-TCH-silver proteinase (Fig. 12), while it is stained darkly with OsO₄-PTA-acetone (Fig. 14) and even more dense with PTA-acetone without OsO₄ (Fig. 15). The latter stain showed the presence of a dark fibrillar network within the Zwischenkörper and the Z-layer. The exine and the units of the aperture stained densely for acidic polysaccharides, while the Zwischenkörper stained weakly (Fig. 16).

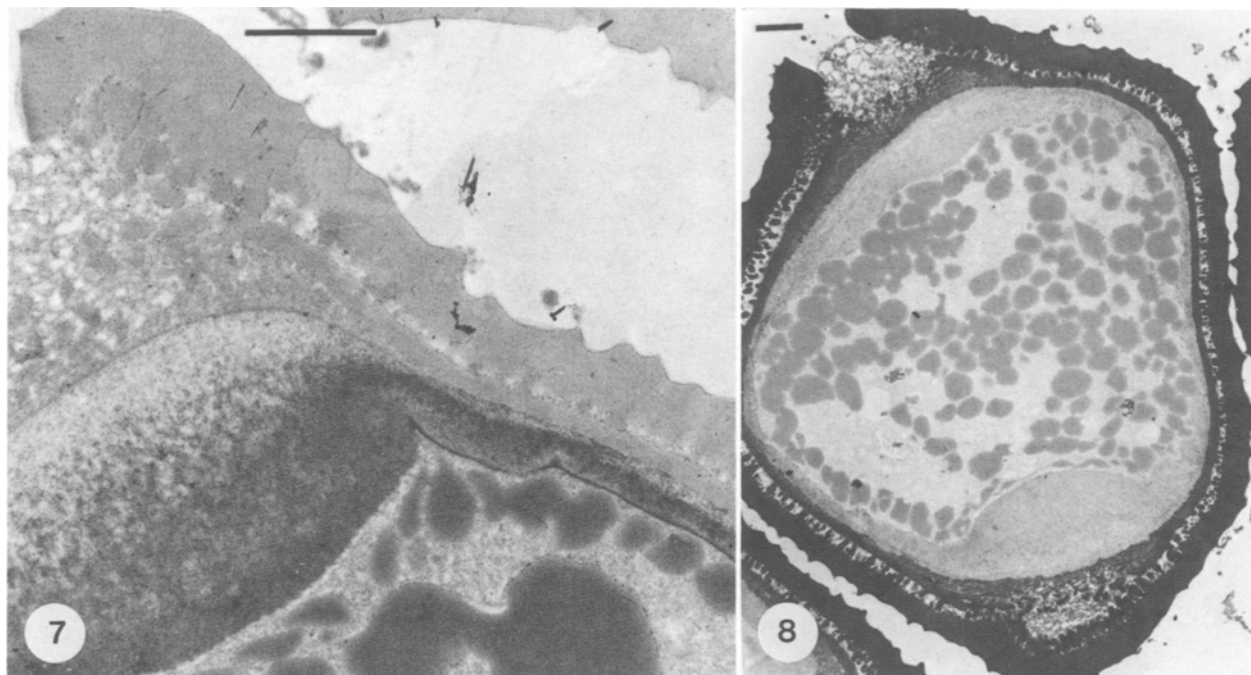
16th January 1990, 3 °C / 4 °C

The microspore's exine showed similar staining as in the above sample. The Zwischenkörper is reduced slightly in size and the fibrillar-flocculent material in the pores became less dense (Figs. 17–20). The test for protein was positive both in the exine and in the aperture (Figs. 19 and 20). The aperture region appeared protuberant (Fig. 19). Several columellae were connected, while some columellae appeared without clear contact with the foot layer, particularly in the mesoapertural regions (Figs. 17 and 18). The microchannels were distinct in the tectum, but were not visible in the foot layer (Figs. 18 and 20).

9th February 1990, -2 °C / 5 °C

The end of dormancy period is probably indicated in this sample. The cytoplasm contained few starch grains and a limited number of lipid globules. The height of the aperture increased, and the fibrillar-flocculent material at the pore expanded (Figs. 21 and 22). Microchannels were evident in the tectum, and spinules were prominent. At this time the deposition of the intine beneath the Zwischenkörper is started (Figs. 21 and 22).





Figs. 7 and 8. Collected on 20th November 1989

Fig. 7. The exine is stained moderately, while the proximal portion of the Zwischenkörper and Z-layer are darkly stained. Few electron dense material is observed in the microchannels of the tectum. Stain: OsO₄, PTA-acetone. $\times 17,000$

Fig. 8. Note intense stainability of the annulus, the exine and the granular-fibrillar layer beneath the exine, for acidic polysaccharides, while Zwischenkörper and Z-layer are weakly stained. Stain: PTA-chromic. $\times 5,800$

9th March 1990, 0.3 °C / 7 °C

This sample included microspores in late vacuolate stage probably just before mitosis (Fig. 23). Anthers with binuclear pollen grains were observed in the same catkin (Fig. 25). The generative cell is associated with the intine (Fig. 25). The fibrillar-flocculent

material at the pore was not visible anymore (Figs. 23 and 24). The cytoplasm is packed with starch grains and lipid globules (Fig. 25). The Zwischenkörper was much reduced in thickness and simultaneously the apertural region reduced in height (Figs. 23 and 24). The intine got thicker at the sites of the apertures and started to develop at interapertural parts. The granu-

Figs. 1–6. Collected on 20th November 1989

Fig. 1. Young microspore. The cytoplasm contains numerous lipid globules. The exine and the annulus are well developed. Zwischenkörper (Z) and Z-layer (ZL) are unstained. Note granular fibrillar layer (arrow) and fibrillar-flocculent material at the pore (arrowhead). Stain: OsO₄, UA-Pb. $\times 5,400$

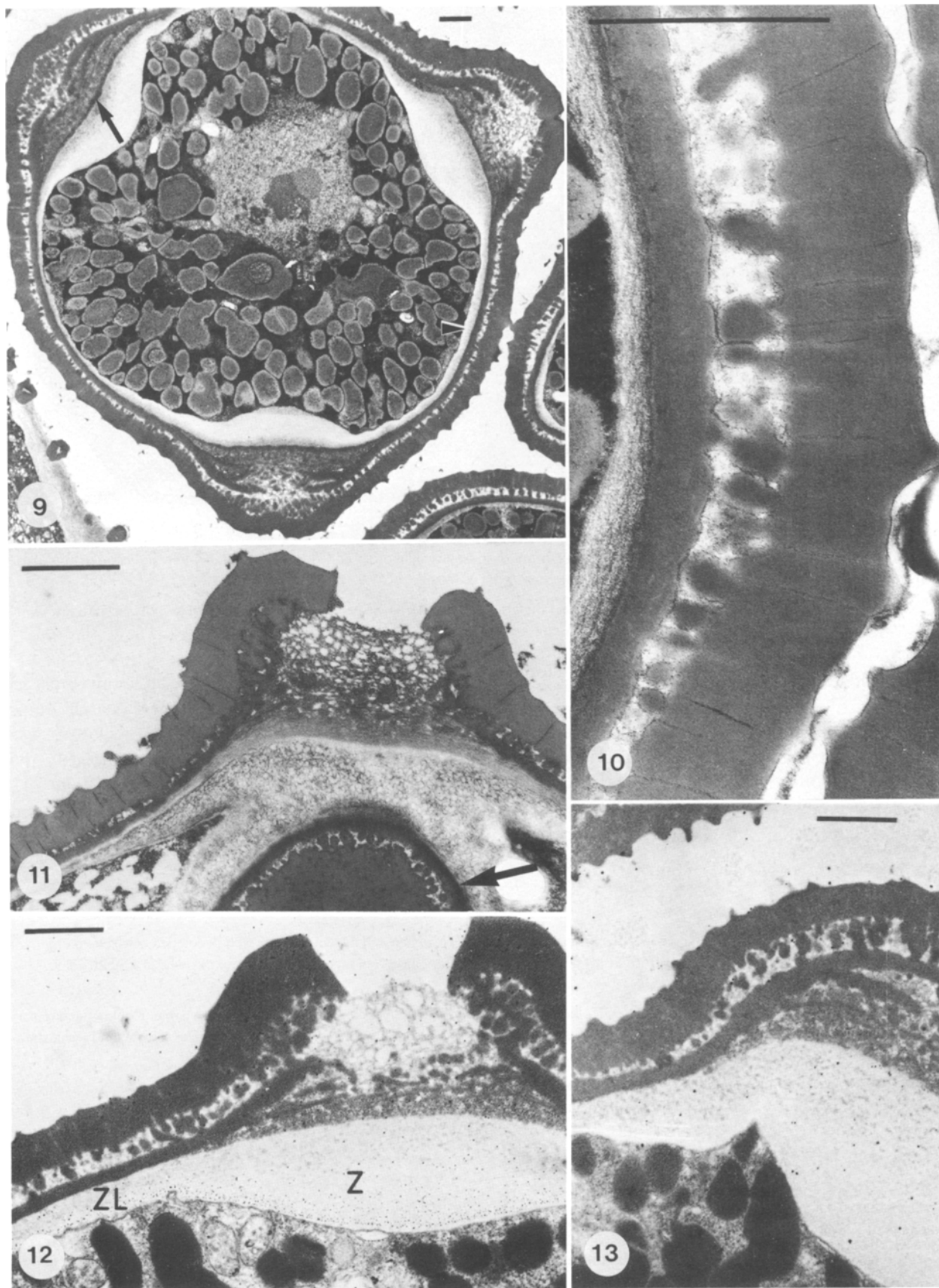
Fig. 2. Detail of the pore, thick annulus with many endexinous lamellae. The microchannels in the tectum are not visible. Columellae are narrowly spaced. The fibrillar-flocculent material is thick at the pore. Note dense stainability of the granular-fibrillar layer (arrow) and weak stainability of Zwischenkörper and Z-layer. Stain: OsO₄, UA-Pb. $\times 12,000$

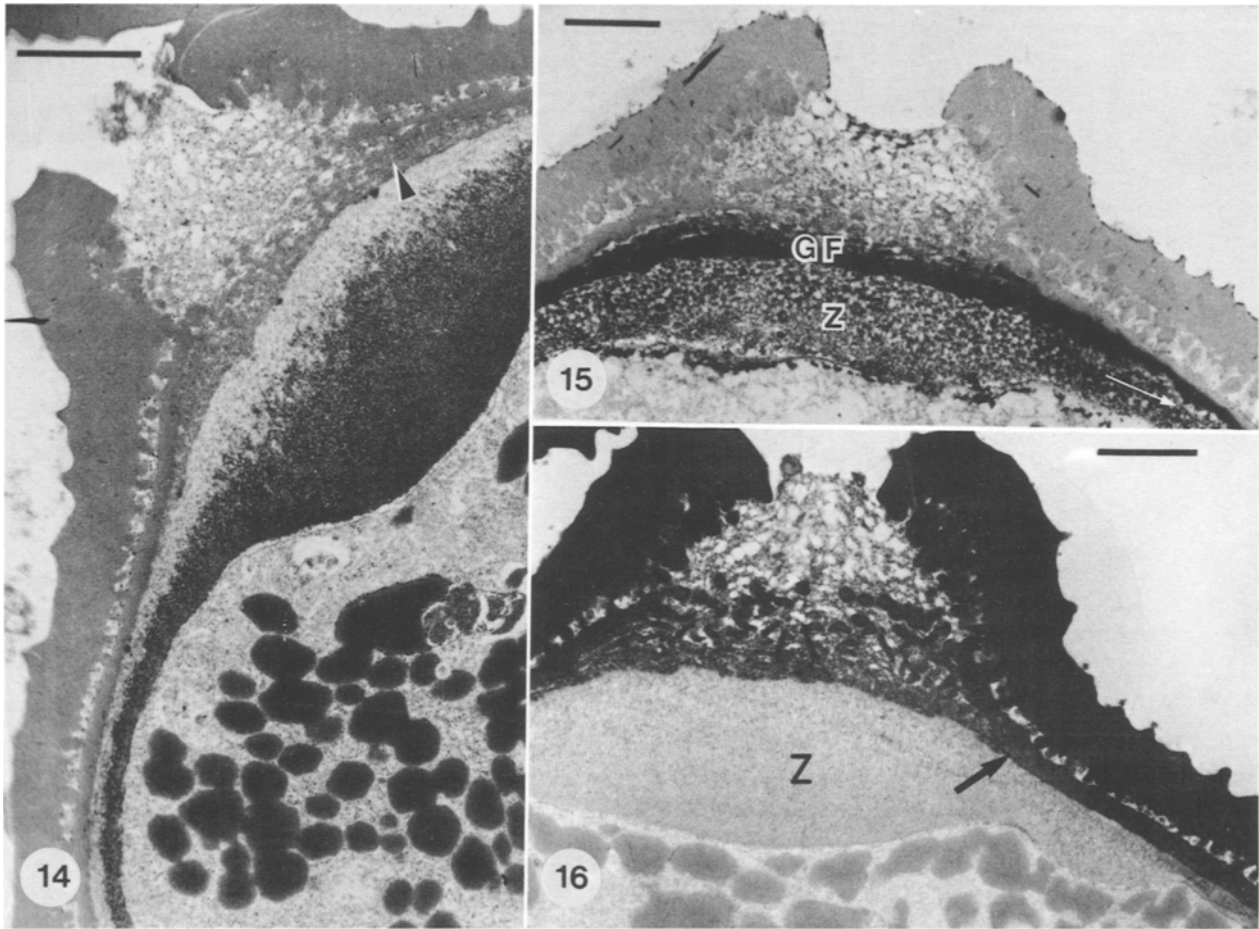
Fig. 3. Detail of interapertural wall showing osmiophilic material in the voids between the columellae and in the microchannels traversing the tectum. Note electron dense coat on the surface of the tectum (arrow) and on distal surface of foot layer (arrowhead). Stain: OsO₄, UA-Pb. $\times 60,000$

Fig. 4. The exine is stained moderately with PA-TCH-silver proteinate for neutral polysaccharides, while Zwischenkörper and Z-layer are faintly stained. Stain: PA-TCH-silver proteinate. $\times 8,500$

Fig. 5. The exine is stained moderately with PA-TCH-silver proteinate for neutral polysaccharides, while Zwischenkörper (Z) and Z-layer (ZL) are stained faintly. Stain: PA-TCH-silver proteinate. $\times 14,000$

Fig. 6. The exine is densely stained by TCH-silver proteinate, while Zwischenkörper and Z-layer are less stained than with PA-TCH-silver proteinate. Stain: TCH-silver proteinate. $\times 8,500$





Figs. 14–16. Collected on 14th December 1989

Fig. 14. The material between the lamellae of the annulus, the granular-fibrillar layer (arrowheads), the proximal portion of Zwischenkörper and Z-layer are densely stained. Note the heterogeneous stainability of Zwischenkörper and Z-layer. Stain: OsO₄, PTA-acetone, $\times 16,000$

Fig. 15. The exine is stained moderately and the compact granular-fibrillar material (GF) beneath the exine is stained very dense. The stain reveals the presence of electron dense fibrillar network within Zwischenkörper (Z) and Z-layer (arrow). Stain: PTA-acetone, $\times 12,000$

Fig. 16. Section of aperture and part of the wall showing very dense stainability of the annulus, the exine and granular-fibrillar layer (arrows). The Zwischenkörper (Z) is stained weakly. Stain: PTA-chromic. $\times 13,000$

Figs. 9–13. Collected on 14th December 1989

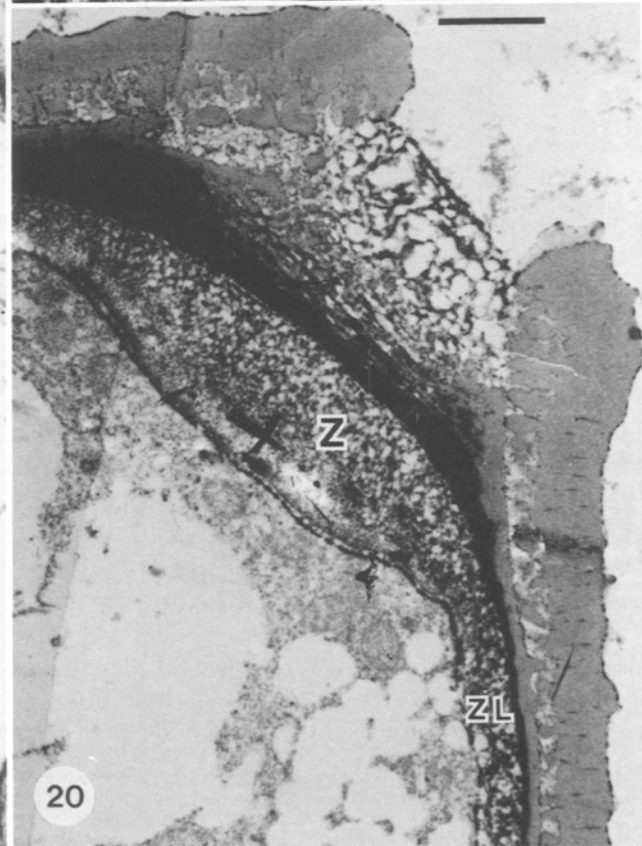
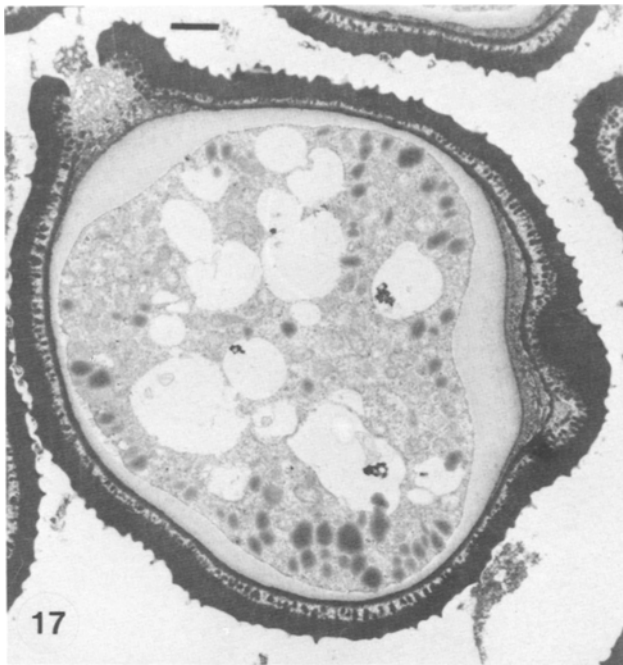
Fig. 9. The exine and the annulus are positively stained, while Zwischenkörper and Z-layer (arrowhead) are weakly stained. Arrow shows compressed fibrillar material beneath the exine. At the pore there is thick fibrillar-flocculent material. The cytoplasm is packed with numerous lipoidal globules. Stain: OsO₄, UA-Pb. $\times 5,600$

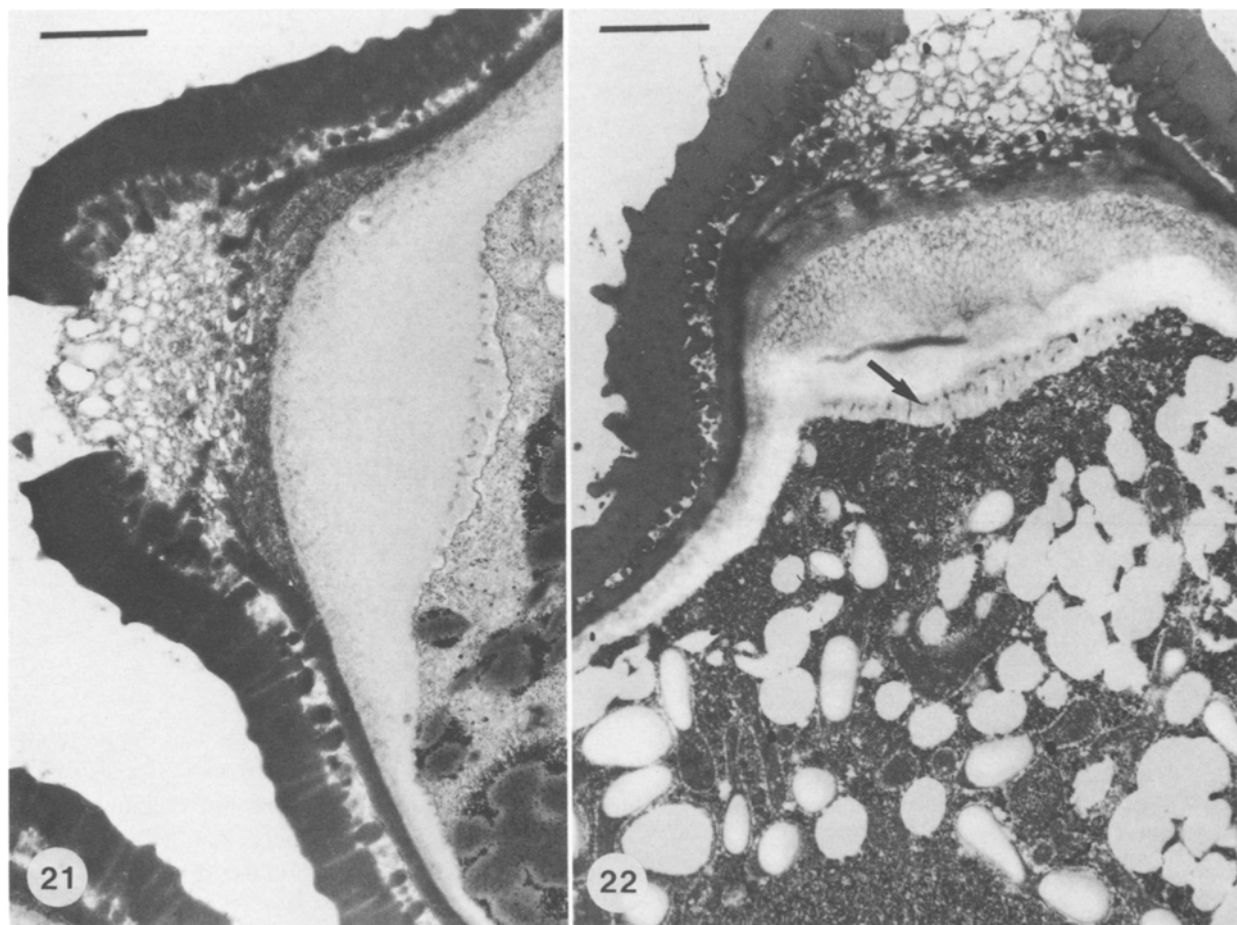
Fig. 10. The microchannels in the tectum are more obvious and contain material dense to electrons. A moderately stained fibrillar material within the voids cavea between the columellae is observed. The columellae are connected with the foot layer, or appear not continuous probably due to plane of section. Note presence of a fine granular-fibrillar layer beneath the foot layer followed by Z-layer. Stain: UA-Pb. $\times 42,000$

Fig. 11. When OsO₄ was not used in fixation, the morphology of the Zwischenkörper and the Z-layer appear fibrillar. The material of the microchannels in the tectum is densely stained. A part of the microspore is sectioned tangentially (arrow). Stain: UA-Pb. $\times 17,000$

Fig. 12. The units of the developed exine are densely stained, while the Zwischenkörper (Z) and the Z-layer (Zl) are almost without contrast. Stain OsO₄, TCH-silver proteinate. $\times 13,600$

Fig. 13. The silver proteinate control stains the exine moderately. Note numerous electron opaque globules in the cytoplasm. Stain: silver proteinate. $\times 14,000$





Figs. 21 and 22. Collected on 9th February 1990

Fig. 21. The distal part of the Zwischenkörper becomes more convex than before, and the granular-fibrillar material above its surface is more compact. The intine starts to develop beneath the Zwischenkörper, but it is not yet apparent in the interapertural parts of the exine. Stain: OsO₄, UA-Pb. $\times 14,000$

Fig. 22. Note fibrillar Zwischenkörper, dense stainability of the microchannels and early development of the intine (arrow). Stain: UA-Pb. $\times 14,000$

Figs. 17–20. Collected on 16th January 1990

Fig. 17. The general morphology and stainability of the wall are as in the former stage except that the annulus is pressed and the fibrillar-flocculent material at the pore decreased in volume. Stain: OsO₄, UA-Pb. $\times 5,000$

Fig. 18. Note that microchannels are obvious in the tectum but not in the foot layer. Several columellae are connected to the foot layer. The endexine lamellae of the annulus are numerous and apparently separated from the proximal surface of the foot layer. Arrow shows compact granular-fibrillar material and arrowheads indicate the Z-layer as a thin continuation of the Zwischenkörper. Stain: OsO₄, UA-Pb. $\times 17,000$

Fig. 19. The material between the lamellae of the annulus, the proximal portion of Zwischenkörper and Z-layer are darkly stained. Note the fibrillar-flocculent material at the pore is slightly reduced. Stain: OsO₄, PTA-acetone. $\times 17,700$

Fig. 20. The exine is stained moderately, while the stainability of the Zwischenkörper, the Z-layer and the compact granular-fibrillar material beneath the exine is increased. If OsO₄ is omitted during fixation, both Zwischenkörper and Z-layer show a fibrillar-network pattern. Stain: PTA-acetone. $\times 13,800$

Table 1. Cytochemistry of pollen wall development

	Free microspores				Young pollen			Mature pollen		
	20/11/89		14/12/89		9/3/90			30/3/90		
Applied stains	E	Z + ZL	E	Z + ZL	E	Z + ZL	I	E	Z + ZL	I
General stain										
UA, Pb	+	±	++	–	++	+	+	++	–	+
Staining for neutral polysaccharides										
PA-TCH-silver proteinate	+	–	+	±	+	++	+	++	±	+
Staining for lipids										
OS-TCH-silver proteinate	++	±	++	±	+	+	+	++	±	+
Staining for protein										
PTA-acetone	+	++	+	++	+	+	++	+	+	++
Staining for acidic polysaccharides										
PTA-chromic acid	+++	±	+++	+	+++	+	+	+++	+	+

Intensity of staining reaction: – no reaction; ± faint; + moderate; ++ dense staining; +++ very dense staining

E Exine; I intine; Z Zwischenkörper; ZL Z-layer

lar-fibrillar material at the proximal surface of the exine became compact (Figs. 24 and 25), this material and the intine stained darkly with OsO₄-PTA-acetone (Fig. 24), while the exine and apertural region were moderately stained. The microchannels were distinct in the tectum (Fig. 25), while the staining of microchannels varied at different stages of development and with different treatment (compare, e.g., Figs. 25 and 10 to Figs. 7 and 18).

30th March 1990, 2 °C / 14 °C

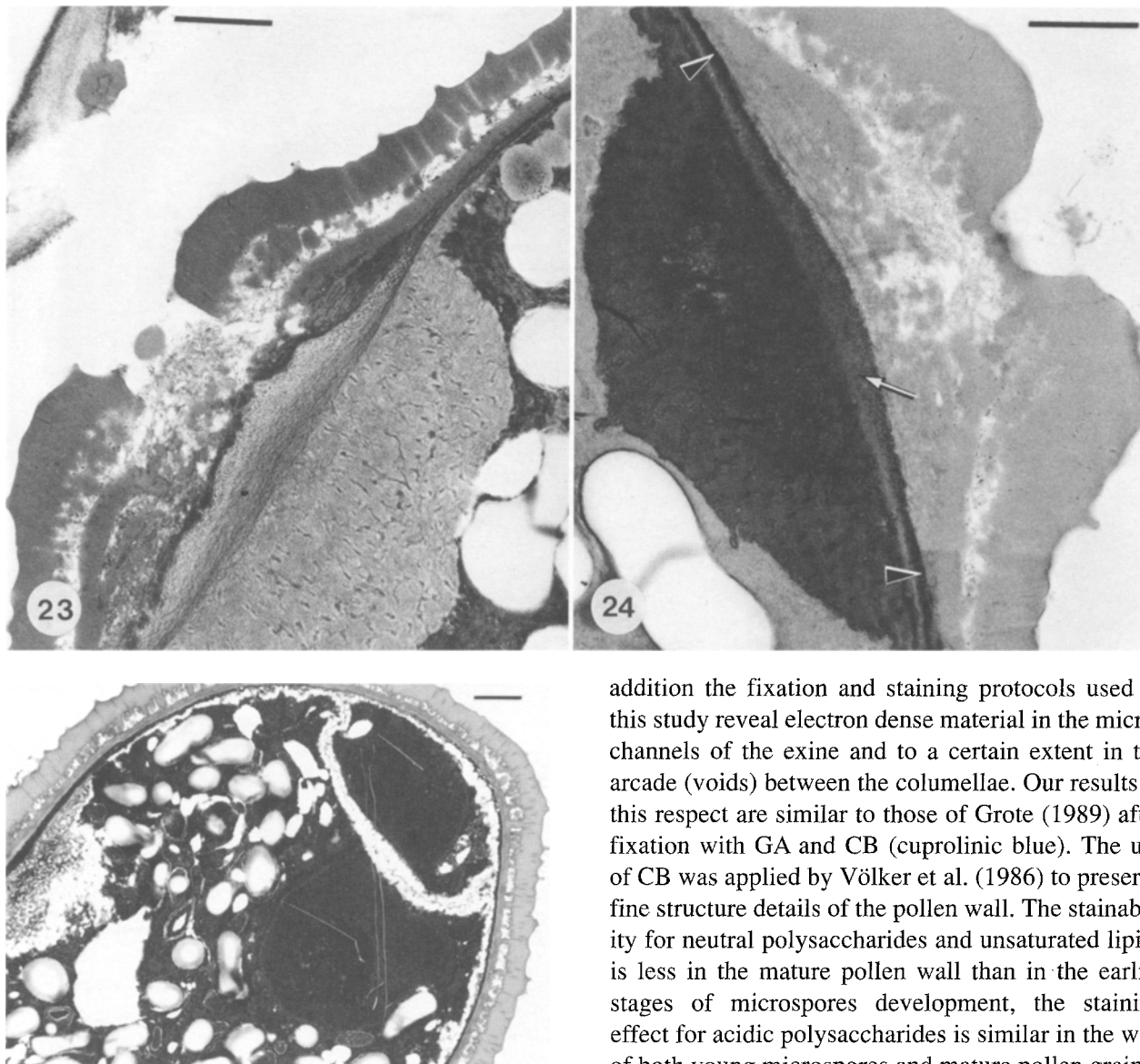
The generative cell migrated towards the center of the young pollen grain developing a thin intine-like wall (Fig. 28). The intine was evident in the interapertural part of the exine and continued to increase in thickness under the aperture (Fig. 26). The Zwischenkörper became much reduced in thickness (Figs. 26 and 30). The exine, the fibrillar material and cytoplasmic channels of the intine were stained darkly with PA-TCH-silver proteinate (Fig. 30) and even darker with TCH-silver proteinate (Fig. 29). The starch grains in the cytoplasm of the engorged pollen stained positively with PA-TCH-silver proteinate but weakly with TCH-silver proteinate, which represents an internal control indicating the oxidising effect of periodic acid (cf. Figs. 29 and 30). Sections treated with TCH-silver proteinate indicated the presence of aldehydes on the surface of the starch grains as osmophilic particles. The exine stained moderately for protein, while

the fibrillar layer beneath the exine and the membranous units of the intine were dense (Fig. 31). The fibrillar material and the cytoplasmic channels of the intine were stained moderately with PTA-chromic, while the exine was electron-dense (Fig. 32).

Discussion

In the present investigation we observed slight variations in the ultrastructure of the *Betula pendula* microspores wall during dormancy and distinct variations before anthesis. The fibrillar layer beneath the pores and the Zwischenkörper were the most variable part of the wall. The stainability of the Zwischenkörper for protein increased during dormancy and decreased before anthesis, however, the final stages of intine maturation showed an increase in its protein. Different chemical composition observed in the layers at the aperture region may reveal different functions for these layers.

At the beginning of dormancy the microspore wall and the apertures appear well developed. The apertural exine is thicker than in the interapertural part. The tectum is beset with spinules and contains numerous microchannels. The spinules are usually arranged on ridges except near the apertures (Pragowski 1962, Erdtman et al. 1962, Takeoka and Stix 1963). The columellae are short and densely spaced. In some sections we observed electron dense material or membrane-like coatings on the surface of the tectum. In



Figs. 23–25. Collected on 9th March 1990

Fig. 23. Shortly before mitosis the fibrillar-flocculent material at the pore is not visible anymore, and the lamellae of the annulus become more compact than before. The Zwischenkörper is much reduced in size and the intine is thick at the pores and starts to develop beneath the interapertural wall. Stain: OsO₄, UA-Pb. $\times 14,200$

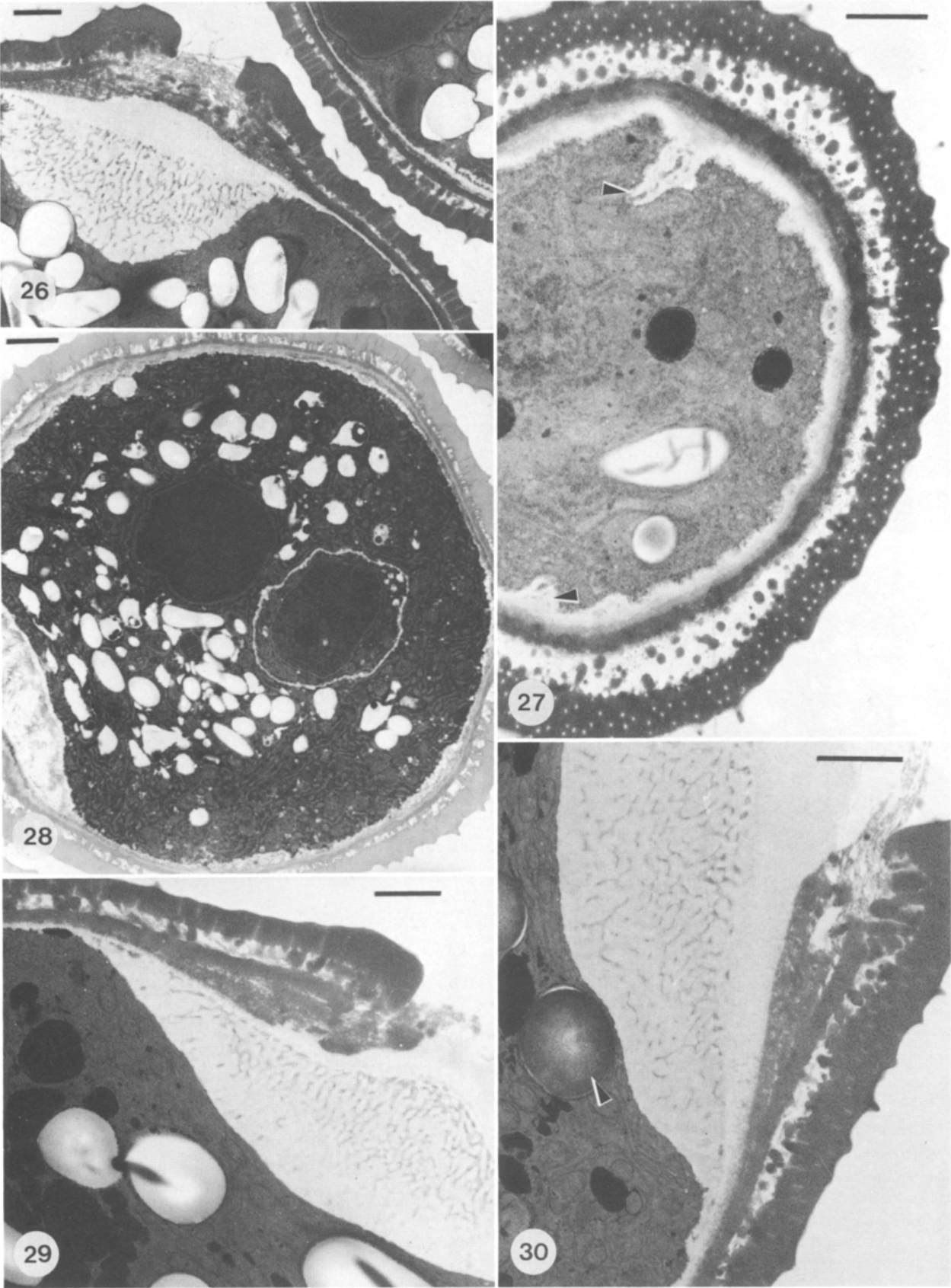
Fig. 24. The granular-fibrillar material beneath the exine (arrow-heads) and the membranous units of the intine are stained densely, while the remaining portion of Zwischenkörper (arrow) and Z-layer are moderately stained. Stain: OsO₄, PTA-acetone. $\times 16,000$

Fig. 25. The generative cell is located close to the pollen wall and an intine-like wall is developed around it. The intine is evident in the interapertural part of the exine. Note low stainability of the wall, the microchannels are evident in the tectum but not observed in the foot layer. The short columellae are cylindrical and generally attached to the foot layer. Stain: UA-Pb. $\times 11,600$

addition the fixation and staining protocols used in this study reveal electron dense material in the microchannels of the exine and to a certain extent in the arcade (voids) between the columellae. Our results in this respect are similar to those of Grote (1989) after fixation with GA and CB (cuproline blue). The use of CB was applied by Völker et al. (1986) to preserve fine structure details of the pollen wall. The stainability for neutral polysaccharides and unsaturated lipids is less in the mature pollen wall than in the earlier stages of microspores development, the staining effect for acidic polysaccharides is similar in the wall of both young microspores and mature pollen grains.

Effect of temperature

Our material was collected in an exceptionally short and warm winter. The effect of temperature on the development, release, and viability of pollen grains was investigated by several authors (e.g., Schrauwen et al. 1986, Kandasamy and Kristen 1989, Nepi and Pacini 1993), however, there are no studies on effect of temperature variation on the histochemistry of pollen wall during developmental stages. Sulkinoja and Valanne (1980) reported that in 1978 the frequency of *Betula pubescens* twin seedling collected from one tree was ca. 28%, and in 1979 the seed material collected from the same tree was ca. 1.5%. According to Sulkinoja and Valanne (1980) this difference could be





Figs. 31 and 32. Collected on 30th March 1990

Fig. 31. Note dense staining of both the intine and the granular-fibrillar layer beneath the exine (arrowheads) and less dense staining of the remaining part of Zwischenkörper and compact Z-layer. Stain: OsO₄, PTA-acetone. $\times 16,000$

Fig. 32. The exine and the tubular ingrowths of the intine are intensely stained, while the reduced Zwischenkörper and the compact Z-layer are weakly stained. Stain: PTA-chromic acid. $\times 14,000$

Figs. 26–30. Collected on 30th March 1990

Fig. 26. The endexine lamellae at the apertural margin are pressed together. The intine is well developed at the aperture and is thin in interapertural part. Starch grains are present in the cytoplasm. Stain: OsO₄, UA-Pb. $\times 8,000$

Fig. 27. Tangential section showing thick tectum with numerous microchannels which appear circular to oval shaped in cross and partially oblique section. The foot layer is apparently devoid of such microchannels. Columellae are arranged close to each other and they show an irregular shape in cross section. The intine is irregular in shape and thickness (arrowheads). Stain: OsO₄, UA-Pb. $\times 13,700$

Fig. 28. The intine increases in thickness in nonapertural part of the exine. The generative cell migrates towards the center of the cell and is surrounded with a thin intinous layer. Microchannels are obvious and contain osmophilic material. Most columellae are radial orientated and are connected with the foot layer. Stain: UA-Pb. $\times 7,800$

Fig. 29. The test for lipids (TCH-silver proteinate) is positive in the wall but almost negative in the Zwischenkörper. Note few electron dense granules near the surface of the starch grains, that indicate the presence of few free aldehydes. Stain: TCH-silver proteinate. $\times 11,500$

Fig. 30. The exine and the tubular ingrowths of the intine are densely stained for neutral polysaccharides, while Zwischenkörper and Z-layer are faintly stained. Pollen cytoplasm, the amyloplasts (arrowhead) and lipid globules are stained intensely. Stain: PA-TCH-silver proteinate. $\times 14,400$

due to the rapid thermal sum increase in 1978, which caused profuse release of pollen at that time when the female flowers of this particular tree were at their receptive stage. The significant effect of variation in temperature on the pollination and fertilization in *Betula* is obvious from the study of Sulkinoja and Valanne (1980). It is also of interest to study the effect of temperature variation on the development of pollen wall, which we started in the present paper. Note that the investigation by Dunbar and Rowley (1984) on development of the *Betula* pollen wall before and after dormancy was based on material collected in a season with a longer and colder winter than the season we collected the material for the present study. Therefore we compare our results with those of Dunbar and Rowley (1984), particularly to find the possible effect of temperature variation on pollen wall development. One clear difference between our observations and those of Dunbar and Rowley (1984) is observed in the distribution of the fibrillar-flocculent material in the microspore wall. This layer, described by Dunbar and Rowley (1984) as wide pericytoplasmic zone which separates the cytoplasm from the exine, while in our study the fibrillar-flocculent material is observed only in the pore sites. Apparently this layer takes part in protecting the young free microspores during the cold climate in dormancy. In our material where the temperature was not so low, this layer is developed only at the pores. The formation of the intine in *Betula pendula* started at the pores just before mitosis, which is earlier than that in *Betula verrucosa* examined by Dunbar and Rowley (1984).

The columellae in our material are frequently radially oriented and in contact with the foot layer. In some cases, probably due to the plane of sectioning the attachment of the columellae with the foot layer is not clear. Our observations are generally in agreement with those of Dunbar and Rowley (1984). In other cases the columellae appear as circular or irregular in cross sections, which may explain the description of columellae as a granular structure, e.g., by Doyle et al. (1975).

Zwischenkörper

In *Betula pendula* the interstitial body (*Zwischenkörper*, sensu Fritsche 1837, Beer 1906) develops in the young free microspores, between the cytoplasm and the granular-fibrillar layer at the sites of the pores. The *Zwischenkörper* is usually described as to

be restricted below the pores (Christensen and Horner 1974; Dunbar and Rowley 1984; El-Ghazaly and Jensen 1986, 1987; Charzynska et al. 1990). In *Betula* the *Zwischenkörper* is continuous in the interapertural wall as a thin layer (Z-layer). The early development and histochemical tests support a discrimination of the *Zwischenkörper* in the apertures and the Z-layer in interapertural wall from the intine. The *Zwischenkörper* shows a negative stainability with UA-Pb and a faint stain for neutral and acidic polysaccharides (Table 1), while the membranous channels and the fibrillar material of the intine are stained positively for these stains. At the free microspore stage, the *Zwischenkörper* is stained positively for protein. When OsO₄ is omitted in the fixation, and PTA-acetone stain for protein is applied, the *Zwischenkörper* stains densely and shows a characteristic network pattern. In mature pollen grains the *Zwischenkörper* is stained moderately for protein, while the intine is densely stained.

Because of the above mentioned morphological and histochemical variations between the intine and the *Zwischenkörper*, we do not agree with Charzynska et al. (1990) in considering both *Zwischenkörper* and Z-layer as outer layer of the intine. The *Zwischenkörper* in *Betula* is reduced much in size just before anthesis and its upper surface stains positively for protein and carbohydrates. These components of the *Zwischenkörper* may indicate a role of this layer in the male-female recognition reaction. This proposed function of *Zwischenkörper* was also mentioned by Grote and Fromme (1984) on birch pollen. In addition, Charzynska et al. (1990) suggested that the *Zwischenkörper* in *Secale* may facilitate the diffusion of solutes from the tapetum to the microspore; the early development of the *Zwischenkörper* in close contact with the tapetum in *Betula* may be in favour of this suggestion. Certainly additional experiments are needed to demonstrate possible routes for transfer of materials between sporophyte and gametophyte.

Our histochemical tests reveal the heterogeneity of the *Zwischenkörper* and indicate a hidden fibrillar network in its structure. The histochemical complexities of the *Zwischenkörper* in *Betula* are widely similar to that of *Corylus avellana* (Heslop-Harrison and Heslop-Harrison 1991). The *Zwischenkörper* in *C. avellana* is distinguished from the intine by its staining properties. Heslop-Harrison and Heslop-Harrison (1991) referred that the pectinous nature of the *Zwischenkörper* reflects its specialization for a criti-

cal early function in germination. The fibrillar nature observed in the Zwischenkörper of *Betula* probably indicates a germination function of this layer, since microfibrillar pectins are associated with the pollen tube. Additional histochemical tests and different techniques for, e.g., freeze substitute fixation at different stages of *Betula* wall development are necessary to solve questions of chemistry and morphology of the Zwischenkörper, and to elucidate its functions.

Development of the intine

The intine starts to develop at around the vernal equinox beneath the apertures, increasing in thickness under the pores, and later on starts to develop under the interapertural parts as a thin layer. The morphology of the Z-layer and the thin intine at interapertural part of the wall appears as irregular ingrowths. The pronounced irregular ingrowths of intine in *Betula pendula* observed in this study is in agreement with the observations of Charzynska et al. (1990) in *Secale cereale*, and may support their conclusion that these ingrowths form a transfer girdle in contact with the cytoplasm of the external pole of the microspore towards the tapetum. The intine beneath the pores becomes comparatively very thick and is provided with fibrillar material and radially arranged tubular ingrowths. The fibrillar and tubular elements of the intine are stained positively for acidic polysaccharides and protein (Table 1). The presence of protein in the intine was reported in birch pollen (*Betula pendula*, *B. nana*, and *B. pubescens*) by many authors, e.g., Knox and Heslop-Harrison (1970) and Grote and Fromme (1984 b). The protein in birch pollen is considered as one of the main causes of type I allergic reaction in Sweden (Wihl 1988) and in large parts of the northern hemisphere (Swoboda et al. 1994). Bet v 1, a polypeptide of 17 kDa, has been identified as the major birch pollen allergen responsible for IgE binding in more than 95% of birch pollen allergic patients (Swoboda et al. 1994). Our study demonstrates that the stainability for protein is positive in the Zwischenkörper of late microspores and in pollen wall and intine of mature pollen grain before anthesis. In their study of major birch pollen allergen during anther development Swoboda et al. (1994) found that Bet v 1 proteins are encoded by late pollen-specific genes, whose transcription is activated only during the final stages of pollen maturation. We conclude that the protein detected in the intine of the mature pollen grain in this study may be the reason for allergic reactions. The earlier formed protein in the

Zwischenkörper of *B. pendula* is apparently not allergenic. This is based also on the study of Seiberler et al. (1994), where Bet v III is expressed preferentially in mature birch pollen. This observation may add a clear histochemical difference between the Zwischenkörper and the intine, regarding the type and timing of protein formation in these structures. It also points towards different functions of the two layers. The deposition of protein in the exine and intine prior to pollination may be a prerequisite for successful pollen-stigma recognition and interactions. On the basis of quantitative immunoassays and immunocytochemistry, Southworth et al. (1988) stated that purified exines are antigenic and that the polyclonal and monoclonal antibodies bind to exines of some selected angiosperms and gymnosperms. From this it seems that allergenic protein may exist in both material of the intine and the exine.

The fibrillar-granular layer observed in this study above the Zwischenkörper and between Z-layer and the exine stains densely for protein. A similar layer was described by Ciampolini et al. (1993) in *Cucurbita pepo* as sporophytic proteins. This layer developed in *Betula* at the early free microspore stage and is thicker at the pore sites. It may be part of the gametophytic proteins, localized in the intine, in pollen-stigma recognition and interaction.

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