

Localization and release of allergens from tapetum and pollen grains of *Betula pendula*

G. El-Ghazaly^{1,*}, R. Moate², M. Cresti³, B. Walles⁴, Y. Takahashi⁵, F. Ferreira⁶, and G. Obermeyer⁷

¹Palynological Laboratory, Swedish Museum of Natural History, Stockholm, ²Electron Microscopy Centre, University of Plymouth, Plymouth, ³Department of Environmental Biology, University of Siena, Siena, ⁴Department of Botany, Stockholm University, Stockholm, ⁵The Yamagata Prefectural Institute of Public Health, Yamagata, and Departments of ⁶Genetics and General Biology and of ⁷Plant Physiology, University of Salzburg, Salzburg

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Summary. Although intact pollen grains are assumed to be the primary carrier of pollen allergens, specific immunoreactive components have been found in other aerosol fractions, e.g., starch grains and remains of tapetal cells. Cryo-scanning-electron-microscopy results demonstrate the presence of a clear network of strands connecting the tapetum with the microspores. The distribution of protein in tapetal orbicules, pollen wall, and pollen cytoplasm was tested by histochemical stains for light microscopy and transmission electron microscopy. The protein is mainly localized at the apertures and starch grains in the cytoplasm of pollen and in the core and on the surface of tapetal orbicules. Monoclonal antibodies Bv-10, BIP3, and BIP4 have been used to locate the cellular sites of pollen and tapetal allergens in *Betula pendula* (syn. *B. verrucosa*). The application of rapid-freeze fixation prevented relocation of allergens from their native sites. The allergens are predominantly found in the starch grains and to lesser extent in the exine. We also tested interactions between mature birch pollen and human fluids: saliva, nostrils fluid, and eyes solution. The aim was to mimic more closely the in vivo situation during allergenic response. In all cases we observed several pollen grains that were burst and had released their cytoplasmic contents. In the nose the allergens are released from the pollen within minutes. In rhinitis, nasal pH is increased from the normal pH 6.0 to 8.0. When we used nasal fluid at pH 8.0, the number of ruptured pollen grains increased. The mechanism that might induce formation of small allergen-bearing particles from living plant cells is discussed.

Keywords: Allergens; Allergy; *Betula pendula*; Immunolabelling; Pollen grains; Tapetum.

Introduction

An allergen is an environmental antigen which can provoke the allergic reaction by interacting with the

immune system in susceptible humans. Aqueous extracts of birch (*Betula pendula*) pollen contain a complex mixture of proteins. Approximately 40 distinct proteins were easily released from pollen grains upon hydration (Jarolim et al. 1989a). Only a small number of the proteins are allergenic; approximately 10–12, out of the released 40, were recognized by immunoglobulin E (IgE) from allergic individuals (Viander et al. 1979, Jarolim et al. 1989b). Loads of airborne pollen have been measured for many years in several countries to get information on daily quantities of allergenic pollen and their relations to symptoms of pollen allergy, and to make forecasts of airborne pollen concentration in the air (El-Ghazaly et al. 1993). Mostly the aeroallergen information service is based on the volumetric sampling of airborne pollen grains on adhesive tape mounted into a Burkard pollen and spore trap. So far, very little is known about the structure and characteristics of sub-microscopic allergens in the air. Earlier studies have indicated the presence of allergens in association with cytoplasm of pollen grains of birch trees (Grote 1991, Grote et al. 1993). The major allergen, Bet v 1, as well as the minor allergens Bet v 3 and Bet v 4 have recently been identified by the technique of gene cloning (Seiberler et al. 1994, Engel et al. 1997). The precise distribution of allergens in the cytoplasm has been rarely investigated. Belin and Rowley (1971) assumed that the allergens in *Betula verrucosa* are stored inside the pollen protoplasm and diffuse through the aperture during germination. Staff et al.

* Correspondence and reprints: Palynological Laboratory, Swedish Museum of Natural History, Roslagsvägen 101, S-10405 Stockholm, Sweden.

(1990), Suphioglu et al. (1992), Taylor et al. (1994), and B. Knox and Suphioglu (1996) localized allergens in the starch granules and cytosol of rye-grass pollen. El-Ghazaly et al. (1996) localized the main allergen Bet v 1 in the starch granules of *Betula pendula* pollen grains.

The main aim of this study is to determine the sites of allergenic proteins in the anther tissue and pollen grains of birch and to define the mechanism of their release to the environment.

Material and methods

Cryo-scanning electron microscopy, rapid-freeze fixation, and immunogold labelling of the major allergen Bet v 1 in pollen wall and cytoplasm

Pollen grains from *Betula pendula* (syn. *B. verrucosa*) were collected at the beginning of April until the end of May in the years 1995–1998 in the Stockholm area. Some anthers at free-microspore stage were prepared for cryo-SEM technique. The anthers were treated with 1% glutaraldehyde, or with 0.05 M sodium cacodylate buffer at pH 7.5 (Maldonado et al. 1986) or deposited on wet filter paper. They were then placed on SEM stubs, attached to the stub by silver paint, frozen in liquid nitrogen and transferred to cryo unit where they were kept at -180°C , fractured, etched, coated with gold, and examined by SEM and photographed under -140°C . In addition mature anthers of *B. pendula* were fixed by rapid-freeze techniques and substituted in an alcohol series and embedded in London Resin White.

For general contrast, sections were stained with 1% aqueous uranyl acetate for 5 min, washed with distilled water, and floated on lead citrate stain for 5 min. To detect protein, sections on gold grids were stained with 5% phosphotungstic acid (PTA) in 10% acetone for 15 min (Benedetti and Bertolini 1963, Marinozzi 1968).

Immunogold labelling was performed by the method of Probert et al. (1981). The ultrathin sections were treated with Bv-10 (IgG1; ALK Laboratories, Hørsholm, Denmark), BIP3 and BIP4 (Department of Genetics, University of Salzburg, Salzburg, Austria) monoclonal antibodies, prepared at dilution of 1:500 in 0.05 M Tris-buffered saline (pH 7.2), and incubated for 24 h at 4°C . Bv-10 is a murine MAb raised in BALB/c mice against the major allergen Bet v1 from birch pollen (Bv-10 is the name of the clone). The antibody has been tested in, e.g., immunoblot and is shown to react only to Bet v 1 (IgG1; ALK Laboratories). BIP3 is a mouse MAb (IgG) specific for a 60 kDa allergen from birch pollen, and BIP4 is a mouse MAb (IgG) specific for the Bet v 1 allergen. The production and specificity of BIP3 and BIP4 were tested by Jarolim et al. (1989a, b).

The treated sections were then incubated in 10 nm colloidal-gold-conjugated goat anti-mouse IgG (GAM, 1:20; Amersham International plc, Amersham, U.K.) for 60 min at room temperature. For control staining, to demonstrate the specificity of immunogold labelling, the antiserum was replaced with normal goat serum. After immunogold staining, ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Zeiss EM 906 at Botany Department, Stockholm University, Sweden.

Immunogold labelling of Bet v 1 in atmospheric samples

A sampler with a tandem filter system was used. It was composed of a $5\text{ }\mu\text{m}$ pore size capillary filter (Nuclepore filter; Nuclepore Cor-

poration, Pleasanton, Calif., U.S.A.) and a $0.3\text{ }\mu\text{m}$ pore size Millipore filter with diameters of 47 mm . The sampler was placed beside the Burkard trap 20 m above ground level in a roof of a building at Stockholm University. Particles with aerodynamic diameter below $5\text{ }\mu\text{m}$, collected daily from 1st May to end of June in 1997 and 1998 were extracted from the filters.

Allergen extraction

Allergens on the filters were extracted with 1 ml of a solution of $0.125\text{ M NH}_4\text{HCO}_3$ and 0.01% bovine serum albumin at 4°C for 24 h.

Bet v 1 allergen detection

The amount of Bet v 1 in the extracts was measured by a fluorometric sandwich enzyme-linked immunosorbent assay. Briefly, microplate wells were coated with rabbit anti-Bet v 1 IgG. Reference Bet v 1 and extracted allergen samples were incubated in the wells. The wells were reacted first with biotinylated anti-Bet v 1 rabbit IgG, and then with β -D-galactosidase-conjugated streptavidin, and finally with 0.1 ml of 0.1 mM 4-methyl umbelliferyl- β -D-galactoside. After incubation at 37°C for 2 h, the fluorescence intensity of the enzyme reaction product, 4-methylumbelliferone, was read on a microplate fluorometric reader (Fluoroskan; Flow Laboratories, McLean, Va., U.S.A.). The sensitivity of this enzyme-linked immunosorbent assay was 0.1 ng of Bet v 1 per ml.

For details of methods of extraction and detection of allergens in the filters, see Takahashi et al. (1991).

Release of allergens from Betula pollen

Fresh pollen grains were mounted in saliva, nostrils fluid, or eye solution, in glass slides and kept for 10 min at room temperature. The slides were then examined and photographed with the light microscope. A drop of I/KI stain was added to each preparation to test the presence of starch grains. The pH of the nostril fluid (7.4) was raised to 8 by adding NaOH, to examine the effect of a higher pH that generally occurs in allergic patients.

Results

Immuno-reactive components in aerosol

The presence of allergens associated with particles sized below $5\text{ }\mu\text{m}$ has been confirmed by using molecular membrane filters that were cleared and examined microscopically or extracted with aqueous solvents for immunochemical assay.

Studies of airborne pollen were carried out by means of Burkard volumetric spore traps at Stockholm region for more than thirty years. In this study we present the amount of *Betula* pollen grains in the Stockholm region during the last five years. Figure 1 shows that the main pollen season occurs in the second week of May in all the years investigated. At the site of the Burkard trap we placed a high-volume filters trap to collect the particles with aerodynamic diameter below $5\text{ }\mu\text{m}$, daily

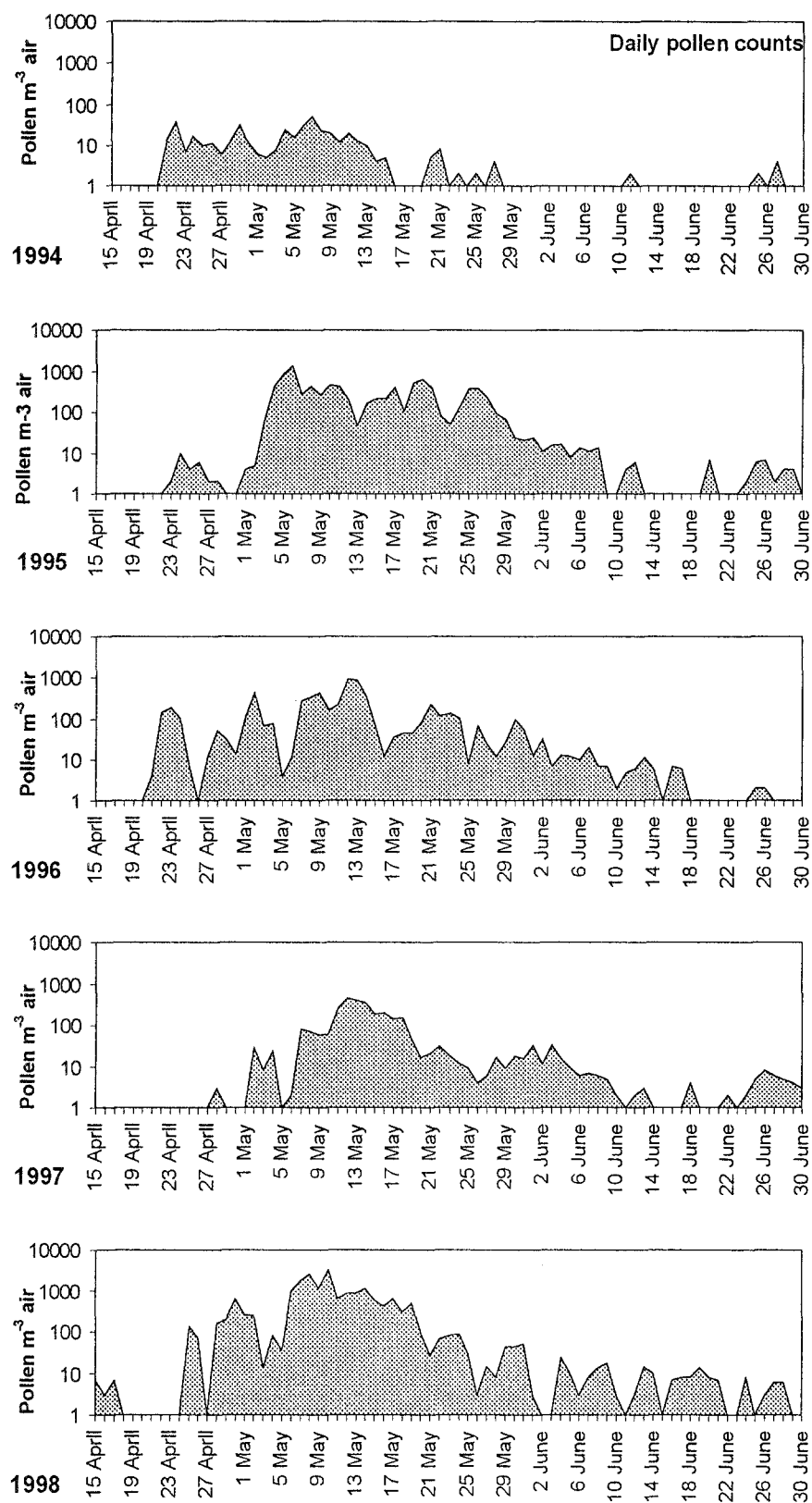


Fig. 1. Daily pollen count of *Betula* pollen per cubic meter of air from start to end of the pollen season in the years 1994–1998. The main pollen season of birch occurred in the second week of May in all the years investigated

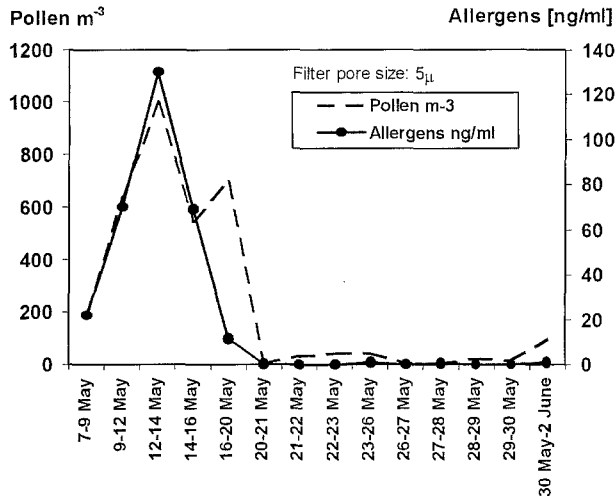


Fig. 2. The amount of allergens (ng/ml) collected, during the birch pollen season 1997, from filters of the trap installed at a site close to the Stockholm city center. The amount of allergens in the atmosphere correlates positively with the amount of pollen grains

from 1st May to end of June in 1997 and 1998. The particles that were trapped in the filters were extracted and their allergenic load was identified by applying the MAb of the major birch pollen allergen Bet v 1. The amount of allergenic particles in the atmosphere (Fig. 2) correlates positively with the number of pollen grains captured by the standard Burkard 7-day volumetric spore trap (Figs. 1 and 2).

Strands extending between tapetal cells and microspores

Our results obtained by applying the cryo-SEM illustrate the presence of strands extending from tapetal cells to the surface of microspores in locules (Figs. 3 and 4). The diameter of such strands is much greater in the cryo-prepared material than in the chemically prepared material.

Stainability for protein

The result of applying Coomassie brilliant blue stain for protein reveals that protein is mainly localized at the apertures and starch grains in the cytoplasm of mature pollen. The pollen wall and the orbicules (Ubish bodies) that are lining the inner surface of the tapetal cells are stained moderately. In transmission electron microscopy, the orbicules are generally irregular in shape and beset with minute pointed excrescences. They have a thick homogenous wall and a narrow, spherical or siltlike central core (Fig. 5). When sections were treated with PTA-acetone for protein and examined by transmission electron microscopy, the orbicules, the exine, and the lower portion of Zwischenkörper were stained moderately, while the exine surface, the microchannels, the fibrillar layer beneath the exine (Fig. 6), and the membranous units of the intine were densely stained (Fig. 6).

Electron-microscopic immunocytochemistry

Monoclonal antibodies have been used to locate allergens in the subcellular sites of *B. pendula* pollen and tapetal cells. We applied rapid-freeze fixation and substitution followed by London Resin White embedding to minimize relocalization effects. When MAb Bv-10 was used, the allergen was predominantly located in the starch grains and to a lesser extent in the exine (Fig. 8). In the case of MAbs BIP3 and BIP4, the allergen was localized moderately in the cytoplasm and pollen wall (Figs. 9 and 10) and minor labelling was observed in the orbicules.

Release of small allergen-bearing particles from birch pollen

Fresh birch pollen grains were collected from open anthers and treated directly for ca. 10 min with human

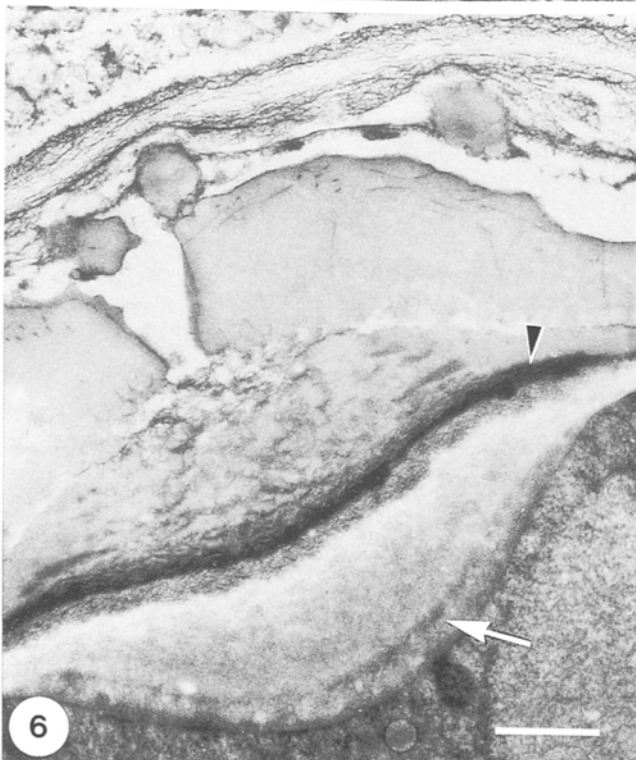
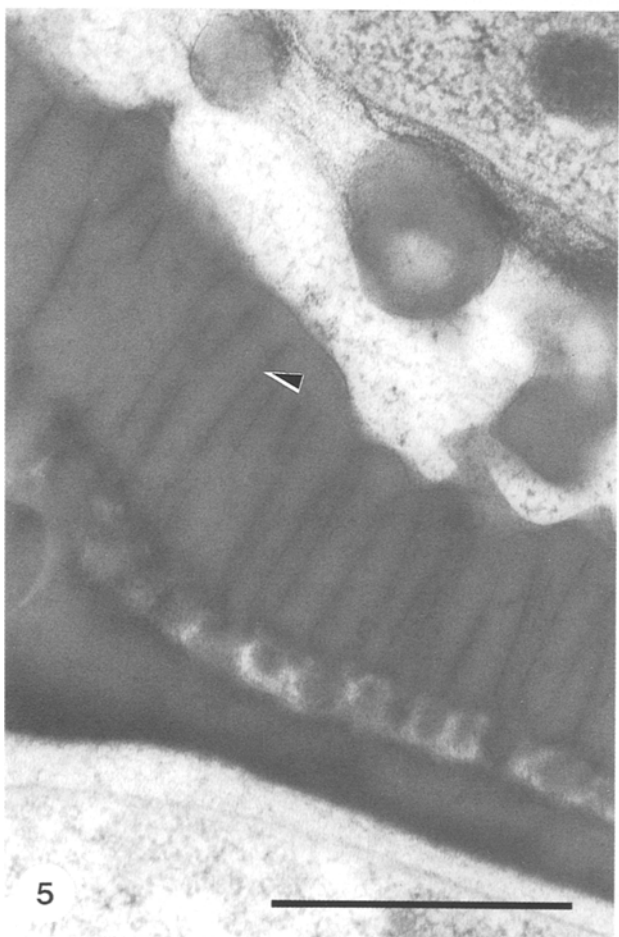
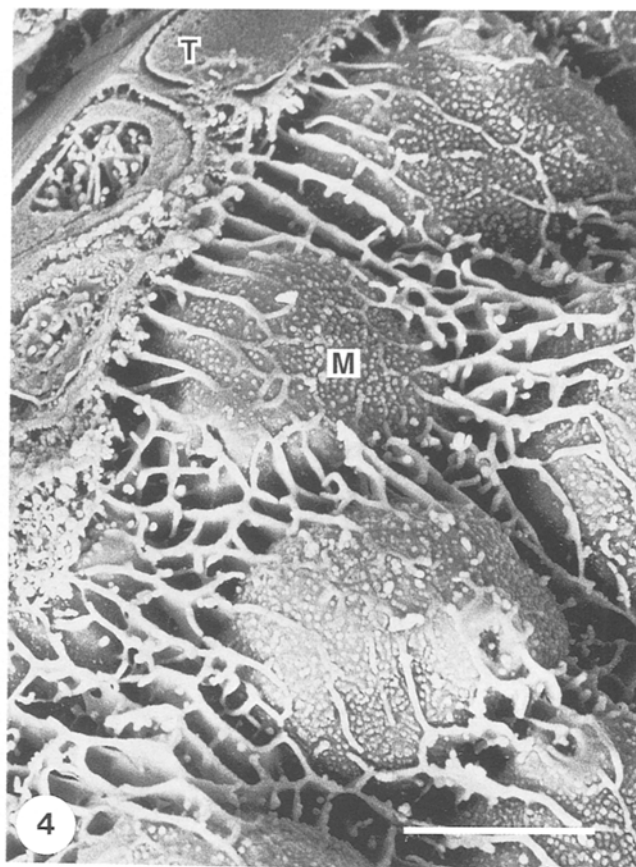
Figs. 3 and 4. Low-temperature (-180°C) preparation of living *B. pendula* microspores followed by cryo-SEM (-180°C)

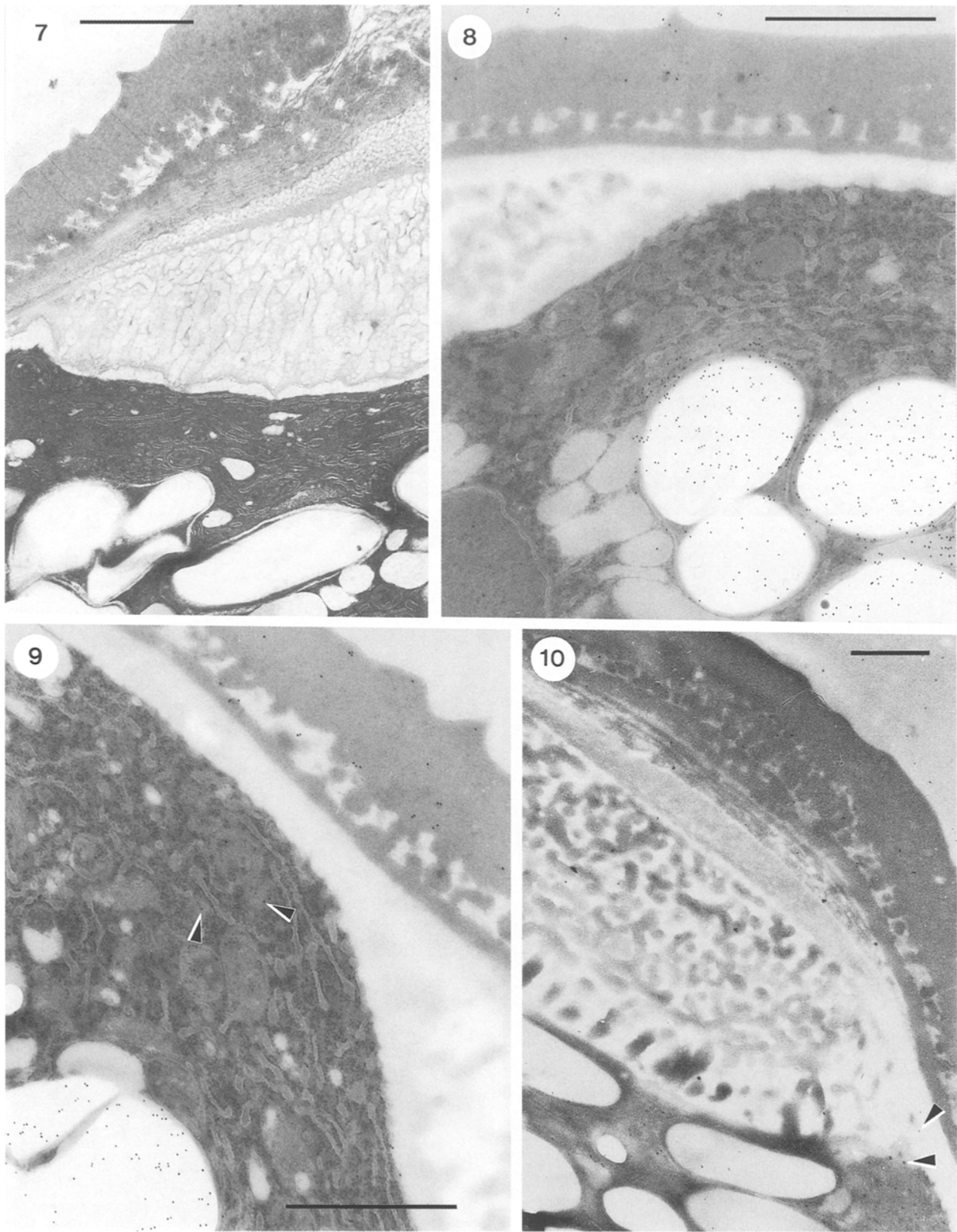
Fig. 3. The microspores are connected with a well organized system of strands. Bar: $10\ \mu\text{m}$

Fig. 4. The tapetal cells (*T*) have been sectioned but the microspores (*M*) show intact exine surfaces. Strands from the surface of tapetal cells are connected to the surface of microspores (presumably to microchannels). Bar: $10\ \mu\text{m}$

Fig. 5. Microchannels are obvious (arrowheads) and contain osmophilic material. The orbicules vary in shape. They have a central core and a thick wall and are connected with a well preserved fibrillar layer. Stain: UA-Pb. Bar: $1\ \mu\text{m}$

Fig. 6. The exine surface, the microchannels, the granular-fibrillar material beneath the exine (arrowhead), and the membranous units of the intine are stained densely, while the orbicules, the exine, and the lower portion of the Zwischenkörper (arrow) are moderately stained. Stain: PTA-acetone. Bar: $1\ \mu\text{m}$





saliva (Fig. 11), eyes solution (Fig. 12), and human nostrils fluid at pH 7.4 (Fig. 13) and pH 8 (Fig. 14). In all cases we observed several pollen grains that were burst having released their cytoplasmic contents including starch grains. When stored pollen grains of *B. pendula* from previous seasons were used, they remained intact.

Discussion

The major allergen, Bet v 1, of birch pollen is considered as one of the main causes of type I allergic reaction in Sweden (Wihl et al. 1988) and in large parts of the Northern hemisphere (Jarolim et al. 1989b, Swoboda et al. 1995). 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 (Jarolim et al. 1989b).

To evaluate the amount of birch pollen in the air we have applied a Burkard trap, which has been used for more than thirty years in different regions in Sweden. In the present work we present the occurrence of birch pollen in the air of Stockholm in the last 5 years during the high pollen season of birch. The histograms confirm that birch pollen grains represent an important potential source of allergens in Sweden.

In the present study we observed that the stainability for protein is positive in pollen wall and intine of mature pollen grain before anthesis. Swoboda et al. (1995) in their study of the major birch pollen allergen during anther development 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 mature pollen grain of *Betula* spp. may be the reason for allergic reactions. The presence of protein in the intine was reported in pollen of birch (*B. pendula*, *B. nana*, and *B. pubescens*) by many authors (e.g., R. Knox and Heslop-Harrison 1970,

Grote and Fromme 1984). On the basis of quantitative immunoassays and immunocytochemistry, Southworth et al. (1988) established that purified exines are antigenic and that the polyclonal and monoclonal antibodies bind to exines of some species of angiosperms and gymnosperms. Due to the rapidity with which the allergens were released on contact with water it was earlier assumed that allergens appear mainly in the exine (Fromme et al. 1985, Grote and Fromme 1986). This theory has not been altogether disregarded. Miki-Hirosige et al. (1994) localized allergenic proteins in the sexine as well as in the dictyosomes in *Cryptomeria japonica* pollen.

During the last years a lot of information has accumulated, suggesting that the pollen allergens are signal substances of importance for the germination of pollen grains on the stigma, and that they are of sporophytic origin, i.e., that they mainly appear in the outer wall layer, the exine. In addition, other functions of the allergens have been suggested. For example, the major allergen Bet v 1 shows structural similarity with a protein protecting against pathogens (Breiteneder et al. 1989). In addition to homology to pathogenesis-related proteins, Bet v 1 shows high sequence similarity to the major pollen allergens from *Alnus glutinosa*, Aln g 1 (Breiteneder et al. 1992), from *Carpinus betulus*, Car b 1 (Larsen et al. 1992), and from *Corylus avellana*, Cor a 1 (Breiteneder et al. 1993). This is in agreement with the clinical observation that patients displaying specific IgE to Bet v 1 also show symptoms during the flowering season of hazel and other trees belonging to the order Fagales (Eriksson 1978).

More recently, cDNA clones coding for other birch pollen allergens (Bet v 2, Bet v 3, and Bet v 4) showing sequence similarities to known proteins have been isolated and characterized. Bet v 2 was identified as the actin-binding protein profilin (Valenta et al. 1992); and Bet v 3 as well as Bet v 4 were found to belong to

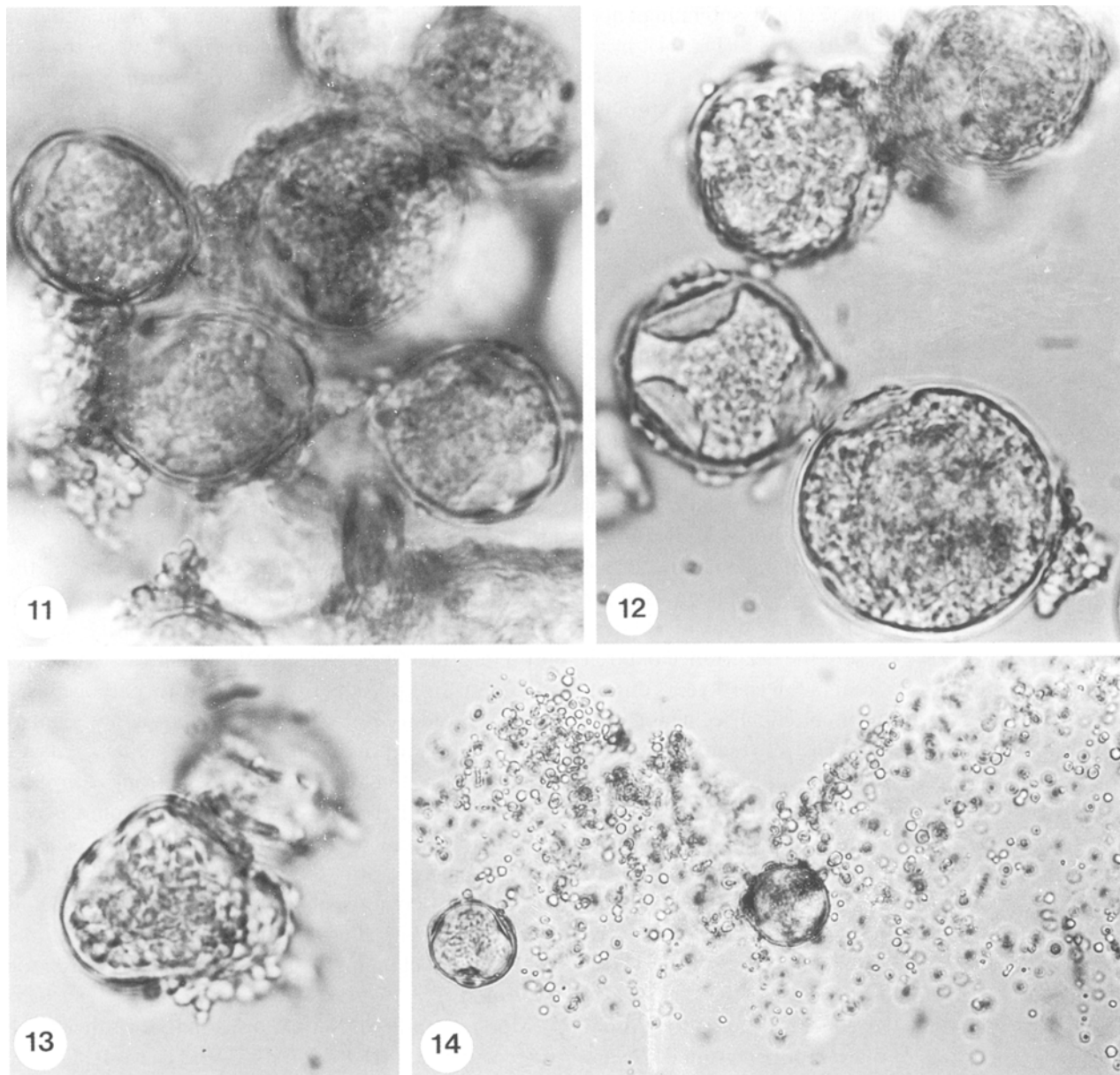
Figs. 7–10. Immunogold localization of allergens with monoclonal antibodies

Fig. 7. Control section with secondary antibody and omitted primary antibody. There is no immunogold associated with starch grains in the amyloplasts or with the pollen wall and intine. Bar: 1 μ m

Fig. 8. The major allergen Bet v 1 detected by monoclonal antibody anti-Bv-10 (localized by 10 nm diameter gold particles). The major birch allergen is located primarily within the starch granules. Bar: 1 μ m

Fig. 9. Localization of *Betula* allergen by MAb BIP3. The allergen is located in the starch granules, cytoplasm (arrowheads), and exine. Bar: 1 μ m

Fig. 10. Localization of Bet v 1 by MAb BIP4. Note labelling of the allergen in the intine at aperture region and in the cytoplasm (arrowheads). Bar: 1 μ m



Figs. 11–14. Fresh mature pollen grains treated with fluid of eyes (Fig. 11), mouth (Fig. 12), and nose at pH 7.4 (Fig. 13) and pH 8 (Fig. 14). Note that in all cases several pollen grains are ruptured and their contents are released. Figs. 11–13, $\times 1,000$; Fig. 14, $\times 400$

a novel class of calcium-binding proteins (Seiberler et al. 1994, Engel et al. 1997). Suphioglu et al. (1997) have cloned and immunologically characterized a calcium-binding allergen (Cyn d 7) from Bermuda grass, which shows high sequence similarity with Bet v 4. These calcium-binding allergens are assumed to affect many functions in the mature pollen grain. So far, Bet v 4 is the only allergen whose putative function was investigated by re-injection of the recombinant Bet v 4 in living pollen tubes (Engel et al. 1997).

The monoclonal antibodies Bv-10 and BIP4 against Bet v 1 reveal a significant pattern of immunostain associated with starch granules and organelles in the cytoplasm of *Betula* pollen grains. We detected in some cases also a minor labelling in the pollen wall and intine. The finding of the main birch allergen, Bet v 1, in starch grains adds new precise data on the occurrence of the allergens in *Betula* pollen. This result with Bet v 1 agrees with our previous finding (El-Ghazaly et al. 1996). Grote et al. (1993) studied birch pollen by

immuno electron microscopy after fixation in vapour phase and found Bet v 1 and Bet v 2 mainly in the cytoplasm. Taylor et al. (1994) used fixation in the presence of 2,2-dimethoxypropane and found two different allergens in rye-grass pollen, one of them in connection with the starch grains of the plastids, and the other one in the cytoplasm.

Release of allergens from birch pollen

It seems as if the main allergens in the dry, mature pollen grains are kept inside the plasma membrane. As soon as the pollen grains come in contact with water, e.g., on mucose membranes inside the human nose, the allergens are rapidly released. Allergenic starch grains have been associated with epidemics of asthma during thunderstorms and the grass pollen season (Packer and Ayres 1985, Bellomo et al. 1992). No similar connections between free starch grains and weather conditions have hitherto been shown for birch pollen allergy. The mechanism of how the allergens are released from birch pollen is not quite clear. Almost all birch pollen grains that are trapped on the tape of Burkard trap are intact. As shown in this study, the burst of *Betula* pollen grains upon direct contact with fluids of mouth, eyes, and nose represents an efficient way of release of allergen-loaded particles. Such burst could be due to an osmotic shock. In some cases it has been shown that the plasma membrane in dry pollen has a micellar structure and is unable to retain cytoplasmic proteins during pollen grain inhibition when the grain is first wetted. It is also possible that for their proposed function in pollen germination it is necessary that they are stored in such a way that they should be easily accessible during germination. In any case they seem to be relatively inaccessible in dry, mature pollen grains.

Concerning the mechanism of release of allergen-loaded fine particles from pollen grains, several hypotheses have been proposed. Sedimented pollen has been suggested to release its allergens when moistened after rainfall and allergenic material may become airborne after drying. Orbicules may act as carriers for birch pollen allergens (El-Ghazaly et al. 1995). Schäppi et al. (1997) mentioned that in Melbourne birch pollen germinated on leaves and each grain produced a pollen tube that was easily ruptured, releasing the cytoplasmic contents of the tube, including up to 400 starch granules.

Our observations indicate the necessity of a complement to the traditional forecasting of allergenic

pollen grains with Burkard traps. A more relevant parameter may be the actual concentration of airborne allergenic particles rather than pollen grains. For this purpose a device for monitoring small allergenic particles such as starch grains and orbicules (ca. 2 µm in diameter) should be used.

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