

Original article

The potential role of orbicules as a vector of allergens

Background: In the locules of anthers of flowering plants, tiny (1.5–2 µm) granules of sporopollenin may occur next to the pollen grains. Those granules, called orbicules, mostly occur on the radial and innermost tangential wall of secretory tapetum cells.

Methods: We have investigated the presence of orbicules in 15 important European allergenic species with scanning electron microscopy (SEM).

Results: Orbicules were present in all species investigated of the families Betulaceae, Chenopodiaceae, Fagaceae, Poaceae, Polygonaceae, and Urticaceae. However, in the Asteraceae and Oleaceae species studied, orbicules were lacking. In all Chenopodiaceae, Poaceae, and Urticaceae species, orbicules were attached to the pollen exine. These observations indicate the possibility of the dispersal of orbicules into the atmosphere during anthesis.

Conclusions: The hypothesis of the potential role of orbicules as possible important vectors of allergens is put forward, based on the comparison of our results with recent literature about the evidence of allergenic activity in the smaller micronic atmospheric aerosol fraction. Our results provide evidence that an in-depth investigation of the sites of allergens across the whole anther is required. We suggest that allergen researchers apply immunoelectron microscopy on whole anthers to determine whether orbicules possess allergens.

S. Vinckier, E. Smets

Laboratory of Plant Systematics, Institute of Botany and Microbiology, K.U. Leuven, Leuven, Belgium

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Dr S. Vinckier
Laboratory of Plant Systematics
Institute of Botany and Microbiology
K.U. Leuven
Kasteel Park Arenberg 31
B-3001 Leuven
Belgium

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In flowering plants, pollen grains develop within the locules of the anthers. At anthesis, the mature pollen grains are released from the locules. On the surface of the locule wall, tiny (1.5–2 µm) granules may occur (Figs. 1–4) that are in close contact with the pollen grains. These granules are called orbicules.

Orbicules develop simultaneously with the growing pollen exine and are composed of sporopollenin, similar to the pollen exine. Pro-orbicules, which are produced by the endoplasmic reticulum of secretory tapetum cells, are the progenitors of orbicules (1). The pro-orbicules are extruded through the radial and innermost tangential wall of the secretory tapetum cells, at the beginning of the tetrad stage (2–5). The pro-orbicules are spherical structures composed of a complex organic mixture. They are delimited by a glycocalyx (4). Deposition of sporopollenin precursors, produced by the protoplast of the secretory tapetum cells, on the glycoprotein filaments of the glycocalyx of the pro-orbicules, and on the growing pollen exine, starts at the late tetrad stage. The orbicule wall of mature orbicules is composed of structural and filling elements (4). Proteins, pectins, polysaccharides, and glycolipids,

organized in a three-dimensional polygonal frame (6), are among the structural elements. The filling elements consist of unsaturated lipids and polyphenols (4).

The function of orbicules remains unknown (7–10). The present knowledge on the distribution of orbicules in angiosperms and their possible functions is reviewed by Huysmans et al. (11, 12). Huysmans et al. (13), Vinckier et al. (14), and Vinckier & Smets (15) have examined the distribution of orbicules and their morphologic as well as their ultrastructural diversity in the order Gentianales.

Orbicules are considered to be a general feature of species characterized by a secretory tapetum. They are absent, however, in several species with such a tapetum (11). Species with an amoeboid tapetum are characterized by the absence of orbicules (16, 17), except for *Gentiana acaulis*, according to Lombardo & Carraro (18). An interesting fact, in the scope of this paper, is that hay-fever-causing angiosperms are characterized by the presence of secretory tapeta (19). Davis (19) first raised the question of whether orbicules also contain allergens. One of the first proofs of this hypothesis was supplied by Miki-

Hirosige et al. (20). Using both monoclonal and polyclonal antibodies produced from the protein, they found that the major allergen responsible for *Cryptomeria japonica* pollinosis is also present in the orbicules. It is assumed that some of the allergenic protein is produced in the tapetum cells, and attached to the orbicules and pollen wall through the accumulation of residual materials during maturation. Later, another part of the allergenic protein is produced in the Golgi bodies of the pollen grains and moves to the generative cell wall through the Golgi vesicles at the maturation stage of the pollen (20). Takahashi et al. (21) investigated the size distribution of the particles emitted from the flowers of allergologically important plants in Japan. Scanning electron microscopy (SEM) revealed that 99% of the micronic particles emitted from the dehiscing anthers of *C. japonica* were orbicules. The results of Takahashi et al. (21) and Miki-Hirosige et al. (20) indicate that, in the case of *C. japonica*, the orbicules are dispersed into the atmosphere, acting as a very effective vector to bring allergens to susceptible individuals. The orbicules, containing the major allergen, are much more numerous than the pollen grains, and they are small enough (0.46–1.40 μm) to penetrate into the lower regions of the respiratory tract. Because of their small size, they may remain in the air for a long period and can be present after the pollen season (21).

In the past, allergy research has focused on allergen presence in pollen grains, although pollen grains are too big to penetrate to the smallest human airways. However, the most recent studies have evidence provided that allergenic activity in the smaller micronic atmospheric aerosol fraction may play an

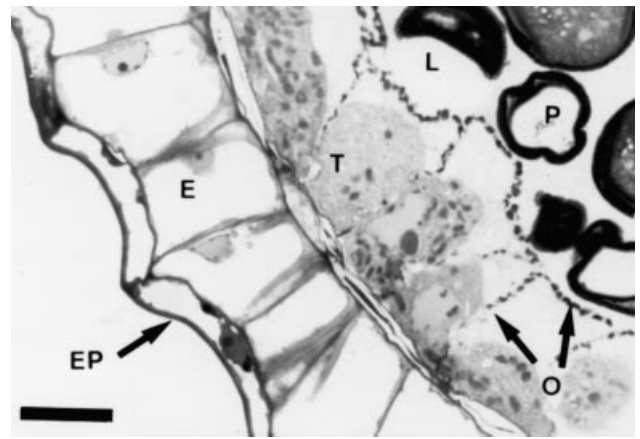


Figure 1. Light microscopic observation of transverse section of part of anther of *Ixora chinensis* “alba” (Rubiaceae). Orbicules (O) are visible on radial and innermost tangential wall of secretory tapetum cells (T). Inside locule (L), we observe pollen grains (P). EP: epidermis; E: endothecium. Scale bar: 20 μm .

important role in causing allergic reactions (21–31). Atmospheric starch granules, released from pollen grains due to osmotic bursts of humidified pollen grains, and even diesel particles might also contribute to the transmission of allergens in the smaller micronic atmospheric aerosol fraction (27, 32–34). Orbicules, if present in allergenic species, can also be part of the micronic fraction, emitted from the anthers during anthesis.

In the present study, we investigated the presence of orbicules in selected European allergologically important plants. The hypothesis of the potential role of orbicules as an important vector of allergens will be put forward, based on the comparison of our

Table 1. Orbicule size, shape, and presence on pollen exine. Species in bold possess orbicules. BR: herbarium of National Botanic Garden of Belgium; LV: herbarium of K.U. Leuven (Belgium); s.n.: without specimen number

Family	Species	Orbicule size (μm)	Orbicule shape	Orbicules attached on the pollen exine	Specimen investigated (country, collector, no., and herbarium)
Asteraceae	<i>Ambrosia coronopifolia</i> (ragweed)	—	—	—	France, A. Schneider 13601 (BR)
	<i>Artemisia vulgaris</i> (mugwort)	—	—	—	Denmark, J. Svendsen (s.n.) (BR)
Betulaceae	<i>Alnus glutinosa</i> (black alder)	0.50-(0.69)-1.00	Spiny	—	Belgium, S. Vinckier (s.n.) (LV)
	<i>Betula pendula</i> (birch)	0.50-(0.73)-1.00	Spiny	—	Denmark, A. Straehler (s.n.) (BR)
	<i>Corylus avellana</i> (hazel)	0.38-(0.83)-1.00	Spiny	—	France, F. T. de Witte (s.n.) (BR)
	<i>Chenopodium ambrosioides</i> (goosefoot)	0.75-(1.19)-1.50	Spiny	—	Denmark, J. Svendsen (s.n.) (BR)
Chenopodiaceae	<i>Chenopodium ambrosioides</i> (goosefoot)	0.75-(1.19)-1.50	Spiny	+	France, J. Lebeau (s.n.) (BR)
Fagaceae	<i>Castanea sativa</i> (sweet chestnut)	0.13-(0.29)-0.50	Spherical	—	France, G. F. de Witte 15149 (BR)
	<i>Quercus robur</i> (oak)	0.25-(0.51)-0.75	Granular-spherical	—	France, B. Bastin (s.n.) (BR)
Oleaceae	<i>Olea europaea</i> (olive)	—	—	—	Portugal, R. Dechamps 7222 (BR)
					France, G. F. de Witte 17.463 (LV)
Poaceae	<i>Cynodon dactylon</i> (Bermuda grass)	0.38-(0.54)-0.75	Spiny	+	Italy, Vandernaeghen (s.n.) (LV)
	<i>Dactylis glomerata</i> (cocksfoot)	0.38-(0.51)-0.75	Spiny	+	Belgium, P. Vande Vyvere (s.n.) (BR)
	<i>Lolium perenne</i> (ryegrass)	0.38-(0.59)-0.88	Spiny	+	Belgium, S. Vinckier (s.n.) (LV)
	<i>Phleum pratense</i> (timothy grass)	0.23-(0.38)-0.46	Spiny	+	Belgium, S. Vinckier (s.n.) (LV)
	<i>Rumex acetosa</i> (sorrel)	0.75-(0.91)-1.25	Spiny	—	Italy, S. Pignatti 11971 (BR)
Polygonaceae	<i>Rumex acetosa</i> (sorrel)	0.75-(0.91)-1.25	Spiny	—	Switzerland, G. Coutrez 480 (BR)
Urticaceae	<i>Parietaria diffusa</i> (pellitory)	0.38-(0.58)-1.00	Irregular	+	Greece, V. Vasak (s.n.) (BR)

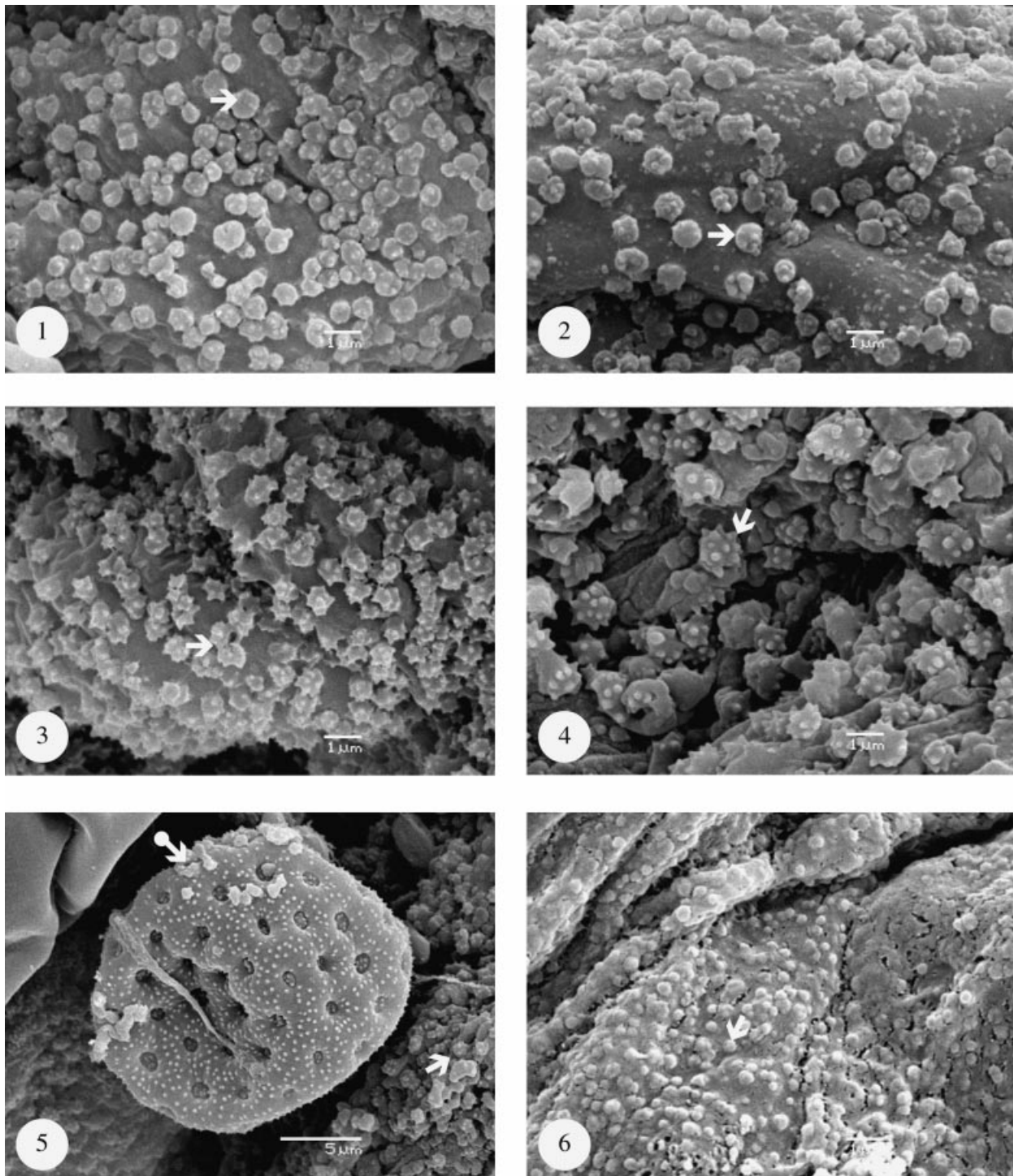


Figure 2. SEM observations of orbicules in Betulaceae (1–3), Chenopodiaceae (4–5), and Fagaceae (6) species. 1) *Alnus glutinosa*. Spiny orbicules (arrow) covering locule wall surface. 2) *Betula pendula*. Spiny orbicules (arrow) lying on locule wall surface. 3) *Corylus avellana*. Locule wall surface, covered by spiny orbicules (arrow). 4–5) *Chenopodium ambrosioides*. 4) Orbicules, possessing many microspines (arrow). 5) Orbicules (arrow) covering locule wall surface. Some orbicules are attached to exine of pollen grain (dotted arrow). 6) *Castanea sativa*. Spherical orbicules (arrow) covering whole locule wall surface.

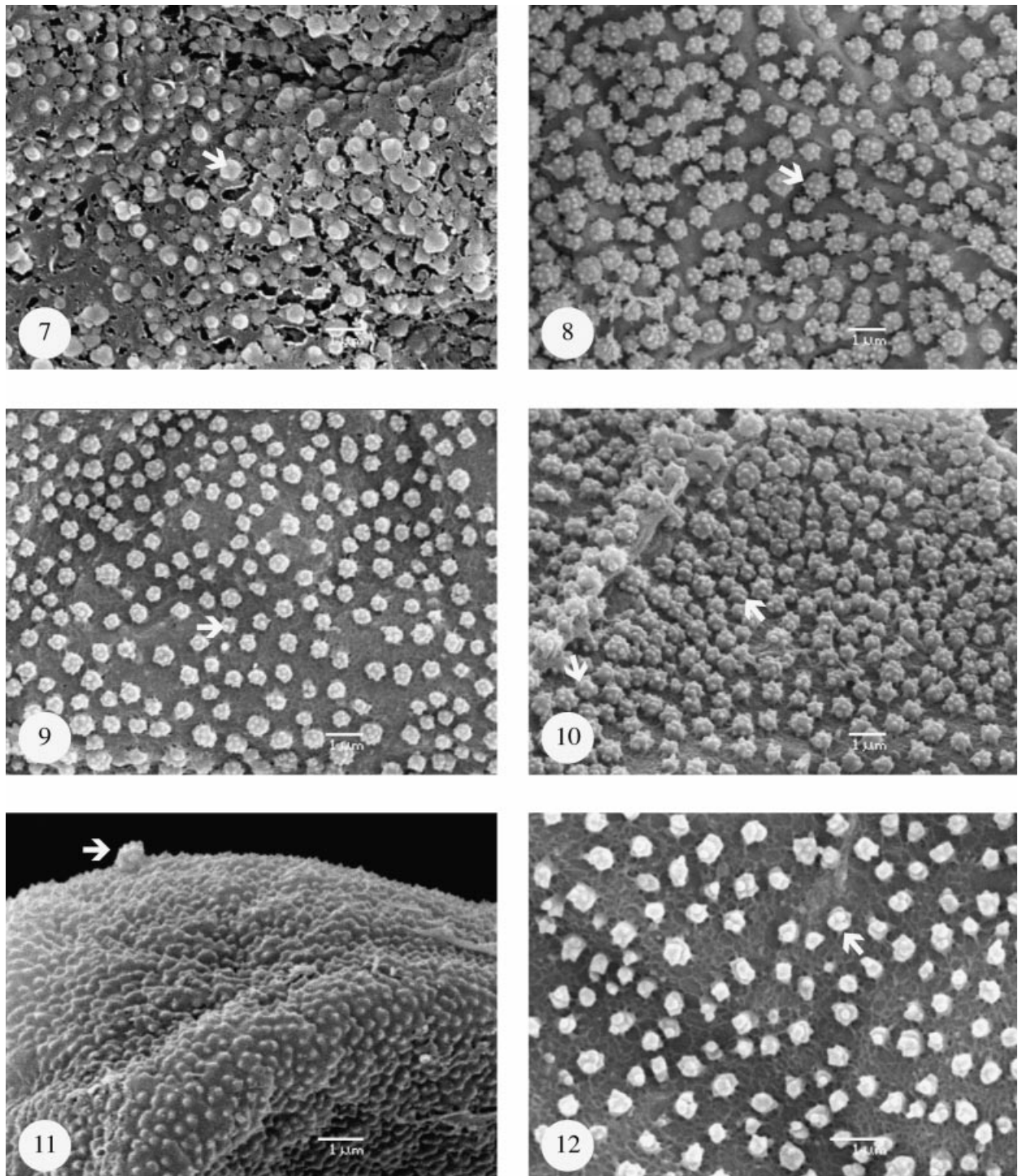


Figure 3. SEM observation of orbicules of Fagaceae (7) and Poaceae (8–12) species. 7) *Quercus robur*. Granular-spherical orbicules (arrow) lying on locule wall surface. 8) *Cynodon dactylon*. Locule wall surface is covered by spiny orbicules (arrow). 9) *Dactylis glomerata*. Small spiny orbicules (arrow) lying on locule wall surface. 10–11) *Lolium perenne*. 10) Small spiny orbicules (arrow) covering locule wall surface. 11) Single orbicule (arrow) is attached to exine of pollen grain. 12) *Phleum pratense*. Spiny orbicules (arrow) covering locule wall surface.

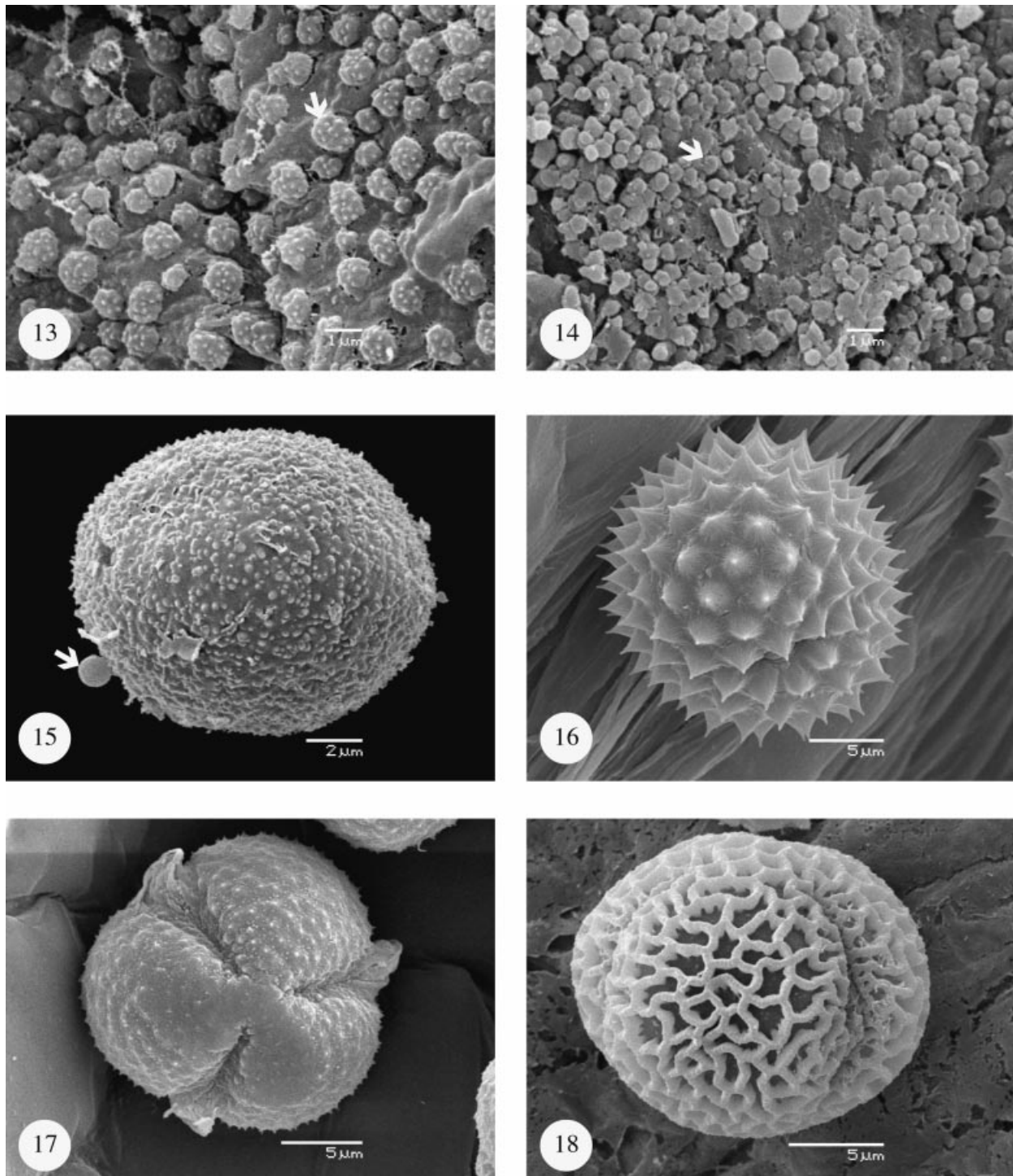


Figure 4. SEM observation of orbicules of Polygonaceae (13) and Urticaceae (14–15) species. 13) *Rumex acetosa*. Orbicules (arrow) possess many microspines on their orbicule wall. 14–15) *Parietaria diffusa*. 14) Irregularly shaped orbicules (arrow). 15) Orbicule (arrow) is attached to exine of pollen grain. 16–18) SEM observation of pollen grains of Asteraceae and Oleaceae species lacking orbicules. 16) *Ambrosia coronopifolia*. Pollen grain lying on smooth locule wall surface. 17) *Artemisia vulgaris*. Pollen grain lying on smooth locule wall surface. 18) *Olea europaea*. Pollen grain lying on locule wall surface.

results with the most recent literature about the evidence of allergenic activity in the smaller micronic atmospheric aerosol fraction.

Material and methods

This study is based on herbarium material (BR and LV) (Table 1). Fifteen species of 15 different genera were selected for observation, covering the families Asteraceae, Betulaceae, Chenopodiaceae, Fagaceae, Oleaceae, Poaceae, Polygonaceae, and Urticaceae (Table 1). The species selection was based on the *Allergy service guide in Europe* (35).

For observations of orbicules, six dried flowers or buds from each specimen were rehydrated in the wetting agent Agepon® (Agfa Gevaert, Leverkusen, Germany) for 1–2 h. All the anthers, at the stage of anthesis, were dissected from each flower, and the anther tips were removed with a razor blade to facilitate rehydration of the locules. After dissection, the anthers remained for another hour in the wetting agent. After dehydration in a graded acetone series, the material was critical point dried (CPD 030, Balzers) and mounted on stubs with double adhesive tape before further preparation. The pollen grains were removed from the opened locules with a fine cactus needle to facilitate observation of the locule surface. The stubs were sputter-coated with gold (SPI-MODULE™ Sputter Coater, SPI Supplies) prior to observation with a Jeol JSM-6400 scanning electron microscope (SEM), at 10–20 kV. Size measurements of orbicules were performed on the SEM micrographs.

Results

We observed orbicules (0.29–1.19 µm, mean values), covering the whole inner locule wall surface, in the anthers of all 12 allergenic species investigated of the families Betulaceae, Chenopodiaceae, Fagaceae, Poaceae, Polygonaceae, and Urticaceae (Figs. 2–4, 1–15) (Table 1). Orbicules were absent in the three allergenic Asteraceae and Oleaceae species investigated. If present, orbicules occur in very high numbers and are supernumerary to pollen grains.

We observed orbicules attached to the exine of the respective pollen grains (Table 1) in *Chenopodium ambrosioides* (Fig. 2, 5), *Parietaria diffusa* (Fig. 4, 15), and all grass species, as in *Lolium perenne* (Fig. 3, 11).

A short description of the orbicule size and shape, and the presence of orbicules on the pollen exine is given in Table 1. A more extensive description of the structure of these orbicules is given in Vinckier & Smets (36).

Discussion

In all allergenic species studied, orbicules were present on the locule wall surface of the anthers, except for *Ambrosia coronopifolia*, *Artemisia vulgaris*, and *Olea europaea*, in which the orbicules were absent (Fig. 4, 16–18).

The occurrence of orbicules attached to the exine (Figs. 2–4, 5, 11, 15) of pollen grains indicates that a percentage of the orbicules occur free in the anther. From our observations, we can conclude that orbicules

of *C. ambrosioides*, *P. diffusa*, and all the grass species investigated can be easily dispersed into the atmosphere during anthesis. Orbicules of these species can thus be part of the smaller micronic fraction emitted from the anthers. We did not observe any orbicules on the pollen exines of the other species studied, but this observation does not exclude the possibility of the occurrence of free orbicules in the anthers of these species. The size of the orbicules in the species studied ranged from 0.29 to 1.19 µm (mean values). If these micronic particles were dispersed into the atmosphere and inhaled, they could easily penetrate into the lower regions of the respiratory tract.

In *Betula* (Fig. 2, 2), we observed tiny orbicules with diameters ranging from 0.5 to 1 µm. Rantio-Lehtimäki et al. (37) found Bet v 1 in aerosol fractions smaller than pollen grains, even when no birch pollen was present. When Matikainen & Rantio-Lehtimäki (23) studied airborne birch-pollen allergens in different particle sizes, they found very high Bet v 1 activities (76% of total Bet v 1 activity) in particles with a size below 2.4 µm in diameter. *Betula* pollen is 21–24 µm in diameter. In the case of mAbs BIP 3 and BIP 4, El-Ghazaly et al. (32) localized the allergen in the cytoplasm and pollen wall. Minor labeling was observed in the orbicules. Comparing their results with ours, we conclude that *Betula* orbicules, containing a few allergens, may be present in the smaller micronic atmospheric aerosol fraction (below 2.4 µm) emitted from the anthers during anthesis.

Takahashi et al. (21) observed that the anthers of *Alnus japonica* and *Quercus serrata* discharged small particles other than pollen grains. The number of those particles was 15 times higher than the pollen count. Their diameter ranged from 0.46 to 2.2 µm, the most common size being 1.0–1.2 µm. We observed orbicules, in addition to pollen, in *Alnus* (Fig. 2, 1) and *Quercus* (Fig. 3, 7), with a diameter of 0.50–1.00 µm in *Alnus*, and 0.25–0.75 µm in *Quercus*. Comparable with the results for *C. japonica* (21), our results suggest that the micronic particles emitted from the dehiscing anthers of *Alnus* and *Quercus* may be orbicules.

The results of Spiekma et al. (29) strongly suggested the existence of airborne grass-pollen allergens associated with the smaller micronic fraction. Takahashi et al. (21) observed that the anthers of *Dactylis glomerata* discharged small particles, other than pollen grains, with a diameter of 0.46–2.2 µm. Our observations of the orbicules present on the pollen exine of all grass species studied, as in *Lolium perenne* (Fig. 3, 10 and 11), with a diameter of 0.23–0.88 µm, suggest that orbicules are among the particles emitted from the anthers of grass species.

Vithanage et al. (38) found glycoproteins, including group 1 allergen, on the orbicules and in the anther locule of *L. perenne*. The orbicules present in *L. perenne* can thus play an important role as a vector of allergens

when they are dispersed into the atmosphere. However, in the study of Taylor et al. (39), using specific monoclonal antibodies after anhydrous fixation, no labeling was shown on the orbicules or in the tapetal cells of *L. perenne*.

Ambrosia coronopifolia, *Artemisia vulgaris*, and *O. europaea* (Fig. 4, 16–18) lack orbicules. Agarwal et al. (22) found that *Ambrosia* antigen activity is present in particles of different size ranges, varying from 0.3 to >6 µm. They suggested that persistent *Ambrosia* plant debris, especially the inflorescence, may be a source of *Ambrosia* antigens found in small particles as well as *Ambrosia* antigen found before and after the *Ambrosia* pollination season. However, the observations of Takahashi et al. (21) did not suggest that airborne micronic *Ambrosia* antigens originate from the dehiscent anther. Takahashi et al. (21) found no micronic particles emitted from the dehiscent anthers of *Ambrosia*. This result corresponds with our observation of the absence of orbicules in the anthers of *Ambrosia*.

Pollen allergens are mainly of sporophytic origin; i.e., they are produced by the tapetum cells and appear in the pollen exine (32). Orbicules are produced by tapetum cells. At maturity, they have the same chemical composition as the pollen exine, and they develop

simultaneously with the pollen exine. If allergens are present in the orbicules, and orbicules are released into the atmosphere, they may act as very effective vectors of allergens.

In summary, we stress the need for allergy research by immunocytochemistry methods on whole anthers of allergenic plants, to reveal the sites of allergens across the whole anther and to determine whether the orbicules possess allergens. If allergens are found in the orbicules of allergenic species, we will have a better understanding of the allergenic activity in the smaller micronic atmospheric aerosol fraction, which causes allergic reactions in the lower respiratory tract.

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