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The manifold characters of orbicules: structural diversity, systematic significance, and vectors for allergens

STEFAN VINCKIER, PASCAL CADOT and ERIK SMETS

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In the anthers of flowering plants, gymnosperms, and seed ferns, tiny (±1 μm) granules might occur on the radial and innermost tangential wall of secretory tapetum cells. These sporopollenin granules develop simultaneously with the pollen exine and are called orbicules or Ubisch bodies. The present paper focuses on two quite different topics associated with orbicules.

The morphological and ultrastructural diversity of orbicules in the order Gentianales is summarized, and it is demonstrated that orbicules are a plesiomorphic feature in the order. Furthermore, orbicule characters seemed to be correlated with evolutionary trends in pollen dispersal unit and tapetum type features.

In the second part, we report on our investigation of *Corylus avellana* L. (Hazel) pollen, using immunogold electron microscopy to gain an insight into the possible role that orbicules may play as a vector of pollen allergens. During the pollen season orbicules are dispersed into the atmosphere along with Hazel pollen grains. The localisation of homologues of the new birch pollen allergen *Bet v* 7 was studied at the subcellular level in Hazel anthers. The results of this study indicate that orbicules and pollen of Hazel might act as very effective vectors for homologues of *Bet v* 7 and that debris of Hazel anthers represent vectors of allergens after the pollen season.

Stefan Vinckier, Erik Smets, Laboratory of Plant Systematics, Institute of Botany and Microbiology, K. U. Leuven, Kasteelpark Arenberg 31, B-3001 Leuven; Pascal Cadot, Laboratory of Experimental Immunology, Department of Clinical Immunology, K. U. Leuven, Herestraat 49, B-3001 Leuven, Belgium.*

**Corresp Author. E-mail: stefan.vinckier@bio.kuleuven.ac.be*

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In the anthers of flowering plants, gymnosperms, and seed ferns, on the radial and innermost tangential wall of secretory tapetum cells tiny (±1 μm) granules might occur. These sporopollenin granules are in close contact with the pollen grains inside the anther locule, and are called orbicules (Erdtman et al. 1961) or Ubisch bodies (Kosmath 1927). Despite their early discovery by Rosanoff (1865), little attention was paid to these structures and in many palynological studies they have been overlooked.

Orbicules are considered to be a general feature of species characterised by a secretory tapetum. Huysmans et al. (1998) reported, however, that orbicules are lacking in several taxa with such a tapetum. Species with an amoeboid tapetum, which is believed to be more derived than the secretory tapetum type, characteristically do not produce orbicules (Sporne 1973, Pacini et al. 1985). During the past 20 years different hypotheses have been suggested to determine the function of orbicules. For a summary of some of the proposed functions see Huysmans et al. (1998). Recent studies on wheat anthers demonstrated that orbicules carry a sporophytic structural protein (RAFTIN) that is targeted to the microspore exine and is essential for pollen development in this species (Wang et al. 2003).

Orbicules develop simultaneously with the growing pollen exine and are composed, like the pollen exine, of sporopollenin. Pre-orbicules, which are produced by the endoplasmic reticulum of secretory tapetal cells, are their progenitors (Echlin & Godwin 1968). The pre-orbicules are extruded through the radial and innermost tangential wall of the secretory tapetum cells, at the beginning of the tetrad stage (Christensen et al. 1972, El-Ghazaly & Jensen 1986, Clément & Audran 1993*a, b, c*; Vijayaraghavan & Chaudry 1993, Suarez-Cervera et al. 1995). Pre-orbicules are spherical structures, which are composed of a complex organic mixture. Sporopollenin deposition on the pre-orbicules can be mediated by ‘white lines’ showing a correlation between endexine and orbicule development as demonstrated by Echlin & Godwin (1968) in *Helleborus foetidus* L. and in *Tarenna gracilipes* (Hayata) Ohwi (Vinckier & Smets 2004). The wall of mature orbicules is composed of structural and filling elements (Clément & Audran 1993*a, b, c*). Proteins, pectins, polysaccharides, and glycolipids, organised in a three dimensional polygonal frame (Rowley 1990) are part of the structural elements. The filling elements consist of unsaturated lipids and polyphenols (Clément & Audran 1993*a, b, c*). At the ultrastructural level, Clément & Audran (1993*a, b, c*) could distinguish four different zones of electron density in mature orbicules of *Lilium* L.: an electron translucent orbicule cavity, a thin and very electron dense orbicule cavity-wall interface, a homogeneous electron dense orbicule wall, and a thin very electron dense peripheral layer.

In angiosperms, the ornamentation of the pollen exine and that of the orbicule wall often show striking parallelisms (Nilsson & Robyns 1974, El-Ghazaly & Jensen 1986, Hesse 1986, Vinckier & Smets 2002*a, b, c*, 2003). These

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parallelisms are explained by Hesse (1986) as being rooted in the homology of tapetum and sporogeneous tissue. The tapetum and the microspores contain homologous, nearly identical genetic information for the production, release and accumulation of sporopollenin (Young et al. 1979, Pacini et al. 1985, Hesse 1986). This may indicate that studies on orbicule wall formation will clarify the underlying factors determining exine and orbicule wall patterning. Furthermore, orbicules are extracellular structures, independent of cytoplasmic control, as opposed to the pollen exine. These observations corroborate the idea proposed by Clément & Audran (1993*b, c*) that orbicule wall development provides a model to study sporopollenin deposition.

Since ornamentation of the pollen exine offers useful characters for systematics, orbicules might also have taxonomic value. In the first part of the present paper we give a summary of our major findings on the evolution of orbicule characters in the order Gentianales based on previously published work (Vinckier et al. 2000, Vinckier & Smets 2002*a, b, c*, 2003; Vinckier 2003).

Orbicules and allergy

The pollen grains of *Betula pendula* Roth (Silver Birch) are one of the main causes of allergic reactions in Middle and Northern Europe, North America, Russia, and in some areas of Australia, with symptoms occurring in early spring (El-Ghazaly 1999, Valenta et al. 1999). Birch allergies are a major threat to public health in these countries, since 10 – 15% of the population suffers from these diseases (El-Ghazaly 1999). Five birch pollen allergens have already been identified (Ipsen & Løwenstein 1983, Valenta et al. 1991, Seiberler et al. 1994, Engel et al. 1997). Cadot et al. (1995) found three new birch allergens, possibly isoforms of the same protein, in birch pollen extracts, which were prepared at controlled basic pH. In a later study (Cadot et al. 2000) they were able to purify and characterise the new birch allergen, which was designated as *Bet v* 7. The internal amino acid sequence analysis shows high homology of *Bet v* 7 to plant cyclophilins (Cadot et al. 2000). Cyclophilins have been defined as a new family of cross-reactive allergens (Flückiger et al. 2002).

In the past, allergy research has been focused on the study of allergen presence in pollen grains, although pollen grains are of a size which does not allow them to reach the smallest human airways. However, in the most recent studies evidence is supplied that allergenic activity in the atmospheric aerosol fraction smaller than pollen grains may play an important role in causing allergic reactions (Agarwal et al. 1984, Spieksma et al. 1990, Takahashi et al. 1991, Takahashi et al. 1995, Schäppi et al. 1997*a, b*, Matikainen & Rantio-Lehtimäki 1998, Schäppi et al. 1999*a, b*, Schumacher et al. 1998, Taylor et al. 2004). Amyloplasts and remains of the pollen cytoplasm, released as particulate aerosol from pollen grains due to osmotic bursts of humified pollen grains followed by drying winds, and even diesel particles might contribute to the transmission of allergens in the aerosol smaller than pollen grains (Grote et al. 1994, El-Ghazaly et al. 1999, Schäppi et al. 1999*b*, Staff et al. 1999, Taylor et al. 2004).

Pollen allergens are mainly of sporophytic origin, i.e., they are produced by the tapetum cells and appear in the pollen exine (El-Ghazaly et al. 1999). Orbicules are also produced by tapetum cells, but at maturity, they have the same chemical composition as the pollen exine, and they develop simultaneously with the pollen exine. An interesting fact is that hay-fever-causing angiosperms are characterised by the presence of secretory tapeta (Davis 1967). Davis (1967), for the first time raised the question of whether orbicules also contain allergens. Orbicules are present in all important allergenic species of Betulaceae, Chenopodiaceae, Fagaceae, Poaceae, Polygonaceae, and Urticaceae (Vinckier & Smets 2001*a, b*). However, orbicules are absent in some clinically important Asteraceae (*Ambrosia coronopifolia* Torr. & A. Gray, *Artemisia vulgaris* L.) and Oleaceae (*Olea europaea* L.) species (Vinckier & Smets 2001*a, b*). If allergens are present in the orbicules, and orbicules are released into the atmosphere, they may act as very effective vectors of allergens (Vinckier & Smets 2001*b*). In the second part of the present study we aim to investigate the localisation of cross-reactive homologues of the cyclophilin *Bet v* 7 in the anthers of *Corylus avellana* L. (Hazel), using immunogold electron microscopy.

MATERIAL AND METHODS

This study is based on light (LM), scanning electron (SEM) and transmission electron microscopy (TEM) on herbarium and living plant material. Our results are partly based on previously published studies (Vinckier et al. 2000, Vinckier & Smets 2001*a, b*, 2002*a, b, c*, 2003), on orbicule diversity in the order Gentianales and on the presence of orbicules in important allergenic species. Orbicules have been studied in 178 species (130 genera) distributed among Loganiaceae *s.l.*, Apocynaceae *s.l.*, Gentianaceae, and the rubiaceous subfamily Ixoroideae. This selection covers all major monophyletic groups recently recognised in Gentianales, supplemented with 15 important allergenic species (15 genera). For collection data of the studied specimens, and for preparation techniques for LM, SEM and TEM of orbicules and pollen from herbarium or fresh material we refer to Vinckier et al. (2000), Vinckier & Smets (2001*a, b*, 2002*a, b, c*, 2003). Previous data have been supplemented with new material and techniques (immunogold electron microscopy) for the second part of this paper.

Capturing emitted particles from Hazel anthers at anthesis

Branches from *Corylus avellana* L. shrubs, with approximately 10 attached flowering catkins at early anthesis, were harvested and supported, in a controlled environment chamber, above stubs coated with double-sided adhesive tape in order to capture the particles emitted from the catkins. The stubs were sputter coated with gold (SPI-MODULE^{TM} Sputter Coater, SPI Supplies, West Chester, PA, USA) and observed with a JSM-6400 scanning electron microscope (JEOL Inc., Peabody, MA, USA) at 25 kV.

Immunogold electron microscopy

For immunogold electron microscopy fresh *Corylus avellana* L. anthers were collected from a shrub growing near the Institute of Botany and Microbiology. The athers were fixed overnight at 4°C with a mixture of 0.25% glutaraldehyde and 4% *p*-formaldehyde at pH 7.4, buffered with 0.1 M sodium cacodylate. The fixed material

Table I. *Pollen dispersal unit and orbicule presence in Gentianales.*

Gentianales taxa studied		Pollen dispersal unit	Orbicules present	References
Apocynaceae *s.l.*	**Rauvolfioideae**	3–4-colporate monads	+	Vinckier & Smets 2002*b*
	Apocynoideae	3–4-porate, occasionally aperturate or polypantoporate monads	+	"
		Porate tetrads in *Apocynum*	+	"
	Periplocoideae	4–6-porate tetrads	+	"
		Multiporate tetrads in *Raphionacme*	+	"
		Two pollinia (massulae) per locule composed of porate tetrads without outer wall enclosing the pollinium in *Hemidesmus* and *Streptocaulon*	+	"
	Secamonoideae	Two pollinia (massulae) per locule composed of inaperturate tetrads without outer wall enclosing the pollinium	–	"
	Asclepiadoideae	One pollinium per locule composed of inaperturate single pollen grains with an outer wall enclosing the pollinium (excl. *Fockea*)	– (excl. *Riocreuxia*)	"
Gentianaceae	***Voyria* (genus *incertae sedis*)**	2-porate monads	–	Vinckier & Smets 2003
	***Anthocleista* (Potalieae)**	3-porate monads	+	"
	Exaceae, Gentianeae, Potalieae, Saccifolieae	3-colporate monads	+/–	"
	Chironieae	3-colporate monads	+/–	"
		Tetrads in *Coutoubea* and *Schultesia*	–	"
	Helieae	3-colporate monads	+/–	"
		Tetrads in *Calolisianthus* and *Prepusa*	+	"
		Polyads in *Irlbachia*	–	"
Loganiaceae *s.l.*		3-colporate monads	+	Vinckier & Smets 2002*a*
Rubiaceae	**Ixoroideae Pavetteae, Octotropideae, Coffeeae, Ixoreae**	3-colporate monads	+	Vinckier et al. 2000
	Gardenieae incl. Aulacocalyceae	3-colporate monads	+/–	"
		Tetrads	–	"

was dehydrated at 4°C in a graded ethanol series, followed by impregnation with LR-White Resin overnight at 4°C, and embedding in LR-White Resin at 49°C for 24 hours. The protein used to immunise the rabbits is a recombinant form of *Bet v* 7 that was produced in *Escherichia coli* at the Laboratory for Experimental Immunology (K. U. Leuven, Belgium). Immunoblots have shown that the rabbit antiserum also detects the natural *Bet v* 7, purified from Silver Birch pollen. The rabbit antiserum specifically recognizes an 18-kDa protein in Hazel pollen extract and this reaction is inhibited by pre-incubation of the antiserum with purified *Bet v* 7.

Incubation of the ultra-thin sections on gold grids was performed with 5% BSA in PBS at pH 7.4 during 15 minutes. The sections were incubated overnight with primary polyclonal rabbit antiserum against *Bet v* 7 diluted 1:200 in PBS at 4°C. After the incubation with the primary polyclonal antibody, the sections were rinsed three times for 5 minutes with PBS (pH 7.4) followed with one rinse for 5 minutes with TBS - 0.1% BSA (pH 8.2). Next, the sections were incubated, for one hour at room temperature, with gold conjugated (10 nm) secondary goat anti-rabbit IgG diluted 1:20 in TBS – 0.1% BSA (pH 8.2). The sections were rinsed once for 5 minutes with TBS - 0.1% BSA (pH 8.2), followed by three rinses with distilled water. After the sections were dried, they were counter-stained with uranyl acetate and lead citrate in LKB 2168 Ultrostainer and were observed in a Zeiss EM 900 transmission electron microscope at 50 kV. As a control, sections were incubated with a secondary antibody, without the prior incubation with the primary antibody. All methods were carried out in duplicate.

RESULTS AND DISCUSSION

Orbicules in Gentianales

Research of orbicule diversity in Gentianales species has revealed new insights into the correlation between orbicule characters and evolutionary trends in the palynology of this order (Vinckier et al. 2000, Vinckier & Smets 2002*a, b, c*, 2003; Vinckier 2003).

In Apocynaceae *s.l.* a trend is observed from the presence of spherical orbicules in the majority of species belonging to the basal group of subfamilies Rauvolfioideae, Apocynoideae and Periplocoideae (Endress & Bruyns 2000) which are characterised by colporate, orporate single pollen grains, or 3–6 porate tetrads, towards embedded orbicules in the more advanced genera of Periplocoideae which have multiporate tetrads or pollinia (Table I; Vinckier & Smets 2002*b*, Nilsson et al. 1993, Verhoeven & Venter 2001). Therefore, we suggest that embedded orbicules are a more derived orbicule type. Lastly, orbicules do not occur in Secamonoideae and the other most advanced Asclepiadoideae (Endress & Bruyns 2000), with the exception of *Riocreuxia* Decne. (Table I).

These observations also fit well with our study of Rubiaceae, subfamily Ixoroideae (Vinckier et al. 2000). The primitive nature of tribe Gardenieae, suggested by Robbrecht & Puff (1986), was not supported in Andreasen & Bremer's (2000) analysis. On the contrary, the monophyletic newly circumscribed tribe Gardenieae is shown to be the most derived group in the Ixoroideae. Absence of orbicules occurs in different Gardenieae genera, especially in those characterised by the presence of tetrads (Table I, Vinckier et al. 2000). Although more research is needed, it is suggested that the absence of orbicules, is probably a derived feature in this group.

In the majority of Gentianaceae genera monads are present, but tetrads and polyads have also evolved within this family (Struwe et al. 2002). Comparable with the palynological trend observed in Apocynaceae *s.l.* and Rubiaceae (Vinckier et al. 2000, Vinckier & Smets 2002*b*), a similar trend is observed in Gentianaceae from genera which have monads possessing orbicules, to species which have more derived pollen dispersal units (tetrads or polyads) which lack orbicules (Table I, Vinckier & Smets 2003). However, no comparable trend in orbicule typology, as recorded in Apocynaceae *s.l.* (spherical orbicule type→ embedded orbicule type→orbicules absent) (Vinckier & Smets 2002*b*), was present in the family Gentianaceae. The Gentianaceae species *Canscora decussata* (Roxb.) Schult. & Schult. f. and *Swertia perennis* L. are characterised by amoeboid tapeta (Maheswari Devi 1962, Sankara Rao & Chinnappa 1983), and produce very small orbicules distributed in very low distribution densities on the locule wall surfaces (Vinckier & Smets 2003). Therefore, we suggest that presence of very small orbicules (mean: 0.2 μm), along with a very low distribution density (mean: 2 orbicules/ 25 μm^2), may indicate the presence of an amoeboid tapetum in a species, as sporopollenin granules have already been reported in taxa with an amoeboid tapetum by Tiwari & Gunning (1986).

Orbicules and allergy

Morphology, ultrastructure, and dispersion of Hazel orbicules

In *Corylus avellana* L. (Hazel) spiny orbicules cover the locule wall surface (Fig. 1A, Vinckier & Smets 2001*a, b*). At the ultrastructural level orbicules of Hazel occur as single entities, indicated by the presence of one electron translucent orbicule cavity (Fig. 1B). Between the cavity and the homogeneous electron dense orbicule wall an electron dense orbicule cavity-wall interface is present (Fig. 1B, *white arrow*). The sporopolleninous spinulae, which are characteristic for this type of orbicule, are part of the homogeneous electron dense orbicule wall. Around this wall a very electron dense peripheral layer is present (Fig. 1B, *black arrow*).

Scanning electron microscopic observation of the particles emitted from the anthers of Hazel at anthesis revealed that spiny orbicules were dispersed, along with the pollen grains, into the atmosphere (Fig. 1C–D). In this species orbicules are also attached to the pollen exine (Fig. 1C–D).

Localisation of homologues of Bet v 7 in Hazel anthers

This paper reports the results of the first localisation study of homologues of *Bet v* 7 in Hazel anthers. The localisation experiments are carried out on mature Hazel anthers at the stage of anthesis; as a consequence tapetum cells are completely degenerated. As a control condition, sections are incubated with secondary gold-labelled antibody, without prior incubation with the primary antiserum, as a

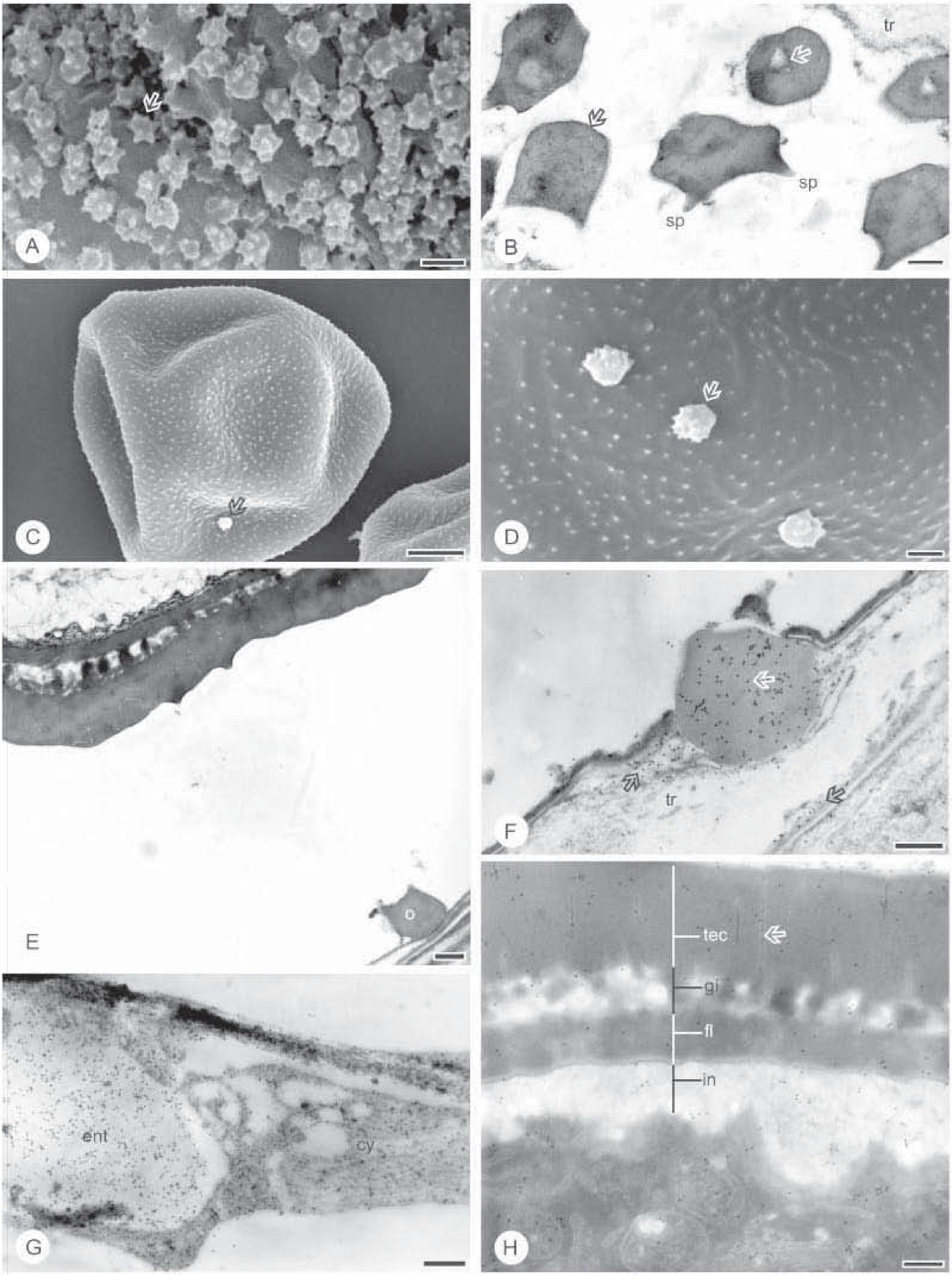
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Fig. 1. **Morphology, ultrastructure (A–D), and localisation experiments of homologues of *Bet v* 7 in orbicules of *Corylus avellana* L. (E–H).** (A) Detailed SEM observation of the locule wall covered by spiny orbicules (***arrow***); (B) TEM observation of Hazel orbicules. Between the electron translucent orbicule cavity and the homogeneous electron dense orbicule wall an electron dense orbicule cavity-wall interface is present (***white arrow***). The sporopolleninous spinulae (***sp***) are part of the homogeneous electron dense orbicule wall. Around this wall a very electron dense peripheral layer is present (***black arrow***); (C) SEM observation of a pollen grain emitted from a Hazel anther during anthesis. An orbicule is attached to the exine (***arrow***); (D) Detailed SEM observation of three orbicules attached to the pollen exine (***arrow***); (E) Control condition of localisation experiments. No labelling is found in the orbicules (***o***) or pollen, TEM; (F) Localisation of homologues of ***Bet v*** 7 in the orbicules (***white arrow***) and the remnants of the tapetal cells (***tr***) (***black arrow***), TEM; (G) Localisation of homologues of ***Bet v*** 7 in the endothecial cytoplasm (***cy***) and thickening (***ent***), TEM; (H) Localisation of homologues of ***Bet v*** 7 in tectum (***tec***), granular infratectum (***gi***), foot-layer (***fl***), intine (***in***), and cytoplasm of the pollen. A microchannel is indicated (***white arrow***). TEM. Scale bars – 1 µm (A, D); 0.4 µm (B, G); 5 µm (C); 0.6 µm (E); 0.25 µm (F, H).

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test for occurrence of false-positive reactions. No labelling of the tissue was observed in the control condition, thus excluding the possibility of false-positive reactions (Fig. 1E).

The spiny orbicules are slightly embedded in the remnants of the tapetal cells (Fig. 1F). In the tectum of the pollen exine microchannels are observed (Fig. 1H, *white arrow*).

Major labelling for cross-reactive homologues of *Bet v* 7 was observed in the orbicules, in the homogeneous electron dense orbicule wall (Fig. 1F, *white arrow*). The remnants of the tapetal cells (Fig. 1F, *black arrows*), endothecium cytoplasm (Fig. 1G), and endothecial thickenings (Fig. 1G) display major labelling for homologues of *Bet v* 7. In the pollen grains labelling was found in the tectum, granular infratectum, foot-layer, intine, and cytoplasm (Fig. 1H).

Vectors for allergens

The occurrence of orbicules attached to the exine of Hazel pollen grains, indicate that a percentage of the orbicules occurs free in the anther and can become airborne during anthesis (Fig. 1C–D). The size of the orbicules, in the species studied, ranges from 0.29 to 1.19 µm (mean values) (Vinckier & Smets 2001*a, b*). If these microscopic particles are dispersed into the atmosphere and are inhaled by individuals, they can easily penetrate into the lower regions of the respiratory organs (Vinckier & Smets 2001*a, b*). Our localisation experiments in Hazel support the hypothesis that orbicules may act as very effective vectors to bring the allergenic homologues of *Bet v* 7 to susceptible individuals (Fig. 1F) (Vinckier & Smets 2001*b*). The occurrence of allergenic homologues of *Bet v* 7 in the Hazel pollen grain cytoplasm (Fig. 1H) confirms that fragments of pollen ruptured under humid conditions can represent a vector of allergens (Taylor et al. 2004). In a study conducted by Canini et al. (2004) the localisation of a carbohydrate epitope recognised by human IgE was found in pollen and orbicules of allergenic Cupressaceae species. These results provide additional support to our hypothesis that orbicules may act as vectors for allergens.

After the Hazel pollen season, the anthers will die and debris from the tapetum and endothecium cells will be dispersed into the environment, acting as vectors of allergenic homologues of *Bet v* 7 (Fig. 1G).

Bet v 7 is characterised as cyclophilin and cyclophilins appear to be involved in plant stress response. Under stress conditions differences are observed in cyclophilin mRNA levels depending upon stress factor and the plant species studied (Marivet et al. 1994). Cyclophilins might function as a 'chaperone-like molecule' in order to decrease the risk of proteolytic degradation or to avoid aggregation of proteins, reactions that take place during stress (Kay 1996). In maize and bean it has been shown that the synthesis of cyclophilins is up-regulated by stress (Marivet et al. 1994). It is also known that *Bet v* 1, the major birch allergen, and its homologue proteins are up-regulated by stress (Breiteneder et al. 1989, Swoboda et al. 1994). As a consequence, it is possible that in Silver Birch trees or Hazel shrubs experiencing stress, higher amounts of the allergenic cyclophilins will be produced. These findings indicate that stress factors, such as pollution, may also play an important role in the increase of allergens present in our environment and in the rising incidence of birch pollen allergy (Cadot et al. 2000).

CONCLUSIONS

Orbicules are a general feature in Gentianales and their characters seemed to be strongly correlated with evolutionary trends in the palynology of Apocynaceae *s.l.*, Gentianaceae, and Rubiaceae. Our observations corroborate the idea that the presence of orbicules is a plesiomorphic feature (Sporne 1973, Pacini et al. 1985). In Gentianales additional research should be carried out on the ontogeny of orbicules, especially on Gentianaceae species which produce very small 'orbicules' and are believed to possess an amoeboid tapetum. Such studies may give an answer to the question whether the small 'orbicules' present in these species are homologous with the sporopollenin granules produced by amoeboid tapeta (cf. *Tradescantia* L., Tiwari & Gunning 1986) or can be considered as orbicules.

Immunocytochemical methods are a very useful tool to identify the allergens carried by particulate matter in the atmosphere such as amyloplasts, remains of the pollen cytoplasm, or orbicules. Our findings contribute to a better understanding of the allergenic activity in the aerosol fraction smaller than pollen grains, causing allergic reactions in the lower region of the respiratory tract. Besides offering an explanation for bronchial asthma symptoms in susceptible patients, a practical implication of this study is that the traditional pollen count may be misleading as an index of outdoor allergen exposure (D'Amato 2001).

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