

Systematic and morphologic survey of orbicules in allergenic angiosperms

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Abstract Orbicules are sub- or pauci-micronic sporopollenin-coated particles associated with pollen grains. Recent studies have highlighted their importance in terms of systematic value or as vectors of allergens that may trigger pollinosis. While orbicules have indeed been reported for most families and genera of allergological importance, their usefulness in systematics remains little explored. Our study aims: (a) to discover the presence/absence of orbicules in species of allergological interest hitherto overlooked regarding these particles; (b) to propose standard descriptors for orbicule morphology; and (c) to link the features of orbicules to a systematic framework. We observed or measured selected orbicule traits in SEM micrographs of pollen sacs and pollen grains of 23 species of 17 genera in Asteraceae, Betulaceae, Fagaceae, Oleaceae, Poaceae, Plantaginaceae and Polygonaceae families. Categorical and continuous traits were included in a matrix and then subjected to NMDS as ordination method to assess the usefulness of orbicule traits as systematic characters. We detected orbicules both on the locule wall and on the pollen exine in almost all investigated species and described unknown nanoparticles smaller than orbicules. Based on orbicules traits, NMDS separated the samples at species level. We detected and described orbicules on

the tapetum and on the pollen exine in 19 target species and observed unknown nanoparticles that might explain (a) the process of deposition of sporopollenin during orbicule development and (b) the onset of pollinosis before and after the dispersion of pollen grains into the air. Finally, within the restricted sample examined, selected orbicule traits allowed to resolve the data set at species level.

Keywords Orbicule traits · Systematic characters · Unknown nanoparticles · Sporopollenin deposition · Allergenic plants · Pollinosis · Asthma

1 Introduction

The major allergenic pollen grains are released by botanical families that are widely distributed in the world (Byng et al. 2016). They represent the most significant biotic particles in the atmosphere, as its allergens are the drivers of airborne allergic diseases. Currently, pollinosis has a significant clinical and economic impact globally, increasing every year with over 400 million people suffering from allergic rhinitis and over 300 million people affected by allergic asthma (Valovirta 2011). Aerobiological monitoring represents the most common monitoring system, which allows the identification and count of airborne pollen grains. However, the size of aerodispersed

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intact pollen grains—usually between 15 and 40 μm —may explain the allergenic reactions in the upper airways but not the symptoms in lower ones: pollen grains cannot reach so deep, because they are larger than the lower airways lumen (D’Amato et al. 2007). Furthermore, it does not explain the pollinosis before the dispersion of pollen grains into the air and after their deposition on the ground (D’Amato et al. 2007). Many studies have revealed that during anthesis, a large number of micronic particles—up to 15 times greater than the pollen count—are dispersed, representing a significant biotic fraction of the atmospheric aerosol (Takahashi et al. 1995; El-Ghazaly et al. 1995; Staff et al. 1999; D’Amato et al. 2002, 2007; Swoboda et al. 2004; Vinckier and Smets 2001a, b; Taylor et al. 2004; Dinis et al. 2007; Sénéchal et al. 2015; Ruggiero and Bedini 2018). Of these particles, orbicules have been identified as important particles in triggering allergic diseases (D’Amato et al. 2002, 2007; Solomon 2002; Swoboda et al. 2004; Sénéchal et al. 2015). Orbicules are sub- and pauci-micronic granules of sporopollenin found on the secretory tapetum on the anthers in several orders of angiosperms and conifers (Verstraete et al. 2014) and in some instances on the pollen surface, e.g. in Cupressaceae (Takahashi et al. 1995; Sénéchal et al. 2015; Ruggiero and Bedini 2018). In many instances, in angiosperms the orbicule wall ornamentations closely resemble those of the pollen exine, thus suggesting their possible value as systematic characters (Vinckier and Smets 2001b). Usually, they are much more numerous than pollen grains, and when dispersed into the atmosphere, they are able to remain in the air much longer than pollen grains and are small enough to reach even the pulmonary alveoli. Ruggiero and Bedini (2018) proved that Cupressaceae species release orbicules in large amounts in the atmosphere, as revealed directly in daily aerobiological samples. Many studies have confirmed the presence of pollen allergens and antigens in association with orbicules and sporopollenin using immunocytochemical methods (Southworth et al. 1988; Miki-Hirosige et al. 1994; Suárez-Cervera et al. 2003; Canini et al. 2004; Vinckier et al. 2005, 2006; Jato et al. 2010). This suggests that orbicules may act as very effective vectors of allergens (Vinckier and Smets 2001a, b; Vinckier et al. 2005).

In the current phylogenetic classification of angiosperms, orbicules are not considered as an apomorphic

feature. They are common in the ANITA grade and monocots, but are absent in the Asteraceae and the majority of Lamiaceae, revealing an evolutionary trend from presence to absence along the phylogenetic tree. Their size, shape and density are useful systematic characters in the tribal characterization within families of Gentianales (Verstraete et al. 2014). However, to the best of our knowledge no previous attempts have been made to assess the systematic value of orbicule traits by any kind of multivariate statistical method.

Previous studies have investigated the presence of orbicules in species of allergological interest (Takahashi et al. 1995; Vinckier and Smets 2001a, b; Vinckier et al. 2005; Dinis et al. 2007). They have shown that orbicules are indeed present and hypothesized their dispersal in the atmosphere, thus supporting their role in the onset of pollinosis.

The aims of this survey of plant species of allergological interest are: (a) to increase the sampling of species in terms of the presence and location of orbicules; (b) to describe the orbicules’ morphology in relation to pollen exine ornamentation; (c) to gain a deeper insight into the systematic importance of orbicules.

2 Materials and methods

2.1 Plant materials

This study is based on specimens collected in the Herbarium Horti Pisani of the University of Pisa (PI), as listed in Table 1. For our survey, 23 species from 17 different genera were selected, covering seven families of allergological interest: Asteraceae, Betulaceae, Fagaceae, Oleaceae, Plantaginaceae, Polygonaceae and Poaceae (Nilsson and Spieksma 1994; D’Amato et al. 2007). Plant nomenclature followed Conti et al. (2005, 2007) and subsequent updates published in the scientific journal *Informatore Botanico Italiano* (2007–2015) and subsequently in the Italian Botanist (2016 onwards). The suprageneric classification followed Byng et al. (2016).

2.2 Scanning electron microscope (SEM)

We excised three ripe inflorescences per specimen and then collected two anthers from each one. We then

Table 1 Summary of orbicules and pollen morphology of the investigated species from PI (Herbarium Horti Pisani, University of Pisa, Italy)

Family	Species	Size (μm)				Shape of orbicule	Orbicular ornamentation	Sexine ornamentation	Density (n° orbicules per μm^2)	Note	Herbarium code
		N	Min.	Mean	Max.						
Asteraceae	<i>Ambrosia maritima</i> L.	—	—	—	—	—	—	Echinate	—	—	9146—1 (PI)
Asteraceae	<i>Artemisia arborescens</i> L.	—	—	—	—	—	—	Echinate	—	—	9358—2 (PI)
Asteraceae	<i>Artemisia annua</i> L.	—	—	—	—	—	—	Echinate	—	—	9358—2 (PI)
Betulaceae	<i>Betula pendula</i> Roth	100	0.619	0.836	0.999	0.103	Irregular polygon	Blunt nanocone*	0.41	emb.— threads— aggr	1887—6 (PI)
Betulaceae	<i>Corylus avellana</i> L.	100	0.546	0.860	1.180	0.123	Cylinder	Blunt nanocone*	0.37	emb.— aggr	1886—5 (PI)
Betulaceae	<i>Carpinus betulus</i> L.	100	0.179	0.873	1.198	0.172	Rounded	Nanogranulate— blunt nanocone*	0.52	emb.— threads— aggr	1884—7 (PI)
Betulaceae	<i>Ostrya carpinifolia</i> Scop.	100	0.255	0.626	0.844	0.148	Rounded	Nanogranulate— blunt nanocone*	0.67	emb.— threads— aggr	1884—18 (PI)
Betulaceae	<i>Alnus cordata</i> (Loisel.) Duby	100	0.447	0.611	0.954	0.095	Cylinder	Nanogranulate— blunt nanocone*	1.16	emb.— aggr	1888—1 (PI)
Betulaceae	<i>Alnus glutinosa</i> (L.) Gaertn.	100	0.420	0.604	0.821	0.084	Cylinder	Nanogranulate— blunt nanocone*	0.87	emb.— aggr	1888—3 (PI)
Fagaceae	<i>Quercus ilex</i> L.	100	0.326	0.577	0.942	0.119	Granule	Psilate	1.46	emb.— aggr.	1830—0001/1 (PI)
Fagaceae	<i>Quercus suber</i> L.	100	0.282	0.505	0.836	0.098	Granule	Psilate	1.57	emb.— aggr.	1893—3 (PI)
Fagaceae	<i>Quercus pubescens</i> Willd.	100	0.365	0.600	0.850	0.079	Granule	Psilate	1.05	emb.— aggr.	1893—18 (PI)
Fagaceae	<i>Castanea sativa</i> Miller	100	0.142	0.349	0.672	0.075	Granule	Psilate	2.97	emb.— aggr.	1891—15 (PI)

Table 1 continued

Family	Species	N	Size (μm)			Shape of orbicule	Orbicular ornamentation	Sexine ornamentation	Density (n° orbicules per μm^2)	Note	Herbarium code
			Min.	Mean	Max.						
Fagaceae	<i>Fagus sylvatica</i> L.	100	0.357	0.697	1.077	0.146	Granule	Psilate–nanogranulate	1.03	emb.–threads–aggr.	1890–4 (PI)
Oleaceae	<i>Fraxinus ornus</i> L.	100	0.128	0.355	0.608	0.102	Wart-like	Psilate–reticulate	3.12	emb.–aggr.	6420–20 (PI)
Oleaceae	<i>Fraxinus excelsior</i> L.	100	0.150	0.247	0.352	0.041	Wart-like	Nanogranulate–reticulate	8.12	emb.–aggr.	0000–0203–2 (PI)
Oleaceae	<i>Fraxinus angustifolia</i> subsp. <i>oxycarpa</i> (Willd.) Franco & Rocha Afonso	100	0.124	0.203	0.286	0.034	Wart-like	Nanogranulate–reticulate	2.22	emb.–aggr.	0000–0119–1 (PI)
Oleaceae	<i>Ligustrum lucidum</i> W.T. Aiton	–	–	–	–	–	–	Microgranulate–reticulate	–	–	6436–4 (PI)
Plantaginaceae	<i>Plantago maritima</i> subsp. <i>serpenitina</i> (All.) Arcang	100	0.442	0.952	1.702	0.246	Irregular polygon	Nanospinulose	0.84	Lightly emb.–threads–aggr.	8116–4 (PI)
Polygonaceae	<i>Persicaria bistorta</i> (L.) Samp.	100	0.178	0.293	0.433	0.063	Wart-like	Psilate	13.01	emb.–aggr.	2201–6 (PI)
Poaceae	<i>Anthoxanthum odoratum</i> L.	100	0.386	0.529	0.698	0.065	Rounded	Nanospinulose–psilate	1.69	Lightly emb.–threads	205–13 (PI)
Poaceae	<i>Paspalum dilatatum</i> Poir.	100	0.401	0.654	0.918	0.097	Rounded	Nanospinulose	1.30	Lightly emb.	161–4 (PI)
Poaceae	<i>Echinochloa crus-galli</i> (L.) P. Beauv.	100	0.254	0.488	0.733	0.096	Rounded	Psilate–nanogranulate	6.78	Lightly emb.–threads–aggr.	166–6 (PI)

N, number of observations; size, minimum–mean–maximum–SD, standard deviation; density, number of orbicules per μm^2 ; emb, embedded in tapetum, lightly emb, partially embedded in tapetum; aggr, aggregated; threads, orbicules connected to each other and/or with tapetum; +, present; –, absent; *, new terminology

shook anthers directly onto aluminium stubs covered with double adhesive carbon tape, in order to release pollen grains from the pollen sacs. Finally, the anthers were fixed on the carbon tape and sputter-coated with gold. The specimens were examined and photographed with a SEM (Verstraete et al. 2014), (JEOL JSM 5410, Jeol Ltd, Tokyo, Japan). Herbarium specimens are commonly used as source in orbicule studies and our own SEM comparisons of orbicules from herbarium and in vivo specimens for selected species allow us to safely exclude that orbicules obtained from herbarium specimens undergo any significant changes in shape and size.

2.3 Measurements and statistical analysis

We checked for the presence of orbicules on pollen exine in at least 15 pollen grains per sample found either in the pollen sac or directly on the stub.

We measured the size of the orbicules on the tapetum in six anthers from each specimen, with 100 random measurements per species.

Density of the orbicules on the tapetum was calculated on the micrographs by counting the number of orbicules in six square sections of $75 \mu\text{m}^2$ each, randomly chosen on flat surfaces of the tapetum included in the micrograph. Therefore, in total we analysed $450 \mu\text{m}^2$ in six anthers of each species, except for *Fraxinus excelsior*, *Persicaria bistorta* and *Anthoxanthum odoratum*, where we sampled $30 \mu\text{m}^2$ for each tapetum for a total of $180 \mu\text{m}^2$. The density was then defined as the mean number of orbicules divided by the unit sampled area.

We described pollen morphology according to Punt et al. (2007) and used the same glossary, with additional terms integrated, to characterize the orbicule morphology with four qualitative parameters, taking into account (1) the general shape; (2) ornamentations visible on the surface; and (3) the links, if any, with one another and with the tapetum surface (Table 1). The orbicule shape can sometimes be obfuscated by the external ornamentations. In these cases, the orbicule shape was inferred by visually subtracting the ornamentation from SEM images, thus resulting in a “naked” orbicule.

Then, we described the association between the orbicules and tapetum wall. In particular, we indicated the level of fusion between the parts of the orbicule in contact with the tapetum wall with two different terms:

(1) fully embedded, when the underneath part of the orbicule was fused, (2) lightly embedded, when it was only partially fused in the tapetum wall. Finally, we characterized orbicules in terms of their capability to form: (1) aggregates among themselves and (2) links—called threads—with one another and/or the tapetum surface (Huysmans et al. 2010).

For all species where orbicules were detected, we assembled a data set as a matrix with species in rows and the following variables in columns: mean size, density (continuous variables), shape, orbicular ornamentation, association between orbicules and tapetum wall, and presence/absence of aggregates or threads (categorical variables).

We chose non-metric multidimensional scaling (NMDS) (Minchin 1987) as the ordination method for the matrix; however, we first had to compute a distance matrix with Gower distance (Gower 1971) on account of the combination of continuous and categorical variables in the data set. The distance matrix was computed with the R package “cluster” (R Development Core Team 2014; RStudio Team 2015). The NMDS was applied with the R package “vegan”. Goodness of fit was also calculated via the “factorfit” function of the same package, and the level of statistical significance was set at $p < 0.05$ based on 999 permutations.

We used Statistica 10 (StatSoft software) to calculate the correlation index between the diameter and density of orbicules for each species. Finally, we performed unpaired t tests between the size of the unknown nanometric particles detached on the pollen surface and/or tapetum wall and the size of the orbicular ornamentations for each species listed in Table 2 ($n = 30$). The level of statistical significance was set at $p < 0.05$.

3 Results

Table 1 summarizes the observed orbicule traits in all target species. Table 2 summarizes the observations of unknown nanometric particles on the tapetum and on pollen sexine.

3.1 Presence of orbicules

In most of the investigated species, orbicules were present both on the tapetum of the anthers and on the pollen surfaces (Figs. 1, 2, 3, 4, 5, 6, 7, 8; Table 1).

Table 2 Summary of sizes of unknown nanometric particles on the tapetum and on pollen sexine

Family	Species	N	Size (µm)—orbicular ornamentations				Size (µm)—unknown nanometric structures				Independent <i>t</i> test			
			Min.	Mean	Max.	SD	Min	Mean	Max	std. dev.	<i>t</i> value	df	<i>p</i> 2-sided	Other ornamentation attached on the pollen exine
Betulaceae	<i>Betula pendula</i> L.	30	0.157	0.197	0.244	0.024	0.160	0.201	0.273	0.026	0.651	57.778	0.517	+
Betulaceae	<i>Corylus avellana</i> L.	30	0.149	0.216	0.299	0.038	0.124	0.201	0.273	0.043	1.409	57.332	0.164	+
Betulaceae	<i>Carpinus betulus</i> L.	30	0.118	0.176	0.254	0.033	0.116	0.171	0.235	0.027	0.676	55.489	0.502	+
Betulaceae	<i>Ostrya carpinifolia</i> Scop.	30	0.127	0.167	0.231	0.025	0.105	0.156	0.205	0.024	1.667	57.825	0.101	+
Betulaceae	<i>Alnus cordata</i> (Loisel.) Duby	30	0.150	0.206	0.264	0.029	0.142	0.206	0.259	0.026	0.028	57.072	0.978	+
Betulaceae	<i>Alnus glutinosa</i> (L.) Gaertn.	30	0.167	0.235	0.298	0.034	0.164	0.224	0.324	0.041	1.152	56.076	0.254	+
Plantaginaceae	<i>Plantago maritima</i> subsp. <i>serpentina</i> (All.) Arcang	30	0.171	0.246	0.325	0.042	0.191	0.265	0.357	0.045	1.733	57.815	0.088	+

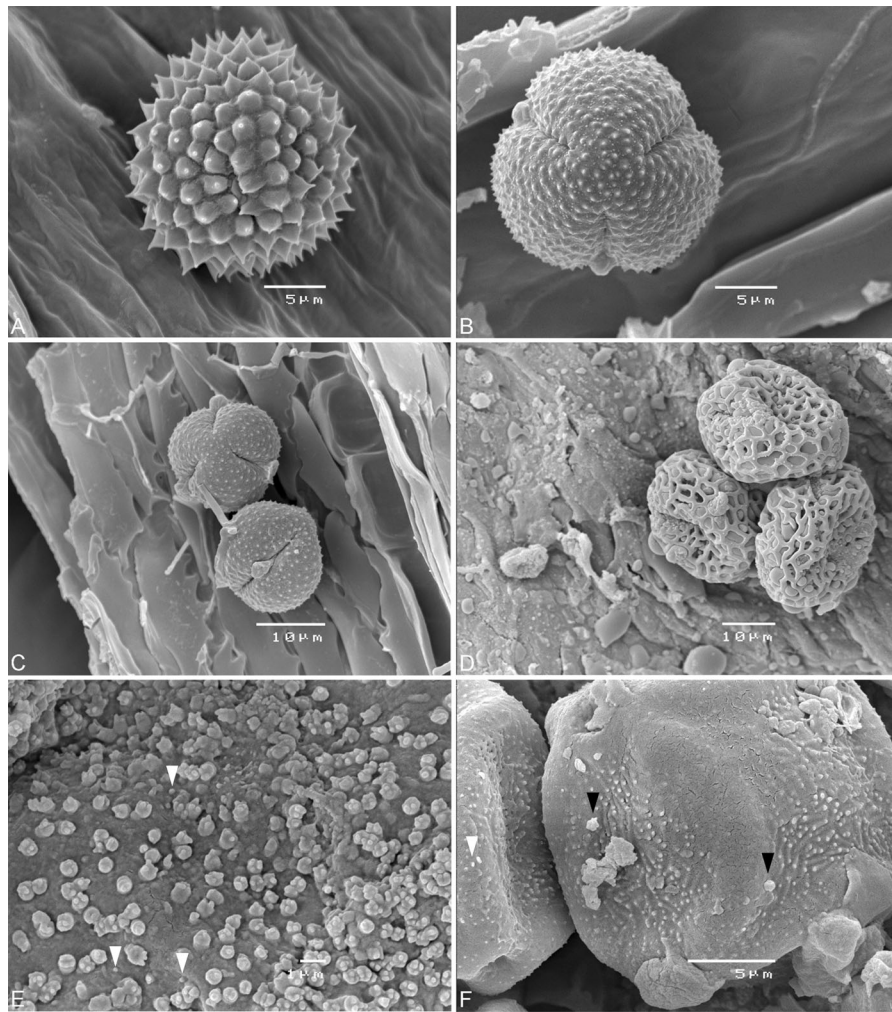


Fig. 1 Tapetum surfaces and pollen grains lacking in orbicules in **a** *Ambrosia maritima*, **b** *Artemisia annua* and **c** *A. arborescens*. **d** Tapetum surface and pollen grains of *Ligustrum lucidum* with pollenkitt. **e**, **f** Orbicules and unknown nanoparticles on the tapetum surface and on pollen grains of *Alnus cordata*, in black and white arrowheads, respectively. Thin

fusion lines can be observed on the structure of the orbicules and their ornamentations in *A. cordata* (**e**). Orbicules have a cylinder shape with nanogranulate—blunt nanocone ornamentations, and they are embedded on the tapetum surface in *A. cordata* (**e**). Sexinic ornamentations in *A. cordata* are nanogranulate—blunt nanocone (**f**)

The only exceptions were *Ambrosia maritima*, *Artemisia arborescens*, *A. annua*, whose tapetum surfaces were smooth and devoid of orbicules (Fig. 1a–c), and *Ligustrum lucidum*, whose locule wall was covered by pollenkitt droplets (Fig. 1d).

3.2 Size

The mean size of the orbicules varied from 0.203 μm (*Fraxinus angustifolia* subsp. *oxycarpa*) to 0.952 μm (*Plantago maritima* subsp. *serpentina*). The smallest

size was found in *F. angustifolia* subsp. *oxycarpa* (0.124 μm), the largest in *P. maritima* subsp. *serpentina* (1.702 μm ; Table 1).

3.3 Distribution, density and location of orbicules

Sometimes, orbicules were connected with one another and/or with tapetal membrane by thin threads (Figs. 2d, 3a, f, 5e, 7a, f, 8d).

When present, orbicules covered the entire locule wall and vastly outnumbered the pollen grains,

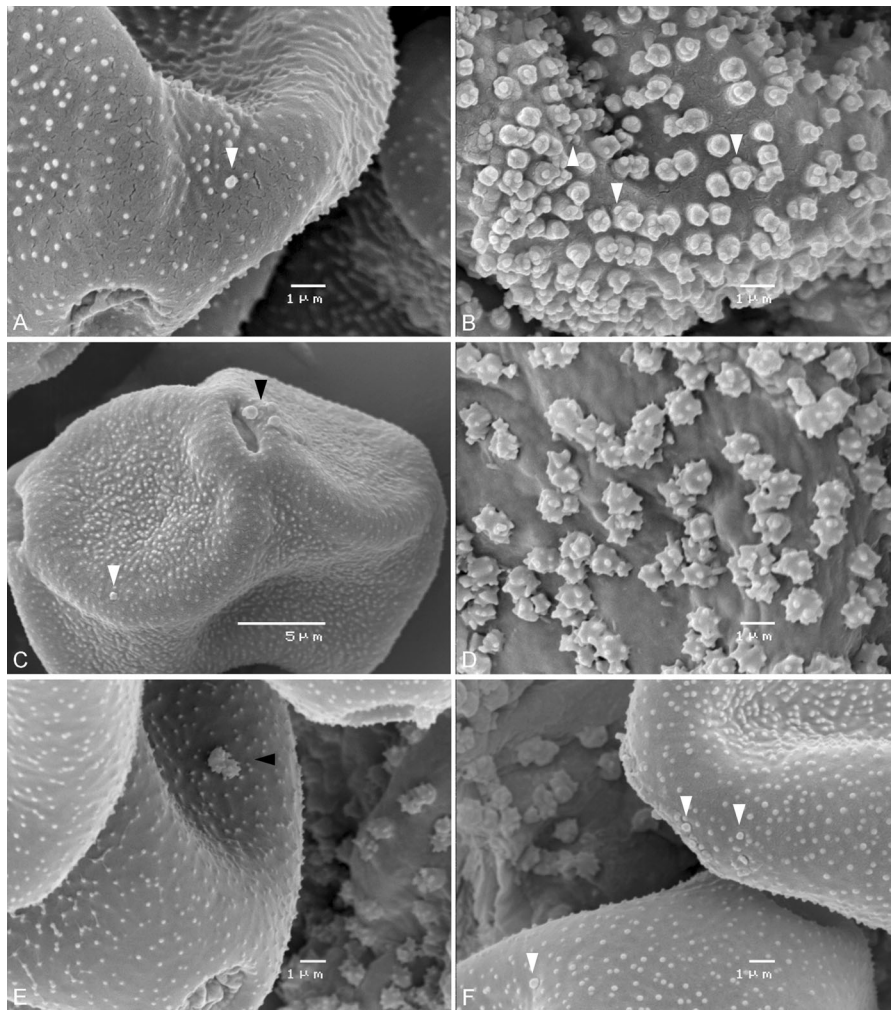


Fig. 2 Unknown nanoparticles on the pollen grains of *Alnus cordata* (a), *A. glutinosa* (c), *Betula pendula* (f), (white arrowheads). Orbicules on the tapetum and on the pollen grains of *A. glutinosa* (b), (c) and *Betula pendula* (d), (e) (black arrowheads). Thin lines of fusion are visible by observing the structure of the orbicules and their ornamentations in *A. glutinosa* (b). In *A. glutinosa*, orbicules have a cylindrical shape with nanogranulate—blunt nanocone ornamentations, and they are

embedded on the tapetum surface and irregularly clustered (b). In *B. pendula*, orbicules have a shape as an irregular polygon with blunt nanocone ornamentations; they are embedded on the tapetum surface, irregularly clustered and thin threads occur, connecting orbicules to each other or with the tapetum surface (d). Sexinic ornamentations in *A. glutinosa* are nanogranulate—blunt nanocone (c) and in *B. pendula* are blunt nanocone (d), (f)

especially in *Pericaria bistorta* (Fig. 7d) where the surface of the tapetum was barely visible, thus registering the highest values of mean density with 13.01 orbicules/ μm^2 (Table 1). Usually, they were irregularly clustered (Figs. 1e, 2b, d, 3a, c, f, 4c, e, 5a, c, e, 6a, c, e, 7a, d, 8d) (Table 1), but rarely in *Paspalum dilatatum* and *Anthoxanthum odoratum* (Figs. 7f, 8b).

In four species, the orbicules were lightly embedded in the tapetum (Figs. 7a, f, 8b, d), and in the

remaining species, they were fully embedded (Figs. 1e, 2b, d, 3a, c, f, 4c, e, 5a, c, e, 6a, c, e; Table 1).

Orbicle size and number of orbicules per μm^2 were negatively correlated ($r = -0.6329$; $p = 0.004$).

3.4 Shape

The species belonging to the Betulaceae family exhibited a considerable variation in the orbicle

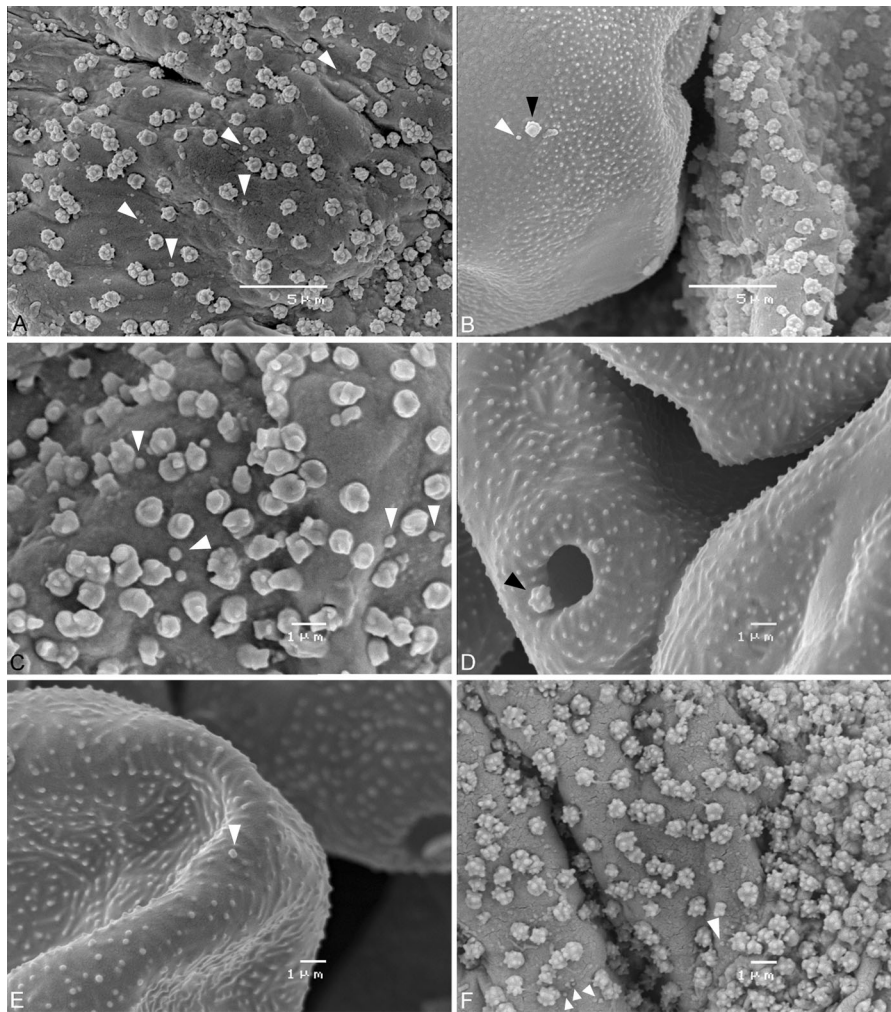


Fig. 3 Tapetum surfaces and pollen grains with orbicules (black arrowheads) and unknown nanometric particles (white arrowheads) in the species *Carpinus betulus* (**a**), (**b**); *Corylus avellana* (**c**), (**d**), (**e**) and *Ostrya carpinifolia* (**f**). Thin lines of fusion are visible observing the structure of the orbicules and their ornamentations of *C. betulus* (**a**); *C. avellana* (**c**) and *O. carpinifolia* (**f**). In *C. betulus* (**a**) and *O. carpinifolia* (**f**), orbicules have a rounded shape with nanogranulate—blunt

nanocone ornamentations, and they are embedded on the tapetum surface and irregularly clustered and thin threads occur, connecting orbicules to each other or with the tapetum surface. In *C. avellana*, orbicules have a cylindrical shape with blunt nanocone ornamentations, and they are embedded on the tapetum surface (**c**). Sexinic ornamentations in *C. betulus* (**b**) are nanogranulate—blunt nanocone and in *C. avellana* are blunt nanocone (**d**), (**e**)

shape, ranging from cylinder shape in the *Alnus* and *Corylus* species (Figs. 1e, 3c) to rounded in *Carpinus* and *Ostrya* (Fig. 3a, f), and an irregular polygon in *Betula* (Fig. 2d). Their orbicular ornamentations were usually developed on the top of the structure in *Alnus* and *Corylus*, while in *Betula*, *Carpinus* and in *Ostrya*, they usually also emerged laterally. However, in *Alnus*, *Carpinus*, *Ostrya*, they were usually nanogranulate and sometimes blunt nanocone, while in *Corylus*

and *Betula*, they were elongated and/or blunt nanocone. Their number varied from one to 11 elements.

In all investigated species of Polygonaceae and Oleaceae, the orbicules were mainly wart-like; in Fagaceae and Poaceae, they were granule and rounded, respectively (Figs. 4c, e, 5a, c, e, 7f, 8b, d). The orbicules of Fagaceae and Oleaceae, as well as those of *Echinochloa crus-galli*, originated from the overlapping and/or fusion of two or more subunits (Figs. 4, 5, 6, 8d). Usually, this feature appears in

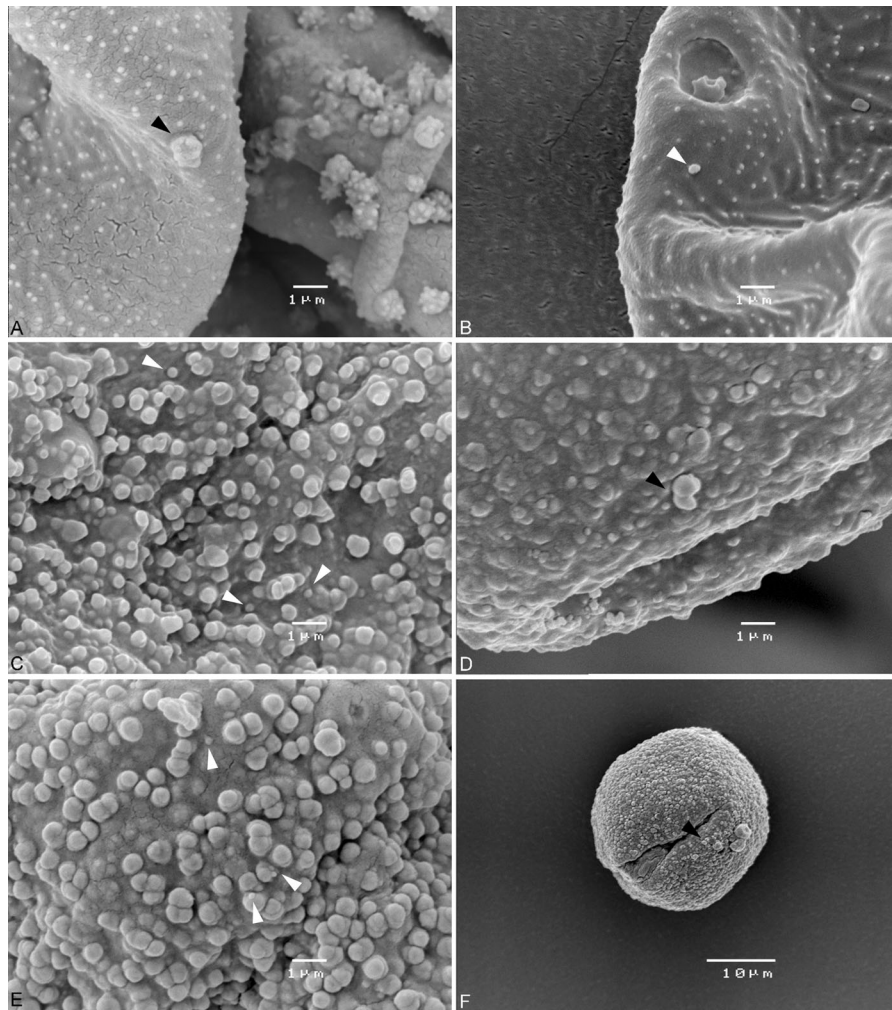


Fig. 4 **a, b** Orbicule (black arrowhead) and unknown nanometric particle (white arrowhead) on the pollen grains of *Ostrya carpinifolia*, respectively. Tapetum surfaces and pollen grains with orbicules in *Quercus ilex* (**c**), (**d**) and *Q. suber* (**e**), (**f**) (black arrowheads). Unknown nanometric particles are present on both the tapetum walls of *Q. ilex* (**c**) and *Q. suber* (**e**). Thin fusion

lines are present on the orbicule surfaces. In *Q. ilex* (**c**) and *Q. suber* (**e**), orbicules have a granular shape with psilate ornamentations; they are embedded on the tapetum surface and irregularly clustered. Sexinic ornamentations in *O. carpinifolia* are nanogranulate—blunt nanocone (**a**), (**b**) and in *Q. ilex* (**d**) and *Q. suber* (**f**) are microgranulate

orbicules with psilate and/or nanogranulate ornamentations as observed in the species cited previously.

In *Plantago maritima* subsp. *serpentina*, orbicules had the shape of an irregular polygon, with orbicular ornamentations attached on all the surfaces (Fig. 7a). Their shape was mainly nanospinulose in *P. maritima* subsp. *serpentina* (Fig. 7a), *Anthoxanthum odoratum* (Fig. 7f) and *Paspalum dilatatum* (Fig. 8b). Sometimes in *A. odoratum*, the orbicule wall appeared smooth and in *P. maritima* subsp. *serpentina* was also present as elongated elements. In *Echinochloa crus-*

galli, the orbicules were also made up of the aggregation of rounded structures with smooth walls, and seldom with tiny granularities (Fig. 8d).

In all the species investigated, several times we noted thin fusion lines developing around the main core and the base of the orbicular ornamentations. These features were more evident in the species with granule-like orbicules or rounded ones with nanogranulate and psilate ornamentations. In particular, in *Fraxinus excelsior*, *F. angustifolia* subsp. *oxycarpa* and *Echinochloa crus-galli*, the orbicules formed by

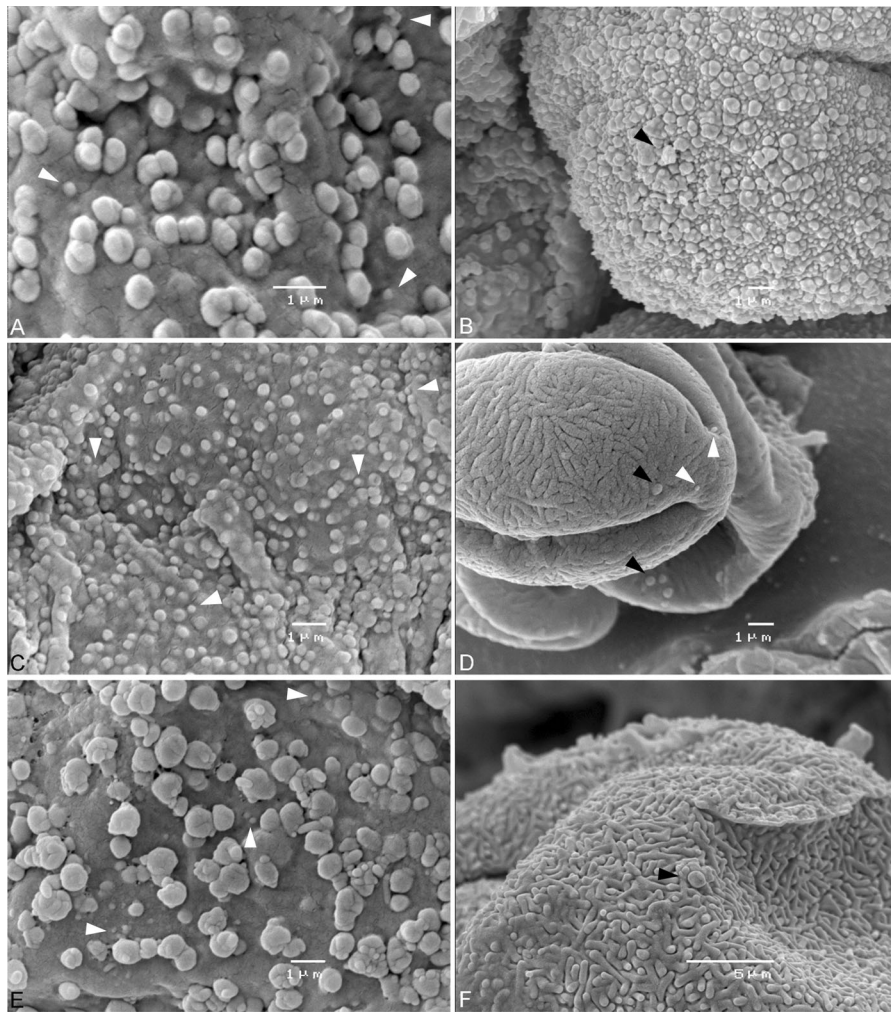


Fig. 5 Orbicules (black arrowheads) on the tapetum walls and on the pollen grains in *Quercus pubescens* (a), (b); *Castanea sativa* (c), (d) and *Fagus sylvatica* (e), (f). Unknown nanometric particles (white arrowheads) on the tapetum surfaces in the previous species (a), (c), (e) and on the pollen exine of *C. sativa*. Thin lines of fusion are visible by observing the structure of the orbicules on the tapetum walls of previous species. In *Q. pubescens* (a), *C. sativa* (c) and *F. sylvatica* (e), orbicules have a

granular shape with psilate ornamentations in (a), (b) and psilate—nanogranulated in (e). They are embedded on the tapetum surface and irregularly clustered, and in *F. sylvatica* (e) thin threads occur, connecting orbicules to each other or with the tapetum surface. Sexinic ornamentations in *Q. pubescens* are microgranulate (b) and in *C. sativa* (d) and *F. sylvatica* (f) are rugulate

aggregations of rounded subunits with highly variable sizes (Figs. 6c, e, 8d).

3.5 Morphology and ornamentation of pollen sexine and orbicule

In our study, the predominant shapes of the orbicules were round and granule-like followed by wart-like. Generally, we observed a similarity between the orbicular and sexinic ornamentations. However, we

found some exceptions in the Fagaceae and Poaceae families. Indeed, in the Fagaceae family, orbicular ornamentations usually appeared psilate, while the sexinic ornamentations were microgranulate or rugulate (Figs. 4d, f, 5b, d, f). In *Echinochloa crus-galli*, the orbicular ornamentation was nanogranulate and psilate, while the sexinic ornamentation was nanospinulose (Fig. 8d, e). In some cases, we found a minority of orbicules lacking ornamentation in species characterized by ornamented orbicules (Figs. 1e, 2b, 3a, c).

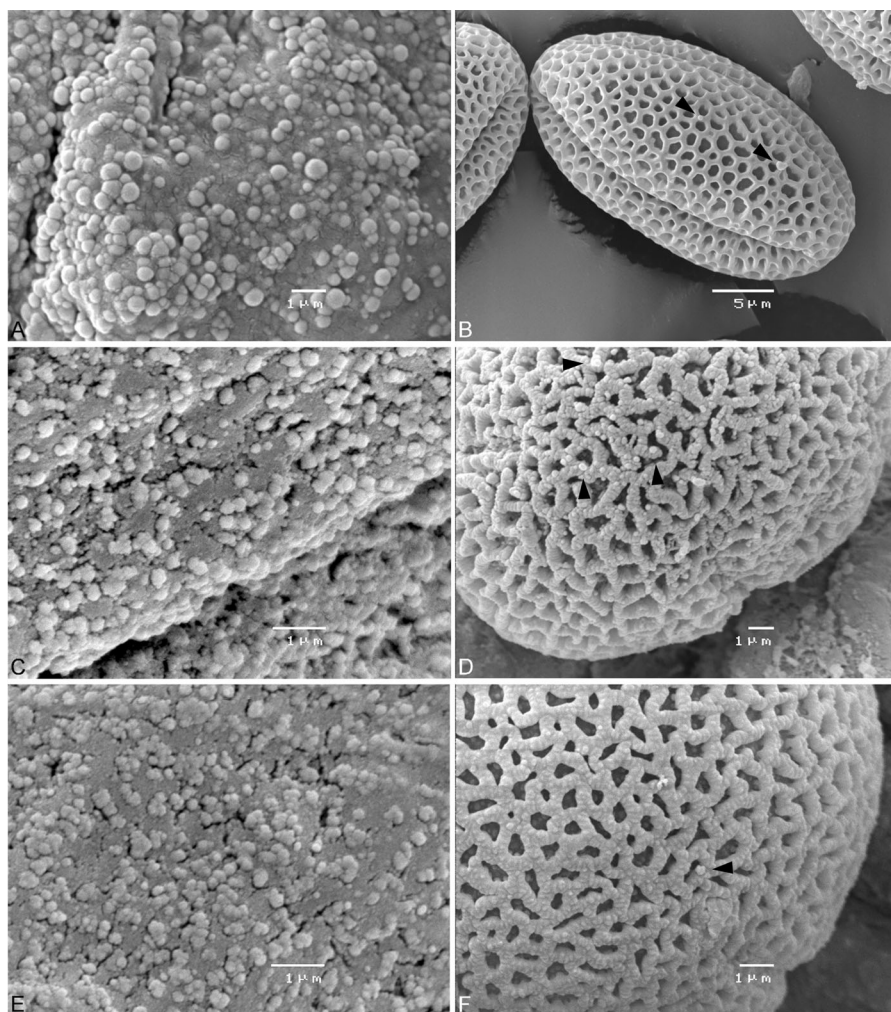


Fig. 6 Orbicules (black arrowheads) on the tapetum walls and on the pollen grains in *Fraxinus ornus* (a), (b); *F. excelsior* (c), (d) and *F. angustifolia* subsp. *oxycarpa* (e), (f). In *F. ornus* (a), *F. excelsior* (c) and *F. angustifolia* subsp. *oxycarpa* (e), orbicules have a wart-like shape with psilate ornamentations

in *F. ornus* (a), and nanogranulate in *F. excelsior* (c), and in *F. angustifolia* subsp. *oxycarpa* (e). They are embedded on the tapetum surface. Sexinic ornamentations in *F. ornus* are psilate—reticulate (b) and in *F. excelsior* (d) and *F. angustifolia* subsp. *oxycarpa* (f) are nanogranulate—reticulate

3.6 Non-metric multidimensional scaling (NMDS)

Figure 9 shows the result of the NMDS. The investigated species are ranked in six distinct groups, corresponding to the respective botanical families. Within each group, genera are clearly distinguished from one another, while the different species of *Quercus*, *Alnus* and *Fraxinus*, with the exception of *F. ornus*, are very close in the plot or totally overlapping (*A. glutinosa* and *A. cordata*). Our findings are

supported by the results of goodness of fit, with $r^2 = 0.8274$, $p = 0.001$, and stress = 0.14.

3.7 Unknown nanometric structures

In the investigated species of Plantaginaceae, Betulaceae (except *Betula*) and Fagaceae, we observed on the tapetum—in addition to the orbicules—unknown nanometric structures, especially in *Carpinus* and in *Ostrya* (Figs. 3a, b, f, 4b). Their sizes are (Table 2) is smaller than that of orbicules and we were unable to observe particles of intermediate size, which led us to

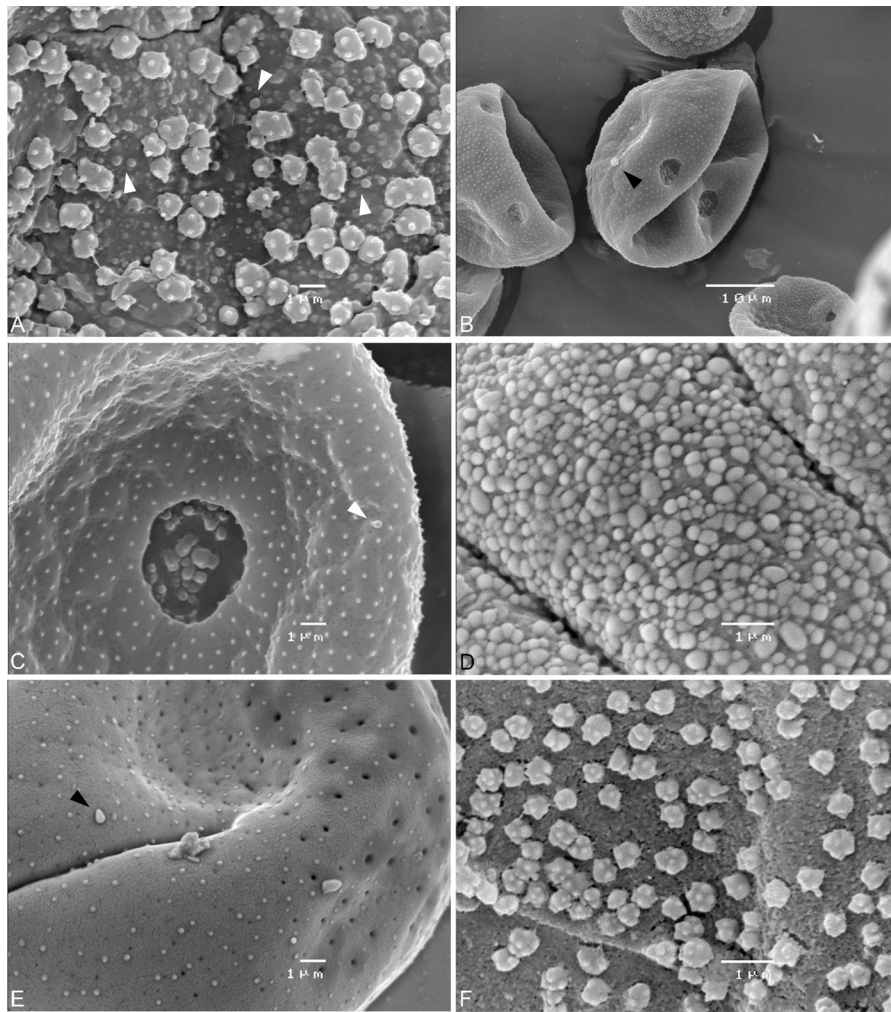


Fig. 7 Tapetum surfaces and pollen grains with orbicules (black arrowheads) and unknown nanometric particles (white arrowheads) in the species *Plantago maritima* subsp. *serpentina*, (a), (b), (c); *Persicaria bistorta*, (d), (e); *Anthoxanthum odoratum*, (f). Thin lines of fusion are visible by observing the structure of the orbicules in *P. maritima* subsp. *serpentina* (a) and *A. odoratum* (f). In *P. maritima* subsp. *serpentina* (a), *P. bistorta* (d) and *A. odoratum* (f), orbicules have a shape as an irregular polygon, a wart-like and rounded, respectively. Orbicular ornamentations in *P. maritima* subsp. *serpentina*

(a) are nanospinulose, in *P. bistorta* (d) psilate and in *A. odoratum* (f) nanospinulose—psilate. Orbicules are light embedded on the tapetum surface in *P. maritima* subsp. *serpentina* (a), *A. odoratum* (f) and embedded in *P. bistorta* (d). In *P. maritima* subsp. *serpentina* (a) and *A. odoratum* (f) thin threads occur, connecting orbicules to each other or with the tapetum surface. Sexinic ornamentations in *P. bistorta* (d) are verrucate and in *P. maritima* subsp. *serpentina* (a) and *A. odoratum* (f) are nanospinulose

exclude that they are in fact small orbicules. The highest mean size (0.265 µm) and maximum size (0.357 µm) were both recorded in *Plantago maritima* subsp. *serpentina*, while the minimum size was recorded in *Ostrya carpinifolia* with 0.105 µm. The same structures were also photographed on the surface of pollen in all the species above cited. The results of unpaired t tests, as listed in Table 2, show that the sizes

of these unknown structures are similar to those of the orbicular ornamentations.

In addition, in *Castanea sativa* and *Echinochloa crus-galli*, we reported these structures both on the tapetum and on the pollen sexine with sizes ranging from 0.124 to 0.216 µm and 0.174 to 0.397 µm, respectively. In both cases, their shapes were rounded.

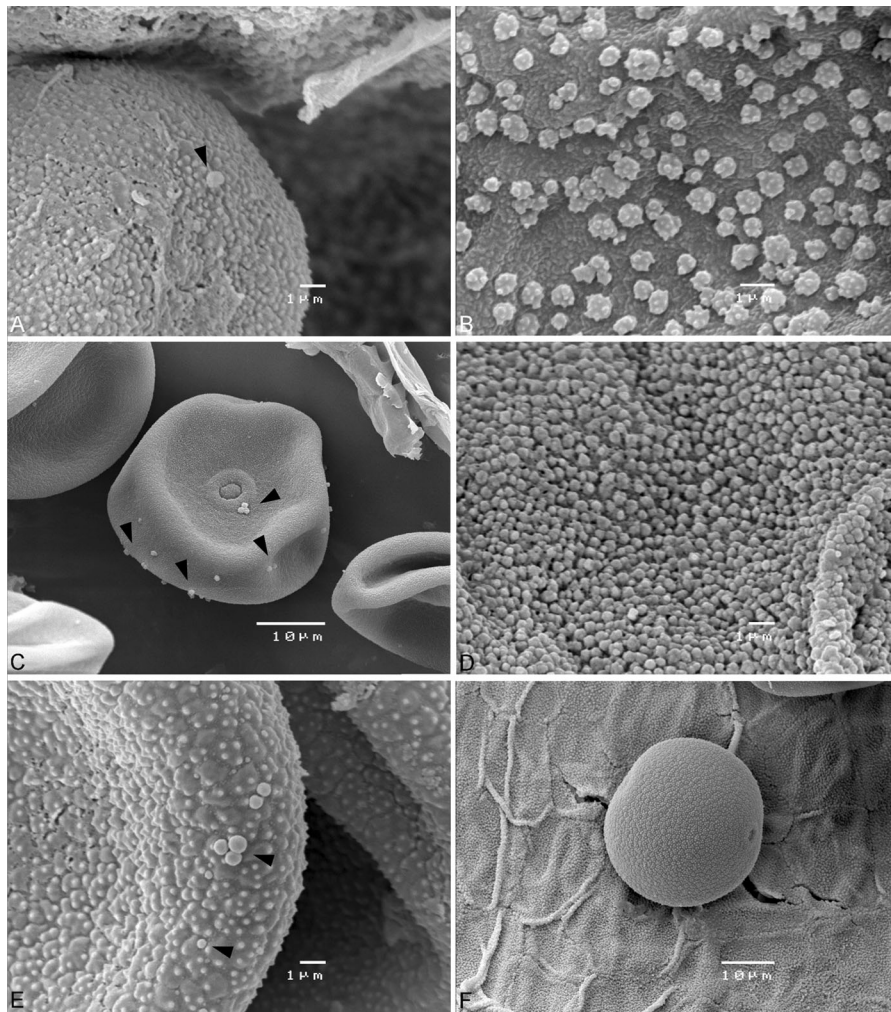


Fig. 8 Tapetum surfaces and pollen grains with orbicules (black arrowheads) and unknown nanometric particles (white arrowheads) in the species *Anthoxanthum odoratum* (a); *Paspalum dilatatum* (b), (c); *Echinochloa crus-galli* (d), (e) and (f). In *P. dilatatum* (b) and *E. crus-galli* (d), orbicules have a rounded shape and their ornamentations are

nanospinulose and psilate—nanogranulate, respectively. Orbicules are light embedded on the tapetum surface in *P. dilatatum* (b), *E. crus-galli* (d) and in (d) thin threads occur, connecting orbicules to each other or with the tapetum surface. Sexinic ornamentations are nanospinulose in *P. dilatatum* (c) and *E. crus-galli* (e)

4 Discussion

Our survey investigated 23 species, 19 of which had not previously been investigated with a specific focus on the orbicules. With the exception of *Ambrosia maritima*, *Artemisia arborescens*, *A. annua* and *Ligustrum lucidum*, we found that the remaining species possess orbicules on the entire locule wall and on the pollen sexine. Our results are somewhat consistent with previous studies by Vinckier and Smets 2001a, b, who did not find orbicules on the

pollen sexine in *Alnus glutinosa*, *Betula pendula*, *Corylus avellana* and *Castanea sativa*, although they reported their presence—but not their density—on the tapetum.

As regards the above-mentioned species, the orbicule mean sizes, ranging from 0.349 to 0.860 μm , are consistent with measurements reported in other studies (Vinckier and Smets 2001a, b). For the other species included in our work, the mean size ranged from 0.203 to 0.952 μm .

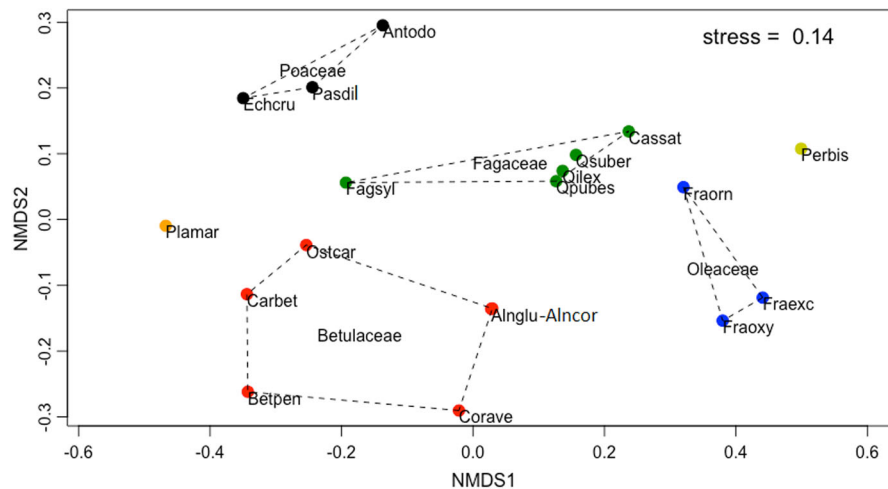


Fig. 9 NMDS ordination—Betpen = *Betula pendula*; Corave = *Corylus avellana*; Alncor = *Alnus cordata*; Alnglu = *Alnus glutinosa*; Ostcar = *Ostrya carpinifolia*; Carbet = *Carpinus betulus*; Fraoxy = *Fraxinus oxycarpa*; Fraexc = *Fraxinus excelsior*; Fraorn = *Fraxinus ornus*; Fagsyl = *Fagus sylvatica*; Qpubes = *Quercus pubescens*;

Qilex = *Quercus ilex*; Qsuber = *Quercus suber*; Cassat = *Castanea sativa*; Echcru = *Echinochloa crus-galli*; Pasdil = *Paspalum dilatatum*; Antodo = *Anthoxanthum odoratum*; Plamar = *Plantago maritima* subsp. *serpentina*; Perbis = *Persicaria bistorta*

We thus confirm the indirect evidence of Takahashi et al. (1995) who measured the size distribution of the particles released into the air from the flowers of five Japanese species of allergological interest, revealing small particles ranging from 0.46 to 2.2 μm and outnumbering pollen grains by 15-fold. Therefore, it seems likely that orbicules scatter in the atmosphere, causing a real allergological risk and which would explain the onset of pollinosis before the detection of pollen grains by traditional aerobiological monitoring (D'Amato et al. 2007).

We confirmed a general correlation between the ornamentations of the pollen grain exine and the ornamentations of the orbicules, as reported in other studies (Huysmans et al. 1997; Vinckier et al. 2000; Vinckier and Smets 2001a, b; Verstraete et al. 2014). In accordance with other authors, the prevailing shape of the orbicules was granule and/or rounded (El-Ghazaly 1989; El-Ghazaly and Nilsson 1991; El-Ghazaly and Chaudhary 1993; Huysmans et al. 1997, 2000; Vinckier et al. 2000; Vinckier and Smets 2001a, b; Gotelli et al. 2012).

Interestingly, the results of the NMDS (Fig. 9) are congruent with the current taxonomic circumscription of the investigated species and with the results obtained from molecular studies (Wallander 2008; Hinsinger et al. 2013; Manos et al. 2001; Coutinho

et al. 2015; Chen et al. 1999; Wang et al. 2016). *Fraxinus excelsior* and *F. angustifolia* subsp. *oxycarpa*, which are grouped together in Sect. *Fraxinus*, are closely associated in the NMDS space and are distant from *F. ornus*, which belongs to Sect. *Ornus* (Wallander 2008; Hinsinger et al. 2013). The three genera of Fagaceae included in the present study were ranked in three sections, and in particular, *Fagus* was well separated from *Quercus* and *Castanea*, which is consistent with the phylogenetic reconstruction proposed by Manos et al. (2001) and Coutinho et al. (2015).

The position of *Corylus avellana*, which was quite close to *Alnus glutinosa* and *A. cordata* but distant from *Carpinus betulus* and *Ostrya carpinifolia*, supports the current taxonomic circumscription of the family Betulaceae. This resulted from the merging of former Corylaceae members, and the phylogenetic position of *Corylus*, identified as the sister group of the *Ostryopsis-Carpinus-Ostrya* clade in the Coryloideae subfamily (Chen et al. 1999; Wang et al. 2016).

In general, our findings regarding the morphological traits of orbicules prove their systematic importance, as suggested by other authors based mainly on their size (Hesse 1986; Raj and El Ghazaly 1987; Huysmans et al. 1997; Vinckier and Smets 2002). Despite the restricted sampling of the present study,

the congruence of the results of the NMDS with the current systematic framework is encouraging and warrants further research, for instance by widening the taxonomic sampling, in order to verify whether our results can be generalized to other angiosperm clades. According to Verstraete et al. (2014), “17 orders remain blank spots on the angiosperm-orbicule map” due to the lack of data, thus highlighting the need for more research in this field.

For the first time, in addition to orbicules, unknown nanometric particles were detected and measured both on the tapetum and on pollen sexine in the species listed in Table 2. These unknown particles were also revealed on the tapetum wall of the Fagaceae species investigated here. However, we hypothesize that in the *Quercus* species, such particles may also be present on the pollen surface but might be obfuscated by the microgranular ornamentations of the sexine. In gymnosperms, similar particles have been reported for Cupressaceae by Ruggiero and Bedini (2018). As regards the unknown nanometric structures reported in the present research, their size and shape closely match those of sporopollenin granules produced by microcytes as shown in Furness and Rudall (2001: Fig. 9E). If indeed these nanometric particles are made up of sporopollenin and are similar to the nanoparticles found in Cupressaceae by Ruggiero and Bedini (2018), then not only do they contribute to the submicron aerosol, but they are likely to carry antigens, thus representing a real risk for sensitized patients (Southworth et al. 1988).

Finally, environmental stress conditions might promote the detachment of orbicular ornamentations through the weakening of the sporopollenin molecule (Sénéchal et al. 2015), which would then become free nanoparticles. This would thus explain the higher frequency of pollinosis and allergic asthma in urban areas than in rural ones (D’Amato et al. 2002; 2007).

Contrary to Taylor et al. (2004), who observed nanoparticles (“inter-orbicular structures”) ranging from 30 to 60 nm on the locule walls of *Betula*, we observed slightly larger nanoparticles only on the pollen surface of *Betula pendula*.

The unknown nanometric particles of *Carpinus betulus* and *Ostrya carpinifolia* are strikingly similar to their respective orbicular ornamentations. Thus, if we assume that these particles are made of sporopollenin, they may derive from the detachment of orbicular ornamentations, probably caused by

particular environmental conditions (Sénéchal et al. 2015). This hypothesis would explain the presence of orbicules that are completely lacking in ornamentations even in species with ornamented orbicules. As part of the same hypothesis, the unknown nanometric structures might be involved in the development of sporopollenin coat of orbicules, which does not proceed through the overlapping layers of sporopollenin as in the pollen wall of Cupressaceae (Kurmman 1994), but through the envelopment and fusion of sporopollenin aggregates around a lipid droplet. We speculate that orbicules’ size interindividual variation may be caused by the exocytosis of lipid droplets of different size, around which a sporopollenin coat is deposited (Verstraete et al. 2014). Indeed, TEM micrographs published by Southworth et al. (1988), Kurmman (1994), Furness and Rudall (2001), Quilichini et al. (2015), Gabarayeva et al. (2017) show the exocytosis of lipid droplets of different sizes and their subsequent coating with small sporopollenin blocks. TEM micrographs in Furness and Rudall (2001: Fig. 5d) clearly show the discrete structure of the sporopollenin coat (but see also Southworth et al. 1988; Kurmman 1994). Dinis et al. 2007 term “microchannel” the thin fusion lines detected among sporopollenin blocks fused around the lipid droplets.

5 Conclusions

Our results report the presence or absence of orbicules and their location in 19 species investigated here for the first time, belonging to the families Asteraceae, Betulaceae, Oleaceae, Fagaceae, Poaceae, Polygonaceae, and Plantaginaceae. We confirm a general correlation between the ornamentations of the pollen grain exine with those of the orbicules and the prevailing granular and/or round orbicule shape. In addition, unknown nanometric structures were recognized both on the tapetum and on the pollen exine in *Echinochloa crus-galli* and in the species belonging to Betulaceae, Fagaceae and Plantaginaceae families. We hypothesize that these nanometric structures are constituted by sporopollenin and have a role in (a) the deposition of sporopollenin in order to shape “mature” orbicules and (b) the onset of pollinosis before and after the dispersion of pollen grains into the air. Finally, we described the morphological traits of the orbicules, with a combination of continuous variables

(mean size, density) and categorical variables (shape, orbicular ornamentation, association between orbicules and tapetum wall, and presence/absence of aggregates or threads). We introduced a new terminology for description of orbicular and sexinic ornamentations, and all variables were analysed with multivariate statistical techniques. Our results are congruent with the current taxonomical framework of the investigated species and highlight the usefulness of orbicules traits as systematic characters within the investigated taxa. Due to the limited taxonomical sampling of the present research, our results do not allow inferences about phylogeny. Orbicules presence is considered a plesiomorphism in angiosperms (Verstraete et al. 2014), but deeper insight into this topic might come from the use of ancestral character reconstruction and phylogenetic analysis tools, especially if new data are obtained to fill the current taxonomical gap in the knowledge of these rather elusive particles.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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