

Free orbicules of Cupressaceae detected in daily aerobiological samples by optical and confocal microscopy

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Abstract Members of Cupressaceae and Taxaceae are known to release large amounts of highly allergenic pollen grains into the atmosphere, which are responsible for the onset of pollinosis in many countries throughout the world. In addition to pollen grains, their pollen sacs produce orbicules, which are submicron particles reported to carry allergens and which are potentially able to reach much further down the respiratory tract than pollen grains. Previous research has postulated the presence of orbicules in the atmosphere; however, direct observations have not yet been reported. The aim of this research was to provide the first direct evidence that Cupressaceae orbicules are released into the atmosphere by detecting them in daily aerobiological samples. We observed pollen sacs, pollen grains, and orbicules of nine species of Cupressaceae using scanning electron microscope (SEM). We then used a light and confocal microscope, to examine daily aerobiological samples. Under SEM, we measured the orbicule size (0.494–0.777 μm) and detected unknown nanometric particles (130–200 nm). Under the light microscope, aerobiological samples showed clusters of stained dots surrounding the pollen grains of Cupressaceae. Under the confocal microscope, the same clusters were resolved into submicron particles with the same

autofluorescence as the pollen grains. These features enabled us to identify them as orbicules. We believe that our findings help to explain the onset of pollinosis and allergic asthma related to Cupressaceae pollen grains in many countries, even before pollen grains are actually detected or after they are no longer observed in aerobiological monitoring samples.

Keywords Allergic asthma · Pollinosis · Free orbicules · Aerobiological samples · Pollen trap

1 Introduction

Cupressaceae include 30 genera with about 130–140 species and represent the largest conifer family in terms of genera and the third largest in terms of species (Earle 2017).

Cupressaceae is the most widely distributed of all gymnosperm families, occurring in different habitats, including extreme environments (Farjon 2010). All genera, other than *Juniperus*, show strongly relictual distributions, with a large number of localized, rare and endangered taxa. Most of the generic diversity is in the southern hemisphere; however, the largest genus, *Juniperus*, is chiefly north-temperate (Earle 2017).

In Italy, Cupressaceae and Taxaceae are diffusely represented by both autochthonous and alien species

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used for reforestation or ornamental purposes. Notable among the autochthonous plants are *Juniperus communis* L., *J. macrocarpa* Sibth. & Sm., *Cupressus sempervirens* L., and *Taxus baccata* L. which are either trees or shrubs, forming part of the natural vegetation in woodlands and thickets throughout the Mediterranean region (Arrigoni et al. 1999). Of the alien species, the most frequent include *C. arizonica* Greene, *Chamaecyparis lawsoniana* (A. Murray) Parl., *Calocedrus decurrens* (Torr.) Florin, and *Platycladus orientalis* (L.) Franco and are used as ornamental plants.

Cupressaceae pollen grains represent the most abundant fraction of the annual pollen spectrum in Mediterranean countries (D'Amato et al. 2007). Thus, the pollen levels registered in certain periods of the year are extremely high, due to both the abundance of these species as well as their mass flowering, although the pollen production varies greatly from year to year (D'Amato et al. 2007; Ruggiero and Bedini 2016; Bedini and Ruggiero 2016). The high concentrations of the pollen of these species cause considerable biological pollution during the winter, which makes this type of pollen the most abundant in this period of the year, reaching about 41% of the pollen spectrum (Ruggiero and Bedini 2016).

The Cupressaceae family is also responsible for winter pollinosis in a period of the year when no other allergenic plants are flowering (D'Amato et al. 2007), and the Cupressaceae pollen grains are a strong risk factor for allergic asthma (Mari et al. 1997; Charpin et al. 2005).

Pollinosis caused by Cupressaceae is on the increase in many countries such as Japan (Fujimura and Kawamoto 2015), Australia (Bass et al. 2000), the USA (Schwietz et al. 2000), Spain, France, Israel, and Italy (D'Amato et al. 2007). An Italian study showed that 14.7% of the Cupressaceae-allergic patients were monosensitized and that their average age was higher than that of the polysensitized patients (Ariano et al. 2002). This suggests that at least in some Cupressaceae-allergic patients, sensitization may be caused by long-lasting exposure to the pollen (Caiaffa et al. 1993).

In a study conducted by Canini et al. (2004), the localization of a carbohydrate epitope recognized by human IgE was found in pollen and orbicules of allergenic Cupressaceae species. These results provide additional support to Vinckier's hypothesis that

orbicules may act as vectors for allergens (Vinckier and Smets 2001).

However, while airborne Cupressaceae pollen grains are commonly observed in aerobiological monitoring, free orbicules have never been reported in aerobiological samples due to their small size, and the limitations of optical resolution, although their presence has been postulated by some researchers based on indirect evidence (Takahashi et al. 1995).

As part of the aerobiological monitoring in Pisa, Italy, set up within the AIS LIFE—Aerobiological Information System and allergic respiratory disease management—LIFE13ENV/IT/001107 project (Ruggiero et al. 2015), we observed fuzzy clusters of stained matter associated with pollen grains of Cupressaceae. Accordingly, the aim of this work was to verify (1) if Cupressaceae's orbicules could break off from microsporophylls and from pollen grains, and (2) if they could be identified in the aerobiological monitoring samples.

2 Materials and methods

2.1 SEM

We collected at least six ripe microsporophylls from one or more populations per species, from the herbarium specimens in Herbarium Horti Pisani of the University of Pisa (PI) and Herbarium of the Agricultural Faculty of the University of Florence (FIAF), listed in Table 1, for nine species of Cupressaceae and Taxaceae, which were represented in the area of Pisa either in the natural vegetation or as cultivated plants. The microsporophylls were shaken directly onto aluminium stubs covered with double adhesive carbon tape, in order to release the pollen grains from the pollen sac. The microsporophylls were then placed on the carbon tape and sputter-coated with gold. Finally, the specimens were observed and photographed with an SEM (Verstraete et al. 2014), (JEOL JSM 5410, Jeol Ltd, Tokyo, Japan). We measured the diameter of the orbicules in each sample with 50 measurements per species.

2.2 Aerobiological samples

For our investigation, we observed daily aerobiological samples obtained from the AIS LIFE project.

Table 1 Voucher specimens

Family	Species	Number of herbarium specimen examined	Collection location	Herbarium code
Cupressaceae	<i>Cupressus sempervirens</i> L.	1	Massa	43.2 (PI)
Cupressaceae	<i>Cupressus sempervirens</i> L.	1	Pisa	43.3 (PI)
Cupressaceae	<i>Cupressus arizonica</i> Greene	1	Pisa	44.1 (PI)
Cupressaceae	<i>Cupressus arizonica</i> Greene	1	Pisa	44.2 (PI)
Cupressaceae	<i>Calocedrus decurrens</i> (Torr.) Florin	1	Pisa	41.1 (PI)
Taxaceae	<i>Taxus baccata</i> L.	1	Massa	19.3 (PI)
Taxaceae	<i>Taxus baccata</i> L.	1	Pisa	19.4 (PI)
Cupressaceae	<i>Juniperus macrocarpa</i> Sibth. & Sm.	1	Massa	45.3 (PI)
Cupressaceae	<i>Juniperus macrocarpa</i> Sibth. & Sm.	1	Massa	45.7 (PI)
Cupressaceae	<i>Juniperus communis</i> L.	1	Lucca	45.10 (PI)
Cupressaceae	<i>Juniperus communis</i> L.	1	Lucca	45.15 (PI)
Cupressaceae	<i>Juniperus phoenicea</i> L.	1	Massa	951 (FIAF)
Cupressaceae	<i>Juniperus phoenicea</i> L.	1	Massa	954 (FIAF)
Cupressaceae	<i>Platycladus orientalis</i> (L.) Franco	1	Florence	640 (FIAF)
Cupressaceae	<i>Platycladus orientalis</i> (L.) Franco	1	Florence	638 (FIAF)
Cupressaceae	<i>Chamaecyparis lawsoniana</i> (A. Murray) Parl.	1	Florence	33886 (FIAF)
Cupressaceae	<i>Chamaecyparis lawsoniana</i> (A. Murray) Parl.	1	Florence	687 (FIAF)

PI Herbarium Horti Pisani, University of Pisa, Italy; FIAF Herbarium of the Agricultural Faculty, University of Florence, Italy

Samples were collected with a Lanzoni VPPS 2000 pollen trap installed in an urban area of Pisa about 17 m from the pavement (Google maps coordinates 43.718343° N, 10.395110° E) and equipped with pre-siliconed Silko-strip sampling tape (Lanzoni). Sections corresponding to 24-h intervals were cut from the tape, placed on microscope slides, and stained with fuchsin jelly (UNI 11108:2004). Among the samples available when the paper was drafted, spanning the period November 2014–September 2016, only those showing clusters of stained matter associated with pollen grains of Cupressaceae were selected.

2.3 Light microscope

The pollen grains in daily aerobiological samples were identified at the family level under a light microscope (Leica Diaplan) at 400× and photographed using a Leica MC170 HD. Species names were allocated based on phenological observations in the area. Plant nomenclature follows the updates published in *Informatore Botanico Italiano* (Conti et al. 2005, 2007), and *Italian Botanist* (<http://italianbotanist.pensoft.net/>).

Pictures were obtained by overlapping and merging at least 25 photographs of the same aerobiological subject taken at different levels of focus in order to create an enhanced image with as many details as possible, using Helicon Focus 6.0 software.

2.4 Confocal microscope

The same daily aerobiological samples that were observed under the light microscope were also examined using a Nikon A1 plus confocal microscope with a 400x Fluotar objective to check whether the emission spectrum and size of the submicron particles could be traced back to orbicules. The excitation wavelength was 561 nm, and fluorescence emitted with a wavelength longer than 595 nm was detected. Images were captured with a lateral resolution of 1024 × 1024 pixels and a vertical distance between individual optical sections of 0.3 μm (Banks et al. 2006). The resulting image stacks were then processed using NIS-Elements AR—version 4.10.03. A total of 30 orbicules were then measured using ImageJ version 1.48v.

3 Results

3.1 SEM observations

Orbicules usually form a dense cover on the exine of pollen grains as well as on the tapetum of microsporophylls; however, some of them are free to move within the pollen sac, as shown on the intine of *Calocedrus decurrens* (Fig. 1a), or on the adhesive carbon tape of all the samples of pollen grains of the species considered (Fig. 1b–i), where they dropped off while gently shaking the microsporophyll.

The diameters of the orbicules are reported in Table 2 and ranged from 0.494 μm for *Juniperus communis* to 0.777 μm for *Chamaecyparis lawsoniana*. In all target species, orbicules had spine-shaped ornamentations on the outer surface (Fig. 1), while the surface of the pollen grains was granulate.

In every sample, some orbicules broke off freely from the tapetum and pollen grains and fell directly on the stubs (Fig. 1). In addition, we observed other smaller particles than orbicules, which ranged from 130 to 200 nm (Fig. 1). Most probably, new nanometric particles were fragments of pollen exine or orbicular ornamentations and/or underdeveloped orbicules whose development may have been stopped due to the insufficient size of the starting oil droplet or to other causes linked to their genesis.

3.2 Light and confocal microscopy

Under the optical microscope, the daily aerobiological samples sometimes exhibited clusters of very small, well-stained dots, surrounding the Cupressaceae pollen grains (Fig. 2).

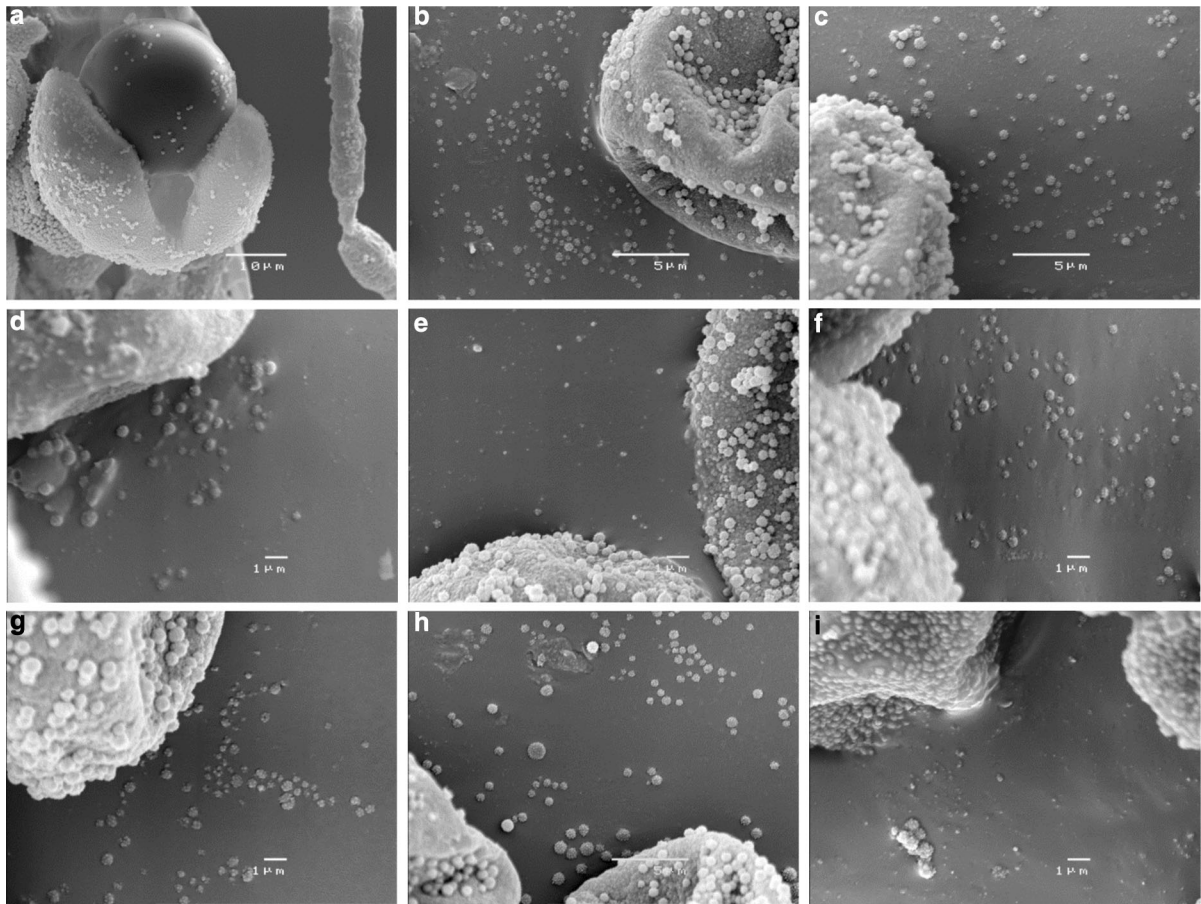
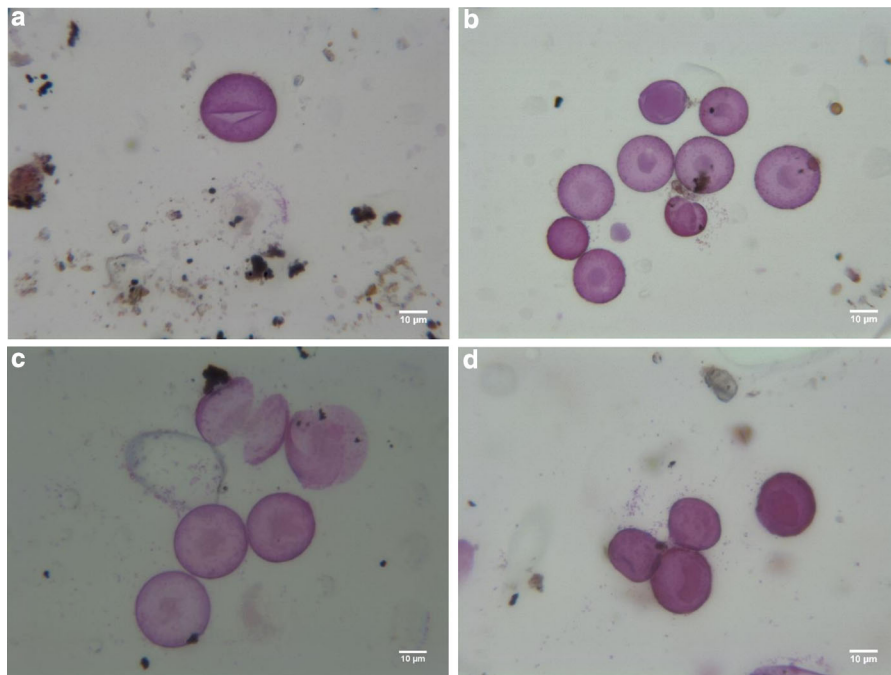


Fig. 1 SEM images of the investigated species **a** *Calocedrus decurrens*, **b** *Cupressus sempervirens*, **c** *Juniperus macrocarpa*, **d** *Cupressus arizonica*, **e** *Juniperus communis*, **f** *Juniperus*

phoenicea, **g** *Platycladus orientalis*, **h** *Chamaecyparis lawsoniana*, and **i** *Taxus baccata*

Table 2 Orbicule diameter in target species

Species	N	Mean diameter (μm)	SD
<i>Cupressus sempervirens</i>	50	0.552	0.077
<i>Cupressus arizonica</i>	50	0.758	0.088
<i>Taxus baccata</i>	50	0.711	0.105
<i>Juniperus macrocarpa</i>	50	0.523	0.051
<i>Juniperus communis</i>	50	0.494	0.074
<i>Juniperus phoenicea</i>	50	0.623	0.070
<i>Chamaecyparis lawsoniana</i>	50	0.777	0.087
<i>Platycladus orientalis</i>	50	0.593	0.078

**Fig. 2** Pollen grains surrounded by clusters of stained dots in daily aerobiological samples collected in Pisa from December 2015 to March 2016, observed with a light microscope**a** *Juniperus macrocarpa* (03/12/2015), **b** *Cupressus arizonica* (11/01/2016), **c** *Platycladus orientalis* (14/02/2016), **d** *Cupressus sempervirens* (03/03/2016)

The Cupressaceae pollen grains cannot be identified at the species level with the optical microscope (Bortenschlager 1990); thus, we identified them by direct phenological observations performed during aerobiological monitoring, considering the time lapse between the observations.

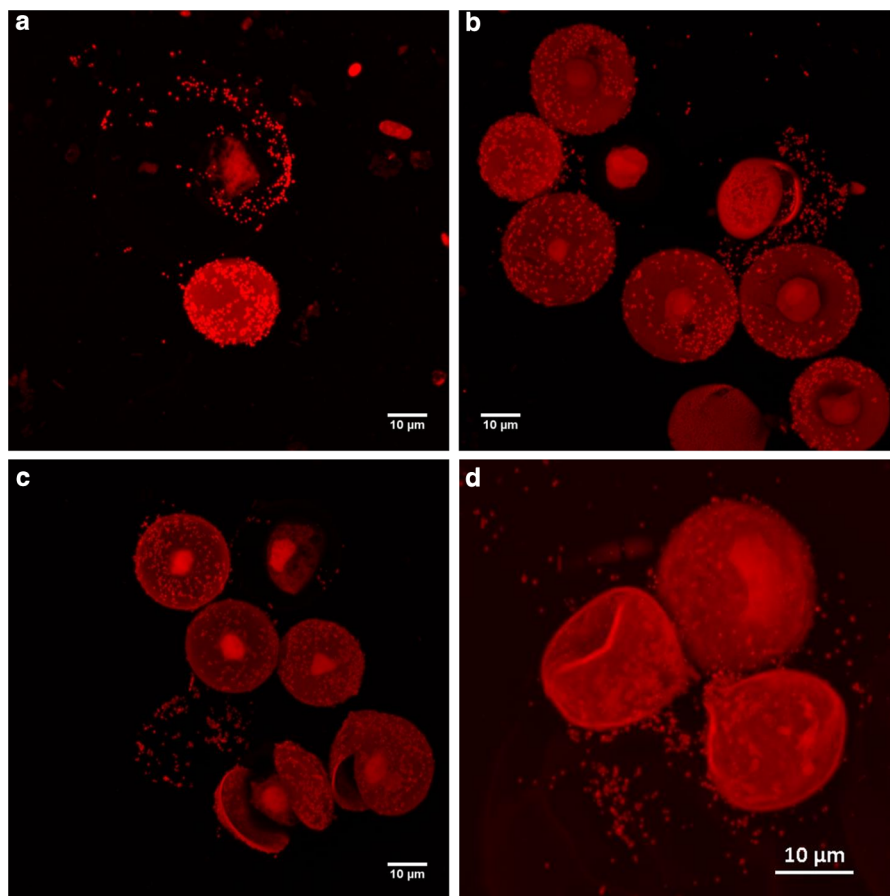
When the same samples were observed with a confocal microscope, the clusters were resolved in submicron particles, ranging from 0.514 to 0.747 μm (Table 3), with the same autofluorescence emission as the exine of the pollen grains, which enabled us to identify them as orbicules. We observed a large

Table 3 Orbicule diameter in daily aerobiological samples observed using confocal microscope

Species	N	Mean diameter (μm)	SD
<i>Juniperus macrocarpa</i>	30	0.514	0.072
<i>Cupressus arizonica</i>	30	0.747	0.079
<i>Platycladus orientalis</i>	30	0.589	0.059
<i>Cupressus sempervirens</i>	30	0.551	0.057

number of orbicules both adhering onto pollen grains and fully disconnected from them (Fig. 3). This finding demonstrates that orbicules are indeed present

Fig. 3 Pollen grains and free orbicules in daily aerobiological samples collected in Pisa from December 2015 to March 2016, observed with confocal microscope **a** *Juniperus macrocarpa* (03/12/2015), **b** *Cupressus arizonica* (11/01/2016), **c** *Platycladus orientalis* (14/02/2016), **d** *Cupressus sempervirens* (03/03/2016). Images appear reversed across a horizontal axis with respect to those in Fig. 2, due to the optical layout of the confocal microscope



in the atmosphere, at least at the same time as the “parental” pollen grains, and can be detected and counted in aerobiological samples.

4 Discussion

Our results show for the first time—to the best of our knowledge, that Cupressaceae and Taxaceae orbicules can be detected as loose particles on daily aerobiological samples at the same time as “parental” pollen grains but vastly outnumbering them. Previous studies (Takahashi et al. 1995) measured the size distribution of the particles released from the flowers of five species of allergological interest placed in a confined environment. The research showed that micronic particles belonging to the gymnosperm *Cryptomeria japonica* (Cupressaceae) were orbicules and that their number was about eightfold greater than that of intact pollen grains. Vinckier and Smets (2001) found that in

12 species of angiosperms orbicules were present in huge numbers and far exceeded the amount of pollen.

In the present study, orbicules were not observed without their parental pollen grains; however, the presence and high number of orbicules in the atmosphere would seem to suggest that they play a role in exacerbating pollinosis in sensitized subjects, as argued by other researchers based on the presence of a carbohydrate epitope recognized by human IgE on the orbicules of allergenic Cupressaceae species (Canini et al. 2004). Due to their small size, orbicules are able to penetrate deeper into the airways than pollen grains, with a potentially higher allergenic impact which should be addressed in specialist studies.

In our target species, orbicules had spine-shaped ornamentations on the outer surface (Fig. 1). Spiny orbicules have been observed also in angiosperms (Vinckier and Smets 2001); however, studies focussing on orbicule morphology showed that the ornamentation of the orbicules is strongly correlated with

and remarkably similar to that of the pollen grain exine (Huysmans et al. 1997, 1998; Vinckier et al. 2000). In contrast, in our target species the spine-shaped ornamentation of the orbicules does not match that of the pollen grains exine which is granulate.

New nanometric particles were observed, and although further studies are required, it can be hypothesized that some of the smaller particles were orbicular ornamentations and/or underdeveloped orbicules, resulting from an insufficient size of the oil droplets around which the sporopollenin wall is arranged (Verstraete et al. 2014), while others may have derived from the degradation of exine. It is well known that in Cupressaceae and Taxaceae the exine is very thin (0.4–0.9 µm in thickness) and can be easily broken (Bortenschlager 1990; Kurmann 1994), especially in particular environmental conditions, by air chemical pollutants (Shahali et al. 2009; Sénéchal et al. 2015).

It is therefore likely that in adverse environmental conditions, the source of the submicron particles potentially released in the atmosphere includes not only regular orbicules but also underdeveloped orbicules and exine fragments. Considering that all these elements have been shown to carry antigens (Southworth et al. 1988), their presence in the atmosphere might explain the onset of pollinosis even before and after the dispersion of pollen grains into the atmosphere (D'Amato et al. 2007).

As regards their adaptive role in plants, this is largely uncharted territory. As a way of directing further much needed research on this topic, it can be speculated that at least in Cupressaceae and Taxaceae, orbicules may have the following functions:

1. Reinforcing the exine so as to protect pollen grains from rupturing, especially in adverse environmental conditions and atmospheric pollution (Shahali et al. 2009; Sénéchal et al. 2015).
2. Facilitating pollination by increasing the surface of contact with pollination drop (Mugnaini et al. 2007). This hypothesis is supported by the unequal distribution of the orbicules, which are less abundant next to the pore (Bortenschlager 1990), the area of the wall which ruptures to release the male gametes (Danti et al. 2011).
3. Improving dispersion in the air so as to travel farther, by providing micro-rugosity on the pollen

surface. Such micro-rugosity might induce the formation of a turbulent boundary layer in the air surrounding the pollen grain. The turbulent boundary layer detaches from the pollen grain surface later than a laminar boundary layer and determines a smaller drag. Therefore, pollen grains with a micro-rugose surface provided by adhering orbicules can travel farther than grains with a smooth surface (Clauser 1956; Choi et al. 2006).

5 Conclusions

Our investigations proved that orbicules of Cupressaceae and Taxaceae can be detected as loose particles on daily aerobiological samples. On account of their smaller size, free orbicules can reach—and carry their allergenic load—much deeper in respiratory systems than pollen grains, triggering exacerbations in pollinosis.

In addition to the orbicules, we observed new nanometric particles, most probably fragments of pollen exine and/or underdeveloped orbicules whose development was stopped due to insufficient size of the starting oil droplet or to other causes linked to their genesis, yet to be clarified.

Finally, we hypothesize that orbicules in Cupressaceae and Taxaceae have important functions: (a) protecting pollen grains from extreme fragility of exine versus environmental conditions; (b) increasing the surface of contact with pollination drop in order to facilitate the fecundation; (c) improving dispersion in the atmosphere.

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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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