Is the quantity of orbicules released by Dactylis glomerata and Cynosurus echinatus (Poaceae) big enough to play an allergenic role?

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Abstract
Orbicule characteristics of *Dactylis glomerata* and *Cynosurus echinatus* (Poaceae) were investigated using light (LM), scanning electron (SEM) and transmission electron microscopy (TEM). Based on SEM micrographs, the number of orbicules per 100 μm^2 of the locule wall surface was determined in both dehisced and undehisced anthers and was further compared statistically. A total of 100 pollen grains were examined using SEM in search for orbicules attached to the pollen exine. Orbicules were not found distributed freely in the anther locules. They were attached to the locule wall surface through sporopolleninous fibrils, the orbicule wall being firmly embedded in, and often in continuity with the thin layer of sporopollenin lining each locule. The orbicule density on the locule wall surface of both the dehisced and undehisced anthers did not differ significantly. Only a few orbicules were seen attached to the pollen exine in both species. It is concluded that orbicules are not easily removed from the surface of the locule wall and, consequently, that the number of orbicules emitted from the two grass species is too low to play a significant role in triggering allergic diseases.

Keywords: *Anthers, orbicules, grasses, allergy, airborne particles, micronic atmospheric aerosol,* Dactylis glomerata, Cynosurus echinatus

Pollinosis (''hay fever'') is an important problem in human pathology all over the world. For this reason, research to identify and localize allergens in pollen grains has been intensified over the past few decades (reviewed by Andersson & Lidholm, 2003; Puc, 2003). Most recently, studies have also been made to analyse the micronic atmosphere aerosol fraction and search for possible allergenic activity (e.g. El-Ghazaly et al., 1995; Staff et al., 1999; D'Amato et al., 2002, 2007; Swoboda et al., 2004; Taylor et al., 2004). Pollen grains are too large to enter into the lower human airways. On the other hand, atmospheric microaerosols are smaller than pollen grains and may play an important role in triggering allergic diseases (D'Amato et al., 2002, 2007; Solomon, 2002; Swoboda et al., 2004).

Orbicules (Ubisch bodies) are minute granules of sporopollenin lining the radial walls and the innermost tangential wall of secretory tapetum cells (reviewed by Huysmans et al., 1998). They are present in many allergenic species and are usually much more numerous than pollen grains.

Besides, they are small enough to pass easily through the pores of most protective masks (Huysmans et al., 1998). Accordingly, orbicules are able to reach the peripheral airways with inhaled air inducing asthma in susceptible individuals if they are loaded with allergens. Recently, the presence of pollen allergens in association with orbicules was verified in some species using immunocytochemical methods (Miki-Hirosige et al., 1994; Suarez-Cervera et al., 2003; Canini et al., 2004; Vinckier et al., 2005, 2006). This suggests that orbicules may act as very effective vectors of allergens (Vinckier & Smets, 2001a, b; Vinckier et al., 2005). However, there are still conflicting reports regarding whether or not the amount of orbicules released into the atmosphere is significant (cf. Miki-Hirosige et al., 1994; El-Ghazaly et al., 1995; Takahashi et al., 1995; Staff et al., 1999; Vinckier & Smets, 2001a, b; Suarez-Cervera et al., 2003; Vinckier et al., 2006).

The family Poaceae is known to include many of the most important and widespread allergenic species.

Usually, grasses release large numbers of pollen during spring and early summer when susceptible individuals develop symptoms of hay fever and bronchial asthma. This is due to particular proteins and glycoproteins present in the pollen grains that can interact with the immune system causing allergic reactions (Staff et al., 1999; Andersson & Lidholm, 2003; Puc, 2003). The existence of orbicules attached to the pollen exine of several grasses (Vinckier & Smets, 2001*a*, *b*) and of small particles emitted from dehiscing anthers, which were analysed with an Aerosizer (Takahashi et al., 1995), led to the assumption that orbicules are among the particles emitted from the anthers of grasses, too. However, there is no conclusive evidence to support this.

In the present work we perform a comparative study of the orbicule characteristics in dehisced and undehisced anthers of two Poaceae species, *Dactylis glomerata* L. and *Cynosurus echinatus* L., to investigate whether the quantity of orbicules emitted from the anthers is significant enough to support their role in causing allergic diseases.

Material and methods

Plant material

Specimens of *Cynosurus echinatus* and *Dactylis glomerata*, of which voucher specimens were deposited at the herbarium of the Department of Botany, University of Coimbra (COI), were collected at three distinct places in the centre of Portugal. For each specimen collected, anthers were allowed to dehisce in the laboratory. After this, dehisced and undehisced anthers were gathered from the same specimen and processed for light and electron microscopy.

Microscopy

For light microscopy (LM), thin sections of undehisced anthers processed as for transmission electron microscopy (see below) were stained with Sudan black B (Bronner, 1975) and observed in a Nikon Eclipse E400 light microscope equipped with a Nikon Digital Sight DS-U1 camera, using the Act-2U informatics program.

For scanning electron microscopy (SEM) whole anthers were fixed in 2.5% buffered glutaraldehyde (see below), dehydrated in a graded acetone series (70–100%) and critical point dried. After cutting the closed anthers longitudinally with a razor blade to expose the anther locule, the anthers were mounted on aluminium stubs and a few pollen grains were gently removed with a fine needle to facilitate observation of the locule surface. Then, both dehisced and undehisced anthers were sputter coated with a 20 nm layer of gold-palladium prior to examination with a JEOL JSM-5400 at 15 kV.

For transmission electron microscopy (TEM), undehisced anthers were placed in a drop of the fixing solution and the tips were gently removed with a razor blade to facilitate the entry of solutions into the locules. The anthers were then fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 6.8) supplemented with 1mM calcium chloride, for 3–4 h at room temperature. After rinsing with the same buffer, the samples were post-fixed in 1% buffered osmium tetroxide and rinsed again. Following dehydration in a graded ethanol series (70–100%), samples were embedded in Spurr's resin. Ultrathin sections were cut on a LKB Ultratome NOVA ultramicrotome equipped with a diamond knife, conventionally stained with uranyl acetate and lead citrate, and observed in a JEOL JEM-100 SX at 80 kV.

Measurements and statistical analysis

In the undehisced anthers of each Poaceae species, a total of 100 pollen grains were examined with SEM to search for orbicules attached to the pollen exine. If orbicules were present on the exine, their number per pollen grain was counted. To determine the mean diameter of the orbicules, 30 orbicules were randomly selected in the respective SEM micrographs and measured. Upon observation of SEM micrographs, the number of orbicules per 100 μm^2 of the tapetal surface was also determined in both dehisced and undehisced anthers belonging to each of the three specimens of the two Poaceae species. Each SEM micrograph was divided into a 100 μm^2 square grid from which five squares were randomly picked and the number of orbicules counted. The total area examined was 1 500 μm^2. The means of orbicule density per 100 μm^2 were obtained and further analysed using SPSS 13.0 statistical package (SPSS inc., USA). Two paired t-tests were performed, one for each species, to compare the orbicule density between dehisced and undehisced anthers. The null hypothesis states that differences in orbicule density between dehisced and undehisced anthers are not significant. The level of statistical significance was set at $p < 0.05$.

Results

Orbicule morphology and distribution

Cross sections of anthers of both *Dactylis glomerata* and *Cynosurus echinatus* showed minute spherical

granules lining the entire locule wall surface, which reacted positively to the Sudan black B staining (Figure 1A). Similar granules were not distributed freely inside of the anther locules (Figure 1A). Examination with SEM revealed that these granules correspond to numerous orbicules covering the entire locule wall surface, randomly distributed (Figures 1B, C & 2A, B). In both species, the

orbicules had a blackberry-like shape with abundant pointed excrescences on the orbicule wall (spiny-type orbicules; Figures 1D, 2C) that resemble those of the spinulose pollen exine surface (Figures 1E, 2D). The mean diameter of the orbicules was 0.532 ± 0.062 μm in *Dactylis glomerata* and 0.494 ± 0.090 μm in *Cynosurus echinatus*. In both species, orbicules were attached to the locule wall
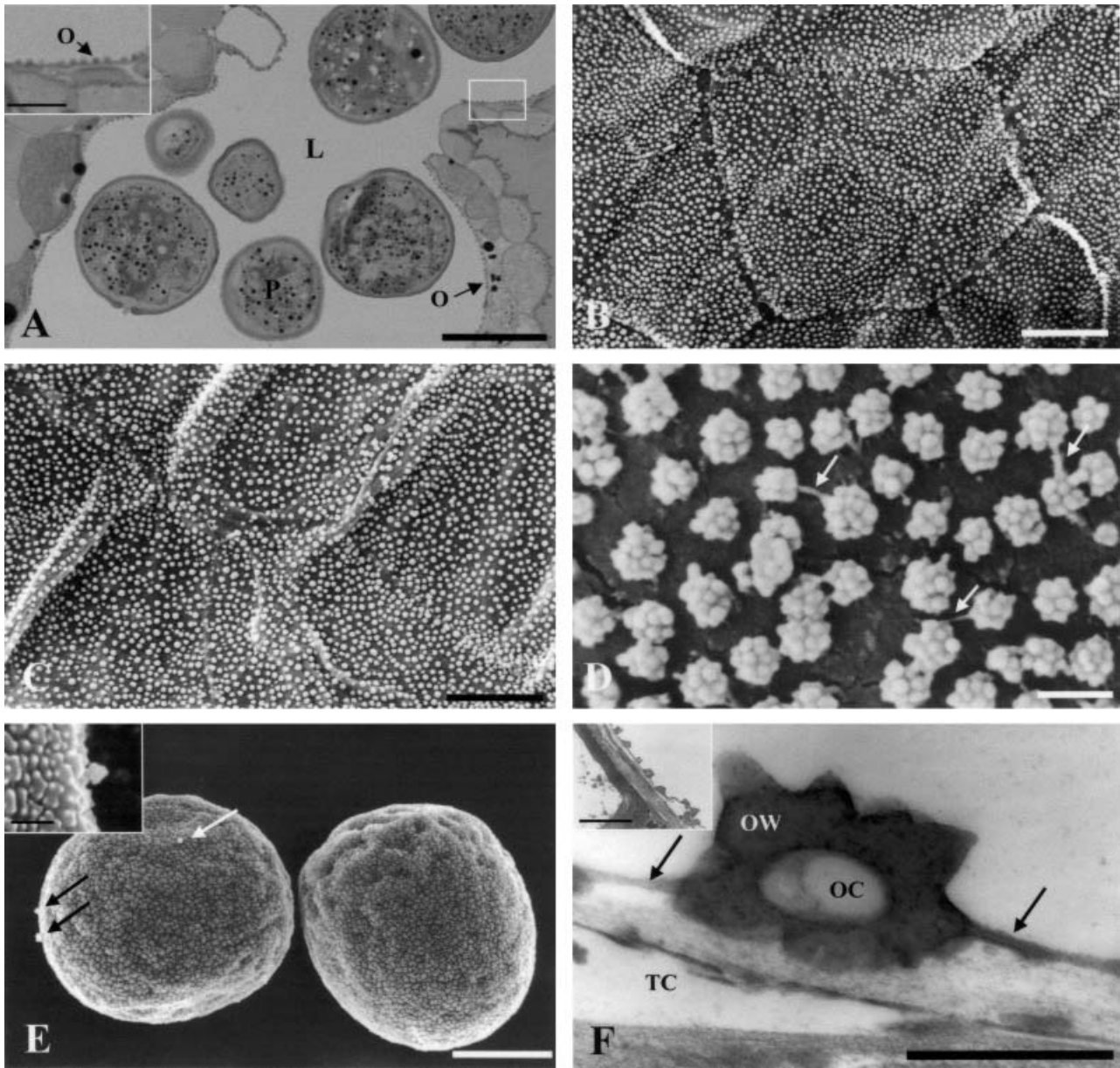

Figure 1. **A–F.** Orbicules and pollen of *Dactylis glomerata*. **A.** Light micrograph of an anther cross section stained with Sudan black B showing orbicules (*O*) lining the locule wall surface. Pollen grains (*P*) are seen in the locule (*L*). The inset is a higher magnification of orbicules attached to the locule wall surface. **B.** SEM micrograph of orbicules covering the locule wall surface in an undehisced anther. **C–D.** SEM micrographs of orbicules covering the locule wall surface in dehisced anthers. Note orbicules attached to the locule wall surface by reasonably large fibrils (*arrows*) that also link orbicules to each other. **E.** SEM micrograph of two pollen grains, of which one shows orbicules (*arrows*) on the pollen exine. The inset is a detail of an orbicule attached to the pollen exine. **F.** TEM micrograph of an orbicule comprising the spherical core (*OC*) and the thick sporopolleninous wall (*OW*). The latter is in continuity (*arrows*) with the layer of sporopollenin covering the tapetal cells remnants (*TC*). The inset is a low magnification of orbicules lining the locule wall surface. Scale bars – 20 μm (A); 10 μm (B, C, E); 5 μm (insets in A, F); 1 μm (D, inset in E); 0.5 μm (F).
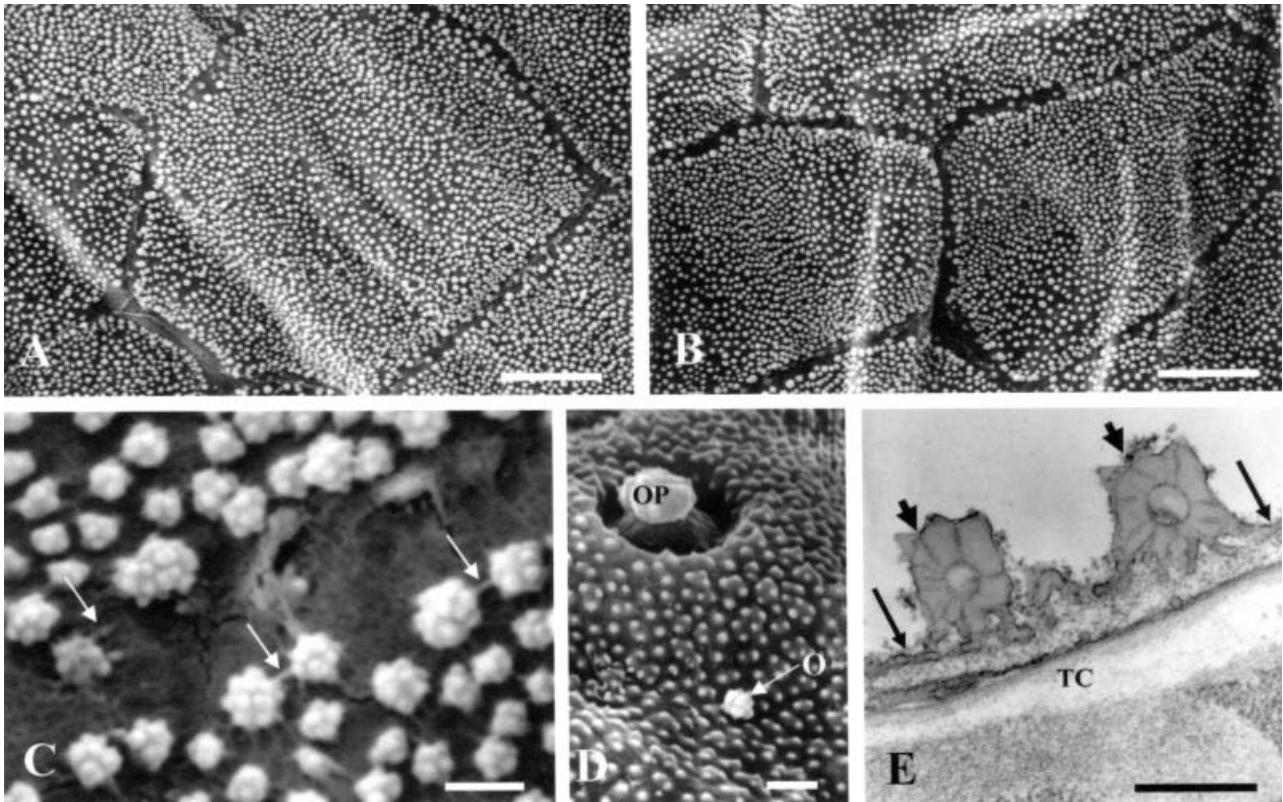
Figure 2. **A–E.** Orbicules of *Cynosurus echinatus*. **A.** SEM micrograph of the locule wall surface in an undehisced anther. **B–C.** SEM micrographs of the locule wall surface in dehisced anthers. Note the spiny orbicules attached to the locule wall surface by sporopolleninous fibrils that also connect orbicules to each other (*arrows*). **D.** SEM micrograph of part of a pollen grain showing the operculum (*OP*) and an orbicule (*O*) attached to the pollen exine. **E.** TEM micrograph of orbicules comprising the spherical core and the thick sporopolleninous wall. The latter is traversed by microchannels (*arrowheads*) and is in continuity (*arrows*) with the layer of sporopollenin covering the tapetal cells remnants (*TC*). Scale bars – 10 μm (A, B); 1 μm (C, D); 0.5 μm (E).

surface through reasonably large fibrils (distended spines?) that radiated from the orbicule wall (Figures 1D, 2C). Similar fibrils were seen connecting orbicules to each other (Figures 1D, 2C).

Before anthers dehiscence, the locules were filled with pollen grains. In these undehisced anthers, the percentage of pollen grains with orbicules attached to the exine surface was 21% in *Dactylis glomerata* and 38% in *Cynosurus echinatus* (Figure 3). In both species, the number of orbicules attached to the same single grain was generally very low (Figures 1E, 2D, 3), but *Cynosurus echinatus* showed a relatively higher number of orbicules (>15 orbicules) attached to a few pollen grains than *Dactylis glomerata* (Figure 3).

Following anther dehiscence, very few pollen grains were found within the anther locules indicating that almost all of them are released from the anthers in both species. In contrast, a very high number of orbicules were still attached and covering the whole locule wall surface (Figures 1C, 2B). The orbicule density on the locule wall surface of both the dehisced and undehisced anthers of the two grass species is indicated in Table I. No statistical differences concerning the orbicule density were found between dehisced and undehisced anthers of *Cynosurus echinatus* ($p=0.06$, $t=3.845$, df$=2$) or between dehisced and undehisced anthers of *D. glomerata* ($p=0.26$, $t=1.544$, df$=2$) (Table I).

Orbicule ultrastructure

Examination with TEM revealed orbicules comprising a reasonably electron-translucent spherical core surrounded by a thin electron-dense interface, which was covered by a thick sporopolleninous wall showing many micro-spines (Figures 1F, 2E). The orbicule wall was penetrated by many microchannels in *Cynosurus echinatus* (Figure 2E), contrary to what was observed in *Dactylis glomerata* (Figure 1F). A material very similar in appearance to that of the orbicule wall was found on the surface of the tapetal cells remnants (Figures 1F, 2E), constituting the so-called sporopollenin reticulum (see Christensen et al., 1972). Noteworthy, the spinulose orbicule wall was always firmly embedded in, and very often in continuity with this thin layer of sporopollenin lining each anther locule (Figures 1F, 2E). Neither
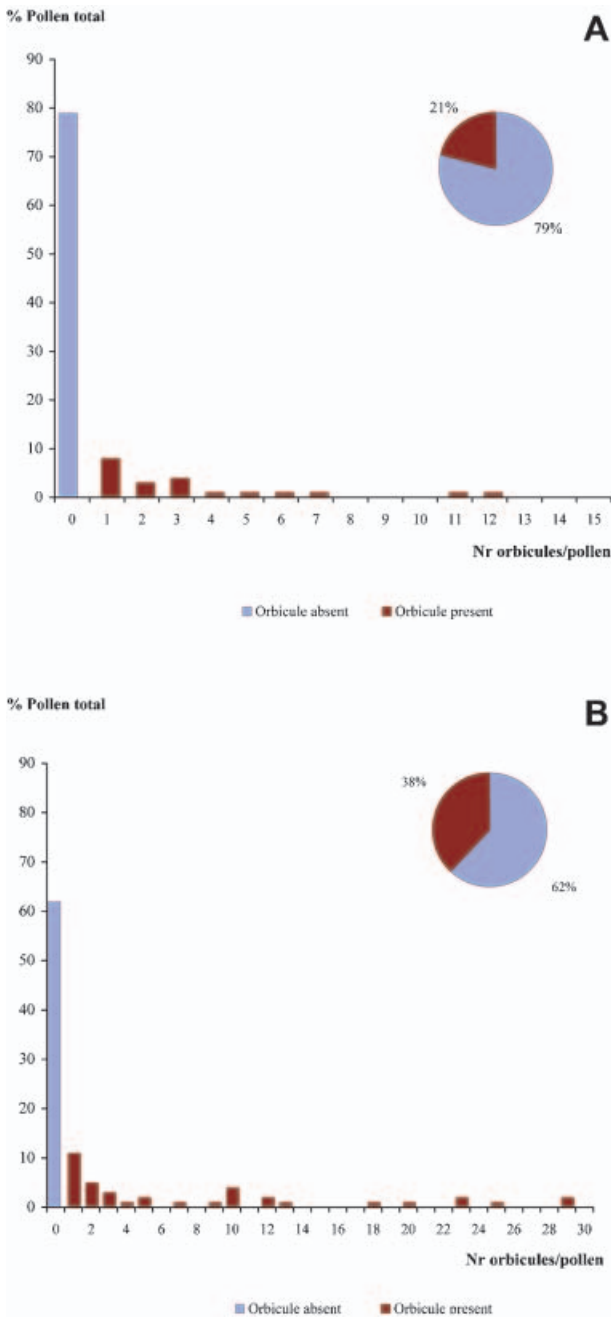
Figure 3. Frequencies of orbicules attached to the pollen wall in *Dactylis glomerata* (**A**) and *Cynosurus echinatus* (**B**). The insets show the percentage of pollen grains with and without orbicules on the exine surface.

of the sections examined with TEM showed orbicules freely occurring in the anther locules or attached to the pollen exine surface.

Discussion

In the last few years, orbicules have been hypothesised as being part of the airborne submicronic fraction that can cause allergic diseases in sensitized patients (e.g. El-Ghazaly et al., 1995; Vinckier &

Smets, 2001*a*, *b*; D'Amato et al., 2002, 2007; Vinckier et al., 2005). They are much more numerous than pollen grains and, unlike these they are small enough to enter the lower human airways. In addition, allergens responsible for pollinosis were already immunolocalized in orbicules of *Betula verrucosa* Ehrh. (syn. *B. pendula* Roth.) (Vinckier et al., 2006), *Corylus avellana* L. (Vinckier et al., 2005), *Cryptomeria japonica* (L. f.) D. Don (Miki-Hirosige et al., 1994), and of several species of *Cupressus* (Suarez-Cervera et al., 2003; Canini et al., 2004). However, they have yet to be found in orbicules of many other clinically important species, including grasses. In *Lolium perenne* L., Vithanage et al. (1982) found group 1 allergens in the orbicules, but no labelling was obtained by Taylor et al. (1994) using monoclonal antibodies. Therefore, it is still unknown whether orbicules are always loaded with allergens and which pollen allergens, i.e., major and/or minor allergens they contain. Major and minor allergens differ in regard to both IgE binding frequency and allergenic potency, the former causing reactions in the sera of more than 50% of sensitive patients. In *Betula verrucosa* it was verified that not all pollen allergens are present in the orbicules since the minor allergen Bet v 7 was immunolocalized in the orbicule wall, but only a weak labelling was found for the major allergen Bet v 1 (Vinckier et al., 2006). Taking into account that reactivity to minor allergens only occurs in small percentages of pollen-allergic patients (e.g. Cadot et al., 2000; Rossi et al., 2003); the authors concluded that orbicules are not important vectors of allergens in birch allergy. The contrary, however, is found in *Cryptomeria japonica* and *Cupressus* spp. where major allergens are present in orbicules (Miki-Hirosige et al., 1994; Suarez-Cervera et al., 2003; Canini et al., 2004). Also noteworthy is that orbicules are absent from some clinically important species, such as those of Asteraceae and Oleaceae (Vinckier & Smets, 2001*a*, *b*).

Despite the fact that orbicules may contain allergens in some species, they will be able to induce allergic diseases only if they are released into the environment in relatively high numbers. This question is pertinent but still controversial. In *Cryptomeria japonica* (Miki-Hirosige et al., 1994; Takahashi et al., 1995), *Cupressus arizonica* Greene and *Cupressus sempervirens* L. (Suarez-Cervera et al., 2003), orbicules were reported to be emitted from the anthers in large quantities. The same was assumed for *Betula verrucosa* since few orbicules were found in the anthers after anthesis (El-Ghazaly et al., 1995). However, Vinckier et al. (2006) showed recently that orbicules in this species are released in very low quantities, therefore not

Table I. Number of orbicules per 100 μm^2 of the locule wall surface in the dehisced and undehisced anthers of *Dactylis glomerata* and *Cynosurus echinatus*.

| | *Dactylis glomerata* | | *Cynosurus echinatus* | |
| | Undehisced | Dehisced | Undehisced | Dehisced |
	X	X	X	X
Specimen A	156.5	147.7	173.9	170.3
Specimen B	135.9	136.1	164.1	159.1
Specimen C	146.6	143.1	149.2	140.6

contributing significantly to the allergenic microaerosol. In a previous study, Vinckier and Smets (2001*a*, *b*) reported orbicules to be among the particles emitted from anthers of some important allergenic plants, including *Betula verrucosa* and grasses such as *Cynodon dactylon* (L.) Pers., *Dactylis glomerata*, *Lolium perenne* and *Phleum pratense* L. This study was based on the observation of orbicules attached to the pollen exine; no quantification of the amount of orbicules attached to the pollen wall was performed. Contrarily, Staff et al. (1999) observed that orbicules of *Lolium perenne* do not shed from anthers either during or after anther dehiscence, implying that this species cannot contribute to the airborne micronic particles. None of these studies provided a detailed comparative investigation of the orbicules in anthers before and after dehiscence. In the present study we performed such investigation and provide evidence that the quantity of orbicules emitted from the anthers of *Cynosurus echinatus* and *Dactylis glomerata* is very low to support their role in bringing allergens to susceptible individuals. This is contrary to the idea that orbicules of *Dactylis glomerata* and other species of Poaceae ''can be easily dispersed into the atmosphere during anthesis'' (Vinckier & Smets, 2001*b*, p. 765). Also, the size of the orbicules of *Dactylis glomerata* (0.532±0.062 μm) is smaller than that of the micronic particles emitted from the dehiscing anthers of this species (Takahashi et al., 1995), which implies that the latter are not orbicules. The size of the orbicules we determined corresponds to that found by Vinckier and Smets (0.431±0.154 μm; Vinckier & Smets, 2001*b*, Table I). Nevertheless, the percentage of the unknown micronic particles reported by Takahashi et al. (1995) is too low (3.3%) to contribute significantly to the respirable aerosol fraction.

Examination of thin sections using TEM and LM revealed no orbicules free in the anther loculus. This is consistent with the fact that orbicules are firmly attached to the locule wall surface. They are lining the entire locule wall surface and are firmly attached to it by numerous sporopolleninous fibrils that also establish bridges between orbicules. Ultrathin sections showed that the orbicule wall is firmly embedded in, and often in continuity with the thin layer of sporopollenin that lines each locule (see also Christensen et al., 1972). Importantly, no statistical differences were found between the orbicule density in the dehisced and undehisced anthers. Altogether, these data show that the number of orbicules released into the anther loculus is very low in both species. It may be argued that free orbicules are not found in the anther locules because they are extracted during the sample processing for microscopic examination. However, this is unlikely to occur. Supposing that such is true, differences would be found in the orbicule density between the anthers before and after dehiscence. Moreover, the fact that orbicules do not shed easily from the locule wall surface during processing of the samples is evidence that they also will be hardly removed during rainfall. Nevertheless, a few orbicules were seen attached to the pollen exine indicating that some of them may be dispersed together with pollen grains. This is in accordance with observation by Vinckier and Smets (2001*a*, *b*). However, we found that only 21% of pollen grains in *Dactylis glomerata* and 38% in *Cynosurus echinatus*, had orbicules attached to the pollen exine. Moreover, the number of orbicules attached to the same single pollen grain was always very low in both species. This further confirms that the amount of orbicules emitted from the two grasses is insignificant.

Obviously, our present study and that of Vinckier et al. (2006) do not exclude the possibility of orbicules being emitted in high quantities from the anthers of other species. As noted above, this seems to be the case in some gymnosperms which may be related to the most tenuous attachment of orbicules to the locule wall surface. However, based on a larger study we are carrying out on the taxonomic significance of orbicules in the Poaceae (A. M. Dinis et al., unpublished results) we have evidence that the orbicule features in other grasses do not differ significantly from those described in the present work. Also, studies of

herbarium specimens of Poaceae held at COI confirm that locule wall surfaces are still covered with orbicules in the dehisced anthers. This indicates that the attachment of orbicules to the locule wall surface is not altered significantly after anther dehiscence. Thus, we consider it unlikely that orbicules are emitted in large numbers from the Poaceae.

Conclusions

The putative allergenic activity of orbicules and their importance in triggering allergic diseases has recently been put forward. However, the question of whether orbicules are released from the anthers is still controversial. According to Thommen's postulates (1930), the allergenic particles must be produced in high quantities. In the present study, we show that the orbicules of grasses are not easily removed from the locule wall surface to which they are firmly attached. Therefore, the amount of orbicules of grasses that might reach the human respiratory system is most likely too small to play a significant role as potential triggers of allergic reactions. In the future, it would be interesting to perform a similar research in anthers of other allergenic plants and search for alternative particles, such as those emitted from bursting pollen (e.g. Staff et al., 1999; D'Amato et al., 2002, 2007; Swoboda et al., 2004; Taylor et al., 2004), which can trigger allergic asthma in pollinosis patients.

Acknowledgements

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