Pollensomes as natural vehicles for pollen allergens (15-00452-FL)

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Abstract

Olive (*Olea europaea*) pollen constitutes one of the most important allergen sources in the Mediterranean countries and some areas of America, South Africa and Australia. Recently, we provided evidences that olive pollen releases nanovesicles of respirable size –named generically pollensomes- during *in vitro* germination. Olive pollensomes contain allergens, such as Ole e 1, Ole e 11 and Ole e 12, suggesting a possible role in allergy. The aim of this study is to assess the contribution of pollensomes to the allergic reaction. We show that pollensomes exhibit allergenic activity in terms of patients' IgE-binding capacity, human basophil activation and positive skin reaction in sensitized patients. Furthermore, allergen-containing pollensomes have been isolated from three clinically relevant non-phylogenetically related species: birch (*Betula verrucosa*), pine (*Pinus sylvestris*) and ryegrass (*Lolium perenne*). Most interesting, pollensomes were isolated from aerobiological samples collected with an eight-stage cascade impactor collector, indicating that pollensomes secretion is a naturally occurring phenomenon. Our findings indicate that pollensomes may represent widespread vehicles for pollen allergens, with potential implications in the allergic reaction.

Word count: 171

***Key words*:** pollen allergen, nanovesicles, allergen release, cross-reactivity

Introduction

The most important outdoor sources of allergens are pollen grains from anemophilous plants, including trees, grasses and weeds. In Mediterranean countries and some areas of America, South Africa and Australia, olive (*Olea europaea*) pollen constitutes one of the most important causes of pollinosis (1). Exposure to its pollen grains leads to a variety of allergic symptoms ranging from seasonal rhinoconjunctivitis to severe asthma in susceptible individuals (2). Olive pollen contains a wide collection of different allergens (3). To date, twelve allergens -Ole e 1 to Ole e 12- have been identified, isolated and characterized.

Allergen release from pollen is a prerequisite for sensitization and elicitation of the allergic symptoms in humans. Several works have reported that allergen liberation from pollen grains occurs in two different compartments: first, outside the individual organism when pollen grains are spreading through the atmosphere and second, on the mucosal surface of the upper respiratory tract after inhaling pollen. Previous studies have shown that the air, in addition to carrying airborne pollens, contains allergenic pollen-derived submicronic (< 10 μm) and paucimicronic (< 1 μm) particles that can reach the lower airways, eliciting allergic symptoms in susceptible subjects (4-12). These particles are mainly composed of starch granules and polysaccharide (P)-particles, structures that are scarce or absent in mature olive pollen (13).

Recently, we reported that fresh olive pollen grains release nanovesicles of respirable size – named pollensomes- during *in vitro* germination (14). In addition to proteins involved in fertilization process, pollensomes contain Ole e 1, Ole e 11 and Ole e 12 allergens, suggesting their possible role in allergy. The aim of this study is to assess the contribution of these nanovesicles to allergic reaction. We study whether pollensomes show IgE-binding capacity and induce specific activation of human basophils and positive skin reactions in olive pollen allergic patients. Moreover, the release of allergen-containing pollensomes from birch, pine and ryegrass pollens, three clinically relevant but non-phylogenetically related pollens, is also analyzed. Finally, to determine if pollensomes release is a naturally occurring phenomenon, we examine their presence

in aerobiological samples collected with a cascade impactor collector. Our findings indicate that pollensomes may represent widespread vehicles for pollen allergens, with a potential role in the natural induction of the allergic reaction.

Materials and Methods

Pollen material

Mature pollen grains were collected from olive (*Olea europaea*) trees in Granada (Spain) during seasons of 2009-2011. Pollen was dried overnight and stored at -20ºC until used. Pollens of birch (*Betula verrucosa*), pine (*Pinus sylvestris*) and ryegrass (*Lolium perenne*) were supplied by ALK-Abelló (Madrid, Spain). Pollen extracts were prepared as described (15).

Antibodies and reagents

Polyclonal sera against Ole e 1, Ole e 2, Ole e 3, Ole e 9, Ole e 11 and Ole e 12 allergens from olive pollen were generated by Dr. F. Vivanco's laboratory (Fundación Jiménez Díaz, Madrid, Spain). A polyclonal serum against the major allergen from birch pollen, Bet v 1, was prepared by immunization of BALBc mice. mAb raised against the purified major group 1 allergen (Phl p 1) from timothy pollen and anti-human IgE mAb were kindly donated by ALK-Abelló. Anti-Phl p1 mAb recognizes other grass group 1 allergens such as Lol p 1, the major allergen from ryegrass pollen. HRP-conjugated goat anti-rabbit IgG (BioRad), goat anti-mouse IgG (Pierce Chemical) were used as secondary antibodies.

Patients

Olive pollen-allergic patients (n = 4), included in this study, were diagnosed on the basis of a clinical history of allergy to olive pollen, a positive skin prick test (SPT) and CAP-FEIA System (Pharmacia AB) classes 3-6 to olive pollen. Non-atopic individuals (n = 2), were used as controls. A written informed consent was obtained from all participants.

In vitro pollen germination and isolation of pollensomes

Olive pollen (0.1 g) was hydrated in a humid chamber at room temperature for 30 min before transferring to Petri dishes (15 cm in diameter) containing 20 ml of germination medium: 10%

sucrose, 0.03% $Ca(NO_3)_2$, 0.01% KNO_3, 0.02% $MgSO_4$ and 0.03% H_3BO_3. Pollen was germinated at 30ºC in dark for 16 h (16). Similar protocol was used for birch and ryegrass pollens. For pine, a gymnosperm, *in vitro* pollen germination was performed in 15% sucrose, 0.02% Ca_2Cl and 0.03% H_3BO_3, for 72 h at 30ºC in dark (17). Pollen grains were considered germinated only when the tube was longer than the diameter of the pollen grain.

Cultured medium was collected and cleared of pollen debris by centrifugation. Pollensomes were isolated from cleared medium as previously described (14). The protein concentration in pollensome preparations was measured by the micro-bicinchoninic acid assay (Pierce Chemical).

Aerobiological samples

Atmospheric particles were sampled in the city of Granada (Spain) during April 2010, using an eight-stage cascade impactor collector (Andersen Inc.) (11). Particles were collected on glass microfiber filters (Type GF/A, Whatman). Every 24 h, filters were removed and frozen at -20ºC until required. Particulate filters from stage 7 and stage F were gently washed out in PBS onto a Petri dish for 1 h and supernatants were subjected to the pollensomes purification protocol.

Scanning electron microscopy

A piece of microfiber filters (0.5 cm^2) were mounted on aluminum stubs and sputter-coated with gold. Images were obtained at 80 kV with a JEOL 6400 scanning electron microscope (JEOL, http://www.jeol.com) and images collected and processed with the software INCA (http://www.oxford-instruments.com).

Electron microscopy

Pollensomes were fixed in 2% paraformaldehyde and loaded onto copper-formvar/carbon-coated 200-mesh electron microscopy grids. Samples were contrasted with 1% uranyl acetate and observed at 80 kV with a transmission electron microscope (JEOL1010, JEOL Instrument).

Western and Dot blot analysis

For Western blot, pollensomes and pollen protein extracts were resolved by 15% SDS-PAGE and transferred onto nitrocellulose membranes (Hybond ECL 0.45 μm, Amersham) as previously described (15). After blocking with 3% skim milk in PBS-0.1% Tween 20, the membranes were incubated with the specific polyclonal serum -or mAb- for each allergen for 1 h at room temperature. The membranes were then washed with PBS-0.1% Tween 20 and incubated with secondary HRP-conjugated goat anti-rabbit IgG Ab (1:3000 dilution) – or HRP-conjugated anti-mouse IgG Ab (1:2500 dilution)- for 1 h, followed by visualization with the enhanced chemiluminescence western blotting detection reagent (Amersham) and the LAS-3000 mini (Fujifilm) imaging system.

For IgE-binding assays, the membranes were incubated with individual sera from patients allergic to olive pollen (1:10 dilution) and, for control purposes, from non-allergic individuals, for 2 h at room temperature. This was followed by incubation with anti-IgE mAb (1:5000 dilution) and HRP-conjugated goat anti-mouse IgG (1:2500 dilution).

For Dot blot experiments, samples were applied onto nitrocellulose membranes using a Minifold I dot-blot system and bound IgG Abs were detected with the secondary HRP-conjugated goat anti-rabbit IgG Ab as described for Western blot.

Skin prick test

Twenty microliter of pollen-derived nanovesicles (50 μg/ml in PBS) was gently pricked on the forearm of patients as described (18). Commercial olive pollen extract (ALK-Abelló) was used as a positive control at a concentration recommended by manufacturer (30 HEP). The prick test was considered positive when the mean diameter ([D + d]/2) of the wheal induced by the allergen was > 3 mm.

Basophil activation test

Whole blood cells from olive pollen allergic patients (n = 4) and non-allergic individuals (n = 2), were challenge with increasing concentrations of pollensomes or Ole e 1 (0.005-5 μg/ml), and for control purpose with anti-IgE Ab, as described (19). Results were expressed in percentage of activated $CD63^+$-basophils detected by flow cytometry.

Results

Allergens are released from olive pollen in pollensomes

Olive pollensomes were isolated from germination medium as previously described (14), with sizes ranging from 28 to 68 nm (Fig. 1A). Western blot analysis revealed that, besides Ole e 1, Ole e 11 and Ole e 12 allergens, Ole e 3 -the olive polcalcin- was also present in these pollensomes (Fig. 1B). By contrast, Ole e 2 and Ole e 9 (a profilin and a 1,3-β-glucanase, respectively) were not detected in pollensomes (Fig. 1B).

Pollensomes play an active role in allergy

The IgE reactivity of pollensomes was analyzed by Western blot using individual sera from two olive pollen-allergic patients, representing two different allergenic sensitization patterns (Fig. 1C). The IgE-binding pattern of olive pollen extract is complex, since it contains a large number of IgE-reactive components with molecular masses ranging from 10 to 60 kDa. Pollensomes also displayed an IgE-binding profile which varied among allergic individuals, but with lower complexity compared to the whole pollen protein extract. IgE reactivity was detected against protein bands which, according to their molecular masses, might represent Ole e 1 (18.5-20 kDa) and Ole e 3 (9.2 kDa). IgE-reactive protein bands of 60-70 kDa were also detected. No significant reactivity was observed using control sera from non-atopic subjects.

The ability of pollensomes to elicit cutaneous reactions *in vivo* was evaluated in four patients with olive pollen allergy (Fig. 1D). The whole group presented positive SPT to both olive pollen extract and pollensomes. In all cases olive pollen extract induced a larger skin reaction than the isolated pollensomes, with mean wheal area values of 22.0 mm^2 and 11.6 mm^2, respectively. These results could be explained in terms of the differences in contents of allergens (number and concentration) in pollen extracts *versus* pollensomes. No positive reaction either to pollensomes or to pollen extracts was observed in control subjects, indicating the high specificity of SPT. No adverse effects were observed in the subjects tested.

In order to obtain a more complete picture of pollensomes' IgE reactivity and, therefore, their potential clinical significance, basophil activation assays were performed with peripheral blood cells of four olive pollen allergic patients (Fig. 1E). In all patients tested, pollensomes induced activation of basophils in a dose-dependent manner, while Ole e 1 reached its maximal effect or plateau at the dose-range represented. Basophils from non-atopic controls did not respond to pollensomes.

Pollensomes represent a new mechanism of allergen release common to different species

To investigate whether pollensomes represent a common mechanism for allergen release, pollens from three clinically relevant and non-phylogenetic related species -birch, ryegrass and pine- were assayed. These pollens released pollensomes during *in vitro* germination with sizes comparable to those ones described for olive: 30-60 nm in diameter (Fig. 2A). The major allergen from birch pollen, Bet v 1, was present in pollensomes derived from this species (Fig. 2B). In contrast, Lol p 1, the major allergen of ryegrass pollen, was not detected in released pollensomes from this pollen. In addition, Ole e 12-homolous allergen could be localized in nanovesicles released from birch, ryegrass and pine pollens. Therefore, it can be defined as a protein marker for pollensomes However, Ole e 1-like protein was only detected in birch pollensomes but not in those from ryegrass and pine.

Pollensomes are present in the atmospheric aerosol

To provide direct evidence that pollensomes are airborne nanoparticles released to the air, atmospheric samples were taken during April 2010 using an eight-stage cascade impactor collector. The study was performed in Granada, a city in the south of Spain. The aerobiological analysis was performed according to the Spanish Aerobiological Network (http://www.uco.es/rea/) guidelines, showing that the dominant pollen types recorded in the studied period were *Plantago* followed by *Pinus*. Olea pollen was registered in low concentration (2 grains pollen/m3). The sizing efficiency of the cascade impactor collector was verified examining air filters by scanning electron microscopy. Pollen grains (17-21 μm in

diameter) were only found in filters of stages 0 and 1, whereas the remaining stage filters of the collector were free of pollen grains (Fig. 3A). Pollensomes-like nanovesicles were collected from stage 7 (0.4-0.7 μm) and stage F (< 0.4 μm) filters of the cascade collector (Fig. 3B). No pollensomes were detected in control ones (unexposed filters). When the airborne pollensomes were compared to those released *in vitro* by transmission electron microscopy, similar features were found for both types of nanovesicles: 27-55 nm in diameter and round-shaped morphology. However, Ole e 12-like proteins were only detected by dot blotting in the nanovesicles collected from stage 7, defining them as pollensomes (Fig. 3C). For Ole e 1-like proteins, the staining was very faint and diffuse in pollensomes of stage 7. This might be explaining by the low levels of airborne pollen grains of *Oleaceae* family at the sampling date, together with the low/or no cross-reactivity among Ole e 1-like proteins from taxonomically unrelated pollens. These results revealed that pollensomes were presented in the atmospheric aerosol samples during the season of 2010 and, therefore, could contribute to the allergenic activity in the atmosphere.

Discussion

Olive pollen allergens are one of the major causes of allergic reactions in the Mediterranean area during the flowering season (1). However, the mechanism by which olive pollen allergens become airborne respirable-sized particles with the ability to reach the respiratory tract and trigger an allergic response in susceptible people, remains unknown. Here, we proved experimental evidences to support that: (ii) pollensomes, defined as allergen-loaded nanovesicles released from olive pollen during *in vitro* germination, exhibited allergenic activity; (ii) they are released from pollens belonging to different taxa; and (iii) their natural occurrence in the atmospheric aerosol.

Our data have shown that allergens, including Ole e 1, Ole e 3, Ole e 11 and Ole e 12, are released in the context of olive pollensomes. Moreover, pollensomes seem to be a mechanism of allergen release common to different clinical relevant pollens, including both gymnosperm (pine) and angiosperm species (birch, olive and ryegrass). To our knowledge, this would be the first report describing allergens as a *cargo* of secreted membrane nanovesicles. On the basis of the results obtained in this study by means of IgE-binding capacity, activation of human basophils and positive skin prick test in patients allergic to olive pollen, pollensomes might exhibit allergenic activity. The potential clinical implication for these findings in allergy is supported by the fact that pollensomes occur in the atmospheric aerosol. Airborne pollensomes were isolated from stage 7 filters of a cascade impactor collector, containing Ole e 1- and Ole e 12-like allergens. In support, De Linares et al. (11) revealed that allergenic activity of Ole e 1 in the atmosphere primarily involves paucimicronic particles. The release of paucimicronic and submicronic particles containing allergens into the atmospheric aerosol has been described for other pollen species including grasses, weeds and trees (4-10, 12). These particles include mainly starch granules, P-particles and other subpollen particles which are scarce or absent in olive pollen (13). Two main mechanisms have been proposed for the release of these particles from pollen grains: (i) a process described for grass (7, 9) and ragweed (10) that involves simply ruptures of pollen grains after contact with rainwater; and (ii) abortive germination described for allergen release from pollen trees of the *Fagales* order

(birch, alder, hazel) (5, 8). In this study, we described a new mechanism by which pollen allergens become aerosols, which involves the secretion of nanovesicles -pollensomes- during pollen germination. The observation that, after rainfall, birch pollen germinates on leaf surfaces (5) supports this proposed mechanism of allergen release in the atmospheric aerosol. Further work is needed to determine the contribution of pollensomes as allergen-carriers on the allergenic aerosol. Because of their small size, pollensomes should be efficiently deposited in lower airways, which cannot be reached by intact pollen grains due to its aerodynamic diameter (19-21 μm). Thus, pollensomes seem suitable to trigger an allergic response in susceptible people, and may explain both the severe asthmatic symptoms associated with olive pollen season (20) and the increased allergen levels during periods when little or no pollen grains are present in the air (11). The latest observation has also been reported for birch (5) and several grasses (4, 6) species. In addition, the particulate nature of the pollensomes and their components likely amplifies the immune response in the airways. Yet, pollensomes can contribute by themselves to the activation of the mucosal epithelium of the respiratory tract. In this context, pollens -beside allergens- contain a range of other compounds that may be contributing to initiation, manifestation or exacerbation of allergic inflammation. Adjuvant factors of pollens include: (i) NADP(H)-oxidases which generate reactive oxygen species provide a signal that enhances allergic airway inflammation (21); (ii) bioactive lipid mediators (PALMS), with chemical and functional similarities to leukotrienes and prostaglandins, that contribute to the generation of a local microenvironment favoring T_{H2} responses (22); and (iii) adenosine, a potent immunoregulator in pollen allergy, that acts on the level of dendritic cells (23). If such compounds are present in pollensomes, they will likely contribute to allergen sensitization and clinic symptoms. In this sense, NADP(H)-oxidases have been detected in subpollen particles released from ragweed pollen (10). Finally, it has been demonstrated that air pollutants (eg., diesel exhaust, dust, fuel combustion) can enhance the immune response to pollen allergens, after interacting in the atmosphere or, more probably, from co-deposition in the human airways (24, 25).

Thus, the existence of pollensomes in the air made feasible their interaction with these pollutants which might also enhance the allergenic potential of these nanovesicles.

These findings open a new dimension in the understanding of early events in both allergic sensitization and exacerbation of the disease prior to allergen-induced mechanisms. Moreover, pollensomes described in this work provide a unique insight into the dynamics of protein secretion during the processes of pollen germination and pollen tube growth, which are critical for successful fertilization in flowering plants.

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Disclosures

The authors have no financial conflicts of interest.

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Footnotes

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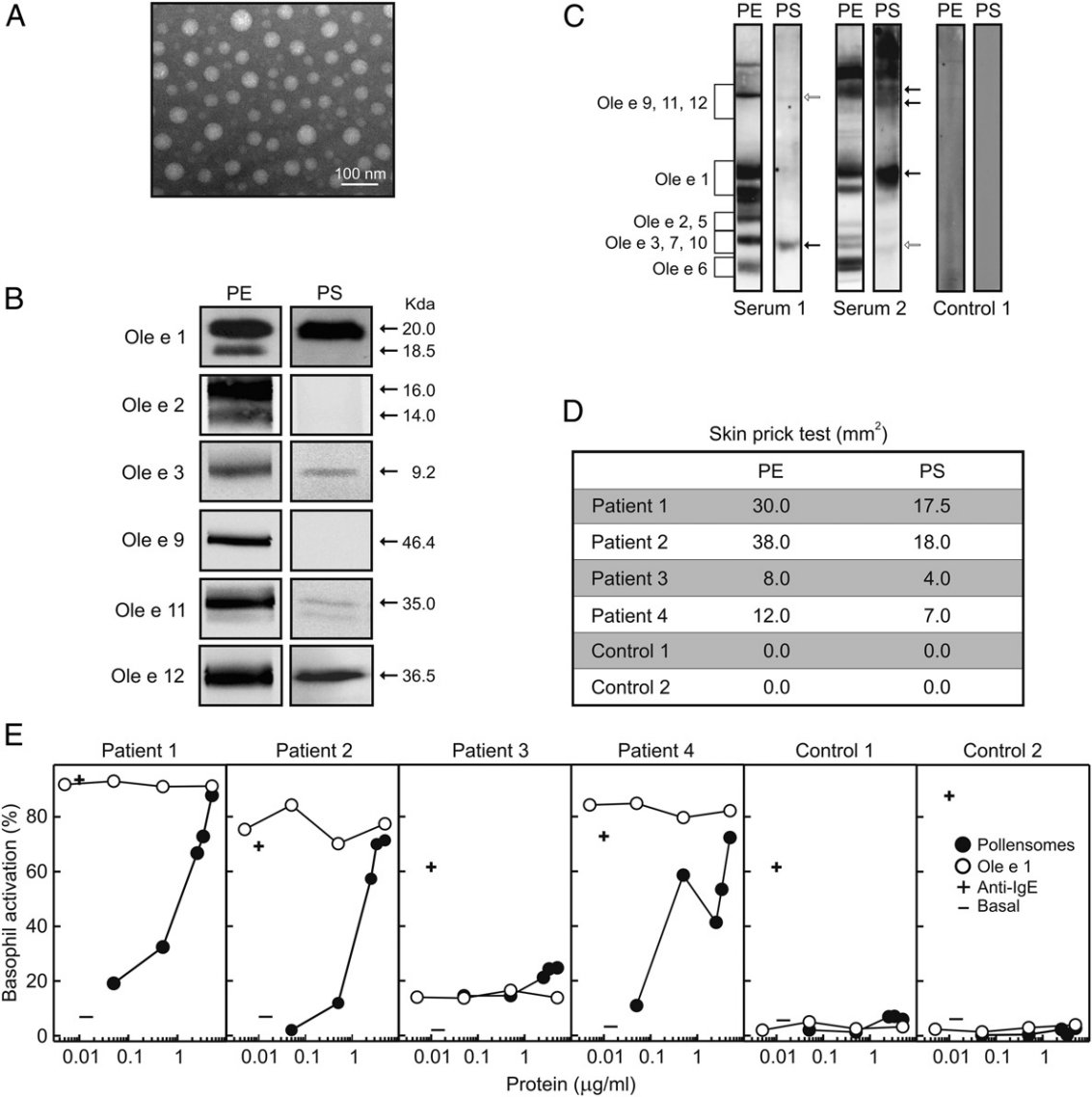
3. *Abbreviations*: SPT, skin prick test.

Figure legends

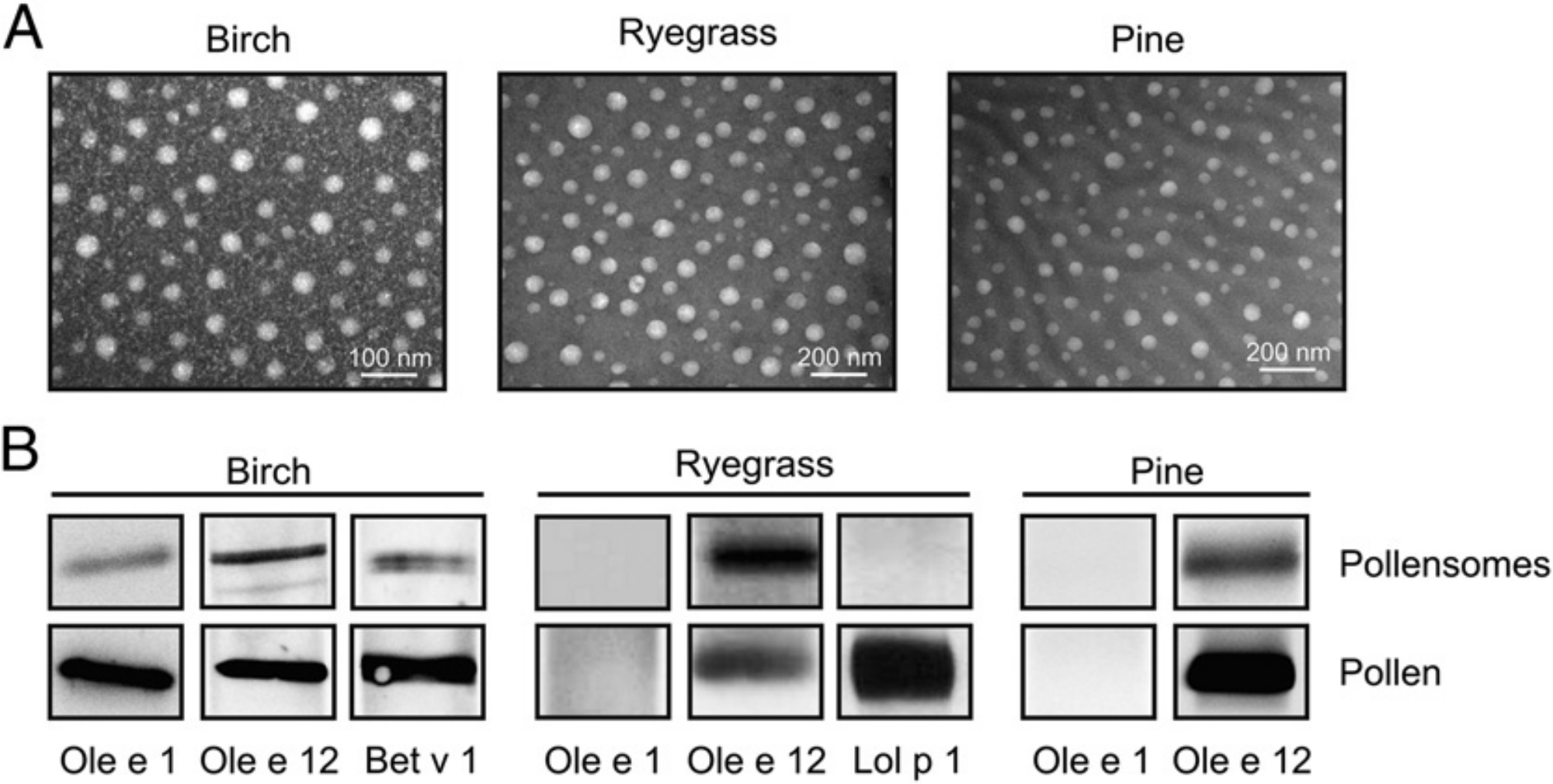
FIGURE 1. Pollensomes, vehicles for olive pollen allergens, play an active role in allergy. (**A**) Transmission electron micrograph of pollensomes released from *in vitro* germinated pollen from olive (*Olea europaea*). (**B**) Western blot analysis of olive pollensomes protein extract (PS, 50 μg) for the presence of Ole e 1, 2, 3, 9, 11 and 12 allergens using specific Ab. Olive pollen extract (PE, 30 μg) was used as positive control. M_r of allergens are indicated in kDa. (**C**) Western blot analysis of olive pollensomes (50 μg) with IgE from serum of two olive pollen allergic-patients and one non-allergic individual (control 1). Olive pollen extract (30 μg) was used as positive control. Arrows indicate allergen positions. Faint bands are marked with white arrows. (**D**) SPT of four olive pollen-allergic patients and two non-allergic individuals (control 1 and 2) with pollensomes and olive pollen extract (as positive control). Values are wheal and fare areas in mm^2. (**E**) Allergenic potency of pollensomes determined by the basophil activation test using flow cytometry. Basophils from four olive pollen-allergic patients and two non-allergic individual were incubated with different doses of pollensomes or Ole e 1. Results are given as percentage of CD63 expression.

FIGURE 2. Pollensomes, a mechanism of allergen release common to a different of species. (**A**) Transmission electron micrographs of pollensomes released from birch, ryegrass and pine pollens during *in vitro* germination. (**B**) Western blot analysis of Ole e 1- and Ole e 12-like proteins in pollensomes protein extracts (50 μg) from birch, ryegrass and pine, using specific antibodies. The same analyses were carried out for the allergens Bet v 1 in birch and Lol p 1 in ryegrass, using an anti-Bet v 1 and anti-Phl p 1 Ab, respectively. Pollen extracts (30 μg) of birch, ryegrass and pine were also probed as controls.

FIGURE 3. Pollensomes occur into the respirable airborne particles. (**A**) Scanning and (**B**) transmission electron micrographs of an air filters and pollensomes-like nanovesicles isolated from stage 7 and stage F filters, respectively. (**C**) Dot blots analysis of atmospheric pollensomes for the presence of Ole e 1- and Ole e 12-like allergens, using specific Ab. Unexposed filter was used as control.



	PE	PS
Patient 1	30.0	17.5
Patient 2	38.0	18.0
Patient 3	8.0	4.0
Patient 4	12.0	7.0
Control 1	0.0	0.0
Control 2	0.0	0.0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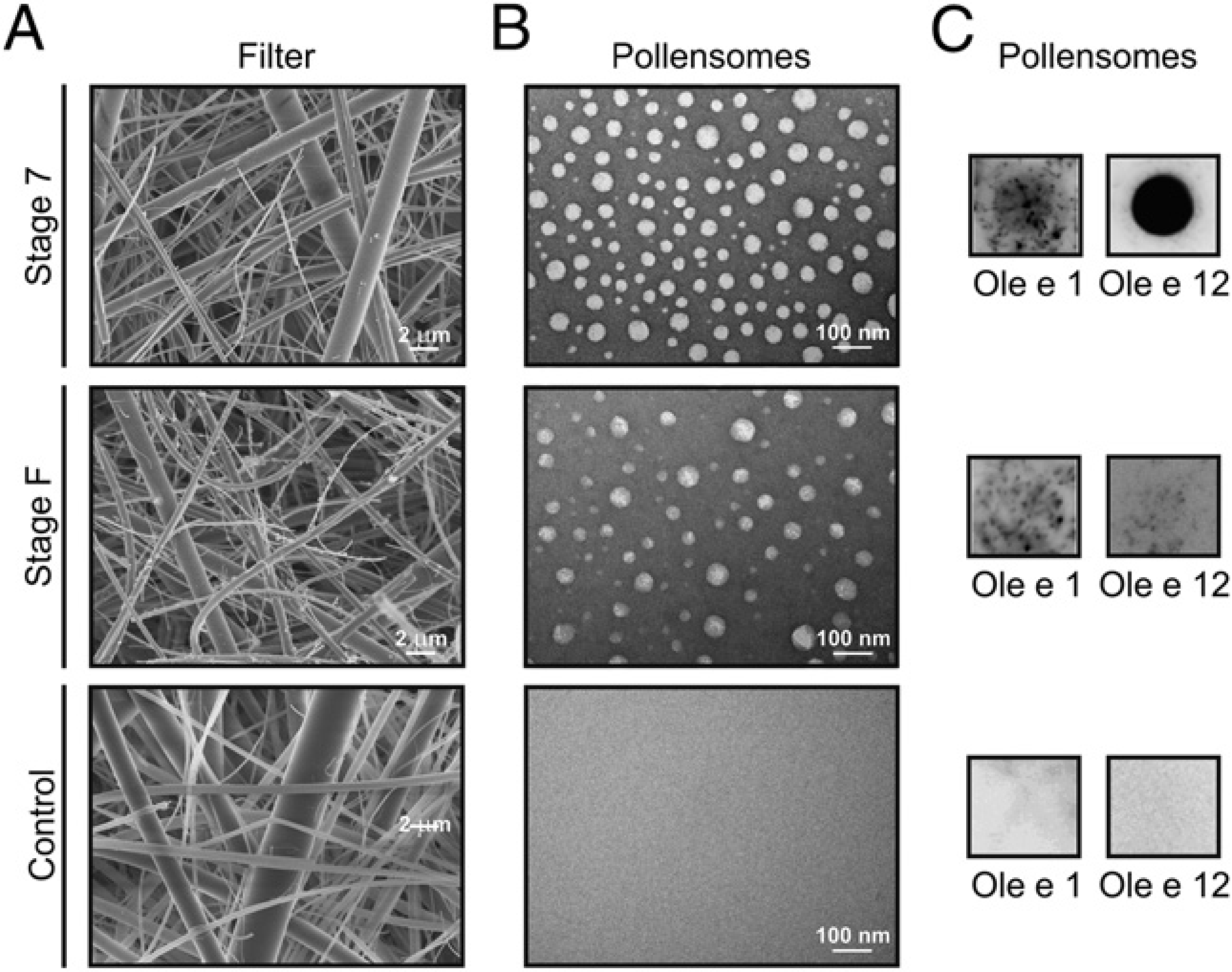
200 nm
B
Birch
Ryegrass
Pine
Pollensomes
Pollen
Ole e 1
Ole e 12
Bet v 1
Ole e 1
Ole e 12
Lol p 1
Ole e 1
Ole e 12


A
Filter
B
Pollensomes
C
Pollensomes
Stage 7
Stage F
Control
2 µm
100 nm
Ole e 1
Ole e 12