

Birch pollen rupture and the release of aerosols of respirable allergens

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Summary

Background Birch pollen allergens have been implicated as asthma triggers; however, pollen grains are too large to reach the lower airways where asthmatic reactions occur. Respirable-sized particles containing birch pollen allergens have been detected in air filters, especially after rainfall but the source of these particles has remained speculative.

Objective To determine the processes by which birch pollen allergens become airborne particles of respirable size with the potential to contribute to airways inflammation.

Methods Branches with attached male catkins were harvested and placed in a controlled emission chamber. Filtered dry air was passed through the chamber until the anthers opened, then they were humidified for 5 h and air-dried again. Flowers were disturbed by wind generated from a small electric fan. Released particles were counted, measured and collected for immuno-labelling and high-resolution microscopy.

Results Birch pollen remains on the dehiscent anther and can rupture in high humidity and moisture. Fresh pollen takes as long as 3 h to rupture in water. Drying winds released an aerosol of particles from catkins. These were fragments of pollen cytoplasm that ranged in size from 30 nm to 4 µm and contained Bet v 1 allergens.

Conclusion When highly allergenic birch trees are flowering and exposed to moisture followed by drying winds they can produce particulate aerosols containing pollen allergens. These particles are small enough to deposit in the peripheral airways and have the potential to induce an inflammatory response.

Keywords birch, particulate aerosol, pollen allergens, pollen rupture, respirable particles

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Introduction

Birch pollen allergens are a major cause of allergic reactions, especially in large parts of the Northern Hemisphere [1, 2]. Outdoor aeroallergens, such as pollen, are also believed to play a role in asthma exacerbations [3–5], but whole pollen grains are too large to penetrate into the lower airways where asthmatic reactions originate [6]. Birch pollen allergens have been recorded in aerosol fractions smaller than pollen grains even when no birch pollen grains are present [7–16]. The concentration of these small allergenic particles increases after rainfall [11–16]. Unlike whole pollen grains, these allergen-loaded particles are small enough to enter the human airways where they would have the potential to cause asthmatic responses in susceptible people [6, 17, 18]. In one of the few quantitative studies performed to date, up to 1.2 ng of Bet v 1 per m³ of air has been measured in particles smaller than 7 µm that were collected on outdoor air filters after rainfall [15]. Improvements in air sampling and allergen elution and

detection are still required to optimize the quantitative analysis of allergens collected from the air.

Researchers have long considered that changes in the weather might cause fragmentation of pollen and spores, generating aerosols that contain allergens as well as those governing their release. Understanding the precise mechanisms involved in this link between asthma and the weather would be of great importance [19]. We have recently shown how two major allergenic grasses released respirable aerosols of cytoplasmic debris containing pollen allergens [20]. Grass pollen grains ruptured while enclosed within moistened anthers. Following drying, winds entrained the debris of pollen cytoplasm into the atmosphere. Recently Grote et al. [21] showed that birch pollen ruptures in rainwater and the particles released onto a glass slide contained Bet v 1 allergens. They described in detail the sequence of events, first observed by Schäppi et al. [14], involving pollen tube rupture and the emission of cytoplasm particles into rainwater; a process they called 'abortive pollen germination'. Although it is well known that hydrated pollen grains secrete, both from their surface and from sites within the cytoplasm, small molecules involved in compatibility recognition interactions at the initial stages of pollination, Grote et al. [21] also showed that allergenic proteins are released from the birch

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pollen tubes predominantly upon rupture. Here we investigated whether a similar mechanism of pollen rupture that we previously found for grasses is also involved in the release of respirable aerosols containing birch pollen allergens.

Methods

Pollen rupture and anther analysis

Branches with male catkins (flowers) were collected from trees of European white birch (*Betula pendula*, Roth) growing in Pasadena. Pollen was collected from open anthers by contact with a glass microscope slide or cover slip. Pollen was collected from trees following both dry and wet weather. Commercially available birch pollen was also purchased (Greer Laboratories, Lenoir, NC, USA). Some pollen grains were stained with Calberla's solution (three drops of aqueous basic fuchsin, three drops of glycerine jelly, 5 mL glycerine, 10 mL ethanol and 15 mL water). This basic dye stains the exine of the pollen. To assess pollen rupture a drop of water or 10% sucrose in water was applied. Percentage rupture was recorded using an Olympus light microscope with bright-field optics (Olympus, Melville, NY, USA). Segments of catkins (flowers) and cover slips were mounted on scanning electron microscopy stubs with carbon conductive paint and then air-dried and sputter coated (Techics Hummer Sputter Coater, Alexandria, VA, USA) with platinum/palladium or gold. Specimens were viewed with an Etec Autoscan SEM (Hayward, CA, USA) at 20 kV.

Controlled emission experiment

Branches from birch trees, with approximately 20 attached flowering catkins that had begun to anthes, were harvested and placed in a controlled environment chamber connected to a Marple cascade impactor (Sierra Instruments Inc., Monterey, CA, USA; flow rate 3.0 L/min), and temperature and relative humidity (RH) probes as described by Taylor et al. [20] except that wind disturbance was generated by a small fan placed inside the chamber. The controlled emission chamber was monitored to optimize flowering by controlling RH, temperature, airflow and lighting. A flow of filtered air was passed through the chamber for 15 h at 2 L/min to eliminate any background aerosol. Next, the air in the chamber was humidified by passing the incoming air at a reduced flow rate of 0.5 L/min through an evaporator. In other experiments, stopping the flow through the chamber allowed transpiration to raise the humidity. The plants were left in high humidity for up to 6 h to allow the flowers to equilibrate with the high humidity prior to air-drying. Flowers were then disturbed by wind generated from a small electric fan, while a 2–4 L/min flow of dry air was drawn through by the sampling instruments. Measurements of nano-sized particles (30–700 nm) in aerosols were performed with a differential mobility analyser (TSI Inc., Shoreview, MN, USA) with a flow rate of 0.3 L/min. In later experiments, the humidified chamber was opened and the plants were wet with a fine mist produced by a water spray prior to drying. All experiments were replicated at least three times. Pollen debris was collected in the cascade impactor in the following size fractions: 0.4–2, 2–4, 4–10 and over 10 µm. Size-classified particles collected on cover slips in the cascade impactor were

observed with light microscopy and processed for scanning electron microscopy.

Ultrastructural analysis and immuno-labelling

For transmission electron microscopy (TEM), cover slips removed from the cascade impactor were anhydrously fixed in the vapour from 1.7% paraformaldehyde in 2,2-dimethoxy propane for 10 min. They were then infiltrated in a drop of LR gold resin for 24 h at 4 °C (London Resin Co., Theale, UK). Each sample was inverted and placed on top of a gelatine capsule filled with LR gold resin containing 1% Benzil. Samples mounted on capsules were inverted and polymerized at 4 °C with a 40 W lamp. Sections, 100 nm thick, were collected on pioloform-coated gold grids and immuno-labelled for birch allergens with rabbit anti-recombinant Bet v 1 (rBet v 1) antibody. These anti-sera were raised against purified rBet v 1. The primary antibody was detected with 15 nm gold-goat anti-rabbit (Sigma Chemical Co., St Louis, MO, USA). For controls, the primary antibody was omitted and antibody pre-incubated with rBet v 1, antibody serially diluted, a pre-immune sera or an irrelevant antibody (rabbit anti-rPhl p 1) were used according to established protocols. For each antibody, immuno-labelling was performed on at least three separate grids with sections attached. Preparations were viewed with a Philips EM 420 transmission electron microscope (Philips, Eindhoven, the Netherlands).

Size-fractionated samples were collected from the controlled emission chamber with a Sierra cascade impactor. Particles released from birch flowers after a cycle of wet and dry were compared with particles released after exposure to dry air only. Size-classified particles were deposited onto glass cover slips in the impactor. The cover slips were removed and particles re-suspended in 100 µL phosphate-buffered saline. They were analysed with immuno-dot blotting as previously described by Miguel et al. [22]. Twofold serial dilutions were applied for the rBet v 1 control. The nitrocellulose membranes were blocked and then incubated with rabbit anti-rBet v1 antibody, overnight at 4 °C. After washing, the membranes were incubated in anti-rabbit IgG alkaline phosphatase conjugate (Sigma) and were developed in Western Blue stabilized substrate (Promega, Madison, WI, USA).

Results

Pollen viability and rupture

Both male (Fig. 1) and female catkins occur on the same birch tree. The male catkin houses tri-porate pollen grains that are about 20 µm in diameter. Fresh birch pollen takes at least 3 h to rupture in water. About 65% of pollen grains grew a pollen tube up to 300 µm long prior to rupture and release of their cytoplasmic contents. Commercially purchased pollen did not rupture nor germinate. Only freshly collected, viable pollen ruptured. Upon dehiscence of the anther, mature pollen grains are metabolically dormant. Dormancy is broken when pollen is exposed to at least 80% RH. If mature dormant pollen is transferred directly to water, pollen might not germinate or rupture. At 70% RH pollen stayed viable for at least 24 h. However, at 40% RH pollen loses viability within

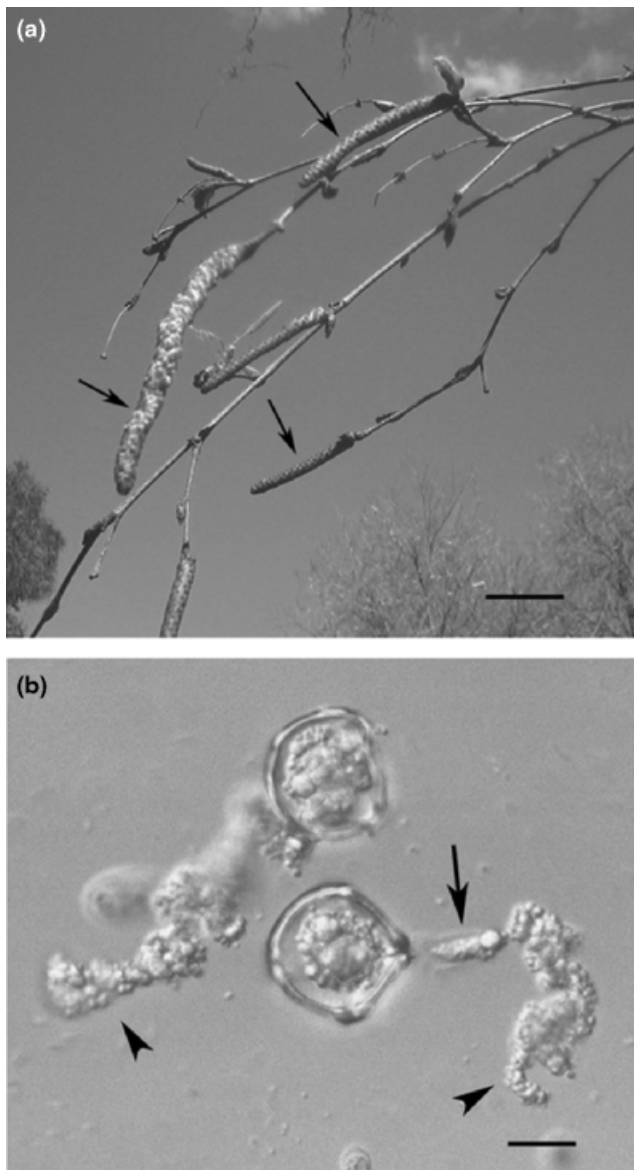


Fig 1. (a) Birch tree flowers. arrows, male catkins. Bars = 20 mm. (b) Ruptured pollen after germination in water for 3 h. Arrows, pollen tubes; arrowheads, cytoplasmic debris. Bars = 10 μ m.

6 h. Viability was determined by exposing pollen to 80% RH for 20 min and then measuring the percentage rupture or germinated upon immersion in water. At 96% RH, many of the birch pollen grains germinated. As with grass flowers, the walls of the dehiscent birch anthers reflexed when the humidity rose above 88% RH and enveloped the pollen remaining on the anther; a cycle of reversible dehiscence that could be experimentally induced three times over a 1 h period by fluctuating the RH. However, the floral bracts of the birch catkin remain anthesed. Early in the morning, pollen was removed from outdoor flowers that had been exposed to overnight moist weather. When viewed under the light microscope most pollen had germinated and formed a short pollen tube or was empty of cytoplasm. Further experiments involving pollen rupture were performed in a chamber to assess the formation of an aerosol.

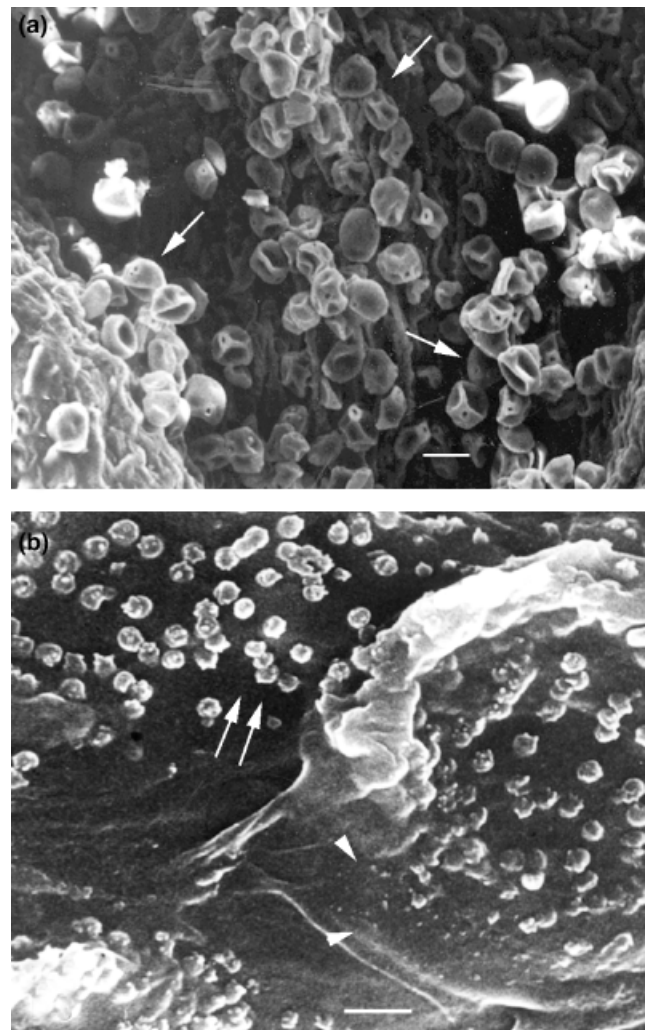


Fig 2. Scanning electron micrographs of structures located within the birch catkin. (a) Bar = 20 μ m. arrows, pollen grains. (b) Bar = 2 μ m. Double arrows, orbicules; arrowheads, inter-orbicular structures.

Chamber release of respirable aerosols

Most birch pollen remained on the anthesed flowers housed within the controlled emission chamber (Fig. 2). We observed that birch catkins are less effective at shedding pollen into the air than grass flowers. This is due to birch anthers being recessed within the floral bracts of the catkin whereas grass anthers are exerted on thin filaments that may extend as much as 1 cm away from the flower. When flowering in a quiescent environment, most of the pollen remained on the birch catkins. Moreover, pollen-laden flowers can be retained on trees for several days until the pollen is dislodged by winds or the flowers fall from the trees. In the present experiments, particles of fragmented cytoplasm were released from birch flowers after a cycle of wetting and drying. Particles were also released from flowers that had been collected from outdoors after wet weather. Particles collected on the lower impactor stages ranged in size from 0.4 to 4 μ m in diameter when examined with scanning electron microscopy (Fig. 3). The fragmented debris released from the pollen formed particles as small as 30 nm in diameter when measured with a differential mobility analyzer (TSI Inc.) (Fig. 4).

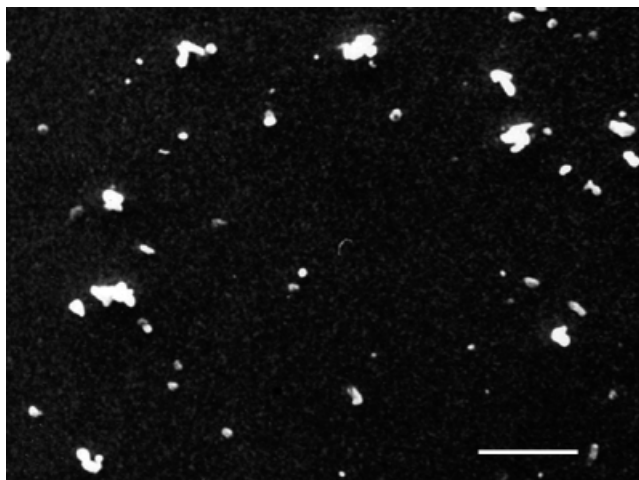


Fig 3. Scanning electron micrographs of particles captured in a cascade impactor after release from birch flowers housed within a controlled emission chamber. Particles of pollen cytoplasm released from flowers exposed to a cycle of wet and dry. Bar = 5 μm .

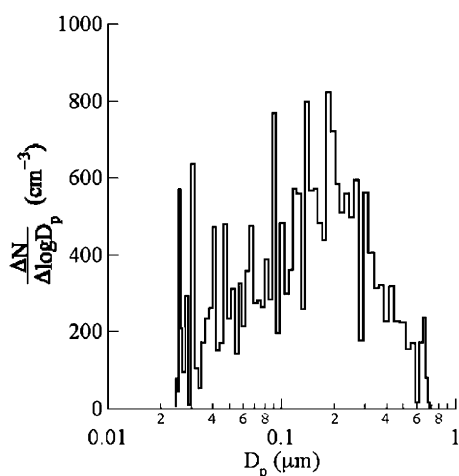


Fig 4. Differential mobility analyser counts of sub-micronic particles released from birch flowers that were exposed to a cycle of wet and dry followed by a wind disturbance while housed within a controlled environment chamber.

The surface of the anther is lined with orbicules that are approximately 0.8 μm in diameter. Orbicules remain attached to the grass anther, but some are released from birch flowers with a wind disturbance (Figs 2b and 3). Also, for birch, small (30–60 nm in diameter) inter-orbicular structures line the anther (Fig. 2b). Some of these particles are released to form an ultra-fine aerosol of fine particles seen in the differential mobility analyser measurements (data not shown). The number of fine particles released from the flowers after initial drying was much lower than observed following a cycle of wet and dry. Scanning electron microscopy analysis revealed that, within some anthers, wind had shed (released) most of the pollen, but many of the anther wall structures remained in place.

Immuno-labelling

Particles that were released from the birch flowers and collected in a cascade impactor were identified as fragments

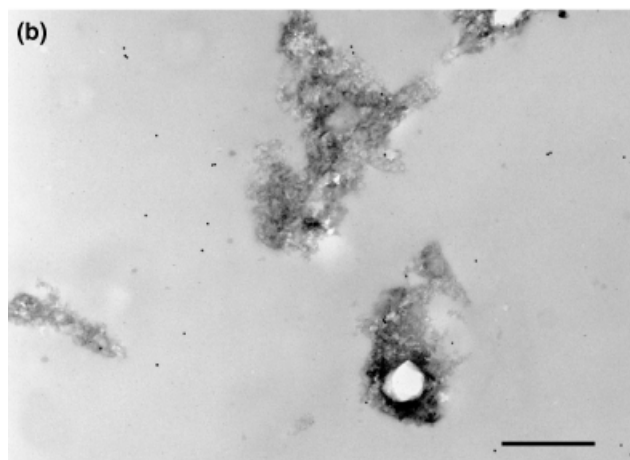
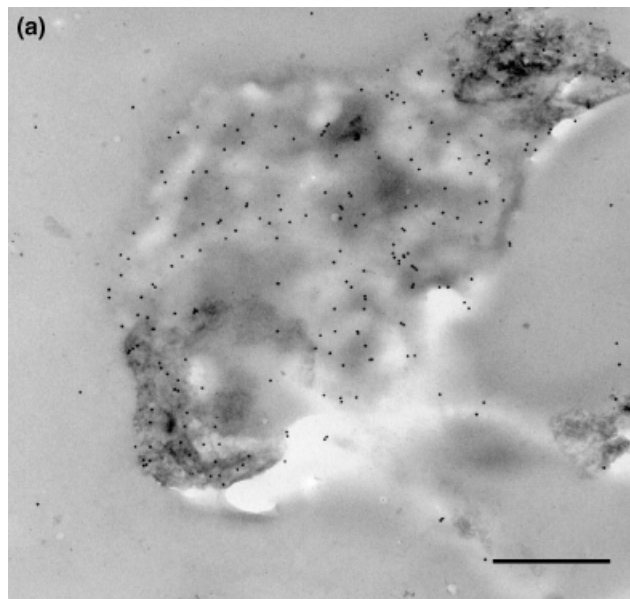


Fig 5. Transmission electron micrographs of sections of pollen cytoplasmic debris collected during controlled emission experiments. (a) Immuno-labelled with rabbit anti-recombinant Bet v 1 antibody and 15 nm gold anti-rabbit probes. (b) Control performed with rabbit anti-rPhl p 1 antibody. Bars = 0.5 μm .

of pollen cytoplasm when viewed with TEM (Fig. 5). Bet v 1 allergens were immuno-localized within these debris particles. Immuno-controls performed were not positively labelled. Immuno-blots of the particles tested positive for Bet v 1 activity in each of the stages except for the 4–10 μm stage (data not shown). A small number of particles were released from flowers that were exposed only to dry air. These particles, which were identified as orbicules, and were collected in the 0.4–2 μm size fraction in the cascade impactor, did not show positive immuno-labelling for Bet v 1. The various immuno-labelling controls performed were also negative.

Discussion

Like pollen from highly allergenic grasses [20], we observed that birch pollen ruptures in moisture to form particles that are readily released directly from the flowers to form a

respirable aerosol. Because of their small size, these particles should be able to efficiently deposit in the human airways. Particles consisted of fragmented pollen cytoplasm and contained pollen allergens. We describe in this article the details of how pollen allergens are released from birch and compare similarities in the mechanism of aerosol formation described for grass.

Many hypotheses have been proposed to explain the appearance of birch allergens in respirable aerosol particles. Germinated and ruptured pollen has been found on birch leaves [14]. It has been suggested that the starch granules that are released into water from the moistened pollen may be capable of forming an aerosol [8, 14–16]. Rain splash or jet bubble dispersal mechanisms have been proposed [13, 23]. Other hypotheses include: the formation of fragments by slow physical degradation of birch pollen grains [24], or formation of Bet v 1-laden aerosols from bark, leaves, or buds of birch trees [11, 25]. It has been suggested that allergens might be transferred from pollen to small particles [9, 26] or that pollutant chemicals or particles that have been detected on pollen grains may modify allergen release from pollen [27]. Grote et al. [21] have localized birch pollen allergens in the fragments of cytoplasm that were released by pollen rupture in water on a glass slide. Observations of orbicules on birch anther walls have led to suggestions that they are released to form a respirable aerosol and speculations that they may be loaded with allergens [12, 28, 29]. But in all these studies the formation of aerosol particles was not investigated.

Wind-dispersed pollen grains and their flower surfaces have evolved to exploit dispersion by air currents. There has been a history of contradictions reported concerning the forces involved in removing pollen from the anther. Many researchers consider that pollen grains are catapulted into the air or fall off the flower during anther opening [30, 31]. Several have described the immediate release of birch pollen from the dehiscing anther and the temporary storage of this pollen on the neighbouring scales of the catkin until blown away [23, 32–34]. One of these articles [32] went on to say that after leaving some birch catkins or grass flowers in a vase in a room with no draught there is an even deposition of pollen on the table below. Taylor et al. [20] observed grass pollen grains remaining on the anther and also found this to be a site for pollen rupture. In this article, we observed that birch pollen grains, as with pollen from the majority of wind-pollinating plants, do remain on the open anthers that are housed within the male flower unless disturbed. Pollen that is dislodged from the anthers can be retained on the scales of the catkin. Little pollen is released from the catkins if it remains undisturbed. A single birch catkin produces about 5–6 million pollen grains. Birch pollen that is retained on the flower is thought to be protected from rain and moisture [23]. In this work we demonstrated that moisture ruptures pollen while still on the catkin and pollen fragments can be released as a respirable aerosol from the flower surface. Immuno-dot blotting experiments showed Bet v 1 located in the pollen debris that was released from the birch flowers and captured in a cascade impactor. Results confirm the TEM observations showing positive immuno-labelling of Bet v 1 in these fragments of pollen cytoplasm.

Anthers have evolved to function as platforms for the effective release of particles, i.e. pollen grains. The orbicules

lining the anther are believed to assist in pollen dispersal by being non-wettable and mutually repulsive to the pollen [35]. Orbicules most likely aid in pollen shedding by reducing the van der Waals forces in the pollen–anther surface interaction. Orbicules also appear effective in aiding the release of pollen fragments from the anther surface. Some orbicules are also released into the air, along with the newly described inter-orbicular structures. These latter structures are obvious from previously published figures [28, 29] but their release from the anther has not previously been considered. Since few orbicules are released and pollen allergens are specifically located within the cytoplasm of mature pollen grains [36, 37] it seems unlikely that orbicules significantly contribute to allergen carriage, especially in dry weather. We propose that the structures lining the anther in birch might become allergenic but only when pollen has been moistened or ruptured prior to release from the anther. Evidence of allergen transfer comes from observations of a clear network of strands connecting the anther surface and the pollen grains in birch [37]. Further work is needed to determine the contribution that small numbers of released orbicules have on the allergenic aerosol.

A source for the release of respirable-sized pollen allergens into the air has now been determined for birch and this involves a mechanism similar to that found in grasses [20]. It is likely that most highly allergenic wind-pollinating plants can produce pollen allergen-loaded aerosols directly from the flowers after exposure to rainfall, fog or dew followed by drying winds. These particles are small enough to be inhaled and might trigger an asthmatic response in susceptible people. Fine particles derived from fossil fuel combustion can enhance the immune response to allergens [38]. Binding of these particles to allergens is possible in the atmosphere; however, they are far more likely to co-deposit in similar regions of the human airways. Thus, an important factor in eliciting disease may be the physical proximity of deposits of these two classes of particles within the airways. The next challenge is to accurately monitor levels of respirable pollen allergens in the atmosphere and determine the potential risk to asthma sufferers.

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