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In vivo biological potency of Fraxinus bee-collected pollen on patients allergic to oleaceae

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ABSTRACT

Oleaceae bee-collected pollen is identified as being potentially at the origin of allergic accidents but the biological potency of Oleaceae bee-collected pollen is not well known.

In this experiment, Fraxinus mass was identified in bee pollen mass and after having done so the proportion of Fraxinus mass using the bee pollen melissopalynology spectrum was calculated. Skin reactivity to Fraxinus was assessed by measuring wheal diameters (W) from skin prick tests using three serial dilutions of bee pollen on 10 patients allergic to Oleaceae pollen, in order to calculate the relationship between Fraxinus mass (Mass fraxinus) in bee pollen and skin reactivity.

The dose–response power regression curve ($W_{fraxinus} = 2.46\ (Mass_{fraxinus})^{0.21}$ $R^2 = 0.99$) and the linear function (Log 10 ($W_{fraxinus}$) = 0.21 (Log 10 [$Mass_{fraxinus}$]) + 0.39 $R = 0.99$) were established using a bee pollen sample with 0.273 mg of Fraxinus pollen per mg.

Fraxinus allergens seem to be little or not altered by bee secretions. Fraxinus bee-collected pollen retains its allergenic capacity.

To the best of our knowledge this is the first time it has been shown that skin reactivity of patients allergic to Oleaceae pollen is proportional to the absolute Fraxinus mass contained in the bee-collected pollen.

Introduction

Pollen is flower sperm. It is the only source of certain macronutrients and is collected by worker honeybees. Collected from floral anthers at the tips of stamens, flower pollen grains stick to bee secretions. They are then assembled by the bee in loads, and placed in the baskets of the hind legs of the insect. Each load has a weight of 5–10 mg [1] and has several hundred thousand grains of a single floral species. Each load requires a visit to at least 80 flowers of the same plant type. The mixture of floral pollen is in the form of pellets and is what is commonly called "bee pollen". It consists of various loads from different plant species.

Bees visit numerous plant flowers. There are, for example, more than 268 species and varieties of plants in England [2]. G. Ricciardelli D'Albore and F. Intoppa have listed all the families of plants in Europe that are visited by bees [3].

Some floral pollen in bee products is responsible for allergies. Anaphylactic accidents related to the use of bee

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products are on the increase. There is substantial literature supporting this observation.

In spring, Oleaceae members represent as pollen suppliers for honeybees at locations where the olive and ash trees are an important pollen source [4].

Oleaceae bee-collected pollen is botanically closely related to common airborne allergenic pollen grains or could cross-react with unrelated allergenic plants. These allergic cross-reactions are caused by proteins sharing important structural homologies with several plant families [5], e.g., Profilin and Polcalcin panallergens from Ash pollen [6].

Oleaceae allergenic proteins appear to retain their allergenic properties in bee pollens from the time they enter the beehive to the harvesting by the beekeeper and their use by the end consumer [7].

To the best of our knowledge, however, there is currently no technical definition of the allergenic potential of Oleaceae in bee-collected pollen. The purpose of this study is to define the biological potency of pollen of Oleaceae in bee-collected pollen *in vivo* by skin prick tests on patients allergic to Oleaceae pollen.

Materials and methods

Analysis of bee pollen spectrum

A pollen analysis of bee products is usually performed in a specialist laboratory by analyzing the beehive products. In our case, we used Honey Expertise Laboratory – Naturalim France Miel, 39330 Port-Lesney, France.

Such an analysis defines the type and frequency of each botanical genus or family floral pollen and determines the total mass of floral pollen. Bee pollen is thoroughly cleaned when intended for human consumption. Cleaning the pollen separates pollen dust balls, bee body fragments, wax or propolis. Melissopalynology is based on the European Maurizo and Louveaux standard without acetolysis, as recommended by the International Commission for Bee Botany [8].

Counting and identifying floral pollen grains are made by examination under a microscope. Identification of pollen types is based on the laboratory pollen reference collection for local plants or guides specialized in the pollen morphology of floral species.

Ten grams of well-homogenized bee-collected pollen was dissolved and washed in distilled water, centrifuged, and then prepared using aqueous glycerine and paraffin for smear preparations. Each smear was studied under a microscope to identify 500 floral pollen grains in order to determine the percentage of each type of flower pollen.

With bee-collected pollen, the floral pollen mass is equated with the bee pollen mass because it is accepted that the bee-collected pollen pellets only contain kneaded floral pollen grains.

Calculation of the floral pollen allergen mass "$Mass_{p\text{-}allergen}$" in bee pollen

1. Calculate the volume "V_{pn}" of each of the 1 to *n* types of floral pollen from the bee pollen spectrum using the formula $V_{pn} = 4/3\pi r^3$ if the pollen grain is spherical or using the formula $V_{pn} = 4/3\pi e^2 l$ if the floral pollen has an ellipsoidal shape.

   The values of the radius *r* and of the mid-equatorial and longitudinal axes *e* and *l* are obtained from the literature from observations made on bee product pollen, including bee pollen (2). It is important to take into account changes in volumes of flower pollen due to orthodox or recalcitrant pollen qualities when pollen grains come into contact with aqueous bee fluids.
2. Calculate the proportion of volume $P_{p\text{-allergen}}$ of flower pollen allergen p-allergen

$$P_{p\text{-allergen}} = \frac{(V_{p\text{-allergen}} \times \%_{p\text{-allergen}})}{((V_{p\text{-allergen}} \times \%_{p\text{-allergen}}) + (V_{p2} \times \%_{p2}) + \cdots + (V_{pn} \times \%_{pn}))}$$

   (%pn is the percentage of flower pollen pn observed in the bee pollen analysis).
3. Calculate the mass of floral pollen allergen $Mass_{p\text{-allergen}}$

$$Mass_{p\text{-allergen}} = P_{p\text{-allergen}} \times Mass_{pollens}$$

Calculation using the equation defining the allergenic potential of flower pollen allergen in bee pollen

Before applying this equation, it is necessary to:
- Use bee pollen with only one floral pollen allergen,
- Calculate the mass of floral pollen allergen as indicated above,
- Use a bee pollen without any floral pollen allergen as a "bee pollen negative control" to eliminate a skin sensitization to bee specific allergens.

1. Preparation of bee pollen extracts:

Samples were prepared with the two types of bee pollen defined above. Five grams of fresh or frozen bee pollen was well homogenized on a glass plate and 450 mg of bee pollen was diluted in 4.5 ml of isotonic 0.4% phenol diluent (dilution weight/volume: 100 mg of bee pollen/ml of diluent) and was homogenized with a stirrer at a maximum speed for one minute. Samples were stored at room temperature for 24 h and homogenized one more time with the stirrer before being removed, then 0.5 ml of the 100 mg/ml diluted solution was diluted in 4.5 ml of isotonic 0.4% phenol diluent to produce a 10 mg/ml diluted solution. These steps were repeated one more time to produce at least three samples of bee pollen, i.e., 100 mg/ml, 10 mg/ml and 1 mg/ml, respectively.

The allergen pollen floral mass contained in per millilitre of each sample was deduced using the mass of floral pollen allergen in the bee pollen. Samples were kept at 5 °C and were used within five days.

2. Measurement of skin reactivity to floral pollen allergen contained in bee pollen:

Skin prick tests were duplicated on the inner side of the forearms of 10 subjects. Patients (three women/seven men) aged between 19 and 33 (mean: 25.3) had been referred for seasonal symptoms (rhino-conjunctivitis and/or asthma) produced in spring. They were recruited in the south of France. They were not hyposensitized and were positive

skin prick tested with a commercially available pollen mixture of three Oleaceae extracts (Ligustrum-Olea-Fraxinus Stallergenes). They were sensitized to Ole e1 by testing for specific IgE-antibodies (>0.53 kui/l). In addition to Oleaceae pollen, they were sensitive to mites [5], cats [3] and cypress [5] but none were sensitive to grasses, birch, salicaceae mixture (willow-poplar Stallergenes), to maple, to oak, to artemisia and to ambrosia and none had a history of bee sting reactions or food allergy.

Informed consent was obtained from each patient.

Skin reactivity was assessed by geometric measuring of the two largest wheal diameters observed twenty minutes after the pollen sample prick tests, positive (histamine 10 mg/ml) and negative (glycerinated saline) controls and commercial extract tests (Stallergenes pollen mixture of three Oleaceae extracts 100 IR/ml).

$W_{p\text{-allergen}}$ was defined by geometric measuring of skin reactivity to floral pollen allergen contained in bee pollen.

3. Analysis of the relationship between skin reactivity to floral pollen allergen in bee pollen $W_{p\text{-allergen}}$ and floral pollen allergen mass $Mass_{p\text{-allergen}}$:

   If the model curve was a power regression:

   $$(W_{p\text{-allergen}}) = b(Mass_{p\text{-allergen}})^a$$

   then the linear function was calculated as follows:

   $$Log_{10}(W_{p\text{-allergen}}) = A(Log_{10}(Mass_{p\text{-allergen}})) + B$$

   where A and B are specific pollen allergen constants.

   Variances analysis was performed by calculating R^2, which records the results of the value dispersions associated with regression. The closer R^2 is to 1, the more the total variance is explained by the linear regression.

Results

Calculation of $Mass_{fraxinus}$ of bee pollen

Our bee pollen has a floral pollen allergen: Fraxinus. It was collected in April 2013 in Contault (France) at GPS location: X 48.91094, Y 4.78943.

Its spectrum includes 30.0% Fraxinus, 31.8% Salix, 13.8% Aceraceae, 8.8% Quercus, 7.9% Prunus, 4.9% Ranunculaceae, 1.9% Brassicaceae (<0.9% undetermined) pollen.

Fraxinus, Salix, Aceraceae, Quercus, Prunus, Ranunculaceae, and Brassicaceae have spherical pollens. Their respective diameters are 23, 18, 25, 25, 35, 26 and 20 μm.

Indeterminate fractions are ignored.

1. Calculation of V_{pn} volumes:
   Fraxinus: $V_{fraxinus} = 4/3\pi\ (23/2)^3 = 6367\ \mu^3$
   Salix: $V_{salix} = 4/3\pi\ (18/2)^3 = 3052\ \mu^3$
   Aceraceae: $V_{aceraceae} = 4/3\pi\ (25/2)^3 = 8177\ \mu^3$
   Quercus: $V_{quercus} = 4/3\pi\ (25/2)^3 = 8177\ \mu^3$
   Prunus: $V_{prunus} = 4/3\pi\ (35/2)^3 = 22438\ \mu^3$
   Ranunculaceae: $V_{ranunculaceae} = 4/3\pi\ (26/2)^3 = 9198\ \mu^3$
   Brassicaceae: $V_{brassicaceae} = 4/3\pi\ (20/2)^3 = 4187\ \mu^3$
2. Calculation of $P_{fraxinus}$ proportion:
   $P_{fraxinus} = (V_{fraxinus} \times \%_{fraxinus})/((V_{fraxinus} \times \%_{fraxinus}) + (V_{salix} \times \%_{salix}) + (V_{aceraceae} \times \%_{aceraceae}) + (V_{quercus} \times \%_{quercus}) + (V_{prunus} \times \%_{prunus}) + (V_{ranunculaceae} \times \%_{ranunculaceae}) + (V_{brassicaceae} \times \%_{brassicaceae})) = (6337 \times 30.0\%)/((6337 \times 30.0\%) + (3052 \times 31.8\%) + (8177 \times 13.8\%) + (8177 \times 8.8\%) + (22438 \times 7.9\%) + (9198 \times 4.9\%) + (4187 \times 1.9\%)) = 191\,021/700\,725 = 0.273$
3. Calculation of $Mass_{fraxinus}$:
   $Mass_{fraxinus} = P_{fraxinus} \times Mass_{pollens} = 0.273 \times 1\ mg = 0.273\ mg$
   There was 0.273 mg of Fraxinus pollen per mg of bee pollen.

Calculation of $Mass_{hedera\ helix}$ of bee pollen

Our bee pollen is a pure, unique, floral pollen, Hedera Helix (99%; indeterminate percentage <0.9%). It was collected in September 2013 in Thezillieu (France) at GPS location: X 45.8833, Y 5.6.

This is a spherical pollen with a diameter of 25 μm.

1. Calculation of volume $V_{pnHedera\ helix}$:
   $V_{hedera\ helix} = 4/3\pi\ (25/2)^3 = 8177\ \mu^3$
2. Calculation of proportion $P_{hedera\ helix}$:
   $P_{hedera\ helix} = (V_{hedera\ helix} \times \%_{hedera\ helix})/(V_{hedera\ helix} \times \%_{hedera\ helix}) = (8177 \times 99\%)/(8177 \times 99\%) = 1$
3. Calculation of $Mass_{hedera\ helix}$:
   $Mass_{hedera\ helix} = P_{hedera\ helix} \times Mass_{pollens} = 1 \times 1\ mg = 1\ mg$
   There was 1 mg of Hedera helix pollen per mg of bee pollen.

Measurements of skin reactivity to Fraxinus and Hedera helix pollen and analysis of the relationship between "$W_{p\text{-allergen}}$" and "$Mass_{p\text{-allergen}}$"

Skin prick test results with three 10-fold dilutions of bee pollen with 0.273 mg of Fraxinus pollen per milligram and with 1 mg of Hedera helix pollen per milligram are shown in Table I.

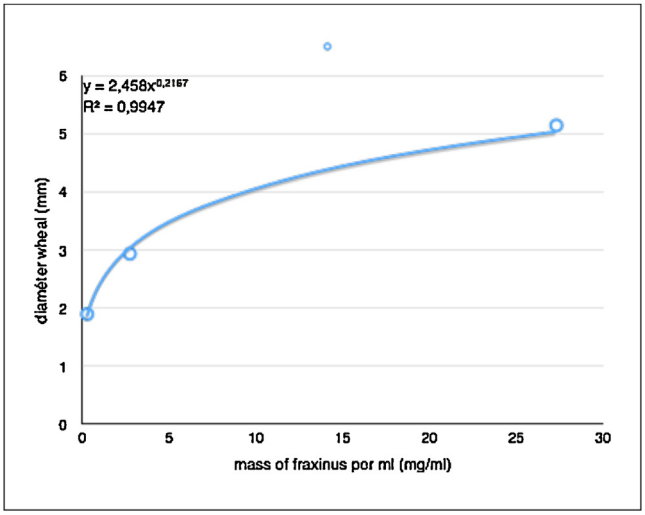

Fig. 1 – Dose–response curve power regression between $W_{fraxinus}$ and $Mass_{fraxinus}$

Table I – Skin prick test results with three 10-fold dilutions of bee pollen with 0.246 mg of Fraxinus pollen per milligram or with 1 mg of Hedera Helix pollen per milligram

Patient	Fraxinus[a]				Control[a]		Hedera helix[b]		
	Fraxinus 27.3 mg/ml	Fraxinus 2.73 mg/ml	Fraxinus 0.27 mg/ml	Oleaceae commercial extract	Positive control	Negative control	Hedera helix 100 mg/ml	Hedera helix 10 mg/ml	Hedera helix 1 mg/ml
P1	5	4	3.97	14.49	8.48	0	0	0	0
P2	4.90	2.45	2	8	3.46	0	0	0	0
P3	4.47	4	1.73	8.94	6.48	0	0	0	0.5
P4	3.87	2.45	1.73	7.93	6	0	0	0	0
P5	3.87	1.73	1	3.87	5.47	0	0	0	0.5
P6	4.90	3	1.73	9.16	8.48	0	0	0	0
P7	4.90	1.73	1	5.91	4	0	0.5	0	0
P8	5	2	1.73	7	6.32	0	0	0	0
P9	9.49	6.92	5.91	10.39	6.70	0	0.5	0.5	0.5
P10	6.92	3.87	1.41	11.83	5.47	0	0	0	0
Mean wheal	5.14	2.93	1.89	8.26	5.87	0	0.10	0.05	0.15

[a] Geometric mean wheal (mm).
[b] Mean wheal (mm).

1. Skin reactivity and Fraxinus:

The model dose–response curve of Fraxinus bee pollen is a power regression:

$$W_{\text{fraxinus}} = 2.46(\text{Mass}_{\text{fraxinus}})^{0.21}, \quad R^2 = 0.99$$

The dose–response curve power regression is shown in Fig. 1 and the linear function is:

$$\text{Log}\,10(W_{\text{fraxinus}}) = 0.21(\text{Log}\,10(\text{Mass}_{\text{fraxinus}})) + 0.39, \quad R = 0.99$$

The dose–response curve linear function is shown in Fig. 2.

2. Skin reactivity and Hedera helix:

The model dose–response curve of Hedera helix bee pollen is not a power regression:

$$W_{\text{hedera helix}} = 0.11(\text{Mass}_{\text{hedera helix}})^{-0.08}, \quad R^2 = 0.13$$

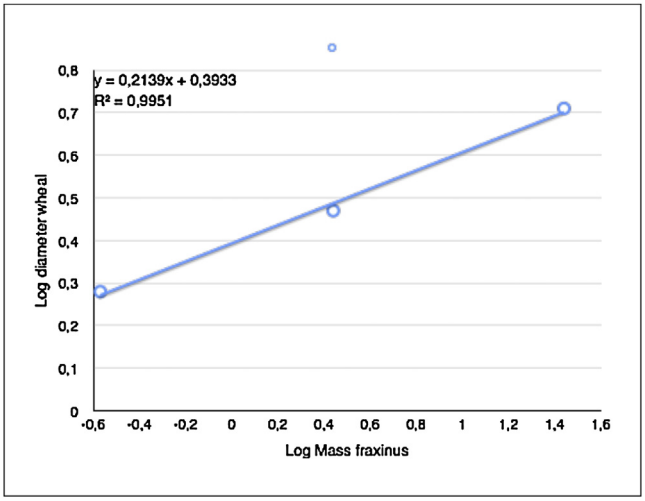

Fig. 2 – Dose–response curve linear regression between Log W_{fraxinus} and Log $\text{Mass}_{\text{fraxinus}}$

Discussion

Olaeceae members are plant species that provide bees with pollen but not nectar. Patients sensitized to Oleaceae pollen who ingested bee products may be at risk to experience an immediate allergic reaction because of cross-reaction between Oleaceae bee product pollen and airborne Oleaceae pollen [7].

However, patients who are allergic to bee products (honey, royal jelly, bee pollen) may be also sensitized to honeybee secretion proteins, pollen proteins contained in bee products [9] or bee venom components [10]. This is why we tested our patients with bee pollen not containing airborne pollen allergens, which was used as a bee pollen negative control. This was to eliminate skin sensitization to allergens other than Oleaceae allergens (i.e., bee specific component allergens). Our bee pollen negative control was 100% Hereda helix bee pollen. Hedera helix pollen is not a common allergic pollen. In some rare cases, it might be responsible for cross-reaction to pollen panallergens among Mexican allergic patients with dermatitis [11].

None of our patients had positive skin prick reactions to Hedera helix bee pollen. No relationship was established between Hedera helix bee pollen and skin reactivity.

A honeybee collects pollen grains at maturity from the male organs of flowers in order to obtain certain proteins or lipids. It gathers using an elaborate strategy based on pollen research of the highest quality for optimal protein and nutrient collection. It takes advantage of the plant fertilization mechanisms in order to attain its objective, which is why the bee is not interested in wet pollen. As with floral nectar, wet pollen swells on contact with the secretions of sugar-water pollen grains that then release the soluble nutrient content. Based on comparisons between hand- and bee-collected pollens, it appears that half or more of the mass of bee-collected pollens can be attributed to the addition of nectar-derived sugars to the pollen [12]. The

protein content of the grain decreases and this causes a leakage of the proteins in the external environment [13].

It seems that our patients are sensitized exclusively to the Fraxinus allergens contained in bee pollen sample

Fraxinus, Salix, Aceraceae, Quercus, Prunus, Ranunculaceae and Brassicaceae pollens were contained in Fraxinus bee pollen sample. They are common in bee pollens [3]. Literature searches in Medline were performed and no paper has described these pollens, except perhaps Willow at one time [14], as being allergic pollens when they are included in bee pollens.

This fact should be compared with what we know of the allergen qualities of these pollens:

- Salix caprea provides bees with a high quantity of pollen. Its genus, Salix, belongs along with the genus Populus to the Salicaceae family. This family contains sensitizing allergens but none have been yet well characterised. The prevalence of sensitization of Salicaceae pollen is moderate, 8% among American atopic patients [15]. Cross-reactivities occur between members of Salicaceae [16] and, to a lesser extent, between Fagales and Salicaceae and between Salix and Saliaceae [17]. One case of anaphylaxis induced by bee pollen was reported [14] with bee pollen which contained pollens of dandelion, mugwort and willow. Asteraceae pollen might cause this allergic reaction because mugwort pollen and, more generally, the Asteraceae family, are implicated in the origin of such accidents [10]. Our patients were not sensitized to Salicaceae, to Fagales (e.g., birch and oak) and to Artemisia pollen.
- Aceraceae allergy is not well known and to our knowledge no allergenic protein has been described. But allergic symptoms and positive skin sensitization have been shown where maple is ubiquitous and produces a lot of pollen, especially in a urban area polluted with SO_2 and NO_2 [18]. Never the local trap of the French aerobiology network has shown maple pollen and our patients were not sensitized to maple.
- Quercus pollen is one of the major airbone pollen recorded in traps of the French aerobiology network in the south of France. It is the first in 2006 and 2007 in Salamanca in western Spain [19]. But the prevalence of sensitization of Quercus pollen is very moderate, 11% among Japanese atopic patients [20]. Allergenicity of oak pollen is highly variable and depends of the Quercus genus. Thus, the prevalence of holm oak pollen allergy is null in Merida in western Spain [21] but Que a1 could cross-react with other group 1 Fagales proteins, e.g., Bet v 1 [22]. In this experience no patient was sensitized to oak and to birch.
- Prunus allergy is related to fruit varieties contained in the *Prunus* genus and is well known in food allergy and pollen-food syndrome because of cross-sensitization to panallergens (e.g., profilin, polcalcin, lipid transfer protein and 1,3-betaglucane) from other common pollens [5]. No patient was sensitive to grasses, birch, artemisia and to ambrosia and none had a history of food allergy.
- Ranunculaceae are ornamental plants. Sensitization to ornamental plant pollens could be frequent in the general atopic population but never public patients exposed to Ranunculaceae pollen have increased allergic symptoms [23]. In contrast, 45% of flower growers described allergic symptoms with occupational exposure [23] but in this experience none patient was flower grower.
- Brassicaceae pollen allergens are well known in cabbage, oilseed rape or mustard (e.g., profilin, calcium-binding protein, lipid transfer protein). They might be responsible for cross-reactivity between foods and pollens [24, 25]. The prevalence of sensitization of rapeseed pollen is correlated to exposure level and is higher (11.8%) among French atopic patients [26].

In contrast, the prevalence of oilseed rape pollen allergy is very low (between 0.2% and 2%) in the United Kingdom, even in areas of high production [27, 28], and the symptoms may be due to both allergens and irritant potentials of oilseed rape [29]. In addition, no patient had a history of food allergy and there was no rapeseed or mustard cultivation in the area.

Furthermore, there is no Salix, Aceraceae, Ranunculaceae or Brassicaceae pollen in the analysis of the contents of the pollen traps of the French aerobiology network in the area neighbouring to Hyères.

It seems that our bee pollen samples with Fraxinus contains Oleaceae (Fraxinus) protein allergens

In the literature, a strong correlation has been noted between cutaneous reactivity to bee pollen containing Oleaceae pollen and the cutaneous reactivity in patients with a positive skin prick test to a Oleaceae commercial extract [7]. Pitsios et al. found that approximately 65% of patients were sensitized to both bee pollen and floral Oleaceae pollen. They considered that it might be due to Oleaceae pollen in their samples, which contained 20 mg of bee pollen per ml of solution.

This correlation was observed in their five bee pollen samples but only three melissopalynology analyses of bee pollen samples have shown major Oleaceae pollen. Furthermore, one of the strongest correlations has been shown with the poorest Oleaceae bee pollen sample (less than 4.6%). This might be due to the qualitative and quantitative methods used to analyse bee pollen. Only five spherules of different tinges were chosen from each bee pollen sample. Tinge loads are subjective. Colours change with time, if the loads are dry or are exposed to sunlight [30]. Many plant species have pollen loads with very similar colours and sometimes up to three colours are observed for a single genus [2]. Oleaceae pollen is often a minor bee pollen and choosing five pellets can raise the risk of non-homogenized samples.

On the contrary, our bee pollen was analyzed using the standard European melissopalynology method recommended by the International Commission for Bee Botany [8]. This method is based on the study of 10 g of well-homogenized bee pollen and 10 g composed of more than 1000 pellets.

Our bee pollen sample is rich in Fraxinus pollen, with 30.0% and 0.273 mg of Fraxinus pollen per mg. Quantifying the absolute mass of Fraxinus pollen with bee pollen per gram requires knowing the pollen spectrum of bee pollen

and measuring pollen grain sizes. More particularly, this requires knowing pollen sizes when in contact with aqueous fluids. In contact with water, the pollen grain is in osmotic shock. Hydrated grain results in a change of its volume and opens pores and fissures [13] depending on the recalcitrance and orthodoxy of the pollen. Fraxinus pollen is orthodox and swells in contact with water [31].

Strong relationships were established between the absolute mass of the Fraxinus pollen in bee pollen and skin reactivity despite our patient group including a small number of individuals sensitized to pollen mixture of three Oleaceae extracst (Ligustrum-Olea-Fraxinus) and to Ole e1.

The dose–response curve was a power regression curve:

$$W_{\text{fraxinus}} = 2.45(\text{Mass}_{\text{fraxinus}})^{0.21}, \quad R^2 = 0.99$$

from which we were able to deduce the linear curve:

$$Log\,10(W_{fraxinus}) = 0.21(\text{Log}\,10[\text{Mass}_{\text{fraxinus}}]) + 0.39, \quad R = 0.99$$

Fraxinus allergens in bee pollen appear to be little or not altered by bee secretions and the allergens retain their allergenic capacity. In fact, the bee secretions contain digestive enzyme sugars [32] but are devoid of proteases. There is no protein digestion, as salivary and hypopharyngeal glands do not produce proteolytic enzymes [33].

Conclusion

To the best of our knowledge this is the first time it has been shown that the skin reactivity of patients who are allergic to Oleaceae pollen is proportional to the absolute Fraxinus mass contained in bee pollen. Further studies are needed to determine how Fraxinus allergens retain their allergenic qualities.

Authors' contributions/Wkład autorów

According to order.

Conflict of interest/Konflikt interesu

None declared.

Financial support/Finansowanie

None declared.

Ethics/Etyka

The work described in this article have been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform Requirements for manuscripts submitted to Biomedical journals.

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