

Antigenicity of the pollen proteins of various cultivars of *Olea europaea*

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The incidence of positive skin test responses among atopic subjects with suspected respiratory allergy was investigated with commercial and autochthonous pollen extracts of various cultivars of *Olea europaea*. Pollen was collected from olive trees of well-defined cultivars, extracted, and separated by SDS–PAGE. Immunoblots were used to identify the various IgE-binding proteins of the pollen extracts of the various cultivars. The results revealed six predominant IgE-binding bands, some of which appear in all the cultivars examined. The 18–20-kDa band (Ole e 1) appeared in only eight of the cultivars, but not in the nine others. The presence of specific IgE-binding bands in the various pollen extracts and their correlation with the incidence of positive skin tests are discussed.

**Y. Waisel¹, C. Geller-Bernstein²,
N. Keynan¹, G. Arad¹**

¹Department of Botany, Tel-Aviv University, Tel-Aviv; ²Kupat Holim, Allergy Clinic, Tel-Aviv, Israel

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Prof. Yoav Waisel
Department of Botany
Tel-Aviv University
Tel-Aviv 69978
Israel

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The importance of olive pollen in provoking respiratory allergy is well established and documented (1–12). Several specific IgE-binding proteins have been identified in olive pollen, two of them, Ole e 1 and Ole e 2, being identified as the major olive allergens. However, in most of the reported cases, identification of the allergens was based on the analysis of commercially available pollen of undisclosed sources. Only very few attempts have been made to investigate the allergenic constituents of the pollen of various cultivars, or to distinguish between their effects on atopic patients (5, 11, 13). In view of the well-known diversity of olive trees, we investigated variations in olive pollen proteins of several cultivars, and deliberated on their differential capability to bind specific IgE.

Material and methods

Pollen collection and preparation

Pollen grains of *Olea europaea* were collected in the arboretum of the Volcani Institute, Israel, from the following cultivars: Barnea, Manzanillo, Muhassan, Merhavia, Sourì, Uovo di Piccione, Kalamata, Kadesh, Koronaiki, one hybrid of Kalamata, and one hybrid of Barnea. The pollen of the cultivars Bosana, Nera di Gonnos, Palma, Pizz'e Carroga, and Tonda di Cagliari, as well as that of *O. oleaster*,

was collected in Sardinia. Pollen was collected by shaking the branches of fully flowering trees into paper bags. The discharged grains were dried at 40°C and then sifted through a silk cloth (Swiss Silk Bolting Cloth Manufacturing Co., catalog no. T-100). The sifted grains were checked microscopically for purity, and then stored dry, in hermetically sealed containers, at –20°C.

Pollen extraction for immunochemical studies

Pollen grains were ground in a ceramic mortar in a buffer solution ("sample buffer" of Laemmli, [15]) of 10% glycerol, 5% 2-mercaptoethanol, 3% sodium dodecyl sulfate (SDS), Tris-HCl buffer at pH 6.8, and 2% phenyl methane sulfonylfluoride (PMSF) (16). After 5 min of grinding, extracts were boiled for 1 min to prevent subsequent changes in the proteins, and centrifuged at 7600 g for 30 min. The supernatant was then put through a filter paper with 0.45-µm pores. Acetone was added to the obtained fluid (×5 v/v) to precipitate the proteins.

The solution was stored at –4°C overnight and recentrifuged the next day. The precipitated proteins were then dissolved in the sample buffer, and stored at –20°C until used. The protein concentration of the extracts was estimated by the spot test (17) with bovine serum albumin (BSA) solution as standard. Proteins were separated by SDS–PAGE,

a 12.5% polyacrylamide gel being used, and 30 µg proteins being loaded into each well. The proteins were detected by Coomassie brilliant blue staining. The molecular weight of the extracted proteins was calculated by comparison with known markers (Sigma SDS-6H and SDS-7). The separated proteins were electroblotted onto nitrocellulose (NC) membranes (18), with the modification of Szewczyk & Kozloff (19). After blotting, the NC membranes were stained with Ponceau red (1% in 5% acetic acid) and freeze-dried. Nonspecific receptors were blocked by coating the NC sheets with 3% BSA with 0.5% Tween in phosphate-buffered saline. The IgE-binding fractions in the extracts were detected by enzyme-linked immunoassay (EIA).

The blotted NC membranes were incubated overnight with human serum (dilution of 1:20) at room temperature. The membranes were then washed in saline Tween and incubated for 2.5 h in monoclonal mouse antibodies to human IgE (MαHIgE, Biomakor, Israel), washed, and incubated with HRP-GαMIg (Jackson, West Grove, PA, USA). Unbound antibodies were washed away. The blots were immersed in an enhanced chemiluminescence solution (Amersham, UK). The specific IgE-binding bands were identified after exposure to an AGFA Curix X-Ray film for 90 s.

Pollen extraction for skin tests

Autochthonous pollen extracts were prepared as follows. Sifted pollen grains were sonicated in an ice-cold, sterile, saline glycerol buffer solution at pH 8.2 (20), containing 2.8% NaHCO₂, 0.5% NaCl, and 50% glycerol; i.e., the standard solution in which commercial allergen extracts were supplied (e.g., Center Laboratories). The solution was then centrifuged for 30 min at 7600 g to allow precipitation of the pollen-grain solid remains. The supernatant was then put through a filter membrane with 45-µm diameter pores. The protein content of the obtained extract was determined by the Lowry procedure (21), with BSA as a standard. After the protein content was determined, each of the autochthonous extracts was diluted to a standard concentration of 5 mg protein/ml solution (5, 22). The extracts were not dialyzed before use. These allergen extracts were then stored frozen.

Patients

The patients investigated were mostly suspected of respiratory pollen allergy by their general practitioners. They were skin prick tested (SPT) with a panel of airborne allergens common in Israel. The tests included the commercial extracts of *O. europaea* (Center Laboratories, USA), as well

as autochthonous extracts, each prepared from pollen of one of the 17 pollen sources (Table 1). A 1 mg/ml histamine solution was used as a positive control and buffered saline glycerin as a negative one. The following scale was used to define the degrees of response: 0: none; 1: wheal diameter of 2–4 mm; 2: wheal of 5–7 mm; 3: wheal 8–10 mm; and 4: wheal diameter larger than 10 mm with developed pseudopodia (23). Venous blood samples were taken from some of the patients, and the sera of six of them were used for the immunoblots. The control subjects were either atopic patients who showed no positive SPT response to olive, or healthy volunteers.

Results

Comparison of pollen proteins of the tested extracts

The electrophoretically separated pollen proteins of the various cultivars of *O. europaea* and of *O. oleaster* revealed a multitude of similar bands (Fig. 1). However, one outstanding difference was found; i.e., in the content of the 18–20-kDa protein cluster. This group appeared in substantial amounts in the commercial extract of *O. europaea* and in the extracts of Muhassan, Souri, Kadesh, Kalamata, Nera di Gonnos, Palma, Bosana, and Pizz'e Carroga. The 18–20-kDa band was undetectable in the pollen extracts of Barnea, Manzanillo, Uovo de Piccione, Koronaiki, Merhavia, Barnea hybrid, Kalamata hybrid, Tonda di Cagliari, and *O. oleaster*. The Kalamata extract showed a unique pattern, with intensive bands in the 17–21-kDa range and in the 35-kDa and 45-kDa bands.

Comparison of the IgE-binding proteins of the tested extracts

Enzyme-linked immunoblots were made with sera of eight patients: six that had positive SPT to olive pollen; one of a subject who was allergic, but not to olive; and one of a healthy, nonallergic volunteer. The sera of the olive-sensitized patients showed individual patterns with specific IgE binding to several protein bands in the range 8–116 kDa. Fig. 2 shows an example of the difference in response in the serum of one subject to the various olive sources. The 18–20-kDa band of the commercial extract showed high affinity to this serum. The intensity of binding by the autochthonous extracts was strong in the following order: Souri, Kadesh, Manzanillo, Kalamata hybrid, Bosana, Merhavia, and Palma. The extracts of Muhassan and Pizz'e Carroga showed very little binding to this band. No apparent IgE binding to the 18–20-kDa band region was detected for the extracts of Barnea, Uovo di

Table 1. Specific IgE-binding protein bands of various olive allergens, probed with sera of six patients with positive SPT to olive. Percentage of sera with substantial binding

Name of cultivar	Protein band (kDa)						Positive SPT	
	18–20	24–26	32–35	44	47	64–66	Intensity*	%**
Group I								
<i>Olea europaea</i>								
(commercial)	100	33.3	66.7	50	100	33.3	2.6	39%
Kalamata (hybrid)	50	50	83.3	50	83.3	50	1.6	39%
Kadesh	100	66.7	50	50	66.7	83.3	2.1	35%
Souri	83.3	33.3	100	66.7	83.3	83.3	1.9	27%
Bosana	50	16.7	50	50	100	66.7	1.2	26%
Manzanillo	50	66.7	66.7	50	50	83.3	1.7	26%
Merhavia	100	66.7	100	50	50	50	2.0	25%
Kalamata	83.3	33.3	16.7	16.7	50	50	1.6	25%
Uovo di Piccione	33.3	33.3	83.3	50	66.7	83.3	1.4	22%
Palma	33.3	0	33.3	50	100	50	1.3	18%
Group II								
Muhassan	?	16.7	83.3	50	83.3	83.3	1.4	31%
Pizz'e Carroga	?	16.7	50	50	100	66.7	–	–
Barnea	ND	66.7	50	33.3	66.7	83.3	1.8	29%
Koronaiki	ND	33.3	83.3	33.3	50	50	1.2	23%
Barnea (hybrid)	ND	16.7	66.7	50	83.3	50	2.0	16%
Nera di Gonnos	ND	16.7	83.3	50	100	66.7	–	–
Tonda di Cagliari	ND	100	33.3	50	100	33.3	–	–
<i>O. oleaster</i>	ND	16.7	16.7	50	83.3	50	–	–

– Not tested; ND: not detected; * mean intensity of SPT; ** % positive SPT in studied population (for details, see Ref. 5).

Piccione, Koronaiki, Barnea hybrid, Nera di Gonnos, Tonda di Cagliari, and *O. oleaster*. Based on such results, two distinct groups of cultivars were compiled: one characterized by potent IgE binding to the 18–20-kDa band, and the other lacking it (Table 1).

The correlation between the quantity of the protein in a band and its IgE-binding capability was not always clear. For example, five intensive protein bands of the Kalamata extract were detected by Coomassie brilliant blue staining (Fig. 1). Nevertheless, only weak binding of IgE to three bands was found in the immunoblots of this extract (Fig. 2).

Some weak IgE binding to the 47- and 66-kDa protein bands was observed for sera of the healthy and of the non-olive-sensitive subjects. This was probably nonspecific and was regarded as beyond the scope of the present contribution.

Six major protein groups with specific IgE-binding capability have appeared in the various sources, though in combinations that varied in various cultivars (Table 1).

The intercorrelation between the incidence of positive SPT and IgE binding to the six principal bands, as revealed by the immunoblots, is presented in Fig. 3. Apparently, two clusters could be identified with a higher IgE binding by the antigens of the first group of cultivars. The extracts of Kalamata and Palma showed odd behavior.

Sensitization to different pollen extracts

Sensitivity of patients to olive pollen was also assessed by SPT (Table 1). Significant differences in incidence and in intensity of the responses were reflected, indicating differences in sensitivity of the subjects to pollen extracts of the various cultivars (5).

Discussion

Olive pollen is an important inducer of clinical hay fever, as assessed by the seasonal symptoms, by skin prick tests, and by provocation tests (6, 10, 24). Nevertheless, as thousands of cultivars of *O. europaea* exist, we wished to know whether the variations in their ecology and in their fruit characteristics are paralleled by their pollen allergenicity. Evidently, our data show differences in SPT responses to various sources of olive pollen, as well as differences in the manifestation of various antigen bands in the immunoblots.

Pollen-grain extracts contain scores of proteins. Why was the number of IgE-binding protein bands so different (Table 2) in different publications? Several reasons may account for such differences. One possible explanation is the use of the two extraction methods. The addition of the proteinase inhibitor PMSF (16) throughout our process of

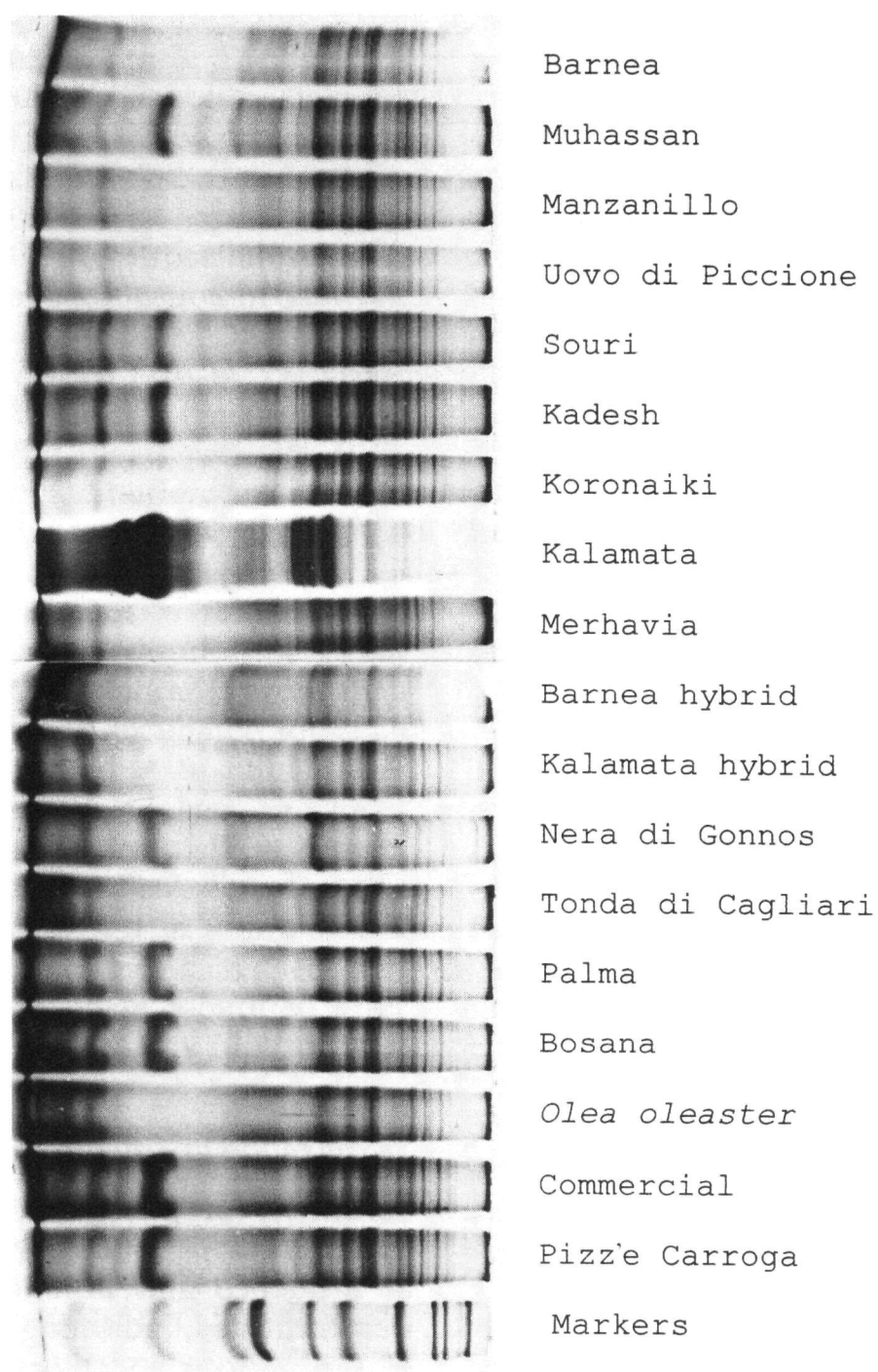


Fig. 1. SDS-PAGE of pollen extracts of various cultivars of *Olea europaea*, of *O. oleaster*, and of commercial olive extract (Center Laboratories, USA) (Coomassie brilliant blue staining).

extraction for the immunoblot analysis may have prevented protein decomposition of the extracts and, thus, preserved the multitude of pollen proteins of our extracts. A second reason could rest in the differences in the sources of pollen that were used by various investigators. Such a possibility is strongly emphasized by the data of the present investigation.

Which of the various proteins of the pollen elicits the allergic response? This is an enigma (25). However, the 18–20-kDa protein cluster has been frequently reported in the literature as constituting the major allergen of olive pollen (26, 27). No doubt, it does. However, in view of the intraspecific variability among plants in general (22, 28), and of the genus *Olea* in particular (29–31), the chance that Ole e 1 is the only major allergen of *Olea* is slight. Indeed, during the last few years, additional

antigens have been identified in olive-pollen extracts (9, 32, 33) (Table 2). Some of those antigens are recognized by antibodies against Ole e 1, whereas others are not. Wheeler et al. (24) have reported that the sera of most of their patients react with the protein bands of the 17–21-kDa cluster (Ole e 1). Nevertheless, reactions were recorded also with 8- (Ole e 2), 41-, and 50–60-kDa allergens (dimers and trimers of Ole e 1?).

Our data confirm those findings. However, they shed additional light on the intraspecific heterogeneity of the genus *Olea*, as expressed by the differences in IgE-binding bands and by the differences in responses of the patients to different pollen sources.

Apparently, the commercial olive extract has a massive content of antigens, and binds specific IgE of most of the patients (33). Indeed, as shown

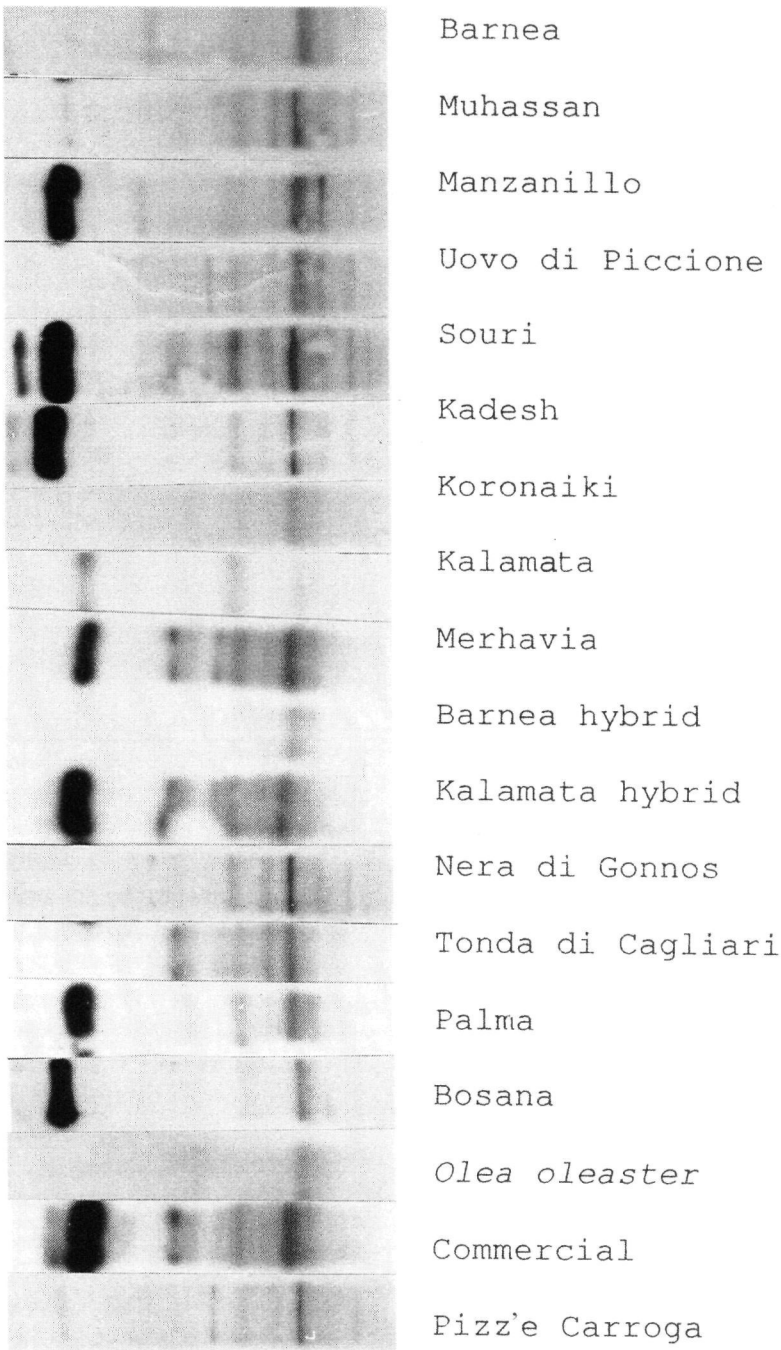


Fig. 2. Immunoblots of various antigens of 18 olive cultivars (same as in Fig. 1), probed by serum of one olive-sensitive patient.

Table 2. Significant antiolive IgE-binding bands of olive pollen extracts, as reported in literature

Source	IgE-binding bands (kDa)											
	8	10-15	17	19-21	30-32	38-39	40-42	44	47	50-60	64-66	> 68
1) Vela et al. (37)											+	
2) Blanca et al. (38)			+	+	+							
3) Lahoz et al. (39)				+			+			+		
4) Rubio et al. (40)			+	+	+							
5) Lauzurica et al. (26, 27)	+		+	+			+			+		
6) Barber et al. (32)			+	+	+	+				+		
7) Wheeler et al. (24, 35)			+	+			+			+	+	
8) Villalba et al. (41)			+	+	+		+					
9) Spinozzi et al. (42)			+	+	+							
10) Baldo et al. (29)	+	+		+		+	+	+		+	+	+
11) Lombardero et al. (43)				+								
12) Obispo et al. (8)				+							+	
13) Rodriguez et al. (9)	+	+		+		+						
14) Lahoz et al. (6)		+	+	+	+	+		+	+	+	+	
15) Martin-Orozco et al. (44)			+	+						+		
16) Present investigation		+	+	+	+			+	+	+	+	

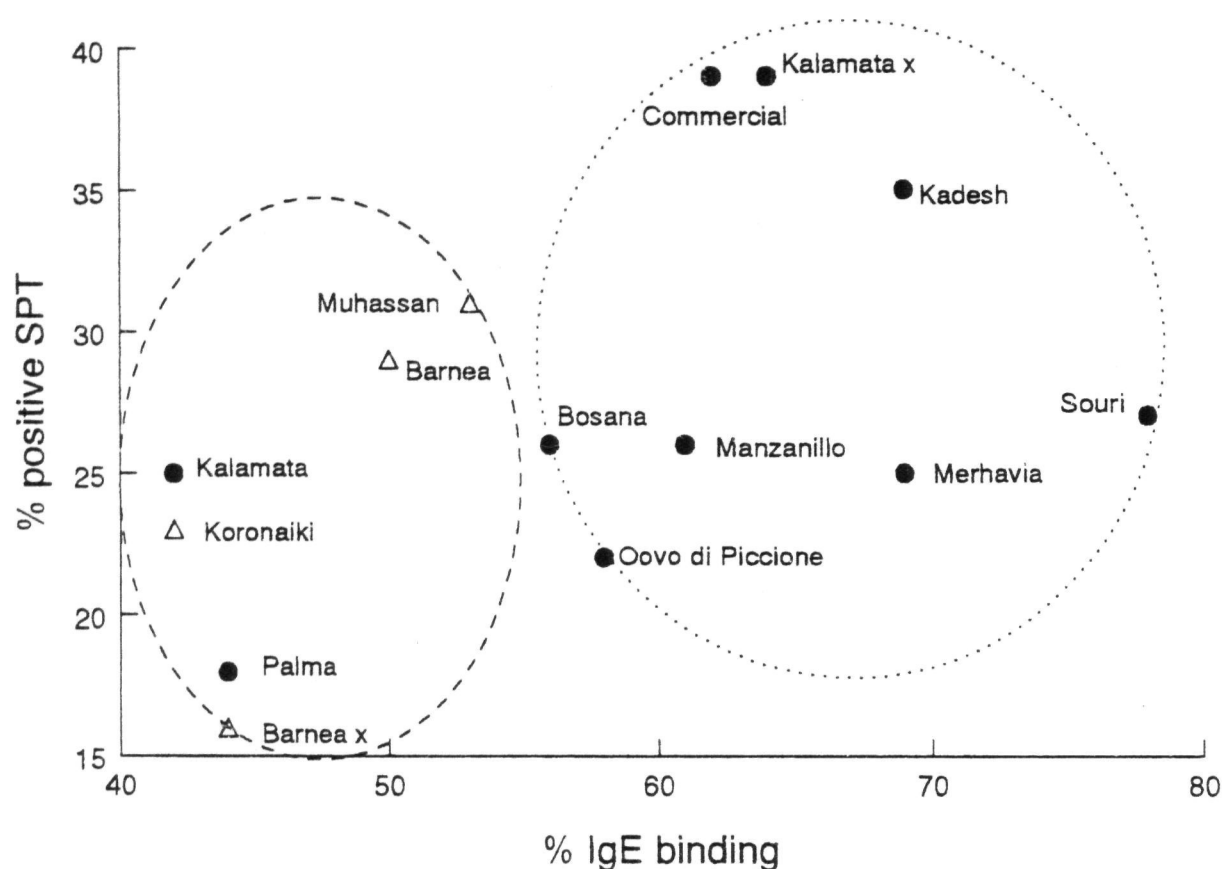


Fig. 3. Intercorrelation between percentage of positive SPT (Ref. 5) and IgE binding to six principal bands of immunoblots by six sera used (average percentage). Closed circles: cultivars with Ole e 1; triangles: cultivars without Ole e 1. Data from Table 1.

before by the matrix TSP analysis (34), this extract gave a good representation of the eight olive cultivars which we have investigated before. However, Ole e 1 is only one factor in the elicitation of olive allergy. Though many of the sera of the tested patients responded to Ole e 1 (35), others did not. As demonstrated by the immunoblots of the present investigation, the latter have responded intensely to other antigens of *Olea*. Thus, the question posed by D'Amato & Liccardi (36) of whether all these varieties of olive tree can produce different allergens received this answer in the present investigation: Yes, their products differ. The questions of which antigens are of clinical relevance in olive allergy, and which cultivar massively presents such antigens need further clarification.

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