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Analysis of the pollen allergen content of twelve olive cultivars grown in Portugal

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Abstract Olive pollen presents intercultivar variability as regards to its antigenic and allergenic composition. In this study, we report the presence of differences among the SDS-PAGE pollen protein profiles of twelve Portuguese olive cultivars. Though most soluble proteins from these extracts seemed similar, three bands of about 18, 20 and 22 kDa presented sharp differences in intensity among the cultivars analyzed. The dissimilarity of patient's sera reactivity to these protein extracts and the presence of several allergens already characterized (Ole e 1, Ole e 2, Ole e 5 and Ole e 9) in the extracts were also investigated. Epidemiological data indicated that 53.3 % out of the 428 patients analyzed with reactivity to pollen extracts, presented specific IgE levels to *Olea europaea*. A representative number of these sera were assayed in immunoblotting experiments. The cultivars 'Galega' and 'Conserva de Elvas' displayed low reactivity to the sera of atopic patients, whereas the extracts corresponding to the cultivars 'Cobrançosa', 'Ascolana' and 'Verdeal de Serpa' led to higher IgE reactivity. The use of antibodies to the allergens Ole e 1, Ole e 2, Ole e 5 and Ole e 9 in immunoblotting experiments also allowed cultivar discrimination. The cultivar 'Verdeal de Serpa' presented the highest Ole e 1, Ole e 5 and Ole e 9 allergen loads but the lowest Ole e 2. 'Carrasquenha' was the second cultivar in terms of the higher allergen content. Oppositely, the lowest allergen loads were those of the cultivars 'Galega' and 'Conserva de Elvas' coincidentally with their low IgE reactivity. These data may help interpret physiological differences in pollen performance for successful olive fertilization and, moreover, to better define future strategies for allergy diagnosis and treatment by specific immunotherapy.

Keywords Allergy · Immunoblotting · *Olea europaea* L. · Ole e

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1 Introduction

Pollen allergens are among the most frequent natural inducers of allergy symptoms. The increase in the area dedicated to olive groves and the use of this tree for ornamental purposes among the Mediterranean countries have led to an escalation of airborne pollen concentration during olive flowering season. This fact potentiates the occurrence of olive pollen–related allergic respiratory diseases.

The olive pollen allergenic profile is complex since it has approximately 40–50 proteins with allergenic activity (Rodriguez et al. 2002). These proteins present high variability and complexity when recognized by IgE antibodies from different serum samples from olive allergic patients (Rodriguez et al. 2002; Esteve et al. 2012).

To date, twelve allergens have been identified, isolated and characterized in olive pollen extracts, being termed Ole e 1 to Ole e 12 (Esteve et al. 2012; Castro et al. 2008). Ole e 1, Ole e 2 and Ole e 10 are considered prevalent allergens of olive pollen. Ole e 1 is frequently described as a protein exhibiting glycosylated (20 kDa) and non-glycosylated (18.5 kDa) forms, being the first one the most abundant (Esteve et al. 2012). However, other bands corresponding to different glycosylation grades of this protein have been reported, such as those of 22 kDa (hyperglycosylated form) and 40 kDa (aggregate produced from the glycosylated and non-glycosylated forms during sample treatment or storing) (Wheeler et al. 1990; Esteve et al. 2012). Ole e 1 is involved in pollen hydration and germination (Alché et al. 2004). Ole e 2 belongs to the profilin family, and in olive pollen extracts, it has been reported at least nine isoforms with molecular masses ranging between 13 and 18 kDa (Ledesma et al. 1998a; Morales et al. 2008). It is involved in the control of the actin cytoskeleton organization and signal transduction (Staiger et al. 1994). Ole e 10 has a molecular weight of 10.8 kDa and is involved in cross-reactivity phenomena and related to asthma induction in allergic patients (Barral et al. 2005).

Ole e 3 and Ole e 8 are polcalcins belonging to the buffering-type Ca^{2+}-binding protein subfamily (Esteve et al. 2012; Ledesma et al. 1998B; Batanero et al. 1996). Ole e 3 has a molecular mass of 9.2 kDa (Batanero et al. 1996), while Ole e 8, a single polypeptide chain of 171 amino acid residues, presents an apparent molecular mass of 20 kDa by SDS-PAGE (Ledesma et al. 2000). Ole e 4 displays an apparent molecular mass of 32 kDa presenting two isoforms, and Ole e 5 has a molecular weight of 16 kDa, with at least five isoforms (Boluda et al. 1998; Esteve et al. 2012). Alché et al. (1998) observed, by isoelectric focusing four isoforms of Ole e 5 with pIs range of 4.6–5.22 and by immunoblot analysis with anti-cytoplasmatic CuZn–SOD antibody, a single polypeptide of 16.5 kDa.

Ole e 6 is a protein with 50 amino acids composed of a single polypeptide chain with unknown function and a molecular weight of 5.8 kDa (Barral et al. 2004). Ole e 7 is a non-specific lipid transfer protein (LTP) from plant tissues with four different isoforms ranging 9.6–10 kDa (Tejera et al. 1999).

Ole e 9 is a glycosylated 1,3-glucanase, which belongs to the pathogenesis-related (PR-2) protein family, with molecular mass of 46 kDa (Huecas et al. 2001). It shows four components with pIs values between 4.8 and 5.4. It is composed by N- and C-terminal domains, immunologically active, with an apparent molecular mass of 36 kDa and 13 kDa, respectively (Palomares et al. 2003; 2006). Ole e 11 is a pectin methylesterase of 342 amino acid length polypeptide with a molecular mass of 37.4 kDa (Salamanca et al. 2008). Finally, Ole e 12 is an isoflavone reductase with a molecular mass of 37.4 kDa (Castro et al. 2008).

Differences in allergens expression levels and in the number and molecular characteristics of the expressed allergen isoforms between the different olive cultivars have been reported (Alché et al. 2007; Hamman-Khalifa et al. 2008). These differences are intrinsic to the genetics of each cultivar and have important clinical and biological consequences. Patients may suffer differentiated levels of sensitization and symptoms depending on the cultivar of the olive pollen inhaled (Alché et al. 2007).

Therefore, the characterization of as much as possible of olive cultivar pollen allergen loads could contribute to differentiate cultivar-dependent allergenicity, enabling improvements in diagnosis and therapy of olive pollen allergy.

The aim of this study was to describe for the first time the pollen protein profiles of twelve Portuguese olive cultivars. Also, it was investigated the dissimilarity regarding the reactivity of the patient's sera and the content of the different pollen extracts in some

already characterized allergens (Ole e 1, Ole e 2, Ole e 5 and Ole e 9).

2 Materials and methods

2.1 Pollen samples

Mature pollen of 12 different Portuguese cultivars was collected, in the same year, during the olive flowering season (beginning of May), on 25-year-old olive trees, under rainfed cultivation grown in the experimental fields of the 'Instituto Nacional dos Recursos Biológicos' (Elvas, Southeast of Portugal). For each cultivar, four trees were randomly chosen (cvs. 'Ascolana'; 'Blanqueta'; 'Carrasquenha'; 'Cobrançosa'; 'Conserva de Elvas'; 'Galega Vulgar', 'Maçanilha de Tavira'; 'Redondil'; 'Verdeal de Serpa'; 'Verdeal de Trás-os-Montes'; 'Negrinha' and 'Maçanilha de Almendral').

Pollen grains were collected into paper bags placed around 15 inflorescences per tree, one per each canopy quadrant, selected at random in the middle position of fruiting shoots as soon as the beginning of dehiscence of each cultivar started. After sieving through 90-μm pore filters, pollen was stored at −20 °C.

2.2 Epidemiological data

Data from patients' sera presenting specific IgE levels to pollen extracts, performed by ImmunoCAP[TM] FEIA test (Phadia AB), were obtained from a central hospital in Porto region between 2006 and 2012. Random patients presenting IgE levels to *Olea europaea* pollen extract were accounted, and a brief descriptive statistical analysis was performed according to different levels of specific IgE recognition: 0.35–0.7 kU_A/L; 0.7–3.5 kU_A/L; 3.5–17.5 kU_A/L; 17.5–50 kU_A/L; 50–100 kU_A/L and >100 kU_A/L. The study was performed with those files obtained from anonymous sensitization test only, without regarding gender, sex or age data.

2.3 Protein extraction and SDS-PAGE

Pollen extracts were prepared by incubating pollen in a 1:20 (w/v) proportion in phosphate-buffered saline at pH 7.4 with continuous stirring for 4 h at 4 °C. The suspension was then centrifuged at $16,100 \times g$ for 30 min at 4 °C. The supernatant was filtered through a 0.45-μm Millipore filter and centrifuged once again. The soluble protein content of pollen extracts was estimated with the Coomassie Protein Assay Reagent by Bradford's method (Bradford 1976). SDS-PAGE was performed in 12.5 % polyacrylamide gels under reducing conditions (Laemmli 1970). The polypeptides of pollen extracts were visualized by Coomassie Brilliant Blue R-250 staining. The molecular mass of bands was estimated by comparison with a protein reference marker of known molecular weights using a Molecular Imager GS800 calibrated densitometer and Quantity One 1-D analysis, v 4.6 software (Bio-Rad Laboratories).

2.4 Immunoblot analysis with patient's sera and immunoblot analysis with Ole e antibodies

For the immunoblots analysis with patient's sera and immunoblot analysis with Ole e 2 antibody, protein bands separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes (Protran, Whatman® Schleicher & Schuell, Germany). The membranes were saturated during 1 h in a blocking solution [5 % non-fat dry milk (w/v), 0.1 % goat serum (v/v) and 0.1 % Tween in 20 mM Tris, 150 mM NaCl (TBS)] and then incubated overnight at 4 °C with the following: (1) 15 individual atopic patient's sera diluted 1:10 in blocking buffer, for the identification of olive allergens; (2) anti-Ole e 2 polyclonal antibody produced by immunization of rabbits with a keyhole limpet hemocyanin (KLH)-linked synthetic peptide (AQSATFPQFKPEEM) designed from the predicted amino acid sequence of an olive profilin (Ole e 2) (Morales et al. 2008), diluted 1:500. After washing, blots incubated with sera were probed with mouse anti-human IgE-HRP (1:2,000). Those blots probed with the anti-Ole e 2 antibody, were incubated with a goat anti-rabbit IgG peroxidase HRP-conjugated secondary antibody, and diluted 1:2,000 in TBS-T during 1 h at 4 °C. An enhanced chemiluminescence solution (ECL—Luminata Crescendo, Interface, Ltd.) was used as a detection system. The chemiluminescent signal was exposed to AGFA medical X-ray film and developed by Fuji medical film processor model FPM 100A. The antigenic profile bands were recorded and quantified by densitometry as described for SDS-PAGE.

For the immunoblot analysis with Ole e 1, Ole e 5 and Ole e 9 antibodies, protein bands separated by SDS-PAGE were electrophoretically transferred to PVDF membranes (Trans-Blot Semi-Dry transfer cell Bio-Rad). The membranes were incubated with a blocking solution containing 0.3 % (v/v) Tween 20 and 10 % (w/v) skimmed dried milk in TBS for 2 h. Primary antibodies used: (1) mouse anti-olive Ole e 1 monoclonal antibody, provided by Fundación Jiménez Díaz, Madrid, Spain (Lauzurica et al. 1988), diluted 1:20,000; (2) commercially available rabbit polyclonal antibody that recognizes a chloroplastic isoform of Cu/Zn-superoxide dismutase (SOD) from *Arabidopsis thaliana* (Agrisera, Sweden, Product No AS06 170) with probed cross-reactivity to Ole e 5, diluted 1:250; (3) rabbit anti-olive Ole e 9 polyclonal antibody, produced as described above using a synthetic peptide (YPYFAYKNQPTPDT) from the Ole e 9 amino acid sequence (Huecas et al. 2001; Duffort et al. 2006), diluted 1:10,000, were diluted in blocking solution and incubated for 2 h at room temperature. After washing, blots were probed with secondary antibody goat anti-mouse IgG antibody Alexa fluor 488-conjugated, diluted 1:10,000 for (1) and goat anti-rabbit IgG antibody Alexa fluor 633-conjugated, diluted 1:10,000 for (2) and (3) in TBS buffer containing 0.3 % (v/v) Tween 20 (TBS-T) and incubated for 1 h at room temperature in the dark. After antibodies' incubation, membranes were rinsed in TBS-T buffer four times for 5 min each and left to dry at room temperature in the dark. Imaging was carried out as described.

3 Results

Out of 428 patients with specific IgE reactivity to at least one or more pollen extracts, 228 patients (53.3 %) presented specific IgE levels to *Olea europaea*. Among the specific IgE levels, the highest percentage of patients had $0.7–3.5kU_A/L$ (28 %) followed by $3.5–17.5kU_A/L$ (11.7 %), $0.35–0.7kU_A/L$ (9.1 %), $17.5–50kU_A/L$ (2.4 %), $50–100kU_A/L$ (1.6 %) and finally $>100 kU_A/L$ (0.5 %). Demographic data were not available due to confidentiality policy.

SDS-PAGE (12.5 %) of soluble proteins from the *Olea* pollen extracts demonstrated similar profiles between the twelve cultivars being observed several

bands ranging from 11 to 100 kDa after Coomassie staining of the gel (Fig. 1a). Three prominent bands of about 18.5, 20 and 22 kDa with different intensity among the cultivars were observed. 'Galega' and 'Conserva de Elvas' presented lower intensity compared with other cultivars. Several other bands distributed between 25 and 75 kDa with similar intensity between cultivars were also visualized.

Allergenicity changes among the cultivars tested were evaluated according to the binding affinity to specific IgE of atopic patient's sera to olive pollen extracts. The IgE-binding pattern of the 15 atopic sera to olive pollen extracts revealed intercultivar differences regarding the number and intensity of IgE-binding proteins (Fig. 1b–f, b'–f', only 5 representative immunoblots to illustrate the results are shown).

Four prominent groups of IgE-reactive bands were observed, namely 14–18 kDa, 18–22 kDa, 30–40 kDa and 45–53 kDa. The percentages of sera reactive to each one of these groups were 40, 86, 86 and 47 %, respectively. Combined reactivity to the 18–22 kDa and 30–40 kDa groups was always observed.

Generally, among the tested cultivars, the less reactive were 'Galega' and 'Conserva de Elvas'. Pollen of 'Cobrançosa', 'Ascolana' and 'Verdeal de Serpa' cultivars lead to higher IgE reactivity, particularly with the lower molecular weight bands and therefore potential higher allergenicity. The cultivar 'Carrasquenha' presented also high IgE reactivity. Furthermore, the cultivar 'Negrinha' presented high reactivity to the protein bands of around 30–40 kDa.

The immunoblot detection of Ole e 1, Ole e 2, Ole e 5 and Ole e 9, using the respective antibodies for these proteins, allowed quantifying its reactive bands in the different cultivars tested (Fig. 2).

On the immunoblot probed with anti-Ole e 1, four protein species were detected: 18.0, 18.5, 20.0 and 22.0 kDa (monomeric forms with different glycosylation grades) with varying intensities between cultivars. 'Verdeal de Serpa', 'Carrasquenha' and 'Cobrançosa' revealed the most intense bands while 'Negrinha', 'Galega' and 'Conserva de Elvas' presented nearly negligible band intensity. The most frequent forms were 18.0 and 20.0 kDa, and the less was 22.0 kDa. The cultivars 'Verdeal de Serpa', 'Cobrançosa' and 'Carrasquenha' presented four forms.

On the immunoblot probed with anti-Ole e 2, four polypeptide species were detected: 13.0, 13.7, 14.0
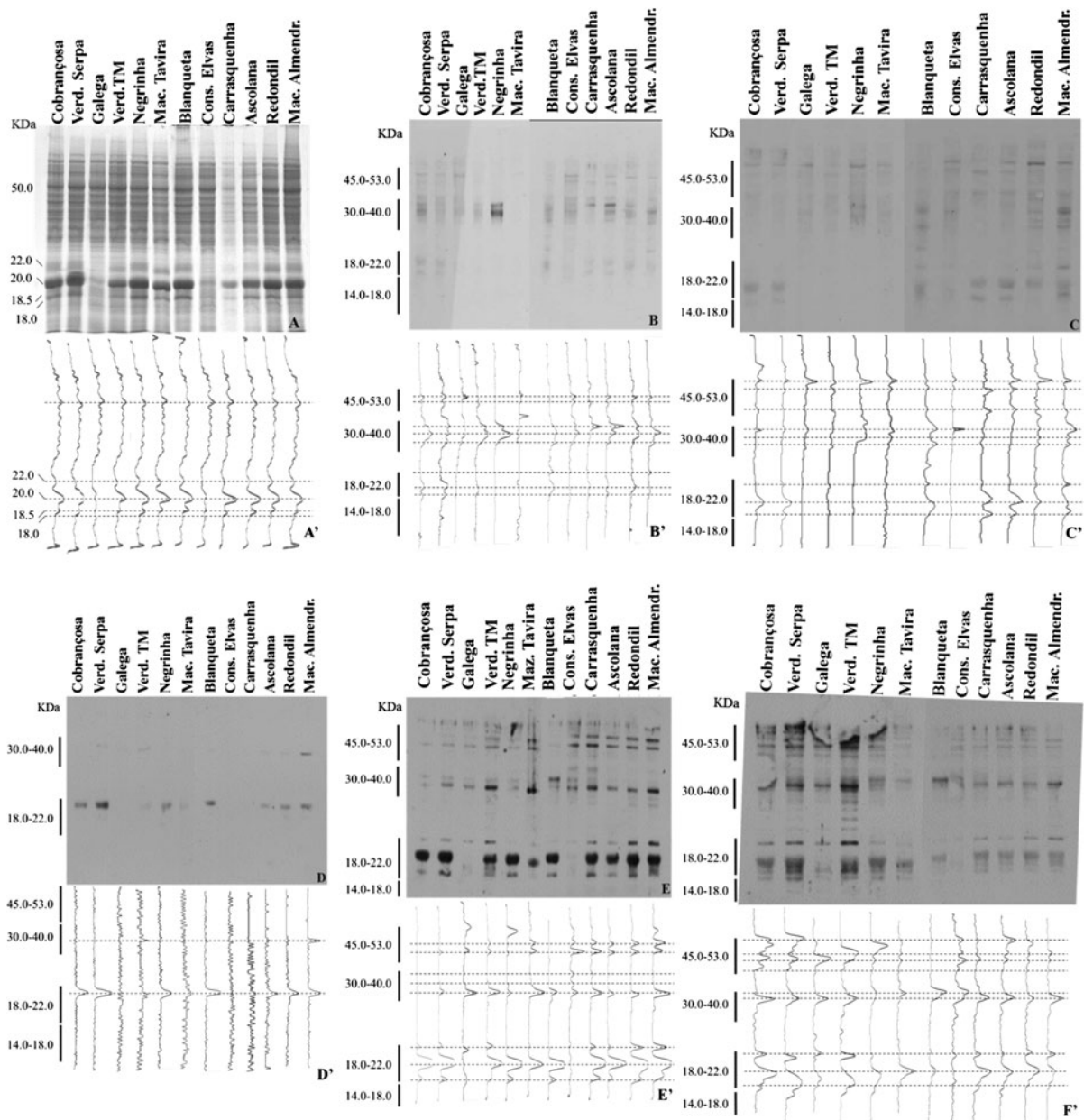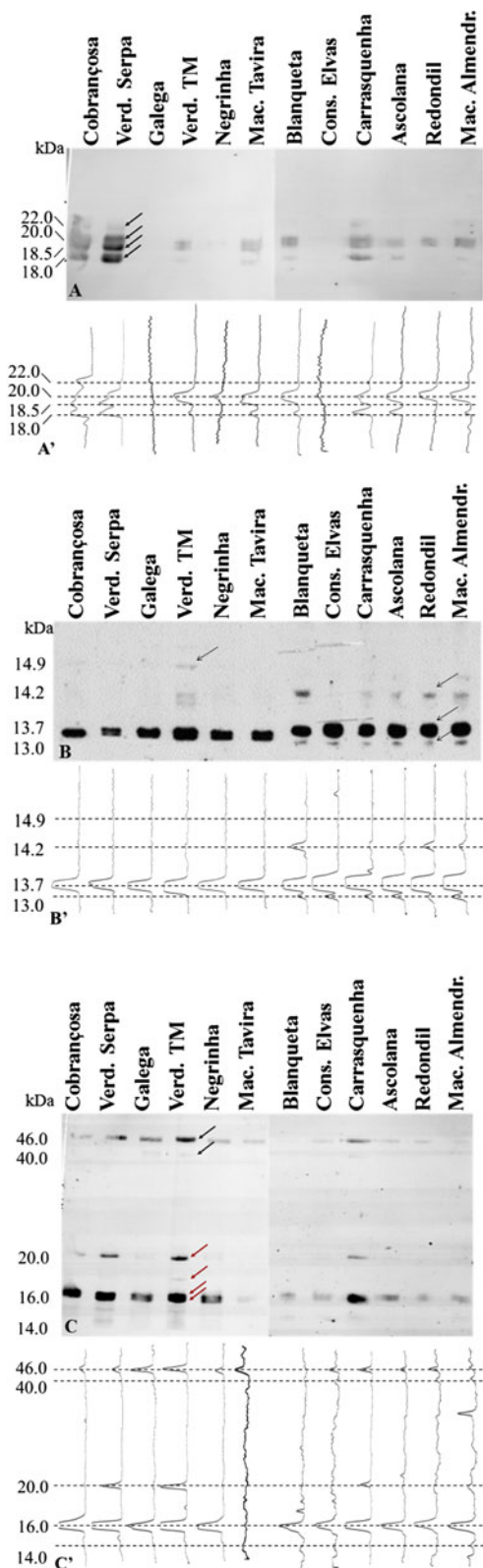
Fig. 1 Soluble protein pattern separated by SDS-PAGE of the 12 olive cultivars analyzed (**a**) and respective lane profile (**a**′). Immunodetection of 12 olive cultivars pollen extracts assayed with sera from patients sensitized to *Olea europaea* L. (**b**–**f**) and respective lane profiles (**b**′–**f**′). Only five representative immunoblots out of 15 are shown

and 14.9 kDa. Some differences in the level of this polypeptide were observed with the 'Verdeal de Serpa' and 'Cobrançosa', presenting the lowest band intensity.

When the twelve olive cultivar pollen extracts were probed with anti-Ole e 5, three main protein species

were detected: 14.0, 16.0 and 20.0 kDa. 'Verdeal de Trás-os-Montes', 'Verdeal de Serpa', 'Carrasquenha' and 'Cobrançosa' were the most reactive. Also, the other cultivars showed similar band intensity, being the cultivar 'Maçanilha de Tavira' the one with the lowest level of this polypeptide.

Finally, on the immunoblot probed with anti-Ole e 9, two protein bands were detected: 40 kDa and 46 kDa. The cultivar 'Verdeal de Trás-os-Montes' revealed the most intense bands followed by 'Galega', 'Verdeal de Serpa' and 'Carrasquenha'. The form with molecular weight of 40 kDa was only detected in 'Verdeal de Trás-os-Montes', 'Galega' and 'Carrasquenha'.

4 Discussion

Although evidence of the divergence of pollen micromorphology, physiological characteristics and allergenic content among varieties/cultivars of a given plant is accumulating, the literature devoted to this topic is still scarce (Alché et al. 2012).

We have observed in this study a large variability among the 12 olive cultivars tested. This variability affects different aspects, which include visible changes in the levels of several protein species within the SDS-PAGE pollen protein profiles, the IgE reactivity of the patient's sera and the contents of specific allergens like Ole e 1, 2, 5 and 9. This intercultivar variability was observed in several other studies performed in Spain, Jordan, USA, Israel and Morocco (Alché et al. 2007; Arilla et al. 2008; Carnés Sánchez et al. 2002; GellerBernstein et al. 1996; Jaradat et al. 2012; Quiralte et al. 2002; Rodriguez et al. 2002; Sanchez et al. 2002). Furthermore, variability in the antigenic and allergenic composition within the same cultivar grown in different countries was observed (Boluda et al. 1998). No information on this topic exists to our knowledge for Portuguese cultivars neither from other cultivars planted in Portugal. This information is important since it has been reported that differences in olive pollen allergenicity are intrinsic to the genetics of each cultivar (Fernandez-Caldas et al. 2007; Hamman-Khalifa et al. 2008) and the polymorphism described in pollen allergens can represent an adaptive advantage in the physiology of the olive reproductive system, for example, the establishment of the compatibility system, pollen germination or defense and adaptation responses of the plant to different environmental

conditions (Alché et al. 2007). Additionally, the geographic location and the environmental conditions have also important influence on the allergen content and on the sensitivity of the individuals to a specific allergen (Casanovas et al. 1997; Morales et al. 2012; Quiralte et al. 2002).

The wide reported polymorphism in olive pollen allergenic proteins was evident in our study. Several protein forms were observed when pollen extracts were probed with Ole e 1, 2, 5 and 9 antibodies. This variability has been attributed to amino acid substitutions, PTMs (e.g. glycosylation) and/or the presence of multimeric forms (Castro et al. 2010; Morales et al. 2012; Salamanca et al. 2010). In the case of Ole e 1, the allergenic forms observed in Portuguese cultivars correspond well with those already described in the literature for cultivars from other origins, although the molecular complexity reported for Ole e 1 was related to the difficulty in determine the exact number of forms present in a particular olive cultivar (Castro et al. 2010).

For Ole e 2, four forms of 13.0, 13.7, 14.2 and 14.9 kDa were observed in the cultivars analyzed here. However, one additional form (15.7 kDa) was detected in Spanish olive cultivars after using the same antibody (Alché et al. 2007). Heterogeneity in Ole e 2 sequence with implications in the 3-D structure of the protein has been reported by some authors (Asturias et al. 1997; Jimenez-Lopez et al. 2012; Martínez et al. 2002).

Ole e 5 was characterized as a highly polymorphic allergen (Boluda et al. 1998; Tejera et al. 1999). In our study, three forms were detected, one equivalent with the 16.0 kDa frequently reported by several authors and the other two (14.0 and 20.0 kDa) likely corresponding to others among the five forms already described in the literature (Boluda et al. 1998; Esteve et al. 2012).

Among the two Ole e 9 forms observed in the present work, the one of 46 kDa has similar molecular mass than the allergen described by Huecas et al. (2001), whereas the other form reactive to the antibody (40 kDa) was less detected among the olive cultivars assayed and can be attributed to a degradation product of Ole e 9.

Allergens with molecular weights of 18–22 kDa and 30–40 kDa showed a high frequency of recognition by the atopic patient's sera, whereas the recognition rate for allergens with molecular weights of 14–18 kDa and 45–53 kDa was lower. The 18–22 kDa bands may correspond to Ole e 1 forms but may also be attributed to other allergens with similar molecular masses such as Ole e 5. The bands around 46 kDa might correspond to Ole e 9 while those around 14 kDa may correspond to Ole e 2. The remaining bands (30–40 kDa and around 50 kDa) did not match any of the bands cross-reacting to the antibodies used in this study. Bands higher than 50 kDa may correspond to other allergenic proteins observed in olive pollen extracts but not characterized yet (Huecas et al. 2001).

After analysis of allergen content, it was observed that the different cultivars produced pollen with a different allergenic load. The cultivar 'Verdeal de Serpa' presented the highest allergen load of Ole e 1, Ole e 5 and Ole e 9 but the lowest of Ole e 2. Also, 'Carrasquenha' was the second cultivar with high allergen content. Therefore, we can sustain that the pollen of these cultivars can cause stronger atopic reactions compared with other cultivars. In fact, these cultivars also presented the highest IgE response from the atopic patient sera. However, the prevalence of olive allergy depends on the pollen concentration and exposure time (Esteve et al. 2012) and, regardless of the allergen load, when olive pollen is inhaled in larger amounts, it can produce severe atopic reactions. On the other hand, it is also noticeable the lowest allergen load and IgE reactivity of the cultivars 'Galega' and 'Conserva de Elvas' which would be important from a preventive and environmental control point of view. Allergenic potency should also be considered a primary criterion in order to choose olive cultivars for ornamental purposes and to avoid an increase in the prevalence of respiratory allergy. It is also important for clinical trials to use pollen extracts that imitate as much as possible the composition of the panel of allergens to which the patient is normally exposed and is reactive (Alché et al. 2007). Therefore, the knowledge of the intercultivar allergenicity can be important to adjust the extracts used for diagnosis or even immunotherapy to the cultivars present in a given region (e.g. discriminating the cultivar used for extract preparation) (Alché et al. 2007).

The differences displayed here among Portuguese cultivars could be further analyzed by using newly developed highly informative methods such as multiplex labeling (Morales et al. 2012), 2-D electrophoresis and immunoblotting (Zienkiewicz et al. 2012) and peptide mapping (Castro et al. 2010).

5 Conclusions

With the exception of several noticeable differences, mainly in the range of 18–22 kDa, similar SDS-PAGE pollen protein profiles were observed in the olive cultivars analyzed. However, clear IgE-binding differences were shown in these pollen extracts. Also, the distribution of olive pollen allergen forms between cultivars showed a wide heterogeneity in both their number and expression level. This heterogeneity was particularly observed for Ole e 1 and Ole e 5. The cultivars 'Galega' and 'Conserva de Elvas' presented the lowest allergen load and IgE reactivity while the cultivars 'Verdeal de Serpa' and 'Carrasquenha', the highest. It would be interesting to study whether this heterogeneity would remain along the years and its correlation with climatic conditions.

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