Differential Characteristics of Olive Pollen From Different Cultivars: Biological and Clinical Implications

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Abstract

The olive tree is grown in many parts of the world. Its germplasm is very broad, with 250 varieties in Spain alone. Variations in the ability of pollen to germinate have been studied in detail and show conspicuous differences between varieties. However, commercial olive pollen from cultivars whose origin is unknown is the material that is commonly used for clinical and biological studies. We aim to assess the putative heterogeneity of olive cultivars with regard to the presence of several pollen allergens and to determine whether these differences have biological and clinical relevance. Previous studies show that most allergens isolated and characterized to date are highly polymorphic. Olive cultivars display wide differences in the expression levels of many allergens and in the number and molecular characteristics of the allergen isoforms expressed. These differences are maintained over the years, and are intrinsic to the genetics of each cultivar. Such broad polymorphism seems to be involved in the physiology of the olive reproductive system, which might include the adaptation of the plant to different environmental conditions, the establishment of the compatibility system, and pollen performance. The differences in allergen composition in cultivars, particularly in the Ole e 1 allergen, are responsible for the important differences in the allergenic potency of the extracts. These findings could have a number of implications for the diagnosis and therapy of olive pollen allergy. We discuss how cultivar differences affect extract quality, diagnostic and therapeutic efficacy and safety, and the development of new vaccines based on the use of recombinant allergens.

Key words: Allergens. Clinical test. Cultivar. Physiology. Olive. Pollen. Polymorphism. Variety.

Resumen

El olivo es un cultivo ampliamente representado en el mundo. Su germoplasma es muy amplio, con 250 variedades sólo en España. La capacidad del polen para germinar, que presenta notables diferencias entre variedades, ha sido estudiada en detalle. El material usado comúnmente para estudios clínicos y biológicos es, sin embargo, polen comercial de cultivares de origen desconocido. Nuestro objetivo es evaluar la posible heterogeneidad de los cultivares de olivo en relación a la presencia de varios alérgenos del polen, y determinar si esas diferencias tienen relevancia biológica y clínica. Estudios previos muestran que la mayor parte de los alérgenos aislados y caracterizados hasta la fecha son altamente polimórficos. Los cultivares de olivo muestran amplias diferencias en los niveles de expresión de muchos alérgenos, así como en el número y características moleculares de las isoformas alergénicas expresadas. Estas diferencias se mantienen a lo largo de años, y son intrínsecas a la genética de cada cultivar. Este amplio polimorfismo parece estar implicado en la fisiología del sistema reproductivo del olivo, en relación con la adaptación de la planta a diferentes condiciones ambientales, el establecimiento de un sistema de compatibilidad, y el dinamismo del polen. Las diferencias en la composición alergénica de los cultivares, particularmente en cuanto al alérgeno Ole e 1, son responsables de las importantes diferencias en la potencia alergénica de los extractos. Estos hallazgos pueden tener numerosas implicaciones en la diagnosis y terapia de la alergia al polen del olivo. Discutimos cómo las diferencias entre cultivares afectan a la calidad del polen, a la eficacia y seguridad del diagnóstico y la terapia, así como al desarrollo de nuevas vacunas basadas en el uso de alérgenos recombinantes.

Palabras clave: Alérgenos. Pruebas clínicas. Cultivar. Fisiología. Olivo. Polen. Polimorfismo. Variedad.

Olive Germplasm and Its Classification

The olive tree was one of the earliest fruit crops to be domesticated. It spread from the Middle East towards the west of Europe approximately 6000 years ago [1,2]. Over time, a large number of cultivars have appeared due to events such as outcrossing, mutation, clonal selection, and selective pressure (including grower requirements) on the original olive germplasm. Controversy surrounds many other aspects of olive genetics including the putative origin of the species (supposed to be an allopolyploid), the phylogenetic relationships between *Olea europaea* and related *Olea* species, and between cultivated and wild forms of the olive [3,4]. Although 2600 different olive cultivars have been recorded [5], the number of olive cultivars throughout the world is uncertain.

In Spain, olive cultivars were initially described in the first century and have reappeared in historical documents until the present. Modern systematic classifications of olive cultivars were first carried out in Andalusia [6] and later in other regions of Spain to provide a picture of the whole country, which includes 272 cultivars. Olive cultivars in Spain are spread throughout continuous regions, where they are predominant. Outside these regions their importance quickly decreases [4]. The classification of olive germplasm is increasingly urgent as a requirement of modern cultivation strategies and the breeding and selection programmes currently in progress. Morphological, biometric, and agronomical characteristics have been widely used to describe olive cultivars. However, biochemical and molecular techniques are emerging as the preferred tools for cultivar identification. They include isozymes, randomly amplified polymorphic DNA markers, amplified fragment length polymorphism markers, inter-simple sequence repeat markers, and, more recently, microsatellites [7].

Both sexual reproduction and asexual reproduction coexist in olive. Vegetative propagation is widely used for agronomical purposes and is one of the principal reasons for the marked genetic homogeneity occurring within cultivated varieties [8]. Sexual reproduction is the main physiological process responsible for olive production, and morphological parameters of the fruit, particularly of the endocarp, are widely used as key distinctive characteristics for olive cultivar discrimination [4,6]. Other characteristics of the reproductive organs, such as inflorescence length, shape, presence of supernumerary flowers, and thickness of flower buds, have also been used for this purpose [6]. As for pollen grains, some authors have proposed pollen morphology as an additional tool for cultivar identification, based on the sporophytic origin of the exine, its stability, independence from environmental conditions, and genetic control [7]. Few publications have made use of this approach to date, and information is limited [9,10].

One of the first biochemical approaches applied for cultivar discrimination was the use of isozymes [11]. For this purpose, pollen rather than leaves was the material of choice, particularly because of its higher degree of enzyme polymorphism [12]. The analysis of isoenzymes has enabled several authors to discriminate successfully between the cultivars assayed using only a limited number of enzyme systems [11,13]. None of the authors observed intracultivar polymorphisms using these methods.

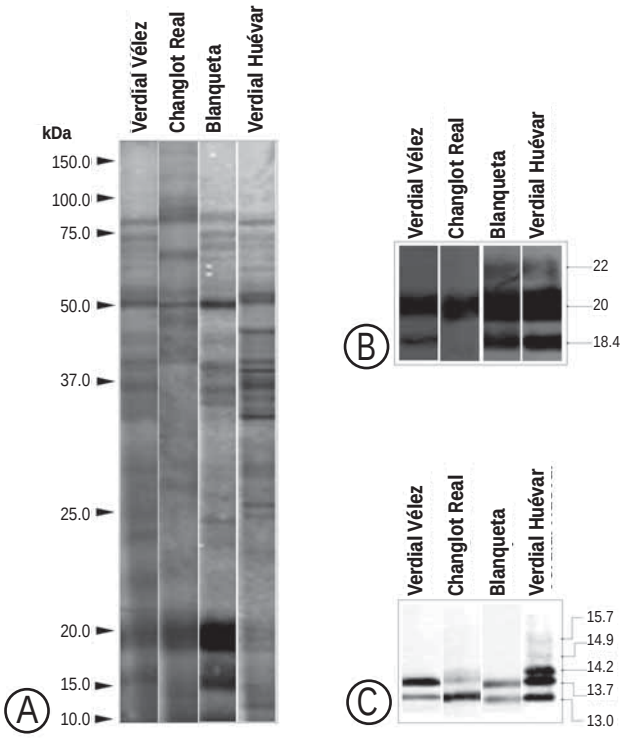

Figure 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12%) and Western blot identification of Ole e 1 and Ole e 2 forms in crude pollen protein extracts from 4 olive cultivars. Panel A. Silver-stained gel. Panel B. Immunoblot tested with an anti-Ole e 1 monoclonal antibody showing 1 to 3 cross-reactive bands with apparent molecular masses of 22.0, 20.0, and 18.4 kDa. Panel C. Immunoblot tested with a polyclonal serum to Ole e 2, showing up to 5 cross-reactive bands with apparent molecular masses of 15.7, 14.9, 14.2, 13.7, and 13.0 kDa apparent molecular masses. Thirty micrograms of total protein was loaded per lane.

Polymorphism Is a General Characteristic of Many Olive Pollen Allergens

Biochemical and molecular studies to characterize the allergenic proteins present in olive pollen have shown that polymorphism is a general feature. In this context, we can say that Ole e 1 presents a high degree of polymorphism in both its nucleotide and amino acid sequences [14-16]. Ole e 1 can be characterized as glycosylated (apparent molecular weight of 20 kDa), non-glycosylated (18.5 kDa), hyperglycosylated (22 kDa), and dimers of the glycosylated form (40 kDa), in addition to the diversity generated by the components of the glucidic chains [17]. For Ole e 2 (profilin), some authors have shown the presence of heterogeneities in its sequence [18,19]. The protein possesses an average molecular weight of 15 kDa, and the analysis of a limited number of available sequences has shown heterogeneity in at least 4 residues of the primary structure, with important implications

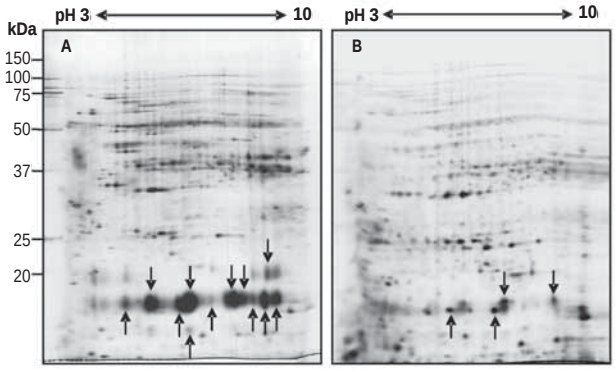

Figure 2. Two-dimensional protein profiles of pollen from 2 olive cultivars (A: Picual, and B: Arbequina) after colloidal Coomassie staining. Conspicuous differences can be observed in both the quantitative expression and the isoform distribution of Ole e 1 allergen in both cultivars (arrows).

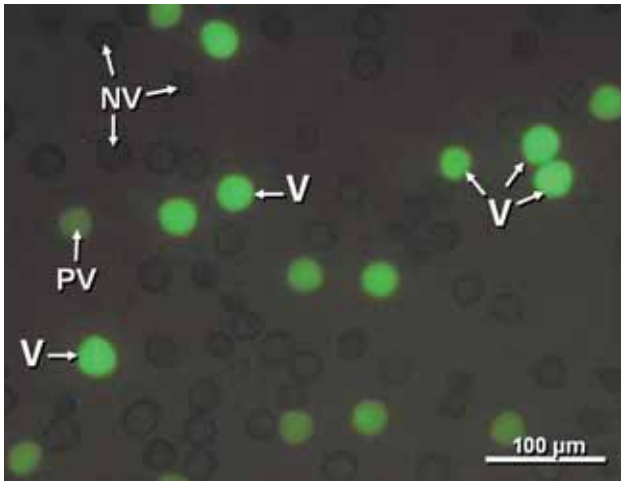

Figure 3. Fluorescein diacetate test of pollen viability. Fluorescent pollen grains are considered viable (V), whereas nonfluorescent pollen grains are considered nonviable (NV). Some pollen grains display slight fluorescence, and are therefore considered partially viable only (PV).

for the 3-D structure of the protein. A recent study describes cloning and sequencing of the complete form of Ole e 5 [20] (GenBank AJ428575). The sequence obtained, together with those already provided by different authors [21,22] (GenBank P80740, AF191342, AF426829), is well conserved, since the percentage of identity with Cu/Zn-superoxide dismutase from other plant sources ranges from 80% to 90%. However, all the aforementioned authors have described the presence of a remarkable degree of polymorphism in this olive protein. Thus, in preliminary work carried out by our group, we managed to detect and localize 4 isoforms of the enzyme in pollen of the variety "Picual," displaying isoelectric points of 4.60, 4.78, 5.08, and 5.22 [23]. Five isoforms of the protein were later described [21]. Butteroni et al [20] reported discrepancies between the amino acid sequences of the native

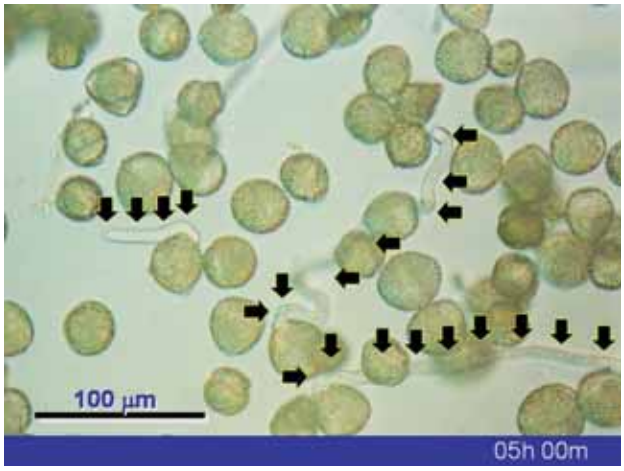

Figure 4. Olive pollen germination after culture in vitro for 5 hours. For statistical purposes, pollen grains are considered germinated when the length of the emerging pollen tube (arrows) is at least equal to the pollen grain diameter (approximately 30 µm).

and the recombinant forms of the protein. For Ole e 7, a high degree of polymorphism has been demonstrated, with molecular weights ranging from 9.87 kDa to 10.29 kDa, and 2 isoforms have been characterized [22]. Ole e 9 shows a relatively low (although still significant) level of polymorphism, which has been detected by high-performance liquid chromatography and later confirmed after nucleotide sequencing [24]. This polymorphism can be attributed to the presence of microheterogeneities in the peptide chain and/or the glycosylated moiety of the allergen, which presents 2 putative N-glycosylation sites, as does Ole e 1.

Allergen Polymorphism Is Closely Related to the Cultivar Origin of Olive Pollen

Molecular evidence regarding the differential composition of the allergens in the pollens from different cultivars is beginning to emerge. Preliminary studies characterizing cDNA sequences of Ole e 1 in a limited number of cultivars showed the existence of a high number of microheterogeneities in the analyzed sequences. Software analysis of microheterogeneities showed that the intercultivar variability detected was higher than the intracultivar variability [25,26,unpublished results]. These studies are presently being extended to several characterized allergens in a significant number of olive cultivars. The numerous sequences obtained have been sent to GenBank.

The analysis of the levels of expression of each allergen and its biochemical characteristics in the major olive cultivars is also beginning to be addressed. In addition to the well established differences in Ole e 1 expression (Figure 1), preliminary results regarding the expression and presence of sequence heterogeneities for Ole e 3, Ole e 5, and Ole e 6 are already available [26,27], indicating the presence of significant differences between olive cultivars. Ole e 2 is also revealing itself as a relatively highly polymorphic allergen in cultivars, displaying at least 5 different isoforms (Figure 1).

Proteomic approaches are promising in this context. A recent study on a number of olive pollen extracts from different cultivars [28] detected significant differences in their allergenic composition. Figure 2 shows 2-D profiles obtained from the pollen of 2 cultivars regarded as very different in their Ole e 1 content.

Biological Implications of Allergen Polymorphism

Differences in pollen production and performance among cultivars are well documented [29]. Two major tests are widely used to assess pollen viability: the fluorescein diacetate test [30] (Figure 3) and the determination of the germinability percentage after in vitro pollen culture [31] (Figure 4). Olive cultivars present wide differences regarding both parameters.

In addition to their allergenic character, allergens are considered key proteins for pollen physiology. An important question is whether these biological functions might differ to some extent according to the cultivars. Although the function of many olive pollen allergens is well studied [17,32-34], a large amount of information is still lacking. We can speculate that the presence of numerous forms for each of the proteins studied in the different varieties represents an adaptive advantage of the plant to different environmental conditions, which could explain the varying abilities of the pollen grains from different varieties to germinate, their different viability, or even the (self) incompatibility/(self) pollination ability of each variety [35]. It could also explain the existence of androsterile varieties or varieties with low/null pollination efficiency [29]. Constitutive accumulation of ROS/H_2O_2 appears to be a feature of angiosperm stigmas [36], which is discussed in terms of a possible role for pollen–stigma interactions and defense. Therefore, pollen antioxidant systems such as Ole e 5 may also play an important role in such processes. An increase in profilin expression has also been described as a response to salinity in some species [37]. Many of these models can be tested using multidisciplinary approaches including biochemical, molecular, and cellular analysis of allergen expression.

Clinical Implications of Allergen Polymorphism

In the past 2 decades, several pioneering papers and research communications have established the presence of differences in the protein composition and allergenic activity of pollen extracts from different origins [38,39]. Studies carried out in Israel [40,41] using olive pollen extracts from autochthonous and foreign varieties showed sharp differences in the quantitative/qualitative allergenic composition of such extracts and the reactivity of patients' sera. These authors suggested that multiple olive extracts should be used in order to improve the reliability of skin-prick tests, particularly in cases of questionable diagnosis where the patients have clinical evidence of olive-induced hay fever but do not have a positive skin-prick test response to one of the commonly used commercial extracts (10% of patients). Conspicuous differences were later observed in the reactivity of the sera from Spanish patients tested against protein extracts from Californian pollen [21]. Quantitative differences in the levels of certain allergens between extracts from different sources of pollen in Spain and California have also been described [42]. Further studies carried out in Spain [43-46] indicated that skin-prick test reactivity to olive pollen extracts varies greatly depending on the olive cultivar. Olive pollen extracts from different cultivars also possess differences in allergenic potency expressed in histamine equivalent prick units per gram of raw material [46]. These studies have made it possible to identify Ole e 1 as one of the major reasons for the differences reported to date (Figure 1), although the role of other allergens cannot be excluded. However, disparities in allergenic potency and Ole e 1 content have been maintained over the years, suggesting that they are due to genetic differences intrinsic to the cultivars [46].

Our increasing knowledge of the variability of allergenic molecules with respect to the genetic origin of the allergens is not exclusive to olive pollen allergens. Similar results have been obtained for other plant allergens, such as *Phoenix dactylifera* [47,48]. In apple (*Malus domesticus*), cultivars differ considerably in allergenicity [49,50]. The genetic basis of polymorphism of Mal d 1 (PR-10), Mal d 2 (thaumatin-like protein), Mal d 3 (nonspecific lipid transfer protein), and Mal d 4 (profilin) genes has been characterized [51-53]. In birch (*Betula pendula*), 13 Bet v 1 putative alleles have been characterized and their occurrence in different cultivars is a matter for future study [54].

The presence of such variability will undoubtedly involve a number of aspects of current clinical practice. Here, we suggest the main concerns that will need to be addressed by future research.

Pollen batches provided by different companies to extract manufacturers have been shown to vary widely in their total protein content, Ole e 1 content, and allergenic potency [55]. In addition, pollen samples commonly show large batch-to-batch variability in several parameters. In most cases, these pollen samples are obtained from undisclosed sources and, in general, no information regarding the cultivar origin is released. The discrepancies observed may be due to the use of different cultivars as the pollen source. Since reliability of pollen extracts used for clinical purposes is a major concern for clinicians, we suggest that such information should be considered as a major criterion for standardization.

The main objective of pollen extracts should be to imitate as much as possible the composition of the panel of allergens to which the patient is normally exposed and is reactive. This can be achieved through increased specialization and personalization of the extracts used for diagnosis and immunotherapy, ie, discriminating the cultivar used for their preparation. The use of appropriately identified and standardized pollen extracts from independent cultivars may also lead to more efficacious diagnosis and immunotherapy, given that some patients have proven particularly sensitive to the extracts from specific cultivars [43,45].

An additional advantage of extracts that have been well characterized by cultivar origin is their increased safety. Adjusting the extracts used for immunotherapy to a patient's reactivity may help to avoid the undesirable de novo immunotherapy-induced sensitizations reported by some authors, even though these are relatively uncommon [56].

Novel diagnostic and therapeutic concepts often include the use of recombinant allergen molecules [57]. Recombinant allergens will undoubtedly offer tremendous advantages over conventional allergen-specific immunotherapy based on extracts from natural sources. However, in our opinion, a reduction in the number of allergenic structural entities in the extracts might result in substantial differences between these preparations and real exposure to an allergen in the patient's environment, unless the recombinant molecules are carefully selected. As the number of isoforms for each allergenic protein represented in the different cultivars is being characterized, it would be interesting to include such information in the putative recombinant formulae.

This strategy could be incorporated into practically all the new developments in allergy diagnosis and therapy, from the new high-throughput diagnosis systems to the preparation of hybrid molecules, use of allergen fragments, allergen multimers, and design of hypoallergens. For instance, detailed analysis of the reactivity of the natural isoforms of a given allergen in different cultivars, combined with the sequence analysis already under way, would help to design hypoallergens, thus complementing current strategies [58]. Moreover, further research on allergen variability through olive germplasm would prove that hypoallergenic and other allergenic forms with putative application in clinical practice are already available as natural allergens in some cultivar sources.

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