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HISTOLOGICAL, PROTEOMIC AND TRANSCRIPTOMIC ANALYSIS OF THE OLIVE SEED

AUTHOR:
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ANÁLISIS HISTOLÓGICO, PROTEÓMICO Y TRANSCRIPTÓMICO DE LA SEMILLA DEL OLIVO

PRESENTADA POR:
ADORACIÓN ZAFRA ÁLVAREZ

DIRIGIDA POR:
JUAN DE DIOS ALCHÉ RAMÍREZ
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PROTEOMIC, TRANSCRIPTOMIC AND HISTOLOGICAL ANALYSIS OF THE OLIVE SEED

Memoria presentada por Dña. Adoración Zafra Álvarez para optar al grado de Doctora
por la Universidad de Jaén

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A mis hijos Adrián, Elsa y Diego
A Vale

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A Vale, de nuevo, el mejor apoyo que puedo haber tenido.

ABSTRACT:

The olive oil industry has a high social and economical importance in the Mediterranean area. Spain is the major producer of olive oil in the world, with a 38% of the world production. The production of extra virgin olive oil is based on a mechanical extraction using mainly the whole fruit. Thus, the obtained juice contains also components from the seed, among which, the 11S-type Seed Storage Proteins (SSPs) are the most abundant ones. However, not many literature is available about the kind/quantity of other proteins in the seed as well as its implication in the olive oil properties. Currently, alternative industrial procedures are being designed to process the olive fruit aimed to increase its added value and to reduce the generation and toxicity of the by-products such as the alpeorujo. Some of these procedures allow the uninterrupted extraction of the stone (also obtained from industries that produce the table olives) and the purification of the seed (a raw material that is considered for multiple purposes such as alimentary and nutraceutical uses) as the knowledge of its components becomes deeper. Thus, the study of the olive seed by using different methodological approaches is the main goal of the present work.

The material used along the whole study is the olive seed. Whole seeds or their integrating parts (endosperm, testa and embryo) were used for a complete histological characterization along its development by means of light microscopy. In some of the cases this material was grinded and defatted by different methodologies at industrial level, including pressing and defatting by means the use of supercritical fluids, aimed to finally obtain a flour. This flour was used for proteomic and transcriptomic analysis. Besides, the flour was used to optimize the generation of protein isolates, to test the anti-inflammatory effects on human cultured cells, and to determine its cross reactivity against the G12 and R5 antibodies for gluten detection. Finally, the presence of another group (7S-type SSPs in this case), was identified in this raw material.

The results from the histological characterization are novel in this field, providing a detailed description of the the distribution/formation of the protein bodies along the seed development in the different tissues. The study of the complete seeds or their derived flour conducted to novel results in all cases. The proteomic approach by means of one- and two-dimensional electrophoresis showed the presence a wide panel of newly described proteins, among which storage proteins as well as enzymatic proteins are present. Focussing on the storage proteins, we used the reproductive transcriptome which is available at the ReprOlive database generated by the working group, aimed to identify and characterize the main SSPs (11S and 7S). Regarding the 11S, the isoform 2 of this protein was identified, then it was subjected to a complete bioinformatic analysis, including alignment with sequences from other species, phylogenetic and allergenic analysis. Similarly, the 7S transcripts were identified in the olive seed through the data mining analysis of the ReprOlive database, and the presence of the protein was validated by using immunoblotting. Analogously to the 11S, the 7S-type SSPs were subjected to a complete bioinformatic analysis, besides to a study using sera from allergic patients, in which cross-reactivity with similar proteins from other alimentary sources was detected in several patients. Concerning to the examination of the olive seed flour as a potentially edible food, the generation and characterization of protein isolates was successfully completed. Besides, the flour was identified to own highly valued characteristics for the human consumption, like being a gluten-free product, with putative positive effects on diabetic patients.

The use of differentially obtained products from the olive fruit reveals a high potential for the near future. On the one hand, the use of the alternative processing of the olive fruit allows us to approach even more to the production of "zero by-products" within the context of a "circular economy". On the other hand, the use of the olive seed flour is an attractive alternative to the consumption of animal proteins as edible food with healthy properties.

RESUMEN:

La industria del aceite de oliva posee una elevada importancia social y económica en el área mediterránea. España es el mayor productor de aceite de oliva en el mundo, con un 38% de la producción mundial. La producción de aceite de oliva virgen extra está basada en una extracción mecánica que utiliza mayoritariamente el fruto completo. Por tanto, el zumo obtenido contiene, además, componentes de la semilla, entre los cuales las Proteínas de Almacenamiento de la Semilla (SSP) de tipo 11S son los más abundantes. Sin embargo, no existe prácticamente bibliografía sobre el tipo/cantidad de otras proteínas en la semilla, así como su implicación en las propiedades del aceite de oliva. En la actualidad se están diseñando procedimientos industriales alternativos para el procesado de la aceituna, destinados a incrementar su valor añadido y disminuir la generación y la toxicidad de subproductos como el alpeorujo. Algunos de estos procedimientos permiten la extracción continuada de hueso (también obtenido en las industrias de elaboración de aceitunas de mesa) y la purificación de la semilla (un material para el que se están diseñando numerosos usos tanto alimentarios como nutracéuticos) a medida que se va progresando en el conocimiento de sus componentes. Por tanto, el estudio de la semilla del olivo mediante diferentes aproximaciones metodológicas es el principal objetivo perseguido en el presente trabajo.

El material usado a lo largo de todo el estudio es la semilla del olivo. Las semillas intactas o sus partes integrantes (endospermo, testa y embrión) fueron usados para la caracterización histológica completa de la semilla a lo largo de su desarrollo mediante microscopía óptica. En algunos de los casos, este material fue triturado y desengrasado usando diferentes metodologías, a nivel industrial, que incluyen el prensado y el desengrasado mediante fluidos supercríticos, con el objetivo de obtener una harina. Esta harina fue usada para su análisis proteómico y transcriptómico. Además, la harina fue usada para optimizar la generación de aislados proteicos, para probar sus efectos anti-inflamatorios sobre células humanas en cultivo y para determinar su reactividad frente a los anticuerpos G12 y R5 para la detección de gluten. Finalmente se identificó la presencia de otro grupo de SSPs (de tipo 7S en este caso) en este material.

Los resultados de la caracterización histológica son novedosos en este campo, aportando una detallada descripción de la distribución/formación de los cuerpos proteicos a lo largo del desarrollo de la semilla en los diferentes tejidos. El estudio de las semillas completas o de su harina derivada conllevó en todos los casos resultados novedosos. La aproximación proteómica mediante electroforesis unidimensional y bidimensional reveló la presencia una amplia variedad de proteínas descritas por primera vez, entre las cuales figuran proteínas de almacenamiento y proteínas enzimáticas. Focalizados en las proteínas de almacenamiento, usamos el transcriptoma reproductivo descrito en la base de datos ReprOlive, generada por el grupo de trabajo, con el objetivo de identificar y caracterizar las SSPs mayoritarias (11S y 7S). Con respecto a las 11S, se identificó la isoforma 2 de esta proteína que fue posteriormente sometida a un completo análisis bioinformático, incluyendo alineamiento con secuencias de otras especies y análisis filogenético y alergénico. De igual modo, los transcritos de 7S fueron identificados en la semilla del olivo a través de análisis de datos en la base de datos ReprOlive, y posteriormente la presencia de la proteína fue validada mediante inmunoblotting. De forma análoga a las 11S, las SSPs de tipo 7S fueron sometidas a un completo análisis bioinformático, además de a un estudio usando sueros de pacientes

alérgicos, en los que se detectó reactividad cruzada en algunos pacientes con otras fuentes agroalimentarias de proteínas similares. En relación a los estudios de la harina de la semilla de olivo como un alimento potencialmente comestible, la generación y caracterización de aislados proteicos fue completada exitosamente. Además, la harina fue identificada como poseedora de características altamente valorables para el consumo humano, ya que es un producto sin gluten y con posibles efectos positivos en pacientes diabéticos.

El uso de productos obtenidos diferencialmente del fruto del olivo posee un alto potencial para el futuro próximo. Por una parte, el uso de un procesamiento alternativo del fruto del olivo nos permite aproximarnos aún más a una producción de "cero residuos" en el contexto de una "economía circular". Por otra parte, el uso de la harina de semilla de olivo es una alternativa atractiva para el consumo de proteínas animales como alimento comestible de carácter saludable.

INDEX

CHAPTER 2. Contribution to the identification of the olive seed proteome by 1D/2D electrophoresis and MS/MS analysis

CHAPTER 3. Identification and bioinformatic analysis of the mayor SSPs transcripts in the olive seed

3.1. Transcriptome-based identification of a seed olive legumin (11S globulin). Characterization of subunits, 3D modelling and molecular assessment of allergenicity. *Zafra A., Jimenez-Lopez J.C., Carmona R., Claros G., Alché J.D. (2016) Transcriptome-Based Identification of a Seed Olive Legumin (11S Globulin). Characterization of Subunits, 3D Modelling and Molecular Assessment of Allergenicity. In: Ortuño F., Rojas I. (eds) Bioinformatics and Biomedical Engineering. IWBBIO 2016. Lecture Notes in Computer Science, vol 9656. Springer, Cham*

3.2. Identification and assessment of the potential allergenicity of the 7S vicilins in olive (*Olea europaea* L.) seeds. *JC. Jiménez-López, A Zafra, L Palanco, JF Florido, and JD Alché, (2016) Identification and Assessment of the Potential Allergenicity of 7S Vicilins in Olive (Olea europaea L.) Seed. BioMed Research International, vol. 2016, Article ID 4946872, 11 pages. https://doi.org/10.1155/2016/4946872*

CHAPTER 4. Evidence of alimentary advantages and nutraceutical properties of olive seed components

Overall discussion

Annexes

ÍNDICE

Agradecimientos

Resumen (inglés)

Resumen (castellano)

INTRODUCCIÓN: Proteínas de interés alimentario y biotecnológico de la semilla del olivo

CAPÍTULO 1. Características histológicas de la semilla del olivo y presencia de Proteínas de Almacenamiento de la Semilla tipo 7S como marcador del desarrollo del fruto.

Zafra A, M'rani-Alaoui M, Lima E, Jimenez-Lopez JC and Alché JD (2018) Histological Features of the Olive Seed and Presence of 7S-Type Seed Storage Proteins as Hallmarks of the Olive Fruit Development. Front. Plant Sci. 9:1481. doi: 10.3389/fpls.2018.01481

CAPÍTULO 2. Contribución para la identificación del proteoma de la semilla del olivo mediante electroforesis 1D/2D y análisis MS/MS

CAPÍTULO 3. Identificación y análisis bioinformático de los principales transcritos de SSPs en la semilla del olivo

3.1. Identificación basada en el transcriptoma de una legumina de semilla de olivo (globulina 11S). Caracterización de subunidades, modelaje tridimensional y evaluación molecular de alergenicidad.

Zafra A., Jiménez-López J.C., Carmona R., Claros G., Alché JD. (2016) Transcriptome-Based Identification of a Seed Olive Legumin (11S Globulin). Characterization of Subunits, 3D Modelling and Molecular Assessment of Allergenicity. In: Ortuño F., Rojas I. (eds) Bioinformatics and Biomedical Engineering. IWBBIO 2016. Lecture Notes in Computer Science, vol 9656. Springer, Cham

3.2. Identificación y evaluación de la potencial alergenicidad de las vicilinas 7S en semillas de olivo (*Olea europaea* L.)

JC. Jimenez-Lopez, A Zafra, L Palanco, JF Florido, and JD Alché, (2016) Identification and Assessment of the Potential Allergenicity of 7S Vicilins in Olive (Olea europaea L.) Seeds. BioMed Research International, vol. 2016, Article ID 4946872, 11 pages, https://doi.org/10.1155/2016/4946872

CAPÍTULO 4. Evidencia de las ventajas alimentarias y propiedades nutraceúticas de los componentes de la semilla del olivo

4.1. Aislados proteicos de semilla de olivo: composición, propiedades funcionales y caracterización proteica. *A. Zafra, A. Zienkiewicz, A. Clemente, S. Al-lach, A. Fernández Márquez, I. Martín Aznarte, A. Rueda, C. Salmerón, J.C. Jiménez López, A.J. Castro, MªI. Rodríguez García, J.D. Alché (2013) Aislados proteicos de semillas de olivo: composición química, propiedades funcionales y caracterización proteica. El Aceite de Oliva. Actas Simposio Expoliva 2013 pp 1-7. I.S.B.N. 978-84-938900-1-8*

4.2. Determinaciónen de la presencia/ausencia de gluten en harinas de semilla de olivo

4.3. Efectos anti-inflamatorios de harinas purificadas de semilla de olivo en pacientes diabéticos. *Lima-Cabello E, Alché V, Zafra, A, Sánchez-Rivas JP, Jiménez-López JC, Marco F, Alché JD. (2017) Efectos anti-inflamatorios de harinas purificadas de semillas de olivo en pacientes diabéticos. El aceite de oliva. Comunicaciones Científicas. Simposium Expoliva 2017. ISBN.978-84-946839-1-6*

Discusión general

Anexos

Introduction

Proteins of Alimentary and Biotechnological Interest in the Olive Seed

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The olive oil industry-derived economy has a high impact in the Mediterranean area. Most of the production of extra virgin olive oil is based on multi-step mechanical oil extraction procedures using the whole fruit as primary raw material. The stone (including the seed) is a rich source of different families of proteins, lipids and other compounds of potential interest. Currently, only few studies have been performed concerning the nature and properties of the proteins present in the olive seed, and their implications in the oil properties, as a result of their presence (as traceable elements) in the olive oil after the whole olive fruit processing, in comparison with oils obtained from de-stoned olive fruits. The aim of the present review is to provide a comprehensive insight about the proteins contained in the olive seed, their biochemical properties, cellular location, and functional implications for olive oil properties as described to date. Also, an update of the methods currently used for such analyses is performed. The potential use of olive seed flours and their isolated or modified components as animal/human food complements is further discussed.

KEYWORDS: Legumins, microscopy, *Olea europaea* L., proteomics, seed storage proteins, transcriptomics.

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INTRODUCTION

Alimentary and nutraceutical properties of plant-derived compounds, particularly seed proteins are becoming of interest for their inclusion in animal and human diet due to a wide range of health benefits (Varzakas et al., 2016; Gul et al., 2016). A balanced combination of seed proteins might provide the human requirements of essential amino acids and other macro- and micro-nutrients, with health benefits. These attributes are getting increasing attention among population, and among researchers in particular (Day, 2013). The study of seed proteins has progressed in the last years mainly due to the advance of proteomic-based techniques. These studies have provided abundant knowledge regarding the protein contents in different crop plants such as legumes (soybeans (Min et al., 2015; Xu et al., 2015b; Fercha et al., 2016; Ma et al., 2015), chickpea (Singh et al., 2016; Bhagyawant et al., 2015), lupins (Kasprowicz-Potocka et al., 2016; Pastor-Cavada et al., 2009; Foley et al., 2015), and other pulses (Pastor-Cavada et al., 2011, 2010), maize (Wu and Messing, 2014; Woo et al., 2001), wheat (He et al., 2015; Yang et al., 2016), rice (Yang et al., 2013; Guo et al., 2016; Xu et al., 2015a; Liu et al., 2016), barley (Kristoffersen and Flengsrud, 2000; Kuczýska et al., 2012; Guo et al., 2016), oilseeds, i.e. canola (Hajduch et al., 2006; Jolivet et al., 2009; Liu et al., 2015), and amaranth (Rastogi and Shukla, 2013). In addition, these studies endeavor searching for functional attributes in the protein extracts and hydrolysates from mixes of several oilseed flours (Radha et al., 2007). Preparation of proteins isolates from oilseed plants has been performed, aiming to minimize the presence of toxic compounds (Rodrigues et al., 2012), which are eliminated in further steps. Multiple applications have derived from the elimination of these anti-nutritional compounds, and for example have promoted the use of proteins from terrestrial crops for fish feeding (Fry et al., 2016).

Although alimentary and nutraceutical studies concerning the use of seed proteins for human food and animal feeding, as well as cosmetic or pharmaceutical applications are common in the literature, most of them focus on the above mentioned crop species (oilseeds, legumes or cereals). However, the olive seed had still low consideration for similar analyses. The olive oil has been used as a food for millennia, particularly in the Mediterranean area, where current production reaches over 95% of the world's olive oil. Spain is the largest olive oil producer in the world with a 28% of the total production (Vossen, 2013). Health benefits of the olive oil include improvement and/or prevention of cardiovascular diseases, cancer, high blood pressure, diabetes, obesity, and dysfunctions of skin and the immune and digestive systems, all through during pregnancy, childhood, and ageing (International Olive Council. http://www.internationaloliveoil.org/estaticos/view/87-mediterranean-diet-pyramid).

As mentioned above, scientific literature is little by little dissecting out the components of the olive oil responsible for such beneficial properties. The olive oil presents a major fraction of triacylglycerides (oleic acid being the main fatty acid), which represent over 98% of the total weight, and a minor fraction formed of a complex set of compounds, including over 230 chemical compounds (aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds, phenols and pigments) (Barjol, 2013). Analysis of over 23 virgin olive oils and one refined oil has evidenced that the protein content in these samples ranged from 0.05 to 2.40 mg/kg (Koidis and Boskou, 2006). Although some studies have detected that such protein content may affect oil stability (Hidalgo and Zamora, 2006; Zamora et al., 2001; Hidalgo et al., 2007; Alu'datt et al., 2013, 2014) as well as oil organoleptic properties (Luaces et al., 2003), knowledge regarding the nature and proportion of these proteins is quite limited. Some available analyses discuss the putative tissue origin of olive oil proteins, driving their attention to the olive seed as the most remarkable source of proteins in comparison to the olive mesocarp (Krebesová, 2014; Krebesová et al., 2015).

Herein, we review proteomic, microscopic biochemical and transcriptomic evidence of the presence of proteins of interest in the olive seed, as a first approach to further determine its contribution to the olive oil.

INTRODUCCIÓN

Las propiedades alimentarias y nutracéuticas de los compuestos derivados de las plantas, particularmente las proteínas de semillas, se están convirtiendo en un tema de interés por su inclusión en la dieta en animales y humanos debido al amplio rango de beneficios que aportan a la salud (Varzakas et al., 2016; Gul et al., 2016). Una combinación equilibrada de proteínas de semillas puede suministrar en humanos los requerimientos de aminoáciods esenciales así como otros macro- y micro-nutrientes con beneficios para la salud. Por tanto, está incrementado el interés hacia estos atributos entre la población, y en particular entre los investigadores (Day, 2013). El estudio de las proteínas de semillas ha progresado en los últimos años, principalmente debido al avance en las técnicas basadas en la proteómica. Estos estudios han aportado un ingente conocimiento sobre los contenidos proteicos en las semillas de diferentes plantas cultivadas que incluyen diversas legumbres como la soja (Min et al., 2015; Xu et al., 2015b; Fercha et al., 2016; Ma et al., 2015), garbanzo (Singh et al., 2016; Bhagyawant et al., 2015), altramuz (Kasprowicz-Potocka et al., 2016; Pastor-Cavada et al., 2009; Foley et al., 2015), y otras semillas (Pastor-Cavada et al., 2011, 2010), maíz (Wu and Messing, 2014) (Woo et al., 2001), trigo (He et al., 2015; Yang et al., 2016), arroz (Yang et al., 2013; Guo et al., 2016; Xu et al., 2015a; Liu et al., 2016), cebada (Kristoffersen and Flengsrud, 2000; Kuczýska et al., 2012; Guo et al., 2016), y semillas oleaginosas como la colza (Hajduch et al., 2006; Jolivet et al., 2009; Liu et al., 2015) y el amaranto (Rastogi and Shukla, 2013). Además, estos estudios se esfuerzan en la búsqueda de atributos funcionales en los extractos de proteínas e hidrolizados procedentes de mezclas de harinas de varias semillas oleaginosas (Radha et al., 2007). La preparación de aislados proteicos de semillas oleaginosas ha sido realizada con el objetivo de minimizar la presencia de compuestos tóxicos (Rodrigues et al., 2012), los cuales son eliminados en pasos posteriores. Múltiples aplicaciones se derivan de la eliminación de estos compuestos anti-nutricionales, y por ejemplo han impulsado el uso de proteínas de cultivos terrestres para alimentación de peces (Fry et al., 2016).

Aunque los estudios sobre alimentación y nutracéutica relativos al uso de proteínas de semillas para alimentación humana y en animales, así como para aplicaciones cosméticas o farmacéuticas son comunes en la bibliografía, la mayoría de ellos se centran en los cultivos mencionados previamente (semillas oleaginosas, legumbres o cereales). Sin embargo, las semillas de olivo han tenido hasta ahora poca consideración para análisis similares. El aceite de oliva ha sido usado como alimento durante milenios, particularmente en el área del Mediterráneo, donde la producción actual supera el 95% a nivel mundial. España es el principal productor de aceite del mundo con un 28% de la producción total (Vossen, 2013). Los beneficios para la salud del aceite de oliva incluyen mejora y/o prevención de enfermedades cardiovasculares, cáncer, hipertensión, diabetes, obesidad y disfunciones de la piel y de los sistemas inmune y digestivo a lo largo del embarazo, infancia y envejecimiento (International Olive Council. http://www.internationaloliveoil.org/estaticos/view/87-mediterranean-diet-pyramid).

Como se ha mencionado previamente, la bibliografía científica está poco a poco desmembrando e identificando los componentes del aceite de oliva responsables de dichas propiedades beneficiosas. El aceite de oliva presenta una fracción mayoritaria de triacilglicéridos (siendo el ácido oleico el principal ácido graso), que representa más del 98% del peso total y una fracción minoritaria formada por un complejo conjunto de compuestos, entre los cuales se incluyen más de 230 compuestos químicos (alcoholes alifáticos y triterpénicos, esteroles, hidrocarbonos, compuestos volátiles, fenoles y pigmentos) (Barjol, 2013). Análisis realizados en 23 aceites de oliva virgen y un aceite refinado han demostrado que el contenido proteico en dichas muestras oscilaban entre 0.05 y 2.40 mg/kg (Koidis and Boskou, 2006). Aunque algunos estudios han detectado que dicho contenido proteico puede afectar a la estabilidad del aceite (Hidalgo and Zamora, 2006; Zamora et al., 2001; Hidalgo et al., 2007; Alu’datt et al., 2013, 2014) así como a las propiedades organolépticas del aceite (Luaces et al., 2003), los datos relativos a la naturaleza y proporción de estas proteínas son bastante limitados. Algunos análisis disponibles discuten el posible origen tisular de las proteínas presentes en los aceites de oliva, dirigiendo su atención a las semillas del olivo como una fuente importante de proteínas en comparación con el mesocarpo del fruto del olivo (Krebesová, 2014; Krebesová et al., 2015).

En el presente documento realizamos una revisión de las evidencias de la presencia de proteínas de interés en la semilla del olivo a nivel proteómico, microscópico, bioquímico y transcriptómico, como una primera aproximación para posteriormente determinar su contribución al aceite de oliva.

THE PROTEIN COMPOSITION OF THE OLIVE SEED

First evidence of the presence of specific proteins in the olive seed was described by Ross et al. (Ross et al., 1993), who reported that Oil Bodies (OBs) from endosperm and embryo tissues contained about 10% (w/w) of the total seed proteins. They associated certain proteins to the oil body membrane on the basis of immunolocalization experiments, and attributed these proteins to the oleosin family, mostly composed of 22- and 50-kDa polypeptides. Further analyses of oleosin gene expression in the olive embryo showed the presence of oleosin transcripts corresponding to a amino acid sequence of 165 residues, with a core hydrophobic domain including a highly conserved ‘‘proline knot’’ sequence and a amphipathic α-helix at the carboxi terminal end. The phylogenetic analysis of these transcripts showed a group, namely “H group”, that included all of them (high molecular weight oleosins) (Giannoulia et al., 2007; Tzen et al., 1990). Oleosins from the H group, previously described (Ross et al., 1993) (aprox. 20 kDa), were the most abundant proteins in the OBs and the microsomal fractions of the olive seeds (Hidalgo et al., 2001). These authors also revealed that the oleosins displayed a similar pattern of gene expression in embryo and endosperm during the olive drupe development. Such expression pattern is characterized by a protein synthesis starting from the late heart embryo stage (11 weeks after flowering -WAF-), reaching the maximum level at mid-torpedo stage (16–19 WAF), to continue with a rapid decrease. No oleosin forms belonging to the L group (low molecular weight oleosins) have been described in the olive seed, nevertheless the presence of low abundance polipeptides of 4.6 kDa have been described (Hidalgo et al., 2001). Such peptides have been proposed to represent oleosin-like proteins (Hidalgo et al., 2001), although no additional experimental or other supporting evidence indicate that these protein fraction would correspond to partially degraded oleosins.

The potential presence of enzymes involved in the desaturation of reserve lipids (Fatty Acid Desaturases: FAD2-1 and FAD2-2) in young and mature seed stages of development has been previously described in the olive tree (Hernández et al., 2005). FAD2-1 is expressed in very young seeds (13 WAF) followed by a decrease in the expression afterwards. FAD2-2 displayed moderated expression levels in seed tissues during the late stages of development, with a small increase of the expression at 31 WAF. Opposite results were found by Banilas et al., (2005), who reported very low level of expression of FAD2 in both, the endosperm and the embryo tissues. In addition, the expression level of FAD-6 started to be noticeable 16 WAF, reaching the highest level at 22 WAF. FAD2 and FAD6 transcripts were detected in both the mesocarp and seed tissues, however, in both cases the level of expression were lower in the seed, with the exception of the FAD-6 at the stage of 22 WAF (Banilas et al., 2005). Other enzymes such as lipase and lipoxygenase (LOX) have been reported in the olive seed as well (Zienkiewicz et al., 2014). At seed maturity, seven bands revealed lipolytic activity on a SDS-PAGE assay, where one of them (53 kDa) was notably more active, lasting until the third day of *in vitro* seed germination. Interestingly, only five bands displayed lipase activity after seed imbibition. Low, but constant activity, was detected for proteins of 40 kDa during the whole course of the *in vitro* seed germination and seedling growth. Expression of two different transcripts and two bands of activity corresponding to LOX protein isoforms were detected in the olive seed (OeLOX1 and OeLOX2). OeLOX1 showed an increased expression pattern after seed imbibition reaching the maximum level at 6 hours of *in vitro* germination, followed by a decrease after the third day, reaching stable levels afterwards. On the other hand, OeLOX2 reached the maximum level of expression at the fourth day of seed germination, with a strong decrease from that point onwards (until day 26 of the *in vitro* seed germination experiments). Immunobloting analyses revealed the presence of two bands (98 kDa and 100 kDa), probably corresponding to two OeLOX2 isoforms. Additionally, the activity over SDS-PAGE gels showed a third LOX, with a molecular weight of approximately 96 kDa. The accumulation of LOX is associated to the oil bodies in the mature olive endosperm (Zienkiewicz et al., 2014).

Seed Storage Proteins (SSPs) are the most abundant proteins in the mature olive seed, and represent approximately 70% of total seed proteins (Alché et al., 2006). Among SSPs, 11S globulins have been described as the most representative. 11S protein analysis in the olive seed (Alché et al., 2006) revealed the presence of two precursors of the 11S globulins, giving rise to individual mature proteins named p1 to p5, with molecular weights of 20.5, 21.5, 25.5, 27.5, and 30 kDa, respectively. A similar analysis performed in both the endosperm and the embryo of the olive seed by using high resolution 2D-gels disclosed the presence of multiple spots, some of them corresponding to the p1 to p5 polypeptides. Furthermore, evidence of the presence of posttranslational modifications was abundantly detected in these forms of 11S polypeptides (Alché et al., 2006). The analysis of 11S proteins presence in the olive seed tissues displayed a developmental pattern, characterized by three periods of protein synthesis: (I) early synthesis (before105 days after anthesis -DAA-), (II) rapid and massive synthesis and accumulation (105-130 DAA) and (III) slow synthesis and accumulation (130 DAA onwards) (Wang et al., 2001). Seed maturity at this regard might be reached between 90 to 105 DAA given the presence of the mature forms of the SSPs in the olive seed at this time (Wang et al., 2007b).

A recent *in silico* analysis allowed the identification of various 11S sequences in different seed tissues of the olive (Zafra et al., 2016). These sequences shared high identity with the isoform 2 of the 11S proteins in other species, as reveled by phylogenetic and structural analyses. In this regard, the predicted 3D structure also highlighted the similarity to the sequence of this isoform 2 of the olive 11S globulin, with a high level of identity. On the other hand, the analysis of molecular signatures for allergy triggering in the alpha and beta subunits of this isoform revealed the presence of predicted epitopes recognized by the T-cells (eight and four for α and ß subunits, respectively), suggesting that 11S proteins may act as a potential source of allergy in humans. Studies of digestibility (also involved in the development of allergy phenomena) of olive 11S seed storage proteins have revealed that 11S proteins are easily degraded during the digestive process in animals (Ben Ali, 2011). The peptides generated in this process were not recognized by anti-11S antibodies, thus suggesting a low probability of their recognition as allergens after digestion. Microscopical and biochemical analyses also showed alterations in the structure of the Protein Bodies (PBs) mainly composed by 11S proteins (Alché et al., 2006; Ben Ali, 2011) following digestion. Consequently, the 11S proteins may be considered as putative food for animals, as they can be easily digested, and the hydrolyzed forms offer low allergenicity (Ben Ali, 2011). Similarly to other plant species giving rise to protein isolates, the olive seed has been studied aimed to take advantage of the high protein content. Different methodologies have been set up for olive seed protein isolation (Zafra et al., 2013), and to provide a solid approach to obtain proteins as potential uses for human food.

An analogous strategy has been recently utilized to investigate the presence of additional SSPs in the olive seed. In this regard, the reproductive transcriptome database ReprOlive (Carmona et al., 2015) was scrutinized in the search of sequences corresponding to 7S globulins belonging to the vicilin family and Cupin superfamily (Jiménez-López et al., 2016). The data retrieved from the olive seed transcriptome allowed performing a phylogenetic analysis and building 3D structures, which revealed high similarity between olive 7S globulins and those from other species. The identification of epitopes and comparative molecular allergy analysis indicated that olive seed 7S proteins display 19 T-cells epitopes along the sequence. Nine out of nineteen epitopes where commonly found in at least one orthologue protein from other plant species. Seventeen B-cells epitopes were identified, where 11 out 17 were also commonly found in other orthologous 7S globulins. These results point to a high probability of cross-reactivity between olive 7S proteins and these orthologues from other species (Jiménez-López et al., 2016). Such cross-reactivity is currently experimentally assessed with the aid of recently raised antibodies to common synthetic peptides (Zafra et al., 2018).

CELLULAR LOCALIZATION OF OLIVE SEED PROTEINS

In mature/imbibed olive seeds the proteins are mainly located in the PBs that are surrounded by OBs (Zienkiewicz et al., 2011,2014). During the first hours of the *in vitro* germination process, the number of PBs is reduced, although they became much larger to finally form one large compartment. The differentiation of the cells of the seed is coincident with a decrease of the PBs (Zienkiewicz et al., 2014). The endosperm and the embryo tissues of the olive seed have shown to be highly enriched in these structures, with a characteristic location, and surrounded by numerous OBs (Zienkiewicz et al., 2011, 2014). Various populations of PBs in terms of size, shape and fluorescence intensity after immunolabeling with anti 11S-proteins were differentiated between endosperm and cotyledon (Jiménez-López and Hernández-Soriano, 2014). Such differences may be representative of functional differences among PBs, based on differential proteins content and/or protein folding and packing. A particular cytological feature of olive cotyledon cells during the first steps of *in vitro* germination process consists in the fusion of PBs, leading to the formation of a vacuole filling up the whole cytoplasm. From this step onwards, different mobilization processes take place (Jiménez-López and Hernández-Soriano, 2014) until cotyledons become green. Once autotrophic state is reached, no PBs are visible (Zienkiewicz et al., 2014).

Interestingly, the biogenesis of PBs in the olive seed is characterized by asynchrony in PBs formation between the cotyledon and the endosperm. Thus, between 60 and 210 DAA, there is a high level of synthesis of 11S protein in the endosperm. Contrarily, the cotyledon displayed low quantity of 11S protein at 60 DAA, and massive synthesis of 11S proteins only started from 90 DAA onwards. Immunolocalization studies in both tissues revealed that small accumulations of proteins started to be visible at the marginal side of the cytoplasm 20 DAA. In the endosperm cells, at 60 DAA the presence of 11S proteins appeared scattered and spread along the cytoplasm to finally form a high abundant population of well-defined, compartmentalized PBs at 145 DAA. The proteins could be synthetized through the ER pathway to pre-vacuolar compartment, ending the mature forms into large storage vacuoles. In the mature stage, (210 DAA), the number of PBs has increased in the endosperm, and they display a regular shape. However, cotyledon PBs exhibited a slight delay in their formation time (Jiménez-López and Hernández-Soriano, 2014). Moreover, and although the endosperm and the cotyledon are considered both storage tissues in the olive, the PBs in both tissues show morphological differences (Jiménez-López and Hernández-Soriano, 2014). Previous studies pointed the endosperm cells to possess an uniform morphology containing numerous and densely packed PBs surrounded by OBs. Alternatively, cotyledon cell were described with a variety of morphological shapes, with a major number of cells presenting low quantity of PBs and OBs (Alché et al., 2006; Jiménez-López and Hernandez-Soriano, 2014). Later studies, have shown a detailed description of the anatomy and histology of both, the embryo and the endosperm, revealing that embryo and endosperm possess a characteristic pattern of distribution of the PBs/OBs which is developmentally stage-dependent. Besides, the size, quantity, pattern of distribution, and intensity of staining of the PBs may change within a tissue, probably related with ontogenetic issues. The same event happens with the size, quantity and pattern of distribution of the OBs. A complementary distribution of both storage materials has been described (Zafra et al., 2018).

Along the seed imbibition and the *in vitro* germinating process, biochemical analyses indicate a correlation between the decrease of the 11S precursor forms and an increment of the presence of 11S subunits, which is associated with the protein breakdown initiated by proteinases. Morphological features observed in both the endosperm and cotyledon PBs (particularly regarding number and density of these organelles) are in good agreement with this suggested mobilization (Zienkiewicz et al., 2011; Wilson et al., 1986; Shutov and Vaintraub, 1987; Bewley and Black, 1994; Müntz, 1998; Schlereth et al., 2000; Wang et al., 2007a).

The presence of key enzymes in the olive cotyledon cells including lipase, PLA and LOX is also mainly restricted to the PBs, as demonstrated by (Zienkiewicz et al., 2014). Lipase activity in mature seeds is mainly accumulated in the PBs After the imbibition, it was detected the presence of spots within the PBs without lipase activity.

After 6 hours of *in vitro* germination, the lipase activity was more intensively detected in the PBs, but also appeared spread in the cytoplasm. However, at the 3th day of *in vitro* germination, the lipase activity was detected only in the matrix of the PBs. During seedling growth (4th day), the enzyme was present surrounding a unique, large PB. Finally, after 15 days of seedling growth, lipase activity was located in the cytoplasm that surrounds the central vacuole. Regarding lipoxigenase, LOX enzyme was detected within the PBs in the mature seeds. After the imbibition stage, the signal intensity of this enzyme increased in the PBs and started to be present in the cytoplasm surrounding the OBs. After 3 days of *in vitro* germination, the enzyme was located in the PBs matrix. One day later (4th day of *in vitro* germination), the enzyme was also detected surrounding the PBs and in the surrounding cytoplasm, and low activity in the rest of the cytoplasm. In the 15th day of the seedling growth, the lipase was detected in chloroplast and the surrounding cytoplasm (Zienkiewicz et al., 2014). Moreover it was suggested that a linoleate 13-LOX is located at the membranes of oil bodies of olive endosperms. The localization of lipase and PLA in living olive cotyledon indicated that in mature seed, the lipase/PLA activities were detected in the area of the PBs. After the imbibition, the activity was also detected in the OBs (Zienkiewicz et al., 2014).

FUNCTIONAL FEATURES OF THE OLIVE SEED PROTEINS

Oleosins in the seeds of different species are small proteins of approximately 15-26 kDa (Murphy, 1993; Huang, 1992). Oleosins have been suggested to be present in all oil-bearing plant tissues suffering a period of desiccation followed by a step of OBs mobilization. They have been proposed to have roles in the formation of the OBs during seed development, during the stabilization of lipid bodies in seed desiccation and afterward mobilization in the germination, or probably in all of the processes (Ross et al., 1993). Oleosins are also implicated in the development of the oilseed by stabilization the OBs during the imbibition stage (Leprince et al., 1998). The oleosins stabilize the OBs surface through a protective layer that firmly anchors thanks to a long hydrophobic stretch (Huang, 2016), shielding phospholipids against the phospholipases (Tzen and Huang, 1992). Regarding signal transduction, the oleosins could also act as receptors during the germination, binding triacylglycerol (TAG)-degrading lipases. They are prone to ubiquitination, therefore becoming tagged for the degradation process (Huang, 2016). Also, oleosins have been proposed to play a key role preventing dehydration, and the rehydratation prior the germination (Leprince et al., 1998). The hydrophilic surface of the OBs of the seed is due to the presence of oleosins, but also to PL that interact between them with a defined molar ratio for maximal effectiveness(Tzen and Huang, 1992). Besides the structural functions, in Arabidopsis seed, oleosins have been related to the promotion/limitation of OB fusion (Miquel et al., 2014).

The enzymes present in the olive seed tissues are implicated in seed germination. The task of the PLA and lipase activities are carried out in the surface of the OBs during the germination process, being firstly the PLA responsible for the phospholipid breakdown, allowing the lipase to mobilize the TAG from the OBs. The lipase activity, firstly present in the PBs and later transferred to the OBs during the seed germination, could facilitate the metabolism of storage lipids in PBs during seed germination. PBs are pointed out as dynamic-multifunctional organelles implicated in the storage of enzymes involved in the mobilization during seed germination (Zienkiewicz et al., 2014). LOX activity, which correlates with PLA and lipase activity on the OBs surface, could be involved in seed germination as they possess a similar activity to a peroxidase (POX) (Zienkiewicz et al., 2014; Yi et al., 2005). Besides, the linoleate 13-LOX from the endosperm OBs could be involved in the oxygenation of free linoleic acid (Georgalaki et al., 1998a). FAD2-1 is proposed to be responsible for the enhanced linoleic content observed in the olive seed at early stages of development (13 WAF) (Hernández et al., 2005). Moreover, the oleate desaturation in this premature stage coincidentally occurs with a quick accumulation of oil in the olive seed (12-22 WAF) if compared with the slow oil deposition occurring in the mesocarp (Hernández et al., 2005; Sanchez, 1994). On the other hand, the alternative isoform of the oleate desaturase described in the microsomal fraction, (OepFAD2-

2) takes part in different physiological roles, and is probably involved in the desaturation of storage lipids in the mature seed and the mesocarp (Hernández et al., 2005).

In advance to seed germination, part of the PBs fuse with degradative compartments with lytic activity such as lytic vesicles (Bewley and Black, 1994). This process has been poorly studied in the olive seed. The post-germination synthesis of new proteases is essential for protein utilization in the cotyledons (Bewley et al., 2013).

Up to date, the 11S and the 7S globulins have been described in the olive seed, where 11S is the most abundant protein in the seed (Alché et al., 2006; Jiménez-López et al., 2016). During germination and seedling growth, the complete degradation of SSPs takes place inside the PBs This process is usually triggered by newly formed proteinases (Shutov et al., 2003). The 7S and 11S include specific sites susceptible to restricted proteolysis, being a key property in the storage process. Two kinds of hydrophilic polypeptide regions (I1 and I2) are responsible for this function. Both, 7S and 11S globulins have the inserts I1 and I2 that act as target to proteolytic attack. Thus, it is suggested that the acquisition of the I1 and I2 sites is essential for the storage function of a protein. However the storage function may be acquired independently for 7S and 11S globulins. Although, with exceptions, the general rule is that the presence of Asn residues within the I1 and I2 sites could be responsible for the protection against the proteolysis attack. It has been also suggested that additional sites must be responsible for unlimited proteolysis (Shutov et al., 2003). SSPs are involved in the supply of aminoacids for protein synthesis for radicle emergence and post-germinative stages. In both, leguminous and non-leguninous seeds, a general pattern of SSPs proteolysis is proposed (Bewley et al., 2013). During the germination there is a breakdown of the SSPs that take place independently in the embryonic axis and the cotyledon, generating an amino acid source that is employed in the protein biosynthesis. However, during the seedling growth process, there is a transfer of amino compounds from the cotyledon into the axis (Schlereth et al., 2000). In the olive seed, the 11S proteins are stored in the endosperm and cotyledon.

Preliminary biochemical (2-D electrophoretic separation) analyses have shown the presence of spots attributed to 11S protein displaying similar molecular weight but different isoelectric point, which may indicate the presence of posttranslational modifications (PTMs) in these proteins (Alché et al., 2006). The PTMs could be involved in regulatory processes, in degradation pathways, or probably in both (Wang et al., 2015). As an example, the mobilization of the SSPs in the olive cotyledon occurs gradually, until the seed becomes autotrophic (Zienkiewicz et al., 2011).

SEED PROTEINS MAY AFFECT OLIVE OIL PROPERTIES

Several studies have been performed aimed to analyze oil components affecting its functional properties. Among these compounds, proteins are key elements (mostly 11S globulins) which can be co-extracted along with the olive oil. Luaces et al., (2003) showed that several enzymes contained in the seed are responsible of the olive oil aroma. Among these enzymes, there are lipoxigenases, alchohol dedydrogenases, alcohol acyltransferases, and hydroperoxide lyases, as well as others enzymes related to 13-hydroperoxide metabolism. In addition, proteins and protein hydrolysates in edible oils seem to function as antioxidants(Hidalgo and Zamora, 2006). Polypeptides present in olive oils may play important roles in conjunction with other olive oil antioxidant components (Zamora et al., 2001). This antioxidant activity seem to increase in oils when proteins interact with lipid oxidation products (Hidalgo and Zamora, 2006), tocopherols (Hidalgo et al., 2007), or phenolic compounds (Alu'datt et al., 2013, 2014).

The scarce number of analyses available regarding the mesocarp indicate that proteins represent between 1.3-1.8% of the dry weigh of the whole fruit, and it has been suggested proteins from mesocarp act as stabilizers for the oil (Zamora et al., 2001).

The analysis of the proteins of the olive oil (originated from both, the seed and the mesocarp) showed the presence of lipoxygenase and polyphenol oxidase activities (Georgalaki et al., 1998b). LOX from olive endosperm may be detectable in virgin olive oil still active (Georgalaki et al., 1998a). The implications in the olive oil stability of the compounds originated from the olive stone is still controversial. Experiments carried out on olive oils from stoned and de-stoned fruits suggest that the resistance to oxidation as well as the main organoleptic and physicochemical parameters are not affected by the compounds present in the stone (Patumi et al., 2003). Alternatively, Angerosa et al., (1999) proposed that oils from de-stoned fruits have positive repercussion on the flavour. Another study reveals that olive oils obtained from de-stoned fruits display positive physico-chemical properties compared to standard processing procedures including a better acidity degree, coefficient value K_{270} and antioxidant capacity (Joorani, 2010). The quantity of 11S globulin that is transferred from the seed to the olive oil is uncertain. Probably, low amounts of 11S proteins pass to the olive oil since the presence of proteins in the olive mill residues has been detected in substantial quantities (Jiménez-López et al., 2007). The similarity in the aminoacidic content and the SDS- PAGE profile of the SSPs from the seed and the alpeorujo, revealed that almost all the protein content of the olive fruit remains in the waste derived from the olive oil production (Jiménez-López et al., 2007). Additional evidence of the presence of other proteins from the olive seed in the olive oil is beginning to emerge (Krebesová, 2014; Krebesová et al., 2015), however, much effort has to be dedicated to this purpose.

METHODOLOGICAL APPROACHES FOR THE DETERMINATION OF OLIVE SEED PROTEINS

A wide variety of methods have been used to study the proteins present in the olive seed. A broad review on this topic was performed by Montealegre et al., (2014). Among the described methods, the SDS-PAGE, Western-blotting and spectrophotometric assays were the most commonly used for the identification of proteins of a specific weight or protein activity. The use of high-sensitivity proteomic techniques to analyse seed protein extracts is frequently surpassed by the problem of the presence of highly abundance proteins, corresponding to SSPs, which mask the potential detection of minor proteins present in the samples. This is currently solved by using proteomic depletion methods (Miernyk, 2014). The use of peptide ligand libraries able to detect low abundance proteins is a new technology that has been used in olive seed in combination to nL-MS/MS, identifying 61 unique gen products (Esteve et al., 2012), including 14 histones, two isoforms of globulins, 3 isoforms of the 11S legumin, 1 isoform of the 7S vicilins, and one isoform of oleosin.

The use of 1-D and 2-D electrophoresis have been successfully performed using complete olive seed tissues without SSPs depletion (Alché et al., 2006). In this case, the use of specific antibodies in Western blotting experiments allowed the recognition of non-mature (pre-proteins) forms, and the alpha and beta subunits of the 11S proteins in individual extracts of the whole seed, the embryo and the endosperm. The pattern of spots obtained after 2-D gel analysis of the extracts also revealed that both, the embryo and the endosperm tissues, present a comparable proteome at the mature stage of development (Alché et al., 2006). The detection of isoforms and PTMs (glycosylation, acetylation, phosphorylation and deamidation) is also feasible by using this methodology (Alché et al., 2006; Rogowska-Wrzesinska et al., 2013). Regarding these methods, the selection of an appropriate extraction buffer is of paramount importance in order to extract the larger quantity of proteins as possiblen(Esteve et al., 2012; Vergara-Barberán et al., 2014).

Identification of different olive cultivars has also been achieved using Capillary Electrophoresis (CE) methodologies combined with SDS-PAGE to analyse the whole olive fruit and the seed tissues, respectivelyn(Vergara-Barberán et al., 2014; Montealegre et al., 2010). Montealegre et al., (2010) described for the first time a method to recognize different protein profiles by CE aimed to identify different olive cultivars using protein extracts from both, mesocarp and stone. As a result, different profiles were clearly obtained, which probably were due to a differential pattern of proteins accumulation in the seeds. Similarly, an optimized method for protein extraction from olive stone followed by SDS-PAGE is also proposed to identify specific electrophoretic profiles and band intensities. This method is also helpful to distinguish stone samples according to their genetic varietal origin (Vergara-Barberán et al., 2014). Capillary electrophoresis–mass spectrometry (CE–MS^2) has also been used to detect non-protein amino acids in the oils, mainly originated in the seed, allowing authentication of the extra virgin olive oil extraction with a level of detection of 2.5-10% of mixture (w/w) (Sánchez-Hernández et al., 2011). Alternatively, the use of HPLC in the study of proteins present in the olive oil may provide amino acids profiles (Hidalgo et al., 2002). At the cellular level, the use of microscopy techniques and immunocytochemistry is also a valuable tool for the identification and localization of seed proteins (Zienkiewicz et al., 2014; Alché et al., 2006; Zienkiewicz et al., 2011; Jiménez-López et al., 2015, 2007). These approaches are highly dependent on the availability of specific antibodies to the required protein (Wang et al., 2007b; Jiménez-López et al., 2016).

FUTURE PERSPECTIVES

Despite the large amount of proteins contained in the olive seeds, current use of the olive seeds in the food industry is still limited. Traditional extraction methods are still massively applied in the production of the olive oil, based in a mechanical extraction using the whole fruit. The process generates large quantities of waste and byproducts, which in one hand, contain high-added value compounds, and toxic and environmental compromising elements at the other side (Roselló-Soto et al., 2015).

Alternative processing of the olive fruit, implementing a prior separation of the stone, and the generation of differential fractions obtained from the later (empty stones, whole seeds, seed oils, flours, protein isolates, purified proteins and many others) would enable the generation of innovative products of interest, reducing waste without compromising olive oil quality.

Pioneer studies of nutritional and nutraceutical use of some of these fractions has revealed very interesting applications in animal feeding (i.e. in fisheries, Alché et al., personal communication), and in *ex vitro* assays with cultured blood (Lima et al., 2015; Lima-Cabello et al., 2017) (chapter 5.3 in the present work). Additional applications require a extensive characterization of the protein fraction (among others characterizations) of the olive seeds, which is approached in different chapters of the present work. Additional and extensive Information can be retrieved from the ReprOlive database (Carmona et al., 2015), which contains extremely relevant information regarding thousands of transcripts expressed in the olive seed tissues. Such analysis should be started by a functional classification of the transcripts, and a selection of the most promising gene products on the basis of parameters like their alimentary interest, demonstrated nutraceutical relevance, and enzyme activity among others, and without forgetting putative antinutritional aspects like allergenicity and toxicity. The information retrieved from these bioinformatics approaches should be further contrasted through biochemical and alimentary validations.

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Objectives

The main purpose of the present work was to deep into the knowledge about the olive seed regarding several aspects not well described until now, which include structural and histological definition of the olive seed, its proteome, the contribution and characteristics of their main protein constituents and the determination of critical aspects for the alimentary use of olive seed flours as healthy food. Four specific objectives were formulated for these purposes:

1. Describe major anatomical and histological features of the olive seeds across their development, and the contribution of the different tissues to the total weight of the seed as well as cultivar variability.

2. Identify the proteome of the olive seed at the mature stage, as key potential contributor to olive seed-derived food products and to the protein composition of the (extra) virgin olive oil.

3. Characterize fundamental properties of the seed storage proteins 7S and 11S as representative and highly abundant protein components of the olive seed.

4. Determine valuable properties of olive seed flours aimed to define alimentary and nutraceutical uses.

Objetivos

El objetivo principal del presente trabajo fue profundizar en el conocimiento de la semilla del olivo en relación a varios aspectos no bien descritos hasta el momento, incluyendo descripciones estructurales e histológicas de la semilla del olivo, su proteoma, la contribución y características de los principales constituyentes proteicos y la determinación de aspectos críticos para uso alimentario de harinas de semillas como alimento saludable. Cuatro objetivos específicos fueron formulados para dichos fines:

1. Describir las principales características anatómicas y funcionales de la semilla del olivo a lo largo de su desarrollo y la contribución de los diferentes tejidos al peso total de la semilla así como la variabilidad entre variedades.

2. Identificar el proteoma de la semilla del olivo en el estadio de madurez como contribuyente potencial clave en los productos alimentarios derivados de la semilla de olivo y a la composición proteica del aceite de oliva virgen (extra).

3. Caracterizar las propiedades principales de las proteínas de almacenamiento 7S y 11S como componentes representativos y altamente abundantes de la semilla del olivo.

4. Determinar propiedades valiosas de las harinas de semillas de olivo para definir usos alimentarios y nutracéuticos.

CHAPTER 1: Histological features of the olive seed and presence of 7S-type Seed Storage Proteins as hallmarks of the olive fruit development

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Histological Features of the Olive Seed and Presence of 7S-Type Seed Storage Proteins as Hallmarks of the Olive Fruit Development

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The production of olive oil is an important economic engine in the Mediterranean area. Nowadays, olive oil is obtained mainly by mechanical processes, by using the whole fruit as the primary raw material. Although the mesocarp is the main source of lipids contributing to olive oil formation, the seed also contributes to the olive oil composition and attributes. The olive seed is also becoming an interesting emerging material itself when obtained after alternative processing of the olive fruit. Such seed is used for the production of differential oil and a unique flour among other bioactive products, with increasing uses and applications in cosmetics, nutrition, and health. However, olive seed histology has been poorly studied to date. A complete description of its anatomy is described for the first time in the present study by using the 'Picual' cultivar as a model to study the development of the different tissues of the olive seed from 60 to 210 days after anthesis. A deep analysis of the seed coats, endosperm storage tissue and the embryo during their development has been performed. Moreover, a panel of other olive cultivars has been used to compare the weight contribution of the different tissues to the seed, seed weight variability and the number of seeds per fruit. In addition to the histological features, accumulation of seed storage proteins of the 7S-type (β-conglutins) in the seed tissues has been assessed by both biochemical and immunocytochemical methods. These hallmarks will help to settle the basis for future studies related to the location of different metabolites along the olive seed and mesocarp development, and therefore helping to assess the appropriate ripening stage for different commercial and industrial purposes.

Keywords: β-conglutins, cotyledon, development, endosperm, olive, radicle, seed, seed storage proteins

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INTRODUCTION

Alimentary industries based in the preparation of table olives and olive oil are of paramount importance for the economy of Mediterranean countries and some areas of America and Australia. The very well valued Extra Virgin Olive Oil (EVOO) is produced exclusively by mechanical processes where the whole fruit is used. Other olive oil qualities [Virgin Olive Oil (VOO) and

Ordinary Virgin Oil (OVO)] are also produced mainly from whole fruits as the primary raw material. Thus, the obtained juices also contain components from the seed, which contribute to olive oil aroma and other potential properties like peroxidase activity (Luaces et al., 2003, 2007). The olive seed itself is also becoming an interesting material with multiple uses beginning to emerge (Rodríguez et al., 2008; Matos et al., 2010; Pattara et al., 2010; Naghmouchi et al., 2015). Morphological characteristics of the olive pit have been used as descriptors of pomological interest for varietal characterization in the olive tree (Barranco and Rallo, 1984). However, differences between the seed have not been reported in detail to our knowledge.

The histology of the different tissues of the seeds have been described in a variety of species other than olive (*Olea europaea* L.). In these studies, the structure of the seed coat was one of the most widely topics described in the literature. Early in the thirties, a deep study on the almond seed surface was performed aimed to easily distinguishing the different varieties of almonds, hence helping identifying misrepresentation or adulteration (Pease, 1930). Examination of *Arabidopsis* seed coat development showed major morphological changes associated with the transition of the integuments into the mature seed coat (Beeckman et al., 2000). Analysis of the seed coat histological distribution has also been performed in *Cucurbita pepo* L. to examine mutations concerning the lignification of the testa (Zraidi et al., 2003). Similarly, the seed coat of *Chenopodium quinoa* was histologically studied aimed to assess and improve quality of the seeds for human and animal consumption (Raamsdonk et al., 2010). The seed coat form of other species such as *Passiflora ligularis* Juss or *Strychnos potatorum* L. has also been analyzed (Cárdenas-Hernández et al., 2011; Mishra and Vijayakumar, 2015). Regarding endosperm anatomy, a new approach in the disclosure of the history of flowering plants has been provided after comparison of the patterns of endosperm development as well as analysis of phylogenetic and ontogenic evolution of this tissue using several basal flowering plants (Floyd and Friedman, 2000). The histology of the seeds from plants like *Vitis vinifera* L., *Paronychia*, *Theobroma cacao* L., *Annona squamosa* L., and *Medicago truncatula* has been described (Cadot et al., 2006; Kaplan et al., 2009; Rangel-Fajardo et al., 2012; Martínez et al., 2013; Verdier et al., 2013). The structure and storage content of *Arabidopsis* and *Cuphea glutinosa* endosperms has also been scrutinized (Li et al., 2006; Di Santo et al., 2012). Finally, the anatomy of the cotyledons has been particularly studied in *Theobroma cacao* L. and *Eurycoma longifolia* seeds (Elwers et al., 2010; Danial et al., 2011), where descriptions of the pattern of distribution of the polyphenolic compounds and the development of the vascular system have been provided. By means of non-destructive techniques, the structure of whole seeds has been also examined. As result, valuable information about the transport system for gas exchange in embryos of the *Arabidopsis* seed has been provided (Cloetens et al., 2006). Similarly, a 3D reconstruction of the compartments present in the maize seed have been performed (embryo, endosperm, nucellus, and pericarp) from 7 to 21 days after pollination (Rousseau et al., 2015).

The number of studies focused on the olive seed histology is still reduced. Initial studies dealt with the description of morphological, histological, and ultrastructural changes in the olive pistil during flowering (Suárez et al., 2012), and the localization of seed storage proteins (SSPs) in the olive seed. SSPs are synthesized in abundance in the developing seeds and are accumulated primarily in the protein storage vacuoles (PSVs) of terminally differentiated cells of embryo and endosperm (Herman and Larkins, 1999). Previous reports indicate that mature olive seeds contains very similar subcellular structure in both the embryo and endosperm tissues, essentially with electro-dense protein bodies (PBs) surrounded by lipid bodies with diameters ranging from 0.5–2.0/μm (Ross et al., 1993; Zienkiewicz et al., 2014). The endosperm and the cotyledon are considered storage tissues, where members of the 11S protein family are the most abundant from the total of seed proteins (Alché et al., 2006). However, asynchrony exists in the formation of PBs between both tissues (Jiménez-López et al., 2015). The analysis of the protein synthesis along the seed formation has determined three periods: (I) a period of early synthesis (before 105 days after anthesis, DAA), (II) a rapid and massive period of synthesis (105–130 DAA), and (III) a period characterized by slow synthesis (from 130 DAA until full ripening) (Wang et al., 2001).

Authors have also fixed their attention to describe the intracellular events occurring during the first hours of the *in vitro* germination process (Zienkiewicz et al., 2011; Jiménez-López and Hernández-Soriano, 2013), drawing their attention particularly to PBs. Zienkiewicz et al. (2011) also revealed that the cellular organization of the olive leaf is achieved after 26 days of germination.

β-Conglutins, vicilins of 7S globulins are also major SSPs in different plants, particularly legumes. Among them, they have been particularly studied in Lupinus species (Jimenez-Lopez et al., 2015, 2016, 2018; Lima-Cabello et al., 2017a,b, 2018). They belong to the Cupin superfamily, and mainly associate (as storage protein function) with plant physiological processes through the supply of amino acids during seedling germination (Monteiro et al., 2010). Primary evidence of the presence of β-conglutins in the olive arise from transcriptomic analyses, as the presence of 7S globulins transcript sequences have been detected in the olive seed (unpublished results). However, direct evidence of the presence and distribution of β-conglutins in the olive seed has not been provided to date.

In spite of these pioneer studies, an overall histological description of the olive seed is yet missing to date. Here we perform a report of the different tissues of the olive seed throughout its development and we use a new molecular tool: the 7S SSPs (β-conglutins) recently described in the olive seed, and a specific antibody developed to evidence the presence of these proteins and their changes as markers along tissue development. The results shown here may serve as a hallmark for analyzing seed (and fruit) maturity and to monitor the presence of these proteins in future biotechnological and alimentary uses due to their increasing interest. Finally, cell localization of these proteins is also reported.

MATERIALS AND METHODS

Plant Material

Seeds used for microscopy analysis were collected from olive trees (*Olea europaea* L. cv. 'Picual') cultivated at the Estación Experimental del Zaidín (Granada). Four stages were considered: (0) small developing fruit, (I) green fruit, (II) fruit at veraison, and (III) mature fruit. The collection took place 60, 105, 130, and 210 DAA, respectively. Seeds from different cultivars were kindly provided by the Protected Certificate of Origin "Poniente de Granada." The cultivars studied were 'Ombliguillo,' 'Llorón,' '3,' 'Lechín,' 'Hojiblanca,' 'Picual,' 'Lucio,' 'Alameño,' 'Nevadillo,' 'Loaime,' 'Azul,' and 'Gordal de Alhama.' Twenty fruits per cultivar were dissected by using a knife, a de-stoning commercial device, and a scalpel to dissect the pulp (mesocarp + epicarp), stones and the seed tissues respectively. Weight measurements were performed individually using 20 samples of the complete mature fruit (210 DAA) and each one of the dissected tissues [mesocarp + epicarp, whole endocarp (stone), testa, endosperm, and embryo]. The number of seeds obtained from each fruit was also counted.

Preparation of Samples for Microscopy

Seeds from olive fruits at four developmental stages were collected. The mesocarps + epicarps (pulp) and the endocarps (stones) were removed with a knife and a de-stoning device, respectively. At the stage 0, the complete seed was used. In the rest of the stages the obtained seeds were carefully dissected into two parts: on the one hand the coat and the endosperm were treated together, on the other hand the embryo was carefully excised. Once the embryo was obtained, the apical part (radicle) and the middle part (cotyledons) were treated separately (**Figure 1**). The plant materials were fixed with 4% (w/v) paraformaldehyde and 0.2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h at 4°C with points of vacuum treatment to improve penetration of the fixative. Samples were dehydrated in ethanol series and embedded in Unicryl resin at −20°C using ultraviolet light. Semithin sections were obtained with a Reichert-Jung Ultracut E microtome using a glass knife. Sections were placed on Biobond-coated slides and used for cytochemical staining.

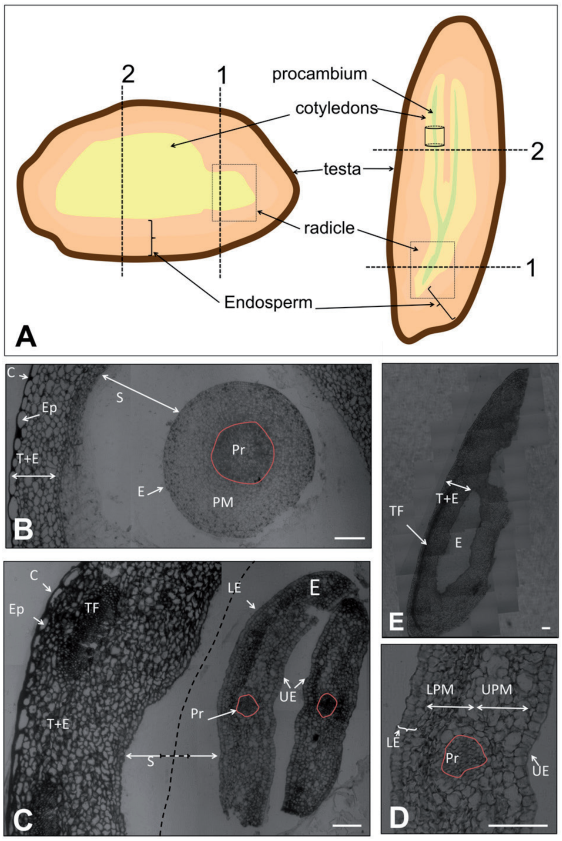

FIGURE 1 | (A) Representation of the tissues of the olive seed in longitudinal-versus cross-sections. Dotted lines/boxes show the orientation of the samples for microscopy analysis. **(B)** Cross section of the complete seed at the radicle site (pointed out with "1" in **A**) at 60 DAA. **(C)** Cross section of the complete seed at the cotyledon site (pointed out with "2" in **A**) at 60 DAA. Dotted lines indicate the shortening of the space between endosperm-embryo. **(D)** Detail of the cotyledon observed in panel **(C)**. **(E)** Longitudinal section of the complete seed at 60 DAA. Pr, procambium; PM, premesophyll; E, embryo; S, space between endosperm-embryo; C, cuticle; Ep, epidermis; T+E, testa and endosperm; TF, transversal fibers; LE, lower epidermis; UE, upper epidermis; UPM, upper premesophyll cells; LPM, lower premesophyll cells. Scale bars: 100 μm.

Histological Study

For histological observations, sections were stained with a mixture of basic dyes [0.05% (w/v) methylene blue and 0.05% (w/v) toluidine blue] aimed to stain the carboxyl groups of proteins, which reveal the presence of such components. Most non-stained structures correspond to lipids. Stained samples were observed in a LM Zeiss Axioplan (Carl Zeiss, Oberkochen, Germany). Photomicrographs were obtained with a ProgRes MF Cool Digital Camera, by using the ProgRes CapturePro 2.6 software (Jenoptik, LaserOptic System).

Development of an Anti-β-conglutin Antibody

Olive transcriptomic information together with sequence information of β-conglutins from different species was used to define potential cross-reactive epitopes of these proteins present in these species (Jimenez-Lopez et al., 2015). The peptide RLENLQNYRIVEFQS was selected as a cross-reactive component on this basis and was synthesized and used to immunize rabbits by Agrisera (Sweden) (Prod. No. AS15 2892). The resulting sera were affinity-purified with the synthetic peptide, and their specificity assessed by Western blotting and ELISA (not shown).

Protein Extraction and Western Blotting Analysis

Plant material (as described) was used to prepare protein extracts by grinding with liquid nitrogen. Proteins were extracted with

40 mM Tris–HCl pH: 7.0, 2% Triton X-100, 60 mM DTT and 10 μl/sample of protease inhibitor cocktail (Sigma). Samples were denatured with Laemmli sample buffer at 95°C for 5 min and separated on 4–20% TGX precast SDS-PAGE mini-gels (Bio-Rad). Protein profiles were determined by means of Stain-free technology using a Gel Doc^{TM} EZ System (Bio-Rad), and normalized for total protein (30 μg/lane). Gels were blotted to supported nitrocellulose using a Trans-Blot Turbo (Bio-Rad) semi-dry device and blocked with 5% skimmed milk in TBS plus 0.05% Tween-20 for 1 h at room temperature (RT) with agitation. Blot was incubated in the anti-β-conglutin primary antibody at a dilution of 1:1000 for 8 h at 4°C with agitation in TBS-T plus 5% skimmed milk. The antibody solution was decanted and the blot was rinsed briefly twice, then washed once for 15 min and 3 times for 5 min in TBS-T at RT with agitation. Blot was incubated in secondary antibody [anti-rabbit IgG horseradish peroxidase conjugated, from Sigma (A-0545)] diluted to 1:2000 in for 1h at RT with agitation. The blot was washed as above and developed for 3 min with Clarity Western ECL substrate (Bio-Rad). Exposure time was 6–12 min in a C-Digit scanner (LI-COR Biotechnology, United States). The intensity of the reacting bands and their approximate Mw was determined with the Image Studio^{TM} software (LI-COR Biotechnology, United States) as the average ± SD of three experiments.

TEM Immunolocalization of Olive 7S SSPs (β-Conglutins)

Ultrathin sections (70 nm) were obtained using a Reichert-Jung ultramicrotome and picked up using 200 mesh nickel grids coated with formvar. The grids were then sequentially treated with a blocking solution [5% (w/v) bovine serum albumin, 0.1% (v/v) Tween 20 in phosphate-buffered saline], a diluted (1:100) solution of the anti-7S antiserum in blocking solution, a 1:1000 solution of the secondary antibody (goat anti-rabbit IgG: 30 nm gold, BB International), and finally contrasted using a 5% (w/v) uranyl acetate alternative solution (Ted Pella Inc., CA, United States) and observed in a JEM-1011 (Jeol) transmission electron microscope (TEM). Negative control sections were treated as above but using preimmune serum instead of the anti-conglutin antiserum. Morphometric measurements were performed using the UTHSCSA ImageTool (version 3.00 for Windows) software.

Statistical Analysis

The Kolmogorov–Smirnov test was used to test the normality of all weight parameters. The Pearson test was performed aimed to determine whether whole fruit and mesocarp weight were correlated. For Western blotting and immunocytochemical analysis, values expressed as mean ± SEM of individual experiments were assessed for statistical significance of the data by analysis of variance followed by Dunnett's analysis. *P*-values ≤ 0.001 were considered statistically significant. All analyses were performed using IBM SPSS statistics v.24 software.

RESULTS

Olive Seed Anatomy at Early Stages of Fruit Development

The complete seed was processed 60 DAA to visualize general structure at a very early stage of development. At this moment, dissection of the seed into its tissues was not achievable without tissue damage due to the small size of the seed and the high compaction of the tissues. In **Figure 1A**, a schematic draw of the different tissues of the olive seed is displayed, as well as the positions selected for longitudinal- and cross-sections performed in this study.

A cross section of the complete seed at the radicle level showed that the testa and the endosperm tissues were immature, without appreciable differentiation among these two tissues (**Figure 1B**). The cells appeared unstained, indicating no clear accumulation of storage material neither in the endosperm nor in the embryo, as previously described (Jiménez-López et al., 2015).

No presence of the aleurone layer was detected. However, the presence of the cuticle and the pro-epidermal layer cells from the testa was visible. The cuticle was evidenced by an intense staining with methylene blue at the outermost site. The pro-epidermal layer of cells was placed under the cuticle, composed of long-shaped cells. Regarding the embryo, isodiametric cells were observed with slight differences among them. In the center of the embryo, the cells appeared intensely stained, this central structure corresponding to the precambium. The premesophyll cells were located surrounding those of the precambium. The pre-dermal cells appeared in the outer part, characterized by the presence of notorious nuclei. The embryo and the endosperm were separated by an ample space that remained unstained (**Figure 1B**). Similarly, a cross section of the embryo at the cotyledon level showed that the testa and the endosperm appeared undifferentiated. However, in this area, the presence of transversal fibers was patent. The thickness of the precursor of the testa and endosperm at the cotyledon level was approximately twofold that at the radicle level. The width increment was due to both, the presence of transversal fibers, and the rise in the number of cells (**Figure 1C**). The embryo cross-section at the level of the cotyledon showed cells with a quite marked differentiation (**Figure 1E**). Four types of cells were observed: those forming the procambium, the upper epidermis, the lower epidermis, and the premesophyll (**Figures 1C,E**). It was observed that the upper and lower epidermis contained one and two layers of cells, respectively, in both cases with a cubic shape. On the other hand, the premesophyll contained non-stained cells with variable shape and size. The presence of the procambium cells was evidenced as a group of small and densely packed cells among the premesophyll. A longitudinal section of the complete seed showed the position of the embryo within the seed as well as the disposition of the transversal fibers (**Figure 1D**).

The Formation of the Seed Coat Throughout Olive Fruit Development

After fertilization, the integuments of the ovule normally develop into the seed coat or testa. The histological analysis of this tissue

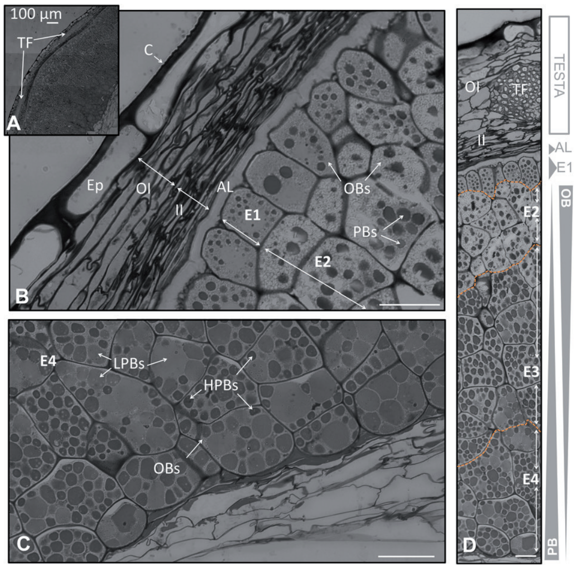

FIGURE 2 | Anatomical structure of the coats and the endosperm of the olive seed 105 DAA, corresponding to green fruit. **(A)** Cross section of the testa and the endosperm. **(B)** Testa and adjacent cells corresponding to the endosperm. **(C)** Cell of the endosperm corresponding to the part in contact with the embryo. **(D)** Testa and endosperm cross section showing the differential content in lipids and proteins along it. Orange dotted lines demarcate the different layers of the endosperm. OBs, oil bodies; PBs, protein bodies; C, cuticle; Ep, epidermis; OI, outer integument; II, inner integument; TF, transversal fibers; AL, aleurone layer; E1, endosperm layer 1; E2, endosperm layer 2; E3, endosperm layer 3; E4, endosperm layer 4.

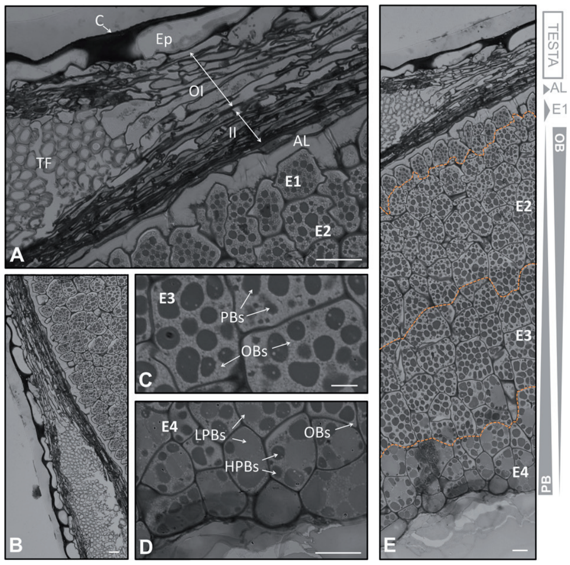

FIGURE 3 | Anatomical structure of the coats and the endosperm of the olive seed 130 DAA, which corresponds to the fruit at the veraison stage. **(A,B)** Cross section of the testa and cells adjacent to the endosperm. **(C)** Cells in the middle part of the endosperm. **(D)** Cells corresponding to the part in contact to the embryo. **(E)** Testa and endosperm showing differential protein/lipid content. Orange dotted lines demarcate the different layers of the endosperm. OBs, oil bodies; PBs, protein bodies; C, cuticle; Ep, epidermis; OI, outer integument; II, inner integument; TF, transversal fibers; AL, aleurone layer; E1, endosperm layer 1; E2, endosperm layer 2; E3, endosperm layer 3; E4, endosperm layer 4.

along three stages of the seed development has revealed that three layers can be distinguished: (i) mucilage or cuticle, (ii) epidermis, (iii) integument (**Figures 2**–**4**).

At the green fruit stage (105 DAA), the cuticle appeared strongly stained, forming a layer that covered evenly the non-stained and long shaped cells from the epidermis. Underneath appeared the integument composed by 8–10 well packed cells in a longitudinal orientation. The integument was divided into two parts: the outer and the inner integument; each one formed by 4–5 layers of cells. In the inner integument the cells displayed a more-flatted form, with minor intracellular spaces in comparison to the outer integument (**Figure 2B**). A cross section of the coat showed the presence of transversal fibers. These fibers crossed the integuments at the line of separation between both integuments causing a prominence of the coat (**Figures 2A,D**). This prominence causes the typical ornamentation of the olive seed that can be macroscopically distinguished.

At the veraison stage (130 DAA), a conspicuous loss of thickness of the cuticle in certain areas was detected. The cells from the epidermis appeared slightly distorted when compared to those at the green fruit stage. Besides, the start of a laxation in the cells from the outer integuments was noticed, whilst in the inner integument the cells appeared more densely packed. The transversal cells crossing the integument were observed to suffer also a light loosening, which also contributed to a progressive loss of compaction of the seed coat (**Figures 3A,B**).

At fruit maturity (210 DAA), the seed coat was characterized by the structure disorganization of the different layers. The cuticle was irregularly disposed over the epidermal cells, with a significant loss of width in some areas. The epidermis cells appeared with a patent loss of the structured disposition described for the previous stages. The same phenomena occurred in the outer, the inner integument, and the transversal fibers (**Figures 4A,B,D**).

The Formation of the Endosperm Throughout Olive Fruit Development

The outermost layer of the endosperm (termed aleurone) was observed to be composed by longitudinal shaped cells that laid over the cells of the endosperm with a high content in lipids. At the green fruit stage (105 DAA), this layer was well developed (**Figures 2A,D**). At the veraison stage (130 DAA), no significant changes were observed in the aleurone layer, with the exception of minor modifications in the shape. The cells set off slight penetrations in the vicinity of the endosperm cells (**Figures 3A,E**). At the mature fruit stage (210 DAA), the aleurone layer seemed with a less-structured disposition compared to the previous stages. The lipid-rich cells forming the upper part of the

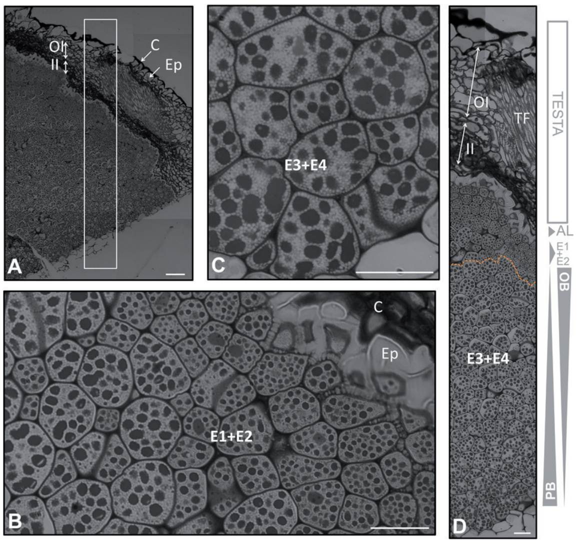

FIGURE 4 | Anatomical structure of the coats and the endosperm of the olive seed 210 DAA, obtained from mature fruits. **(A)** Cross section of the testa and endosperm. **(B,C)** Cell of the endosperm close to the testa, **(C)** cells in the middle part of the endosperm. **(D)** Testa and endosperm cross section. Orange dotted lines demarcate the different layers of the endosperm. OBs, oil bodies; PBs, protein bodies; C, cuticle; Ep, epidermis; OI, outer integument; II, inner integument; TF, transversal fibers; AL, aleurone layer; E1, endosperm layer 1; E2, endosperm layer 2; E3, endosperm layer 3; E4, endosperm layer 4.

endosperm appeared interweaved with those from the aleurone (**Figures 4A,D**).

In the olive endosperm two main types of reserve material were detected along the seed/fruit development: lipids and proteins. These substances have been already described during the olive seed formation and in the olive seedling, where they have been related in unspecified areas of the endosperm and embryo. The proteins have been reported to accumulate forming PBs, surrounded by lipids that form oil bodies (OBs) (Jiménez-López and Hernández-Soriano, 2013; Jiménez-López et al., 2015).

In the present study we have observed that the endosperm was composed by isodiametric cells with uneven distribution of PBs/OBs (**Figures 1E**, **2D**, **3D**). The gradient of PBs/OBs accumulation followed a similar pattern during the three stages considered (from 105 to 210 DAA). The cells enriched in OBs were present predominantly near the testa, with a gradual decrease of lipids in the area near the embryo. The opposite tendency was observed in the PBs. It was detected the presence of differentially stained PBs within the endosperm cells. Thus, even when considering one single cell, differential types of PB staining was noticed. Cytokinesis phenomena occurred along the tree stages of development as phragmoplasts were detected.

Attending to the disposition and the PBs/OBs content within the endosperm cells, a classification of this tissue into four layers was performed. The first layer (adjacent to the aleurone) was named endosperm 1 (E1). It was detected as a monolayer of isodiametric cells with an arranged disposition. These cells contained small PBs surrounded by small OBs (**Figures 1C,E**). Following the E1, the cells were bigger and with an untidy disposition. This area was named as endosperm 2 (E2) and was the most lipid-enriched layer (**Figures 1C,E**). The area named as endosperm 4 (E4) was highly enriched in PBs, with an increment in their size. The area named as endosperm 3 (E3) was considered as a transition between E2 and E4 as regard to the size and quantity of PBs/OBs.

Noticeable modifications in the pattern of accumulation of reserve substances were observed in the endosperm 130 DAA, corresponding to the veraison stage. The E1 layer was not so clearly differentiated from the E2 as it was in the green fruit stage. The E1 cells lost their arrangement and contained larger PBs (**Figures 3A,E**). The differences between E1, E2, and E3 were not so apparent (**Figure 2D**). However, the transition between the E3 and E4 layers was still perceptible (**Figures 3C,D**).

At the mature stage, the main characteristic of the endosperm was an increment in the homogeny of the cellular size and PB/OBs composition. The aleurone and the E1 layers were interweaved. A conspicuous differentiation could be observed, with the E1+E2 representing a single layer and E3+E4 another one (**Figures 4A,C,D**). The distribution of the storage material was similar to that described by other authors at the same stage of development (Jiménez-López and Hernández-Soriano, 2013).

The Formation of the Cotyledon Throughout Olive Fruit Development

As described for the endosperm, the olive embryo also stocks two main kinds of storage material: lipids and proteins (Jiménez-López and Hernández-Soriano, 2013; Jiménez-López et al., 2015) that build up OBs and PBs, respectively. A deep scrutiny on the embryo histology showed an uneven distribution of this storage material mainly in the cotyledon and the radicle.

Observation of cross sections of the embryo at the cotyledon level (lines named "2" at **Figure 1**) 105 DAA showed the presence of a storing premesophyll tissue that appeared to consist of two zones with cells differing in shape, OBs/PBs distribution and intracellular spaces. Taking into account the orientation of the cotyledon sections, the two areas were identified as the future abaxial/adaxial sites of the leaf. In both zones, the premesophyll was covered by one/two layer of cells corresponding to the upper and lower pro-epidermis, respectively (**Figure 5**).

The pro-epidermal cells of the adaxial side, called upper pro-epidermis were cubic in shape and possessed small and strongly stained PBs surrounded by OBs. On the other hand, the under pro-epidermis was a monolayer of long shaped cells with transversal disposition. Underneath, it was noticed the presence of three layers of isodiametric cells forming the upper premesophyll (UPM). The UPM had densely packed cells with larger PBs compared to the upper pro-epidermal cells. The PBs were also surrounded by OBs. The lower premesophyll (LPM) cells occupied approximately two third parts of the cotyledon section, and their PBs were larger than those from the UPM cells. In the abaxial side it was noticed the presence of intracellular spaces. Interestingly, the PBs showed different stain intensity in both parts of the premesophyll. The lower pro-epidermis was

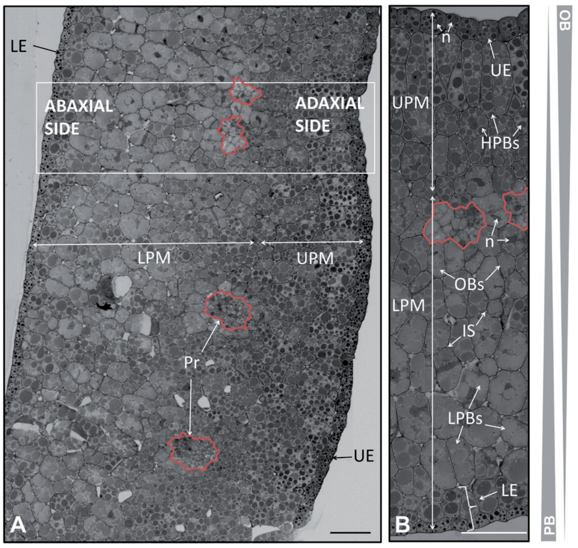

FIGURE 5 | Anatomical structure of the cotyledon of the olive seed 105 DAA. **(A)** Low magnification picture of the cotyledon. **(B)** Large magnification of the cotyledon revealing the precursors of the vascular system (pointed out with a red line). OBs, oil bodies; PBs, protein bodies; UE, upper epidermis; LE, lower epidermis; LPM, lower premesophyll cells; UPM, upper premesophyll cells; Pr, procambium; IS, intracellular spaces; n, nucleus.

composed by two layers of cells with different characteristics. The outermost layer possessed cubic cells with small and intensely stained PBs. Next, a layer of cells with half-way characteristics of the outermost epidermal cells and the LPM cells was observed. This layer was considered a transition as regard the cell size, PBs size, PBs stain intensity, and cell shape. The presence of nucleus was detected in all the cells along the cotyledon section (**Figure 5B**).

In between the UPM and the LPM cells, the presence of clusters of cells with irregular shape and size were distinguished, corresponding to the precambium. These cells appeared as densely packed, with nucleus, and without storage material within them (**Figure 5**).

The analysis of the histology of the olive seed cotyledon at the veraison stage of the fruit revealed changes in the premesophyll, precambium, and epidermal cells, which were characterized by changes in the disorganization of the storage material. The nucleus was observed in the cells of all the tissues. At this stage, the presence of structures considered precursors of stoma was detected (**Figure 6**).

The upper pro-epidermis was formed at 130 DAA by a single layer of cubic cells with parallel disposition and with small and intensely stained PBs surrounded by OBs. The layer of cells under the upper pro-epidermis had suffered transversal divisions giving rise to isodiametric cells similar to those of the rest of the UPM below. The cells from the adaxial side appeared densely packed with a tendency toward homogeneity in the cell size, PBs size, and PBs staining intensity. Concerning the LPM cells, the presence of subtle changes in the size and PBs/OBs disposition was detected, leading to a homogenization of the internal organization from the UPM and LPM. The two zones were not so clearly differentiated as in the previous stage with the exception of the presence of the precambium. Moreover, in the UPM cells there was noticed a combination of low- and highly stained PBs within the same cell, being predominant the latest ones. This phenomenon also was evident in the LPM cells, where the low-stained PBs were the most abundant ones in this case. It was observed that the LPM possessed several distinctive attributes: the intracellular spaces, cells slightly bigger than those from the UPM, and PBs occupying most of the volume of the cell.

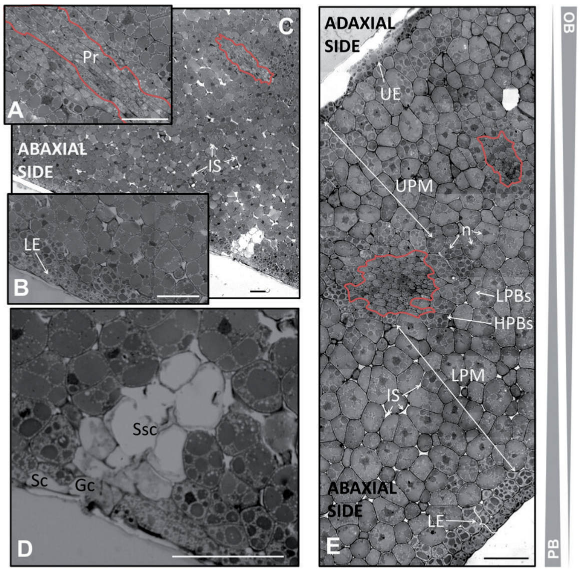

FIGURE 6 | Anatomical structure of the cotyledon of the olive seed 130 DAA. **(A)** Low magnification of the cotyledon. **(B,C)** Large magnification of the cotyledon at the procambium (pointed out with a red line) and lower pro-epidermal cells, respectively. **(D)** Stoma. **(E)** Cotyledon section showing the abaxial and adaxial sites and the cells encompassing them. OBs, oil bodies; PBs, protein bodies; HPBs, highly stained protein bodies; LPBs, low stained protein bodies; UE, upper epidermis; LE, lower epidermis; LPM, lower premesophyll cells; UPM, upper premesophyll cells; Pr, procambium; IS, intracellular space; n, nucleus; Gc, guard cells; Sc, subsidiary cells; Ssc, substomatal cavity.

The cells forming the precambium were detected in the center of the cotyledon section. Noticeable changes in the total area of the UPM and LPM were detected in comparison with the previous stage, with an increment in the UPM and a drop in the LPM area, respectively.

Modifications in the lower pro-epidermis at the veraison stage were detected. Two layers were distinguished, both of them composed by cubic, parallelly arranged cells, and with small PBs intensely stained within them. However, the sizes of the PBs were smaller in the outermost layer than in the internal one. In both cases, PBs size, stain intensity and OBs quantity were clearly different from the cells from LPM (**Figure 6**).

Precursors of stomata were observed at the abaxial side. The lower epidermis was interrupted by the guard and the subsidiary cells. Below the stoma, a mass of non-stained cells with intracellular spaces was identified. The structure was similar to that described in *Zea mays* (Mauseth, 1988; **Figure 6D**).

The study of the anatomy of the olive cotyledon in the mature seed corresponding to 210 DAA showed a defined structure, with clear precursors of the spongy and palisade mesophyll. The imbalanced distribution of the storage material within the cells of the mesophyll was observed to be the main characteristic of this stage (**Figure 7**).

The upper pro-epidermis contained a monolayer of flattened cells disposed parallel in the plane to the surface. These cells were observed to be highly enriched in OBs and small PBs (**Figure 7C**).

The UPM cells occupied half of the cross section of the cotyledon and they were filled by numerous OBs surrounding the PBs. There was a mixture of highly stained and low stained PBs within the cells, mainly dominated by the highly stained ones. The cells from the LPM had a lower OBs content that surrounds the large PBs. Poles apart, the low stained PBs were dominant over the high stained ones within the cells of the LPM (**Figures 6B,D**).

The precambium appeared among the UPM as a group of long shaped cells without storage material within them (**Figure 7B**). At the mature stage the procambium did not show mature xylem or phloem elements. The lower epidermis was detected to be composed by two layers of cells with a non-arranged disposition, with highly stained PBs and elevated quantities of OBs (**Figure 6D**). The nucleus was observed in all the layers of cells across the cotyledon section.

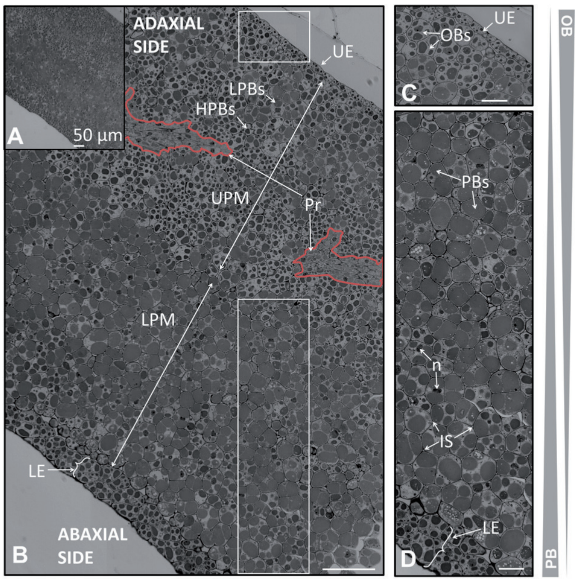

FIGURE 7 | Anatomical structure of the cotyledon of the olive seed 120 DAA. **(A)** Low magnification of the cotyledon. **(B)** Large magnification of the cotyledon. **(C)** Detail of the upper pro-epidermis and UPM cells. **(D)** Detail of the lower pro-epidermis and the LPM cells. OBs, oil bodies; PBs, protein bodies; HPBs, highly stained protein bodies; LPBs, low stained protein bodies; UE, upper epidermis; LE, lower epidermis; LPM, lower premesophyll cells; UPM, upper premesophyll cells; Pr, precambium; IS, intracellular space; n, nucleus.

The Formation of the Radicle Throughout Olive Fruit Development

Sections of the radicle taken form seeds at the green fruit stage showed the presence of three different kind of cells corresponding to the protoderm, ground meristem, and procambium, respectively (**Figure 8A**). The cells from the apex, which form to the ground meristem had a high degree of compaction and possessed large nuclei. The PBs were also large being surrounded by small OBs. The apical ground meristem cells suffered anticlinal divisions (**Figure 8D**). Regarding the protoderm, two layers of long shaped cells were observed, being the PBs small and intensely stained (**Figure 8B**). Underneath, a gradual change in the cell shape and the characteristics of the stored material within the cells was detected, giving rise to isodiametric cells with low-stained and large PBs. These cells were bigger than those forming the protoderm and the presence of intracellular spaces among them was detected. The procambium cells were long-shaped, devoid of storage material, and lacking intracellular spaces among them, which allowed differentiating them from the meristem cells. In between the procambium and the meristem, cells appeared as a transition concerning to the shape and the PBs/OBs content (**Figure 8C**).

At the veraison stage, few changes in the histology of the radicle were observed (**Figures 8E–G**). These changes corresponded mainly to the meristem, which appeared less packed. The cells at the apex of the meristem were an exception, with a high degree of compaction among the cubic-shaped cells.

At the mature fruit stage (210 DAA), we observed the presence of notorious changes in the organization and differentiation of the cells of the radicle (**Figures 8H–K**). The procambium appeared as a central bundle in the midpoint of the radicle. At the distal end of the procambium, the quiescent center was visible, mainly characterized by the disposition of the cells around a central point. In the above part, the cells of the columella and root apex displayed an arranged organization (**Figure 8I**). On the left and right sides of the quiescent center it was noticed that the cells suffered a progressive change in the shape and content of storage material to finally give rise to the meristematic cells. Below the quiescent center, the procambium comprises two areas. The area located in the middle was composed by isodiametric cells containing low-stained PBs. The external area was comprised of long-shaped cells without OBs, nor PBs. In both cases, the presence of the nucleus within the cells was perceptible, as well as the absence of storage material (**Figure 8J**). The protoderm was identified as two layers of long-shaped cells longitudinally arranged. These cells were differentiated from the adjacent meristematic cells since the latter possessed intracellular spaces, large cellular size, large PBs, and non-well-structured cells (**Figure 8K**).

SDS-PAGE protein profiles of whole seeds, isolated endosperm (+testa) and embryo at different DAA were resolved by SDS-PAGE under reducing conditions, as displayed in **Figure 9A**. Conspicuous bands of proteins appear corresponding

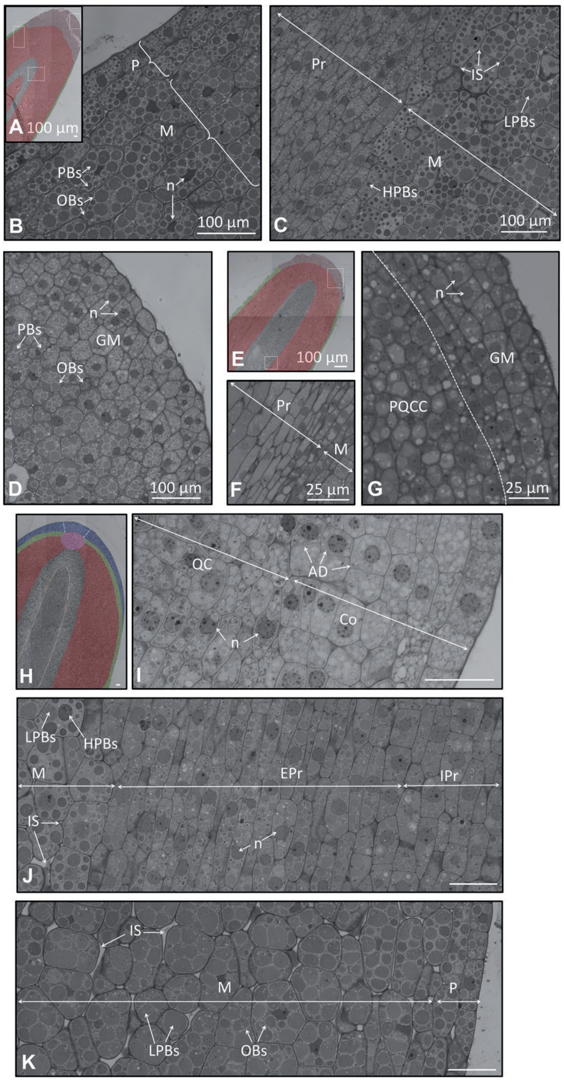

FIGURE 8 | (A–G) Longitudinal section of the embryo at the radicle part in a seed 105 and 130 DAA. **(A)** Low magnification radicle showing the different kind of cells 105 DAA. In light red appears the grown meristem at the apex; in green the protoderm; in gray the procambium. **(B)** Large magnification of the radicle showing a detail of the protoderm and the meristem 105 DAA. **(C)** Large magnification of the cell of the procambium and the meristem 105 DAA. **(D)** Large magnification of the ground meristem at the very apex 105 DAA. **(E)** Low magnification radicle showing the different kind of cells 130 DAA. In light red appear the grown meristem at the apex; in green the protoderm; in gray the procambium. **(F)** Cell of the procambium and the meristem 130 DAA. **(G)** Cell of the procambium and the meristem 130 DAA. **(H–K)** Longitudinal section of the embryo at the radicle in a seed 210 DAA. **(H)** Low magnification radicle showing the different types of cells. In red appears the meristem; in green the protoderm; in blue the root apex, in gray the procambium. Orange dotted line demarcates two different types of cells within the procambium; blue dotted line demarcates the columella. **(I)** Detail of the radicle at the root apex and quiescent center. **(J)** Large magnification picture of the meristem and the procambium. **(K)** Cells of the protoderm and the meristem. P, protoderm; M, meristem; Pr, procambium; QC, Quiescent Centre; C, columella.

to the peptides p1 to p5 as described by Alché et al. (2006), which represent different peptides integrating the highly abundant 11S SSPs. The Western blotting profile after probing with the anti-β-conglutin primary antibody showed two reactive bands of c.a. 45 and 49 kDa, respectively present in all extracts, although with different relative intensities (**Figure 9B**). Relative quantification of each one of the reactive bands in all samples showed bands of 49 kDa evenly distributed in the endosperm and embryo tissues, with little changes in their intensity through the time developmental course. Contrary, the bands of 45 kDa presented noticeable changes in their intensity, particularly along the developmental stages for a given tissue (endosperm and embryo). The added intensities of both bands for each stage exhibited an increasing trend in the overall amount of β-conglutin along endosperm, embryo, and whole seed development (**Figure 9C**). Relative amount of β-conglutins was higher in the embryo compared to the endosperm.

Immunolocalization studies using the anti-β-conglutin primary antibody yielded an intense labeling by gold particles specifically located in the PBs present in the endosperm and the embryo all-through the seed developmental stages (**Figures 10A–C,A'–C'**). Labeling in the lipid bodies, any other cell structures (cell wall, nucleus, and testa) and in the negative controls processed by either omitting the primary antibody or using the pre-immune serum (not shown) was negligible. A statistically significant and progressive increase of labeling density in the PBs present in both the endosperm and the embryo was observed (**Figure 10D**). The overall density of labeling was significantly higher in the embryo than in the endosperm (**Figure 10D**).

Seed Weight Variability Among Olive Cultivars

The weight of the whole fruit was a variable parameter in the cultivars submitted to the present study (**Supplementary Figures S1A,B**). They ranged from an average of 1.28 g in 'Lechin' to 4.91 g in 'Ombliguillo.' Similarly, the average weights of the mesocarps were comprised between 0.89 g in 'Lechín' to 4.00 g in 'Ombliguillo.' Besides, the data obtained from the average weight of the endocarp oscillated from 0.39 g in 'Lechín' to 0.99 g in 'Gordal de Alhama.' The weights of the whole fruit and that of the pulp (mesocarp + epicarp) showed to have a positive correlation (**Supplementary Figure S1C**). As regard to the number of seeds found within each endocarp, six of the cultivars showed just one seed, whereas in the other six cultivars we managed to observe the presence of two seeds per endocarp in some of the fruits.

Focusing on the seed tissues we identified that the average weights of the complete seeds measured in the 12 cultivars ranged from 0.040 in 'Lechín' to 0.101 g in 'Azul.' The testa ranged from to 0.005 g in the cultivar '3' to 0.030 g in the cultivar 'Hojiblanca.' The endosperm ranged from 0.024 g in 'Picual' to 0.059 g in 'Loaime.' The embryo weights were comprised among 0.011 g in 'Picual' to 0.032 g in 'Nevadillo' (**Supplementary Figure S1D**).

As regard to the olive yield, measured as the ratio complete fruit/pulp weight, the obtained data showed the lowest ratio for 'Llorón' (1.20) while the highest one corresponded

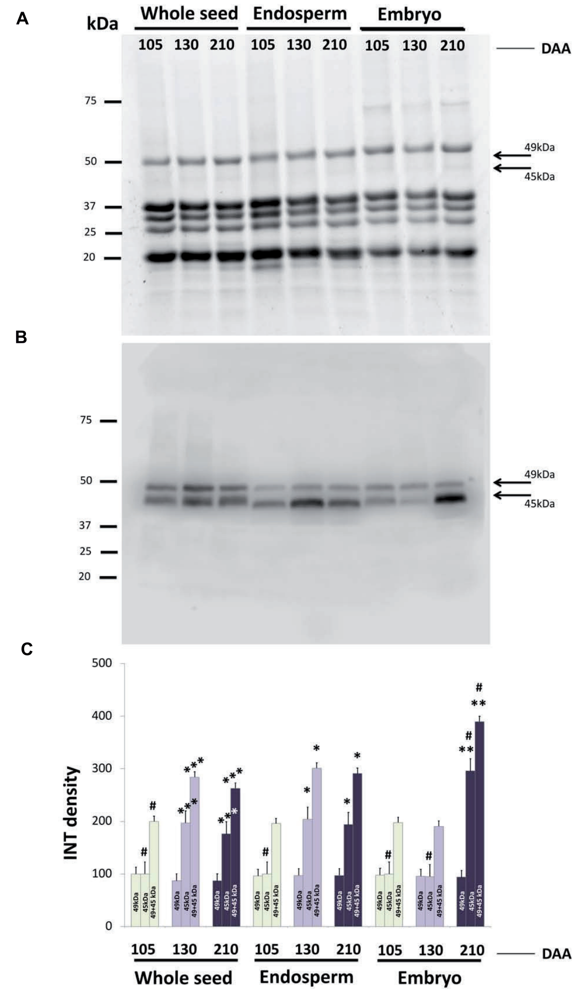

FIGURE 9 | SDS-PAGE profiles of and Western blotting analysis of β-conglutins in samples of whole seeds, isolated endosperm plus testa and embryo from olive fruits at different developmental stages. **(A)** SDS-PAGE profiles showing conspicuous bands corresponding to 11S peptides under denaturing, reducing running conditions. **(B)** Western blotting after using the anti β-conglutin antibody. **(C)** Densitometry of the reactive bands to the antibody. Green bars: green fruit (105 DAA). Light purple bars: veraison fruit (130 DAA). Dark purple bars: mature fruits (120 DAA). $^{*}p < 0.001$ versus the corresponding endosperm sample at 105 DAA; $^{**}p < 0.001$ versus the corresponding embryo sample at 105 DAA; $^{***}p < 0.001$ versus the corresponding whole seed sample at 105 DAA; $^{\#}p < 0.001$ among all samples of the same band category (49, 45, or 49+45 kDa).

to ‘Picual’ with a ratio of 1.47. On the other hand, the fruit/endocarp ratio oscillated between 3.15 in ‘Picual’ to 5.77 in ‘Llorón’ (**Supplementary Figure S1E**). The endocarp/seed ratio showed values among 4.83 in ‘Azul’ to 14.72 in ‘Picual.’ The seed/embryo ratios oscillated between 2.15 in ‘Nevadillo’ to 4.58 in ‘Ombliguillo.’ In the case of the seeds containing two seeds per endocarp, each seed was weighted as independent sample. Spearman correlation between the weights of endosperm/embryo, seed/testa, seed/endosperm, and seed/cotyledon for different olive cultivars, as well as the registered presence of some fruits of the cultivar containing more than 1 seed is displayed in **Supplementary Figure S1F**.

DISCUSSION

Endocarp morphology is a widely accepted pomological signature for olive tree identification and classification of cultivars based on the presence of morphological differences (Barranco and Rallo, 1984; Rallo et al., 2005), which has been later evidenced to be in accordance with results obtained by molecular methods like simple sequence repeat (SSR) screening (Fendri et al., 2010). Within the endocarp, the olive seed represents a potential source of nutrients and biological elements of high interest, in addition of representing an additional varietal mark as demonstrated in the present work. Such designed potential will allow increasing the added value of this material, which is frequently disposed of concomitantly with olive processing residues. The olive seed can also be used as a source of genetic variability of interest for the development of breeding programs, in combination with the vegetative propagation of the resulting individual of interest (Morales-Sillero et al., 2012).

Histological structure of the olive seed doesn’t substantially differ from those of dicots as described here; however, the distribution of the different tissues and their development has to be assessed in order to gain knowledge and establish parameters of maturity, which make easier the analysis of the expression and the presence of the compounds of interest, as is the case of 11S proteins (Alché et al., 2006; Jiménez-López et al., 2015) and the present case of 7S proteins described here. Such studies may help to define further technological developments, i.e., for sub-fractioning olive seed in order to enrich certain components, which could be majority present in a particular fraction. Also, these analyses may help to identify histological parameters relevant for seed and fruit physiology. Thus, the seed coats from different species have been analyzed for a variety of purposes such as the generation of a dichotomous key (Kaplan et al., 2009), or to analyze implications in key physiological roles like viability, dormancy and early control of germination (De Giorgi et al., 2015). The seed coat from the olive tree contains a well-defined cuticle covering the epidermis, which could be involved in key physiological roles. The intense staining might indicate a major presence of proteins, analogously as described in the grape seed coat, which is also rich in polysaccharides (Cadot et al., 2006). Proteome analysis in *Arabidopsis* has revealed the presence of proteins unique to mucilage responsible of alterations of its structure and mechanical alteration of the primary cell wall (Arsovski et al., 2010; Haughn and Western, 2012; Tsai et al., 2016). The key role of the seed cutin has also been associated to soil erosion (Engelbrecht et al., 2014). However, the protein complexity of the seed coat in the olive seed is still unrevealed.

We observed a general laxation and disarrangement of the coats that could be involved in the need to have access to oxygen

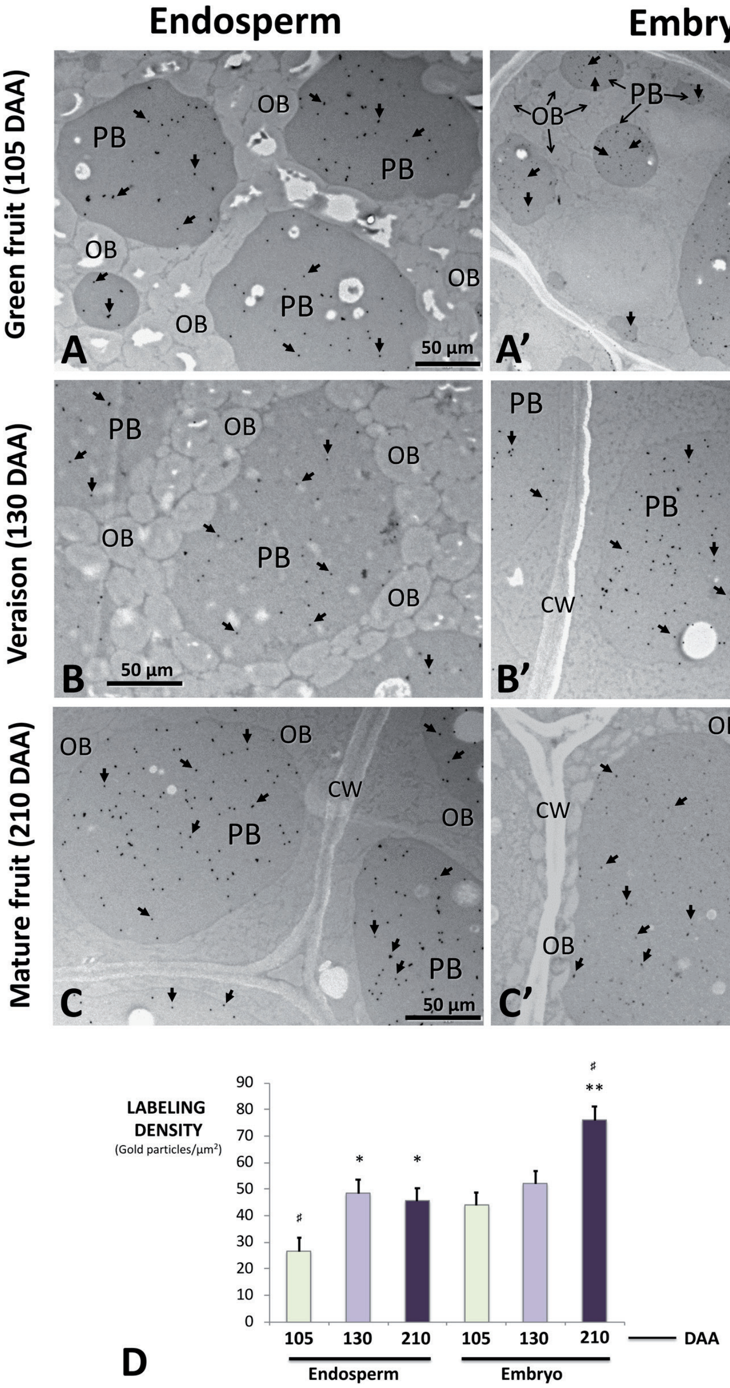

FIGURE 10 | Transmission electron microscope (TEM) immunolocalization of β-conglutins in samples of endosperm plus testa **(A–C)** and embryo **(A′–C′)** from olive fruits at different developmental stages. Gold particles (arrows) are specifically decorating protein bodies of different sizes. Lipid bodies, cell wall, and the nucleus are devoid of gold particles. **(D)** Quantification of labeling density in the protein bodies of both tissues at the different stages analyzed. Green bars: green fruit (105 DAA). Light purple bars: veraison fruit (130 DAA). Dark purple bars: mature fruits (210 DAA). CW, cell wall; OBs, oil bodies; N, nucleus; PBs, protein bodies. Magnification bars: 50 μm. $^{*}p < 0.001$ versus the corresponding endosperm sample at 105 DAA; $^{**}p < 0.001$ versus the corresponding embryo sample at 105 DAA; $^{\#}p < 0.001$ among all samples.

needed in the germination process. The three-dimensional study of the *Arabidopsis* seed revealed a putative network of intercellular air space that allows gas exchange for germination (Cloetens et al., 2006). The X-ray in-line phase tomography performed in maize is as practical tool for the detection of other characteristic non-detectable by conventional microcopy methods, like the metabolic state and the water content (Rousseau et al., 2015). The olive seed presents intracellular spaces and discontinuous in their structures putative involved in the need for the gas exchange, water intake or metabolic activity mainly in the mature stage and prior to the germination process. The presence of a well-defined aleurone layer in the olive seed has been described in the present study. This structure changes form a well-structured disposition in the green stage to disorganization in the mature stage that could be involved in some way in the easy removal of the seed coat at the mature stage.

As regards to the endosperm, Floyd and Friedman (2000) provided the first insight into how different endosperm developmental patterns are evolutionarily and developmentally related. The study of the endosperm possesses an increasing interest further than the long-established role of the endosperm as nourishment and mechanical barrier. The endosperm is capable of sensing environmental signals and interacts with the embryo establishing a bidirectional communication (Yan et al., 2014). The endosperm in the olive tree showed a clear change as regard to the organization and quantity of the OBs/PBs. These data were similar to those of the embryo. Moreover, it was found the presence of differentially stained PBs in both tissues. Thus, the composition of both, the proteins and the lipids could be differentially accumulated. The analysis of the fatty acid composition of the endosperm and embryo was detected to be different in *Arabidopsis* probably due to an hormonal regulation (Penfield et al., 2004) and later confirmed in both, *Arabidopsis* and *B. napus* (Li et al., 2006). These authors also observed that the fatty acid profile was different among embryo tissues. Finally, the apical meristems consist in three types of tissues that correspond to protoderm, ground meristem and procambium. The procambium is differentiated early in the development (60 DAA), however, the proper phloem and xylem did not appear differentiated in the mature seed. These events correlates to those previously described by Zienkiewicz et al. (2011) that pointed out the complete cellular organization of the leaf olive mesophyll is achieved 16 days after germination.

The study of the olive embryo and endosperm reveals the 11S protein as the most abundant one in these tissues (Alché et al., 2006). However, no studies about the presence of other specific proteins are available till the moment, whereas the present study confirms that 7S-type SSPs (β-conglutins) are also relevant constituents of both the endosperm and the embryo. Both proteomic and transcriptomic analysis aimed to agronomical improvements have shown for example the seed coat to function as a specialized secondary cell wall (Haughn and Western, 2012), to be involved in endosperm permeability, seed viability, and seed dormancy which correlates with higher levels of seed lipid oxidative stress (De Giorgi et al., 2015), with implication in specific functions that affects the seed composition, seed permeability, and hormonal regulation (Verdier et al., 2013). The study of both the proteome and the transcriptome of the olive seed (currently being approached) could represent interesting tools for multiple purposes, including the study of specific proteins involved in organoleptic properties of the olive oil. Thus, the presence of seed enzymes involved in the lipoxygenase pathway, enzymatic activities metabolizing 13-hydroperoxides other than hydroperoxide lyase, alcohol dehydrogenase, and alcohol acyltransferase activities among others would provide multiple esters in the olive oil (Luaces et al., 2003, 2007).

Within the increasing demand for plant-derived proteins as components of functional foods in the nutraceutical industry and as an alternative to expensive and less-environmental-friendly production of animal protein, β-conglutins are considered an economical dietary source of good quality protein. Also, they have positive effects on many human health dysfunctions, as many of the seeds containing β-conglutins are protein- and fiber-rich, low in fat and starch, and have a very low glycemic index (Arnoldi, 2008; Duranti et al., 2008). Positive effects have been described for these proteins on blood pressure, risk of cardiovascular disease and the prevention and treatment of type 2 diabetes, by modulating the insulin signaling pathway and diminishing inflammation (Lima-Cabello et al., 2017a). For the olive seed, preliminary work (unpublished) indicates the presence of anti-inflammatory components in the flours derived from this material. However, the direct involvement of β-conglutins in these effects is yet to be analyzed.

Expression of β-conglutins in the olive seed tissues is remarkable as shown here, with at least two forms of the protein reactive to the antibody, which might indicate the presence of a protein maturation process, as it is the case of the olive 11S SSPs (Alché et al., 2006), and has been proposed for β-conglutins (Duranti et al., 2008). The 49 kDa form of the protein shows a constitutive presence in the endosperm, embryo, and whole seed, whereas the 45 kDa form displays developmental changes as well as slight tissue differences. The accumulated presence of both β-conglutin forms indicates that this protein is progressively accumulated in the seed, through the developmental process, and that the relative amount of β-conglutins was higher in the embryo compared to the endosperm. Olive seed development was already characterized as a tissue-dependent process characterized by differential rates of legumin accumulation and PB formation in the main tissues integrating the seed (Jimenez-Lopez et al., 2016) on the basis of the accumulation of 11S legumin proteins. Such developmental pattern is then shared by β-conglutins as well. The relationships between the 45 and 49 kDa forms of the protein must be stablished through future work. Proteomic and transcriptomic work in course will serve the basis for this information, and will help to determine whether these correspond to maturation forms of the protein or the result of the expression of differential genes. These studies will also determine the presence of embryo- and endosperm-specific proteins, as have been recently identified in *Phoenix dactylifera* (Sekhar and DeMason, 2017).

Lupin β-conglutins are located in the endosperm and cotyledonary PBs, as shown by immunocytochemical experiments carried out here, and as it is also the occurrence with olive 11S legumins. As showed by Duranti et al. (2008), the covalent integrity is not apparently a pre-requisite for β-conglutin to be correctly deposited in these cellular structures, since the mature β-conglutin from lupin dry seeds appeared already proteolytically cleaved in a number of sites, giving rise to complex SDS-PAGE patterns. Immunolocalization of β-conglutins in the olive PBs likely reflects the localization of both the 45 and 49 kDa forms of the protein, as they are both recognized by the antibody. Quantification of the labeling in the PBs is consistent with the quantification of the signal of the 49+45 kDa bands in Western blotting experiments, showing an incremental presence of these proteins through the maturation process, analogous to that of 11S proteins, which is concomitant with the increased presence of PBs in all the tissues analyzed here. Also, the higher presence of ß-conglutins in the embryo compared to the endosperm was verified in the immunocytochemical experiments.

Both the histological features and analytical characteristics and the localization of the olive seed β-conglutins were also determined at longer times after anthesis (240 DAA). Such parameters did not differ substantially from those displayed here for 210 DAA in the cultivar 'Picual' and therefore were not shown in the present work. This may suggest that maturation of the seed ends before the maturation of the pulp in the olive fruit.

The distinctive character of the olive endocarp morphology and size amongst olive cultivars, previously reported by Barranco and Rallo (1984), was also verified in the present work. However, in this case the differences were also assessed as regard to the main parameters of the different constituents of the seed. Although differences among cultivars exist, some general directions can be detected. As an example, the weights of the whole fruit and that of the mesocarp were detected to have a positive correlation for all cultivars, whereas the weight of the whole seed was positively correlated with the weights of the individual components (endosperm and cotyledon) for most cultivars, and on the contrary, no correlation was detected between the weight of the whole seed and the weight of the testa for most cultivars. Such relationships may have particular meaning for future and potential uses of particular cultivars for the extraction of seed derived components, as it is the case of polyphenols (work in progress). In addition, the endocarp is considered to represent an evolutionary strategy for seed protection and dispersal (Dardick et al., 2010). Therefore, their size, and that of the different components of the seed should be further analyzed in relation to their dispersion efficiency, viability, ability of germination and vigor for the different olive cultivars, and particularly for wild olives, which are mainly propagated by seeds. This is one of the objectives of several research projects funding the present work. Also, moderate and severe reductions in water availability proportionately decrease endocarp expansion and prolong the sclerification, delaying the date of physically perceived hardening but not affecting the final degree of endocarp sclerification (Hammami et al., 2013). Therefore, the analysis of the hardening dynamics of the endocarp and the final size of the endocarps might be used as a marker for biological studies and crop management, as well as a marker for cultivar tolerance to water availability.

CONCLUSION

The described anatomy and histological distribution of the olive seed of the 'Picual' cultivar, allows identifying the main features typical of dicots within a developmental time frame. Cell storage structures (PBs and OBs) present a well-defined pattern of accumulation, with complementary distribution in the olive seed tissues.

Seed storage proteins of the 7S-type (β-conglutins) are relevant components of all olive seed tissues, displaying an accumulative pattern concurrent with the development of the seed and fruit. These proteins are present in at least two peptide forms, and are subcellularly associated to PBs in the different tissues analyzed.

Moreover, a panel of other olive cultivars has been used to compare the weight contribution of the different tissues to the seed, seed weight variability, and the number of seeds per fruit.

These hallmarks will help to settle the basis for future studies related to the location of different metabolites along the olive seed and mesocarp development, and therefore helping to assess the appropriate ripening stage for different commercial and industrial purposes.

AUTHOR CONTRIBUTIONS

AZ and JA designed the experimental structure of the work and redacted the manuscript. AZ performed the experiments, observations, image capture, and analysis of the results, whereas MM'-A performed TEM immunocytochemical detection and signal quantitation. JJ-L was particularly involved in the work with the databases and tools on the web servers for prediction of synthetic peptide and the generation of the antibody. EL was responsible for Western blotting experiments and analysis. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01481/full#supplementary-material

FIGURE S1 | (A) De-stoned seeds from 18 fruits cv. 'Picual' as an example. The endocarp and seeds are shown. **(B)** Dissection of the tissues from a 'Picual' seed. Up to down: endocarp, testa, endosperm, and embryo. **(C–E)** Representation of the different parameters in a total of 12 olive cultivars, including 'Picual' (arrow). Scale bars: 20 mm. **(F)** Spearman correlation of several weights of the seed tissues form different olive cultivars. (+/−) indicate positive/negative correlation ($p < 0.01$). Y/N (Yes/No) indicates whether some fruits of the cultivar contained more than 1 seed.

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CHAPTER 2: Contribution to the identification of the olive seed proteome by 1D/2D electrophoresis and MS/MS analysis

Contribution to the identification of olive seed proteome by 1D/2D electrophoresis and MS/MS analysis

INTRODUCTION:

The study of the olive seed proteins is still an unexplored area of knowledge. However, the implications of this protein compositionon and their contribution to olive oil stability and quality, as well as to human health are probably high. On the other hand, the increasing problem of human allergy to foods, and the cross-reactivity with allergens from other species is rising interest in the study of these proteins.

Up to date, a few proteins from the olive seed have been described, and are detailed in the following table (Table 1). More detailed information can be found at the introduction of the present Ph. D. report.

Protein	Reference
Oleosin family	(Ross et al. 1993), (Giannoulia et al. 2007)
Low abundance polipeptides	(Hidalgo et al. 2001)
Fatty Acid Desaturases	(Hernández et al. 2005) (Banilas et al. 2005)
Lipases	(Zienkiewicz et al. 2014)
Lipooxigenases	(Banilas et al. 2005) (Zienkiewicz et al. 2014)
SSP-11S	(Alché et al. 2006) (Ben Ali 2011)(Zafra et al. 2016)
SSP-7S	(Jimenez-López et al.2016) (Zafra et al. 2018)

Table 1: Proteins from the olive seed described to date. In green and red appear enzimatic and storage proteins, respectivelly.

MATERIALS AND METHODS:

Plant material:

Seeds were collected from olive trees (*Olea europaea* cv. 'Picual') cultivated at the Estación Experimental del Zaidín (Granada). The last stage of development was considered for the experiments, corresponding to seeds from mature fruits (210 days after anthesis -DAA- as determined by Wang et al. (2001)). To obtain the material, the mesocarp was removed by using a knife. Next, a de-stoning device was used to obtain the seed. Finally, the seeds were frozen in liquid nitrogen and stored at -80°C.

Protein extraction:

Proteins for 1-D and 2-D electrophoresis extracts were prepared in a similar way, as follows: olive seeds were crushed in liquid nitrogen to make them a fine powder and then re-suspended in extraction buffer [7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 60 mM DTT, 10 µL/mL plant protease inhibitor cocktail and 0.002 % (w/v) bromophenol blue] and shaked for 3.5 h at 4ºC. After that, samples were centrifuged at 20000 x g for 30 min at 4ºC. The aqueous supernatant was recovered and filtrated trough PD-10 Desalting Columns (BioRad). The elution obtained was precipitated with 20% TCA and 0.2% DTT in chilled acetone overnight at -20ºC followed by a centrifugation at 20000 x g for 30 min at 4ºC. The pellet was washed with 0.2% DTT in chilled acetone for 3 times and finally was let to partially dry. Semidry pellet was resuspended in an appropriate volume of 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 40 mM DTT, 0.5 % (v/v) carrier ampholytes pH 3–10, and 0.002 % (w/v) bromophenol blue.

Protein quantification:

Protein extracts were quantified by using the 2D Quant Kit (Amersham Biosciences) according to the manufacturer's instructions.

1-D gel electrophoresis:

SDS- PAGE was performed in a 4-20% stain-fee pre-cast gel (BioRad). One lane was loaded with the sample (25 µg of total protein). The molecular marker used were the Precision Plus Protein Unstained Protein Standards (BioRad). The electrophoresis was carried out at 100 V for 10 min and then,120 V for 60 min. The proteins were visualized with the aid of a GelDoc EZ Imager (BioRad).

2-D gel electrophoresis:

Rehydration of 11 cm IPG strips (pH 3-10 NL, BioRad) was performed overnight at RT including the sample (200µg of total protein). The IEF was carried out at 20ºC in a Ettan IPGphor Isoelectric Focusing System (Amersham Biosciences) as follows: 300 V for 1 min, 1000 V for 10 min, 8000 V for 90 min, and finally a total of 30 kVh. Reduction and alkylation steps were performed as described by (Görg et al. 1988). The second dimension was performed in a CriterionTM Cell (BioRad). The gels were stained with Sypro Ruby Protein Gel Stain (BioRad).

Gel spot/band excision and (nLC-MS) analysis:

The whole well from the 1-D gel was divided into 13 bands (0.5 cm length per band) that were sistematically scised and stored at 4ºC until analysis.

For the 2-D gel and aimed to avoid contamination of the samples the spots were excised by using a gel spot picker equipment at the facilities of the Proteomic Services at the López Neyra Institute (Granada, Spain). The obtained spots were stored at -20°C until analysis.

The analysis of a part of the spots was performed by liquid chromatography on-line coupled to tandem mass spectrometry (nLC-MS) at CIC bioGUNE (Bizkaia). LC was performed using an NanoAcquity nano-HPLC (Waters), equipped with a Waters BEH C18 nano-column (200mm x 75 um ID, 1.8um), A chromatographic ramp of 120 min. (5 to 60% ACN) was used with a flow rate of 300 nl/min. Mobile phase A was water containing 0.1% v/v formic acid, while mobile phase B was ACN containing 0.1% v/v formic acid. A lock mass compound [Glu1]-Fibrinopeptide B (100fmol/ul) was delivered by an auxiliary pump of the LC system at 500 nl/min to the reference sprayer of the NanoLockSpray (Waters) source of the mass spectrometer. 0.5 ug of each sample were loaded for each run. The Mass spectrometry was carried out in a Synapt G2Si ESI Q-Mobility-TOF spectrometer (Waters) equipped with an ion mobility chamber (T-Wave-IMS) for high definition data acquisition analyses. All analyses were performed in positive mode ESI. Data were post-acquisition lock mass corrected using the double charged monoisotopic ion of [Glu1]-Fibrinopeptide B. Accurate mass LC-MS data were collected in HDDA mode that enhances signal intensities using the ion mobility separation step.

Database search:

Database searching was performed using MASCOT 2.2.07 (Matrixscience, London, UK) against the specified databases. For protein identification the following parameters were adopted: carbamidomethylation of cysteines (C) as fixed modification and oxidation of methionines (M) as variable modifications, 15 ppm of peptide mass tolerance, 0.2 Da fragment mass tolerance and up to 3 missed cleavage points, Peptide charges of +2 and +3.

Bioinformatic analysis:

The identified peptides were analysed by using the Blast2Go software (Conesa et al. 2005). The mentioned software was also used to construct the multilevel charts of the GO terms.

RESULTS:

The 1D gel showed 6 major bands intensively stained that correspond to the acidic and basic forms of the 11S proteins (Figure 1). The molecular weights of these bands ranged between ~45 to ~10 kDa. Besides, minor bands as well as a light smear was observed along the whole well.

The analysis of all the scised bands (Figure 1) retrieved a total of 690 proteins identified from different databases. After skip of redundant

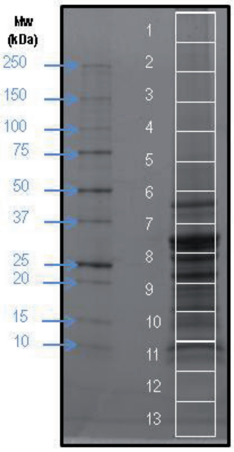

Figure 1: Electrophoretic separation of proteins from the olive seeds (cv. 'Picual') at the mature stage. The squares demarcate the 13 scised bands. Molecular weights (Mw) are indicated on the left

accession number, a total of 415 proteins were left for further bioinformatic analysis (Table 2).

The identification of the proteins retrieved from different databases as well as the total coverage from all the peptides, the number of unique peptides and the numer of peptides obtained for each protein are listed in Supplementary tables 1-11. The further bioinformatic analysis of the data will provide a complete panorama of the proteins harboured in the seed at the mature stage.

Band	# proteins
2	71
3	67
4	67
5	38
6	92
7	73
8	35
9	106
10	22
11	47
12 and 13	72
TOTAL	690 (415 non redundant)

Table 2: Summary of the results from the 1D gel and nLC-MS after searching using MASCOT against several databases. More data are available at Supplementary tables 1 to 11.

Regarding the 2D gel, a total of 189 sports were obtained (86 spots from the A1 area, 44 from A2, 72 from A3, and 84 from A4) (Figure 2). The use of a non-lineal gel for electrofocusing did not allow to determine the p*I* of the proteins from the spots detected. The use of standards simultaneolsy with the sample will help to know the p*I*. We observed the presence of highly stained spots in both, the acidid and the basic area of the gel, with a poor presence of spots in the central area. The A3 area showed the most intensivelly stained spots, corresponding to the acidic part of the gel (pH ranging from 17 to 35 kDa).

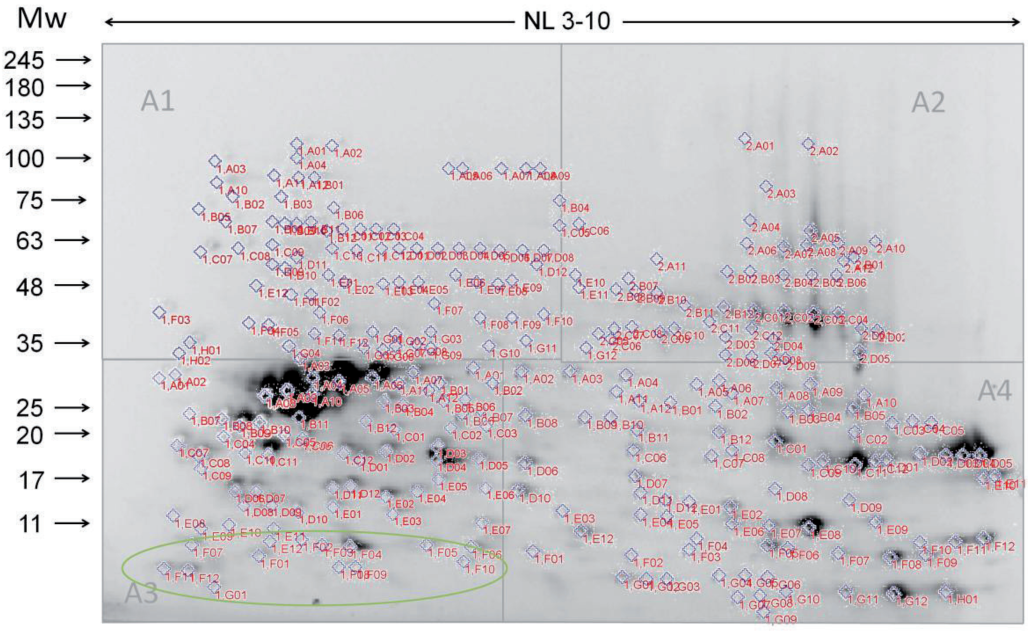

Figure 2: Two-dimensional map of the olive seed protein distribution. A1 to A4 show the areas of the gel aimed to identify the picked spots. The green oval demarcates the spots analysed in the present work (A3F01 to A3F12 and A3G01). Molecular weights (Mw) and pIs are indicated on the left and upper part respectively.

From the picked spots, a total of 31 ID Uniprot entries were retrieved. The presence of repeated peptide sequences was usually determined in different spots. We performed clustering of peptides that retrieved the same ID Uniprot (Table 3).

From this approach, four IDs were identified with enzymatic function, 4 IDs were classified as storage proteins, and 2 IDs corresponded to a fungus. Among the proteins with enzymatic function, a variety of processes were detected as those involved in regulation of endopeptidase activity, alkaloid metabolic process, response to biotic/abiotic stress or storage material biosynthetic process (carried out by alpha-amylase inhibitor, perakine reductase, pyridoxal 5'-phosphate synthase, or glucose-1-phosphate adenylyltransferase respectively). The four mentioned enzymes have been reported for the first time in the olive seed in the present study.

Among the SSPs, the 12S seed storage protein CRA1 was a newly described storage protein in the olive. A total of 3 peptides were identified in a single spot matching with the 12S SSP. The proteins identified as 11S globulin seed storage protein-2 (ID: Q9XHP0), and Glycinin G3 (ID: P11828), enclose several peptides, that in most of the cases were repetitive. Although, the bioinformatic algorisms output them as different UniprotIDs, they correspond to the 11S family. Another proteins identified as 11S and legumin A were identified with the ID:A0A1J6L9S7 (11S globulin subunit beta), ID: A0A022PUJ3 (legumin A). In both cases the peptide sequence displayed several changes when compared to the other 11S proteins previously identified. The presence of such variability in IDs corresponding to the 11S was not surprising, even more when the size of the analysed spots is greater than those already described. This is due to the presence of several isoforms of the 11S (Hsiao et al. 2006; Zafra et al. 2016). Moreover, massive presence of peptides from the 11S-type is expected from future analysis corresponding to the remaining spots to be analysed as this proteins have been revealed as the most abundant in the olive seed (Alché et al. 2006).

The existence of the fungus *Aureobasidum pullulans* is suggested as two different peptides from two enzymes were identified. On the one hand, it was detected the presence of a DNA-directed RNA polymerase, on the other hand, it was identified an ubiquitin-conjugating enzyme. The later was identified in 4 different spots, in all the cases, the same peptide sequence was detected. *Aureobasidum pullulans* has been described as an endophyte fungus found in twigs, buds, leaves and seeds of several plants species (Pugh and Buckley 1971). Fungal infections in olive fruits (and therefore likely seeds) are frequent, including many different fungal species like *Colletotrichum*, *Verticullium* and *Epicoccum* (Romero et al. 2017; Berardo et al. 2018; Trapero et al. 2018).

A preliminary analiyisis of the Gene Ontology terms related to biological processes, molecular function and cellular component was performed. Although, the picked spots represents less than a 7% of the total of spots to be analysed, they represent a first approach of the metabolism of the olive seed at the mature stage (Figure 2).

Focusing on the biological process, most of the proteins are devoid of protein oligomerization. Besides, a diversity of biological processes are ocurring at the mature stage such as those related to alkaloid metabolic process, response to a variety of stimulus, seed maturation or biosynthtetic processes among others.

The score distribution as regard to the molecular function showed a lower variety, with most of the proteins involved in the nutrient storage or in the formation of multimeric forms.

The cellular component representation is in good agreement with the previous charts, with a major number or peptides corresponding to the formatión of protein bodies. The protein bodies (PBs) are de main storage component in the olive seed (Jiménez-López and Hernández-Soriano 2013; Jiménez-López et al. 2015; chapter 1 in the present doctoral thesis).

UniProt ID	Name	Identified peptides	Spots
P01083 *	Alpha-amylase inhibitor 0.28	VVQQQQQEEGSYGAQEMEEIVEK	A3F1
Q3L181 *	Perakine reductase	FGVAGYDASGVIVK	A3F1
Q39963 *	Probable pyridoxal 5'-phosphate synthase subunit PDX1	XTGAAMIR	A3F4
		XTGAAMIR	A3F6
A0A1A7MDX6 *	DNA-directed RNA polymerase OS=Aureobasidium pullulans	SRVVDNDK	A3F4
A0A1J6L9S7	11S globulin subunit beta	TNDNAMISPLAGR	A3F8
		TNDNAMISPLAGR	A3F9
Q9XHP0	11S globulin seed storage protein 2	GLIVNVQGR	A3F2
		LSVSQPSR GLIVNVQGR	A3F3
		GLIVNVQGR	A3F7
		GLIVNVQGR LSVSQPSR	A3F11
		GLIVNVQGR	A3F12
		GLIVNVQGR	A3G1
P11828	Glycinin G3	LSVSQPSR	A3F1
		LSVSQPSR	A3F4
		LSVSQPSR	A3F5
		LSVSQPSR	A3F8
		LSVSQPSR	A3F9
		LSVSQPSR	A3F10
A0A022PUJ3	Legumin A	IQSEGGVTESYDYNDDQFR	A3F1
		IQSEGGVTESYDYNDDQFR	A3F2
		IQSEGGVTESYDYNDDQFR	A3F4
		IQSEGGVTESYDYNDDQFR	A3F6
		IQSEGGVTESYDYNDDQFR KLQGENDQR LQGENDQR FFLAGNPR	A3F7
		KLQGENDQR LQGENDQR FDNVFK	A3F12
P15455 *	12S seed storage protein CRA1	IQSEGGVTESYDYNDDQFR GLPVDVIVNAYQVSR KVAEQLK	A3F3
A0A1A7MMV3 *[1]	Ubiquitin-conjugating enzyme OS=Aureobasidium pullulans	MSSSDMAFVTK	A3F5
		MSSSDMAFVTK	A3F8
		MSSSDMAFVTK	A3F12
		MSSSDMAFVTK	A3G1
P55243 *[1]	Glucose-1-phosphate adenylyltransferase large subunit 3, chloroplastic/amyloplastic	LSPALSXR	A3F11

Table 3: Identified peptides from the 2D gel. In green and red appear the enzymatic and storage proteins, respectively. Asterisks mark the proteins non previously described in the olive seed.[1]Point out sequences from a fungus.

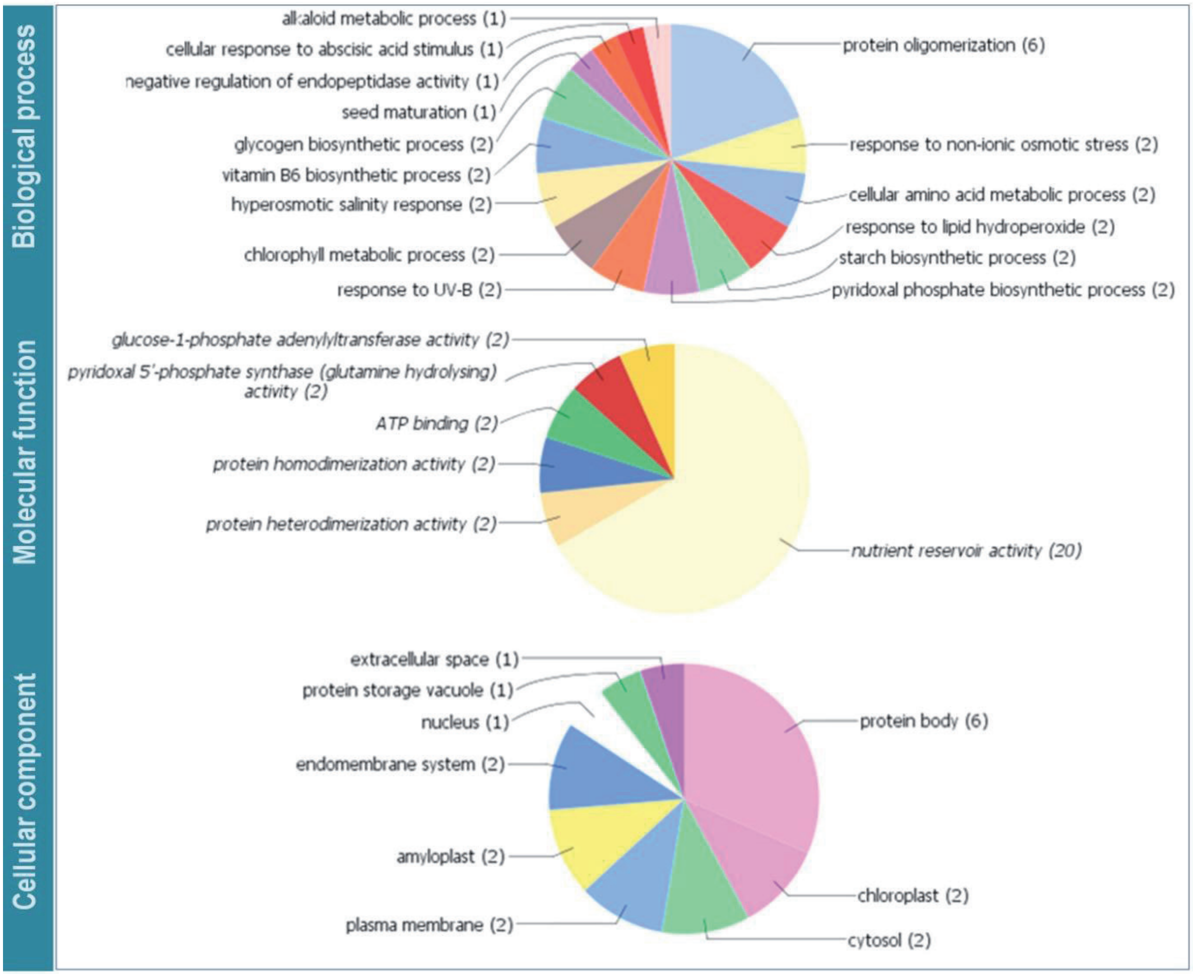

Figure 2: Score distribution as regard to the biological processes, molecular function, and cellular component by using the Blast2Go software and the peptides from the MS/MS as input data**.**

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	Accession no.	ΣCoverage	Σ# Unique Peptides	Σ# Peptides
1	se11_olive_001385	50.10	6	19
2	st11_olive_044888	45.54	1	4
3	se11_olive_004187	45.35	16	16
4	OE6A086946P1;se11_olive_032407;Oeu047135.1	40.38	5	5
5	po11_olive_012949	38.91	3	7
6	se11_olive_001935	38.83	3	16
7	OE6A025388P1	38.65	4	12
8	po11_olive_006047	37.04	7	10
9	se11_olive_003475	29.97	2	10
10	se11_olive_002407	28.11	9	9
11	OE6A060083P1	26.03	5	5
12	OE6A048872P1;Oeu025418.1	25.50	5	9
13	Oeu005907.1	21.56	6	6
14	se11_olive_038672	21.52	1	1
15	se11_olive_021540	21.05	1	1
16	se11_olive_009672	20.00	1	1
17	OE6A096590P1	15.94	2	2
18	se11_olive_061119	15.71	2	2
19	st11_olive_018218	15.00	1	1
20	OE6A079934P2	14.29	2	2
21	st11_olive_049264;OE6A067874P2;se11_olive_028153	14.06	2	2
22	se11_olive_012561	13.92	3	3
23	Oeu053751.1	13.82	2	2
24	se11_olive_008577	13.77	2	5
25	st11_olive_023635;OE6A042027P1;Oeu056242.1	13.70	2	2
26	Oeu056399.1	12.82	2	2
27	se11_olive_011371	12.75	1	2
28	st11_olive_042761	12.24	1	1
29	se11_olive_033389	12.05	2	2
30	se11_olive_049224	11.70	2	2
31	OE6A053952P2;Oeu023438.1	10.48	1	1
32	se11_olive_068035	10.00	1	1
33	OE6A033183P1;Oeu021666.1	9.79	1	1
34	OE6A062144P1;se11_olive_062869	9.68	1	1
35	OE6A072702P1	9.57	4	4
36	OE6A116843P1	9.22	1	1
37	se11_olive_010359	9.18	2	2
38	se11_olive_013355	8.67	2	2
39	se11_olive_060405	8.67	1	1
40	OE6A061739P1	8.60	4	4
41	po11_olive_021315	8.22	2	2
42	se11_olive_023818	8.08	1	1
43	po11_olive_020759	7.87	1	1
44	st11_olive_020379	7.85	2	2
45	se11_olive_009355	7.56	1	2
46	st11_olive_003259	7.45	3	3
47	st11_olive_003679	7.43	2	2
48	OE6A011535P1	7.38	4	4
49	OE6A092657P1	7.38	1	1
50	se11_olive_065900	7.29	1	1
51	se11_olive_035581	7.28	1	1
52	se11_olive_045553	7.26	1	1
53	st11_olive_002519;OE6A033723P1	7.06	3	3
54	se11_olive_010165	5.58	1	1
55	po11_olive_013895	5.17	1	1
56	st11_olive_008733	4.76	1	1
57	st11_olive_006413;OE6A041235P1	4.24	1	1
58	st11_olive_002817;OE6A105640P2;se11_olive_003793	4.15	1	1
59	st11_olive_006821	4.01	1	1
60	OE6A021975P1;Oeu045264.1	3.91	1	1
61	OE6A065898P1;Oeu038387.1	3.88	1	1
62	po11_olive_018119	3.38	1	1
63	Oeu022788.1	3.37	1	1
64	Oeu006216.1	3.35	1	1
65	st11_olive_003499;OE6A071181P1	3.23	1	1
66	st11_olive_003291;OE6A020079P1	3.15	1	1
67	st11_olive_005047;OE6A006603P1;Oeu005904.1	2.45	1	1
68	st11_olive_000513;OE6A005975P1;Oeu039535.2	1.36	1	1
70	A0A1A7MDX6	1.10	1	1
71	OE6A014885P2;Oeu006728.1	1.10	1	1

Supplementary table 1: Raw data from the MS/MS analysis obtained from band 2. The accession indicates the correspondence of the retrieved peptides to proteins identified from several databases. The total coverage of the peptides sequences, the number of unique peptides, and the number of peptides for each protein are indicated.

	Accession no.	ΣCoverage	Σ# Unique Peptides	Σ# Peptides
1	st11_olive_044888	45.54	1	4
2	OE6A025388P1	44.69	5	13
3	B2BGV3	42.86	1	1
4	OE6A085162P2;Oeu059422.1	42.11	6	17
5	OE6A086946P1;se11_olive_032407;Oeu047135.1	40.38	5	5
6	OE6A042009P1	35.93	13	13
7	OE6A001876P1;Oeu058096.1	32.66	7	11
8	se11_olive_001935	30.59	2	13
9	se11_olive_003475	30.23	2	10
10	OE6A048872P1;Oeu025418.1	29.80	5	11
11	Oeu020988.1	28.74	1	4
12	se11_olive_047909	27.59	2	2
13	OE6A060083P1	22.73	4	4
14	OE6A094761P1	22.42	2	3
15	se11_olive_002407	21.20	7	7
16	po11_olive_012740	21.19	2	2
17	se11_olive_021540	21.05	1	1
18	OE6A063781P1	20.43	3	4
19	OE6A074162P1;Oeu051074.1	20.29	3	3
20	po11_olive_021343	18.07	2	3
21	se11_olive_008577	18.03	1	5
22	st11_olive_023635;OE6A042027P1;Oeu056242.1	16.44	2	2
23	OE6A096590P1	15.94	2	2
24	Oeu005907.1	15.83	5	5
25	Oeu033907.1	15.32	1	2
26	Oeu003096.1	15.28	16	16
27	st11_olive_018218	15.00	1	1
28	st11_olive_049264;OE6A067874P2;se11_olive_028153	14.06	2	2
29	se11_olive_044659	13.50	2	2
30	OE6A033183P1;Oeu021666.1	13.29	2	2
31	OE6A102177P1;Oeu020138.1	12.29	1	1
32	se11_olive_009225	12.00	2	2
33	se11_olive_016546	10.88	1	1
34	OE6A011535P2	10.73	4	4
35	se11_olive_061437	10.29	1	1
36	OE6A061739P1	10.22	5	5
37	OE6A062144P1;se11_olive_062869	9.68	1	1
38	se11_olive_062400	9.38	1	1
39	se11_olive_014983	9.27	1	1
40	OE6A116843P1	9.22	1	1
41	Oeu029688.1	8.86	1	2
42	OE6A072702P1	8.85	3	3
43	se11_olive_060405	8.67	1	1
44	Oeu053751.1	8.55	1	1
45	se11_olive_023818	8.08	1	1
46	se11_olive_011244	8.03	1	1
47	se11_olive_025179	7.90	1	1
48	se11_olive_011371	7.52	1	1
49	OE6A092657P1	7.38	1	1
50	se11_olive_065900	7.29	1	1
51	se11_olive_045553	7.26	1	1
52	se11_olive_033389	6.63	1	1
53	Oeu003852.1	5.59	1	1
54	Oeu031872.1	4.92	1	1
55	se11_olive_005137	4.79	1	1
56	Oeu008540.1	4.53	3	3
57	st11_olive_022165	4.17	1	1
58	OE6A121079P1	4.07	3	3
59	OE6A021975P1;Oeu045264.1	3.91	1	1
60	OE6A065898P1;Oeu038387.1	3.88	1	1
61	Oeu022788.1	3.37	1	1
62	A0A1A7MIJ5	2.47	1	1
63	st11_olive_002217;OE6A060547P1;se11_olive_000143	2.46	1	1
64	st11_olive_005047;OE6A006603P1;Oeu005904.1	2.45	1	1
65	st11_olive_000553	1.67	1	1
66	st11_olive_002391	1.35	1	1
67	OE6A119104P1	0.97	1	1

Supplementary table 2: Raw data from the MS/MS analysis obtained from band 3. The accession indicates the correspondence of the retrieved peptides to proteins identified from several databases. The total coverage of the peptides sequences, the number of unique peptides, and the number of peptides for each protein are indicated.

	Accession no.	ΣCoverage	Σ# Unique Peptides	Σ# Peptides
1	OE6A085162P2;Oeu059422.1	47.57	8	19
2	se11_olive_004187	42.56	15	15
3	OE6A025388P1	35.51	3	11
4	se11_olive_001935	32.97	2	13
5	se11_olive_047909	27.59	2	2
6	se11_olive_010141	26.51	1	7
7	OE6A011535P2	26.18	14	14
8	OE6A048872P1;Oeu025418.1	25.50	5	10
9	OE6A063781P1	24.26	4	6
10	se11_olive_003475	24.18	1	8
11	OE6A074162P1;Oeu051074.1	24.15	3	3
12	Oeu033907.1	23.42	1	3
13	OE6A060083P1	22.73	4	4
14	Q8LGL8	22.42	2	3
15	se11_olive_067225	21.62	1	1
16	se11_olive_002407	21.20	7	7
17	OE6A012987P2	21.13	5	12
18	OE6A001876P1;Oeu058096.1	17.48	5	8
19	OE6A096590P1	15.94	2	2
20	po11_olive_012949	14.48	3	3
21	st11_olive_049264;OE6A067874P2;se11_olive_028153;Oeu004985.1	14.06	2	2
22	Oeu031872.1	14.02	1	3
23	OE6A061739P1	13.44	6	6
24	se11_olive_008577	13.11	1	4
25	OE6A102177P1;Oeu020138.1	12.29	1	1
26	st11_olive_042761	12.24	1	1
27	se11_olive_009225	12.00	2	2
28	Oeu001009.1	11.64	1	8
29	se11_olive_066903	11.49	1	1
30	se11_olive_035869	11.33	1	1
31	Oeu006088.1	11.01	1	1
32	OE6A075944P1	10.80	3	5
33	po11_olive_023518	10.49	1	1
34	se11_olive_061437	10.29	1	1
35	OE6A086946P1;se11_olive_032407;Oeu047135.1	10.26	1	1
36	se11_olive_068035	10.00	1	1
37	OE6A033183P1;Oeu021666.1	9.79	1	1
38	se11_olive_008959	9.38	2	2
39	se11_olive_062400	9.38	1	1
40	se11_olive_014983	9.27	1	1
41	OE6A110575P1	9.18	4	4
42	Oeu005907.1	9.17	3	3
43	se11_olive_054150	8.42	1	1
44	se11_olive_023818	8.08	1	1
45	st11_olive_055511	7.78	1	1
46	OE6A056675P1	7.46	2	2
47	se11_olive_065900	7.29	1	1
48	OE6A048146P4	7.16	2	2
49	se11_olive_033389	6.63	1	1
50	OE6A096205P3	6.60	1	3
51	st11_olive_001089;OE6A080379P2	6.21	1	3
52	po11_olive_018637	5.85	1	1
53	se11_olive_037565	4.69	1	1
54	po11_olive_000259;OE6A086846P1	4.63	2	2
55	st11_olive_002519;OE6A033723P1;se11_olive_000159	4.45	2	2
56	se11_olive_008199	4.33	1	1
57	po11_olive_006581	4.32	1	1
58	se11_olive_046362	4.32	1	1
59	st11_olive_006821	4.01	1	1
60	OE6A068610P1;Oeu023741.1	3.93	1	1
61	OE6A021975P1;Oeu045264.1	3.91	1	1
62	Oeu038387.1	3.88	1	1
63	OE6A075835P4;Oeu033834.2	3.46	1	1
64	se11_olive_010053	3.21	1	1
65	OE6A031045P1	2.86	1	1
66	st11_olive_005047;OE6A006603P1;Oeu005904.1	2.45	1	1
67	A0A1A7MDX6	1.10	1	1

Supplementary table 3: Raw data from the MS/MS analysis obtained from band 4. The accession indicates the correspondence of the retrieved peptides to proteins identified from several databases. The total coverage of the peptides sequences, the number of unique peptides, and the number of peptides for each protein are indicated.

	Accession no	ΣCoverage	Σ# Unique Peptides	Σ# Peptides
1	OE6A025388P1	38.65	2	12
2	OE6A085162P2;Oeu059422.1	38.26	7	17
3	se11_olive_004187	32.79	11	11
4	po11_olive_007534	30.34	2	2
5	se11_olive_010141	30.26	1	8
6	se11_olive_003475	27.46	1	9
7	se11_olive_001935	26.25	1	11
8	se11_olive_002407	21.20	7	7
9	po11_olive_008599	20.34	1	5
10	se11_olive_009672	20.00	1	1
11	se11_olive_008391	18.38	2	5
12	po11_olive_026298	17.16	1	3
13	Oeu024223.1	14.50	5	5
14	OE6A120877P1	13.48	5	6
15	se11_olive_010359	13.44	2	3
16	OE6A005775P1	13.27	3	6
17	se11_olive_066903	11.49	1	1
18	OE6A068610P1;Oeu023741.1	11.09	3	3
19	st11_olive_044888	10.89	1	1
20	st11_olive_017602	10.89	1	1
21	se11_olive_008959	9.38	2	2
22	se11_olive_014983	9.27	1	1
23	st11_olive_024157	9.24	1	1
24	se11_olive_036452	7.25	1	1
25	OE6A048872P1;Oeu025418.1	7.16	2	2
26	OE6A075944P1	6.97	2	3
27	se11_olive_052864	6.88	1	1
28	st11_olive_008979;se11_olive_005275	6.80	1	2
29	st11_olive_046179	6.72	1	1
30	OE6A063781P1	4.68	1	1
31	st11_olive_005855;OE6A060488P2	4.05	1	1
32	OE6A001876P1;Oeu058096.1	3.15	1	1
33	OE6A061739P1	3.05	2	2
34	st11_olive_003679;po11_olive_002119;Oeu028498.1	2.65	1	1
35	st11_olive_005047;OE6A006603P1;Oeu005904.1	2.45	1	1
36	A0A1A7MN35	1.99	1	1
37	A0A1A7MHK4	1.28	1	1
38	A0A1A7MDX6	1.10	1	1

Supplementary table 4: Raw data from the MS/MS analysis obtained from band 5. The accession indicates the correspondence of the retrieved peptides to proteins identified from several databases. The total coverage of the peptides sequences, the number of unique peptides, and the number of peptides for each protein are indicated.

	Accession no.	ΣCoverage	Σ# Unique Peptides	Σ# Peptides
1	se11_olive_003475	49.87	5	14
2	OE6A025388P1	48.79	3	16
3	OE6A048872P1;Oeu025418.1	48.14	7	16
4	st11_olive_002051_split_1;Oeu040532.1	46.22	3	12
5	se11_olive_004187	41.86	15	15
6	Oeu056399.1	37.82	8	8
7	se11_olive_001385	36.05	9	18
8	se11_olive_006407	35.53	5	8
9	OE6A001876P1;Oeu058096.1	34.67	4	10
10	st11_olive_013543;po11_olive_004495;OE6A013240P3	33.33	3	9
11	se11_olive_002407	32.72	10	10
12	se11_olive_047909	31.90	3	3
13	OE6A116538P2	31.84	2	9
14	OE6A013474P2	31.42	3	8
15	st11_olive_008979;se11_olive_005275	31.36	2	8
16	OE6A005218P1;Oeu052149.1	31.20	9	10
17	se11_olive_010141	31.12	1	9
18	po11_olive_007037	30.56	4	4
19	se11_olive_017527	28.47	1	5
20	OE6A084267P1	26.86	4	8
21	se11_olive_008577	25.57	2	7
22	Oeu051123.1	24.87	7	7
23	po11_olive_012949	24.43	5	5
24	se11_olive_008959	23.58	3	6
25	Oeu004925.1	23.08	3	5
26	OE6A083712P2;Oeu038666.2	22.78	2	5
27	st11_olive_006413;OE6A041235P1	22.69	1	6

Supplementary table 5: Raw data from the MS/MS analysis obtained from band 6. The accession indicates the correspondence of the retrieved peptides to proteins identified from several databases. The total coverage of the peptides sequences, the number of unique peptides, and the number of peptides for each protein are indicated.

28	se11_olive_001935	22.56	2	11
29	st11_olive_012867;po11_olive_003769	22.09	4	4
30	st11_olive_009893	20.80	6	6
31	se11_olive_059400	20.59	1	2
32	Oeu021861.1	20.35	2	7
33	Oeu059241.1	20.27	1	4
34	se11_olive_011371	20.26	2	5
35	st11_olive_059151	18.28	1	1
36	po11_olive_002412	17.39	1	1
37	OE6A094965P1	17.14	4	4
38	st11_olive_005855;OE6A060488P2	17.12	5	5
39	se11_olive_008391	17.03	2	5
40	st11_olive_002817;OE6A105640P2;se11_olive_003793	16.91	2	4
41	Oeu051278.1	15.71	4	4
42	st11_olive_013057;se11_olive_007487	15.43	1	5
43	st11_olive_003679;Oeu028498.1	14.85	3	3
44	Oeu020283.1	14.49	2	2
45	OE6A011279P1	14.44	1	3
46	se11_olive_062504	14.02	1	1
47	st11_olive_009056	13.70	1	1
48	st11_olive_011233	13.61	3	4
49	po11_olive_014354	13.27	1	1
50	st11_olive_034739	13.21	1	1
51	Oeu005907.1	12.16	5	5
52	OE6A017058P1	12.11	4	4
53	se11_olive_012561	12.09	1	3
54	OE6A074162P1;Oeu051074.1	11.59	1	1
55	st11_olive_011831;se11_olive_004169	11.57	1	3
56	se11_olive_024389	11.50	1	2
57	OE6A072702P1	11.24	4	4
58	Oeu028188.3	11.07	2	2
59	se11_olive_040365	10.55	2	2
60	se11_olive_017619	10.12	1	1
61	Oeu045134.1	9.09	2	2
62	se11_olive_054150	8.42	1	1
63	OE6A061739P1	8.06	4	4
64	OE6A106759P1	7.94	1	1
65	st11_olive_011349;po11_olive_003129	7.76	2	2
66	OE6A086946P1;se11_olive_032407;Oeu047135.1	7.69	1	1
67	se11_olive_065900	7.29	1	1
68	se11_olive_029590	7.28	1	1
69	OE6A107141P1	7.26	2	2
70	se11_olive_030941	7.20	1	1
71	Oeu004268.2	7.17	1	1
72	se11_olive_030712	6.99	1	1
73	se11_olive_052864	6.88	1	1
74	st11_olive_028095	5.36	1	1
75	po11_olive_000659	5.26	1	2
76	OE6A098222P1	4.94	2	2
77	OE6A048146P4	4.37	1	1
78	OE6A068227P1	4.14	1	1
79	po11_olive_001765	3.64	1	1
80	A0A1A7MRV9	3.55	1	1
81	st11_olive_014043;OE6A052971P3;se11_olive_009481	3.48	1	1
82	OE6A075835P4;Oeu033834.2	3.46	1	1
83	se11_olive_032213	2.82	1	1
84	OE6A021130P1	2.48	1	1
85	st11_olive_005047;OE6A006603P1;Oeu005904.1	2.45	1	1
86	Oeu004625.1	2.29	1	1
87	st11_olive_001575;OE6A069005P1;Oeu030728.1	2.00	1	1
88	F4MKN1	1.58	1	1
89	A0A1A7MH00	1.55	1	1
90	Oeu015239.1	0.83	1	1
91	OE6A016475P2	0.74	1	1
92	A0A1A7MFH2	0.61	1	1

Supplementary table 5 (continued): Raw data from the MS/MS analysis obtained from band 6. The accession indicates the correspondence of the retrieved peptides to proteins identified from several databases. The total coverage of the peptides sequences, the number of unique peptides, and the number of peptides for each protein are indicated.

	Accession no.	ΣCoverage	Σ# Unique Peptides	Σ# Peptides
1	po11_olive_012949	54.30	1	11
2	OE6A085162P2;Oeu059422.1	52.43	1	24
3	se11_olive_001385	52.14	2	25
4	OE6A085162P3	50.83	1	17
5	se11_olive_025791	48.98	1	11
6	se11_olive_004187	46.28	6	15
7	Oeu029688.1	42.80	4	9
8	se11_olive_048524	41.95	2	5
9	po11_olive_027602	38.51	2	5
10	se11_olive_001935	36.44	2	15
11	se11_olive_002407	36.18	11	11
12	OE6A032471P1	34.27	2	11
13	se11_olive_047909	31.90	3	3
14	OE6A074178P1	31.62	11	11
15	se11_olive_055883	28.57	1	1
16	po11_olive_007194	27.59	1	1
17	OE6A025388P1	27.54	2	9
18	se11_olive_003475	26.45	2	8
19	Oeu021563.1	25.13	4	4
20	st11_olive_008657	25.08	6	6
21	OE6A060083P1	22.73	4	4
22	se11_olive_008299	21.95	4	4
23	po11_olive_009623	19.75	3	3
24	OE6A059955P1	19.08	1	1
25	po11_olive_023606	16.67	3	3
26	se11_olive_010039	15.35	3	3
27	OE6A006678P2	15.20	1	2
28	OE6A053566P2;Oeu053846.1	14.96	1	3
29	Oeu053844.1	14.96	1	3
30	po11_olive_013428	14.88	1	1
31	se11_olive_001825	14.71	2	4
32	st11_olive_023635;OE6A042027P1;Oeu056242.1	13.70	2	2
33	se11_olive_026087	13.60	2	2
34	se11_olive_044659	13.50	2	2
35	J7FSH5	12.77	1	1
36	se11_olive_047541	12.66	2	2
37	po11_olive_006047	12.12	2	2
38	B3V758	11.38	1	1
39	OE6A018645P1	11.11	2	2
40	se11_olive_008391	11.08	1	3
41	Oeu002036.1	10.81	1	1
42	st11_olive_035394	9.79	1	1
43	se11_olive_018922	9.57	1	1
44	st11_olive_015925;OE6A021769P1	8.70	2	2
45	po11_olive_018329	8.33	1	1
46	se11_olive_023818	8.08	1	1
47	OE6A088855P2;Oeu044186.1	7.98	1	1
48	se11_olive_025179	7.90	1	1
49	se11_olive_038452	7.76	1	1
50	OE6A086946P1;se11_olive_032407;Oeu047135.1	7.69	1	1
51	po11_olive_010244	7.62	1	1
52	st11_olive_021891;OE6A046514P1;se11_olive_015397	7.33	1	1
53	se11_olive_065900	7.29	1	1
54	se11_olive_030712	6.99	1	1
55	Oeu036539.1	6.78	1	1
56	po11_olive_004083	6.55	1	1
57	st11_olive_032715	5.88	1	1
58	OE6A087816P3;Oeu019044.1	5.71	1	1
59	Oeu003852.1	5.59	1	1
60	po11_olive_025880	5.36	1	1
61	po11_olive_006405	5.32	1	1
62	Oeu004925.1	4.95	1	1
63	po11_olive_025019	4.89	1	1
64	st11_olive_033095;OE6A102039P1	4.86	1	1
65	se11_olive_040365	4.64	1	1
66	OE6A048146P4	4.37	1	1
67	st11_olive_006821	4.01	1	1
68	st11_olive_012625;OE6A090959P2	3.85	1	1
69	OE6A048872P1;Oeu025418.1	3.15	1	1
70	OE6A077722P1	3.00	1	1
71	Oeu026061.1	2.97	1	1
72	OE6A016373P1;Oeu047010.2	2.91	1	1
73	OE6A074073P1	1.75	1	1

Supplementary table 6: Raw data from the MS/MS analysis obtained from band 7. The accession indicates the correspondence of the retrieved peptides to proteins identified from several databases. The total coverage of the peptides sequences, the number of unique peptides, and the number of peptides for each protein are indicated.

	Accession no.	ΣCoverage	Σ# Unique Peptides	Σ# Peptides
1	se11_olive_001385	38.09	9	18
2	OE6A042009P1	34.63	15	15
3	se11_olive_002407	30.88	8	10
4	se11_olive_048390	29.35	3	3
5	Oeu005907.1	28.67	1	9
6	se11_olive_055883	28.57	1	1
7	st11_olive_027499;OE6A082544P1	27.83	3	3
8	se11_olive_001935	26.03	2	11
9	OE6A074178P1	24.02	1	9
10	OE6A025388P1	20.29	5	5
11	Oeu021563.1	19.60	3	3
12	po11_olive_009744	19.30	1	1
13	Oeu002293.2	16.67	3	3
14	se11_olive_016113	15.00	2	2
15	se11_olive_040125	14.62	1	3
16	OE6A058019P1	12.15	2	2
17	po11_olive_007037	11.51	1	1
18	st11_olive_002051_split_1;Oeu040532.1	11.48	2	2
19	st11_olive_012889;se11_olive_005163	8.27	1	1
20	se11_olive_023818	8.08	1	1
21	se11_olive_025179	7.90	1	1
22	st11_olive_021113;OE6A021385P2;Oeu028103.1	7.21	1	1
23	OE6A066249P1;Oeu059728.1	6.92	1	1
24	OE6A064543P1;se11_olive_060253;Oeu016643.1	5.73	1	1
25	se11_olive_061866	5.71	1	1
26	st11_olive_024415;po11_olive_009189;se11_olive_013353;Oeu000017.1	5.70	1	1
27	st11_olive_002817;OE6A105640P2	5.64	1	1
28	Oeu003852.1	5.59	1	1
29	OE6A021130P1	4.96	1	1
30	po11_olive_025019	4.89	1	1
31	po11_olive_016325	4.79	1	1
32	OE6A081892P1;Oeu020283.1	4.67	1	1
33	st11_olive_025807	4.44	1	1
34	st11_olive_006821	4.01	1	1
35	st11_olive_002221	2.91	1	1

Supplementary table 7: Raw data from the MS/MS analysis obtained from band 8. The accession indicates the correspondence of the retrieved peptides to proteins identified from several databases. The total coverage of the peptides sequences, the number of unique peptides, and the number of peptides for each protein are indicated.

	Accession no.	ΣCoverage	Σ# Unique Peptides	Σ# Peptides
1	OE6A086946P2;se11_olive_016481	71.15	6	13
2	se11_olive_004187	68.37	5	26
3	po11_olive_000924	64.29	2	2
4	se11_olive_001385	57.03	13	28
5	st11_olive_013785_split_0;se11_olive_034859;Oeu060418.1	56.98	3	8
6	OE6A066249P1;Oeu059728.1	55.97	7	7
7	OE6A027104P1	55.41	1	7
8	Oeu002036.1	55.41	1	7
9	OE6A072000P1	55.13	1	8
10	OE6A032471P1	53.80	1	22
11	se11_olive_007982	46.53	4	4
12	se11_olive_048390	44.57	2	2
13	se11_olive_002407	44.24	14	14
14	Oeu032666.1	42.77	2	4
15	se11_olive_001935	42.73	2	17
16	st11_olive_052659	41.89	1	5
17	st11_olive_034509;OE6A112336P1;se11_olive_037863	40.70	1	6
18	st11_olive_040375;Oeu018441.1	39.51	3	3
19	st11_olive_047607;OE6A019703P1;Oeu016801.1	36.88	2	5
20	OE6A024639P1	36.84	3	3
21	OE6A029561P1;Oeu059464.1	35.45	4	4
22	se11_olive_010756	29.51	2	3
23	OE6A094761P1	29.09	3	4
24	OE6A010084P1;se11_olive_051534;Oeu048272.1	28.89	2	2
25	po11_olive_021315	28.77	3	3
26	se11_olive_060405	28.67	2	2
27	se11_olive_030712	27.97	1	2
28	OE6A025388P1	27.78	4	8
29	st11_olive_042579;OE6A091095P1	27.63	4	4
30	se11_olive_047909	27.59	2	2
31	st11_olive_049461;OE6A011073P1	27.39	3	3
32	Oeu005907.1	26.83	2	10
33	OE6A048070P1	26.22	2	2
34	OE6A101982P1	26.11	2	2
35	OE6A087246P1	25.56	1	1
36	OE6A027236P1	25.41	4	4
37	OE6A074162P1;Oeu051074.1	24.15	3	3
38	se11_olive_060849	24.00	1	2
39	OE6A064543P1;se11_olive_060253;Oeu016643.1	23.96	4	4
40	se11_olive_039273	23.46	1	5
41	st11_olive_031511;se11_olive_002699	23.33	3	3
42	se11_olive_030653	22.63	3	3
43	st11_olive_044011;OE6A026823P1;se11_olive_047535;Oeu059975.1	22.29	3	3
44	st11_olive_002051_split_1;Oeu040532.1	22.13	2	6
45	se11_olive_067225	21.62	1	1
46	Oeu047085.1	21.43	1	1
47	po11_olive_013381	20.34	2	3
48	J7FXB3	20.27	1	1
49	OE6A074178P1	19.71	2	10
50	st11_olive_048091	18.57	1	1
51	st11_olive_013543;po11_olive_004495;OE6A013240P3	18.49	1	5
52	se11_olive_003618	18.39	1	1
53	OE6A108400P2	18.07	2	3
54	Oeu031399.1	17.27	1	2
55	se11_olive_009138	16.67	1	1
56	Oeu021563.1	16.58	4	4
57	se11_olive_047260	15.65	1	1
58	Oeu052371.1	15.65	1	1
59	st11_olive_027321;OE6A080235P2;Oeu032743.1	15.25	3	3
60	OE6A033183P1;Oeu021666.1	13.29	2	2
61	st11_olive_031797	13.16	2	2
62	st11_olive_039335;OE6A025809P1;se11_olive_057211;Oeu017263.1	12.60	1	1
63	OE6A032059P1	11.81	1	1
64	se11_olive_020464	11.59	1	1
65	OE6A109181P2;Oeu051062.1	11.18	1	1
66	po11_olive_012095	11.17	1	2
67	st11_olive_017602	10.89	1	1
68	Oeu023471.1	10.88	3	3
69	OE6A041586P1	10.56	2	2
70	se11_olive_017619	10.12	1	1
71	OE6A006678P2	10.00	1	1
72	OE6A014686P1;se11_olive_062085;Oeu023227.1	9.84	1	1
73	po11_olive_016325	9.58	1	2
74	st11_olive_044289	9.25	2	2
75	OE6A116843P1	9.22	1	1
76	st11_olive_024217;po11_olive_020023;OE6A052316P1;se11_olive_053519;Oeu030409.1	9.09	1	1
77	st11_olive_003893	8.84	3	3
78	OE6A034909P1;se11_olive_041645	8.78	1	1

Supplementary table 8: Raw data from the MS/MS analysis obtained from band 9. The accession indicates the correspondence of the retrieved peptides to proteins identified from several databases. The total coverage of the peptides sequences, the number of unique peptides, and the number of peptides for each protein are indicated.

79	se11_olive_017745	8.59	1	1
80	Oeu063292.1	8.46	1	1
81	st11_olive_041281;OE6A040074P1;se11_olive_055959;Oeu049196.1	8.39	1	1
82	st11_olive_043325;po11_olive_023045;OE6A096419P1;se11_olive_040721;Oeu033984.	8.28	1	1
83	se11_olive_023818	8.08	1	1
84	se11_olive_003119	7.82	2	2
85	st11_olive_028501;po11_olive_010961;OE6A004278P1;se11_olive_028827;Oeu022145.	7.73	1	1
86	se11_olive_043259	7.49	1	1
87	se11_olive_065900	7.29	1	1
88	se11_olive_045553	7.26	1	1
89	st11_olive_031941;Oeu052020.1	7.23	1	1
90	st11_olive_002817;OE6A105640P2;se11_olive_003793	6.82	2	3
91	st11_olive_037573;po11_olive_013983;OE6A018431P1;se11_olive_048517	6.77	1	1
92	se11_olive_033389	6.63	1	1
93	st11_olive_021703;se11_olive_037095	6.31	1	1
94	OE6A098105P1	6.11	1	1
95	st11_olive_036811	5.77	1	1
96	st11_olive_009877;OE6A030293P1	5.40	2	2
97	st11_olive_013045;OE6A017323P1	5.06	1	1
98	st11_olive_006821	4.01	1	1
99	se11_olive_000185	3.30	1	1
100	Oeu004925.1	3.02	1	1
101	st11_olive_005855;OE6A060488P2	2.70	1	1
102	po11_olive_000881	2.44	1	1
103	OE6A061739P1	2.15	1	1
104	st11_olive_001575;OE6A069005P1;Oeu030728.1	2.00	1	1
105	A0A1A7MRV1	1.92	1	1
106	OE6A016475P2	0.74	1	1

Supplementary table 8 (continued): Raw data from the MS/MS analysis obtained from band 9. The accession indicates the correspondence of the retrieved peptides to proteins identified from several databases. The total coverage of the peptides sequences, the number of unique peptides, and the number of peptides for each protein are indicated.

	Accession no.	**ΣCoverage**	**Σ# Unique Peptides**	**Σ# Peptides**
1	se11_olive_046105	25.76	1	1
2	se11_olive_018423	23.79	2	2
3	st11_olive_031875_split_0;OE6A016618P1	22.78	1	1
4	OE6A042009P1	21.65	10	10
5	OE6A085162P2;Oeu059422.1	20.85	1	9
6	se11_olive_001385	19.35	1	9
7	se11_olive_001935	13.45	1	5
8	se11_olive_007362	12.50	1	1
9	Oeu008613.1	9.71	1	1
10	st11_olive_054435	9.29	1	1
11	OE6A083307P1	8.41	4	4
12	po11_olive_021315	8.22	1	1
13	se11_olive_025179	7.90	1	1
14	se11_olive_065900	7.29	1	1
15	se11_olive_030712	6.99	1	1
16	po11_olive_001293	4.87	2	2
17	se11_olive_046362	4.32	1	1
18	OE6A093070P1;Oeu011144.1	4.23	1	1
19	se11_olive_044633	3.35	1	1
20	st11_olive_003679;po11_olive_002119;OE6A022576P2;Oeu028498.1	2.65	1	1
21	OE6A061739P1	2.15	1	1
22	A0A1A7MND2	1.23	1	1

Supplementary table 9: Raw data from the MS/MS analysis obtained from band 10. The accession indicates the correspondence of the retrieved peptides to proteins identified from several databases. The total coverage of the peptides sequences, the number of unique peptides, and the number of peptides for each protein are indicated.

	Accession no.	ΣCoverage	Σ# Unique Peptides	Σ# Peptides
1	OE6A085162P2;Oeu059422.1	47.77	7	20
2	se11_olive_001935	41.00	3	16
3	se11_olive_020464	57.97	4	4
4	se11_olive_004187	42.09	15	15
5	se11_olive_002407	30.88	9	9
6	OE6A086946P1;Oeu047135.1	17.95	3	3
7	st11_olive_032659;Oeu010915.1	39.02	2	2
8	po11_olive_001293	10.92	4	4
9	OE6A025388P1	18.12	7	7
10	st11_olive_025862;OE6A063938P1	24.62	2	2
11	se11_olive_063194	43.12	4	4
12	st11_olive_038555;OE6A021038P1;Oeu033115.1	10.17	1	1
13	se11_olive_009672	32.00	1	2
14	st11_olive_005855;OE6A060488P2	4.05	1	1
15	st11_olive_008979;se11_olive_005275	2.96	1	1
16	st11_olive_038369;Oeu000056.1	10.81	1	1
17	st11_olive_006821	4.01	1	1
18	OE6A034909P1;se11_olive_041645	10.81	1	1
19	OE6A031096P3;Oeu063154.1	9.40	1	1
20	OE6A054427P1	7.53	1	1
21	OE6A119560P1;Oeu007646.1	6.34	1	1
22	se11_olive_007982	22.77	2	2
23	se11_olive_007362	41.67	1	2
24	st11_olive_044137	5.23	1	1
25	st11_olive_005076	18.87	1	1
26	po11_olive_009639	4.30	1	1
27	po11_olive_000881	2.44	1	1
28	po11_olive_002877	1.88	1	1
29	po11_olive_025880	5.36	1	1
30	G0YLZ7	8.46	1	1
31	A0A1A7MT72	2.70	1	1
32	OE6A056866P1	2.78	1	1
33	OE6A032862P1	15.19	1	1
34	OE6A096413P1	10.34	1	1
35	se11_olive_065900	7.29	1	1
36	se11_olive_010165	5.58	1	1
37	se11_olive_030712	6.99	1	1
38	se11_olive_007866	14.81	1	1
39	se11_olive_054150	8.42	1	1
40	se11_olive_034297	5.38	1	1
41	Oeu022788.1	3.37	1	1
42	Oeu005907.1	3.21	1	1
43	Oeu008613.1	9.71	1	1
44	Oeu025923.1	5.73	1	1
45	Oeu039737.1	13.83	1	1
46	Oeu023816.1	1.66	1	1
47	Oeu000829.1	11.76	1	1

Supplementary table 10: Raw data from the MS/MS analysis obtained from band 11. The accession indicates the correspondence of the retrieved peptides to proteins identified from several databases. The total coverage of the peptides sequences, the number of unique peptides, and the number of peptides for each protein are indicated.

	Accession no.	ΣCoverage	Σ# Unique Peptides	Σ# Peptides
1	se11_olive_002407	51.15	15	15
2	se11_olive_004187	50.70	18	18
3	OE6A085162P2;Oeu059422.1	48.99	1	21
4	se11_olive_001385	48.68	1	21
5	se11_olive_067019	40.00	1	1
6	se11_olive_003475	38.04	3	11
7	OE6A025388P1	36.96	3	12
8	OE6A017058P1	33.88	5	13
9	se11_olive_001935	32.75	1	12
10	Oeu036098.1	30.48	2	10
11	st11_olive_015733	30.38	6	7
12	st11_olive_005855;OE6A060488P2	28.83	9	9
13	se11_olive_047909	27.59	2	2
14	st11_olive_058707	25.00	2	2
15	OE6A061739P1	23.48	10	10
16	st11_olive_003259	21.42	9	9
17	se11_olive_010141	21.04	1	7
18	po11_olive_011055	20.50	3	3
19	se11_olive_027790	19.18	1	2
20	OE6A049397P2	19.09	7	7
21	po11_olive_014770	17.82	1	1
22	po11_olive_009303	15.85	2	2
23	OE6A048872P1;Oeu025418.1	15.47	5	5
24	se11_olive_008391	14.59	1	4
25	po11_olive_001405	12.80	3	5
26	se11_olive_009355	12.79	3	3
27	OE6A068610P1;Oeu023741.1	12.70	4	4
28	st11_olive_018017	12.65	3	3
29	OE6A074178P1	12.53	4	4
30	Oeu012522.2	12.35	4	4
31	OE6A074432P3	11.66	3	3
32	st11_olive_005047;OE6A006603P1;Oeu005904.1	11.36	5	5
33	po11_olive_018980	10.96	1	1
34	Oeu057617.1	10.96	3	3
35	se11_olive_045130	10.57	1	1
36	OE6A086946P1;se11_olive_032407;Oeu047135.1	10.26	1	1
37	se11_olive_000669	10.07	1	2
38	OE6A108943P1	10.02	3	3
39	st11_olive_058492	9.94	1	1
40	Oeu021666.1	9.79	1	1
41	OE6A098222P1	9.70	1	5
42	se11_olive_000631	9.66	3	3
43	se11_olive_010359	9.51	2	2
44	st11_olive_002997	9.17	1	5
45	Oeu029688.1	8.86	1	2
46	se11_olive_023818	8.08	1	1
47	st11_olive_003679	7.69	1	2
48	st11_olive_020545	7.53	1	1
49	se11_olive_001457	7.36	2	2
50	se11_olive_046923	7.35	1	1
51	se11_olive_065900	7.29	1	1
52	se11_olive_035581	7.28	1	1
53	OE6A048880P6	7.19	2	2
54	st11_olive_005967;OE6A003027P1;Oeu014452.1	7.16	2	2
55	OE6A011535P2	7.16	3	3
56	se11_olive_030712	6.99	1	1
57	st11_olive_037573;po11_olive_013983;OE6A018431P1;se11_olive_048517	6.77	1	1
58	st11_olive_003899;OE6A096445P1;Oeu044039.1	6.08	1	2
59	Oeu011395.1	5.71	1	1
60	po11_olive_002717;OE6A047380P1	5.57	1	2
61	OE6A053387P1	4.75	1	1
62	st11_olive_003499;OE6A071181P1	4.74	1	1
63	st11_olive_004011;Oeu018515.1	4.73	1	1
64	OE6A063781P1	4.68	1	1
65	OE6A074162P1;Oeu051074.1	4.35	1	1
66	st11_olive_006821	4.01	1	1
67	st11_olive_033009	3.91	1	1
68	OE6A005218P1;Oeu052149.1	3.21	1	1
69	st11_olive_002217;OE6A060547P1;se11_olive_000143	2.46	1	1
70	OE6A087904P1;Oeu011087.1	2.43	1	1
71	st11_olive_000351_split_1;OE6A053478P1;Oeu029163.1	1.99	1	1
72	st11_olive_003047	1.74	1	1

Supplementary table 11: Raw data from the MS/MS analysis obtained from band 12 and 13. The accession indicates the correspondence of the retrieved peptides to proteins identified from several databases. The total coverage of the peptides sequences, the number of unique peptides, and the number of peptides for each protein are indicated.

3.1. Transcriptome-based identification of a seed olive legumin (11S globulin). Characterization of subunits, 3D modeling and molecular assesment of allergenicity

3.2. Identification and assesment of the potential allergenicity of 7S vicilins in olive (*Olea europaea* L.) seeds

CHAPTER 3: Identification and bioinformatic analysis of the mayor SSPs transcripts in the olive seed

Transcriptome-Based Identification of a Seed Olive Legumin (11S Globulin). Characterization of Subunits, 3D Modelling and Molecular Assessment of Allergenicity

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Abstract. Seed storage proteins (SSPs) are fundamental molecules for seed germination as an important source of carbon and nitrogen. Among the main four protein families that integrate SSPs, legumins (11S globulins) are widely distributed in dicots, and represent the major contribution to the pool of seed proteins in olive. In the present study, we have used an olive seed transcriptome generated de novo by 454/Roche Titanium+ sequencing to identify a broad panel of 11S protein sequences. Among these identified legumin sequences, five were selected using their presence within the output results from the BLASTP alongside the whole NCBI database, and their clustering with previously-characterized 11S sequences in the phylogenetic analysis as the criteria. The selected sequences were identified as corresponding to the isoform 2 of the 11S protein precursor, and one of the sequences was used for further analysis. Individual acidic and basic subunits within this sequence were recognised, 3D-modeled and assessed as regard to their potential molecular interaction by docking methods. Furthermore, T-cell epitopes were forecast by using predictive software in order to evaluate the putative implications of the olive 11S proteins in food allergy. The potential use of this protein highly present in the olive seed as a food source is discussed.

Keywords: Electrostatic potential · Globulin · Legumin · Molecular modelling · Olive · T-cell epitopes · Transcriptome · 11S

1 Introduction

The olive tree (*Olea europaea* L.) is agriculturally and economically very important, mainly in the Mediterranean area where it represents 95 % of the total world production of green and black table olives (16 million tons) and olive oil (2.7 million tons). Among Mediterranean countries, Spain is the major producer of olive oil (44 % of the world production) [1, 2]. Olive oil is unavoidably rooted to a genuine diet that has been associated to protective features against cardiovascular disease and cancer [3–7].

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The use of new olive by-products and specific procedures envisaged to improve economic returns from olive drupes is currently under development. Among these, the use of olive stones and seeds is an alternative with a high potential.

Seed proteins are synthesized at high levels and stored in specific tissues (endosperm and cotyledon) at certain stages of development [8], particularly during the maturation process. After their synthesis, seed storage proteins (SSPs) are deposited in membranous specialized organelles namely protein bodies (PBs) inside of cotyledon and the endosperm cells [9]. They are further mobilized and hydrolysed during seed germination in order to supply amino acids used as nitrogen, carbon and sulphur source [10].

On the basis of their solubility properties [11], SSPs are classified in four major families: albumins, globulins (legumin and vicilin), prolamins, and glutelins.

Legumin and vicilin globulins belong to the cupin superfamily, mainly characterized by displaying a β-barrel structural feature [12], with sedimentation coefficients of 11S and 7S, respectively. Legumins and vicilins are originated from a common ancestral gen [13], which is under positive selection for 11S globulins in dicot plants only [14]. Thus, 11S globulins are widely distributed in dicots [15], and represent the most abundant protein form (70 % of the total seed nitrogen) in dicot seeds [8]. 11S proteins are normally in an oligomeric state, composed of up to six subunits situated at the vertices of an octahedron, with a symmetry 32 [16]. Each subunit is composed of an acidic and a basic chain derived from a single precursor of 50–60 kDa [17–20].

According to canonical synthesis and maturation pathways, their integrating polypeptides enter the endoplasmic reticulum (ER) because of the presence of an N-terminal signal peptide [21], where inter-chain disulphide bridges are established between both the basic and the acidic subunits [22], followed by a cleavage step directed by a asparaginyl endopeptidase [22–24] to finally yield the mature hexameric 11S protein integrated by α and β subunits associated into trimmers, through covalent and non-covalent interactions [25, 26]. This process (11S globulins endoproteolytic cleavage into two subunits linked by a disulphide bridge) is evolutionarily conserved in seeds of conifers, monocots and dicots [22].

After a desiccation period in seeds, SSPs are mobilized under seed hydration by *de novo* synthesis of cysteine proteinases [27, 28] required for the breakdown of peptides, allowing seed germination and seedling growth [29].

The 11S proteins have been widely studied in numerous monocot and dicot plant species [14]. However only few studies about 11S and other SSPs are available as regard to the olive tree [9, 30–33]. Based on proteomic studies, it was proposed the presence of 3 precursors of the 11S protein, obtained from combinations of 3 α subunits and 2 β subunits [9]. However, the identification of the nucleotide and amino acid sequences of one of these subunits is reported here for the first time. We have also performed *in silico* analysis of the structure of one of these precursors by homology modelling and molecular docking analysis to show the α/β subunits interacting surface, and the identification of T-cell epitopes.

2 Materials and Methods

2.1 *De Novo* Construction and Functional Annotation of an Olive Seed Transcriptome

For the construction of the transcriptome, mature seeds of the olive cultivar ‘Picual’ were used. Samples were thoroughly grinded with a pistil and liquid N_2 followed by the extraction of the total RNA as RNeasy Plant Mini Kit (Qiagen) manual instructions recommends. RNA integrity was checked by formaldehyde gel analysis [34]. The mRNAs were purified using the Oligotex mRNA mini kit (Qiagen). The concentration and quality of the mRNAs were determined by the Ribogreen method (Quant-it Ribo-Green RNA Reagent and kit) and the Agilent RNA 6000 Pico assay chip (Bioanalizer 2100). The isolated mRNAs were subjected to 454/Roche Titanium+ sequencing. Reads were processed, assembled and the functional annotation was performed as described by Zafra *et al.* [35].

2.2 Alignment and *in Silico* Analysis of the Sequences

Different strategies were defined to select 11S transcripts. First, semantic and name searches were carried out in the generated annotations using key words. BLASTP searches of known, conserved sequences for 11S were also performed in the annotated database. Finally, annotations were manually screened for specific sequences selected from well-established bibliography resources.

Partial amino acid sequences from olive 11S proteins were compiled and aligned using Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/). Alignments visualization was performed by using Bioedit V7.0.5.3 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Paired sequence (overlapping) reads were matched and joined to complete full-length sequences. Various mismatches in the overlapping sequence reads were allow in order to obtain these full sequences. Protein sequences derived from these final alignments were further analyzed for the presence of putative functional (biologically meaningful) motifs by the ScanProsite program (http://www.expasy.org/tools/scanprosite), and to confirm the identity of the final (mature) sequences. Protparam tool (http://web.expasy.org/protparam/) was used to identify theoretical pI and molecular weight.

2.3 Phylogenetic Tree

Phylogenetic tree was constructed with the aid of the software Seaview [36] using the maximum likelihood (PhyML) method implemented with the LG model of the most probable amino acid substitution calculated by the ProtTest 2.4 server [37]. The branch support was estimated by bootstrap resampling with 100 replications.

Based on the results of the phylogenetic tree, five sequences among all identified legumin sequences in the seed transcriptome were selected. As the criteria for selection, we designated their presence within the output results from the BLASTP (E-value = 0.0 to e-14) alongside the whole NCBI database, and their clustering with previously characterized 11S sequences in the phylogenetic analysis.

2.4 3D-Modeling

The sequences of two subunits taking part of one of the five 11S globulin precursors selected as indicated above (ID: 002407), were subjected to 3D homology modeling (http://swissmodel.expasy.org/workspace/) [38–41] by using 3ehk, 2e9q, 1od5, 3c3v, 3 kgl and 2d5f protein templates (alpha subunit) and 1fxz, 2e9q, 1od5, 3c3v, 3qac and 3 kgl protein templates (beta subunit) available in PDB (http://www.pdb.org). Models were visualized by PyMol software (https://www.pymol.org/).

2.5 Identification of T-cell Epitopes

Identification of linear T-cell epitopes was performed as described by Jiménez-López *et al.* [42].

3 Results

3.1 Searching the Olive Seed Transcriptome for 11S Sequences

A total of five inputs corresponding to 11S seed storage proteins were obtained from the database generated (Table 1).

Table 1. Selected sequences from the olive seed transcriptome identified as 11S seed storage proteins. Lengths and identity of the coding proteins, as well as those of the alpha and beta subunits are indicated, and compared to other species.

Olive sequences			Identity with other species		
Identifier	Length (aa)	Description (α/ß-subunits)	Percent	Accession number	Description
040125	253	α: lack N-terminal	51 %	NP_001291336.1	11S globulin seed storage protein 2 precursor [*Sesamum indicum*]
		ß: lack C-terminal			
067540	165	α: not sequenced	56 %	XP_012831308.1	11S globulin seed storage protein 2-like [*Erythranthe guttatus*]
		ß: lack N-terminal			
012745	291	α: lack N-terminal	70 %	NP_001291336.1	11S globulin seed storage protein 2 precursor [*Sesamum indicum*]
		ß: complete			
002407	435	α: complete	67 %	NP_001291336.1	11S globulin seed storage protein 2 precursor [*Sesamum indicum*]
		ß: complete			
052095	52	α: lack N-terminal and C-terminal	67 %	XP_009761684.1	11S globulin seed storage protein 2-like [*Nicotiana sylvestris*]
		ß: not sequenced			

3.2 Alignment of the NGS-Retrieved Sequences of 11S Proteins to the NCBI Database and Phylogenetic Analysis

BLASTP query of the individual sequences and ScanProsite analysis of motifs confirmed all 11S sequences as seed storage proteins. These sequences exhibited high identity to the 11S globulin precursor 2 (Table 1). Alignment of the sequences with high identity allowed us to identify the presence of both conserved and hyper-variable regions [43] (Fig. 1). Four cysteines involved in the establishment of disulphide bridges between subunits were also identified. From these, C14 and C47 were implicated in intra-chain disulphide bonds [25], while C90 and C251 were involved in the disulphide bridge between the α and ß subunits [44].

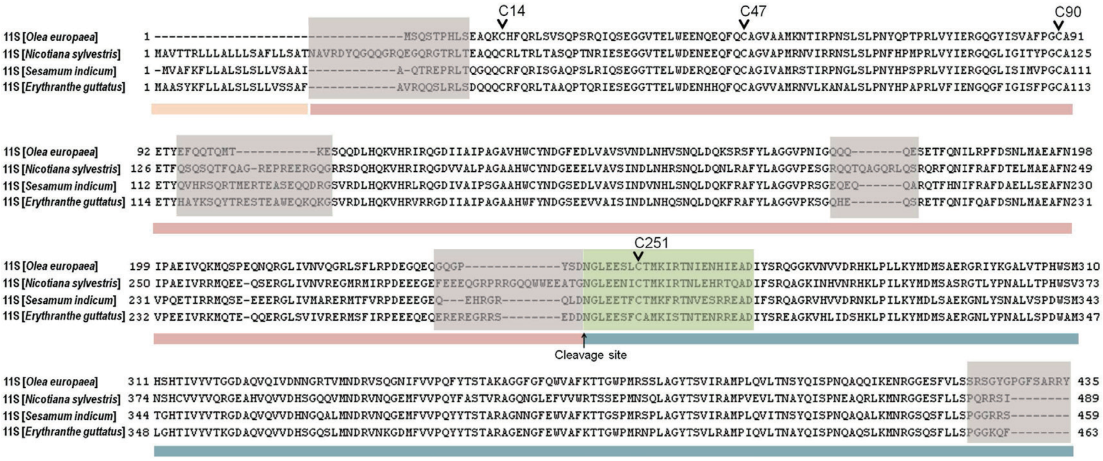


Fig. 1. Sequences alignment of the olive 11S (sequence id: 002407) and the 11S isoform 2 from tobacco (accession number: XP_009761684.1), sesame (accession number: NP_001291336.1) and common yellow monkey flower (accession number: XP_012831308.1). Hyper-variable regions are highlighted with gray boxes. The consensus pattern was located within a green box. The signal peptide and the acid/basic subunits (α/ß) are underlined in orange/red/blue colours, respectively. The four cysteine residues involved in disulphide bridges are pointed out with black arrows (Color figure online).

A total of 39 sequences were used to build a phylogenetic tree, which included a selection of the 11S sequences retrieved from the olive seed transcriptome, as well as the most relevant sequences (best BLAST scores) from other plant species. The olive retrieved sequences grouped together, and close to a cluster including the isoform 2 of the 11S precursor protein from other different plant species (named as "Group 1") (Fig. 2). Among such sequences, we found some from plants members of the following orders: Solanales, Gentianales, Sapindales, Ericales, Cariophyllales, Myrtales, Cucurbitales, Malgiphiales, Caryophyliales, Asterales, Rosales, and Lamiales, these later displaying the larger similarity with the olive sequences.

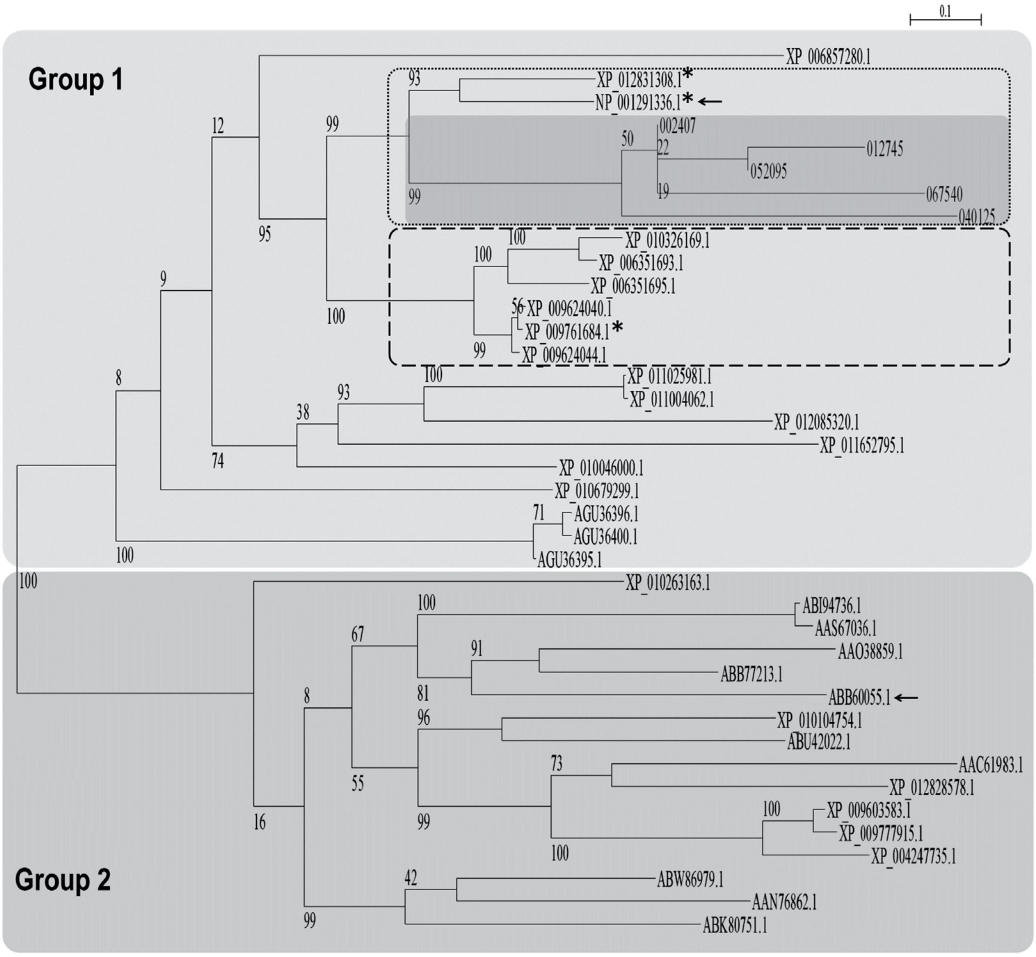
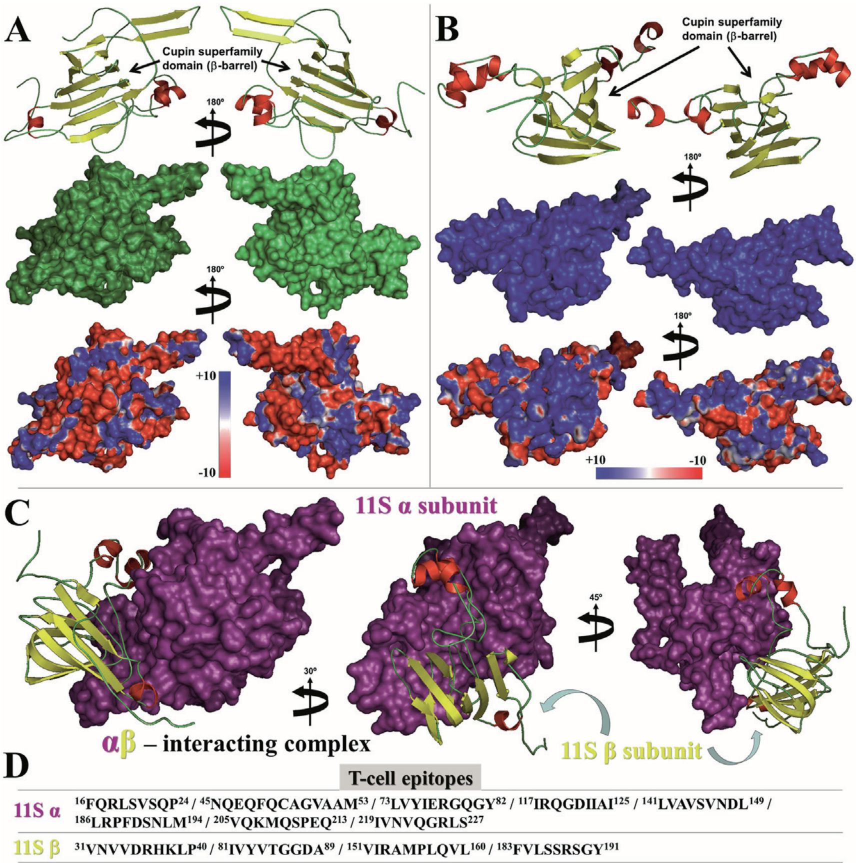


Fig. 2. Phylogenetic analysis of olive 11S seed storage protein sequences. Olive sequences obtained by NGS and most relevant orthologous identified in the NCBI database were used. Tree is divided in two big clusters, named Group 1 and Group 2. Group 2 contains sequences less-related to olive from a variety of plant species. Sequences from group 1 included all retrieved olive sequences (dark grey box). Sequences from Lamiales/Solanales are framed with doted and dashed lines. Asterisks (*) highlight the sequences from tobacco, sesame and common yellow monkey flower used for the alignment in Fig. 1. Arrows point out two sequences from group 1 and 2 corresponding to 11S isoform 2 and 4 from the same species (sesame). Measurements of support for the nodes are represented as percentages.

3.3 3D Modeling, Docking Analysis and Identification of T-cell Epitopes in the Olive 11S Globulin Subunits α and β

The olive 11S sequence identified as 002407 was used for homology modeling. Two models, built individually for the α subunit (Fig. 3A) and the β subunit (Fig. 3B), showed a β-barrel structure integrated by 11 antiparallel β-strands, which has been described as an specific feature of the cupin superfamily.



T-cell epitopes

11S α	^{16}FQRLSVSQP^{24} / ^{45}NQEQFQCAGVAAM^{53} / ^{73}LVYIERGQGY^{82} / ^{117}IRQGDIIAI^{125} / ^{141}LVAVSVNDL^{149} / ^{186}LRPFDSNLM^{194} / ^{205}VQKMQSPEQ^{213} / ^{219}IVNVQGRLS^{227}
11S β	^{31}VNVVDRHKLP^{40} / ^{81}IVYVTGGDA^{89} / ^{151}VIRAMPLQVL^{160} / ^{183}FVLSSRSGY^{191}

Fig. 3. Structural analysis of the olive (Olea europaea L.) 11S protein. Three-dimensional structure of olive 11S protein corresponding to the α (A) and ß (B) subunits. Structures were depicted as a cartoon diagram. α-helices, β-sheets and coils are depicted in red, yellow and green, respectively. Two views rotated 180 degrees round the x-axis are provided for both subunits of surface (green and blue) and electrostatic potential representation. The electrostatic potential surface colors are clamped at red (−10) or blue (+10). (C) Representation of the 11S α/β – interacting complexes. Three views rotated 30 and 45 degrees around the x-axis are provided (α in purple and ß as a cartoon diagram). (D) T-cell epitopes identified for the 11S individual α and ß subunits. Position of the epitope in each subunit is numbered at the beginning and end of the sequence (Color figure online).

The analysis of the surface electrostatic potential revealed several prominent charged residues, with more than half of the side exhibiting extensive number of negative residues in the α-subunit (Fig. 3A), and positive residues in the β-subunit (Fig. 3B). Docking analysis revealed predicted features for the heterodimeric association of α/β subunits (Fig. 3C). The models generated are in good agreement with the models present in the PDB database, corresponding to ortologous forms from other species. Both, the IE and IA faces, as well as the covalent and non covalent interactions between subunits were also predicted to occur properly in the olive forms.

The identification of T-cell epitopes in olive 11S (Fig. 3D) showed a large difference between both subunits in the number of epitopes predicted (eight and four for α and ß subunits, respectively).

4 Discussion

Although evidence of the presence of 11S proteins in the olive seed is available throughout biochemical approaches [9, 30–33], the sequences of olive seed 11S globulin transcripts have been identified in the current work for the first time. The results retrieved from the BLASTP analyses revealed that all the sequences obtained from olive transcriptomic approaches shared high identity to the isoform 2 of these proteins in other plant species. Furthermore, the phylogenetic analysis indicated that olive isoform 2 sequences grouped with orthologous sequences from other species as well. Structural analysis of both α/β subunits conforming the isoform 2 sequence showed a comparable 2-D distribution of elements, overall 3D folding and surface and charges to other described cupin proteins. All these above results lead to confirm the identity of the isoform 2 as belonging to a seed 11S globulin or legumin family. It has been largely described that the primary structure of the 11S globulin is well-conserved [45, 46]. Our results confirm that the 11S isoform 2 is also conserved in the olive.

Such high level of identity, together with the wide presence of these proteins in the olive seed represent convincing arguments favouring the potential use of olive seeds for animal and/or even human feeding purposes, in a similar way to other legumin containing plants like pea, lupin, chicken pea, pigeon pea, almond, coffee, lentil and others. In the case of the olive seed, massive amounts of olive seeds are available in those producing countries. However, technological approaches to isolate and purify the seeds from whole fruits and stones have yet to be developed. Moreover, additional studies have to be conducted in order to determine additional and important aspects of these proteins like digestibility, presence of different forms, variability among cultivars, or allergenicity. Knowledge of the corresponding transcript sequences is of particular interest as a way to determine these aspects, or to generate recombinant proteins able to easy the design and improve the reliability of numerous assays.

Food allergy is a growing world concern. Seed proteins are of special relevance since a high percentage of allergies diagnosed in atopic patients are caused by proteins present in seeds (i.e., sesame, mustard, grains as cereals), legumes (i.e., soybean, peanut, lupin) or nuts, (i.e., hazelnut and almond) almond [47–52]. Some of the proteins responsible for these effects are also responsible for cross-allergenicity (i.e., between tree nuts and peanut and mustard [50]), and include 11S and 7S SSPs. The identification of T-cell epitopes in the olive 11S, as reported here, revealed that these proteins might also putatively trigger allergy phenomena in humans. Moreover, the model proposed here indicates that alpha subunits from olive 11S may be more reactive than beta subunits, as a larger number of T-epitopes was predicted in the former. These analyses could help to identify hyper- and low- reactive forms of the protein, and for extension, forms with a higher or lower potential of cross-reactivity to other species. This knowledge would help to prevent allergy problems among the population providing that the olive seeds could become a source of proteins form human use, either on the whole, or as elaborated foods (i.e., flours, protein isolates, protein hydrolisates, etc.) [53].

5 Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

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Research Article

Identification and Assessment of the Potential Allergenicity of 7S Vicilins in Olive (*Olea europaea* L.) Seeds

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Olive seeds, which are a raw material of interest, have been reported to contain 11S seed storage proteins (SSPs). However, the presence of SSPs such as 7S vicilins has not been studied. In this study, following a search in the olive seed transcriptome, 58 sequences corresponding to 7S vicilins were retrieved. A partial sequence was amplified by PCR from olive seed cDNA and subjected to phylogenetic analysis with other sequences. Structural analysis showed that olive 7S vicilin contains 9 α-helixes and 22 β-sheets. Additionally, 3D structural analysis displayed good superimposition with vicilin models generated from *Pistacia* and *Sesamum*. In order to assess potential allergenicity, T and B epitopes present in these proteins were identified by bioinformatic approaches. Different motifs were observed among the species, as well as some species-specific motifs. Finally, expression analysis of vicilins was carried out in protein extracts obtained from seeds of different species, including the olive. Noticeable bands were observed for all species in the 15–75 kDa MW interval, which were compatible with vicilins. The reactivity of the extracts to sera from patients allergic to nuts was also analysed. The findings with regard to the potential use of olive seed as food are discussed.

1. Introduction

The olive tree is a vital element in the environment and agriculture of much of the Mediterranean basin, particularly in Spain. In addition to the high production levels of olive fruit and the quality of its oil [1], extra-virgin olive oil (EVOO) is reputed to have a number of health benefits [2–4].

The specialised olive oil industry is mainly based on crushing olive drupes, which is generally followed by further treatment of the raw material to extract the lipid fraction. However, an increasing body of evidence has highlighted the potential of olive stones as a complementary source of biomolecules with multiple uses and also a role in the biogenesis of EVOO [5, 6]. This has led to the generation of alternative processing techniques such as destoning processes prior to olive milling.

The mature olive fruit, or drupe, is composed of an epidermis surrounding a fleshy mesocarp, in addition to a woody endocarp containing an embryo enclosed by an endosperm with two half-sector hemispheres [7]. The tissues encompassing the olive embryo and endosperm contain a large amount of storage lipids and proteins in the form of compartmentalised structures surrounded by membrane, named oil bodies and protein bodies, respectively [8].

Seed storage proteins (SSPs) in the Poaceae family of monocots and in the dicots plant such as legumes have been widely studied [9]. SSPs have been classified with regard to their molecular masses expressed in terms of their sedimentation coefficients (S) [10] and classically on the basis of their solubility in different solutions. Thus, albumins are soluble in water, globulins in diluted saline, prolamins in alcohol, and glutelins in diluted acid or basis [11]. Previous studies by our research group have demonstrated that 11S SSPs similar to legumins are extremely abundant in the olive endosperm and embryo and are localized in numerous protein bodies present in the cytoplasm of cells, which are part of these tissues [8, 12]. In the olive seed, 11S proteins have been biochemically characterised, and the evolution of their olive seed formation and germination has been determined [8, 13, 14].

SSPs are among the principal food allergens [15, 16], the most important of which within this family are members of 11S, 7S, and 2S SSPs. Their allergenicity is dependent on thermal processing, salt, and high-pressure treatments [17, 18], and particularly gastrointestinal digestion [19]. Many of these proteins have a high level of identity between plant species, resulting in a high level of cross-reactivity [20].

Although more information on olive 11S SSPs has begun to emerge, no up to date data exists about the presence of 7S vicilins in olive. This type of SSPs, widely known as vicilins, can vary in their proportion to 11S SSPs [21]. Neither of these groups is characterised by clear sequence similarity, although they have similar holoprotein structures and a common evolutionary origin [22]. They are both members of the cupin superfamily and have evolved from bacterial enzymes [23]. This study aimed to identify signs of the presence of 7S vicilins in olive seeds at both the transcriptomic and biochemical level and also to assess the putative involvement of these proteins in triggering potential allergenic reactions. This information is considered to be highly useful for investigating the physiology of these seeds and for determining their use as a source of food in the future.

2. Materials and Methods

2.1. Plant Material. Seeds from 4 species, including *Olea europaea* L., *Helianthus annuus* L., *Arachis hypogaea* L., and *Anacardium occidentale*, were used. Olive seeds from the cultivar "Picual" were kindly provided by Elayotecnia S. L. (Castillo de Locubín, Jaén, Spain). Sunflower, peanut, and cashew seeds were purchased from a grocery store.

2.2. Retrieval of Sequences from Olive Seed Transcriptome. The search of sequences potentially corresponding to 7S vicilins was carried out in the *de novo* assembled and annotated transcriptome related to olive seeds at different stages of development as well as seed tissues. This transcriptome (unpublished) was generated by the Plant Reproductive Biology group from olive seed cDNA libraries subjected to 454/Roche Titanium+ sequencing reactions. The sequences obtained were preprocessed by using SeqTrimNext to eliminate incorrect, low-quality, and low-complexity readings, linkers, adapters, vector fragments, polyA/polyT tags, and contaminated sequences. The assembly strategy used a combination of different algorithms to correct bias and to produce the best available assembly. To generate primary contigs, MIRA3 (based on overlap-layout-consensus-based algorithms) and de Brujin graph-based Euler-SR software was used. Contigs were further exposed to consensus using CAP3 software. Such strategy was similar to that designed to generate a reproductive transcriptome in olive [24]. Preliminary annotation of the seed transcriptome was used to localise and analyze sequences corresponding to the SSPs of 7S vicilins.

2.3. Identity Analysis of Olive Vicilin Sequences. In order to calculate sequence identity, nucleotide and amino acid sequences were aligned by using the ClustalW software program [25–28]. Alignments were carried out among sequences of the same species and between different species. For this purpose, the BLOSUM- (BLOck SUbstitution Matrix-) type substitution/identity matrices [29] were calculated by using the BioEdit software program [30].

2.4. Experimental Cloning of Olive 7S Vicilins: Sequencing and Identification of Consensus Sequences. Total RNA was extracted from 0.1 g of olive seeds by following standard methods of extraction and purification using phenol/LiCl [31]. Reverse transcription was performed by using first strand cDNA synthesis (RevertAid Reverse Transcriptase, ThermoScientific) according to the manufacturer's instructions. PCR amplification was carried out in a Mastercycler pro S thermocycler (Eppendorf, Hamburg, Germany) using the following primers designed on the basis of the aligned sequences with the aid of the PRIMER3 program [32]: forward (Oe7S33F) 5′-CAACTTATTTAACAATAG-3′ and reverse (Oe7S11R) 5′-CTAATTGATTGATTATATTC-3′. The PCR products were analysed in 1% TAE-agarose gels, and the unique band of amplification observed was excised, eluted with the MinElute Gel Extraction Kit (Qiagen), cloned into the pGEM-T Easy (Promega) vector, and used to transform competent *E. coli* cells (strain DH5α). Blue/white colony selection enabled several colonies harbouring the vicilin sequence to be selected. Plasmid DNA was isolated with the aid of the Real Miniprep Turbo Kit (REAL) and was used for sequencing at the DNA sequencing facilities of the Estación Experimental del Zaidín (CSIC, Granada, Spain). The sequencing readings were again processed with ClustalW in order to generate a consensus sequence, which was used for further bioinformatic analysis.

2.5. Phylogenetic Analysis. Olive vicilin consensus sequences were used together with sequences across species to analyze phylogenetic relationships. The amino acid sequence alignment generated by ClustalW was used to generate phylogenetic trees according to the NJ (Neighbour Joining) method [33] based on the BLOSUM-type matrix. Tree visualization was performed using the Treedyn software program [34].

2.6. Identification and Selection of Templates for the Generation of 3D Structures. The olive vicilin amino acid sequence was used to search for and select homologous sequences in the PDB (Protein Data Bank) database [35] using the BLAST [36] and SWISS-MODEL [37] servers to identify possible structural patterns. Structural assessment was performed using stereochemical and structural energy parameters [38], and structure comparisons between olive 7S vicilins and 7S vicilin proteins from other plant species were carried out by superimposition of α carbon residues in order to calculate the average distances between these Cα backbones [39].

An initial structural model was generated and checked for error recognition in 3D structures using ProSA (https://prosa.services.came.sbg.ac.at/prosa.php) and also for an initial o verall quality estimation of the model using QMEAN (http://swissmodel.expasy.org/qmean/cgi/index.cgi). The final structures of the olive 7S vicilin protein and other proteins were subjected to energy minimization with GROMOS96 force field energy implemented in DeepView/Swiss-PDBViewer v3.7 (http://spdbv.vital-it.ch/) to improve the van der Waals contacts and to correct the stereochemistry of the model. For each sequence analyzed, the quality of the model was assessed by QMEAN, with protein stereology being checked using PROCHECK (http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/) and ProSA (https://prosa.services.came.sbg.ac.at/prosa.php) programs, and protein energy being verified with the aid of ANOLEA (http://protein.bio.puc.cl/cardex/servers/anolea/). The Ramachandran plot statistics for the models were also calculated to show the number of protein residues in the favored regions.

2.7. Building 3D Structures Corresponding to 7S Vicilin Proteins. The best structural template (1dquA) was retrieved from the PDB and used to model the 3D structure of the olive seed's 7S vicilin protein by means of the homology modeling approach with the aid of the Workspace application and the SWISS-MODEL automated modeling facility [37]. Visualisation of the 3D model was carried out using the SWISS-PDB Viewer/DeepView program.

2.8. Analysis of 2D, Surface, and Electrostatic Potential of Olive 7S Vicilins. The secondary structure was analysed using the Segmer algorithm [40], which compares homologous sequences retrieved from PDB in order to identify conserved substructures. The secondary structure was identified and then compared with the results obtained by the SSpro8 (Scratch Protein Predictor) which adopts the full class output classification of the DSSP [41], PredictProtein [42], NetSurfp [43], and PSIPRED [44] servers.

2.9. Identification and Analysis of T- and B-Cell Epitopes. Prediction of regions to the most common alleles of the HLA-DR (human major histocompatibility complex MHC) of an antigenic sequence (first residue of each nonapeptide) was generated by the T-EPITOPE software program [45] based on quantitative matrices and neuronal networks covering a large proportion of the peptides binding to class II human HLA. For the present study, the following most frequent HLA-DR alleles in the caucasian population were selected: DRB1*0101 (DR1), DRB1*0301 (DR3), DRB1*0401 (DR4), DRB1*0701 (DR7), DRB1*0801 (DR8), DRB1*1101 (DR5), and DRB1*1501 (DR2). The T-EPITOPE algorithm, which predicts nonapeptides binding to these alleles, was used under 5% prediction threshold conditions. In addition, in order to identify the HLA-DR ligands in the 7S vicilin proteins, only peptides binding to at least 3 different haplotypes in the majority of the sequences analyzed were selected [46].

To identify B-cell lineal, or continuous, epitopes, the vicilin sequences were analysed using the Ellipro (http://tools.immuneepitope.org/), ABCpred (http://www.imtech.res.in/raghava/), and COBEpro (http://scratch.proteomics.ics.uci.edu/) programs based on different algorithms [46].

2.10. Protein Extraction. The olive seeds were first ground and then defatted by means of supercritical fluid technology (at 300 bar and 55°C) from Elayotecnia S. L. The protein extracts were obtained by stirring 0.1 g of the flour obtained in 1.5 mL of extracting buffer [50 mM phosphate buffer (pH7.8) and 0.2% (p/v) SDS] for 2 h at 4°C. Samples were centrifuged at 1200 ×g for 20 min at 4°C and supernatants were filtered through a 0.22 μm filter (MillexGP, Millipore) and stored at −80°C until use.

Sunflower, peanut, and cashew seeds were first ground and then defatted using hexane. 40 g of ground seeds was mixed with 100 mL of hexane and shaken for 20 min, and the samples were then filtered to remove excess hexane. This step was repeated 3 times. Samples were washed by shaking with distilled water for 20 min and then filtered. This step was repeated a further 3 times. Finally, the flour was washed with 20% ethanol and left to totally dry. Protein extracts were prepared by stirring 0.2 g of flour in 5 mL of extracting buffer [40 mM Tris-HCl (pH7.0), 2% (v/v) Triton-X100, 60 mM DTT, and 10 μL of protease inhibitor cocktail (Sigma)] for 2 hours at 4°C. Samples were centrifuged at 1200 ×g for 20 min at 4°C and stored at −80°C until use.

2.11. Protein Quantification. Extracts from olive seeds were quantified using the Bradford method [47]. Alternatively, extracts from sunflower, peanut, and cashew were quantified by using the 2D Quant Kit (Amersham Biosciences) according to the manufacturer's instructions.

2.12. SDS-PAGE and Immunoblotting. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a horizontal ECL system (Amersham) and 4–20% precast gels. 30 μg of total protein was loaded per lane. Gels were stained with Coomassie Blue or transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% defatted milk in TBST for 1 h at room temperature (RT) and then incubated individually with sera from six different patients. Sera were diluted 1/100 in TBST and 5% defatted milk, and the membranes were incubated overnight at 4°C. Five patients were previously identified as allergic to nuts on the basis of their medical record and complementary assays (ImmunoCAP), with one nonallergic patient being used as the control. Immunodetection was performed using a goat anti-human IgE H&L (HRP) secondary antibody (Abcam) diluted 1/5000 for 1 h at RT. Bands were revealed by using the Clarity Western ECL Substrate (BioRad) and visualized in a ChemiDoc XRS system (BioRad). Images were gathered using a 12-bit CCD camera after 30 minutes of exposure and analyzed with Quantity One software v.4.6.2 (BioRad).

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                    5        15        25        35        45        55        65        75        85        95       105       115
Olive vicilin   ----------------------------------------------------------------------------------------LFPGCALRDADPHNVCFFGSIANLLVSVICKE
Q9AUD0_SESIN    ------MSCGGRLCLVLFALLLASAVVASESKDPELKQCKHQCKAQQQISKEQKEACIQACKEYIRQKHQGEHGRGGGDILEEEVWNRKSPIERLRECSRGCEQQHGEQREECLRRCQEE
B4X640_PISVE    ------------------------------KTDPELKQCKHQCKVQ-----------------------------------------RQYDEEQKEQCAKGCEKYYKEKKGREQEEEEEE
Q8L5L5_ANAOC    ----MGPPTKFSFSLFLVSVLVLCLGFALAKIDPELKQCKHQCKVQ-----------------------------------------RQYDEQQKEQCVKECEKYYKEKKGREREHEEEE
Q8S4P9_CORAV    ---------------------------MLPKEDPELKKCKHKCRDE-----------------------------------------RQFDEQQRRDGKQICEEKARERQQEEGNSSEES
F5B8V9_LUPAN    MAKMRVRLPMLILLLGVVFLLAASIGIAYGEKDFTKNPPKEREEEEHEPRQQPRPRQQEEQEREHRREE----------------KHDGEPSRGRSQSEESQEEEHERRREHHREREQEQ
P43237_ARAHY    ---MRGRVSPLMLLLGILVLASVSATQAKSPYRKTENPCAQRCLQSCQ--QEPDDLKQKACESRCTKLE----------------YDPRCVYDTGATNQRHPPGERTRGR-------QPG
Consensus

                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
                  125       135       145       155       165       175       185       195       205       215       225       235
Olive vicilin   EKQRKSLRVGGSRLLTTDKPHDLRLDLLKVDSPG----------------------------------------------------------------------------------MKYR
Q9AUD0_SESIN    YQREKGRQDDDNPTDPEKQYQQCRLQCRRQGEGGGFSREHCERRREEKYREQQGREGGRGEMYEGREREEEQEEQGRGRIPYVFEDQHFITGFRTQHGRMRVLQKFTDRSELLRGIENYR
B4X640_PISVE    EWGSGRGRGDEFSTHEPGEKRLSQCMKQCERQDGGQQKQLCRFRCQEKYKKERREHSYSRDEEEEEEGDEEQEEEDEN--PYVFEDEHFTTRVKTEQGKVVVLPKFTKRSKLLRGLEKYR
Q8L5L5_ANAOC    EE-WGTGGVDEPSTHEPAEKHLSQCMRQCERQEGGQQKQLCRFRCQERYKKERGQHNYKREDDEDEDEDE-AEEEDEN--PYVFEDEDFTTKVKTEQGKVVLLPKFTQKSKLLHALEKYR
Q8S4P9_CORAV    YG----------------------------------------------------------------------KEQEEN--PYVFQDEHFESRVKTEEGRVQVLENFTKRSRLLSGIENFR
F5B8V9_LUPAN    QPRPQRRQEEEEEEEEWQPRRQRPQSRREEREEREQEQGSSSGSQRGSGDERRQHRERRVHREEREQEQDSRSDSRRQRNPYHFSSNRFQTYYRNRNGQIRVLERFNQRTNRLENLQNYR
P43237_ARAHY    DYDDDRRQPRREEGGRWGPAEPREREREEDWRQPREDWRRPSHQQ-----PRKIRPEGREGEQEWGTPGSEVREETSRNNPFYFPSRRFSTRYGNQNGRIRVLQRFDQRSKQFQNLQNHR
Consensus                                                                                                                            :.*

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                  245       255       265       275       285       295       305       315       325       335       345       355
Olive vicilin   VAVLEAQPQTFVVPNHLDADALVYVAQGMGTVSLMSEDNRESFNIREGDFFWISAGTPTYLINRQKNQNLVIAKLLQPVAVPGRFEAYFGAGGENPESFYNAFSDEILDAALNAKSDKWR
Q9AUD0_SESIN    VAILEAEPQTFIVPNHWDAESVVFVAKGRGTISLVRQDRRESLNIKQGDILKINAGTTAYLINRDNNERLVLAKLLQPVSTPGEFELFFGAGGENPESFFKSFSDEILEAAFNTRRDRLQ
B4X640_PISVE    LAFLVANPQAFVVPNHMDADSIFFVSWGRGTITKIRENKRESMNVKQGDIIRIRAGTPFYIVNTDENEKLYIVKLLQPVNLPGHYEVFHGPGGENPESFYRAFSREVLEAALKTPRDKLE
Q8L5L5_ANAOC    LAVLVANPQAFVVPSHMDADSIFFVSWGRGTITKILENKRESINVRQGDIVSISSGTPFYIANNDENEKLYLVQFLRPVNLPGHFEVFHGPGGENPESFYRAFSWEILEAALKTSKDTLE
Q8S4P9_CORAV    LAILEANPHTFISPAHFDAELVLFVAKGRATITMVREEKRESFNVEHGDIIRIPAGTPVYMINRDENEKLFIVKILQPVSAPGHFEAFYGAGGEDPESFYRAFSWEVLEAALKVRREQLE
F5B8V9_LUPAN    IIEFQSKPNTLILPKHSDADFILVVLNGRATITIVNPDKRQVYNLEQGDALRLPAGTTSYILNPDDNQNLRVAKLAIPINNPGKLYDFYPSTTKDQQSYFSGFSKNTLEATFNTRYEEIE
P43237_ARAHY    IVQIEARPNTLVLPKHADADNILVIQQGQATVTVANGNNRKSFNLDEGHALRIPSGFISYILNRHDNQNLRVAKISMPVNTPGQFEDFFPASSRDQSSYLQGFSRNTLEAAFNAEFNEIR
Consensus       :  : :.*:::: * * **: :. :  * .*::    :.*:  *: .*. . : :*   *: * ..*:.* :.::  *:  **.   :. .  .: .*:  .** : *:*:::.  :  .

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                  365       375       385       395       405       415       425       435       445       455       465       475
Olive vicilin   RLFRK--------------------QRQGAILKASEEQIRAMS--REE-GGIWPFGGQTKRSVINIHDQRPSQFNQYGQLYEVD-SSQHWQLQDHDVSISLANITQGAMTTPYYNSKATK
Q9AUD0_SESIN    RIFGQ--------------------QRQGVIVKASEEQVRAMS--RHEEGGIWPFGGESK-GTINIYQQRPTHSNQYGQLHEVD-ASQYRQLRDLDLTVSLANITQGAMTAPHYNSKATK
B4X640_PISVE    KLFEK--------------------QDEGAIVKASKEQIRAMSRRGEGP-SIWPFTGKST-GTFNLFKKDPSQSNNYGQLFESE-FKDYPPLQELDIMVSYVNITKGGMSGPFYNSRATK
Q8L5L5_ANAOC    KLFEK--------------------QDQGTIMKASKEQIRAMSRRGEGP-KIWPFTEEST-GSFKLFKKDPSQSNKYGQLFEAE-RIDYPPLEKLDMVVSYANITKGGMSVPFYNSRATK
Q8S4P9_CORAV    KVFGE--------------------QSKGSIVKASREKIRALSQHEEGPPRIWPFGGESS-GPINLLHKHPSQSNQFGRLYEAH-PDDHKQLQDLDLMVSFANITKGSMAGPYYNSRATK
F5B8V9_LUPAN    RVLLGDDELQENEK--QRRGQEQSHQDEGVIVRVSKKQIQELRKHAQSS-SGEGKPSE--SGPFNLRSNKPIYSNKFGNFYEITPDIN-PQFQDLNISLTFTEINEGALLLPHYNSKAIF
P43237_ARAHY    RVLLEENAGGEQEERGQRRRSTRSSDNEGVIVKVSKEHVQELTKHAKSV-SKKGSEEEDITNPINLRDGEPDLSNNFGRLFEVKPDKKNPQLQDLDMMLTCVEIKEGALMLPHFNSKAMV
Consensus       :::                      : :* *::.*.:::: :    .          :   . :::    *   *::*.:.*     .   :.. :: :: .:*.:*.:  *.:**:*

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                  485       495       505       515       525       535       545       555       565       575       585       595
Olive vicilin   ISIVVDGEGYFEMACPHLS------QQQQQR-----------GPSYQKVSSPLKRGTVVVVPAGHPYVAVASNNQNLQLLCFEINSKNNKRYPLAG-RRNIINQLEREAKELAFGVSARE
Q9AUD0_SESIN    IALVVDGEGYFEMACPHMSRSRGSYQGETRG-----------RPSYQRVASRLTRGTVVIIPAGHPFVAVASSNQNLQVLCFEVNANNNEKFPLAG-RRNVMNQLEREAKELAFGMPARE
B4X640_PISVE    IAIVVSGEGRLEIACPHLSSSKNSGQEKS-------------GPSYKKLSSSIRTDSVFVVPAGHPFVTVASGNQNLEILCFEVNAEGNIRYTLAG-KKNIIEVMEKEAKELAFKTKGEE
Q8L5L5_ANAOC    IAIVVSGEGCVEIACPHLSSSKSS------------------HPSYKKLRARIRKDTVFIVPAGHPFATVASGNENLEIVCFEVNAEGNIRYTLAG-KKNIIKVMEKEAKELAFKMEGEE
Q8S4P9_CORAV    ISVVVEGEGFFEMACPHLSSSSGS---------------------YQKISARLRRGVVFVAPAGHPVAVIASQNNNLQVLCFEVNAHGNSRFPLAG-KGNIVNEFERDAKELAFNLPSRE
F5B8V9_LUPAM    IVVVDEGEGNYELVGIRDQQRQQDEQEEEYEQGEE---------EVRRYSDKLSKGDVFIIPAGHPLSINASS--NLRLLGFGINANENQRNFLAGSEDNVIKQLDREVKELTFPGSIED
P43237_ARAHY    IVVVNKGTGNLELVAVRKEQQQRGRREQEWEEEEEDEEEEGSNREVRRYTARLKEGDVFIMPAAHPVAINASS--ELHLLGFGINAENNHRIFLAGDKDNVIDQIEKQAKDLAFPGSGEQ
Consensus       * :* .* *  *:.  : .                           ::    :  . *.: **.**    **   :*.:: * :*:. * :  *** . *::. ::::.*:*:*    .:

                ....|....|....|....|....|....|....|....|....|....|
                  605       615       625       635       645
Olive vicilin   VDEVFGSQNDEGFVKGP--RQQQQGRADA---------------------
Q9AUD0_SESIN    VEEVSRSQQEEFFFKGP--RQQQQGRADA---------------------
B4X640_PISVE    VDKVFGKQDEEFFFQGPKWRQHQQGRADE---------------------
Q8L5L5_ANAOC    VDKVFGKQDEEFFFQGPEWRKEKEGRADE---------------------
Q8S4P9_CORAV    VERIFKNQDQAFFFPGPNKQQEEGGRGGRAFE------------------
F5B8V9_LUPAN    VERLIKNQQQSYFANAQPQQQ------QQREKEGRRGRRGPISSILNALY
P43237_ARAHY    VEKLIKNQRESHFVSARPQSQSPSSPEKEDQEEENQGGKGPLLSILKAFN
Consensus       *:.:  .* :  *  .    :
```

Figure 1: Amino acid sequence alignment of the PCR-validated olive 7S vicilin gene and vicilins from *Sesamum indicum* (SesIn_Q9AUDO), *Pistacia vera* (PisVe_B4X640), *Anacardium occidentale* (AnaOc_Q8L5L5), *Corylus avellana* (CorAv_Q8S4P9), *Lupinus angustifolius* (LupAn_F5B8V9), and *Lupinus albus* (LupAl_Q6EBC1).

3. Results

3.1. Identification and Validation of 7S Vicilin Sequences. A *de novo* assembled and annotated olive seed transcriptome (unpublished data) was used to search for sequences allocated to 7S vicilin proteins, vicilins, and vicilin-like proteins. A total of 58 entries were detected, all of which corresponded to partial transcripts (not shown). Further alignment and analysis of the retrieved sequences enabled us to design specific primers, which were used to amplify cDNA from mature olive seeds. PCR resulted in the amplification of a single band of ca. 720 bp. Sequencing of different cloned PCR products facilitated the identification of a partial consensus sequence of 240 amino acids that was used for additional bioinformatic analysis.

3.2. Bioinformatic Analysis of the Consensus Olive Vicilin Sequence and Vicilins from Other Species. The olive 7S vicilin sequence obtained was analysed by using the BLAST (Basic Local Alignment Search Tool) program [48] in order to identify considerable homology with vicilins from other sources. The amino acid sequences selected were used to generate sequence alignments with the aid of the ClustalW program (Figure 1).

The partial 7S vicilin gene sequence from olive was also used to carry out a phylogenetic analysis aimed at

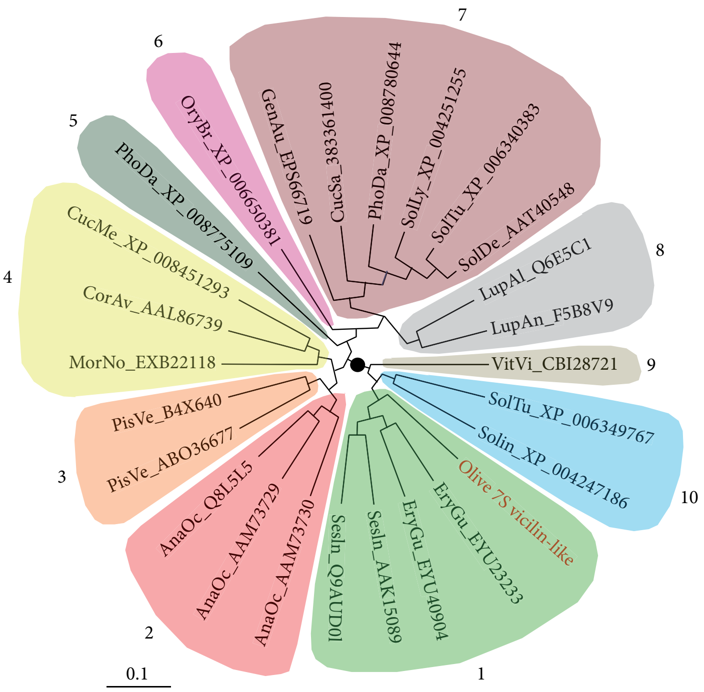


Figure 2: Phylogenetic analysis of 26 amino acid sequences of vicilins from olive and the following plant species: EryGu, *Erythranthe gutta*; SesIn, *Sesamum indicum*; AnaOc, *Anacardium occidentale*; PisVe, *Pistacia vera*; MorNo, *Morus notabilis*; CorAv, *Corylus avellana*; CucMe, *Cucumis melo*; PhoDa, *Phoenix dactylifera*; OryBr, *Oryza brachyantha*; GenAu, *Genlisea aurea*; CucSa, *Cucumis sativa*; SolLy, *Solanum lycopersicum*; SolTu, *Solanum tuberosum*; SolDe, *Solanum demissum*; LupAl, *Lupinus albus*; LupAn, *Lupinus angustifolium*; VitIn, *Vitis vinifera.*

determining its relationship to 7S vicilin proteins from other species (Figure 2). The phylogenetic analysis was performed using the NJ (Neighbour Joining) method [33] based on the BLOSUM-type matrix. A total of 26 amino acid sequences from different plants were used. Ten different phylogenetic groups were identified, with the olive 7S vicilin integrated in Group 1, jointly to four additional sequences from *Erythrante guttata* and *Sesamum indicum*.

A structural assessment to verify the accuracy of the olive 7S vicilin protein model was carried out through a comparative analysis of the template crystallographic structure used to build the model, using stereochemical and energy minimization parameters showing the following data.

Analysis of the best template 1dqu gave a value of 0.636 for the Qmean parameter (linear combination of six terms, including stereology and energy, to estimate model reliability ranging from 0 to 1) and 0.598 for the olive 7S vicilin model. ProSA, another parameter used to check overall structural quality, showed a *Z*-score of −9.06 and −7.95 for the crystallographic structural template and for the olive 7S vicilin structure constructed, respectively. These two parameters show quite similar values when compared to the natural, crystallographic structure and the structure of the olive 7S vicilin model; this means that the olive 7S vicilin model constructed is accurate and close to its template in structural quality terms.

Thus, we also checked the stereochemistry of the model using Procheck analysis, which showed that 80.5, 18.6, 0.7, and 0.2% of template structure residues were located in favorable regions, allowed regions, generally allowed regions, and disallowed regions in the Ramachandran plot, respectively; for the olive 7S vicilin model, these values were 91.1, 7.2, 1.0, and 0.6%, respectively, with even more residues being located in favourable regions and a similar situation for residues in nonfavourable regions.

Taking the comparisons of all these parameters together, it is possible to state that the olive 7S vicilin model built from its crystallographic template is sufficiently accurate to be used in further structural analyses. A similar assessment was made for the other 7S vicilin protein structures built in comparison with their templates.

A comparative structural analysis of the partial sequence of the olive vicilin and vicilins from other species was also carried out using homology modeling. In the protein structure of the olive 7S vicilin, a total of 9 α-helixes and 22 β-sheets were identified (Figure 3, upper panel). We also obtained a representation of the protein fragment surface and its electrostatic potential (Figure 3, middle and lower panels, resp.). Analysis of the latter revealed that prominent charged residues are present in the structure, with over 50% of the side chains exhibiting positive values (Figure 3, lower panel).

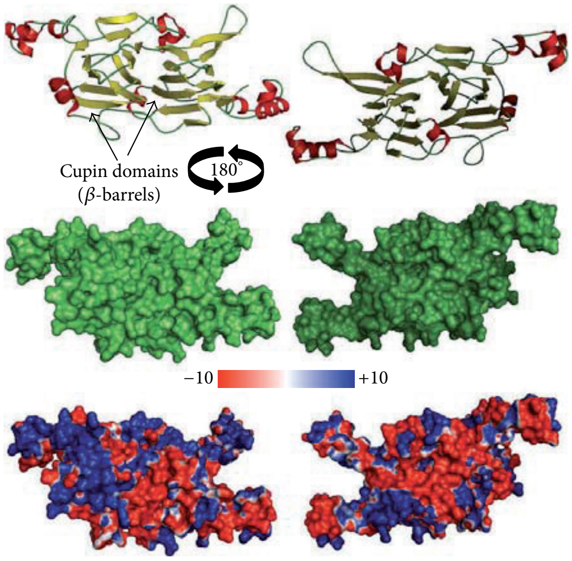


Figure 3: 2D structural elements, surface structure, and electrostatic potential analysis of the partial structure of the olive 7S vicilin. Upper panel: 3D structure showing α-helixes in red, β-sheets in yellow, and coils in green. Middle panel: representation of the protein surface. Lower panel: representation of electrostatic potential. The surface colors represent the charge density, with positive values in blue and negative values in red.

Surface electrostatic potential analysis revealed several prominent charged residues, with over half of the side chains showing high positive values in the blue region. Interestingly, a large number of positively charged residues were concentrated on one side of the structure. By assigning a value of +1 to basic residues (Arg, Lys) and −1 to acidic residues (Asp, Glu), the net protein charge was calculated to be +4 for the olive 7S vicilin.

Structural comparison of olive vicilin fragments, through 3D structure superimposition, showed superimposition values ranging from 0.318 Å to 1.717 Å (Figure 4). The best superimposition matches were those corresponding to *Pistacia vera* (0.318 Å) and *Arachis hypogea* (0.374 Å).

All vicilin-like proteins shared a common feature, two b-barrels, each of which is composed of 11 antiparallel β-sheets. Both barrels are part of the main globular domain of the protein. This commonly shared feature among vicilin-like proteins is also a characteristic of the whole cupin superfamily. The olive 7S vicilin is a seed storage protein, which, being a nonenzymatic protein, does not exhibit a catalytic domain. Comparison with other vicilin-like proteins showed that most of these 7S vicilin proteins are quite similar in terms of their core structure, or globular domain; major differences between the protein structures compared in this study have been found in the special distribution and the length of some coils/turns and α-helixes connecting antiparallel β-sheets in the same domain as the 2D structures.

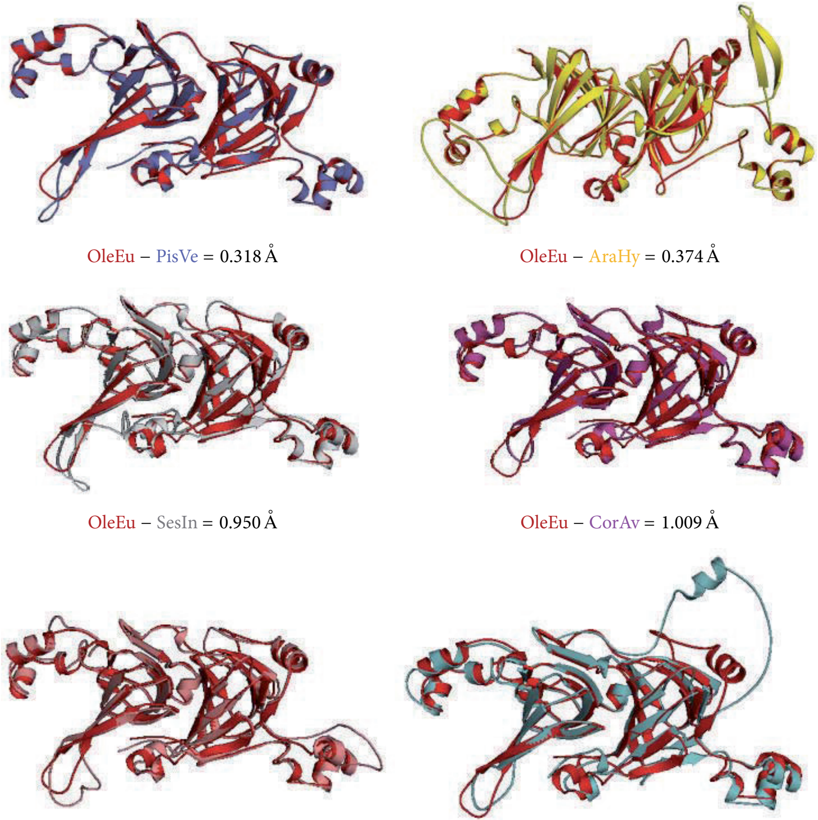
Table 1: T-cell epitopes common to more than one of the vicilins analysed.

Epitope name	Sequence	Shared species
T1	LVIAKLLQP	**Olive_002829** *Corylus avellana*_Q8S4P9 *Arachis hypogea*_P43237
T2	FEMACPHLS	**Olive_002829** *Sesamum indicum*_Q9AUD0 *Corylus avellana*_Q8S4P9
T3	INLLHKHPS	*Corylus avellana*_Q8S4P9 *Lupinus angustifolius*_F5B8V9 *Lupinus albus*_Q6EBC1
T4	YVAVASNNQ	**Olive_002829** *Sesamum indicum*_Q9AUD0
T5	LVIAKLLQP	**Olive_002829** *Sesamum indicum*_Q9AUD0 *Pistacia vera*_B4X640 *Corylus avellana*_Q8S4P9 *Arachis hypogea*_P43237
T6	VVLNGRATI	*Lupinus angustifolius*_F5B8V9 *Lupinus albus*_Q6EBC1, *Arachis hypogea*_P43237
T7	VVLLPKFTQ	*Anacardium occidentale*_Q8L5L5 *Lupinus albus*_Q6EBC1
T8	LRGIENYRV	*Sesamum indicum*_Q9AUD0 *Pistacia vera*_B4X640 *Arachis hypogea*_P43237
T9	LVSVLVLCL	*Anacardium occidentale*_Q8L5L5 *Lupinus albus*_Q6EBC, *Lupinus angustifolius*_F5B8V9

3.3. Identification and Analysis of T- and B-Epitopes. Cross-allergenicity and modulation of the allergenic response are highly dependent on the presence of epitopes recognised by the T cells of the human immune system. These epitopes may be present in different orthologs and therefore be responsible for cross-reactivity. In order to determine these putative epitopes and their variability, the method described by Burastero et al. [49] was used with the aid of the T-EPITOPE program [45].

The use of this software program enabled us to identify 19 T-epitopes, 9 of which were shared with more than one of the vicilins analysed (Table 1). Two epitopes (T2 and T9) were ubiquitous and were detected in all vicilins analyzed. The other T-epitopes, which were detected in only one of the vicilins analysed, were therefore considered to be species-specific (Table 2). The olive vicilin fragment showed 7 T-epitopes, 4 of which were found in other species, with 3 T-epitopes being unique to this species.

Analysis of the presence of immunodominant epitope regions recognised by IgG and IgE (B-cell epitopes) in the different vicilins resulted in the identification of 17 epitopes, 11 of which were shared by several 7S vicilin proteins (Table 3). Six other B-cell epitopes, present in particular species, were thus considered to be species-specific (Table 4). The olive vicilin fragment displayed 5 of these epitopes, 2 of which



OleEu − PisVe = 0.318 Å

OleEu − AraHy = 0.374 Å

OleEu − SesIn = 0.950 Å

OleEu − CorAv = 1.009 Å

OleEu − LupAn = 1.088 Å

OleEu − AnaOc = 1.717 Å

Figure 4: Structural comparison of the olive vicilin fragment with 7S vicilin proteins from other species. Superimpositions of the 3D structure of the olive vicilin (in red) and 3D models generated for the other proteins species are shown. *Pistacia vera* (purple), *Arachis hypogea* (yellow), *Sesamum indicum* (grey), *Corylus avellana* (pink), *Lupinus angustifolius* (light pink), and *Anacardium occidentale* (light blue). Numerical values for the structural differences are also displayed (in Å).

Table 2: Species-specific T-cell epitopes in the different species analyzed.

Epitope name	Sequence	Specific species
Ts1	FFGSIANLL	**Olive_002829**
Ts2	YLINRQKNQ	**Olive_002829**
Ts3	WRRLFRKQR	**Olive_002829**
Ts4	IVIVSKKQI	*Lupinus albus*_Q6EBC1
Ts5	MRVRLPMLI	*Lupinus angustifolius*_F5B8V9
Ts6	YVNITKGGM	*Pistacia vera*_B4X640
Ts7	YRLAVLVAN	*Anacardium occidentale*_Q8L5L5
Ts8	VKILQPVSA	*Corylus avellana*_Q8S4P9
Ts9	FVSARP QSQ	*Arachis hypogea*_P43237
Ts10	MVIVVVNKG	*Arachis hypogea*_P43237

were shared with several other vicilins. Interestingly, 3 of the epitopes were only present in the olive vicilin.

3.4. SDS-PAGE Analysis of Protein Extracts from Nuts and Reactivity of Sera from Allergic Patients. Figure 5(a) shows the SDS-PAGE profiles of the 4 extracts used in this analysis under denaturing and reducing conditions for the separation of peptides. The 4 profiles are essentially different and contain numerous bands of different intensities. It is important to note the presence of high-intensity bands in the 15–75 kDa range.

The allergograms obtained after probing the immunoblots with the sera of the different patients (Figure 5) are characterized by a wide variability in the reactivity to the extracts. Thus, patient #2 showed lower overall reactivity as compared to the other sera. With regard to the individual extracts, the cashew extract showed low reactivity to any of the patients used to generate the allergograms. On the other hand, sunflower and peanut extracts displayed IgE reactive bands for all the patients tested, with the exception of the nonallergic (control) extract. The olive seed extract contained IgE reactive bands in relation to three of the patients (#3, #4, and #5). The sizes of the highly reactive bands coincided with respect to the olive (ca. 22 kDa), sunflower (ca. 35 kDa),

Table 3: B-cell epitopes identified common to more than one of the vicilins analysed.

Epitope name	Sequence	Shared species
B1	ASESKDPELK	*Sesamum indicum*_Q9AUD0, *Corylus avellana*_Q8S4P9, *Arachis hypogea*_P43237
B2	QQQISKEQKE	*Sesamum indicum*_Q9AUD0, *Pistacia vera*_B4X640, *Anacardium occidentale*_Q8L5L5, *Corylus avellana*_Q8S4P9
B3	RGCEQQHGEQR	*Sesamum indicum*_Q9AUD0, *Pistacia vera*_B4X640, *Anacardium occidentale*_Q8L5L5, *Corylus avellana*_Q8S4P9
B4	RQGEGGGFSR	*Sesamum indicum*_Q9AUD0, *Pistacia vera*_B4X640, *Anacardium occidentale*_Q8L5L5
B5	CERRREEKYREQQGREGGRGEMYEGREREEEQEEQGRGR	*Sesamum indicum*_Q9AUD0, *Pistacia vera*_B4X640, *Anacardium occidentale*_Q8L5L5
B6	GAGGENPESFY	**Olive_002829** *Pistacia vera*_B4X640 *Anacardium occidentale*_Q8L5L5, *Corylus avellana*_Q8S4P9, *Lupinus angustifolius*_F5B8V9
B7	EKQDEGAIVK	*Pistacia vera*_B4X640, *Anacardium occidentale*_Q8L5L5, *Corylus avellana*_Q8S4P9, *Lupinus albus*_Q6EBC1
B8	EDEQEYEEQRRGQEQSDQDE	*Lupinus angustifolius*_F5B8V9, *Lupinus albus*_Q6EBC1, *Arachis hypogea*_P43237
B9	SREEGGIWPFGGQTKR	**Olive_002829** *Corylus avellana*_Q8S4P9 *Lupinus angustifolius*_F5B8V9, *Lupinus albus*_Q6EBC1
B10	DQQRQQDEQEEEYEQGEEEVRR	*Lupinus angustifolius*_F5B8V9, *Lupinus albus*_Q6EBC1, *Arachis hypogea*_P43237
B11	QSYFANGQPQQQQQQQSEKEGRRGRRGSSL	*Lupinus angustifolius*_F5B8V9, *Lupinus albus*_Q6EBC1, *Arachis hypogea*_P43237

Table 4: Species-specific B-cell epitopes in the different species analysed.

Epitope name	Sequence	Specific species
Be1	KHQGEHGRGGGDIL	*Sesamum indicum*_Q9AUD0
Be2	DQRPSQFNQ	**Olive_002829**
Be3	QGAMTTPYYNSKA	**Olive_002829**
Be4	EITPDRNPQVQ	*Lupinus albus*_Q6EBC1
Be5	KNNKRYPLA	**Olive_002829**
Be6	CQQEPDDLKQK	*Arachis hypogea*_P43237

and peanut extracts (ca. 70 kDa), although other minor bands (e.g., 20.5 and 21.5 kDa) were visible in all the allergograms. Immunoblots probed with the sera of a nonallergic patient displayed no reactive bands.

4. Discussion

The use of up-to-date transcriptomic data available on olive seeds enabled us to clearly determine that 7S vicilin proteins, or at least vicilin-like proteins, are present in this seed. However, the information available is still scarce: this transcriptome contains only a limited number of transcripts, as it was built on the basis of 454/Roche Titanium+ technology, which was used in order to obtain long readings suitable for building a transcriptome in the absence of genomic data [24]. A larger number of 7S vicilin protein sequences might therefore be present in the olive seeds. In addition,

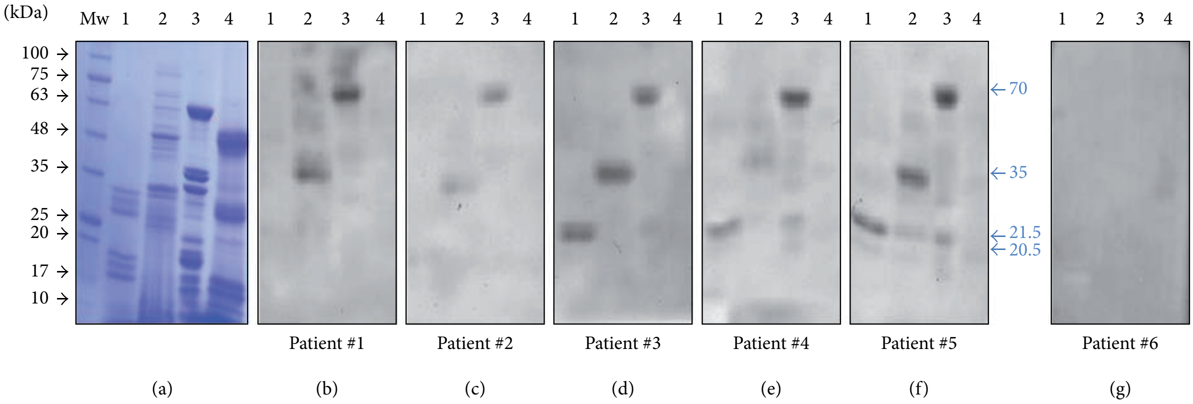


Figure 5: (a) Protein profiles of extracts from different seeds after SDS-PAGE and Coomassie staining. Lane 1: olive seed, Lane 2: sunflower, Lane 3: peanut, and Lane 4: cashew. (b)–(f) Immunoblots containing the same protein extracts as (a), probed with the sera from patients #1, #2, #3, #4, and #5 allergic to nuts. (g) Immunoblot containing the same protein extracts as (a) probed with the serum from a nonallergic patient (negative control).

variability among these sequences is likely to be underrepresented in the current data. Next generation sequencing (NGS) developments, currently underway, will provide a better understanding of these proteins in the olive seed and also provide data concerning their variability and expression levels.

To the best of our knowledge, the present study reports for the first time the presence of vicilins in olive seeds. Experimental validation provided us with a partial, though highly significant, sequence of this protein family. The sequence obtained was good enough to make an *in silico* comparative analysis of vicilins from other species. Thus, structural data, genetic relationships, and 3D structural superimposition with the models of other vicilins from different species were obtained. One of the preconditions to obtain 3D PDB models [35] is that, to model the protein, it must share considerable identity (usually over 50%) with templates in the database, a requirement which was widely fulfilled by the olive vicilin sequence obtained. For this reason, the superimpositions carried out can be regarded as reliable. Moreover, the superimposition values are sufficiently low to ensure a good coincidence rate between the molecules tested.

Allergy is a very important issue with regard to vicilin proteins which are regarded as important allergens involved in food allergies [15, 16, 21]. Although olive seed consumption has been highly limited to date, the implementation of new and alternative olive processing methods may lead to this material being used as a food ingredient. It is therefore important to determine the allergenic potential of this material. The *in silico* analysis carried out in this study clearly identifies T- and B-epitopes which are likely to be involved in allergies. Most of the epitopes identified are shared with other vicilins, thus highlighting the similarities between the olive vicilin and other vicilins. Moreover, specific epitopes are also present in this olive protein and other proteins. This finding is consistent with the results obtained following immunoassays of olive seed protein extracts with patients' sera, which may reflect predicted *in silico* heterogeneity. Nevertheless, the differences observed in allergograms cannot be unequivocally attributed to the presence of vicilins, as many other potentially allergenic proteins, such as 11S and 2S SSPs, are present in the assayed seed extracts. Although 7S vicilin proteins range in size from 60 to 75 kDa, other sizes are possible as a result of SDS-PAGE separation carried out under both denaturing and reducing conditions prior to immunoassays. These assays could benefit from using specific antibodies in addition to sera from patients [50, 51] in order to focus on the specificity of the IgE targets. However, to our knowledge, no commercially available antibodies to vicilins are available. Future studies need to address the generation of such antibodies, which would be highly useful molecular tools for diagnostic and research purposes. Other biochemical approaches, including the purification of vicilins, with the aid of chromatographic methods followed by SDS-PAGE and MALDI-TOF/MS identification have not yet been used [52].

5. Conclusions

Evidence of the presence of vicilins in olive seeds is supported by the identification of at least 58 sequences in the mature olive seed transcriptome, displaying considerable annotation identity with 7S vicilins from other species. Furthermore, one of these sequences was validated by experimental methods, enabling *in silico* comparative analysis of other vicilins. Although the olive sequence was incomplete, it showed characteristic structural details, a close genetic relationship, and good 3D structural superimposition with the built models of vicilins from different species, suggesting that olive vicilins are, to a great extent, similar to other vicilins. Moreover, both unique and/or commonly shared T- and B-epitopes among other 7S vicilins were present in the olive sequence. These epitopes are likely to be responsible for the reactivity of olive seed extracts to the sera from several patients allergic to nuts despite the fact that olive seeds are not commonly used as a food ingredient.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jose C. Jimenez-Lopez and Adoración Zafra contributed equally to this work.

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4.1. Aislados proteicos de semillas de olivo: composición química, propiedades funcionales y caracterización proteica

4.2. Determination of the presence/absence of gluten in olive seed flours

4.3 Efectos anti-inflamatorios de harinas purificadas de semillas de olivo en pacientes diabéticos

CHAPTER 4: Evidence of alimentary advantages and nutraceutical properties of olive seed components

XVI simposio científico-técnico del aceite de oliva
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Aislados proteicos de semillas de olivo: composición química, propiedades funcionales y caracterización proteica

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RESUMEN

La industria alimentaria produce rutinariamente aislados proteicos a partir de harinas desengrasadas de diversas fuentes vegetales. Estos aislados proteicos se utilizan en la elaboración de productos alimenticios de calidad y con propiedades funcionales mejoradas respecto a las harinas iniciales. Las fuentes proteicas más utilizadas son la soja, cacahuete, trigo y diversas leguminosas. El olivo posee semillas que no son hasta el momento utilizadas por estas industrias de forma rutinaria, a pesar de su alto contenido proteico. El presente trabajo explora diversos métodos para la preparación de aislados proteicos a partir de esta fuente, y describe su posterior caracterización bioquímica mediante electroforesis en geles de poliacrilamida (SDS-PAGE) y su contenido en proteínas de tipo 11S similares a leguminas. Finalmente, se describe la localización celular y subcelular de los lugares de acumulación de estos componentes. Los resultados obtenidos son de gran utilidad para establecer el potencial de esta fuente alimentaria y servirán de base a posteriores análisis nutricionales en distintos animales de experimentación.

Palabras clave: *aislados proteicos, cuerpos proteicos, globulinas, leguminas, Olea europaea L., olivo, proteínas, semillas, tripsina.*

INTRODUCCIÓN

El olivo y sus industrias asociadas generan gran cantidad de residuos agroalimentarios cuyo procesamiento y gestión son actualmente susceptibles de optimización y valorización, de forma que contribuyan a generar valor añadido y a disminuir su impacto ambiental. Los sistemas de elaboración de aceite más comunes en España utilizan en la actualidad la aceituna en su conjunto, a pesar de que históricamente se han desarrollado sistemas para el deshuesado de la aceituna previo a la molturación, como el clásico "trapetum" de origen griego (S II A.C.) o el "Acapulco-Quintanilla" de principios del siglo XX. En la actualidad existe un renovado y creciente interés por el deshuesado previo, que ha motivado la existencia de diversos estudios que describen su efecto sobre el aroma del aceite de oliva virgen (Luaces et al., 2004), o sobre factores de calidad como el perfil fenólico, estabilidad y amargor (Joorani, 2010). Existen en la actualidad algunas almazaras que realizan deshuesados previos a la molturación de manera rutinaria, gracias a deshuesadoras de diseño propio o modificadas sobre sistemas de tipo "Ulrich" (Luaces et al., 2004). Por otra parte diversas industrias como las de elaboración de aceituna de mesa, generan igualmente ingentes cantidades de hueso, que cada vez es utilizado en una mayor variedad de aplicaciones (Rodríguez et al., 2007).

A diferencia de otras plantas como la soja, colza, garbanzo, girasol, trigo, guisante, altramuz, haba, etc., la semilla del olivo no ha sido tradicionalmente utilizada para alimentación animal, aunque diversos estudios han demostrado que las proteínas más abundantes en las semillas maduras del olivo son las denominadas "proteínas de almacenamiento de semillas" (SSPs) de tipo 11S, similares a leguminas (Alché et al., 2006; Wang et al., 2007; Castro et al., 2007). En gran parte de las oleaginosas, la extracción del aceite genera ingentes cantidades de harina desengrasada, cuyos principales componentes son fibra y proteína, y que son por tanto utilizados como complemento en alimentación animal (Vioque et al., 2001). Con objeto de incrementar el valor añadido de estos subproductos y permitir aplicaciones de mayor interés como la alimentación humana, las harinas son sometidas a procesos de extracción y mejora de estas proteínas que culminan en la obtención de los denominados concentrados y aislados proteicos. En este trabajo se describen procedimientos preliminares para la generación de estos aislados proteicos a partir de la semilla del olivo, mediante la utilización de métodos clásicos de precipitación isoeléctrica (Sánchez-Vioque et al., 1999; Vioque et al., 2001; Robles y Mora, 2012)

MATERIAL Y MÉTODOS

Material

Se utilizaron almendras de olivo de la variedad 'Picual', cedidas por las empresas Elayotecnia S.L. (Castillo de Locubín, Jaén) y almazara "el Picón" (Albanchez de Mágina, Jaén). Estas almendras fueron obtenidas a partir de aceitunas maduras mediante deshuesado y posterior fractura y separación de los endocarpos (Fig. 1A, B). El material se conservó a -20ºC hasta su uso.

Preparación, desengrasado y pre-tratamiento de la harina

Se trituraron 40 g de almendras, y la harina resultante fue desengrasada utilizando hexano (Figura 1C) (Sánchez-Vioque et al., 1998 con ligeras modificaciones). Brevemente, el triturado se agitó con 100 ml de hexano durante 20 min y continuación se eliminó la fase superior (hexano). Este proceso se repitió otras 3 veces más, dejándose secar finalmente el triturado resultante. A continuación se realizaron 4 lavados de 20 min con agua mediante agitación y posterior filtración. Finalmente, la harina se lavó con etanol al 20% (v/v) y se dejó secar.

Extracción de proteínas

Se probaron dos métodos de extracción alcalina (Sánchez-Vioque et al., 1998). El método 1 incluyó la suspensión de 20 g de harina desengrasada en 200 ml de NaOH al 0,2 % (p/v), pH 12. Se agitó durante 1 h y a continuación se centrifugó a 8.000 g durante 15 min. El sobrenadante se guardó a 4ºC y el pellet obtenido se volvió a resuspender en NaOH al 0.2%, pH 12, repitiéndose el proceso otras dos veces más hasta obtener el producto de tres sobrenadantes, que fueron finalmente combinados. El método 2 se ejecutó de igual forma, pero la solución utilizada para la extracción de proteínas fue en este caso Na2SO3 al 0,25% (p/v), pH 10,5. Las proteínas obtenidas en las distintas extracciones fueron cuantificadas mediante el método Bradford (Bradford, 1976).

Determinación del punto isoeléctrico de las proteínas de harina de semillas de olivo

Se llevó a cabo mediante una titulación de alícuotas del primero de los tres sobrenadantes arriba mencionados para cada procedimiento. Para el cambio de pH se utilizó HCl 0,5 M hasta conseguir un total de 11 alícuotas con pHs desde 7,0 hasta 2,0, con una diferencia de media unidad entre ellas. Estas alícuotas fueron centrifugadas a 8.000g durante 30 min a 4ºC y tanto los sobrenadantes como los precipitados fueron almacenados para posteriores determinaciones. La cantidad de proteínas de los sobrenadantes obtenidos fue igualmente cuantificada por el método Bradford.

Separación electroforética de las proteínas

Las proteínas presentes en los sobrenadantes y precipitados resultantes del método 1 fueron separadas mediante SDS-PAGE en gradiente 4-20% (Sistema CriterionTM, BioRad) en condiciones desnaturalizantes y reductoras (tampón de muestras 50 mM Tris-HCl pH 6,8, 2% SDS, 50% glicerol, 0,1 M DTT, 0,03% azul de bromofenol). El volumen de muestra cargado fue igual en todas las calles, teniendo en cuenta el factor de dilución ocurrido durante la titulación. La electroforesis se realizó por duplicado para las 11 muestras de pellet y para las 11 de sobrenadante. Una de las réplicas se utilizó para la visualización del perfil proteico mediante tinción con sales de plata (Rabilloud et al., 1992) y la otra para transferencia y posterior inmunomarcado. El anticuerpo utilizado en el procedimiento de inmunomarcado fue un antisuero anti-proteínas 11S de olivo desarrollado en nuestro laboratorio (Alché et al., 2006). Como anticuerpo secundario se utilizó un anticuerpo anti-conejo IgG marcado con Alexa-488 (Molecular Probes) y el revelado se realizó en un equipo Pharos FX (Bio-Rad). Para la cuantificación de las bandas se utilizó el software Quantity One 4.6.2 (Bio-Rad). La intensidad de las bandas fue expresada en CNT (unidades arbitrarias devueltas por el software).

Microscopia óptica y electrónica

Las semillas fueron obtenidas de aceitunas maduras de la variedad Picual. Estas semillas fueron procesadas de forma paralela para microscopia óptica y electrónica de transmisión (Alché et al., 2006) en resina Unicryl (BBInternational). Las muestras fijadas fueron cortadas en secciones semifinas de 1µm (que fueron teñidas con azul de Toluidina), y ultrafinas de 70 nm (que en este caso fueron contrastadas con acetato de uranilo y nitrato de plomo), y fueron visualizadas respectivamente en un microscopio óptico Zeiss Axioplan y un microscopio electrónico JEOL TEM1011.

RESULTADOS Y DISCUSIÓN

Los huesos de olivo suministrados como resultado del deshuesado de la aceituna (Fig. 1A) están integradas por el endocarpo leñoso (que procede del lóculo) y la almendra, semilla o embrión localizada en su interior, y que puede ser aislada del hueso tras la fractura del endocarpo y su separación (Fig. 1B). El resultado de la trituración de estas almendras fue una pasta rica en componentes lipídicos (Fig. 1C) que fue sometida a un desengrasado mediante disolventes orgánicos, lavado posterior y desecado para generar la harina desengrasada que consideramos el material original de partida para la generación de los aislados proteicos (Fig. 1D). El proceso indicado puede considerarse simple y efectivo, y está destinado a evitar la formación de complejos lípido-proteína que puedan deteriorar la calidad del producto final (Clemente, 1998). Sin embargo, podrían ser utilizados otros métodos alternativos que pueden mejorar los rendimientos posteriores. Estos procedimientos incluyen la utilización alternativa de harinas ya desengrasadas parcialmente, obtenidas por prensado de las almendras en factorías especializadas mediante condiciones adecuadas, y destinado a la obtención de aceite de almendra de olivo. Otro procedimiento alternativo ampliamente descrito en la literatura es el uso del extractor Soxhlet con el mismo propósito (Clemente, 1998; Sánchez-Vioque et al., 1999; Robles y Mora, 2012).

Tras el desengrasado de la harina, la preparación de aislados proteicos se realiza generalmente en el caso de leguminasas siguiendo alguno de los siguientes procedimientos: i) precipitación isoeléctrica de las proteínas y posterior separación de las mismas del resto de las moléculas solubles mediante centrifugación, ii) concentración proteica por ultracentrifugación, o iii) extracción de proteínas con cloruro de sodio y precipitación por micelación (Clemente, 1998 ; Vioque et al., 2001; Robles y Mora, 2012). En el presente trabajo se ha utilizado el primero de estos procedimientos. En este caso se han preparado dos tipos de aislados de la almendra de olivo, usando sulfito sódico en el medio de extracción o sin él. La extracción con NaOH es económicamente más barata que la extracción con sulfito sódico, aunque este segundo tipo tiene ventajas como el uso de condiciones menos severas de extracción (pH 10,5 frente a pH 12), y el hecho de que el sulfito inhibe la oxidación de polifenoles presentes en la muestra, eliminando posibles reacciones secundarias de éstos. En relación a la cantidad de proteínas extraídas mediante ambos métodos, dicha cantidad es sustancialmente superior usando NaOH (Fig. 2). Las extracciones sucesivas (rondas 2 y 3 de extracción) de la harina desengrasada no mejoran esencialmente el rendimiento en proteína obtenido en la primera extracción (Fig. 2).

A lo largo de las titulaciones correspondientes, la solubilidad de las proteínas de semillas de olivo fue decreciendo de forma exponencial desde pH 7,0 hasta llegar a un mínimo entre los pHs 4 y 4,5 (Fig. 3). Las titulaciones a pHs inferiores (entre 4,0 y 2,0), aunque también produjeron lecturas inferiores de la cantidad de proteína medida mediante el método de Bradford, supusieron cambios en la tendencia de las curvas, por lo que es probable la presencia de algún tipo de interferencia en la lectura espectrofotométrica que limite la fiabilidad del método Bradford a esos pHs. Estas mediciones deberían ser por tanto contrastadas mediante métodos de determinación proteica alternativos (ej. Lowry) o la determinación de nitrógeno in los sobrenadantes en relación al nitrógeno total extraído (Sánchez-Vioque et al., 1999). Las tendencias son idénticas en el caso de ambos métodos de extracción (NaOH y Na2SO3), aunque la curva de disminución de solubilidad es más regular en el caso del Na2SO3. Como se observa en la Fig. 2, la capacidad extractiva total de proteínas es superior en el caso del método 1 (NaOH) que en el 2 (Na2SO3) (Fig.3). El pI mayoritario de las proteínas presentes en los extractos es por tanto próximo a 4,0-4,5, lo que es compatible con estudios anteriores realizados en distintas fuentes de harina desengrasada como la colza y el garbanzo (Gonçalvez et al., 1997; Sánchez-Vioque et al., 1999).

Los análisis de los perfiles proteicos mediante SDS-PAGE de los sobrenadantes y precipitados obtenidos a diferentes pHs durante la titulación (Fig. 4), confirman los resultados obtenidos mediante el método de Bradford mencionado anteriormente (Fig.3). Es por ejemplo claro el aumento de la cantidad de proteína presente en los precipitados exactamente a pH 4,0 (Fig 4B).

Por otra parte, los inmunoblots permiten ratificar que las especies peptídicas presentes en los perfiles a distintos pHs corresponden en una elevada proporción a proteínas de almacenamiento de tipo 11 similares a leguminas (Fig. 4C y D, y cuantificación en Fig. 4), dado su reconocimiento por el anticuerpo específico anti-11S y el patrón característico de los péptidos obtenidos en condiciones desnaturalizantes y reductoras (Alché et al., 2006; Wang et al., 2007; Castro et al., 2007). Este patrón está bien conservado en diferentes subproductos del procesado de la semilla del olivo y de la producción del aceite (Jiménez-López et al., 2007; Ben Ali, 2011) y es incluso similar en otras especies vegetales (Allach et al., 2009).

Finalmente, el análisis de la estructura celular y subcelular de los tejidos de la semilla permite identificar los lugares de acumulación de los componentes lipídicos y proteicos, respectivamente en cuerpos lipídicos y proteicos, con una distribución subcelular altamente organizada y repetitiva. Esta distribución sufre una serie de modificaciones como respuesta a los cambios fisiológicos que experimenta la semilla por ejemplo durante la germinación (Zienkiewicz et al., 2011a).

Los resultados obtenidos representan una primera aproximación a la generación de aislados proteicos en el caso de la harina de semillas de olivo. Estos resultados deben ser complementados con otros numerosos parámetros que incluyen la determinación de la composición química de los aislados (proteína total por diversos métodos, fibra, grasa residual, azúcares…), presencia de factores antinutritivos como la sinapina de colza, gosipol de algodón, ácido clorogénico en girasol y de aflatoxinas (provenientes de contaminación por Aspergilus por condiciones de almacenamiento en humedad). Al igual que la mayoría de leguminosas sería razonable determinar la presencia de inhibidores de la tripsina y otras proteasas, así como lectinas, que generalmente están presentes en cantidades moderadas. Igualmente sería conveniente testar la presencia de taninos en la testa, palatabilidad de la harina, y contenido en metales. Otros factores a tener en cuenta para el uso potencial de estos aislados serían la uniformidad de la disponibilidad de la fuente primaria de material (harina) a lo largo del año. y la posible existencia de diferencias entre variedades o entre métodos de obtención. Es previsible la presencia de algunas diferencias en los perfiles proteicos entre harinas de semillas procedentes de deshuesado previo a molturación o de deshuesado de aceitunas de mesa, tras el proceso de cocción. Finalmente, sería interesante conocer la digestibilidad de las harinas y aislados proteicos en comparación con la de los extractos completos de semillas (Zienkiewicz et al., 2011b).

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Figura 1. Semillas maduras de O. europaea cv. 'Picual' y productos de su transformación. A: semillas resultantes del deshuesado de aceitunas maduras. B: almendras obtenidas tras la ruptura y separación del endocarpo. C: pasta oleaginosa generada mediante triturado de las semillas. D: harina resultante del desgrasado de la pasta mediante tratamiento con hexano y posterior secado de la muestra.

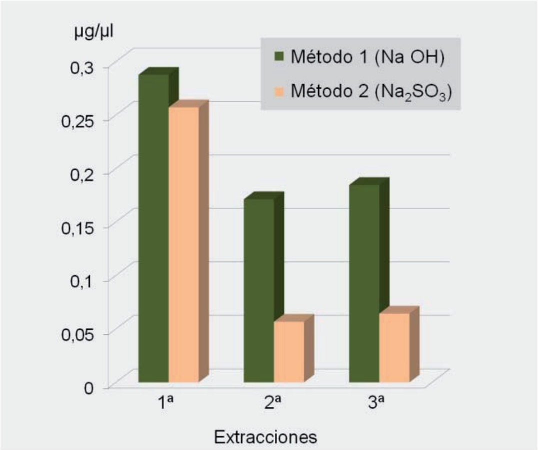

Figura 2. Contenido en proteína total recuperada de la harina de semilla utilizando dos soluciones básicas alternativas. Concentración de proteínas (µg/µl) en cada una de las tres extracciones realizadas para cada solución, calculada mediante el método de Bradford.

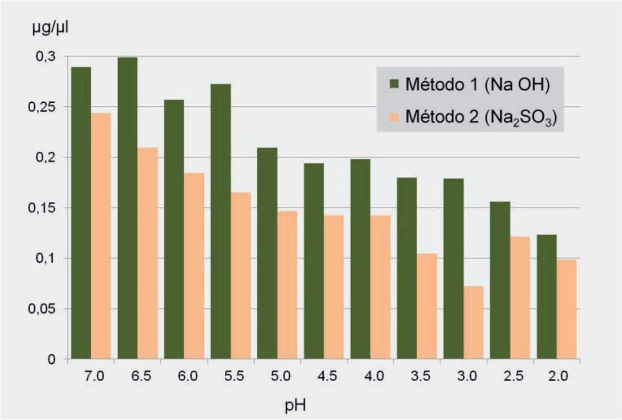

Figura 3. Contenido de proteína total soluble tras ajustar el pH de las soluciones básicas alternativas de extracción a valores entre 2.0 y 7.0. Concentración de proteínas calculada mediante el método de Bradford (µg/µl) en los sobrenadantes, tras la titulación de los extractos a distintos valores de pH para cada uno de los dos métodos de extracción utilizados.

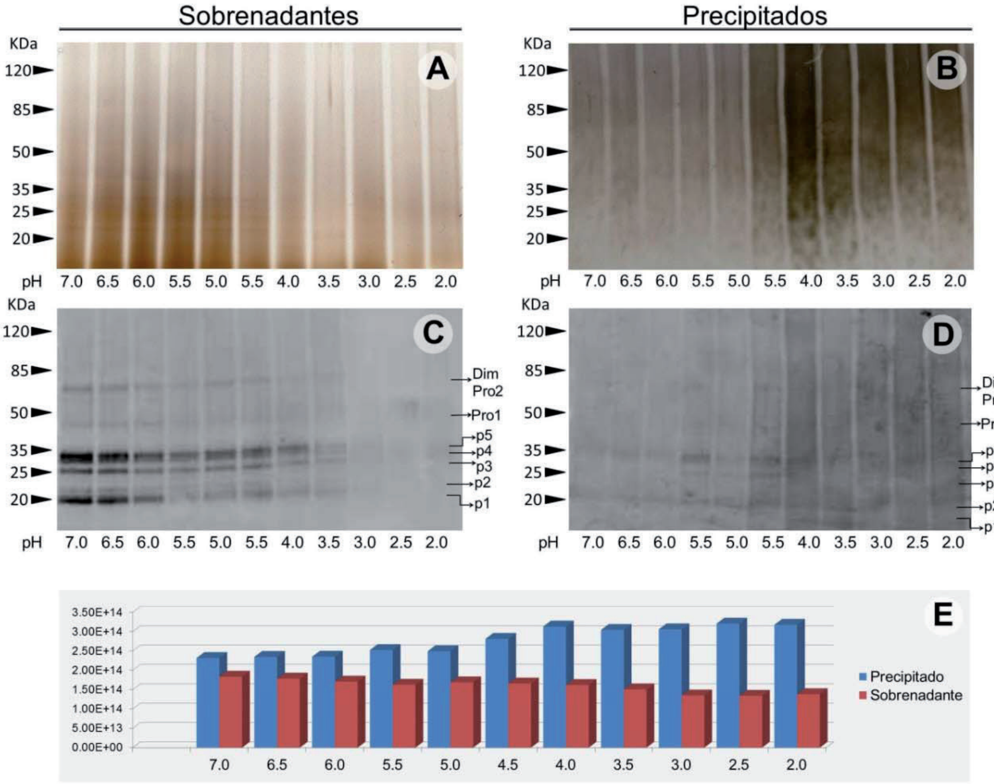

Figura 4. Perfiles proteicos SDS-PAGE (A y B) e inmunoblots correspondientes a proteínas 11S (C y D) de las fracciones solubles (sobrenadantes) e insolubles (precipitados) obtenidos tras la titulación del extracto obtenido mediante el método 1 a diferentes pHs. E: cuantificación de las bandas reactivas al anticuerpo anti-proteínas de almacenamiento de tipo 11S de olivo. Los péptidos p1 a p5 corresponden a las distintas cadenas de tipo α y β que integran los precursores Pro 1 y Pro 2, generalmente observados en condiciones no reductoras (Alché et al., 2006). Se observa una progresiva disminución en la cantidad de proteína soluble, de forma coincidente con la disminución del pH de la solución. Alternativamente se produce un incremento en la cantidad de proteína 11S presente en los precipitados, con un pico alrededor de pH 4.0.

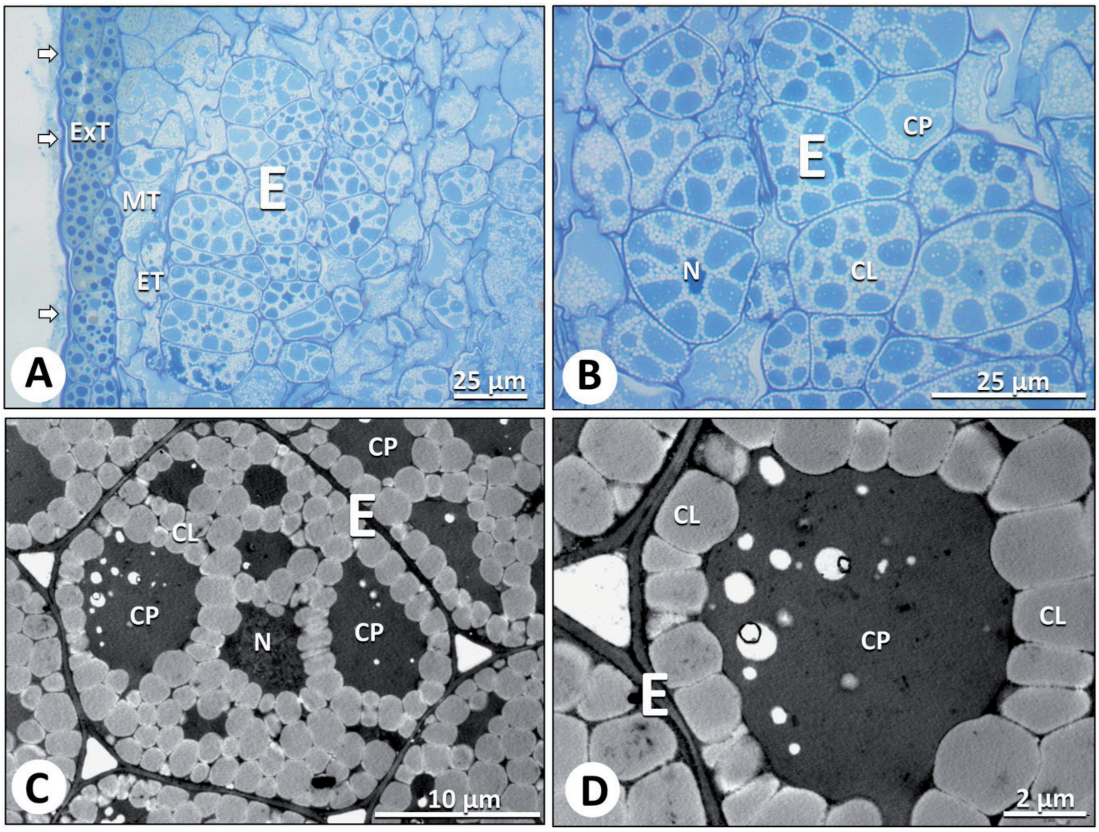

Figura 5. Estructura tisular y subcelular del endospermo de la semilla de olivo.
A y B: secciones semifinas observadas a microscopía óptica en la que se observan los tegumentos, compuestos por una cutícula (flechas), y una testa formada por varios estratos (exo-, meso- y endotesta) que rodea a las células del endospermo (E). Las células del endospermo contienen numerosos cuerpos densamente teñidos por azul de Toluidina en el citoplasma (cuerpos proteicos: CP), rodeados por estructuras birrefringentes no teñidas (cuerpos lipídicos: CL). En algunas células del endospermo se observa el núcleo intensamente teñido, y con localización central. Las estructuras mencionadas son claramente distinguibles en secciones ultrafinas observadas a microscopía electrónica de transmisión (C y D). En este caso, los cuerpos lipídicos, de unas 2 µm de diámetro, contienen material con baja densidad electrónica en comparación con los cuerpos proteicos a los que rodean, y que presentan en su interior zonas translúcidas a los electrones.

Determination of the presence/absence of gluten in olive seed flours

Adoración Zafra and Juan de Dios Alché

The present report corresponds to a technical assessment of gluten presence in olive seed flours which was disclosed to Acer Campestres S.L. in the frame of collaborative project NUTRAOLEUM (ITC-20131031).

BACKGROUND:

Coeliac disease (CD) is one of the most common genetic diseases, resulting from both environmental (gluten) and genetic (HLA-DQ2 or -DQ8) factors (Gujral et al. 2012). Current knowledge indicates that different gluten peptides are involved in the disease process in a different manner, some fragments being 'toxic' and others 'immunogenic'. Those defined as 'toxic' are able to induce mucosal damage, while those defined as 'immunogenic' are able to specifically stimulate HLA-DQ2- or DQ8-restricted T cell clones (Ciccocioppo et al. 2005). Moreover, the ubiquitous enzyme tissue transglutaminase (tTG) has been identified as autoantigen of the celiac disease (Dieterich et al. 1997).

For many societies, the seeds constitute an important source of nutrients. The plant proteins can be classified attending to their function (structural/metabolic proteins, protective proteins and functional proteins. However, they have traditionally categorized following the Osborne´s classification (Osborne 1924) attending to their solubility. Nowadays, this classification is still prevailing. Five groups can be considered (Verdier and Thompson 2012):

- **Albumins** (soluble in water).
- **Glutelins** (soluble in alkali solution). Are the second most prevalent storage protein, comprising 21–27% of the total groat protein (Zwer 2017). In the case of rice, the glutelins are the predominant proteins (~70%). Glutelins are classified into two groups (high-molecular-weight and low-molecular-weight, HMW and LMW, respectively). Glutelins from wheat are called glutenins.
- **Globulins** (soluble in dilute salt solutions). They are sub-classified into 7S (known as vicilins), 11S and 12S attending to their sedimentation coefficient. The 11S globulins are the major storage proteins in most legumes, being mainly studied in these plants, therefore are commonly named as legumins. The 11S represent among 70-80% of the total seed proteins in olive (Alché et al. 2006).
- **Prolamins** (soluble in 70:30 ethanol/water). They are the major Seed Storage Proteins (SSPs) in most of the cereals. Prolamins from wheat (gliadins), rye (secalins), barley (hordeins), maize (zeins), oat (avenins) are characterized by the presence of repetitive blocks of short-peptide motifs enriched in nitrogen-rich amino acid residues and embedded in non-repeated sequences (Shewry and Halford 2002). Among them, the gliadins has been the best studied.

Gliadin is an heterogeneous mixture of proteins that contains at least 40 components that can be classified into four major groups (α-, β-, γ-, and ω-gliadins) attending to their electrophoretic mobility. Attending to their N-termilal amino acid sequence they are classified into three major types, designated as α-, γ- and ω-types. Although the correspondence between both nomenclature is not complete, it may be assumed that the electrophoretically separated α- and β-gliadins mainly constitute the α-type gliadins, while γ- and ω- gliadins include the γ- and ω-types, respectively (Ciccocioppo et al. 2005).

Wheat gluten is composed by gliadin and glutenin, being the mixture responsible for the visco-elastic properties of dough and its ability to retain gas during fermentation (Verbruggen et al. 1998).

Antigen analysis:

Ciccocioppo et al. (2005) listed a high number of gliadin peptides previously purified and tested for their immunogenicity (Table 1). The presence of immunodominant peptides were designated as +++, being able to elicit a strong T cell response. The degree of immunogenicity was clasiffied as $^{-}$/+/++/+++, corresponding to non-immunogenicity, low immunogenicity, medium immunogenicity and high immunogenicity, respectively.

Peptide	Amino acid sequences	Position	Immunogenicity
	VRVPVPQLQPQNPSQQQPQ	α-gliadin: 1–19	+
	QNPSQQQPQEQVPLVQQQ	α-gliadin: 11–28	+
	QVPLVQQQQFPGQQQPFPPQ	α-gliadin: 21–40	+
	PGQQQPFPPQQPYPQPQPF	α-gliadin: 31–49	+
	FPGQQQPFPPQQPYPQPQPF	α-gliadin: 30–49	+
	QPYPQPQPFPSQQPYLQL	α-gliadin: 41–58	+
	PQPFPSQQPYLQLQPFPQ	α-gliadin: 46–63	+
	PQPQLPYPQPQLPY	α-gliadin: 62–75/(a)	+/++ +
	QLQPFPQPQLPY	α-gliadin: 57–68 (a)	+/++ +
	QLQPFPQ	α-gliadin: 57–63 (a)	+ + +
33 mer	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	α-gliadin: 57–89/(a)	+/++ +
	QLQPFPQPQLPY	α-gliadin: 58–69/(a)	+/++ +
	PQPQLPYPQPQLPY	α-gliadin: 63–76/(a)	+/++ +
α20	PFRPQQPYPQPQPQ	α-gliadin: 93–106 (a)	+
	LIFCMDVVLQ	α-gliadin: 123–132	+
α20	QQPLQQYPLGQGSFRPSQQNPQAQG	α-gliadin: 198–222	+
	QYPLGQGSFRPSQQNPQA	α-gliadin: 203–220/(a)	+/+
	PSGQGSFQPS	α-gliadin: 205–214	–
	PSGQGSFQPSQQ	α-gliadin: 205–216/(a)	+/++ +
	SGQGSFQPSQQN	α-gliadin: 206–217/(a)	+/++ +
	QGSFQPSQQN	α-gliadin: 208–217/(a)	–/++ +
	LQPQQPFPQQPQQPYPQQPQ	γ-gliadin: 60–79	+
	FPQQPQQPYPQQPQ	γ-gliadin: 66–78	+
	FSQPQQQFPQPQ	γ-gliadin: 102–113/(a)	–/+
	OQPQQSFPEQQ	γ-gliadin: 134–153/(a)	+/++ +
	VQGQGIIQPQQPAQL	γ-gliadin: 222–236/(a)	+/+
	QQQQPPFSQQQQSPFSQQQQ	glutenin: 40–59/(a)	–/+
	QQPPFSQQQQPLPQ	glutenin: 46–60/(a)	–/+
	SGQGQRPGQWLQPGQGQQGYYPTSPQQSGQGQQLGQ	glutenin: 707–742/(a)	+/+
	PGQGQQGYYPTSPQQSGQ	glutenin: 719–736	+
	GYYPTSPQQSGQGQQLGQ	glutenin: 725–742	+
	GYYPTSPQQSG	glutenin: 725–735	+
	QGYYPTSPQQS	glutenin: 724–734/(a)	+ –
	QQGYYPTSPQQSG	glutenin: 723–735	+
	GQQGYYPTSPQQSG	glutenin: 722–735	+
	GQQGYYPTSPQQS	glutenin: 722–734	+

Table 1: Immunogenic gliadin peptides. Adapted from Ciccocioppo et al. (2005).

Antibody development:

Investigations aimed to find the best test to detect gliadins have been focused on the search for antibodies. A first monoclonal antibody was developed by Shewry and Halford (2002) in the mid 1980´s against wheat gliadin from an Australian wheat variety which recognizes HMW (high molecular weight) glutenin subunits and the heat stable subfraction called ω-gliadins. The different tests carried out with this antibody produced differences in the results obtained, like differences in the quantity of ω-gliadins among species. Moreover, the hordeins are not detected using this antibody.

Next, the R5 antibody (Valdés et al. 2003) was raised against rye secalin, although it also showed strong cross reactivity to wheat gliadin. However, it also detects proteins from soy and lupin that are not harmful prolamins. This assay is based on the use of R5 antibody, a R5 conjugated antibody, that bind to different sites on the antigen. The R5 antibody recognizes potential toxic-celiac epitopes which occur repeatedly in prolamins (mainly QQPFP, QQQFP, PQPFP, LQPFP, QQPYP, QLPYP) that are contained in toxic-celiac peptides such as Gliadin 33 mer peptide, Gliadin 26 mer peptide and Gliadin 25 mer peptide45 (Mena and Sousa 2015). The use of the R5 monoclonal antibody allows an accurate quantification in both intact and split gluten. The Codex Alimentarius Commission states that a modification of the R5 assay has to be applied for the detection of hydrolyzed gluten (Mena and Sousa 2015).

After that, it was designed an antibody raised against the immunotoxic 33-mer peptide, called G12 antibody (Morón et al. 2008). This peptide is a gliadin fragment of 33 amino acids in length identified as the primary initiator of inflammatory responses in Celiac Disease (Shan 2002). Recent investigations carried out on flours of 23 hexaploid modern and 15 old common (bread) wheat as well as two spelt cultivars has revealed that all flours contained the 33-mer peptide at levels ranging from 91–603 µg/g flour. In contrast, the 33-mer was absent (<limit of detection) from tetra- and diploid species. Therefore, it is necessary to go on focused in the study of the 33-mer (Schalk et al. 2017).

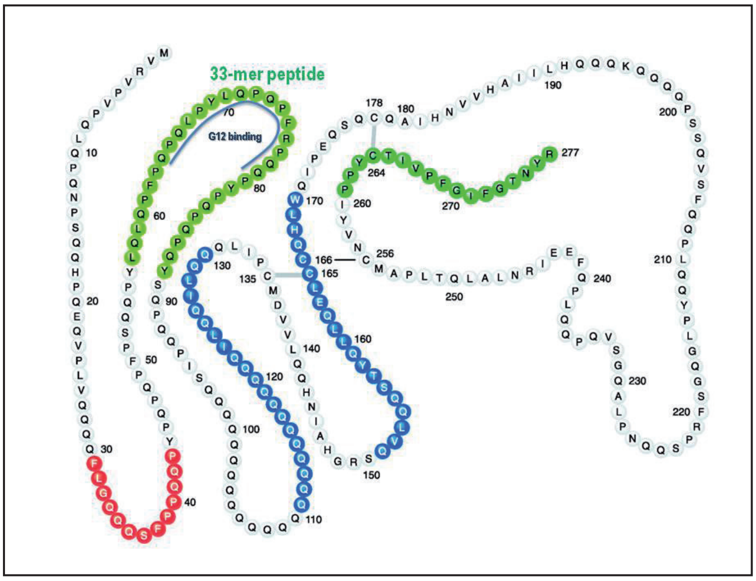

Figure 1: Gliadin motifs. Mapping of α-gliadin motifs exerting cytotoxic activity (red), immunomodulatory activity (light green), zonulin release and gut-permeating activity (blue), and CXCR3-IL-8 release in CD patients (dark green). The binding to the G12 antibody is show pointed out with a blue line. Adapted from Fasano (2011).

Antibody	Skerrit	R5	G12	α-20
Specificity	HMW glutenin and w-gliadin (wheat)	QQPFP (rye secalin)	QPQLPY (wheat gliadin)	wheat
Also detects	Rye, barley	Wheat, barley	Rye, barley	oat

Table 2: Summary of the antibodies developed to detect gluten in foods.

The study of van den Broeck et al. (2010) compared the genetic diversity of the gliadin epitopes (Glia-α9 y Glia-α20) in 36 modern European wheat varieties and in 50 landraces. They found Glia-α9 an immunodominat epitope in most of the CD patients. Glia- α20 is a minor epitope that is recognized by a minority of patients, being used as technical reference (van den Broeck et al. 2010).

Table 2 summarizes the main antibodies used for the detection of gluten in foods. Apart from the cited studied and improved antibodies, other studies have been carried out to develop polyclonal antibodies (e.g. Morinaga Wheat Protein ELISA method).

Legislation and commercially available tests:

Several factors may affect the results of gluten analysis, such as the modifications of proteins produced during manufacturing of foods, the interference of the mixture of ingredients, and the use of the appropriate standard for gluten analysis (Mena and Sousa 2015).

The comparison of gluten contents in different samples by using a variety of commercially available ELISA test kits revealed considerable differences between the kits. The use of different extraction procedures, test formats, and reference materials as well as the specificities and sensitivities of the mAbs and pAbs are involved in such differences (Scherf 2017).

Special attention has to be paid also to the extraction system aimed to recover both, prolamins and glutelins. The commercially available kit provided by Biomedal Diagnostics (Seville, Spain) provides an extraction buffer that has a high extraction efficiency of both, simple, complex, processed and unprocessed samples (Mena and Sousa 2015).

Immunoassays like ELISAs (enzyme-linked immunosorbent assays) and LFDs (lateral flow devices) have many advantages, and are therefore frequently used in the food industry to track allergen contamination. However, one big issue in allergen quantification is that results vary from kit to kit. The lack of an official reference method and official reference materials are factors contributing to this issue (Hochegger et al. 2015).

The R5 ELISA has been authorized by the Codex Committee on Methods of Analysis and Sampling as a type 1 method for determination of the gluten content in "gluten free" (GF) foods (Codex Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten). Gliadins are detected with an R5 ELISA in the range of 2–5 mg/kg food, having a Limit of Detection (LoD) of 3 ppm gluten (1.5 ppm gliadin) and a Limit of Quantification (LoQ) of 5 ppm (Haraszi et al. 2011). The Codex Standard 118-1979, establish that the “gluten-free” products must obey with gluten levels (including prolamin fractions from rye, barley and oats) under 20 mg/kg. In the case of "foods specially processed to reduce gluten content” must contains levels between 20 and 100 mg/kg.

During the processing of some foods, proteins are treated at high temperatures in a dry state at a neutral pH, forming isopeptide bonds between lysine and asparagine and glutamine residues. Furthermore, the heat-treatment of cooked and baked products leads to the formation of proteins aggregates in an insoluble matrix that makes analyses even more difficult (Mena and Sousa 2015).

To ensure correct labeling and safe product for CD patients in the future, test methods that are able to detect multiple gluten proteins and peptides simultaneously should be investigated (Bruins Slot et al. 2015).

All the provided data reveals the vital importance in the choice for the analysis of the foods. Subsequently, a compilation of the commercially available test as well as the fundamental procedure used are presented in table 3.

MATERIALS AND METHODS:

Attending to the wide literature, the suggestions of the Codex Alimentarius Commission as well as the Protein & Enzymes Technical Committee of AACC International, and the scrutiny of the commercially available test we decided to use the following kits:

- **GutenTox Sticks (Biomedal, Seville, Spain):** Technical information of this hit offers the detection of gluten in food, beverages and on surfaces, specially designed for gluten free food manufactures and handlers. The limit of detection is down to 3 ppm. The kit contains the **G12 anti-gliadin antibody** that specifically recognizes the most immunogenic fraction of gluten that triggers the Celiac Disease.
- **Ingezim gluten hidrolizado (Ingenasa, Madrid, Spain):** Is a direct immunoenzymatic assay which uses the **R5 monoclonal antibody** specific of prolamins. The assay performs a quantitative analysis of hyrolyzed gluten in food samples with a limit of detection of 0.25 ppm. The test has been designed for samples with a content of gluten between 0-300 ppm. This assay is **endorsed by the CODEX Alimentarius as Type I Method** for the determination of gluten in food samples.

Two types of samples were used for the gluten determination, seeds from green and mature fruits (105 and 210 DAA-Days After Anthesis-respectively). Briefly, the fruits were dissected by using a knife, a de-stoning commercial device, and a scalpel to remove the pulp (mesocarp + epicarp), stones and the seed tissues respectively. The seeds were crushed to obtain a fine powder. The gluten determination was performed was performed following the kits instructions in both cases.

Test Kit	Manufacturer	Antibody	Test format	LoD	LoQ
GutenTox ELISA Competitive	Biomedal	G12	Competitive	1.5	1.5
GutenTox ELISA Sandwich	Biomedal	G12	Sandwich	0.3	0.3
Guten-Check ELISA	Bio-Check (UK) LTD.	Skerrit	Sandwich	0.3	2.5
ALLER-TEK Gluen ELISAAssay	ELISA Technologies	Skerrit	Sandwich	2.5	2.5
GLUTEN-TEC ELISA	EuroProxima	α-20	Competitive	2.5	2.0
Gliadin/Gluten	Immunolab	Polyclonal (pAb1)	Sandwich	0.3	2.5
GlutenAlert ELISA	InCura	Polyclonal	Competitive	1.0	1.5
Ingezim Gluten	Ingenasa	R5	Sandwich	1.5	0.1
Ingezim Gluten Hidrolizado	Ingenasa	R5	Direct	0.1	1.5
Biokits Gluten assay	Neogen	Skerrit	Sandwich	0.5	2.5
Veratox for Gliadin R5	Neogen	R5	Sandwich	2.0	2.5
RIDASCREEN Gliadin	R-Biopharm	R5	Sandwich	1.5	5.0
RIDASCREEN Gliadin Competitive	R-Biopharm	R5	Competitive	1.4	2.0
AgraQuant Gluten G12	Romer Labs	G12	Sandwich	1.0	4.0
Wheat/Gluten (Gliadin) ELISA	Moringa Institute of Biological Sciences, Japan	pAb2	Sandwich	0.26*	0.78**

Table 3: List of commercially available test for the detection of gluten. LoD: Limit of detection. LoQ: Limit of Quantification (expressed as mg/kg gliadin). *µg/g food **ng/ml wheat protein. Adapted from (Bruins Slot et al. 2015).

RESULTS AND DISCUSSION:

Table 4 shows the results obtained after testing samples of seeds at two developmental stages with both kits. A slight positive identification was obtained with the high-sensitivity test, Ingezim, in the sample of 105 DAA only, whereas the GlutenTox test was unable to identify gluten in the samples.

Therefore, the analyzed samples can be designated as gluten free following the indication of the Food and Drug Administration (FDA) of the United States based on Codex standards, and the European Commission Regulation (EC) No 41/2009 concerning the composition and labeling of foodstuffs suitable for people intolerant to gluten. The use of two different procedures (based on the G12- and the R5-antibody), in the present work authenticates the endorsement by both, the Protein & Enzymes Technical Committee of AACC International as well as the Codex Alimentarius Commission that establish in both cases a labeling of "gluten free" in foods when the presence of gluten is less than 20 pmm. Besides, the detected limits are under 10 pmm that it is established by the Federation of Associatios of Coeliac Patients of Spain ("Asociaciones de Celíacos de España") (F.A.C.E.).

Material	GlutenTox Sticks (Biomedal) LoD: 3ppm	Ingezim Gluten Hidrolizado (Ingenasa) LoD: 0.25ppm
Seeds 105 DAA	-	+
Seeds 210 DAA	-	-

Table 4: Results of the gluten detection analysis in seeds from green and mature fruits (105 and 210 DAA respectively). The results are negative in all the cases except for the seeds at the green stage with a gluten content of 1.7 ppm. LoD: Limit of detection.

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Efectos anti-inflamatorios de harinas purificadas de semillas de olivo en pacientes diabéticos

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RESUMEN

La deconstrucción o fraccionamiento de la aceituna de forma previa a su procesado en las almazaras puede proporcionar nuevos y valiosos productos. En este contexto hemos centrado nuestra atención fundamentalmente en las harinas procedentes de la semilla del hueso y su posterior valoración como ingrediente nutracéutico. Varios estudios confirman la idea de que en el desarrollo y la progresión de la diabetes mellitus tipo 2 (DT2) subyacen mecanismos inmunológicos e inflamatorios. El objetivo del presente estudio consiste en valorar los potenciales efectos beneficiosos sobre el sistema inmune de este novedoso ingrediente. El estudio se realizó exclusivamente *ex vitro* mediante cultivos sanguíneos de sujetos controles sanos y con DT2 que se sometieron a co-cultivo con dichas harinas como "challenge", posteriormente se procedió a la cuantificación de moléculas marcadoras de actividad anti inflamatoria: la interleuquina 1β (IL-1β) y la óxido nítrico sintasa inducible (iNOS). A la vista de los resultados obtenidos podemos concluir que la adición de extractos de harina de semilla de oliva a los cultivos produce signos inequívocos de modificación del complejo de reacción inflamatoria subclínica de los pacientes diabéticos que cursa con una reducción de los niveles y/o la actividad biológica de ambos marcadores (IL-1β e iNOS).

Palabras clave: *cultivo de sangre, diabetes, inducción de citoquinas, inflamación, marcadores, olivo, semilla.*

INTRODUCCIÓN

La dieta Mediterránea con el aceite de oliva como fuente principal de grasa, ha demostrado ser un protector potencial frente a diversas patologías (enfermedad cardiovascular, procesos inflamatorios de bajo grado, daños oxidativos, cáncer etc.), principalmente debido a su contenido en compuestos bioactivos, y mayoritariamente a su fracción fenólica (oleuropeína, oleocantal, hidroxitirosol, tirosol...) caracterizada por sus propiedades antioxidantes (1). Las diferentes partes de la aceituna poseen diferencias en la cantidad y tipo de los diferentes componentes menores. Así, por ejemplo, el aceite de orujo, extraído en su mayoría de la piel y de las semillas, es muy rico en ácidos triterpénicos, que han mostrado actividades anti-inflamatorias y vasodilatadoras en estudios con modelos celulares y animales (2). En la actualidad el único producto que se obtiene mayoritariamente de la aceituna tras su procesamiento en almazaras es el aceite, si bien algunas experiencias previas han demostrado que la deconstrucción o fraccionamiento de la aceituna puede proporcionar nuevos y valiosos productos. En este sentido, tan sólo una pequeña parte de los compuestos fenólicos (metabolitos con propiedades beneficiosas para la salud ampliamente demostradas) presentes en las aceitunas completas se transfieren al aceite de oliva. La búsqueda de nuevos aprovechamientos industriales para el 75-80% de los subproductos no utilizados de la aceituna constituye una tarea vital para el sector olivarero español. Unos de estos productos infrautilizados son las harinas semillas de olivo, cuyo uso potencial está comenzando a ser explotado (3). En este sentido, el sector de alimentación animal es uno de los recientemente propuestos para la aplicación de varias biomoléculas presentes en las semillas procedentes de los huesos de aceitunas (4). En este trabajo hemos centrado nuestra atención fundamentalmente en las harinas procedentes de la semilla del hueso y su posterior valoración como ingrediente nutracéutico con aplicaciones en el ámbito alimentario.

La diabetes mellitus (DM) representa un conjunto de enfermedades metabólicas que se caracterizan por presentar una hiperglucemia provocada por defectos en la secreción de insulina, en la acción de la misma o por ambas causas. La hiperglucemia crónica de la DM se asocia a la presencia a largo plazo de lesiones a nivel ocular, renal, nervioso, cardíaco y vascular.

La diabetes mellitus tipo 2 (DT2) representa el 90% de todos los casos de diabetes, con una prevalencia en torno al 13,8% en población mayor de 18 años, y con todavía un 6% de los sujetos sin diagnosticar, según un estudio reciente realizado en España (5). Como es bien conocido, esta prevalencia está en aumento y la DT2 se está convirtiendo en una auténtica epidemia, sobre todo en países desarrollados, debido al importante incremento de la obesidad, al sedentarismo y al progresivo envejecimiento de la población. La estimación de crecimiento de la prevalencia de la DT2 entre los años 2010 y 2030 supone un 72% en todo el mundo (6), lo que se asociará también a un incremento paralelo del gasto sanitario.

A pesar de los tratamientos farmacológicos disponibles, una gran proporción de los pacientes DT2 no cumplen con las recomendaciones para el control de la glucemia, o los tratamientos no consiguen controlar su glucemia. Todo ello da lugar a complicaciones crónicas asociadas como defectos en la señalización de la insulina que conducen a la hiperglucemia. Hay un flujo continuo de nuevos resultados de investigaciones que evidencian que las especies de oxígeno reactivo (ROS) realizan una contribución significativa a la progresión de la diabetes y sus complicaciones. El término estrés oxidativo se refiere a la situación en la que se produce un desequilibrio entre la producción de ROS y las defensas antioxidantes, a favor de la generación de especies oxidadas, lo que conlleva a un daño potencial de los tejidos (5,7). La mayoría de las biomoléculas esenciales (lípidos, proteínas y ácidos nucleicos) constituyen dianas de las ROS generadas durante la hiperglucemia. Como consecuencia de este estado de inflamación, se pueden causar daños oxidativos en el páncreas, hígado y riñón (8). Es por ello que la identificación de nuevos productos alimenticios que ayuden potencialmente al control de la glucemia, y a la reducción de la actividad antiinflamatoria producida por dicha enfermedad con trasfondo inmunológico, puede permitir el diseño de estrategias de complementación de los tratamientos farmacológicos tradicionales.

Como se ha comentado anteriormente, varios estudios confirman la idea de que en el desarrollo y la progresión de la DT2 subyacen mecanismos inmunológicos e inflamatorios (9,10). Hay evidencias claras de que la inflamación participa de manera importante a través de factores como el factor de necrosis tumoral (TNF), y las citoquinas IL1 y IL6 entre otros. Otro de los marcadores ampliamente utilizados para cuantificar el estrés oxidativo en los tejidos es la determinación de los niveles de óxido nítrico sintasa inducible (iNOS). El presente trabajo pretende evaluar el efecto antiinflamatorio potencial de las harinas de semilla de olivo en pacientes diabéticos, en comparación con pacientes no diabéticos, a través de un sistema ex vitro de cultivo de sangre, en el que se analizan algunos de los marcadores indicados anteriormente. Entender los mecanismos que reducen la inflamación utilizando este ingrediente de excelentes características y con moléculas bioactivas podría tener importantes implicaciones no sólo para diseñar novedosas terapias, sino para prevenir la asociación de enfermedades crónicas inflamatorias.

MATERIAL Y MÉTODOS

Recogida de muestras

Los pacientes diabéticos de tipo 2 y controles no diabéticos procedían del área de cobertura del área básica “Pedro Martínez” (A.G.S. noreste Granada, España). Para dicho diagnóstico se aplicaron los criterios de clasificación de la Organización Mundial de la Salud. Se seleccionaron 6/8 pacientes de cada tipo, que cumplían los criterios de inclusión y exclusión establecidos.

a) Criterios de inclusión:Pacientes varones diabéticos tipo 2 (y no diabéticos como control) entre 18-30 años. No tratados y no resistentes a la insulina.Aceptación y comprensión del consentimiento informado.
b) Criterios de exclusión: No aceptación del consentimiento informado. Tabaquismo.

Se procedió a la extracción de sangre periférica de vena cubital en tubos heparina-Li de 4ml. La sangre total, tanto de sujetos sanos como pacientes con DT2, se distribuyó en placas de 24 pocillos (1,5ml por pocillo), dentro de las primeras horas de muestreo. Las sangres fueron inducidas con varios agentes antigénicos y mitogénicos como lipopolisacárido (LPS), fitohemoaglutinina (PHA), y forbol-12-miristato-13-acetato junto con ionomicina (PMA+IO). También fueron co-inducidas con distintas concentraciones de extractos proteicos de harinas de semillas (10,5 y 2.5 mg/ml). Como control negativo se dejó un pocillo sin estimular con fines comparativos. Los cultivos se incubaron en cámara húmeda en una atmósfera con CO_2 al 5 % a 37^{o}C por 24 h. Posteriormente las placas se centrifugaron a 1,200 xg 15 min, y se recolectó el plasma de los diferentes grupos experimentales. Las muestras de plasma se congelaron a -80^{o}C para su posterior uso.

Determinación de marcadores inflamación mediante RT-PCR en plasma.

Se procedió a la determinación de los marcadores de inflamación en plasma mediante RT-PCR. La extracción de ARNm de las muestras de plasma se realizó usando un kit específico Qiagen® miRNA Purification Kit. La calidad y concentración del ARN fue evaluada mediante la razón de absorbancias 260/240nm (relación entre ácidos nucleicos y fenoles) y razón de absorbancias 260/280nm (relación entre ácidos nucleicos y proteínas) usando un microespectrofotómetro Nanodrop^{TM} 2000 (Thermo). Para la síntesis de cDNA se empleó el kit High-Capacity cDNA (InvitrogenTM), proceso en el que, empleando una polimerasa de origen retroviral (la denominada transcriptasa inversa), se sintetiza cDNA a partir de un molde de RNA. El siguiente paso fue emplear el cDNA molde obtenido en la retrotranscripción para realizar una PCR cuantitativa. Se empleó el kit TaqMan® Universal Master Mix (Applied Biosystems). Este método se caracteriza por realizar una cuantificación a tiempo real de la amplificación de un gen en concreto (en este caso, IL-1β, iNOS y β-actina respectivamente). En la reacción se añadieron unas sondas comerciales (sondas TaqMan®) y una Taq polimerasa con actividad 5'-3' exonucleasa. A continuación, se preparó la placa de PCR MicroAmp® Fast Optical 96-Well Reaction Plate (Applied Biosystems). Por último, se centrifugó la placa, que fue introducida en el termociclador Ligut Cycler Roche 480 siguiendo el programa detallado. Cada experimento además incluyó un control negativo de cada una de las muestras de ARN que no fueron sometidas a la transcripción reversa. Los cambios relativos en la expresión génica se determinaron mediante el cálculo del $2^{-\Delta\Delta Ct}$ (tal como fue descrito por Livak y Schmittgen, 2001(11).

Análisis estadístico

Se llevó a cabo el análisis de homogeneidad de varianza (Bartlett-Box) seguido de un ANOVA de clasificación simple. La comparación de medias entre los diferentes parámetros fue realizada a través de la utilización de la prueba t de Student de muestras no pareadas. Los datos experimentales fueron expresados como la media ± la desviación estándar. El nivel de significación estadística fue asumido como $p<0,05$. El paquete estadístico utilizado para el procesamiento de los resultados fue el SPSS.

RESULTADOS Y DISCUSIÓN

Información de los participantes en el estudio.

Los parámetros clínicos del grupo de sujetos que participaron en el estudio están recogidos en la siguiente Tabla (Tabla 1). Los sujetos controles y los pacientes con DT2 no mostraron diferencias significativas en los parámetros de edad. Sí que se observaron diferencias significativas en relación al índice de masa corporal (IMC). Existen también diferencias significativas en los niveles de glucemia ($P<0,001$), presión sanguínea ($P<0,001$), frecuencia cardiaca ($P<0,001$) y hemoglobina glucosilada (HbA1c) ($P<0,001$).

Expresión de los genes relacionados con la inflamación en sujetos sanos y pacientes con DT2

Actualmente existe una gran evidencia de que en el desarrollo y la progresión de la diabetes tipo 2 subyacen mecanismos inmunológicos e inflamatorios. Una dieta rica en aceite de oliva no sólo es una buena alternativa en el tratamiento de la diabetes; también podría ayudar a prevenir o retrasar la reacción inflamatoria de la enfermedad. Es por ello que analizamos la interleuquina 1β (IL-1β) y la forma inducible de óxido nítrico sintasa (iNOS) como marcadores de inflamación.

Como se puede apreciar en la Figura 1, la producción de estos marcadores por parte de individuos sanos y en ausencia de estímulos fue insignificante. El tratamiento tanto con LPS, PHA, como con PMA+IO estimuló significativamente tanto la producción de IL-1β (Figura 1A) como de iNOS (Figura 1B). Cuando se añadió al cultivo de sangre entera de forma concomitante tanto LPS, PHA, ó PMA+IO y la harina de semilla, se observó un claro descenso de estos dos marcadores, llegando al nivel basal (Figura 1A, Figura 1B, respectivamente). Para ver si esta harina de semilla tenía algún efecto sobre estos dos marcadores, dicha harina fue añadida de forma aisladamente, en ausencia de inducción, no observándose ningún cambio con respecto al control (Figura 1A, Figura 1B). A la vista de estos resultados podemos concluir que esta proteína podría tener propiedades antiinflamatorias.

Cuando se realizaron experimentos paralelos en pacientes diabéticos, observamos niveles elevados de los marcadores IL-1β (Figura 2A) e iNOS (Figura 2B) aún en muestras no inducidas (Figura 2, muestras DT2), lo que refleja la existencia de fenómenos inflamatorios basales con respecto a sujetos sanos (Figura 2, muestras C). Las muestras de pacientes diabéticos en cultivos de sangre completa también fueron hipersensibles a la inflamación inducida por PHA, LPS y PMA + IO, como se demuestra por la presencia de ligeros aumentos de los niveles de IL-1β y e iNOS en plasma (Figura 2A, Figura 2B, respectivamente). La adición de extractos de harina de semilla de oliva produce signos inequívocos de reducción de la respuesta inflamatoria, probablemente consistentes en la modificación del complejo de reacción inflamatoria subclínica de los pacientes diabéticos mediante la reducción de los niveles y/o la actividad biológica de IL-1β, iNOS (Figura 2A, Figura 2B, respectivamente).

Podríamos concluir que los componentes específicos de la harina de semilla de oliva son en última instancia responsables de estos efectos antiinflamatorios. Dichos componentes están aún por ser caracterizados de forma precisa. Tal caracterización ayudará a diseñar nuevos enfoques terapéuticos para estos pacientes.

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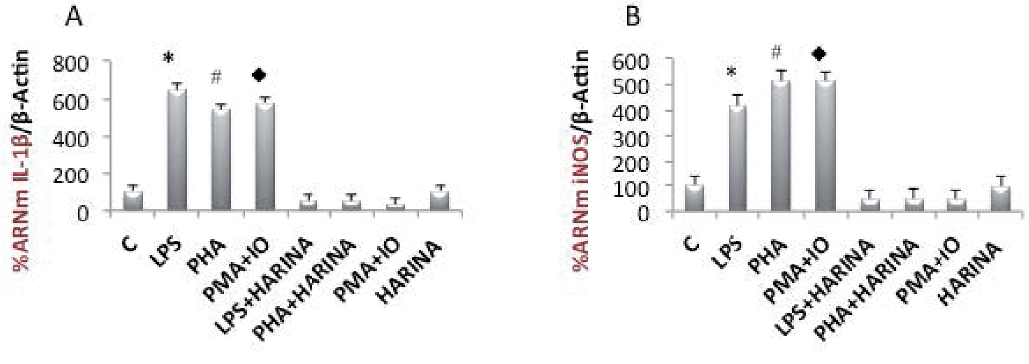

Figura 1. La sangre completa de sujetos sanos fue incubada con LPS, PHA, PMA+IO de manera individual, y de manera concomitante con LPS, PHA, PMA+IO y las harinas de semilla de olivo. Las barras representan la IL-1β (figura A) y la iNOS (figura B) respectivamente en sujetos sanos. C: muestras no inducidas.

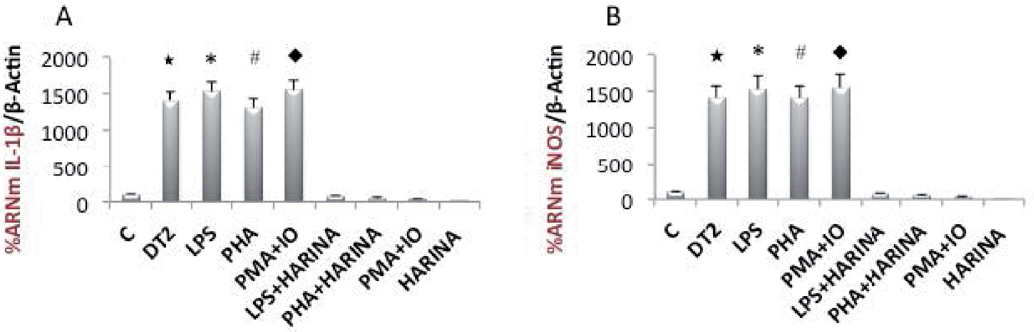

Figura 2. La sangre completa de pacientes diabéticos (DT2) fue incubada con LPS, PHA, PMA+IO de manera individual, y de manera concomitante con LPS, PHA, PMA+IO y las harinas de semilla de olivo. Las barras representan la IL-1β (figura A) y la iNOS (figura B) respectivamente en DT2. C: muestra sin inducir de sujeto sano.

Tabla 1. Características del grupo de estudio. La comparación de medias entre los diferentes parámetros fue realizada a través de un ANOVA. El nivel de significación estadística fue asumido como $p<0{,}01$ (representado en negrita).

	Sujetos Control	DT2	*P*-valor
Hombres	6	8	
Edad	50 (40,53)	48 (45,58)	**0.007**
Glucemia (mg/dL)	83 (80, 91)	165 (132, 198)	**<0.001**
Presión Sanguínea (mmHg)	12/7 (11/7, 12/7)	15/8 (14/8, 15/7)	**<0.001**
IMC (kg/m^2)	24.8 (23, 26.6)	33.3 (27.5, 46.7)	**<0.001**
Frecuencia Cardiaca (bpm)	64 (65, 76)	84 (96, 80)	**<0.001**
HbA1c %	5.5 (5.4, 5.7)	6.7 (6.0, 7.4)	**<0.001**

Overall discussion

The traditional manner in which the farmers/industries treat the olive fruit in order to mainly obtain olive oil and olive table fruits has evolved greatly along the time, with a plethora of literature available about new methods to obtain or improve such productions. However, much effort can still be done in order to improve the available methods and to develop new ones able to cope with emerging topics like circular economy, energy improvements, development of novelty products, and limitation of the generation of wastes among many others.

ANCIENT AND NEW TECHNOLOGIES FOR THE USE OF THE OLIVE FRUIT

It has been described that the virgin olive oil quality and organoleptic properties depend on different factors such as olive cultivar, olive tree cultivation and the operations during the olive picking, storage and processing (Giovacchino and Sestili, 2002; Servili and Montedoro, 2002; Frankel et al., 2013). Factors such as the presence of leaves in the mix with olives or the use of metal crushers may also affect the organoleptic properties of the olive oil. The presence of by-products from the oil extraction process is unavoidable at present. This waste consists in parts of pits and damaged fruit. Besides, the wastewater also represents an important source of by-products. Depending on the kind of procedure used for the extraction (pressure and continuous- two or -three phases), the quantity of waste is variable. The solid defatted pomace is another by-product obtained after extraction of olive oil with solvents (Toscano and Montemurro, 2012). The optimisation of the conventional methods in the last decades has considerably reduced the olive oil waste (Kapellakis et al., 2008). Currently, it is not easy to estimate the volume of waste from the olive oil industries produced worldwide, which is depending on several factors such as olive variety, olive seed maturity, cultivation techniques, and geological-climatic conditions (Kapellakis et al., 2008). We also have to highlight the current absence of a legal framework policy among the EU members. In Spain, in 1981, it was established a law prohibiting the discharge of untreated olive oil waste into rivers.

New procedures are constantly appearing, like the enzyme-assisted extraction processing (EAEP), a newly developed methodology that includes the use of enzymes in the oil-extraction. The mix of enzymes has proven to be effective in the increment of the quality and the yield of the olive oil (De Faveri et al., 2008; Aliakbarian et al., 2008; Sharma et al., 2015). In seeds, the EAEP has been pointed out to be more eco-friendly than the solvent extraction. However, the specificity of the enzymes to be used has yet to be determined for each material. The main drawback of this procedure is the high cost, however, the environmental advantages make this area of research a goal for the future (Liu et al., 2016). Better understanding of the enzymatic machinery present in the olive seed could help to improve the olive oil quality. At this regard, the information regarding enzyme expression described in the present work, and the use of transcriptomic information as those included in ReprOlive database would be a feasible tool to start the research in this area.

The use of alternative technologies aimed to produce zero by-products is now a fact developed by companies such as Grupo Elayo. The aim of this company is to use all the fractions of the whole olive fruit (exocarp, mesocarp, stone and seed) to generate among others, a flour obtained from the olive seed. The seed storage proteins are key elements (Alché et al., 2006) to be analyzed as they are the major component of the olive seed.

On the other hand, other modified, alternative products, like de-stoned dried olive fruits for the animal diet is becoming an attractive alternative, mainly due to the chemical composition of the residual fat content (with elevated quantities of antioxidants and oleic acid, besides to a balanced composition in polyunsatured fatty acids), that have been described to have beneficial effects on the milk quality (Servili et al., 2015).

All the new methodologies and alternatives for the olive fruit utilization are increasing nowadays, that together with the rising research in the area, the ancient crop of the olive tree, with the well known properties of its fruit, is currently up-valued.

OLIVE SEEDS AND SEED STORAGE PROTEINS AS A MATTER OF STUDY: ORGANOLEPTIC PROPERTIES

Studies carried out in coffee beans point to the 11S seed storage proteins to have a physiological role providing a source of amino acids and nitrogen for seed germination. Similarly to olive, under reducing conditions, the mature precursor form of the 11S generates two subunits (high: R-component and low molecular weight: a component) (Rogers et al., 1999). On the other hand, the seed storage proteins have also a role at the cup quality level. at this regard, amino acids react with sugars moieties through Maillard reactions to produce aromatic compounds (de Castro and Marraccini, 2006). Also, some differences exist in the composition of free amino acids in green coffee beans of different species (Arnold et al., 1994). The 11S storage proteins include a high content in glutamine, glycine, leucine, and glutamic amino acids, and a low content in sulphur amino acid cysteine and methionine, therefore the implication on aroma and flavour in coffee is suggested to be limited (Grosch, 2001; De Castro and Marraccini, 2006). In coffee, no clear proofs of the involvement of the seed storage proteins on flavour and aroma are available. However, proteomic studies show the presence of abundant peptides of degradation corresponding to 11S storage proteins (Rogers et al., 1999; Ludwig et al., 2000).

In cocoa, the role of the 7S as responsible for the specific aroma by the action of an aspartic protease and a carboxypeptidase (Voigt et al., 1994) has been suggested. Recently, the characterization of protease enzymatic activity and the presence of isoenzymes in two cultivars of cacao has been shown under various conditions (Silveira et al., 2017). Besides, extrinsic factor such as yeast presence, can affect final cocoa properties. The presence of the *K.marxianus* yeast during fermentation produced chocolate with a more acceptable sensorial evaluation in comparison to the one from cocoa obtained through natural fermentation (Leal et al., 2008). Besides, it is suggested the dependency of the flavour formation on the degree of acidification of the cocoa beans during the fermentation (Schwan and Fleet, 2014).

Analogous investigations to cocoa and coffee are open to be carried out in the olive seed as several databases are available nowadays, as well as proteomic approaches to elucidate the effects of the environmental conditions, the cultivar, the harvesting period, the post-harvests treatments, etc., which provide a deep knowledge of the flavour/aroma properties provided by the olive seed storage proteins to the final product commercialized. In these sense, products derived from olive seeds as for example infusions would be also valuable products to be studied and commercialized. Such products might also contain high quantities of polyphenols and other valuable components, apart from proteins. Organoleptic properties of many alimentary samples are also quite depending on the redox state as described by Montavon et al., 2003. These authors report that the ripening state of the coffee bean represents a key factor of the oxidation of the chlorogenic acids and the 11S seed storage proteins digestions. In the olive seed, scarce investigations have been focused on the oxidative metabolism, with the exception of some emerging work on glutathione metabolism (Patón Álvarez, 2017; Martínez-Beas, 2017).

OLIVE SEEDS AND SEED STORAGE PROTEINS AS A MATTER OF STUDY: NUTRITIONAL CHARACTERISTICS

The European Food Safety Authority (EFSA) indicates that food products can only be considered functional if together with the basic nutritional impact, they have beneficial effects on one or more functions of the human organism, thus either improving the general and physical conditions or/and decreasing the risk of the evolution of diseases (Martirosyan and Singh, 2015).

The virgin olive oil is *per se* considered as a functional food -as also stated by the EFSA because of its content in healthy compounds (Reboredo-Rodríguez et al., 2017). However, the potential contribution of the olive seed proteins to this matter is still undetermided. In the case of rice, it has been suggested that alteration of prolamins can contribute to improving the nutritional quality indicating that a regulation of endogenous storage protein content is a promising tool for improvement of the nutritional quality of the grains (Mandal and Mandal, 2000). In the case of sorghum seeds, the high concentration of soluble lysine and threonine is at the basis of their proposal as potential food source due to the better balanced amino acids profile (Vendemiatti et al., 2008). Proteomic approaches have revealed the proteome profiling of seed storage proteins and the nutritional potential of *Salicornia brachiata,* an halophyte that grows in salty marshes (Jha et al., 2012). A similar approach has been preliminary performed in the present doctoral thesis (chapter 2). These previous results were very promising as regards to the nutritional aspect: presence of seed storage proteins, as well as endopeptidase activity.

Seed storage proteins are considered to provide functional an nutritional properties with a relevant value in the food industry. Biotechnology tools are nowadays used for producing proteins with better commercial properties. Therefore, improvements in the genetics or the seed crop of seed storage proteins from crops is an future issue of study (Tandang-Silvas et al., 2011). Seed technology is a promising field, as seeds maintain a wide and stable space for the accumulation of recombinant products without loss of activity. Thus, investigations on the molecular mechanisms that regulate the expression and accumulation of seed storage proteins may provide helpful information for the production of recombinant products (Kawakatsu and Takaiwa, 2010). In the olive seed, investigation at the transcriptional, translational and pos-translational levels are still on their beginnings. However, the tools available nowadays are increasing, which encourage to researchers to draw their attention on this crop, also taking into account its well known beneficial effects on human health.

The nutrigenomics/nutrigenetics (how dietary nutrients affect gene expression and how genes affect nutrient metabolism) are tools to be used in the future to study the effects of the olive derived compounds in health. Scientific reports are available about the nutrigenomic effect of the virgin olive oil (Khymenets et al., 2009; Konstantinidou et al., 2010). However, nutrigenomic/nutrigenetic focussed on the effects of the proteins obtained from the olive seeds is still a challenge for the future. Preliminary studies (chapter 2 of the present doctoral thesis) present a panel of proteins with putative implications in gene expression and nutrient metabolism). After fulfilling the whole experiments, a huge amount of information would be available for the designing and accomplishment of specific studies on omics sciences. Chapter 4.3 of the present thesis also provides a preliminary example of the anti-inflammatory effects of olive seed flours on diabetic patients. Further studies on this direction are currently underway, within the frame of several research projects listed as funding sources of the present work.

OLIVE SEEDS AND SEED STORAGE PROTEINS AS A MATTER OF STUDY: ALLERGENICITY

The prevalence of food allergy is increasing in the recent years (Kattan, 2016). Among grains and legumes, wheat and soybean are the most frequent and well-characterized allergenic food. The characterization of the allergenic proteins as well as the antinutritional factors in soybean and wheat have described and reviewed widely (Tatham and Shewry, 2008; Wang et al., 2014; Pasha et al., 2016). Besides, the classification of plant food allergens as well as cross-reactivity studies have been performed (Jenkins et al., 2005), resulting a total of 20 families, 4 of them the most relevant ones (cupin, prolamins, profilins and pathogenesis-related proteins). The common legumes described with the ability to elicit allergenic response are peanut, soybean, lentil, lupine, pea, mung, chickpea, red gram, green gram, French gram, and blackgram (Verma et al., 2013) among others. Besides to the cross-reactivity described among allergens from unrelated species, it has been described the cross-reactivity between legumes and pollen. The study of this phenomena offers a helpful guide to allergenic patients about the decision of avoiding certain foods sharing the IgE epitope/s to other pollen/food allergens to which they are sensitized (Verma et al., 2013).

Considering the increasing number of literature as regard the allergenicity of the seed storage proteins in different species, as well as the cross-reactivity described, the allergenic characterization of the olive seed storage proteins is a need for their implementation as food ingredient. The present doctoral thesis provides a first approach to the characterization of the allergenic potential of the main SSPs in the olive (7S and 11S) (chapter 3), together with an detailed report about the absence of gluten, as reported in chapter 4. To our knowledge no other literature is found in this area.

NEW PERSPECTIVES/INVENTIONS IN THE OLIVE OIL FIELD

The outstanding functional properties of plant proteins in food applications are widely described in the scientific literature. Therefore, it is not surprising the appearance of new inventions/patents related to this area. For instance, a method to modify the flavour profile of a plant protein preparation for use in foodstuffs is described in the patent "Method for modifying the flavour profile of a plant protein preparation US 8309160 B2". This patent provides a method of protein preparation for leguminous plants which has the most neutral flavour profile possible, without the flavours typical of leguminous plants. Protein preparations made from raw materials such as soya, rice, wheat, peas, lupines or other protein-containing plant seeds are used in foodstuffs as water binders, oil binders, gel-forming agents, emulsifiers or foaming agents, for example (https://www.google.com/patents/US8309160?cl=en). The incorporation of olive seeds for protein preparation is a challenge, that could be possible as soon as the knowledge on seed proteins boosts. As a preliminary step, we describe in chapter 4 a procedure for the preparation of protein isolates from olive seeds. Proteins isolates allow (among many other applications) the elimination of anti-nutritional substances, improvement of flavour, digestibility, concentration, etc. of foods rich in proteins, increasing their potential uses.

On the other hand, the EcoPROLIVE (Ecofriendly PROcessing System for the full exploitation of the OLIVE health potential in products of added value) project is focussed on sustainability of the olive oil industry (http://cordis.europa.eu/result/rcn/198133_en.html). The aim of this project is the waste-minimization to a virtually "zero process". However, the ECOLIVE project has recently finished the experimental phase and it is now moving towards the application phase. This project is an example proposing a process to profit of the bioactive potential of olives in different products with minimal loss of valuable compounds and improved environmental impact.

The already mentioned company "Grupo Elayo" is a pioneer in the achievement of alternative processing of the olive fruit, with the commercialization of the olive seed flour that has been the focus of the study of the present doctoral thesis.

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Conclusions

1. The described **anatomy and histological distribution of the olive seed** of the ‘Picual’ cultivar, allowed identifying the **main features typical of dicots** within a **developmental time frame**. Cell **storage structures** (PBs and OBs) presented a **well-defined pattern of accumulation**, with **complementary distribution** in the olive seed tissues. Moreover, a panel of other olive cultivars has been used to compare the **weight contribution of the different tissues to the seed**, seed **weight variability**, and the **number of seeds** per fruit. These hallmarks will help **to settle the basis for future studies** related to the location of different metabolites along the olive seed and mesocarp development, and therefore will help to **assess the appropriate ripening stage** for **different commercial and industrial purposes**.

2. 1D/2D electrophoresis followed by nLC-MS/MS strategies allowed the **identification of numerous proteins** in the olive seed, with a large **predominance of SSP proteins** (mainly of the 11S and 7S type), as well as **numerous structural and enzymatic proteins**, revealing the large **complexity of this proteome** and thus confirming the potential **interest of this material** for alimentary, biotechnological and nutraceutical purposes.

3. An olive seed transcriptome generated by *de novo* sequencing, assembly and annotation was used to **identify a broad panel of 11S and 7S protein** sequences in this material. Bioinformatic assessment of the identified transcripts allowed analysing their **clustering with previously-characterized 11S sequences** and their identification as corresponding to the isoform 2 of the 11S protein precursor. **Individual acidic and basic subunits** within this sequence were **recognised, 3D-modeled** and **assessed as regard to their potential molecular interaction** by docking methods. Furthermore, **T-cell epitopes were forecast** by using predictive software in order to **evaluate the putative implications** of the olive 11S proteins in **food allergy**.

4. **Evidence of the presence of vicilins** in olive seeds was supported by the identification of **at least 58 sequences** in the mature olive seed transcriptome, displaying **considerable annotation identity** with 7S vicilins from other species. Furthermore, **one of these sequences was validated by experimental methods**, enabling *in silico* comparative analysis with other vicilins. Although the olive sequence was incomplete, it showed **characteristic structural details**, a **close genetic relationship**, and **good 3D structural superimposition** with the built models of **vicilins from different species**, suggesting that olive vicilins are, to a great extent, similar to other vicilins. Moreover, both **unique** and/or **commonly shared T- and B-epitopes** among other 7S vicilins were present in the olive sequence. These epitopes are likely to be responsible for the **reactivity of olive seed extracts to the sera from several patients allergic** to nuts despite the fact that olive seeds are not commonly used as a food ingredient.

5. **Preparation of protein isolates** from defatted olive seed flours **is feasible** through standard procedures, which aim to the generation of these products with improved quality and composition when compared to the raw flour material. Such protein isolates **maintain a protein composition similar** to the original flours, **with a large proportion of 11S SSPs** of **good nutritional character** and **low allergenicity**, together with the **absence of antinutritional characteristics**.

6. Based on the use of two procedures with differential sensitivity and ability to detect gluten, **olive seed flours can be designated as gluten-free ingredients**, widely exceeding the indications of the FDA of the USA and the EC regulations.

7. Plasma samples from diabetic patients in *ex vitro* whole blood cultures are hyper-responsive to inflammation induction by PHA, LPS and PMA+IO, as demonstrated by the increased levels of IL-1β and iNOS markers in plasma. However, **challenge of these cultures with extracts from olive seed flour produced unequivocal signs of reduced inflammatory response, mainly in diabetic patients**. These symptoms are likely mediated **through modifications of the complex subclinical inflammatory reaction**, and are witnessed as reduced levels and/or biological activity of IL-1β and iNOS. The **specific components** of the olive seed flour ultimately responsible for these anti-inflammatory effects **are yet to be characterized**. Such characterization will help to design **new therapeutical approaches** for these patients.

Conclusiones

1. La descripción de la **anatomía y la distribución histológica de la semilla del olivo** de la variedad "Picual" permitió identificar las **principales características de una dicotiledónea típica** dentro de un **marco temporal** de desarrollo. Las **estructuras de almacenamiento** (Cuerpos Proteicos -PBs- y Cuerpos Lipídicos -OBs-) presentaron **un patrón de acumulación bien definido**, con una **distribución complementaria** en los tejidos de la semilla del olivo. Además, se usaron otras variedades para comparar la **contribución del peso de los diferentes tejidos a la semilla, variabilidad del peso** de la semilla y el **número de semillas** por fruto. Estos sellos distintivos ayudarán a **sentar las bases de futuros estudios** relacionados con la localización de diferentes metabolitos durante el desarrollo de la semilla y el mesocarpo del olivo, y por tanto serán de ayuda para **evaluar el adecuado estado de madurez** con **diferentes propósitos comerciales e industriales**.

2. La electroforesis 1D y 2D seguidas de un análisis mediante nLC-MS/MS permitieron la **identificación de numerosas proteínas** en la semilla del olivo, con una gran **predominancia de las proteínas SSPs** (principalmente las de tipo 11S y 7S), además de **numerosas proteínas con función estructural y enzimática**, lo que revela la gran **complejidad de este proteoma** y por tanto confirma el potencial **interés de este material** para propósitos alimentarios, biotecnológicos y nutracéuticos.

3. Se ha utilizado un transcriptoma se semilla de olivo que fue generado mediante secuenciación *de novo,* ensamblaje y anotación para la **identificación un amplio número de proteínas 11S y 7S** en este material. Los estudios bioinformáticos de los transcritos identificados permitieron analizar su **agrupamiento con secuencias 11S previamente caracterizadas** y su identificación con la isoforma 2 del precursor de la proteína 11S. Las **subunidades individuales** ácida y básica de esta secuencia fueron **reconocidas, modelizadas** a nivel 3-D y fueron además **evaluadas por su potencial interacción molecular** mediante métodos de "anclaje". Además, los **epítopos de las células T fueron predichos** mediante el uso de programas informáticos predictivos con el objetivo de **evaluar las posibles implicaciones** de las proteínas 11S del olivo en **alergias alimentarias**.

4. Las **evidencias de la presencia de vicilinas** en las semillas del olivo fueron apoyadas por la identificación de **al menos 58 secuencias** en el transcriptoma de semilla madura de olivo, que mostraron una considerable identidad en la anotación con vicilinas 7S de otras especies. Además, **una de estas secuencias fue validada experimentalmente**, permitiendo un análisis comparativo *in silico* con otras vicilinas. Aunque la secuencia de olivo estaba incompleta, mostró **detalles estructurales característicos**, una **relación genética próxima** y una **buena superposición de la estructura 3D** con los modelos construidos de **vicilinas procedente de otras especies**, lo que sugiere que las vicilinas del olivo son, en gran parte, similares a otras vicilinas. Además, tanto **epítopos únicos** como los epítopos T y

B comúnmente **compartidos** con otras vicilinas 7S estaban presentes en las secuencias de olivo. Estos epítopos son probablemente responsables de la **reactividad de los extractos de la semilla del olivo con sueros de diferentes pacientes alérgicos** a frutos secos a pesar de que las semillas del olivo no son usadas normalmente como ingrediente alimentario.

5. La **preparación de aislados proteicos** de harinas de semilla de olivo desgrasada **es posible** mediante procedimientos estándar, con el objetivo de la generación de estos productos con una calidad y composición mejorada si se compara con harina obtenida del materia original. Estos aislados proteicos **mantienen una composición proteica similar** a la de las harinas originales, con una **gran proporción de proteínas de almacenamiento 11S** con **buenas propiedades nutricionales** y **baja alergenicidad**, junto con la **ausencia de características antinutricionales**.

6. Basado en el uso de dos procedimientos con sensibilidad y capacidad diferencial para detectar gluten, **las harinas procedentes de semillas de olivo pueden ser clasificadas como ingredientes libres de gluten**, con indicadores muy por debajo de los indicados por la normativa FDA de los EE.UU. y de la Comisión Europea.

7. Las muestras de plasma procedente de sangre completa de pacientes diabéticos en cultivos *ex vitro* presentan una hiper-respuesta a la inducción de inflamación mediante PHA, LPS y PMA+IO, como ha sido demostrado por los niveles elevados de los marcadores de IL-1β e iNOS en plasma. Sin embargo, **la incubación conjunta de estos cultivos con extractos de harina de semilla de olivo, produjo signos inequívocos de una reducción de la respuesta inflamatoria, principalmente en pacientes diabéticos**. Estos síntomas son probablemente mediados a **través de modificaciones de la compleja reacción de inflamacion subclínica**, y son detectados como niveles reducidos y/o actividad biológica reducida de IL-1β e iNOS. Los **componentes específicos** de la harina de semilla de olivo que constituyen los responsables últimos de estos efectos antiinflamatorios **están todavía por caracterizar**. Dicha caracterización ayudará al diseño de **nuevas aproximaciones terapéuticas** para estos pacientes.

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ANOLEA (http://protein .bio.puc.cl/cardex/servers/anolea/)

Bioedit V7.0.5.3 (http://www.mbio.ncsu.edu/bioedit/bioedit.html)

BLAST: Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih .gov/Blast.cgi)

Center for Biological Sequence Analysis, Technical University of Denmark DTU, Kongens Lyngby, Denmark (http://www.cbs .dtu.dk/)

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PDB (http://www.pdb.org)

Primer3 Pickprimers from a DNA sequence (http://bioinfo.ut .ee/primer3-0.4.0/)

PROCHECK (http://www.ebi.ac.uk/thorn-ton-srv/software/PROCHECK/)

ProPred, MHC Class-II Binding Peptide Prediction Server (http://www.imtech.res.in/raghava/propred/index.html)

ProSA (https://prosa .services.came.sbg.ac.at/prosa.php)

Protparam tool (http://web.expasy.org/protparam/)

PyMol software (https://www.pymol.org/)

QMEAN (http://swissmodel.expasy.org/qmean/cgi/index.cgi)

ScanProsite program (http://www.expasy.org/tools/scanprosite)

Scratch Protein Predictor, http://scratch.proteomics.ics.uci.edu/.

SWISS-MODEL (http://swissmodel.expasy.org/workspace/)

The European Bioinformatics Institute, Multiple Sequence Alignment, http://www.ebi.ac.uk/Tools/clustalw/index.html.

The PSIPRED Protein Sequence Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/)

World Wide Protein Data Bank (PDB) (http://www.wwpdb .org/)

- List of abbreviations
- List of tables and figures
- Publications and works presented to conferences arising from this thesis

Annexes

2D gel: Bidimensional gel

3D: Three-dimensional

AACC: American Association of Cereal Chemists

A.C.: (=B.C.): Before Christ

A.G.S.: Área de Gestión Sanitaria

Å: Angstrom

aa: Aminoacid

AL: Aleurone layer

AnaOc: *Anacardium occidentale*

ANOVA: Analysis of variance

AraHy: *Arachis hypogea*

Arg: Arginine

ARNm: see mRNA

Asp: Aspartic acid

BLAST: Basic local alignment search tool

BLASTP: Basic local alignment search tool with proteins

BLOSUM: BLOck SUbstitution Matrix

bp: Base pair

Co: Columella

C: Cuticle (Chapter 1)

C: Non induced samples (control) (Chapter 4.3)

CCD: Charge-coupled device

CD: Coeliac disease

cDNA: Complementary deoxyribonucleic acid

CE: Capillary Electrophoresis

CE–MS2: Capillary electrophoresis–mass spectrometry

CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

CLs: Cuerpos lipídicos

CNT: Arbitrary Units (BioRad)

CorAv: *Corylus avellana*

CPs: Cuerpos proteicos

CW: Cell wall

Da: Dalton

DAA: Days after anthesis

DM: Diabetes mellitus

DNA: Desoxyribonucleic acid

DT2: Type 2 diabetes

DTT: Dithiothreitol

E: Embryo

E1: Endosperm layer 1

E2: Endosperm layer 2

E3: Endosperm layer 3

E4: Endosperm layer 4

EAEP: Enzyme-assisted extraction processing

EC: European commission

EcoPROLIVE: Ecofriendly PROcessing System for the full exploitation of the OLIVE health potential in products of added value

EFSA: European food safety authority

ELISA: Enzyme-linked immunosorbent assay

Ep: Epidermis

ER: Endoplasmatic reticulum

Et: Endotesta

EU: European union

EVOO: Extra virgin olive oil

Ext: Exotesta

F.A.C.E.: Federation of associatios of coeliac patients of Spain

FAD: Fatty acid desaturase

FDA: Food and drug administration

g: Relative centrifugal force

Gc: Guard cells

GF: Gluten free

Glu: Glutamic acid

GO: Gene ontology

HLA: Human leukocyte antigens

HMW: High molecular weight

HPBs: Highly stained protein bodies

HPLC: High-performance liquid chromatography

HRP: Horseradish peroxidase

I1: Hydrophilic polypeptide region 1

I2: Hydrophilic polypeptide region 2

ID: Identification

IEF: Isoelectric focusing

IgE: Immunoglobulin E

IgG: Immunoglobulin G

II: Inner integument

IL-1ß: Interleukin- 1ß

IMC: Índice de masa corporal (= BMI) Body mass index

iNOS: Inducible nitric oxide sinthase

INT density: Densitometry of the reactive bands to the antibody (measurement of intensity)

IO: Ionomycine

IPG: Immobilized pH gradient

IS: Intracellular spaces

kDa: Kilodalton

KVh: Kilovolts/hour

LC: Liquid chromatography

LE: Lower epidermis

LFDs: Lateral flow devices

LG model: Model after the authors Le & Gascuel (2008)

Li: Lithium

LiCl: Lithium chloride

LM: Light microscopy

LoD: Limit of Detection

LoQ: Limit of Quantification

LOX: Lipoxigenase

LPBs: Low stained protein bodies

LPM: Lower premesophyll

LPS: Lipopolysaccharide

LupAl: *Lupinus albus*

LupAn: *Lupinus angustifolius*

Lys: Lysine

M: Meristem (Chapter 1)

M: Molar

MALDI-TOF: Matrix-assisted laser desorption/ionization-time of flight

mL: Mililiter

mM: Milimolar

mRNA: Messenger ribonucleic acid

MS: Mass spectrometry

Mt: Mesotesta

Mw: Molecular weight

N: Nucleus

NCBI: National center for biotechnology information

NGS: Next generation sequencing

NJ: Neighbour joining

NL: Non-linear

nLC-MS: Liquid chromatography on-line coupled to tandem mass spectrometry

OBs: Oil bodies

Oe: *Olea europaea*

OI: Outer integument

OleEu: *Olea europaea*

OVO: Ordinary virgin oil

p/v: Peso/volumen

P: Protoderm

PAGE: Polyacrylamide gel electrophoresis

PBs: Protein bodies

PCR: Polymerase chain reaction

PDB: Protein data bank

pH: Power of Hydrogen (measure of Hydrogen ion concentration)

PHA: Phytohemaglutinin

PhyML: Maximum likelihood

p*I*: Isoelectric point

PisVe: *Pistacia vera*

PL: Phospholipase

PLA: Phospholipase A

PM: Premesophyll

PMA: Phorbol 12-myristate 13-acetate

POD=POX: Peroxidase

ppm: parts per million

PPO: Polyphenol oxidase

Pr: Procambium

Pro 1: Precursor 1 de 11S

Pro 2: Precursor 2 de 11S

PSV: Protein storage vacuoles

PTMs: Post-translational modifications

PVDF: Polyvinyldene difluoride

PVPP: Polyvinylpolypyrrolidone

QC: Quiescent centre

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RT: Room temperature

RT-PCR: Real time-polymerase chain reaction

S: Space between endosperm-embryo

Sc: Subsidiary cells

SD: Standard deviation

SDS: Sodium dodecyl sulphate

SEM: Standard error of the mean

SesIn: *Sesamum indicum*

SPSS: Statistical package for the social sciences

Ssc: Substomatal cavity

SSpro8: Scratch Protein Predictor

SSPs: Seed storage proteins

SSR: Simple sequence repeat

T+E: Testa and endosperm

TAE: Tris-acetate-EDTA

TAG: Triacylglyceride

TBS: Tris-buffered saline

TBS-T: Tris-buffered saline + Tween

TCA: Trichloroacetic acid

TEM: Transmission electron microscopy

TF: Transversal fibers

TGX: Tris-Glycine eXtended

TNF: Tumor necrosis factor

Tris–HCl: TRIS hydrochloride

tTG: Tissue transglutaminase

UE: Upper epidermis

UPM: Upper premesophyll

v/v: Volume/volume

V: Volts

VOO: Virgin olive oil

w/v: Weight/volume

w/w: Weight/weight

WAF: Weeks after flowering

Tables

Figures

INTERNATIONAL CONFERENCES:

Title: Perfil nutricional y componentes nutracéuticos de la semilla del olivo
Authors: D Barrionuevo; D Maestri; R Bodoira; **Adoración Zafra**; José Carlos Jiménez López; Juan de Dios Alché.
Conference: VII Congreso Internacional de Ciencia y Tecnología de Alimentos (CICYTAC)
Type of participation: Speech
Place: Córdoba, Argentina
Date: 01-3/10/2018
Organizing entity: Ministerio de Ciencia y Tecnología de la provincia de Córdoba, Argentina

Title: Transcriptome-based identification of a seed olive legumin (11S globulin). Characterization of subunits, 3D modelling and molecular assessment of allergenicity
Authors: Rosario Carmona; José Carlos Jiménez López; **Adoración Zafra**; M. Gonzalo Clarós; Juan de Dios Alché
Conference: 4rd International work-conference on bioinformatics and biomedical engineering
Type of participation: Speech
Place: Granada, Spain
Date: 20-22/04/2016
Organizing entity: Universidad de Granada

Title: Identification of proteins of agro-alimentary interest in flours and protein isolates derived from olive seeds.
Authors: **Adoración Zafra**; Juan Pedro Sánchez Rivas; Rebeka Krebesová; Elena Lima; José Carlos Jiménez López; Antonio Jesús Castro; Juan de Dios Alché.
Conference: VII Food Technology International Symposium
Type of participation: Poster
Place: Murcia, Spain
Date: 14/05/2015
Organizing entity: Satin. Satiety Innovation

Title: Transcriptome-mining approach in the seek of non-described olive tree allergens
Authors: Rosario Carmona; Pedro Seaone; **Adoración Zafra**; Antonio Jesús Castro; José Carlos Jiménez López; M. Gonzalo Claros; Juan de Dios Alché.
Conference: III Plant Genomics Congress
Type of participation: Poster
Place: London, UK
Date: 11-12/05/2015
Organizing entity: Global Engage

Title: De novo assembly of the olive (*Olea europaea* L.) seed transcriptome during germination and seedling growth
Authors: **Adoración Zafra**; Rosario Carmona; Estela Domingo; María José Jiménez Quesada; Antonio Jesús Castro; Rocío Bautista; M.Gonzalo Claros; Juan de Dios Alché.
Conference: II Plant Genomic Congress
Type of participation: Poster
Place: London, UK
Date: 12-13/05/2014
Organizing entity: Royal Society of Biology/UK Plant Sciences Federation

PUBLICATIONS:

Zafra A, M'rani-Alaoui M, Lima E, Jiménez-López JC and Alché JD (2018) Histological Features of the Olive Seed and Presence of 7S-Type Seed Storage Proteins as Hallmarks of the Olive Fruit Development. Front. Plant Sci. 9:1481. doi: 10.3389/fpls.2018.01481.

Lima-Cabello E, Alché V, **Zafra, A**, Sánchez-Rivas JP, Jiménez-López JC, Marco F, Alché JD. (2017) Efectos anti-inflamatorios de harinas purificadas de semillas de olivo en pacientes diabéticos. El aceite de oliva. Comunicaciones Científicas. Simposium Expoliva 2017. ISBN.978-84-946839-1-6.

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Title: Enzyme activities regulating ROS metabolism in olive (*Olea europaea* L.) seeds
Authors: A Zienkiewicz; María José Jimémez Quesada; José Ángel Traverso; **Adoración Zafra**; Eduardo López Huertas; K Zienkiewicz; Antonio Jeús Castro; María Isabel Rodríguez García; Juan de Dios Alché
Conference: 11th International POG Conference. Reactive Oxygen and Nitrogen Species in Plants
Type of participation: Participativo - Poster
Place: Varsaw, Polonia
Date: 17-19/07/2013
Organizing entity: Warsaw University of Life Sciences-SGGW

Title: Characterization and potential uses of protein isolates from olive and argan seeds
Authors: Andrea Rueda; Irene Martín Aznarte; Ada Fernández Márquez; Sara Al-lach; **Adoración Zafra**; A Zienkiewicz; Alfonso Clemente; Antonio Jesús Castro; Juan de Dios Alché.
Conference: PIIISA 2013
Type of participation: Poster
Place: Granada, Spain
Date: 23/05/2013
Organizing entity: Universidad Grandada/CSIC

POPULARIZATION OF SCIENCE:

Title: Caracterización y usos potenciales de aislados proteicos de semillas de olivo y argán. **Adoración Zafra**; Agnieszka Zienkiewicz; Antonio Jesus Castro; Juan de Dios Alché.
Name of the event: Programa PIIISA 2013
Type of event: Programa de iniciación a la investigación de alumnos de secundaria
Organizing entity: EEZ-CSIC

NATIONAL CONFERENCES:

Title: Characterization and functional analysis of enzymes involved in glutathione metabolism in olive reproductive biology
Authors: José Carlos Jiménez López; Elena Lima Cabello; Estefanía García Quirós; Rosario Carmona; **Adoración Zafra**; Antonio Jesús Castro; Juan de Dios Alché.
Conference: Olivebioteq 2018
Type of participation: Speech
Place: Sevilla, Spain
Date: 15-19/10/2018
Organizing entity: Instituto de Investigación y Formación Agraria y Pesquera

Title: Histological Features of the Olive Tree Seed. A LM Structural Analysis Throughout Seed Development
Authors: **Adoración Zafra;** Juan de Dios Alché.
Conference: Microscopy at the frontiers of science 2017
Type of participation: Poster
Place: Zaragoza, Spain
Date: 05-8/09/2017
Organizing entity: Sociedad de Microscopía Española (SME)

Title: Study on reproductve biology of the olive tree, from pollen to seed
Autors: **Adoración Zafra**.
Conference: Young Researchers´Science Symposium
Type of participation: Speech
Place: Granada, Spain
Date: 19/12/2016
Organizing entity: Estación Experimental del Zaidín (CSIC)

Title: Automatic workflow for the identication of constitutively-expressed genes based on NGS reads mapping
Authors: Rosario Carmona; Pedro Seoane; **Adoración Zafra**; María José Jiménez Quesada; Juan de Dios Alché
Conference: 4rd International work-conference on bioinformatics and biomedical engineering
Type of participation: Speech
Place: Granada, Spain
Date: 20-22/04/2016
Organizing entity: Universidad de Granada

Title: Perfiles proteicos de harinas de semilla procedentes de procesados alternativos de la aceituna
Authors: **Adoración Zafra**; Juan Pedro Sánchez Rivas; Rebeka Krebesová; Antonio Jesús Castro; Juan de Dios Alché.
Conference: IV Jornadas Nacionales del grupo de Olivicultura de la SECH
Type of participation: Poster
Place: Baeza (Jaén), Spain
Date: 25-26/09/2014
Organizing entity: Sociedad Española de Ciencias Hortícolas