UNIVERSIDAD DE CÓRDOBA

DEPARTAMENTO DE AGRONOMÍA

ANÁLISIS DE PROCESOS MORFOGENÉTICOS DEL CRECIMIENTO, DESARROLLO Y PRODUCCIÓN DEL OLIVO

ANALYSIS OF MORPHOGENETIC PROCESSES OF OLIVE TREE GROWTH DEVELOPMENT AND PRODUCTION

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TESIS DOCTORAL

CORDOBA Junio 2012

Photo Olive Tree Dogga Tunisia
Ulrich Münstermann

TÍTULO: *Análisis de procesos morfogenéticos del crecimiento, desarrollo y producción del olivo*

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إهداء خاص إلى أبي ,أمي ,زوجتي و إبني

Dedicada a mis padres y mi pequeña familia

AGRADECIMIENTOS

Quiero expresar mis sinceros agradecimientos a la directora de este trabajo la Dra. Hava Rapoport, por su incondicional disponibilidad y por su gran contribución para desarrollar mis conocimientos, por transmitirme sus conocimientos académicos y científicos, y sobre todo por concederme su amistad.

Al Dr. Raúl de la Rosa, por todo lo que he aprendido de el, por su apoyó para llevar a cabo todos los ensayos y por todo lo que me ha enseñado en el campo de la ciencia y de la mejora.

Al tutor de mi tesis Pr. Elías Fereres, por su ayuda, preocupación y apoyó a lo largo de todo el desarrollo de la tesis.

Al Dr. Lorenzo León, por su continua ayuda y apoyó para llevar a cabo todos los ensayos asimismo por su incondicional disponibilidad.

A Esther García Cuevas por su especial ayuda y apoyo a lo largo de este trabajo en todos los aspectos del laboratorio de histología.

A mis amigos Dra. Angjelina Belaj, Dr. Milad riachy, José Ortiz, Olga, Mustafa Bani, Dr. Yamen Hamdan, Manoli, Onyeda, Dori, Dr. Durayd, Bochra, Dr. Mehdi Fendri, Antonio, Victor del Río, Dra. Victoria, Rafaela, Ahmed, por su cariñoso apoyo y por su animación durante toda mi estancia en España.

A mis amigos en Túnez, Aymen Mahfoudi, Walid Salhi, Mohamed-Ali, Moez Nafti, Moez Grami, Ridah, Mohamed Kabouchi, Hamdi, Mehdi Trad, por estar siempre a mi lado y atenderme en todos los momentos.

A mis amigos y directores del INAT Pr. Abdelrahman Jebari, Dr. Karim Aounallah, Pr. Hechmi Mehri por todo lo que me han enseñado y por tanto apoyo que mi brindaron en toda mi carrera

A mis amigos del Instituto del Olivo Pr. Msallem, Dr. Ajmi, Dr. Hessouna, Mr. Abichou, Abdel Elrazak, Ali y Dra. Mouna, por el apoyo y la ayuda que mi proporcionaron durante el desarrollo de este trabajo

Al Consejo Superior de Investigación Científica y Consejo Oleícola Internacional (COI), por financiar mi estancia en España y ofrecerme la oportunidad de realizar la tesis doctoral. Este trabajo ha sido financiado en parte por los proyectos AGL2008-02570/AGR (Procesos morfogenéticos clave de la fructificación en genotipos de olivo) y AGL2009-07248 (Procesos morfogenéticos clave del desarrollo del fruto en genotipos de olivo) financiados por el Ministerio de Ciencia e Innovación, y los proyecto RTA2008-00033-C02-00 del Instituto Nacional de Investigaciones Agrarias y PTR 1995-1015-0P del Ministerio de Educación.

A todos los miembros del programa de mejora de Córdoba, especialmente a, Mari Carmen, Dr. Aurelio, Estrella, David, Julillo, Manolo y Enrique por su ayuda y por crear tan buen ambiente de trabajo en el programa.

A la Dra. Inmaculada Moreno-Alias por revisar el español y por toda su ayuda y apoyo.

A todos los miembros del laboratorio de Crecimiento y Desarrollo del IAS, sobre todo a Walaa, Rocío, Ayman, Guillermo, Franco, Mª Angeles por el apoyo que mi brindaron durante la elaboración de este trabajo.

Aunque decir gracias, no es suficiente, quiero agradecer, todas las familias Hammami, Grami y Sghaier, especialmente a mis queridos padres y hermanos, mi esposa y mi niño por tanto amor, apoyó y confianza que en mi depositaron.

Sofiene

Resumen

La presente tesis doctoral tiene como objetivo global estudiar algunos aspectos del desarrollo vegetativo y reproductor para avanzar en nuestro conocimiento sobre estos procesos y proporcionar la información científico-técnica necesaria para mejorar el manejo del cultivo y facilitar la selección de nuevas variedades de olivo. Este objetivo global se aborda mediante objetivos específicos organizados en relación con tres aspectos principales del crecimiento de la planta: desarrollo vegetativo, desarrollo floral y desarrollo del fruto. En la parte del desarrollo vegetativo, se evaluó la arquitectura de plantas jóvenes de semilla de olivo de dos formas diferentes, una cuantitativa y otra cualitativa (visual). En base de diferentes criterios, como la baja correlación entre ellos y su alta heredabilidad, se eligieron los parámetros más relevantes de cada tipo, que fueron 4 cuantitativos y 5 cualitativos, para la caracterización y la evaluación de la arquitectura en los programas de mejora. Esta evaluación se puede realizar desde los 9 meses, según los resultados cuantitativos, la edad a partir de la cual las características arquitectónicas del genotipo parecen ser más constantes en el tiempo. Los parámetros cualitativos relevantes, que se revelaron fiables de igual forma que los cuantitativos, permitieron la identificación y la caracterización de 8 tipos arquitectónicos dominantes. La segunda parte de la tesis se dedicó a estudiar la respuesta de diferentes fases del desarrollo floral a un riesgo de déficit hídrico. Los resultados mostraron distintas respuestas según el momento de aplicación del déficit. Durante el periodo de desarrollo de las inflorescencias, el déficit hídrico provoca una importante reducción en los parámetros de floración, mientras que en el periodo final de formación de flores dificulta la polinización y el proceso de fecundación. Una reducción en la disponibilidad de agua en el periodo de floración y cuajado inicial parece impedir la polinización, debido a la obstaculización de la apertura de los pétalos. La aplicación de un déficit hídrico en cualquier momento del desarrollo floral activo conduce a una reducción en la producción final de frutos en peso y número totales. En la última parte de la tesis, se examinó la implicación de los tejidos (exocarpo, mesocarpo y endocarpo) y de los procesos celulares (división y expansión) en el crecimiento del fruto y la variabilidad genotípica de su tamaño. La división celular en el mesocarpo de la aceituna experimenta una primera fase de actividad intensa hasta las 8 semanas después de plena floración, seguida por una segunda fase más larga y de actividad reducida pero que produce alrededor del 25% del

número total de células. La expansión celular ha sido continua y algo lineal durante todo el crecimiento del fruto. Las diferencias genotípicas de tamaño de fruto observadas en seis cultivares con un gran rango de variabilidad se deben a diferencias en el tamaño del mesocarpo y del endocarpo. A nivel celular, estas diferencias, se deben principalmente al número de células del mesocarpo y no a su tamaño. La variación de las dimensiones y número de células entre las capas celulares de la zona externa del fruto y su relación con la variabilidad del tamaño del fruto, reveló la existencia de dos regiones subepidérmicas muy diferentes: 1) formada por las 4 primeras capas adyacentes a la epidermis (1-4), con un comportamiento similar a dicha capa, y 2) formada por las siguientes cinco capas (5-9), que mostraron un comportamiento más similar al mesocarpo. Estos resultados proporcionan una nueva perspectiva sobre las pautas celulares en la zona externa del fruto y sugieren que las células de las capas 1-4 forman, junto con la epidermis, un exocarpo multiseriado, mientras que las capas 5-9 constituyen un 'mesocarpo exterior' o una región transitoria.

Abstract

This thesis explores selected aspects of olive tree vegetative and reproductive development, in order to advance our knowledge regarding the morphogenetic processes involved, and to provide useful scientific and technical information for improving crop management and selecting new varieties. This overall objective is addressed with specific objectives organized in three categories based on the type of plant growth: vegetative development, flower development and fruit development.

With respect to vegetative development we evaluate the plant architecture of young olive seedlings by both quantitative and qualitative (visual) methods. In each study we use criteria such as the low correlation among parameters and their high heritability to determine the most relevant parameters for plant architecture characterization and evaluation in breeding programs. According to the analysis of the quantitative measurements, this evaluation can be performed as early as 9 months after planting, the age at which the phenotypic architectural features seem to become consistent over time. Together, the selected relevant qualitative parameters, which were demonstrated to be as reliable as the quantitative ones, led to the identification and characterization of eight dominant architectural types.

The second section involves the response of the different flower development stages to water deficit. The results showed different responses depending on the moment the deficit is applied. Deficit during inflorescence development reduced many different flowering parameters, including inflorescence number, flower number, perfect flower number and percentage, and ovule development. When applied two weeks prior to bloom little change was noted in floral development, but ovary and ovule starch content were reduced as well as fruit set. Deficit during bloom-initial fruit set produced a drastic effect in which many flowers remained closed and fertilization was prevented.

In the final section we examine the role of the different fruit tissues (exocarp, mesocarp and endocarp) and cellular processes (division and expansion) in fruit development and in the genetically based variation in fruit size. In olive cultivars with a wide range of fruit size the mesocarp cell size increased constantly and substantially from bloom to maturity. In contrast to cell size, cell number initially increased rapidly until 8 weeks after bloom, then, until fruit maturity, occurred at a much slower rate, under which 25% of the final cell number was produced. The genotypic differences in

fruit size are due to differences in both mesocarp and endocarp sizes. For the mesocarp, these differences are mainly due to cell number and not to cell size. In the fruit exterior, variation of cell dimensions among the subepidermal layers and the implied cellular contributions to fruit expansion revealed the existence of two very different subepidermal regions: 1) the first four cell layers (1-4) with similar behavior to the epidermis and 2) the following five (5-9) which were more similar to the mesocarp. The results, consistent among the different cultivars studied, suggest that layers 1-4 form, together with the epidermis, a multiseriate exocarp, and layers 5-9 constitute an outer mesocarp.

Índice General

B. Índice de Figuras

B. Índice de Tablas

I. INTRODUCCIÓN Y OBJETIVOS GENERALES

I.1. Introducción general

El olivo es uno de los cultivos más antiguos e importantes del mediterráneo a nivel social y económico. En los últimos años, este cultivo ha experimentado marcados cambios en las prácticas agronómicas, destacando el aumento de la densidad de plantación, el regadío y la mecanización de la cosecha (Rallo y Muñoz Diez, 2010; Tous, 2010). Estos cambios han hecho imprescindible profundizar el conocimiento en los diferentes procesos morfogenéticos del crecimiento y del desarrollo vegetativo y reproductor del olivo, que determinan el potencial agronómico del genotipo y de los cuales depende el manejo del cultivo (Connor y Fereres, 2005). Este desarrollo se puede enfocar a diferentes niveles de organización de la planta o de unidad de crecimiento: planta entera, órganos, tejidos y/o células, según los aspectos de crecimiento y los procesos biológicos que se pretende explorar. En este sentido el crecimiento vegetativo inicial produce y determina la estructura global de la planta (Barthélémy y Caraglio, 2007), que sirve de base estructural y fuente de asimilados para el desarrollo de los órganos reproductores, cuales condicionan el potencial productivo del árbol (Andreini et al., 2008). Las características finales del fruto, formado mediante estos órganos, y que representa la unidad de producción, están determinadas por el crecimiento de sus tejidos y de los procesos celulares de división, expansión, diferenciación y almacenamiento (Gillaspy, 1993; Corelli-Grappadelli y Lakso, 2004).

La arquitectura estudia y describe la organización espacio-temporal del los componentes de la estructura vegetativa del árbol (Hallé et al., 1978; Barthélémy y Caraglio, 2007), lo que también se conoce en Inglés como “plant form” (Bell and Bryan, 2008). En los frutales, la arquitectura de la planta se ha visto implicada en la duración del periodo juvenil, la forma y el tamaño del árbol, el método de cosecha, y el hábito de fructificación (Lauri et al., 2001; Costes et al., 2006). Considerando estas implicaciones, diferentes autores plantearon recientemente incluir la arquitectura en la selección de nuevas variedades adaptadas a altas densidades de plantación y/o a la mecanización (un solo eje, con un vigor reducido) (Laurens et al., 2000; L. Rallo et al., 2008), además de buscar criterios de selección para un corto periodo juvenil (Hartmann y Engelhorn, 1992; Pritsa et al., 2003; De la Rosa et al., 2006; Moreno-Alías et al., 2010). Sin embargo, poco avance se ha conseguido hasta el momento, debido principalmente al escaso conocimiento disponible sobre

la relación entre los diferentes parámetros, su heredabilidad y diversidad genética, aspectos necesarios para elegir los parámetros más relevantes a evaluar en los programas de mejora (Costes et al., 2006; Segura et al., 2006).

En el caso del olivo es aún más complicado estudiar la arquitectura, a causa del alto potencial de brotación que suele producir un sistema ramificado más complejo, la ambigüedad que existe sobre su naturaleza arbórea o arbustiva (Gucci y Cantini, 2000), y a la escasez de conocimiento preciso sobre los factores fisiológicos implicados en su crecimiento. Los trabajos de arquitectura en olivo, empezados con retraso en comparación con otros frutales, han surgido principalmente en el contexto de de los programas de mejora y relacionado con ello han constado principalmente en medir pocos y simples parámetros (Villemur, 1995; Mezghani y Trigui, 1998; Lauri et al., 2001, De la Rosa et al., 2006; P. Rallo et al., 2008). A la hora de desarrollar criterios de selección es muy importante tener en cuenta las condiciones de un programa de mejora, donde se evalúa normalmente un número muy alto de plantas y durante distintas edades o etapas. Adicionalmente se debe considerar los conceptos morfo-fisiológicos que determinan la arquitectura del árbol (Caraglio y Barthélémy, 1997). En consecuencia, el desafío más importante es desarrollar medidas lo más posiblemente simplificadas, repetibles y sólidas, y que permitieran caracterizar la arquitectura de la planta entera, el nivel de organización más interesante en este caso.

Una vez formada la estructura vegetativa básica del árbol durante el periodo juvenil o improductivo, la transición al estado adulto es solo asegurada a través del desarrollo de las primeras yemas reproductoras. En el olivo, la inducción floral, que determina el futuro de la yema como reproductora, parece ocurrir durante el mes de julio en las yemas formadas en el mismo año (Fernández-Escobar et al. 1992; Andreini et al., 2008). Estas yemas, a diferencia de las de la mayoría de los frutales, quedan indiferenciadas durante el periodo de reposo invernal. Al comienzo de la primavera con el aumento de las temperaturas, las yemas reproductoras, se diferencian y se desarrollan en inflorescencias (Lavee et al. 1996; De la Rosa et al. 2000). El potencial productivo del olivo depende, además del número de yemas reproductoras, del número de inflorescencias y flores en la planta, de la calidad individual de estas flores.

La calidad de la flor del olivo, que puede influir la fecundación y el cuajado del fruto esta determinada principalmente por el desarrollo de los órganos y tejidos que la componen (Fernandez-Escobar et al. 2008). El olivo es una especie andromonoica con dos tipos de flores: las flores perfectas, con estambres (órgano masculino) y pistilo (órgano femenino) bien desarrollados, y las flores imperfectas, con estambres bien formados pero un pistilo rudimentario o ausente (Ghrisi et al., 1999; Cuevas y Polito et al. 2004). El frecuente aborto pistilar que ocurre en el olivo es un componente definitivo de la calidad de flor, definida como la capacidad para producir un fruto, y por lo tanto afecta directamente la producción (Rapoport, 2005).

El desarrollo de flor perfecta o hermafrodita, con un pistilo normal, es un paso necesario pero no siempre suficiente para asegurar la calidad de la flor (Rapoport et al., 2006). En cada ovario de la flor del olivo, se encuentran normalmente cuatro primordios seminales, donde por norma, tan solo uno será fecundado, prosiguiendo su desarrollo para formar la semilla (Rapoport, 2005). En algunos casos el desarrollo del primordio seminal es incompleto o ausente, algo frecuentemente observado en el olivo (Rallo et al. 1981; Martins et al. 2006). La presencia de un numero bajo de primordios seminales bien desarrollados en el ovario, dos o menos, puede limitar la probabilidad de fecundación, el requisito para el desarrollo del ovario en un fruto. La calidad de flor se ha visto influenciada por el cultivar (Rosati et al. 2011; Rapoport y Rallo, 1991; Martins et al. 2006), y también por factores fisiológicos (Reale et al., 2009) y ambientales, como son el agua (Uriu, 1960) y el nitrógeno (Fernández-Escobar et al., 2008).

El proceso de desarrollo floral, desde la diferenciación de las yemas reproductoras, el desarrollo de la inflorescencia y las flores hasta la fecundación y el cuajado, dura varios meses, aproximativamente desde el inicio de marzo hasta el mes de junio en la región del mediterráneo donde se localiza más del 90% del área cultivada (Lavee, 1996; Rapoport y Rallo, 1991; De la Rosa et al. 2000). Durante este largo periodo existe el riesgo de déficit hídrico en años de sequía en esta región, y donde esta previsto un aumento de la aridez en un futuro próximo de acuerdo a los modelos climáticos globales (IPCC, 2007), lo que puede comprometer diferentes aspectos del desarrollo de los órganos reproductores y por consecuencia la producción.

Un buen desarrollo floral seguido por una polinización exitosa tiene que terminar por la formación del fruto, el órgano que representa el objetivo económico del cultivo. La aceituna, el fruto de olivo, es considerada una drupa típica, compuesta por un exocarpo, que es la capa exterior fina y protectora, un mesocarpo o pulpa carnosa y un endocarpo endurecido, llamado frecuentemente hueso (King, 1938). El crecimiento de la aceituna, descrito en base del diámetro transversal y del peso freso, comienza a partir de la fecundación con un aumento rápido del tamaño del mesocarpo y del endocarpo, tal y como ocurre en otras drupas. Prontamente, el endocarpo pone fin a su crecimiento y el fruto sigue creciendo solo mediante el mesocarpo hasta alcanzar su máximo tamaño (Hartmann, 1949; Lavee, 1986).

La división y la expansión celular son los principales procesos celulares responsables de la determinación del tamaño final del fruto carnoso (Gillaspy *et al.*, 1993). La información disponible sobre los procesos celulares del mesocarpo al principio del crecimiento de la aceituna muestra que, durante este periodo, el crecimiento del fruto es el resultado de los procesos de división y expansión celular en conjunto (Lavee, 1986; Rallo y Rapoport, 2001). En los principales frutos comerciales tipo drupa, como son el melocotón, la cereza, la ciruela o el albaricoque, se ha descrito que la división celular del mesocarpo ocurre básicamente durante un breve periodo inmediatamente después de antesis, representando aproximadamente el diez por ciento del periodo del desarrollo del fruto, seguido por un periodo solamente de expansión celular que dura hasta la maduración (Bollard, 1970). No obstante, tanto en el olivo, como en las otras drupas, la división y la expansión celular se ha evaluado generalmente en una sola dimensión mediante el número y el tamaño de las células a lo largo del radio del mesocarpo. Esta metodología puede limitar la información adquirida sobre los procesos debido a que el área o volumen total del mesocarpo no se debe solamente a su grosor, sino también al espacio (el endocarpo) que rodea. Otra dificultad en evaluar las contribuciones de la división y la expansión celular al crecimiento se debe a la interrelación de estos dos procesos en producir el tamaño celular, a su variabilidad en el tiempo y espacio, y también a la interacción entre tamaño y número en los métodos de medida (Green, 1976).

El tamaño final del fruto se ve influenciado y determinado tanto por factores exógenos, como la disponibilidad de agua y la temperatura ambiental, como endógenos, como el nivel de carga y la diferencias genéticas (Corelli-Grappadelli y Lakso, 2004), pero el tamaño potencial del fruto está genéticamente controlado. Este tamaño es el resultado del crecimiento y desarrollo de los diferentes tejidos del fruto y que varían en tipo, número y porcentaje entre las especies (Bollard, 1970; Coombe, 1976). Sin embargo, la mayoría de los estudios de la variabilidad genotípica de tamaño de los frutos tipo drupa, se centran en evaluar el mesocarpo, y no considerado la contribución del exocarpo y del endocarpo a esta variabilidad por diferentes razones.

En las drupas comerciales comunes, como el melocotón, el ceruela y el albaricoque, el endocarpo ha sido generalmente negligido debido al porcentaje bajo que representa del peso fresco y seco del fruto (Bollard, 1970; Coombe, 1976). En contraste, en el olivo, el endocarpo destaca por representar una proporción bastante alta del peso seco y freso del fruto (Bianchi, 2003), y la relación mesocarpo/endocarpo entre cultivares es un criterio de calidad muy importante en la aceituna de mesa y su variabilidad es más notable en este fruto que en el resto de las drupas (Del Río y Caballero, 2008). Recientemente, el hueso (endocarpo lignificado) se ha propuesto como una fuente de biodiesel atractiva, mediante el aprovechamiento de la gran cantidad de hueso que generan las industrias de aceituna de mesa que practican el deshuesado (Rodríguez et al. 2008). Estos datos demuestran una importancia económica del endocarpo, y sugieren una notable implicación del endocarpo en el tamaño y final del fruto y de su calidad en el caso del olivo.

La contribución del excarpo o epicarpo, la zona más externa del fruto, al tamaño final del fruto y su variabilidad no ha sido considerado en los estudios llevados acabo en los frutos carnoso, debido en parte al muy bajo porcentaje que representa en el fruto, pero sobre todo a la falta de precisión en definir las capas celulares que lo componen. De hecho, el exocarpo esta considerado diversamente compuesto de la epidermis solo o junto con un número variable de capas celulares subepidérmicas en la misma especie (Roth, 1977), caso que ocurre también en el olivo (King 1938; Lavee 1986; Mulas 1994). Sin embargo, recientes trabajos indican la importante actividad de división celular en este tejido a lo largo del

crecimiento del fruto (Lemaire-Chamley et al. 2005; Schlosser et al. 2008), uno de los procesos que determinen su tamaño final (Gillaspy et al., 1993). Además, las capas celulares del exocarpo juegan un papel clave en la interacción entre el fruto y su medio ambiente biótico y abiótico, así como en la determinación de su calidad (Knee et al, 2002; Mintz-Oron et al, 2008). Estudios recientes sobre patógenos (Wang et al., 2009) y los daños producidos por la cosecha mecánica (Ferguson et al., 2010) indican que la interacción con el exterior puede estar relacionada con el tamaño de la aceituna, lo que sugiere la posibilidad de diferentes características celulares de las capas externas en frutos de tamaños distintos. La resolución de estas posibilidades requiere como primer paso examinar más a fondo la estructura del exocarpo y determinar si esto se relaciona con el tamaño varietal del fruto.

I.2. Objetivos generales

La presente tesis doctoral tiene como objetivo global estudiar algunos aspectos del desarrollo vegetativo y reproductor para avanzar nuestro conocimiento de estos procesos y proporcionar información científico-técnica necesaria para mejorar el manejo del cultivo y facilitar la selección de nuevas variedades de olivo. Este objetivo se usa como la base para plantear enfoques de estudio a diferentes niveles de la organización de la planta, con el fin de dar la oportunidad al doctorando para elegir y afrontar la hipótesis y el nivel de organización adecuado, y las implicaciones teóricas y metodológicas de cada enfoque.

Este objetivo global se abordará en objetivos más específicos organizados en relación con tres aspectos principales del crecimiento de la planta: Desarrollo vegetativo, desarrollo floral y desarrollo del fruto.

PARTE 1: DESARROLLO VEGETATIVO

Capitulo 1: Se evalúan parámetros cuantitativos de la arquitectura de la planta en una población numerosa de plantas de semilla jóvenes procedentes de diferentes cruzamientos. La variabilidad de estos parámetros se analiza en relación con la edad de la planta y los genitores. Los resultados obtenidos sirven para determinar los parámetros más relevantes en la evaluación y caracterización de la arquitectura en los programas de mejora.

Capitulo 2: Se definen criterios visuales para evaluar complejas características arquitectónicas en un gran número de plantas de semilla de olivo y en sus padres. Basado en la baja correlación entre ellos, y de un alto nivel de diversidad y de heretabilidad, se seleccionan los criterios más relevantes para la descripción de la arquitectura de planta. Finalmente, se evalúa la fiabilidad de los criterios cualitativos mediante su comparación con medidas cuantitativas.

PARTE 2: DESARROLLO FLORAL

Capitulo 3: Se evalúa la influencia del déficit hídrico en diferentes fases del desarrollo floral. Para ello, se aplica un déficit hídrico controlado en períodos sucesivos del desarrollo floral, desde el reposo de las yemas reproductoras, hasta el momento inicial del desarrollo del fruto.

PARTE 3: DESARROLLO DEL FRUTO

Capitulo 4: En este capitulo se examina la contribución de los tejidos (mesocarpo y endocarpo) y de los procesos celulares (división y expansión) al crecimiento del fruto y la variabilidad genotípica de su tamaño. Estos parámetros se evalúan en área transversal de fruto (dos dimensiones), en lugar del radio (una dimensión) comúnmente usado en este tipo de estudios.

Capitulo 5: Se evalúan las dimensiones y del número de células en la epidermis y en 20 capas subepidérmicas sucesivas, y su relación con la variabilidad genotípica del tamaño del fruto. Los resultados se utilizan para explorar de la organización celular en la zona externa del fruto y su posible implicación en la interacción del fruto con los factores externos.

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II. PARTE 1. DESARROLLO VEGETATIVO

II.1. Capítulo 1:

Early growth habit and vigour parameters in olive seedlings

Scientia Horticulturae 129 (2011) 761–768

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Early growth habit and vigour parameters in olive seedlings

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II.1.1. Abstract

Fruit tree growth habit and vigour are important traits for orchard management and production, yet are difficult to select for in breeding programs because the parameters for their evaluation are complex, and their expression often requires sufficient plant size and development, entailing valuable time. Thus, in olive (*Olea europaea* L.) breeding, as for other fruit crops, it is critical to define growth habit and vigour parameters with selection potential, and to determine the earliest age at which they can be measured. Furthermore seedling growth habit traits are important in themselves, particularly in their relation to juvenile period length and management. To explore these issues we evaluated a series of new and standard growth habit parameters during the first year of growth in a population of unpruned olive seedlings originating from six different crosses. The influence of plant age on growth habit traits was determined by comparing measurements at two or three different times. Both parent genotype and plant age significantly affected the vigour and growth habit of the olive seedlings, and 9 months was the most appropriate age for evaluating seedling growth parameters. 'Picual', 'Arbosana' and Sikitita' were shown to be promising cultivars for use as genitors, because of their tendency to produce offspring with desirable growth habit traits such as high vigour, weeping habit and low lateral shoot number. From amongst the 17 parameters studied, based on parent influence and the absence of correlation among parameters, five were identified which best described olive seedling growth habit: Primary Shoot Top Diameter, Primary Shoot Conicity, Secondary Shoot Number, Secondary Shoot Insertion Angle and Longest Secondary Shoot Internode Length.

II.1.2. Introduction

Growth habit, which determines plant architecture, describes plant form, size and branching behaviour (Djouvinov, 2004; Legave et al., 2006; Scorza et al., 2006). In woody fruit crops growth habit traits have an important impact on crop management, intensification and orchard design (Sansavini and Musacchi, 1994; Stephan et al., 2007; Lauri et al., 2001). Furthermore, the growth habit of young seedlings has been associated with the length of their juvenile period (Visser et al., 1976; Alston and Bates, 1979; Hartmann and Engelhorn, 1992; Thompson and Grauke, 2003), indicating its importance both for breeding programmes and the prompt entry of new orchards into fruit production. As a consequence, these traits have recently been included in fruit breeding schemes to breed for early bearing, low vigour and adaptation to mechanical harvesting (Janick and Moore, 1996; Laurens et al., 2000; Carrillo-Mendoza et al., 2010), and an adequate choice of genitors has been shown to have a significant influence on the growth habit traits of their descendants (Visser et al., 1976; Hjeltnes, 1988; Hodge and White, 1992; Lawson et al., 1995; Segura et al., 2006; Ledbetter et Sisterson, 2008; Rezaee et al., 2009).

Among growth habit traits of interest for the selection of new olive genotypes, the weeping growth habit is desirable due to its utility in the new high-density and hedge-row plantations (Rallo et al., 2008b). In contrast to other tree fruit crops (Hjeltnes, 1988; Djouvinov, 2004; Werner and Chaparro, 2005; Scorza et al., 2006), little information is available in olive about parent genotype influence in this trait, nor regarding the heritability of other growth habit characteristics.

In order to reduce costs and labour in the breeding programmes, it is important evaluate traits as early as possible. However the plant age at which growth habit is evaluated may frequently influence the results obtained (Osario et al., 2003). For instance De Wit et al. (2004) reported distinct differences in apple seedling growth habit for evaluations carried out at one and two years. In apricot, a direct relation was observed between the degree of genotypic differences in early growth habit and the plant age (Legave et al., 2006).

Seedling growth habit traits, as well as being potential indicators of adult characteristics, may also be important in themselves, that is, as seedling properties. In olive trees, early seedling vigour, an aspect closely integrated with

growth habit, has been associated with the length of the juvenile period (Pritsa et al., 2003; Santos-Antunes et al., 2005; Rallo et al., 2008a). In fact, selection criteria for vigour, based on height and primary shoot (trunk) diameter, have been put into practise in order to discard those plants with a long juvenile period early during greenhouse growth rather later in the field (De la Rosa et al., 2006). Those studies, however, have produced few and in some cases contrasting results (Pritsa et al., 2003; Santos-Antunes et al., 2005; De la Rosa et al., 2006; Rallo et al., 2008a). Such confusion is not surprising, as the plants were measured in diverse ways, at diverse ages, grown under different conditions and obtained from different parent genotypes. To mention one example, little attention has been given to the height at which primary shoot diameter has been measured and its influence on the evaluation of vigour, whereas evidence from apple seedlings indicates different behaviour between basal and top primary shoot diameters (Segura et al., 2006; Segura et al., 2007.

Another growth habit characteristic which is desirable in olive seedlings is a low number of lateral branches, which, when present, require elimination in order to achieve rapid growth for juvenility period reduction (Santos-Antunes et al., 2005). Other seedling lateral-shoot growth-habit parameters, such as length and insertion height, can influence in first flowering position and management in olive seedlings (Moreno Alias et al., 2010).

The aim of our study is to describe the growth habit and vigour parameters in unpruned young olive seedlings and to explore their variability as influenced by both plant age and parent genotype. We compare the values obtained from different parameters and evaluate the relations and interactions among them. Finally, the relevance of the different parameters for the evaluation of growth habit in order to improve the breeding selection process is addressed.

II.1.3. Materials and methods

II.1.3.1. Plant Material

We evaluated 837 olive seedlings from six different crosses: ‘Sikitita’ x ‘Arbosana’ (134 plants), ‘Picual’ x ‘Hojiblanca’ (82 plants), ‘Picual’ x ‘Jabaluna’ (132 plants), ‘Frantoio’ x ‘Arbosana’ (190 plants), ‘Frantoio’ x ‘Manzanilla’ (131 plants) and ‘Frantoio’ in open pollination (168 plants). The crosses were performed at the

experimental farm of IFAPA in Córdoba, Spain. Seeds extracted from fruits collected in the fall were cold-treated and subsequently germinated as described by Santos-Antunes et al. (2005). Recently germinated seedlings were transplanted to 3 L pots in the greenhouse, where they were grown with drip fertirrigation, at controlled temperature (22ºC on average) and under continuous light using a sodium lamps. No pruning was performed in order not to interfere with the natural growth habit. A paternity test using SSR markers was performed following a previously developed protocol (De la Rosa et al., 2004) in order to confirm the authenticity of the crosses.

II.1.3.2. Measurements

All direct measurements and the branching order used for shoot evaluation are shown in Figure 1.1. The first order (Primary Shoot, Pr Sht) was assigned to the trunk; the first lateral branches were thus Secondary Shoots (Sec Sht); and so on. The evaluated parameters, their abbreviations, the plant age at which the measurements were made and the plants (crosses) which were measured are summarized in Table 1.1. The indicated abbreviations will be used in all following text. Additional parameters were calculated from the direct measurements, and their formulas are also presented in Table 1.1. The calculated parameters were Pr Sht Internode Lgth, Pr Sht Conicity, Sec Sht per Node, Longest Sec Sht Internode Lgth and Internode Lgth to 1st Sec Sht.

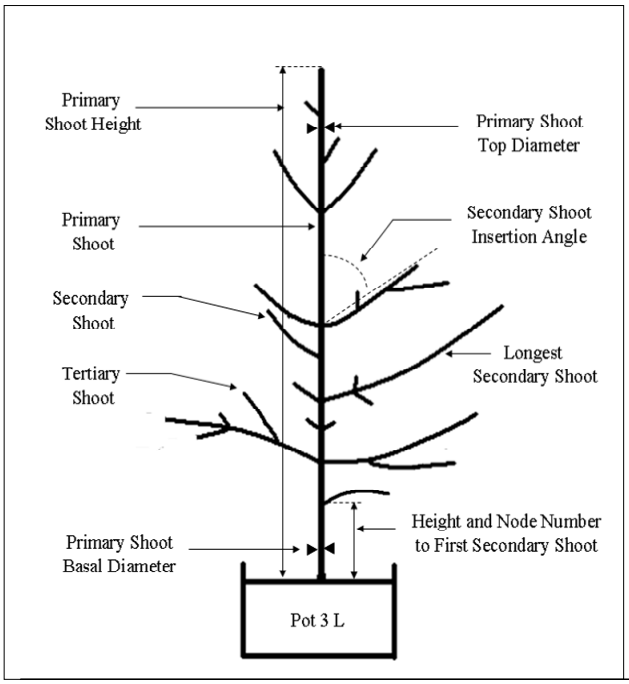

Fig.1.1. Schematic representation of olive seedling showing the direct measurements and the different orders assigned to studied shoots. Basal and Top Diameters are measured respectively at 5 and 100 cm height of the primary shoot.

Table 1.1 List of the different measured and calculated parameters and the plant age when they were determined. The principal parameters describe the characteristics of initial seedling shoot growth and branching. The secondary parameters characterize aspects of secondary shoot branching which appear as plant development progresses. Formulas are shown for calculated parameters. Positions on the plant shown in Fig.1.1.

Parameters	Abbreviation	Formula or measurement details	Plant Age (month)		
Principal parameters [a]			6 mo.	9 mo.	12 mo.
Primary Shoot Height	Pr Sht Ht		X	X	X
Primary Shoot Basal Diameter	Pr Sht Basal Diam	Measured at 5 cm height	X	X	
Primary Shoot Node Number	Pr Sht Node No		X	X	
Primary Shoot Internode Length	Pr Sht Internode Lgth	Average internode length of Pr Sht	X	X	
Primary Shoot Top Diameter	Pr Sht Top Diam	Measured at 100 cm height		X	
Primary Shoot Conicity	Pr Sht Conicity	(Basal Diameter-Top Diameter)/L [c]		X	
Secondary Shoot Number	Sec Sht No			X	X [b]
Secondary Shoots per Node	Sec Sht per Node	Sec Sht Number / Pr Sht Node Number		X	
Tertiary Shoot Number	Ter Sht No			X	
Secondary parameters [b]					
Secondary Shoot Length	Sec Sht Lgth	Average of all Sec Sht lengths			X
Accumulated Secondary Shoot Length	Accm Sec Sht Lgth	Sum of all Sec Sht lengths			X
Secondary Shoot Insertion Angle	Sec Sht Insert Angle				X
Longest Secondary Shoot Length	Longest Sec Sht Lgth				X
Longest Secondary Shoot Internode Length	Longest Sec Sht Internode Lgth	Average internode length for the longest Sec Sht			X
Height to 1st Secondary Shoot	Ht to 1st Sec Sht				X
Node Number to 1st Secondary Shoot	Node No to 1st Sec Sht				X
Internode Length to 1st Secondary Shoot	Internode Lgth to 1st Sec Sht	Average of Pr Sht internode length below the 1st Sec Sht			X

[a] Recorded in all crosses: 'Sikitita' x 'Arbosana' (134 plants), 'Picual' x 'Hojiblanca' (82 plants), 'Picual' x 'Jabaluna' (132 plants), 'Frantoio' x 'Arbosana' (190 plants), 'Frantoio' x 'Manzanilla' (131 plants).

[b] Determined in three crosses: 'Sikitita' x 'Arbosana' (40 plants), 'Picual' x 'Hojiblanca' (40 plants), 'Picual' x 'Jabaluna' (40 plants).

[c] Formula from Segura et al. (2006). L is Length between the two points of measurement; in our case 100cm - 5cm = 95cm.

At 9 months after germination, all principal parameters were measured in all crosses (Table 1.1). These parameters describe the principal aspects of initial seedling shoot growth and branching. Among them, the parameters Pr Sht Ht, Pr Sht Top Diam (measured at 100 cm height in plants exceeding this height) and Pr Sht Basal Diam (measured at 5 cm height) were considered to indicate vigour.

At 12 months, a second group of parameters, the secondary parameters, was used to describe more detailed aspects of secondary shoot branching which appear as plant development progresses (Table 1.1). Those measurements were carried out in three crosses that had demonstrated high vigour and similarity in the principal parameters, and were designed to both describe further development and test its relationship with the initial parameters. A random sample of 40 seedlings per cross of Sikitita' x 'Arbosana', 'Picual' x 'Jabaluna' and Picual' x 'Hojiblanca' was used for those measurements.

To determine the influence of the age of evaluation on primary shoot growth, Pr Sht Ht, Node No and Basal Diam were measured at two times: 6 months and 9 months. Pr Sht Ht was measured again at 12 months due to its demonstrated importance as a vigour parameter and an early selection criterion for short juvenile period in the olive tree (De la Rosa et al., 2006). To investigate the influence of evaluation age on lateral shoot growth, which was limited or absent at 6 months, Sec Sht No was counted at 9 months in all crosses and at 12 months in the three crosses studied at that age.

II.1.3.3. Statistical analysis

Analysis of variance (ANOVA) was used to test differences among crosses. Means were compared by Tukey's test at $P<0.05$. Pearson's correlation coefficient was calculated between principal vigour parameters. ANOVA analysis and Pearson coefficient were determined by *Statistix 9* (Analytical Software, Tallahassee, USA). The data of all evaluated parameters were used to perform a principal components analysis (PCA) using the statistical package The Unscrambler (CAMO A/S, Trondheim, Norway).

II.1.4. Results

II.1.4.1. Principal parameters

The analysis of variance of the principal parameters measured indicates that a low proportion of the total variability is due to differences among crosses (Table 1.2). In fact

more than 70% of the sums of squares correspond to the error term. Also, high coefficients of variation were obtained. Therefore, most of the variability for the principal parameters seems be due to differences among seedling genotypes independent of the crosses. However, significant differences among crosses were also observed in all cases (Table 1.2).

For all parameters measured at both 6 and 9 months the coefficient of variation was lower for 9 months (Table 1.2). Also, means comparison provided a higher number of subsets at 9 than at 6 months, except for Pr Sht Basal Diam. This result indicates that seedlings at 9 months tended to be more homogeneous within crosses and more different among crosses than at the younger age. However, subsequently, the values for the parameters Pr Sht Ht and Sec Sht No at 12 months did not show different behaviour from those at 9 months.

For the measurements carried out at 9 months, 'Sikitita' x 'Arbosana' descendants showed the highest values for Pr Sht Basal Diam and Pr Sht Node No, while descendants of 'Picual' tended to have higher Pr Sht Ht, Pr Sht Top Diam and Pr Sht Internode Lgth than 'Frantoio' descendants (Table 1.2). The Pr Sht Internode Lgth and Pr Sht Conicity revealed a high proportion of variance (24 and 31%) attributed to differences among crosses (Table 1.2). 'Frantoio' descendants showed higher Pr Sht Coinicity than 'Picual' x 'Hojiblanca' and 'Picual' x 'Jabaluna', in contrast to results obtained in Pr Sht Ht and Pr Sht Top Diam.

Significant differences were obtained for Sec Sht No at 9 months, with 'Picual' x 'Hojiblanca' showing the highest values (Table 1.2). This cross also showed higher Ter Sht No than the rest, and is the only one showing fourth order shoots (data not shown). Sec Sht per Node presented a similar order and differences among crosses as Sec Sht No, with 'Picual' x 'Hojiblanca' and 'Frantoio' x 'Arbosana' having the highest and lowest values respectively (Table 1.2).

Correlations were carried out between the vigour-indicating parameters measured at different ages. Pr Sht Ht was only slightly correlated with Pr Sht Basal Diam, but highly correlated with Pr Sht Top Diam (Fig.1.2). In fact, Pr Sht Top and Pr Sht Basal Diameters were not correlated. Pr Sht Top Diameter seems to be more adequate for vigour estimation due to its high correlation with Pr Sht Ht. Pr Sht Height measured at 9 months was highly correlated with that measured at 12 months, but both showed a low

Table 1.2 Comparison of means between six crosses for principal parameters. The percentage of sums of squares (SS) between (Cross) and within crosses (Error) and the coefficient of variation (CV) are also indicated. Different letters in each column represent significant difference at $p < 0.05$ level based on Tukey's Test.

Crosses	Pr Sht Ht (cm)			Pr Sht Basal Diam (mm)		Pr Sht Node No		Pr Sht Internode Lgth (cm)		Pr Sht Top Diam (mm)	Pr Sht Conicity (%)	Sec Sht No		Sec Sht per Node	Ter Sht No
	6 mo.	9 mo.	12 mo.	6 mo.	9 mo.	6 mo.	9 mo.	6 mo.	9 mo.	9 mo.	9 mo.	9 mo.	12 mo.	9 mo.	9 mo.
'Sikitita' x 'Arbosana'	35.7 a	104.2 bc	134.6 bc	2.50 b	5.12 a	22.0 a	48.6 a	1.60 cd	2.12d	2.33 bc	0.35 ab	19.2 b	21.85 b	0.38 b	1.44 b
'Picual' x 'Hojiblanca'	36.1 a	111.9 ab	144.6 ab	2.21 cd	5.05 ab	18.0 b	41.0 c	1.97 a	2.66 a	2.86 a	0.24 d	30.5 a	37.92 a	0.66 a	3.85 a
'Picual' x 'Jabaluna'	31.4 ab	115.1 a	148.8 a	2.38 bc	4.53 c	18.5 b	46.4 ab	1.67 bc	2.46 b	2.49 b	0.22 d	17.4 b	25.57 b	0.36 bc	1.64 b
'Frantoio' x 'Arbosana'	31.6 ab	85.0 d	116.4 d	2.82 a	4.70 c	19.1 b	43.1 c	1.52 d	1.96 e	1.73 d	0.38 a	11.8 c	-	0.26 d	1.29 b
'Frantoio' x 'Manzanilla'	32.3 b	94.5 cd	122.5 d	2.95 a	4.75 bc	19.7 b	44.6 bc	1.57 cd	2.09 d	2.07 cd	0.33 bc	17.1 b	-	0.39 b	1.53 b
'Frantoio' open pol.	29.6 b	97.1 c	126.6 cd	2.11 d	4.53 c	18.2 b	41.5 c	1.72 b	2.31 c	2.13 c	0.31 c	12.4 c	-	0.28 cd	0.99 b
SS (%) Cross	4	13	13	10	7	8	10	12	24	17	31	18	17	18	4
Error	96	87	87	90	93	92	90	88	76	83	69	82	83	82	96
CV (%)	34	28	29%	21	18	22	19	21	17	29	27	29	51	64	49

correlation with 6 months Pr Sht Ht. The seedling vigour at 9 and 12 months manifested a similarly tendency, but differed when evaluated sooner, at 6 months.

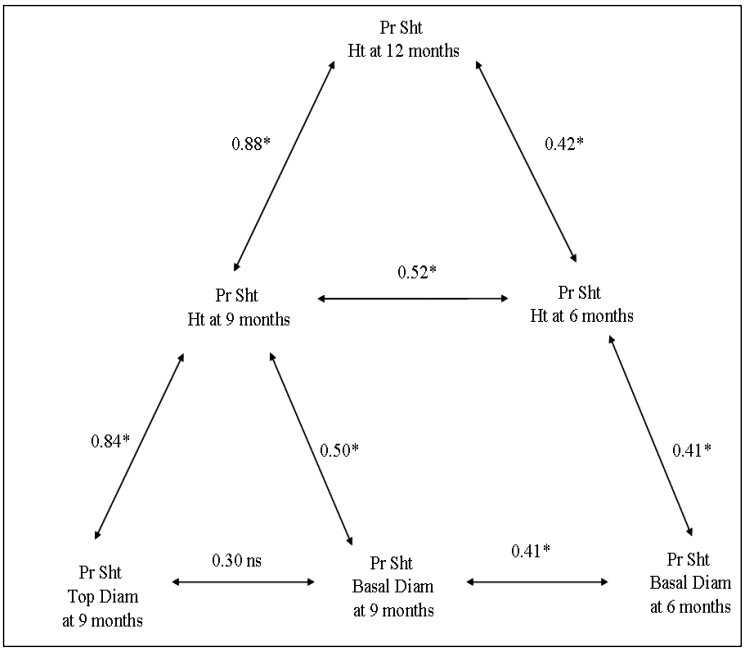

Fig.1.2 Correlations between parameters used to evaluate vigour (Pr Sht Ht, Pr Sht Basal Diam and Pr Sht Top Diam) and their values at different times. * Significant correlation at $p<0.01$. ns, non-significant correlation.

Principal component analysis (PCA) was performed for all of the principal parameter values obtained in all crosses and times (Fig.1.3A). This analysis projects the data onto a coordinate system so that the greatest data variance lies on the first coordinate, called the first principal component, and the second greatest variance on the second coordinate. The first and the second principal components (horizontal and vertical axes, respectively) accounted for 59% of the total variance. All principal parameters (Table 1.1) except Ter Sht No and Pr Sht Conicity were positively associated with the first principal component. This component thus reflects the basically negative relationship between Pr Sht Coincity and the plant vigour parameters Pr Sht Ht and Pr Sht Diameters. The second principal component was positively determined mainly by Pr Sht Internode Lgth and Sec Sht per Node, and negatively by Pr Sht Basal Diam and Pr Sht Node No. Therefore, this component principally indicates a negative relationship between the Pr Sht Internode Lgth and Pr Sht Node No. A different behaviour was observed for Pr Sht Ht measurements at different times, in that Pr Sht Ht was positively associated with the second principal component at 9 and 12 months but negatively at 6. The analysis also indicated a strong relationship between the morphologically related Sec Sht No at 9 months and Sec Sht per Node at the same age.

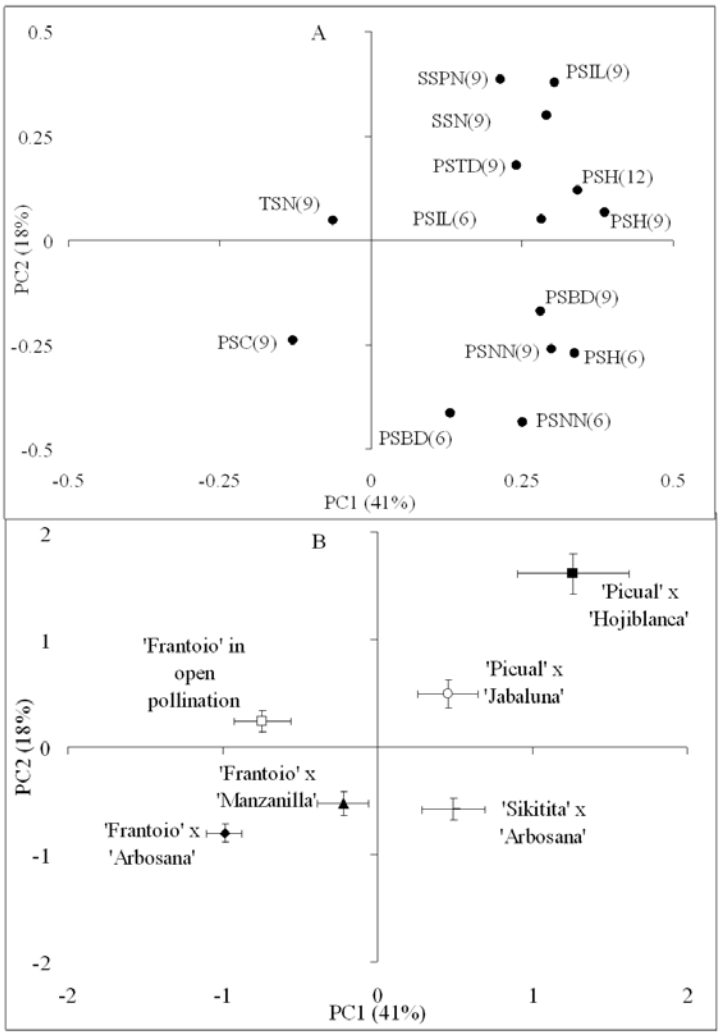

Fig.1.3 Principal Component Analysis for principal parameter values measured in all crosses at different times. Parameters are abbreviated as follows: Pr Sht Ht (PSH), Pr Sht Basal Diam (PSBD), Pr Sht Node No (PSNN), Pr Sht Internode Lgth (PSIL), Pr Sht Top Diam (PSTD), Pr Sht Conicity (PSC), Sec Sht No (SSN), Sec Sht per Node (SSPN), Ter Sht No (TSN), and (measurement age in months). Parameters loading plot is shown in A, and the crosses score plot is presented in B. Each point represents the average score value of seedlings per cross ± Standard Error.

The PCA biplot of mean seedling scores by cross for principal components 1 and 2 showed higher standard error values across the first principal component, i.e. a higher variability was observed for this component (Fig.1.3B). 'Picual' crosses were located in the first quadrant (positive values for both components) while 'Frantoio' x 'Arbosana' and 'Frantoio' x 'Manzanilla' were located in the third quadrant (negative values for both components). This indicates that the descendants of these two groups of crosses have opposite behaviours for the principal parameters. Thus 'Picual' descendants tended to have higher vigour and low Pr Sht Conicity, the inverse of what occurred in 'Frantoio' x 'Arbosana' and 'Frantoio' x 'Manzanilla'. In any case, 'Picual' x 'Hojiblanca' descendants tended to present very different growth habit than the rest of the crosses. Sikitita' x 'Arbosana' showed an opposed growth habit tendency to 'Frantoio' in open pollination, characterized by the highest Pr Sht Basal Diam and Pr Sht Node No (Fig.1.3B).

II.1.4.2. Secondary parameters

Significant differences among crosses were observed in most of the secondary parameters (Table 1.3). As with the principal parameters, differences between crosses accounted for less than 30 % of the total sum of squares in the analysis of variance for all the secondary parameters and a high coefficient of variation was obtained in all cases, indicating again that most of the variability seems to be attributable to differences within crosses. Longest Sec Sht Internode Lgth and Sec Sht Insert Angle revealed high percentages (31 and 25%) of sum of squares due to differences among crosses.

Differences among crosses for Sec Sht parameters varied among the parameters. The descendants of 'Picual'x'Hojiblanca' showed the highest value of Accm Sec Sht Lgth (Table 1.3), but no differences were found among the three crosses for Sec Sht Lgth, ie the average length of all secondary shoots. However when individual secondary shoots are considered, Longest Sec Sht Lgth was highest for 'Picual' x 'Hojiblanca' descendants and lowest for descendants of' 'Sikitita' x 'Arbosana', as was Longest Sec Sht Internode Lgth. A different value for Sec Sht Insert Angle was observed among all crosses, with 'Sikitita' x 'Arbosana' having the highest value, i.e. a more pronounced weeping habit.

The parameters Ht to 1st Sec Sht, Node No to 1st Sec Sht and Internode Lgth to 1st Sec Sht characterize the position where the first secondary shoot formed. 'Picual' x 'Jabaluna' descendants showed the highest Ht and Node No to 1st Sec Sht (Table 1.3). 'Sikitita' x 'Arbosana' showed the lowest values in all these parameters.

A combined PCA was performed including both principal and secondary parameters with the data of the three crosses in which secondary parameters was studied (Fig.1.4A). The two first principal components explained 44% of the total variance. A similar association among principal parameters as that previously observed for the six crosses data was obtained (Fig.1.3A). Comparing the secondary parameters with the previously studied principal parameters, Accm Sec Sht Lgth is the parameter most associated with the vigour parameters (Pr Sht Ht and Pr Sht Diameters). In contrast, Node No to 1st Sec Sht and Sec Sht Lgth seem to be negatively associated with high vigour. For Longest Sec Sht Internode Lgth and Pr Sht Internode Lgth, two morphologically related parameters, a positive relationship is indicated. A high similarity was shown for Pr Sht Ht at 9 and 12 months (Fig.1.2, 3A, 4A), as well as for Sec Sht No at those two times (Fig.1.4A).

Table 1.3 Comparison of means between crosses for the secondary parameters (measured at 12 months). The percentage of sums of squares (SS) between (Cross) and within crosses (Error) and the coefficient of variation (CV) are also indicated. Different letters in each column represent significant difference at $p < 0.05$ level based on Tukey's Test.

Crosses		Sec Sht Lgth (cm)*	Accm Sec Sht Lgth (cm)*	Sec Sht Insert Angle (º)	Longest Sec Sht Lgth (cm)*	Longest Sec Sht Internode Lgth (cm)	Ht to 1st Sec Sht (cm)*	Node No to 1st S. Sht.*	Internode Lgth to 1st Sec Sht (cm)*
'Sikitita' x 'Arbosana'		15.83 a	317.28 b	70.5 a	41.35 b	1.84 c	8.92 b	6.17 b	1.40 b
'Picual' x 'Hojiblanca'		13.41 a	456.98 a	58.9 b	51.47 a	2.55 a	10.55 ab	5.87 b	1.75 a
'Picual' x 'Jabaluna'		15.12 a	311.90 b	51.1 c	45.55 ab	2.12 b	13.41 a	8.12 a	1.54 b
SS (%)	Cross	3	12	25	5	31	6	7	14
	Error	97	88	75	95	69	94	93	86
CV (%)		55	54	23	34	20	61	55	23

* Data were transformed for comparison of means using logarithm or root square and back-transformed for presentation.

The scores biplot of the PC1 and PC2 showed a wide variability of all seedlings, although some grouping can be observed which relates to the different crosses (Fig.1.4B). Differences between crosses were more clearly observed for PC2: 'Picual' x 'Hojiblanca' descendants showed positive values for this component, 'Sikitita' x 'Arbosana' negative values and 'Picual' x 'Jabaluna' seedlings were located in an intermediate position.

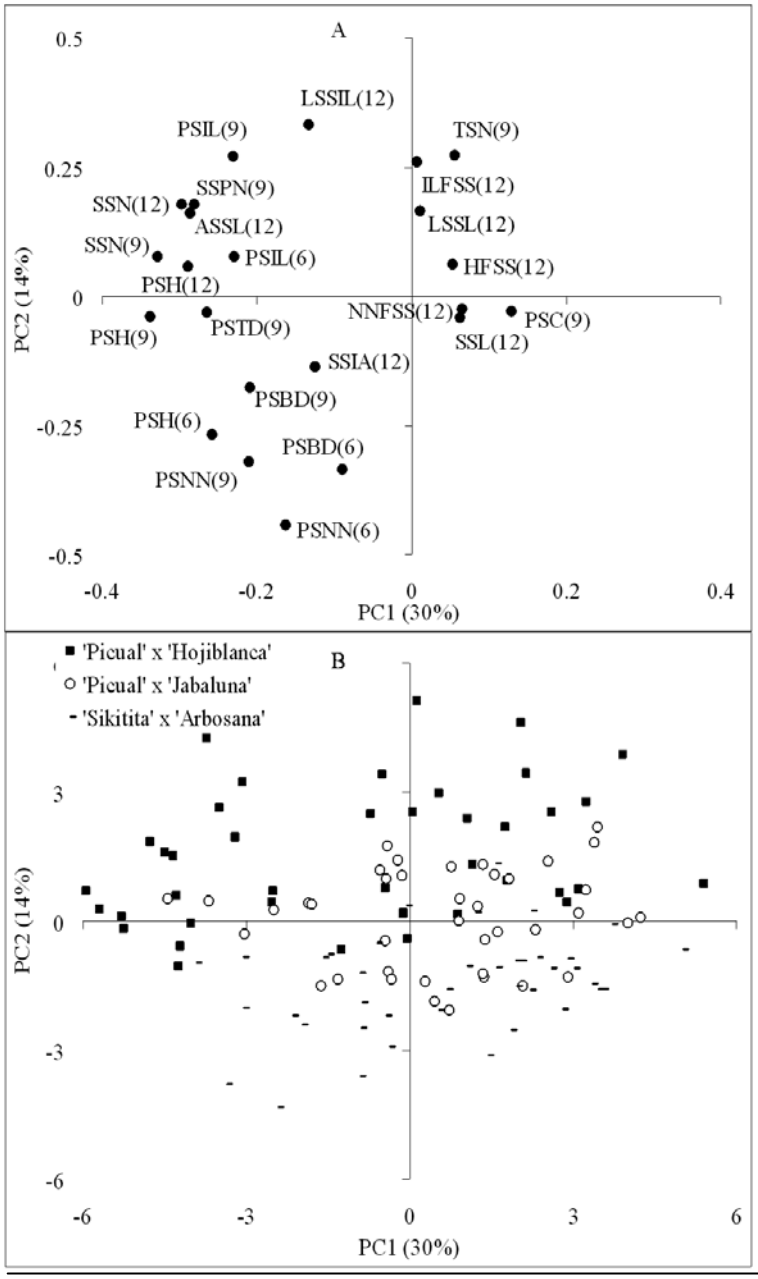

Fig.1.4 Principal Component Analysis for principal and secondary parameters values at different times. Parameters are abbreviated as follows: Pr Sht Ht (PSH), Pr Sht Basal Diam (PSBD), Pr Sht Node No (PSNN), Pr Sht Internode Lgth (PSIL), Pr Sht Top Diam (PSTD), Pr Sht Conicity (PSC), Sec Sht No (SSN), Sec Sht per Node (SSPN), Ter Sht Number (TSN), Accm Sec Sht Lgth (ASSL), Sec Sht Lgth (SSL), Sec Sht Insert Angle (SSIA), Longest Sec Sht Lgth (LSSL), Longest Sec Sht Internode Lgth (LSSIL), Ht to 1st Sec Sht (HFSS), Node No to 1st Sec Sht (NNFSS) and Internode Lgth to 1st Sec Sht (ILFSS), and (age in months). Parameters loading plot is shown in A, and seedlings score plot by cross is presented in B.

II.1.5. Discussion

II.1.5.1. Parent genotype influence

With the exception of Sec Sht Lgth, consistent differences among crosses were found in all principal and secondary growth habit parameters, indicating that olive seedling early growth habit has a significant genetic component. This has been reported for other fruit crops (Conner et al., 1998; Kim et al., 2003; Liebhard et al., 2003; Segura et al., 2007). However, higher differences among individuals than among crosses were observed for these parameters.

For example, clear differences among crosses were showed for Pr Sht Ht and Internodes Lgth, but more than 75% of their variances were attributed to within-cross differences. This is similar to results of previous studies for growth habit traits in prune (DeBuse et al., 2005), walnut (Rezaee et al., 2009), and also to fruit and oil characteristics in olive (León et al., 2004) for which most of the phenotypic variance is attributable to differences among seedlings within rather than among crosses.

Vigorous early growth has usually been considered a favourable seedling trait for obtaining a short juvenile period in fruit tree species such as pear (Visser et al., 1976), apple (Visser et al., 1976), plum (Hartmann and Engelhorn, 1992), pecan (Thompson and Grauke, 2003) and recently in olive tree (De la Rosa et al., 2006; Rallo et al., 2008a). Seedling vigour, apart from its evaluation by Pr Sht Ht, is also frequently estimated by Pr Sht Diam. Unfortunately, however, little attention is generally given to the height at which the diameter measurements to estimate plant vigour are carried out. In this study Top Diam (measured at 1 m height), in contrast to Basal Diam (measured at 5 cm height), showed a high correlation with Pr Sht Ht. Furthermore, Top Diam showed a higher sum of squares percentage attributed to the crosses effect than did Basal Diam, which could indicate a stronger genetic effect on the top measurement. Taken together these results indicate that Pr Sht Diam measured at 1 m height (Top) could be a better estimate of plant vigour than when measured at the more standard 5 cm height (Basal).

Those crosses with ‘Picual’ as a genitor had significantly higher values for most growth parameters than those with 'Frantoio'. That difference in magnitude is confirmed by the PCA analysis when analysing the loadings and scores plots of the two first components. These results agree with previous studies which found

that 'Picual' transmitted a shorter juvenile period to descendants than did 'Frantoio' (Santos-Antunes et al., 2005), as short juvenile period and high seedling vigour seem to be positively related (De la Rosa et al., 2006). It is also worthy to note that the cross between 'Sikitita' and 'Arbosana', two cultivars characterized by low vigour as adult trees (Rallo et al., 2008a; Del Río et al., 2005), produced progeny with vigorous growth at the seedling stage, a result which coincides with the lack of correlation found between vigour measured at seedling and adult stages by León et al. (2007). All these observation indicate that 'Picual', 'Sikitita' and 'Arbosana' can be recommended as genitors in olive breeding programmes as they transmit initial high vigour, a desirable growth habit trait (De la Rosa et al., 2006), to their offspring.

'Sikitita' x 'Arbosana', 'Picual' x 'Hojiblanca' and 'Picual' x 'Jabaluna', the crosses selected to explore the secondary parameters related to branching growth, showed clear differences for the majority of those parameters. 'Picual' x 'Hojiblanca' descendants showed a higher tendency to branching. Abundant lateral growth is undesirable due to the high cost of pruning required for its control, particularly in seedlings (Santos-Antunes et al., 2005; Carrillo-Mendoza et al., 2010). Therefore the use 'Hojiblanca' as a parent is not recommended in olive breeding, because of its tendency to transfer high lateral shoot number to its descendents.

The weeping habit is considered a desirable growth habit aspect for high planting density due to the compact and reduced canopy volume that it confers to the plant (Rallo et al., 2008b). Lateral branch angle indicated a greater tendency to a weeping habit in Sikitita' x 'Arbosana' descendants compared with the other crosses. Indeed, both genitors have a weeping habit (Rallo et al., 2008b), suggesting the heritability of this feature. This result suggested the possibility of using both cultivars in breeding to obtain genotypes with a weeping habit.

II.1.5.2. Plant age influence

The influence of plant age at the time of measurement on seedling growth habit parameters has been observed in other woody species (De Wit et al., 2004; Osario et al., 2003; Legave et al., 2006; Ledbetter and Sisterson, 2008). Our results confirm that in olive seedlings as well, the variability and significance of early

growth habit and vigour traits clearly depend on the moment of evaluation. In fact, for the majority of the parameters, a higher proportion of variability was attributable to differences among crosses at 9 months than at 6 (average Pr Sht Ht of 99.6 and 31.3 cm respectively), that is the parent genotype effect on growth habit was more clearly observed at the later plant age. Similar results have been observed in pear, with differences among crosses in vigour parameters more evident at two years than in one-year-old seedlings (Hjeltnes, 1988).

In different olive breeding programmes the optimal time to transplant the plants to the field has varied from an average height of 30 cm, corresponding approximately to 6 months plant age (Lavee et al., 1996) to 12 months (Santos-Antunes et al., 2005). In our results, vigour at 9 months age (average height of 99.6 cm) is different from that observed at 3 months (average height of 31.3 cm), but similar to that manifested later at 12 months (average height of 133 cm). This result suggests that 3 months is too soon to evaluate olive seedling vigour, while 9 months it is an appropriate time for evaluation.

The observed influence of plant age on vigour characters could explain some of the contradictions among previously reported results concerning the correlation between olive seedling height and juvenile period duration. De la Rosa et al. (2006) and Rallo et al. (2008a) found a clear-cut relationship between these two parameters but Pritsa et al. (2003) did not, probably due to the different ages at which seedlings were evaluated. The action of different sets of genes regulating growth at different ages has been suggested as a possible the basis for the differences in growth habit values at different times (Hodge and White, 1992; Jansson et al., 2005).

We did not observe an age effect on Sec Sht No. While 6 months was too early in development to conduct lateral shoot growth evaluation, a high correlation was found between values at 9 and 12 months. It remains to be tested whether differences in this parameter will occur as plant growth continues beyond 12 months.

II.1.5.3. Parameter relevance

Two criteria could be used to assess the relevance of the different parameters for describing the growth habit of olive progeny. In the first case a

greater relevance can attributed to parameters with a higher capacity for differentiating among crosses, what can be considered as an estimation of the parent genotype effect. Based on the 9 month values, the principal parameters with the highest percentages of sums of square attributable to differences among crosses are Pr Sht Internode Lgth, Pr Sht Top Diam, Pr Sht Conicity, Sec Sht No and Sec Sht per Node, and the secondary parameters Sec Sht Insert Angle and Longest Sec Sht Internode Lgth.

A second criterion, based on the principal component analysis results, uses the degrees of correlation among parameters, and was applied to those parameters which showed a high parent genotype effect according to the first criterion. For highly correlated parameters, those with a higher capacity for differentiating among crosses and fewer difficulties in their evaluation could be considered to have greater relevance or utility. Following this procedure, Sec Sht No and Sec Sht per Node are highly correlated, so the first one could be chosen due to its less tedious evaluation. Similarly, a strong positive correlation was shown between Pr Sht Internode Lgth and Longest Sec Sht Internode Lgth, so the second, Longest Sec Sht Internode Lgth is chosen as more relevant due its higher percentage of sum of squares attributed to differences among crosses.

As a result of this process five parameters of those studied were considered the most relevant for describing the growth habit of olive seedlings. These were Pr Sht Top Diam, Pr Sht Conicity and Sec Sht No, from the principal parameters, and Sec Sht Insert Angle and Longest Sec Sht Internode Lgth from the secondary parameters. In evaluating apple progenies Segura et al. (2006) found high heritability in some of the same parameters, shoot internode length, lateral shoot number and shoot conicity, and thus proposed them as good parameters for evaluating growth habit in breeding.

The primary shoot height is considered as a good early selection criteria for short juvenile period in olive seedlings (De la Rosa et al., 2006; Rallo et al., 2008). The present study, however, did not indicate that the Pr Sht Ht is a relevant parameter because the parent genotype effect was only moderate. But this parameter was strongly correlated with the Pr Sht Top Diam, a relevant parameter, so that the use of the more relevant Pr Sht Top Diam can also provide a good estimation of Pr Sht Ht.

II.1.6. Conclusions

In conclusion we observed a clear effect of both parent genotype and plant age on the vigour and growth habit of olive seedling. The influence of parent genotype could be used in breeding to promote desirable growth habit traits, such as high early vigour, low number of lateral shoots and weeping habit, by parent selection. In this sense 'Picual', 'Arbosana' and Sikitita' seem to be the most interesting cultivars, of those used in the present study, for use as parents in olive breeding. On the other hand, seedling evaluation at 9 months of plant growth in greenhouse conditions, with an average height of 99.6 cm, seems to be an adequate and appropriate moment for the evaluation of primary shoots and their lateral growth. Finally, Pr Sht Top Diam, Pr Sht Conicity, Sec Sht No, Sec Sht Insert Angle and Longest Sec Sht Internode Lgth were considered the parameters that best reflect the genetic variability of olive seedling growth habit, and which thus could be useful to enhance evaluation process, as suggested based on their influence by parent genotype and the lack of correlation among them.

II.1.7. Acknowledgements

This evaluation was performed with plant material from the olive breeding program carried out jointly by the University of Córdoba, Spain and the IFAPA, Junta de Andalucia, Spain. The work was partly funded by project RTA2008-00033-C02-01, National Institute of Agricultural Research, Spain, partially funded by European Social Fund and represents part of a Masters Thesis by S. B. M. H. in Olivicultura and Eleotecnia, for which he was supported by a scholarship from the International Olive Oil Council.

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II.2. Capítulo 2:

Reliable and relevant qualitative descriptors for evaluating complex architectural traits in olive progenies

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(Submitted to Scientia Horticulturae February 2012)

II.2.1. Abstract

Architectural characteristics play an important role in the agronomic performance of fruit tree genotypes. However quantifying such traits in large numbers of individuals represents an important challenge, and little is known regarding their diversity and inheritance. This study evaluates the occurrence, variability, relevance and robustness of visual descriptors, which we propose and test for assessing ten major plant architectural traits in a large number of young olive seedlings and their parents. Our results revealed high phenotypic plant architecture diversity in the studied 825 seedlings from directed crosses, as well as significant parent genotype influence. From ten initially proposed traits, five ('Main vertical axis', 'Preferential distribution of lateral shoots', 'Dominant length of lateral shoots', 'Branch orientation' and 'Branch bending') were found to have the most relevant descriptors for olive seedling architecture based on the high capacity to indicate diversity, strong influence of parent genotype, lack of correlation with each other, and demonstrated value for agronomical performance. All of the descriptors of these five most relevant traits were then combined to generate 105 plant phenotypes, eight of which predominated and showed a clear dependence on parental characteristics. Validity of the results obtained from visual evaluation was verified by their correspondence to quantitative measurements at different stages of analysis. The synthetic characterization of plant-form provides new insights regarding olive seedling description, variability, and parent genotype influence, and represents a significant advance for measuring complex plant architectural traits.

II.2.2. Introduction

Tree architecture describes plant form by defining the spatial organization of the different plant structural components (Godin et al., 1999). This structural organization may significantly influence the agronomic performance of fruit tree genotypes, such as by affecting the fruit-bearing habit, tree size and form, light penetration and capture by the canopy and water transport pathways (Costes et al., 2006). Breeders have recently started using architectural traits for the selection of new tree fruit varieties with increased productivity and reduced management costs (Laurens et al., 2000; Costes et al., 2004). A better knowledge of the genetic variability of architectural traits, the relationships among them, and their inheritance is essential to achieving this objective (Wang and Li, 2006; Segura et al., 2009).

Significant insights regarding the genetic diversity of perennial plant architectural organization have been provided by the descriptive models, largely botanical and morphological in their focus, used to catalogue the general architectural variation among plant species (Hallé and Oldeman, 1970; Hallé et al., 1978; Barthélémy and Caraglio, 2007; Bell and Bryan, 2008). However, intra-specific variability, that which is of greatest interest for breeding purposes, has been little studied for architectural traits due to multiple methodological difficulties, none the least of which are the details involved and the multiple levels of organization at which they occur. In fact, current researchers suggest that finding rapid methods to systematically evaluate the complex branching systems of perennial plants in large numbers of individuals is one of the major challenges for breeding progress in this area (Laurens et al., 2000; Costes et al., 2006; Segura et al., 2008).

During recent decades, significant progress has been achieved in developing mathematical models, analytical software and image analysis tools to make the complexity of perennial plant architecture more legible (Costes et al., 2006; Guo et al., 2011). These tools have advanced our understanding of the involvement of morphogenetic processes in whole-plant organization, the role of structure in interaction with abiotic factors, the quantification of genetically based variability, and the simulation of plant growth (Barthélémy and Caraglio, 2007; DeJong et al., 2011). These methods for evaluating architectural variability, however, can be limited by the requirement of large amounts of quantitative data in order to do so.

Olive, *Olea europaea* L., is an example of a tree-fruit crop in which the analysis of the phenotypic variability of architectural traits and the influence of parent genotype have advanced rapidly in recent years (Pritsa et al., 2003; P. Rallo et al., 2008; Hammami et al., 2011). These studies in olive, similar to the majority of those in other species (Apple: De Wit et al., 2002; Segura et al., 2006; Peach: Carrillo-Mendoza et al., 2010; Walnut: Rezaee et al., 2009), have been conducted using young progenies, which provide certain methodological advantages. First of all, the use of one to two-year-old plants is sooner and facilitates evaluation due to the relative simplicity of their branching system in comparison with mature trees (Segura et al., 2006), while their development is sufficiently advanced to appreciate the inheritance and the genetic variability of growth habit parameters (Hjeltnes, 1988; Segura et al., 2006; Hammami et al., 2011). Secondly, in heterozygote species such as the olive tree the use of seedling populations produced by directed crosses provides good indications regarding the genetic variability within the species (Brown and Caligari, 2008). In many of these studies, however, analysis has mainly been limited to easily measured traits such as tree size and the dimensions and number of the different axes, and a few qualitative traits such as axis form.

Tree architecture can be evaluated using qualitative visual criteria based on morphological and botanical concepts (Edelin 1991; Lespinasse, 1992; Segura et al., 2009). Visual criteria can be very useful in assessing plant form in high numbers of genotypes (Segura et al., 2009) and often integrate multiple information to provide a simplified representation of whole-plant architecture variation (Lespinasse, 1992). However, visual criteria can present some disadvantages. The robustness of qualitative data has frequently been questioned, and their subsequent analysis is difficult (Hansche et al., 1972a). These impediments can be resolved by the use of powerful and adapted statistical procedures for this type of data, and by comparison with the quantitative evaluation of a subsample of the studied population (Patton, 2002; Sofaer, 2002). In addition, the vague or highly complicated definitions sometimes found in describing complex plant form traits contribute to increased error and reduced credibility of collected data (Caraglio and Barthélémy, 1997). Defining simple and concise descriptor for each complex trait is essential for minimizing these difficulties, and can furthermore provide a framework for using quantitative measurements for their verification.

This study evaluates the occurrence, variability, relevance and robustness of visual descriptors, which we propose to assess ten major plant architectural traits in a

large number of young olive seedlings and their parents. We initially define and evaluate ten qualitative traits, each of which with two to four morphologically-based descriptors. Following evaluation of the seedling population, four different criteria were used to identify the traits with the most relevant descriptors: 1) low correlation among traits; 2) highly informative regarding diversity (based on polymorphic information content); 3) strong parent genotype influence; 4) Preferential choice of traits with demonstrated value for agronomical performance. Then, for the identified traits, descriptor robustness was tested against quantitative measurements. Our results simplify, validate and advance the use of visually-based morphological criteria to evaluate complex architectural traits previously not considered in fruit tree breeding programs.

II.2.3. Materials and methods

II.3.1. Plant Material

We evaluated 825 olive seedlings from six different crosses: 'Sikitita' x 'Arbosana' (136 plants), 'Picual' x 'Hojiblanca' (76 plants), 'Picual' x 'Jabaluna' (132 plants), 'Frantoio' x 'Arbosana' (189 plants), 'Frantoio' x 'Manzanilla' (131 plants), 'Frantoio' in open pollination (161 plants). We also assessed 12 young plants (from cuttings) of each parent cultivar, with the exception of 'Jabaluna' due to the unavailability of adequate material. The procedures for crossing, seed germination and initial seedling growth have been reported previously by Hammami et al. (2011).

II.2.3.2. Definition and evaluation of traits

Ten initial plant-form traits were chosen and evaluated in all 825 seedlings and their parents at one year old. Each trait consisted of two to four simply and concisely defined descriptors based on morphological concepts (Table 2.1), used to characterize each plant by the choice of one descriptor per trait. The traits and their descriptors were chosen based on a combination of descriptions present in published reports and preliminary visual impressions of the morphology of the olive seedlings. In order to simplify nomenclature, as the architecture of young plants consists of relatively few degrees of branching, the terms "main shoot" and "lateral shoots" (LS) were used to refer to the first order (trunk) and secondary shoots (branches), respectively, similar to the nomenclature adopted by De Wit et al. (2002). To minimize the human error involved in visual evaluation, descriptor choices were carried out separately by two

Table 2.1 Descriptions and reference sources for the ten initial plant-form traits and the descriptors used to characterize them. Each olive seedling is described by the choice of one descriptor per trait. The main shoot(s) and lateral shoots (LS) refer to the first order (trunk) and the secondary shoots, respectively. Definitions and nomenclature of the traits and descriptors were based either mainly or partially on the cited references.

Trait	Descriptors	Definition	Reference
Main vertical axis	Mono-axis	Only one vigorous main shoot present	(De Wit et al., 2002; Pritsa et al., 2003)
	Multi-axis	Two or more vigorous main shoots	
	No main axis	No well-developed or vertically oriented main shoot	
Branching rhythm	Continuous	All nodes of the main shoot form at least 1 branch	(Robinson, 1996; Barthélémy and Caraglio, 2007)
	Rhythmic	Regular alternation of branched and unbranched nodes of the main shoot	
	Diffuse	A part of nodes are branched, but without a regular pattern of distribution along the main shoot	
Node sprouting	Single	Only one shoot is developed per branched node of the main shoot	(Bell and Bryan, 2008; Hammami et al., 2011)
	Paired	Two shoots are developed per branched node of main shoot	
	Mixed	One or two shoots are developed per branched node of the main shoot	
Branching system	Simple	Branching does not exceed the tertiary order	(Barthélémy and Caraglio, 2007; Bell and Bryan, 2008)
	Complex	Branch order exceeds the tertiary order	
Preferential distribution of lateral shoots	Basitony	Basal shoots on the main shoot are clearly longer and more vigorous than the rest of the shoots	(Bell and Bryan, 2008; Barthélémy and Caraglio, 2007)
	Mesotony	Central shoots on the main shoot are clearly longer and more vigorous than the rest of the shoots	
	Acrotony	Distal shoots on the main shoot are clearly longer and more vigorous than the rest of the shoots	
	No preference	No clear preferential zone along the main shoot with vigorously growing shoots	
Unbranched zone	Basal zone	No shoots in the basal zone of the main shoot	(Courbet et al., 2007; Segura et al., 2006)
	Central zone	No shoots in the central zone of the main shoot	
	Distal zone	No shoots in distal zone of main shoot	
	Absent	Shoots present in all zones along the main shoot	
Dominant length of lateral shoots	Short	LS Length < 10 cm	(Hammami et al., 2011; Castillo-Llanque and Rapoport, 2011)
	Medium	10 cm < LS length <20 cm	
	Long	LS length > 20 cm	
	Varied	Presence of the 3 previous LS lengths	
Extreme leaf size	Very small	Leaf length < 2 cm	(P. Rallo et al., 2008; Moreno-Alias et al., 2009)
	Extreme absent	2 cm < leaf length < 4 cm	
	Very large	Leaf length > 4 cm	
Branch orientation	Upright	LS growing in vertical direction; orthotropic	(Hallé et al., 1978; Bell and Bryan, 2008)
	Horizontal	LS growing in horizontal direction; plagiotropic	
Branch bending	Bent up	LS are curved upwards	(Costes et al., 2006; Segura et al., 2008)
	Straight	LS uncurved	
	Bent down	LS are curved downwards	

experienced observers, the results were compared, and in the few cases where a difference occurred, the observers returned to the plant and reached a consensus opinion.

Four criteria were used to identify the traits with the most relevant plant-form descriptors among the initially evaluated ten traits. That is, for each group of closely correlated traits we chose the one with the highest PIC value and the greatest variance attributed to cross effect (an indicator of inheritance and genetic control), and also considered the importance of demonstrated value for agronomical performance. The selected traits and their component descriptors were then used to describe the whole plant-form diversity present in the seedling population, and to identify the most frequent architectural types.

The robustness of the five chosen qualitative plant-form descriptors was then tested by comparison with quantitative parameters. Two weeks after the initial observations, quantitative parameters describing the selected relevant traits were defined and measured in a subsample consisting of 100 seedlings chosen randomly within the population. The corresponding qualitative traits and quantitative parameters are shown in Table 2.2. Four of the qualitative traits were assessed with one corresponding quantitative parameter. One particularly complex trait, "Preferential distribution of LS", was best assessed by three quantitative parameters, but a formula integrating the three (SDI; Shoot Distribution Index) was used for the analyses requiring a single parameter (Table 2.2).

II.2.3.3. Data analysis

The associations among the ten qualitative traits and the degree of separation among crosses and parents based on those traits was determined by the Categorical Principal Components Analysis (CATPCA) using IBM SPSS Statistics 19 (IBM Company, New York USA). The significance of the partitioning of total genetic variance of plant-form among and within crosses was carried out by adapting AMOVA (Analysis of Molecular Variance) analysis using GenAlEx 6.4 (Peakall and Smouse, 2006). To perform this statistical procedure for categorical data, a binary matrix of the data was generated (Belaj et al., 2011; Benor et al., 2011). As each seedling can have only one descriptor for a given trait, the existence or absence of each descriptor (per trait) was coded 1 or 0, respectively, to generate this matrix. The informativeness of the employed traits was

also evaluated using methodology more commonly applied in molecular studies, by determining the effective number of descriptors per trait ($N_e = 1/\Sigma P^2_i$, where P_i is the frequency of the *i*th descriptor; Berg and Hamrick, 1997) and the polymorphic information content (PIC) ($PIC_i = 1-\sum P^2_{ij}$, where P_{ij} is the frequency of the *j*th descriptor for the *i*th trait, summed over n descriptors; Weir, 1990).

Table 2.2 Quantitative parameters and the related qualitative traits they were used to assess. The main shoot(s) and lateral shoots (LS) refer to the first order (trunk) and the secondary shoots, respectively.

Quantitative parameter	Measurements and comments	Formula	Qualitative trait assessed
Number of main shoots	All well-developed main shoots on each plant were counted		Main vertical axis
Average length of LS in basal zone[b] (B)	Length of all lateral shoots in the basal zone of the main shoot(s) of each plant		[a] Preferential distribution of LS
Average length of LS in central zone [b](M)	Length of all lateral shoots in the central zone of the main shoot(s) of each plant		[a] Preferential distribution of LS
Average length of LS in distal zone [b] (D)	Length of all lateral shoots in the distal zone of the main shoot(s) of each plant		[a] Preferential distribution of LS
Shoot distribution index (SDI)	α is negative for plants with similar values of B, D and M	SDI= α ((M-D) / B)	[a] Preferential distribution of LS
Median of the LS Length	Determined statically using the length of all lateral shoots in each plant		Dominant Length of LS
Deviation of LS from vertical	Angle of three lateral shoots from the vertical were recorded in each plant		Branch orientation
Bending index (BI)	Chord [c] (C) of three lateral shoots and their length (L) for each plant. α is negative for bent down shoots	BI = α (1- C / L) [d]	Branch bending

[a] Preferential distribution of LS was assessed by the parameters B, M and D for ANOVA analysis, and the formula SDI for all other analyses (Categorical regression, and PCA).
[b] Zones were determined by dividing main shoot(s) length into three equal sections
[c] Chord is the line segment between two points on a given curve, in this case the initial and final ends of each shoot
[d] According to Segura et al., 2008.

The robustness of the five chosen qualitative plant-form traits was tested by comparing them with quantitative parameters using different analytical procedures. 1) Categorical Regression (CATREG) procedure was performed to evaluate the ability of the categorical data to predict the variability of the quantitative parameters measured (Van der Kooij et al., 2006). 2). ANOVA analysis was performed to test the differences between descriptors of each trait and to determine variance components. 3) Finally, Principal Component Analysis (PCA) was carried out for the plants with the most frequent whole-plant form types in the quantitatively evaluated subsample in order to appraise the ability of the quantitative parameters to differentiate these types established by the visual evaluations. All of these analyses were performed using the IBM SPSS Statistics 19 (IBM Company, New York USA).

II.2.4. Results

II.2.4.1. Initial qualitative traits

For all ten traits, the descriptor frequency clearly varied among crosses, but dominant descriptors occurred for many of the traits (Fig.2.1). For example '*mono-axis*' was the predominant main vertical axis type (Fig.2.1a), *'diffuse'* was the major branching rhythm (Fig.2.1b), the predominant branching system was '*simple'* (Fig.2.1d), and the major branch orientation was *'upright'* (Fig.2.1i). Other descriptors were only present at extremely low frequency, such as the absence of a main vertical axis *('no main axis'*; Fig.2.1a), the preferential development of vigorous shoots in the distal zone (*'acrotony'*; Fig.2.1e) and the dominance of *'short'* LS (Fig.2.1g). For the majority of the traits, parent architecture corresponded to the dominant descriptor(s) of their descendants.

The CATPCA of the ten plant-form traits produced two first components (PC1 and PC2), together accounting for 46% of the total variation (Fig.2.2). This analysis showed a clear grouping of parents and their descendants, indicating an evident influence of parents on the architectural characteristics of their descendents. For example, 'Picual' and its descendants ('Picual' x 'Jabaluna' and 'Picual' x 'Hojiblanca') were distinguished by the tendency for more than one main axis (*'multi-axes'*), absence of a preferential distribution of LS (*'no preference'*), and higher proportion of *'medium'* LS length. On the other hand 'Sikitita' and its offspring were characterized by the dominant presence of '*basitony'* and by a strong tendency to produce *'horizontal'* and '*bent down'* LS.

Fig.2.1 Percentage of Descriptors for each plant form trait evaluated in six young olive seedling populations from different crosses: SxA: 'Sikitita' x 'Arbosana', FxA: 'Frantoio' x 'Arbosana', F o.p.: 'Frantoio' in open pollination, FxM: 'Frantoio' x 'Manzanilla', PxH: 'Picual' x 'Hojiblanca', PxJ: 'Picual' x 'Hojiblanca'. Descriptors for parents are indicated by the first letter of their name above the descriptor (for 'Extreme Leaf Size' all parents showed 'Very large' leaves except 'Picual').

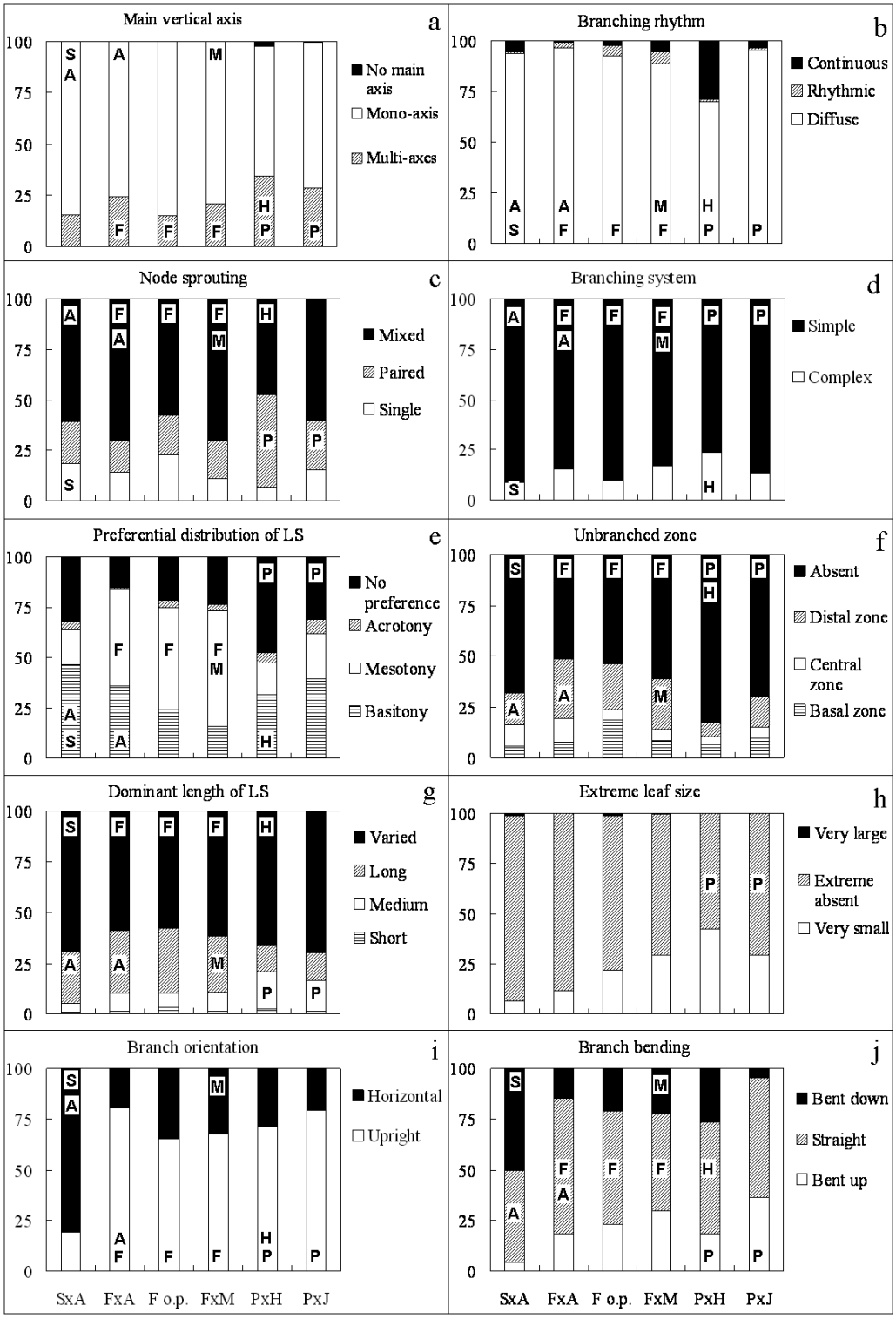
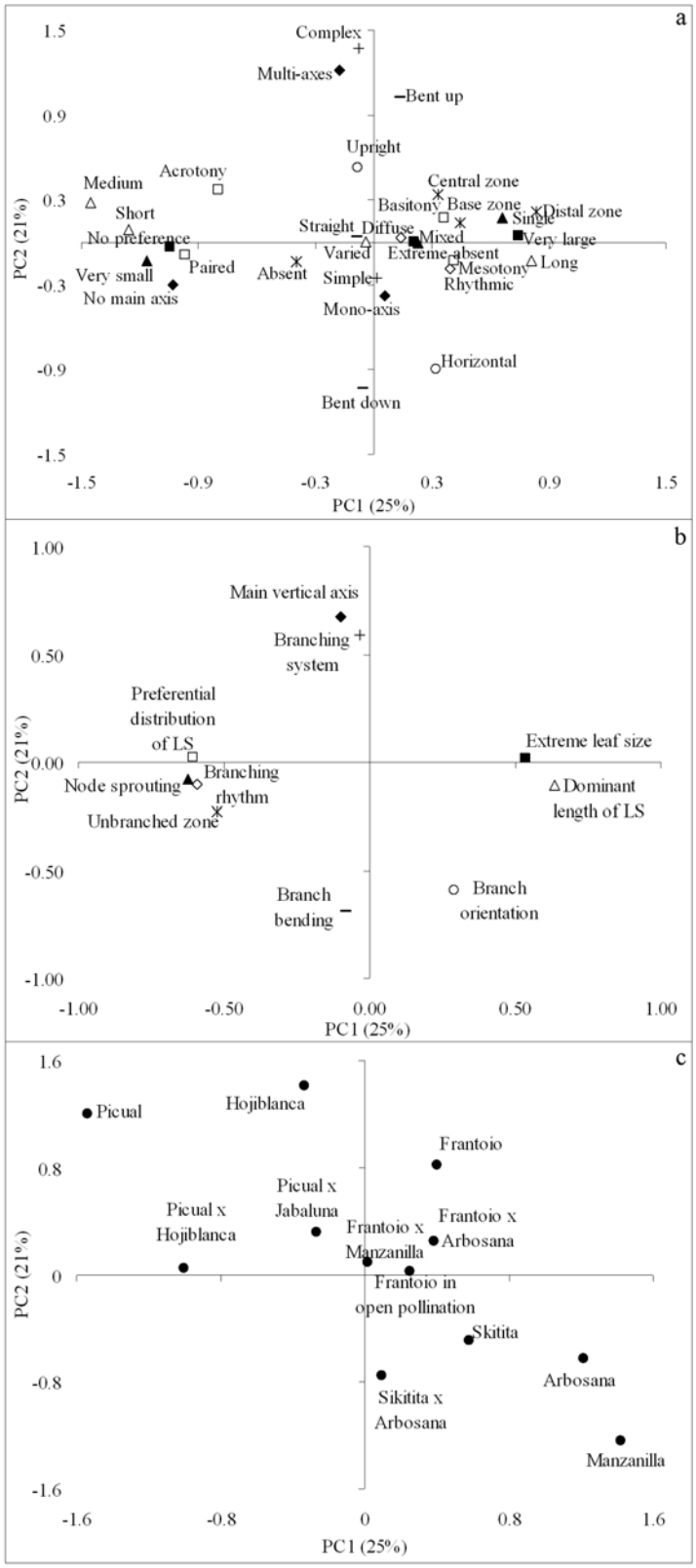


Fig.2.2 Categorical Principal Components Analysis (CATPCA) for the ten plant-form traits evaluated in young offspring of six olive crosses and parent cultivars. a) Descriptors loading plot (descriptors of the same trait are represented by the same symbol). b) Trait loading plot. c) Plot of mean score for each cross (female parent x male parent) and parents.



In addition to the progeny tendencies, the CATPCA revealed three evident groups of correlated architectural traits. The first group included four traits related to LS organization along the trunk (the main shoot): 'Preferential distribution of LS', 'Branching rhythm', 'Node sprouting' and 'Unbranched zone'. In the second group 'Main vertical axis' and 'Branching system' were highly associated, mainly due to the strong relationships found between their component descriptors *'mono-axis'* and *'simple'*, and *'multi-axes'* and *'complex'*. The third group was composed of the traits that describe shoot and leaf organ sizes ('Dominant length of LS' and 'Extreme leaf size'). In contrast, the two traits describing branch form ('Branch bending' and 'Branch orientation') were only slightly associated and did not obviously group together.

All ten plant-form traits were polymorphic, having from 2 to 4 descriptors (1.20 to 3.14 effective descriptors) that express the multiple forms in the studied population (Table 2.3). Based on these traits and their component descriptors, 455 different architectural phenotypes were found among the 825 olive seedlings. A wide range was found in the PIC value, being 'Branching rhythm' (PIC 0.16) and 'Branching system' (PIC 0.22) the least informative traits for plant-form diversity, while 'Preferential distribution of LS' (PIC 0.62) and 'Branch bending' (PIC 0.52) the most informative.

Table 2.3 Total number of descriptors (N_c), effective number of descriptors (N_e) and the polymorphic information content (PIC) for the ten initial plant-form traits evaluated in 825 olive seedlings from six crosses.

Trait	N_c	N_e	PIC
Main vertical axis	3	1.58	0.32
Branching rhythm	3	1.20	0.16
Node sprouting	3	2.16	0.48
Branching system	2	1.32	0.22
Preferential distribution of LS	4	3.14	0.62
Unbranched zone	4	2.26	0.51
Dominant length of LS	4	2.06	0.46
Extreme leaf size	3	1.52	0.28
Branch orientation	2	1.84	0.37
Branch bending	3	2.44	0.52
Mean	3.1	1.96	0.40

AMOVA analysis indicated that most of the phenotypic plant-form variability was due to differences within crosses rather than among them, although significant

differences among crosses (P<0.05, obtained after 999 permutations) were obtained in all evaluated traits. The degree of parental influence, indicated by the variance components, varied widely among traits, ranging from 1% of the total variance for 'Branching system', to 22 % for 'Branch orientation'. Together with 'Branch orientation', 'Branching rhythm' (8%), 'Preferential distribution of LS' (8%), 'Branch bending' (6%), 'Dominant length of LS' (5%) and 'Extreme leaf size' (5%) showed the highest percentages of parental influence in the total variance.

II.2.4.2. Most relevant qualitative traits and their quantitative assessment

'Main vertical axis', 'Preferential distribution of LS', 'Dominant length of LS' , 'Branch orientation', and 'Branch bending' were chosen as the traits with the most relevant plant-form descriptors. The qualitative descriptors of these five traits were tested against related quantitative parameters (Table 2.4) by categorical regression, which showed a strong and highly significant capacity of these qualitative descriptors to predict the variability of the quantitative parameters in the olive seedling subsample (Table 2.4). The lowest R^2 value (0.71) was found between the 'Dominant length of LS' (qualitative) and the 'Median LS length' (quantitative), and the highest R^2 (0.83) between 'Main vertical axis' (qualitative) and the 'Number of main shoots' (quantitative).

ANOVA indicated that most of the variance of the quantitative evaluation was found among rather than within the descriptors of each trait (Table 2.5). The different descriptors were all well defined by the measurement values, confirming the close associations between qualitative traits and quantitative parameters. For example, longer LS were found in the basal, central and distal zones of the basitonic, mesotonic and acrotonic plants respectively, while no significant LS length differences were observed among the different branching zones of the *'no preference'* descriptor. Similarly, LS have an acute angle with the vertical for plant of the descriptor *'upright'* orientation, but that angle was significantly greater and practically perpendicular (90º) for plants with the descriptor '*horizontal*' orientation.

II.2.4.3. Whole-plant architectural types

The qualitative results, i.e. the visual descriptor decision for each trait, of the most relevant traits were integrated to explore the whole-plant architectural variability

Table 2.4 Summary of Categorical Regression Model to evaluate the ability of qualitative traits (Predictor) in estimate differences in the architectural features assessed by quantitative parameters (Predict). LS is Lateral Shoots. Evolution used a 100 plants subsample of the initial olive seedling population.

		Parameter				
Predictor		Main vertical axis	Preferential distribution of LS	Dominant length of LS	Branch orientation	Branch bending
Predict		Number of main shoots	Shoot distribution index	Median of the LS length	Deviation of LS from vertical	Bending index
Model information	R^2	0.83	0.81	0.71	0.78	0.81
	Apparent prediction error	0.18	0.20	0.30	0.23	0.20
	F [a]	403.95***	140.63***	75.55***	336.10***	193.17***

[a] F-statistics used for the test significance
***Significant at $P<0.001$

Table 2.5 Comparison of means of quantitative parameters among descriptors of 'Main vertical axis', 'Dominant length of LS', 'Branch orientation' and 'Bending index' traits and among the three branching zones (BZ) used to quantify each descriptor of 'Preferential distribution of LS'. The percentages of sums of squares of the correspondent ANOVA between and within descriptors (or BZ) are also indicated. Different letters within columns indicate significant differences between means of descriptors (or BZ) at $P< 0.01$.

Descriptor		Number of main shoots [a]	BZ	Average length of LS per zone, for plants of each Descriptor (cm) [b]				Descriptor	Median of LS length (cm) [b]	Descriptor	Deviation of LS from vertical (°) [a]	Descriptor	Bending index [b]
				Basitony	Mesotony	Acrotony	No preference						
Mono-axis		1.03 b	Basal zone	28.31 a	15.80 b	15.71 b	15.09 a	Short	5.83 d	Upright	38.15 a	Bent down	-0.13 c
Multi-axes		2.27 a	Central zone	12.98 b	27.78 a	12.03 b	13.54 a	Medium	14.07 c	Horizontal	84.28 b	Straight	0.05 b
No main axis		-	Distal zone	7.47 c	7.51 c	34.49 a	12.95 a	Long	46.00 a			Bent up	0.18 a
								Varied	22.04 b				
Sum of Squares (%)	Descriptor or BZ	78		59	55	56	2		73		77		89
	Error	22		41	45	45	98		27		23		11

[a] Differences between means was tested using a t test for unequal variance

[b] Differences between means was tested using Games-Howell test for unequal variance

in the olive progenies. By combining the descriptors of the five most relevant qualitative traits finally selected, 105 phenotypes were found among the 825 offspring of the six different crosses, in contrast to 455 phenotypes when all ten initial traits were used. Furthermore, a large proportion of the plants, almost half, presented only eight principal phenotypes, drawn schematically in Fig.2.3. The frequency of these types varied from 11% for Type 1, characterized by one vertical axis with vigorous LS in the central zone (*'mesotony'*), a great variation in the length (*'varied'*) of LS which grow vertically (*'upright'*) and uncurved (*'straight'*), to 3.2% for Type 8, which differed from Type 1 by *'no preference'* in the distribution of LS, which are *'horizontal'* and *'bent down'*. Type 7 was the only frequent type which had multiple main axes (*'multi-axes'*) and LS *'bent up'*, while LS *'long'* were only found for Type 6.

The frequency of the eight plant-form types varied greatly among crosses, but a similar trend occurred among those with the same parents, indicating their inheritance (Fig.2.4). For example, similar to their parents, 'Sikitita' x 'Arbosana' descendants showed a greater tendency to plant-form Type 3, which is distinguished from the other types by combining *'basitony'* with *'horizontal'* branch orientation. In contrast, 'Frantoio' descendants tended to Type 1 and 6, integrating *'mesotony'* and *'upright'* branch orientation, characteristic of their mother. 'Picual' is characterized by*'upright'* LS with *'no preference',* and *'multi-axes'*, and its descendants tended to Types 4 and 7.

Principal Component Analysis of the quantitative parameters for the subsample plants with the eight most frequent plant-form types gave two first components (PC1 and 2) accounting for 70% of the total variation (Fig.2.5). The evaluated seedlings were clearly grouped according their plant-form types (Fig.2.5). The separation among these groups confirms the differences in the architectural features reported by the visual evaluations.

II.2.5. Discussion

II.2.5.1. Olive seedling architecture: tendency and parent genotype influence

The initial evaluation of ten architectural traits revealed high diversity in the olive seedling population, identifying 455 phenotypes (combining descriptors) within the 825 genotypes tested. Various traits, however, clearly presented dominant descriptors, which provide insights regarding olive tree architecture. The seedlings showed a strong tendency to one main vertical axis (*'mono-axis'*) and *'simple'* branched system. Along

Fig.2.3 Schematic representation of the most frequent whole plant-form types for young olive seedlings, integrating the most frequent descriptor(s) of five traits: (a) 'Main vertical axis', (b) 'Preferential distribution of LS', (c) 'Dominant length of LS', (d) 'Branch orientation', and (e) and 'Branch bending'. The percentage of each architectural type in the total population is indicated under each drawing.

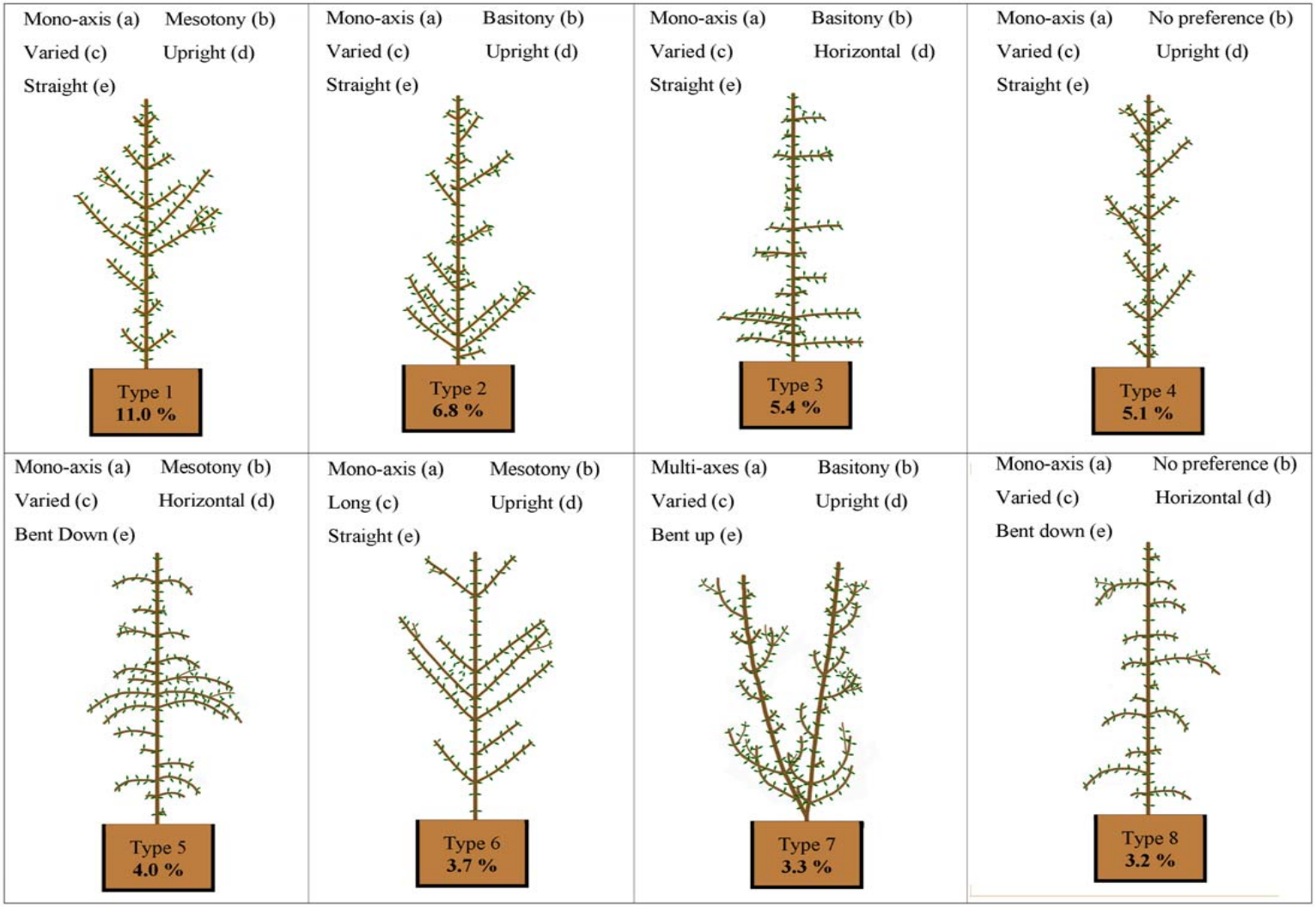


Fig.2.4 Percentage of the most frequent whole plant-form types among 825 olive seedlings from six crosses. Crosses are abbreviated as: SxA: 'Si kitita' x 'Arbosana', FxA: 'Frantoio' x 'Arbosana', F: 'Frantoio' in open pollination, FxM: 'Frantoio' x 'Manzanilla', PxH: 'Picual' x 'Hojiblanca',

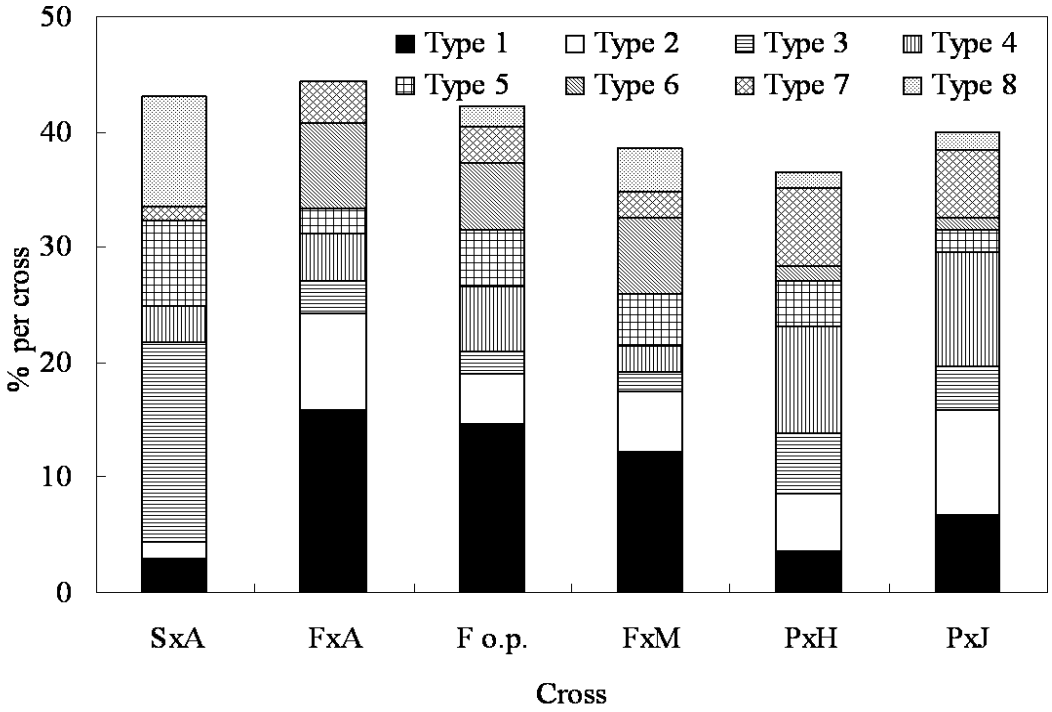
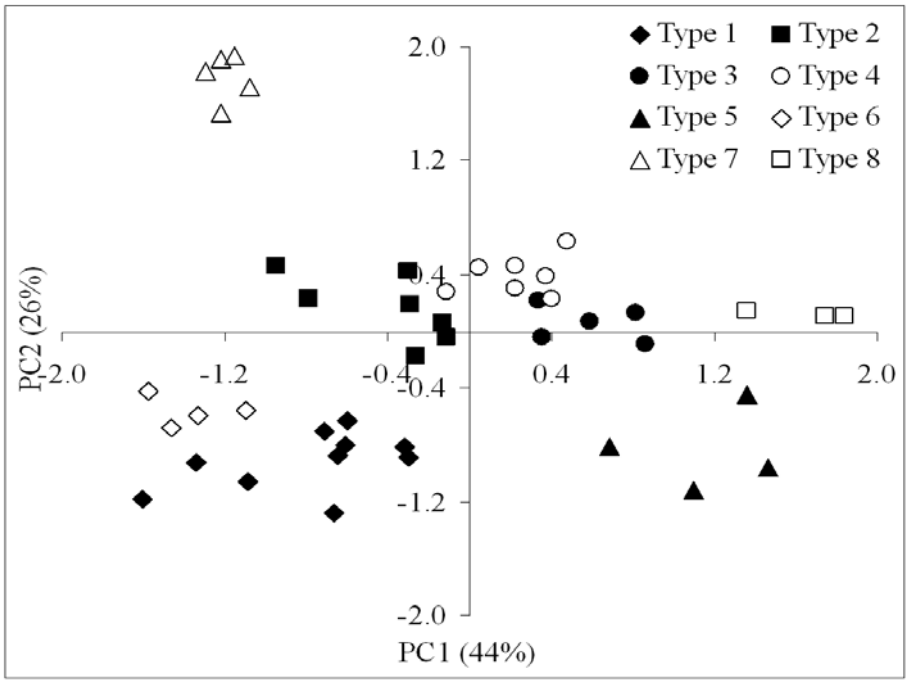


Fig.2.5 Principal Component Analysis (PCA) for quantitative parameters in olive seedlings in relation to plant-form type. Analysis is based on the 46 seedlings, in the quantitatively measured subsample group of 100, which exhibited the eight principal plant-form types. LS are the abbreviation of Lateral Shoots.



with *'mono-axis',* however, '*basitony'* predominated in a large number of the young seedlings, and could possibly indicate genotypes which lose their mono-axial nature as the plants grow and develop. The development of dominant and vigorous shoots in the distal zone (*'acrotony'*), observed only in few seedlings, is considered fundamental in allowing the formation of a trunk (Crabbé, 1987), while the development of vigorous basal shoots (*'basitony'*) is responsible of the formation of a bushy habit (Barnola and Crabbé, 1991). Gucci and Cantini (2000) observed a definite tendency towards bushy growth in mature unpruned olive trees, which could be a reflection of strong *'basitony'* although it contrasts with the dominance of *'monoaxis'* in seedlings.

The major part of the plant-form diversity occurred within rather than among crosses. These results follow the same trend as those obtained for other morphological and agronomical traits studied in different olive seedling populations (León et al., 2004; Hammami et al., 2011; Belaj et al., 2011) as well as in other species (Prune: DeBuse et al., 2005; Walnut: Rezaee et al., 2009). However although less than within the crosses a significant influence of parent genotype was also shown for all plant-form traits.

At this early age the traits 'Branch orientation', 'Branch bending', 'Preferential distribution of LS', and 'Branching rhythm' all presented a significant parent genotype influence, suggesting a important genetic component determining these architectural traits. The CATPCA analysis also indicated a clear separation among crosses, with those with the same parent showing marked similarities among themselves and with their parents. Segura et al. (2006), evaluating plant architecture quantitatively in young apple tree offspring, have reported the significant heritability and genetic control of lateral branch bending and angle of insertion. Our findings demonstrate the possibility of evaluating genetically based architectural diversity in olive at very early seedling stage.

For the other parent cultivars, however, the knowledge regarding adult tree architecture features is principally limited to their vigor (low to high) or by global habit (Lauri et al., 2001). In breeding programs it is essential to determine the influence of parent cultivars on the architectural features of their descendants, and to identify new potential parent genotypes. In fact the most frequent plant-form types showed clear parent genotype influence. 'Skitita' and 'Arbsoana' parent

cultivars are characterized by bent-down branches (weeping habit) and by the development of one dominant vertical trunk (monocone or mono-axis), characteristics apparently transmitted to their descendants as plant-form types 5 and 8. These features are considered desirable in the selection of new cultivars for high planting density due to the reduced tree size that they confer (Preziosi et al., 1994; L. Rallo et al., 2008). 'Picual' as female parent contributes strongly to plant-form type 4, characterized by a *'mono-axis'*, *'upright'* LS, and *'no preference'* in LS distribution. This type is very similar to the well-known columnar habit in apple and peach considered highly applicable for high density plantations in those species due to its compact lateral growth (Miller and Scorza, 2002; Costes et al., 2004). In contrast, 'Picual' as female parent also tends to produce a high frequency of genotypes with type 7, characterized by the presence of various dominant vertical axes (*'multi-axes'*) and basitonic growth, both potentially inconvenient for the monocone formation usual in hedgerow orchards. The above examples indicate the high value of identifying principal plant-form types in order to facilitate selection for useful plant architectural features.

II.2.5.2. Plant-architecture descriptor relevance

Corresponding with the diversity of research objectives, different strategies have been developed to describe plant architecture, ranging from direct measurements to the construction of virtual plant representations. Independent of the approach which is used, however, it is coherent only when the basic morphological concepts of plant development are taken into account (Godin et al., 1999; Barthélémy and Caraglio, 2007). The present study evaluates the diversity of architectural traits in a large number of olive seedlings, introducing visually simplified and concisely defined morphological criteria. This approach represents a significant advance over the previous methods used in fruit crop breeding programs, which have mainly evaluated seedling architecture using laborious quantitative traits (De Wit et al., 2002; Pritsa et al., 2003; Segura et al., 2006; P. Rallo et al., 2008; Rezaee et al., 2009; Carrillo-Mendoza et al., 2010; Moreno-Alias et al., 2010; Hammami et al., 2011), in both simplifying the evaluation procedure and introducing previously unevaluated traits.

In genetic studies and breeding for plant architecture, two of the most important concerns are which traits should be measured to identify diversity and how they can be chosen (Costes et al., 2004; Segura et al., 2006). In this study four different criteria were used for selecting the most relevant descriptors for plant architecture in olive progenies. The first one consisted in selecting traits little correlated with each other, reducing the number of traits to assess; in this case the unassessed traits are still included indirectly due to their high correlation with those which are assessed (Hansche et al., 1972b; Segura et al., 2006). The second criterion was based on using the PIC value to maximize the information provided about plant-form diversity, similarly as is done for molecular markers (Belaj et al., 2011). The third criterion was the variance attributed to parent genotype effect was used to select the traits most genetically determined and inheritable (León et al., 2004; Hammami et al., 2011). Finally, we preferentially chose traits considered to be relevant for canopy formation and agronomic performance. Based on these criteria 'Main vertical axis', 'Preferential distribution of LS', 'Dominant length of LS', 'Branch orientation' and 'Branch bending' were found to be the traits with the most relevant descriptors. These traits have been identified as having an important physiological and agronomic role in fruit tree crops related to light interception, hydraulic conductance, planting design and density, tree size and fruit-bearing habit (Preziosi et al., 1994; Solar and Štampar, 2003; Costes et al., 2006; Han et al., 2007; Lauri, 2007; L. Rallo et al., 2008).

In previous studies, olive plant architectural features have principally been evaluated and analyzed as separate morphological traits, without considering their integration into whole-plant structure (Pritsa et al., 2003; P. Rallo et al., 2008; Moreno-Alias et al., 2010; Hammami et al., 2011). Integrating that kind of information to define more comprehensive architectural descriptors has permitted us to evaluate whole plant morphological diversity and identify the most frequent plant-architecture types. This approach also provides the possibility of defining architectural types related to specific agronomic goals such as finding genotypes best adapted to different planting density, orchard design and harvesting methods. A final benefit of defining the plant-form types is that their schematic representation can facilitate their prompt identification in genotype screening in breeding programs.

II.2.5.3. Evaluation strategy robustness

The possible extension and applicability of simplified visual approaches in breeding programs has been directly related to the degree of their accuracy in comparison with more precise and consistent but highly laborious quantitative measurements (Riday, 2009; Kim and Tai, 2011). Thus we tested the reliability of our visual criteria in comparison with the quantitative measurements by different statistical procedures. Categorical Regression analysis demonstrated a high ability (R^2 approximately 0.80 for the majority of traits) of the visual traits to predict the quantitative differences in the architectural features in a large number of seedlings. This high predictability was furthermore either similar to or much higher than that found for other, more simple traits frequently appraised visually in breeding programs, such as yield scores (Riday, 2009) and pathogen-produced damage level (Burd et al., 1993). Other quantitative measurements not included here, however, could be difficult to relate with qualitative traits, a difficulty which Segura et al. (2009) found for apple. Further investigation should thus evaluate the possibility of developing additional qualitative traits able to explain other important quantitative measurements or vice versa.

The high association between the qualitative descriptors and the quantitative parameters was confirmed by ANOVA analysis. The quantitative differences coincided with the initial definitions of the visual descriptors, that is, the qualitatively defined differences among descriptors were verified by the quantitative measurements. Additionally, PCA analysis using the quantitative parameters revealed seedling grouping according to plant-form type, verifying the accuracy of these qualitative types. All of these analytical results demonstrate the reliability of our visual methods to accurately evaluate early plant-form diversity in olive progenies.

II.2.6. Conclusion

In summary, our study revealed high phenotypic plant architecture diversity in the studied 825 young olive seedlings from directed crosses, as well as significant parent genotype variability and influence. ‘Main vertical axis’, ‘Preferential distribution of LS’, ‘Dominant length of LS’, ‘Branch orientation’ and

'Branch bending' were identified as the traits with the most relevant plant architecture descriptors. Qualitative descriptor robustness was verified by quantitative measurements and eight predominant plant-form phenotypes which described nearly half of the seedlings evaluated and showed a clear parent influence, were identified based on the descriptors. The results provide new insights regarding olive seedling description, variability, and inheritance, while the methods represent a significant advance for rapidly measuring and screening complex architectural traits.

II.2.7. Acknowledgements

The authors wish to thank Dr. M. El Riachy for his participation in the data collection and Dr. J. F. Sánchez Sevilla and Dr. A. Belaj for their contribution to the statistical analysis. The study was performed with plant material from the olive breeding program carried out jointly by the University of Córdoba, Spain and IFAPA, Junta de Andalucia, Spain. The work was partly funded by project RTA2008-00033-C02-01, National Institute of Agricultural Research, Spain partially funded by European Regional Development Funds (ERDF).

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III. PARTE 2. DESARROLLO FLORAL

III.1. Capítulo 3:

Influence of water deficits at different times during olive tree inflorescence and flower development

Environmental and Experimental Botany 77 (2012) 227–233

Contents lists available at SciVerse ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Influence of water deficits at different times during olive tree inflorescence and flower development

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III.1.1. Abstract

Sufficient flower number and quality is a prerequisite for subsequent fruit set, and, in the case of commercial fruit crops, for fruit production. In the olive tree, *Olea europaea* L., flower development constitutes an extensive process which requires two to three months and includes the elongation and branching of the inflorescence axis and the formation of the individual flowers. We applied controlled water deficit to young olive trees in successive periods from winter dormancy until flowering and initial fruit set, and determined inflorescence, flower, ovary and ovule development and final fruit yield at both morphological and histological levels. Water deficit during winter dormancy had no effect on either flowering or fruiting parameters. Fruit production was reduced in all other treatments, but due to different timing-related causes: Deficit during inflorescence development reduced many different flowering parameters, including inflorescence number, flower number, perfect flower number and percentage, and ovule development. When applied in the two weeks prior to bloom little change was noted in floral development, but ovary and ovule starch content were reduced as was fruit set. Finally, deficit during bloom-initial fruit set produced a drastic effect in which many flowers remained closed and fertilization was prevented. Overall the results indicated developmental plasticity and compensation among flowering and fruiting parameters, and high sink priority for the ovary tissues.

III.1.2. Introduction

Formation of flowers in sufficient number and of sufficient quality is a prerequisite for subsequent fruit set, and for fruit production in the case of commercial fruit crops. In the olive tree, *Olea europaea* L., the flowers are born on paniculate inflorescences which develop from buds in the leaf axils of the previous season's shoot growth. Following a period of winter dormancy in which they are still undifferentiated as reproductive structures, the axillary buds reinitiate growth and commence inflorescence differentiation (Lavee, 1996; De la Rosa et al., 2000). Consequently, flower number is determined by the number of axillary buds which differentiate into inflorescences and the number of flowers per inflorescence.

Flower quality comprises all of the structural and developmental characteristics of the flower which influence its capacity for fertilization and fruit set (Williams, 1965) and has two critical developmental limitations in the olive tree: flower gender and ovule development (Fernández-Escobar et al., 2008). Olive inflorescences bear a mixture of hermaphrodite (perfect) and functionally staminate (imperfect) flowers caused by varying degrees of pistil abortion (Cuevas and Polito, 2004; Reale et al., 2009). Only the hermaphroditic perfect flowers contain a pistil and thus the capability for forming a fruit. There is a cultivar-related tendency for producing imperfect flowers (Rallo and Frenández Escobar, 1985; Rosati et al., 2011), as well as a noted influence of growing conditions such as moisture availability and nutritional status (Uriu, 1960).

Once a complete, ovary-containing pistil is assured by the presence of a perfect flower, the transformation of an olive ovary into a fruit requires fertilization and the development of a seed from at least one of the four ovules present in the ovary. That capacity may be limited by the occurrence of incompletely differentiated ovules, a phenomenon characteristic of the ornamental olive cultivar Swanhill but also present to lesser degrees in normally fruiting cultivars (Rallo et al., 1981¸ Rapoport and Rallo, 1991a). Thus, as a consequence of incomplete differentiation, the embryo sac, the entity in which the final steps of fertilization are carried out and the embryo is initiated, may be absent in any number of the four ovules normally present in the olive ovary (Martins et al., 2006).

Following winter dormancy, the subsequent changes from axillary bud to blooming inflorescence constitute an extensive growth and development process which requires two to three months and includes the elongation and branching of the inflorescence axis and the formation and development of the individual flowers (King, 1938; Hartmann, 1951; De la Rosa et al., 2000; Cuevas and Polito, 2004). At any time during that period the growing conditions, including water availability, could influence the different aspects of differentiation and development. Hartmann and Panetsos (1961) found both fewer flowers per inflorescence and reduced percentage of perfect flowers for olive trees in pots when they severely reduced soil moisture during both inflorescence formation and flower development. Generally, though, under the Mediterranean climatic conditions in which olive trees have been traditionally grown, fall and/or winter rains provide sufficient water in the soil during floral development and bloom, and the effect of water deficit has been of little concern (Connor and Fereres, 2005). However the potential economic risks from drought years in traditional growing regions, the threat of global warming, and the different weather patterns found in new areas where olive trees are now being planted require information regarding water deficit effects during the different developmental phases.

Just as water stress at different times during fruit development differently affects the growth of olive fruit tissues (Rapoport et al., 2004; Gucci et al. 2009), water deficits at different times during the development of floral organs should produce different effects on floral biology. In this study we applied controlled water deficit to young olive trees for successive periods from winter dormancy until flowering, and also at the time of flowering and initial fruit set. Inflorescence, flower, ovary and ovule development were observed and final fruit yield determined.

III.1.3. Material and methods

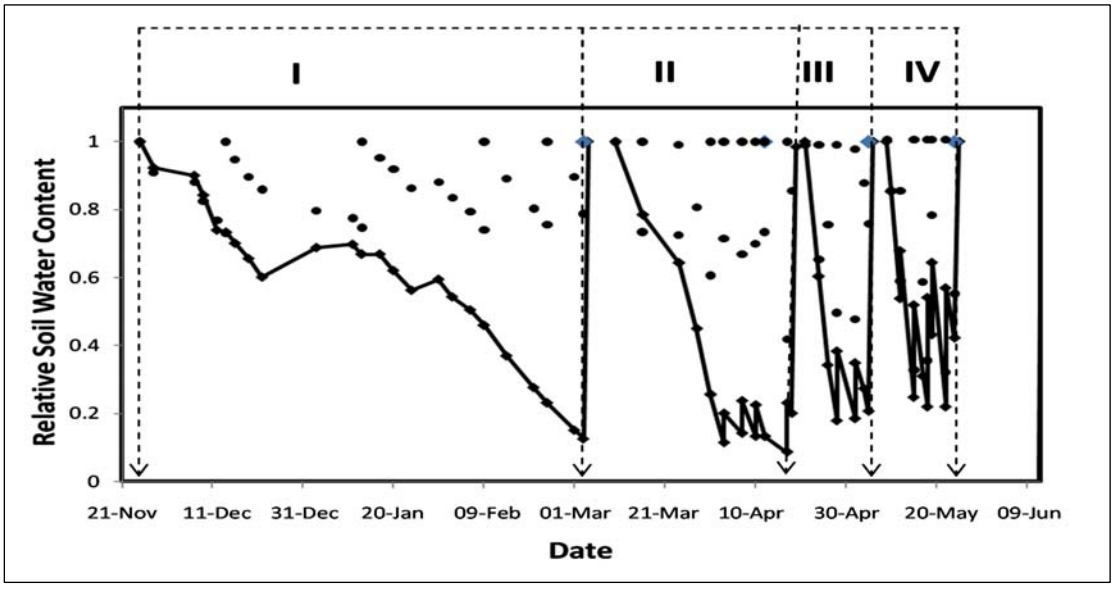
III.1.3.1. Plant material, growing conditions and irrigation treatments

The experiment used three-year-old self-rooted 'Picual' olive trees grown outside in 50 L containers, in a 1:1 soil (sandy loam): peat mixture. The plants had been transplanted to those containers in November, one year previous to initiating the experiment, when they were 18 months old. Any fruits which appeared in that

pre-treatment year were removed in order to eliminate any possible influence on flowering during the treatment year.

Four trees were assigned to each of five treatments, four with different deficit periods and a control. Full bloom was May 5 and the deficit periods were I. Winter dormancy (November 25 - March 3); II. Inflorescence formation (March 10 - April 18); III. Final floral development (April 21 – May 7) and IV. Flowering and initial fruit set (May 8 – May 24). The control treatment and the control periods of the deficit treatments were irrigated to replace evapotranspirative loss, and for the deficit periods irrigation was restricted to 25% of the control amount during the designated period. Soil water content and transpiration were monitored periodically by weighing, as described below and presented in Fig.3.1. The container soil surfaces were covered with plastic to exclude entrance of any water from rain.

Fig.3.1 Relative soil water content for deficit treatments (solid lines) and control plants (separate points) in the successive deficit periods: I. Winter dormancy, II. Inflorescence formation, III. Floral development and IV. Flowering and initial fruit set. The control treatment and the control periods of the deficit treatments were irrigated to replace evapotranspirative loss, determined by weighing as explained in materials and methods. For the deficit-period treatments, irrigation was restricted to 25% evapotranspiration during the designated period. For each period, RSWC values are presented for the control and the deficit treatment corresponding to that period. Symbols represent the means of 4 plants.



In the month of April the water retention characteristics of the soil substrate plus root system were determined for four additional plants growing in the same conditions as those of the experiment. Irrigation was discontinued, and when the leaves were completely dry all above-ground plant material was removed in order to determine soil water content at field capacity (0.38 $\pm$ 0.006 cm^3/cm^3) and soil water content at permanent wilting point (0.08 $\pm$ 0.009 cm^3/cm^3). During the experiment the relative soil water content (RSWC) was determined periodically by weighing each plant in its container. At the beginning and end of each stress period all plants were irrigated to excess and then allowed to drain until constant weight. The possible effect of increased plant weight on RSWC, determined as the difference between those initial and final weights, was found to be negligible (0.012% of total volume of water). RSWC during the successive deficit periods is presented in Fig.3.1.

Due to the seasonal changes in evapotranspirative demand the irrigation frequency varied from every 10 days during winter dormancy to alternate days in April and May. Transpiration ranged from approximately 0.15 l/plant/day minimum to 3.5 l/plant/day maximum. Furthermore, at the beginning of the experimental period six additional plants were harvested and dried in order to determine representative total leaf area (1.78 $\pm$ 0.29 m^2/plant) and total above-ground dry weight biomass (855 $\pm$ 119 g/plant).

III.1.3.2. Inflorescence, flower and ovary observations

Flowering and fruiting were evaluated on marked shoots which remained on the tree throughout the experiment, and by more detailed morphological and anatomical observations of flowers from inflorescences sampled at bloom. Four flowering shoots were marked per tree, on which node number, inflorescence number and flower number per inflorescence were determined prior to bloom, and fruit number determined when fruit set was final. At fruit maturity total fruit number and fruit weight per plant were measured.

At bloom 30 inflorescences containing a mixture of open and closed flowers were collected around the tree from central positions on the flowering shoots, and fixed in FAE (formalin: acetic acid: 60% ethanol= 2:1:17 v/v/v). The number of flowers per inflorescence and number and percentage of perfect flowers were

determined, and twelve to fifteen pistils per tree were obtained, utilizing a maximum of two pistils per inflorescence. Since floral development timing within the inflorescence is not uniform, and in order that the pistils all coincided in developmental stage, they were acquired only from recently opened perfect flowers, chosen on the basis of petal position and condition. The pistils were processed in Histosec® embedding paraffin melting point 56-58ºC (Merck, Darmstadt, Germany) according to standard paraffin procedures (Berlyn and Miksche, 1976) and sectioned transversely at 12 µm. Sections were stained with toluidine blue prior to paraffin removal (Sakai, 1973) for ovary tissue measurements and ovule evaluation, and with Fast Green/ I_2KI (iodine – potassium iodide) after deparaffinization for starch (Ruan et al., 1997).

The ovules pertaining to ten ovaries per plant were observed and characterized as having normal or anomalous development. As the olive ovary contains four ovules (Fig.3.2), the ovaries were each rated according to the number of normal, fully developed ovules of the four, that is x/4, with x presenting values from 1 (one fully developed ovule) to 4 (four fully developed ovules). Taking the ovule evaluation into account, ovary and ovary tissues were measured for ten pistils which had either three (3/4) or four (4/4) fully developed ovules, that is ovaries considered to have good, complete development (Rapoport and Rallo, 1991a; Martins et al, 2006). In this way we could evaluate the direct treatment effects on ovary growth, eliminating any possible reduction in ovary size as a consequence of incomplete ovule development. When ovaries with less than three developed ovules were present in the initial group of ten per tree, additional pistils were sectioned in order to obtain ten which met the ovule development criteria. Ovary, mesocarp and endocarp size were determined as the transverse area of these tissues measured at the point of widest ovary diameter with an image analysis system (Leica QWIN 5001, Leica Microsystems Cambridge, UK) connected to either a stereo microscope (Leica MZ12, Leica Microsystems Weitzlar, Germany) or optical microscope (Nikon Labophot, Izasa S.A., Sevilla, Spain). The ovary mesocarp and endocarp were distinguished by the circle of vascular bundles which separates those two tissues (Rallo and Rapoport, 2001), and total ovary area and mesocarp areas were determined as described by Martins et al., 2006 (Fig.3.2).

Standard errors were calculated and used to compare mean values for the flowering parameters of the marked shoots, ovary size, and fruit parameters (Figs.2.3, 4, 7). Analysis of variance (ANOVA) was performed for flowering parameters of sampled inflorescences and ovary classification (Tables 3.1, 3.2), for which the data variance was sufficiently homogeneous to do so, and means were compared by LSD test at $p \leq 0.05$ using Statistix 9 (Analytical Software, Tallahassee, Florida, USA).

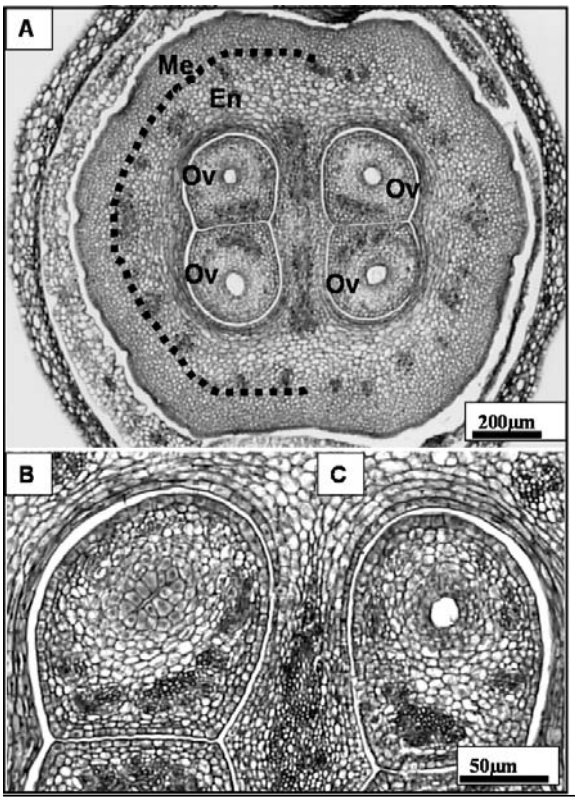


Fig.3.2 Transverse sections of the olive ovary and ovules at anthesis, showing developed and undeveloped ovules. Toluidine blue stain. A. Complete ovary consisting of mesocarp(Me) and endocarp (En), separated by a ring of vascular bundles (dotted line), and containing four developed ovules (Ov). B. Detail of an undeveloped ovule in which embryo sac differentiation was only partial, forming a closed channel within the ovule integument, observed in transverse section as a narrow groove. C. Detail of a fully developed ovule, distinguished by the round, open appearance of the embryo sac in the ovule center.

III.1.4. Results

Flowering parameters (Inflorescences per node, flowers per inflorescence and flowers per node) and the percent fruit set (fruits per flower x 100) for the marked shoots are presented in Figure 3.3. For winter dormancy deficit (Period I) no significant effect was observed on any of those parameters with respect to the control. For Period II, the time of inflorescence formation, the flowering parameters (Fig.3.3 A, B, C) were reduced, and fruit set (fruits/flowers %) increased (Fig.3.3 D). In contrast, for Period III there was no difference with the control for the inflorescence and flowering parameters, but fruit set was reduced.

For the sampled inflorescences (Table 3.1), flower number, perfect flower number and perfect flower percent were similar among the control and Periods I

and III, and reduced for Period II. Although flower number was slightly higher for the collected inflorescences (Table 3.1) than those on the marked branches (Fig.3.3B), a T test (not shown) indicated that the differences were not significant and, more important, the relative behavior among treatments was consistent in both sample types.

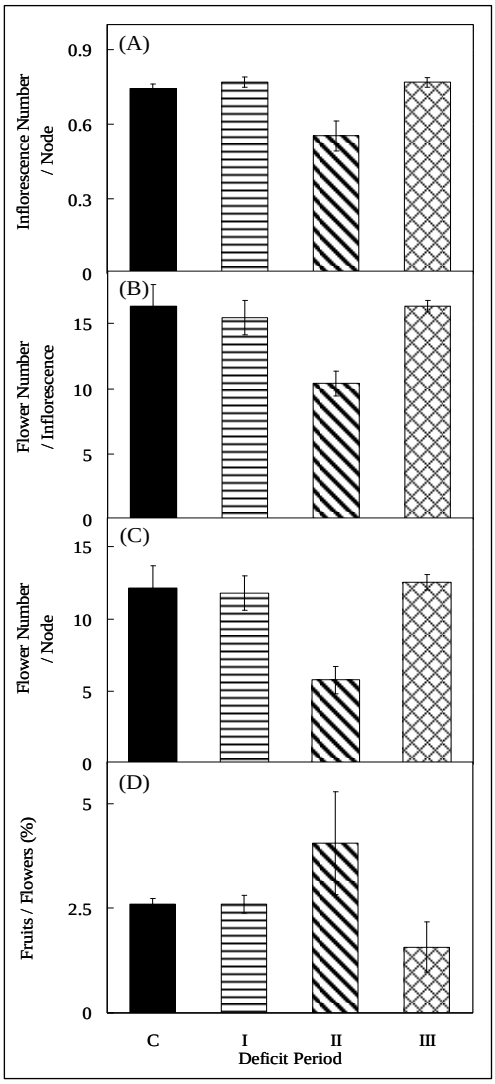


Fig.3.3 Flowering parameters (A. Inflorescences per node, B. flowers per inflorescence and C. flowers per node) and percent fruit set (D. fruits per flower x 100) on selected flowering shoots of olive plants subjected to different deficit periods: I. Winter dormancy (November 25 - March 3); II. Inflorescence formation (March 10 - April 18); III. Floral development (April 21 – May 7) and C. Control – irrigated to replace evapotranspiration. Columns represent means and standard errors for 4 shoots/plant, 4 plants/treatment.

Table 3.1 Flowers and perfect flowers per inflorescence for different deficit periods, measured on the sampled inflorescences. C: control (no deficit); I: winter dormancy; II: inflorescence formation; III: floral development.

Deficit period	Flower number	Perfect flower number	Perfect flower %
C	17.86 a	16.26 a	90.80 a
I	18.27 a	17.27 a	94.71 a
II	12.08 b	7.53 b	64.00 b
III	16.62 a	14.04 a	83.96 a

Different letters indicate significant differences ($p \leq 0.05$) within columns according to the LSD test.

Ovary quality at bloom, based on the percentage of ovaries with four (4/4), three (3/4) and two or less developed ovules per ovary is shown in Table 3.2. Relative to the control, ovary quality was reduced for the Period II deficit, as indicated by the lower proportion of ovaries with all four ovules fully developed, and by the higher proportion with two or less. Ovary quality for the Period III deficit showed a similar tendency but was not significantly different either from the control or Period II. Ovary and ovary mesocarp transverse areas were greatest for Period II deficit (Fig.3.4). Starch grains (Fig.3.5) were numerous in all ovary and ovule tissues, but their presence was less for the Period III deficit than the others. That difference was noted both in concentration and distribution, so that for Period III ovary starch was reduced in the mesocarp and largely absent in the endocarp (Fig.3.5 C, D) and in the ovules the central zone midway between the micropylar and chalazal ends was most affected (Fig.3.5 E, F).

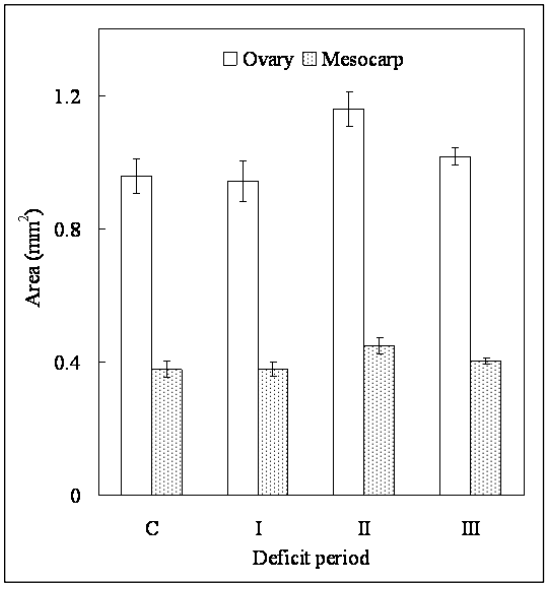


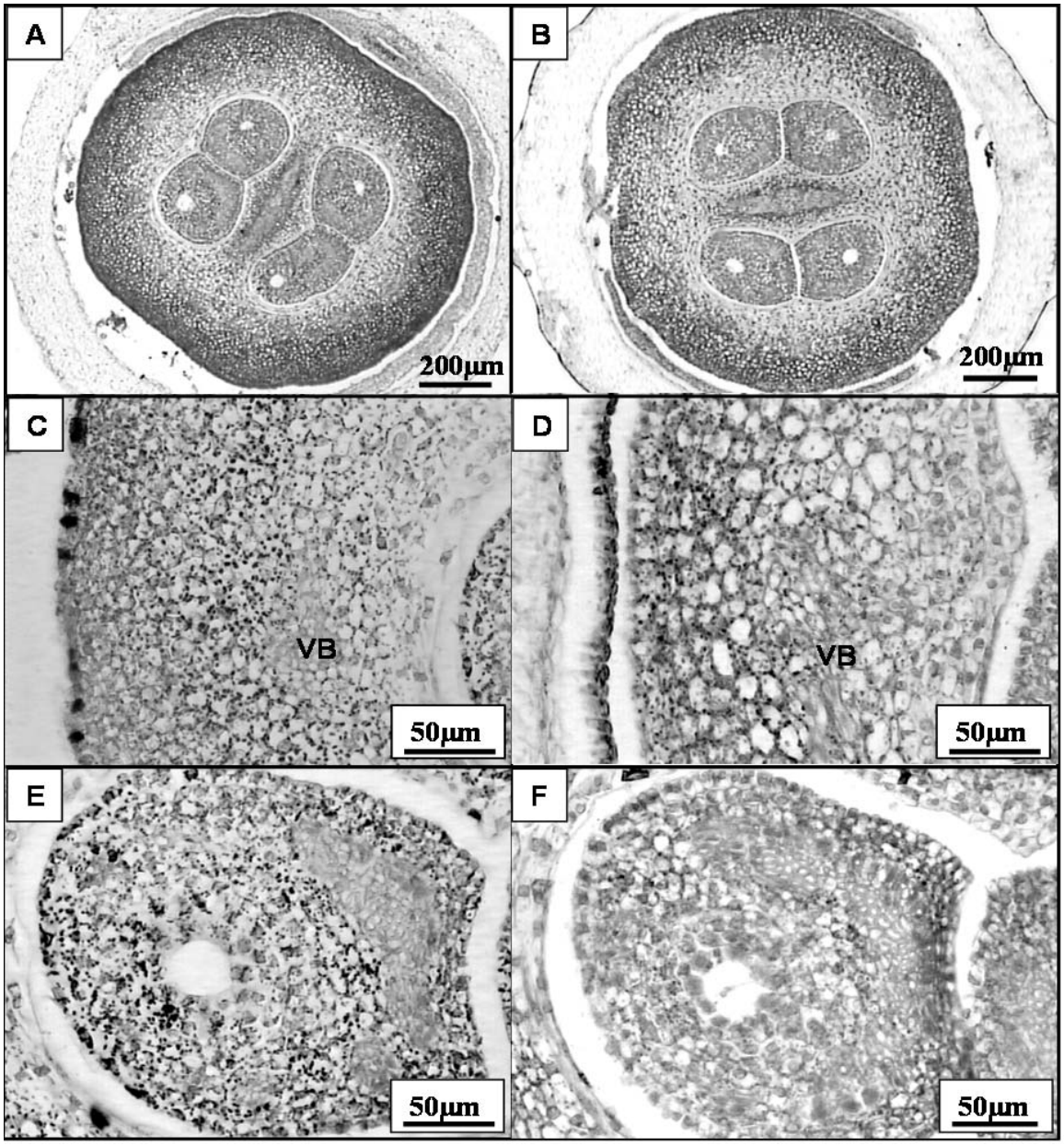
Fig.3.4 Equatorial transverse area of olive ovary and ovary mesocarp at anthesis for different deficit treatments: I. Winter dormancy (November 25 - March 3); II. Inflorescence formation (March 10 - April 18); III. Floral development (April 21 – May 7) and C. Control – irrigated to replace evapotranspiration. Columns represent means and standard errors for 10 ovaries/plant, 4 plants/treatment.

Table 3.2 Classification of ovaries based on the proportion of fully developed ovules for different deficit periods, measured in ovaries at anthesis from sampled inflorescences. C: control (no deficit); I: winter dormancy; II: inflorescence formation; III: floral development.

Deficit period	Proportion of developed ovules/ovary (% ovaries)		
	4/4	3/4	2/4 or less
C	73.3 a	21.6 a	5.0 b
I	55.0 ab	35.0 a	10.0 b
II	35.0 b	33.3 a	31.6 a
III	52.0 ab	28.0 a	20.0 ab

Different letters indicate significant differences ($p \leq 0.05$) within columns according to the LSD test. N = 40 (10 ovaries/plant, 4 plants/treatment)

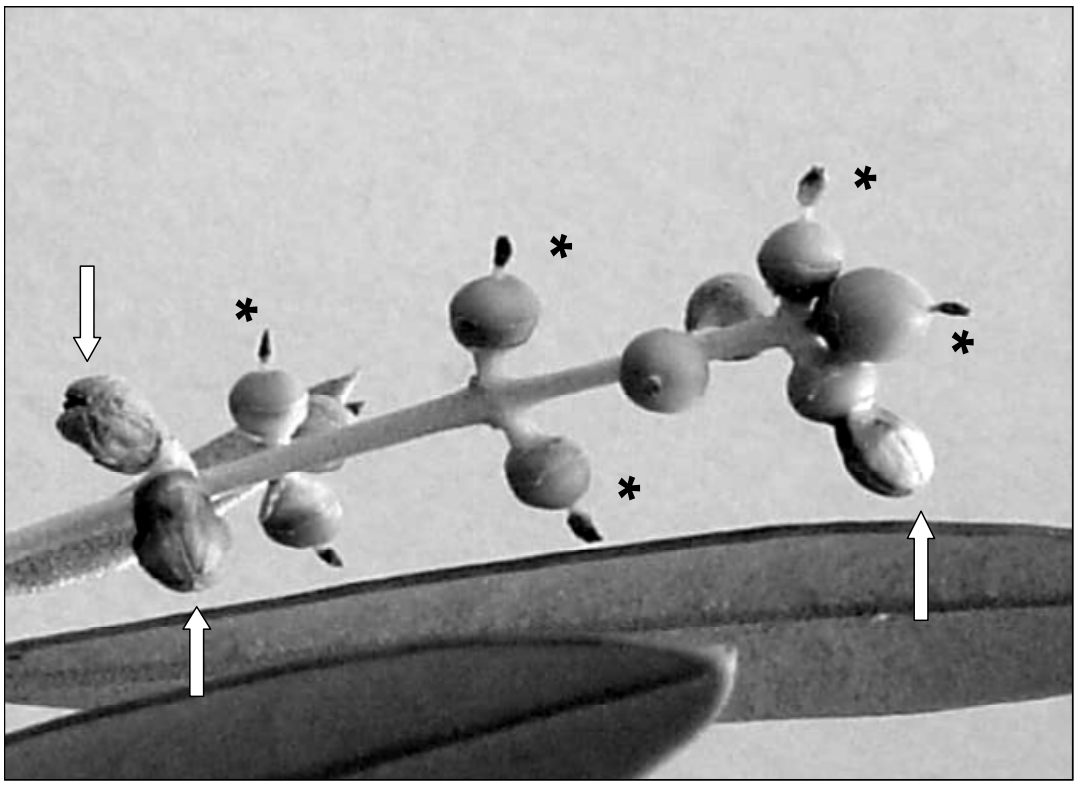
Fig. 3.5 Transverse sections of the olive ovary and ovules at anthesis. Fast Green/I2KI stain – starch grains are stained black. A, C, E. Control treatment; B, D, F. Period III (immediately prior to bloom) deficit. A; B. equatorial transverse section of complete ovary. C, D. Detail of mesocarp and endocarp separated by vascular bundles (VB); the ovary exterior is to the left, the interior to the right of each image. E, F. Detail of ovule at approximate midpoint (midway between micropyle and chalaza).



The Period IV deficit was applied from flowering until initial fruit set, after the flowering parameters were already established in the plants. Thus that deficit could not have affected any flowering parameters, and neither flowering nor ovary

data are presented. Flowering branches and inflorescences of that treatment were measured before treatment, however, to verify that there were no differences between the trees about to be subjected to deficit and the control trees (data not shown). Following deficit onset, the petals of many of the Period IV flowers dried and remained closed. The closed, dry petals abscised as a unit, exposing a dark, senescent and presumably unreceptive stigma (Fig.3.6). Many small shotberries or swollen, unfertilized ovaries were formed, a typical response in olive when pollination and fertilization are prevented (Rapoport and Rallo, 1991b).

Fig.3.6 Typical inflorescence of a plant subjected to water deficit during bloom – initial fruit set (Period IV). The petals of many of the flowers dried and remained closed (arrows); subsequently if the corolla (all petals) abscised a swollen ovary with a darkened, senescent stigma was exposed (asterisks).



Fruit production (Fig.3.7A) was highest and similar for the control and Period I, whereas both fruit number and yield (Kg/ tree) were reduced for Periods II, III and IV. There was some compensation in fruit size, so that weight of individual fruits was greater for the treatments (II, III and IV) with lower fruit number (Fig.3.7B).

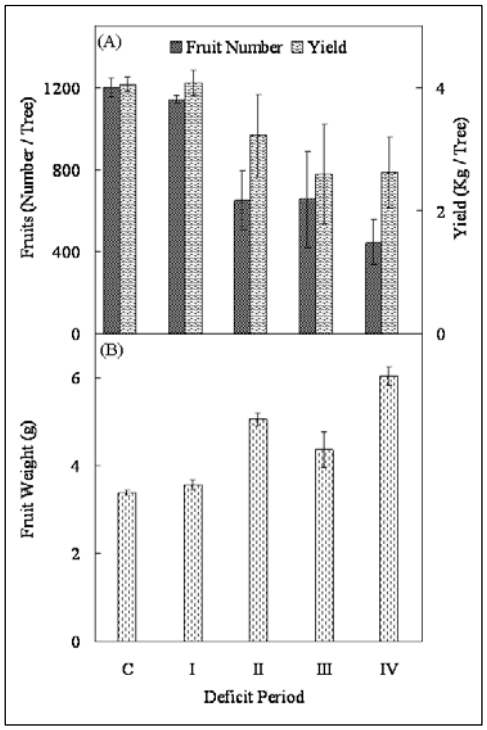


Fig.3.7 Fruit parameters for control plants (C) and successive deficit treatments periods: I. Winter dormancy, II. Inflorescence formation, III. Floral development, IV. Flowering and initial fruit set. A. Fruit number and yield per tree (total fruit fresh weight). B. Fresh weight/fruit. Columns represent means and standard errors for 4 plants/treatment.

III.1.5. Discussion

For the Period I deficit during winter bud dormancy no effect was observed on subsequent flowering. That response is not unexpected, as there is little physiological activity at that time (De la Rosa et al., 2000), but to our knowledge it has not been previously tested.

When the water deficit occurred during inflorescence development (Period II) many different flowering parameters were reduced, including inflorescence number, flower number, perfect flower number and percentage, and ovule development, as also shown in a preliminary report (Rapoport et al., 2011). Their plasticity and the compensation among the multiple flowering parameters are consistent with the olive tree reproductive strategy to achieve fruit set (Cuevas and Polito, 2004; Lavee et al., 1999). Hartmann and Panetsos (1961) found an even more marked response for olive inflorescence, flower and perfect flowers under extremely severe deficit in small pots. Period II encompasses the time of inflorescence formation and individual flower initiation, so it is logical for flower number and perfect flower percent to be affected by water deficit during that time. Imperfect flowers in the olive tree are caused by pistil abortion (Cuevas and Polito, 2004; Reale et al., 2009), which could have been provoked either directly by reduced water status or, very likely indirectly by reduced assimilate supply. The important role of competition for assimilates in olive pistil abortion has been

recently confirmed by thinning within the inflorescence Seifi et al., 2008) and comparisons among cultivars with differences in ovary mass (Rosati et al. 2011).

The Period II deficit affected ovule development as well as inflorescence and flower parameters. However megagametogenesis, the development of the embryo sac, occurs after that deficit period, in the 2 weeks immediately prior to flowering (Extremera et al., 1988), and was also affected. Thus the decreased ovule quality observed for Period II must be due to either residual stress, or to an indirect effect, such as reduced assimilate supply in the stressed plants.

For Period III, the deficit immediately prior to bloom, we observed no significant reductions in the flowering parameters (inflorescence number, flower number, perfect flowers), and a small but insignificant tendency for diminished ovule development. That lack of response could result from the majority of the flowering parameters already having been established, the time required for the plant to sense the deficit, or the high sink strength of the developing flowers for assimilates which made them strong sinks for water as well. In spite of little reduction observed in flowering parameters, however, fruit set and yield for Period III were much lower than the control. Thus, even though flower structures appeared normal, the imposed stress could have produced limitations to fertilization such as reduced stigma receptivity or ovule longevity (Fernandez-Escobar et al., 2008), but those parameters were not measured in this study.

Ovary and ovule starch, observed by histochemical staining, were lower for the Period III treatment, but were still present. Rodrigo et al. (2000) demonstrated the important role of ovary and ovule starch for flower reproductive function in apricot. Drought stress reduces ovary carbohydrates and consequently reproductive performance in maize (Westgate and Boyer, 1985; Zinselmeier et al., 1995), but drought-tolerant wheat germplasm is able to maintain ovary carbohydrate accumulation (Ji et al., 2010). De la Rosa et al. (2000) demonstrated the importance of starch at the onset of olive inflorescence differentiation and Reale et al. (2009) showed that olive ovary and ovule starch is closely related to abortive development. The lower starch grain presence which we observed in the ovaries and ovules for Period III is consistent with the drought effect noted in other species, and may have contributed to the reduced fruit set. Those observations also suggest that water deficit immediately prior to bloom mainly

affected photoassimilate supply, so that the ovaries either stored less starch in preparation for bloom or consumed starch which was already present.

The extent to which the proportion of developed ovules will influence fruit set is not certain, nor the degree to which this structural feature responds to developmental conditions. Due to very large flower number in the olive tree in relation to final fruit set, ovule development is apparently a critical factor only in extreme cases of stress or low flower number (Rapoport and Rallo, 1991a). Because only one fertilized ovule is required to form the olive seed and initiate fruit development, and also because it is usual to find a small number of ovules with incomplete development (Rallo et al., 1981; Rapoport and Rallo, 1991a), one can consider that the 4/4 and 3/4 ovaries both have a good potential for fertilization and fruit formation. Thus one can globally evaluate ovary quality by grouping those two categories, or, in the same manner observe the inverse data, that is the number of poor quality ovaries which have two or less developed ovules. Using this approach ovary quality was reduced for Periods II and III, although the percentage of 4/4 ovaries was significantly lessened only for the Period II deficit.

The Period IV deficit effect was quite drastic, producing numerous flowers with dried, closed petals. At bloom, the petals open to expose the stamens and pistil and permit fertilization, a process in which turgor pressure acts directly, although assimilate supply is also involved because the mechanism is triggered by increased sucrose concentration (Reid, 2005). However not only was petal opening blocked, but the effect was apparently so severe that petal drying and senescence occurred as well, suggesting a critical nature of water status at this time, and perhaps a particularly rapid response to stress of the container-grown plants brought on by the increased evaporative demand in this period relative to the earlier ones.

Fruit number was reduced for Periods II, III and IV. Period II had fewer flowers, Period III appeared to have difficulties in fertilization, and in Period IV many flowers dried and were unable to form fruits. The observed response of increased fruit size with lower fruit load is typical for olive (Gucci et al., 2007) as well as other fruit trees (Corelli-Grappadelli and Lakso, 2004). However fruit growth did not fully compensate for the considerable reduction in fruit number

which occurred for the Periods II, III and IV deficits, in which total yield dropped even though individual fruits were larger (Fig.3.7). Ovary and ovary mesocarp sizes were also greatest for the Period II deficit, in which flower and perfect flower number were lowest. Possibly ovary size affects fruit size, a relationship which has been demonstrated among olive cultivars of different fruit size (Rosati et al., 2009), but has yet to be tested as a physiological response within the same genotype.

III.1.6. Conclusion

These experiments were carried out in 50L containers, so deficit application was more precisely controlled but could also have been more severe than for trees growing in the field. Nevertheless the results provide valuable information regarding olive tree reproductive biology and irrigation needs in olive crop production. In summary water deficit during winter dormancy (Period I) showed no effect on inflorescence or flower formation. The greatest reductions in flowering parameters occurred for the period II deficit, most likely related to both its length and the multiple inflorescence and flower development events which occur during that time. Deficit during final floral development (Period III) produced lesser reductions in flowering parameters but hampered the pollination and fertilization process, with probable high ovary sink priority at that moment. Deficit during flowering and initial fruit set (Period IV) reduced pollination by hindering flower opening. Some compensation in fruit size occurred when the deficit treatments resulted in lower fruit number, but it was insufficient for maintaining full fruit production. The results of this study complement recent information on the effect of water deficit on cellular processes of olive fruit at different times during development (Rapoport et al. 2004; Gucci et al. 2009).

III.1.7. Acknowledgements

The authors wish to thank Ester García-Cuevas for excellent technical assistance. This study was financed by ERDF co-financed Spanish Ministry of Science and Innovation grants AGL2008-02570 and AGL2009- 07248, and Andalusian Excellence grant PO6-AGR-01791.

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IV. PARTE 3. DESARROLLO DEL FRUTO

IV.1. Capítulo 4:

Cultivar-based fruit size in olive depends on different tissue and cellular processes throughout growth

Scientia Horticulturae 130 (2011) 445–451

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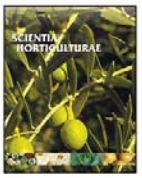

Cultivar-based fruit size in olive depends on different tissue and cellular processes throughout growth

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IV.1.1. Abstract

In drupe fruits, in addition to fruit size, the proportion of mesocarp and endocarp tissues are critical objectives for fruit quality, crop production and management. The olive fruit is a typical drupe, with cultivars which show a wide range in both fruit size and the proportions of mesocarp and endocarp. Characterizing the role of tissue and cellular processes in producing genetically based fruit size variability is necessary for crop improvement, as well as deepening our understanding of fruit developmental physiology. This study used microscope image analysis to evaluate cell number and size, the growth of mesocarp and endocarp tissues, and their developmental timing in producing fruit size among six olive cultivars with a large range of fruit size. We founded that cultivar mesocarp and endocarp size increased linearly with fruit size, with larger sizes favoring an increasingly greater mesocarp/endocarp ratio. Within the mesocarp, cultivar-based fruit size related directly to cell number and was established soon after bloom by cell division rate. In spite of different cell division rates, all cultivars showed similar timing of cell division activity, with the majority of cells produced in the two months after bloom but, surprisingly, a substantial number of cells formed during the following 6 months. Cell expansion was high throughout fruit growth and an important factor in achieving final fruit size, but cell size did not differ among cultivars at any time. We can conclude that fruit size differences among olive cultivars are due at the tissue level to both mesocarp and endocarp sizes and at the cellular level to cell division throughout fruit growth. Furthermore, since cell size is consistent among cultivars in spite of variable cell division, it is likely that cultivar differences in cell expansion accompany those in cell division.

IV.1.2. Introduction

Fruit size is influenced and regulated by both exogenous factors, such as water availability and ambient temperature, and endogenous ones, such as crop load and genetic differences (Corelli-Grappadelli and Lakso, 2004). Potential fruit size is genetically controlled and has been a critical character for selection and breeding of commercial fruit crops. Final fruit size results from the sum of the growth of the different tissues that compose the fruit, which vary in type, number and proportion among fruits (Coombe, 1976). Characterizing the role of the tissue and cellular processes in producing genetically based fruit size variability is necessary for crop improvement, as well as deepening our understanding of fruit developmental physiology and molecular controls (Seymour et al., 2008; Bertin et al., 2009; Galla et al., 2009; Banilas et al., 2011).

The drupe, a type of fleshy fruit, consists of a thin protective exocarp or epicarp, a fleshy mesocarp, and an inedible stony endocarp surrounding the seed (Roth, 1977). The mesocarp and endocarp are the two largest tissues in size, but their proportions depend on both species and variety or cultivar. In major commercial drupe fruits, such as peach (*Prunus persica* L.), apricot (*Prunus armeniaca* L.) and cherry (*Prunus avium* L.), the mesocarp comprises a far superior proportion of mature fruit fresh weight and volume than the endocarp (Bollard, 1970). Consequently experimental studies exploring genotypic fruit size differences in those fruits (Scorza et al., 1991; Yamaguchi et al., 2002; Yamaguchi et al., 2004; Olmstead et al., 2007; Quilot and Génard, 2008), have examined mesocarp growth and size. The endocarp, however, can represent an important proportion of fruit dry weight (Bollard, 1970) and is also considered as strong sink competitor with the mesocarp (Barabé and Jean, 1995). Furthermore the proportion of endocarp is often greater in species of smaller fruit size or higher dry-weight content such as olive (Bianchi, 2003; Del Rio and Caballero, 2008) and Saskatoon (McGarry et al., 2001).

The olive, *Olea europaea* L., fruit has the basic drupe structure consisting of an exocarp, mesocarp and endocarp (King, 1938), but presents certain morphologic and physiological differences that distinguish it from other drupes. In fact, drupes typically form from a single carpel ovary (Roth, 1977), while the olive fruit originates from a bicarpellate ovary in which each carpel has two ovules (King, 1938; Rallo and Rapoport, 2001). The olive fruit is distinguished by a considerable portion of the fruit occupied by

the endocarp (Del Rio and Caballero, 2008), the accumulation of a significant amount of oil as a storage product, the high energy cost of that storage component, and the relatively low water content (50-67%) (Nergiz and Engez, 2000; Bianchi, 2003). Those characteristics contrast with other more common commercial drupes, such as peach and apricot, with a low endocarp proportion, accumulation principally of carbohydrates in the form of sugars, and containing a high amount of water (82-85%) (Bollard, 1970). Additionally, the large range in fruit size (Barranco, 2004) and in mesocarp:endocarp ratio among cultivars (Del Rio and Caballero, 2008) are particularly salient in the olive fruit. These features of the olive fruit suggest an important contribution of endocarp growth to final fruit size and fruit-size variability among cultivars.

The morphogenetic process of fruit growth and development is accomplished by four different activities of the fruit cells: cell division, expansion and differentiation, and the accumulation of storage components. Cell division and expansion are the principle cellular processes that produce the final size of fleshy fruits (Gillaspy et al., 1993). In the majority of commercial drupes, such as peach, sweet cherry and apricot, cell division occurs in an initial phase that represents approximately ten percent of the total fruit growth duration, followed by only cell expansion until fruit maturation (Bollard, 1970). Studies performed in these drupes (Scorza et al., 1991; Yamaguchi et al., 2004; Olmstead et al., 2007; Quilot and Génard, 2008) have determined that genotypic fruit size differences are principally due to mesocarp cell number and not to cell size. Final cell number differences may be produced at different times during fruit development, such as in the peach ovary (Scorza et al., 1991) or during varied periods of post anthesis cell division in apricot (Yamaguchi et al., 2004).

In most fruit cell studies of drupes (Scorza et al., 1991; Yamaguchi et al., 2002; Yamaguchi et al., 2004; Olmstead et al., 2007) as well as other fleshy fruits (Higashi et al., 1999; Harada et al., 2005; Zhang et al., 2005; Zhang et al., 2006) measurements of the mesocarp and its cells were carried out in only one dimension, along the radius that crosses the mesocarp in a transverse plane. This form of evaluation provides only partial information, however, because mesocarp size is due not only to growth in thickness, but also to expansion around any internal space, i.e. the endocarp, that it surrounds. Another difficulty in evaluating the contributions of cell division and expansion to fruit growth derives from the interaction of both processes in determining, cell size, the variability in

time and space, and the dependency between cell size and number generated by measurement methods (Green, 1976; Evans, 2000).

The objective of this study was to examine the contributions of mesocarp and endocarp tissues, and mesocarp cell number and size to olive fruit growth and development. We determined these parameters by using image analysis to measure transverse equatorial sections, thus permitting measurements based on cell and tissue areas rather than just radius. We evaluated the roles of both the temporal pattern and final values in determining fruit size differences among cultivars.

IV.1.3. Material and methods

IV.1.3.1. Plant material and experiment design

The experiments used six olive tree (*Olea europaea* L.) cultivars with a wide range of fruit size (Barranco, 2004): very large ('Gordal Sevillana'), large ('Manzanilla de Sevilla' and 'Hojiblanca'), average ('Picual' and 'Lechin de Sevilla') and small fruit ('Arbequina'). The trees were twenty years old and planted at standard density of 270 trees ha^{-1} at the experimental farm 'Alameda del Obispo' of the Andalusian Institute for Research and Training in Agriculture, Food and Fisheries (IFAPA) in Cordoba, Spain (37º53' N, 4º45' O). A completely randomized design (CRD) was used for fruit sampling. That is, in the orchard there were six randomly distributed rows per cultivar, each with six plants. At bloom 24 trees, one per row and four per cultivar, were selected based on similar canopy size and uniformly abundant flowering level. The trees were grown with irrigation under standardized cultivation conditions.

IV.1.3.2. Fruit preparation and measurement

Ten ovaries or fruits per tree were sampled around the canopy at bloom, every two weeks from 4 to 8 weeks after bloom, then every four weeks until fruit maturity 32 weeks after bloom. Fruits were fixed in FAE (formalin: acetic acid: 60% ethanol = 2:1:17 v/v). Structural observations and measurements were performed on transverse sections at the point of widest fruit diameter. Slices of the fixed fruits were processed according to standard paraffin procedures, sectioned at 10-12 μm, and stained with toluidine blue O prior to paraffin removal (Sakai, 1973). Although these histological procedures, i.e. fixation in FAE and embedding in paraffin are known to produce some tissue shrinkage (Ruzin, 1999), we considered them to be valid for the cultivar comparisons because the

material was similar in nature, and because previous testing of the methodology indicated that the shrinkage effect is consistent for olive fruits of different size, development and water status (Gucci et al., 2009). Until 8 weeks, 3-4 mm equatorial slices of the entire fruit were utilized. After that time, it was necessary to first remove the hardened endocarp from the fruit and to section slices of only mesocarp tissue.

Quantitative observations of tissue growth and differentiation were made with an image analysis system (Leica QWIN 5001, Leica Imaging Systems Ltd., Cambridge, UK) connected to a stereo microscope (Leica MZ12, Leica Microsystems, Wetzler, Germany). For the 8 weeks and younger samples, total fruit and endocarp cross-sectional areas of the histological preparations were measured with the stereo microscope, and mesocarp area was calculated as the difference between those two tissues. For the pitted fruits, caliper measurements of the pit diameter were used to calculate endocarp area, assuming a circular shape. Then, mesocarp area was determined by subtracting that calculated endocarp area for each fruit from the total fruit area measured in the preparations.

With the ocular microscope, a field of 200 cells was counted and its area determined for each fruit mesocarp. These data were used to determine average cross-sectional area per cell and, in combination with mesocarp area, to calculate total mesocarp cell number for the transverse sections. Thus cell area values refer to mean cell area contribution to mesocarp transverse area, and cell number to number of cells in mesocarp equatorial transverse section.

IV.1.3.3. Data analysis

The experimental design was completely randomized with four replications (trees) per cultivar, and the value of each replication the average of the values obtained for 10 fruits. Results related to fruit growth from bloom to maturity are expressed as mean and standard error of mean (±SE). The contribution of fruit tissues and cellular processes to genotypic mature fruit size variation was determined by linear regression. The correlations of final fruit area and cell number with their values at successive moments during fruit growth were obtained by the Pearson Coefficient.

The contributions of mesocarp cell size and number at different times during fruit growth were evaluated by dividing the 32-weeks of fruit growth into two periods based on the intensity of cell division. Period I, the period of active cell division,

occurred from bloom (0 weeks) to 8 weeks and Period II, in which cell division was less, from 8 to 32 weeks. A one-factor CRD ANOVA was performed to test the influence of growth period and cultivar on the rate of increase and the percentage of final value achieved during growth of fruit area, cell number and cell area. Prior to ANOVA, data normality and variances homogeneity assumptions were tested using the Shapiro-Wilk and Bartlett's tests, respectively. All analysis was performed using *Statistix 8* (Analytical Software, Tallahassee, USA).

IV.1.4. Results

For all cultivars, fruit and mesocarp transverse areas showed similar seasonal patterns in which growth was initially slow, was more rapid for the majority of the growth period, and then slowed again as maturity was reached (Fig.4.1a and b). Also for all cultivars fruit and mesocarp growth took place during approximately 28 weeks. Endocarp transverse area also expanded exponentially from soon after bloom, but, in contrast to the mesocarp ceased abruptly at 8 weeks (Fig.4.1c).

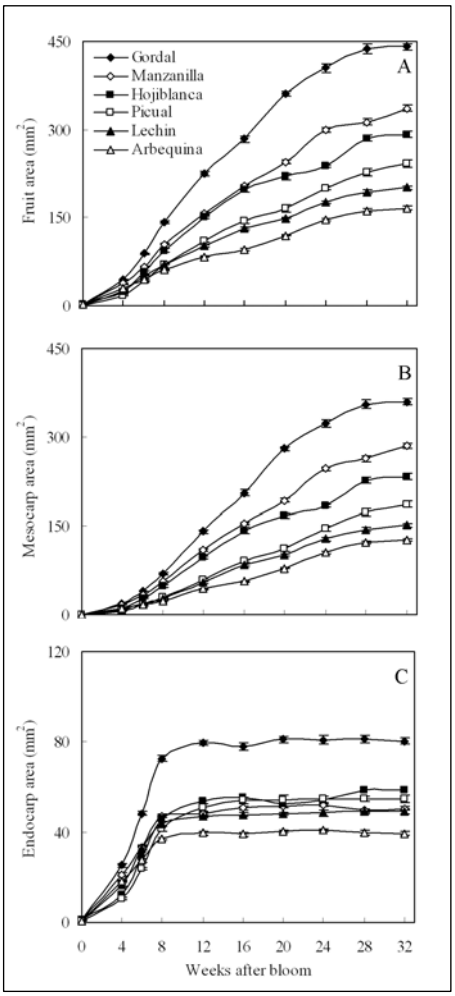

Fig.4.1 Transverse fruit (a), mesocarp (b) and endocarp (c) areas during fruit development for six olive cultivars. Bars show standard errors of means of four replications (n=4), each replication is the average value of 10 fruits.

Mesocarp transverse area at fruit maturity showed significant differences among all cultivars and followed an order consistent with that of fruit size (Fig.4.1a and b). The hierarchy of fruit and mesocarp areas was established by 8 weeks, from which time the differences among cultivars increased with successively increasing fruit size. Those differences successively attained statistical significance, so that by 20 weeks all cultivar fruit areas were significantly different, and 24 weeks all mesocarp areas. Endocarp area at maturity was significantly highest for 'Gordal Sevillana', the cultivar of greatest fruit size and lowest for 'Arbequina', the smallest fruited cultivar, but 'Manzanilla de Sevilla', 'Hojiblanca', 'Picual' and 'Lechin de Sevilla', cultivars with average to large fruit were grouped together (Fig.4.1c). In 'Gordal Sevillana', mesocarp and endocarp area were successively three and two times greater than in 'Arbequina'.

Linear regression analysis showed that mature fruit area strongly correlated with both final mesocarp and endocarp tissue areas (Fig.4.2), although the correlation was slightly less for the endocarp. The greater slope of the mesocarp area /fruit area regression (Fig.4.2a) than that for endocarp area /fruit area (Fig.4.2b) indicated that larger cultivar fruit area corresponds much more to mesocarp than endocarp area. A decreasing contribution of the endocarp to fruit size as fruit size increases was shown by the negative linear correlation found among cultivars between final endocarp proportion and final fruit area (Fig.4.3a). Consistent with those observations fruit size correlated with mesocarp/endocarp ratio (Fig.4.3b).

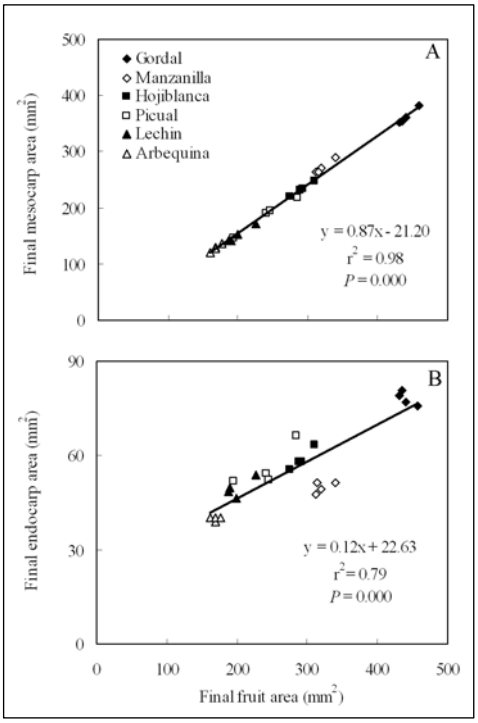

Fig.4.2 Correlations of mesocarp (a) and endocarp (b) tissues areas with fruit area at maturity for six olive cultivars. Each data point is the mean of 10 fruits/tree.

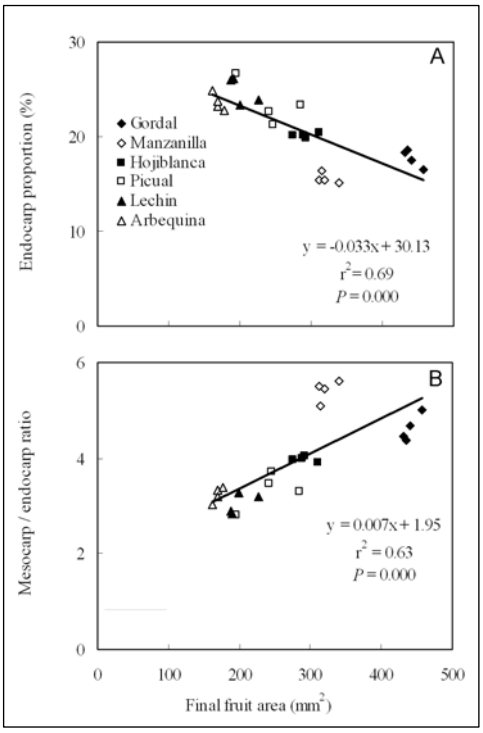

Fig.4.3 Correlations of endocarp proportion (a) and mesocarp/endocarp ratio (b) with fruit area at maturity for six olive cultivars. Each data point is the mean of 10 fruits/tree.

Mesocarp cell size increased constantly and substantially from bloom to maturity (Fig.4.4a). Cell size increase was initially moderate, more accelerated from 8 weeks until 20-24 weeks, and then slowed again as maturity was reached. All cultivars showed a similar pattern and similar dimensions, with only 'Picual' fruits having a significantly different final mesocarp cell size.

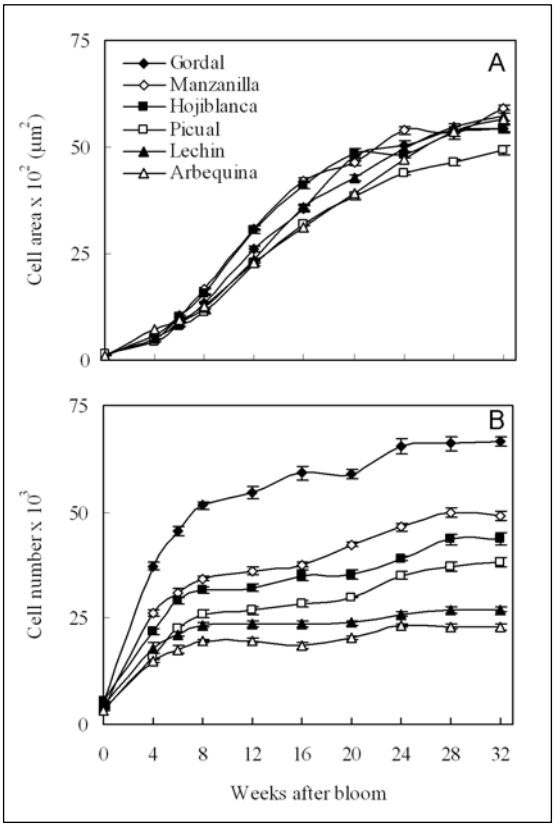

Fig.4.4 Mesocarp cell area (a) and number (b) during fruit development for six olive cultivars, listed in order of decreasing fruit size. Cell area is mean cell area contribution to mesocarp transverse area; cell number is total number of cells in one mesocarp equatorial transverse section. Bars show standard errors of means of four replications (n=4), each replication is the average value of 10 fruits.

In contrast to cell size the increase in cell number was initially rapid, then followed by a much slower stage until fruit maturity (Fig.4.4b). The principal period of cell division represented approximately 25% of total fruit growth. Although all cultivars showed a similar pattern of cell division, the cell number values differed widely and significantly among all cultivars.

Linear regression analysis revealed no significant relationship (P> 0.05) between cell area and transverse fruit area at maturity (Fig.4.5a). A strong linear correlation, however, was found between final cell number and fruit size (Fig.4.5b), indicating that fruit size increased linearly with cell number but not with cell area.

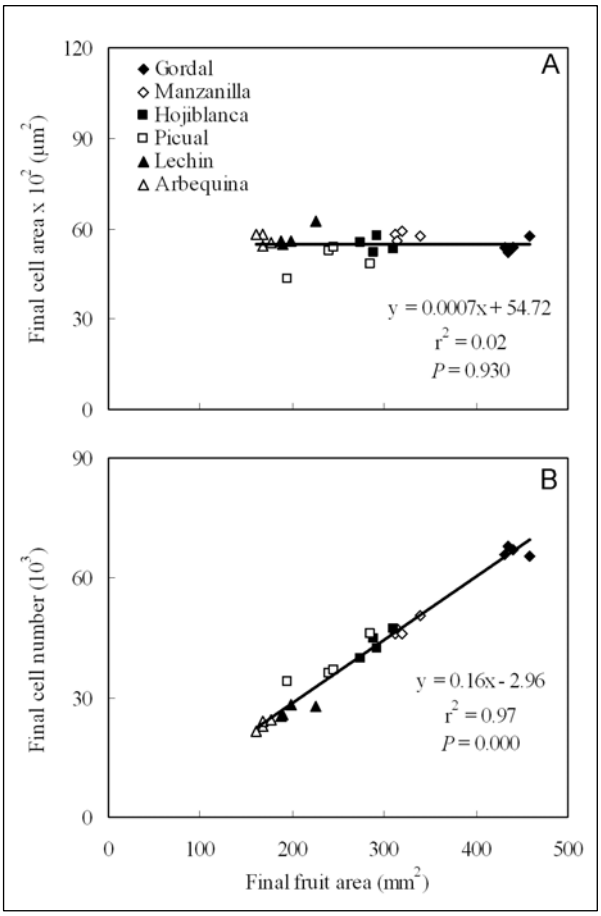

Fig.4.5 Correlations of mesocarp cell area (a) and number (b) with fruit area at maturity for six olive cultivars. Cell area is mean cell area contribution to mesocarp transverse area; cell number is total number of cells in one mesocarp equatorial transverse section. Each data point is the mean of 10 fruits/tree.

The Pearson correlation coefficients indicated that fruit area and cell number differences among cultivars at maturity were established early in fruit growth and were highly significant during the majority of the season (Fig.4.6). For both of those parameters the values for the early dates showed a low correlation with values at maturity, but by 8 weeks after bloom the correlations with maturity values were highly significant.

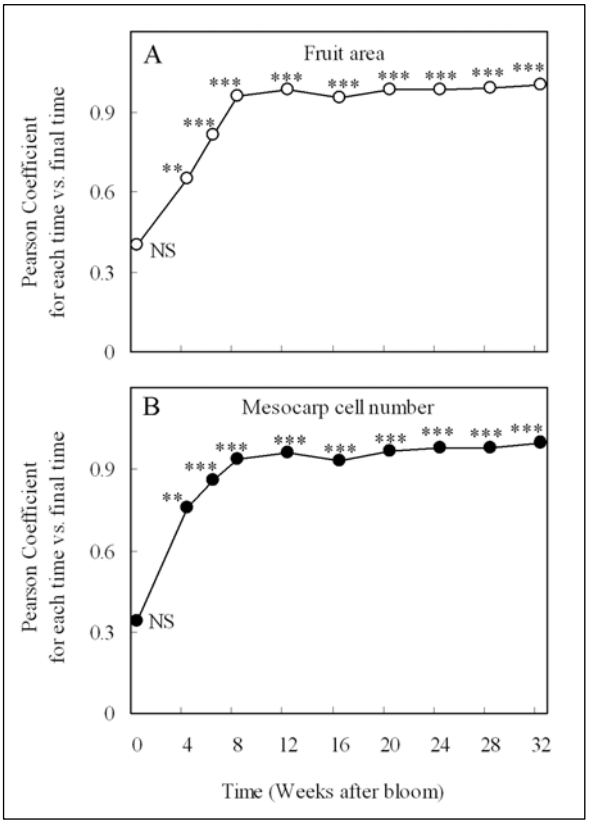

Fig.4.6 Pearson coefficient correlations between the values at different successive times during fruit growth and the value at maturity of (a) fruit area and (b) cell number for the six studied olive cultivars. Each data point is based on the mean of four trees, 10 fruits per tree. NS no significant correlation. ** and *** indicate significant correlations at $P<0.01$ and at $P< 0.001$, respectively.

The contributions of mesocarp cell size and number before bloom, during initial and later fruit growth differed somewhat among cultivars but showed consistent trends (Table 4.1). Ovary area and cell size were quite small relative to those in the mature fruit, and ovary mesocarp cell number represented 7-16% of the final value. During Period I the fruits achieved 31-37 % of transverse area, 61-71 % of final cell number, and approximately 25% (20-29%) of final cell size. In Period II the remaining 69-78% of the increase in cell size and 16-30% in cell number took place. Fruit area growth rate was greater in Period I than II, but always followed the same order among cultivars as final fruit size (Table 4.1). For cell number, the rate of increase in Period I was five to 11 times greater than in Period II, showed an order among cultivars strictly consistent with fruit size during Period I, and a similar tendency in Period II. For cell size, the rate of increase was slightly greater in Period II than I in the majority of cultivars, but in neither period related to final cultivar fruit size order.

Table 4.1 Contributions of total area, mesocarp cell area and mesocarp cell number at different times during fruit growth: the ovary, active (Period I) and slow (Period II) cell division stage. The amount of change during each period is expressed as percent of final value achieved in that period, and overall rates of change are shown for Periods I and II.

Cultivar		Ovary or fruit area		Mesocarp cell area		Mesocarp cell number	
		% final size	R[a] (mm^2/week)	% final size	R[a] (μm^2/week)	% final number	R[a] (Cells/week)
Gordal Sev.	Ovary	0.4±0.02	-	2.3±0.1	-	7.6±0.4	-
	Period I	32.3±0.8	17.60±0.27	22.8±0.6	154.5±2.9	70.6±2.0	5825.6±71.6
	Period II	67.3±0.8	14.73±0.24	74.9±0.5	202.2±5.4	21.8±2.3	726.5±10.7
SED_P		1.19***	0.62**	0.77***	6.24***	3.05***	109.69***
Manzanilla Sev.	Ovary	0.4±0.03	-	2.1±0.1	-	7.5±0.2	-
	Period I	33.0±1.3	12.88±0.23	29.1±0.4	195.3±2.0	62.4±3.2	3817.5±113.2
	Period II	66.6±1.4	10.43±0.33	68.8±0.4	182.1±3.2	30.1±3.3	769.6±39.0
SED_P		1.81***	0.63**	0.20***	3.23**	5.31***	158.33***
Hojiblanca	Ovary	0.5±0.02	-	2.2±0.2	-	12.4±1.3	-
	Period I	32.8±1.0	11.59±0.28	26.9±0.6	177.4±2.8	61.4±4.1	3291.1±79.5
	Period II	66.7±1.1	9.55±0.31	70.9±0.8	189.7±6.6	26.2±4.9	595.1±37.8
SED_P		1.50***	0.39***	0.96***	9.25 NS	6.41***	162.82***
Picual	Ovary	0.4±0.02	-	3.1±0.2	-	8.0±0.5	-
	Period I	31.3±2.4	8.70±0.20	20.9±1.0	121.2±3.9	63.1±5.2	2838.3±68.4
	Period II	68.2±2.4	7.12±0.69	76.0±1.2	176.1±11.6	28.9±5.5	561.5±52.8
SED_P		3.53***	1.03 NS	1.59***	8.18***	7.59**	172.23***
Lechin Sev.	Ovary	0.6±0.01	-	2.2±0.1	-	16.0±0.7	-
	Period I	35.9±0.8	8.71±0.25	20.1±0.6	136.1±3.7	68.1±1.2	2287.0±97.8
	Period II	63.5±0.8	6.13±0.33	77.7±0.5	212.7±8.4	15.9±1.4	199.7±27.1
SED_P		1.17***	0.37***	0.93***	5.32***	2.52***	93.49***
Arbequina	Ovary	0.7±0.03	-	2.3±0.1	-	14.7±1.1	-
	Period I	36.8±1.3	7.44±0.17	21.7±1.5	145.6±9.1	65.0±3.7	1867.7±77.1
	Period II	62.5±1.4	5.06±0.15	76.0±1.6	203.8±4.9	20.3±4.9	239.9±34.1
SED_P		1.96***	0.28***	2.22***	11.94**	5.71***	97.59***
SED_Cv.	Ovary	0.03***		0.17***		1.15***	
	Period I	2.02 NS	0.33***	1.25***	6.60**	5.10 NS	118.50***
	Period II	2.04 NS	0.59***	1.31***	8.98*	5.69 NS	152.11**

[a] overall rate of increase. Each value represents the mean ± SE (n=4), each replicate is the average value of 10 fruits per tree. SED_Cv. and SED_P are the standard error of the differences between cultivars and Periods (I and II), respectively. Significant difference at P<0.05, P<0.01 and P<0.001 are indicate by *, **, ***, respectively. NS no significant difference.

IV.1.5. Discussion

Differences in the amount of mesocarp tissue growth contributed markedly to cultivar fruit size variation, from early in fruit growth. An important genetically-based contribution of the mesocarp has also been described for other drupes such as sweet cherry (Olmstead et al., 2007), peach (Yamaguchi et al., 2002; Quilot and Génard, 2008) and apricot (Yamaguchi et al., 2004). In spite of the different mesocarp sizes among cultivars, however, all cultivars showed comparable continuous expansion of the mesocarp and fruit transverse area until the final period of fruit growth. The continuous growth of the mesocarp which we observed contrasts with the double sigmoid growth pattern commonly attributed to drupes (Bollard, 1970; Coombe, 1976; Barabé and Jean, 1995) and originally observed in olive (Hartmann, 1949), but is consistent with recent reports for olive, in which a double sigmoid pattern was not found (Lavee et al., 2007; Trentacoste et al., 2010; Martín-Vertedor et al., 2011).

Among the olive cultivars, endocarp size was also strongly correlated with fruit size, although slightly less than mesocarp size. That weaker correlation is likely related to the much shorter period of endocarp growth as compared to the mesocarp, the greater portion of the fruit occupied by the mesocarp and the successively smaller proportion of the endocarp with increasing fruit size. McGarry et al. (2001) attributed cultivar differences in fruit size equally to both mesocarp and endocarp tissues, in the Saskatoon, a fruit in which both tissues occupy equal proportions of the whole fruit and have similar growth duration.

Maximized mesocarp/endocarp ratio is a priority trait for olive breeding (Barranco, 2004; Del Rio and Caballero, 2008) because the mesocarp is the edible part of olive fruit and the tissue of oil accumulation. Our study suggests that among current commercial genotypes the endocarp contribution is generally reduced with increased fruit size, favoring a greater mesocarp/endocarp ratio, and that very likely future selection for fruit size will produce similar tendencies. One cultivar, 'Manzanilla de Sevilla', showed values which were slightly distant from the regression line for those parameters, but whether that distance indicates potential morphogenetic differences remains to be tested.

The fruit size differences among the studied *Olea europaea* cultivars were principally due to cell number, and not to cell size. Cell number has also been

shown to be the major determining factor for fruit size differences among cultivars of drupes such as peach (Scorza et al., 1991; Yamaguchi et al. 2002), Japanese apricot (Yamaguchi et al., 2004) and sweet cherry (Olmstead et al., 2007) as well as other fruits such as apple (Harada et al., 2005), blueberry (Johnson et al., 2011) and Japanese pear (Zhang et al., 2006). When wild genotypes are included in the comparisons, cell size also plays a role along with number (Yamaguchi et al., 2002; Harada et al., 2005).

In some species the fruit cell numbers which produce the genotypic variation in fruit size are established in the ovary by anthesis (tomato: Frary et al., 2000; peach: Scorza et al., 1991; strawberry: Cheng and Breen, 1992), while for Japanese apricot (Yamaguchi et al., 2004), Japanese pear (Zhang et al., 2005; Zhang et al., 2006), melon (Higashi et al., 1999) and blueberry (Johnson et al., 2011) the varietal difference in fruit size was attributed to the duration of the active cell division stage after bloom. In the examined olive cultivars, mesocarp cell division was substantial after bloom, producing a four- to thirteen-fold cell number increase depending on the cultivar. Rosati et al. (2009), based on dry weight, reported a significant ovary contribution to olive cultivar fruit size differences, suggesting ovary cell number could also be a factor in olive fruit size differences. The observed ovary cell numbers, however, were far less than the considerable cell number increase following bloom.

All cultivars showed similar timing of cell division activity with cell division dropping drastically at 8 weeks after bloom. Although that moment can only be calculated approximately with the current data, cell division period does not appear to be a major factor for fruit size differences among cultivars. Those differences were principally due to cell division rate, and occurred mainly during the rapid cell division period (Period I). In period II, in which a smaller but still considerable number of cells were formed, differences among cultivars tended to relate, although not as strictly, to final fruit size.

To our best knowledge the moderate but continued cell division during Period II, producing approximately one third of the final cell number, has not been shown previously in drupes. Such an amount of cell division subsequent to the intensive cell division phase may be a unique characteristic of olive fruits, or could have been overlooked in other fruits when cells were measured only in one

dimension, along the radius, a methodological limitation which has also been mentioned by Bollard, (1970), Coombe (1976) and Correlli-Grapadelli and Lakso (2004). Counting and measuring cells on an area basis adds a second dimension and refines our perceptive capacity, even though the cellular processes really occur in three dimensions (on a volume basis).

Mesocarp cell size, although not a determinant of fruit size differences, increased fifty times from bloom to fruit maturity, reflecting the importance of cell expansion as a driving force in fruit growth. Cells reached 20-29% of their final area in Period I, 69-78% in Period II. Due to the interaction of cell division and expansion in producing cell size (Green, 1976; Evans, 2000), and with cell division occurring simultaneously, the observed increases in cell size indicate active cell expansion in both periods of olive fruit growth. It is interesting to note, though, that even with quite different cell division rates among cultivars the cell size was similar throughout fruit development, suggesting a relationship or interaction between cell division and expansion processes in fruit growth and thus also implying differences in cell expansion among cultivars.

IV.1.6. Conclusion

In conclusion both mesocarp and endocarp tissues contributed to final olive fruit size, with a greater contribution by the mesocarp. As fruit size increased the mesocarp/endocarp ratio, a particularly important feature for table olives, increased due to a proportionately greater increase of the mesocarp than the endocarp. Within the mesocarp, genotypic fruit size differences were produced principally by cell division after bloom, consisting of different division rate, but not timing or duration, among cultivars. All cultivars produced the majority of their cells in the first two months after bloom, followed by previously unreported 15-30 % more cells in the following 6 months. Mesocarp cell expansion, although not a determinant of cultivar fruit size differences, was active and important throughout fruit growth. However since cell size showed consistent values among cultivars in spite of variable cell division, it is likely that cultivar differences in cell expansion accompany those in cell division.

IV.1.7. Acknowledgements

We thank Ester García-Cuevas for excellent technical assistance and the Andalusian Institute for Research and Training in Agriculture, Food and Fisheries (IFAPA) for use of the olive trees. This work was supported by Spanish Ministry of Science and Innovation ERDF co-financed funds [grant number AGL2009-07248].

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IV.2. Capítulo 5:

Quantitative analysis of cell organization in the external region of the olive fruit

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(Submitted to International Journal of Plant Sciences February 2012)

IV.2.1. Abstract

The definition of the cells which constitute the exocarp of fleshy fruits is often vague, with contradictory descriptions in the same species consisting of the epidermis plus none or varying numbers of underlying cell layers. This study investigates how cell dimensions and number per cell layer, and their relation with genetically based fruit size differences, can contribute to characterizing tissue organization in the external fruit region, using the olive drupe as a model. We determined cell area, radial and tangential widths, and cell number in the epidermis and 20 subepidermal cell layers in mature fruits of four olive cultivars ranging in fruit size. Variation of these measurements among cell layers and the implied cellular contributions to fruit expansion revealed the existence of two very different subepidermal regions, but with constant widths and layer numbers for all cultivars: 1) the first four cell layers (1-4) with similar behavior to the epidermis and 2) the following five (5-9) which were more similar to the mesocarp. The results provide new insights about external fruit region cell patterns and suggest that layers 1-4 form, together with the epidermis, a multiseriate exocarp, and layers 5-9 constitute an outer mesocarp.

IV.2.2. Introduction

Drupe fruits, a type of fleshy fruit, are characterized by a thin protective exocarp or epicarp, a fleshy mesocarp, and an inedible stony endocarp surrounding the seed (Roth 1977). Of these tissues, fruit developmental biology research has mainly evaluated the mesocarp and endocarp, the two principal tissues in size and energetic cost, while the exocarp has been frequently neglected (Bollard 1970; Coombe 1976). Recent studies indicate the fundamental role of the exocarp in fruit growth and development, particularly mentioning the high cell division and metabolic activity observed in this tissue (Lemaire-Chamley et al. 2005; Bargel and Neinhuis 2005; Schlosser et al. 2008; Fu et al. 2010). The exocarp tissue also is thought to play key roles in fruit quality and in the interaction between fruits and their environment (Knee 2002; Jeffree 2006; Mintz-Oron et al. 2008). One of the obstacles to exploring and accurately interpreting the role of the exocarp in fruit biology is the difficulty in defining the limit between the exocarp and mesocarp, in contrast, for example, to the ease in visually distinguishing between parenchymatous mesocarp and sclerified endocarp tissues.

The fleshy fruit exocarp has been defined by some authors as the epidermis plus one or more subepidermal layers, but many others have considered the exocarp strictly as only the epidermis, formed by the epidermal cells and their cuticle (Roth 1977). Multiseriate exocarp definitions are usually based on the interpretation of a morphologically distinct subepidermal tissue, sometimes called a hypodermis, which differs from the mesocarp in cell size (either larger or smaller), metabolism and/or form (shape, cell wall characteristics) (Sterling 1953; Archibald and Melton 1987; Considine and Knox 1981; Lavee 1986; King et al. 1987; Yamaguchi et al. 2003; Mintz-Oron et al. 2008). In contrast, those authors who propose the strictly epidermal definition of the exocarp interpret the subepidermal layers as a part of the outer mesocarp or as a transitionary zone (King 1938; Bain and Robertson 1951; Roth 1977; Bobrov et al. 2005). In the same manner, even when the exocarp is considered to be multiseriate, different interpretations of the number of participating subepidermal layers occur. For example, in the sweet cherry fruit the number of subepidermal layers included in the exocarp varies between three to four (Demirsoy and Demirsoy 2004) and two to eight (Sekse 1995). Similar contradictions are also found in other (non drupe) fleshy fruits, such as the two (Homutová and Blazek 2006) to twelve (Simons 1980) subepidermal layers reported for the apple. In fleshy fruits, except for a tendency to relatively smaller

cell size, the external fruit cells are often quite similar in appearance to those of the rest of the fruit fleshy tissue, (Roth 1977), so definitions based principally on subjective observations are likely to vary. Modern image analysis tools for cell measurement, however, provide the means of precisely examining cell dimensions and number and could contribute valuable information regarding cellular patterns in this region.

Potential fruit size is genetically determined and is a desirable character in the selection and breeding of commercial fruit crops. Cell division and expansion are the principle cellular processes that produce the final size of fleshy fruits (Gillaspy et al. 1993). Comparative studies attribute cultivar fruit size variation to cell number (Scorza et al. 1991; Yamaguchi et al. 2004; Olmstead et al. 2007; Quilot and Génard 2008; Hammami et al. 2011), or to both cell number and size when wild genotypes are included in the comparisons (Yamaguchi et al. 2002; Harada et al. 2005). However, although exocarp cell activity is considered a relevant component of fruit development (Gillaspy et al. 1993; Lemaire-Chamely et al. 2005; Fu et al. 2010) it has generally not been considered in studies of fruit growth or with respect to genetically based size differences. Size has been found to be important for the interaction of fruits with environmental factors (Lescourret et al. 2001; Zeebroeck et al. 2006; Opara 2007; Wang et al. 2009), raising questions about whether or how the structure of the exocarp, considered the principal tissue involved in those interactions, varies in relation to fruit size. Considine and Brown (1981) used theoretical fruit-growth calculations to determine that the internal forces generated by fruit expansion increase with greater fruit size, and suggested that those forces directly influence the external fruit tissue structure.

The olive, *Olea europaea* L., fruit has basic drupe structure consisting of an exocarp, mesocarp and endocarp, and for which differing interpretations exist concerning exocarp cell layers (King 1938; Lavee 1986; Mulas 1994). Olive cultivars present a wide range of fruit size (Del Rio and Caballero 2008), and cultivar fruit size has been indicated in susceptibility to external biotic (Wang et al. 2009) and abiotic (Ferguson et al. 2010) factors. These features make the olive fruit a good model candidate for exploring exocarp structural characteristics and their relation to fruit size, as well as identifying the potential horticultural significance of this tissue.

In this study, using image analysis and statistical tools, we explore the variability of cell dimensions and number among the most external fruit cell layers and in relation

with cultivar fruit sizes. The results indicate important morphogenetic differences in the epidermis and particularly in the subepidermal tissues, which we use to reexamine the concept of the exocarp and suggest a new characterization of the relevant cellular layers. Furthermore, the comparison of fruits from cultivars of different fruit size provides useful insight into overall fruit developmental processes and, on a practical level, fruit susceptibility to environmental impact. To our knowledge, this is the first evaluation of its kind conducted on a fleshy fruit.

IV.2.3. Material and methods

IV.2.3.1. Plant material and experiment design

We evaluated fruits of four olive tree (*Olea europaea* L.) cultivars with a range of mature fruit size, which, in order from large to small are 'Manzanilla de Sevilla', 'Hojiblanca', 'Picual' and 'Arbequina' (Barranco 2004). The trees were twenty years old and planted at standard density of 270 trees ha^{-1} at the experimental farm 'Alameda del Obispo' of the Andalusian Institute for Research and Training in Agriculture, Food and Fisheries (IFAPA) in Cordoba, Spain. Four trees per cultivar were randomly distributed in each of four rows. They had a similar moderately high crop load, and were grown with irrigation under standardized cultivation conditions.

IV.2.3.2. Fruit preparation and measurement

At fruit maturity, determined by the onset of color change, five fruits per tree were sampled around the tree circumference and fixed in FAE (formalin: acetic acid: 60% ethanol = 2:1:17 v/v). Structural observations and measurements were performed on transverse sections of the mesocarp at the point of widest fruit diameter, obtained following rehydration and pitting (removal of the stony endocarp) of the fixed fruits. Complete 5 mm mesocarp slices were processed according to standard paraffin procedures, sectioned at 10-12 µm, and stained with toluidine blue O prior to paraffin removal (Sakai 1973). Although these methods are known to produce some tissue shrinkage (Ruzin 1999), we considered the procedures to be valid for the cultivar comparisons because the material was similar in nature, and because previous tests indicated that the shrinkage effect is consistent for olive fruits of different size, development and water status (Gucci et al. 2009).

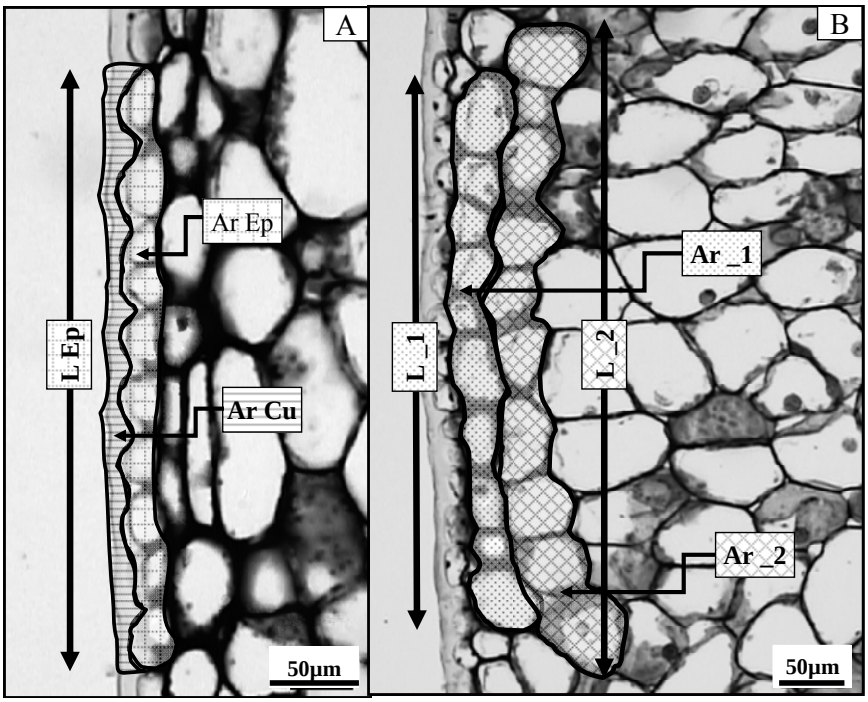
Quantitative observations of the external fruit tissue were made on the prepared sections with an image analysis system (Moticam 2500 and Motic Images Plus 2.0 ML, Motic China Group Co. Ltd., Xiamen, China) connected to ocular microscope (Motic BA 310, Motic China Group Co. Ltd., Xiamen, China). Measurements were carried out in three different locations around the transverse fruit section. In each location the combined area and length of ten cells forming a tangential layer, and separately for the cuticle covering the ten–cell group of epidermal cells, were measured in the epidermis and twenty successive subtending cell layers (Fig.5.1). Average cell area and average tangential cell width per layer were calculated based on combined area and combined tangential length of the ten measured cells, respectively. Likewise, cuticle thickness was calculated by dividing the combined cuticle area by the combined tangential length of the ten measured epidermal cells. The mean radial cell width was determined similarly, dividing the area of each ten-cell layer by its length, and in the case of the epidermis the cuticle was not included.

Fruit equatorial transverse diameter was measured before pit removal. Fruit diameter and the radial widths of the measured cell layers were used to calculate the circumference of each layer, which was then used to determine the total number of cells composing each layer in the fruit transverse section. The total cell number exterior to the pit was determined in the transverse sections according Hammami et al. (2011). In analyzing and discussing fruit tissue size we used the term fruit flesh for all fruit tissue exterior to the pit, in order to avoid the terms mesocarp and exocarp prior to our interpretations.

IV.2.3.3. Data analysis

The experimental design was completely randomized with four replications (trees) per cultivar and five fruits per replication. For each fruit the means of the values of the three zones were used. All analysis was performed using *Statistix 8* (Analytical Software, Tallahassee, USA). A one-factor ANOVA was performed to test the influence of cultivar on the cuticle and epidermal cell dimensions, and means were compared by LSD test at $P<0.05$. Prior to ANOVA, data normality and variances homogeneity assumptions were tested using the Shapiro-Wilk and Bartlett's tests, respectively.

Fig.5.1 Micrographs of the olive fruit exterior cells in transverse section showing the measurements which were made. The combined cell area (Ar) and length (L) of groups of ten cells in tangential layers were determined for the epidermis (Ep), cuticle (Cu) and twenty centripetally successive subtending cell layers (numbered consecutively towards the fruit interior). Those values were used to calculate mean cell area, tangential cell width, radial cell width and cuticle thickness as described in the text. (A) Measurements of the epidermis. (B) Measurements of the first two subepidermal layers, _1 and _2.



The twenty successive tangential cell layers which were measured were identified by an order number (N) from 1 to 20, starting with the first subepidermal layer and progressing centripetally. Cell size and number values were expressed as means plus standard error of the mean (±SE). Pearson coefficient correlation procedure was used to determine the relationship between fruit size and different structural parameters.

Mean cell-size of each layer was contrasted with that of the previous (more external) and succeeding layers, and with the maximum cell area observed in the twenty evaluated cell layers using Comparison with Largest Value statistical procedure.

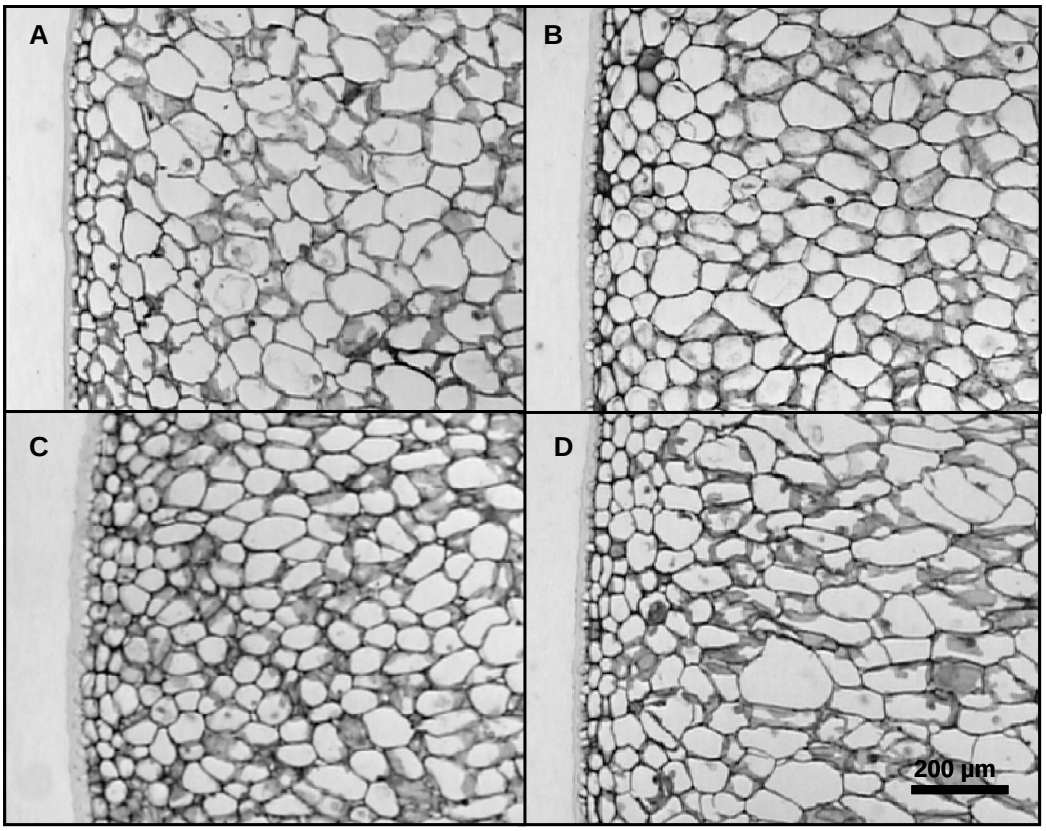
Comparison with Smallest Value statistical procedure was used to compare the relative increase of cell area between each two successive layers. Linear regression analysis was used to test the relationships of the cell measurements to cell layer order and to fruit size. To perform this analysis data normality was confirmed using the Shapiro-Wilk test.

IV.2.4. Results

IV.2.4.1. Visual observation

The epidermal cells, observed in transverse sections, are small, wider tangentially than radially, and covered by a continuous and conspicuous cuticle (Fig.5.2). All cells interior to the epidermis are parenchymatic in nature, and their cell walls generally similar in appearance with toluidine blue staining (Sakai 1973). In the transverse sections the most external cells are isodiamteric in shape and smaller than the more internal cells, which also tend to elongate in the radial dimension. Although there are different cell sizes and shapes, no clear visual distinction indicates different tissues or cell types.

Fig.5.2 Micrographs of the external fruit region the four studied olive cultivars, presented in decreasing fruit size order. A, 'Manzanilla'. B, 'Hojiblanca'. C, 'Picual'. D, 'Arbequina'.



IV.2.4.2. Measurements of the epidermis

Epidermal cell number was similar for all cultivars, but a definite cultivar influence was observed in all other epidermal parameters (Table 5.1). 'Picual' and 'Hojiblanca' presented thick cuticles and high cuticle area per cell, 'Arbequina' had a thick cuticle but low cuticle area per cell, and 'Manzanilla' had both a thin cuticle and low cuticle area per cell. The three cell-size parameters, radial width, tangential width and area, differed substantially among cultivars, but tangential width was always greater than radial width. Significant correlations were found between cultivar fruit diameter and some of the epidermal dimensions (Table 5.1). Thus thinner cuticles were found with greater fruit size, but the amount (area) of cuticle per epidermal cell was not affected. Epidermal cell area and tangential cell width increased linearly with fruit size, while the radial cell width was reduced.

Table 5.1 Epidermal cell and cuticle parameters and their correlation with transverse fruit diameter for the different olive cultivars. Cultivars are listed in descending fruit size order. Different letters indicate significant differences within columns at $P< 0.05$ by LSD test.

Cultivar	Cuticle		Epidermal cell size[1]			Epidermal cell number[2]
	Thickness (μm)	Area/cell (μm²)	Radial width (μm)	Tangential width (μm)	Area (μm²)	
Manzanilla	10.12 c	291.40 b	15.61 c	29.60 a	462.86 b	2216.7 a
Hojiblanca	13.46 b	379.89 a	17.11 b	27.51 b	470.90 ab	2130.4 a
Picual	14.50 a	362.04 a	20.34 a	24.99 c	508.94 a	2126.1 a
Arbequina	13.23 b	279.68 b	19.12 a	21.33 d	409.11 c	2134.5 a
Correlation with fruit size	-0.59*	0.22 ns	-0.58*	0.96***	0.52*	0.07 ns

[1]Excluding cuticle;

[2]Total number of epidermal cells in the fruit median transverse section

ns, not significant; *, Significant at $P<0.05$; ***, Significant at $P<0.001$

IV.2.4.3. Cell dimensions in successive subepidermal layers

The cell areas observed in the first twenty subepidermal cell layers showed a consistent pattern in all cultivars (Fig.5.3). Cell area increased centripetally towards the fruit interior, forming a gradient of increasing cell size between successive cell layers. In the first nine layers the gradient was notable and cell size was significantly lower than the highest value found in the twenty evaluated layers. In subsequent layers cell size was similar among layers and to the highest value. In all cultivars cell size increased five fold from approximately 1000 to 5500μm² between the first and the ninth layer.

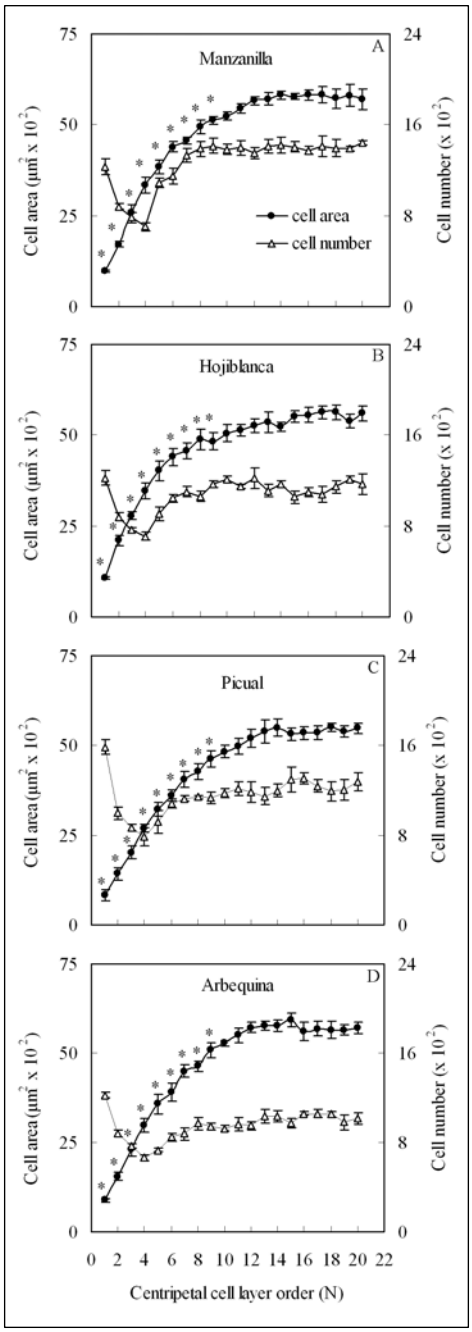


Fig.5.3 Cell area (●) and cell number (Δ) for each of the twenty first successive subepidermal cell layers, in four olive cultivars. Cell number is the total number of cells in each cell layer in the fruit transverse section. Vertical bars represent the standard error of 20 replicates (Five fruits per tree, and four trees per cultivar). * indicates significant differences with the largest cell area value in the twenty layers ($P< 0.05$). N indicates the cell layer order, with number 1 closest to the exterior.

In the first twenty cell layers total cell number per layer in the complete fruit transverse section was similar among cultivars in its pattern, although it differed somewhat in value (Fig.5.3). Centripetally, starting with the first subepidermal layer, cell number decreased rapidly for layers 1-4. From the fifth layer, cell number started to increase, then became constant by the ninth layer. For the subsequent internal layers, both cell size and number remained unchanged. Based on these results and those of cell size above, for subsequent analysis we considered only the first nine subepidermal layers.

The relative cell area increase between layers, calculated as cell area $_{N+1}$/ cell area $_{N}$, where N is the cell layer order, at first decreased rapidly and then reached a stable value in all cultivars (Fig.5.4). Comparison of the successive values with the lowest observed value indicated that relative cell area increase between layers was significantly higher in layers 1-4 but not in layers 5-9.

Cell tangential and radial width increased linearly in relation to centripetal cell layer order in layers 1-4 (Fig.5.5). In the following five layers (layers 5-9) the relationship changed: radial cell width continued to increase linearly, although to a lesser degree than at first, and, in contrast, tangential cell width became uniform. In general, the linear regressions (Fig.5.4) were stronger for radial than tangential growth.

IV.2.4.4. Cell dimensions in relation to fruit size

The relationships between cell parameters and cultivar fruit size varied according to cell layer order, with layers 1-4 behaving very differently from layers 5-9 (Table 5.2). Linear correlations were found between fruit diameter and both tangential cell width and cell area for layers 1-4, while this relationship was absent for layers 5-9. Those correlations were higher for tangential cell width than for cell area, but in both cases decreased with centripetal layer order. In direct contrast to cell area and tangential width, cell number per layer increased with cultivar fruit size only in layers 5-9. Radial cell width, however, was not related to cultivar fruit size in any layers. Thus, overall, fruit size was associated with cell size, specifically in the tangential sense, in layers 1-4, whereas in layers 5-9 it was associated with cell number.

Transverse fruit diameter was significantly different among all cultivars (Table 5.3). The percentages of fruit flesh cell number and area pertaining to layers 1-4 and to layers 5-9 both decreased significantly in relation to cultivar fruit size (Table 5.3). These

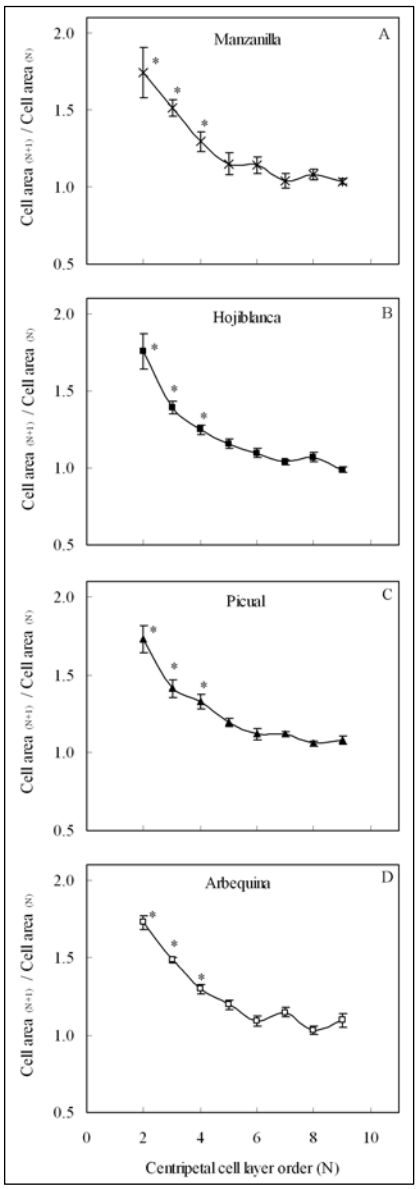


Fig.5.4 Relative increase of cell area between each two successive cell layers, calculated as Cell area (N) / Cell area (N+1) for each of 9 first successive subepidermal cell layers, in four olive cultivars. Vertical bars represent the standard error of 20 replicates (Five fruits per tree, and four trees per cultivar). N indicates the cell layer order.* indicates significant differences with smallest observed value (P< 0.05).

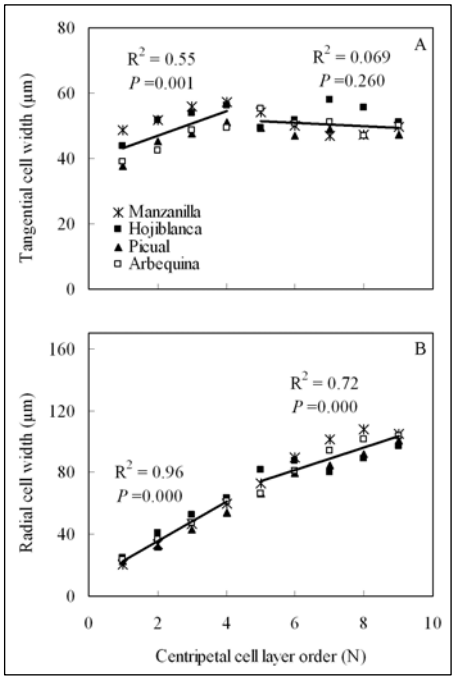


Fig.5.5 Linear regressions of the tangential (A) and the radial (B) cell widths with the centripetally cell layers order, for subepidermal layers 1-4 and 5-9. Correlation is significant when P<0.05. Each point represents the average value per cultivar.

Table 5.2 Relationship between cell measurements, in each one of the first nine subepidermal layers, and the cultivar fruit size. ns, not significant; *, significant at $P<0.05$; ***, significant at $P<0.001$.

Cell measurements	Centripetal cell layer order								
	1	2	3	4	5	6	7	8	9
Cell area	0.57*	0.58*	0.56*	0.54*	0.39 ns	0.40 ns	0.31 ns	0.41 ns	0.21 ns
Tangential cell width	0.75**	0.83***	0.77***	0.55*	-0.04 ns	0.14 ns	0.07 ns	0.31 ns	0.24 ns
Radial cell width	-0.23 ns	0.09 ns	0.27 ns	0.26 ns	0.38 ns	0.25 ns	0.05 ns	0.17 ns	-0.02 ns
Cell number[1]	-0.08 ns	-0.05 ns	0.38 ns	0.43 ns	0.78***	0.75***	0.76***	0.66**	0.87***

[1]Total number of cells in the corresponding cell layer in the fruit transverse section

two layer groups together contributed 28% of the cell number, but only occupied 12% of the fruit flesh area. Neither the combined radial width of layers 1-4 (Fig.5.6B) nor that of layers 5-9 (Fig.5.6C) was correlated with fruit size, but a significant inverse correlation was found between fruit size and epidermis width (Fig.5.6A).

Table 5.3 Fruit size and percentages of fruit flesh area and fruit flesh cell number pertaining to the first four (1-4) and the internal five (5-9) subepidermal cell layers, for the different olive cultivars. Cultivars are listed in descending fruit size order. Different letters indicate significant differences within columns at $P< 0.05$ by LSD test.

Cultivar	Fruit diameter (mm)	Layers 1-4		Layers 5-9	
		% of fruit flesh [1] area	% of fruit flesh [1] cell no.	% of fruit flesh [1] area	% of fruit flesh [1] cell no.
Manzanilla	20.22 a	3.37 c	10.17 c	6.82 c	12.88 c
Hojiblanca	18.66 b	3.98 bc	10.12 c	7.26 c	13.95 c
Picual	16.94 c	4.40 b	13.70 b	8.70 b	15.51 b
Arbequina	14.48 d	5.59 a	16.78 a	10.21 a	18.45 a

[1]The term fruit flesh indicates all fruit tissue exterior to the pit (explained in materials and methods)

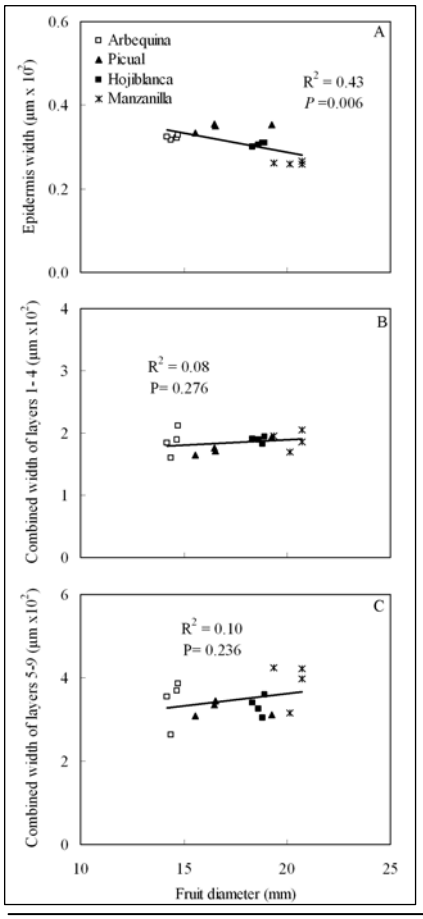


Fig.5.6 The relationships of transverse fruit diameter with the epidermis (A) and subepidermal layers 1-4 (B) and 5-9 (C) widths. Each point represents the average for 5 fruits per tree.

IV.2.5. Discussion

IV.2.5.1. Cellular dimensions and numbers in the external fruit zone

The olive epidermis showed a similar general structure to that of other drupe fruits (Roth 1977). That is, the epidermal cells, observed in transverse sections, present small size (about 450μm^2), are covered by a continuous cuticle and are wider tangentially than radially. The cuticle of olive fruits, however, is much more developed than in other commercial drupes with larger fruits. For example cuticle thickness is between 3 to 4μm in plum fruit (Sterling 1953) and between 2 to 5 μm in the nectarine fruit (King et al. 1987), whereas it is 10 to 14 μm in the measured olive fruits. Furthermore the cuticle occupies a greater proportion of the epidermis, about 40%, as compared to approximately 20% for sweet cherry fruit (Demirsoy and Demirsoy 2004), a drupe of similar size. These results suggest an important role of the cuticle in olive fruit biology.

Smaller cell size in the zone directly beneath the epidermis than in the rest of the mesocarp has been commented for different fleshy fruits (Apple: Bain and Robertson 1951; Avocado: Schroeder 1953; Cucumber: Marcelis and Hofman-Eijer 1993). Our measurements in olive fruits revealed that the subepidermal region was not only composed of small cells, but also that cell size increased centripetally, to forming a clear gradient among successive cell layers. The zone in which this gradient occurred was limited to an equal nine subepidermal layers in all genotypes, representing only 4 to 8% of the total fruit radius. Since cell size is the combined result of cell division and expansion (Green 1976; Evans 2000), the observed differences in cell size suggest differences in the activities of these two cellular processes.

Observations of fleshy fruit cell form have usually reported elongation in the tangentially direction for the cells directly subjacent to the epidermis, while elongation in the radial sense is more typical for the internal fruit mesocarp cells (King 1938; Sterling 1953; Skene et al. 1966; Archibald and Melton 1987), apparently related to physical pressures in fruit expansion. However, the tangential and radial cell width changes we found across the most external nine subepidermal layers, suggest a dimensional complexity of both cell division and expansion in those layers. Thus the cell size increases among layers 1-4 resulted from cell expansion in both tangential and radial directions, while increasing cell size in layers 5-9 was produced principally by radial expansion.

Differences among the first nine subepidermal layers were not limited to cell dimensions, but also were found for fruit cell number per layer. Three successive patterns, that are centripetally decreasing, then increasing, then constant cell number, occurred radially across the nine cell layers. High cell division activity has been frequently attributed to the outer zone of fleshy fruit tissue, by both anticlinal divisions that determine the cell number per layer, and periclinal divisions that determine the number of layers (Skene et al. 1966; Considine and Knox 1981; King et al. 1987). The high proportion of fruit flesh cell number (28%) pertaining to the first nine subepidermal layers indicates high cell division, while the cell number differences we observed among layers directly reflect differences in anticlinal cell division.

IV.2.5.2. Relationships of fruit size to epidermal and subepidermal cell patterns

Cell size in the epidermis and in subepidermal layers 1-4 increased linearly with cultivar fruit size, but cell number did not. In contrast, cultivar fruit size variability is generally due to mesocarp cell number rather than cell size in olive (Hammami et al. 2011) and other drupes (Scorza et al. 1991; Yamaguchi et al. 2004; Olmstead et al. 2007; Quilot and Génard 2008), a pattern which also occurred in layers 5-9. This contrasting behavior indicates a morphogenetic difference between layers 1-4, which behave in a similar manner to the epidermis, and layers 5-9, which behave in the same manner as the mesocarp.

Similar to cell area, tangential width of the epidermal cells increased linearly with fruit size, but greater fruit size was associated with a thinner epidermis, due to both thinner cuticle and reduced radial cell width. Thus increased fruit size involves reduction in the radial dimension and increase in the tangential dimension, suggesting thinning from physical stretching of the epidermis. This mode of action is supported by Considine and Brown (1981), who used calculations to demonstrate that with greater fruit size internal fruit expansion forces increase, modifying cell geometry as a consequence of tangentially oriented mechanical stress along the fruit surface. Epidermis thickness is considered to protect fruits against biotic and abiotic external factors (Manandhar et al. 1995; Romig 1995; Kubo and Hiratsuka 1999; Hong et al. 2008; Ghafir et al. 2009). In this sense, the high sensibility of 'Manzanilla de Sevilla' fruits to mechanical damage (Jiménez et al. 2012) could probably be due in part to this cultivar's thinner epidermis providing less protection.

The decreasing proportion of area occupied by layers 1-4 in increasingly larger fruit, as well as cell size differences in that region among cultivars, could impact interaction with external factors and consequently fruit quality. Indeed, cell dimensions in the external fruit zone appear to influence fruit quality and susceptibility to physical damage in a number of fruit crops (Sweet cherry: Yamaguchi et al. 2003; Pear: Hong et al. 2008; Apple: Ouattara et al. 2011), but further experimental testing is necessary for confirmation and general applicability of that proposition.

IV.2.5.3. Tissue organization in the external fruit region

In fleshy fruits, the identification of a subepidermal tissue which is different from the mesocarp in size or form, frequently considered a hypodermis, has been the main criterion for determining whether the exocarp is multiserate or uniserate in nature (Roth 1977). Our quantitative approach demonstrates the existence of definitive differences in cell size and number across the most external region of the fruit which can provide valuable information regarding exocarp tissue composition. For the four cultivars and all cell layers we evaluated (epidermis and 20 subepidermal layers), the cellular patterns changed centripetally at two consistent positions along the fruit transverse radius, thus defining three different regions: 1) the epidermis plus the first four immediately subepidermal layers (layers 1-4), 2) the five centripetally internal layers (layers 5-9), and 3) the following eleven internal cell layers (layers 10-20).

A number of characteristics indicate that the most internal layers we studied, layers 10-20, are part of and form a continuum with the rest of the mesocarp tissue. First of all, cell size is constant among all eleven cell layers and higher than in the first nine subepidermal layers, in agreement with the high cell expansion activity characterizing mesocarp cells in comparison with those located in fruit exterior (Lemaire-Chamely et al. 2005; Schlosser et al. 2008; Fu et al. 2010). Secondly, cell area in these internal eleven cell layers was very similar to that found in a previous study for cells throughout the mesocarp in the same olive cultivars (Hammami et al. 2011). Finally, when a multiseriate exocarp has been described in olive fruits, a maximum of four subepidermal layers has been reported (Lavee 1986; Mulas 1994), while no further internal layers have ever been attributed to any other tissue than mesocarp.

The region composed of layers 5-9 was much more similar to the mesocarp than to the external layers 1-4. In fact, the main difference observed between those five layers

and the mesocarp in general was cell size, but both behave in a similar manner in relation to cultivar fruit size, and exhibit similar cell radial elongation tendency. These five cell layers could be considered to compose an outer mesocarp, using the name given by King (1938) to the smaller cells he observed in the outer zone of olive fruit flesh.

The first four (1-4) subepidermal layers were unmistakably distinct. In those layers the cell size gradient was produced by both radially and tangentially oriented cell expansion, in contrast to only to radial cell expansion in the remaining layers. In the grape berry the exocarp and mesocarp tissues also differ in the orientation of cell expansion, as well as in its timing (Huang and Huang 2001; Schlosser et al. 2008). Cell size was clearly smaller than that of the mesocarp cells, coinciding with cell size as an important criterion for identifying a hypodermis (Roth 1977). Besides, as we indicated above, in layers 1-4 cell size but not cell number was associated with genetically based fruit size differences, in direct contrast with the more internal layers and the general behavior of fruit mesocarp tissue (Scorza et al. 1991; Yamaguchi et al. 2004; Olmstead et al. 2007; Quilot and Génard 2008; Hammami et al. 2011). The greater tangential cell expansion we observed in the layers 1-4 has also been noted in grape berries by Schlosser et al. (2008), who found that internal expansion produced pressure which forced the hypodermal cells tangentially. Besides, tissue expansion forces of this nature are consistent with the high correlation between fruit size and tangential cell width in the layers 1-4. All of these distinctive cellular characteristics of the layers 1-4 indicate behavior as an independent tissue, different from the mesocarp.

The epidermal cells were similar to subepidermal layers 1-4 in their tendency to tangential elongation. They were also similar in that cultivar fruit size variability was related to cell size rather than the cell number. In other fleshy fruit Considine and Knox (1981) found similarities in epidermal and hypodermal cellular development during fruit growth, which they interpreted as suggesting a common nature or origin of the epidermis and hypodermis, an assessment which strengthens the interpretation of the hypodermal nature of the layers 1-4 in olive fruit. All of these results provide strong evidence that olive exocarp is multiseriate in nature, composed of, the epidermis plus four hypodermal layers, independent of cultivar and fruit size. Further testing of this hypothesis could be aimed at examining cell wall thickness and cell composition, found to differ between the grape berry exocarp and mesocarp (Huang and Huang 2001;

Schlosser et al. 2008), and it would also be of interest to evaluate more olive genotypes as well as other species of drupes.

IV.2.6. Conclusion

The results obtained in this study and the tissue organization they suggest are summarized schematically in Figure 5.7. Successively increasing cell size was found in the first nine subepidermal cell layers in all cultivars. These nine layers are divided in two parts, the first 1-4 layers and the following 5-9 layers, which showed consistent differences. Cell size and number distinguish the first zone as a hypodermis, and indicate a multiseriate exocarp formed by the hypodermis plus the epidermis. The number of subepidermal layers forming the hypodermis in olive fruit, four layers, was consistent among cultivars. In the exocarp tissue, genetically based fruit size difference correlated with cell size but not with cell number, in contrast to current and previous observations in the mesocarp. The five cell layers immediately internal the exocarp, composed of smaller cells than the mesocarp but otherwise similar in their behavior, appears to be an outer mesocarp. This analytical focus provides new insights regarding tissue organization in the olive fruit, and merits testing during the course of fruit development and in other fleshy fruit species.

IV.2.7. Acknowledgements

The authors wish to thank Trinidad Manrique and Ester García-Cuevas for excellent technical assistance in fruit sampling and histological preparation. The study was funded by Spanish Ministry of Science and Innovation grant AGL2009-07248 and accompanying FEDER funds.

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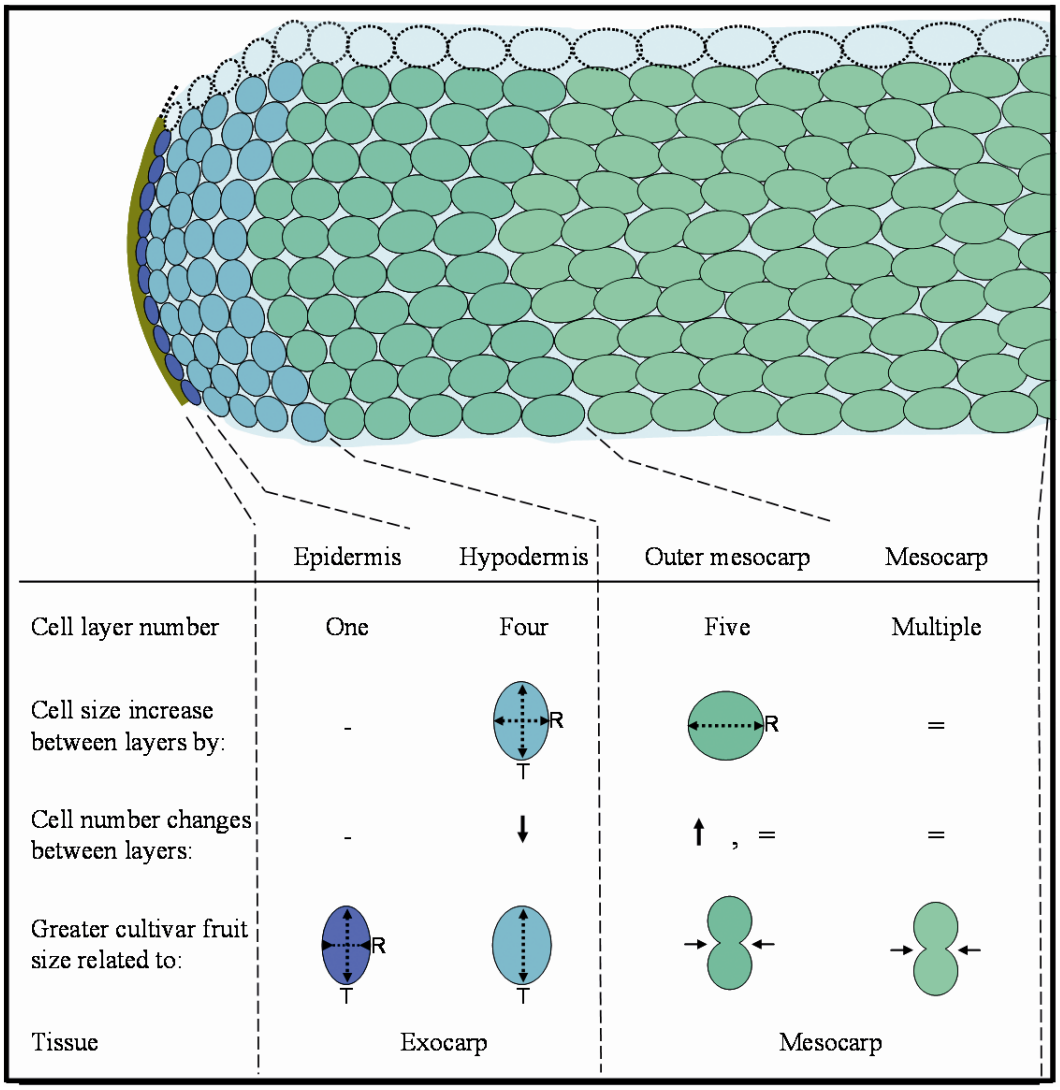
Fig.5.7 Diagram of proposed cellular organization in the external region of the olive fruit. Successive cell layers are shown from the epidermis (left) proceeding centripetally within the fruit, and their observed characteristics summarized below. Letters T (tangential) and R (radial) and arrows indicate orientation of cell activities: Dotted arrows inside the cell show the orientation of size change (cell expansion or shape modification). Simple external arrows show cell division, with orientation only for the outer mesocarp (not evaluated in the other mesocarp layers). Measurements were based on groups of ten cells per layer as explained in the text.



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V. DISCUSIÓN GENERAL

V.1. Discusión general

En esta tesis se han realizado estudios de un amplio rango de órganos y estructuras de la planta, desde la arquitectura de la joven planta vegetativa hasta las flores y el fruto. También se han estudiado un rango amplio de niveles de organización, entre células y su contenido, tejidos, órganos y agrupación de órganos en la planta completa. Con todo ello, se ha pretendido profundizar en los procesos del crecimiento y desarrollo del olivo en base de un análisis preciso y de nuevos enfoques metodológicos.

V.1.1. Desarrollo vegetativo

Diferentes investigadores, según el objetivo de su estudio, han desarrollado distintas estrategias para describir la arquitectura de la planta, desde medidas simples y directas hasta la simulación y la reconstrucción virtual de la misma (Godin, 1999; Barthélémy y Caraglio, 2007). Para reducir la complejidad de las medidas implicadas en la arquitectura y permitir la evaluación de un alto número de genotipos, el enfoque principal adaptado hasta el momento en los programas de mejora ha sido evaluar parámetros cuantitativos directos y simples, como el volumen de la copa, altura de la planta y diámetro del tronco. Dichos parámetros, además, han sido utilizados no tanto como descripciones estructurales sino principalmente para caracterizar el vigor del árbol, tanto en los frutales en general (Zimmerman, 1972; Visser, 1976; Hartmann y Engelhorn, 1992) como en el olivo (Villemur, 1995; Lauri et al., 2001; Mezghani et al., 2001; De la Rosa et al., 2006; Rallo et al., 2008). Con el fin de comprobar y avanzar sobre el uso de estas formas de evaluación, en la presente tesis se ha propuesto usar dos metodologías diferentes de evaluación, una cuantitativa y una cualitativa, y comparar la información que proporciona cada una.

En el primer trabajo de la tesis se evaluaron parámetros cuantitativos, unos básicos como los previamente descritos y otros nuevos para evaluar características complejas de la arquitectura de la planta. Estas medidas cuantitativas permitieron determinar una edad mínima, a partir de los 9 meses, para la evaluación temprana de la arquitectura y del vigor de las plantas de semilla de olivo. Sin embargo, hemos notado dos principales dificultades en este primer trabajo que son: la

cantidad de tiempo y esfuerzo que se necesita para evaluar todos los parámetros de forma cuantitativa y la imposibilidad de usar la información obtenida para la caracterización de la arquitectura completa de la planta.

En el segundo trabajo, se usaron criterios cualitativos basados en los conceptos morfogenéticos y en las medidas cuantitativas, con el objetivo de presentar una metodología de evaluación sencilla y sólida a la vez.

Utilizando ambos tipos de características, los individuos evaluados se agrupan de un modo parecido según sus padres, lo que no solo indica la heredabilidad de la arquitectura sino también que las dos metodologías proporcionan una información muy similar. Teniendo en cuenta esta similitud, la facilidad y la rapidez de la evaluación cualitativa, en contraste con la evaluación cuantitativa del primer trabajo, y su capacidad de reportar una caracterización sólida de la planta completa, la hacen mucho más interesante para los mejoradores.

Para alcanzar el avance deseado de considerar la arquitectura en la selección de nuevas variedades, no es suficiente usar una forma de evaluación sencilla y sólida. También es esencial elegir los parámetros más eficientes e interesantes para describir la arquitectura en base de diferentes criterios como la baja correlación entre ellos, su alta heredabilidad y su capacidad de reflejar la diversidad fenotípica (Costes et al., 2006, Segura et al., 2006). Los resultados de los dos estudios realizados en esta tesis permiten diseñar unos criterios simplificados que integran los dos tipos de parámetros, teniendo en cuenta la relevancia indicada por los análisis y la simplicidad relativa de las observaciones cualitativas frente a las medidas cuantitativas. De cada estudio se eligieron 5 parámetros como los más relevantes, que entre los cuantitativos fueron (1) 'Primary shoot top diameter', (2) 'Primary Shoot Conicity', (3) 'Longest Secondary Shoot Internode Length', (4) 'Secondary Shoot Number' y (5) 'Secondary Shoot Insertion Angle'; y entre los cualitativos (1) 'Main vertical axis', (2)'Preferential distribution of Lateral Shoots', (3)'Dominant length of Lateral Shoots', (4) 'Branch orientation' y (5) 'Branch bending'. De estos 10 parámetros elegidos, se puede eliminar la última medida cuantitativa 'Secondary Shoot Insertion Angle' porque se evalúa cualitativamente a través de 'Branch orientation'. Los otros parámetros cuantitativos relevantes no están reflejados en los cualitativos, y los primeros tres

de ellos ('Primary Shoot Top Diameter', 'Primary Shoot Conicity', y 'Longest Secondary Shoot Internode Length') son considerados difíciles de evaluar de forma visual (Segura et al., 2009). Por consecuencia, proponemos mantener los parámetros cuantativos (1) – (4) en conjunto con los cinco parámetros cualitativos para realizar la evaluación de la arquitectura en los programas de mejora. Estas medidas integradas pueden ser comprobadas en futuro estudios, particularmente en relación con criterios de importancia agronómica como la duración del periodo juvenil o improductivo y la adaptación a diferentes diseños de plantación.

V.1.2. Desarrollo reproductor

La productividad del olivo como cultivo comienza una vez superado el periodo juvenil o improductivo, cuando el árbol desarrolla la capacidad de reproducción sexual manifestada en la formación de flores y después frutos. El potencial productivo de cada año empieza por la formación de las flores, que se determina a diferentes niveles de organización morfogenética y en diversos momentos, desde la diferenciación de las yemas, el desarrollo de las inflorescencias y flores en cantidad (número por planta) y calidad (desarrollo de órgano y tejidos), hasta la fertilización del óvulo y su desarrollo en fruto (Martins y Rapoport, 2006). El objetivo de la parte del desarrollo floral de esta tesis fue estudiar la respuesta de estos procesos a un riesgo de déficit hídrico en diferentes momentos del desarrollo floral. El estudio llevado a cabo ha proporcionado una información importante a nivel metodológico, en relación con la biología del desarrollo floral, y también a nivel práctico agronómico.

Planificar tratamientos de déficit hídrico controlado en el periodo de desarrollo floral del olivo en el campo es muy complicado debido al alto riesgo de lluvia. Hartmann y Pantesos (1961) utilizaron macetas de pequeño tamaño para evitar este problema, sin embargo, esto ha conducido a un estrés muy severo y a condiciones de ensayo muy diferentes a las del campo. Para reducir esta complicación hemos recurrido a realizar el ensayo en macetas de gran tamaño y protegidas de la lluvia. Por otra parte, se han comparado dos formas de muestro de las flores e inflorescencias, en brotes previamente marcados o mediante un muestro al azar, generalmente utilizadas en este tipo de estudio (Martins et al.,

2006; Castillo-Llanque y Rapoport, 2011). Los resultados obtenidos validaron las dos metodologías de muestreo.

El desarrollo floral se ha visto afectado de modo diferente según el momento de aplicación del déficit hídrico. Durante el periodo de desarrollo de las inflorescencias el déficit hídrico provoca una importante reducción en los parámetros de floración, mientras que en el periodo final de formación de flores dificulta la polinización y el proceso de fecundación. Una reducción en la disponibilidad de agua en el periodo de floración y cuajado inicial parece impedir la polinización, debido a la obstaculización de la apertura de los pétalos. Sin embargo, todos estos tratamientos conducen a una reducción en la producción final de frutos en peso y número totales, a pesar del aumento de tamaño de los frutos mediante los mecanismos de compensación conocidos en el olivo (Gucci et al., 2007) y en los frutales en general (Corelli-Grappadelli y Lakso, 2004)

El tamaño final del fruto y su variabilidad genotípica es el resultado del crecimiento y desarrollo de los diferentes tejidos que lo componen, mediante los procesos de división y expansión celular (Gillaspy, 1993; Seymour et al., 2008; Gucci et al., 2009). Sin embargo, en la mayoría de los estudios del crecimiento de los frutos carnosos tipo drupa (cereza: Olmstead et al., 2007, melocotón: Yamaguchi et al., 2002; Quilot y Génard, 2008; albaricoque: Yamaguchi et al., 2004), este tamaño ha sido analizado en base del crecimiento del mesocarpo, como tejido dominante, sin considerar la contribución del endocarpo y del exocarpo. Los resultados de la última parte de la presente tesis indican la importancia de considerar todos los tejidos para llegar a comprender de modo integrado el crecimiento y desarrollo de la aceituna, su calidad y la variabilidad genética de su tamaño. También, como en los capítulos anteriores, los resultados obtenidos demostraron la relevancia de la metodología adaptada para profundizar nuestro conocimiento en estos aspectos.

La evolución temporal del número de células observadas en los cortes transversales indicó que la división celular en el mesocarpo de la aceituna experimenta una primera fase de actividad intensa hasta aproximadamente las 8 semanas después plena floración. Esta fase está seguida por una segunda fase más larga y de actividad reducida, pero en la cual se produce alrededor del 25% del número total de células. El aumento en tamaño celular ha sido continuo durante

todo el crecimiento del fruto. Sin embargo, los frutos carnosos, incluyendo las drupas, se han caracterizado generalmente por una expansión celular lenta con una alta división celular durante un corto periodo, directamente después antesis, seguido por una considerable aceleración de la expansión, mientras que se detiene la división (Bollard, 1970, McGarry et al., 2001; Scorza et al., 1991; Yamaguchi et al., 2004). Este contraste de interpretación puede deberse a la forma de medir el número y el tamaño celular, ya que generalmente se ha medido en una sola dimensión a lo largo de un radio (Scorza et al., 1991; Yamaguchi et al., 2004; Zhang et al., 2006), o puede reflejar una diferencia morfogenética entre la aceituna y otras drupas. La medición de las células a lo largo del radio es un procedimiento experimental directo y factible, pero está limitado porque únicamente representa la contribución de una dimensión del crecimiento y división celular.

Las medidas que generalmente se realizan usando el radio del fruto no solamente pueden limitar la percepción del tamaño y número celular del tejido sino también la del tamaño del mesocarpo. Esta limitación, inicialmente notada en estudios de la aceituna por Rallo y Rapoport (2001), se debe al hecho de no tener en cuenta la forma completa del mesocarpo, un tejido que crece al exterior de otro, el endocarpo. En realidad, la limitación metodológica no reside en la dimensión que se mide, es decir, en la forma de medir, sino en la de calcular o analizar sin compensar teniendo en cuenta el radio interno (radio del endocarpo). Teniendo en consideración todos estos factores, la realización de las medidas sobre el área transversal como se ha hecho en este trabajo reduce una serie de faltas y mejora mucho la percepción del crecimiento. Sin embargo, las medidas por área todavía pueden estar limitadas, particularmente por usar solamente cortes transversales y de la zona central del fruto, y, por consecuencia, en cómo reflejan las contribuciones de expansión y división celular al volumen del mesocarpo. También existen limitaciones metodológicas debido a basar la medida del número y tamaño celular en el conteo de células en un área determinada, en lugar de poder usar medidas totalmente independientes para cada caso.

Para estudiar los procesos celulares en la zona externa del fruto hemos desarrollado, usando un sistema de análisis de imagen, una metodología basada en evaluar el número y las dimensiones celulares de la epidermis y las 20 capas subepidérmicas sucesivas siguientes, en la sección transversal del fruto. Los

resultados obtenidos han permitido definir los diferentes componentes de la zona externa del fruto. La epidermis y las primeras 4 capas subepidérmicas (1-4), que manifiestan un comportamiento celular muy diferente al de las células del interior del mesocarpo, forman el exocarpo. Estas serán las capas celulares que hay que incluir en los estudios del comportamiento del fruto frente a los factores externos, como los daños por cosecha y por patógenos. Las 5 siguientes capas subepidérmicas (5-9) parecen formar una zona de mesocarpo exterior (Outer mesocarp) caracterizado por células pequeñas pero con un comportamiento similar al de las células del mesocarpo. Esta nueva y más precisa definición de la zona externa del fruto indica la necesidad de reexaminar las zonas del fruto en donde ocurren los daños por molestado y revisar la definición legal de los daños externos (Real decreto 1230/2001 sobre aceitunas de mesa), donde se habla de 'daños de 'epidermis' en contraste con 'daños internos'.

V.2. Bibliografía

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VI. CONCLUSIONES GENERALES

VI. CONCLUSIONES GENERALES

1. Se ha observado una alta variabilidad en la mayoría de las medidas cuantitativas y cualitativas de la arquitectura de la planta, lo que refleja la complejidad y las dificultades que supone su estudio en programas de mejora.

2. La arquitectura y el vigor de la planta, evaluados de forma cuantitativa, se han visto afectados por la edad de la planta. Los resultados obtenidos permitieron establecer una edad mínima (9 meses) para la evaluación temprana del vigor y de la arquitectura de las plantas de semilla jóvenes en los programas de mejora.

3. Los criterios cualitativos desarrollados para la evaluación se han mostrado fiables y repetibles, y permitieron la identificación y la caracterización de 8 tipos arquitectónicos dominantes. Esta metodología y los resultados que ha proporcionado representan un paso avanzado en la consideración de la arquitectura en los procesos de evaluación y selección de los programas de mejora.

4. Combinando los resultados de los estudios cuantitativo y cualitativo se ha podido determinar los parámetros más relevantes (9 parámetros) para la descripción de la arquitectura en el olivo: 'Primary Shoot Top Diameter', 'Primary Shoot Conicity', 'Secondary Shoot Number', 'Longest Secondary Shoot Internode Length', 'Main vertical axis', 'Preferential distribution of Lateral Shoots', 'Dominant length of Lateral Shoots', 'Branch orientation' y 'Branch bending'.

5. El desarrollo floral se ha visto afectado de forma distinta según el momento de aplicación del déficit hídrico. Durante el periodo de desarrollo de las inflorescencias, el déficit hídrico provoca una importante reducción en los parámetros de floración, mientras que en el periodo final de formación de flores dificulta la polinización y el proceso de fecundación. Una reducción en la disponibilidad de agua en el periodo de floración y cuajado inicial parece impedir la polinización, debido a obstaculización de la apertura de los pétalos.

6. La aplicación de un déficit hídrico en cualquier momento del desarrollo floral activo conduce a una reducción en la producción final en frutos en número y peso total, a pesar del aumento de tamaño de los frutos mediante los mecanismos de compensación.

7. La división celular en el mesocarpo de la aceituna experimenta una primera fase de actividad intensa hasta las 8 semanas después de plena floración,

seguida por una segunda fase más larga y de actividad reducida pero que produce alrededor del 25% del número total de células. La expansión celular ha sido continua y algo lineal durante todo el crecimiento del fruto.

8. Las diferencias genotípicas en tamaño de fruto observadas en seis cultivares con un gran rango de variabilidad, se deben a diferencias en el tamaño del mesocarpo y del endocarpo. A nivel celular, estas diferencias, se deben al número de células del mesocarpo y no a su tamaño.

9. La variación de las dimensiones y número de células entre las capas celulares de la zona externa del fruto, y su relación con la variabilidad genotípica del tamaño del fruto, reveló la existencia de dos regiones subepidérmicas muy diferentes: 1) las 4 primeras capas adyacentes a la epidermis (1-4), con un comportamiento similar a la epidermis, y 2) las siguientes 5 capas (5-9) que mostraron un comportamiento más similar al mesocarpo.

10. Los resultados obtenidos proporcionan una nueva perspectiva sobre las pautas celulares en la zona externa del fruto y sugieren que las células de las capas 1-4 forman, junto con la epidermis, un exocarpo multiseriado, mientras que las capas 5-9 constituyen un 'mesocarpo exterior' o una región transitoria.

VII. ANEXOS

VII.1. Anexo 1: Informe Journal Citation Reports

Capitulo 1:

Autores: **Sofiene B.M. Hammami**, Lorenzo León, Hava F. Rapoport y Raul De la Rosa
Título: Early growth habit and vigour parameters in olive seedlings

Revista : SCIENTIA HORTICULTURAE, Volumen: 129 Páginas, inicial: 761, final: 768, Fecha: 2011

Área temática: Horticulture
Impact factor 2010: 1.045
Cuartil dentro del área temática: Segundo

Capitulo 3:

Autores: Hava F. Rapoport, **Sofiene B.M. Hammami**, Paula Martins, Oscar Pérez-Priego y Francisco Orgaz
Título: Influence of water deficits at different times during olive tree inflorescence and flower development

Revista: ENVIRONMENTAL AND EXPERIMENTAL BOTANY, Volumen: 77, Páginas, inicial: 227 final: 233 Fecha: 2012

Área temática: Plant Sciences
Impact factor 2010: 2.699
Cuartil dentro del área temática: Primero

Capitulo 3:

Autores: **Sofiene B.M. Hammami**, Trinidad Manrique y Hava F. Rapoport
Título: Cultivar-based fruit size in olive depends on different tissue and cellular processes throughout growth

Revista: SCIENTIA HORTICULTURAE, Volumen: 130, Páginas, inicial: 445, final: 451, Fecha: 2011
Área temática: Horticulture
Impact factor 2010: 1.045
Cuartil dentro del área temática: Segundo

VII.1. Anexo 2: Otras actividades y aportaciones asociadas a la tesis

Apellidos: HAMMAMI Nombre: SOFIENE

Situación profesional actual

Organismo: CSIC
Facultad, Escuela o Instituto: INSTITUTO DE AGRICULTURA SOSTENIBLE
Depto./Secc./Unidad estr.: PROTECCION DE CULTIVOS
Dirección postal: FINCA ALAMEDA DEL OBISPO, APARTADO DE CORREO 4084, 14080 CORDOBA, ESPAÑA.

Teléfono (indicar prefijo, número y extensión): 957499216
Fax: + (34) 957 499252
Correo electrónico: sofienehammami@hotmail.com

Líneas de investigación

Breve descripción, por medio de palabras claves, de la especialización y líneas de investigación actuales.
Factores genéticos y ambientales en calidad de flor y desarrollo de los tejidos del fruto del olivo
La arquitectura del olivo y su utilidad en la mejora

Formación Académica

Titulación Superior	Centro	Fecha
Diploma de Estudios Avanzados (DEA)	Universidad de Córdoba	2008/2010
Master of science en Olivicultura y Eleotecnia	Universidad de Córdoba	2005-2007

Publicaciones o Documentos Científico-Técnicos

Autores (p.o. de firma): Adolfo Rosati, Silvia Caporali**,** **Sofiene B.M. Hammami**, Inmaculada Moreno-Alías,
Andrea Paoletti y Hava F. Rapoport
Título: Differences in ovary size among olive (Olea europaea L.) cultivars are mainly related to cell number, not to cell size

Revista: SCIENTIA HORTICULTURAE, Volumen: 130, Páginas, inicial: 185, final: 190
Fecha: 2011

Participación en Proyectos de I+D financiados en Convocatorias públicas.
(nacionales y/o internacionales)

Título del proyecto: ISAFRUIT

Entidad financiadora: UNION EUROPEO
Entidades participantes: CSIC
Duración: 2006-2009 Cuantía de la subvención: 13.8 millones €
Investigador responsable: Dr. Javier Abadía, responsable en España
Número de investigadores participantes: 61 en Europa

Título del proyecto: Procesos morfogenéticos claves del desarrollo del fruto en genotipos de olivo

Entidad financiadora: Ministerio de Ciencias
Entidades participantes: CSIC
Duración: 1/01/2010-31/12/2012 Cuantía de la subvención: 130000 €
Investigador responsable: Dra. Hava Rapoport
Número de investigadores participantes: 5

Título del proyecto: Control del vigor en plantaciones de olivar en seto, RTA2008-00033-C02-00
Financiación: INIA
Investigador principal: Raul de la Rosa.
Duración: 1/1/2008-31/12/2010.

Título del proyecto: Efecto de la variedad y del porte en la productividad de plantaciones superintensivas de olivar, A/9613/06-07.
Financiación: AECI
Investigador principal: Raúl de la Rosa.
Duración: 1/1/2006-31/12/2007.

Título del proyecto: Selección de material vegetal y manejo de plantaciones superintensivas de olivar, PTR1995-1015-OP.
Financiación: Ministerio de Ciencias
Investigador principal: Raúl de la Rosa.
Duración: 1/1/2006-31/12/2008.

Participación en contratos de I+D de especial relevancia con Empresas y/o Administraciones (nacionales y/o internacionales)

Título del contrato/proyecto: Evaluación del efecto de Goemar Olivo sobre floración y fructificación en variedades de olivo.

Tipo de contrato: convenio
Empresa/Administración financiadora: Aragonesas Agro. S. A:
Entidades participantes: IAS, CSIC; IFAPA Junta de Andalucía
Duración, desde: 9/2008 hasta: 3/2010
Investigador responsable: Dr. Hava Rapoport
Número de investigadores participantes: 4
PRECIO TOTAL DEL PROYECTO: 14.780€

Nota: Si necesita más casos, añádalos utilizando las funciones de copiar y pegar con el 2º caso.

Contribuciones a Congresos

Autores: **Hammami Sofiene** y Rapoport F. Hava

Título: **Olive fruit external cell and tissue structure**
Tipo de participación: Oral communication

Congreso: ***4th Olivebioteq Congress***

Publicación:

Lugar celebración: Grecia (Chania) Fecha: 31 octubre 2011

Autores: **Hammami Sofiene**, De La Rosa Navarro Raúl, León Moreno Lorenzo y Rapoport F. Hava

Título: **Architecture and Juvenility Period for Early Selection**
Tipo de participación: Poster

Congreso: ***28th Internacional Horticultural Congress***

Publicación:

Lugar celebración: Lisboa (Portugal) Fecha: 22 agosto 2010

Autores: Hava Rapoport, **Sofiene B.M. Hammami**, Ayman Abdellatife y Antonio de Haro

Título: **New Information Concerning the Pulp-to-pit Ratio in Olive Fruit from a Developmental Perspective and Using Different Parameters**

Tipo de participación: Poster

Congreso: III International Table Olive Conference

Publicación:

Lugar celebración: Sevilla (España) Fecha: 10 Marzo 2010

Nota: Si necesita más casos, añádalos utilizando las funciones de copiar y pegar con el 2º caso.

Autores: Moreno-Alías, **Sofiene Hammami**, F. Luque, H. F. Rapoport, L. León and R. De la Rosa
Título: **Overcoming juvenility in an olive breeding program**
Tipo de participación: Poster
Congreso: The Sixth International Symposium on Olive Growing

Publicación:

Lugar celebración: Évora, Portugal Fecha: 9-13 Septiembre 2008

Autores: **Hammami Sofiene** ; De La Rosa Navarro R.; León Moreno L. y Rapoport H. F.

Título: Observaciones sobre el comportamiento arquitectónico en plantas de semilla del olivo

Tipo de participación: Articulo
Congreso: IV CONGRESO DE MEJORA GENÉTICA DE PLANTAS

Publicación:

Lugar celebración: CORDOBA, ESPAÑA Fecha: 14-16 OCTUBRE 2008

Otros méritos o aclaraciones que se desee hacer constar
(utilice únicamente el espacio equivalente a una página).

Conocimiento en Informática y estadístico: SAS, Statistix, SPSS, Cervus, GenAlex, PAST Palaeontological Statistics
Manejo de programas de análisis de Imagen en el entorno científico: ImageJ, Fiji Image, Pro Image Plus Cybernetics, ImagePlus Motic, PhotoShop.
Curso análisis de imagen para microscopia (Universidad de Málaga, 2009).