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Desarrollo de métodos biotecnológicos aplicados a la mejora genética del níspero japonés (*Eriobotrya japonica* (Thunb.) Lindl.)

TESIS DOCTORAL

presentada por

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Para Elena,
carnaval toda la vida
y una noche junto a vos
si no hay galope se nos para el corazón

Para Jorge y Lucía,
conèixer-vos a tots és tan bonic
que em fa viure
que em fa créixer i ser petit alhora

Para mis padres y mi hermano,
Bon cop de falç!

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ABSTRACT

In the context of the breeding program carried out at IVIA, in this thesis we have developed tools for implementing the program and increase its efficiency. Being the genetic resources the main breeding tool, the genetic diversity of a germplasm collection has been studied using microsatellites molecular markers. We used Factorial Correspondence Analisys, Bayessian analysis and UPGMA cluster analysis to determine the population structure of the collection. We obtained 5 subpopulations related to the origin of the accession. Genetic distances and the grouping analysis suggest that accessions introduced in the Mediterranean Basin would come from Asia. On the other hand, we have obtained 3 subpopulations that include accessions from European origin which demonstrated the high varietal diversity and adaptability reached by this species although its late introduction in Europe. Additionally, the self-incompatible alleles provided valuable information about the germplasm movements and contributed to know the inter-compatibility groups into the collection. The genetic information gathered completes the phenotypic characterization made previously at IVIA, all together the results will be a valuable tool for planning the future crosses in the breeding program.

Another biotechnology tool developed for implementing the program was the techniques set up for increasing the diversity with new genotypes with different ploidy levels. We have applied chemical mutagenesis using colchicine and *in vitro* selection, aimed at obtaining polyploids, which are of high interest in loquat species, due to its potential for producing varieties with bigger fruits (tetraploids) o seedless fruits (triploids). We obtained stable polyploids soaking the seeds in a colchicine buffer. Two triploids (3*x*) were obtained, probably already present by natural mutation in the hybrid seed lot, and one tetraploid (4*x*). The ploidy level was determined by flow cytometry, the results were confirmed by chromosome counting in leaves and roots, and morphological analysis.

On the other hand, aimed at obtaining haploids and double haploids (DH), we studied the potential gametophytic embryogenesis induction in both gametes type, male (by isolated microspores culture and anthers) and female (*in situ* parthenogenesis induced by irradiated pollen). Homozygous lines obtained in a unique generation by biotechnology methods are very useful in long juvenile period species as loquat. The haploid genotypes allow obtaining of homozygous genotypes in one step, they simplify

genetic studies, allow alignment of sequences, and to exploit the hybrid vigor. The experiments made on isolated microspores succeeded for calli induction in several accessions used, which is the first morphogenetic step. The anther culture resulted in a triploid plant (3*x*) probably explained by a natural chromosome duplication during the regeneration process. Results demonstrated that induction of embryogenesis in loquat is possible; however it depends on many variables that need to be analyzed. Gynogenesis *in situ* by irradiated pollen with gamma rays and embryo rescue *in vitro* allowed obtaining four haploid plants. The ploidy level was determined by flow cytometry, the results were later confirmed by chromosome counting in leaves.

RESUMEN

En el contexto del programa de mejora genética que se lleva a cabo en el IVIA, en esta tesis se han desarrollado una serie de herramientas que implementen el programa y permitan aumentar la eficacia del mismo. En primer lugar, se ha estudiado la diversidad genética de la colección de germoplasma del IVIA, por medio de marcadores moleculares tipo microsatélites, ya que los recursos fitogenéticos son la principal herramienta de la mejora. Combinando 3 tipos de análisis: Análisis Factorial de Correspondencia, método de agrupamiento Bayesiano y UPGMA se ha determinado la estructura poblacional de la colección. Se han obtenido 5 subpoblaciones relacionadas con los orígenes de las accesiones. Las distancias genéticas obtenidas y los análisis de agrupación sugieren que las accesiones introducidas en la cuenca mediterránea procederían de Asia. Por otra parte, se han obtenido 3 subpoblaciones formadas por accesiones de origen europeo que demuestran la alta diversificación varietal y la adaptación de la especie en los países mediterráneos a pesar de su tardía introducción en Europa. También el análisis de los alelos de compatibilidad ha aportado información sobre el movimiento de germoplasma y ha contribuido a conocer los grupos de intercompatibilidad dentro de la colección. La información genética generada complementa la fenotípica obtenida previamente por el IVIA y será de gran ayuda en la planificación de los futuros cruzamientos del programa.

Otra herramienta biotecnológica para implementar el programa de mejora ha sido la puesta a punto de técnicas para aumentar la diversidad basada en genotipos con diferentes niveles de ploidía. Por una parte, se ha utilizado la mutagénesis química con colchicina y posterior selección *in vitro* con el objetivo de obtener poliploides, de gran interés en níspero, ya que puede dar lugar a variedades con frutos de mayor tamaño (tetraploides) o frutos sin semilla (triploides). Se obtuvieron poliploides estables sumergiendo las semillas sin germinar en una solución de colchicina, dos triploides ($3x$) posiblemente ya presentes en el lote de semilla híbrida de partida, y un tetraploide ($4x$). El nivel de ploidía se determinó primero mediante citometría de flujo, y los resultados se confirmaron posteriormente por conteo cromosómico en hoja, ápice radicular, y evaluación morfológica.

Por otro lado, con la finalidad de obtener haploides y doble-haploides (DH), se ha estudiado la capacidad de inducción de embriogénesis gametofítica en ambos tipos de gametos, masculinos (cultivo de microsporas aisladas y anteras) y femeninos

(partenogénesis *in situ* inducida por polen irradiado). La producción de líneas puras mediante técnicas biotecnológicas en una única generación, es especialmente útil en especies de largo periodo intergeneracional como el níspero. Los genotipos haploides permiten obtener individuos homocigotos en un solo paso, facilitan estudios genéticos, alineamiento de secuencias y explotar el vigor híbrido. En los experimentos de cultivo de microsporas aisladas se consiguió inducir callogénesis en diversas accesiones de la especie, siendo el primer paso hacia la respuesta morfogénica. El cultivo de anteras ha dado lugar a una plántula triploide (3*x*), posiblemente debido a una duplicación cromosómica espontánea durante el proceso de regeneración, y ha permitido demostrar que es posible la inducción de embriogénesis en níspero aunque hay muchos factores que influyen en la respuesta. Mediante partenogénesis *in situ* con polen irradiado con rayos gamma, y posterior rescate y cultivo de embriones *in vitro* ha sido posible obtener cuatro plantas haploides. El nivel de ploidía se determinó primero mediante citometría de flujo, los resultados se confirmaron posteriormente por conteo cromosómico en hoja.

RESUM

En el context del programa de millora genètica que es duu a terme en l'IVIA, en aquesta tesi s'ha desenvolupat una sèrie d'eines que implementen el programa i en permeten augmentar l'eficàcia. En primer lloc, s'ha estudiat la diversitat genètica de la col·lecció de germoplasma de l'IVIA, per mitjà de marcadors moleculars tipus microsatèl·lit, ja que els recursos fitogenètics són la principal eina de la millora. Combinant 3 tipus d'anàlisi: Factorial de Correspondència, mètode d'agrupament Bayesià i UPGMA, s'ha determinat l'estructura poblacional de la col·lecció. S'han obtingut 5 subpoblacions relacionades amb els orígens de les accessions. Les distàncies genètiques obtingudes i les anàlisis d'agrupació suggereixen que les accessions introduïdes en la conca mediterrània procedirien d'Àsia. D'altra banda, s'han obtingut 3 subpoblacions formades per accessions d'origen europeu que demostren l'alta diversificació varietal i l'adaptació de l'espècie als països mediterranis malgrat la seua tardana introducció a Europa. També l'anàlisi dels al·lels de compatibilitat ha aportat informació sobre el moviment de germoplasma i ha contribuït a conèixer els grups d'intercompatibilitat dins de la col·lecció. La informació genètica generada complementa la fenotípica obtinguda prèviament per l'IVIA i serà de gran ajuda en la planificació dels futurs creuaments del programa.

Una altra eina biotecnològica per implementar el programa de millora ha estat la posada a punt de tècniques per augmentar la diversitat basada en genotips amb diferents nivells de ploïdia. D'una banda, s'ha utilitzat la mutagènesi química amb colquicina i posterior selecció *in vitro* amb l'objectiu d'obtenir poliploides, de gran interès en nesprer, ja que pot donar lloc a varietats amb fruits de major grandària (tetraploides) o fruits sense llavor (triploides). Es van obtenir poliploides estables submergint les llavors sense germinar en una solució de colquicina, dos triploides (3*x*) possiblement ja presents en el lot de llavor híbrida de partida i un tetraploide (4*x*). El nivell de ploïdia es va determinar primer mitjançant citometria de flux, els resultats es van confirmar posteriorment per recompte cromosòmic en fulla, àpex radicular i avaluació morfològica.

D'altra banda, amb la finalitat d'obtenir haploides i doble-haploides (DH), s'ha estudiat la capacitat d'inducció de embriogènesi gametofítica en ambdós tipus de gàmetes, masculins (cultiu de microspores aïllades i anteres) i femenins (ginogènesi *in situ* induïda per pol·len irradiat). La producció de línies pures mitjançant tècniques

biotecnològiques en una única generació és especialment útil en espècies de llarg període intergeneracional com el nesprer. Els genotips haploides permeten obtenir individus homozigots en un sol pas, faciliten estudis genètics, alineament de seqüències i explotar el vigor híbrid. En els experiments de cultiu de microspores aïllades es va aconseguir induir callogènesis en diverses accessions de l'espècie, sent el primer pas cap a la resposta morfogènica. El cultiu d'anteres ha donat lloc a una plàntula triploide (3*x*), possiblement a causa d'una duplicació cromosòmica espontània durant el procés de regeneració, i ha permès demostrar que és possible la inducció de embriogènesi en nesprer encara que hi ha molts factors que influeixen en la resposta. Mitjançant ginogènesi *in situ* amb pol·len irradiat amb raigs gamma i posterior rescat i cultiu d'embrions *in vitro* ha estat possible obtenir quatre plantes haploides. El nivell de ploïdia es va determinar primer mitjançant citometria de flux, els resultats es van confirmar posteriorment per recompte cromosòmic en fulla.

INTRODUCCIÓN GENERAL

1. NÍSPERO JAPONÉS: DESCRIPCIÓN BOTÁNICA, ORIGEN E IMPORTANCIA ECONÓMICA

1.1 Descripción botánica

La primera descripción botánica del níspero japonés la realizó el naturalista y físico alemán Engelbert Kaempfer en 1712 quién lo denominó *Amoenite Exotic* (Badenes et al. 2013). Sin embargo, el primero en clasificar el níspero japonés, fue el botánico sueco Carl Peter Thunberg en 1784, que lo clasificó como *Mespilus japonica* (Calabrese 2006; Morton 1987). No fue hasta 1822, cuando John Lindley revisó el género *Mespilus* y clasificó al níspero japonés dentro de un nuevo género llamado *Eriobotrya*, del griego *erio* que significa 'lanoso' y *botrys* 'grupo', referido a las panículas (Lin et al. 1999). Dentro de los centros de origen descritos por Vavilov en 1926, el centro del Sur de China sería el centro de origen del níspero japonés (Calabrese 2006). El número de especies dentro del género *Eriobotrya* ha sido revisado por varios autores pero sigue en discusión (Vidal 1965; Calabrese 2006; Huang et al. 2007; Lin 2007).

El níspero japonés (*Eriobotrya japonica* (Thunb.) Lindl.) pertenece a la familia *Rosaceae*, subfamilia *Maloideae*, compuesta por diploides funcionales ($2n = 2x = 34$) con un posible origen alopoliploide (Chevreau et al. 1985). Es un frutal subtropical vigoroso, de buen porte (5 - 6 m de altura) y raíz principal pivotante (2.5 m de profundidad). Sus hojas son perennes, coriáceas, elíptico-lanceoladas de 18 - 45 cm de longitud y bordes aserrados. Las flores son olorosas, blancas y pequeñas, se disponen en panículas de 10 - 19 cm de longitud y forma piramidal, pudiendo contener entre 30 y 260 flores cada una. La especie florece en otoño. El polen es amarillento tricolpado, llegando al 70% de germinación en óptimas condiciones de temperatura (20 ºC). Comparte junto al resto de especies de rosáceas el sistema de autocompatibilidad gametofítico (GSI) basado en *S*-RNasas (Igic y Kohn 2001). Es un árbol de rápida entrada en producción (2 - 3 años), los frutos se desarrollan durante el invierno, adquiriendo un color de epidermis variable entre amarillo pálido y naranja oscuro, la pulpa es de color blanco o amarillo (Figura 1.1). Son pubescentes y albergan entre 1 y 5 semillas de gran tamaño, pudiendo contener hasta diez (Figura I.1). El periodo de cosecha tiene lugar durante la primavera y se extiende durante un mes y medio (Quinzà et al. 1972; Badenes et al. 2006; Reig y Agustí 2006).

Figura I.1. Frutos de níspero del cultivar Algerie. Fruto maduro en árbol y sección horizontal y vertical de fruto mostrando las semillas

El níspero se incluye dentro de las especies de frutales menores, por volumen de producción y consumo (Llácer 1996; Bellini 2002). En España está muy bien adaptado a un nicho agro-ecológico muy concreto, el Valle de los ríos Algar - Guadalest en la provincia de Alicante (Rodríguez 1983), donde se ha desarrollado un cultivo muy intensivo que combina métodos artesanales (cultivo en pequeños bancales y terrazas) y tecnificación (cubiertas de malla, riego localizado, fertilizantes y mano de obra especializada), que ha dado lugar a uno de los cultivos más rentables de la fruticultura española (Fernández y Caballero 2002).

1.2 Origen y domesticación

La especie es originaria de China, más concretamente de la zona situada entre el curso medio y bajo del río Dadhue, en la provincia de Sichuan (Zhang et al. 1990a), donde se han encontrado pruebas de su cultivo de más de 2000 años de antigüedad (Lin et al. 1999). Yang et al. (2005) sugirieron que la provincia de Yunnan, podía ser un centro de diversificación secundario. En Japón se halla documentado desde 1180 (Ichinose 1995), lugar desde el cual en el s. XVIII se distribuyó a Europa como árbol ornamental. Algunos ejemplares se plantaron en los jardines botánicos de París (Francia) en 1784 y Kew (Inglaterra) en 1787 (Liu 1982). Posteriormente, se extendió por países de toda la cuenca mediterránea como Argelia, Chipre, Egipto, Grecia, Italia, España, Túnez y Turquía (Demir 1983; Morton 1987), adaptándose muy bien a su clima. En España, fue introducido por los marinos mercantes en la zona de Sagunto, expandiéndose después a la región Este y Sudoeste de la Península Ibérica (Rodríguez 1983). A finales del s. XIX se introdujo en Florida desde Europa y en California desde

Japón (Morton 1987) y finalmente en el s. XX se extendió a la India, el sureste de Asia, Australia (Goubran y El-Zeftawi 1983), Nueva Zelanda (Burney 1980), Madagascar y Sudáfrica. En la actualidad, se cultiva entre las latitudes 20 y 35º Norte o Sur, aunque en los climas marítimos puede llegar hasta los 45º de latitud, donde comparte áreas medioambientales con cítricos (Lin et al. 1999; Soriano et al. 2005).

1.3 Importancia económica

Actualmente España produce unas 30.500 toneladas anuales (MAAMA 2012) lo que la sitúa como el primer productor europeo y segundo mundial, detrás de China, superando, por orden decreciente de producción, a Turquía, Japón, Pakistán, Marruecos, Italia, Israel, Grecia, Brasil, Portugal y Chile (Lin 2007). Casi la mitad de esta producción es exportada, ocupando España el liderazgo en el ámbito mundial y siendo los principales destinos países de la UE (Caballero y Fernández 2004). Alrededor del 55% de la producción española se localiza en la provincia de Alicante, el resto se produce en Andalucía, en las provincias de Granada y Málaga (Figura I.2; MAAMA 2012). La difusión de plantaciones regulares fue tardía, en el año 1950 la superficie era inferior a 100 ha. No obstante, la producción se duplicó entre 1985 y 1995, pasando de 18.308 a 36.520 t, alcanzando su máximo histórico en 2002 con un total superior a las 45.000 t producidas (Figura I.3).

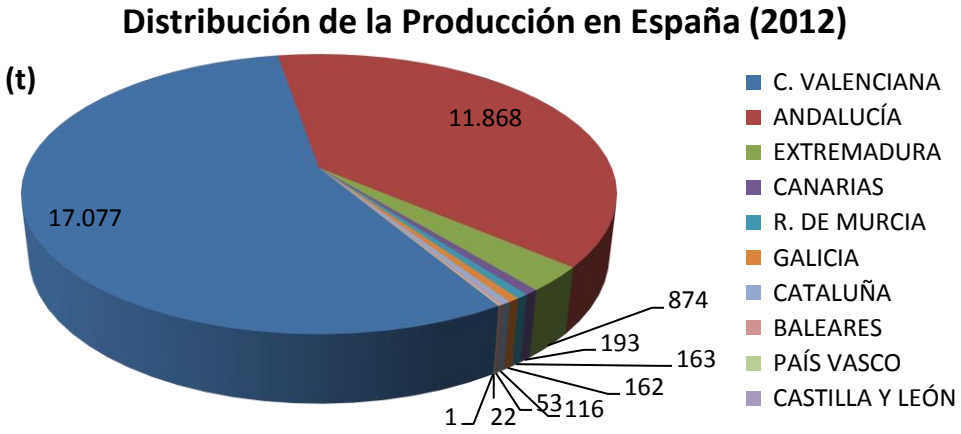

Figura I.2. Distribución de la producción de níspero japonés en las distintas zonas productoras de España en el año 2012 (MAAMA).

La mayor parte de la producción mundial de níspero se comercializa para su consumo en fresco, aunque también en almíbar, mermeladas y zumos. En China se

considera una especie con alto valor medicinal donde todos los órganos son utilizados con este fin (Feng et al. 2007; Hong et al. 2007; Lu et al. 2007; Zheng 2007), aunque también es muy valorado como vino, licor o infusión (Jiang et al. 2007; Lin et al. 2007; Xu et al. 2007). En las zonas productoras españolas, la miel obtenida es otro de los productos más apreciados (Llácer y Soler 2001; Caballero y Fernández 2004).

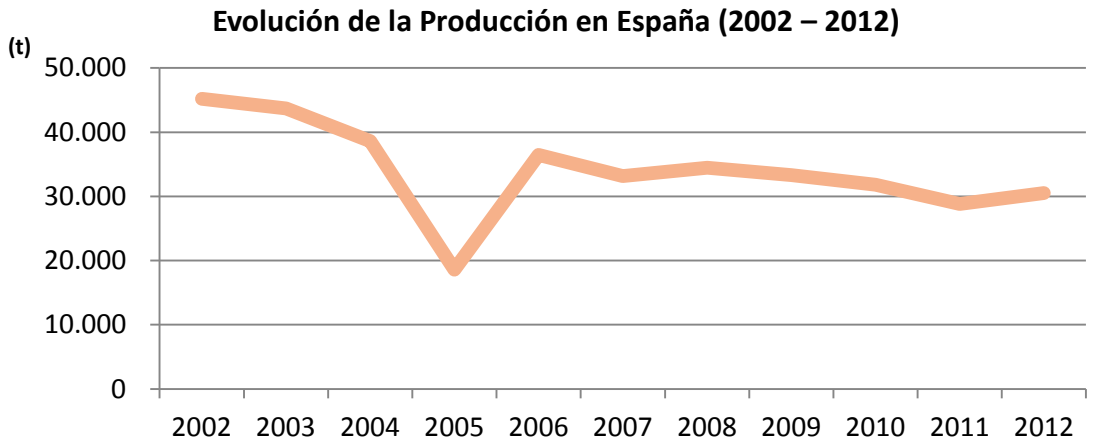

Figura I.3. Evolución de la producción de níspero japonés en España durante el periodo de 2002 a 2012 (MAAMA 2012).

Aunque el níspero japonés es un frutal menor, cada vez recibe más atención debido a su época de recolección, ya que es el primer frutal de primavera (Cui et al. 2007). En España, concretamente en la Comunidad Valenciana, los estudios sobre este frutal se iniciaron en el IVIA y en el IAM-UPV en 1992 y 1997, respectivamente. Los principales problemas detectados desde entonces son (Gisbert et al. 2006; 2007a):

- Cultivo prácticamente monovarietal, más del 90 % de la producción en la provincia de Alicante corresponde al cultivar 'Algerie' y sus mutaciones, con el riesgo comercial y sanitario que supone depender de una sola variedad.
- Los costes de cultivo son muy elevados, el 66 % de éstos corresponde a la mano de obra necesaria para el aclareo de frutos y la recolección.
- El moteado producido por el hongo *Spilocaea eriobotryae* que requiere numerosos tratamientos para su control.
- Recientemente la amenaza del fuego bacteriano, enfermedad presente en España.

2. MEJORA GENETICA Y RECURSOS FITOGENETICOS

2.1 Mejora genética

Gran parte de los problemas que presenta este cultivo se pueden solucionar mediante mejora genética y optimización de las técnicas de cultivo. La mejora genética está detrás de los grandes avances que se han realizado en agricultura. El inicio de la mejora frutal data del s. XIX y sus orígenes se encuentran en la selección masiva para mejorar los cultivos de la fresa y el peral (Janick 2006). Ya a comienzos del s. XX se desarrollaron programas de mejora en frutales, como en manzano (Crosby et al. 1992; Janick et al. 2000), uva de mesa, con los primeros cruzamientos en 1923 en el USDA-Fresno (Hinrichsen et al. 2006), o cerezo en 1940, en el John Innes Centre de Inglaterra (Lichou et al. 1990). En la actualidad se pueden encontrar gran número de programas de mejora genética de frutales distribuidos alrededor del mundo (revisado en Badenes y Byrne 2012).

Los principales objetivos de los programas de mejora de frutales son la obtención de un producto de mayor calidad, en la que los aspectos beneficiosos para la salud del consumidor junto con la uniformidad del producto, mayor duración poscosecha, reducción de los costes de producción, expansión a nuevas zonas productoras y resistencia a factores abióticos y bióticos son fundamentales (Byrne 2012).

En el caso concreto del níspero japonés, los objetivos de mejora se centran, además de en lo citado anteriormente, en obtención de frutos sin semillas, introducción de nuevos colores en el epicarpio y mesocarpio del fruto, además de un mayor tamaño del mismo, mayor contenido en sólidos solubles, resistencia al frío y ampliar la época de maduración, al igual que la productividad (Badenes et al. 2013). Se llevan a cabo programas de mejora en China (Lin et al. 2007), en Japón (Terai 2002) y selección de germoplasma autóctono en Turquía (Karadeniz y Senyurt 2007; Polat 2007; Polat y Caliskan 2007). En España existe un programa de mejora en colaboración entre el IVIA y la Cooperativa Ruchey de Callosa d'en Sarrià. Los objetivos de este programa son la obtención de variedades más competitivas por su mayor calidad, resistencias a factores bióticos y abióticos y mayor productividad (Gisbert et al. 2009a).

Por otro lado, no existen programas de mejora de portainjertos de níspero. Dada la importancia que éstos tienen sobre la variedad injertada y la adaptación a distintas

condiciones edafológicas, sería necesario disponer de portainjertos tolerantes a suelos calcáreos y a enfermedades como *Phytophthora* y *Armillaria*; además de patrones enanizantes que mejoren el manejo del cultivo.

En cuanto a la mejora de técnicas de cultivo se ha actuado sobre el aclareo de frutos y en la actualidad se dispone de la tecnología necesaria para realizar aclareo químico complementario al manual y también se han realizado estudios para estimular el desarrollo del fruto y obtener frutos de mayor tamaño (Gariglio y Agustí 2005; Agustí et al. 2007). También se han mejorado prácticas culturales en temas de irrigación y fisiología así como métodos para incrementar la calidad del fruto y/o aumentar la productividad. (Cañete et al. 2007; Cuevas et al. 2007; Hueso et al. 2007; Pinillos et al. 2007; Rodríguez et al. 2007).

2.2 Recursos fitogenéticos

2.2.1 Recursos fitogenéticos como herramienta de mejora

Una de las principales herramientas para mejorar un cultivo son los recursos genéticos. A pesar de su importancia, la riqueza fitogenética disponible que se aprovecha es muy baja (Hernández 1999). En parte es debido a que se han seleccionado para el cultivo un reducido número de especies más promisorias, a la reducida base genética utilizada en la mejora de los cultivos existentes y a la utilización en la agricultura de material vegetal mejorado con base genética estrecha (Paarlberg 1990). A su vez, las prácticas agrícolas intensivas demandan el uso restringido de variedades. Todos estos factores ligados a la agricultura han supuesto una alta erosión genética.

Una forma de paliar en parte esta erosión es la preservación de los recursos genéticos en colecciones de germoplasma con el objetivo de conservar la variabilidad genética en el ámbito de cada cultivo (Jaramillo y Baena 2000). Existen organismos internacionales cuyo fin es la promoción de la conservación de los recursos genéticos como el IPGRI (International Plant Genetic Resources Institute) a nivel internacional o el ECPGR (European Cooperative Program for Plant Genetic Resources) a nivel europeo.

Así, dentro de las especies leñosas existen bancos de germoplasma de prácticamente todas las especies de importancia a escala mundial, situadas en las áreas ecogeográficas de dichas especies. En el caso del níspero, al ser un frutal menor el

número de bancos de germoplasma es más reducido, pero se encuentran representadas todas las áreas de origen y dispersión de la especie. La mayor variabilidad se encuentra en China, su centro de origen, seguida de Japón y Europa. Existen bancos de germoplasma estudiados y caracterizados en China y Japón (Lin et al. 1999; Lin 2007), mientras que la colección europea se haya localizada en el IVIA, Valencia, España (Blasco et al. 2014a).

En China se dispone de varias colecciones que suman más de 1.000 accesiones de especies del género *Eriobotrya* (Badenes et al. 2006), por lo que algunos grupos de investigación están haciendo especial hincapié en el estudio de dichos recursos genéticos (Cai et al. 2007; He et al. 2007a; Xie et al. 2007; Xu et al. 2007). Los estudios se orientan hacia la identificación y clasificación de las accesiones y posterior uso de las mismas en obtención de nuevas variedades, también se incluyen estudios de citología, orientada a la obtención y explotación de la ploidía en mejora genética del níspero (Guo et al. 2007; He et al. 2007b; Wu et al. 2007; Zheng 2007). En Japón, al introducirse su cultivo hace más de 1000 años, se ha desarrollado una enorme diversidad, haciendo posible el establecimiento de la segunda colección de germoplasma más importante a escala mundial de este género, ubicada en el Nacional Institute of Fruit Tree Science. No obstante, la mayor parte de las variedades destacadas de esta especie proceden de hibridación como, por ejemplo, el cultivar 'Tanaka' (Lin 2007). En Pakistan existen colecciones de variedades locales bien adaptadas a la zona, algunas de las cuales se cultivan y se destinan a mercados locales (Hussain et al. 2007a).

La única colección europea se localiza en el IVIA. Este banco se estableció como resultado de un proyecto europeo GENRES29. Este proyecto permitió realizar prospecciones en los países mediterráneos y generar una colección de germoplasma que actualmente cuenta con 126 accesiones (Llácer et al. 1995), lo que la ha convertido en la mayor colección fuera de Asia. Estas accesiones provienen de 8 países, Brasil, China, EEUU, Italia, Japón, Pakistán y Portugal, aunque la mayoría proceden de prospecciones en zonas de cultivo españolas.

En conclusión, el proceso de domesticación del níspero ha dado lugar a numerosos ecotipos en diferentes lugares en el transcurso de su cultivo y aclimatación. A partir de estos ecotipos iniciales, surgieron un gran número de variedades en diversos países donde el níspero era cultivado. Muy a menudo, las variedades actuales son seleccionadas como mutaciones o nísperos de semilla obtenidos por hibridación natural de variedades élite (Badenes et al. 2013).

2.2.2 Caracterización de los recursos fitogenéticos y su diversidad

La caracterización de los materiales conservados en el banco del IVIA se inició mediante la utilización de la lista de descriptores adaptada de la UPOV (Martínez-Calvo et al. 2000). A partir de los datos obtenidos se han elaborado fichas pomológicas de gran parte de las accesiones (Martínez-Calvo et al. 2006; 2008).

La caracterización morfológica y fenológica de las accesiones del banco de germoplasma se ha ido ampliando con la caracterización molecular, puesto que el genotipado de las mismas permite la identificación de homonimias, sinonímias o subgrupos (Mohammandi y Prasanna 2003). El primer genotipado de la colección de níspero del IVIA se realizó con marcadores tipo RAPDs, Random Amplified of Polymorphic DNA (Vilanova et al. 2001). Posteriormente, Badenes et al. (2004) demostraron que marcadores codominantes como los tipo SSRs (Simple Sequence Repeat) procedentes del género *Malus* (Gianfranceschi et al. 1998) son transferibles a *Eriobotrya*. Soriano et al. (2005) confirmaron la utilidad de los marcadores SSR como herramienta para estudios genéticos en níspero y Gisbert et al. (2009b), desarrollaron y caracterizaron 21 loci microsatélites polimórficos obtenidos de una librería genómica de níspero enriquecida para CT/AG.

Además de la información generada por el genotipado de cada una de las accesiones que componen un banco del germoplasma con fines de identificación de la diversidad presente, también es importante conocer la estructura poblacional de dichas colecciones. La distinta procedencia de las accesiones de una colección (especies silvestres, cultivares locales, variedades élite, variedades procedentes de prospecciones, etc.) y su número a veces elevado, que proporcionan toda la diversidad alélica, también reducen la eficacia con que estos recursos genéticos pueden ser explotados. Para resolver este problema se propuso la creación de colecciones nucleares, definida como el menor número de accesiones que, con una repetividad mínima, representasen la diversidad genética de una especie cultivada y sus especies silvestres relacionadas (Frankel 1984). Para ello, la determinación de la estructura genética (partición) de las colecciones de germoplasma es un aspecto fundamental en la toma de muestras, ya que asegura que tanto el espectro genético como el ecológico de la especie estén plenamente representados en las colecciones de referencia (Brown 1995; van Hintum et al. 2000). Además este tipo de análisis han demostrado ser clave en la realización de estudios de

asociación, como la búsqueda de QTLs (Quantitative Trait Locus) o el mapeo asociativo (Wang et al. 2005; Shriner 2007).

Ya sea para su uso en el muestreo de nuevas accesiones que mejoren las colecciones de referencia o para estudios de asociación, la elección del método que determine la estructura genética de tales colecciones representa un factor clave. En el pasado, la determinación de la estructura genética de las colecciones se realizaba mediante análisis estadísticos multivariantes, como el análisis por componentes principales, por lo general sobre la base de los datos agronómicos (Peeters y Martinelli 1989; Franco et al. 1997; 2005; 2006; revisado en Odong et al. 2011). Actualmente se han desarrollado nuevas herramientas informáticas para el estudio de estructuras poblacionales en poblaciones silvestres que cada vez están siendo más utilizadas en el análisis de bancos de germoplasma (Odong et al. 2011). En cuanto a cultivos leñosos se refiere, esta metodología ha demostrado ser eficaz en diversas especies, como en el análisis de diversidad y estructura poblacional de *Malus sieversii*, ancestro silvestre del actual manzano (Richards et al. 2009), en el estudio de variedades obsoletas de peral español (Miranda et al. 2010), o en el estudio de 2273 accesiones de uva compuestas por variedades cultivadas (*V. vinifera* ssp. *sativa*), sus especies silvestres relacionadas (*V. vinifera* ssp. *sylvestris*), híbridos interespecíficos y diversos patrones (Emanuelli et al. 2013).

Dada la diversidad de orígenes de los materiales que componen el actual Banco de Germoplasma de níspero del IVIA y las herramientas analíticas de las que se disponen en la actualidad, es de gran interés conocer la posible estructura poblacional de las accesiones que componen dicho banco, ya que proporcionará información valiosa para la planificación de futuras estrategias de mejora en níspero. En la presente tesis se ha determinado la estructura de la colección de germoplasma del IVIA en el capítulo 1 con vistas a un mejor manejo de las potenciales ampliaciones así como a la optimización del uso de las accesiones actuales.

3. POLIPLOIDES EN LA MEJORA GENÉTICA

3.1 Definición y tipos de poliploides

Se definen como poliploides aquellos organismos cuya dotación cromosómica excede del número diploide (Comai 2005). La poliploidía es bastante común en plantas,

proporcionando un mecanismo natural de adaptación. Así, cerca del 70% de las angiospermas han tenido algún proceso de poliploidía durante su evolución (Chen et al. 2007). En la actualidad, importantes cultivos son poliploides como el trigo hexaploide, la banana triploide, la patata y la alfalfa tetraploide o la fresa y la caña de azúcar octoploide.

Las plantas poliploides en función de su origen genómico pueden dividirse en autopoliploides, especies que poseen genomas derivados de una única especie ancestral, y alopoliploides, especies procedentes de la hibridación de dos genomas distintos, seguida de multiplicación genómica. La alopoliploidía ha ejercido una mayor influencia en el desarrollo del proceso evolutivo que la autopoliploidía, de hecho la mayoría de especies poliploides observadas en la naturaleza tienen origen híbrido. Los poliploides pueden también dividirse en naturales y sintéticos, siendo los naturales resultado de una duplicación espontánea del genoma, y los sintéticos el resultado de una duplicación del genoma inducida con o sin hibridación previa. Basado en el número de grupos de cromosomas homólogos post duplicación, los poliploides pueden clasificarse en triploides, tetraploides, hexaploides, octoploides, etc (Yang et al. 2011). Los aneuploides, en cambio, son poliploides que contienen algún cromosoma de más o menos no siendo su dotación cromosómica múltiplo de la básica (Acquaah 2007).

3.2 Poliploides naturales

Aunque la ‘duplicación cromosómica’ en sentido estricto puede ser un método de formación de poliploides, la formación y fusión de gametos no reducidos es considerado el mecanismo más común de poliploidización (de Wet 1980). La producción de gametos masculino y femenino (grano de polen y óvulo) es un proceso complejo, lo que ocasionalmente provoca fallos en la reducción cromosómica durante la meiosis en muchas especies de plantas (Tate et al. 2005). Por tanto, la unión de estos gametos sin reducir resultaría en un poliploide directo, triploide natural si la unión es entre un gameto no-reducido y uno normalmente reducido, y tetraploide natural si la unión es entre dos gametos no reducidos (Figura 4). Este último mecanismo se detectó en progenies F_1 de polinización abierta en manzano, que condujeron a manzanos tetraploides naturales (Einset 1959). Cuando se fusionan gametos sin reducir en cruzamientos interespecíficos se originan los alopoliploides (Figura I.4).

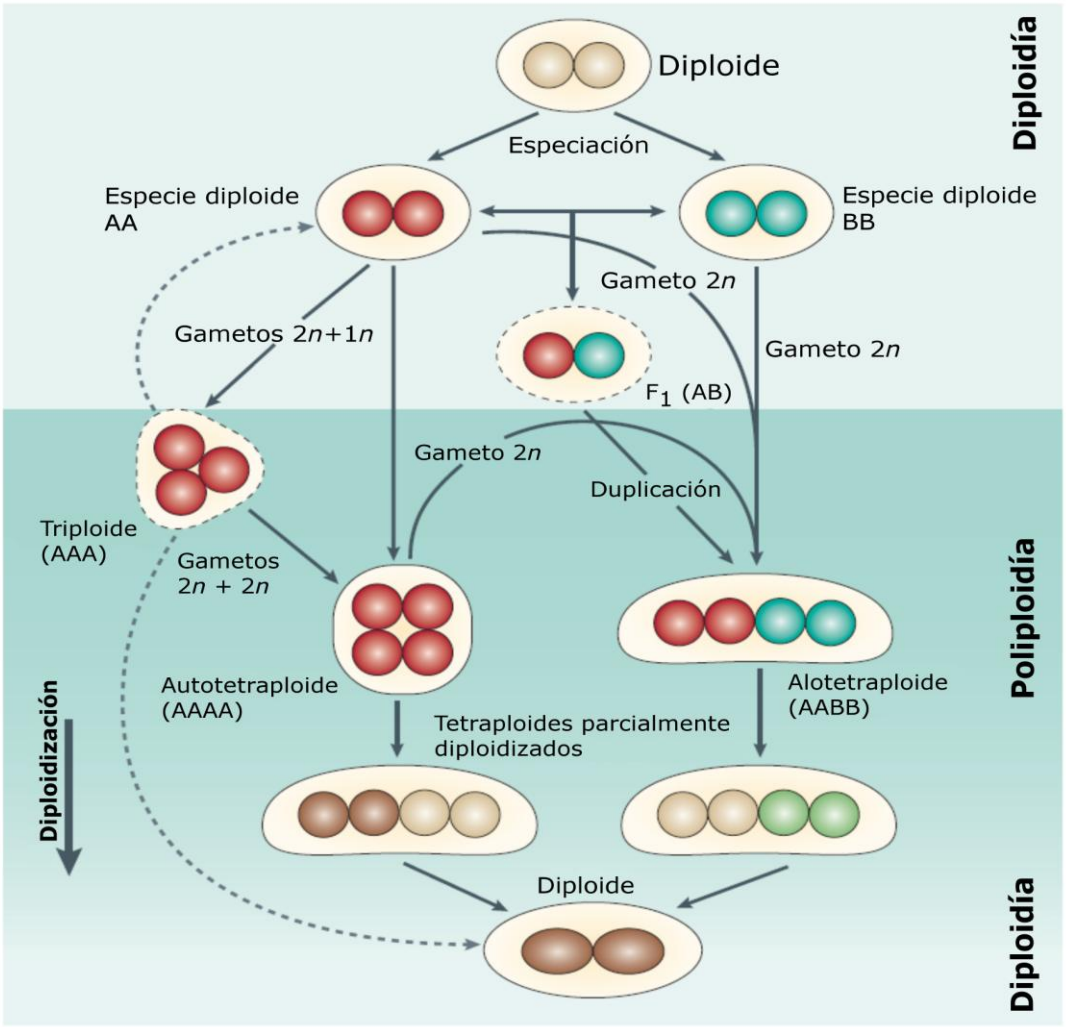

Figura I.4. Alternancia evolutiva entre diploidía y poliploidía. La figura muestra los posibles caminos que conducen a la transición repentina de diploidía a poliploidía y la transición gradual de poliploidía a diploidía (Comai 2005).

Sin embargo, debido a que la probabilidad de producirse gametos sin reducir es bastante baja dentro del total de gametos producidos, algunos autores indican que parece más probable que en la formación de plantas poliploides actúe un triploide de intermediario (Ramsey y Schemske 1998).

Una vía poco común de formación de poliploides se da cuando un óvulo es fertilizado por varios gametos masculinos, como sucede en algunas ornamentales como las orquídeas (Ramsey y Schemske 1998). Otros procesos de duplicación natural de los cromosomas se han registrado en células meristemáticas por alteraciones durante la mitosis, por ejemplo en tejidos meristemáticos de tomate (Ramsey y Schemske 1998).

Durante el proceso de formación de poliploides se produce la ‘diploidización’, es el proceso por el cual se producen los cambios genómicos necesarios para eliminar genes repetidos y evitar el silenciamiento génico (Clarkson et al. 2005; Comai 2005;

Ozkan y Feldman 2009) de forma que el tetraploide se comporta como un diploide normal a nivel genético. Este fenómeno ha sido descrito ampliamente en el género *Nicotiana* and *Cucumis* (Chen et al. 2007; Comai 2005).

3.3 Poliploides artificiales

La inducción artificial de poliploidía a través de la inhibición de la mitosis es una práctica muy extendida desde la descripción de los primeros agentes antimitóticos como la colchicina o las dinitroanilinas. La colchicina (N-((7S)-5,6,7,9-tetrahidro-1,2,3,10-tetrametoxi-9-oxobenzo(a)heptalen-7-il)-acetamida) fue la primera en descubrirse (Blakeslee y Avery 1937) y ha sido utilizada para inducir poliploides en un amplio rango de especies tales como arándanos, centeno, sandía, remolacha azucarera, trigo y tabaco (revisado en Hancock 1997), además de haber resultado eficaz en numerosas especies frutales como *Actinidia chinensis* (Wu et al. 2011), *Punica granatum* L. (Shao et al. 2003), *Pyrus pyrifolia* (Kadota y Níimi 2002), *Vitis vinifera* L. (Yang et al. 2006) y *Zizyphus jujuba* (Gu et al. 2005).

La orizalina (4-(Dipropylamino)-3,5-dinitrobenzenesulfonamida) también ha sido utilizada con éxito y es considerada menos tóxica que la colchicina, aunque induce niveles más bajos de tetraploidía (Aleza et al. 2009a; Contreras et al. 2011).

Ambos agentes tienen un modo de acción similar, inhiben la polimerización de los microtúbulos en metafase evitando de este modo que los cromosomas replicados se separen en las células hijas. Posteriormente, cuando la concentración del agente disminuye, se reanuda el crecimiento normal de la planta, volviendo a producirse la mitosis en el grupo de células poliploides generadas. Aplicaciones en concentraciones relativamente bajas pueden ser toleradas por muchas especies sin resultar letales.

La fuerte interacción existente entre los distintos agentes antimitóticos y las especies estudiadas, hace que experimentos previos no puedan ser extrapolados al níspero japonés (Dhooghe et al. 2011).

3.4 Aplicación de la poliploidía en la mejora

La producción de poliploides ya sean naturales o artificiales conlleva una serie de cambios a nivel genético y fisiológico en las plantas que en muchas ocasiones implican una serie de ventajas que se pueden aprovechar para mejora genética.

Algunos poliploides presentan mayor tamaño de hojas y flores, densidad estomática y mayor número de cloroplastos, este fenómeno ha sido ampliamente explotado en mejora de plantas ornamentales (Dhawan y Lavania 1996, Blasco et al. 2014b). También se han descrito plantas tetraploides que presentan mayor calidad del fruto, en este caso asociado a cambios en el balance hormonal del tetraploide (Wu et al. 2013), incremento en la resistencia a enfermedades (Predieri 2001), mayor producción y mejor adaptabilidad (Liu et al. 2009). Los cambios fisiológicos en los poliploides también se han aprovechado en la producción de plantas con mayor rendimiento en la producción de principios activos de aplicación farmacológica o insecticida (Liu y Gao 2007).

El vigor híbrido presente en los alopoliploides representa una ventaja respecto a los diploides ya que mantienen un doble set de cromosomas homólogos impidiendo la pérdida de este vigor híbrido por recombinación (Comai 2005). Un ejemplo de la explotación de aloploidía fue la obtención del triticale, híbrido interespecífico donde se combina la calidad del grano del trigo y la rusticidad del centeno (Acquaah 2007; Chen 2010). Por otra parte, en esquemas de mejora donde se busca maximizar la heterocigosidad, el número de combinaciones alélicas obtenidas a partir de autofecundación de tetraploides es mayor que en diploides (Acquaah 2007).

De la misma forma, en programas de mejora donde se explota la obtención de mutaciones como método de generar diversidad, los poliploides representan una ventaja ya que su genoma duplicado permite tolerar una mayor tasa de mutación, y por tanto obtener diversidad genética en condiciones donde a partir de un diploide no se lograría. En orquídeas, autotetraploides obtenidos por tratamientos con colchicina pudieron resistir dosis de producto 20 veces superiores al original diploide (Broertjes 1976).

Una aplicación de los poliploides muy importante en horticultura es la obtención de frutos sin semillas por medio de la obtención de tetraploides y posterior hibridación de los mismos con diploides, dando como resultado genotipos triploides sin semilla. Se han obtenido por este método triploides en sandía (Wehner 2008) y en uva de mesa (Ledbetter y Ramming 1989) con mucha aceptación por parte del consumidor.

3.5 Poliploidía en árboles frutales

En cítricos se han hecho muchos esfuerzos con el fin de obtener híbridos triploides que no producen semillas ni inducen la formación de las mismas en otros

genotipos por polinización cruzada. Se han seleccionado cítricos triploides naturales (Luro et al. 2004), procedentes de cruces 4*n* × 2*n* (Ollitrault y Navarro 2012) y procedentes de cruces con restitución meiótica de uno de los parentales (Chen et al. 2008). Sin embargo, el método más utilizado para obtener tetraploides en cítricos que luego sirvan como parentales en los programas de obtención de triploides, es la utilización de tratamientos con colchicina en brotes microinjertados *in vitro* (Juárez et al. 2004; Navarro y Juárez 2007).

En manzano la unión de gametos no reducidos condujo a la obtención de tetraploides naturales, este mecanismo se detectó en progenies F_1 de polinización abierta. En manzano la tasa de no reducción de gametos se ha estimado en 0.28 % (Einset 1959).

En níspero se han descrito triploides naturales procedentes de hibridaciones entre gametos no reducidos de la planta madre con gametos masculinos normales. En níspero la frecuencia de gametos no reducidos varía entre 0.18 % y 1.62%, ya que es altamente genotipo-dependiente (Guo et al. 2007).

En este sentido, la obtención de triploides artificiales por otras vías como son la obtención de tetraploides artificiales (tratamientos con colchicina) a partir de variedades élite y posteriores hibridaciones, es una alternativa interesante a la producción de frutos sin semillas que no depende de la tasa de no reducción gamética del genotipo.

4. EL MÉTODO HAPLO-DIPLOIDE EN LA MEJORA GENÉTICA

4.1 Plantas haploides y doble-haploides

Las plantas haploides son aquellas cuya dotación cromosómica corresponde a la de los gametos (*n*) y por procesos de duplicación cromosómica se pueden obtener doble-haploides (DH, 2*n*) homocigóticos. La obtención de haploides se da por inducción de embriogénesis gamética pero también se han identificado haploides generados de forma natural. Actualmente la obtención de plantas haploides o DH, ya sea mediante el uso de métodos espontáneos o artificiales, se ha descrito en más de 200 especies, que pertenecen a cada una de las familias que conforman todo el reino vegetal (revisado en Forster et al. 2007).

La primera especie haploide natural fue descrita en *Datura stramonium* por Blakeslee et al. en 1922, a la que se le sumaron descubrimientos similares en *Nicotiana*

tabacum (Clausen y Mann 1924) y *Triticum aestivum* (Gains y Aase 1926). Posteriormente fueron descritos haploides espontáneos en distintas especies de cereales, hortícolas y ornamentales (Dunwell 2010). En lo referente a especies frutales leñosas se han recuperado plantas haploides en albaricoque, ciruelo, manzano, melocotonero y peral, pero con una frecuencia tan baja que lo hace inviable para su aplicación en mejora (Zhang et al. 1990b; Bouvier et al. 1993). En total, se ha descrito la obtención de individuos haploides espontáneos en más de 100 especies angiospermas (Kasha 1974).

El origen de los haploides espontáneos es variado. Los procesos de partenogénesis espontánea, poliembrionía y androgénesis espontánea durante la reproducción, pueden ser los responsables de la formación de los mismos. La partenogénesis natural se encuentra muy extendida. Después de la polinización, un haploide partenogenético (o ginogenético) puede desarrollarse a partir de un óvulo sin fertilizar. Haploides espontáneos partenogenéticos ocurren con una frecuencia estimada del 1 por 1000 plantas en maíz (Chase 1969) y 1 por 1100 plantas en tabaco (Burk 1962). El porcentaje de obtención de haploides partenogénicos en especies angiospermas aumenta cuando de una única semilla germinan dos o más plántulas (Clayton y Yawney 1972). Este fenómeno ha sido descrito en frutales como el kiwi (Crètè 1944), mango (Sobrinho y Gurgel 1953), melocotón (Toyama 1974), almendra (Gulcan 1975) y especialmente en diferentes cítricos (Leroy 1947; Sobrinho y Gurgel 1953; Koltunow et al. 1996).

Desde su descubrimiento se han considerado de interés en los procesos de mejora y en estudios genéticos, por lo que se han desarrollado métodos para su identificación así como para su obtención por diversas técnicas biotecnológicas.

4.2. Obtención de plantas haploides *in vivo*

Además de la partenogénesis natural también se pueden obtener haploides *in vivo* por partenogénesis inducida mediante el uso de una amplia gama de técnicas: polinización con polen de la misma especie (maíz), polinización con polen irradiado, polinización con polen de una especie relacionada (cebada, patata) o especie no relacionada (trigo) (Murovec y Bohanec 2012). La polinización puede ir seguida de la fertilización del óvulo y el desarrollo de un embrión híbrido, en el cual la eliminación del cromosoma paterno ocurre al principio de la embriogénesis o no tiene lugar la

fertilización del óvulo y el desarrollo del embrión haploide es consecuencia de la polinización del núcleo polar y el desarrollo del endospermo.

La polinización con polen irradiado induce la formación de haploides maternos utilizando la polinización intra-específica. Ésta se logra por medio de tratamientos de irradiación del polen seguidos de polinización *in situ* y posterior rescate de los embriones mediante técnicas de cultivo *in vitro*. Dicho polen se irradia normalmente con rayos gamma provenientes de una fuente de Cobalto-60 (^{60}Co), aunque también puede tratarse con rayos X o luz ultravioleta. Este polen puede germinar en el estigma, crecer dentro del estilo y alcanzar el saco embrionario, pero es incapaz de fecundar el óvulo y los núcleos polares, sin embargo estimula a los óvulos hacia el desarrollo de embriones haploides (Musial y Pzrywara 1998). El éxito de esta técnica depende de numerosos factores como la dosis de irradiación aplicada, el estadio de desarrollo del embrión en el momento de su puesta en cultivo *in vitro*, la composición del medio de cultivo y las condiciones de cultivo (Germanà 2012). A pesar de las dificultades experimentales, este método es de los más utilizados con éxito en numerosas especies. En frutales se ha aplicado en cítricos (Froelicher et al. 2007; Aleza et al. 2009b), ciruelo europeo (Peixe et al. 2000), kiwi (Chalak y Legave 1997), manzano (Nicoll et al. 1987; Zhang et al. 1988; Zhang y Lespinasse 1991) o peral (Bouvier et al. 1993).

Otro método por el que se induce la formación de individuos haploides *in vivo* es la hibridación entre especies genéticamente distantes, siendo considerada como una de las metodologías más eficaces para la obtención de plantas haploides (Wędzony et al. 2009). Hibridaciones entre individuos de la misma o distinta especie, o hibridaciones entre especies con diferentes niveles de ploidía también han producido individuos haploides (Dunwell 2010).

Los mecanismos que regulan este proceso todavía son desconocidos, pero se cree que aunque en la mayoría de los casos la doble fecundación se realiza con normalidad formándose un cigoto híbrido y un endospermo, en la subsiguiente división celular se eliminan los cromosomas masculinos dejando un embrión haploide. El endospermo también sufre dicha eliminación cromosómica, lo cual provoca normalmente el aborto de la semilla, por lo que el embrión haploide debe ser rescatado por medio de técnicas de cultivo *in vitro* (Forster 2007). En algunos otros casos la fecundación va seguida de la eliminación parcial de cromosomas del parental masculino del embrión híbrido (Dunwell 2010). Este fenómeno fue descubierto por primera vez en cebada con los cruces entre *Hordeum vulgare* y *H. bulbosum*, y la posterior

identificación de haploides de *H. vulgare* (Lange 1971). De este modo se han obtenido también plantas haploides en *Citrus* a partir de cruzamientos entre diploides y triploides (Germanà y Chiancone 2001).

Cuando en el proceso de hibridación, el gameto masculino penetra dentro de la ovocélula pero no se fusiona con el gameto femenino, ambos evolucionan independientemente por lo que se produce un individuo haploide que presenta tejidos originales de ambos parentales. A este proceso se le denomina semigamia y ha sido descrito en distintas especies del género *Solanum*, *Brassica*, *Fragaria*, *Populus* (revisado en Rowe 1974) o en alfalfa (*Medicago sativa* L.) por cruzamientos con *M. falcata* (revisado en Lacadena 1996).

Existen otro tipo de técnicas menos conocidas para la obtención de haploides *in vivo*. Una de ellas es la castración y aislamiento de las flores, técnica descrita por Kihara y Katayama (1932) con la que consiguieron haploides en *Triticum monococcum*. Por otra parte, el método de la polinización retrasada, que consiste en la polinización al límite de la madurez receptiva del estigma y que permitió obtener monoploides y haploides en maíz y trigo harinero respectivamente polinizando entre 5 ó 9 días después de la fecha idónea (revisado en Lacadena 1996).

4.3 Obtención de plantas haploides *in vitro*

El proceso de embriogénesis puede ser inducido a partir de cualquier tipo de tejido de la planta. Cuando ésta se induce a partir de tejidos somáticos, se generan plantas de la misma ploidía y composición genética que la planta donante, por lo tanto, cuando la embriogénesis es inducida a partir del gameto masculino (embriogénesis del polen) o femenino (ginogénesis) los embriones regenerados representarán la progenie haploide de la planta donante, denominándose a este proceso embriogénesis gamética (Soriano et al. 2013). Durante las últimas décadas el uso de la embriogénesis gamética ha ido creciendo exponencialmente gracias a los avances producidos en el área de cultivo *in vitro* y regeneración de plantas, considerándola distintos autores la vía biotecnológica más adecuada para la obtención de plantas haploides y DH. Dicha vía es: 1) La más rápida para obtener individuos homocigotos. 2) Pueden obtenerse en una sola generación. 3) Pueden producir cientos (potencialmente miles) de un solo donante y de una sola vez. 4) Proporciona nuevas vías para la mejora genética. 5) Los cultivos

embriogénicos pueden ser manipulados para mutación o transformación. 6) Las plantas producidas son sanas y libres de enfermedades (Pintos et al. 2013).

4.3.1 Embriogénesis del polen

La obtención de plantas haploides por embriogénesis a partir de gametos masculinos es la técnica más eficiente y más ampliamente utilizada. Hay tres rutas androgénicas que pueden originar individuos haploides o DH (Figura I.5; Seguí-Simarro 2010): Ruta 1, desarrollo de un embrión haploide dentro del saco embrionario. Tras la fecundación, el núcleo femenino del cigoto ha sido inactivado o eliminado, quedando un cigoto unicelular haploide, con cromosomas únicamente masculinos, que continúa su desarrollo normal. Ruta 2, reprogramación de las microsporas de la ruta gametofítica a la esporofítica, provocando la división celular a nivel haploide seguido de la formación de callos o embriones. Ruta 3, obtención de callos haploides o DH a partir de meiocitos. La Ruta 2 es la más eficiente y utilizada, de hecho es la ruta en la que se basan la mayoría de trabajos orientados a la obtención de haploides y DH.

La embriogénesis del polen se ha descrito en más de 200 especies de las familias de Solanáceas, Crucíferas y Gramíneas (Dunwell 1986; Hu y Yang 1986; Maluszynsky et al. 2003), mientras que en especies de árboles frutales se ha ensayado con éxito en *Citrus madurensis* Lour., [*Musa balbisiana* (BB)], *Poncirus trifoliata* L. Raf., *Annona squamosa* L. y *Carica papaya* L. (revisado en Germanà 2006a).

La embriogénesis del polen puede inducirse a partir del cultivo *in vitro* de anteras inmaduras o de microsporas aisladas,

Cultivo de anteras: consiste en el cultivo *in vitro* de anteras enteras extraídas de yemas florales en condiciones de esterilidad. El cultivo de anteras fue la primera técnica de inducción de haploides que se utilizó con éxito (Maluszynski et al. 2003), y aún hoy en día es la más utilizada ya que está puesta a punto en un mayor número de especies que el cultivo de microsporas aisladas.

Cultivo de microsporas aisladas: consiste en la eliminación del tejido somático de las anteras antes de proceder al cultivo *in vitro.* Tiene las ventajas sobre el cultivo de anteras que se eliminan los efectos negativos del tejido esporofítico, permitiendo un mejor conocimiento sobre el proceso de embriogénesis. Sin embargo, requiere un mayor control de todo el proceso de cultivo *in vitro* y sus factores. Existe por tanto un menor número de trabajos de cultivo de microsporas en árboles frutales, entre los que se

pueden citar en manzano (Höfer 2004) y olivo (Bueno et al. 2005). Progresos en este sentido se han dado en otras especies como níspero (Padoan et al. 2011).

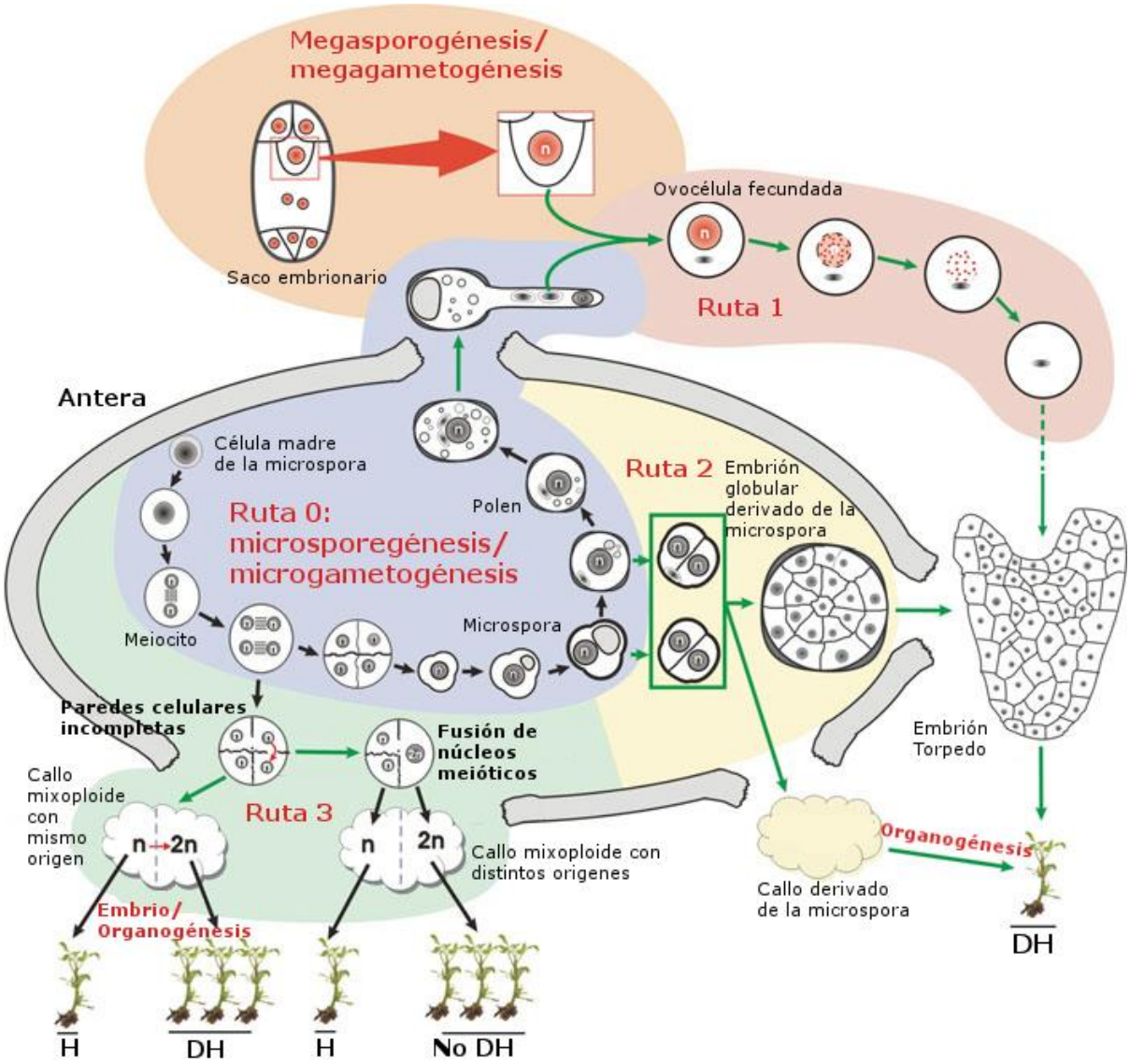

Figura I.5. Las diferentes rutas androgénicas (Seguí-Simarro 2010).

La respuesta androgénica puede verse afectada por distintos factores endógenos y exógenos. Entre los principales factores endógenos se encuentra el genotipo. Diferencias intraespecíficas en la respuesta androgénica se han observado en numerosas especies frutales como *Carica papaya* (Tsay y Su 1985), *Citrus* (Germanà et al. 1997) y *Malus domestica* (L.) Borkh. (Milewska-Pawliczuk 1990, Höfer 1999). El estadio de desarrollo del polen más adecuado para inducir la embriogénesis también varía ampliamente. Se han obtenido embriones tanto en estadios muy tempranos de desarrollo como tardíos (Dunwell 2010; Seguí-Simarro 2010). Por otra parte, factores exógenos

como estreses abióticos también tienen una influencia importante en la embriogénesis del polen (Duncan y Heberle 1976; Heberle-Bors y Reinert 1981). Otros factores exógenos como temperatura, contenido en nitrógeno de la planta durante la floración, tratamientos de choques de frío o calor y composición del medio de cultivo, sobre todo el tipo y concentración de reguladores del crecimiento, tienen un papel destacado en la respuesta androgénica (Germanà 2011a; Dunwell 2010).

No existen por tanto métodos únicos recomendables para lograr haploides por medio de embriogénesis del polen, ya que el número de factores que influyen en el resultado final es muy amplio y deben de analizarse y optimizarse para cada uno de los casos.

A pesar de que el cultivo de anteras se utiliza primeramente para la obtención de haploides, las plantas regenerantes a veces resultan poliploides por duplicaciones espontáneas de los cromosomas. Así, se han obtenido embriones y plantas poliploides en *Anthurium andreanum* L. (Winarto et al. 2011), *Citrus clementina* (Germanà et al. 2005), *Datura metel* L. (Narayanaswamy y Chandy 1971), álamo (*Populus* × *beijingensis*) (Li et al. 2013), *Pyrus pyrifolia* (Kadota y Niimi 2004) y *Triticum turgidum* L. (Doğramacı-Altuntepe et al. 2001). El origen de estos individuos triploides y tetraploides ha sido estudiado por Sunderland et al. (1974), quienes demostraron que los triploides se forman por la fusión de dos núcleos generativos endorreduplicados más el núcleo vegetativo, mientras que la fusión de dos núcleos generativos endorreduplicados mas dos núcleos vegetativos hijos forman el embrión tetraploide.

4.3.2 Ginogénesis

La inducción *in vitro* de haploides maternos (ginogénesis) es otra ruta para la producción de embriones haploides únicamente a partir de un gametofito femenino. Aunque los regenerantes ginogenéticos muestran alta estabilidad genética y bajo porcentaje de plantas albinas comparado con los androgenéticos, la ginogénesis es utilizada principalmente en especies en las cuales otras técnicas de inducción, como embriogénesis del polen y los métodos de polinización antes descritos han fallado, o en plantas que presentan esterilidad masculina o son de naturaleza dioica (Bhat y Murthy 2007; Chen et al. 2011).

Esta técnica consiste básicamente en el cultivo *in vitro* de diferentes partes de la flor sin polinizar, tales como óvulos, óvulos con la placenta adherida, ovarios o flores

enteras. El éxito del método y su eficacia depende de distintos factores abióticos y bióticos, siendo los más importantes el genotipo y las condiciones de cultivo de la planta donante. Los otros factores que afectan a la respuesta embriogénica de los gametos en cultivo son: estadio de desarrollo de los gametos, pretratamiento de las yemas florales y las condiciones y el medio de cultivo *in vitro* utilizados. Al contrario que sucedía en la embriogénesis del polen, al principio del cultivo el gametofito femenino es inmaduro, continuando su desarrollo durante el cultivo *in vitro*, llegando a saco embrionario maduro (Musial et al. 2005). De todas las células haploides que contiene el saco embrionario, los óvulos en condiciones óptimas en especies con respuesta ginogenética son los que experimentan desarrollo esporofítico (Bohanec 2009). Sin embargo, el cultivo de óvulos es un procedimiento muy complejo debido a que la manipulación del material es complicada, dado que se trata de una estructura delicada, muy pequeña y altamente hidratada que puede ser dañada con suma facilidad. Es imprescindible realizar la microcirugía con rapidez para evitar que los tejidos sufran desecación y/u oxidación durante el proceso (Cardone et al. 2010).

La regeneración de haploides a partir de los gametofitos femeninos sin fertilizar, ya sean óvulos, ovarios o botones florales, ha sido desarrollado con éxito en diversas especies de interés agronómico (Bohanec 2009). Desde que fue descrita por primera vez por San Noeum (1976) en *Hordeum vulgare*, se ha logrado inducir ginogénesis *in vitro* en más de 25 especies, de entre las cuales se destacan cultivos de gran interés como *Alluim cepa*, *Brassica oleracea*, *Oryza sativa*, *Solamun tuberosum*, *Triticum aestivum*, *Zea mays* y leñosos como *Hevea brasiliensis*, *Morus alba* y *Vitis* × *labruscana* (Dennis Thomas et al. 1999; Nakajima et al. 2000; Bhojwani y Dantu 2013).

Actualmente la ginogénesis es la técnica menos favorable debido a su baja eficiencia, pero el valor de las líneas puras en especies que no responden al resto de técnicas hace que valga la pena investigar sobre el método (Forster 2007).

4.4 Determinación del nivel de ploidía

Como en cualquier otra técnica de cultivo *in vitro*, los regenerantes obtenidos a partir de la inducción embriogénica de los gametos pueden presentar ploidías diferentes, siendo necesario el recuento del número de juegos cromosómicos para confirmar el éxito de la misma.

En general las plantas haploides suelen ser más pequeñas que sus progenitores diploides. El espacio que ocupa el núcleo de un genoma haploide dentro de la célula es menor que en el diploide, por tanto las células que lo contienen deben ser menores, lo que hará los tejidos y/u órganos también lo sean.

El nivel de ploidía puede estimarse por otros métodos indirectos, como el tamaño de los plástidos o los basados en la densidad y tamaño de los estomas y el recuento de cloroplastos en sus células guarda (revisado en Germanà 2010). El número de estos cloroplastos está altamente correlacionado (r=0,92) con el nivel de ploidía (Rotino 1996).

Por otra parte, existen diferentes métodos directos para conocer la ploidía de las plantas regeneradas. El nivel de ploidía de los embriones o las plántulas regeneradas puede conocerse mediante el uso de técnicas citológicas convencionales como el conteo cromosómico en ápices radiculares u hojas jóvenes. En estas células, los cromosomas están condensados en las placas metafásicas permitiendo así su conteo.

El nivel de ploidía también puede evaluarse más fácilmente por análisis de citometría de flujo (Bohanec 2003). Hoy en día, es la técnica más utilizada para conocer la ploidía, ya que permite medir grandes conjuntos de células en un periodo de tiempo pequeño permitiendo una gran precisión y rapidez a la hora de estimar la ploidía de las plantas regeneradas (Doležel y Bartoš 2005). De esta forma, se pueden distinguir haploides de diploides, triploides, aneuploides o mixoploides, entre otros.

4.5 Diploidización

Las plantas haploides son en su mayoría estériles. En ausencia de cromosoma homólogo, la meiosis es anormal y por lo tanto no se forman gametos viables. A fin de obtener diploides homocigóticos fértiles, el genoma de los haploides debe ser duplicado. En algunas plantas, la duplicación espontánea del número cromosómico se produce en una alta frecuencia (> 50%), sobre todo en especies de plantas donde la embriogénesis es inducida a través de la formación de callo, como es el caso de muchos cereales. Seguí-Simarro y Nuez (2008), a partir de distintas evidencias, indican que el mecanismo principal de duplicación cromosómica es la fusión nuclear. Durante la mitosis, la citocinesis no llega a completarse y los núcleos ya separados vuelven a unirse, lo cual parece estar asociado a un retraso en la formación de la pared celular (Kasha 2005).

Si el proceso espontáneo de duplicación cromosómica no tiene lugar, se puede entonces recurrir a las sustancias antimitóticas. Las de mayor uso son la colchicina seguida del amiprophos-methyl que presentan una eficacia del 70%, en menor proporción se utilizan la trifluralina y la orizalina de menor eficacia de duplicación. El tipo de explante utilizado, la sustancia duplicadora, la duración del tratamiento y las condiciones bajo las cuales se realiza el mismo son factores que afectan a las distintas técnicas de duplicación cromosómica (Foschi et al. 2009).

Aún sabiendo que la colchicina es altamente tóxica, debido a las altas dosis que se requieren para lograr una alta tasa de duplicación, hasta el momento es la sustancia más utilizada (Nowak 2000). En cultivo de microsporas de *Brassica napus* existen métodos eficaces de duplicación cromosómica con colchicina que han hecho que se utilice este sistema como sistema modelo, presenta una muy alta inducción de embriones y una embriogénesis sincronizada, lo cual hace posible obtener cerca del 100% de duplicación cromosómica en las plantas regeneradas (Möllers et al. 1994; Zhao et al. 1996). No obstante, a pesar de todos los trabajos llevados a cabo de duplicación cromosómica con distintos agentes antimitóticos, no existe hasta el momento un rango definido de concentraciones y tiempos de aplicación de los mismos para optimizar el proceso de duplicación.

4.6 Aplicaciones de los doble-haploides en la mejora

El interés de los mejoradores en la obtención de haploides o DH radica en la posibilidad de acortar el tiempo necesario para producir líneas completamente homocigotas a una sola generación, en comparación con los métodos convencionales que necesitan varias generaciones de autofecundaciones para alcanzar el 98% de homocigosis. Además, no siempre es posible obtener líneas homocigotas mediante ciclos de autofecundación, ya que en algunos casos existen barreras de autoincompatibilidad y en otros tantos se provoca depresión genética por consanguinidad.

En plantas leñosas caracterizadas generalmente por un largo ciclo reproductivo, un alto grado de heterocigosidad y a veces autoincompatibilidad, no es posible obtener líneas homocigotas a través de métodos convencionales basados en repetidos ciclos de autofecundación (Germanà 2011a). Por ello, la obtención de DH en estas especies tiene mucho interés.

Los genotipos homocigotos representan muchas ventajas en estudios genéticos que posteriormente se pueden aprovechar en mejora genética. Por ejemplo, permiten la identificación de mutaciones de interés en casos de herencia recesiva. Permiten la creación de líneas F_1 que exploten la heterosis o vigor híbrido. Simplifican los análisis genéticos necesarios para el alineamiento de secuencias, construcción de mapas genéticos y físicos, así como la identificación de zonas del genoma ligadas a genes de interés. También permiten simplificar los estudios genómicos y los estudios de los mecanismos de expresión génica, así como la comparación entre genomas (Kamle et al. 2011).

4.7 Haploides y doble-haploides en frutales

El número de especies frutales en las que se ha conseguido regenerar plantas haploides o DH abarca un amplio número, entre las cuales se encuentran algunas de las más importantes económicamente a nivel mundial. Dentro de las especies del género *Citrus*, el primer haploide inducido se obtuvo en *C. natsudaidai* por medio de irradiación del polen seguido de fecundación y posterior rescate de embriones inmaduros (Karasawa 1971). Mediante la técnica del cultivo *in vitro* de anteras se obtuvieron plántulas en *C. madurensis* Lour. y *Poncirus trifoliata* L. Raf. (Hidaka et al. 1979; Chen et al. 1980). Assani et al. (2003) y Li et al. (2008) también obtuvieron plantas haploides en [*Musa balbisiana* (BB)] y *Eriobotrya japonica* L. respectivamente mediante esta técnica.

En *Malus domestica* (L.) Borkh se han obtenido plantas haploides mediante partenogénesis espontánea e inducida por el uso de polen irradiado (Zhang et al. 1988). Del mismo modo ocurre con *Pyrus communis* L. donde se han obtenido haploides y DH mediante partenogénesis, tanto inducida como espontánea (Bouvier 1993). Dentro del género *Prunus*, en melocotonero se han obtenido haploides y DH partenogénicos espontáneos (revisado en Germanà 2006a) e inducidos en cerezo (Höfer y Grafe 2003).

Por todo lo descrito con anterioridad, sería un gran avance para la mejora del níspero japonés la obtención de haploides y DH de cultivares de interés agronómico, lo cual supondría un progreso en los estudios genéticos de los mismos. Entre las múltiples aplicaciones a las que puede dar lugar se encuentran, estudios de genética de asociación con los caracteres objetivo de la mejora y el mapeo genómico.

OBJETIVO GENERAL

Dentro del marco del programa de mejora por cruzamientos del níspero japonés que se lleva a cabo en el IVIA, el objetivo general de esta tesis es aumentar la eficacia del mismo mediante la aplicación de distintas técnicas biotecnológicas con el fin de producir e identificar nuevo material vegetal de interés.

Objetivos específicos

Objetivo I

Caracterización molecular de las accesiones del Banco de Germoplasma del IVIA como herramienta para la selección de genotipos de interés en el proceso de mejora genética.

Objetivo II

Obtención de poliploides del cv. ‘Algerie’ mediante tratamientos con colchicina, con el fin de incorporarlos posteriormente al programa de mejora.

Objetivo III

Obtención de plantas y/o callos de distintos cultivares de níspero mediante cultivo de anteras y/o microsporas aisladas, para incorporarlos al programa de mejora.

Objetivo IV

Obtención de plantas haploides de níspero del cv. ‘Algerie’ mediante partenogénesis inducida con polen irradiado y posterior uso de las mismas en el programa de mejora o en estudios genéticos.

CAPÍTULO 1:

Genetic variation and diversity among loquat accessions

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Genetic variation and diversity among loquat accessions

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Abstract

Loquat (*Eriobotrya japonica* (Thunb.) Lindl., Maloideae, Rosaceae) is a subtropical evergreen fruit tree indigenous of China, where is located the center of origin of the species. Loquat is grown in all subtropical areas and was introduced in the Mediterranean basin in late 18th century. In Europe, the largest germplasm bank is located at Instituto Valenciano de Investigaciones Agrarias (IVIA, Valencia, Spain). Thirteen microsatellites and a conserved region of *S*-allele were used to assess the genetic diversity of 102 accessions of the IVIA collection. A total of 38 SSR alleles and 11 putative *S*-alleles were used to study the genetic structure of the loquat germplasm bank using the STRUCTURE software, Factorial Correspondence Analysis (FCA) and unweighted pair-group method (UPGMA) cluster analyses. The total diversity was H_T = 0.5682, the genetic differentiation G_{ST} = 0.1660 and the standardized G_{ST} reached a much higher value of G'_{ST} = 0.4948. The Evanno's test indicated that the most informative number of populations was five, with accessions distributed according to their geographic origin in two, one and two groups of Spanish, Italo-Spanish and non-European origin, respectively. Knowledge of the substructure and diversity of the IVIA loquat collection and the self-incompatibility genotype data will allow us to select and incorporate useful materials into the loquat breeding program.

Introduction

Loquat (*Eriobotrya japonica* Lindl., Maloideae, Rosaceae) is a subtropical, evergreen fruit tree indigenous to China that was described as early as 1189 in Japan (Zhang et al. 1993; Ding et al. 1995). Loquat was introduced in the Mediterranean basin in late 18th century, later in the 19th century from Europe to Florida and from Japan to California (Lin et al. 1999). Currently, it is grown throughout the world's subtropic. As would be expected, the highest species diversity of *Eriobotrya* is found in China (Zheng 2007). There are numerous studies on the collection, identification, distribution, and classification of *Eriobotrya* genus (Cai 2000; Lin et al. 2004), but the number of species is still under dispute. *Eriobotrya japonica* is the only species from the genus cultivated for its fruit. There are more than 500 cultivars from China conserved in the National Fruit Germplasm Repository in Fuzhou (Fujian, China), most of which are seedling selections no longer in production. Beside of the late introduction in non-Asian

countries the diversity kept by the species is notable (Gisbert et al. 2009a). There are native cultivars in Brazil (Atayde et al. 1992), Italy (Baratta et al. 1995), Spain and Turkey (Badenes et al. 2000). The major cultivars grown worldwide are revised in Badenes et al. (2013).

Loquat has been grown in 10 different regions on China, and there are more than 1000 accessions described in the various Chinese germplasm collections (Zheng 2007).The largest loquat germplasm bank is located in Fuzhou (Badenes et al. 2009). In Europe, the largest germplasm bank is located at Instituto Valenciano de Investigaciones Agrarias (IVIA, Valencia, Spain). This collection was iniciated from surveys made in the Mediterranean countries under the framework of the European project GENRES, section of underutilized fruits (Badenes et al. 2004) and completed with non-European accessions, being the most diverse according to origins and characteristics. Analysis of genetic diversity in germplasm collections facilitates identification of subsets or core accessions with possible utility for breeding purposes (Mohammandi and Prasanna 2003).

Codominant markers as simple sequence repeat (SSR) have been used for loquat diversity studies. Badenes et al. (2004) used SSRs cloned from *Malus* species (Gianfranceschi et al. 1998) to study a set of loquat accessions, and found a good degree of transferability between both genuses. Soriano et al. (2005) confirmed the usefulness of microsatellite markers as a suitable tool for genetic studies on loquat. Afterwards Gisbert et al. (2009b) developed and characterized the first 21 polymorphic microsatellite loci from a CT/AG enriched loquat genomic library. In the present study we used 13 microsatellites from this enriched library aimed at assessing the genetic diversity of the largest collection outside Asia, maintained at IVIA germplasm bank (Valencia, Spain). For further breeding purpose we included in the analysis primers designed from the self-incompatibility locus aimed at assessing the groups of inter-compatibility. All together, we increased the powerfulness of the diversity analysis while providing useful information for planning breeding strategies.

Material and Methods

Plant material

The plant material included 102 loquat accessions from different sources that belong to the European loquat germplasm collection located at IVIA, Valencia, Spain (latitude: 37º 45' 31.5 N; longitude: 1º 01' 35.1 O). The accessions came from Brazil, China, Japan, Italy, Pakistan, Portugal, Spain and USA. Table 1.1 summarizes information of loquat accessions evaluated, passport data and a selection of main characteristics.

Table 1.1. Loquat accessions evaluated in this study: name of cultivar, passport data and main characteristics.

Country	Name of cultivar	Origin	Main characteristics
Brazil	1. Ronda Brasil[b]	Unknown. Introduced to Spain in 1999	F +18; R +11; FW 53,80 g; EQ good
	2. Saval Brasil[c]	Unknown. Introduced to Spain in 1999	F +2; R +10; FW 102,50 g; EQ good
China	3. Changhong-3[a,d]	Selected from Changhong seedling in 1990	F -8; R -11; FW 59,80 g; EQ good
	4. Dazhong[a,d]	Unknown	F +3; R +5; FW 84,56 g; EQ acceptable
	5. Hongganben[a,d]	Unknown	F -11; R -5; FW 35,62 g; EQ good
	6. Jiefanghzhong[a,d]	Selected from Dazhong seedling in 1950	F +6; R +1; FW 86,27 g; EQ acceptable
	7. Meihuaxia[d]	Unknown	F -12; R +6; FW 42,94 g; EQ medium
	8. Puxinben[d]	Unknown	F -3 ; R +7; FW 62,50 g; EQ acceptable
	9. Taicheng-4	Unknown	Data not available. Currently under evaluation
	10. Xiangzhong-11	Unknown	Data not available. Currently under evaluation
	11. Zaozhong-6[a,d]	Jiefangzhong × Moriowase	F -18; R -21; FW 52,84 g; EQ medium
Japan	12. Mogi[a,b]	Chance seedling of Chinese loquat. Introduced from China in 1840	F -16; R -22; FW 30,77 g; EQ medium
	13. Mogi Wasse	Unknown	F +13; R +6; FW 36,10 g; EQ good
	14. Tanaka[b,e]	A seedling originated in Japan. Introduced to the USA in 1902	F +11; R +13; FW 52,62 g; EQ excellent
Italy	15. Bianco[b]	Unknown	F +13; R +7; FW 45,01 g; EQ low
	16. Italiano-1[b]	Unknown. Introduced to Spain in 2002	F +10; R -1; FW 51,38 g; EQ excellent
	17. Marchetto[c]	Unknown	F +12; R +-0; FW 54,89 g; EQ medium
	18. Ottaviani[b]	Unknown	F +14; R +11; FW 44,01 g; EQ low
	19. Rosa	Unknown	Data not available. Currently under evaluation
	20. Rosa Tardío[c]	Unknown	F +43; R +22; FW 63,37 g; EQ low
	21. Sanfilippara[b]	Unknown	F -32; R +19; FW 52,03 g; EQ low
	22. Vaniglia Dulce	Unknown	F -4; R -14; FW 42,08 g; EQ medium
	23. Virticchiara[b]	Unknown	F +16; R +3; FW 54,25 g; EQ medium
Pakistan	24. Ikramullah-1	Unknown	Data not available. Currently under evaluation

	25. Ikramullah-2	Unknown	Data not available. Currently under evaluation
	26. Khyber-1	Unknown	Data not available. Currently under evaluation
	27. Saeed-1	Unknown	Data not available. Currently under evaluation
Portugal	28. Almargem[d]	Unknown	F +11; R +4; FW 56,01 g; EQ low
	29. Mata Mouros Regional[d]	Unknown	F +18; R +16; FW 61,50 g; EQ medium
	30. Rolhâo II[d]	Unknown	F +3; R +3; FW 69,30 g; EQ good
	31. Tavira[d]	Unknown	F ±0; R -3; FW 73,65 g; EQ acceptable
Spain	32. Al-Ama	Unknown	Data not available. Currently under evaluation
	33. Alcácer	Unknown	F +6; R ±0; FW 55,83 g; EQ medium
	34. Alfons Gregori-1	Unknown	F -28; R -32; FW 61,60 g; EQ good
	35. Alfons Gregori-2	Unknown	Data not available. Currently under evaluation
	36. Algerie (Altea)[b]	Unknown. Seedling selected in Algeria. Introduced to Spain in the 60s	F 8th of November; R first week May ; FW 54,98 g; EQ good
	37. Algerie clon tardío[b]	Introduced like a later Algerie clon at the IVIA. Local cultivar (Alicante province)	F +4; R +6; FW 57,85 g; EQ good
	38. Barret[d]	Unknown. Local cultivar (Alicante province)	F -7; R +3; FW 75,37 g; EQ good
	39. Benimelli[d]	Unknown. Local cultivar (Alicante province)	F -6; R +3; FW 74,54 g; EQ good
	40. Beniaratx	Unknown. Local cultivar (Valencia province)	Data not available. Currently under evaluation
	41. Borde[b]	Unknown. Local cultivar (Alicante province)	F +9; R +3; FW 45,00 g; EQ good
	42. Cabelo	Unknown	F +6; R +11; FW 81,00g; EQ good
	43. Cambrils[c]	Unknown. Local cultivar (Catalonia)	F -17; R -15; FW 45,00 g; EQ good
	44. Chirlero	Unknown	Data not available. Currently under evaluation
	45. Cort[c]	Unknown. Local cultivar (Catalonia)	F +5; R +8; FW 56,17 g; EQ medium
	46. Cox[c]	Unknown. Local cultivar (Alicante province)	F -10; R –6; FW 41,67 g; EQ good
	47. Cremaor[d]	Unknown. Local cultivar (Alicante province)	F -5; R +3; FW 79,19 g; EQ medium
	48. Dama	Unknown	Data not available. Currently under evaluation
	49. Dulce Pera[b]	Unknown. Local cultivar (Bétera, Valencia)	F +4; R +10; FW 39,85 g; EQ good
	50. Estrada Blanc[c]	Unknown. Local cultivar (Catalonia)	F -6; R –13; FW 44,12 g; EQ medium
	51. Estrada Groc[c]	Unknown. Local cultivar (Catalonia)	F -11; R –11; FW 38,97 g; EQ medium
	52. Francisco el Gordo	Unknown	F -5; R +4; FW 86,36 g; EQ medium
	53. IRTA[c]	Unknown. Local cultivar (Catalonia)	F -12; R +1; FW 57,26 g; EQ good
	54. Ismael[b]	Unknown. Local cultivar (Alicante province)	F +9; R +1; FW 50,54 g; EQ good
	55. Javierín[b]	Unknown. Local cultivar (Alicante province)	F +2; R +7; FW 75,90 g; EQ good
	56. Joaquín Giner[d]	Unknown. Local cultivar (Alicante province)	F -3; R +5; FW 75,44 g; EQ acceptable

57. Linares	Unknown	Data not available. Currently under evaluation
58. Magdal	Unknown. Local cultivar (Andalusia)	F -17; R –17; FW 45,52 g; EQ low
59. Magdal Carne Blanca	Unknown	F +2; R –2; FW 40,10 g; EQ medium
60. Maite[d]	Selected seedling from Algerie. Local cultivar (Alicante province)	F -4; R ±0; FW 73,37 g; EQ good
61. Manises[d]	Unknown. Local cultivar (Valencia province)	F +12; R +11; FW 48,54 g; EQ medium
62. Marc[b]	Unknown. Local cultivar (Alicante province)	F -4; R +3; FW 90,10 g; EQ medium
63. Mas Vagué[c]	Unknown. Local cultivar (Catalonia)	F -5; R –16; FW 47,92 g; EQ medium
64. Masía Cañera[d]	Unknown. Local cultivar (Alicante province)	F +1; R +13; FW 48,10 g; EQ good
65. Menera[c]	Unknown. Local cultivar (Alicante province)	F -7; R -1; FW 68,23 g; EQ medium
66. Mercedes	Unknown	F -8; R +4; FW 57,84 g; EQ medium
67. Mil Homens	Unknown	Data not available. Currently under evaluation
68. Miquel d'Aixarà[b]	Unknown. Local cultivar (Alicante province)	F -5; R +6; FW 70,36 g; EQ good
69. Miquel Nucier[d]	Algerie mutation. Local cultivar (Alicante province)	F -3; R +3; FW 72,55 g; EQ good
70. Nadal Tardío[c]	Unknown. Local cultivar (Alicante province)	F +25; R +14; FW 35,59 g; EQ good
71. Nadal Temprano[c]	Unknown. Local cultivar (Alicante province)	F -8; R -12; FW 37,09 g; EQ excellent
72. Peluches[b]	Probably Algerie mutation. Local cultivar (Alicante province)	F -12; R +4; FW 95,00 g; EQ medium
73. Peix	Unknown	Data not available. Currently under evaluation
74. Pere Esquena	Unknown. Local cultivar (Catalonia)	F -6; R -8; FW 36,12 g; EQ medium
75. Piera[d]	Unknown. Local cultivar (Alicante province)	Ever flowering and ripening; FW 52,32 g; EQ low
76. Polop-1	Unknown	F +4; R +5
77. Raúl[d]	Selected seedling from Algerie. Local cultivar (Alicante province)	F +4; R +1; FW 76,00 g; EQ médium
78. Redonet[c]	Algerie mutation. Local cultivar (Alicante province)	F +10; R -1; FW 62,63 g; EQ good
79. Requina[d]	Unknown. Local cultivar (Alicante province)	F +1; R +3; FW 82,61 g; EQ excellent
80. Ronda Gruesos[d]	Unknown. Local cultivar (Alicante province)	F +5; R +8; FW 156,18 g; EQ good
81. Sacós	Unknown	F -5; R ±0; FW 68,04 g; EQ excellent
82. Saguntí	Unknown. Local cultivar (Sagunto, Valencia)	F -11; R -3; FW 78,57 g; EQ excellent
83. Sally[d]	Selected seedling from Algerie. Local cultivar (Alicante province)	F -22; R -13; FW 72,72 g; EQ acceptable
84. Samper-1[d]	Selected seedling from Algerie. Local cultivar (Alicante province)	F -5; R -2; FW 66,48 g; EQ good
85. Samper-2[d]	Selected seedling from Algerie. Local cultivar	F -23; R -13; FW 66,58 g;EQ good

		(Alicante province)	
	86. Saval-1[b]	Unknown. Local cultivar (Alicante province)	F -2; R -1; FW 53,46 g; EQ good
	87. Saval-2[b]	Unknown. Local cultivar (Alicante province)	F +3; R +3; FW 53,71 g; EQ excellent
	88. Saval Moreno[b]	Unknown. Local cultivar (Alicante province)	F -4; R -2; FW 42,26 g; EQ médium
	89. Saval Nerviado[b]	Unknown. Local cultivar (Alicante province)	F +4; R ±0; FW 58,46 g; EQ acceptable
	90. Sisantanou	Unknown. Local cultivar (Alicante province)	F +-0; R -3; FW 60,85 g; EQ good
	91. Siscar	Unknown. Local cultivar (Alicante province)	F -3; R -1; FW 71,90 g; EQ acceptable
	92. Susana[d]	Unknown. Local cultivar (Alicante province)	F +4; R +14; FW 69,30 g; EQ medium
	93. Temprano de Petrés[c]	Unknown. Local cultivar (Petrés, Valencia)	F -10; R -3; FW 61,92 g; EQ acceptable
	94. Toni Tomaca	Unknown	Data not available. Currently under evaluation
	95. Vila	Unknown	F -36; R -32; FW 47,05 g; EQ medium
USA	96. Advance[a,e]	Seedling selected in California in 1897	Data not available. Currently under evaluation
	97. Champagne[a,e]	Selected and introduced to California around 1908	Data not available. Currently under evaluation
	98. Golden Nugget	Unknown	F +13; R +1; FW 55,54 g; EQ acceptable
	99. McBeth[a,e]	Chance seddling, 1966	Data not available. Currently under evaluation
	100. Mrs Cooksey	Unknown	Data not available. Currently under evaluation
	101. Sabroso	Unknown	Data not available. Currently under evaluation
	102. Vista White	Unknown	Data not avaiilable. Currently under evaluation

[a] Lin et al. (1999), [b] Martínez-Calvo et al. (2000), [c] Martínez-Calvo et al. (2006), [d] Martínez-Calvo et al. (2008), [e] Morton (1987), *F* Flowering date expressed as days from cv.'Algerie' used as reference, *R* Ripening date using cv.'Algerie' as reference , FW average fruit weight, *EQ* eating quality

DNA isolation

DNA was extracted from 200 mg of young leaves following the method of Doyle and Doyle (1987) with some modifications according to Gisbert et al. (2009a). Two adult trees for each accession were sampled independently.

Obtaining of molecular markers and identification of self-incompatibility alleles

Thirteen SSR polymorphic microsatellite loci developed from *E. japonica* by Gisbert et al. (2009b) were tested using two replicates per genotype. The polymerase chain reaction (PCR) was performed with three primers: the specific forward primer of each microsatellite with M13(-21) tail at its 5′ end, the sequence-specific reverse primer,

and the universal fluorescent-labeled M13(-21) primer (Schuelke 2000). SSR amplifications were performed in a GeneAmp®PCR System 9700 thermal cycler (Perkin-Elmer Corp, Freemont, CA) in a final volume of 20 µl, containing 1× PCR, 1.5 mM MgCl2, 0.1 mM of each dNTP, 0.8 µM of each primer, 20 ng of genomic DNA and 1 U of Taq polymerase (Invitrogen), using the following temperature profile: 94 °C for 2 min, then 35 cycles of 94 °C for 30 s, the optimized annealing temperature for 60 s and 72 °C for 1 min and 30 s, finishing with 72 °C for 7 min, as described by Gisbert et al. (2009a). Allele lengths were determined using an ABI Prism 3130 Genetic Analyzer with the GeneMapper software, version 4.0 (Applied Biosystems).

The *S*-allele fragments were amplified using the partial degenerated primers SC/C2-F [5'-GTT YAC BGT TCA CGG WTT GTG GCC-3'] and SC/R [5'-CGG CAA AAT WAT TTY CAA CTG-3'] designed from conserved regions of *S*-allele sequences of *Malus* x *domestica* (Borkh.) and *Pyrus* spp. (Gisbert et al. 2009a). The PCR conditions used were those described for SSR analysis. Table 1.2 sumarizes combination of primers used.

Table 1.2. Acronym of primers used, repeat motif, linkage group, number of alleles, fragment length, expected heterozigosity (He), observed heterozygosity (Ho), fixation index (*F*) and PIC value.

Acronym	Repeat	Linkage Group	No. of allele	Range (bp)	H_e	H_o	*F*	PIC
*ssr*EJ005[1]	$(AG)_{12}$	Unknown	2	214-216	0.346	0.343	0.007	0.357
*ssr*EJ037[1]	$(AG)_{19}$	14	2	233-248	0.462	0.461	0.002	0.459
*ssr*EJ039[1]	$(GA)_{12}$	Unknown	2	236-242	0.400	0.333	0.167	0.396
*ssr*EJ042[1]	$(GA)_{28}$	11	3	202-214	0.510	0.564	-0.107	0.627
*ssr*EJ075a[1]	$(GA)_{11}$	Unknown	2	222-228	0.490	0.706	-0.440	0.634
*ssr*EJ086[1]	$(GA)_{15}$	2	3	231-245	0.646	0.696	-0.078	0.487
*ssr*EJ088[1]	$(GA)_{15}$	3	3	215-245	0.541	0.686	-0.269	0.644
*ssr*EJ095b[1]	$(GA)_{23}$ $(CTGTGT)_3$	Unknown	2	239-243	0.452	0.446	0.014	0.523
*ssr*EJ104[1]	$(GA)_{21}$	7	3	162-168	0.639	0.716	-0.120	0.443
*ssr*EJ271[1]	$(AG)_{24}$	8	5	192-247	0.666	0.696	-0.045	0.510
*ssr*EJ282[1]	$(AG)_{12}AA$ $(AG)_{11}$	5	4	133-187	0.674	0.720	-0.069	0.545
*ssr*EJ324[1]	$(AG)_{23}$	Unknown	4	233-258	0.646	0.657	-0.018	0.490
*ssr*EJ329[1]	$(AG)_{19}$	10	3	154-175	0.513	0.647	-0.263	0.644
SC/C2[2]		Unknown	11	270-600	0.809	0.930	-0.149	0.805

[1] Gisbert et al. (2009a), [2] Raspé and Khon (2002)

Data analysis

The following parameters were calculated from marker data: number of alleles per locus; expected heterozygosity ($He = 1 - \sum p_i^2$, where p_i is the frequency of the *i*th allele) (Nei 1973); observed heterozygosity (*Ho*, calculated as the number of heterozygous genotypes divided by the total number of genotypes); fixation index ($F = 1 - (Ho / He)$) (Wright 1965); and the polymorphism information content (PIC), calculated according to Weir (1990) based on allele frequencies of all cultivars analyzed as: $PIC_i = 1 - \sum P_{ij}^2$, where P_{ij} is the frequency of the *j*th allele for the *i*th marker locus and summation extends over *n* alleles. Putative *S*-alleles were named in alphabetical order starting with the largest size.

Possible population structure and likelihood of classification of each accession were estimated using the Bayesian-based model procedure implemented in the software STRUCTURE v2.3.3 (Pritchard et al. 2000). The analysis was carried out using a burning period of 10000 iterations. A continuous series of *K* were tested, from 1 to 12, in 20 independent runs (Falush et al. 2003). No prior knowledge about the population of origin was introduced. The most informative *K* was identified using the Evanno's test (ΔK) (Evanno et al. 2005). Subsequently, population structure was inferred for $K = 5$ and using 50000 iterations.

A Factorial Correspondence Analysis (FCA) of genetic data was performed with GENETIX software version 4.05.2 (Belkhir et al. 2000), in order to examine genetic relationships among individual samples. The distance used was Chi-square distance:

$$d^2(j, j') = \left[\sum_{j=1}^{p} \left(\frac{f_{ij}}{f_{i.}} - \frac{f_{i'j}}{f_{i'.}} \right)^2 \times \frac{1}{f_{.j}} \right]^{\frac{1}{2}}.$$

Genetic diversity was estimated with the total diversity (H_T) (Nei 1973). The proportion of total variation that is distributed among populations (G_{ST}) was calculated as $G_{ST} = 1 - (H_S / H_T)$, where H_S is the mean heterozigosity within populations. The standardized G_{ST} (G'_{ST}), showing the maximum value that G_{ST} could reach according to the observed diversity, was calculated as $G'_{ST} = G_{ST}(1 + H_T)/(1 - H_S)$. Genetic distances and identities among groups were calculated according to Nei (1972).

Genetic distances between loquat accesions for phylogenetic tree construction were calculated according to Nei (1972), and the distance matrix obtained was

processed through the unweighted pair-group method (UPGMA) cluster analysis using the software POPULATIONS v.1.2.32 (Langella 2002). The stability of the UPGMA tree was tested using 1000 bootstrapped data matrices.

Results

Genotype characterization and genetic similarities

All SSRs loci analyzed resulted polymorphic in the 102 accessions studied. Putative *S*-allele fragments amplified from degenerated primers resulted also highly polymorphic. A total of 38 SSR alleles were clearly differentiated using the capillary electrophoresis sequencer (Table 1.2; Table S1). No discrepancies were found in the banding pattern of the duplicate analysis of each DNA sample. Number of alleles ranged from 2 to 5 per locus with a mean value of 2.9. The allelic frequencies (*p*) ranged from 0.78 to 0.02 with a mean value of 0.34. Six out of the 38 alleles might be considered rare (i.e., $p \leq 0.1$). The mean values estimated were: $H_e = 0.54$, $H_o = 0.59$, and PIC = 0.55. Regarding the *S*-locus, a total of 11 putative *S*-allele differing in length were identified, allowing genotype identification of the *S*-allele locus of loquat accessions analyzed (Table 1.3). In this case, *p* varied from 0.005 (‘*S*a’ and ‘*S*g’) to 0.24 (‘*S*i’) with a mean value of 0.09. Seven putative *S*-alleles might be considered rare (i.e., $p \leq 0.1$). H_e, H_o, and PIC values were significantly higher than those calculated for the SSRs, being 0.81, 0.83, and 0.80 respectively. The PIC values for all markers ranged from 0.357 (*ssr*EJ005) to 0.805 (SC/C2), with and average value of 0.551. The variability found using SSRs along with putative *S*-allele fragments was enough to distinguish 83 genotypes out of 102 accessions. The genotype characterization showed that 16 accessions share the same genotype as ‘Algerie’, so henceforth this group will be named ‘Algerie and its relatives’. This group includes bud mutations and related seedlings corresponding to accession’s number: 32, 34, 35, 36, 37, 38, 54, 55, 62, 65, 72, 80, 82, 83, 85, 92 and 95 (Table 1.1).

Table 1.3. Putative *S*-allele fragment composition detected by PCR analysis.

Name of cultivar	Putative *S*-alelle[a]	Name of cultivar	Putative *S*-alelle[a]
Advance	*Sc Sk*	Mercedes	*Sf Sk*
Al-Ama	*Sb Si*	Mil Homens	*Sb Si*
Alcácer	*Si Sk*	Miquel d'Aixarà	*Sb Si*
Alfons Gregori-1	*Sb Si*	Miquel Nucier	*Sb Si*
Alfons Gregori-2	*Sb Si*	Mogi	*Si Sk*
Algerie (Altea)	*Sb Si*	Mogi Wasse	*Se Sk*
Algerie clon tardío	*Sb Si*	Mrs Cooksey	*Sg Si*
Almargem	*Si Sk*	Nadal Tardío	*Sb Sc*
Barret	*Sb Si*	Nadal Temprano	*Sb Sc*
Beniaratx	*Sb Sd*	Ottaviani	*Sk*
Benimelli	*Sb Sc*	Peix	*Sb Sc*
Bianco	*Sc Sf*	Peluches	*Sb Si*
Borde	*Sh*	Pere Esquena	*Sc Si*
Cabelo	*Sb Si*	Piera	*Sk*
Cambrils	*Sd Sk*	Polop-1	*Sd Sf*
Champagne	*Sc Sf*	Puxinben	*Sj Sk*
Changhong-3	*Sf*	Raúl	*Sc Si*
Chirlero	*Sb Si*	Redonet	*Sb Sk*
Cort	*Sb Si*	Requina	*Sb Sc*
Cox	*Sd Sk*	Rolhâo II	*Sc Si*
Cremaor	*Sb Sc*	Ronda Brasil	*Sb Si*
Dama	*Sk*	Ronda Gruesos	*Sb Si*
Dazhong	*Si Sk*	Rosa	*Sc Sf*
Dulce Pera	*Sc Sf*	Rosa Tardío	*Sb Sc*
Estrada Blanc	*Sc Si*	Sabroso	*Si Sk*
Estrada Groc	*Sb Sk*	Sacós	*Sb Sk*
Francisco el Gordo	*Sb Sc*	Saeed-1	*Sb Si*
Golden Nugget	*Sk*	Saguntí	*Sb Si*
Hongganben	*Sf Sj*	Sally	*Sb Si*
Ikramullah-1	*Sd Sf*	Samper-1	*Sb Sc*
Ikramullah-2	*Sd Sf*	Samper-2	*Sb Si*
IRTA	*Sf Si*	Sanfilippara	*Sb Sc*
Ismael	*Sb Si*	Saval Brasil	*Sj Sk*
Italiano-1	*Sb Sk*	Saval Moreno	*Sb Sc*
Javierín	*Sj Sk*	Saval Nerviado	*Sc Si*
Jiefanghzhong	*Sb Si*	Saval-1	*Sb Sc*
Joaquín Giner	*Sb Si*	Saval-2	*Sb Sc*
Khyber-1	*Se Sk*	Sisantanou	*Sb Sc*
Linares	*Sb Si*	Siscar	*Sb Sc*
Magdal	*Sf Si*	Susana	*Sb Si*
Magdal Carne Blanca	*Sd Sk*	Taicheng-4	*Sb Sc*
Maite	*Sb Sc*	Tanaka	*Si Sk*
Manises	*Sb Si*	Tavira	*Sb Si*
Marc	*Sb Si*	Temprano de Petrés	*Sk*
Marchetto	*Sd Sf*	Toni Tomaca	*Sb Sk*
Mas Vagué	*Sb Sk*	Vaniglia Dulce	*Sb Sc*
Masía Cañera	*Sb*	Vila	*Sb Si*
Mata Mouros Regional	*Si Sk*	Virticchiara	*Sj Sk*
McBeth	*Sc Si*	Vista White	*Sc Sk*
Meihuaxia	*Sa Sk*	Xiangzhong-11	*Si Sk*
Menera	*Sb Si*	Zaozhong-6	*Si Sk*

[a] Approximate size (in bp) of the putative *S*-allele fragments amplified were: *S*a (550), *S*b (467), *S*c (459), *S*d (437), *S*e (399), *S*f (322), *S*g (308), *S*h (300), *S*i (294), *S*j (280) and *S*k (270)

Some SSR alleles are present in specific accessions and sets of cultivars. For instance, '*ssr*EJ282-d' allele is only present in 'Italiano-1', 'Redonet' and 'Xiangzhong-11' and '*ssr*EJ282-e' is absent in Chinese and Japanese accessions excepting 'Taicheng-'4. '*Ssr*EJ042-b' allele is present in 9 out of 102 cultivars, six Spanish, two Italian and one North American cultivars. '*Ssr*EJ088-b' is in 10 out of 102 cultivars, one Chinese, 4 Italian and 5 Spanish cultivars. Likewise, '*ssr*EJ271-e' allele is privative to nine accessions, from China (2), Japan (1), Italy (4), Portugal (1) and Spain (1). '*Ssr*EJ086-a' is presented in at least one accession from each area of origin, except in Italian and Portuguese accessions where it is not present in any of them. Regarding the *S*-alleles, '*S*a' is privative for 'Meihuaxia', '*S*e' is just found in 'Khyber' and 'Mogi Wasse', '*S*g' is present in 'Mrs Cooksey' and accession 'Borde' is the only one having the allele '*S*h'.

Population structure and genetic diversity

Genotype data from 13 SSR markers and *S*-allele were used to determine population structure among 102 accessions from the germplasm bank. The software STRUCTURE v2.3.3 uses a Bayesian clustering approach to make a probabilistic assignment of individuals to populations based on genotype. Individuals are assigned to multiple populations if their genotype indicates admixture. This analysis makes no assumption about the particular mutation process and not closely linked markers. The Evanno's test indicated that the most informative number of populations (*K*) was 5. The inferred population structure for $K = 5$ (Figure 1.1) showed that most of the accesions (71,6%) have a membership coefficient (q_i) to one of the population higher than 0.8, while the rest (28.4%) could be considered as admixted ($q_i \leq 0.8$). According the origin region there are two groups of Spanish, one group of Italo-Spanish, and two groups of non-European accessions. The 1st STRUCTURE group, named Spanish I, includes a total of 37 cultivars containing 'Algerie and its relatives', the Brasilian 'Ronda Brasil', the Italian 'Italiano-1', the Portuguese 'Tavira', the Pakistani 'Saeed-1', and Chinese 'Taicheng-4' and 'Xiangzhong-11'. The 2nd STRUCTURE group, named Spanish II, includes 18 cultivars from Spain and the Portuguese 'Rolhâo II'. The 3rd STRUCTURE group, named Italo-Spanish, consists of 15 cultivars, including 6 from Italy, the Portuguese 'Mata Mouros Regional' and 8 from Spain. The 4th STRUCTURE group consists of a mixture of 17 cultivars from the eight geographical areas, with a higher number of non-European accessions (58.8%), so named as Non-European I. Similarly,

the 5th STRUCTURE group consists mainly of a set of non-European cultivars (71.4%): North America (4), China (3) and Pakistan (2), so it was named as Non-European II.

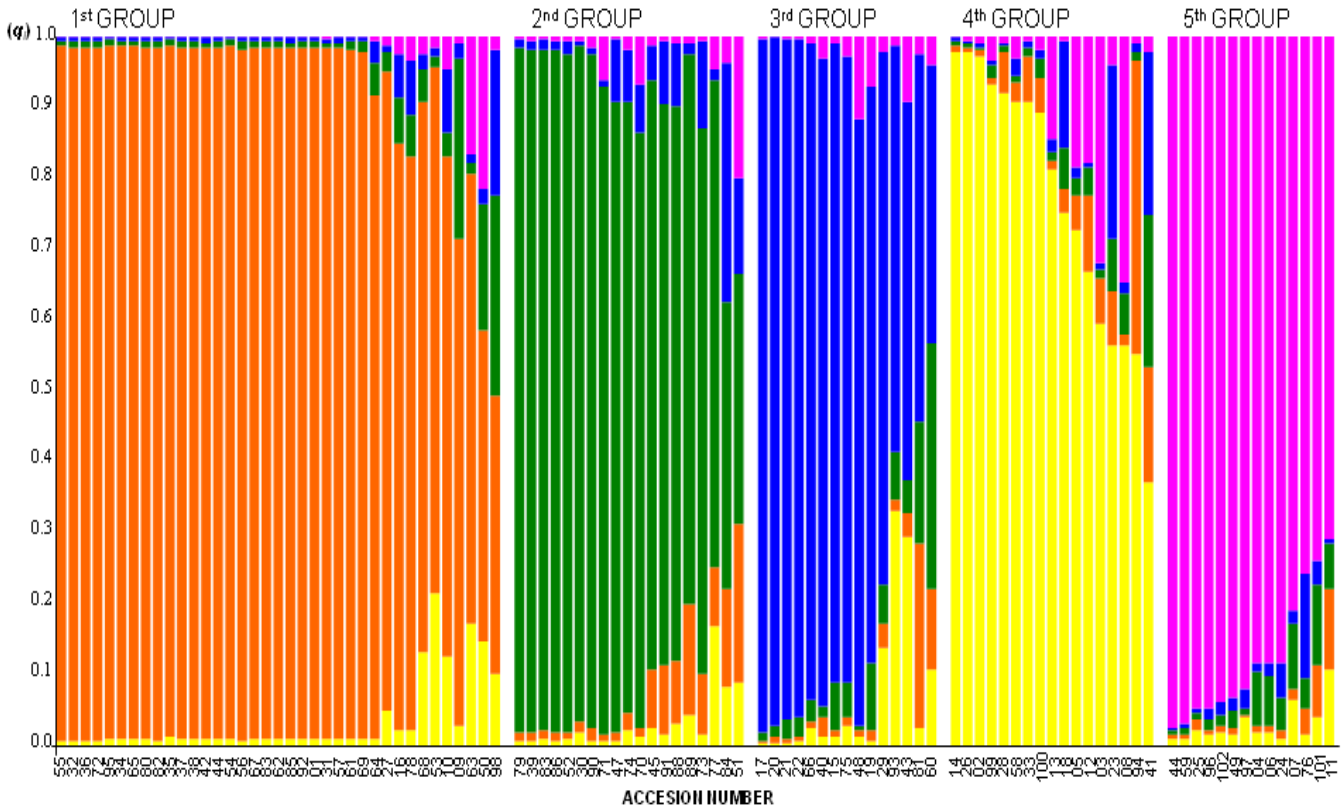

Fig.1.1. Assignment of 102 accessions to 5 STRUCTURE groups. The Y-*axis* displays the estimated membership (q_i) of each individual to a particular group, which is partitioned into colored segments (Orange = 1st GROUP, Green = 2nd GROUP, Blue = 3rd GROUP, Yellow = 4th GROUP, Pink = 5th GROUP). X-*axis* shows accessions numbered according to the order shown in Table 1.1.

In order to avoid the bias produced by a high number of identical genotypes over the genetic diversity calculation, just one accession from the 'Algerie and its relatives' group was kept, remaining 21 accessions in this 1'st STRUCTURE group. The five groups present a total genetic diversity (H_T) ranging from H_T = 0.4593 (the 5th STRUCTURE group) to H_T = 0.5241 (the 1'st STRUCTURE group) (Table 1.4). The total diversity within five STRUCTURE groups (H_T = 0.5682) results in a moderate value of the relative magnitude of genetic differentation (G_{ST} = 0.1660). However, the standardized G_{ST} (G'_{ST}) reached a much higher value (G'_{ST} = 0.4948) (Table 1.4). The values of heterozigosity per individual accession were high, with and average value of 0.62, reaching a maximum of 0.86 in 4 out of 102 cultivars.

The genetic distance between groups generated by STRUCTURE software is summarized in Table 1.5. The lowest value (0.130) is found between the 2nd and 3rd STRUCTURE groups, which corresponded to the Spanish II and Italo-Spanish ones.

The maximum value obtained (0.369) corresponds to Italo-Spanish and Non-European I groups. It is interesting the genetic distance (0.152) between the Spanish I and Non-European I groups.

Table 1.4. Total genetic diversity (H_T), genetic diversity within groups (H_S), relative magnitude of genetic differentiation (G_{ST}) and standarized G_{ST} (G'_{ST}), calculated from SSR and *S*-allele data according to the STRUCTURE analysis.

GROUP	Nº OF CULTIVARS	H_T	H_S	G_{ST}	G'_{ST}
TOTAL	102	0.5682	0.4739	0.1660	0.507
1 – Spanish I	21	0.5241			
2 – Spanish II	19	0.4754			
3 – Italo-Spanish	15	0.4892			
4 – Non-European I	17	0.4957			
5 – Non-European II	14	0.4593			

Table 1.5. Genetic distances (Nei 1972) between the groups obtained by STRUCTURE analysis.

GROUP	2 – Spanish II	3 – Italo-Spanish	4 – Non-European I	5 – Non-European I
1 – Spanish I	0.170	0.291	0.152	0.317
2 – Spanish II		0.130	0.252	0.226
3 – Italo-Spanish			0.369	0.295
4 – Non-European I				0.194

Factorial correspondence analysis

The first and second axis of the FCA using SSR and *S*-allele data account for 17.4% and 13.8% of the total contribution, respectively. The results showed a good agreement with the others analyzes conducted. Accessions assigned to each population ($q_i \geq 0.8$) appear grouped in specific areas in the plot (Figure 1.2). First axe separates 1st and 4th STRUCTURE groups, from 2nd and 5th STRUCTURE group, with 3rd STRUCTURE group in an intermediate possition. Regarding to the second axis, accessions from 1st, 2nd, and 3rd STRUCTURE groups, were located in the upper part, while 4th and 5th ones appear in the bottom part.

Cluster analysis

Based on SSRs and *S*-allele data, genetic distances among loquat accessions were used to generate an UPGMA cluster analysis (Figure 1.3). The dendrogram has eight major clusters mainly in agreement with the groups formed with STRUCTURE

software. Cluster 1 (C1) matches with 1st STRUCTURE group plus 'Toni Tomaca'. This accession could be considered as admixted for 1st and 4th STRUCTURE groups (q_1 = 0.413, q_4 = 0.549). The second cluster (C2) comprises 14 out of 19 accessions corresponded mainly to the 2nd STRUCTURE group. Accession 'Maite' (q_1 = 0.113, q_2 = 0.345, q_3 = 0.389 and q_4 = 0.108) was included into this cluster. The third and the fourth cluster (C3, C4) include twelve varieties of 4th STRUCTURE group. The fifth cluster (C5) is divided into 3 subclusters.

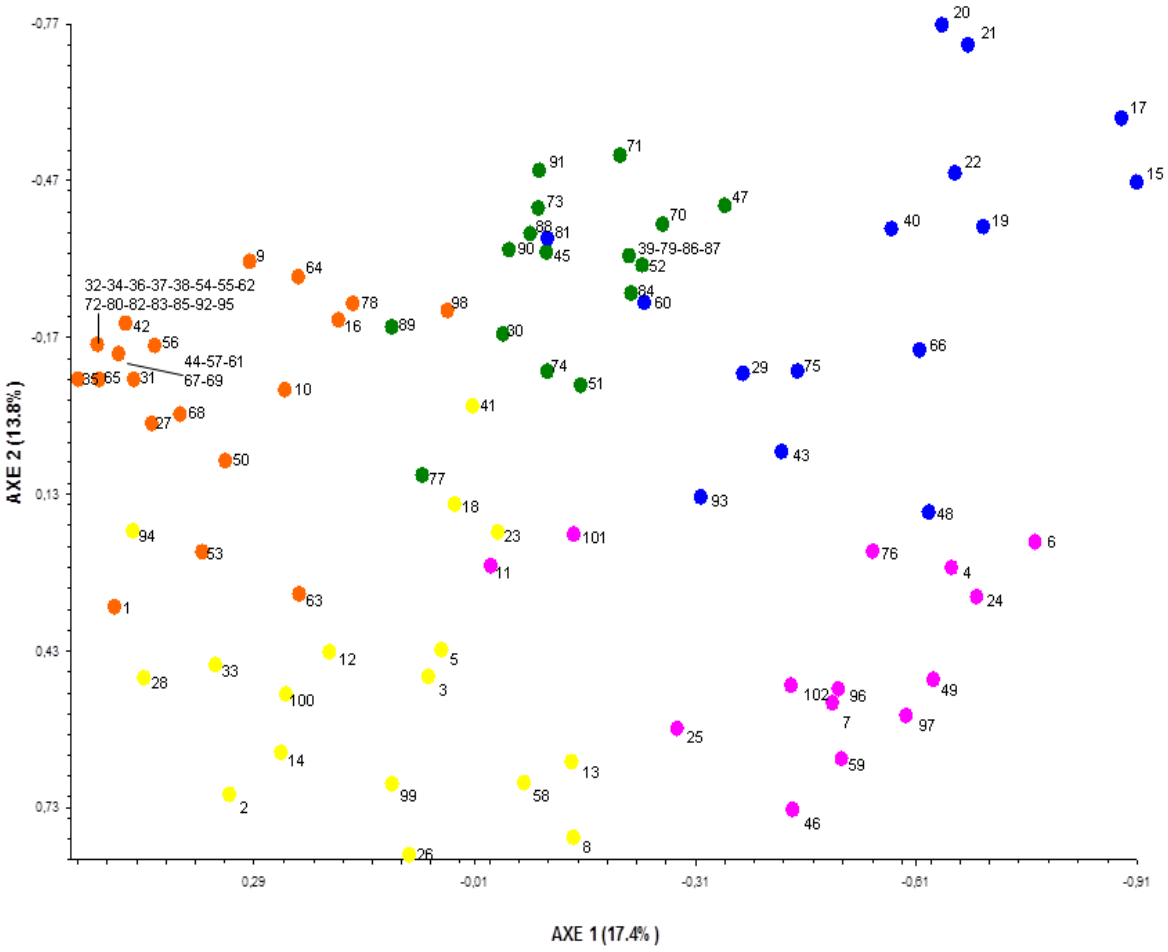

Fig.1.2. Factorial correspondence analysis (FCA) based on SSR and *S*-allele data. Scatterplot shows the projections of the 102 accessions on the first and second factors of the FCA (which accounted 17.4% and 13.8% of the total percentage of inertia, respectively). Accessions were numbered according to the order shown in Table 1.1, colors corresponded to the STRUCTURE groups (Orange = 1st GROUP, Green = 2nd GROUP, Blue = 3rd GROUP, Yellow = 4th GROUP, Pink = 5th GROUP). Accessions considered as admixted ($q_i \leq 0.8$), have been colored with the color corresponding to the group of its higher q_i.

The C5a and C5c subclusters correspond to cultivars belonging to the 5th STRUCTURE group, while C5b is composed by four Spanish and one Portuguese accession. The sixth cluster (C6) is mainly formed by admixed cultivars mostly

clustered in 3rd STRUCTURE group. The seventh cluster (C7) was composed of a mixture of six Asian accessions, four Chinese and two Japanese accessions. The eighth cluster (C8) was formed by 7 accessions belonging to 3th STRUCTURE group. Six out of nine Italian studied accessions were grouped in this cluster.

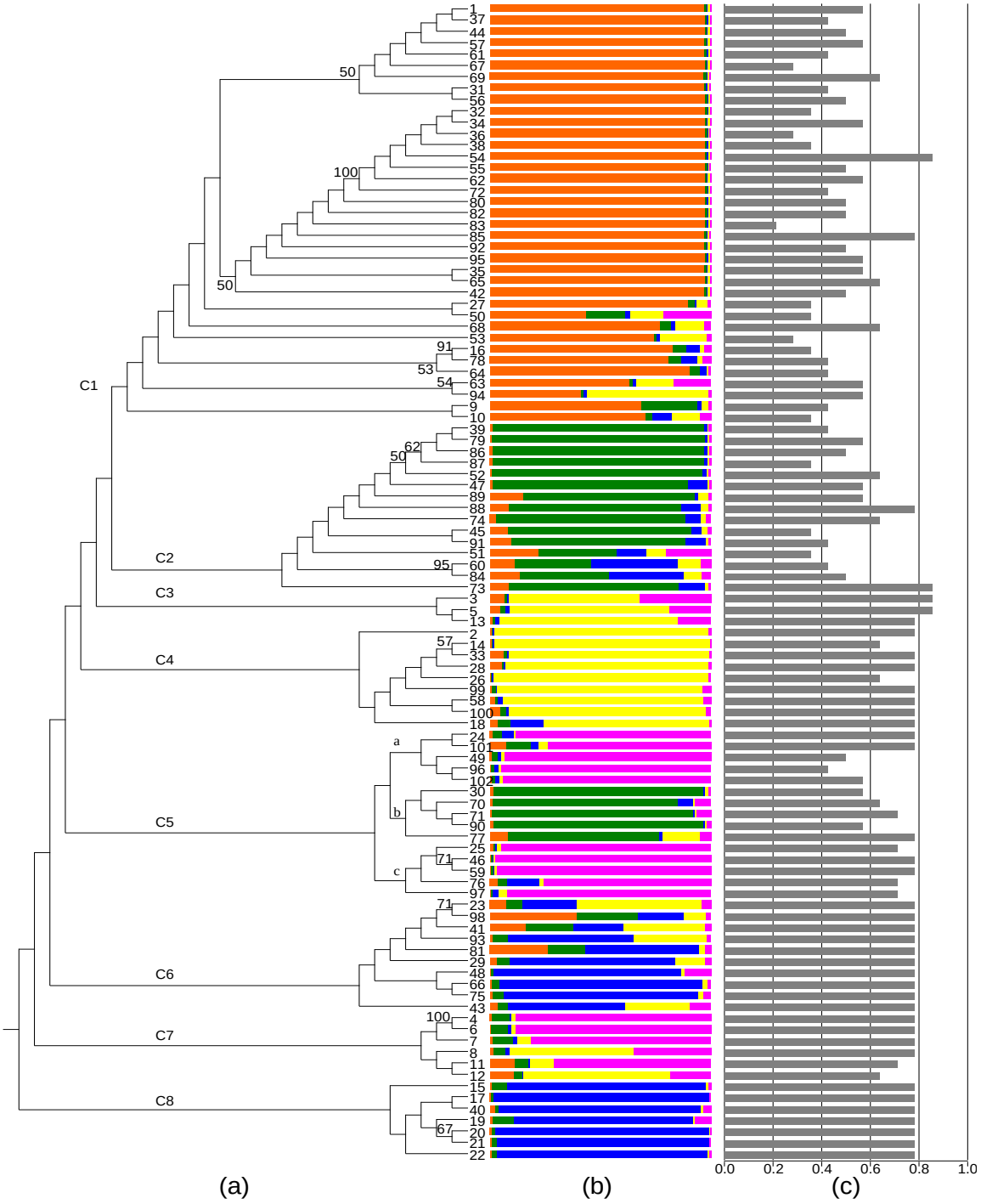

Fig.1.3. (a) UPGMA cluster analysis based on SSR and *S*-allele markers of the 102 loquat cultivars using Nei's (1972) genetic distance. Bootstrap values greater than 50% are shown. Accessions are numbered according to the order shown in Table 1.1. (b) For each accessions the estimated membership (q_i) to each group is shown (Orange = 1st GROUP, Green = 2nd GROUP, Blue = 3rd GROUP, Yellow = 4th GROUP, Pink = 5th GROUP). (c) Heterozigosity level of each accession.

Discussion

The IVIA loquat germplasm collection is the largest outside China and contains accessions from almost all countries where the crop has been established (Brazil, China, Japan, Italy, North America, Pakistan, Portugal and Spain). The main purpose of the study was to enlarge the information about the genetic diversity of the collection and to determine the genetic relationships among accessions, aimed at defining the best crossing strategies for future breeding programs, also to solve questions of identity and potential origin of some accessions. The use of SSR markers for diversity analysis has been performed with positive results in other woody plants such as persimmon (Naval et al. 2010), citrus (Barkley et al. 2006) and also it has proved to be effective in other crops such as eggplant (Hurtado et al. 2012) and maize (Frascaroli 2013). The SSR primers used were previously tested in loquat (Gisbert et al. 2009b), and displayed high polymorphism. These authors also included alleles from the *S*-locus, which were useful for detemining groups of intercompatibility. The present work increases the previous study including accessions not previously studied and also genotypes of the self-incompatibility alleles, which is of great importance for future breeding activities.

The genetic structure of the loquat germplasm bank was analyzed for the first time using jointly STRUCTURE software, FCA and UPGMA cluster analyses. Combination of these approaches was already tested with satisfying results as in genetic diversity studies of grape (Emanuelli et al. 2013), elite wheat (Couviour et al. 2011) and olive (Breton 2006).

The Bayesian-based analysis without a priori assignment of accessions to population resulted in five groups. As a result, seventy-three accessions were clasified into a single group, since they presented a membership coefficient q_i to group higher than 0.8 (Burle et al. 2010; Hurtado et al. 2012). The rest of cultivars could be considered as admixed, being most of them a mixture of two or three populations.

The groups of accessions observed were consistent in all three analyzes, the groups obtained using the STRUCTURE are used for consistency. European accessions showed a strong substructure, with 3 groups clearly defined: two with materials originated mainly from Spain, and another one mainly from both Italy and Spain. The remaining accessions grouped into two non-European groups. Within the Spanish I group (1st STRUCTURE group), 16 accessions share the same genotype as ‘Algerie’, both using SSRs and *S*-alleles, we named this group as ‘Algerie and its relatives’.

According to the records, these varieties share a common origin and were selected in a specific region by growers, either by seedlings or sport mutation in a small loquat crop area in the Southeast of Spain (Llácer et al. 2002). Using just one representative of the 'Algerie and its relatives' group, this 1'st STRUCTURE group showed the highest genetic diversity of the five deduced STRUCTURE groups. Within in the Spanish I group, 11 out 37 accessions were admixed showing some alleles from other groups, mainly from the Spanish II group and the Non-European group I, as can be shown both in FCA and STRUCTURE analyses. The Spanish II group is genetically closer to the Italo-Spanish group, but also close to the Spanish I group that contains 'Algerie and its relatives'. Interestingly, Morton (1987) reported that in the late 18th century loquat was planted in the Botanical Gardens of Paris (France) and Kew (England), and subsequently, the tree was grown on the Riviera, in Malta and French North Africa (Algeria) and the Near East. The substructure observed in the Spanish materials could suggest a relationship to this historical distribution of the species. Accession of Spanish II group could be related with material from other places not present in the IVIA germplasm collection.

Accessions included in Non-European materials, mainly come from China, Japan and North America. Plant material may have been exchanged between these regions, due to their geographical proximity and their relationships throughout history (Lin 2004). However, the lack of complete passport documentation about the real procedence of accessions, and not just the genebank where they are mantained, make difficult to analyze in more detail whether there are geographical differentiation between these two groups. As known, loquat is native to the South East of China. The medium and low region of Dahube river and South East of the Gongga Mountains is considerate the primary center of origin for *E. japonica* (Zhang et al. 1993), with the Yunnan region as a secondary center (Yang et al. 2005). The highest diversity based on pomological traits is located in its center of origin, there are thousands accessions described in the various Chinese germplasm collections (Lin et al. 2004; Zheng 2007). Interestingly, the Non-European I group shows high genetic diversity, with the accessions having a great dispersion in the graph, so it could be really interesting to know about the real origin of these materials and discuss their relationships with the center of origin of the species. On the other hand, there is more genetic distance between Non-European I and II groups, than between Non-European I and the Spanish I. This could suggest a genetic relationship between these two groups and be related

with the hypothesis that the cultivars introduced in the Mediterranean Basin were obtained from China at the end of the 18th century (Lin 2004).

Distribution of *S*-alleles and frequencies gave clues about the movement of germplasm during the spread of the species outside of Asia (Badenes et al. 2013). For instance, the *S*-allele '*S*b' is restricted to the Mediterranean area with four exceptions: 'Jiefanghong', 'Ronda Brasil', 'Saeed-1' and 'Taicheng-4'. From them, the genotypes '*S*b*S*c' and '*S*b*S*k' are only present in Spanish and Italian cultivars, and interestingly the Chinese 'Taicheng-4' is the only one containing the '*S*b*S*c' genotype suggesting a relationship with the Mediterranean accessions. The remained *S*-alleles identify in the genotypes studied are widely distributed among regions, thus, they could not be assigned to a specific geographical area.

The genetic diversity and structure of germplasm collections must be described before they can be exploited for plant breeding. New information about genetic diversity of loquat and its dispersion in the Mediterranean Basin was obtained. The substructure of loquat germplasm bank at IVIA will allow to select and to incorporate the most suitable plant materials into the breeding program of the species.

CAPÍTULO 2:

Colchicine-induced polyploidy in loquat (*Eriobotrya japonica* (Thunb.) Lindl.)

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Colchicine-induced polyploidy in loquat (*Eriobotrya japonica* (Thunb.) Lindl.)

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Abstract

The induction of polyploids in loquat (*Eriobotrya japonica* (Thunb.) Lindl.) is of great interest for producing larger and seedless fruits according to market demands. Under this premise, this work was aimed to obtain tetraploid plants of loquat using the antimitotic agent colchicine. Experiments consisted in applying colchicine on shoot apex from *in vitro* grown plants, *in vitro* grown whole plants and ungerminated seeds. Treatments on the shoot apex or submerging whole plants produced no stable polyploids. Conversely, subjecting ungerminated seeds to colchicine produced two triploids in the 0.5 % (w/v) solution after 24 h and one tetraploid after 48 h. The triploids obtained among treated seeds make us believe that these plants were present in the hybrids original seedlot. Polyploidy levels were firstly detected by flow cytometry and later confirmed by chromosome counting and morphological characteristics. The relative fluorescence was 1.5-fold higher in triploids and twofold higher in tetraploids as compared to diploids. As expected, the chromosome number was 2*n* = 34 in diploids, 2*n* = 51 in triploids and 2*n* = 68 in the tetraploid. Moreover, differences in morphological characteristics between diploid and polyploid plants were significant. The tetraploid plant was more compact than triploids or diploids. Particularly, stomata of polyploids were larger with lower density than diploids. Results indicate that induction of polyploidy in loquat species is a reliable tool for breeding new loquat varieties.

Introduction

Loquat (*Eriobotrya japonica* (Thunb.) Lindl., Rosaceae) is a non-climacteric fruit native to subtropical regions of China, and currently cultivated widely. Production in Spain is located in the Southeast coast, where 'Algerie' is the most important cultivar, accounting for 98% of the total production. Loquat is dormant during the summer, blooms in autumn, develops its fruit in the winter and is harvested in early spring. The juicy fruit is delicious and appreciated by consumers (Tian et al. 2007).

The major European loquat germplasm collection is held at the 'Instituto Valenciano de Investigaciones Agrarias' (IVIA) of Spain. The IVIA collection includes 127 cultivars, including 80 selected in Spain (Soriano et al. 2005; Gisbert et al. 2009a; Blasco et al. 2014a). The IVIA initiated a loquat breeding program in 2002 to obtain

new cultivars with the positive agronomic features of ‘Algerie’ but with more diversity in ripening date and fruit characteristics (Gisbert et al. 2006).

A tremendous effort has been made in the field of the conservation and evaluation of loquat biodiversity to date, but other ways to increase variability (as polyploidy) have not yet been sufficiently explored. Polyploidy has an important role in genetic and phenotype diversity as well as in plant evolution and breeding (Ramsey and Schemske 1998; Dhooghe et al. 2011; Xing et al. 2011). Natural polyploidy is widespread in the plant kingdom and is one of the most frequent ways for generating new species or lineages, mainly among ferns and flowering plants. Moreover, polyploid genotypes from fruit species include commercially successful cultivars because of their favourable characteristics, such as fruit quality (Wu et al. 2013), large fruit size (Rugini et al. 1996), seedless fruits (Kagan-Zur et al. 1991), disease-resistance (Predieri 2001), high yield and good adaptability (Liu et al. 2009).

In addition to the natural polyploidy, artificially induced polyploids has been considered a routine method for introducing variability. In order to obtain polyploid genotypes the chromosome number should be duplicated and duplication can be induced by several antimitotic agents, the most common used is colchicine. Since its discovery by Blakeslee and Avery (1937), colchicine has been successfully used to obtain polyploid plants from many fruit species (Sanford 1983).

Within the polyploidy, triploidy is an important feature of many fruit crops. Apple and pear triploids tend to be more vigorous than diploids (Einset and Imhofe, 1951). Furthermore, triploidy can lead to seedlessness as observed in banana (Simmonds and Shepherd 1955), citrus (Ollitrault et al. 2008), loquat (Guo et al. 2007) and watermelon (Kihara 1951). This objective is especially relevant in loquat species when the fruit has a relatively small size (30 to 40 g) and the edible proportion is lower than 70% (He et al. 2012). Loquat seedlessness has been achieved in China by selection of naturally occurring triploid clones, followed by applications of gibberellic acid for fruit set. The clones selected are currently under study to determine if they have characteristics for commercial uses (Badenes et al. 2013). The natural rate of polyploidy in loquat is 0.35% (He et al. 2012) hence more efforts are needed to develop alternative methodologies more efficient.

In this context of developing new cultivars and breeding lines to be used in plant production, the aim of our study was to asses an efficient methodology for obtaining

tetraploids in loquat. Here, we report for the first time the induction of polyploid plants from ‘Algerie’ loquat.

Material and Methods

Plant Material

Seeds from open-pollinated ‘Algerie’ loquat grown in Callosa d´Ensarrià (Alicante, Spain) were collected and stored at 4 ºC.

Colchicine treatment

1. Whole plant treatments

In vitro-grown seedlings (28 days after germination) were immersed into 0.1% (w/v) colchicine solution for 15, 30, 45 or 60 min at room temperature (RT). Forty eight plants were used for each treatment. Plants treated only with distilled water were used as control samples. After the treatments, the plants were cultured in test tubes containing 20 ml of liquid medium consisted in Murashige and Skoog (MS) (1962) inorganic basal salts (Duchefa Biochemie), vitamin mixture (200 $mg.l^{-1}$ glycine, 1 $g.l^{-1}$ thiamine hydrochloride, 1 $g.l^{-1}$ nicotinic acid, 500 $mg.l^{-1}$ pyridoxine hydrochloride, 500 $mg.l^{-1}$ ascorbic acid), sucrose (20 $g.l^{-1}$), *myo*-inositol (100 $mg.l^{-1}$) and thiamine hydrochloride (0.1 $mg.l^{-1}$) and maintained in a tissue-culture chamber at 24 ºC ± 2 ºC under cool-white fluorescent and Gro-Lux light (60-100 µE m^{-2} s^{-1}; 16-h light/8-h dark photoperiod).

2. Shoot apex treatments

In vitro-grown seedlings (28 days after germination) were used as source of explants. Developed leaves and axillary buds were removed and colchicine solution was applied to the shoot apex. One drop of 0.01 or 0.05% (w/v) colchicine solution was applied to the shoot apex for one, two or three consecutive days. Forty eight seedlings were used per treatment. Seedlings treated with distilled water were used as control samples. Plants were then cultured in test tubes containing liquid medium and maintained in a tissue-culture chamber under the conditions described above.

3. Seed treatments

Colchicine solution was applied directly to ungerminated seeds. Seeds were surface-sterilized by immersion in a diluted commercial bleach solution (4% w/v sodium hypochlorite) with 0.1% (v/v) of Tween-20® for 10 m, followed by three rinses in sterile distilled water. After sterilization, seeds were treated with 0.1%, 0.3% or 0.5% (w/v) colchicine solution for 24 or 48 h at RT. Forty eight seeds were used for each treatment. Seeds treated only with distilled water were used as controls. Treated and control seeds were germinated in the darkness (150 × 25 mm test tubes) on germination medium consisting of MS supplemented with 2% (w/v) sucrose. This medium was solidified with 0.8% (w/v) purified agar (Pronadisa) and its pH ajusted to 5.7 before autoclaving at 120 ºC for 30 min. After 3 weeks, the tubes were transferred to a tissue-culture chamber as described above.

Acclimatization

After *in vitro* culture for two months, the plants were transferred to pots (250 cc) containing a mixture of peat and perlite (1:1). The medium was carefully washed out of the roots. The potted plantlets were covered with transparent plastic for 7 days which was gradually perforated to allow slow adaptation of plantlets to lower humidity. All plants were grown under natural photoperiod and greenhouse conditions (18 – 28 ºC).

Determination of ploidy level

1. Flow Cytometry Analysis

DNA content was determined using a flow cytometer with fluorescence excitation provided by a mercury arc lamp (PA-II Ploidy Analyzer; Partec). Each sample comprised a leaf-piece of the analysed plant (approx 0.5 mm^2) with a similar leaf-piece from a diploid control plant. Samples were chopped on a 50 mm petri dish with a sharp razor blade in 1.0 ml of nuclei isolation buffer (Partec, Münster, Germany). The samples were re-suspended and filtered through a 50 μm nylon filter (Nybolt, Zürich, Switzerland), and 3.0 ml of coloration solution (Partec, Münster, Germany) containing 1 $mg.l^{-1}$ of DAPI (4,6-diamino-2-phenyl-indole) for fluorescent DNA staining were added. The plot of data on a semilogarithmic scale resulted in a histogram with peaks from 2C to 50C evenly distributed along the abscis axe. Calibration was

carried out using the 2C-peak of nuclei of leaves from diploid loquat of ‘Algerie’. About 500 to 1000 nuclei were measured per sample.

2. Chromosome Counting

Leaf pieces (5 mm^2) and root tips (6 mm long) were collected and pretreated with 0.04% of 8-hydroxyquinoline for 4h at RT, followed by 3.5 h at 4 ºC in the dark for metaphases accumulation. After removing the 8-hydroxiquinoline, samples were treated in a small vial of fixative solution (3 parts 100% ethanol: 1 part glacial acetic acid) for 72 h at RT in the dark. Samples were rinsed thoroughly in distilled water to remove fixative. For mitotic analysis, samples were hydrolysed in 0.25 N HCl for 10 min at RT, washed in distilled water and placed in digested citrate buffer (0.01 M trisodium citrate-dihydrate + 0.01 M citric acid monohydrate), for 10 min at RT. Before enzyme solution treatment, leaf pieces and root tips were cut obtaining 2 mm^2 and 0.5-1.5 mm long pieces respectively. The explants were then incubated at 37 °C in an enzymatic solution (5% Cellulase Onozuka R10 + 1% pectolyase Y23) for 30 to 40 min (D´Hont et al. 1996). Then, samples were rinsed in distilled water for 15 minutes, removed the excess of water and 1 or 2 drops of fixative solution added. Followed by spread with a fine forceps. The slides were air dried. Chromosome slides were coloured with 1 µg/ml DAPI and viewed with a fluorescence microscope at oil immersion objective 100×.

3. Morphological measurements

The 2-year-old diploid and polyploid plants were compared for morphological traits including plant height (cm), number of internodes and internodal distance of main stem (cm). Leaves were evaluated for leaf length (cm) and width (cm), and leaf index (length/width). Size and density of stomata from diploids and polyploids were also recorded.

Statical analysis

Survival rate of all colchicine treatments and polyploid induction efficiency were evaluated. Polyploid induction efficiency was measured with the method described by Bouvier et al. (1994), where % efficiency = % seedling survival × % polyploid induction. Efficiency gives a range from 0 to 100, which 100 would indicate that all

treated seedling survived showing polyploidy. Zero would indicate that all treated seedling died or no polyploidy induction occurred. Multifactorial ANOVA and multiple range tests (Student's *t* test) were performed using the software Statgraphics Plus 5.1 (Statistical Graphics Corporation, USA).

Results and Discussion

Survival rate and pre-screening of polyploids

The colchicine treatment applied to shoot apex decreased survival rate. While the control showed a survival rate of 100%, under different colchicine treatments ranged from 69 to 81% (Table 2.1). Survival was significantly lower in all treated plants independently of the treatment duration and colchicine dose. Whole in vitro-grown seedlings showed higher survival rates, ranging from 100% (15 min treatment) to 75% (60 min treatment). On the other hand, treatments on ungerminated seeds exhibited significant differences in survival depending on colchicine dose and time of exposure. In this case, the survival rate ranged from 77 to 94%. As colchicine concentration and time of exposure increased, survival decreased. The highest lethality was observed in the treatments with the 0.5% solution for 48 h. High concentrations of colchicine are associated with plant cell death because of the highly toxic effect of this antimitotic agent, which blocks spindle fiber development and modifies the differentiation process (Pintos et al. 2007). The higher level used here is higher than the common doses used in other fruits, that usually range between 0.02% and 0.1% (Dhooghe et al. 2011), but the lethality observed with this dose in loquat is lower than the observed in citrus (Aleza et al. 2009a), which caused 100% lethality using micrografted shoot tips.

Two months after the colchicine treatments, one leaf per seedling and treatment was subjected to flow cytometry to quantify the DNA content and confirm the presence of putative polyploids (pre-screening). Independently of the type of explant, concentration dose and time of exposure, the induction of putative polyploids was negatively correlated with the survival rate (R^2 = -0.57).

The number of putative polyploids differed between the treatments (Table 2.1). Forty-three putative polyploids were obtained in seedlings treated in the shoot apex including all concentrations and exposure times. With this procedure, 0.05% colchicine concentration applied with 1 drop during 3 days induced the highest number of putative

polyploids (12). Seedlings treated by immersion of the whole plant in 0.1% colchicine resulted in 16 putative polyploids including 4 exposure times (15, 30, 45 and 60 min). The highest rate was obtained with 30 minutes of exposure.

Table 2.1. Effect of colchicine treatments on different organs. Survival rate and induction of polyploid plants

Treatment	Colchicine concentration (% w/v)	Treatment duration	Nº treated explants	Nº survival treated explants[a] (%)	Nº putative polyploids[b] (%)	Nº polyploids identified[c] (%)
Shoot apex	0 (control)	1 drop/1 day	48	48 (100.0)	0 (0.0)	0 (0.0)
		1 drop/2 days	48	48 (100.0)	0 (0.0)	0 (0.0)
		1 drop/3 days	48	48 (100.0)	0 (0.0)	0 (0.0)
	0.01%	1 drop/1 day	48	38* (79.1)	2 (4.2)	0 (0.0)
		1 drop/2 days	48	39*(81.3)	7 (14.6)	0 (0.0)
		1 drop/3 days	48	37*(77.1)	6 (12.5)	0 (0.0)
	0.05%	1 drop/1 day	48	35*(72.9)	5 (10.4)	0 (0.0)
		1 drop/2 days	48	33* (68.8)	11 (22.9)	0 (0.0)
		1 drop/3 days	48	34* (70.8)	12 (25.0)	0 (0.0)
Whole plant	0 (control)	15 min	48	48 (100)	0 (0.0)	0 (0.0)
		30 min	48	48 (100)	0 (0.0)	0 (0.0)
		45 min	48	48 (100)	0 (0.0)	0 (0.0)
		60 min	48	48 (100)	0 (0.0)	0 (0.0)
	0.10%	15 min	48	48 (100)	2 (4.2)	0 (0.0)
		30 min	48	42 (87.5)	8 (16.7)	0 (0.0)
		45 min	48	42 (87.5)	2 (4.2)	0 (0.0)
		60 min	48	36* (75.0)	4 (8.3)	0 (0.0)
Ungerminated seed	0 (control)	24h	48	48 (100)	0 (0.0)	0 (0.0)
		48h	48	48 (100)	0 (0.0)	0 (0.0)
	0.10%	24h	48	44 (91.6)	0 (0.0)	0 (0.0)
		48h	48	40 (83.3)	1 (2.1)	0 (0.0)
	0.30%	24h	48	45 (93.7)	0 (0.0)	0 (0.0)
		48h	48	38* (79.2)	0 (0.0)	0 (0.0)
	0.50%	24h	48	41 (85.4)	5 (10.4)	2 (4.2)
		48h	48	37* (77.1)	3 (6.3)	1 (2.1)

[a] Data recorded 1 month after colchicine treatment,
[b] Flow cytometric analysis in 2-month-old plants
[c] Flow cytometric analysis in 2-year-old plants
* Significantly different to the control (Student's *t* test, $P < 0.05$)

Treatments on ungerminated seeds were the least efficient in terms of recovery of putative polyploids. No polyploid was obtained with the 0.3% dose and only one putative polyploid was obtained using 0.1% colchicine and 48 h of exposure. With this type of material the highest dose (0.5%) was the most efficient, with 8 induced putative polyploids, most of them obtained with the 24 h treatment (Table 2.1). The lower induction obtained with ungerminated seeds may be related with an effect of the seed coat over the penetration of the compound. It is true that the viability obtained with

0.1% was similar to the one observed with whole plants, but with 24 times higher exposition duration. The behaviour observed in this work differs from the observed in other species. For example, Rubuluza et al. (2007) in *Colophospermum mopane* Kirk ex Benth. J. Léonard observed a higher polyploidy induction in ungerminated seeds at 0.1% concentration of colchicine and 48 h of exposure, indicating that higher concentrations were harmful to growth and survival. In other species, the use of shoot tip as treated tissue has also proved to be more efficient in the recovery of putative polyploids (Kulkarni and Borse 2010).

Despite mixoploidy being described as relatively common in putative polyploids obtained after colchicine treatments in other species (Sun et al. 2009; Zhang et al. 2010; Harbard et al. 2012), in our case, no putative polyploid could be classified as mixoploid.

Flow cytometric analysis

Putative polyploids obtained and control plants were successfully acclimatized and transplanted in the greenhouse. Confirmation of ploidy level was carried out by flow cytometric analysis in a combined sample of four leaves from different parts of the plant two years after the transfer to the greenhouse. The shoot apex treatments despite of proving to be the most efficient method in terms of putative polyploidy induction rates, failed to generate whole polyploid plants (Table 2.1). This may be explained by the production of chimeras, a common effect of colchicine treatments (Schifino and Moraes-Fernandes 1987; Tel-Zur et al. 2011). In meristem treatments only part of the cells or cell layers resulted affected, and reversions to diploid status would be expected (Väinölä 2000; Harbard et al. 2012). In loquat tetraploid tissues were not identified in any of the potential polyploid mature plants. In other species such as citrus, the recovery of cytochimeras with variable proportions of 2*x* and 4*x* tissues is even higher than stable tetraploids, and different methodologies can be applied to recover tetraploid plants from them (Aleza et al. 2009a). In our case, it seems that a preponderance of the diploid tissue in the meristems giving raise to the whole plants should have occurred in an early stage.

The use of oryzalin has been proposed as an alternative to colchicine treatments, claiming that reversion of putative tetraploids to diploid state can be lower than in the case of colchicine, though oryzalin-induced seedling stunting may delay plant evaluation (Lehrer et al. 2008). Nevertheless, considering the survival rates obtained

after colchicine treatments the use of oryzalin could be considered as an alternative. In this sense, in programs aimed at the development of tetraploids in citrus oryzalin proved be less phytotoxic, although it induced lower levels of tetraploidy (Aleza et al. 2009a). Still, a strong interaction between antimitotic agents and the species is expected and previous results obtained in other species might not apply in loquat (Dhooghe et al. 2011). In fact, in contrast with the results reported by Aleza et al. (2009a) in citrus, Lehrer et al. (2008) working with Japanese barberry (*Berberis thunbergii* DC.) obtained higher lethality with oryzalin and the levels of polyploidy were more complete and stable than with colchicine.

The method of immersion in colchicine of seedlings also failed to produce mature whole polyploid plants. In this case, the 16 putative polyploids showed poor growth potential, the elongation of the radicle was inhibited and the plants finally died. This deleterious effect of colchicine on subsequent root growth was also observed in colchicine treatments of ungerminated seeds of *Platanus* × *acerifolia* (Liu et al. 2007).

Out of the 9 putative polyploid plants obtained from ungerminated seed treated with colchicine, two were triploid, one was tetraploid and six were, in fact, diploid (Figure 2.1). The triploid plants were recovered with 0.5% colchicine for 24 h and the tetraploid plant with 0.5% colchicine for 48 h (Table 2.1). Thus, the optimum polyploid induction rate was achieved with the higher concentration of colchicine independently of the exposure time. Therefore, more aggressive treatments, caused higher lethality but also a higher rate of stable tetraploid recovery.

Triploids have been recovered from interspecific crosses in different species (Hahn et al. 1990) or from selfing in the same species (Ollitrault et al. 2007). Usually, the spontaneous polyploids came from the union of reduced and unreduced gametes, as somatic mutation generally only doubles the base chromosome number (Ramsey and Schemske 1998). Thus, it is rather unusual to obtain triploids without the mediation of diplod × tetraploid crosses or spontaneous gamete non reducted in diploid × diploid crosses.

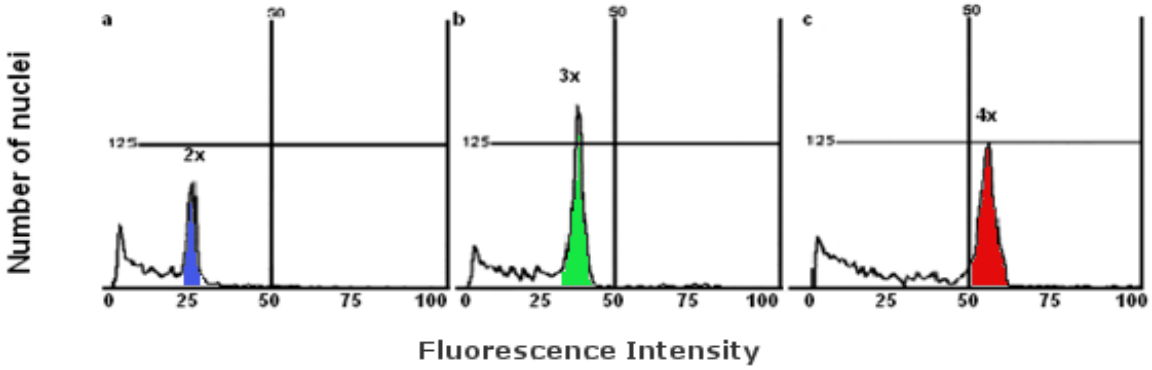

Fig.2.1. Flow cytometry histogram of 2-year-old loquat plants from ungerminated seeds treated with colchicine. a. Diploid control plant (2*x*=34), b. induced triploid plant (3*x*=51), c. induced tetraploid plant (4*x*=68).

In our case, triploids recovered from ungerminated seeds treated with colchicine should have been originated during the previous hibridization. Previous reports on spontaneous triploid recovery in diploid x diploid crosses have proved a genotypic effect with triploid recovery rates between 0.18% and 1.62% (Guo et al. 2007). Nevertheless it remains unclear why a relatively high spontaneous triploid generation rate (4.2%) was only observed in a certain treatment.

Chromosome counting in leaf and root-tip

Flow cytometry is an easy and efficient tool for ploidy analysis and it is regarded as one of the most accurate tool for ploidy determination (Gamiette et al. 1999; Loureiro et al. 2005). Nevertheless, in order to corroborate the data obtained by this technique, we proceeded to count the chromosomes in the polyploid plants obtained as a direct method. Chromosome counting on leaf and root-tip nuclei confirmed the results of the flow cytometry analysis. The chromosome number of the diploid control plant was $2n$ = 34 (Figure 2.2a), whereas that of triploid plants was $2n$ = 51 (Figure 2.2b) and tetraploid plant was $2n$ = 68 (Figure 2.2c). The use of an alternative methodology on different tissue samples helped to discard any possible chimeric artifact.

Morphological and stomata characteristics

The effects of ploidy level were evaluated in morphological and stomata characteristics, using traits usually affected by ploidy level. In fact, height, number of nodes and internodal distance, and stomatal differences have been used to provide data

for early identification (pre-screening) of polyploids, specially when a large amount of putative polyploids need to be evaluated (Mishra 1997; Aryavand et al. 2003; Khazaei et al. 2010).

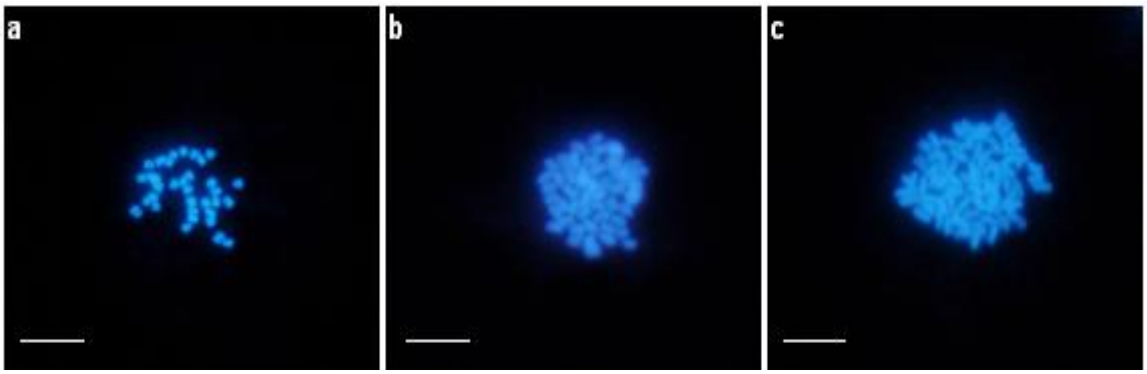

Fig.2.2. Chromosomes of root tip cell. a. Cuttings of diploid control plant (2*x*=34), b. induced triploid plant (3*x*=51), c. induced tetraploid plant (4*x*=68). *Bar* 10 µm.

Polyploid plants grew normally in the greenhouse, but the tetraploid obtained showed a slower growing rate than the triploid and control diploid plants. Two years after the colchicine treatment, the tetraploid plant still showed a reduced plant height, higher number of internodes and shorter internodal distances compared with control plant (Table 2.2; Figure 2.3a). On the other hand, the triploids exhibited similar increased height and internodal distances than diploid plants. They also showed larger leaves than those of the diploid and tetraploid plants (Table 2.2; Figure 2.3b).

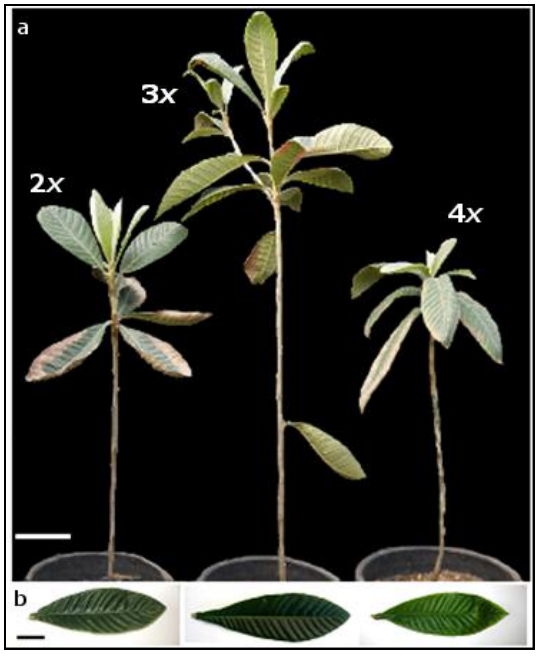

Fig.2.3. Morphological characteristics of polyploids obtained. a. 2-year-old plant diploid control (left), triploid (middle) and tetraploid (right) loquat (*bar* 10cm). b. fully expanded leaves of 2-year-old diploid (left), triploid (middle) and tetraploid (right) (*bar* 5 cm).

Table 2.2. Comparison of morphological characters and stomata characteristics of 2-year-old polyploid and diploid plants of *Eriobotrya japonica* (Tunb.) Lindl.

Characters	Sample					S
	2*x*		3*x*		4*x*	
	Plant 1	Plant 2	Plant 1	Plant 2	Plant 1	
Plant height (cm)	70.80	74.8	83.50	84.50	54.00	
Number of internodes	25	27	28	27	35	
Internodal distance of main stem (cm)	2.90	2.77	2.98	3.13	1.60	
Length of leaf (cm)	25.00 ± 1.58	26.02 ± 1.10	28.11 ± 3.52	31.19 ± 2.51	21.40 ± 2.79	*
Width of leaf (cm)	9.84 ± 0.15	9.58 ± 0.23	8.85 ± 1.25	10.24 ± 0.74	9.00 ± 1.22	
Leaf index (length/width)	2.55	2.72	3.18	3.05	2.37	*
Length of stomata (μm)	14.13 ± 0.88	14.88 ± 0.80	17.93 ± 0.60	17.95 ± 0.63	20.0 ± 0.20	*
Width of stomata (μm)	13.19 ± 0.88	13.32 ± 0.8	16.91 ± 0.82	17.08 ± 0.82	17.0 ± 0.10	**
Stomata index (length/width)	1.07	1.12	1.06	1.05	1.18	*
Number of stomata per unit area ($n.mm^{-2}$)	57.4 ± 3.91	56.4 ± 6.50	43.60 ± 4.10	42.40 ± 3.29	35.7 ± 1.1	*

S (SIGNIFICANCE): * indicated means significantly different within three homogenous group and ** indicated means significantly different within diploid and polyploid group according to Student's *t* test $P < 0.05$

Stomatal length and width and mean stomatal frequency per square millimetre were calculated. For stomatal length, there were significant differences between diploid (mean 14.5 μm), triploid (mean 17.9 μm) and tetraploid plants (mean 20.0 μm). For stomatal width triploid and tetraploid plants were significantly larger than diploids but the difference between triploids and tetraploid were not significant (Table 3.2; Figure 3.4).

For stomatal frequency there were significant differences among diploid, triploid and tetraploids plants (Table 2.2). The average of stomata frequency in diploids was $56.9/mm^2$, in triploids was $43.0/mm^2$ and that in tetraploids was $35.7/mm^2$ (Table 2.2; Figure 2.4).

Leaves from polyploid plants showed more chloroplasts per stomata than leaves from diploid plants (Figure 2.5). Our results were in agreement with previously studies of stomatal characteristics at different ploidy levels in the genus *Coffea* (Mishra 1997), in *Aegilops neglecta* Req. ex Bertol. (Aryavand et al. 2003) and wheat landraces

(Khazaei et al. 2010) where authors noted that diploid species had the highest stomatal frequency and the lowest stomatal length and width.

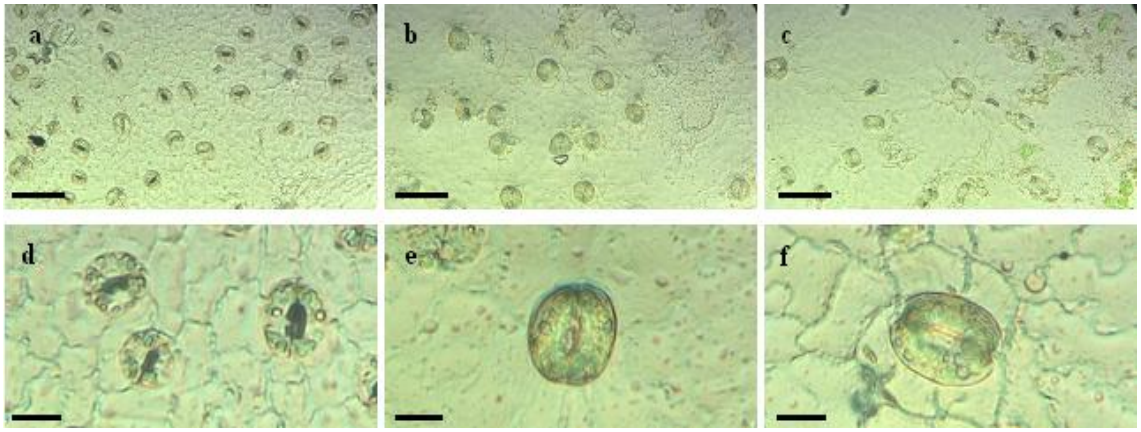

Fig.2.4. Stomata characteristics of 2-year-old polyploid and diploid plants. Stomatal density in loquat diploid (a), triploid (b) and tetraploid (c) (*bar* 50µm). Stomata size in loquat diploid (d), triploid (e) and tetraploid (f) (*bar* 10µm).

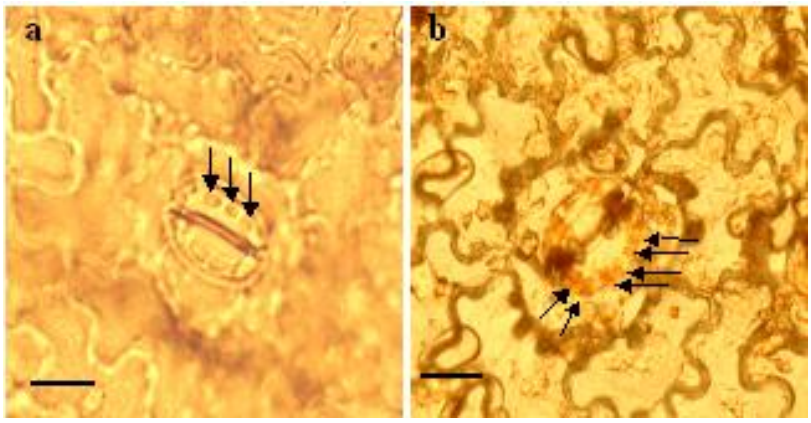

Fig.2.5. Differences in chloroplast number in guard cell. (a) Diploid and (b) triploid loquat (*Bars* 10 µm).

All of them conclude that stomatal observation represents a rapid and efficient method for screening putative polyploid plants. However, precautions should be taken into account, since age and leaf position could influence the size of stomata, resulting in a no reliable and consistent indicator of ploidy (Sakhanokho et al. 2009).

Among the methods for analysis of polyploidy, flow cytometry is the most efficient and precise method for detecting changes in ploidy level (Doležel 1997). Flow cytometry equipment presents important advantages, e.g. samples are easily and rapidly prepared, there is no need to divide cells and only a few milligrams of tissue are needed, and it is a quick and reproducible method for determining the ploidy levels of large numbers of samples (Sakhanokho et al. 2009).

In conclusion, it is possible to obtain induced tetraploids in loquat using colchicine treatments, although high colchicine doses show higher lethality, they are more reliable to develop stable tetraploid plants. The efficiency in tetraploid development might be susceptible of improvement. In this sense higher doses on shoot apex and whole plants might help to generate stable polyploids. The high rate of triploid recovery observed in a certain treatment also deserves a more detailed analysis in future works. The polyploids obtained are currently growing in the field for further use in breeding programs.

CAPÍTULO 3:

Pollen embryogenesis

CAPÍTULO 3.1:

Embryogenic response from anther culture of loquat (*Eriobotrya japonica* (Thunb.) Lindl.) cultivars from different origin

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Euphytica. Submitted.

Embryogenic response from anther culture of loquat (*Eriobotrya japonica* (Thunb.) Lindl.) cultivars from different origin

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Abstract

Production of haploid plants in loquat (*Eriobotrya japonica* (Thunb.) Lindl.) is of great interest for obtaining homozygous lines in a single step. Homozygous lines are very useful for genetic studies applied to plant breeding in higher plants. In this study, pollen embryogenesis by anther culture has been used to regenerate plants. Different variables related to embryogenesis induction, such as temperature pre-treatment of flower buds, growth regulators in the culture medium and the effect of genotype were studied. Eight cultivars of loquat from different origins were used. The first step was to unravel the association between floral bud size and the corresponding pollen microspore developmental stage for the different cultivars. Microspores at the uninucleate stage were the right stage for induction to produce calli. Cold pre-treatments at 4 ºC for 4, 8 and 12 days on flower buds were applied resulting in callus formation only when the pre-treatment lasted 4 days; however, the response was lower than on anther without cold treatment. The highest percentage of morphogenic calli was obtained on medium supplemented with 4.56 μM Z and 5.36 μM NAA in cvs. 'Changhong-3' (27%), 'Jiefanghong' (30%) and 'Moggi Wase' (36%). When produced calli were transferred to induction embryo medium, six embryos were obtained from cv. 'Jiefanghong'; and one of them developed a plantlet. Flow cytometry and chromosome counting results revealed that the plantlet was triploid.

Introduction

The main objective of the loquat (*Eriobotrya japonica* (Thunb.) Lindl.) breeding program is to develop new varieties that meet consumer requirements including fruit quality, seedlessness and larger fruit size, while meeting the growers demands of high yield, longer ripening season and resistance or tolerance to biotic and abiotic stresses, that reduced the cost of orchard management along with dwarfing rootstocks with graft compatibility (Badenes et al. 2013).

Loquat is a perennial fruit tree with a long juvenil period and high heterozygosity due to its self-incompatibility trait in many cultivars. Therefore, breeding programs, based on conventional methods such as hybridization followed by seedling selection, has become a long term procedure (Gisbert et al. 2007b; Zheng 2007; Tepe et al. 2011). In perennial species, fixation of the most important target genes

became a difficult and time-consuming task due to the need of a high number of cycles of backcrosses and selection. In the last few decades, new biotechnology methods that can improve and speed crop breeding in woody species have become available. For instance, development of markers targeting the most important traits, somatic hybridization to overcome the cross barriers, embryo culture, genetic transformation and haploidization through gametic embryogenesis. Among the different techniques available for increasing breeding efficiency, obtaining haploids and double haploids (DH) individuals has a great interest, since these procedures allow the fixation of traits in homozygous state in a single step. Additionally, haploids are important in genetic studies towards plant breeding goals since they offer higher efficiency in the selection of desired recombinants, selection of recessive mutants and exploitation of heterosis (Germanà 2009).

Anther culture objective is focused on production of haploid plants, but sometimes the regenerants grown from anthers showed changes in chromosome number (Germanà 2011a). Several processes such as endomitosis, nuclear fusion and endoreduplication of unreduced microspores may be the cause of polyploidization (Shim et al. 2006; Li et al. 2013).

Biotechnological methods offer different ways to obtain haploid or DH individuals, involving the male or female gametophytes. Among them, *in vitro* anther or isolated microspore embryogenesis has become successful technique for the breeding industry in the last few decades (Maluszynski et al. 2003; Germanà 2009). But despite the potential impact of the development of DH in woody species, the progress in this area and its application in breeding new varieties have been limited; mainly due to slow progress in the development of efficient methods for haploid isolation (Dunwell 2010). Regeneration from male gametes has been reported in about 200 species belonging to different families, such as *Solanaceae*, *Cruciferae* and *Gramineae* (Dunwell 1986; Hu and Yang 1986; Maluszynsky et al. 2003), while in fruit crops, anther culture has been successfully applied in *Annona squamosa* L., *Carica papaya* L., *Citrus madurensis* Lour., [*Musa balbisiana* (BB)], and *Poncirus trifoliata* L. Raf. (Germanà 2006a). Nevertheless, not all the crops or varieties offer a high efficiency via pollen embryogenesis.

There are several endogenous and exogenous factors affecting pollen embryogenesis. The genotype is one of the main endogenous factors affecting sporophytic pathway induction. Differences in androgenic response within species have

been widely reported in fruit trees including *Carica papaya* (Tsay and Su 1985), *Citrus* (Germanà and Reforgiato 1997) and *Malus domestica* (L.) Borkh. (Milewska-Pawliczuk 1990; Höfer et al. 1999). The stage of microspore development also plays a critical role. Although for most species the only period where the microspore is most readily diverted to a sporophytic pathway corresponds to the first pollen mitosis, it varies among species. Induction has been achieved at early stages, just divided pollen, and even later stages (Dunwell 2010; Seguí-Simarro 2010). Exogenous factors, including abiotic stresses, also play an important role in androgenic induction (Duncan and Heberle 1976; Heberle-Bors and Reinert 1981). Environmental factors such as temperature and nitrogen status at the flowering stage affect the efficiency of anther-derived callus induction. Additionally, heat or cold shocks applied as pretreatments at the early stages of induction result in a substantial increase in efficiency (Germanà 2011a; Dunwell 2010). Nutrient composition and plant growth regulators also result in different androgenic responses among species. Generally embryo induction is stimulated by addition of mineral salt mixture and a carbon source to the culture medium, usually N6 medium (Chu 1978) or Murashige and Skoog (1962) mineral salts, and sucrose (Germanà 2006a; Dunwell 2010). Additionally, to use anti-oxidant agents and activated charcoal to prevent the tissue browning caused by the presence of oxidized phenols has proved to be beneficial in several species such as *Malus domestica* and *Pyrus pyrifolia* Nakai (Germanà 2006a; 2011a). Consequently, there is not a standard methodology for the optimum progress of pollen embryogenesis. Therefore, it is necessary to set up the most efficient conditions for each species and genotype. Anther-derived embryos from cultures in water agar-solidified medium rarely occur.

In the case of loquat species, there is limited information available concerning the conditions that maximize haploid isolation efficiency (Germanà 2006a). In this context, the objective of this study was to investigate the effect of the genotype of the donor plant, pre-culture treatment and culture medium on anther culture-derived regenerants of loquat, and to characterize potential changes in chromosome number in the regenerated plants.

Materials and methods

Plant material

Terminal racemose inflorescences (panicles) of eight cultivars of loquat from different origins were used (Table 3.1.1). The plant materials belong to the European loquat germplasm collection located at IVIA, Valencia, Spain (latitude: 37º 45' 31.5 N; longitude: 1º 01' 35.1 O). All plants in the collection received standard cultural techniques.

Table 3.1.1. Loquat accessions evaluated in this study: country, origin, and flowering date.

Cultivar	Country	Origin	Flowering Date*
Algerie	Spain	Unknown. Seedling selected in Algeria. Introduced to Spain in the 1960s	First 10 days of November
Changhong3	China	Selected from Changhong seedling in 1990	-8
Jiefanghong	China	Selected from Dazhong seedling in 1950	+6
Moggi Wase	Japan	Unknown	+13
Raúl	Spain	Unknown. Local cultivar (Alicante province, Spain)	+4
Sanfilippara	Italy	Unknown	+32
Tavira	Portugal	Unknown	±0
Zaozhong6	China	Jiefangzhong x Moriowase	-18

* Algerie flowering date is used as reference (±days)

Microspore's development stage analysis

Microscopic evaluation of microspore development stage within anthers was performed to ensure that the selected flower buds contained mostly polarized uninucleate microspores. Flower buds were clustered in four groups according to their morphology and size: A. 5.0-5.3 mm, B. 6.5-7.1 mm, C. 8.0-8.5 mm, D. 9.2-10.1 mm. Five anthers from 5 buds per size group and cultivar were used for microscopic analysis. Anthers were fixed in 3:1 ethanol-glacial acetic acid for 10 min, rinsed with distilled water and stained with 4'-6-diamidino-2-phenylindole (DAPI, Partec, Münster, Germany) solution (1 $mg.l^{-1}$) for fluorescent microscope observation. The percentage of microspores at each stage of development was calculated over the total number of

microspores observed under three different fields for each slide as described by Telmer et al. (1992).

Pretreatment of flower buds and anther culture

The flower buds were placed in a flask containing an antioxidant (2.8 mM ascorbic acid and 3.9 mM citric acid) and stored in a refrigerator at 4 ºC for 4, 8 or 12 days. The control buds were not subjected to cold pretreatments.

After the pretreatment, flower buds were surface-sterilized with 70% (v/v) ethanol for 5 min and 4% (w/v) sodium hypochlorite with 0.1% (v/v) of Tween-20® for 10 min, followed by three rinses in sterile distilled water under aseptic conditions. Once the plant material was sterilized, sepals and petals were withdrawn, and anthers without stamen filaments were cultured in the darkness (60-mm Petri dish) on a callus induction medium consisting of half-strength MS inorganic basal salts, cysteine (16.5 μM), mixture vitamins (2.6 mM glycine, 2.9 mM thiamine hydrochloride, 8.1 mM nicotinic acid, 2.4 mM pyridoxine hydrochloride and 2.8 mM ascorbic acid), *myo*-inositol (1.1 mM) and sucrose (2% w/v). Also, the solid medium was supplemented with growth regulators, 4.56 μM zeatin (Z) versus four doses of 1_naphtalene acetic acid (NAA, 0.54, 1.34, 2.68 and 5.36 μM) in order to determine optimum conditions for callus induction. Cultures were incubated at 24 ºC for four weeks in the dark.

The morphogenic calli were divided and cultivated in two embryo induction media (Li et al. 2008) containing MS basal salts supplemented with 0.23 μM Z, 0.05 μM NAA and 0.05 μM Indole-3-butyric acid (IBA; medium A) or with 0.23 μM Z, 0.11 μM NAA and 0.1 μM IBA (medium B). Cultures were incubated in a growth chamber under 16 h light photoperiod at 24 ± 2 ºC.

Developed embryos were transferred onto MS basal salts with 20 $g.l^{-1}$ sucrose, and regenerants were subcultured onto a shoot multiplication and rooting medium described by Wang et al. (2013).

Two evaluations were carried out to determine the optimal conditions. In the first one 1000 isolated anthers of seven cultivars were used, and in the second 600 anthers of eight cultivars. ‘Zaozhong-6’ was included in the second evaluation only.

Flow Cytometry Analysis and Chromosome Counting

The ploidy level of the regenerated plantlets was analyzed using flow cytometry. Leaf samples were chopped in 1.0 ml of nuclei isolation buffer (Partec), filtered through a 50 μm nylon filter (Nybolt, Zürich, Switzerland), and stained with 3.0 ml of coloration solution containing 1 $mg.l^{-1}$ of DAPI. The fluorescence intensity of the nuclear mixture was measured using a CyFlow® Counter (Partec). Nuclei obtained from a loquat cv. Algerie were used as a diploid control. The plot data on a semi logarithmic scale resulted in a histogram with peaks from 2C to 100C evenly distributed along the abscissa axe.

Chromosome counts were performed on leaf pieces pre-treated with 0.04% 8-hydroxyquinoline for 4 h at room temperature (RT) and 3.5 h at 4 ºC in the dark. Samples were fixed in 3:1 ethanol-glacial acetic acid for 72 h at RT in darkness, hydrolyzed in 0.25 N HCl for 10 min and washed in distilled water. Each sample was placed in a digested citrate buffer (0.01 M Trisodium citrate-dihydrate + 0.01 M Citric acid monohydrate) for 10 min at RT and limited to 2 mm^2. The explants were incubated at 37 °C in enzymatic solution (5% cellulase Onozuka R10 + 1% pectolyase Y23) for 20 to 30 min (D'Hont et al. 1996) and then immersed in distilled water for 15 minutes. Thereafter a single sample was placed on a slide, the excess of water was removed and one drop of fixative solution was added and spread with fine forceps. Chromosomes slides were stained with 1 $mg.l^{-1}$ DAPI and viewed with a fluorescence microscope.

Statistical analysis

The success rate of anther culture was calculated as the percentage of anthers showing callus and the percentage of anthers with organogenic calli one month after culture initiation. In order to detect differences among treatments and genotypes, a binomial test was applied using the S-PLUS v. 8.01 statistical packages (Insightful Corp., Seattle).

Results and discussion

Study of correlation between flower bud size and microspore development stage

The pollen development stage is a complex factor that strongly affects the anther culture results; the appropriate gametophyte stage is critical to induce pollen embryogenesis. The exact stage of pollen development most readily diverted to a sporophytic pathway varies among species (Dunwell 2010). Cytological analysis of some fruit tree species shows that the optimum moment for pollen response lies between the first pollen mitosis and early bicellular stage (Touraev et al. 2001; Germanà 2006a). Exact determination of the microspore stage requires a cytological analysis but for large-scale tests an external morphological indicator such as corolla length is most used. Since no previous information was available about the microspore development stage and flower size in loquat, this study was focused on determining the relationship between the flower bud size and the different microspore developmental stages (tetrad, uninucleate microspore and bicellular and mature pollen grains) in order to establish the most convenient flower size for loquat pollen response.

The results demonstrated that different microspore stages can be found according to the bud size. Four groups were defined (Table 3.1.2 and Figure 3.1.1). In group A, divisions were observed in isolated microspores from buds 5 mm long. This size corresponds to closed flower buds with pale green and semitransparent anthers containing microspores at tetrad stage (Figure 3.1.1a). In group B, buds reached a length of approximately 7.0 mm long. This size corresponds to elliptical flower buds; the petals begin to grow among the sepals, and anthers turn fully opaque bright yellow containing microspores at uninucleate-early binucleate pollen (Figure 3.1.1b). In group C, buds were approximately 8.5 mm long. This size corresponds to flower buds with the petals protruding above the sepals, anthers losing brightness and filament beginning to turn white, containing microspores at binucleate pollen stage (Figure 3.1.1c). In group D, flower buds were approximately 9.5 mm long. This size corresponds to flower buds about to open with pale yellow-brown anthers, containing mature pollen (Figure 3.1.1d). Similar results were obtained by other authors in rape (Telmer et al. 1992), soybean (Lauxen et al. 2003), tobacco (Kasperbauer and Wilson 1979), and tomato (Summers et al. 1992). Results showed an evident correlation between floral bud size and pollen developmental stage in loquat.

Assuming that the microspores from uninucleate stages are the most responsive for the anther culture of loquat (Germanà et al. 2006b; Li et al. 2008), the bud size promoting embryogenesis in loquat should be 6.5 – 7.0 mm (group B), which was confirmed for all cultivars studied (Table 3.1.2).

Table 3.1.2. Microspore developmental stage distribution in anthers of each floral bud of different loquat cultivars. Bud size groups: A. 5.0-5.3 mm, B. 6.5-7.1 mm, C. 8.0-8.5 mm, D. 9.2-10.1 mm. Five buds from each size group and cultivar were sampled.

Cultivar	A		B		C		D	
	Flower Size (mm)	Stages (%)[a]	Flower Size (mm)	Stages (%)[a]	Flower Size (mm)	Stages (%)[a]	Flower Size (mm)	Stages (%)[a]
Algerie	5.2 ± 0.5	90% Tetrade	7.0 ± 0.3	90% Uni 10% EBP	8.5 ± 0.5	70% EBP 30% LBP	10.1 ± 0.4	100% MP
Changhong3	5.0 ± 0.4	100% Tetrade	6.5 ± 0.4	85% Uni 15% EBP	8.2 ± 0.5	75% EBP 25% LBP	9.4 ± 0.3	100% MP
Jiefanghong	5.0 ± 0.2	90% Tetrade	7.0 ± 0.4	98% Uni 2% EBP	8.5 ± 0.3	50% EBP 50% LBP	9.8 ± 0.4	100% MP
Moggi Wase	5.1 ± 0.3	90% Tetrade	7.1 ± 0.4	70% Uni 30% EBP	8.5 ± 0.3	90% EBP 10% LBP	9.6 ± 0.3	100% MP
Raúl	5.3 ± 0.4	80% Tetrade	6.8 ± 0.5	40% Uni 60% EBP	8.0 ± 0.3	70% EBP 30% LBP	9.3 ± 0.3	100% MP
Sanfilippara	5.1 ± 0.7	95% Tetrade	6.9 ± 0.3	100% Uni	8.2 ± 0.2	80% EBP 20% LBP	9.3 ± 0.2	100% MP
Tavira	5.2 ± 0.4	90% Tetrade	6.8 ± 0.6	60% Uni 40% EBP	8.0 ± 0.5	80% EBP 20% LBP	9.5 ± 0.5	100% MP
Zaozhong6	5.2 ± 0.4	85% Tetrade	6.7 ± 0.3	90% Uni 10% EBP	8.4 ± 0.2	75% EBP 25% LBP	9.6 ± 0.2	100% MP

[a] Stages: Uni = Uninucleate; EBP=Early binucleate pollen; LBP = Late binucleate pollen; MP = Mature pollen

Anther culture

Effect of cold pre-treatment

From the previous results on bud size, flower buds from group B (6.5-7.0 mm) were selected for testing different factors affecting anther culture using seven loquat cultivars. Cold pretreatments at 4 ºC for 0, 4, 8 and 12 days on flower buds were applied, resulting in callus formation only when the pre-cultivation treatment lasted 4 days (Table 3.1.3).

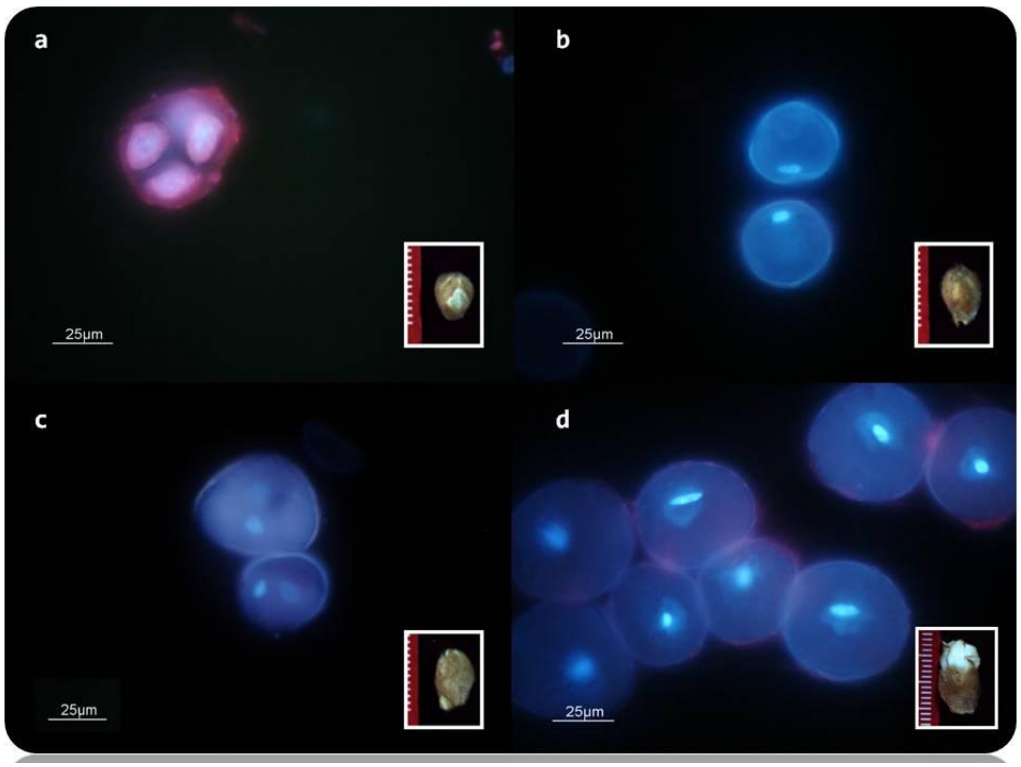

Fig.3.1.1. Flower bud and microspore development stages in loquat (cv. 'Algerie'). Tetrad (a) polarized uninucleate (b) binucleate pollen (c) and mature pollen (d), stained by DAPI. *Bars* 25 µm.

Table 3.1.3. Frequency of callus induction in anthers of loquat cultivars grown on MS media supplied with different naphthaleneacetic acid (NAA) doses with or without previous cold pre-treatment (4 ºC, 4 days) of excised buds (100 anthers were cultured for each condition in ten Petri dishes).

Cold pre-treatment	NAA ($mg.l^{-1}$)	Algerie		Changhong-3		Jiefanghong		Moggi Wase		Raúl		Sanfilippara		Tavira	
		AA (%)	MC (%)	AA (%)	MC (%)	AA (%)	MC (%)	AA (%)	MC (%)	AA (%)	MC (%)	AA (%)	MC (%)	AA (%)	MC (%)
No treat.	0	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}
	0.10	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}
	0.25	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}
	0.50	6_{c}	0_{a}	31_{b}	11_{b}	30_{b}	23_{cd}	49_{d}	15_{c}	0_{a}	0_{a}	60_{c}	9_{b}	3_{bc}	0_{a}
	1.00	4_{bc}	0_{a}	60_{c}	27_{d}	47_{c}	30_{d}	38_{c}	36_{d}	6_{c}	0_{a}	57_{c}	0_{a}	7_{c}	0_{a}
4 days	0	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}
	0.10	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}
	0.25	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}
	0.50	1_{b}	0_{a}	31_{b}	21_{cd}	26_{b}	9_{b}	39_{c}	21_{c}	1_{b}	0_{a}	21_{b}	9_{b}	2_{b}	0_{a}
	1.00	4_{bc}	0_{a}	26_{b}	15_{bc}	49_{c}	18_{c}	10_{b}	8_{b}	4_{bc}	0_{a}	51_{c}	9_{b}	4_{bc}	0_{a}

Different letters indicate significant differences (Bionomial test, P = 0.05)
AA: Androgenic anthers, MC: Morphogenic callus

Results revealed that prolonged cold pretreatments (over four days) resulted in browning, and finally the tissue became necrotic without obtaining callogenic response. Based on these results we compared the response to anther culture using a pre-treatment at 4 ºC for 4 days and a control (no pre-treatment). All genotypes tested showed anthers

producing calli (Figure 3.1.2a), but the cold pretreatment did not have a significant effect in cvs. ‘Algerie’, ‘Jiefanghong ‘, ‘Raúl’, ‘Sanfilippara’ and ‘Tavira’ or it did have a significant negative effect on the response in ‘Changhong-3’ and ‘Moggi Wase’ (Table 3.1.3). The highest percentage of morphogenic calli (Figure 3.1.2b) was obtained in cvs. ‘Changhong-3’, ‘Jiefanghong’ and ‘Moggi Wase’ with no pre-treatment. On the other hand, anthers of cultivars ‘Algerie’, ‘Raúl’ and ‘Tavira’ showed no morphogenic calli, and there was a very low response in the ‘Sanfilippara’ cultivar regardless of the pretreatment applied (Table 3.1.3).

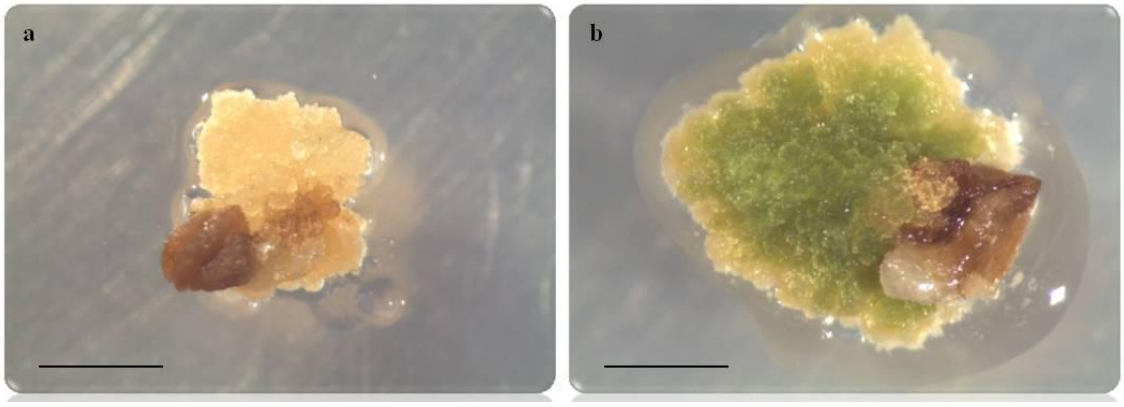

Fig.3.1.2. Callogenesis induction in loquat anthers (cv. ‘Jiefanghong’). Callus bursting open anther wall and growing out (androgenic anther) (a) and 2-month-old callus showing organized tissues (morphogenic callus). *Bars* 5 mm.

Cold pretreatment of flower buds before excising anthers for culture is used widely to induce the androgenic response in many plant species such as apple (Zhang et al. 2013), barley (Huang and Sunderland 1982; Szarejko 2003), citrus (Germanà and Chiancone 2003), maize (Barnabás 2003), rice (Zapata-Arias 2003), strawberry (Shahvali-Kohshour et al. 2013) and wheat (Kasha et al. 2003). In our study, cold pretreatment (4 ºC) of flower buds resulted in total inhibition or a lower number of androgenic anthers, the response being genotype dependent. Similar to our results, other authors obtained anther culture without cold treatments. Li et al. (2013) got haploids from poplar (*Populus* × *beijingensis*) without cold pre-treatment, and Kadota and Niimi (2004) saw similar rates of pollen embryogenesis in pear with and without cold treatment. The diversity of results obtained in tests of the effects of cold on angrogenesis can be explained by the role of the physiological condition of donor plants in anther development. Studies on barley (Foroughi-Wehr and Mix 1979), oilseed rape (Keller and Stringham 1978; Dunwel and Cornish 1985), turnip (Keller et al. 1983) and

wheat (Lazar et al. 1984) showed that the temperature in which the donor plants are grown can also markedly affect the culture response.

Results of anther culture in woody plants are very season dependent even when the same protocols are applied. Different responses to the culture depending on the experimental year were previously reported in *Citrus* (Germanà et al. 2005; Germanà 2009). This may be explained by the fact that besides all the factors affecting pollen embryogenesis discussed above, the growth conditions have an effect on the physiological status of donor plants, mainly if the plants are cultivated in open-air, affecting the final results (Germanà 2011a). In our case, the loquat donor plants are grown in fields, under different climatic conditions and cultural practices than those used in other studies, which resulted in differences in the physiological state of the anthers. For example, Li et al. (2008) in a loquat study carried out in China found that the pre-treatment with low temperatures (4 ºC for 2 days) promoted callus induction from anthers. The effect of the physiological status of the donor plant in anther development has been reported in many species (Dunwell 2010; Germanà 2011a; Seguí-Simarro et al. 2011; Islam and Tuteja 2012).

Effect of media composition

The nutritional requirements of the excised anthers are a key factor that affects the success of anther culture. In order to test this factor in the initial evaluation, a total of 1000 anthers per cultivar were cultured *in vitro* on callus induction medium with four different doses of NAA (0.54, 1.34, 2.68 and 5.36 μM). Development of callus was achieved only when the nutrient medium was supplemented with 2.68 and 5.36 μM NAA (Table 3.1.3). No response was observed when anthers were cultured on basal MS medium or on a MS medium supplemented with low concentrations of NAA (0.54 and 1.34 μM). The percentage of androgenic anthers obtained with two doses of NAA varies according to the loquat cultivar. Cultivars ‘Algerie’, ‘Tavira’ and ‘Sanfilippara’ did not show a significant difference between 2.68 and 5.36 μM NAA doses, while cvs. ‘Changhong-3’, ‘Jiefanghong’ and ‘Raúl’ showed a significantly higher percentage using 5.36 μM NAA dose, the cv. ‘Moggi Wase’ gave better response using a 2.68 μM dose (Table 3). The highest percentage of morphogenic callus was obtained on medium supplied with 5.36 μM NAA in cvs. ‘Changhong-3’ (27%), ‘Jiefanghong’ (30%) and ‘Moggi Wase’ (36%) without cold pretreatment (Table 3.1.3).

Since callus induction was achieved only for 2.68 and 5.36 μM NAA doses, a second experiment with a total of 600 anthers per cultivar cultured on callus induction medium with both doses was carried out. The cv. 'Zaozhong-6' was added to the experiment by including a genotype used by Li et al. (2008), being the callus induction rate obtained similar in both studies.

Callus formation (androgenic anthers) was achieved with both doses in all cultivars tested. As in the initial experiment, the cold pretreatment did not have a significant effect on the percentage of morphogenic callus (Table 3.1.4). The highest percentage of morphogenic callus was obtained using medium supplied with 5.36 μM NAA in cv. 'Zaozhong-6' (56%). The response to the callus induction was highly genotype dependent.

Table 3.1.4. Second experiment of callus induction in anthers of loquat cultivars grown on MS media complemented with two naphthaleneacetic acid (NAA) doses with or without previous cold pre-treatment (4 ºC, 4 days) of excised buds (100 anthers were cultured for each condition in ten Petri dishes).

Cold pre-treatment	NAA ($mg.l^{-1}$)	Algerie		Changhong-3		Jiefanghong		Moggi Wase		Raúl		Sanfilippara		Tavira		Zaozhong-6	
		AA %	MC %	AA %	MC %	AA %	MC %	AA %	MC %	AA %	MC %	AA %	MC %	AA %	MC %	AA %	MC %
No treat.	0	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}
	0.50	0_{a}	0_{a}	11_{c}	3_{b}	35_{d}	30_{c}	77_{d}	41_{d}	1_{b}	0_{a}	64_{c}	8_{b}	0_{a}	0_{a}	41_{b}	23_{c}
	1.00	1_{b}	0_{a}	23_{d}	12_{c}	6_{b}	6_{a}	75_{d}	39_{d}	2_{b}	0_{a}	48_{b}	12_{bc}	2_{b}	2_{b}	83_{d}	56_{d}
4 days	0	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}
	0.50	1_{b}	0_{a}	1_{b}	0_{a}	22_{c}	6_{b}	16_{b}	6_{b}	5_{b}	1_{b}	52_{b}	12_{bc}	0_{a}	0_{a}	32_{b}	12_{b}
	1.00	1_{b}	0_{a}	5_{b}	0_{a}	11_{b}	2_{b}	48_{c}	22_{c}	4_{b}	2_{b}	48_{b}	15_{c}	5_{b}	2_{b}	57_{c}	27_{c}

Different letters indicate significant differences (Bionomial test, P = 0.05)
AA: Androgenic anthers, MC: Morphogenic callus

The effect of type and concentration of plant growth regulators on the pollen embryogenesis in anther culture has been widely investigated (Germanà et al. 1994; Xie et al. 1995; Ouédraogo et al. 1998; Trejo-Tapia et al. 2002; Kumar et al. 2003; Oleszczuk et al. 2004; Perera et al. 2009; Smýkalová et al. 2009). Most *Solanaceae* species do not require auxin addition; however, for *Gramineae* and *Cruciferae* 2,4-Dichlorophenoxyacetic acid (2,4-D) is usually added to the media (Dunwell 2010). In fruit trees, different auxin types and concentrations have been used to induce callus formation from anther, 28.55 μM Indole-3-acetic acid (IAA) in *Annona squamosa* (Nair et al. 1983), 0.54 μM NAA in *Carica papaya* (Rimberia et al. 2007), 0.11 μM NAA and 0.09 μM 2,4-D in *Citrus clementina* (Germanà and Chiancone 2003), but not auxin addition in *Malus domestica* (Höfer et al. 1999; Höfer 2004) and 2.26 μM 2,4-D in

Eriobotrya japonica (Li et al. 2008). In the present study, we applied the same concentrations of 2,4-D used previously by Li et al. (2008) and under the same experimental conditions. Nevertheless, we did not observe any androgenic response in any of the genotypes analysed (data not shown). It seems that the uncontrolled environmental factors may indeed play a relevant role in the androgenic response.

Embryogenesis and plant regeneration

The morphogenic calli obtained in the second experiment were subcultured in the two embryogenesis inducing media evaluated by Li et al. (2008). Only in cv. Jiefanghong on medium B (MS basal salts supplemented with 0.23 μM Z, 0.11 μM NAA and 0.1 μM IBA) the calli grew and developed. Embryos initials, which developed and were observed 1.5 months after culture (Figure 3.1.3a), continued to grow, producing embryos (Figure 3.1.3b). Six embryos formed on one of callus, while the remaining other callus did not show any response. Four of the six embryos stopped growing and ended up necrotic, probably because of the intra-competition among embryos from the same cluster. As the remaining two embryos grew, they were transferred onto MS basal medium but only one of them progressed into a plantlet (Figure 3.1.3c). Formed shoots were subcultured in shoot multiplication medium and rooting medium in order to obtain clonal replicates and thus increase the material available for further analysis (Figure 3.1.3d).

One month after the subcultivation, one leaf per shoot was subjected to flow cytometry to quantify the DNA content. The results revealed that the plantlet was triploid (Figure 3.1.4a). Chromosome counting on leaf pieces nuclei confirmed the results of the flow cytometry analysis. The chromosome number of the diploid control plant was $2n = 2x = 34$ (Figure 3.1.4b) and of the triploid plant $2n = 3x = 51$ (Figure 3.1.4c).

Cytological variations have previously revealed different ploidy levels of plants derived from *in vitro* anther culture (Dunwell 2010). These alterations could be originated by the use of plant growth regulators during tissue culture, which may cause an imbalance in the mitotic activity of cells (Winarto et al. 2010). In this and other studies, triploids have been obtained from anther culture of woody trees such as *Citrus clementina* (Germanà et al. 2005), poplar (*Populus* × *beijingensis*) (Li et al. 2013) and *Pyrus pyrifolia* (Kadota and Niimi 2004).

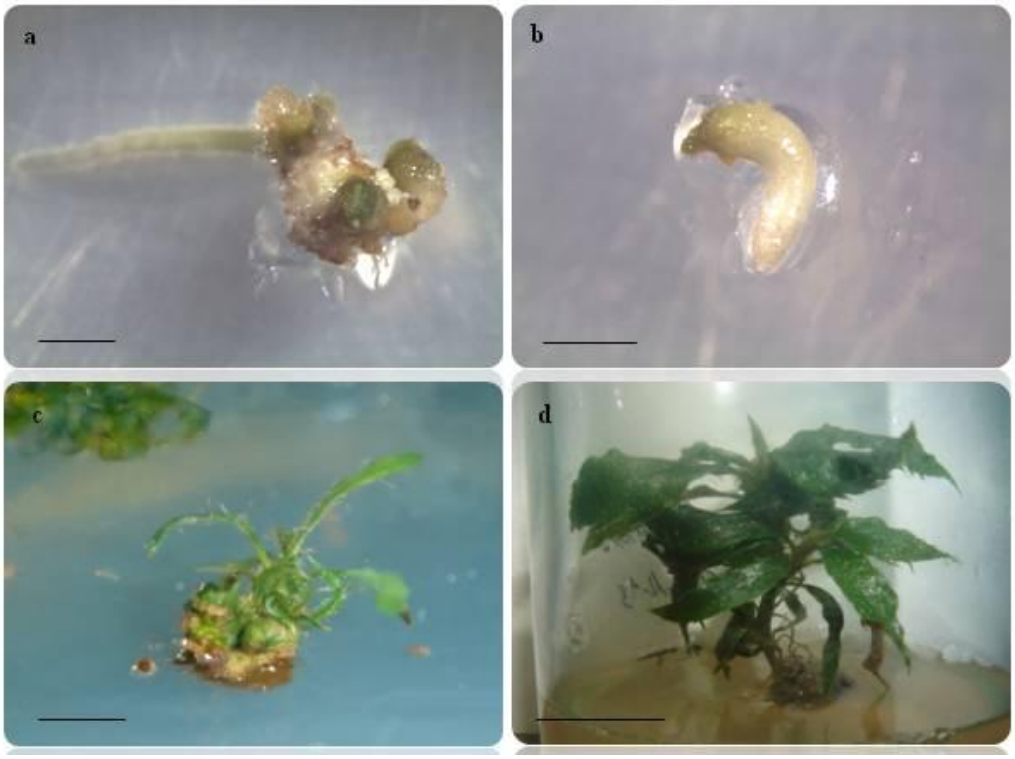

Fig.3.1.3. Embryogenesis in cultured anthers of loquat (cv. 'Jiefanghong'). Clump of embryos in different development states (a, *Bar* 1 cm); isolated embryo (b, *Bar* 5 mm); plantlet after 4 months (c, *Bar* 5 mm) and shoot development after ten months of culture (d, *Bar* 1 cm).

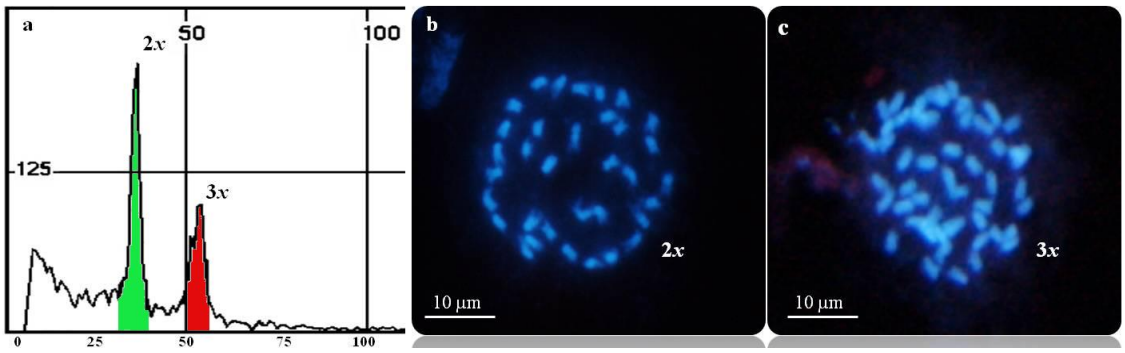

Fig.3.1.4. Flow cytometry histogram of diploid control loquat plant (2*x*) and anther-derived triploid plant (3*x*) (a). Chromosomes count on loquat leaf piece of diploid control cell (2*n* = 2*x* = 34) (b) and triploid cell (2*n* = 3*x* = 51) (c). *Bars* 10µm.

Polyploid fruit species include commercially successful cultivars because of their favorable horticultural characteristics, such as large fruit size, sturdiness, high productivity, disease-resistance and fewer or no seeds (Sandford 1983; Predieri 2001). In many species triploid genotypes resulted in seedless fruits with great commercial interest, such as citrus (Ollitrault et al. 2008), grape (Ledbetter and Ramming 1989) and watermelon (Kihara 1951).

In loquat, triploids have been recovered from $2n \times 4n$ crosses, where the pollen parent is tetraploid (Huang 1984, 1989). Also naturally occurring triploids have been obtained as a result of cross-pollination, when unreduced gametes are spontaneously developed in the seed parent (Liang et al. 2011).

In conclusion, the results obtained in this study show that anther culture at the vacuolated microspore stage might be considered as the most appropriate stage to induce the sporophytic pathway in loquat microspores. Results indicated that embryogenesis from anther culture on loquat is possible, although a strong genotypic effect exists. In fact, considering the influence of genotype and medium composition on the final success of the culture, it is necessary to enlarge the number of genotypes analyzed and broaden experimental conditions in order to obtain a standard protocol maximizing the efficiency of pollen embryogenesis.

In the present study we reported, for the first time, the triploid plant production of *Eriobotrya japonica* (Thunb) L. ($2n = 3x = 51$) by microspore/pollen embryogenesis, which indicates that anther culture might be a method for the production of artificial triploids. Triploidy can lead to seedlessness, which will be an important goal in loquat breeding since the edible proportion of the fruit is lower than 70%.

CAPÍTULO 3.2:

Isolated microspore culture in eleven cultivars of *Eriobotrya japonica* (Thunb.) Lindl.

IV International Symposium on Loquat. Palermo (Italy), May 12-15 of 2014. Poster.

Isolated miscrospore culture in eleven cultivars of *Eriobotrya japonica* (Thunb.) Lindl.

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Abstract

In plant breeding, microspore embryogenesis via isolated microspore culture is a more and more employed method to obtain in a single-step doubled haploids and homozygosity. In this study, isolated microspore culture of eleven cultivars of loquat (*Eriobotrya japonica* (Thunb.) Lindl.) has been carried out.

Before microspore isolation, a previous analysis of anthers from different flower bud sizes has been carried out, to identify and to select the flower bud size with anthers containing the highest quantity of vacuolated microspores. After a pre-treatment of the panicles at 4 ºC in dark for 4 days, flower buds were sterilized and microspores were isolated and cultured in two different media (P and MB). Moreover, only the isolated microspores cultured in P Medium were subjected to two additional thermal shocks: cold shock (1 h at -20°C) and heat shock (7 days at 33°C). All the cultures were placed in a growth cabinet at 26 ± 1ºC in the dark for 30 days and later placed in the light. Samples from cultures were processed for microscopy analysis at specific times. Observations on microspore development were carried out by fluorescence and light microscopy. The sporophytic development of isolated microspores in culture has been confirmed by the presence in the cultures of binucleated with two equal-size vegetative-type nuclei (that had just started their sporophytic pathway), multinucleated, pollen-derived multicellular structures and calli.

Introduction

Loquat (*Eriobotrya japonica* (Thunb.) Lindl) is a subtropical evergreen tree, belonging to the *Maloideae* subfamily of the *Rosaceae*. It was originated in China, in the medium and low region of Dadhue River which is considered the primary center of origin for *Eriobotrya japonica* (Zhang et al. 1990a). In the 18th century, loquat was introduced from Japan into Europe as ornamental tree in the National Botanical Garden of Paris and the Royal Botanical Gardens at Kew, England. Thereafter, loquat was distributed around the Mediterranean countries (Morton 1987), and in the United States, Brazil, Venezuela and Australia (Badenes et al. 2000; Vilanova et al. 2001). Loquats are found between latitudes 20º and 35º North or South, but can be cultivated up to latitude 45º under marine climates (Lin et al. 1999).

In general, temperate fruit trees are high heterozygous due to self-incompatibility (Kadota and Niimi 2004). Microspore embryogenesis via isolated microspore culture is a more and more employed method to obtain in a single-step doubled haploids and homozygosity. A male-derived haploid or DH plant can be obtained by microspore embryogenesis through anther or isolated microspore culture (Germanà 2011a). In this study, isolated microspore culture of eleven cultivars of loquat has been studied to induce the sporophytic pathway.

Material and Methods

Plant Material

Panicles of eleven cultivars of loquat: ‘Algerie’, ‘Bueno’, ‘Claudia’, ‘El Buenet’, ‘Fiore’, ‘Marcenò’, ‘Nespolone di Trabia’, ‘Peluche’, ‘Sanfilippara’, ‘Tanaka’ and ‘Virticchiara’, were selected and collected to isolate microspores.

Development of microspores

After flower bud sterilization, the anthers of 80 flowers per cultivar were excised and microspores were isolated following the protocol reported by Kuhmlen et al. (2006). Isolated microspores were cultivated on two media, medium P (Germanà et al. 1996) and MB (Blasco et al. 2014c, submitted). Moreover, isolated microspores cultured in P Medium were subjected to two thermal shocks: Heat (7 days at 33 °C) (PH) and Cold (1 h at –20°C) (PF). Development of microspores was monitored every month, under fluorescence microscope (Zeiss, Axiophot, Germany) after 4’,6’-diamidino-2-phenylindole dihydrochloride (DAPI) staining. The number of Petri dishes containing calli and the number of calli per each Petri dish after 6 months of culture was registered.

Results and discussion

During the culture, different structures were observed: tetrades (Figure 3.2.1a), uninucleated microspores with no development (Figure 3.2.1b), binucleated with two asymmetrical nuclei which is the normal gametophytic pathway resulted from one

vegetative and one generative nucleus (Figure 3.2.1c). The last structure sometimes followed the regular gametophytic pathway, consisted in germination and development of the pollen tube (Figure 3.2.1d). However, also binucleated microspores with two equal-size vegetative-type nuclei were observed (Figure 3.2.2a), following a sporophytic pathway. As a result, trinucleated, tetranucleated, multinucleated (Figure 3.2.2b) and microspore-derived calli and proembryos were detected (Figure 3.2.2c).

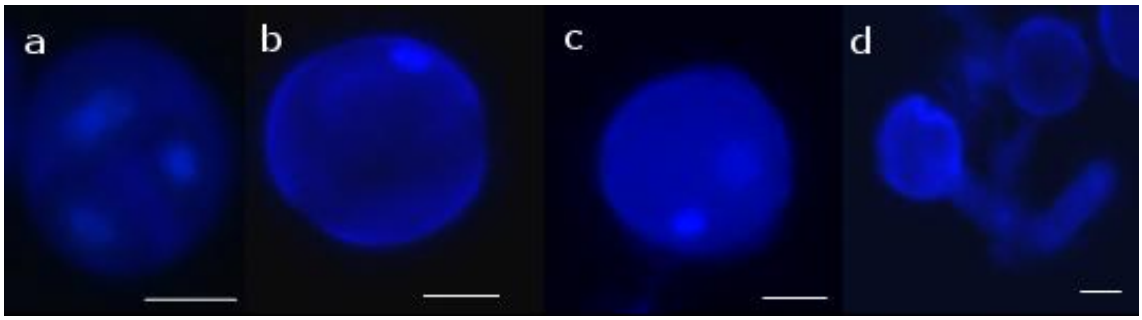

Fig.3.2.1. Gametophytic pathway in loquat. a) tetrad; b) vacuolated microspore; c) binucleated microspore, with asymmetrical division; d) germinated pollen grain. *Bars* 10 µm.

After three months in culture, microspores started to produce two types of calli: hard yellow-light brown (Figure 3.2.2d) and friable yellowish-white. In both types, the cell nuclei were visible at the fluorescent microscope (Figure 3.2.2 e and f). The number of calli increased progressively during the culture. However differences within a cultivar were detected, depending on media and treatments applied (Figure 3.2.3). The genotype of the donor plants affects deeply the response of the cultured microspores to callogenesis and embryogenesis induction (Zagorska et al. 1998).

Six out of eleven cultivars responded positively to the culture, producing both types of calli (Figure 3.2.3). Comparison among genotypes regarding the response of the isolated microspores to the culture resulted in a high genotype-dependence. Particularly, two genotypes responded very well to the callogenesis and embryogenesis (‘Peluche’ and ‘Tanaka’), while four (‘Bueno’, ‘El Buenet’, ‘Nespolone di Trabia’ and ‘Fiore’) had a low development and five (‘Algerie’, ‘Claudia’, ‘Marcenò’, ‘Sanfilippara’ and ‘Virticchiara’) did not produce any calli.

Comparison of the two basal media resulted in P medium inducing the production of calli in a higher percentage than MB, so it could be argued that the presence of an appropriate concentration of PGRs in the medium plays a critical role in microspore-derived callus or embryo formation (Sopory and Munshi 1996).

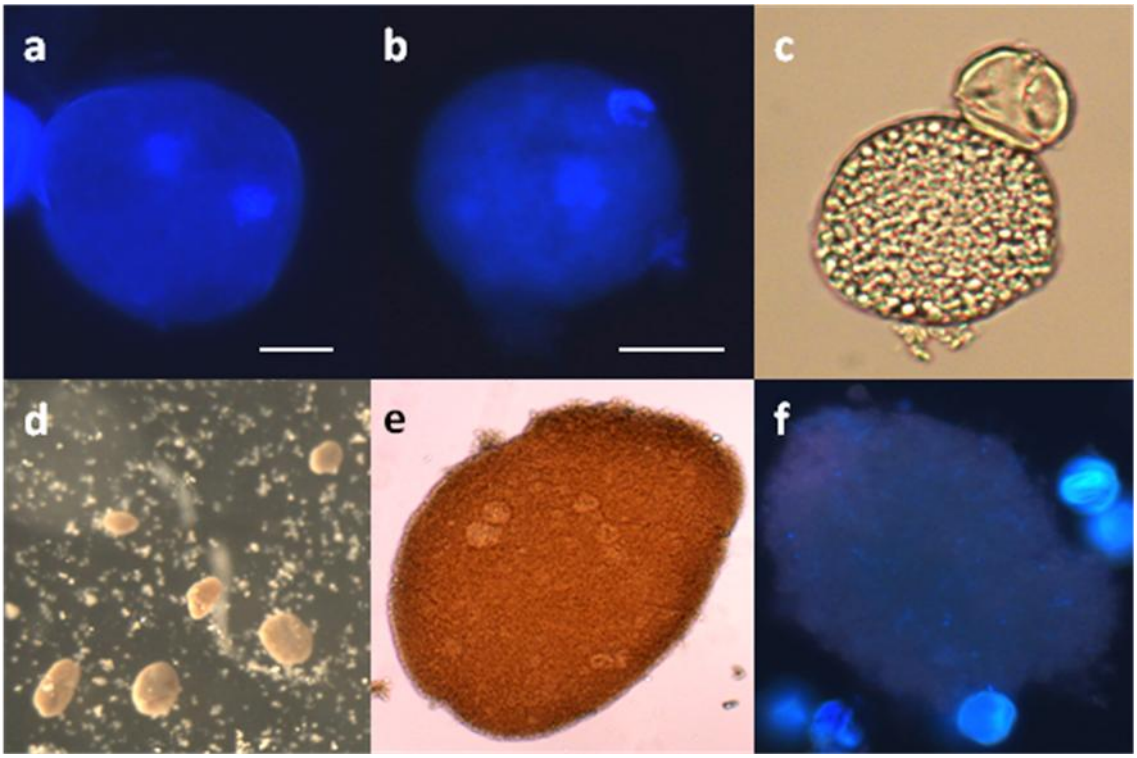

Fig.3.2.2. Sporophytic pathway in isolated microspores culture of loquat. a) binucleated microspore, with symmetrical division; b) multinucleated microspore; c) microspore producing new structures d-e) hard yellow-light brown calli; f) friable yellowish-white. callus. *Bars* 10 µm.

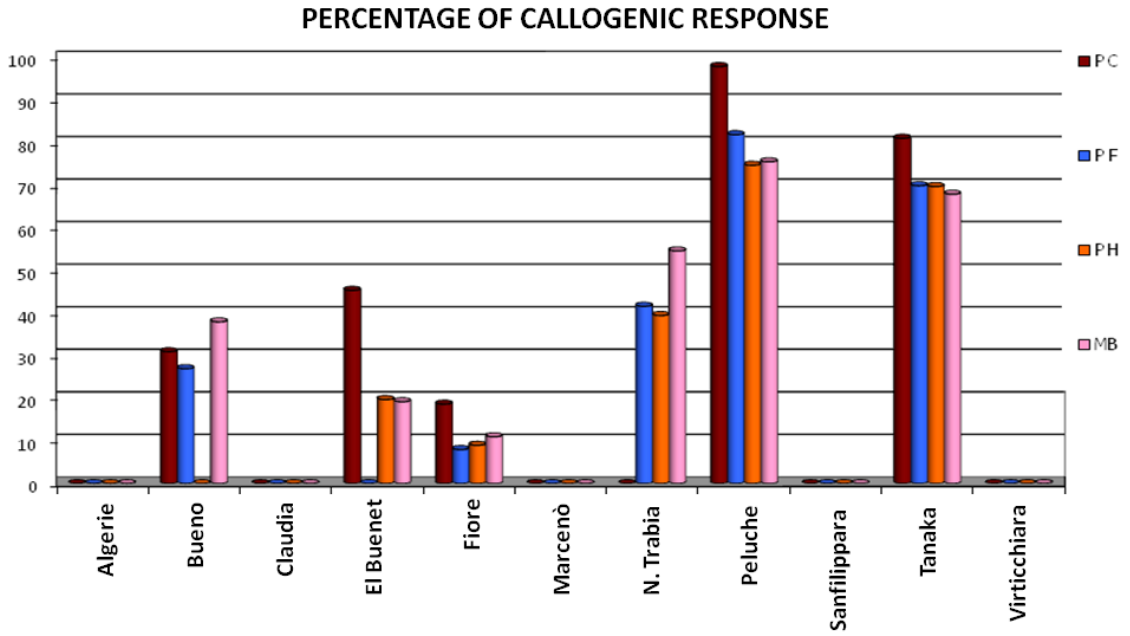

Fig.3.2.3. Influence of cultivar, medium composition and thermal shock, on the formation of microspore-derived calli of loquat, after six months of culture.

According the thermal pretreatments, generally, the control PC gave the best results inducing the highest formation of calli when compared with the cold and heat shocks, which agrees with Blasco et al. (2014c, submitted) who obtained a better response of anther-derived callus in non treated anthers.

These results represent an advancement in the knowledge of pollen embryogenesis in loquat towards the regeneration of homozygous plants.

CAPÍTULO 4:

Induced parthenogenesis in loquat (*Eriobotrya japonica* (Thunb.) Lindl) for haploid production

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Scientia Horticulturae. In prep.

Induced parthenogenesis in loquat (*Eriobotrya japonica* (Thunb.) Lindl) for haploid production

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Abstract

First successful haploid induction in *Eriobotrya japonica* (Thunb.) Lindl. through *in situ*-induced parthenogenesis with gamma-ray irradiated pollen has been achieved. Female flowers of cultivar Algerie were pollinated using pollen of cultivars 'Changhong-3', 'Cox' and 'Saval Brasil' that had been irradiated with two doses of gamma rays, 150 and 300 Gy. The fruits were harvested 90, 105 and 120 days after pollination. Percentage of germinated seeds was influenced by source pollen, irradiation dose and fruit harvest time (days after pollination). Haploid plantlets were obtained from the cross 'Algerie' × 'Saval Brasil' with a 300 Gy gamma-ray dose, with fruits harvested 105 days after pollination. The haploid origin was confirmed by flow cytometry and chromosome count. The haploids showed a very weak development compared to the diploid plants.

Introduction

Loquat (*Eriobotrya japonica* (Thunb.) Lindl) is a subtropical evergreen tree, belonging to the Maloideae subfamily of the Rosaceae. This non-climacteric fruit, originated in China (Zhang et al. 1990), is cultivated between latitudes 20º and 35º North or South (Lin et al, 1999). The crop is grown in subtropical areas sharing environmental requirements similar to citrus. Being a minor fruit crop, the breeding activities and genetic studies focused on this species is scarce. Increasing knowledge on genetics of loquat requires to the optimization of biotechnological techniques in this specie. Among them, exploring the ploidy manipulation to develop new cultivars is of great interest in woody plants such as loquat.

There are different techniques for the alteration of chromosome number. Among them, the obtention of haploids has many advantages for the progress of genetic studies, especially in perennial fruit trees that have a long reproductive cycle and high heterozigosity due to self-incompatibility. Haploids and double haploids lines, are very interesting since allow in a single step to fix traits in homozygous state. These homozygous individuals are very useful for genome mapping, providing reliable information about the location of major genes and quantitative trait loci for economically important traits (Khush and Virmani 1996). In this sense, haploids have been used for physical mapping (Leeuven et al. 2003), genetic mapping (Hussein et al.

2007; Zhang et al. 2008; Chu et al. 2008), integration of physical maps (Zhebentyayeva et al. 2008) and for the implementation of the reference whole genome sequence in fruit tree species such as citrus (Aleza et al. 2009b) or peach (Verde et al. 2012). Additionally, haploids allow the development of lines that exploit F_1 heterosis, contribute to studies on the process of differentiation of plants and allow studies of totipotency mechanisms in plant cells (Germanà 2009; 2011a; 2011b).

Spontaneous haploid individuals have been identified in several fruit species as kiwi, mango, peach and almond (Crètè 1944, Sobrinho and Gurgel 1953, Toyama 1974; Martínez-Gómez et al. 2003). However, spontaneous evidence is a rare event which resulted in limited application; hence artificial haploid induction is necessary for potential use in breeding. Haploid plants can be achieved using several methods: *in vitro* pollen embryogenesis (anther-isolated microspore culture) and gynogenesis (ovule-ovary culture), *in situ* parthenogenesis (pollen irradiation or treatment with chemicals), wide hybridization (chromosome elimination, 'bulbosum' method), selection of twin seedlings, etc. (Germanà and Chiancone 2001). Although pollen embryogenesis through *in vitro* anther culture is the most practical method, alternative methods should be evaluated to improve the response.

Induction of female-derived haploid embryos following pollination with irradiated pollen has been used successfully in different fruit species as apple (Nicoll et al. 1987; Zhang et al. 1988; Zhang and Lespinasse 1991), pear (Bouvier et al. 1993; Inoue et al. 2004), kiwi (Chalak and Legave 1997) and citrus (Froelicher et al. 2007; Aleza et al. 2009b). Pollen commonly is irradiated with gamma rays because of their simple application, good penetration, reproducibility, high mutation frequency, and low disposal (lethal) problems (Chahal and Gosal 2002). Gamma-irradiated pollen can germinate on the stigma, grow though the style and reach the embryo sac. Despite, being unable to fertilize the egg cell and the polar nuclei, it stimulates the development of haploid embryos (Musial and Pzrywara 1998). This method requires immature embryos rescue under *in vitro* conditions. Among the different factors influencing the efficiency of the progress, the irradiation dose applied, the developmental stage of the embryo during *in vitro* culture, the culture media composition and the culture conditions are especially important (Germanà 2011a).

The objective of the present study was to evaluate the response of 'Algerie' loquat to *in situ*- induced parthenogenesis by pollination with gamma irradiated pollen from 'Changhong-3', 'Cox' and 'Saval Brasil' loquat, followed by *in vitro* embryo

rescue. We present results on the viability of irradiated pollen, percent fruit set and parthenogenetic embryos formation according to the irradiation doses. Regenerants were characterized using flow cytometry.

Material & Methods

Plant Material

The experiments were conducted in 2012 and 2013. Cv. 'Algerie' was used as female parent and cvs. 'Changhong-3', 'Cox' and 'Saval Brasil' were used as the pollen parents. The pollinators were chosen for their intercompatibility and flowering date (Table 4.1). Field experiments were conducted at the Ruchey Cooperative experimental orchards in Callosa d'en Sarrià (Alicante, Spain).

Table 4.1. Loquat accessions used in this study: cultivar, *S*-allele genotype and flowering date.

Cultivar	*S*-allele genotype	Flowering date*
Algerie	*Sb Si*	November 8th(F)
Changhong-3	*Sf*	F –8
Cox	*Sd Sk*	F -10
Saval Brasil	*Sj Sk*	F +2

*Flowering date expressed the difference in days from the flowering date of cv. 'Algerie'

Pollen collection and irradiation

Flower buds were collected in autumn just before anthesis. Anthers were excised before pollen dehiscence and dried at 30°C for 24 h. Dried anthers were crushed and divided in three samples for irradiation at 0, 150 and 300 Gy doses of gamma rays by a ^{60}Co source. After irradiation, pollen viability was evaluated on a nutrient medium containing 1.5% sucrose solidified with 0.12% agar at pH 5.7 in Petri dishes (100 mm) at 26 ºC. Five replications of 100 pollen grains in each irradiation pollen were observed after 24 h.

Pollination

Three pollen sources were evaluated:'Changhong-3', 'Cox' and 'Saval Brasil'. Pollen samples were irradiated at 150 and 300 Gy in 2012, and 300 Gy in 2013. Flowers from Algerie were emasculated before anthesis, hand-pollinated with the different pollen sources and covered with paper bags to avoid contamination by foreign pollen. Control pollinitations with non-irradiated pollen were also performed.

Seeds extraction and in vitro embryo rescue

Fruits were harvested 90, 105 and 120 days after pollination (dap). Seeds were surface-sterilized by immersion in a diluted commercial bleach solution (4% w/v sodium hypochlorite) with 0.1% (v/v) of Tween-20® for 10 min, followed by three rinses in sterile distilled water under aseptic conditions.

Seed-coat was removed and embryos were cultured on solid medium composed of half-strengh Murashige and Skoog (MS, 1962) mineral salts supplemented with glycine 200 $mg.l^{-1}$, thiamine hydrochloride 1 $g.l^{-1}$, nicotinic acid 1 $g.l^{-1}$, pyridoxine hydrochloride 500 $mg.l^{-1}$, ascorbic acid 500 $mg.l^{-1}$, 200 $mg.l^{-1}$ *myo*-inositol, 20 $g.l^{-1}$ sucrose and 7 $g.l^{-1}$ agar. The pH was adjusted to 5.7 prior to addition of agar. Samples were placed in darkness for 3 weeks and then transferred to 16-h photoperiod in a tissue-culture chamber at 24 ºC ± 2 ºC.

Acclimatization

After *in vitro* culture, plantlets were transferred to pots (250 cc) containing a mixture of peat and perlite (1:1). The solid medium was carefully washed out of the roots. The potted plantlets were covered with transparent plastic for 7 days in which the cover was gradually removed to allow smooth adaptation of plantlets to lower humidity. Once the plants became acclimatized, they were transferred to a greenhouse at 18-28 ºC under natural early fall photoperiod.

Flow Cytometry Analysis and Chromosome Counting

The plantlets obtained were analyzed by flow cytometry to asses the ploidy level. Leaf samples were chopped in 1.0 ml of nuclei isolation buffer (Partec), filtered through a 50 μm nylon filter (Nybolt, Zürich, Switzerland), and stained with 3.0 ml of coloration solution containing 1 $mg.l^{-1}$ of DAPI (4'-6-diamidino-2-phenylindole.HCl). The fluorescence intensity of the nuclear mixture was measured using a CyFlow® Counter (Partec). Nuclei obtained from 'Algerie' were used as diploid control. The plot data on a semi logarithmic scale resulted in a histogram with peaks from 2C to 100C evenly distributed along the abscissa axe.

Chromosome number was counted in leaf piece cells obtained from *in vitro*-grown haploid plantlet. The leaf sample was pre-treated with 0.04% 8-hydroxyquinoline for 4 h at room temperature (RT) and 3.5 h at 4 ºC in the dark. Sample was fixed in 3:1 ethanol-glacial acetic acid for 72 h at RT in darkness, hydrolyzed in 0.25 N HCl for 10 min and washed in distilled water. Fixed leaf piece was placed in digested citrate buffer (0.01 M Trisodium citrate-dihydrate + 0.01 M Citric acid monohydrate) for 10 min at RT and limited to 2 mm^2. The explant was incubated at 37 °C in enzymatic solution (5% cellulase Onozuka R10 + 1% pectolyase Y23) for 20 to 30 min and then immersed in distilled water for 15 min (D´Hont et al. 1996). Thereafter a single sample was placed on a slide, the excess of water was removed and 1 drop of fixative solution were added before spread it with fine forceps. Chromosomes slides were colored with 1 $mg.l^{-1}$ DAPI and viewed with a fluorescence microscope.

Results

Pollen germination

In vitro pollen germination of irradiated pollen was determined for all male parents, 'Changhong-3', 'Cox' and 'Saval Brasil' in 2012. The highest germination rate was found using control (non-irradiated) pollen (≈ 70.0%) with no significant differences among cultivars (Table 4.2). With the highest irradiation dose (300 Gy) the germination capacity was significantly reduced (≈ 45%) compared with the lowest irradiation dose (≈ 60% for 150 Gy) in all cultivars (Table 4.2).

Table 4.2. Mean *in vitro* germination percentages of pollen samples used for in situ induced parthenogenesis in 2012.

Cultivar	Irradiation dose (Gy)	Pollen germination ± SE (%)
Changhong-3	0	69.2 ± 1.2 a*
	150	62.4 ± 1.0 b
	300	50.6 ± 1.6 c
Cox	0	70.8 ± 1.9 a
	150	60.8 ± 0.9 b
	300	42.2 ± 0.9 c
Saval Brasil	0	70.4 ± 1.0 a
	150	54.4 ± 1.6 c
	300	45.2 ± 1.6 c

*For each cv. percentages followed by the same letters are not significantly different (Newman and Keuls test, $P = 0.05$)

Fruit set

The fruit set in flowers pollinated with non-irradiated pollen was 100% for all cross combinations in 2012 (Table 4.3). Pollen irradiated at doses of 150 Gy significantly reduced fruit set (≈ 60%) compared with the control. The degree to which irradiated pollen affected fruit set was dependent on the cross and irradiation dose. The number of fruit set decreased for high dose of irradiation (300 Gy) when compared with the low dose (150 Gy) but the sensitivity depended on the male parent (Table 4.3). The lowest percentage of fruit was obtained with irradiated pollen at 300 Gy for the three crosses (51.5% for 'Algerie' × 'Changhong-3', 35.5% for 'Algerie' × 'Cox' and 50.3% for 'Algerie' × 'Saval Brasil'.

Table 4.3. Effect of pollen irradiation on fruit set in 2012.

Cross	Irradiation dose (Gy)	Flowers pollinated (no.)	Fruit set (%) [no. (%)]
Algerie × Changhong-3	0	45	45 (100) a*
	150	400	256 (64.0) b
	300	400	206 (51.5) cd
Algerie × Cox	0	45	45 (100.0) a
	150	400	276 (69.0) b
	300	400	142 (35.5) d
Algerie × Saval Brasil	0	45	45 (100) a
	150	400	210 (52.5) c
	300	400	201 (50.3) c

* For each cross percentages followed by the same letters are not significantly different (Newman and Keuls test, $P = 0.05$)

Influence of fruit-harvest times in embryo rescue

In order to assess the most convenient stage of the embryo for successful embryo rescue, inmature fruits were harvested at 90, 105 and 120 days after pollination (dap), which corresponded to seed development and fruit enlargement stages (Figure 4.1). The same number of fruits was selected for embryo rescue at 90, 105 and 120 dap for every condition (cross and pollen irradiation dose) in 2012 (Table 4.4).

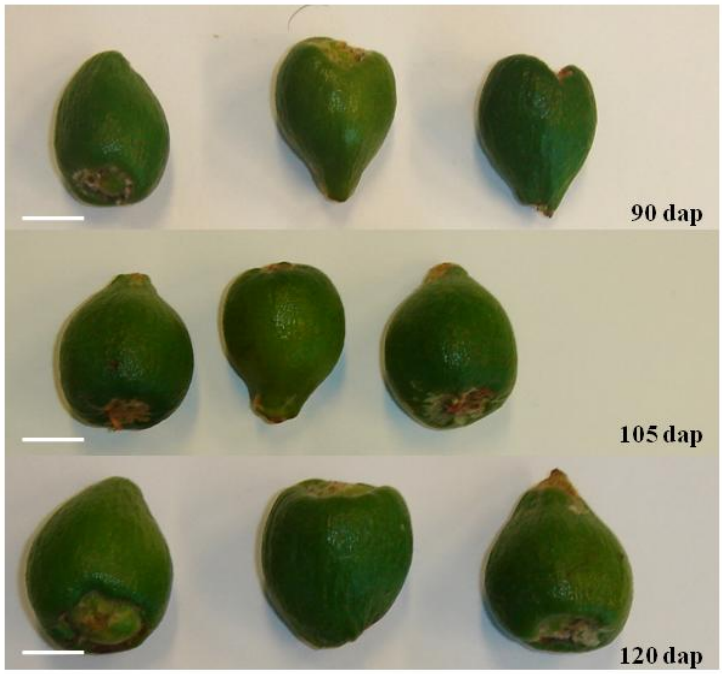

Fig.4.1. Fruits harvested 90, 105 and 120 days after pollination (dap), which corresponded to fruit enlargement stages. *Bars* 1 cm.

The percentage of seed germination in control conditions ranged from 75% in 'Algerie' × 'Changhong-3' to 97 % in 'Algerie' × 'Saval Brasil' (Table 4.4). Increasing the number of dap for proceeding with the embryo rescue improved the percentage of seed germination in control conditions. For both irradiation doses (150 and 300 Gy) a reduction of the seed germination percentage similar to the effect on fruit set (average value for 150 and 300 Gy doses were 29.2% and 18.4% respectively) was observed. There was no difference in embryo germination rates between fruit harvested 105 and 120 dap in radiated pollen samples (Table 4.4). However, we could not conclude that embryo rescue at later stages (120 dap) resulted in recovery of more plants that at shorter times (90 dap).

In 2013, the percentage of seed germination in control condition was approx. 100%. The rate of germinated seeds in fruits harvested 105 and 120 dap at 300 Gy

treatment decreased compared with fruits harvested 90 dap (Table 4.5). As in the previous year, embryo rescue at later stages did not result in increased number of recovered plants.

Table 4.4. Effect of pollen irradiation and harvest time (days after pollination) on embryo rescue and parthenogenetic plant production of three loquat crosses in 2012.

Cross	dap*	Irradiation dose (Gy)	Fruits harvested (no.)	Germinated seeds [no. (%)]	Haploid (no.)	Diploid (no.)
Algerie × Changhong-3	90	0	15	27 (75.0)	0	27
		150	85	80 (45.2)	0	80
		300	68	52 (28.7)	0	52
	105	0	15	26 (76.5)	0	26
		150	85	41 (25.0)	0	41
		300	69	26 (14.8)	0	26
	120	0	15	30 (81.1)	0	30
		150	86	43 (23.9)	0	43
		300	69	23 (12.1)	0	23
Algerie × Cox	90	0	15	28 (84.8)	0	28
		150	92	87 (45.1)	0	87
		300	47	27 (27.6)	0	27
	105	0	15	30 (85.7)	0	30
		150	92	40 (20.1)	0	40
		300	47	25 (23.4)	0	25
	120	0	15	33 (91.7)	0	33
		150	92	57 (31.8)	0	57
		300	48	23 (22.1)	0	23
Algerie × Saval Brasil	90	0	15	30 (85.7)	0	30
		150	70	42 (29.6)	0	42
		300	67	23 (17.0)	0	23
	105	0	15	32 (97.0)	0	32
		150	70	25 (18.8)	0	25
		300	67	12 (8.3)	3	9
	120	0	15	31 (91.2)	0	31
		150	70	35 (23.3)	0	35
		300	67	16 (11.2)	0	16

*dap: days after pollination

Ploidy level

A precise estimation of nuclear DNA content of the recovered plantlets was carried out by flow cytometry. A first analysis was made 2 months after sowing, when the plants developed at least 2-3 true leaves. Individual plants were analyzed, including those obtained from non-irradiated pollen.

In 2012, the ploidy level of the plants obtained was diploid for all the conditions except the cross ‘Algerie’ × ‘Saval Brasil’. In this cross, with a dose of 300 Gy and embryo rescue performed at 105 dap resulted, three haploid plants out of 12 were

recovered (Table 4.4). However, the plants died during acclimatization due to a toxic phytosanitary spray. No further ploidy analysis was carried out.

Table 4.5. Effect of pollen irradiation at 300 Gy and harvest time (days after pollination) on embryo rescue and parthenogenetic plant production of three loquat crosses in 2013.

Cross	dap*	Irradiation dose (Gy)	Fruits harvested (no.)	Germinated seeds [no. (%)]	Haploid (no.)	Diploid (no.)
Algerie × Changhong-3	90	0	10	22 (95.7)	0	22
		300	25	21 (84.0)	0	21
	105	0	10	22 (100)	0	22
		300	40	15 (36.6)	0	15
	120	0	10	22 (100.0)	0	21
		300	38	9 (29.9)	0	9
Algerie × Cox	90	0	10	21 (100)	0	21
		300	24	23 (62.2)	0	23
	105	0	10	18 (100)	0	18
		300	83	20 (23.8)	0	2
	120	0	10	23 (100)	0	23
		300	105	38 (80.9)	0	38
Algerie × Saval Brasil	90	0	10	19 (100)	0	19
		300	31	14 (51.9)	0	14
	105	0	10	20 (90.9)	0	20
		300	83	10 (20.0)	1	9
	120	0	10	21 (100)	0	21
		300	97	15 (40.5)	0	15

*dap: days after pollination

In 2013, for the same condition ‘Algerie’ × ‘Saval Brasil’, 300 Gy irradiation dose and embryo rescue at 105 dap) one haploid was obtained from the cross ‘Algerie’ × ‘Saval Brasil’ out of 10 recovered plants. The haploid level was confirmed by flow citometry and chromosome count in a leaf sample (Figure 4.2).

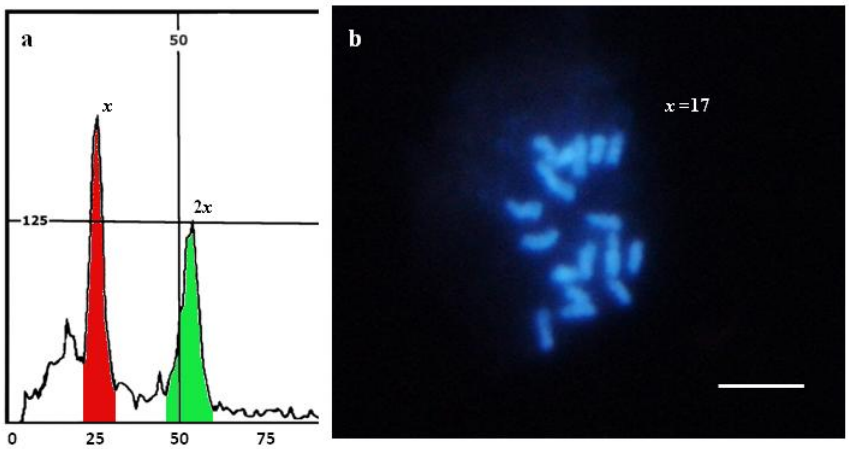

Fig.4.2. Ploidy analysis. (a) Flow cytometry histogram of diploid control loquat plant (2*x*) and the haploid obtained by induced partheogenesis (*x*); (b) Chromosomes count on loquat leaf piece of the haploid cell (2*n* = *x* = 17). *Bar* 10µm.

Seventeen chromosomes were visible at the cytological analysis. The haploidy was confirmed again by flow cytometry once the plants were transferred to greenhouse. The haploid plant presented very small leaves and a reduced growth habit when compared with diploid plants (Figure 4.3). Unfortunately, the weak growth resulted in death of the plant during the procedure of acclimatization.

Fig. 4.3. Haploid plant subcultured in test tube. *Bars* 1 cm.

Discussion

Effect of pollen irradiation on pollen viability and fruit set

Irradiation did affect pollen viability for the two doses tested. The effect of the radiation dose on pollen germination rate is a specie-dependent parameter. In some species, this effect is limited, as in the case of apple (Zhang and Lespinasse 1991), mandarin (Froelicher et al. 2007) and sweet cherry (Höfer and Grafe 2003), while in some others, as European plum, pumpkin, winter squash and walnut, the gamma-ray dose affects significantly the pollen germination (Peixe et al. 2000; Kurtar et al. 2009; Grouh et al. 2011). We have obtained a significant reduction of pollen germination in irradiated pollen at both doses (150 and 300Gy), being more reduced at higher doses. Results indicated that loquat is more sensitive to ionizing radiation than other fruit trees. Additionally, a strong genotype influence is detected in this specie. In this case, the cv. 'Cox' showed an especially high sensitivity.

Fruit set was affected by pollen irradiation in all essayed crosses, the degree to which irradiated pollen affected fruit set was dose-dependent. Similar results were obtained in apple (Zhang and Lespinasse 1991), pear (Bouvier et al. 1993), citrus (Froelicher et al. 2007), cacao (Falque et al. 1992), melon (Lotfi et al. 2003) and European plum (Peixe et al. 2000), kiwifruit (Chalak and Legave 1997). The effect of irradiation of pollen on fruit set can be explained by the damage cause by low levels of irradiation on the generative nucleus while maintaining its capacity to fertilize the egg cell leading to a successful hybridization (Sestili and Ficcadenti 1996). In citrus, higher doses of irradiation resulted in an increase of small seeds (Ollitrault et al. 1996). The small seeds corresponded to empty seeds without embryo. In other species, the irradiation causes a decrease in the number of seeds, mainly due to the aborted seeds. In *Arabidopsis* the abortion rate at 400 Gy was 50% (Yang et al. 2004). In loquat we obtained a reduction of fruit set varying from 30% when a dose of 150 Gy was used to 50% for a dose of 300 Gy. Besides of reduction of fruit set, pollination with irradiated pollen causes early fruit drop in some species (Peixe et al. 2000; Grouh et al. 2011), an explanation could be that the pollen tube cannot reach the egg cell, inducing an early embryo abortion and fruit drop (Peixe et al. 2000).

Induced parthenogenesis

The ratio of haploid per embryo obtained was 3:422 and 1:146 in the first and second experiment respectively, both in the cross ‘Algerie’ × ‘Saval Brasil’. These results are higher than those obtained in pear trees with 2/594 (Bouvier et al. 1993) but lower than those on kiwifruit with 18/44 and 21/21 for different doses (Chalak and Legave 1997) and citrus with 1/11 and 1/26 for Clementine and ‘Fortune’ (Froelicher et al. 2007).

The effect of irradiation dosage of pollen for inducing parthenogenesis varies among species. In this experiment, we found that 300 Gy was the optimum dose for recovery haploid embryos, in fact, none haploids were recovered when lower dose as 150 Gy were used. Doses between 25 and 50 Gy were the best ones for squash (Kurtar et al. 2002), 250 Gy for pear (Bouvier et al. 1993), 300 Gy for citrus (Froelicher et al. 2007), 200 and 500 Gy for apple (Zhang and Lespinasse 1991) and 1.500 Gy for kiwifruit (Chalak and Legave 1997).

In conclusion, in our study parthenogenesis induced by irradiated pollen has allowed to obtain haploid plants from the cv. ‘Algerie’. The efficacy of embryo induction in loquat is determined by several factors, such as radiation dose and genotype. A low dose of gamma radiation, about 300 Gy, was shown to be effective. Haploids have been produced at low frequencies but this paper reports the first results of a programme to obtain haploids using irradiated pollen. We have shown that use of parthenogenesis induced by irradiated pollen is more effective than anther culture (Blasco et al. 2014c, submitted) for production of haploid loquat plants, at least for our cultivars

DISCUSIÓN GENERAL

El banco de germoplasma del IVIA, es la colección de esta especie más importante fuera de Asia, conteniendo accesiones procedentes de casi todos los lugares del mundo donde la especie se ha domesticado y adaptado. Una gran parte corresponde a accesiones prospectadas en los países de la cuenca del mediterráneo, donde a pesar de su introducción tardía se han producido y diversificado un gran número de variedades de las cuales muchas se cultivan actualmente.

Dado que los recursos genéticos representan una de las herramientas más importantes de la mejora genética, nos planteamos en este trabajo realizar una caracterización molecular de la colección, que complementará la caracterización fenotípica que se está llevando a cabo en el grupo (Martínez-Calvo et al. 2008). Los marcadores utilizados fueron los SSRs desarrollados por el equipo del IVIA (Gisbert et al. 2009b), pues a pesar de que se ha demostrado una alta transferibilidad de dichos marcadores entre especies próximas genéticamente, siempre es más efectivo utilizar los procedentes de la misma especie (Soriano et al. 2005). La caracterización molecular ha permitido la identificación de todas las accesiones, conocer la diversidad genética de la colección, calcular distancias genéticas entre accesiones, así como dilucidar el posible origen híbrido o mutacional de algunas de las prospectadas en la zona de cultivo del níspero. Esta información es muy relevante en la planificación de las hibridaciones en un programa de mejora. Además, el genotipado de los alelos de autoincompatibilidad ha proporcionado información sobre los grupos de intercompatibilidad. La autocompatibilidad es un carácter agronómico muy crítico para asegurar la producción, y por tanto muy importante durante la evaluación de nuevas selecciones en un programa de mejora (Gisbert et al. 2009; Socias i Company et al. 2011).

Para conocer la estructura genética del banco de germoplasma se han analizado los genotipos mediante Análisis Factorial de Correspondencias (FCA), análisis de agrupación Bayesiano mediante el programa STRUCTURE y UPGMA. Estos análisis se habían aplicado de forma satisfactoria en estudios de diversidad de olivo (Breton 2006) trigo (Couviour et al. 2011) y vid (Emanuelli et al. 2013). El estudio indicó que, según la composición alélica de las accesiones, el Banco de Germoplasma del IVIA se estructura en 5 grupos o subpoblaciones, donde más del 70% de las accesiones que lo componen tienen un coeficiente de pertenencia (q_i) superior a 0.8, siendo el resto una mezcla entre dos o tres grupos (Burle et al. 2001; Hurtado et al. 2012). Los grupos encontrados corresponden a 3 grupos de accesiones europeas y 2 de origen distinto al europeo.

Las accesiones europeas forman 3 grupos claramente definidos: dos con materiales provenientes principalmente de España y otro con materiales de Italia y España. Como describió Morton (1987) a finales del s. XVIII, el níspero se introdujo en Europa como planta ornamental en los jardines botánicos de París y Kew, desde donde se distribuyó a distintas zonas de la cuenca Mediterránea. A pesar de esta tardía introducción, se produjo una buena adaptación al clima mediterráneo, acompañada de una alta diversificación que dio como resultado un elevado número de accesiones autóctonas que concuerda con la subestructura observada de los materiales europeos.

Dentro del grupo Español I hay que destacar que 16 accesiones comparten el mismo genotipo que 'Algerie'. Estos datos están de acuerdo con el origen de estas variedades, que fueron seleccionadas por los agricultores en una pequeña zona de cultivo de níspero en el sureste de España (Llácer et al. 2002), donde el cultivo se apoya en el cv. 'Algerie', por lo que procederían por mutación de esta variedad. También destacar que las accesiones introducidas en la colección desde China, Japón y América del Norte, se han agrupado en una subpoblación, lo que se explica por la proximidad geográfica y también por la introducción del níspero en Norteamérica desde Japón (Lin et al. 1999; Badenes et al. 2013). Por otra parte, la distribución y frecuencia de los alelos *S* nos ha proporcionado datos sobre el movimiento de germoplasma durante la propagación de la especie fuera de Asia (Badenes et al. 2013). Por ejemplo el alelo '*S*b' aparece sólo en accesiones mediterráneas y en algunas chinas como 'Jiefanghong' y 'Taicheng-4', lo que podría indicar que estas variedades están relacionadas con los ancestros procedentes de China que dieron lugar a la diversificación del níspero en el Mediterráneo. En cambio, el resto de alelos identificados se distribuyen en todos los grupos o subpoblaciones. Otro hecho que sugiere que el origen de las accesiones mediterráneas se sitúa en China, es la menor distancia genética entre el grupo no europeo I y el grupo I de accesiones españolas, corroborando lo indicado por Lin et al. (1999). Sin embargo, la falta de documentación completa de los movimientos de germoplasma, que en nuestro caso se limita únicamente el banco de germoplasma donde son mantenidos, hace difícil conocer con más detalle la relación entre los orígenes del material y las agrupaciones obtenidas.

A pesar de estas deficiencias en la documentación y origen de las accesiones, este trabajo ha proporcionado información relevante sobre la diversidad genética del níspero y su propagación en la cuenca mediterránea**.** La subestructura de la colección de níspero del IVIA descrita, conjuntamente con la diversidad observada y los datos

relativos a los alelos de autoincompatibilidad, permitirá seleccionar e incorporar los materiales más convenientes a los programas de mejora de la especie.

Entre las distintas técnicas que se han estudiado con el fin de implementar el programa de mejora del níspero, están aquellas destinadas a aumentar la diversidad creando nuevos genotipos con diferentes niveles de ploidía. Uno de los estudios ha tenido como objetivo la obtención de poliploides por medio de mutagénesis química con colchicina y posterior selección *in vitro*. La inducción de poliploides tiene un gran interés en la mejora del níspero ya que pueden dar lugar a variedades con frutos de mayor tamaño, alta productividad y resistencia a enfermedades (tetraploides), o producir frutos sin semilla (triploides). Este último objetivo es especialmente importante en especies como el níspero, con frutos relativamente pequeños (30 a 40 g) y donde la parte comestible es menor al 70% (He et al. 2012). Dado que no existía información previa en esta especie, se diseñó un estudio donde se evaluó *in vitro* el efecto de la dosis y tiempo de exposición a colchicina en diferentes explantes de níspero tales como ápice terminal, plántulas enteras y semillas sin germinar. Independientemente del explante tratado, a medida que aumentaba la concentración y tiempo de exposición a colchicina la tasa de mortalidad se incrementaba. La mayor tasa de letalidad se observó en los tratamientos con soluciones de colchicina al 0.5% durante 48 h. En este trabajo se han ensayado dosis de colchicina más altas de las que normalmente se utilizan en especies frutales, que oscilan entre 0.02% y 0.1% (Dhooghe et al. 2011), y con estas altas dosis la tasa de letalidad observada en níspero ha sido inferior a la observada en los cítricos, donde fue de 100% en yemas microinjertadas (Aleza et al. 2009a).

La aplicación de colchicina sobre el ápice terminal, a pesar de ser el método más eficiente en términos de tasas de inducción de poliploides putativos, no logró generar plantas poliploides adultas. Esto puede explicarse por la producción de quimeras, un efecto común en los tratamientos con colchicina (Schifino y Moraes-Fernandes 1987; Tel-Zur et al. 2011). En los tratamientos sobre ápices terminales sólo parte de las células o capas de células resulta afectada, produciéndose posteriores reversiones al estado diploide (Väinölä 2000; Harbard et al. 2012). Por otra parte, el tratamiento de inmersión en colchicina de plántulas enteras tampoco produjo plantas poliploides adultas. En este caso, los 16 poliploides putativos obtenidos mostraban crecimiento anómalo e inhibición de la elongación de la radícula, lo que produjo la muerte de los mismos. Este efecto de la colchicina sobre el crecimiento de la raíz también ha sido descrito en tratamientos con colchicina de semillas de *Platanus* × *acerifolia* (Liu et al. 2007).

Por último, el tratamiento de las semillas sin germinar con distintas concentraciones de colchicina causó mayor letalidad y menor número de poliploides putativos. Sin embargo, de los 9 posibles poliploides, 2 fueron triploides (3*x*) y 1 tetraploide (4*x*). El nivel de ploidía se determinó mediante citometría de flujo, los resultados se confirmaron posteriormente por conteo cromosómico en hoja y ápice radicular y por evaluación de caracteres morfológicos. En nuestro caso, dado que la colchicina lo que provoca es la 'duplicación cromosómica', los triploides obtenidos posiblemente se originaran en el proceso previo de hibridación, por la no reducción de uno de los gametos de uno de los parentales. Ya anteriormente en níspero se han obtenido triploides espontáneos a partir de cruzamientos entre plantas diploides. La tasa de no reducción gamética observada en níspero varía con el genotipo entre 0.18% y 1.62% (Guo et al. 2007). En otras especies se han obtenido triploides a partir de hibridaciones interespecíficas (Hahn et al. 1990) o mediante autofecundación (Ollitrault et al. 2007).

La caracterización morfológica de los poliploides reveló un mayor tamaño y menor densidad de estomas en sus hojas. Este efecto se observó también en estudios llevados a cabo en especies del género *Coffea* (Mishra 1997), en *Aegilops neglecta* Req. ex Bertol. (Aryavand et al. 2003) y en líneas de trigo (Khazaei et al. 2010), donde los autores observaron una mayor frecuencia de estomas en los diploides pero de un menor tamaño. Todos los autores concluyeron que el tamaño y la densidad de los estomas en las hojas es un carácter fenotípico que puede servir para identificar los poliploides.

En níspero la mutagénesis química mediante colchicina ha permitido la obtención de poliploides, pero teniendo en cuenta las tasas de supervivencia y recuperación de tetraploides estables después de los tratamientos, el uso de otros agentes antimitóticos como la orizalina podría considerarse una alternativa. En este sentido, con el uso de la orizalina, la reversión de tetraploides putativos a estado diploide podría ser menor que en el caso de la colchicina, aunque esta pueda inducir un retraso del crecimiento de las plántulas que dilate su evaluación (Lehrer et al. 2008). En programas dirigidos al desarrollo de tetraploides en cítricos resultó ser menos fitotóxica, aunque indujo niveles más bajos de tetraploidía (Aleza et al. 2009a). Teniendo en cuenta los resultados obtenidos, la generación de poliploidía en níspero puede mejorar su eficiencia mediante una ampliación de los métodos de aplicación de colchicina o por medio del ensayo de otros agentes antimitóticos. No obstante, los poliploides obtenidos

tienen un alto valor, se han injertado en campo con el fin de caracterizarlos y estudiarlos para su incorporación al programa de mejora.

Por otro lado, con la finalidad de obtener haploides y doble-haploides (DH), se ha estudiado la capacidad de inducción de embriogénesis gamética en ambos tipos de gametos, masculinos (cultivo de microsporas aisladas y anteras) y femeninos (partenogénesis inducida por polen irradiado). La producción de líneas puras mediante técnicas biotecnológicas es especialmente útil en especies de largo período intergeneracional como el níspero, ya que permite la fijación de caracteres en homocigosis en una sola generación. Los genotipos homocigóticos ofrecen nuevas posibilidades para estudios genéticos y de mejora, incrementando la eficacia de la selección.

En los experimentos de cultivo de microsporas aisladas se consiguió inducir callogénesis en diversas de las accesiones estudiadas, siendo el primer paso hacia la respuesta morfogénica. El genotipo de la planta donante afectó a la inducción de callogénesis, 6 de los 11 cultivares produjeron respuesta. El elevado efecto del genotipo de partida sobre la respuesta ya se había observado con antelación en otro trabajo de cultivo de microsporas de níspero (Padoan et al. 2011). En el cultivo *in vitro* de anteras se determinó en primer lugar la etapa de desarrollo del gameto más apropiada para que tenga lugar la reprogramación de la miscrospora de níspero. Los resultados revelaron que en la etapa de microspora vacuolada, cuando el botón floral mide entre 6.5 – 7.0 mm de longitud, puede considerarse el momento idóneo para que la microspora abandone la ruta gametofítica en la formación de polen hacia la ruta esporofítica que puede conllevar a la formación de embriones haploides. Estos resultados permitieron establecer un criterio de selección rápido del estadio de desarrollo del polen en las yemas florales (González-Melendi et al. 2005; Gemanà y Chiancone 2003; Peixe et al. 2004). En la mayoría de especies estudiadas, al igual que sucede en níspero, los estadios óptimos para inducir embriogénesis son microspora vacuolada y polen bicelular temprano (Raghavan et al. 1986; Smýkal et al. 2000; Pechan et al. 2001; Germanà y Chiancone 2003; Peixe et al. 2004). Sin embargo, la embriogénesis del polen depende de un amplio número de factores como los pretratamientos aplicados a las anteras o a los botones florales, el genotipo empleado y la composición del medio de cultivo, entre otras. Los pretratamientos de frío de los botones florales son muy utilizados para inducir la embriogénesis en muchas especies tales como arroz (Zapata-Arias 2003), cebada (Huang y Sunderland 1982; Szarejko 2003), cítricos (Germanà y Chiancone 2003), maíz

(Barnabás 2003), fresa (Shahvali-Kohshour et al. 2013), manzano (Zhang et al. 2013) y trigo (Kasha et al. 2003). En nuestro estudio, el pretratamiento de frío (4 ºC) aplicado a las yemas florales inhibió o redujo la respuesta respecto al control sin pretratamiento, siendo la repuesta dependiente del genotipo. Otros autores han obtenido respuesta sin pretratamiento de frío en olmo (Li et al. 2013) o peral (Kadota y Niimi 2004). La diversidad de resultados obtenidos podría explicarse por el gran efecto de las condiciones fisiológicas del material de partida sobre la respuesta androgénica (Germanà 2011a), que en el caso del níspero donde los árboles están en campo es difícil de estandarizar. El efecto del tipo y concentración de regulador del crecimiento en el medio de cultivo también fue evaluado, observándose una respuesta altamente dependiente del genotipo y de nuevo, de las condiciones de cultivo de la planta donante. El mayor porcentaje de anteras con callo se obtuvo en el medio base suplementado con 4.56 μM Z y 5.36 μM ANA en los cvs. 'Changhong-3' (27%), 'Jiefanghong' (30%) y 'Moggi Wase' (36%). En árboles frutales, diferentes tipos y concentraciones de auxinas han sido utilizados para inducir la formación de callos a partir de anteras, 28.55 μM AIA en *Annona squamosa* (Nair et al. 1983), 0.54 μM ANA en *Carica papaya* (Rimberia et al. 2007), 0.11 μM ANA y 0.09 μM 2,4-D in *Citrus clementina* (Germanà y Chiancone 2003), sin adición de auxina en *Malus domestica* (Höfer et al. 1999; Höfer 2004) y 2.26 μM 2,4-D en *Eriobotrya japonica* (Li et al. 2008). Hay que señalar que, en este estudio se aplicaron bajo idénticas condiciones experimentales, las mismas concentraciones de reguladores de crecimiento empleado por Li et al. (2008) en un trabajo de cultivo de anteras de níspero, nosotros al contrario que ellos no observamos respuesta androgénica en ninguno de los cultivares analizados. Cuando los callos diferenciados se transfirieron a medio de inducción de embriogénesis, se formaron 6 embriones a partir de anteras del cv. 'Jiefanghong', uno de ellos se desarrolló en planta adulta, mientras el resto se necrosó y murieron. Los resultados de la citometría de flujo y el conteo cromosómico determinaron que la planta era triploide (3*x*). Estas alteraciones citológicas con modificación de los niveles de ploidía de plantas obtenidas a partir de cultivo *in vitro* de anteras, podría deberse al uso de reguladores del crecimiento en el medio de cultivo, lo cual puede causar un desequilibrio en la actividad mitótica de las células (Winarto et al. 2010). Se han obtenido triploides a partir de cultivo de anteras de distintas especies leñosas, *Citrus clementina* (Germanà et al. 2005), *Populus* × *beijingensis* (Li et al. 2013) y *Pyrus pyrifolia* (Kadota y Niimi 2004).

Por tanto, el trabajo realizado supone un paso importante en la embriogénesis del polen *in vitro* de níspero ya que demuestra que ésta es posible mediante el cultivo de anteras, por lo que cabe esperar la obtención de individuos haploides o DH en futuros experimentos. No obstante, teniendo en cuenta los factores que influyen en el éxito final del cultivo, la técnica necesita optimizarse para llevar a plenitud las múltiples aplicaciones a las que puede dar lugar.

Otro método de obtención de haploides, alternativo a la embriogénesis del polen, es la partenogénesis inducida con polen irradiado con rayos gamma y posterior rescate y cultivo de embriones *in vitro***.** El factor clave para la obtención de individuos haploides mediante partenogénesis se centra en ajustar la dosis de irradiación que recibe el polen y el ratio de haploide/diploide recuperados. En el caso concreto del níspero japonés, la dosis óptima que permitió obtener embriones haploides fue de 300 Gy, pero produjo una reducción de la tasa de germinación del polen destacada. El efecto de la dosis de irradiación sobre la germinación del polen varía según especies. En algunas especies como cerezo (Höfer y Grafe 2003), cítricos (Froelicher et al. 2007) o manzano (Zhang y Lespinasse 1991) no se ha registrado efecto alguno sobre la viabilidad del polen, mientras que en otras especies como calabaza, ciruelo o nuez la dosis de rayos gamma si afecta significativamente (Peixe et al. 2000; Kurtar 2009; Grouh et al. 2011). También afecta al cuajado del fruto, en nuestro estudio la irradiación redujo el cuajado en una media de 30% y 50% cuando el polen se irradió a dosis de 150 y 300 Gy respectivamente. Posiblemente porque el tubo polínico no fue capaz de alcanzar el óvulo, causando el aborto del embrión, lo que provocó la caída prematura del fruto. Resultados similares se han obtenido en cacao (Falque et al. 1992), ciruelo europeo (Peixe et al. 2000), cítricos (Froelicher et al. 2007), manzano (Bouvier et al. 1993) y melón (Lotfi et al. 2003).

En este estudio del cv. ‘Algerie’ respondió a la inducción de haploides por medio de partenogénesis inducida por polen irradiado, aunque el número de haploides obtenido fue más bajo que en otras especies. Se obtuvieron 4 haploides a partir de hibridaciones con polen irradiado del cv. ‘Saval Brasil’ a dosis de 300 Gy. Estos resultados son similares a los obtenidos en peral, donde se obtuvieron 2 haploides procedentes de polen irradiado a 250 Gy (Bouvier et al. 1993) pero más bajos que los obtenidos en kiwi (Chalak y Legave 1997).

El trabajo muestra que la partenogénesis inducida con polen irradiado es una herramienta útil para la inducción de haploides. En nuestro caso, ha sido posible obtener

haploides del cv. ‘Algerie’ de gran interés para la obtención de líneas puras. Sin embargo, dado el ratio obtenido entre embriones haploides y embrión rescatado, es necesario mejorar las técnicas de rescate de embriones y los procesos de aclimatación de las plántulas.

CONCLUSIONES

1. El análisis de diversidad del Banco de Germoplasma de níspero del IVIA ha revelado que las accesiones que lo componen pueden subdividirse en cinco grupos geográficamente distintos, donde el 71% de las accesiones poseen un coeficiente de pertenencia (q_i) a un único grupo superior a 0.8. Los grupos observados fueron consistentes en los tres distintos análisis realizados.
2. Se han obtenido poliploides, un tetraploide (4*x*) y dos triploides (3*x*), a partir de la inmersión de semillas sin germinar de polinización abierta del cv. 'Algerie' en una solución acuosa de colchicina (0.5%). Se ha observado que la colchicina afecta a la viabilidad de las semillas y a la tasa de desarrollo de las mismas.
3. La planta tetraploide (4*x*) se ha incorporado al Programa de Mejora de Níspero del IVIA como parental masculino para la posterior obtención de híbridos triploides. A su vez, se está llevando a cabo la evaluación morfológica, agronómica y molecular tanto del tetraploide (4*x*) como de los triploides (3*x*).
4. Se ha conseguido la inducción de callogénesis mediante el cultivo de microsporas aisladas y embriogénesis mediante el cultivo de anteras de diferentes cultivares de níspero japonés.
5. Se ha establecido una correlación entre el estado morfológico de las anteras y las diferentes etapas de desarrollo del polen en los diferentes cultivares. La fase de microspora vacuolada fue la más adecuada para la inducción de respuesta.
6. Se han estudiado diversos factores y su influencia en el cultivo *in vitro* de anteras: pretratamiento con frío de los botones florales, genotipo y medio de cultivo de las anteras, determinándose las condiciones idóneas para inducir la embriogénesis. No obstante, se ha podido comprobar la gran influencia que tiene el estado fisiológico de la planta donante.
7. Se ha regenerado con éxito una planta a partir de los embriones (6) derivados de polen y originados a partir del cultivo de anteras del cv. 'Jiefanghong'. El análisis cromosómico mediante citometría de flujo y conteo cromosómico en hoja ha determinado que se trata de una planta triploide (3*x*).
8. La obtención de la planta triploide en níspero mediante embriogénesis del polen, indica que la técnica de cultivo de anteras puede considerarse un método alternativo a la obtención de poliploides sintéticos/artificiales.
9. La partenogénesis inducida con polen irradiado con rayos gamma ha demostrado ser eficaz en la inducción de haploides ginogénicos. Se han obtenido plantas haploides (4) del cv. 'Algerie' procedentes de semillas extraídas de frutos obtenidos

a partir de la polinización con dicho polen. Se ha establecido la dosis de 300 Gy como la radiación óptima del polen.

MATERIAL SUPLEMENTARIO

Table S1. Alleles obtained of the 102 accessions using 13 microsatellite and a conserved region of *S*-allele

Name of Cultivar	No	ssrEJ005		ssrEJ037		ssrEJ039		ssrEJ042		ssrEJ075a		ssrEJ086		ssrEJ088	
Ronda Brasil	1	214	214	248	248	242	242	202	214	222	228	238	245	215	245
Saval Brasil	2	216	216	248	248	242	242	202	202	228	228	231	231	245	245
Changhong-3	3	214	214	248	248	242	242	202	202	222	228	231	245	215	245
Dazhong	4	214	214	233	233	236	242	202	202	222	222	231	231	231	245
Hongganben	5	214	214	233	233	242	242	202	202	222	222	245	245	215	245
Jiefangzhong	6	214	214	233	233	236	236	202	202	222	222	231	231	231	245
Meihuaxia	7	214	214	233	233	236	236	202	202	222	228	231	245	215	245
Puixnben	8	214	214	233	233	242	242	202	202	222	228	231	238	215	215
Taicheng-4	9	214	214	248	248	242	242	214	214	222	222	238	245	215	245
Xiangzhong-11	10	214	214	233	248	236	242	202	214	222	228	238	245	215	245
Zaozhong-6	11	214	214	233	233	242	242	202	202	222	228	231	245	215	215
Moggi	12	214	214	233	233	242	242	202	202	228	228	231	245	245	245
Moggi Wasse	13	214	214	233	233	242	242	202	202	222	228	245	245	215	245
Tanaka	14	214	216	248	248	242	242	202	202	222	228	238	245	245	245
Bianco	15	216	216	233	233	236	236	205	214	222	222	238	238	215	245
Italiano-1	16	214	214	233	233	236	242	202	214	222	228	238	245	215	245
Marchetto	17	216	216	233	233	236	236	214	214	222	228	238	238	215	231
Ottaviani	18	216	216	233	248	236	242	202	214	222	228	238	245	245	245
Rosa	19	214	214	233	233	236	242	214	214	222	228	238	238	215	231
Rosa Tardío	20	214	214	233	233	236	236	214	214	222	228	238	238	215	231
Sanfilippara	21	214	214	233	233	236	236	214	214	222	228	238	238	215	231
Vaniglia	22	214	216	233	233	236	236	202	214	222	222	238	238	215	231
Virtichiara	23	214	214	233	248	236	242	202	205	222	222	238	245	215	245
Ikramullah-1	24	214	216	233	233	236	242	202	202	222	222	231	231	215	215
Ikramullah-2	25	214	216	233	233	242	242	202	202	228	228	231	231	215	215

Name of Cultivar	No	ssrEJ005		ssrEJ037		ssrEJ039		ssrEJ042		ssrEJ075a		ssrEJ086		ssrEJ088	
Khyber	26	214	216	248	248	242	242	202	202	228	228	231	245	245	245
Saeed-1	27	214	214	233	248	242	242	202	202	222	228	238	245	215	245
Almargem	28	214	214	248	248	242	242	202	202	222	222	238	245	245	245
Manta Mouros	29	214	214	233	248	236	242	214	214	222	222	238	238	215	215
Rolhâo	30	214	214	233	233	242	242	202	214	222	222	245	245	215	245
Tavira	31	214	214	233	248	242	242	202	214	222	228	238	245	215	215
Al-Ama	32	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Alcacer	33	214	216	248	248	242	242	202	202	222	228	238	245	215	245
Alfons Gregori-1	34	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Alfons Gregori-2	35	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Algerie	36	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Algerie clon tardío	37	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Barret	38	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Benimelli	39	214	216	233	233	236	242	202	214	222	228	238	245	215	245
Beniaratx	40	214	216	233	233	236	236	205	214	222	228	231	238	215	215
Borde	41	214	214	233	248	236	242	-99	-99	222	228	238	245	215	245
Cabelo	42	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Cambrils	43	214	216	233	248	236	242	202	205	222	228	238	245	215	215
Chirlero	44	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Cort	45	214	216	233	248	236	242	214	214	222	222	238	245	215	245
Cox	46	214	216	233	233	242	242	202	202	228	228	231	231	215	215
Cremaor	47	214	214	233	233	236	242	202	214	222	222	238	245	215	215
Dama	48	214	216	233	233	236	236	202	202	222	228	231	238	215	215
Dulce Pera	49	216	216	248	248	236	242	202	202	222	228	231	231	215	215

Name of Cultivar	No	ssrEJ005		ssrEJ037		ssrEJ039		ssrEJ042		ssrEJ075a		ssrEJ086		ssrEJ088	
Estrada Blanc	50	214	214	233	248	242	242	202	202	222	228	231	245	215	231
Estrada Groc	51	214	214	233	248	236	242	202	214	222	228	231	238	215	231
Francisco el Gordo	52	214	216	233	233	236	242	202	214	222	228	238	245	215	245
IRTA	53	214	214	248	248	242	242	202	202	222	228	238	245	215	245
Ismael	54	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Javierín	55	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Joaquín Giner	56	214	214	233	248	242	242	202	214	222	228	238	245	215	215
Llinares	57	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Magdal	58	214	214	248	248	236	242	202	202	222	228	231	238	215	245
Magdal Blanco	59	214	216	233	233	242	242	202	202	222	228	231	231	215	215
Maite	60	214	216	233	233	236	242	202	214	222	228	238	238	215	245
Manises	61	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Marc	62	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Mas Vagué	63	214	214	233	248	242	242	202	202	222	228	231	245	215	245
Masía Cañera	64	214	214	233	233	236	242	202	214	228	228	238	245	215	245
Menera	65	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Mercedes	66	214	216	233	233	236	236	202	205	222	222	238	245	215	215
Mil Homens	67	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Miquel d'Aixarà	68	214	216	233	248	242	242	202	214	222	228	238	245	245	245
Miquel Nucier	69	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Nadal Tardío	70	214	216	233	233	242	242	202	205	222	222	231	231	215	231
Nadal Temprano	71	214	216	233	233	242	242	202	202	222	222	231	231	215	231
Peluches	72	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Peix	73	214	216	233	233	236	242	214	214	222	228	238	238	215	215
Pere Esquena	74	214	216	233	233	242	242	202	214	222	228	231	238	215	245

Name of Cultivar	No	ssrEJ005		ssrEJ037		ssrEJ039		ssrEJ042		ssrEJ075a		ssrEJ086		ssrEJ088	
Piera	75	214	216	233	233	236	242	202	214	222	228	238	238	215	215
Polop-1	76	214	216	233	233	236	242	202	202	222	228	231	238	215	215
Raúl	77	214	216	233	248	242	242	202	202	222	222	238	245	215	245
Redonet	78	214	214	233	233	236	242	202	214	222	222	238	245	215	245
Requina	79	214	216	233	233	236	242	202	214	222	228	238	245	215	245
Ronda Gruesos	80	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Sacós	81	214	214	233	233	242	242	205	214	222	228	238	238	215	231
Saguntí	82	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Sally	83	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Samper-1	84	214	216	233	233	236	242	202	214	222	228	238	238	215	245
Samper-2	85	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Saval 1	86	214	214	233	233	236	242	202	214	222	228	238	245	215	245
Saval 2	87	214	216	233	233	242	242	202	214	222	228	238	245	215	245
Saval Moreno	88	214	216	233	248	236	242	202	214	222	222	238	238	215	245
Saval Nerviado	89	214	216	233	248	242	242	202	214	222	228	238	245	215	245
Sisantanou	90	214	214	233	233	242	242	202	202	222	222	231	245	215	215
Siscar	91	214	216	233	248	236	242	214	214	222	228	238	245	215	245
Susana	92	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Temprano Petrés	93	214	214	233	233	236	236	202	205	222	222	238	245	245	245
Toni Tomaca	94	214	216	233	248	242	242	202	202	222	228	245	245	215	245
Vila	95	214	214	233	248	242	242	202	214	222	228	238	245	215	245
Advance	96	214	216	233	248	236	242	202	202	222	228	231	231	215	215
Champagne	97	214	216	233	248	236	242	202	202	228	228	231	231	215	215
Golden Nugget	98	214	214	233	248	236	242	205	214	222	222	238	245	215	245
McBeth	99	214	216	248	248	242	242	202	202	222	228	231	245	215	245

Name of Cultivar	No	ssrEJ005		ssrEJ037		ssrEJ039		ssrEJ042		ssrEJ075a		ssrEJ086		ssrEJ088	
Mrs Cooksey	100	214	214	248	248	242	242	202	214	222	228	238	245	245	245
Sabroso	101	214	214	233	248	236	242	202	202	222	222	231	231	215	215
Vista White	102	214	214	248	248	236	242	202	202	222	228	231	231	215	215

Name of Cultivar	No	ssrEJ095b		ssrEJ104		ssrEJ271		ssrEJ282		ssrEJ324		ssrEJ329		SC/C2	
Ronda Brasil	1	243	243	162	165	192	197	162	187	242	246	154	175	467	294
Saval Brasil	2	243	243	162	165	197	197	162	162	233	242	175	175	280	270
Changhong-3	3	239	243	168	168	192	192	154	162	242	242	154	175	322	322
Dazhong	4	239	239	162	168	192	247	154	154	258	258	154	154	294	270
Hongganben	5	239	243	165	165	192	197	154	154	242	242	154	175	322	280
Jiefangzhong	6	239	239	162	168	192	247	154	154	258	258	154	154	294	270
Meihuaxia	7	239	243	162	165	192	192	154	154	233	258	154	175	600	270
Puixnben	8	239	239	162	168	192	197	154	154	233	233	154	175	280	270
Taicheng-4	9	239	243	168	168	192	192	162	187	242	246	154	175	467	459
Xiangzhong-11	10	239	243	168	168	192	197	162	185	242	246	154	175	294	270
Zaozhong-6	11	239	243	162	168	192	197	154	162	258	258	154	154	294	270
Moggi	12	243	243	162	168	192	197	154	162	233	258	154	175	294	270
Moggi Wasse	13	239	239	162	168	194	247	154	154	242	242	154	175	399	270
Tanaka	14	239	239	162	168	194	197	154	162	233	242	154	175	294	270
Bianco	15	239	243	165	165	231	247	154	187	246	258	154	175	459	322
Italiano-1	16	243	243	162	168	192	192	162	185	242	246	154	175	467	270
Marchetto	17	243	243	168	168	231	231	154	187	246	246	175	175	437	322
Ottaviani	18	239	243	168	168	194	197	162	187	242	242	175	175	270	270
Rosa	19	243	243	165	168	192	231	154	154	246	258	157	175	459	322
Rosa Tardío	20	243	243	168	168	231	247	154	162	246	258	175	175	467	459
Sanfilippara	21	243	243	165	165	231	247	154	162	246	258	175	175	467	459
Vaniglia	22	239	243	168	168	194	247	162	187	246	246	157	175	467	459
Virtichiara	23	239	243	162	165	192	194	162	162	242	246	175	175	280	270
Ikramullah-1	24	239	243	168	168	192	192	133	133	246	246	157	175	437	322
Ikramullah-2	25	239	243	162	162	192	192	154	162	242	242	154	175	437	322

Name of Cultivar	No	ssrEJ095b		ssrEJ104		ssrEJ271		ssrEJ282		ssrEJ324		ssrEJ329		SC/C2	
Khyber	26	239	243	162	165	194	194	154	162	233	242	154	175	399	270
Saeed-1	27	239	243	162	168	192	197	162	187	242	246	154	175	467	294
Almargem	28	239	243	162	168	192	197	154	162	233	242	154	154	294	270
Manta Mouros	29	239	239	162	165	231	247	154	162	233	246	175	175	294	270
Rolhâo	30	239	239	162	168	192	192	154	187	246	258	175	175	459	294
Tavira	31	243	243	162	165	192	197	162	187	242	242	154	175	467	294
Al-Ama	32	243	243	162	168	192	197	162	187	242	246	154	175	467	294
Alcacer	33	239	243	162	168	194	197	162	187	233	242	154	175	294	270
Alfons Gregori-1	34	243	243	162	168	192	197	162	187	242	246	154	175	467	294
Alfons Gregori-2	35	243	243	162	168	192	197	162	187	242	242	154	175	467	294
Algerie	36	243	243	162	168	192	197	162	187	242	246	154	175	467	294
Algerie clon tardío	37	243	243	162	165	192	197	162	187	242	246	154	175	467	294
Barret	38	243	243	162	168	192	197	162	187	242	246	154	175	467	294
Benimelli	39	239	243	162	168	192	192	154	187	246	258	175	175	467	459
Beniaratx	40	243	243	168	168	231	231	162	187	242	242	175	175	467	437
Borde	41	-99	-99	162	165	192	194	162	187	242	246	175	175	300	300
Cabelo	42	243	243	168	168	192	197	162	187	242	246	154	175	467	294
Cambrils	43	239	239	168	168	197	231	154	162	242	246	175	175	437	270
Chirlero	44	243	243	162	165	192	197	162	187	242	246	154	175	467	294
Cort	45	239	243	162	162	192	231	154	187	246	258	154	175	467	294
Cox	46	239	243	162	165	192	192	133	154	242	242	154	157	437	270
Cremaor	47	239	243	162	165	192	231	154	187	246	258	175	175	467	459
Dama	48	243	243	165	165	194	231	162	162	246	246	157	175	270	270

Name of Cultivar	No	ssrEJ095b		ssrEJ104		ssrEJ271		ssrEJ282		ssrEJ324		ssrEJ329		SC/C2	
Dulce Pera	49	239	243	162	165	192	192	154	154	246	246	157	175	459	322
Estrada Blanc	50	239	243	162	168	192	197	162	187	242	246	154	175	459	294
Estrada Groc	51	239	243	162	168	192	197	154	187	242	246	175	175	467	270
Francisco el Gordo	52	239	243	162	165	192	192	154	187	246	258	175	175	467	459
IRTA	53	243	243	162	162	192	194	162	187	242	246	154	175	322	294
Ismael	54	243	243	162	168	192	197	162	187	242	246	154	175	467	294
Javierín	55	243	243	162	168	192	197	162	187	242	246	154	175	467	294
Joaquín Giner	56	243	243	162	165	192	197	162	187	242	246	154	175	467	294
Llinares	57	243	243	162	165	192	197	162	187	242	246	154	175	467	294
Magdal	58	239	243	162	162	194	194	133	162	242	242	175	175	322	294
Magdal Blanco	59	239	243	162	165	192	192	133	154	242	246	154	157	437	270
Maite	60	239	243	162	165	197	231	154	162	242	258	154	175	467	459
Manises	61	243	243	162	165	192	197	162	187	242	246	154	175	467	294
Marc	62	243	243	162	168	192	197	162	187	242	246	154	175	467	294
Mas Vagué	63	243	243	162	162	192	197	133	162	242	246	157	175	467	270
Masía Cañera	64	243	243	162	165	192	192	162	187	242	246	154	175	467	467
Menera	65	243	243	162	168	192	197	162	187	242	242	154	175	467	294
Mercedes	66	239	243	162	165	194	247	162	187	246	246	175	175	322	270
Mil Homens	67	243	243	162	165	192	197	162	187	242	246	154	175	467	294
Miquel d'Aixarà	68	243	243	162	168	192	197	154	162	242	246	154	175	467	294
Miquel Nucier	69	243	243	162	165	192	197	162	187	242	246	154	175	467	294
Nadal Tardío	70	239	243	168	168	192	192	162	187	246	246	175	175	467	459
Nadal Temprano	71	239	239	168	168	192	192	187	187	246	246	175	175	467	459

Name of Cultivar	No	ssrEJ095b		ssrEJ104		ssrEJ271		ssrEJ282		ssrEJ324		ssrEJ329		SC/C2	
Peluches	72	243	243	162	168	192	197	162	187	242	246	154	175	467	294
Peix	73	239	239	162	162	192	197	162	187	246	246	154	175	467	459
Pere Esquena	74	239	243	165	165	192	194	187	187	246	246	175	175	459	294
Piera	75	239	243	162	168	194	231	-99	-99	246	246	175	175	270	270
Polop-1	76	243	243	162	168	192	231	154	187	242	242	157	175	437	322
Raúl	77	239	243	168	168	192	192	154	187	233	246	154	175	459	294
Redonet	78	243	243	162	168	192	192	162	185	242	246	154	175	467	270
Requina	79	239	243	162	168	192	192	154	187	246	258	175	175	467	459
Ronda Gruesos	80	243	243	162	168	192	197	162	187	242	246	154	175	467	294
Sacós	81	239	243	165	168	192	192	162	162	242	246	175	175	467	270
Saguntí	82	243	243	162	168	192	197	162	187	242	246	154	175	467	294
Sally	83	243	243	162	168	192	197	162	187	242	246	154	175	467	294
Samper-1	84	239	243	162	168	197	231	154	162	242	258	154	175	467	459
Samper-2	85	243	243	162	168	192	197	162	187	242	246	154	175	467	294
Saval-1	86	239	243	162	168	192	192	154	187	246	258	175	175	467	459
Saval-2	87	239	243	162	168	192	192	154	187	246	258	175	175	467	459
Saval Moreno	88	239	243	162	165	192	197	162	187	246	258	175	175	467	459
Saval Nerviado	89	243	243	162	168	192	197	154	187	246	258	175	175	459	294
Sisantanou	90	239	243	168	168	192	192	187	187	246	246	175	175	467	459
Siscar	91	239	243	162	165	192	231	187	187	246	246	154	175	467	459
Susana	92	243	243	162	168	192	197	162	187	242	246	154	175	467	294
Temprano Petrés	93	239	239	162	162	194	194	162	162	246	246	175	175	270	270
Toni Tomaca	94	243	243	162	162	197	197	162	162	242	242	175	175	467	270

Name of Cultivar	No	ssrEJ095b		ssrEJ104		ssrEJ271		ssrEJ282		ssrEJ324		ssrEJ329		SC/C2	
Vila	95	243	243	162	168	192	197	162	187	242	246	154	175	467	294
Advance	96	243	243	162	168	192	194	154	154	246	246	154	157	459	270
Champagne	97	239	243	162	165	194	231	133	133	242	242	157	175	459	322
Golden Nugget	98	239	243	162	165	192	192	162	162	242	246	175	175	270	270
McBeth	99	239	243	162	162	194	197	133	154	233	242	154	175	459	294
Mrs Cooksey	100	239	239	162	165	192	197	133	154	242	242	157	175	308	294
Sabroso	101	239	243	162	168	192	192	133	162	246	246	175	175	294	270
Vista White	102	239	243	162	168	192	231	133	154	242	246	157	175	459	270

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